2005

Attenuation of bromobenzene-induced hepatotoxicity by poly(adp-ribose) polymerase inhibitors

Kelly Waggoner Hall
University of South Florida

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Attenuation of Bromobenzene-Induced Hepatotoxicity by Poly(ADP-ribose) Polymerase Inhibitors

by

Kelly Waggoner Hall

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Environmental and Occupational Health College of Public Health University of South Florida

Major Professor: Raymond D. Harbison, Ph.D.
Ira S. Richards, Ph.D.
Philip P. Roets, Sc.D.
Skai W. Schwartz, Ph.D.

Date of Approval: July 15, 2005

Keywords: poly(ADP-ribose) polymerase, nicotinamide, 6(5H)-phenanthridone, Bromobenzene, hepatotoxicity

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DEDICATION

In memory of my mother, Earliene Waggoner, who died of a degenerative neurological disorder. Research will find the cause and cure, so others will not have to suffer.
ACKNOWLEDGEMENTS

I would like to thank each of my committee members for their support through this endeavor. First I would like to thank my major professor, Dr. Raymond D. Harbison, for his guidance and motivation throughout this project. Without his support, this project would never have seen a successful end. I feel fortunate to have had such a dedicated individual as my major professor and mentor.

I would like to thank Dr. Ira Richards. His tough questions and courses have led me to study areas that have helped me understand what I needed to. Many thanks to Dr. Phil Roets for his guidance through my masters degree and for being a great professor in many courses that I took. Thanks also go to Dr. Skai Schwartz for helping me understand Epidemiology, and learning to think outside the box.

Many other individuals have helped me throughout this project. I would like to thank Dr. Carlos Muro-Cacho for taking time out of his very busy schedule to assess my pathology slides. To Drs. Vasyl Sava and Adriana Velasquez, thank you lending your equipment and expertise in fluorescence work. I would also like to thank my fellow doctoral candidates and students in the EOH department for their support and encouragement: Paul Grivas, Robin DeHate, Scott Dotson and Marilyn Williams.

Lastly, I would like to thank my family, Tom Hall, Jerry and Jan Waggoner and Karen Straus for all of the encouraging words that have helped me make it to this point. Thank you for being there for me throughout this endeavor.
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LIST OF ABBREVIATIONS

ALT  Alanine aminotrasferase
APAP  Acetaminophen
ATP  Adenosine triphosphate
BHQ  2-bromohydroquinone
BB  Bromobenzene
DMSO  Dimethyl sulfoxide
DTNB  5-5’-Dithiobis(2-nitrobenzoic acid)
GR  Glutathione Reductase
GSH  Glutathione
H&E  Hematoxylin-eosin
IACUC  Institutional Animal Care and Use Committee
IHC  Immunochemistry
IP  Interperitoneal
kDa  Kilodaltons
NAD  Nicotinamide Adenine Dinucleotide
NADPH  β-Nicotinamide Adenine Dinucleotide Phosphate
NAPQI  N-aceyl-p-benzoquionone imide
NMN  Nicotinamide Mononucleotide
PARP-1  Poly(ADP-Ribose)polymerase-1
Phe  Phentolamine
Phen  6(5H)-Phenanthridinone
TCA  Trichloroacetic Acid
TUNEL  Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
MW  Molecular Weight
Attenuation of Bromobenzene-Induced Hepatotoxicity by Poly(ADP-ribose) Polymerase Inhibitors

Kelly Waggoner Hall

ABSTRACT

Previous studies have shown extensive cellular damage can activate poly(ADP-ribose) polymerase-1 (PARP-1) and cause a rapid decrease in the levels of NAD$^+$ and ATP, thereby preventing apoptosis and promoting necrosis and inflammation. The purpose of this study was to extend previous observations that inhibitors of PARP-1 could alter acetaminophen and carbon tetrachloride-induced hepatotoxicity. Bromobenzene (BB) a glutathione dependent hepatotoxicant was tested. Groups of male mice were treated with a single dosage of 112mg/kg (0.075 ml/kg) BB by the intraperitoneal (ip) route. All animals were maintained in a controlled environment and provided food and water ad libitum. This dosage of BB resulted in hepatotoxicity as measured by an increase in serum alanine transferase (ALT). BB treatment resulted in a 5-fold increase in ALT. Moderate hepatotoxicity was detected with this treatment regime. Subsequently, another group of mice were treated with three treatments of nicotinamide at 0.5, 1 and 2 hours following BB treatment. Serum ALT elevations were reduced by 90% at 24 hours following BB and nicotinamide treatments. BB-induced liver pathology was also blocked by nicotinamide. Mortality among BB treated animals was also significantly
reduced by nicotinamide treatment. Mortality among mice treated with BB and
nicotinamide was near control. The model was verified with a more potent and specific
inhibitor, Phen. BB treatment was kept at the same level as in the previous study, and
Phen was administered concomitantly. Serum ALT elevations were reduced by 75%.
Phen also blocked BB-induced liver pathology. Mortality among mice treated with BB
and Phen was reduced 75%. PARP-1 inhibitors appear to alter chemical-induced
hepatotoxicity that has either a glutathione dependent or independent mechanism. PARP-
1 inhibitors may have pharmacological application for modifying chemical-induced liver
injury.
CHAPTER 1
INTRODUCTION

Previous studies investigating hydrocarbon-induced hepatotoxicity have provided a basis for the experimental hypothesis of the current investigation. Bromobenzene (BB) is a pungent smelling organic solvent that is used for large-scale crystallizations where the use of a heavy liquid is preferred. (PPRTV, 1999) The compound is a common component of motor oils and has been used as an intermediate in industrial chemical syntheses. These uses have led to occupational exposures, dermal and inhalation, and the release of BB into the environment as a contaminate (Van Vleet and Schnellmann, 2003). The liver, kidney and lung are identified histologically as the primary target organs. The liver is the most sensitive target organ following acute oral exposure (PPRTV, 1999).

BB (Figure 1) has been studied extensively in the literature. BB has been known to cause necrosis of the centrilobular parenchymal cells in the liver (Brodie et al., 1971). Jollow et al. (Jollow et al., 1974) determined that BB-induced hepatic necrosis was due to the formation of a reactive metabolite that arylates vital cellular macromolecules, thereby causing the hepatic damage. This metabolite is BB 3,4-oxide which is formed by a cytochrome P-450 mixed function oxidase system (Monks et al., 1982).
Figure 1. Structure of bromobenzene (BB).

Many drugs and aromatic hydrocarbons are metabolized to reactive epoxide intermediates by the hepatic cytochrome P-450 mixed function oxidase system (Monks et al., 1984). Chemically reactive metabolites of BB, 2,3- and 3,4-BB epoxides are formed via this system (Lau and Zannoni, 1981). The toxic reactive metabolite of BB, 3,4-epoxide subsequently rearranges nonenzymatically to form p-bromophenol or is converted to the 3,4-dihydrodiol by epoxide hydrolase. Most of the 3,4-epoxide is detoxified to two glutathione conjugates by glutathione transferases in the liver. It has been postulated that o-bromophenol occurs from a nontoxic precursor, BB-2,3-oxide (Figure 2) (Monks et al., 1984). Human liver microsomes generate a much greater proportion of the hepatotoxic metabolite (3,4-epoxide) than the nonhepatotoxic metabolite (2,3-epoxide). Humans may experience a greater hepatotoxicity at lower doses of BB than do mice (Kerger et al., 1988c).
Figure 2. Bromobenzene is metabolized via the hepatic mixed function oxygenase system to reactive intermediates 2,3- and 3,4-bromobenzene epoxides. The toxic metabolite, 3,4-epoxide rearranges nonenzymatically to form $p$-bromophenol or is converted to 3,4-dihydrodiol by epoxide hydrolase. Most of the 3,4-epoxide is detoxified to two glutathione conjugates by glutathione (GSH) transferases in the liver. It has been postulated that $o$-bromophenol is a nonenzymatic rearrangement of a nontoxic precursor, BB-2,3-oxide.
Jollow et al. showed that administration of BB to rats resulted in a rapid and extensive depletion of glutathione from the liver. After approximately 5 hours about 15% of the initial level remains. Hepatic glutathione remained depleted during the metabolism of BB (Jollow et al., 1974). This had led to the understanding that BB forms conjugates with hepatic glutathione and that under these conditions the GSH level is decreased, making the liver cells more susceptible to the development of lipid peroxidation (Casini et al., 1985). It has been shown that the magnitude of the BB induced hepatotoxicity is dependent on the hepatic glutathione present during its liver detoxification (Jollow et al., 1974; Kerger et al., 1988a).  

BB has been used in various experiments as a model compound inducing liver and kidney impairment (Szymanska, 1998). Symanska stated that no lethal dose of BB could be found, and used a statistical method to determine the approximate lethal dose (ALD). The ALD dose for BB was set at 900 mg/kg. Doses used in this study were 200, 400 and 800 mg/kg. The %ALD was 22.2, 44.4 and 88.9. These doses were for outbred male Imp Balb/cJ mice, 23 – 30 g body weight. Mice used in the current study are male ICR mice, 25 – 30 g body weight. Past experiments have used B6C3F1 mice at a lower dose of BB (0.05 ml/kg) due to the fact that this strain had been used in a number of studies of hepatotoxic and carcinogenic agents (Kerger, 1988). Roberts et al. stated that BB toxicity occurs in both the inbred B6C3F1 and outbred ICR mice, but the toxicity occurred at lower doses with the B6C3F1 mice (Roberts et al., 1997). This led to experimentation using a higher dose of BB to cause hepatotoxicity.  

Past studies have shown that BB hepatotoxicity could be reduced. Phentolamine (Kerger et al., 1988a; Kerger et al., 1988b) and cystamine (De Ferreyra et al., 1979) have
been studied in the past. In all cases the conclusions were that BB hepatotoxicity could be reduced by treatment with these agents. A new novel method for reducing hepatotoxicity and centrilobular necrosis using PARP-1 inhibitors is the focus of this current study.

Current research of BB has studied the toxicity of BB in mice and rats at the molecular level using transcriptomics. Transcriptomics is the determination of the expression of genes (Heijne et al., 2003). Bartosiewicz, et al. administered male swiss-webster mice at a level of 2.5g/kg BB. All mice died before the 48-hour time point. They found that there were 14 genes that were expressed in the liver. These genes were involved in DNA damage response, oxidative stress and phase II metabolism (Bartosiewicz et al., 2001). Rat studies using male Wistar Rats receiving an i.p. injection of BB also analyzed gene expression. (Heijne et al., 2003). The gene expressions that Heijne et al. found to be significantly changed upon BB metabolism included those involved in glutathione conjugation.

In 1963, the enzyme poly(ADP-ribose) was discovered by Dr. Paul Mandel’s (Chambon et al., 1963) laboratory in France. It was originally thought to be poly(A), a DNA dependent enzyme induced by nicotinamide mononucleotide (NMN). Poly(A) polymerase catalyzes the addition of adenosine to the 3’ end of RNA (Alberts et al., 2002). By 1966 it was ascertained that the enzyme was slightly structurally different than poly(A), it was a novel structure now termed poly(ADP-ribose). It was discovered that poly(ADP-ribose) was not a nucleic acid, because unlike DNA and RNA which have a phosphodiester bond joining the polymer units, there is a ribose (1”→ 2’) ribose-phosphate-phosphate backbone. The anomic carbon of one ADP-Ribose molecule is
bound to the adenosine moiety of the next via a 1”→2’ glycosidic linkage (Althaus and Richter, 1987).

Poly (ADP-Ribose) polymerase (PARP-1) is a 113 kDa nuclear enzyme that catalyzes the transfer of the ADP-ribose moiety of NAD$^+$ to various nuclear acceptor proteins and then to the protein bound ADP-ribose (Ueda and Hayaishi, 1985). There are 3 main domains: the N-terminal DNA-binding domain (DBD) containing two zinc fingers, the auto modification domain and the C-terminal domain (Figure 3) (Southan and Szabó, 2003).

Figure 3. The structure of PARP-1. The DNA-binding domain corresponds to regions A,B and C. Section A contains two zinc fingers (F1 and F2) that are involved in DNA strand break recognition. Section B is the nuclear location signal (NLS). The NLS includes the caspase cleavage site. Region C has an unknown function. Region D consists of several glutamic acid residues, which are sites of automodification. Regions E and F encompass the catalytic domain. Figure adapted from (de Murcia et al., 1991; de Murcia and Shall, 2000)
The two zinc fingers (F1 and F2) in the amino (N) terminal end of PARP-1 are important for DNA binding and the enzyme activity of PARP-1. PARP-1 is activated by the presence of both single and double DNA strand breaks. F1, closed to the N terminal end, is essential for PARP-1 enzyme activity in response to nicked DNA or double strand breaks (Ikejima et al., 1990; Uchida and Miwa, 1994).

Automodification leads to the formation of long, branched PARPs on target proteins, primarily itself, using NAD$^+$ as a substrate, which leads to a depletion of NAD$^+$ (Herceg and Wang, 2001).

By manipulating the NAD concentration or the chemical nature of the poly(ADP-ribosyl)ation substrate, Alvarez-Gonzalez and Mendoza-Alvarez were able to dissect the C-terminal end of ADP-ribose polymerase into individual reactions of initiation, elongation and branching (Alvarez-Gonzalez and Mendoza-Alvarez, 1995). Initiation is the attachment of an ADP-ribose moiety to an acceptor protein. Elongation is where additional ADP-ribose moieties are attached to protein-bound ADP-ribosyl residues. Branching is the introduction of an ADP-ribose residue via a linkage that initiates a branch along the linear portion of the polymer. Another component of the C terminal end is Abortive N A Dase, which cleaves NAD$^+$ into nicotinamide and free ADP-ribose (de Murcia and Shall, 2000). Figure 2 is a graphical representation of PARP-1.

With excessive activation of PARP-1, its substrate NAD$^+$ is depleted and in efforts to resynthesize NAD$^+$, ATP is also depleted (Figure 6) (Ha and Snyder, 1999). PARP-1 is cleaved by caspases (primary caspase-3 and caspase-7) during apoptosis. This cleavage separates the DNA binding domain from the catalytic domain, resulting in the inactivation of PARP-1 (Affar et al., 2001).
Figures 4 and 5 are the chemical structures of the inhibitors chosen for this study. Isoquinoline derivates (Figure 5) have polyaromatic heterocyclics with a carboxyl group in the second ring. This and the third aromatic ring also appears to be critical for inhibition to occur (Weltin et al., 1997)

![Chemical structure of nicotinamide](image)

Figure 4. Chemical structure of nicotinamide, a nonspecific PARP inhibitor.

![Chemical structure of Phen](image)

Figure 5. Chemical structure of Phen. Phen is an isoquinoline derivative and is a very potent inhibitor.

Inhibition of PARP-1 has been investigated due to the fact that PARP activation has been found to contribute to an energy-consuming cellular process, which leads to cellular NAD and ATP depletion, mitochondrial dysfunction and an overall cellular dysfunction. This process can lead to cell death through necrosis (Southan and Szabó, 2003). PARP is involved in the regulation of many cellular processes such as DNA
repair, gene transcription, cell cycle progression, cell death, chromatin functions and genomic stability. (Figure 6) (Tentori et al, 2002).

As of 2003, NIC is the only PARP-1 inhibitor that has been administered to humans. Two clinical trials have utilized NIC, one was a pilot trial in osteoarthritic patients, the other was a trial in Type I diabetes. The diabetes trial showed promise in Phase II studies. Phase III trials did not show efficacy. This was attributed to the concentration of the inhibitor. Any amount over 3 g/day to humans begins to exert varial toxic effects (Southan and Szabó, 2003).

This laboratory has conducted the only studies using Phen in vivo as a PARP-1 inhibitor. The first study was attenuation of CCl$_4$-induced hepatotoxicity (Su et al., 2003; Banasik et al., 2004) and this current study showing the attenuation of BB-induced hepatotoxicity. Both BB and CCl$_4$ cause centrilobular (zone 3) necrosis.
Figure 6. Overview of PARP-1 activation to DNA damage. Inhibition of the overaction of PARP will change the pathway to the unrepairable pathway.
Research Question

It has been shown previously that BB causes centrilobular hepatotoxicity. BB causes hepatotoxicity through glutathione depletion. The research hypothesis includes:

- A non-specific PARP-1 inhibitor can attenuate BB-induced hepatotoxicity
- A specific PARP-1 inhibitor can attenuate BB-induced hepatotoxicity
- PARP-1 inhibitors can attenuate hepatotoxicity independent of BB metabolism.
CHAPTER 2

METHODS

Animals and Treatment: Male, ICR mice, 25-30 g, were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Animals were housed 4 per cage, had free access to food and water and were exposed to a 12-hour light/dark cycle. Animals were acclimated for 7 days prior to testing. Drugs and chemicals were administered by intraperitoneal injection (i.p.) at a volume of 0.05-ml/10 kg body weight. The animals were cared for in accordance with Guide to the Care and Use of Experimental Animals and the University of South Florida Institutional Animal Care and Use Committee (IACUC) approved this study. All test solutions were prepared fresh immediately preceding animal treatment. BB, Nic and Phen were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were of the best quality commercially available. BB was dissolved in corn oil prior to administration. Nic was dissolved in saline. 6(5-H)-Phen was dissolved in 100% DMSO. Groups for comparison were treated with either (1) corn oil vehicle, (2) PARP-1 inhibitor, (3) BB or (4) BB and PARP inhibitor.

Inhibitor Efficacy: PARP-1 inhibitors were screened for efficacy in reducing ALT. BB was administered to all mice with the exception of controls. PARP-1 inhibitors were administered subsequent to BB-administration.
Experimental Design: Animals were randomized into groups as shown in Tables 1 and 2. BB was administered as a single, i.p. dosage at 112 mg/kg (0.075 mg/kg). Animals were randomly assigned to groups with the number of animals in each of the treated groups being approximately double that of the number in the control groups. The n for each treatment group ranged from 6 to 121 depending on the size of the experiment. Control groups ranged from 4 to 8 mice.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Chemicals</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Vehicle only</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group II</td>
<td>Bromobenzene (112mg/kg)</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group III</td>
<td>NIC (PARP-1 inhibitor)</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group III</td>
<td>BB + NIC</td>
<td>50 μl/10 g body weight</td>
</tr>
</tbody>
</table>

Table 1. Experimental Design for control and treatment group for the non-specific inhibitor study

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Chemicals</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Vehicle only</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group II</td>
<td>Bromobenzene (112mg/kg)</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group III</td>
<td>Phen (PARP-1 inhibitor)</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group IV</td>
<td>DMSO (vehicle for Phen)</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group V</td>
<td>BB + Phen</td>
<td>50 μl/10 g body weight</td>
</tr>
<tr>
<td>Group V</td>
<td>BB + DMSO</td>
<td>50 μl/10 g body weight</td>
</tr>
</tbody>
</table>

Table 2. Experimental design for controls and treatment groups for specific inhibitor
Time Study: This study was performed to find the optimum time for BB toxicity. Mice were administered with BB and asphyxiated by carbon dioxide at 12-hour, 24-hour and 36-hour time frames. Blood was drawn by cardiac puncture.

Serum Biochemistry: Serum Alanine Amino Transferase (ALT) was determined by the method of Reitman et al. (Reitman and Frankel, 1957) using a commercially prepared reagent kit from TECO Diagnostics (Anaheim, CA). Whole blood was collected in micro centrifuge tubes and allowed to clot at room temperature for a minimum of 5 minutes. Serum was obtained after centrifugation at 6000 g for 10 minutes. ALT assays were obtained as directed in the diagnostics kit.

Gross Pathology: Necropsy was performed on all mice and visual findings were recorded to correlate with ALT. Photographs were taken of random samples from BB, BB/NIC, BB/Phen and controls.

Histopathology: Livers were preserved in 10% neutral buffered formalin (Fisher Scientific, Fair Lawn, NJ). The livers were sectioned (5 - 6 mm), dehydrated with ethyl alcohol, cleared with xylene and embedded in paraffin. Sections of 5- to 6-mm were mounted, dried and stained with hemotoxylin/eosin to assess parenchymal histopathological changes. The methodology used to evaluate hepatotoxicity have been described previously (Price et al., 1999). Briefly, the parameters evaluated were proliferation, apoptosis, necrosis and fibrosis. Proliferation was determined according to the presence of four parameters: hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation. To determine proliferation intensity, the sum of the individual intensity of each of the four parameters was used to reach a total score of no observable
pathological changes (0), mild intensity (1), moderate intensity (2) or prominent intensity (3).

Apoptosis was evaluated by using ApopTag® Plus In Situ Apoptosis Detection, according to a standard protocol. This method allows visualization of apoptotic bodices and also intact apoptotic nuclei. It is a useful method for detecting apoptosis at the earliest stages. A solid brown nuclear staining identified apoptotic hepatocytes. The number of apoptotic cells was expressed as a percentage of total number of cells (Apoptotic Index, AI). The apoptotic index was then converted to a numerical score. No observable apoptosis is given a score of zero. This equates to AI 0%. Mild intensity, given a score of 1, represents and AI <25%. Moderate intensity, given a score of 2, represents AI 25% to 50%. Prominent intensity, given a score of 3 represents AI 50%.

Cleaved Caspase-3 histopathology was performed using Cleaved Caspase-3 (Asp175) (5A1) Rabbit Monoclonal Antibody from Cell Signaling Technology. The method of immunochemistry was used. Cleaved caspase-3 (Asp175) detects levels of large fragments (17-19 kDa) of activated caspase 3 resulting from cleavage adjacent to Asp 175. Expression of cleaved caspase 3 stains brown. The scale for expression is 0 to 4. A score of 0 is no expression of cleaved caspase 3. A score of 1 is <25% of the centrilobular region expressing cleaved caspase 3. A score of 2 is 26-50% expression, a 3 is 51-75% expression and a 4 is 76-100% expression.

Glutathione: Total glutathione (oxidized and reduced) was measured using a kinetic assay measured at wavelength 405 nm. Briefly, livers were removed and homogenized (1 in 10 w/v in 7.4 pH phosphate buffered saline). Supernate was separated by centrifugation of an aliquot of liver homogenate. The supernate was deprotienated
with tricloroacetic acid (TCA). After deproteination, the supernate was decanted into a microcentrifuge tube and DTNB and GR reagents were added. Immediately before analysis, NADPH was added. GSH was measured for 3 minutes at intervals of 15 seconds. A standard curve was a plot of increase in optical density (OD)/min as a function of the concentration of GSH. Values were calculated from the standard curve and normalized to the weight of the liver to report values as μM GSH/mg liver.

Lipid Peroxidation: Lipid Peroxidation was measured using a spectrophotometric method. The method used is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid. Livers were removed and homogenized (1 in 10 w/v in 7.4 pH phosphate buffered saline). Supernate was separated by centrifugation of an aliquot of liver homogenate. Butylated hydroxytoluene in methanol was added to an aliquot of supernate. 1M phosphoric acid and 2-thiobarbituric acid were added. The sample was incubated for 60 minutes at 60° C and analyzed. Values were calculated from the standard curve and ratioed to the weight of the liver to report the values as μM MDA/mg liver.

Statistical Analysis: Comparisons among groups were made using a one-way analysis of variance (ANOVA), with a Student Neuman-Keuls post test. In all cases statistical differences were considered significant with p ≤ 0.05. All statistical analysis were performed with GraphPad Prism® version 4.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).
CHAPTER 3

RESULTS

Mice were treated with BB with or without a cotreatment with a PARP-1 inhibitor. Table 3 lists the PARP-1 inhibitors that were screened for efficacy. Nic and Phen were selected as the nonspecific and specific inhibitors for this study. BB treatment alone resulted in a 5-fold increase in ALT. Mice treated with Nic at time intervals of 1.2, 1 and 2 hours following BB decreased serum ALT activity by 90%. Mice treated concomitantly with BB and Phen showed a 75% reduction in serum ALT activity.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (μM)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzamide</td>
<td>22</td>
<td>No positive findings</td>
</tr>
<tr>
<td>4-Aminobenzamide</td>
<td>1800</td>
<td>Solubility issues – did not use</td>
</tr>
<tr>
<td>Aminophylline</td>
<td>3.3</td>
<td>No reproducible results</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>210</td>
<td>Inhibitor used in this study</td>
</tr>
<tr>
<td>6(5H)-Phenanthridinone</td>
<td>0.30</td>
<td>Inhibitor used in this study</td>
</tr>
</tbody>
</table>

Table 3. Screening of inhibitors. Five inhibitors were screened for this study. Of those screened, only Nic and Phen had positive results. Benzamide showed no significant reduction in ALT. 4-Aminobenzamide was soluble in saline upon heating, but precipitate was observed after 12 hours. Aminophylline was screened at concentrations from 1 mg/kg to 100 mg/kg with no reproducible results.
Serum ALT activity was measured at 12-hour intervals to find the point of maximum activity. At the 12-hour time point, there was no statistically significant difference in serum ALT activity expressed by BB and controls. At the 24-hour time point, there was a 345% increase in serum ALT activity compared to the 12-hour time point for BB. Serum ALT activity decreased 22% between 24 hours and 36 hours. The maximum serum ALT activity was expressed at the 24-hour time point. All experimental data will be collected at the 24-hour time point for the duration of the study.

BB and Nic were co-administered concomitantly (Figure 8). There was no statistically significant difference in serum ALT activity of BB with a concomitant administration of Nic when compared to administration of BB alone. The timing of the subsequent administration of Nic in a single i.p. injection was varied from 1 hour to 3 hours. Administration of Nic at 1 hour after BB (Figure 9) reduced serum ALT activity by 53%. Administration of Nic at 3 hours after BB shows no statistically significant decrease in serum ALT activity.

Administration of Nic at 3 time intervals, ½ hour, 1 hour and 2 hours after BB showed a 90% reduction in serum ALT (Figure 11). To confirm these findings, the experiment was repeated (Figure 12). There was an 85% reduction in serum ALT activity.
Figure 7. ALT measurements (IU/L) measured at 12-hour intervals. Mice were sacrificed every twelve hours and ALT was measured to find the time point where the ALT for BB was at the maximum. Serum ALT level is expressed for controls (C) and BB (BB) as mean ± SEM. An * represents a value that is statistically different from the starting point at 12 hours. Statistical differences of ALT data were determined using one-way analysis of variance (p<0.05).
Figure 8. BB and Nicotinamide administered concomitantly. Controls were administered corn oil. Nic was administered to a group of mice to verify that no elevation of ALT would result. Results are expressed as mean $\pm$ SEM. Statistical differences of ALT data were determined using one-way analysis of variance (p<0.05). There is no significant lowering of ALT when BB and Nic are administered concomitantly.
Figure 9. Nicotinamide was administered 1 hour after BB dose. Serum ALT level is expressed for controls, bromobenzene (BB), nicotinamide (NIC) and bromobenzene/nicotinamide (BB/NIC) as mean ± SEM. An * represents a value that is statistically different from BB. Statistical differences of ALT data were determined using one-way analysis of variance (p<0.05).
Figure 10. Nicotinamide treatment 3 hours after BB treatment. Serum ALT level is expressed for controls, bromobenzene (BB), nicotinamide (NIC) and bromobenzene/nicotinamide (BB/NIC) as mean ± SEM. There is no statistically significant difference in ALT is seen between BB and BB/NIC at 3-hour post treatment.
Figure 11. Nicotinamide (NIC) administration given at ½, 1 and 2 hours. Serum ALT level is expressed for controls, bromobenzene (BB), nicotinamide (NIC) and bromobenzene/nicotinamide (BB/NIC) as mean ± SEM. An * represents a value that is statistically different from the BB. A statistical difference of ALT data was determined using one-way analysis of variance (p<0.05).
Figure 12. Confirmation of BB and 3x dose, where 3x refers to Nic administered ½, 1 and 2 hours after BB. Serum ALT level is expressed for controls, bromobenzene (BB), nicotinamide (NIC) and bromobenzene/nicotinamide (BB/NIC) as mean ± SEM. Results are expressed as mean ± SEM. An * represents that BB/NIC is statistically different from BB. Statistical differences of ALT data was determined using one-way analysis of variance (p<0.05).
Gross anatomical findings showed hemorrhagic and necrotic damage in mice treated with BB. When Nic was administered subsequent to BB, no pathological changes were observed. Visual observations were recorded on all mice that blood was drawn on. A correlation with ALT was performed. Findings showed that for those mice having gross pathological changes, there was an increase in ALT. Because the mice selected were male ICR mice, there was some interspecies variation. This is shown in graphs by error bars.

As indicated by the data in figure 13, Nic administered in 3 subsequent administrations after BB, has the ability to reduce serum ALT activity for up to 72 hours. ALT activity was measured every 12 hours and compared to the ALT activity of BB at 24 hours, which is the time point at which the maximum ALT is expressed (Figure 7). The reduction in ALT was approximately 80% at every time point. Controls with corn oil and Nicotinamide are shown as a reference. All BB + Nic ALT responses were near that of controls.

A seven day mortality study was performed for both mice administered BB, and mice that received multiple administrations of Nic subsequent to BB administration. Nic completely protected against mortality, while 56% of those mice administered BB alone died within the 7 day period (Figure 14).
Figure 13. 72 hour time study for BB/NIC. Serum ALT level is expressed for controls (C), bromobenzene (BB) and bromobenzene/nicotinamide (BB/NIC) BB/NIC at 12, 24, 36, 48, 60 and 72-hours was near that of the control at 24 hours. Previous experiments (figure 6) showed that ALT for controls does not change significantly over time. All responses are reported as mean ± SEM. An * represents a value that is statistically different from the BB at 24 hours. Statistical differences of ALT data was determined using one-way analysis of variance (p<0.05).
Figure 14. 7-day mortality for mice administered BB only and mice administered BB + Nic. BB alone showed a 56% mortality, while BB + Nic showed a 0% mortality. NIC was protective against BB-induced mortality.
Livers from mice treated with BB, with or without subsequent Nic treatment, were removed and sectioned for histopathologic examination. One mouse from each treatment group was selected for macroscopic examination (Figures 15 – 17). Figure 15 shows the liver treated with saline. There were no abnormalities in the tissue. Figure 16 shows the liver treated with BB. There was hemorrhagic damage on the outer edges of the liver. Figure 16B is a close up of the necrotic damage. Figure 17 is a macroscopic view of a liver treated with BB and Nic. No tissue abnormalities are seen. Macroscopic examination of liver samples were in accordance with the biochemical findings.
Figure 15. Macroscopic view of liver from a mouse administered saline only. Liver was removed immediately following necropsy. No tissue abnormalities were apparent.
Figure 16. Macroscopic view of liver from a mouse administered bromobenzene. Liver was removed immediately following necropsy. Liver is hemorrhagic around edges with necrosis visible on upper surface (A). Close up of the necrotic damage on the upper surface (B).
Figure 17. Macroscopic view liver from a mouse treated with BB and Nic. Liver was removed immediately following necropsy. No tissue abnormalities are apparent.
Table 4 lists the histopathological evaluation of necrosis, apoptosis and cleaved caspase 3 (Caspase IHC). The values reported are the averages of the findings. Figures 18 – 20 are hematoxylin-eosin stained to assess parenchymal histopathological changes. Figures 21 – 23 are TUNEL stained to assess apoptosis. Figures 24 – 26 are stained for cleaved caspase 3 to assess the evidence of expression.

<table>
<thead>
<tr>
<th>Administered Substance</th>
<th>H&amp;E</th>
<th>TUNEL</th>
<th>Cleaved Caspase 3 (Caspase IHC)</th>
</tr>
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<tbody>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bromobenzene</td>
<td>3+</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td></td>
<td>1+ necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-3 apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromobenzene/ Nicotinamide</td>
<td>50% of samples showed no necrosis, no apoptosis reversible 1+</td>
<td>Focal 3+ reversible</td>
<td>0</td>
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Table 4. Histopathologic evaluation of H&E, TUNEL and Immunochemistry. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation (no expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (no expression of apoptosis = AI 0% or a score of 0; mild intensity = AI < 25% or a score of 1; moderate intensity = AI 25% to 50% or a score of 2; prominent intensity = AI > 50% or a score of 3). Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 –75%; 4 = 75 – 100%).
Figure 18. H&E staining of saline control mouse liver. An arrow indicates a centrilobular vein. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation (no expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). Histopathology expression was given a score of 0.
Figure 19. H&E staining of liver from mouse administered bromobenzene. An arrow indicates a centrilobular vein. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binucleation (No expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). Centrilobular damage (    ) indicates swelling of the cells, termed ballooning. Histopathology expression was given a score of 3+. 
Figure 20. H&E stain of liver tissue from mouse administered with bromobenzene and nicotinamide. An arrow indicates a centrilobular vein. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation (No expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). Histopathology expression was given a score of 1+. Fifty percent of samples showed no necrosis, no apoptosis and reversible hypertrophy.
Figure 21. TUNEL staining of saline control. An arrow indicates a centrilobular vein. TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (no expression of apoptosis = AI 0% or a score of 0; mild expression = AI < 25% or a score of 1; moderate expression = AI 25% to 50% or a score of 2; prominent expression = AI > 50% or a score of 3). TUNEL expression was given a score of 0.
Figure 22. TUNEL staining of BB induced damage. An arrow indicates a centrilobular vein. Apoptosis is indicated by (↓ or ↑). TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (no expression of apoptosis = AI 0% or a score of 0; mild expression = AI < 25% or a score of 1; moderate expression = AI 25% to 50% or a score of 2; prominent expression = AI > 50% or a score of 3). TUNEL expression was given a score of 3+. 
Figure 23. TUNEL stain of liver with a BB and NIC dose. An arrow indicates a centrilobular vein. TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (no expression of apoptosis = AI 0% or a score of 0; mild expression = AI < 25% or a score of 1; moderate expression = AI 25% to 50% or a score of 2; prominent expression = AI > 50% or a score of 3). TUNEL expression was given a score of 0.
Figure 24. Cleaved Caspase 3 staining of a liver from a mouse that was administered saline. Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 – 75%; 4 = 75 – 100%). Immunochemistry evaluation was negative (a score of 0). An arrow indicates the centrilobular vein.
Figure 25. Cleaved Caspase 3 staining of a liver from a mouse that was administered bromobenzene. Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 – 75%; 4 = 75 – 100%). Immunochemistry evaluation rated the damage as a 1+. An arrow indicates the centrilobular vein. Cleaved caspase 3 stains brown.
Figure 26. Cleaved Caspase 3 staining of a liver from a mouse that was administered BB and Nic. Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 – 75%; 4 = 75 – 100%). Immunochemistry evaluation was negative (a score of 0). An arrow indicates the centrilobular vein.
Histopathologic evaluation of the liver sections were in accordance with the biochemical findings. Mice administered only saline showed no expression of abnormalities, no expression of apoptosis and no expression of cleaved caspase-3. With the administration of BB, there is histopathologic expression of swelling of cells, or ballooning. Apoptosis is evident, and there is expression of cleaved Caspase-3. Administration of Nic subsequent to BB attenuated the hepatotoxicity.

BB and Phen (10 mg/ml) were administered concomitantly (Figure 27). Due to Phen being soluble in 100% DMSO, BB and DMSO were administered concomitantly to assess the inhibitory effects of DMSO. Phen administered concomitantly with BB reduced serum ALT activity by 54%. DMSO administered concomitantly with BB reduced serum ALT activity by 40%. Reductions in ALT activity were statistically significant when compared to the activity expressed by BB.

BB and Phen (20 mg/ml) administered concomitantly (Figure 28) decreased serum ALT activity by 58% compared to animals treated with BB alone. BB and Phen (40 mg/ml) administered concomitantly (Figure 20) decreased serum ALT activity by 73% compared to animals treated with BB alone. Phen at a concentration of 40 mg/ml showed the largest reduction in serum ALT activity. This level of inhibitor was repeated (Figure 29) in a subsequent study to verify results. The reduction in serum ALT activity in the subsequent experiment was 74% compared to animals treated with BB alone.
Figure 27. Phen was administered concomitantly with BB. Phen is soluble in 100% DMSO. The dose of BB/DMSO showed a statistically significant lowering of ALT. The dose of 10 mg/ml also showed a statistically significant lowering of ALT. Results are expressed as mean ± SEM. An * represents statistically significant difference when compared to BB. Statistical differences of ALT data was determined using one-way analysis of variance (p<0.05).
Figure 28. Screening of 20, and 40 mg/ml concomitant administration of BB and Phen. Results are expressed as mean ± SEM. An * represents statistically significant difference when compared to BB. Statistical differences of ALT data was determined using one-way analysis of variance (p<0.05).
Figure 29. Confirmation experiment to show repeatability at the BB/40 mg/mL Phen level. The reduction in ALT was comparable to the initial experiment at this level. An * represents statistically significant difference when compared to BB. Statistical differences of ALT data was determined using one-way analysis of variance (p<0.05).
A 7-day mortality study was conducted using four groups of mice. One group received only BB, one group received BB + Phen at a concentration of 40 mg/kg, one group received BB + DMSO and one group received DMSO only. The sample size for all groups was n = 8 (Figure 30). There was a 50% mortality in mice that were administered BB. This is comparable to the 56% seen in the previous experiment (Figure 14). There was 12.5% (1 in 8) mortality in mice administered BB + Phen, Phen only and DMSO only. Due to Phen solubility in 100% DMSO, mice were administered DMSO alone as a control.
Figure 30. 7 day mortality for BB, BB/Phen, BB/DMSO and DMSO only. There was 50% mortality for BB only, and 12.5% for other groups. There was a 4-fold decrease in mortality. All groups with one death were administered DMSO, either alone, or as the vehicle for Phen. N=8 for all groups.
Histopathology was performed for BB/Phen, Phen and DMSO by a certified pathologist.

Table 5 lists the histopathological evaluation of necrosis, apoptosis and cleaved caspases 3 (Caspase IHC). Macroscopic pathology was also performed (Figure 31). Figures 32 – 34 are hematoxylin-eosin stained to assess parenchymal histopathological changes. Figures 35 – 37 are TUNEL stained to assess apoptosis. Figures 38 – 405 are stained for cleaved caspase 3 to assess the evidence of expression. No abnormalities were seen any of the pathology samples for this experiment.

<table>
<thead>
<tr>
<th>Administered Substance</th>
<th>H&amp;E</th>
<th>TUNEL</th>
<th>Cleaved Caspase 3 (Caspase IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromobenzene/Phen</td>
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<td>0</td>
</tr>
<tr>
<td>Bromobenzene/DMSO</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DMSO Control</td>
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</table>

Table 5. Histopathologic evaluation of H&E, TUNEL and Immunochemistry. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation (no expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (no expression of apoptosis = AI 0% or a score of 0; mild intensity = AI < 25% or a score of 1; moderate intensity = AI 25% to 50% or a score of 2; prominent intensity = AI > 50% or a score of 3). Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 –75%; 4 = 75 – 100%).
Figure 31. Macroscopic view of livers from mice administered Phen (A), DMSO (B) and BB/Phen (C). Liver was removed immediately following necropsy. No tissue abnormalities were apparent.
Figure 32. H&E stain of liver tissue from a mouse administered bromobenzene and Phen concomitantly. An arrow indicates a centrilobular vein. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation (No expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). Histopathology was given a rating of 0.
Figure 33. H&E stain of liver tissue from a mouse administered bromobenzene and DMSO concomitantly. An arrow indicates a centrilobular vein. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binculeation (No expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). Histopathology was given a rating of 0.
Figure 34. H&E stain of liver tissue from a mouse administered DMSO. An arrow indicates a centrilobular vein. H&E was graded using a scale 0 to 3 and is a sum of four parameters – hepatocyte hyperplasia, hepatocyte hypertrophy, mitotic activity and binucleation (No expression of histological changes = 0; mild intensity = 1; moderate intensity = 2; prominent intensity = 3). Histopathology was given a rating of 0.
Figure 35. TUNEL stain of liver tissue from a mouse administered bromobenzene and Phen concomitantly. An arrow indicates a centrilobular vein. TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (No expression of apoptosis = AI 0% or a score of 0; mild expression = AI < 25% or a score of 1; moderate expression= AI 25% to 50% or a score of 2; prominent expression = AI > 50% or a score of 3). TUNEL expression was given a score of 0.
Figure 36. TUNEL stain of liver tissue from a mouse administered bromobenzene and DMSO concomitantly. An arrow indicates a centrilobular vein. TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (No expression of apoptosis = AI 0% or a score of 0; mild expression = AI < 25% or a score of 1; moderate expression = AI 25% to 50% or a score of 2; prominent expression = AI > 50% or a score of 3). TUNEL expression was given a score of 0.
Figure 37. TUNEL stain of liver tissue from a mouse administered DMSO. An arrow indicates a centrilobular vein. TUNEL is expressed as the number of apoptotic cells as a percentage of total number of cells (Apoptotic Index or AI). AI is expressed on a scale of 0 to 4. (No expression of apoptosis = AI 0% or a score of 0; mild expression = AI < 25% or a score of 1; moderate expression = AI 25% to 50% or a score of 2; prominent expression = AI > 50% or a score of 3). TUNEL expression was given a score of 0.
Figure 38. Cleaved Caspase 3 staining of a liver from a mouse that was administered BB and Phen. Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 – 75%; 4 = 75 – 100%). Immunochemistry evaluation was negative (a score of 0). An arrow indicates the centrilobular vein.
Figure 39. Cleaved Caspase 3 staining of a liver from a mouse that was administered bromobenzene and DMSO. Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunochemistry expression; 1 < 25%; 2 = 26 – 50%; 3 = 51 – 75%; 4 = 75 – 100%). Immunochemistry evaluation was negative (a score of 0). An arrow indicates the centrilobular vein.
Figure 40. Cleaved Caspase 3 staining of a liver from a mouse that was administered DMSO. Immunochemistry was used to evaluate caspase expression using a scale of 0 to 4 (0 = no immunohistochemistry evaluation; 1 < 25%; 2 = 26 – 50%; 3 = 51 – 75%; 4 = 75 – 100%). Immunohistochemistry evaluation was negative (a score of 0). An arrow indicates the centrilobular vein.
Total glutathione (reduced and oxidized) was measured in liver samples that were extracted 24 hours after BB administration (Figure 41). Livers were stored at –80°C until analysis. Administration of BB alone resulted in an 81% reduction in GSH. The reduction in GSH is in accordance with the biochemical and histopathological findings. Administration of BB and subsequent injections of Nic resulted in a 36% reduction in GSH. A concomitant administration of Phen and BB resulted in a 91% reduction in GSH. This confirms that PARP-1 inhibition of BB-induced hepatotoxicity is independent of the metabolism of BB.

Lipid peroxidation in the liver was assessed (Figure 42). Lipid peroxidation is used as an indirect measure of liver injury. The method used was a measurement of malondialdehyde (MDA) accumulation (TBARS). There was no statistically significant difference in any of the groups when compared to either the controls, which received no treatment, or BB alone. This shows that either BB administration at 112 mg/kg is at a concentration that is insufficient to cause lipid peroxidation or reductions in GSH are not correlated to lipid peroxidation.
Figure 41. Total glutathione (uM GSH/mg liver) measured for BB, BB/NIC and BB/Phen. Livers were extracted 24 hours after BB was administered. Livers were kept at –80 C until analysis. An * represents a statistically significant depression of GSH as compared to the level measured in the control. In all groups there was a significant depression of glutathione as measured by one-way ANOVA. The level of significance is $p \leq 0.05$. 


Figure 42. Lipid Peroxidation (TBARS) measured for BB, BB/NIC, BB/Phen BB/DMSO. Values reported are in uM MDA/mg liver. There was no statistical significant between any groups as measured by one-way ANOVA. Results are expressed as mean ± SEM. Significance was set at p ≤ 0.05.
CHAPTER 4
DISCUSSION

This experiment has shown that nicotinamide attenuates BB-induced hepatotoxicity. Because nicotinamide is a non-specific inhibitor, this experiment was repeated using a potent specific inhibitor to verify that the results could be replicated. The inhibitor selected was Phen. Phen is not soluble in saline, but was found to be soluble in 100% DMSO. Dimethyl sulfoxide (DMSO) has beneficial properties as an anti-inflammatory agent and as a treatment for ischemia. It has been shown to protect the liver of rats from BB when given up to 24 hours after oral administration (Lind and Gandolfi, 1999b). In a follow-up study, Lind and Gandolfi determined that DMSO looses its effectiveness if administered after the twenty four hour time point (Lind and Gandolfi, 1999a). Past evidence has shown that DMSO (IC\textsubscript{50} = 4.791 M) is capable of inhibiting PARP-1 (Banasik et al., 2004). In this experiment Phen, a potent PARP-1 inhibitor, was dissolved in 100% DMSO. This laboratory has shown that Phen was protective against carbon tetrachloride (CCl\textsubscript{4}) hepatotoxicity (Banasik et al., 2004). Animals were administered CCl\textsubscript{4} and Phen concomitantly. CCl\textsubscript{4} is bioactivated mainly by cytochrome P450 2E1 via a reductive dehalogenation to form the trichloromethyl free radical (Cl\textsubscript{3}C•) (Banasik et al., 2004). CCl\textsubscript{4} and BB cause centrilobular necrosis by different pathways. The CCl\textsubscript{4} experiment described in Banasik, et al. was repeated using BB as the toxicant (Figure 27). The concentration of the inhibitor used in the CCl\textsubscript{4}
experiment, 10 mg/kg was used in this study. BB and Phen were administered concomitantly and showed a significant decrease in serum ALT. The experiment was repeated using concentrations of 20 mg/kg and 40 mg/kg (Figure 28) to test for a further decrease in ALT. A concentration of 20 mg/kg showed a 2.6 fold decrease in serum ALT, a concentration of 40 mg/kg showed a 4-fold decrease. The experiment was repeated at 40 mg/kg concentration for confirmation (Figure 29).

The first studies concentrated on nicotinamide as the PARP-1 inhibitor. Work involving nicotinamide has been focused on acetaminophen. Acetaminophen (APAP) overdose causes hepatic and renal failure. The use of nicotinamide alone or in combination with N-acetylcysteine, an antioxidant, or l-methione, an amino acid, suppressed hepatotoxicity (Kröger et al., 1996; Kröger et al., 1997). Nicotinamide was shown to prevent APAP-induced lipid peroxidation, DNA damage and the associated apoptotic and necrotic cell deaths (Ray et al., 2001).

One function of PARP-1 is to participate in cellular recovery from DNA damage (Durkacz et al., 1980). At the site of a DNA strand break, PARP-1 catalyzes the transfer of the ADP-ribose moiety from its substrate NAD+ to either a protein acceptor, or to the enzyme itself (automodification) (de Murcia and Menissier-de Murcia, 1994). PARP-1 activation has been found to contribute to an energy-consuming cellular process, which leads to NAD and ATP depletion, mitochondrial dysfunction and an overall cellular dysfunction. This process can eventually lead to cell death (Southan and Szabó, 2003).

PARP-1 inhibition by chemicals can prevent a drop in NAD. Preserving cellular energy level appears to be the main effect that PARP inhibitors exhibit in reducing necrotic cell death (Zhang and Li, 2000). BB, the toxicant used in this study, is a well-
known and model chemical for causing hepatic centrilobular necrosis. The purpose of this study was to show BB hepatotoxicity was present and to show that PARP-1 inhibitors could attenuate that hepatotoxicity. The first inhibitor chosen was NIC, a non-specific inhibitor. The second inhibitor chosen was Phen, a specific and potent inhibitor.

Serum ALT is a measure of liver damage. NIC resulted in a 90% reduction in serum ALT, PHEN resulted in a 75% reduction. To confirm that the reduction in ALT was a measure of attenuation of hepatotoxicity, histopathology was analyzed. H&E, TUNEL and cleaved caspase 3 were assessed and graded by a certified pathologist. PARP is cleaved by caspases, predominantly caspase-3, during apoptosis (Affar et al., 2001). The pathology findings showed that BB induced damage including ballooning of cells, hyperplasia and apoptosis. Cleaved caspase 3 was expressed. Saline and DMSO were analyzed controls. NIC is soluble in saline and PHEN is soluble in DMSO. The control samples showed no abnormal pathology. BB/NIC and BB/PHEN were assessed for the same parameters as BB. Because DMSO has some inhibitory properties, BB/DMSO was also assessed. The pathological evaluation showed that apoptosis was not present in any of the samples that contained inhibitors, including the BB/NIC, BB/PHEN and BB/DMSO. TUNEL and cleaved caspase 3 were not expressed.

Most of the toxic metabolism of BB is by way of the formation of the 3,4-epoxide. Detoxification of the epoxide is by conjugation with GSH under the influence of glutathione-S-transferase. When the rate of formation of the 3,4-epoxide exceeds the rate of the detoxifying reaction and the available GSH is depleted, the epoxide begins to bind covalently to tissue macromolecules and thereby cause necrosis (Zimmerman, 1999). Glutathione is most concentrated in the liver. GSH levels were significantly reduced for
BB, BB/NIC and BB/Phen. This finding showed that although GSH levels were reduced, the PARP-1 inhibitor treated animals had reduced ALT and no abnormal histopathology. This leads to a conclusion that the attenuation of BB-induced hepatotoxicity is totally independent of metabolism.

Bromobenzene forms conjugates with hepatic GSH. This reduces GSH levels and the liver cells are made more susceptible to the development of lipid peroxidation (Comporti, 1985). In primary cultures of hepatocytes, BB induced a rapid depletion of GSH followed by the appearance of lipid peroxidation (Casini et al., 1982). The methodology used to measure lipid peroxidation was a thiobarbituric acid test. It is a spectrophotometric quantitation of the pink complex formed after reaction of MDA with two molecules of TBA (Botsoglou et al., 1994). There was no statistical difference in level of MDA between BB alone and BB when administered with NIC, PHEN or DMSO. The findings for no lipid peroxidation are contrary to the work of Casini, et. al. The work done by Casini, et. al. was in vitro. This would lead to a conclusion that either this work is not reproducible in vivo, or that the level of BB used was insufficient to produce lipid peroxidation. When a PARP-1 inhibitor is used, it would be expected that lipid peroxidation would not be expressed. Further study in the area of BB-induced in vivo lipid peroxidation using a higher concentration may show that lipid peroxidation may occur.

The major pharmaceutical uses of PARP-1 inhibitors have focused on acetaminophen (APAP). APAP is a common analgesic that is safe at therapeutic doses. Overdoses of APAP result in fulminant hepatic and renal tubular necrosis (Mitchell et al., 1973a). The toxicity results from the formation an arylating metabolite of APAP
(Mitchell et al., 1973b). The metabolite is N-aceyl-p-benzoquionone imide (NAPQI). Cytochromes 2E1, 1A2, 4A4 and 2A6 have been reported to oxidize APAP to NAPQI (James et al., 2003). APAP is a classic example of a compound detoxified by GSH (James et al., 1993). PARP-1 inhibition of acetaminophen has been studied using both specific and non-specific inhibitors. The inhibitors studied include nicotinamide, benzamide, caffeine, theophylline, thymidine, l-tryptophan (Kröger et al., 1996) N-acetylcysteine, l-Methionine (Kröger et al., 1997), chlorpromazine and 4-aminobenzamide (Ray et al., 2001).

Of the inhibitors used in APAP studies, nicotinamide, benzamide, theophylline (in the form of aminophylline hydrate) and 4-aminobenzamide were assessed. Aminophylline is 78% theophylline with the addition of ethylenediamine for saline solubility. No reproducible results were obtained with this inhibitor. Benzamide gave no positive findings. 4-aminobenzamide had solubility problems in saline. It is soluble in hot saline with constant stirring (Ray, personal communication). Solubility was obtained at the boiling point of saline. Upon sitting, over a period of approximately 12 hours, precipitate was observed. This inhibitor was not used as the heating to an elevated temperature may have reduced the efficacy or changed the chemical structure of the inhibitor.

This laboratory has conducted research in the area of PARP-1 inhibition of an environmental chemical, CCl₄. Because of its propensity to cause hepatotoxicity, CCl₄ is routinely used as a model compound for generating centrilobular necrosis. Su et al. tested for polymer formation. The study found that there were significant quantities of PARP-1 in areas of sever necrosis in the centrilobular region (Su et al., 2003). The
conclusion of this study was that the necrogenic effects of CCl₄ were in part explained by a rapid decrease in cellular energy due to overactivation of PARP-1. A follow-on study assessed the usage of a PARP-1 inhibitor to attenuate the CCl₄-induced hepatotoxicity. The PARP-1 inhibitor selected for this study was Phen. Phen was dissolved in 5.5% DMSO and concomitantly administered with CCl₄. Serum ALT was reduced significantly for CCl₄ and Phen. There was no significant difference in ALT for those treated with CCl₄ and those treated with CCl₄ + DMSO (5.5%) (Banasik et al., 2004). Animals treated with CCl₄ or CCl₄ + DMSO exhibited severe necrotic centrilobular lesions with pronounced areas of apoptosis (unpublished results). In the current experiment, Phen was dissolved in 100% DMSO. There was a statistically significant difference in ALT for BB, BB + Phen and BB + DMSO. The decrease in ALT for BB + DMSO is due to the concentration of DMSO. A concentration of 5.5% was insufficient to dissolve Phen in the current study. After vortexing, centrifugation and sonication, a slurry was formed. No positive results were observed using this standard at this concentration. It was concluded that the Phen was not being administered, but had precipitated. A solubility study confirmed that Phen was soluble in 100% DMSO.

When a PARP-1 inhibitor is administered with BB, there was a reduction of ALT, no histopathological findings, glutathione depletion and no lipid peroxidation. This leads to a conclusion that the attenuation of BB-induced hepatotoxicity is independent of its metabolism. It is also evidence that PARP-1 inhibitors, both specific and non-specific can attenuate BB-induced hepatotoxicity.

Two areas of further study of PARP-1 inhibition have been identified. The first is nephrotoxicity. A model compound for nephrotoxicity is 2-bromohydroquinone (BHQ).
BHQ is a metabolite of BB. BB is metabolized to the 3,4-oxide and further metabolized to o-bromophenol. In the liver o-bromophenol has been shown to form toxic metabolites which are then transported to the kidney (Lau et al., 1984). BHQ forms a diglutathione conjugate which has been shown to be nephrotoxic (Monks et al., 1985). This laboratory has shown that Phentolamine (Phe), an adrenergic antagonist, could modulate BHQ-induced nephrotoxicity (Harbison et al., 2000). Phe was shown in the past to attenuate the hepatotoxicity of BB (Kerger et al., 1988a).

Ethylene dibromide (EDB) is a model component for eliciting hepato- and nephrotoxicity. Conjugation with GSH has been shown to play a role in the bioactivation of EDB. This laboratory has shown that Phe attenuated the nephrotoxicity of EDB (Harbison et al., 2003).

PARP-1 inhibition attenuated the hepatotoxicity of BB. Based on past research using Phe in BB-induced hepatotoxicity, it is conjectured that PARP-1 can attenuate nephrotoxicity.

Another area of research is to test the hypothesis that PARP-1 inhibitors can attenuate hepatotoxicity in the midzonal region of the liver. A model hepatotoxicant that causes apoptosis in this region is cocaine. Apoptosis contributes significantly to cocaine-induced liver damage (Price et al., 1999). In the current study, PARP-1 attenuated apoptosis in the centrilobular region of the liver.

The results of this study extend previous observations of PARP-1 attenuation of CCl₄-induced hepatotoxicity (Banasik et al., 2004). The role of PARP-1 attenuation of BB-induced hepatotoxicity has been described. The importance of these findings have important public health implications because there are pharmaceuticals and industrial
chemicals that cause hepatotoxicity. This study has shown that a non-specific PARP-1 inhibitor attenuates BB-induced hepatotoxicity at a concentration of approximately half of the toxic level to humans. A specific inhibitor attenuates BB-induced hepatotoxicity at a concentration of 3,000% less than that of a non-specific PARP-1 inhibitor.
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ABOUT THE AUTHOR

Kelly W. Hall received her B.S. in Chemistry from the University of Florida in 1991. She was employed at Honeywell, Inc. in St. Petersburg, FL. She then went to work at Environmental Science and Engineering in Gainesville, FL, where she worked as a Senior Staff Laboratory Scientist. She joined Bausch & Lomb in Tampa, FL in 1997 as a Chemist. While employed at Bausch & Lomb, she completed her MPH in Safety Management at the University of South Florida. After completing her MPH, she entered the Ph.D. program in Toxicology at the University of South Florida. She completed her Ph.D. while continuing to work full time at Bausch & Lomb. Bausch & Lomb generously provided tuition assistance for both the MPH and Ph.D. programs.