EFFECT OF DT-DIAPHORASE INDUCTION ON THE ANTI-TUMOR ACTIVITY OF RH1 AND MITOMYCIN C

BY

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

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Abstract

DT-diaphorase is a reductive enzyme that is important for the activation of many bioreductive agents and is a target for an enzyme directed approach to cancer therapy. It can be selectively induced in many tumor types by a number of compounds including dimethyl fumarate (DMF) and sulforaphane. Mitomycin C (MMC) is a bioreductive agent that is used clinically for treatment of solid tumors. RH1 (2,5-diaziridinyl-3-3[hydroxymethyl]- 6-methyl-1,4-benzoquinone) is a new bioreductive agent currently in clinical trials. We have shown previously that inducers of DT-diaphorase can enhance the antitumor activity of MMC in tumor cells *in vitro* and *in vivo*. As RH1 is activated selectively by DT-diaphorase while MMC is activated by many reductive enzymes, we investigated whether induction of DT-diaphorase would produce a greater enhancement of the antitumor activity of RH1 compared with MMC.

HCT116 human colon cancer cells and T47D human breast cancer cells were incubated with, or without, DMF or sulforaphane followed by MMC or RH1 treatment. Cytotoxic activity was measured by a clonogenic (HCT116) or MTT assay (T47D). DMF and sulforaphane treatment increased DT-diaphorase activity by 1.9 to 2.8 fold and resulted in a significant enhancement of the antitumor activity of MMC, but not RH1. The lack of enhancement of RH1 activity appeared to be due to the presence of a sufficient constitutive level of DT-diaphorase activity in the tumor cells to fully activate the RH1. When mice implanted with HL60 human promyelocytic leukemia cells were fed DMF and treated with RH1, DT-diaphorase activity in the tumor increased but there was no enhancement of RH1 antitumor activity. These results suggest that the ease of

reduction of RH1 by DT-diaphorase makes it a poor candidate for an enzyme directed approach to cancer therapy.

The ability of RH1 to cross-link DNA in the presence and absence of DT-diaphorase was examined. Double stranded DNA was incubated with RH1 with, and without, DT-diaphorase and cross-linking was assessed by a gel assay. RH1 produced DNA cross-links only in the presence of DT-diaphorase. These results indicate that RH1 must be reduced to form cross-links and that DT-diaphorase is capable of carrying out this reduction.

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Abbreviations

ADEPT	Antibody-directed enzyme prodrug therapy
ANOVA	Analysis of variance
AP2	Activator protein 2
ARE	Antioxidant response element
AZQ	2,5-diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone (diaziquone)
DMEM-F12	Dulbecco's modified eagle medium: nutrient powder F-12 (Ham)
DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
DT-diaphorase	NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1)
EO9	3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
GDEPT	Gene-directed enzyme prodrug therapy
GSH	Glutathione
GST	Glutathione S-transferase
HBSS	Hank's buffered salt solution
MeDZQ	2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone
MMC	Mitomycin C
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH	β--Nicotinamide adenine dinucleotide
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NQO1	NAD(P)H:quinone acceptor oxidoreductase 1 (DT-diaphorase)
NSCLC	Non-small-cell lung carcinoma
PBS	Phosphate buffered saline
P450R	NADPH:cytochrome P450 reductase
RH1	2,5-Diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone
ROS	Reactive oxygen species
SCF	Surviving cell fraction
WBC	White blood cells
XRE	Xenobiotic response element

INTRODUCTION

Cancer

General Description

Cancer is one of the most dreaded diagnoses a person may receive from their doctor. It is a diagnosis that carries devastating emotional effects on both the patient and their loved ones. Cancer will be the cause of death for roughly 1 in 5 persons in the developed world; only heart disease will be responsible for more (Alberts *et al*, 1994). In an effort to gain an understanding of this very complicated disease vast resources have been dedicated to research. This has lead to better early detection systems and treatments resulting in an improved prognosis for many patients. Despite the successes of the past there is still much that is not understood and many types of cancer are still associated with a poor prognosis.

Cancer is really a broad term describing many different diseases with similar characteristics. By definition a cancer cell must have two properties, it must reproduce without obeying normal growth restrictions and must infiltrate and subsequently colonize areas that are reserved for other cells (Alberts *et al*, 1994). Cancerous cells arise from normal cells within the body due to mutations in the genes of the cell. A single mutated gene will not lead to cancer as there are several redundancies in the cell growth control system; however, if multiple complementary mutations accumulate, the cell may become cancerous (Griffiths *et al*, 1997). These mutations often accumulate sequentially, as the cells lose cell growth control and DNA repair genes, their mutation rates increase, creating more mutations and resulting in a progressively more aggressive disease.

In general, mutations are rare; spontaneous mutations occur at an estimated rate of 10^{-6} mutations per gene per cell division. With about 10^{16} cell divisions occurring in the human body, each gene will theoretically be mutated on 10^{10} separate occasions during a lifetime. As a result the chance of acquiring the complementary mutations in the same cell at the same time increases with increasing age making age a risk factor for cancer (Alberts *et al*, 1994). Other risk factors for cancer include a hereditary predisposition due to the presence of an inheritable mutation that increases the chance of cells becoming malignant, or exposure to environmental mutagens that result in increased mutation rates.

Treatment

Treatments for cancer can be grouped into one of three main modalities: surgical excision, radiation, and pharmacological treatment. Other approaches to cancer therapy do exist but are still in their infancy and require much further research before they become widely accepted treatments. Some examples of these include gene therapy, immunotherapy, and viral therapy.

Surgical excision of the tumor and surrounding tissue is the preferred and most successful treatment for all primary solid tumors (Elias, 1989). This treatment may result in complete remission of the disease if the tumor is discovered early enough, before it has had a chance to infiltrate nearby tissue and metastasize. This approach is also an effective way to help reduce tumor burden in cancer patients with advanced disease, but will not result in a successful elimination of the malignant disease. To help ensure the treatment is a success, surgery is often combined with radiation therapy and/or pharmacological

agents to deal with any malignant cells that may have already left the confines of the primary tumor.

In addition to being a form of treatment for cancer, surgery is also very important in the initial diagnosis of the disease. For most suspected cancer patients a biopsy is performed to confirm that the tumor is indeed malignant. The exception to this is for tumors located in areas of the body that would require invasive surgery to access. In these cases the tumor is resected and the diagnosis made following the operation (Elias, 1989).

Radiation therapy is also an approach more specifically geared towards confined solid tumors. Radiation therapy may be used as the primary mode of therapy and is also an integral part of combination therapies. For many solid tumor types surgery is followed up by radiation therapy of the surrounding tissue, to ensure that any cancer cells which may have already infiltrated nearby tissue have been treated. The choice between radiation and surgery as the primary therapy is generally dependent on the size of the tumor as radiation is only effective when the overall tumor burden is minimal (Elias, 1989). Radiation is ineffective against larger tumors as the cells in the center of the tumor exist in a low oxygen tension environment and oxygen is required for radiation to kill the cells (Gray *et al*, 1953). Attempts have been made to improve the outcome of radiation therapy by overcoming tumor hypoxia; however, these methods have met with limited success (Overgaard, 1992).

Of the three major treatment modalities the pharmacological approach is the only one that acts systemically rather than locally; this has both positive and negative implications. A negative effect is that the drugs employed must have some degree of specificity for tumor cells to avoid toxicity. On a positive note, it is the only approach

with the potential to effectively deal with advanced metastatic disease and hematologic cancers such as lymphomas and leukemias. Chemotherapy also plays a very important role in combination with surgery and radiation to eliminate any potential micrometastases when dealing with solid tumors (Brenner, 2000). In addition chemotherapy is often used for palliative care of patients with advanced metastatic disease (Elias, 1989) or inoperable primary tumors (Brenner, 2000).

Pharmacological Treatments

Successful pharmacological agents used for the treatment of cancer must be capable of targeting fundamental differences between malignant cells and normal, healthy cells. In reality this is a very difficult task to accomplish as cancer cells are really normal cells of the body with relatively few minor alterations, and as a result, pharmacological treatments commonly cause adverse, toxic effects.

The most commonly targeted cancer characteristic is the increased rate of cell division. As a result the major toxicities associated with drugs that target rapidly dividing cancer cells are due to the death of normally fast growing cells such as bone marrow stem cells, gastrointestinal epithelial cells and hair follicles resulting in myelosuppression, gastrointestinal problems and hair loss, respectively.

Current cancer chemotherapy agents have met with limited success as they often fail to destroy all of the malignant cells leading to a high rate of relapse. This is due to the heterogeneity of the tumor cell population that arises from the frequent mutations occurring within tumor cells. To help reduce the risk of treatment failure combinations of anti-tumor drugs are often employed. The combinations are carefully selected to target

different malignant cell characteristics that are likely to be present. It is also important to try to avoid using drugs with similar sites of major toxicity as additive or even synergistic toxic effects must be avoided. Despite the best efforts of physicians the selected combination of therapeutic agents often fails to attack all of the clonal sub-populations making up the tumor. The cells that survive are those that are resistant to the drugs and these will be selected for and a subsequent treatment with the same drugs will be ineffective. The mechanisms for drug resistance that can contribute to this problem are various and include decreased entry of drugs into the cell or increased efflux of the drugs, altered target proteins with decreased affinity for the drugs or increased activity of repair systems within the cell (Brenner, 2000).

Another factor that may contribute to the poor success rates is the inability of the drugs to gain access to all areas within the tumor. The vascular system of most tumors is very poorly developed and as a result the drugs cannot access many areas of the tumor. In addition to affecting drug distribution to the tumor, the poor vascular system also provides other challenges to traditional chemotherapy drugs. Areas of the tumor with poor blood flow become hypoxic, resulting in decreased rates of cell division. As most anti-tumor agents target fast growing cells, they are often ineffective against these oxygen poor regions of the tumor. In addition responses of these hypoxic regions to radiation therapy are also very poor.

Currently Employed Pharmacological Agents

Despite all these limitations there are many pharmacological agents approved by the FDA and Health Canada for the treatment of cancer. These agents provide curative

therapy for many forms of leukemia and lymphoma, and for some solid tumors, but also increase survival and improve the quality of life for many other cancer patients. Anticancer agents are classified based on their mode of action. Although a detailed description of each of the classes is beyond the scope of this thesis, each is briefly described below along with an example given in parenthesis.

DNA synthesis inhibitors are drugs that are structurally similar to the molecular precursors for DNA base synthesis. They act by inhibiting key enzymes in the synthesis process (methotrexate) or by terminating the DNA chain when incorporated (fluorouracil). These drugs are only active against actively dividing cell populations; dormant cells within the tumor will not be affected (Brenner, 2000).

DNA intercalating drugs include natural antibiotic compounds and synthetic derivatives. They all have a planar structure that allows them to insert between the base pairs in the double stranded DNA chain. This intercalation deforms and uncoils the DNA. In some cases the intercalation exerts the only cytotoxic effect of the drug as the deformed DNA cannot be efficiently used as a template for DNA synthesis or transcription or may cause cell death by triggering an apoptotic signalling pathway (dactinomycin). Other drugs have a more complex mechanism of action as the intercalation not only deforms the DNA but also acts to localize a cytotoxic element in close proximity to the DNA. This cytotoxic element may generate free radicals that then cleave the DNA strands (doxorubicin) (Brenner, 2000).

Mitotic inhibitors work by interfering with the dynamic construction and degradation of the mitotic spindle that is required to separate the chromosomes during mitosis. The mitotic spindle apparatus is made up of polymers of tubulin known as

microtubules. Vinca alkaloids (vincristine) bind to the tubulin monomers and prevent polymerization while the taxanes (paclitaxel) bind to assembled microtubules and prevent depolymerization. As the mitotic spindle is involved only in the final stages of mitosis, these drugs are only effective against actively dividing tumor cells (Brenner, 2000).

The topoisomerase inhibitors act against the topoisomerase enzymes. These enzymes are responsible for releasing the torsional stress of supercoiled DNA to allow replication and transcription enzymes to access the DNA and utilize it as a template. There are two main types of topoisomerase enzymes, the type 1 topoisomerases act by cleaving one strand of the DNA and then rotating that loose end around the other intact strand before ligating the two loose ends of the cleaved DNA strand. In contrast the type 2 topoisomerases cleave both strands of DNA simultaneously and allow the DNA to unwind prior to ligation (Lehninger *et al*, 1993). In parallel there are two types of topoisomerase inhibitors, one for each of the two types of topoisomerases. The type 1 topoisomerase inhibitors are all synthetic analogues of a natural product camptothecin (irinotecan). The type 2 topoisomerase inhibitors are natural products (doxorubicin) or synthetic analogues of another natural product known as podophyllin (etoposide) (Brenner, 2000). Without the ability to relax supercoiled DNA, enzymes for DNA transcription and replication cannot access the DNA required as a template for DNA and RNA synthesis.

Hormone based therapies are also employed to treat cancers that arise from tissues within the body that are normally responsive to hormones. The most common scenario is to administer antagonists of the natural hormone. The best known examples of hormone dependent cancers are those of the breast in women and of the prostate in men. It is

worth mentioning that not all cancers of the breast or prostate are hormone dependent and in these cases hormone antagonists will not be effective. Some of these cancers may have originated from a cell type that is not hormone dependent or through mutation may have lost the requirement of the hormone for growth. If the tumor is hormone dependent then estrogen antagonists (tamoxifen) or androgen antagonists (flutamide) may be effective against breast and prostate cancer, respectively. In addition tamoxifen has been shown to be effective in preventing breast cancer in women from families with a strong history of breast cancer (Brenner, 2000).

Immunomodulating drugs are a group of drugs that alter immune system function. There are two main groups of immunomodulators, those that enhance the immune system and those that suppress it. In terms of cancer treatment the immune system enhancers are the drugs of interest. Recombinant interferons (interferon alfa) and cytokines (aldesleukin) have the ability to increase the activity of cytotoxic cells of the immune system (Brenner, 2000). This results in a greater immune response to tumor cells in some cases. Monoclonal antibodies also have the ability to enhance the immune response against tumor cells by binding to surface antigens on tumor cells and then directing the immune response against those cells (Rituximab). Monoclonal antibodies may also have a direct anti-tumor activity by binding to receptors for growth factors and blocking the receptor stimulation (Trastuzumab) (Brenner, 2000). They may also serve a very important role in future cancer treatment as it is believed that they can be attached to anti-tumor drugs to provide a mechanism of specifically targeting malignant cells.

The focal drug for this thesis belongs to the final group of anti-tumor drugs to be discussed, the DNA alkylating drugs. These drugs all have the ability to bind covalently

to proteins, membranes and DNA. The interaction between the drug and the guanine residues of DNA strands is the most important in terms of the anti-tumor activity of these drugs as the alkylation of the DNA induces apoptosis of the cell (Sun *et al*, 1996).

The nitrogen mustards (discovered in the early 1940's) are considered to be the first effective anti-tumor drugs (Brenner, 2000) and are still used in many treatment regimens (Pratt *et al*, 1994). Nitrogen mustards (chlorambucil) are bifunctional alkylating agents, meaning they are capable of forming two covalent bonds to DNA. If the two bonds are formed with residues on separate DNA strands, the resulting interstrand cross-link prevents separation of the strands for replication and transcription leading to cell death. Other cross-linking potentials include intrastrand cross-links, inter-helix cross-links or even DNA-protein cross-links; however, none of these appear to be as toxic as the interstrand cross-links (Hargreaves *et al*, 2000). Cross-linking is not required for the cytotoxicity of alkylating agents as even mono-alkylation of the DNA can result in base pair mismatches, apurinic sites and the formation of DNA single strand breaks (Hargreaves *et al*, 2000).

A number of other drugs act in a similar fashion to the nitrogen mustards and these include the nitrosoureas (carmustine) and busulfan (Brenner, 2000). The platinum compounds (cisplatin) are not alkylating agents but act in the same manner as they are capable of forming cross-links between guanine residues through an ionic mechanism (Brenner, 2000). There are other common examples of DNA alkylating agents (mitomycin C, cyclophosphamide) that are not active in their administered form and must be metabolized by naturally occurring enzymes to their active forms. This leads to a

potential targeting mechanism known as enzyme directed therapy to promote anti-tumor activity while at the same time minimizing systemic toxicity.

Enzyme Directed Therapy

The basic goal of enzyme directed therapy is to identify differences in the enzymatic profiles of malignant and normal cells within the body and use these differences as a targeting mechanism. Enzymes that are over-expressed by malignant cells are the ideal candidates for this type of therapy. To successfully achieve this goal, basic research must identify as many enzymes as possible that are commonly over-expressed in cancer and then design pro-drugs which are selectively metabolized by these enzymes to cytotoxic forms. This ensures that the highest concentration of active drug in the body is found within the tumor. In this way, the therapeutic effect of the treatment is maximized while the associated toxicities are minimized.

A second element is required to make the enzyme directed approach to therapy successful and involves development of screening processes to create enzyme profiles that would be used to select the optimal drugs for each patient (Workman, 1994). This step is necessary as each tumor, and each patient has unique enzyme expression levels that will affect the outcome of the treatment.

Bioreductive Agents

One of the more promising enzyme directed therapies involves a group of drugs known as bioreductive agents; an approach that was first introduced in 1990 (Workman and Walton, 1990). The drugs found within this group require metabolic reduction by

cellular reductases to become active cytotoxic agents. Some of the more notable bioreductive drugs include the prototype drug, mitomycin C (MMC) and it's analogue porfiromycin, the indoloquinone, 3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol (EO9), tirapazamine, 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (AZQ), and 2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone (MeDZQ) (See Fig. 1).

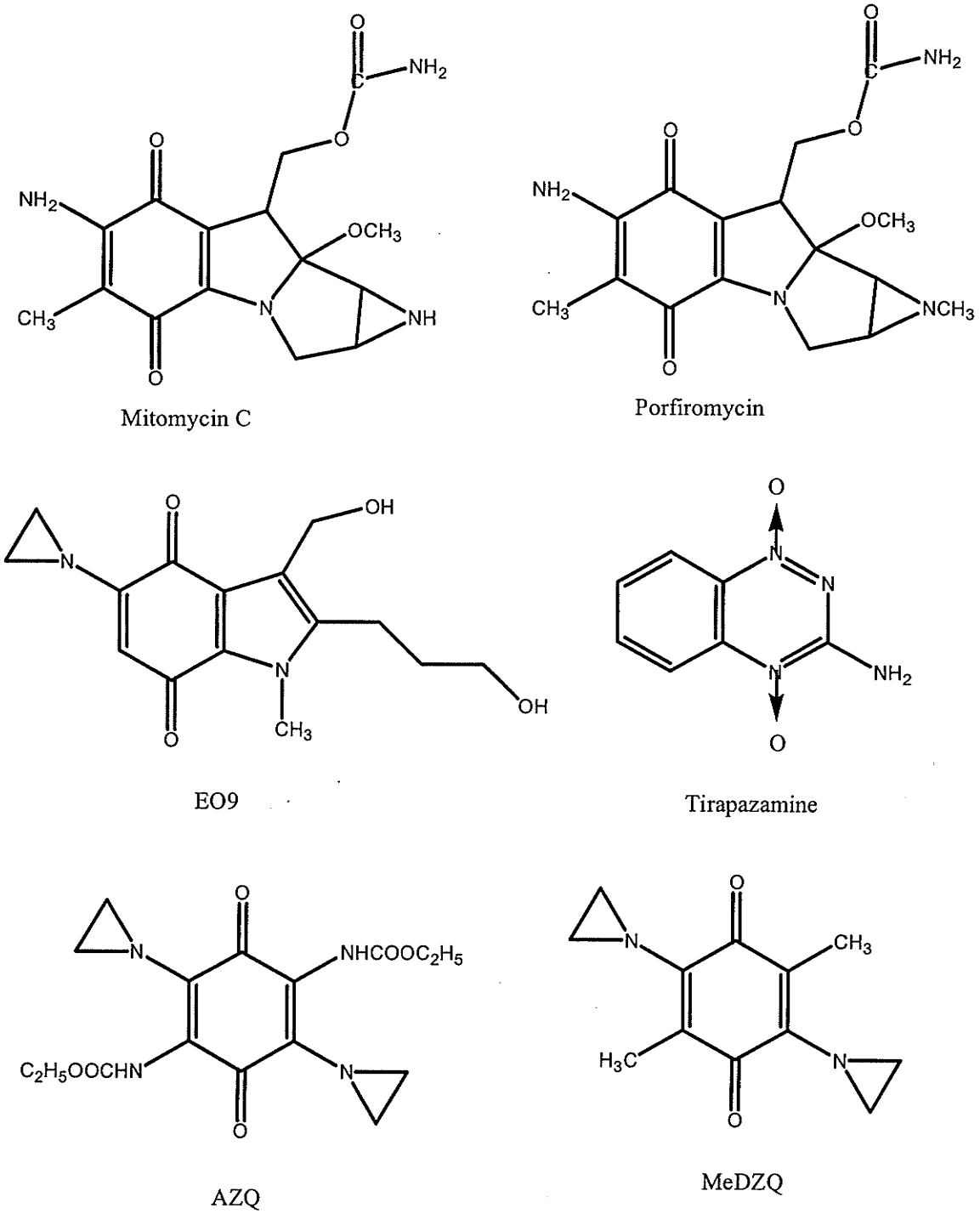

Figure 1 Molecular structures of some of the more notable bioreductive agents

A number of different enzymes are potential targets for this type of therapy including, but not limited to, NADPH:cytochrome P450 reductase (P450R) (EC 1.6.2.4),

NADH:cytochrome b5 reductase (EC 1.6.2.2), xanthine oxidase (EC 1.1.3.22), xanthine dehydrogenase (EC 1.1.1.204) and NAD(P)H:quinone acceptor oxidoreductase 1 (DT-diaphorase, NQO1) (EC 1.6.99.2). Of these DT-diaphorase and P450R have received the majority of the research interest for this application. P450R is likely the most important activating enzyme for the majority of bioreductive agents studied to date; however, DT-diaphorase is a significant contributor (Riley and Workman, 1992). DT-diaphorase has a number of other characteristics that make it more interesting as a target for enzyme directed therapy. These will discussed in detail later in this chapter.

In addition to enzyme directed targeting, bioreductive agents also have a second potential tumor targeting mechanism, the specific microenvironmental conditions within the interior of the tumor mass. As a tumor grows from the original cell to a tumor mass, the tumor cells are forced away from existing capillaries. It has been estimated that under most circumstances oxygen cannot diffuse more than approximately 150μm through respiring tissue before it is consumed (Brown and Giaccia, 1998). This would imply a maximum tumor size of about 300μm in diameter without the development of a tumor vasculature system, a concept introduced in the early 1970s (Folkman, 1971). The cells of the growing tumor secrete angiogenesis factors that stimulate the growth of new blood vessels (Folkman and Klagsburn, 1987). However, the newly developed vasculature system is not as orderly and efficient as the vasculature within healthy tissues of the body (Vaupel *et al*, 1989, Jain, 1988). As a result the blood flow within the tumor is irregular, sluggish and leaky as shown in Fig. 2. This results in areas of the tumor that are hypoxic or even necrotic. The presence of areas of hypoxia within a tumor appears to correlate

with more aggressive forms of disease with increased incidence of metastasis (Graeber *et al*, 1996, Brizel *et al*, 1996).

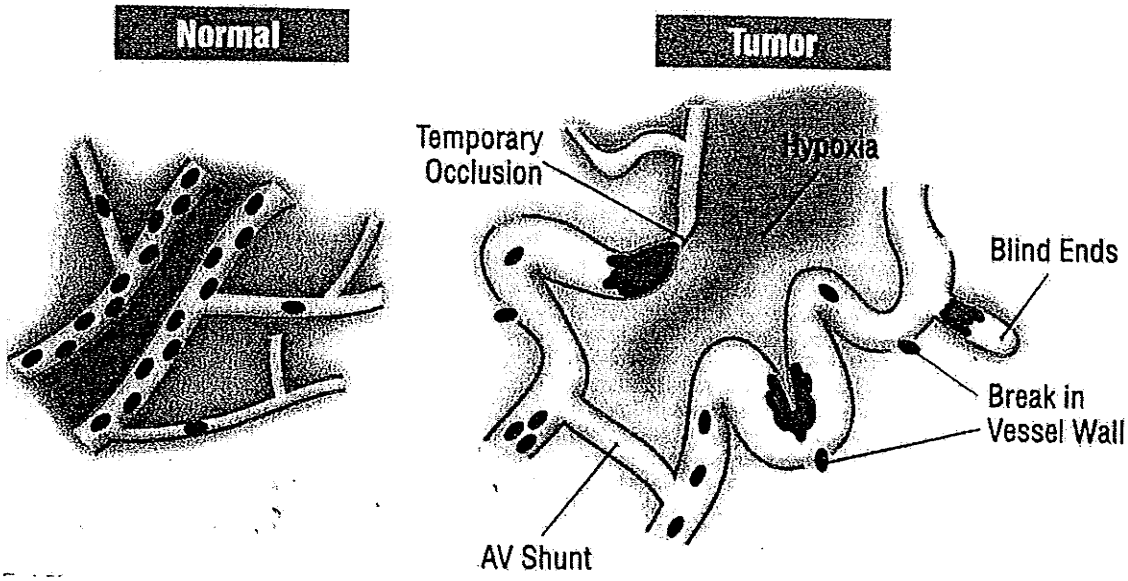

Figure 2 Diagrammatic comparison of normal vs. tumor tissue vasculature (Brown, 1998; with permission)

By their nature, bioreductive drugs should be most effective against cells within the hypoxic regions of tumors (Lin *et al*, 1972; Workman and Stratford, 1993). The low oxygen tension within these regions would allow the reductive activation of the drugs to occur more readily, and would decrease the rate of back oxidation to the parent compound. Many bioreductive drugs have been studied in terms of their hypoxia selective ratio, the ratio of cytotoxicity in hypoxic vs. aerobic cells. While hypoxic areas of the tumor are targeted by bioreductive agents, they are generally resistant to conventional therapy. When conventional chemotherapy and radiotherapy treatments are employed, they often destroy the regions of the tumor with good supplies of oxygen without harming the cells from the hypoxic region (Coleman *et al*, 1988). These hypoxic cells remain viable (Moulder *et al* 1984, Moulder and Rockwell, 1987), and this is likely

a leading cause of failure for many treatments. Theoretically then, bioreductive agents could play a very crucial role in combination with conventional chemotherapy agents by attacking these hypoxic cells and improving the response to the treatment (Melton *et al*, 1998; Naylor *et al*, 1997, Workman and Stratford, 1993).

All bioreductive agents have two functional portions, a bioreductive portion of the drug and a cytotoxic portion that does not become active until the bioreductive portion has been reduced. The bioreductive portion may include such moieties as quinones (MMC), nitrogen oxides (tirapazamine), and nitro groups (RSU 1069) (Workman and Stratford, 1993). The cytotoxic group is generally an alkylating group although for some drugs, such as tirapazamine, the bioreductive group acts as the cytotoxic group (Patterson *et al*, 1994). For tirapazamine, a one-electron reduction yields a radical that causes double-strand DNA breaks, or that may inhibit the essential enzyme topoisomerase 2 (Peters and Brown, 2002), the major mechanisms of cytotoxicity for this drug under hypoxic conditions (Brown, 1993).

The basic goal of bioreductive drug development is to design compounds which are activated solely by one of the enzymes that are commonly over-expressed in tumors as this would make the drug more selective and theoretically result in a wide therapeutic window. This is not an easy task to accomplish as the reductase enzymes have overlapping substrate profiles. An additional goal is to find ways to further expand the therapeutic window by selectively increasing the level of enzyme in the tumor, without increasing expression levels in the rest of the body.

Bioreductive Enzymes

The two most actively studied enzymes in terms of bioreductive drug activation are P450R and DT-diaphorase. These two enzymes are both capable of reducing many of the same potential bioreductive drugs; however, the mechanisms that they use to accomplish the reductions are distinct. The quinone based bioreductive drugs require two electrons to convert them to the active hydroquinone form. P450R accomplishes this reduction in two steps; first a single electron transfer to form a semiquinone intermediate and then a second single electron transfer to form the active hydroquinone form. DT-diaphorase accomplishes this same task with one step, a two-electron transfer that eliminates the semiquinone intermediate. Both the semiquinone and hydroquinone are capable of donating single electrons to molecular oxygen resulting in the formation of a superoxide radical anion; however, the hydroquinone is generally more stable than the semiquinone (Gutierrez, 2000). By eliminating the semiquinone intermediate, the production of superoxide and the reactive oxygen species (ROS) that may be generated is minimized (see Fig. 3).

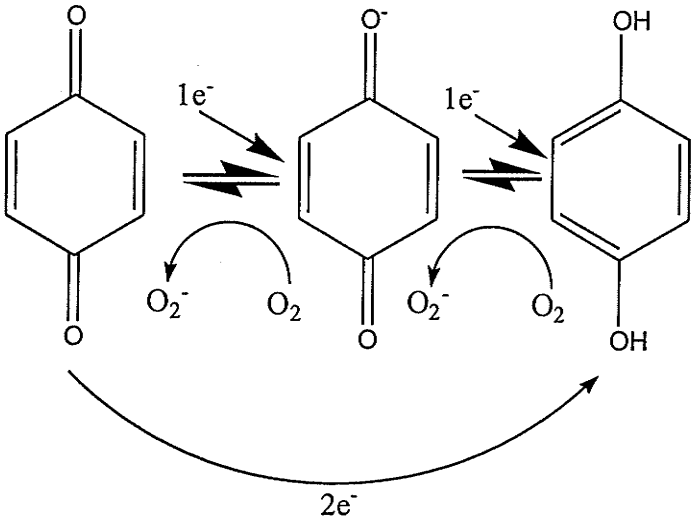

Figure 3 Mechanisms of reduction of quinone group.

Cytochrome P450 Reductase

P450R, a phase I enzyme, is a 68 KDa protein found in the microsomal fraction of cell homogenates (Williams and Kamin, 1962; Phillips and Langdon, 1962). The primary function of P450R in tissues is to provide electrons to the cytochrome P450 enzymes. These enzymes, in turn, use the electrons for the metabolism of diverse substrates including steroids (Masters *et al*, 1973), fatty acids (Wada *et al*, 1968), carcinogens (Prough and Burke, 1975) and drugs (Strobel *et al*, 1980; Goepter *et al*, 1995). Although originally discovered in the liver (Horecker, 1950) P450R is now known to be present throughout the body (Masters and Okita, 1980). P450R is known to exist throughout the plant, animal, and bacterial worlds (Nebert *et al* 1991). P450R likely evolved at the same time as the rest of the cytochrome P450 superfamily of enzymes, about 1 billion years ago (Goeptar *et al*, 1995).

The electrons required for reduction by P450R are obtained from NADPH in pairs and are then transferred one at a time to the cytochrome P450 enzymes (Backes and Reker-Backes, 1988). The activity of P450R is dependent on the presence of FAD and FMN cofactors in equal concentrations. Studies have shown that FAD accepts the electrons from NADPH. This is followed by an intramolecular transfer of the electrons to FMN which then acts as the electron donating portion transferring the electrons to the final electron acceptor (Vermillion and Koon, 1978). The complete mechanism of P450R electron transfer is very complex involving, 1, 2, 3 and 4 electron reduced forms of the enzyme with electron transfers occurring between the various reduced forms of the enzyme (Backes, 1993). Only the 2-4 electron reduced forms of the enzyme have redox potentials great enough to act as electron donors (Iyanagi *et* al, 1974). A detailed

description of this mechanism is beyond the scope of this paper but can be found in Backes (1993).

DT-diaphorase

DT-diaphorase was first identified by Ernster and Navazio in 1958 (Ernster *et al*, 1958). It is classified as a phase II detoxification enzyme with this being a source of some debate. By the classical definition phase I enzymes catalyze oxidative, hydrolytic and reductive reactions, while phase II enzymes catalyze conjugation reactions (Brenner, 2000). DT- diaphorase is a reductive enzyme by nature and as such should be classified as a phase I enzyme based upon the classical definition. The explanation for the classification of DT-diaphorase as a phase II enzyme is based on the similarity in regulation and function (to detoxify compounds) to other phase II detoxifying enzymes (Perason *et al*, 1988; Prestera *et al*, 1993).

DT-diaphorase is a flavoprotein involved in the reduction of a wide variety of compounds including quinones, quinone imines, quinone epoxides, azo dyes and nitrogen oxides (Riley *et al*, 1992). Electrons for the reduction of these compounds are obtained from the oxidation of NADH or NADPH (Ernster, 1967). The mechanism of reduction of quinone containing compounds is carried out by a ping-pong kinetic mechanism (Hosoda *et al*, 1974). The first step in the process is binding of the electron donor (NADH or NADPH) to the enzyme and transfer of the two electrons to FAD. After the electron donor leaves the quinone containing substrate binds to a separate catalytic site and electrons are transferred from the FAD to the quinone (Ross *et al*, 1994). The molecular characteristics of anti-tumor agents that determine binding efficiency in the

active site are not yet fully known, however binding appears to be dependent on the size of the substrate (Chen *et al*, 1999; Fourie et al, 2002).

The natural role of DT- diaphorase is to detoxify quinones and chemicals via a pathway that avoids the production of harmful free radicals and ROS (Lind *et al*, 1982, Ross, 1997) and the depletion of intracellular reserves of glutathione (Dinkova-Kostova and Talalay, 2000). Once DT-diaphorase has reduced a quinone to the hydroquinone form, the xenobiotic can undergo conjugation reactions making elimination possible. The major mechanisms involved in this process are conjugation with glutathione (GSH) by glutathione transferase (GST) (Talalay *et al*, 1987) and with glucuronic acid catalyzed by UDP-glucuronosyl transferases (Kasper and Henton, 1980). The importance of this process is illustrated by studies that have shown mice with no DT-diaphorase activity are more susceptible to quinone toxicity (Radjendirane *et al*, 1998) and that humans with a mutant form of DT-diaphorase are more susceptible to the toxic and neoplastic effects of benzene (Smith, 1999).

Although for the most part, DT-diaphorase detoxifies carcinogens, the enzyme activates some xenobiotics (Smith *et al*, 1987). Some of these xenobiotics become active carcinogens following activation and others become toxic molecules. It is these compounds, which become toxic, that are of interest as they may be employed as bioreductive anti-tumor agents. It is interesting to note that DT-diaphorase has been implicated in the development of resistance of tumors to some non-bioreductive treatments (Farber, 1987) and even some bioreductive treatments such as tirapazamine (Patterson *et al*, 1994), menadione (Atallah *et al*, 1988), and benzoquinone mustards (Begleiter and Leith, 1990).

DT-diaphorase is found in many tissues of the body but the highest levels exist in the liver, kidney, and gastrointestinal tract (Riley *et al*, 1992). More importantly, DT-diaphorase is found in very low levels within the bone marrow (Siegel *et al*, 1991), a site of major toxicity for most chemotherapy agents. Drugs that require activation by DT-diaphorase should theoretically then have very little bone marrow toxicity. In addition it has been demonstrated that DT-diaphorase is often over-expressed in tumors when compared to the normal tissue from which they arose making it an excellent candidate for enzyme-directed bioreductive drug therapy (Cresteil *et al* 1991; Fitzsimmons *et al*, 1996). The levels of DT-diaphorase activity are highly variable among tumor cell lines and appear to be higher in malignant cells *in vitro* than *in vivo* (Fitzsimmons *et al*, 1996). DT-diaphorase activity levels have been shown to increase with malignant progression of colon cancer (Mikami *et al*, 1998). Studies also found that DT-diaphorase expression may be related to the expression of the pro-apoptotic protein BAD, indicating a further justification for the use of DT-diaphorase as a target for enzyme-directed therapy (Tudor *et al*, 2003; Kitada *et al* , 1998). No explanation is currently available for why DT-diaphorase expression is so variable in tumors and why it tends to be overexpressed in tumors when compared to the normal tissues from which they arose.

One limiting factor for the use of DT-diaphorase as a targeting mechanism for therapy is the existence of a mutant form of the protein in some individuals. The frequency of this mutation in *Homo sapiens* ranges from 4-22% of the population depending on the ethnic group studied (Kelsey *et al*, 1997). The mutation is a point mutation, C to T, at base 609 of the gene and results in the expression of a protein that is rapidly ubiquinated and degraded (Siegel *et al*, 2001). The affected individuals have

essentially no active DT-diaphorase and as a result neither will their tumors making them poor candidates for a DT-diaphorase directed therapy strategy.

85-95% of all DT diaphorase activity is found within the cytoplasm of the cell (Danielson *et al*, 1960, Winski *et al*, 2002) with subcellular organelle and nuclear fractions believed to contain the remaining activity (Riley and Workman, 1992). In the past many studies have demonstrated significant levels of DT-diaphorase activity in the mitochondria with smaller amounts in the Golgi apparatus and microsomes (Edlund *et al*, 1982). A more recent study using an immunoelectron microscopy technique has shown that the observation of DT-diaphorase in these sub-cellular organelles may be an artifact of the methods used. This study concluded that DT-diaphorase that is not cytoplasmic is nuclear in origin (Winski *et al*, 2002). It is this nuclear DT-diaphorase that is of most interest in terms of enzyme directed therapy as the drugs that have been examined to date are DNA damaging agents. The reduction to their active forms within the nucleus is probably important if not necessary for their cytotoxicity (Winski *et al*, 2002).

DT-diaphorase Induction

The regulation of expression of DT-diaphorase occurs primarily at the transcriptional level. The *NQO1* (NAD(P)H quinone oxidoreductase I) gene that encodes DT-diaphorase is found on chromosome 16q22.2. It is 20 kb in length with 6 exons and five introns (Jaiswal, 1991). Within the promoter region of the gene is a xenobiotic response element (XRE) (Favreau *et al*, 1991), an antioxidant response element (ARE) (Rushmore *et al*, 1991), and activator protein 2 (AP2) elements (Jaiswal, 1994) which are believed to control the transcriptional regulation of the gene. The ARE is important for

both base and inducible DT-diaphorase expression while the role of the XRE is not certain but appears to be mainly involved in inducible expression (Belinsky and Jaiswal, 1993).

The induction pathway via the ARE is not clearly understood although it is believed to act through transcription factors such as Jun, Nrf, Maf, Fas, and Fra (Venugopal and Jaiswal, 1996; Kepa and Ross, 1999; Nguyen *et al*, 2000). One of the better understood mechanisms involves Nrf2. Normally Nrf2 is found in the cytoplasm bound to Kcap1. When the cells are exposed to oxidative stress an unknown signal results in the release of Nrf2 from Kcap1. The free Nrf2 is then translocated to the nucleus where it forms a heterodimer with c-Jun. This heterodimer may then bind to the ARE resulting in increased expression of NQO1 and other phase II enzymes (Venugopal and Jaiswal, 1996).

Little is known about the mechanism of induction involving the XRE in terms of NQO1. It appears to involve the binding of a ligand to the aromatic hydrocarbon receptor (AHR). This receptor/ligand complex is then able to dimerize with members of the PAS (Per, Arnt, Sim) family of proteins, and subsequently interact with the XRE resulting in increased expression of the NQO1 gene (Landers and Bunce, 1991; Ross *et al*, 2000).

There is little evidence that DT-diaphorase activity is regulated by post-translational mechanisms, although the protein can be ubiquinated and degraded by proteosomes (Siegel *et al*, 2001).

The known inducers of DT-diaphorase have been categorized into two main groups, monofunctional and bifunctional inducers (Prochaska and Talalay, 1988). Monofunctional inducers are those which induce only the phase II enzymes and are

generally highly electrophilic in nature (Prestera *et al*, 1993). The bifunctional inducers induce both phase I and phase II enzymes; they are generally aromatic hydrocarbons and exert their effect by binding to the aromatic hydrocarbon receptor and then interacting with the XRE (Prestera *et al*, 1993). Phase II enzyme inducers consist of a large number of very diverse compounds many of which are found in the human diet including 1,2-dithiole-3-thiones, quinones, and isothiocyanates (Prestera *et al*,1993). The natural role for this mechanism in cells is to prepare the cell to protect itself from the toxic and carcinogenic nature of most of the compounds in this group (Prestera and Talalay, 1995).

The importance of the induction of phase II enzymes in cancer prevention is illustrated by a number of controlled studies, with the bulk of the interest centered on colorectal cancer (O'Dwyer *et al*, 1996). Other epidemiological studies have shown that individuals who consume large quantities of fruits and vegetables, many of which contain known phase II enzyme inducers, have lower cancer rates (Steinmetz and Potter, 1996). The genus *Brassica* of the plant family *Cruciferae*, which includes vegetables such as broccoli, cauliflower, and brussel sprouts have been shown to reduce the risk of epithelial cell based cancers in a number of different organs (Beecher, 1994; Verhoeven *et al*, 1997). The compounds found within these vegetables that are believed to be responsible for these observations are the isothiocyanates, specifically sulforaphane (Zhang *et al*, 1994; Zhang *et al*, 1992). Studies have shown that these compounds are capable of increasing the expression of a number of phase II enzymes, including DT-diaphorase, *in vivo* and that this induction is responsible for the chemopreventative effects (Fahey *et al*, 1997).

Aside from cancer prevention, the inducible nature of DT-diaphorase makes it very interesting in terms of enzyme directed bioreductive therapy. The magnitude of induction seen in malignant cells is often greater than that seen in the non-malignant tissues of the body, especially those tissues which are the site of major toxicity of many bioreductive drugs (Begleiter *et al*, 1997). A recent study demonstrated that a known inducer, dimethyl fumarate (DMF) had no significant effect on the levels of DT-diaphorase in the bone marrow, lung, or heart tissues of mice (Begleiter *et al*, 2004). As the dose limiting toxicity for a number of bioreductive agents, including the prototype MMC is myelosuppression (Doll *et al*, 1985), the lack of induction in the bone marrow could have significant clinical implications. The tissues that did show significant induction of DT-diaphorase were the kidney, liver, and forestomach (Begleiter *et al*, 2004). The induction of DT-diaphorase in tumors, but not in tissues prone to the toxic effects of bioreductive drugs, may represent a useful strategy in the treatment of cancer (Doherty *et al*, 1998).

DT-diaphorase is not inducible in all malignant cells, with one study showing a non-inducible phenotype in approximately 25% of human tumor cell lines treated with the known inducer 1,2-dithiole-3-thione (Doherty *et al*, 1998). Subsequent experiments suggest that the lack of induction in this group may be due to the presence of the base 609 mutation in the DT-diaphorase gene or defects at the transcriptional or pre-transcriptional level (Begleiter *et al*, 2001). The exact cause of these latter non-inducible phenotypes has not be elucidated; however, mutations in the promoter region of the gene have been ruled out (Begleiter and Lange, 2002).

Quinone Alkylating Agents

A subgroup of bioreductive agents is the quinone alkylating agents. These compounds all contain quinone groups as the bioreductive element and alkylating groups that act as the cytotoxic element. For the drug to act effectively as a bioreductive drug, the cytotoxicity of the alkylating agent must depend on the oxidation-state of the compound (Workman and Stratford, 1993). As a result the compounds of interest require that the quinone be in the reduced form before the alkylating group(s) become active. Two well-known examples of this are MMC and EO9 (Workman and Stratford, 1993). Once the compound has been reduced the active alkylating group can form covalent bonds with cellular macromolecules including protein and DNA. The interactions with DNA are likely the major cause of toxicity to the cells (Riley and Workman, 1992). Toxicity of the quinone alkylating agents may also be caused by a mechanism involving the quinone group of the compound. In the presence of oxygen, the semiquinone and hydroquinone forms of the drug can be reoxidized leading to a cycle of reduction and oxidation (redox cycling, see Fig. 4). With each oxidation, superoxide anions are produced, which, in the presence of hydrogen ions and metal ions go on to produce reactive oxygen species (ROS), including peroxide and hydroxyl radicals that can damage cellular macromolecules (Bachur *et al*, 1978, Begleiter, 1985). The hydroxyl radical is the most harmful of these to the cells (Öllinger and Kågedal, 2002). The degree of redox cycling which occurs is dependent on a number of factors including the enzymes present, oxygenation level, pH, and the particular drug (Gutierrez, 2000). The cytotoxic effect of these ROS is generally minimal in comparison to the alkylation. This is believed to be due to the cells ability to defend against ROS using enzymes such as

superoxide dismutase, catalase and glutathione peroxidase (Beall and Winski, 2000). Only when these cellular defense mechanisms have been overwhelmed, leading to oxidative stress, do they contribute to the observed cytotoxicity (Halliwell and Gutteridge, 1999).

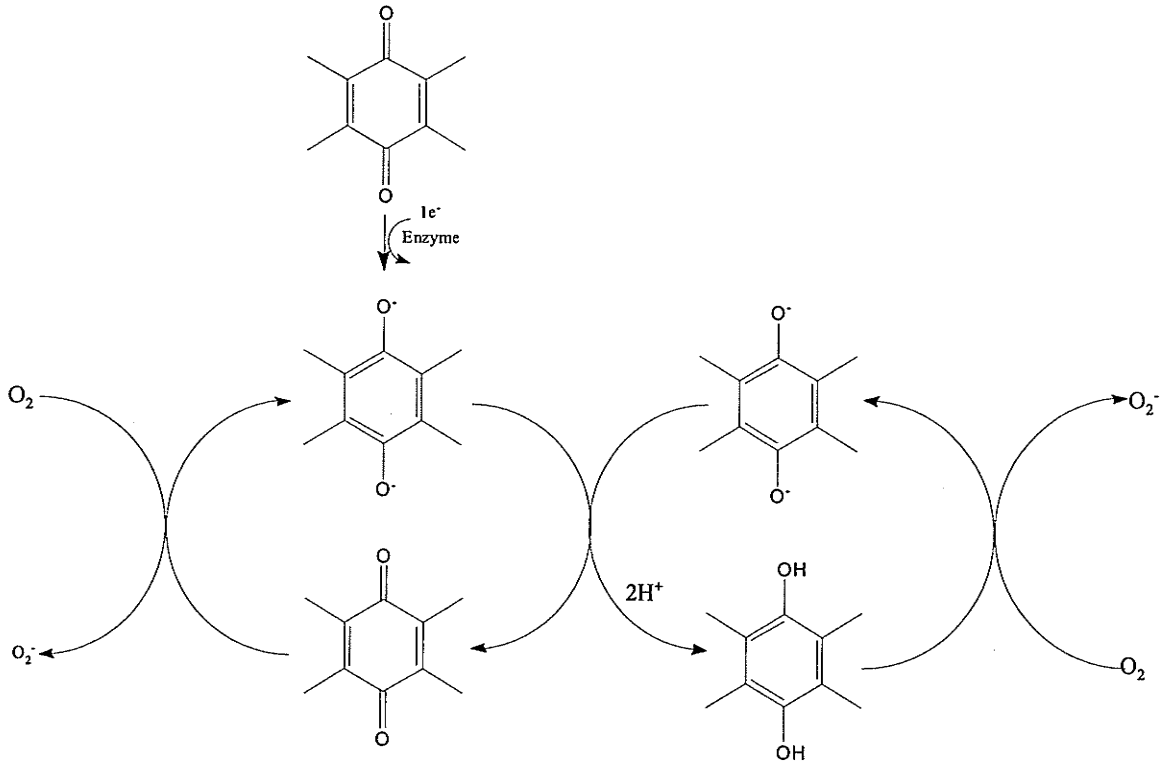

Figure 4 Redox cycling that occurs following a single electron reduction of the quinone group to the semiquinone. The hydroquinone can be oxidized back to the semiquinone, and the semiquinone to the quinone with each of these oxidations resulting in the production of a superoxide anion. (Gutierrez, 2000)

Mechanisms of cytotoxicty of quinone alkylating agents

As mentioned, the quinone alkylating agents can exert their toxic effects through two distinct mechanisms, production of ROS and DNA alkylation. Most bioreductive alkylating agents are able to invoke both of these mechanisms under the right circumstances. For MMC the single electron reduction (eg. by P450R) of this drug will result in the production of ROS while the two-electron reduction (eg. by DT-diaphorase)

will result in the activation of the alkylating group (Riley and Workman, 1992). The relative contribution of each of these mechanisms to the observed cytotoxicity of the agent is dependent on the drug, pH, oxygen level, and enzyme profile of the affected cells.

The ROS produced by redox cycling of the quinone group attacks both the sugar and the base portions of DNA. These attacks lead to DNA fragmentation, base loss and strand breaks (Imlay and Linn, 1988) leading to cell death through inhibition of replication and transcription. In addition oxidative stress is known to induce apoptosis in cells (Simon *et al*, 2000). The induction of apoptosis by ROS species is extremely complex with a number of activation pathways involved. One study using menadione as the redox cycling agent showed that at low doses the major mechanism was Fas/FasL induced apoptosis (Laux and Nel, 2001). This is confirmed by the observation that ROS are involved in the induction of both the Fas receptor (Delneste *et al*, 1996) and Fas ligand (Bauer *et al*, 1998) genes in T cells. At higher doses of menadione Laux and Nel (2001) noted that the induction of apoptosis appeared to be Fas independent. They proposed that the induction of apoptosis under these circumstances is due to the release of cytochrome c from the mitochondria in response to decreased ATP production. The loss of ATP production is due to disruption of the integrity of the inner mitochondrial membrane that occurs because of oxidation of the mitochondrial pores by the ROS (Zamzami *et al*, 1995).

The alkylation of DNA is believed to be the most significant contributor to toxicity for a number of different bioreductive anti-tumor agents including MMC (Iyer and Szybalski, 1963). The simple alkylation of DNA by alkylating agents results in a

number of types of DNA lesions including base loss, base-pair mismatches, and single strand breaks. DNA damage is known to stimulate a p53 dependent apoptotic response (Williams and Smith, 1993); however, DNA alkylating agents can also induce apoptosis in cells with mutant p53. This indicates the involvement of a p53-independent pathway that appears to be related to the mismatch repair process. The mechanism involved in this process is not yet clearly understood (Hickman and Samson, 1999). Of greater significance in terms of observed cytotoxicity is the formation of cross-links when a single drug molecule alkylates bases on opposite strands of the helix, preventing the strands from separating for replication and transcription. (Hargreaves *et al*, 2000). Although interstrand cross-links represent only about 27% of the total DNA alkylation observed in cells treated with MMC (Palom *et al*, 2002) they are believed to be the primary cause of cell death (Iyer and Szybalski, 1963). Although not clearly understood yet, it appears that the net result of interstrand cross-links is the induction of necrotic cell death rather than apoptosis (Hickman and Samson, 1999).

Mitomycin C

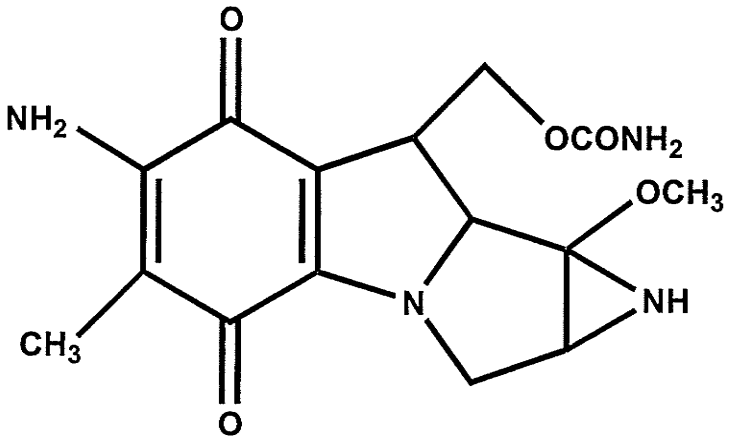

Figure 5 Molecular structure of the prototype bioreductive agent Mitomycin C (MMC)

MMC shown in Fig. 5 is a naturally occurring antibiotic produced by *Streptomyces caespitosus* that was first identified in the late 1950's (Wakaki *et al*, 1958) and has been used in the clinic for the treatment of solid tumors since that time. Despite a great deal of research on other potential bioreductive agents, it remains the only bioreductive agent currently approved for general use (Beall and Winski, 2000). As such it is viewed as the prototypical bioreductive anti-tumor agent to which all potential new agents are compared (Sartorelli *et al*, 1994).

The use of MMC in the clinic is quite controversial due to the extensive toxicity associated with the drug. In 1974, the US Food and Drug Administration (FDA) approved MMC for the treatment of a number of malignancies including head and neck, lung, breast, cervix, colon, stomach, pancreas, liver and skin. In 2001, following discussions between the FDA and the manufacturer the product was relabelled for the treatment of only stomach and pancreatic cancers due to concerns of associated toxicities and the emergence of many more effective treatments (Bradner, 2001). Despite these

changes the use of MMC for the treatment of many other cancers has continued (Bradner, 2001). Toxicity concerns aside, MMC is viewed as one of the best single agent therapies for the treatment of non-small-cell lung carcinoma (NSCLC) (Spain, 1993).

MMC shows a great deal of promise when combined with radiation therapy for the treatment of a number of solid tumor types (Boyer, 1997). The contribution of MMC to this treatment combination is two-fold. First MMC may act as a radiosensitizer making the cells more susceptible to radiation damage (Rockwell *et al*, 1988). Secondly, MMC targets the hypoxic cells of the tumor that are largely unaffected by ionizing radiation (Adams *et al*, 1992). The cytotoxic effects of radiation are dependent upon the formation of reactive oxygen species that requires the presence of oxygen at the time of irradiation (Gray *et al*, 1953).

The cytotoxicity of MMC is due to two separate mechanisms, DNA cross-linking (Ross *et al*, 1997), and DNA strand breaks (Pritsos and Sartorelli, 1986) caused by the production of ROS. Of these two mechanisms, the DNA cross-linking is probably the largest contributor to MMC toxicity (Rockwell *et al*, 1993; Tomasz *et al*, 1987). Studies have shown that the formation of DNA cross-links correlates well with the observed anti-tumor activity of MMC (Dorr *et al*, 1985; Kennedy *et al*, 1985).

For the alkylating group of MMC to become active, the quinone must be reduced fully to the hydroquinone form (Iyer and Szybalski, 1964). This reduction can occur enzymatically or non-enzymatically, however the non-enzymatically reduced MMC is not capable of forming DNA cross-links and thus is not likely responsible for a significant amount of the observed cytotoxicity of MMC (Joseph *et al*, 1996). The enzymatic reduction of MMC can be carried out by at least 5 known enzymes including

NADH:b5 reductase, P450R, xanthine dehydrogenase, xanthine oxidase, and DT-diaphorase (reviewed in Workman, 1994). Under aerobic conditions DT-diaphorase is believed to be a significant contributor to reductive activation while under hypoxic conditions P450R and other single electron reductases play a much greater role in the enhanced cytotoxicity (1.5 – 3.0 fold, Rauth *et al*, 1983) of the drug (Sartorelli *et al*, 1994; Ross *et al*, 1996). The ability to be reduced by such a wide variety of enzymes makes MMC somewhat difficult to study as a bioreductive agent as it is difficult to predict the outcome of the treatment based on the expression levels of any one enzyme. This is demonstrated by the inconsistency of different studies attempting to correlate cytotoxicity to DT-diaphorase activity in human tumor cell lines. One study showed no significant correlation (Robertson *et al*, 1992) in a large panel of cell lines while another study using the National Cancer Institute Tumor Cell Line Panel showed a significant correlation (Fitzsimmons *et al*, 1996). This could very easily be explained by the relative contributions of each of the other activating enzymes found within the cell along with the competing cytotoxicity mechanisms (alkylation, oxidative stress) (Cummings *et al*, 1995). It has also been proposed that these results may be better explained by attempting to correlate MMC activity to nuclear DT-diaphorase levels rather than overall levels (Winski *et al*, 2002).

When fully reduced to the hydroquinone form, MMC has the ability to form two covalent bonds with DNA and as a result can both alkylate and cross-link DNA resulting in cell death (Tomasz *et al* 1993). MMC alkylation of DNA occurs through the aziridine and the carbamate groups of the drug. These alkyl bonds can form with many different

sites on the DNA but the preferred site for MMC alkylation is the N-2 position of guanine residues (Tomasz *et al*, 1987).

As mentioned earlier, one of the major drawbacks to MMC treatment is the toxicity of the drug. The dose-limiting toxicity is myelosuppression that is reversible. Other associated toxicities include anorexia, nausea, vomiting and diarrhea (Doll *et al*, 1985). These toxicities are all dose-related; however, there are other very severe and potentially fatal toxicities that occur rarely and do not appear to be dose-related. These include pulmonary toxicity in approximately 5% of patients (Castro *et al*, 1996) and hemolytic-uremic syndrome in 4-15% of patients (Doll *et al*, 1985, Lesesne *et al*, 1989).

EO9

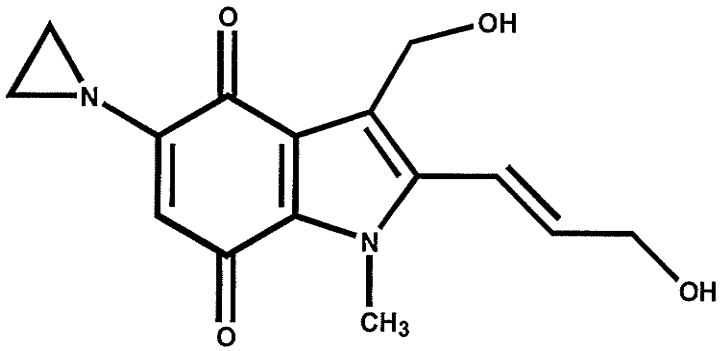

Figure 6 Molecular structure of the indoloquinone, EO9

3-Hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol (EO9) shown in Fig. 6 is an indoloquinone that was synthesized as part of a large study

attempting to create more efficacious analogues of MMC (Speckamp and Oostveen, 1992). In early studies this drug appeared to be very promising as it showed minimal bone marrow toxicity, activity against a number of tumor types in preclinical work (Hendriks *et al*, 1993) and was a good substrate for DT-diaphorase (Walton *et al*, 1991). Phase I trials showed partial responses to the treatment, leading to phase II trials (Schellens *et al*, 1994). The results of the phase II clinical trials were disappointing as no anti-tumor activity was observed against breast, colon, pancreatic, and gastric cancer (Dirix *et al*, 1996) or non-small cell lung cancer (Pavlidis *et al*, 1996). A possible explanation for the failure of these trials was the rapid clearance of the drug from patients ($t_{1/2}$ of 10 minutes) (Verweij *et al*, 1994). It has been shown that the rate of clearance of a drug from circulation will affect the ability of the drug to penetrate a solid tumor mass, and as a result, influence the anti-tumor activity of the drug (Phillips *et al*, 1998). A second possible explanation for the failure of EO9 in these trials is that the trials were not designed to measure appropriate end-points for a bioreductive agent. EO9 is hypoxia selective, and would therefore have little effect on the aerobic cells, and thus the size of the tumor (Connors, 1996). The dose-limiting toxicity of EO9 was determined to be kidney toxicity manifested in the form of proteinuria that was reversible (Pavlidis *et al*, 1996).

Like MMC, EO9 is capable of both DNA cross-linking and DNA strand-breaks, with the strand breaks appearing to be the major mechanism of DNA damage in contrast to MMC (Bailey *et al*, 1997). The most interesting difference between MMC and EO9 is the observation that EO9 is more selective for DT-diaphorase based activation, probably because it is a better substrate for the enzyme (Plumb *et al*, 1994). Under aerobic

conditions DT-diaphorase is responsible for nearly all of the activation; however, under hypoxic conditions other enzymes begin to take on a greater role (Bailey *et al*, 1997). Overall, the correlation of EO9 activity and DT-diaphorase activity is stronger than with MMC, particularly under aerobic conditions (Plumb *et al*, 1994).

Despite the reduced selectivity of EO9 for DT-diaphorase activation under hypoxic conditions, the contribution of the other reductases makes it selective for hypoxic cells (Hendriks *et al*, 1993). As a result, EO9 may still play an important role in the clinic in combination with aerobic selective treatments such as radiation (Connors, 1996).

Diaziridinylbenzoquinones

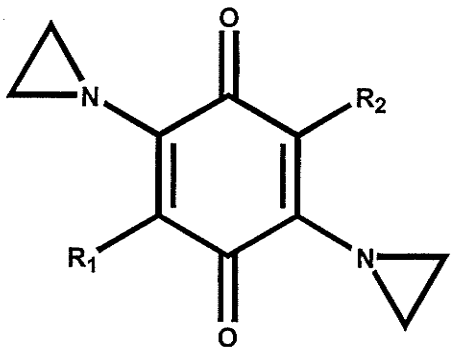

Figure 7 Base molecular structure of diaziridinylbenzoquinones. R_1 and R_2 represent different chemical groups depending on the particular drug.

A large number of diaziridinylbenzoquinones have been examined as potential anti-tumor agents, with some having been tested in clinical trials. One of the best studied of these is the drug AZQ (diaziquone, R_1 and $R_2 = NHCO_2CH_2CH_3$). AZQ was

synthesized in an attempt to design drugs for use against tumors of the central nervous system (Khan and Driscoll, 1976). Clinical trials for AZQ for treatment of central nervous system tumors were disapointing with response rates of only 15-20%; however, some complete remissions were observed (Bender et al, 1983). AZQ is not used in the clinic for the treatment of these tumors as other agents have been shown to be more effective (Malkin et al, 1994). Two other aziridinylbenzoquinones, triaziquone (R_1 = H, R_2 = aziridinyl) and carbazilquinone ($R_1 = CH_3$, $R_2 = CH(OCH_3)CH_2OCONH_2$) have also been studied in the clinic for the treatment of central nervous system tumors but have failed to become accepted treatments (Chou *et al*, 1976). The major toxicity observed for all three of these compounds was found to be myelosuppression (Hargreaves *et al*, 2000)

Following the discovery that DT-diaphorase is overexpressed in many tumor types (Phillips, 1996) and that AZQ was reduced by the enzyme, attempts were made to create drugs that were good substrates for DT-diaphorase (Gibson *et al*, 1992). One of the most promising of these compounds was MeDZQ (R_1 and $R_2 = CH_3$), which proved to be a very efficient substrate for DT-diaphorase. MeDZQ was found to be much more potent than AZQ (Gibson *et al* 1992) and required reduction by DT-diaphorase to the hydroquinone form for DNA cross-linking (Lee *et al*, 1992; Hargreaves *et al*, 1999). Although these observations made MeDZQ appear to be an excellent candidate for cancer therapy, poor solubility in aqueous solutions (<0.05 mg/ml at 25°C) was a significant drawback (Khan *et al*,1999).

RH1

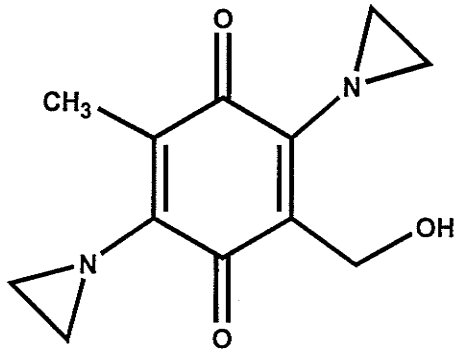

Figure 8 Molecular structure of the novel bioreductive agent RH1

2,5-Diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1), shown in Fig. 8, is a relatively new bioreductive anti-tumor agent. It was designed based upon the structure of MeDZQ, which was known to be an excellent substrate for DT-diaphorase. The addition of the hydroxyl group to the 3-methyl of MeDZQ had some surprising affects aside from increased solubility in aqueous solutions (>0.5 mg/ml at 25°C) (Khan *et al*, 1999). RH1 was found to be a better substrate for DT-diaphorase than MeDZQ; in fact, of all the bioreductive agents studied, it is one of the best substrates for DT-diaphorase (Beall *et al*, 1994). There is no clear explanation why RH1 is such an efficient substrate for this enzyme. The importance of DT-diaphorase activation of RH1 is illustrated by the observation that RH1 demonstrates greater anti-tumor activity against human tumor cells with elevated levels of the enzyme (Winski *et al*, 1998, Sharp *et al*, 2000). The addition of the hydroxy group also made RH1 a more potent drug than its predecessor. RH1 has demonstrated significant anti-tumor activity against malignant cell lines *in vitro* (Loadman *et al*, 2000) and *in vivo* (Cummings *et al*, 2003).

A two-electron reduction of the benzoquinone to the hydroquinone form of RH1 is required for RH1 to be cytotoxic. When reduced to the hydroquinone form, the two aziridine groups may become protonated allowing them to open up and alkylate DNA (Fig. 9), as a result increased DNA alkylation activity is observed at acidic pH (Hartley *et al*, 1991; Lee *et al*, 1992).

The preferred site of DNA alkylation by the diaziridinylbenzoquinones is the N7 position of guanine residues. Upon reduction, the hydroxyl groups on the hydroquinone are capable of forming hydrogen bonds with the cytosine on the opposite strand, placing the aziridine groups in close proximity to N7 of the guanine residue (Berardini *et al*, 1993). These alkylations can result in DNA cross-links, preferably where the nucleotide sequence is 5' GNC 3' with 5' GCC 3' being the ideal sequence for RH1 (Fig. 9), as this places the two guanine residues the ideal distant apart (Khan *et al*, 1999, Berardini *et al*, 1993). Cross-linking by the diaziridinylbenzoquinones can occur to a lesser degree at 5' GC 3' and 5' GNNC 3' sequences (Berardini *et al*, 1993).

The hydroquinone form of RH1 is relatively stable in comparison to MMC and undergoes little redox cycling with a reoxidation half-life of 40 minutes (Nemeikaite-Ceniene *et al*, 2003). As a result RH1 cytotoxicity is almost exclusively due to alkylation as opposed to oxidative stress caused by redox cycling (Nemeikaite-Ceniene *et al*, 2003). This infers that a requirement for low oxygen tensions within the tumor is not as important as for many other bioreductive agents (Xing and Skibo, 2000).

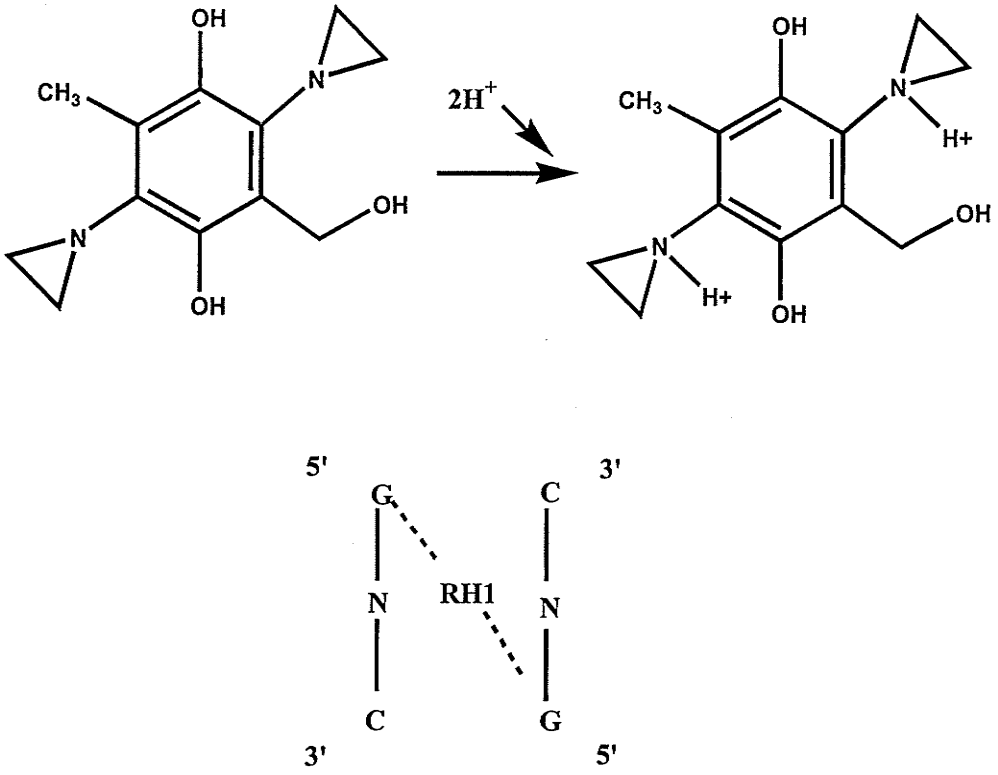

Figure 9 Mechanism of protonation of RH1 following reduction and subsequent cross-linking of guanine residues at 5' GNC 3' DNA sequences.

Unfortunately few studies have been conducted on the efficacy, toxicity, and other pharmacological properties of RH1. No clinical data is available in the literature although the drug is currently involved in clinical studies (Cummings *et al*, 2003). In an effort to determine if RH1 will suffer the same fate as EO9, pharmacological parameters for the two have been determined and compared (Loadman *et al,* 2000). RH1 has a relatively rapid rate of clearance, though not nearly as fast as that of EO9. The elimination of RH1 is biphasic in nature with an initial half-life of 2.9 minutes and a terminal half-life of 23 minutes compared to the 1.8 minute half-life of EO9 (Loadman *et al*, 2000). The metabolism of RH1 is slower in the kidney than EO9, and may help to explain the slower elimination of the drug. It may also prove to be less kidney toxic than EO9, which would be important clinically. The studies also suggest the metabolism of

RH1 by the liver is relatively rapid; however, this metabolism seems to be correlated with the levels of one-electron reducing enzymes rather than those of DT-diaphorase, and this may have implications for liver toxicity (Loadman *et al*, 2000).

A recent study examined the efficacy of RH1 against 3 NSCLC and 1 colon human xenograft (Cummings *et al*, 2003). In this study, RH1 exhibited moderate tumor growth inhibition in the three NSCLC xenografts (tumors sizes were 43-81% of the control tumor volume 16 days after the first treatment). The effect on the colon xenograft was more profound with the tumors on the treated mice being only 38% of the control tumor volume 16 days after the initial treatment. When the antitumor activity observed for each of these cells lines was compared to the levels of DT-diaphorase expression no correlation was seen (Cummings *et al*, 2003).

Combination therapy: DT-diaphorase inducers and bioreductive agents

The inducible nature of DT-diaphorase, along with its ability to act as an activating enzyme for a number of bioreductive anti-tumor agents makes it an ideal target for enzyme directed drug therapy. Although the field of bioreductive anti-tumor therapy is well established, the attempt to increase the effectiveness of bioreductive drugs by selectively inducing the enzymes that activate these agents has received very little attention to date. Studies have demonstrated that MMC and EO9 activities are indeed increased when combined with known inducers of DT-diaphorase (Doherty, 1999; Begleiter and Leith, 1995; Begleiter *et al*, 1997). The use of dicoumarol (a specific DT-diaphorase inhibitor) was able to reverse the effect of the inducer, confirming that the increased cytotoxicity of MMC and EO9 was due to DT-diaphorase rather than the other

phase II enzymes that are induced concurrently (Begleiter *et al*, 1997). A recent unpublished study by Begleiter *et al* (2004) has also demonstrated a significant enhancement of the anti-tumor activity of MMC against human colon tumor xenografts in mice following DT-diaphorase induction by DMF. Although the observed enhancement of MMC by induction of DT-diaphorase is significant, this drug is not an ideal candidate for this approach due to the contributions of other reductive enzymes to its activation (Workman, 1994). As RH1 is highly selective for activation by DT-diaphorase, this drug should theoretically be better suited to this approach than MMC. This is the theory that was examined as the basis for this thesis project.

Two similar approaches to increase the activity of a particular reductase enzyme within the tumor environment are currently being examined. ADEPT (Antibody-directed enzyme prodrug therapy) is an approach that targets the activating enzyme to the tumor via an antibody specific for a tumor antigen (Bagshawe, 1993). The main problem seen with this approach is the ability of the very large conjugated molecules to penetrate the tumor tissue (Connors, 1995). Gene-directed enzyme prodrug therapy (GDEPT) attempts to transform cells with a gene that encodes the activating enzyme and is under the control of a tumor-specific promoter (Connors, 1995; Roth and Cristiano, 1997). The main problem facing GDEPT, as with all gene therapy, is finding an ideal vector with the ability to efficiently transform the tumor cells (Roth and Cristiano, 1997).

Specific Objectives of the Research

1. To determine if the induction of DT-diaphorase can enhance the anti-tumor activity of the new bioreductive alkylating agent RH1 *in vitro* and *in vivo*.

   Our lab has previously established that the induction of DT-diaphorase results in an enhancement of the anti-tumor activity of the prototype bioreductive agent MMC *in vitro* and *in vivo*. In this research we used the same approach to determine if the anti-tumor activity of RH1 could be enhanced to a greater degree by induction of DT-diaphorase as RH1 is more selective for activation by DT-diaphorase than MMC. We tested this hypothesis *in vitro* using two known inducers of DT-diaphorase, in two cell lines known to have an inducible form of the *NQO1* gene. To determine if RH1 anti-tumor activity could be enhanced *in vivo* we used a human tumor xenograft nude mouse model. The mice were fed a diet with or without a DT-diaphorase inducer and then were treated with or without RH1. Tumor volumes were measured to determine the anti-tumor effect, and body weight, histological examination of organ, serum biochemistry and blood counts were measured to monitor any toxic effects.

2. To confirm the proposed mechanism of action of RH1 cytotoxicity.

   Several labs have suggested that the major mechanism of RH1 cytotoxicty is through the formation of DNA interstrand cross-links (Khan *et al*, 1999; Berardini *et al*, 1993). In this research we attempted to determine if the formation of these cross-links is dependent on the reductive activation of RH1 by DT-diaphorase using a cell free system.

EFFECT OF DT-DIAPHORASE INDUCTION ON THE ANTITUMOR ACTIVITY OF RH1

Materials

Cell culture and in vitro cytotoxicity

RPMI 1640, RPMI 1640 with HEPES and DMEM-F12 (1:1) media along with Hanks Buffered Salt Solution (HBSS), trypsin and trypan blue were obtained from Invitrogen Life Technologies (Burlington, ON, Canada). Sodium bicarbonate, streptomycin, penicillin and MMC were from Sigma-Aldrich (Oakville, ON, Canada). Fetal Bovine Serum (FBS) was obtained from Cansera (Etobicoke, ON, Canada). All plasticware for tissue culture and the Wright-Giemsa stain used for the clonogenic assays was obtained from VWR (Mississauga, ON, Canada). RH1 was a gift from Dr. David Ross (University of Colorado Health Sciences Center, Denver, CO, USA).

DT-diaphorase induction

Bio-Rad DC kit including γ-globulin protein standard was obtained from Bio-Rad (Mississauga, ON, Canada). DMSO and disposable microcuvettes used for the protein and DT-diaphorase assays were from Fisher Scientific (Nepean, ON, Canada). All reagants for the DT-diaphorase cycling assay; (NADH, FAD, glucose-6-phosphate, glucose-6-phosphate hydrogenase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), menadione, and dicoumarol, along with dimethyl fumarate (DMF) and sucrose were obtained from Sigma-Aldrich (Oakville, ON, Canada). Sulforaphane was obtained from LKT Labs (St. Paul, MN, USA). Absolute ethanol was obtained from Manitoba Liquor Control Commission (Winnipeg, MB, Canada)

In vivo efficacy and toxicity

Female CD-1 nude mice were obtained from Charles River (Montreal, QC, Canada) at 6-8 weeks of age and given 2 weeks to adjust to their surroundings before studies were initiated. All mice were handled and cared for following Canadian Council on Animal Care (Olfert *et al*, 1993) and University of Manitoba Central Animal Care guidelines. Ketalean and rompum were obtained from Central Animal Care, University of Manitoba (Winnipeg, MB). Blood collection capillary tubes were from Sarstedt (St. Leonard, QC, Canada). Specialized autoclavable mouse diet with anti-oxidant free corn oil and vitamin K instead of menadione was obtained by custom order from ICN Biochemical Division (Aurora, OH, USA). Cyclophosphamide was obtained from Sigma-Aldrich (Oakville, ON, Canada). Zap-oglobin II lytic reagent and Isoton II were from Beckman Coulter (Mississauga, ON, Canada). 10% Buffered formalin phosphate was obtained from Fisher Scientific (Nepean, ON, Canada).

Methods

Cell culture

HCT116 human colon carcinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM-F12 (1:1) + 10% FBS. T47D human breast ductal carcinoma cells were obtained from Dr. S. Mai (Manitoba Institute of Cell Biology, Winnipeg, MB) and grown in RPMI 1640 with hepes + 10% FBS. HL-60 promyelocytic leukemia cells were obtained from Dr. A.H. Greenberg (Manitoba Institute of Cell Biology, Winnipeg, MB) and grown in RPMI 1640 with HEPES + 10% FBS.

Induction of DT-diaphorase

Cells from cultures in log phase were obtained, washed with phosphate buffered saline (PBS) and resuspended in fresh media supplemented with 10% FBS. The DT-diaphorase inducers (sulforaphane dissolved in DMSO, DMF dissolved in EtOH) were added to the culture such that concentration of solvent was <1%, and allowed to incubate for 48 hours at 37°C in an atmosphere containing 5% CO_2. The final concentration of sulforaphane was 1.5μM and of DMF was 5μM as these doses resulted in less than 10% cell kill as measured by MTT assay. Following the 48-hour incubation the cells were washed with PBS and resuspended in fresh media for the cytotoxicity assays or harvested into 0.25M sucrose, sonicated, and stored at –80°C for measurement of DT-diaphorase activity.

Measurement of DT-diaphorase activity

Total protein concentration in each of the samples was measured using the Biorad DC kit, which is based upon the Lowry method of protein determination. A standard curve was created using a γ-globulin solution 1 mg/ml and diluting to give concentrations from 0.125 mg/ml to 1 mg/ml. Samples were added in appropriate volumes to give protein concentrations within the range of the standard curve. Solution A (copper tartrate) solution was added and mixed well, followed by the addition of solution B (Folin's reagent). The resulting solution was allowed to incubate 15 minutes or longer before absorbance at 750 nm was determined using a Cary 1 UV spectrophotometer (Varian, Mississauga, ON, Canada).

DT-diaphorase activity was measured using menadione as the electron acceptor, by the procedure described by Prochaska and Santamaria, 1988. Known amounts of protein were added to disposable cuvettes. To these were added a Tris buffer solution (25 mM Tris HCl, pH 7.4) containing 0.06% bovine serum albumin, 0.01% Tween-20, 30 μM NADP, 5 μM FAD, 1 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, 7.2 μM MTT, and 50 μM menadione. The solution was maintained at a temperature of 25°C, shielded from light in the Cary 1 UV spectrophotometer and the increase in absorbance at 610 nm was measured over 6 minutes. This was repeated with the addition of dicoumarol (final concentration 0.13 μM) to the cuvette. The difference between the two measurements was the dicoumarol-inhibitable activity and was expressed as nanomoles of menadione reduced per minute per mg of protein (nmol min^{-1} mg $protein^{-1}$). Control and inducer treated groups were compared using two-tailed t-tests.

In vitro cytotoxicity assays

Cytotoxicity of RH1 and MMC on T47D cells was determined by MTT assay as described by Johnston *et al*, 1994. Cells were grown in 100 mm plates to a concentration of 2 X 10^6 cells/plate and then incubated with or without DT-diaphorase inducers for 48 hours. Media was then removed and replaced with 4 ml of fresh pre-warmed media containing the drug at the appropriate concentrations and allowed to incubate for 1 hour at 37°C, 5% CO_2. Following the incubation the media containing the drug was removed and the cells were washed with citrate saline. Trypsin solution (1ml) was added and the plates were returned to the incubator until cells became loose (5-10 minutes). Media with FBS (3 ml) was added to each plate to stop trypsin activity and a pasteur pipette was used

to remove the cell suspension to 17 X 100 mm cloning tubes. The cell suspension was aspirated several times to break up any cell clumps. Cells from each sample were then counted using a Z2 Coulter Counter (Beckman Coulter, Mississauga, ON, Canada) to ensure cell concentrations were uniform. Cell suspensions were serially diluted to concentrations of 4000, 3200, 2400, 1600, and 800 cells/ml in media without hepes buffer and 250 µl from each was plated in quadruplicate in 96 well plates. Plates were incubated at 37°C, 5% CO_2 for 10-12 days at which time MTT was added to a final concentration of 0.2 mg/ml and allowed to incubate at 37°C for approximately 3 hours. Plates were spun at 1300 x g (Centra GP8R centrifuge, IEC) for 10 minutes and the media was removed. DMSO was added and the plates were placed on a plate shaker (Minishaker, Dynatech) for a few minutes to dissolve the crystals. Absorbance at 540 nm was determined using a spectrophotometric plate reader (Multiskan MCC/340, Titertek). Surviving cell fraction (SCF) was determined by dividing absorbance values for the treated group by absorbance values for an untreated control group. Each experiment was repeated a minimum of three times or until the standard errors of the SCF values was below 20%. From the regression lines obtained by comparing the mean SCF to drug concentration the D_{10} (negative reciprocal of the regression slope) was determined. The significance of the difference of cytotoxicities between induced and non-induced groups was assessed using a paired two-tailed t-test comparing the slopes of the regression lines.

Cytotoxicity of RH1 and MMC on HCT116 cells was determined using a clonogenic assay described by Begleiter *et al*, 1989. DT-diaphorase inducer and drug treatments were performed in the same manner as with the T47D cells in the MTT assay. Cells were then serially diluted to 200, 100 and 50 cells/ml for control; higher cell

concentrations were used for drug treated cells and depended on the expected SCF. 1 ml of each dilution was then added to 6 well plates along with 3 ml media and allowed to incubate at 37°C, 5% CO_2 until most of the colonies observed contain a minimum of 50 cells (8 or 9 days). Media was removed and the plates were washed with PBS, followed by staining with Wright-Giemsa stain for a few minutes. Stain was removed, and plates washed gently with water to remove excess stain. Plates were inverted and allowed to dry before counting. Only colonies with >50 cells were counted. Cloning efficiency was determined by dividing the observed number of colonies in the control plate by the number of cells plated and multiplying the result by 100 to give a percent value. SCF for each treatment group was determined by calculating the cloning efficiency for the treated cells in the same manner as for the controls and dividing by the cloning efficiency of the control group. Each experiment was repeated a minimum of three times or until the standard errors of the SCF values was less than 20%. The D_{10} and statistical significance were determined in the same manner as that described for the T47D cells.

In vivo DT-diaphorase induction by DMF

HL60 cultures in log phase were counted, sedimented by centrifugation, washed three times in HBSS and resuspended in HBSS at a concentration of 5 X 10^7 viable cells/ml in HBSS. Viability was determined using the trypan blue dye exclusion method and counting using a haemocytometer. 100 µl (5 X 10^6 cells) of this cell suspension was injected subcutaneously into the right and left flanks of 8-10 week old female CD-1 nude mice, injected 3 days previously with cyclophosphamide (150 mg/kg i.p.) to facilitate tumor take (Potter *et al*, 1984).

Ten days following tumor implantation mice were placed on an experimental diet containing antioxidant corn oil and vitamin K instead of menadione supplemented with or without 0.3% (w/w) DMF. Following 2 weeks on this diet the mice were sacrificed and samples of the tumor and kidney were collected and stored in 0.25M sucrose at -80°C until ready to measure DT-diaphorase activity. Whole kidney or tumor samples were mechanically separated using dissection scissors while still submerged in 0.25M sucrose. The samples were further disrupted by sonication and the resulting suspension was tested for protein concentration and DT-diaphorase activity as described for the *in vitro* studies. Statistical significance of the difference between the control and the samples from mice receiving 0.3% DMF were determined by two-tailed t-test.

In vivo RH1 efficacy studies

HL60 cells were prepared and implanted as described above. Mice were fed standard irradiated mouse diet for the first ten days after tumor implantation. At this time the mice were randomly separated into four experimental groups and placed onto the experimental diet containing antioxidant corn oil and vitamin K instead of menadione. Two of the groups were given the experimental diet, the other two groups were given the same experimental diet supplemented with 0.3% (w/w) DMF. Tumor volume was monitored 3 times weekly using calipers to measure the length, width and depth of the tumor and tumor volume was calculated using the following formula, l x w x d x 0.5236 (Rockwell *et al*, 1972). When one of the tumors in each mouse reached a volume of 200 mm^3 the mice were started on a series of five daily i.p. injections. One group of mice on the experimental diet and one group of mice on the DMF supplemented diet received

HBSS. Similarly, one group of mice on the experimental diet and one group of mice on the DMF supplemented diet received 0.4 mg/kg RH1 in HBSS i.p. (total cumulative RH1 dose 2 mg/kg). Tumor volumes were continuously monitored until the tumor became ulcerated or caused mobility problems for the mice, at which time the mice were sacrificed. The four experimental groups were: Group A – control diet and saline, Group B – DMF diet and saline, Group C – control diet and RH1, Group D – DMF diet and RH1. Tumor volumes for each of the four groups were compared by ANOVA to determine significance of the differences observed for each of the days.

In vivo RH1 toxicity studies

Mice from the same four groups as mentioned above for the efficacy studies were used for the measurement of a number of toxicity markers. Body weight and blood counts were continuously monitored until tumors became ulcerated or caused mobility problems in the mice at which time the mice were sacrificed. Mean body weights for each of the four groups were compared for days 0, 1, 2, 4, 5, 7, 9, 11, and 13 using ANOVA to determine significance of the differences seen.

White blood cell (WBC) and platelet counts were determined using the method of Begleiter et al, (2004) on days 0, 3, 6, 9, and 12 (day 0 = first RH1 treatment). Blood from the saphenous vein (25-30 μl) was collected in blood collection capillary tubes coated with EDTA. 10μl of blood was added to 240μl isoton solution and centrifuged at 83 x g for 3 min to pellet red blood cells. Supernatant was collected and diluted 1 in 100 in isoton and counted with the Coulter Z2 particle counter (Beckman Coulter, Mississauga, ON, Canada) with upper limit set at 24.4 fl and lower limit at 1.4 fl (70

micron aperture tube) and data analyzed with Coulter AccuComp software. For WBC determinations 10 µl of blood was diluted to 10 ml in isoton and treated with Zap-oglobin II (50 µl) to lyse red blood cells. The resulting solution was counted using the Coulter Z2 with a lower limit of 3 microns (100 micron aperture tube) and results analyzed with Coulter AccuComp software. WBC and platelet counts for the four groups of mice were compared using ANOVA for each of the days measurements were obtained.

Seven days after the last HBSS or RH1 injection a few mice were randomly selected from each group and euthanized by CO_2 asphyxiation. From these the tumor, kidney, heart, lung, liver, and forestomach were collected and preserved in formalin. Organs were sectioned and stained with hematoxylin and eosin and examined histologically (Dr. JA Thliveris, Department of Human Anatomy and Cell Science, University of Manitoba, Winnipeg, MB). Several mice in each group that failed to develop tumors were sacrificed seven days after the final HBSS or RH1 injection and blood obtained by cardiac puncture (while anesthetized with Ketalean and Rompum) for clinical chemistry analysis. The mice were then euthanized by CO_2 asphyxiation. Serum was prepared by allowing the blood obtained by cardiac puncture to clot and sediment, facilitated by centrifugation (1500 g, 15 min.) and stored at -80°C. Clinical chemistry analysis was performed by the Department of Clinical Chemistry (Health Sciences Centre, Winnipeg, MB.) using a Roche Hitachi 917. Clinical chemistry analysis included tests for dehydration, liver function and kidney function. Liver function was examined by looking at serum levels of the liver enzymes alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT), and lactase dehydrogenase (LD). Kidney performance was evaluated using

serum levels of creatinine and blood urea nitrogen. The state of hydration of the animals was assessed using serum concentrations of sodium, potassium and chloride. Serum clinical chemistry values obtained for the 4 groups of mice were compared using ANOVA to determine statistical significance.

Results

Effect of DT-diaphorase inducers on the activity of MMC and RH1 in vitro

Following 48-hour exposure of HCT116 and T47D cells to either 5 μM DMF or 1.5 μM sulforaphane the levels of DT-diaphorase increased significantly in both cell lines (see insets from figures 10-13 and table 1). In the HCT116 cells DMF increased DT-diaphorase activity 2.1 fold from 94.0 ± 3.0 to 194.0 ± 5.3 nmol min^{-1} mg^{-1} ($p < 0.001$), and sulforaphane increased the activity 2.2 fold from 121.0 ± 14.2 to 268.0 ± 17.7 nmol min^{-1} mg^{-1} ($p < 0.001$). In the T47D cells DMF increased DT-diaphorase activity 1.9 fold from 37.2 ± 11.8 to 69.0 ± 12.3 nmol min^{-1} mg^{-1} ($p < 0.05$) while sulforaphane increased the activity 2.8 fold from 26.0 ± 4.1 to 72.4 ± 5.7 nmol min^{-1} mg^{-1} ($p < 0.0001$).

Figures 10 and 11 show the effects of 48-hour exposure to 5 μM DMF and 1.5 μM sulforaphane, respectively, on the cytotoxic activity of MMC and RH1 in HCT116 cells *in vitro* as determined by MTT assay. The increases in DT-diaphorase activity was associated with a decrease of the D_{10} for MMC from 2.12 ± 0.13 to 1.55 ± 0.05 μM ($p<0.001$) and from 2.62 ± 0.19 to 1.59 ± 0.11 μM ($p<0.001$) for DMF and sulforaphane, respectively. The D_{10} for RH1 remained unchanged at 0.15 ± 0.01 μM (NS) when

HCT116 cells were pretreated with DMF and increased from 0.16 ± 0.01 to 0.20 ± 0.01 μM (NS) when pretreated with sulforaphane (see table 2).

Comparable results were obtained using T47D cells in the clonogenic assay, as shown in figures 12 and 13. 48-hour exposure to 5 μM DMF and 1.5 μM sulforaphane resulted in a decrease in the D_{10} for MMC from 10.80 ± 0.96 to 4.60 ± 0.31 μM ($p<0.001$) and from 6.1 ± 0.7 to 3.2 ± 0.3 μM ($p<0.001$), respectively. With RH1 the D_{10} remained unchanged at 0.20 ± 0.02 to 0.22 ± 0.01 μM (NS) and from 0.18 ± 0.01 to 0.19 ± 0.01 μM (NS) for 5 μM DMF and 1.5 μM sulforaphane, respectively (see table 2).

Table 1: Summary of the effects of DMF and Sulforaphane on DT-diaphorase activity in HCT116 and T47D cells *in vitro*

Tumor	Inducer	Mean DT-diaphorase activity (± SE)		P value	Control/ Induced
		Control	Induced		
HCT116	DMF	94.0 ± 3.0	194.0 ± 5.3	<0.001	2.1
	Sulforaphane	121.0 ± 14.2	268 ± 17.7	<0.001	2.2
T47D	DMF	37.2 ± 11.8	69.0 ± 12.3	<0.05	1.9
	Sulforaphane	26.0 ± 4.1	72.4 ± 5.7	<0.0001	2.8

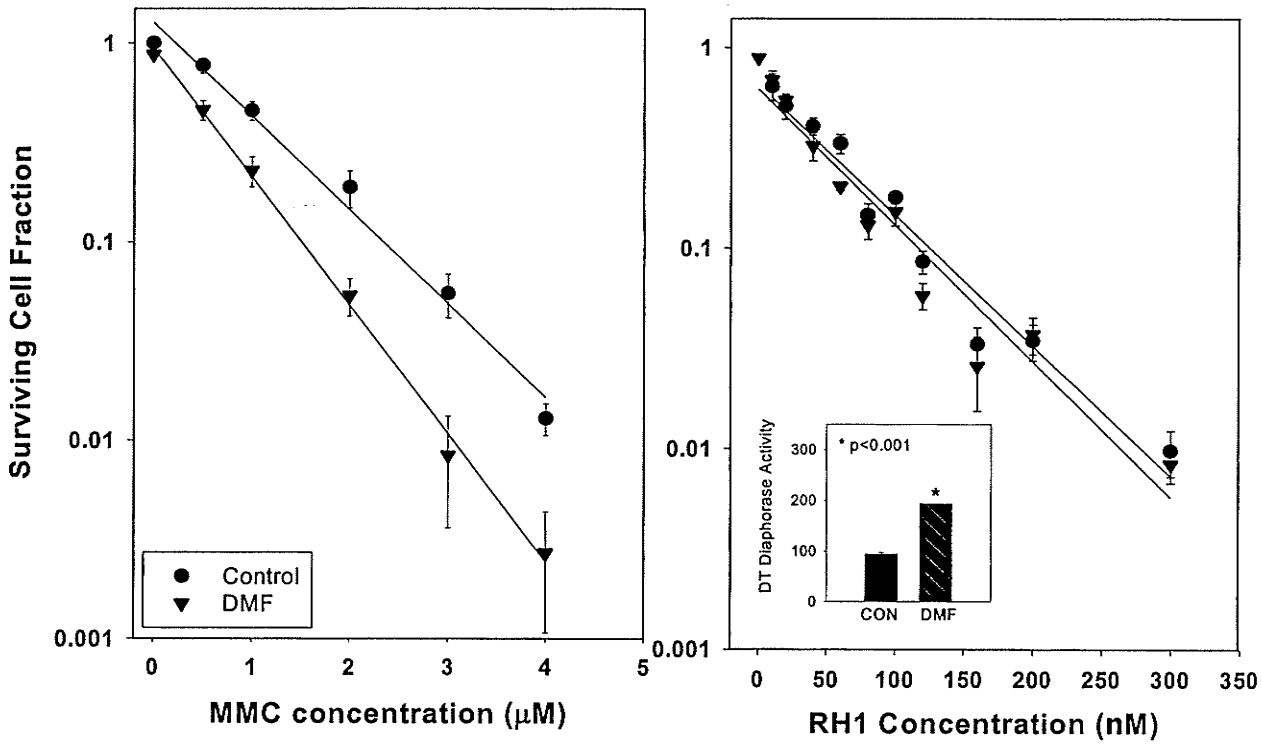

Figure 10 Effect of DMF on the activity of MMC and RH1 in HCT116, human colon tumor cells. Cells were incubated at 37°C with or without 5 μM DMF for 48 hours. Cells were then treated with various concentrations of MMC or RH1 for 1 hour. Surviving cell fraction was determined using a clonogenic assay (Begleiter *et al*, 1989). DT-diaphorase activity (inset) was determined using menadione as the electron acceptor (Prochaska and Santamaria, 1988) and is expressed as nmol min^{-1} mg $protein^{-1}$. The points represent the mean surviving cell fraction ± standard error of 4-22 determinations. The lines are linear regression lines. Inset, level of DT-diaphorase in control cells and cells treated with DMF. The bars represent the mean DT-diaphorase activity ± standard error of 26-27 determinations. The means were compared using a t-test to determine the significance of the difference between control and DMF treated cells.

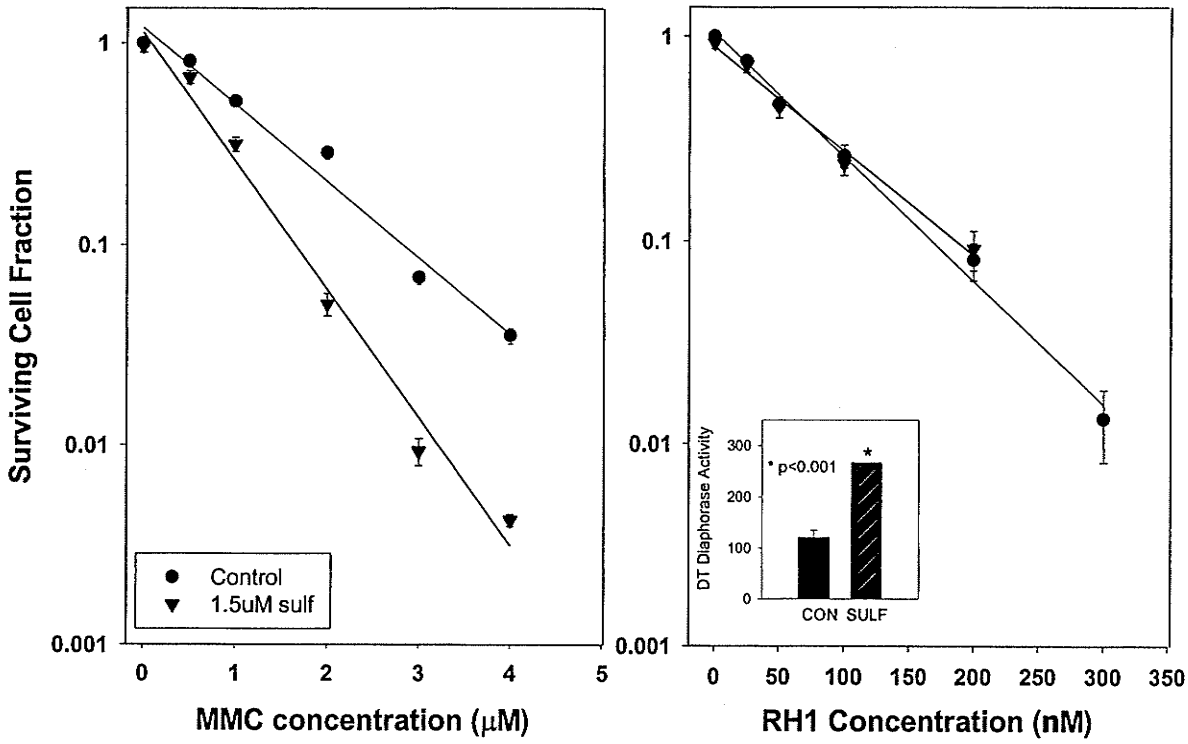

Figure 11 Effect of sulforaphane on the activity of MMC and RH1 in HCT116, human colon tumor cells. Cells were incubated at 37°C with or without 1.5 μM sulforaphane for 48 hours. Cells were then treated with various concentrations of MMC or RH1 for 1 hour. Surviving cell fraction was determined using a clonogenic assay (Begleiter *et al*, 1989). DT-diaphorase activity (inset) was determined using menadione as the electron acceptor (Prochaska and Santamaria, 1988) and is expressed as nmol min^{-1} mg $protein^{-1}$. The points represent the mean surviving cell fraction ± standard error of 6-12 determinations. The lines are linear regression lines. Inset, level of DT-diaphorase in control cells and cells treated with sulforaphane. The bars represent the mean DT-diaphorase activity ± standard error of 10 determinations. The means were compared using a t-test to determine the significance of the difference between control and sulforaphane treated cells.

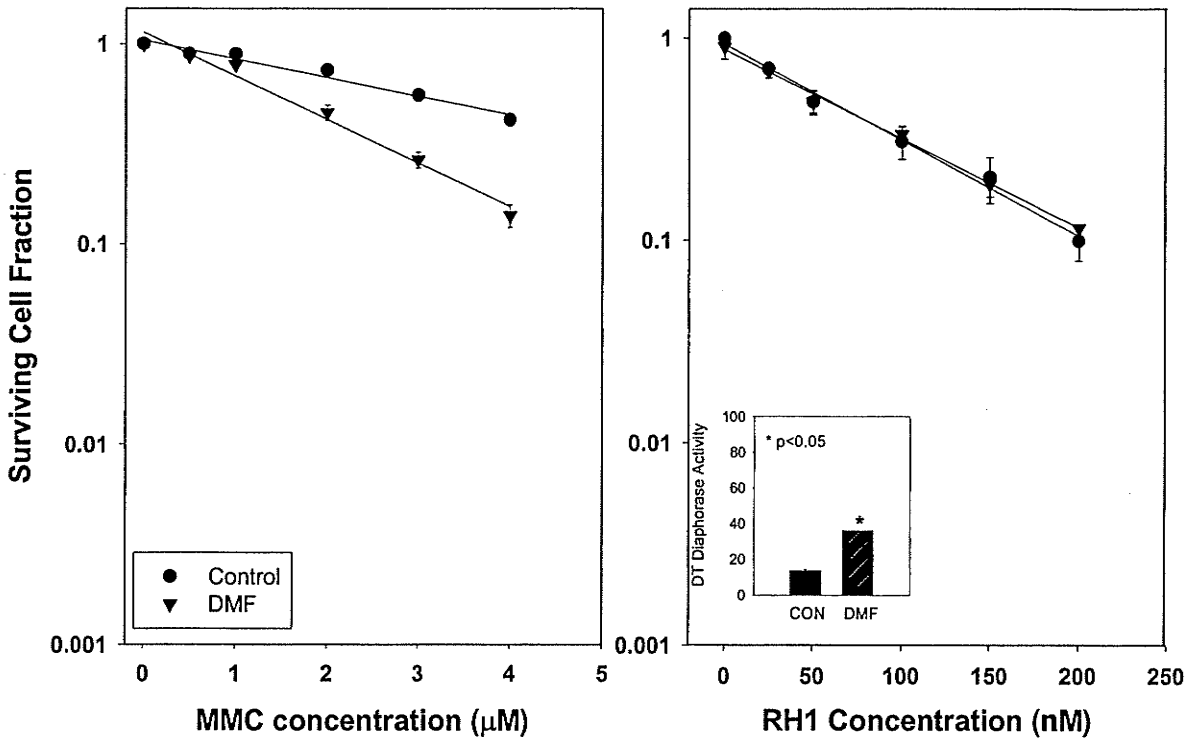

Figure 12 Effect of DMF on the activity of MMC and RH1 in T47D, human breast cancer cells. Cells were incubated at 37°C with or without 5 μM DMF for 48 hours. Cells were then treated with various concentrations of MMC or RH1 for 1 hour. Surviving cell fraction was determined using the MTT assay (Jonhston *et al*, 1994). DT-diaphorase activity (inset) was determined using menadione as the electron acceptor (Prochaska and Santamaria, 1988) and is expressed as nmol min^{-1} mg $protein^{-1}$. The points represent the mean surviving cell fraction ± standard error of 4 determinations. The lines are linear regression lines. Inset, level of DT-diaphorase in control cells and cells treated with DMF. The bars represent the mean DT-diaphorase activity ± standard error of 7 determinations. The means were compared using a t-test to determine the significance of the difference between control and DMF treated cells.

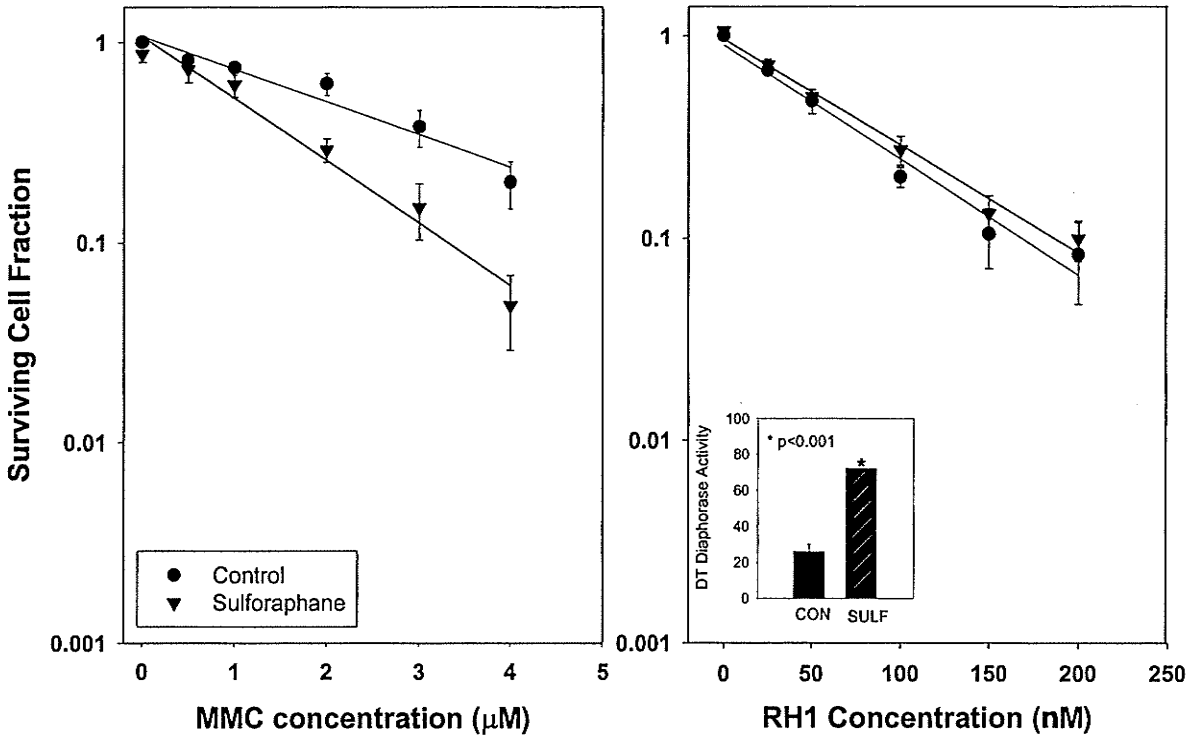

Figure 13 Effect of sulforaphane on the activity of MMC and RH1 in T47D, human breast cancer cells. Cells were incubated at 37°C with or without 1.5 μM sulforaphane for 48 hours. Cells were then treated with various concentrations of MMC or RH1 for 1 hour. Surviving cell fraction was determined using the MTT assay (Jonhston *et al*, 1994). DT-diaphorase activity (inset) was determined using menadione as the electron acceptor (Prochaska and Santamaria, 1988) and is expressed as nmol min^{-1} mg $protein^{-1}$. The points represent the mean surviving cell fraction ± standard error of 4-5 determinations. The lines are linear regression lines. Inset, level of DT-diaphorase in control cells and cells treated with sulforaphane. The bars represent the mean DT-diaphorase activity ± standard error of 6 determinations. The means were compared using a t-test to determine the significance of the difference between control and sulforaphane treated cells.

Table 2: Summary of the effects of DMF and Sulforaphane on the Cytotoxicity of RH1 and MMC *in vitro*

Tumor	Drug	Inducer	D_{10} (μM) ± SE		P value	Control/ Induced
			Control	Induced		
HCT116	MMC	DMF	2.12 ± 0.13	1.55 ± 0.05	<0.001	1.37
		Sulforaphane	2.62 ± 0.19	1.59 ± 0.11	<0.001	1.65
	RH1	DMF	0.15 ± 0.01	0.15 ± 0.01	NS	1.00
		Sulforaphane	0.16 ± 0.01	0.20 ± 0.01	NS	0.80
T47D	MMC	DMF	10.80 ± 0.96	4.60 ± 0.31	<0.001	2.35
		Sulforaphane	6.10 ± 0.71	3.20 ± 0.26	<0.001	1.91
	RH1	DMF	0.20 ± 0.02	0.22 ± 0.01	NS	0.91
		Sulforaphane	0.18 ± 0.01	0.19 ± 0.01	NS	0.95
NS = not significant						

Effect of DMF diet on DT-diaphorase activity in vivo

HL60 human promyelocytic leukemia cells were implanted subcutaneously in the flanks of female CD-1 nude mice and mice were fed standard irradiated rodent chow for 10 days. Mice were then randomly assigned to two groups of three animals per group, one group was fed control experimental diet and the other experimental diet supplemented with 0.3% DMF for 7 days. Mice were euthanized and tumors and kidneys were excised and the level of DT-diaphorase determined. The 0.3% DMF supplemented diet significantly increased the DT-diaphorase activity in both the tumors (from 3.6 ± 0.3 to 5.5 ± 0.6 nmol min^{-1} mg^{-1}, $p < 0.02$) and the mouse kidneys (from 100 ± 9 to 368 ± 36 nmol min^{-1} mg^{-1}, $p<0.00005$), the results are illustrated in figure 14.

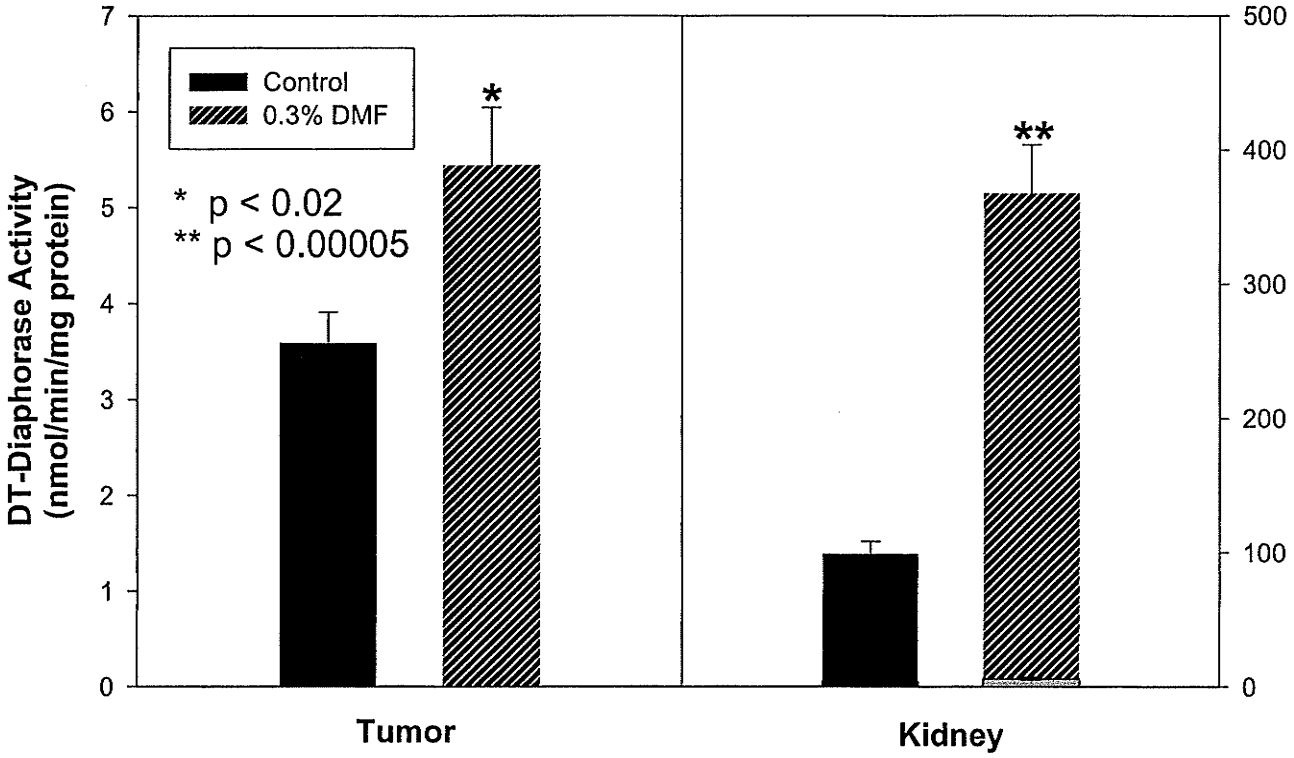

Figure 14 Effect of 0.3% DMF diet for 7 days on the DT-diaphorase activity in HL60 human promyelocytic tumors and mouse kidney tissues. DT-diaphorase activity was determined using menadione as the terminal electron acceptor (Prochaska and Santamaria, 1988). The bars represent the mean DT-diaphorase activity ± standard error of 4-6 determinations. The means were compared using a t-test to determine the significance of the difference between control and DMF treated samples.

Effect of DMF on antitumor activity of RH1 in vivo

HL60 human promyelocytic leukemia cells were implanted subcutaneously in both flanks of female CD-1 nude mice and mice were fed standard irradiated rodent chow for 10 days. Mice were randomly assigned to 4 groups with 12 mice per group. Two of the groups were fed control experimental diet and the other two were fed experimental diet supplemented with 0.3% (w/w) DMF. When either tumor on a mouse reached 200 mm^3, the mouse was given 5 daily i.p. injections with mice in one of the control and one of the DMF fed groups receiving HBSS and the other two groups receiving 0.4 mg/kg RH1 in HBSS daily. This day was designated as day 0. Tumor dimensions were determined daily for the first three days and then every second day up until day 13 when

mice were euthanized. Growth of the tumors during this time is shown in figure 15. Tumor volumes for each of the groups were compared using ANOVA to determine statistical significance for each of the days; only day 9 showed a significant difference.

On day 9 the control tumors were 356 ± 53% of their day 0 size, the DMF alone group 512 ± 66%, RH1 alone group 320 ± 39% and the DMF + RH1 combination group was 319 ± 35%. ANOVA showed a significant difference ($p < 0.05$) and further analysis using Tukey's test determined that the DMF alone group had tumors that were significantly larger than any of the other groups.

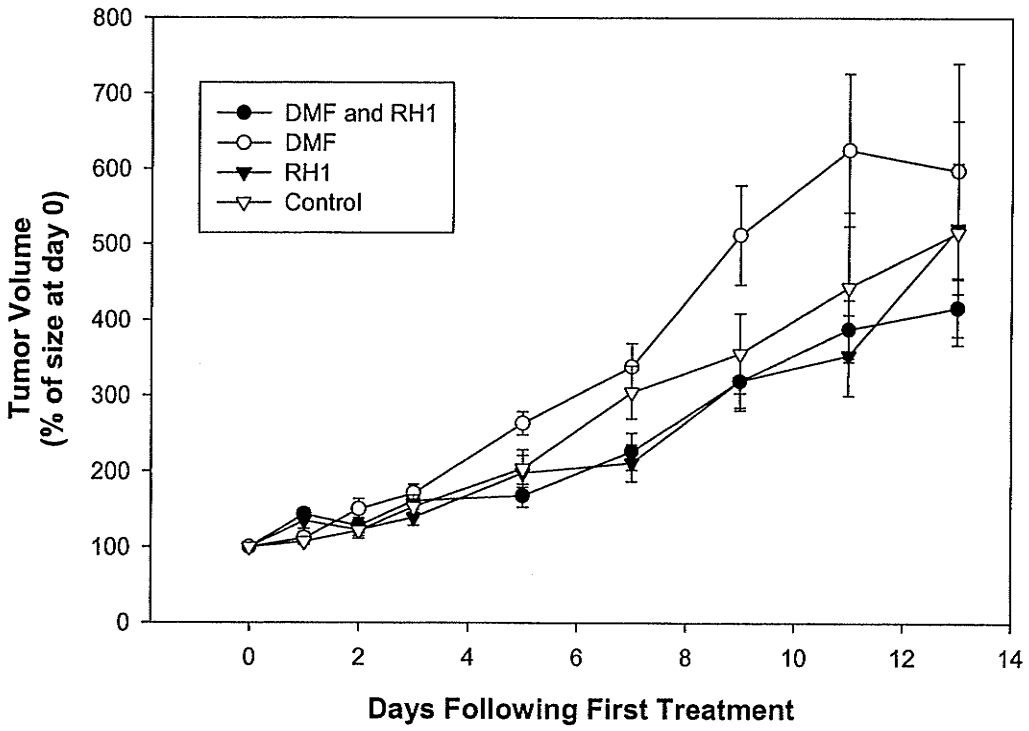

Figure 15 Effect of DMF diet and RH1 on HL60 tumor volumes in female CD-1 nude mice. CD-1 nude mice were implanted with 5 x 10^6 viable cells subutaneously into both flanks of each mouse. After 10 days the mice were fed a custom experimental diet containing 0 or 0.3% DMF (w/w) for 8-18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received 5 daily i.p. injections of saline or 0.4 mg/kg RH1. On days 0, 1, 2, 3, 5, 7, 9, 11, and 13 mice were weighed. The points represent the mean tumor volume ± standard error for 4-12 mice.

Toxicity of RH1 and DMF in CD-1 nude mice

Blood samples were collected from the saphenous vein of mice from each of the four groups described previously in the *in vivo* antitumor activity study. Samples were taken every three days, with day 0 being the day of the first HBSS or RH1 injection, and white blood cell and platelet counts were determined. The presence or absence of DMF in the diet did not have any significant effect on the white blood cell (WBC) levels for either the RH1 treated or saline treated groups at any of the time points (see figure 16). There was a significant reduction ($p < 0.01$ for days 3, 6 and 9) of WBC counts in the two groups that received RH1 injections beginning at day 3 with the nadir occurring at day 6 (one day after the final injection). The concentrations of WBC in the circulating blood of the RH1 and RH1 + DMF treated mice were 2.5 and 3.3 X 10^6 cells/ml or 27 and 36% of the control value of 9.3 x 10^6 cells/ml, respectively. The observed leukopenia was reversible with WBC counts returning to control levels by day 12 (7 days following the final RH1 injection). Again only data up to 12 days have been analyzed because of the small sample size following this time point due to the required euthanasia.

Similarly platelet concentration in the blood was determined. There was no statistical significance between any of the four groups for this measurement, for any of the days measured (figure 17).

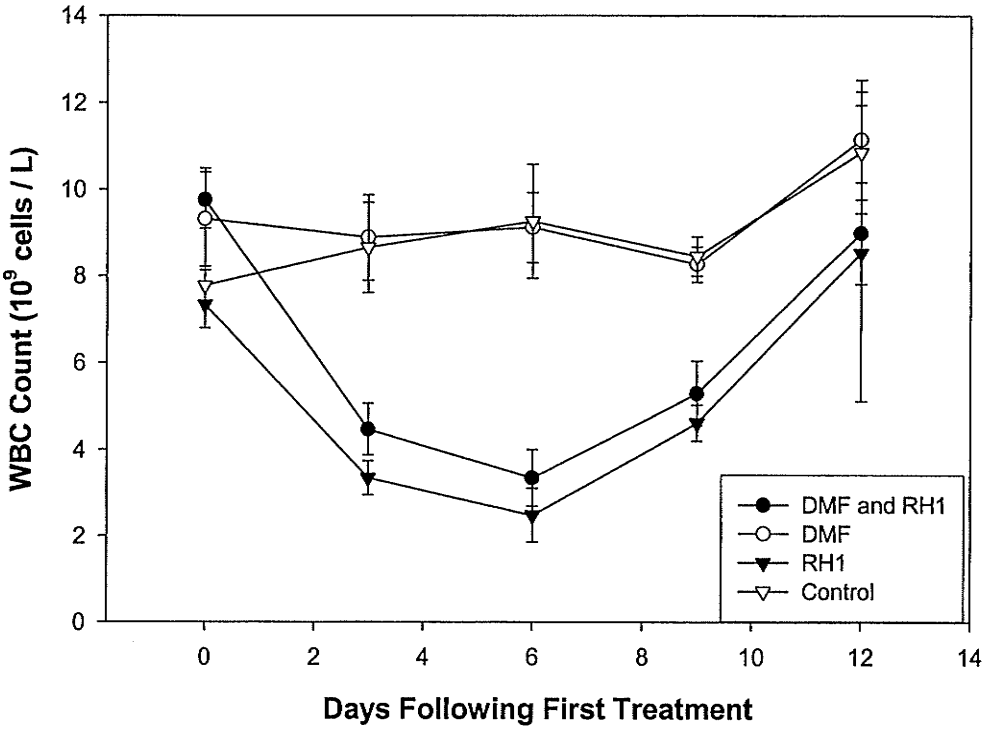

Figure 16 Effect of DMF diet on RH1 toxicity to WBC in CD-1 nude mice. CD-1 nude mice were implanted with 5 x 10^6 viable cells subcutaneously in both flanks of each mouse. After 10 days the mice were fed an experimental diet containing 0 or 0.3% DMF (w/w) for 8-18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received 5 daily i.p. injections of saline or 0.4 mg/kg RH1. On days 0, 3, 6, 9, and 12 approximately 25 µl of blood was collected from the saphenous vein of some of the mice in each treatment group (Hem et al, 1998). The blood was collected into a microvette CB300 with potassium EDTA and 10 µl aliquots were used for the determination of WBC counts. The counts were determined with a Coulter Z2 particle counter and cell analyzer. The points represent mean white blood cell counts ± standard error for 5-11 mice. Differences in the WBC counts for the different treatment groups were analyzed statistically by ANOVA for each time point.

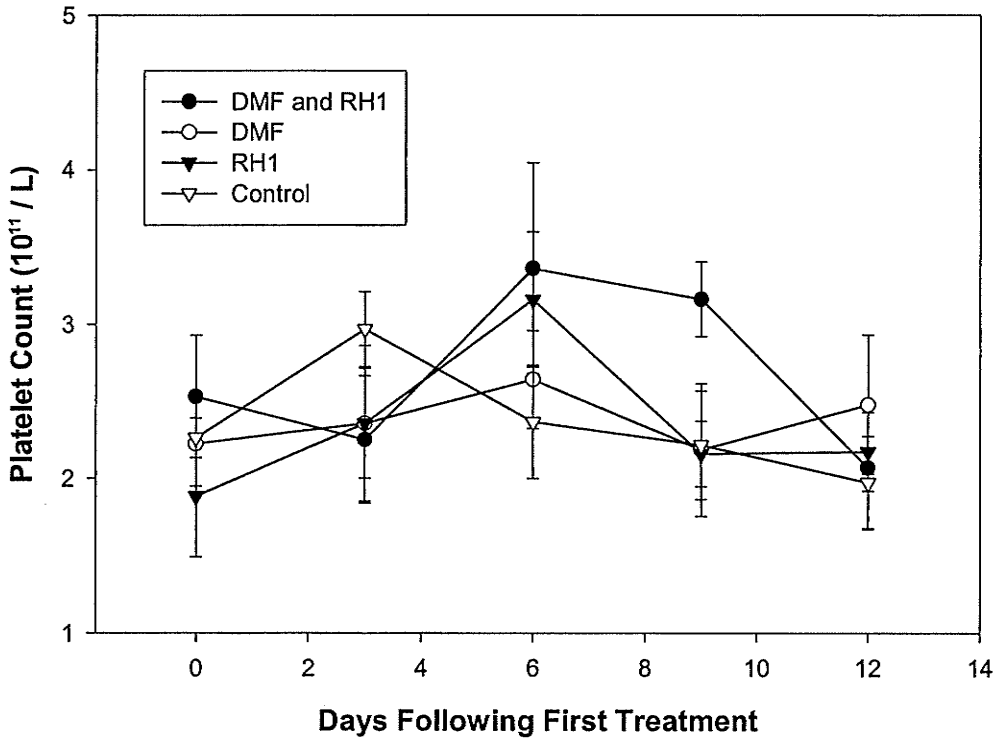

Figure 17 Effect of DMF diet on RH1 toxicity to platelets in CD-1 nude mice. CD-1 nude mice were implanted with 5 x 10^6 viable cells subutaneously into both flanks of each mouse. After 10 days the mice were fed a custom experimental diet containing 0 or 0.3% DMF (w/w) for 8-18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received 5 daily i.p. injections of saline or 0.4 mg/kg RH1. On days 0, 3, 6, 9, and 12 approximately 25 µl of blood was collected from the saphenous vein of some of the mice in each treatment group (Hem et al, 1998). The blood was collected into a microvette CB300 with potassium EDTA and 10 µl aliquots were used for the determination of platelet counts. The counts were determined with a Coulter Z2 particle counter and cell analyzer. The points represent the mean platelet counts ± standard error for 6-10 mice. Differences in the platelet counts for the four treatment groups were analyzed statistically by ANOVA for each time point.

Mice from each of the four groups described above in the *in vivo* antitumor activity studies that failed to develop tumors were used for histological examination of the major organs and to conduct clinical chemistry analysis. 7 days following the last HBSS or RH1 injection the mice were anaesthetized with Ketalean and Rompun and blood samples obtained by cardiac puncture. The mice were then euthanized by CO_2

asphyxiation and tumor, kidney, heart, lung, liver, and forestomach were collected and preserved in formalin for histological examination. Serum was prepared by allowing the blood to sit for 30 minutes to clot, followed by centrifugation. Histological examination of the organs showed no visible damage in any of the four treatment groups (figure 18). Similarly, the clinical chemistry analysis of the serum obtained from each of the four experimental groups showed no significant changes in blood chemistry (figure 19). The levels of gamma-glutamyl transpeptidase and serum creatinine were too low to be measured.

Mice from the *in vivo* antitumor activity study were weighed every day for the five days they received injections of HBSS or RH1, and every second day thereafter. Evaluation of mouse body weight (see figure 20) showed a reduction in body weight for the mice given RH1 throughout the five day treatment period. On day 5 the RH1 group had lost 3% of their body weight and the RH1 + DMF group had lost 5% of their body weight. Immediately following cessation of the RH1 injections the mice body weights began to increase again. Comparison of each of the groups on each day using ANOVA showed that from day 3 to day 11 the group receiving the combination therapy was significantly lighter than both the control group and the group receiving DMF in their diet. Although not statistical significant at the $p = 0.05$ level, a trend existed suggesting that RH1 alone may also result in treatment associated weight loss.

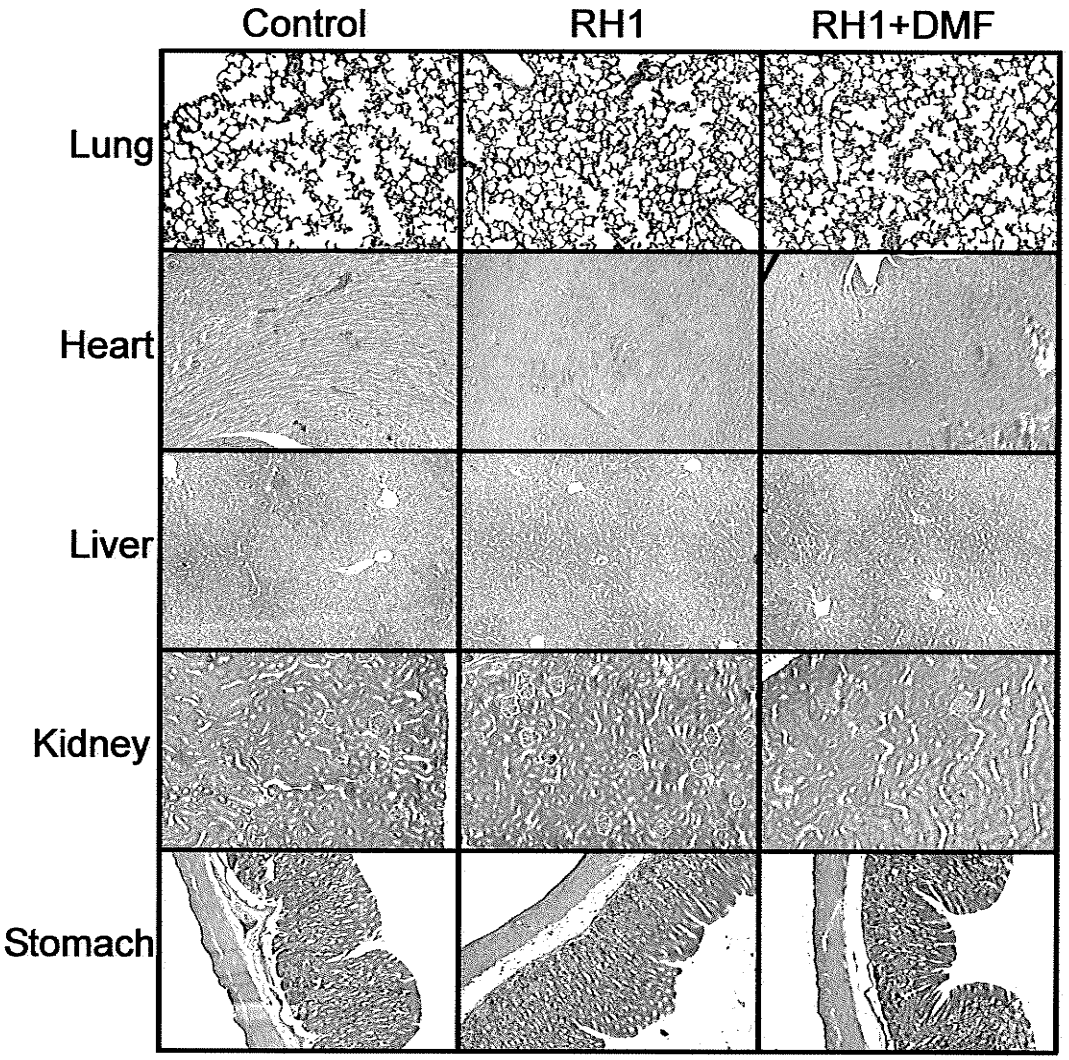
Control
RH1
RH1+DMF
Lung
Heart
Liver
Kidney
Stomach

Figure 18 Effect of DMF diet and RH1 treatment on tissues from female CD-1 nude mice. CD-1 nude mice were fed custom experimental diet containing 0 or 0.3% DMF (w/w) for 10 days. The mice were weighed and received 5 daily i.p. injections of HBSS or 0.4 mg/kg RH1. Seven days after the final injection the mice were euthanized by CO_2 asphyxiation and the tissues were removed and fixed in neutral buffered formalin. The organs were sectioned, stained with hematoxalin and eosin and examined histologically. Shown are light micrographs of various tissues from mice fed control diet and treated with saline (Control), mice fed control diet and treated with RH1 (RH1) and mice fed 0.3% DMF diet and treated with RH1 (RH1 + DMF).

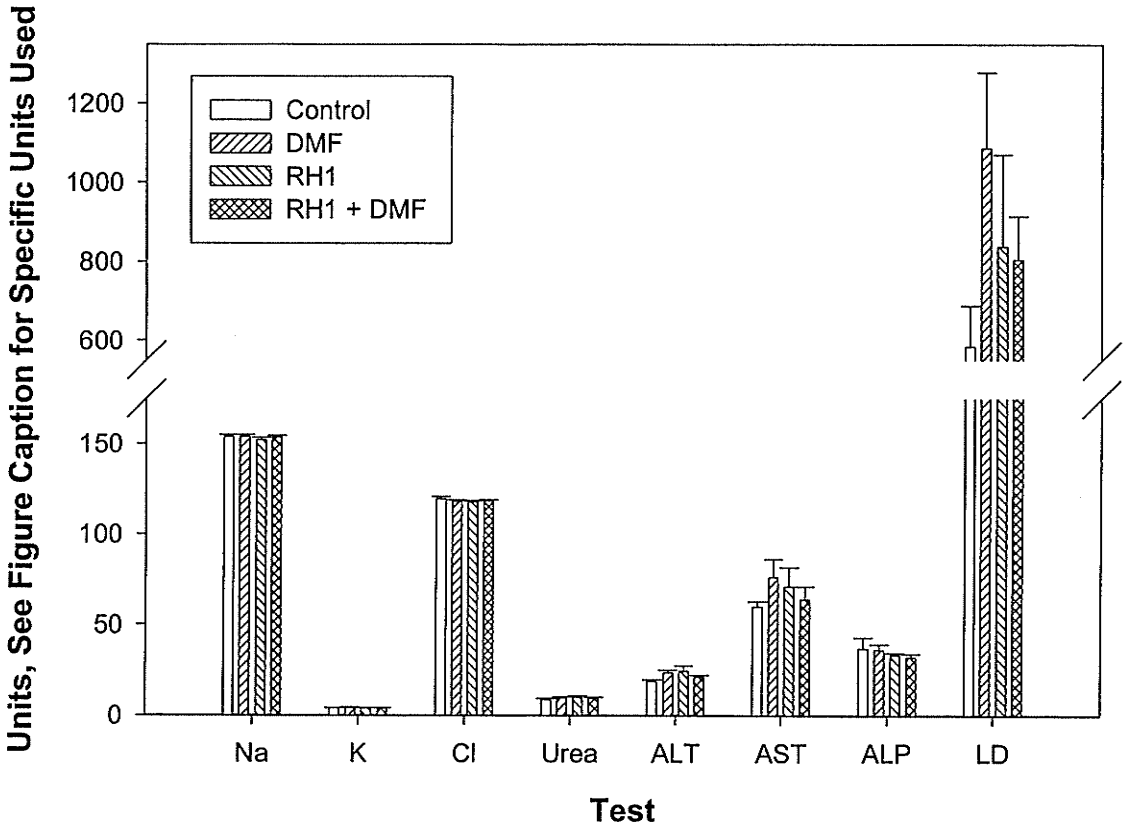

Figure 19 Effect of five daily 0.4 mg/kg RH1injections and 0.3% DMF diet on serum biochemistry of female CD-1 nude mice. Blood was obtained from mice by cardiac puncture, 7 days after the final injection of RH1 or HBSS. The bars represent values for each of the tests ± standard error for 5-11 determinations. Units are mmol/L for Na, K, Cl, and Urea, units/L for ALT (Alanine Amino-transferase), AST (Aspartate Amino-transferase), ALP (Alkaline Phosphatase) and LD (lactate dehydrogenase).

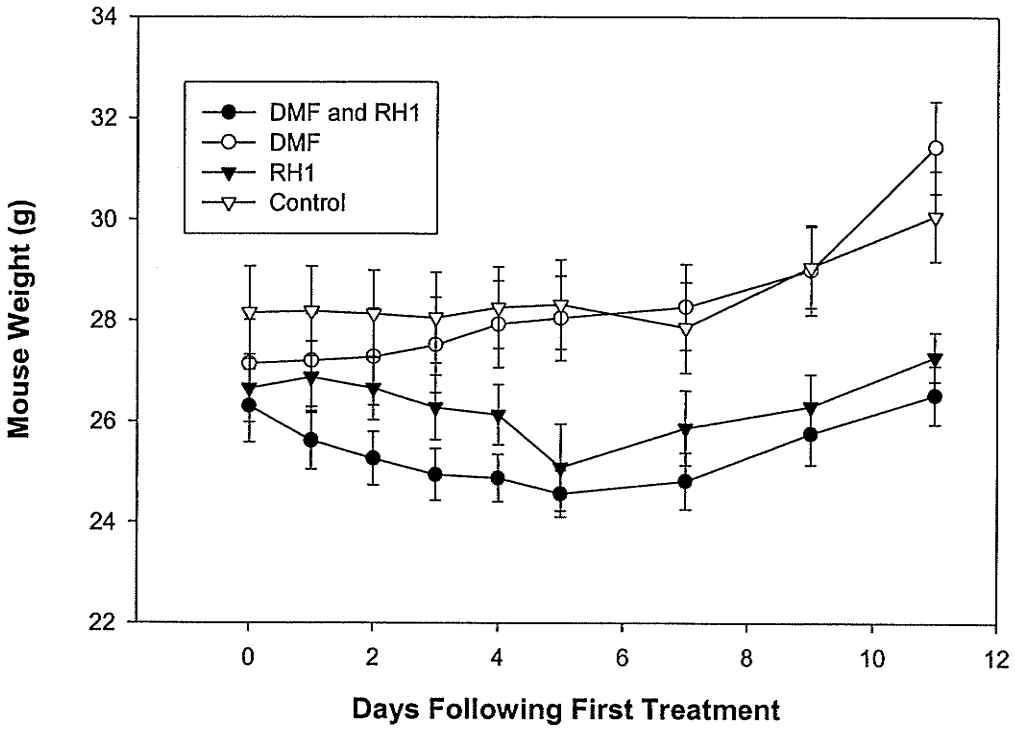

Figure 20 Effect of DMF diet and RH1 on body weights of female CD-1 nude mice. CD-1 nude mice were implanted with 5 x 10^6 viable cells subutaneously into both flanks of each mouse. After 10 days the mice were fed a custom experimental diet containing 0 or 0.3% DMF (w/w) for 8-18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received 5 daily i.p. injections of saline or 0.4 mg/kg RH1. On days 0, 1, 2, 3, 4, 5, 7, 9, 11, and 13 mice were weighed. The points represent the mean body weight ± standard error for 5-12 mice.

DT-DIAPHORASE INVOLVEMENT IN THE FORMATION OF DNA CROSS-LINKS BY RH1

Materials

Purified linear PBR322 plasmid was obtained from Jeanne Fourie (Department of Pharmacology and Therapeutics, University of Manitoba, Canada) (Fourie *et al*, 2004). Nick columns, EcoR1 and (α-^{32}p)dATP (6000 Ci/mmol) were from Amersham Biosciences (Baie d'Urfe, QC, Canada). FAD, NADH, Tris HCl, trizma base, Na_2EDTA, sucrose, bromophenol blue, xylene cyanol were from Sigma-Aldrich (Oakville, ON, Canada). Novagen pellet paint coprecipitant and sodium acetate were from VWR (Mississauga, ON, Canada). Ethidium bromide (EtBr), Klenow fragment of DNA polymerase I and agarose were obtained from Invitrogen Life Technologies (Burlington, ON, Canada). Purified DT-diaphorase was a gift from Dr. Sushu Pan (University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA).

Methods

DNA cross-linking was determined using the method of Hartley *et al* (1991) with reaction buffers modified to ensure DT-diaphorase was active (Fourie *et al*, 2004). PBR322 plasmid DNA was linearized by incubating 20 µg of DNA with 80 units of EcoR1 for 1 hr at 37°C. Purified linear PBR322 plasmid (400ng) was incubated with 2 µl react 2 buffer, 1 µl 5mM dTTP, 2 µl Klenow fragment of DNA polymerase 1, 5 µl (α-^{32}P) dATP (6000 Ci/mmol) and water to a final volume of 20 µl for 30 minutes at 37°C to radioactively label the DNA at the 3' end. Excess dATP was removed from the labelled

DNA by running through a Nick column (Sephadex G-50 DNA Grade with 0.15% Kathon as a preservative) with pH 7.5 TE buffer (10 mM trizma, 1 mM Na_2EDTA) sterilized by 0.2 μM filtration. The final volume of elutant was 400 μl giving a labelled DNA concentration of 1 ng/μl.

500 μl Tris HCl buffer (25 mM Tris HCl, pH 7.4) containing 0.1 mM FAD and 0.4 mM NADH was made anaerobic by purging with nitrogen for 30 minutes. To this was added 6.3 ng/ml (final concentration) DT-diaphorase activated by 0.01% Tween 20 and 30 ng labelled DNA. The desired concentration of RH1 was added last to initiate the reaction and the reaction mixture was allowed to incubate in a 37°C water bath to allow cross-links to form. The reaction mixture was kept anaerobic by purging with oxygen free nitrogen in the reaction tubes. Following 15 minutes of incubation the reactions were terminated by the sequential addition of 2 μl Novagen pellet paint coprecipitant, 5μl sodium acetate (pH 5.3) and 100 μl 100% ethanol to a 50 μl volume of the reaction mixture. The reaction mixture was agitated gently following the addition of each of the above. After allowing the mixture to sit for 3 minutes at room temperature the DNA was sedimented by centrifugation (14,000 x g, 10 min), the pellet washed with 100 μl 70% ethanol, and centrifuged at 14,000 x g for 5 minutes. Supernatant was removed and the pellet allowed to dry inverted at room temperature for 30 minutes before being resuspended in loading buffer. Loading buffer for the double stranded (DS) DNA controls was 0.3 M sucrose with 0.05% bromophenol blue (BB) and 0.05% xylene cyanol (XC). Loading buffer for all other controls and the samples contained 35% DMSO, 10 mM Tris, 1 mM EDTA, 0.05% BB and 0.05% XC. All samples except the DS DNA controls were placed in a 70°C water bath for 3 minutes and immediately cooled in an ice

water bath for 30 minutes to prevent reannealing of the DNA. Samples were loaded into a 1.6% agarose gel containing 0.4 μg/ml EtBR and allowed to run for 1 hour at 125 V with TAE buffer (40 mM Trizma base, 40 mM acetic acid, 1 mM EDTA, pH adjusted to 7.0 using glacial acetic acid). Following electrophoresis the gel was dried in a Bio-rad gel dryer (Mississauga, ON, Canada). The dried gel was used to expose a storage phosphor cassette and the DNA bands were visualized using a Storm 860 image-scanning system (Molecular Dynamics, Sunnivaly, CA, USA) and quantified with ImageQuant software.

The amount of cross-linking was determined as the percentage of DNA found within the DS DNA band. The amount of DS DNA observed in the single stranded (SS) DNA control lane is due to spontaneous reannealing. To determine if cross-linking occurred the percentage of DS DNA in each test lanes was compared to the amount of DS DNA in the SS DNA control lane using a two-tailed t-test. A significant result indicated that cross-linking of the DNA had occurred.

Results

Purified linear PBR322 plasmid was incubated with RH1 at various concentrations with or without DT-diaphorase activity. Following a 15 minute incubation the reactions were terminated and the DNA precipitated and washed. DNA was then placed in a strand-separating buffer at 70°C to separate double stranded DNA into single strands. The resulting preparations were run for 1 hour at 125V in a 1.6% agarose gel to separate the double stranded DNA from the single stranded. DNA that has

been cross-linked will appear in the DS DNA band while non-cross-linked DNA will appear in the SS DNA band.

RH1 by itself resulted in no significant formation of cross-links when compared to the amount of DS DNA created by spontaneous reannealing in the SS control DNA lane. When DT-diaphorase was added to the reaction mixtures the amount of cross-linked DNA was significantly greater than both the single stranded DNA control and the RH1 without DT-diaphorase. Figure 21 shows a representative gel scan and table 2 shows a summary of the percentage of DS DNA in each lane. The double-stranded DNA control (DS) is used to indicate the position of DS DNA bands on the gel, the single-stranded DNA controls (SS) indicate the position of the SS DNA bands. Additionally, the amount of DS DNA observed in the SS control wells indicates the amount of reannealing that occurred spontaneously. The amount of DS DNA observed in the 10, 25 and 50 μM RH1 wells was not significantly different that the amount observed in the SS DNA control wells. When DT-diaphorase was added to the reaction mixture the amount of DS DNA observed was significantly greater than the amount of spontaneously forming DS DNA observed in the SS control wells indicating the formation of cross-links.

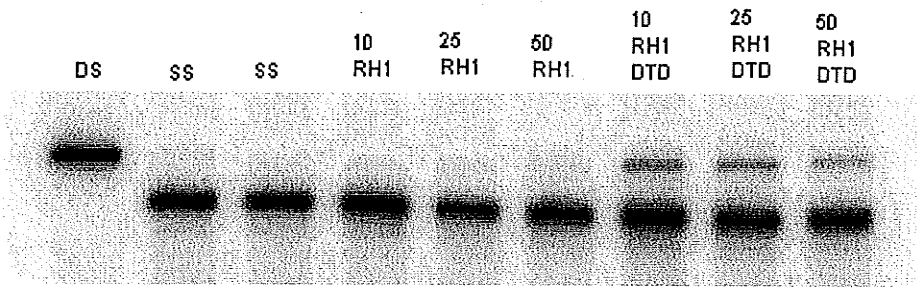

Figure 21 Agarose gel showing the effects of various concentrations of RH1with or without DT-diaphorase on the formation of DNA cross-links. The assay was performed using the method of Fourie *et al*, 2004. Cross-linked DNA remains as DS DNA following strand separation while non-cross-linked DNA

has been separated into single strand DNA. The upper bands are the slower travelling DS DNA while the lower bands are the SS DNA. Abreviations used: DS = double-stranded DNA control, SS = single-stranded DNA control, RH1 concentrations of 10, 25, and 50 are in μM, DTD = DT-diaphorase, the concentration of DT-diaphorase used was 6.3 ng/ml.

Table 3: Summary of the amount of cross-linking observed for 10, 25, and 50 μM RH1 with or without the presence of DT-diaphorase

SAMPLE	% DS DNA	P VALUE	PAIRED
SS control	2.84 ± 1.96		
10μM RH1	2.97 ± 1.75	0.963	0.012
10μM RH1 + DTD	28.03 ± 5.49	0.002	
25μM RH1	5.15 ± 2.75	0.51	0.004
25μM RH1 + DTD	36.70 ± 5.63	0.0005	
50μM RH1	2.96 ± 1.00	0.96	0.013
50μM RH1 + DTD	38.02 ± 8.19	0.003	

P value column is the p value obtained by comparing each of the samples against the SS control by two-tailed t-test, the paired column is the p value obtained by comparing the RH1 and RH1 + DTD groups for each drug concentration with a paired two-tailed t-test. % DS DNA is the mean amount of double stranded DNA in each lane ± SE. DTD= DT-diaphorase

DISCUSSION

MMC, the prototype bioreductive agent was first isolated in1958 from the bacteria *Streptomyces caespitosus* (Wakiki *et al*, 1958) and has been used in the clinic since the early 1960's (Begleiter, 2000). Since then, the use of MMC has decreased due its toxicity and the emergence of more effective treatments (Bradner, 2001). The dose limiting toxicity observed for MMC is delayed myelosuppression appearing three to four weeks after treatment. This effect is reversible and full recovery occurs within 8 weeks (Begleiter, 2000, Doll *et al*, 1985). To help overcome this problem MMC is generally administered in widely spaced dosing strategies with the second treatment occurring only after WBC and platelet counts have recovered (generally 6-8 weeks). It would be possible to increase the maximum dose of MMC by combining with autologous bone marrow transplant if not for other rare but potentially fatal non-haemotologic toxicities (Bradner, 2001). The most serious of these is haemolytic uremic syndrome (HUS) that occurs in 4-15% of patients. The occurrence of this toxicity appears to be dose related and is most common in patients receiving a total cumulative dose greater than 60 mg/m^2 (Begleiter, 2000; Lesesne *et al*, 1989). A maximum total dose of 30 mg/m^2 is considered to be safe (Bradner, 2001). A second rare but serious side effect is pulmonary toxicity seen in approximately 5% of patients, the occurrence of this toxicity is not believed to be dose-related (Begleiter, 2000).

It has long been recognized that the benzoquinone group of MMC must be reduced before DNA cross-linking can occur (Iyer and Szybalski, 1964). Despite this the concept of a bioreductive enzyme-directed approach to cancer therapy was not introduced until 1990 (Workman and Walton, 1990). Over the years many bioreductive agents have

been examined in the laboratory and several have even undergone clinical trials. Some of the more notable examples are porfiromycin, EO9, tirapazamine, and diaziquone. Despite the efforts of researchers around the world MMC remains the only clinically approved drug in this group (Beall and Winski, 2000).

DT-diaphorase is one of the enzymes involved in the activation of MMC and a number of other bioreductive agents (Riley and Workman, 1992). DT-diaphorase has a number of characteristics that make it a very promising target for enzyme directed therapy. DT-diaphorase is often overexpressed in tumor tissue (Cresteil *et al* 1991; Fitzsimmons *et al*, 1996), its levels increase with malignant progression (Mikami *et al*, 1998), it is found at low levels in the bone marrow (Siegel *et al*, 1991), and it can be selectively induced in tumor tissues (Begleiter *et al*, 1997). Studies have demonstrated that the anti-tumor activity of MMC can be enhanced by increasing the level of expression of DT-diaphorase (Begleiter *et al*, 2004). Due to the obvious potential of DT-diaphorase as a target enzyme for enzyme directed tumor targeting a number of studies have attempted to identify structural features of bioreductive agents that affect interactions with, and reduction by DT-diaphorase (Fourie *et al*, 2002; Chen *et al*, 1999; Li *et al*, 1995). As DT-diaphorase is able to catalyze the reduction of a wide variety of substrates it is difficult to single out characteristics required to confer DT-diaphorase specificity (Beall and Winski, 2000).

A number of promising compounds that are highly selective for activation by DT-diaphorase have been developed and examined in *in vitro* and *in vivo* situations with a few having been tested in the clinic. One of the most promising of these, EO9, was first identified in a study testing a large number of MMC analogues in the search for a

compound with lower toxicity (Speckamp and Oostveen, 1992). EO9 displayed good anti-tumor activity against human cancer cell lines *in vitro* as well as mouse tumors and human tumor xenografts *in vivo* (Smitskamp-Wilms *et al*, 1996; Hendriks *et al*. 1993). An important observation of preclinical and phase I trials was the absence of bone marrow toxicity (Hendriks *et al*, 1993; Schellens *et al*, 1994). Phase II clinical trials of this drug turned out to be a disappointment as no tumor response was observed (Dirix *et al*, 1996; Pavlidis *et al*, 1996). The most likely explanation for these results was the rapid elimination of the drug that has been seen both in animals and humans (Begleiter, 2000; Loadman *et al* 2000). The majority of the metabolism responsible for the clearance of EO9 from mice occurred in the liver and kidneys (Loadman *et al*, 2000). The major observed toxicity of EO9 was to the kidney manifested as proteinurea (Schellens *et al*, 1994).

Another recently developed DT-diaphorase selective drug, RH1, is currently in clinical trials (Cummings *et al*, 2003). RH1 is a very good substrate for DT-diaphorase but is also metabolized by P450R (Nemeikaitė-Čėniene *et al*, 2003). The efficiency of DT-diaphorase reduction of RH1 is so great that nearly all of the activation of the drug is due to DT-diaphorase activity (Beall *et al*, 1994). Recent studies involving RH1 have shown significant anti-tumor activity against NSCLC and colon cancer human xenografts (Cummings *et al*, 2003). RH1 is eliminated relatively rapidly from blood of mice; however, the rate of elimination is 10 times slower than that of EO9 and this may allow the drug to exist in circulation long enough to exert an anti-tumor effect (Loadman *et al*, 2000). One likely explanation for the lower rate of clearance of RH1 in comparison to EO9 is the reduced metabolism in the kidney; however, metabolism in the liver is more

rapid for RH1 than for EO9 (Loadman *et al*, 2000). The lower rate of metabolism by the kidney may have important implications for the use of RH1 in the clinic, as theoretically it should have reduced kidney toxicity in comparison to EO9. Even more interesting is the potential for combining RH1 therapy with DT-diaphorase inducers.

In this study we proposed that the use of a DT-diaphorase inducer would result in a greater enhancement of the anti-tumor activity of RH1 than was seen for MMC (Begleiter *et al*, 2004). As RH1 requires reductive activation by DT-diaphorase, myelosuppression should not be a major toxicity of this drug due to the very low levels of this enzyme in the bone marrow (Begleiter *et al*, 2004). In addition the reduced rate of kidney metabolism of RH1 should avoid the dose-limiting toxicity, proteinurea, observed for EO9. Since a recent study has shown that the dietary DT-diaphorase inducer DMF significantly increases the levels of the enzyme in kidney but not liver tissue (Begleiter *et al*, 2004) the rapid metabolism of RH1 in the liver should be unaffected by the addition of a DT-diaphorase inducer to the treatment. As a result, enhancement of RH1 toxicity by DT-diaphorase inducers should be minimal.

To determine if induction of DT-diaphorase enhances the anti-tumor affect of RH1 *in vitro*, the HCT116 human colon tumor cell line was selected as the experimental model as it was previously used in our laboratory to show that MMC antitumor activity is increased by inducing DT-diaphorase. The RH1 studies were paired with MMC for comparison purposes as enhancement of the anti-tumor activity of MMC has previously been shown in these cells when DT-diaphorase activity was induced by pretreatment with 1,2-dithiole-3-thione (D3T) (Wang *et al*, 1999). When treated for 48 hours with two known DT-diaphorase inducers, DMF and sulforaphane, a significant increase in DT-

diaphorase activity was observed with the magnitude of induction being 2.1 and 2.2-fold, respectively. This increase in DT-diaphorase activity resulted in a 1.37 and 1.65-fold increase in the anti-tumor activity of MMC for DMF and sulforaphane, respectively; however, no increase in the anti-tumor activity of RH1 was observed with either inducer. As RH1 is such a good substrate for DT-diaphorase and the concentrations of RH1 used were very low (0-300 nM) it is likely that the levels of DT-diaphorase found in the non-induced HCT116 cells (94-121 nmol min^{-1} mg^{-1}) were sufficient to fully activate the drug. Thus, increasing the level of DT-diaphorase in these cells by induction would not increase anti-tumor activity.

To test this hypothesis a second cell line was selected as the experimental model; T47D human breast cancer cells. The base levels of DT-diaphorase reported for these cells (27.9 ± 1.2) was 3-4 fold lower than that of the HCT116 cells (89.8 ± 11.9) in a previous study (Doherty *et al*, 1998). Pretreatment of the cells with DMF and sulforaphane for 48 hours resulted in a significant increase in DT-diaphorase activity of 1.9 and 2.8-fold respectively. These levels of induction resulted in a 2.35 and 1.91-fold increase in the anti-tumor activity of MMC respectively. Similar to the experiments with the HCT116 cells no enhancement of the RH1 anti-tumor effect was observed with either inducer.

The possibility that the lower base levels of DT-diaphorase in the T47D cells were still high enough for full activation of the drug still existed and was confirmed by results from a parallel study conducted by Winski *et al* (2001). They created a series of stably transfected BE cells (human colon tumor cells with no significant DT-diaphorase activity due to a mutation in the NQO1 gene) that expressed variable levels of DT-

diaphorase. They used this model to show that DT-diaphorase levels above 77 nmol/min/mg measured using the DCPIP method resulted in maximum activation of RH1. The DCPIP method measures the dicoumarol-inhibitable fraction of DCPIP reduction by cellular homogenates. It differs from the DT-diaphorase cycling assay we employed which measures the amount of dicoumarol inhibitable activity using menadione as the terminal electron acceptor. Studies in our lab using the two separate methods indicate that the activities observed with the DCPIP assay are approximately 2-3 times greater than the values observed using the cycling assay (unpublished data). Therefore the DCPIP measured activity of 77 observed by Winski *et al* (2001) would be equivalent to approximately 25-40 nmol/min/mg as measured by the cycling assay. As the base levels observed in the T47D cells were 26-37 nmol/min/mg (using the cycling assay) it is possible that maximum RH1 activation could occur in these cells without DT-diaphorase induction.

In light of this new evidence a third cell line with an even lower base DT-diaphorase level than the T47D cells was selected, HL60 human promyelocytic leukemia cells. Attempts to conduct *in vitro* cytotoxicity assays with these cells were unsuccessful as these cells grow as a suspension making MTT assays difficult. In addition the observed cloning efficiencies of these cells were less than 1% making them poor candidates for the clonogenic assay. A variation of the MTT assay using XTT as the substrate also proved to be unsuccessful as the HL60 cells did not metabolize this compound. As HL60 cells do grow well as xenografts in CD-1 nude mice we decided to conduct an *in vivo* study.

When mice implanted with HL60 tumors were placed on a diet containing 0.3% (w/w) DMF for a period of 7 days, a significant increase in DT-diaphorase activity was observed in both the tumor and kidneys. The observed 3.7 fold induction (from 100 ± 8.7 to 368 ± 35.8 nmol min^{-1} mg^{-1}) in the kidneys was higher than the 2.4 fold reported previously by Begleiter *et al* (2004). The 1.5 fold increase (from 3.6 ± 0.3 to 5.5 ± 0.6 nmol min^{-1} mg^{-1}) in DT-diaphorase activity in the HL60 tumors was considerably less than the 2.5 fold enhancement observed by Begleiter *et al* (2004) in the HCT116 cells *in vivo*.

The induction of DT-diaphorase in the HL60 tumors did not produce the expected increase in the anti-tumor activity of RH1 against these tumors. On day 9 of the study the tumors from the RH1 treated mice were 320% ± 39% of their day 0 size while the tumors from the DMF + RH1 combination groups were 319% ± 35%. It is likely that the small increase in DT-diaphorase activity in the tumors of the mice on the DMF supplemented diet is a probable explanation for the lack of RH1 enhancement. More surprising however was the observation that RH1 had no discernible anti-tumor activity on these tumors. A recent study by Cummings *et al* (2003) showed that RH1 had significant anti-tumor activity against 3 NSCLC and 1 colon cancer xenograft with final tumor volumes for the RH1 treated groups being 38-81% of the tumor volumes of the untreated mice after 16-21 days. The dose of RH1 they used in these studies was 2 X 0.75 mg/kg for a total dose of 1.5 mg/kg compared to the 5 X 0.4 mg/kg for a total dose of 2 mg/kg that was used in our study. The most likely explanation for this difference is that the HL60 cells are resistant to RH1. The DT-diaphorase levels may be responsible for this potential resistance if they are too low to activate the RH1. The study by Winski *et al*,

(2001) showed that at DT-diaphorase levels below a level of 23 nmol/min/mg as measured by DCPIP, no RH1 toxicity was observed in the transfected BE cells. Recall that this same study suggested a DT-diaphorase level of 77 nmol/min/mg as measured by DCPIP would result in maximum activation of RH1. As the Winski group did not have any transfected BE cells lines that expressed DT-diaphorase levels between 23 and 77 nmol/min/mg as measured by DCPIP it is quite possible that window between the upper and lower thresholds for RH1 activation could be considerably narrower. If the lower threshold for observed toxicity was significantly higher than the 23 nmol/min/mg as measure by DCPIP (approximately 8-12 nmol/min/mg as measured by cycling assay) it could explain the lack of RH1 activity against these tumors. This could also explain the lack of enhancement of RH1 activity observed with the addition of DMF to the diet, if the lower threshold is above the DMF induced levels of DT-diaphorase in the HL60 tumors there would be no effect.

The increased tumor growth rates observed in the group fed DMF diet and receiving saline injections is not a novel finding. This has been seen in a several tumor xenografts with a number of different DT-diaphorase inducing compounds in our lab, however these observations have not been published. No studies have been conducted on the reason for this phenomenon, however it may indicate that DT-diaphorase is acting as a tumor promoter.

Taken together, the studies using the HCT116 and T47D cells *in vitro* and the HL60 cells *in vivo* suggest that the strategy of inducing DT-diaphorase in tumor cells is not practical for RH1. The reason being that RH1 is too efficient a substrate for DT-diaphorase and too potent a drug. The added possibility that the window between the

upper and lower thresholds of RH1 activation may be too narrow further limits the use of the DT-diaphorase induction strategy for this drug. For the DT-diaphorase induction strategy to work, changes in DT-diaphorase activity must result in different levels of reduced RH1. If the window between lower and upper thresholds of activation is narrow the potential level of enhancement of RH1 would be small. The observation that this window exists at such a low DT-diaphorase level also severely restricts the potential of this approach as most tissues and tumors will have DT-diaphorase levels that fully activate all the RH1 present. The use of DT-diaphorase as the target in an enzyme directed approach to cancer therapy is due in part to the very low levels of the enzyme found within the bone marrow, a site of toxicity for the majority of anti-tumor agents (Siegel *et al*, 1991). The observation of leukopenia in our studies indicates that the levels of DT-diaphorase in the bone marrow are above the upper threshold of RH1 activation.

This is a very important point to consider now that RH1 is in clinical trials. With RH1 being fully activated in tissues such as the bone marrow it indicates that this agent may not be suitable for an enzyme directed tumor targeting approach. RH1 will be activated in nearly every tissue of the body. If RH1 does not have any other mechanism to make it selective against tumor cells it will likely result in toxicity to the actively dividing cells of the body including the bone marrow, gastrointestinal tract lining and hair follicles. These toxicities may limit the dose of drug that can be administered to the patient to ineffective levels. Very little information on the toxicity of RH1 has been made available to the scientific community and as such this is a good area for further research.

We used the CD-1 nude mouse model to measure a number of toxicity markers following 7 days on a diet with or without DMF supplement and treatment with or without RH1. Serum clinical chemistry tests revealed no obvious effect of RH1 or DMF, alone or in combination on liver and kidney function and no signs of dehydration. Histological examination of the kidney, liver, lung, heart, colon and forestomach showed no evidence of toxicity to any of these organs. Evaluation of body weights of the mice during and after treatment with RH1 did show signs of treatment associated weight loss with body weights decreasing throughout the treatment period. Immediately following cessation of RH1 treatment, body weights began to increase indicating that this weight loss is reversible. When compared by ANOVA, the DMF and RH1 combination group had body weights significantly lower than the control group from day 3 through day 11. Although not statistically significant, the RH1 alone group exhibited a strong trend towards reduced body weight. In addition there was a trend suggesting that DMF increased the weight loss associated with RH1 treatment but this difference was not statistically significant based on this experiment.

Platelet counts obtained from the four groups of mice showed no thrombocytopenia; however, leukopenia was an obvious toxicity due to RH1 and was not influenced by the presence or absence of DMF in the diet. The nadir occurred 6 days after the onset of RH1 treatment (1 day following cessation of RH1 treatment) with total WBC counts decreasing by 73% and 64% of the control group counts for the RH1 and RH1 + DMF groups, respectively. The reduction in WBC counts also appeared to be a reversible toxicity with the WBC levels increasing to normal levels 7 days after the fifth and final RH1 treatment. The myelosuppression observed in these experiments was far

greater than the 25% and 45% observed for 2.0 and 3.5 mg/kg single doses of MMC by Begleiter *et al* (2004). The nadir occurred 3 days after treatment with MMC and 1 day after the last dose of RH1. These differences are likely due to the 5 daily doses of RH1 rather than the single dose of MMC. In the case of both MMC and RH1 the WBC counts took about one week to recover to control levels. These results strongly disprove the original hypothesis that RH1 would be less toxic to the bone marrow than MMC and would suggest that this will be a major toxicity observed in the phase I trials currently underway.

Berardini *et al* (1993) and others have demonstrated that DNA cross-linking is the major mechanism through which RH1 exerts its cytotoxic effects. Using a cell free *in vitro* system we determined the amount of cross-linking produced by RH1 in the presence and absence of DT-diaphorase. The results clearly indicated that RH1 without DT-diaphorase activation was unable to produce DNA cross-links. When DT-diaphorase and its required cofactors were introduced to the system there was a very significant amount of observed cross-linking. These results confirm that RH1 acts as a bioreductive agent and that DT-diaphorase is capable of activating RH1 by the reduction of the benzoquinone group. Although these results provide evidence that DT-diaphorase can act as an activating enzyme for RH1 they do not rule out the possible involvement of other reductases in this process. If the hypothesis that HL60 cells are resistant to RH1 because of their low DT-diaphorase activity is correct this would then indicate that the involvement of other reductase enzymes that are expressed by the HL60 cells do not contribute significantly to the activation of RH1.

Conclusion

Based upon the results obtained in our experiments, RH1 does not appear to be a suitable drug for use in combination therapies with inducers of DT-diaphorase. This is due to the very narrow range of DT-diaphorase activity where only a portion of the RH1 is activated. In addition the low levels of DT-diaphorase that are required to fully activate RH1 indicate that this drug will be converted to the active hydroquinone form in most tissues of the body making it very non-selective. As a result, RH1 is likely not a suitable agent for an enzyme-directed form of therapy, and may even prove to be toxic enough to the bone marrow that it will not be an effective agent for the treatment of cancer. The answer to this question will come from the clinical trials currently underway. Although RH1 may not be a suitable drug for this approach, a few minor changes to structural elements of the drug could have a drastic effect on the reduction of the quinone by DT-diaphorase. The studies of Fourie *et al* (2002 and 2004) showed that the addition of functional groups to benzoquinone mustards affected the rate of reduction by DT-diaphorase. Therefore by altering the structure of RH1 it may be possible to produce a compound that has the upper and lower thresholds of DT-diaphorase activation of RH1 in a range suitable for this enzyme-directed approach to cancer therapy.

Future Directions: Increasing the Effectiveness of Bioreductive Antitumor Agents

Despite the work that has been done in the past, the lack of widespread use of bioreductive drugs in the clinic indicates that a great deal more must still be done. The most obvious future pursuit is the development of new bioreductive agents. A single

reductase enzyme, preferably one that is commonly overexpressed in human tumors to help minimize toxicity would selectively activate the ideal new agent. These new compounds may be novel structures or they may be analogues of currently known bioreductive agents. The use of structure-activity studies will play an important role in this process as they seek to identify specific functional groups that make the compound more specific for activation by a particular enzyme. Structure-activity studies such as those conducted by Fourie *et al* (2002), Phillips *et al* (1999), and Beall *et al* (1998) for DT-diaphorase have already provided some insight into the types of groups that provide specificity. In addition the drug must also be stable under physiological conditions and not quickly eliminated or metabolized, as was the case with EO9, to allow enough time for the drug to act upon the tumor cells.

Additionally more successful methods (induction, ADEPT, GDEPT) of selectively increasing the activity of the target enzyme within the tumor would further minimize toxicity. The same effect may also be achieved by developing patient-tumor profiling techniques to help identify the best possible target enzymes for a particular patient, and then selecting the appropriate drug, or drug combination (Workman and Stratford, 1993).

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MARGARET FOTI, Ph.D., M.D. (h.c.)
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May 11, 2004

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