THE EFFECT OF FUNCTIONAL GROUP SUBSTITUTION ON THE REDUCTION AND ACTIVATION OF BENZOQUINONE MUSTARDS BY NADPH: CYTOCHROME P450 REDUCTASE

By

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A Thesis submitted to the Faculty of Graduate Studies of

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ABSTRACT

Introduction: Bioreductive antitumour agents are an important class of anticancer drugs due to their suitability for enzyme-directed tumour targeting. Determining the effects of structural factors on the activation of bioreductive agents will allow the development of bioreductive agents that are specific for activation by a single enzyme.

Objective: To identify the effect of functional groups on the activation of the Benzoquinone Mustard (BM) bioreductive agents mediated by the reductive enzyme NADPH: cytochrome P450 reductase (P450 reductase).

Methods: A series of model BM analogs were used, including analogs substituted with electron donating groups: MBM, MeBM, m-MeBM, with electron withdrawing groups:CBM, and with sterically bulky groups:PBM, m-PBM, m-TBM. The effect of the functional groups on the rate of reduction of BM analogs by P450 reductase was determined by: a) EPR and b) spectrophotometric assay. Cytotoxicity of BM analogs was determined in two human cancer cell lines by means of MTT assays. DNA damage subsequent to P450 reductase-mediated reduction of the BM analogs was measured using gel electrophoresis assays.

Results: Reduction studies suggested that substitution of functional groups in to the parent BM structure increase the rate of reduction of the quinone group compared to BM in cell-free assays. Cytotoxicity studies suggested that electron donating groups seem to increase the cytotoxicity of BM analogs, while sterically bulky groups seemed decrease the cytotoxicity of BM analogs in whole cell

systems. Inhibition studies using the P450 reductase inhibitor DPIC seemed to decrease the overall cytotoxicity of the analogs MeBM, m-MeBM, CBM, m-PBM, and m-TBM, but seemed to have a negligible effect of DPIC on the activation of BM, MBM, and PBM in MDA-MB-468 cells. DNA single strand break formation was significantly decreased by substitution of electron donating *methyl* and *phenyl* groups on BM. The *chloro* electron withdrawing group on CBM and the strong electron donating group *methoxy* on MBM do not seem to affect the production of DNA strand breaks mediated by P450 reductase. Substitution of the parent BM with electron donating group *methyl* seemed to increase the formation of DNA cross-links subsequent to reduction by P450 reductase.

Conclusion: These results demonstrated that P450 reductase plays only a minor role in the activation of benzoquinone mustards. Although P450 reductase seemed to mediate the reduction some of the BM analogs, cytotoxicity and inhibition studies suggest that P450 reductase does not contribute to the activation of BM analogs in breast cancer cells with normal P450 reductase activity.

To my family:

I will always be grateful for your unconditional love and support, and for allowing me to pursue my aspirations. You have instilled in me a continued interest in life and the strength to endure many of the challenges it has to offer.

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ABBREVIATIONS

AZQ	2,5-diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone
BM	2-(di(chorloethyl)amino)1,4-benzoquinone
BSA	bovine serum albumin
CBM	5-chloro-2-(di(chorloethyl)amino)1,4-benzoquinone
D_{10}	Dose of drug reducing surviving cell fraction to 10%
DFM	N,N,-dimethyl formamide
DMEM/F12	Dubelcco's modified Eagle medium: nutrient powder F-12(Ham)(1:1)
DMSO	dimethyl sulfoxide
DPIC	Diphenyliodonium chloride
E_{10}	The percent of DNA damage caused by 10uM of drug
E_{25}	The percent of DNA damage caused by 25uM of drug
EO9	3-hydrosymenthyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β–en–α–ol
EPR	Electron paramagnetic resonance
FAD	flavin adenine dinucleotide
FBM	5-fluoro-2-(di(chorloethyl)amino)1,4-benzoquinone
FBS	fetal bovine serum
FMN	flavin mononucleotide
IC_{50}	Dose of drug reducing surviving cell fraction to 50% of control
LB Media	Luria broth media
MBM	5-methoxy-2-(di(chorloethyl)amino)1,4-benzoquinone

MeBM	5-methyl-2-(di(chorloethyl)amino)1,4-benzoquinone
MeDZQ	2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone
MMC	Mitomycin C
m-MeBM	6-methyl-2-(di(chorloethyl)amino)1,4-benzoquinone
m-PBM	6-phenyl-2-(di(chorloethyl)amino)1,4-benzoquinone
m-TBM	6-*t*-butyl-2-(di(chorloethyl)amino)1,4-benzoquinone
MTT	3-[4,5-dimethlythiazol-2-yl]-2,5diphenyltetrazoliumbromide
NADH	β-nicotimanide adenine dinucleotide (reduced form)
NADPH	β-nicotinamide adenine dinucleotide phosphate(reduced form)
NQO1	NAD(P)H dehydrogenase quinone 1
P450 Reductase	NAD(P)H:cytochrome P450 reductase (EC 1.6.2.4)
PBM	5-phenyl-2-(di(chorloethyl)amino)1,4-benzoquinone
PBS	phosphate buffered saline
RH1	2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone
ROS	reactive oxygen species
T½	Time required to reduce the drug concentration by one-half
TPZ	Tirapazamine

1 INTRODUCTION

1.1 Cancer, hypoxia and cellular metabolism

Extensive research has revealed cancer to be a disease with a multi-step process in which genetic alterations drive the progressive transformation of normal cells in to highly malignant derivatives. Tumourogenesis basic philosophy suggests that cancer cells have defects in the regulatory pathways that control cell proliferation and homeostasis. Various reviews suggest there are six essential alterations in cell physiology that collectively dictate malignant growth both individually and in combination (Hanahan and Weinberg 2000). These acquired capabilities include:

- Independent production of intrinsic growth signals. Normal Cells require growth signal before advancing into an active proliferative state. Tumour cells on the other hand show reduced dependence on stimulation from their microenvironment. This self-sufficiency disrupts mechanisms that ensure proper behaviours of cell within tissues.
- Insensitivity to antigrowth signals. Anti-proliferative signals help maintaining cellular and tissue homeostasis. Cancer cells are able to avoid these signals allowing normal cells to spread.
- Ability to avoid Apoptosis. Resistance toward apoptosis is considered the hallmark of most types of cancers, causes expansion of tumour cell populations, and limits the attrition of these malignant cells. Resistance to apoptosis is associated to the loss of pro-apoptotic regulation by p53

tumour suppressor gene, which monitors DNA damage and induces apoptotic pathways (Harris 1996).

- Unlimited replication potential. Cells possess internal mechanisms that limit cell replication. In tumour cells these regulatory mechanisms appear to be disrupted, leading to formation of macroscopic tumours. Telomere maintenance is associated to all types of malignants cells and plays a central role in replication of tumour proliferation (Shay and Bacchetti 1997).
- Maintained Angiogenesis. The growth of new blood vessels in new tissue is required to supply oxygen and nutrients and its normally transient and highly regulated. In order to grow, tumour masses must develop angiogenic capabilities, which are commonly associated with production of VEGF inducer.
- Metastasis and tissue invasion. When primary tumour masses proliferate they release cells able to invade neighbouring tissues and metastasize to distant areas of the body to grow and form new tumour masses. The mechanisms involved in invasion and metastasis are complex and not completely understood, but they are often associated with the inactivation of molecules involved in cell-to-cell interactions such as E-cadherins, which allow the transmission of antigrowth and other regulatory signals (Christofori and Semb 1999).

The efficacy of anticancer treatments is significantly affected by the microenvironment surrounding tumour cells. Tumour microenvironment is defined by the local and systemic architecture surrounding a cancer cell, and includes other cells, growth factors, enzymes and parts of the blood, lymphatic and immune systems. The microenvironment surrounding cancer cells can greatly influence the access of therapeutic agents, the ability of the body to activate and metabolize therapeutic agents, and the development of resistance to cancer treatments. Human solid tumours are consistently less oxygenated than normal tissues, a difference currently being exploited to develop more efficient cancer treatments. Hypoxia is a characteristic feature of locally advanced solid tumours resulting from an imbalance between oxygen supply and consumption, where tumour areas with pO_2 values ≤2.5 mmHg are considered hypoxic (Vaupel, Briest et al. 2002). Several responses are developed by cells in upon the onset of hypoxia (Michiels 2004):

- Systemic increase in ventilation and cardiac output to prevent myocardial and cerebral ischemia that can lead to heart infarction or stroke.
- Cellular switch from aerobic to anaerobic metabolism, which involves and increase in anaerobic glycolysis activity to meet energetic demands and a decrease of energy-consuming processes.
- Promotion of improved vascularization to protect against obstruction in oxygen delivery.

- Enhancement of oxygen carrying capacity through the activation of genes involved in angiogenesis, red blood cell production, and enzymes involved in glycolysis.

Activation of these adaptive processes is mediated mainly by the action of hypoxia-inducible factor-1 (HIF-1). HIF-1 is activated by low oxygen conditions and induces changes to equal oxygen supply and demands. HIF-1 is a heterodimer transcription factor made up of HIF-1α and HIF-1β (ARNT). ARNT is constitutively expressed and it is localized in the nucleus. HIF-1α levels are constant in normal oxygen conditions and under hypoxia, however under normal conditions HIF-1α is degraded, where areas it accumulates in hypoxia. When HIF-1α and ARNT bind, the heterodimer can the bind to hypoxia responsive elements within the promoters of hypoxia responsive gene. Activation of such genes allows cells to undergo the biological changes to adapt to low oxygen levels. HIF-1α has been shown to upregulate the expression of all the enzymes of the glycolytic pathway, glucose transporters, oxygen transport, and iron metabolism. A second group of HIF-1 regulated genes contributes to the formation of new blood vessels in an attempt to maintain oxygen levels (Semenza 1999). Lastly HIF-1 has been shown to promote p53 dependant apoptosis that is mediated by APAF-1 and caspase 9. Following activation of p53 initiates the transcription of pro-apoptotic Ccl-2 family member which in turn induces cell death (Ravi, Mookerjee et al. 2000).

HIF-1 has a profound effect on tumour biology and contributes to tumour progression by:

- Mediating angiogenesis through the activation of VEGF inducer necessary for metastasis and tumour growth.
- Mediating adaptation to hypoxia through up regulation of glycolysis and glucose transport necessary for tumour growth.
- Selecting for genes resistant o hypoxia and apoptosis.

Tumour hypoxia is a therapeutic concern since it can reduce the effectiveness of radiotherapy, and oxygen-dependent cytotoxic agents because these require oxygen to be maximally cytotoxic. (Coleman, Bump et al. 1988; Vaupel, Kelleher et al. 2001). Poor perfusion in hypoxic fractions of tumours not only prevents delivery of anticancer drugs, but also limits the concentration of oxygen available to produce reactive oxygen species that induce damage to cellular components, thus limiting the cytotoxic capacity of chemotherapeutic agents. Gray et al. (1953) demonstrated the presence of oxygen at the time of irradiation caused radiation sensitivity. However, this sensitivity was not the effect of the metabolites produced. Instead oxygen was able to react with radicals on the DNA produced by the ionizing radiation, fixing the damage to the molecule. In the absence of oxygen the damage produced by radical species can be restored to its native form with the help of hydrogen atoms donated by molecules such as sulfhydryls (Gray, Conger et al. 1953). Hypoxia can result in drug resistance indirectly if under this condition cells more effectively detoxify the drug

molecules. Several methods have been designed to overcome the resistance of hypoxic tumour cells to radiation and chemotherapy, such as radiosensitive and agents that selectively kill hypoxic cells (Adams and Stratford 1986; Stratford, O'Neill et al. 1986; Zeman, Brown et al. 1986; Stratford 1992).

1.2 Enzyme-Directed Tumour Targeting

A primary focus of cancer chemotherapy is to kill rapidly proliferating cancer cells by targeting general cellular metabolic processes, including DNA, RNA, and protein synthesis. Consequently, these treatments affect not only malignant cells but normal cells as well. Growing understanding of the molecular characteristics underlying the etiology of different cancers, as well as the signalling events involved in the proliferation of cancer cells, provides opportunities to develop more efficient agents. In anticancer drug design, the goal is to develop drugs which are selectively toxic to tumour cells with minimal toxicity to normal cells.

One approach to achieving selective toxicity anticancer agents is to identify compounds which are substrates for, and bioactivated by specific enzymes. This concept is known as "enzyme directed" tumour targeting. An enzyme-directed approach to drug development, first proposed by Workman and Walton, suggests that hypoxic cells could be better targeted if differences in the levels of reductases in various cell types were considered in order to direct appropriate agents to particular human tumour based on their differential expression of enzymes (Workman 1994; Fitzsimmons, Workman et al. 1996). This approach to cancer treatment allows increased targeting of cancer cells

while minimizing the damage caused to normal cells, and addresses the indiscriminating toxic effects of current chemotherapeutic agents, such as cyclophosphamide, carboplatin, carmustine, and melphan (Teicher, Holden et al. 1990; Littlewood 2001).

Enzyme directed tumour targeting involves the exploitation of biochemical processes that result in the selective reductive activation of chemotherapy agents in low oxygen concentrations(Kennedy 1987; Workman and Stratford 1993). More specifically low levels of oxygen present in solid tumours could allow reduction of a drug to a product more toxic than the parent compound (Lin, Cosby et al. 1972). The agents are now identified as bioreductive compounds.

1.2.1 Activation of bioreductive agents

Traditional methods for the treatment of cancer are based on a combination of surgery, radiation, and chemotherapy drugs. These approaches bear limitations in their ability to control cancer, including limited accessibility of the tumour to the treatment, dose-limiting systemic toxicities of chemotherapeutic agents which are toxic to normal tissues, such as bone marrow, and the development of tumour subpopulations that are resistant to drugs.

The intracellular activation of bioreductive alkylating agents requires enzymatic reduction by bioreductive enzymes. Bioreductive agents can be activated by either one- electron reducing enzymes including P450 reductase, xanthine oxidase (Pan, Andrews et al. 1984; Rockwell, Sartorelli et al. 1993), cytochrome b5 reductase (Hodnick and Sartorelli 1993); and by two-electron electron reducing enzymes like NQO1 (Ross, Beall et al. 1996) and xanthine

dehydrogenase (Gustafson and Pritsos 1992). The activity of these reductases varies in different tumours and cell lines, and is also dependent on pH and oxygenation. In recent studies, P450 reductase and NQO1 have received considerable attention as target enzymes for activation of bioreductive alkylating agents, because of their relative activity and expression levels in tumours.

1.2.2 NADPH: cytochrome P450 Reductase

NADPH: cytochrome P450 reductase (EC 1.6.2.4) is a flavoprotein, that participates as an electron carrier in the hydroxylation of various endogenous and foreign compounds (Williams 1976). This enzyme was previously referred to as cytochrome c reductase or reductase, due to its involvement in the reduction of cytochrome c (Horecker 1950). It is now commonly called NADPH: cytochrome P450 reductase, referring to the endogenous electron acceptor, cytochrome P450. The enzyme will be referred to here on as P450 reductase. The first report P450 reductase in animal tissue was made by Horecker in 1949. This newly discovered enzyme was isolated from pig liver after acetone extraction and trypsin treatment. It had a molecular weight of 68,000, used NADPH as its source of reducing equivalents, and was reported to contain flavine adenine dinucleotide (FAD) as a prosthetic group (Horecker 1950). The reductase was reported have microsomal localization (Phillips and Langdon 1962; Williams and Kamin 1962). Both research groups speculated about the potential role of the enzyme in the hydroxylation of steroids and demethylation of drugs. Preliminary evidence indicated the possible participation of P450 reductase in a microsomal electron transport system that did not involve cytochrome c (Phillips and Langdon

1962). Later, direct evidence for the participation in microsomal hydroxylation reactions came from reconstitution of laurate ω-hydroxylase activity from a detergent-solubilised preparation of cytochrome P450, NADPH-cytochrome c reductase, and a heat-stable component, that was later identified as phosphatidylcholine (Lu and Coon 1968; Lu, Junk et al. 1969; Strobel, Lu et al. 1970).

One of the difficulties in the identification of the physiological role of the reductase was due to the methods for purification. In early studies the enzyme was purified by either trypsin (Horecker 1950; Phillips and Langdon 1962; Iyanagi and Mason 1973) or by treatment with lipase (Williams and Kamin 1962; Masters, Bilimoria et al. 1965). These treatments isolated a protein with molecular weight of 68,000, that was able to reduce a series of electron acceptors, including cytochrome c (Horecker 1950), ferricyanide, 2,6-dichloroindophenol, menadione, nitro blue tetrazolium, and 3-acetyl pyridine adenine dinucleotide phosphate (Masters, Bilimoria et al. 1965; Vermilion and Coon 1978). However, this enzyme was unable to reduce microsomal proteins such as cytochrome b_5 (Masters, Bilimoria et al. 1965) or cytochrome P450 (Lu, Junk et al. 1969; Black, French et al. 1979). Black and Coon (1982) reported that the small hydrophobic NH_2-terminal region of the reductase molecule is responsible for the binding to both cytochrome P450 and phospholipids (Black and Coon 1982). This hydrophobic region (molecular weight, 4800) was cleaved by trypsin treatments, thereby explaining the inability of trypsin-treated reductase to reduce microsomal proteins. The non-hydrolized enzyme was isolated using detergent solubilization

of liver microsomes, and it retained its ability to participate in the reduction of cytochrome P450, and its associated reactions (Lu, Junk et al. 1969; Dignam and Strobel 1975; Yasukochi and Masters 1976). The isolated enzyme had a molecular weight of about 78,000 (76,000 to 80,000), and it was reported to contain one molecule of FMN and FAD in each molecule of protein (Yasukochi and Masters 1976; Dignam and Strobel 1977).

1.2.2.1.1 Biological significance of P450 reductase

P450 reductase belongs to the cytochrome P450 superfamily of heme-containing monooxygenases that hydroxylate a wide range of physiological and xenobiotic compounds (Strobel 1995). The general reaction for hydroxylation catalyzed by P450 reductase along with the cytochrome P450 system in the endoplasmic reticulum is:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

Here, RH represents a variety of substrates including *N*- and *O*- alkyl drugs, polycyclic aromatic hydrocarbons, alkanes, fatty acid, pesticides and chemical carcinogens (Strobel 1995). In some instances the product of hydroxylation ROH may be more or less active than the substrate RH. This demonstrates the potential of the cytochrome P450 system to inactivate some xenobiotics and therapeutic agents, as well as activation of other chemicals, environmental contaminants and therapeutic agents to potential carcinogenic metabolites or active drugs. The expression of the P450 enzymes has been reported in almost all mammalian tissues including the brain (Bergh and Strobel 1992; Hodgson, White et al. 1993), lung (Guengerich and Strickland 1977), small intestine (Stohs,

Grafstrom et al. 1976), kidney (Ellin and Orrenius 1971), and colon (Fang and Strobel 1978). However the highest concentration of these enzymes seems to be the liver. To date more than 400 forms of cytochrome P450 are known to exist, and although not every tissue expresses all of them, each tissue contains a spectrum of forms (Strobel 1995).

There are four classes of P450 proteins. They are classified by the manner in which electrons are transferred to the catalytic site from NAD(P)H. Class I of P450 enzymes is associated with the inner membrane of the mitochondria. In this system, the electrons are derived from NAD(P)H and are transferred from the FAD-containing NAD(P)H ferredoxin reductase to the P450 via a small iron-sulfur-containing ferredoxin (Sligar 1976; Munro and Lindsay 1996). This class of P450 enzymes are involved in several steps of the biosynthesis of steroid hormones and vitamin D_3 in mammals (Werck-Reichhart and Feyereisen 2000).

Class II of P450 enzymes are the most common class in eukaryotes and most importantly, they receive electrons from the diflavin enzyme P450 reductase (Iyanagi and Mason 1973). These enzymes are dissociated from P450 reductases, and independently anchored on the outer face of the endoplasmic reticulum (ER) by amino-terminal hydrophobic anchors. They are involved in many physiological functions in mammals, including biosynthesis and catabolism of signalling molecules, steroid hormones, retinoic acid and oxilipins (Nelson; Feyereisen 1999; Hasler 1999). Class I and II P450 enzymes participate in the detoxification, or in some circumstances the activation of xenobiotics (Lu and

Coon 1968; Hasler 1999). P450 enzymes from class III are considered to be self-sufficient and do not require and external source of electrons, or oxygen. They are involved in the dehydration of alkylperoxide generated by dioxygenases, and in the synthesis of signalling molecules such as prostaglandins in mammals (Mansuy 1998). P450 enzymes belonging to class IV receive their electrons directly from NADH. This class is unique to fungi, it is soluble and reduces NO generated by denitrification to N_2O (Mansuy 1998).

All cytochrome P450s require the sequential transfer of two reducing equivalents from and electron donor by means of accessory enzymes. The specific accessory enzyme utilized is determined by the type of cytochrome P450 involved in a given reaction. NADPH and P450 reductase are the preferred electron donors and accessory enzyme required for reaction involving microsomal cytochrome P450s (Danielson 2002).

1.2.2.2 Characterization of P450 Reductase

The development of efficient methods to purify P450 reductase allowed the characterization of this enzyme. The monomeric molecular weight of the purified P450 reductase was determined by SDS gel electrophoresis technique in the presence of standards of known molecular weight. This technique indicated that the molecular weight of P450 reductase is 79,000 with a range of 76,000-80,000 reported by various investigators (Yasukochi and Masters 1976; Dignam and Strobel 1977; Strobel and Dignam 1978; Vermilion and Coon 1978). P450 reductase contains one molecule each of FMN and FAD (Iyanagi and Mason 1973). The transfer of electrons from NADPH through the reductase to acceptors

has been of great interest to investigators for many years. Studies suggest that the electron flow through the reductase follows the pathway NADPH → FAD → FMN → electron acceptor (Masters, Bilimoria et al. 1965; Vermilion, Ballou et al. 1981; Kurzban and Strobel 1986).

1.2.2.3 Expression of P450 Reductase

The cytochrome P450-dependent metabolism system is known to be widely distributed in mammalian tissues. The presence and properties of this drug metabolizing system have been studied in lung, kidney, small intestine, heart, adrenals, mammary glands, skin, pancreas , brain, male reproductive organs including testis and prostate and other tissues (Rydstrom 1983). P450 reductase has been shown to be present in all these drug metabolizing systems with no isoforms of this enzyme being identified to date (Strobel 1995). P450 reductase is most abundant in the liver, where cytochrome P450 is highly expressed, and its distribution is thought to be correlated with the P450 enzyme system. The molar ratio of total P450 content to P450 reductase in microsomes is about 5-10 to 1 (Estabrook, Franklin et al. 1971; Shephard, Phillips et al. 1983). The expression of P450 reductase has been quantified by RNase protection assays using human tissues. This study established the presence of P450 reductase mRNA in the liver, prostate, adrenal, colon, placenta, and in the human breast cancer line MCF-7 (Shephard, Palmer et al. 1992). Immunological staining assays established the presence of P450 reductase in various human tissues (Baron J 1983; Hall, Stupans et al. 1989). High levels of staining were observed in the liver, lung and small intestines, while lower levels of staining

where evident in the colon and stomach. The presence of P450 reductase was further established in pancreas, gall bladder, appendix, adrenal gland, skin, breast and prostate (Baron J 1983).

P450 reductase activity has also been established in various cancer cell lines, including leukemia, CNS, colon, lung, melanoma, ovarian, prostate and kidney (Fitzsimmons, Workman et al. 1996; Lopez de Cerain, Marin et al. 1999; Yu, Matias et al. 2001). P450 reductase is present in a variety of tumour cell lines including cells from leukemia, melanoma, central nervous system, breast, colon, lung, ovarian, prostate, and renal tumours (Yu, Matias et al. 2001). Data on the levels of P450 reductase between normal and tumour cells are diverse. In general, P450 reductase activity is lower in tumour tissue than in the corresponding normal tissue and correlates with P450 activity (Forkert, Lord et al. 1996). Only a small variation in P450 reductase in tumour tissues versus normal tissues of human breast and lung (Lopez de Cerain, Marin et al. 1999). The activity of P450 reductase in human liver cancer tissue is 1.8 fold higher than in normal liver tissue (Plewka, Plewka et al. 2000). Overall, the level of P450 reductase activity in tumour cells does not necessarily reflect that of the corresponding tumour tissue due to loss of enzyme activity as a result of cell culturing.

1.2.2.4 The P450 Reductase gene

Although P450 reductase is ubiquitously expressed in most mammalian tissues, in humans the protein is encoded by a single gene located on chromosome 7q11.2 and spans 50kb (Porter and Kasper 1985). The

organization of the exons in the coding region, specifically exons 2-16, correlates with the domain structure of the protein (Figure 3). For instance, analysis of the gene structure revealed that the hydrophobic amino terminal segment that anchors the enzyme to the phospholipids bilayer is encoded by exon 2 (Porter, Beck et al. 1990). The first exon residing 30.5kb upstream of the coding region remains untranslated. The first intron in the P450 reductase gene is 30kb, and it contains multiple transcription start sites. The P450 reductase enzyme is encoded by a GC-rich promoter lacking 'TATA box' and 'CCAAT box' elements, which is coincides with the low-levels of expression of this enzyme in many tissues. The promoter contains nine copies of the Sp1 binding element; the first two Sp1 sites are the most important for the transcription of the reductase gene. Loss of a 35-bp fragment containing the distal Sp1 consensus produced a 90% decrease in promoter function (O'Leary, McQuiddy et al. 1996). However, the remaining seven distal sites can compensate for the loss of the first two. Three elements were identified as essential for the basal regulation of the reductase gene. The first element is a distal Sp1 site, made up of three overlapping motifs: an inverted Sp1 consensus, an Egr-1 site and a CACC box, namely SEC. The second element is an OxidoReductase Upstream element (ORU), which appears to be unique to the P450 reductase gene, and essential to the basal transcription and cell-specific expression of the P450 reductase promoter (O'Leary and Kasper 2000). Lastly the third element is a thyroid response element (TRE) at position -564. This site binds thyroid hormone receptors and signals to reporter gene constructs (O'Leary, Li et al. 1997).

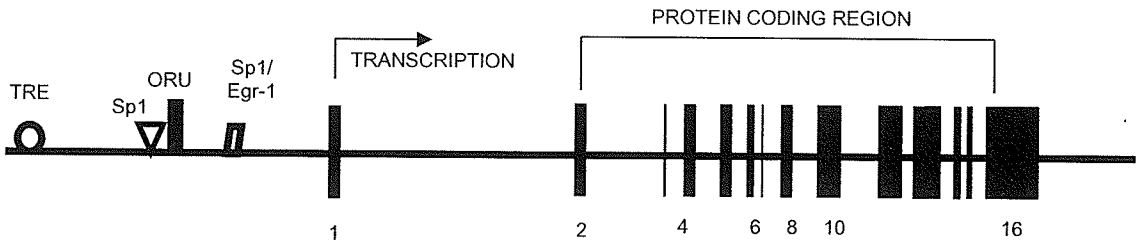


Figure 1. Structure of the P450 reductase gene. Gene is located in chromosome 7q11.2 and spans 50 kb. [Figure adapted from Porter et al. 1990 (Porter, Beck et al. 1990)].

1.2.2.5 Regulation of P450 Reductase gene

Due to its many physiological roles, P450 reductase is expressed in all tissues at some level. P450 reductase expression is transcriptionally regulated by the pituitary-thyroid axis, and thyroid hormone (T3-triiodothyronine) is required to maintain expression. Rats with a T3-triiodothyronine deficiency displayed a decrease of ≥75% in liver P450 reductase mRNA, protein, and activity (Waxman, Morrissey et al. 1989; Ram and Waxman 1992; Li, Liu et al. 2001). Replacement of T3-triiodothyronine has been demonstrated to substantially restore the levels of P450 reductase in hypophysectomized or hypothyroid rats, indicating that T3-triiodothyronine is essential for the full expression of P450 reductase activity. P450 reductase is also found at high levels in the adrenal

glands. The expression of the enzyme in these tissues is regulated by adrenocorticotrophic hormone (ACTH) (Dee, Carlson et al. 1985).

1.2.2.6 Induction of P450 reductase

The levels of P450 reductase, unlike other cytochrome P450s, are not easily induced. Phenobarbital and related barbiturates are well known as powerful inducers of a broad range of P450 genes and P450 reductase (Danielson 2002). However, only a 2-3 fold increase in P450 reductase levels are observed after treatment with a strong inducer such as phenobarbital or pregnenolone-16a-carbonitrile (Taira, Greenspan et al. 1980; Hardwick, Gonzalez et al. 1983). Inducers such as ethanol and methylcholanthrene, do not show a significant effect on the levels of P450 reductase.

1.2.2.7 Disruption of the P450 reductase gene

P450 reductase is essential for the transfer of electrons to other P450 systems; no other physiological electron acceptor has been identified in vertebrates. Disruption of the P450 reductase gene has been associated with the production of truncated cytoplasmic protein. P450 reductase mutant (-/-) mice, display a series of embryonic defects including bone friablility, cell adhesion defects, neural tube, cardiac, eye and limb abnormalities, and retardation of development (Shen, O'Leary et al. 2002). Many P450 reductase-dependent pathways are required for normal development of embryos, such as biosynthesis and degradation of retinoic acid, sterols, prostaglandins, and steroids. Inadequate activity of multiple steroid enzymes has been associated

with mutations in the P450 reductase gene in patients with disordered steroidogenesis and Antley-Bixler syndrome (ABS) (Otto, Henderson et al. 2003). The Antley-Bixler syndrome, first reported in 1975 by Antley and Bixler, is a disorder causing musculoskeletal, craniofacial, and urogenital anomalies (Antley 1975). This is a rare disorder, of which only 30 cases had been reported up to the year 2000. Antley-Bixler syndrome is also known to develop due to the toxic effects of the antifungal agent fluconazole during early pregnancy (Pursley, Blomquist et al. 1996).

1.2.2.8 Structure and reduction properties of P450 Reductase

P450 reductase is a multi domain protein, made up of three separable domains: 1) a hydrophobic N-terminal domain, known to anchor the enzyme to the endoplasmic reticulum membrane, 2) an FMN-binding domain, and 3) a FAD/NADPH-binding domain (Porter and Kasper 1986; Porter 1991; Smith, Tew et al. 1994). The FMN-binding domain shows sequence homology to numerous bacterial FMN-containing flavodoxins (Porter and Kasper 1986), while the FAD/NADPH-binding site show sequence homology to ferredoxin-NADP+ reductase and NADH-cytochrome b_5 reductase (Porter and Kasper 1986; Karplus, Daniels et al. 1991). Previous studies have elucidated the roles of the flavin moieties in P450 reductase (Vermilion and Coon 1978; Vermilion, Ballou et al. 1981; Oprian and Coon 1982). Studies determined that FAD accepts electrons from NADPH, while FMN donates electrons to acceptor proteins such as the cytochromes. FMN appears to be the more electronegative of the two flavins, which correlates with the thermodynamic concept where the more electronegative species provides a stronger force for the

transfer of electrons to the acceptor protein. For rabbit P450 reductase, $FMNH_2$ has a redox midpoint potential of -270mV, compared to the -110mV of the semiquinone form (Iyanagi, Makino et al. 1974). Mammalian P450 reductase forms an air stable semiquinone on the FMN moiety. The resting state of the enzyme is the species with the more electropositive potential, which in the case of P450 reductase is the FMN semiquinone (Iyanagi and Mason 1973; Yasukochi, Peterson et al. 1979). P450 reductase cycles between reduction states of 1-2-3-1 electrons (Figure 4) during reduction of acceptor proteins. The enzyme is reduced by the transfer of two-electrons from NADPH to FMNH/FAD, producing a 3-electron $FMNH/FADH_2$ intermediate of the enzyme. Rearrangement of the electrons within the enzyme produces the $FADH/FMNH_2$ 3-electron intermediate. Subsequent oxidation of $FMNH_2$ through two one-electron transfers to cytochrome P450, returns the enzyme to its normal resting FMNH/FAD semiquinone form (Vermilion, Ballou et al. 1981; Munro, Noble et al. 2001).

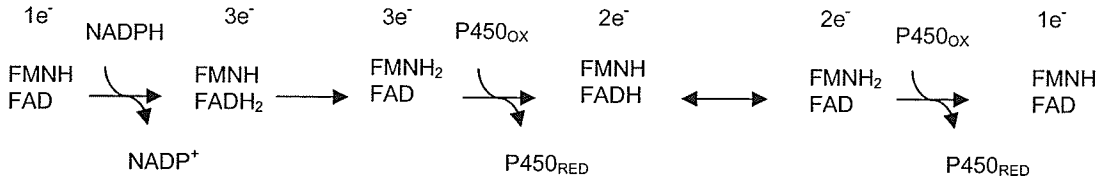


Figure 2. Electron distribution and cycling of P450 reductase flavoproteins. P450 reductase cycles between reduction states of 1-2-3-1 electrons during reduction of acceptor proteins. The enzyme is reduced by the transfer of two-electrons from NADPH to FMNH/FAD, producing a 3-electron $FMNH/FADH_2$ intermediate of the enzyme. Rearrangement of the electrons within the enzyme produces the $FADH/FMNH_2$ 3-electron intermediate. Subsequent oxidation of $FMNH_2$ through two one-electron transfers to cytochrome P450, returns the enzyme to its normal resting FMNH/FAD semiquinone form adapted from Munro et al. 2001 (Vermilion, Ballou et al. 1981; Munro, Noble et al. 2001).

1.2.2.9 Interaction of P450 reductase with electron acceptors

Electrostatic charge pairing plays a primary role in the interaction of P450 reductase and electron acceptors such as cytochrome P450 (Inano and Tamaoki 1985; Nadler and Strobel 1991). Chemical cross linking and modification studies established that P450 reductase contains numerous carboxylate groups, made up of acidic amino acids aspartate and glutamate (Bernhardt, Pommerening et al. 1987; Nadler and Strobel 1988). These groups couple with basic amino acids (leucines, and arganines) on the electron acceptor proteins. Electron transfer from P450 reductase to cytochrome P450 is thought to occur due to the formation of a dipole across the cytochrome P450 molecule, with the positive charge formed at the proximal face of the protein where the heme is closest to the surface (Figure 5). Non-polar amino acids allow the two proteins to be close enough for electron transfer (Inano and Tamaoki 1985; Nadler and Strobel 1991; Hasemann, Kurumbail et al. 1995). A similar mechanism of interaction is predicted for other electron acceptor proteins like cytochrome c, cytochrome b_5, heme oxidase, and squalene monooxygenase (Wang, Roberts et al. 1997). Site-directed mutagenesis studies show that mutation of acidic amino acid clusters in FMN, disrupt the interaction between P450 reductase with cytochrome and cytochrome c in different fashions, suggesting that both of these acceptor proteins have different interaction mechanisms with P450 reductase (Shen and Kasper 1995; Zhao, Modi et al. 1999)

1.2.2.10 Kinetic mechanism of the P450 Reductase electron transfer to substrates

Characterization of the kinetic mechanism of P450 reductase remains ambiguous due to several factors including the number of electron acceptors, enzyme isoforms, reduction states of the flavins in P450 reductase, indistinguishable kinetic steps, and location of enzymes on the ER membrane. Several kinetic mechanisms have been suggested for P450 reductase, based on steady-state kinetic analyses, including Ping-Pong, random and ordered Bi-Bi, as well as hybrid mechanisms (Phillips and Langdon 1962; Masters, Bilimoria et al. 1965; Dignam and Strobel 1977; Sem and Kasper 1994). However, some researchers argue a sequential Bi-Bi kinetic mechanism for P450 reductase (Murataliev, Feyereisen et al. 2004). Sequential Bi-Bi kinetic mechanisms require two substrates capable to bind the enzyme at separate and independent sites. Evidence suggests that in fact P450 reductase follows sequential Bi-Bi kinetics: 1) P450 reductase is able to bind and become reduced by NADPH in the absence of a terminal electron acceptor. Further more, NADPH is able to bind and reduce P450 reductase while covalently attached to cytochrome c in rat microsomes (Nisimoto 1986; Nisimoto and Otsuka-Murakami 1988). 2) NADPH reduces the cross-linked cytochrome b_5-P450 reductase- CYP2B4 complex during mixed function oxidase activity (Tamburini and Schenkman 1987). This indicates that NADPH can reduce both free and bound forms of P450 reductase. 3) Cytochrome c is able to bind reduced P450 reductase (Masters, Bilimoria et al. 1965), indicating that cytochromes are capable of interaction with oxidized and

reduced P450 reductase (Murataliev, Feyereisen et al. 2004). Further kinetic studies are required using other electron acceptors, both physiological and artificial, to determine the preferred kinetic mechanism of P450 reductase.

1.2.2.11 Relevance of P450 reductase in the activation of bioreductive alkylating agents.

Bioreductive activation of anticancer agents mediated by P450 reductase, exemplifies the usefulness of the "enzyme-directed" approach to bioreductive drug development. This flavoenzyme can donate electrons to pro-drugs with the appropriate redox potentials, and over expression can confer sensitivity to various classes of bioreductive agents including Tirapazamine, E09, and Mitomycin C. Sensitivity of breast cancer cells to Tirapazamine has been found to correlate strongly with activity of P450 reductase in these cell lines under hypoxic conditions, where cell lines with higher levels of P450 reductase were more sensitive to this agent (Patterson, Barham et al. 1995; Patterson, Saunders et al. 1997).

1.2.3 NAD(P)H dehydrogenase quinone 1

NAD(P)H dehydrogenase quinone 1 enzyme was first described in 1958 by Ernster and Navazio (Ernster L. 1958) and is also known as NQO1 but will be referred to from here on as NQO1. NQO1 is an obligate two-electron reductase which bioactivates chemotherapeutic quinones. It is characterized by its ability to utilize either NADH or NADPH as sources of reducing equivalents, and it is inhibited by dicoumarol (Ernster 1967). NQO1 levels are elevated in a number of

tumour types, including non-small cell lung carcinoma, colorectal carcinoma, liver cancers and breast carcinomas, when compared to normal tissues. The differential expression of NQO1 between normal and tumour tissue allows specific activation of various antitumour quinones in tumours, while minimizing toxic damage to normal tissues. NQO1 has multiple cellular roles, it is often characterized as a Phase II detoxifying enzyme, and it protects the cell from various chemically reactive metabolites (Riley and Workman 1992). Further more, NQO1 is able to reduce quinone compounds directly to hydroquinones, avoiding the formation of potentially toxic semiquinone radical intermediates (Lind, Hochstein et al. 1982; Thor, Smith et al. 1982). Reduction by NQO1 can lead to bioreductive activation of quinone-containing alkylating agents, such as Mitomycin C, AZQ, EO9, RH1, and model agents such benzoquinone mustards (Siegel, Gibson et al. 1990; Siegel, Gibson et al. 1990; Walton, Bibby et al. 1992; Fourie, Oleschuk et al. 2002). Differential expression of NQO1 has been documented, where the enzyme levels of various tumour cells lines were compared, and significant correlation between NQO1 expression levels and drug sensitivity was established (Fitzsimmons, Workman et al. 1996). Further more, the levels of NQO1 have been determined in both preneoplastic tissues and established tumours (Schlager and Powis 1990; Cresteil and Jaiswal 1991).

Three NQO1 polymorphic variations have been identified in humans: the wild type NQO1*1, NQO1*2 (C609T), and NOQ1*3 (C465T) allele. The NQO1*2 variant, is associated with a point mutation at base pair 609 in exon six produced in a cytosine to thymine replacement. This homozygous variant resulted in a null

phenotype due to the rapid degradation of the mutant protein, leading to the lack of activity of the NQO1 enzyme (Siegel, Anwar et al. 2001). The NQO1*3 variant is associated with a point mutation at the 465 base pair also produced a cytosine to thymine replacement. Heterozygous or homozygous variants resulted in low protein content in mutated cell line HTC-116. This mutation did not seem affect the enzymatic activity of NQO1, but rather it decreased the overall concentration of NQO1 present (Pan, Han et al. 2002). These NQO1 polymorphism seem have a major impact not only on bioreductive role of NQO1 by decreasing the activation of clinically used bioreductive agents to cytotoxic metabolites, but also on its ability to detoxify toxic metabolites from the body.

The potential for activation of quinone-containing alkylating agents by NQO1, along with differential levels of enzyme expression in tumour tissues and between tumour and normal tissues has encouraged the search for agents which can be specifically activated by this enzyme.

1.3 Bioreductive chemotherapy agents

Bioreductive agents are a group of anticancer drugs that utilize an enzyme-directed approach to confer their cytotoxic effects on tumour cells. The underlying principle for the development of bioreductive drugs that hypoxic cells near the necrotic core are more capable of reductive metabolism than aerobic cells (Lin, Cosby et al. 1972). Increased reductive metabolism of hypoxic cells can be used to reduce anticancer agents, which upon reduction would confer their cytotoxic effects resulting in selective killing of hypoxic cell. Redox cycling in well oxygenated areas would regenerate the less toxic "prodrug", and cellular

defences, such as glutathione peroxidase, superoxide dismutase and catalase, would limit toxicity due to reactive oxygen species to normal cells. Bioreductive alkylating agents were first described in 1972 (Lin, Agrawal et al. 1972). Although bioreductive agents have diverse chemical structures, they share the common requirement for reductive activation. Bioreductive agents are potent electrophiles and react with (or "alkylate") electron-rich molecule in cells to form covalent bonds. The most important reaction with respect to antitumour activity is their reaction with DNA bases. Nitrogen mustards were the first alkylating agents that demonstrated clinical antitumour activity. Studies published in 1946 demonstrated the regression of tumours, especially lymphomas after treatment with alkylating agents (Goodman L.S. 1946; Jacobson L.P. 1946; Rhoads 1946). One of the first clinically effective nitrogen mustards, mechloroethamine, is still used today in combination therapy for the treatment of Hodgkin's lymphoma (Pratt W.B. 1994). Some alkylating agents react with only one strand of DNA. On the other hand bifunctional alkylating agents react with one atom on each of the two DNA strands to produce a covalent link, or "cross-link", between the two strands of DNA. This lesion prevents effective cell replication if it is not repaired. The toxicity of alkylating agents is mainly dependent on the efficiency of cellular repair mechanisms.

Bioreductive agents have varied chemical structures and mechanisms of cytotoxicity. The structure of bioreductive agents consists of two essential elements: 1) a bioreductive element, and 2) a cytotoxic element. In Mitomycin C for instance the bioreductive element is a quinone group, which regulates the

activation of the cytotoxic alkylating element (Lown, Begleiter et al. 1976; Tomasz, Lipman et al. 1987). In other cases, the bioreductive element itself acts as the cytotoxic element, as established for the nitrogen-oxide group found in tirapazamine (Patterson, 1993). Reduction of bioreductive elements can occur through the sequential transfer of single electrons, or through a direct two-electron transfer (Figure 1).

A single electron reduction results in the formation of a radical intermediate that can damage cellular components such as proteins, lipids and DNA (Bachur, Gordon et al. 1979), by adding across double bonds or by removing a hydrogen (Trush, Mimnaugh et al. 1982). A second one-electron reduction or a direct two-electron reduction of the bioreductive element, results in the formation of a two-electron reduced product. Reduction of the bioreductive element to either the semiquinone/radical intermediate or to the fully reduced form, may lead to the activation of the cytotoxic element. In the case of the bioreductive agents Mitomycin C and EO9 the cytotoxicity is conferred by an alkylating group (Lown, Begleiter et al. 1976; Riley and Workman 1992). Following activation, the alkylating group can bind covalently to cellular components such as proteins and DNA, disturbing normal cellular processes including DNA replication, which in turn lead to cellular death by apoptosis (Lown, Begleiter et al. 1976; Trush, Mimnaugh et al. 1982; Tomasz, Lipman et al. 1987; Begleiter, Leith et al. 1994). In the presence of molecular oxygen, both the fully reduced and radical forms of the drug consume oxygen generating superoxide radical anions, hydrogen peroxide, and hydroxyl radicals (reactive oxygen

species, ROS) via redox cycling. ROS are detrimental to cellular macromolecules in that they are capable of causing DNA strand breaks in addition to protein oxidation. (Lown, Begleiter et al. 1976; Riley and Workman 1992).

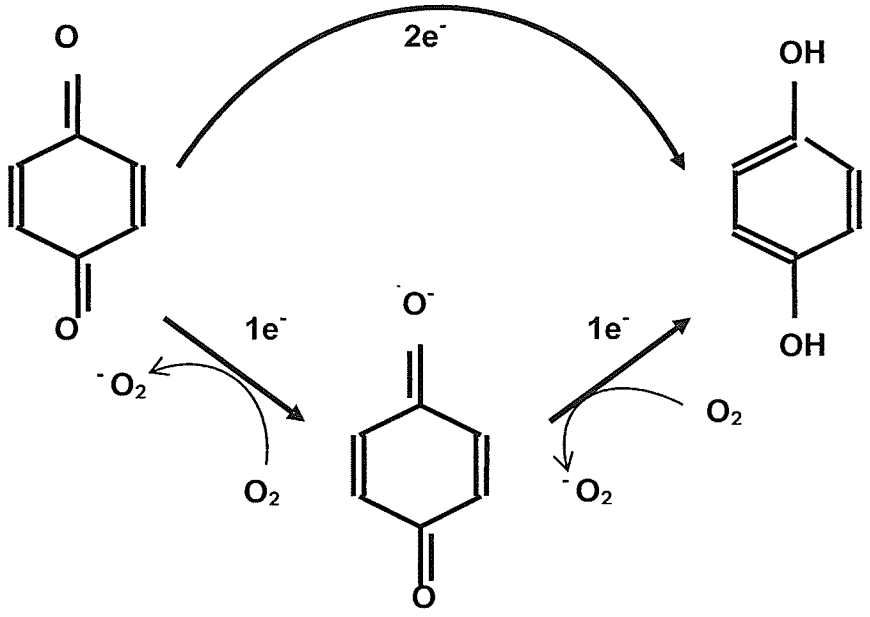


Figure 3. Reduction of bioreductive agents. A single electron reduction results in the formation of a radical intermediate. A second one-electron reduction or a direct two-electron reduction of the bioreductive element, results in the formation of a two-electron reduced product. (Adapted from Fourie et al, 2002)

1.3.1 Mitomycin C

Quinone-containing alkylating agents were first reported in the late 1950's, with the isolation of Mitomycin C from *Streptomyces* species (Beijnen J.H. 1987). Mitomycin C is a naturally occurring antitumour quinone (Figure 1), derived from *Streptomyces caespitosus*, a strain of actinomyces. It has been used as a cytotoxic agent since the 1960's, and antitumour activity has been reported against pancreas, lung, stomach, head and neck, prostate, breast and bladder tumours (Pratt W.B. 1994; Teicher 1997). The principal toxicity associated with Mitomycin therapy is bone marrow suppression, and it is dose-dependent. Mitomycin C is considered the prototype bioreductive drug (Sartorelli, Hodnick et al. 1994), given that it is inactive until it becomes reduced by either one- or two-electron reductases.

Mitomycin C is a major substrate for reductive activation by the enzymes NADPH: cytochrome P450 reductase and NAD(P)H: quinone oxidoreductase 1, NQO1 (Pan, Andrews et al. 1984; Siegel, Gibson et al. 1990). Reduction of the Mitomycin prodrug causes the formation of DNA cross-links and strand breaks, thus inhibiting DNA replication (Begleiter, Robotham et al. 1989; Begleiter, Robotham et al. 1992; Begleiter, Leith et al. 2004). Activation of Mitomycin C can be influenced by pH and hypoxia, it demonstrates higher cytotoxic effects at acidic pH, and it has been shown to kill preferentially hypoxic tumour cells (Sartorelli, Hodnick et al. 1994). Under reductive conditions Mitomycin C alkylates the 2-amino group of guanine nucleosides in the minor groove of DNA. The alkylating moieties in Mitomycin C are the aziridine at C1 and the carbamate

at C10. The discovery of Mitomycin C led to the development of numerous other analogs, some of which conserve the basic structure of Mitomycin, while other research groups employ only the basic indolequinone framework of Mitomycin C to develop novel agents. Additional quinone alkylating agents include the indolequinone E09, the benzoquinone diaziquone (AZQ), and its analogs MeDZQ, and RH1 (Figure 2).

1.3.2 EO9

The indolequinone EO9 (3-hydroxy-5aziridinil-1-methyl-2(1H-indole-4,7-dione)-prop-beta-en-al-pha-ol) is a synthetic analog of Mitomycin C (Figure 2). Hendriks et al (1993) found EO9 had a different spectrum of antitumor activity than Mitomycin C; it demonstrated greater cytotoxicity against solid tumors, and did not show significant myelosuppression in animal toxicology studies (Hendriks, Pizao et al. 1993). In phase I clinical trials tumours showed partial responses to EO9 (Schellens, Planting et al. 1994; Aamdal, Lund et al. 2000). However, it did not show significant antitumour activity in phase II clinical trials in breast, pancreatic, colon and gastric cancer (Dirix, Tonnesen et al. 1996). The failure of EO9 in clinical trials has been associated primarily to a short half-life, poor tissue penetration and dose-limiting kidney toxicity. EO9 was demonstrated to be a better substrate for reductive activation by NQO1 compared to Mitomycin C, because it can undergo more efficient redox cycling and its activation is not dependent on pH (Bailey, Lewis et al. 1998). The activation of this agent by NQO1 resulted in the production of metabolites able to induce DNA strand breaks along with DNA interstrand cross-links (Bailey, Wyatt et al. 1997), thus

inhibiting DNA replication. A strong association has been observed between high NQO1 activity and sensitivity to EO9 (Robertson, Haigh et al. 1994; Collard, Matthew et al. 1995). P450 reductase has recently been implicated in the reduction of EO9. Studies performed with breast cancer cells expressing P450 reductase, established that P450 reductase contributes to the bioactivation of EO9 under aerobic and hypoxic conditions (Saunders, Jaffar et al. 2000). Furthermore, studies by Bailey et al (2001) reported the activation of EO9 to free radical and DNA-damaging species using purified rat P450 reductase (Bailey, Lewis et al. 2001). Due to the failure of EO9 during clinical trials, work is proceeding on developing EO9 analogs with improved activity and delivery, while retaining the specificity of activation in tumour cells and toxicities to normal tissues.

1.3.3 AZQ and its analogs

Diaziquone (AZQ) (2,5-bis(carbethoxyamino)-3,6-diaziridinyl1,4-benzoquinone) is an aziridinylbenzyquinone (Figure 2), designed for tumors of the central nervous system (Chou, Khan et al. 1976). This agent can be reduced by P450 reductase to produce semiquinones that in turn generate reactive oxygen species in the presence of oxygen through redox cycling. AZQ is also a substrate for reduction by NQO1, leading to increased DNA cross linking, and thus cytotoxicity (Fisher, Donis et al. 1992). A correlation has been observed between levels of NQO1 activity and cytoxicity of AZQ (Gibson, Hartley et al. 1992). In clinical trials AZQ demonstrated activity against malignant brain tumors, in particular gliomas and refractory lymphomas. Toxicities associated

with AZQ treatment include myelotoxicity, in particular thrombocytopenia (Feun, Yung et al. 1984). In spite of the observed antitumour activity, AZQ did not exceed the activity of currently used agents. Due to the failure of AZQ to surpass the activity of other chemotherapeutic agents, researchers focused on developing analogs with improved targeting. One such analog is MeDZQ (2,5-diaziridiny-3,6-dimethy-1,4benzoquinone) (Figure 2), an excellent substrate for activation by NQO1, and more toxic than AZQ against colon carcinoma (Gibson, Hartley et al. 1992). However, the activity of MeDZQ is largely limited by its poor solubility. In view of this challenge a water soluble analog was developed, RH1 (2,5-diaziridinyl-3-hydroxymethy-6-methyl-1,4-benzoquinone) (Figure 2). This agent has increased water solubility due to the hydroxyl group, is a better substrate for NQO1 than MeDZQ, and its antitumour activity has been demonstrated both *in vitro* and *in vivo* (Winski, Hargreaves et al. 1998). The involvement of P450 reductase in the reduction of RH1 has been assessed on breast cancer cells (Kim, Patterson et al. 2004). Further more research by Begleiter et al (Begleiter, Leith et al. 2007), determined RH1 to be a substrate for activation by P450 reductase, causing DNA damage upon activation. However the activation of RH1 by P450 reductase is of little significance in comparison to its activation by NQO1, further emphasizing RH1 specificity for this enzyme.

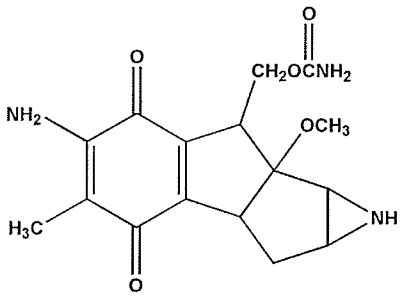
RH1 phase I clinical trials in human patients with solid tumours in the UK involved 18 patients of WHO performance status 0-1 with advanced refractory solid malignancies. 14 patients were male and eight patients had colorectal adenocarcinoma. The maximum tolerated dose used was 1,430 mcg/m2/day and

the dose-limiting toxicity was bone marrow suppression. Tested doses were associated with DNA cross-linking in both peripheral blood lymphocytes and tumour cells. DNA cross-linking after RH1 was observed in tumour samples, even with low levels of NQO1(Danson, Ranson et al. 2007). The maximum tolerated dose of 1,430 mcg/m2/day has been recommended for phase II clinical trials (Danson S. 2007).

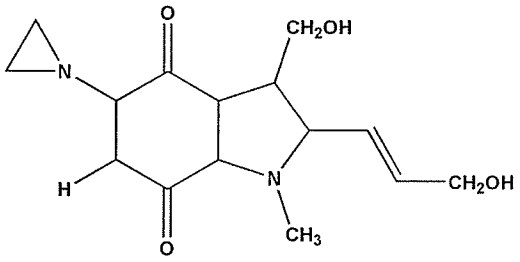
1.3.4 Tirapazamine

Tirapazamine (TPZ, 3-amino-1,2,4-benzootriazine-1,4di-N-oxide, SR4233) is a clinically used bioreductive agent that is activated at low oxygen levels typically found in solid tumours (Figure 2). It was originally developed as an adjunct to radiation therapy due to the known failure of radiotherapy towards hypoxic tumour fractions. TPZ is activated through one-electron reduction catalyzed by P450 reductase, producing a highly damaging nitroxide radical intermediate; further reduction of this active intermediate produces the non-toxic metabolite SR4317. In the presence of molecular oxygen the TPZ radical becomes fully reduced, and therefore inactivated (Brown 1993). The antitumour effects of TPZ are mainly due to DNA alkylation and the formation of DNA damaging hydroxyl radicals in the absence of molecular oxygen, which accounts for its selective toxicity towards hypoxic cells (Brown 1993). Although TPZ is predominately activated by P450 reductase, NQO1 is known to play a role in the activation of this agent (Patterson, Saunders et al. 1998). Preclinical studies have established a correlation between the antitumour activity of TPZ and the activity of P450 reductase in breast and non-small cell lung cancer cell lines (Patterson,

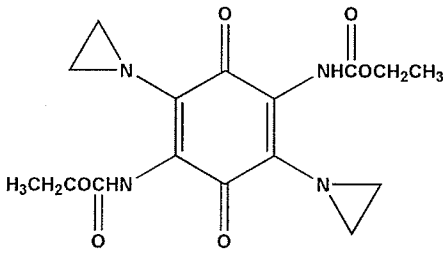
Barham et al. 1995; Patterson, Saunders et al. 1997; Chinje, Patterson et al. 1999). These results led to the clinical evaluation of TPZ against head and neck, and lung cancers in phase I clinical trials (Rischin, Peters et al. 2001; Le, McCoy et al. 2004). Further more, TPZ is currently undergoing phase II and III clinical trials, as part of combination therapy with cisplatin or radiation therapy (Treat, Johnson et al. 1998; Rischin, Peters et al. 2005).



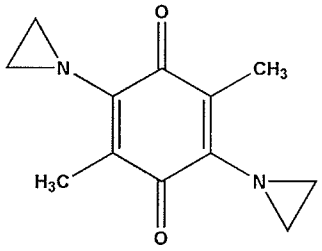
Mitomycin C



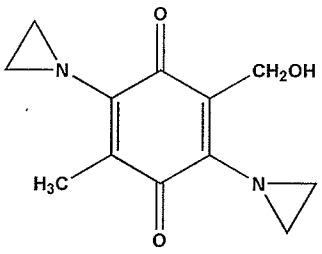
Indolequinone EO9



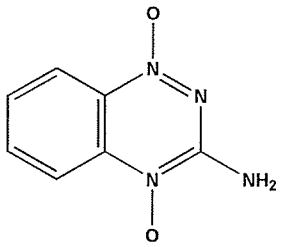
Diaziquone (AZQ)



Methyl-DZQ



RH1



Tirapazamine

Figure 4. Quinone alkylating agents: Mitomycin C, indolequinone E09, the benzoquinone diaziquone (AZQ), and its analogs MeDZQ, and RH1.

1.3.5 Benzoquinone Mustards (BM)

Benzoquinone mustards are newly developed model bioreductive alkylating agents with relatively simple structures, and are good model compounds for investigating the effects of structural alterations. The parent compound 2-(Di(chloroethy)amino)-1,4-benzoquinone (BM), has been previously studied by several groups including our laboratory (Begleiter 1983; Begleiter and Leith 1990). Similar to other bioreductive agents, the structure of BM is made up of a quinone bioreductive element like that found in MMC, EO9, and AZQ (Figure 5). The cytotoxic element in BM is a nitrogen mustard alkylating group. The nitrogen mustard alkylating group in BM has been shown to increase the cytotoxic activity of this agent by 30,000 times when compared to the hydrolyzed benzoquinone mustard, and 600 times when compared to the aniline mustard in a L5178Y mouse lymphoma cell line (Begleiter 1983; Begleiter and Leith 1990).

A series of model BM analogs were prepared, which included compounds substituted with electron donating, electron withdrawing, or sterically bulky groups (Table 1). Each functional group substituted on the BM moiety illustrates a specific chemical property, such that specific effects can be identified for each BM analog. Specifically, electron donating groups such as *methoxy* in MBM and *methyl* on MeBM have been substituted at either C5 or C6 of the quinone moiety. Also substituted at the C5 position are the electron withdrawing groups *chloro* in CBM, and a *fluoro* in FBM. The purpose for selecting electron withdrawing and electron donating groups is to determine how they affect the electron density around molecules to which they are bound. For instance, electron donating

groups are able to increase electron density around the quinone group, while electron withdrawing groups decrease electron density. Alterations in electron density can modify the redox potential of BM analogs, changing the rates of reduction and subsequent activation of the mustard group, along with enzyme-substrate interactions. In contrast, sterically bulky groups are used to determine how the size, position and more electron-dense molecules alter enzyme-substrate interactions, and in some cases electron potentials. The sterically bulky groups studied include a *t-butyl* group substituted on C6 in m-TBM, and *phenyl* group substituted on either C5 in PBM and on C6 in m-PBM.

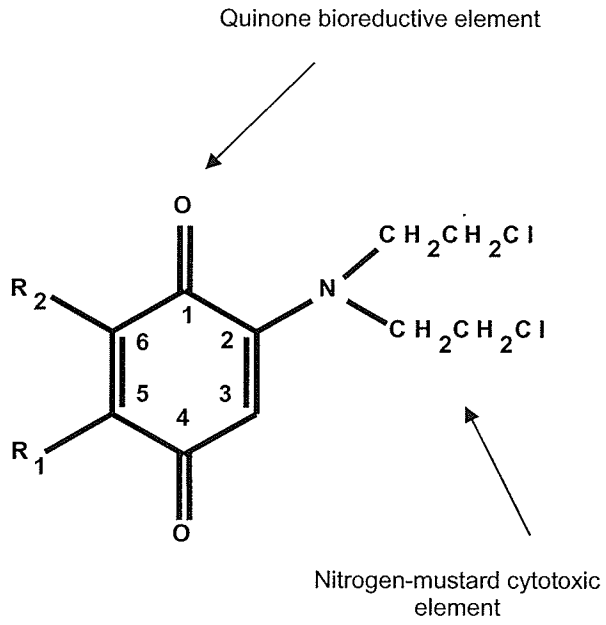


Figure 5. Structure of the parent benzoquinone mustard (BM). Benzoquinone mustard is made up of a quinone bioreductive element and a nitrogen mustard cytotoxic element.

Table 1. Functional groups substituted in BM analogs. Electron donating groups *methoxy* in MBM and *methyl* on MeBM have been substituted at either C5 or C6 of the quinone moiety. Electron withdrawing groups *chloro* in CBM, and a *fluoro* in FBM have been substituted at the C5 position. Sterically bulky groups include a *t-butyl* group substituted on C6 in m-TBM, and *phenyl* group substituted on either C5 in PBM and on C6 in m-PBM.

BM ANALOG	R_1	R_2
BM	H	H
FBM	F	H
CBM	Cl	H
MeBM	CH3	H
m-MeBM	H	CH3
MBM	OCH3	H
PBM	Phenyl	H
m-PBM	H	Phenyl
m-TBM	H	C(CH3)3

BM and its analogs are believed to be activated by P450 reductase and by NQO1 (Begleiter and Leith 1990; Fourie, Oleschuk et al. 2002). Consequently two pathways for the reduction of these compounds have been stipulated (Figure 6). The first pathway is mediated by P450 reductase, where the enzyme reduces the compound by a series of one-electron transfers. The first electron transfer reduces the quinone form of BM to a semiquinone radical intermediate, and a second one-electron transfer that further reduces the semiquinone to a fully reduced hydroquinone product. The second pathway of reduction involves a single two-electron transfer that is mediated by NQO1, which leads to the direct formation of the fully reduced hydroquinone product. Reduction of BM to either the semiquinone or the hydroquinone products are thought to activate the cytotoxic nitrogen mustard element. The activated nitrogen mustard element can covalently bind to cellular components including DNA and proteins, and can generate DNA cross-links. In the presence of molecular oxygen, the hydroquinone and semiquinone forms of BM are rapidly oxidized back to the quinone form, and in the process generates ROS. The BM semiquinone radicals and ROS can cause further damage to cellular DNA and proteins by introducing DNA strand breaks and other oxidative damage to cellular components. The significance of the various mechanisms of toxicity of BM is primarily dependent on tumour microenvironment, which includes oxygen levels, pH, and activity levels of reductive enzymes in tumour cells.

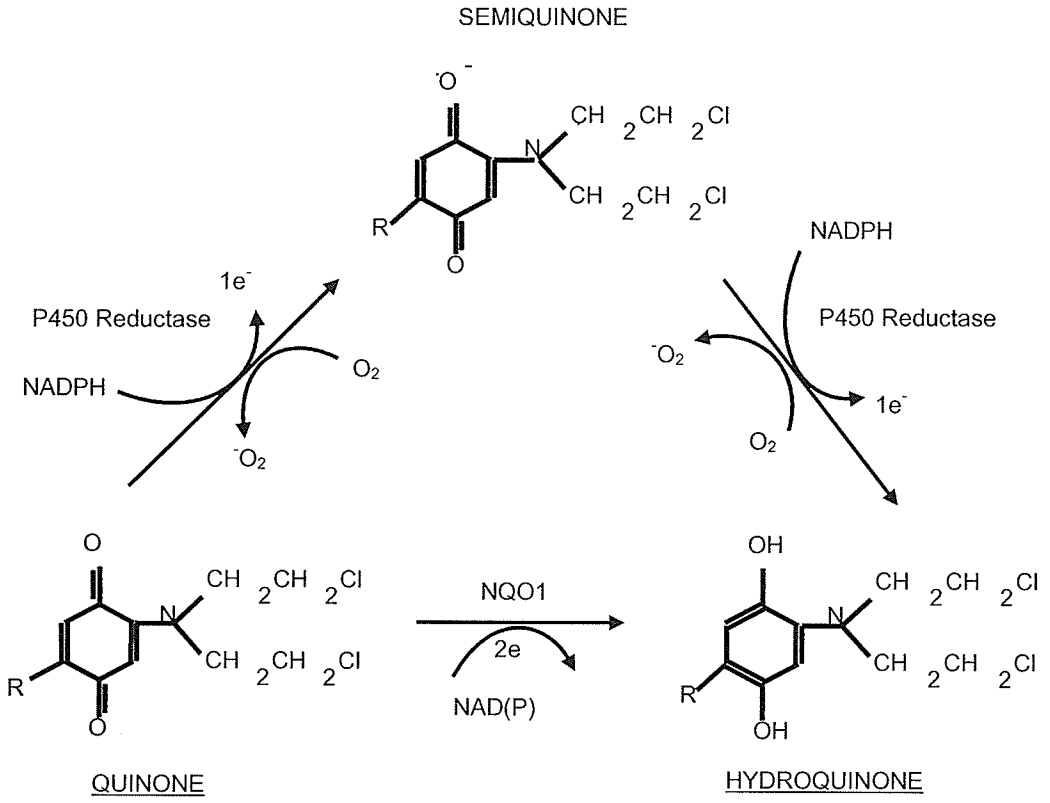


Figure 6. Reduction pathways of benzoquinone mustard bioreductive alkylating agents. One pathway is mediated by P450 reductase, where the enzyme reduces the compound by a series of one-electron transfers. The first electron transfer reduces the quinone form of BM to a semiquinone radical intermediate, and a second one-electron transfer that further reduces the semiquinone to a fully reduced hydroquinone product. The second pathway of reduction involves a single two-electron transfer that is mediated by NQO1, which leads to the direct formation of the fully reduced hydroquinone product.

Structure-activity studies by Fourie et al (2002) of benzoquinone mustards established the role of NQO1in the reduction of these agents, a long with the effect of functional groups on the reduction and activation of these agents by NQO1 (Fourie, Oleschuk et al. 2002). The research focused on establishing the ability of specific functional groups to modify reduction and subsequent activation of these agents mediated by NQO1 (Fourie, Oleschuk et al. 2002). They also investigated the effect of functional groups on DNA cross-link and DNA strand-break formation as mechanisms of cytotoxicity of these agents (Fourie, Guziec et al. 2004). Reduction studies suggest that functional groups decrease the rate of reduction of the quinone compared to the parent BM. Experimental data shows that electron donating groups substituted at C5 in MBM and MeBM enhance redox cycling of reduced quinone groups. Steric hindrance was observed in analogs with functional groups substituted at C6 position, where reduction of PBM was slower than BM, but faster than its positional isomer m-PBM. Electron withdrawing groups substituted at C5 and the electron withdrawing *chloro* group in CBM displayed similar rates of reduction, indicating that electron density surrounding the quinone moiety does not have a significant effect on reduction by NQO1.

Overall, the reduction studies by Fourie et al. demonstrated that functional groups can significantly affect the reduction and activation of quinones mediated by NQO1, where steric effects of functional groups play a more important role in modifying the rate of reduction by NQO1, rather than electronic effects.

NQO1 mediated DNA damage is caused mainly by two mechanisms, alkylation mediated DNA cross-linking, and DNA strand-break formation due to the presence of radical species. Both mechanisms of DNA damage have been positively correlated to cytotoxicity of BM analogs on two cell lines expressing distinct levels of NQO1. Results from DNA cross-linking studies indicate that the electron donating groups *methoxy* and *methyl*, substituted at C5 of the quinone structure increase the formation of DNA cross-links mediated by NQO1 compared to BM. Electron withdrawing groups such as *chloro* and *fluoro* on substituted at C5 of the quinone structure decreased the formation of DNA cross-links mediated by NQO1 compared to BM. The formation of DNA strand-breaks mediated by NQO1 increased in analogs substituted with electron donating groups, such as those in MBM and MeBM, compared to BM. The increase in DNA strand-break is attributed to an increase in the rated of redox cycling as well as to the formation of quinone radical intermediates.

Although these structure-activity studies demonstrate the participation of NQO1 in the reduction and subsequent activation of some BM analogs, they do not fully account for all of the cytotoxic activity displayed by these analogs. Alternate mechanisms of reduction and activation of BM analogs may be involved, thus further research is required to investigate these alternate mechanisms of activation.

2 RATIONALE AND PURPOSE OF RESEARCH

Current bioreductive agents are activated by more than one bioreductive enzyme. The "enzyme directed" approach to the development of bioreductive drugs requires the identification of bioreductive enzymes which are either over expressed or exhibit elevated activity in tumour cells compared to normal tissues. Moreover, this approach requires the development of agents which are substrates for these bioreductive enzymes. Determining the manner in which structural factors affect the activation of bioreductive agents will allow the development of agents that can be selectively activated by a single bioreductive enzyme, leading to enhanced tumour targeting and decreased toxicity to normal tissues.

The present study aims to establish the effects of selected functional group substitutions on 1) the ability of P450 reductase to reduce and activate Benzoquinone mustard analogs, and 2) the cytotoxic activity and mechanisms through which these agents confer cytotoxicity following reduction by P450 reductase.

The objective is to identify the manner in which structural factors confer selectivity for activation of BM analogs by P450 reductase can help improve targeting of these anticancer agents towards tumour cells over expressing P450 reductase. Activation of BM by P450 reductase is hypothesized to be most relevant under hypoxic conditions (e.g., solid tumours), where accumulation of semiquinone radical intermediates along with the fully reduced hydroquinone in can lead to increased tumour-selective toxicity.

3 MATERIALS AND METHODS

3.1 Materials

The fetal bovine serum (FBS) and cell media for the cell lines SK-Mel 28 and NCI H661, and MDA-MB-231 were obtained from GibcoBRL (Grand Island, NY, USA). For the MDA-MB-468 cell line both the media and fetal bovine serum was from American Type Culture Collection (ATCC) (Manassas, VA, USA). All the reagents for the cytochrome P450 reductase assay, Cytochrome C, NADPH, diphenyliodonium chloride (DPIC), phosphate buffer, and KCN were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purified, recombinant human NADPH-P450 reductase was purchased from GENTEST through BD Biosciences Discovery Labware (Bedford, MA, USA), with activity of 61umole of Cytochrome c reduced per min per mg protein at 37°C. *RC DC* Protein Assay Kit I, was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Dichloromethane, methanol, hexanes, 95% ethanol, ethyl acetate, silica gel (1000 mesh), glacial acetic acid, anhydrous ether, hydrogen peroxide solution and potassium fluoride were purchased from Mallinkrodt and Baker Inc. (Phillipsburg, NJ). N,N,-dimethyl formamide (DMF), dimethyl sulfoxide (DMSO) and cupric acetate were from Fisher Scientific (Fair Lawn, NJ). Vanillin, di(chloroethyl)amine hydrochloride, 1,4-benzoquinone, chromic acid,2-methyl-1,4-benzoquinone, 2-phenyl-1,4-benzoquinone, 2-chloro-1,4-benzoquinone, fluorobenzoquinone, tert-butyl benzoquinone, SYBR Green I nucleic acid gel stain, ampicillin (sodium salt), cholramphenicol, and Sigma-Aldrich GenElute^{TM} High Performance (HP) Plasmid Kits were from Sigma-Aldrich (St. Louis, MO).

Standard rectangular cells, screw cap with septum were obtained from Starna Cells (Atascadero, CA, USA). Luria-Bertani Broth, Miller and Luria-Bertani Agar, Miller were from Becton Dickindon and Co. (Sparks, MD, USA). Klenow fragment of DNA polymerase I, 10x BlueJuice gel loading buffer, High DNA Mass ladder, and agarose were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). EcoRI, [α-^{32}P] dATP (6000Ci/mmol), and Nick columns (used to remove unincorporated ^{32}P-labelled nucleotides) were obtained from Amersham Biosciences Corp. (Baie d'Urfe, PQ, Canada). Perfectprep® Gel Cleanup Kit was purchased from Eppendorf Int. through Brinkmann Instruments, Ltd. (Mississauga, ON, Canada).

3.1.1 Cells and Culture

MDA-MB-468 metastatic breast adenocarcinoma, and MDA-MB-231 breast adenocarcinoma cell lines, were obtained from the American Type Culture Collection (Rockville, MD, USA). The MDA-MB-468 cells were grown in Leibovitz's L-15 Medium plus 10% fetal bovine serum (FBS), both MDA-MB-231 and NCI-H661 cells were grown in RPMI-1640 medium plus 10% FBS, and SK-Mel28 cells were grown in DMEM/F12 1:1 plus 10% FBS. Sodium bicarbonate was added to all the media to buffer pH changes during incubation periods.

3.2 Methods

3.2.1 Preparation of BM analogs

Benzoquinone Mustard and its analogs were prepared by Dr. F. Guziec, at Southwestern University, Georgetown, Texas, USA. BM was synthesized using the method developed by (Makarova 1967). The methods for synthesis of the BM analogs has been previously described (Fourie, Oleschuk et al. 2002). A purple precipitate was collected by filtration, dried and re-crystalized from hexanes to give BM. The purity and structure of BM was confirmed by ^{1}H nmr analysis and by melting point.

MBM was prepared using the method described by Crosby and Lutz (1956) for oxidative amination of 1,4-benzoquinones and the method developed by Makarova and Berlin (1967) for synthesis of BM. Di(chloroethyl)amine hydrochloric, potassium fluoride and cupric acetate were added to a solution of 3-methoxy-1,4-benzoquinone in 95% ethanol. The 3-methoxy-1,4-benzoquinone was prepared as described by Corral (1957). The reaction mixture was stirred at room temperature for 72hrs in the dark and then filtered. The filtrate was washed four times with ethyl acetate. The pooled solution (consisting of extracts and supernatants) were washed with an equal volume of 0.1 HCl to remove remaining cupric acetate. The ethyl acetate layer was separated, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to give a red precipitate. The red precipitate was re-crystallized using 95% ethanol to yield

MBM. The structure and purity of MBM was determined by ^{1}H nmr analysis and by melting point.

MeBM was prepared using the methods developed by Crosby and Lutz (1956) and Makarova and Berlin (1967) starting once again with 2-methyl-1,4-benzoquinone as with MBM. The mixture was filtered using a vacuum and the resulting precipitate was washed with ethyl acetate four times. The pooled solution was washed with an equal part 0.1 HCl and then dried with anhydrous sodium sulfate. A crude solid was produced with was re-crystallized using methanol yielding MeBM. The compounds structure and purity were determined by ^{1}H nmr analysis and by melting point.

m-MeBM was prepared using a combination of previously described methods (Crosby 1956; Makarova 1967). Di(chloroethyl)amine hydrochloride, potassium fluoride and cupric acetate were added to a solution of 2-methyl-1,4-benzoquinone in 95% ethanol (Corral 1957). The mixture was stirred for 72hrs in the dark, and filtered. The precipitate was washed with ethyl acetate. The pooled extract was concentrated and the residual oil dissolved in ethyl acetate. This solution was washed using 0.15 M HCl and then brine. Drying over anhydrous sodium sulfate, concentration and re-crystallization from hot methanol, yielded m-MeBM crystals. The structure of m-MeBM was confirmed by ^{1}H nmr analysis and by X-ray crystallography.

CBM was prepared using the methods developed by (Crosby 1956; Makarova 1967), starting from 2-chloro-1,4-benzoquinone, as previously

described for MBM. The resulting purple precipitate was re-crystallized using methanol to yield CBM.

FBM was prepared beginning with the synthesis of fluorobenzoquinone. Fluorobenzoquinone was added to a solution of ceric ammonium nitrate, and distilled water. The mixture was stirred for 1hr at room temperature and extracted using ethyl ether three times. The extracts were dried over sodium sulfate and concentrated to yield yellow crystals of fluorobenzoquinone. Di(chloroethyl)amine hydrochloride and cupric acetate were mixed with a solution of fluorobenzoquinone in 95% ethanol. Potassium fluoride was added, and the combination was stirred at room temperature for 72hrs. The mixture was filtered, and the precipitate washed with three times with ethyl acetate. The pooled extracts were washed with 0.1M HCl and then brine. The mixture was dried over anhydrous sodium sulfate and concentrated to yield red oil, which was re-crystallized from 95% ethanol yielding red FBM crystals. The structure of FBM was confirmed using ^{1}H nmr analysis.

PBM and m-PBM were prepared again by using the methods developed by (Crosby 1956; Makarova 1967) starting from 2-phenyl-1,4-benzoquinone, as previously described for MBM. The mixture was vacuum filtered and the precipitate was washed four times with ethyl acetate. The pooled extract was washed with water, followed by an equal volume of 0.1 HCl, and then dried over anhydrous sodium sulfate. Concentration yielded a combination of phenyl benzoquinone mustard isomers. Re-crystallization from ethyl acetate-petroleum ether generated the predominant m-PBM isomer's red crystals. The structure

and purity of m-PBM was determined by ^{1}H nmr and X-ray analysis. Concentration of the crude mother liquor from above generated a red precipitate rich in 5-phenyl isomer. This mixture was dissolved in ethyl acetate and then separated using radial chromatography with a Chromatoron apparatus on a silica plate, using ethyl acetate-hexanes as solvent. PBM was concentrated and dried using a vacuum, yielding PBM red crystals. The structure and purity of PBM was determined using ^{1}H nmr, X-ray, and melting point analysis.

m-TBM was prepared using the methods of (Crosby 1956; Makarova 1967), beginning from tert-butylbenzoquinone as explained for m-MeBM. The reaction mixture was stirred at room temperature for 72hrs in the dark. The mixture was filtered and the precipitate washed four times using ethyl acetate. The pooled extract was washed with 0.1M HCl and then brine. Red oil formed after drying over anhydrous sodium sulfate and concentrating. The oil was purified using flash chromatography on silica gel using ethyl acetate-hexanes as solvent. This produced the pure m-TBM as red oil. The structure was confirmed by NMR spectroscopy.

3.2.2 EPR studies

Electron paramagnetic resonance experiments were carried out using a Varian Associates model E-12 EPR spectrometer, a Nicollet Instruments computer model 1180, and a digital oscilloscope model 2090. The instruments settings were as follow: scan range 505-1000 G, microwave power 19.873 mW, modulation amplitude 2.0 G, modulation frequency 100 kHz, and microwave frequency 9.253 GHz.

All reactions were carried out at 37°C, under anaerobic conditions, and in a cell-free system. Anaerobic conditions were maintained by flushing the system with Argon gas. Standard reaction constituents included: P450 reductase (0.244ug), drug (1mM) dissolved in DMSO, and diluted in buffer, and NADPH (1mM) made up to a total volume of 75ul in 0.05M phosphate buffer (pH 7.4). The concentrations given are the final reagent concentrations in the reaction. The reaction was initiated by the addition of P450 reductase. 15ul of the complete reaction mixture was loaded in a glass or Teflon capillary for analysis by EPR. The controls for the reactions included: buffer, drug, and NADPH, in the absence of P450 reductase. Recording of the first-derivative EPR spectra was started about one minute after the reaction system was prepared (42 sec/ scan). The signal amplitude was expressed as relative EPR signal intensity (mV), (arbitrary units).

3.2.3 Spectrophotometric studies

The reduction experiments were conducted under anaerobic conditions in a hypoxic chamber flushed with anaerobic gas mixture. The reactions were carried out using Screw-cap septum-sealed quartz cuvettes to maintain hypoxic conditions throughout the experiment. 0.05M potassium phosphate buffer (pH 7.8), and DMSO were placed inside the hypoxic chamber and allowed to become hypoxic for 5-10 days before each experiment. NADPH and BM analogs were dissolved in hypoxic buffer and DMSO inside the chamber immediately before each experiment.

Each experiment comprised 30ul of BM analog (for a final concentration of 100uM), 1.5ul of P450 reductase stock (for a final concentration of 2.4ug/ml), made up to 1485ul with phosphate buffer. The control experiments included control with the complete reaction system excluding P450 reductase, and a control comprised of BM analog and phosphate buffer alone. The cuvettes were sealed inside the hypoxic chamber. The cuvettes were immediately placed in the spectrophotometer heated to 37°C, and the reaction was initiated by the addition of 15ul of NADPH (for a final concentration of 300uM) using a Hamilton syringe inserted through the cuvette's septum. The reductions were monitored for 45-60min by taking absorbance readings every 2-5 min. Reduction rate of the BM analogs was determined by measuring the decrease of the quinone absorbance maxima. The absorbance values obtained at each time point for each of the BM analogs were plotted in a rate curve (Abs. vs. time). The half-time of reduction of each analog was determined from the linear portion of each curve. The half-time of reduction values are the mean of 3-4 experiments. The means of the individual analogs were compared to the mean half-time of reduction of the parent compound BM by ANOVA.

3.2.4 NADPH: Cytochrome P450 reductase activity

P450 reductase activity was determined in MDA-MB-468 and MDA-MB-231 from cell sonicates by spectrophotometric assay as the NADPH-dependent reduction of cytochrome c (Strobel and Dignam 1978). Cell lysates were prepared by harvesting up to $1x10^7$ cells in exponential growth phase. The cells were centrifuged at 8000 rpm for 10 min. and washed twice with phosphate-

buffered saline (PBS). The cell pellet was re-suspended in 200ul of 20% glycerol and sonicated 3 x 10 sec. The samples were kept at 4°C between each sonication, and then stored at -80°C. The protein concentration of the cell lysates was determined using the Bio-rad *RC-DC* protein assay kit I, and using gamma-globulin as the standard. The standard assay was carried out in semi-micro cuvettes containing 0.3M potassium phosphate buffer (pH 7.7) containing KCN, 40umol of cytochrome c, 12umol of NADPH, and up to 100ug of lysate protein, in a final volume of 1.0ml. The reaction was initiated by the addition of NADPH to the cuvette. The rate of reduction of cytochrome c was monitored at 550nm for 6min against a blank without NADPH. The rate of reaction was calculated based on the extinction coefficient of $21mM^{-1}cm^{-1}$and expressed as nmol cytochrome c reduced per min per mg lysate protein.

3.2.5 Cytotoxicity studies

The cytotoxic activity of BM analogs was determined using the MTT proliferation assay. This assay is based on the ability of viable cells to convert soluble tetrazolium salt MTT, into purple formazan crystals by mitochondrial dehydrogenase. Cytotoxicity is presented as the optical density of the purple formazan crystals at 540nm of drug-treated cells as a fraction of the optical density of control cells, as previously described by (Doherty, Leith et al. 1998).

The MTT assays for the dose-response curves for MDA-MB-468 and MDA-MB-231 cells were carried out by first plating the required cell dilutions in 96-well plates, and incubated for 24hrs at 37°C, to allow cell adhesion to the plates. After 24hrs, the media in the 96-well plates was removed and replaced

by RPMI media containing a BM analog, and incubated for 1hr at 37°C. Tirapazamine was used as a positive control for cytotoxicity. In addition, control experiments in the absence of BM analogs were incubated with RPMI media alone for 1hr at 37°C. Following the 1hr incubation the media was removed, the cells were washed using Hank's balanced salt solution. Fresh media was added to the plates, and the cells were allowed to grow for at least four cell doublings, corresponding to six to nine days. RPMI-1640 media without hepes, containing MTT (0.04mg MTT/ ml media) was replaced. The cells were allowed to incubate for two to four hours at 37°C. The 96-well plates were then centrifuged at 4°C for 10min at 15000 rpm. The RPMI media containing MTT was removed, followed by the addition of 150ul of DMSO to each well. The plates were shaken for one minute to dissolve the formazan crystals. The absorbance was measured at 540nm.

The resulting dose response curves for each of the analogs and each of the cell lines were the means of 3-16 determinations. The D_{10} value for each drug on each of the cell lines was calculated. The D_{10} value refers to the concentration of BM analog that will reduce the surviving cell fraction to 10% (0.1) of the control (i.e. cells incubated in the absence of BM analog). The D_{10} values for each BM analog were calculated from the inverse of the slope of the linear regression lines of the optical density against drug concentration curves. The Cytotoxicity of each of the BM analogs was compared to that of the parent compound BM by determining the difference between the slopes of the linear

regression lines of the optical density against drug concentration curves as previously described (Doherty, Leith et al. 1998).

For the inhibition studies, MDA-MB-468 cells were incubated for 1hr. in the presence of 5uM of the P450 reductase inhibitor diphenyliodonium chloride (DPIC) prior to treatment with the respective IC_{50} concentration for each BM analog. The IC_{50} refers to the concentration of drug that will reduce the surviving cell fraction (SCF) to 50% of the original cell fraction, and is determined from the linear portion of drug-response curves of each BM analog. The concentration of DPIC chosen (5uM) produced the maximum inhibitory effect of P450 reductase with the least toxicity to MDA-MB-468 cells over the total incubation time of 120 minutes. After treatment with DPIC for 60 minutes, the cells were incubated in the presence or absence of BM analogs for an additional 60 minutes in RPMI media containing 5uM DPIC. The same steps were followed as for determining the dose-response curves. The statistical significance of the difference between the surviving cell fraction means of the control cells and cells treated with DPIC were compared. The effect of DPIC on the cytotoxic activity of each of the BM analogs at the IC_{50} on MDA-MB-468 was compared using two-tailed t-tests.

3.2.6 Preparation and transformation of competent E.coli by high-voltage electroporation

E.coli bacteria (DH5 alpha strain) were made competent for electroporation using the method described by (Sambrook 1989). The competent *E.coli* culture was grown to mid-log phase, then chilled and centrifuged. A low salt buffer was used to wash the cell pellet to help decrease its ionic strength. The cell pellet was resuspended in 10% glycerol and stored at -70°C. An aliquot of bacteria was thawed for electroporation at 0°C with a mini-electrode from Bio-Rad Laboratories (Mississauga, ON, Canada).

3.2.7 Purification of pBR322 plasmid from E.*coli*

Luria-Bertani (LB) agar plates containing ampicillin (100ug/ml) were streaked with the transformed *E.coli* (DH5 alpha) culture containing the pBR322 plasmid, to obtaine single colonies. The streaked plates were incubated at 37°C for 24hrs. A single colony on the plate was used to inoculate 5ml of Luria-Bertani (LB) broth containing 100ug/ml ampicillin (100ug/ml). The inoculated broth was incubated at 37°C in a rotary shaker until the *E.coli* reached the late-log phase (O.D.600 $\approx$ 0.6). A volume of 400ml LB broth containing ampicillin was inoculated with 3.5ml of the *E.coli* culture and incubated at 37°C in a rotary shaker until the O.D.600 reached 1.0. Chloramphenicol was added to the culture (final concentration 170ug/ml). The culture was the incubated for an additional 16hrs at 37°C in a rotary shaker to allow the amplification of the plasmid. pBR322 is a low copy plasmid, and will only achieve moderate copy numbers in the DH5

alpha bacteria. The cells were harvested by centrifugation at 8000g for 15min at 4°C, followed by the removal of all the supernatant liquid. The plasmid was isolated and purified using the GenElute HP Plasmid Maxiprep kit from Sigma-Aldrich. The concentration and conformation of the plasmid was determined by gel electrophoresis in a 1% agarose gel using a high-DNA mass ladder, subsequent to enzymatic digestion by ECOR1. A band at approximately 4.3 kbp was detected, corresponding to the length of pBR322 plasmid DNA. Using a comparison of the intensity of DNA band at 4.3 with the intensity of the High DNA mass ladder fragments, the concentration of pBR322 plasmid was calculated.

3.2.8 Measurement of DNA strand-break formation

The formation of DNA single strand breaks following the activation of BM analogs was measured using the method described by (Walton, Smith et al. 1991). This method measures the conversion of supercoiled DNA form of a plasmid to its open, relaxed circular conformation. The present assay is a modification of the assay previously described by (Fourie, Guziec et al. 2004). The isolated pBR322 plasmid was purified to obtain supercoiled plasmid DNA by gel electrophoresis and subsequent extraction using the Perfectprep® Gel Cleanup Kit from Eppendorf. The strand break reactions were carried out in clear 1.5ml microfuge tubes, in a final volume of 50ul of potassium phosphate buffer (0.05M, pH 7.8). The reagents were added as follows: BM analog (1-300uM in final DMSO concentration of <2%), 0.5ug P450 reductase, and 150ng of supercoiled DNA. The reaction was initiated with the addition of 10mM NADPH, followed by incubation at 37°C for 45 min. The control reactions consisted of: 1)

buffer, P450 reductase and NADPH in the absence of drug, 2) buffer, NADPH and drug in the absence of P450 reductase. The reaction was terminated by placing the tubes in a iced-water bath, and by the addition of 0.5mM DPIC. A 25ul aliquot of the reaction mixture was taken and mixed with 3ul of BlueJuice loading buffer. The samples were loaded in a 1% agarose gel, followed by electrophoresis (90V for 1.5hrs); in TAE buffer (pH 7.0). The gel was subsequently stained with SYBR Green I nucleic acid stain (0.01% in TAE buffer pH 8.0), and dried at 45°C for 6hrs. DNA was quantified by densitometry using the STORM fluorescence scanning system (Molecular Dynamics Inc. Sunnyvale, CA, USA). The bands for supercoiled DNA were multiplied by 1.6 to correct for decreased binging of SYBR Green to supercoiled DNA compared to relaxed and linear DNA. This correction factor was previously reported by (Jones and Weinfeld 1996). The concentration of relaxed DNA was determined as a percent of the total DNA loaded in each well, for each of the BM analogs. The data is expressed as mean values ± SEM of 3-7 experiments. The results are expressed as E_{10} values, which refer to the maximum concentration of cross-links formed after treatment of DNA with 10uM of BM analog. The E_{10} value was used because it fell within the linear portion of the DNA cross-links vs. BM analog concentration curves. The E_{10} values for each of the BM analogs were compared by ANOVA to assess the statistical significance of the differences between the means of the BM analogs ($P = <0.001$). The Holm-Sidak method was used to compare the E_{10} of the analogs vs. the parent BM to identify which

functional groups have a significant effect on the ability of the parent drug to induce DNA damage via single strand breaks (P=<0.05).

3.2.9 Determination of DNA cross-links formation

The cross-linking activity of BM and its analogs following activation was measured based on the method developed by (Hartley, Berardini et al. 1991). The following method is a modified version of the method described previously by (Fourie, Guziec et al. 2004). The isolated plasmid PBR322 DNA was linearized by digestion with ECOR1. The linearized plasmid was radio-labeled with [α-^{32}P] dATP (5ul of 6000Ci/mmol), and Klenow fragment. A reaction mixture consisting of 0.25 dTTP, 5ul of 6000Ci/mmol of [α-^{32}P] dATP, 2U of Klenow fragment, 400ng of pure linear pBR322 plasmid DNA, and React 2 buffer corresponding to 10% of the final volume of the reaction. The mixture was incubated at 37°C for 30 min. Excess dATP was removed by column filtration using a 3ml NICK™ Column (a pre-packed Sephadex G-50 DNA grade column). The ^{32}P labeled DNA was eluted with TE buffer (10mM Tris pH7.5, 1mM Na_2EDTA). The final concentration of DNA was 1ng/ul. The experiments were carried out under anaerobic conditions, in clear 1.5ml microfuge tubes, in a final volume of 200ul of potassium phosphate buffer (0.05M, pH 7.8). The buffer was purged with Nitrogen gas for 15 min at 37°C to create anaerobic conditions. The reagents for the reaction were added in the following order: BM analog dissolved in DMSO (5-100uM drug in final DMSO concentration <2%), 0.05ug P450 reductase, and 30ng of end-labeled DNA. The reaction was initiated by the addition of 500uM of NADPH, followed by incubation for 45 min at 37°C. Nitrogen continued to be

flushed over the reaction mixture to maintain anaerobic conditions. Controls of the BM analogs included: 1) buffer, NADPH, radio-labeled DNA, and P450 reductase in the absence of BM analog. 2) Buffer, NADPH, radio-labelled DNA, and BM analog, in the absence of P450 reductase. The reaction was terminated by placing the microfuge tubes in iced-water bath, and by the addition of 0.5mM DPIC. 15ul of strand-separation buffer (53% DMSO, 15mM Trisma pH 7.0, 1.5mM EDTA pH 7.0, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added to a 15ul aliquot of the reaction mixture. The DNA strands were separated by incubating the DNA at 70°C for 5min, and immediately cooling in an iced-water bath for 10min. The DNA was loaded onto a 1.6% agarose gel containing 0.4ug/ml ethidium bromide, followed by electrophoresis (120V for 30min) in TAE buffer (pH 7.0). The single-stranded control was generated by placing 70ng of radio-labelled pBR322 plasmid DNA in an equal volume of strand-separation buffer, followed by incubation at 70°C for 5min, and cooling in iced-water for 10min. The double-stranded control was prepared by placing 70ng of radio-labelled pBR322 plasmid DNA in strand-separation buffer and directly loading it onto the agarose gel. The gel was dried at 45°C for 6hrs, and exposed to a phospho-imaging screen for 3hrs. The double stranded and single stranded DNA were visualized and quantified by densitometry using the STORM image-scanning system (Molecular Dynamics Inc. Sunnyvale, CA, USA). The concentration of double stranded DNA was determined as a percent of the total concentration of DNA loaded per well. The data is expressed as mean values ± SEM of 3-7 experiments. The results are expressed as E_{25} values, which refer to

the maximum concentration of cross-links formed after treatment of DNA with 25uM of BM analog. The E_{25} value was used because it fell within the linear portion of the DNA cross-links vs. BM analog concentration curves. The E_{25} values for each of the BM analogs were compared by ANOVA to assess the statistical significance of the differences between the means of the BM analogs ($P = <0.001$). The Holm-Sidak method was used to compare the E_{25} of the analogs vs. the parent BM to identify which functional groups have a significant effect on the ability of the parent drug to induce DNA damage via single strand breaks ($P=<0.05$).

4 RESULTS

4.1 Studies on the effect of functional groups on Reduction catalysed by P450 reductase

The series of analogs of benzoquinone mustard (BM) were prepared by the Dr. Frank Guziec (Southwestern University, TX). Each BM analog illustrates a specific property inherent to each functional group. The series of analogs were prepared to determine the effect of each functional group on the reduction of the quinone ring by P450 reductase, as well as on the activity and mechanisms of cytotoxicity of these analogs after activation by P450 reductase (Table 1).

The analogs MeBM, m-MeBM, and MBM have electron donating properties. More specifically the *methyl* group in MeBM and m-MeBM is a weaker electron donor compared to the *methoxy* group in MBM. The *chloro* and *fluoro* groups in CBM and FBM respectively display electron withdrawing properties. Alternatively the *phenyl* and *tertiary-butyl* groups in PBM, m-PBM and m-TBM are large sterically bulky groups. In the *m-* isomer analogs (m-MeBM, m-PBM, and m-TBM) the functional groups have been substituted at the C6 position, instead to the C5 position. These analogs were used to study the effect of each functional group substituted on the quinone moiety, on the reduction by the bioreductive enzyme P450 reductase.

4.1.1 Measurement of free radical formation using EPR

EPR spectra were obtained following incubation of BM analogs with NADPH and P450 reductase under anaerobic conditions. All reactions were

carried out at 37°C, and in a cell-free system. Anaerobic conditions were maintained by flushing the system with Argon gas. Standard reaction constituents included: P450 reductase (0.244ug), drug (1mM) dissolved in DMSO, and diluted in buffer, and NADPH (1mM) made up to a total volume of 75ul in 0.05M phosphate buffer (pH 7.4). The negative controls for the reactions included: buffer, drug, and NADPH, in the absence of P450 reductase. The indolequinone EO9 was used as the positive control for these studies. Figure 7 shows the spectra obtained for EO9, BM, CBM, MBM and PBM. Other benzoquinone mustard analogs, namely MeBM, m-MeBM, m-PBM and m-TBM were not available for EPR analysis.

The EPR spectrum for EO9 shows radical formation in the presence of P450 reductase, which suggested the reaction system was working (Figure 7.A). The parent compound BM was the only agent to produce a significant free radical signal (Figure 7.C). However in the presence of P450 reductase the BM free radical signal decreased (Figure 7.D). Similar results were observed for the analogs CBM and MBM, where the free radical signal was larger in the absence of P450 reductase. Upon addition of P450 reductase to the reaction system, the already small free radical signal disappeared (Figure 7. F, H). No free radical signal was detected for PBM in the absence or presence of P450 reductase.

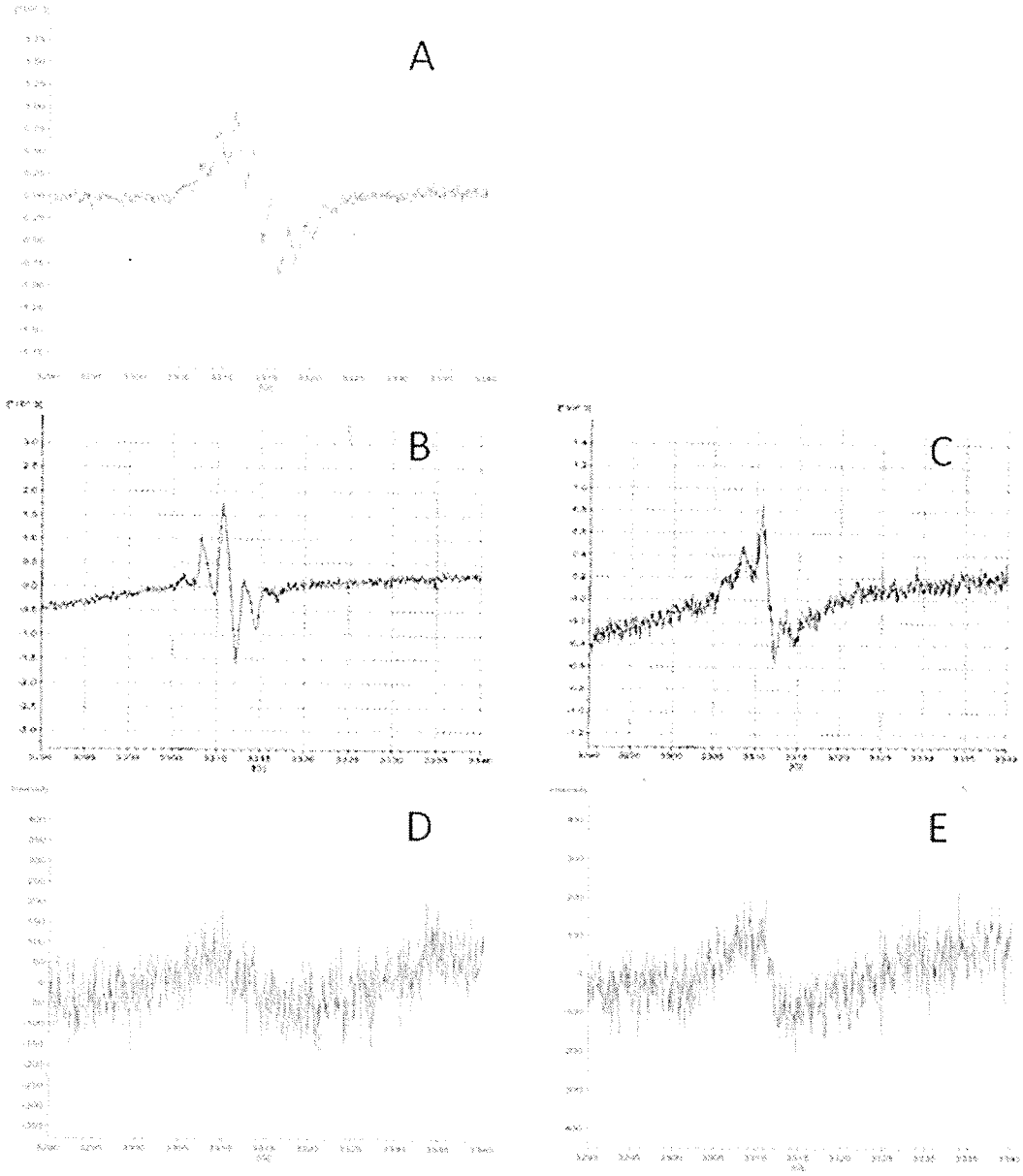


Figure 7.1. EPR spectra obtained for EO9, BM, and CBM, following incubation with NADPH and P450 reductase under anaerobic conditions. All reactions were carried out at 37°C, and in a cell-free system under anaerobic conditions.Standard reaction included: P450 reductase (0.244ug), drug (1mM) in DMSO, and NADPH (1mM) in 0.05M phosphate buffer (pH 7.4). 15ul of the complete reaction mixture was loaded in a Teflon capillary for analysis. The negative controls for the reactions included: buffer, drug, and NADPH, in the absence of P450 reductase. The signal amplitude was expressed as relative EPR signal intensity (mV), (arbitrary units). **A** EO9 (used as the positive control); **B** BM; **C** BM+P450 reductase; **D** CBM; **E** CBM+P450 reductase.

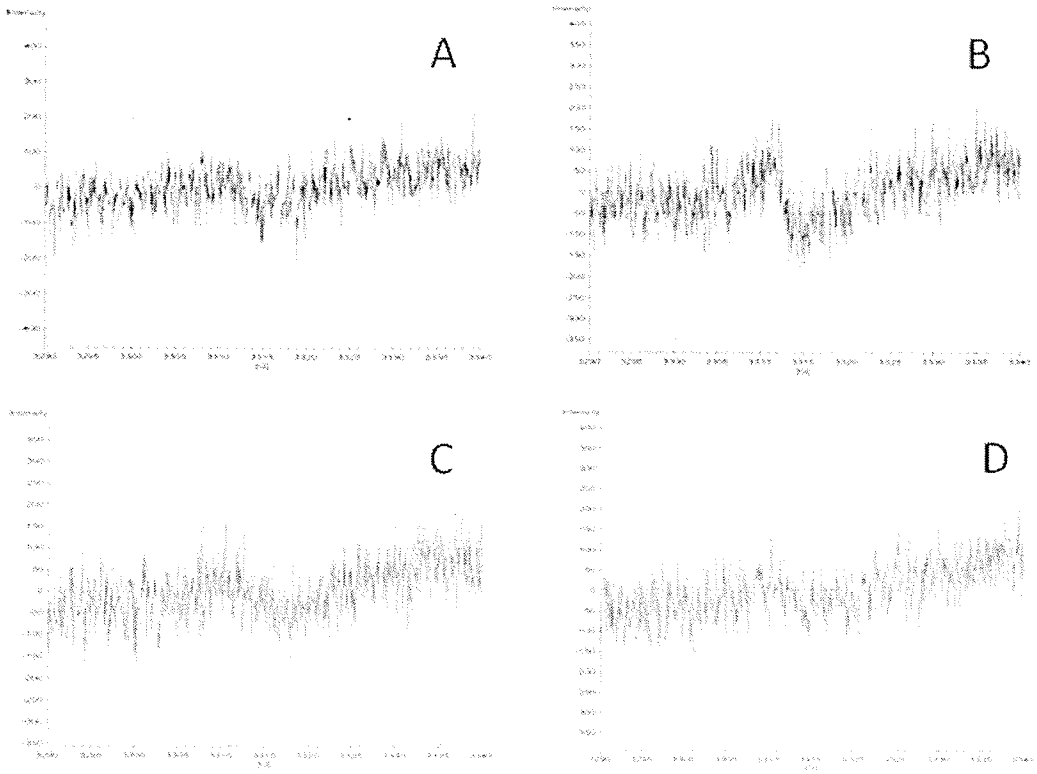


Figure 7.2. EPR spectra obtained for MBM and PBM, following incubation with NADPH and P450 reductase under anaerobic conditions. All reactions were carried out at 37°C, and in a cell-free system under anaerobic conditions.Standard reaction included: P450 reductase (0.244ug), drug (1mM) in DMSO, and NADPH (1mM) in 0.05M phosphate buffer (pH 7.4). 15ul of the complete reaction mixture was loaded in a Teflon capillary for analysis. The negative controls for the reactions included: buffer, drug, and NADPH, in the absence of P450 reductase. The signal amplitude was expressed as relative EPR signal intensity (mV), (arbitrary units). **A** MBM ; **B** MBM+P450 reductase; **C** PBM; **D** PBM+P450 reductase.

4.1.2 Measurement of the decrease in quinone absorbance

The rate of reduction of BM analogs was determined by measuring the decrease in absorbance of the quinone maximum peak characteristic to the coloured oxidized BM analog, using a spectrophotometric assay (Figure 8). The underlying assumption in this study is that the decrease of the absorbance of the maximum over time is attributed to the formation of the hydroquinone product, which is characteristically colorless and undetectable in the visible spectrum. Reduction of BM analogs was carried out under hypoxic conditions in a cell-free system. The reaction system consisted of 100uM BM analog, 2.4ug/ml P450 reductase, 300uM NADPH in a final volume of 1500ul of 0.05M potassium phosphate buffer pH 7.8. The reaction was allowed to proceed for 45 minutes or until the entire drug became reduced (no absorbance detected in the visible range).

The linear portion of each rate curve (Absorbance vs. time), was used to determine the t½ of reduction for the analogs (Figure 9). T½ refers to the length of time required to reduce one-half the concentration of the drug, in this case the time required to reduce 50uM of analog. The t½ values of the various BM analogs were compared by means of ANOVA.

Overall, the t½ of m-PBM < m-MeBM ≈ MeBM ≈ MBM ≈ PBM ≈ m-TBM << CBM, with t½ values of 3.18 ± 0.37, 3.67 ± 0.27, 3.80± 0.29, 3.83 ± 0.12, 3.93 ± 0.20, 4.20 ± 0.31, and 56.7 ± 8.9 minutes (Table 2). The t½ of BM could not be determined since its reduction took longer than 60 minutes, by which time the enzyme had become inactive. Control experiments showed a simultaneous

decrease in the absorbance of NADPH, indicating that the decrease in quinone absorbance resulted from the reduction of the BM analogs by P450 reductase. No change in the absorbance of the oxidized quinone compounds was detected in the absence of NADPH. No change in absorbance of the oxidized quinone compounds was detected in the absence of P450 reductase.

Reduction studies showed that substitution of BM with electron donating groups increases the rate of reduction by P450 reductase of the benzoquinone mustard parent molecule. On the other hand substitution with *chloro* electron withdrawing group does not seem to affect the rate of reduction of these compounds by P450 reductase. EPR studies showed that the parent BM was able to produce free radical species in the absence of P450 reductase, while none of the analogs seemed to produce free radical species.

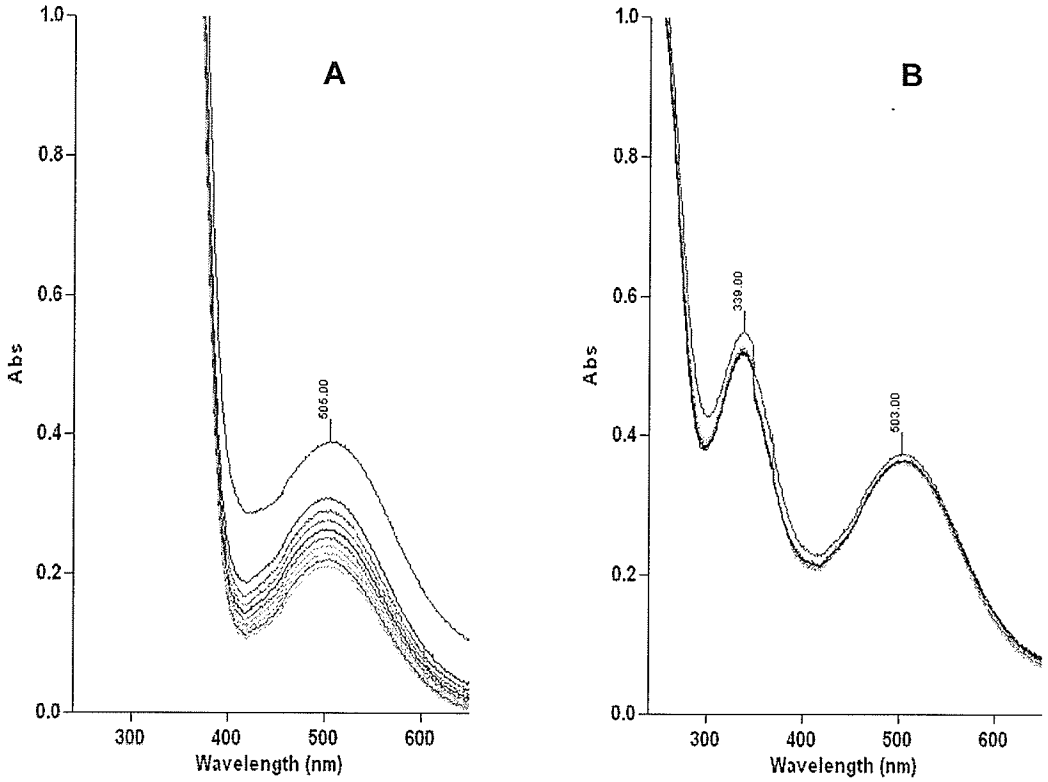


Figure 8. Spectrophotometric scan of m-PBM in the presence (A) and absence (B) of P450 reductase. For m-PBM the quinone absorbance maximum was established to be 503mn (B). This study measured the decrease of the quinone absorbance maximum for each of the BM analogs in the presence of P450 reductase. The reaction mixture consisted of purified P450reductase, 300uM NADPH and 100uM each of the BM analogs in potassium phosphate buffer pH7.8. The assay was carried out under hypoxic conditions using a hypoxic chamber flushed with anaerobic gas mixture (5% CO2, 10% H2, balanced with N2).

Table 2. Reduction of BM analogs by P450 reductase. $T_{1/2}$ refers to the time in minutes required to reduce the concentration of BM analog by one-half. (*NS*= not significant)

BM ANALOG	$T_{1/2}$	*P*-value
BM	>60.0	
CBM	56.7 ± 8.9	NS
MBM	3.83 ± 0.12	<0.001
MeBM	3.80± 0.29	<0.001
m-MeBM	3.67 ± 0.27	<0.001
PBM	3.93 ± 0.20	<0.001
m-PBM	3.18 ± 0.37	<0.001
m-TBM	4.20 ± 0.31	<0.001

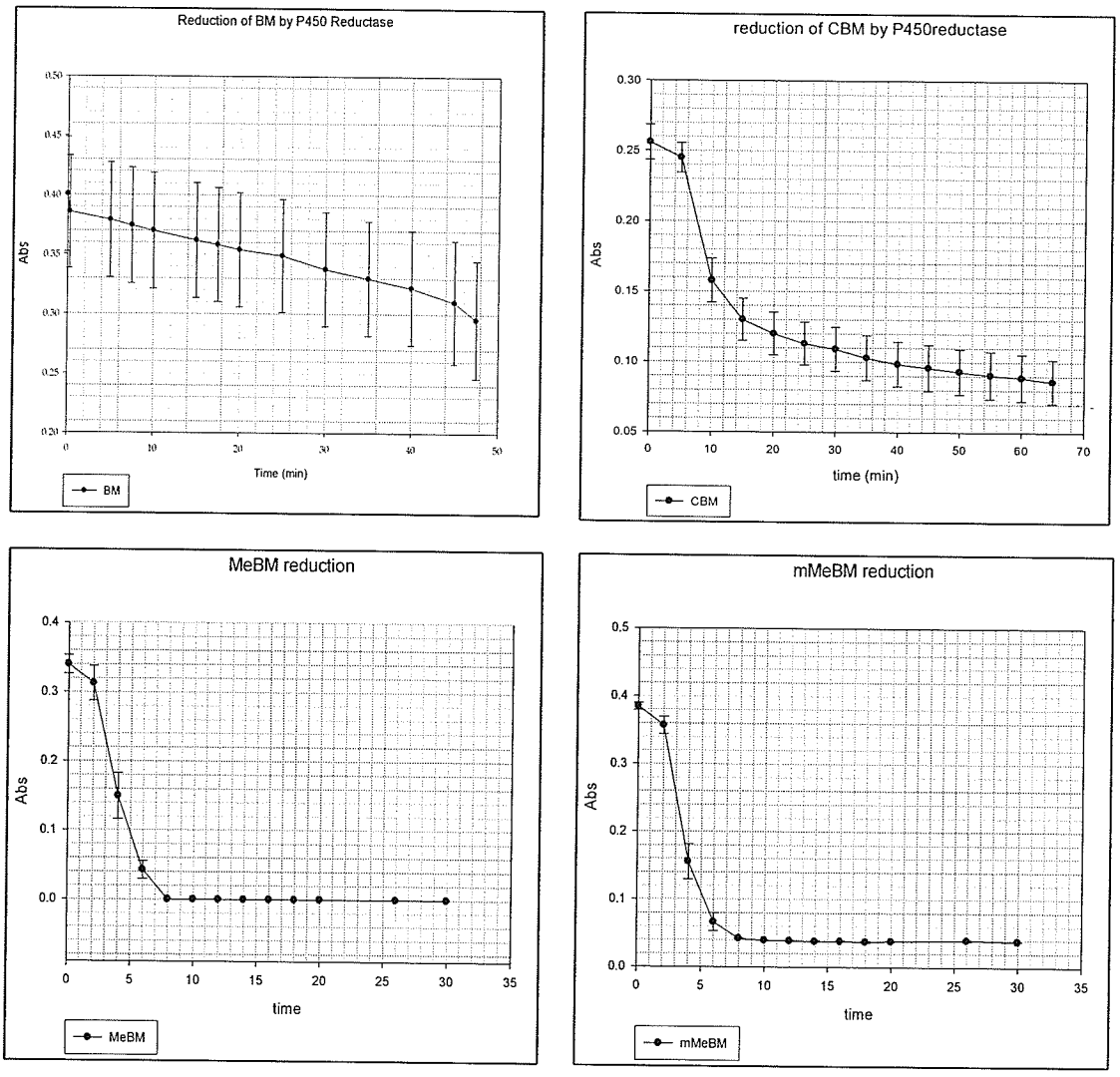


Figure 9.1. Time course plots monitoring the decrease in absorbance of the maximum peak over time for BM, CBM, MeBM, m-MeBM subsequent to addition of P450 reductase. Reduction experiments were conducted under anaerobic conditions, using screw-cap septum-sealed quartz cuvettes to maintain hypoxic conditions throughout the experiment. Each experiment included BM analog (100uM), P450 reductase (2.4ug/ml), in phosphate buffer. The controls included: complete reaction system excluding P450 reductase, and BM analog and phosphate buffer alone. The reaction was initiated by the addition of NADPH (300uM) using a Hamilton syringe inserted through the cuvette's septum. The rate reduction was determined by measuring the decrease of the quinone absorbance maxima (~500nm). Reductions were monitored using the scanning kinetics module in a Varian Cary 1 spectrophotometer. Each point represents the mean ±SE from three to 6 determinations.

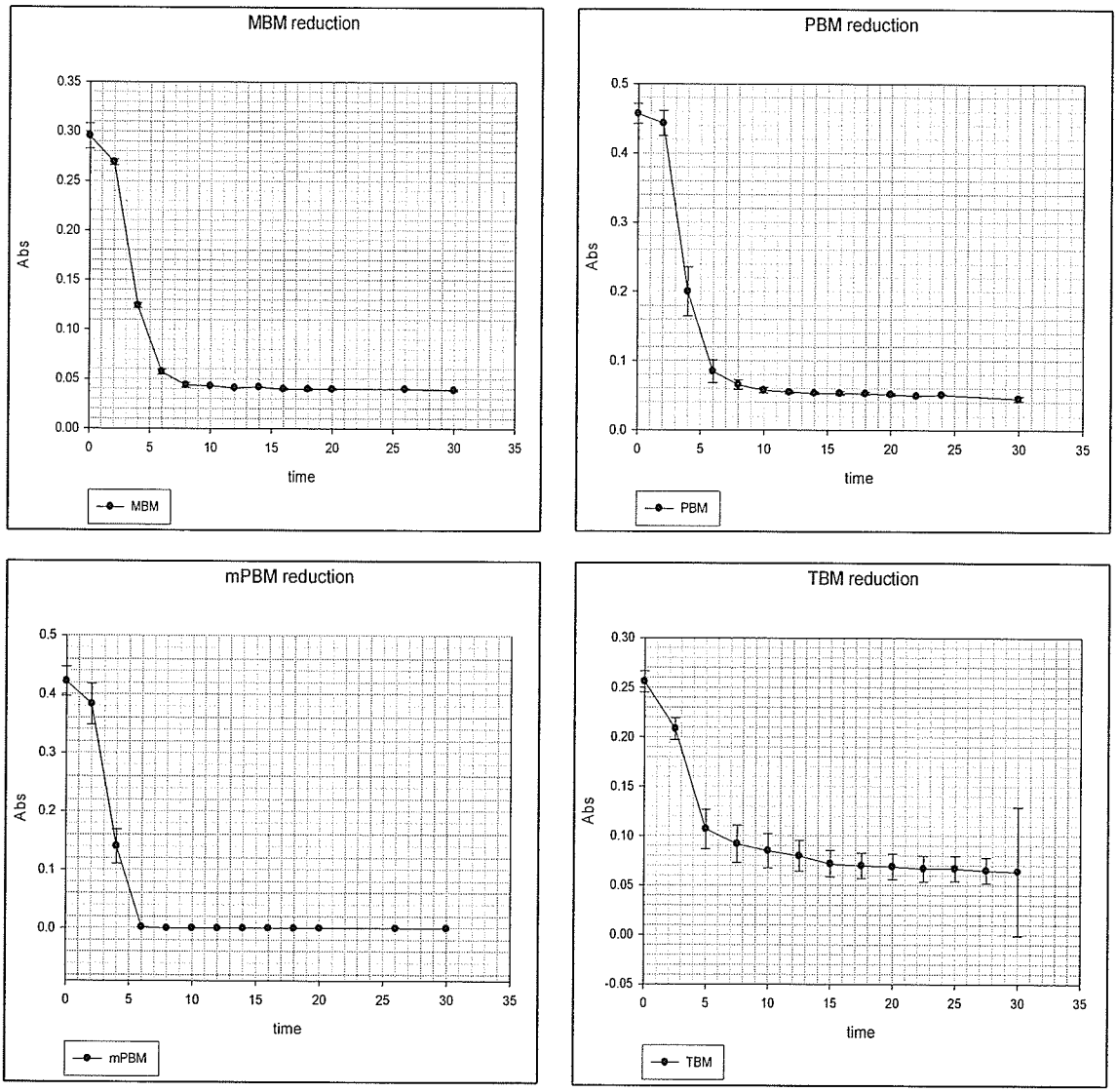


Figure 9.2. Time course plots monitoring the decrease in absorbance of the maximum peak over time for MBM, PBM, m-PBM, m-TBM subsequent to addition of P450 reductase. Reduction experiments were conducted under anaerobic conditions, using screw-cap septum-sealed quartz cuvettes to maintain hypoxic conditions throughout the experiment. Each experiment included BM analog (100uM), P450 reductase (2.4ug/ml), in phosphate buffer. The controls included: complete reaction system excluding P450 reductase, and BM analog and phosphate buffer alone. The reaction was initiated by the addition of NADPH (300uM) using a Hamilton syringe inserted through the cuvette's septum. The rate reduction was determined by measuring the decrease of the quinone absorbance maxima (~500nm). Reductions were monitored using the scanning kinetics module in a Varian Cary 1 spectrophotometer. Each point represents the mean ±SE from three to 6 determinations.

4.2 Studies on the effect of functional groups on cytotoxicity of BM analogs

The breast cancer cell lines MDA-MB-468 and MDA-MB-231 were chosen for this study because they display moderate and low levels of P450 reductase activity respectively. In addition, both of these cancer cell lines are known to be NQO1 deficient (Yu, Matias et al. 2001). This deficiency in NQO1 helps to isolate the effect of P450 reductase on the reduction of BM and its analogs and is significant because NQO1 is thought to be a good catalyst in the reduction and subsequent activation and toxicity of Benzoquinone mustards (Fourie, Oleschuk et al. 2002).

Enzyme activity studies on P450 reductase in the cancer cell lines MDA-MB 468 and MDA-MB 231 established enzymatic activities of 11.21 ng/min/mg of protein and 5.2 ng/min/ng of protein respectively (Table 3). Enzyme activity was determined using the NADPH-dependent reduction of cytochrome C described by Strobel et al. 1978 (Strobel and Dignam 1978). The dependence of the cytotoxicity of BM analogs on P450 reductase activity was established by obtaining complete dose-response curves for each of the analogs. Cytotoxicity of each of the BM analogs was compared to that of the parent compound BM by determining the difference between the slopes of the linear regression lines of the optical density against drug concentration curves as previously described (Doherty, Leith et al. 1998). The results are presented as D_{10} values for each drug used to treat each of the cell lines (Table 4).

The D_{10} value refers to the concentration of BM analog that will reduce the surviving cell fraction to 10% (0.1) of the control (i.e. cells incubated in the absence of BM analog). The D_{10} values for each BM analog were calculated from the inverse of the slope of the linear regression lines of the optical density against drug concentration curves.

The analogs m-MeBM and MeBM displayed the highest cytotoxic activity against MDA-MB-468, with D_{10} values of 0.34 ± 0.11 uM and 0.45± 0.1 uM respectively. Sterically bulky analogs m-PBM and PBM, with D_{10} values 2.0 ± 0.2 uM, and 2.2 ± 0.24 uM respectively, were more cytotoxic than BM with a D_{10} of 4.07 ± 0.20 uM. However, PBM and m-PBM were less cytotoxic than the *methyl*-substituted analogs. Substitution of *methoxy* on MBM produced D_{10} values of 7.49 ± 1.0, a lower cytotoxic activity than the parent BM. Finally, the analog substituted with a *t-butyl* group m-TBM displayed the lowest cytotoxic activity against MDA-MB-468 cells with D_{10} value of 70.8± 7.27 uM. Data analysis showed that *methyl*-substituted analog MeBM was most cytotoxic against MDA-MB-231 cells with a D_{10} value 1.14 ± 0.03 uM when compared to the parent BM with a D_{10} of 7.24 ± 0.77. The analogs CBM, m-MeBM, and MBM followed with D_{10} values of 3.47± 0.34 uM, 3.68 ± 0.39 uM, 3.76 ± 0.9 uM respectively. The *phenyl*-substituted analogs PBM, and m-PBM had cytotoxic activities similar to that of BM, with D_{10} values of 6.56 ± 0.59 uM, 7.24 ± 1.03 uM. Finally m-TBM had the highest D_{10} value of 157.0± 18.8 uM when compared to the parent BM and to the other analogs.

Cytotoxicity studies on MDA-MB-468 cells showed that substitution of benzoquinone mustard with weak electron donating groups *methyl, phenyl* and weak electron withdrawing group *chloro* increase the cytotoxic activity of these agents. Substitution of strong electron donating group *methoxy* and larger *t-butyl* group decreased the cytotoxic activity of the analogs. Cytotoxicity studies on MDA-MB231 cells showed that substitution of electron donating groups *methoxy, methyl* and *phenyl*, as well as the *chloro* electron withdrawing group increased the overall cytotoxic activity the benzoquinone mustard parent compound. On the other hand, substitution of sterically bulky groups *phenyl* and *t-butyl* at the C6 position and decreased the cytotoxic activity of these agents.

Table 3. Activity of P450 reductase on the cells lines MDA-MB-468 and MDA-MB-231 in the presence, and absence of 5uM of the P450 reductase inhibitor diphenyliodonium chloride (DPIC). The rate of reaction was calculated based on the extinction coefficient of 21mM-1cm-1 and expressed as nmol cytochrome c reduced per min per mg lysate protein.

	P450 reductase activity (ng/min/mg protein)	
Dose DPIC (uM)	**MDA-MB-468**	**MDA-MB-231**
0	11.21±0.32	5.19±0.63
1	6.27±0.23	2.63±0.10
5	3.10±0.07	3.01±0.09
10	3.13±0.42	1.65±0.09
100	2.7±0.31	1.41±0.05

Table 4. Cytotoxic activity of benzoquinone mustard analogs on MDA-MB-468 and MDA-MB-231. D_{10} refers to the dose of BM analog reducing the surviving cell fraction to 10%, calculated from the slope of the linear regression line of surviving cell fraction *vs.* dose curve. * Indicates $p<0.05$ compared to BM

BM ANALOG	MDA-MB-468 D_{10} (uM)	MDA-MB-231 D_{10} (uM)
BM	4.07 ± 0.20	7.24 ± 0.77
CBM	2.6 ± 0.18*	3.47± 0.34*
MBM	7.49 ± 1.0 *	3.76 ± 0.9 *
MeBM	0.45± 0.1 *	1.14 ± 0.03 *
m-MeBM	0.34 ± 0.11 *	3.68 ± 0.39 *
PBM	2.2 ± 0.24 *	6.56 ± 0.59 *
m-PBM	2.0 ± 0.2 *	7.24 ± 1.03
m-TBM	70.8± 7.27 *	157.0± 18.8 *

4.2.1 Studies of cytotoxicity of BM analogs mediated by P450 reductase

The cytotoxicity of BM analogs was studied in the presence and absence of 5uM of the P450 reductase inhibitor diphenyliodonium chloride (DPIC) prior to treatment with BM analogs. An inhibitor of P450 reductase was used in order to isolate role of this enzyme in the cytotoxic effects of BM analogs. Studies showed that 5uM of DPIC produceed the maximum inhibitory effect of P450 reductase with the least toxicity to the cells. Treatment of cells with 5uM of DPIC

for 2 hours decreased the overall intracellular activity of P450 reductase from 11.21±0.32 ng/min/mg protein in MDA-MB-468 and 5.19±0.63 ng/min/mg protein in MDA-MB-231, to ≈3.0 ng/min/mg protein in both cell lines (Figure 10).

Using the dose-response curves, we identified the dose of BM analog which will reduce the surviving cell fraction (SCF) by one-half of the original cell number. This concentration of drug is referred to as the IC_{50} (Table 5). We then used the IC_{50} values to determine the effect of the P450 reductase inhibitor DPIC on the cytotoxic effects of the BM analogs. The effect of DPIC on the cytotoxic activity of each of the BM analogs on MDA-MB-468 and MDA-MB-231 was compared using two-tailed t-tests. Here, the statistical significance of the difference between the surviving cell fraction means of the control cells and cells treated with DPIC were compared.

This study showed that incubation of the MDA-MB-468 with DPIC decreased the overall cytotoxic activity of the analogs MeBM, m-MeBM, CBM, m-PBM, and m-TBM ($P<0.05$), (Table 5). However, DPIC did not affect the cytotoxicity of MBM, PBM, or the parent compound BM (Figure 11). These results suggest that P450 reductase may not mediate the activation of these analogs in whole cell systems.

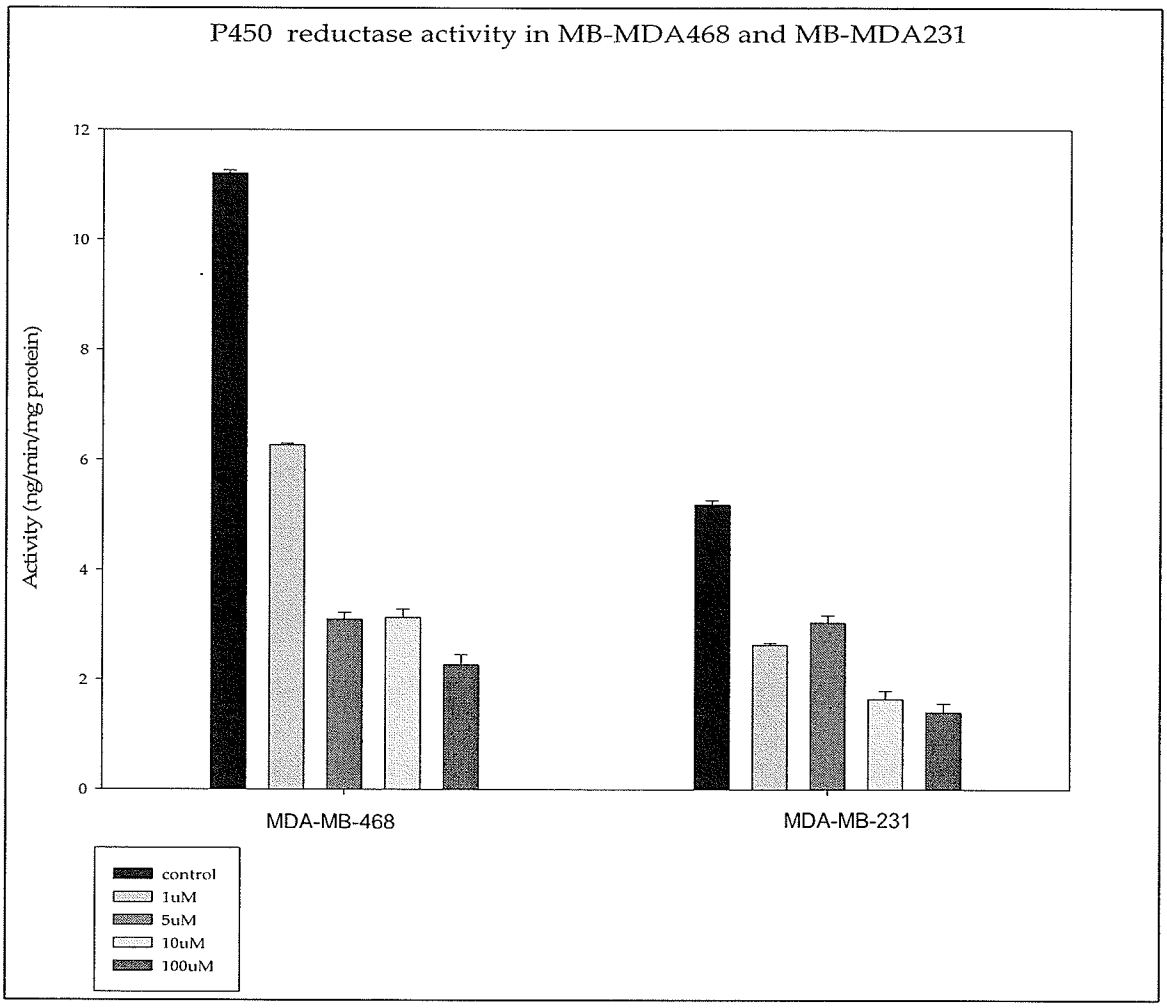


Figure 10. Inhibition of P450 reductase activity by DPIC in MDA-MB-468 and MDA-MB-231 cells. Cells were incubated in the absence and in the presence of 1uM, 5uM, 10uM, or 100uM of DPIC for 180 minutes. The observed P450 reductase enzyme activity was measured using the method by Strobel and Dignam (Strobel and Dignam 1978), and reported as nanograms cytochrome c reduced per minute per milligram of protein. The data is presented as means ± SE of 3-12 experiments.

Table 5. Cytotoxicity of BM analogs in MDA-MB-468 in the presence of 5uM of P450 reductase inhibitor DPIC. IC_{50} refers to the dose of BM analog reducing the surviving cell fraction (SCF) to 50%; calculated from the slope of the linear regression line of surviving cell fraction *vs.* dose curve. (*NS* = not significant).

BM ANALOG	IC_{50} (uM)	SCF	SCF (+DPIC)	*P*- value
BM	1.0	0.39 ± 0.08	0.41 ± 0.08	NS
CBM	1.5	0.61 ± 0.007	0.80 ± 0.042	<0.05
MBM	0.75	0.35 ± 0.039	0.41 ± 0.017	NS
MeBM	0.1	0.441± 0.01	0.54 ± 0.015	<0.05
m-MeBM	0.075	0.36 ± 0.031	0.88 ± 0.013	<0.05
PBM	0.75	0.74 ± 0.035	0.83 ± 0.036	NS
m-PBM	0.75	0.32 ± 0.022	0.95 ± 0.014	<0.05
m-TBM	25.0	0.44± 0.022	0.54± 0.022	<0.05

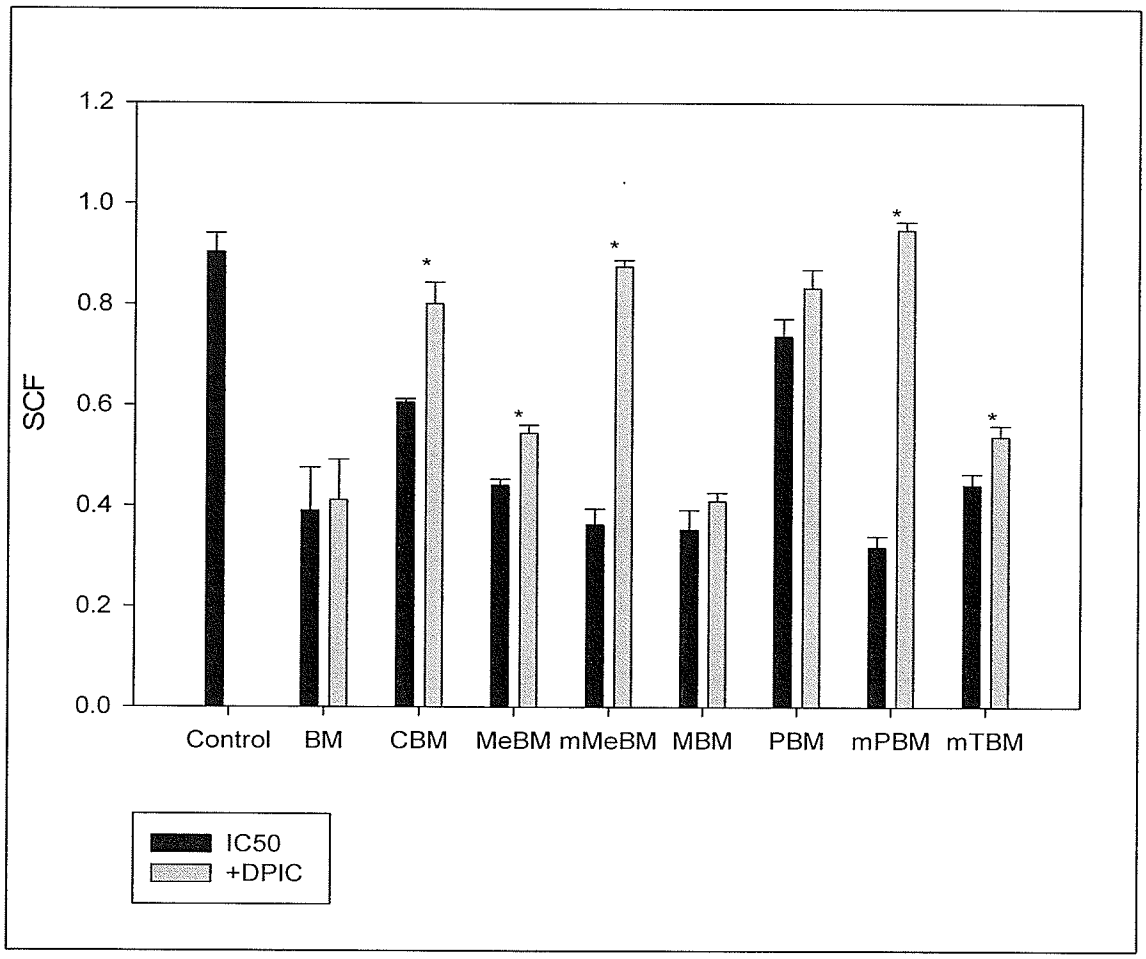


Figure 11. Effect of DPIC on the cytotoxic activity of BM analogs in MDA-MB-468 cells measured using MTT assay. The control cells were incubated with IC_{50} BM analog for 60 minutes, and the experiment cells were incubated with 5uM of DPIC for 60 min, followed by additional 60 minute incubation with IC_{50} BM analog+ 5uM DPIC. The results represent means ± SE of 4-16 determinations. The effect of DPIC on the cytotoxicity of BM analogs in MDA-MB-468 was compared by two-tailed *t*-tests comparing the significance of the differences between the mean surviving cell fraction (SCF) of control cells, cells treated in presence of DPIC and cells treated in the absence of DPIC. *Denotes $p<0.05$ compared to cytotoxicity of each analog in the absence of DPIC.

4.3 Determination of DNA strand-break formation in the presence of P450 reductase

Single strand break formation following the activation of BM analogs by P450 reductase was determined using the method described by Walton et al. (Walton, Smith et al. 1991). This method measures the change of supercoiled DNA in a plasmid to the open, relaxed circular conformation of the DNA. The assay used is a modification of the cell-free gel assay previously used by Fourie et al. (Fourie, Guziec et al. 2004). The strand break reactions were carried out in the presence of air to allow formation of the semiquinone radical via redox cycling. DNA in the gel was quantified by densitometry using the STORM fluorescence scanning system (Molecular Dynamics Inc. Sunnyvale, CA, USA) (Figure 12). The concentration of relaxed DNA was determined as a percent of the total DNA loaded in each well, for each of the BM analogs. The data is expressed as mean values ± SE of 3-7 experiments (Table 6). The results are expressed as E_{10} values, which represent the maximum concentration of cross-links formed after treatment of DNA with 10uM of BM analog. The E_{10} value was used because it fell within the linear portion of the percent DNA strand breaks vs. BM analog concentration curves. The E_{10} values for each of the BM analogs were compared by ANOVA to assess the statistical significance of the differences between the means of the BM analogs ($P = <0.001$). The Holm-Sidak method was used to compare the E_{10} of the analogs vs. the parent BM to identify which functional groups have a significant effect on the ability of the parent drug to induce DNA damage via single strand breaks ($P=<0.05$).

Following reduction by P450 reductase, all the BM analogs produced dose dependent single-strand breaks in supercoiled pBR22 plasmid DNA (Figure 13). E_{10} values for BM analogs showed that the parent compound BM produced the greatest percentage of DNA single strand breaks at 37.7 ± 8.11, followed by CBM with E_{10} of 19.0 ± 3.21. The E10 of CBM was not statistically different than BM. The E_{10} for the other BM analogs are: MBM > m-MeBM ≈ m-PBM > PBM ≈ MeBM, with E10 values of 23.14 ± 5.27, 9.50 ± 1.50, 6.67 ± 0.88, 3.33 ± 0.33, 3.17 ± 1.16 (mean ± SE), respectively. The analogs, MBM, MeBM, m-MeBM, PBM and m-PBM produced significantly fewer DNA single-strand breaks than the parent BM ($P<0.001$), (Table 6). None of the BM analogs caused DNA single-strand breaks without the presence of P450 reductase.

Overall, the parent benzoquinone mustard BM caused the greatest percent of DNA strand break damage in this cell-free system. Substitution of BM with electron donating groups namely *methyl, methoxy* and *phenyl* decreased the P450 reductase-mediated formation of strand breaks in DNA. Substitution of BM with the weak electron withdrawing group *chloro* did not have a significant effect on the formation of DNA strand breaks compared to the parent compound. These results suggest that DNA strand breaks formation resulting from the production of reactive oxygen species (ROS) and/or free radicals subsequent to reduction by P450 reductase is the preferred mechanism of DNA damage for the parent BM.

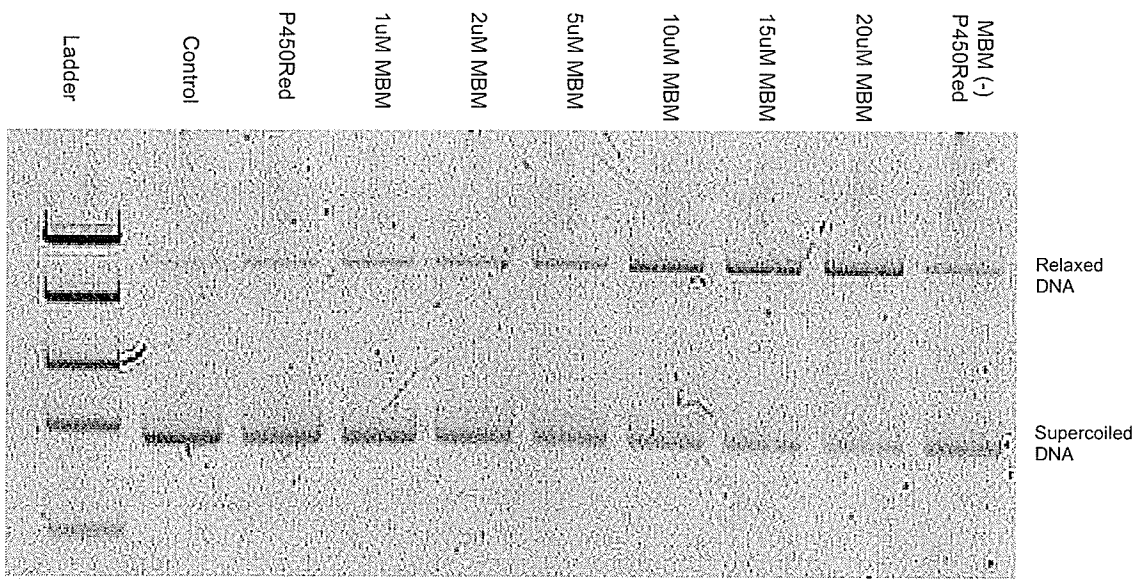


Figure 12. Scan of a 1% agarose gel illustrating dose-dependent single strand break formation in supercoiled DNA following treatment with MBM. The pBR322 plasmid was incubated with various concentrations of MBM for 45 min. at 37°C at pH 7.8 under aerobic conditions. The control reactions consisted of: 1) P450 reductase and NADPH in the absence of drug, 2) NADPH and drug in the absence of P450 reductase, and 3) DNA alone. The molecular weight ladder (Ladder) used was *HindIII* digested λDNA.

Table 6. DNA single strand breaks after treatment with BM analogs following reduction by P450 reductase in a gel assay. DNA strand breaks formation was quantified by densitometry using the STORM fluorescence scanning system. E_{10} refers to the percent of DNA strand breaks caused by 10uM of drug. The data represents means ±SE of 3-7 experiments. The E_{10} of the BM analogs were compared to that of BM by one-way ANOVA, followed by Holm-Sidak tests. (NS= not significant).

BM ANALOG	BM analog concentration (uM)	E_{10}	P value
BM	10	37.7 ± 8.11	
CBM	10	19.0 ± 3.21	NS
MBM	10	23.14 ± 5.27*	P<0.05
MeBM	10	3.17 ± 1.16 *	P<0.05
m-MeBM	10	9.50 ± 1.50*	P<0.05
PBM	10	3.33 ± 0.33 *	P<0.05
m-PBM	10	6.67 ± 0.88*	P<0.05

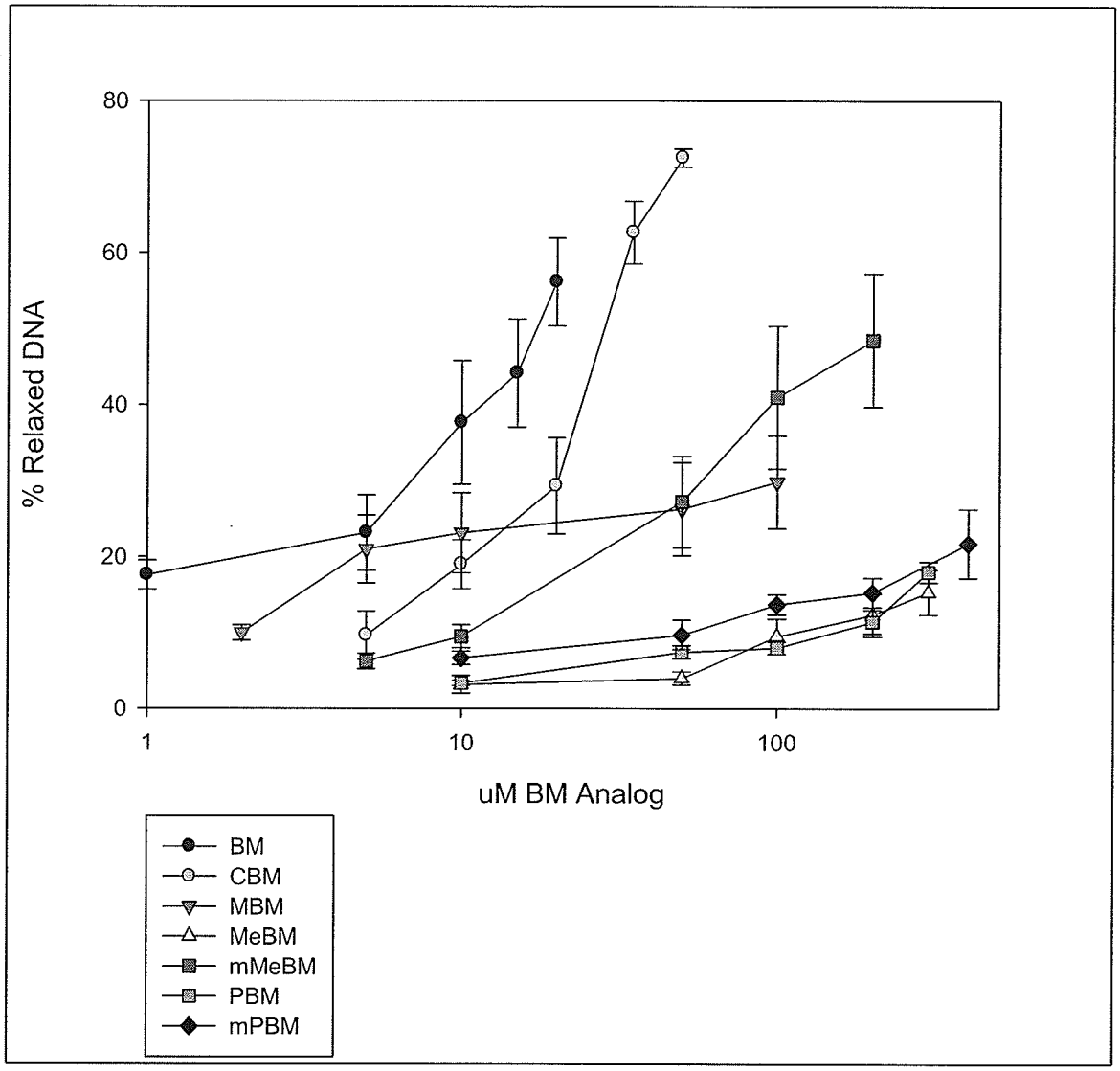


Figure 13. Concentration-dependent curves of DNA single-strand break formation in pBR322 by BM analogs following activation by P450 reductase. Plasmid pBR322 was incubated for 45 minutes with increasing concentrations of each BM analog and purified P450 reductase in potassium phosphate buffer (0.05M, pH 7.8) under aerobic conditions. The DNA was analyzed using a 1% agarose gel. The concentration of relaxed DNA was determined as a percent of the total DNA loaded in each well, for each of the BM analogs using densiometric analysis. The data is expressed as mean values ± SE of 3-7 experiments.

4.4 Determination of DNA interstrand cross-links formation in the presence of P450 reductase

The interstrand cross-linking activity of BM and its analogs following activation was measured using the method developed by (Hartley, Berardini et al. 1991). The following assay is a modified version of the assay described previously by (Fourie, Guziec et al. 2004). The DNA interstrand cross-linking experiments were carried out under anaerobic conditions, where potassium phosphate buffer (0.05M, pH 7.8) was purged with Nitrogen gas throughout the experiment to maintain anaerobic conditions. Linearized plasmid pBR322 DNA was radio-labelled with [α-^{32}P] dATP. Each reaction mixture consisted of: BM analog dissolved in DMSO (5-100uM drug), P450 reductase, end-labelled DNA, and NADPH to start the reaction. Controls included: 1) buffer, NADPH, radio-labelled DNA, and P450 reductase in the absence of BM analog. 2) Buffer, NADPH, radio-labelled DNA, and BM analog, in the absence of P450 reductase. The reaction was terminated by placing the reaction tubes in iced-water, and addition of DPIC. The DNA strands were separated by adding strand-separation buffer followed by incubation of the DNA at 70°C, and immediately cooling in an iced-water bath for 10min; and subsequent electrophoresis in TAE buffer (pH 7.0). The single-stranded control was generated by placing 70ng of radio-labelled pBR322 plasmid DNA in strand-separation buffer, followed by incubation at 70°C for 5min, and cooling in iced-water for 10min. After the gel was dried at and exposed to a phospho-imaging screen the double-stranded and single-stranded DNA was visualized and quantified using the STORM image-scanning

system (Molecular Dynamics Inc., Sunnyvale, CA, USA) by means of densitometry. The concentration of double stranded DNA was determined as a percent of the total concentration of DNA loaded per well. The data is presented as mean values ± SEM of 3-7 experiments. The results are expressed as E_{25} values referring to the concentration of interstrand cross-links formed after treatment of DNA with 25uM of BM analog. The E_{25} value was used because it fell within the linear portion of the DNA interstrand cross-links vs. BM analog concentration curves (Figure 14). The E_{25} values for each of the BM analogs were compared by ANOVA to assess the statistical significance of the differences between the means of the BM analogs (P= <0.001). The Holm-Sidak method was used to compare the E_{25} of the analogs vs. the parent BM to identify which functional groups have a significant effect on the ability of the parent drug to induce DNA damage via interstrand cross-links (P=<0.05).

Following reduction by P450 reductase, all BM analogs produced dose dependent DNA interstrand cross-links in linearized plasmid pBR322 DNA radio-labelled with [α-^{32}P] dATP under anaerobic conditions. The resulting autoradiograms of the1.6% agarose gel were quantified by densitometry using the STORM fluorescence scanning system (Figure 15). E_{25} for the BM analogs displayed the rank order: MeBM > MBM ≈ BM ≈ PBM ≈ m-MeBM ≈ CMB > m-PBM, with E_{25} values of 54.5 ± 7.8, 53.5 ± 7.9, 31.25 ± 7.6, 25.5 ± 5.69, 24.66 ± 2.6, 15.33 ± 0.88, 4.5 ± 1.04 (mean ± SE), respectively (Table 7). These E_{25} values indicate that MeBM produced the greatest percent of DNA interstrand cross-links 54.5 ±7.8. Namely, the effect of the electron donating functional group

in MeBM on DNA interstrand cross-links is statistically greater than the parent BM with E_{25} of 31.25 ± 7.6. On the other hand, m-PBM caused the least concentration of DNA interstrand cross-links with E_{25} of 4.5± 1.04, which is statistically lower than the parent BM.

Overall, cross linking studies showed that electron donating group *methyl* on MeBM significantly increased the P450 reductase-mediated formation of cross links in DNA. Substitution of BM with the bulky group *phenyl* at the C6 significantly decreased the formation of DNA strand breaks compared to the parent compound BM. These results indicate that DNA cross link formation subsequent to reduction by P450 reductase is the preferred mechanism of DNA damage by the MeBM compared to DNA strand breaks.

Table 7. DNA interstrand cross-links caused by BM analogs following reduction by P450 reductase. DNA cross link formation was quantified by densitometry using the STORM fluorescence scanning system. E_{25} represent the percent of DNA interstrand cross-links caused by 25uM of drug. The data represents means ±SE of 3-4 experiments. The E_{25} of the BM analogs were compared to that of BM by one-way ANOVA, followed by Holm-Sidak tests. (NS = not significant).

BM ANALOG	BM analog concentration (uM)	E_{25}	P value
BM	25	31.3 ± 7.61	
CBM	25	15.3 ± 0.88	NS
MBM	25	53.5 ± 7.96	NS
MeBM	25	54.5 ± 7.86 *	P<0.05
m-MeBM	25	24.6 ± 2.60	NS
PBM	25	25.5 ± 5.69	NS
m-PBM	25	4.50 ± 1.04*	P<0.05

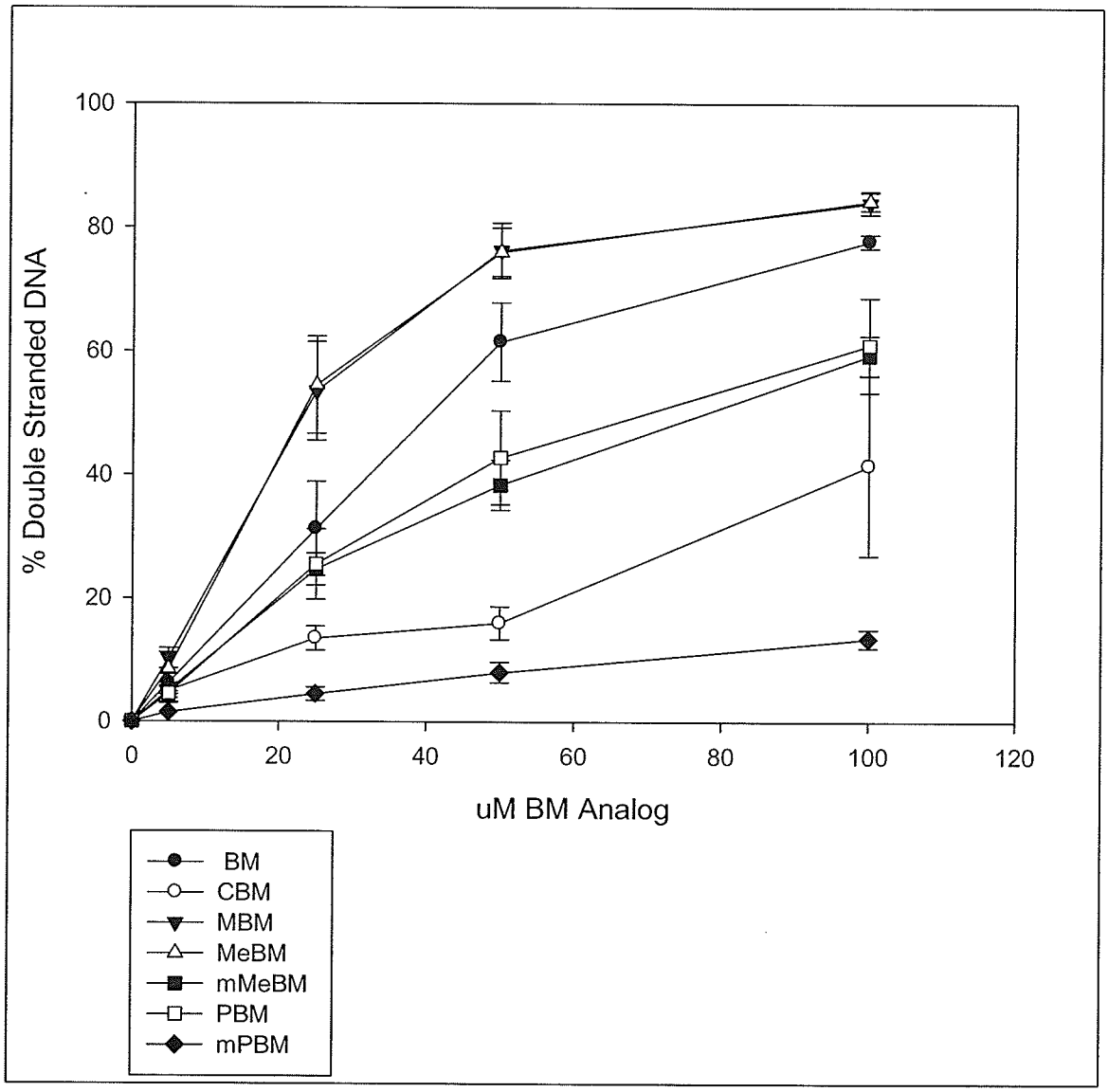


Figure 14. Concentration-dependent curves of interstrand cross-link formation in linearized DNA by BM analogs following activation by P450 reductase under anaerobic conditions. Linearized plasmid pBR322 DNA was radio-labelled with [α-^{32}P] dATP. Each reaction mixture consisted of: BM analog dissolved in DMSO (5-100uM drug), P450 reductase, end-labelled DNA, and NADPH to start the reaction. The double-stranded and single-stranded DNA found in the phosphor-imaging screen was visualized and quantified using the STORM image-scanning system (Molecular Dynamics Inc., Sunnyvale, CA, USA). The concentration of double stranded DNA was determined as a percent of the total concentration of DNA loaded per well. The data is presented as mean values ± SEM of 3-7 experiments.

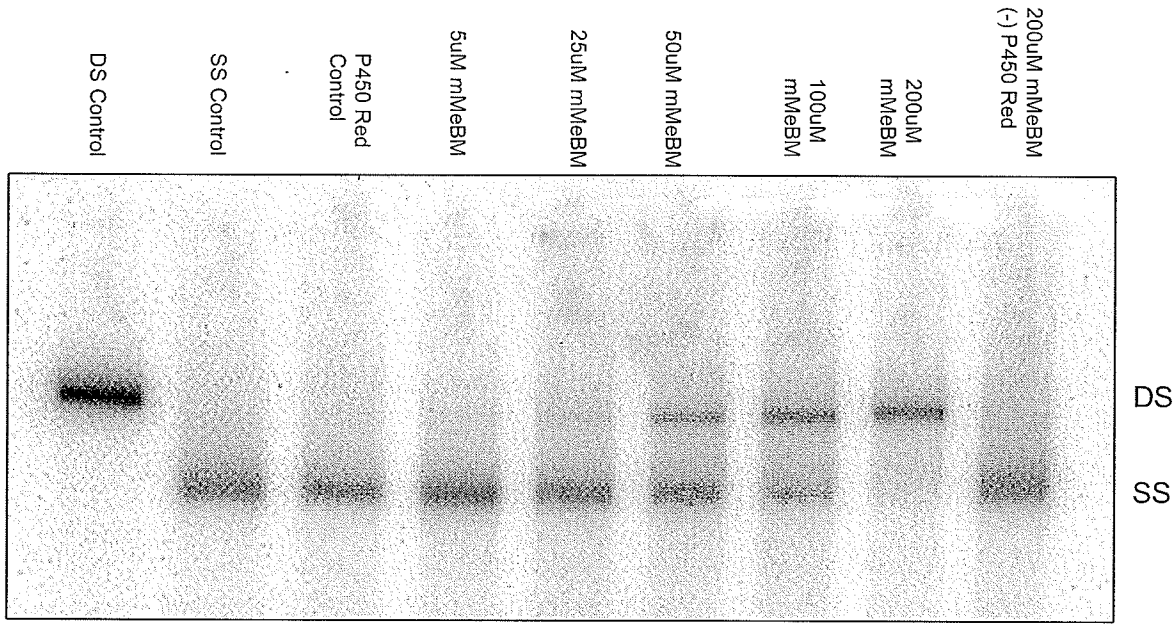


Figure 15. Autoradiogram 1.6% agarose gel containing linear and cross-linked DNA following treatment with various concentrations of m-MeBM. Linearized end-labelled DNA was incubated with various concentrations of m-MeBM, P450 reductase, and NADPH to start the reaction. Controls included: 1) buffer, NADPH, radio-labelled DNA, and P450 reductase in the absence of BM analog. 2) Buffer, NADPH, radio-labelled DNA, and BM analog, in the absence of P450 reductase. The single-stranded control was generated by placing 70ng of radio-labelled pBR322 plasmid DNA in an equal volume of strand-separation buffer, followed by incubation at 70˚C for 5min, and cooling in iced-water for 10min. The double-stranded control was prepared by placing 70ng of radio-labelled pBR322 plasmid DNA in strand-separation buffer and directly loading it onto the agarose gel. **DS** = double stranded DNA, **SS** = single stranded DNA.

4.5 SUMMARY OF RESULTS

- Reduction studies provided some evidence that P450 reductase may mediates the reduction of MBM, MeBM, m-MeBM, PBM, m-PBM and m-TBM to their hydroquinone forms, while reduction of BM and CBM may not fully depend on reduction by P450 reductase.

- Cytotoxicity studies indicated that :
    - The *methyl* group substituted at either C5 in MeBM or C6 in m-MeBM significantly increased the cytotoxic activity of the bioreductive agents in both cell lines.
    - The *t-butyl* group substituted at C6 in *m*-TBM had the least cytotoxic effect of all the analogs.

- BM conferred the highest percent of DNA strand breaks:
    - MeBM, m-MeBM, PBM and m-PBM produced significantly less DNA single strand-breaks compared to the parent BM. CBM and MBM did not affect DNA strand break formation.

- P450 reductase-mediated DNA cross-link formation is significantly increased by substitution of the weak electron donating *methyl* group on MeBM.

5 DISCUSSION

The efficacy of anticancer drugs is highly dependent on the tumour's microenvironment. The physical characteristics of the tumour fraction dictate the efficiency of drug-delivery methods, the mechanisms of cytotoxicity and ultimately the specificity of the drugs towards tumour cells. One crucial challenge for chemotherapy agents is poor perfusion in hypoxic fractions of solid tumours given that it limits drug delivery and the mechanisms through which agents can confer cytotoxic effects due to alterations in the cell cycle. In response to these challenges the development of anticancer drugs now involves targeting of hypoxic cells and exploiting the tumour's inherent characteristics, such as the expression and activity patterns of accessory enzymes that can modulate the activity of antitumour agents. This approach to anticancer drug development is referred to as enzyme-directed tumour targeting (Workman 1990; Workman and Stratford 1993; Workman 1994).

Bioreductive antitumour agents are an important class of anticancer drugs due to their suitability for enzyme directed tumour targeting. This class of anticancer drugs have a common requirement for activation by reductive enzymes such as NADPH: cytochrome P450 reductase (P450 reductase) or NQO1 (Pan, Andrews et al. 1984; Rockwell, Sartorelli et al. 1993). Mitomycin C, is a bioreductive quinone-containing alkylating agent widely used clinically. Other quinone-containing alkylating agents including, porfiromycin, diaziquone, carbazilquinone, triaziquone and EO9, have also been used for the treatment of cancer (Begleiter 2000). The general structure of these compounds includes a

bioreductive element, such as a quinone group, and a cytotoxic element, such as nitrogen mustard alkylating group that is activated by the reduction of the bioreductive element. Reduction of the quinone moiety can occur through two main pathways (Figure 6). This study focuses on the pathway mediated by P450 reductase. P450 reductase mediates a series of one-electron transfers that lead to the sequential formation of a semiquinone reactive intermediate, followed by the formation of a hydroquinone. Formation of the semiquinone species or the hydroquinone product can lead to activation of the cytotoxic element of the compound. In the presence of oxygen, the hydroquinone and the semiquinone intermediate can undergo redox cycling to produce reactive oxygen species that can cause damage to the cellular components (Goeptar, te Koppele et al. 1992).

Benzoquinone mustards are newly developed model bioreductive alkylating agents. A series of derivatives of the parent compound 2-(Di(chloroethy)amino)-1,4-benzoquinone (BM) were used to study the effect of various functional groups on the specificity for reduction of these quinone containing agents by the reductive enzyme P450 reductase (Table 1). The derivatives used in this study included: CBM which was substituted with the electron withdrawing *chloro* group; MeBM, and MBM were substituted at C5 with electron donating *methyl* and *methoxy* groups respectively; PBM, and TBM where substituted with sterically bulky groups *phenyl* and *t-butyl* at either C5 or C6 respectively; lastly positional isomers m-MeBM and m-PBM where substituted at C6.

Cytotoxic activity following treatment with BM analogs is likely influenced by:

1) Affinity of P450 reductase towards the quinone bioreductive element

2) The reactive drug species produced, namely semiquinones, hydroquinones and reactive oxygen species

3) The resulting DNA damage that occurs

4) The cell's response to that damage.

The present study addresses points 1-3, and provides evidence for the participation of P450 reductase in the reduction, and in some cases the activation of benzoquinone mustards.

5.1 Studies on the effect of functional group on reduction mediated by P450 reductase

The redox potential can predict the stability of semiquinone and hydroquinone states of BM analogs. Redox potentials are related to the difference in free energy between the quinone and semiquinone, and the semiquinone and hydroquinone, as described by the Nerst equation (Driebergen, Holthuis et al. 1986). Greater negative redox potential of the semiquinone and hydroquinone forms results in less stable semiquinone and hydroquinone, compared to the quinone state.

Previous unpublished studies by Oleschuck and Begleiter determined the first and second redox potentials for the BM analogs to be: BM -0.165 and -0.600, MBM -0.120 and -0.240, PBM -0.200 and -0.680, CBM -0.200 and -0.600, and TBM -0.200 and -0.650 respectively. These redox potentials suggest that the

hydroquinone is more stable than the semiquinone and less likely to redox cycle once it has been fully reduced. On the other hand the semiquinone radical is more likely to react and redox cycle in the presence of oxygen to produce reactive oxygen species. Given that both reduction products are generated by P450 reductase through single electron-transfers, and both products cause damage to cellular components through diverse mechanisms, detecting the formation of each product can define the effects of functional groups on reduction and predict the prevailing mechanisms of cytotoxicity for each analog.

Electron paramagnetic resonance (EPR) analysis of BM analogs was conducted using the method developed by Bailey et al. 2001 for EO9 as described in the methods section. Attempts to use this method to detect radical species formed from the reduction of BM analogs by P450 reductase did not provide quantitative evidence of the reduction of BM analogs by P450 reductase. The parent compound BM was the only agent to produce a significant free radical signal. No EPR signal was detected for the analogs of BM. The formation of free radical species from the parent BM can be attributed to its redox potentials. BM has an intermediate first redox potential which favours the first electron transfer (-0.165 Volts). The second redox potential of BM is the highest of the analogs, implying that the formation of the hydroquinone product would take a lot more energy than the semiquinone product. Results also suggest that BM may have the greatest effect on DNA strand-breaks due to the production of semiquinone radicals.

Several attempts were made to optimize this assay for the other BM analogs. The indolequinone EO9 was used as a positive control to determine if the reaction system was functioning. The rate of formation of semiquinone from BM analogs was hoped to help determine how functional groups substituted on the various BM analogs affected the rate of which P450 reductase transferred electrons to the quinones to produce semiquinone radicals. Due to the cost of reagents and labour to operate the EPR spectrometer, we were unable to optimize the protocol for other BM analogs.

A large EPR signal would indicate:

1) Fast 1^{st} electron transfer to quinone, leading to formation of semiquinone.

2) Fast 2^{nd} electron oxidation from hydroquinone, leading to formation of semiquinone.

A small EPR signal would indicate:

1) Fast 2^{nd} electron transfer to semiquinone, leading to formation of hydroquinone.

2) Fast reoxidation of the hydroquinone back to the quinone form.

3) Lack of reduction of the BM analogs by P450 reductase.

The overall lack of free radical formation from BM analogs in the presence of P450 reductase suggest: 1) that the CBM, MBM, m-PBM may not be substrates for reduction by P450 reductase; or 2) reduction of the semiquinone

by P450 reductase is faster than the reduction the quinone, and cannot be measured using electron paramagnetic resonance.

Ultimately EPR did not provide quantitative evidence of the reduction of BM analogs by P450 reductase and an alternative method to detect reduction had to be optimized.

A spectrophotometric assay was used to determine the rate of reduction of BM analogs by P450 reductase, and to help identify the participation of P450 reductase in the reduction of these agents by following the decrease in absorbance of the coloured quinone which is typically detected at 600nm. The decrease in absorbance at the quinone peak is correlated to the formation of hydroquinone for which absorbance is not detected in the visible spectrum.

Structure activity studies of these analogs using NQO1 as the reducing enzyme also established that functional groups may increase specificity for activation by NQO1 (Fourie, Oleschuk et al. 2002). Unpublished work by Curtis Oleschuk found the parent compound BM displayed a lower overall rate of reduction by P450 reductase, compared to its BM analogs, which agrees with our findings where the slowest reduction rates were for BM and CBM with t½ close to 60 minutes (Oleschuk 1998). Statistical analysis of the t½ of the MBM, MeBM, m-MeBM, PBM, m-PBM and m-TBM indicates the reduction of these agents is significantly faster than that of BM and CBM. These findings argue that P450 reductase mediates the reduction of MBM, MeBM, m-MeBM, PBM, m-PBM and m-TBM to their hydroquinone forms, while activation of BM and CBM may not

fully depend on reduction by P450 reductase. Given the similarities in redox potentials of PBM to BM, PBM was expected to favour a semiquinone radical formation, except PBM favoured the formation of hydroquinone subsequent to reduction by P450 reductase, suggesting that the *phenyl* group acts to stabilize the hydroquinone.

In general the results for this study suggest that substitution of functional groups in to the parent benzoquinone mustard structure increase the overall rate of reduction of the quinone group compared to BM.

Enzyme activity studies have determined the levels of P450 reductase vary in different normal tissues and their tumour counterparts, but no significant differences have been demonstrated (Lopez de Cerain, Marin et al. 1999). Low P450 reductase activity suggests the participation of other potential mediators of bioreductive activation of BM analogs. Additional bioreductive enzymes that may be involved in the activation of these agents include: NADH cytochrome b5 reductase, xanthine oxidase, and cytochrome P450. These one-electron reductases are known to produce reactive oxygen species under both aerobic and hypoxic conditions. More information is required on the expression and interaction of the full range of bioreductive enzymes in cell lines and human tumour tissues to identify the major players involved in activation of benzoquinone mustards, or any drug dependent on enzymatic reduction.

Cytotoxicity studies using RB90740 on a panel of human cancer cell lines, showed no relationships between cytotoxicity and enzyme activity, and

suggested that relationships may be obscured, in part, by the interaction of P450 reductase with cytochrome b5 reductase in order to activate RB90740 (Barham and Stratford 1996). This finding implies the potential involvement of multiple enzymes on the activation of bioreductive drugs in cell-based assays. Cytochrome b5 reductase is reportedly able to reduce Mitomycin C in EMT6 tumour cell sonicates under hypoxia (Hodnick and Sartorelli 1993). Mitomycin C along with EO9 are also activated by xanthine oxidase via single electron transfers (Pan, Andrews et al. 1984; Maliepaard, Wolfs et al. 1995). In addition, the bioreductive agent RH1 is reportedly activated by other reductases including cytochrome b5 reductase, and xanthine oxidase (Ward, Danson et al. 2005). Enzyme profiling of cell and tissues, along with a measure of tumour hypoxia may provide a useful screen for predicting the activity of bioreductive agents *in vivo* (Rampling, Cruickshank et al. 1994) and should be considered in future studies.

5.2 Studies on the effect of functional groups on cytotoxicity of BM analogs

A comparison of the dose-response cytotoxicity studies were carried out using MDA-MB-468 and MDA-MB-231 cell lines. Enzyme activity studies on P450 reductase established differential P450 reductase activities of 11.21 ng/min/mg of protein in MDA-MB-468 and 5.2 ng/min/mg of protein in MDA-MB-231 (Table 3). These cell lines were chosen due to their varying levels of P450 reductase and their low of NQO1 activity. MDA-MB-468 cell line reported activity of NQO1 is less than 5nmol DCPIP/min/mg of protein (Dehn, Siegel et al. 2003).

MDA-MB-231 has negligible NQO1 activity reported as less than 1nmol DCPIC/min/mg of protein (Begleiterabc, Leith et al. 2001). Low levels of NQO1 are important to isolate the effects of functional groups of BM analogs on reduction mediated by P450 reductase. Ultimately the cytotoxic activity of BM analogs in these cells lines should be proportional to the activity of P450 reductase assuming that P450 reductase is the main catalyst in the reduction of these compounds.

Lower overall D_{10} values observed for the cytotoxicity of BM analogs in MDA-MB-468 cells, compared to MDA-MB-231 cells indicate that less drug is required to produce a toxic effect in the cell line with higher concentration of P450 reductase; and suggests the potential activation of the BM analogs by P450 reductase. In addition, this group of studies suggests that functional groups affect the cytotoxic activity of these compounds. The *methyl* electron donating group substituted at either C5 in MeBM or C6 in m-MeBM significantly increased the cytotoxic activity of the bioreductive agents in both cell lines. The analog m-MeBM showed the most toxicity towards MDA-MB-468 while MeBM displayed the most toxicity towards MDA-MB-231 cells with D_{10} of 1.14±0.03 uM. On the other hand m-TBM had the least cytotoxic effect of all the analogs.

In MDA-MB-468 cells, the analogs CBM, MeBM, m-MeBM, PBM and m-PBM, showed significantly higher cytotoxic activity compared to the parent BM, suggesting that reduction mediated by P450 reductase activates these agents. The analogs MBM and m-TBM showed lower cytotoxic activities compared to BM, suggesting that *methoxy* and *t-butyl* functional groups decrease the cytotoxic

activity of these agents following activation by P450 reductase. Given that reduction studies show that both MBM and m-TBM are reduced by P450 reductase, we can deduce that reduction by P450 reductase may lead to inactivation of these compounds in MDA-MB-468 cells

In MDA-MB-231 cells, CBM, MBM, MeBM, m-MeBM, and PBM exhibited significantly higher cytotoxicity compared to the parent BM. These results indicate that reduction mediated by P450 reductase leads to the activation of these analogs. The analog m-PBM did not have significantly different cytotoxic effects than BM, and m-TBM lowered cytotoxic activity significantly compared to the parent analog BM, indicating that large groups substituted at the C6 position inhibit the overall activity of these agents.

Overall, electron donating groups seemed to increase the cytotoxicity of BM analogs, while large sterically bulky functional groups decreased the cytotoxicity of BM analogs in whole cell systems.

5.2.1 Studies on the cytotoxicity of BM analogs on MDA-MB-468 cells mediated by P450 reductase.

We used the P450 reductase inhibitor DPIC in order to isolate role of this enzyme in the cytotoxic activity of BM analogs. Incubation of the MDA-MB-468 with DPIC decreased the overall cytotoxic activity of the analogs MeBM, m-MeBM, CBM, m-PBM, and m-TBM (Table 5). The increase in surviving cell fraction (SCF) suggests that P450 reductase may mediate the activation m-PBM, m-MeBM, CBM, and MeBM. Using the same level of significance, DPIC did not

seem to have an effect on the cytotoxicity of MBM, PBM, or the parent compound BM (Figure 11).

The behaviour of MBM in this study is consistent with the conclusions by Oleschuck et al. and Fourie et al, both suggested that MBM became inactivated upon reduction by NQO1 (Fourie, Oleschuk et al. 2002). Our reduction studies indicate that MBM is a substrate for reduction by P450 reductase with at t½ of 3.83 ± 0.12 min. However, reduction by P450 reductase did not lead to a significant increase in cytotoxicity compared to the parent BM. In addition treatment with DPIC did not decrease the cytotoxicity of MBM towards cells, indicating potential independence of MBM from the activity of P450 reductase.

Cytotoxicity studies provided evidence of the efficacy of BM analogs to cause cell death in tumour cells. However, these results may be affected by the activity of NQO1 in MDA-MB-468. After reviewing the literature, it is evident that these cells possess low activity of NQO1 (Barham and Stratford 1996; Dehn, Siegel et al. 2003), which may interfere with the interpretation of the results. In fact, the patterns of cytotoxic activity for BM analogs on MDA-MB-468 cells resemble those found on SK-MEL-28 cells, where analogs substituted with electron donating groups displayed greater cytotoxic activity and analogs substituted with sterically bulky groups had lower cytotoxic activity all compared to the parent BM (Fourie, Oleschuk et al. 2002). Due to the overall low enzymatic activity of P450 reductase in the cell lines chosen for these cytotoxicity studies, the results do not provide sufficient evidence to support the role of P450 reductase in the activation of BM analogs.

The negligible effect of DPIC on the activation of BM, MBM, and PBM suggests that P450 reductase may not mediate the activation of these analogs in whole cell systems and other enzymes may play a more significant role in the cytotoxic activity of these agents.

P450 reductase is essential for the transfer of electrons to other P450 systems in particular to cytochrome c; no other physiological electron acceptor has been identified in vertebrates. Inhibition of P450 reductase would shut down the electron transport chain and cellular function. Due to the house keeping role P450 reductase performs in cellular metabolism, it is not possible to carry out nock down studies with P450 reductase. As an alternative, future studies should be carried out using cell lines transfected with P450 reductase. For instance, T47D human breast cancer cells (11.5 nmol/min/mg protein) have been transfected with the human P450 Red gene, resulting in P450 reductase activity of 311.8nmol/min/mg protein (Begleiter, Leith et al. 2007). This would help to amplify the effects of P450 reductase activity on the reduction and subsequent activation of bioreductive drugs.

5.3 Determination of DNA damage by BM analogs mediated by P450 reductase

BM analogs are predicted to confer their cytotoxic effects by means of two main mechanisms: DNA strand break formation in the presence of reactive oxygen species, and DNA cross-linking due to alkylation of DNA and other cellular molecules.

Analysis of DNA strand-break formation by BM analogs was carried out to assess how the different functional groups affect DNA damage subsequent to P450 reductase-mediated activation of BM analogs. To summarize results, none of the BM analogs caused DNA single strand breaks in the absence of P450 reductase. The parent analog BM had the percent of DNA strand breaks. The analogs CBM and MBM showed similar profiles of DNA strand break formation compared to BM (Table 6). The analogs MeBM, m-MeBM, PBM and m-PBM produced significantly less DNA single strand-breaks compared to the parent BM.

In general DNA single strand break formation is significantly decreased by the characteristics and position of electron donating methyl and phenyl functional groups substituted on BM. The *chloro* electron withdrawing group on CBM and the strong electron donating group *methoxy* on MBM do not seem to affect the production of DNA single strand breaks mediated by P450 reductase.

Observing that these experiments were carried out in the presence of oxygen, we can suggest two the mechanisms for formation of the radical species:

1) Free radical species can be formed after the first one-electron transfer from P450 reductase to the quinone pro-drug to form a reactive semiquinone radical, which is further reduced to the hydroquinone by a second one-electron transfer. A similar mechanism of bioreductive activation was proposed for Tirapazamine (TZP). This agent can be reduced by one-electron transfer to a highly-reactive free radical that is able to react with DNA to remove hydrogen, leading to strand breaks. (Sartorelli, Hodnick et al. 1994; Rauth, Melo et al. 1998).

2) The fully reduced hydroquinones can redox cycle with molecular oxygen to produce reactive oxygen species. Mitomycin C for example, when reduced in the presence of oxygen, can generate damaging superoxide radicals, peroxide or hydroxyl radicals (Bachur, Gordon et al. 1979; Komiyama, Kikuchi et al. 1982; Riley and Workman 1992).

Looking back to our reduction studies, the formation of DNA single strand breaks by BM is expected due to the detection of free radical species in our EPR analysis. Production of DNA strand breaks by MBM can be partially explained by a lower second redox potential for MBM (-0.240 Volts) which would favour formation of the hydroquinone. The fully reduced hydroquinone can react with molecular oxygen to produce reactive oxygen species (ROS) which can cause DNA strand breaks. EPR analysis could not have detected ROS production by redox cycling of MBM hydroquinone because no spin trap was used in the study. For CBM, the slow reduction observed in the absorbance reduction experiments indicate that the fully reduced product is difficult to form due to greater redox potential (-0.600 Volts) as previously determined by the work of Curtis Oleschuck. It is important to note that CBM has a similar second redox potential to that of BM (-0.670 Volts) which may help explain the similarity in the rate of formation for DNA single strand breaks.

Not all the cytotoxicity conferred by BM analogs can be associated to DNA single strand-break formation. Alkylating agents such as nitrogen mustards are potent electrophiles and react with many electron-rich molecules within the cell to

become inactivated. Reduction of BM analogs by P450 reductase may lead to stabilization of the hydroquinone which is known to cause DNA cross-linking (Begleiter and Blair 1984; Begleiter 1986; Tomasz, Lipman et al. 1987; Fourie, Guziec et al. 2004). Stabilization of the hydroquinone moiety may cause the activation of the nitrogen mustard group and lead to the formation of an aziridinium ion, which can react with the N(7)-position of guanines found in the same or opposing DNA strands leading to cross-linking of the DNA strand(s) (Ojwang, Grueneberg et al. 1989).

Following reduction by P450 reductase, all the BM analogs produced dose-dependent DNA cross-links in linearized pBR22 plasmid DNA (Figure 14).

P450 reductase-mediated DNA cross-link formation is significantly influenced by the characteristics and position of functional groups substituted on the benzoquinone molecule of BM, as outlined by the E_{25} values (Table 7). Substitution of the weak electron donating functional group *methyl* on MeBM significantly increased the formation of DNA cross-links subsequent to reduction by P450 reductase, compared to the parent compound BM. On the other hand, substitution of the large *phenyl* group at the C6 position on m-PBM significantly decreased the formation of DNA strand breaks. A less significant trend was observed with BM analogs substituted at the C6 position, that is m-MeBM and m-PBM decreased the overall formation of DNA cross-links compared to their C5-substituted isomers MeBM and PBM (Table 7).

Overall, substitution of BM with electron donating group *methyl* seemed to increase the overall formation of DNA cross-links subsequent to reduction by P450 reductase.

Cytotoxicity of BM analogs is mainly attributed to the formation of both oxygen-derived species and drug-derived species. Cellular damage conferred by these species can be mainly attributed to DNA, RNA and protein synthesis inhibition is associated with the formation of DNA strand breaks and alkylation. However, other types of cellular damage may play more significant roles in the cytotoxic activity of benzoquinone mustard and its analogs. The nitrogen mustard Chlorambucil has been known to cause a decrease in the thymidine incorporation into genomic DNA following incubation of 0-10uM of Chlorambucil for 18 hours, and sufficient to inhibit DNA synthesis by 85% (Wang, Dziegielewski et al. 2003). Disruption of cell cycle, are also associated with alkylating agents, which cause arrest in the G2/M checkpoint of the cell cycle. In other studies, dose-response Chlorambucil treatment of 293 cells showed an increase in the peak in DNA content in flow cytometry studies suggesting an increase in the content of DNA in 293 cells associated with mitosis (Konopa 1988; O'Connell, Walworth et al. 2000). To protect cellular apparatus mammalian cells possess defence mechanisms such as superoxide dismutase, glutathione reductase, catalase, peroxidase, and reactive structural cellular components. The activity of each of these enzymes may affect the degree of DNA damage caused by bioreductive agents. Glutathione (GSH) is an electron rich tripeptide containing a free cysteine sulfhydryl, present in millimolar

concentrations in cells. GSH has been associated with the inactivation of clinically used alkylating agents. Further more, elevated cellular concentrations of GSH has been correlated with resistance to nitrogen mustards (Suzukake, Petro et al. 1982). These effects should be considered in future studies involving whole cells—such as cell viability testing, given the activity of these detoxifying enzymes will affect the cytotoxic response towards bioreductive drugs.

Given the findings from the preceding studies we propose the analog MeBM to be suitable for further development as a clinically utilizable chemotherapy agent.

CONCLUSION

The results from structure activity studies of Benzoquinone Mustards (BM), established that P450 reductase plays only a minor role in the activation of these compounds. Only the analog MeBM would be suitable for further develop as a clinically relevant antitumor agent. Although P450 reductase seemed to mediate the reduction some of the BM analogs, cytotoxicity and inhibition studies suggested that P450 reductase does not contribute to the activation of BM analogs in breast cancer cells with normal P450 reductase activity, suggesting that other enzymes may play a more significant role in the cytotoxic activity of these agents. The present study also confirmed that BM analogs cause DNA damage through production strand breaks and cross links, supporting results from previous studies by Fourie et al., information which can be utilized to predict the behaviour of new pro-drugs with similar structures.

6 FUTURE DIRECTIONS

Based on the work accomplished, future directions for the development of effective bioreductive cancer chemotherapy should also include:

1. Further examination of analog MeBM. Cytotoxicity assays showed that MeBM and m-MeBM were most effective in decreasing the SCF of MDA-MB-468 and MDA-MB-231 breast cancer cell lines. Inhibition studies with DPIC suggest that the cytotoxic effects of MeBM can be attributed in part to P450 reductase mediated activation of this analog in whole cells. In addition MeBM produced the highest percent of DNA cross-links following reduction by P450 reductase, suggests that P450 reductase-mediated activation could contribute to the cytotoxic activity of MeBM and m-MeBM. Previous studies by Fourie et al., also found that analogs containing electron donating groups, namely MeBM, MBM and m-MeBM, produced the most DNA cross-links (Fourie, Guziec et al. 2004). Future studies should include cytotoxicity assays using MeBM on various cancer cells lines, both under aerobic and anaerobic conditions, such as MTT assays.

2. Studies on P450 reductase mediated tumour targeting should include cytotoxcicity assays on cell lines transfected with P450 reductase to amplify the effects of P450 reductase activity on the reduction and subsequent activation of bioreductive drugs. Cytotoxicity can be tested for using MTT assays. For instance, T47D human breast cancer cells (11.5

nmol/min/mg protein) have been transfected with the human P450 Red gene, resulting in P450 reductase activity of 311.8nmol/min/mg protein (Begleiter, Leith et al. 2007).

3. Enzyme profiling for individual tumours (Workman and Stratford 1993), will allow the identification of effective "matching" of pro-drug to activating enzyme. We can define the tumours by reactivity of MeBM on different cell lines, and then look at the activity of P450 reductase and other possible activating enzymes.

4. Studies using a range of bioreductive enzymes will help isolate the effects of diverse enzymes and elucidate to novel activation pathways for bioreductive agents, by determining the enzyme activity of different bioreductive enzymes in cell lysates. For example, NADH cytochrome b5 reductase, xanthine oxidase, and cytochrome P450 are reductases also known to mediate reduction of bioreductive drugs.

5. Studies on the activity of detoxifying antioxidant enzymes that act to decrease damage by ROS, such as superoxide dismutase, glutathione reductase, catalase, peroxidase, and reactive structural cellular components, may help to tailor drugs whose activity is faster or out of range from cellular detoxifying mechanisms. Determination of the presence of detoxifying enzymes can be carried out by Western blotting.

6. The design of new pro-drugs with specificity of activation by a single enzyme through manipulation of the chemical structure of compounds to alter electronegativity potentials, electron densities, and/or improve the physical interaction between enzyme and substrate. Ideally new drugs should include better electron donating groups, as these seem to increase the toxicity of current pro-drugs.

Ultimately, identifying the manner in which structural factors confer selectivity for activation of bioreductive agents will allow the further development of clinically utilizable agents that can be selectively activated by a single bioreductive enzyme, leading to enhanced tumour targeting and decreased toxicity to normal tissues.

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