A Structure-Activity Approach to Drug Action in Pharmacology:

A) Structure-Activity Relationships Involved in DT-diaphorase (NQO1) Mediated Reduction Kinetics and Mode of Action of Anticancer Bioreductive Benzoquinone Alkylating Agents

B) Structural Characteristics of Novel NMDA Receptor Antagonists Required for Renal Tubule Organic Cation Secretion

BY

JEANNE FOURIE

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Therapeutics
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Objectives: *Study 1 and 2:* To investigate the effects of functional group substitutions on the benzoquinone ring of the bioreductive benzoquinone alkylating agent (BM), on DT-diaphorase (DTD) mediated reduction kinetics, cytotoxicity and DNA cross-link or strand break formation. *Study 3:* To evaluate the inhibitory potency of NMDA receptor antagonists on energy-dependent amantadine uptake by renal organic cation transporters.

Methods: *Study 1:* Using purified human DTD, the rates of DTD mediated reduction of BM analogs with electron-donating (MBM, MeBM, m-MeBM), electron-withdrawing (CBM, FBM) and sterically bulky group (PBM, m-PBM, m-TBM) substitutions were determined *in vitro.* Cytotoxic activity was measured via the MTT assay with/without the DTD inhibitor, dicoumarol. *Study 2:* DNA cross-link and strand break formation were assessed by agarose gel assays after reduction of the BM analogs by DTD. *Study 3:* Inhibition of [^{3}H]amantadine uptake by structurally similar NMDA receptor antagonists were determined in rat proximal and distal renal tubules.

Results: *Study 1:* Steric, rather than electronic effects, were more important in modifying the rate of reduction by DTD. Electron-donating groups increased redox cycling of the reduced products vs. BM. Reduction by DTD was an activating pathway for MBM. *Study 2:* DNA damage produced by the BM analogs displayed a rank order of MeBM≈MBM>m-MeBM≈PBM>BM>CBM>FBM>m-PBM≈m-TBM for DNA cross-link formation, and MeBM>MBM>m-MeBM>PBM>BM≈CBM>FBM>m-PBM≈m-TBM for strand break

formation. *Study 3:* Steric hindrance around the ionized amino group of the cyclohexane ring appeared to prevent bicarbonate–mediated organic cation transport, and MRZ 2/579 displayed a novel distal tubule selectivity of inhibition.

Conclusions: *Study 1 and 2:* Steric effects were more important than electronic effects in decreasing the rate of reduction by DTD. Sterically bulky group substitutions at the C6 position of the quinone were more important in decreasing the rate of reduction by DTD than substitutions at the C5 position. DTD mediated DNA cross-link and strand break formation positively correlated with the cytotoxic activity of the BM analogs. *Study 3:* A steric mechanism of bicarbonate-mediated transport inhibition is proposed, while the selective distal tubule inhibition of MRZ 2/579 may be utilized to determine the relative importance of distal vs. proximal renal tubule organic cation transporters.

This thesis is dedicated to my family, Sophia Fourie, Ockert Fourie, Ockie Fourie, Ockert J. Fourie, Johanna P. Fourie and Maria M. Viljoen.

I will always be grateful to you for a magical childhood filled with wonder.

I continue to learn from you, I admire you and I love you.

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As always I am also extremely grateful to my parents and brother for their love, support, and encouragement in all that I endeavor.

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List of abbreviations:

ABC: ATP-binding cassette

AGT: alkylguanine-DNA alkyltransferase

Amantadine: 1-adamantanamine HCl

ANOVA: analysis of variance

ARE: antioxidant response element

ARH019: 3-hydroxymethyl-5-(2-methylaziridin-1-yl)-1-methyl-2-phenylindole-4,7-dione

AQ4N: 1,4-bis{[2-(dimethylamino-N-oxide)ethyl]amino}5,8-dihydroxyanthracene-9,10-dione.

AZQ: diaziquone or 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone

BM: benzoquinone mustard or 2-[di(2'-chloroethyl)amino]1,4-benzoquinone

CB-1954: 5-(aziridin-1-yl)-2,4-dinitrobenzamide

CBM: 5-chloro-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone

CT: Cross-Taggart

DCPIP: 2,6-di-chlorophenolindophenol

DIC: dicoumarol

DMF: dimethylformamide

DNA: deoxyribonucleic acid

DTD: DT-diaphorase or NAD(P)H:quinone oxidoreductase I

DZQ: 2,5-diaziridinyl-1,4-benzoquinone

EO9:	3-hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4,7-indione)-propenol
ES936:	5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione
FAD:	flavin adenine dinucleotide
FBM:	5-fluoro-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
GSH:	glutatione
GSTs:	Glutathione S-transferases
HBM:	2-[di(2'-hydroxyethyl)amino]-1,4-benzoquinone
KHS:	Krebs-Henseleit solution
MBM:	5-methoxy-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
MeBM:	5-methyl-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
MeDZQ:	2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone
Memantine:	2,5-dimethyl-1-adamantanamine
m-MeBM:	6-methyl-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
MRZ 2/579:	1-amino-1,3,3,5,5-pentamethylcyclohexane HCl
MRZ 2/600:	1-amino-1-ethyl-3,3,5,5-tetramethylcyclohexane HCl
MRZ 2/615:	1-amino-1,3,5,5-tetramethyl-3-ethylcyclohexane HCl
NADH:	nicotinamide adenine dinucleotide
NADPH:	nicotinamide adenine dinucleotide phosphate
NMDA:	N-methyl-D-aspartate
NMR:	Nuclear magnetic resonance
m-PBM:	6-phenyl-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
MRP:	multidrug resistance associated protein

m-TBM:	6-t-butyl-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
NQO1:	NAD(P)H:quinone oxidoreductase1
OCT:	organic cation transporter
PBI:	pyrrolo[1,2-a]benzimidazole
PBM:	5-phenyl-2-[di(2'-chloroethyl)amino]-1,4-benzoquinone
RH1:	2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone
SAR:	structure-activity relationships

CHAPTER 1

DT-diaphorase (NAD(P)H:quinone oxidoreductase I), Quinone-based Bioreductive Alkylating agents and Enzyme-Directed Drug Targeting

PROLOGUE

The study of molecular structure-activity relationships (SARs) is an important concept in pharmacology and therapeutics with multiple applications in qualitative and quantitative aspects of enzyme-substrate and drug-transporter interactions. Specifically, these include uses in the:

a.) Determination of pharmacokinetics and pharmacodynamics of prodrug compounds that undergo enzyme mediated bioactivation in order to exert their therapeutic effects.

b.) Investigation of structural characteristics which may be applied in the rational design of such prodrugs in order to increase the efficiency of bioactivation through certain enzymes, or increase their therapeutic index.

c.) Prediction of the safety profile of new therapeutic agents in terms of drug-drug interactions at common transporter sites.

d.) Forecast of structural characteristics necessary for drug transport via a specific drug transporter type, with the view that this information may contribute to the development of probe compounds selective for certain drug transporter isoforms.

The overall goal of this thesis is to apply SAR studies in relation to the above listed concepts. Specifically, Chapter 2 and Chapter 3 will illustrate the use of SAR studies to understand mechanisms of enzyme mediated activation and action of model anticancer bioreductive alkylating agents. An important concideration with respect to the originality of the studies in Chapter 2 and Chapter 3, is that the present set of investigations were conducted in an

integrated manner to capture SARs at three different levels including reduction kinetics, and pharmacodynamics at thel level of the DNA and at the whole cell level. Chapter 4 applies SAR studies to investigate the characteristics of three novel NMDA receptor antagonists, which are required for drug transport through specific transporter sites, as well as drug-drug interactions at common transporter types.

Overall, SAR studies do not directly test a specific hypothesis, but can uncover critical and original information. Such information can lead to specific and novel hypotheses for testing in future investigations. Moreover, SAR studies can reveal the mode of action of model bioreductive alkylating agents, as well as determine factors that are important in renal tubule organic cation transport.

Section 1:

DT-diaphorase (NAD(P)H:quinone oxidoreductase I): Historical perspective and biological/pharmacological significance

DT-diaphorase (DTD), also known as NAD(P)H:quinone oxidoreductase I (NQO1) (EC 1.6.99.2), is a cytosolic flavoenzyme that was first described by Ernster and Navazio in 1958 as the enzyme responsible for metabolism of 2,6-dichlorophenolindophenol (Ernster & Navazio 1958). This group was also the first to recognize that this enzyme can utilize both NADH and NADPH as the cofactor from which electrons are donated in its reduction reactions. In fact, it is the earlier nomenclature for NADH and NADPH, which were diphosphopyridine nucleotide reduced (DPNH) and triphosphopyridine nucleotide reduced (TPNH), respectively, which led to the naming of this enzyme as DTD (Ernster et al., 1960). Early studies suggested that the biological role for DTD was in the biosynthesis of the quinone containing vitamin K (K2) and coenzyme Q, however to date there is no direct evidence indicating a direct role for DTD in these processes (Ernster, 1987). Indirectly however, it is thought that DTD may function to keep these quinone factors in their reduced form where vitamin K can act in posttranslational processes of protein carboxylation and coenzyme Q can play a role as an antioxidant factor in cellular membranes (Wallin & Martin, 1987, Beyer et al., 1996).

It was not until the 1960s that one of the main physiological roles of DTD was discovered: its ability to detoxify a variety of xenobiotics including quinones, azo-dyes and nitro products (Ernster, 1987). Subsequently, in the early 1970s

Iyanagi and Yamazaki (1970) further characterized DTD as an obligatory two-electron reductase. This was a critical finding which led to the discovery of the biological importance of DTD in the detoxification of quinones biologically, as the two-electron reduction may lead to formation of the relatively stable hydroquinone product, and bypass the production of semiquinone and other reactive oxygen species (formed by reoxidation of the reactive semiquinone) which would form through one-electron reduction pathways of quinone-containing compounds (Ernster, 1987).

Overall, to date, DTD has been found to have three major roles biologically: (1) It acts in catalyzing the bioactivation of certain quinones such as those found in cooked foodstuff (Sugimura et al., 1966), (2) It has a role as a detoxifying enzyme, by catalyzing an obligatory two-electron reduction of a broad range of quinone substrates, leading to their detoxification (Ernster 1967, Ross 1997) and (3) It is important in its function as an antioxidant enzyme (Siegel et al, 1997). These roles of DTD will be discussed individually in more detail in subsequent sections of the thesis.

A more recent and emerging significant research issue is the exploitation of DTD in cancer chemotherapy through its use in the activation of antitumor quinones (bioreductive alkylating agents) into DNA damaging species (Ross et al., 1994). The term 'bioreductive alkylating agent' was first used by Sartorelli and coworkers in the mid 1970s. It describes a specific class of anti-tumor agents, which require bioreductive activation, by cellular reductase enzymes, to form electrophilic species, which can covalently bind to cellular macromolecules

to produce their anti-tumor effects (Lin et al, 1972, Sartorelli, 1988). Subsequently, this bioreductive approach to activation of bioreductive alkylating agents was investigated for its use in the selective targeting of radiation resistant hypoxic cells in solid tumors. This particular line of research is based on the premise that the resistant hypoxic cells should have a greater potential for reductive metabolism (activation) of bioreductive alkylating agents than their aerobic counterparts (Rockwell, 1992). To this end, the first drug to be used in these studies was the clinically available quinone containing natural product from *Streptomyces caespitosus*, mitomycin C (Sartorelli, 1988). This compound requires reductive activation by either one- or two-electron reductase enzymes, which leads to its rearrangement to form a bifunctional alkylating agent, which can cross-link DNA (Workman 1994, Sartorelli et al., 1994). As well, this approach has led to the development of the aromatic N-oxide bioreductive agent, tirapazamine, which is selectively activated in the hypoxic fraction of solid tumors by NADPH:cytochrome P450 reductase (Patterson 1993, Brown & Wang, 1998).

The main focus of this thesis encompasses a second line of research, named as the enzyme-directed approach to bioreductive drug development, where bioreductive drug activation is also utilized. With this approach, the aim is to design cytotoxic agents with increased tumor selectivity through activation by enzymes such as DTD, which are over-expressed in tumor cells compared to normal cells (Malkinson et al., 1992, Workman 1994). DTD has been a central target in the enzyme directed approach to bioreductive alkylating agent development. Within this line of research, the principal aims of the SAR studies

include the determination of structural characteristics which may influence DTD mediated reduction kinetics and mechanisms of DNA damage as well as the specificity of cytotoxicity to tumor cells across different types of quinone-based experimental bioreductive alkylating agents (Beall & Winski 2000).

1.1 Bioactivation of quinones by DTD

Quinone-based bioreductive alkylating agents may be reduced via one- or two-electron reductase enzymes to form semiquinone and hydroquinone products, respectively. There are two main mechanisms depending on characteristics of the hydroquinone reduced product, by which DTD mediated reduction can lead to compounds with increased cytotoxicity. In the presence of oxygen, hydroquinones (which are redox-labile) can autoxidize and redox cycle to produce reactive intermediates and reactive oxygen species which may produce oxidative damage. For example DTD mediated reduction has been shown to be the dominant pathway of activation of 4-nitroquinoline-1-oxide, found in cooked foodstuff, to its DNA damaging metabolite 4-hydroxyaminoquinoline-1-oxide (Benson, 1993). As well, this metabolite has been indicated to produce oxidative damage which may underlie mutational events involved in 4-hydroxyaminoquinoline-1-oxide-induced rat acinar cell carcinogenesis (Nakae et al., 1994). In addition to the redox-labile metabolites, the reduction by DTD may also lead to compounds that can undergo rearrangements to generate alkylating species as shown by the DNA damaging metabolite 4-hydroxyaminoquinoline-1-

oxide produced by reduction by DTD of the parent structure 4-nitroquinoline-1-oxide in rat liver (Benson, 1993).

1.2 DTD as an antioxidant enzyme

The reduction of endogenous quinones by DTD has been implicated in the protection of cell membranes from oxidative damage. Studies using rat liver DTD have indicated that reduction of ubiquinone analogs (coenzyme Q) to their ubiquinol forms occurs in liposomes and rat hepatocytes, and that these products act as antioxidants, protecting membrane phospholipids from oxidative damage. In addition, the oxidation of vitamin E (α-tocopherol) leads to the product α-tocopherolquinone. The subsequent reduction, of α-tocopherolquinone by human DTD, leads to the formation of α-tocopherolhydroquinone, and this product has also been shown to have antioxidant properties (Siegel et al., 1997). In support of the antioxidant role of DTD, immunohistochemical studies in humans have indicated that this protein is expressed in a variety of tissues requiring antioxidant protection, including epithelial cells of the lung, breast, and colon and vascular endothelium (Siegel et al., 1998, Siegel & Ross, 2000).

1.3 Detoxification of quinone substrates by DTD

In addition to the redox-labile hydroquinone products formed following reduction by DTD which lead to increased cytotoxicity mediated by oxidative damage, DTD mediated reduction may also result in the formation of stable hydroquinone products that can be readily conjugated and eliminated. This

suggests a possible role of DTD in modulating sensitivity to the toxicity of quinone-based xenobiotics and carcinogenesis. This phenomenon is illustrated by the compound menadione, which when reduced by DTD forms a stable hydroquinone product that is conjugated and excreted (Losito et al, 1967). Furthermore, disruption of the *NQO1* gene in mice leads to an increase in menadione mediated toxicity (Radjendirane et al., 1998).

1.4 Enzyme directed drug targeting: DTD directed antitumor agents

In addition to being expressed ubiquitously in normal human tissue including epithelial and endothelial tissue, DTD has been shown to be over-expressed in a variety of solid tumors including those of the breast, colon, ovary, cornea and lung (Cresteil and Jaiswal 1991, Schlager and Powis 1990, Malkinson et al., 1992) when compared to normal tissue of the same origin. Furthermore, DTD is differentially induced in L5178Y murine lymphoma cells compared to normal mouse bone marrow cells (Begleiter et al., 1996) and DTD mediated reduction leads to bioactivation of bioreductive agents such as mitomycin C (Siegel et al., 1990b) and the aziridinylbenzoquinone RH1 (Winski et al., 1998) to species with increased cytotoxic activity. Specifically, the cytotoxic activity of these bioreductive alkylating agents subsequent to reduction by DTD may either be due to the production of alkylating species or active oxygen species producing oxidative damage, and depends on the chemical properties of the quinone-based bioreductive agent undergoing enzymatic reduction (Cadenas 1995). Taken together, these observations have lead to

DTD being considered central to the enzyme-directed approach to bioreductive drug development. With this approach, the aim is to design cytotoxic agents with increased tumor selectivity through activation by enzymes such as DTD, which are over-expressed in the tumor tissue compared to normal cells (Malkinson et al., 1992, Workman 1994). Currently, there are several quinone-based bioreductive agents which are under development for DTD-directed bioactivation, and these include aziridinylbenzoquinones, indolequinones, mitosenes, pyrolo-benzimidazolequinones, aziridinylcyclopent[b]indolequinones and cyclopropamitosenes (Ross et al., 2000, Xing et al., 2000). Current approaches under which these agents are investigated include determination of SARs that predict enzyme specific reduction or cytotoxic activity (Beal & Winski 2000), gene targeting (Yoshida & Tsuda, 1995) and different mechanisms of enzyme induction (Doherty et al., 1998). Each of the different quinone-based bioreductive agent types will be discussed in more detail in later sections with respect to enzyme directed drug targeting.

Section 2:

Quinone Chemistry

Quinone-based compounds play an important role in many biological systems. Specifically, quinones are found in plants, fungi and bacteria, where biochemically they are elements of the electron transport chains and function in processes such as cellular respiration and photosynthesis (Nohl & Jordan, 1986, Kagan, et al., 1990). Human exposure to quinones may occur through diet

(Lindsey, 1974), exposure to industrial pollutants containing polyaromatic hydrocarbons (Smart and Zannoni, 1984) and chemotherapeutic agents such as bioreductive alkylating agents (Gibson et al., 1994).

The basic quinoid structure is shown in Figure 1, with different subtypes varying in the substitution of oxygen (-C=O), sulfur (-C=S), carbon ($-C=CH_2$) or nitrogen (-C=NH) at the terminal positions (X) (Brunmark and Cadenas, 1989). Furthermore, as observed from the chemical structure, the quinone is a diketone [$O=C-(C=C-)_n-C=O$] with carbonyl groups conjugated to the double bonds of the ring or to additional rings. Due to the diketone structure, quinone chemistry is based on that of $\alpha - \beta$ unsaturated ketones (Figure 2). In addition, their redox character is based on the electrophilic reactivity determined by the carbonyl groups, and the reaction of the polarized double bonds with nucleophiles (Brunmark and Cadenas, 1989).

Figure 1 Quinoid structure

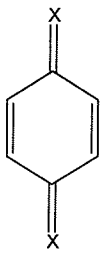
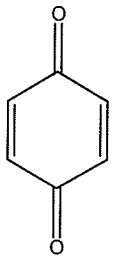


Figure 2 Quinone structure



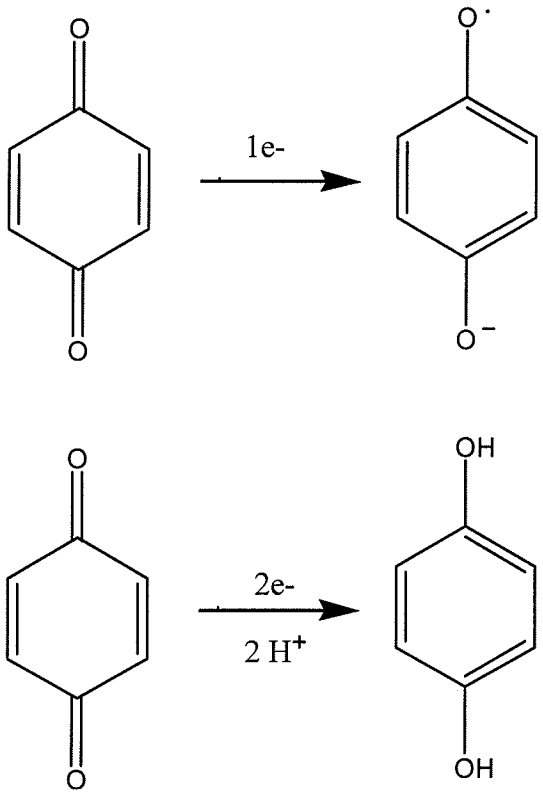
The quinone is often incorporated as the bioreductive element of bioreductive alkylating agents. In order to fully understand the overall contribution of the quinone structure to the activation of these prodrugs, and its contribution to the mechanisms of toxicity of bioreductive alkylating agents, it is

important to understand the redox features of quinones. The following sections will summarize the main redox transitions involving quinones. Specifically, they will describe enzymatic reduction of quinones, non-enzymatic formation of the semiquinone and nucleophilic addition reactions leading either to oxidation or reduction of the quinone ring.

2.1 Enzymatic reduction of quinone compounds

Quinone reduction may occur either as a one-electron transfer, or an obligatory two-electron transfer mediated by cellular reductase enzymes (Figure 3).

Figure 3 Enzymatic reduction of quinones



In the first pathway, a single electron is transferred from the reduced pyridine nucleotide to the flavoprotein reductase enzyme, which can then catalyze the single electron reduction of the quinone to form the semiquinone free radical product. Such one-electron reduction enzymes include NADH-cytochrome b_5 reductase, xanthine oxidase and NADPH:cytochrome P450 reductase (Iyanagi, 1987, Pan et al., 1984).

The second pathway of quinone reduction is an obligatory two-electron transfer mediated by reductases such as xanthine dehydrogenase (Gustafson & Pritsos, 1992a,b) and DTD (NAD(P)H:quinone oxidoreductase I) (Ernster, 1967, Jaiswal et al., 1999), and results in the formation of a hydroquinone product. Reduction by DTD has been studied extensively, especially in the design of quinone containing bioreductive anticancer agents. DTD is unique in that this flavoenzyme can utilize either NADH or NAD(P)H as a cofactor from which electrons are donated for reduction reactions (Ernster, 1967). This direct two-electron reduction forming the hydroquinone is often thought to be a detoxification mechanism as it circumvents one-electron reduction of the quinone, and thus the formation of the free radical semiquinone product. It is important to note however that whether or not formation of the hydroquinone is an activation, or a detoxification pathway depends on the character of the hydroquinone formed. Specifically, reduction by DTD may lead to *activated or inactivated* hydroquinone products. The activated products may be classified into two types: a.) redox-labile hydroquinones which may autoxidize in the presence of oxygen to form reactive oxygen species and the semiquinone

reactive intermediate, and b.) hydroquinones that rearrange to form electrophiles which can alkylate cellular nucleophiles, (bioreductive alkylation) (Lin et al., 1976). Potentially, characteristics of both types (a) and (b) may be found in a single hydroquinone product. In contrast, reduction by DTD may also lead to the formation of *inactivated* redox-stable hydroquinone products, which can be detoxified through conjugation.

2.2 Activation of quinones by DTD: Formation of redox-labile hydroquinones

Subsequent to enzymatic reduction of the quinone to a hydroquinone, cytotoxicity may be induced through redox cycling. Redox cycling involves the autoxidation of the hydroquinone to form reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$) and semiquinone reactive intermediates which can undergo subsequent reduction in a cycling mechanism. Reactive oxygen species and reactive intermediates may then produce oxidative damage to proteins, nucleic acids and lipids. For example, damage to nucleic acids includes DNA strand breakage which involves hydrogen abstraction from one of the carbons of the deoxyribose sugar backbone of DNA, followed by base release (Goldberg, 1987, Giulivi & Cadenas 1994, Goin et al., 1995). DTD mediated reduction has been shown to facilitate the redox cycling of the diaziridinylbenzoquinone antitumor agent diaziquone for example. Specifically it has been shown that diaziquone is reduced to its hydroquinone form by purified DTD, and by the S9 fraction (consisting of both microsomal and cytosolic cellular fractions) of MCF-7 human

breast carcinoma cells (Fisher et al., 1992, Fisher and Gutierrez 1991a,b). Furthermore, addition of the DTD inhibitor dicoumarol (DIC) to the S9 fraction of the MCF-7 cells prevented the formation of the $HO^{\cdot}$ radical, which suggests that reduction of the quinone form by DTD is important in allowing for the formation of reactive oxygen species through autoxidation of the hydroquinone (Fisher et al., 1992).

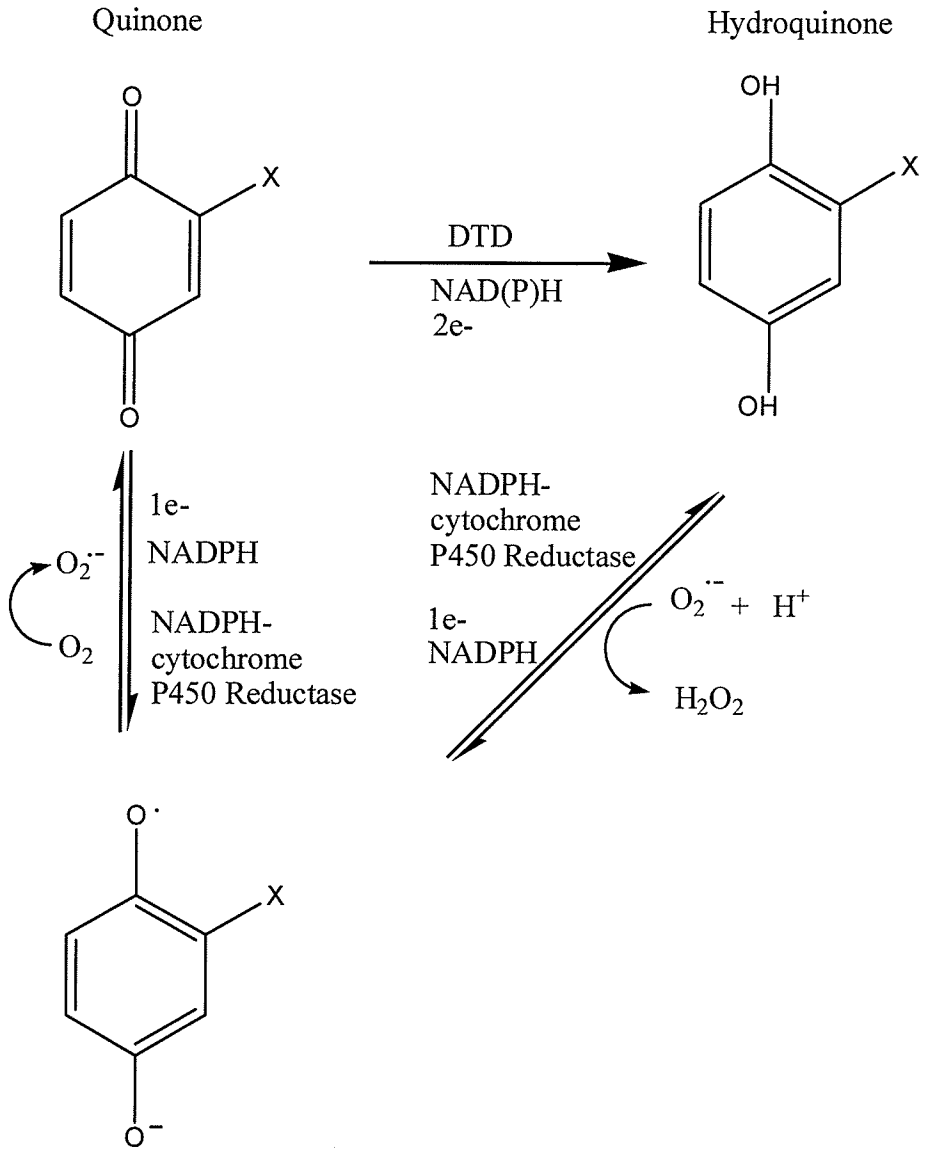
Importantly, autoxidation of the hydroquinone depends on the specific characteristics of the hydroquinone produced through reduction by DTD. It has previously been shown, for example, that the nature of functional groups or substituents, and the position of substitution on the quinone affect the potential of the hydroquinone to autoxidize. Specifically, menadione (2-methyl-1,4-naphthoquinone) forms a stable hydroquinone product following reduction by DTD, while its analog 2-methyl-3-glutathionyl-1,4-naphthoquinone undergoes rapid autoxidation (Buffinton et al., 1989).

2.3 DTD mediated activation of bioreductive alkylating agents

In addition to the redox-labile hydroquinone products formed through reduction by DTD, quinone-based bioreductive alkylating agents, may also be reduced by DTD and other reductases previously mentioned. (Lin et al., 1972). Bioreductive alkylating agents are defined as antitumor agents which undergo bioactivation or rearrangement reactions to generate electrophilic species which can bind to cellular nucleophiles following reduction by cellular reductases such as NADPH:cytochrome P450 reductase and DTD (Lin et al., 1972). Structurally,

bioreductive alkylating agents consist of two main functional elements, a bioreductive element, and a cytotoxic element. In the case of quinone-based bioreductive alkylating agents, including mitomycin C, (Lown et al., 1976, Tomasz et al., 1987) the bioreductive element regulates a separate cytotoxic element. Examples of typical bioreductive elements include the n-oxide moiety and quinone moiety, in the case of the clinically used mitomycin C. Upon reduction of the bioreductive element, the cytotoxic element becomes activated to an electrophilic species. Cytotoxicity of these agents is mediated by the electrophilic products, which can be mono-and bifunctional alkylating agents that can alkylate or cross-link DNA. Furthermore, under aerobic conditions, redox cycling of the hydroquinone or semiquinone products leads to reformation of the prodrug or quinone parent structure, generating reactive oxygen species, which can produce oxidative damage including DNA strand breaks (Figure 4). Current quinone-based bioreductive alkylating agents under review for potential clinical use have included structures such as aziridinylbenzoquinones, indolequinones, mitosenes, pyrrolobenzimidazolequinones, aziridinylcyclopent[b]indolequinones and cyclopropamitosenes. These quinone-based bioreductive agents as well as other agents will be reviewed in greater detail in subsequent sections.

Figure 4 Enzyme mediated activation of benzoquinone bioreductive akylating agents and mechanisms of redox cycling



Semiquinone

X- cytotoxic element

2.4 Non-enzymatic production of semiquinones

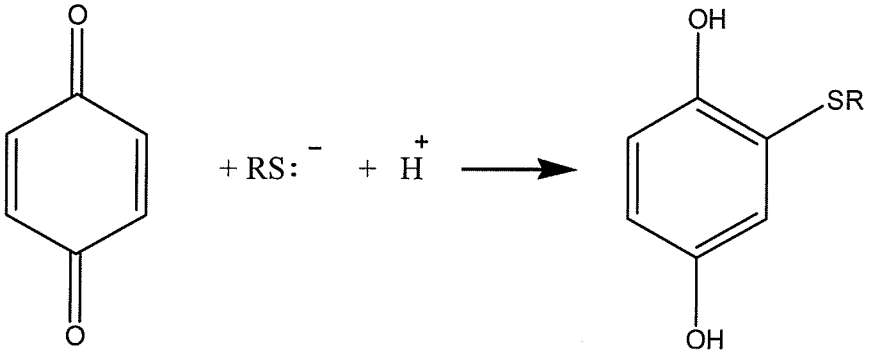
Semiquinones are produced through one-electron enzymatic reduction, or through redox-cycling of the hydroquinone form by autoxidation pathways. Furthermore, semiquinones may also form non-enzymatically. One such mechanism to generate the semiquinone is through the comproportionation reaction (reaction 1). If for instance the comproportionation for the quinone form of the molecule prevails over disproportionation for the semiquinone (*kf* > *kb*), this would lead to an increase in the steady-state concentration of the semiquinone. In the case of diaziquone for example, the disproportionation for the semiquinone radicals occur at very slow rates when compared to that of mitomycin C. Therefore, the semiquinone form of diaziquone may be important in the redox transitions of diaziquone and in the formation of DNA damage via reactive oxygen species and reactive intermediates, or DNA alkylation reactions (Butler et al., 1987).

$$Q + Q^{2-} \underset{b}{\overset{f}{\rightleftharpoons}} Q^{\cdot-} + Q^{\cdot-} \qquad \text{(reaction 1)}$$

2.5 Addition of sulfur nucleophiles to quinoid compounds

As described previously, quinone chemistry is similar to that of α,β–unsaturated ketones, which undergo Michael addition reactions. Michael addition (1,4-reductive addition) is defined as addition of nucleophiles (Michael donors) to the β carbon atom of the unsaturated ketone structure ($-C_\alpha=C_\beta-$) in the quinone ring (Michael acceptor). One common example of Michael addition is

the addition of sulfur nucleophiles to the polarized double bond of the quinone ring, leading to the formation of a thioether product (reaction 2).



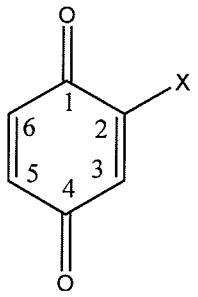
(reaction 2)

Intracellular sulfur nucleophiles are represented as protein and nonprotein based sulfhydryls. Glutathione (GSH) is the predominant non-protein sulfhydryl present in cells, and previously was shown to occur abundantly in tumor and normal mouse tissue with concentration ranges of 1-2 mmol kg^{-1} and 0.8-8 mmol kg^{-1}, respectively (Minchinton et al., 1984, Griffith & Meister 1979). Several factors may influence the addition of sulfur nucleophiles to quinones. These include, in particular, the type and orientation of substituents on the quinone and the ratio of [GSH]/[quinone].

The orientation of functional groups on p-benzoquinones has been shown to influence the position of the sulfur nucleophile addition to the quinone moiety. Specifically, electron-donating functional groups ($-CH_3$, -OH and $-OCH_3$) decrease the electron-withdrawing character of one of the unsaturated ketones of p-benzoquinones. This effect of the electron-donating functional groups results in nucleophilic addition to the β carbon of the other, more electron-deficient

unsaturated ketone. For example, in the case where an electron-donating group (X) is substituted at the C_2 position (Figure 5), the rank order of reactivity towards the nucleophile has been reported as: $C_5>C_6>C_3$ by Brunmark and Cadenas (1989). In contrast, electron-withdrawing groups substituted on the quinone moiety at position X activate the β-position and produce a reactivity profile of $C_3>>C_6>C_5$ (Brunmark and Cadenas, 1989).

Figure 5 p-Benzoquinone



The addition of GSH to quinones has been studied extensively, and a number of the product thioether hydroquinone derivatives have been shown to contribute to the overall cytotoxicity of quinones by undergoing autoxidation and oxidation reactions with unreacted quinones (Figures 6, 7a, 7b). These oxidation reactions lead to the formation of semiquinone thioethers, which can undergo redox cycling and cause oxidative damage. For example, 1,4-naphthoquinones have previously been shown to undergo 1,4-reductive addition reactions with GSH to form glutathionyl hydroquinone conjugates (Figure 6) (Finley, 1974). Furthermore, glutathionyl-semiquinone detection via electron spin resonance

spectroscopy has shown that glutathionyl hydroquinone conjugates autoxidize in a similar manner as hydroquinones (Takahashi et al., 1987). In particular, the glutathionyl thioether 2-methyl-3-glutathionyl-1,4-naphthoquinone derivative of 2-methyl-1.4-naphthoquinone undergoes rapid autoxidation (Buffinton et al., 1989). In addition aziridinylbenzoquinones activated through glutathione nucleophilic substitution, have been shown to autoxidize and are associated with DNA strand break formation which is inhibited by superoxide dismutase (Goin et al., 1995).

Figure 6 Addition of GSH to naphthoquinone

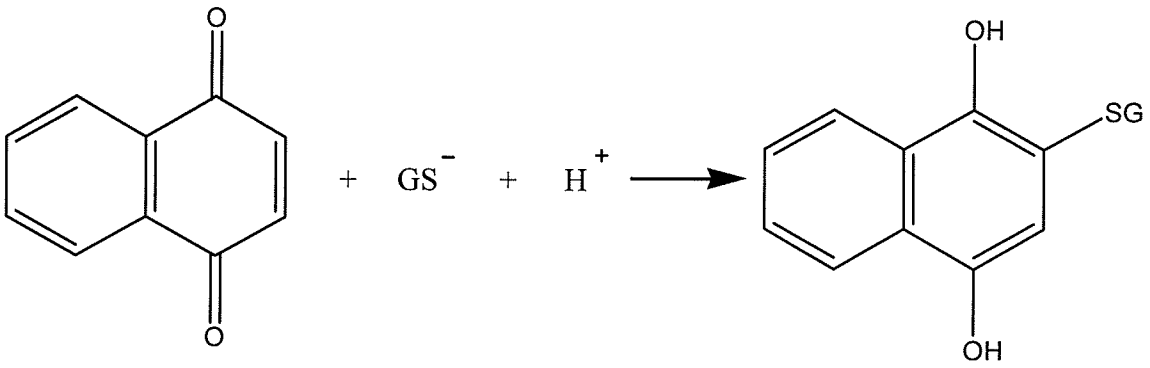
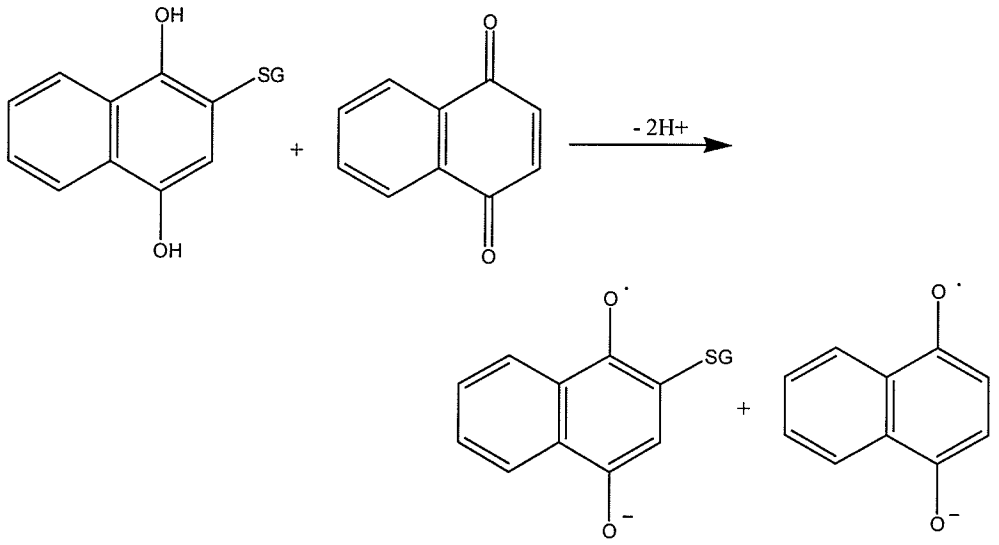
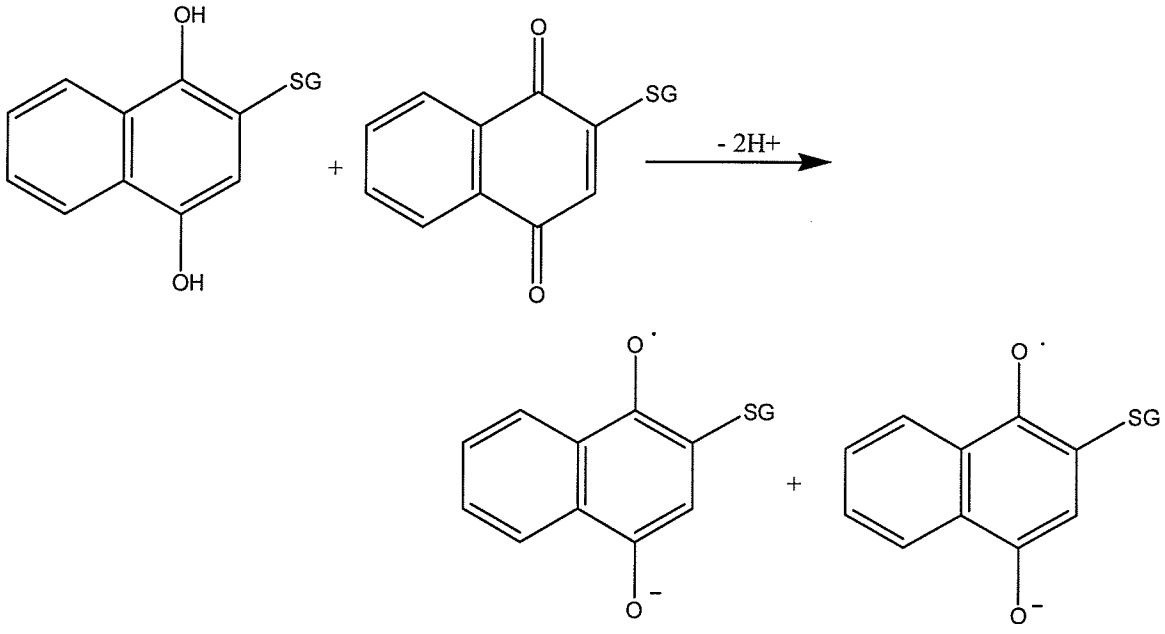


Figure 7a,b Formation of semiquinone via oxidation of the glutathionyl-hydroquinone with unreacted quinone (A) or coproportionation (B). Reproduced with permission from Brunmark and Cadenas, 1989.

7a.



7b.



Another characteristic of some thioether derivatives of quinones is their ability to act as substrates for reduction by enzymes such as DTD. For example, the glutathionyl conjugates of 1,4-naphthoquinone and 2-methyl-1-4,naphthoquinone are substrates for DTD, and autoxidize more rapidly than their unsubstituted parent structures (Buffinton et al., 1989). Therefore, the cytotoxicity of quinones may not solely be due to the transition between parent quinone, semiquinone and hydroquinone, but also may encompass cytotoxicity produced through the redox cycling and reduction of quinone products that have undergone sulfur nucleophilic addition.

2.6 Cellular detoxification of quinones

Detoxification of quinone-based hydrophobic chemotherapeutic agents involves metabolism to more hydrophilic metabolites that are more easily eliminated from the body. DTD mediated two-electron reduction of the quinone may facilitate this process, as it leads to the introduction of hydroxy groups, which are needed for conjugation reactions with glucuronate or sulfate. The ease of conjugation however may further depend on the characteristics of the hydroquinone product as suggested by Brunmark and Cadenas (1989). Specifically, conjugation and detoxification would be more preferable, provided that the reduction to the hydroquinone does not lead to activation, such as in the case of the bioreductive alkylating agent mitomycin C. Furthermore, redox stable hydroquinone products, which do not undergo rapid redox cycling leading to cytotoxicity, would be more prone to undergo conjugation intracellularly.

Quinones have been shown to undergo conjugation reactions with GSH leading to the formation of glutathionyl-hydroquinone products (Brunmark and Cadenas, 1988). It still needs to be elucidated, whether this is a detoxification pathway, as these products may autoxidize at higher rates than their hydroquinone counterparts (Brunmark and Cadenas, 1988). On the other hand, it has been suggested that GSH conjugation would act as a detoxification pathway. In particular, the conjugates may have increased hydrophilicity compared to the quinone, and therefore may be more easily eliminated. As well, conjugation may decrease the number of electrophilic sites on the quinone that can react with nucleophilic sites on DNA and protein and therefore influence the cytotoxic activity of the compound (Brunmark and Cadenas 1989).

2.7 Superoxide dismutase and DTD: A concerted antioxidant activity

One of the main mechanisms of cytotoxicity of quinone-based bioreductive alkylating agents is through redox cycling of the reduced hydroquinone product which results in the formation of reactive semiquinones and the formation of superoxide radical ($O_2^{\cdot-}$). Subsequent reactions of superoxide radical can lead to the formation of the highly mutagenic hydroxyl radical. For instance, the two-electron reduction of quinones such as 1,4-naphthoquinones by DTD leads to the formation of redox labile hydroquinones, which can autoxidize readily (Brunmark et al., 1988, Brunmark et al., 1987). Oxidation of the hydroquinone first involves formation of superoxide via semiquinone autoxidiation (reaction 3), and is

followed by superoxide consumption during hydroquinone oxidation (reaction 4) as follows:

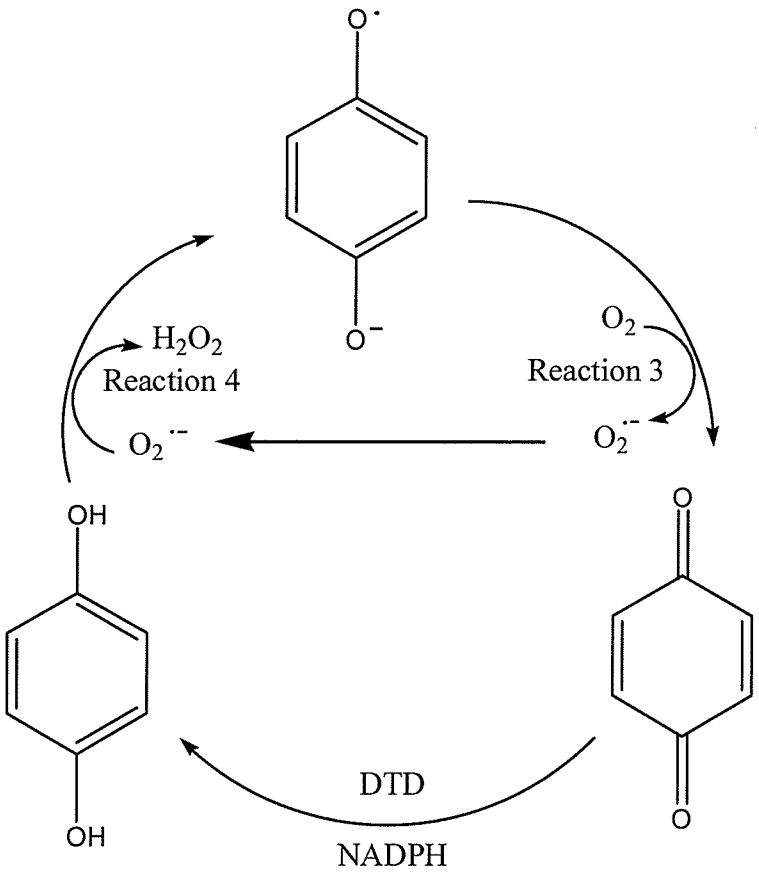
$$Q^{\cdot -} + O_2 \underset{b}{\overset{f}{\rightleftharpoons}} Q + O_2^{\cdot -} \qquad \text{(reaction 3)}$$

$$HQ^- + H^+ + O_2^{\cdot -} \rightarrow Q^{\cdot -} + H_2O_2 \qquad \text{(reaction 4)}$$

The relative cytotoxicity mediated through this mechanism is related to such factors as the oxygen tension in the cell, the characteristics of the hydroquinone which would affect its ability to redox cycle and cytoprotective antioxidant mechanisms within the cell, including enzymes such as glutathione peroxidase and superoxide dismutase. As an example, the metalloenzyme superoxide dismutase, which functions in the protection of cells against oxidative damage, may inhibit the propagation of the hydroquinone oxidation (reaction 4) through catalyzing the rapid disproportionation of the superoxide radical to form H_2O_2 (reaction 5) (Figure 8, Cadenas, 1995).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(reaction 5)}$$

Figure 8 Superoxide radical-mediated propagation of hydroquinone autoxidation. (See text above for descriptions of reactions 3,and 4.)



Reproduced with permission from Cadenas, 1995.

Section 3:

Mechanisms of Resistance to Chemotherapy

One major problem encountered with the treatment of cancer with current chemotherapies is the rapid development of resistance to these agents. Studies focused on understanding the mechanisms of resistance to chemotherapeutic drugs including alkylating agents are critical in the identification of future

molecular targets for the development of new chemotherapeutic drugs. The following section will provide a brief overview of some of the mechanisms by which resistance to clinically used alkylating agents may occur as there is limited or no data available for specific experimental bioreductive alkylating agents. Future studies are required with specific bioreductive alkylating agents which are bound for development as clinically used agents, to determine whether these mechanisms which have been described for alkylating agents also apply to bioreductive alkylating agents.

3.1 Role of ATP-binding cassette (ABC) superfamily transporter genes

Broad-based resistance to chemotherapy frequently develops from the enhanced cellular efflux of chemotherapeutic agents due to over-expression of members of the ATP-binding cassette (ABC) superfamily of membrane transporters, including P-glycoprotein and several multidrug resistance associated protein (MRP) family proteins (Kuwano et al., 2003). Over-expression of these proteins is responsible for resistance against many anticancer agents including *Vinca* alkaloids, anthracyclines (doxorubicin, daunomycin), taxanes and epipodophylotoxins (Germann, 1996). These proteins appear more important in the efflux of natural product anticancer agents due to their weakly basic hydrophobic character and large molecular weights (>600Da). As most bioreductive alkylating agents have low molecular weights, and are moderately hydrophilic, ABC protein mediated efflux by its self is thought not to be a major mechanism by which resistance occurs to these agents (Penketh et al., 1996),

but may be important in combination with other cellular detoxification pathways, as discussed in the following section.

3.2 Glutathione S-transferases (GSTs)

Glutathione S-transferases (GSTs) are a super family of phase II isoenzymes with broad overlapping substrate specificity and low substrate affinities. These enzymes catalyze the reaction of GSH with reactive electrophilic carcinogens and xenobiotics (Paumi et al., 2001). GSTs are also thought to be important in the inactivation and production of resistance for specific alkylating agents such as nitrogen mustards (Puchalski and Fahl, 1990). As well, in a recent study it was shown that MRP isoform 1 (MRP1) is required for the human GST A1-1 dimer mediated resistance to chlorambucil. Specifically MRP1 allows for efflux of the intracellular chlorambucil-SH conjugate, which can act to inhibit GST via potent product-inhibition. This efflux of the chlorambucil-SH conjugate subsequently allows for efficient inactivation of chlorambucil by GST, and drug resistance (Paumi et al., 2001).

3.3 Enhanced DNA repair

Increased resistance to alkylating agents due to increased mechanisms of DNA repair has been investigated with nitorsoureas, which mediate their genotoxicity via bifunctional alkylating species which can form DNA cross-links through alkylation at the O^6 position of guanine (Erickson et al., 1980) on opposite strands of DNA. The DNA repair enzyme O^6-alkylguanine-DNA

alkyltransferase (AGT) is one mechanism by which cells may prevent the formation of DNA cross-links by nitrosoureas, and data suggest that this may be an important mechanism by which resistance to this class of agents occurs clinically. In a study by Erickson and colleagues, (1980), it was shown for example that cell lines with low AGT expression and deficiency in repair of O^6-alkylation of guanine produced greater amounts of DNA cross-links subsequent to treatment with nitrosourea than cells with higher levels of this repair enzyme. Further studies are needed to determine if this enzyme is important in the repair of DNA damage and cytotoxicity due to bioreductive alkylating agents, and if there are cellular resistance mechanisms in which this pathway or other repair pathways are affected.

Homologous recombination is utilized both in DNA double strand repair and DNA interstrand cross-link repair in mammalian cells, and is a process by which the homologous chromosome, or chromatid, is used as a template to repair broken strands of DNA (McHugh et al., 2001). DNA interstrand cross-link repair occurs first via nucleotide excision repair which involves the formation of a DNA double strand break at a location before the cross-link via ERCC-1/XPF endonuclease activity which makes an incision 5' to the cross-link. Subsequently, this is followed by homologous recombination (Bessho et al., 1997, Thompson and Schild, 2001). An important nuclear serine/threonine kinase associated with DNA double strand break repair and joining steps in recombination mechanisms is DNA-dependent protein kinase (DNA-PK) (Jin et al., 1997). This protein is a trimeric nuclear complex consisting of a large

catalytic subunit, (protein kinase) and a DNA binding subunit (Ku heterodimer) that regulates kinase activity by its association with DNA. It functions by the Ku heterodimer binding to discontinuous DNA which is then followed by recruitment of the catalytic subunit, which can phosphorylate many mammalian DNA repair complexes involved in homologous recombination (Falzon et al., 1993, Gottlieb and Jackson, 1993, Lees-Miller et al., 1990).

Currently some of the most effective anticancer agents are those that produce DNA cross-links. Agents which produce their cytotoxic activity through DNA cross-link formation include nitrogen mustards (melphalan, chlorambucil, cyclophosphamide and ifosfamide), platinum compounds (cisplatin and carboplatin), chloroethylnitrosoureas (carmustine and lomustine), alkylalkanesuphonates (busulfan) and mitomycin C. The repair of DNA cross-links via nucleotide excision repair and homologous recombination appears to be important mechanism, which protects cells from this type of damage. Increased DNA repair through this pathway may therefore be an important mechanism of acquired clinical resistance to these agents. In support of this, recent work indicated that the sensitivity to melphalan in plasma cells correlated with DNA interstrand cross-link repair in patients with multiple myeloma; however, the exact mechanism by which this occurred, was not elucidated (Spanswick et al., 2002). To address this issue, Panasci and colleagues have conducted several studies which investigated the specific mechanisms by which homologous recombinational repair may influence resistance to nitrogen mustards in chronic lymphocytic leukemia (Bello et al., 2002, Christodoulopoulos et al., 1999, 1998,

Muller et al., 1998). Specifically, DNA-PK activity in lymphocytes of untreated patients with chronic lymphocytic leukemia, or patients resistant to chlorambucil, was determined through the capacity of the kinase to phosphorylate a peptide substrate *in vitro*. It was found that the phosphorylation activity of DNA-PK was increased significantly in the resistant samples compared to the samples from untreated patients, and that there was a significant correlation between the level of DNA-PK activity and the inherent resistance of the lymphocytes to cholorambucil *in vitro*. In addition, this increased DNA-PK activity in the resistant samples was attributed to an increased DNA-binding activity of the large Ku heterodimer. Overall, these studies suggest that DNA-PK activity may contribute to the mechanism of resistance to nitrogen mustards observed in chronic lymphocytic leukemia (Muller et al., 1998). Further studies are required to determine whether this kinase may play a role in creating resistance to bioreductive alkylating agents, which produce DNA interstrand cross-links, as well as its role in resistance to agents that produce DNA cross-links in other types of cancers.

3.4 Apoptosis

Apoptosis is a programmed energy dependent process which leads to cell death. It involves internucleosomal DNA degradation executed by selectively activated caspases. The morphologic characteristics of apoptosis include nuclear condensation and fragmentation, and condensation of the cell with preservation of organelles. Subsequently, this is followed by fragmentation of the

cell into membrane-bound apoptotic bodies which undergo phagocytosis by nearby cells without associated inflammation (Saraste, 1999).

Many chemotherapeutic agents, including mitomycin C are known to induce apoptosis in tumor cells, but resistance to these agents may occur through mechanisms which involve impaired apoptosis. Bioactivation by DTD of quinone-based bioreductive alkylating agents including MeDZQ, streptonigrin and mitomycin C results in active metabolites which can produce oxidative damage through their redox cycling and formation of reactive oxygen species as well as activated alkylating agents which can cross-link DNA. Alkylating agents and free radicals are known to induce apoptosis, however the precise mechanisms by which bioreductive alkylating agents induce apoptosis are largely unknown and are under investigation (Hickman, 1992, Sarafian and Bredesen, 1994, Wolfe et al., 1994). It would be beneficial to determine the mechanisms by which these agents induce apoptosis, as disregulation of apoptosis following chemotherapy may be an important mechanism by which drug resistance could occur. An understanding of such mechanisms of drug resistance would be beneficial in the future design of chemotherapeutic agents which can effectively induce cytotoxicity and cell death. Furthermore, the mechanisms by which apoptosis is induced and or disregulated following therapeutic intervention may lead to the identification of new molecular targets for cancer chemotherapeutic drug design.

Bcl-2-like family members and caspase family members regulate apoptosis in human cells either positively or negatively. The human Bcl-2-like

family of proteins includes gene products such as Bcl-2 (Reed, 1994), Bax (Oltvai et al., 1993), Bcl-x (Boise et al., 1993), Mcl-1 (Kozopas et al., 1993), Bak (Chittenden et al., 1995), Bag (Takayama et al., 1996), Bfl-1 (Choi et al., 1995), Bik (Boyd et al., 1995), Bcl-w (Gibson et al., 1996), Brag-1 (Das et al., 1996) and Bid (Wang et al., 1996). Specifically, Bcl-2-like family members all share one or several Bcl-2 homology regions and behave as pro- or anti apoptotic proteins. For example, the anti-apoptotic Bcl-2-like family members include Bcl-2 and Bcl-xL. These proteins act to preserve mitochondrial outer membrane integrity, thus preventing the release of soluble pro-apoptotic molecules and cytochrome C (Bettaieb et al., 2003). The Bcl-2-like family members, which promote apoptosis, act to sense cellular damage, and initiate apoptosis. Examples of a pro-apoptotic Bcl-2 members are the BAD and Bax-α proteins (Bettaieb et al., 2003). Some of these Bcl-2 family proteins have been studied in considerable detail in relation to chemotherapy induced apoptosis and resistance. For example, over expression of Bcl-2 has been implicated with protection from apoptosis induced by anticancer compounds including mitomycin C (Ohmori, 1993, Reed, 1994, 1995a, 1995b). Furthermore, Bcl-xL has been shown to act in a protective role against anticancer agents (Datta et al., 1995), whereas Bax-α increases apoptosis induced by anticancer agents (Chresta et al., 1996). These effects were demonstrated in studies using human leukemic monocyte-like U-937 control cells and U-937 cells transfected with Bcl-xL or Bax-α to have increasing exogenous expression of either protein. Subsequently, the cells were treated continuously for 8 h with increasing concentrations of the DNA alkylating agent

cis-platinum, and Bcl-xL was shown to protect the cells against apoptosis in a mechanism which is thought to act downstream of the primary mechanism of action of this drug. In contrast, Bax-α promoted apoptosis induced by cis-platinum (Schmitt et al., 1998). The mechanism by which this occurs is still under investigation, however it appears that Bax accelerates caspase-3 activation, and sufficient levels of Bax may bypass the requirement for the tumor suppressor protein p53, a protein which is known to trigger chemotherapy induced apoptosis (Schmitt et al., 1998, Strobel et al., 1996).

The caspase family of proteins consists of cysteine proteases (caspases), and these proteins contain an active cysteine residue. Activation of these proteases as well as serine proteases such as granzyme B, lead to activation of the apoptotic process (Greenberg and Litchfield 1995, Schmitt et al., 1998). For example, caspase-3 activation correlates with the occurrence of DNA fragmentation, a hallmark of apoptosis (Schmitt et al., 1998). Furthermore, studies using cell extracts and purified recombinant Bcl-xL protein have shown that Bcl-xL acts upstream from serine proteases and caspase-3, by blocking or delaying signals that would lead to the activation of these proteases and induction of apoptosis (Schmitt et al., 1997). Further studies are needed to determine if these mechanisms are important in triggering apoptosis mediated by specific bioreductive alkylating agents.

DTD mediated bioactivation of MeDZQ, streptonigrin and mitomycin C has been shown to result in the induction of apoptosis in human cancer cell lines. Specifically, these agents induced marked apoptosis in cells with high DTD

activity (HT29), which was inhibited by DIC. On the other hand, apoptosis was markedly less in BE cells with no DTD activity, and furthermore was not affected by DIC. As well, there was a positive correlation between the substrate affinity of these agents to DTD and induction of apoptosis, with streptonigrin being the most efficient substrate and most potent in the induction of apoptosis (Sun and Ross 1996). Further evidence linking DTD to mechanisms of apoptosis induction was a correlation between the expression of the pro-apoptotic Bcl-2 family protein BAD, which produces apoptosis via the mitochondria involving cytochrome C release, and DTD in a 60 cell line panel (Kitada et al., 1998). Furthermore, Tudor and colleagues (2003) recently reported comparisons between two cell line panels: 1.) MCF-7 human breast carcinoma cells (high DTD activity and BAD protein levels) and, 2). HL-60 myeloid leukemia cells (no DTD activity and low BAD protein levels). Specifically, they correlated the ability of a series of aziridinylbenzoquinone bioreductive alkylating agents to induce cytotoxicity and apoptosis in these cell lines. Good correlations were observed between the cytotoxic activity of the aziridinylbenzoquinones, which were good substrates for DTD, and cellular DTD activity. Furthermore in a subset of these compounds that were good substrates for DTD, there were further good positive correlations between cytotoxicity and increased BAD protein levels as well as cytotoxicity and induction of apoptosis. In contrast, in the other subset of good substrates for DTD, there was a correlation between cytotoxicity and induction of apoptosis, but not BAD protein levels. This suggested that aziridinylquinones, which are good substrates for DTD, may induce apoptosis through different

mechanisms (mitochondrial and non-mitochondrial). On the other hand, poor substrates for DTD produced both decreased cytotoxic activity and deficient induction of apoptosis in the cell line panels (Tudor et al., 2003). Differences in the relative proportions of the different forms of BAD protein (dephosphorylated (active) and phosphorylated (inactive)) may have implications in the overall interpretation of results observed by Tudor and colleagues (2003). Therefore, further studies are required to determine the significance of potential changes in the amounts of active and inactive forms of BAD protein in these experiments. Additional studies are also warranted to determine the importance of these findings in relation to differential cytotoxicity observed with bioreductive alkylating agents.

Section 4:

The Human *NQO1* gene

The *NQO1* gene which encodes DTD (NQO1) is localized to chromosome 16q22 and is approximately 20 kb pairs in length (Figure 9). Moreover, *NQO1* consists of six exons interrupted by five introns, and encodes a 274 amino acid residue protein (Jaiswal, 1991). The sixth exon in the human *NQO1* gene contains four polyadenylation sites (AATAAA) and a single copy of human Alu repetitive sequence between the second and third polyadenylation signal site. It is thought that the three transcripts originating from the human *NQO1* gene are as a result of the use of three of the four polyadenylation sites in exon six (Jaiswal, 1991).

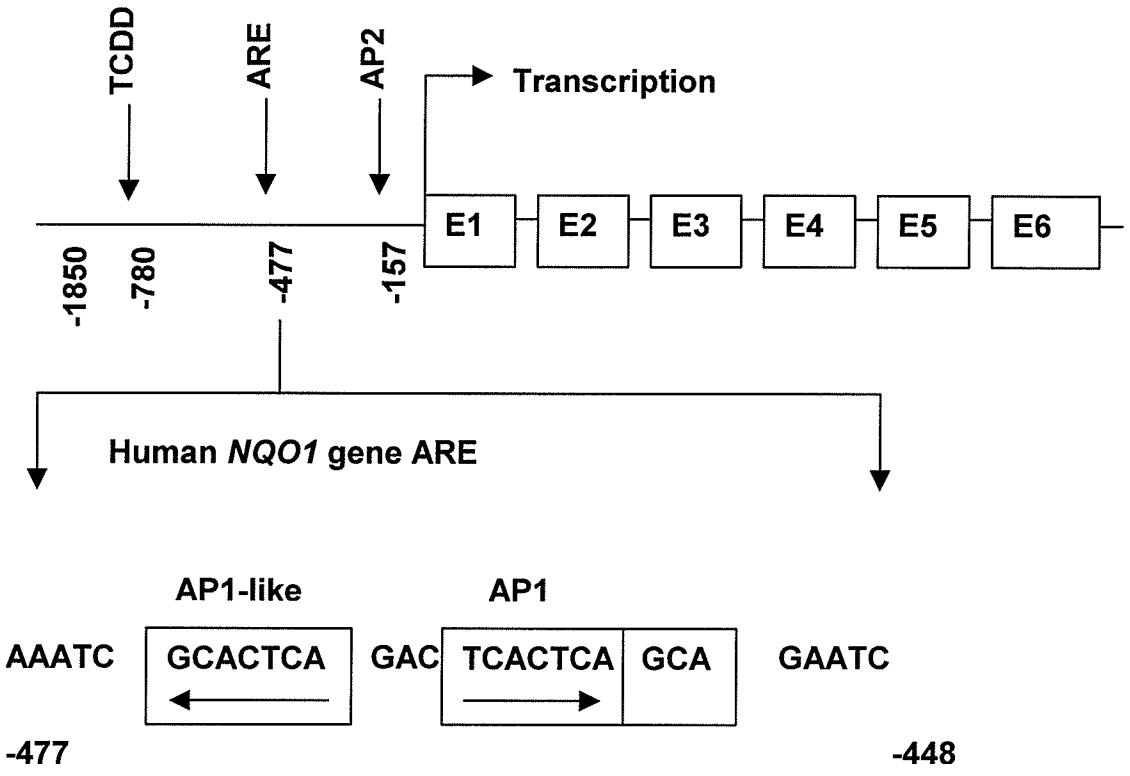
4.1 Induction of *NQO1* gene expression

There are two classes of chemical inducers of DTD: 1) bifunctional inducers, which induce both cytochrome P-450 genes and genes encoding phase II detoxification enzymes including DTD. These consist of polycyclic aromatic hydrocarbons including β-naphthoflavone (Jaiswal, 1991), 3-methylcholantherene (Richardson and Cunningham, 1951) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Beatty and Neal, 1976). 2) Monofunctional inducers induce phase II drug metabolizing enzymes exclusively, and include such compounds as 1,2 dithiole-3-thiones, coumarins (Prochaska et al., 1985) and R-1-isothiocyanato-4-methylsulfinylbutane (sulforaphane) (Thimmulappa et al., 2002).

4.2 Mechanisms for the regulation of the *NQO1* gene

As determined by deletion mutagenesis experiments, there are several essential cis-elements within the human *NQO1* gene promotor region, which are required for the expression and induction of this gene (Jaiswal, 1991, Li and Jaiswal, 1992, Xie et al., 1995). These elements include an antioxidant response element (ARE), a DNA fragment essential for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) mediated induction of *NQO1* gene expression (nucleotide position –780 to –365), and an AP-2 element required for the cAMP-induced expression of the gene (Figure 9).

Figure 9 Human *NQO1* gene. Reproduced from Jaiswal, 2000 with permission.



NQO1 Gene: E1-6: exons 1-6; ARE: antioxidant response element; AP-2: AP-2 binding site; TCDD: fragment needed for 2,3,7,8-tetrachlorodibenzo-p-dioxin mediated induction; ARE: antioxidant response element; AP-1: TPA response element, or binding site for Jun and Fos proteins; AP-1-like: imperfect AP-1 element.

The following is a brief discussion on the regulation of the *NQO1* gene by the ARE element (Figure 9), which is necessary for basal expression as well as xenobiotic (Li and Jaiswal, 1994), antioxidant (Dhakshinamoorthy and Jaiswal,

2001) and oxidant (Joseph et al., 1994) induced *NQO1* gene induction. The nucleotide sequence of the NQO1 ARE is depicted in Figure 9. Specifically, it contains one perfect and one imperfect AP-1 (TPA response element) element, which are arranged as inverse repeats of each other, and are separated by three base pairs followed by a "GC" box (Radjendirane et al., 1997, Jaiswal 1994). TPA response elements were first identified in the promoter region of genes other than those containing ARE elements, which require TPA to activate their expression. The human NQO1 gene and other detoxifying enzymes gene AREs are however unique in that these cis-elements do not require TPA, but rather respond to xenobiotics and antioxidants (Radjendirane et al., 1997, Jaiswal 1994). The reason for the distinct function of the NQO1 ARE from those genes that respond to TPA is thought to be in part due to the other nucleotide sequences. These include the CTGAC***CG core sequence present in the ARE, as well as additional cis-element and nucleotide sequences flanking the core sequence of the ARE, sequences which through mutational analysis have been shown to contribute to the ARE-mediated expression and induction of NQO1 (Radjendirane et al., 1997, Xie et al., 1995, Prestera et al., 1993, Wasserman and Fahl, 1997).

4.3 Positive regulation of the ARE of the *NQO1* gene

Several nuclear transcription factors have been identified to be part of the human ARE-nuclear protein complex, which binds to the ARE, and so regulates transcription of the human *NQO1* gene. The transcription factors which bind to

the human *NQO1* ARE include c-Jun, Jun-B, Jun-D, c-Fos, Fra1, Nrf1, and Nrf2 (Venugopal and Jaiswal, 1996, Venugopal and Jaiswal, 1998).

It has been shown that Nrf1 and Nrf2, which are b-zip (leucine zipper) proteins that do not heterodimerize with each other, but rather require a second leucine zipper protein for activation, and are positive regulators of ARE-induced expression and induction of the *NQO1* gene in response to oxidants and xenobiotics (Venugopal and Jaiswal, 1996). There have been several studies aimed at broadening our understanding of the mechanisms of Nrf1 and Nrf2 mediated regulation of *NQO1*. Nrf2 has been implicated in the *in vivo* regulation of *NQO1* gene expression by knockout studies in mice. Particularly, Nrf2-/- mice lacking the expression of Nrf2 showed significantly decreased expression and induction of the *NQO1* gene (Itoh et al., 1997). Furthermore, a cytosolic protein Kcap1 is suggested to act as an oxidative stress sensor protein, as it was shown to retain Nrf2 in the cytoplasm under physiological conditions, but upon exposure to xenobiotics and antioxidants the association was terminated, allowing for nuclear translocation of Nrf2 (Itoh et al., 1999). In addition, Venugopal and Jaiswal (1998) have shown that nuclear transcription factors Nrf2 and Nrf1 heterodimerize with c-Jun, Jun-B and Jun-D proteins. In this study, they provided evidence that in response to antioxidants and xenobiotics, these Nrf-Jun complexes (in addition to unknown cytosolic factors), bind to the ARE, which is followed by subsequent expression and synchronized induction of other detoxifying enzymes genes in response to antioxidants and xenobiotics.

4.4 Negative regulation of the ARE of the *NQO1* gene

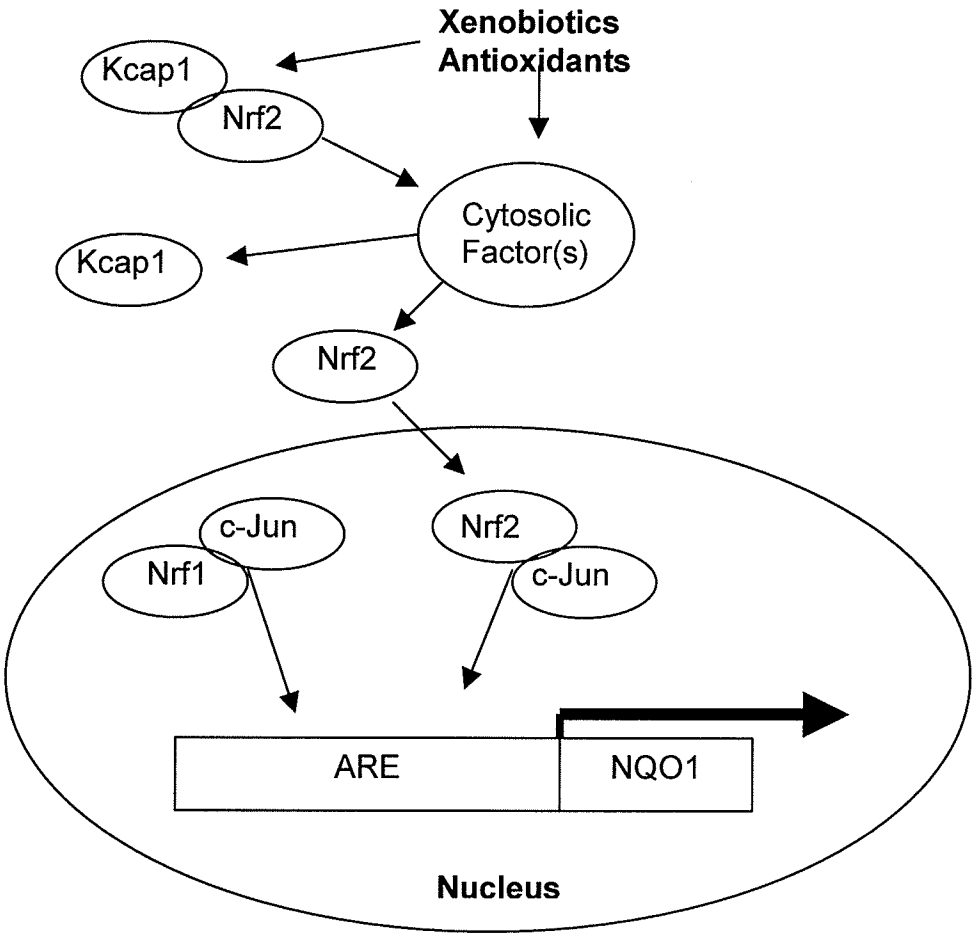
ARE mediated expression of the *NQO1* gene is thought to be controlled by a balance between positive regulatory factors such as Nrf2 and proteins which repress *NQO1* gene expression. For instance, c-Fos is thought to act in the repression of ARE-mediated gene expression as shown with c-Fos-/- knockout mice, which demonstrated increased expression of NQO1 as compared to wild-type c-Fos+/+ mice (Wilkinson et al., 1998). This repression of detoxifying enzymes such as DTD is hypothesized to be required to modulate the significant and potent effects on decreasing free radicals beyond base line physiological concentrations by genes such as *NQO1*.

4.5 Mechanism of signal transduction for ARE-mediated expression of NQO1

Although many positive and negative regulators of ARE-mediated NQO1 expression have been identified, the precise mechanisms involved in basal expression and coordinated induction and repression of NQO1 expression still need to be fully elucidated. Recently, Jaiswal has described a hypothesized model by which ARE-mediated expression might occur (Jaiswal, 2000) (Figure 10). Briefly, it is known that xenobiotics and antioxidants undergo metabolism to produce superoxide and related reactive oxygen species (De long et al., 1987). It is thought that the superoxide serves as a trigger that activates a battery of defensive genes such as *NQO1*, which protect cells against oxidative stress. This is supported by the finding that hydrogen peroxide leads to induction of the

ARE-mediated expression of rat and human *NQO1* genes (Favreau and Pickett 1991, Li and Jaiswal, 1994). It is thought that the superoxide signal involves first, interactions with intermediary proteins, which ultimately lead to interactions with cytosolic factors which may be important in the modification of interactions between transcription factors such as Nrf2 and the Kcap1 cytosolic factor which sequesters it in the cytoplasm. Subsequent to this proposed dissociation between Nrf2 and Kcap1, Nrf2 may translocate to the nucleus, and form Nrf2/c-Jun heterodimers, which may finally bind to the ARE, resulting in the induction of NQO1 expression. Further research is needed to identify all the players involved in these pathways, including the identification of unknown cytosolic factors. This information will ultimately lead to the elucidation of the signal transduction pathways involved in xenobiotic, antioxidant and endogenous physiological mediated expression of NQO1, and enhance our understanding of the mechanisms by which DTD protects the cell against free radical damage and oxidative stress.

Figure 10 Mechanism of signal transduction for ARE-mediated expression of *NQO1*



Reproduced from Jaiswal, 2000 with permission.

4.6 Disruption of the *NQO1* gene in mice

Gene knockout experiments in mice is one of the main methodologies used to investigate the role of specific genes *in vivo*. Previously, *NQO1* knockout mice were generated by the use of homologous recombination in embryonic stem cells. These animals are currently used to identify the physiological and molecular pathways in which DTD plays a role (Radjendirane et al., 1998). When compared to *NQO1* +/+ mice, the *NQO1* null mice have thus far been found to have an increased sensitivity to menadione induced hepatic toxicity (Radjendirane et al., 1998), and have a decreased accumulation of lower abdominal adipose tissue (Gaikwad et al., 2001). Such findings provide further evidence for the role of DTD in the detoxification of xenobiotics, and suggests a possible role in metabolic pathways.

Disruption of the *NQO1* gene in mice has also recently been shown to cause myelogenous hyperplasia (i.e. increase in myeloid cells in the bone marrow) (Long et al., 2002). Investigators further suggested potential mechanisms by which DTD may play a protective role and guard against myelogenous hyperplasia. It was observed that the bone marrow in the null mice had a reduction in tumor suppressor proteins p53 and p73, as well as a decrease in apoptosis compared to the wild type litter mates. These changes were suggested to occur due to the reduced p53 and p73 tumor suppressor proteins, as these accumulate in cells subsequent to stress signals, and can cause either growth arrest or apoptosis under normal circumstances. In this study there was also a reported the accumulation of NAD(P)H cofactors for DTD, however, these

differences were not supported by statistical analysis, and therefore should be interpreted with caution. The results from this study and earlier studies, led to the suggestion that the mechanism by which the tumor suppressor proteins were decreased in the null mice involved a redox mechanism which was dependent on DTD-mediated NAD(P)H oxidation to NAD. This hypothesis was based on the fact that NAD is known to act as a substrate for poly(ADP-ribose) polymerase, a DNA binding protein that catalyzes the transfer of ADP-ribose residues from NAD to p53 (Gaikwad et al., 2001). Poly(ADP-ribose) polymerase is also suggested to play a role in the stabilization of p53, as human cancer cells with reduced NAD and deficient poly(ADP-ribose) polymerase exhibit decreased basal levels of p53 (Jacobson et al., 1999, Whitacre et al., 1995). Furthermore, DTD was suggested to be important in the stabilization of the tumor suppressor protein p53, in studies in which wild type (*NQO1*) or mutant (*NQO1*2) NQO1* were transfected in human colon cancer HCT-116 cells. With these studies it was shown that wild type but not mutant DTD protein increased the stability of p53 in HCT-116 cells, and this increased stability was inhibited by the DTD inhibitor DIC (Asher et al., 2002). Overall, these data provided some evidence that DTD may stabilize p53 through a redox mechanism, however further studies are needed to support this hypothesis in light of a recent study which suggests an alternative mechanism of stabilization (Anwar et al., 2003).

In contrast to the redox mechanism suggested to be involved in the stabilization of p53, a recent study provides strong evidence suggesting that the stabilization of p53 occurs via a protein-protein interaction between p53 and

DTD. Specifically, co-immunopricipitation techniques in human primary cell cultures and cell free systems demonstrated a specific interaction between full-length p53 and DTD (Anwar et al., 2003).

Taken together, the evidence of DTD mediated stabilization of p53, and the finding that *NQO1* null mice, which display myelogenous hyperplasia, have a reduction in p53 protein, as well as a decrease in apoptosis compared to wild type litter mates, suggest that DTD may act to protect against neoplasia via a mechanism other than through its function as a reductase enzyme. In particular it has been hypothesized that the increase in susceptibility to various forms of neoplasia in individuals which are homozygous for the C609T mutation in *NQO1* (Smith et al., 2001) may be explained in part through the decreased stability of p53, leading to decreased p53 mediated cell cycle arrest or apoptosis subsequent to DNA damage in these individuals (Anwar et al., 2003).

4.7 NQO1 genetic polymorphism and its clinical implications

A genetic polymorphism in drug and xenobiotic metabolism is defined as a "Mendelian or monogenic trait that exists in the population in at least two phenotypes (and presumably at least two genotypes), neither of which is rare-that is, neither of which occurs with a frequency of less than 1 to 2%" (Vogel and Motulsky, 1986). DTD has been shown to be genetically polymorphic in humans, with three allelic variants defined as follows: the wild type NQO1*1 allele, the C609T variant (NQO1*2) allele and, the C465T (NQO1*3) variant allele.

The NQO1*2 single nucleotide polymorphism constitutes a point mutation resulting in a cytosine to thymine transition at base pair 609 in exon six of the NQO1 cDNA. This point mutation confers a proline to serine substitution at codon 187 of the DTD protein (Ross et al., 1996, Traver et al., 1997). As characterized by inborn genetic polymorphisms, there are considerable inter-individual and inter-ethnic differences in phenotype due to this C609T mutation (Table 1) (Kelsey et al., 1997). Specifically, the homozygous C609T variant results in a null phenotype due to the rapid polyubiquitination and proteosomal degradation of the mutant protein (Siegel et al., 2001).

Table 1

Ethnic group	***NQO1 T/T genotype prevalence (%)***
Caucasian	4.4
African-American	5.2
Hispanic	15.5
Korean Asian	18.8
Chinese Asian	22.4

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The second NQO1*3 single nucleotide polymorphism is defined as a point mutation resulting in a cytosine to thymine transition at base pair 465 of the NQO1 cDNA (Pan et al., 1995, Hu et al., 1996). It confers an arginine to tryptophan substitution at codon 139 of the DTD protein, and the allele frequency of this mutation in humans ranges from 1-5%, depending on ethnic origin (Pan et al., 1995, Hu et al., 1996, Gaedigk et al., 1998). The potential significance of this polymorphism was assessed in human colon carcinoma HCT-116R30A and

HCT-116 cells which are homozygous (NQO1*3/*3) and heterozygous ((NQO1*1/*3) for the C465T mutation, respectively (Pan et al., 2002). In this study it was found that the C465T variant produced an alteration of a normal pre-mRNA 5'-splice site of intron-4 that is present in NQO1*1 and NQO1*2. This alteration resulted in the generation of exon-4-deleted mRNA in both cell lines and subsequent low DTD protein content in HCT-116R30A cells to 5% of the quantity observed in HCT-116 cells (Pan et al., 2002). An earlier study further showed that when compared to HCT-116 cells, there was a conciderable decrease in the ability of the HCT-116R30A cells to reduce mitomycin C (Pan et al., 1995). This decrease in reduction of mitomycin C did not apprear related to a lower enzyme activity of the mutant DTD, as the C465T mutation did not alter the enzymatic activity of DTD compared to wild type DTD protein (Hu et al., 1996, Pan et al., 1995). Rather, the decrease in the ability of the HCT-116R30A cell line to reduce mitomycin C appeared to be attributed to the significant decrease in DTD protein in this cell line (5% of HCT-116) (Pan et al., 2002, Pan et al., 1995). Further studies are required to assess the clinical significance of this mutation in the predisposition to different cancers and in the pharmacological treatment with bioreductive alkylating agents.

As reviewed, DTD is important in several biological processes including its role in the detoxification or bioactivation of environmental and endogenous quinone substrates and its function as an antioxidant enzyme. It is also being explored for its potential to selectively activate bioreductive alkylating agents in the enzyme directed drug development approach to tumor targeting. An

adequate understanding of the genotype-phenotype relationships associated with DTD is central to epidemiological studies. Such studies would determine the importance of DTD in a variety of disease states associated with the biological pathways in which DTD is implicated. As well these studies would determine its role in the activation of clinically used bioreductive agents to cytotoxic metabolites and the impact on drug efficacy. To support the importance of DTD in the pathophysiology of disease states, recent studies have indicated that a lack of DTD activity is associated with increased incidence of benzene-induced hemotoxicity (Rothman et al., 1997). The metabolic activation of benzene in humans involves metabolism by cytochrome P4502E1 (CYP2E1) to benzene oxide, followed by spontaneous phenol formation, and further oxidative metabolism to the hydroquinone form via CYP2E1 (Kalf, 1987, Koop et al., 1989). Subsequently, the hydroquinone form is converted to potent hematoxic and genotoxic benzoquinones by myeloperoxidase in the bone marrow (Eastmond and Smith 1987, Smith et al., 1996). It was suggested that DTD may play a protective role in this pathway by converting the toxic benzoquinones back to the less toxic hydroxy metabolites (Ross, 1996, Rothman et al., 1997). In fact, this hypothesis was supported by the finding that the *NQO1* C609T mutation was associated with and increased risk of benzene poisoning as assessed by the increased incidence of hematological malignancy among humans with a history of occupational benzene exposure. Other diseases states impacted by the *NQO1* C609T mutation include Benzo(a)pyrene-induced skin carcinogenesis as shown in mice deficient in NQO1 RNA and protein (Long et al., 2000),

chemotherapy-related myeloid malignancies (Larson et al., 1999), leukemias (Smith et al., 2001), renal and urological cancers (Schulz et al., 1997) and basal cell carcinoma (Clairmont et al., 1999).

Section 5:

DTD: Enzyme structure and mechanisms of reduction

5.1 DTD subcellular localization

DTD is a mainly cytosolic flavoprotein, with this compartment containing 84% or more of the total amount of DTD as determined by studies using rat liver (Conover and Ernster, 1962, Edlund et al., 1982). Similar analysis revealed that the remaining fractions of this enzyme were located in the mitochondria (13%), golgi apparatus (1%) and microsomes (2%) (Edlund et al., 1982). This distribution is thought to hold in the case of the human DTD enzyme, as the protein shares 85% amino acid homology with that of the rat, however some discrepancies have been identified. In a recent study, confocal immunoelectron microscopy in NCI-H661 human non small cell lung carcinoma cells and HT-29 human colon carcinoma cells, DTD could not be identified in the mitochondrial fraction. This finding was further attributed to possible methodological constraints, or the possibility that mitochondrial localization only occurred under cellular stress (Winski et al., 2002). This study was also the first to indicate, through confocal immunoelectron microscopy and cell fractionation methodology, that a significant proportion of DTD is found in the nuclei of NCI-H661 and HT-29 cancer cell lines. The significance of nuclear DTD is unknown at present,

however, it may have implications in the possible nuclear activation or inactivation of quinone-based bioreductive alkylating agents and other quinone substrates. Furthermore, this study was conducted in cancer cell lines, and it would be important to confirm these observations in normal human cells.

5.2 Structure of the DTD enzyme

The structure of recombinant human DTD has been reported in the presence and absence of bound substrates (Faig et al., 2000, 2001). This information has greatly improved our understanding of the exact mechanisms by which this enzyme interacts with quinone substrates including anticancer agents, and will be of great importance in molecular modeling studies and the possible structure-based development of bioreductive anticancer agents. In this section, the main features of the DTD flavoenzyme structure as well as the mechanisms by which it interacts with its substrates will be discussed.

DTD is a 61 kDa protein consisting of two interlocked 30.7 kDa homo-monomeric subunits. Each subunit is comprised of two separate domains: a catalytic domain, and a small C-terminal domain. The catalytic domain consists of a site that binds flavin adenine dinucleotide (FAD), a site which binds the adenine ribose portion of nicotinamide adenine dinucleotide (phosphate) (NAD(P)), and the substrate donor/acceptor binding site that binds either the hydride acceptor (substrate) or the hydride donor (the nicotinamide moiety of NAD(P)H).

At the FAD binding site, the interaction of FAD with DTD requires primary amino acid residues from one of the two monomers of the dimer. Furthermore, the isoalloxazine moiety of FAD (three ring structure, Figure 11) is planar when bound to human DTD, and is required for making binding contacts with the substrate when it occupies the catalytic substrate binding site.

The substrate donor/acceptor binding site of human DTD is comprised of a catalytic substrate binding pocket and alternatively binds the nicotinamide portion of NAD(P)H and the substrate (Figures 12, 13). The pocket consists of: residues Phe-178, Tyr-126 and Tyr-128 from the first monomer which form the roof of the pocket; the isoalloxazine ring of FAD which forms the floor; and His-161, Gly-149, Gly 150 (amino acid residues from the second monomer) and two conserved water molecules which form the side of the pocket.

Figure 11 Chemical structure of FAD

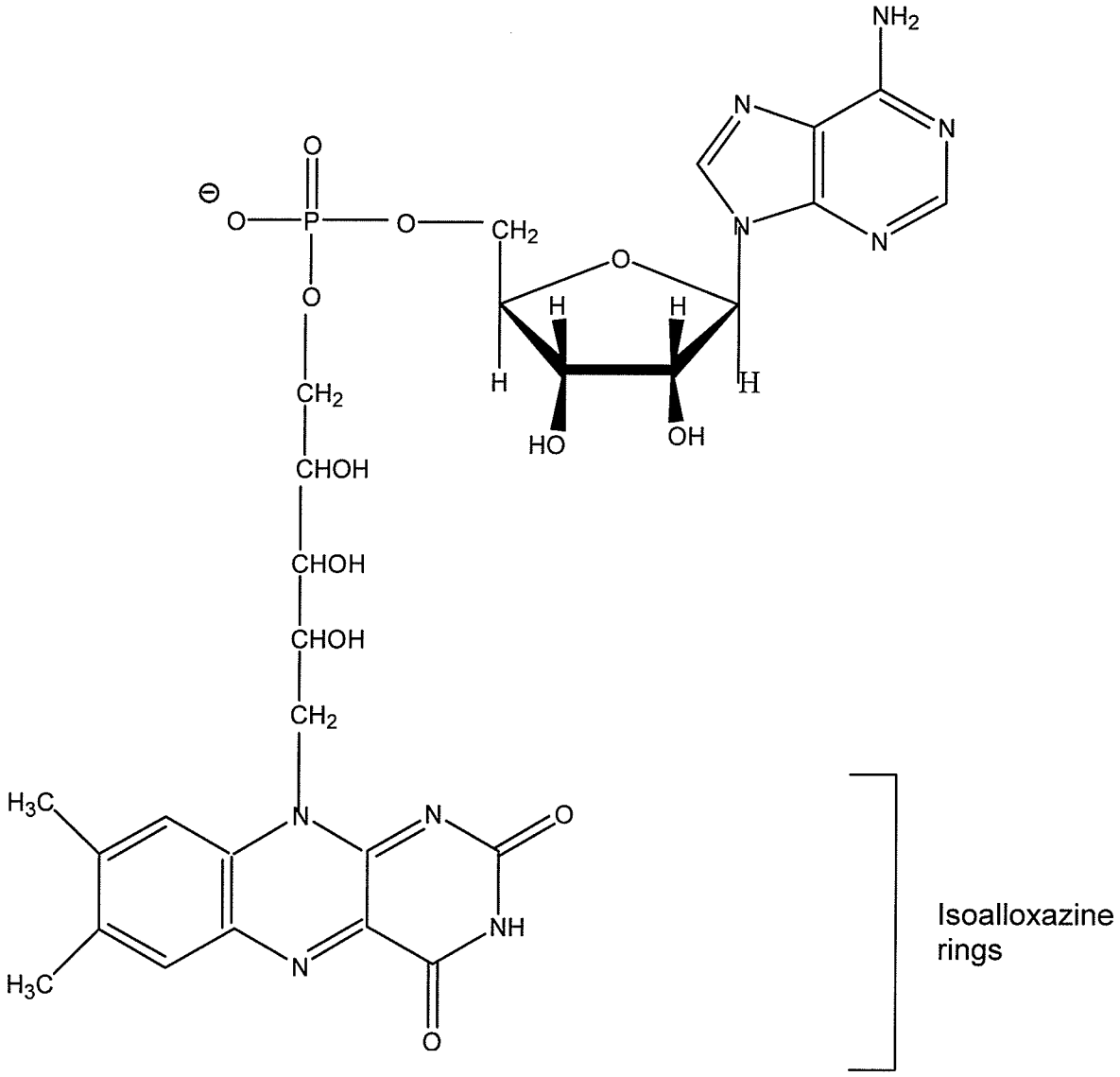


Figure 12 Chemical structure of NADH

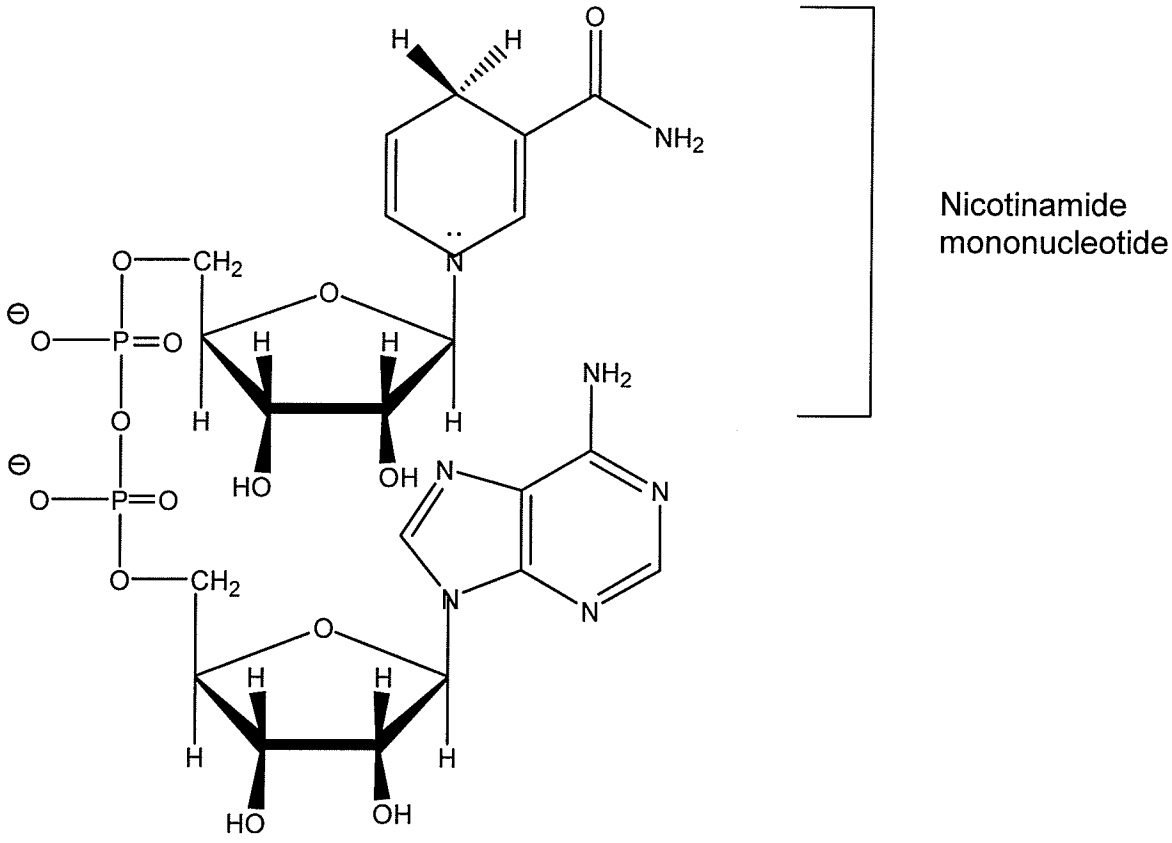
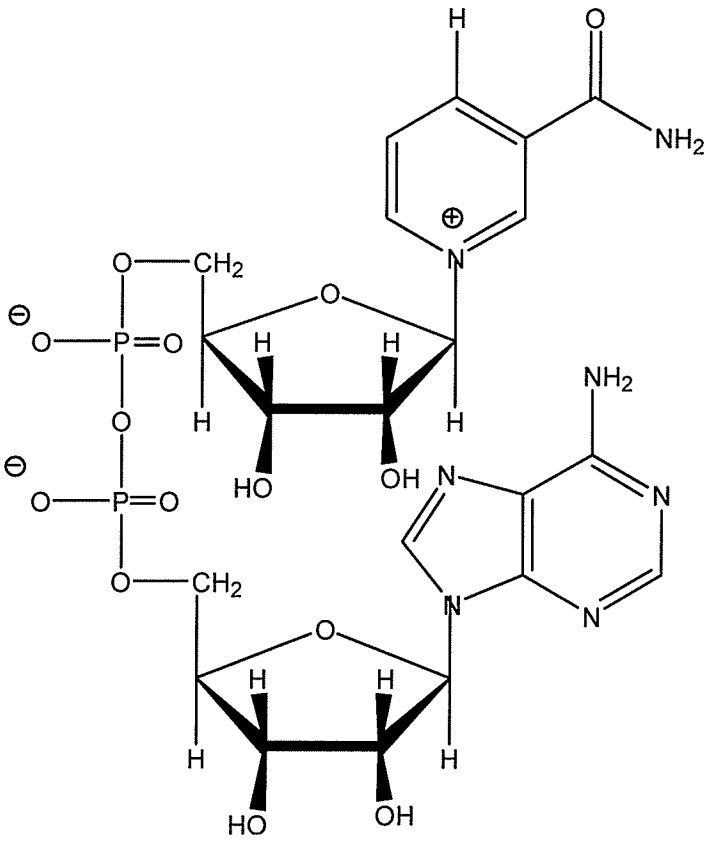


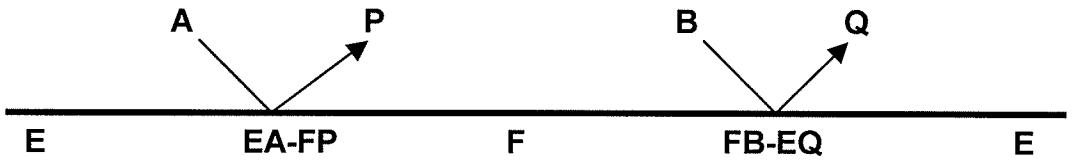
Figure 13 Chemical structure of NAD+



5.3 DTD mediated reduction

DTD follows a ping-pong mechanism of reduction kinetics (Figure 14) (Horton et al., 1993). In ping-pong kinetics, substrate A combines with free enzyme E to yield the complex EA from which product P departs. The departure of P leaves behind the covalently substituted enzyme F, which can then combine with substrate B to produce the FB complex. Subsequently this FB complex decomposes and forms product Q and free enzyme E.

Figure 14 Ping-pong reaction kinetics



Specifically, in terms of DTD, ping-pong mediated reduction kinetics occurs as follows: NAD(P)H binds DTD to yield the complex DTD-NAD(P)H. Subsequently, the FAD molecule is reduced by NAD(P)H to form $FADH_2$, which is followed by the release of $NAD(P)^+$ from the catalytic site. Leaving of the cofactor $NAD(P)^+$ from the catalytic binding pocket then allows for the binding of the substrate to this site. This is then followed by the hydride transfer from $FADH_2$ to the substrate, and release of the reduced substrate product from the enzyme catalytic binding pocket (Faig et al., 2000). Recently, in the case of quinone substrates, the His-161 residue, which lines the catalytic binding pocket, was also been shown to be important in the reduction of quinone substrates.

Specifically, this residue first acts as a hydrogen bond donor for the binding of the quinone substrate, and subsequent to the hydride transfer from $FADH_2$, the proton from His-161 is tranferred to the reduced quinone to produce the neutral hydroquinone product (Faig et al., 2001).

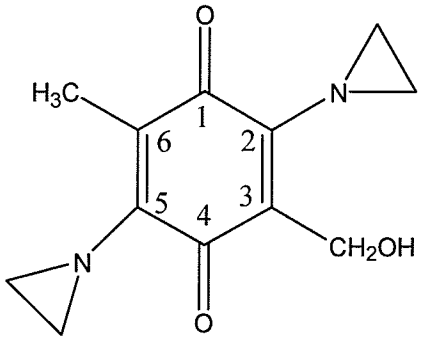
5.4 Inhibition of DTD

The anticoagulant, DIC is a competitive inhibitor of DTD with a K_i value of 10 nM in rat liver cytosolic DTD in the presence of the DTD substrate dichlorophenolindophenol (DCPIP) (Hollander and Ernster, 1975). In particular, it competes with NAD(P)H for binding to the oxidized form of the enzyme at the NAD(P)H binding site (Hollander and Ernster, 1975, Hosoda et al., 1974). Due to the competitive nature of this inhibition by DIC, as well as its ability to inhibit other cellular reductases, there has been interest in the development of a more selective inhibitor for this enzyme. Winski and colleagues reported the development of the DTD inhibitor, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936), which inhibits DTD through the formation of an ES936-DTD adduct (Winski et al., 2001). The specific and selective inhibitory characteristics ES936 will be beneficial in future studies aimed at the investigation of the precise role of DTD in the reduction and cytotoxic effects of experimental bioreductive alkylating agents.

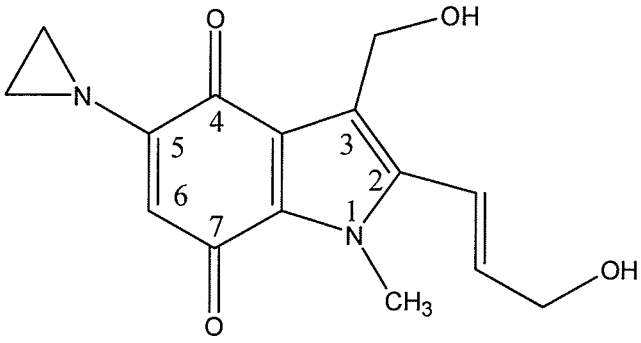
5.5 Substrate interaction with the binding pocket of DTD

The crystal-structures of complexes of DTD bound to the aziridinylbenzoquinone RH1, aziridinylindolequinones EO9 and ARH019 and the aziridinyldinitrobenzamide CB-1954 substrates have identified the precise spatial interaction between DTD and different substrates. In addition, the characteristics of these interactions has been shown to correlate well with structure-activity studies which examined the effects of functional groups on DTD mediated reduction (Figure 15) (Faig et al., 2001, Skelly et al., 1999). Overall, this information would be important to consider in the design of new bioreductive alkylating agents through the enzyme-directed drug targeting approach.

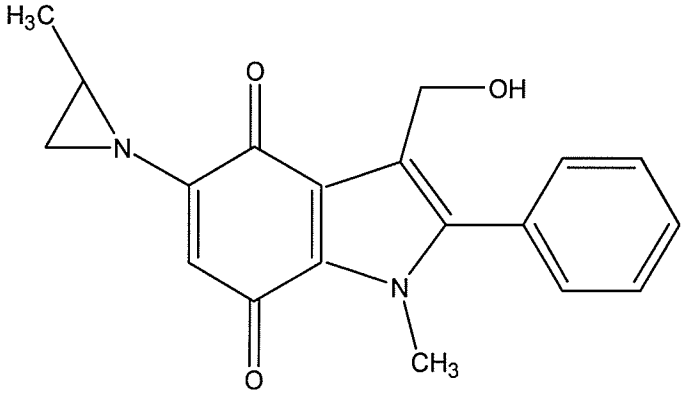
Figure 15



RH1



EO9



ARH019

In terms of aziridinylbenzoquinones, it was previously shown that these compounds only tolerate large functional group substitutions at either the 3 position or 6 position. In particular, the rates of DTD mediated reduction of compounds with large functional groups at either one of these positions were increased in comparison with compounds that had large functional group substitutions at both the 3 and 6 positions (Figure 15) (Gibson et al., 1992). This restriction appears to be consistent with the crystal structure of DTD bound to RH1. In particular, in the case of RH1, one of these two positions is directed to the outside of the binding pocket where large groups can be accommodated, while the other position faces the inside of the pocket where only small functional group substitutions are tolerated.

The crystal structures of the aziridinylindolequinones E09 and ARH019 bound to DTD revealed that even though these compounds had very similar structures, they bound to the enzyme in opposite ways (Faig et al., 2001). In the case of ARH019, the indolequinone departs from the exact aromatic stacking with the the isoalloxazine rings of FAD, the methyl-aziridine group at the 5 position stacks against Trp-105-indole and the 2-phenyl group points out of the active site pocket. On the other hand, the plane of the E09 molecule is parallel to the isoalloxazine rings of FAD and the 5-aziridinyl group is located at the Phe-106 in the binding pocket (Faig et al., 2001). Structure-activity studies in addition to the crystal-structure studies with these aziridinylindolequinones appeared to indicate that there was a relationship between binding orientation of the substrate and reduction by DTD. Specifically, the rates of reduction by DTD of analogs of

EO9 appeared to be decreased with larger functional groups at the 5 position (Bailey et al., 1992). On the other hand, analogs of ARH019, with larger functional groups at the 5 position, are reduced efficiently by DTD (Faig et al., 2001, Beall et al., 1998b). This observation was suggested to occur as a result of the alternate binding conformation of the ARH019 analogs compare to that of E09. As well, it suggests that DTD can accommodate bulky 5 position substituents with greater ease when these substrates are bound in the orientation of ARH019 versus the binding orientation of EO9. (Faig et al., 2001).

Another important finding from studies which analyzed the crystal structure of DTD bound to the quinone-based bioreductive agents is that the substrate binding pocket is capable of modifying its shape to accommodate the binding of a wide variety of compounds through movements of the side chains of specific amino acid residues in both monomers of the enzyme complex (Faig et al., 2001).

Overall, the crystal structure of DTD bound to substrate bioreductive alkylating agents indicates that compounds with similar structures may bind to the enzyme in different conformations, which can have a profound effect on the SARs observed, as well as the rate of reduction by DTD. Information obtained through molecular modeling and crystal structure analysis therefore appears to be a critical tool in the design of new bioreductive alkylating agents and in the design of analogs with increased specificity of reduction by DTD. In addition differing binding orientations may explain changes in rates of reduction by DTD observed with different compounds that have similar structures.

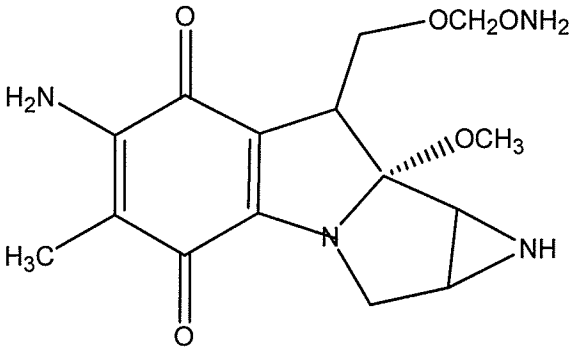
Section 6:

Prototype bioreductive alkylating agent, mitomycin C

6.1 Mitomycin C: Activation and environmental factors influencing cytotoxicity

Mitomycin C is considered the prototypical quinone-based bioreductive alkylating agent and has been used clinically in the treatment of solid tumors of the breast (Kalofonos et al., 2001), urinary bladder (superficial) (Crawford, 1996), stomach (Schnall and Macdonald 1993), pancreas (Kelsen, 1994), gastro-oesophagus (Slater et al., 2002) as well as in non small cell lung cancer (Spain, 1993) (Figure 16). Many enzymes have been implicated in the reductive activation of mitomycin C under aerobic and/or anaerobic conditions, and include NADPH: cytochrome P450 reductase, NADH cytochrome b5 reductase, xanthine oxidase and DTD (Bligh et al., 1990, Hodnick and Sartorelli 1993, Gustafson and Pritsos 1993, Ross et al., 1993).

Figure 16 Mitomycin C



DTD mediated reduction of mitomycin C was suggested to be important in the activation of mitomycin C to cytotoxic metabolites. Specifically, an early study showed that mitomycin C was 3-4-fold more cytotoxic to L5178Y/HBM10 cells which have a 24-fold increased DTD activity compared to the L5178Y parent cell line. Furthermore, the DNA damage produced in these cells through DNA cross-link formation could be partially reversed by the DTD inhibitor, DIC, suggesting that reduction by DTD of mitomycin C led to products which could cross-link DNA (Begleiter et al., 1989). In addition to inhibition of DTD having an effect on mitomycin C cytotoxicity, induction of the enzyme by 1.2-dithiole-3-thione in human cancer cell lines has also enhanced the cytotoxic potency of this compound (Wang et al., 1999). The *in vitro* reduction of mitomycin C by DTD was further supported by a study comparing DNA cross-link formation and cytotoxic activity in two different colon carcinoma cell lines; HT29 cells with very high DTD activity, and BE cells with no DTD activity. It was found that mitomycin C was 6-fold more cytotoxic to the HT29 cells than the BE cells, where no DNA cross-link formation was observed. As well, mitomycin C induced DNA cross-links and cytotoxicity could be inhibited by pretreatment with DIC (Siegel et al., 1990b).

Although DTD is important in the activation of mitomycin C, there are several cellular environmental factors, which have been shown to influence the activation of mitomycin C by this enzyme. DTD mediated reduction of mitomycin C is of greater significance under aerobic than hypoxic conditions. In a study with L5178Y/HBM10 cells which have high DTD activity, mitomycin C mediated

cytotoxicity and DNA cross-link formation were selectively decreased by the DTD inhibitor, DIC, under aerobic conditions, but not under hypoxic conditions (Begleiter et al., 1992). These results and that from other studies (Dulhanty and Whitmore 1991) therefore suggested that the one-electron reduction of mitomycin C by enzymes such as NADPH:cytochrome P450 reductase is more important in the activation of mitomycin C under hypoxic conditions as the semiquinone reactive intermediate is not back-oxidized under hypoxia, and therefore is able to form alkylating species, which are thought to be the most important mechanism by which mitomycin C induces cytotoxicity. In contrast, in the presence of oxygen, reduction by one-electron reductases may be less important, as now the semiquinone is able to back-oxidize and re-form the parent structure. Therefore, under aerobic conditions, reduction of mitomycin C by DTD to a more stable hydroquinone, which can alkylate DNA, or redox cycle to produce oxidative damage, appears to play a more important role in mediating cytotoxicity.

Another factor which contributes to the overall cytotoxicity of mitomycin C is pH. Data from a cell free system using purified rat DTD showed that metabolism of mitomycin C was increased as reaction buffer pH was decreased from 7.8 to 5.8, an effect not dependent on acid-mediated decomposition of mitomycin C (Siegel et al., 1990b). As well, it was shown that under aerobic conditions, but not hypoxic conditions, DIC completely reversed the enhanced cytotoxicity of mitomycin C under acidic pH (6.6) in Chinese hamster ovary cells to an activity similar to that observed at neutral pH (7.2). Therefore, it appears

that under acidic conditions, the enhanced cytotoxic potency of mitomycin C is due to increased activation via DTD, an effect that only seems relevant under aerobic conditions (Begleiter and Leith, 1993). The mechanism by which pH enhances DTD mediated activation of DTD has been studied extensively, and is suggested to occur due to preferential inhibition of DTD by mitomycin C under neutral pH (6.5-7.4), compared to acidic pH (5.8) by studies using purified rat DTD and cell culture studies using HT29 cells (Siegel et al., 1993). The precise pharmacological mechanism of inhibition was suggested to occur due to cross-link formation between a reduced mitomycin C metabolite and DTD at alkaline but not acidic pH (Siegel et al., 1993). From recent data it also appears that the inhibition may be tissue specific, but to date there is no strong evidence suggesting that inhibition of DTD by mitomycin C occurs in patients at therapeutic concentrations. Therefore, further studies are needed to determine if this proposed mechanism of inhibition of DTD has any clinical significance (Gustafson et al., 2003).

Collectively, the cytotoxicity of mitomycin C appears to be influenced by multiple factors, including the types of intracellular reductases present within the tumor environment, and their respective activities, intracellular pH and oxygen tension. These factors may not only be important in the determination of the cytotoxicity of mitomycin C, but may be important to consider in the determination of antitumor activity, as well as augmentation of therapeutic efficacy of other bioreductive alkylating agents currently investigated. These factors may also be

important to consider in the design of novel and more selective bioreductive alkylating agents.

6.2 Mitomycin C: Mechanisms of cytotoxicity

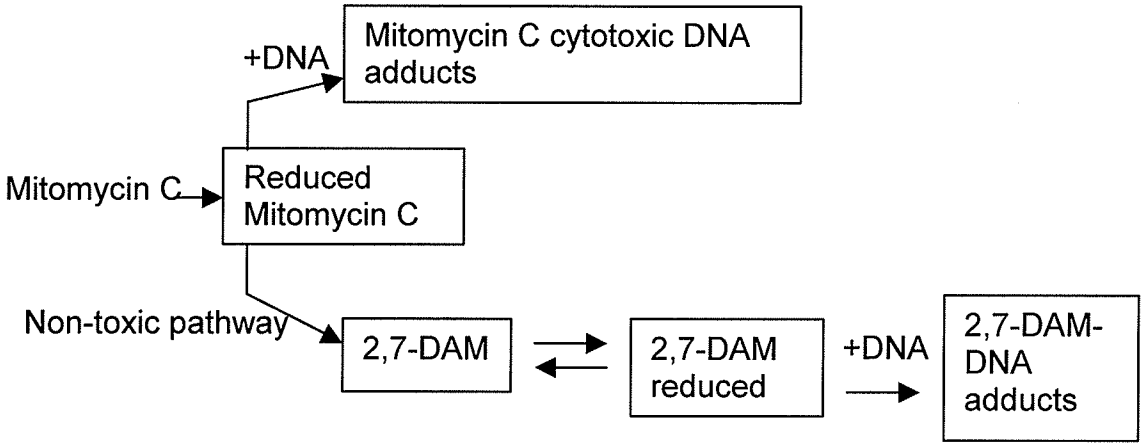
Reductive bioactivation of mitomycin C by either one- or two-electron reductase enzymes leads to metabolites capable of cytotoxic activity through DNA alkylation or DNA cross-link formation. Furthermore, the semiquinone reactive intermediate formed by one-electron reduction of the quinone and reactive oxygen species formed via redox cycling of the reduced products are capable of oxidative damage including DNA strand breakage.

Several major mitomycin C-DNA adducts formed in cell-free systems following chemical and enzymatic reduction (e.g. DTD) have been characterized (Pan et al., 1986, Tomasz et al., 1986, 1887, Tomasz and Palom 1997, Suresh Kumar et al., 1996, Sartorelli et al, 1994). As well, reduction of mitomycin C by DTD results in the formation of DNA adducts *in vitro* (Prakash et al., 1993). Recently, the adducts formed in cell free systems were found to correspond to the six major mitomycin C-DNA adducts formed in EMT6 mouse mammary tumor cells subsequent to mitomycin C treatment, and supports a role for these adducts in the mechanism of action of mitomycin C (Palom et al., 1998).

The chemical structure of these adducts indicated that there are two main pathways of reduction of mitomycin C in tumor cells (Palom et al., 2001) (Figure 17). One pathway, involves the formation of four cytotoxic adducts from reduced mitomycin C and results in mitomycin C mono-adducts, and DNA interstrand or

intrastrand cross-linked adducts. The second pathway involves the formation of 2 non-toxic adducts from the major metabolite of mitomycin C, 2,7-diaminomitosene (2,7-DAM), and can thus be considered as an inactivation pathway (Palom et al., 1998, 2001).

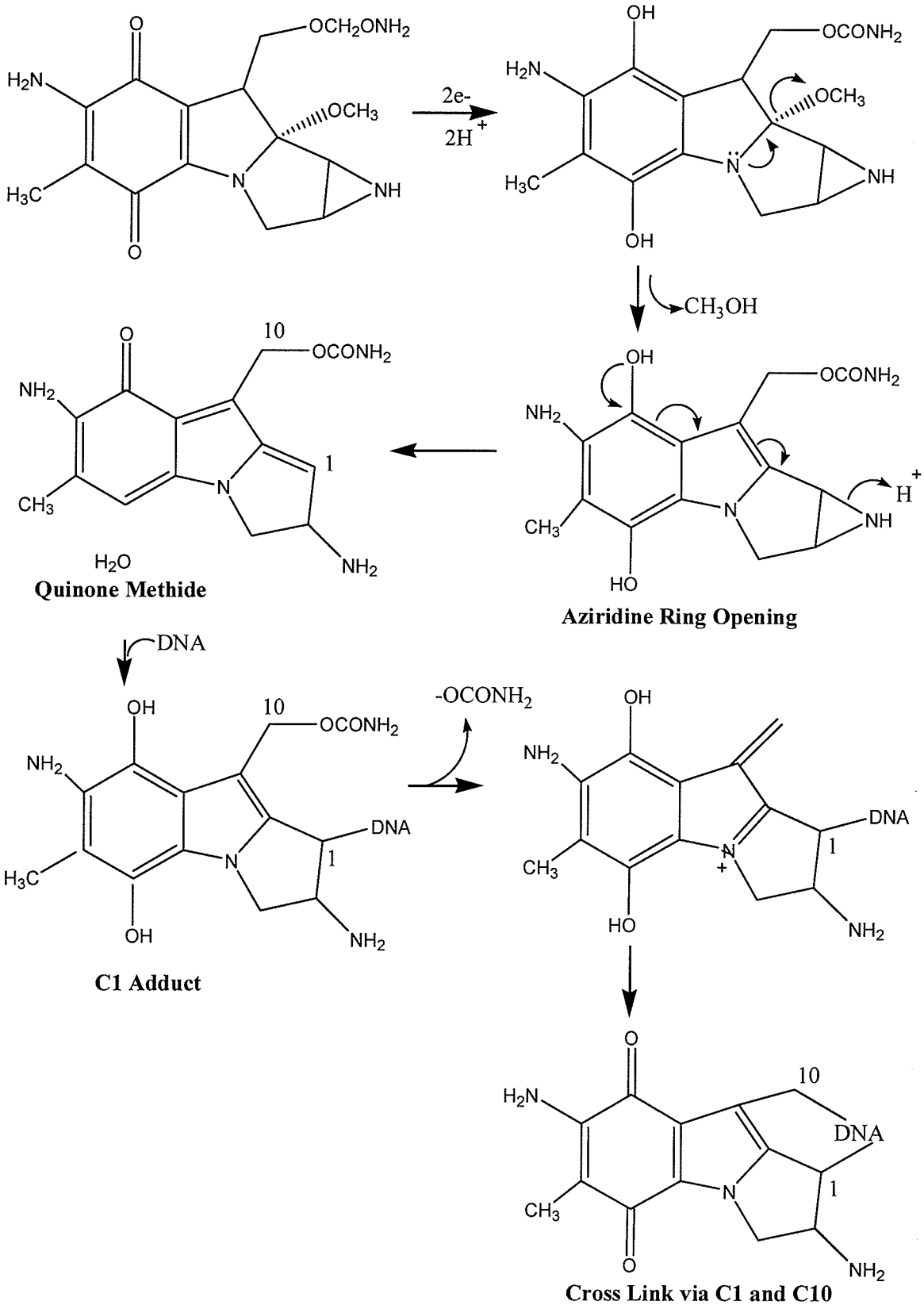
Figure 17



Reproduced from Palom et al., 2001 with permission.

DNA interstrand cross-link formation is considered as one of the most cytotoxic lesions formed by mitomycin C. DTD mediated reduction leads to the activation of two electrophilic sites located at the C(1) and C(10) positions of the molecule. Cross-link formation involves first, the formation of a covalent bond between one of these electrophilic sites on mitomycin C and a nucleophilic NH_2 at the 2 position of guanine. Subsequently, cross-link formation is completed through a second alkylation reaction of the other electrophilic site to the NH_2 at the 2 position of an adjacent guanine (Tomasz et al., 1987) (Figure 18).

Figure 18


2e-
2H+
CH3OH
H2O
DNA
-OCONH2
Quinone Methide
Aziridine Ring Opening
C1 Adduct
Cross Link via C1 and C10

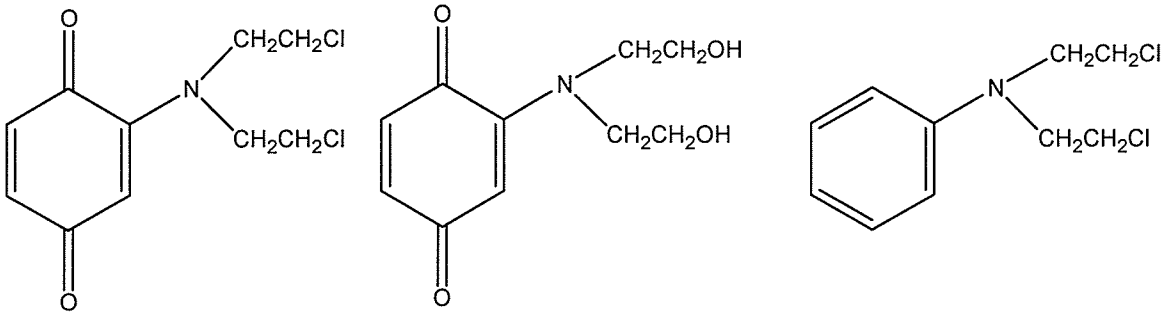
Section 7:

Benzoquinone mustard (BM) and its structural analogs

7.1 Impact of the benzoquinone bioreductive element on the cytotoxic activity of BM and its structural analogs

Structure-activity studies with the model bioreductive alkylating agent benzoquinone mustard (BM) have led to an increased overall understanding of the mechanisms by which bioreductive alkylating agents mediate cytotoxicity and anti-tumor effects. Both free radical/reactive intermediate induced oxidative damage and DNA alkylation through reductive activation of the cytotoxic group of quinone-based bioreductive alkylating agents have been shown to be important in mediating the cytotoxicity of these compounds. It has also been shown that these two different mechanisms of cytotoxicity, within the same compound, can act synergistically to enhance its cytotoxic potency. This was indicated through comparisons between BM, with both a quinone-based bioreductive element and a cytotoxic mustard group, hydrolyzed benzoquinone mustard with only a quinone-based bioreductive element, and aniline mustard with only a cytotoxic mustard group (Figure 19).

Figure 19



Specifically, the cytotoxic potency of BM in L5178Y murine lymphoblasts was 30,000 times greater than that of hydrolyzed benzoquinone mustard, and 600 times greater than that of aniline mustard. As well, BM was 200 times more cytotoxic than equal molar concentrations of both hydrolyzed benzoquinone mustard and aniline mustard combined, indicating a synergistic mechanism of cytotoxicity for BM. The improved cytotoxicity was suggested to occur through an enhanced activity of the quinone group which may produce reactive oxygen species in close proximity to the target DNA by the ability of this agent to bind to DNA (Begleiter, 1983).

The increased cytotoxic activity observed with BM compared to hydrolyzed benzoquinone mustard and aniline mustard was further investigated by development of strains of L5178Y cells, (L5178Y/HBM2 and L5178Y/HBM10), which were resistant to hydrolyzed benzoquinone mustard (Begleiter et al., 1988). The mechanism of resistance of these cells has not been fully elucidated, but may be due to the combined or singular effects of an elevated intracellular glutathione concentration and increased superoxide dismutase, catalase, and DTD activity observed in these cells compared to the parent cell line. For example, DTD activity was 3-fold increased in the L5178Y/HBM2 cells and 24-fold increased in the L5178Y/HBM10 cells, compared to the parent cell line (Begleiter et al., 1988). Furthermore, relative to the parent cell line, the cytotoxic activities of the quinone containing BM and hydrolyzed benzoquinone mustard were significantly less in the resistant cell lines. Interestingly, the decreased cytotoxicity observed for BM was partially reversed with the DTD inhibitor, DIC.

As well, the 7-fold decrease in cytotoxicity observed with BM in the L5178Y/HBM2, compared to the parent cell line, was paralleled with a 3-fold decrease in DNA interstrand cross-link formation and a 2-fold decrease in DNA strand break formation (Begleiter et al., 1990). In contrast, for the non-quinone containing aniline mustard analog, the cytotoxic activity, and the mechanisms by which DNA damage was produced, were not altered when comparing the parent and resistant cell lines (Begleiter et al., 1990).

Overall, these studies suggested that the decreased cytotoxicity of quinone-containing alkylating agents may be related to a decrease in DNA cross-link and strand break formation and the altered regulation of the quinone moiety in the resistant cell line, compared to the parent cell line. This is supported by the finding that in the case of the non-quinone containing aniline mustard analog, its cytotoxic activity and DNA cross-link formation were not altered in the resistant cell line (Begleiter et al., 1990). Suggested mechanisms by which this altered regulation of the quinone may occur include increased reduction of the quinone to the hydroquinone product as a result of increased DTD activity in the resistant cell lines compared to the parent cell line. This increased reduction by DTD may also result in relatively inactive hydroquinone reduced products compared to possible semiquinone reduced products formed by other reductase enzymes. Thus, DTD may inactivate BM via increased formation of the hydroquinone product in the resistant cell lines compared to the parent cell line. The increased reduction via DTD may lead to an increased activity of cellular inactivation pathways through glutathione conjugation of the hydroquinone.

Furthermore, there may also be changes in the balance between hydroquinone formation and the formation of other semiquinone intermediates which could impact the mechanisms of cytotoxicity of quinone containing agents in the resistant cell line. Alternatively, there may be decreased reactive oxygen species due to elevated concentrations of superoxide dismutase and catalase. Further studies are needed to elucidate the exact mechanisms important in determining the cytotoxic activity of these benzoquinone mustard bioreductive alkylating agents.

7.2 The benzoquinone bioreductive element and cytotoxic mustard groups: Effects on mechanism of cytotoxicity of BM and its analogs

Differences in cytotoxic potency of BM and its structural analogs have also been accompanied by alterations in the precise mechanisms by which cytotoxicity is induced (i.e. DNA cross-link or strand break formation). These observed changes have been important in increasing our understanding of the exact mechanisms by which either the cytotoxic element or the bioreductive element induces DNA damage. In these studies, the potential to induce DNA cross-link and strand break formation was assessed through alkaline elution assays for BM, hydrolyzed benzoquinone mustard and aniline mustard (Begleiter and Blair, 1984). The quinone structure within these model compounds was shown to be responsible for the production of DNA single strand and double strand breaks in the L5178Y lymphoblast cells, and was inhibited by free radical and reactive oxygen species scavengers, superoxide dismutase and

catalase. In particular, BM was shown to produce approximately 15,000 fold more DNA double strand breaks than hydrolyzed benzoquinone mustard. This difference again appears to be attributed to the ability of BM to bind to DNA. On the other hand, aniline mustard did not produce any DNA strand breaks, consistent with the absence of the quinone moiety in this compound. The cytotoxic mustard groups found in BM and aniline mustard were also indicated to be important in the concentration dependent increase in formation of DNA cross-links, where BM was most efficient. The studies with these compounds provided some evidence that both DNA strand break and cross-link formation may be important in mediating the cytotoxicity of BM and hydrolyzed benzoquinone mustard. For example, DNA strand break formation at the whole cell level correlated positively with the cytotoxic activity of both compounds. Furthermore, in a study with L5178Y/HN2 cells, which are resistant to alkylating agents, and L5178Y cells which are not, it was found that the resistant cells were 2-fold more resistant to the alkylating quinone BM compared to the non resistant cells (Begleiter, 1985). In contrast, no resistance was observed towards the non-alkylating hydrolyzed benzoquinone mustard (Begleiter, 1985). As well, the alkylating quinone, BM, showed a 2-fold decrease in DNA cross-link formation in the resistant cells compared to the sensitive cells, while the induction of double strand breaks by BM was reduced 5-fold. On the other hand, DNA strand break formation was similar for the hydrolyzed benzoquinone mustard when comparing the resistant and sensitive cells. These data suggest that the alkylating activity cannot directly explain all of the enhanced cytotoxic activity of the akylating

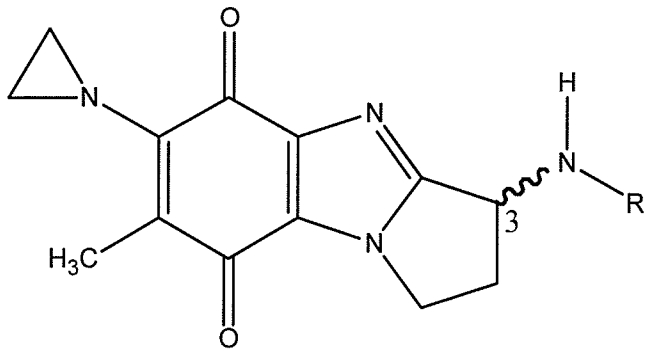
quinone BM. Furthermore, it suggests that the enhanced formation of DNA double strand breaks by alkylating quinones compared to non alkylating quinones (Begleiter and Blair, 1984) is in part related to the ability of BM to bind DNA. These conclusions would have however been strengthened by increasing the number of analogs studied, to include compounds with very low or high cytotoxic activity compared to BM.

Section 8:

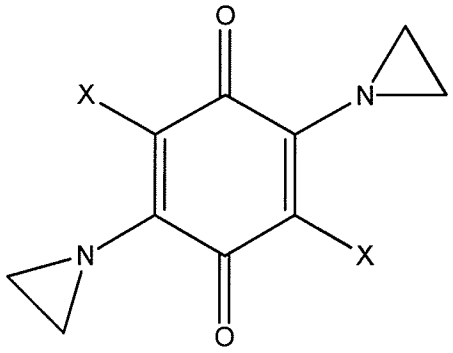
Structure-activity studies with quinone-based bioreductive alkylating agents: Effects on DNA alkylation and/or DNA strand-break formation

Subsequent to bioreductive activation by enzymes such as DTD and NADPH:cytochrome P450 reductase, quinone-based bioreductive alkylating agents may produce cytotoxic activity at the target DNA level through alkylation by the cytotoxic elements of the reduced products, or by oxidative damage (e.g. DNA strand break formation) mediated by reactive oxygen species and reactive intermediates. Within the enzyme-directed approach for bioreductive alkylating agent development, several different types of quinones have been investigated for efficient bioactivation by DTD via SAR studies. These potential quinone-based antitumor agent classes include aziridinylbenzoquinones, indolequinones, pyrrolobenzimidazoles, indoles, cyclopent[b]indoles and cyclopropamitosenes (Figure 20).

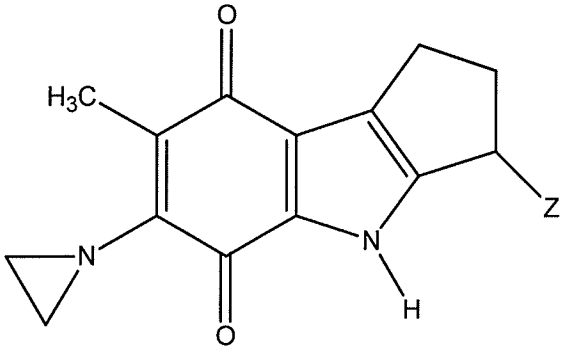
Figure 20



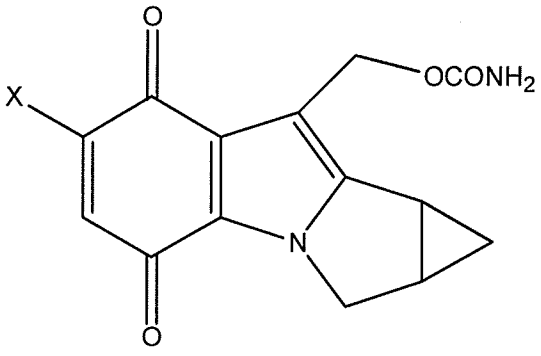
Pyrrolo[1,2-*a*]benzimidazole



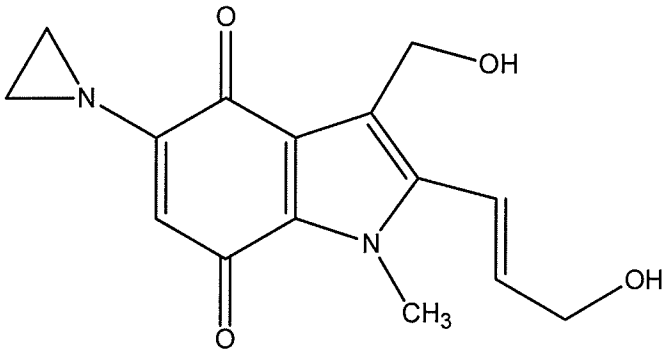
Aziridinylbenzoquinone



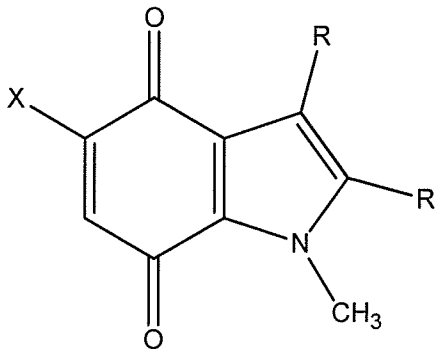
Cyclopent[b]indole



Cyclopropamitosene



Indolequinone EO9

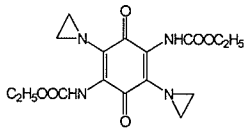
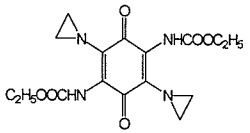


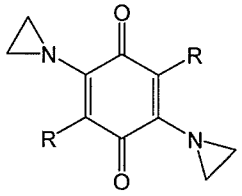
Indolequinone

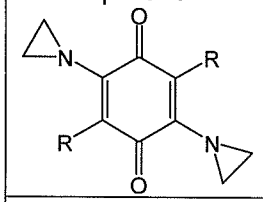
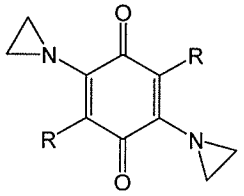
Although the cytotoxic effects of quinone-based bioreductive agents are thought to be due to DNA cross-link and strand break formation, there are limited data demonstrating a direct association between the cytotoxic activity of these agents and DTD mediated reduction kinetics or the extent and type of DNA damage. Furthermore, it is unclear at present how varying functional group substitutions on the quinone moiety of bioreductive alkylating agents may influence their cytotoxic activity and ability to form DNA cross-links or strand breaks. SAR studies with a focus on determining structural characteristics of quinone-based bioreductive alkylating agents which impact DTD mediated reduction, have also investigated the impact of structural changes on mechanisms of DNA damage and cytotoxicity. These studies have been summarized in Table 2, and focus on the effects of structural alterations to aziridinylbenzoquinones, indolequinones, cyclopent[b]indoles and benzoquinone mustards on DTD mediated activation in relation to mechanisms of DNA damage. Several different *in vitro* methodologies have been used to assess DTD mediated DNA alkylation, cross-link formation and strand break formation. These techniques include those that measure DNA cross-link or strand break formation at the whole cell level (e.g. alkaline elution technique; comet assay) and agarose gel assays which assess DNA cross-link or strand break formation to plasmid DNA by specific bioreductive alkylating agents. Whole cell assays are advantageous to determine the overall potential of experimental agents to produce DNA damage at the whole cell level in the presence of complex cellular factors such as DNA repair mechanisms, and detoxification pathways. On the

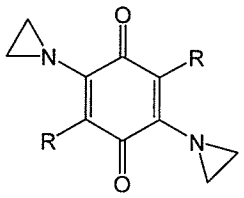
other hand, agarose gel assays may be conducted in the absence of such cellular factors in order to specifically assess the effects of structural modifications to model bioreductive agents on the precise mechanism of cytotoxicity.

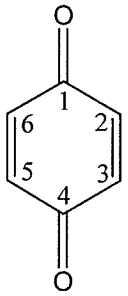
Table 2. Effects of functional group substitutions to bioreductive alkylating agents in relation to DNA alkylation, DNA cross-link formation or DNA strand break formation.

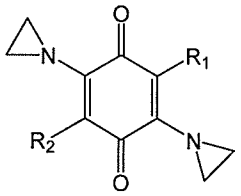
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Aziridinyl-benzoquinone AZQ 	None	*In vitro* alkaline elution assays to assess DNA interstrand cross-link and strand break formation in human HT-29 cells (high DTD activity)	Used concentration -effects study design in all assays	No DNA strand break formation Concentration dependent increase in DNA cross-link formation	Szmigiero et al., 1984
Aziridinyl-benzoquinone AZQ 	None	*In vitro* alkaline elution assays to assess DNA interstrand cross-link and strand break formation in human HT-29 cells (High DTD activity) and BE cells (low DTD activity)	Used concentration -effects study design in all assays	Concentration dependent increase in DNA cross-link formation by AZQ in the HT-29 cells. Effect inhibited by DIC Small amounts of DNA cross-links formed in BE cells No DNA strand break formation observed	Siegel et al., 1990a

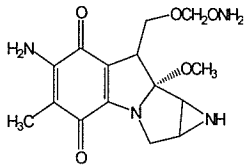
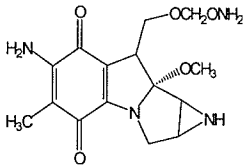
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Aziridinyl-benzoquinones 	**Drug** **R** BZQ $NHCH_2CH_2OH$ DZQ H MeDZQ CH_3	*In vitro* alkaline elution assays to assess DNA interstrand cross-link and strand break formation in human HT-29 cells (High DTD activity) and BE cells (low DTD activity)	Alkaline elution assays performed under aerobic conditions Used concentration -effects study design in all assays	Concentration dependent increase in DNA strand break formation by DZQ in the HT-29 cells. Effect inhibited by DIC Concentration dependent increase in DNA cross-link formation by MeDZQ in the HT-29 cells. Effect inhibited by DIC DZQ and MeDZQ less cytotoxic in BE cells than HT-29 cells Correlation between DTD mediated reduction and cytotoxicity in the HT-29 cells BZQ induced DIC-insensitive DNA interstrand cross-links in both cell lines	Gibson et al., 1992

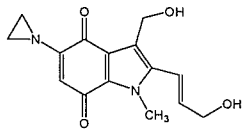
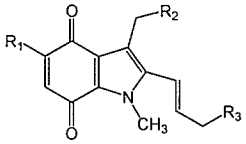
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Aziridinyl-benzoquinone 	**Drug** **R** AZQ $NHCOOC_2H_5$ BZQ $NHCH_2CH_2OH$	*In vitro* comet assay	Used concentration -effect study design in all assays	Concentration dependent increase in DNA strand break formation by AZQ in the K562 cells, and small amounts of strand breaks in the K562R cells	Ward et al., 1997
Aziridinyl-benzoquinone (con'd) 	**Drug** **R** AZQ $NHCOOC_2H_5$ BZQ $NHCH_2CH_2OH$	Assessed DNA cross-link and strand break formation in human K562 cells and a K562R cells resistant to AZQ and BZQ		Cross-link formation by AZQ could not be assessed as it would have been masked by DNA strand break formation Concentration dependent increase in DNA cross-link formation by BZQ in the K562 cells, and was reduced in the K562R cells No strand breaks produced by BZQ in K562 or K562R cells	Ward et al., 1997

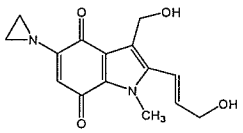
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Aziridinyl-Benzoquinones 	**Drug** **R** BZQ HCH_2CH_2OH DZQ H MeDZQ CH_3 D1(AZQ) $NHCOOCH_3$ D2 $NHCOOC_2H_5$ D3 $NHCOOC_3H_7$ (*n*-propyl) D4 $NHCOOC_3H_7$ (*i*-propyl) D5 $NHCOOC_4H_9$ (*n*-butyl) D6 $NHCOOC_4H_9$ (*i*-butyl) D7 $NHCOOC_4H_9$ (*sec*-butyl)	*In vitro* agarose gel assay - DNA interstrand cross-link formation	Reduction by rat DTD DZQ, MeDZQ used concentra-tion-effect study design All other analogs used a single concentra-tion design	Analogs produced different amounts of DNA cross-links following DTD mediated reduction: DZQ > MeDZQ >> D1 > D3 > D2 > D5 > D7 > D4 > D6 BZQ was not substrate for DTD Extent of DNA cross-link formation decreased as size of alkyl substituents increased Ease of DNA cross-link formation followed reduction rate by DTD and cytotoxicity *in vitro* DTD mediated reduction affected sequence selectivity of alkylation by analogs	Lee et al., 1992

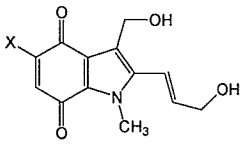
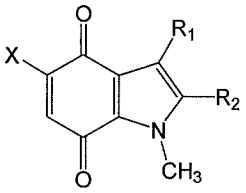
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Aziridinyl-benzoquinones (Quinone Methides) 	**Drug 2 3 5 6** 1 Me H H H 2 Me Me H H 3 Me H Me H 4 Me H H Me 5 Me Me Me H 6 Me Me Me Me 7 Az H Me H 8 Az H Me Me 9 Az Me Me H 10 Az H Et H 11 Az H Pr H 12 Azet H Azet H 13 Azet H Me H 14 NH2 H Me H 15 NH2 H NH2 H 16(DZQ) Az H Az H Az = aziridinyl. Axet = azetidinyl	*In vitro* agarose gel assay - DNA interstrand cross-link formation DNA cross-link formation assessed at 10 μM and 100 μM concentrations of each analog	Reduction by ascorbic acid	Analog 16 (DZQ) was very efficient DNA cross-linking agent Analogs 1-6 and 12-15 did not cross-link DNA Analog 7 was more efficient at cross-linking than compounds 8 and 9 Cross-link formation decreased with an increase in length of the methylene chain at position 5 in compounds 7, 10 and 11	Mayalarp et al., 1996

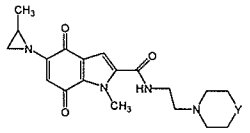
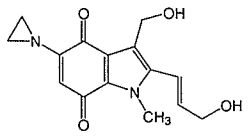
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Aziridinyl-benzoquinones 	**Drug** **R_1** **R_2** 1 CH_3 CH_3 2 CH_3 C_2H_5 3 CH_3 *n*-C_3H_7 4 CH_3 *i*-C_3H_7 5 C_2H_5 C_2H_5 6 *n*-C_3H_7 *n*-C_3H_7 7 *i*-C_3H_7 *i*-C_3H_7 8 H H	*In vitro* agarose gel assay - DNA interstrand cross-link formation	Chemical reduction in agarose gel assay DNA cross-link formation assessed using 1μM, 10μM and 100 μM concentra-tions of each analog	DNA cross-link efficiency decreased as size of alkyl group side chain increased A methyl group allows for more efficient DNA cross-linking than the disubstituted analogs (2 vs. 5, or 3 vs. 6). This is as a result of less steric hindrace to prevent DNA cross-link formation Analog 7 did not produce DNA cross-links Rate of reduction by human DTD correlated with DNA cross-link formation through chemical reduction of the analogs Cross-link efficiency followed DTD mediated reduction and cytotoxicity in the HT-29 and H460 cells vs. the BE and H596 cells, respectively	Hargreaves et al., 1999

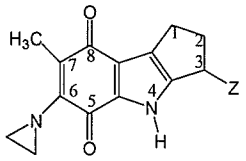
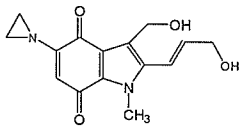
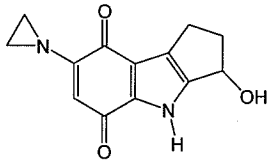
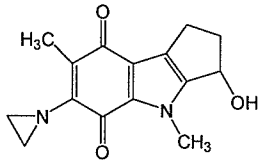
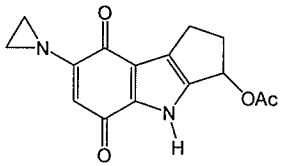
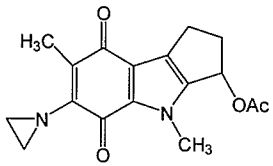
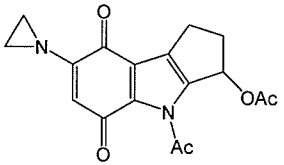
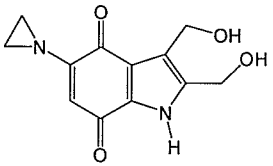
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Mitomycin C 	None	*In vitro* alkaline elution assays to assess DNA interstrand cross-link and strand break formation in human HT-29 cells (High DTD activity) and BE cells (low DTD activity)	Assays performed in presence and absence of the DTD inhibitor, DIC Used concentration -effect study design	Mitomycin C induced DNA cross-links in the HT-29 cells, but not in the BE cells Cross-link formation inhibited by DIC Metabolism of mitomycin C increased as pH was lowered to 5.8 No DNA single strand breaks formed in either cell line	Siegel et al., 1990b
Mitomycin C 	None	*In vitro* agarose gel assay - DNA interstrand cross-link formation	Reduction by rat DTD and human DTD in agarose gel assay Used 10μM mitomycin C with increasing concentrations of DTD	DTD concentration dependent increase in DNA cross-link formation	Beall et al., 1994

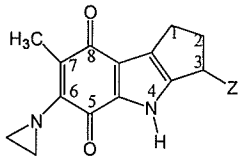
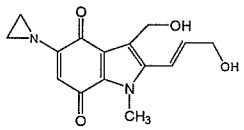
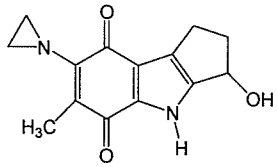
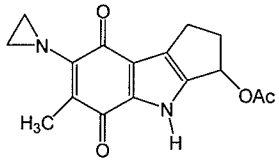
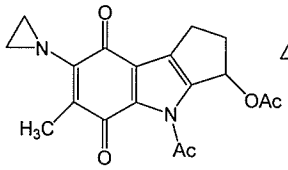
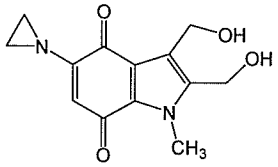
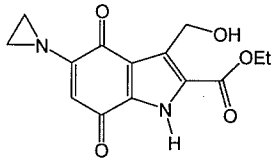
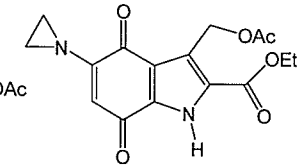
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Indolequinone EO9 	None	*In vitro* agarose gel assay - DNA single strand break formation	Reduction by rat DTD in agarose gel assay Concentration-effect study design	Concentration dependent increase in DNA single strand breaks	Walton et al., 1991
Indolequinone EO9 analogs 	**Drug** **R_1** **R_2** **R_3** EO1 OCH_3 $OCOCH_3$ $OCOCH_3$ EO2 OCH_3 OCO_2CH_3 OCO_2CH_3 EO4 Az $OCOCH_3$ $OCOCH_3$ EO9 Az OH OH EO15 NHC_6H_5 OH OH EO68 Az NHC_6H_5 OH EO72 Az OCO_2CH_3 H Az = aziridinyl	*In vitro* agarose gel assay - DNA interstrand cross-link formation	Reduction by human DTD in agarose gel assay DNA cross-link formation assessed using single 100 μM concentration of each analog except EO4 which followed a concentration -effect study design	EO9, EO68 and EO4 were substrates for DTD mediated reduction EO9, EO68 and EO4 preferentially toxic to the H490 cells (high DTD activity), vs. H596 cells (low DTD activity) No DNA cross-link formation in absence of DTD No DNA cross-link formation in presence of DTD, except for EO4 Concentration dependent cross-link formation with EO4 inhibited by DIC	Phillips et al., 1996

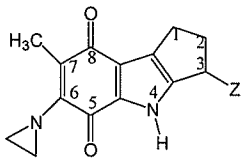
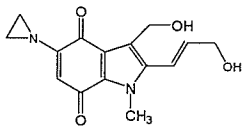
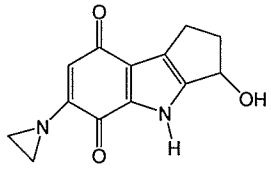
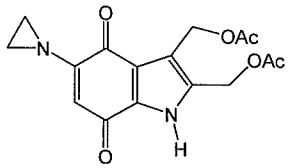
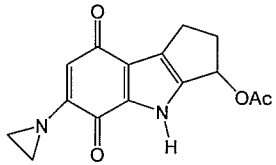
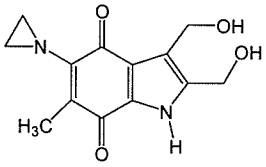
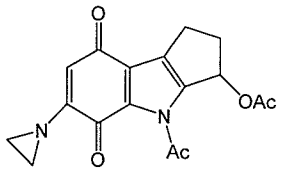
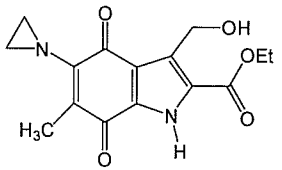
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Indolequinone EO9 	None	*In vitro* alkaline elution assays to assess DNA interstrand cross-link and strand break formation in human HT-29 cells (High DTD activity) and BE cells (low DTD activity) *In vitro* agarose gel assay - DNA interstrand cross-link formation	Alkaline elution assays performed under aerobic and anaerobic conditions Used concentration -effects study design in all assays Reduction by rat DTD in agarose gel assay	Cytotoxicity of EO9 increased in HT-29 cells versus BE cells under aerobic conditions Under hypoxia, cytotoxicity of EO9 in the BE cell line was increased versus under aerobic conditions. Hypoxia did not affect the cytotoxicity of EO9 in the HT-29 cells EO9 induced DNA strand breaks and cross-links in the HT-29 cells in air, and this correlated with cytotoxicity DNA cross-link formation potentiated in the BE cell line under hypoxia versus air. (Possibly due to activation by one-electron reductase enzymes) Concentration dependent increase in DNA cross-link formation observed in agarose gel assay	Bailey et al., 1997

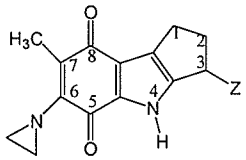
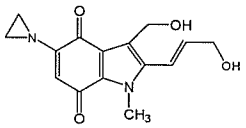
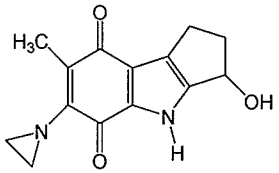
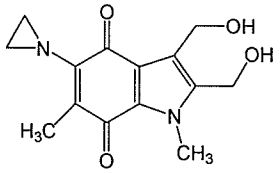
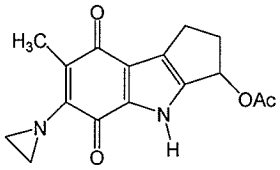
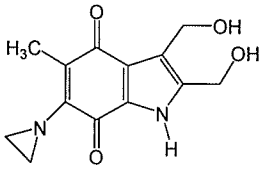
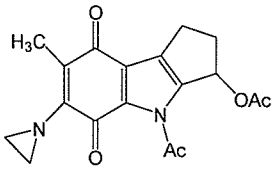
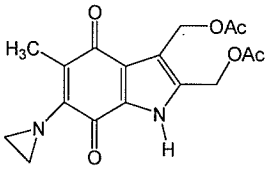
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Indolequinones EO9 analogs (2)  Indolequinones (3) 	**Drug X R_1 R_2** 2a Az 2b 2-MeAz 2c OCH_3 3a OCH_3 CH_2OH Ph 3b Az CH_2OH H 3c Az H $CH=CHCH_2OH$ 3d OCH_3 CH_2OH H 4a (Y = O) 4b (Y = CH_2) 3e Az CH_2OH C-Pr 3f Az CH_2OH CH_3 3g 2-MeAz CH_2OH Ph 3h 2-MeAz CH_2OH CH_3 3i OCH_3 OH CH_3 3j 2,3-Me2Az OH CH_3 3k OCH_3 CH_2OCONH_2 $CH(CH_3)_2$ 3l OCH_3 CH_2OCO-o-F-Ph H Az = aziridinyl	*In vitro* agarose gel assay - DNA single strand break formation	Reduction by DTD DNA strand break formation assessed using single 4 μM concentration of each analog	No correlations between DTD mediated reduction, cytotoxic potency *in vitro*, or DNA single strand break formation	Phillips et al., 1999

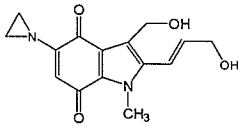
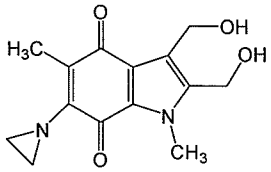
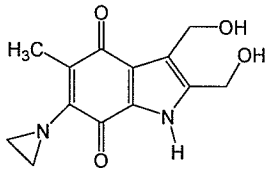
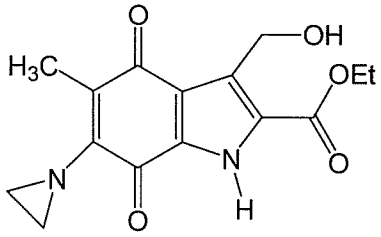
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Indolequinones (con'd) EO9 Indole-2-carboxamides (4) 	**Drug X** **R_1** **R_2** 3m OCH_3 CH_2O-2,4,6-tri-Cl-Ph H 3n OCH_3 CH_2OCONH_2 c-Pr 3o OCH_3 CH_2OCOPh H 3p OCH_3 CH_2OCOPh-p-F H 3q OCH_3 CH_2Cl H 3r OCH_3 CH_2OCO-p-F CH_3 3s OCH_3 CH_2CO-p-FPh Ph			No DNA strand break formation in absence of DTD	Phillips et al., 1999
Indolequinone EO9 	None	*In vitro* agarose gel assay - DNA single strand break formation	Reduction by cytochrome P450 reductase Used concentra-tion-effect study design	Concentration dependent DNA single strand break formation	Bailey et al., 2001

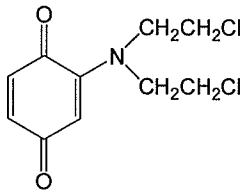
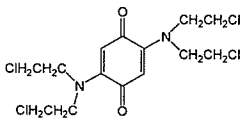
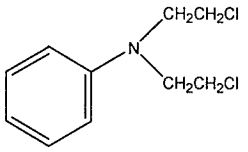
Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Cyclopent [b] indoles (1-5)  Indoles (6-9) 	**Drug Structure Drug Structure** 1a  5a  1b  5b  1c  6a 	*In vitro* spectrophotometic analysis of drugs bound to DNA (DNA alkylation)	Assay performed under anaerobic conditions	6-methyl group in quinone ring of series 1 reduces alkylation compared to analogs with only an aziridinyl group (series 2) N-Methylation of series 5, or indoles (7c, 8c) results in complete loss of DNA alkylation N-Acetylation of 1c-4c decreases DNA alkylation Acetate leaving group at 3α-position of the indole (6a, 6e, 8b) leads to high DNA alkylation An ethoxycarbonyl at the 2-postion of the indole leads to high DNA alkylation, if there is also an acetate or hydroxide leaving group at the 3α-position. 6c and 6d are excellent DNA alkylating agents	Skibo et al., 2001

Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Cyclopent [b] indoles (1-5) (con'd)  Indoles (con'd) (6-9) 	**Drug Structure Drug Structure** 2a  2b  2c  6b  6c  6d 				Skibo et al., 2001

Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Cyclopent [b] indoles (con'd) (1-5)  Indoles (6-9) (con'd) 	**Drug Structure Drug Structure** 3a  6e  3b  7a  3c  7b 				Skibo et al., 2001

Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Cyclopent [b] indoles (con'd) (1-5)  Indoles (6-9) (con'd) 	**Drug Structure Drug Structure** 4a  7c  4b  8a  4c  8b 				Skibo et al., 2001

Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Indoles (6-9) (con'd) 	**Drug Structure Drug Structure** 8c  9a  9b 				Skibo et al., 2001

Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Benzoquinone Mustard (BM)  Benzoquinone dimustard (BDM)  Aniline mustard (AM) 	BM BDM AM	*In vitro* alkaline elution assays to assess DNA interstrand cross-link and strand break formation Used parent murine L5178Y lymphoblast cells and L5178Y /HBM10 and L5178Y HBM/2 cells (higher DTD activity than the L5178Y parent cell line, and resistant to hydrolyzed BM)	Alkaline elution assays under aerobic conditions Used concentration -effects study design in all assays	L5178Y/HBM2 and L5178Y/HBM10 cells were 7- and 9- fold less sensitive to BM and 2- and 4- fold less sensitive to BDM, respectively *vs.* L5178Y cells DIC increased the cytotoxic activity of BM and BDM in the L5178Y/HBM10 cells The resistant cells showed no resistance to the alkylating AM analog DNA double strand breaks formed by BM was reduced by 2-and 8- fold in the L5178Y/HBM2 and L5178Y/HBM10 cells respectively *vs.* the L5178Y cells DNA cross-link formation produced by BM was 3- and 6-fold lower in the L5178Y/HBM2 and L5178Y/HBM10 cells respectively *vs.* the L5178Y cells	Begleiter & Leith, 1990

Compound Class and Structure	***Analogs***	***Type of Assay***	***Study Design***	***Results***	***Reference***
Benzoquinone Mustard (con'd)	BM BDM AM			DNA cross-link formation produced by BDM was 35% and 2-fold lower in the L5178Y/HBM2 and L5178Y/HBM10 cells respectively *vs.* the L5178Y cells Cross-link formation by AM was similar in all cell lines	Begleiter & Leith, 1990

8.1 Cytotoxic activity in relation to DTD mediated DNA damage in bioreductive alkylating agents

8.1.1 Aziridinylbenzoquinones and indolequinones

Studies with the clinically used aziridinylbenzoquinone, diaziquone (AZQ), as well as its structural analogs have suggested qualitative associations between their cytotoxic activity in cells with either high or low DTD activity, and DNA cross-link formation as measured by the alkaline elution technique or comet assay (Szmigiero et al., 1984, Gibson et al., 1992, Ward et al., 1997). As well, in a study using a single concentration of a series of aziridinylbenzoquinones, the ease of reduction by DTD was shown to closely follow both their *in vitro* cytotoxicity and their ability to cross-link DNA (Lee et al., 1992).

In determining possible relationships between the cytotoxic activity of indolequinones and DTD mediated reduction kinetics or extent and type of DNA damage, inconsistent results were observed with the indolequinone, EO9. Specifically, in a study using a single-concentration of EO9 and its analogs, Phillips and colleagues (1999) observed no measurable DNA cross-links with EO9, but did observe DNA cross-link formation with the EO9 analog, EO4, which was inhibited by DIC. In contrast, a concentration dependent increase in both DNA strand break and cross-link formation was observed with EO9 by Bailey and colleagues (1997), which correlated with the cytotoxic potency of this agent. In a later study it was also reported that for EO9, there were no correlations between DTD mediated reduction, cytotoxic potency or DNA single strand break formation (Phillips et al., 1999).

Overall, the inconsistent results observed in the assessment of associations between cytotoxic activities of certain quinone-based bioreductive alkylating agents and either DNA damage formation or DTD mediated reduction may be due to several factors. These factors include potentially different results which may be observed by assessment of DNA damage in whole cell systems compared to agarose gel assays. In addition, differences may also be due to assessment of outcome measures under different pH or oxygen tension conditions. As well, many studies assessed outcome measures at a single concentration of investigational compound. To this end, it would be more informative to test structure-activity relationships for DNA cross-link or strand break formation using a broad range of concentrations. This would ensure that correlations are based on drug concentrations within the linear range of the dose-response relationships, thus avoiding conclusions based on measurements in the saturation or floor portions of the dose response curves.

8.2 Effects of structural alterations to bioreductive alkylating agents on mechanisms of DNA damage: The need for further SAR studies

8.2.1 Aziridinylbenzoquinones, and cyclopent[b]indoles

Currently, there are limited data on how varying functional group substitutions on quinone-based bioreductive alkylating agents may influence their cytotoxic activity and ability to alkylate DNA or form DNA cross-links or strand breaks. In studies with different aziridinylbenzoquinones, the efficiency of DNA cross-link formation was found to be impaired with an increased length of alkyl

group substituted on the benzoquinone moiety (Hargreaves et al., 1999, Lee et al., 1992, Table 2). Similarly, in the case of quinone methide aziridinylbenzoquinone analogs, DNA cross-link formation decreased with an increase in length of the methylene chain at position 5 of the analogs (Mayalarp et al., 1996, Table 2). Furthermore, functional group substitutions on cyclopent[b]indole bioreductive alkylating agents, have led to the identification of several SARs and the design of new bioreductive alkylating agents with increased DNA alkylating ability subsequent to reduction by DTD (Skibo et al., 2001, Table 2). The successful design of such new bioreductive alkylating agents, by the use of SAR studies, warrants further studies with other quinone-based bioreductive alkylating agents to determine how functional group substitutions may impact on their mechanisms of cytotoxic activity. This information would be essential in the development of future generations of quinone-based anti-tumor agents with increased efficacy.

Section 9: The role of DTD in the bioactivation of bioreductive alkylating agents

DTD has been an important target in the enzyme directed drug targeting approach for new bioreductive alkylating agent development. Due to the broad range of substrates reduced by DTD, there have been many different types of quinone-based experimental compounds proposed for development as putative bioreductive alkylating agents. Specifically, studies have focused on the establishment of whether compounds are substrates for reduction by DTD, and

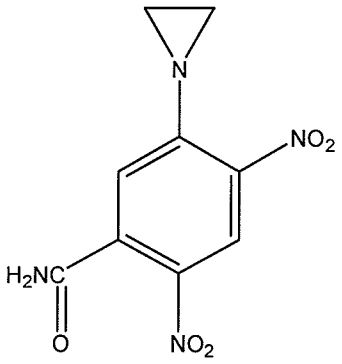
followed up with determination of potential relationships between reduction by DTD and cytotoxic potency in tumor cell panels with varying levels of DTD activity (Robertson et al., 1992, Fitzsimmons et al., 1996) or paired cell lines with high DTD activity and low or non-detectable activity (Siegel et al., 1990a,b, Beall et al., 1996). The potential confounding factor in such non-isogenic paired cell line models is the potential variability in other bioactivation enzymes and detoxification pathways. This has led to the development of new experimental models based on isogenic human cancer cell lines, where the wild-type *NQO1* gene is stably transfected into the NQO1-mutant BE human colon tumor cell line (Sharp et al., 2000) to investigate the mechanisms involved in bioactivation and cytotoxic activity of experimental bioreductive alkylating agents. Specifically, these models have allowed for comparisons between NQO1-transfected versus vector control BE lines with parallel *in vitro* studies to determine the role of DTD in the bioactivation of model bioreductive alkylating agents and *in vivo* studies to determine the role of DTD in the anti-tumor effects of these agents through the establishment of tumor xenografts wit these cell lines (Sharp et al., 2000). Furthermore, SAR studies and studies utilizing the crystal structure of human DTD have investigated the effects of functional group substitutions on DTD mediated reduction and cytotoxicity, in order to determine potential functional group substitutions or structural alterations to investigational agents that might lead to specificity of reduction by DTD. The following sections will provide an overview of quinone-based and nitroaromatic-based bioreductive agents which are currently under investigation for DTD-directed drug development. This will be

followed by a discussion of some of the specific structural alterations and functional groups which modify DTD mediated reduction.

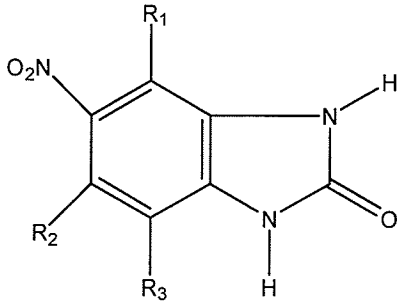
9.1 Nitroaromatic compounds

The nitroreductase activity of DTD has led to the design of several different nitroaromatic compounds as proposed bioreductive anticancer agents. The nitrobenzamide CB-1954 (5-(aziridinyl-1-yl)-2,4-dinitorbenzamide) has been shown to have cytotoxic activity against cell lines with high DTD activity (Knox et al., 1993), and to be a substrate for rat DTD, where reduction led to metabolites capable of producing DNA cross-links (Figure 21) (Knox et al., 1993). Unfortunately, CB-1954 did not have any activity against human tumors. The lack of human anti-tumor effect was determined to be as a result of CB-1954 being a poor substrate for the human DTD, but not the rat form of DTD, which was utilized for the earlier developmental work with this agent. (Knox et al., 1993, Boland et al.,1991).

Figure 21



CB 1954



Nitrobenzimidazole

Studies with dinitropyrenes have in large been unsuccessful as a result of the low nitroreductase activity of DTD. As well, experimental agents have been poor substrates for DTD, a factor which has further hindered DTD mediated reductive activation (Hajos and Winston, 1991, Knox et al., 1993).

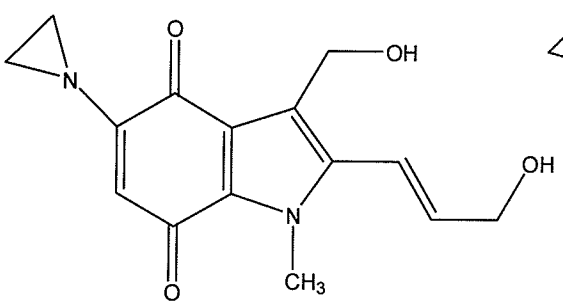
Recently, there has been interest in the design of nitroaromatic compounds which are more selectively reduced by DTD compared to nitropyrenes and nitrobenzamides. The polynitrobenzimidazoles is a new class of nitrocompounds (Figure 21). These agents contain multiple nitro groups on the benzene ring, and are much better substrates for DTD than the preceding nitroaromatic compounds (Sarlauskas et al., 1997, Knox et al., 1993, Knox et al., 1988, Cenas et al., 1995, Anusevicius et al., 1998). Furthermore, data have indicated that DTD mediated reduction of these compounds is in part responsible for their cytotoxic activity against bovine leukemia virus-transformed fibroblast culture (Sarlauskas et al., 1997). As these experiments were conducted using rat DTD, it would be of importance to establish whether these relationships are applicable to human DTD.

9.2 Indolequinones

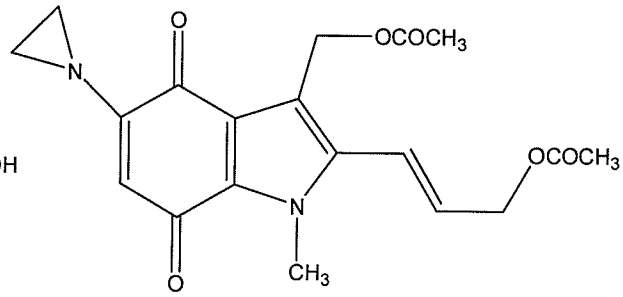
EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4,7-indione)-propenol) was originally synthesized as a indolequinone analog of mitomycin C (Figure 22). However, unlike mitomycin C, this compound is a very good substrate for DTD (Bailey et al., 1998), but is also metabolized by other

reductases, which become important under hypoxic conditions (Bailey et al., 2001).

Figure 22



EO9



EO4

Specifically, in a study which utilized DTD purified from rat Walker tumor cells, EO9 was metabolized to a hydroquinone metabolite under both hypoxic and aerobic conditions (Bailey et al., 1998). This metabolite was further characterized, using electron spin resonance spectrometry, to be highly sensitive to oxygen, and very rapidly autoxidized to produce the semiquinone form and reactive oxygen species. Due to the rapid detection of the semiquinone under aerobic conditions, it was suggested that the cytotoxicity of EO9 occurs through a redox mechanism involving a coproportionation:disproportionation reaction mediated by the concerted action of superoxide radical and the EO9-hydroquinone autoxidation as described in Sections 2.5 and 2.7 (Bailey et al., 1998). The exact molecular mechanisms involved in determining the cytotoxic activity of EO9 were further elucidated in a study which employed xenografts of HT29 (high DTD activity) and BE (low DTD activity) human colon carcinoma cells

(Cummings et al., 1998). Using these models, the antitumor activity of EO9 *in vivo*, as well as its metabolism *in vitro* using tumor homogenates were studied in parallel. Results indicated no relationship between the kinetics of *in vitro* metabolite formation and antitumor activity, but did show a correlation between the rate of removal of the parent compound and antitumor activity ($r = 0.43$) (Cummings et al., 1998). Overall, these data suggested that for EO9, the overall capacity of the tumor to metabolize the compound is the most significant determinant of antitumor activity and that the metabolites do not lead to the active forms of the drug. Rather, it is proposed that the active forms are the parent EO9 compound and reactive intermediates which form possibly through the coproportionation:disproportionation reaction mechanism described by Bailey and colleagues (1998). It is thought that this proposed mechanism of cytotoxicity, as well as the rapid clearance of EO9 in humans are factors that contributed to the disappointing Phase II clinical trials with this compound (Pavlidis et al., 1996).

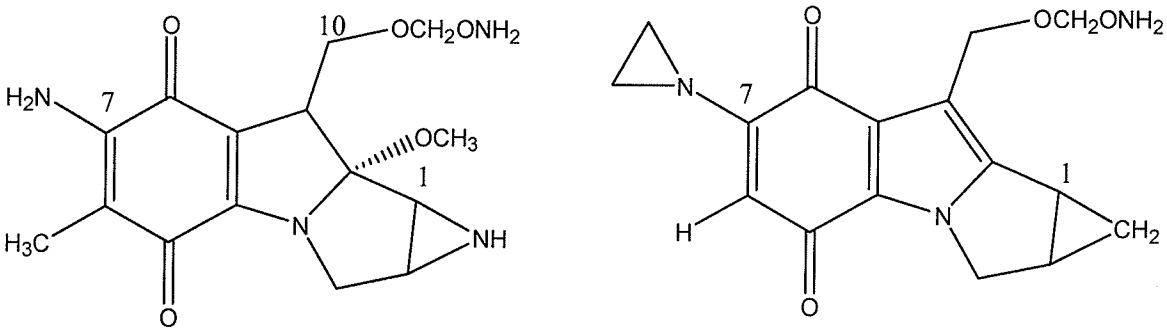
Several analogs with relatively minor structural alterations from the EO9 compound have been shown to be substrates for DTD (EO68 and EO4) (Figure 22). Furthermore, DTD has been shown to activate EO4 to metabolites capable of DNA cross-link formation (Phillips, 1996). As well, the EO9 analogs have been shown to possess preferential toxicity towards DTD rich H490 human non small cell lung cancer cells compared with DTD-deficient H596 counterparts, and this cytotoxicity was inhibited by DIC (Phillips, 1996). These findings warrant further studies in a wide panel of cell lines as well as human tumor xenografts to

determine the overall potential use of these agents as bioreductive alkylating agents.

9.3 Cyclopropamitosenes

Cyclopropamitosenes are analogs of mitomycin C, in which the aziridine ring at C1 is replaced by a cyclopropane to reduce the electrophilicity at this site (Figure 23).

Figure 23



Mitomycin C

7-aziridinyl cyclopropamitosene analog

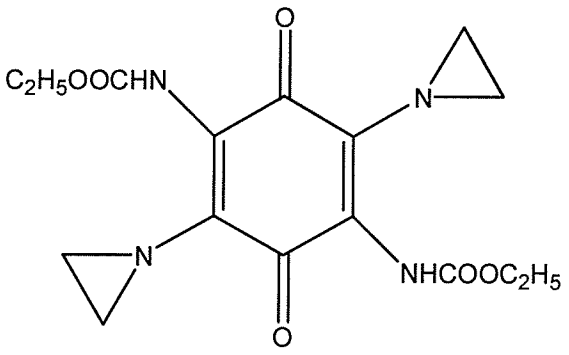
The 7-methoxycyclopropamitosene and its 7-substituted derivatives have been shown to be substrates for purified Walker rat tumor DTD (Moody et al., 1994). Cytotoxicity of these agents is thought to involve bioactivation of the parent structures by DTD to potent electrophiles which may then alkylate DNA. As well, one-electron mediated reductive activation of these compounds is suggested to lead to reactive intermediates which could produce DNA strand breaks (Moody et al., 1994). The role of DTD mediated reduction in the cytotoxic

activity of these agents has been assessed under hypoxic and aerobic conditions in Chinese hamster V79 cells (Moody et al., 1994). Data from this study suggested that the 7-aziridinyl analog was significantly more toxic than mitomycin C or EO9 and that DIC decreased the cytotoxicity of this agent under aerobic conditions (Figure 23). From these initial findings it was suggested that DTD mediated reduction is important in the bioactivation of the 7-aziridinyl analog under aerobic conditions. However, further studies are needed to determine the overall importance of these agents as bioreductive alkylating agents for clinical use (Moody et al., 1994).

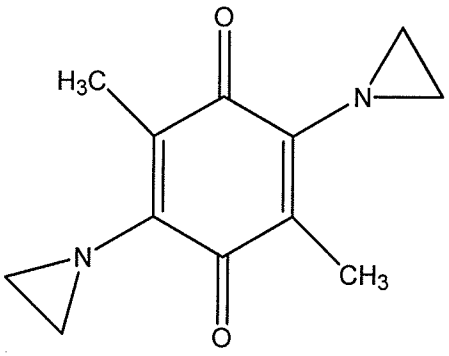
9.4 Aziridinylbenzoquinones

Diaziquone (AZQ) (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone) was the first aziridinylbenzoquinone to be approved for clinical use in tumors of the central nervous system (Figure 24) (Haid et al., 1985).

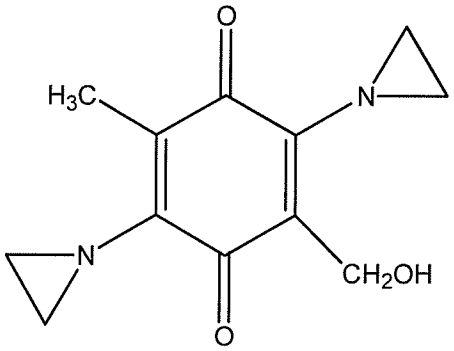
Figure 24



AZQ



MeDZQ



RH1

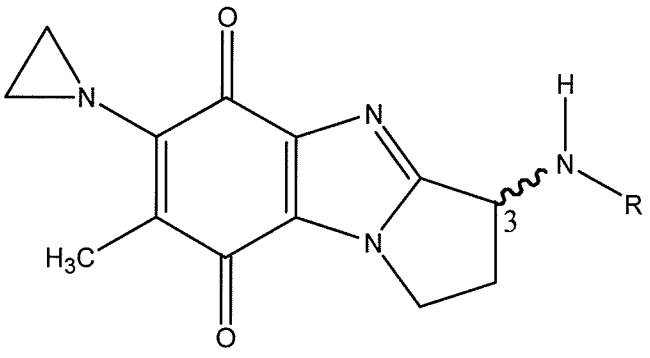
AZQ and several other investigational aziridinylbenzoquinones, produce their antitumor effects subsequent to bioactivation by one-electron reductases and DTD. Specifically, their two cytotoxic aziridinyl groups can produce bifunctional alkylation, or DNA cross-links subsequent to bioactivation of the benzoquinone. Semiquinones formed by redox cycling of the hydroquinone products, or one-electron reduction of the quinone also produce reactive oxygen species and intermediates which results in oxidative damage mediated by these agents (Powis, 1987, Riley and Workman, 1992, Gutierrez, 1989, Ross et al., 1990, Fisher and Gutierrez, 1991a, Siegel et al., 1990a, Gibson et al., 1992).

In studies with a series of DZQ (2,5-diaziridinyl-1,4-benzoquinone) analogs, it was found that the dimethyl derivative, MeDZQ (2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone), was a very efficient substrate for DTD and it produced significantly increased cytotoxicity to HT29 cells (human colon carcinoma cells with high DTD activity) compared to other analogs (Gibson et al., 1992). Furthermore, this analog produced DNA interstrand cross-links in HT29 cells, which along with cytotoxicity were inhibited by DIC (Gibson et al., 1992). However, MeDZQ was not sufficiently water-soluble for formulation. This problem led to the synthesis of its water-soluble analogue RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) (Figure 24) (Winski et al., 1998). RH1, as well as MeDZQ, are both metabolized efficiently by DTD –expressing cell lines and in cell-free systems, with RH1 being a better substrate for DTD and possessing increased cytotoxicity in cell lines with high DTD activity, compared to MeDZQ (Beall et al., 1995, Winski et al, 1998). Currently, RH1 is slated to begin clinical trials as a bioreductive alkylating agent selectively bioactivated by DTD, and is an example of the successful application of the enzyme directed approach to bioreductive alkylating agent development.

9.5 Pyrrolo[1,2-a]benzimidazoles

This new class of experimental bioreductive antitumor agents has its structure based on the pyrrolo[1,2-a]benzimidazole ring system (PBI) and it possesses several features which distinguishes it from other previously designed bioreductive agents (Figure 25). These features include a novel mechanism of alkylation which involves the DNA phosphate backbone. This alkylation results subsequent to reduction by DTD and produces a hydrolytically labile phosphotriester complex (Schulz et al., 1995). Furthermore, these agents, when reduced to the hydroquinone, can produce hydrogen bonds in the major groove of DNA at the DNA bases, and this facilitates the alkylation reactions at the phosphate backbone (Skibo et al., 1997).

Figure 25



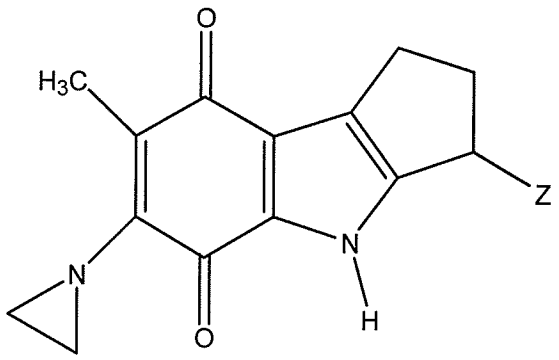
Pyrrolo[1,2-*a*]benzimidazole

Huang and colleagues (2000) have shown that there are differences in the antitumor effects of R- and S-enantiomers of PBI analogs. Structure-activity studies and modeling studies using the X-ray crystal structure of DTD with these compounds have indicated that the higher DTD substrate specificity of the S-enantiomer compared to the R-enantiomer of specific PBI analogs is due to steric interactions between the R-enantiomer and DTD which do not occur in the case of the S-enantiomer. This group further suggested that this steric effect contributes to the differences in antitumor effects observed with enantiomers of the same analogs (Huang et al., 2000). Overall, these features of the PBIs may therefore function as unique parameters which could lead to the design of future analogs with DTD selective activation and tumor specific toxicity.

9.6 Aziridinylcyclopent[b]indoles and indoles

The indolequinone bioreductive antitumor agents discussed in previous sections typically have alkyl groups substituted at the N-indole system. Recently, new indolequinone-based anticancer agents were proposed which possess the cyclopent[b] indole or N-unsubstituted indole system (Xing et al., 2000, Skibo et al., 2001) (Figure 26).

Figure 26



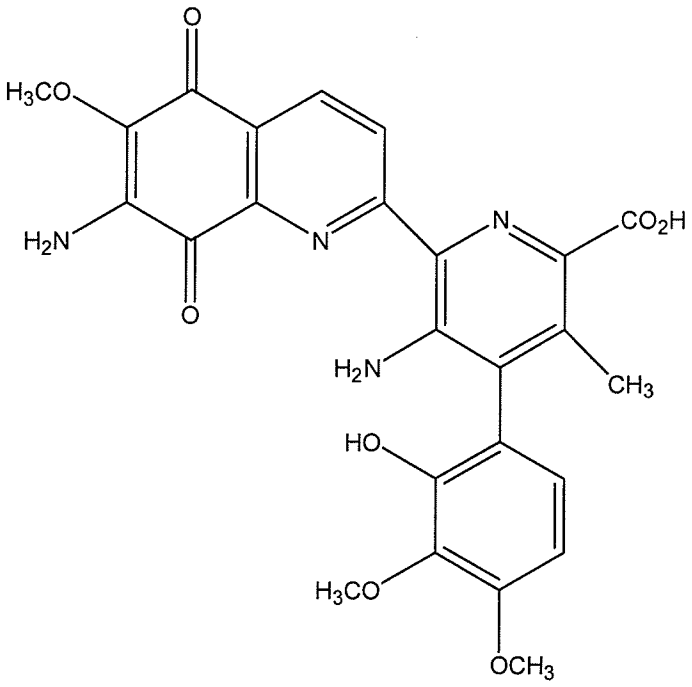
Cyclopent[b]indole

SAR studies with a large number of these analogs have led to the identification of several characteristics which impact DTD substrate specificity, DTD mediated cytotoxicity and DNA alkylation (Skibo et al., 2001). Important findings from these studies revealed that high DTD substrate specificity is not warranted in the design of indole and cyclopent[b]indole bioreductive agents, as this leads to a loss of cancer selectivity as well as increased toxicity. Currently, it is not clear whether this finding is specific only for these classes of agents, or whether it may be implicated in the rational design of other quinone-based bioreductive alkylating agents.

9.7 Quinolinequinones

The quinolinequinone streptonigrin is metabolized efficiently by human DTD, and displayed increased cytotoxic activity in H460 non small cell lung carcinoma cells (high DTD activity) compared to H596 non small cell lung carcinoma cells (low DTD activity) (Figure 27) (Beall et al., 1994, Beall et al., 1995). Furthermore, there is a good correlation between DTD activity and cytotoxicity of streptonigrin as determined from an NCI human tumor cell line panel (Paull et al., 1994). Recent studies have also identified some functional group substitutions to streptonigrin which result in altered DTD substrate specificity (Fryatt et al., 1999). Further investigations are required to determine whether this compound, or its analogs may be useful for further development as anticancer agents for use in tumors with high DTD expression.

Figure 27



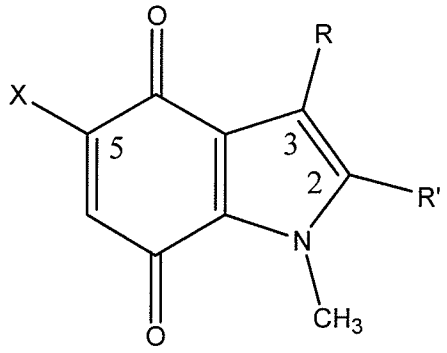
Streptonigrin

Section 10: Effects of structural alterations to bioreductive alkylating agents on reduction kinetics: The need for further SAR studies

SAR studies have investigated the effects of functional group substitutions on DTD mediated reduction in order to determine potential characteristics which may lead to specificity of reduction by DTD resulting in potential specificity of cytotoxicity to tumor tissue. The following section will provide an overview of some of the studies with indolequinone-based bioreductive anticancer agents which investigated the effects of functional groups and structural alterations on DTD mediated reductive activation.

Previously, different functional group substitutions at the C5 position, indole 3- position, or indole 2-position of a series of indolequinone anticancer agents were studied for their effects on DTD mediated reduction (Figure 28) (Beall et al., 1998a,b, Swann et al., 2001).

Figure 28



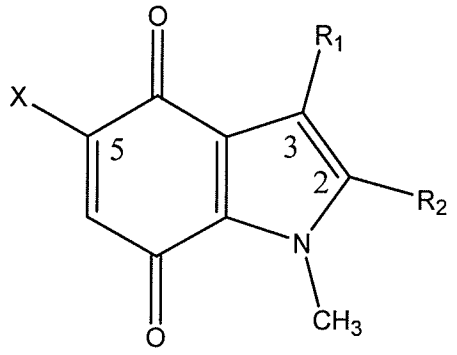
Indolequinone analogs with functional group substitutions at the 2-, 3-and 5-positions

Several SARs were established with these specific indolequinone analogs. Specifically, compounds with an amine group (other than an aziridine) at the C5 position of the quinone were not efficiently reduced by DTD. This inhibition of DTD mediated reduction was described to be due to either steric effects in the case of large pyrrolidine or morpholine groups, or as a result of electron-donating effects from the nitrogen lone pair into the quinone ring, thus hindering the gain of electrons through reduction by DTD. Compounds with an aziridinyl group at the C5 position would be unable to introduce electrons into the quinone, and were most easily reduced by DTD, followed by analogs with methylaziridine and methoxy group substitutions. On the other hand, substitution of a carbamate at the indole 3-postion led to compounds that were not good substrates for DTD. Furthermore, compounds with electron-withdrawing groups at the indole 3-postion were most effectively reduced by DTD, while compounds with no functional group substitution at this position, or a hydroxymethyl substitution at the indole 3-position were reduced slightly less efficiently. Interestingly, inactivation of DTD was produced by compounds which had a leaving group at the 3-indolyl methyl position. Parallel studies with a series of mitosenes indicated similar SARs as observed with the indolequinone series (Beall et al., 1998a, b).

EO9 is a good substrate for human DTD, and there is a good correlation between DTD activity and chemosensitivity to EO9 under aerobic conditions (Phillips, 1996, Fitzsimmons et al., 1996, Robertson et al., 1994). This led Phillips and colleagues (1999) to design a series of indolequinones, including

analogs of EO9, to determine the structural features which confer substrate specificity to human DTD in these agents (Figure 29).

Figure 29



Indolequinone analogs with functional group substitutions at the 2-, 3-and 5-positions

SAR studies with this series of indolequinones found that a large variety of modifications including sterically bulky groups such as a phenyl moiety at the 2-position of the indolequinone nucleus, did not affect DTD mediated reduction kinetics. Further molecular modeling studies suggested, that these groups were located within the binding site entrance, and therefore were accommodated by the enzyme binding pocket. In contrast, substitutions at the (indol-3-yl)methyl position with sterically bulky leaving groups, or groups with a chlorine atom, produced compounds that were poor substrates for DTD. Molecular modeling studies indicated that these groups were close to important amino acids within the binding pocket of DTD, and may interfere with DTD mediated reduction through mechanisms which involve alkylation or disruption of the mechanism by which electrons are transferred in the reduction reaction (Phillips et al., 1999).

The substitution of large sterically bulky groups at the 5-position of the quinone decreased the ability of the quinone to be reduced by DTD, mainly because these groups were located at the rear wall of the DTD binding pocket which is thought to have limited ability to accommodate large functional groups (Phillips et al., 1999).

In conclusion, the results from these structure-activity studies have led to the discovery of several structural features, which allow for increased substrate specificity and reduction by DTD. Currently, however, this type of structure-activity relationship information is not known for a wide variety of quinone-based bioreductive alkylating agents, and it would be beneficial to design future studies to determine the effects of functional groups and structural alterations on these compounds.

The overall goal of this thesis is to use SAR studies to provide a better understanding of some of the mechanisms of enzyme mediated activation and action of the model anticancer bioreductive alkylating agent BM. Specifically, in Chapter 2 and Chapter 3 we will determine the effects of a wide range of functional group substitutions to the quinone moiety of BM on: 1) DTD mediated reduction kinetics in vitro and cytotoxic activity in human cancer cell lines and, 2) DTD mediated DNA cross-link and strand break formation in vitro. Furthermore, Chapter 4 will apply SAR studies to investigate the characteristics of three novel NMDA receptor antagonists in terms of their transport through specific renal tubule transporter sites, as well as drug-drug interactions at common renal tubule transporter types.

CHAPTER 2

The Effect of Functional Groups on Reduction and Activation of Benzoquinone Bioreductive Alkylating Agents by DTD

Section 1: Background and Rationale

1.1 Bioreductive alkylating agents and enzyme directed drug targeting

Bioreductive drugs have become an important class of antitumor agents (Rockwell et al., 1988, Workman & Stratford, 1993). The prototype drug in this class, mitomycin C (Rockwell et al., 1993), is used in the treatment of bladder, breast, stomach, head and neck, rectal and other solid tumors (Begleiter et al., 2000). Another agent that has been used in the clinic is diaziquone (Chamberlain et al., 1988), while the indolequinone EO9 (Dirix et al., 1996) and tirapazamine have been tested in clinical trials (Begleiter et al., 2000). In addition, the new bioreductive agent, RH1, (Winski et al., 1998) has demonstrated good antitumor activity *in vitro*, and there has been considerable interest in the synthesis of new agents (Denny et al., 1996).

Bioreductive agents have varied chemical structures but share a common requirement for reductive activation (Workman & Stratford, 1993). They produce their antitumor effects by different mechanisms, and while active under oxygenated conditions, many of these agents are preferentially toxic to hypoxic cells (Workman & Stratford, 1993). Bioreductive agents can be activated by one electron reducing enzymes such as NADPH:cytochrome P450 reductase [EC 1.6.2.4] (Pan et al., 1984, Rockwell et al., 1993) and NADH:cytochrome b_5 reductase [EC 1.6.2.2] (Hodnick & Sartorelli, 1993), and by two electron reducing enzymes like DTD [EC 1.6.99.2] (Riley & Workman, 1992, Ross et al., 1993) and xanthine dehydrogenase [EC 1.1.1.204] (Gustafson & Pritsos, 1992a,b). NADPH:cytochrome P450 reductase is the most notable activating enzymes for

many of these agents (Hoban et al., 1990, Riley & Workman, 1992), but DTD is also a significant contributor to the activation of bioreductive agents (Begleiter et al., 1992, Riley & Workman 1992, Begleiter & Leith, 1993, Ross et al., 1993). Drug activation is strongly influenced by the level of oxygen (Hoban et al., 1990, Begleiter et al., 1992) and pH (Siegel et al., 1990b, Begleiter & Leith, 1993). The relative importance of each activating enzyme varies with the agent, the type and origin of the cell and the tumor environment.

All bioreductive agents contain a bioreductive element and a cytotoxic element. In some cases, like tirapazamine and AQ4N (Patterson, 1993), the bioreductive element may also act as the cytotoxic element; while in others, like mitomycin C (Lown et al., 1976, Tomasz et al., 1987), the agents have additional cytotoxic elements that are regulated by the reductive element. Quinone and nitrogen-oxide groups are the bioreductive elements most commonly found in current bioreductive antitumor agents. One-electron reduction of these elements results in the formation of radical species that can add across double bonds or can abstract a hydrogen from cellular molecules (Trush et al., 1982), leading to degradation of cellular components such as proteins, lipids and DNA. In addition, in the presence of oxygen, the radical species can undergo redox cycling resulting in the generation of reactive oxygen species (Kappus, 1986) that can also degrade cellular components and produce DNA strand breaks (Lown et al., 1976, Trush et al., 1982). Direct two-electron reduction of the bioreductive element, or further one-electron reduction of the initial one-electron reduced product, results in a two-electron reduced product that may or may not have

cytotoxic activity. Depending on the stability of the two-electron reduced state, it may also undergo redox cycling to generate reactive oxygen species.

Alternatively, reduction of the bioreductive element may result in activation of a cytotoxic element, generally an alkylating group. For example, mitomycin C (Lown et al., 1976, Tomasz et al., 1987), EO9 (Riley & Workman, 1992) and the model bioreductive agent, BM (Begleiter & Leith, 1990) all have alkylating groups that are activated by reduction of a quinone bioreductive element. The alkylating groups can bind covalently to cellular components such as DNA and proteins and can produce DNA cross-links (Lown et al., 1976, Tomasz et al., 1987, Begleiter & Leith, 1990, Riley & Workman, 1992), which can lead to cell death by apoptosis (Begleiter et al., 1994). While the quinone group of these bioreductive agents can also undergo redox cycling (Kappus, 1986), and these agents do produce DNA strand breaks (Lown et al., 1976, Riley & Workman, 1992), the alkylating activity is thought to be most important for their antitumor effect (Rockwell et al., 1993). However, the relative importance of these various mechanisms is highly dependent on the tumor environment including the levels of reductive enzymes within the cells (Hoban et al., 1990, Begleiter et al., 1992), the level of oxygen (Hoban et al., 1990, Begleiter et al., 1992) and the pH (Siegel et al., 1990b, Begleiter & Leith, 1993).

A major focus for improving cancer chemotherapy has been to increase the selectivity and targeting of antitumor drugs to tumor cells, relative to the normal healthy cell populations in the patient. The bioreductive class of antitumor agents is ideally suited to improve tumor selectivity by an "enzyme-

directed" approach to tumor targeting (Riley & Workman, 1992, Workman & Stratford, 1993). For example, studies have found higher levels of DTD in tumor cells than in normal cells (Belinsky & Jaiswal, 1993, Riley & Workman, 1992, Smithskamp-Wilms et al., 1995). Thus, bioreductive agents that are specifically activated by DTD could be used to target tumors that have high levels of this enzyme. This approach is illustrated by the development of the bioreductive agent RH1, which is selectively activated by DTD (Winski et al., 1998). EO9 showed very good antitumor activity against tumors that had high levels of DTD but caused little bone marrow toxicity (Hendriks et al., 1993). Consistent with this observation, bone marrow has been shown to have very low levels of DTD (Begleiter et al., 1989, Begleiter et al., 1996).

An alternative paradigm has been developed for bioreductive agents selectively activated by NADPH:cytochrome P450 reductase. The activity of these agents is generally lower under aerobic conditions because, in the presence of oxygen the initially formed one-electron reduction product can be re-oxidized, resulting in a lower concentration of activated drug (Workman & Stratford, 1993). These agents normally have greater activity under hypoxic conditions because the re-oxidation process cannot occur under these conditions. Most solid tumors have regions of hypoxia resulting from poor vascularization, bioreductive agents that are primarily activated by NADPH:cytochrome P450 reductase have been used to target these hypoxic cells either as cytotoxic agents or as radiosensitizers (Workman & Stratford, 1993). This approach to tumor targeting is illustrated by bioreductive agents

tirapazamine and AQ4N which have high hypoxic/oxic cytotoxicity ratios (Patterson, 1993).

There has also been considerable interest in the development of new bioreductive agents. Denny and colleagues (1996) have developed new bioreductive molecules and methods for modifying the enzyme selectivity of these agents. A number of new bioreductive agents are currently under investigation, and include AQ4N, which is based on the aliphatic nitrogen-oxide bioreductive element (Patterson, 1993). Quinone-based bioreductive alkylating agents which are currently under investigation for effective bioactivation by DTD include aziridinylbenzoquinones, indolequinones, pyrrolobenzimidazolequinones, quinolinequinones and cyclopropamitosenes (Bailey et al., 1998, Moody et al., 1994, Gibson et al., 1992, Huang et al., 2000, Fryatt et al., 1999). Despite a number of structure-activity studies (Bailey et al., 1992, Naylor et al., 1997, Beall et al., 1998a,b, Fryatt et al., 1999, Phillips et al., 1999), a major problem has been a lack of knowledge of structural factors that produce selectivity for activation of bioreductive agents by reductive enzymes like DTD and NADPH:cytochrome P450 reductase.

Previously, it was shown that BM, which contains a quinone bioreductive element and a nitrogen mustard cytotoxic element, is more toxic to tumor cells than the non-quinone alkylating agent, aniline mustard (Begleiter, 1983). BM produced both DNA cross-links and strand breaks, but the cross-links were the major contributor to the cytotoxic activity (Begleiter & Blair, 1984, Begleiter, 1986). Both the cytotoxic and cross-linking activities of BM were increased by

reduction of the quinone (Begleiter et al., 1991). The cytotoxic activity of BM and BM mediated DNA strand break and cross-link formation were lower in mouse lymphoma cells with elevated DTD, compared with similar cells with low enzyme activity. DIC increased BM activity in the cells with high enzyme activity, suggesting that reduced activated products formed through DTD mediated reduction may be less cytotoxic than reduced products which may form due to activation by one-electron reducing enzymes such as NADPH:cytochrome P450 reductase (Begleiter & Leith, 1990).

In the present study BM analogs were investigated to determine the effect of a number of functional groups on the reduction and activation of these model bioreductive agents by DTD. The SARs established may improve our understanding of the structural characteristics of benzoquinone–based bioreductive alkylating agents which influence DTD mediated reduction and cytotoxic activity.

Section 2: Materials and Methods

2.1 Materials

All media and fetal bovine serum were obtained from GibcoBRL (Grand Island, NY). Dichloromethane, methanol, hexanes, 95% ethanol, ethyl acetate, silica gel (1000 mesh), glacial acetic acid, anhydrous ether, hydrogen peroxide solution and potassium fluoride were from Mallinkrodt (Paris, KY) and Baker Inc. (Phillipsburg, NJ). All reagents for the DTD assay, NADH, FAD, DIC, di(chloroethyl)amine hydrochloride, Tris HCl, vanillin, 1,4-benzoquinone, chromic

acid, 2-methyl-1,4-benzoquinone, 2-phenyl-1,4-benzoquinone, 2-chloro-1,4-benzoquinone, fluorobenzoquinone, tert-butyl benzoquinone were from Sigma-Aldrich (St. Louis, MO). N, N,-dimethyl formamide, dimethyl sulfoxide and cupric acetate was from Fisher Scientific (Fair lawn NJ). Purified recombinant human wild-type DTD with activity of 880 µmol/min per mg protein, (as measured by DIC-sensitive reduction of 2,6-dichlorophenolindophenol (DCPIP)) (Benson et al., 1986), was obtained from Dr. D. Ross, University of Colorado Health Sciences Center, Denver, Colorado, USA.

2.2 Preparation of BM Analogs

The BM analogs were prepared in collaboration with the laboratory of Dr. F. Guziec, Southwestern University, Georgetown, Texas, USA. BM was synthesized using the method developed by Makarova and Berlin (1967). The purple precipitate was collected by filtration, dried and re-crystallized from hexanes to give BM. The structure was confirmed and purity was assessed by ^{1}H NMR spectroscopy and melting point.

MBM was synthesized using a combination of the method described by Crosby and Lutz (1956) for oxidative amination of 1,4-benzoquinones and Makarova and Berlin (1967) for synthesis of BM. Di(chloroethyl)amine hydrochloride, potassium fluoride and cupric acetate were added to a solution of 2-methoxy-1,4-benzoquinone in 95% ethanol. 2-Methoxy-1,4-benzoquinone was prepared as previously described (Corral, 1957). The reaction mixture was stirred at room temperature for 72h with minimum light exposure. The mixture

was filtered and the filtrate was washed 4 times with ethyl acetate. The combined organic phases were washed with an equal volume of 0.1 M HCl to remove any remaining cupric acetate. The ethyl acetate layer was separated, dried with magnesium sulfate and rotary evaporated *in vacuo* to dryness to give a red solid. The red solid was re-crystallized using 95% ethanol to give MBM. The structure was confirmed and purity was assessed by ^{1}H NMR spectroscopy and by melting point.

MeBM was synthesized using the methods of Crosby and Lutz (1956) and Makarova and Berlin (1967) starting from 2-methyl-1,4-benzoquinone, as described above for MBM. The mixture was filtered using vacuum and the resulting precipitate was washed with ethyl acetate (4 x 20 ml). The combined organic phases were washed with an equal volume of 0.1 M HCl and then dried with anhydrous sodium sulfate. Concentration afforded a crude solid which was recrystallized from methanol affording MeBM. The structure was confirmed and purity was assessed by ^{1}H NMR spectroscopy and X-ray analysis.

m-MeBM was synthesized using a combination of previously described methods (Crosby & Lutz 1956, Makarova & Berlin 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 reaction mixture was stirred in the dark at room temperature for 72 h, at which time no methylbenzoquinone remained as determined by thin layer chromatography. The mixture was filtered and the precipitate washed with ethyl acetate (30 ml). The combined organic phases were concentrated and the residual oil taken up in

ethyl acetate (150 ml). This solution was washed with 0.15 M HCl (100 ml) and then brine. Drying over anhydrous sodium sulfate, concentration and crystallization from hot methanol afforded the 6-methylbenzoquinone mustard as red crystals, (2.40 g, 22% yield), mp 90-91°C. The structure was confirmed by ^{1}H NMR spectroscopy and X-ray crystallographic analysis.

CBM was synthesized using the methods of Crosby and Lutz (1956) and Makarova and Berlin (1967) starting from 2-chloro-1,4-benzoquinone, as described above for MBM. The resulting purple solid was re-crystallized using methanol to give CBM.

FBM was prepared starting from fluorobenzoquinone synthesized as follows: Fluorohydroquinone (1.00g, 7.6 mmol) was added to a solution of ceric ammonium nitrate (8.67 g, 15.3 mmol) in distilled water (50 ml). After stirring for 1 h at room temperature, the mixture was extracted with ethyl ether (3 x 30 ml). The extracts were dried over sodium sulfate and concentrated affording yellow crystals of fluorobenzoquinone (760 mg), mp 77 °C. The crystals rapidly darkened in the presence of light but were of satisfactory purity to be used directly in the preparation of the corresponding mustard. Di(chloroethyl)amine hydrochloride and cupric acetate were added at room temperature to a stirred solution of fluorobenzoquinone in 95% ethanol. Potassium fluoride was added, and the mixture was stirred in the dark at room temperature for 72 h, at which time no starting fluorobenzoquinone remained as determined by thin layer chromatography. The mixture was filtered, and the precipitate washed with ethyl acetate (3 x 100 ml). The combined organic phases was washed with 0.1 M HCl

(100 ml) and then brine. Drying over anhydrous sodium sulfate and concentration afforded a red oil which was crystallized from 95% ethanol affording red crystals of 5-fluorobenzoquinone mustard, (170 mg, 11 % yield) mp. 142-144 °C. The structure was confirmed by ^{1}H NMR spectroscopy .

m-PBM and PBM were synthesized using the methods of Crosby and Lutz (1956) and Makarova and Berlin (1967) starting from 2-phenyl-1,4-benzoquinone, as described above for MBM. The mixture was filtered using vacuum and the resulting precipitate was washed with ethyl acetate (4 x 25 ml). The combined organic phases were washed with water, with an equal volume of 0.1 M HCl and then dried with anhydrous sodium sulfate. Concentration afforded a crude mixture of isomeric phenyl benzoquinone mustards. Recrystallization from ethyl acetate -petroleum ether afforded the major isomer, m-PBM as red crystals. The structure was confirmed and purity was assessed by ^{1}H NMR spectroscopy and X-ray analysis. Concentration of the crude mother liquor from above afforded a red solid enriched in the 5-phenyl isomer. About 200 mg of this mixture was dissolved in ethyl acetate (2.5 ml) and the mixture separated using radial chromatography with a Chromatotron apparatus on a 4 mm silica plate. Ethyl acetate - hexanes (2:3) was used as a solvent. PBM was less polar than the m-PBM isomer. Concentration and drying under vacuum afforded the minor isomer PBM as red crystals. The structure was confirmed and purity was assessed by ^{1}H NMR spectroscopy, X-ray analysis and by melting point.

m-TBM was synthesized using the previously described methods (Crosby & Lutz 1956, Makarova & Berlin 1967), and starting from tert-butylbenzoquinone

as described for m-MeBM. The reaction mixture was stirred in the dark at room temperature for 72 h, at which time no tert-butylbenzoquinone remained as determined by thin layer chromatography. The mixture was filtered and the precipitate washed with ethyl acetate (4 x 20 ml). The combined organic phases were washed with 0.1 M HCl (100 ml) and then brine. Drying over anhydrous sodium sulfate and concentration afforded a red oil. This oil was purified by flash chromatography on silica gel using ethyl acetate-hexanes (1:2) affording the pure 6-tert-butylbenzoquinone mustard as a red oil (400 mg, 16% yield). The structure was confirmed by NMR spectroscopy.

2.3 Cells

NCI-H661 human non-small-cell lung carcinoma cells and SK-MEL-28 human malignant melanoma cells were obtained from American Type Culture Collection (Rockville, MD). The NCI-H661 cells were grown in RPMI-1640 plus 10% fetal bovine serum, and the SK-MEL-28 human malignant melanoma cells were grown in DMEM/F12 1:1 plus 10% fetal bovine serum. The media used contained a bicarbonate-based buffer system which prevented significant changes in the pH during the incubation times and MTT assays. The DTD activities of the NCI-H661 and SK-MEL-28 cell lines were 112.7 ± 12.4 nmol/min per mg protein and 586.7 ± 19.6 nmol/min per mg protein, respectively (Doherty et al., 1998).

2.4 Reduction of BM Analogs by Purified DTD

Reduction reactions were carried out in 1.5 ml microfuge tubes in 1 ml of a reaction buffer (25 mM Tris HCl, pH 7.4). Freshly prepared NADH and FAD were added to the reaction buffer to give final concentrations of 100 μM and 0.5 μM, respectively. The tube was sealed and the cover was perforated twice with a needle to create an inlet and outlet hole. A 1.5-inch stainless steel needle was placed through the inlet into the reaction buffer. Using this needle, the reaction buffer was purged with nitrogen gas, or air, for 3 hrs at 37 °C. Excess DTD at a final activity of 880 nmol/min per mg protein, followed by the BM analog at a final concentration of 50 μM, were added through the outlet hole to the reaction buffer. DTD was prepared fresh on the day of the experiment from -80 °C frozen stock and activated with 0.01% Tween 20. BM analogs were prepared fresh on the day of the experiment in DMF. The final concentration of DMF in the reaction buffer was 1%. Nitrogen, or air, was bubbled into the reaction vessel for the entire time course. At various times, 10 μM DIC was added through the outlet hole to stop the reaction, and the reaction solution was immediately frozen at -80°C for HPLC analysis. Control reaction mixtures ran for each experiment consisted of reaction mixture in the absence of BM analog. Preliminary control experiments consisted of reaction mixtures in the absence of DTD and in the presence of BM analog incubated for 90 minutes under aerobic or anaerobic conditions. These control experiments indicated no consumption of NADH cofactor (i.e. reduction of the quinone), as measured via HPLC analysis, during the incubation times.

2.5 HPLC analysis

Samples were kept frozen, prior to analysis, for no longer than 5 days. Reduction of the BM analogs was determined by measuring consumption of NADH. An aliquot of the reaction solution was removed and consumption of NADH was quantified by reverse-phase HPLC, as described by Gibson and colleagues (1992). Briefly, reduction of NADH was analyzed with a Phenomenex Prodigy 5 μm ODS 150 x 4.6 mm analytical column. A linear solvent gradient program of 10 mM potassium phosphate buffer (pH 6.0) and HPLC grade methanol was used to isolate the NADH (Table 3). NADH was detected at 340 nm, at a retention time of 15 min, and was quantified by measuring the area under the curve. The half-times of reduction should reflect the initial rates of reduction, as they were determined from the initial linear portion of the mean values of NADH concentration versus time curves using the least-squares regression method. While it would have been preferred to determine the initial rates of reduction of the BM analogs by DTD, methodological constraints prevented this approach from being utilized.

Table 3. Solvent Gradient

Time (min)	***Flow (ml/min)***	***% Potassium Phosphate Buffer***	***% Methanol***
Initial	1.00	100	0
5.00	1.00	95	5
15.00	1.00	20	80
25.00	1.00	20	80
30.00	1.00	95	5
35.00	1.00	100	0
45.00	1.00	100	0

2.6 Cytotoxicity Studies

Cells were plated in six well plates and incubated until 50% confluent. Media was removed and the cells were washed with citrate-saline, followed by incubation with, or without, 100 μM DIC for 20 minutes at 37°C in fresh media.

A relatively high concentration of DIC was chosen for these studies (100 μM), as the K_i values for DIC inhibition of reduction of the BM analogs are currently unknown, and may be greater than those values reported for compounds such as DCPIP (see chapter 1, page 56). Furthermore due to the competitive mechanism of DTD mediated reduction by DIC, the K_i values for DIC mediated inhibition of BM analog reduction would vary depending on the specific compound or substrate that is reduced by DTD. Moreover, the concentration of DIC used in these studies was not associated with any cytotoxic activity, as control experiments of cells incubated in the presence of DIC, but in the absence of BM analog, produced surviving cell fractions that were similar to those found in control cells incubated with neither BM analog or DIC (data not shown).

Subsequently, the cells were treated with BM analog and incubated for 1 h at 37°C in a CO_2 incubator. Control experiments consisted of cells in the absence of BM analog incubated at 37°C in a CO_2 incubator for 1h. This incubation was followed by removal of the media, and the cells were washed with sterile citrate-saline. The cells were trypsinized with 0.5 ml trypsin, resuspended in 2 ml fresh media and 10% FBS, counted and 250 μl aliquots of cells were plated in quadruplicate into 96-well microplates. Cytotoxicity was determined by MTT assay (Kirkpatrick et al., 1990) after a four to nine day incubation at 37°C in

a CO_2 incubator to allow for at least four cell doublings. Briefly, the MTT assay involved removal of the media and the addition of 200 μl of fresh RPMI media containing 10 μl of MTT stock to each well. Cells were incubated for 3 h, and then centrifuged for 10 min at 1300 g. The media was removed and 150 μl DMSO was added to each well. The plate was shaken for 2-3 min to dissolve the purple crystals of formazan and the absorbance was measured at 540 nm. The surviving cell fraction of each drug concentration and each cell line were the means of 4-16 determinations. The cytotoxic activities of MeBM, MBM, m-MeBM, CBM, FBM, PBM, m-PBM and m-TBM and were compared to BM through assessment of the differences between the slopes of the linear regression lines of the optical density versus drug concentration curves as described previously (Doherty et al., 1998). The effect of DIC on the cytotoxic activity of each BM analog in each cell line was compared by two-tailed t-tests comparing the significance of the differences of the mean fraction of control cells for cells treated without, or with, DIC.

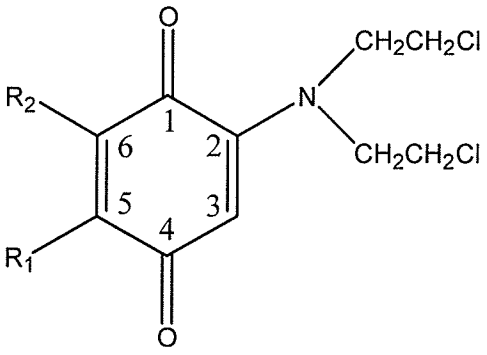
The dose of each BM analog which reduced the surviving cell fraction to 0.1 (D_{10} value) of control cells (i.e. cells not incubated with BM analog), was calculated in the NCI-H661 and the SK-MEL-28 cell lines respectively. The D_{10} value was calculated form the inverse of the slope of the linear regression lines of the optical density versus drug concentration curves in the absence of DIC. Data are expressed as the mean ± S.E.M. of four or more experiments.

Section 3: Results

3.1 BM Analogs

The series of BM analogs having different functional groups attached to the quinone bioreductive element was prepared by the laboratory of Dr. Frank Guziec (Southwestern University TX) (Figure. 30). The analogs were synthesized as model compounds to determine the effects of a wide range of functional group substitutions to the quinone moiety of BM on: 1.) DTD mediated reduction kinetics, and 2.) cytotoxic activity in human cancer cell lines.

Figure 30 Molecular Structures of BM analogs



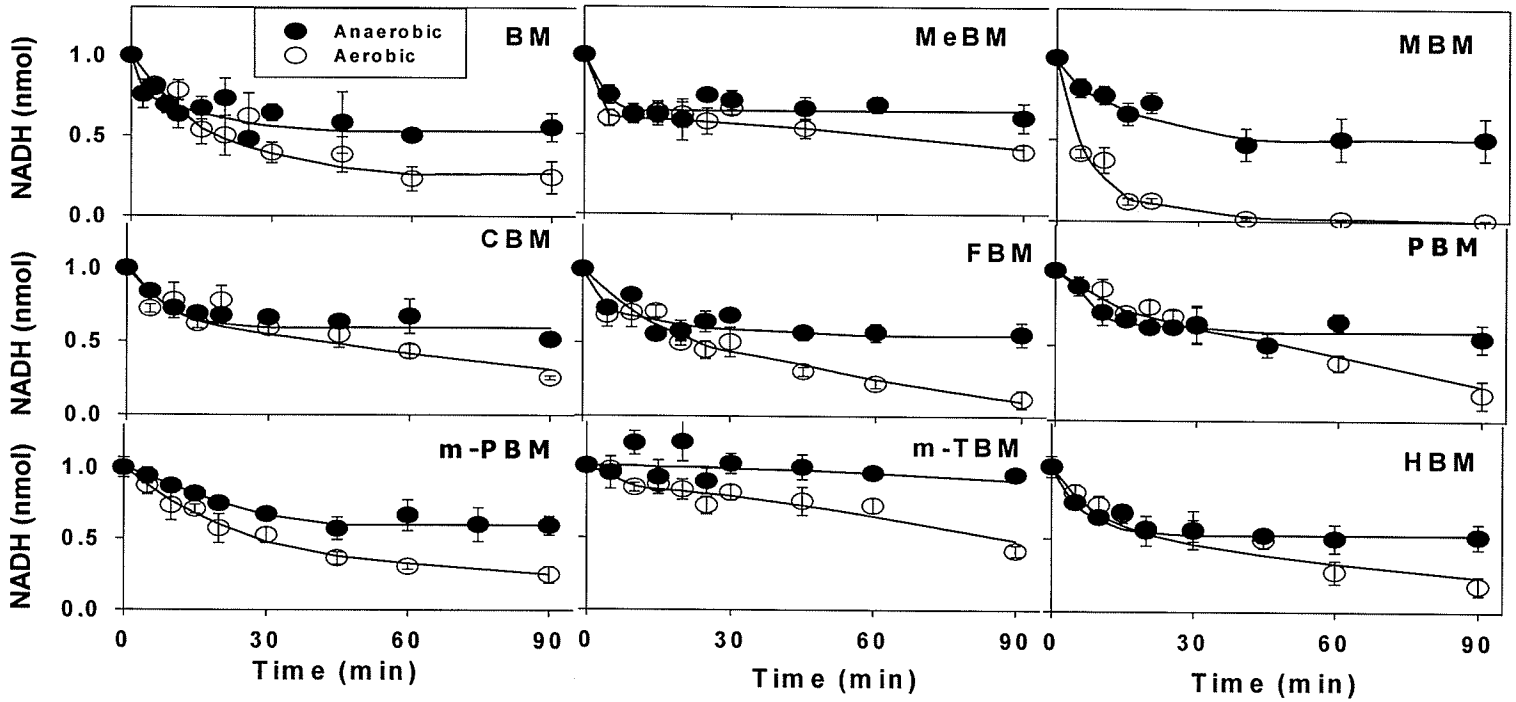
BM analog	R_1	R_2
BM	H	H
MeBM	CH_3	H
MBM	OCH_3	H
m-MeBM	H	CH_3
CBM	Cl	H
FBM	F	H
PBM	Phenyl	H
m-PBM	H	Phenyl
m-TBM	H	$C(CH_3)_3$

MeBM, MBM, CBM, FBM and PBM all have functional group substitutions at the C5 position of the benzoquinone. Specifically, MeBM has a weak electron-donating methyl group, MBM has a strong electron-donating methoxy group, CBM and FBM have chloro and fluoro electron-withdrawing groups, respectively, and PBM has a sterically bulky phenyl group substituted on the benzoquinone. In contrast, m-MeBM, m-PBM and m-TBM have functional group substitutions at the C6 position of the benzoquinone. m-MeBM has a weak electron-donating methyl group, m-PBM has a sterically bulky phenyl group, and m-TBM has a very large sterically bulky tertiary-butyl group attached to the benzoquinone moiety. The analogs were studied to identify the effect of functional groups on the reduction kinetics of the benzoquinone group by DTD and on the cytotoxic activity of the analogs. HBM is an analog of BM in which the chlorines on the nitrogen mustard group have been replaced by hydroxyl groups making the mustard unable to alkylate cell components.

3.2 Reduction of BM Analogs by Purified DTD

Reduction of the BM analogs by purified DTD *in vitro* was carried out under hypoxic or aerobic conditions in solutions containing 880 nmol/min per mg protein purified DTD, 100 μM NADH, 0.5 μM FAD and 50 μM BM analog. At various time points, 10 μM DIC was added to stop the reaction, and the extent of reduction was measured by using HPLC to follow the loss of the enzyme co-factor, NADH, which acts as the electron donor (Figure 31).

Figure 31. Reduction of BM analogs by purified DTD *in vitro*. Air or nitrogen was bubbled through a solution of 100 µM NADH and 0.5 µM FAD in 1 ml of 25 mM Tris HCl (pH 7.4) buffer for 3 hr at 37^{0}C. Purified DTD (880 nmol/min per mg protein) and 50 µM BM analog were added and bubbling was continued. Reactions were stopped by addition of 10 µM DIC. Reduction of BM analog was determined by HPLC, which quantified the loss of NADH. The data for each point represent the mean ± S.E.M. of 4 to 10 determinations. Control reactions consisted of reaction mixture in the absence of BM analog.



Under hypoxic conditions, approximately half (one equivalent) of the NADH was consumed with each of the BM analogs, except m-TBM. The rank order of the rates of reduction of the analogs was BM = FBM > MeBM > CBM ≈ MBM ≈ PBM >> m-PBM >> m-TBM, with $t_{½}$ of 4, 4, 6, 8, 9, 10 and 21 min and >>21 min, respectively. When the BM analogs were reduced by purified DTD under aerobic conditions, greater than one equivalent of NADH was consumed for all the BM analogs except m-TBM (Figure 31). Initial loss of NADH under aerobic conditions was rapid with MBM and MeBM, slower with BM, FBM, CBM, PBM and m-PBM, and very slow with m-TBM.

HBM and BM had identical rates of reduction by DTD under hypoxic conditions ($t_{½}$ of reduction of 4 min), as well as a similar rate under aerobic conditions (Figure 31).

3.3 Cytotoxic Activity of BM Analogs

NCI-H661 human non-small cell lung cancer cells, which have a moderate level of DTD, were pretreated at 37 °C for 20 min without, or with, 100 μM DIC, and then were incubated with BM, MeBM, MBM, m-MeBM, CBM, FBM, PBM, m-PBM or m-TBM for 1 h. Cytotoxic activity was determined by MTT assay (Figure 32). m-PBM and FBM had similar cytotoxic activities to BM in the NCI-H661 cells in the absence of DIC, while MeBM, MBM, m-MeBM, CBM, and PBM had significantly greater activities ($p < 0.05$) (Figure 32 and Table 4) and m-TBM had significantly less activity in these cells, compared to BM ($p < 0.05$) (Fig. 32 and Table 4).

Figure 32. Cytotoxic activity of BM, MeBM, MBM, CBM, PBM and m-PBM in NCI-H661 human non-small cell lung cancer cells in the absence of dicoumarol (DIC). Cells were incubated without, or with 100 μM DIC for 20 min and then with BM analog for 1 hr. Cytotoxicity was determined by MTT assay and is presented as the optical density in BM analog treated cells as a fraction of the optical density in control cells not treated with BM analog. Points represent the mean ± S.E.M. of 4 to 16 determinations, and lines are linear regression lines. Slopes of the linear regression lines of the optical density versus concentration of BM analog were compared by two-tailed t-tests comparing the differences in the slopes between BM and its analogs (* $p < 0.05$).

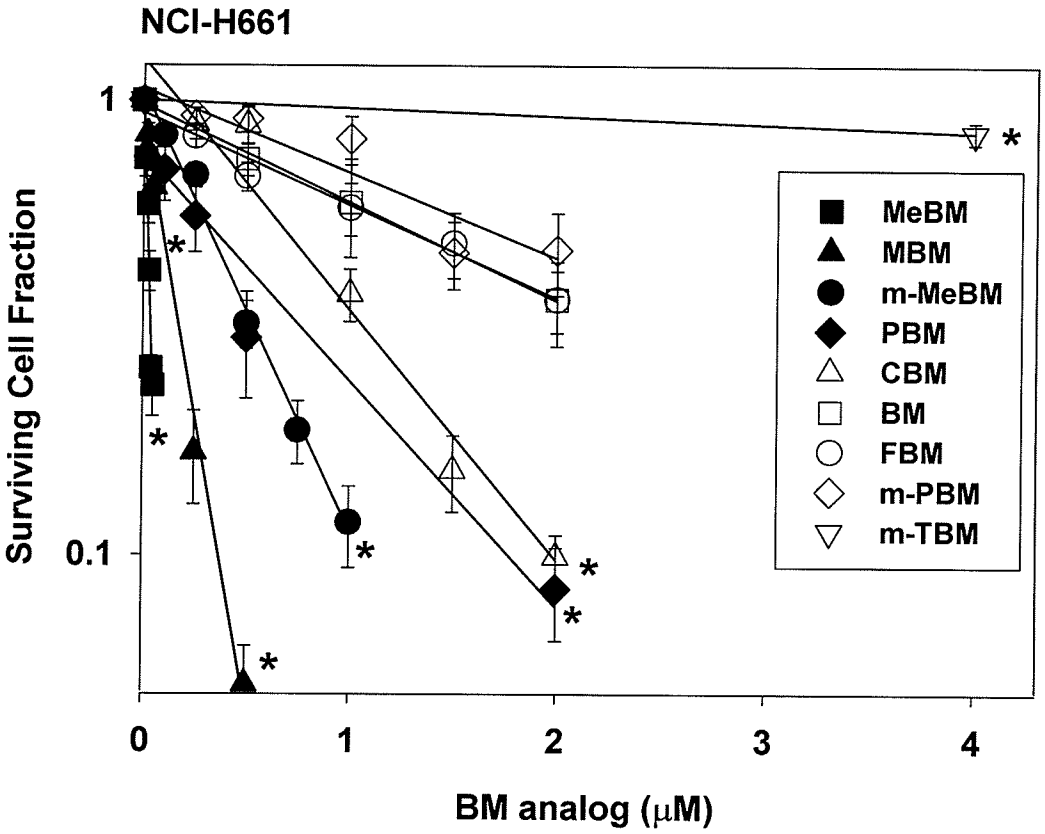


Table 4 Cytotoxic activity of the BM analogs in NCI-H661 human non-small cell lung cancer cells and SK-MEL-28 human melanoma cells. Cells were incubated with BM analog for 1 h. Cytotoxicity was determined by the MTT assay and is presented as the D_{10} value (concentration of each BM analog which reduced the surviving cell fraction to 0.1 of control cells not treated with BM analog). D_{10} was calculated from the inverse slope of the linear regression lines of the optical density versus BM analog concentration curves. Slopes of the linear regression lines were compared by two-tailed t-tests comparing the differences in the slopes between BM and its analogs. Data are represented as the means ± SEM of 4 to 16 experiments (NS non-significant)

BM analog	NCI-H661 cell line		SK-MEL-28 cell line	
	D_{10} (μM)	P-value	D_{10} (μM)	P-value
BM	4.87 ± 0.14		7.57 ± 0.76	
MeBM	0.56 ± 0.18	<0.05	0.76 ± 0.02	<0.001
MBM	0.40 ± 0.02	<0.001	1.39 ± 0.04	<0.05
m-MeBM	0.45 ± 0.02	<0.001	2.31 ± 0.13	<0.05
CBM	1.67 ± 0.15	<0.05	4.71 ± 0.45	NS
FBM	5.30 ± 0.41	NS	17.70 ± 2.92	<0.05
PBM	2.16 ± 2.65	<0.05	3.11 ± 0.27	<0.05
m-PBM	5.05 ± 1.37	NS	7.32 ± 0.37	NS
m-TBM	40.7 ± 3.97	<0.001	106 ± 9.42	<0.001

Pretreatment of the NCI-H661 cells with DIC significantly increased the cytotoxic activity of BM and m-MeBM ($p < 0.05$) (Figure 33 and Table 5) and decreased the cytotoxic activity of MBM, when compared to the cytotoxic activity in the absence of DIC ($p < 0.05$, Table 5). DIC did not significantly affect the cytotoxicity of MeBM, CBM, FBM, PBM, m-PBM and m-TBM in these cells (Figure 33 and Table 5).

Figure 33. Cytotoxic activity of the BM analogs in NCI-H661 human non-small cell lung cancer cells in the absence and presence of dicoumarol (DIC). Cells were incubated without or with 100 μM DIC for 20 min and then with BM analog for 1 h. Cytotoxicity was determined by the MTT assay and is presented as the surviving cell fraction (optical density in BM analog-treated cells as a fraction of the optical density in control cells not treated with BM analog). The results represent the means ± SEM of 4 to 16 determinations. The effect of DIC on the cytotoxic activity of each BM analog in each cell line was compared by two-tailed t-tests comparing the significance of the differences of the mean surviving cell fraction of cells treated without or with DIC (* $p < 0.05$).

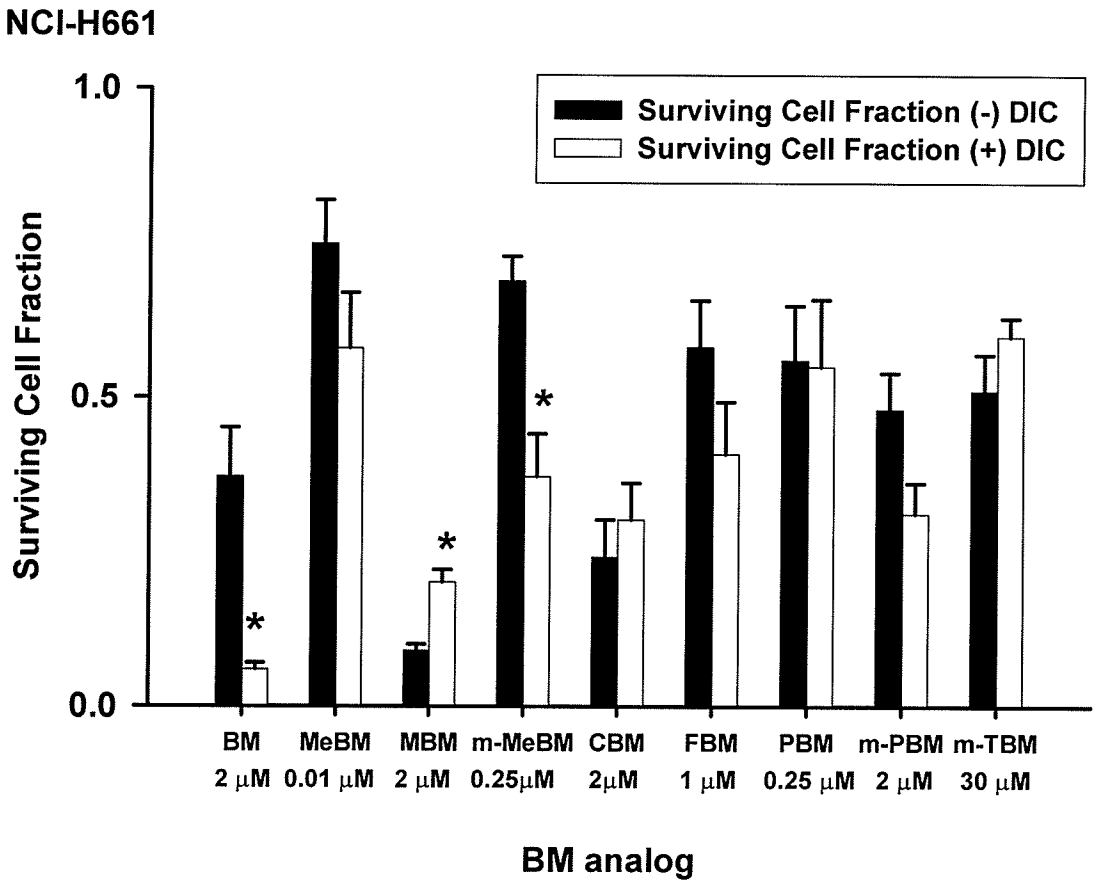
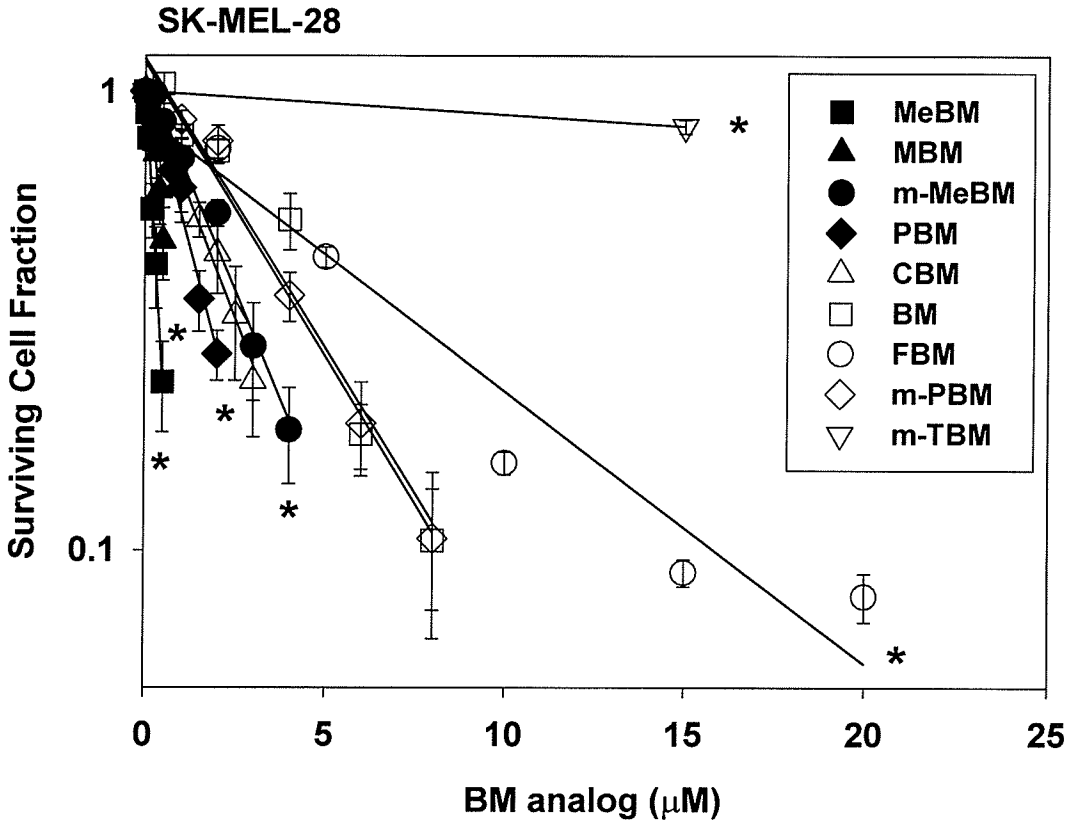


Table 5 Cytotoxic activity of the BM analogs in NCI-H661 human non-small cell lung cancer cells and SK-MEL-28 human melanoma cells in the absence and presence of dicoumarol (DIC). Cells were incubated without or with 100 μM DIC for 20 min and then with BM analog for 1 h. Cytotoxicity was determined by the MTT assay and is presented as the surviving cell fraction (optical density in BM analog-treated cells as a fraction of the optical density in control cells not treated with BM analog). The results represent the means ± SEM of 4 to 16 determinations. The effect of DIC on the cytotoxic activity of each BM analog in each cell line was compared by two-tailed t-tests comparing the significance of the differences of the mean surviving cell fraction of cells treated without or with DIC. (NS non-significant)

BM analog	**NCI-H661 cell line**				**SK-MEL-28 cell line**			
	Drug dose (μM)	**Surviving cell fraction - DIC**	**Surviving cell fraction + DIC**	**P-value**	**Drug dose (μM)**	**Surviving cell fraction - DIC**	**Surviving cell fraction + DIC**	**P-value**
BM	2	0.37 ± 0.08	0.06 ± 0.01	<0.05	2	0.74 ± 0.05	0.20 ± 0.09	<0.001
MeBM	0.01	0.75 ± 0.07	0.58 ± 0.09	NS	0.2	0.56 ± 0.07	0.28 ± 0.08	<0.05
MBM	2	0.09 ± 0.01	0.20 ± 0.02	<0.05	2	0.10 ± 0.03	0.28 ± 0.06	<0.05
m-MeBM	0.25	0.69 ± 0.04	0.37 ± 0.07	<0.05	2	0.54 ± 0.04	0.06 ± 0.01	<0.001
CBM	2	0.24 ± 0.06	0.30 ± 0.06	NS	2	0.54 ± 0.06	0.38 ± 0.10	NS
FBM	1	0.58 ± 0.08	0.41 ± 0.09	NS	5	0.44 ± 0.02	0.29 ± 0.06	<0.05
PBM	0.25	0.56 ± 0.09	0.55 ± 0.11	NS	1	0.62 ± 0.10	0.63 ± 0.10	NS
m-PBM	2	0.48 ± 0.06	0.31 ± 0.05	NS	2	0.77 ± 0.06	0.70 ± 0.10	NS
m-TBM	30	0.51 ± 0.06	0.60 ± 0.03	NS	30	0.70 ± 0.05	0.68 ± 0.04	NS

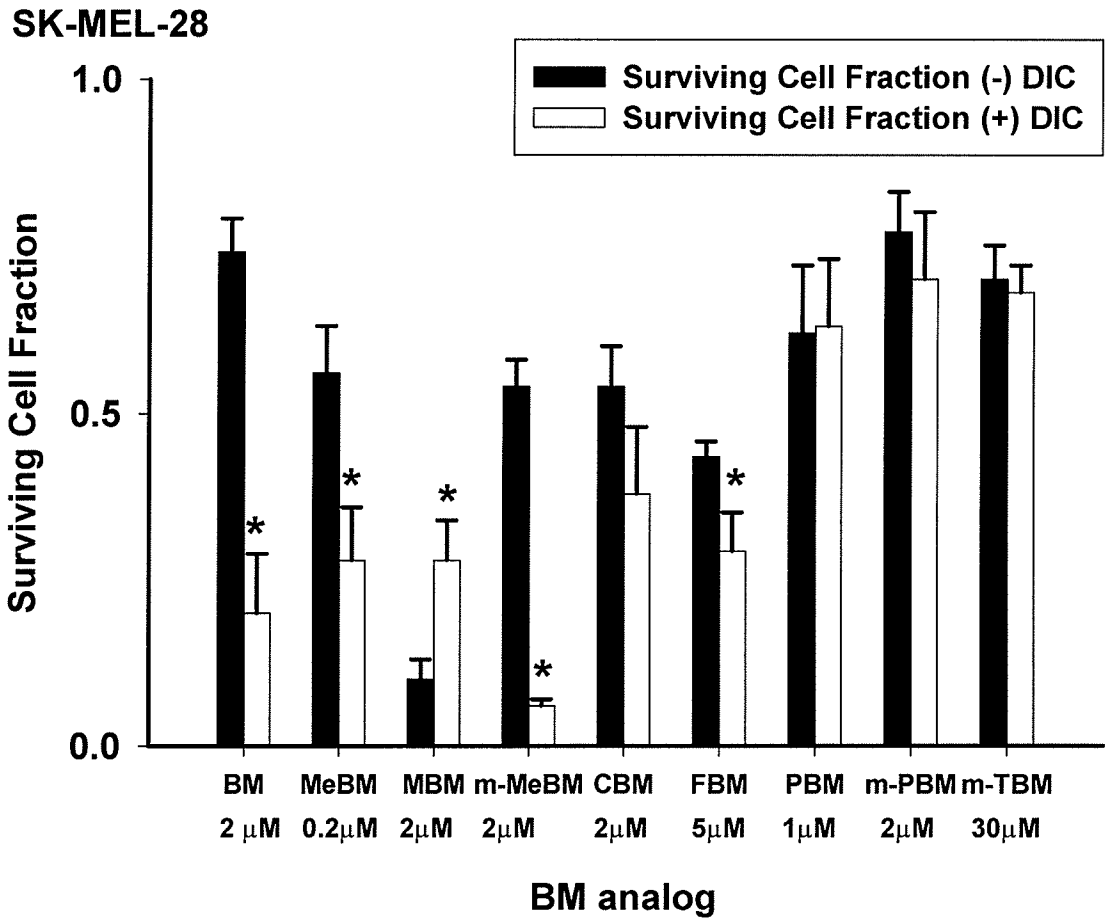
SK-MEL-28 human melanoma cells, which have a high level of DTD, were pretreated at 37 °C for 20 min without, or with, 100 μM DIC, and then were incubated with BM, MeBM, MBM, m-MeBM, CBM, FBM, PBM, m-PBM or m-TBM for 1 h. The surviving cell fractions were determined by MTT assay (Figure 34). CBM and m-PBM had similar cytotoxic activities to BM in the SK-MEL-28 cells in the absence of DIC, while MeBM, MBM, m-MeBM and PBM had significantly greater activities in these cells compared to BM ($p < 0.05$) (Fig. 34 and Table 4). In contrast, FBM and m-TBM had significantly less activity than BM in the SK-Mel-28 cells ($p < 0.05$) (Fig. 34 and Table 4).

Figure 34. Cytotoxic activity of BM, MeBM, MBM, CBM, PBM and m-PBM in SK-MEL-28 human melanoma cells in the absence of dicoumarol (DIC). Cells were incubated without, or with 100 μM DIC for 20 min and then with BM analog for 1 hr. Cytotoxicity was determined by MTT assay and is presented as the optical density in BM analog treated cells as a fraction of the optical density in control cells not treated with BM analog. Points represent the mean ± S.E.M. of 4 to 16 determinations, and lines are linear regression lines. Slopes of the linear regression lines of the optical density versus concentration of BM analog were compared by two-tailed t-tests comparing the differences in the slopes between BM and its analogs (* $p < 0.05$).



Pretreatment with DIC significantly increased the cytotoxic activity of BM, MeBM, m-MeBM and FBM in these cells, when compared to the cytotoxic activity in the absence of DIC ($p < 0.05$) (Figure 35 and Table 5). In contrast, pretreatment with DIC decreased the cytotoxic activity of MBM ($p < 0.05$) (Figure 35 and Table 5). DIC did not significantly affect the cytotoxicity of CBM, PBM, m-PBM and m-TBM in these cells (Figure 35 and Table 5).

Figure 35. Cytotoxic activity of the BM analogs in SK-MEL-28 human melanoma cells in the absence and presence of dicoumarol (DIC). Cells were incubated without or with 100 μM DIC for 20 min and then with BM analog for 1 h. Cytotoxicity was determined by the MTT assay and is presented as the surviving cell fraction (optical density in BM analog-treated cells as a fraction of the optical density in control cells not treated with BM analog). The results represent the means ± SEM of 4 to 16 determinations. The effect of DIC on the cytotoxic activity of each BM analog in each cell line was compared by two-tailed t-tests comparing the significance of the differences of the mean surviving cell fraction of cells treated without or with DIC (* $p < 0.05$).



Section 4: Discussion

Enzyme directed targeting of antitumor agents is a novel strategy for increasing the effectiveness of cancer chemotherapy. This approach is based on the premise that the selectivity of antitumor agents can be increased by the use of agents that are activated by specific activating enzymes in tumors that have high levels of these enzymes (Riley & Workman, 1992, Workman & Stratford, 1993). This would result in increased tumor kill and decreased toxicity to normal tissues which do not have elevated levels of the activating enzyme. Bioreductive agents are ideally suited for enzyme-directed tumor targeting since they require activation by reductive enzymes. Although there has been considerable effort to develop new bioreductive agents with greater enzyme selectivity (Patterson, 1993, Denny et al., 1996), a major problem encountered in developing agents with specificity of activation has been a lack of knowledge of structural factors that produce selectivity for activation of bioreductive agents by reductive enzymes such as DTD and NADPH:cytochrome P450 reductase. In the present study, SARs were determined to identify the effect of functional groups on reduction and activation of bioreductive agents by DTD.

Previously, the antitumor activity and mechanisms of action of the model bioreductive agent, BM were investigated (Begleiter, 1983, Begleiter & Blair, 1984, Begleiter, 1986, Begleiter & Leith, 1990, Begleiter et al., 1991). It was shown that BM was 600-fold more toxic to mouse lymphoma cells than the non-quinone alkylating agent, aniline mustard, and was 30,000-fold more toxic to these cells than HBM, a benzoquinone analog with an inactive nitrogen mustard

group (Begleiter, 1983). BM produced both DNA cross-links and strand breaks, but the cross-links were the major contributor to the cytotoxic activity (Begleiter, 1986, Begleiter & Blair, 1984). Both the cytotoxic and cross-linking activities of BM were increased by reduction of the quinone (Begleiter et al., 1991). The cytotoxic activity and DNA damage produced by BM were lower in mouse lymphoma cells which have high DTD, compared with similar cells which have low enzyme activity. The DTD inhibitor, DIC, increased BM activity in the cells with high enzyme activity, suggesting that DTD mediated reduction of BM leads to products which are less cytotoxic than reduced products which may form via activation by other reductases (Begleiter & Leith, 1990).

Because of their relatively simple structure, analogs of BM are good models to investigate the effects of various functional groups on the reduction of the benzoquinone group by DTD and on the activity of the reduced products. MBM and MeBM were used to study the effects of strong and weak electron-donating groups, respectively. CBM and FBM were used to study the effects of electron-withdrawing groups. FBM was also utilized to investigate the effect of an electron-withdrawing group which has no steric effects, as sterically, the fluorine group in FBM is similar in size to the hydrogen at the C5 position of BM. The positional isomer of MeBM, (m-MeBM) was utilized to study the effect of an electron-donating group at the C6 position of the benzoquinone on DTD mediated reduction and cytotoxicity. Electron-donating and -withdrawing functional groups would be expected to affect the electron density around the benzoquinone, and thus might alter the ability of DTD to reduce this bioreductive

element. These functional groups could also influence the reactivity of the nitrogen mustard group after reduction of the benzoquinone. PBM was used to study the effect of a sterically bulky functional group on reduction by DTD, as such groups may influence the substrate-enzyme affinity. The positional isomer of PBM, (m-PBM), as well as m-TBM, which has a very large sterically bulky group at the C6 position of the benzoquinone, were also used to study the effects of sterically bulky groups on the reduction by DTD, as both the size and position of such a group may affect the substrate-enzyme affinity and cytotoxic activity.

Reduction of the BM analogs by purified DTD was measured *in vitro* to determine the effect of the functional groups on the rate of reduction of the benzoquinone under hypoxic and aerobic conditions. Reduction under hypoxic conditions provides a true measure of the rate of reduction of the analogs since redox cycling is prevented. Under these conditions, the half-times ($t_{½}$) for reduction of the analogs by DTD were 4, 4, 6, 9, 8, 10 and 21 >>21 min for BM, FBM, MeBM, MBM, CBM, PBM, m-PBM and m-TBM, respectively. All functional groups, except the fluorine group, decreased the rate of reduction of the benzoquinone compared to the parent compound BM. The sterically similarly sized electron-donating methyl and methoxy groups in MeBM and MBM, and the electron-withdrawing chloro group in CBM, decreased the rate of reduction to a similar extent. This suggested that the electron density around the benzoquinone is not a major factor influencing reduction by DTD. The half-time of reduction of PBM was slower than that of the BM analogs with smaller electron-donating and –withdrawing functional group substituted at the C5

position, however it was not as slow as that observed with m-PBM. This suggests that sterically bulky groups at the C5 position may not interfere with the substrate-enzyme interaction as much as sterically bulky group substitutions at the C6 position. The relatively small effect of the phenyl group at C5 on the reduction of the benzoquinone may be due to the ability of the benzene ring to rotate and take on a position that would interfere minimally with the enzyme-substrate interaction when substituted at the C5 position. This accommodation of the substrate at the binding pocket of DTD may not be possible at the more critical site for the enzyme-substrate interaction at the C6 position. To confirm the finding that steric effects are more critical in affecting DTD mediated reduction kinetics, FBM was synthesized. This compound has an electron-withdrawing fluorine group substitution, but no steric effects, as the fluorine is sterically similar in size to the hydrogen at the C5 position of the benzoquinone moiety in BM. The half-time of reduction of FBM was equivalent to that of the parent structure BM, providing further evidence that electronic effects are not important in affecting the rate of reduction by DTD. Moreover, m-TBM, which has a very large sterically bulky group substituted at the benzoquinone, was a poor substrate for DTD as the quantity of NADH consumed over the 90 min reaction time was insignificant and did not allow for the determination of a half-time of reduction for this analog. This result further suggested that steric effects at the C6 position of the benzoquinone are important in decreasing the rate of reduction by DTD. Similar results were observed in studies with a series of indolequinones, where large functional group substitutions at the 5 position

(equivalent to the C6 position in our analogs), led to compounds that were poor substrates for DTD (Phillips et al., 1999). In the current study, the results indicated that very large sterically bulky groups at the C6 position may, in fact, prevent significant reduction by DTD, presumably by steric interactions in the active site.

When the BM analogs were reduced by purified DTD under aerobic conditions, there was a greater loss of NADH for all compounds, compared with hypoxia, indicating that the initially produced reduction products could undergo redox cycling. The initial loss of NADH (indicated by the initial slope of the NADH versus time plots under aerobic conditions) was faster with MBM compared with BM, MeBM, FBM, CBM, PBM and m-PBM, which produced similar but slower losses of NADH. This suggests that the methoxy group may enhance the rate of redox cycling. The loss of NADH under aerobic conditions was very slow for m-TBM compared to the other analogs, which is consistent with it being a poor substrate for DTD.

The rate of reduction or redox cycling of the benzoquinone is not affected by small structural alterations to the cytotoxic element. The structure of HBM is identical to that of BM except that the chloro groups on the cytotoxic mustard have been replaced by hydroxyl groups. Reduction of the BM analog, HBM, by DTD and its ability to redox cycle were similar to BM.

The cytotoxicity studies in human tumor cell lines with moderate and high levels of DTD suggested that the phenyl group of m-PBM had little effect on the overall cytotoxic activity of the bioreductive agent, since BM and m-PBM

produced similar tumor cell kill in both cell lines. In contrast, the methoxy, methyl and phenyl group at the C5 position and the methyl group at the C6 position significantly increased the cytotoxic activity, with MBM, MeBM, PBM and m-MeBM, respectively, all of which showed greater activity than BM in both cell lines. The chloro group at the C5 position significantly increased the cytotoxic activity of the bioreductive agent in the NCI-H661 cell line, however, the cytotoxicity of CBM in the SK-MEL-28 cell line was similar to that of BM. The cytotoxic activity of FBM was similar or decreased when compared to BM, in the NCI-H661 and SK-MEL-28 cell lines, respectively. The tertiary-butyl group at the C6 position significantly decreased the cytotoxic activity of m-TBM in both cell lines, which may be explained by a poor activation of this analog by DTD.

As observed earlier, in mouse lymphoma cells with high DTD activity (Begleiter & Leith, 1990), DIC increased the cytotoxicity of BM in both NCI-H661 and SK-MEL-28 cells, compared to in the absence of DIC, suggesting that DTD was an inactivating enzyme for this agent. Similarly, DTD mediated reduction appeared to be an inactivating pathway for m-MeBM, as its cytotoxicity was increased in both cell lines in the presence of DIC, compared to in the absence. DIC only increased the cytotoxic activity of MeBM and FBM in the SK-MEL-28 cell line, however, similar trends were observed in the NCI-H661 cell line which has lower DTD activity. This suggests that the role of DTD as an inactivating enzyme for MeBM and FBM may only be important in the presence of relatively high DTD activity. On the other hand, DIC decreased the cytotoxic activity of MBM in both human tumor cell lines, indicating that DTD was an activating

enzyme for MBM. The enzyme inhibitor did not significantly affect the cytotoxic activities of CBM, PBM, m-PBM and m-TBM in both cell lines, which suggested that DTD did not play an important role in the activation of these agents.

The effect of DTD on the cytotoxic activity of the BM analogs may, at least in part, be explained by the effect of the functional groups on reduction of the quinone by DTD and the subsequent redox cycling of the reduced products. Reduction of BM by DTD was shown to be an inactivation pathway, and the hydroquinone product appeared less active than the semiquinone or other reduced products which formed in the absence of DTD mediated reduction. Such products may form through reduction of BM by alternate reductases such as NADPH:cytochrome P450 reductase, and further studies are required to assess this possibility. BM appears to be a very good substrate for DTD, with a half-time of reduction of 4 min, thus DTD mediated reduction of BM may decrease the amount of drug available for reduction by other reductases, leading to an overall detoxifying effect. Alternatively, apparent activation and inactivation of different BM analogs by DTD may be due to variable balances between inactivation pathways (e.g. glucuronidation) and activation pathways, between the compounds, and further studies are required to investigate this hypothesis.

The increased cytotoxicity observed with MBM may be due to the electron-donating group conferring an increased cytotoxicity through activation of the cytotoxic elements of the reduced products, as the increased electron density within the hydroquinone may facilitate the loss of the chloride leaving groups of the cytotoxic mustard group. Furthermore this compound has an increased rate

of redox cycling compared to BM and its analogs, which may allow for elevated formation of reactive oxygen species leading to increased DNA and other cellular oxidative damage.

MeBM had an increased cytotoxic activity compared to BM, but DTD mediated reduction only appeared important as an inactivation pathway for this analog in the SK-Mel-28 cells with high DTD activity. The increased cytotoxicity of this compound may be due to the activation of the cytotoxic group in the reduced products formed via reduction by DTD and other enzymes. These other reductase enzymes may produce reduced products with increased cytotoxicity compared to those produced by DTD, and therefore may be more important in the activation of MeBM relative to DTD. Furthermore, compared to MBM, MeBM did not undergo as much redox cycling, as not all the NADH was consumed over 90 minutes, as was observed with MBM. This may reflect the weaker electron-donating character of the methyl group compared to the methoxy group. The methoxy group may therefore allow for fast redox cycling, and quantitatively more reactive oxygen species to form compared to the weaker electron donating methyl group.

The positional isomer of MeBM, m-MeBM, had an increased cytotoxicity compared to BM, but unlike MBM, DTD mediated reduction appeared to be an inactivating pathway for this compound, as its cytotoxic activity was increased in the presence of DIC compared to in the absence of DIC. Therefore, similar to the results with BM, the reduced products which form via DTD mediated reduction may be less cytotoxic than those that form via other reductases for m-

MeBM. As well, its increased cytotoxic activity, compared to BM, may be due to the electron-donating group conferring an increased cytotoxicity, through activation of the cytotoxic elements of the reduced products, which may allow for efficient DNA cross-link formation.

DTD mediated reduction was an inactivation pathway for FBM in the SK-Mel-28 cells with high DTD activity. This analog is reduced rapidly by DTD with an identical half-time of reduction as BM. Therefore, cells with high DTD activity may reduce this compound to the hydroquinone relatively rapidly, and therefore less compound may be available for reductive activation via other enzymes. The cytotoxicity of FBM was similar to BM in the NCI-H661 cell line, which has lower DTD activity compared to the SK-Mel-28 cell line. Therefore, in the presence of lower DTD activity, DTD mediated inactivation may be less important, and reduction via other one-electron reductase enzymes may play a role in the activation of this analog.

PBM displayed an increased cytotoxicity compared to BM, but reduction by DTD did not appear to be important in the activation of PBM, as its cytotoxic activity was similar in the presence and absence of DIC. Other reductases such as NADPH:cytochrome P450 reductase may therefore be important in the activation of this compound.

The cytotoxicity of CBM was increased compared to BM in the NCI-H661 cell line, but similar to that of BM in the SK-Mel-28 cell line. As in the case of PBM, reduction by DTD did not appear to be important in the activation of this compound, and hence, other reductases may be more important in the activation

of CBM. The differences in the cytotoxicity of CBM in the two cell lines may be due to the relative balance between DTD activity and the activity of other reductase enzymes which could activate this compound. For example, the NCI-H661 cell line has relatively low DTD activity compared to the SK-Mel-28 cell line. Therefore, in the presence of low DTD activity, more of the compound may be activated via other reductase enzymes, and this may lead to an increased cytotoxicity of CBM, compared to BM in the NCI-H661 cells. On the other hand, such an activation effect through other reductases may be less important in the SK-Mel-28 cell line, with high DTD activity. Furthermore, the chloro group, in addition to the other functional group substitutions, may affect the alkylating and cross-linking activities of the DTD mediated reduction metabolites, and this may further influence the cytotoxic activity of the BM analogs.

In the case of m-PBM, the sterically bulky phenyl group significantly decreased the rate of reduction by DTD, and did not alter the cytotoxicity of this agent compared to BM. Reduction of m-PBM by DTD appeared relatively unimportant in its activation, as the cytotoxic activity of m-PBM was similar in the presence and absence of DIC. Due to the large amount of steric hindrance produced by the C6 phenyl group, this compound may not be effectively activated by DTD. Furthermore, the cytotoxic activity of PBM and its rate of reduction by DTD were more efficient than that of m-PBM, further suggesting that the steric effect at the C6 position is important in decreasing the reduction and cytotoxicity of m-PBM. Further studies are required to determine if reduction by

other enzymes like NADPH:cytochrome P450 reductase are more important that DTD in activating this BM analog.

The results with m-TBM indicated that very large sterically bulky groups such as the tertiary butyl group at the C6 position, might significantly inhibit reduction by DTD, presumably by steric interactions in the active site of DTD. This decreased activation of m-TBM by DTD was supported by its decreased cytotoxic activity compared to BM in both cell lines. The cytotoxic potency for m-TBM in the presence of DIC was similar to that in the absence of DIC, indicating that DTD does not contribute to its activation.

m-TBM had a decreased cytotoxic activity compared to BM in the presence and absence of DIC, which may indicate that in addition to its poor activation by DTD, this compound may not be a good substrate for other cellular reductase enzymes.

The results of the present study are consistent with earlier structure-activity studies, which examined reduction of a quinone group by DTD (Bailey et al., 1992, Beall et al., 1998a,b, Naylor et al., 1997, Phillips et al., 1999). Studies with indoloquinones (Bailey et al., 1992, Beall et al., 1998a,b, Phillips et al., 1999) suggested that functional groups at the 5-position in these compounds, which is equivalent to the C6 position of the functional groups in the current study, influenced reduction of the quinone by DTD through a steric effect. In addition, similar to our finding that the methoxy group increased redox cycling of reduced MBM, it was suggested that a 5-methoxy group in the indolequinone models may increase the rate of reoxidation of the reduced quinone group

(Naylor et al., 1997). The studies with indolequinones also showed that an aziridinyl group appeared to be particularly advantageous for increasing cytotoxic activity (Bailey et al., 1992, Beall et al., 1998a,b, Phillips et al., 1999). Functional group substitutions at the indole 3- position of a series of indolequinone anticancer agents have also been shown to further affect DTD mediated reduction (Beall et al., 1998a,b, Swann et al., 2001). Overall, certain functional group substitutions to the quinone moiety of benzoquinone and indolequinone compounds appear to produce similar effects on DTD mediated reduction. On the other hand, other structural modifications, produce unique effects on specific classes of quinone-based bioreductive alkylating agents such as the indolequinones. This indicates that structure activity relationships, or the effects of functional groups on the cytotoxicity and reduction of quinone-based bioreductive alkylating agents can be very complex.

Taken together, these findings have demonstrated that functional groups can significantly affect the reduction and activation of bioreductive agents by DTD. The methoxy, methyl, chloro, phenyl and tertiary-butyl functional groups decreased the rate of reduction of the benzoquinone group by DTD. Steric, rather than electronic, effects of the functional groups appeared to be most important for decreasing the rate of reduction by DTD, and very large functional groups at the C6 position may significantly decrease DTD mediated reduction. DTD appeared to be an inactivating enzyme for BM and m-MeBM in both cell lines and for FBM and MeBM in the SK-MEL-28 cell line. It was an activating enzyme for MBM but had little effect on the cytotoxic activity of FBM and MeBM

(in the NCI-H661 cell line), and CBM, PBM, m-PBM and m-TBM in both cell lines. This may result, in part, from the formation of a less reactive hydroquinone product following reduction of BM by DTD, an effect of the methoxy group on the ability of the reduction product of MBM to redox cycle or alkylate DNA, the slow rate of reduction for m-PBM and m-TBM by DTD. Electron-donating groups also increased the rate of redox cycling MBM. The increased cytotoxic effects of MeBM, m-MeBM, CBM and PBM may be due to activation of the compounds by other reductases, redox cycling of the reduced product, as well as direct effects of the functional groups on the activity of the cytotoxic elements of the reduced products. The decreased cytotoxicity observed with FBM and m-TBM may be due to the formation of relatively inactive hydroquinone products via DTD mediated reduction, or decreased of bioactivation, respectively.

Other mechanisms involving drug uptake or efflux, detoxification pathways and DNA repair could also have effects on the overall observed cytotoxic activities of the BM analogs. Whether such mechanisms, or other mechanisms through which the functional groups may exert their effects on the cytotoxic activity of the BM analogs are important, need to be investigated in future studies.

CHAPTER 3

A Structure-Activity Study with Bioreductive Benzoquinone Alkylating Agents: Effects on DTD Mediated DNA Cross-link and Strand Break Formation in Relation to Mechanisms of Cytotoxicity

Section 1: Background and Rationale

1.1 Bioreductive Alkylating Agents: Mechanisms of cytotoxicity and the role of DTD mediated activation

Bioreductive alkylating agents have two main structural elements, a bioreductive element and a cytotoxic element. For some agents, such as tirapazamine, (Patterson, 1993) the bioreductive and cytotoxic elements are the same moiety, whereas for other agents, including mitomycin C, (Lown et al., 1976 Tomasz et al., 1987) the bioreductive element regulates a separate cytotoxic element. In the case of mitomycin C, and aziridinylquinones such as AZQ, for example, the bioreductive element is a quinone moiety, which can undergo both one- and two-electron reduction, and the cytotoxic element is an alkylating group that is activated when the quinone is reduced (Bender et al., 1883, Pan et al., 1984, Gutierrez, 1989, Dzielendziak et al., 1990, Gibson et al., 1992, Beall et al., 1995). One-electron reduction of the quinone moiety is mediated by enzymes such as NADPH:cytochrome P450 reductase (Bachur et al., 1979, Powis, 1987), and results in the formation of semiquinone reactive intermediates which can react with oxygen to produce superoxide and other reactive oxygen species. These reactive intermediates in turn can undergo nucleophilic additions with DNA, and produce DNA strand breaks (Szmigiero et al., 1984, Gutierrez, 1989). Two-electron reduction, converts the parent quinone moiety to its hydroquinone form, which may react with oxygen to produce oxidative damage, and cross-link DNA through the activated cytotoxic element (Walton et al., 1991, Bailey et al., 1997).

The obligatory two-electron reductase DTD (EC1.6.99.2) is elevated in many tumors such as non small cell lung carcinoma (Malkinson et al., 1992). Bioactivation of bioreductive agents by DTD to species with increased cytotoxic activity is considered central to the enzyme-directed approach to bioreductive drug development (Workman, 1994).

DTD is pivotal in the reductive activation of mitomycin C, (Siegel et al., 1990b) aziridinylbenzoquinones such as RH1, (Winski et al., 1998) AZQ and MeDZQ (Siegel et al., 1990a, Lee et al., 1992) and the indolequinone, EO9 (Plumb et al., 1994, Beall et al., 1995, Bailey et al., 1997) to cytotoxic species capable of alkylating and cross-linking DNA. DNA interstrand cross-link formation is considered one of the most cytotoxic lesions formed by bioreductive alkylating agents. In the case of mitomycin C, DTD mediated reduction leads to the activation of two electrophilic sites located at the C(1) and C(10) positions of the molecule. Cross-link formation involves first, the formation of a covalent bond between one of these electrophilic sites on mitomycin C and a nucleophilic NH_2 at the 2 position of guanine. Subsequently, cross-link formation is completed through a second alkylation reaction of the other electrophilic site to the NH_2 at the 2 position of an adjacent guanine (Tomasz et al., 1987).

Depending on the ambient oxygen tension, the structure of the drug and its redox potential, and the characteristics of reduction of the quinone, both DNA strand break and cross-link formation may be important mechanisms of cytotoxicity for a given agent, and may produce synergistic effects in terms of the overall cytotoxic potency of the drug (Begleiter, 1983).

Although the cytotoxic effects of benzoquinone-based bioreductive agents are thought to be due to DNA cross-link and strand break formation, there is limited data demonstrating a direct association between the cytotoxic activity of benzoquinone bioreductive agents and DTD mediated reduction kinetics or, the extent and type of DNA damage. Several reports have suggested qualitative associations between cytotoxicity of the diaziridinylbenzoquinones in different cell lines and DNA cross-link formation (Szmigiero et al., 1984, Dzielendziak et al., 1990, Hargreaves et al., 1999). As well, in experiments using a single concentration of a series of aziridinylbenzoquinones, the ease of reduction by DTD was shown to closely follow both their *in vitro* cytotoxicity and their ability to cross-link DNA (Lee et al., 1992). To this end, it would be more informative to test structure-activity relationships for DNA cross-link or strand break formation using a broad range of concentrations. This would ensure that the correlations were based on drug concentrations within the linear range of the dose-response relationships, thus avoiding conclusions based on measurements in the saturation or floor portions of the dose-response curves.

Moreover, it is at present unclear how varying functional group substitutions on the benzoquinone moiety of bioreductive alkylating agents may impact their cytotoxic activity and ability to form DNA cross-links or strand breaks. Previously, we provided evidence that electron-donating groups substituted at the C5 position of our model benzoquinone mustard (BM) produced compounds with increased cytotoxic potency compared BM, while sterically bulky groups at the C6 position decreased both the *in vitro* rate of

reduction by DTD and the cytotoxic activity of the analogs compared to BM (Chapter 2, Fourie et al., 2002). As well, alkyl group substitutions to the quinone moiety of aziridinylquinones affected their cross-linking efficiency subsequent to chemical reduction (Hargreaves et al., 1999), while functional group substitutions to cyclopent[b]indoles have led to the identification of several structure-activity relationships and the design of new bioreductive alkylating agents with increased DNA alkylating ability subsequent to reduction by DTD (Skibo et al., 2001).

Currently it is unknown whether differences in kinetic parameters of reduction by DTD or alternatively, differences in effects at the DNA drug target are most important in determining the cytotoxic potency. This information would be crucial for an increased understanding of the mechanisms of cytotoxic activity of these agents, and for the development of future generations of quinone-based anti-tumor agents with increased efficacy.

The aim of the present study was to investigate SARs between a series of benzoquinone mustard analogs with a wide range of functional group substitutions and varying levels of cytotoxicity. This study investigated the effects of these functional group substitutions on DTD mediated DNA cross-link or strand break formation by employing a concentration-effect study design, which would ensure that comparisons were made within the linear range of the concentration effect profiles, in order to characterize true structure-activity relationships. Furthermore, it was determined whether, and to what extent, differences produced by functional group substitutions on DTD mediated

reduction kinetics or DNA cross-link and strand break formation related to the cytotoxic activity of the BM analogs in human tumor cell lines.

Section 2: Materials and Methods

2.1 Materials

Purified recombinant human wild-type DTD was obtained from Dr. Sushu Pan, University of Pittsburgh Cancer Institute (Pittsburgh, PA). The DTD from Dr. Sushu Pan was used for the DNA cross-link and strand break experiments, and its activity was determined to be 33 μmol/min per mg protein, as measured by the dicoumarol-sensitive reduction of 2,6-di-chlorophenolindophenol (DCPIP) (Benson et al., 1986). NADH, FAD, DIC, Tris-HCl, vanillin, chromic acid, Tris (hydroxymethyl)amino-methane, Na_2EDTA, SYBR Green I nucleic acid gel stain, ampicillin (sodium salt) and chloramphenicol were from Sigma-Aldrich Inc. (St. Louis, MO). Luria-Bertani Broth, Miller and Luria-Bertani Agar, Miller were from Becton Dickinson and Co. (Sparks, MD). Qiagen Plasmid Purification Kits were from Qiagen Pty. Ltd. (Mississauga, ON, Canada). Novagen Pellet paint co-precipitant and sodium acetate were from VWR International Ltd. (Mississauga, ON, Canada). Klenow fragment of DNA polymerase I and agarose were obtained from Invitrogen Canada Inc. (Burlington, ON, Canada). [α-^{32}P]dATP (6000 Ci/mmol), EcoRI and Nick columns (to separate unincorporated ^{32}P-labelled nucleotides) were purchased from Amersham Biosciences Corp. (Baie d'Urfe, PQ, Canada), while N,N,-dimethyl formamide and dimethyl sulfoxide were from Fisher Scientific (Fair Lawn, NJ).

2.2 Preparation of BM analogs

The syntheses of the model compound BM and its analogs MeBM, MBM, m-MeBM, CBM, FBM, PBM, m-PBM and m-TBM were reported in full in Chapter 2.

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

Competent cultures of *E. coli* (DH5 alpha) were obtained for electroporation via the procedure outlined by Sambrook and colleagues (1989). The competent bacteria were grown to mid-log phase, chilled, centrifuged and then washed with low-salt buffer in order to decrease the ionic strength of the cell suspension. Subsequently, the cells were suspended in 10% glycerol, and stored at –70 °C. Prior to electroporation, an aliquot of bacteria was thawed and electroporation was carried out at 0 °C using a mini-electrode and sample holder obtained from Bio-Rad Laboratories (Mississauga, ON, Canada).

2.4 Purification of pBR322 plasmid from *E. coli*

A starter bacterial culture was prepared by placing 1 μl of stock *E. coli* (DH5 alpha) containing PBR322 plasmid into a 5 ml volume of Luria-Bertani (LB) broth containing ampicillin at a final concentration of 50 μg/ml. The starter culture was incubated for 6 h at 37°C in a rotary shaker at 60 rpm. Luria-Bertani (LB) Agar (17.5 g/500 ml double distilled H_2O) was autoclaved and a 100 ml aliquot was placed in a water bath and cooled to 42°C. Ampicillin, at a final

concentration of 100 μg/ml, was added to the cooled agar, and it was poured into sterile plates. Subsequently, the plates were streaked with the starter culture of E. coli (DH5 alpha) containing PBR322 plasmid using a sterile inoculating loop, and the plates were incubated in a 37°C incubator for 16 h to obtain single bacterial colonies.

A single bacterial colony was picked from the streaked selective plate to inoculate 10 ml of LB broth containing 50 μg/ml ampicillin, and the culture was grown to late log phase (i.e., to an OD 600 of approximately 0.6) 37°C in a rotary shaker. Subsequently, a 400 ml volume of LB broth (50 μg/ml ampicillin) was inoculated with 7 ml of the 10 ml culture, and grown at 37°C in a rotary shaker with vigorous shaking (approximately 300 rmp) for 3 h until the OD 600 was 1.0. 150 μl of a 34 mg/ml chloramphenicol stock was added to the 400 ml LB media growth. The culture was grown for 12-16 h at 37°C in a rotary shaker with vigorous shaking (approximately 300 rmp) to allow for amplification of the low copy PBR233 plasmid, which only replicates to moderate copy numbers in the DH5 alpha bacteria.

The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C, followed by removal of all traces of supernatant through inverting the open centrifuge tube until all medium has drained. Next, the plasmid was purified by using the Qiagen plasmid maxi Prep technique as described in the Maxi prep kit (Qiagen Pty. Ltd,), followed by the determination of the plasmid DNA concentration.

2.5 Measurement of DNA cross-link formation

DNA interstrand cross-link formation, subsequent to bioreductive activation of BM and its analogs, was assessed based on a previously developed assay (Hartley et al., 1991). In brief, pBR322 plasmid DNA was isolated from *E. coli* (DH5 alpha) by large-scale plasmid preparations. The plasmid DNA was linearized by digestion with EcoRI and 3'-end-labeled with [α-^{32}P]dATP (5 µl, 6000 Ci/mmol) and Klenow fragment of DNA polymerase I. Unincorporated dATP was removed by column filtration and DNA was eluted in TE buffer (10mM Tris pH 7.5, 1mM Na_2EDTA) at a final concentration of 1 ng/µl. Reactions were performed in 1.5-ml microfuge tubes in a final volume of 500 µl of reaction buffer (25 mM Tris HCl, pH 7.4). Freshly prepared NADH and FAD were added to the reaction buffer to give final concentrations of 400 µM and 0.1 µM, respectively. The reaction mixture was purged with nitrogen at 37°C for 30 min to create anaerobic reaction conditions (Chapter 2, Fourie et al., 2002). DTD (prepared fresh for each experiment from –80°C frozen stock and activated with 0.01% Tween 20), at a final activity of 9.243 nmol/min per mg protein, followed by 30 ng end-labeled DNA were added to the reaction buffer. Experimental conditions were such that reaction mixtures contained excess amounts of DTD and its cofactors. Preliminary experiments indicated that very large amounts of protein present in the reaction mixtures that were loaded into the gels caused the loaded samples to be retained in the loading wells subsequent to electrophoresis. Therefore, DNA cross-link gel assays did not allow for DTD protein levels to exceed the concentrations present in our reaction mixtures. Reactions were

initiated by the addition of freshly prepared BM analog in DMF (1 nM to 75 μM, final DMF concentration of 0.2%), followed by incubation at 37°C for 15 min while anaerobic reaction conditions were maintained. Subsequently the reactions were terminated by the addition of 2 μl Novagen Pellet Paint co-precipitant and 5 μl sodium acetate (3 M, pH 5.2) to a 50 μl aliquot of the reaction mixture. This was followed by the addition of 100 μl of 100% ethanol, and incubation at room temperature for 3 min. The reaction mixture was then centrifuged at 14, 000 g for 10 min and the supernatant was removed. The DNA pellets were washed with 70% ethanol, centrifuged for 5 min at 14, 000 g, followed by removal of the supernatant. The microfuge tubes were inverted and the DNA pellets were dried for 30 min. The DNA pellets were dissolved in 20 μl strand separation loading dye (35% DMSO, 1 mM Na_2EDTA pH 7, 10 mM Tris pH 7, 0.05% bromophenol blue, 0.05% xylene cyanol). DNA strand separation consisted of incubation of the DNA in a 70°C water bath for 3 min, followed by immediate cooling in an ice-water bath for 30 min. The DNA was subsequently loaded onto a 1% agarose gel containing 0.4 μg/ml ethidium bromide, followed by electrophoresis (125 V for 30 min) in TAE buffer (40 mM Tris base, 40 mM acetic acid, 1mM EDTA). The gel was dried at 45°C for 6 h, and the double-stranded and single-stranded conformations of DNA were visualized and quantified by densitometry using the STORM image-scanning system (Molecular Dynamics Inc. Sunnyvale, CA). The quantity of the cross-linked double stranded DNA as a percent of the total amount of DNA loaded per well on the agarose gel was determined for each BM analog, and data are expressed as mean values ± SEM of four to seven

experiments. Visual inspection of the DNA cross-link versus BM analog concentration curves indicated a hyperbolic function. Hence data were fitted to a hyperbola equation through non-linear regression analysis using the Statistical Package for Social Sciences (SPSS, Chicago, IL) version 11.0 to determine the maximum amount of DNA cross-links formed by each BM analog.

2.6 Measurement of DNA strand break formation

DNA single-strand break formation, subsequent to bioreductive activation of BM and its analogs, was assessed based on the DNA supercoiled relaxation method which measures the conversion of the supercoiled form of plasmid to its open relaxed circular conformation (Walton et al., 1991). Briefly, pBR322 plasmid DNA was isolated from *E. coli* (DH5 alpha) by large-scale plasmid preparations. This was followed by purification of the supercoiled conformation of the plasmid DNA by gel electrophoresis. Reactions were performed in 1.5-ml microfuge tubes in a final volume of 200 µl of reaction buffer (10 mM Tris pH 7.5, 1 mM Na_2EDTA). Freshly prepared NADH and FAD were added to the reaction buffer to give final concentrations of 1mM and 0.25 µM, respectively. A 1.5-inch stainless steel needle through which air was blown was inserted into the reaction tube to a position just above the reaction buffer to create aerobic reaction conditions for 30 min at 37°C. DTD (prepared fresh from –80°C frozen stock and activated with 0.01% Tween 20), at a final activity of 23.2 nmol/min per mg protein, followed by 150 ng DNA were added to the reaction buffer. Experimental conditions were such that reaction mixtures contained excess amounts of DTD

and its cofactors. Preliminary experiments indicated that very large amounts of protein present in the reaction mixtures that were loaded into the gels caused the loaded samples to be retained in the loading wells, as well as smearing, subsequent to electrophoresis. Therefore, DNA strand break gel assays did not allow for DTD protein levels to exceed the concentrations present in our reaction mixtures. Reactions were initiated by the addition of freshly prepared BM analog in DMF (1 nM to 100 µM, final DMF concentration of 0.2%), followed by incubation at 37 °C for 15 min under aerobic conditions. Control reactions incubated at 37 °C for 15 min under aerobic conditions consisted of: 1) buffer, DTD, NADH, FAD, with no BM analog and, 2) buffer, NADH, FAD, BM analog, with no DTD. Reactions were terminated by the addition of 2 µl of Novagen Pellet Paint co-precipitant and 20 µl of sodium acetate (3 M, pH 5.2). This was followed by the addition of 400 µl of 100% ethanol, and incubation at room temperature for 3 min. The reaction buffer was then centrifuged at 14, 000 g for 10 min and the supernatant was removed. The DNA pellets were washed with 70% ethanol, centrifuged for 5 min at 14, 000 g, followed by removal of the supernatant. The microfuge tubes were inverted and the DNA pellets were dried for 30 min. The DNA pellets were dissolved in 15 µl loading dye ([30% sucrose in 10mM Tris HCl (pH 8)-1mM EDTA] containing 0.1% bromophenol blue) and the total volume of dissolved DNA was loaded into a 2.5% agarose gel. Following electrophoresis (30 V for 40 h) in TAE buffer (40 mM Tris base, 40 mM acetic acid, 1mM EDTA), the gel was stained with SYBR Green I nucleic acid stain (0.01% in TAE buffer, pH 8) and dried at 45°C for 6 h. The DNA was

quantified by densitometry using the STORM fluorescence scanning system (Molecular Dynamics Inc. Sunnyvale, CA). To account for the lower binding constant of SYBR green I nucleic acid stain to the supercoiled plasmid, compared to the relaxed circular conformation, values for the supercoiled bands were multiplied by a factor of 1.6. This correction factor was determined by densitometry through measuring the fluorescent emissions of equal concentrations of linearized and supercoiled pBR322 plasmid DNA, subsequent to agarose gel electrophoresis and staining with SYBR green I stain (Jones & Weinfeld, 1996). The quantity of the relaxed circular conformation of DNA as a percent of the total amount of DNA loaded per well on the agarose gel for each of the BM analogs was determined, and data are expressed as mean values ± SEM of four to seven experiments.

2.7 Statistical analysis for DNA strand break and DNA cross-link assays

The data analysis was conducted using the Statistical Package for Social Sciences (SPSS, Chicago, IL) version 11.0. One-way analyses of variance (ANOVA) were performed to test the contribution of the functional group substitutions to variability in E_{10} values (extent of DNA damage at a 10 μM concentration of BM analog) for DNA cross-link or DNA strand break formation *in vitro*. E_{10} values were used for this analysis, because the extent of DNA cross-link or strand break formation at a concentration of 10 μM was within the linear portion of both the DNA cross-link *versus* BM analog concentration and the DNA strand break *versus* BM analog concentration curves, respectively. The

independent variable in the ANOVA was the BM analog (BM, MeBM, MBM, CBM, FBM, PBM, m-MeBM, m-PBM or m-TBM), and the dependent variable was either E_{10} for DNA cross-link formation, or E_{10} for DNA strand break formation. The normal distribution of the dependent variable (BM analog) and homogeneity of variance were tested using the Kolmogorov-Smirnov and the Levene tests, respectively. When a deviation from normal distribution or lack of homogeneity of variances for the dependent variable was found, the dependent variable was transformed (square root) and analyzed again for normal distribution and homogeneity of variances prior to ANOVA. Subsequent to ANOVA, paired multiple comparisons were performed using the Least Significant Difference (LSD) test to identify individual differences between BM and its analogs with different functional group substitutions. To assess the mechanism of variability in cytotoxicity among the BM analogs, Spearman rank-order nonparametric correlation analyses were performed between the half-times of reduction of the BM analogs by DTD (Chapter 2), or D_{10} values for cytotoxicity and the E_{10} values for DNA cross-link formation or DNA strand break formation. Due to the rank order correlations, this non-parametric analysis would not be affected by differences in the specific activities of DTD used in the DTD reduction experiments (Chapter 2) and the DNA cross-link or strand break experiments. A p-value of <0.05 was accepted as statistically significant in all analyses.

Section 3: Results

3.1 Description of the BM analogs

The syntheses and structures of the benzoquinone mustards, BM, MeBM, MBM, m-MeBM, CBM, FBM, PBM, m-PBM and m-TBM were reported in Chapter 2 (Figure 30). In brief, MeBM, MBM, CBM, FBM and PBM all have functional group substitutions at the C5 position of the benzoquinone. Specifically, MeBM has a weak electron-donating methyl group, MBM has a strong electron-donating methoxy group, CBM and FBM have chloro and fluoro electron-withdrawing groups, respectively, and PBM has a sterically bulky phenyl group substituted on the benzoquinone. In contrast, m-MeBM, m-PBM and m-TBM have functional group substitutions at the C6 position of the benzoquinone. m-MeBM has a weak electron-donating methyl group, m-PBM has a sterically bulky phenyl group, and m-TBM has a very large sterically bulky tertiary-butyl group attached to the benzoquinone moiety. The analogs were studied to identify the effect of functional groups on the mechanisms of cytotoxicity of the analogs subsequent to activation by DTD and the cytotoxic activity of the analogs.

3.2 DNA cross-link formation by the BM analogs

All BM analogs except m-PBM and m-TBM produced a concentration dependent increase in DNA interstrand cross-link formation in linearized pBR322 plasmid DNA, as measured by an agarose gel assay (Figure 36), following reduction by DTD under anaerobic conditions (Figure 37). Functional group substitutions produced differences in the maximum levels of DNA cross-links that

ranged from 0% to 89.3% (Table 6). E_{10} values displayed a rank order of MeBM ≈ MBM > m-MeBM ≈ PBM ≈ BM > CBM > FBM > m-PBM ≈ m-TBM (Table 7). DTD mediated reduction of MeBM and MBM led to the formation of DNA damaging species with an increased ability to produce DNA cross-links compared to BM, with E_{10} values for MeBM and MBM of 53.6 ± 2.8% and 46.0 ± 2.5%, respectively, compared to an E_{10} value of 23.5 ± 1.8% for BM ($p < 0.05$, Table 7). Bioactivation of m-MeBM and PBM by DTD resulted in DNA cross-link formation that was similar to BM, while CBM and FBM produced fewer DNA cross-links compared to BM ($p < 0.05$, Figure 37). In contrast, incubation of m-PBM and m-TBM with DTD produced no DNA cross-links. The mean amounts of background cross-linked DNA in control reaction mixtures with no DTD and in the presence of BM analog, at the highest concentration used in the concentration-effect studies, were not different from the small amounts of cross-linked DNA present in the control reaction mixtures with no BM analog ($p > 0.05$). Therefore it was concluded, for the purposes of the data analysis, that no significant DNA cross-links were produced by the BM analogs in the absence of DTD.

Figure 36. Autoradiogram of a 1% neutral agarose gel showing concentration dependent DNA interstrand cross-link formation in linearized pBR322 DNA by m-MeBM. DNA was incubated for 15 min in 25 mM Tris-HCl, pH 7.5, with increasing concentrations of m-MeBM and DTD (final activity of 9.243 nmol/min per mg protein) under anaerobic conditions. DNA markers were: linearized pBR322 plasmid not subjected to strand separation at 70 ^{0}C (Control DS) and; linearized pBR322 plasmid subjected to strand separation at 70 ^{0}C (Control SS). Control reaction mixtures were: incubated in the absence of DTD in the presence of m-MeBM (75 μM), or incubated in the presence of DTD without m-MeBM. (DS, double stranded DNA; SS, single stranded DNA)

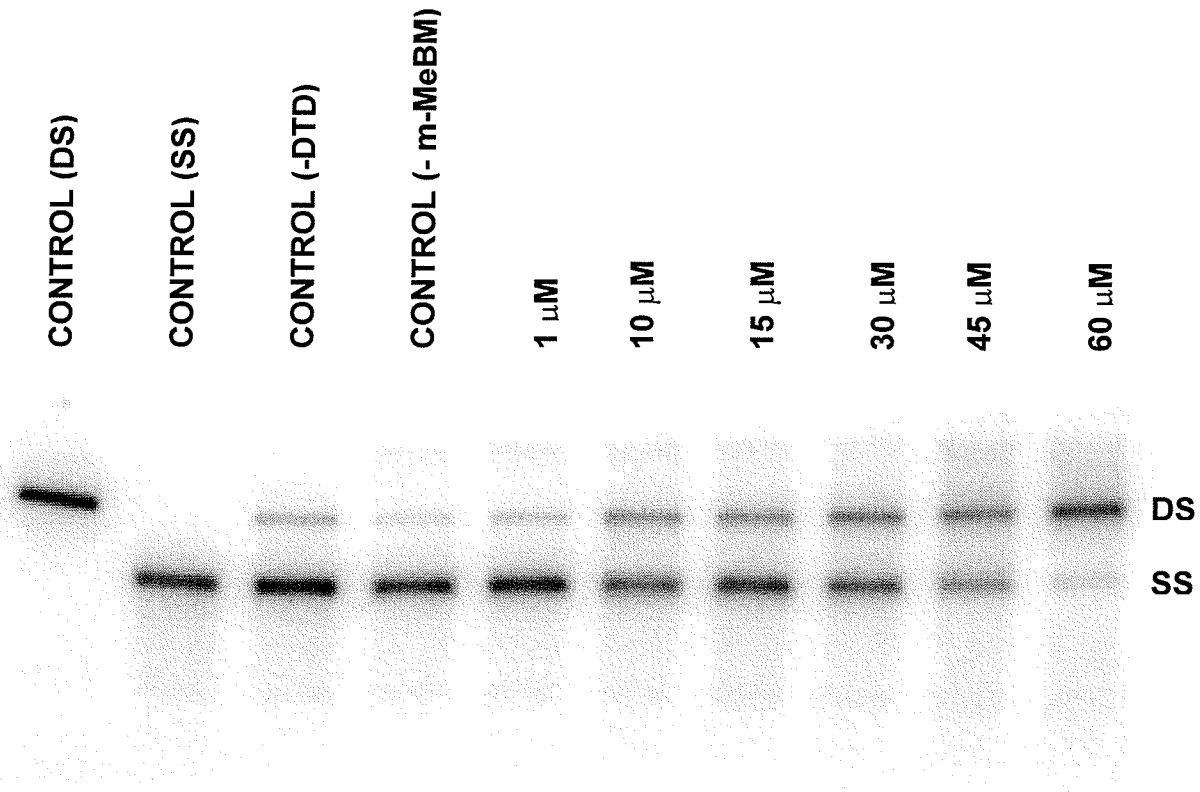


Figure 37. Concentration dependence of DTD mediated interstrand DNA cross-link formation by the BM analogs in linearized pBR322 DNA. Linearized pBR322 plasmid was incubated for 15 min with increasing concentrations of each of the BM analogs, and purified recombinant human DTD (9.243 nmol/min per mg protein) in 25 mM Tris-HCl buffer under anaerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was denatured at 70 ^{0}C and run on a 1% neutral agarose gel and the percentage of cross-linked DNA was determined by densitometric analysis. The quantity of the cross-linked double stranded DNA as a percent of the total amount of DNA loaded per well on the agarose gel was determined for each BM analog, and data are expressed as mean values ± SEM of four to seven experiments.

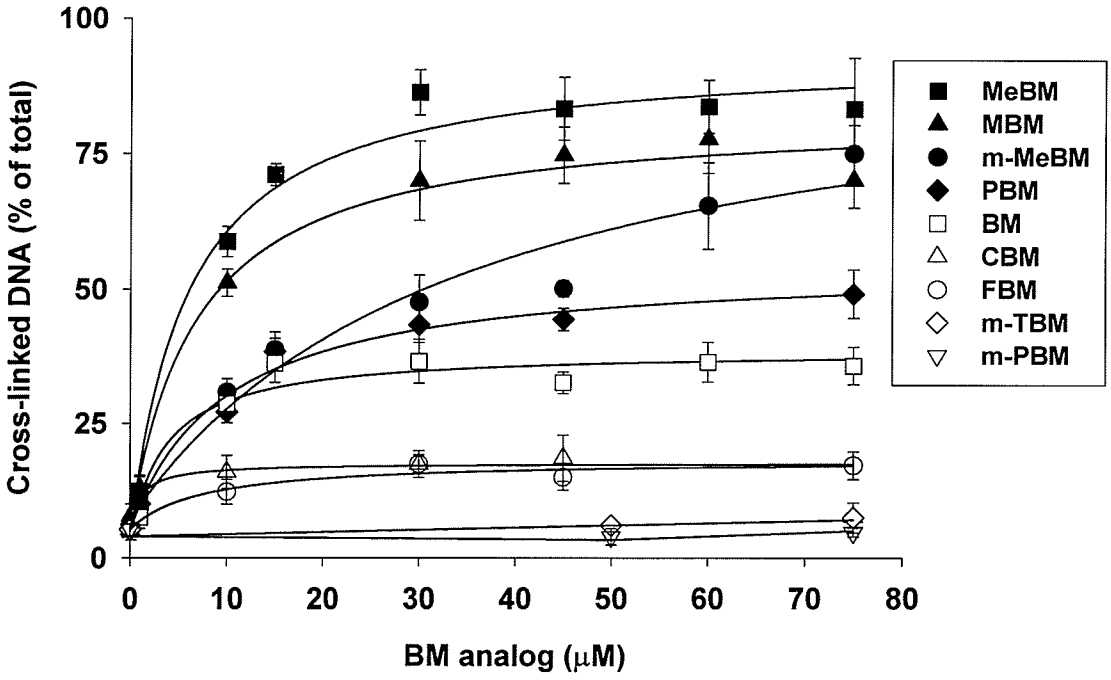


Table 6 Maximum levels of DNA interstrand cross-link formation in linearized pBR322 DNA following reduction of the BM analogs by DTD. Linearized pBR322 plasmid was incubated for 15 min with increasing concentrations of each of the analogs, and purified recombinant human DTD (9.243 nmol/min per mg protein) in 25 mM Tris-HCl buffer under anaerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was denatured at 70 ^{0}C and run on a 1% neutral agarose gel and the percentage of cross-linked DNA was determined by densitometric analysis. Data were fitted to a hyperbola equation through non-linear regression analysis to determine the maximum amount of DNA cross-links formed by each BM analog. The maximum amount of DNA cross-links formed by each analog was corrected for baseline values of DNA cross-link formation.

BM analog	**Maximum levels of cross-linked DNA (% of total)**
BM	32.1
MeBM	88.9
MBM	77.2
m-MeBM	89.3
CBM	11.5
FBM	10.9
PBM	49.6
m-PBM	0
m-TBM	2.2

Table 7 DTD mediated inter-strand DNA cross-link formation by the BM analogs in linearized pBR322 DNA. Linearized pBR322 plasmid was incubated for 15 min with increasing concentrations of each of the analogs, and purified recombinant human DTD (9.243 nmol/min per mg protein) in 25 mM Tris-HCl buffer under anaerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was denatured at 70 ^{0}C and run on a 1% neutral agarose gel and the percentage of cross-linked DNA was determined by densitometric analysis. Data are presented as the level of cross-linked DNA formed by each of the BM analogs at a 10 μM concentration (E_{10} value) in the presence of DTD. The E_{10} value is equal to the percent of the total amount of DNA loaded per well on the agarose gel that is in the cross-linked conformation and is corrected for baseline values of DNA cross-link formation. The E_{10} values of the BM analogs were compared to BM by one-way ANOVA, followed by post-hoc LSD tests. Data are expressed as mean values ± SEM of four to seven experiments. (NS non-significant)

BM analog	BM analog concentration (μM)	DNA cross-link formation (% of total)	P value
BM	10	23.5 ± 1.8	
MeBM	10	53.6 ± 2.8	< 0.05
MBM	10	46.0 ± 2.5	< 0.05
m-MeBM	10	25.6 ± 2.6	NS
CBM	10	14.1 ± 4.0	< 0.05
FBM	10	7.0 ± 2.3	< 0.05
PBM	10	21.9 ± 2.0	NS
m-PBM	10	1.5 ± 0.7	< 0.05
m-TBM	10	1.4 ± 0.7	< 0.05

3.3 DNA strand break formation by the BM analogs

All BM analogs, except m-TBM, produced concentration dependent increases in DNA single strand break formation in supercoiled PBR322 plasmid DNA, as measured by an agarose gel assay (Figure 38), following reduction by DTD under aerobic conditions (Figure 39). E_{10} values among the BM analogs displayed a rank order of MeBM > MBM > m-MeBM > PBM > BM ≈ CBM > FBM > m-PBM ≈ m-TBM. MeBM, MBM, m-MeBM and PBM, with E_{10} values of 72.6 ± 4.3%, 62.8 ± 1.3%, 48.7 ± 4.1% and 39.6 ± 8.7% (mean ± SEM), respectively, produced DNA single strand breaks more readily than the parent compound BM (24.4 ± 2.7%) ($p < 0.05$, Table 8). The E_{10} values for DNA strand break formation were similar for BM and CBM (Table 8); FBM and m-PBM produced fewer DNA strand breaks compared to BM ($p < 0.05$, Table 8), while m-TBM did not produce any DNA strand breaks. The mean amounts of background DNA strand breaks present in control reaction mixtures with no DTD and in the presence of BM analog, at the highest concentration used in the concentration-effect studies, were not different from the small amounts of DNA strand breaks present in the control reaction mixtures with no BM analog ($p > 0.05$). Therefore it was concluded, for the purposes of the data analysis, that no significant DNA strand breaks were produced by the BM analogs in the absence of DTD. At high concentrations of the BM analogs, smearing of the DNA occurred which produced decreases in the sum of total amounts of DNA quantified in the relaxed circular and supercoiled conformations. For example, in the case of m-PBM, this smearing accounted for a decrease in the sum of supercoilled and relaxed

circular DNA of approximately 30% compared to baseline reaction mixtures that contained no m-PBM. Despite of the smearing that occurred, potentially due to DNA fragmentation as a result of high amounts of DNA strand break formation, one could still observe a concentration dependent increase in the relaxed circular conformation of DNA with all analogs studied.

Figure 38. Autoradiogram of a 2.5% neutral agarose gel showing concentration dependent DNA single strand break formation in supercoiled pBR322 DNA by MeBM. DNA was incubated for 15 min in 10 mM TAE buffer, pH 7.5, with increasing concentrations of MeBM and DTD (final activity of 23.2 nmol/min per mg protein) under aerobic conditions. Control reaction mixtures were: incubated in the absence of DTD in the presence of MeBM (10 μM), or incubated in the presence of DTD without MeBM. Molecular weight markers are λDNA digested with *Hind* III. (RL, relaxed circular DNA; SC, supercoiled DNA).

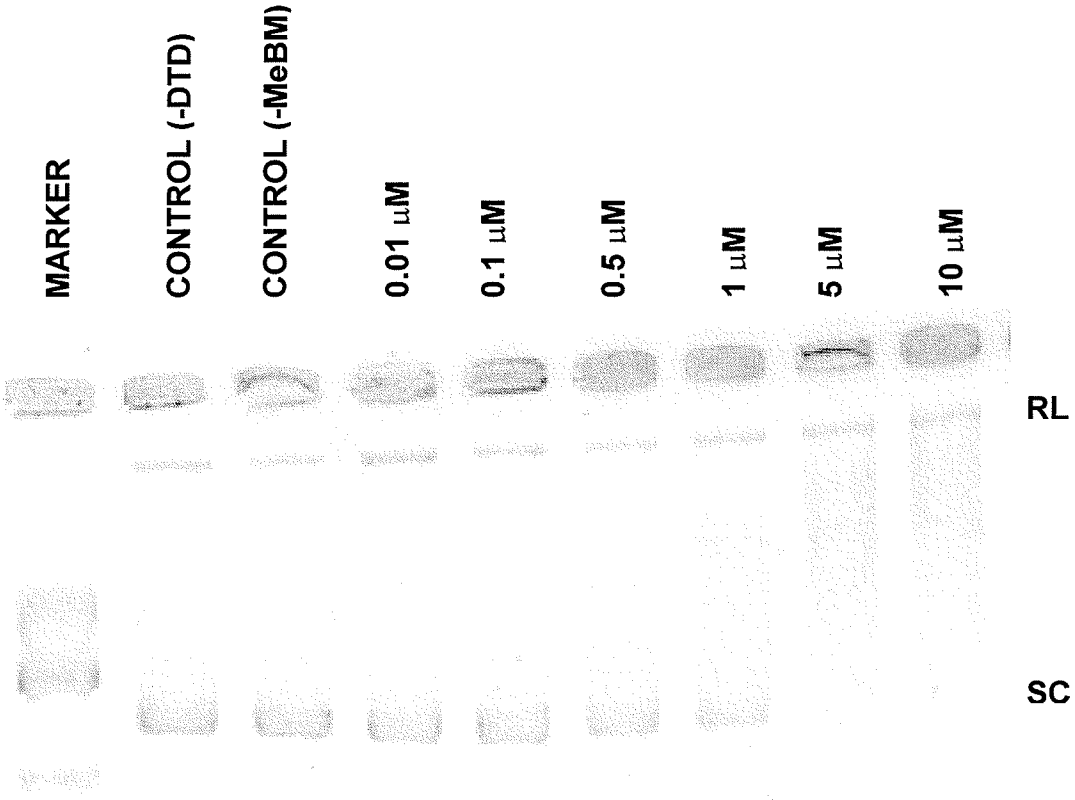


Figure 39. Concentration dependence of DTD mediated single strand break formation by the BM analogs in supercoiled pBR322 DNA. PBR322 plasmid was incubated for 15 min with increasing concentrations of each of the BM analogs, and purified recombinant human DTD (23.2 nmol/min per mg protein) in 10 mM TEA buffer (pH 7.5) under aerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was run on a 2.5% neutral agarose gel and the percentage of relaxed circular DNA was determined by densitometric analysis. The quantity of the relaxed circular conformation of DNA as a percent of the total amount of DNA loaded per well on the agarose gel for each of the BM analogs was determined, and data are expressed as mean values ± SEM of four to seven experiments.

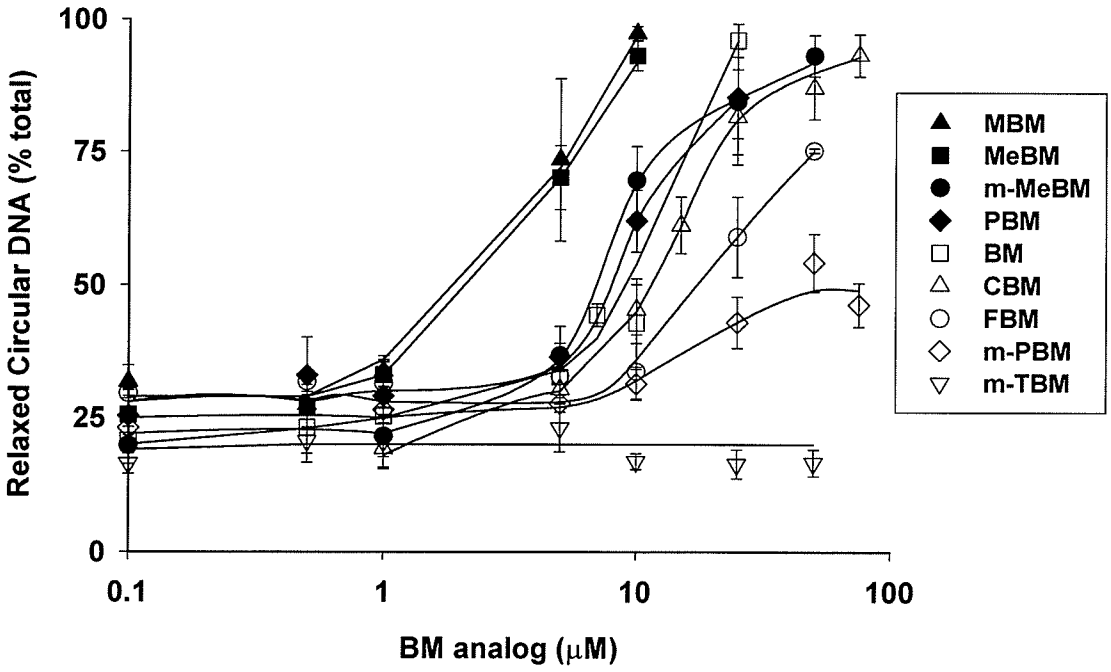


Table 8 DTD mediated single strand break formation by the BM analogs in supercoiled pBR322 DNA. PBR322 plasmid was incubated for 15 min with increasing concentrations of each of the analogs, and purified recombinant human DTD (23.2 nmol/min per mg protein) in 10 mM TEA buffer (pH 7.5) under aerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was run on a 2.5% neutral agarose gel and the percentage of relaxed circular DNA was determined by densitometric analysis. Data are presented as the amount of relaxed circular DNA formed by each of the BM analogs at a 10 μM concentration (E_{10} value). The E_{10} value is equal to the percent of the total amount of DNA loaded per well on the agarose gel that is in the relaxed circular conformation and is corrected for baseline values of DNA strand break formation. The E_{10} values of the BM analogs were compared to BM by one-way ANOVA, followed by post-hoc LSD tests. Data are expressed as mean values ± SEM of four to seven experiments. (NS non-significant)

BM analog	BM analog concentration (μM)	DNA strand break formation (% of total)	P value
BM	10	24.4 ± 2.7	
MeBM	10	72.6 ± 4.3	< 0.05
MBM	10	62.8 ± 1.3	< 0.05
m-MeBM	10	48.7 ± 4.1	< 0.05
CBM	10	29.0 ± 4.0	NS
FBM	10	7.5 ± 5	< 0.05
PBM	10	39.6 ± 8.7	< 0.05
m-PBM	10	5.7 ± 2.0	< 0.05
m-TBM	10	2.2 ± 2.2	< 0.05

3.4 Cytotoxic activity of the BM analogs and the relationship to DNA cross-link and strand break formation

The E_{10} values of the BM analogs for DNA cross-link and DNA strand break formation correlated significantly with the D_{10} values for cytotoxic activity in both the NCI-H661 and the SK-MEL-28 cell lines, as determined in Chapter 2 (Table 4 & Table 5 in Chapter 2). For the SK-MEL-28 cell line, the correlation coefficients (r_s) were -0.87 ($p < 0.05$, Fig. 40) and -0.95 ($p < 0.05$, Fig. 41) for DNA cross-link and strand break formation, respectively, while for the NCI-H661 cell line, the r_s values were: –0.87 ($p < 0.05$, Fig. 42) for cross-link formation and -0.92 ($p < 0.05$, Fig. 43) for strand break formation. In addition, a significant correlation was observed between the maximum levels of DNA cross-links formed and the D_{10} values for cytotoxicity, with r_s values of -0.85 ($p < 0.05$) for the NCI-H661 cell line, and -0.81 ($p < 0.05$) for the SK-MEL-28 cell line. No significant correlations were found between the half-time of reduction of the BM analogs by DTD and DNA cross-link formation, DNA strand break formation or cytotoxic potency of the analogs ($p > 0.05$).

Figure 40. Association between cytotoxic potency for each BM analog in the SK-MEL-28 cell line and DNA interstrand cross-link formation.

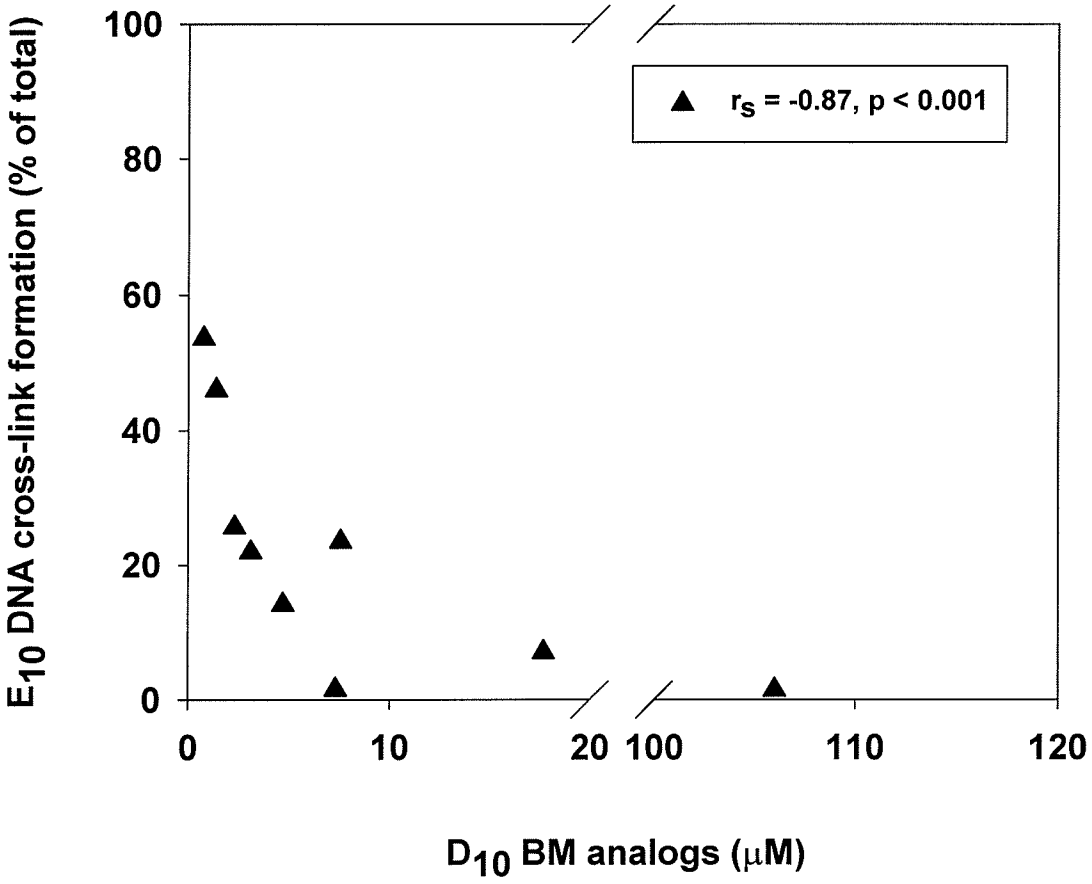


Figure 41. Association between cytotoxic potency for each BM analog in the SK-MEL-28 cell line and DNA single strand break formation.

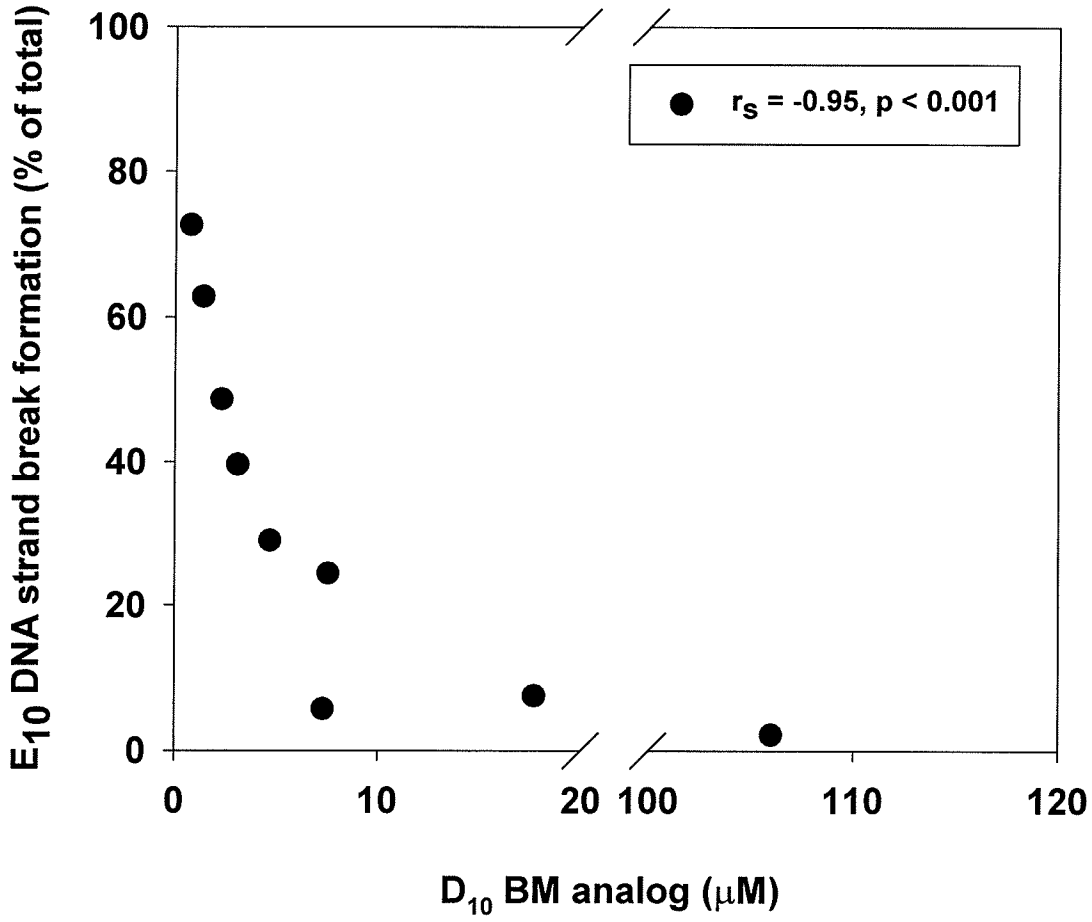


Figure 42. Association between cytotoxic potency for each BM analog in the NCI-H661 cell line and DNA interstrand cross-link formation.

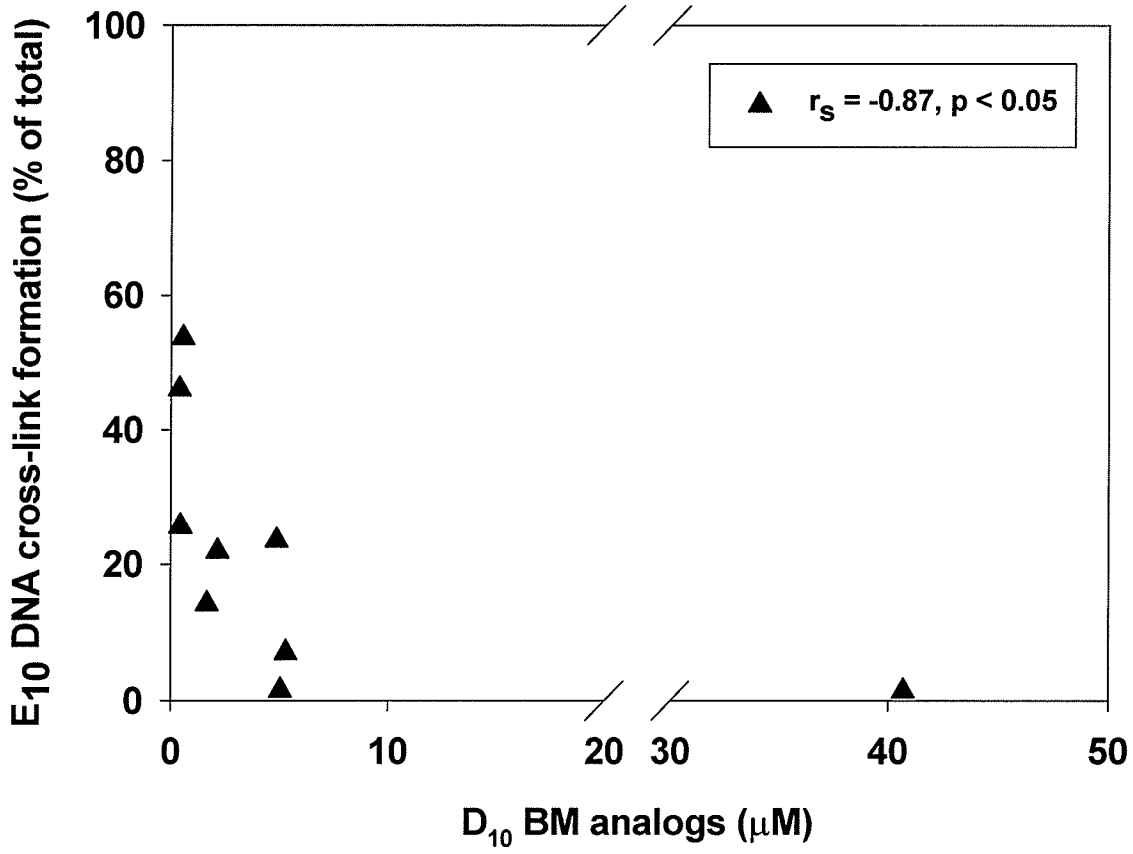
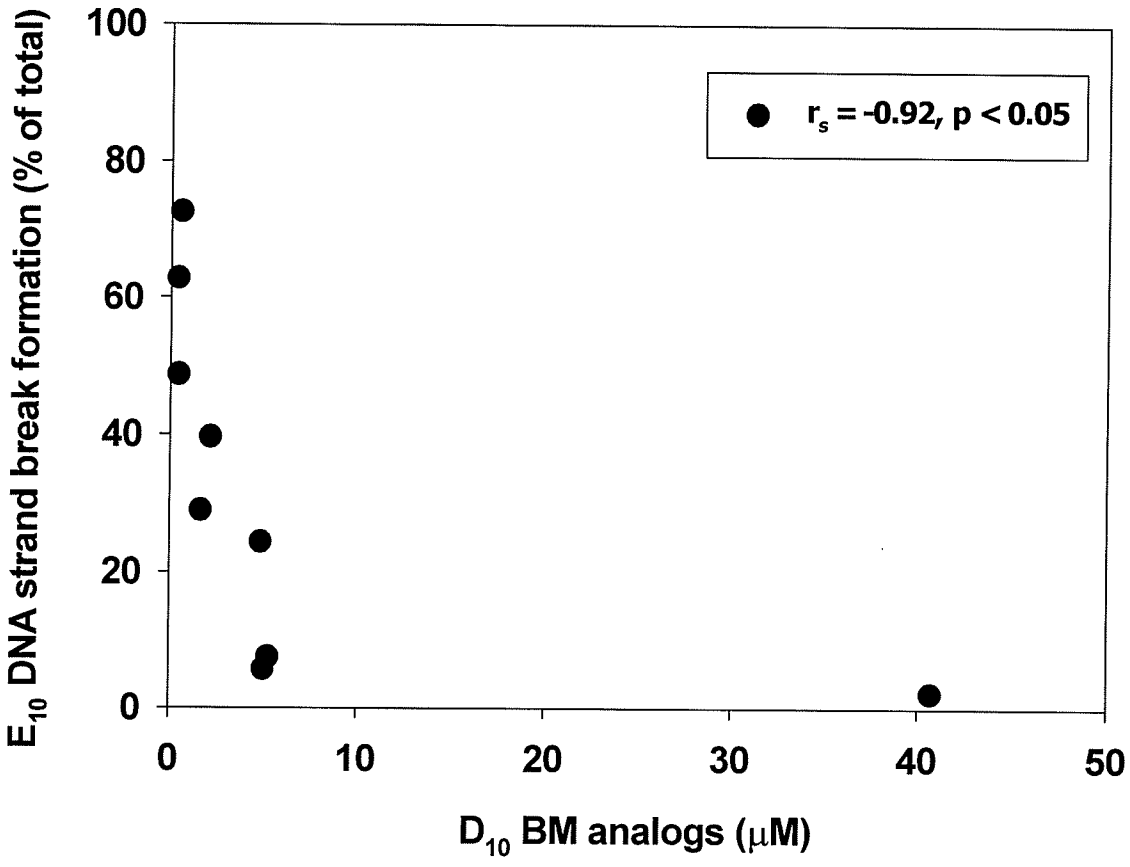


Figure 43. Association between cytotoxic potency for each BM analog in the NCI-H661 cell line and DNA single strand break formation.



Section 4: Discussion

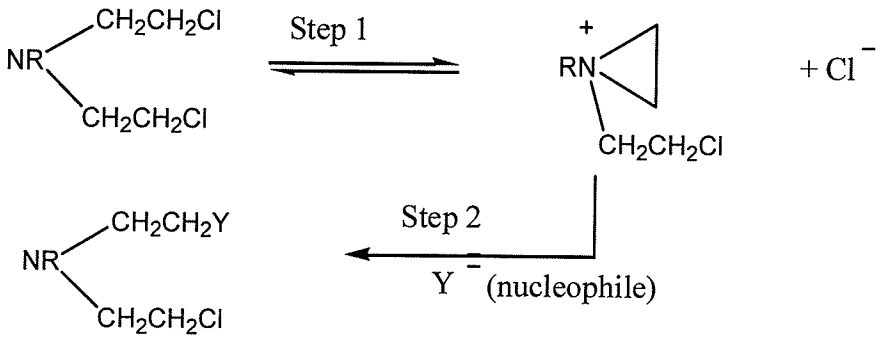
The characterization of quantitative structure-activity relationships across a range of compounds and biological endpoints requires the use of a concentration-response study design, which allows comparisons between compounds within the linear range of the concentration-effect profiles. Otherwise, the study conclusions may be subject to potential confounding due to, for instance, saturation of the target biological system at excessively high drug concentrations (ceiling effect) or alternatively, due to a floor effect secondary to a concentration threshold which may need to be surpassed in order to produce the desired biological effect. With this in mind, DNA cross-link and DNA strand break formation produced by a range of concentrations of BM analogs, having a wide variety of functional groups, were measured following reduction by DTD. As well, the effects of the functional groups on DTD mediated DNA cross-link and strand break formation at a 10 μM concentration of the BM analogs, which was within the linear range of the concentration-effect profiles were compared. The data indicated that DTD mediated DNA cross-link and strand break formation were significantly influenced by the characteristics and position of functional group substitutions on the benzoquinone moiety of BM.

Although the effects of functional group substitutions on quinone-based bioreductive alkylating agents have been previously reported in relation to effects on DNA sequence specific alkylation (Lee et al., 1992, Mayalarp et al., 1996, Hargreaves et al., 1997), there are limited quantitative data on the effects of various functional groups on DNA cross-link formation. For instance, the level of

DNA cross-links produced following reduction of quinone methide aziridinylquinones decreased, as the length of the methylene chain at the C5 position increased. However, it was not clear whether these relationships were determined at concentrations within the linear portion of the respective concentration-effect profiles (Mayalarp et al., 1996).

The mechanism by which nitrogen mustards may form either DNA intra- or inter-strand cross-links involves aziridinium ion formation by each nitrogen mustard group which can subsequently react with the N7 position on guanine residues within opposite or the same strands of DNA (Ojwang et al., 1989), (Figure 44). In the present study, DTD mediated reduction was required in order to observe the dose dependent increase in cross-linked DNA products with the BM analogs. Specifically, the mean amounts of background DNA cross-links present in control reaction mixtures with no DTD and in the presence of BM analog at the highest concentration used in the concentration-effect studies, were not different from the small amounts of background DNA cross-links present in the control reaction mixtures with no BM analog ($p > 0.05$). Therefore it was concluded, for the purposes of the data analysis, that no significant DNA cross-links were produced by the BM analogs in the absence of DTD.

Figure 44



The results indicated that the electron-donating methoxy and methyl groups, when substituted at the C5 position of the benzoquinone moiety, increased DTD mediated cross-link formation compared to BM at the 10 μM concentration. The electron-donating effects of these functional groups may enhance activation of the nitrogen mustard groups, by facilitating leaving of the chloride groups, thus allowing for aziridinium ion formation with greater ease. In fact, the electron-donating effects appear also to facilitate the intrinsic efficacy for cross-link formation, as the maximum levels of DNA cross-link formation by MeBM, MBM and m-MeBM, were increased by more than two-fold compared to BM (Table 6). The E_{10} value for cross-link formation for m-MeBM was similar to that of BM, and therefore, the activating effect observed appears more effective when the electron-donating groups are substituted in the para-position versus the meta-position to the cytotoxic element. In contrast, there was no difference observed between maximum levels of DNA cross-link formation by m-MeBM, MBM and MeBM, and therefore this parameter does not appear to be influenced by the position of the functional group substitution.

Electron-withdrawing groups, substituted at the C5 position of the benzoquinone moiety, decreased DTD mediated cross-link formation compared to BM at the 10 μM concentration. Furthermore, there was a decrease in maximum levels of DNA cross-link formation by CBM and FBM, with values approximately one third of that observed with BM. It is likely that these results are due to the inactivating effects of electron-withdrawing groups on the nitrogen mustard; an effect which would hinder leaving of the chloro groups, and thus, the formation of the aziridinium ion and cross-link formation.

The maximum levels of cross-linked DNA formed by analogs with sterically bulky groups at the C6 position of the benzoquinone were similar to the baseline values. Therefore, limited or no cross-linked DNA is formed following incubation of these analogs by DTD. This observation was not surprising for m-TBM, as this analog was a very poor substrate for DTD, and therefore should not have been activated through this pathway. m-PBM was a better substrate for DTD than m-TBM, but was reduced more slowly than BM (Chapter 2, Fourie et al., 2002). Although its rate of reduction was slow compared to BM, some DNA cross-links would have been expected after the 15 min incubation of m-PBM with the DNA, as this compound did produce DNA strand breaks, which indicated redox cycling of the reduced product. Therefore, the substitution of the phenyl group at the C6 position on the benzoquinone may produce steric interference, preventing cross-link formation. Substitution of the phenyl group at the C5 position allowed for DNA cross-link formation, with a concentration-effect profile similar to that of BM, and therefore, the steric hindrance which prevented DNA

cross-link formation with m-PBM appeared to be limited to the C6 position of the benzoquinone.

The mechanism of induction of DNA strand breaks by DZQ is thought to be through reoxidation of the hydroquinone formed by reduction via DTD, and subsequent generation of reactive oxygen species (Gibson et al., 1992, O'Brien et al., 1990, Walton et al., 1991). Previously, we reported that the strong electron-donating methoxy group substitution on the benzoquinone moiety leads to a compound with increased potential to redox cycle subsequent to reduction by DTD, compared to BM and its analogs with weak electron-donating, electron-withdrawing or sterically bulky group substitutions (Chapter 2, Fourie et al., 2002). The enhanced DNA strand break formation may result from the electron-donating groups modifying the hydroquinone moiety to enhance its interaction with oxygen, leading to increased reactive intermediates. These intermediates include the semiquinone, which is formed due to one-electron oxidation, and reactive oxygen species. The electron-donating groups may presumably also affect the stability and the potential of the semiquinone to alkylate DNA. For instance, electron-donating groups, especially at the C5 position, could act to push the unpaired electron towards the cytotoxic groups at the C2 position, an effect which would lead to increased DNA alkylation. The observation that electron-donating groups led to compounds with an increased potential to produce DTD mediated cross-links and strand breaks, contributes to the hypothesis that DNA strand break formation may be enhanced by the close proximity of the analog to DNA following cross-link formation. This mechanism

may be important in increasing the DNA strand-break formation by analogs with electron-donating functional groups in particular. For example, MeBM does not redox cycle as fast as MBM, possibly due to its weaker electron-donating group compared to MBM. Therefore, the increased DNA strand break formation observed with MeBM, and m-MeBM (maximum DNA strand break formation) compared to BM, may be explained by the increased ability of these compounds to form DNA cross-links due to the activating effect of the electron-donating groups on the reduced cytotoxic element. (Chapter 2, Fourie et al., 2002).

The electron-withdrawing chloro group of CBM did not affect DNA strand break formation, which was similar to that of BM at the 10 μM concentration. In contrast, there was a decrease in DNA strand break formation observed with FBM, compared to BM at the 10 μM concentration. Qualitatively, there was a decrease in the maximum levels of DNA cross-link formation by CBM and FBM, compared to BM, and a significant decrease in DNA cross-link formation by these compounds at the 10 μM concentration, versus BM. Therefore it is suggested that the similar or decreased ability of CBM and FBM to produce DNA strand breaks, compared to BM, may be due to the inactivating effect of the electron-withdrawing groups on the reduced cytotoxic element, which would decrease DNA cross-link formation. This decrease in DNA cross-link formation may in turn lead to less efficient DNA strand break formation due to redox cycling and the generation of reactive oxygen species at positions further removed from the target DNA. Further studies with stronger electron-donating and -withdrawing groups are required in order to further investigate this finding.

There was an increase in DNA strand break formation with PBM, however, analogs with sterically bulky groups at the C6 position were less efficient in producing DNA strand breaks compared to BM, subsequent to reduction by DTD. The increase in DNA strand break formation observed with PBM was unexpected, and may be due to stabilization of the semiquinone by an electron-donating effect of the phenyl group when substituted in the para- position to the mustard groups. However, this hypothesis needs further study. Substitutions of sterically bulky groups at the C6 position of the benzoquinone led to compounds with decreased DNA strand break formation. This was expected for m-TBM, as reduction of this analog by DTD appeared to be insignificant. In the case of m-PBM, this compound did produce DNA strand breaks, but the level was decreased when compared to its positional isomer PBM. Several factors may be responsible for this observation. The absence of DNA cross-link formation by m-PBM may lead to less efficient DNA strand break formation as a result of redox cycling and reactive oxygen species generation at positions further removed from the DNA. As well, the rate of reduction of m-PBM was slower than PBM (Chapter 2, Fourie et al., 2002), which may lead to a decrease in the amount of reduced hydroquinone available to redox cycle and produce DNA strand breaks.

Previously, it was reported that the cytotoxicity of CBM was equivalent to that of BM in the SK-MEL-28 cell line, but increased in the NCI-H661 cell line (Chapter 2, Fourie et al., 2002), while the cytotoxic activity of FBM was similar or decreased when compared to BM, in the NCI-H661 and SK-MEL-28 cell lines, respectively (see Chapter 2). As well, DNA cross-link formation was decreased

for both CBM and FBM compared to BM. Furthermore, DNA strand break formation was decreased for FBM but not altered for CBM, compared to BM. The contrast in effects of CBM and FBM on DNA strand break formation, as well as the relative importance of DTD mediated reduction to act as an inactivating pathway for FBM, and its lack of importance in the activation of CBM, may explain the differing cytotoxic potencies of compounds. Further studies with analogs that have stronger electron-withdrawing group substitutions on the benzoquinone moiety are required to further explore the effects of electron-withdrawing groups on the cytotoxicity and mechanisms of DNA damage of BM.

Although it is suggested that the cytotoxic effects of quinone-based bioreductive agents are due to DNA cross-link and strand break formation, there is limited experimental data demonstrating a direct association between the cytotoxic activity of benzoquinone-based bioreductive agents and DTD mediated reduction kinetics or, the extent and type of DNA damage. The two-electron reduction of mitomycin C and a series of aziridinylbenzoquinones was shown to be a bioactivation process, and led to the formation of cytotoxic metabolites that produced DNA cross-links and strand breaks. Such DNA damage was inhibited by DIC, and the compounds were less toxic in DTD-deficient BE cell line (Siegel et al., 1990b, Gibson et al., 1992). In the case of a series of indolequinones, it was concluded that DNA strand break formation, following reduction by human DTD, was not a major mechanism by which a series of indolequinones produced cytotoxicity, as cytotoxicity did not correlate with the extent of DNA strand break formation (Phillips et al., 1999). Although these reports provide some evidence

on the mechanism of cytotoxicity associated with quinone analogs, correlations were often established without adequate attention to concentration-effect relationships, which might have led to confounding in those earlier studies.

A number of factors that may impact the cytotoxic effects of specific classes of bioreductive alkylating agents include functional group characteristics, rate of reduction by bioactivating enzymes, oxygen radical generation, importance of detoxification or inactivation pathways, rate and extent of DNA alkylation and other types of DNA damage. It is not clear from current knowledge which factors are important in determining the overall cytotoxicity. In this study, it was found that both DTD mediated cross-link formation and single strand break formation positively correlated with the cytotoxic potency in two different cell lines for a broad range of BM analogs. This provides further quantitative evidence that DNA strand break and cross-link formation by the reduced products of BM analogs are important factors in the mechanism of cytotoxicity of these agents.

Electron-donating functional group substitutions at the C5 and C6 position of the quinone, led to analogs with an increased cytotoxicity compared to BM, and an increased production of DNA cross-links and strand breaks. Substitution of electron-withdrawing groups produced compounds that were less cytotoxic than compounds with electron-donating groups, while these groups either decreased or had no effect on the levels of DNA cross-link and strand break formation compared to BM.

PBM had an increased cytotoxic potency compared to BM, but its concentration-DNA cross-link profile was similar to that of BM. In contrast, PBM

did have an increase in DNA strand break formation when compared to BM, which could explain the increase in cytotoxicity *versus* BM. Sterically bulky groups at the C6 position of the benzoquinone led to compounds with decreased cytotoxic potency compared to BM, and decreased DNA cross-link and strand break formation.

Despite the correlation between cytotoxic effects and both DNA cross-link and DNA strand break formation, there was no correlation between DTD mediated reduction and cytotoxicity or between reduction and DNA cross-link or strand break formation. This suggests that further downstream events, following activation of analogs to cytotoxic metabolites are most important in influencing the cytotoxic character of the analogs. The SARs identified in this study may be helpful in the design of new bioreductive agents with the right balance between efficacy in production of DNA damage and affinity to bioreductive activating enzymes such as DTD for improved antitumor activity.

CHAPTER 4

NMDA Receptor Antagonists to Characterize Rat Renal Organic Cation Transporter Function

Section 1: Background and Rationale

1.1 Renal tubule organic cation transporters

The kidney plays an important role in drug elimination and contributes to the clearance of organic cationic drugs through glomerular filtration and tubular secretion (Rennick, 1981; Goralski and Sitar, 1999). Organic cation renal tubule secretion is characterized by transporter-mediated movement from the peritubular capillaries, across the renal tubule cell basolateral membrane, and subsequent transport into the lumen of the kidney tubule. There are multiple organic cation transporters in the basolateral membrane of the renal tubule cell. Tetraethylammonium and amantadine are substrates for different organic cation transporters in the basolateral membrane, and have been utilized as probe substrates to characterize distinct renal organic cation transporters (Takano et al., 1984; Escobar and Sitar, 1995; Goralski and Sitar, 1999).

Cellular uptake of tetraethylammonium across the basolateral membrane of the proximal renal tubule is mediated by members of the organic cation transporter (OCT) family. These include rOCT1, rOCT1a, rOCT2 and rOCT3, all of which are expressed in rat kidney (Takano et al., 1984; Sokol and McKinney, 1990; Grundemann et al., 1994; Okuda et al., 1996; Zhang et al., 1997; Kekuda et al., 1998; Inui et al., 2000). Subsequent to basolateral transport, luminal transport of tetraethylammonium is facilitated by a saturable H^+/organic cation exchanger that uses a proton gradient derived from the luminal membrane Na^+/H^+ exchanger (Takano et al., 1984; Rafizadeh et al., 1987).

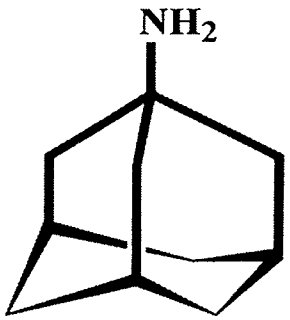
Tetraethylammonium transport studies in rat proximal and distal renal tubule segments identified two basolateral tetraethylammonium bicarbonate-independent organic cation transport sites, a high-affinity/low-capacity and a lower-affinity/higher-capacity tetraethylammonium transporter (Goralski and Sitar, 1999).

The organic cation drug amantadine, clinically used in the treatment of influenza A, undergoes electrogenic transport by hOCT2 using Xenopus laevis oocytes expressing hOCT2 (Busch et al., 1998). In addition, amantadine has been used as a probe substrate to characterize renal tubule organic cation transport systems that are distinct from the OCT family of renal tubule transporters characterized by tetraethylammonium. These studies have shown that amantadine accumulates in proximal and distal renal tubules in rat and in human renal cortical tissue slices in an energy-dependent manner (Wong et al., 1990; 1992b). Furthermore, this process is subject to competitive inhibition by other organic cation substrates (Wong et al., 1990; 1991; 1992a; 1992b). In humans, quinine and quinidine inhibit amantadine renal clearance exclusively in males (Gaudry et al., 1993). However, interaction of these two inhibitors has been observed in rat renal cortical tissue from both sexes (Wong et al., 1992a). The basolateral energy-dependent amantadine transport in rat proximal and distal renal tubule segments is not yet fully characterized at a molecular level, but is known to be comprised primarily of high affinity-capacity, bicarbonate-dependent and a lower affinity-capacity, bicarbonate-independent site(s)

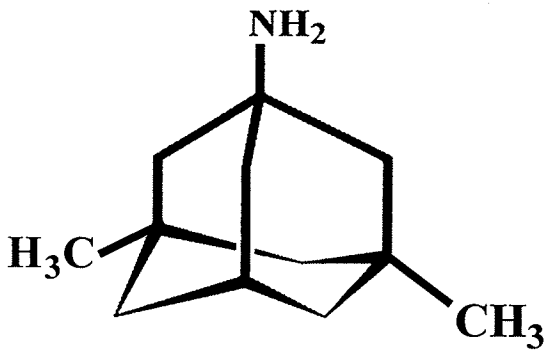
(Escobar et al., 1994; Escobar and Sitar, 1995; Goralski and Sitar, 1999). In contrast to electrogenic basolateral uptake of tetraethylammonium, amantadine transport appears to be mediated mainly by a nonelectrogenic step at the basolateral membrane (Goralski and Sitar, 1999). Hence, amantadine basolateral transport may be subject to regulation by OCTs listed above, as well as other unidentified cation transporters. Similarly, the molecular basis of luminal transport of amantadine has not yet been characterized in full, and may occur through mechanisms similar and/or alternative to that for tetraethylammonium.

Uncompetitive N-methyl-D-aspartate (NMDA)-type glutamate receptor antagonists such as memantine (2,5-dimethyl-1-adamantanamine), and more recently developed aminoalkyl cyclohexane compounds MRZ 2/579 (1-amino-1,3,3,5,5-pentamethyl-cyclohexane HCl), MRZ 2/600 (1-amino-1-ethyl-3,3,5,5-tetramethyl-cyclohexane HCl) and MRZ 2/615 (1-amino-1,3,5,5-pentamethyl-3-ethyl-cyclohexane HCl) are derivatives of the achiral primary aliphatic amine amantadine (1-adamantanamine HCl) (Fig. 45) (Kornhuber et al., 1991; Parsons et al., 1993; 1995; Danysz et al., 1997; Parsons et al., 1999). These compounds are under investigation for their neuroprotective effects in the brain. Memantine is used in the treatment of Parkinson's disease, and is being evaluated for neuroprotective effects in a variety of other diseases (Parsons et al., 1995; Danysz et al., 1997), while MRZ 2/579, MRZ 2/600 and MRZ 2/615 are under investigation as putative antiepileptic agents (Parsons et al., 1999).

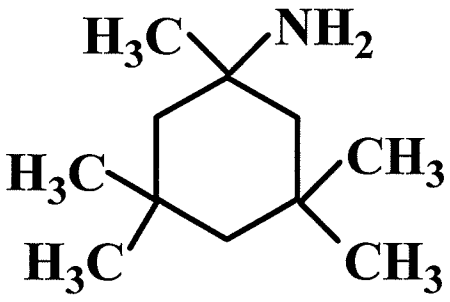
Figure 45. Molecular structures for amantadine, memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615.



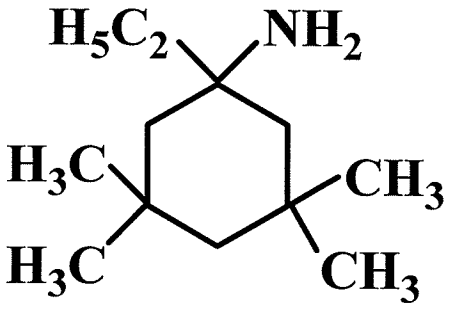
Amantadine



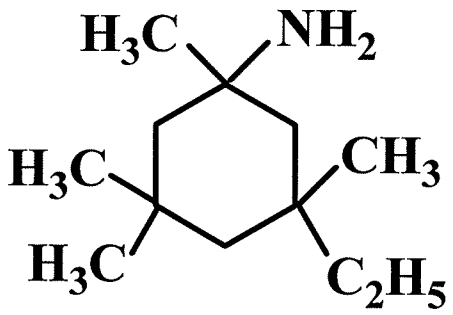
Memantine



MRZ 2/579



MRZ 2/600



MRZ 2/615

It was proposed that the structural similarity of these NMDA receptor antagonists to amantadine would allow them to inhibit organic cation transport through the transporters for which amantadine is a prototype substrate. The objectives of this study were twofold: 1.) to evaluate the potential of memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 to be used as pharmacological tools to further characterize organic cation transporters for amantadine, and 2.) to investigate the safety of these compounds from a drug-drug interaction perspective in the kidney. Hence, the ability of memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 to inhibit energy-dependent amantadine uptake into rat renal tubules using an *in vitro* model was determined.

Section 2: Materials and Methods

2.1 Renal tubule preparation

This study was approved by the University of Manitoba Protocol Management and Review Committee. Proximal and distal renal tubule segments were purified based on the Percoll density-gradient centrifugation method (Vinay et al., 1981, Gesek et al., 1987), as modified and described previously (Wong et al.,1991, 1993). In brief, the modified protocol was as follows: Four male Sprague-Dawley rats (Charles River breeding stock; University of Manitoba, Canada) weighing 200 to 300 g were anaesthetized with a single dose of sodium pentobarbital (50 mg/kg, i.p.), and killed by severing the aorta. Kidneys were removed, decapsulated and placed in ice-cold oxygenated (95% O_2; 5% CO_2)

Krebs-Henseleit solution (KHS), (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 11 mM glucose. Dissection of the renal cortical tissue involved sectioning the medullary tissue approximately 1 mm from the corticomedullary boundary. Subsequently, the cortical tissue was finely minced using a tissue chopper (Mickle Lab. Engineering Co. Ltd., Gomshall, Surrey, UK) and placed in 10 ml of ice-cold KHS. The tissue-KHS mixture was then added to a KHS-collagenase solution (15 ml of KHS, 1 ml of 10% bovine serum albumin and 10 mg low-trypsin collagenase A (0.23 U/mg lysozyme) and oxygenated with 95% O_2/5% CO_2 for 2 min. The oxygenated tissue was incubated at 31°C with shaking (100 oscillations/min) in a Dubnoff incubator (Precision Scientific Co., Chicago, IL, USA) for 35 min. During digestion (35 min in total), the tissue was gently pipetted for 5 min intervals with a large bore (5 ml) pipette tip at 15 min, 25 min and 30 min into the digestion period, which aided in tissue breakup. Tissue was monitored by light microscopy (100 x magnification) of a small aliquot of the digestion mixture, starting at 30 min after the start of the incubation to insure that tissue was not over digested. Digestion was terminated by the addition of 30 ml of ice-cold KHS, and the tissue was filtered through a polyethylene mesh filter (pore size 292 µm) to remove undigested tissue. Subsequently, the tissue was washed three times by sequential resuspension in ice-cold KHS, followed by low-speed centrifugation (4°C, 60 x g for 1 min). The final pellet was resuspended in 40 ml of a 50% Percoll solution (20 ml each of Percoll and double-strength KHS

at pH 7.4) and centrifuged for 30 min at 27,000 x g (4°C). Bands IV and II, consisting of proximal and distal tubules respectively, were then removed from the gradient, washed three times by sequential resuspension in ice-cold oxygenated KHS, followed by low-speed centrifugation (4°C, 60 x g for 1 min).

Following the final wash, proximal and distal tubule fractions were routinely resuspended in the desired volume of oxygenated KHS. However, if the transport assays included measurements in the absence of bicarbonate, the last wash and the final resuspension of the tubule fragments would be done with Cross-Taggart (CT) buffer. CT buffer consisted of 135 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 15 mM sodium phosphate buffer (pH 7.4), 1.0 mM $CaCl_2$ and 11 mM glucose, and the pH was adjusted with NaOH. Preliminary experiments in the laboratory of Dr. Daniel Sitar have shown that differences in calcium concentrations, such as those that exist between the KHS and CT buffers, do not affect renal tubule organic cation transport. The tissue protein concentration was determined using the Biuret method (Gornall et al., 1949), and the resuspension volume was adjusted to give a final concentration of 6-8 mg/ml. The proximal and distal tubule suspensions were kept on ice and warmed to room temperature by a 20 min incubation in a 25°C water bath just prior to the start of the transport assays. The purity of the tubule fractions was assessed by measuring the activity of enzyme markers (Guder and Ross, 1984), as well as by microscopic examination as previously described (Scholer and Edelman, 1979; Vinay et al., 1981; Wong et al., 1991).

2.2. Experimental protocol

Inhibition of [^{3}H]amantadine uptake (10 or 50 µM) by memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 (10-1000 µM) was determined in proximal and distal tubules in the presence of bicarbonate in oxygenated KHS buffer (pH 7.4), and in the absence of bicarbonate in phosphate based CT buffer (pH 7.4). Reactions were performed in KHS or CT buffer, to investigate bicarbonate-dependent or bicarbonate-independent renal tubule organic cation transport, respectively. Reactions were performed in triplicate in borosilicate tubes. Reaction mixtures consisted of a fixed amount of [^{3}H]amantadine (1 nM), unlabeled amantadine (final concentrations of 10 or 50 µM) and increasing concentrations of memantine, MRZ 2/579, MRZ 2/600 or MRZ 2/615 in a total volume of 150 µl of KHS or CT buffer. The proximal or distal tubule suspension (50 µl in the appropriate buffer) was added to each assay tube (final volume of 200 µl) to initiate the transport reaction. Subsequent to addition of the tubule suspension, the reaction mixture was incubated for 30 s in a 25°C shaking water bath (100 oscillations/min). The transport assays were performed at 25°C, as this ensures that transport occurs within the linear portion of the velocity versus log [S] curve. The reactions were terminated by addition of 2 x 4 ml of ice-cold oxygenated KHS, followed by rapid filtration under negative pressure through glass fiber filters (no. 32; Schleicher and Schuell, Inc., Keene, NH, USA). The filters were immediately placed into scintillation vials containing 4 ml of Ready

Safe scintillation fluid (Beckman Instruments Inc., Fullerton, CA, USA) and counted using a Beckman model LS5801 scintillation counter.

Nonspecific uptake of [^{3}H]amantadine to the tissue and filters was determined by measuring uptake of [^{3}H]amantadine in the presence of unlabeled amantadine at a saturating amount (10 mM). The proximal or distal tubule suspension (50 µl in the appropriate buffer) was added to each assay tube (final volume of 200 µl) to initiate the transport reaction. Subsequent to addition of the tubule suspension, the reaction mixture was incubated for 30 s in a 25°C shaking water bath (100 oscillations/min). Specific energy-dependent uptake of amantadine was then calculated by subtracting nonspecific uptake from the total radioactivity.

2.3 Drugs

Unlabeled amantadine was obtained from Dupont Canada (Mississauga, Ontario, Canada). Memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 were supplied by Merz and Co (Frankfurt, Main, Germany).

Amantadine is an achiral primary aliphatic amine. Similar to amantadine, memantine has an amino group substituted at the C1 position, however two hydrogen atoms on the amantadine structure have been replaced by methyl groups at the C2 and C5 positions. The aminoalkyl cyclohexane derivatives, MRZ 2/579, MRZ 2/600 and MRZ 2/615 all have amino groups substituted at the C1 position, similar to amantadine. Unlike amantadine, MRZ 2/579 has five

methyl groups substituted at the C1, C3 and C5 positions of the molecule. Similar to MRZ 2/579, MRZ 2/600 also has methyl groups substituted at the C3 and C5 positions, but unlike amantadine and the other analogs, MRZ 2/600 also has an ethyl group substituted at the C1 position. This ethyl group is sterically larger than the hydrogen or methyl groups substituted at the C1 position in amantadine memantine, MRZ 2/579 and MRZ 2/615. Similar to MRZ 2/579, MRZ 2/615 has methyl group substitutions at the C1, C3 and C5 positions, however unlike amantadine and its other analogs, MRZ 2/615 also has an ethyl group substitution at the C3 position.

2.4 Chemicals

[^{3}H]Amantadine (350 mCi/mmol) was purchased from Amersham International (Buckinghamshire, UK). Collagenase was obtained from Boehringer Mannheim (Laval, PQ, Canada). Percoll was obtained from Pharmacia Biotechnology (Baie D'urfe, PQ, Canada). All other chemicals were of the highest grade available from commercial suppliers.

2.5 Statistical analysis

The data analysis was conducted using the Statistical Package for Social Sciences (SPSS, Chicago, IL) version 10.0. A two-way analysis of variance (ANOVA) with two-way interaction was performed to test the contribution of the tubule and the buffer type to variability in inhibition constants *in vitro* (K_i). The two

independent variables in the ANOVA were tubule (proximal versus distal) and buffer (bicarbonate versus phosphate), and the dependent variable was K_i value at 10 μM or 50 μM amantadine concentrations. When a deviation from normal distribution or lack of homogeneity of variances between each factor was found, the data were log transformed prior to ANOVA. The normal distribution of the dependent variable (K_i) and homogeneity of variance were tested using the Kolmogorov-Smirnov and the Levene tests, respectively. In cases where the Levene test suggested non-homogeneity of variances, the results of the ANOVA were also ascertained with nonparametric tests (Kruskal-Wallis and Mann-Whitney U). A P-value of <0.05 was accepted as statistically significant in all analyses. Data points for each individual inhibition experiment are the mean of three individual determinations, using the same proximal or distal tubule preparations from four rats. Data are expressed as means ± S.E.M. of 4 to 7 individual experiments. Transport rates are reported as specific uptake, which were determined by subtracting nonspecific uptake from total uptake of amantadine by the tubules in nmol/min/mg of protein. IC_{50} values were determined from the amantadine inhibition profiles by increasing inhibitor concentrations using regressive probit analysis (Cheng and Prusoff, 1973). Dixon (1953) and Cornish-Bowden (1974) analyses were used to determine the nature of inhibition. The inhibition studies involved measuring amantadine accumulation in the KHS or CT buffers at different concentrations of amantadine (10 μM or 50 μM), under increasing concentrations of memantine, MRZ 2/579, MRZ 2/600 or

MRZ 2/615. Representative K_i values were determined by using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Section 3: Results

3.1 Amantadine transport

In the absence of the NMDA receptor antagonists, and in the presence of bicarbonate (KHS), the rate of amantadine transport into proximal renal tubules was 0.55 ± 0.04 nmol/min/mg of protein (mean ± S.E.M.) at the therapeutic (10 μM) concentration of amantadine, and 1.89 ± 0.10 nmol/min/mg of protein at the toxic (50 μM) concentration of amantadine. In the absence of bicarbonate (CT buffer), in the proximal tubules, these values were 0.20 ± 0.01 nmol/min/mg of protein and 0.77 ± 0.06 nmol/min/mg of protein at the therapeutic and the toxic concentrations of amantadine, respectively. The rate of amantadine transport into the distal renal tubules, in the absence of the NMDA receptor antagonists, and in the presence of bicarbonate (KHS), was 0.34 ± 0.02 nmol/min/mg of protein at the therapeutic (10 μM) concentration of amantadine, and 0.87 ± 0.09 nmol/min/mg of protein at the toxic (50 μM) concentration of amantadine. Similarly, in the absence of bicarbonate, (CT buffer), these values were 0.13 ± 0.01 nmol/min/mg of protein and 0.40 ± 0.04 nmol/min/mg of protein at the therapeutic and toxic concentrations of amantadine, respectively.

3.2 Amantadine transport inhibition studies

The present *in vitro* study evaluated memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 with respect to their inhibitory potency on energy-dependent uptake of [^{3}H]amantadine into rat proximal and distal renal tubule segments in the presence of bicarbonate-based KHS, or phosphate-based CT buffer. Each compound displayed a concentration-dependent inhibition of amantadine uptake in both the proximal and distal renal tubule segments in the presence and absence of bicarbonate at pH 7.4 (Figs. 46-49). Figures 46 to 49 represent the total data and are presented mainly for a comparative value between the two amantadine concentrations. The important calculations for IC_{50} and K_i values were determined from individual experiments and the data were analyzed according to a single dominant rate limiting transport process present in the presence in bicarbonate-based KHS and phosphate-based CT buffer, as previously determined (Goralski & Sitar, 1999). The observation, that at the lowest inhibitor concentration amantadine transport is not 100%, is a process that appears to equally affect all the inhibitors studied, and therefore should not be a confounding factor (Figs. 46-49). As well, this observation reflects our concentration on the main inhibitory process that is reflected in the terminal slope of the amantadine uptake versus inhibitor concentration profiles. Therefore, only this terminal linear portion of the amantadine uptake versus inhibitor concentration curve for each individual experiment was used in regressive probit analysis to determine the IC_{50} values.

Figure 46. Concentration dependent inhibition of energy-dependent amantadine uptake by memantine in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to five experiments.

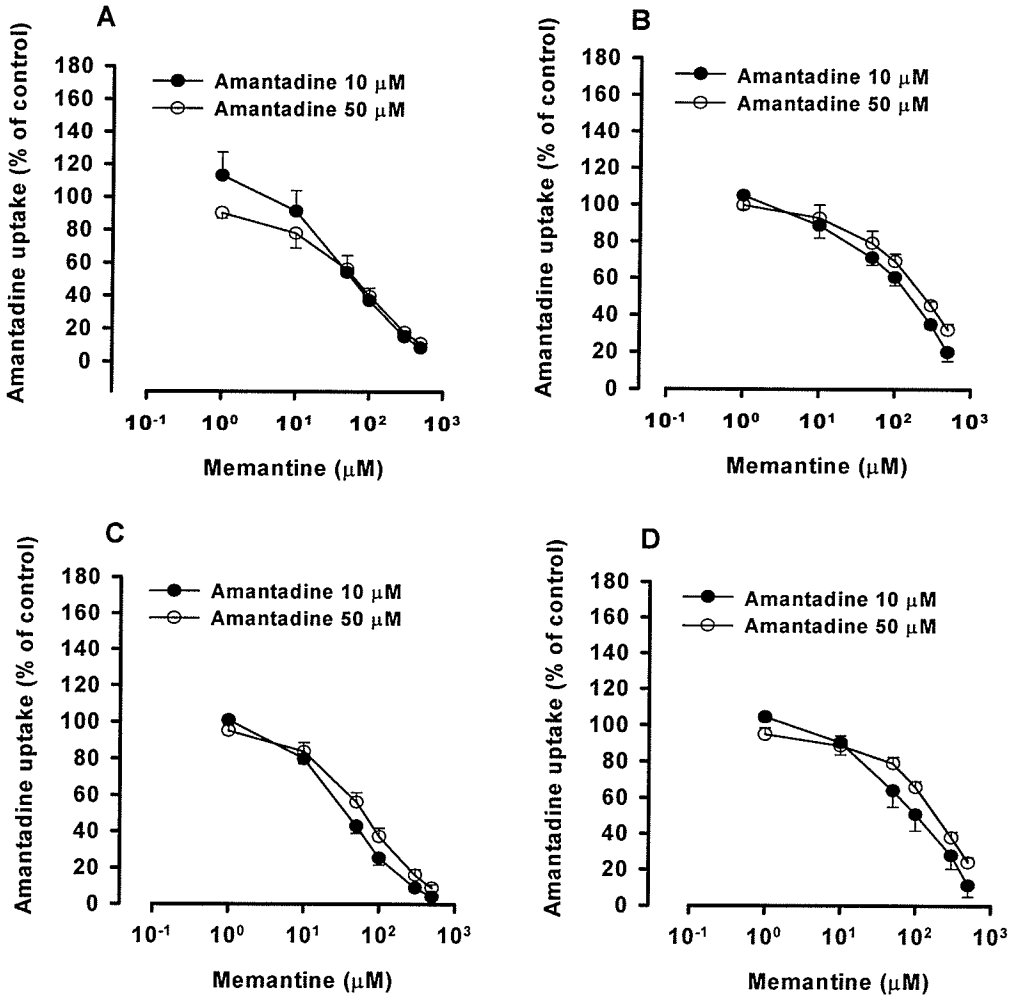


Figure 47. Concentration dependent inhibition of energy-dependent amantadine uptake by MRZ 2/579 in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to seven experiments.

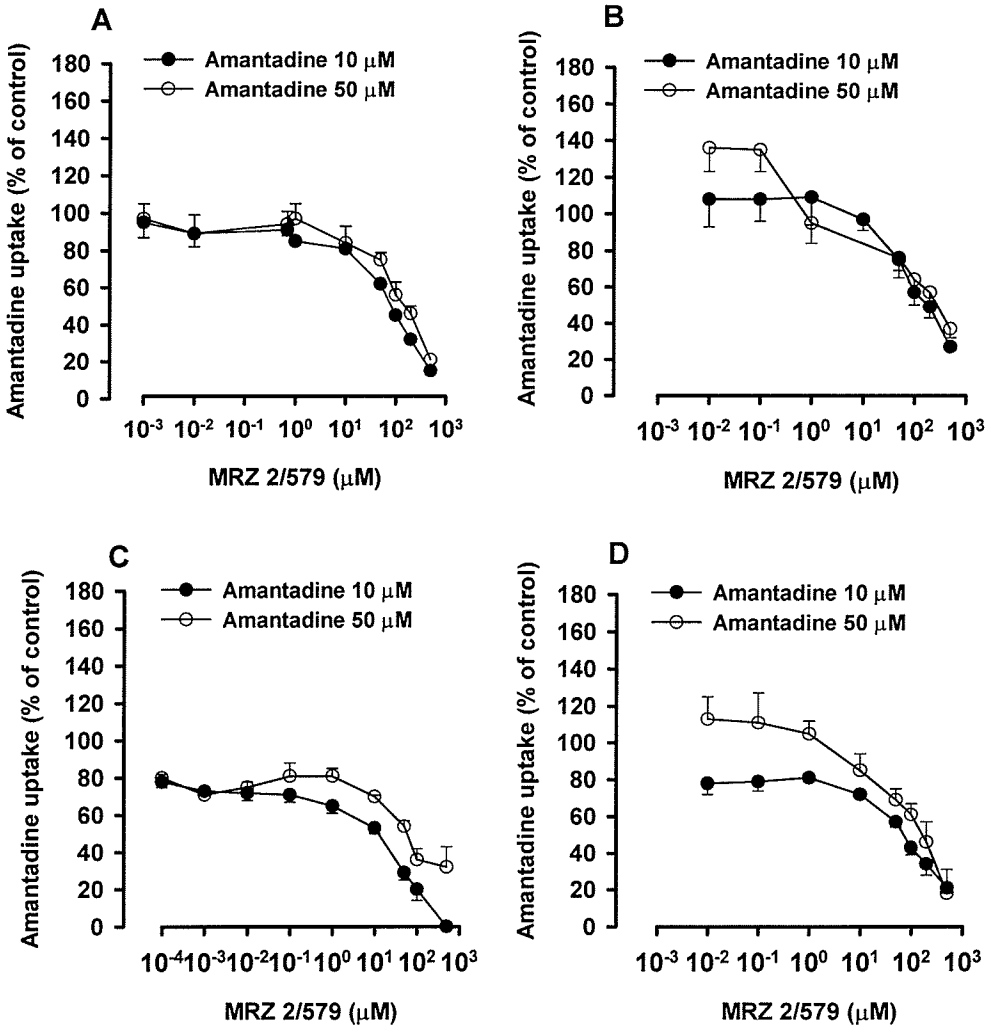


Figure 48. Concentration dependent inhibition of energy-dependent amantadine uptake by MRZ 2/600 in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to seven experiments.

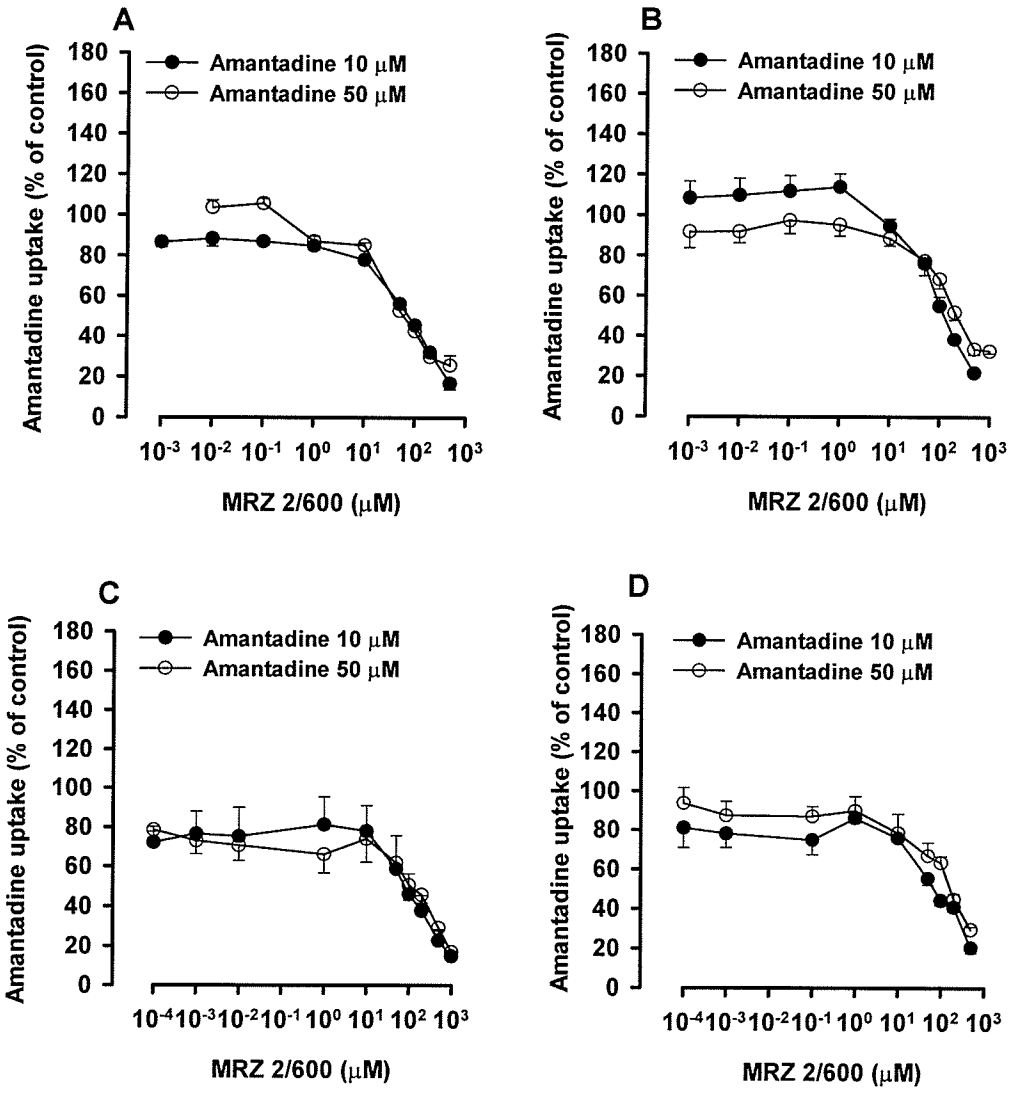
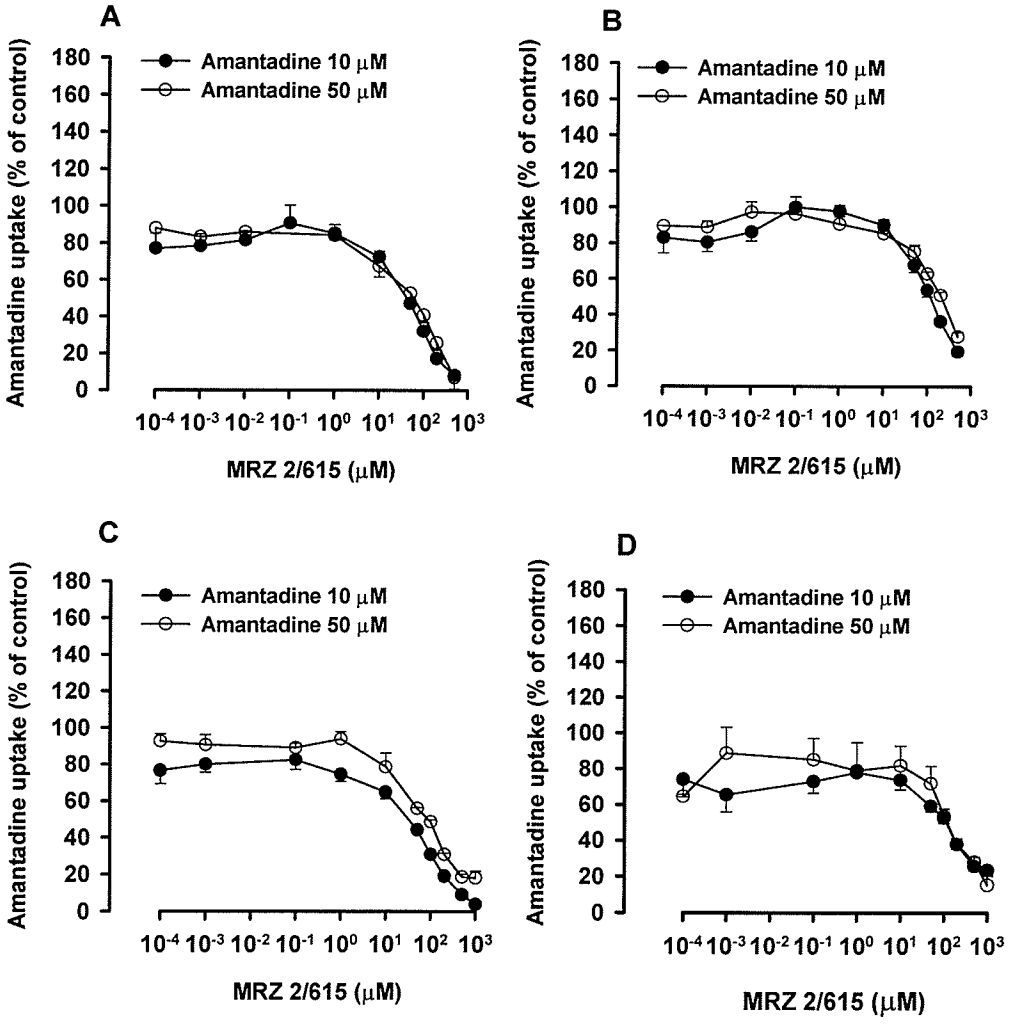


Figure 49. Concentration dependent inhibition of energy-dependent amantadine uptake by MRZ 2/615 in: (A) proximal tubule segments in bicarbonate-based KHS buffer; (B) proximal tubule segments in phosphate-based CT buffer; (C) distal tubule segments in bicarbonate-based KHS buffer; (D) distal tubule segments in phosphate-based CT buffer. Data are mean ± S.E.M. of four to seven experiments.



Dixon and Cornish-Bowden analyses confirmed that all the NMDA receptor antagonists inhibited amantadine uptake in a competitive manner. For example, a representative Dixon plot (1/V vs. *i*) for MRZ 2/579 in the proximal tubules in the absence of bicarbonate is supportive of a competitive mode of inhibition of amantadine uptake (Fig. 50). Further regression analysis of the component slopes yielded an apparent K_i of 184 ± 29 μM for MRZ 2/579 in the proximal tubules in the absence of bicarbonate. These observations justified the determination of K_i values from IC_{50} values by utilizing the Cheng-Prusoff competition method (Cheng and Prusoff, 1973). The IC_{50} and inhibitor dissociation constant (K_i) values for memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 inhibition of amantadine energy-dependent uptake at both therapeutically relevant (10 μM) and clinically toxic (50 μM) concentrations of amantadine are presented in Tables 9 and 10.

Figure 50. Dixon plot of MRZ 2/579 inhibition of energy-dependent amantadine uptake by proximal rat renal tubule segments in the absence of bicarbonate, using amantadine concentrations of 10 and 50 μM.

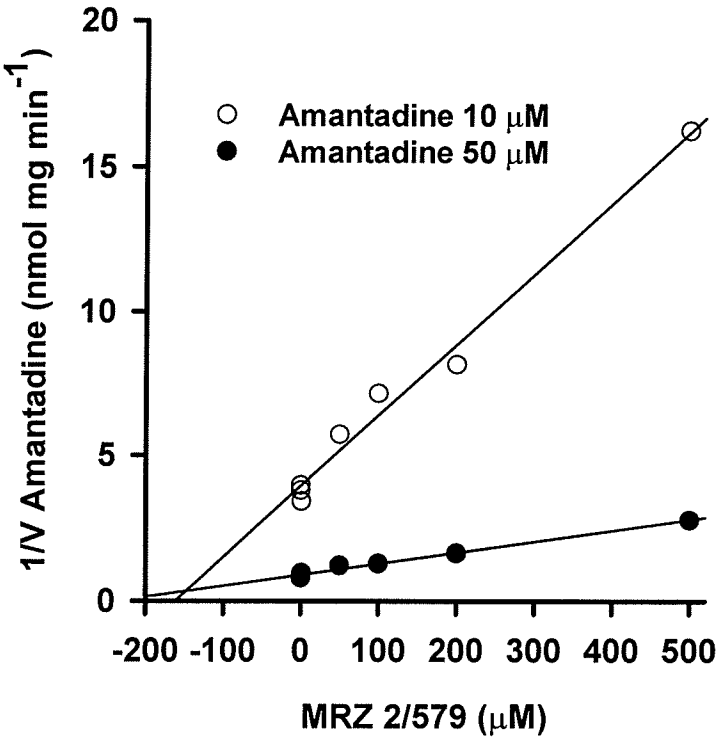


Table 9 IC_{50} values for memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 inhibition of energy-dependent amantadine accumulation in the proximal and distal renal tubule segments in Krebs-Henseleit solution (KHS) and Cross-Taggart (CT) buffer. IC_{50} values were determined at therapeutic (10 μM) and toxic (50 μM) concentrations of amantadine. Values are means ± S.E.M. of four to seven experiments. Statistical analysis was performed on the derived K_i values, which are presented in Table 10.

Tubule	*Buffer*	*Amantadine (μM)*	*Memantine IC_{50} (μM)*	*MRZ 2/579 IC_{50} (μM)*	*MRZ 2/600 IC_{50} (μM)*	*MRZ 2/615 IC_{50} (μM)*
Proximal	KHS	10	61 ± 21	75 ± 5	65 ± 7	37 ± 5
Proximal	CT	10	148 ± 29	175 ± 49	136 ± 15	117 ± 18
Distal	KHS	10	45 ± 8	10 ± 3	101 ± 78	29 ± 4
Distal	CT	10	66 ± 26	76 ± 16	84 ± 13	102 ± 25
Proximal	KHS	50	54 ± 9	112 ± 23	74 ± 8	49 ± 12
Proximal	CT	50	209 ± 44	328 ± 121	209 ± 24	187 ± 18
Distal	KHS	50	53 ± 19	80 ± 38	117 ± 33	75 ± 1
Distal	CT	50	129 ± 27	213 ± 75	167 ± 25	153 ± 34

Table 10 Calculated K_i values for memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 inhibition of energy-dependent amantadine accumulation in the proximal and distal renal tubule segments in Krebs-Henseleit solution (KHS) and Cross-Taggart (CT) buffer. K_i values were determined at therapeutic (10 μM) and toxic (50 μM) concentrations of amantadine. Values are means ± S.E.M. of four to seven experiments.

[1] $P < 0.05$, [2] $P < 0.01$, [3] $P < 0.001$.

[a] KHS versus CT buffer for memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 inhibition of amantadine uptake.

[b] Proximal versus distal tubule for memantine and MRZ 2/579 inhibition of amantadine uptake.

[c] Tubule by buffer interaction for memantine, MRZ 2/579 and MRZ 2/600 inhibition of amantadine uptake.

Tubule	*Buffer*	*Amantadine (μM)*	*Memantine K_i (μM)*	*MRZ 2/579 K_i (μM)*	*MRZ 2/600 K_i (μM)*	*MRZ 2/615 K_i (μM)*
Proximal	KHS	10	$54 \pm 18^{a2,c2}$	$71 \pm 5^{a3,c1}$	61 ± 7	35 ± 5^{a3}
Proximal	CT	10	168 ± 17^{c2}	163 ± 45^{c1}	127 ± 14	110 ± 17
Distal	KHS	10	39 ± 6^{c2}	$9 \pm 3^{af, b3, c1}$	90 ± 69	26 ± 3^{a3}
Distal	CT	10	$45 \pm 16^{b3, c2}$	66 ± 14^{c1}	74 ± 12	89 ± 22
Proximal	KHS	50	33 ± 5^{a3}	85 ± 17^{a1}	$56 \pm 6^{a2,c1}$	37 ± 9^{a3}
Proximal	CT	50	177 ± 38	242 ± 89	154 ± 18^{c1}	138 ± 13
Distal	KHS	50	38 ± 10^{a3}	50 ± 24^{a1}	72 ± 20^{c1}	47 ± 1^{a3}
Distal	CT	50	99 ± 22	124 ± 44	98 ± 15^{c1}	89 ± 20

For memantine, both a tubule (Table 10, $P < 0.001$) and buffer (Table 10, $P < 0.01$) effect were noted, as well as a tubule by buffer interaction (Table 10, $P < 0.01$) at the therapeutic (10 μM) concentration of amantadine (Fig. 46). A comparison of the 95% confidence intervals (CI) showed that the inhibition by memantine in the proximal tubule was more potent in the presence of bicarbonate (KHS) (95% CI: 3-105 μM in KHS versus 114-222 μM in CT). Furthermore, in the presence of phosphate (CT buffer), inhibition was more potent in the distal tubules (95% CI: 0-94 μM distal tubules in CT versus 114-222 μM proximal tubules in CT). In the presence of a toxic amantadine concentration (50 μM), a clear buffer effect was demonstrated ($P < 0.001$) with greater potency in the presence of bicarbonate.

For MRZ 2/579 inhibition of amantadine uptake, both a tubule and a buffer effect were observed at a therapeutic concentration of amantadine (Fig. 47, $P < 0.001$), as well as a tubule by buffer interaction (Fig. 47, $P < 0.05$). Further analysis using the 95% CI of the tubule effect at the therapeutic concentration of amantadine indicated that inhibition was most potent in the distal tubules, and in the presence of bicarbonate (KHS) (95% CI: 2-15 μM, distal tubules in KHS; 29-130 μM distal tubules in CT; 55-99 μM, proximal tubules in KHS; 62-344 μM, proximal tubules in CT). Notably, the K_i value for MRZ 2/579 inhibition of amantadine uptake was 9 ± 3 μM in the distal renal tubule segments in the presence of bicarbonate. (Fig. 47 and Table 10, $P < 0.001$). In the presence of a clinically toxic concentration of amantadine, a buffer effect was indicated for

MRZ 2/579 (Table 10, $P < 0.05$), with more potent inhibition in the presence of bicarbonate.

The initial two-way ANOVA demonstrated that the pattern of energy-dependent amantadine uptake inhibition displayed by MRZ 2/600 was distinct from that of the other compounds studied. Specifically, there were no significant tubule or buffer effects observed at the therapeutic concentration of amantadine (Fig. 48). However, at the clinically toxic concentration of amantadine, a buffer effect (Table 10, $P < 0.01$) and a tubule by buffer interaction (Table 10, $P < 0.05$) were found. Further analysis using the 95% CI indicated that the inhibition of amantadine uptake was more potent in the proximal tubules in the presence of bicarbonate (95% CI: 36-76 μM in KHS, versus 97-211 μM in CT).

For MRZ 2/615 inhibition of amantadine uptake, a consistent buffer effect was demonstrated for both the therapeutic and the clinically toxic concentrations of amantadine (Fig. 49 and Table 10, $P < 0.001$) with increased potency of inhibition in the presence of bicarbonate.

Section 4: Discussion

There are several basolateral renal tubule organic cation transporter sites that mediate the clearance of organic cations. Amantadine has been used to identify two distinct basolateral organic cation transport sites. Specifically, amantadine energy-dependent uptake into rat proximal and distal renal tubule segments consists of a high-affinity-capacity, bicarbonate-dependent transport

site, and a lower affinity-capacity, bicarbonate-independent transport site (Escobar et al., 1994; Escobar and Sitar, 1995; Goralski and Sitar, 1999). All four of the NMDA receptor antagonists produced concentration dependent inhibition of amantadine uptake in rat proximal and distal renal tubules in the presence and absence of bicarbonate based buffer. The present study was conducted at pH 7.4 and the NMDA receptor antagonists were mostly in the protonated form. The pKa values for memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615 were 10.72, 10.72, 10.84 and 10.74 respectively, as calculated by use of the Pallas 3.0 program (CompuDrug Chemistry Ltd., Hungary). The pKa values of these NMDA receptor antagonists are comparable amongst each other as well as to that of amantadine (10.1) (Aoki and Sitar, 1988). Thus, variability in pKa values of the compounds is unlikely to confound the determination of the K_i values or the amount of protonated antagonist concentration.

However, a point to keep in mind is that the extent of inhibition of the renal tubule amantadine transporters *in vivo* depends on both the K_i values and the concentrations of the inhibitors in the kidney during treatment with typical clinical doses. Therefore, these results should be interpreted with caution in terms of the potential for drug-drug interactions *in vivo*, as the clinically relevant concentrations of these NMDA receptor antagonists in the protein bound and free forms at the transporter sites in humans are unknown at the present time.

Even though all four of the NMDA receptor antagonists inhibited energy dependent transport of amantadine, there were important differences in the

inhibition profiles when specific buffer or tubule environments were considered. At a therapeutically relevant concentration of amantadine (10 μM), the potency of inhibition of amantadine uptake was most efficient on the bicarbonate-dependent organic cation transporter by memantine, MRZ 2/579 and MRZ 2/615. This may be of clinical relevance, as 80% of amantadine is transported through the bicarbonate-dependent, high affinity-capacity transporter site (Goralski and Sitar, 1999). Bicarbonate-dependent organic cation transporter selectivity of inhibition was also observed at the clinically toxic concentration of amantadine (50 μM) by memantine, MRZ 2/579, MRZ 2/600 and MRZ 2/615, but this observation may not be of clinical importance.

MRZ 2/579 displayed a novel distal tubule selectivity of inhibition at a therapeutic concentration of amantadine. In particular, the most potent inhibition of all compounds studied, was observed with MRZ 2/579 in the distal tubule segments in the presence of bicarbonate, with a K_i value of 9 ± 3 μM (mean ± S.E.M.). This unique selectivity of inhibition by MRZ 2/579 to the distal renal tubule segments has not been observed with any compound previously studied. The distal tubule selectivity of MRZ 2/579 may potentially be attributable to differences in molecular configuration of the organic cation transporters in different renal tubule segments and further molecular studies are therefore warranted. Alternatively, it is possible that MRZ 2/579 may inhibit unidentified amantadine transporters that are selectively expressed in the distal (but not the proximal) renal tubule. Distal renal tubule organic cation transport may be of

physiological importance as it was previously indicated that amantadine accumulation does occur in both proximal and distal renal tubule segments in rat kidney (Escobar & Sitar, 1995). Thus, MRZ 2/579 may be a novel tool to assess the relative importance of proximal tubule versus distal tubule organic cation transport systems. Moreover, MRZ 2/579 may therefore be useful to identify pharmaceutical compounds whose pharmacokinetics is significantly influenced by distal tubule organic cation transporters. As the molecular identity of organic cation transporters contributing to amantadine transport and their relative expression in rodents and humans are not fully characterized at the present time, caution should be exercised in extrapolation of the present results to humans.

MRZ 2/600 displayed a buffer effect only at a clinically toxic concentration of amantadine. Notably, most potent inhibition was observed on the bicarbonate-dependent organic cation transporter site in the proximal tubule segments. This observation may suggest the presence of multiple amantadine transporter sites, which are distinguished only by a clinically toxic concentration of amantadine.

MRZ 2/600 was unique in having similar potencies of inhibition at therapeutic concentrations of amantadine in both the proximal and distal renal tubule segments, irrespective of buffer environment. Unlike memantine, MRZ 2/579, and MRZ 2/615, this compound did not display selectivity of inhibition to the bicarbonate-dependent organic transporter sites at a therapeutic concentration of amantadine. This observation leads to the novel hypothesis that

bicarbonate-dependent organic cation transport selectivity requires specific structural characteristics from the substrate. The molecular structure of MRZ 2/600 is distinct from the other three NMDA receptor antagonists, in that this compound has a sterically bulky ethyl group close to the ionized amino group. It is suggested that this ethyl group may produce steric hindrance around the ionized amino group, which may prevent bicarbonate-dependent organic cation transport selectivity. Due to the bulky ethyl group, MRZ 2/600 may not be a good substrate for the bicarbonate-dependent organic cation transporter site. Therefore, this steric hindrance may explain the observation that MRZ 2/600 does not exhibit bicarbonate-dependent organic cation transporter selectivity of inhibition. Data from previously published studies are consistent with this observation. Specifically, inhibition studies in rat proximal and distal renal tubule segments indicated that tetraethylammonium did not inhibit amantadine transport through its bicarbonate-dependent transport site, suggesting that tetraethylammonium does not undergo transport through the bicarbonate-dependent organic cation transporter characterized by amantadine (Goralski and Sitar, 1999). Tetraethylammonium has four ethyl groups around its nitrogen, and these may produce a large amount of steric hindrance, preventing transport through the bicarbonate-dependent organic cation transport site. This interpretation is consistent with the hypothesis that bicarbonate-dependent organic cation transport selectivity is not affected by steric hindrance around the

ionized amino group of the cyclohexane ring structures in memantine, MRZ 2/579 and MRZ 2/615 (Fig. 45).

In summary, these data demonstrate that all of the NMDA receptor antagonists studied displayed a concentration-dependent inhibition of amantadine uptake in both the proximal and distal renal tubule segments in the presence and absence of bicarbonate. At a therapeutically relevant concentration of amantadine, the potency of inhibition of amantadine uptake was most efficient on the bicarbonate-dependent organic cation transporter by memantine, MRZ 2/579 and MRZ 2/615, which may have clinical implications. MRZ 2/579 displayed a novel distal tubule selectivity of inhibition. This unique selectivity by MRZ 2/579 may provide a new tool to assess the relative importance of proximal versus distal tubule organic cation transport mechanisms in future studies. At a therapeutic concentration of amantadine, MRZ 2/600 was unique in having similar potencies of inhibition in both proximal and distal renal tubule segments, irrespective of buffer environment. This observation leads us to a new hypothesis, which suggests that steric hindrance around the ionized amino group of the cyclohexane ring structure appears to prevent bicarbonate-dependent organic cation selectivity of inhibition. Further structure-activity studies are warranted to evaluate the initial observations in support of this hypothesis.

CHAPTER 5

Overall Significance and Ramifications for Future Research

– Overall Significance and Limitations –

SAR Studies with Benzoquinone Bioreductive Alkylating Agents

The SAR studies presented in Chapter 2 and Chapter 3 of this thesis were aimed at determining the effects of functional group substitutions to the benzoquinone moiety of the model benzoquinone bioreductive alkylating agent on DTD mediated reduction, DTD mediated mechanisms of cytotoxicity (DNA cross-link and strand break formation) and overall activation by DTD and cytotoxic potency *in vitro*.

1.1 SARs identified in relation to DTD mediated reduction kinetics:

- Steric effects were more important than electronic effects of functional groups in decreasing DTD mediated reduction
- Steric effects at the C6 position were more important than substitutions at the C5 position in decreasing the rate of reduction by DTD
- Electron-donating groups increased redox cycling of the quinone compared to all other analogs

1.2 Significance of findings and future studies

The rate of DTD mediated reduction was decreased by all functional group substitutions except the fluoro group in FBM, where no effect was observed compared to the parent compound, BM. We suggest that these decreases in rates of reduction were due to steric effects of functional groups and not electronic effects, as functional groups with opposite electronic effects but similar

steric effects produced comparable decreases in DTD mediated reduction. Furthermore, the inability of electron-withdrawing groups to affect DTD mediated reduction was shown with FBM where the fluoro group had no steric effect as it is sterically similar in size to hydrogen, but yet had an electron-withdrawing character, and its rate of reduction by DTD was identical to that of BM. The magnitude of the decreases observed in rate of reduction by DTD due to steric effects of functional groups was dependent on the position as well as the size of the functional group on the benzoquinone moiety.

These findings provide a better understanding of the factors that are important in affecting DTD mediated reduction. One could postulate that electron-donating groups, for example, may donate electrons into the quinone moiety which may interfere with the electron transfer in DTD mediated reduction of the quinone. In contrast to this hypothesis, our studies provide evidence that electronic effects do not affect DTD mediated reduction. This finding is generally consistent with a similar SAR study with indolequinones, by Phillips and colleagues (1999) where sterically bulky groups were found to be an important factor in decreasing DTD mediated reduction. The latter study also showed that functional groups with chlorine atoms led to compounds which were poor substrates for DTD, a finding that was attributed not to electronic effects, but rather due to steric effects interfering with the enzyme-substrate interaction. Further SAR studies with indolequinones have also indicated that electron-withdrawing groups at the indole 3-position (see Chapter 1 for chemical structure) are very good substrates for DTD and that these groups do not affect

DTD mediated reduction significantly when compared to analogs with hydroxymethyl or no substituents at this position (Beall et al., 1998a,b). The finding that analogs with electron-withdrawing groups at this position do not alter DTD mediated reduction compared to other analogs with no substituents at this position suggests that it is not the electronic effects which allow for these analogs to be good substrates for DTD, but rather the lack of steric hindrance present which may hinder the substrate-enzyme interaction. Moreover, when sterically bulky groups are considered, our findings indicated that sterically bulky groups at the C6 position of the quinone are most important in decreasing reduction by DTD. This is similar to studies with indolequinones, where sterically bulky groups at equivalent positions of the quinone moiety also provide steric hindrance and produces poor substrates for DTD (Phillips et al., 1999).

Collectively, these observations suggest that similar structure activity relationships may exist among different types of quinone-based bioreductive alkylating agents. This may have important implications in the rational design of new bioreductive alkylating agents, or modifying existing agents for more enzyme specific bioreduction. These SARs may have application in the modification of existing quinone-based bioreductive alkylating agents to agents with increased specificity of reduction by DTD if these compounds contain sterically bulky groups at positions that interfere with DTD mediated reduction. Alternatively, one may design agents, with decreased specificity of reduction by DTD by adding sterically bulky groups at positions within the molecule which would decrease reduction by DTD. This strategy may lead to compounds which are more

specifically reduced and activated by other enzymes that are targets in enzyme directed drug targeting (e.g. cytochorome P450 reductase), and possibly prevent their inactivation by DTD. It is noteworthy that there are currently no quinone-based bioreductive alkylating agents that are specifically inactivated when reduced to the hydroquinone form by DTD and activated through reduction via single one-electron reductase to the semiquinone. Therefore, this theoretical approach may only be potentially useful when such theoretical agents are designed.

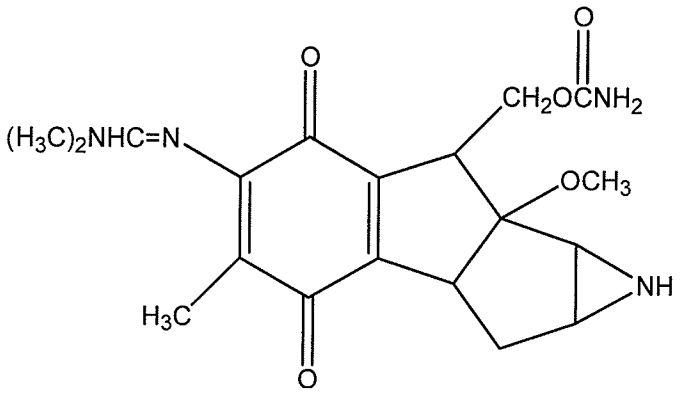
Another potential application of the steric hindrance approach to decrease reduction by DTD may be utilized in the case of very good substrates for DTD, where potentially these agents may be very cytotoxic to non-tumor tissue due to activation of the agent in cells with low DTD activity. The selectivity of these agents might be increased by adding steric bulk at the C5 or C6 position of the quinone to decrease the substrate specificity to a desired value, which may allow for preferential activation by tumor tissues with high DTD activity.

It is also important to note that the experiments in the absence and presence of DIC indicated that a subset of the BM analogs including BM, are inactivated by DTD (i.e. the reduced products formed through reduction by DTD are less cytotoxic than those produced via reduction through other pathways). Therefore, the formation of these less cytotoxic reduced products might be prevented by substitution of sterically bulky groups at the C6 position of the quinone moiety. Furthermore, when considering these analogs with sterically bulky groups at the C6 position, the further substitution of an electron-donating

methoxy group at the C5 position may further act to activate the cytotoxic group of the reduced benzoquinone products. It is suggested that such dual substitutions to the quinone moiety may potentially lead to compounds which are activated selectively by reductases other than DTD, as well has having improved cytotoxic activity.

- Further studies are also required to determine whether the increased redox cycling observed with a methoxy group substitution can also be observed in other investigational quinone-based bioreductive agents, and if this may have implications in affecting the quinone-based bioreductive alkylating agents in which cytotoxicity under aerobic conditions is in part mediated through oxidative damage mechanisms. It may be possible, for example, to design analogs of mitomycin C, streptonigrin or EO9 with a methoxy group at the C5 or C6 position of the quinone, and observe possible altered cytotoxicity under aerobic conditions due to increased redox cycling of the analog following reduction by DTD. This concept may be supported by the mitomycin C analog, 7-dimethylamidinomitosane (BMY-25282), which has an electron-donating dimethylaminomethylene group at the N7 position (Figure 51).

Figure 51



BMY-25282

In particular, BMY-25282 was more toxic to EMT6 mouse mammary tumor cells under aerobic conditions than mitomycin C (Pritsos & Sartorelli, 1986). This finding suggested an oxidative free radical mechanism of cytotoxicity by BMY-25282, which may occur as a result of a possible increased redox cycling potential as a result of the electron-donating group substitution. To support this mechanism, BMY-25282 was shown to produce significantly greater quantities of oxygen free radicals in a biological reducing system, which consisted of EMT6 cell sonicate, reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase, and mitochondria when compared to mitomycin C (Pritsos & Sartorelli, 1986). As well, in experiments where superoxide dismutase activity in EMT6 cells was significantly decreased by the superoxide dismutase inhibitor diethyldithiocarbamate, the cytotoxicity of BMY-25282 was potentiated; however, the cytotoxicity of mitomycin C was not affected (Pritsos et al., 1989). Another characteristic of BMY-25282, which may

occur as a result of the electron-donating group substitution, is that this compound produces cardiotoxicity, as indicated in neonatal rat-heart myocytes, an effect not observed with mitomyicin C (Dorr et al., 1992). Overall, these studies provide a rationale for the substitution of electron-donating groups on the quinone moiety to increase redox cycling potential and oxidative damage by currently used bioreductive alkylating agents. Furthermore, such groups may also explain the cardiotoxicity observed with BMY-25282, and additional structure activity studies with weaker or stronger electron-donating group substitutions are required to investigate this effect further.

- Future studies should include similar investigations with additional analogs that have stronger electron-donating or –withdrawing group substitutions such as the amino or ester groups, respectively. Such studies would be important to fully conclude that electronic effects are not important in affecting the rate of reduction by DTD.
- The importance of steric effects in decreasing the rate of reduction by DTD could also be further investigated with additional BM analogs where there is a gradual increase in steric bulk. This may be achieved through the substitution of different alkyl groups with a gradual increase in chain length.

1.3 SARs identified in relation to DTD mediated cytotoxic activity

- DTD mediated reduction was a bioactivation pathway for MBM
- DTD mediated reduction was an inactivation pathway for BM and m-MeBM in both the NCI-H661 and SK-Mel-28 human cancer cell lines, as well as for FBM and MeBM in the SK-Mel-28 cells
- DTD did not appear important in augmenting the cytotoxicity of CBM, PBM, m-PBM and m-TBM in the human cancer cell lines
- Electron-donating groups at the C5 or C6 position of the quinone and the sterically bulky phenyl group at the C5 position increased the cytotoxic activity of the BM analogs compared to BM in the human tumor cell lines, whereas electron-withdrawing groups and sterically bulky groups at the C6 position either had no effect or decreased the cytotoxicity versus BM.

1.4 Significance of findings and future studies

The *in vitro* cytotoxicity data of the BM analogs in the NCI-H661 and SK-Mel-28 human cancer cell lines in the absence of DIC indicate that functional groups with different characteristics can significantly increase or decrease the cytotoxicity of these quinone-based bioreductive alkylating agents. Electron-donating groups and the phenyl group at the C5 position of the quinone led to compounds with increased cytotoxicity compared to BM, but further studies are required to elucidate the exact mechanisms involved, as well as determination of the redox potentials of the analogs. In the case of the electron-donating groups, the increased cytotoxic activity compared to BM may be as a result of the singular or

combined effects of activation of the reduced product cytotoxic element and increased potential to redox cycle. In contrast to the strong-electron donating group in MBM, the weaker electron-donating methyl group substitution did not show an increase in redox cycling of MeBM and m-MeBM compared to BM. This suggests that the increase in cytotoxicity observed with electron-donating groups is more likely due to the mechanism involving activation of the cytotoxic element in the reduced product. It would be beneficial to conduct cytotoxicity studies with the BM analogs under hypoxia as this may allow for determination of the overall effect of redox cycling of the analogs on cytotoxic potency and for comparisons with cytotoxicity under aerobic conditions.

DTD mediated reduction was observed to be an inactivating pathway for BM. This is consistent with earlier comparisons between L5178Y cells and their alkylating agent resistant L5178Y/HBM2 or L5178Y/HBM10 counterparts. As reviewed earlier, cytotoxic activity of BM was decreased in the L5178Y/HBM2 and L5178Y/HBM10 cell lines compared to the parent cell line, which was suggested to be as a result of the increase in DTD activity in these cell lines compared to the parent cell line. These earlier studies in combination with the findings of experiments from this thesis provide evidence suggesting that the reduction mediated by DTD may act to inactivate DTD through the production of reduced products which may be less cytotoxic than those produced via other activation mechanisms in the cell. Further studies are warranted to identify other pathways of activation that are involved in mediating the cytotoxic activity of BM and reduced products formed by BM intracellularly. Such unidentified pathways

may lead to the observed increased cytotoxic activity of BM in the absence of DTD mediated reduction, resulting from the presence of DIC.

Taken together, the following intracellular factors may play a role in the overall cytotoxic activity of BM and its analogs:

- Alternative mechanisms of the bioactivation via other reductase enzymes may produce changes in the balance between the hydroquinone and semiquinone intermediates formed.
- Variability in the rates of redox cycling may influence cytotoxic activity mediated by reactive oxygen species, as well as the overall role of super oxide dismutase and catalase in the cellular protection against oxidative damage.
- Hydroquinone reduced products may have differences in their detoxification by enzymes such as UDP-glucuronosyl-transferases, glutathione S-transferases and other conjugative enzymes that act to detoxify hydroquinones (Talalay, 2000). This may produce differences in the balance of activation via DTD and detoxification via conjugation, which would affect the overall cytotoxic activity of these analogs. As an example, if the hydroquinone product of BM is a substrate for conjugation by UDP-glucuronosyl-transferase, but that of MBM is not, then the reduced product of MBM may be more cytotoxic than that of BM.
- Alternatively, benzoquinones may also undergo addition reactions with intracellular thiols. This could lead to reduced products with increased or decreased abilities to redox cycle, or which may have a differential

cytotoxic activity when compared to reduced hydroquinone products which did not undergo thiol addition reactions.

1.5 SARs identified in relation to DTD mediated mechanisms of cytotoxic activity

- Electron-donating groups increased DTD-mediated DNA cross-link and strand break formation, whereas electron-withdrawing groups and sterically bulky groups at the C6 position of the quinone had no effect or decreased DTD-mediated DNA cross-link and strand break formation
- DNA cross-link and strand break formation appear to importantly impact the cytotoxicity of by the BM analogs in human tumor cell lines.

1.6 Significance of findings and future studies

The effects of functional group substitutions on quinone-based bioreductive alkylating agents have been previously reported in relation to DNA cross-link and/or strand break formation at the whole cell level. However, there are limited quantitative data on the effects of functional groups on these specific mechanisms of genotoxicity in isolated systems, without possible confounding results due to cellular factors present such as DNA repair or detoxification mechanisms. Our SAR studies were undertaken to delineate the effects of a wide range of functional group substitutions characteristics to the BM quinone moiety on DTD mediated DNA cross-link or strand break formation in isolated systems through using agarose gel assays.

Notably, previous SAR studies have utilized functional group substitutions with a limited variability in chemical characteristics (e.g. variations in methylene chain length) and used concentrations of analogs which may not be suitable for determination of appropriate SARs (Mayalarp et al., 1996). There may also be differences in mechanisms of DNA damage if assessed by methodologies which use chemical reduction versus biological mechanisms of reductive activation, that may lead to the determination of different SARs (Hargreaves et al., 1999). In our studies we assessed the effects of functional groups with a wide range of chemical characteristics, and employed a concentration-effect study design in order to ensure that the SARs are assessed within the linear portion of the concentration-effect profile, and therefore, minimized the risk for confounding due to floor (e.g. very low drug concentration incapable of producing DNA damage) effect or, ceiling effects where excessively high drug concentrations may obscure the variability in the extent of DNA damage that is attributable to differences in chemical structures.

We found that DTD mediated reduction of analogs with electron-donating group substitutions led to compounds with increased DNA cross-link and strand break formation versus BM. As discussed earlier, the cytotoxic activity studies in the presence and absence of DIC suggested that DTD mediated reduction was an inactivation pathway for MeBM and m-MeBM, but an activation pathway for MBM. Our studies suggest that at the whole cell level, DTD mediated reduction of MeBM and m-MeBM leads to products which may be less cytotoxic than products produced via other pathways (e.g. possibly one-electron reduction).

However, the DTD reduced products are still very efficient in their production of DNA cross-link and strand breaks. Furthermore, MeBM and m-MeBM are cytotoxic even in the absence of DTD mediated reduction as shown by the cytotoxicity studies in the presence and absence of DIC.

Further studies are required to determine what other pathways are involved in the activation/detoxification of these analogs at the whole cell level, and which mechanisms of cytotoxicity are most important in determining cytotoxic activity of the BM analogs subsequent to activation by these DTD-independent mechanisms. In the case of analogs which produced similar or less DTD mediated DNA damage than BM, the precise mechanisms by which these decreases occur are unknown at present.

In addition, future studies also appear to be warranted to address the following issues:

- Identification of mechanisms involved in increased/decreased DNA cross-link formation. For example are there differences in the ability of the analogs to interact with DNA and produce alkylation reactions due to factors such as steric hindrance?
- Does an inability to produce DNA cross-links by some BM analogs directly affect DNA strand break formation? For example, if a compound is unable to alkylate DNA, the reactive intermediates and oxygen species which occur due to redox cycling of the hydroquinone may be positioned too far from the target (DNA) to produce strand breaks.

- Are there differences in the rate at which the hydroquinone products may be inactivated through for example hydration of the cytotoxic elements, thus preventing DNA cross-link formation?
- It may also be beneficial to assess potential relationships between the cytoxic activity of these analogs under hypoxia and DTD-mediated DNA cross-link or strand break formation to determine if both mechanisms of cytotoxicity are important under hypoxia. Under hypoxic conditions, redox cycling of the quinone may not occur to a significant extent in compounds with strong electron-donating groups (MBM). Therefore under hypoxic conditions, one may expect a decreased cytotoxic activity with analogs such as MBM compared to the cytotoxicity under aerobic conditions. Such a decrease in cytotoxicity may be caused by a potential decrease in DNA strand break formation as a result of lower rates of redox cycling under hypoxic conditions.

We observed a positive correlation between the cytotoxic potency of the BM analogs and DNA cross-link formation or DNA strand break formation, which suggests that these pathways of DNA damage formation are important in determining the overall cytotoxicity of these bioreductive alkylating agents. There were no significant correlations between half-times of DTD mediated reduction and cytotoxic activity, DNA cross-link formation or DNA strand break formation. The lack of correlation of cytotoxicity or mechanisms of DNA damage with DTD mediated reduction kinetics suggests that further downstream events (DNA

cross-link or strand break formation), following activation of analogs to cytotoxic metabolites are likely most important in influencing the cytotoxic character of the analogs. Thus, the ability of a BM analog to produce DNA cross-links or strand breaks may potentially be used for the rational prediction of the cytotoxic properties of new BM analogs.

Overall, the SARs identified in these studies may be helpful in the design of new bioreductive agents with the right balance between efficacy in production of DNA damage and affinity to bioreductive activating enzymes such as DTD for improved antitumor activity.

SAR Studies with Novel NMDA Receptor Antagonists

SAR studies presented in Chapter 4 of this thesis were conducted with amantadine derivative NMDA receptor antagonists to determine their safety profile in terms of drug-drug interactions at common kidney organic cation transporters, as well as the elucidation of structural characteristics, which would confer transport through specific renal tubule organic cation transporter subtypes.

2.1 SARs identified with novel NMDA receptor antagonists

- All NMDA receptor antagonists produced concentration dependent inhibition of amantadine uptake in rat proximal and distal renal tubules in the presence and absence of bicarbonate.
- MRZ 2/579 displayed a novel distal renal tubule selectivity of inhibition of energy dependent amantadine transport at a therapeutic concentration.
- Unlike MRZ 2/579, MRZ 2/600 and memantine, MRZ 2/600 did not display selectivity of inhibition to the bicarbonate-dependent organic transporter sites at therapeutic concentrations of amantadine.

2.2 Significance of findings and future studies

The significance of the above SARs was discussed in detail in Chapter 4. Future studies are warranted to investigate the following issues:

- Determination of the clinical significance of the inhibition of amantadine uptake by the NMDA receptor antagonists

- The mechanism behind the possible modest inhibition of energy-dependent amantadine uptake at lower concentrations of the MRZ 2/579, MRZ 2/600 and MRZ 2.615 needs to be further investigated with transfected pure renal tubule organic cation transporter molecules in tissue culture.

Recommendations for future research:

- Application of the SARs determined in the present thesis to the modification of existing experimental or clinically used bioreductive alkyting agents, or in design of new bioreductive alkylating agents to determine their clinical utility.
- Further studies using isogenic models with human tumor cell lines or xenograft models to confirm if SARs determined in this thesis hold (e.g. relationship between cytotoxic activity in the presence and absence of DTD mediated activation, and DTD mediated mechanisms of DNA damage).
- Further studies are warranted to determine whether analogs which are good substrates for reduction by DTD also have selectivity of cytotoxicity to tumor tissue compared to normal tissue.
- Studies to determine the mechanisms by which the BM analogs induce cytotoxicity and cell death following activation by DTD and induction of DNA damage. Such studies may include the determination of specific mechanisms by which apoptosis may be induced (e.g. mitochondrial or non mitochondrial mechanisms). This may lead to an overall better understanding of the mechanisms involved in mediating cell death and cytotoxicity by these agents. The information generated by such studies may be useful in the discovery of potential new molecular targets which could explain the activation and cytotoxic activity of bioreductive alkylating

agents, and lead to the development of future bioreductive alkylating agents targeted to tumor cells.

- Identification of alternate mechanisms of activation and detoxification of the model benzoquinone bioreductive alkylating agents.
- Determination of the characteristics of the precise interactions between the BM analogs and the binding pocket of human DTD with molecular docking studies using the crystal structure of human DTD.
- The distal tubule selectivity of inhibition of amantadine uptake by MRZ 2/579 may be useful as a new tool to assess the importance of proximal versus distal tubule organic cation transport as well as the importance of bicarbonate versus bicarbonate-independent renal tubule transport at the distal renal tubule transporters.
- Future studies are required to evaluate the hypothesis that steric hindrance around the ionized amino group is required to prevent bicarbonate-dependent organic cation selectivity of inhibition. Such studies would include further SAR studies with MRZ 2/600 and new analogs with increased amounts of steric hindrance around the ionized amino group compared to MRZ 2/600.

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