THE EFFECTS OF HYDROQUINONE AND BENZOQUINONE ON THE TRANSCRIPTION FACTORS PU.1, AML-1, C/EBP, C-MYB AND GATA-2 IN HL-60 CELLS

by

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Abstract

Benzene, a common chemical solvent and component of cigarette smoke, is a ubiquitous environmental pollutant that most Canadians are routinely exposed to by inhalation. Benzene is classified as a group 1A carcinogen by the International Agency for Research on Cancer (IARC) and exposure to benzene is linked to both myeloid leukemia and aplastic anemia in humans. Benzene is metabolized to toxic reactive metabolites by CYP2E1 enzymes in the body and many studies have concluded that benzoquinone and hydroquinone are the most toxic of benzene metabolites. There are several transcription factors that play a significant role in hematopoietic differentiation. Of these transcription factors, PU.1, AML-1, C/EBP-α, c-Myb, and GATA-2 are considered the most crucial to differentiation. PU.1, AML-1, C/EBP-α, c-Myb, and GATA-2 are specific to cells in the hematopoietic system and play a critical role in hematopoietic differentiation as knockout mice deficient in any of these transcription factors possess impaired hematopoietic cells, eventually resulting in death of the animal. To test the hypothesis, that exposure of HL-60 cells to hydroquinone and benzoquinone will result in altered DNA binding activity and relative protein expression of PU.1, AML-1, C/EBP-α, c-Myb and GATA-2, HL-60 cells were plated at $5.0 \times 10^5$ cells/ml in 10 ml plates and exposed to either phosphate buffered saline, or equal parts hydroquinone or benzoquinone totaling 5, 10, 15, or 25 µM parts for 20 hours. Following exposure, cells were harvested and nuclear fractions were isolated and then used in a filter plate assay to determine DNA binding of the transcription factor. Western blotting was also performed on nuclear extract samples in order to confirm protein presence. Filter plate assays revealed no significant differences in DNA binding amongst the different treatment groups when assaying for PU.1, AML-1 and C/EBP-α.
DNA binding. However, c-Myb and GATA-2 DNA binding assays revealed a decrease in DNA binding when the 25 µM benzoquinone and hydroquinone treatment group was compared to the vehicle control. Western blotting performed on the nuclear extracts demonstrated the presence of PU.1, AML-1, C/EBP-α, c-Myb and GATA-2 with no observable difference in relative protein expression. This data suggests that altered DNA binding of c-Myb and GATA-2 may play a role in benzene-mediated toxicity.
Co-Authorship

The research conducted in this thesis was performed by the candidate, Joseph Cozzarin, under the supervision of Dr. Louise M. Winn.
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## List of Abbreviations

ALL: acute lymphocytic leukemia  
AML: acute myeloid leukemia  
AML-1: acute myeloid leukemia protein  
B-cell: B lymphocyte  
BER: base excision repair  
BQ: benzoquinone  
BT: benzotriol  
C/EBP-α: CAAAT enhancer binding protein  
CLP: common lymphoid progenitor cells  
CMP: common myeloid progenitor cells  
c-Myb: c Myb proto-oncogene protein  
CNS: central nervous system  
DTT: dithiothreitol  
EGF: epidermal growth factor  
EC: erythrocytes  
ES: eosinophil  
FBS: fetal bovine serum  
GATA-1: GATA binding protein 1  
GATA-2: GATA binding protein 2  
GMP: granulocyte monocyte progenitor  
GSH: glutathione  
GST: glutathione-S-transferase  
HQ: hydroquinone  
IARC: International Agency for Research on Cancer  
IKZF1: Ikaros family zinc finger protein 1  
LMO2: LIM only protein 2  
LT-HSC: long term hematopoietic stem cells  
MA: muconic acid  
MC: mast cell
MD: muconaldehyde
MEP: myeloid erythrocyte progenitor
MK: megakaryocyte
MPO: myeloperoxidase
MN/MP: monocyte/macrophage
MW: molecular weight
NQO1: NAD(P)H quinone oxidoreductase 1
NER: nuclear excision repair
NP: neutrophil
PDGF-α/ PDGF-β: platelet derived growth factor alpha/beta
PPB: parts per billion
PPM: parts per million
PU.1: purine rich box 1
RBC: red blood cell
ROS: reactive oxygen species
RPM: rotations per minute
SPM: S-phenyl mercapturic acid
ST-HSC: short term hematopoietic stem cells
SULT: sulphotransferase
TAL1: T-cell acute lymphocytic leukemia protein 1
TBP: TATA-box binding protein
T-cell: T lymphocyte
UGT: uridine 5’-diphospho-glucuronosyl transferase
VOH: volatile organic hydrocarbon
Chapter 1

Introduction

1.0 Benzene

Benzene, an aromatic hydrocarbon characterized by a six member ring, is a common environmental pollutant. It is a colourless, volatile and flammable substance with a sweet aroma. Benzene has been classified by the International Agency for Research on Cancer (IARC) as a group 1A carcinogen, meaning it is carcinogenic to humans (1, 2). Benzene has had many different applications in society that has led to its widespread use in many different industries such as rubber manufacturing, plastic synthesis, pesticides, pharmaceutical, adhesives, paint and petrol industries (1, 2, 3).

Benzene was initially discovered as a byproduct of the illuminating gas industry in the early nineteenth century. At this point in history it was common practice to use gases from whale oil and cod oil to provide lighting for homes. Benzene is released from whale and cod oil when it is distilled to produce these illuminating gases. It was observed that the gases produced oil after standing (3). In 1825 Michael Faraday isolated benzene from this oil and determined that it’s chemical structure was C₆H₆; it was not until 1865 that August Kekule inferred that benzene possessed its characteristic cyclic structure (3).

Later in 1845 it was discovered that benzene could also be isolated from coal at approximately 2 gallons of benzene per one ton of coal. Up until the 1940’s, coal refining was the major source of benzene production; however, after that time isolation of benzene from crude oil refinement became a significant source of benzene production. Petroleum refining eventually surpassed benzene production from coal refinement in the
early 1960’s giving yield to 600 million gallons (3). Today, benzene is primarily used as a chemical precursor for the production of organic chemicals such as styrene, phenol, cyclohexane, aniline, maleic anhydride, alkyl benzenes and chlorobenzenes. Benzene is also currently used as a precursor for the synthesis of compounds that are important in pharmaceutical and pesticide synthesis such hydroquinone (HQ), benzene sulfonic acid and benzene hexachloride (4). For example, HQ based creams are the most widely prescribed depigmenting agent for treating melisma, a skin pigmentation condition (5). Although benzene remains useful to society, its use has drastically been reduced in first world countries due to toxicity concerns.

1.1 Benzene and Human Exposure

1.1.1 Routes of Benzene Exposure

Benzene’s ubiquitous nature warrants the need for establishing safe environmental exposure concentrations as well as occupational exposure concentrations. Benzene is a volatile organic hydrocarbon (VOH) that partitions to its gas form at ambient room temperature. As a result, inhalation is the main route of human exposure, although ingestion and dermal absorption are also routes of exposure, albeit less significant (1). Regardless that benzene exposure in the environment is quite low, exposure can be increased significantly depending on certain factors such as geographical location, indoor air quality, smoking and occupational exposure (3).

1.1.2 Benzene Geographical Exposure

Because of benzene’s ubiquitous nature, humans can be exposed due to various factors. For example, geographical location can impact the amount of benzene an individual is exposed to. Individuals who reside in areas that are in close proximity to
high volume automobile traffic freeways/highways, gasoline filling stations or industrial parks are exposed to markedly higher airborne concentrations of benzene than individuals that don’t (6,7). Benzene levels in ambient air analyzed from 1989-1998 across Canada demonstrated this concept when rural, urban and industrial ambient air was analyzed for the presence of benzene. Rural ambient air contained 0.16 parts per billion (ppb) benzene, meanwhile in urban areas benzene was measured at 0.56-1.13 ppb. Benzene levels were found to be the highest in industrial ambient air with levels of 4.1 ppb. The reasoning behind this can be found by analyzing the components of gasoline. Benzene is an additive in gasoline which functions as an anti-knock agent to prevent engine knocking in automobiles. Federal guidelines state that benzene concentrations in gasoline cannot exceed 1.5% in Canada (9). When gasoline undergoes combustion in the engines of cars, benzene is released into the environment as a byproduct (6,7). Individuals employed in service stations are chronically exposed to levels of benzene ranging from 0.0002- 0.8 parts per million (ppm), while individuals refueling cars are exposed to acute concentrations ranging from 0.04-1.42 ppm. Health risk scenarios were determined from this exposure data at gas stations using hazard quotients and it was determined that gas station service workers may be at risk for adverse health effects, while customers experiencing acute exposure during refueling had minimal health risk (9).

1.1.3 Indoor Air Benzene Exposure

Another factor that must be examined in regards to non-occupational benzene exposure is indoor versus outdoor air exposure. Canadians spend approximately 90% of their time indoors and as a result inadvertently increase their exposure to benzene. Median ranges of benzene concentrations in indoor air have been measured as 0.5-2.2
µg/m$^3$ relative to 0.2-0.9 µg/m$^3$ in outdoor air. The increased concentrations can be attributed to attached garages as benzene from automobile exhaust, stored gasoline or other chemical solvents can migrate from garage to home if the garage has not been sealed appropriately (11). In addition, ordinary household products can contain benzene; some examples of household products that can contribute to indoor air concentrations of benzene are paints wax, or glues (12).

**1.1.4 Benzene and Cigarette Smoke**

Finally, the most significant source of non-occupational benzene exposure is from cigarette smoke. Benzene is contained within tar that is produced during the combustion of tobacco. Smokers experience the highest exposure levels of benzene; in fact, the amount of benzene emitted into ambient air per cigarette is 120-480 µg (13). The actual inhaled quantity of benzene ranges from 6-73 µg per cigarette. Importantly, individuals in close proximity to smoking can also inhale benzene due to the second hand smoke produced (14). The amount of cigarettes smoked is directly proportional to blood benzene levels as each cigarette smoked increased blood concentrations by 12 ng/L (15). Average levels of benzene in the blood of active smokers are 365 ± 178 ng/L as opposed to 176 ± 62 ng/L in non-smokers or 211 ± 85 ng/L in non-active smokers (16). Thus, smoking is one of the highest sources of benzene exposure for non-occupationally exposed individuals.

**1.1.5 Benzene Exposure in the Workplace**

Benzene is well renowned historically for its high exposure levels in various occupational settings. Benzene exposure in industries such as rubber manufacturing, printing presses, petroleum refining and shoemaking have been surrounded by
controversy almost indefinitely. Airborne benzene concentrations in these settings are renowned for possessing excessive levels of benzene in the work environment. It was noted that airborne benzene concentrations in Italian shoe factories (1942-1975) could range from 200-500 ppm due to the high benzene content in the glues that were utilized and handled. In Italian rotogravure plants (1942-1975) airborne benzene concentrations could reach 200-400 ppm with occasional peak exposures of 1500 ppm (17). Benzene’s application as a solvent has largely been eliminated at the current time in North America and Europe, however in countries with poorly developed health and safety legislation, benzene continues to be used at high levels and thus risk of exposure continues to occur. Although benzene has been replaced with less toxic solvents such as xylene and toluene, these solvents still contain trace amounts of benzene as an impurity (18). Canadian workplaces that utilize benzene are much safer than in the past as the current acceptable time weighted average exposure limit in Ontario workplaces is 0.5 ppm over the span of an 8 hour workday along with a ceiling limit of 2.5 ppm; these values have been implemented as there has been no reported toxicity associated with these levels (19).

1.2 Hematopoiesis

Benzene is known to disrupt hematopoiesis by targeting the bone marrow, which is a critical organ responsible for the process of adult hematopoiesis (20). Hematopoiesis describes the process of the formation of various mature blood components such as red blood cells (RBC) and white blood cells from immature multipotent stem cells. The formation of these mature blood cells begins in the bone marrow, specifically in the stroma, where rare hematopoietic stem cells (HSCs) are either replicating to ensure self-
renewal or committing to various blood cell lineages such as granulocytes (GC), macrophages (MP), erythrocytes (EC), megakaryocytes (MK), and more (Figure 1) (20).

These primary stem cells can be classified as long term and short term HSCs. The long term hematopoietic stem cells (LT-HSC) are pluripotent cells responsible for replenishing the population of HSCs in the bone marrow to ensure an appropriate amount for future differentiation (20). Short term hematopoietic stem cells (ST-HSC) are multi potential cells that have become committed to differentiation and lost their pluripotent nature (20). In this stage of hematopoiesis the ST-HSC have the potential to commit to either the myeloid or lymphoid lineage. The myeloid lineage is primarily responsible for the body’s innate immunity and the lymphoid system is primarily responsible for the body’s adaptive immunity (20). The initial cells in myeloid and lymphoid development are referred to as common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP). The common myeloid progenitor cells can further differentiate to either a megakaryocyte/erythroid precursor (MEP) or a granulocyte/monocyte precursor (GMP). Lymphoid progenitor cells are able to terminally differentiate into either a B-lymphocyte (B-cell) or T-lymphocyte (T-cell) cell, which play an important role in adaptive immunity as well as the production of antibodies. Finally, MEPs are able to differentiate to RBCs or MKs. The GMP cells are unique cells in that they have the ability to terminally differentiate into a variety of different cells such as mast cells (MC), eosinophils (ES), neutrophils (NP), and monocytes/macrophages (MN/MP), which all play critical roles in the innate immunity (20).
1.3 Benzene Toxicity

1.3.1 Benzene Acute and Chronic Toxicity

The recognition of toxicity following chronic and acute exposure to benzene has been dated back to the late nineteenth century. Benzene toxicity is able to manifest in the central nervous system (CNS) as well as the hematopoietic system. Acute exposure to high doses of benzene results in signs of CNS depression such as dizziness, ataxia, or in severe cases impaired breathing or even death. These effects are caused directly by benzene as it is a highly lipophilic compound that can easily partition to nervous system tissue (21). Although benzene causes CNS depressing effects acutely, it is better known for its role in disrupting hematopoietic processes. Acute exposure to benzene at low or high concentrations results in a decrease of circulating ECs, leukocytes and thrombocytes. It is known that chronic benzene exposure can lead to the development of various blood cancers and disorders described below (22).

1.3.2 Benzene and Blood Disorders

There are three hematological disorders associated with benzene exposure: pancytopenia, aplastic anemia and acute myeloid leukemia (AML). Firstly, pancytopenia is a broad term that describes a decrease in any type of blood cell. There are multiple types of cytopenias that are related to benzene exposure such as: leukopenias, thrombocytopenias and anemias. Leukopenia describes a condition in which there is a lack of white blood cells in circulating blood, while thrombocytopenia is a lack of platelets in circulating blood and anemia describes a decrease in RBCs in circulating blood (23). Additionally, aplastic anemia is a disease that is characterized by a damaged resident HSCs in the bone marrow. The lack of healthy progenitor cells results in a lack
of blood cells in circulation. In bone marrow that has been affected by this disease the HSCs are replaced by fat cells (24). Unlike the inhibition of hematopoietic cells in aplastic anemia, leukemia is marked by an acute or chronic increase in replication of dysfunctional HSCs inside the bone marrow. Thus, leukemia is characterized by an increase in proliferation of immature cells that are unable to perform their respective function. The cells proliferate inside the bone marrow eventually outcompeting healthy HSCs for space and resources. This proliferation of unhealthy cells leads to symptoms of impaired immune function, lethargy, high counts of abnormal white blood cells and fever (25). Benzene exposure is associated with AML, a type of leukemia characterized by an accelerated replication of immature myeloid cells in the bone marrow (24).

1.3.3 Benzene as a Carcinogen

The association between benzene and leukemia was difficult to demonstrate due to the lengthy lag times between exposure and onset of disease, which could reach a timespan of years before leukemia was able to manifest. Benzene gained group 1A carcinogen status by the IARC in 1970. Though early cases of benzene exposure and toxicity were reported as anecdotal evidence in the late 1800’s (26, 27), it was not until 1938 that the relationship between occupational benzene exposure and toxicity was first recorded. In the first reporting, which originated from benzene exposed workers evaluated from 1928-1938, 60 cases of aplastic anemia and 10 cases of leukemia were reported (28). Another study evaluating workers exposed to benzene, was conducted in Milan over the span of 1942-1975. In this report, 11 cases of aplastic anemia and 7 cases of leukemia were discovered out of 66 cases of hemopathy reported (17). Furthermore, from 1967-1973, 26 patients with AML were observed in a population of 28, 500 shoe
workers exposed to benzene in Istanbul (29). The observed rate of leukemia amongst workers chronically exposed to benzene was 13/100,000 compared to 6/100,000 in the general population (29). Another study evaluating American workers in a rubber manufacturing plant reported significantly elevated cases of myeloid leukemia in workers exposed to benzene (30). This study is especially powerful due to the fact that these workers were exposed exclusively to benzene solvents; the more solvents being used in the workplace the more difficult it becomes to associate benzene with leukemia as the use of other solvents creates confounding variables. Another issue to note in this study is the fact that the exposure concentrations were recorded at 10-15 ppm. The American workers exposed to benzene had much lower exposure levels compared to Turkish and Italian shoe workers (200-400 ppm), yet a 5-fold increase in the risk of leukemia was observed in this study. Concerningly enough, these concentrations of benzene were considered safe at the time (30). One of the largest studies conducted with respect to benzene exposure and leukemia, examined Chinese workers exposed to benzene during the time span of 1972-1987. This study included a cohort of 74,828 benzene exposed workers and 35,805 unexposed workers marking it the largest benzene exposure cohort study currently published. The findings of the study demonstrated that the benzene exposed group had increases in AML, aplastic anemia, myelodysplastic syndrome, and malignant lymphoma (31). This study reinforced benzene as a carcinogen and aided in classification of benzene as a group 1A carcinogen by the IARC.

1.3.4 Leukemia in Canada

Cancer is a prolific disease in the Canadian public. Approximately 2 in 5 Canadians will be diagnosed with cancer and 1 in 4 Canadians will die from cancer (32).
The lifetime probability of developing cancer in males and females from 2010 data was 45% and 42% respectively. Of new cancer cases predicted to be diagnosed in Canada in 2016, leukemia was estimated to comprise 3.4% of all cancers in males and 2.4% in females. The lifetime probability of developing leukemia in Canadian males was 1.9% and 1.4% in Canadian females for the year of 2016. Leukemia has a rare incidence of occurrence in adults compared to children (32).

1.3.5 Childhood Leukemia

The most common cancers diagnosed in children are leukemia, lymphoma and CNS cancers (32). Between 2006 to 2010, children in the age group 0-14 were the most likely to be diagnosed with leukemia accounting for 32% of all diagnoses. Of all leukemia’s diagnosed in children, 78% are classified as acute lymphocytic leukemia (ALL) and 22% as AML (32). Leukemia rates in children have remained fairly static in Canada. On the other hand, increases in incidences of leukemia have been observed in Europe by 0.6% per decade over the span of 1978-2004 (33). The USA has also seen increases in cases of leukemia amongst children by as much as 1.6% annually from 1974-1991 (34). One of the possible factors responsible for this increasing trend in leukemia could be due to increasing exposure of children to chemicals found in cigarette smoke, pesticides, automobile exhaust, paint and household cleaners (35). Of all the environmental risk factors identified, only ionizing radiation and benzene have been demonstrated to be linked to acute leukemia. Determining environmental risk factors for acute leukemia is arduous as quantifying exposures of chemicals retrospectively is unreliable. Furthermore, insufficient sample sizes due to childhood leukemia’s infrequency can also cause difficulties, as well as variants of leukemia, and lack of
knowledge in regards to mechanistic toxicology (36). Future improvements in analytical techniques to determine exposure levels in children as well as prospective cohort studies may reveal risk factors currently not understood.

1.4 Benzene Metabolism
1.4.2 Metabolism and Toxicity

Benzene metabolism plays a considerable role in benzene toxicity as metabolites have been determined to cause detrimental effects in tissues (3). In general, exposure of mice to benzene results in a lowered incorporation of iron in ECs, as well as increased levels of benzene metabolites in bone marrow (39). Mice treated with both toluene and benzene, did not display any apparent hallmarks of benzene toxicity such as lowered iron intake in erythorcytes (EC) or the presence of benzene metabolites in the bone marrow. This particular study demonstrated that toluene, a competitive inhibitor of benzene metabolism, is capable of preventing benzene toxicity. These findings display that benzene must undergo metabolism in order to cause toxicity (39). To further reinforce this concept, rats which had 70-80% of their liver removed had lower levels of hematotoxicity as well as lower levels of metabolism, following exposure to benzene (40). Pre-treatment of mice with 3-methylcholanthrene, beta-naphthoflavone, ethanol or acetone increased benzene metabolism as well as benzene toxicity due to their stimulating effects on pathways responsible for benzene metabolism (41, 42). CYP2E1, a cytochrome P450 enzyme, is the primary enzyme responsible for inducing benzene toxicity via the production of toxic metabolites (42, 43). Blocking CYP2E1 activity with a specific inhibitor, propylene glycol, resulted in lowered levels of genotoxicity in mice dosed with benzene by half compared to mice exposed to benzene that did not receive
propylene glycol (44). The most convincing argument for the role of benzene metabolism in benzene toxicity originates from the use of transgenic mice. CYP2E1 knockout mice exposed to benzene had a marked reduction of benzene metabolites in urine by 90% alongside a complete block in hematotoxicity compared to wildtype mice (45). This study ultimately confirmed that CYP2E1 was the primary enzyme responsible for metabolism and toxicity of benzene in mice.

1.4.3 Pathway of Benzene Metabolism

In order to understand the metabolism of benzene, it is important to consider where it is metabolized in the body and what metabolites are produced (Figure 2). Benzene metabolism occurs primarily in the liver with small amounts of metabolism occurring in the lung. Benzene intermediate metabolites can also be further metabolized inside the bone marrow (53). CYP2E1 is highly expressed in liver, specifically in the hepatic triad in proximity to the central vein. CYP2E1-mediated metabolism of benzene is a very complex pathway that includes many different metabolites and enzymes (Figure 2). The initial step of benzene biotransformation includes the insertion of an oxygen into the benzene ring via the CYP2E1 enzyme. This results in the formation of benzene oxide, a very unstable electrophilic compound. Benzene oxide can undergo phase 2 transformation via glutathione-S-transferase (GST). Glutathione (GSH) conjugated to benzene oxide is metabolized by gamma-glutamyltransferase to facilitate the removal of the glutamyl and glycine groups. The remaining product is a cysteine conjugate that can then be acetylated on the amino group of the cysteine residue by N-acetyl-transferase. The N-acetyl S-conjugate, a non-toxic metabolite, is termed mercapturic acid and is excreted by the kidney into the urine. In the case of benzene metabolism, the mercapturic
acid metabolite is termed, S-phenylmercapturic acid (SPM), a urinary metabolite (37, 54). Typically, GST metabolism results in non-toxic metabolites, however there are some cases where GST metabolism can produce reactive metabolites. For example, 2-glutathion-S-acyl HQ can be easily oxidized to form a GSH-1,4-BQ. This reductive addition reaction can continue until many products are formed such as: GS-HQ, 2,3-(GS)-HQ, 2,5-(GS)-HQ, 2,6-(GS)-HQ, 2,3,5-(GS)-HQ, and 2,3,4,5-(GS)-HQ. This cycle depletes GSH in the body which can contribute to hematotoxicity (54, 55).

Another fate of benzene oxide is to spontaneously form equilibrium with its valence tautomer, benzene oxepin. This tautomer can undergo ring opening through a mechanism that remains unclear. From what is understood, the ring opened product forms a reactive α-β-unsaturated aldehyde termed, cis-cis-muconaldehyde. This intermediate then isomerizes to cis-trans and then trans-trans. The muconaldehyde (MD) must then undergo oxidation via aldehyde dehydrogenase to yield trans-trans muconic acid (MA), a major urinary metabolite (56, 57). All isomers of MA are thought to be hematotoxic due to their electrophilic nature. As much as 0.05% of administered radiolabeled MA has been shown to reach the bone marrow in mice. However, the mechanism of MA’s journey to the bone marrow is not well characterized (58).

Another pathway that benzene oxide can follow is metabolism by epoxide hydrolase. The enzyme epoxide hydrolase facilitates the conversion of the epoxide group to two hydroxyl groups called a dihydrodiol group. The resultant product from the epoxide hydrolase reaction is the benzene dihydrodiol (59). Benzene dihydrodiol can then be metabolized by dihydrodiol dehydrogenase, which results in the production of a catechol (60).
Lastly, the final option for benzene oxide is to spontaneously rearrange to form phenol. Phenol is considered a toxic substance due to its corrosive nature as well as CNS-depressing effects. Although phenol may cause toxic effects, it is not considered to be directly hematotoxic (61). Phenol is able to cause hematotoxicity indirectly through metabolism by CYP2E1 to yield quinone metabolites including HQ or catechol. Catechol is toxic mainly due to its ability to form reactive metabolites that can promote the formation of reactive oxygen species (ROS). Catechol in the bone marrow can be metabolized by MPO to form 1, 2- BQ. This process is reversible in the presence of the enzyme, NAD(P)H quinone oxidoreductase 1 (NQO1). Catechol can also undergo metabolism by CYP2E1 to yield 1, 2, 4- benzenotriol (BT). If the phenol molecule is converted to HQ through CYP2E1, the HQ can be metabolized by MPO to form a 1, 4-BQ which can be reversed by NQO1. Similar to catechol, HQ can be metabolized by CYP2E1 to yield 1, 2, 4- BT. All phenolic metabolites have the potential to be conjugated via sulfotransferase (SULT) or UDP-glucoronyltransferase (UGT), resulting in detoxification of these reactive metabolites. Eventually, the metabolites phenol, catechol, HQ, MA, and SPM can be found excreted in urine (Figure 2) (37, 62).
Figure 2: Schematic of benzene metabolism. CYP2E1, cytochrome P450 enzyme 2E1; GST, glutathione S-transferase; MPO, myeloperoxidase; NQO1, NAD(P)H:quinone oxidoreductase 1; ADH, aldehyde dehydrogenase.
1.5 Mechanism of Benzene Toxicity

1.5.1 Reactive Oxygen Species

Although it is understood that metabolites of benzene play a key role in benzene toxicity, the mechanism of how this occurs is a matter of speculation. From what is understood of benzene toxicity, it is thought that ROS play a powerful part in the mechanism of toxicity. ROS defines a category of chemically reactive molecules containing oxygen. These ROS can contain one or more unpaired electrons indicating their reactive nature. There are a number of ROS: superoxide, peroxyl, hydroperoxyl, hydroxyl, hydrogen peroxide, and hypochlorous acid (63, 64). ROS are an endogenous product of metabolism, cellular respiration, and other cellular processes (63). Although ROS are naturally occurring in the body, xenobiotics such as benzene are capable of generating increased levels of ROS in vitro and in vivo (65). ROS are highly reactive molecules that can target macromolecules such as DNA, RNA, proteins, and lipids, promoting oxidative damage (66). Different ROS have varying levels of oxidative activity. For example, superoxide is considered to have the weakest oxidative potential as it can only inactivate a select few enzymes, including nicotinamide adenine dinucleotide phosphate dehydrogenase (67). In addition, protective enzymes such as superoxide dismutase are able to convert superoxide into the even less reactive hydrogen peroxide. Hydrogen peroxide does not contain an unpaired electron and thus is not a strong oxidant. Hydrogen peroxide is able to be metabolized by another protective endogenous enzyme, catalase. Catalase facilitates the conversion of hydrogen peroxide into harmless water and oxygen. However, if hydrogen peroxide evades catalase it may eventually react to form the highly reactive hydroxyl radical. The formation of hydroxyl from hydrogen
peroxide, termed the fenton reaction, is catalyzed by the presence of Fe (II). The
hydroxyl radical is a highly reactive and short lived molecule that can react with virtually
any macromolecule in the cell. This ROS is unable to be metabolized by protective enzymes such as catalase and superoxide dismutase. The only antioxidants capable of quenching this reaction are free radical scavenging proteins like GSH (65, 69).

As indicated above, ROS can cause oxidative damage to various macromolecules. These oxidative events can be categorized as lipid peroxidation, protein oxidation, and DNA oxidation. Firstly, lipid peroxidation describes the chain reaction of repeating fatty acid damage. Lipid peroxidation must be initiated by a free radical extracting an electron from an unsaturated fatty acid. This is typically carried out by either hydroxyl radicals or a lipid peroxyl radical. After the free radical has reacted with the fatty acid chain the chain is left with an unpaired electron that can now react with a dioxygen group to yield a lipid peroxyl radical. This lipid peroxyl radical is free to react with another fatty acid chain to form another lipid radical and a lipid peroxide. This radical chain reaction can be terminated by two fatty acid chain radicals reacting with one another, or interaction with either GSH or vitamin E (70).

Proteins are also common targets for free radical damage due to being present in high levels in cells as well as their high affinity for free radicals. Proteins have many sites where free radical damage can occur such as side chains and the backbone of an amino acid. Oxidative damage of protein side chains can impact a number of factors such as polarity, protein folding and substrate binding. Changes in the structure of proteins can ultimately lead to changes in function. (71, 72). Finally, the most important target of oxidative damage is considered to be DNA due to the potential for mutagenesis. DNA
oxidation describes the concept of the formation of oxidized DNA bases from free radical species. The most commonly oxidized DNA base is deoxyguanosine as it has the highest oxidation potential. This oxidized DNA base is named, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and is a suitable marker for DNA oxidation (73). This oxidative damage can induce genotoxic effects that may lead to mutagenic events if not repaired by DNA repair mechanisms. Some of these DNA repair mechanism include base excision repair (BER), nucleotide excision repair (NER), recombinant repair, and mismatch repair (73).

1.5.2 Benzene Metabolites and Production of ROS

Benzene and its metabolites are capable of producing ROS which are believed to be one of the underlying causes of benzene toxicity (74). The benzene metabolites BQ, HQ, BT, MD, catechol and phenol have all been shown to produce ROS in HL-60 cells (74, 75). Certain metabolites have been demonstrated to be more toxic than others. For example, BQ was found to display the most toxicity among metabolites due to its ability to induce the most DNA damage (76). BQ has also been shown to cause the most cell death and DNA strand breaks of all metabolites (77). In addition to causing DNA damage, BQ has also been shown to initiate the highest levels of ROS as well as lipid peroxidation in HL-60 cells (75).

The mechanism by which benzene metabolites form ROS is currently not well understood. It was initially thought that redox cycling between BQ and HQ metabolites generated ROS. It was later disproven as HQ and BQ redox cycling can only occur at a pH ≥ 9.85; this redox cycling occurs at a pH value that is ≥ than the PKₐ of HQ (80). Another, theory for benzene ROS production involves the fenton reaction. Specific benzene metabolites including HQ, catechol and BT are capable of releasing free Fe(II)
from the carrier protein, ferritin. Unbound Fe(II) is then able to interact with hydrogen peroxide produced by benzene metabolites. This would lead to the production of hydroxyl radicals due to the fenton chemistry (Figure 3) (81). Another proposed mechanism of ROS generation involves GSH. In this case phenol reacts with MPO to yield a phenoxy radical which then abstracts a hydrogen from GSH resulting in a phenol and a thyl radical. This thyl radical then forms a disulfide bridge with any encountered thiol group. As a result of the disulfide bridge, a superoxide anion is formed (Figure 3) (82).

Figure 3: A-Illustration of the Fenton reaction. B- Mechanism of GSH mediated production of ROS involving thyl radical (GS●) and glutathione disulfide bridge (GSSG).
1.5.4 Benzene Genotoxicity

Genotoxicity describes the ability of a xenobiotic to induce some form of DNA damage that can lead to mutations and ultimately cancer. Benzene metabolites have been shown to induce genotoxic events in human lymphocytes and HL-60 cells as evidenced by increased levels of 8-OHdG, a marker of genotoxicity (84). Additionally, benzene has been shown to cause the formation of micronuclei in mouse bone marrow cells as well as in human cells (85, 86). Hydroxy benzene metabolites such as phenol, HQ, catechol, and BT have also been shown to increase micronuclei formation in benzene-treated CD-1 mice ECs and lymphocytes. In addition, the benzene metabolites HQ and phenol caused chromosomal aberrations in vivo and in vitro (88). The ability of benzene to covalently bind to DNA and cause DNA adduct formation has also been assessed in mice exposed acutely and chronically to benzene (89). Studies employing $^{32}$P-postlabelling consistently failed to detect adduct formations in bone marrow and liver from benzene exposed mice suggesting that benzene DNA adducts do not play a large role in benzene toxicity.

Although, micronuclei formation was present in rodent benzene exposure studies, human population studies displayed a lower rate of micronuclei formation following benzene exposure (89-91). This is likely due to the fact that humans are exposed to lower levels of benzene than experimental animals as humans exposed to greater than 1 ppm benzene displayed results consistent with rodent and human cell line studies. In vitro studies using human cells demonstrated similar micronuclei results to rodent in vitro studies (91). It is important to recognize that genotoxicity does not necessarily translate to mutagenicity. Currently there is inconclusive evidence to suggest that benzene is a mutagen in humans.
In conclusion, animal and human *in vitro* studies indicate that benzene metabolites display a similar genotoxicity, specifically exhibiting clastogenic effects (91).

**1.5.5 Benzene Metabolites and Inhibition of Differentiation**

Another proposed mechanism of benzene toxicity involves the inhibition of differentiation of hematopoietic progenitor cells. *In vitro* studies using mice stromal cells exposed to benzene demonstrated an inhibition of cell differentiation into functional hematopoietic cells (93). Another *in vitro* study in mice stromal cells showed a decrease in the formation of mature granulocyte and monocyte cells following exposure to the benzene metabolites HQ and BQ (93, 94, 95). The benzene metabolites HQ and BQ, have also been shown to inhibit differentiation of human HL-60 cells into granulocyte and monocyte cells (96). Inhibition of differentiation may lead to high amounts of immature blood cells, ultimately reducing amounts of mature functioning blood cells and leading to a compromised immune system.

**1.6 Transcription Factors**

**1.6.1 Transcription Factors and Hematopoiesis**

Hematopoiesis is a highly regulated process that involves a fine balance of production and breakdown of blood components (102). During this process, it is critical that cells maintain proper levels of proliferation, quiescence, and differentiation in order to facilitate hematopoiesis. Transcription factors govern all stages of differentiation and can influence the type of blood cell that will be produced depending on which transcription factors are expressed in the cell. Transcription factors can communicate with other transcription factors through protein-protein interactions and post translational modifications, which can attenuate transcriptional activity leading to changes in cell
morphology (102). Disruptions in transcription factor signaling from inappropriate activity, expression, and formation of transcription factors can trigger leukemic events (102). In particular, the five transcription factors that were examined in this study were PU.1, C/EBP-α, AML-1, GATA-2, and c-Myb.

1.6.2 PU.1 Transcription Factor

The purine rich box 1 protein (PU.1) is an essential transcription factor that is encoded by the Sfpi1 gene and plays a powerful role in hematopoiesis as it is required at both early and late stages of lymphoid and myeloid development (104). PU.1 contains 272 amino acids with a molecular weight (MW) of 31kDa and belongs to the E26 transformation-specific family transcription factor family (103). PU.1 possesses certain functional domains such as an Ets domain that harbors a winged helix-turn-helix, which recognizes DNA containing the GGAA motif otherwise known as “the purine rich box”. In order to demonstrate the importance of PU.1, knockout alleles of Sfpi1 in mice were studied. Sfpi1 null mice studies demonstrate that PU.1 is required in order to produce most myeloid lineages such as monocytes, granulocytes, dendritic cells as well as lymphoid cells like B cells (105, 106). PU.1 is highly expressed in the myeloid lineage with expression increasing dramatically during differentiation to granulocyte-monocyte cells and decreased expression observed during differentiation to megakaryocyte, EC, B cell, and T cell lineage (107). The importance of maintaining appropriate levels of PU.1 is clear, as reduced PU.1 expression inhibits hematopoietic cells from communicating with other cells inside the microenvironment of the stroma. This lack of communication can lead to irregular proliferation and immature cell phenotypes as seen in AML patients (119).
1.6.2 C/EBP-α Transcription Factor

Another transcription factor that plays an important role in hematopoiesis is C/EBP-α. This transcription factor belongs to the CCAAT/enhancer binding protein family. C/EBP-α is an isoform of the C/EBP family that is encoded by the CEBPA gene and has a MW of 42kDA (120, 121). C/EBP-α contains a leucine zipper domain as well as a DNA binding domain that recognizes the CCAAT consensus sequence (122). C/EBP-α appears to be particularly important during myeloid development as demonstrated by the inhibition of granulopoiesis in 32D cl3 cells when C/EBP-α was blocked (123). Similarly, in C/EBP-α deficient mice there was a lack of differentiated granulocytes measured in blood samples compared to wild-type animals (124). It is thought that the loss or downregulation of expression of C/EBP-α could contribute to the progression of AML (132). Patients with AML have been observed to exhibit impaired C/EBP-α transcription and translation mainly from protein-protein interactions due to the acute myeloid leukemia-eighty-twenty one fusion protein (AML1-ETO) and breakpoint cluster region- Abelson murine leukemia fusion protein (bcr-abl) (130, 131). Post-translational modifications are another mechanism for disruption of C/EBP-α as extracellular signalled kinase 1/2 (ERK1/2) mediated phosphorylation has been demonstrated to inhibit C/EBP-α function (132). In addition, there have been heterozygous mutations in the C/EBP-α locus identified in AML patients. It is evident that interfering with C/EBP-α can lead to disruptions in maintaining proper cell growth and proliferation in hematopoiesis, which may ultimately lead to leukemia (133).
1.6.3 AML-1 Transcription Factor

Transcription factor acute myeloid leukemia 1 protein (AML-1) is another important constitutively expressed protein involved in the complex process of hematopoiesis. AML-1 is composed of 453 amino acids that are encoded by the \textit{AML1/RUNXI} gene. AML-1 is composed of a number of sub units including a conserved DNA binding motif termed, Runt, which is able to target the PEBP2 consensus sequence (134). This DNA binding is achieved through a protein-protein interaction with CBFβ protein as well as other co-activators or co-repressors such as p300 and CREB binding protein (135). AML-1 is key transcription factor in hematopoiesis as disruption of this protein is associated with \textit{in utero} death in mice and hematopoietic disorders such as AML in humans(136). Furthermore, AML-1 knockout mice die at midgestation by embryonic day 12.5 (E12.5) due to a complete hematopoietic failure to produce innate immune cells as well as high levels of hemorrhage in the CNS (137). In addition, bone marrow cells from AML-1 deficient mice bone marrow cells demonstrated an inhibition of megakaryocytic development, increased presence of immature hematopoietic progenitor cells and irregular T- and B-lymphocyte development (139). Finally, AML-1 disruptions are present in patients suffering from AML as translocations at breakpoint t(8;21) are the most commonly reported mutations in AML patients (140). This translocation leads to the formation of the fusion protein, AML1-ETO, which acts to inhibit regular AML-1 as well as other transcription factors essential to hematopoiesis. This suggests that loss of function of AML-1 is required for leukemogenesis (141).
1.6.4 c-Myb Transcription Factor

c-Myb is yet another transcription factor known for its important role in 
hematopoiesis. C-Myb belongs to a small family of transcription factors in the Myb 
proto-oncogene family. This family is comprised of three similar transcription factors: a, 
b, and c-Myb (142). The c-Myb transcription factor is a 78-kDA protein that is encoded 
by the c-Myb gene and binds to its consensus sequence, PyAACG/TG (143, 148). 
Hematopoietic progenitor cells have been shown to possess the highest amounts of c- 
Myb expression with levels decreasing as cells differentiate to more mature phenotypes 
(149). It is important to recognize the c-Myb expression gradient among differentiating 
cells, as overexpression halts differentiation of myeloid cells (150). In addition, c-Myb is 
not expressed in terminally differentiated myeloid cells (151). Multilineage blood cell 
studies in fetal mice displayed hematopoietic defects caused by knockout c-Myb alleles. 
These cells were unable to differentiate into myeloid and erythroid cells even though 
there were ample supplies of immature HSCs (152). The key defect in c-Myb 
upregulation appears to be a lack of differentiation as well as altered proliferation of 
cells, which could play a role in the onset of a leukemic event as maintenance of 
progenitor cells in an immature state is a pathway to AML (152, 153).

1.6.5 GATA-2 Transcription Factor

GATA binding protein 2 (GATA-2) is another transcription factor that is essential 
to the differentiation and proliferation of HSCs. This transcription factor is composed of 
480 amino acids collectively weighing 50.5 kDA. This protein belongs to the GATA 
gene family and is encoded by the GATA2 gene (156). The GATA family proteins target 
similar consensus sequences in target gene promoters, (T/A)GATA(A/G), from which
their name is derived. This DNA binding occurs through a highly conserved DNA binding domain that consists of an amino and carboxy terminal zinc fingers (157). GATA-2 is expressed in primitive erythroid, mast and megakaryocyte cells, but more importantly high levels of expression are present in early pluripotent adult and fetal HSCs. This was demonstrated in in vivo mice studies which addressed the requirement for GATA-2 in the survival of early progenitor cells. In these studies, GATA-2 null mouse embryos were reported to die at embryonic day 10 or 11, as a result of anemia due to hematopoietic failure (158, 159). Furthermore, embryonic stem cells with a GATA2 knockout demonstrated impaired production of all blood cell types with similar results observed in adult hematopoiesis GATA-2 knockout studies (160). Another in vitro study examined forced expression of GATA-2 and its effects on differentiation. Upon addition of GATA2/GFP vector in gene-modified bone marrow Sca-11 Lin2 cells, cell differentiation and proliferation was blocked in a dose dependent manner (162). It appears that GATA-2 is highly expressed in immature phenotypes and expressed at low levels in mature phenotypes supporting its role in proliferation and maintenance of HSCs (162). Due to its role in proliferation, GATA-2 has been found to be overexpressed in individuals suffering from leukemia. In fact, individuals with AML that overexpress GATA-2 display a poorer prognosis than those with regular expression (171). In general, transcription factors that impact survival, proliferation and differentiation will naturally play a role in leukemia if they display erratic behavior (172).
1.7 Research Hypothesis And Objectives

1.7.1 Hypothesis

The hypothesis for this study was that *in vitro* exposure to the benzene metabolites, BQ and HQ, will alter transcription factor DNA binding and relative expression of PU.1, C/EBP-α, AML-1, c-Myb, and GATA-2 in HL-60 cells. Specifically, I hypothesize that exposure to HQ and BQ will decrease DNA binding and relative expression of the transcription factors PU.1, C/EBP-α and AML-1, while simultaneously increasing DNA binding and relative expression of c-Myb and GATA-2.

1.7.2 Objectives

Objective 1:
To determine the impact of BQ and HQ exposure on the DNA binding activity of PU.1, C/EBP-α, AML-1, c-Myb, and GATA-2 in HL-60 cells.

Objective 2:
To determine whether BQ and HQ exposure altered the relative expression levels of PU.1, C/EBP-α, AML-1, c-Myb, and GATA-2 in HL-60 cells.
Chapter 2

Materials And Methods

2.1 Materials

Cell culture media was obtained from Cedar Lane (Burlington, Ontario, Canada). Fetal bovine serum (FBS) was obtained from GE Healthcare Life Sciences (Logan, Utah, USA). Filter plate assay kits were obtained from Signosis (Santa Clara, California, USA). BQ and HQ were obtained from Sigma-Aldrich Chemical CO. (St. Louis, Missouri, USA).

2.2 Cell Culture

HL-60 cells were obtained from American Type Culture Collection (Manassas, Virginia, USA). HL-60 cells are a human promyelocytic cell line derived from a 36 year old Caucasian female. This cell line possesses the ability to commit to either granulocyte or monocyte cell lineages when exposed to various inducing agents. As a result, this cell line serves as a valuable model for studying differentiation of HSCs. HL-60 cell cultures were maintained in Isocove’s Modified Dulbecco’s Media supplemented with 20% FBS as well as 10 U/ml Penicillin/Streptomycin from Sigma-Aldrich Chemical CO (St. Louis, Missouri, USA). Cell cultures were maintained in a humidified incubator at 37 °C and 5% carbon dioxide.

2.3 BQ and HQ Exposure

BQ and HQ were dissolved in phosphate buffered saline (PBS). Cells were plated at a concentration of 5.0 x 10⁵ cells/ml and exposed to either vehicle control (PBS) or equal parts BQ and HQ totaling 5, 10, 15, 25 µM for a period of 20 hours.
2.4 Nuclear Extraction

Following exposure, cells were harvested and nuclear content was extracted using a nuclear extraction kit obtained from Signosis. Briefly, cells were centrifuged at 500 x g for 5 minutes and washed with 1X cold PBS. Rinsed cells were again centrifuged at 3,000 rpm for 5 minutes. A 1 X lysis buffer was diluted from a 10X stock. In addition, a proprietary protease inhibitor and dithiothreitol (DTT) solution were diluted in the 1X lysis buffer to a 1:100 dilution. Lysis buffer was added to cells and centrifuged at 4 °C for 10 minutes at 200 rpm. Cells were then centrifuged at 12,000 rpm for 5 minutes at 4 °C. The supernatant was discarded and the cell pellet was added to a 1X nuclear extract buffer along with an added proprietary protease inhibitor (1:100) and DTT solution (1:100). Cell extract with nuclear extraction buffer was then incubated at 4 °C for 2 hours at 200 rpm. Extract was then centrifuged at 12,000 rpm for 5 minutes at 4 °C. The supernatant nuclear extract was collected and stored at -80°C until further analysis. The obtained nuclear extracts were analyzed for protein content by employing the Bradford assay according to the manufacturer’s instructions (Bio-Rad, Mississauga, Ontario, Canada). An Ultrospec 3100 Pro scanning spectrophotometer (BiochromLimited, Cambridge, UK) was used to quantify Bradford calibration curve and sample data.

2.5 Filter Plate Assay

Filter plate assays from Signosis were employed to quantify DNA binding in HL-60 cell nuclear extracts. These assays were performed using the Signosis filter plate protocol. Briefly, a master mix was prepared containing 2 µg of protein from nuclear extracts of various treatment groups was prepared. The extract was incubated for 30 minutes at 16°C with various biotin labeled probes for the proteins: PU.1, C/EBP-α,
AML-1, c-Myb, and GATA-2. The master mix was applied to a filter plate with incubation over ice for 30 minutes. The filter plate was then centrifuged for 2 minutes at 600 x g in order to rinse unbound probe as well as other particulates. The filter plate was then washed with filter wash buffer by centrifugation at 600 x g for 2 minutes. This wash was repeated 3 more times. In order to elute probe bound to transcription factor, the filter plate was incubated with elution buffer and centrifuged at 600 xg for 2 mins. The eluent was then heated at 95°C for 3 minutes and immediately placed over ice. Hybridization buffer was added to a hybridization plate and previous sample was transferred into each corresponding well of hybridization plate with hybridization buffer. The plate was sealed and allowed to incubate overnight at 42°C. Buffer was removed from the wells and each well was washed with warm plate hybridization buffer. Blocking buffer was then added and incubated for 15 minutes at room temperature. Streptavidin-Horseradish peroxidase was added in a 1:500 dilution to each well and incubated for 45 minutes at room temperature. The plate was then rinsed with detection wash buffer. Proprietary substrate A and B was diluted in substrate dilution buffer and added to each well and incubated for 1 minute. The plate was then placed in a LUMIstar Galaxy luminometer (BMG Labtech, Ortenberg, Germany) and equilibrated for 5 minutes. Samples were analyzed with the following luminometer parameters: integration time 1 second and gain 130. Samples were analyzed in triplicate and the average was displayed as the relative light value unit per well.

2.6 Western Blotting

Nuclear extracts were resolved in a 8% acrylamide gel for 1 hour and 45 minutes at 80V immersed in 1X running buffer. Proteins were transferred overnight with a voltage
of 20V in transfer buffer onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 3% skim milk (PU.1, C/EBP-α, c-Myb antibodies) or 5% bovine serum albumin (BSA) (AML-1 and GATA-2) in tris-buffered saline containing tween (TBST) for 1 hour. Primary antibodies, PU.1 (#2266), AML-1(#4336), C/EBP-α (# 8178), GATA-2 (#4595), c-Myb (#12319) were obtained from Cell Signaling Technologies (Danvers, Massachusetts, USA) and were diluted in respective blocking buffer at 1:1000 dilution. Antibodies in TBST were incubated with the membrane for a period of 1 hour and then membranes were rinsed three times with TBST. Secondary antibodies, obtained from GE Healthcare Sciences (Mississauga, Ontario, Canada), were diluted (1:10000) in respective buffer (3% milk or 5% BSA). Secondary antibody consisted of a Horseradish peroxidase tagged antibody specific to either mouse IgG (used for TATA-box binding protein (TBP) antibody) or rabbit IgG (used for PU.1, C/EBP-α, AML-1, c-Myb, and GATA-2). Secondary antibodies diluted in TBST were incubated with membranes for 1 hour and rinsed with TBST. The membranes were then exposed to Western Lightning Plus 40 ECL, Enhanced Chemiluminescence Substrate, from Perkin Elmer (Waltham, Massachusetts, USA), and allowed to incubate for 1 minute. Membranes were then exposed to X-ray film in order to visualize results, which were quantified using densitometry. Membranes were reprobed with the loading control antibody TBP (#51841) diluted at 1:2000, obtained from Abcam (Cambridge, Massachusetts, USA).

2.7 Trypan Blue Exclusion Assay

Cell death was measured 20 hours following exposure of cells to BQ and HQ using trypan blue dye and a hemocytometer. An aliquot of cells was taken and diluted to
a 1:1 ratio with 0.4% trypan blue dye obtained from Sigma-Aldrich Chemical CO (St. Louis, Missouri, USA). Viable cells (unstained) were counted amongst blue stained cells. Total viability count was determined from the ratio of blue cells to unstained cells.

2.8 Statistical Analysis

Statistical analysis was performed using a one way analysis of variance test for filter plate, western blot, and trypan blue exclusion results. Post Hoc analysis was conducted by performing a Dunnet’s multiple comparison test. All statistical tests were carried out using Prism 7.0, GraphPad Software Inc (San Diego, California, USA).
Chapter 3

Results

3.1 Transcription Factor DNA Binding

There were no significant differences in DNA binding amongst the transcription factors: PU.1, C/EBP-α and AML-1 in HL-60 cells treated with either 5, 10, 15, or 25µM BQ-HQ compared to vehicle exposed cells (Figure 4). There were statistically significant decreases in DNA binding of the transcription factors c-Myb (46%, p=0.002) and GATA-2 (97%, p=0.016) in cells treated with 25 µM BQ-HQ compared to vehicle exposed cells (Figure 4).

3.2 Protein Expression

There were no statistically significant differences between treatment groups in terms of relative protein expression of PU.1, C/EBP-α, AML-1, c-Myb, GATA-2 transcription factor in cells treated with any concentration of BQ-HQ when compared to cells exposed to the vehicle control (Figure 5).
Figure 4: Filter plate DNA binding assay results of nuclear extracts from HL-60 cells exposed to either the vehicle control or varying concentrations of equal parts BQ and HQ in (A) PU.1 filter plate, (B) AML-1 filter plate, (C) C/EBP-α filter plate, (D) c-Myb filter plate, (E) GATA-2 filter plate. DNA binding is expressed in light units relative to the vehicle control treatment, PBS. * indicates a statistically significant difference from vehicle control (p<0.05) (n=3)
Figure 5: Relative protein expression of (A) PU.1, (B) C/EBP-α, (C) AML-1, (D) c-Myb, (E) GATA-2 in HL-60 cells exposed to either the vehicle control or varying concentrations of BQ and HQ in nuclear extracts. Results are normalized to the loading control, TBP (n=3).
3.3 Cell Viability

There were no statistically significant differences in cell death among cells treated with either 5, 10, or 15 µM BQ-HQ compared to cells treated with the vehicle control, however cells treated with 25 µM BQ-HQ had a statistically significant increase in cell death compared to cells exposed to the vehicle control (11.3%, P=0.01). (Figure 6).

Figure 6: (A) Cell viability of HL-60 cells exposed to either the vehicle control (PBS) or 5, 10, 15, 25 µM BQ-HQ using the Trypan blue exclusion assay. The * indicates a statistically significant difference compared to vehicle control (n=3)
Chapter 4
Discussion

4.1 Summary

The objectives of this study were to determine if there were any differences in DNA binding and relative protein expression of the transcription factors: PU.1, C/EBP-α, AML-1, c-Myb, and GATA-2 following exposure to BQ and HQ. Given their important roles in mediating cell differentiation, it was hypothesized that exposure to BQ and HQ would decrease DNA binding and relative protein expression of PU.1, C/EBP-α, AML-1 and increase DNA binding and relative protein expression of c-Myb and GATA-2. Based on results from the filter plate assays measuring DNA binding and western blot data, it is clear that the hypothesis was not supported. There were no significant differences in DNA binding of PU.1, C/EBP-α, or AML-1 in HL-60 cells exposed to BQ and HQ compared to cells exposed to the vehicle control. However, c-Myb and GATA-2 DNA binding was decreased in cells exposed to 25 µM BQ and HQ, which is contrary to the hypothesis. Furthermore, there were no statistically significant differences in relative protein expression of PU.1, C/EBP-α, AML-1, c-Myb, and GATA-2 in HL-60 cells following BQ and HQ exposure. A trypan blue exclusion assay was also performed in order to determine whether a decrease in transcription factor DNA binding was due to increased cell death from HQ and BQ exposure. Although there was a statistically significant difference in cell death in the 25 µM BQ and HQ treatment group, the increase in cell death was a modest 11.3% compared to vehicle control (Figure 4). This small increase in cell death is likely not sufficient enough to contribute to a 46% and 97% reduction of DNA binding in c-Myb and GATA-2 respectively.
4.2 Decreased c-Myb DNA Binding

The results demonstrating decreased DNA binding of c-Myb are not consistent with previous research. Previous research in the Winn lab has established that c-Myb possesses increased activity in HD3 and K562 cells following exposure to the benzene metabolites: BQ, catechol, BT and phenol (179, 180). Although, an increase in c-Myb activity was reported in previous studies, no changes in c-Myb protein expression were observed, which is similar to the protein expression results of our current study (179, 180). Past results are consistent with the notion that increased c-Myb activity, in theory, could promote leukemic events due to the ability of c-Myb to promote cell proliferation and expansion (181, 182, 183). Although studies have demonstrated a requirement for downregulation of c-Myb during differentiation they have not assessed transcriptional activity of c-Myb during differentiation (181). Future work regarding differentiation and c-Myb activity would add valuable information to the mechanism of benzene toxicity. While the decreased c-Myb DNA binding observed in this study does not parallel previous studies, a decrease in c-Myb DNA binding could impart hematological disruptions that could lead to conditions such as aplastic anemia and leukemia. Lowering activity of c-Myb in early progenitor cells could interrupt proliferation and expansion of early pluripotent HSCs. c-Myb knockdown studies in mice demonstrate irregular lineage commitment and an increase of differentiation in HSCs (184). This increase in differentiation, from decreased c-Myb transcriptional activity, could rapidly deplete early HSC numbers in bone marrow due to reduced proliferation of early pluripotent HSC populations. Depletion of early HSC leads to difficulties in replenishing supplies of all blood cells eventually leading to pancytopenias and even aplastic anemia (184). This
decrease in c-Myb DNA binding could be important in the mechanism of benzene-mediated aplastic anemia as decreased early pluripotent HSCs are a characteristic of this disease.

Possible explanations for the observed decrease in DNA binding of c-Myb include post-translational modifications. c-Myb activity is able to be modified by various post translational modifications such as phosphorylation, ubiquitination and sumoylation (145, 146, 147). Enzymes, like mitogen activated protein kinase (MAPK), play a negative regulatory role through phosphorylation of c-Myb. When serine 528 on c-Myb is phosphorylated by MAPK, transcriptional activity is lowered, thus demonstrating the negative regulatory role of phosphorylation on c-Myb (145). The lowered c-Myb DNA binding observed in this study may be caused by an increase in phosphorylation due to the negative regulatory role of MAPK and c-Myb. Previous studies have demonstrated that exposure of CD34+ cells to benzene metabolites, such as HQ, have resulted in hyperphosphorylation of transcription factor PU.1 (188). This suggests that benzene metabolite exposure can interfere with transcription factor phosphorylation patterns in vitro. These changes in c-Myb phosphorylation could be present in cells exposed to benzene metabolites and warrants further investigation to confirm this idea.

4.3 Decreased GATA-2 DNA Binding

This is the first study to assess DNA binding of GATA-2 in HL-60 cells exposed to benzene metabolites. Previous studies investigating effects of benzene metabolites (phenol, HQ and BT) on GATA-2, evaluated protein expression and mRNA production in the cell line, K562, a chronic myelogenous leukemia cell line. These studies demonstrated a decrease in protein expression and mRNA production when K562 cells
were exposed to metabolites of benzene (185). In our study, it was predicted that DNA binding would increase for GATA-2 due to the increased role of GATA-2 in cell proliferation and survival in leukemia. Our study revealed that GATA-2 may be more involved in benzene mediated aplastic anemia than AML. This is because human aplastic anemia cells have been demonstrated to possess aberrant protein expression of GATA-2 compared to cells from control subjects (186, 187). The effects of a decrease in GATA-2 DNA binding include a steep decrease in the proliferation and maintenance of pluripotent HSCs. Lower levels of GATA-2 protein leads to a loss of HSC proliferation in bone marrow as high levels of this protein are required for maintenance of HSCs. (186,187). A loss in pluripotent HSCs could lead to aplastic anemia as bone marrow that is not able to produce enough blood cells is consistent with the characterization of aplastic anemia. Currently there is only protein expression data available for GATA-2 and benzene studies; transcriptional activity of GATA-2 exposed to benzene metabolites has not been assessed. Although results from our DNA binding studies imply decreased GATA-2 transcription factor activity, it is still important for this to be tested directly via luciferase assays. The results of this experiment warrant further research to be conducted in order to confirm the transcriptional activity of GATA-2 in benzene exposed HSCs. Future studies should evaluate the effects of benzene and its metabolites on GATA-2 transcriptional activity during differentiation as this information is lacking. Information regarding GATA-2 activity and differentiation would yield insight on benzene’s inhibitory role in the process of differentiation.

Similar to other transcription factors, post-translational modifications such as: phosphorylation, acetylation, ubiquitination and sumoylation can all modify activity of
GATA-2 (167, 168, 169, 170). In fact, DNA binding studies of GATA-2 have demonstrated that phosphorylation of serine 401 inhibits DNA binding activity of GATA-2 (192). As previously mentioned, benzene metabolites could possibly be initiating phosphorylation of GATA-2, leading to a decrease in DNA binding. Future studies could address the role of phosphorylation or other post-translational modifications of GATA-2 in cells exposed to benzene metabolites. These studies would lend insight to the mechanism of the observed decrease in GATA-2 DNA binding in this study.

4.4 PU.1, C/EBP-α, and AML-1 DNA Binding

The relationship between benzene metabolite exposure and the activity of the transcription factors PU.1, C/EBP-α, and AML-1 are poorly understood; there has only been one study, which assessed the effects of HQ exposure on PU.1 phosphorylation patterns and differentiation (188). This study found that exposure of HQ to CD34+ hematopoietic progenitor cells induced a dosage dependent alteration in the pattern of hyperphosphorylation of PU.1; furthermore, these phosphorylation modifications were expressed in immature CD34+ cells that failed to differentiate after exposure to a differentiation agent (188).

In our current studies, we found no significant effects of BQ-HQ exposure on the DNA binding activity of these transcription factors, however future studies should investigate post translational modifications of these proteins. For example, phosphorylation alters transcription in all three proteins and thus impacts the progression of hematopoiesis (189, 190, 191). Since DNA binding of PU.1, C/EBP-α, and AML-1 does not appear to be modified by benzene metabolite exposure in undifferentiated cells, it may be important to further analyze DNA binding during induction of differentiation in
HL-60 cells. This is because protein expression and activity of PU.1, C/EBP-α, and AML-1 can be increased as cells progress through the differentiation process (191). It is possible that these transcription factors will be more vulnerable to benzene metabolites during differentiation as studies have shown that benzene metabolites can inhibit HL-60 cell differentiation, which is known to be a transcription factor reliant process (89).

4.5 Future studies

Future studies should evaluate DNA binding/activity and protein expression of other transcription factors involved in hematopoiesis in the presence of benzene metabolites. Transcription factors governing hematopoiesis are very complex as many of them are able to interact with one another through protein-protein interactions, resulting in alteration of transcriptional activity. It is very unlikely that one single transcription factor will solely dictate the effects of benzene toxicity. It is more practical to examine many different transcription factors involved in hematopoiesis, as transcription factors work in concert to facilitate and regulate hematopoiesis. There are several transcription factors involved in hematopoiesis that have yet to be studied regarding the effects of benzene metabolite exposure and DNA binding. These transcription factors include: GATA binding factor 1 (GATA-1), LIM only protein 2 (LMO2), T-cell acute lymphocytic leukemia protein 1 (TAL1) and Ikaros family zinc finger protein 1 (IKZF1) (193, 196, 197). GATA-1, similarly to GATA-2, is an important component of hematopoiesis as it is essential for production of RBCs, MKs and ESs (193). The importance of GATA-1 in fetal hematopoiesis was demonstrated by in vivo knockout GATA-1 studies. GATA-1 knockout studies have shown that GATA-1 deficient embryos are unable to generate RBCs (193). Furthermore, transcription factors, LMO2 and TAL1,
are incredibly important to hematopoiesis as they are essential for the development of RBCs (194, 195). LMO2 and TAL1 regulate promotion of immature HSCs to mature RBCs. This was shown by in vivo studies with fetal, LMO2 and TAL1 knockout mice (194, 195). Similar to GATA-1, knockout mice did not survive embryogenesis due to the lack of fetal erythropoiesis. The similar results of null phenotypes of GATA-1, LMO2, and TAL1 in mice suggests that these three transcription factors have a closely related function. In fact, these three transcription factors may have synergistic activity as LMO2 is able to bind to TAL1 and GATA-1 (196). This demonstrates that each protein could possibly influence one another through protein-protein interactions, however their relationship with one another is currently not well understood. Lastly, the IKZF1 transcription factor plays a key role in lymphoid and myeloid cell development and is considered a master regulator of lymphoid differentiation (197). In addition, IKZF1 has also been reported to modulate self renewal and proliferation of LT-HSCs (198). The role of IKZF1 has been indicated in leukemia, as focal deletions and heterozygous alterations of the IKZF1 gene have both been shown to lead to acute lymphoblastic leukemias (199). Studying the effects of benzene on these transcription factors would help to elucidate the mechanism of toxicity of benzene, as the mechanism is most likely orchestrated by many different factors as opposed to a single factor.

4.6 Additional Future Studies

In addition to the future studies mentioned above, the role of ROS in benzene toxicity is not to be underestimated. Transcription factors can be influenced by a number of mechanisms previously mentioned, such as other protein-protein interactions and post-translational modifications. However, these mechanisms are comprised of complex
signaling cascades that can be altered via ROS from endogenous or exogenous sources (173). It is well known that ROS-mediated cell signaling occurs in cells. For example, H$_2$O$_2$ has been demonstrated to activate platelet derived growth factor alpha/beta (PDGF-$\alpha$/PDGF-$\beta$) and epidermal growth factor (EGF) receptors due to induction of phosphorylation of tyrosine residues (174). Another ROS molecule that has been shown to interfere with signaling cascades is superoxide. Superoxide is capable of interrupting phosphorylation-dephosphorylation homeostasis of PDGF receptors (175). Endogenous ROS appears to be ineffective at stimulating changes in signaling cascades in comparison to exogenous ROS (176). Some transcription factors are considered to be directly oxidant moderated such as NF-κB, however the exact mechanism behind this is not completely understood (177, 178). Previous studies have revealed that benzene metabolite generation of ROS has been shown to impact transcription factors involved in hematopoiesis such as c-Myb (187). It is certainly possible that the observed changes in DNA binding could be ROS-mediated. Future experiments should be conducted to address this question by evaluating the effects of the addition of antioxidant enzymes, superoxide dismutase and catalase, to treatment groups. If the effect is ROS-mediated, the treatment groups containing antioxidants and BQ-HQ will have no difference in DNA binding between vehicle control due to the removal of ROS by antioxidant enzymes.

4.7 Conclusion

This study did not support our hypothesis that DNA binding and relative protein expression of PU.1, C/EBP-$\alpha$, and AML-1 in HL-60 nuclear extracts decreases following exposure to BQ and HQ. Additionally, the hypothesis that DNA binding and relative protein expression of c-Myb and GATA-2 would increase was also not supported. In
contrast, this study demonstrated that in HL-60 cells exposed to 25 µM BQ and HQ, there were statistically significant decreases in c-Myb and GATA-2 DNA binding, but no changes in the DNA binding of the other three transcription factors. Further research into post-translational modifications and the effects of benzene and its metabolites on other transcription factors that play pivotal roles in hematopoiesis is warranted. Given the association between benzene and leukemia, the understanding of the molecular mechanisms involved will help in developing novel therapeutic strategies for the prevention of leukemia.
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