Accelerated Cytotoxicity Mechanism Screening of 4-Aminobiphenyl in an in vitro Hepatocyte Inflammation Model

By:

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University of Toronto

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Abstract

ACCELERATED CYTOTOXICITY MECHANISM SCREENING OF 4-AMINOBIPHENYL IN A HEPATOCYTE INFLAMMATION MODEL

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Masters of Science, 2011
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4-Aminobiphenyl is an aromatic amine compound that is present in cigarette smoke, diesel exhaust, cooking oil fumes and dye intermediates. It is a well-known human bladder carcinogen and liver carcinogen in experimental animals that is metabolically activated by liver CYP1A1/2. We have used the “Accelerated Cytotoxicity Mechanism Screening” (ACMS) techniques to analyze the molecular cytotoxic mechanisms of 4-aminobiphenyl. Hepatocyte exposure to an inflammatory system significantly increased hepatocyte susceptibility to 4-aminobiphenyl. 4-Aminobiphenyl-induced cytotoxicity and lipid peroxidation were both prevented by altering cellular redox status and with the addition of antioxidants. Toxicity was increased with the depletion of hepatocyte GSH levels and by inhibiting N-acetyltransferase. These results will provide more insight into the cytotoxic and genotoxic mechanisms of 4-aminobiphenyl and also suggest that inflammation may be responsible for an increase in arylamine carcinogenesis.
Dedications

I would like to dedicate this thesis to Mommy and Douglas. Thank you for your unconditional love, support and motivation. I am forever grateful for everything you have done for me and could not have completed this work without your encouragement. I would also like to dedicate this thesis to the late Grandpa Aboud who provided me with unparalleled inspiration, enthusiasm and “joie de vivre”.
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<tbody>
<tr>
<td>ABT</td>
<td>1-Aminobenzotriazole</td>
</tr>
<tr>
<td>ACMS</td>
<td>Accelerated cytotoxicity mechanism screening</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ArNH₂</td>
<td>Aromatic amine</td>
</tr>
<tr>
<td>ArNHOH</td>
<td>Hydroxylamine</td>
</tr>
<tr>
<td>ArNO</td>
<td>Nitrosamine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BOHB</td>
<td>β-Hydroxybutyrate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C₂₀:₅</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>C₂₂:₆</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>Cupric copper</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper/zinc-containing superoxide dismutase</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450 enzymes</td>
</tr>
<tr>
<td>DETAPAC</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>ECSOD</td>
<td>Extracellular Superoxide Dismutase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>F.I</td>
<td>Fluorescence intensity</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>Ferrous iron</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>Ferric iron</td>
</tr>
<tr>
<td>G/Go</td>
<td>Glucose/ glucose oxidase</td>
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</table>
GP₃  Glutathione peroxidase
GR   Glutathione reductase
GS•  Thiyl radical
GSH  Glutathione (reduced)
GSSG Glutathione (oxidized)
H₂O₂ Hydrogen peroxide
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC High performance liquid chromatography
HRP  Horseradish peroxidase
IARC International Agency for Research on Cancer
IC₅₀ Concentration inhibiting 50% of enzymes
LC₅₀ Concentration causing 50% cell death at 2h
LPS  Lipopolysaccharide
MDA  Malondialdehyde
MMP Mitochondrial membrane permeability
MnSOD Manganese-containing superoxide dismutase
MPO  Myeloperoxidase
NAD⁺ Nicotinamide adenine dinucleotide (oxidized)
NADH Nicotinamine adenine dinucleotide (reduced)
NADP⁺ Nicotinamine adenine dinucleotide phosphate (oxidized)
NADPH Nicotinamine adenine dinucleotide phosphate (reduced)
NAT1/2 N-Acetyltransferase 1/2
NOX  NAD(P)H oxidase
NQO1 NAD(P)H quinone oxidoreductase 1
P450  Cytochrome P450 enzyme
PABA  p-Aminobenzoic acid
PUFAs Polyunsaturated fatty acids
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PUFA•</td>
<td>Polyunsaturated fatty acid radical</td>
</tr>
<tr>
<td>PUFAO₂•</td>
<td>Peroxy radical</td>
</tr>
<tr>
<td>PUFAOOH</td>
<td>Reactive aldehydes from lipid peroxidation</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂•⁻</td>
<td>Superoxide anion radical</td>
</tr>
<tr>
<td>OH’</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>R•</td>
<td>Free radical</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive species</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMPOL</td>
<td>4-hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine 5’-diphospho-glucuronosyltransferase</td>
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</table>
 CHAPTER 1- GENERAL INTRODUCTION

1.1 Project Overview

The purpose of this thesis is to determine the molecular cytotoxic mechanisms of the aromatic amine, 4-aminobiphenyl, with and without the presence of an inflammatory system. In an attempt to define mechanisms of 4-aminobiphenyl-induced cell death, we have combined Accelerated Cytotoxicity Mechanism Screening (ACMS) techniques (Chan et al., 2005; O’Brien, 2006; O’Brien and Siraki, 2005) with and without an in vitro hepatocyte inflammation model to simulate an increase in xenobiotic-induced hepatotoxicity during an episode of subclinical inflammation (Tafazoli, 2008; Tafazoli and O’Brien, 2009). In an effort to further characterize the importance of inflammation in changes in xenobiotic metabolism, our lab has developed a hepatocyte system that is able to mimic the release of H$_2$O$_2$ produced by NADPH oxidase (NOX) and myeloperoxidase (MPO) released from activated immune cells. By assessing various parameters of oxidative stress, I have been able to characterize oxidative-stress mediated toxicity of 4-aminobiphenyl. These methods provide a more robust technique for analyzing the mechanism of 4-aminobiphenyl in inducing cell injury and facilitates the identification of preventative and protective therapies. With the knowledge gained from these data, it is hoped to further define the role of cytotoxicity in induction of cancer. 4-Aminobiphenyl is classified as a group 1 carcinogen that can cause bladder, lung and breast cancer in humans. It is an excellent model toxin for the analysis of the role of oxidative stress- and radical- induced cytotoxicity in relation to carcinogenesis.
### 1.2 Aromatic Amines

Aromatic amines are a group of compounds that are widely known for their highly mutagenic and electrophilic metabolites that covalently bind to nucleophilic cellular macromolecules, such as DNA (Kato, 1986 and O’Brien, 1988). Aromatic amines are also substrates for phase I monooxygenases, namely CYP1A1/2, which results in their bioactivation to cytotoxic N-hydroxylamines (Guengerich et al., 1995). Furthermore, aromatic amine (ArNH₂) metabolites, hydroxylamines (ArNHOH) and nitrosamines (ArNO) have been identified as potent redox-cyclers in intact mitochondria, which lead to the generation of highly reactive superoxide radicals and can subsequently lead to mitochondrial toxicity and cell death (Klöhn et al., 1995). Specifically, electrophilic metabolites of aromatic amines are responsible for inhibiting the mitochondrial electron transport chain, where oxidative phosphorylation is partially uncoupled and the redox pair facilitates the removal of electrons from the respiratory chain (Chan et al., 2005; Neumann, 2007). Additionally, aromatic amine oxidation, catalyzed by myeloperoxidase, generates radicals that are able to cooxidize GSH and NADH, accompanied by oxygen activation. These radicals have been known to dismutate or undergo a subsequent one-electron oxidation to an electrophilic two-electron derivative, which can go on to alkylate DNA (O’Brien, 1988). A skeleton structure of a basic aromatic amine is shown in figure 1 below, along with the structure of the xenobiotic of interest, 4-aminobiphenyl.
1.3 Accelerated Cytotoxicity Mechanism Screening (ACMS)

The Accelerated Cytotoxicity Mechanism Screening (ACMS) method is an important technique for determining the molecular cytotoxic mechanisms of a drug or xenobiotic in an *in vitro* model. This technique is useful in determining the cytotoxic effectiveness of a drug or xenobiotic over a 2-3 hour time period in a freshly isolated Sprague-Dawley rat hepatocyte suspension. A major assumption that needed to be examined using these ACMS techniques was to determine whether a high drug dose over a short time (2-3 hours) *in vitro* simulated the relative amount of hepatotoxicity found using a lower drug dose over a longer period of time (24-48 hours), that is used to measure *in vivo* hepatotoxicity. It was found that the relative order for the toxicity of 16 halobenzenes towards isolated hepatocytes *in vitro* found in 2 hours of exposure was the same as hepatotoxicity found *in vivo* in 24hr (Chan et al., 2005). The ACMS method also allowed us to prioritize the hepatocyte metabolizing enzymes and identify the reactive/unreactive metabolites formed. Furthermore, this method enabled us to use known

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Figure 1. Structure of a basic aromatic amine and 4-aminobiphenyl
metabolizing enzyme inhibitors to prioritize the various drug/xenobiotic metabolizing enzymes involved in the metabolism or activation of a specific drug or xenobiotic by comparing the effects of enzyme inhibitors or activators on cell viability. This functionomic approach was also useful for determining the molecular cytotoxic mechanism e.g., the effects of enzyme inhibitors or substrates on the induction of cytotoxicity caused by the drug or xenobiotic under investigation. Table 1 below outlines various enzyme modulators applied in the ACMS method that enable the identification of bioactivation and detoxification pathways. The ACMS method is used to:

1. Determine the concentration of drug or xenobiotic that caused 50% cell death over a 2 hour time period (LC$_{50}$) using the trypan blue exclusion assay. Cell death is observed as a loss in membrane integrity and hence a decrease in trypan blue exclusion.

2. Examine the cytotoxic effectiveness of a drug or xenobiotic by inhibiting or inducing drug metabolizing enzymes. This will allow for the identification of activating or detoxifying pathways involved in the metabolism or induction of hepatotoxicity induced by the compound in question.

3. Identify which enzyme cofactors, nutrients or antioxidants inhibit drug or xenobiotic-induced cytotoxicity in order to further elucidate the molecular cytotoxic mechanism.

4. Determine the drug or xenobiotic- induced mechanism of hepatotoxicity by measuring changes in bioenergetics (e.g. adenosine triphosphate (ATP) levels and mitochondrial membrane potential), production of reactive oxygen species (ROS) and cellular redox status (oxidized or reduced) by analyzing GSH/GSSG levels, lactate/pyruvate ratios and markers for oxidative stress.
<table>
<thead>
<tr>
<th>Enzyme/ Small Molecule</th>
<th>Inhibitors (-) or Inducers (+)</th>
</tr>
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<tbody>
<tr>
<td>Cytochrome P450s</td>
<td>1- Aminobenzotriazole (-)</td>
</tr>
<tr>
<td>CYP1A1/2</td>
<td>3-Methylcholanthrene (+)</td>
</tr>
<tr>
<td>Catalase</td>
<td>Azide (-)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1-Bromoheptane (-)</td>
</tr>
<tr>
<td>Glucuronidation (UGT)</td>
<td>Borneol (-)</td>
</tr>
<tr>
<td>N-Acetyltransferase</td>
<td>p-Aminobenzoic Acid (-), 2-Bromoacetanilide (-)</td>
</tr>
<tr>
<td>NAD(P)H/Quinone Oxidoreductase</td>
<td>Dicumarol (-)</td>
</tr>
</tbody>
</table>

**Table 1.** Enzyme Modulators used in Accelerated Cytotoxicity Mechanism Screening Techniques

### 1.4 Hepatocytes and Studying Xenobiotic-Induced Toxicity

Isolated hepatocytes are an ideal model for xenobiotic toxicity screening, as the liver is the chief organ responsible for metabolism. Liver parenchymal cells contain an abundance of drug-metabolizing enzymes, which are responsible for removal of a xenobiotic from the system (Guillouzo et al., 1993). While *in vivo* models may be a good method of investigating drug effects in terms of kinetics and systemic parameters, isolated primary hepatocytes are a superior technique for examining molecular mechanisms in a controlled environment (Ulrich et al., 1995). Hepatic metabolism is a complex set of processes, divided into phase I and phase II drug metabolism. Phase I reactions consist mainly of oxidation, reduction and hydrolysis reactions and catalyze the
formation of functional groups required for phase II processes to occur. The endoplasmic reticulum contains cytochrome P450-dependent monoxygenases embedded in its membrane, where oxidation of xenobiotics takes place. Phase II reactions generally involve conjugation reactions carried out by transferases. By modulating hepatic metabolism through phase I and phase II processes, it is possible to determine the bioactivating (toxifying) and detoxifying mechanisms of a xenobiotic. Primary isolated hepatocytes are an excellent model for determining molecular cytotoxic mechanisms, as they are able to retain their regular enzyme activities and are also able to be induced or inhibited by other xenobiotics (Davila and Morris, 1999). Furthermore, in such in vitro studies allow for the prediction of hepatic metabolism and hepatotoxicity of candidate xenobiotics and aid in defining metabolic stability, and drug-drug interactions (Galati and O’Brien, 2004).

1.5 Biochemistry of Free Radicals and Reactive Oxygen Species (ROS)

Free radicals are defined as unstable chemical species, which contain an odd number of electrons (one or more unpaired electrons) and may be neutral, positively or negatively charged (Pryor, 1976). Free radicals are also characterized by their strong electrophilic properties, which allow them to easily react with cellular constituents. These free radicals are usually centered on carbon, nitrogen, sulfur or oxygen atoms. There are two major reaction pathways that are able to generate free radicals: homolytic bond fission or electron- transfer reaction, respectively shown in equation 1 and 2 below (Slater, 1984):
(1) Homolytic Bond Fission \[ A: B \rightarrow A\cdot + B\cdot \]

(2) Electron-Transfer Reactions \[ A^-: + B \rightarrow A\cdot + B^-\cdot \]

Equation (1) seen above can occur through absorption of various types of radiation: ionizing, ultraviolet, visible or thermal. In contrast, reaction (2) can occur through redox reactions, including non-enzymatic electron transfer, metal catalyzed reactions or enzyme-catalyzed processes, all which can occur in biological systems. In addition, oxygen can readily accept electrons from free radicals to form partially reduced, short-lived and highly reactive species. These oxygen free radicals, along with non-radical reactive oxygen species can be produced through many biological redox reactions, some of which are seen below.

(i) Superoxide anion radicals (O$_2$•) are formed through the one electron reduction of oxygen (O$_2$) in equation 3 below:

\[ (3) \quad O_2 + e^- \rightarrow O_2^- \]

(ii) Hydrogen peroxide (H$_2$O$_2$) is considered a non-radical ROS, as it contains no unpaired electrons. It can be produced through several cellular metabolic processes (including immune cell reactions), likely through the reduction of other reactive
oxygen radicals (Pryor, 1976). H₂O₂ ultimately results from the dismutation reaction of O₂•⁻ catalyzed by superoxide dismutase (SOD), with an intermediary hydroperoxyl radical species (HO₂•) in equation 4 and 5:

(4) O₂•⁻ + H⁺ → HO₂•
(5) HO₂• → H₂O₂ + O₂

(iii) The extremely reactive hydroxyl radicals (OH⁻) can be formed from H₂O₂ via the Fenton reaction catalyzed by transition metals, such as Fe²⁺ or Cu²⁺ and is shown in figure 6 below (Chance et al., 1979).

**Fenton Reaction:**

(6) H₂O₂ + Fe²⁺ + H⁺ → OH⁻ + Fe³⁺ + H₂O

Hydroxyl radicals are highly reactive and are considered the most toxic reactive oxygen species. They are so energetic that they are able to react at the same rate as their collision with other molecules, as fast as 10⁹ M⁻¹ sec⁻¹ (Anbar and Neta, 1967). Due to the extremely high rate constant, the OH⁻ radical readily reacts with nearly all cellular components, including: sugars, amino acids, phospholipids, DNA bases and organic acids (Halliwell and Gutteridge, 1984), leading to cell injury as a result of hydroxyl radical catalyzed lipid peroxidation and protein or DNA oxidation. Other reactive species
produced through cellular metabolism and biological redox reactions can also cause tissue damage and cytotoxicity. These non-radical cytotoxic species include: hypochlorous acid, formed from myeloperoxidase/H₂O₂/chloride that can be released from immune cells, fatty acid hydroperoxides and reactive aldehyde species, such as alkoxy and peroxy radicals (Halliwell and Gutteridge, 1985).

1.6 Lipid Peroxidation

The peroxidation of polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (C₂₀:₅) and docosahexaenoic acid (C₂₂:₆), has been implicated in various types of tissue injury and is caused by a free radical-mediated degradation process (Slater, 1984). The process of lipid peroxidation begins with an initiating radical (R•) that starts a series of reactions resulting in the formation of damaging end products, such as the PUFA free radical (PUFA•), which is formed from the abstraction of hydrogen atoms from phospholipid membranes. The reaction with oxygen causes the formation of the peroxy radical (PUFAO₂•), leading to the propagation step, which further degrades the lipid and yields fatty acid hydroperoxides and other reactive aldehydes (PUFAOOH) (Slater 1984; Cheeseman, 1993). This reaction is shown in figure 2 below:
Figure 2. Schematic diagram of the process of lipid peroxidation (Modified from Cheeseman, 1993).
R•: reactive free radical, RH: drug/xenobiotic, PUFA: polyunsaturated fatty acid, PUFA•: lipid radical, PUFAO₂•: lipid peroxy radical.
R• are derived from xenobiotic metabolism stars a chain reaction by abstracting hydrogen atoms from PUFAs, leading to the formation of a PUFA•, which reacts with oxygen to form PUFAO₂• and causes propagation of the chain reaction.

Peroxidation of biological membranes leads to tissue injury by eventually leading to the destruction of membrane integrity and fluidity. Furthermore, endogenous enzymatic processes and functions of subcellular organelles, namely the mitochondria and endoplasmic reticulum, become compromised with lipid peroxidation-induced cell damage (Poli et al., 1987). In addition, the production of lipid peroxidation end products, such as 4-hydroxyalkenals or aldehydes interfere with cellular and enzymatic functions leading to cytotoxic and mutagenic effects (Marnett et al., 1985). These highly reactive substances are also able to react with thiol groups on GSH and amino groups on proteins to form Schiff bases, both compromising cell integrity (Poli et al. 1987).
1.7 Cellular Detoxification Systems

Reactive oxygen species and free radicals are able to cause adverse chemical modifications and damage to lipids, proteins and nucleic acids, which have been associated with the development of various diseases, including cardiovascular disease, neurodegenerative diseases, chronic inflammatory diseases and cancer (Droge, 2002). However, ROS are a natural by-product of biological metabolism and are required for various cell processes including immune function, cellular signaling and gene expression (Kamata and Hirata, 1999). Under normal cell function, there is a well-maintained balance between pro- and anti-oxidant levels. In the instance that the cells endogenous antioxidant capacities cannot cope with an increase in pro-oxidants, oxidative stress ensues. Cells are equipped with endogenous enzymatic and non-enzymatic antioxidant defense systems that protect cells from toxic processes from ROS and radical-induced cellular damage. The antioxidant cellular defense system includes the following small molecules and enzymes and a schematic of detoxification pathways is shown at the end of section 1.7 in figure 4.

(i) Glutathione (L-\(\gamma\)-glutamyl-l-cysteinylglycine; GSH) is a tripeptide and an endogenous thiol, which scavenges ROS and free radicals and detoxifies xenobiotics by conjugating electrophilic metabolites with GSH, by glutathione-S-transferase (Meister, 1995; Hayes and McLellan, 1999). Figure 3 below adapted from O’Brien, 1988, shows GSH reacting with a free radical species. GSH donates a hydrogen atom and is converted to a thyl radical (GS•). This radical can then react with another thyl
radical to form glutathione disulfide (GSSG). GSSG is reduced back to GSH by glutathione reductase, using NADPH as a necessary cofactor.

Figure 3. GSH-mediated detoxification of a peroxidase-generated free radical (Modified from O’Brien, 1988 and Casarett & Doull’s Toxicology, 6th ed., 2001.)

Glutathione peroxidases (GPx) are a group of selenoenzymes located in the cytosol and mitochondria that reduce H$_2$O$_2$ to water. Organic hydroperoxides are reduced to the corresponding alcohol (Ursini et al., 1985; Ng et al., 2007). GPx uses GSH as a cofactor for its reducing equivalents, in order to catalyze the conversion of H$_2$O$_2$ to water, as shown in the reaction below (Ng et al., 2007).

\[
\text{GPx} \quad \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

(ii) Catalase is an enzyme that also detoxifies H$_2$O$_2$ to water and oxygen, but is mainly located in peroxisomes and does not require any cofactors in its catalytic mode (Chance et al., 1979).
(iii) **Superoxide dismutase (SOD)** is one of the most important enzymes involved in free radical detoxification (Fridovich, 1995). Although the major source of superoxide production is controversial, there is evidence suggesting that it is formed from NADPH oxidase and xanthine oxidase. However, other evidence indicates the major site of superoxide production is the mitochondrial electron transport chain, through electron leakage from complexes I and III. It has been suggested that superoxide is formed and released into the matrix side of the inner membrane from complex I, whereas complex III likely generates superoxide in the matrix and intermembrane space (St. Pierre et al., 2002). Due to the highly reactive nature of superoxide, it must be detoxified in the compartment in which it is generated. Due to this necessity, there are three forms of SOD in the cell: manganese-containing SOD (MnSOD), which is located in the mitochondrial matrix, copper and zinc-containing SOD (CuZnSOD), located in the cytosol, extracellular space and mitochondrial inner membrane and lastly extracellular SOD (ECSOD), which is located on the cell membrane and also contains copper and zinc (Fridovich, 1995). The SOD enzyme detoxifies superoxide to hydrogen peroxide, as shown below:

$$4 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$
Figure 4. Schematic of cellular detoxification pathways. (Adapted from Kappus, 1985; Hayes and McLellan 1999).
1.8 Peroxidases

Peroxidases are heme-containing enzymes that oxidize a variety of xenobiotics using hydrogen peroxide. There are a variety of peroxidases found in plants and humans, such as horseradish peroxidase and myeloperoxidase, respectively (O’Brien, 2000). The following cycle of reactions illustrate the oxidation of xenobiotics that are considered peroxidase substrates:

\[
\text{Peroxidase} + \text{ROOH} \rightarrow \text{Compound I} + \text{ROH} \\
\text{Compound I} + \text{XOH} \rightarrow \text{Compound II} + \text{XO}^• \\
\text{Compound II} + \text{XOH} \rightarrow \text{Peroxidase} + \text{XO}^• + \text{H}_2\text{O}
\]

The catalytic activation of peroxidase involves the reaction of the heme iron group, Fe (III) protoporphyrin, with \( \text{H}_2\text{O}_2 \) to give a ferryl porphyrin cation radical. This radical is two oxidation equivalents above the resting ferric state and is represented as compound I. Compound I is reduced by a sequence of one electron transfers from different peroxidase substrates. The first reduction forms compound II, which is the intermediate that is one oxidation equivalent above the ferric state. The second reduction restores the resting ferric state of peroxidase, while the substrates that provided the electrons are oxidized to radical products. Efficient peroxidase substrates typically contain good electron donating groups (O’Brien, 2000; Ozaki and Ortiz de Montellano, 1995).
1.9 Oxidative Stress and Inflammation

Although xenobiotics have a major influence on directly or indirectly generating ROS in the cell, an episode of subclinical inflammation may also contribute to ROS-induced oxidative stress. The inflammatory response has found to be a source of cellular stress by increasing cell susceptibility to drug-induced oxidative stress (O’Brien et al., 2004). Kupffer cells are specialized macrophages that reside in the sinusoidal areas of the liver and become activated through contact between membrane receptors and xenobiotic particles (Wisse et al., 1996; Klaunig et al., 2007). Another key immune cell involved in mediating the hepatic inflammatory process is the neutrophil. Activated Kupffer cells and neutrophils produce large amounts of ROS (O₂•⁻ and H₂O₂) via the phagocyte isoform of the NADPH oxidase (NOX) complex. This considerable and rapid production of ROS is called the “respiratory burst”, which consumes large amounts of oxygen in order to destroy invading microbes. The following reaction illustrates the one-electron reduction of oxygen to superoxide, catalyzed by NADPH oxidase:

\[
2O_2 + 2\text{NADPH} \rightarrow 2\text{NADP}^+ + 2H^+ + 2 O_2\cdot^- \rightarrow \text{H}_2\text{O}_2 + O_2
\]

Generation of H₂O₂ provides an oxidizing substrate for peroxidases contained within the phagosome and plays a key role in host defense mechanisms against microbial pathogens. Furthermore, the O₂•⁻ formed from the “respiratory burst” also establishes the starting materials for the production of free radicals, reactive oxidants and singlet oxygen,
which facilitates the immune response. This process is highly regulated, as the production of these reactive compounds can result in damage to nearby tissues and can also engage in oxidative metabolism of xenobiotics (Babior, 1999). There is also a myriad of evidence suggesting that activated Kupffer cells contribute to the pathogenesis of various liver diseases involving liver injury, especially due to their constant stimulus by bacterial endotoxins from the gut and debris in the sinusoids (Blizer et al., 2006). The inflammatory cell-hepatocyte model for hepatitis is illustrated in the figure 5 below (Modified from Tafazoli and O’Brien, 2005) and outlines the large increase in liver susceptibility to drugs.
Figure 5. Inflammatory cell- hepatocyte model. (Modified from Tafazoli and O’Brien, 2005). Neutrophils and Kupffer cells residing in the liver can release H₂O₂ and myeloperoxidase during an inflammatory response. It has been suggested that this H₂O₂-peroxidase system is able to oxidize drugs/xenobiotics to form reactive pro-oxidant radical, which are toxic to hepatocytes. (See previous text for full description of figure).
1.10 Hypothesis

1. *The molecular cytotoxic mechanisms of 4-aminobiphenyl involve mitochondrial oxidative stress and redox cycling, leading to an increase in cellular ROS production and eventually cell death.*

2. *Exposure to an inflammation model will markedly increase hepatocyte susceptibility to 4-aminobiphenyl-induced cytotoxicity, through radical mechanisms.*

**Figure 6.** Summary of the *in vitro* hepatocyte inflammation model and ACMS hypotheses (H1: hypothesis 1, H2: hypothesis 2).
1.11 Experimental Design

Figure 7. Flowchart of experimental design: Isolated rat hepatocytes were exposed to various conditions. Hepatocyte viability and oxidative stress parameter were examined.
1.12 Organization of Thesis

This thesis is organized as follows, with Chapter 1 as the general introduction, followed by Chapter 2, which is the major data chapter. Chapter 2 consists of an introduction, materials and methods, results and discussion. This section will also be submitted for publication. Chapter 3 compiles the general conclusions and future perspective research from the knowledge gained from the thesis. 4-Aminobiphenyl was selected in order to increase knowledge and further our understanding on how oxidative- or radical- induced cytotoxicity contributes to the induction of cancer.
CHAPTER 2- ACCELERATED CYTOTOXICITY MECHANISM SCREENING OF 4-AMINOBIPHENYL IN A HEPATOCYTE INFLAMMATION MODEL

2.1 Abstract

4-Aminobiphenyl is an aromatic amine compound that is present in cigarette smoke, diesel exhaust, cooking oil fumes and dye intermediates. It is a well-known human bladder carcinogen and liver carcinogen in experimental animals that is metabolically activated by liver CYP1A1/2. We have used the “Accelerated Cytotoxicity Mechanism Screening” (ACMS) techniques to analyze the molecular cytotoxic mechanisms of 4-aminobiphenyl. Hepatocyte exposure to an inflammatory system significantly increased hepatocyte susceptibility to 4-aminobiphenyl. 4-Aminobiphenyl-induced cytotoxicity and lipid peroxidation were both prevented by altering cellular redox status and with the addition of antioxidants. Toxicity was increased with the depletion of hepatocyte GSH levels and by inhibiting N-acetyltransferase. These results will provide more insight into the cytotoxic and genotoxic mechanisms of 4-aminobiphenyl and also suggest that inflammation may be responsible for an increase in arylamine carcinogenesis.
2.2 Introduction

Aromatic amine exposure through occupational hazards, smoke inhalation or the consumption of charred meats is a major public safety concern. 4- Aminobiphenyl is an arylamine present mainly in cigarette sidestream and mainstream smoke at levels ranging from 0.2 ng to 140 ng per cigarette (Hoffman and Hoffman, 1997; Patrianakos and Hoffman, 1979). It is also found in dye intermediates, diesel exhaust and cooking oil fumes (Turesky et al., 2003). This well-known bladder pro-carcinogen is thought to require metabolic activation by CYP1A1/2 in order to exert its mutagenic and DNA binding effects (Kim and Guengerich, 2005). In addition to being oxidized, 4-aminobiphenyl can also undergo N-acetylation (NAT) or glucuronidation, which are both competing pathways of metabolism (Ciotti et al., 1999). NAT2 is considered to be a detoxification pathway of 4-aminobiphenyl by forming an N-acetyl derivative. NAT2 competes with N-hydroxylation- catalyzed cytochrome P450 enzymes (e.g., CYP1A1/2). The N-hydroxy products of 4-aminobiphenyl can further undergo O-acetylation to an unstable N-acetoxy derivative, which has the ability to form a highly mutagenic arylnitrenium ion (Kim and Guengerich, 2005). The N-hydroxylated derivative of 4-aminobiphenyl may also be able to autooxidize to form 4-nitrosobiphenyl. It has been previously demonstrated that metabolites of a structurally similar arylamine to 4-aminobiphenyl, 2-acetylaminofluorene, can redox cycle in the mitochondria, removing electrons from the respiratory chain and forming superoxide (Klöhn et al., 1995; Neumann, 2007). Figure 8 below outlines those possible metabolic pathways of 4-aminobiphenyl.
Figure 8 Metabolic pathways for 4-aminobiphenyl. (Modified from Bendaly et al., 2009; Karreth and Lenk, 1991). See text for a detailed description of figure.
In the following, an attempt has been made to understand the molecular cytotoxic mechanism of 4-aminobiphenyl towards isolated rat hepatocytes by using the technique “Accelerated Cytotoxic Mechanism Screening (ACMS)” (Chan and O’Brien, 2005). Previously our laboratory showed that hepatocyte CYP1A2 metabolically activated 4-aminobiphenyl. Evidence suggesting this route of metabolism was shown using CYP1A2-induced rats (with 3MC), which were two-fold more susceptible to AB compared to un-induced hepatocytes. This increase in cytotoxicity was prevented by the CYP1A2 inhibitor isosafrole. 4-aminobiphenyl added to control hepatocytes also caused hepatocyte GSH depletion and mitochondrial toxicity (Siraki and O’Brien 2002).

Recently, it was also found that hepatotoxic drugs such as isoniazid (Tafazoli et al., 2008), hydralazine (Tafazoli and O’Brien, 2008), amodiaquine (Tafazoli and O’Brien, 2009) and troglitazone (Tafazoli et al., 2005), became markedly more toxic if the hepatocytes are exposed to nontoxic concentrations of peroxidase and/or H₂O₂.

Previously it was found that 4-Aminobiphenyl was oxidized by H₂O₂/peroxidase to form a radical, which can either dimerize to form 4, 4’azobisphenyl (biphenyl), co-oxizide to a GSH-AB conjugate or can also form a glutathionyl free radical product (Eling, 1992). In the following it is shown that in the hepatic inflammation model i.e. a low non-toxic continuous infusion of H₂O₂ in the presence of nontoxic peroxidase levels, (such as could be released by activated immune cells that formed H₂O₂ from the NADPH oxidase “respiratory burst” from infiltrated neutrophils or activated resident Kupffer
cells) exposed hepatocytes were much more susceptible to 4-aminobiphenyl than control hepatocytes.
2.3 Materials and Methods

Chemicals:

Type II collagenase was purchased from Worthington (Lakewood, NJ, USA). All other chemicals and enzymes were purchased from Sigma Aldrich Corp (Oakville, Ont., Canada).

Animal Treatment and Hepatocyte Preparation:

Male Sprague-Dawley rats weighing 275-300 grams were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in ventilated plastic cages over PWI 8-16 hardwood bedding. The environmental temperature was kept at 21- 23°C with a relative humidity of 50- 60%. Twelve air changes occurred per hour and a photoperiod of 12 hours (from 0800h) was maintained. Rats were fed ad libitum with a diet consisting of standard chow and water. Animal care and treatment were in accordance with the Canadian Council of Animal Care guidelines and the University of Toronto Animal Care Committee approved the experimental protocol. Hepatocytes were isolated by collagenase perfusion using methods described by Moldeus et al. (1978). Once isolated, hepatocytes were suspended (10 mL, $10^6$ cells/mL) in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in continually rotating 50 mL round-bottom flasks under an atmosphere of 95% O₂ and 5%
CO₂ in a 37°C water bath. Hepatocytes were allowed to acclimatize for 15 minutes before chemicals were added.

**Cell Viability:**

Hepatocyte cell viability was assessed microscopically by plasma membrane disruption as determined by the Trypan blue (0.1% w/v) exclusion test (Moldeus et al. 1978). Hepatocyte viability was determined immediately after isolation and every hour for three hours. Cell preparations over 80% cell viability were used in experiments.

**Hepatocyte Inflammation Model:**

Hepatocytes susceptibility to hepatotoxin was found to be markedly increased when exposed to the products of activated immune cells such as H₂O₂ generated by their activated NADPH oxidase (Tafazoli et al. 2008; Tafazoli and O’Brien 2008). Direct hepatocyte exposure to H₂O₂ is an inefficient system, as it will take seconds for catalase to metabolize H₂O₂ (Ou and Wolff, 1996). A H₂O₂-generating system (Antunes and Cadenas, 2001) was employed using 10 mM glucose and glucose oxidase (1 unit) to the hepatocyte suspension. This glucose/glucose oxidase system allows for a continuous supply of H₂O₂ to be generated over the experimental period (3h) and does not affect GSH levels.
Measurement of GSH oxidation:

To determine the rate of GSH co-oxidation, the reaction mixture contained 1 mL of 100 mM potassium phosphate buffer (pH 8) with ethylenediamine tetraacetic acid (EDTA), 10 µM substrate (4-aminobiphenyl), 100 µM GSH, 100 µM H₂O₂ and 0.1 µM HRP. 100 µL of 0.01g/mL of 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to each tube at each time point and absorbance was followed over a period of 60 minutes at 412 nm using a Shimadzu UV-240 spectrophotometer.

Mitochondrial Membrane Potential Determination:

Estimating the mitochondrial membrane potential is determined by measuring the uptake and retention of the cationic fluorescent dye, rhodamine 123. This dye selectively accumulates in the mitochondria by facilitated diffusion. When the mitochondrial membrane potential is decreased, facilitated diffusion does not occur and the amount of rhodamine 123 that enters the cell is decreased. Therefore, the amount of rhodamine 123 that remains in the suspension is increased and the amount in the cell pellet is decreased when the mitochondrial membrane is compromised. Samples (500 µL) were removed from the cell suspension and centrifuged at 50xg for 1 minute. The supernatant was discarded and the pellet re-suspended in fresh incubation medium containing 1.5 µL and allowed to incubate for 10 minutes. Suspension was re-centrifuged and the remaining rhodamine 123 in the incubation medium was measured fluorimetrically with an excitation of 490 nm and an emission of 520 nm. The difference in fluorescence intensity
between control and treated cells was used to determine the capacity of the mitochondria to uptake the dye (Andersson et al., 1987)

**Preparation of Enzyme- Inhibited Hepatocytes:**

Glutathione (GSH)-depleted hepatocytes were obtained by pre-incubating hepatocytes with 1-bromoheptane (200 µM) for 30 minutes prior to the start of the experiment. Glucuronidation- inhibited hepatocytes were obtained by incubating the cell with 700 µM borneol (a UGT inhibitor), preincubated for 15 minutes before the addition of the other agents. Catalase inhibition was achieved by incubating with sodium azide (4 mM) for 30 minutes prior to adding other chemicals. Stock solutions of all chemicals were prepared in Millipore water or dimethylsulfoxide (DMSO).

**Hepatocyte Lipid Peroxidation:**

Lipid peroxidation was determined by measuring levels of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides. At various time points, 1mL aliquots of hepatocyte suspension (10^6 cells/mL) were added to test tubes containing 250 µL of TCA (70% w/v), followed by 1 mL of thiobarbituric acid (0.8% w/v) and 750 µL of double distilled water. The suspension was then boiled for 20 minutes and read at an absorption of 532 nm using a Pharmacia Biotech Ultrospec 1000. An extinction coefficient of 156 mM^{-1} cm^{-1} was used to determine the concentration of malondialdehyde produced (Smith et al., 1992).
**Bovine Serum Albumin (BSA) Protein Carbonylation Assay**

BSA (2 mg/ml) was prepared in 100 mM phosphate buffer (pH 7.4). BSA (0.5 ml) was incubated for 1 hour at room temperature with 0.5 ml of 2,4-dinitrophenylhydrazine (DNPH) (0.1% w/v) in 2 N hydrochloric acid. 1 ml of trichloroacetic acid (TCA) (20% w/v) was added to the suspension to stop the reaction. The sample was centrifuged at 50xg to obtain the cellular pellet, and the supernatant was removed. DNPH was removed by extracting the pellet three times using 0.5 ml of ethyl acetate: ethanol (1:1) solution. After the extraction, the pellet was dried under a gentle stream of nitrogen and dissolved in 1 ml of Tris-buffered 8 M guanidine-HCL (pH 7.2). The solubilized hydrazones were measured spectrophotometrically using an extinction coefficient of 22000 M$^{-1}$ cm$^{-1}$ at 374 nm (Hartley et al., 1997).

**Statistical Analysis:**

The statistical significance was determined by performing a one-way analysis of variance (ANOVA), with a post hoc Tukey’s analysis to determine differences between treatments. Values were considered statistically significant when $p < 0.05$. Data are presented as mean ± standard error of the mean (SEM) (n = 3).
2.4 Results

2.4.1 The LC$_{50}$ of 4-aminobiphenyl under various treatments

As shown in Table 2, 4-aminobiphenyl (AB) toxicity towards isolated rat hepatocytes was concentration dependent, with 700 µM AB causing approximately 50% cell death at 2h, (LC$_{50}$) measured using the trypan blue exclusion test. Furthermore, hepatocyte cytochrome P450 1A1/2 induced with 3-methylcholanthrene (CYP-induced) caused a decrease in the LC$_{50}$ to 600 µM (Siraki et al., 2002). The addition of our model H$_2$O$_2$-generating system caused a greater than a two-fold increase in 4-aminobiphenyl cytotoxicity, lowering the concentration to 300 µM at 2 h. Moreover, a 17.5-fold increase in toxicity was observed with the addition of a non-toxic dose of peroxidase to 4-aminobiphenyl and an H$_2$O$_2$-generating system. In order to determine the molecular cytotoxic mechanisms of 4-aminobiphenyl, various compounds and enzyme modulators were added to the cell suspension to determine their role in bioactivation or detoxification, and hence change in cytotoxicity. The LC$_{50}$ of AB, 700 µM, was used as a baseline concentration for the purpose of determining the ability of various compounds to modulate its toxic response. Using a mildly toxic dose of AB, 200 µM, we were able to determine the roles of phase I and phase II enzymes in modulating 4-aminobiphenyl toxicity.
**Table 2.** Concentrations of 4-aminobiphenyl that induce 50% cell death at 2h in isolated rat hepatocytes under different experimental conditions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LC$_{50}$ at 2h</th>
<th>Fold Increase in Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Aminobiphenyl</td>
<td>700 µM</td>
<td>-</td>
</tr>
<tr>
<td>+ CYP-induced</td>
<td>600 µM</td>
<td>1.2 x</td>
</tr>
<tr>
<td>+ H$_2$O$_2$-generating system</td>
<td>300 µM</td>
<td>2.3 x</td>
</tr>
<tr>
<td>+ Peroxidase</td>
<td>40 µM</td>
<td>17.5 x</td>
</tr>
</tbody>
</table>
2.4.2 Altering toxic response of a non-toxic dose of 4-aminobiphenyl using enzyme modulators

As shown in figure 9, hepatocyte incubation with 200 µM 4-aminobiphenyl resulted in a marginal increase in cytotoxicity compared to control, which allowed for the determination of possible detoxification or bioactivation pathways. GSH depletion by bromoheptane resulted in a significant increase in hepatocyte toxicity to 4-aminobiphenyl (Kaderlik et al., 1994; Khan and O’Brien 1991). Furthermore, when hepatocytes were pre-incubated with borneol, a glucuronidation inhibitor, for 30 minutes, a significant increase in cytotoxicity occurred (Gregus et al., 1983; Kretz-Rommel and Boelsterli, 1993). Hepatocyte susceptibility to 200 µM 4-aminobiphenyl also significantly increased hepatocytes within a 30-minute pre-incubation with p-aminobenzoic acid (PABA), an N-acetyltransferase inhibitor. In contrast, the non-selective P450 inhibitor, 1-aminobenzotriazole (Balani et al., 2002) caused no significant change in hepatocyte susceptibility when co-incubated with a non-toxic dose of 4-aminobiphenyl (200 µM).
Figure 9. Modulating 4-aminobiphenyl-induced cytotoxicity in isolated rat hepatocytes. Modulating with glucuronidation, NAT or P450 inhibitors and GSH depletion. Refer to methods section for a description of experiments performed. 1-aminobenzotriazole: P450 inhibitor; bromoheptane: depletes GSH; p-aminobenzoic acid (PABA): NAT inhibitor; borneol: glucuronidation inhibitor all pre-incubated in hepatocyte suspension for 30 minutes prior to the addition of other agents. Means ± SEM for three separate experiments are given. *Significant as compared to control and 4-aminobiphenyl treated hepatocytes (p <0.05).
N.B. Concentrations of enzyme modulators are non-toxic to hepatocytes. Ideal IC$_{50}$ concentrations were used.
2.4.3 Modulating cytotoxicity induced by an LC$_{50}$ dose of 4-aminobiphenyl using enzyme/small molecule inhibitors

As shown in Table 3, pre-incubation with the non-selective P450 inhibitor, 1-aminobenzotriazole caused a significant delay in 4-aminobiphenyl cytotoxicity, suggesting a major role for P450 enzymes in bioactivating 4-aminobiphenyl to a toxic or reactive metabolite. Furthermore, inhibiting the glucuronidation pathway with borneol resulted in a significant increase in hepatocyte susceptibility to 4-aminobiphenyl. Depleting GSH from the cell caused the most rapid increase in cytotoxicity, while 4-aminobiphenyl co-incubation with dicumarol, a selective NAD(P)H/quinone oxidoreductase inhibitor (Preusch et al., 1991; Edwards et al., 1980), ultimately resulted in the highest increase in cytotoxicity. Pre-incubation with 2-bromoacetanilide (NAT inhibitor) also resulted in an increased hepatocyte susceptibility to an LC$_{50}$ dose of 4-aminobiphenyl. Catalase inactivation with azide resulted in an increase in hepatocyte susceptibility to 4-aminobiphenyl, while the direct addition of catalase to the hepatocyte suspension delayed cytotoxicity, suggesting H$_2$O$_2$ may be generated through the metabolism of 4-aminobiphenyl.
Table 3. Modulating 4-aminobiphenyl- induced cytotoxicity with various enzyme inhibitors or with the addition of enzymes.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (% Trypan Blue Uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation time (min)</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>700 µM 4-Aminobiphenyl</td>
<td>46 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM Aminobenzotriazole (CYP-inhibitor)</td>
<td>28 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 700 µM Borneol (glucuronidation inhibitor)</td>
<td>57 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ GSH Depleted Hepatocytes</td>
<td>52 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 20 µM Dicumarol (NQO&lt;sub&gt;1&lt;/sub&gt;inhibitor)</td>
<td>54 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM 2-Bromoacetanilide (NAT inhibitor)</td>
<td>54 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 4 mM Azide (catalase inhibitor)</td>
<td>55 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 0.25 mg/ml Catalase</td>
<td>42 ± 2</td>
</tr>
</tbody>
</table>

Means ± SEM for three separate experiments are given. Hepatocytes were preincubated with 1-bromohexane to deplete endogenous GSH, 1-aminobenzotriazole, P450 inhibitor and PABA, NAT inhibitor, for 30 minutes prior to the addition of other agents. Refer to Experimental Procedures for a description of the experiments performed.

<sup>a</sup>Significant as compared to control (p <0.05).
<sup>b</sup>Significant as compared to 700 µM 4-aminobiphenyl alone (p<0.05).

N.B. Concentrations of enzyme modulators are non-toxic to hepatocytes. Ideal IC<sub>50</sub> concentrations were used.
2.4.4 Modulating cytotoxicity induced by the LC$_{50}$ dose of 4-aminobiphenyl using antioxidants, radical scavengers, metal chelators and redox therapy

Table 4 demonstrates redox therapy or antioxidants modulate hepatocyte susceptibility to the LC$_{50}$ concentration of 4-aminobiphenyl. Ascorbate is a known antioxidant and cofactor for many enzymes and resulted in a significant delay in cytotoxicity when added to the hepatocyte suspension with 4-aminobiphenyl. The vitamin E analogue, trolox, also significantly reversed 4-aminobiphenyl-induced cytotoxicity. TEMPOL, a known ROS scavenger and superoxide dismutase mimic, also protected the hepatocyte suspension from cell death. Scavenging free ferrous iron with desferoxamine protected hepatocytes, likely by not allowing for the Fenton reaction to take place. In addition, redox therapy using the NAD(P)H generator, $\gamma$-hydroxybutyrate (GHB), significantly prevented cytotoxicity, while the addition of fructose allowed for the direct generation of ATP and also significantly protected the hepatocyte against 4-aminobiphenyl-induced cytotoxicity.
Table 4. Preventing 4-aminobiphenyl-induced cytotoxicity with various antioxidants, radical scavengers, chelators and redox therapy.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (% Trypan Blue Uptake)</th>
<th>Incubation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>21 ± 1</td>
</tr>
<tr>
<td>700 µM 4-Aminobiphenyl</td>
<td></td>
<td>46 ± 1</td>
</tr>
<tr>
<td>+ 200 µM Ascorbate</td>
<td></td>
<td>30 ± 1</td>
</tr>
<tr>
<td>+ 200 µM Trolox</td>
<td></td>
<td>30 ± 3</td>
</tr>
<tr>
<td>+ 200 µM TEMPOl</td>
<td></td>
<td>32 ± 1</td>
</tr>
<tr>
<td>+ 10 mM Fructose</td>
<td></td>
<td>28 ± 1</td>
</tr>
<tr>
<td>+ 200 µM Desferoxamine</td>
<td></td>
<td>32 ± 3</td>
</tr>
<tr>
<td>+ 10 mM GHB</td>
<td></td>
<td>27 ± 1</td>
</tr>
</tbody>
</table>

Means ± SE for three separate experiments are given.

*Significant as compared to control (p < 0.05).

*Significant as compared to 700 µM 4-aminobiphenyl alone (p<0.05)
2.4.5 4-Aminobiphenyl- induced mitochondrial membrane potential collapse

As shown in table 5, an increase in fluorescence (FI) units (representing mitochondrial membrane potential) is seen with 250 µM 4-aminobiphenyl, suggesting this dose is toxic to mitochondria. A higher fluorescence intensity denotes a more compromised mitochondrial membrane potential, as the difference in fluorescence intensity between control and treated cells was used to determine the capacity of the mitochondria to uptake the dye. A significant protection resulted with the non-selective P450 inhibitor, 1-aminobenzotraiazole. Additionally, the NAD(P)H generator, GHB, superoxide dismutase mimic, TEMPOL and NAD(P)H/quinone oxidoreductase inhibitor, dicumarol all provided mitochondrial protection. This suggests that 4-aminobiphenyl intermediates from the parent compound might generate reactive species capable of compromising mitochondrial membrane integrity. However, 50 µM 4-aminobiphenyl in an inflammatory system did not lead to mitochondrial toxicity, suggesting an alternate route for toxicity, likely oxidative stress generated through radical-mediated processes.
Table 5. Modulating 4-aminobiphenyl-induced mitochondrial membrane potential collapse by redox therapy, radical scavengers or enzyme inhibitors.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Mitochondrial Membrane Potential (FI Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time (min)</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>495 ± 6</td>
</tr>
<tr>
<td>+ 250 µM 4-Aminobiphenyl</td>
<td>590 ± 33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 700 µM 4-Aminobiphenyl</td>
<td>549 ± 27</td>
</tr>
<tr>
<td>+ 200 µM 1-Aminobenzotriazole</td>
<td>496 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 mM GHB</td>
<td>529 ± 13</td>
</tr>
<tr>
<td>+ 200 µM TEMPOL</td>
<td>512 ± 19</td>
</tr>
<tr>
<td>+ 20 µM Dicumarol</td>
<td>499 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 50 µM 4-Aminobiphenyl</td>
<td>497 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ H_{2}O_{2} generating system + 0.1 µM HRP</td>
<td></td>
</tr>
</tbody>
</table>

1-aminobenzotriazole, a P450 inhibitor, pre-incubated for 30 minutes prior to the addition of 4-aminobiphenyl. H_{2}O_{2} generating system, 10 mM glucose and 1 U/mL glucose oxidase, <sup>b</sup> Hepatocytes were preincubated with horseradish peroxidase (HRP) for 15 minutes prior to the addition of other agents. Means ± SEM for three separate experiments are given. 
<sup>a</sup> Significant as compared to control (p < 0.05)
<sup>b</sup> Significant as compared to 250 µM 4-aminobiphenyl (p < 0.05).
N.B. Concentrations of enzyme modulators are non-toxic to hepatocytes. Ideal IC<sub>50</sub> concentrations were used.
2.4.6 Bioactivation of 4-aminobiphenyl in the hepatocyte inflammation model: effects of antioxidants, radical scavengers and redox therapy

As shown in table 6 the nontoxic H$_2$O$_2$-generating system with peroxidase markedly increased hepatocyte cytotoxicity induced by 50 µM 4-aminobiphenyl. The LC$_{50}$ of 4-aminobiphenyl with the H$_2$O$_2$-generating system and peroxidase is 40 µM (table 2), which represents a 17.5 fold increase in toxicity. The antioxidant ascorbate and ROS scavenger TEMPOL, both significantly delayed cytotoxicity. Trolox is a vitamin E analogue and radical scavenger that resulted in a significant decrease in hepatocyte susceptibility to 4-aminobiphenyl + H$_2$O$_2$ generating system + peroxidase, likely due to its ability to trap radicals. Redox therapy using GHB, a NAD(P)H generator, BOHB, a mitochondrial NADH generator and sorbitol, a glycolytic intermediate and cytosolic NADH generator, all significantly delayed toxicity induced by 4-aminobiphenyl + H$_2$O$_2$ generating system + peroxidase.
**Table 6.** Bioactivation of 4-aminobiphenyl in the hepatocyte inflammation model and prevention with various antioxidants and redox therapy.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (% Trypan Blue Uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation time (min)</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>+ 50 µM 4-Aminobiphenyl</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>+ H₂O₂ generating system + 0.1 µM HRP</td>
<td>64 ± 5&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM Ascorbate</td>
<td>36 ± 3&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM Trolox</td>
<td>37 ± 3&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 mM GHB</td>
<td>32 ± 1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 mM BOHB</td>
<td>27 ± 2&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 mM Sorbitol</td>
<td>39 ± 1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM TEMPOLO</td>
<td>38 ± 2&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>H₂O₂ generating system, 10 mM glucose and 1 U/mL glucose oxidase, b Hepatocytes were preincubated with horseradish peroxidase (HRP) for 15 minutes prior to the addition of other agents. Refer to Experimental Procedures for a description of the experiments performed. Means ± SEM for three separate experiments are given.</sup>

<sup>d Significant as compared to control (p < 0.05).</sup>

<sup>e Significant as compared to 50 µM 4-aminobiphenyl alone (p<0.05).</sup>

<sup>f Significant as compared to 50 µM 4-aminobiphenyl + H₂O₂ generating system + 0.1 µM HRP (p<0.05)</sup>
2.4.7. Bioactivation of 4-aminobiphenyl in the hepatocyte inflammation model: effects of enzyme inhibition

Table 7 demonstrates changes in hepatocyte susceptibility to 4-aminobiphenyl + H₂O₂ generating system + peroxidase when exposed to various enzyme modulators. In contrast to 4-aminobiphenyl alone (table 2), a greater increase in cytotoxicity occurs when hepatocytes are depleted of endogenous GSH, suggesting the formation of a highly reactive intermediate when 4-aminobiphenyl is incubated with a H₂O₂ generating system and peroxidase. Furthermore, inhibiting N-acetyltransferase with PABA resulted in a slight inhibition of hepatocyte toxicity. The non-selective P450 inhibitor 1-aminobenzotriazole also resulted in a delay in 4-aminobiphenyl toxicity in an inflammatory system. These data suggest a shift in detoxification metabolism of 4-aminobiphenyl in an inflammatory environment, compared to 4-aminobiphenyl alone. N-acetylation of the peroxidase-catalyzed intermediate may be toxifying, in contrast to 4-aminobiphenyl under normal metabolic conditions.
Table 7. Bioactivation of 4-aminobiphenyl in a hepatocyte inflammation model and modulating cytotoxicity with enzyme inhibitors.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cytotoxicity (% Trypan Blue Uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation time (min) 60 120 180</td>
</tr>
<tr>
<td>Control</td>
<td>25 ± 1 28 ± 1 33 ± 2</td>
</tr>
<tr>
<td>+ 50 μM 4-Aminobiphenyl</td>
<td>33 ± 1 37 ± 2 39 ± 1</td>
</tr>
<tr>
<td>+ H₂O₂ generating system(^a) + 0.1 μM HRP(^b)</td>
<td>64 ± 5(^{d,e}) 81 ± 3(^{d,e}) 84 ± 2(^{d,e})</td>
</tr>
<tr>
<td>+ GSH- Depleted Hepatocytes(^c)</td>
<td>59 ± 1(^{d,e}) 83 ± 4(^{d,e}) 93 ± 1(^{d,e})</td>
</tr>
<tr>
<td>+ 2 mM PABA(^c) (NAT inhibitor)</td>
<td>47 ± 3(^d) 68 ± 1(^d) 73 ± 1(^d)</td>
</tr>
<tr>
<td>+ 200 μM 1-Aminobenzotriazole(^c) (CYP inhibitor)</td>
<td>44 ± 1(^f) 51 ± 1(^f) 51 ± 1(^f)</td>
</tr>
</tbody>
</table>

\(^a\)H₂O₂ generating system, 10 mM glucose and 1 U/mL glucose oxidase, \(^b\)Hepatocytes were preincubated with horseradish peroxidase (HRP) for 15 minutes prior to the addition of other agents. \(^c\)Hepatocytes were preincubated with 1-bromoheptane to deplete endogenous GSH, 1-aminobenzotriazole, P450 inhibitor and PABA, NAT inhibitor, for 30 minutes prior to the addition of other agents. Refer to Experimental Procedures for a description of the experiments performed. Means ± SEM for three separate experiments are given.

\(^d\)Significant as compared to control (p < 0.05).
\(^e\)Significant as compared to 50 μM 4-aminobiphenyl alone (p<0.05).
\(^f\)Significant as compared to 50 μM 4-aminobiphenyl + H₂O₂ generating system + 0.1 μM HRP (p<0.05).

N.B. Concentrations of enzyme modulators are non-toxic to hepatocytes. Ideal IC\(_{50}\) concentrations were used.
2.4.8 Lipid peroxidation induced by 4-aminobiphenyl in the hepatocyte inflammation model

Table 8 demonstrates lipid peroxidation levels induced by 4-aminobiphenyl. 4-aminobiphenyl along or with the addition of the H$_2$O$_2$-generating system without peroxidase did not cause a significant increase in thiobarbituric acid reactive substances (TBARS) levels, represented by the concentration of malondialdehyde (MDA). However, hepatocyte lipid peroxidation was markedly increased by the addition of the nontoxic H$_2$O$_2$-generating system with peroxidase (HRP). Ascorbate, an antioxidant and trolox, a radical scavenger, both significantly protected against lipid peroxidation induced by 4-aminobiphenyl + H$_2$O$_2$ generating system + peroxidase. Furthermore, the superoxide scavenger TEMPOL also significantly protected against lipid peroxidation. GHB, a NAD(P)H generator and desferoxamine, an iron chelator significantly protected against lipid peroxidation, while the direct addition of catalase had little effect on lipid peroxidation levels.
Table 8. Lipid peroxidation induced by 4-aminobiphenyl in the hepatocyte inflammation model.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Lipid Peroxidation (µM MDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time (min)</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td>+ 250 µM 4-Aminobiphenyl</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>+ H₂O₂ generating system&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>+ 50 µM 4-Aminobiphenyl</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>+ H₂O₂ generating system + 0.1 µM HRP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99 ± 0.09&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM Ascorbate</td>
<td>0.49 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM Trolox</td>
<td>0.45 ± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM TEMPOL</td>
<td>0.43 ± 0.06&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 mM GHB</td>
<td>0.49 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 200 µM Desferoxamine</td>
<td>0.57 ± 0.08&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 0.25 mg/ml Catalase</td>
<td>0.85 ± 0.03&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> H₂O₂ generating system, 10 mM glucose and 1 U/mL glucose oxidase, <sup>b</sup> Hepatocytes were preincubated with horseradish peroxidase (HRP) for 15 minutes prior to the addition of other agents. Refer to the Experimental Procedures for a description of the experiments performed. Means ± SEM for three separate experiments are given.
<sup>c</sup> Significant as compared to control (p <0.05).
<sup>d</sup> Significant as compared to 50 µM alone (p <0.05).
<sup>e</sup> Significant as compared to 50 µM 4-Aminobiphenyl + H₂O₂ generating system + 0.1 µM HRP (p <0.05).
2.4.9 Protein carbonylation induced by 4-aminobiphenyl in a cell- free inflammation system

Figure 10 outlines the extent of protein carbonylation cell free system containing 2mg/ml bovine serum albumin (BSA). A dose of 1 mM 4-aminobiphenyl in this cell-free system (greater than the LC₅₀ in hepatocytes, which is 700 µM alone) resulted in little or no protein carbonylation. Additionally, a treatment of or H₂O₂ + peroxidase alone did not affect protein carbonyl levels in a cell free system. However, BSA protein carbonylation was markedly increased with the addition of the H₂O₂-generating system with peroxidase (inflammatory system), when co-incubated with 4-aminobiphenyl. Protein carbonylation was significantly delayed with the addition of the ROS scavenger/superoxide dismutase mimic TEMPOL, antioxidant ascorbate and radical scavenger, trolox, suggesting a role for an initiating radical generating reactive protein carbonyls.
Figure 10: Modulating 4-aminobiphenyl + H$_2$O$_2$-generating system + HRP- induced BSA protein carbonylation. Trolox: an antioxidant; ascorbate: a radical scavenger and TEMPOL: a superoxide dismutase mimic. Means ± SEM for three separate experiments are given. *Significant as compared to control (p < 0.05). **Significant as compared to AB 1 mM + HRP 0.5 µM + H$_2$O$_2$ 1 mM.

*Work in collaboration with Cyndi Ngo
2.4.10 Glutathione depletion induced by 4-aminobiphenyl in a cell-free inflammation system

Figure 11 demonstrates the rate of glutathione oxidation by radical mediated processes, induced by 4-aminobiphenyl in a cell-free inflammatory system. Reduced glutathione (GSH) is read at an absorbance of 412 nm. A slight decrease in GSH levels was observed with 4-aminobiphenyl alone, while peroxidase + H₂O₂ alone added to a cuvette containing GSH, did not cause any GSH oxidation. However, when 50 µM 4-aminobiphenyl + peroxidase + H₂O₂ were added to the system, the radical formed directly caused GSH depletion via direct conjugation or oxidation.
Figure 11: GSH depletion: 4-aminobiphenyl in a cell-free inflammation system. Means ± SEM for three separate experiments are given. *Significant as compared to control (p <0.05).
2.5 Discussion

In the present study, we have assessed whether cytotoxicity could precede or contribute to the development of carcinogenesis by assessing various parameters of oxidative stress and hepatocyte susceptibility to the aromatic amine, 4-aminobiphenyl. The formation of DNA adducts is thought to be the major cause of aromatic amine-induced carcinogenesis. Carcinogenic aromatic amines generally produce tumors in specific target tissues (Neumann, 2007). The highly carcinogenic aromatic amine, 4-aminobiphenyl, is considered a group 1 carcinogen by the International Agency for Research on Cancer (IARC) and has been known to cause bladder, lung and breast cancer in humans, while 4-aminobiphenyl administration produces hepatocellular and bladder carcinomas in mice and bladder carcinomas in rats (IARC, 1972). The formation of 4-aminobiphenyl-induced hepatic tumors in experimental animals is consistent with the detection of hemoglobin and DNA adducts, suggesting a strong link between 4-aminobiphenyl and carcinogenesis (Sarkar et al., 2006; King et al., 1976). A structurally similar aromatic amine, 2-acetylaminofluorene also produces liver tumors in rats. This compound is thought to have both genotoxic and non-genotoxic events contributing to its carcinogenicity, with its cytotoxic effects being of particular importance (Neumann, 2007). The cytotoxic effects of 2-acetylaminofluorene are similar to 4-aminobiphenyl, as they share analogous metabolic pathways. ACMS techniques allow for the assessment of different parameters of oxidative stress and toxicity that may contribute to carcinogenesis (Siraki and O’Brien, 2004).
The present study has assessed the concentrations of 4-aminobiphenyl that are able to cause 50% cell death at 2 hours and the effects of introducing continuous, non-toxic concentrations of H$_2$O$_2$, with and without peroxidase to the hepatocyte suspension. These conditions were introduced in an effort to further characterize the importance of NADPH oxidase (NOX) in the phagosome of inflammatory cells for forming H$_2$O$_2$ and releasing myeloperoxidase from the azurophil granules of inflammatory cells. Both H$_2$O$_2$, and/or peroxidase markedly increased 4-aminobiphenyl cytotoxicity as well as the metabolism of other carcinogenic aromatic amines (O’Brien, 1988). We have demonstrated that the LC$_{50}$ of 4-aminobiphenyl in normal hepatocytes is 700 µM. By inducing hepatic cytochrome P450, namely CYP1A2, cell viability was compromised at a dose of 600 µM 4-aminobiphenyl (Siraki et al, 2002).

There was also evidence suggesting that N-hydroxylation by CYP1A2 was the essential step in 4-aminobiphenyl bioactivation, leading to the formation of cytotoxic and carcinogenic intermediates (Vineis et al, 1990). We have also demonstrated that 4-aminobiphenyl-induced hepatocyte toxicity was increased greater than two-fold in the presence of a continuous, non-toxic infusion of H$_2$O$_2$ using glucose and glucose oxidase. We have previously reported a marked increase in the toxicity of various drugs when hepatocytes were exposed to this H$_2$O$_2$-generating system at doses that did not affect cell viability or endogenous glutathione levels (Tafazoli and O’Brien, 2005). This suggests that the drug metabolites formed could compromise hepatocyte resistance to H$_2$O$_2$. Additionally in this study, we have demonstrated a 17.5-fold increase in cytotoxicity when horseradish peroxidase (HRP) was added to the system, decreasing the LC$_{50}$ to 40
µM 4-aminobiphenyl. The one-electron peroxidative oxidation of 4-aminobiphenyl by HRP leads to the formation of a free-radical metabolite capable of forming reactive oxygen species, oxidizing GSH and forming DNA adducts (Hughes et al, 1992; O’Brien, 1991).

Cytochrome P450 inhibited hepatocytes with 1-aminobenzotriazole incubated with a non-toxic dose of 4-aminobiphenyl (200 µM), caused no change in cytotoxicity compared to control hepatocytes. In contrast, hepatocytes became increasingly more susceptible to cytotoxicity with that same dose of 4-aminobiphenyl when phase II detoxification pathways were inhibited. Specifically, inhibiting glucuronidation and N-acetylation with p-aminobenzoic acid (PABA) both significantly increased hepatocyte vulnerability to a non-toxic dose of 4-aminobiphenyl. Inhibitors of N-glucuronidation resulted in inactivation of 4-aminobiphenyl, which leads to excretion (Dutton, 1980). This pathway also competes with the toxifying N-hydroxylation (CYP1A2) pathway, which can explain why inhibition of glucuronidation shunts 4-aminobiphenyl metabolism to form toxic N-hydroxylated metabolites. N-acetyltransferase 2 catalyzed N-acetylation of 4-aminobiphenyl, which is also considered a detoxification pathway that competes with P450 catalyzed N-hydroxylation (Bendalay et al., 2009). Inhibiting the N-acetylation pathway could also be attributed to the substantial increase in hepatocyte susceptibility to 4-aminobiphenyl-induced cytotoxicity. Furthermore, reactive metabolites formed by 4-aminobiphenyl metabolism likely conjugated with GSH, as hepatocytes depleted of GSH were significantly more susceptible to 4-aminobiphenyl toxicity (figure 11).
It has also been demonstrated how various enzyme modulators can affect hepatocyte susceptibility to 4-aminobiphenyl. The non-selective P450 inhibitor, 1-aminobenzotriazole caused a significant protection against 4-aminophenyl-induced hepatocyte toxicity, using the LC$_{50}$ dose with and without peroxidase. The CYP1A2 catalyzed oxidation of 4-aminobiphenyl to an N-hydroxy intermediate is the most important step in assessing genotoxic potential and is considered an ultimate carcinogen (Sabbioni, 1993). Furthermore, HPLC analysis of 4-aminobiphenyl metabolism in isolated rat hepatocytes indicated major metabolites being N-hydroxy 4-aminobiphenyl, 4-nitroso biphenyl and various ring hydroxylated metabolites of 4-aminobiphenyl (Jatoe and Gorrod, 1987). By inhibiting P450 oxidation, reactive intermediates cannot be formed and the parent compound can be eliminated via detoxifying pathways. The addition of catalase to the LC$_{50}$ dose of 4-aminobiphenyl, 700 µM, caused a decrease in cytotoxicity, whilst inhibiting endogenous catalase with azide significantly increased hepatocyte susceptibility to 4-aminobiphenyl. This is consistent with the findings by Wang et al, 2006, as the addition of catalase to their system caused a decrease in DNA damage induced by 4-aminobiphenyl, indicating H$_2$O$_2$ involvement in 4-aminobiphenyl-induced DNA damage.

The two electron reduction pathway of NAD(P)H quinone oxidoreductase (NQO$_1$) was considered to be a detoxification pathway, as inactivating NQO$_1$ with dicumarol, caused the most significant increase in hepatocyte susceptibility to 4-aminobiphenyl toxicity. This indicates that a quinone could contribute to the cytotoxic mechanism of 4-aminobiphenyl. When this enzyme is inhibited, the cell loses the ability
to reduce the quinone intermediate back to 4-aminobiphenyl. Previously, we have shown that NQO₁ is responsible for hepatocyte protection against quinone toxicity (Sood et al., 1997; Pourahmad et al., 2008). By inhibiting this enzyme, a marked increase in GSH oxidation was observed, likely by direct GSSG formation by the quinone intermediate (Pourahmad et al., 2008). NAD(P)H generators, such as γ-hydroxybutyrate (GHB) significantly prevented 4-aminobiphenyl- induced hepatocyte toxicity, likely by activating or re-generating NQO₁ and allowing for the reduction of the quinone intermediate.

The inhibition of mitochondrial respiration is an important molecular mechanism of toxicity and lethality of many xenobiotics and their metabolites. Antioxidants and ROS/radical scavengers all protected against 4-aminobiphenyl- induced toxicity, indicating the generation of oxidative stress through 4-aminobiphenyl metabolism. A non-toxic dose of 4-aminobiphenyl, 250 µM, and the LC₅₀ dose, 700 µM, both caused significant mitochondrial toxicity. TEMPOL, a superoxide dismutase mimic protected against mitochondrial toxicity induced by 4-aminobiphenyl, suggesting that the collapse in mitochondrial membrane potential was ROS-mediated. The N-hydroxylated and nitroso metabolites of 4-aminobiphenyl may act as a redox couple and remove electrons from the mitochondrial respiratory chain (Neumann, 2007). This redox cycling can generate reactive oxygen, namely the superoxide anion radical (O₂⁻), causing mitochondrial oxidative stress (Klöhn et al., 1995). TEMPOL is able to scavenge the superoxide produced from the redox cycle and restore mitochondrial membrane potential. 1-Aminobenzotriazole, a general P450 inhibitor, acts to prevent the formation of N-
hydroxy 4-aminobiphenyl from the parent compound, not allowing it to engage in redox cycling, and thus protecting the integrity of the mitochondrial membrane. When hepatocytes were exposed to a non-toxic dose of 4-aminobiphenyl, 50 µM, in an inflammatory system, mitochondrial toxicity did not ensue and was likely not the cause of cell death.

Peroxidases are able to catalyze one- or two-electron oxidations of organic substrates to form reactive radicals or oxidized electrophilic intermediates such as quinoneimines, dimines or nitrenium cation radicals (Gorlewska-Roberts et al., 2004;). Covalent interaction of these electrophiles with a nucleophilic region of a cellular macromolecule is thought to be the basis for initiating carcinogenesis. However evidence supporting free-radical mediated covalent or non-covalent DNA damage has become widely accepted as an important mechanism of aromatic amine-induced carcinogenesis. Furthermore, radicals formed from 4-aminobiphenyl metabolism are able to react with oxygen and form reactive oxygen species, which are able to cause further damage to lipids, proteins and DNA (O’Brien, 1985).

The cytotoxicity of 50 µM 4-aminobiphenyl + inflammatory system was likely directly radical-mediated, and resulted from oxygen activation, lipid peroxidation and GSH oxidation in the hepatocyte (O’Brien 1988). Ascorbate is a potent reducing agent for oxygen-, nitrogen- and sulfur-centered radicals and was able to significantly protect the hepatocyte from 4-aminobiphenyl-induced toxicity and lipid peroxidation in an
inflammatory system (Niki, 1999). Ascorbate is also able to scavenge the superoxide radical, which may have been produced via radical-mediated oxygen activation and contributed to cell death. Trolox, the vitamin E analogue may have played a role in preventing 4-aminobiphenyl radical-induced cytotoxicity and lipid peroxidation by acting as a chain-breaker and inhibiting the propagation step. Lipid peroxidation end products, such as malondialdehyde (MDA), are considered to be highly mutagenic and could contribute to the carcinogenic capacities of 4-aminobiphenyl. MDA is able to react with DNA to form deoxyguanosine and deoxyadenosine adducts (Marnett, 1999).

Altering the cellular redox state using NAD(P)H generating compounds also delayed cytotoxicity and lipid peroxidation under inflammatory conditions. γ-Hydroxybutyrate (GHB), β-hydroxybutyrate (BOHB) and sorbitol all provide reducing equivalents to the respiratory chain in the form of NADH to maintain normal cell homeostasis. Nutrients that are able to generate NADPH are of particular importance during oxidative stress and inflammation, as NADPH provides the reducing potential for most antioxidant and redox regulatory enzymes, including glutathione (Holmgren, 1989). Hepatocyte toxicity induced by 4-aminobiphenyl with and without peroxidase was prevented using the NADPH-generating compound, GHB (Kaufman and Nelson, 1991).

Using the LC₅₀ of 4-aminobiphenyl (50 µM) in an inflammatory system, we have also demonstrated how various enzyme modulators can affect hepatocyte toxicity. Consistent with 4-aminobiphenyl in the absence of peroxidase, inhibiting cytochrome
P450s with 1-aminobenzotriazole significantly delayed cytotoxicity. This suggests that a peroxidase-catalyzed reaction can occur from an N-hydroxy intermediate, leading to the formation of a highly reactive radical product. Glutathione is fundamental for protecting the cell against oxidative stress and can play a key role in detoxification of free radicals (Hayes and McLellan, 1999). Depleting the hepatocyte of endogenous GSH levels beforehand caused a significant increase in 4-aminobiphenyl-induced cytotoxicity, as the cell became more susceptible to radical-mediated damage. Glutathione oxidation was also demonstrated in this study, verifying the role for the small molecule in scavenging the reactive metabolite of 4-aminobiphenyl formed from peroxidase oxidation. In contrast to 4-aminobiphenyl-induced cytotoxicity in the absence of peroxidase, inhibiting N-acetylation actually delayed cytotoxicity of 50 µM 4-aminobiphenyl in an inflammatory system. The N-hydroxy intermediates from P450 or peroxidase metabolism may be subsequently O-acetylated by NAT2, forming a highly toxic N-acetoxy derivative, which is why protection is seen with the inhibition of acetylation (Culp et al., 1997; Bendaly et al, 2009). This suggests that the molecular mechanism of 4-aminobiphenyl-induced cytotoxic pathways differ in the presence of inflammation, which is outlined in figure 12.

In addition, the extent of cell-free protein carbonylation induced by 4-aminobiphenyl + H₂O₂/peroxidase was also examined to correlate with 4-aminobiphenyl binding to other nucleophilic cell components, such as DNA. Measuring protein carbonyl content is considered an excellent marker of severe oxidative protein damage, which can also contribute to the development of chronic diseases, such as cancer (Dalle-Donne, 2003). 1 mM of 4-aminobiphenyl alone did not cause any protein carbonylation.
However, a significant amount of protein carbonylation was noted when non-toxic concentrations of H\textsubscript{2}O\textsubscript{2} and peroxidase were present. Protein carbonylation was almost completely reversed with the addition of antioxidants and radical scavengers, namely trolox, ascorbate and TEMPOL, confirming a radial-mediated mechanism of protein oxidation.
Figure 12- Different routes of 4-aminobiphenyl metabolism (P450 bioactivation vs. peroxidase)
CHAPTER 3- GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

3.1. Hypotheses Revisited

**Hypothesis 1:** The molecular cytotoxic mechanisms of 4-aminobiphenyl involve mitochondrial oxidative stress and redox cycling, leading to an increase in cellular ROS production and eventually cell death.

The aromatic amine, 4-aminobiphenyl, has been identified as the major tobacco carcinogen, which causes mutagenicity, DNA damage, and likely initiates carcinogenesis. It has been hypothesized that inflammation, oxidative stress and the oxidation of other macromolecules contribute to carcinogenesis. However it is still not known whether 4-aminobiphenyl causes oxidative stress cytotoxicity and collapse of mitochondrial membrane potential.

In this study, I have investigated hepatocyte susceptibility to 4-aminobiphenyl-induced cytotoxicity and oxidative stress parameters. This allowed for the elucidation of the molecular cytotoxic mechanisms of 4-aminobiphenyl in an isolated rat hepatocyte model. By modulating various drug metabolizing enzymes, evidence supporting a P450-catalyzed bioactivation pathway was established as a major toxifying route. The N-hydroxylated derivative of 4-aminobiphenyl caused a significant loss in mitochondrial membrane potential and dramatically affected cell viability. This was confirmed with the inhibition of P450 metabolism, it resulted in a decrease in cell death and protected against mitochondrial membrane potential collapse. This mitochondrial collapse was attributed
to the N-hydroxylated derivative of 4-aminobiphenyl formed by P450 metabolism, while general P450 inhibitors prevented mitochondrial toxicity.

Furthermore, TEMPOL, a superoxide dismutase mimic, likely protected against mitochondrial damage by detoxifying the superoxide produced from 4-aminobiphenyl redox cycling in the mitochondria. Surprisingly, restoring redox homeostasis with GHB best protected against 4-aminobiphenyl-induced cytotoxicity, as GHB is able to regenerate NADPH (Kaufman and Nelson, 1991). Thus, reducing equivalents were provided to help regenerate GSH (Holmgren, 1989). GSH is essential to protect against electrophilic and ROS-mediated cell damage caused by 4-aminobiphenyl metabolites. In addition to GSH, glucuronidation, NAT and NQO₁ detoxification were all important in detoxifying various toxic metabolites produced from 4-aminobiphenyl metabolism. Inhibiting catalase-mediated H₂O₂ detoxification with azide also increased hepatocyte vulnerability to 4-aminobiphenyl and indicated that oxidative stress contributed to cell death. The production of H₂O₂ could have resulted from redox cycling and inhibition of mitochondrial respiration by 4-aminobiphenyl metabolites. The oxidative stress induced by 4-aminobiphenyl is an ideal environment for initiating DNA damage and carcinogenesis. DNA mutation through oxidation is a critical step in initiating carcinogenesis (Valko et al., 2006).
**Hypothesis 2:** Exposure to an inflammation model will markedly increase hepatocyte susceptibility to 4-aminobiphenyl-induced cytotoxicity, through radical mechanisms.

Oxidative stress has been shown to act as a general tumor promoter (Hui, 2009). 4-aminobiphenyl induced oxidative DNA damage in tumor cells and reactive oxygen species were formed (Wang et al., 2006). However, protein, lipid oxidation and oxidative stress were not measured. Peroxidase/H\textsubscript{2}O\textsubscript{2} in an *in vitro* cell free system oxidized 4-aminobiphenyl (Hughes et al., 1992) but no cellular systems were tested.

As described in the introduction, our lab has previously shown that hepatocyte vulnerability to xenobiotics may increase in the presence of an inflammatory response. Hepatocyte susceptibility to 4-aminobiphenyl-induced cytotoxicity and oxidative stress were examined in the presence of peroxidase and H\textsubscript{2}O\textsubscript{2} to simulate conditions of subclinical inflammation. Specifically, our system simulated the release of myeloperoxidase from activated Kupffer cells and H\textsubscript{2}O\textsubscript{2} formed by the activation of NADPH oxidase by infiltrated neutrophils during the inflammatory response in the liver. It has also been demonstrated that inactivating neutrophils and Kupffer cells can prevent drug-induced hepatotoxicity (Ganey and Roth, 2001; Roth et al., 1997).

This suggests that the large increase in hepatocyte toxicity to 4-aminobiphenyl could be attributed to the H\textsubscript{2}O\textsubscript{2} and peroxidases released by activated immune cells that
can catalyze the oxidation of xenobiotics and their cytochrome P450 metabolites. In turn, this one-electron oxidation can form reactive pro-oxidant radicals that can cause lipid peroxidation, protein carbonylation and cytotoxicity. Furthermore, a decrease in 4-aminobiphenyl-induced cytotoxicity was observed when P450 metabolism was inhibited with 1-aminobenzotriazole, suggesting a toxic radical product could have been formed from a P450 metabolite. Hepatocyte treatment with antioxidants and radical scavengers, ascorbate, trolox and TEMPOL all resulted in a decrease in 4-aminobiphenyl-induced cytotoxicity (table 6), lipid peroxidation (table 8) and protein carbonylation (figure 10), further supporting the proposed radical mediated cell damage in the presence of an inflammatory response.

3.2 Conclusion

In summary, it has been established that the molecular cytotoxic mechanism of 4-aminobiphenyl differs with the presence of non-toxic concentrations of H$_2$O$_2$ and peroxidase. Hepatocyte susceptibility to 4-aminobiphenyl greatly increased under conditions simulating an episode of subclinical inflammation. However, oxidative stress is the underlying factor for 4-aminobiphenyl-induced cytotoxicity, with and without the presence of inflammation. Oxidative stress (radical- or ROS-mediated) induced by 4-aminobiphenyl via lipid peroxidation, protein carbonylation and collapse of the mitochondrial membrane potential causes a state of redox imbalance that may stimulate a carcinogenic environment, especially in the presence of inflammation.
3.3 Future Perspectives

The hepatocyte inflammation model developed by the O’Brien lab provides a more robust drug/xenobiotic screening method to compliment our traditional ACMS techniques. This model allows for the analysis of the molecular cytotoxic potential of drugs/xenobiotics in an \textit{in vitro} system. However, as is the issue with other \textit{in vitro} systems, it is difficult to correlate toxicity with \textit{in vivo} conditions. Developing an animal system to mimic conditions in isolated rat hepatocytes may further clarify mechanisms and consequences of toxicity. Triggering a mild inflammatory response in an animal and then introducing the drug/xenobiotic in question may be a good technique. Lipopolysaccharide (LPS), or bacterial endotoxin, at smaller doses may activate an inflammatory response and influence response and metabolism of hepatotoxins (Sneed et al., 1997). It would be valuable to determine changes in liver morphology to determine extent of damage to the tissues in an \textit{in vivo} scenario and correlate it back to \textit{in vitro} findings.

In the case of 4-aminobiphenyl, the aforementioned \textit{in vivo} proposal may be effective at correlating cytotoxicity to carcinogenesis. Analyzing DNA adduct formation and levels of oxidative DNA damage could be useful in determining mutagenic potential of 4-aminobiphenyl in an \textit{in vivo} inflammatory system in a more chronic context. Furthermore, performing \textit{in vitro} experiments, such as lipid peroxidation and protein carbonylation on LPS-induced and mildly inflamed hepatocytes may provide evidence to strengthen the oxidative stress/ radical-mediated hypothesis of 4-aminobiphenyl.
By expanding our knowledge from *in vitro* to *in vivo* systems, it will allow for stronger evidence to determine mechanisms of cytotoxicity and liver damage and their potential link to initiating carcinogenesis.

3.4 Limitations

Although ACMS techniques are effective at predicting the molecular cytotoxic mechanisms of xenobiotics, these methods only permit for the study of acute toxicity. Primary isolated rat hepatocytes only remain viable for a few hours and are quite sensitive and dependent on proper isolation practices. The isolation methods described by Moldeus et al. (1978) that are currently used in the O’Brien lab involves multiple steps, which may theoretically cause mechanical disruptions to hepatocytes and affect cell viability. Furthermore, concentrations of the xenobiotic in question are generally much higher in the isolated hepatocyte system and are do not correlate with therapeutic concentrations or chronic levels of exposure. In addition, enzyme modulators used in the present study, although non-toxic to hepatocytes and at an inhibitory concentration, they may not be “clean” and could possibly interfere with other cell functions. That being said, every experimental system has specific limitations, however our system has proven to be effective at predicting molecular cytotoxic mechanisms of drugs/xenobiotics.
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