Chemical Biology of 6-Nitrochrysene Induced Deoxyadenosine DNA Adduct and Formamidopyrimidine DNA Lesion: Mutagenesis and Genotoxicity in E. coli and Human Cells

Brent V. Powell
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Chemical Biology of 6-Nitrochrysene Induced Deoxyadenosine DNA Adduct and Formamidopyrimidine DNA Lesion: Mutagenesis and Genotoxicity in *E. coli* and Human Cells

Brent Valentine Powell, Ph.D.

University of Connecticut, 2020

The environmental pollutant, 6-nitrochrysene (6-NC) is the most potent carcinogen evaluated by the newborn mouse assay. The genotoxicity of 6-NC is derived from its ability to form electrophilic species in cells which can react with nucleophilic sites of 2'-deoxyguanosine (dG) and 2'-deoxyadenosine (dA) in DNA to generate DNA-carcinogen adducts. DNA lesions derived from 6-NC can play important roles in the development of human cancer by inducing mutations in crucial genes resulting in the disruption of gene expression. Mutations in an oncogene, a tumor-suppressor gene such as *p53*, or a gene that controls the cell cycle can lead to uncontrolled cell growth, resulting in carcinogenesis, a process which ultimately gives rise to human cancer. Most of these mutations arise through error-prone mutagenic bypass of the lesions which is enabled by low fidelity translesion synthesis (TLS) DNA polymerases.

6-NC is metabolically activated by nitroreduction and a combination of ring oxidation and nitroreduction pathways. The nitroreduction pathway yields major DNA adducts at the C8 and N² positions of dG, *N*-(dG-8-yl)-6-AC and 5-(dG-N²-yl)-6-AC. The nitroreduction pathway also yield adduct at the C8 position of dA, *N*-(dA-8-yl)-6-AC and a 2'-deoxyinosine (di) adduct, *N*-(di-8-yl)-6-AC; which is believed to be a product of deamination of the adenine adduct *N*-(dA-8-yl)-6-AC. The *N*-(dA-8-yl)-6-AC adduct is particularly interesting, a nucleotide excision repair assay demonstrated that it is repaired much more slowly than many other bulky DNA adducts, including other DNA adducts formed by 6-NC. Neither the total synthesis nor cellular replication properties of the *N*-(dA-8-yl)-6-AC or other adducts derived from 6-NC have ever been reported.
Chapter 2 describes synthetic methods developed to access 6-NC modified nucleosides, phosphoramidites and oligodeoxynucleotides of the C8-dA adduct, N-(dA-8-yl)-6-AC employing an optimized Buchwald-Hartwig palladium catalyzed cross-coupling strategy, which provided a high yield of the protected N-(dA-8-yl)-6-AC adducted nucleoside. The protected N-(dA-8-yl)-6-AC adduct was converted to the protected 3’-phosphoramidite monomer and site specifically incorporated into 12 and 15-mer oligodeoxynucleotides (ODNs) via automated solid-phase DNA synthesis. These oligodeoxynucleotides were purified by reverse phase HPLC followed by denaturing polyacrylamide gel electrophoresis and characterized by high resolution mass spectrometry.

Chapter 3 employs 2D NMR spectroscopy and DFT calculations to investigate the conformational preference of N-(dA-8-yl)-6-AC adducted nucleoside. We further investigated the helical structures of N-(dA-8-yl)-6-AC adduct in 12 and 15-mer oligodeoxynucleotide duplexes using circular dichroism (CD) spectroscopy. The stability of N-(dA-8-yl)-6AC adducted nucleoside was also investigated under biologically relevant conditions using UV/Vis spectroscopy and HPLC assays.

Chapter 4 describes investigation of the replication bypass efficiency of N-(dA-8-yl)-6-AC adduct in Escherichia coli and in human embryonic kidney (HEK293T) cells. The 15-mer containing N-(dA-8-yl)-6-AC modified oligodeoxynucleotide was used to construct single-stranded shuttle vectors containing a single N-(dA-8-yl)-6-AC adduct site using recombinant DNA technology. These recombinant vectors were replicated in Escherichia coli and HEK293T cells. The roles of several translesion synthesis (TLS) replication polymerases in adduct bypass was also
investigated via conducting replication experiments in isogenic cells where individual TLS DNA polymerases were depleted by the CRISPR/Cas9 genome editing method or siRNA knockdown approach.

Chapter 5 evaluates the mutagenicity and replication bypass efficiency of the ring opened formamidopyrimidine (Fapy.dG) adduct that is produced via oxidative stress in two interesting p53 mutation hotspot sequences that include codons 248 and 249. Fapy.dG modified oligodeoxynucleotides was used to construct single-stranded shuttle vectors containing a single adducted site and replicated in HEK293T cells.
Chemical Biology of 6-Nitrochrysene Induced Deoxyadenosine DNA Adduct and Formamidopyrimidine DNA Lesion: Mutagenesis and Genotoxicity in E. coli and Human Cells

Brent Valentine Powell

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Chemical Biology of 6-Nitrochrysene Induced Deoxyadenosine Adduct and Formamidopyrimidine DNA Lesion: Mutagenesis and Genotoxicity in *E. coli* and Human Cells

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University of Connecticut, 2020
Dedicated to my mother

Claudine Cleopatra Powell

for her unyielding support and confidence in me.
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The past five and half years at UConn have been productive and have truly made me more resilient. Here, I would like to acknowledge the people who I am deeply indebted to for their advice, friendship, encouragement, and support.

<table>
<thead>
<tr>
<th>Name</th>
<th>Message</th>
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<tbody>
<tr>
<td>Prof. Ashis Basu</td>
<td>I am very grateful for the opportunity to work in your research lab and for your mentorship. Over the years, you have given me insightful feedback and guidance on my work in the lab which have enabled my growth as a scientist. I have also benefitted from your immense knowledge on DNA damage and repair through the many scientific discourse we’ve shared.</td>
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<tr>
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<td>Prof. Amy Howell</td>
<td>I have been motivated by your passion for chemistry and your commitment to training and mentoring students. Thank you for serving on my thesis committee and also for giving me advice before my BI internship.</td>
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<tr>
<td>6-AC</td>
<td>6-aminochrysene</td>
</tr>
<tr>
<td>6-NC</td>
<td>6-nitrochrysene</td>
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<tr>
<td>N-OH-6-AC.</td>
<td>N-hydroxy-6-aminochrysene</td>
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<tr>
<td>2-deoxyribose</td>
<td>2-deoxy-D-erythro-pentose</td>
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<tr>
<td>8-Me-dG</td>
<td>8-methyl-2’-deoxyguanosine</td>
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<tr>
<td>8-Oxo-dG</td>
<td>7,8-dihydro-8-oxo-2’-deoxyguanosine</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DFT</td>
<td>Density functional theory</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>ESI</td>
<td>Electron spray ionization</td>
</tr>
<tr>
<td>HEK293T cells</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>KD</td>
<td>Knock down</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Pols</td>
<td>Polymerases</td>
</tr>
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<td>Polymerase κ (kappa)</td>
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<td>Polymerase ι (iota)</td>
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<td>Pol ζ</td>
<td>Polymerase ζ (zeta)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TLS</td>
<td>Translesion DNA synthesis</td>
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<td>Ultraviolet</td>
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Chapter 1

Introduction
Chapter 1 – Introduction

1.1 DNA Damaging Agents

DNA is the repository of all the genetic information in living cells. Thus, its structural integrity and stability are essential to life. However, DNA is not an inert entity, and therefore may become damaged by reactive chemical modalities that are generated in cells or that may enter cells from exogenous sources. Exposure to a variety of DNA damaging agents can lead to an estimated rate of up to 100,000 lesions per cell per day. This status quo requires a constant excision and replacement of damaged residues by DNA repair machinery in order to counteract potentially genotoxic and mutagenic outcomes. The reaction of genotoxic agents with DNA result in a plethora of lesions, commonly referred to as adducts. Endogenous agents include chemicals generated from normal cellular processes such as respiration and lipid peroxidation. These processes generate reactive oxygen species (ROS) that may damage DNA. Genomic DNA may also be assaulted by exogenous or environmental agents. The main perpetrators are ionizing radiation and genotoxic chemicals that may enter cells. Genotoxic chemicals are often metabolically activated in cells to form reactive metabolites that engage DNA in chemical reactions resulting in covalent linkages to specific sites of the four nucleotides (dNTPs) of DNA to generate DNA adducts or lesions.

Figure 1. Types of DNA damages from endogenous and exogenous agents
1. 2 Carcinogenicity of Nitroaromatic compounds

DNA damage by genotoxic chemicals can become important in the development of biologically relevant events and human cancers.\textsuperscript{7,9} Many nitroaromatic hydrocarbons (NO\textsubscript{2}-PAH) are known to induce tumors in experimental animals.\textsuperscript{7,10,11} Nitroaromatic compounds are environmental pollutants that are present in many mixtures such as cigarette smoke, coal fly ash, diesel exhaust and have also been detected in burnt grill food. Notably, drugs containing nitroaromatic motifs have also been marketed worldwide. Thus, human exposure to nitroaromatic compounds is inevitable.\textsuperscript{12}

![Examples of nitroaromatic compounds](image)

Figure 2. Examples of nitroaromatic compounds

In the postindustrial era, food appears to be the most significant source of NO\textsubscript{2}-PAH exposure. In addition, simultaneous exposure to PAH and nitrogen oxide gas appears to lead to endogenous formation of NO\textsubscript{2}-PAH.\textsuperscript{8,12}

In cells, most nitroaromatic compounds are metabolically activated to reactive modalities via nitroreduction. However, oxidative pathways also contribute to the metabolism of many of these compounds. Notably, significant difference in the enzymes responsible for nitroreduction and
oxidation has been observed in different organisms. In humans, xanthine oxidase and microsomal NADPH-cytochrome c have been identified as the enzymes responsible for nitroreduction, while the cytochrome P450 family of enzymes is predominantly responsible for the oxidative metabolism of these compounds.9,12,13

1. 3 6-Nitrochrysene

6-Nitrochrysene (6-NC) belongs to the family of NO2-PAH above. 6-NC is a potent mutagen in bacteria. It is an exceptionally potent carcinogen in newborn mice, and it induces mammary carcinoma in rats. The genotoxicity of 6-NC is derived from its ability to chemically react with 2’-deoxyguanosine (dG) and 2’-deoxyadenosine (dA) in DNA to generate DNA-carcinogen adducts (Scheme 1).13–15

Scheme 1. Metabolic activation of 6-nitrochrysene via nitroreduction and ring oxidation
Several *in vivo* studies in mice and rats have demonstrated that 6-NC can be activated by two major pathways. These include a nitroreduction and a nitroreduction-ring oxidation tethered pathway (Scheme 1). The nitroreduction pathways involves a simple nitroreduction of 6-NC to form the corresponding N-hydroxy-6-aminochrysene (N-OH-6-AC). This N-OH-6-AC electrophilic species reacts with the nucleophilic C8-site of purines bases in dG and dA to generate DNA adducts, N-(dG-8-yl)-6-AC (1), and N-(dA-8-yl)-6-AC (3). Adduct 3 is believed to give rise to the corresponding 2'-deoxyinosine adduct N-(dl-8-yl)-6-AC (3'), but whether the process of deamination is enzymatic or non-enzymatic has never been established. N-OH-6-AC electrophilic species also reacts at the $N^2$ site of dG to form 5-(dG-$N^2$-yl)-6-AC (2). Additional adducts are formed by a combination of ring oxidation and nitroreduction (Scheme 1).

**Scheme 2.** Generation of nitrenium ion as ultimate carcinogen
As with many nitroaromatic compounds, metabolic activation via nitroreduction of 6-nitrochrysene to its N-hydroxy-6-aminochrysene, N-OH-6-AC (Scheme 1) is a proximate carcinogen which can react with nucleobases in cellular DNA to induce DNA adducts. However, it is believed that the N-hydroxy-6-aminochrysene species is further activated by O-esterification or N,O-diesterification enabled by cellular enzymes such as the acetyl- and sulfotransferases (Scheme 2). The sulfonylhydroxyamines (Scheme 2) spontaneously undergo heterolytic N-O fission to form highly reactive nitrenium ions, which is the ultimate carcinogen species that react with DNA molecules to form the corresponding DNA adducts.17,18

Intramammary administration of 6-NC metabolites resulted in the formation of mammary tumors at the site of injection (Table 1). The carcinogenic potency of 6-NC and its nitroreduction and ring-oxidation metabolites toward the mammary gland of rats was ranked in the following order: 6-NC > 1,2-DHD-6-NC > 6-AC > 6-NCDE > 1,2-DHD-6-AC (Figure 3). Taken together, these results suggest that metabolites derived from both ring-oxidation and nitroreduction contribute to the overall carcinogenicity of 6-NC in the rat mammary gland.7,10

![Chemical structures](image)

**Figure 3.** Carcinogenicity scale of 6-nitrochrysene (6-NC) and reactive metabolites.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Tumor Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 6-NC</td>
<td>95.8</td>
</tr>
<tr>
<td>2. 1,2-DHD-6-NC</td>
<td>37.5</td>
</tr>
<tr>
<td>3. 6-Ac</td>
<td>48</td>
</tr>
<tr>
<td>4. 6-NCDE</td>
<td>16</td>
</tr>
<tr>
<td>5. 1,2-DHD-6AC</td>
<td>28</td>
</tr>
<tr>
<td>6. Control DMSO</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Mammary tumor induction by 6-NC and reactive metabolites.\textsuperscript{10}

6-Nitrochrysene is noticeably less abundant than other NO\textsubscript{2}-PAHs in the environment. However, it is the most potent NO\textsubscript{2}-PAH compound ever tested in the newborn mouse assay.\textsuperscript{10} 6-NC is much more active than chrysene, and any of the other mononitro-chrysene isomers and benzo[a]pyrene (B[a]P). It has been shown that 6-NC is a powerful mammary carcinogen in rats. In this study it was more potent than the extensively investigated ultimate carcinogen, the bay region diol epoxide of B[a]P. 6-NC is not only carcinogenic in mammary gland, it is tumorigenic in the colon of rats, and the lung and skin of mice.\textsuperscript{7,9,13} It is known that 6-NC can become metabolically activated in human liver and lung to form genotoxic metabolites.\textsuperscript{7,15}

DNA lesions derived from 6-NC can play important roles in the development of human cancer if they are not repaired via cellular repair pathways such as nucleotide excision repair prior to DNA replication. These lesions can induce mutations in crucial sequences of cancer genes. Mutations in an oncogene, a tumor-suppressor gene such as $p53$, or a gene that controls the cell cycle can lead to uncontrolled cell growth, resulting in tumorigenesis.\textsuperscript{19,20} A large fraction of mutations in most human cancers involves mutations in the G:C base pairs of critical genes like $p53$ and a relatively smaller fraction of mutations occurs at the A:T base pairs.\textsuperscript{20–22} Indeed,
carcinogen-induced mutational signatures also predominantly involve G:C base pairs.\textsuperscript{23} Even so, mutations at A:T base pairs occur. An example of a nitroaromatic compound derived mutations at the A:T base pair include aristolochic acid (AA), which forms \( N^6 \)-dA adducts. Mutational spectra of AA is dominated by A:T→T:A transversions in the \( p53 \) gene in urothelial tumors.\textsuperscript{22,24}

Recently, an \textit{in vitro} repair study by El-Bayoumy and coworkers showed that the efficiency of nucleotide excision repair (NER) of the C8-dA adduct, \( N -(dA-8-y1)-6-AC \) (3) is \(~8\) times lower than that of the C8-dG adduct, \( N -(dG-8-y1)-6-AC \) (1). In fact, \( N -(dA-8-y1)-6-AC \) was estimated to be more resistant to NER than all other adducts formed by 6-NC and benzo[\( a \)]pyrene diol epoxide.\textsuperscript{14}

\textbf{1. 4 DNA Repair Pathways and Genoprotective Networks}

\textbf{1. 4. 1 Overview}

DNA damages challenge the integrity of the genome and can stall replication and transcription machinery thereby inducing mutations and the onset of diseases. However, the process of evolution has equipped cells with the tools to identify and rectify DNA damages prior to DNA replication. In most cases, assaults on DNA are identified and cells elicit complex repair mechanisms that rectify damages. This mechanism is known as DNA damage response (DDR) and involves a series of coordinating enzymes, which removes DNA damages and reassemble DNA fragments.\textsuperscript{1} Thus, deleterious and mutagenic consequences of DNA damages are either minimized or completely eliminated. DNA damage signaling and cellular checkpoints coordinate DNA repair with cell-cycle transitions and can effectively halt the growth of a cell with damaged DNA until its DNA is repaired. The types of DNA lesion that results from a DNA damaging event controls the repair pathway elicited by cell’s repair machinery. DNA damages that results in
nucleobase alterations are usually removed by error-free pathways such as base excision repair (BER) and nucleotide excision repair (NER).\textsuperscript{3,4}

1.4.2 Biological Consequences of DNA Damage

Despite cell’s robust and precise ability to identify and repair damaged DNA, some damages to DNA may escape cell’s repair machinery, particularly when the damages are induced at high frequency. Replication of damaged DNA that eludes cellular defense mechanisms poses a challenge to DNA replication.\textsuperscript{1,2} First, replication of unrepaired lesions cause mutations, which can result in genetic diseases. Second, DNA adducts can block replicative DNA polymerases, which are cellular workhorses responsible for copying DNA with high fidelity during replication. High fidelity replicative polymerases can fit only one templating nucleotide in their active site.\textsuperscript{25,26} This favors accurate Watson-Crick-Franklin base pairing with incoming nucleotide of the newly made complementary strand of DNA. RNA and DNA polymerases blockages at replication forks can cause genome instability by chromosomal aberrations and can lead directly or indirectly to DNA strand breaks, which tend to be lethal in most cells.\textsuperscript{26,27}

The detrimental biological consequences that DNA damage can present cells with is evident by the large commitment of genome protection expended by cell’s genoprotective networks including electrophile scavengers, recombination complexes that permit DNA lesion tolerance, specialized polymerases that enable lesion bypass and a large arsenal of DNA repair proteins.\textsuperscript{3} The inability of this protective network to effectively protect the genome can result in the onset of diseases.

1.4.3 Base Excision Repair (BER)

In human cells, base modifications that’s are caused by endogenous agents such as reactive oxygen species (ROS) are generally removed by the base excision repair pathway.\textsuperscript{1–3} BER is
initiated by DNA glycosylases that hydrolytically cleave the base–deoxyribose glycosyl bond of a damaged nucleotide residue. This results in formation of an abasic site. The coordinated activities of AP endonuclease (APE1) and polymerase β then insert the correct base and the nicks are sealed by XRCC1-ligase 3 complex.\textsuperscript{28,29} The insertion of the appropriate base by polymerase β, can be accomplished in two different mechanisms, short-patch and/or long-patch repair. In short-patch repair DNA pol β performs a one nucleotide gap filling reaction and removes the AP site via lyase activity.\textsuperscript{29}

1. 4. 4 Nucleotide Excision Repair (NER)

The nucleotide excision repair pathway is mainly involved in the direct repair of bulky DNA lesions such as those induced by nitroaromatic chemical carcinogens, UV radiation and DNA damaging drugs. These distorting lesions thermodynamically destabilize DNA double helix and the enzymes involve in global NER utilize these perturbations to recognize and repair these lesions (Figure 4).\textsuperscript{30}

As depicted in Figure 4, distorting lesions are directly recognized by the enzyme XPC-RAD23B, which binds the nondamaged strand opposite the lesion. TFIIH interacts with XPC-RAD23B through protein-protein interaction and effectively forces the DNA helix wide open with its XPB subunit allowing XPD to slowly moves along DNA until its stalls at the damage site. This stalling of XPD at the lesion allows for the formation of the pre-incision complex by recruitment of other enzymes such as XPA, RPA, and XPG which cooperatively makes an incision 5’ to the lesion. DNA repair synthesis is then initiated by Pol δ and Pol κ or Pol ε and associated factors, followed by 3’ incision by XPG. The newly synthesized strand is stitched to the undamaged strand by DNA ligase which seals the nick and completes the process.\textsuperscript{30,31}
Figure 4. Mechanism of nucleotide excision repair in eukaryotes. Adapted from reference 28 with permission from Cold Spring Harbor Laboratory Press.

1. 5 Translesion Synthesis (TLS)

In order to evade the lethal consequences of excessive DNA damage, cells have developed a specialized mechanism known as translesion DNA synthesis (TLS), a pathway that is able to tolerate DNA damages in the genome by briefly replacing high fidelity replicative DNA polymerases with TLS polymerases.27,32 These specialized enzymes can bypass DNA lesions by inserting the correct or incorrect base opposite the lesion site.27,32,33 However, TLS polymerases are low fidelity enzymes which makes TLS an inherently error-prone process. Unlike replicative polymerases with tight binding sites, translesion polymerases have a solvent-exposed active site that is spacious enough to accommodate bulky DNA lesions, while enabling low fidelity and potential mutagenic replication.25
1. 5. 1 Translesion Synthesis in Mammalian Cells

When replicative polymerases such as eukaryotic Pol ε (Figure 5) stalls at the replication fork upon encountering a DNA lesion site, TLS polymerases are recruited to the site. TLS polymerases are recruited through protein-protein interactions with the replicative sliding processivity clamp (yellow).²⁶,²⁷

![Figure 5. Mechanism of Translesion DNA synthesis (TLS) in mammalian systems. Adapted from reference 24 with permission from Cold Spring Harbor Laboratory Press.²⁶](image)

This is known as proliferating cell nuclear antigen (PCNA) in eukaryotes. In eukaryotic systems stalling of replicative polymerase causes the proliferating cell nuclear antigen (PCNA) to becomes ubiquitinated by the protein ubiquitin.²⁶ This effectively signals the release of replicative polymerase from the sliding clamp of the replisome and recruit TLS polymerases to bind to the
replication fork. These polymerases include Y family polymerases such as pol η, pol κ, pol ι, Rev1 and the B family polymerase Pol ζ.26,27,33 TLS polymerases insert a correct or an incorrect base opposite the lesion. Translesion DNA synthesis is subsequently extended in the 5’ to 3’ direction past the lesion by the same TLS polymerase or a second TLS polymerase.26,33 After synthesizing a short sequence of DNA, all TLS polymerases are dissociated from the replication fork and replaced with high fidelity replicative polymerase to resume DNA synthesis.

**Polymerase η**

Polymerase η (Pol η) is a Y-family polymerase and is the most extensively studied TLS polymerase. Pol η bypasses UV-induced thymine-thymine cyclobutane pyrimidine dimers (CPD) with high efficiency and high fidelity.34 In fact, Pol η is capable of bypassing these lesions with higher accuracy than even undamaged DNA. In humans, the presence of mutations in the POLH, gene responsible for the expression of Pol η, is known to result in the variant form of xeroderma pigmentosum (XP)33,35, a disease associated with an increased incidence of skin cancer. Pol η has been labeled as CPD bypass polymerase due to its ability to bypass the CPD lesions with high fidelity. However, Pol η have been shown to play important roles in mutagenic and error free bypass many other lesions, such as to bypass 8-oxodG,36 O6-MedG,37 benzo[a]pyrene-N2-dG,37 and DNA lesions derived from cisplatin.38

**Polymerase κ**

Polymerase κ (Pol κ) is found in all domains of life and is the most highly represented and strongly conserved of all the TLS Y-family polymerases.26 Pol κ is considered to be the most accurate y-family polymerase on processing undamaged DNA.27,34 Notably, Pol κ has the unique
capability in accurately bypassing various dG lesions, including C8-guanyl, N\textsuperscript{2}-furfuryl-dG and 8-oxo- guanine.\textsuperscript{33}

**Polymerase ζ**

Unlike most mammalian TLS polymerases which are low fidelity Y-family polymerases, polymerase ζ (Pol ζ) belongs to B-family class of polymerases. Notably, other B-family polymerases includes high fidelity replicative polymerases such as Pol delta (δ), epsilon (ε) and alpha (α).\textsuperscript{26,33,34} However, unlike these replicative polymerases, Pol ζ is error-prone and lacks 3’-5’ exonuclease activity which makes it categorically a TLS polymerase. Pol ζ is a heterodimer composed of the Rev3 catalytic subunit and the Rev7 accessory subunit. Many researchers have shown that Pol ζ participates in a variety of error-free and error-prone bypass of many DNA lesions.\textsuperscript{41,42}

**1. 5. 2 DNA Damage Response in *Escherichia coli***

**SOS Response**

In *E. coli* DNA repair is regulated by the expression of a network of genes which are referred to as the SOS response. Induction of the SOS response involves more than forty independent SOS genes, most of which are responsible for the expression of proteins responsible for protection, DNA repair and TLS lesion bypass.\textsuperscript{43,44} Under normal growth conditions the SOS genes are expressed at a basal level. These levels increase significantly when the SOS genes box is turned on in response to genome damage. Thus, in bacterial systems the expression of TLS polymerases is regulated by SOS response.\textsuperscript{45}

The expression of genes in the SOS box is regulated by the LexA protein. Under normal cellular conditions, LexA dimer is bound to the promoter region of the SOS box thereby inhibiting
gene expression (Figure 6).\textsuperscript{45,39} Upon encountering a replication blockage at the replication fork during DNA replication the RecA protein is activated to become a coprotease by formation of a filament of RecA on single-stranded DNA (ssDNA). RecA is a protease and facilitates the self-cleavage reaction of LexA. As LexA repressor levels decrease, the SOS genes are expressed and induces DNA repair mechanisms, tolerance of DNA damage, and induced delay of the cell cycle.\textsuperscript{44,45}

![Image of DNA repair mechanisms](image.png)

**Figure 6.** Induction of SOS response in *E. coli*. Adapted from reference 43.

**Translesion Synthesis in *Escherichia coli***

In *E. coli*, TLS low fidelity polymerases are Pol II, Pol IV and Pol V. When replicative polymerase Pol III (Figure 7) stalls at the replication fork upon encountering a site of a DNA lesion, TLS polymerases are recruited to the site.\textsuperscript{26,32} TLS polymerases are recruited through interactions with the replicative sliding processivity clamp (yellow). This is known as β-Clamp in *E. coli*. This signals the release of replicative Pol III from the sliding clamp of the replisome and
recruit TLS polymerases to bind to the replication fork. These polymerases include low fidelity polymerases are Pol II, Pol IV and Pol V. TLS polymerases insert a correct or an incorrect base opposite the lesion. Translesion DNA synthesis is subsequently extended past the lesion by the same TLS polymerase or a second TLS polymerase. After synthesizing a short sequence of DNA, all TLS polymerases are dissociated from the replication fork and replaced with high fidelity replicative polymerase to resume DNA synthesis.26

Figure 7. Mechanism of Translesion DNA synthesis (TLS) in E. coli. Adapted from reference 30 with permission from Cold Spring Harbor Laboratory Press.
1. 6 Scope of Dissertation

It is now clear that carcinogen-DNA adducts can interfere with DNA structure and affect the accuracy of DNA replication leading to heritable genetic changes and cancer. The overarching goal of my thesis research is to investigate the mechanisms of mutagenesis and DNA repair induced by DNA lesions in cells. The multi-faceted nature of this goal requires tools in organic synthesis, analytical chemistry and molecular and cell Biology.

Chapter 2 of this thesis focus on the synthesis of site-specifically modified oligodeoxynucleotides containing C8-deoxyadenosine DNA adduct derived from 6-nitrochrysene (6-NC) modification. This lesion derives from the nitroreduction 6-NC to its reactive metabolite which subsequently reacts at the C8 site of 2′deoxyadenosine in DNA to generate N-(dA-8-yl)-6-AC DNA adduct. This adduct is referred to as N-(dA-8-yl)-6-AC throughout this thesis. 6-NC is the most potent carcinogen ever tested in newborn mouse assay and is a potent mutagen in bacteria. DNA lesions resulting from 6-NC modifications can inhibit DNA replication and are likely to induce mutations if they are not removed by cellular defense pathways prior to DNA replication. The N-(dA-8-yl)-6-AC DNA adduct is particularly interesting; it has been showed to be poorly repaired in nucleotide excision repair assay in HeLa cells extract. Using phosphoramidite chemistry and solid phase DNA synthesis, I have successfully synthesized this N-(dA-8-yl)-6-AC adduct. The key step in the synthesis of N-(dA-8-yl)-6-AC DNA adduct is a palladium-catalyzed Buchwald-Hartwig cross coupling reaction wherein carcinogenic 6-aminochrysene is coupled at the C8 site of protected bromo-dA. The 6-NC modified adduct, N-(dA-8-yl)-6-AC was converted to its corresponding 3′-phosphoramidite precursor and used to synthesize 12-mer and 15-mer oligodeoxynucleotides. These oligodeoxynucleotides were designed to mimic parts of the p53 tumor suppressor gene with biologically relevant mutation hotspots. N-(dA-8-yl)-6-AC adduct
containing oligodeoxynucleotides were purified by reversed phase HPLC and denaturing polyacrylamide gel electrophoresis and characterized by high resolution mass spectrometry.

**Chapter 3** address the biophysical characterization of the 2’-deoxyadenosine DNA adduct, \( N-(dA-8-y1)-6-AC \). In this section, 2D NMR spectroscopy and DFT calculations were used to investigate the conformational preference of \( N-(dA-8-y1)-6-AC \) adducted nucleoside. We further investigated the helical structure of \( N-(dA-8-y1)-6-AC \) adduct in the 12 and 15-mer oligodeoxynucleotide duplexes using circular dichroism (CD) spectroscopy. The stability of \( N-(dA-8-y1)-6AC \) adducted nucleoside was also investigated under biologically relevant conditions using UV/Vis spectroscopy and HPLC.

The focus of **Chapter 4** is to investigate the replicative bypass of \( N-(dA-8-y1)-6-AC \) adduct in *E. coli* and human embryonic kidney cells, HEK293T cells. The 15-mer oligodeoxynucleotide (5’-GCCCTCAA*CAAGATG-3’) where \( A^* = N-(dA-8-y1)-6-AC \) adduct was used to construct a single-stranded shuttle vector containing a single adducted site using recombinant DNA technology and replicated in cells. The DNA sequence of the 15-mer was chosen from TP53 gene codon 129-133, because crops contaminated with another nitroaromatic carcinogen aristolochic acid (AA) causes A→T mutation in codon 131 in patients with urothelial tumors. In Escherichia coli, viability of \( N-(dA-8-y1)-6-AC \) modified vector was ~60% of the control, indicating slower translesion synthesis of the adduct, which increased to nearly 90% upon induction of the SOS functions. \( N-(dA-8-y1)-6-AC \) DNA adduct is mutagenic in *E. coli*. The major type of targeted mutation is \( A^* \rightarrow G \) in both uninduced and SOS-induced cells.

The roles of translesion synthesis DNA polymerases in bypassing the 6NC modified C8-2’-deoxyadenosine adduct, \( N-(dA-8-y1)-6-AC \) in HEK293T were also investigated in chapter 4.
Replication of plasmid containing a single site-specific N-(dA-8-yl)-6-AC adduct in HEK293T provided ~12 % progeny plasmid with mutations largely A*→T transversions. A 4-fold decrease in mutation frequency was observed when the plasmid is replicated in polymerases κ or ζ deficient cells. Similar experiments in polymerase η KO cells showed a 1.5-fold increase in mutation frequency. We also noted that plasmid replication in polymerases ζ KO and κ siRNA KD cells resulted in a 10-fold decrease in mutation frequency. Taken together, these results suggest that polymerases κ and ζ are cooperatively involved in the error-prone TLS of N-(dA-8-yl)-6-AC, while pol η is performs error-free bypass.

**Chapter 5** evaluates the mutagenicity and replication bypass efficiency of the ring opened formamidopyrimidine (Fapy.dG) adduct that is produced via oxidative stress. DNA sequences: 5’-AAC CG*G AGG CCC-3 and 5’-AAC CGG AG*G CCC-3 with codon 248 and 249 (underlined) respectively of the tumor suppressor gene p53 was incorporated into a plasmid and replicated in human embryonic kidney 293T cells, which showed that Fapy.dG DNA adduct is mutagenic inducing largely G*→T transversions. Furthermore Fapy.dG adduct in CG*G sequence context (codon 248) induce mutations at ~50% greater frequency than AG*G in codon 249.
Chapter 2

Total Synthesis of Site-Specific Oligodeoxynucleotides Containing C8-2’-Deoxyadenosine Adduct Formed by 6-Nitrochrysene

2. Abstract

The environmental pollutant, 6-nitrochrysene (6-NC) is a potent mutagen in bacteria and it induces mammary carcinoma in rats. It is an exceptionally potent carcinogen in newborn mice. 6-NC is metabolically activated by two pathways, which include only nitroreduction and both nitroreduction and ring oxidation. The nitroreduction pathway generates dG-C8, dG-N², and the dA-C8 adduct, N-(dA-8-yl)-6-AC. Here, we report the synthesis of the phosphoramidite monomer of the C8-dA adduct of 6-NC from dA in 10 steps and its incorporation into oligodeoxynucleotides. The key step in the synthesis of oligodeoxynucleotides containing N-(dA-8-yl)-6-AC was palladium-catalyzed Buchwald-Hartwig cross coupling chemistry, which was optimized to achieved in 85% yield. The protected N-(dA-8-yl)-6-AC derivative was deprotected and then converted to appropriately protected phosphoramidite monomer for solid-phase DNA synthesis. The resulting phosphoramidite was used to synthesize a 12-mer and a 15-mer oligonucleotide. The adduct-containing oligodeoxynucleotides were purified by reversed phase HPLC followed by denaturing polyacrylamide gel electrophoresis and characterized by mass spectrometry.
2.1 Introductions

2.1.1 Buchwald-Hartwig Cross-Coupling Chemistry

Scheme 3. Examples of arylamine C8-dA Buchwald-Hartwig cross coupling reactions
The Buchwald-Hartwig palladium catalyzed C-N bond formation strategy has been successfully applied by many researchers to synthesize various DNA adducts (Scheme 3).\textsuperscript{46–50} This strategy involves protection of the reactive functionalities on the nucleoside substrate and any necessary functionalization that facilitates smooth coupling. The Buchwald–Hartwig reaction is a cross-coupling reaction where C-N bond of an arylamines or heteroarylamines is formed by the reaction of an aryl halide or triflate with a primary or secondary amine in the presence of a palladium (0) catalyst and a base (Scheme 4).\textsuperscript{51–53}

![Buchwald-Hartwig Reaction Scheme]

\(X = \text{Cl, Br, I, OTf}\)

\(\text{Amine} = 1^\circ, 2^\circ\) and \(R_1/R_2 = \text{aromatic or aliphatic substituent}\)

**Scheme 4.** Buchwald-Hartwig cross coupling reaction overview

Typically for carcinogen-DNA coupling, C-N bond connecting the NO\textsubscript{2}-PAHs carcinogen and the nucleoside base is installed via a Buchwald-Hartwig coupling reaction. The point of adduction of the purine bases varies. Nitroaromatic carcinogens-DNA attachments can occur at the C8 and \(N^2\) site deoxyguanosine (dG) or at the C8 and \(N^6\) deoxyadenosine (dA) as shown in Figure 8.
The Buchwald-Hartwig cross coupling reaction begins with oxidative addition of the aryl halide or triflate species to the palladium which increases the oxidation state of Pd from 0 to +2. This is followed by coordination of the amine to the palladium. An attack of amine on the palladium (II) complex kicks out the halide as a leaving group and react with a strong base in solution. Reductive elimination then produces the final aryl amine product and regenerates the catalyst (Figure 9).52,53

Figure 9. (A) Buchwald-Hartwig cross-coupling catalytic cycle. (B) Commonly used ligands.
The most common chelating ligand used in nitroaromatic carcinogen-nucleobase Buchwald-Hartwig cross coupling reactions is 2,2’-bis(diphenylphosphino)-1,1’-binaphthyl (BINAP).\textsuperscript{54} However, other ligands have also been used successfully. Generally, these ligands are bulky electron-rich dialkylbiaryl phospines.\textsuperscript{55,56}

2. 1. 2 Synthesis of Carcinogen Modified Oligodeoxynucleotides

**Biomimetic Postoligomerization**

To investigate mutation outcomes in cells carcinogen modified oligodeoxynucleotides are often synthesized via a biomimetic postoligomerization strategy or using phosphoramidite chemistry. In a postoligomerization approach an unmodified oligodeoxynucleotide is directly reacted with a carcinogen or its reactive metabolite. The resulting oligomer is then purified and characterized. The recovery of these reactions is inherently low yielding and only a single reactive base is accommodated in the starting unmodified oligodeoxynucleotide. Multiple reactive bases in the unmodified oligo substrate increases the number of possible products exponentially which makes purification difficult and further decreases the yields.\textsuperscript{13,14,50}

**Phosphoramidite Chemistry Method**

The synthesis of site-specifically carcinogen modified oligodeoxynucleotides utilizing phosphoramidite chemistry and solid phase DNA synthesis is currently the gold standard for synthesizing modified oligodeoxynucleotides.\textsuperscript{57} Phosphoramidite chemistry, developed in the 1980s and later enhanced with solid-phase supports and automation, is the method of choice for DNA oligonucleotide synthesis.\textsuperscript{58–60} Unlike biosynthesis, which proceeds in 5' → 3' direction, solid phase DNA synthesis proceeds in the 3' → 5' direction according to the steps outlined in Figure 10. The synthesis cycle begins with detritylation, the 5'-DMT protecting group is removed from the first, solid-support-linked nucleoside. In step 2, coupling, the free 5'-OH of the
first, solid-support-linked nucleoside attacks the phosphorus of the incoming second nucleoside, displacing its diisopropylamino group. This is the step in which modified phosphoramidites can be introduced. In step 3, oxidation, the resulting phosphite triester is unstable and is converted to a stable phosphate triester, this allows the next cycle to proceed to step 1. However, before moving to the next cycle, unreacted nucleosides with 5'-OH on the solid support are acetylated or capped, thereby preventing elongation of sequences with deletion mutations.

Figure 10. Synthesis cycle for solid-phase oligonucleotides synthesis.
2. 2 Material and Methods

All reagents were purchased from commercial chemical suppliers and used without further purification unless otherwise noted. All reactions were performed in round bottomed flasks sealed with rubber septa, under an atmosphere of nitrogen, and stirred with a TeflonTM-coated magnetic stir bar unless otherwise noted. Temperatures above 23 °C were controlled by a temperature modulator. Pre-dried tetrahydrofuran (THF), benzene, toluene, acetonitrile (MeCN), methanol (MeOH), and triethylamine (Et3N), were degassed with argon for 60 min. Reactions were monitored by thin layer chromatography (TLC) using J.T Baker 2.5 x 7.5 cm silica gel flexible TLC sheets and visualized using UV (254 nm). Volatile solvents were removed using a rotary evaporator under reduced pressure. Silica gel chromatography was performed using Sorbent Technologies 60 A, 230 x 400 mesh silica gel (40-63 μm).

1H NMR and 13C NMR were obtained in CDCl3 and DMSO-d6 on 400 MHz spectrometers with 13C operating frequencies of 100 MHz and analyzed using MestReNova. Chemical shifts are reported in parts per million (δ) relative to residual chloroform (7.26 ppm for 1H and 77.16 ppm for 13C). Data for 1H NMR spectra are reported as follows: chemical shift (multiplicity, number of hydrogens and assignment). Multiplicity is designated as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), or m (multiplet). High-resolution mass spectral (HRMS) data was obtained using electrospray ionization (ESI) and the data was analyzed using MassLynx.
2. 2. 1 Experimental Methods

8-Bromo-2'-deoxyadenosine (4)

Bromine (9.6 ml, 194.72 mmol) was added to freshly prepared 1M acetate buffer, pH 5.4 (190 ml) and stirred for 24 hours until completely dissolved. The bromine solution was added drop-wised to a slurry of 2'-deoxyadenosine (10.0 g, 37.13 mmol) in acetate buffer (100 ml). The reaction was monitored by TLC and was completed after 90 min. Solid sodium bisulfite was added to destroy excess bromine, then the reaction mixture was neutralized with 2N NaOH. The reaction mixture was then allowed to cool at -20 °C for 30 minutes. After this time the reaction mixture was filtered and washed with cold water and dried to yield a beige powder 2 (8.5 g, 69 %). TLC (CH₂Cl₂/MeOH, 80:20): Rf 0.56.

HRMS (ESI⁺) m/z calc. for C₁₀H₁₂BrN₅O₃ [M+H]⁺, 330.1380; found, 330.0188.

¹H NMR (400 MHz, DMSO-d₆) δ 8.12 (s, 1H), 7.50 (s, 2H), 6.30 (t, J = 8.0, 6.4 Hz, 1H), 5.34 (d, J = 4.2 Hz, 1H), 5.28 (dd, J = 7.7, 4.4 Hz, 1H), 4.49 (dt, J = 6.6, 3.2 Hz, 1H), 3.89 (td, J = 4.7, 2.5 Hz, 1H), 3.66 (dt, J = 11.8, 4.5 Hz, 1H), 3.49 (ddd, J = 12.2, 7.8, 4.9 Hz, 1H), 3.25 (ddd, J = 13.6, 8.0, 6.0 Hz, 1H), 2.20 (ddd, J = 13.1, 6.5, 2.7 Hz, 1H).

¹³C NMR (101 MHz, DMSO) δ 155.38, 152.63, 150.38, 127.21, 120.17, 88.80, 86.83, 71.62, 62.58, 37.46.

8-bromo-3', 5'-O-bis(tert-butylidimethylsilyl)-2'-deoxyadenosine (5)

8-bromo-2'-deoxyadenosine, 4 (5.8 g, 17.508 mmol) and imidazole (5.685 g, 83.5175 mmol) were suspended in 15 ml of dry DMF. The reaction mixture was allowed to stir for 10 minutes. After this time, tert-butylidimethylsilyl chloride (7.26 g, 48.15 mmol) was added to the stirring mixture at room temperature. The solution turned transparent within 10-15 minutes and was allowed to stir for an additional 12-16 hours at room temperature. The reaction was monitored by TLC (90/10...
CH₂Cl₂/MeOH). The solvent was evaporated under pressure and purified on silica gel column chromatography with a step gradient of methanol (0-6%) in DCM as the mobile phase. The product was isolated as light yellow solid (8.9 g, 90%).

**HRMS (ESI⁺)** m/z calc. for C₂₂H₄₀Br₅N₅O₅Si₂ [M+H]⁺, 559.1833; found 560.0100

**¹H NMR (400 MHz, Chloroform-d) δ** 8.23 (s, 1H), 6.38 (s, 2H), 6.34 (t, 1H), 4.86 (dt, J = 6.1, 3.9 Hz, 1H), 4.06 – 3.77 (m, 2H), 3.77 – 3.45 (m, 2H), 2.22 (ddd, J = 13.1, 7.0, 4.2 Hz, 1H), 0.93 (s, 9H), 0.81 (s, 9H), 0.13 (s, 6H), -0.02 (s, 3H), -0.06 (s, 3H).

8-Bromo-3', 5'-O-bis(tert-butyldimethylsilyl)-N⁶-dimethoxytrityl-2'-deoxyadenosine (6)

8-Bromo-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine 5 (9.8 g, 17.442 mmol) and 4-dimethylaminopyrine (42.2 mg, 0.3488 mmol) was dissolved in 20 ml of anhydrous pyridine. 4,4'-dimethoxytrityl chloride (8.9 g, 26.24 mmol) was added to the solution at room temperature. The reaction mixture was then allowed to stir at room temperature for 18 hours. After this time, TLC analysis indicated complete consumption of 5. The reaction mixture was concentrated in vacuo and the crude reaction mixture was purified on Al₂O₃ column chromatography with a step gradient of (0-12%) ethyl acetate (EtOAc) in hexanes. The product was isolated as a foamy and white solid (13.83 g, 92%).

**HRMS (ESI⁺)** m/z calc. for C₄₃H₅₈Br₅N₅O₅Si₂ [M+H]⁺, 861.0423; found 862.3206

**¹H NMR (400 MHz, CDCl₃) δ** 7.97 (s, 1H), 7.46 – 7.06 (m, 8H), 6.94 – 6.66 (m, 5H), 6.34 (t, J = 6.7 Hz, 1H), 4.98 – 4.76 (m, 1H), 4.05 – 3.84 (m, 2H), 3.80 (s, 7H), 3.77 – 3.52 (m, 2H), 2.22 (ddd, J = 13.0, 7.0, 4.3 Hz, 1H), 0.95 (s, 9H), 0.83 (s, 9H), 0.15 (s, 6H), -0.00 (s, 3H), -0.06 (s, 3H).**¹³C NMR (101 MHz, CDCl₃) δ** 158.53, 158.41, 158.32, 152.99, 152.90, 152.56, 151.96, 151.83, 149.83, 149.52, 145.32, 145.14, 144.90, 142.33, 138.29, 137.37, 136.37, 136.11, 130.26, 130.15, 130.12, 128.83, 128.31, 128.23, 127.88, 127.76, 127.72, 126.82, 126.79, 120.05, 113.20,
General procedure for Optimization of Buchwald-Hartwig cross coupling reaction.

8-bromo-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine 5 (0.098 g, 0.1745 mmol) or 8-bromo-3',5'-O-bis(tert-butyldimethylsilyl)-N6-dimethoxytrityl-2'-deoxyadenosine 6 (0.150 g, 0.1745 mmol), 6-aminochrysene (0.064 mg, 0.263 mmol), Pd (0) catalyst (4 mol %) and rac-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP, ) (12.5 mg, 0.02 mmol) were suspended in 5 ml of anhydrous solvent (see Table 1). The solution was degassed by purging with argon for 1h. The reaction flask was then heated under argon at 85 – 100 °C for 1h. After this time sodium tert-butoxide, NaOtBu or cesium carbonate, Cs2CO3 (0.263 mmol) was added and the reaction stirred at 85 – 100°C for an additional 1-2 hours. After this time, the reaction was monitored via TLC for consumption of compound 5 or 6. The reaction was then cooled, diluted with CH2Cl2 and filtered through Celite ®. The filtrate was allowed to evaporate in vacuo. For substrate 5, the crude mixture was purified on silica gel column chromatography with a step gradient (0 – 5%) methanol in dichloromethane. For substrate 6, the crude mixture was purified on Al2O3 column chromatography with a step gradient of EtOAc (0-10%) in hexanes as the mobile phase.

8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-N6-dimethoxytrityl-2'-deoxyadenosine (7b)

8-Bromo-3',5'-O-bis(tert-butyldimethylsilyl)-N6-dimethoxytrityl-2'-deoxyadenosine 6 (0.595 g, 0.693 mmol), 6-aminochrysene (0.250g, 1.04 mmol), palladium(II) acetate Pd(OAc)2 (6 mg, 0.0276 mmol) and rac-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) (50 mg, 0.080 mmol) were suspended in 10 ml anhydrous toluene. The solution was degassed by purging with
argon for 1 h. The reaction flask was then heated under argon at 90°C for 1 h. After this time cesium carbonate, Cs₂CO₃ (0.366 g, 1.04 mmol) was added and the reaction stirred at 100°C for an additional 1 hour. The reaction was cooled, diluted with ether and filtered through Celite ®. The filtrate was allowed to evaporate under reduced pressure. The crude mixture was purified on aluminum oxide column chromatography with a step gradient of EtOAc (0-10%) in hexanes as the mobile phase. The product was isolated as a dark green solid (0.603 g, 85%).

**HRMS (ESI⁺)** m/z calc. C₆₁H₇₀N₁₆O₅Si₂ [M+H]⁺, 1023.5025; found, 1023.5056

**¹H NMR (400 MHz, CDCl₃)** δ 9.60 (s, 1H), 8.88 (d, J = 8.2 Hz, 1H), 8.74 (d, J = 8.3 Hz, 1H), 8.70 (d, J = 9.2 Hz, 1H), 8.61 (s, 1H), 8.16 (dd, J = 8.1, 1.5 Hz, 1H), 8.03 – 7.92 (m, 3H), 7.81 – 7.54 (m, 4H), 7.42 – 7.35 (m, 2H), 7.33 – 7.29 (m, 2H), 7.28 – 7.17 (m, 5H), 6.85 – 6.70 (m, 4H), 6.65 (s, 1H), 6.53 (t, J = 7.8, 5.4 Hz, 1H), 4.23 (q, J = 3.3 Hz, 1H), 3.99 (dd, J = 11.4, 3.6 Hz, 1H), 3.91 – 3.80 (m, 1H), 3.77 (s, 6H), 3.61 (s, 6H), 3.21 (ddd, J = 13.4, 7.8, 5.9 Hz, 1H), 2.51 (ddd, J = 13.0, 5.5, 2.9 Hz, 1H), 0.96 (s, 8H), 0.61 (s, 8H), 0.16 (d, J = 4.6 Hz, 6H), -0.18 (s, 3H), -0.30 (s, 3H).

**¹³C NMR (101 MHz, Chloroform-d)** δ 158.07, 151.59, 149.54, 149.20, 145.89, 138.18, 133.40, 132.49, 131.49, 130.09, 128.87, 128.83, 128.43, 127.73, 126.82, 126.70, 126.54, 126.51, 126.30, 126.11, 125.33, 123.99, 123.53, 121.38, 121.01, 118.71, 113.20, 113.04, 88.35, 85.60, 72.43, 70.31, 63.02, 55.16, 39.79, 25.84, 25.65, 18.24, 18.07, -4.51, -4.72, -5.71, -5.76.

**8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine (7a)**

**Method 1**

Fully protected coupled substrate, 8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-N⁶-dimethoxytrityl-2'-deoxyadenosine, 7b (2.0 g, 1.95 mmol) was dissolved in 5.0 mL dichloromethane and to this added 1M zinc bromide solution in methanol: dichloromethane (1:1)
(6 mL). The reaction was stirred for 0.5 h and was monitored for completion by TLC (90/10 DCM/MeOH, v/v). The reaction was quenched with aqueous sodium bicarbonate and extracted with dichloromethane. The organic layer was combined and dried with sodium sulfate. The solvent was evaporated under reduced pressure and crude product was purified by silica gel flash column chromatography with a step gradient of 0-10% methanol in dichloromethane to afford compound 6 as a beige powder (1.05 g, 75%).

**Method 2**

8-Bromo-3', 5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine 5 (0.098 g, 0.1745 mmol), 6-aminochrysene (0.064 mg, 0.263 mmol), tris(dibenzyldieneacetone)dipalladium (Pd2(dba)3 (6.4 mg, 0.007 mmol) and rac-2,2'-bis(diphenylphosphino)-1,1'-binaphthy (BINAP) (12.5 mg, 0.02 mmol) were suspended in 8 ml diethoxy ethane (DME). The solution was degassed by purging with argon for 1 h. The reaction flask was then heated under argon at 85°C for 1 h. After this time cesium carbonate, Cs2CO3 (86 mg, 0.263 mmol) was added and the reaction stirred at 85°C for an additional hour. The reaction was cooled, diluted with dichloromethane and filtered through Celite®. The filtrate was allowed to evaporate under reduced pressure. The crude mixture was purified on silica gel column chromatography with a step gradient (0 – 5%) methanol in dichloromethane. The product 7a was isolated as a beige powder (71 mg, 56%).

**HRMS (ESI+) m/z calc. C40H52N6O3Si2 [M+H] +, 721.37; found, 721.3729**

**1H NMR (400 MHz, CDCl3) δ** 9.23 (s, 1H), 8.86 (d, J = 8.4 Hz, 1H), 8.74 (dd, J = 23.0, 8.6 Hz, 3H), 8.42 (s, 1H), 8.25 (d, J = 1.9 Hz, 1H), 8.16 (d, J = 7.9 Hz, 1H), 8.00 (t, J = 9.4 Hz, 3H), 7.81 – 7.60 (m, 5H), 6.64 (dd, J = 8.4, 5.4 Hz, 1H), 5.30 (d, J = 15.9 Hz, 1H), 4.67 (dd, J = 5.9, 2.9 Hz, 1H), 4.22 (d, J = 2.9 Hz, 1H), 4.05 (dd, J = 11.5, 3.0 Hz, 1H), 3.88 (dd, J = 11.5, 2.8 Hz, 1H), 3.12 (dt, J = 14.0, 7.1 Hz, 1H), 2.54 – 2.40 (m, 1H), 0.98 (s, 9H), 0.56 (s, 9H), 0.17 (d, J = 8.7 Hz, 6H),
-0.24 (s, 3H), -0.33 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 158.53, 158.32, 152.99, 151.96, 145.32, 142.33, 137.37, 136.11, 130.15, 130.12, 128.83, 128.23, 127.88, 127.76, 126.82, 126.79, 120.05, 113.20, 113.16, 113.05, 110.22, 108.06, 87.70, 85.01, 72.12, 70.66, 62.50, 59.01, 55.21, 36.89, 25.90, 25.78, 25.74, 18.36, 18.08, -4.63, -4.68, -5.39, -5.45.

8N-(aminochrysene)-2'-deoxyadenosine (3)

8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine 7a (200 mg, 0.2772 mmol) was dissolved in 3 ml of anhydrous tetrahydrofuran. 0.416 ml of 1M tetrabutylammonium fluoride in tetrahydrofuran was added and the reaction was allowed to stir for 24 hours under N$_2$. After this time, the solvent was evaporated under reduced pressure and the crude product was purified via silica gel column chromatography with methanol (0-5%) in dichloromethane. The product was isolated as a light brown solid (116 mg, 85%).

MS (ESI$^+$) m/z calc. C$_{28}$H$_{24}$N$_6$O$_3$ [M+H]$^+$, 493.53; found, 493.53

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.32 (s, 1H), 9.01 (d, $J = 8.4$ Hz, 1H), 8.89 (d, $J = 7.4$ Hz, 2H), 8.84 (d, $J = 8.2$ Hz, 3H), 8.19 (d, $J = 8.2$ Hz, 1H), 8.10 (t, $J = 6.8$ Hz, 2H), 8.02 (s, 1H), 7.84 – 7.66 (m, 4H), 4.14 – 3.99 (m, 1H), 3.81 (d, $J = 11.9$ Hz, 1H), 3.10 (td, $J = 15.5, 14.4, 8.3$ Hz, 1H), 2.30 (dt, $J = 12.9, 6.9$ Hz, 1H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 153.75, 150.12, 150.02, 149.66, 135.98, 132.38, 131.56, 130.00, 128.90, 128.53, 127.64, 127.43, 127.24, 127.12, 126.32, 124.26, 124.18, 124.04, 121.74, 117.26, 116.87, 88.27, 84.32, 71.90, 62.20, 58.00, 55.38, 38.79.

$N^6$-Benzoyl-8N-(aminochrysene)-2'-deoxyadenosine (8)

8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine, 7a (445mg, 0.617 mmol) was dissolved in anhydrous pyridine (5 mL) under an atmosphere of nitrogen and benzoyl chloride (0.108 mL, 0.9255) was added dropwise. The reaction mixture was stirred for 4 h at room temperature. It was then diluted with dichloromethane (10 mL) and washed
with saturated sodium hydrogen carbonate solution, and the aqueous layer was extracted twice with dichloromethane. After removal of the dichloromethane under reduced pressure, morpholine (0.134 mL, 1.5425 mmol) was added and the resulting mixture was stirred at room temperature for 2 h. After this time, the reaction mixture was diluted with dichloromethane (10mL) and then washed twice with 0.5M sodium dihydrogenphosphate solution. The aqueous layer was extracted three times with dichloromethane. After complete removal of the dichloromethane under reduced pressure, 100 mg, 0.1386 mmol of crude product was dissolved in 3 ml of anhydrous tetrahydrofuran. 0.416 ml of 1M tetrabutylammonium fluoride in tetrahydrofuran was added and the reaction was allowed to stir for 24 hours under N₂. After this time, the solvent was evaporated under reduced pressure and the crude product was purified via silica gel column chromatography with methanol (0-10%) in dichloromethane. The product was isolated as a yellow solid (70 mg, 85%).

**HRMS (ESI⁺)** m/z calc. C₃₅H₂₈N₆O₄ [M+H]⁺, 597.2250; found, 597.2234 and [M+Na]⁺

**¹H NMR (300 MHz, DMSO-d₆)** δ 10.89 (s, 1H), 9.74 (s, 1H), 9.61 (s, 1H), 8.99 (d, J = 7.8 Hz, 1H), 8.83 (d, J = 8.9 Hz, 2H), 8.58 (s, 1H), 8.29 (d, J = 7.6 Hz, 1H), 8.02 (t, J = 6.6 Hz, 5H), 7.79 (s, 0H), 7.59 (dt, J = 14.5, 7.4 Hz, 1H), 7.43 (dt, J = 14.4, 7.6 Hz, 3H), 6.82 (t, J = 7.1 Hz, 1H), 5.48 (d, J = 4.2 Hz, 2H), 4.57 (s, 1H), 4.12 (s, 1H), 3.91 – 3.59 (m, 3H), 3.27 – 2.97 (m, 1H), 2.42 (dd, J = 12.9, 6.0 Hz, 1H).

**¹³C NMR (75 MHz, DMSO-d₆)** δ 151.51, 148.95, 145.54, 134.07, 134.00, 132.54, 132.42, 131.26, 130.18, 128.84, 128.75, 128.36, 127.60, 127.35, 127.20, 127.05, 126.96, 125.69, 124.33, 124.06, 123.33, 121.64, 115.19, 88.45, 84.85, 71.77, 62.02
N\textsuperscript{6}-Benzoyl-8N-(6-aminochrysene)-5\textprime-O-dimethoxytrityl-2\textprime-deoxyadenosine (9)

The N\textsuperscript{6}-benzoylated C8-arylamidine-dA adduct 8 (0.295g, 0.494 mmol) was dissolved in anhydrous pyridine (6 mL) under a nitrogen atmosphere and 4,4\textprime-dimethoxytrityl chloride (0.177g, 0.522 mmol) and silver nitrate (0.089g, 0.522 mmol) were added. The mixture was stirred at room temperature until the reaction was complete (18 h). The reaction mixture then diluted with dichloromethane (10 mL) and washed with saturated sodium hydrogencarbonate solution and brine. The aqueous layer was extracted twice with dichloromethane. The organic layers were combined, dried over sodium sulfate, and filtered, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with 0-3% methanol in dichloromethane to afford the desired DMT protected 7 as a yellow foam, (328 mg, 74%).

**HRMS (ESI\textsuperscript{+}) m/z calc. C\textsubscript{56}H\textsubscript{46}N\textsubscript{6}O\textsubscript{6} [M+H\textsuperscript{+}], 899.3552; found, 899.3532**

**\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3})** δ 9.31 (s, 1H), 8.76 (dd, J = 7.5, 1.3 Hz, 1H), 8.72 – 8.73 (m, 1H), 8.44 (s, 1H), 8.23 (s, 1H), 8.16 (dd, J = 7.8, 1.3 Hz, 1H), 8.05 – 8.03 (m, 2H), 8.00 – 7.94 (m, 1H), 7.89 (d, J = 9.2 Hz, 1H), 7.72 – 7.66 (m, 2H), 7.66 – 7.62 (m, 2H), 7.54 – 7.42 (m, 3H), 7.42 – 7.38 (m, 2H), 7.37 (d, J = 1.4 Hz, 1H), 7.33 – 7.27 (m, 2H), 7.27 – 7.20 (m, 1H), 7.13 – 7.07 (m, 4H), 6.87 – 6.81 (m, 4H), 6.60 (ddd, J = 4.0, 2.4, 0.9 Hz, 1H), 6.30 (s, 1H), 4.23 – 4.18 (m, 2H), 3.82 (s, 6H), 3.68 (qd, J = 12.0, 3.1 Hz, 2H), 2.56 (dt, J = 12.5, 3.3 Hz, 1H), 2.31 (dt, J = 12.3, 5.1 Hz, 1H), 1.91 (s, 1H). **\textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3})** δ 165.57, 165.06, 158.21, 153.17, 152.09, 151.83, 149.38, 144.96, 144.53, 143.84, 136.17, 135.10, 135.01, 134.11, 132.71, 132.45, 132.10, 131.22, 130.09, 129.83, 129.77, 129.33, 128.49, 128.38, 128.24, 128.15, 127.68, 127.64, 127.01, 126.80, 126.73, 126.41, 125.91, 125.50, 124.80, 123.68, 123.51, 123.13, 122.01, 121.90, 121.10,
This reaction was performed in a glove bag. \(N^6\)-Benzoyl-8\(N\)-(6-aminochrysene)-5'\(O\)-dimethoxytrityl-2'-deoxyadenosine-3'\(O\)-(cyanoethyl-\(N\),\(N\')-diisopropyl-phosphoramidite (10)

This reaction was performed in a glove bag. \(N^6\)-Benzoyl-8\(N\)-(6-aminochrysene)-5'\(O\)-dimethoxytrityl-2'-deoxyadenosine 9 (0.400 g, 0.444 mmol), \(N\), \(N\)-diisopropylethylamine (0.155 mL, 0.888 mmol) was dissolved in anhydrous dichloromethane (6 mL) under argon. 2-cyanoethyl-\(N\), \(N\)-diisopropylethylchlorophosphoramidite (0.105 mL, 0.444 mmol) was added and the reaction mixture stirred for 15 minutes. After this time, additional \(N\), \(N\)-diisopropylethylamine (0.155 mL, 0.888 mmol) was added to the solution and the reaction stirred for an additional 0.5 h. After this time, the reaction mixture was diluted with dichloromethane (20 mL) and saturated sodium bicarbonate (25 mL). The organic layer was retained, and the aqueous layer was extracted twice with dichloromethane (2 x 15 mL). The organic layers were combined, washed with brine and dried over sodium sulfate. The solvent was concentrated under reduced pressure (2 mL). The solution was added dropwise to a stirring solution of 220 mL hexanes. The resulting precipitate was collected and further purified on basified silica gel flash chromatography with a step gradient (0-5%) methanol in dichloromethane containing 1% triethylamine. The product was isolated as a yellow solid (0.297 g, 61%)

HRMS (ESI\(^+\)) m/z calc. \(C_{65}H_{63}N_8O_7P\) [M+H]\(^+\), 1099.4636; found, 1099.4607

\(^{31}\)P NMR (162 MHz, \(CD_2Cl_2\)) \(\delta\) 149.06, 149.01, 148.57, 148.51.
2. 2. 2 Synthesis of Site-Specifically Modified Oligodeoxynucleotides
Phosphoramidite 10 was used to synthesize modified oligodeoxynucleotides as per manufacture’s instruction. However, the time of the coupling step was repeated extended to 15-min for the incorporation of modified phosphoramidite with total coupling efficiencies of > 95%. The unmodified and complementary oligodeoxynucleotide sequences were purchased from Integrated DNA Technologies, Inc in Coralville, Iowa, USA.

2. 2. 3 Purification of Site-Specifically Modified Oligodeoxynucleotides
The modified oligonucleotides were purified by reverse-phase HPLC and denaturing polyacrylamide gel electrophoresis (PAGE). The homogeneity of the purified modified and unmodified (control) oligodeoxynucleotides were confirmed via phosphorylation with T4 polynucleotide kinase in the presence of $[^{32}\text{P}]$ adenosine triphosphate and subsequent PAGE analysis. The modified oligodeoxynucleotides were further characterized by ESI mass spectrometry. HRMS data are given in Table 3.

HPLC Purification
The crude oligodeoxynucleotides were purified using reversed phase HPLC utilizing a C18 – semi prep column (Phenomenex Gemini –NX 5μ C18 (250 × 10 mm)) with an eluent of 0.1 M NH₄OAc in ACN-H₂O (solvent A) and 80% ACN-20% H₂O (solvent B) with a linear gradient (0 to 50 min, 2% to 30%) at a flow rate of 3.0 mL/ min monitored at both 254 nm and 340 nm. The purified oligomers were collected in several 1.5 mL microcentrifuge tubes and filtered through Amicon ultracel 3K filters. The filtrates were collected and dried in a SpeedVac.

PAGE Purification
After HPLC purification, the oligomers were purified by denaturing polyacrylamide gel electrophoresis. Modified and unmodified oligomers were loaded onto 20% PAGE (50 cm length)
containing 8M urea. The gel was run at 2250 V (45V/cm²) for 5~6 hours at room temperature until
the leading dye bromophenol blue (which runs approximately with a 6mer) reached the bottom of
the gel. The desired oligomer-containing band was visualized using a UV (254 nm) light and
excised from the gel. The oligonucleotides were extracted from the excised gel by Spin-X (Costar
Corning) columns and eluted with water, and subsequently desalted by passing through Sep-Pak
C18 cartridges (Waters) with acetonitrile:water (60:40). The elute from each sample was dried in
a speed-vac and the concentration was determined by UV absorbance measurements. The
homogeneity of the purified modified and unmodified (control) oligodeoxynucleotides were
confirmed via phosphorylation with T4 polynucleotide kinase in the presence of [γ³²P] adenosine
triphosphate and subsequent PAGE analysis.
2. 3 Results and Discussion

In order to synthesize the C8-dA adduct of 6-NC in any desired DNA sequence, we employed a total synthesis approach. This approach involves the synthesis of the modified 3’ phosphoramidite monomer which can be site-specifically incorporated in any desire oligodeoxynucleotide sequence via solid-phase DNA synthesis. While many C8-dG adducts by different polycyclic aromatic amines and NO2-PAHs have been synthesized and incorporated in DNA by this method, there are only a few reports on the synthesis of dA adducts. Notably, synthesis of several polycyclic aromatic hydrocarbons (PAH) derived DNA adducts at the 6 position of dA have been accomplished, but only Meier and Takamura have incorporated C8-arylamine-modified dA adducts in DNA by total synthesis. They used the Pd-catalyzed Buchwald-Hartwig C-N bond forming strategy to incorporate phenylamine derivatives and 4-aminobiphenyl at the C8 position of dA.

Scheme 3. Preparation of Fully Protected Br-dA.

Selective bromination at C8 position of dA was achieved using Br2 in acetate buffer (pH 5.4.) Protection of the 3’, 5’ hydroxyls and exocyclic amine is necessary to avoid unwanted reaction in the Buchwald-Hartwig cross coupling reaction. Thus, silylation of 3’, 5’ hydroxyls moieties were achieved using tert-butyldimethylsilyl chloride. The N6-exocyclic amine was protected using 4,4’-dimethoxytrityl chloride (DMTCl) in pyridine with catalytic amounts of 4-dimethylaminopyridine (DMAP), which provided fully protected dA substrate 6 for coupling.
2. 3. 1 Buchwald-Hartwig Cross-Coupling Reaction Optimization

We envisioned performing Buchwald-Hartwig cross-coupling reaction without protection of the \( N^6 \) exocyclic amine as previously reported\(^{46-49} \). In order to increase the efficiency of the key palladium catalyzed Buchwald-Hartwig cross coupling reaction; a series of reaction conditions with respect to \( N^6 \) protected or unprotected substrate, catalyst, base, solvent and temperature were screened to determine the optimal reaction conditions (Table 2). Previous work have demonstrated that \( \text{Pd}_2(\text{dba})_3/\text{NaO}t\text{Bu} \) were good catalyst/base combo; while combination of \( \text{Pd(OAc)}_2/\text{Cs}_2\text{CO}_3 \) work best together\(^{46,50} \). Thus, Buchwald-Hartwig optimization were done with this framework in mind. Initially \( N^6 \) exocyclic free amine substrate, 5 reacted with 6-aminochrysene (6-AC) with combinations of \( \text{Pd}_2(\text{dba})_3/\text{NaO}t\text{Bu} \) in toluene as solvent at 100 °C. The desired product was isolated with 45\% yield (Table 2, entry 1). Identical reaction conditions except with combination of \( \text{Pd(OAc)}_2/\text{Cs}_2\text{CO}_3 \) furnished the desired product with increased yield of 56\% (Table 1, entry 2). Each reaction shown here was carried out for 3-4 h. Longer reaction times (up to 24 h) led to significant tailing on TLC for reactions carried out at 100 °C in toluene, which is usually indicative of degradation of the product. In 1,2-DME (performed at 85°C) this tailing was not observed, and the reactions were allowed to run overnight. However, longer reactions times did not result in improvement in reaction yields.

For the Pd-catalyzed coupling of protected dG with polyaromatic amines, Gillet and Schärer showed that \( N^2-\text{DMT} \)-protected dG provides excellent yield of the adduct,\(^{47} \) which we have used successfully for the synthesis of dG-1-aminopyrene adduct.\(^{50} \) So, to improve the coupling efficiency, the \( N^6 \) exocyclic amine was protected using 4,4'-dimethoxytrityl chloride (DMT-Cl) in pyridine with catalytic amounts of 4-dimethylaminopyridine (DMAP) and then the \( N^6 \) DMT protected Br-dA nucleoside, 6 was subjected to the Pd-catalyzed coupling. Gratifyingly, \( N^6 \) exocyclic amine DMTr protected substrate, 6 coupled with 6-AC with combination of
Pd(OAc)$_2$/Cs$_2$CO$_3$ in toluene to furnish the desired product with 85% yield at 100 °C for 3 h (Table 2). For the DMT protected Br-dA nucleoside (6) also longer reaction time did not improve the yield. We believe that the improved yields with N$^6$ DMT protected substrate in the Buchwald-Hartwig cross coupling reactions can be attributed to the increased solubility of the substrate in toluene (and DME) facilitated by the non-polar DMT group.

![Chemical structure](image)

**Table 2.** Optimization of Buchwald-Hartwig cross coupling reaction.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Catalyst (4 mol %)</th>
<th>Base (1.5 equiv)</th>
<th>Ligand (12 mol%)</th>
<th>Solvent</th>
<th>Temp. (˚C)</th>
<th>%Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>Pd$_2$(dba)$_3$</td>
<td>NaOrBu</td>
<td>BINAP</td>
<td>Toluene</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td>Pd(OAc)$_2$</td>
<td>Cs$_2$CO$_3$</td>
<td>BINAP</td>
<td>Toluene</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td>Pd$_2$(dba)$_3$</td>
<td>NaOrBu</td>
<td>BINAP</td>
<td>DME</td>
<td>85</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>Pd(OAc)$_2$</td>
<td>Cs$_2$CO$_3$</td>
<td>BINAP</td>
<td>DME</td>
<td>85</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>DMT</td>
<td>Pd$_2$(dba)$_3$</td>
<td>NaOrBu</td>
<td>BINAP</td>
<td>Toluene</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>DMT</td>
<td>Pd(OAc)$_2$</td>
<td>Cs$_2$CO$_3$</td>
<td>BINAP</td>
<td>Toluene</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>7</td>
<td>DMT</td>
<td>Pd$_2$(dba)$_3$</td>
<td>NaOrBu</td>
<td>BINAP</td>
<td>DME</td>
<td>85</td>
<td>68</td>
</tr>
<tr>
<td>8</td>
<td>DMT</td>
<td>Pd(OAc)$_2$</td>
<td>Cs$_2$CO$_3$</td>
<td>BINAP</td>
<td>DME</td>
<td>85</td>
<td>61</td>
</tr>
</tbody>
</table>

*All reactions were carried out for 3-4 h.*
Figure 11. Mechanism of Buchwald-Hartwig cross coupling reaction of protected 8-bromo-2′-deoxyadenosine and 6-aminochrysene (6-AC)

2.3.2 Synthesis of Modified Phosphoramidite Monomer

With the optimized conditions in hand and an efficient strategy to access coupled product 7b; N°-exocyclic amine was deprotected using a 1 M solution of ZnBr₂ to furnish 7a. To enable solid phase DNA synthesis, N° amine was then protected with base-labile benzoyl group using benzoyl chloride in pyridine followed by treatment with morpholine to afford N°-benzoylated product; desilylation of the 5′- and 3′-hydroxyl was achieved using tetrabutylammonium fluoride (TBAF) in THF to furnish 8. To initiate DNA synthesis the 5′postion was protected with acid-labile DMTr, 9. The corresponding 3′-phosphoramidite was prepared using 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite to give phosphoramidite monomer 10 (Scheme 6).
Scheme 4. Synthesis of Corresponding 3’-Phosphoramidite 10 a) 1M ZnBr₂ in 1:1 solution. MeOH:CH₂Cl₂, 30 mins, 75%; b) 1M TBAF in THF, RT, 24h, 85%; c) Benzoyl chloride (BzCl), pyridine, RT followed by treatment with morpholine, 2h d) 1M TBAF in THF, RT, 24h, 85%; e) 4,4-dimethoxytrityl chloride (DMTCl), pyridine, AgNO₃, RT 24h, 74% f) 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite, DIEA, CH₂Cl₂, RT, 1h, 61%.

2.3.3 Synthesis of Oligodeoxynucleotides Containing N-(dA-8-yl)-6-AC Adduct

Fully protected N-(dA-8-yl)-6-AC phosphoramidite (10) was incorporated in the 12 and 15-mer oligodeoxynucleotides with total coupling efficiency of > 95%. Oligodeoxynucleotide deprotection was done at 55°C with ammonium hydroxide for 24h to furnished modified oligodeoxynucleotides containing N-(dA-8-yl)-6-AC adduct (Scheme 7). During deprotection, 0.25M 2-mercaptoethanol was added to avoid oxidative degradation. These modified oligodeoxynucleotides were purified by reverse-phase HPLC and PAGE and characterized by high resolution electrospray ionization mass spectrometry in negative mode (Table 3).
Scheme 5. Modified oligodeoxynucleotides deprotection and purification

<table>
<thead>
<tr>
<th>Oligodeoxynucleotides</th>
<th>Calculated (M-3H)³⁻</th>
<th>Observed (M-3H)³⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-GTG CAT GTT TGT-3'</td>
<td>3679.622</td>
<td>3679.627</td>
</tr>
<tr>
<td>5'-GTG CA*T GTT TGT-3'</td>
<td>3921.922</td>
<td>3921.7274</td>
</tr>
<tr>
<td>5'-GCCCTCAACAAGATG-3'</td>
<td>4542.800</td>
<td>4542.8010</td>
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<tr>
<td>5'-GCCCTCAA*CAAGATG-3'</td>
<td>4784.100</td>
<td>4784.9298</td>
</tr>
</tbody>
</table>

Table 3. HRMS-ES mass for modified and unmodified (control) oligodeoxynucleotides

The 12-mer sequence was taken from part of p53 tumor suppressor gene, 5’-GTG CG*T GTT TGT-3’ where the underlined part is codon 273, which is frequently mutated in many different types of human cancers. We replaced G* with A* so that mutations from A* can be compared with the G adduct. The 15-mer sequence (Table 3), 5'-GCC CTC AA*C AAG ATG-3' was taken from another part of p53, which include codon 131 (underlined). Codon 131 is one of the rare mutation hotspots in which an adenine is mutated. This was discovered from mutation spectrum by aristolochic acid, a naturally occurring nitroaromatic compound and a dietary contaminant.²²,²⁴,⁶²
**Figure 12.** Structures of modified oligodeoxynucleotides.

### 2. 4 Conclusion

In summary, we have reported an efficient strategy for the synthesis of C8-6-nitrochrysene adduct of 2’-deoxyadenosine. The 6-aminochrysene carcinogen was introduced at the C8 site of 2’-dA using Buchwald–Hartwig palladium catalyzed cross-coupling chemistry. This optimized strategy provides efficient and convenient access to \( N-(dA-8\text{-yl})-6-\text{AC} \) adduct. The corresponding 3’phosphoramidite adduct of \( N-(dA-8\text{-yl})-6-\text{AC} \) were site-specifically incorporated into oligodeoxynucleotides via. solid-phase DNA synthesis.
Chapter 3

Conformational Preference and Stability of 6-Nitrochrysene C8-2’Deoxyadenosine Adduct

Carcinogen-DNA adducts that derive from covalent modifications of DNA nucleobase by nitro polycyclic aromatic compounds are known to form bulky DNA adducts. These adducts can assume various conformations in DNA helix which can be responsible for inducing a variety of mutagenic outcomes. We investigated the conformational preference of the C8-2’deoxyadenosine nucleoside that derives from 6-nitrochrysene modification, \( N-(\text{dA-8-yl})-6-\text{AC} \). The adducted nucleoside showed \textit{syn} conformational preference by NMR spectroscopy and DFT calculations. We also investigated the stability of \( N-(\text{dA-8-yl})-6-\text{AC} \) adducted nucleoside using UV/Vis spectroscopy and HPLC. We determined that \( N-(\text{dA-8-yl})-6-\text{AC} \) adduct is stable under \textit{in vitro} biologically relevant conditions. We further investigated the helical structure of \( N-(\text{dA-8-yl})-6-\text{AC} \) oligodeoxynucleotide containing duplexes using circular dichroism spectroscopy. The CD curves of the \( N-(\text{dA-8-yl})-6-\text{AC} \) containing 12 and 15mer duplexes showed only slight deviation from the B type DNA helical structure of the unmodified duplex.
3.1 Introduction

Nitrogen-linked arylamine C8-dG lesions can adopt three distinct conformations (Figure 13) depending on the orientation about the glycosidic (sugar-base) bond and the location of the bulky aryl ring system within the duplex.66 These adducts are known to possess conformational heterogeneity. This refers to the ability of nucleotides to adopt more than one conformational posture about the glycosidic bond.67–69 The two orientation nucleotides can typically adopt are anti and syn of the nucleobase to the sugar.66 The ability of nucleotides to assume different conformations is believed to be an important factor in mutation outcomes. Carcinogen-DNA adduct possesses altered biophysical properties compared to the precursor nucleoside.70–72 It is believed that the correlation between aryl ring size, adduct planarity, and sequence context regulate distribution in conformational preference. Wetmore and coworkers purports that the distribution in conformational preference of adduct greatly impacts repair propensity and mutational outcomes.66

**Figure 13.** Depictions of three major conformations induced in N linked C8-aryl-dG adducts

The nucleotide excision repair (NER) efficiency of \( N-(dA-8-yl)-6-AC \) adduct (Figure 14) (3) is eight times less that the \( N-(dG-8-yl)-6-AC \) (1) in identical sequence context.14 This suggests that \( N-(dA-8-yl)-6-AC \) adduct (3) is potentially more mutagenic than \( N-(dG-8-yl)-6-AC \) (1).
Figure 14. Major DNA adducts derived from 6-NC via nitroreduction pathway

In human cells, bulky DNA lesions derived from nucleobase modifications are subjected to NER that attempt to restores the integrity of DNA prior to DNA replication through a ‘cut and patch’ mechanism.4,31 NER in humans involve recognition of helix distorting DNA damages that recruits NER enzymes.73,74 This process involves as many as 30 distinct proteins in a large complex known as nucleotide excision repairosome. It is believed that local thermodynamic destabilization plays an important role in determining lesion recognition and subsequent excision efficiency.30 Therefore, non-distorting conformers of adducts may escape NER repair, while distorting conformers may be more efficiently repaired. Thus, the equilibrium between conformations might help determine their potential carcinogenicity.68 We hypothesized that \( N-(dA-8-yl)-6-\text{AC} \) (3) adduct is less distorting that \( N-(dG-8-yl)-6-\text{AC} \) (1) in an identical sequence context and therefore results in less destabilization and so is poorly recognized by NER enzymes.

To test this hypothesis, we studied the intrinsic conformation of the adducted free nucleoside to establish the preferred conformation of the adducted nucleoside. Interestingly, Sproviero et. al. demonstrated that bulky C8-arylamine-dG prefers \textit{syn} conformation as adducted nucleosides but prefers \textit{anti} conformation in duplex DNA. Furthermore, we studied the conformation of \( N-(dA-8-\text{yl})-6-\text{AC} \) (3) using circular dichroism spectroscopy and molecular dynamics. Furthermore it has been suggested that \( N-(dA-8-\text{yl})-6-\text{AC} \) adduct (3) undergoes spontaneous deamination to produce
a deoxyinosine derivative \( N-(dI-8-yl)-6-AC \).\textsuperscript{14} We were interested in understanding this facile deamination. To this end, we’ve studied the stability of \( N-(dA-8-yl)-6-AC \) under physiological temperature and pH.
3. 2 Materials and Methods

3. 2. 1 Synthesis of \(N\)-(dA-8-yl)-6-AC nucleoside
See 2.2.1 [Synthesis of compound 3]

3. 2. 2 Synthesis of site-specifically modified oligodeoxynucleotides
See 2. 2. 4

3. 2. 3 DFT Calculations

![Figure 15. Structures and chemical numbering of N-linked DNA adduct at the C8-site of 2'-deoxyadenosine](image)

To analyze the intrinsic conformational preferences of the adducts, the potential energy surface was mapped as a function of the \(\theta\) \((\angle(N9-C8-N10-C11))\) and \(\phi\) \((\angle(C8-N10-C11-C12))\) dihedral angles using nucleobase models and as a function of \(\chi\) \((\angle(O4'-C1'-N9-C4))\) and \(\theta\) using nucleoside \(N\)-(dA-8-yl)-6-AC adducts.\(^{75}\) Specifically, surfaces were generated using B3LYP/6-31G(d) optimizations by altering and constraining each dihedral angle from 0° to 350° in 10° increments. B3LYP/6-31G(d) has been successfully used to study the conformational landscape of several DNA adducts.\(^{66,68}\) However, the extended \(\pi\)-system in the adducts investigated in the present work suggest that more accurate modeling of dispersion forces may be required.\(^{68}\) Therefore, all minima identified from the scans were subsequently fully optimized with both B3LYP and B3LYP-D3(BJ) using the 6-31G(d) basis set for comparison. The similarities in the key structural parameters obtained with both functionals support our use of B3LYP for the
computationally intensive scans. Regardless, more accurate relative energies were obtained for the fully B3LYP-D3 optimized minima with B3LYP-D3(BJ)/6-311+ G(2df,p), which include scaled (0.9813) zero-point vibrational energy (ZPVE) corrections.\(^7\)

The initial nucleobase models were built using GaussView 5.0 by appending the bulky groups to the template guanine. The nucleoside models were built from the lowest energy conformation obtained for the nucleobase models by replacing H9 with 2'-deoxyribose in the B-DNA conformation obtained in a previous conformational analysis (i.e., C2'-endo pucker, \(\beta (\angle (H-O5'-C5'-C4'))\) dihedral angle fixed to 180° and the dihedral angle at the 3' terminus \(\angle (H-O3'-C3'-H3')\) locked to 60°). All DFT calculations were performed using Gaussian 09 (revision D.01).

### 3. 2. 4 Circular Dichroism Studies

The concentration of oligodeoxynucleotides were determined using NanoDrop 2000 spectrophotometer. Equal amount of two complementary strands (5 nmol) were dissolved in buffer [1 mL, NaH\(_2\)PO\(_4\) buffer (10mM), NaCl (140 mM), EDTA (1mM), pH 6.6] and heated to 70°C and then slowly allowed to cool to room temperature to enable annealing of the two strands. CD measurements were carried out at 25 °C and samples were scanned from 400 to 200 nm at 0.5 nm intervals averaged over 5 s on a Chirascan\textsuperscript{TM} V100.

### 3. 2. 5 UV/Vis Spectroscopy Stability Studies

In 50 mm phosphate-buffered saline (PBS), pH 7.3 (containing DMSO, which was necessary to enhance the solubility). While incubating both compound 3 and 2'deoxyadenosine as control at concentrations of 6mM at 37 °C, aliquots were taken at various time points, diluted with water to a final concentration of 0.3 mM and analyzed by their UV/Vis absorption spectra.\(^7\)
3. 3 Results and Discussion

Figure 16. Proton NMR spectrum of $N$-(dA-8-yl)-6-AC adducted nucleoside (3) highlighting conformationally relevant peaks

NMR data on C8-arylamine-dA adducts showed that with a single phenyl ring, the preferred conformation of the $N$-glycosidic bond is *anti*, but addition of an $N$-acetyl group replacing the amino hydrogen rotates it to *syn* conformation.\(^46\) To determine *syn* or *anti* preference of $N$-(dA-8-yl)-6-AC, its conformation was investigated using ROESY-NMR spectroscopy. Figure 16 shows $^1$H NMR spectrum with of $N$-(dA-8-yl)-6-AC adducted nucleoside with conformationally relevant peak assignment, while Figure 17 shows the ROESY NMR spectrum with a cross-peak between H1' proton of the sugar and 6-AC amine tethered to the C8 site of dA, indicating a preference for *syn* conformation. Furthermore, no cross-peaks were observed between the H2 proton on nucleobase and the H1' proton on the sugar, typically observed for *anti*-conformation. These results indicate a preference of $N$-(dA-8-yl)-6-AC to exist in the *syn* conformation of the $N$-glycosidic bond, which was also reported for other bulky C8-arylamine modified nucleosides.\(^78,79\)
To determine the conformational preference of the adducted nucleoside, we attached the 6-aminochrysene moiety to the template for adenosine in the Gaussian quantum chemistry software and scanned over the O4’–C1’–N9–C4 dihedral in 10° increments.\textsuperscript{69,80} Consistent with the ROESY NMR data, we found that the syn conformation is the lowest energy conformer, and that the lowest energy anti conformer is significantly higher in energy (Figure 18). Interestingly, both are stabilized by an intramolecular hydrogen bond. At still considerably higher energy, there is a mix of syn and anti-conformers. These results qualitatively confirms the assignment of the syn conformation preference from experiment, which is also in agreement with similar adducted nucleosides.\textsuperscript{46,81}
Figure 18. Potential energy surface scan around the deoxyribose-nucleobase bond of N-(dA-8-yl)-6-AC adduct (3)

Circular Dichroism (CD) Studies

The 15mer (5’-GCCCTCAACAAGATG-3’) unmodified and N-(dA-8-yl)-6-AC modified oligodeoxynucleotides (5’-GCCCTCAA*CAAGATG-3’) were allowed to anneal to their complementary strand, and the resultant duplexes were evaluated on a circular dichroism spectrometer (Chirascan™ V100). As shown in Figure 19 (Panel A), the CD spectra confirm overall B type DNA conformation of the unmodified control duplex and the N-(dA-8-yl)-6-AC containing duplex. For control duplex a positive Cotton Effect was observed at 280 nm with
negative one at 245 nm typically found in B type DNA. However, CD curve of the \( N-(dA-8-yl)-6-AC \) containing

\[ \text{Figure 19. CD curves of unmodified (red solid line) and modified (black solid line) 15-mer (Panel A) and 12-mer (Panel B) oligonucleotides (sequence shown in Table 2) after annealing with their complementary strand. The CD spectra of the single-stranded oligonucleotides are shown in black dotted lines.} \]

Similarly, the 12mer (5’-GTGCATGTTTGT-3’) unmodified and \( N-(dA-8-yl)-6-AC \) modified oligodeoxynucleotides (5’-GTGCA*TGTTTGT-3’) were allowed to anneal to their complementary strand, and the resultant duplexes were evaluated on a circular dichroism spectrometer. Likewise, the positive and negative Cotton Effect consistent with canonical B-type DNA helical structure were observed for 12mer control (unmodified) and \( N-(dA-8-yl)-6-AC \) modified duplex. However, both 12mer unmodified and modified duplexes showed less structured helices than that of the 15mer duplexes.
Study of the Stability of \( N-(dA-8\text{-yl})\)-6-AC Adducted Nucleoside

\( N-(dA-8\text{-yl})\)-6-AC DNA adduct is believed to give rise to the corresponding 2'-deoxyinosine adduct, but whether the process of deamination is enzymatic or non-enzymatic has never been established.\(^{14,13}\) We investigated the stability of \( N-(dA-8\text{-yl})\)-6-AC adducted nucleoside under biologically relevant conditions; in aqueous solution of PBS buffer at nearly neutral pH at 37 °C. \( N-(dA-8\text{-yl})\)-6-AC adducted nucleoside was incubated in 50 mm phosphate-buffered saline (PBS), pH 7.3 containing 30% v/v DMSO, which was necessary to enhance the solubility. As shown in Figure 20, \( N-(dA-8\text{-yl})\)-6-AC adducted nucleoside had an absorption maximum at \( \lambda = 268 \) nm. During incubation aliquots were taken at various time points, diluted with water to a final concentration of 0.3 mM, and analyzed by their UV/Vis absorption spectra. The absorption maxima remained constant over 180 hours suggesting that \( N-(dA-8\text{-yl})\)-6-AC adducted nucleoside is stable under the experimental conditions. Samples from the UV/Vis experiments were injected into the HPLC to determine the presence of new peaks. However, only a single peak corresponding to \( N-(dA-8\text{-yl})\)-6-AC adducted nucleoside standard was observed. Thus, our biochemical \textit{in vitro} assay suggests that \( N-(dA-8\text{-yl})\)-6-AC is stable in aqueous solution at a pH of 7.3, which is the pH detected inside the nucleus and cytoplasm of healthy cells.\(^{82}\) \( N-(dA-8\text{-yl})\)-6-AC adducted nucleoside does not undergo spontaneous deamination to deoxyinosine derivative under the conditions described. However, further studies need to be performed in order to unequivocally confirm the stability of \( N-(dA-8\text{-yl})\)-6-AC.
Figure 20. A) Overlay of the absorption spectra of N-(dA-8-yl)-6-AC adducted nucleoside measured at the indicated time points; incubation of the compound (6 mM) in water at 37°C; for each measurement aliquots were taken and diluted with water to a final concentration of 0.3 mM. B) Plotted data taken from the absorption measurement: intensity of absorption at $\lambda = 268$ nm against incubation time.
3. 4 Conclusion

Using ROESY NMR spectroscopy, we determined that the adducted purine prefers syn conformation of its glyosidic bond of $N$-(dA-8-yl)-6-AC in solution. The CD spectra of $N$-(dA-8-yl)-6-AC 15-mer and 12-mer containing duplexes were consistent with B-DNA, although $N$-(dA-8-yl)-6-AC modified duplexes showed small deviations from the control duplexes, suggesting less structured helices for both 12 and 15-mer. In addition, we determined that $N$-(dA-8-yl)-6-AC nucleoside is stable under in vitro biologically relevant conditions.
Chapter 4

Translesion Synthesis and DNA Repair of 6-Nitrochrysene-Derived C8-2’-Deoxyadenosine Adduct in \textit{E. coli} and Human Cells

\textsuperscript{†}Powell, B.V.; Basu, A.K. “Translesion Synthesis and DNA Repair of 6-Nitrochrysene Derived C8-2’-Deoxyadenosine Adduct in Human Cells.” [\textit{Manuscript in preparation}]
4. Abstract

DNA lesions resulting from 6-NC modifications can inhibit DNA replication and are likely to induce mutations if they are not removed by cellular defense pathways prior to DNA replication. A previous in vitro study in HeLa cell extracts have shown that the C8-2'-deoxyadenosine DNA adduct derived from 6-nitrochrysene is more resistant to nucleotide excision repair (NER) than the corresponding C8-2'-deoxyguanosine DNA adduct. In this study, we investigated the replication bypass efficiencies and the roles of translesion synthesis (TLS) DNA polymerases in bypassing the 6NC modified C8-2'-deoxyadenosine adduct, N-(dA-8-yl)-6-AC in human embryonic kidney cells (HEK293T). We further investigated the mutagenicity and translesion synthesis (TLS) efficiency of N-(dA-8-yl)-6-AC in E. coli to compare mutation signature in E. coli with mutations induced in human cells. Replication of plasmid containing a single site-specific N-(dA-8-yl)-6-AC adduct in HEK293T provided ~12% progeny plasmid with mutations largely A→T transversions. We observed 4-fold decrease in mutation frequency when the plasmid is replicated in polymerases κ or ζ HEK293T KO cells. Similar experiments in polymerase η KO cells showed a 1.5-fold increase in mutation frequency and a 38% decrease in TLS bypass efficiency. We also noted that plasmid replication in polymerases ζ KO and κ siRNA KD cells resulted in a 29% decrease in TLS bypass efficiencies and 10-fold decrease in mutation frequency. Taken together, these results suggest that polymerases κ and ζ are involved in the error-prone TLS of N-(dA-8-yl)-6-AC, while pol η is performs error-free bypass. In Escherichia coli, viability of the adducted construct was ~60% of the control, indicating slower translesion synthesis of the adduct, which increased to nearly 90% upon induction of the SOS functions. Without SOS, the mutation frequency (MF) of the adduct was 5.2%. With SOS, the targeted MF increased 3-fold to 9.0%. The major type of targeted mutation was A*→G in both uninduced and SOS-induced cells.
Chapter 4 – Introduction

4.1 Introduction

Ubiquitous environmental contaminants such as nitropolycyclic aromatic hydrocarbons (NO2-PAH) are known to induce mammary cancer in rats and must therefore be regarded as potential human carcinogens. A representative example of NO2-PAH is 6-nitrochrysene (6-NC). It is now clear that 6-NC is activated in cells not only from simple nitroreduction but also from a combination of ring oxidation and nitroreduction.

![Chemical structures](image)

*Figure 21.* Nucleotide excision repair of 6-NC DNA adducts in HeLa cell extracts.14
El-Bayoumy and coworkers evaluated the nucleotides excision repair (NER) propensity of DNA adducts that is form from 6-NC in an *in vitro* study. They demonstrated that the \(N-(dG-8-yl)-1,2\)-DHD-6-AC adduct is more resistant to NER than the \(N-(dG-8-yl)-6\)-AC adduct by a factor of \(\sim 2\) and that the \(N-(dA-8-yl)-6\)-AC is much more resistant to repair since its NER efficiency is \(\sim 8\)-fold lower than that of the \(N-(dG-8-yl)-6\)-AC adduct (Figure 20). Thus, the 6-NC-derived adenine adduct represents an example of the resistance of bulky DNA adducts to the human nucleotide excision repair system.\(^{14}\) This suggests that although \(N-(dA-8-yl)-6\)-AC adduct is formed in less abundance, it is more persistent in human cells. As a result, it could be potentially more deleterious and mutagenic than the other more abundant DNA damages. DNA adducts that are resistant to NER are persistent in cells can stall replication machinery and might induce mutation at higher frequencies.

For over three decades it has been known that 6-NC is potent carcinogen in laboratory animals.\(^{13}\) However, little is known about the types and frequencies\(^9,20\) with which 6-NC induces mutations or the role of TLS enzymes in bypassing DNA lesions that derive from 6-NC in cells.
4. 2 Materials and Methods

4. 2. 1 Materials

Chemicals, enzymes, plasmid DNA and cells

All chemicals and reagents and enzymes were purchased from commercial suppliers and used without purification, unless stated otherwise. Chemicals for gel electrophoresis such as agarose, Tris base, boric acid, Na2EDTA, urea, acrylamide, N,N’-methylene bisacrylamide, TEMED, APS), and preparation of biological media (e.g., agar, yeast extract, bactotryptone, sodium chloride) were purchased from Fisher Scientific (Agawam, MA). Phenol (Ultrapure<sup>TM</sup> Buffer-Saturated Phenol) for phenol-chloroform extraction was obtained from Invitrogen. Hybridization buffer (PerfectHyb<sup>TM</sup> Hybridization Buffer 1X) chloroform and isoamyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO). [γ-<sup>32</sup>P] ATP for gel assays and hybridization were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA).

The media required for mammalian cell culture such as fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), non-essential amino acids (NEAA), penicillin/streptomycin (PS), Dulbecco’s phosphate buffer saline (D-PBS) solution, 0.25% trypsin-EDTA, Opti-MEM I Reduced Serum Medium and transfection reagent (Lipofectamine<sup>TM</sup> 2000) were purchased from Invitrogen Corp. (Carlsbad, CA).

All enzymes needed for construct preparation and radiolabeling including EcoRV, EcoRI T4 DNA ligase, T4 polynucleotide kinase (PNK), uracil DNA glycosylase (UDG), and exonuclease III (exo III) were obtained from New England Biolabs (Beverly, MA).

pMS2 plasmid was a gift from M. Moriya (SUNY, Stony Brook, NY).<sup>84</sup>
**Escherichia coli** strain DH10B and DH12S were purchased from Invitrogen (Carlsbad, CA). HEK 293T cells were obtained from ATCC (Manassas, VA).

The *E. coli* strains used were AB1157 [F thr-1 araC14 leuB6(Am) Δ(gpt-proA)62lacY1 tsx-33 supE44(AS) galK2(Oc) hisG4(Oc) rfbD1 mgl51 rpoS396(Am) rpsL31(Str') kdgK51 xylA5 mtl-1 argE3(Oc) thi-1] were provided by G. Walker (MIT, Cambridge, MA).

The unmodified oligodeoxynucleotides were purchased from Integrated DNA Technology (Coralville, IA) and Midland Certified Reagent Company (Midland, TX). The modified oligonucleotides were prepared as reported. Oligonucleotides were desalted using a Sep-Pak C18 cartridge from Waters Corp. (Milford, MA).

Synthetic siRNA duplex against *POLK* (SI04930884), *POLI* (SI03033310) was purchased from Qiagen (Valencia, CA).

### 4.2.2 Methods

**Agarose gel electrophoresis for single-stranded vector analysis**

Agarose gel electrophoresis was performed to analyze purity of the plasmid preparations and to determine the construct concentration. The ss-pMS2 vector analysis was performed in 1.1% agarose gels. The agarose gel (1.1% w/v, 0.66 mg agarose in 60 mL of 1x TBE) was casted on a horizontal gel apparatus and gel was allowed to solidify for 30 min. The sample was prepared by mixing 0.2 μg (2 μL) of the plasmid DNA with 2 μL of 6x loading dye (10mM Tris-HCl (pH7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA) diluted to 10 μL with water. ss-pMS2 analysis gel was run at 110 V for 3 hours at room temperature. Following electrophoresis, the gel was stained with ethidium bromide (25 μg/μL in 500 mL water) for five minutes, de-stained for 30 min in 1x TBE, and the DNA bands were visualized by UV-light.
**Bacterial electro-competent cell preparation and SOS induction**

Four milliliters of overnight *E. coli* culture, prepared from a single colony, was transferred to 200 mL of 1x LB media. *E. coli* cells were grown to $1 \times 10^8$ cells/mL (~ 2 h) and then harvested by centrifugation at 5000g for 15 min at 0 °C. This procedure was repeated twice except the cells were resuspended in 40 mL ice-cold deionized water. The bacterial pellet was resuspended in 1 mL of glycerol/water (10% v/v) and kept on ice until further use. For SOS response, the following additional steps were introduced after the first centrifugation. The cells were resuspended in 50 mL 10 mM MgSO$_4$ and treated with 50 J/m$^2$ or 20 J/m$^2$ of UV light (254 nm) in 25 mL aliquots in 150 × 50 mm plastic petri dishes. The cultures were incubated in Luria broth at 37 °C for 40 min in order to express SOS functions maximally. Following SOS induction, these cells were centrifuged, deionized, and resuspended in glycerol/water in a similar manner as described earlier except all manipulations were carried out in subdued light.

**Large scale preparation of single-stranded pMS2 (sspMS2) vector**

Single-stranded phagemid pMS2 DNA was prepared from *E. coli* JM109 with the aid of the helper phage M13K07 (NEB, Beverly, MA) as reported by Moriya. The pMS2 vector contains origins of replication for f1, CoIE1, and SV40, which can be used for single stranded replication in *E. coli*, double stranded replication in *E. coli* and mammalian cells. It also contains neomycin-resistant (neo) and ampicillin-resistant (amp) genes, which can be used for the selection of the cells containing the vector in mammalian and bacterial cells, respectively. In addition, it has the SV40 early promoter, SV40 small tumor (T) antigen splice sites, SV40 early polyadenylylation sites, and a multiple cloning site with a hairpin loop, that allow digestion of the single-stranded vector with *EcoRV*
Electrocompetent *E. coli* DH12S (Invitrogen) cells were thawed on ice and 50 μL of cells were mixed with 50 ng of double stranded replicative form (RF) of pMS2. Then this mixture was transferred to a pre-chilled 1-mm gapped electroporation cuvette and the electroporation was carried out under following conditions: 1.8 kV, 25 μF and 200 Ω. Immediately after electroporation 1 mL of pre-warmed (37 °C) SOC medium (Invitrogen) was added, transferred to 14 mL culture tube and was grown at 37 °C in an orbital shaker at 225-250 rpm for 1 hour. From the resulting culture, 5.0 μL aliquot was plated on lx YT + ampicillin (100μg/ml) and incubated overnight at 37 °C. The following day, about 100 colonies were scraped from the 1x YT plate using a sterile loop, inoculated to 2 L of ampicillin containing 2xYT media along with 1 mL of M13K07 helper phage (Invitrogen) and incubated at 37 °C. After two hours of growth, 100μg/mL kanamycin was added and the culture was incubated overnight for another 16 hours.
Figure 22. Workflow for the large-scale preparation of ssPMS2 plasmid.

The phage-infected culture was transferred to 250 mL centrifuge bottles, spun down at 10,000 rcf (20 min, 15°C), the resulting supernatant was transferred to another 250 mL centrifuge bottle and centrifuged at the same condition. Supernatant from the second spin was transferred 2L flask containing 4% (w/v) polyethylene glycol (PEG) and 3.5 M NaCl. The mixture was stirred in ice for at least one hour to precipitate the phage particles. The resulting cloudy solution was centrifuged at 10,000 rcf (20 min, 4 °C) and the pellet obtained was re- suspended in the minimum volume (6 mL) of 1x Tris-EDTA (pH 7.6) buffer. Then the phage particles were re-precipitated with ice-cold 4% (w/v) PEG and 4 M NaCl for an hour and centrifuged at 10,000 rcf (20 min, 4°C). The pallet obtained was re-suspended in 2.4mL of freshly prepared 1x Tris-EDTA (pH 7.6)
buffer and centrifuged for 10 min at 4°C and 10000 rpm. The resulting supernatant containing the phage particles was incubated with 0.625 μg/mL proteinase K (Invitrogen) for 16 hours and single-stranded pMS2 was purified from the digested phage solution by a series of phenol: chloroform extractions, followed by ethanol precipitation. Finally, sspMS2 was re-suspended in 200 μL sterile deionized water and quantified on the UV spectrometer. After purification, the purity of the ssp-pMS2 was determined by the absorbance ratio of 260 nm to 280 nm followed by analysis on 1.1 % agarose gel.

**Single-Stranded pMS2 modified vector construction**

![Diagram of vector construction](image)

**Figure 23.** Workflow for construction of modified recombinant vector.

The construction of modified pMS2 vectors containing site-specifically incorporated adducts were performed following the method developed by Moriya. For construction of the pMS2 with modified or unmodified 12mer insert, 100 μg (58 pmols) of sspMS2 DNA was digested with 800
units of EcoRV at 37 °C for 3 h followed by room temperature overnight (16h). An aliquot of the reaction mixture was run on an 1% agarose gel to confirm the complete linearization of the circular vector. An equimolar ratio of a 58-mer scaffold containing deoxyuridinies in place of deoxythymidines was annealed to linear ssDNA in 50 mM NaCl by heating at 90 °C for 5 min followed by slow cooling to 9 °C over 16 h. Annealing of the scaffold generated a gapped duplex that contained a 12mer-gap with complementary sequence of the lesion to be incorporated. An aliquot of the gapped duplex was run on 1% agarose gel with a known amount of control plasmid (ss-pMS2). The amount of the gapped duplex was determined by comparing the net-intensity of the gapped duplex band to the net-intensity of the known control plasmid using the KODAK Digital ScienceTM-1D program. After determining the gapped duplex concentration, a 20-fold molar excess of the modified or unmodified 5’-phosphorylated 12mer insert was added and ligated at 16°C overnight with 2800 units of T4 DNA ligase. Unligated oligonucleotides were removed by being passed through Centricon 100 apparatus. Any residual uncut-circular or religated DNA was removed by additional round of EcoRV digestion. The scaffold was removed by enzymatic digestion with uracil DNA glycosylase (UDG, 05 units/μl) and exonuclease-III (0.5 units/μL) at 16°C overnight. The resulting constructs were extracted with phenol: chloroform and precipitated with ethanol. The final constructs were resuspended in deionized distilled-water and were run on 1% agarose gel. The amount of construct was quantified in a manner similar to the way gapped duplex was quantified.
Figure 24. Agarose gel analysis for recombinant DNA construction

Replication of modified vector in HEK293T cells

The HEK293T wild type and specific polymerase knock out (KO) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4 mM L-glutamine, and adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum in. When it was about ~80% confluent, cells were washed with PBS (Invitrogen), harvested with 6 mL of Accutase and was seeded in 24-well plate (approximately 1 x 10^5 cells). The cells were grown to ~80% confluency in the 24 well plate and transfected with 100 ng of each construct using Lipofectamine cationic lipid reagent (Invitrogen) according to manufacturer’s instruction. Subsequent to transfection with control or lesion containing pMS2, the cells were allowed to grow at 37°C, 5% CO2 for 24 hours and the plasmid DNA was harvested using Qiagen DNA extraction kit.

TLS Assay in Human Cells

The lesion-containing or control pMS2 vector was mixed with a single-stranded pMS2 DNA vector (internal control); a 23-mer oligodeoxynucleotide sequence different from the N-(dA-8-yl)-
6-A containing sequence (or control) DNA sequence. The molar ratio of internal control vector to control vector was 1:1, and those for the internal control vector to \(N-(dA-8-y1)-6-AC\) containing vector was 1:3. The a 23-mer oligodeoxynucleotide vector was used as an internal control and gave equal number of progeny as the control vector. TLS efficiency was determined as the percentages of the colonies originating from the \(N-(dA-8-y1)-6-A\) containing plasmid relative to the internal control.

**Mutant screening by oligonucleotide hybridization**

Dot-blot hybridization was performed to identify the possible mutations at the lesion-site or at nearby locations in the progeny. In brief, appropriate amounts of liquid culture were plated on \(1\times\) YT + ampicillin (100\(\mu\)g/mL) plates and incubated at 37 °C overnight (16-18 h). The resulting individual colonies were picked using sterile toothpicks into single wells of a 96-well plate (Fisher Scientific) containing 200 \(\mu\)l \(1\times\) YT + ampicillin (100 \(\mu\)g/mL). The inoculated 96-well plate was then incubated at 37°C for a minimum of 3 hrs before blotting colonies onto filter paper. Using a 96-well replicator (Boekel Scientific, Feasterville, PA), cultures in the 96-well plate were blotted onto sterile labeled Whatmann chromatography papers placed on \(1\times\) YT + ampicillin (100 \(\mu\)g/mL) plates, in triplicates. The plates were incubated at 37 °C for 16 h for colonies to be replication on the filter paper. After overnight incubation, following procedure was carried out to fix DNA onto the filter paper. The filters were carefully lifted from the plates and subsequently transferred to (colony side up) 0.5 N NaOH, denaturing solution, for 12 mins, and to 0.5 M Tris-HCl (pH 7.5), neutralizing solution for 7 mins. Then the filters were washed twice with \(1\times\) SSC for 4 mins with slow agitation (50 rpm) and once with 100% ethanol for 30 seconds before they were baked for 2 hours at 80 °C. In-between each washing steps, except for two SSC washes, filters were drained and damped dry on paper towels (colony side up). Thereafter, each filter was placed separately in
a heat sealable bag and sealed with 6 mL of PerfectHybeTM hybridization buffer (Sigma) containing corresponding left, right or wild-type $^{32}$P-radiolabeled probe (10 pmol of probe/filter). Liquid in the sealed bags were spread evenly to entirely cover the filters and were allowed to hybridize to corresponding probe at its optimum hybridization temperature for a minimum of 4 hours in the hybridization oven (ProblotTM 12). Then the filters were carefully removed from the bags and gently agitated (60 rpm) twice with 2x SSC+0.1% SDS, preheated to the hybridization temperature ($T_{Hyb}$), for 10 mins. Finally, the filters were removed from the SSC solution, dried and exposed to autoradiography. Two left and right probes were used to select phages containing the correct insert, and transformants that did not hybridize with both the left and right probes were omitted. Any transformants that hybridized with the left and right probes but failed to hybridize with the wild-type probes were subjected to DNA sequence analysis.

**Transformation of modified vector in *E. coli* and progeny analysis**

Competent *E. coli* cells were prepared as described earlier. To induce SOS, the cells were re-suspended in 10 mM MgSO$_4$ solution and aliquots were treated with UV light (254 nm) (20 J/m$^2$) in plastic Petri dishes. The combined aliquots were incubated in Luria broth at 37° C for 40 min for maximum SOS induction. Following SOS induction, these cells were processed in a similar manner, except the procedure was carried out in subdued light. Then transformation of cells was carried out in a Bio-Rad Gene-Pulser apparatus by mixing vector DNA and un-induced cells or SOS-induced cells. Cells were grown for 1 h at 37° C after adding SOC medium and then plated on a 1X YT plates containing Ampicillin and the transformants were analyzed by hybridization.
4. 3 Results and Discussion

The strategy for construction of the adduct containing single stranded vector was developed by Moriya\textsuperscript{84} and has used been successfully by our lab\textsuperscript{85–88} and many other researchers.\textsuperscript{89–91} We used pMS2 shuttle vector plasmid containing the origins of replication for f1, ColE1, and SV40, and the genes for neo and amp resistance.

Figure 25. A general scheme for construction of the lesion-containing vector and replication in \textit{E. coli} or HEK 293T cells, and analysis of the progeny.
This single-stranded pMS2 vector also contain a hairpin region that, upon digestion with the EcoRV restriction enzyme followed by scaffolding with an oligonucleotide, generates the desired gapped duplex (Figure 25). The 15-mer oligodeoxynucleotide (5’-GCCCTCAA*CAAGATG-3’) containing N-(dA-8-yl)-6-AC adduct is ligated to this gap and the scaffold was enzymatically removed before replication. The strategy for construction and mutant screen assay is shown in Figure 25.

**Mutational analyses of N-(dA-8-yl)-6-AC in *E. coli***

The DNA sequence of the 15-mer was chosen from *TP53* gene codon 129-133, because crops contaminated with another nitroaromatic carcinogen aristolochic acid (AA) causes A→T mutation in codon 131 in patients with urothelial tumors. While many polyaromatic amine and nitroaromatic compounds form adducts at the C8 position of dG, which induce frameshifts and G→T transversions as the dominant mutations, the major adducts by AA are formed at the N6 position of dA. The N-(dA-8-yl)-6-AC containing plasmid was replicated in uninduced and SOS-induced *E. coli* AB1157 cells. In uninduced cells, the number of progeny colonies from the adduct containing construct was reduced to ~60% to that of the control (Figure 26A). This suggests that though replication was inhibited, N-(dA-8-yl)-6-AC is less toxic than the C8 dG adduct of 1-nitropyrene, which gives less than 30% progeny. Upon induction of SOS, viability of the adducted genome increased to nearly 90%, indicating significantly more facile translesion synthesis (TLS) of N-(dA-8-yl)-6-AC by the SOS proteins (Figure 26A).

Without SOS, 5.2% of the replicates were mutants, which comprised of 2.9% targeted and 2.3% semi-targeted mutants (Figure 26B). Of the targeted mutations, 80% was A*→G transitions. With SOS, overall MF increased to 12.2%, showing three-fold increase of the targeted mutations to 9% frequency, whereas semi-targeted mutation increased only marginally to 3.2% (Figure 26C).
Although A*→G was also the prevalent (55%) targeted mutation with SOS, nearly half as many A*→C was detected.

**Figure 26.** (A) The bypass efficiencies in *N*-(*dA-8-y1)-6-AC construct in *E. coli* (-) SOS (WT) and (+) SOS cells. The data represent the means and standard errors of the mean of results from 3 independent replication experiments. (B) The frequencies of total targeted mutations (C) induced in progeny from *N*-(*dA-8-y1)-6-AC construct in (-) SOS (WT) and (+) SOS *E. coli* cells. The data represent the means and standard errors of the mean of results from 3 independent replication experiments. *P < 0.05; ***P < 0.001. The P-values were calculated by using unpaired two-tailed Student’s t-test.
**Translesion Synthesis of N-(dA-8-yl)-6-AC in Human Cells**

To investigate the replication properties of N-(dA-8-yl)-6-AC adduct in human cells and the role of TLS polymerases in bypassing this lesion in cells; we conducted replication experiments in HEK293T cells and in HEK293T isogenic cells where individual TLS DNA polymerases were depleted by the CRISPR/Cas9 genome editing method and siRNA knock down approach.\(^{79,92}\) To study the role of specific TLS polymerase in the bypass of a lesion many researchers have conducted replication experiments in cells where specific TLS polymerase have been knockdown by siRNA approach.\(^{85,86}\) However, the siRNA method often results in incomplete depletion of TLS polymerases. The presence of residual amounts of TLS Pols makes is difficult to draw clear conclusions about the role of specific polymerases in lesion bypass.\(^{92,93}\) The recent advances in CRISPR-Cas9 genome editing method has enabled the facile knockout of individual genes in cultured human cells.\(^{94,95}\) To determine the TLS efficiency of N-(dA-8-yl)-6-AC adduct in HEK 293T cells, we mixed 3:1 ratio of N-(dA-8-yl)-6-AC vector and unmodified plasmid that contained a different unmodified sequence. The unmodified plasmid was used as an internal control. The percentages of the colonies originating from the N-(dA-8-yl)-6-AC lesion-containing construct relative to the unmodified plasmid, reflecting the percentage of TLS, were determined by oligonucleotide hybridization.

In contrast to TLS efficiency in *E. coli* WT cells which was 60%; TLS replication efficiency was 83% in HEK293T WT cells in which all TLS polymerases are expressed (Figure 27). The largest decrease in TLS efficiency was observed in Pol η KO cells (45%). In Pol κ and Pol ζ KO cell TLS efficiencies were ~60 % and ~70 % respectively. TLS efficiency decreased only marginally in Pol ι KO cells. TLS efficiencies in Pol η/κ and Pol η/ζ double KO cells were ~25 % and ~35% respectively. In Pol ζ KO and Pol κ KD cells TLS efficiency was ~54%. On the basis
of TLS replication bypass efficiencies, the role of TLS Pols in the bypass of N-(dA-8-yl)-6-AC can be rank as follows Pol η > Pol κ > Pol ζ > Pol ι.

**Figure 27.** TLS bypass efficiencies of the N-(dA-8-yl)-6-AC containing construct in HEK293T cells and various TLS polymerase KO HEK 293T cells. The data represent the means and standard
errors of the mean from 2-4 independent replication experiments. *P < 0.05; **P < 0.01; ***P < 0.001. The P-values were calculated by using unpaired two-tailed Student’s t-test.

**Mutational analyses of N-(dA-8-yl)-6-AC in Human Cells**

DNA sequence analysis showed that N-(dA-8-yl)-6-AC is mutagenic in HEK293T cells. Replication of plasmid containing a single site-specific N-(dA-8-yl)-6-AC adduct in HEK293T wild type cells provided ~12% progeny plasmid with mutations that included ~11% at the targeted site and ~1% occurred at the 3’ or 5’ end of the target site and are referred to as semi-targeted mutations (Figure 28). In HEK293T wild type cells, mutations at the target site that were largely A→T transversions. We observed 4-fold decrease in mutation frequency when the plasmid is replicated in polymerases κ or ζ HEK293T individually KO cells. Similar experiments in polymerase η KO cells showed a 1.5-fold increase in mutation frequency. We also noted that plasmid replication in polymerases ζ KO and κ siRNA knock down cells resulted in 10-fold decrease in mutation frequency.
Figure 28. Targeted and semi-targeted mutations induced in the progeny from the $N$-(dA-8-yl)-6-AC construct in HEK 293T and various polymerase knockout cells. The data represent the mean and the standard deviation (of the total MF) from 2-4 independent experiments. The statistical significance of the difference in MFs between HEK 293T and TLS pols knockouts was calculated using two-tailed, unpaired Student’s t test. (*P < 0.05; **P < 0.01; ***P < 0.001).
Figure 29. The types and frequencies of targeted mutations induced in the progeny from the $N$-(dA-8-yl)-6-AC construct in HEK 293T cells and various polymerase knockout HEK293T cells. The data represent the mean and the standard deviation (of the total targeted MF) from 2-4 independent experiments. The statistical significance of the difference in targeted MFs between HEK 293T and TLS pols knockouts was calculated using two-tailed, unpaired Student’s t test. (*P < 0.05; **p < 0.005).

The Role of Specific TLS Polymerases in $N$-(dA-8-yl)-6-AC TLS Bypass

The results from TLS replication efficiency assay and DNA sequencing analysis (Figure 29) suggest that Pol η is strongly involved in bypassing $N$-(dA-8-yl)-6-AC DNA lesion in an error free manner. Neither TLS bypass efficiency nor mutation frequency differed significant from the wild type results when $N$-(dA-8-yl)-6-AC vector was replicated Pol ı KO cells. This suggests that Pol ı does not play a significant role in bypass of $N$-(dA-8-yl)-6-AC DNA lesion. We observed 4-fold decrease in mutation frequency when the plasmid is replicated in polymerases κ or ζ HEK293T KO cells. A*→T transversions were the main mutation induced by $N$-(dA-8-yl)-6-AC adduct in
HEK293T cell. However, in Pol κ KO cells A*→G transitions were induced at a higher frequency than A*→T transversions. This suggests that Pol κ in responsible for inducing a significant amount of A*→T transversions when N-(dA-8-yl)-6-AC vector is replicated in HEK293T cells. Furthermore, the decrease in mutation frequency in the absence of polymerases κ and ζ suggests that polymerases κ and ζ are strongly involve in the error-prone or mutagenic bypass of N-(dA-8-yl)-6-AC. Replication experiments in cells where κ and ζ are simultaneously depleted resulted in 10 fold decrease in mutation frequency suggesting that κ and ζ are cooperatively involved in the error-prone bypass of N-(dA-8-yl)-6-AC adduct.
4. 4 Conclusion

In conclusion, \(N-(dA-8-yl)-6-AC\) is mutagenic in \(E. coli\) and HEK293T cells. In \(E. coli\), \(N-(dA-8-yl)-6-AC\) DNA adduct induces \(~5\%\) mutant progeny in wild type cells. The induction of SOS response increases lesion bypass efficiency from \(~60\%\) (wild type cells) to \(~90\%\) in SOS induced cells and increased targeted mutation in progeny 3-fold. This can be attributed to increase expression of low fidelity TLS polymerases pol II, pol IV and pol V. The major type of targeted mutation was \(A^*\rightarrow G\) in both uninduced and SOS-induced cells. In contrast, in HEK293T cells \(N-(dA-8-yl)-6-AC\) DNA adduct induces \(~12\%\) progeny plasmid with mutations largely \(A^*\rightarrow T\) transversions. In HEK293T cells, polymerases \(\kappa\) and \(\zeta\) are cooperatively involved in the error-prone bypass of \(N-(dA-8-yl)-6-AC\) adduct while pol \(\eta\) in strongly involved in the error-free bypass. Thus, our study provided important new insight into cellular replication properties of \(N-(dA-8-yl)-6-AC\) lesion and defined the roles of TLS polymerases in bypassing this lesion in \(E. coli\) and human cells.
Chapter 5

Mutagenicity and Genotoxicity of Fapy.dG DNA Adduct in Human Cells
5. Abstract

Fapy·dG are formed in DNA by hydroxyl radical damage. In order to study replication bypass efficiency and mutation outcomes of Fapy.dG lesion in HEK293T cells, we constructed a single-stranded shuttle vector containing the lesion. Two plasmid vectors containing Fapy.dG adduct at the G1- or G3-positions of a part p53 sequence, 5’-AAC CG1G AG3G CCC-3’ containing mutation hotspot codon 248 (red) or codon 249 (green). Replication of vectors in human embryonic kidney HEK293T cells, showed that Fapy.dG DNA adduct is mutagenic inducing largely G*→T transversions. Furthermore Fapy.dG adduct in \textit{CG*G} sequence context (codon 248) induce mutations at ~50% greater frequency than \textit{AG*G} in codon 249. The roles of several translesion synthesis (TLS) replication polymerases in adduct bypass was also investigated via conducting replication experiments in the isogenic cells where individual TLS DNA polymerases were depleted by the CRISPR/Cas9 genome editing method. Mutational analyses in HEK293T KO cells are in progress.
5.1 Introduction

Oxidative DNA damage from reactive metabolites produced in cells can play important roles in aging and various diseases.\textsuperscript{96,97} Ionizing radiation generates many lesions in DNA that are formed by oxidative stress. It is now clear that oxidative DNA damage induces many different lesions in DNA, of these lesions 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) has received the most of attention.\textsuperscript{98} However, oxidative DNA damages also generates ring-opened formamidopyrimidine derivative, Fapy.dG. It is believed that 8-oxo-dG and Fapy.dG derives from a common radical intermediate (Scheme 8).\textsuperscript{99–102}

\textbf{Scheme 6.} Postulated hydroxyl radical mediated pathway to Fapy.dG and 8-oxo-dG

Fapy·dG DNA lesions is subject to repair by excision repair enzymes. Formamidopyrimidine glycosylase (fpg, MutM) is known to excise several oxidative damages such as Fapy.dG.\textsuperscript{101} The site-specific synthesis of Fapy.dG DNA adduct and incorporation in oligodeoxynucleotides have been accomplished.\textsuperscript{103,104} These advances in the synthesis of oligodeoxynucleotides containing

\[ \text{8-oxo-dG} \rightarrow \text{Fapy.dG} \]
Fapy·dG have enabled biological studies that investigate biological endpoints of Fapy·dG in cells and the role TLS polymerases in bypassing Fapy·dG adduct in cells. Kalam et. al. showed that that Fapy·dG is mutagenic when the modified vector containing Fapy·dG is replicated in simian kidney (COS-7) cells, inducing primarily targeted Fapy·G→T transversions. In the 5’-TGT sequence mutational frequency of Fapy·dG was ~30%, whereas in the 5’-TGA sequence it was ~8%. Using a similar approach, Pande et. al. reported that DNA polymerase λ is responsible for a significant portion of Fapy·dG induced G → T mutations in human cells, whereas it conducts predominantly error-free bypass of 8-oxo-dG.

\[
\begin{align*}
5'-\text{AAC CG*G AGG CCC-3'} & \\
5'-\text{AAC CGG AG*G CCC-3'} &
\end{align*}
\]

Figure 30. Oligonucleotide sequences from the p53 hotspots that include codons 248 and 249

In contrast, this work aims at determining the mutational signatures of Fapy·dG in interesting p53 hotspots sequences that include codons 248 and 249. The mutations in p53 gene in cancer are distinct and have been noted in several hot spots such as at codons 157, 175, 248, 249, and 273, which correspond to amino acids within the DNA binding domain of p53. In human breast cancer, p53 codon 248 is most frequently mutated. Nearly 50% of all colon cancers contain mutations at the three CpG hot spots at codons 175, 248, and 273. By contrast, in hepatocellular carcinoma, G → T transversion of the second G in codon 249 occurs frequently, in which the mutated G appears in a GpG sequence. Indeed, GpG sequences are hot spots for oxidative damage. Furthermore, oxidative damage of human fibroblasts with H₂O₂ and
FeCl₃ induced G:C → T:A transversions at p53 codons 249₁⁰⁹, which is also a susceptible site in DNA isolated from the liver tissues of Wilson’s disease patients₁¹⁰.

5. 2 Material and Methods

5. 2. 1 Synthesis of Fapy·dG containing oligonucleotides

The synthesis and characterization of Fapy·dG 12mers, 5’-AAC CG*G AGG CCC-3’ and 5’-AAC CGG AG*G CCC-3 were synthesized in the laboratory of Prof. Marc Greenberg and have been reported.₃₆,₉₈,₁₀⁴ The unmodified and complementary oligodeoxynucleotide sequences were purchased from Integrated DNA Technologies, Inc in Coralville, Iowa, USA.

5. 2. 2. Single stranded vector construction and replication studies.

Procedure for construction and characterization of pMS2 vectors containing a single Fapy·dG site are reported in section 4. 2. 2 of thesis.
5.3 Results and Discussion

To investigate the mutation outcome of Fapy.dG in p53 hotspot sequences, single-stranded pMS2 vectors bearing a single Fapy.dG adducted site were constructed with different modified oligodeoxynucleotides. Modified vectors were constructed with 5’-AAC CG\(^*\)G AGG CCC-3’ (dG\(_1\)-Fapy) where codon 248 is underlined and with 5’-AAC CGG AG\(^*\)G CCC-3 (dG\(_3\)-Fapy) where mutation hotspot codon 249 is underlined. These adduct containing oligodeoxynucleotides and unmodified control oligodeoxynucleotides were inserted into plasmids to construct site-specifically modified vectors using recombinant DNA technology. These vectors were replicated human embryonic kidney (HEK293T) cells. The resulting double stranded plasmid DNA was isolated and analyzed via oligonucleotide hybridization and DNA sequencing (Figure 30).

![Oligonucleotide Hybridization](image)

**Figure 31.** Progeny plasmid processing via oligonucleotide hybridization and DNA sequencing

Oligonucleotide hybridization and DNA sequencing showed that Fapy.dG is mutagenic in HEK293T cells, inducing largely G\(^*\)→T transversions. Furthermore Fapy.dG adduct in CG\(^*\)G sequence context (codon 248) induce mutations at ~50% greater frequency than AG\(^*\)G in codon
TLS replication bypass efficiencies of the Fapy.dG containing vector in HEK293T cells were \(~90\%\) and \(~88\%\) respectively for dG1-Fapy (codon 248) and dG3-Fapy (codon 249) sequences. Thus, bypass efficiency for Fapy.dG in G\(_1\) or G\(_3\) sequence were essentially the same in both sequence context.

Figure 32. (A) The bypass efficiencies of Fapy.dG constructs (G\(_1\) and G\(_3\)) in HEK293T cells. The data represent the means and standard errors of the mean of results from 3 independent replication experiments. (B) The frequencies of total targeted mutations induced in progeny from Fapy.dG construct in dG1-Fapy and dG3-Fapy.

DNA sequencing analysis showed that Fapy.dG in codon 248 induces \(~12\%\) mutant progeny and induces \(~5\%\) mutant progeny in codon 249. In both sequences the main mutation is G*→T.
transversions at the target site. In similar work from our lab, Pande et al. showed that Fapy.dG induces 10–22% mutations, predominantly G → T transversions, in human embryonic kidney 293T cells in four TG*N sequence contexts, where N = C, G, A, or T.85 This current study aims at investigating and defining the mutation signature of Fapy.dG in p53 mutation hotspots sequences realized from cancer patients.

5. 4 Conclusion

In conclusion, Fapy.dG is mutagenic in HEK293T cells inducing mainly G→T transversions. In the p53 sequence, the lesion is most mutagenic when located at G1 (codon 248). Nonetheless, Fapy.dG is also mutagenic when located at G3 position (codon 249).

5. 5 Future Direction

The effect of specific TLS replication proteins in bypassing Fapy.dG lesion in codon 248 and 249 p53 sequences will be assessed by performing TLS assay in CRISPR/Cas9 genome edited knockout human embryonic kidney cells (HEK293). TLS efficiency will be assessed with pol η, pol κ, pol ι, Rev 1 single knock out cells; followed by TLS assay in double/ triple knock out cells.
References


(16) Rundle, A. Carcinogen-DNA Adducts as a Biomarker for Cancer Risk. *Mutat. Res.* -


(23) Vom Brocke, J.; Krais, A.; Whibley, C.; Hollstein, M. C.; Schmeiser, H. H. The


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(64) Yagi, H.; Frank, H.; Seidel, A.; Jerina, D. M. Revised Assignment of Absolute Configuration of the Cis-and Trans-N 6-Deoxyadenosine Adducts at C 14 of (()-11,12r-Dihydroxy-13r,14r-Epoxy-11,12,13,14-Tetrahydrodibenzo[a,l]Pyrene by Stereoselective Synthesis. https://doi.org/10.1021/tx800268f.


(102) Candeias, L. P.; Steenken, S. Reaction of HO. with Guanine Derivatives in Aqueous


Appendix A: Chapter 2 Supporting Information – Spectral Data
Figure S1. $^1$H NMR spectrum of 8-bromo-2’-deoxyadenosine
Figure S2. $^{13}$C NMR spectrum of 8-bromo-2’-deoxyadenosine
Figure S3. MS spectrum of 8-bromo-2’-deoxyadenosine
Figure S4. $^1$H NMR spectrum of 8-bromo-3’, 5’-O-bis(tert-butyldimethylsilyl)-2’-deoxyadenosine (4)
**Figure S5.** MS spectrum of 8-bromo-3’, 5’-O-bis(tert-butyldimethylsilyl)-2’-deoxyadenosine (4)
Figure S6. $^1$H NMR spectrum of 8-bromo-3', 5'-O-bis(tert-butyldimethylsilyl)-N$^6$-dimethoxytrityl-2'-deoxyadenosine (5)
Figure S7. $^{13}$C NMR of 8-bromo-3’, 5’-O-bis(tert-butyldimethylsilyl)-N$^6$-dimethoxytrityl-2’-deoxyadenosine (5)
Figure S8. MS spectrum of 8-bromo-3’, 5’-O-bis(tert-butyldimethylsilyl)-N⁶-dimethoxytrityl-2’-deoxyadenosine (5)
Figure S9. $^1$H NMR spectrum of 8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-N$^6$-dimethoxytrityl-2'-deoxyadenosine (6b)
Figure S10. \(^1\)H NMR spectrum of 8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-\(N^6\)-dimethoxytrityl-2'-deoxyadenosine (6b)
Figure S11. MS spectrum of 8N-(6-aminochrysene)-3’,5’-O-bis(tert-butyldimethylsilyl)-N6-dimethoxytrityl-2’-deoxyadenosine (6b)
Figure S12. $^1$H NMR spectrum of 8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine (6a)
Figure S13. $^{13}$C NMR spectrum of 8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine (6a)
Figure S14. MS spectrum of 8N-(6-aminochrysene)-3',5'-O-bis(tert-butyldimethylsilyl)-2'-deoxyadenosine (6a)
Figure S15. $^1$H NMR spectrum of 8N-(6-aminochrysene)-2’-deoxyadenosine (3)
Figure S16. $^{13}$C NMR spectrum of 8N-(6-aminochrysene)-2’-deoxyadenosine (3)
Figure S17. $^1$H-$^1$H ROESY NMR spectrum of 8N-(6-aminochrysene)-2’-deoxyadenosine (3)
Figure S18. MS spectrum of 8N-(6-aminochrysene)-2’-deoxyadenosine (3)
Figure S19. $^1$H spectrum of $N^6$-Benzoyl-8N-(aminochrysene)-2'-deoxyadenosine (7)
Figure S20. $^{13}$C spectrum of $N^6$-Benzyol-8$N$-(aminochrysene)-2$'$-deoxyadenosine (7)
Figure S21. MS spectrum of $N^6$-Benzoyl-8$N$-(aminochrysene)-2'$-deoxyadenosine (7)
Figure S22. MS spectrum of $N^6$-Benzoyl-8N-(6-aminochrysene)-5'-$O$-dimethoxytrityl-2'-deoxyadenosine
Figure S23. $^{31}$P spectrum of $N^0$-Benzoyl-8N-(6-aminochrysene)-5'-O-dimethoxytrityl-2'-deoxyadenosine-3'-O-(cyanoethyl-N,N'-diisopropyl-phosphoramidite (8)
**Figure S24.** MS spectrum of $N^6$-Benzoyl-8N-(6-aminochrysene)-5'-O-dimethoxytrityl-2'-deoxyadenosine-3'-O-(cyanoethyl-N,N'-diisopropyl-phosphoramidite (8)
**Figure S25.** ESI-MS (negative mode) spectrum of \(N-(dA-8-yl)-6-\text{AC}\) modified 12-mer oligodeoxynucleotide

\[
5\'-\text{GTGCA}^*\text{TGTTTGT}-3'
\]

(M-3H)\(^3\): 1306.2352 m/z yields an overall MW of 3921.7274 Da
Figure S26. ESI-MS (negative mode) spectrum of $N$-(dA-8-yl)-6-AC modified 15-mer oligodeoxynucleotide

5'-GCCCTCAA*CAAGATG-3'

(M-4H)$^4$: 1195.2252 m/z yields an overall MW of 4784.9298 Da
Figure S27. 20% PAGE Gel of Pure N-(dA-8-yl)-6-AC modified 12-mer and 15-mer oligodeoxynucleotides
Appendix B: Chapter 4 Supporting Information
Table S1. Mutational frequency in wild type (-) SOS and (+) SOS *E. coli* (AB1157) cells

<table>
<thead>
<tr>
<th>Exp #</th>
<th>Total colonies screened</th>
<th>Total no. of mutants</th>
<th>MF (%)</th>
<th>% Survival</th>
<th>Targeted Mutations</th>
<th>Semi-Targeted Mutations [no. of mutants] (%)</th>
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</thead>
<tbody>
<tr>
<td>1 (-) SOS</td>
<td>149</td>
<td>9</td>
<td>6.0</td>
<td>65**</td>
<td>0</td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.68)</td>
<td>--</td>
</tr>
<tr>
<td>2 (-) SOS</td>
<td>98</td>
<td>3</td>
<td>3.0</td>
<td>62*</td>
<td>0</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>(2.04)</td>
<td>--</td>
</tr>
<tr>
<td>3 (-) SOS</td>
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<td>58***</td>
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<td>6</td>
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<td>(0.75)</td>
<td>(2.26)</td>
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<td>4 (-) SOS</td>
<td>16 (-) SOS</td>
<td>4 (+) SOS</td>
<td>1 (+) SOS</td>
<td>2 (+) SOS</td>
<td>(+) SOS TOTALS</td>
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<td>183</td>
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<td>8</td>
<td>35</td>
<td>25</td>
<td>13</td>
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<td>5.0</td>
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<td>13.6</td>
<td>10.23</td>
<td>12.2%</td>
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<td>3 (0.45%)</td>
<td>89***</td>
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<td>87</td>
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<td>4</td>
<td>1 (2.53)</td>
<td>16 (2.4%)</td>
<td>9 (4.92)</td>
<td>7 (3.82)</td>
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<td>16 (2.4%)</td>
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<td>7 (0.78)</td>
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<td>A1 or A*2 del [1] (0.6)</td>
<td>G1→ C [1] (0.54)</td>
<td>G3→ A [1] (0.54)</td>
<td>G3 del [1] (0.54)</td>
<td>C3T1 C4 → TT1A [1] (0.54)</td>
<td>5'C3T1 C4A1 del [2] (1.09)</td>
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*3 Plates Average; **3 – 6 Plates Average; ***6 – 12 Plates Average
**Table S2.** TLS efficiencies and mutational frequencies in HEK WT and KO cells

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<tr>
<th>Exp #</th>
<th># of Colonies Screened</th>
<th>Mutants</th>
<th>MF (%)</th>
<th>% Survival</th>
<th>Targeted Mutations</th>
<th>Semi-Targeted Mutations [# of mutants] (%)</th>
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<td>5</td>
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<td>14</td>
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<td>WT Totals</td>
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<td>60%</td>
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Replication in HEK 293T Pol Z KO Cells

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Replication in HEK 293T Pol I KO Cells

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<td>81*</td>
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Replication in HEK 293T Pol H KO Cells

Replication in HEK 293T Pol H/Z DKO Cells
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<th>32***</th>
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<th>0</th>
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<td>2</td>
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Replication in HEK 293T Pol H/K DKO Cells

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<th>C₄A₁ del [2] (0.8)</th>
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<td>25**</td>
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<td>0</td>
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<td>0</td>
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Replication in HEK 293T Pol K/Z KD Cells
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Appendix C: Chapter 5 Supporting Information
<table>
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<tr>
<th>Exp #</th>
<th>No. of colonies screened</th>
<th>Mutants</th>
<th>MF (%)</th>
<th>% Survival</th>
<th>Targeted Mutations</th>
<th>Semi-Targeted Mutations</th>
<th># of mutants</th>
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<td><strong>G1- Codon 248</strong></td>
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<td>33</td>
<td>13.5</td>
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<td><strong>87</strong></td>
<td><strong>12 %</strong></td>
<td><strong>90%</strong></td>
<td><strong>53</strong> [7.1 %]</td>
<td><strong>24</strong> [3.2 %]</td>
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<tr>
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**Note:** The numbers in brackets represent percentages.
Fapy.dG PAGE Gel

1-p53 CT 5’-GTG CGT GTT TGT
2-p53 CT 5’-GTG CGT GTT TGT
3-HY2169-1 5’-AAC CXG AGG CCC
4-HY2169-2 5’-AAC CGG AXG CCC
5-HY2169-3 5’-GTG CXT GTT TGT

X = Fapy\_dG