Liquid Chromatography- Mass Spectrometry for Detection and Characterization of DNA Biomarkers and Reactive Metabolites

A dissertation presented by

Dayana Argoti

to The Department of Chemistry and Chemical Biology

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

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for
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ABSTRACT OF DISSERTATION

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ABSTRACT

This dissertation presents advances in liquid chromatography-mass spectrometry for the analysis of reactive metabolites, DNA adducts and the evaluation of adult stem cell mechanisms.

Chapter 1 provides an introduction to mass spectrometry, including electrospray, triple quadrupole and three-dimensional ion trap mass analyzers. In addition, human drug metabolism and specific metabolic processes relevant to the work presented in this dissertation are discussed.

Chapter 2 deals with idiosyncratic or adverse drug reactions that are typically not detected until a large population has been studied, mostly during or after phase III clinical trials. Methods have been introduced in the pre-clinical phase of drug development to help identify compounds from a large library that form reactive metabolites. A commonly used technique for this purpose is glutathione (GSH) trapping assay, however, this assay only detects reactive metabolites that form stable GSH adducts. A high throughput LC-MS/MS method for the detection and characterization of iminium ion reactive intermediates via constant neutral loss of 27 in potassium cyanide microsomal incubations is presented. The method shows complementarity to GSH trapping results and provides preliminary structural information on detected cyano-adducts. Reported is a comprehensive triple quadrupole mass spectrometric investigation on the formation of cyanide adducts on a total of fourteen compounds.
Chapter 3 describes the development of a multidimensional chromatography-mass spectrometric method for the quantification of DNA adducts of cigarette smoke constituent, 4-aminobiphenyl (4-ABP) in human bladder RT-4 cells. The method presented in this chapter represents an important improvement on the current methodologies for the analysis of 4-ABP-DNA adducts. The methodology was successfully applied to the evaluation of chemopreventive agents for the modulation of 4-ABP DNA adduct: \( N^-\text{(deoxyguanosin-8-yl)}\)-4-aminobiphenyl (dG-C8-ABP).

Chapter 4 presents the utilization of LC-MS/MS to corroborate the theory of immortal DNA strands in adult stem cells (ASCs). Immortal DNA strands in ASCs were tagged with stable labeled thymidine, \( ^{15}\text{N-dT} \), and allowed to replicate for approximately eight generations. The amount of \( ^{15}\text{N-dT} \) in immortal DNA strands was measured as a means to evaluate the degree of geometric label dilution.

Chapter 5 provides insights for further improvements on the methodology presented in the previous chapters. In addition, suggestions for future research opportunities are discussed.
ACKNOWLEDGEMENTS

‘Unselfish and noble actions are the most radiant pages in the biography of souls.’

~ David Thomas ~

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Para mi Mama y mis abuelitos Eloy y Zoila

To my Mom and Grandparents
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<td>Acetyl CoA</td>
<td>Acetyl Coenzyme A</td>
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<td>4-aminobiphenyl</td>
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<td>ACN</td>
<td>Acetonitrile</td>
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<td>Antioxidant Response Element</td>
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<td>ATP</td>
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<td>capLC</td>
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<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>L</td>
<td>Column length</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>M</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>Maf</td>
<td>muscle aponeurotic fibrosarcoma</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>Mef</td>
<td>Murine embryo fibroblast</td>
</tr>
<tr>
<td>m_p</td>
<td>Mass of a proton</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MS^n</td>
<td>Multiple stage mass Spectrometry</td>
</tr>
<tr>
<td>µESI-MS</td>
<td>Micro-electrospray mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NL</td>
<td>Neutral Loss</td>
</tr>
<tr>
<td>N-OH-AABP</td>
<td>N-hydroxy-acetylaminobiphenyl</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450 monooxygenases</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PSI</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>Q</td>
<td>Quadrupole</td>
</tr>
</tbody>
</table>
q    Ion charge
QIT Quadrupole ion trap mass spectrometer
QqQ Triple quadrupole mass spectrometer
R    Resolving Power
RF    Radio frequency
RP    Reversed phase
RSD Relative standard deviation
RT Retention time
SIM single ion monitoring
S/N Signal-to-noise ratio
SPE Solid phase extraction
SRM Selected Reaction Monitoring
t    Time
TIC Total Ion Current
TIS TurboIon Spray
ToF Time of Flight
TRIS™ tris(hydroxymethyl)-aminomethane
TRIZMA™ tris(hydroxymethyl)-aminomethane
UDPG1A UDP-glucuronosyltransferase 1A
UGTs Uridine diphosphate glucuronosyl transferases
V    Volts
ω    Angular frequency
z    Charge
Chapter 1:

Introduction to Mass Spectrometry and Human Drug Metabolism
1.1 Introduction

The main theme of this dissertation revolves around the toxicological effects of toxic xenobiotic metabolites in the development of cancer and their evaluation by liquid chromatography-mass spectrometric techniques. An introduction to mass spectrometric methods and background of human drug metabolism relevant to the work presented in this dissertation are found in this introductory chapter.

1.2 Fundamentals of Mass Spectrometry(1, 2)

Although the name of the methods leads the user to believe that actual mass measurements are involved, mass spectrometry rather separates analytes based on their mass-to-charge \((m/z)\) ratio and reports the abundance of each analyte ion. Typical mass spectrometric data are shown as a plot of ion abundance vs. \(m/z\) for all ions detected. All mass spectrometric data are based on the analysis of analyte ions in the gas-phase. It is important to note that the mass spectrometer measures isotopic masses as opposed to the molecular weight, which is a weighted average of all isotopes present in a compound. For example, in the electrospray (discussed later) mass spectrum for a compound containing multiple nitrogen atoms, such as the nucleoside thymidine, the \(m/z\) ion measured includes an additional proton. Therefore, the mono-isotopic mass of 242 for the \(^{12}\text{C}^{14}\text{N}\) isotopes is detected at \(m/z\) 243, the mass for the \(^{13}\text{C}\) isotopic contribution is detected at \(m/z\) 244 and the mass for the contribution of \(^{13}\text{C}^{15}\text{N}\) isotopes is detected at \(m/z\) 245 in the the mass spectrum corresponding for each isotope as shown in Figure 1.1.
Figure 1.1. Electrospray mass spectrum of deoxynucleoside thymidine.
All mass spectrometer systems have four main components, the source where ionization takes place, the mass analyzer where the analyte ions are separated according to their $m/z$ ratios, the detector where ion abundance is determined and the data system where data are analyzed.

1.3 Ion Source-Electrospray Ionization (ESI)

The most critical reaction in mass spectrometry is the conversion of analytes into gas-phase ions. ESI is one of two primary ionization techniques widely used today. The other is matrix assisted laser desorption ionization, otherwise known as MALDI. The development of these techniques for the analysis of a great majority of large, thermally labile and non-volatile compounds opened up the applicability of mass spectrometry to a large number of fields, most importantly the analysis of biomolecules such as proteins and oligonucleotides.

ESI operates under the basic principles of an electrochemical cell and occurs at atmospheric pressure. Typically the eluent from a liquid chromatograph enters the source through an electrospray needle and into the electric field created between the high voltage applied to the needle and the counter electrode forming an electrolytic cell as shown in Figures 1.2 and 1.3. The most common charging method used today involves either the addition or removal of a hydrogen ion to produce a charged species. The addition of a hydrogen ion occurs at a low pH and produces the positively charged molecule ion $[M+H]^+$. Negatively charged ions are produced by the removal of a hydrogen ion, at high pH, and consequently forms the molecule ion $[M-H]^-$. While a single charge is typical for small molecules, compounds whose mass
**Figure 1.2.** Electrospray source schematic

Adapted from Ref (5)

**Figure 1.3.** Electrospray Taylor cone mechanism.

Adapted from Ref (5)
is <1,000 Da, multiply charged analyte ions are typical for large biomolecules such as proteins, peptides, oligonucleotides and other biopolymers. These large biomolecules have multiple ionization sites which produce either a \([M+nH]^n+\) or \([M-nH]^n-\) ion, where \(n\) is the number of hydrogens added or removed. It is typical for these analytes to produce more than one ion because, although the analyte mass remains the same, the \(m/z\) ratio will be different depending on the number of charges acquired during ionization\(^{(1)}\). The \(m/z\) ratio can be determined by the following formulas:

- For positively charged ions:
  \[
  m/z = \frac{M + zm_p}{z}
  \]

- For negatively charged ions:
  \[
  m/z = \frac{M - zm_p}{z}
  \]

where \(M\) is the molecular mass of the analyte in Daltons (Da), \(z\) is the charge, and \(m_p\) is the mass of a proton in Daltons, nominally 1 or 1.008. For brevity and relevance, the remainder of this section will focus on singly charged positive ions.

When a positive potential is applied to the electrospray needle positive ions emerge from the tip initially forming a Taylor cone as they are attracted to the counter electrode as shown in Figure 1.3 \(^{(3)}\). As the positive charge builds up at the tip of the Taylor cone fine charged droplets are formed. Typically at flow rates of 300-800 \(\mu\)L \(\text{min}^{-1}\) a nebulizing gas, \(N_2\), is used to assist in the desolvation process generating aerosol charged droplets. The droplet eventually gets desolvated until the Rayleigh limit is reached and a coulombic explosion occurs leading to the formation of positively charged gas-phase ions \(^{(3, 5, 6)}\). The positive gas-phase analyte ions then continue to be attracted to and travel towards the counter electrode and into the mass spectrometer.
A. Nanoelectrospray and Microelectrospray (3)

A mathematical and theoretical ESI model was developed by Wilm and Mann in 1994 that first introduced the concept of nanoelectrospray. The model allowed for the optimization of ion source dimensions, specifically the use of a narrow electrospray needle internal diameter, as small as 1 µm, leading to the formation of microdroplets as small as 100 nm in diameter. The formation of smaller droplets is key to the advantages offered by nanoelectrospray, including a high tolerance for buffer salts, higher sensitivity and the elimination of the need for the use of a sheath gas or heat. A smaller droplet reduces the amount of solvent that must evaporate, the number of coulombic explosions needed and the amount of background contaminants in the sample. Nanoelectrospray typically operates at flow rates of ~25 nL min$^{-1}$ that are usually achieved by the electroosmotic flow of ions and mobile phase toward the electrospray tip and into the electric field, eliminating the use of a mechanical pump to force liquid through the tip. Although nanoelectrospray and microelectrospray terms are used interchangeably, each technique is different. Microspray differs from nanoelectrospray in that a mechanical pump is used to deliver solvent at flow rates between 50 and 500 nL min$^{-1}$. In addition the microelectrospray needle internal diameter is in the order of 10 µm (7).
1.4 Mass Analyzers (2)

The component responsible for separating analytes according to their \( m/z \) ratio is the mass analyzer. There are five main types of mass analyzers, time of flight (TOF), magnetic sector, fourier transform ion-cyclotron resonance (FTICR), quadrupole and quadrupole ion trap. Each of these mass analyzers separate ions in a different manner, thus a specific mass analyzer may be more suitable for a particular type of analysis. Characteristics specific to each mass analyzer, also known as the mass spectrometric figures of merit, include mass resolving power, mass accuracy, mass range, linear dynamic range, abundance sensitivity, precision, efficiency, duty cycle, speed, ionizer compatibility, cost and size (7). Mass resolving power is defined as the number that determines the capacity of the instrument to separate ions at different \( m/z \) values. Resolving power (R) is determined from peak profiles distributed along the \( m/z \) scale for two ions differing in mass by a known value (\( \Delta m \)) and the \( m/z \) value of one of the two peaks (M). These values are mathematically related as: \( R = \frac{M}{\Delta m} \). Mass accuracy is the ratio of the mass-to-charge measurement error (i.e. the difference between the measured \( M \) and the true \( M \)) divided by the true mass-to-charge ratio and is usually stated in parts per million (ppm). The mass range of an instrument is the range of mass-to-charge ratios amenable to the analysis by a given analyzer. The linear dynamic range is the range over which ion signal is linear with analyte concentration. The reproducibility with which ion abundances can be determined is called the precision of an instrument, whereas an instrument’s efficiency is the product of the transmission of the analyzer and its duty cycle. The duty cycle is the fraction or percentage of the ions of interest (formed in the
ionization step) that enter the mass analyzer and are actually detected. The last figure of merit to be noted is the speed of analysis of a given instrument that is the time frame of the experiment; ultimately used to determine the number of spectra per unit time that can be generated (spectral generation rate described in Hertz). These figures of merit are shown for various mass analyzers in Table 1.1 (6). The remainder of this section will focus only on the mass analyzers utilized in the research presented in this dissertation, specifically the quadrupole and the quadrupole ion trap.
<table>
<thead>
<tr>
<th></th>
<th>Quadrupole</th>
<th>Ion Trap</th>
<th>Time-of-Flight</th>
<th>Time-of-Flight</th>
<th>Magnetic Sector</th>
<th>FTMS</th>
<th>Quadrupole-TOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>0.01% (100 ppm)</td>
<td>0.01% (100 ppm)</td>
<td>0.02 to 0.2% (200 ppm)</td>
<td>0.001% (10 ppm)</td>
<td>&lt;0.0005% (&lt;5 ppm)</td>
<td>&lt;0.0005% (&lt;5 ppm)</td>
<td>0.001% (10 ppm)</td>
</tr>
<tr>
<td>Resolution</td>
<td>4,000</td>
<td>4,000</td>
<td>8,000</td>
<td>15,000</td>
<td>30,000</td>
<td>100,000</td>
<td>10,000</td>
</tr>
<tr>
<td>m/z Range</td>
<td>4,000</td>
<td>4,000</td>
<td>&gt;300,000</td>
<td>10,000</td>
<td>10,000</td>
<td>10,000</td>
<td>10,000</td>
</tr>
<tr>
<td>Scan Speed</td>
<td>~a second</td>
<td>~a second</td>
<td>milliseconds</td>
<td>milliseconds</td>
<td>~a second</td>
<td>~a second</td>
<td>~a second</td>
</tr>
<tr>
<td>Tandem MS</td>
<td>MS(^2) (triple quad)</td>
<td>MS(^n)</td>
<td>MS</td>
<td>MS(^2)</td>
<td>MS(^2)</td>
<td>MS(^n)</td>
<td>MS(^2)</td>
</tr>
</tbody>
</table>
| Precursor ion selection is limited to a wide mass range; growing number of applications | Limited resolution, High-energy collisions | Excellent accuracy and resolution of product ions | Excellent accuracy, Low-energy collisions;
| General Comments     | Low cost   | Low cost   | Low cost       | Good accuracy  | High resolution, MS\(^n\) high vacuum, super conducting magnet, expense | Known for high sensitivity and accuracy when used for MS\(^2\) |
|                      | Ease of switching pos/neg ions | Ease of switching pos/neg ions | Well-suited MS\(^n\) | Good resolution | legible |

**Table 1.1** Selected figures of merit for mass analyzers. Adapted from (6)
A. Quadrupole

The quadrupole mass analyzer is the most widely used type of mass spectrometer because of the low cost and facile automation. It consists of four parallel surfaces, usually in the shape of a cylindrical rod, composed of either molybdenum or gold plated ceramic as shown in Figure 1.4 (2). Opposing poles are electronically linked together where radio frequency (RF) and direct current (DC) power sources create a two-dimensional electrical field that affects ion trajectory and therefore also ion detection (8). Mass separation is based strictly on the $m/z$ ratio of the analyte ions. A constant RF/DC ratio is maintained as the RF amplitude and the DC potentials are changed (ramped), allowing only ions with stable trajectories to travel down the z-axis toward the detector (9). The resulting mass spectrum generated, when the RF/DC ratio is ramped, is referred to as the total ion chromatogram.

The physical variables that govern ion trajectories are based on second order differential equations that are also referred to as Mathieu equations. A graphical representation of the solutions to these equations has been summarized in the Mathieu stability diagram shown in Figure 1.5 (10). The diagram is a plot of parameter $a$ vs. $q$, where $a$ is related to the DC voltage and $q$ is related to the RF voltage. At a constant RF/DC ratio the instrument resolution is established. The higher the slope of the operating line, shown in red in the Mathieu stability diagram in Figure 1.5, the higher the resolving power of the instrument the lower the sensitivity. A consequence of higher resolution, however, is loss of sensitivity.
Figure 1.4. Quadrupole mass analyzer schematic.

(A) Adapted from Ref. (8) (B) Adapted from Ref. (9)
Figure 1.5. Mathieu stability diagram for quadrupole mass analyzer.

Adapted from Ref. (10).
Two important variations of the quadrupole were utilized for the MS/MS work presented in this dissertation. The first, the triple quadrupole, is a tandem-in-space and the second, the three dimensional quadrupole ion trap is tandem-in-time.

**B. Triple Quadrupole**

The triple quadrupole (QqQ) is the most common tandem-in-space instrument. It arranges three quadrupole mass analyzers in a series where the first and third quadrupoles (Q1 and Q3) function as transmission quadrupoles, while the second usually serves as a collision cell where fragment ions are produced, Q2 operates in RF-only mode in order to transmit all ions to the third quadrupole where product ions are analyzed and transmitted to the detector. In addition to the three quadrupoles, there are several ion guides often in the form of quadrupoles, hexapoles, octapoles, and lenses which are utilized to transmit ions formed in the source to the mass analysers. Illustrated in Figure 1.6 is a schematic of Thermo Fisher Corporation’s (formerly Thermo Electron Corporation) triple quadrupole mass spectrometer, the Finnigan TSQ Quantum that was employed in the work presented in Chapter 4 of this dissertation (11). In contrast to other QqQ instruments used today, the TSQ Quantum has a collision cell that is bent at a 90° angle, allowing for a more compact configuration, making it feasible as a benchtop instrument in contrast to bulky laboratory space instruments.

By changing the operation of each quadrupole, a variety of scanning techniques can be used for analysis, identification and characterization of analyte ions as shown in Figure 1.7 (11). The six scanning techniques illustrated in
**Figure 1.6.** Schematic for the Thermo Fisher TSQ Quantum.

Adapted from Ref. (11).
<table>
<thead>
<tr>
<th>Technique</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Scan</td>
<td>Scanning</td>
<td></td>
<td>ions pass</td>
</tr>
<tr>
<td>Single Ion Monitoring (SIM)</td>
<td>Fixed</td>
<td></td>
<td>ions pass</td>
</tr>
<tr>
<td>Product Ion Scanning</td>
<td>Fixed</td>
<td></td>
<td>Scanning</td>
</tr>
<tr>
<td>Precursor Ion Scanning</td>
<td>Scanning</td>
<td></td>
<td>Fixed</td>
</tr>
<tr>
<td>Constant Neutral Loss (CNL)</td>
<td>Scanning</td>
<td></td>
<td>Scanning</td>
</tr>
<tr>
<td>Selected Reaction Monitoring (SRM)</td>
<td>Fixed</td>
<td></td>
<td>Fixed</td>
</tr>
</tbody>
</table>

**Figure 1.7.** Triple quadrupole scanning techniques.
the figure are the full scan, single ion monitoring (SIM), product ion scanning, precursor ion scanning, constant neutral loss (CNL), and selected reaction monitoring (SRM), which is typically used for quantification purposes. In full scan and SIM modes, the second quadrupole is used only as a transmission quadrupole. Therefore, in full scan mode all analyte ions formed in the source that are within the specified $m/z$ range are detected. This type of scan is also known as a Q1 scan. In SIM mode only a specific $m/z$ value is allowed to enter the first quadrupole and ultimately be detected.

The other types of scans are tandem, MS/MS, scans where the second quadrupole is used as a collision cell. In product ion scanning, a specific $m/z$ is selected and transmitted to the collision cell where the inert gas, typically Argon, causes collision induced dissociation (CID) and the resulting fragment ions are scanned in Q3. Precursor ion scanning allows all ions within a selected $m/z$ range to go through the first quadrupole, into the second quadrupole for fragmentation and into the third quadrupole where only the selected $m/z$ value will be allowed to travel down to the detector. In CNL, both the first and third quadrupoles allow ions within the selected $m/z$ range to be scanned, the unique aspect of this scan mode is that Q1 and Q3 are linked so that only ions that differ by a specific neutral loss are transmitted to the detector. The most common type of scan used for quantification is SRM, where only a specific $m/z$ ion is allowed to travel through Q1 into Q2 for fragmentation and only a selected fragment ion is allowed to reach the detector. Additionally, more than one SRM transition can be monitored at the same time and this technique is known as multiple reaction monitoring (MRM).
There are advantages and disadvantages to each of these techniques, therefore the user must take into account what type of information is desired in order to choose the appropriate technique. For example, in the analysis of unknowns the product ion scan is useful in identification of structural features; when a mixture of related compounds needs to be analyzed precursor ion scanning is the best choice. Additionally, the CNL scan is useful in the analysis of a compound mixture with a common structural feature and fragment ion, such as in the analysis of cyano adducts which is presented in Chapter 2. In the case where sensitivity is desired, the SIM scan is useful; when both sensitivity and selectivity are required SRM is best. While a form of SRM is also available in the quadrupole ion trap, which is discussed next, SRM in the triple quadrupole is considered more sensitive because only a selected \( m/z \) ion is detected while all other masses are excluded because they crash into the poles.
C. Quadrupole Ion Trap (2)

The quadrupole ion trap (QIT), otherwise referred to as the 3D QIT in order to differentiate this mass analyzer from the linear quadrupole ion trap, that has emerged in recent years, was introduced in the mid-1980’s. The use of quadrupole electric fields to manipulate ions was initially investigated by Wolfgang Paul et al., in the early 1950’s; the research resulted in the joint Physics Nobel Prize in 1989 with Hans Georg Dehmelt. The ion trap is composed of two end-cap electrodes and one ring electrode as shown in Figure 1.8 (12). The cross-sectional area of all electrodes has a hyperbolic shape. A three-dimensional electric field is generated that is dependent on the geometric arrangement of the electrodes and the magnitude of the applied alternating current (AC) and DC fields. The primary component of the electrical field that surrounds the ions is based on radio frequency (RF). Ion stability is described by the Mathieu equations in a similar manner as in the quadrupole, as shown in Equations 1.1 and 1.2 (2). The ion stability is dependent upon the charge defined as m, the internal radius of the ring electrode defined as r₀, the sinusoidal RF potential applied is defined as V, the DC potential (if any) applied to the endcap electrodes, ω_RF the angular frequency, which are shown in the following equation:

\[
a_z = -2a_r = -16 \frac{eU}{mr_0^2 \omega_{RF}^2}
\]

\[
q_z = -2q_r = -8 \frac{eV}{mr_0^2 \omega_0^2}
\]

Equation 1.1  
Equation 1.2

The region within the red plot area in the Mathieu stability diagram shown in Figure 1.9 represents the values at which ions are stable in the ion trap (8, 12, 13).
Figure 1.8. 3D Quadrupole ion trap schematic.

Adapted from Ref. (12).
Figure 1.9. Mathieu stability diagrams for the 3D quadrupole ion trap.

Adapted from Refs. (12, 13).
Ions that are trapped in the electrical field continually oscillate along a three-dimensional trajectory and are stored in concentric orbital-like layers as shown in Figure 1.10 (6). Each layer represents a specific $m/z$ value, where the outermost layer contains the lowest $m/z$ ion and the innermost layer the highest $m/z$ ion. Ion detection is based on the loss ion trajectory stability that causes the ion to move toward the end-cap electrode that has an opening, allowing ions to move into the detector component of the mass spectrometer.
Figure 1.10. Schematic for ion trajectory within the quadrupole ion trap, ring electrode not shown.

Adapted from Ref. (6).
1.5 Human Drug Metabolism (14)

In general when a xenobiotic (a drug or foreign chemical), is ingested it is absorbed, distributed, metabolized and excreted. During oral ingestion, the xenobiotic is absorbed into the gut wall, enters hepatic portal circulation and is carried to the liver where it is metabolized and conjugated for transportation and excretion as shown in Figure 1.11 (15). Metabolism occurs by a series of biotransforming enzymes, known as cytochrome P450 monooxygenases (CYPs or P450). This series of enzymes are the body’s main protection system against aromatic hydrocarbons, aromatic amines, exogenous hormone like molecules and other xenobiotics.

There are two main biotransformation phases, the first phase often referred to as Phase I metabolism and the second, known as Phase II metabolism. CYPs are involved during Phase I metabolism and their roles in living systems include biomodulation, endogenous steroid synthesis, dietary toxin clearance and drug clearance through biotransformation, usually in the form of mono-oxygenation. Although the role of CYPs is to protect the body from foreign chemicals, by introducing a functional group that makes the molecule more polar and water soluble to enhance clearance, these processes are flawed. Biotransformation is a double-edged sword because there is also the potential to form metabolites that are more toxic and reactive than the xenobiotic itself. Yet the body’s second barrier, Phase II metabolism, readily reacts with these metabolites to enhance transport and facilitate clearance.
Figure 1.11. Traditional route of oral drug metabolism.

Adapted Ref. (15).
Phase II metabolism involves conjugative and transport processes that make xenobiotic metabolites even more water soluble by orchestrating the addition of more polar groups, through acetylation, methylation, and sulphation, or conjugation to transport biomolecules such as glucose, in glucuronidation, and glutathione (GSH). Other Phase II related processes include epoxide hydrolases, esterases, amidases and amino acid conjugation to either glycine or glutamate. Only relevant Phase II metabolic pathways, which relate to the work presented in this dissertation specifically: acetylation, glucuronidation and GSH conjugation, will be discussed next.

A. Glutathione System

GSH evolved as a means for cells, specifically liver hepatocytes, to deal with toxic reactive products of the oxidative processes that form ATP. It is a tripeptide composed of cysteine, glycine and glutamate whose structure is shown in Figure 1.12. The thiol of cysteine is at the center of GSH’s function of trapping highly reactive electrophilic metabolites.

In addition to GSH’s role as an electrophile scavenger, it is an integral part of cellular maintenance, including the restoration of oxidized antioxidants like Vitamin E and ascorbic acid. This process leads to the formation of a glutathionyl radical (GS•) that is oxidized to form GSSG, which can be reduced, to replenish GSH levels in the cells, by GSSG reductase. In hepatocytes, the levels of GSH are maintained between 8-10 mM even when under electrophilic metabolite attack. The process of GSH reduction of a reactive species is assisted by a series of enzymes known as
**Figure 1.12.** Structure of Glutathione with reactive thiol group highlighted in blue and the subsequent fragment used in CNL experiments to detect GSH adducts.
glutathione-S-transferases (GSTs), which promote the reaction between GSH and electrophilic metabolites. GSTs bind both the reactive species and GSH through hydrogen bonding facilitating the formation of a GSH conjugate as shown in Figure 1.13 (16).

The function of GSH has been exploited to screen large drug candidate libraries for the formation of reactive metabolites through LC-MS/MS by utilizing a CNL scan of 129 as shown in Figure 1.12. Work related to this type of screening is presented in Chapter 2 of this dissertation.
Figure 1.13. Glutathione reaction scheme.

Adapted from Ref. (16).

Figure 1.14. Acetylation reaction scheme.
B. Acetylation

Acetyltransferases are a family of enzymes responsible for the acetylation of xenobiotics and contribute to many cellular functions such as the acetylation of DNA histone proteins and those involved in cell cycle regulation. In regards to the metabolic function of xenobiotic acetylation, the addition of an acetyl group is accomplished by two families of acetyltransferases, N-acetyltransferase 1 (NAT-1) and N-acetyltransferase 2 (NAT-2) along with the co-factor acetyl Coenzyme A (acetyl CoA) as shown in Figure 1.14 (17).

Acetylation works well in conjunction with P450s in the metabolism of xenobiotics to activate and detoxify as shown in Figure 1.15 (18). However, the level of efficient detoxification is dependent on the interindividual variation of NAT phenotypes. There is little evidence for the differential expression of NAT-1, however, there is significant evidence for the positive relationship between the slow NAT-2 phenotypes and the development of diseases such as diabetes and cancer. Therefore, the rate of acetylation becomes an important genetic factor in an individual’s predisposition for cancer since the process of acetylation involves two steps. First, the movement of the acetyl group from acetyl CoA to the acetyltransferase followed by the acetylation of the xenobiotic and the subsequent release of CoA as shown in Figure 1.14.
Figure 1.15. Phase I and Phase II metabolism relationships in the formation of reactive intermediates that lead to mutations.

Adapted from Ref. (18).
C. Glucuronidation

Glucuronidation is accomplished by a series of enzymes known as uridine diphosphate glucuronosyl transferases (UGTs) to activate and convert a wide variety of chemicals to glucuronides through a nucleophilic substitution as shown in Figure 1.16 (17). UGTs are typically found in the liver. The process of glucuronidation typically occurs after Phase I oxidative metabolism and is a major xenobiotic clearance pathway. The formation of a glucuronide conjugate makes the xenobiotic virtually inert and significantly more water soluble. Of particular relevance to the work presented on Chapter 3 of this dissertation, is the glucuronidation of aromatic amines, which tend to be either directly conjugated on the nitrogen of the amine forming an N-glucuronide or after Phase I metabolism. The resulting hydroxylamine from oxidation can be conjugated at either the nitrogen or the oxygen to form either an N-glucuronide or O-glucuronide conjugate.

N-glucuronides have been shown to be extremely susceptible to acid hydrolysis in the presence of β-glucuronidases. Such an event can occur in the urine of a person whose diet is rich in meat and dairy products which make urine more acidic. The presence and activity of β-glucuronidases in urine is enhanced by an acidic pH, making acid hydrolysis highly likely (14, 19). Therefore, either the parent aromatic amine or the hydroxylamine, that were transported to the bladder as N-glucuronide conjugates, are likely released into the urine and enter cells lining bladder wall, eurothelial cells, leading to the formation of DNA adducts and ultimately the development of cancer.
Figure 1.16. The glucuronidation process for amines and mono-oxygenated metabolites

Adapted from Ref. (17).
Chapter 2:

Trapping and High Throughput Screening for Iminium Ion Reactive Intermediates with Potassium Cyanide Using Constant Neutral Loss Liquid Chromatography Mass Spectrometry.

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2.1 Introduction

Over the years, reactive metabolites are believed to have played an important role in the safety profile of pharmaceuticals. Cytochrome P450 mediated bioactivation of the drug to reactive metabolites has been reported to be the first step in many adverse drug reactions (20). The covalent binding theory has linked toxicity to the formation of reactive metabolites, where bioactivation is a critical step in the process leading to an electrophilic metabolite that can bind to cellular macromolecules, e.g., proteins and DNA (21). The resulting drug-protein adduct may cause partial or complete loss of the biochemical function of the protein, or act as a hapten that elicits an immune response and results in cellular and organ damage. The drug-DNA conjugates are potentially carcinogenic and may lead to undesired effects on gene expression and gene mutation. Currently, no animal model can accurately predict adverse drug reactions in human.

Adverse drug reactions usually have a low occurrence rate and typically are not observed until a large population has been studied, most likely during or after phase III clinical trials (22). Therefore, reactive metabolite detection has become a vital topic of interest for early screening to help prevent safety surprise or compound failure at a later stage. A few methods have been introduced in the pre-clinical phase of drug development to help identify compounds from a large library that carry such potential risk. One of the most commonly used techniques is glutathione (GSH) trapping assay with hepatic subcellular fractions in the presence of P450 cofactor NADPH. In this assay GSH, a naturally occurring tripeptide is used to trap electrophilic metabolites via the cysteine sulfhydryl group and form a conjugate. The resulting GSH conjugate, when
fragmented by collision-induced decomposition, gives a common loss of the pyroglutamic acid moiety from the structure, which can be easily detected and characterized by a neutral loss scan of 129 using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (23), (24). However, GSH trapping assay only detects those reactive metabolites that form stable adducts with GSH and can not trap some types of reactive intermediates, e.g., iminium ions, acyl glucuronides and aldehydes.

Metabolic activation of alicyclic amines often generates a number of oxidative products including N-dealkylation, ring hydroxylation, α-carbonyl formation, N-oxygenation and ring opening metabolites that can be formed through an iminium ion intermediate(25). Therapeutic pharmaceuticals and the metabolites containing an alicyclic amine structure have the potential to form iminium ions that are reactive towards nucleophiles. An extensively studied example is nicotine, which has been shown to form an iminium ion that can mediate covalent binding of nicotine to cellular macromolecules (25). The metabolism of compounds containing an alicyclic amine structure has been studied with radiolabeled cyanide in radiometry assays, where radiolabeled cyanide was used to react with the iminium ion intermediates (26). The method was relatively time consuming and no structural information was directly available. Recently, it was briefly noted by Evans, et al. that the cyanide conjugates can be detected using the neutral loss scan of 27 on LC-MS/MS, where non-radiolabeled cyanide was used (22). However, no detailed description of the method was reported. We present in this chapter a relatively high-throughput LC-MS/MS method for screening iminium ion formation in rat and human liver microsomes using potassium cyanide as the
trapping reagent. The cyanide trapping experiments, along with GSH trapping, were performed on fourteen compounds containing an alicyclic amine structure. Furthermore, the MS/MS fragmentation pathway of cyanide adducts of nefazodone and prochlorperazine was investigated, and the structures of the detected iminium ion intermediates were proposed.
2.2 Experimental

A. Chemicals and reagents

All test compounds, Potassium Cyanide (KCN, K\(^{13}\)C\(^{15}\)N) and Glutathione (GSH) were obtained from Sigma-Aldrich Corporation (MO, USA). Pooled mixed gender human liver microsomes were purchased from XenoTech, LLC (KS, USA). Methanol (HPLC grade) was purchased from JT Baker (NJ, USA), and ammonium acetate was purchased from Sigma/Aldrich (MO, USA). Distilled water was purified “in-house” using a MilliQ system Millipore (MA, USA). Other chemicals and reagents were of the highest quality available.

B. Cell Culture Incubations

Cyanide trapping experiments with the each test compound were incubated with rat or human liver microsomes in the presence of an NADPH regenerating system and cyanide in 50 mM potassium phosphate buffer (pH 7.4). Two separate incubations were performed, one with KCN and the other with K\(^{13}\)C\(^{15}\)N. Each incubation set entailed three controls, the first one without test compound, the second one without NADPH and the third one without KCN. The incubations were conducted in 96-deepwell refill tubes at 37°C in a water bath shaking at 45 rpm. The final concentrations of microsome, test compound and KCN/K\(^{13}\)C\(^{15}\)N in the incubation mixture (500 µL in total volume) were 1 mg/mL, 100 µM and 1 mM, respectively. The incubation mixture also contained a final concentration of 0.5 % DMSO, the solvent used to make the test compound stock solution. After 3 minutes pre-incubation, the reaction was initiated by an NADPH-generating system (100 µL solution consisting of 2.22 mM NADP\(^{+}\), 27.65 mM glucose-
6-phosphate and 6.0 units/mL glucose-6-phosphate dehydrogenase together with 15 mM MgCl₂ in phosphate buffer). The incubation was terminated at 90 minutes by the addition of 100 µL of acetonitrile containing 5% ammonium hydroxide. The samples were placed on ice for approximately 10 minutes, followed by centrifugation at 2,250 g at 4°C for 20 minutes. The supernatant was directly injected onto LC/MS/MS.

The same experiments described above for cyanide incubations were also performed for GSH trapping except the trapping agent is 10 mM GSH (final concentration) and the quenching solution is 100 µL of acetonitrile containing 6% of acetic acid.

C. Liquid Chromatography (LC)

LC was performed on a Flux Instruments Rheos 2000 (Leap Technology, NC, USA) and CTC HTS PAL autosampler (Leap Technology). The separations were performed on a 2.1 x 50mm Waters YMC ODS-AQ C₁₈ 5µm column. A gradient elution was employed on the column at 300µL/min with mobile phase A (10 mM ammonium acetate, pH adjusted to 4 with acetic acid) and mobile phase B (methanol) running a linear gradient in which the percent of mobile phase B increased from 4 to 90% during the first three minutes and was held at 90% for two minutes and then immediately returned to 4%. The analysis time was 8 minute per sample. The typical injection volume was 5 µL.
D. Mass Spectrometry

All the mass spectra were collected on an Applied Biosystems/MDS Sciex API-4000 triple quadrupole mass spectrometer (ON, Canada). TurboIonspray (TIS) interface was operated in positive ion mode. The mass spectrometric conditions for cyanide trapping samples were optimized using the nicotine cyanide conjugate. The MS/MS experiments performed included the neutral loss scan (NL scan) of 27 (or 29), and the sequential product ion scan and SRM (selective reaction monitoring) experiments. The mass spectrometric conditions for GSH conjugates detection using the NL scan of 129 was optimized using the benzoquinone-GSH adduct, formed by mixing benzoquinone and GSH directly. The sequential SRM experiments were set up by monitoring the loss of pyroglutamate moiety (loss of 129), and glycine moiety (loss of 75). The TurboIonspray source temperature was maintained at 600 °C. The ionspray voltage was set at 4000 V. The curtain gas was set at 20, the declustering potential (DP) at 56 V, and the nebulizer (GS1) and TIS (GS2) gases at 50 and 70 psi, respectively. The CID gas was set at 4 Torr, and the collision energy was set at 29 eV. Unit mass resolution was used in all the experiments. LC-MS/MS data were acquired using Analyst™ Software Version 1.1 (MDS Sciex, Canada).
2.3 Results and Discussion

The experimental conditions for cyanide incubation were modified from the previously established GSH trapping method (23), (26). Using nicotine as the test compound, the final KCN/K$^{13}$C$^{15}$N concentration in the incubation mixture was optimized at 1 mM which yielded the highest cyanide adduct amount. Acetonitrile containing 5% ammonium hydroxide was chosen as the quenching solution to prevent the potential formation of toxic HCN gas. Two separate cyanide incubations were performed for each study, one with KCN and the second with K$^{13}$C$^{15}$N, in order to firmly establish the formation of cyano adducts detected in the incubations. The cyanide conjugates were detected by LC-MS/MS analysis via a constant neutral loss scan (NL) of 27 Da and 29 Da on a triple quadrupole mass spectrometer, for incubations with KCN and K$^{13}$C$^{15}$N, respectively. Each incubation set entailed three controls, one without test compound to rule out any potential interference from endogenous compounds, the second without NADPH, a cofactor for P450 activities, to assess the metabolism dependence of each cyano-adduct formation, and the third without KCN/ K$^{13}$C$^{15}$N.

A. Nicotine

The scheme for formation of the iminium ion, in equilibrium with $\alpha$-carbinolamine, through hydrogen or electron abstraction by P450 isozyme and the subsequent trapping with cyanide is shown in Figure 2.1. Nicotine has often been used as the standard in these investigations due to its thoroughly studied cyano adduct formation using radiolabeled cyanide (26), (27). The LC-MS/MS chromatograms
Figure 2.1. Iminium ion intermediate formation from alicyclic amines, in equilibrium with α-carbinolamine, through hydrogen or electron abstraction by P450 isozyme and subsequent trapping via cyanide adduction during drug metabolism in liver microsomes.
from the constant neutral loss scan (NL) of 27 Da and 29 Da for incubations of nicotine in human liver microsome with KCN and $^{13}\text{C}^{15}$N are shown in the Figures 2.2A and 2.2B, respectively. The detection of the two isomeric cyano adducts of nicotine reflects the formation of two iminium ion intermediates resulting in direct cyanide addition to each of the two carbons alpha to the endocyclic nitrogen. The mass to charge (m/z) ratio for the protonated cyanide adduct was found to be 188 for incubation with KCN and 190 for incubation with $^{13}\text{C}^{15}$N. The difference in m/z ratio corresponded to the mass difference between KCN and $^{13}\text{C}^{15}$N. The finding here is consistent with the known metabolic activation of the compound (26), (27). Nicotine was therefore also chosen as the positive control compound included in each set of cyanide trapping studies described in this paper. On the other hand, in a parallel incubation of nicotine with GSH followed by neutral loss scan of 129 Da LC-MS/MS experiment, no GSH adducts were detected, thus confirming that iminium ion intermediates were in fact trapped more efficiently by cyanide.

**B. Nefazodone**

Nefazodone, an antidepressant drug with known hepatotoxicity (28), has been reported to be extensively metabolized via N-dealkylations and hydroxylation (29). It was therefore of interest to investigate the metabolism of nefazodone, which is typical of alicyclic amines via an iminium ion intermediate with KCN trapping assay. As shown in Figure 2.3, LC-MS/MS analysis of the rat liver microsome incubation sample using NL scan of 27 Da identified a cyano adduct of nefazodone and several cyano adducts with parent masses consistent with those of metabolic products as
Figure 2.2. Total Ion Chromatogram (TIC) of Neutral Loss Scan (NL) of 27 (panel A) and 29 Da (panel B) for cyano adducts of nicotine in human liver microsomes with KCN and $^{13}\text{C}^{15}\text{N}$ respectively. The two peaks on the chromatogram reflect the formation of two iminium ion intermediates and are subsequently correlated by the formation of two isomeric cyano adducts.
Figure 2.3. TIC of NL 27 of Nefazodone KCN trapping assay in rat liver microsomes. Cyano adducts detected in this sample include a parent cyano adduct (NM6) and several metabolites (NM1-NM5).
**Figure 2.4.** Proposed structures of cyano adducts of nefazodone and mono-oxygenated metabolites. **NM1** cyano adducts of N-dealkylated/di-oxygenated metabolite. **NM2** cyano adduct of N-dealkylated mono-oxygenated metabolite. **NM3** cyano adduct of di-oxygenated metabolite. **NM4** and **NM5** cyano adducts of mono-oxygenated metabolites. **NM6** cyano adduct of parent compound. In cyano adducts **NM1** and **NM2** the additional 14 Da has two possibilities that cannot be distinguished by the current methodology. The possibilities include a carbon-carbon double bond in conjunction with a mono-oxygenation or α-carbonyl formation.
previously reported. The proposed structures of detected metabolites were shown in Figure 2.4. Further MS/MS analyses of these metabolites confirmed this initial assessment for each product as well as the cyano adduct of nefazodone.

The product ion mass spectrum of the parent drug nefazodone (Figure 2.5A), was used as a template to characterize the structures of the various nefazodone metabolites, whose protonated parent masses were detected in the NL scan as shown in Figure 2.3. Comparison of MS/MS spectra of the parent compound with those of the mono-oxygenated cyano adducts at m/z 511 revealed two mono-oxygenated isomers, NM5 and NM4. The prominent fragment at m/z 290, in the mass spectrum of mono-oxygenated metabolite, NM5 (Figure 2.5B), reflects a 16 Da shift from the m/z 274 ion in the MS/MS spectrum of parent drug, which is consistent with mono-oxygenation on the portion of the molecule containing the phenoxyethyl triazolone (Figure 2.4, MN5). We propose that the immediate neutral loss of HCN (27 Da) from the cyano adduct of the mono-oxygenated metabolite, and subsequent fragmentation gives rise to a fragment of m/z 194 in the MS/MS spectrum of NM5. The structure of the second mono-oxygenated metabolite, NM4 (Figure 2.6A), can be inferred by comparison of its MS/MS spectrum with that of NM5. The fragment of NM5 at m/z 194 shifts by 16 Da, to m/z 210, in the MS/MS spectrum of NM4 suggesting likely mono-oxygenation of the chlorophenyl piperazine (Figure 2.4, MN4). Additionally MS/MS spectra of the cyano adduct of NM5 formed in the presence of K\(^{13}C^{15}N\), did not show any 2 Da shifts in the fragmentation pattern, indicating the immediate neutral loss of HCN (27 Da) from the cyano adducts under CID conditions (Figure 2.5C). Further investigation of the MS/MS spectra of NM5 at m/z 513, the \(^{37}\)Cl
Figure 2.5. MS/MS spectra of nefazodone and cyano adducts of mono-oxygenated metabolites [M+H] = 511, NM5. Panel A: MS/MS spectrum of nefazodone m/z 470. Panel B: MS/MS spectrum of mono-oxygenated cyano adduct NM5 [M+H] = 511. Panel C: MS/MS spectrum of mono-oxygenated cyano adduct NM5 formed in K^{13}C^{15}N incubation [M+H] = 513. Panel D: MS/MS spectrum of^{37}Cl isotope of mono-oxygenated cyano adduct NM5 [M+H] = 513.
Figure 2.6. **Panel A:** MS/MS spectrum of mono-oxygenated cyano adduct NM4 \([\text{M+H}]^+ = 511\). **Panel B:** MS/MS spectrum of di-oxygenated cyano adduct, NM3 \([\text{M+H}]^+ = 527\). **Panel C:** MS/MS spectrum of N-dealkylated plus mono-oxygenated cyano adduct NM2 \([\text{M+H}]^+ = 399\). **Panel D:** MS/MS spectrum of N-dealkylated plus di-oxygenated cyano adduct NM1 \([\text{M+H}]^+ = 415\).
isotope of the protonated molecule (Figure 2.5D), confirmed the presence of a chlorine atom in fragment ion at $m/z$ 194 in NM5 from the respective shift to $m/z$ 196. The presence of a chlorine atom in fragment ion at $m/z$ 210 in MS/MS spectra of NM4 was subsequently correlated in the same manner (data not shown). Remaining metabolites at parent masses 527 (NM3), 399 (NM2), 415 (NM1), and 495 (NM6) corresponding to di-oxygenation, N-dealkylation plus mono-oxygenation, N-dealkylation plus di-oxygenation (Figures 2.6B through 2.6D), and nefazodone cyano adduct respectively, were identified in a similar manner and proposed sites for modification are shown in Figure 2.4. These results are consistent with published metabolite data in terms of oxygenation sites and metabolites formed (29).

**C. Prochlorperazine**

Prochlorperazine, a second example of the study is a currently marketed drug used to treat dizziness due to labyrinthine disorder. It contains the characteristic alicyclic amine moiety, and has been reported to form a number of metabolites via N-oxidation, N-alkylation, sulfoxidation and aromatic ring hydroxylation (30). The expected metabolism of the alicyclic amines, as mentioned above, was confirmed in the KCN trapping assay. The NL profile of prochlorperazine incubates revealed a number of metabolites, including two mono-oxygenated metabolites at $m/z$ 415 (PM1 and PM4), a mono-oxygenated plus N-demethylated metabolite at $m/z$ 401 (PM2), one mono-oxygenated plus an unidentified 14 Da metabolite at $m/z$ 429 (PM3), and a cyano adduct of prochlorperazine at $m/z$ 399 (PM5) and (Figure 2.7). The proposed structures and key MS/MS fragments of the cyano adducts detected were shown in Figure 2.8. MS/MS analyses on the cyano-adduct of parent drug and metabolites
Figure 2.7. TIC for NL scan of prochlorperazine KCN trapping assay in human liver microsomes. Cyano adducts detected in this sample include a parent cyano adduct (PM5) and several metabolites (PM1-PM4).
**Figure 2.8.** Proposed structures of cyano adducts of prochlorperazine metabolites. **PM1** and **PM4** cyano adducts of mono-oxygenated metabolites. **PM2** cyano adduct of N-demethylation/mono-oxygenated metabolite. **PM3** cyano adduct of mono-oxygenated plus unknown 14 Da metabolite. **PM5** cyano adduct of parent compound. In cyano adduct **PM3** the additional 14 Da has two possibilities that cannot be distinguished by the current methodology. The possibilities include a carbon-carbon double bond in conjunction with a hydroxylation or α-carbonyl formation.
confirmed initial metabolism assessment for each product and revealed modification sites within the compound parent structure (Figures 2.9 and 2.10). The details are described as follows.

A strategy similar to that for nefazodone was used to determine the structures of the cyano adducts of metabolites detected in NL scans. The CID mass spectrum of the parent drug, as shown in Figure 2.9A, was used as a template to determine sites of biotransformation of prochlorperazine. The structure of the cyano adduct of the parent drug at a mass of 399 Da, PM5, was thus characterized as follows. A comparison of the MS/MS data for the parent compound and the cyano adduct of PM5 (Figures 2.9A and 2.9B), reveals a 25 Da shift of the fragment ions at \( m/z \) 113 and 141, to \( m/z \) 138 and 166 respectively, indicative of cyanide addition on the piperazine ring (Figure 2.8, PM5). This assignment was further confirmed by examination of the MS/MS spectra of the \( ^{37} \text{Cl} \) isotope of PM5 and the cyano adduct of PM5 formed by incubation in the presence of K\(^{13}\text{C}^{15}\text{N} \) (Figures 2.9C and 2.9D). Specifically, the MS/MS data for the cyano adduct formed in the presence of K\(^{13}\text{C}^{15}\text{N} \), showed a 27 Da mass shift for parent fragments at \( m/z \) 113 and 141, to \( m/z \) 140 and 168 respectively (Figures 2.9A and 2.9D). This shift confirmed the presence of stable labeled cyanide on the parent structure, as mentioned above. The MS/MS spectrum of the \( ^{37} \text{Cl} \) isotope of the protonated parent ion at \( m/z \) 401, shown in Figure 2.9C, subsequently correlated the presence of Cl on parent fragment \( m/z \) 246, by exhibiting a 2 Da shift to \( m/z \) 248. This information in turn further confirmed the presence of a cyano-adduct on the piperazine ring containing fragment of the parent structure.
Figure 2.9. MS/MS spectra of prochlorperazine and its cyano adduct PM5. **Panel A:** MS/MS spectrum of prochlorperazine [M+H]$^+$ = 374. **Panel B:** MS/MS spectrum of cyano-adduct, PM5, [M+H]$^+$ = 399. **Panel C:** MS/MS spectrum of $^{37}$Cl isotope of cyano adduct, PM5, [M+H]$^+$ = 401. **Panel D:** MS/MS spectrum of cyano-adduct, PM5, in K$^{13}$C$^{15}$N incubation, [M+H]$^+$ = 401.
Figure 2.10. MS/MS spectra for cyano-adducts of prochlorperazine hydroxylation metabolite isomers at [M+H]$^+$ = 415, PM1 and PM4. Panel A: hydroxylation metabolite PM1 in KCN incubation. Panel B: hydroxylation metabolite PM4 in KCN incubation.
For the structural characterization of mono-oxygenated metabolite PM1 at retention time of 3.94 min, a comparison of MS/MS spectrum of parent compound with that of PM1 showed that a major parent fragment ion at m/z 141 had shifted by 25 Da to a minor fragment ion at m/z 166 indicative of cyanide presence on the piperazine ring (Figure 2.10A). The prominent fragment ion at m/z 139 observed in Figure 2.10A was formed by a loss of HCN from the fragment ion at m/z 166. This information also suggested that the mono-oxygenation occurred on the phenothiazine.

For metabolite PM4 at retention time of 4.49 min, fragment ion at m/z 388 was formed by the loss of HCN (27 Da) from cyano adduct of mono-oxygenated metabolite [M+H]+ = 415 (Figure 2.10B). The major fragment ion at m/z 155 was generated by the loss of the phenothiazine from m/z 388. Further loss of 17 Da from m/z 388 to yield a fragment ion at m/z 371 suggested the formation of N-oxide on the piperazine ring.

The remaining cyano adducts of prochlorperazine, mono-oxygenated plus unknown 14 Da and desmethyl plus mono-oxygenated, were characterized in a similar manner and proposed modification sites are shown on Figure 2.8, for PM3 and PM2 respectively. The unidentified additional 14 Da in PM3 has a couple of possibilities that cannot be distinguished by the current methodology. The possibilities include a carbon-carbon double bond in conjunction with a hydroxylation or α-carbonyl formation on the phenothiazine.

**D. Other Compounds with Alicyclic Amine Core Structure**

A total of 14 compounds with the characteristic alicyclic amine core structure were studied by both KCN and GSH trapping assays. The neutral loss scan (NL) of 27 Da for cyanide adduct and NL of 129 Da for GSH adduct were performed to detect the
formation of cyanide or GSH conjugates, respectively. In general, the results for all 14 compounds screened with the KCN trapping assay were complementary to results obtained with the GSH trapping assay. For a majority of the compounds, no, or few GSH adducts, were detected, while a number of cyano adducts were detected in KCN assays (Table 1). In some cases, interesting (unusual) metabolites were trapped and identified with the KCN assay, as will be discussed. The formation and subsequent trapping of these metabolites may be attributed to metabolism inhibition effects due to the presence of cyanide in the system as was noted by Gorrod et al., for nicotine metabolism (26). Nevertheless, overall metabolism for the compounds screened with the KCN assay was generally consistent with published metabolism studies performed on the compounds screened (29), (30).

A brief discussion on the results for the remaining compounds screened is reported below. The cyanide trapping assay detected two α-carbonyl iminium ion intermediates in the metabolism of triprolidine, corresponding to similar metabolites previously reported (31). Indinavir iminium ion intermediates were detected in the cyanide trapping assay and correspond to ring opening mono-oxygenated metabolites that are similar to metabolites previously reported (32). Metabolites reported for ketoconazole indicate formation of an N-oxide among other metabolites (33). A mono-oxygenated iminium ion intermediate was detected in the cyanide trapping assay for ketoconazole. Metabolism reports for mianserin indicate an iminium ion intermediate and the formation of an N-oxide in the metabolism of the drug, both of which were identified with the cyanide trapping assay (34). More importantly, the
Table 2.1. Result summary for imminium ion reactive intermediate assessment with KCN assay. Overall compounds with known toxicity profiles containing an alicyclic amine structure were effectively detected in the KCN assay, when not detected in GSH trapping assay.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CN Adduct Detected (proposed metabolic reaction)</th>
<th>GSH Adduct Detected (proposed metabolic reaction)</th>
</tr>
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<tbody>
<tr>
<td>Nicotine</td>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Nefazodone</td>
<td>P</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P+16 (mono-oxygenation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P+32 (di-oxygenation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-112+14 (desalkylation+mono-oxygenation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-112+30 (desalkylation+di-oxygenation)</td>
<td></td>
</tr>
<tr>
<td>Prochlorperazine</td>
<td>P</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P+16 (mono-oxygenation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P+2 (desmethylation+mono-oxygenation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P+30 (mono-oxygenation+unknown)</td>
<td></td>
</tr>
<tr>
<td>Triprolidine</td>
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<td>P</td>
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<td></td>
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<td></td>
<td>P+ CN (bis cyano adduct)</td>
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<tr>
<td>Mianserin</td>
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<td>ND</td>
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<tr>
<td></td>
<td>P+16 (mono-oxygenation)</td>
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<tr>
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<tr>
<td></td>
<td>P-77 (unknown)</td>
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<tr>
<td></td>
<td>P-77+16 (unknown + mono-oxygenation)</td>
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<td>Ketoconazole</td>
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<td>P-14 (desmethylation)</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>ND</td>
<td>P+16 (mono-oxygenation)</td>
</tr>
</tbody>
</table>

<sup>a</sup>: P: parent drug  
<sup>b</sup>: ND: not detected

Table 2.1. Result summary for imminium ion reactive intermediate assessment with KCN assay. Overall compounds with known toxicity profiles containing an alicyclic amine structure were effectively detected in the KCN assay, when not detected in GSH trapping assay.
iminium ion of mianserin has been reported as the reactive intermediate that causes agrunulocytosis (20)(1 ADR). Thioridazine metabolism reports indicate the formation of several sulphoxides, sulphones and an N-oxide in the bioactivation of the drug (35)(Blake et al. 1995). Iminium ion metabolism intermediates consistent with these oxidative metabolites were identified in the cyanide trapping assay. In addition, there have been several reports of idiosyncratic reactions and a couple hepatotoxicity incidents associated with thioridazine (36).

Clozapine poses an interesting metabolism which has been reported to cause idiosyncratic agranulocytosis at a rate of 0.8% and isolated cases of hepatotoxicity (37). Reactive metabolites resulting from the formation of radical intermediates of oxidative processes were detected with GSH (37, 38). Other metabolites reported for the metabolism of clozapine include a desmethyl, an N-oxide and hydroxylation metabolites. These metabolites were detected in the cyanide trapping assay and thus are presumed to form via an iminium ion intermediate. In this case, all metabolites related with the metabolism of clozapine were detected with the combination of both GSH and cyanide trapping assays, demonstrating the complementarity of the trapping methods used in detecting reactive metabolites. Most importantly the reactive metabolite reported to be directly associated with toxicity of the drug was identified as a nitrenium intermediate formed during bioactivation of clozapine (39). The nitrenium ion intermediate has very similar chemical characteristics to an iminium ion and thus was detected in the cyanide trapping assay.

In the compound library screened there were several compounds which were expected to give positive results in the cyanide trapping assay, which gave negative
results in our assay. The negative results for phencyclidine and clemastine can be attributed to a number of documented reasons. There have been several publications on the inhibitory effects of phencyclidine on metabolism of P450 2B1 (40). Also, the concentration of cyanide used in the system has been shown to exhibit an 87% metabolism inhibition of phencyclidine at a 1.0 mM NaCN in a microsomal system (41). All previously reported metabolites for clemastine lack the alicyclic amine structure where the iminium ion is expected to form (42). However, in a recent report phase I metabolites containing the alicyclic amine structure were detected in excreted urine of dogs, horses and humans after hydrolysis(43). Although, the formation of an iminium ion is expected for this compound, our results suggest a bioactivation pathway that does not involve an iminium ion intermediate or that our incubation conditions are not favorable for the reaction of the iminium ion intermediate and cyanide. Metabolism rates are often different in vivo accounting for the difference between previous reports and our results. Negative results for rifampin and ticlopidine in the cyanide trapping assay may be a result of the metabolic pathway of each drug does not form an iminium or nitrenium ion intermediate. This is in fact the case for ticlopidine, where the metabolic pathway reported does not show an iminium ion intermediate as the reactive species (44). However, the reactive metabolites formed for each of these drugs were trapped with the GSH assay, once again demonstrating the complementarity of the two trapping assays.

2.4 Conclusions

The power and promise of the cyanide trapping assay and the complementarity to GSH trapping assays was demonstrated above. However, there are a couple of caveats related to this technique. First, the concentration of cyanide used may inhibit
bioactivation of some drugs, as was seen in phencyclidine. Second, the incubation conditions may not be favorable for the formation of an iminium ion cyano adduct in the bioactivation of some drugs that are expected to form an iminium ion intermediate. In these cases, it may be necessary to find optimal incubation conditions for these compounds.

Although, the system may have some problems tracking phase I metabolites that do not go through an iminium ion intermediate but from reactive metabolites. These oxidative metabolites can often be detected in GSH trapping assays as was shown for the bioactivation of clozapine. Thus, the complementarity of both trapping assays is clearly demonstrated in clozapine results, where oxidative reactive metabolites were detected with GSH and those metabolites formed though an iminium ion where detected with cyanide.

Finally, there are several major advantages to the cyanide trapping technique in comparison to radiometry assays. First, the throughput of the assay in comparison to radiometry assays is considerably improved, with chromatographic separations at 8 min per sample. Longer separations, > 20 min per sample, are required for the identification of metabolites in radiometry assays. A second considerable advantage over radiometric techniques is the power to determine the sites of bioactivation and cyanide adduction on the parent molecule, with information generated in preparation for high-throughput analysis.
2.5 Acknowledgement

The authors thank Matt Cyronak, Jacob Dunbar, Harma Ellens, Steve Clarke, and Roberto Tolando at Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Pharmaceuticals for their support and help with this project.
Chapter 3:
3.1 DNA Adduct Significance

The formation of DNA adducts has long been associated with carcinogenesis. It has been postulated that when DNA adducts are not efficiently repaired, alterations in the DNA sequence may occur during replication that lead to mutation and ultimately cancer. Although there has been significant evidence linking DNA adducts to cancer, the specific molecular effects on specific genes have yet to be elucidated. This is further complicated by the multistage nature of carcinogenesis and interindividual metabolic variation. DNA adducts associated with cigarette smoke xenobiotics have been linked to lung, colon, breast and bladder cancers. Two major analytical challenges in DNA adduct analysis have been the sample requirements and the need to reach detection levels between 1 and 10 adducts per $10^9$ unmodified DNA bases using less than 40 µg of DNA, i.e., the equivalent found in 1 mL of blood. An analytical challenge that has been met in this chapter is the detection level of 5 adducts per $10^9$ unmodified DNA bases using as little as 10 µg of DNA, i.e., the equivalent of only 250 µL of blood or approximately 5 drops of blood. But in a given analysis only a fraction of this is utilized for analysis.

3.2 Bladder cancer

Cigarette smoking has long been linked with both the formation of DNA adducts (45-47) and bladder cancer (19, 45, 46, 48-58). In the United States there have been an estimated 68, 810 new cases and 14, 100 bladder cancer related deaths in 2008 alone (59). While statistics for bladder cancer may not appear as significant when compared to other cancer types, in comparison other cancer types often require the use of invasive procedures to obtain samples for analysis. The study of bladder cancer allows for the
unique opportunity to easily collect exfoliated bladder cells that have been excreted in urine and easily isolate genomic DNA in the range of 1 to 100 µg for DNA adduct content analysis.

3.3 4-Aminobiphenyl(19)

One prominent carcinogen present in cigarette smoke, 4-Aminobiphenyl (4-ABP) has been identified as a human urinary bladder carcinogen (19, 60-63). The postulated mode of action for 4-ABP is metabolic activation, DNA adduct formation followed by mutation leading to carcinogenesis (53, 64-66). Metabolism of 4-ABP leads to the formation of DNA adducts via both Phase I and Phase II metabolism as illustrated in Figure 3.1. Phase I metabolism by CYP1A2 produces largely hydroxylated metabolites at various positions along the aromatic rings and most importantly forms the reactive N-hydroxy-4-ABP (N-OH-ABP) metabolite that is subject to Phase II metabolic conjugation, including N-glucuronidation and N-sulfation, for transport and excretion (67, 68). The N-glucuronide-4-ABP conjugate has been closely associated with bladder carcinogenesis (19). It has been postulated that in the bladder at low pH the glucuronide is easily released resulting in the formation of N-OH-ABP which further ‘decomposes’ down to the nitrenium ion reactive intermediate, ultimately leading to the formation of 4-ABP-DNA adducts (69, 70).
Figure 3.1. Metabolism of 4-ABP that leads to the formation of DNA adducts.

Adapted from Ref. (19).
The three most prominent adducts formed in the reaction of the nitrenium reactive intermediate with DNA, \(N\)-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP), \(N\)-(deoxyadenosin-8-yl)-4-aminobiphenyl (dA-C8-ABP) and \(N\)-(deoxyguanosin-N\(^2\)-yl)-4-azoaminobiphenyl (dG-N2-azo-ABP), are shown in Figure 3.2 (71, 72). The research described herein focuses only on the dG-C8-ABP isomer which has been shown to reach steady state levels with a linear dose response rate (73), suggesting the formation of stable adducts. The most common types of mutations associated with 4-ABP-DNA adducts are frameshifts and base pair substitutions (45, 74). In animal dosing studies, 4-ABP-DNA adduct content has been correlated to tissue susceptibility and tumor induction. Recent research has shown that carcinogenesis can be prevented (75). Of particular interest to the area of chemoprevention are active compounds that are found in broccoli and other cruciferous vegetable extracts along with other analogs that have been found to inhibit bladder cancer development (76).

### 3.4 Chemoprevention

Active compounds found in plants are known as phytochemicals. Isothiocyanates (ITCs) are a well known class of such chemicals (77). Sulforaphane, \([1\text{-isothiocyanato-4-}(\text{methylsulfinyl})\text{butane}]\), an ITC, found in broccoli sprouts at an estimated level of 14 µg mg\(^{-1}\) of broccoli, has been known to protect cells against a wide variety of carcinogens (78-81). ITCs have a direct enzymatic inhibition of Phase I enzymes that activate procarcinogens like 4-ABP and up-regulation of Phase II conjugation enzymes(82, 83) through the activation of the antioxidant response element (ARE) via the nuclear factor erythroid 2-related factor 2 (Nrf-2)- Kelch-like ECH-associated protein 1 (Keap-1) signaling pathway as shown in Figure 3.3 (75, 77, 84-87).
Figure 3.2. The three most prominent 4-ABP-DNA adduct isomers formed as a result of metabolic activation of 4-ABP are shown above. Top left: dG-C8-ABP. Top right: dA-C8-ABP. Bottom: dG-N2-azo-ABP. Adapted from (71).
A second class of chemopreventive agents are dithiolethiones. One of these compounds, oltipraz, was investigated in Phase I and II clinical trials as a chemopreventive agent, however, the compound failed due to significant toxicity (88). Several analogues of oltipraz are still under investigation, particularly 5,6-Dihydrocyclopenta[c]-1,2-dithiole-3(4H)-thione (CPDT). Shown in Figure 3.3, CPDT a potent inducer of Phase II enzymes was used as second chemopreventive agent in the work presented in this chapter (89-91).

Many Phase I enzymes are modulated by sulforaphane either through direct inhibition or transcriptional down-regulation, the P450 isoform CYP1A2 among them being the most important since it is this specific P450 isoform that is responsible for the formation of hydroxylated metabolites of 4-ABP (48, 92, 93). Of significant interest is the up-regulation of Phase II conjugation enzymes and carcinogen detoxifying enzymes including glutathione-S-transferase (GST) (84, 94) and UDP-glucuronosyltransferase 1A (UDPG1A) (95) through the activation of the ARE via the Nrf2-Keap1 signaling pathway which is stimulated by sulforaphane and CPDT (48, 93, 96).

Nrf2, a 66 kDa protein has been shown to bind to ARE with high affinity as a heterodimer with a small muscle aponeurotic fibrosarcoma (Maf) protein, as shown in Figure 3.3 (75). Interestingly the Nrf2 gene also carries an ARE that is stimulated by ARE mediated inducers giving rise to the possibility of positive autoregulation (75). However, studies done by McMahon et al. showed only a marginal increase in Nrf2 mRNA when exposed to sulforaphane which is considered a potent ARE inducer (97).
Figure 3.3. Nrf2-Keap1 signaling pathway in the activation of ARE and up regulation of Phase II enzymes.

Adapted from Ref. (75).
The second element in the signaling pathway, Keap1, is a 69 kDa protein that is found in the cytoplasm attached to actin and is typically associated with Nrf2 (98-100). The Nrf2/Keap1 complex is subject to modification by sulforaphane, initiating the disassociation of Nrf2 from Keap1 allowing it to enter into the nucleus and forms a heterodimer with Maf thereby activating the ARE region of many carcinogen detoxifying and conjugating enzymes as illustrated in Figure 3.3 (75, 98, 101).

Stimulation of the Nrf2-Keap1-ARE signaling pathway with a chemopreventive phytochemical such as sulforaphane will allow for the investigation of the metabolic pathways shown in the boxed area of Figure 3.1. By increasing the amount of Phase II reactions the pathway shown at the bottom right of Figure 3.1 will be forced in the direction of conjugate formation and ultimately lead to the excretion of the carcinogen. Alternatively, by inhibiting Phase I metabolism, the amount of reactive N-OH-ABP metabolite produced will be significantly reduced.

In this chapter we present improved DNA adduct quantification methodology, where sample requirements for the analysis of DNA adducts have been reduced by as much as 60 fold. This method was successfully applied to the evaluation of chemopreventive agents for the prevention of 4-ABP adducts.
3.5 Experimental Design

A cell dosing experiment was designed to test the carcinogen detoxifying potential of two chemopreventive agents, sulforaphane and CPDT, by quantifying the number of dG-C8-ABP adducts formed in cells treated with a chemopreventive agent. Generally, human bladder RT-4 cells were dosed at three conditions. Condition 1 was the positive control where the cells were pretreated for 24 hours only with solvent either ACN or DMSO for sulforaphane and CPDT experiments respectively. In conditions 2 and 3, cells were pretreated with a low and high amount of inhibitor respectively for 24 hours. After 24 hours of pretreatment all cells were exposed for 3 hours to either a reactive metabolite of 4-ABP, N-hydroxy-4-acetaminobiphenyl (N-OH-AABP), or dosed directly with 4-ABP that was metabolically activated by S9 liver microsomes. In treating the cells with only the active metabolite, it was possible to isolate the effects of the chemopreventive agent to the effects of up-regulation of Phase II enzymes. Consequently, in the metabolically activated case, we can measure the total effect of both down-regulation of Phase I metabolically activating enzymes and up-regulation of Phase II enzymes by measuring the total number of adducts formed in each condition.

A. On-line Sample Clean up

An on-line sample clean up method was developed in order to minimize sample handling and reduce sample loss. The design of the Agilent Chip cube allows for the enrichment of analyte onto a 40 nL trap column while washing off unwanted salts and unmodified nucleosides, eliminating the need for solid phase extraction enrichment in a 2-dimensional LC system as illustrated in Figure 3.4. In order to prove the feasibility of
this approach a model nucleoside solution was prepared in order to simulate the components (nucleosides and adducts) in the actual sample prior to on-line enrichment and analysis. To this end, a solution containing the equivalent of 0.5 μg of DNA digest in nucleosides and 41.1 fg μL⁻¹ dG-C8-ABP was injected in order to optimize enrichment conditions. As shown in Figure 3.5A, injection of the model matrix solution without enrichment condition optimization resulted in a matrix factor of 0.23, i.e., 23% of neat response, when compared to signal obtained with neat dG-C8-ABP standard injection. Conditions were optimized to a final loading mobile phase composition of 90% water, 7% Methanol and 3% Acetonitrile (ACN) with 0.1 % Acetic Acid Figure 3.5B, resulting in a matrix factor of 0.85.
Figure 3.4. Agilent HPLC Chip schematic for 2-Dimensional chromatography.
Figure 3.5. Evaluation of loading conditions for on-line sample clean up. (A) Model matrix solution without optimization. (B) Optimized on-line sample clean up conditions with the same model matrix solution as above.
3.6 Experimental

A. Materials

The following chemicals were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO): nuclease p1 from *penicillium citrinium*, deoxyribonuclease 1 (DNase I) from bovine pancreas, alkaline phosphatase from *Escherichia coli*, tris (hydroxymethyl)-aminomethane hydrochloride (TRIS-Cl), ammonium acetate, formic acid, ammonium bicarbonate, magnesium chloride and ethanol. Phosphodiesterase 1 (crotalus adamanteous venom) was received from USB Corporation (Cleveland, OH). dG-C8-ABP and the deuterium labeled analog dG-C8-ABP-\textsubscript{d9} synthetic standards were previously synthesized and characterized by Dr. Elaine Ricicki and Dr. John Soglia in our laboratory (102). Blood and Cell Culture DNA Maxi Kits were purchased from Qiagen Inc. (Valencia, CA). Burdick and Jackson solvents were obtained from Thermo Fischer Scientific (Pittsburgh, MA) and were HPLC grade, unless otherwise noted.

B. Cell Culture

Human bladder RT-4 cells were pretreated with a chemopreventive agent for 24 hours then exposed to 4-ABP for 3 hours. DNA was extracted using a Qiagen blood and cell culture kit and quantified using an Invitrogen Quant-iT dsDNA BR kit (Eugene, OR).
C. DNA Digestion

Samples and calibration standards were taken through a modification of a previously described DNA digestion procedure (103). Briefly samples were incubated at 98 °C for 3 min, and then chilled in freezer down to room temperature for about 5 min. Then, 0.3 units of Nuclease P1 (0.3 units µL⁻¹ solution of 5 mM TRIS-Cl, pH= 7.4) and 3.1 Kunits of DNase I (1 µg µL⁻¹ solution in 5mM TRIS/10 mM MgCl₂, pH=7.4) were added per µg of DNA and incubated at 37°C for 5 hours. Followed by 0.003 units of phosphodiesterase (100 ng µL⁻¹ in 5mM TRIS/10 mM MgCl₂, pH=7.4), and 0.002 units of Alkaline phosphatase (straight) per µg of DNA. After an overnight incubation for 18 hours at 37 °C, the digestion was terminated by the addition of 5 volumes of ice cold ethanol (-80°C). Digest proteins were pelleted by centrifugation at 7500 x g (Thermo Fischer Scientific, model Marathon 21000R) for 15 min at 4°C. The supernatant was recovered and lyophilized. The sample was then reconstituted with 20 µL 90:10 (V/V) water:methanol.

D. Liquid Chromatography-Mass Spectrometry

Liquid chromatography was performed on an Agilent 1100 system, equipped with a microwell-plate autosampler. Chromatographic separations were performed on a small molecule Chip RP-C18, 5 µm particle size with a 40 nL trap and 0.5 x 0.075 mm analytical column (Agilent Technologies, Wilmington, DE). The sample was loaded at a flow rate of 4.00 µL min⁻¹ with 93 % mobile phase A (3% acetonitrile, 0.1% acetic acid in water) and 7% mobile phase B (0.1% acetic acid in methanol). The typical injection volume was 5 µL. Chromatographic separations were conducted at a flow rate of 300 nL.
min⁻¹ with mobile phase A (0.1% acetic acid in water) and mobile phase B (0.1% acetic acid in methanol). Mobile phase B, 10% was held for 4.21 min, then linearly increased to 90% over 2.8 min and held for 2 min, then stepped down to 10% B for re-equilibration for 5 min.

All mass spectrometric analyses were performed on an Agilent Technologies XCT Ultra Ion Trap mass spectrometer, operated in ultra scan mode with 1.5 mass isolation width. Capillary voltage was -1675 V, dry gas temperature was 325 °C, dry gas flow was set to 3.0 L min⁻¹ and nebulizer gas was set to 2.0 psi. MS/MS spectra were collected with a 290-375 m/z scan window with a maximum accumulation of 50 ms and fragmentation amplitude of 1.5 Volts. Extracted ion chromatograms were used to monitor the transitions, 435 →319 and 444 →328 for the characteristic loss of deoxyribose for dG-C8-ABP and dG-C8-ABP-d₉ respectively.
3.7 Results

A. MS Analysis and Optimization of dG-C8-ABP Synthetic Standard

Prior to sample analysis, a 10 ng mL\(^{-1}\) solution of dG-C8-ABP in 70:30:0.1 methanol:water:acetic Acid (v/v%) was infused at 300 nL/min to auto-tune skimmer, octopoles 1 and 2 DC, trap drive and octopole Rf settings. The resulting MS/MS spectrum is shown in Figure 3.6, where the [M+H]\(^{+}\) ion appears at \(m/z\) of 435 and the most prominent product ion, resulting from the loss characteristic of deoxyribose, appears at \(m/z\) 319. The optimization of these settings allowed for the transition [M+H]\(^{+}\)\(\rightarrow\) [M+H-116]\(^{+}\) for both the dG-C8-ABP standard and dG-C8-ABP-\(d_9\) internal standard to be detected with high sensitivity.

B. LC-MS/MS Analysis of Human Bladder RT-4 Cells Dosed \textit{in vitro}

Based on preliminary feasibility results for on-line sample clean up, synthetic standards dG-C8-ABP and dG-C8-ABP-\(d_9\), representing 0.3 and 1.1 fmol on column respectively, were spiked into 10 µg of blank RT-4 genomic DNA, digested as previously described and final analyte recovery was determined. The corresponding extracted ion chromatograms (EICs) and mass spectra from the spiked digest are shown in Figure 3.7. The final analyte recovery determined was approximately 95\% for both dG-C8-ABP and dG-C8-ABP-\(d_9\).

Next, RT-4 cells samples pretreated with sulforaphane for 24 hours and dosed with 4-ABP along with S9 microsomes after pretreatment with sulforaphane were used to develop final procedure conditions (DNA isolation, digestion, appropriate
Figure 3.6. (A) Structure of dG-C8-ABP, most prominent fragment is highlighted by the dashed arrow. (B) MS/MS spectrum of dG-C8-ABP
Figure 3.7. (A) EIC for MS/MS of dG-C8-ABP shown in pink for 435 ➔ 319 transition and dG-C8-ABP-$d_9$ shown in purple for 444 ➔ 328 transition spiked into 10 µg DNA digest. (B) MS/MS spectrum of dG-C8-ABP. (C) MS/MS spectrum of dG-C8-ABP-$d_9$. 

amount of internal standard, etc.) while allowing for the qualitative measurement of DNA adduct inhibition by sulforaphane. An example of sample preparation reproducibility is shown in Figure 3.8, where cells were treated with 4-ABP plus S9 liver microsomes in triplicate, isolated genomic DNA was taken through the digestion procedure previously described also in triplicate and injected twice per sample. In addition, a 6 µL aliquot from each sample was pooled and injected three times, in an attempt to normalize analyte signal within a specific condition shown in aqua in Figure 3.8.

In addition the aforementioned RT-4 cell doings experiments were used as an initial probing to determine the best 4-ABP dosing conditions that would yield enough adducts to be detected reproducibly in 5 or 10 µg of DNA. An example of two sets of inhibition study dosings that were analyzed as described above, are shown in Figure 3.9. The first inhibition study set is shown in purple, while the second set is shown in red. In general, there was at least a 50% reduction in the number of adducts formed when compared to the positive control, see Condition 1 on left hand side of chart shown in Figure 3.9. Interestingly, the number of adducts formed was slightly higher for the highest concentration of sulforaphane, see Condition 3 on right hand side of bar graph in Figure 3.9, in comparison to Condition 2, suggesting the potential stimulation of other signaling pathways.
Figure 3.8. RT-4 nuclear DNA from individual dosings with 4-ABP shown in blue purple and yellow. Pooled digest samples 1 through 3 are shown in aqua.
Figure 3.9. Two preliminary inhibition study sets dosed with 4-ABP by S9 activation. Qualitative relative analysis, set 1 shown in purple, set 2 shown in red. Conditions 2 and 3 show a relative adduct reduction of approximately 50% when compared to the positive control, Condition 1.
C. Determination of Calibration Curve Linearity and dG-C8-ABP Limits of Quantification

Once all experimental conditions had been established, standard calibration curves were prepared and run in triplicate during three separate periods to determine method reproducibility and linearity over three orders of magnitude as shown in Figure 3.10A. Raw data and statistics for each of these curves shown in Figure 3.10A are summarized in Table 1. Data analysis of standard curves showed a linear response in proportion to the amount of standard added to the digest within a range of 0.038 to 9.4 fmol on column. The resulting regression based on pooled data is shown in Figure 3.10B, where the overall $R^2 = 0.997$ and the slope is $0.009 \pm 1 \times 10^{-4}$. The EIC for the mean limit of quantification representing 38 amol on-column with a signal to noise ratio of 10:1 is shown in Figure 3.11.
Figure 3.10. (A) Independent calibration curve data from three independent preparations. (B) Pooled calibration curve regression.

\[ y = 0.0009x + 0.1464 \]
\[ R^2 = 0.997 \]
Table 3.1. Raw calibration curve data

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*Rejected with 90% confidence based on Q-test

Linear Regression values

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Table 3.1. Raw calibration curve data
Figure 3.11. EICs for the mean limit of quantification (green trace), solvent/system blank (pink trace), procedure blank (blue trace).
D. Quantification of dG-C8-ABP in Human Bladder RT-4 Cell Line for the Evaluation of Chemopreventive Agents

After 24 hour pretreatment with sulforaphane at two different levels, 4 and 8 µM, RT-4 cells were dosed with the active metabolite N-OH-AABP at two different levels 50 and 100 µM. Following sulforaphane/N-OH-AABP treatment, genomic DNA from these cells was extracted, analyzed and adduct content was determined and expressed as the number of adducts per $10^7$ unmodified DNA bases as shown in Figure 3.12. The number of adducts present in these samples ranged from 1 to 24 adducts in $10^7$ unmodified DNA bases. On average for the 50 µM N-OH-AABP dosing set there was a 52% and 20% reduction in the number of adducts formed for Conditions 2 and 3 respectively, see red bar series in Figure 3.12. Compare Conditions 2 and 3 to the positive control, condition 1, found on the left hand side of Figure 3.12. When dosed with 100 µM N-OH-AABP, there was a 92% and 33% reduction in the total number of adducts formed for Conditions 2 and 3 respectively, see purple bar series in Figure 3.12.

Given the success of these analyses, a different chemopreventive agent CPDT was also investigated as shown in Figure 3.13. Cell treatment conditions were the same as for sulforaphane inhibition, with CPDT 24 hour pretreatment concentrations of 12.5 and 25 µM for Conditions 2 and 3 respectively. Generally, quantitative analysis showed a 30-80% reduction in the number of adducts formed after pretreatment with CPDT in comparison with the positive control; compare Condition 1 to Conditions 2 and 3 in Figure 3.13. Samples dosed with 4-ABP along with S9 liver microsomes for activation, shown in the teal bar series in Figure 3.13. A 30% and 60% reduction in the number of adducts for Conditions 2 and 3 respectively within this set of samples was observed.
Figure 3.12. Quantitative analysis for N-OH-AABP dosing at 50 and 100 µM with 24 hour pretreatment at either 4 or 8 µM sulforaphane (SF).
Figure 3.13. Quantitative analyses for DNA adduct prevention by CPDT. First set (Green bar series), dosing with 4-ABP by S9 activation. Second set dosing with 50 µM N-OH-AABP (shown in red series). Third set, dosing with 100 µM N-OH-AABP (shown in purple series).
In addition samples dosed with 50 µM N-OH-AABP, the red bar series in Figure 3.12, showed a 45% and 82% reduction in number of adducts for Conditions 2 and 3 respectively. At the higher 4-ABP metabolite concentration, 100 µM N-OH-AABP, there was a 40% reduction in the number of adducts for Condition 2. However, for Conditions 1 and 3, the positive control and 25 µM CPDT respectively, the number of adducts detected were not statistically different, see purple bar series in Figure 3.12. This result is in line with the trend seen for the phytochemical chemopreventive agent sulforaphane, again suggesting the stimulation of other signaling pathways.
3.8 Discussion

Initially the Agilent Chip Cube LC-MS system was to be employed as a second enrichment step following solid-phase extraction (SPE) as had previously been established in our laboratory for the analysis of 4-ABP-DNA adducts (104). However, because of the relatively large injection volume used for the enrichment of analyte from a dilute solution, SPE related polymers were also being enriched causing a host of unforeseen problems such as ion suppression, microfluidic flow path blockage and carry over. Key differences between the previous methods used in our laboratory and the current method presented in this chapter were the injection volume and required amount of DNA. In the previous method only a very small volume, 500 nL, was injected from a 300 µg DNA digest that had been cleaned-up by SPE, thus avoiding the potential for microfluidic path blockage and most importantly the concentration of SPE excipients that cause severe ion suppression.

The method presented in this chapter eliminates the need for SPE, thereby reducing sample preparation time by approximately 50% in comparison to previous methods. In addition, an extremely important difference between this method and other methods in the literature (103, 104) is the amount of DNA required for analysis for the detection of adducts in the range of $10^9$ normal nucleosides. Previously published methods require between 100 and 300 µg of DNA for analysis, making the applicability of these methods plausible but not practical for the analysis of human samples. In contrast the method presented in this chapter has a DNA amount requirement 10 to 60 fold lower with the same levels of sensitivity as the methods discussed above. Furthermore, statistical analysis of individually prepared and analyzed calibration
standard curves showed excellent linearity and reproducibility over three orders of magnitude.

In the investigation of two chemopreventive agents for their potential to reduce the number of adducts formed by the urinary bladder carcinogen 4-ABP, a maximum reduction of 92% was observed with sulforaphane at lower concentrations and 82% for CPDT at higher concentrations. CPDT showed a linear relationship between the number of adducts formed and the concentration of CPDT used. This, resulted in a significant reduction in the number of adducts formed at higher concentrations of CPDT, as seen for all series in Figure 3.12. Specifically the red bar series shows an 82% drop in the number of adducts detected, compare **Conditions 1 and 3**.

The phytochemical sulforaphane, however, was shown to have a lower effective concentration. An unexpected yet interesting trend was observed at higher concentrations of sulforaphane. Namely, a rise in the number of adducts formed was observed in comparison to the number of adducts formed at lower concentrations of sulforaphane, indicative of chemopreventive agent saturation. Additionally, it must be noted that this result occurred at high dosing levels of N-OH-AABP, therefore, cells were most likely saturated with reactive intermediates beyond the effectiveness of the chemopreventive agent. We can estimate the same was true in the 4-ABP by S9 activation dosing, where the approximate reactive metabolite concentration of 200 µM was based on the number of adducts detected when dosed with N-OH-AABP as shown in Figure 3.12.
Scheme 3.1. Suggested metabolic pathway modifications and their effect on DNA adduct formation.
It is of interest to note that the highest observed effect for both of the chemopreventive agents investigated occurred with the N-OH-AABP metabolite dosing, instead of dosing directly with 4-ABP by S9 activation, highlighted by the red and blue boxed areas and text respectively in Scheme 1. This is consistent with the known effectiveness of these agents in the up-regulation of Phase II detoxifying enzymes, which consequently reduces the amount of reactive metabolite available to form the reactive nitrenium ion intermediate and ultimately reducing the number of adducts formed, as shown within the red box of Scheme 1. Measurement of the number of adducts formed the 4-ABP by S9 microsome activation condition, highlighted by the green boxed area and text in Scheme 2, allowed for the determination of the overall effect on both Phase I and Phase II metabolism of this carcinogen. Although, the exact conversion of 4-ABP to the reactive metabolites N-OH-ABP or N-OH-AABP when activated by S9 liver microsomes is variable, a significant decrease, between 50% and 60%, in the number of adducts formed was observed for both sulforaphane and CPDT.

3.9 Conclusions

A sensitive method was developed for the quantification of 4-ABP DNA adduct isomer dG-C8-ABP in small amounts, usually less than 10 μg, of DNA. The method was successfully applied to the measurement of DNA adducts in a human bladder cell line to evaluate the effectiveness of chemopreventive agents, such as sulforaphane and CPDT, in the reduction of adduct formation.
Chapter 4:

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Elucidation of Immortal DNA Strands in Mouse Cell Models for Adult Stem Cell Mechanism of Repair and Asymmetric Self-Renewal.
4.1 Cell Cycle

The cell cycle can be grouped into two major phases: the interphase, where the cell prepares for cell division and the mitotic phase where the nucleus and cytoplasm are divided (105, 106). In the longest part of the cell division cycle, the interphase, there are three main stages: the $G_1$ phase or first gap, the $S$ phase or synthesis phase and the $G_2$ phase or second gap (105). As interphase progresses, the cell duplicates cytoplasmic organelles, proteins and chromosomes. The rest of this section will center in describing chromosome replication as this phase directly pertains to the focus of this chapter.

In preparation for cell division the cell must duplicate all of its chromosomes. Each chromosome consists of chromatin fibers that are made up of DNA-protein complexes known as nucleosomes (Figure 4.1) (107, 108). In each nucleosome DNA is tightly associated with histone proteins which organize and compact long DNA double-helix molecules. Each nucleosome is then compacted into progressively higher ordered structures leading up to the chromosome structure visible with a light microscope. Once all chromosomes have been copied they exist as sister chromatids joined at the centromere region (Figure 4.2) (105, 109). In theory, the resulting DNA double-helix molecules are “identical” as a result of the DNA replication process.
Figure 4.1 Nucleosomes the main building block of chromatin fibers and chromosomes. DNA molecules wrap around histone proteins to form nucleosomes which are then compacted further into a chromatin fibre.

Adapted from Ref. (108).
Figure 4.2. Sister chromatid joined at the centromere region highlighted by the blue circle.

Adapted from Ref. (109).
A DNA Replication

Generally the DNA double helix molecule unwinds and each single DNA strand serves as a template for the synthesis of new strands resulting in two DNA molecules. According to the Watson and Crick model for DNA replication, the process follows a semi-conservative mechanism where each new DNA molecule consists of a new strand and an old strand (Figure 4.3)(110-113). This semi-conservative model was supported by the experiments of Meselson and Stahl in 1957 (114), where E. coli cells were grown in heavy (\textsuperscript{15}N) media for many generations resulting in a fully labeled DNA molecule(115). The cells were then transferred to grow in light (\textsuperscript{14}N) media for one generation, i.e., until the cell population doubled, resulting in hybrid DNA molecules consisting of one heavy strand and one light strand. Finally, these cells were allowed to grow for another generation, i.e., two generations from the fully labeled DNA molecule, resulting in a mixture of hybrid and light DNA molecules (Figure 4.4)(116). A similar strategy is discussed at the end of this section for the investigation of the adult stem cell cycle, specifically the theory of immortal DNA strand co-segregation.
**Figure 4.3.** DNA semi-conservative replication, new strands are shown in red while parent strands are shown in blue.

Adapted from Ref. (116).
**Figure 4.4.** Fully labeled DNA from first phase of the experiment appears as a single band at the bottom of the CsCl gradient illustrated in the test tubes at the bottom of the figure. DNA isolated from the second phase of the experiment appears at a higher position in the gradient and DNA from the third phase of the experiment appears as two bands. One band appears at the same position as the DNA from the second phase of the experiment and one band that is higher than this band, indicating the generation of both hybrid and light DNA molecules. Adapted from Ref. (116)
4.2 Stem Cells

Stems cells have long been scrutinized by both scientists and society due to their regenerative potential yet controversial sources. However, there are two types of stem cells: embryonic stem cells (ESCs) and adult stem cells (ASCs) (117-119). ESCs are derived from epiblast cells in a blastocyst, a group of cells in embryos at an early developmental stage (118, 119). These cells have the potential to give rise to cells of all 3 germ layers, a characteristic otherwise known as pluripotency (120). In contrast, ASCs arise later in fetal development and are responsible for renewal of postnatal tissues (117).

One of the first ASCs populations to be discovered was in hematopoetic tissue (118, 119). In comparison to ESCs, ASCs have a limited differentiation potential and are responsible for tissue renewal by providing a renewable source of cells to form new tissues in developing organisms and supply cells for tissue growth and renewal (119). The limits of ASCs in comparison to ESCs are also linked to tissue aging which may be due to accumulation of mutations in ASCs. Other characteristics unique to ASCs and their study are important to the fields of gene therapy, aging development, cancer and tissue engineering (119).

Two processes contribute to the ability of ASCs to segregate to themselves the set of chromosomes with the oldest DNA templates otherwise known as immortal DNA strands (121). The first asymmetric self-renewal limits the number of ASCs that exist in any given tissue by producing one ASC and one transient daughter cell with the potential to differentiate into adult tissue. The second, DNA strand co-
Figure 4.5. ASC specific cell kinetics, one progenitor cell produces one transient daughter cell and a renewed ASC containing the original cell DNA strands of the progenitor cell for many subsequent cell generations.
segregation, involving immortal DNA strands, limits the number of mutations due to replication errors (Figure 4.5). Both of these processes are meant to restrict carcinogenesis by limiting the number of ASCs and replication errors that may lead to the accumulation of mutations, however, they may also allow for the accumulation of covalent modifications.

One important aspect of immortal DNA strands is that, in theory, they remain unchanged as a result of cell proliferation, thus the term immortal DNA strands. Because immortal DNA strands follow conservative replication, mutations caused by replication errors are less likely. It has been postulated that in order to maintain immortal DNA strand integrity there may be a lack of repair enzymes in ASCs. However, lack of repair enzymes does not protect the immortal DNA strand from covalent modifications. The build up of covalent modifications would result in DNA aging, which in turn also puts at risk new DNA strands that are synthesized from the covalently modified parent immortal DNA strands. The resulting new daughter DNA would have a higher degree of replications errors due to the interference of covalent modifications on the parent strands, resulting in gene mutations that may lead to a mutated transient daughter cell. Furthermore, the key processes unique to ASCs described above are regulated by the p53 cancer gene (120).

4.3 Experimental Design

Based on the information presented above, further investigation and verification of the self-renewal process in ASCs would allow for a better understanding of similar processes in other types of stem cells. To this end, a DNA labeling experiment was
designed using p53 inducible and p53-null, isogenic control, mouse embryo fibroblast (MEF) cells. MEF cells were engineered to undergo symmetric self-renewal under p53 non-inducing conditions, allowing for the incorporation of DNA tracers such as isotopically labeled nucleosides during the S-phase of the cell cycle (4.6). MEF cells were labeled for one generation under conditions for symmetric self-renewal resulting in cells with hemilabeled DNA. The first generation of hemilabeled cells was then placed under p53 inducible conditions by the addition of the co-factor Zn\(^+\). Under these conditions p53-induced MEF cells underwent asymmetric-self renewal with immortal DNA strand co-segregation and were allowed to grow for approximately 8 generations (Figure 4.7). Samples from each critical phase, first generation hemilabeled cells, and the final resulting progenitor and daughter cells, were analyzed for DNA label content and evaluated for consistency with the theory of immortal DNA strand co-segregation.

Analysis of the proposed experiments by LC-MS/MS will accomplish three goals. Firstly, it will provide the first independent physiochemical demonstration of immortal DNA strand co-segregation. Secondly, the quantitative data generated will allow for the generation of theoretical cell kinetic modeling providing valuable information in regards to cell culture growth and expected degree of label dilution in control cells. Finally, LC-MS/MS can be used to look for signs of chemical aging and degradation of immortal DNA strands.
Figure 4.6. p53 inducible mouse embryo fibroblast cells under non-inducing conditions undergoing symmetric self-renewal with semi-conservative DNA replication and random chromosome segregation.
Mouse embryo fibroblast cells under p53 inducing conditions exhibit asymmetric self-renewal, where one progenitor cell, or ASC, produces one transient daughter cell and a renewed ASC containing one complete set of original DNA strands that is retained for subsequent cell divisions.
4.4 Experimental

A. Materials

The following chemicals were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO): thymidine (dT), 2’-deoxycytidine (dC), 2’-deoxyadenosine (dA), 2’-deoxyguanosine (dG), nuclease p1 from *penicillium citrinium*, alkaline phosphatase from *Escherichia coli*, tris (hydroxymethyl)-aminomethane hydrochloride (TRIS-Cl), ammonium acetate, formic acid, ammonium bicarbonate, magnesium chloride and ethanol. Phosphodiesterase 1 (crotalus adamanteous venom) was received from USB Corporation (Cleveland, OH). Thymidine $^{13}$C$_{10}$ $^{15}$N$_2$, 98% and 96-98% respectively ($^{13}$C$^{15}$N-dT), Thymidine $^{15}$N$_2$, 98% ($^{15}$N-dT), and 2’deoxyadenosine $^{15}$N$_5$, 96-98% ($^{15}$N-dA) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Blood and Cell Culture DNA Maxi Kits were purchased from Qiagen Inc. (Valencia, CA). Solvents were obtained from Thermo Fischer Scientific (Pittsburgh, MA) and were HPLC grade, unless otherwise noted.

B. Preparation of $^{15}$N-labeled Nucleoside Standard Curves

A three-fold calibration curve was prepared for the quantification of $^{15}$N-dT, dT and dA by the addition of 50 µL 5 mM TRIS-Cl to eight microcentrifuge tubes, 30 µL of each previously prepared serial dilution of $^{15}$N-dT, dT and dA and 2 nmol of $^{15}$N-dA and $^{13}$C$^{15}$N-dT, 25.6 µL working solution each were mixed. The mass range spanned 3 orders of magnitude for each of the analytes. Specifically the total amounts added for $^{15}$N-dT were 0.025, 0.25, 0.5, 4.0, 8.0, 12.0 and 16 nmol, for dT and dA 0.25, 1.0, 4.0, 8.0, 12.0, 16.0, and 24.0 nmol each. Standards were taken through a modification of a previously
described DNA digestion procedure (122). Briefly samples were incubated at 98 °C for 3 min, and then chilled on ice down to room temperature. A 1/10 volume equivalent of a 0.1 M ammonium acetate solution (pH=5.3) was added, then 0.3 units of Nuclease P1 (0.3 unit/µL solution of 5 mM TRIS-Cl, pH= 7.4) were added per µg of DNA and incubated at 45 °C for 2 hours. Then, a 1/10 volume equivalent of 1 M ammonium bicarbonate solution was added, followed by 0.002 units of phosphodiesterase (100 ng/uL in 5 mM TRIS/10 mM MgCl₂, pH=7.4) and incubated for 2 hours at 37 °C. Alkaline phosphatase (straight, 0.06 units/uL) was added and the reaction solution was incubated for an additional hour. The digestion was terminated by the addition of 3 volumes of ice cold ethanol (-20 °C). Digest proteins were pelleted by centrifugation at 5000xg (Thermo Fischer Scientific, model Marathon 21000R) for 15 min at 10 °C. The supernatant was evaporated to dryness and reconstituted with 500 µL water. Solutions were filtered through PTFE minispike (Waters, 0.45 µm pore size) to remove any particulates prior to analysis.

C. Cell Culture

Murine embryo fibroblast cell lines 4-3 and 5-8 were split to 1/20 and 1/10 confluency respectively in Dulbecco’s modified eagle medium (Gibco) supplemented with 10% dialyzed fetal bovine serum, 1% penicillin/streptomycin and 5 µg/mL puromycin. Media was replaced when cells reached 1/4 confluency and after 24 hours cells were trypsinized. Approximately 60,000 trypsinized cells were plated in the same culture medium in 150-cm² flasks and cultured for 24 hours. Thereafter, ¹⁵N-dT was added to the cultures to a 10 µM concentration. After 24 hours of culture, this labeling medium was removed, cells were washed with label-free medium, and cultures were
replaced with medium containing 65 µM ZnCl₂ to induce immortal DNA strand co-segregation. After the initial 24 hour labeling period, one culture of each cell type was harvested by trypsinization; and the cells were pelleted by centrifugation for 5 min at 1000 rpm to create first generation cell pellets for day 0. After 4 days for 4-3 cells and after 8 days for 5-8 cells, 1/2 of the cell culture medium was removed and spun down for 5 minutes at 1500 rpm, supernatant was removed and 0.1 µg/mL colcemid was added. After 16 hours, the remaining cell culture was spun down for 5 min at 1000 rpm to create cells pellets for colcemid-arrested cells. Attached cells were trypsinized, neutralized with media and spun down for 5 min at 1000 rpm to create cell pellets. DNA was extracted using a Qiagen blood and cell culture kit and quantified using an Invitrogen Quant-iT dsDNA BR kit (Eugene, OR).

D. Liquid Chromatography-Mass Spectrometry

Liquid chromatography was performed on a Agilent 1100 system, equipped with an autosampler. Chromatographic separations were performed on a Waters Atlantis dC₁₈ column, 2.1 mm x 50 mm, 3.5 µm particle size (Waters, Milford, MA). A gradient elution at a flow rate of 0.300 µL/min with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in methanol) was used to separate all nucleosides present in sample. Mobile phase B 0% was held for 1 min, the linearly increased to 8% over 3.3 min and further increased to 70% over 2.7 min and held for 1 min, then immediately returned to 0% B for equilibration for 5 min. The typical injection volume was 5 µL.
All mass spectrometric analyses were performed on a Thermo Electron Quantum triple quadrupole mass spectrometer, operated in positive ion detection mode with 0.2 mass resolution in the first and third quadrupoles. Prior to sample analysis, a 10 ng/mL solution of each analyte, $^{15}$N-dT, dT and dA, was infused to auto-tune sheath gas, collision energy and lens values. Capillary temperature was set at 300 °C, capillary voltage was 2.7 kV and sheath gas pressure was 36 PSI. Selected reaction monitoring (SRM) was used to monitor the transitions for the characteristic loss of deoxyribose for all nucleosides.
4.5 Results

The goal of these experiments was to utilize LC-MS methodology to quantitatively illustrate the degree of label retention in ASCs undergoing asymmetric self-renewal immortal DNA strand co-segregation. The analytical approach involved two phases. The first phase was a preliminary assessment of both experimental and analytical conditions, where an external calibration curve was employed to evaluate LC-MS system requirements, experimental concentrations of DNA tracers and to qualitatively determine ASC kinetics in cell culture conditions. The first phase of the experiment was also used as the basis for the development of a quantitative calibration curve with the addition of appropriate internal standards. In order to normalize the amount of label detected, the results were normalized to the percent of $^{15}$N-dT based on the total number of dT bases, labeled plus normal, or the total amount of dA detected for the quantitative analysis.

A. Preliminary Assessment

An external calibration curve with a range of 0.25 pmol to 50 pmol on-column was used to evaluate system requirements. Linear regression analysis for the measurement of $^{15}$N-dT gave a slope of 11,238 and a correlation coefficient of 0.996 (Figure 4.8). Generally the amount of label detected in all samples was above baseline levels found in both control unlabeled cell lines (Table 4.1).
Figure 4.8. External calibration curve for the analysis of $^{15}$N-dT.
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>DNA Amount (µg)</th>
<th>% Label Detected ± RSD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N1</strong>: Ind-8 Colcemid cycling mitotic fraction</td>
<td>10.8</td>
<td>6.7 ± 0.33</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>N2</strong>: Ind-8 24-hr labeled total cell pellet</td>
<td>2.2</td>
<td>37.0 ± 2.28</td>
<td>5.5</td>
</tr>
<tr>
<td><strong>N3</strong>: Ind-8 Colcemid adherent cell fraction</td>
<td>5.4</td>
<td>10.4 ± 0.63</td>
<td>5.3</td>
</tr>
<tr>
<td><strong>Control 1</strong>: Ind-8 unlabeled cells</td>
<td>14.2</td>
<td>0.6 ± 0.08</td>
<td>11.7</td>
</tr>
<tr>
<td><strong>N4</strong>: Con-3 Colcemid cycling mitotic fraction</td>
<td>6.0</td>
<td>1.1 ± 0.14</td>
<td>4.0</td>
</tr>
<tr>
<td><strong>N5</strong>: Con-3 24-hr labeled total cell pellet</td>
<td>2.4</td>
<td>9.0 ± 1.3</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>N6</strong>: Con-3 Colcemid adherent cell fraction</td>
<td>4.8</td>
<td>8.2 ± 1.3</td>
<td>10.2</td>
</tr>
<tr>
<td><strong>Control 2</strong>: Con-3 unlabeled cells</td>
<td>21.1</td>
<td>0.8 ± 0.15</td>
<td>18.5</td>
</tr>
</tbody>
</table>

**Table 4.1.** Preliminary result summary of % label detected in each sample consisting of p53-inducible (Ind-8) and p53-null (Con-3) cell lines.
Analysis of endpoint colcemid cycling mitotic symmetrically self-renewing (Con-3) cells, sample N4, demonstrated a nine-fold decrease in label, when compared to hemi-labeled first generation cells, sample N5, after approximately four generations of cell growth (Table 3.1, compare sample N5 to N4). On the other hand analysis of endpoint colcemid cycling mitotic asymmetrically self-renewing (Ind-8) cells, sample N1, demonstrated a five-fold decrease in label, when compared to hemi-labeled first generation cells, sample N2, after approximately eight cell generations (Table 3.1, compare sample N2 to sample N1).

B. Quantitative Analysis

A three-fold standard curve was prepared by running nucleoside solutions in varying amounts of dA, dT and $^{15}$N-dT and internal standards, as described in the previous section, through the enzymatic digestion of DNA down to nucleosides. A 7-point curve using peak height ratios of labeled nucleoside to internal standard versus amount of nucleoside added was developed with a range of 250 pmol to 24 nmol. Linear regression for the quantification of $^{15}$N-dT gave a slope of 43.409 and a correlation coefficient of 0.997 (Figure 4.9).

Analysis of incubations labeled with $^{15}$N-dT, showed a sixteen-fold decrease in label after approximately four generations in control (Con-3), symmetrically self-renewing, cells in the colcemid mitotic fraction, with a relative standard deviation of $\leq 10\%$ (Table 3.2, compare sample N5 to N4). In contrast to a five-fold decrease in label after approximately eight generations in asymmetrically self-renewing cells.
Ind-8), i.e., ASCs, in the colcemid mitotic fraction (compare sample N2 to N1 in Table 3.2). When colcemid cycling mitotic fractions, samples N1 and N4 for Ind-8 and Con-3 cell lines respectively are compared to their respective colcemid adherent cell fractions, sample N3 and N6 for Ind-8 and Con-3 cell lines respectively, Ind-8 cells show a higher degree of label retention than Con-3 cells.

C. Cell Kinetics Modeling

Based on the modeled constant division fraction of new daughter cells ($F_d$), where the theoretical values of 1.0 and 0.5 correspond to ideal symmetric and asymmetric self-renewal respectively, the experimental value of 1.0 for Con-3 cells indicated ideal symmetric self-renewal conditions in this phase of the experiment. An experimentally modeled $F_d$ value of 0.7 for Ind-8 cells, however, indicated a mixture of asymmetric and symmetric self-renewal.

Theoretical calculations based on the number of cell division in symmetrically self-renewing cells, predicted a 0.19% label retention for Con-3 cells and a 0.21% label retention for Ind-8 cells. Experimental label retention percentage observed was 0.12% for Con-3 cells and 0.67% label retention for Ind-8. These results were also indicative of a mixture of symmetric and asymmetric self-renewal kinetics in the Ind-8 cell experiment.
Figure 4.9. Quantitative calibration curve for the analysis of $^{15}$N-dT
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>DNA Amount (µg)</th>
<th>% Label Detected ± RSD</th>
<th>% RSD</th>
<th>Relative Label Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1: Ind-8 Colcemid cycling mitotic fraction</td>
<td>10.8</td>
<td>0.67 ± 0.03</td>
<td>4.48</td>
<td>1/5</td>
</tr>
<tr>
<td>N2: Ind-8 24-hr labeled total cell pellet</td>
<td>2.2</td>
<td>3.18 ± 0.18</td>
<td>5.51</td>
<td></td>
</tr>
<tr>
<td>N3: Ind-8 Colcemid adherent cell fraction</td>
<td>5.4</td>
<td>1.38 ± 0.07</td>
<td>5.29</td>
<td>1/2</td>
</tr>
<tr>
<td>N4: Con-3 Colcemid cycling mitotic fraction</td>
<td>6.0</td>
<td>0.12 ± 0.01</td>
<td>4.01</td>
<td>1/16</td>
</tr>
<tr>
<td>N5: Con-3 24-hr labeled total cell pellet</td>
<td>2.4</td>
<td>1.89 ± 0.13</td>
<td>6.71</td>
<td></td>
</tr>
<tr>
<td>N6: Con-3 Colcemid adherent cell fraction</td>
<td>4.8</td>
<td>1.07 ± 0.11</td>
<td>10.24</td>
<td>1/2</td>
</tr>
</tbody>
</table>

Table 4.2. Quantitative label retention measurements for $^{15}$N-dT as the label.
4.6 Discussion

In the preliminary assessment phase of the experiment it was determined that the amount of label used was detectable at quantifiable levels. In addition the amount of label calculated by the external calibration curve was suggestive of ASC kinetics. Furthermore, results obtained during preliminary and quantitative analyses were in agreement. During preliminary assessment, the amount of label detected in Con-3 cells after approximately 4 generations, sample N4, was nine-fold lower than the amount of label detected in hemilabeled cells, sample N5. Quantitative analysis for these samples showed a sixteen-fold decrease in label. This high rate of label dilution was expected for cells undergoing symmetric self-renewal, i.e., exponential cell growth and random chromosome segregation.

Analysis of Ind-8 cells after approximately 8 generations, demonstrated a five-fold relative amount of label dilution in hemilabeled cells for both preliminary and quantitative analyses performed, compare samples N1 and N2. At this point, it is important to note that Ind-8 cell analysis was performed after twice as many generations than Con-3 cells, yet the amount of label retention was approximately two-fold higher. The high degree of label retention is indicative of immortal DNA strand co-segregation.

Quantitative analysis of these samples allowed for the verification of observations drawn from the preliminary assessment as was discussed above and the generation of cell kinetic modeling data. In addition the amount of label retained in comparison to the colcemid adherent cell fraction in both analyses (compare sample N1 to sample N3 in Table 3.2) indicated a higher degree of label retention that is suggestive of immortal
DNA strand co-segregation. Cell kinetic modeling was employed to further investigate these results.

The constant division factor experimental values for Con-3 cells were in perfect agreement with the theoretical values. In contrast, the experimental value for Ind-8 cells was above the theoretical value, suggesting imperfect asymmetric self-renewal conditions due to a mixture of both symmetric and asymmetric self-renewal. Contributions of symmetric self-renewal in this phase of the experiment may have compromised the overall amount of DNA strand label retention measured due to geometric dilution. Predicted percents of label retention were based on symmetric self-renewal, i.e., random chromosome segregation, thus were not ideal for the comparison of asymmetric self-renewal, i.e., non-random chromosome co-segregation. However, based on these theoretical numbers we can conclude that the observed percent of label retention in Con-3 cells was in agreement with the theoretical percent retention of 0.19. It was also determined that the percent of label retained for asymmetrically self-renewing cells was three-fold higher than the theoretical amount, but it is important to emphasize at this point that the theoretical amount was based on symmetric self-renewal. In addition, the percent of label observed was five-fold greater, in Ind-8 cells, than the percent label observed for Con-3 cells, which grew ideally. The higher degree of label retention observed in Ind-8 is consistent with the theory of immortal DNA strand co-segregation in association with asymmetric self-renewal.
4.7 Conclusions

The feasibility of a mass spectrometry method to trace labeled ASCs through several cell cycles and quantitatively illustrate the existence of immortal DNA strands was demonstrated, with DNA quantities as little as 2.2 µg of DNA. Significant degrees of label retention were observed for ASCs when compared to control cells. Additionally, the control phase of the experiment was in perfect accordance with all theoretical data generated by cell kinetic modeling.

The experimental data for Ind-8 cells, however, was not in complete agreement with the theoretical data. We can justify for this discrepancy by noting that predicted label retention for these cells was based on symmetric self-renewal, i.e., exponential cell growth. In the case of the $F_d$ value, we can argue that with each asymmetric self-renewal cycle a transient non-cycling cell is produced, this cell has the potential to grow with symmetric self-renewal kinetics. Therefore, we would expect to see a small deviation from the theoretical $F_d$ value of 0.5 for asymmetric self-renewal due to the symmetric self-renewal contribution by the transient daughter cells produced in each asymmetric self-renewal cycle. These symmetric divisions are estimated to constitute about 15% of all divisions in ASCs cultures (123).
Chapter 5:

Future Research Perspectives
5.1 Analysis of DNA adducts and monomers

A. On-line Digestion

In order to bring the methodology for DNA adduct analysis to a clinical study setting, sample throughput must be further improved. Currently, in the work presented in Chapter 3 of this dissertation, sample throughput is limited to 10 samples per day, not including DNA isolation and digestion time. While LC-MS parameters have little room for improvement as far as analysis time, improvements can be made on the sample preparation end. The DNA digestion process can be streamlined in such a way that it can be carried out on-line; much like protein digestions have been previously done (124). Two important enzymes, nuclease P1 and alkaline phosphatase, have been successfully immobilized without a loss of enzymatic activity (125-127). Immobilized nuclease P1 was successfully utilized for the continuous hydrolysis of RNA down to nucleotides (125). Conceptually, the resulting nucleotides that are in solution can be either bound to an immobilized metal affinity chromatography column (128) or go onto a second column where alkaline phosphatase has been immobilized (126). In order to assure digestion completeness, the enzyme phosphodiesterase can be titrated into the mobile phase during the first in-column digestion. Finally, remaining digest proteins can be removed from the solution by the employment of a zirconia based C18 column (ZirChrom Separations, Anoka, MN), ProTain®, which has been shown to irreversibly bind proteins while allowing small molecules such as nucleosides to pass through. The use of ProTain® can be applied during LC-MS analysis where the trapping column on the Agilent chip, described in Chapter 3, is packed with this stationary phase.
B. Further Improvements on 4-ABP adduct detection limits

Currently, the method presented in Chapter 3 of this dissertation was limited by the size of the enrichment column available. The small volume of the enrichment column limited the amount of DNA digest load to a maximum of 2.5 μg of DNA digest on-column. Recently, Agilent technologies has developed a high capacity chip with a 500 nL trapping column. This represents a 12.5-fold increase in enrichment capacity which in turn would allow for the realization of even lower relative adduct content detection limits, by allowing for the enrichment of larger amounts of DNA digests.

It must be noted that only a small increase, approximately 2-3 fold, in the amount of DNA required for analysis would bring the methodology presented in Chapter 3 to a limit of quantification range in the order of approximately 5 adducts in $10^{10}$ nucleosides. Allowing for the reliable measurement of background adduct levels of major adducts, the significance of which has yet to be investigated, and detection of minor adduct lesions which constitute about 15% of 4-ABP adducts formed.

C. Enzymatic Synthesis of DNA adduct standards

The formation of reactive metabolites in microsomal cultures has been utilized to screen for toxicity potential as was discussed in Chapter 2 of this dissertation, where typically nucleophiles such as GSH and the cyanide anion are utilized to trap reactive metabolites. The electrophilic species produced during microsomal activation have the potential to react with individual nucleosides as shown in Figure 5.1 (129). Therefore, microsomal incubations can be utilized to generate the reactive electrophilic species that readily bind to DNA, as was discussed in Chapters 2 and 3. The same strategy can be
utilized with the exception that nucleosides will be used as the trapping agent. In this manner, by only adding one nucleoside base such as dG, only adduct isomers of dG will be easily generated. Reaction clean-up and adduct characterization would be straightforward as the bulk proteins can be precipitated by the addition of organic solvent, as described in previous chapters. Collection and characterization of the resulting adducts can be achieved through the employment of the integrated nanosplitter LC-MS-NMR system developed in our laboratory in collaboration with Dr. Kautz. Residual proteins can be removed through the use of the Protain column as was described earlier in this section.
Figure 5.1 (A) Formation of aryl nitrenium ion. (B) Mechanism for the formation of dG adducts.

Adapted from Ref. (129).
5.2 Investigation of ASC Repair Mechanisms

In the characterization of ASCs, experimental verification of DNA repair mechanism is lacking. Although, it has been postulated that ASCs are the most likely candidate for the formation of cancer stem cells, the exact processes leading to this event are unclear. One step in elucidating this process is the investigation of DNA repair mechanisms, which can be achieved by reacting immortal DNA strands with a carcinogen like 4-ABP and monitor for both oligonucleotide gene sequence selectivity and rate of repair, if any. The proposed experiment is essentially the same as those presented in Chapter 4 of this dissertation, with the exception that our DNA tracer or label will be 4-ABP DNA adducts, specifically the isomer dG-C8-ABP for which a sensitive method has been developed as was discussed in Chapter 3 of this dissertation.

A. Cancer Stem Cells

Current theory in cancer development points to the existence of cancer stem cells (CSCs) that have been discovered in association with acute myeloid leukemia, breast cancer and brain tumors (130). CSCs may originate as mutated adult stem cells (130, 131). A key mechanism to both ASCs and CSCs is asymmetric self-renewal (Figures 4.5 and 5.2) (130-132). The implications of CSCs are threefold: first, the CSC may be the source of all malignant cells in the primary tumor; second, CSCs may be the small population of cells responsible for cancer relapse after chemotherapy; and third, if exfoliated, CSCs may give rise to distant metastases (130).
Figure 5.2. Asymmetric self-renewal is a key mechanism of both ASCs and CSCs. CSCs may originate from mutated adult stem cells, shown on the left hand side and indicated by the purple cell to green cell pathway. Alternatively their first generation transient daughter cell may acquire mutations, indicated by the blue cell to green cell pathway (dashed arrow).

Adapted from Ref. (130)
These implications have been best exemplified in leukemia, where CSCs were initially discovered.

Currently, there are two types of leukemia where CSCs have been identified (130, 132). These include chronic myelogenous leukemia and acute myelogenous leukemia. While chronic myelogenous leukemia CSCs have a well-characterized cell cycle, acute myelogenous leukemia CSCs have a similar cell cycle to that of chronic myelogenous leukemia. Thus, typical anti-proliferative cytotoxic strategies have not been effective in eradicating these types of cells. However, characterization of these CSCs has demonstrated cell surface markers that are unique to CSCs associated with leukemia, which have been explored for treatment development.

On the other hand, the CSCs associated with brain tumors have a different morphology, as these have been discovered to exist within neurospheres, a ball of cells in non-adherent cell cultures (130). Recent studies have shown that a new neurosphere can be generated by single cells extracted from other neurospheres. Yet the origin of CSCs remains elusive. Some studies suggest the possibility of progenitor cells to acquire stem-cell like properties during mutation to cancer.
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