QUANTIFICATION OF 4-AMINOBIPHENYL-INDUCED GENOTOXICITY BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY: EVALUATION OF CHEMOPREVENTIVE AGENTS FOR THE INHIBITION OF BLADDER CARCINOGENESIS

by

Kristen L. Randall

to

The Department of Chemistry and Chemical Biology

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

in the field of Chemistry

Northeastern University
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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate School of Arts and Sciences of Northeastern University, May 2011
This dissertation demonstrates the significance of liquid chromatography-mass spectrometry (LC-MS) methods to the development of targeted early intervention chemopreventive strategies against bladder cancer. Here, LC-MS techniques facilitate a quantitative analysis of DNA adducts of the human bladder carcinogen 4-aminobiphenyl (4-ABP). These DNA adducts are a measure of exposure and risk, and chemopreventive agents can mitigate this risk by inhibition of adduct formation or promotion of adduct repair. Accordingly, DNA adducts are effective biomarkers of the efficacy of chemopreventive agents.

Chapter 1 provides an overview of the metabolism of 4-ABP and methods for quantifying its genetic damage. Chapter 2 reviews the application of LC-MS in the quantitative analysis of DNA adducts for the specific evaluation of chemopreventive agents. Many of the findings in this dissertation contribute to this new and exciting field.

The development of a validated LC-MS/MS method for quantification of 4-ABP-induced genotoxicity was crucial for this research and is described in chapter 3. This method overcomes analytical challenges that are often encountered in the quantitative analysis of DNA adducts from human samples, including limited sample availability and the need to reach detection limits approaching the part-per-billion threshold. By operating at nano-flow rates and incorporating a capillary analytical column in addition to an automated
sample enrichment step, we have developed a sensitive HPLC-MS/MS method appropriate for quantifying dG-C8-4-ABP. The Limit of Detection (LOD) of 5 adducts in $10^9$ nucleotides (20 attomol dG-C8-4-ABP and 1.25 $\mu$g DNA nucleosides on column) and low sample requirement of 5 $\mu$g DNA per sample makes this method an improvement from the current methods. Subsequent chapters describe our efforts to expand this research by considering DNA adducts not only as biomarkers of exposure but as significant to the discovery of chemopreventive agents.

In Chapter 4, we investigate the widespread chemopreventive strategy of activating the cytoprotective protein nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as a potential mechanism for 4-ABP DNA adduct inhibition in bladder cells and tissues. We show that Nrf2 inhibits 4-ABP DNA adduct formation locally in human bladder RT-4 cells. However, in vivo research comparing 4-ABP DNA adduct levels in Nrf2$^{+/+}$ and Nrf2$^{-/-}$ C57BL/6 mice revealed that in the process of detoxifying 4-ABP and its metabolites from the liver, Nrf2 increased the bioavailability of 4-ABP in the bladder. Subsequent experiments showed that UDP-glucuronosyltransferase (UGT) was at least partly responsible for the detoxification and transport of 4-ABP from the liver to the bladder: Nrf2 up-regulated UGT, promoted conjugation of 4-ABP with glucuronic acid in the liver, and increased urinary excretion of the conjugate. We concluded that Nrf2 activation that doesn’t result in up-regulation of liver UGT should be considered and investigated further as a chemopreventive strategy against 4-ABP induced bladder carcinogenesis.
In Chapter 5, two cancer chemopreventive agents Sulforaphane (SF) and 5,6-Dihydrocyclopenta[c][1,2]-dithiole-3(4H)-thione (CPDT) were evaluated for their inhibition of 4-ABP DNA adducts. SF and CPDT, both derived from compounds found in cruciferous vegetables, are well known chemopreventive agents that induce Nrf2 and have been shown to be specific for bladder tissue. We found that SF and CPDT both activate Nrf2 and the Nrf2 cytoprotective signaling pathway in human bladder carcinoma RT-4 cells and C57BL/6 mouse bladder tissue. Both agents inhibit 4-ABP DNA adduct formation in bladder cells and tissues and require Nrf2 to exert substantial cytoprotective effects. Furthermore, neither agent induced UGT liver enzymes that are highly significant in the transport of 4-ABP to the bladder. These results suggest that SF and CPDT are promising chemopreventive agents against 4-ABP-induced bladder carcinogenesis and provide mechanistic insight into their cytoprotective effects.

Future directions of this research are considered in Chapter 6. This includes quantification of the isomers of 4-ABP DNA adducts, a comprehensive human smoke-quit study involving the quantitative monitoring of 4-ABP DNA adducts in urothelial cells over an extended period of time, an analysis of additional metabolites of 4-ABP, and the evaluation of structural analogs of SF and CPDT.
ACKNOWLEDGEMENTS

As I complete my degree requirements, I owe many thanks to many people and most importantly, to my research advisor Dr. Paul Vouros. Dr. Vouros accepted me into his well respected lab providing me with tremendous learning opportunities. He has given me invaluable guidance and advice in my research and I have learned so much from him. He truly cares about his students and I am thankful for his continuous patience and compassion.

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Thank you to everyone who has helped me along the way.
to my mother and father, Barbara and Robert
## TABLE OF CONTENTS

Abstract 2
Acknowledgements 6
Dedication 9
Table of Contents 10
List of Figures 14
List of Tables 19
List of Abbreviations 20

### Chapter 1: DNA adducts of the human bladder carcinogen 4-aminobiphenyl as biomarkers of exposure 26

1.1 Bladder cancer and its causes 27
1.2 Metabolism of 4-ABP 30
1.3 Formation of 4-ABP DNA adducts 36
1.4 4-ABP mutagenicity and link to bladder cancer 38
1.5 Quantification of 4-ABP DNA adducts 40
1.6 Significance of dose-response studies as representations of real-life exposure 49
1.7 4-ABP DNA adducts in smokers versus non-smokers 50
1.8 Conclusions 51
Chapter 2: Quantification of bulky DNA adducts by liquid chromatography-mass spectrometry for the evaluation of early intervention chemopreventive agents

2.1 Early intervention chemoprevention 67
2.2 LC-MS/MS of modified nucleotides 71
2.3 Mass spectrometric detection of modified bases 75
2.4 Additional applications of LC-MS to chemopreventive agent analysis 83
2.5 Conclusions 84

Chapter 3: An improved liquid chromatography-tandem mass spectrometry method for the quantification of 4-aminobiphenyl DNA adducts in urinary bladder cells and tissues

3.1 Introduction 92
3.2 Project goals 97
3.3 Experimental 97
3.4 Results and Discussion 105
3.5 Conclusions 134
Chapter 4: Activation of Nrf2 as a chemopreventive strategy against 4-ABP-induced bladder cancer

4.1 Introduction

4.2 Project goals

4.3 Experimental

4.4 Results

4.5 Discussion

4.6 Conclusions

Chapter 5: Inhibition of 4-ABP DNA adduct formation by Sulforaphane and 5,6-dihydrocyclopenta[c][1,2]-dithiole-3(4H)-thione in bladder cells and tissues

5.1 Introduction

5.2 Project goals

5.3 Experimental

5.4 Results

5.5 Discussion

5.6 Conclusions
Chapter 6: Summary and Future Directions

6.1 Summary

6.2 Detection and quantification of dG-ABP isomers

6.3 Investigation into 4-ABP metabolism

6.4 Comprehensive human study

6.5 Evaluation of analogs of SF and CPDT

Biographical data
# LIST OF FIGURES

## Chapter 1

Figure 1.1 The chemical structures of arylamines associated with human bladder cancer. 29

Figure 1.2 4-ABP and some of its metabolites. 31

Figure 1.3 Schematic of 4-ABP metabolism. 33

Figure 1.4 4-ABP DNA adducts. 37

Figure 1.5 A good correlation was observed in a comparison of 4-ABP DNA adduct levels between a GC/MS analysis and an immunohistochemical method with fluorescence detection. 48

## Chapter 2

Figure 2.1 Selected phytochemicals that have been identified as potential chemopreventive agents. 69

Figure 2.2 MS/MS spectrum of dG-C8-4-ABP showing the loss of deoxyribose [M+H-116]^+. 73

Figure 2.3 Three isomers from the B[a]P treated MCF-7 cells were detected in an LC-MS/MS analysis. 74

Figure 2.4 Co-treatment of cells with B[a]P and chrysoeriol reduced BPDE-DNA adduct levels. 75

Figure 2.5 LC-MS of aflatoxin-N7-guanine isolated from urine. 77

Figure 2.6 Calibration curve of AFB1 dose versus AFB1-N7-gua detected in rat urine. 78

Figure 2.7 LC-MS detection of AFB1-N7-gua in rat urine of rats treated with AFB1. 79

Figure 2.8 Chemical structures of Resveratrol, NAcCys, melatonin, and lipoic acid. 81
Chapter 3

Figure 3.1 Typical offline SPE workflow. 93

Figure 3.2 A. Isolation of precursor ion, no fragmentation.  B. Fragmentation amplitude: 0.10 volt.  C. Fragmentation amplitude: 0.15 volt.  D. MS$^3$ 435→319→302. 106

Figure 3.3 The proposed fragmentations for dG-C8-4-ABP. 107

Figure 3.4 HPLC-MS of dG-C8-4-ABP standards. 108

Figure 3.5 Typical workflow in DNA adduct analysis by LC-MS. 111

Figure 3.6 A detailed view of the microfluidic chip used in our automated sample enrichment method. 113

Figure 3.7 A detailed view of the Agilent valve of the microfluidic chip used in our automated sample enrichment method. 114

Figure 3.8 Heat treatment stability assessment. 115

Figure 3.9 Internal standard degradation during digest. 117

Figure 3.10 Standard curve dG-C8-4-ABP of fmol analyte on column versus the ratio of analyte to internal standard peak areas were generated in triplicate over an 8-month period. 119

Figure 3.11 Zoom of the lowest five points of the calibration curve. 121

Figure 3.12 LOD and LOQ. Extracted ion chromatograms (435→319) of the LOD, 20 amol on column in 1.25 µg DNA or approximately 5 adducts in 10⁹ nucleosides, the LOQ, 70 amol on column in 1.25 µg DNA or 2 adducts in 10⁸ nucleosides, and the procedure blank, 1.25 µg calf-thymus DNA. 122
Figure 3.13 Logarithmic scale plot of dG-C8-4-ABP levels observed in dosed RT-4 human bladder carcinoma cells.

Figure 3.14 Plot of 4-ABP dose versus detected dG-C8-4-ABP quantity in bladder tissue from rats treated with 4-ABP.

Figure 3.15 Zoom in of the EIC (435→319 for the analyte and 444→328 for the IS) from the lowest dose rat and control samples.

Figure 3.16 Analysis of dG-C8-4-ABP adducts by digesting 1 µg of DNA.

Chapter 4

Figure 4.1 Structure of 4-ABP-N-glucuronide.

Figure 4.2 Schematic of glucuronidation in the metabolic pathways of 4-ABP induced human bladder cancer.

Figure 4.3 An example EIC in the time course study.

Figure 4.4 4-ABP-induced DNA damage in human bladder carcinoma RT-4 cells in a dose duration study.

Figure 4.5 4-ABP-induced DNA damage in human bladder carcinoma RT-4 cells in a dose concentration study.

Figure 4.6 A western blot of Nrf2 in whole cell lysates from RT-4 cells treated with control siRNA and Nrf2 siRNA for 48 hours.

Figure 4.7 dG-C8-4-ABP DNA adduct levels in human bladder carcinoma RT-4 cells.

Figure 4.8 dG-C8-4-ABP adduct levels in wild type and Nrf2 knockout mouse bladder tissue following treatment with 50 mg/kg 4-ABP.
Figure 4.9  dG-C8-4-ABP adduct levels in wild type and Nrf2 knockout mouse liver tissue following treatment with 50 mg/kg 4-ABP.

Figure 4.10  Western blots of liver homogenates from Nrf2<sup>+/+</sup> mice and Nrf2<sup>−/−</sup> mice.

Figure 4.11  S9 fractions were prepared from liver tissues of Nrf2<sup>+/+</sup> mice and Nrf2<sup>−/−</sup> mice and measured for UGT activity in catalyzing 4-ABP conjugation with glucuronic acid.

Figure 4.12  Total amounts of 4-ABP-N-glucuronide were measured in 24 h urine collected from Nrf2<sup>+/+</sup> mice and Nrf2<sup>−/−</sup> mice given 50 mg/kg 4-ABP and were adjusted by urinary creatinine levels.

Figure 4.13  Full scan of 1 ng 4-ABP-N-glucuronide synthetic standard. A. Full scan. B. EIC 346.1 C. Mass spectrum.

Figure 4.14  LC-MS/MS of 4-ABP-N-glucuronide standard. The top panel shows the extraction ion chromatogram m/z 346 → 170 and the bottom panel shows the mass spectrum under this peak.

Figure 4.15  Injection of mouse urine sample. 200 µL of urine from two wild type mice that were administered 50 mg/kg 4-ABP was taken through an acetonitrile precipitation and injected for detection of 4-ABP-N-glucuronide.

Chapter 5

Figure 5.1  Chemical structures of SF and CPDT.

Figure 5.2  Conversion of glucoraphanin to SF or the SF nitrile.

Figure 5.3  SF and PEITC reduced AFB<sub>1</sub> DNA adduct formation in human hepatocyte cultures.
Figure 5.4 The structures of three dithiolethiones D3T, Olitpraz, and ADT.

Figure 5.5 SF activates Nrf2 and the Nrf2 signaling pathway in human bladder carcinoma RT-4 cells.

Figure 5.6 CPDT activates Nrf2 and the Nrf2 signaling pathway in human bladder carcinoma RT-4 cells.

Figure 5.7 Wild-type C57BL/6 mice and Nrf2 knocked out C57BL/6 mice were treated with vehicle or SF by gavage once a day for 5 days.

Figure 5.8 Nrf2<sup>+/+</sup> mice and Nrf2<sup>-/-</sup> mice were treated with CPDT or vehicle by gavage once daily for 5 days; 24 h later after the last CPDT dose, animals were killed, and phase 2 enzymes in the bladders and livers were measured by Western blotting.

Figure 5.9 4-ABP-induced DNA damage in human bladder cells and the protective effect of SF.

Figure 5.10 4-ABP-induced DNA damage in human bladder cells and the protective effect of SF in sprout form.

Figure 5.11 4-ABP-induced DNA damage in rat bladder cells and the protective effect of SF in sprout form.

Figure 5.12 CPDT inhibits 4-ABP DNA adduct formation in human bladder carcinoma RT-4 cells.

Figure 5.13 SF requires Nrf2 to inhibit 4-ABP DNA adduct formation in mouse bladder tissue.

Figure 5.14 CPDT inhibits 4-ABP DNA adduct formation in wild type mouse bladder tissue.

Figure 5.15 CPDT does not inhibit 4-ABP DNA adduct formation in mouse liver tissue.
LIST OF TABLES

Chapter 3

Table 3.1  LC-MS methods for quantifying dG-ABP adducts. 96
Table 3.2  Dose–response data in human cells and rat tissue. 128
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degree Centigrade</td>
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<tr>
<td>1,6-DNP</td>
<td>1,6-dinitropyrene</td>
</tr>
<tr>
<td>³H</td>
<td>Tritium</td>
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<td>4-ABP</td>
<td>4-aminobiphenyl</td>
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<td>4-OHE₂</td>
<td>4-hydroxyestradiol</td>
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<td>ACN</td>
<td>Acetonitrile</td>
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<td>ADT</td>
<td>5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione</td>
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<td>AFB₁</td>
<td>Aflatoxin B₁</td>
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<td>Aflatoxin B₂</td>
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<tr>
<td>amol</td>
<td>Attomole</td>
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<tr>
<td>AMS</td>
<td>Accelerator mass spectrometry</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>B[a]P</td>
<td>Benzo[a]pyrene</td>
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<tr>
<td>BBI</td>
<td>Bowman-Birk Inhibitor</td>
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<td>BBN</td>
<td>N-butyl-N-(4-hydroxybutyl)nitrosamine</td>
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<tr>
<td>BPDE</td>
<td>Benzo[a]pyrene diol epoxide</td>
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<td>CE</td>
<td>Capillary electrophoresis</td>
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<td>CH$_3$OH</td>
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<td>Chlorophyllin</td>
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<td>CID</td>
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<tr>
<td>CNL</td>
<td>Constant neutral loss</td>
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<td>CPDT</td>
<td>5,6-dihydrocyclopenta[c][1,2]-dithiole-3(4H)-thione</td>
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<tr>
<td>ct DNA</td>
<td>Calf thymus DNA</td>
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<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
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<td>Differential mobility spectrometry</td>
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<td>DMSO</td>
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<tr>
<td>EIC</td>
<td>Extracted ion chromatogram</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Ethoxyresorufin-O-deethylase</td>
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<td>Epithiospecifier protein</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GC</td>
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<td>GCS_c</td>
<td>Catalytic subunit of glutamate cysteine ligase</td>
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<td>GST</td>
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<td>HPLC</td>
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<td>i.p.</td>
<td>Intraperitoneal injection</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>ID</td>
<td>Internal diameter</td>
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<td>IQ</td>
<td>2-amino-3-methylimidazo[4,5-f]quinoline</td>
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<td>Keap1</td>
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<td>LC</td>
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<td>LC-MS</td>
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<td>LIF</td>
<td>Laser-induced fluorescence</td>
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<td>N-acetylcysteine</td>
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<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>N-acetyltransferase 1</td>
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<td>Nrf2</td>
<td>NF-E2 related factor 2</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEITC</td>
<td>Phenylethyl isothiocyanate</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>ppb</td>
<td>Part per billion</td>
</tr>
<tr>
<td>QIT</td>
<td>Quadrupole ion trap</td>
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<tr>
<td>QR</td>
<td>NAD(P)H-quinone reductase</td>
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<tr>
<td>R²</td>
<td>Correlation coefficient</td>
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<td>RF</td>
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<td>Radioimmunoassay</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RP</td>
<td>Reverse phase</td>
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<tr>
<td>Abbreviation</td>
<td>Term</td>
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<td>RSD</td>
<td>Relative standard deviation</td>
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<td>Retention time</td>
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<td>Standard deviation</td>
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<td>SERMS</td>
<td>Selective estrogen receptor modulators</td>
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<td>Sulforaphane</td>
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<td>SIM</td>
<td>Single ion monitoring</td>
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<td>SPE</td>
<td>Solid phase extraction</td>
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<td>Selective reaction monitoring</td>
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<td>Sulfotransferase</td>
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<td>Uridine 5’-diphospho (UDP)-glucuronosyltransferase</td>
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<td>Ultra performance liquid chromatography</td>
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<td>Volume to volume ratio</td>
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<td>Microgram</td>
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CHAPTER 1

DNA ADDUCTS OF THE HUMAN BLADDER CARCINOGEN

4-AMINOBIPHENYL AS BIOMARKERS OF EXPOSURE
1.1 Bladder cancer and its causes

Bladder cancer is a widespread health problem. The National Cancer Institute (NCI) estimates that as one of the most common types of malignancies, the United States alone saw 70,530 new cases and 14,680 deaths in 2010 (1). This prevalence, compounded by the frequently prolonged treatments and high recurrence rate, makes it the most expensive cancer to treat and monitor in the United States (2). Thus, there is a need for a greater understanding of the causes of bladder cancer, its progression, and methods for treatment and prevention.

Smoking is the primary cause of bladder cancer but other risk factors include age, occupation, race, gender, certain infections, treatment with cyclophosphamide or arsenic, family history, personal history, low fluid intake, and diets low in fruits and vegetables and high in fat (1-7). Certain polyaromatic hydrocarbons and arylamines have been also implicated in bladder cancer (8-11). For most individuals, the primary source of exposure to arylamines is through cigarette smoke. Certain industries are also associated with high levels of exposure to arylamines, including rubber, plastics, cable, and wood manufacturing, but scientists and technicians who directly handle arylamines are at the greatest risk for occupational exposure (12). Additional sources of arylamine exposure include colorants, pesticides, fumes from heated oils and fuels, drugs, fugitive emissions, and the environment as a result of the combustion of nitrogen containing organic material (5, 11, 13-21).
Several arylamines have been assessed by the International Agency for Research on Cancer (IARC) and categorized based on carcinogenicity. 4-aminobiphenyl (4-ABP), 2-naphthylamine, and benzidine are known human bladder carcinogens and classified in Group I as carcinogenic to humans (22-24). Other arylamines are strongly associated with bladder cancer: O-toluidine is classified in Group 2A as probably carcinogenic to humans and 2,6 dimethylaniline and 4,4’-methylenebis(2-chloroaniline) are classified in Group 2B as possibly carcinogenic to humans (25). The structures of these arylamines are shown in Figure 1.1.

Certain structural characteristics of an arylamine can be an indication of the extent of its carcinogenicity. All arylamines contain at least one aromatic hydrocarbon and one amine, but the positions of the amine substituents and the number of aromatic rings vary, affecting hydrophobicity and electronic and steric properties. Arylamines that have more amine substituents and that are more hydrophobic are more likely to be absorbed and permeate biological membranes for faster distribution to the target tissues (26). The placement of amine group in the ortho or para position of phenyl compounds and the β or 2 position of polycyclic aromatic compounds increases the chance that the arylamine will be N-oxidized and activated (27). 4-ABP for example, which has an amine at the para position is more carcinogenic than 3-ABP and 2-ABP (28, 29).
Figure 1.1. The chemical structures of arylamines associated with human bladder cancer.
1.2 Metabolism of 4-ABP (17)

4-ABP is a procarcinogen and is not genotoxic and mutagenic until it is converted to an active metabolite that will react with DNA. The resulting DNA adducts are recognized as an initiating step in carcinogenesis (21). The structures of 4-ABP and some of its metabolites are shown in Figure 1.2. The metabolism of 4-ABP can be described as a series of activating and deactivating reactions, several of which are shown in Figure 1.3 (17). Activating reactions lead to the formation of the active metabolite and ultimate carcinogen while detoxifying and deactivating reactions lead to the excretion of unreactive metabolites of 4-ABP.

4-ABP is activated to N-hydroxy-4-ABP (N-OH-4-ABP) primarily in the liver. The hepatic enzymes that catalyze this reaction are cytochrome P450 1A2 (CYP1A2) in rats and humans (30) and another unidentified form of cytochrome P450 (CYP450) in mice (31, 32). Following activation, N-OH-4-ABP then either remains in the liver or is transported through the blood to other organs. N-OH-4-ABP however is also known to form in the bladder from 4-acetylaminobiphenyl, N-hydroxy-4-acetylaminobiphenyl, and acetylCoA (33-36). N-OH-4-ABP can then undergo further activation via Phase 2 conjugation reactions such as sulfation, acetylation or glucuronidation. Sulfation of N-OH-4-ABP is catalyzed by hepatic sulfotransferases (SULT) enzymes and is associated with liver DNA adducts. In the bladder, N-acetyltransferase 1 (NAT1) catalyzes the O-
Figure 1.2. 4-ABP and some of its metabolites.
or N-acetylation of N-OH-4-ABP to form N-acetoxy-4-aminobiphenyl and N-hydroxy-4-acetyl-aminobiphenyl (N-OH-AABP). N-OH-AABP and N-acetoxy-4-acetylaminobiphenyl can be then converted to N-acetoxy-4-aminobiphenyl by an N,O-acetyltransferase (33, 37). Under acidic conditions the acetyl group of N-acetoxy-4-aminobiphenyl will be oxidized to form the nitrenium ion, the reactive electrophilic ion that ultimately adds to DNA.

These 4-ABP activation reactions are in constant competition with detoxification reactions. Early detoxification reactions of 4-ABP in the liver, such as ring hydroxylation, N-acetylation, and N-glucuronidation involve formation of metabolites that can not be directly converted to N-OH-4-ABP by the activating CYP450 enzymes. Although N-acetyl-4-ABP metabolites can be oxidized to N-OH-AABP and metabolized to an O-glucuronide which is transported to the bladder, this is not acid labile and is excreted. Thus, these 4-ABP detoxification reactions compete with the activating N-oxidation of 4-ABP. If N-OH-4-ABP is formed in the liver there are deactivation reactions that can occur and these include Phase 2 reactions such as acetylation, glucuronidation, sulfation, and glutathione addition. These conjugates can be transported from the liver to the bladder for excretion. However, many of the resulting metabolites may be converted back to the active metabolite or ultimate carcinogen before excretion occurs. For example, N-glucuronides of 4-ABP that are transported to the bladder are acid labile and can result in the formation of DNA adducts (36).
Figure 1.3. Schematic of 4-ABP metabolism. (Reproduced from (32))
Differences in response to 4-ABP exposure explain variations in cancer risk. While the target organ in humans, rabbits and dogs is the bladder, 4-ABP targets the liver and bladder in mice, and mammary glands and intestine in rats (12, 38-40). Differences in 4-ABP metabolism however, are apparent not only among species but also among individuals within a species. This is due to a number of factors that affect the activation and deactivation of 4-ABP including genetic polymorphisms, variations in conjugation reactions, urinary pH, and urothelial cell proliferation.

Rate of 4-ABP acetylation has a large impact on whether or not the procarcinogen will be activated and is determined by genetic polymorphism. Humans either have the phenotype of a rapid or slow acetylator, and this is referring specifically to the activity of N-acetyltransferase-2 (NAT2). NAT2 is expressed primarily in the liver and intestinal epithelium (41) and catalyzes the formation of 4-acetylnobiphenyl from 4-ABP before it can undergo CYP450 catalyzed oxidation to N-OH-4-ABP. In slow acetylators, 4-ABP remains in the liver for a relatively long time, increasing the possibility of the formation of the active metabolite. Indeed, slow acetylators have been identified as being more susceptible to bladder cancer than rapid acetylators because they are exposed to more active carcinogens in the bladder (42, 43). Genetic polymorphisms of NAT1, another acetylator but one which is primarily expressed in the bladder and catalyzes 4-ABP detoxification reactions, have not been identified as having significant effects on bladder cancer development.
Variations in the activities of Phase 2 enzymes also affect the outcome of 4-ABP exposure. These enzymes catalyze competing reactions where inhibition of one type of conjugation reaction may result in enhancement of the others, and vice-versa (44). Phase 2 metabolism of N-OH-4-ABP is typically thought of as a detoxification but the resulting conjugates may be oxidized to form the nitrenium ion that adducts to DNA and some are more susceptible to this than others. Additionally, certain Phase 2 reactions are associated with organ-specific tumors. Sulfation of N-OH-4-ABP for example, is linked to liver cancer. Human sulfotransferases will catalyze the formation of DNA adducts from N-OH-4-ABP in the liver, possibly even reducing adduct formation in the bladder (45). Glucuronidation of N-OH-4-ABP is associated with bladder cancer (46, 47). In the liver, uridine 5'-diphospho (UDP)-glucuronosyltransferase (UGT) catalyzes the formation of a 4-ABP N-glucuronide conjugate, a metabolite that is more water soluble than 4-ABP. It is transported from the liver to the urinary bladder where it is either excreted or at low pH, dissociates to form the 4-ABP nitrenium ion capable of adducting to DNA. Hence, competing activities of Phase 2 enzymes can influence whether or not 4-ABP will be excreted or genotoxic, and if genotoxic, which tissues are affected.

If 4-ABP metabolites are transported to the bladder, urinary pH, mostly a consequence of diet, affects the dissociation of 4-ABP esterified compounds and whether or not the nitrenium ion will form. If other bladder carcinogens are present, there may be a synergistic response resulting in a greater risk of bladder cancer development. Furthermore, exposure to these carcinogens may be even more damaging if cells are
already proliferating. Risk of developing bladder cancer therefore, is not only dependent on exposure but also on a number of modulating factors specific to an individual.

1.3 Formation of 4-ABP DNA adducts

The 4-ABP nitrenium ion is electrophilic and covalently binds to nucleophilic sites on DNA. More than 70% of these 4-ABP DNA adducts are located at the C8 position of guanine to produce 4-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-4-ABP) (48-55), but other adducts have been identified and characterized: 4-(deoxyadenosin-8-yl)-4-aminobiphenyl (dA-C8-4-ABP) and a hydrazo linked adduct, 4-(deoxyguanosin-N2-yl)-4-aminobiphenyl (dG-N2-N4-4-ABP) were observed in hepatic cells that were treated with 4-ABP (56) and 3-(deoxyguanosin-N2-yl)-4-aminobiphenyl (dG-N2-4-ABP) was observed in human uroepithelial cells treated with N-OH-4-ABP (57). The structures of these 4-ABP DNA adducts are shown in Figure 1.4.
Figure 1.4. 4-ABP DNA adducts.
1.4 4-ABP mutagenicity and link to bladder cancer

If these 4-ABP-DNA adducts are not repaired, mutations can occur that ultimately may lead to bladder cancer (58-65). The genetic events of carcinogenesis have been classified into the three distinct phases of initiation, promotion and progression, although this process may not actually transpire in such a well defined sequence. The initiation phase is usually associated with carcinogen binding and damage to DNA. We are constantly exposed to carcinogens and DNA adducts are constantly forming but the majority of DNA damage is repaired. However, over time genetic mutations accumulate and the cell enters the promotion phase. Cell proliferation begins and the cancer advances to the progression phase. While 4-ABP DNA adducts form within hours of exposure, 4-ABP-induced mutations arise within two weeks of exposure in vivo and urinary bladder tumors take years to develop (66-68).

Some researchers have focused on the effect of 4-ABP on the highly significant tumor suppressor gene $p53$. 4-ABP has been shown to induce $p53$ mutations in the bladder (20, 69-72) and several studies have found a link between 4-ABP induced $p53$ mutations and bladder tumor grade and invasiveness (73). Additionally, common codons of mutational hotspots in the $p53$ gene were observed in human bladder cancer and cell cultures treated with 4-ABP (74). For example, codon 285 on the $p53$ gene is a mutational hot spot in human bladder cancer and also the preferential binding site for 4-OH-ABP (75).
However, the relationship between 4-ABP exposure, the resulting genetic damage, and bladder cancer risk is not completely understood and results have been conflicting (12). In some cases, a clear relationship was identified between 4-ABP adduct levels and tumorogenesis in bladder tissue (19, 76, 77) and several studies have shown that after cases were adjusted for exposures, higher levels of DNA adducts were observed in cancer patients than non-cancer patients (78-82). However, in another study adduct levels were not correlated with tumor grade (83). These researchers hypothesized that the time between the adduct formation and the initiation of tumors may account for these differing results; the quantified adduct levels might reflect the average 4-ABP exposure at the moment the measurement is made while the tumor grade reflects the accumulation of genetic damage (83).
1.5 Quantification of 4-ABP-DNA adducts (84)

4-ABP DNA adducts are important biomarkers in 4-ABP-induced bladder cancer. Quantitative relationships can be evaluated between mechanisms of carcinogenesis ranging from initiation to promotion to progression, but the measurement of DNA adducts has several advantages over others as a gauge of exposure and risk. For example, because DNA adduct formation is the initiating step in carcinogenesis, their quantification is an early detection measurement that can be made within hours of exposure. Other measurements of later stages of carcinogenesis are not early detection measurements and might only be detectable weeks or years after the initiation stage (85, 86). Another advantage of 4-ABP DNA adduct quantification is that the source of the DNA damage is inherent in the measurement, while in DNA mutagenesis studies or tumor grade analyses the cause may not be as clear. Indirect measurements of 4-ABP exposure can only serve as estimates of potential DNA damage but are often easier to make because of the relative high concentrations of the analyte. Such measurements include the quantification of 4-ABP in cigarette smoke or the quantification of 4-ABP hemoglobin adduct levels. Direct measurements of 4-ABP DNA adducts account for inconsistencies in adsorption, metabolism, detoxification, and DNA repair and are consequently more relevant to assessing the effect of exposure on bladder cancer (87). Accordingly, DNA adducts serve as both biomarkers of exposure and susceptibility to cancer and for that reason their measurement has significant implications for disease risk assessment (88).
DNA adducts also have a specific niche as biomarkers of the efficacy of chemopreventive and chemotherapeutic agents. As a measurement of genotoxic risk and present in all individuals in at least background levels, DNA adducts can be used to identify agents that decrease this risk by inhibition of their formation or promotion of their repair. Chemoprevention studies are aimed at preventing carcinogenesis and require biomarkers of cancer initiation rather than progression and DNA adducts meet this criterion (40). Chemotherapeutic agents are evaluated based on their efficacy in decreasing malignancy (85) and as DNA adducts have been detected in tumor tissue, they can also serve as biomarkers of therapeutic intervention in later stages of carcinogenesis.

Several techniques appropriate for the quantification of 4-ABP DNA adducts are discussed below (84). Important factors to consider include sensitivity, selectivity, and amount of DNA required. In the analysis of DNA adducts from human samples, adduct levels are expected to be in the range of 1 adduct in $10^8$ nucleosides. Furthermore, these adducts will likely be present in complex mixtures and typically only μg quantities of DNA are available. As an example of one human study the GC-MS analysis of bladder cancer biopsies had a limit of detection of 0.1 fmol of 4-ABP DNA adduct per ug DNA and DNA adducts were detected in 37 out of 75 patients. The average DNA adduct level was $2.7 \pm 0.7$ fmol/ug DNA adducts ($86 \pm 22$ adducts in $10^8$ nucleosides) (76). These adduct quantities have been observed in a number of studies employing a wide array of
distinct analytical methodologies that include $^{32}\text{P}$-postlabeling, immunoassays, fluorimetry, and mass spectrometry (89).

1.5.1 $^{32}\text{P}$-postlabeling (90)

$^{32}\text{P}$-postlabeling involves radiolabeling adducted nucleosides and measuring the $^{32}\text{P}$-decay. In this method, DNA is enzymatically digested to 3’ mononucleotides and adducted nucleotides are isolated and radiolabeled at the 5’-position with $[\gamma-^{32}\text{P}]\text{ATP}$ by polynucleotide kinase-mediated phosphorylation. The resulting [5’-32P]-3’-biphosphates are chromatographically separated and $^{32}\text{P}$-decay is measured.

$^{32}\text{P}$-postlabeling can be combined with High Performance Liquid Chromatography (HPLC) or Thin Layer Chromatography (TLC) for greater specificity. These methods also have high sensitivity, requiring only 10 µg DNA or less for the analysis of samples containing only 1 adduct in $10^{10}$ nucleotides. The technique is particularly useful when analyzing adducted nucleosides in complex mixtures.

However, $^{32}\text{P}$-postlabeling requires working with radioactive material and does not provide structural information. In addition, the technique generally requires extensive sample preparation. Errors can occur in quantification: false negatives reflect the loss of adducts during sample preparation, incomplete digestion, and inefficiency of labeling and false-positive results result from polynucleotide kinase labeling of non-nucleic acid
components and contamination of DNA with RNA. Improvements in sensitivity are limited because the technique is dependent on the specific activity of $[^{32}\text{P}]-\text{ATP}$.

1.5.2 Immunoassays (91)

In immunoassay based DNA adduct quantification, antibodies react with specific DNA adducts and are subsequently detected by radiometry, fluorometry, coulorometry, or chemiluminesence (92-94). There are a large variety of immunoassay techniques for the analysis of DNA adducts each with their own advantages and drawbacks. Immunohistochemical techniques for example can be used to detect adducts in specific cell types in small amounts of tissue, either frozen or paraffin preserved. However, these assays have low sensitivity and quantification is relative. Absolute quantification is possible using immuno-dot/slot-blot methods, which involve detection of enzyme-labeled secondary antiserium by colorimetric or chemoluminescent techniques. However, although less than a $\mu\text{g}$ of DNA per sample is analyzed, the assay is not sensitive enough for the analysis of human samples. Radioimmunoassays (RIAs) are sensitive and reproducible but have been largely replaced by the enzyme-linked immunosorbent assay (ELISA), which doesn’t require the use of handling radioactive material and is considered to be sensitive enough for the analysis of human samples. Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) uses the same antisera as ELISA and has greater sensitivity. The chemiluminescence immunoassay (CIA) is even more sensitive than DELFIA and is almost as sensitive as $^{32}\text{P}$-postlabelling. Furthermore, there is a lower background, higher signal-to-noise, and standard curves are more reproducible.
In general, immunoassays have the advantage of being high-throughput, rapid, straightforward, reproducible and highly sensitive.

However, immunoassays have a number of drawbacks. These techniques require raising specific antibodies from rabbits or mice, characterizing the antiserum, and validating the assay. They rely on antibodies to react specifically with certain DNA adducts, but in practice these antibodies cross-react with different structurally similar DNA adducts and are therefore considered to be class specific rather than adduct specific. Furthermore, those antibodies that react with DNA adducts also react with the corresponding RNA adducts.

Monoclonal antibodies and rabbit sera have already been developed for 4-ABP DNA adducts (95). The monoclonal antibody 3D6, for example is specific for dG-C8-4-ABP (96) and has been used as an enrichment step before LC-MS/MS analysis of human bladder samples (97).

1.5.3 Fluorescence spectroscopy

Fluorescence assays have been developed that either exploit the fluorescent characteristics of some adducts or attach a fluorescent label to non-fluorescent adducts. HPLC combined with fluorescence detection allows for the detection of stereoisomers. They are fast and inexpensive, and although they require large quantities of DNA (about 100 to 1000 µg), the method has a detection limit of about 1 adduct in $10^8$ nucleotides. In
synchronous fluorescence spectrophotometry, excitation and emission wavelengths are scanned at a fixed wavelength difference. Theoretically, this technique can be applied to resolve multiple components in mixtures without separation. For the detection of non-fluorescent adducts, capillary electrophoresis and laser-induced fluorescence (CE-LIF) can be an ultrasensitive assay. Fluorescent dyes can also be chemically linked to DNA adducts and then separated and detected by CE-LIF. This approach achieved a detection limit of 2 adducts in $10^6$ nucleotides for PAHs. Electrostacking further improved the detection limit to about 1 adduct in $10^7$ nucleotides. Hydroxyl-specific fluorescence labeling of dG-ABP has been achieved with BODIPY (98).

1.5.4 Mass spectrometry

The main advantage of mass spectrometry in DNA adduct quantification is that in addition to the quantitative determination of adduct levels, it provides structural characterization of the analyte. This is useful for confirmation that the correct compound is being analyzed, identification of unknown compounds, and structural characterization of standards. These standards may then be used to determine adduct levels by mass spectrometry or other methods. Mass spectrometry methods can also have high sensitivity and low sample requirements (99). Furthermore, this technology is continually improving in sensitivity due to advances in efficiency of ionization, transmission and detection of ions. Accurate quantification of DNA adduct levels is achieved with stable isotope internal standards which account for losses during sample preparation and variations in response of the mass spectrometer, including ion
suppression. Typical detection limits are at about 1 adduct in $10^8$ nucleotides using about 50 µg of DNA. Gas chromatography and liquid chromatography are separation methods commonly performed before mass spectrometric analysis. The use of these methods decreases interferences in the mass spectrometric analysis of the analyte and also provides an important characteristic for identification of the analyte, retention time.

GC-MS requires DNA adducts to be volatile and sample preparation therefore involves de-glycosylation and derivatization. Because de-glycosylation is required however, RNA interferences can be a problem. Chemical derivatization methods, such as silylation and electrophore labeling introduce problems such as the production of artifacts, interferences and sample to sample variability in derivatization (100).

In LC-MS analysis, DNA adducts can be analyzed in their nucleoside, nucleotide, and oligonucleotide forms. LC however, usually has lower peak resolution than GC. In LC analysis, the sample must be only soluble in the mobile phase before injection, rather than be thermally labile as in GC-MS analysis. Mass spectrometric analysis requires that the analyte is in the gas phase, and two ionization methods that are commonly used are ESI and APCI (99). In the analysis of bulky DNA adducts such as 4-ABP, reversed phase separation is often employed. If this is too hydrophilic, ion-pairing methods may used but this introduces ion suppression effects in electrospray ionization, decreasing overall sensitivity.
1.5.4.a. Accelerator mass spectrometry (AMS) (101)

AMS measures isotopes with a low natural abundance and a long half-life and is the most sensitive analytical method for detecting DNA adducts. Measurements are precise and detection limits can be as low as 1 adduct in $10^{12}$ nucleotides. However, cross-contamination of isotopes between samples is a huge problem. In addition, the technique may not differentiate between different metabolites of the same labeled carcinogen so sample preparation must isolate the analyte from possible interferences.

1.5.5 Summary of techniques for the quantification of 4-ABP DNA adducts

Sensitivity, selectivity, and the amount of DNA required for an analysis are significant parameters in a method for the quantitative analysis of DNA adducts; the most sensitive technique is AMS, the most selective methods are mass spectrometry based, and the method requiring the lowest quantity of DNA for an analysis is usually $^{32}$P-postlabeling.

One study compared absolute 4-ABP DNA adduct levels detected between ESI-LC-MS, $^{32}$P-postlabeling, DELFIA, and specific binding of $^3$H-radiolabeling. In this research, $^{32}$P-postlabeling underestimated adduct levels (3-5% relative level), DELFIA overestimated adduct levels (260-550% relative level) and ESI-LC-MS gave levels of adducts similar to those obtained in the specific binding of the $^3$H-radiolabel assay (102). Furthermore, they found that LC-MS was the most precise method.
However, it can be difficult to compare methods based on the absolute number of adducts detected because often the recovery of the adduct during sample preparation is unknown and the accuracy in quantification can not always be determined. One study was designed to compare immunohistochemistry-fluorescence and GC-MS methods for detection of 4-ABP DNA adducts in liver tissue of 4-ABP treated mice. A good correlation was observed and is shown in Figure 1.5. Rather than comparing absolute adduct levels, it focused on relative increases in fluorescence intensity or adduct levels within each method (103).

**Figure 1.5.** A good correlation was observed in a comparison of 4-ABP DNA adduct levels between a GC/MS analysis and an immunohistochemical method with fluorescence detection. (Reproduced from (103))
1.6 Significance of dose-response studies as representations of real-life exposure

(104, 105)

Cell and animal dose response studies are useful models for carcinogen-induced genotoxicity and tumor formation in humans. In DNA adduct dose response studies, cells or animals are treated with a carcinogen and the resulting formation of DNA adducts is measured. All of the pathways involved in the formation and repair of DNA adducts can be saturated. If no pathway is saturated, a linear dose-response relationship is expected. However, if activation is saturated, a supralinear response occurs and if detoxication or DNA repair is saturated, a sublinear response occurs. Exposure in humans generally occurs chronically and at low doses reaching steady-state concentrations. Therefore, a linear relationship is observed between carcinogen exposure and DNA adduct formation (106). Steady-state levels can also be reached under experimental conditions if exposure continues at low levels for several weeks allowing adducts to accumulate (39, 107-110).

The synergistic effect of exposure to multiple carcinogens can result in greater damage in real-life exposure than in experimental models at the same exposure levels. For example, other chemicals in cigarette smoke increase the genotoxicity of 4-ABP; reactive oxygen species (ROS) or free radicals hydroxylate DNA bases, causing single strand breaks or oxidative DNA damage. Furthermore, fifty of the approximately 4000 hazardous chemicals in cigarette smoke have been directly implicated in cancer (IARC) (111).
Increased cell proliferation caused by the many chemicals in cigarette smoke provides more DNA on which 4-ABP can cause damage, potentiating its genotoxicity.

### 1.7 4-ABP DNA adducts in smokers versus non-smokers

Although several studies have not established a relationship between detected DNA adducts and the formation of tumors, many have found that adduct levels are higher in smokers than nonsmokers (49, 76, 112-117). Exposure to cigarette smoke is the main source of 4-ABP and the main cause of bladder cancer (118-120). Cigarette smoke is also known to contribute to many cancers including lung, larynx, pharynx, esophagus, bladder, uterine, kidney, cervical and pancreatic cancers (120, 121). Mainstream cigarette smoke, composed of both inhaled and exhaled smoke, contains 2.4 to 4.6 ng 4-ABP per filtered cigarette and 0.2 to 23 ng per filtered cigarette and sidestream smoke emitted directly from the end of a lit cigarette, contains up to 140 ng 4-ABP per cigarette (122).
1.8 Conclusions

4-ABP is implicated in bladder cancer, a serious health problem. Its DNA adducts reflect exposure and tumor development and can be used as a biomarker for risk assessment and for the evaluation of chemopreventive agents. LC-MS/MS is one of the most sensitive and specific techniques available for the measurement of DNA adducts. In the next chapter, LC-MS techniques in chemoprevention research involving DNA adducts will be discussed. This is followed by a record of our own research developing a highly sensitive LC-MS/MS method for 4-ABP DNA adduct formation, the evaluation of a chemopreventive strategy for inhibition of DNA adduct formation, and the evaluation of two chemopreventive agents against 4-ABP-induced bladder cancer.
References


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CHAPTER 2

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC ANALYSIS OF BULKY DNA ADDUCTS FOR THE EVALUATION OF EARLY INTERVENTION CHEMOPREVENTIVE AGENTS
2.1 Early intervention chemoprevention

The significance of cancer prevention research is well recognized and this is manifested in its high priority level in research funding by the National Cancer Institute (NCI) (1). Although many studies are focused on the later stages of carcinogenesis for slowing, preventing, or reversing tumor progression (2), measures taken before cancer initiates may actually have the greatest potential for reducing cancer incidences and costs (3). While minimizing exposure to known carcinogens is one effective approach to decreasing the likelihood of cancer initiation, carcinogens are ubiquitous and impossible to completely avoid (4-6). Accordingly, an additional precaution against cancer initiation may involve administration of a chemopreventive agent to inhibit genetic damage caused by carcinogens in people at high risk for tumor development.

An ideal chemopreventive agent is inexpensive, effective, easily and infrequently administered and has minimal side effects (7, 8). This is especially important in primary prevention in which subjects have no symptoms of cancer development. Agents can be either natural or synthetic but those that are in foods consumed by people are often desired because it is already known that they are safe at least at the levels at which they are typically consumed. Fruits and vegetables contain many potential chemopreventive agents and in fact the International Agency for Research on Cancer (IARC) has associated their consumption with reduced risk of cancer (9). Furthermore, researchers have identified specific compounds in these foods that are responsible for their anti-
carcinogenic properties. Many anticancer studies are focusing on non-traditional nutrients like phytochemicals and other bioactive plant chemicals that have potential chemopreventive activity, such as those shown in Figure 2.1 (10). Plant derived agents being investigated for their chemopreventive potential include the Bowman-Birk Inhibitor (BBI), carotenoids, curcumin, indoles, isothiocyanates, tea polyphenols, resveretrol, retinoids, soy isoflavones, selenium compounds, sulfur containing antioxidants, and vitamin D. Other agents being investigated include anti-androgens, epidermal growth factor receptor-tyrosine kinase (EGFR-TK) inhibitors, NO-releasing NSAIDS, NSAIDS, ornithine decarboxylase, PPAR gamma agonists, p53 suppressors or gene modulators, resquimod, rexanoids, selective estrogen receptor modulators (SERMS), and statins (11).

The analysis of carcinogen-DNA adduct biomarkers is one approach that has been taken for measuring the efficacy of early therapeutic intervention. The formation of DNA adducts is recognized as an initiating step in the development of carcinogenesis and therefore mitigation of adduct quantities by a chemopreventive agent may demonstrate its potential to reduce risk of cancer development. Adducts present in tissue from healthy individuals may indicate risk of cancer development and need for chemopreventive intervention. The biological relevance to DNA adducts in chemoprevention has been reviewed (12, 13).
Figure 2.1. Selected phytochemicals that have been identified as potential chemopreventive agents. (Reproduced from (14))
Many of these agents exert their protective effects through multiple mechanisms which can be elucidated through various biochemical techniques combined with DNA adduct quantification. These biochemical techniques include knocking out key genes, analyzing multiple analogs of a class of chemopreventive agents to determine the important structural features, or exposing samples to several carcinogen metabolites to determine the stage at which the chemopreventive agent acts. Mechanisms by which chemopreventive agents can decrease DNA adduct levels include detoxification of the carcinogen, eliciting antioxidant activity or electrophile scavenging activity, inducing Phase 2 carcinogen-detoxification enzymes, inhibiting Phase 1 carcinogen-activating enzymes, enhancing DNA repair systems, or inducing cell apoptosis (15, 16). Understanding these mechanisms is useful in the discovery of other chemopreventive agents and in drug modification and development.

Sensitivity, selectivity, and low sample requirement are important parameters in the quantification of DNA adducts. Many preliminary studies on chemoprevention of DNA adducts incorporate high doses of carcinogens and chemopreventive agents. While these studies are meaningful and may reveal important characteristics about chemopreventive agents, those that involve low amounts of chemical treatments resembling typical human exposure are most relevant and are often the best indicators of an agent’s chemopreventive capacity. Several studies have shown that the degree of protection by a chemopreventive agent is dependent on the carcinogen dose and that often greater protection is observed at lower carcinogen doses (17). Furthermore, in the analysis of
human samples, limited sample may be available, necessitating not only high sensitivity but also a low sample requirement. Widely used techniques for the analysis of DNA adducts include $^{32}$P-postlabeling, immunoassays, fluorimetry, accelerator mass spectrometry and mass spectrometry and comparisons between them is the focus of many reviews (12, 18-24).

2.2. LC-MS/MS of modified nucleotides

LC-MS/MS quantification of DNA adduct formation is generally achieved by detection of the adducted monomeric nucleoside. In this type of analysis, DNA is enzymatically digested to mononucleosides and the adducted nucleosides are enriched, often through solid phase extraction (SPE). Quantification of adduct levels is accomplished through the addition of a stable isotope internal standard prior to enzymatic digestion and the subsequent analysis of a standard curve. Samples prepared in the absence of chemopreventive agents are used as a positive control and percent inhibition of a particular chemopreventive agent can be calculated based on the adduct levels of these samples. The analyte is identified by retention time and MS/MS loss of deoxyribose [M+H-116]$^+$ (25).

Tandem mass spectrometry provides increased specificity and due to the universal MS/MS behavior of the nucleosides’ loss of the deoxyribose group, it is well suited for
their analysis. MS/MS quantification of these monomeric nucleosides is usually performed on a triple quadrupole because of these instruments’ large dynamic range, high duty cycle, sensitivity, precision, and accuracy, especially with multiple analytes. Selective reaction monitoring (SRM) is typically used for quantification. In this case, the protonated adduct [M+H]^+ is selectively transmitted through the first analyzer Q1, fragmented in Q2 by collision-induced dissociation (CID), and the fragment exhibiting the loss of the deoxyribose, [M+H-116]^+ is selectively transmitted in Q3.

An example mass spectrum of the MS/MS analysis of dG-C8-4-ABP is shown in Figure 2.2. Here, the loss of the deoxyribose group from the molecular ion m/z 435.1 results in the transmission of the aglycone ion m/z 319.1. Higher collision-energies can further fragment the aglycone ion to obtain structural information. Constant neutral loss (CNL) can be used to screen a complex sample for an overall study of the adducts present. However due to the slow scanning rate of the triple quadrupole, approximately 100-fold lower sensitivity is observed in a CNL analysis than in an SRM analysis and lower abundance adducts are less likely to be detected. For this same reason, lower sensitivity is also observed in product ion scans.
A sensitive LC-MS/MS method based on the characteristic loss of deoxyribose was developed for detection of benzo[a]pyrene (B[a]P) DNA adduct formation and this was applied to the evaluation of methoxy flavone chrysoeriol (3’-methoxy-4’,5,7-trihydroxyflavone) as an inhibitor of these adducts. In this analysis, three isomers from the B[a]P treated MCF-7 cells were detected and are shown in the chromatogram in Figure 2.3 as one major peak and two smaller peaks 1 and 2. The major peak was identified as (+)-trans-BPDE-\( N^2 \)-dG. This was known because it was the standard used in the study. The other smaller peaks were assumed to be (+)-anti-cis-BPDE-\( N^2 \)-dG (peak 1) and syn-BPDE-\( N^2 \)-dG (peak 2). The retention times of the three isomers
differed, but all showed the characteristic loss of m/z 116 (m/z 570→454). Interestingly, co-treatment of cells with 2 µM B[a]P and 5 or 10 µM chrysoeriol inhibited (+)-trans-BPDE-N²-dG and (+)-anti-cis-BPDE-N²-dG formation but not syn-BPDE-N²-dG formation (Figure 2.4). The dominant adduct formed, (+)-trans-BPDE-N²-dG is considered to be the most mutagenic and was the adduct most inhibited by chrysoeriol (27). However, it has been hypothesized that the high abundance isomer is not always the isomer with the greatest contribution to the development of cancer, possibly because high abundance isomers are more likely to be repaired (28-32). Accordingly, the analysis and inhibition of (+)-anti-cis-BPDE-N²-dG and syn-BPDE-N²-dG is of great interest.

Figure 2.3. Three isomers from the B[a]P treated MCF-7 cells were detected in an LC-MS/MS analysis. (Reproduced from (27))
Figure 2.4. Co-treatment of cells with B[a]P and chrysoeriol reduced BPDE-DNA adduct levels. Solid bars represent adduct levels in cells treated only with 2 μM B[a]P. Lined bars represent adduct levels in cells treated with 2 μM B[a]P and 5 μM chrysoeriol and dotted bars represent cells that were treated with 2 μM B[a]P and 10 μM chrysoeriol. While (+)-trans-BPDE-N\textsuperscript{2}-dG and (+)-anti-cis-BPDE-N\textsuperscript{2}-dG levels decreased significantly following treatment with chrysoeriol, syn-BPDE-N\textsuperscript{2}-dG levels did not significantly change. (Reproduced from (27))

2.3. Mass spectrometric detection of modified bases

DNA damage can also be quantified for the purposes of chemopreventive agent evaluation by a surrogate method of detecting modified bases that have been excised from DNA. When a carcinogen binds to nucleophilic sites of adenine or guanine, the carcinogen-purine conjugate may be hydrolyzed from the DNA strand leaving a
depurinated site. This depurinated site can then contribute to mutations, including error prone repair. Accordingly, depurinating adducts can play a major role in the initiation of cancer making them valuable biomarkers of the need for chemopreventive intervention. A major advantage of using depurinating adducts as biomarkers is that samples are readily accessible; rather than having to isolate genomic DNA from tissues, modified bases can be extracted from urine or blood. A drawback however is that the measurement of the carcinogen-purine conjugate is removed from genetic damage. It is not known whether or not the DNA is repaired and if it is repaired correctly. Furthermore, detection of depurinating adducts is only a measure of recent exposure and the base may not necessarily be from DNA but could be from RNA.

Before LC-MS/MS analysis of depurinating adducts, DNA is usually removed from the sample by precipitation and the carcinogen-purine conjugates are concentrated by SPE. If the sample source is urine, adduct levels can be normalized to creatinine levels. Just like in the quantitative analysis of nucleoside adducts, the use of calibration curves and internal standards enable quantification and the analytes are characterized based on retention time and fragmentation patterns. Both absolute amount of DNA adducts and percent inhibition information can be obtained.

An example LC-MS/MS of a depurinating adduct, aflatoxin-N7-guanine is shown in Figure 2.5. This analysis was done on a quadrupole ion trap and the ions from both the base (m/z 152) and the aflatoxin adduct (m/z 329) are visible in the mass spectrum.
LC-MS/MS quantification of depurination by the human liver carcinogen aflatoxin B$_1$ (AFB$_1$) was used to determine the efficacy of Oltipraz inhibition. Rats were treated with AFB$_1$ and their urine was analyzed for depurinated products. The two major compounds they observed were aflatoxin B$_1$-N$^7$-guanine (AFB$_1$-N$^7$-gua) and its imidazole ring opened derivative 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy-aflatoxin B$_1$ (AFB-FAPyr). Aflatoxin B$_2$ (AFB$_2$) was used as an internal standard for recovery and for quantification because it is not naturally produced. The calibration curve for this study is shown in Figure 2.6 and plots AFB$_1$ dose as a function of pg AFB$_1$-N$^7$-gua/mg creatinine. For increased analyte enrichment following SPE, the samples were purified on a monoclonal antibody immunoaffinity column. MS analysis was performed on an LCQ. LC-MS and LC-MS/MS chromatograms and mass spectra of their detection of AFB$_1$-N$^7$-gua are shown in Figure 2.7. As expected, a
significantly better signal-to-noise ratio was observed in the tandem MS experiment. Treatment with Oltipraz resulted in significantly reduced adduct levels (34).

**Figure 2.6.** Calibration curve of AFB₁ dose versus AFB₁-N⁷-gua detected in rat urine. (Reproduced from (34))
Figure 2.7. Panel A shows the LC-MS detection of AFB$_1$-$N^7$-gua in rat urine of rats treated with AFB$_1$. Panel B shows the LC-MS/MS scan of the same sample. Panel C shows the full MS scan from the chromatogram of panel A and panel D shows the MS/MS scan from the chromatogram of panel B. (Reproduced from (34))
A study measuring depurinating estrogen adducts was designed to evaluate several chemopreventive agents, the structures of which are shown in Figure 2.8. Several studies have found that healthy women have lower estrogen-purine adducts in their urine than women at high risk or with breast cancer. Accordingly, decreasing these adduct levels by chemopreventive agents may reflect a decrease in risk or occurrence of breast cancer. Depurination of DNA following exposure to estrogens has been shown to be inhibited by several natural compounds using an LC-MS based technique. In this case, calf-thymus DNA was treated with estradiol-3,4-quinone (E$_2$-3,4-Q) or lactoperoxidase (LP)-activated 4-hydroxyestradiol (4-OHE$_2$) and a potential chemopreventive agent. Following DNA precipitation and SPE purification, the most predominant adducted bases 4-OHE$_2$-1-N$_3$Ade and 4-OHE$_2$-1-N$_7$-Gua were detected and quantified by Ultraperformance LC-MS/MS analysis with a triple quadrupole mass spectrometer. N-acetylcysteine (NAcCys) and reduced lipoic acid reduced the formation of E$_2$-3,4-Q adducts but resveratrol and melatonin did not. Adduct formation by LP-activated 4-OHE$_2$ was inhibited by all of the compounds, the greatest by resveratrol followed by NAcCys, reduced lipoic acid, and finally melatonin (35). In a subsequent manuscript, this same group showed that resveratrol also inhibited E$_2$-3,4-Q or 4-OHE$_2$-induced adduct formation in human breast epithelial cells (MCF-10F) (36).

Comparison between estrogen-purine adduct levels resulting from E$_2$-3,4-Q or 4-OHE$_2$ enabled the researchers to gain insight into the mechanisms of action of chemopreventive agents. They speculated that the chemopreventive agents could either prevent the
formation of E$_2$-3,4-Q and its reaction with DNA or modulate estrogen activating or deactivating enzymes. However, as their research was with calf thymus DNA and did not include these enzymes, they were essentially evaluating the antioxidant effects of the chemopreventive agents on E$_2$-3,4-Q or 4-OHE$_2$. NAcCys’s or dihydrolipoic acid’s inhibition of depurinating adducts increased more significantly with increasing concentration of the chemopreventive agent when the DNA was treated with 4-OHE$_2$ than E$_2$-3,4-Q. In contrast, melatonin or resveratrol only reduced depurination when DNA was treated with 4-OHE$_2$ but not E$_2$-3,4-Q. This indicated that all four agents were involved in reducing the E$_2$-3,4-semiquinone to 4-OHE$_2$, but only NAcCys and dihydrolipoic acid directly interacted with E$_2$-3,4-Q (35). In later work, Resveratrol was shown to inhibit depurination when MCF-10F cells were treated with E$_2$-3,4-Q but this was attributed to its induction of quinone reductase (36).

Figure 2.8. Chemical structures of Resveratrol, NAcCys, melatonin, and lipoic acid.
Clinical trials involving LC-MS analysis of depurination by AFB$_1$ have been carried out to investigate the chemopreventive potential of chlorophyllin (CHL) and SF. These studies were done in an area where people are at a high risk for the development of hepatocellular carcinoma due to consumption of foods contaminated with aflatoxins. Both studies measured the urinary levels of AFB$_1$-N$^7$-gua and used AFB$_2$ as an internal standard. SPE enriched the aflatoxins and this was followed by further enrichment on an AFB$_1$-specific preparative mAb immunoaffinity column. AFB$_1$-N$^7$-gua and AFB$_2$ were quantified by measuring the peak area of specific MS/MS daughter fragments MH$^+$ 152.1 and 259.1 from the parent ions 480.1 and 315.1.

CHL was found to reduce urinary levels of these adducts. This CHL intervention trial involved 180 healthy adults who ingested 100 mg CHL or a placebo three times a day for 4 months. AFB-N$^7$-gua was detected in 105 of 169 samples and Chlorophyllin consumption led to 55% lower urinary adduct levels. The researchers concluded that consumption of CHL could reduce the development of hepatocellular carcinoma (33).

In the SF inhibition clinical trial, 200 healthy adults drank hot water infusions of 3-day-old broccoli sprouts every night for two weeks. The hot water infusions of one group contained 400 µmol glucoraphanin while those of the placebo group contained less than 3 µmol glucoraphanin. Participants were not allowed to eat any green vegetables that contain glucosinolates. Although there was not a significant difference in AFB$_1$-N$^7$-gua between people taking broccoli sprouts and people taking the placebo, this was attributed
to the large variation in the bioavailability of SF in the broccoli sprout preparation. Bioavailability of SF was monitored through the urinary levels of dithiocarbamates, which are metabolites of SF. The researchers did observe an inverse association between dithiocarbamate and AFB$_1$-N$^7$-gua concentrations in the group taking the broccoli sprouts but not in the placebo group (8).

2.4. Additional applications of LC-MS to chemopreventive agent analysis.

In addition to DNA adduct quantification research, LC-MS is useful for characterizing chemopreventive agents, determining their bioavailability, identifying and quantifying their metabolites, and investigating their interaction with carcinogens. Unlike UV, fluorescence, and electrochemical detection, mass spectrometry-based methods provide structural information about a compound which is especially important in the analysis of metabolites of chemopreventive agents.

Many chemopreventive agent metabolites exist in biological fluids as Phase 2 metabolites, including glucuronide and sulfonate conjugates and their detection would provide insight into their metabolism and bioavailability. An analysis of the carcinogen metabolites can be used to determine the ability of a chemopreventive agent to modulate the carcinogen’s metabolism. Typical sample preparation for this analysis involves incorporation of an isotopically labeled internal standard and analyte enrichment, such as
by SPE. However, dilution-only methods have also been shown to be effective and eliminate both time-consuming sample preparation steps and the chance that metabolites were lost during these steps. Hydrolysis of these metabolites would be useful in understanding their bioavailability, while isolation and analysis of the entire conjugate would provide insight into their metabolism. Metabolite profiling usually involves a full scan which provides information on the high abundance metabolites, but lower abundance metabolites are not detected. Constant neutral-loss is useful for detecting glucuronides (loss of 176), sulfates (loss of 80) and glutathione (GSH) adducts (loss of 129). Furthermore, the neutral loss of 56 Da (2 x CO) is a common feature of all isoflavones (37).

2.5. Conclusions

DNA adducts are valuable biomarkers of the efficacy of chemopreventive agents. As a measurement of genotoxic risk and present in all individuals, they can be used to identify agents that decrease this risk by inhibition of their formation or promotion of their repair (12, 38). Furthermore, in the evaluation of chemopreventive agents, it is important to have a biomarker that can be measured before cancer develops (13). In the case where carcinogenesis has been initiated, DNA adducts can potentially serve as biomarkers of chemopreventive intervention (12).
Comprehensive detection of these adducts for chemoprevention presents several analytical challenges. A sensitive method is required for research that involves the detection of low abundance isomers. The importance of a highly sensitive method is even greater in research that most closely resembles typical human exposure, an important feature of an experiment as carcinogen dose affects the metabolism and formation of adducts. Moreover, if the detection of adducts is for the evaluation of chemopreventive agents, adduct levels will be decreased even further. LC-MS based techniques are well suited for these analyses because it provides structural information and sensitivity is continually increasing.

These analyses in combination with others are useful in identifying and evaluating chemopreventive agents. Other important measurements include measuring antioxidant activity, inhibition of Phase 1 enzymes such as Cyp450 carcinogen activating enzymes through the EROD assay, measuring the induction of Phase 2 enzymes, protein adduct analysis and metabolite analysis.


phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China, *Cancer Epidemiol Biomarkers Prev* 14, 2605-2613.


CHAPTER 3

AN IMPROVED LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD FOR THE QUANTIFICATION OF 4-AMINOBIPHENYL DNA ADDUCTS IN URINARY BLADDER CELLS AND TISSUES
3.1 Introduction

A major challenge in the analysis of bulky DNA adducts such as 4-ABP has been the relatively low levels at which they typically occur in vivo. They are found in a complex matrix of protein, ribonucleic acid (RNA), and salt as well as excess unmodified bases, which in the case of 4-ABP is a million to a billion-fold (1, 2). Moreover, often only a small quantity of DNA may be obtainable for analysis from in vivo samples, necessitating minimal analyte loss during sample handling and a sensitive analytical method. Detection at the part-per-billion threshold has become achievable with impressive advances in the trace level detection of bulky DNA adducts by liquid chromatography-mass spectrometry (LC-MS); improvements in sample preparation techniques and in instrumentation have led to reduced sample requirements and greater overall sensitivity.

An enrichment step prior to LC-MS analysis is desired for maximum sensitivity in the measurement of DNA adducts because the matrix in which these adducts are found will interfere with their detection (2). Typical techniques employed for purifying adducted nucleosides following isolation and digestion of genomic DNA include liquid-liquid partitioning, solid-phase extraction (SPE), HPLC, further enzymatic digestion and immunoaffinity chromatography. Liquid-liquid extraction involves handling hazardous organic materials and immunoaffinity chromatography is expensive. SPE has been successfully used in our laboratory to extract DNA adducts from their complex matrix.
A typical manual SPE workflow is demonstrated in **Figure 3.1**. The cartridge is first washed, generally with the same solvent used for elution. The sample is then loaded onto the column and washed to remove unadducted nucleosides and salts. Finally, the analyte is eluted and prepared for analysis.

![Figure 3.1. Typical manual SPE workflow. (Reproduced from (3))](image)

There are a number of drawbacks to manual SPE. The procedure can be labor-intensive and time-consuming, especially if a large number of samples are involved. Impurities from the extracts, which were either in the sample initially or added to the sample during SPE, can lead to pressure build-up on HPLC columns in the LC-MS analysis, ion suppression, and reduced recoveries (4). Analyte loss and the hydrolysis of modified nucleosides both contribute to measurement errors that may also be introduced. A major limitation of many studies using manual SPE is the requirement of relatively large (100 \( \mu \)g) quantities of DNA in order to compensate for the inevitable analyte losses during sample processing.
For these reasons, automated enrichment, which minimizes sample handling by incorporating a trapping column before the analytical column, is preferred. This requires two HPLC pumps and a valve. Because enrichment is carried out within the system, less analyte is lost. Not only are results more reproducible, sample preparation time is significantly decreased and the method can be high throughput (5).

The overall sensitivity of the analysis can be improved by reducing flow rates to the nanoliter (nL) per minute range and accordingly, incorporating smaller internal diameter (ID) analytical columns and ionizing by nanoESI (6). Sources under atmospheric pressure, such as in ESI, are particularly susceptible to ion loss during transfer of ions from the source to the analyzer and decreasing the flow rate leads to more efficient transfer. Because only a very small fraction of the molecules are actually ionized, it is important to maximize ion transfer. Use of nanoESI, instead of ESI or microESI, improves ionization efficiency and allows for a smaller sample volume without decreasing signal to noise ratio; Because mass spectrometry is concentration dependent, low concentrations in low volumes and low concentrations in high volumes produce the same signal to noise ratio (7).

Developments in mass analyzer technology have also led to improvements in sensitivity by reducing ion losses, more efficiently transferring ions from the source to the detector, and improving detection sensitivities. Various devices such as radio frequency (RF) ion
guides, lenses, and ion funnels have been developed to facilitate efficient ion transfer and reduce losses in ion current between major components in a mass spectrometer (7). Developments in technology that improve efficiency and therefore sensitivity have been made in ionization, transmission and detection of ions (4).

With the increase in sensitivity and decrease in sample loss during analyte enrichment and analysis, the amount of DNA required per analysis is reduced. This is particularly relevant in the analysis of in vivo samples where limited sample is available. In many cases only limited sample is available in experimental systems too, such as those that involve small rodents with small tissues. The amount of DNA in different types of tissues vary; from 20 mg of liver tissue, one may expect to isolate 60 µg DNA while 20 mg of bladder tissue may only yield 20 µg DNA and 200 mL of urine may only yield 1 µg urothelial cell DNA. With lower yields of DNA, it is particularly important that the method used for detection is sensitive.

As quantitative LC-MS methods have been developed for the analysis of 4-ABP DNA adducts, there is a general trend of decreasing sample requirement and limit of detection (LOD). This can be attributed to the optimization of enrichment conditions or use of an automated clean-up step, use of reduced flow rates and capillary ID columns coupled to nanoESI, and development of more sensitive mass analyzer technology. Some of these methods are presented in Table 3.1 (2, 8-13).
<table>
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<th>Paper</th>
<th>Quantity of DNA processed (µg)</th>
<th>Flow rate (µL/min)</th>
<th>LOQ (adducts/nucleosides)</th>
<th>Enrichment method</th>
<th>Analytical column ID (mm)</th>
<th>Type of mass spectrometer</th>
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<td>Single quadrupole</td>
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<td>Protein precipitation, online trap column</td>
<td>0.075</td>
<td>QIT</td>
</tr>
</tbody>
</table>

**Table 3.1.** LC-MS methods for quantifying dG-ABP adducts.
The method we present here integrates a 75 µm i.d. analytical column with automated sample enrichment on a trap column prior to analysis, reducing both the quantity of DNA required and detection levels. This improved DNA adduct quantification methodology has a detection level of 5 adducts per $10^9$ nucleosides using a total of 5 µg of DNA and the equivalent of only 1.25 µg of DNA per analysis.

3.2 Project Goals

The goal of this project was to develop a sensitive LC-MS/MS method for the quantification of dG-C8-4-ABP in human cells and tissues. Furthermore, this method was designed to quantify dG-C8-4-ABP adducts from genomic DNA in human urothelial cells to establish a procedure for a future comprehensive human smoke-quit study.

3.3 Experimental

All cell and animal dosing was done in Dr. Yuesheng Zhang’s laboratory at Roswell Park Cancer Institute, Buffalo, NY by Joseph Paonessa and Yi Ding.
3.3.1 Chemicals and Standards

McCoy’s 5A medium supplemented with 10% v/v fetal bovine serum from Life Technologies (Grand Island, NY), rat liver S9 from Moltox (Boone, NC), Nicotinamide adenine dinucleotide phosphate (NADP) from Amresco, Inc. (Solon, OH) and Harlan 7012 Nature Ingredient diet from Harlan Laboratories (Bartonsville, IL) were required during cell and animal dosing periods. The following chemicals were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO): D-Glucose-6-phosphate disodium hydrate, calf thymus DNA, nuclease p1 from penicillium citrinium, deoxyribonuclease 1 (DNase I) type 2 from bovine pancreas, alkaline phosphatase from Escherichia coli (type III), ethanol, magnesium chloride, dimethyl sulfoxide (DMSO), and 4-Aminobiphenyl (4-ABP). Hydrochloric acid was purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphodiesterase 1 (crotalus adamanteous venom) was purchased from USB Corporation (Cleveland, OH). For HPLC-MS/MS analysis, acetic acid (glacial, 99.99+%), ethanol, acetonitrile, and water were obtained from Thermo Fischer Scientific (Pittsburgh, MA) and were HPLC grade. N-(2-deoxyguanosine-8-yl)-4-ABP (dG-C8-4-ABP) was acquired from Toronto Research Chemicals (North York, ON) and the deuterium labeled internal standard dG-C8-4-ABP-D_9 was previously synthesized and characterized in our laboratory (14).
3.3.2 Cell study

RT-4 human bladder carcinoma cells were grown in McCoy’s 5A medium supplemented with 10% v/v fetal bovine serum and maintained in a humidified incubator at 37°C with 5% CO2. Cells were plated at a concentration of 2.0 million cells per 10-cm dish with 10 mL growth medium overnight and then treated with 0, 0.5, 5.0, or 50. µM 4-ABP dissolved in DMSO for 3h in the presence of 6% rat liver S9, 10 mM D-Glucose-6-phosphate disodium hydrate, and 5 mM NADP. Triplicate cultures were completed at each condition. Following treatment, cells were harvested by trypsinization, washed once with ice cold PBS and stored at -80°C until analysis. DNA was extracted using Qiagen Blood and Cell Culture DNA Midi Kits according to the manufacturer’s instructions. Cell cultures of roughly 5 million cells yielded 20 to 40 µg of nuclear DNA.

3.3.3 Animal study

Twelve two-month old male F/344/NHsd rats (approximately 180 g of body weight) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The protocol was reviewed and approved by the Animal Care and Use Committee at Roswell Park Cancer Institute. Animals were acclimated for one week and were fed the Nature Ingredient diet (Harlan 7012) and water ad libitum. 4-ABP was prepared in DMSO and administered by IP injection at doses of 0, 25, 100, or 250 mg/kg of body weight in a final volume of 0.1 mL per rat. Cell and animal dosings were completed in triplicate at each concentration. Animals were sacrificed 24 h after the dosing, and the urinary bladders were obtained immediately and stored at -80°C. DNA was extracted with an Invitrogen Easy-DNA kit.
according to the manufacturer’s instructions. Approximately 10 to 30 µg of DNA was isolated from rat bladder specimens of about 30 mg each.

3.3.4 Exfoliated human urothelial cell study

Four 150 - 250 mL urine samples from a lifetime non-smoker were collected and stored at -80°C. Samples were thawed and urothelial cells were pelleted by centrifugation at 8,000 x g for 10 minutes at 4°C (Thermo Scientific, Sorvall RT-1). DNA was isolated using a Qiagen Blood and Cell Culture DNA Midi Kit with the following yields: 0, 1.3, 2.2 and 2.8 µg. One µg of DNA was removed from each sample, digested according to the procedure described below and reconstituted in 20 µL 10% methanol for three 5 µL injections per sample. DNA from two of the samples was pooled for a 1 µg digest to be spiked with IS and 2.24 fmol dG-C8-4-ABP before protein precipitation. The remainder of DNA from the third sample was reserved for testing digestion efficiency. Specifically, 1 µg urothelial cell DNA was pooled with 10 ng DNA isolated from 4-ABP dosed RT-4 cells. For comparison, 1 µg calf thymus DNA was also pooled with 10 ng of the same adducted RT-4 cell DNA. These two samples were digested according to the protocol described below and reconstituted in 20 µL 10% methanol for three 5 µL injections.

3.3.5 DNA quantification, enzymatic digestion and protein precipitation

DNA isolated from cells and tissues was dissolved in 10 mM MgCl₂/5 mM Tris-HCl buffer (pH 7.2) to about 1 mg/mL. An Invitrogen Corporation (Carlsbad, CA) Quant-IT™ double strand (ds) DNA BR Assay kit with a Qubit fluorometer was used for DNA
quantification. One or 5 µg aliquots were removed for digestion and analysis, and the remainder was stored at -80°C. DNA was hydrolyzed according to a previously described procedure (15) with some modifications. Specifically, samples were incubated at 98°C for 3 to 5 minutes and chilled on ice during the addition of 0.3 units of nuclease P1 (0.3 units µL⁻¹ solution of 5 mM tris(hydroxymethyl)amino methane (Tris-HCl, pH 7.4) and 3.1 Kunits of DNase I (1 µg µL⁻¹ solution in 5 mM Tris-HCl/10 mM MgCl₂, pH 7.4) per µg of DNA. Following a 5-hour incubation at 37°C, 0.003 units of phosphodiesterase (100 ng µL⁻¹ in 5 mM Tris-HCl/10 mM MgCl₂, pH 7.4), and 0.002 units of alkaline phosphatase per µg of DNA were added and the reaction mixture was held at 37°C for 18 hours. At the end of the incubation period, 6 fmol of internal standard, dG-C8-4-ABP was added per µg DNA. The digestion was terminated with the addition of five volumes of ice-cold ethanol (-20°C). Proteins were pelleted by centrifugation at 7500 x g (Thermo Scientific, Sorvall RT-1) for 15 min at 4°C. Following lyophilization, samples were reconstituted in 20 µL of 10% methanol. Samples at replicate doses were pooled and analyzed in triplicate.

3.3.6 Preparation of dG-C8-4-ABP standard curves

Calf-thymus DNA was weighed out and dissolved in 10 mM MgCl₂/5 mM Tris-HCl buffer (pH 7.2) to an approximate concentration of 1 mg/mL. DNA concentration was verified using the Invitrogen Corporation (Carlsbad, CA) Quant-IT™ double strand (ds) DNA BR Assay kit with the Qubit fluorometer. Following DNA digestion but before protein precipitation, dG-C8-4-ABP was spiked into 5 µg aliquots of calf-thymus DNA in
the following amounts: 0, 0.27, 0.54, 1.1, 2.2, 4.3, 8.6, 17., 35., 70., 140, and 280 fmol. Each aliquot was also spiked with 6 fmol dG-C8-4-ABP-D$_9$ per µg DNA. For accurate quantification, the calibration curve was prepared in triplicate.

3.3.7 Preparation of standards for heat treatment stability assessment

To determine the stability of 4-ABP adducts during heat treatment at 98°C for three minutes, four sets of mixtures were prepared in which 8.4 fmol of dG-C8-4-ABP and 30 fmol dG-C8-4-ABP-D$_9$ were added prior to or post-heat treatment to a final volume of 20 µL in 10% methanol. Triplicate mixtures were completed for each set and samples within each set were pooled before analysis.

3.3.8 Capillary liquid chromatography and nESI-MS/MS

Liquid chromatography was performed using ChemStation for LC 3D Systems operating an Agilent 1100 Series system (Agilent Technologies, Wilmington, DE) equipped with a capillary pump, a nano pump, a 1200 series micro well-plate autosampler, and a chip cube interface. Chromatographic separations were performed on an Agilent Technologies small molecule microfluidic chip containing a 40 nL trap and 75 µm i.d. x 43 µm analytical column of reverse phase Zorbax SB-C18 and 5 µm particles. Each injection loaded 5 µL of sample onto the trap column at a flow rate of 4.00 µL min$^{-1}$ to be washed for 4 minutes with 93% mobile phase A (3% acetonitrile, 0.1% acetic acid in water) and 7% mobile phase B (0.1% acetic acid in methanol). Chromatographic separations were conducted at a flow rate of 300 nL min$^{-1}$ with mobile phase A (water with 0.1% acetic
acid) and mobile phase B (methanol with 0.1% acetic acid). Mobile phase B was held at 10% for 4.21 min, then linearly increased to 90% over 2 min, held for 2 min, then stepped down to 10% for a 5 min re-equilibration period.

To reduce carryover and prevent plugging of the narrow i.d. capillaries used for connections between the autosampler and chip cube, several cleaning procedures were implemented. An inline prefilter (0.5 µm) was placed between the autosampler and the loading capillary to prevent particulates in the sample from plugging the loading capillary and enrichment column inlet screen. During analysis mode, a needle wash program cleaned the needle seat, the needle seat capillary, and rotor seal with acetonitrile, methanol, and water while the loading capillary was washed with 90% B. One methanol blank and two 10% methanol blanks were injected between samples. In addition, nano pump and capillary pump pressures, system suitability standards and internal standards were used to monitor system performance.

Mass spectrometric data were acquired on an Agilent 6330 Ion Trap mass spectrometer (Santa Clara, CA) operated with 6300 Series Ion Trap HPLC-MS Software Ver. 6.1 and 6300 Series Trap Control Software Ver. 6.1 from Bruker Daltonik GmbH (Bremen, Germany). The mass spectrometer was calibrated by infusing tuning solution and 10 ng/mL dG-C8-4-ABP. For all analyses, the N₂ drying gas was set to 3.0 L min⁻¹ at 325°C and the capillary voltage was held at -1900 V with an end-plate offset of -500 V. Ion optics and trap parameters were as follows: skimmer 1: 40.5 V, capillary exit: 163.5 V,
trap drive: 54.2 V, ultra scan mode (24,000 m/z per scan), and positive ion mode. Ion charge control parameters were set to a maximum accumulation of 50 ms or 500,000 ions and 3 spectral averages per scan. CID pressure was 3 mT He and voltage ramped from 0.45 to 3 V. MS/MS spectra were collected within a scan window of 290-475 m/z and precursor ions 435.4 ± 1.0 and 444.0 ± 1.5 Da were isolated and fragmented with a cutoff fragmentation of 27% of the precursor ion mass.

Quant Analysis (Ver. 1.8) and Data Analysis (Ver 3.4) for 6300 Ion Trap LC/MS (Bruker Daltonik GmbH) were used for data processing. Extracted ion chromatograms selected to monitor for the characteristic loss of deoxyribose were 435→319 for the analyte, dG-C8-4-ABP and 444→328 for the internal standard, dG-C8-4-ABP-D₉. Gaussian smoothing was set to a width of 0.65 m/z.

3.3.9 Quantification of dG-C8-4-ABP in biospecimens

The standard curve of dG-C8-4-ABP plots the mass of dG-C8-4-ABP vs. the mean analyte to internal standard peak area ratio and was generated in triplicate over an 8 month period. Triplicate analyses were also conducted for every point in each calibration plot. Linear regression data derived from the average of all three plots enabled the quantification of dG-C8-4-ABP in 4-ABP-exposed human bladder cells and rat bladder tissues.
3.4. Results and Discussion

3.4.1 Mass spectrometric analysis of dG-C8-4-ABP synthetic standard

A 10 ng mL$^{-1}$ solution of dG-C8-4-ABP in 70:30:0.1 (v%) methanol:water:acetic acid was infused at 300 nL/min to optimize the trap parameters and ion optics for the dG-C8-4-ABP 435$\rightarrow$319 transition. This fragmentation is due to the characteristic loss of deoxyribose, [M+H]$^+$\$\rightarrow$[M+H-116]$^+$.

Fragmentation amplitude was optimized and its adjustment provided further structural confirmation. Figure 3.2 shows the mass spectra of the infusion of the synthetic standard at various fragmentation amplitudes. In the first panel, the mass spectrometer was set to isolate the precursor ion 435.1 ± 1.0 and the fragmentation voltage was turned off, resulting in a mass spectrum showing only the parent ion. A fragmentation amplitude of 0.1 volts only fragmented some of the analyte, and a significant precursor ion peak was still present. Increasing the voltage to 0.15 fragmented most of the analyte. At 1.5 V, the precursor ion was no longer present in the mass spectrum and the ion of greatest abundance was 319. The MS$^3$ spectrum, in which the precursor ion 435.1 was isolated and fragmented followed by the isolation and fragmentation of the fragment ion 319, is shown in the bottom panel. The major fragment ion shown is m/z 302. The proposed fragmentations are shown in Figure 3.3 (16).
Figure 3.2. A. Isolation of precursor ion, no fragmentation. B. Fragmentation amplitude: 0.10 volt. C. Fragmentation amplitude: 0.15 volt. D. MS$^3$ 435$\rightarrow$319$\rightarrow$302.
Figure 3.3. The proposed fragmentations for dG-C8-4-ABP.

3.4.2 LC-MS and LC-MS/MS of synthetic standard.

Following infusion, both the dG-C8-4-ABP standard and dG-C8-4-ABP-\textsubscript{D9} internal standard were injected on column and the retention time was determined to be 8.9 min. The chemical structures and extracted ion chromatograms (435→319 and 444→328 for the analyte and IS respectively) of 1.89 fmol dG-C8-4-ABP (in red) and 1.74 fmol IS (in blue) are shown in Figure 3.4. The chromatograms are shown as time (min) versus relative peak intensity. The internal standard is isotopically labeled with deuterium around the biphenyl moiety.
Figure 3.4. HPLC-MS of dG-C8-4-ABP standards. The top panel shows the chemical structure of the dG-C8-4-ABP adduct in red. The dG-C8-4-ABP-D$_9$ internal standard (IS) in blue is isotopically labeled with deuterium around the biphenyl moiety. The extracted ion chromatograms (435$\rightarrow$319 and 444$\rightarrow$328 for the analyte and IS respectively) of 1.83 fmol dG-C8-4-ABP and 1.74 fmol IS are shown above. Retention time for the standard and the internal standard is 8.9 min. The MS/MS spectrum of dG-C8-4-ABP is displayed in the middle panel. The MS/MS spectrum of the IS is displayed in the bottom panel.
As expected, the internal standard elutes very slightly ahead of the standard but the peaks overlap considerably. It is not uncommon for the internal standard to co-elute with the analyte in this type of analysis. Because the analyte dG-C8-4-ABP and internal standard dG-C8-4-ABP-D₉ co-elute, we know that they are subject to identical conditions during analysis, making quantification more accurate than if they were chromatographically resolved.

The retention time of the analyte was 8.9 minutes. The gradient begins 4.2 minutes into the run, reaches 90% methanol at 6.2 minutes and is held at 90% methanol until 8.2 minutes. During the first four minutes, the capillary pump loads the analyte onto the trap column, sending whatever is not retained to waste. At four minutes, the valve in the chip switches so that the nano pump can wash the analyte from the trap column to the analytical column for separation. The dead volume on the nano pump is 0.27 µL, which takes 1.1 minutes at a flow rate of 0.3 µL/minute. Including the 40 nL trap column and the 160 nL analytical column, the beginning of the gradient is detected by the mass spectrometer 1.6 minutes after it has started. The gradient should reach the mass spectrometer from 5.8 to 7.8 minutes and stay at 90% methanol until 9.8 minutes. The dG-C8-4-ABP analyte is eluting at 8.9 minutes at the top of the gradient.

dead volume: 0.11 µL (25 µm x 23 cm) + 0.16 µL (15 µm x 90 cm) = 0.27 µL
column volume: 0.04 µL trap + 0.16 µL analytical (75 µm x 4.3 cm) = 0.20 µL
3.4.3 SPE matrix interference and cartridge comparisons: recovery of analyte in matrix

Sample preparation can have a substantial impact on sensitivity in the quantification of DNA adducts by mass spectrometry \((1)\). A typical workflow in the analysis of DNA adducts is shown in Figure 3.5. Injection of the dG-C8-4-ABP standard in the matrix from an SPE cartridge resulted in a loss in signal. A 70% loss was observed when the matrix was from Isolute C18 cartridges and an 84% loss was observed with Waters tC18 cartridges. By designing the experiment this way, possible loss of the analyte was eliminated and it is known that the lower signal is caused by interference from the SPE matrix. This is most likely due to ion suppression from materials introduced from the SPE cartridge. Because the loss of analyte during SPE sample preparation would contribute to an even lower signal, it was necessary to find an alternative method for sample enrichment.

Figure 3.5. Typical workflow in DNA adduct analysis by LC-MS.
3.4.4 Automated adduct enrichment

The automated sample clean-up method presented here eliminates the need for solid-phase extraction (SPE), reducing sample preparation time, sample handling, and analyte loss as well as eliminating the introduction of SPE related artifacts which can cause ion suppression. The microfluidic chip (Figure 3.6) used in this method incorporates both a trap and analytical column, enabling the automated adduct enrichment step. The sample was loaded onto the 40 nL reversed phase C18 trap column and salts and unmodified nucleosides were washed off with 93% water and 7% methanol for four minutes before placing the trap column inline with the analytical column during analysis mode. The analyte was then washed from the trap column to the analytical column for separation.

The chip integrates a 40 nL trap column, a 75 μm i.d. x 43 μm analytical column of reverse phase Zorbax SB-C18 and 5 μm particle size, and a nanospray emitter. In enrichment mode, the sample was washed with 7% methanol, removing unwanted salts and excess unmodified nucleosides. In analysis mode, the nano pump moved the analyte from the trap column to the analytical column for separation. The chip can be set in back-flush or forward-flush mode, but back-flushing sharpens the peak profile. The valve configuration in enrichment mode and analysis mode is illustrated in Figure 3.7.
Figure 3.6  A detailed view of the microfluidic chip used in our automated sample enrichment method. The chip integrates a 40 nL trap column, a 75 µm id x 43 mm analytical column of reverse phase Zorbax SB-C18 and 5 µm particle size, and a nanospray emitter (courtesy of Agilent Technologies, Inc.).
Figure 3.7. A detailed view of the valve of the Agilent microfluidic chip used in our automated sample enrichment method (courtesy of Agilent Technologies, Inc.). The top panel shows the valve in enrichment mode, during which the sample was washed with 7% methanol, removing unwanted salts and excess unmodified nucleosides. The bottom panel shows the valve in analysis mode, during which the analyte was removed from the trap column to the analytical column for separation.
3.4.5 *dG-C8-4-ABP stability assessment during heat treatment*

Samples are subject to heat treatment during the digestion procedure to denature the DNA for efficient hydrolysis by Nuclease P1. Therefore, to determine the stability of 4-ABP adducts towards heat treatment at 98°C for three minutes, we compared standards that were exposed to heat treatment with standards that were not. Four sets of triplicate mixtures were evaluated in which 8.4 fmol of dG-C8-4-ABP and 30 fmol dG-C8-4-ABP-$D_9$ were added prior to or post-heat treatment. The precision within each set was below 20% RSD. As shown in **Figure 3.8**, the standard error bars of all sets overlap, indicating that their difference is not statistically significant.

**Figure 3.8.** Heat treatment stability assessment. Four sets of mixtures were compared in which 8.4 fmol of dG-C8-4-ABP and 30 fmol dG-C8-4-ABP-$D_9$ were added prior to or post-heat treatment at 98°C for 3 min. Each set was taken through the protocol in triplicate and the precision of all sets was below 20% RSD. The standard error bars of all sets overlap, indicating that their difference is not statistically significant.
3.4.6 Assessment of internal standard degradation during digest

In our method, the internal standard was added immediately following the enzymatic digestion of DNA. Although addition of the internal standard post-digestion does not account for oxidation/hydrolysis during digestion, we have found that degradation of the dG-C8-4ABP adduct during the digestion is minimal. To evaluate degradation of the analyte and internal standard during the DNA digestion procedure, we compared four sets of digests in which the standards were added prior to or post-digestion. 8.4 fmol of dG-ABP and 39.6 fmol dG-C8-4-ABP-D9 were added to each digest. 5 μg aliquots of calf-thymus DNA were digested in triplicate for each set and taken through a protein precipitation before analysis. The standard error bars of all sets overlap with each set having a precision under 15% RSD (Figure 3.9). It can therefore be concluded that their difference is not statistically significant.
Figure 3.9. Internal standard degradation during digest. To evaluate degradation of the analyte and internal standard during the DNA digestion procedure, we compared four sets of digests in which the standards were added prior to or post-digestion. 8.4 fmol of dG-C8-4-ABP and 30 fmol dG-C8-4-ABP-\textit{D}_9 were added to each digest. 5 µg aliquots of calf-thymus DNA were digested in triplicate for each set and taken through a protein precipitation before analysis. The standard error bars of all sets overlap with each set having a precision under 15% RSD. It can therefore be concluded that their difference is not statistically significant.
3.4.7. Calibration curve and linearity

The standard curve of the number of fmol dG-C8-4-ABP versus the mean analyte to internal standard peak area ratios was prepared in triplicate in a matrix of 5 µg calf-thymus DNA to simulate the conditions of the real samples. Every point from each calibration curve was also analyzed in triplicate. The isotopically labeled internal standard was added to correct for variations in sample preparation and instrument operation. A linear regression analysis of the data incorporating all three curves was generated in Microsoft Excel using the least squares fit to the line $y = mx + b$. Each point was equally weighted and the line was not forced through the origin. Values derived from the standard curves (Figure 3.10) were as follows: slope = 0.100 ± 0.001, y-intercept = 0.05 ± 0.03, and correlation coefficient $R^2 = 0.99 ± 0.08$. A zoom of the low end of the calibration curve is shown in Figure 3.11. The LOD was determined to be 20 amol on column or approximately 5 adducts in $10^9$ nucleosides and the LOQ, the lowest point on the calibration curve, was 70 amol on column or 2 adducts in $10^8$ nucleosides.

Representative chromatograms of dG-C8-4-ABP analysis at the LOD and LOQ levels are shown in Figure 3.12. Extracted ion chromatograms (435→319) of the LOD, 20 amol on column in 1.25 µg of DNA or approximately 5 adducts in $10^9$ nucleosides and the LOQ, 70 amol on column in 1.25 µg of DNA or 2 adducts in $10^8$ nucleosides. The extracted ion chromatogram (435→319) of 1.25 µg calf thymus DNA on column is also shown as the procedure blank.
The diagram depicts a linear regression analysis with the equation:

\[ y = (0.100 \pm 0.001)x + (0.05 \pm 0.03) \]

and the coefficient of determination \( R^2 = 0.99 \pm 0.08 \). The data points are color-coded, with Sep-08 represented by blue diamonds, Jan-09 by green squares, and Apr-09 by red triangles. The black line represents the linear trend, with the mean values indicated for each category.
Figure 3.10. Standard curve dG-C8-4-ABP of fmol analyte on column versus the ratio of analyte to internal standard peak areas were generated in triplicate over an 8-month period. Every point from each calibration curve was also analyzed in triplicate. The linear regression line incorporates all three curves and has a slope of 0.100±0.001, a y-intercept of 0.05±0.03, and a correlation coefficient R² of 0.99±0.08.
Figure 3.11. Zoom of the lowest five points of the calibration curve. dG-C8-ABP calibration curves of fmol analyte on column versus the ratio of analyte to internal standard peak areas were generated in triplicate over an 8 month period. Every point from each calibration curve was also analyzed in triplicate. The linear regression line incorporates all three curves and has a slope of $0.100 \pm 0.001$, a y-intercept of $0.05 \pm 0.03$, and a correlation coefficient $R^2$ of $0.99 \pm 0.08$. 
Figure 3.12. LOD and LOQ. Extracted ion chromatograms (435→319) of the LOD, 20 amol on column in 1.25 µg DNA or approximately 5 adducts in $10^9$ nucleosides, the LOQ, 70 amol on column in 1.25 µg DNA or 2 adducts in $10^8$ nucleosides, and the procedure blank, 1.25 µg calf-thymus DNA.

3.4.8 Reproducibility

Reproducibility was determined with replicate doses of RT-4 cell and rat bladder DNA digests. RT-4 cells were treated in triplicate with 4-ABP plus an S9 activation system, taken through the sample preparation procedure that was previously described, and each sample was analyzed twice. Subsequently, 6 µL aliquots from each sample were pooled and injected three times. To test reproducibility in the rat specimens, 5 µg aliquots of rat bladder DNA from three rats dosed at 25 mg/kg 4-ABP were taken through the sample preparation procedure and the equivalent of 1.25 µg of DNA from each digest was injected three times. The average number of adducts was determined to be $6 \pm 2$ adducts.
in $10^7$ nucleosides when the samples were run individually and 8 ± 3 adducts in $10^7$ nucleosides when pooled. The precision of the pooled and individually run methods was determined to be statistically the same by the F test (the calculated F-value 1.83 was less than the tabulated value of 4.45 at the 95% confidence level). Applying the T test, there was no statistical difference between running the samples individually or pooled (the calculated value of T, 1.43 was less than the tabulated value of 2.23 at the 95% confidence level). Because replicate dose concentrations resulted in similar adduct quantities in both cells and rats, samples were pooled.

### 3.4.9 Quantification of dG-C8-4-ABP adducts in human bladder RT-4 cells and rat bladder tissue

The quantity of dG-C8-4-ABP adducts detected in dosed cells and tissues was calculated from the peak area ratio of dG-C8-4-ABP to IS and the standard curve linear regression values (Figure 3.13, Figure 3.14). These findings are summarized in Table 3.2. Plots of 4-ABP dose versus observed adduct content and extracted ion chromatograms of the lowest dose rat sample and the control sample are displayed in Figure 3.15. As indicated, no adduct was detected in control samples. Because the ratio of analyte to IS by peak area of the highest dosed cell sample was greater than that of the last point on the calibration curve, the regression values were linearly extrapolated to calculate the adduct quantity in this sample. The observation that adduct content increased with increasing 4-ABP dose in the RT-4 cell study reinforces the implication of this carcinogen in DNA adduct formation (17, 18). This trend may have been more apparent in the rat study had
the 4-ABP dose extended over a wider range, for example two orders of magnitude as in the cell experiment, rather than only one. The ambiguity of this trend may also be due to the complicated processes involved in DNA adduction; the quantification of DNA adducts is not simply a measurement of exposure, but also the metabolism of the carcinogen, including activation and deactivation, enzymatic esterification processes, and detoxification and repair pathways, which may differ among species, tissues, and even phenotypes (1, 19). In comparison to an experimental cell line, the steps leading to adduction are even more complex in an organism, where certain processes are specific to distinct tissues. For example, following exposure to 4-ABP, and certainly with many competing reactions along the way, it is generally accepted that the carcinogen is converted to N-hydroxy-4-ABP in the liver and then to a reactive electrophile in the target tissue (20).
Figure 3.13. Logarithmic scale plot of dG-C8-4-ABP levels observed in dosed RT-4 human bladder carcinoma cells. Cell dosings were completed in triplicate at each concentration 5 µg DNA was digested from each sample and samples from the same dose concentration were pooled before triplicate analysis. Only 1.25 µg DNA was utilized per injection.
Figure 3.14. Plot of 4-ABP dose versus detected dG-C8-4-ABP quantity in bladder tissue from rats treated with 4-ABP. Animal dosings were completed in triplicate at each concentration 5 µg DNA was digested from each sample and samples from the same dose concentration were pooled before triplicate analysis. Only 1.25 µg DNA was utilized per injection.
**Figure 3.15.** Zoom in of the EIC (435$\rightarrow$319 for the analyte and 444$\rightarrow$328 for the IS) from the lowest dose rat and control samples.
### A. 4-ABP Dosed Human Bladder RT-4 Cells

<table>
<thead>
<tr>
<th>Concentration of 4-ABP in DMSO (µM)</th>
<th>Analyte/Internal Standard by Peak Area</th>
<th>fmol dG-ABP on column</th>
<th>Number of Adducts Detected in $10^8$ Nucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.5</td>
<td>0.065 ± 0.005</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5.0</td>
<td>0.14 ± 0.03</td>
<td>0.9 ± 0.5</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>50.</td>
<td>4.0 ± 0.8</td>
<td>40 ± 8</td>
<td>1100 ± 200</td>
</tr>
</tbody>
</table>

*Error discredits value

### B. 4-ABP Dosed F/344/NHsd Rats

<table>
<thead>
<tr>
<th>Concentration of 4-ABP in DMSO IP Injection (mg 4-ABP/kg rat)</th>
<th>Analyte/Internal Standard by Peak Area</th>
<th>fmol dG-ABP on column</th>
<th>Number of Adducts Detected in $10^8$ Nucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>25</td>
<td>0.34 ± 0.09</td>
<td>3 ± 1</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>100</td>
<td>0.49 ± 0.08</td>
<td>4.4 ± 0.9</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>250</td>
<td>0.5 ± 0.1</td>
<td>4 ± 1</td>
<td>120 ± 30</td>
</tr>
</tbody>
</table>
Table 3.2. Dose–response data in human cells and rat tissue. (A) dG-ABP adduct content and absolute standard deviation from RT-4 human bladder carcinoma cells that were treated with 4-ABP dissolved in DMSO. Cells were cultured in triplicate, pooled, and analyzed in triplicate. (B) Adduct quantity and absolute standard deviation from bladder tissue DNA extracted from twelve 2-month old F/344/NHsd rats that were administered 4-ABP prepared in DMSO. DNA from replicate doses was pooled before analysis and injections were completed in triplicate.
3.4.10 Analysis of Urothelial Cell DNA adducts for a smoker quit study

Given the well established propensity of 4-ABP to target the bladder urothelium and the implications associated with bladder cancer, an ultimate objective would be the application of the methodology described here to the analysis of urothelial cell DNA. A particular challenge presented here is the low quantity of exfoliated urothelial cell DNA typically isolated from urine samples. This quantity may vary widely, with often only 1 µg or less extracted from about 200 mL of urine (20-23). In view of this, we scaled down the method to be more suitable for the analysis of these samples by digesting only 1 µg of DNA from these samples rather than 5 µg. This approach would then consume the equivalent of 250 ng rather than 1.25 µg DNA per analysis. Assuming the mass LOD remains at 20 amol, this method would correspond to a detection limit of 2.5 adducts in $10^8$ nucleotides and would still be well suited for the analysis of human samples.

Initially, the 1 µg digestion process was tested by analyzing a bladder DNA sample from a rat exposed to the lowest level of 4-ABP (25 mg/kg 4-ABP). The amount of adduct in the 1 µg digest was found to be $7 \pm 2$ adducts in $10^7$ nucleotides (Figure 3.16, A), which was not significantly different from that determined previously using a 5 µg digest (the calculated value of $T$, 0.7 was less than the tabulated value of 2.5 at the 95% confidence level).

Following confirmation of capability of our method to handle reduced amounts of DNA, three 1 µg aliquots of urothelial cell DNA from the lifetime non-smoker were digested
and the equivalent of 250 ng DNA was analyzed three times per sample. As shown in the representative chromatogram of Figure 3.16, B, no dG-C8-4-ABP adduct was detected in these samples and this should not be surprising since they were obtained from a never-smoker; several studies have linked smoking to 4-ABP adducts in bladder biopsy specimens or exfoliated urothelial cells (21-23). Significantly, however, the response of the internal standard did not appear to be compromised by the urothelial cell matrix. Therefore, the remainder of the DNA isolated from two of the exfoliated urothelial cell samples was pooled for a 1 µg digest and spiked with IS and 2.24 fmol dG-C8-4-ABP. A chromatogram of the analysis showing the detection of 14 ± 3 adducts in 10^7 nucleotides is shown in Figure 3.16, C. Based on back-calculating the mass of dG-C8-4-ABP from the ratio of analyte to internal standard peak areas, this value corresponds to an accuracy of 113%.

Furthermore, to test the digestion efficiency in exfoliated urothelial cell DNA, 4-ABP-modified DNA was mixed with exfoliated urothelial cell DNA prior to digestion. Accordingly, digests of 10 ng 4-ABP dosed RT-4 cell DNA mixed with 1 µg of urothelial cell DNA and 10 ng of 4-ABP dosed RT-4 cell DNA mixed with 1 µg of calf thymus DNA were compared. In each case, approximately 1.5 adducts in 10^6 nucleotides were detected indicating similar digestion efficiencies. These results are summarized in a bar graph form in Figure 3.16, D. These experiments demonstrate that this LC-MS method is capable of measuring dG-C8-4-ABP from exfoliated urothelial cells.
Figure 3.16  Analysis of dG-C8-4-ABP adducts by digesting 1 µg of DNA. In each chromatogram, the internal standard is shown in blue and the analyte is shown in red. A. Chromatogram of dG-C8-4-ABP from the processing of 1 µg DNA (equivalent of 250 ng injected on-column) in bladder sample of a rat treated with 25 mg/kg 4-ABP. Signal corresponds to 7±2 adducts in $10^7$ nucleotides, which is the same as that obtained from the analysis of a 5 µg digest. B. Representative chromatogram from the analysis of a 1 µg urothelial cell DNA digest of a lifetime non-smoker. C. Analysis of dG-C8-4-ABP from two pooled urothelial cell samples; 1 µg DNA was digested and spiked with IS and 2.24 fmol dG-C8-4-ABP. On column injection of 250 ng corresponds to injection of 14±3 adducts in $10^7$ nucleosides. D. Determination of digestion efficiency of urothelial cell DNA. Comparison of dG-C8-4-ABP adduct levels in 1 µg exfoliated urothelial cell DNA and 1 µg calf-thymus DNA each spiked with 10 ng 4-ABP-modified DNA prior to digestion. No statistical differences are observed.
3.5 Conclusions

The HPLC-MS/MS method presented in this paper is suitable for the quantification of 4-ABP DNA adducts in human samples. High sensitivity was accomplished with nano-flow rates, capillary columns and automated sample enrichment prior to HPLC-MS/MS analysis. Only 1.25 µg of DNA is needed per analysis to reach the detection limit of 20 amol (5 adducts in $10^9$ nucleosides) and the linear range spanned from 70 amol to 70 fmol.

Although high reproducibility, accuracy, and precision have been demonstrated on an ion trap mass spectrometer, we expect the use of a triple quadrupole mass analyzer would even further improve the method’s robustness. Traditionally, triple quadrupole mass spectrometers have been used for quantitative measurements because they are more robust than ion trap instruments for reproducibility and precision for trace, quantitative analytical work. In an ion trap, the number of scans acquired is restricted by the slow duty cycle of the scanning events and because fewer data points are acquired for each peak, quantitative measurements may be imprecise. However, adjustments can be made to find a balance between sensitivity, precision and reproducibility and we were able to achieve sufficient sensitivity with high reproducibility in our analyses. Although the linear dynamic range of a triple quadrupole is greater than that of an ion trap, we have produced a linear calibration curve that will allow for quantification. Additionally, by using an ion trap, we were able to perform MS$^3$ for characterization of the analyte. There
are several examples of the use of ion traps in quantitative DNA adduct measurements (24-27).

Once the methodology was established, the assay was applied to the quantification of dG-C8-4-ABP adducts in human bladder cells and rat bladder tissue. Human bladder carcinoma cells were treated with 0.5 to 50 µM 4-ABP and rats were administered 25 to 250 mg/kg 4-ABP, with analytical replicates at each dose. Replicate doses were shown to be precise and reproducible, and the direct relationship between dose and DNA adduct quantity confirmed that 4-ABP exposure led to adduct formation. The sample requirements were further reduced to the equivalent of 250 ng DNA per injection in the analysis of exfoliated human urothelial cells and it was demonstrated that low levels of dG-C8-4-ABP could be detected in this matrix.

The described method can be applied to explore the relevance of 4-ABP DNA adducts to bladder cancer and its relationship to smoking or other exposures. In combination with related research projects such as those analyzing carcinogen metabolites (28), determining the impact of sequence context on the formation of DNA adducts (29-31), profiling gene expression (32, 33), elucidating complex 3-D nucleic acid structures (34) or evaluating chemopreventive agents (35-37), this method can have a considerable impact on the current understanding of carcinogenesis induced by 4-ABP or other structurally related carcinogens.
References


Their Relationship to Mutation, Toxicity, and Gene Expression Profiling, *Analytical Chemistry* 78, 6422-6432.


CHAPTER 4

ACTIVATION OF NRF2 AS A CHEMOPREVENTIVE STRATEGY
AGAINST 4-ABP-INDUCED BLADDER CANCER
4.1 Introduction

4.1.1 Nrf2 induction as a chemoprevention strategy (1)

Activation of the ubiquitous transcription factor NF-E2 related factor 2 (Nrf2) is a widespread strategy for cancer chemoprevention. Nrf2 stimulates the expression of genes involved in many aspects of cytoprotection and many potential chemopreventive agents mediate their cytoprotective effects through Nrf2. Nrf2 has protected cells against carcinogens, including N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), a bladder carcinogen in rodents (2). It has also been shown to reduce the formation of carcinogen DNA adducts including inhibition of the formation of 7,12-dimethylbenz[a]anthracene (DMBA) DNA adducts in MCF-7 cells (3) and Benzo[a]pyrene (B[a]P) (4-6) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (7) DNA adducts in mice. Accordingly, we investigated Nrf2 as a target for the inhibition of 4-ABP DNA adduct formation in bladder cells and tissues.

Nrf2 induces Phase 2 carcinogen detoxifying genes. In the basal state, Nrf2 is bound to a Keap1 dimer, ubiquitinated, and targeted for proteasomal degradation. However, chemopreventive and pharmacological agents can activate Nrf2: their interaction with the sulfhydryl groups of Keap1 bring about a conformational change of Keap1 so that Nrf2 is no longer ubiquitinated and therefore no longer targeted for proteasomal degradation. Nrf2 then accumulates in the cytosol and moves into the nucleus. There, it binds as a heterodimer to the antioxidant response element (ARE) in the 5’-untranslated
region of its target genes (8, 9). This results in the transcription of upstream genes which include Phase 2 genes. Some Phase 2 enzyme catalyzed reactions include glutathione addition, glucuronidation, and sulfation. These are often detoxification reactions of carcinogens such as 4-ABP (10, 11).

There is a great deal of evidence suggesting that Phase 2 enzyme induction is crucial for cytoprotection against carcinogens by many chemopreventive agents. That a chemopreventive agent can detoxify a wide variety of carcinogens and that a carcinogen can be detoxified by a wide variety of chemopreventive agents suggests that low specificity mechanisms, such as induction of Phase 2 enzymes are involved. Furthermore, greater efficacy from the chemopreventive agent is usually observed when it has been administered prior to or at the same time as carcinogens instead of after, suggesting that inhibition of DNA damage is occurring rather than repair. Finally, Phase 2 enzymes have been shown to both inactivate and alter the metabolism of carcinogens and be inducible by chemopreventive agents.

Probing Phase 2 enzymes and their activities can therefore facilitate the identification and evaluation of chemopreventive agents. Extent of carcinogen conjugation with Phase 2 metabolites can be quantified to provide an estimation of carcinogen detoxification. The approximate efficacy of a chemopreventive agent can then be gauged based on the relative change in levels of these conjugates following treatment with the
chemopreventive agent. Measurement of enzyme activities is a more direct measurement of Phase 2 enzyme induction by chemopreventive agents (12-15).

4.1.2 Glucuronidation of 4-ABP is implicated in bladder cancer (16)

The Nrf2-regulated Phase 2 enzymes, Uridine 5'-diphospho (UDP)-glucuronosyltransferases (UGTs), catalyze the conjugation of 4-ABP or its metabolites with glucuronic acid (17-19). Although an elimination reaction, glucuronidation of 4-ABP has been implicated in bladder cancer and has been shown to facilitate transport of the active metabolite from the liver to the bladder (20). The structure of the 4-ABP-N-glucuronide is shown in Figure 4.1. The glucuronidation reaction involves the transfer of the glucuronosyl group of uridine 5'-diphospho-glucuronic acid (UDPGA) to an oxygen, nitrogen, sulfur or carboxyl moieties of its substrates. UGTs metabolize many compounds, endogenous and xenobiotic, but there is a specific human UGT that catalyzes the formation of 4-ABP glucuronide conjugates (21). UGT enzymes are classified into two families, UGT1 and UGT2 (22). In humans, the UGT1A family incorporates nine isoforms (UGT1A1, UGT1A3-UGT1A10) and the UGT1A contributes more to the metabolism of arylamines than the UGT2B family. 4-ABP in particular is glucuronidated mostly by UGT1A4 and UGT1A9 (23, 24). There are species and individual differences in glucuronidation based on individual genes, type of isoforms, quantities of UGTs, substrate specificities, age and extent of exposure to inducers. Many UGT isoforms that are expressed in mice are not detected in humans, or vice versa (25, 26). A recent study showed that multiple UGT isoforms might be up-regulated by Nrf2 in mouse liver,
including UGT1A6, UGT1A7, UGT2B1, UGT2B35 and UGT2B36 (2, 17). A different set of UGT isoforms appear to be up-regulated by Nrf2 in humans, including UGT1A1, UGT1A8 and UGT1A10 (19, 27).

![Structure of 4-ABP-N-glucuronide.](image)

**Figure 4.1.** Structure of 4-ABP-N-glucuronide.

The glucuronidation pathways that affect 4-ABP metabolism and the formation of 4-ABP DNA adducts are illustrated in **Figure 4.2** (16). 4-ABP and its oxidized metabolite N-OH-4-ABP may be N-glucuronidated becoming more water soluble and therefore more easily transported out of the liver and through the bloodstream to the bladder. There, low pH results in hydrolysis and the formation of DNA adducts (16, 20). Although N-hydroxy-N-acetyl-4-aminobiphenyl will also undergo glucuronidation, this glucuronide conjugate is not acid labile and will not result in bladder cancer.
Figure 4.2. Schematic of glucuronidation in the metabolic pathways of 4-ABP induced human bladder cancer. (Reproduced from (16))
4.2 Project Goals

We investigated Nrf2 as a target for inhibition of 4-ABP DNA adduct formation. The interesting effect of Nrf2 on 4-ABP DNA adduct formation in bladder tissue initiated an investigation into Nrf2 expression and the Nrf2 signaling pathway. This led us to consider Nrf2-induced glucuronidation of 4-ABP as a significant mechanism for increasing the bioavailability of 4-ABP in bladder tissue.

4.3 Experimental

All cell and animal dosings, western blots, HPLC-UV measurements, and enzyme activity experiments were carried out and analyzed in Dr. Yuesheng Zhang’s laboratory at Roswell Park Cancer Institute, Buffalo, NY by Joseph Paonessa and Yi Ding.

4.3.1 Chemicals and Standards

4-ABP and N-hydroxy-N-acetyl-4-aminobiphenyl (N-OH-AABP) were purchased from LKT Laboratories (St. Paul, MN), Sigma (St. Louis, MO), and Midwest Research Institute (Kansas City, MO), respectively. Rat liver S9 (36-43 mg protein/ml) was purchased from Moltox (Boone, NC). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), including antibodies specific for Nrf2, and from Millipore (Billerica, MA) for the antibody for glyceraldehyde 3-phosphate
dehydrogenase (GAPDH). 4-ABP-glucuronide and the corresponding internal standard were purchased from Toronto Research Chemicals (North York, Ontario).

4.3.2 Cell Study

RT-4 cells were purchased from American Type Culture Collection (ATCC), stored in liquid nitrogen, and passaged in Dr. Zhang’s lab at Roswell Park Cancer Institute for fewer than 6 months (28). The cell line was characterized by ATCC by antigen expression, DNA profile, cytogenetic profile and isoenzymes. RT-4 cells were cultured and treated with 4-ABP (plus 6% S9) or N-OH-AABP (29, 30). To silence Nrf2, cells were treated with a Nrf2 siRNA or a control siRNA for 48 h (30). To induce DNA damage, RT-4 cells (2-3 x 10^6 cells) were grown in each 10-cm dish with 10 ml medium 24-48 h, followed by treatment with either 4-ABP or N-OH-AABP for 3 h in 5 ml fresh medium per dish. In cases where cells were co-treated with 4-ABP and S9, the medium also contained 6% S9 (v/v), 10 mM glucose-6-phosphate and 5 mM NADP. Cells were harvested by trypsin treatment and low-speed centrifugation, and cell pellets were washed with ice-cold PBS and used for DNA adduct analysis and Western blotting of Phase 2 proteins.

4.3.3 Mouse Study

Wild type C57BL/6 mice (6 weeks of age) (NCI, Frederick, MD) and their Nrf2-deficient counterparts (31) (bred at the Roswell Park Cancer Institute animal facility; the breeders were kindly provided by Dr. Thomas W. Kensler (Johns Hopkins Bloomberg School of
Public Health) (31)) were treated with a single dose of 4-ABP i.p. and sacrificed 24 h later for quantification of dG-C8-4-ABP in the liver and bladder tissues (29). Groups of 3-4 mice were randomized and treated with a single dose of 4-ABP or vehicle i.p. The mice were sacrificed 24 h later, from which the livers and bladders were promptly removed for measurement of dG-C8-4-ABP. Liver and bladder tissues were stored at -80°C. To measure urinary levels of 4-ABP-glucuronide, mice were given a single dose of 4-ABP i.p. and immediately moved to metabolism cages (2 mice/cage) for 24-h urine collection. The specimens were stored at -80°C. All animal protocols and procedures were approved by the Roswell Park Cancer Institute Animal Cancer and Use Committee.

4.3.4 Measurement of dG-C8-4-ABP

Procedures of DNA isolation from bladder cells and tissues and the detailed protocol for the quantitative analysis of dG-C8-4-ABP by capillary liquid chromatography and nESI-MS/MS have been described in chapter 3 of this dissertation and a recent publication (29, 32).

4.3.5 Western blot analysis

Cellular and tissue levels of Nrf2 and/or several Phase 2 enzymes were measured by Western blotting (30). All antibodies were purchased from Santa Cruz Biotechnology, except for anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which was purchased from Millipore.
4.3.6 Measurement of liver UGT activity and urinary levels of 4-ABP-glucuronide and creatinine

The assay protocols for UGT activity and 4-ABP-glucuronide were based on those by Al-Zoughool and Talaska (24) with some modifications. Briefly, liver tissues were homogenized in ice-cold 50 mM potassium phosphate buffer in a glass homogenizer; the homogenates were cleared by centrifugation for 20 min at 9,100g at 4°C to prepare the S9 fraction. UGT activity toward ABP was measured by an HPLC-based biochemical assay coupled with reverse phase HPLC. Each liver S9 sample (0.35 mg protein) was first incubated with 17.5 μg alamethicin in 0.171 ml of 50 mM potassium phosphate buffer (pH7.4) containing 8.75 μL methanol on ice for approximately 20 min. The entire mixture was then transferred to a final 1 ml reaction solution, containing 50 mM potassium phosphate (pH 7.4), 10 mM magnesium chloride, 5 mM saccharalactone and 0.5 mM 4-ABP (containing 4 μL methanol). The reaction was initiated by adding 5 mM UDP-glucuronic acid and continued for 30 min in a 37°C water bath. The reaction was stopped by adding 0.2 ml methanol to each reaction solution. A preliminary experiment showed that the formation of 4-ABP-glucuronide in the reaction was linear for at least 60 min. The solutions were then centrifuged at 16,000g for 5 min at 4°C, and the supernatant portions were analyzed by HPLC. HPLC analysis of 4-ABP-glucuronide was carried out using an Agilent system with a diode-array detector. The mobile phase consisted of 50 mM potassium phosphate (pH 6.8) and methanol. The system was operated at a flow rate of 1.75 ml/min and a partisil 10 ODS-2 reverse phase column (4.6 mm x 250 mm, Whatman) was used. A linear gradient gradually increased methanol
concentration: from 0-7 min, 35% to 55% and from 7-14 min, 55% to 80%. The 4-ABP-glucuronide was monitored at 280 nm and eluted at 5.1 min, and the peak area was integrated using the Agilent’s ChemStation software. A calibration curve of 4-ABP-glucuronide was established using a pure standard (Y = 0.421X + 12.266, R² = 1.0, where Y is the peak area and X is pmol of 4-ABP-glucuronide injected to HPLC). Urinary creatinine was measured using a creatinine kit purchased from Kaymen, according to manufacturer’s instruction.

4.3.7 Capillary liquid chromatography and nESI-MS/MS of 4-ABP glucuronide

The presence of 4-ABP-N-glucuronide in the urine of mice treated with 4-ABP was confirmed by LC-MS. 4-ABP-N-glucuronide was first enriched in 200 µL of mixed urine from two wild type mice treated with 50 mg/kg 4-ABP. Briefly, 1 mL of acetonitrile was added and the sample was centrifuged (Thermo Scientific, Sorvall RT-1) at 10,000 x g for 10 minutes at 4ºC. The supernatant was removed, lyophilized, and reconstituted in 10 µL of 10 mM ammonium acetate. 10 pg of the 4-ABP-N-glucuronide standard was taken through the same sample processing steps to verify minimal loss of the analyte. Capillary liquid chromatography and nESI-MS/MS was performed using the same instrumentation as previously described in chapter 3(32) but with modifications to the method as follows: Following 1 µL sample injections, mobile phase A (10 mM ammonium acetate) was held at 100% for 4.3 minutes. Mobile phase B (acetonitrile) was then linearly increased to 65% over 2 min, held for 0.5 min, and stepped back down to 0% for a 5 min re-equilibration period. MS/MS spectra were collected within a scan window of m/z 100-
400 and the precursor ion m/z 346 ± 1.0 was isolated and fragmented. In data analysis, the extracted ion chromatogram selected to monitor for the loss of the glucuronide was m/z 346→170.

4.3.8 Statistical analysis

The numerical results are expressed as means ± SD. Unpaired two-tailed Student t-test was used for data analysis, with a P value <0.05 being considered significant (GraphPad Version 5.00, GraphPad Software, San Diego, CA).

4.4 Results

We began this research by establishing the experimental dose conditions in human bladder cells and mouse bladder tissue. To determine the effect of Nrf2 on 4-ABP DNA adduct levels, we compared these levels in cells and tissues with Nrf2 to those without Nrf2. Our findings that adduct levels were higher in wild type mice than Nrf2 knockout mice initiated an investigation into the expression of Nrf2-regulated proteins, with a focus on UGT enzymes that are implicated in bladder carcinogenesis.
4.4.1 Nrf2 protects human bladder carcinoma RT-4 cells from 4-ABP and N-OH-AABP

4.4.1a Establishing experimental dosing conditions

In a dose-duration study, human bladder carcinoma RT-4 cells were treated with 50 µM 4-ABP plus S9 for 3, 6, or 24 hours and 4-ABP DNA adduct levels were measured. An example extracted ion chromatogram of the standard dG-C8-4-ABP 435→319 and isotopically labeled internal standard 444→328 from the 6 h set is shown in Figure 4.3.

In the first three hours, 4-ABP DNA adduct levels reached 430±40 adducts in $10^8$ nucleosides, after 6 hours, the adduct level was measured to be 590±50 adducts in $10^8$ nucleosides and after 24 hours, adduct levels fell back down to 51±4 adduct in $10^8$ nucleosides (Figure 4.4).

![Figure 4.3](image_url)

**Figure 4.3.** An example EIC in the time course study. 4-ABP-induced DNA damage in human bladder carcinoma RT-4 cells. RT-4 cells were treated with 4-ABP for 6 hours and harvested for measurement of dG-C8-4-ABP levels by LC-MS/MS. The standard is shown in red and the internal standard in blue.
Figure 4.4. 4-ABP-induced DNA damage in human bladder carcinoma RT-4 cells in a dose duration study. RT-4 cells were treated with 4-ABP and harvested for measurement of dG-C8-4-ABP levels by LC-MS/MS.

Human bladder carcinoma RT-4 cells were exposed to 4-ABP at 0.05, 0.1, 0.25 or 0.5 mM for 3 h in the presence of a rat liver S9 and adduct levels were measured (Figure 4.5). The adduct level was below the limit of detection in cells not treated with S9 in the presence 0.5 mM 4-ABP. Dose dependent DNA damage was detected: adduct levels at 0.05, 0.1, 0.25, and 0.5 mM 4-ABP were measured to be 520±50, 2530±200, 5100±800, and 20000±3000 in 10^8 nucleotides, respectively.
Figure 4.5. 4-ABP-induced DNA damage in human bladder carcinoma RT-4 cells in a dose concentration study. RT-4 cells were treated with 4-ABP and harvested for measurement of dG-C8-4-ABP levels by LC-MS/MS.

4.4.1 b  Effect of Nrf2 specific siRNA treatment

Nrf2 was silenced in RT-4 cells by treatment with a Nrf2-specific siRNA for 48 h. Western blots of whole cell lysates from these Nrf2 siRNA treated cells were compared to those from control siRNA treated RT-4 cells. GAPDH served as the loading control. This western blot, shown in Figure 4.6 confirms that the control cells are expressing Nrf2 while the Nrf2 siRNA cells are not.
Figure 4.6. A western blot of Nrf2 in whole cell lysates from RT-4 cells treated with control siRNA and Nrf2 siRNA for 48 hours. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a loading control.

Both Nrf2 siRNA treated cells and control siRNA treated cells were then exposed to 4-ABP at 0.5 mM (plus S9) or N-OH-AABP at 0.1 mM for 3 h and adduct levels were quantified (Figure 4.7). As expected, in both cases higher levels of dG-C8-4-ABP were detected in cells treated with Nrf2 siRNA. Number of adducts detected in $10^8$ nucleosides in cells treated with 0.5 mM 4-ABP were 29000±5000 in Nrf2 siRNA cells and 16000±1000 in control cells. Number of adducts detected in $10^8$ nucleosides in cells treated with 0.1 mM N-OH-AABP were 5600±400 in Nrf2 siRNA cells and 3200±200 in control cells.
Figure 4.7. dG-C8-4-ABP DNA adduct levels in human bladder carcinoma RT-4 cells. After 48 h treatment with control siRNA or Nrf2 siRNA, RT-4 cells were exposed to 4-ABP plus S9 or N-OH-AABP for 3 h before dG-C8-4-ABP measurement.

4.4.2 Nrf2 potentiates 4-ABP DNA adduct formation in mouse bladder but inhibits 4-ABP DNA adduct formation in mouse liver

Wild type and Nrf2 knockout C57BL/6 mice (6-8 weeks of age) were administered a single dose of 50 mg/kg 4-ABP by i.p. injection and 4-ABP-DNA adduct levels in bladder and liver tissues were measured (Figure 4.8, Figure 4.9). Bladder tissue dG-C8-4-ABP levels in terms of adducts in $10^8$ nucleotides were as follows: female wild type:
Liver tissue dG-C8-4-ABP levels in terms of adducts in $10^8$ nucleotides were as follows: female wild type: 1900±300; female Nrf2$^{-/-}$: 2700±300; male wild type: 480±50; male Nrf2$^{-/-}$: 830±40. Dose-response data from male mice administered a single dose of 0, 5, 10, and 50 mg/kg was linear and for each dose, wild type mice had higher adduct levels than Nrf2 knockout mice.

![Graph showing dG-ABP adduct levels in wild type and Nrf2 knockout mouse bladder tissue following treatment with 50 mg/kg 4-ABP. After 24 hours, they were sacrificed and dG-C8-4-ABP levels in bladder tissue were measured by LC-MS/MS.](image-url)
Figure 4.9. dG-C8-4-ABP adduct levels in wild type and Nrf2 knockout mouse liver tissue following treatment with 50 mg/kg 4-ABP. After 24 hours, they were sacrificed and dG-C8-4-ABP levels in bladder tissue were measured by LC-MS/MS.

Our observations that adduct levels in the bladder tissue of male wild type mice were higher than those in Nrf2 knockout mice required further study. Accordingly, the next phase of this project involved an examination of the expression of key Nrf2-target genes with particular attention to those that are known to be involved in 4-ABP metabolism. As glucuronidation facilitates transport of 4-ABP from the liver to the bladder and is associated with 4-ABP-induced bladder carcinogenesis, it was not surprising to find that Nrf2-induced UGT enzymes may be partially responsible for increasing 4-ABP DNA adduct levels in bladder tissue of male mice.
4.4.3 *Nrf2* promotes UGT-mediated Phase 2 metabolism and urinary excretion of 4-ABP

Western blot analyses of the liver enzymes *N*-acetyltransferases (NAT1 and NAT2) and UGTs were compared between male wild type mice and Nrf2 knockout mice (*Figure 4.10*). Concentrations of NAT1 and NAT2 were not affected by the presence of Nrf2. One UGT1A and one UGT2B were up-regulated in Nrf2<sup>+/+</sup> mice compared to Nrf2<sup>−/−</sup> mice.

**Figure 4.10.** Western blots of liver homogenates from Nrf2<sup>+/+</sup> mice and Nrf2<sup>−/−</sup> mice. Arrows point to the UGT isozymes that were up-regulated by Nrf2. GAPDH is a loading control.
In view of the liver homogenate western blot analysis, UGT enzymes were further examined for their response to Nrf2 in an enzyme activity assay. Enzymatic activity in catalyzing the conjugation of 4-ABP with glucuronic acid was 1.94 fold higher in wild type mice than in Nrf2-deficient mice (Figure 4.11).

**Figure 4.11.** S9 fractions were prepared from liver tissues of Nrf2\(^{+/+}\) mice and Nrf2\(^{-/-}\) mice and measured for UGT activity in catalyzing 4-ABP conjugation with glucuronic acid.
Wild type and Nrf2 deficient mice were treated with 4-ABP at 50 mg/kg and 24-h urinary level of 4-ABP-N-glucuronide (adjusted by urinary creatinine) was measured. 4-ABP-N-glucuronide level was 34% higher in the wild type mice than in the Nrf2-deficient mice (Figure 4.12).

**Figure 4.12.** Total amounts of 4-ABP-N-glucuronide were measured in 24 h urine collected from Nrf2^{+/+} mice and Nrf2^{-/-} mice given 50 mg/kg 4-ABP and were adjusted by urinary creatinine levels.
The 4-ABP-N-glucuronide was analyzed by LC-MS for structural confirmation. The full scan of 1 ng 4-ABP-N-glucuronide standard (Figure 4.13) showed one obvious peak at 8.8 min with a mass spectrum base peak of m/z 346.1.

**Figure 4.13.** Full scan of 1 ng 4-ABP-N-glucuronide synthetic standard. A. Full scan. B. EIC 346.1 C. Mass spectrum.
The LC-MS/MS of the 4-ABP-N-glucuronide standard is seen in Figure 4.14 in which the mass spectrometer was set to isolate and fragment the precursor ion m/z 346.1 ± 1.0. The extracted ion chromatogram 346→170 represents the loss of the glucuronide, [M+H]^+→[M+H-176]^+.

**Figure 4.14.** LC-MS/MS of 4-ABP-N-glucuronide standard. The top panel shows the extraction ion chromatogram m/z 346→170 and the bottom panel shows the mass spectrum under this peak.
The chromatogram of the mouse urine sample had a major peak at 8.8 minutes, matching the retention time of the neat standard (Figure 4.15). Furthermore, the mass spectrum under this peak showed the same major fragments as those observed in standard mass spectrum.

**Figure 4.15.** Injection of mouse urine sample. 200 µL of urine from two wild type mice that were administered 50 mg/kg 4-ABP was taken through an acetonitrile precipitation and injected for detection of 4-ABP-N-glucuronide.
4.5 Discussion

4.5.1 Dose-response

High 4-ABP DNA adduct levels were preferred in these experiments because forthcoming chemoprevention research will use high concentrations of chemopreventive agents. Although such levels of exposure are not typically observed in humans, the same level of exposure by one carcinogen in real life leads to greater DNA damage than would be observed in an experimental setting. This is because typical human exposure occurs over a much longer time period, allowing for an accumulation of genetic damage. Furthermore, in real life, exposure to one carcinogen is combined with exposure to other carcinogens, instigating a synergistic effort to cause DNA damage. Based on our dose-response results, for the majority of our experiments we chose to treat cells with 500 µM 4-ABP plus S9 for 3 hours.

4-ABP treatment of human bladder carcinoma RT-4 cells for 3, 6 and 24 hours led to rapid dG-C8-4-ABP formation followed by rapid repair of these adducts. In the first 3 hours, DNA adduct levels reached 5 adducts in $10^6$ nucleotides but only increased 1.4 fold to 8 adducts in $10^6$ nucleotides. This indicated that the majority of the adducts had already formed in the first three hours. However, after 24 hours, adduct levels fell substantially to 1 adduct in $10^6$ nucleotides, 9% of the level observed at the 6 hour mark. We attributed this decrease in adduct level to repair as RT-4 cells carry the normal p53
and p53 has been shown to be required for repair of 4-ABP-induced genomic DNA damage in human bladder cells (34).

Dose dependent DNA damage was observed in human bladder carcinoma RT-4 cells after exposure to 4-ABP at 0.05, 0.1, 0.25, or 0.5 mM for 3 hours in the presence of rat liver S9 activation system. Adduct levels were below the limit of detection in cells that were treated with 0.5 mM 4-ABP not in the presence of S9. This confirmed that the bladder cells were deficient in the enzymes that activate 4-ABP and that S9 was required for 4-ABP activation. The occurrence of a linear dose-response curve suggests that sufficient S9 was being added to activate 4-ABP in the 3 hour dosing experiment and that neither activation, detoxification nor repair was saturated.

4.5.2 Nrf2 protects bladder cells in vitro

Nrf2 provided protection against 4-ABP and N-OH-AABP in human bladder carcinoma RT-4 cells. Compared to control cells containing Nrf2, dG-C8-4-ABP levels in Nrf2-silenced cells following treatment with either 4-ABP or N-OH-AABP were about 2-fold higher. This suggests that Nrf2 is involved in the detoxification of both 4-ABP and N-OH-AABP.

4.5.3 In vivo detection of dG-C8-4-ABP in mouse liver and bladder tissue

The C57Bl/6 mouse strain used in the research presented in this dissertation is a rapid acetylator strain. Therefore, a significant portion of the 4-ABP dose is expected to be
acetylated and detoxified from the liver before activation occurs. This acetylated conjugate can be oxidized, glucuronidated, and transported to the bladder where it is excreted even in acidic conditions (20). In wild type mice, however we still observed high adduct levels ranging from about 5 to 50 adducts in 10^6 nucleotides.

Our animal study involved comparisons between adduct levels in male and female mice because gender may affect bladder cancer risk. Epidemiological studies have indicated that men are more likely to develop bladder cancer than women, and this has been found to be true even among smokers who are exposed to more bladder carcinogens such as 4-ABP (24, 35). In one study, male mice that were chronically exposed to 4-ABP developed bladder cancer and had higher levels of DNA adduct levels in the bladder than females that developed liver cancer and had higher levels of DNA adducts in the liver (36). 4-ABP targets the liver in female BALB/c mice and the bladder in BALB/c male mice (36-38). It has been suggested that this difference may be due to variations in N-glucuronidation or N-acetylation, leading to differences in effective dose delivered to the bladder. Because 4-ABP targets the liver in female mice and because 4-ABP is primarily metabolized and activated in the liver in both genders, we chose to examine the effect of Nrf2 on dG-C8-4-ABP levels in liver tissue in addition to bladder tissue.

As expected, dG-C8-4-ABP levels were higher in bladder tissue of male mice than female mice, and higher in liver tissue of female mice than male mice. Furthermore, more adducts formed in the bladder than in the liver in male mice, and more adducts
formed in the liver than in the bladder in female mice. The presence of Nrf2 had a protective effect in both types of tissue in female mice, but only in the liver in male mice.

Interestingly, higher levels of dG-C8-4-ABP were detected in bladder tissue of wild type male mice than Nrf2\(^{-/-}\) male mice. This is the opposite effect that was observed in female mice and human bladder RT-4 cells. This reversed effect from bladder cells indicates that Nrf2 is acting outside the bladder to potentiate 4-ABP-induced DNA damage in the bladder. Knowing that 4-ABP is metabolized in the liver and that \(N\)-glucuronidation and \(N\)-acetylation are important in the metabolism of 4-ABP, subsequent experiments examined the effect of Nrf2 on the key catalyzing enzymes in these reactions.

Nrf2 activating compounds have in fact been shown to shift organ carcinogenicity from the liver to the bladder in previous studies. For example, treating rats with the Nrf2-activating compound butylated hydroxytoluene (BHT) has been shown to reduce N-2-fluorenylacetamide (FAA)-induced liver tumors but increase FAA-induced bladder tumors (39). Moreover, only liver tumors were observed in mice not treated with BHT. Accordingly, the observation that Nrf2 is involved in bladder carcinogenesis is not unprecedented. However, that Nrf2 potentiates DNA adduct levels caused by 4-ABP specifically is novel.
4.5.4 *Nrf2 promotes UGT-mediated Phase 2 metabolism and urinary excretion of 4-ABP*

Western blot experiments were carried out to investigate the contrasting in vitro and in vivo results in bladder cells of male mice. Because knocking out Nrf2 in male mice treated with 4-ABP resulted in fewer DNA adducts than their wild-type counterpart, we examined the effect of Nrf2 on NAT and UGT enzymes in male mice. Western blots of liver homogenates from male Nrf2\(^{+/+}\) mice and Nrf2\(^{-/-}\) mice showed that Nrf2 did not have an effect on the concentrations of NAT1 and NAT2 but that it up-regulated one UGT1A enzyme and one UGT2B enzyme. While neither NAT1 nor NAT2 have been shown to be regulated by Nrf2, a number of UGT isoforms are up-regulated by Nrf2 (17-19). The specific UGT isoforms that were up-regulated by Nrf2 in our research were not identified, but this is important to consider in future research in view of the potentially important role of UGT in bladder cancer. We confirmed that UGT activity and 4-ABP-\(N\)-glucuronide levels were increased in Nrf2\(^{+/+}\) mice compared to Nrf2\(^{-/-}\) mice. These results support the hypothesis that UGT enzymes are induced by Nrf2 and are involved in the detoxification and transport of 4-ABP in male mice.

Similarly, it was suggested that glucuronidation of benzidine was involved in benzidine-induced bladder cancer. The authors proposed that the \(N\)-glucuronide of benzidine in the liver resulted in the transport and accumulation of benzidine in the bladder (40).
4.6 Conclusions

Nrf2 is known as a cytoprotective protein involved in defense against chemical carcinogens and oxidants and as a promising target in chemoprevention, it is widely studied. Our research has shown that Nrf2 locally protects cells against DNA damage caused by 4-ABP. In vivo systems are complex, however, and our results have indicated that Nrf2-induced 4-ABP detoxification in one tissue may lead to an increase in its bioavailability in another. In fact, we found that Nrf2 induction of liver glucuronosyl transferases leads to high levels of bladder DNA adduct formation.

We observed differences in 4-ABP DNA adduct formation based on gender, which has previously been reported in the literature (36-38). In female mice, the effect of Nrf2 on 4-ABP DNA adduct formation in the bladder and liver was similar to that in bladder cells; Nrf2 protected against 4-ABP-induced damage and thus its activation would be expected to be an effective strategy against 4-ABP. While this was also true in liver tissue of male mice, the opposite effect occurred in male bladder tissue. This may be due in part to Nrf2 induced up-regulation of UGT enzymes that metabolize and transport 4-ABP.

Therefore, induction of liver UGT may be harmful to the bladder in humans exposed to 4-ABP, particularly in smokers who are exposed to greater quantities of the carcinogen. It is important that a cancer chemopreventive agent that activates Nrf2 does not
significantly induce liver UGT. Accordingly, activation of Nrf2 is a suitable chemopreventive strategy against 4-ABP provided that the chemopreventive agent is specific to bladder tissue or does not up-regulate UGT in liver tissue.
References


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CHAPTER 5

INHIBITION OF 4-ABP DNA ADDUCT FORMATION BY SULFORAPHANE AND 5,6-DIHYDROCYCLOPENTA[C][1,2]-DITHIOLE-3(4H)-THIONE IN BLADDER CELLS AND TISSUES
In chapter 4 we concluded that activation of Nrf2 should be considered as a strategy for chemoprevention of 4-ABP-induced bladder cancer, provided that the UGT enzymes in the liver tissue are not up-regulated. The promising chemopreventive agents Sulforaphane (4-methylsulphinylbutyl isothiocyanate, SF) and 5,6-dihydrocyclopenta[c][1,2]-dithiole-3(4H)-thione (CPDT) have been shown to mediate some of their cytoprotective effects through Nrf2 and moreover they have shown specificity to the bladder. Furthermore, it is well recognized that SF and CPDT both induce Phase 2 enzymes which are involved in carcinogen detoxification. We hypothesized that SF and CPDT inhibit 4-ABP DNA adduct formation and act through mechanisms involving Nrf2 to carry out these cytoprotective effects.

SF, an isothiocyanate and CPDT, a dithiolethione are derived from compounds found in cruciferous vegetables (plants of the family Brassicaceae or Cruciferae) (1). Their chemical structures are shown in Figure 5.1. Both have shown promise as anticancer and anticarcinogenic compounds, acting on all stages of carcinogenesis and interfering with several cell-signaling pathways (2, 3).
5.1.1 Sulforaphane, an isothiocyanate (4)

SF is a well-known chemopreventive phytochemical derived from the glucosinolate Glucaraphanin (β-thioglucoside N-hydroxysulfate), in which broccoli sprouts are especially rich (~ 1000 µmol SF per kg fresh broccoli). The conversion of Glucuraphanin to SF is shown in Figure 5.2. When damage to the plant occurs, such as that from food processing, chewing, or digestion, glucaraphanin comes in contact with myrosinase (thioglucoside glucohydrolase) and is deglycosylated. Glucuraphanin that reaches the intestinal tract without coming in contact with myrosinase can be hydrolyzed by microflora. Following hydrolysis, the intermediate thiohydroximate-O-sulfonate can either be spontaneously converted to the chemopreventive agent SF or catalytically converted to the SF nitrile by Epithiospecifier protein (ESP).

The glucuraphanin content of a particular vegetable depends on a number of factors including variety, age, and growth conditions (5). Broccoli sprouts, for example are
much richer in glucuroaphanin than mature florets. In addition to this, the SF yield varies depending on storage conditions, cooking conditions, metabolism of the devouring individual, and enteric bacteria in an individual (4, 6, 7). High heat, such as that during steaming or boiling denatures myrosinase and decomposes SF. Heating at 60-70°C, however, can denature ESP without inactivating myrosinase, resulting in a high SF yield. The sulfur in the sulfinyl functional group is a chiral center of Sulforaphane, but R-SF and S-SF show the same chemopreventive properties. Broccoli however, yields only R-SF (8).

**Figure 5.2.** Conversion of glucoraphanin to SF or the SF nitrile. (Reproduced from (4))
SF has been reported to have a number of chemopreventive properties; it has been shown to inhibit carcinogen-activating enzymes, induce carcinogen deactivating enzymes, induce apoptosis, arrest cell cycle progression, inhibit inflammation and angiogenesis, inhibit the growth of cancer cells, prevent cancer development, reduce damage caused by carcinogens, induce cell death, and affect the cell cycle, invasion and metastasis (4, 9, 10).

SF may also be particularly important in bladder cancer chemoprevention and several studies have shown that SF is selectively delivered to bladder tissue through urinary excretion (9, 11, 12). Furthermore, epidemiological research has found a link between consumption of SF-rich foods and decreased human bladder cancer risk (12-14).

5.1.2 SF inhibits DNA adduct formation and induces Phase 2 enzymes

Several studies have shown that SF inhibits the formation of DNA adducts in a dose-dependent manner and induces Phase 2 enzymes (15-19). In one study, SF inhibited 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) DNA adduct formation in both human HepG2 cells and human hepatocytes as measured by Accelerator Mass Spectrometry and induced UDP-glucuronosyltransferase (UGT) 1A1 and glutathione S-transferase (GST) A1 mRNA expression. In this study, SF did not appear to affect DNA repair (15). In another study, SF was found to inhibit the formation of benzo[a]pyrene (B[a]P) and 1,6-dinitropyrene (1,6-DNP) DNA adducts in MCF-10F cells as measured by 32P-postlabeling and increase the expression of NAD(P)H-quinone reductase (QR) and
GST proteins (16). In addition, several studies have shown that SF inhibits DNA adducts of the human liver carcinogen aflatoxin B$_1$ (AFB$_1$) and these observations have also been made in conjunction with experiments showing its induction of Phase 2 enzymes.

In vitro, SF inhibited AFB$_1$ DNA adduct formation in human hepatocyte cultures as observed by liquid scintillation counting, and GSTM1 was shown to be involved in AFB$_1$ DNA adduct inhibition. SF did not increase GSTM1 expression but it did decrease CYP3A4 mRNA. SF, however was only effective in inhibiting the formation of AFB$_1$ DNA adducts when cells were treated with it before the carcinogen rather than at the same time or after, indicating that its protective effects were dependent on changes in gene expression rather than inhibition of catalytic activity. Compared to its analog phenylethyl isothiocyanate (PEITC), SF was more effective in reducing AFB$_1$ DNA adduct levels, although both were effective inhibitors (Figure 5.3) (19).
Figure 5.3. SF and PEITC reduced AFB\textsubscript{1} DNA adduct formation in human hepatocyte cultures. Hepatocytes were treated with the phytochemical and then were co-incubated with \textsuperscript{3}H-AFB\textsubscript{1}. The mean control value from cells only treated with \textsuperscript{3}H-AFB\textsubscript{1} was 5.9 adducts in 10\textsuperscript{7} nucleotides. (Reproduced from (19))

In vivo, SF reduced AFB\textsubscript{1} DNA adduct formation and increased GST levels in two strains of rats. In this study, AFB\textsubscript{1}-N\textsuperscript{7}-gua was hydrolyzed from liver tissue DNA and quantified by UPLC-MS. Interestingly, both chemopreventive agents had a greater effect on Sprague Dawley rats, which are more resistant to AFB\textsubscript{1} adduct formation than Fischer rats in reducing both adduct formation and increasing GST levels. Although the researchers also investigated gender-specific responses to SF, no differences were observed (17).
A human study was also conducted to measure the chemopreventive potential of SF in people at high risk of developing AFB$_1$-induced hepatocellular carcinoma. Participants consumed 3-day-old broccoli sprouts or a placebo and their urinary levels of AFB$_1$-N$^7$-gua were measured by LC-MS/MS. Although no correlation between broccoli sprout consumption and reduced levels of AFB$_1$-N$^7$-gua was found, this was attributed to the large variation in glucosinolate concentration in the broccoli sprout mixtures. An inverse correlation however was found between the SF metabolites dithiocarbamates and AFB$_1$-N$^7$-gua (18).

5.1.3 CPDT, a dithiolethione (3, 20)

Many dithiolethiones have shown promise as chemopreventive agents, particularly in their induction of cytoprotective proteins that are involved in the detoxification of carcinogens. 3H-1,2-dithiole-3-thione (D3T) represents the basic structure of these chemopreventive dithiolethiones and a number of its analogs have been synthesized. Both 5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione (ADT) and 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (Oltipraz) are well studied and are shown in Figure 5.4. Oltipraz has been shown to decrease N-nitrosobutyl(4-hydroxybutyl)amine (BBN) induced bladder cancer in vivo and is effective against AFB$_1$, B[a]P, and PhIP. A number of clinical trials have also been conducted on Oltipraz but it has exhibited limited efficacy with substantial side effects.
Several different dithiolethiones were compared based on their mitigation of \( \text{AFB}_1 \) DNA adduct levels. In this study, rats were pre-treated with dithiolethiones followed by administration of \( \text{AFB}_1 \), and \( \text{AFB}_1 \) DNA adducts in liver tissue were measured by radiometric methods. All dithiolethiones, including CPDT reduced adduct levels compared to the control levels from rats that were not pre-treated with a dithiolethione \((21)\).

CPDT was shown to be a highly effective Nrf2 activator and Phase 2 enzyme inducer in cultured bladder cells, and its induction of Phase 2 enzymes was shown to depend on Nrf2. In a rat study in vivo, CPDT was particularly effective in the bladder, and more significantly, Nrf2 activation and induction of Phase 2 enzymes in the bladder by CPDT
occurred exclusively in the epithelium, which is the principal site of bladder cancer development (22). Its activation of Nrf2, induction of Phase 2 enzymes and bladder tissue specificity was confirmed in a separate study (23).

5.2 Project Goals

The ultimate goal of this research was to determine whether or not SF and CPDT inhibit 4-ABP DNA adduct formation in bladder cells and tissues. Because we have found that Nrf2 is a promising target for inhibition of 4-ABP DNA adducts, we first investigated whether or not we could observe an inductive effect of SF and CPDT on Nrf2. Following our observations that Nrf2 was induced by both SF and CPDT, we examined the effect of these agents on adduct levels in cells and animals with and without Nrf2.

5.3 Experimental

All cell and animal dosings and western blots were carried out and analyzed in Dr. Yuesheng Zhang’s laboratory at Roswell Park Cancer Institute, Buffalo, NY by Joseph Paonessa and Yi Ding.
5.3.1 Chemicals

SF, 4-ABP, and N-hydroxy-N-acetyl-4-aminobiphenyl (N-OH-AABP) were purchased from LKT Laboratories (St. Paul, MN), Sigma (St. Louis, MO), and Midwest Research Institute (Kansas City, MO), respectively. CPDT was synthesized (24). Rat liver S9 (36-43 mg protein/ml) was purchased from Moltox (Boone, NC). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), including antibodies specific for the catalytic subunit and the regulatory subunit of glutamate cysteine ligase (GCSc and GCSm), NAD(P)H:quinone oxidoreductase 1 (NQO1) and Nrf2, from Alpha Diagnostic International (San Antonio, TX), including antibodies specific for the α isoform of glutathione S-transferase (GST), and from Millipore (Billerica, MA) for the antibody of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Another antibody against NQO1 purchased from Cell Signaling Technology (Danvers, MA) was used in some of the Western blot analyses.

5.3.2 Cell study

Human bladder carcinoma RT-4 cells were cultured (25). The cell line was purchased from ATCC (Manassas, VA), stored in liquid nitrogen, and passaged in our laboratory for fewer than 6 months. To induce DNA damage, RT-4 cells (2-3 x 10^6 cells) were grown in each 10-cm dish with 10 ml medium 24-48 h, followed by treatment with either 4-ABP or N-OH-AABP for 3 h in 5 ml fresh medium per dish. In cases where cells were co-treated with 4-ABP and S9, the medium also contained 6% S9 (v/v), 10 mM glucose-6-phosphate and 5 mM NADP. In experiments involving pretreatment with a
chemopreventive agent, cells were first treated with SF, CPDT or vehicle and after 24 h or 48 h, exposed to the carcinogen. All test agents were dissolved in DMSO, and the final concentration of DMSO in medium was \( \leq 5\% \). Cells were harvested by trypsin treatment and low-speed centrifugation, and cell pellets were washed with ice-cold PBS and used for DNA adduct analysis.

5.3.3 Animal study

Wild type and Nrf2\(^{-/-}\) C57BL/6 mice (male, 6 weeks of age) were used. The wild type mice were purchased from National Cancer Institute (Frederick, MD). The Nrf2-deficient mice were bred at the animal facility at Roswell Park Cancer Institute; the breeders were kindly provided by Dr. Thomas W. Kensler (Johns Hopkins Bloomberg School of Public Health) (26). Groups of 3-4 mice were randomized and treated with SF or vehicle by gavage once daily for 5 days. Three hours after the last SF dose, the mice were each administered a single dose of 4-ABP i.p. and were sacrificed 24 h after 4-ABP treatment to collect the livers and bladders for measurement of dG-C8-4-ABP. For CPDT intervention, the mice were treated with CPDT (in soy oil) or the vehicle by gavage once daily for 5 days; 4-ABP was given 3 h after the last CPDT dose. To determine the effect of SF or CPDT on Phase 2 enzymes by Western blotting, mice were treated with SF or CPDT by gavage once daily for 5 days and sacrificed 24 h later. Liver and bladder tissues were stored at -80\(^\circ\)C. All animal protocols and procedures were approved by the Roswell Park Cancer Institute Animal Care and Use Committee.
5.3.4 Quantification of dG-C8-4-ABP

Procedures of DNA isolation from bladder cells and tissues and the detailed protocol for the quantitative analysis of dG-C8-4-ABP by capillary liquid chromatography and nESI-MS/MS have been described in Chapter 3 of this dissertation and in a recent publication (27).

5.3.5 Western blot analysis

Cellular and tissue levels of Nrf2 and/or several Phase 2 enzymes were measured by Western blotting (22). All antibodies were purchased from Santa Cruz Biotechnology, except for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which was purchased from Millipore.

5.3.6 Statistical analysis

The numerical results are expressed as means ± SD. Unpaired two-tailed Student t-test was used for data analysis, with a P value <0.05 being considered significant (GraphPad Version 5.00, GraphPad Software, San Diego, CA).
5.4 Results

Before investigating the effect of SF and CPDT on 4-ABP DNA adduct levels, we examined their effect on the expression of Nrf2 and Nrf2-regulated proteins. After observing that both agents did indeed induce Nrf2 and the Nrf2 signaling pathway, we compared the effect of these agents on cells with and without Nrf2 with the goal of evaluating the compounds as chemopreventive agents and determining the function of Nrf2 in their chemopreventive activities.

5.4.1 SF and CPDT activate Nrf2 and the Nrf2 signaling pathway in human bladder cells and mouse bladder tissue

The effect of SF treatment on Nrf2 and Nrf2-regulated enzymes was examined in human bladder carcinoma RT-4 cells. Western blot analyses of RT-4 cells that were treated with 0, 2, and 4 μM SF for 24 h were conducted on Nrf2 and several Phase 2 enzymes including the catalytic subunit of glutamate cysteine ligase (GCS\textsubscript{C}), the regulatory subunit of GCS (GCS\textsubscript{M}) and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Figure 5.5). Treatment with SF increased expression of Nrf2 and Nrf2-regulated GCS\textsubscript{C}, GCS\textsubscript{M} and NQO1. Western blots of UDP-glucuronosyltransferases (UGTs) and N-acetyltransferases (NATs) however, did not indicate a change in expression levels following treatment with SF.
Figure 5.5. SF activates Nrf2 and the Nrf2 signaling pathway in human bladder carcinoma RT-4 cells. RT-4 cells were treated with SF for 24 h and then harvested for measurement of Nrf2 and selected Phase 2 proteins by Western blotting. GAPDH is a loading control. The data are representative of two experiments.

Human bladder carcinoma RT-4 cells were then treated with 0, 12.5, and 25 µM CPDT for 48 h and activation of Nrf2 and Nrf2-regulated proteins was measured by western blot analyses (Figure 5.6). Treatment with CPDT resulted in a significant increase in the expression of Nrf2, NQO1, GCSc and GCSm. Two UGT1A bands and four UGT2B bands were detected in RT-4 cells, but CPDT had no effect on UGT1A and only slightly elevated two UGT2B isoforms. The identities of these isoforms have not been determined.
Figure 5.6. CPDT activates Nrf2 and the Nrf2 signaling pathway in human bladder carcinoma RT-4 cells. RT-4 cells were treated with CPDT for 24 h. Nrf2 and Phase 2 enzymes in whole cell lysates were measured by Western blotting. GAPDH is a loading control. The arrows indicate two UGT2B bands (isoforms) induced by CPDT.

Wild-type C57BL/6 mice and Nrf2 knocked out C57BL/6 mice were treated with vehicle or SF by gavage once a day for 5 days. The mice were killed 24 h later for measurement of selected Phase 2 proteins by Western blotting (Figure 5.7). Mice treated with 0 µmol/kg SF showed significantly lower Phase 2 enzyme expression in both the bladder and liver tissue in Nrf2<sup>−/−</sup> mice compared to Nrf2<sup>+/+</sup> mice. In both the bladder and liver of wild type mice, SF treatment resulted in increased expression of glutathione S-transferase
(GST), GCS, and NQO1 but this did not occur in Nrf2\textsuperscript{−/−} mice. SF treatment did not affect the expression levels of UGTs and NATs in either tissue as indicated by western blots.

**Figure 5.7.** Wild-type C57BL/6 mice and Nrf2 knocked out C57BL/6 mice were treated with vehicle or SF by gavage once a day for 5 days. The mice were killed 24 h later for measurement of selected Phase 2 proteins by Western blotting. GAPDH is a loading control. The data are representative of mice in each group.
Nrf2\(^{+/+}\) mice and Nrf2\(^{-/-}\) mice were treated with CPDT or vehicle by gavage once daily for 5 days; 24 h later after the last CPDT dose, animals were killed, and Phase 2 enzymes in the bladders and livers were measured by Western blotting (Figure 5.8). Mice treated with 0 \(\mu\)mol/kg CPDT showed significantly lower Phase 2 enzyme expression in both the bladder and liver tissue in Nrf2\(^{-/-}\) mice compared to Nrf2\(^{+/+}\) mice. In the bladder of wild type mice, CPDT activated Nrf2, GCSc, GCSm, NQO1, one isoform of UGT1A, and one isoform of UGT2B. In the liver of wild type mice, CPDT induced NQO1, one isoform of UGT1A, several isoforms of UGT2B, but not GCSc, GCSm, and the remaining isoforms of UGT1A and UGT2B. CPDT did not induce any of the enzymes examined in both the bladder and liver of Nrf2\(^{-/-}\) mice. GCSc and GCSm were undetectable in the bladder of Nrf2\(^{-/-}\) mice but significant levels of these enzymes were detected in their liver tissues. Multiple bands of both UGT1A and UGT2B were detected in the bladder and liver, reflecting their multi-isomeric nature, but only one band in each family was up-regulated by Nrf2. The arrows indicate the UGT bands (isoforms) that were regulated in an Nrf2-dependent manner. The Nrf2-regulated UGT bands were slightly elevated by CPDT in the bladder tissues, and to a lesser extent in the liver tissues.
Figure 5.8. Nrf2\(^{+/+}\) mice and Nrf2\(^{-/-}\) mice were treated with CPDT or vehicle by gavage once daily for 5 days; 24 h later after the last CPDT dose, animals were killed, and Phase 2 enzymes in the bladders and livers were measured by Western blotting. GAPDH is a loading control. The arrows indicate the UGT bands (isoforms) that were regulated in an Nrf2-dependent manner.
5.4.2 **SF inhibits dG-C8-4-ABP formation in bladder carcinoma cells**

RT-4 cells were pretreated with 0, 2 or 4 μM SF for 24 h and then exposed to 0.5 mM 4-ABP plus S9 for 3 h. They were harvested and dG-C8-4-ABP levels were examined (Figure 5.9). Samples prepared in the absence of SF are used as a positive control and percent inhibition is measured based on the adduct levels of these samples. Compared to the positive control, cells treated with 2 and 4 μM SF had 70±6% and 59±9% lower adduct levels, respectively. RT-4 cells that were pretreated with SF for 24 h and then exposed to 0.5 mM 4-ABP plus S9 for only 1 h showed 52±3% and 45±6% lower adduct levels than the positive control following 2 and 4 μM SF pretreatment. When RT-4 cells were pretreated with only 1 and 2 μM SF for 24 h and then exposed to 0.5 mM 4-ABP plus S9 for 3 h, 68±4% and 67±4% lower adduct levels were observed than the positive control following 1 and 2 μM SF pretreatment.
Figure 5.9. 4-ABP-induced DNA damage in human bladder cells and the protective effect of SF. RT-4 cells were pretreated with SF for 24 h and then exposed to 0.5 mM 4-ABP plus S9 before measurement of dG-C8-4-ABP levels. In the top panel cells were exposed to 2 and 4 µM SF pretreatment and followed by 4-ABP treatment for 3 hours. In the middle panel cells were exposed to 2 and 4 µM SF pretreatment and followed by 4-ABP treatment for 1 hours. In the bottom panel, cells were exposed to 1 and 2 µM SF pretreatment and followed by 4-ABP treatment for 3 hours.
Human bladder carcinoma RT-4 cells and rat bladder carcinomaa NBT-II cells were treated with 0, 2, or 4 µM SF in sprout form for 24 h and then exposed to 0.5 mM 4-ABP plus S9 for 3 h. Subsequent measurement of dG-C8-4-ABP showed a decrease in adduct levels following SF treatment in both cell types (Figure 5.10, Figure 5.11). Compared to the positive control, RT-4 cells treated with 2 and 4 µM SF in sprouts had 55±7% and 45±2% lower adduct levels, respectively. Compared to the positive control, NBT-II cells treated with 2 and 4 µM SF in sprouts had 39±7% and 51±7% lower adduct levels, respectively.

Figure 5.10. 4-ABP-induced DNA damage in human bladder cells and the protective effect of SF in sprout form. RT-4 cells were pretreated with SF in sprout form for 24 h and then exposed to 0.5 mM 4-ABP plus S9 for 3 h before measurement of dG-C8-4-ABP levels.
Figure 5.11. 4-ABP-induced DNA damage in rat bladder cells and the protective effect of SF in sprout form. NBT-II rat bladder carcinoma cells were pretreated with SF in sprout form for 24 h and then exposed to 0.5 mM 4-ABP plus S9 for 3 h before measurement of dG-C8-4-ABP levels.

5.4.3 CPDT inhibits dG-C8-4-ABP formation in RT-4 bladder carcinoma cells

RT-4 cells were pretreated with CPDT at 12.5 and 25 μM for 48 h; cells were then washed with fresh medium and exposed to 0.5 mM 4-ABP plus rat liver S9 for 3 h. dG-C8-4-ABP levels in these cells were then measured. The CPDT concentrations as well as the treatment conditions for 4-ABP were based on previous findings (22, 28). Treatment with 12.5 and 25 μM CPDT to significant and dose-dependent inhibition of dG-C8-4-ABP formation: 24±6% and 61±6% inhibition respectively (Figure 5.12).
Figure 5.12. CPDT inhibits 4-ABP DNA adduct formation in human bladder carcinoma RT-4 cells. RT-4 cells were treated with CPDT or solvent for 48 h and then exposed to 4-ABP plus S9 for 3 h, followed by dG-C8-4-ABP analysis by LC-MS/MS.

5.4.4 SF and CPDT inhibit dG-C8-4-ABP formation in mouse bladder tissue and Nrf2 is essential for this chemopreventive activity

To assess the effect of SF on 4-ABP in vivo, both wild type mice and Nrf2 knockout mice were administered SF at 0, 10, 40 µmol/kg by gavage once daily for 5 days. A single i.p. dose of 4-ABP at 50 mg/kg was given 3 h after the last SF dose. The mice were sacrificed 24 h after 4-ABP treatment, and the bladders were processed for measurement.
of DNA damage. SF caused dose-dependent inhibition of dG-C8-4-ABP formation in the bladder of the wild type mice, achieving 50±10% and 61±7% inhibition at 10 and 40 µmol/kg dose levels, respectively, as compared to the positive control, mice treated only with 4-ABP. SF did not inhibit adduct formation in the Nrf2 knockout mice (Figure 5.13).

The chemopreventive activity of CPDT was also assessed in mouse bladder and liver tissue based on its inhibition of 4-ABP DNA adducts. Wild type and Nrf2 knockout mice were administered CPDT at 0, 5, 20 and 80 mg/kg by gavage once daily for 5 days; 3 h after the last CPDT dose, each mouse was given 4-ABP at 50 mg/kg i.p.; the mice were killed 24 h after 4-ABP injection for measurement of tissue dG-C8-4-ABP levels. 27±4%, 59±2%, and 67±4% lower adduct levels were observed in the bladder tissue of wild type mice pretreated with 5, 20, and 80 µmol/kg CPDT respectively as compared to the positive control (Figure 5.14). 11±6%, 9±6%, and 33±6% lower adduct levels were observed in the bladder tissue of Nrf2 knockout mice pretreated with 5, 20, and 80 µmol/kg CPDT respectively as compared to the positive control.
Figure 5.13. SF requires Nrf2 to inhibit 4-ABP DNA adduct formation in mouse bladder tissue. Wild-type and Nrf2-knocked out C57BL/6 mice were treated with vehicle or SF by gavage once a day for 5 days. Three hours after the last SF dose, each mouse was given a single i.p. dose of 4-ABP. The mice were killed 24 h later for measurement of dG-C8-4-ABP in bladder tissues.
Figure 5.14. CPDT inhibits 4-ABP DNA adduct formation in wild type mouse bladder tissue. Nrf2<sup>++</sup> mice and Nrf2<sup>−/−</sup> mice were treated with CPDT or vehicle by gavage once daily for 5 days; 3 h after the last CPDT dose, each mouse was given 4-ABP i.p.; 24 h later, animals were killed, and dG-C8-4-ABP levels in the bladders and livers were quantified by LC-MS/MS.
CPDT did not inhibit 4-ABP DNA adduct formation in the livers of wild type mice (Figure 5.15).

Figure 5.15. CPDT does not inhibit 4-ABP DNA adduct formation in mouse liver tissue. Nrf2+/+ mice were pretreated with CPDT or vehicle by gavage once daily for 5 days; 3 h after the last CPDT dose, each mouse was given 4-ABP i.p.; 24 h later, animals were killed, and dG-C8-4-ABP levels in the bladders and livers were quantified by LC-MS/MS.
5.5 Discussion

5.5.1 SF and CPDT activate Nrf2 and the Nrf2 signaling pathway

The effect of treatment by the chemopreventive agents SF and CPDT on Nrf2 and Nrf2-regulated enzymes was examined by western blot analyses. Expression levels of GCS\textsubscript{C}, GCS\textsubscript{M}, NQO1, UGTs, NATs and GSTs were evaluated. Although UGTs and NATs are known to be involved in 4-ABP metabolism and NQO1 and GCS may modulate 4-ABP detoxification (29), changes in their expression levels in these experiments only indicates Nrf2 transactivation and is not necessarily related to 4-ABP metabolism.

SF and CPDT treatment led to significant increases in Nrf2 levels in human bladder carcinoma cells and this is consistent with previous reports (22, 30). As both agents induced Nrf2, increased expression levels of the Nrf2-regulated GCS\textsubscript{C}, GCS\textsubscript{M} and NQO1 were observed, indicating activation of the Nrf2 signaling pathway. Interestingly, SF did not induce two Phase 2 enzymes that are thought to be very significant in 4-ABP-induced cancer, UGTs and NATs. CPDT induced expression of one isoform of UGT1A and one isoform of UGT2B out of several UGTs that were observed.

Effect of SF and CPDT on Nrf2 and Nrf2 regulated proteins was also examined in mouse bladder tissue. In general, lower enzyme levels were observed in western blots of Nrf\textsubscript{2}\textsuperscript{-} mice than Nrf\textsubscript{2}\textsuperscript{++} mice indicating that these enzymes are at least partly regulated by Nrf2. Importantly, both SF and CPDT clearly elevated expression of GST, GCS and NQO1 of
wild type mice, but not Nrf2 knockout mice, showing activation of the Nrf2 cytoprotective signaling pathway in the bladder. This is consistent with the results from bladder cells. Also in agreement with bladder cell results was that CPDT pretreatment resulted in higher levels of one isoform of UGT1A and one isoform of UGT2B and that SF pretreatment did not affect the levels of UGT or NAT.

4-ABP is known to be metabolized mainly in the liver and therefore hepatic levels of these Phase 2 enzymes were also analyzed. Because hepatic levels of GST, GCS and NQO1 were significantly elevated in SF-treated wild type mice, but not in the Nrf2 knockout mice, SF was determined to be not bladder specific. This is inconsistent with previous reports showing that SF is selectively delivered to the bladder through urinary excretion (9, 11, 12). However, the fact that SF did not induce UGTs which are important for 4-ABP transport to the bladder, indicates that its induction of other Phase 2 enzymes in the liver may not necessarily increase the bioavailability of 4-ABP in the bladder. Therefore, despite the apparent lack of organ specificity, SF should not be counted out as a potential chemopreventive agent against 4-ABP-induced bladder cancer. On the other hand, CPDT appears to target the bladder but not the liver; it only induced NQO1 out of the three Nrf2 regulated genes examined and its inducing effect on UGT in the liver was limited. CPDT therefore seems to be relatively bladder-specific and this is in agreement with previous data (22).
Interestingly, while both GCSc and GCSm were undetectable in the bladder of Nrf2−/− mice, significant levels of these enzymes were detected in their liver tissues, implying that these genes are subjected to regulation by Nrf2-independent mechanisms in the liver.

In view of these positive western blot results indicating the SF and CPDT activate Nrf2, we proceeded with our research examining the effect of these agents on 4-ABP DNA adduct formation.

5.5.3 SF and CPDT inhibit 4-ABP DNA adduct formation in bladder cells and tissues

Inhibition by chemopreventive agents in these studies is not likely to be caused by the chemopreventive agent preventing S9 from activating 4-ABP or by direct interaction between the chemopreventive agent and 4-ABP. This is because cells are pretreated with the chemopreventive agent which is removed before exposure to the carcinogen. The occurrence of adduct inhibition is therefore more likely to be caused by induction of Phase 2 cytoprotective proteins.

Both CPDT and SF were determined to be highly effective in inhibiting 4-ABP-induced genotoxicity in bladder cells. Compared to positive control cells that were only treated with 4-ABP, cells treated with 2 and 4 µM SF had 70% and 59% lower adduct levels, respectively. Interestingly, the low SF dose was somewhat more effective. CPDT treatment led to dose-dependent inhibition of dG-C8-4-ABP formation; 24% and 61%
fewer adducts were measured in cells treated with 12.5 and 25 µM CPDT compared to the positive control.

The decrease in adduct levels following SF in sprout form was unexpectedly less pronounced than that following pure SF treatment. A study involving SF in sprout form more closely resembles typical human consumption of broccoli, and cruciferous vegetables containing bioactive components other than glucosinolates that may play a role in anti-carcinogenesis. For example, brussel sprouts which contain a large variety of glucosinolates, also contain a number of protective compounds including glucobrassicin, crambene, S-methyl cysteine sulfoxide, and dithiolethione. In broccoli however, SF only has been shown to be responsible for the up-regulation of detoxification enzymes. It is possible that another compound in the sprout mixture is interfering with SF’s induction of cytoprotective enzymes or is inducing 4-ABP activating enzymes (31).

5.5.4 *SF and CPDT inhibit dG-C8-4-ABP formation in mouse bladder tissue but not in mouse liver tissue*

Pretreatment with either SF or CPDT resulted in dose-dependent inhibition of dG-C8-4-ABP formation in the bladder of wild type mice that were treated with 4-ABP: 10 and 40 µmol/kg SF pretreatment led to 50% and 61% inhibition of 4-ABP DNA adducts, respectively and 5, 20, and 80 µmol/kg CPDT pretreatment led to 27%, 59%, and 67% inhibition of 4-ABP DNA adducts respectively. SF did not inhibit dG-C8-4-ABP formation in the bladder tissue of Nrf2 knockout mice that were treated with 4-ABP and
therefore Nrf2 is required by SF to inhibit 4-ABP-induced DNA damage in the bladder. CPDT only slightly inhibited adduct formation in Nrf2 knockout mice treated with 4-ABP. These very much reduced chemopreventive effects of CPDT in Nrf2 knockout mice indicate that the main cytoprotective pathway for this agent is through Nrf2. However, the occurrence of some chemopreventive activity by CPDT in Nrf2 knockout mice suggests that CPDT may act through Nrf2-independent pathways as well.

Liver tissue from the mice treated with CPDT was also examined for 4-ABP DNA adduct formation and in contrast to the inhibition that was observed in the bladder tissue, CPDT pretreatment had virtually no effect on 4-ABP-induced dG-C8-4-ABP formation in the livers of wild type mice. Previously, researchers have shown that CPDT and several other dithiolethiones are specific for bladder tissue (23). Our observations that CPDT did not inhibit 4-ABP DNA adduct formation in mouse liver tissue supports this prior research. The inability of CPDT to protect liver tissue against 4-ABP in Nrf2 knockout mice suggests that detoxification and therefore glucuronidation of 4-ABP and its metabolites is diminished without Nrf2.
5.6 Conclusion

We have shown that SF and CPDT are highly effective in blocking 4-ABP-induced DNA damage in human bladder cells in vitro and in mouse bladder tissue in vivo. Furthermore, we have shown that not only do these agents activate the well-known cytoprotective protein Nrf2 and the Nrf2 signaling pathway, but they require Nrf2 to substantially inhibit 4-ABP DNA damage. Although SF was not bladder specific, it did not induce UGTs which were determined to be significant enzymes in the transport of 4-ABP from the liver to the bladder. Showing even more promise as a bladder chemopreventive agent, CPDT did not induce Nrf2 in the liver nor did it protect the liver against the formation of 4-ABP DNA adduct formation.

SF and CPDT are promising cancer chemopreventive agents, as they strongly protect cells and tissues against 4-ABP. Both agents have high potential as bladder chemopreventive agents because they do not induce UGTs in the liver. Accordingly, our research suggests that SF and CPDT should continue to be investigated as chemopreventive agents against 4-ABP induced bladder cancer.
References


CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS
6.1 Summary

Quantification of DNA adducts can have significant implications in risk assessment. Moreover, they are important biomarkers in the analysis of chemopreventive agents. Detection of these adducts in healthy tissue can indicate the need for administration of a chemopreventive agent. A decrease in adduct levels following treatment with a chemopreventive agent can reflect the efficacy of the agent in reducing bladder cancer risk. Detection of 4-ABP DNA adducts in human samples is a challenging undertaking due to the minimal sample often available and the low adduct levels expected. In the case of chemoprevention research these levels may be even lower and a sensitive method for detection is therefore required. Cell and animal experiments in this field of research are particularly important for elucidating the mechanisms of action of these agents and to achieve high enough adduct levels for detection and clear signs of inhibition. In view of this, we developed an LC-MS/MS method sensitive enough for detection of adducts in human samples and designed cell and animal experiments for the purpose of evaluating chemoprevention strategies and agents.

This LC-MS/MS method enabled quantification of dG-C8-4-ABP in human bladder cells and animal tissues, and most significantly human urothelial cells. Our chemopreventive research focused on targeting the cytoprotective protein Nrf2. The findings that Nrf2 potentiated 4-ABP DNA adduct formation in mouse bladder tissue in vitro but protected mouse liver tissue and human bladder cells against 4-ABP initiated an investigation into
the effect of Nrf2 on key Phase 2 enzymes involved in 4-ABP metabolism. We observed that UTG, a protein that has already been implicated in 4-ABP-induced bladder carcinogenesis, is at least partly responsible for increasing the bioavailability of Nrf2 in the bladder. Nrf2 protects cells locally against 4-ABP and accordingly, is an effective target for a chemopreventive agent against bladder carcinogenesis provided that the chemopreventive agent does not up-regulate UGT in the liver. In support of these results, SF and CPDT were shown to induce Nrf2 and the Nrf2 signaling pathway and inhibit 4-ABP DNA adduct formation in human bladder cells and mouse bladder tissue. Neither compound induced UGT enzymes in the liver, and both were determined to be possible chemopreventive agents against bladder cancer.

This study provided convincing evidence that administration of chemopreventive agents can result in lower DNA adduct levels and therefore reduce risk of bladder carcinogenesis. These promising results in conjunction with the limitations of our study provide a direction for future research in this area. The ultimate goal is to reduce risk of 4-ABP-induced bladder carcinogenesis, and to do this a greater understanding of 4-ABP metabolism and the mechanisms of action of potential chemopreventive agents is important. What follows are some suggestions for future research.
6.2 Detection and quantification of dG-ABP isomers

Our research focused on detection and quantification of dG-C8-4-ABP, but 4-ABP binds to DNA to form several different isomers and these isomers may be highly significant. Researchers have hypothesized that the biological effect of exposure to 4-ABP is not shown just in the quantification of the major adduct, but that the minor low abundance adducts may have a greater effect on cancer initiation; a high abundance adduct may be the preferred substrate for a repair enzyme leaving the minor adduct to cause mutations and possibly make it the greatest contributor to cancer initiation (1, 2). Indeed, some studies on other small molecule-induced genetic damage have found that the C8 adduct is not as biologically important as the $N^2$ adduct (3-5). We have detected both the dA-C8-4-ABP DNA and the dG-$N^2$-4-ABP adduct in 4-ABP dosed human bladder cells and mouse bladder tissue but further confirmation with characterized standards is required. Furthermore, dose-response curves should be generated and the effect of chemopreventive agents on their abundances should be investigated.

Detection of these minor adducts presents several analytical challenges. The importance of a highly sensitive method is even greater as they are often present in significantly lower quantities. Moreover, if their detection is for the evaluation of chemopreventive agents, the chemopreventive agent will decrease these already low adduct levels even further.
6.3 Investigation into 4-ABP metabolism (6, 7)

An analysis of the metabolites of 4-ABP is important to understanding 4-ABP DNA adduct formation and inhibition. A comprehensive scan of 4-ABP metabolites in urine of experimental animals treated with 4-ABP may point to key detoxifying reactions. The extent of carcinogen conjugation with Phase 2 metabolites can be quantified to provide an estimation of carcinogen detoxification. Furthermore, the approximate efficacy of a chemopreventive agent can be gauged based on the relative change in levels of these conjugates following treatment with the chemopreventive agent. In chapter 4, we examined the effect of Nrf2 on urinary glucuronidation of 4-ABP in mice treated with 4-ABP. Glucuronidation, however is only one significant reaction in the metabolism of 4-ABP and an analysis of sulfation, glutathione conjugation, and acetylation might provide more insight into the detoxification of 4-ABP.
6.4 Comprehensive human study

Following the development of our validated and sensitive LC-MS/MS method appropriate for the quantification of dG-C8-4-ABP in human samples, our laboratory, in conjunction with the Roswell Park Cancer Institute will be conducting a comprehensive human smoke-quit study. In this study, the variation in 4-ABP DNA adduct levels in urothelial cells will be monitored as smokers quit smoking. This will be an extensive 7 week study involving 100 subjects and therefore this research will require a high throughput method. A limiting factor of the methodology in this dissertation is the time required for sample enrichment and separation. If SPE is involved in this next study, use of SPE plates could significantly expedite sample preparation. An ultrafast separation method such as that achieved with the use of differential mobility spectrometry (DMS) may also be feasible.
6.5 Evaluation of analogs of SF and CPDT

In chapter 5, we evaluated SF and CPDT as potential chemopreventive agents against bladder carcinogenesis based on their inhibition of 4-ABP DNA adduct formation and induction of Nrf2 and Nrf2 regulated genes. Activation of Nrf2 in bladder tissue but not liver tissue was determined to be a promising strategy against bladder carcinogenesis in chapter 4. Although our research suggests that both SF and CPDT are promising chemopreventive agents against 4-ABP-induced bladder carcinogenesis, it would be interesting to compare their cytoprotective characteristics to those of their analogs which may be even more effective in inhibiting 4-ABP DNA adduct formation. Indeed, derivatives of 3H-1,2-dithiole-3-thione (D3T) have been evaluated for effectiveness in inducing Phase 2 enzymes and some were found to be more effective than CPDT (8). Analogs of SF have also been investigated, including phenylethyl isothiocyanate (PEITC) (9, 10). Furthermore, it might also be interesting to measure the effect of these chemopreventive agents on other carcinogens implicated in bladder carcinogenesis, such as 2-naphthylamine or in rodents, N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) (11).
References


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