N-(Deoxyadenosine-8-YL)-4-Aminobiphenyl Adduct Formation in Vitro on TP53 EXON7 Genome: Quantitation by LC-ESI-MS/MS

Knoll
N-(DEOXYADENOSINE-8-YL)-4-AMINOBIPHENYL ADDUCT FORMATION
IN VITRO ON TP53 EXON7 GENOME: QUANTITATION BY LC-ESI-MS/MS

by

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Paul René Knoll
N-(DEOXYADENOSINE-8-YL)-4-AMINOBIPHENYL ADDUCT FORMATION IN VITRO ON TP53 EXON7 GENOME: QUANTITATION BY LC-ESI-MS/MS

Paul René Knoll, M.S.

Western Michigan University, 2008

The p53 protein has been documented to be mutated in more than 50% of late stage bladder cancers and tobacco causes 50% of all bladder tumors. The 4-aminobiphenyl (ABP) carcinogen comprises 5 ng per cigarette in the smoke and is a known bladder carcinogen. The research goal in the present study was to compare the reactivity of rat liver S-9 fraction activated 4-aminobiphenyl with the 45-mer sense strand of the TP53 exon7 gene to the respective anti-sense strand. The specific and sensitive technique of LC-tandem mass spectrometry (LC-MS/MS) was used for quantitation. The authentic standard of N-(adenine-8yl-)-4-aminobiphenyl (A-ABP) adduct was prepared by Buchwald-Hartwig coupling, used for MS tuning and LC-MS/MS calibration. The LC-MS/MS (n = 4, p = 0.10) results were 6 ± 3 and 10 ± 6 fmol on the column or reaction results of 5 ± 2 and 9 ± 4 N-(deoxyadenosine-8-yl)-4-aminobiphenyl (dA-ABP) adducts / 10^6 nucleotides in the oligo for the sense and anti-sense strands, respectively. There were 8 vs. 5 dA and 9 vs. 4 dG non-hydrogen-bonded purines available for the sense and anti-sense strands, respectively, in the computationally folded oligos. The decreased reactivity measured for the sense strand is corroborated by computationally derived ssDNA secondary structure results that suggest that the sense strand ratio of available reactive dA to dG loci is less when compared to the anti-sense strand.
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CHAPTER I

INTRODUCTION

Tobacco smoking is involved in 90% of the cases of lung cancer which is roughly 30% of the cancer cases in developed countries. Smoking is involved in other human cancers including: oral cavity, naso-, oro-, and hypopharynx, nasal cavity and paranasal sinuses, larynx, esophagus, stomach, pancreas, liver, kidney, ureter, urinary bladder, uterine cervix, and bone marrow (myeloid leukemia). 1 500 million of those alive today will die due to tobacco use. 2 There are 81 International Agency for Research on Cancer (IARC) classified carcinogens in cigarette “mainstream” smoke, 3 the smoke that is directly inhaled by the smoker. The organic compounds classified as IARC Group 1 carcinogens, i.e., known human carcinogens are: 4-aminobiphenyl, benzene, 2-napthylamine, vinyl chloride and ethylene oxide. 3 Historically occupational sources of urinary bladder cancer have included 2-napthylamine and benzidine used in the dye industry. The use of these compounds has been banned since 1950 and 1962, respectively. The 4-aminobiphenyl (ABP) non-protected commercial production was stopped in 1949 when increased bladder cancer was observed in workers. 4 The ABP carcinogen comprises 5 ng per cigarette in the smoke. 5 ABP may also be present in some permanent hair dyes due to batch to batch variations in the synthesis of the main colorant 1,4-phenylenediamine. 6 ABP has been shown to be a human bladder carcinogen and is now regulated. 7, 8
Tobacco causes 50% of all bladder tumors. ABP forms carcinogen-DNA adducts in urothelial cells. Compared to those who have never smoked, ex-smokers are 1.9 times as likely to have bladder cancer, and current smokers 3.3 times as likely. In an epidemiological review Silverman et al. summarized that smokers have two to three times the risk compared to non-smokers of developing bladder cancer and that avoiding cigarette smoking was the best way to prevent bladder cancer.

The “somatic mutation hypothesis” for cancer holds that cancer forms because of mutations on specific genes called “oncogenes and tumor suppressor genes.” The TP53 tumor suppressor gene has mutations in 5 to 80% of all types of human cancers. The p53 protein has mutations in 30% of bladder cancers overall and has been documented to be mutated in more than 50% of late stage bladder cancers. ABP is 50 times as mutagenic in single stranded DNA (ssDNA) compared to double stranded DNA (dsDNA). The N-(deoxyguanosin-8-yl)-ABP (dGuo-8yl-ABP) adduct and a few other adducts were found to be the result of smoking. The objective of this research project was to quantitate one of those other adducts, N-(deoxyadenosin-8yl)-ABP (dAdo-8yl-ABP) and to explore the relative potential of the adenine residues in each ssDNA strand to form ABP-adducts. The ssDNA substrates used for the in vitro analyses were the coding (sense) and the transcriptional (anti-sense) strands of the synthetic 45 nucleotide long (45-mer) TP53 exon 7 gene.
Function of p53 Tumor Suppressor Protein and Bladder Cancer

Overview

The p53 protein causes cell cycle arrest or apoptosis.\textsuperscript{21-23} It functions by inhibiting the cycle of abnormal, or stressed cells allowing damaged DNA to be repaired or by stopping growth permanently. Apoptosis, meaning programmed cell death, happens when the abnormality / stress continues or occurs when the cell is in replication. Another function of the p53 protein may be to inhibit angiogenesis and metastasis.\textsuperscript{22} Stress signals upstream to the p53 protein include: DNA damage, hypoxia, ribonucleotide triphosphate depletion, telomere damage, nitric oxide (NO), and oncogene activation.\textsuperscript{21,22} The p53 protein has been called the "guardian of the genome" because of these functions.\textsuperscript{21,24}

Domains of p53

The 393 amino acid residue p53 protein binds to DNA as a dimer of dimers, or a tetramer and is constitutively expressed in almost all cell types.\textsuperscript{13} The p53 protein either activates or represses transcription of a large number of genes.\textsuperscript{23} In the un-stressed cell the p53 levels remain relatively low due to rapid turnover.\textsuperscript{23,25} There is a constant
degradation of the p53 protein giving it a very short half-life.\textsuperscript{21} The $N$-terminus of the p53 protein where it interacts with target genes is blocked by the nuclear protein MDM2, the central regulator of p53.\textsuperscript{25} MDM2 functions as an E3 ubiquitin ligase marking p53 for monoubiquitination thus promoting p53 nuclear export and proteomic destruction in the cytosol.\textsuperscript{23,25} But MDM2 is itself a transcriptional target of p53 making an autoregulatory feedback loop between the two.

The p53 protein initiates growth arrest and/or apoptosis through upregulation of p53-mediated transcription functions. Post-translational modifications are the mediator of these changes. Modifications include phosphorylation by kinases on the $N$-terminus to regulate p53 stability and on the C-terminal Ser 392 to activate DNA binding and transcriptional function. Acetylation at C-terminal Lys 382 activates DNA binding and sumoylation at one of the p53 Lys residues enhances transcription.\textsuperscript{21} The C-terminus maintains the p53 protein in its latent form by physical interactions with parts of the $N$-terminus and/or the DNA binding domain.\textsuperscript{13}

The DNA binding domain of p53 binds to DNA consensus sequences of two copies of RRRCWWGYYY in which $R$ is a purine, $W$ is either A or T and $Y$ is a pyrimidine both separated by a 0-21 base pair spacer. There are 30-35 of these response sequences in the human genome.\textsuperscript{22} The main p53 contact in the minor groove with DNA is via Arg 248 and in the major groove to DNA are via Lys 120, Ser 241, Arg 273, Ala 276, Cys 277 and Arg 283. A zinc atom is held by Cys 176, His 179, Cys 238 and Cys 242 through chelation preventing the DNA binding domain from unfolding.\textsuperscript{13} 3-D modeling studies indicated that p53 bends the DNA toward the major groove at the pentamer junction allowing optimal binding in the minor groove.\textsuperscript{13}
The DNA binding domain is flanked by the proline-rich domain on its N-terminus and the oligomerization domain on its C-terminus. The proline-rich domain is necessary for p53 regulated apoptosis but not growth arrest. The oligomerization domain is where the four monomers associate to form the tetramer.  

**Mutations**

The DNA binding domain of *TP53*, exons 5 through 8, contains 90% of the known p53 mutations in human cancer and 83% of these are single nucleotide missense mutations. The two arginine codons 248 and 273 mentioned above are in direct contact with the DNA. Arginines 175, 249 and 282 provide stability to the DNA binding surface. Including glycine at codon 245, these account for 28% of the missense mutations in *TP53*. They are called hot spots. Mutated p53 fails to bind to the DNA response elements efficiently. Genetically inherited *TP53* mutations are associated with the rare Li-Fraumeni Syndrome which has the diagnostic criteria of early onset of cancer with both first, and another first- or second-degree relative with < 45 year old onset of cancer.

There are two types of base substitution mutational events that can take place in DNA: transversions and transitions. Transversions occur when a purine converts to the other purine or a pyrimidine to the pyrimidine (G ↔ A or C ↔ T). Transitions are when purine converts to pyrimidine or vice versa. (G ↔ C, G ↔ T, A ↔ C or A ↔ T). In the nucleotide sequence CpG, the cytosine is often methylated at the C-5 position. Without methylation the frequent deamination of cytosine produces uracil which can be repaired routinely by uracil glycosylases. But when cytosine is methylated
the 5-methyl cytosine (5mC) becomes thymine, un-repairable by the enzymes. This accounts for a 10 to 50 fold increase for the C → T transition over the other three transitions.\textsuperscript{14}

Table 1 is a curated list of the IARC database hot spot mutations in bladder cancer as of March 2007.

Table 1. Human Bladder Cancer Hotspots

Hotspots on exon7 in Italics


<table>
<thead>
<tr>
<th>Codon</th>
<th>Codon Mutation</th>
<th>Residue Mutation</th>
<th>Mutation Type</th>
<th>CpG</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>285</td>
<td>GAG → AAG</td>
<td>Glu → Lys</td>
<td>Transition</td>
<td>No</td>
<td>56</td>
</tr>
<tr>
<td>280</td>
<td>AGA → ACA</td>
<td>Arg → Thr</td>
<td>Transversion</td>
<td>No</td>
<td>35</td>
</tr>
<tr>
<td>248</td>
<td>CGG → CAG</td>
<td>Arg → Gln</td>
<td>Transition</td>
<td>Yes</td>
<td>30</td>
</tr>
<tr>
<td>175</td>
<td>CGC → CAC</td>
<td>Arg → His</td>
<td>Transition</td>
<td>Yes</td>
<td>27</td>
</tr>
<tr>
<td>273</td>
<td>CGT → CAT</td>
<td>Arg → His</td>
<td>Transition</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>280</td>
<td>AGA → AAA</td>
<td>Arg → Lys</td>
<td>Transition</td>
<td>No</td>
<td>22</td>
</tr>
<tr>
<td>248</td>
<td>CGG → TGG</td>
<td>Arg → Trp</td>
<td>Transition</td>
<td>Yes</td>
<td>21</td>
</tr>
<tr>
<td>220</td>
<td>TAT → TGT</td>
<td>Tyr → Cys</td>
<td>Transition</td>
<td>No</td>
<td>19</td>
</tr>
<tr>
<td>271</td>
<td>GAG → AAG</td>
<td>Glu → Lys</td>
<td>Transition</td>
<td>No</td>
<td>18</td>
</tr>
<tr>
<td>245</td>
<td>GGC → AGC</td>
<td>Gly → Ser</td>
<td>Transition</td>
<td>Yes</td>
<td>15</td>
</tr>
</tbody>
</table>

The TP 53 exon7 extends from codons 235 to 249.\textsuperscript{18} The p53 hot spot codons 245, 248 and 249 fall within this range. All of the exon7 bladder cancer point mutations listed in Table 1 were transitions. The mutations at codon 248 of exon7 were G-C → A-T and C-G → T-A transitions with 2.7 and 1.9 \% respectively, while codon 245 G-C → A-T transitions were 1.4 \% of the total number of mutations.\textsuperscript{14}

Another source stated that bladder cancer was dominated by G-C → A-T transitions and G-C → C-G transversions on TP53.\textsuperscript{28} In comparison the transversion
G-C → T-A accounted for one third of the mutations in lung cancers of smokers and was associated with the carcinogen benz[a]pyrene (B[a]P), the precursor of activated benz[a]pyrene diol epoxide (BPDE). In a study of 110 patients with bladder tumors 38% of the TP53 mutations in smokers were between codons 241 and 249 on exon7.

When one dimer of the p53 tetramer is mutant and the other wild-type, the entire heterotetramer becomes dominant negative. In this way heterozygotes lose p53 function. Without a functioning TP53 gene mutant cells that would normally undergo apoptosis will remain to increase the frequency of the mutation and thus tumors are not suppressed. Also p53 malfunction hinders transcription at target genes and thus response to DNA damage. When compared to BPDE formed mutations in the lung, bladder mutations caused by ABP are more evenly distributed along the gene. Bladder mutations occur at the CpG hotspots, common to other cancers, i.e. codons 175 and 248. But they also occur at non-CpG spots such as codons 280 and 285. According to Professor Soussi with bladder cancer it may not be the TP53 that is mutated, or on the other hand because the transitions are “localized predominately outside of the CpG dinucleotide suggesting a carcinogen is still involved”. The reasoning was that mutations at CpG spots are more likely due to spontaneous deamination of 5mC to allow the C → T transition, vide supra.

In murine test subjects for G C → T A transversions BPDE preferred 5mC on the 5' side of a guanine base adduct. N-OH-acetyl-ABP preferred A and T on the 5' side. For the same transversion A was preferred for both BPDE and N-OH-acetyl-ABP on the 3' side. The G-C → A-T transition predominant in p53 human bladder cancer was not observed in the study and was thus a likely cause for Professor Soussi’s equivocation.
Feng et al. indicated the chromatin structure surrounding the p53 genome within a cell was not to be a factor in the distribution pattern of ABP-DNA adducts when comparing naked genomic DNA reacted with N-OH-ABP and bladder cells with N-OH-acetyl-ABP. The pattern was determined by the "binding specificity" of N-OH-ABP and the "human genomic content." In exon 8, ABP-DNA adducts were found clustered around codons 280 to 285, with codon 280 giving the highest level of adducts (compare to Table 1). This region contains no CpG sites, except codon 282. ABP adduct formation was barely detected at codon 273, a CpG site and a major mutational hotspot in many human cancers including bladder cancer.

Metabolic Pathways for 4-Aminobiphenyl

Overview

The arylamine 4-aminobiphenyl (ABP) in cigarette smoke, or from other sources is activated in the liver by cytochrome P450 1A2 to the hydroxyl amine N-hydroxyl-4-aminobiphenyl (N-OH-ABP). In red blood cells (RBC) N-OH-ABP, via a 4-nitrosobiphenyl intermediate binds with hemoglobin's sulfhydryl groups of cysteine to form the hemoglobin adduct ABP-Hb. The ABP-Hb adducts persist for up to 4 months throughout the lifetime of the RBC. The ABP-Hb adduct is a biomarker of smoking exposure but is not in the metabolic pathway towards bladder cancer; rather it is a detoxification pathway.

When these ABP-Hb adducts were detected they were statistically related to bladder cancer risk. Smokers had higher levels of these Hb adducts than non-smokers,
the ABP-Hb level could be used to determine the recent smoking history of a patient. Conversely other studies indicated ABP-Hb adducts though in themselves related to tobacco smoke exposure were only weakly associated with bladder cancer. The correlation to bladder cancer was stronger in women but still only weakly associated. Kadlubar et al. and Babu et al. cited others that show up to 5 to 10% of the ABP given to rats ended up bound to Hb.

Jones and Sabbioni performed a series of LC-MS/MS experiments related to ABP. In vivo they found that rats dosed with ABP had both N-(deoxyguanosin-8-yl)-ABP (dGuo-8yl-ABP) and N-(deoxyadenosin-8-yl)-ABP (dAdo-8yl-ABP), yet quantified only dGuo-8yl-ABP. But in vitro, reacting N-OH-ABP with calf thymus DNA (ct-DNA), the nucleophilic deoxyadenosine produced multiple products not just at the C8 configuration. The majority of the literature described the major DNA adduct formed as dGuo-8yl-ABP comprising 70% of the adducts. 20% of the adducts formed were described as either N-(deoxyguanosin-N2-yl) ABP (dGuo-N2-ABP) or dAdo-8yl-ABP. Yet another citation listed dGuo-8yl-ABP as 80% and dAdo-8yl-ABP as 15% with an in vitro reaction with ct-DNA. When reacting N-OH-[3H]ABP with calf thymus three adducts were observed by the 32P-postlabeling technique. The major adduct was dGuo-8yl-ABP but the other spots were not characterized. Other manuscripts discussed the existence of the minor deoxyadenosine adduct as well.

**Metabolism of 4-Aminobiphenyl**

In humans N-acetyltransferases 1 and 2 (NAT1 and NAT2) perform N-acetylation of ABP, O-acetylation on hydroxyl groups of the P450 1A2 hydroxylated product N-OH-
ABP and \textit{N,O}-transacetylation.\textsuperscript{39-41} NAT2 was cited as found primarily in the liver and intestinal epithelium whereas NAT1 was found throughout the human body.\textsuperscript{40} The P450 1A2 activity (\textit{N}-hydroxylation) was in competition with the detoxification of ABP by NAT2.\textsuperscript{32} NAT2 and NAT1 both \textit{O}-acetylated the P450 1A2 product \textit{N}-\textit{OH}-ABP to \textit{N}-acetoxy-ABP (\textit{N}-OAc-ABP).

The \textit{N}-\textit{OH}-ABP, and to a lesser extent ABP reacted with glucuronic acid in the liver to form their glucuronides (see Figure 1). In the hepatic microsomal fraction glucuronides formed.\textsuperscript{34} The \textit{N}-OH-ABP (and ABP) glucuronides travel in the blood to be passed from the body in urine. But in the mildly acidic urine of pH 5.5 they hydrolyze back to the unconjugated amine or hydroxylamine.\textsuperscript{34} These amines were presumed to be then metabolized by \textit{O}-acetyltransferases and peroxidases in the bladder epithelium to form adducts with DNA.\textsuperscript{34} The half life of the ABP glucuronide in the bladder was 10.5 minutes at pH 5.5. The half life of the \textit{N}-OH-ABP glucuronide was 32 minutes, whereas the \textit{N}-acetyl-\textit{N}-\textit{OH}-ABP glucuronide under the same conditions had a half life of 55 minutes.\textsuperscript{34} This again indicated that NAT acetylation metabolism tended more towards detoxification; the latter compound being more likely to pass in the urine and not dissociate before urination. Acidic urine played some part in the formation of bladder cancer, but frequency of urination played a greater role in a computer model. When urination increased from every 8 hours to "continuously", the amount of ABP-DNA adducts decreased 17-fold.\textsuperscript{33}

In their study Tsuneoka \textit{et al.}, on finding that CYP1A2 knock out mice had more DNA adducts than CYP1A2 wild-type mice came to the conclusion that the P450 1A2 did not activate ABP.\textsuperscript{48} Their resolution to this apparent contradiction with the bulk of
Figure 1. 4-Aminobiphenyl Metabolism in the Human

NAT2* slow acetylation pathway in double arrow above is a detoxification pathway. Dotted pathways determined by Babu et al. occurred in human liver microsomal fractions but not human liver slices. Burger and Swaminathan et al. both reported the existence of dG-N2-4-AABP.

Sources: Kadlubar 1987; Lasko 1988; Bartsch 1993; Meyer 1994; Skipper 1994; Babu 1996; Burger 2001; Swaminathan 2002; Falvey 2004; Nakajima et al. 2006;
the literature was that other enzymes metabolically activate ABP namely by the conjugation with UDP-glucuronic acid by UDP-glucuronide transferase (UGT). Murine P450 1A2 is peculiar in that, unlike in humans and rats it was not the only P450 enzyme to activate ABP to the hydroxylamine.\textsuperscript{49} A somewhat related finding was that in an Asian human population CYP1A2 status was related to bladder cancer risk.\textsuperscript{50} Therefore studies with mice to determine a human parallel for ABP metabolism were not recommended.

\textbf{Nitrenium Cation Intermediate}

The labile nitrenium cation forms when the N-OAc-ABP sheds its acetate anion. It acts as a potent electrophile that is then attacked by nucleophilic sites in DNA.\textsuperscript{41,46} The purine C-8 of guanosine in the bladder urothelial lining is the most prevalent reaction site. Adenine is 10\% as reactive as guanine.\textsuperscript{46} The mechanism is either of direct electrophilic substitution at C-8 or an initial adduct with N-7 and then migration to the C-8 position.\textsuperscript{46} Falvey noted that aryl nitrenium ions formed C-8 adducts with free bases and nucleosides of guanine, but in DNA the N-7 and O-6 adducts were more prevalent\textsuperscript{46} which may explain Jones and Sabbioni’s observation of a variety of adducts (\textit{vide supra}) yet contradicts the evidence that the dGuo-8yl-ABP is the most prevalent ABP adduct.\textsuperscript{36,38,51} Falvey himself presented an N-7 to C-8 migration mechanism for the ABP-nitrenium ion as did Guengerich \textit{et al.} to explain the formation of the C-8 adduct.\textsuperscript{46,52} Falvey also stated in contradiction that the migration mechanism did not occur in DNA.\textsuperscript{46}
Other Pathways Related to Bladder Cancer Research

Kadlubar showed that 10% of the DNA adducts in canine urothelial walls were from the peroxidation of ABP by prostaglandin H-synthase (PHS). Adducts formed from PHS peroxidation were different than those from P450 N-oxidation. The remaining 90% of the adducts were from N-OH-ABP. Burger suggested other minor adduct formation by way of reactive oxygen species formed by peroxidases and arylamidonium ions. The resulting adduct was thought to be 3-(deoxyguanosin-N2-yl)-4-acetylamino-3-biphenyl (see structure in box in Figure 1 in the upper right corner) at less than 5% of the major adduct dGuo-8yl-ABP.

N-OH-ABP was known to react in vitro with DNA to form DNA adducts and was used in studies to simulate the enzymatic pathways. But because of its excessive reactivity it tended to react indiscriminately. Yet in the work of Jones and Sabbioni it was necessary to extract excess N-OH-ABP prior to further analyses indicating its persistence. Verghis et al. used N-OAc-ABP, the metabolically occurring intermediate in their study. Besaratinia used N-OH-4-acetylaminoazobiphenyl (N-OH-AABP). The P450 enzymes associated with genes CYP2A6 and CYP2E1 RsaI had no correlation with urothelial cancer risk in a Japanese population. In another study, polymorphism in CYP2E1 was not correlated to bladder cancer. CYP2E1 does take part in the metabolism of ethanol and acetaminophen, the formation of free radicals from peroxides and the activation of toxic chemicals like CCl4 and N-nitrosamines. The GSTMnull genotype was associated with a higher risk in bladder cancer regardless of smoking or detoxification of tobacco smoke.
CHAPTER III

CYTOCHROME P450 MECHANISM RESEARCH

Introduction

P450 enzymes are a family of heme-containing proteins. They are named for the 450 nm absorption of their carbon monoxide adducts, therefore are called pigment-450. Hemoglobin, typical of other heme proteins, absorbs in the presence of CO at 420 nm. The cause of this red-shift in absorbance of P450s is due to the fifth proximal axial ligand, a thiolate ligand from cysteine whereas in hemoglobin the fifth ligand is histidine. This thiolate ligand donates electron density to the heme iron.

The chemical reactions performed by P450s are: nucleophilic oxidation, incorporation of OH, incorporation of O and/or abstraction of H. Sligar called P450s a "blowtorch". Sligar meant that P450s can accomplish difficult reactions under neutral pH, mild conditions and in aqueous media. P450s are found in mammalian tissues, insects, plants, yeasts and bacteria.

The nomenclature of human P450s is defined by the Human Cytochrome P450 Allele Nomenclature Committee, as such: the first number after CYP designates the family, the letter the subfamily and the second number the particular protein. Those in a family have > 40 % sequence homology; those in a subfamily have > 55 % homology. The eight human P450 families named on the allele nomenclature website are: CYP1, CYP2, CYP3, CYP4, CYP5, then CYP8, CYP19 and CYP21. In this chapter CYP is
used to name the human counterpart of other P450s, but in the literature CYP and P450 are generic terms and used interchangeably.63

Among highly conserved features throughout all P450s are the pair of Thr and carboxylate residues: Thr 321 / Thr 319 / Thr 252 and Asp 320 / Glu 318 / Asp 251, the codons for human CYP1A2 / Saccharomyces cerevisiae P450 1A2 / Pseudomonas putida P450CAM respectively.64 The diversity of the P450 enzymes lie in the delivery system used to supply electrons to the P450 active site.65 P450CAM found in P. putida which 5-hydroxylates camphor, uses electrons from NADH through the flavoprotein putidaredoxin reductase and the iron sulfur protein putidaredoxin.66 Microsomal human CYP1A2 from the endoplasmic reticulum uses membrane bound NADPH-P450 reductase with NADPH as the electron source.67

The P450 1A2 catalyzes the N-hydroxylation of aromatic and heterocyclic amines,52 the first step in the metabolic activation of these carcinogens.68 The P450 1A2 is liver constitutive.69 P450 1A2 was the P450 of interest in this research as it catalyzes the activation of 4-aminobiphenyl.31 The CYP1A2 is the human P450 counterpart that catalyzes this reaction and is the dominant enzyme in the N-hydroxylation of arylamines and heterocyclic amines.52,63 The CYP1A2 enzyme catalyzes carcinogen activation, estradiol hydroxylation70 and metabolic clearance of caffeine, melatonin, and a number of marketed medications.63
Studies on P450 1A2 Compared to P450\textsubscript{CAM}

Before the most recent release of the structure of human CYP1A2 by X-ray diffraction crystallography\textsuperscript{63} no P450 1A2 structure had been determined. The active site structure of rat liver P450 1A2 was probed instead with phenyldiazone and biphenylhydrazine to form adducts with the pyrrole rings of the cytochrome heme.\textsuperscript{69} The structure of P450\textsubscript{CAM} on the other hand was known at that time. Thr 252 and Asp 251 of

![Figure 2. \textit{P. putida} P450\textsubscript{CAM} Protein Structure](source: PDB ID: 1DZ6, Schlichting \textit{et al.} The Catalytic Pathway of Cytochrome P450\textsubscript{CAM} at Atomic Resolution, \textit{Science} 2000, 287, 1615-1622)
P450\textsubscript{CAM} with side chains on the I-helix (the shaded α helix in the center of the lower right domain in Figure 2) were known to be associated with the active site. Tuck \textit{et al.} set out to show how variations of the side chains reflected changes in the ratios of adducts with the above mentioned phenyl probes.

Tuck \textit{et al.} accomplished this through mutations of the respective rat liver P450 1A2 Thr 319 and Glu 318 residues\textsuperscript{69}. The P450 1A2 was observed to give pyrrole ring A adducts through a phenyl probe when Thr 319 was mutated. These observations were consistent with those found with Thr 252 mutations of P450\textsubscript{CAM} but to a greater extent. Glu 318 mutations in the mammalian P450 caused little change in adducts. The Asp 251 of P450\textsubscript{CAM} sits in proximity to the heme group but with the intervening Thr 252. Therefore since Glu 318 mutation, the P450\textsubscript{CAM} Asp 251 counterpart produced no phenyl adducts it was concluded to be further away from the pyrrole rings in P450 1A2 as well\textsuperscript{69}.

Another study performed on \textit{S. cerevisiae} P450 1A2 showed that mutating Glu 318 and Thr 319 led to changes in dissociation constant, \(K_d\) values for \(R\) and \(S\)-enantiomeric axial ligands when measured spectroscopically\textsuperscript{64}. The inference was taken that Glu 318 and Thr 319 are involved with ligand binding to the heme group.

The Structure of Human CYP1A2

The protein structure 2HI4 of human CYP1A2 has been released in the Protein Data Bank (PDB)\textsuperscript{63}. For the structure see Figure 3. Note the similarity of the α-helix, discussed by Tuck \textit{et al.}\textsuperscript{69} named the I-helix shaded in the center of the structure compared to that of P450\textsubscript{CAM} in Figure 2. This human CYP1A2 protein was expressed in \textit{Escherichia coli} for crystallization.
Figure 3. Human CYP1A2 Protein Structure

Prepared with Swiss-Pdb Viewer $^{71}$

Source: PDB ID: 2H14, Sansen et al., Adaptations for the Oxidation of Polycyclic Aromatic Hydrocarbons Exhibited by the Structure of Human P450 1A2, J. Biol. Chem. 2007 282(19) 14348-14355

Note the N-terminus in the Swiss PDB Deep View started at Arg 34, not Met 1. The CYP1A2 protein was expressed for residues 26 to 515 and preceded by Met and Ala. Additionally nine residues at the N-terminus and six at the C-terminus could not be traced in the electron density maps of the synchrotron. $^{63}$ The N-terminus is not necessary for catalytic activity, $^{59}$ but gives it its transmembrane binding character. The removal of the N-terminus of P450 1A2 in the past has not made it soluble enough for crystallization. $^{67}$ Uniform aqueous or organic solubility is the quality that allows a protein to be
crystallized. P450\textsubscript{CAM} is soluble in aqueous solution.\textsuperscript{72} Sansen \textit{et al.} were able to solublize the CYP1A2 protein by expressing it with the chaperones GroEL and GroES.\textsuperscript{63} The substrate in Figure 3 represented in stick figures in black was the polyaromatic hydrocarbon \(\alpha\)-napthoflavone and is not to be confused with the iron containing protoporphyrin IX heme which lies perpendicular to the substrate in the same plane of the viewer's perspective.\textsuperscript{63}

Sansen \textit{et al.} described the CYP1A2 active site architecture as being flat and uniformly narrow, well suited for planar compounds such as those mentioned in the Introduction of this chapter.\textsuperscript{63} The comparison was made with the CYP2A6 active site due to the similar shape to that of the CYP1A2 active site. But the CYP2A6 active site is 50\% larger. CYP2A6 also oxidizes small planar molecules.\textsuperscript{63}

**Kinetic Studies on CYP1A2**

In human’s CYP1A2 substrate \(O\)-dealkylation of 7-ethoxycoumarin, the kinetic isotope effect of \(1,1\)-\(d_2\)-ethyl substrates indicated that C-H bond-breaking was the rate limiting step and not the production of the reactive enzyme-oxygen complex in both purified P450s and human liver microsomes.\textsuperscript{73} Another product of the CYP1A2 reaction was the result of 3-hydroxylation. Only the dealkylation was studied but in order to determine if hydroxylation C-H bond-breaking was rate limiting, a deuterated 7-ethoxycoumarin at the 3-position would have to have been employed to compare directly to the P450\textsubscript{CAM} mechanism of hydroxylation.

In another kinetic isotope effect (KIE) hydroxylation study rabbit P450 1A2 was investigated.\textsuperscript{74} The substrates for P450 hydroxylation were 4-nitroanisole, 4-
cyanoanisole, 1,4-dimethoxybenzene and methacetin. The deuterium KIE ranged from 1.9 ± 0.1 for intermolecular competitive to 14.9 ± 2.1 for intermolecular noncompetitive. The latter value was high, but KIE values of > 10 in P450 reactions are frequent. In mammalian P450 1A2 bond-breaking of C-H was rate limiting. In P450CAM when camphor was the substrate C-H bond-breaking was not the rate limiting step. Only when C-H bond-breaking became a factor with modified rapidly oxidized substrates was it a factor. Mammalian P450s in general had slower KIE rates than bacterial P450s in which quantum mechanical tunneling might occur. Guengerich et al. concluded C-H bond breaking was the rate limiting step in rabbit P450 1A2 and partially rate-limiting in many mammalian P450s including human CYP1A2.74
CHAPTER V

MATERIALS AND METHODS

Chemicals

Aniline, 4-aminobiphenyl (ABP), and 8-bromoadenosine were purchased from Sigma-Aldrich (St. Louis, MO). Tris(dibenzylideneacetone)dipalladium(0) (Pd$_2$(dba)$_3$) was purchased from Sigma-Aldrich (Milwaukee, WI). Sodium tert-butoxide and tert-butyldimethylchlorosilane were purchased from Sigma-Aldrich (Allentown, PA). (S)-(−)-2,2′-Bis(diphenylphosphino)-1,1′-binaphthyl (S-BINAP) was obtained from ACROS Organics (Fair Lawn, NJ). US Filter Pure-LabPlus filtered H$_2$O was used. The filtered H$_2$O had a minimum 18 MΩ resistance. The Sprague-Dawley rat liver S-9 fraction from male rats contained\(^{75}\) 0.197 nmol / mg protein Cytochrome P450 was obtained from XenoTech, LLC (Lenexa, KS) and stored at -80° C until the time of use. The NADPH generating system components were D-glucose 6-phosphate disodium hydrate (G6P), β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP$^+$) and 228 units / mg solid, with 1.2 mg solid glucose-6-phosphate dehydrogenase (G6PDH) from S. cerevisiae; all obtained from Sigma-Aldrich (St. Louis, MO). A unit G6PDH converted 1 µmol G6P to 6-phospho-gluconate in 1 minute per Sigma-Aldrich specifications.

Caffeine and paraxanthine were obtained from Sigma-Aldrich (St. Louis, MO). The 45-mer TP53 exon7 coding (sense) and transcriptional (anti-sense) single stranded DNA (ssDNA) were synthesized for these experiments by the Michigan State University-
Macromolecular Structure Facility (East Lansing, MI). The sequences are drawn in Equations 1 and 2. The molar absorptivity coefficient equations were provided from the ssDNA supplier MSU-MSF.

**Equation 1. Sense Strand Sequence and Molar Absorptivity Coefficient**

\[
AAC\ TAC\ ATG\ TGT\ AAC\ AGT\ TCC\ TGC\ ATG\ GGC\ GGC\ ATG\ AAC\ CGG\ AGG
\]

\[
\varepsilon_{260} = \left[ (12A \times 15,480) + (14G \times 11,760) + (10C \times 7,340) + (9T \times 8,850) \right] \times 0.89 = 503,450 \times 0.89 = 448,070.5 \text{ (M cm}^{-1})
\]

**Equation 2. Anti-Sense Strand Sequence and Molar Absorptivity Coefficient**

\[
CCT\ CCG\ GTT\ CAT\ GCC\ GCC\ CAT\ GCA\ GGA\ ACT\ GTT\ ACA\ CAT\ GTA\ GTT
\]

\[
\varepsilon_{260} = \left[ (9A \times 15,480) + (10G \times 11,760) + (14C \times 7,340) + (12T \times 8,850) \right] \times 0.89 = 465,880 \times 0.89 = 414,633.2 \text{ (M cm}^{-1})
\]

**Reagents**

The buffer for the DispoEq reactions consisted of 50 mM potassium phosphate pH 7.4, 3 mM MgCl₂ and 1 mM EDTA designated as KPhos. KPhos was prepared in 2x concentration to be diluted to 1x by the other reagents prepared in 18 MΩ H₂O. It must be noted that in order to autoclave the KPhos to sterilize the solution for preservation and to remove DNases each component of the buffer was prepared separately, autoclaved, combined and brought to the mark, in this case of 200mL. When combinations of components were combined prior to autoclaving a precipitate formed and the buffer was useless. The EDTA was added to the DNA to minimize the Fenton Reaction formation of OH radicals by Fe²⁺.
The NADPH generating system (G.S.) purpose was to provide the reducing equivalents for the P450 in the S-9 fraction. The G.S. ratios used were approximations of those recommended by the S-9 supplier XenoTech, LLC\textsuperscript{76} namely: 1mM NADP\textsuperscript{+}, 5 mM G6P and 1 unit G6PDH (the G6PDH was prepared in 50% v/v glycerol). (The initially advised G.S. ratios were 0.5 mM NADP\textsuperscript{+}, 10 mM G6P and 0.5 unit G6PDH but were abandoned.\textsuperscript{77} When the UV spectrophotometer was discovered as the source of difficulties instead, the original ratios were not resumed).

**Equipment**

A Mettler-Toledo 3-place balance (Columbus, OH) accurate to 1 mg was used for weighing reagents. A Sartorius CP2P microbalance (Göttingen, Germany) was used to weigh the Adn-8yl-ABP adduct standard. At the maximum 2.1 g weight capacity of the microbalance it had an accuracy of ± 0.005 mg. The infusion for electrospray (ES) mass spectrometry was performed with a Harvard Apparatus (Holliston, MA) geared syringe pump calibrated to a 250 µL syringe to deliver 10 µL per minute. The liquid chromatography tandem mass spectrometer (LC-MS/MS) pump and autosampler were a Waters Alliance 2695 Separations Module. The MS had a Z-spray electrospray ionization (ESI) interface to the Micromass Quattro II MS equipped with MassLynks 3.2 software (Milford, MA). For LC-UV-MS/MS method development a Waters 2487 UV/Vis detector was in-line just ahead of the ESI source. The LC-MS/MS column was a YMC™ Phenyl 2 x 150 mm (5 µm, 120 Å pore size) column. All tubing for method development was 0.010 inches inner diameter (i.d.). The PEEK tubing after the LC column was changed for the final analyses to that of 0.004 inches i.d. A Shimadzu
Prominence LC-UV system (Columbia, MD) with a Waters Xterra-MS C-18 2.1 x 50 mm column with 3.5 µm particle size (Milford, MA) was used for the DispoEq and S-9 fraction system characterizations with caffeine / paraxanthine. The DispoEquilibrium DIALYZERS™ (DispoEq) for ABP activation reactions were obtained from Harvard Apparatus (Holliston, MA). The final UV spectrophotometer used for ssDNA quantitation was a Cary-14/OLIS (Bogart, GA). Ultra-micro and semi-micro disposable BRAND UV-cuvettes (Wertheim, Germany) with 70 – 850 µL and 1.5 – 3.0 mL fill volume range used for ssDNA quantitation and G.S. activity checks respectively. Silver Shield gloves impervious to chlorinated solvents were obtained from Seneca Research (Penn Yan, NY). A protective lab coat and latex gloves were worn when handling ABP and ABP adduct materials.

Synthesis of Adduct Standard

Protection of 8-Bromoadenosine by Silylation

The protected tert-butyldimethylsilyl-8-bromoadenosine substrate for the subsequent coupling reaction was prepared as described in the literature by Schoffers et al. using an oxygen- and moisture-free Schlenk-line technique.78

A 105°C oven dried 2-neck 50 mL round bottom flask and condenser were cooled under vacuum. The 8-bromoadenosine (600 mg, 1.74 mmol) and imidazole (946 mg, 13.92 mmol) were placed in the flask. Dimethylformamide (10 mL) and tert-butyldimethylchlorosilane (1050 mg, 6.96 mmol) were added. The flask was sealed with a glass stopper, put under positive argon pressure and allowed to stir 6 days at room
Reaction 1. Protection Reaction of 8-Bromoadenosine

temperature. The product was purified by flash column chromatography on 30 mL dry-packed silica conditioned with 3:1 hexanes/ethyl acetate into 10 cm glass screw top culture tubes. One row of spots eluted on thin layer chromatography (TLC) at Rf of 0.26. Tubes 4 ➔ 16 were taken and combined. The final product was characterized by $^1$H-NMR Figure 5.

Coupling Reaction of ABP and Silylated 8-Bromoadenosine

The Buchwald-Hartwig coupling reaction was performed as outlined in the literature by Schoffers et al.\textsuperscript{78} Aniline was used as the less toxic arylamine before the final coupling with ABP. A Schlenk-line was used to exclude oxygen and moisture from the reaction flask. A 50 mL 2-neck round bottom flask and condenser were heated in a 105° C oven and cooled under vacuum with the ground glass fittings (with the exception of the glass stopper) wrapped with Parafilm\textsuperscript{®}. The 5 mL glass syringe was placed in a desiccator to cool. The dry ingredients were passed through the glass stoppered side neck sequentially in this order: silylated 8-bromoadenosine (40 mg, 0.06 mmol), sodium tert-
butoxide (9 mg, 0.094 mmol), Pd_{2}(dba)_{3} (17 mg), S-BINAP (18 mg) and 4-aminobiphenyl (31 mg, 0.18 mmol). The S-enantiomer of BINAP was chosen.

Reaction 2. Buchwald-Hartwig Coupling Reaction

because it had provided a better yield. A rubber septum was positioned to replace the glass stopper and the system was alternately vacuumed and purged with argon three times, ending with the argon. Anhydrous toluene (5 mL) was injected through the septum to start the reaction. A preheated silicone oil bath (80 °C) was placed under the flask and the reaction was left stirring under positive argon pressure for 24 hours.

At 24 hours the co-spot TLC method with 5 % methanol / chloroform eluent was used to determine if the reaction had gone to completion. The product had no spot at the R_{f} values of the starting material and the reaction was considered to have been complete. A preliminary $^1$H-NMR spectrum was taken and gave the characteristic terminal proton ABP doublet signal at 6.19 ppm. (Free ABP has a signal at 6.6 ppm for the same proton).

Flash column chromatography was performed with 30 mL silica slurry packed with 1 % methanol / chloroform. The product was found to elute in the colored band. TLC was performed with 5 % methanol / chloroform eluent. The product was in the
tubes with the $R_f$ 0.24 and 0.28 spots. See Figure 6 for the final $^1$H-NMR spectrum and Table 5 for proton assignments.

**Hydrolysis and Purification of Adn-8yl-ABP**

The method of hydrolysis and purification of adenosine adducted with 2-aminofluorene described by Olsen in his dissertation was used here with adaptation to yield the $N$-(adenine-8-yl)-4-aminobiphenyl (Adn-8yl-ABP) adduct. Silylated $N$-(adenosine-8-yl)-4-aminobiphenyl (3 mg) was dissolved in two aliquots of absolute ethanol (750 µL) and acidified with another two aliquots 1.0 N HCl (750 µL) in a 4 mL vial. The vial was heated 1 hr in a H$_2$O bath (75° C). The hydrolyzed adduct was not neutralized but kept acidic prior to solid phase extraction (SPE) to prevent base catalyzed hydrolysis of the arylamine-purine bond. A Grace-Pure C-18 Low 500 mg bed size 3 mL SPE cartridge was conditioned with 3 aliquots, 100 % acetonitrile and then with 4 aliquots, 10 % acetonitrile. The sample was loaded onto the conditioned SPE cartridge and the SPE washed 4 times with the conditioning solvent. The cartridge was vacuumed to dryness 30 seconds and 5 separate aliquots of eluent were collected with 60 % acetonitrile. The 100 µL portion from each aliquot eluent collected was diluted 10 fold with 60 % acetonitrile and taken to infusion electrospray MS (ES-MS) for characterization. A drop formic acid was added to each sample vial to protonate the analyte for positive mode ES-MS. See Figure 7 for the relative composition of the 5 aliquots. (Use of a latex glove during SPE was found imperative because the Adn-8yl-ABP solution caused delayed sensitive skin irritation).
A contamination (and likely a decomposition) peak had been observed earlier at 260 nm on LC-UV\textsubscript{260/310}-MS/MS characterization of the sample after its being dried down. Precautions were taken to shield the adduct standard from excessive heat, light and oxygen and the oven temperature was reduced from 80° to 45° C. The contents of aliquots 1 and 2 were dried down separately with a nitrogen stream in actinic vials and they were placed in beakers covered with latex gloves filled with argon gas and dried for 16.5 hr in the oven.

Although chloroform was the solvent used consistently to this point, it was observed that the hydrolyzed adduct was only very minimally soluble in chloroform. See the LogP values in Table 2. Deuterated methanol was finally used and with success to solvate the adduct standard. See Figure 8 for the \textsuperscript{1}H-NMR spectrum in deuterated methanol of the adduct standard. Figure 9 is the triplicate ES-MS scan of the standard and Figure 10 is the LC-UV\textsubscript{260/310}-MS/MS chromatogram of the standard.

A clear 2 mL LC vial was tared repetitively to obtain an average weight. The contents of the NMR tube were transferred to the vial and dried down under a nitrogen stream. Once dry, the vial was filled with argon and capped until weighed. The contents of the weighed vial were rinsed with 100 % methanol into a 50 mL volumetric flask containing 30 % methanol. The appropriate amount H\textsubscript{2}O was added to give 30 % methanol and then the flask was filled to the mark. This procedure had been followed previously with a chloroform extracted adduct standard but because of poor solubility and the use of a 100 mL volumetric flask a lower concentration check standard was obtained.
Table 2. LogP Values of Adduct Standard Compared to Solvents

Source: The predicted value of LogP was obtained using the ACD/I-Lab Web service (ACD/LogP 8.02)

<table>
<thead>
<tr>
<th>Solvent / Compound</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>2.86 ± 0.41</td>
</tr>
<tr>
<td>Adn-8yl-ABP</td>
<td>2.80 ± 1.09</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>-0.45 ± 0.19</td>
</tr>
<tr>
<td>Methanol</td>
<td>-0.72 ± 0.18</td>
</tr>
<tr>
<td>Water</td>
<td>-1.38 ± 0.21</td>
</tr>
</tbody>
</table>

MS Methodology (ES, LC-MS/MS)

**Tuning of the ESI Interface Settings**

The determination of the proper settings for the electrospray ionization (ESI) interface was made by infusion electrospray mass spectrometry (ES-MS) run in positive mode. A solution of polyethylene glycol (PEG) prepared in 50:50 acetonitrile / 1 mM ammonium acetate was infused to observe the calibration of the MS. PEG has a molecular ion pattern of \([M] = 44n + 18\). The sputtering encountered was eliminated by lowering the desolvation temperature from 200° to 120° C. Other initial tune properties were: a cone temperature of 120° C, a capillary voltage of 3.20 kV and a sample methanol composition of 90 %.
The [M+H]^+ for the adduct was roughly 303 m/z. Continuum scans were made 6 amu wide from 300 to 306 m/z. To determine the optimum cone voltage, triplicate scans were made every 3 or 4 volts from 7 to 72 volts. The relative ion count (RIC) maxima for each scan were averaged for their respective voltages and plotted in Figure 11. To tune the capillary voltage the cone voltage was set at 48 volts and continuum scans were made in triplicate every 200 volts for the first few scans and then every 100 volts from 1.60 to 4.20 kV (see Figure 12). The cone voltage was fine tuned at the capillary voltage setting of 3.30 kV with triplicate scans every 1 volt from 42 to 50 volts (see Figure 13). All settings were saved as a tune method in MassLynks.

The Waters online protocol for optimizing a multiple reaction monitoring (MRM) MS method was followed for the most part as prescribed. While infusing the adduct standard solution; the MS1, Daughter and MS2 windows were opened in the MassLynks tune page. The average molecular mass was used for the parent ion mass. After the completion of the research project the data file for the median optimum scan of the cone coarse tune was re-processed to determine the actual [M+H]^+ of the parent peak for MRM (see Figure 14).

Otherwise the MRM was subsequently optimized as described in the online document as follows: The MS1 low molecular weight / high molecular weight (LM/HM) resolution values were lowered to allow more of the parent ion and first isotope signal showing in the Daughter ion window. The MS1 resolutions were again raised to just eliminate the first isotope peak. Finally the MS2 LM/HM values were lowered to increase the parent ion peak in the Daughter window. The LM/HM final settings were 13.5 and 12.3 for MS1 and MS2 respectively.
To determine the optimal collision energy necessary for fragmentation the collision energy was first set at 120 volts and the argon collision gas pressure adjusted to reduce the parent ion centroid signal. Triplicate daughter ion scans were made. The 152 amu daughter ion signal was the major fragment but 153 and 193 amu fragments were also detected. Triplicate daughter ion scans for collision energies at every 10 then 5 volts were made from 0 to 130 volts (see Figure 15A and Figure 15B). The optimum collision energy settings were saved as a tune method in the software.

**LC-MS/MS Calibration Curve for Analyses**

The LC-MS/MS injection volume was 10 µL. Two check standards and the first 3 levels of authentic adduct standard were run singly to verify the accuracy of the setup before running the 7 levels again completely in triplicate, sequentially from low concentration to high concentration. A check standard was run between each new standard level. The minimum of three runs prior to the LC-MS/MS analyses and a check standard bracketing each triplicate or duplicate data point the analysis protocol throughout the following stages of the research project.

For the final calibration curve used for quantitation (Figure 17) the solvents were pipetted first, the proper quantity of adduct standard solution was added but most importantly the pipette tip of the pipetter was rinsed of adduct to complete the transfer.
Activation of ABP

DispoEquilibrium DIALYZERS™ System

The longevity of the generating system (G.S.) was determined by monitoring \( \text{Abs}_{340} \) of the above ratio diluted 1:1 with 2xKPhos to a volume of 300 µL in a disposable ultra-micro cuvette. Figure 20 demonstrates the initial rise and plateau of the G.S. activity. The spectrophotometer used for Figure 20 did not have a working reference cell. Data readings were taken at \textit{circa} 1 minute intervals starting at 6 minutes. The first data point at 1 minute was at an estimation \( \pm 0.1 \) AU. The activity of the G.S. was tested prior to its use by monitoring a rise in absorbance at 340nm. When using the G.S. with the S-9 fraction initially for the caffeine / paraxanthine studies and the control studies, the complete G.S. including the G6PDH was pre-warmed at 37°C 3 minutes prior to adding to the DispoEq tubes. This protocol was revised for the final ssDNA analyses to pre-warming the G.S. substrates for 3 minutes, after that adding G6PDH with mixing and then pipetting the now complete G.S. to the DispoEq to initiate the final P450 reaction. The initial recipe of 0.75 µL of 10 mM G6P with 0.5 units G6PDH would have supplied a flow of NADPH for 20 minutes. But the G.S. change to the proportions given by the supplier limited the NADPH production to only 5 minutes. The biochemical coupling reaction starting materials and product were nearly the same as those given above in Reaction 2 in the Synthesis of Adduct Standard subchapter (compare to Reaction 3 below). The difference lies in that by organic synthesis the coupling is \textit{via} P450 1A2 ABP Activation mediated with a BINAP / palladium catalyst and the brominated C-8
position of Adn, but biochemically ABP undergoes P450 1A2 enzymatic N-oxidation to N-OH-ABP which eventually reacts to form DNA adducts.

![Chemical reaction diagram]

**Reaction 3. ABP and Deoxyadenosine Coupling**

DispoEq dialysis tubes are constructed with two opposing 75 µL chambers separated by either a 5 kDa or 10 kDa MWCO membrane. The white-capped end was arbitrarily designated the Sampling Side and the black-capped end the Reaction Side for these experiments. The use of Parafilm to insure water tightness for the 37°C incubation in the H₂O bath caused the DispoEq tube to break in half. It was found on inspection that no H₂O entered an unwrapped DispoEq floating over the weekend in the H₂O bath. Therefore the manufacturer's statement that they were leak proof was substantiated. Table 3 indicates the setup proportions used for the result reactions of this research.
Table 3. DispoEq Setup for ABP Activation and Reaction with DNA

<table>
<thead>
<tr>
<th>Analysis 1-2 - Reaction of ABP/TP53 exon7 sense strand</th>
<th>Sampling Side - White</th>
<th>Reaction Side - Black</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.10 µL (25.6 pmol ssDNA) in 2xKPhos</td>
<td>37.5 µL 2xKPhos</td>
<td></td>
</tr>
<tr>
<td>25.4 µL 2xKPhos</td>
<td>7.5 µL 200ppm ABP</td>
<td></td>
</tr>
<tr>
<td>37.5 µL 18 MΩ H2O</td>
<td>7.5 µL S-9 Fraction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.5 µL G.S.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analysis 2-2 - Reaction of ABP/TP53 exon7 anti-sense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling Side - White</td>
</tr>
<tr>
<td>5.94 µL (24.6 pmol ssDNA) in 2xKPhos</td>
</tr>
<tr>
<td>31.6 µL 2xKPhos</td>
</tr>
<tr>
<td>37.5 µL 18 MΩ H2O</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

The ABP and the S-9 fraction 7.5 µL volumes were pipetted with a P-100 pipetter. It was determined with 15 sequential pipettings of 7.5 µL H2O that the RSD was ± 3 % for the P-100. The ssDNA solutions were pipetted with a P-20 and the remaining solutions with an R-200 pipetter. All weighings were verified against standardized weights.

Characterization of the S-9 Fraction

For the purpose of determining the P450 1A2 activity of the S-9 fraction the caffeine / paraxanthine transition was characterized. The caffeine / paraxanthine HPLC method was performed and adapted from Furge et al. to work on the Shimadzu LC-UV system.\(^85\) The run time for the isocratic 200 µL / minute method was 6 minutes and the UV detection was set for 260 nm. The mobile phase composition was 92 % (10 mM ammonium acetate; 2.5 % acetic acid) / 8 % acetonitrile. See Figure 21 for the chromatogram. The elution order on this column was reverse of that demonstrated in the
Separate parallel LC methods with separate calibration curves were created for caffeine and paraxanthine. If a unified method in the Shimadzu Classic VP software were desired it required that the standards be mixed in the same LC sample vial for a composite injection. The retention time windows for caffeine and paraxanthine were verified on each day by injection of the high point standards (4.0 ppm and 8.32 ppm for paraxanthine and caffeine respectively).

The DispoEq protocol was performed as in Table 3 for the caffeine / paraxanthine S-9 characterization. In this case ssDNA was not present on the Sampling Side and 7.5 µL 240 ppm caffeine was placed on the Reaction Side (with and without the 7.5 µL ABP present). The DispoEq tubes were incubated for 1 hour in the H2O bath at 37° and transferred for the remainder of the 24 hours to the rotating stir tray in a 37° convection oven. In the final 150 µL DispoEq volume after full equilibration there was 12 ppm caffeine possible on the Sampling Side.

The 240 ppm caffeine was prepared as a solution in 10 % ethanol. The order of additions was altered such that the caffeine was added to the 37.5 µL 2xKPhos and then the S-9 was added to the newly diluted 1.67 % ethanol solution. This was to prevent the outside chance that the ethanol was precipitating the S-9 proteins essential for reaction. The reference provided by XenoTech, LLC the S-9 fraction used methanol as a solvent for a P450 substrate and documented it did not decrease enzyme metabolism at levels of up to 2 % methanol.86
Control Studies

The inhibition of the S-9 fraction by ABP was tested by inhibition of the caffeine / paraxanthine transition in the same manner in which the S-9 fraction itself was characterized. In addition 7.5 µL of 200 ppm ABP was added to the Reaction Side of the DispoEq (to give an overall final 10 ppm ABP in 150 µL for the protocol) along with 7.5 µL caffeine solution.

For the ssDNA analyses the DispoEq tubes were incubated for 1 hour at 37°C in the H2O bath, agitating them ca. every 10 minutes to insure adequate mixing of the contents on the Reaction Side. An additional 23 hours was spent on a rotating gravity stir tray in a 37°C convection oven (the digital temperature setting had been verified by a thermometer placed on the shelf inside). The former incubation was to insure adequate heat transfer during the G6PDH production of NADPH. The latter 23 hours of incubation was to allow proper agitation for dialysis. The contents of the Sampling Side were quantitatively removed to respective 1.8 mL actinic screw topped vials (with septa) containing 75 µL 60 % methanolic 0.1 N HCl / 40 % 1.0 N HCl. The resulting composition was 30 % methanol in the vial. The vials were suspended in a rack for 2 hours in a 75°C H2O bath. These were the hydrolysis conditions for the Control Studies. The acid composition was changed to 100 % aqueous for the final analyses, i.e. 75 µL 1.0 N HCl. This change was made to insure a more quantitative transfer of adduct, its being more soluble in H2O relative to methanol (see LogP Table 2).

The final control study undertaken was to determine if ABP reacted to form an adduct with ssDNA in the absence of the S-9 fraction. Concurrent 48 and 24 hour duplicate DispoEq incubations were performed with non-calibrated sense strand DNA in
the Sampling Side and G.S. / ABP on the Reaction Side. The concept for both the final
control study and the later final analyses was that the ABP would be transformed by the
S-9 into the reactive N-OH-ABP on the Reaction Side in the first hour and then traverse
the membrane during the remaining 23 hours (here also 47 hours) to react with ssDNA
restricted to the Sampling Side by its larger MW. In this particular control study the S-9
was absent.

The amount of ssDNA used on the Sampling Side was a 37.5 µL aliquot of the
lyophilized amount supplied from MSU-MSF in a microfuge tube reconstituted with 300
µL 2xKPhos and heated at 37°C in the H2O bath. (This dilution and heating became the
protocol for reconstituting the ssDNA). The G.S. and ABP were placed on the Reaction
Side and the DispoEq was placed in the H2O bath for 1 hour at 37°C. The first duplicate
DispoEq were agitated on the rotating gravity stir tray in the 37°C oven for another 47
hours. After 24 hours into the first sample’s incubation / agitation, the second pair of
DispoEq were placed in the H2O bath for 1 hour incubation and transferred to the stir tray
in the oven for the remaining 23 hours. In the first run of this control experiment the
hydrolysis was performed in methanolic HCl. The experiment was repeated under
similar conditions but with 1.0 N HCl hydrolysis for 24 hours (but not 48 hours). The 1.0
N HCl hydrolysis became protocol with the subsequent ssDNA analyses. The resulting
LC-MS/MS chromatograms are shown in Figure 23.

In an attempt to explain the retention time change of the Adn-8yl-ABP adduct
produced in the DispoEq tubes compared to that of the adduct standard as was used for
calibration, the 1195 pmol / µL sense strand DNA was spiked with 86.1 ng / 10 µL
adduct standard. The thymine was also spiked with adduct standard. The amount of
thymine used was equivalent to the 9 thymidine in the sense strand DNA, namely 10.8 nmol / µL. The thymine was spiked at low (check standard) and calibration curve (high point) levels of Adn-8yl-ABP. See Figure 25 and Figure 26 for the chromatograms of the Adn-8yl-ABP spikes of DNA and thymine respectively. If Watson-Crick hydrogen bond pairing was the cause of the prolonged retention times for the adducts in the DispoEq analyses it was expected that the thymine spiked with standard would also have eluted later. It was also expected that the three peaks between 9.8 and 11.0 minutes in the DNA spike chromatogram were the relative concentrations of the match and mismatch base pairing with the Adn adduct, i.e. A-T > G/A > A/A as is mentioned in the Computational Determinations subchapter.

Final Laboratory Analyses

For the final analyses lyophilized sense and anti-sense ssDNA (coding and transcription respectively) in microfuge tubes was reconstituted as per the protocol discussed in the Control Studies subchapter with 300 µL 2xKPhos. The Abs$_{260}$ readings were taken with the Cary14/OLIS (Bogart, GA) spectrophotometer to determine the concentration of DNA using Equation 1 and Equation 2 for the respective strands. The dilution ratios necessary to provide equivalent 5 pmol / µL ssDNA in the eventual 75 µL on each Reaction Side of the DispoEq were determined. See Table 4 for the Abs$_{260}$ readings taken to verify the volumes used. The LC injection volume was 10 µL.

After 24 hours in the DispoEq the 75 µL were diluted 1:1 with 1.0 N HCl for 2 hours of hydrolysis and taken to the LC Column. The two DispoEq tubes for each of the
TP53 exon7 sense and anti-sense strands were themselves run in duplicate by LC-MS/MS.

Table 4. Quantitation of ssDNA Strands at Absorbance 260 nm

<table>
<thead>
<tr>
<th>TP53 exon7 Sense Strand</th>
<th>PRK 1C - 12.10µL to 300µL for Abs260  / /  12.10µL to DispoEq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs260</td>
<td>E260</td>
</tr>
<tr>
<td>0.573</td>
<td>448070.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TP53 exon7 Anti-Sense Strand</th>
<th>PRK 2D - 5.94µL to 300µL for Abs260  / /  5.94µL to DispoEq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs260</td>
<td>E260</td>
</tr>
<tr>
<td>0.511</td>
<td>414633.2</td>
</tr>
</tbody>
</table>

Computational Determinations

VisualOMP6.0 Software Application

The computational determination of the secondary structures of the sense and anti-sense strands was performed with VisualOMP6.0 at DNA Software, Inc (Ann Arbor, MI). VisualOMP6.0 (oligonucleotide modeling platform) uses Watson-Crick base pair nearest neighbor parameters for its calculations derived from an experimental dataset compiled from multiple laboratories. The database of thermodynamic parameters includes structures such as Watson-Crick base pairs, internal mismatches, terminal mismatches, terminal dangling ends, hairpins, bulges, internal loops and multibranched loops.87

VisualOMP6.0 was developed to assist in DNA polymerase chain reaction (PCR) primer design.88 For PCR VisualOMP6.0 not only optimizes more accurately the melting temperature (Tm) of oligos than other primer design software, but optimizes the annealing
temperatures for the highest percentage oligo binding as well. Input parameters necessary for VisualOMP6.0 are the concentration of each oligo, the magnesium ion content and the total concentration of monovalent ions (sodium, potassium and ammonium ions combined). VisualOMP did not as of 2004 have the capability to account for calcium ions such as would be necessary with in vivo simulations. The computational determinations have been shown accurate in the range of 0.05 to 1 M sodium ion. The input values for the TP53 exon7 folding computations were: 0.333 µM concentration ssDNA, 37°C temperature, 91 mM K⁺ monovalent ion and 3 mM for the Mg⁺ ion. (The 1xKPhos buffer was 50 mM at pH 7.4 which gave a K⁺ ion concentration of 91 mM). The following progression is the order of decreasing pairing strength as determined in the database:

$$G-C > A-T > G\!G > G\!T \geq G\!A > T\!T \geq A\!A > T\!C \geq A\!C \geq C\!C.$$  

G was described as being a promiscuous base, binding with most any other base.

![Guanine / Adenine Mismatch Pairing](image)

Figure 4. Guanine / Adenine Mismatch Pairing
GNA Triloop at Codon Arg 248

Recall from Chapter III that Arg 248 is the codon of the DNA binding domain that contacts the consensus sequence of target DNA in the minor groove. The folded sense strand of TP53 exon7 as seen in Figure 29A contained a three base hairpin loop from G41 to A43 in the form of GNA, where N was any possible nucleotide. (The codon Arg 248 was C40, G41 and G42). This type of hairpin of three has been found to be especially stable and was given a computational stability bonus in the calculations. The free energy ΔG and enthalpy ΔH for the sequence CGGAG were -2 and -2 kcal / mol respectively at 37° C in 1 M NaCl. (When GGNAC or CGNAG were the nucleotide combinations flanking a triloop, the bonus was -2 kcal / mol and when AGNAT or TGNAA it was -1.5 kcal / mol.)
CHAPTER VI

RESULTS

Adduct Standard Synthesis Results

Pure protected 8-bromo-adenosine was isolated in 83 % yield as seen in the $^1$H-NMR spectrum (see Figure 5).

Figure 5. $^1$H-NMR Spectrum of Silyl-Protected 8-Bromoadenosine
The refined yield for the protected Adn-8yl-ABP adduct standard was 10 mg (22% yield). See Figure 6 for the $^1$H-NMR spectrum and Table 5 for the proton assignments.

![1H-NMR Spectrum of Silyl-Protected Adn-8yl-ABP](image)

**Figure 6.** $^1$H-NMR Spectrum of Silyl-Protected Adn-8yl-ABP

**Table 5.** Proton Assignments for Figure 6.

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>K</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm Shift</td>
<td>8.23</td>
<td>7.74</td>
<td>7.59-7.25</td>
<td>6.17</td>
<td>5.20</td>
<td>4.87</td>
<td>4.28-3.89</td>
<td>0.95-0.73</td>
<td>(-)0.16-0.31</td>
</tr>
</tbody>
</table>
See Figure 7 for the relative composition of the 5 SPE aliquots to purify the adduct standard. Aliquots 1 and 2 were used in Authentic Standard preparation.

Figure 7. ES-MS Aliquot RIC Comparison

The LC-UV$_{260/310}$-MS/MS characterization chromatograms showed no UV$_{260}$ contamination for the prepared authentic standard. See Figure 8 for the $^1$H-NMR spectrum in deuterated methanol and Table 6 for the proton assignments of the standard used to calibrate the MS. The shift at 4.87 was a water peak and at 3.31 ppm in Figure 8 was the deuterated methanol solvent signal. The solvent shift reference values originated from PalmOne software.$^{89}$
Figure 8. $^1$H-NMR of Adn-8yl-ABP Authentic Standard

Table 6. Proton Assignments for Figure 8.

<table>
<thead>
<tr>
<th>Group</th>
<th>mm Integration</th>
<th>number protons</th>
<th>Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A = br s</td>
<td>10</td>
<td>1</td>
<td>8.14</td>
</tr>
<tr>
<td>D = s</td>
<td>43</td>
<td>4</td>
<td>7.65</td>
</tr>
<tr>
<td>E = d</td>
<td>25</td>
<td>2</td>
<td>7.60</td>
</tr>
<tr>
<td>F = t</td>
<td>24</td>
<td>2</td>
<td>7.42</td>
</tr>
<tr>
<td>G = ? t</td>
<td>11</td>
<td>1</td>
<td>7.31</td>
</tr>
<tr>
<td>K = ? Exchangeable</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>
In the Spectral Database proton A on adenine was low and broad at 12.8 ppm.\textsuperscript{90} In the assignments by Schoffers \textit{et al.} on the silylated compound, proton C was described as a singlet but not broad and was also not broad in the previous Figure 8. Therefore the signal at 8.14 ppm was determined to be proton A. In the literature proton B was suspected to be an exchangeable proton due to $pK_a$ changes in adducted dsDNA.\textsuperscript{91} K was also exchangeable and not apparent in the $^1$H-NMR spectrum because methanol was the solvent. Proton C was also suspected as exchangeable. Although the neighboring F protons should have been equivalent, G was a triplet and not a doublet as was seen with D of the previous Table 5. The D proton of Table 5 corresponding proton G was at 6.17 ppm and more up field. The use of DMSO as an NMR solvent would have given a more resolute $^1$H-NMR spectrum but would have been terminal as far as subsequent use. More confirmation towards the ID of the authentic adduct standard came from the infusion ES-MS spectrum which had the appropriate [M+H]$^+$ centroid signal at 303 $m/z$. seen below in Figure 9 and the UV normalized larger 310 nm vs. 260 nm LC peak shown in Figure 10 (see also Figure 16 and accompanying discussion).
Figure 9. Triplicate ES-MS Centroid Scans of Adn-8yl-ABP
Figure 10. LC-UV\textsubscript{260/310}-MS/MS Chromatogram of the Adn-8yl-ABP Authentic Standard

The weight of the adduct standard to be diluted was 0.430 ± 0.009 mg and the concentration adduct in the 50 mL 30 % methanol in the volumetric flask was 86 ± 2 ng / 10 µL neat LC injection volume. In determining the precision of the LC authentic standard and its influence on the calibration curve, only the error in weighing was taken into account and not from any other real sources such as the volumetric flask, the pipetters and the LC syringe injection loop.
MS Methodology Results

For the [M+H]^+ range of 239 to 591 m/z, the analysis range of this research, all the PEG centroid signals were observed. Before the final pure Adn-8yl-ABP adduct was isolated for weighing and LC-MS/MS calibration the ES was tuned. Ion suppression and ion enhancement by DNA hydrolysis products in the LC-MS/MS analyses were minimized by the inherent characteristics of LC separation. The adduct standard alone eluted at 70 % methanol or the activated ABP product with DNA eluted at 100 % methanol (see Chapter VII). The steps necessary to tune the ES-MS were: cone voltage coarse tuning, capillary voltage tuning, cone voltage fine tuning and collision energy tuning. Triplicate MS scans were averaged for each voltage setting. The maximum coarse cone voltage RIC was at 48 volts. See Figure 11 for the coarse cone tune maximum RIC scans.
The optimum capillary RIC intensity was achieved at 3.30 kV.

Figure 11. Coarse Cone Tune to Adn-8yl-ABP, Maximum 48 Volts

Figure 12. Electrospray Capillary Voltage Tune to Adn-8yl-ABP, Maximum 3.30 kV
The optimum capillary voltage was 44 volts.

![Cone Voltage Fine Tuning](image)

Figure 13. Cone Fine Tune to Adn-8yl-ABP, Maximum 44 Volts

With the cone and capillary voltage at 44 volts and 3.30 kV respectively the extractor was optimized to 7 volts while the Source RF Lens and Lenses 6, 8, and 9 were optimized to their response on the tune page scope of the MS. The average molecular weight\(^9\) of 302.3396, plus a proton was chosen as the parent peak for the adduct standard \(i.e.\) 303.3 \(m/z\) for the Daughter window. After the completion of the research project the data file for the median optimum scan of the cone coarse tune was re-processed\(^8\) to determine the actual \([M+H]^+\) of the parent peak 303.1 \(m/z\) for MRM (see Figure 14).
Figure 14. Molecular Weight of the Parent Peak

Having used the [M+H]$^+$ derived from the average mass or 303.3 $m/z$ instead of the monoisotopic mass 303.1 $m/z$ for LC-MS/MS incurred a slight loss of sensitivity. The span was set at 0.60 amu to allow the passage of the parent peak. Thus only 303.00 to 303.60 $m/z$ were allowed to pass. The full trailing portion of the parent signal was allowed to enter the MS$_2$ quadrupole, but the leading edge of the peak was excluded for the most part. The full sensitivity potential for MRM mass spectroscopy was not realized in this research due to this fact. But the use of a narrow span disallowed the passage of the [M+H]$^+$ signal 304.1 $m/z$ of another possible adduct, N-(guanine-$N^2$)-ABP with the
similar but different transition of 304.1 $\rightarrow$ 152 $m/z$. Note this point for Table 11 presented in Chapter VII. The optimum collision energy of 90 volts for the 303.3 $\rightarrow$ 152 $m/z$ MRM transition was used for the LC-MS/MS calibration and further analyses.

Figure 15A. Collision Energy Tuning to Daughter Ion Fragments

Figure 15B. Parent Molecule and Daughter Ion Fragments of Adn-8yl-ABP

For LC method development the UV wavelengths of 260 and 310 nm were monitored as well as the MRM 303.3 $\rightarrow$ 152 $m/z$ transition (see Figure 16). DNA absorbs at 260 nm.$^{95}$
Figure 16. LC-UV$_{260/310}$-MS/MS Chromatograms for Method Development

The 310 nm wavelength is the characteristic absorbance of aromatic amine-substituted DNA species.$^{15}$ The LC gradient was characterized and the initial calibration curve (Figure 19) was determined as linear throughout the calibration range of 1.3 to 86 ng adduct on the column (with the exception of a dip to the 10.8 ng calibration point). The injection volume was 10 µL. The Savitzky-Golay method of moving quadratic smoothing of chromatograms was performed. Variations in peak shape would have been undesirably minimized by the moving means method of smoothing.

For the final calibration curve the LC parameters were optimized to obtain the best MS sensitivity and reduce the 32 minute chromatographic run times. The UV detector was no longer necessary and was removed from the flow path. The column was
placed in a column heater set at 35° C to raise its temperature above ambient. The result of which was a 27 % increase in peak height for the 86 ng high point and a reduction of 37.5 % in run time to 20 minutes. See Table 7 for the final mobile phase gradient.

Table 7. Optimized Mobile Phase Gradient for LC-MS/MS Analyses

<table>
<thead>
<tr>
<th>Run Time in Minutes</th>
<th>% Methanol in H₂O (and 0.2 % Formic Acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 → 7</td>
<td>30 % → 100 %</td>
</tr>
<tr>
<td>7 → 10</td>
<td>100 %</td>
</tr>
<tr>
<td>10 → 13</td>
<td>100 % → 30 %</td>
</tr>
<tr>
<td>13 → 20</td>
<td>30 %</td>
</tr>
</tbody>
</table>

The calibration curve (Figure 17) established for the quantitation of adenine adducts formed by S-9 fraction activation of ABP to react with TP53 exon7 ssDNA had a coefficient of determination of $R^2 = 0.995684$ and the equation $y = 1447.20 x$ with forced zero. The value for the calibrated check standard was $5.9 ± 0.3$ ng / $10\mu$L, $n = 8$. The pipetting for the adduct standard concentrations was the critical factor in the residuals of the calibration curve.

Starting with 86 ng / $10\mu$L the adduct standard solution was pipetted with an R-200 pipetter for the highest 4 levels. The transition was made to a P-20 pipetter for pipetting the remaining 3 lower levels. The peak height was used to quantitate because LC-MS is a concentration dependent detection compared to other mass-dependent MS. Therefore fluctuations in flow would not alter response as they would if peak area were to be used to quantitate. The peak height of one of the low points for the calibration curve is shown in the Figure 18 chromatogram and was $1.95E03$ RIC. This compared to the peak heights of the adduct ($n = 4$, $p = 0.10$) in the final analyses of $58 ± 21$ and $99 ± 37$ RIC for the sense and anti-sense strands respectively.
Figure 17. LC-MS/MS Calibration Curve for Analyses

Figure 18. Lowest Calibration Point Chromatographic Peak Height

In the first calibration curve established (Figure 19) the standard contents of the R-200 were simply expelled and the tip not rinsed. The negative dip in the plot of the
residuals in Figure 19 at the lowest volume of the R-200, the 10.8 ng level gave evidence that the pipetter was 'To Contain' and not 'To Dispense'. To avoid this systematic error in pipetting, the analyte of interest was pipetted last and rinsed form the tip with the solvents.

![Figure 19. Preliminary Calibration Curve](image)

ABP Activation Results

The duration of NADPH production by the G.S. was determined. The G.S. was essentially spent in 60 minutes (see Figure 20).
Figure 20. Longevity of the Generating System

The RSD for 15 sequential pipettings of 7.5 µL H₂O with the P-100 pipetter was determined as ± 3 %. This was the pipetter used to pipette the S-9, the caffeine and the ABP solutions. When checking membrane permeability it was assessed by LC-UV analysis that only 8 % of the caffeine passed through a 5 kDa MWCO membrane and 57 % passed a 10 kDa MWCO membrane in 24 hours. The latter membrane was chosen for these experiments.

As Figure 21 indicates the amount of paraxanthine generated from caffeine by the S-9 was too little to be integrated for quantification. There was a perceptible peak in the retention time window for paraxanthine. Because of this peak it was concluded that the S-9 fraction did indeed contain P450 1A2 and it was active to some extent. From the 7.5 µL 50 mM G6P present in the DispoEq system and 1 unit G6PDH, the NADPH G.S. was calculated to only have 5 minutes duration for the caffeine experiment and subsequent ssDNA experiments.
To determine if ABP inhibited the G.S., ABP levels of 0 ppm (control), 1 ppm and 20 ppm were used in the cuvette while performing the G.S. check. There was no observable trend between the levels of ABP (see Figure 22). Because the results were comparable for the control, 1 and 20 ppm levels with little difference, ABP was concluded not to inhibit the G.S. at these levels.

The LC-UV$_{260}$ chromatograms were indistinguishable with and without ABP. It was concluded the ABP did not perceptibly inhibit P450 1A2 of the S-9 fraction. The transfer RSD of duplicate vials of adduct spikes measured by LC-MS/MS of 100 % aqueous contents were 9 %, 3 % and 2 % for peak heights averaging 92, 1479 and 22,803 total ion counts respectively. The lowest peak height of the three was for adduct standard 'hydrolyzed' without any thymine nucleoside.

It was postulated that the adenine of NADP$^+$ may be labile towards attack by the activated ABP. But the LC-MS/MS chromatograms of the hydrolyzed Sampling Side
Figure 22. ABP Non-Inhibition of G.S. Testing Results

contents after 24 hour incubation of the S-9, G.S. and ABP together, without ssDNA were unremarkable. It was concluded that ABP and NADP⁺ did not react with each other under these conditions to form an adduct.

The final control study undertaken was to determine if ABP reacted to form an adduct with ssDNA in the absence of the S-9 fraction. The findings of this control study were neither as expected nor were the results completely understood. The resulting LC-MS/MS chromatograms are shown below in Figure 23. It was expected that the S-9 would be essential to activate the ABP to react with the ssDNA, but this was apparently not the case. The retention time for the adduct standard was in the neighborhood of 5.6
minutes when the calibration curve was established (see Figure 18). The retention time for the unique to the Adn-8yl-ABP adduct 303.3 $\rightarrow$ 152 m/z transition varied for the different experiments of this final control test. With an undetermined amount of sense strand DNA and methanolic HCl hydrolysis the retention time for 24 and 48 hours agitation in the DispoEq ranged from the multiple peaks at 2, 9 and 11 minutes and a single peak at 2.4 minutes respectively for the agitation time (see Figure 23). (24 hour agitation: 9.91 minutes, 26 ± 6 ng on the column (n = 4, p = 0.10), 2.40 minutes, 0.72 ± 0.14 ng on the column (n = 4, p = 0.10) and 11 minutes not quantitated; 48 hour agitation: 2.41 minutes, 1.26 ± 0.04 ng on the column (n = 2, p = 0.10)). With a 1.0 N HCl hydrolysis and 24 hour agitation an equal concentration sense strand DNA as was used in the final analyses resulted in a 2.9 minute retention time (see Figure 24). (24 hour agitation: 2.89 minutes, 0.13 ng on the column (n = 2, p = 0.10)) For the results obtained without S-9 fraction ABP activation see Table 8 (compare to Table 10).

After finishing the ssDNA analyses it was investigated if the adduct may Watson-Crick base pair with its complementary thymine present in the hydrolyzed DNA yet

| TP53 exon7 Sense Strand (25.6 pmol) without S-9 |  |
|---|---|---|
| x | p = 0.10 | n = 2 |
| 0.13 ± 0.03 | ng / 10µL Injection Volume |
| 17.9 ± 5 | fmol Adduct on Column |
| 16 ± 4 | mol Adducts per E06 mol nucleo |
Figure 23. Chromatograms of ssDNA, ABP and No S-9 Fraction

Figure 24. Chromatogram of 25.6 pmol ssDNA, ABP and No S-9 Fraction

disassociate in the ES of the MS to allow the proper MRM transition. It was proposed base pairing may have been the cause of the observed prolonged retention time seen in the ssDNA analyses and in one of the control study chromatograms in Figure 23 when compared to the synthetically prepared standards. The longer retention time could not be duplicated with thymine of an equivalent molar amount as in the sense strand DNA of the final analyses. The thymine / Adn-8yl-ABP hydrolyzed solution retention time was 5.9 minutes and comparable to the retention time of the calibration adduct standard alone.
The recoveries of the adduct were 125% and 138% for 5.9 minutes and 9.8 minutes respectively. This was an increase of 10%, or within the range of the confidence limits of the final analyses relative to the final results. The LC column for the thymine tests was at ambient instead of the previous 35°C as was the case with the calibration standard and all of the final analyses. For the comparison of conditions between the DNA and thymine spikes See Table 9.

Table 9. Conditions Comparison for Adn-8yl-ABP Spikes of Sense Strand DNA and Thymine

<table>
<thead>
<tr>
<th>Spike of Sense Strand DNA</th>
<th>Spike of Thymine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssDNA conc. = 1,195 pmol/µL in 2xKPhos</td>
<td>Thymine conc. = 10,800 pmol/µL in 2xKPhos</td>
</tr>
<tr>
<td>Spike conc. = 86.1 ng/10 µL in 30% MeOH</td>
<td>Spike conc. = 86.1 &amp; 5.9 ng/10 µL in 30% MeOH</td>
</tr>
<tr>
<td>Hydrolyzed 1:1 in 1.0 N HCl 2 hr at 75°C</td>
<td>'Hydrolyzed' 1:1 in 1.0 N HCl 2 hr at 75°C</td>
</tr>
<tr>
<td>HPLC column at 35°C</td>
<td>HPLC column at ambient</td>
</tr>
<tr>
<td>ES capillary same as on calibration</td>
<td>ES capillary cleaned prior to chromatography</td>
</tr>
</tbody>
</table>

See Figure 25 and Figure 26 for the chromatograms of the Adn-8yl-ABP spikes of DNA and thymine respectively.

Figure 25. Chromatogram of Heated DNA Spiked with Adn-8yl-ABP Adduct Standard
Final Analyses of DNA Adduct Formation

The signal to noise ratio (S/N) was 2 for the adduct LC peaks. These peaks were 5 % of the low point on the calibration curve (Figure 17). See Figure 27 for the chromatograms of the final analyses.
Figure 27. LC-MS/MS Final Chromatograms

The retention times of the final analyses were verified by comparing with analyses performed at higher concentrations of the sense and anti-sense strands. Figure 28 shows the chromatograms for the 5975 and 359 pmol ssDNA on the column of the sense and anti-sense strands respectively. Because the DNA concentrations of the sense and anti-sense strands in the verification analyses were not in parity, their quantitative results could not be used for comparison with each other. Their peaks along with those of the Adn-8yl-ABP standard spike of DNA (Figure 25.) did prove valuable to designate the retention time of the peaks used for quantitation in the Figure 27 chromatograms.
Figure 28. Chromatograms of Sense and Anti-Sense Strands with Unequal Concentrations

The results for the duplicate LC vials for each strand, run each in duplicate by LC-MS/MS were given in Table 10. It was noted that the sense strand, the coding, the non-transcriptional strand had fewer Adn-8yl-ABP adducts than the anti-sense, transcriptional strand (p = 0.10). See Chapter VII.

The 'fmol on the column' values in Table 10 were not rounded to the appropriate one significant Figure 2ut the 'mol Adducts per 10^6 nucleotides' end results were rounded. The quantitated entity was mol of Adn-8yl-ABP adduct but corresponded directly to the desired result of mol N-(deoxyadenosine-8yl)-4-aminobiphenyl (dAdo-8yl-ABP). The 90 % confidence interval levels were calculated in order to give a significant difference using a pooled sample deviation.
Table 10. LC-MS/MS Results

<table>
<thead>
<tr>
<th>TP53 exon7 Sense Strand (25.6 pmol)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>p = 0.10</td>
<td>n = 4</td>
<td></td>
</tr>
<tr>
<td>0.04 ± 0.01 ng / 10µL Injection Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.7 ± 2 fmol Adduct on Column</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 ± 2 mol Adducts per E06 mol nucleotides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TP53 exon7 Anti-Sense Strand (24.6 pmol)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>p = 0.10</td>
<td>n = 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.07 ± 0.03 ng / 10µL Injection Volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.2 ± 4 fmol Adduct on Column</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 ± 4 mol Adducts per E06 mol nucleotides</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The value for the check standard when the calibration curve was established as 5.85 ± 0.08 ng on the column (n = 8, p = 0.10) and at the time of the final analyses the value was 3.55 ± 0.44 ng on the column (n = 7, p = 0.10). The concentration of the check standard against the calibration curve was a constant throughout the quantitation, acting as a reference value through system optimizations. Because the sensitivity of the ES detector was decreasing, the final results were actually greater than shown in Table 10. To correct for this change the check standards could be used to approximate a correction. The propagation of uncertainty was done through calculating relative error. The corrected results were 8 ± 37 % and 15 ± 43 % for the sense and anti-sense strands respectively.

Computational Results

Figure 29A and Figure 29B were the results of the computational calculations for TP53 exon7 sense and anti-sense strands respectively. Internal single mismatches such as the GA mismatch (Figure 4) in the hairpin of the folded anti-sense strand in Figure 29B
may occur. The bases that were found in Watson-Crick base pairs, as well as the GA mismatch were much less reactive because of their lower energy state compared to the unpaired bases.\textsuperscript{95} Therefore on the sense strand in Figure 29A there were 8 dA (from the 5' end: 1-2-5-7-13-37-38-43) and there were 9 dG (11-17-23-28-29-31-32-41-42) available for reaction. On the anti-sense strand shown in Figure 29B there were 5 dA available (20-34-36-38-42) and 4 dG (16-31-40-43) for reaction with activated ABP. It was computationally determined that the total number of purines available for reaction on the sense strand was greater than on the anti-sense strand. Further, the ratio of available dA to dG on the sense strand was less than on the anti-sense strand. These observations corroborate the experimental evidence that there was less Adn-8yl-ABP found by LC-MS/MS in the DispoEq anti-sense strand compared to the sense strand reactions.
\[ dG \ (37.00^\circ C) = -3.49 \text{ kcal/mole} \]
\[ dH = -68.70 \text{ kcal/mole} \]
\[ dS = -210.25 \text{ cal/mole K} \]
\[ Tm = 53.60^\circ C \]
\[ [\text{monovalent}] = 0.0910 \text{ mol/L} \]
\[ [\text{Mg}^-] = 3.000e-003 \text{ mol/L} \]

Figure 29A. *TP53 exon7*
Folded Sense Strand

Figures Determined Computationally with *VisualOMP6.0*, DNA Software, Inc., Ann Arbor, MI
Figure 30 shows the stacking of the purine bases in the triloop as generated by the RNA-123 Software Suite (Ann Arbor, MI).

Figure 30. Triloop at the Arg 248 Hotspot Codon of TP53 exon7

The Emphasis is on the C-8 Carbon of A43, Loop is 5’ to 3’ Right to Left

Prepared with Swiss-Pdb Viewer

CHAPTER VII

DISCUSSION

Control Studies

One could ask whether N-OH-ABP being highly reactive does remain stable long enough under in vitro conditions to migrate across the DispoEq dialysis membrane over the 24 hour period before degradation. Jones and Sabbioni took precautions to extract remaining unreacted N-hydroxy arylamines after an overnight reaction. Therefore the activated ABP was considered stable enough over the course of the 24 hour DispoEq studies discussed below to remain reactive.

An earlier study using the caffeine / paraxanthine transition had 19 ppm as their low point caffeine level and 155 ppm their high point level in their experiments. The study performed here had 24 ppm caffeine available for reaction. Therefore the poor quantitative results here may likely have been due to not using a high enough concentration of caffeine. Also when the present caffeine metabolism studies were performed the active G.S. was incubated in the H₂O bath for 3 minutes and then added to the DispoEq tubes. This may have been another likely cause for the low paraxanthine results. The protocol order of adding G6PDH to the G.S. before pre-heating the G.S. may have affected the outcome of this caffeine study. The most probable cause for poor results was that the G.S. was actively producing NADPH for only 5 minutes instead of the 20 as was the original design. With only 2 out of the 5 minutes of NADPH
production remaining with G.S. substrates present, the reaction time for paraxanthine production was limited.

As mentioned in Chapter III there was some dispute if it were P450 1A2 that actually performed the N-oxidation, but P450 1A2 was one of the P450 enzymes in the Sprague-Dawley rat liver S-9 fraction activating heterocyclic aromatic amines in a similar reaction.

The 24 hour permeability experiment to determine if ssDNA (13.9 kDa and 13.7 kDa for the sense and anti-sense strands respectively) would pass the 10 kDa MWCO membrane was not successfully completed due to a malfunctioning UV spectrophotometer. It was reasoned that if 194 Da caffeine passed the 10 kDa MWCO membrane with only 57 % success, the 13+ kDa ssDNA would have less success passing. As seen in Figure 29A and Figure 29B the folded ssDNA under the DispoEq conditions was bulkier than simply random coil ssDNA. It was concluded not likely to pass the membrane efficiently. It would theoretically be hindered from passage through the pores of the membrane by such folding.

The removal of NADPH by the S-9 as observed by a sudden increase and then decrease in Abs\textsubscript{340} over 15 minutes would have been a simpler experiment compared to refining, calibrating and performing the caffeine / paraxanthine LC-UV method as described in Chapter V. The NADPH monitoring method would have been non-specific, pertaining to any enzyme that consumes NADPH and not exclusively P450 1A2.

The reason for the variability in retention times of the 303.3 \( \rightarrow \) 152 m/z MRM detected adduct was thought to possibly be the result of three different variables: incomplete hydrolysis of the ribosyl of deoxyadenosine, Watson-Crick base pairing of the
hydrolyzed monomers with the Adn-8yl-ABP adduct and base stacking of the aromatic biphenyl rings of ABP with nucleoside bases. The shorter retention time was surmised to be due to incomplete hydrolysis of the ribosyl sugar from deoxyadenosine. Because of its increased hydrophilic character the adducted nucleoside would elute earlier, retaining with less hydrophobic interactions on the phenyl column. The ribosyl sugar would possibly be unstable and undergo source fragmentation to the detected Adn-8yl-ABP.

The retention time configuration seen between 9.8 and 11 minutes was either due to Watson-Crick base pairing with monomer bases (not likely, see next paragraph) or due to base stacking interactions adduct with various hydrolyzed free bases in the solution. The latter possibility for the protracted elution on the phenyl column was that aromatic purine rings had pi-pi interactions with the biphenyl rings and had thus greater interactions with aromatic LC column. Those aromatic interactions would also not be strong enough to survive the ‘source fragmentation’ and the MRM transition of the adduct would be detected. Guckian et al., when comparing changes in $T_m$ of a 6-mer duplex core with dangling ends composed of pyrimidines, purines and synthetic nucleotides of aromatic compounds such as benzene, naphthalene, phenanthrene and pyrene found that the greater the increase in aromatic character the greater the $T_m$.99 (The definition of $T_m$ is when half of the oligos are in duplex state and half are in random coil).88

There did not appear to be any correlation between the varying hydrolysis conditions and the length of agitation. In any case since the results under similar ssDNA and ABP conditions were greater than with the S-9 present, the S-9 was not essential for
the activation of ABP to react with ssDNA. The retention time observed for the final ssDNA analyses was 9.8 minutes.

It was likely that because the oligonucleotide backbone was absent that the Adn adduct did not pair with monomeric thymine pyrimidine base in the experiment for Figure 26. The $T_m$ for the monomer base pair of A-T was only $2^\circ$ C when calculated under the same conditions as used to calculate the folded structures. The $T_m$ for the sense strand was $53.6^\circ$ C (see Figure 29A). The affinity of the Adn-adduct for the sense strand in oligo form may have been greater than that of the thymine monomer individually but the HCl hydrolyzed the oligo to free bases, and possibly nucleosides ruling out this line of reasoning for the protracted retention times.

The mobile phase composition for the 9.8 minute retention time eluting peak (see Table 7) was 100 % methanol. The mobile phase was 100 % methanol from 7 to 10 minutes. The last analyte peak eluted at 11.0 minutes. The column was just beginning reequilibration when the last peak eluted.

Specificity of MRM Transition and Validity of Quantitation

Table 11 lists the possible known DNA-ABP adducts cited in the literature and indicates that $303.3 \rightarrow 152 \text{ m/z}$ transition was specific to Adn-8yl-ABP. As was mentioned in Chapter VI the $N$-(guanine-$N^2$-yl)-4-aminobiphenyl had a MW close to that of the intended adduct of quantitation, Adn-8yl-ABP. But the span of 0.6 amu (that is $\pm 0.3$ amu) would not allow the passage of the 304.1 [M+H]$^+$ parent ion of $N$-(guanine-$N^2$-yl)-ABP. Any of the other possible ABP adducts were excluded even more so from passage into the second collision quadrupole and towards detection in the third
quadrupole as a 152 m/z daughter ion fragment. The MRM transition of 303.3 → 152 m/z chosen for these analyses was specific to the desired Adn-8yl-ABP adduct.

For the chromatograms in Figure 27, S/N = 2. It is generally accepted that for the lower limit of detection (LLOD) the S/N ratio should be 3 or greater. When looking in Table 10 the 90 % confidence intervals for the results were roughly 50% of the reported values. These two factors did not bode well for the accuracy and precision of the results respectively in terms of quantitation. The tabulated t for the 90 % confidence level for n = 4 was 2.353 and the calculated t was 3.133 for comparing the results of the two tests. Because the calculated t was greater than the tabulated value, there was a significant difference between the results at this confidence level. In order to come to a result with greater certainty more chromatograms would have been necessary to increase the degrees of freedom allowing a higher confidence level tabulated t. (For a 95 % confidence level 4 dof tabulated is 2.776 and less than 3.133) There was a significant difference at the 90 % confidence level at the experimental 3 dof with a tabulated of 2.353. When the results were corrected for the loss of MS sensitivity their difference was no longer significant due to increased relative error. The corrected results were not valid for comparison.

Laboratory and Computational Results Comparison

From the computational determinations there were 8 dA and 9 dG, or a total of 17 purines non-Watson-Crick paired and available for reaction on the sense strand. The anti-sense strand had 5 dA and 4 dG, or 9 purines available for reaction. The competition for the same amount of 10 ppm ABP available was greater among the purines on the
Table 11. Literature Cited DNA-ABP Adducts

Source: The predicted masses were calculated using the ACD/Labs Web service* and Product Version 10.0#

<table>
<thead>
<tr>
<th>Adduct Name and Structure</th>
<th>Monoisotopic Molecular Weight</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$-(adenine-8-yl)-4-aminobiphenyl</td>
<td>302.127994*</td>
<td>Kadlubar 1987(^{44}); Lasko et al. 1988(^{36})</td>
</tr>
<tr>
<td><img src="image1" alt="Diagram" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N$-(guanine-N(^2)-yl)-4-aminobiphenyl</td>
<td>303.11201#</td>
<td>Kadlubar 1987(^{44}); Lasko et al. 1988(^{36})</td>
</tr>
<tr>
<td><img src="image2" alt="Diagram" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N$-(guanine-8-yl)-4-aminobiphenyl</td>
<td>318.122909*</td>
<td>Kadlubar 1987(^{44}); Lasko et al. 1987(^{102}); Lasko et al. 1988(^{36}); Bartsch et al. 1993(^{10})</td>
</tr>
<tr>
<td><img src="image3" alt="Diagram" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-(guanine-N(^2)-yl)-4-aminobiphenyl</td>
<td>318.33266#</td>
<td>Swaminathan 2002(^{43})</td>
</tr>
<tr>
<td><img src="image4" alt="Diagram" /></td>
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</table>
sense strand compared to those on the anti-sense strand. Furthermore since the adduct forming with dA was quantitated and the ratio of available reactive dA to dG was less for the sense strand than for the anti-sense strand. It followed therefore that there was less Adn-8yl-ABP adduct quantitated on the sense strand than on the anti-sense strand. See Figure 31 for a bar graph representation comparison.

Figure 31. Reactant / Product Comparison of DispoEq Coupling Reaction

Implications of Results for Bladder Cancer

The dGuo-8yl-ABP adducts in dsDNA were known to be repaired more effectively than those of their ssDNA counterparts and the other minor adducts of ABP were found to be more mutagenic. The implication was that dAdo-8yl-ABP adducts were more mutagenic. The same mutagenicity of dAdo-8yl-ABP adducts had been suggested by Hatcher et al. Hainaut et al. cited speculations in their review that adenine was affected by tobacco carcinogens. Shapiro et al. found the dAdo-8yl-ABP adduct to lie more favorably in the minor groove thermodynamically than the dGuo-8yl-
ABP adducts for whom the sequence was more of a determining factor of minor / major groove preference. In Figure 32 the upper arrow points to the minor groove and the lower arrow to the major groove (the original propamidine drug in the minor groove is not shown here). B[a]P formed an adduct with guanine in the minor groove which disturbed the B. stearothermophilus DNA polymerase’s minor groove contacts and blocked primer extension in replication.

The mutational spectrum of dGuo-8yl-ABP adducts in embryonic fibroblasts of the Big Blue mouse was not found to be in agreement with the patterns of TP53 mutations in human bladder cancer. The former was predominantly G → T transversions.

Figure 32. Major and Minor Grooves of dsDNA

Source: PDB ID: 102D
Nunn et al., Sequence-dependent drug binding to the minor groove of DNA: crystal structure of the DNA dodecamer d(CGCAAAATTGCG)2 complexed with propamidine, J. Med Chem. 1995 (38) pp. 2317-2325
while the latter was G → A transitions. But the authors cited predominance of G → T mutational spectrum of BPDE adducts matched the literature for the TP53 gene in lung cancer.

For studying the fluorinated 7-fluoro-2-aminoflourene (FAF) analogue of the DNA adduct N-(deoxyguanosine-8-yl)-2-aminofluorene (dG-C8-AF), NOESY 2-dimensional 19F-NMR was used to determine if the dG-C8-FAF was affected by its neighbor in a 12-mer duplex. It was found that when purine bases flanked the adduct on the 3’ side, the stacking (S-conformer) of FAF was preferred. The alternative position for the base was to bind (B-conformer) to the Watson-Crick complementary base pair with its FAF adduct in the major groove. The greater the Van der Waals forces of stacking the greater the S-conformer. Moreover, the higher the % S-conformer, i.e. the % stacking, the higher the nucleotide excision repair. Cho et al. described that in NOESY 1H-NMR studies the major conformer of the dGuo-8yl-ABP adduct lies in the major groove but there were minor conformers. The minor conformers were with ABP stacking and as an adduct in the minor groove. The minor conformers were to have more significance because they occurred at physiological temperatures. Blas-Machado et al. did not observe any induced upregulation of p53 in the livers of Fundulus grandis fish subjected to 2-AF in their feed.

The computationally derived configuration of the Arg 248 codon and overlapping GNA triloop is depicted in Figure 25. The two guanine bases of the CGG codon stacked anti-parallel to the adenine base 3’ to them. If dA were to have the ABP substituent in the C-8 position, the bound ABP would theoretically be able to stack with the two
neighboring guanine for stabilization. Stabilization by Van der Waals forces between the adduct and the DNA backbone could occur in instances of stacking.\textsuperscript{103} There was a documented strand bias of 60\% for G-C $\rightarrow$ A-T transitions at CpG sites on the human bladder $TP53$ sense strand vs. 40\% on the anti-sense strand.\textsuperscript{13} Codons 245 and 248 were CpG sites and had mutations with this transition. An unexplained bias for the anti-sense strand in BPDE binding affinity was observed in the literature, but it was also noted that repair of BPDE adducts was 2 to 4 times slower on the sense strand.\textsuperscript{106} Strand bias was later found to be associated with the G-C $\rightarrow$ A-T transition, transcription-couple repair preferentially repairing the bulky adducts of ABP and BPDE on the anti-sense, that is the transcribed strand.\textsuperscript{107} Pfeifer \textit{et al.} stated that the $TP53$ mutational pattern was determined by both initial DNA adduct levels and strand bias in repair.\textsuperscript{108}

During replication \textit{in vivo} bulky adducts at the replication fork caused polymerases to pause and stall.\textsuperscript{109} Stalled replication forks were thought to result in significant ssDNA. But no ssDNA was observed in EM.\textsuperscript{110} As was said in Chapter I, ABP is 50 times as mutagenic in ssDNA compared to dsDNA\textsuperscript{15} Compared to the free nucleoside, dsDNA is 2\% as reactive and ssDNA is 30\% as reactive to the nitrenium ion intermediate.\textsuperscript{46} Greenblatt \textit{et al.} described how $TP53$ gene frameshift mutations could be explained by quasi-palindromes of one strand being folded back on itself for excision or replication, using the other strand as a template.\textsuperscript{111} Greenblatt \textit{et al.} implied that secondary structure of ssDNA had a bearing on the mutational profile of adducts.
CHAPTER VIII

CONCLUSIONS

The *in vitro* relative adduct level of dAdo-8yl-ABP adducts was determined to be on the order of 5 to 15 adducts / $10^6$ nucleotides. This value compares to the literature value\(^3\) for dGuo-8yl-ABP formed on DNA modified *in vitro* of 450.0 adducts / $10^6$ nucleotides. Furthermore the sense strand had fewer dAdo-8yl-ABP adducts compared to the anti-sense strand with 5 ± 2 vs. 9 ± 4 per $10^6$ nucleotides respectively ($p = 0.10$). By computational determination the sense strand had 17 non-hydrogen bonding purines available for reaction while the anti-sense strand had 9. The dA to dG ratio available for reaction was 8 to 9 and 5 to 4 for the sense strand and the anti-sense strand, respectively. The values of these three ratios in the *VisualOMP6.0* determinations corroborated the results found by LC-MS/MS of fewer adducts on the sense (coding) strand when the complementary 45-*mer* oligos of *TP53 exon7* were compared. The strand bias observed was explained by simple, common mass balance relationships elucidated by the computational calculations in contrast to the literature reported *in vivo* transcription coupled repair on the anti-sense (transcription) strand of guanine adducts\(^1\) These results may also infer that ssDNA secondary structure and purine base ratios have an influence on the “binding specificity” and “human genomic content” variables mentioned by Feng *et al.*\(^1\)
An LC-MS method to determine the composition of the late eluting peaks is necessary. The present LC-MS/MS method was tuned specifically to the $303.3 \rightarrow 152\ m/z$ transition. The LC-MS method would be less sensitive, but scan for ions other than $303.3\ m/z$ and determine the accompanying analyte composition at those retention times. Table 12 gives a suggested experimental setup. In order to accommodate the decrease in sensitivity the sense strand DNA concentration used would be the greatest available or $5975\ \text{pmol}$ on the LC column.

Table 12. Experimental Paradigm to Investigate Late Eluting LC-MS/MS Peaks

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<thead>
<tr>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$37.5\ \mu\text{L}\ ssDNA$ in $2\times \text{KPhos}$</td>
<td>$37.5\ \mu\text{L}\ ssDNA$ in $2\times \text{KPhos}$</td>
</tr>
<tr>
<td>$37.5\ \mu\text{L}\ 10\ \text{ppm ABP}$</td>
<td>$37.5\ \mu\text{L}\ 86\ \text{ng} / 10\ \mu\text{L}\ \text{Adn-8yl-ABP}$</td>
</tr>
<tr>
<td>$75\mu\text{L}\ 1.0\ \text{N HCl}$</td>
<td>$75\mu\text{L}\ 1.0\ \text{N HCl}$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test 3</th>
<th>Test 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$37.5\ \mu\text{L}\ 10\ \text{ppm Ado}$</td>
<td>$37.5\ \mu\text{L}\ 10\ \text{ppm Ado}$</td>
</tr>
<tr>
<td>$37.5\ \mu\text{L}\ 10\ \text{ppm ABP}$</td>
<td>$37.5\ \mu\text{L}\ 86\ \text{ng} / 10\ \mu\text{L}\ \text{Adn-8yl-ABP}$</td>
</tr>
<tr>
<td>$75\mu\text{L}\ 1.0\ \text{N HCl}$</td>
<td>$75\mu\text{L}\ 1.0\ \text{N HCl}$</td>
</tr>
</tbody>
</table>

A possible area of research extending from the computational findings would be to investigate the characteristics of the GNA triloop associated with the Arg 248 hotspot
codon on the sense strand as depicted in Figure 25. A NOESY \textsuperscript{1}H-NMR structural characterization could be made such as performed by Cho \textit{et al.}\textsuperscript{91} Only bases C39 through G45 at the 3’ end of \textit{TP53} exon7 sense strand would be necessary for the determinations and would reduce the analysis to the triloop area. The objective would be to observe the stacking of the three purine bases in the triloop to verify the RNA-123 computational rendition of the structure. From there the potential stability of an ABP moiety of a dAdo-8yl-ABP stacking with the neighboring guanines in the loop and further stabilizing the configuration could be projected.

Another study would be to characterize the stacking of the adducted dAdo in the GNA Triloop. Guckian \textit{et al.} cite that naphthalene – adenine stacking was stronger than adenine – adenine stacking.\textsuperscript{99} To achieve this NOESY \textsuperscript{19}F-NMR could be used with a terminally mono-fluorinated ABP.\textsuperscript{104} Unfortunately all of the three literature references obtained for possibly incorporating an adducted base into an oligo coupled the adduct to the guanine after the purine was already in place. Steps were taken to allow only one guanine, the one of interest to be available for reaction.\textsuperscript{104,112,113} When multiple guanines were available, multiple adduct products were also encountered.\textsuperscript{104,113}

A second set of experiments would be to determine products obtained through the activity of the PCR polymerase on \textit{TP53} exon7 sense strand at progressively increasing temperatures approaching the annealing temperature of the fully random coil template oligo. The sequence would be analyzed at the complementary ends of the synthesized product that had paired with the original 3’ end of the template exon7. Of interest would be how strongly the triloop blocks the action of the polymerase as it progresses up the exon7 template. For that matter the bulge at Gly245 would also hinder the action of the
polymerase (see Figure 33). If an dAdo-8yl-ABP adduct could be positioned in some way at the A43 position of a duplex of exon 7's sense strand a study could be performed to determine if the adduct lay in the minor groove. The effect of blocking the polymerase during PCR in the full temperature range of the cycle could be determined.\(^\text{16, 103}\)

Figure 33. GNA Triloop and Bulge-Looped TP53 exon 7 Sense Strand

Codon 248 Circled at the 3' End – dG and dA Available for Attack from Activated ABP

Codon 245 Circled in Middle – Two dG Available for Attack from Activated ABP

Source: Structure Determined Computationally with VisualOMP6.0, DNA Software, Inc., Ann Arbor, MI
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Synthesis, characterization, and applications of a fluorescent probe of DNA damage.

ABBREVIATIONS

ABP, 4-aminobiphenyl; Adn and A, adenine; Ado, adenosine; dA and dAdo, deoxyadenosine; Gua and G, guanine; Guo, guanosine; dG and dGuo, deoxyguanosine; C, cytosine; 5mC, 5-methyl cytosine; T, thymine

$N$-OH-ABP, $N$-hydroxyl-4-aminobiphenyl; $N$-OH-AABP, $N$-OH-4-acetylamino-phenyl; dA-C8-4-ABP and dAdo-8yl-ABP, $N$-(deoxyadenosin-8-yl)-4-aminobiphenyl; Adn-8yl-ABP, $N$-(adenine-8yl)-4-aminobiphenyl; dG-C8-4-ABP and dGuo-8yl-ABP, $N$-(deoxyguanosin-8-yl)-4-aminobiphenyl; dG-N2-4-ABP and dGuo-N2-ABP, $N$-(deoxyguanosin-N2-yl)-4-aminobiphenyl; dG-N2-4-AABP, 3-(deoxyguanosin-N2-yl)-4-acetylamino-phenyl; FAF, 7-fluoro-2-aminofluorene; dG-C8-AF, $N$-(deoxyguanosine-8-yl)-2-aminofluorene

Ar, biphenyl; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronide transferase; Gluc, glucuronide; PHS, prostaglandin H-synthase; NO, nitric oxide; B[a]P, benz[a]pyrene; BPDE, benz[a]pyrene diol epoxide; NAT, $N$-acetyltransferase

ssDNA, single stranded DNA; dsDNA, double stranded DNA; ct-DNA, calf thymus DNA; PCR, polymerase chain reaction; $T_m$, melting temperature (for DNA); S-conformer, stacking conformer; B-conformer, binding conformer

ES, electrospray; ESI, electrospray ionization; LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; ES-MS, infusion electrospray mass spectrometry; NOESY, Nuclear Overhauser Effect Spectroscopy; KIE, kinetic isotope effect
MRM, multiple reaction monitoring; RIC, relative ion count, AU, absorbance units; V, volts; Da, Dalton; m/z, mass/charge; amu, atomic mass unit; i.d., inner diameter; S/N, LC signal to noise ratio; LLOD, lower limit of detection; LM/HM, low molecular weight / high molecular weight; SPE, solid phase extraction; G.S., generating system; DispoEq, DispoEquilibrium DIALYZERS™; RBC, red blood cell

IARC, International Agency for Research on Cancer; PDB, Protein Data Bank; MSU-MSF, the Michigan State University-Macromolecular Structure Facility

P450, pigment at 450 nm; CYP, cytochrome P450; p53, protein at ‘53 kDa’; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; PEG, polyethylene glycol; (see also chemicals subchapter for abbreviations)
PERMISSIONS

Table 1. Human Bladder Cancer Hotspots


The p53 Mutation handbook, available online; http://p53.free.fr

Data / figures included in this handbook can be used and reproduced freely by anyone as long as the reference to the p53 mutation handbook is indicated.

Figure 30. Triloop at the Arg 248 Hotspot Codon of TP53 exon7

From: John SantaLucia [mailto:jsl@chem.wayne.edu]
Sent: Sunday, March 02, 2008 2:56 PM
To: chemist@paulrkncoll.com
Subject: Re: GNA Triloop

Dear Paul,
You ave my permission to use the structure in your Thesis and your presentation. No need to do anything other than give the following citation:

Sincerely,
John