The effects of 1,4-benzoquinone on c-Myb and topoisomerase II in K-562 cells

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Abstract

Exposure to benzene, a ubiquitous environmental pollutant, has been linked to leukemogenesis, although the mechanism of benzene initiated carcinogenesis remains unclear. It has been proposed that benzene can be bioactivated to toxic metabolites such as 1,4 benzoquinone (BQ), which can alter signalling pathways and affect chromosomal integrity. BQ has been shown to increase the activity of c-Myb, which is an important transcription factor involved in hematopoiesis, cell proliferation, and cell differentiation. The c-Myb protein also increases topoisomerase IIα (topo IIα) promoter activity specifically in cell lines with hematopoietic origin. Topo II is a critical nuclear enzyme that removes torsional strain by cleaving, untangling and religating double-stranded DNA. Since topo II mediates DNA strand breaks, aberrant topo II activity or increased protein levels may increase the formation of DNA strand breaks, leaving the cell susceptible to mutational events. I hypothesize that BQ increases c-Myb activity, which in turn increases topo IIα promoter activity resulting in increased DNA strand breaks. Using luciferase reporter assays in K-562 cells (human chronic myeloid leukemic cells) I confirmed that BQ exposure (25 and 37 μM) caused an increase in c-Myb activity after 24 hours. Contradictory to previous findings, overexpression of exogenous c-Myb or a polypeptide consisting of c-Myb's DNA binding domain (DBD), which competitively inhibits the binding of endogenous c-Myb to DNA, did not affect topo IIα promoter activity. However, BQ exposure (37 μM for 24 hours) caused a significant increase in topo IIα promoter activity, which could be blocked by the overexpression of the DBD polypeptide. Western immunoblotting analysis did not show any significant increases in topo IIα protein levels in cells exposed to 37 μM BQ for 24 hours. Overall, this study

suggests that BQ exposure increases topo IIα promoter activity through the c-Myb signalling pathway and furthers our understanding of BQ-mediated toxicity.

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List of Abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
AML	Acute myelogenous leukemia
AMV	Avian leukemia viruses
ANOVA	Analysis of variance
ATBF1	AT motif-binding factor 1
ATF	Activating transcription factor
BQ	1,4-benzoquinone
CCME	Canadian Council of Ministers of the Environment
CFU	Colony forming unit
c-Myb	Myeloblastoma
CNS	Central nervous system
CYP	Cytochrome P450
CYP2E1	Cytochrome P450 2E1
DBD	DNA binding domain
DTT	Dithiothreitol
ECL	Enhanced chemical luminance
ERK1/2	Extracellular-regulated kinases 1 and 2
GD	Gestational day
GSH	Gluthathione
GSSH	Oxidized glutathione
GST	Gluthathione-S-transferase
GSTT1	Glutathione S-transferase T1 null variant
GSTM1	Glutathione S-transferase M1 null variant
HQ	Hydroquinone
HSE	Heat shock element
IARC	International Agency for Research on Cancer
ICB	Inverted CCAAT box
ICB-90	Inverted CCAAT box Binding Protein of 90 kDa
KD	Knock down
KO	Knockout
LTD4	Leukotriene D(4)
LZ	Leucine zipper
mEH	Microsomal epoxide hydrolase
MLL	Mixed lineage leukemia
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-Y	Nuclear Factor Y
NQO1	NADPH quinone oxidoreductase
ODN	Oligodeoxynucleotide
OEL	Occupational exposure limit
OSHA	Ontario Occupational Health and Safety Act
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
ROD	Relative optical density
ROS	Reactive oxygen species
SDS	Sodium docecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SP	Specificity protein
SPTR	Serine, proline, threonine rich region
SOD	Superoxide dismutase
SULT	Sulfotransferase
TAD	Trans-activation domain
Topo II	Topoisomerase II
Topo IIα	Topoisomerase IIα
Topo IIβ	Topoisomerase IIβ
UGT	UDP-glucuronosyl transferase

1. Introduction

1.1. Benzene

Benzene, an environmental pollutant, is classified as an IARC Group I human carcinogen (International Agency for Research on Cancer, 1998). Historically, occupational exposure to benzene has been linked with the development of leukemia, which raised many health concerns. Benzene (CAS Registry Number: 71-43-2) is a six carbon aromatic hydrocarbon (C_6H_6) with a molecular weight of 78.1. It is colorless, volatile, flammable and liquid at room temperature (Canadian Centre for Occupational Health and Safety, 2005). Due to its chemical and physical properties, benzene is an important industrial compound. It is used as an organic solvent and as a precursor to manufacture various synthetic compounds such as rubber, plastics, dyes, and pharmaceuticals (Environment Canada, 1993). Additionally benzene is a component of petro-chemical fuel (Environment Canada, 1993).

1.1.1. Sources of Exposure

Average background levels of benzene range from 0.189 ppb to 1.7 ppb in Canadian urban cities (Analysis and Air Quality Division of Environment Canada, 2004) and from 0.094 – 0.31 ppb in rural areas (Canadian Council of Ministers of the Environment, 2001). Benzene is released from various anthropogenic and environmental processes. In Canada, major sources of benzene released into the environment are from transportation, wood combustion, natural gas dehydrators, and industrial processes (Environment Canada, 2002).

Personal levels of benzene exposure vary with activity, time period and microenvironment (Duarte-Davidson et al., 2001; Johnson et al., 2007). The major source of personal exposure to benzene is through cigarette smoking (Duarte-Davidson et al., 2001; Johnson et al., 2007). It is estimated that an average smoker is exposed to approximately 6 to 10 times more benzene than a non-smoker (Gordon et al., 2002; Wallace, 1996). Other important sources of benzene exposure include traffic, driving and refueling (Duarte-Davidson et al., 2001; Johnson et al., 2007). Benzene concentrations were found to be approximately 3.1 – 14 ppb within 20 meters of busy roads in London but were higher near heavy traffic (eg. 9.3 – 11.5 ppb near the Cromwell Road) (Mann et al., 1997). Furthermore, due mainly to fuel evaporation and incomplete combustion, concentrations inside automobiles can be up to 1.5 to 10 times higher than outdoor background levels (Chan et al., 1991; Wallace, 1996). Finally, while monitoring personal air levels during automobile refueling, it was noted that individuals are exposed to benzene concentrations between 0.02 - 11 ppm (Egeghy et al., 2002). Given that these events occur repeatedly throughout a lifetime, it is important to remember that cumulative exposure may increase the risk for toxicity associated with benzene exposure (Duarte-Davidson et al., 2001; Johnson et al., 2007).

Exposure to higher concentrations of benzene occurs primarily in occupational settings among people who work with benzene related products. It is estimated that workers may be exposed to 100 to 1000 times more benzene occupationally than through ambient exposures (Turteltaub and Mani, 2003). The occupational exposure limit (OEL) over an 8 hour work day for a 40 hour work week has been set at 0.5 ppm benzene by the Ontario Occupational Health and Safety Act (OSHA) (Ministry of Ontario, 2005). The

maximum exposure limit for short term exposure over a period of 15 minutes is 2.5 ppm (Ministry of Ontario, 2005). Failure to comply with occupational standards or accidents may increase the risk of exposure to benzene (Wang et al., 2006). For example, a survey performed in shoe factories in China found that 65% of the factories had benzene levels higher than the national OEL of 12.4 ppm, and 20% of the factories had levels higher than 155 ppm (Wang et al., 2006). Workers within these factories had a higher risk for leukemia compared to non-exposed workers (Wang et al., 2006).

Given the health risks associated with exposure to benzene (described below), world-wide efforts are taking place with the goal of reducing benzene emissions (Environment Canada, 1993; European Commission, 1999; U.S. Environmental Protection Agency, 2006; Wang et al., 2006). The Canadian Council of Ministers of the Environment (CCME) has set up the Canada Wide Standard for Benzene to implement economically feasible changes to reduce emissions (Canadian Council of Ministers of the Environment, 2001). For example, the endorsement of the Benzene in Gasoline Regulations (in 1999), which limited benzene content to 1.0% of the total gasoline volume, has played a large role in the reduction of ambient benzene levels over the last decade (Analysis and Air Quality Division of Environment Canada, 2004; Thompson and Froese, 2006). The Canadian government has also approved a Phase II plan to further decrease benzene emissions an additional 6 kilotonnes by 2010 (Canadian Council of Ministers of the Environment, 2001).

1.1.2. Route of Exposure

Although benzene can be found in different environmental matrices (air, soil, water and food), it primarily exists in the atmosphere due to its volatile nature (Nielsen et al., 1991). Therefore, approximately 99% of benzene exposure occurs through inhalation (Johnson and Rappaport, 2007). Dermal absorption of benzene from the air is slow and minimal (McDougal et al., 1990; van Wendel de Joode, B. et al., 2005). However, dermal absorption may become an important route of exposure among people who are in direct contact with liquid benzene, such as shoe factory workers using benzene-based glue or people spilling gasoline while refuelling vehicles (Adami et al., 2006; Duarte-Davidson et al., 2001; Wang et al., 2006). Normally water and food contain negligible amounts of benzene (Duarte-Davidson et al., 2001). It is estimated that 1.4 µg of benzene is ingested per day (Environment Canada, 1993). However, when ingested, most of the benzene is rapidly absorbed (Oak Ridge National Laboratory, 1989) making this route of exposure a concern during accidental contamination of food and water resources.

1.1.3. Health Effects After Benzene Exposure

Benzene is a non-threshold toxicant, meaning that it may exert toxic effects at all levels of exposure (Canadian Centre for Occupational Health and Safety, 2005) (Table 1). Acute exposure to 250 to 500 ppm results in headache, dizziness, confusion, loss of coordination and fatigue implying central nervous system (CNS) depression (Canadian Centre for Occupational Health and Safety, 2005). Inhaling 19840 ppm for 5 to 10 minutes (Thienes and Haley, 1972) or ingesting 40 – 132 ppm of benzene can be lethal (Oak Ridge National Laboratory, 1989).

Table 1: Health effects after benzene exposure in humans (Duarte-Davidson et al., 2001; Lan et al., 2004; Verma and des Tombe, 1999).

Effects	Concentration (ppm)
Acute Toxicity	
Death (5 – 10 minutes)	≥19840
Death (hours)	≥10000
Narcosis	4000
Vertigo, drowsiness, headache, nausea	250 - 500
Chronic Toxicity	
Risk for severe pancytopenia	200 – 400
Considerable risk for leukemia	125 – 200
Milder forms of pancytopenia and other cytopenias	65 – 125
Hemocytopenia	40 – 65
Myelodysplastic syndrome and cytopenia	20 – 40
Probably the critical level for the risk of leukemia	10 – 20
Chromosomal aberrations	1 – 100
Reduced peripheral blood counts	0.1 – 1

Given that acute exposure to large concentrations of benzene are rare, the more significant potential adverse effects of benzene exposure are likely due to long term exposures to low concentrations of benzene, which may damage the bone marrow resulting in hematotoxicity (Snyder and Hedli, 1996; Whysner et al., 2004). Decreased white blood cells, platelets and hematological values have been noted among certain people who were chronically exposed to 1 ppm of benzene (Lan et al., 2004). Furthermore, repeated exposure to benzene levels more than 1 ppm can lead to the formation of pancytopenia, aplastic anemia, immune suppression and myelodysplastic syndrome (MDS) (Kipen et al., 1989; Rothman et al., 1996). Serious health consequences can be seen with the disruption of blood cell production as this decreases the body's ability to fight infection, prevent hemorrhage and/or carry out vital gaseous exchanges (Aksoy, 1989; Goldstein, 1977).

1.1.4. Benzene and Leukemia

Chronic exposure to benzene may lead to a variety of cancers such as leukemia, lung cancers and nasopharyngeal cancers, with leukemia being the most prevalent (Aksoy, 1989; Goldstein, 1977; Snyder, 2000; Snyder, 2002). Hematopoiesis is a highly controlled process in which pleuripotent hematopoietic stem cells mature to lymphoid, erythroid or myeloid precursors, which then differentiate to terminal blood cell types (Bellantuono, 2004). In leukemia, the proliferation and differentiation processes are disturbed and the precursor cells fail to transform to their terminal cell types. The body then becomes deficient in normal blood cells and is unable to carry out the necessary circulatory and immunological functions (Sawyers et al., 1991).

Long term exposure to benzene leads mainly to the development of acute myelogenous leukemia (AML) (Schnatter et al., 2005; Snyder, 2002). Other types of hematological disorders that occur less frequently include acute lymphocytic leukemia, non-Hodgkin's lymphoma, multiple myeloma, aplastic anemia and MDS (Hayes et al., 2001; Schnatter et al., 2005). Variability in concentration and duration of benzene exposure may account for the occurrence of different disorders.

The association between benzene exposure and leukemia was established in studies that found a higher prevalence of leukemia among workers who were chronically exposed to high levels of benzene within their occupational setting (i.e. shoemaking, printing, petrochemical, coke and rubber industries) compared to the general population (Bloemen et al., 2004; Clapp and Coogan, 1999; Glass et al., 2003; Gun et al., 2006; Rinsky et al., 1987). However, the exact levels of benzene needed to induce leukemia have not been clearly established (Duarte-Davidson et al., 2001; Hayes et al., 2001; Johnson et al., 2007). Pliofilm rubber workers who were exposed to 1 – 5 ppm benzene for 8 hours a day, 5 days a week over 40 years had a 3-fold increased risk for acquiring myeloid leukemia compared to non exposed workers (Rinsky et al., 1987). On the other hand, Hayes et al., (1997, 2000), found that exposure to 1 - 2.5 ppm benzene over 40 years increased the relative risk for AML combind with MDS by 6-fold. Hematotoxicity has also been observed with chronic exposure to 0.5 ppm of benzene (Lan et al., 2004). Taken together these studies demonstrate that chronic exposure to benzene increases the risk for leukemia. Therefore it is important to understand the etiology of benzene-mediated carcinogenesis.

1.2. Benzene Metabolism

1.2.1. Phase I Metabolism

The importance of benzene bioactivation in benzene toxicity has been noted in many studies. For example, when toluene, a competitive inhibitor of benzene metabolism, is co-administered with benzene, there is decreased benzene biotransformation and reduced toxicity (Andrews et al., 1977). Furthermore, rats with partial hepatectomy also experience decreased benzene toxicity due to decreased hepatic biotransformation (Sammett et al., 1979). Therefore, hepatic biotransformation strongly influences the formation of toxic metabolites, whereas secondary biotransformation in the bone marrow appears to play a role in determining the degree of hematotoxicity (Snyder and Hedli, 1996; Snyder, 2004).

The first step in the hepatic biotransformation process is mediated by cytochrome P450 (CYP) 2E1, which oxidizes benzene to benzene oxide-oxepin intermediate (Guengerich et al., 1991) (Figure 1.1). Benzene oxide-oxepin spontaneously rearranges to phenol, is hydrolyzed by microsomal epoxide hydrolase (mEH) to benzene dihydrodiol or undergoes ring opening to form *trans, trans*-muconaldehyde. Phenol, the principle metabolite, can be hydroxylated to hydroquinone (HQ) or catechol (Golding and Watson, 1999; Snyder and Hedli, 1996). Subsequently, catechol and HQ may be further oxidized by CYP2E1 to 1,2,4-benzenetriol. Benzene dihydrodiol is also converted to catechol while *trans, trans*-muconaldehyde rearranges to *trans, trans*-muconic acid (Snyder and Hedli, 1996; Snyder, 2004). These primary metabolites may circulate around the body and bioaccumulate in the bone marrow where they can be further bioactivated (Irons and

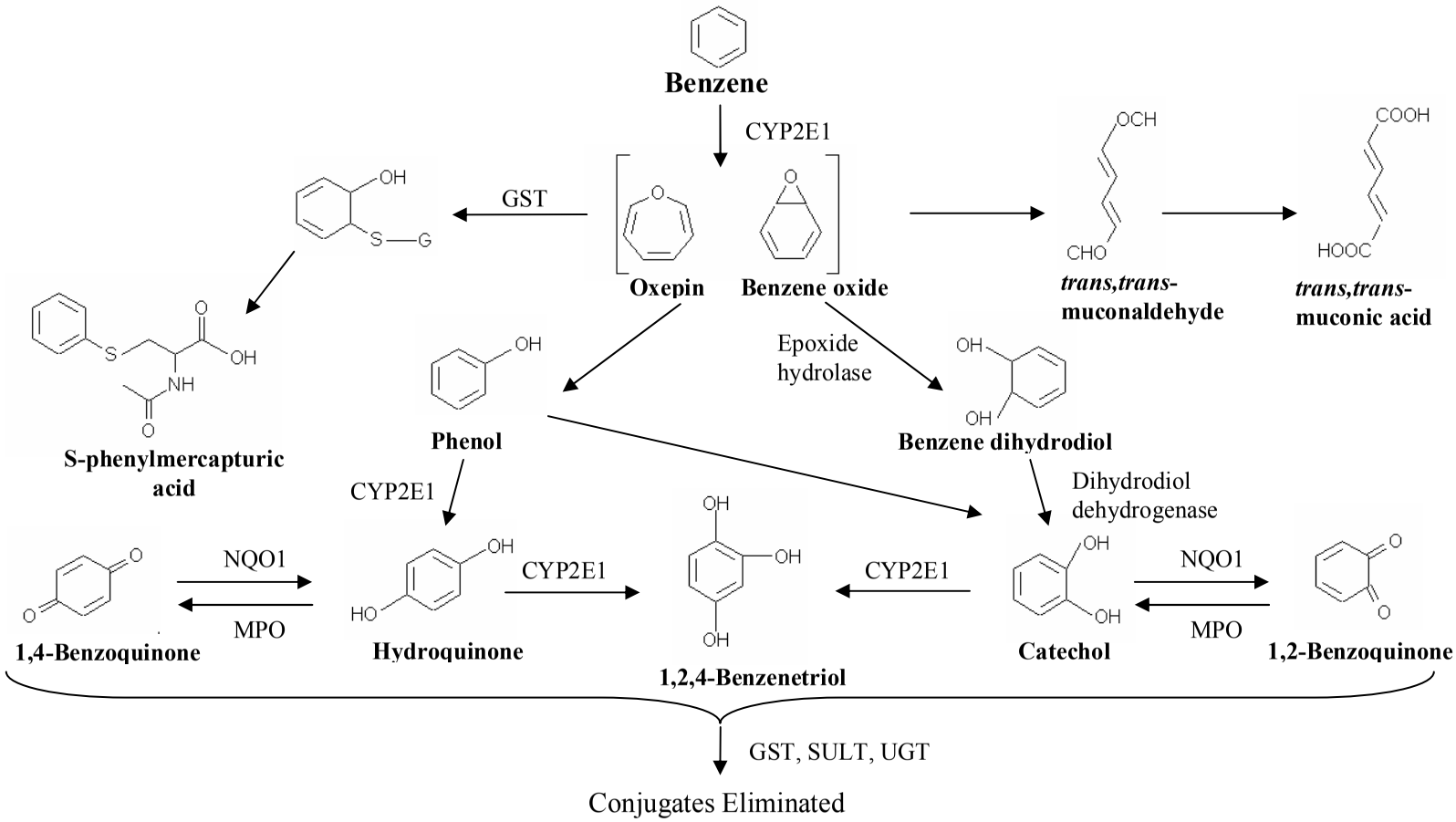

Figure 1.1. Scheme of benzene metabolism.
Benzene is first oxidized by cytochrome P450 (CYP) 2E1 to benzene oxide-oxepin. This intermediate can be further oxidized by CYP2E1, epoxide hydrolase or myeloperoxidase (MPO) to different metabolites. Most of the metabolites are detoxified by glutathione-S-transferase (GST), sulfotransferase (SULT) and UDP-glucuronosyl transferase (UGT). NADPH quinone oxidoreductase (NQO1) converts BQ and 1,2-benzoquinone back to HQ and catechol, respectively (modified from Kim et al., 2006 and Snyder and Hedli, 1996).

Stillman, 1996). The bone marrow is rich in myeloperoxidase (MPO), which oxidizes HQ to 1,4-benzoquinone (BQ) and catechol to 1,2-benzoquinone. The highly toxic BQ and 1,2-benzoquinone metabolites may then be detoxified by the enzyme NADPH quinone oxidoreductase (NQO1) back to the less toxic HQ and catechol respectively (Ross et al., 1996). The site specific production of harmful quinones is important for inducing bone marrow toxicity (Golding and Watson, 1999; Snyder and Hedli, 1996).

1.2.2. Reactive Oxygen Species

During the conversion of HQ to BQ and catechol to 1,2-benzoquinone via semiquinone radical intermediates, redox cycling may lead to the formation of reactive oxygen species (ROS) (Figure 1.2) (Eastmond et al., 1987; Kolachana et al., 1993; Laskin et al., 1995). ROS may also be generated during the re-autooxidation of 1,2,4 benzenetriol (Zhang et al., 1993). Hydrogen peroxide (H_2O_2), superoxide oxygen ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$) are the major ROS that are formed during these processes and have been shown to cause toxicity within the hematopoietic system (Recio et al., 2005).

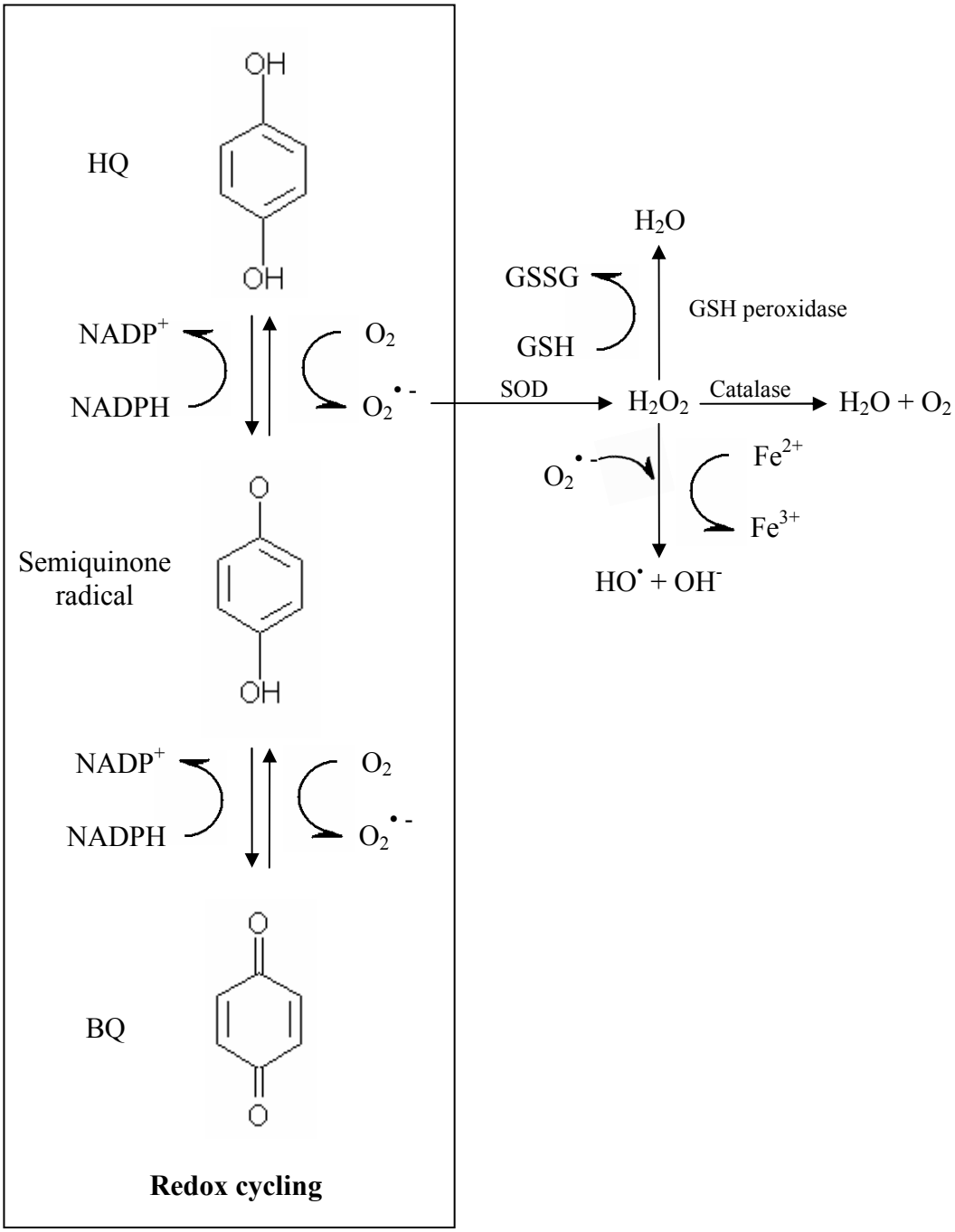

Figure 1.2. Redox cycling and ROS production.
In redox cycling, HQ and BQ interconvert through a semiquinone radical intermediate and produce superoxide anion ($O_2^{\bullet -}$) which is hydroxylated to hydrogen peroxide (H_2O_2) by superoxide dimutase (SOD). H_2O_2 can be detoxified by catalase or glutathione peroxidase (GSH peroxidase) to H_2O and O_2. Further oxidation of H_2O_2 via the Fenton reaction produces different ROS such as the highly reactive hydroxyl radical ($OH^{\bullet}$) and hydroxyl ion (OH^-) (figure from Wan, 2007).

1.2.3. Phase II Metabolism

The formation of reactive quinones may be reduced by numerous detoxification processes. In the liver, the enzyme GSH S-epoxide transferase converts benzene oxide-oxepin to S-phenylmercapturic acid, which can be eliminated in urine (Golding and Watson, 1999) (Figure 1.1). Phenol, catechol, HQ, and 1,2,4-benzenetriol can be detoxified via sulfation, glucuronidation and glutathione conjugation (Orzechowski et al., 1995; Schrenk et al., 1996). At low levels of benzene exposure, more of the benzene escapes phase II detoxification and is converted to HQ and ring opened metabolites (the more toxic metabolites) compared to higher exposure levels (Rothman et al., 1998).

1.2.4. Susceptibility to Toxicity

The balance between bioactivation and detoxification determines the levels of toxic metabolites and the degree of toxicity (Golding and Watson, 1999; Snyder and Hedli, 1996). Genetic polymorphisms, dietary or environmental factors controlling enzyme expression and activity strongly influence biotransformation rates (Golding and Watson, 1999; Snyder and Hedli, 1996). Due to the 13- to 50-fold variability in CYP2E1 activity in humans, people who express highly active forms of the CYP2E1 enzyme are more likely to form toxic metabolites (Bolt et al., 2003; Seaton et al., 1994). Smoking or alcohol intake can also induce CYP2E1 expression and increase the rate of oxidative metabolism in the liver (Tyndale, 2003; Jimenez-Lopez and Cederbaum, 2005). Conversely, reduced expression of bioactivating enzymes decreases benzene-meditated toxicity. For example CYP2E1 (Valentine et al., 1996) and mEH (Bauer et al., 2003) knockout mice have decreased toxicity after benzene exposure compared to wild-type

mice. Furthermore, people who express wild-type MPO have higher sensitivity to benzene toxicity compared to people expressing an inactive recessive mutant (Xu et al., 2003; Zhang et al., 2007). On the other hand, reduced expression of detoxifying enzymes can increase the risk of benzene toxicity. People who express less active mutant forms of glutathione S transferase (GSTT1 null and GSTM1 null), have increased risk of adult acute leukemia (Morgan and Smith, 2002). Individuals expressing the NQO1 c.609C>T (C-to-T substitution in the cDNA at nucleotide 609) variant, which has reduced enzyme activity, have a greater risk for acquiring benzene-induced leukemia, which is likely due to a build up of the more toxic BQ metabolite (Nebert et al., 2002; Smith, 1999). Thus, altered enzyme activity, overexpression of bioactivating enzymes and/or deficiency of detoxifying enzymes significantly affect susceptibility to benzene toxicity.

1.3. Animal Toxicity

Although human pharmacokinetics and benzene metabolism is different from most animals, investigating benzene biotransformation in different animal models increases our understanding of benzene metabolite distribution and helps identify potential mechanisms of toxicity (Whysner et al., 2004). Mice develop malignant lymphoma/leukemia, and lung, Zymbal gland and mammary gland tumours after benzene exposure (Benigni, 1990; Farris et al., 1993; Harper et al., 1989). Rats often develop Zymbal gland tumours, papillomas, and squamous cell carcinomas in the oral cavity and skin but rarely any hematological tumours (Craig, 1992; Huff et al., 1989; Snyder et al., 1984). Differences in toxicity among animals may be explained by differences in excretion, bioaccumulation and tissue and species specific metabolism (Whysner et al.,

2004; Henderson et al., 1992). For example, mice form more quinone and open-ring compounds in the bone marrow than do rats (McDonald et al., 1994; Medinsky et al., 1989). Unlike rats and chimpanzees, mice and monkeys also convert more benzene to HQ (Henderson, 1996). Differences in metabolite distribution in tissues are mostly noted at low doses (Medinsky et al., 1989; Sabourin et al., 1989). Overall, mice are most sensitive to benzene toxicity primarily because they form and retain more of the toxic metabolites in susceptible organs like the bone marrow (Henderson, 1996; Whysner et al., 2004).

1.4. Mechanisms of Toxicity

In benzene-induced leukemia, carcinogenic changes are mediated by benzene metabolites (Smith, 1996; Snyder, 2004; Whysner et al., 2004). The metabolites induce genotoxicity via DNA alkylation, alteration of protein activity and/or ROS production. These processes may then lead to DNA strand breaks and improper DNA repair resulting in chromosomal translocation, deletions and aneuploidy. Such genetic changes may activate oncogenes, inactivate tumour suppressor genes and disrupt intracellular signalling that then leads to leukemogenesis (Smith, 1996; Snyder, 2004; Whysner et al., 2004).

Evidence for DNA alkylation was first noted when DNA adducts were detected in the livers of rats, which had been exposed to radiolabelled benzene via inhalation (Lutz and Schlatter, 1977). After intraperitoneal administration of low doses of benzene in mice, the highest levels of DNA adducts were formed in the liver within 30 minutes and in the bone marrow between 12 and 24 hours after exposure (Creek et al., 1997). *In vitro*

studies have been helpful in identifying the metabolites that bind to DNA. For example, BQ forms exocyclic adducts on the DNA bases adenine, cytosine and guanine, which are highly mutagenic by predominantly inducing deletions (Chenna and Singer, 1995; Xie et al., 2005). HQ and BQ can also form 3'-hydroxy-1,N_2-benz-2'deoxyguanosine adducts *in vitro* and *in vivo* (Bodell et al., 1996; Levay et al., 1996; Pongracz and Bodell, 1996). However, in general only low levels of DNA adducts have been detected, even after exposure to high levels of benzene suggesting that DNA alkylation may not be the primary mechanism involved in leukemogenesis (Golding and Watson, 1999; Snyder, 2004).

Rather than producing site directed mutations, benzene metabolites more commonly cause clastogenic changes such as chromosomal aberrations, sister chromatid exchange and micronuclei (Golding and Watson, 1999; Snyder, 2004). The persistence of specific chromosomal abnormalities in hematopoietic cells implies that only certain chromosomal changes are non-lethal and continue to exist in specific cells (Sivikova et al., 2005). Although aberrations may occur in chromosomes 1, 5-9, 11, 12, 14, 18 and 21, anelopoidy, translocation or deletions involving chromosomes 5, 7, and 8 occur more frequently after exposure to different benzene metabolites (Smith et al., 2000; Zhang et al., 1998; Zhang et al., 2005; Zhang et al., 2007). Furthermore, fluorescent *in situ* hybridization analysis of cultured human blood cells confirm that BQ and HQ are the most potent benzene metabolites when assessing alterations in chromosomes 5, 7, 8 and 21 (Zhang et al., 2005). Such clastogenic changes may arise due to the formation of DNA-metabolite adducts, induction of oxidative stress, inhibition of topo II or disturbance of the mitotic apparatus (Golding and Watson, 1999; Snyder, 2004).

ROS, which are generated by redox cycling and during benzene bioactivation, can bind to DNA, proteins or lipids to initiate carcinogenic processes (Snyder and Hedli, 1996). Exposure to benzene or benzene metabolites (catechol, HQ, BQ, 1,2,4, benzenetriol) induces oxidative damage in human HL-60 leukemic cells and in the bone marrow of mice (Kolachana et al., 1993; Shen et al., 1996). Using antioxidative treatments including electron spin trapping agents, catalase or superoxide dismutase (SOD), revealed that semiquinones, $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ are the major ROS that are formed after exposure to benzene metabolites (Hiraku and Kawanishi, 1996; Rao and Snyder, 1995; Shen et al., 1996). The highly reactive $OH^{\bullet}$ can bind to DNA to form 8-hydroxy-2'-deoxyguanosine (8-OHdG), which has been detected both *in vitro* and *in vivo* (Kolachana et al., 1993; Pilger and Rudiger, 2006). The 8-OHdG adduct can cause point mutations especially G-T and A-C base substitutions, which may induce strand breaks if they are not repaired (Boiteux and Radicella, 1999). Moreover, ROS-induced DNA double strand breaks may be repaired by homologous recombination and have an increased possibility for erroneous repair (Haber, 1999; Slupphaug et al., 2003). Benzene metabolites (phenol, catechol, HQ and BQ) have been shown to increase the frequency of homologous recombination, which may be abolished with catalase pre-treatment (Winn, 2003). Furthermore, several studies have demonstrated that benzene metabolites have different potencies for inducing ROS and that BQ is one of the most potent inducers (Rao and Snyder, 1995; Shen et al., 1996; Wan and Winn, 2007; Winn, 2003).

Benzene and its metabolites may also directly bind to proteins or through other mechanisms alter protein activity to disturb cellular processes (Smith, 1996). For example, HQ can interfere with granulocyte colony stimulating factor, while BQ interacts

with LTD4 receptor protein to stimulate differentiation of myeloid stem cells (Hazel et al., 1996; Niculescu et al., 1995). BQ can also block apoptosis by inhibiting caspase 3 (Kalf et al., 2001). Quinone metabolites have also been shown to covalently bind to spindle fibre proteins and disrupt intracellular structure which can lead to improper chromosomal segregation during mitosis (Pfeiffer and Metzler, 1996; Zhang et al., 1994). Breakage in chromosome 11 and chromosomal t(8,21) translocations in certain cases of benzene-induced leukemia implies that the chromosomal damage may be mediated by disturbances in topo II (Holeckova et al., 2004; Zhang et al., 2007). Topo II-mediated toxicity will be discussed in further detail in a later section. Finally, via ROS signaling, benzene and its metabolites can activate c-Myb (Wan et al., 2005; Wan and Winn, 2006; 2007; Wan, 2007), which is an important hematopoietic transcription factor, and ERK1/2, which regulates entry into S phase during the cell cycle (Ruiz-Ramos et al., 2005). These examples show that benzene exposure can alter the activity of multiple proteins, which may disrupt important biological processes including differentiation, proliferation, cell cycling and apoptosis (Smith, 1996; Snyder, 2004).

Elucidating epigenetic changes will also enhance our understanding of benzene-mediated toxicity. Recently, bisulfite-PCR pyrosequencing analysis revealed alterations in DNA methylation patterns in workers who were exposed to low doses of benzene (Bollati et al., 2007). Additionally, microarray analysis showed significant differences in mRNA expression profiles in mice exposed to benzene compared to controls (Hirabayashi et al., 2004). It has also been shown that radiolabeled benzene can bind to several histones, which could interfere with the ability of histones to fold and package

DNA (Williams et al., 2002). The toxicological impact of benzene-mediated changes in methylation, mRNA expression and histone activity still need to be further investigated.

In summary, benzene-mediated leukemogenesis is a complex process due to the formation of numerous metabolites that induce cellular toxicity through multiple pathways. As outlined above, this toxicity may be characterised by five major mechanisms, which include DNA alkylation, chromosomal damage, oxidative stress, protein alterations and epigenetic changes. This study focused on the effects of BQ on c-Myb signalling and topo II expression.

1.5. c-Myb

Benzene metabolites have been shown to alter an important hematopoietic signalling pathway involving the transcription factor c-Myb (myeloblastoma). The c-Myb gene, located on chromosome 6q22-24, encodes a 78 kDa protein that regulates important biological processes such as proliferation, differentiation and apoptosis (Ness, 2003; Oh and Reddy, 1999). It is mainly expressed in proliferating tissues such as immature progenitor hematopoietic cells, thymus and epithelia of the rapidly dividing colon (Ess et al., 1999; Lieu et al., 2004; Thomas et al., 2005; Zorbas et al., 1999).

Normally c-Myb is abundantly present in immature hematopoietic cells but c-Myb protein expression decreases as cells differentiate to their final stages (Jieping et al., 2007; Sakamoto et al., 2006). Precursor cells can be blocked from differentiating to the terminal myeloid, lymphoid and erythroid cells by overexpressing full-length or ectopic c-Myb protein (Kaspar et al., 2005; Press et al., 1995; Sala et al., 1995; Sandberg et al., 2005). Studies using anti-sense oligodeoxynucleotides (ODN) or dominant negative

forms of c-Myb, which block c-Myb expression or activity, demonstrate that decreased c-Myb induces differentiation in normal myelomonocytes and leukemic cell lines (Kamano et al., 1990; Melotti et al., 1996; Szczylik et al., 1996).

On the other hand, overexpression of a dominant negative form of c-Myb protein induced cell death in human K-562 cells (Yi et al., 2002) and murine T-cells (Taylor et al., 1996). These cells were noted to have decreased Bcl-2 (an anti-apoptotic protein) promoter activity and protein levels, which imply that c-Myb was needed to block the signalling mechanisms that committed the cells to apoptosis (Yi et al., 2002; Taylor et al., 1996). Furthermore, anti-sense mRNA treatment used to ablate c-Myb expression also decreased the life span of leukemic cells (Kitanaka et al., 1994; Waki et al., 1994). These results suggest that c-Myb also plays an important role in apoptosis.

1.5.1. Hematopoiesis

The importance of the c-Myb protein in hematopoiesis is mainly noted in studies conducted on c-Myb knockout (KO) or knockdown (KD) animals (Mucenski et al., 1991; Sumner et al., 2000; Vegiopoulos et al., 2006). The embryos of c-Myb KO (-/-) mice die *in utero* around gestational day (GD) 15 from severe anemia (Mucenski et al., 1991). These mice fail to carry out fetal liver erythropoiesis since they have very few lymphoid and myeloid cells (Mucenski et al., 1991). Moreover, colony forming unit (CFU) assays have shown that hematopoietic cells from GD 8.5 c-Myb KO mice were less proliferative compared to cells from wild-type embryos (Sumner et al., 2000). Finally, Vegiopoulos et al., 2006, analyzed fetal liver from c-Myb KD/KD to show that c-Myb promotes the commitment and progression of erythropoiesis. These studies demonstrate that

hematopoiesis is not possible without functional c-Myb protein (Mucenski et al., 1991; Sumner et al., 2000; Vegiopoulos et al., 2006).

1.5.2. Carcinogenesis

c-Myb's control of cell proliferation and hematopoiesis implies that disturbance in c-Myb signalling and protein expression can lead to leukemia (Gonda, 1998). c-Myb's oncogenic potential was first noted when two different avian leukemia viruses (AMV and E26) expressed truncated but constitutively active versions of the protein (v-Myb), which transformed immature myeloid cells and induced leukemia in chickens (Graf, 1992). Up-regulation of the *c-Myb* gene occurs in various types of leukemia, lymphomas and cell lines derived from hematopoietic tumours (Beck et al., 1998; Calabretta and Gewirtz, 1991). Moreover, AML patients that express high levels of c-Myb are less likely to respond to chemotherapy (Gopal et al., 1992). Changes in gene arrangements and gene duplications leading to abnormal c-Myb expression have also been noted in other types of cancers including glioblastomas, pancreatic tumours, breast tumours and colon tumours (Ness, 2003). Due to c-Myb's role in apoptosis (Gewirtz, 1999; Yi et al., 2002) there is hope that anti-sense ODN against c-Myb could be used as an anti-cancer therapy to induce cell death in tumour cells (Ramsay et al., 2003; Abaza et al., 2003; Pastorino et al., 2003). In a pilot study, ODNs were used to inhibit c-Myb in ex-vivo bone marrow purging; however, the effectiveness of the therapy remained inconclusive. Further improvements in ODN delivery, half-life and tissue specificity are needed (Luger et al., 2002). With necessary modifications, it is hoped that this therapy can be taken to Phase I clinical studies on patients with a variety of hematological malignancies (Gewirtz, 2007).

1.5.3 Protein Structure

The c-Myb protein contains three main domains: the N-terminal DNA binding domain (DBD), the central transactivation domain (TAD) and the C-terminal negative regulatory domain (Sakura et al., 1989) (Figure 1.3). The highly conserved N-terminus region has three imperfect repeats (R1, R2 and R3), which fold into a helix-turn-helix motif (Ogata et al., 1994). Deletion analysis of the subunits demonstrated that the R2 and R3 subunits are necessary for c-Myb's binding to DNA (Howe et al., 1990). The R1 subunit stabilizes the DNA-R2R3 complex (Tanikawa et al., 1993). The TAD has clusters of acidic amino acids which are required for protein-protein interactions (Ptashne, 1988). A leucine zipper, which lies near carboxy end of the TAD, may promote homodimerization (Dash et al., 1996; Nomura et al., 1993).

Much attention has been given to the study of the C-terminus domain since this region seems to be important for the negative regulation of the c-Myb protein (Nomura et al., 1993). The C-terminus contains an EVES domain that may inhibit c-Myb's interaction with DNA (Dash et al., 1996; Nomura et al., 1993). At high levels, c-Myb forms intramolecular interactions which compromise its DNA-binding and trans-activating abilities in a self-limiting mechanism (Hu et al., 1991; Nomura et al., 1993). Protein interactions and phosphorylation of serine residues on the C-terminus can also negatively regulate the c-Myb protein (Favier and Gonda, 1994; Nomura et al., 1993).

Disturbances in the C-terminus can increase c-Myb activity and its oncogenic effects. For example, mutated c-Myb protein constructs lacking the C-terminus, bind more readily to DNA and have increased c-Myb activity (Grasser et al., 1991; Hu et al.,

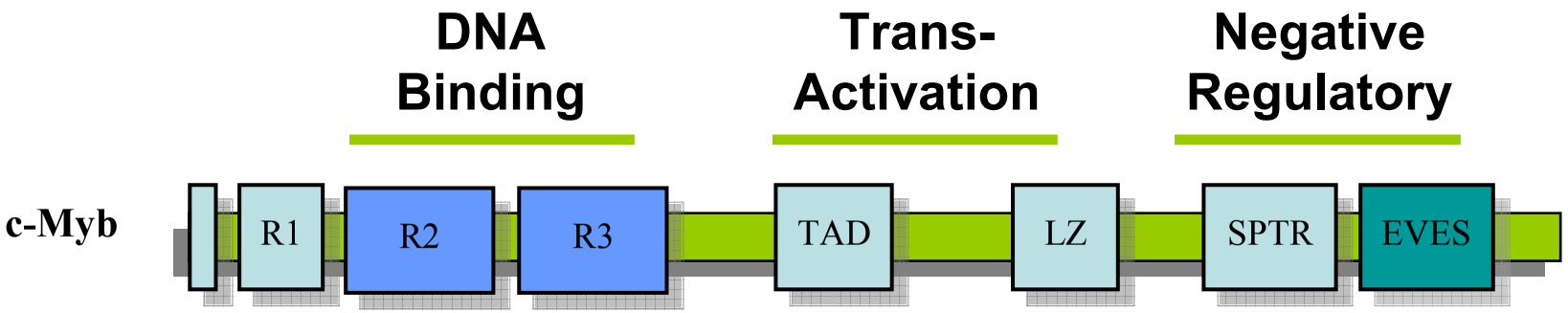

Figure 1.3. Functional domains of the c-Myb protein.
At the N-terminus, the repeat sequences (R1, R2 and R3) regulate the function of the DNA binding domain. The transactivation domain (TAD) is involved in protein-protein interaction and the leucine zipper (LZ) assists in the formation of inhibitory homodimers. The negative regulatory domain at the C-terminus has a serine, proline, threonine rich region (SPTR) and an EVES domain, which assists in the folding of the protein during self-inhibition (modified from Ness, 1999 and Gonda, 1997) .

1991; Ramsay et al., 1991). Secondly, truncating the C-terminus of murine c-Myb increased its half-life from 45 to 120 minutes by inhibiting protein ubiquitinization (Bies and Wolff, 1997). As mentioned earlier, avian viruses express a constitutively active form of c-Myb protein (v-myb), which induces leukemia in chickens (Graf, 1992). This protein lacks the N and C terminus of c-Myb and thereby, it lacks the auto-inhibitory mechanism.

1.5.4 Regulation

In addition to the C-terminus, protein interactions with other transcription factors can also affect c-Myb activity. For example, Pim-1 (a serine/threonine protein kinase that is important for hematopoiesis and has oncogenic potential) cooperates with the p100 protein to enhance c-Myb activity (Leverson et al., 1998). The AT motif-binding factor 1 (ATBF1), a transcriptional regulatory protein, binds to the negative regulatory domain and decreases c-Myb activity (Kaspar et al., 1999). Some other important proteins that have prominent effects on c-Myb include C/EBP proteins, PU.1 and Ets-1 (Ness, 1999).

Posttranslational modifications such as reduction, oxidation (Bergholtz et al., 2001; Myrset et al., 1993), phosphorylation (Bies et al., 2000; Cures et al., 2001; Luscher et al., 1990; Winn et al., 2003), acetylation (Sano and Ishii, 2001; Tomita et al., 2000) and sumoylation. (Bies et al., 2002; Dahle et al., 2003) can also affect c-Myb activity. Pim-1 can also directly regulate c-Myb by phosphorylating the DBD (Winn et al., 2003). Finally, histone acetyltransferase p300 was shown to acetylate the C-terminus and increase c-Myb's transactivation of different promoters (Tomita et al., 2000).

1.5.5. Down Stream Targets

The c-Myb protein binds to the sequence AACTGAC (Tanikawa et al., 1993) and regulates many genes in mammalian cells (Ness, 1996). Some of the prominent genes that play important roles in proliferation, differentiation, function or survival of hematopoietic cells include *c-myc, Bcl-2, c-kit, CD34* and *T-cell receptor delta* (Ness, 2003). Furthermore, microarray analysis shows that c-Myb overexpression leads to different gene expression profile in different cell types (Ness, 1996). Therefore, c-Myb transcriptional regulation of different genes may be dependent on the cellular environment (Ness, 1999).

1.5.6. Benzene and c-Myb

In vitro and *in vivo* studies conducted by Wan and Winn (Wan and Winn, 2004; 2005; 2006; 2007; Wan, 2007) suggest that benzene-mediated carcinogenesis may involve c-Myb signalling. *In vitro* exposure to catechol, HQ and BQ caused a time- and concentration-dependent increase in c-Myb activity and c-Myb phosphorylation but not protein expression (Wan et al., 2005; Wan and Winn, 2007). However, exposure to phenol and benzene did not affect c-Myb activity, phosphorylation or protein levels (Wan and Winn, 2004). Like other studies, they too noted metabolite differences in benzene-mediated toxicity (Chapman et al., 1994; Moran et al., 1996). Of the metabolites, BQ and HQ were the strongest activators of c-Myb signalling (Wan et al., 2005; Wan and Winn, 2007).

Moreover, *in vitro* investigation showed that BQ and HQ mediated increases in c-Myb activity via ROS signalling (Wan and Winn, 2007). Pre-treating cells with the antioxidant superoxide dismutase linked to polyethylene glycol (PEG-SOD) efficiently

blocked BQ's and HQ's effect on c-Myb (Wan and Winn, 2007). In addition, hematopoietic tissues from embryos, which had been exposed *in utero* to benzene during the critical period when embryonic hematopoiesis switches from yolk sac to the liver (GD 10 and 11), had elevated c-Myb and Pim-1 protein compared to non-exposed controls (Wan, 2007). Furthermore, embryonic tissue from mothers pre-treated with the antioxidant catalase conjugated to PEG prior to benzene exposure, did not have elevated c-Myb and Pim-1 proteins levels (Wan, 2007). Therefore, these findings strongly support the hypothesis that benzene-mediated leukemogenesis may act via disturbances in c-Myb signalling (Wan, 2007). This current project examined the downstream effects of BQ-mediated increases in c-Myb activity.

1.6. Topoisomerase II

Topoisomerase II (topo II) is an important homodimermic ATP dependent nuclear protein that is ubiquitously present in all organisms (Wang, 2002; Watt and Hickson, 1994). It is an important nuclear enzyme that modulates DNA topology by removing torsional strain during transcription, cell replication and cell division. Each subunit of the homodimer consists of three domains: a N-terminal ATP domain, a central cleaving/religating domain and a sequence divergent C-terminus. Catalytically, topo II covalently binds to double-stranded DNA and cleaves both strands to form an "open cleaved complex" (also called DNA-topo II complex as shown in Figure 1.4). The binding of ATP stimulates the enzyme to pass another double-stranded duplex through the cleaved site to detangle the two duplexes. ATP hydrolysis then stimulates topo II to

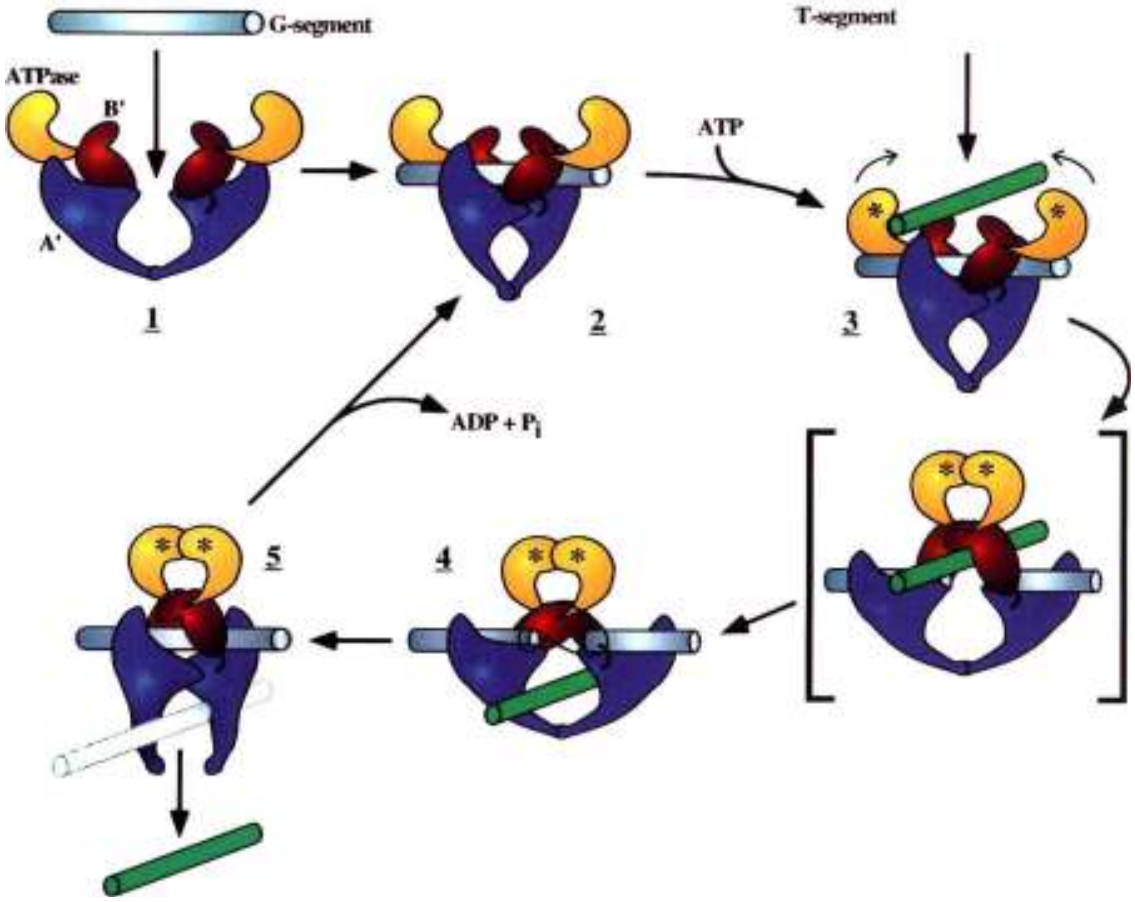

Figure 1.4. Catalytic activity of topoisomerase II.
Topoisomerase II (topo II) is an important nuclear enzyme that modulates the DNA structure during various cellular processes. In the homodimer, the ATP binding domain (yellow) is connected to the DNA binding domain (B'- red) which is bound to the A' segment (blue). The A' segment has the active site for DNA cleavage and joins the two monomers together. Catalytically topo II first covalently binds to a double-stranded DNA (G-segment) which induces a conformational change (1). ATP (*) then binds to the enzyme and (2) facilitates the binding of the second segment of double-stranded DNA duplex (T - segment) (3). The first DNA duplex is cleaved (4) and the second duplex passes through the cleaved section (5). After ATP hydrolysis, the cleaved DNA segment is religated, and both ADP and the attached DNA strand are released. There are two types of compounds that inhibit topo II activity. Topo II poisons stabilize the DNA-topo II complexes (at stages 2 - 5) while topo II catalytic inhibitors distort the topo II enzyme before it binds to DNA (figure from Berger et al., 1996).

religate the cleaved strands and regain its original conformation (Wang, 2002). Although, these transient strand breaks are tolerated by cells, any disturbance in topo II protein activity or levels may lead to unwanted DNA damage (Figure 1.4) (Kingma and Osheroff, 1998; Wang, 2002).

1.6.1. Topo II Isoforms

In vertebrates, there are two different isoforms of topo II. The *topo IIα* gene is located on chromosome 17q21-22 and encodes a 170 kDa protein, whereas the *topo IIβ* gene located on chromosome 3p24 encodes a protein of 180 kDa (Austin and Marsh, 1998). Analysis of the genomic sequence suggests that the two genes arose from a gene duplication event (Lang et al., 1998). The proteins have greater than 70% amino acid sequence homology and differ at their C and N termini (Austin et al., 1993). Although the two enzymes carry out the same catalytic activities, differences in their expression profiles during the cell cycle is consistent with their different physiological functions (Austin and Marsh, 1998; Isaacs et al., 1998).

The topo IIα protein seems to be the primary isoform that regulates DNA structure during DNA replication, transcription and chromosomal segregation for several reasons (Austin and Marsh, 1998; Isaacs et al., 1998). Immunolocalization studies have shown that topo IIα protein is located on the chromosomes while topo IIβ resides within the cytosol of cells during mitosis (M) (Grue et al., 1998; St Pierre et al., 2002a). Secondly, topo IIα is differentially expressed during the cell cycle such that it is hardly present in G0/G1 and is maximal at G2/S/M (Brown et al., 1995; Prosperi et al., 1992; Tanoguchi et al., 1998). On the other hand, topo IIβ expression remains constant

throughout the cell cycle (Brown et al., 1995; Prosperi et al., 1992) and the protein is thought to play a more general role in RNA and/or DNA metabolism (Isaacs et al., 1998). Topo IIα expression is also high in proliferating cells such as the thymus, spleen, bone marrow, intestines, embryonic tissues, and tumours (Juenke and Holden, 1993; Tsutsui et al., 1993; Turley et al., 1997). Upregulation of topo IIβ has also been noted in some tumours, but normally, it is ubiquitously expressed in all cells including fully differentiated tissues (Bauman et al., 1997; Turley et al., 1997).

Topo IIα is an essential protein as demonstrated by the fact that knockout mice die at the 4 to 8 cell stage during fetal development (Akimitsu et al., 2003). Additionally, mouse embryos in which topo IIα protein was depleted using anti-sense ODNs were not able to develop beyond the 2nd cell division *in vitro* (St Pierre et al., 2002b). On the other hand, topo IIβ knockouts die soon after birth due to poor neural development, which implies that topo IIα may substitute topo IIβ's role during early gestation (Bakshi et al., 2001; Yang et al., 2000). *In vitro* studies using anti-sense RNA against either mRNA have shown that topo IIα and topo IIβ can, to some extent, replace each other's function (Sakaguchi and Kikuchi, 2004; St Pierre et al., 2002b). However, depletion or disruption of either protein induces abnormal cell growth of somatic cells, gametes and embryos suggesting that both proteins have distinct functions and cannot fully substitute for one another (Akimitsu et al., 2003; Bizzaro et al., 2000; Sakaguchi and Kikuchi, 2004; St Pierre et al., 2002b).

1.6.2. Topo II Mediated DNA Damage

Due to topo II's catalytic nature, changes in the topo II activity and/or protein expression that enhance the formation of DNA strand breaks may increase the risk of genotoxicity (Wang, 2002). There are two classes of drugs, natural toxins or bioactivated metabolites that disrupt topo II activity (Topcu, 2001). Members of the first class of topo II disruptors are known as "topo II poisons" and include etoposide, doxorubicin and teniposide (Topcu, 2001). These poisons convert topo II into a cellular toxicant by stabilizing the DNA-topo II cleaved complexes that results in increased double strand breaks (Topcu, 2001). These complexes may also interfere with the movement of ribosomes during transcription, affect the ability of DNA polymerase to add nucleotides at the replication fork, or affect chromosomal segregation. Any of these outcomes can be cytotoxic to the cell (Chen and Liu, 1994; Pommier et al., 1994; Topcu, 2001). Furthermore, the complexes may misalign DNA strands during religation. This may increase the risk of erroneous repair as homologous and non-homologous recombination mechanisms try to restore DNA integrity (Cahill et al., 2006; Malik et al., 2006; Sabourin et al., 2003).

On the other hand, drugs such as the fostriecin analogues, merbarone, suramin or topostin (Topcu, 2001) are known as "catalytic inhibitors" and inhibit topo II catalytic activity without trapping the DNA-topo II complex. If topo II is unable to carry out its activity, torsional strain created during transcription, DNA replication or chromosomal segregation may induce random breakage within the DNA (Larsen et al., 2003). It is thought that of the two classes of topo II disruptors, topo II poisons are more potent in damaging DNA (Topcu, 2001). The ability of topo II poisons to induce cell death is

exploited in cancer therapy. Doxorubicin, etoposide and mitoxantrone are some of the most effective chemotherapeutic agents in treating malignant lymphoma, central nervous system tumours, and small cell lung cancers (Fortune and Osheroff, 2000; Wilstermann and Osheroff, 2003).

Since topo II protein expression is important for topo II poison treatment during cancer therapy, changes in topo II protein expression may also affect genotoxicity (Dingemans et al., 1998). With increased topo II protein levels, more strand breaks are created and more mutational events may occur (Topcu, 2001).

1.6.3. Topo IIα Protein Expression

Although both topo II isoforms carry out the same catalytic activity, disturbances in topo IIα protein has a higher risk for altering DNA. This is primarily due to topo IIα's predominant role during DNA replication, chromosome condensation/decondensation, and sister chromatid segregation and since it is abundantly expressed in proliferating cells (Wang, 2002). As DNA undergoes tremendous structural changes during these processes, any disturbances in topo IIα protein level or activity will have a significant impact on DNA integrity and mutational rates. Given these differences, the present study focused on examining changes in the expression of topo IIα.

Fluctuations in topo IIα protein levels (low at G0/G1 and high at G2/M) during the cell cycle are mainly attributed to changes in mRNA transcription and stability (Austin and Marsh, 1998; Bronner et al., 2002). For example, in HeLa cells by late S/G2/M stage topo IIα mRNA is elevated 15-fold due to a 2-fold increase in transcriptional rate and 8-fold increase in mRNA stability (Goswami et al., 1996; Isaacs

et al., 1998). In G1 topo IIα mRNA levels are extremely low and the mRNA has a half-life of 30 minutes compared to 4 hours during S stage (Goswami et al., 1996). Ubiquitinization also helps to rapidly degrade topo IIα protein after mitosis (Nakajima et al., 1997).

Variations in topo IIα mRNA expression during the cell cycle are primarily influenced by changes in activity of the topo IIα promoter (Isaacs et al., 1996; Sandri et al., 1996a). The human topo IIα promoter lacks a functional TATA box and has a high frequency of CpG dinucleotides, features that are characteristic of house keeping genes (Hochhauser et al., 1992). Maximal promoter activity is found within 617 bp before the transcription start site in *in vitro* studies, which includes a putative Myb binding site, two GC rich sequences, five inverted CCAAT boxes (ICB) and putative binding sites for other regulatory elements (Figure 1.5) (Bronner et al., 2002; Hochhauser et al., 1992).

The CCAAT sequence in either orientation is a common element in eukaryotic promoters and is a sequence to which many transcription factors can bind to (Benoist et al., 1980). Site-directed mutational studies show that at least two of the three ICB 1, 2 and 3 are needed to fully activate topo IIα promoter during proliferation (Adachi et al., 2000). ICB 1, located within 90 bp upstream of the transcription start site, is thought to be responsible for the minimal promoter activity since it exhibits 58% of the basal promoter activity in HeLa cells (Hochhauser et al., 1992). Mutation of distal ICB 4 and 5 does not significantly decrease topo IIα promoter activity (Adachi et al., 2000), but they are required to obtain maximal activity (Joshi et al., 2003; Wang et al., 1997).

NF-Y, a heterotrimeric transcription factor, and ICB-90, a nuclear binding protein, both bind to ICBs to alter topo IIα promoter activity (Herzog and Zwelling,

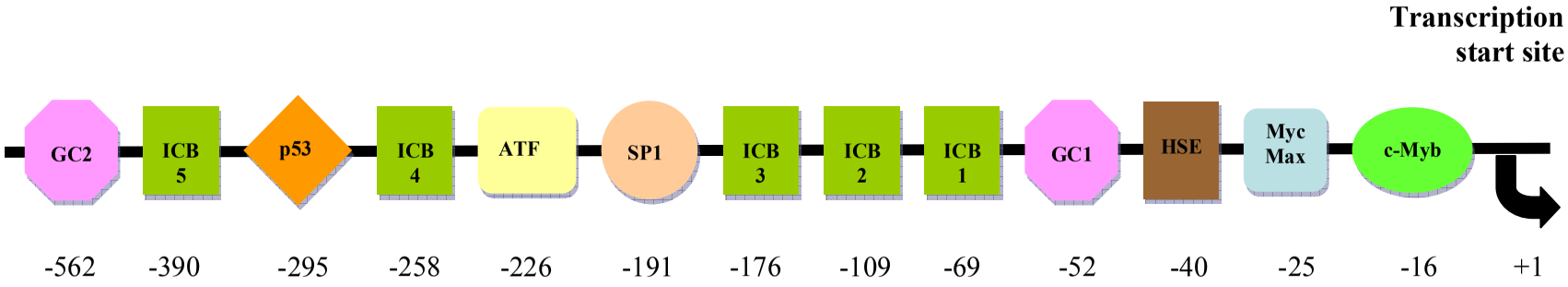

Figure 1.5. Regulatory elements in the human topo II *α* promoter.
The transcription start site is marked as +1 and base numbers refer to 5' of each element. The putative regulatory binding sites include Myb, Myc/Max, Heat shock element (HSE), SP1, ATF and p53. The regions rich in GC sequences and CCAAT boxes in inverted orientation (ICB) have also been marked on the promoter (Figure modified from Bronner et al., 2002 and Adachi et al., 2000).

1997; Hopfner et al., 2002; Isaacs et al., 1996; Morgan and Beck, 2001). Issacs et al., (1996) noted that ICB 2 acted as a negative regulator of topo IIα promoter activity during confluency but NF-Y binding negated ICB 2's suppressive role and allowed topo IIα promoter upregulation in proliferating cells. On the other hand, overexpression of ICBP90 in COS-1-transfected cells enhanced endogenous topo IIα expression (Hopfner et al., 2000).

Specificity proteins (Sp) 1 and 3 are ubiquitous transcription factors that bind to GC sequences (Bronner et al., 2002) on the topo IIα promoter (Allen et al., 2004; Magan et al., 2003; Saxena et al., 2004; Yoon et al., 1999). Sp 1 binds to GC1 as a transcriptional activator but becomes a transcriptional repressor when bound to GC2 via NF-Y interactions (Allen et al., 2004; Magan et al., 2003). Differential Sp3 expression in tumour cells with low topo IIα protein levels suggests that Sp3 acts a repressor of the topo IIα promoter in etoposide/teniposide-resistant human cancer cells (Kubo et al., 1995) or an activator in a merbarone-resistant CEM cell line (Mo et al., 1997).

Heat shock, p53 and histone modification can also affect topo IIα regulation (Bronner et al., 2002, Isaacs et al., 1998). The effects of heat shock on topo IIα expression are dependent on the duration of the heat treatment and cell type. For example, with incubation at 45ºC for 15 minutes, topo IIα mRNA rapidly degrades in HeLa cells (Goswami et al., 1996) but increases in human epidermoid cancer KB cells (Matsuo et al., 1993). The p53 protein is thought to be a negative regulator since studies overexpressing wild-type p53 demonstrated a decrease in topo IIα expression (Joshi et al., 2000; Sandri et al., 1996b). Inhibiting histone deacetylase in mouse embryonic fibroblasts (NIH3T3 cells) causes an ICB-dependent increase in topo IIα promoter

activity, showing that histone deacetylation is important for down regulating topo IIα promoter activity (Adachi et al., 2000).

Due to the proximity of putative c-Myb binding site to the topo IIα transcription site, it is thought that changes in c-Myb may impact topo IIα promoter expression (Figure 1.5.) (Bronner et al., 2002). In co-transfection studies, overexpression of c-Myb increased topo IIα promoter activity and expression of a dominant negative c-Myb polypeptide decreased topo IIα promoter activity. These changes were mainly noted in cells of hematopoietic origin (Brandt et al., 1997). On the other hand, mutation of the Myb binding site in topo IIα promoter reporter constructs had little effect on promoter activity compared to normal constructs (Adachi et al., 2000; Falck et al., 1999). These latter studies utilized non-hematopoietic cells, which may not have been sensitive to effects of c-Myb. Since a partial Myc/Max site lies upstream of the Myb binding sites, the authors of this study hypothesized that c-Myb may have altered the topo IIα promoter indirectly by binding to other transcription factors. However, in co-transfection experiments c-Myc and Max were unable to transactivate the topo IIα promoter implying that this site was not involved in topo II activation (Brandt et al., 1997). In summary, there are many questions remaining on the role of c-Myb on topo IIα promoter regulation.

Despite the current knowledge on topo IIα regulation, further investigation is needed to characterize the transcriptional control of the gene during the cell cycle. Such understanding may help to identify the mechanisms of down-regulating topo IIα protein in tumours resistant to topo II-targeting drugs and will help in designing therapeutics to overcome resistance (Bronner et al., 2002; Isaacs et al., 1998).

1.6.4. Secondary Leukemia and Topo II Poisons

It is known that a small population of patients undergoing cancer therapy involving topo II poisons will develop secondary leukemia (Andersen et al., 2001; Leone et al., 2001; Ng et al., 2005). Most of these patients develop AML and have characteristic translocations involving the *MLL* (mixed lineage leukemia) gene located on the chromosomal band 11q23 (Andersen et al., 2001; Aplan, 2006). The *MLL* gene encodes a protein that is important for transcription and differentiation of hematopoietic cells (De Braekeleer et al., 2005; Kohlmann et al., 2005). The gene fuses with other genes to form oncogenic fusion proteins or block tumour suppressor genes (Aplan, 2006; De Braekeleer et al., 2005). Furthermore, childhood leukemias involving similar 11q23 translocations have been linked to maternal consumption of environmental, nutraceutical or pharmaceutical topo II poisons (Ross, 1998; Spector et al., 2005). These findings imply that abnormal topo II activity may be responsible for initiating AML in some cases.

1.6.5. Benzene and Topo II

Disturbances in topo II may be an important mechanism in the development of benzene-induced leukemia for several reasons. First, certain subgroups of benzene-initiated leukemia share similar manifestations of chromosomal damage (such as translocations on chromosome 11q23 and t(8,21)) with leukemia arising from the use of topoisomerase II inhibitors (Eastmond et al., 2001; Holeckova et al., 2004; Zhang et al., 2005;). Secondly, pediatric leukemic patients expressing defective forms of NQO1 (the detoxifying enzyme that converts BQ back to HQ) have increased risk of forming *MLL*

gene alterations (Alexander et al., 2001; Eguchi-Ishimae et al., 2005; Smith et al., 2002). Finally, earlier studies showed that the benzene metabolites BQ and HQ could act as topo II inhibitors (Baker et al., 2001; Chen and Eastmond, 1995; Hutt and Kalf, 1996), most likely due to the fact that these studies were conducted in environments rich in reducing agents such as DTT, which destroy topo II catalytic activity (Bender et al., 2004; Wang et al., 2001; Zhou et al., 2003). Later studies conducted by Lindsey et al., (2004) showed that BQ acts as a topo II poison. In fact these studies demonstrated that BQ was more potent than etoposide (a known topo II poison that is extensively used in chemotherapy (Topcu, 2001) in inducing topo II-mediated DNA damage (Lindsey et al., 2004). HQ was also found to be a topo II poison but was less potent in stabilizing DNA-topo II complexes compared to BQ (Lindsey et al., 2005). Other benzene metabolites such as catechol, 4,4-biphenol, 2,2-biphenol and 1,2,4-benzenetriol had no effect on topo II activity (Lindsey et al., 2005). In summary, cytogenetic characterizations and *in vitro* enzymatic assays support the hypothesis that BQ and HQ are potent inhibitors of topo II and induce DNA damage similar to that observed in topo II therapy-related secondary leukemia.

1.7 Research Hypothesis and Objectives

Rationale

Based on previous findings, the benzene metabolite BQ may increase topo II-mediated chromosomal damage in two ways. Firstly, BQ can directly bind to the enzyme leading to increased DNA-topo II complexes (Lindsey et al., 2005). Secondly, BQ can increase c-Myb protein phosphorylation, which results in increased c-Myb activity (Wan and Winn, 2007). Since c-Myb may regulate topo IIα transcription (Brandt and Kroll, 1997), an increase in topo IIα protein levels might be anticipated if c-Myb activity is increased. If this is true then BQ exposure could increase topo II protein levels (mediated through the c-Myb pathway) and increase DNA damage.

Hypothesis

I hypothesize that BQ increases c-Myb activity, which in turn increases topo IIα promoter activity. As a result, there will be an increase in topo IIα protein expression which is expected to increase the amount of DNA strand breaks.

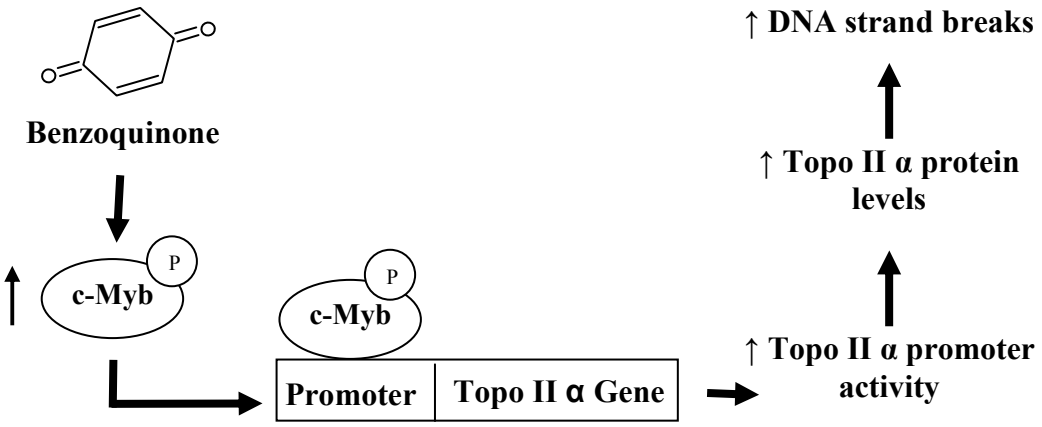

Figure 1.6. Hypothesis: Exposure to BQ increases c-Myb activity, which in turn increases topo IIα promoter activity.

Objectives

To address this hypothesis, the following objectives were investigated:

Objective 1: To examine the effects of BQ on c-Myb activity in human K-562 leukemic cells.

Objective 2: To examine the effects of BQ on topo IIα promoter activity and determine whether the effects are dependent on c-Myb signalling.

Objective 3: To examine the effects of BQ on topo IIα and c-Myb protein levels.

2. Materials and Methods

2.1. Cell Culture

The human chronic myeloid leukemia K-562 cell line (CCL-243, ATCC, Manassas, VA, USA) was maintained at 37ºC/5% CO_2 in RPMI-1640 media containing L-glutamine which was supplemented with 10% fetal bovine serum, 23.4 mM sodium bicarbonate and 10 units/ml penicillin-streptomycin. All cell culture media reagents were obtained from Gibco^{TM} Invitrogen Corporation (Burlington, ON, Canada) except for fetal bovine serum which was acquired from Fisher Scientific (Whitby, ON, Canada).

2.2. Cell Growth

To characterize the growth properties of K-562 cells during the transfection procedure, K-562 cells were plated according to the transfection protocol outlined in Figure 2.1. Cell densities (as $x10^4$ cells/ml) were measured using a Hausser Scientific Levy hemacytometer and Leica DM IL microscope. This experiment was conducted in duplicates and replicated three times.

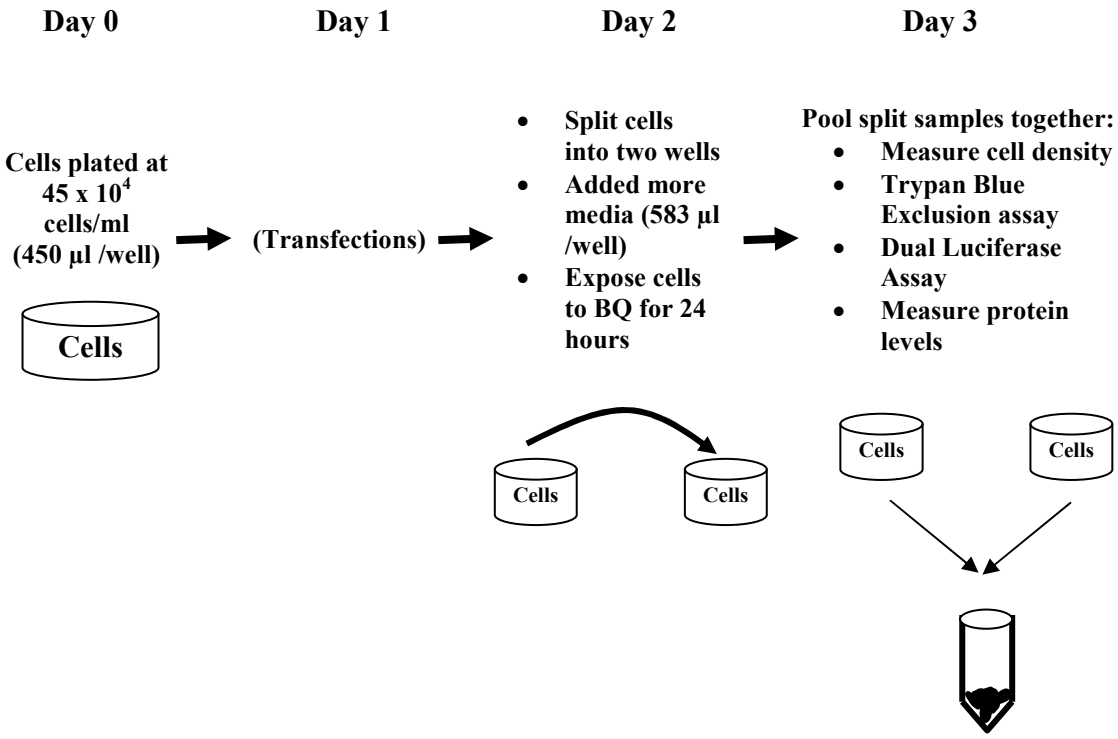

Figure 2.1. Tranfection protocol.
This transfection protocol was used in all of the experiments. K-562 cells were plated at a density of 45 x 10^4 cells/ml on Day 0. Transfections were carried out 24 hours later (Day 1). On Day 2, cells were divided into two parts, nourished with more media and exposed to BQ (0, 5, 10, 25, 37, 50 or 75 µM). Finally, on Day 3 the divided cells were recombined together and assays were performed.

2.3. Trypan Blue Exclusion Assays

The Trypan Blue Exclusion assay was used to measure the effects of BQ on cell survival. Following the transfection protocol (Figure 2.1), K-562 cells were exposed to 0, 25, 37, 50 or 75 μM BQ for 24 hours. After washing the cells in phosphate-buffered saline (PBS), the cells were resuspended in a small volume of PBS. Equal volumes of the cell suspension and a 0.4% solution of trypan blue dye (Sigma) were mixed together. The number of live cells (white cells that did not take up the dye) and dead cells (blue cells which did take up the dye) were then counted. Cell viability was expressed as the percentage of live cells compared to the total number of cells. To control for day-to-day differences between experiments, the cell viability values of non-exposed controls were set to 100% and the cell viability values of BQ treated samples were then expressed as a percentage of the non-exposed groups. This experiment was conducted in duplicates and replicated three times (therefore n=3).

2.4. Transfections for Luciferase Assays

Plasmids expressing the *mim-1* promoter linked to the luciferase gene (ΔEluc) and c-Myb were acquired from Dr. Scott Ness (Department of Molecular Genetics and Microbiology, University of New Mexico, NM, USA). The plasmid expressing the DNA binding domain of the c-Myb (DBD) was obtained from Dr. Giuseppe Raschella (Section of Toxicology and Biomedical Sciences, ENEA Research Center Casaccia, Italy). The plasmid expressing the topo IIα promoter linked to the *luciferase* gene (-620TOP2A-pGL3) was obtained from Dr. Susan P. C. Cole (Department of Pharmacology & Toxicology, Queen's University, ON, Canada) and was used to examine the changes in

topo IIα promoter activity. Plasmids expressing the renilla luciferase thymidine kinase (RLTK) and an empty vector (pGEM) were bought from Promega Inc. (Madison, WI, USA).

K-562 cells were plated at a density of 45 x 10^4 cells/ml (450 µl /well) in 24 well plates. After 24 hours, the cells were transfected using FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. For each well, 1 µl of FuGENE 6 reagent was used along with the appropriate amount of DNA as described below for each treatment.

2.4.1. Effects of BQ on c-Myb Activity

To measure the effects of BQ on c-Myb activity, K-562 cells were first transfected with 0.3 µg of ΔEluc DNA and 0.02 µg of RLTK DNA per well. Negative controls included wells that were transfected with 0.3 µg of empty vector (pGEM), 0.3 µg of ΔEluc only or no foreign DNA. Twenty-four hours after transfection, cells were split into 2 fractions, topped with more media and exposed to BQ (0, 25 or 37µM) as described earlier in section 2.2. In each round of experiments, the transfections were carried out in triplicates for each BQ concentration and in duplicates for the controls. This experiment was replicated 4 times.

2.4.2 Effects of BQ on Topo IIα Promoter Activity

To examine the effects of BQ on topo IIα promoter activity, 0.2 µg of pGEM, 0.1 µg of -620TOP2A-pGL3 and 0.02 µg of RLTK were transfected into each well. K-562 cells were then exposed to BQ (0, 5, 10, 25 or 37 µM) as described in section 2.4.1. Cells

that were transfected with 0.3 μg of empty vector or without any foreign DNA were used as negative controls.

2.4.3 Effects of Overexpressing c-Myb or DBD on Topo IIα Promoter Activity

To examine the effects of overexpressing c-Myb and DBD on topo IIα promoter activity, 0.1 μg of -620TOP2A-pGL3, 0.02 μg of RLTK and 0, 0.05, 0.1 or 0.2 μg of c-Myb or DBD expressing plasmids were transfected into cells. Appropriate amounts of empty vector pGEM were also added to ensure a total amount of 0.302 μg of DNA was added into each well. Cells that were transfected with 0.3 μg of empty vector or without any foreign DNA were used as negative controls.

2.4.4 Effects of Overexpressing c-Myb on c-Myb Activity

To examine the effects of overexpressing c-Myb on c-Myb activity, all cells were transfected with 0.25 μg of ΔEluc, 0.02 μg RLTK and 0, 0.025, 0.05 or 0.1 μg of c-Myb expressing plasmid. Luciferase activity was then measured 48 hours later. The same controls were used as described in section 2.4.1.

2.4.5 Effects of Overexpressing DBD on Topo IIα Promoter Activity in the Presence of BQ

To examine the effects of BQ and DBD on topo IIα promoter activity, 0.1 μg of -620TOP2A-pGL3, 0.02 μg of RLTK and 0, 0.05 or 0.2 μg of DBD expressing plasmids were transfected into each well. Changes in media and BQ exposure (0 or 37 μM) were

carried out as described in section 2.4.1. Cells that were transfected with 0.3 µg of empty vector were used as negative controls.

2.5 Dual Luciferase Assay

To determine luciferase activities, 48 hours after transfection, K-562 cells were washed twice in cold PBS and then lysed in 100 µl of 1x Passive Lysis Buffer (Promega Corp., Madison, WI). Cells were then alternatively incubated at -20ºC, room temperature, -20ºC and finally at room temperature for 10 minutes at each condition. Dual luciferase activities were measured using 20 µl of the cell lysates using a nonproprietary dual luciferase assay (Dyer et al., 2000) and a Lumat LB 9507 Variable Injector luminometer (Berthold Technologies GmbH & Co., Germany). The results are expressed as relative luciferase activity (RL1/RL2) where firefly luciferase readings were normalized to the renilla luciferase readings (internal control). To control for day-to-day differences between experiments, the relative luciferase activities of non-exposed controls were set to 100% and the relative luciferase activities for the experimental groups were expressed as a percentage of the values from nonexposed cells or from cells transfected with an empty vector instead of plasmids expressing c-Myb or DBD.

2.6. Western Immunoblotting

To investigate the effects of BQ on topo IIα and c-Myb protein levels, 90 x 104 K-562 cells were plated into each well of a 6 well plate. After 48 hours, cells were split in a 1:1 ratio, topped up with 2.332 ml of media and exposed to 0, 25 or 37 µM of BQ. After 24 hours, the cells were pooled together as shown in Figure 2.1, washed twice in

cold PBS and lysed by suspension in 300 µL of RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 1 mM EDTA) containing protease inhibitors (1mM PMSF, 1mM benzamidine, 1 µM chymostatin, 1 µM leupeptin, 1 µM, antipain and 1 µM pepstatin A). The lysates were then sonicated on ice twice for 25 seconds using a Fisher Scientific Sonic Dismembrator 60 and then incubated on ice on a rocker for 30 minutes. The homogenates were then centrifuged at 15000 g for 10 minutes at room temperature and the supernatant was collected for each sample. Protein concentrations were measured using the Bio-Rad Laboratories Bradford Protein Assay (Mississauga, ON) and an Ultrospec 3100 Pro scanning spectrophotometer (Biochrom Ltd., UK). Samples containing equivalent amounts of protein were boiled in loading buffer (0.05 M Tris-HCl, 2.5 mM EDTA, 2% (w/v) sodium docecyl sulphate (SDS), 7% (v/v) glycerol, 0.002% (w/v) bromophenol blue) for 5 minutes.

The proteins were separated by SDS-PAGE on a 6% polyacrylamide gel using an Owl Separation Systems P9DS-1 Penguin Dual-Gel water cooled electrophoresis apparatus. Proteins were then transferred on to a polyvinylidene difluoride, carrier membrane (Milllipore Co, Bedford, MA) using the Owl Separation Systems' HEP-1 Panther Semi-Dry Electroblotter at 1 mA/cm^2 of gel for 3 hours. Subsequently, the membranes were blocked with 5% (w/v) skim milk dissolved in a Tris based buffer containing Tween 20 (TBST - 25 mM Tris-HCl, 140 mM NaCl, 2 mM KCl and 0.05% (v/v) Tween 20). To probe for the topo IIα protein, the membrane was incubated overnight at 4ºC with a 1:1000 dilution of 8D2 (a mouse monoclonal antibody with an epitope mapping to the C-terminus of the human topo IIα protein which was obtained from Dr A. Kikuchi (Nagoya University, Japan) in 5% (w/v) milk-TBST solution. Then

the membrane was washed 5 times for 5 minutes in TBST and incubated in horseradish peroxidase conjugated anti-mouse IgG secondary antibody (GE Biosciences Corp., Baie D'Urfe, QC) in 5% (w/v) milk-TBST at room temperature for an hour. The membrane was washed again in TBST and developed using the ECL chemiluminescene detection system (Perkin Elmer, Boston, MA) and the Kodak X-OMAT Blue scientific imaging film (Perkin Elmer, Boston, MA).

To probe for c-Myb protein, the membranes were incubated in a stripping solution (0.7 % (v/v) β-mercaptoethanol, 2% (v/v) SDS, 62.5 mM Tris-HCl, pH 6.7) for 15 minutes at 37ºC. Afterwards, they were blocked with 5% (w/v) milk-TBST solution for 1 hour at room temperature and then incubated in a 1:5000 dilution of an anti-c-Myb rabbit polyclonal IgG antibody that recognizes an epitope on the C-terminus of murine c-Myb (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4ºC. As described previously, the membranes were washed in TBST, incubated in a 1:5000 dilution of sheep anti-rabbit IgG secondary antibody (GE Biosciences Corp., Baie D'Urfe, QC) in 5% (w/v) milk-TBST at room temperature and blots were then developed.

The membrane was once again reprobed for α-tubulin to verify equal protein loading of the wells. The same stripping and blocking processes were carried out as described earlier. Membranes were incubated with a 1:5000 dilution of mouse monoclonal anti-α-tubulin IgG antibody overnight at 4ºC. A 1:5000 dilution of anti-mouse IgG secondary antibody in 5% (w/v) milk-TBST solution was used to detect the α-tubulin antibody. The membrane was developed as described above.

The immunoblot bands were scanned and quantified by measuring their relative optical density (ROD) using Image J acquired from the National Institutes of Health

(NIH). The ROD values for topo IIα and c-Myb were normalized to the band densities of α-tubulin content for each well. To control for day-to-day differences, the ROD ratios were normalized to values from non-exposed cells.

2.7. Data Analysis

Statistical analysis was conducted using GraphPad Prism 3.0 software (GraphPad Inc.). Results for all experiments except experiment 2.4.5 were analyzed using one-away Analysis of Variance (ANOVA) followed by Dunnett post-hoc test to compare treatment groups to the control. The interaction between DBD and BQ in experiment 2.4.5 was investigated using two-way ANOVA followed by Bonferroni post-hoc test. The minimum level of significance was set to $p<0.05$. Each experiment was carried out either in duplicates or triplicates and then replicated multiple times. Therefore, the mean of means was used during statistical analysis, and the values are presented as means ± standard error (SE). The following representation was used to denote the level of significance: * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

3. Results

3.1. Growth of K-562 cells

Since topo IIα protein is abundantly expressed in proliferating cells and is reduced during confluency (Brown et al., 1995; Isaacs et al., 1996; Prosperi et al., 1992), it was essential to establish whether K-562 cells were in proliferative phases over the course of the transfection experiments. Cell growth was assessed by measuring cell densities (x 10^4 cells/ml) at different time points. As noted in Figure 3.1, the cell density approximately triples between Day 0 to Day 2 and doubles after the cells are split and nourished with more media on Day 2. The changes in cell density imply that the cells were in proliferative phases during the period of transfections (days 1- 3) and BQ treatment (days 2 – 3).

3.2. Cytotoxicity and Cell Growth

Cellular toxicity was measured using the Trypan Blue Exclusion assay. Exposure to concentrations above 37 μM BQ for 24 hours significantly increased ($p<0.05$) cell death compared to non-treated controls (Figure 3.2). Furthermore, the effects of BQ on cell growth were assessed by counting the cell density after 24 hours of BQ exposure. Once again concentrations above 37 μM BQ caused a significant decrease ($p<0.01$) in cell density compared to the non-exposed cells (Figure 3.3).

Day 0: Plated 45 x 10^4 cells/ml in each well

Day 1: Cells were incubated at 37ºC, 5% CO_2

Day 2: After determining the cell densities, the cells were spilt into two samples and diluted in more media.

Day 3: Last day of transfection protocol

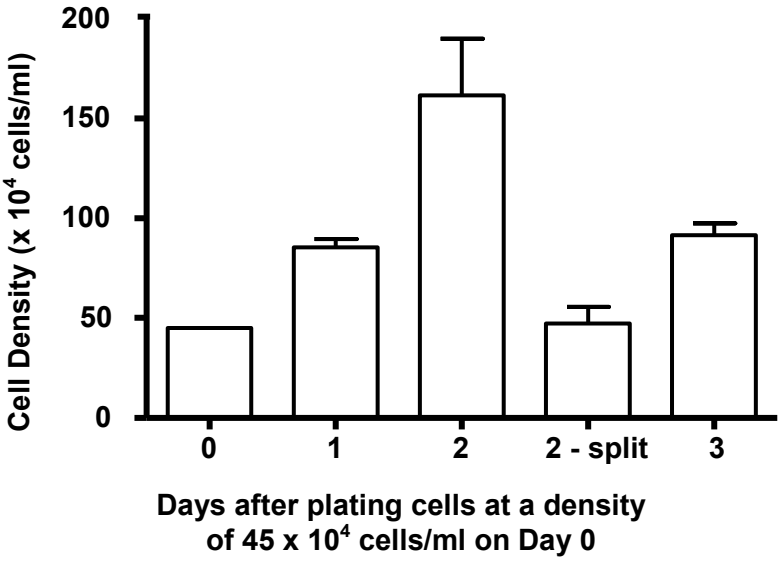

Figure 3.1. Growth profile of K-562 cells over the course of transfection experiments.
K-562 cells were plated at density of 45 x 10^4 cells/ml and cell densities were measured over the next 3 days. (Day 2 split shows the calculated cell density based on the values measured on Day 2) (n=3).

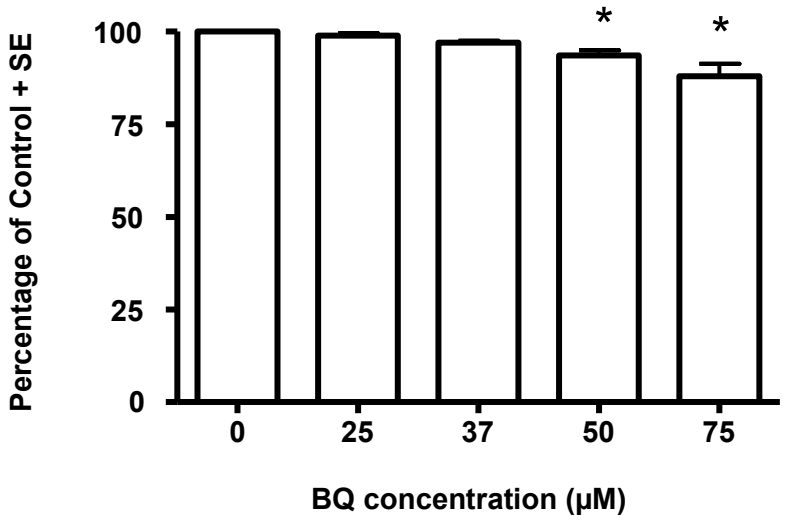

Figure 3.2. BQ cytotoxicity in K-562 cells measured using Trypan Blue Exclusion assay.
K-562 cells were exposed to 0, 25, 37, 50 or 75 μM BQ for 24 hours before cytotoxicity was measured. Cytotoxicity was assessed by calculating the percentage of live cells compared to the total number of cells. To control for day-to-day differences, the data was expressed as a percentage of the viability values of non-exposed cells (control). * indicates statistical difference from 0 μM controls ($p<0.05$) (n=3).

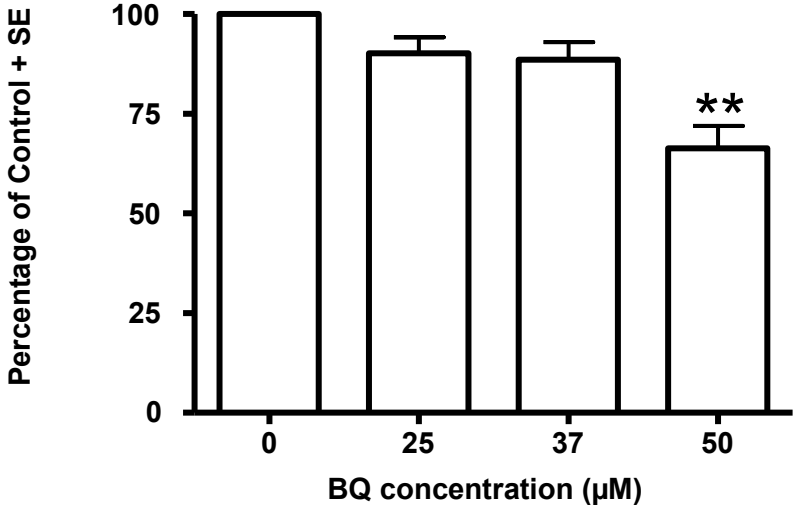

Figure 3.3. Effects of BQ on K-562 cell growth.
Changes in cell growth were measured by counting the cell density (x 10^4 cells/ml) after exposure to 0, 25, 37, or 50 μM BQ for 24 hours. To control for day-to-day differences, data was expressed as a percentage of the cell density values of non-exposed cells (control). * indicates statistical difference from 0 μM controls ($p<0.01$) (n=3).

3.3. Effects of BQ on c-Myb Activity

The ΔEluc c-Myb reporter construct contains the *mim-1* gene linked the *luciferase* gene. The *mim-1* gene is regulated by the c-Myb protein such that expression of the *luciferase* gene provides an indirect assessment of c-Myb's transcriptional activity. Therefore, transient transfections of this reporter construct and dual luciferase assays were used to examine the effects of BQ on c-Myb transcriptional activity. As noted in Figure 3.4 (or Figure A.1 in Appendix) exposure to 25 and 37 μM BQ for 24 hours caused a statistically significant increase ($p<0.05$) in endogenous c-Myb activity.

3.4. Topo IIα Promoter Activity

Changes in topo IIα promoter activity were indirectly assessed by measuring the activity of a topo IIα promoter-linked-luciferase reporter construct. Exposure of K-562 cells to BQ concentration 5, 10 and 25 μM for 24 hours did not change topo IIα promoter activity compared to the control (Figure 3.5). However, exposure to 37 μM BQ caused a statistically significant increase ($p<0.01$) in topo IIα promoter activity.

When c-Myb protein was overexpressed within the cells, no significant increase in topo IIα promoter activity was observed (Figure 3.6). In order to verify that the cells were expressing the c-Myb plasmids, the c-Myb reporter assay was used. Transfection of 0.1 μg of c-Myb plasmid DNA caused an increase in c-Myb activity (Figure 3.7 or Figure A.2 in Appendix) indicating that the cells were overexpressing exogenous c-Myb protein.

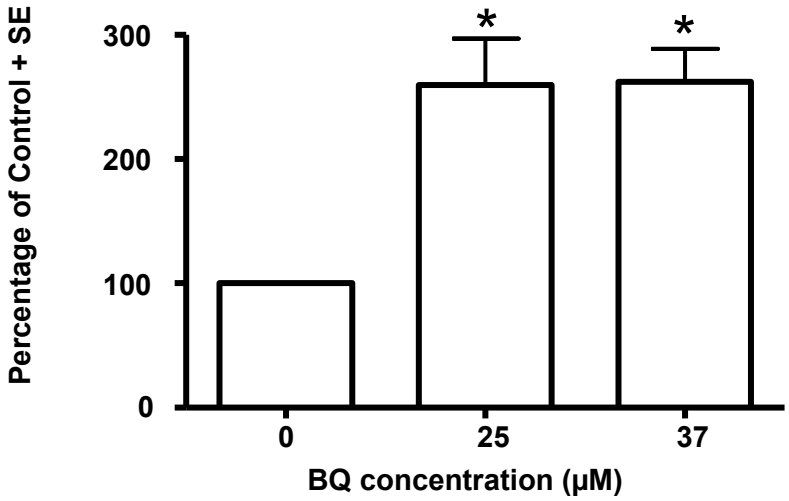

Figure 3.4. Effects of BQ on c-Myb activity.
K-562 cells were exposed to 0, 25 or 37 μM BQ for 24 hours and c-Myb activity was measured using the dual luciferase assay. The results are expressed as a percentage of the relative luciferase units (RL1/RL2) of non-exposed cells. * indicates statistical difference from 0 μM controls ($p<0.05$) (n=4).

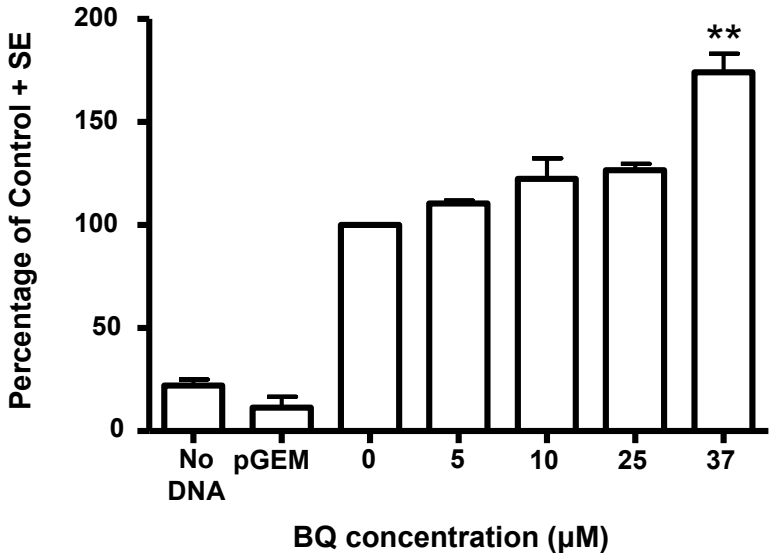

Figure 3.5. Effects of BQ on topo IIα promoter.
K-562 cells were exposed to 0, 25 and 37μM BQ for 24 hours and topo IIα promoter activity was measured using the dual luciferase assay. The results are expressed as a percentage of relative luciferase units (RL1/RL2) of non-exposed cells. Negative controls include cells that were transfected with no foreign DNA (No DNA) or 0.3 μg of empty vector (pGEM). ** indicates statistical difference from 0 μM controls ($p<0.01$) ($n\geq3$).

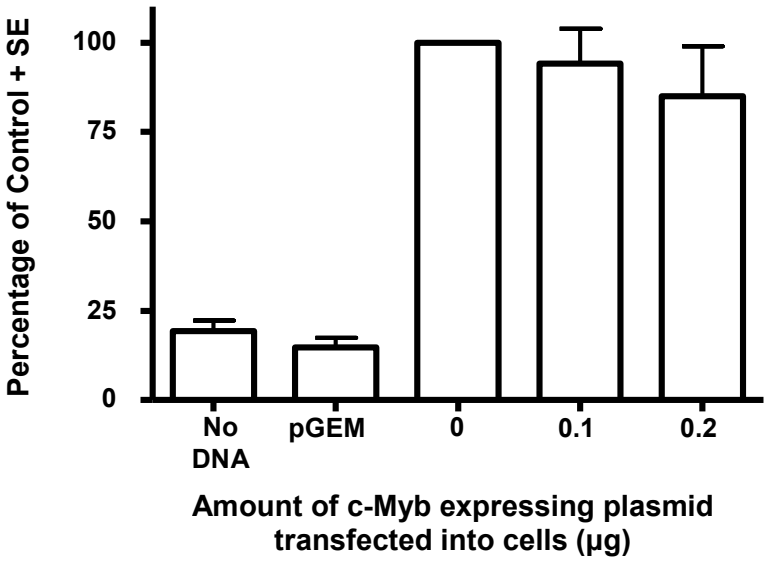

Figure 3.6. Effects of overexpressing c-Myb on topo IIα promoter.
Changes in topo IIα promoter activity were measured using the dual luciferase assay in K-562 cells that were overexpressing c-Myb protein. The results are expressed as a percentage of the relative luciferase units (RL1/RL2) of cells expressing 0 µg exogenous c-Myb (control). Negative controls include cells that were transfected with no foreign DNA (No DNA) or 0.3 µg of empty vector (pGEM) (n=4).

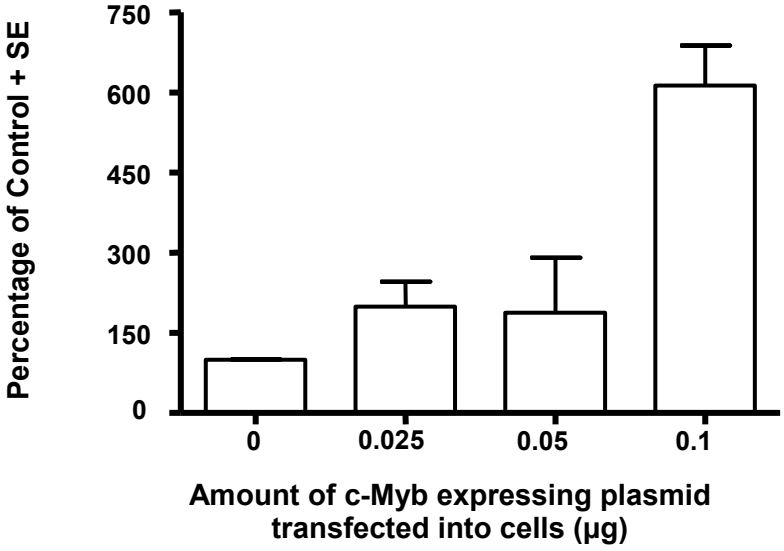

Figure 3.7. Effects of overexpressing c-Myb on c-Myb activity.
Changes in c-Myb activity were measured using the dual luciferase assay in K-562 cells that were overexpressing c-Myb protein. The results are expressed as a percentage of the relative luciferase units (RL1/RL2) of cells expressing 0 µg exogenous c-Myb (control) (n=2).

The cells were then transfected with a plasmid that expresses the DBD subunit of c-Myb in order to block endogenous c-Myb transcriptional activity. The DBD competes with endogenous c-Myb for the c-Myb binding sites on DNA, which leads to decreased c-Myb transcriptional activity. As noted in Figure 3.8, overexpressing DBD did not affect topo IIα promoter activity compared to the controls.

To determine the role of c-Myb in BQ-mediated increase in topo IIα promoter activity, the DBD was overexpressed in the K-562 cells which were also exposed to BQ. Two-way Anova (Table 3.1) indicates that the main effects of BQ, DBD and their interaction are statistically significant ($p<0.01$). The presence of significant interaction implies that the effects of BQ vary with the level of DBD and vice versa. As noted in Figure 3.9, BQ-mediated increase in the topo IIα promoter activity decreases with the expression of the DBD plasmid. To further characterize the nature of interaction, the Bonferroni post-hoc analysis indicated that BQ increased the topo IIα promoter activity significantly ($p<0.001$) in the absence of DBD. However, the effect of BQ was not significant when 0.5 μg or more DBD was expressed.

3.5. Effects of BQ on Topo IIα and c-Myb Protein Levels

The cell growth protocol that was used for transfection experiments was also used to examine the effects of BQ on topo IIα and c-Myb protein levels. After exposure to 25 and 37 μM BQ for 24 hours, while increasing protein trends were observed, no significant changes in topo IIα or c-Myb protein levels were noted compared to the controls (Figure 3.10).

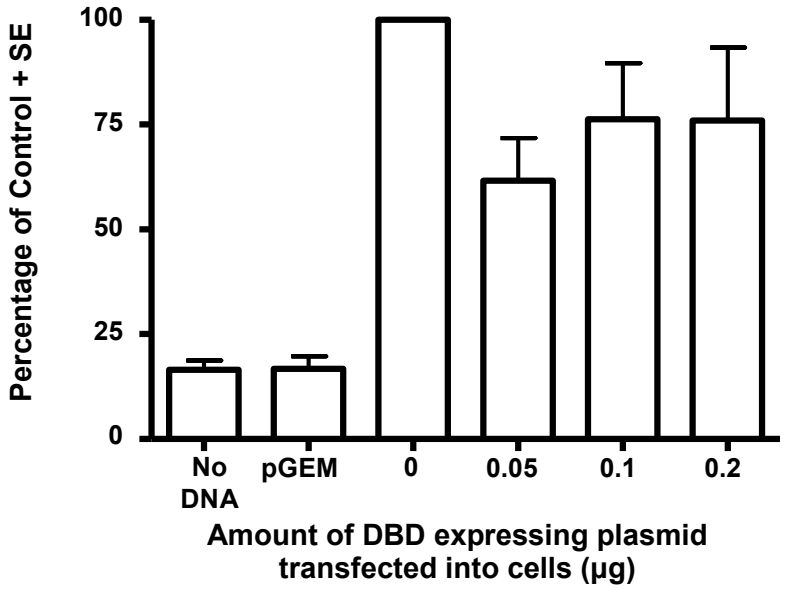

Figure 3.8. Effects of overexpressing DBD on topo IIα promoter.
Changes in topo IIα promoter activity were measured using the dual luciferase assay in K-562 cells that were overexpressing DBD polypeptide. The results are expressed as a percentage of relative luciferase units (RL1/RL2) of cells expressing 0 µg of the DBD plasmid (control). Negative controls include cells that were transfected with no foreign DNA (No DNA) or 0.3 µg of empty vector (pGEM) (n=6).

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F	P value	% of total variation
Interaction	2	6474	3237	6.055	0.0098	18.68
BQ	1	5503	5503	10.29	0.0049	15.88
DBD	2	13060	6530	12.21	0.0004	37.68
Residual	18	9623	534.6			

Table 2. Analysis of variance of topo IIα promoter activity to examine the interactive effects of BQ and DBD.

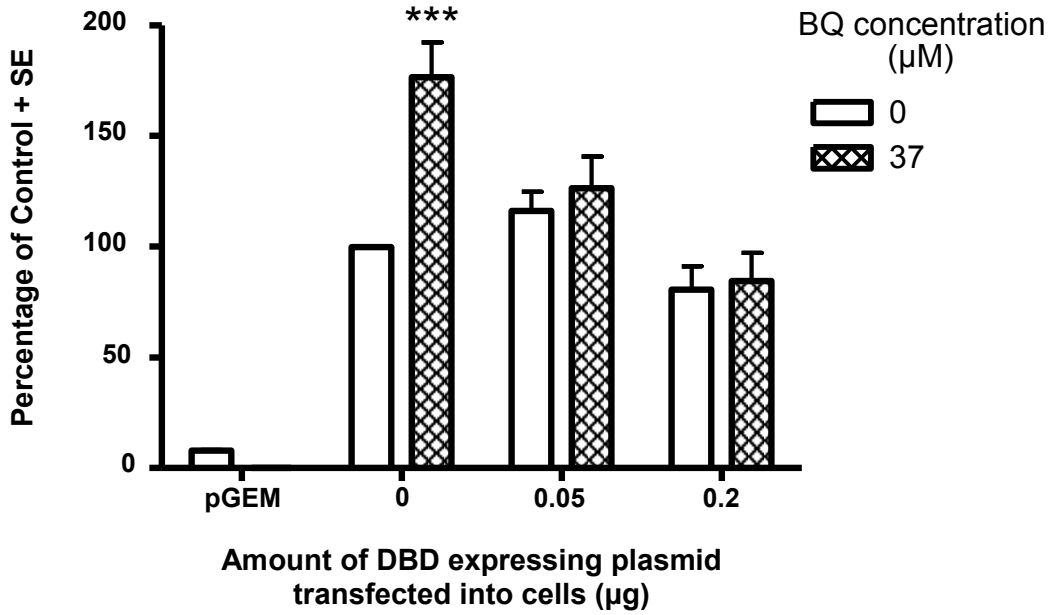

Figure 3.9. Effects of overexpressing DBD and BQ exposure on topo IIα promoter activity.

Changes in topo IIα promoter activity were measured using the dual luciferase assay cells that were overexpressing DBD protein with or without BQ (37 μM) exposure for 24 hours. The results are expressed as a percentage of the relative luciferase units (RL1/RL2) of cells expressing that were expressing 0 μg of the DBD plasmid and that were not exposed to BQ. Negative controls include cells that were transfected 0.3 μg of empty vector (pGEM). *** indicates statistical difference between cells exposure to 37 μM BQ compared to non-exposed cells in the absence of DBD expression ($p<0.001$) ($n=4$).

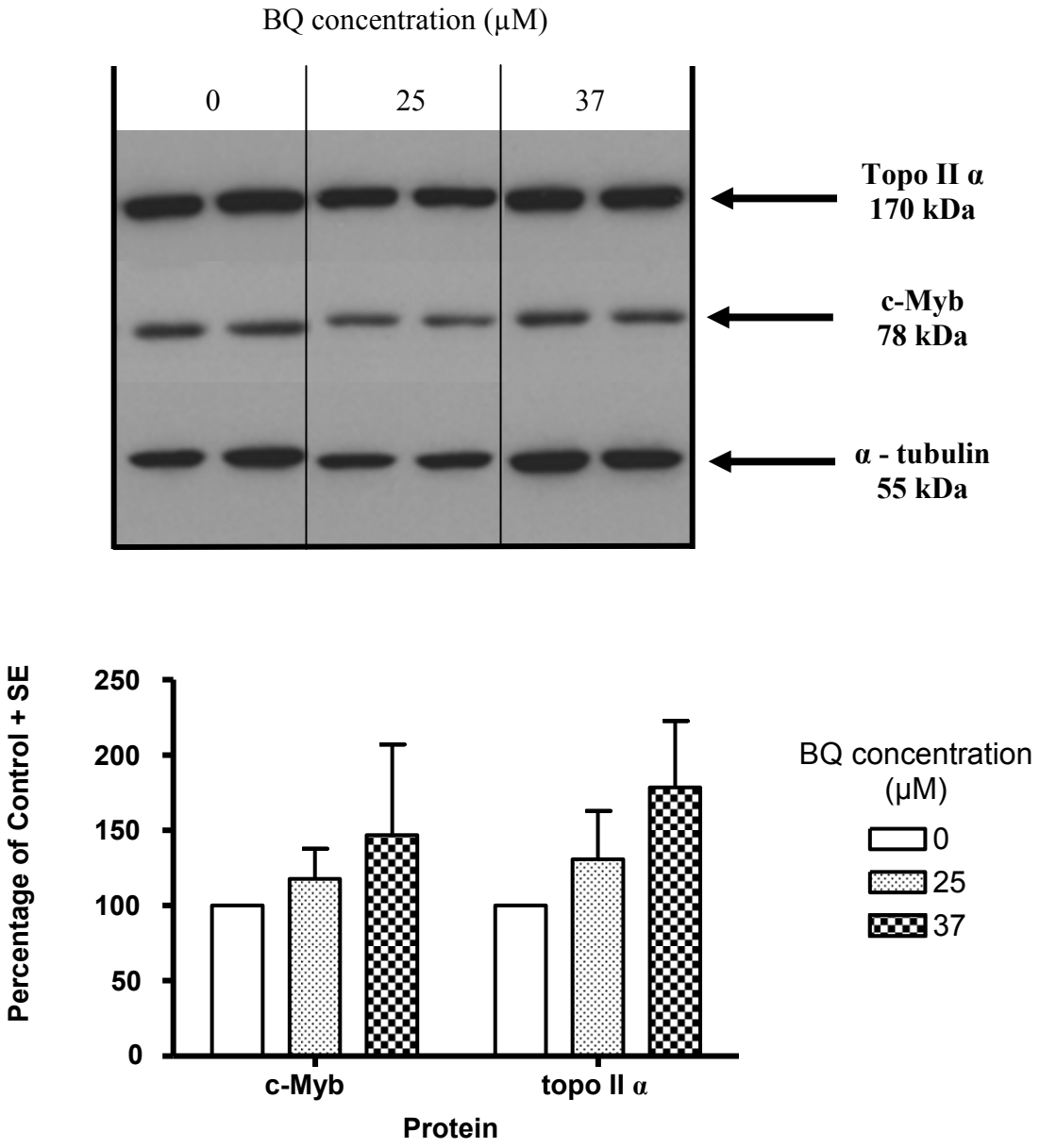

Figure 3.10. Effects of BQ on topo IIα and c-Myb protein levels.
Western immunoblotting was used to detect changes in c-Myb and topo IIα protein levels in K-562 cells treated with or without BQ for 24 hours. All bands were normalized to α-tubulin levels. To control for day-to-day differences, the protein/α-tubulin ratios were expressed as a percentage of the values of non-exposed cells (control) (n=3).

4. Discussion

Although BQ is a known leukemogen, the basis of its toxicity is not well defined. It is thought that BQ induces carcinogenicity by producing ROS, altering protein activity and disturbing chromosomal integrity (Whysner et al., 2004). This thesis investigated the potential for BQ to alter the activity of the transcription factor c-Myb and the DNA modulating enzyme topo IIα. The c-Myb protein plays an important role during hematopoiesis by regulating cell proliferation, differentiation and apoptosis (Gonda, 1998). It is abundantly expressed in immature hematopoietic cells and repressed when cells differentiate to their terminal cell types (Jieping et al., 2007). The c-Myb protein is considered to be oncogenic since overexpression maintains erythroid, lymphoid and myeloid cells in an immature and proliferative state as seen in many cases of AML, T-cell leukemia, melanomas and other forms of leukemia (Gonda, 1998; Weston, 1999).

Recently, Wan and Winn, (2004, 2005, 2006, and 2007) showed that the benzene metabolites catechol, HQ and BQ could induce changes in the c-Myb pathway. In addition, *in vitro* studies demonstrated that BQ mediated an increase in c-Myb activity by increasing c-Myb phosphorylation rather by than up-regulating c-Myb protein levels (Wan and Winn, 2007). Since these experiments were conducted in non-leukemic, chicken erythroblasts (HD3 cell line), the first objective of the current study was to determine whether or not BQ induced changes in c-Myb activity in K-562 cells.

The human chronic myeloid leukemic K-562 cell line was used in this study to examine BQ-mediated changes for several reasons. It is well known that *in vitro* cell models are useful tools to investigate mechanistic changes without the confounding factors normally associated with *in vivo* assays. Furthermore, c-Myb's role in erythroid

differentiation and proliferation has been well characterised in K-562 cells (Miyamoto et al., 1990). These cells also express high levels of c-Myb mRNA (Walker et al., 1998) and were previously used in our laboratory to examine the effects of thalidomide on c-Myb signalling (Thadani et al., 2006). Finally, numerous studies have used the K-562 cell line and its variants to investigate the effects of anticancer drugs (actinomycin-D, etoposide, doxorubicin and cisplantin) on topo IIα mRNA and protein expression (Chen et al., 2002; Kurz et al., 2001; Laurand et al., 2004; Ma et al., 2005; Hargrave et al, 1995). Furthermore, K-562 cells express many of the biotransforming enzymes like CYP2E1, GST, and NQO1 (Nagai et al., 2002; Nagai et al., 2004; Hsieh et al., 2006). However, MPO is poorly expressed within this cell line (Baker et al., 2002).

Using a non-proprietary reporter assay and western immunoblotting, the current study demonstrated that exposure to 25 and 37 μM BQ for 24 hours caused an increase in c-Myb activity (Figure 3.4) but did not alter c-Myb protein levels (Figure 3.10). This increase in activity was not due to changes in cell growth (Figure 3.2) or cytotoxicity caused by BQ exposure (Figure 3.3). These results are consistent with those of Wan and Winn, (2007) and suggest that BQ-mediated changes in c-Myb signalling are similar in both human K-562 cells and chicken HD3 cells.

The c-Myb protein controls different biological processes by regulating the transcription of many genes such as *mim-1, GBX-2, cdc-2, bcl-2* and *topo II α*. (Brandt et al., 1997; Introna and Golay, 1999; Ness, 2003). The *topo II* gene encodes an important nuclear enzyme that removes torsional stress when the DNA undergoes structural changes (Isaacs et al., 1998). Disturbances in topo II activity or protein level may lead to aberrant DNA strands breaks, which may trigger mutagenic events (Topcu, 2001). Of the

two isoforms, the present studies were focused on the changes in topo IIα primarily because this isoform predominantly regulates the DNA structure during transcription, replication and cell division (Austin and Marsh, 1998). Transcriptional regulation of the topo IIα promoter mainly controls topo IIα protein expression (Bronner et al., 2002).

The proximity of a c-Myb's regulatory binding site to the transcription start site (Figure 1.5) suggests that the c-Myb protein may strongly influence topo IIα promoter activity (Brandt et al., 1997). However, c-Myb's regulation of the topo IIα promoter remains controversial (Adachi et al., 2000; Falck et al., 1999; Furukawa et al., 1998). Brandt et al., (1997) used co-transfection experiments to show that c-Myb overexpression increased topo IIα promoter activity specifically in hematopoietic cells. Moreover, they found that overexpression of a dominant negative c-Myb polypeptide decreased topo IIα promoter activity in the HL-60 leukemic cell line but not in HeLa cervical carcinoma cells (Brandt et al., 1997). Other studies have shown that c-Myb does not play an important role in regulating the topo IIα promoter (Adachi et al., 2000; Falck et al., 1999; Furukawa et al., 1998). For example, Furukawa et al., (1998) used truncated topo IIα promoter-luciferase constructs to show that c-Myb binding to the promoter only increased basal promoter activity to 1.6% of the maximum activity in urinary bladder carcinoma cells. Falck et al., (1999) and Adachi et al., (2000) used human and mouse topo IIα promoter-luciferase reporter constructs (respectively) with site-directed mutagenic Myb sites and found insignificant decreases in promoter activity compared to full-length promoter constructs. These studies imply that c-Myb does not influence topo IIα promoter activity. Since these studies were conducted in non-hematopoietic cells, it

is possible that the cell models used were not sensitive to c-Myb mediated changes in topo IIα promoter activity (Adachi et al., 2000; Falck et al., 1999; Furukawa et al., 1998).

Given these conflicting results, the second objective of this thesis was to determine whether c-Myb increased topo IIα promoter activity in K-562 cells. As noted in Figure 3.6, transfecting different amounts of a plasmid expressing c-Myb did not increase topo IIα promoter activity in K-562 cells. These transfection results are contradictory to those reported by Brandt et al., (1997). The difference in results cannot be attributed to poor transfection of the c-Myb plasmid since preliminary results showed that overexpression of c-Myb increased c-Myb activity in K-562 cells (Figure 3.7). Furthermore, competitive inhibition of endogenous c-Myb activity by overexpressing the DBD did not affect topo IIα promoter activity either (Figure 3.8). These results indicate that, under normal conditions, c-Myb does not play an important role in regulating topo IIα promoter activity and that this holds true in a cell model with a hematopoietic origin.

To test the hypothesis that BQ initiates toxicity by binding to the topo IIα promoter, I then determined whether BQ could affect topo IIα promoter activity, and if so, whether this effect was dependent on the c-Myb pathway. Figure 3.5 shows that exposure to 37 μM BQ for 24 hours caused a statistically significant increase in topo IIα promoter activity compared to the control (non-exposed cells). Secondly, two-way ANOVA analysis (Table 2) indicated that the interaction between BQ and DBD was statistically significant implying that BQ-mediated increase was blocked by the overexpression of DBD polypeptide (Figure 3.9). These results imply that the BQ-associated increase in topo IIα promoter activity is dependent on c-Myb signalling.

It is known that phosphorylation of the c-Myb protein at different sites can affect the protein's ubiquitinization, DNA binding and/or transcriptional activation of different promoters (Aziz et al., 1993; Bies et al., 2000; Cures et al., 2001; Luscher et al., 1990; Miglarese et al., 1996; Winn et al., 2003). Recently, Wan and Winn, (2007) showed that BQ exposure increased c-Myb activity, which correlated with an increase in c-Myb phosphorylation. Moreover, phosphorylation of serines 11 and 12 by casein kinase 2 (CK2) alters c-Myb's ability to bind to DNA (Ramsay et al., 1995). Furthermore, phosphorylation of serine 528 differentially affects c-Myb's transcriptional control of different promoters (Miglarese et al., 1996). Therefore, I propose that BQ exposure stimulated the phosphorylation of c-Myb causing the protein to become activated, resulting in increased transcriptional regulation of the topo IIα promoter. Additionally, since increased promoter activity was only observed when cells were treated with a high concentration of BQ, a threshold level of phosphorylation of c-Myb may be required for c-Myb to activate the topo IIα promoter. Under normal conditions, when c-Myb is not hyperactivated, c-Myb does not affect the topo IIα promoter activity (as noted in Figure 3.6). Furthermore, I propose that the DBD does compete with endogenous c-Myb for DNA binding sites, but that under normal conditions (ie. without exposure to BQ) this effect is not apparent. This is demonstrated by the results showing effects with the DBD only under conditions when cells were exposed to BQ. It would therefore be interesting to examine the effects of BQ exposure on topo IIα promoter activity in the presence of c-Myb overexpression, and in particular, evaluating the phosphorylation status of c-Myb.

Since BQ exposure increased topo IIα promoter activity, the third objective of this thesis was to determine whether BQ exposure resulted in increased protein

expression of topo IIα. Normally topo IIα mRNA and protein levels are differentially expressed during the cell cycle (Brown et al., 1995; Fogt et al., 1997; Kim et al., 1999; Prosperi et al., 1992). The protein is abundantly expressed in proliferative cells and repressed during confluency (Isaacs et al., 1996). Measuring the cell densities over the course of the transfection protocol confirmed that the cells were in the proliferative phase when they were exposed to BQ (Figure 3.1). Although the results were not statistically significant, they suggest that there was a dose-dependent increase in topo IIα protein levels with BQ exposure (Figure 3.10). It is possible that BQ caused an increase in topo IIα protein levels; however, the cell rapidly degraded the protein. It is well known that topo IIα protein levels are tightly regulated during the cell cycle (Bronner et al., 2002; Isaacs et al., 1998). For example, soon after mitosis, topo IIα protein is rapidly degraded via ubiquitinization while mRNA levels remain elevated (Nakajima et al., 1995; Nakajima et al., 1996). Furthermore, studies attempting to overexpress the human topo IIα protein using an advenoviral vector, only observed increased topo IIα mRNA and protein expression in tumour cells that expressed low levels of topo IIα (Asano et al., 2005; Zhou et al., 1999). Zhou et al., (1999) hypothesised that normal cells were able to tightly regulate topo IIα protein levels, thereby preventing overexpression of topo IIα, possibly by down regulating endogenous topo IIα.

People are continuously exposed to low concentrations of benzene, which through biotransformation processes ultimately leads to exposure to BQ (Duarte-Davidson et al., 2001; Johnson et al., 2007). Sustained exposure to BQ may induce permanent changes such that topo IIα protein may eventually become up-regulated. Since topo II works by cleaving DNA, with increased topo IIα protein there is a greater potential for the

formation of DNA strand breaks (Wang, 2002). Since DNA strand breaks are repaired by DNA repair mechanisms, which are not error free, increases in the number of strand breaks will also increase the risk of genomic instability (Chen and Liu, 1994; Pommier et al., 1994; Topcu, 2001). Secondly, if more DNA-topo II complexes are present, BQ then can act as a topo II poison (Lindsey et al., 2004) and further increase the amount of DNA strand breaks. Moreover, other metabolites such as catechol and HQ, which also increase c-Myb phosphorylation (Wan et al., 2005; Wan and Winn, 2007) may also indirectly affect topo IIα promoter activity. Future studies evaluating the role of increased DNA strand breaks in BQ toxicity and the role of topo IIα and c-Myb in this process would further elucidate the mechanism of BQ-induced toxicity.

5. Conclusions

In summary, the results of this study show that overexpression of normal c-Myb does not affect topo IIα promoter activity in a cell line of hematopoietic origin. However, BQ exposure activates the topo IIα promoter through a pathway which involves c-Myb. I hypothesize that BQ-mediated increases in c-Myb phosphorylation hyperactivates c-Myb's transcriptional control of the topo IIα promoter. Obtaining a greater understanding of the underlying mechanistic basis of how BQ induces toxicity may answer fundamental mechanistic questions on the biological effects of BQ exposure.

6. Future Directions

Based on the findings of this study, BQ-mediated disturbances in topo IIα promoter activity through c-Myb signalling may be an important step in benzene-mediated carcinogenesis. As other metabolites such as catechol and HQ also increase c-Myb activity and c-Myb phosphorylation (Wan et al., 2005; Wan and Winn, 2007), it would be interesting to examine the effects of these metabolites on topo IIα. HQ and catechol both have longer half-lives than BQ (Qu et al., 2003); therefore, these metabolites may have an effect on topo IIα promoter activity, mRNA transcription and protein expression, which could be evaluated using reporter constructs, reverse transcription PCR and western immunoblotting.

Furthermore, Wan and Winn, (2007) showed that BQ exposure increased phosphorylation of the c-Myb protein; however, the phosphorylation sites were not determined in the study. As mentioned earlier phosphorylation of serine 11 and 12 decreases c-Myb's ability to bind to DNA (Oelgeschlager et al., 1995) and phosphorylation of serine 528 differentially affects the transcriptional regulation of various gene promoters (Miglarese et al., 1996). Another future direction would be to map the phosphorylation sites on the c-Myb protein by mass spectroscopy of digested c-Myb protein extracted from cells exposed to benzene metabolites (Loyet et al., 2005). Determining the phosphorylated sites on the c-Myb protein would help clarify c-Myb's role in BQ-mediated increase in topo IIα promoter activity.

Knowledge of the influence of phosphorylated c-Myb on the topo IIα promoter could have potential therapeutic value by up-regulating topo IIα protein expression in cancers. Many anti-cancer drugs exert their toxicity by acting as topo II poisons to induce

DNA strand breaks with the end goal of stimulating apoptosis (Topcu, 2001). It is known that certain tumour types become resistant to topo II poison therapy by reducing topo II protein levels (Coon et al., 2002; Kang and Chung, 2002; Scandinavian Breast Group Trial 9401 et al., 2006; Williams et al., 2007). Therefore, increasing topo IIα protein expression could overcome this resistance.

Finally, since BQ also acts as a topo II poison, it would be important to quantify the amount of DNA damage caused by up-regulation of topo IIα protein or BQ's action as a topo II poison. The single cell gel electrophoresis assay (comet assay) (Moller, 2006) is a sensitive technique, which could be used to measure DNA damage within a single cell. Reducing agents such as DTT could be used to block BQ's topo II-blocking abilities (Lindsey et al., 2004) while a recombinant adenovirus containing the human *topo IIα* gene (Zhou et al., 1999) could be used to increase topo II protein in cells. Overexpression of topo IIα protein is difficult in normal cells (Zhou et al., 1999) but possible in cell models with reduced topo II protein expression (eg. the etoposide-resistant K562/MX2 human leukemic cell line (Asano et al., 2005)). Hence, overexpression of topo IIα in K562/MX2 cell line could be performed in order to examine the effects of topo IIα protein levels on the formation of DNA strand breaks. With the use of appropriate controls, these types of experiments will characterize the DNA damage caused by BQ and up-regulation of topo II protein.

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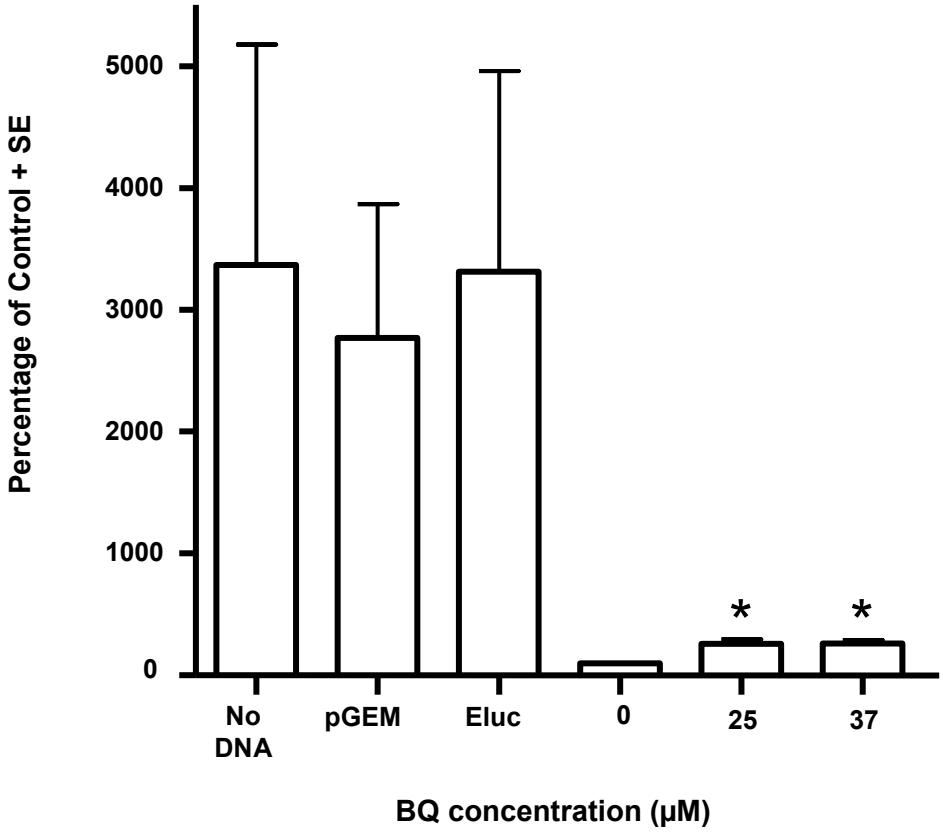

Figure A.1. Effects of BQ on c-Myb activity.
K-562 cells were exposed to 0, 25 or 37 μM BQ for 24 hours and c-Myb activity was measured using the dual luciferase assay. The results are expressed as a percentage of relative luciferase units (RL1/RL2) compared to the values of non-exposed cells. Negative controls included cells that were transfected with 0.3 μg of empty vector (pGEM), 0.3 μg of ΔEluc only (Eluc) or no foreign DNA (No DNA). * indicates statistical difference from 0 μM controls ($p<0.05$) (n=4). (NB. For the experimental data, the RL1 values were much lowerd than the RL2 values. However, within the negative controls, the RL1 values were relatively similar to RL2 values. Therefore the ratio between RL1/RL2 for the negative controls is higher than the ratio for the RL1/RL2 for the experimental data).

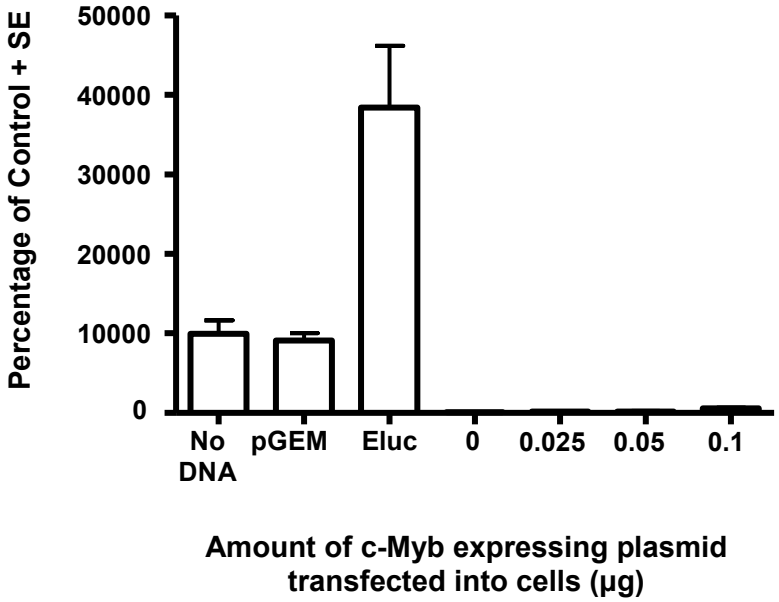

Figure A.2. Effects of overexpressing c-Myb on c-Myb activity.
Changes in c-Myb activity were measured using the dual luciferase assay in K-562 cells that were overexpressing c-Myb protein. The results are expressed as relative luciferase units (RL1/RL2) compared to the values of cells expressing the empty vector (pGEM) instead of a c-Myb expressing plasmid (n=2). (NB. For the experimental data, the RL1 values were much lowerd than the RL2 values. However, within the negative controls, the RL1 values were relatively similar to RL2 values. Therefore the ratio between RL1/RL2 for the negative controls is higher than the ratio for the RL1/RL2 for the experimental data).