ENHANCEMENT OF ANTITUMOUR ACTIVITY AND STRUCTURE-ACTIVITY STUDY OF BIOREDUCTIVE AGENTS

BY

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

MASTER OF SCIENCE

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Abstract

DT-diaphorase is a two-electron reducing enzyme that activates the bioreductive antitumour agent, mitomycin C (MMC). Cell lines having elevated levels of DT-diaphorase are generally more sensitive to MMC. We have shown that DT-diaphorase can be induced in human tumour cells by a number of compounds including 1,2-dithiole-3-thione (D3T) and many dietary inducers. In this study, we investigated whether induction of DT-diaphorase by inducers could enhance the cytotoxic activity of MMC in 6 human tumour cell lines representing 4 tumour types. Cytotoxicity of MMC was significantly increased in 4 tumour lines with the increase ranging from 1.4- to 3-fold. There was a 50% increase in normal human bone marrow toxicity by using this combination approach, but the increase is small in comparison with 3-fold increase in cytotoxicity to tumour cells. This study demonstrates that induction of DT-diaphorase can increase the cytotoxic activity of MMC in human tumour cell lines and suggests that it may be possible to use non-toxic inducers of DT-diaphorase to enhance the efficacy of bioreductive antitumour agents.

Bioreductive antitumour agents are uniquely suited to the improvement of tumour selectivity by an "enzyme-directed" approach to tumour targeting. However, none of the bioreductive agents developed to date have been specific for activation by a single reductive enzyme, in part, due to a lack of knowledge of structural factors that produce selectivity for activation by reductive enzymes. We used a series of model benzoquinone mustard bioreductive agents to investigate the role of functional groups in modifying the specificity for drug activation by DT-diaphorase. We compared the parent agent, benzoquinone mustard (BM), with a series of analogues having different functional groups in their structures to

identify structure-activity relationships. We found that methoxy, phenyl and chloro functional groups decreased the rate of reduction of the quinone group by DT-diaphorase. The methoxy group resulted in DT-diaphorase becoming an activating enzyme for 5-methoxy-BM (MBM) compared to an inactivation enzyme for BM. The functional groups also affected the ability of the reduced product to undergo redox cycling.

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Abbreviations

AQ4N	1,4-bis-{[2-(dimethylamino-N-oxide)ethyl]amino}5,8-dihydrozy-anthracene-9,10-dione
ARE	Antioxidant response element
AZQ	2,5-Diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone
BHA	2(3)-*tert*-Butyl-4-hydroxyanisole
BM	2-(Di(chloroethyl)amino-1,4-benzoquinone
CB 1954	5-Aziridin-1-yl-2,4-dinitrobenzamide
CBM	5-Chloro-2-(di(chloroethyl)amino-1,4-benzoquinone
CDNB	1-Chloro-3,4-dinitrobenzene
D3T	1,2-Dithiole-3-thione
DMEM/F12 (1:1)	Dulbecco's modified eagle medium: nutrient powder F-12 (Ham) (1:1)
DMSO	Dimethyl sulfoxide
DT-diaphorase	NAD(P)H:(quinone acceptor) oxidoreductase
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
GST	Glutathione *S*-transferase
HPLC	High performance liquid chromatography
MBM	5-Methoxy-2-(di(chloroethyl)amino-1,4-benzoquinone

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 (reduced form)
NADP	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate (reduced form)
NSCLC	Non small-cell lung cancer
PBM	6-Phenyl-2-(di(chloroethyl)amino-1,4-benzoquinone
PBS	Phosphate buffered saline
PHMB	P-hydrooxymercuribenzoic acid
PMSF	Phenylmethyl sulfonylfluoride
SCLC	Small cell lung cancer
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
XRE	Xenobiotic response element

INTRODUCTION

Bioreductive antitumour agents

Background

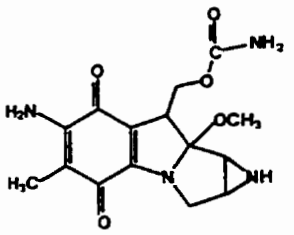
Bioreductive antitumour agents are a relatively new class of anticancer drugs. These agents exert their antitumour functions by various mechanisms but are characterized by a requirement for reductive activation by intracellular enzymes (Workman and Stratford, 1993: Adams et al, 1994). The prototype drug in this class is mitomycin C (MMC) which has been used in the clinic for over 20 years in treating various solid tumours (Rockwell et al, 1993). This class of bioreductive agents also includes porfiromycin, 3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol (EO9) (Schellens, 1994), tirapazamine (Bedikian et al, 1997: Miller et al, 1997), 5-aziridin-1-yl-2,4-dinitrobenzamide (CB 1954), 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone (MeDZQ) (Beall et al, 1995), streptonigrin(Beall et al, 1996), 1,4-bis-{[2-(dimethylamino-N-oxide)ethyl]amino}5,8-dihydrozy-anthracene-9,10-dione (AQ4N) (Wilson et al, 1996) and diaziridinylbenzoquinone (AZQ) (Gibson et al 1992).

The three most important types of bioreductive agents are: (1) the benzotriazine-di-N-oxides (such as tirapazamine) (Workman et al, 1993), (2) the dual function alkylating nitro compounds (such as RSU 1069/RB 6145), and (3) quinone derivatives (such as MMC, porfiromycin, EO9, AZQ and MeDZQ) (Rauth et al, 1993; Workman and Stratford, 1993; Siegel et al, 1990; Gibson et al, 1992). Bioreductive agents contain a bioreductive element and a cytotoxic element. In some cases, like tirapazamine (Patterson, 1993), the bioreductive element may also act as the cytotoxic element, while some other agents, like

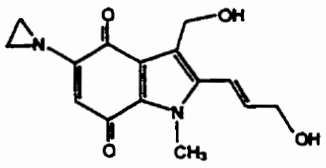
MMC, have additional cytotoxic elements that are regulated by the bioreductive element (Marshall *et al*, 1989; Tomasz et al, 1987). The three principle reductive elements are; nitro, N-oxide and quinone groups (Workman and Stratford, 1993). Structures of some bioreductive quinones, nitro compounds and tirapazamine are given in Figure 1.

There has been much interest in studying which enzymes are involved in the activation of bioreductive agents, particularly the clinically-used quinone drug, MMC, and related compounds (Sartorelli, 1988). These agents can be activated by one electron reducing enzymes such as NADPH:cytochrome P450 reductase (EC 1.6.2.4) (Joseph et al, 1994; Pan et al, 1994), NADH:cytochrome b5 reductase (EC 1.6.2.2) (Hodnick and Sartorelli, 1993) and xanthine oxidase (EC 1.1.3.22) (Pan et al, 1994), and by two electron reducing enzymes such as NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase) (EC 1.6.99.2) (Riley and Workman, 1992; Ross et al, 1993; Begleiter et al, 1989) and xanthine dehydrogenase (EC 1.1.1.204) (Gustafson and Pritsos, 1992). NADPH:cytochrome P450 reductase appears to be the most important activating enzyme involved in bioactivation of many of the drugs, but DT-diaphorase is also an important contributor for the activation of many bioreductive antitumour agents (Rockwell, 1993; Ross et al, 1993; Begleiter et al, 1992).

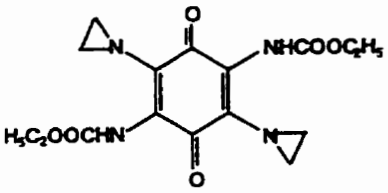
The reduction of the bioreductive agents by one-electron reducing enzymes can form radical intermediates which are reactive and can directly bind to DNA and protein to cause cell damage. The reduction of the bioreductive agents can also activate the cytotoxic element resulting in cellular damage. The intermediates can also go back to original form by back oxidation under aerobic condition (redox cycling) and generate superoxide anion radical, which can contribute to the cytotoxicity of the bioreductive agents by producing



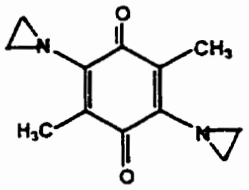
Mitomycin C



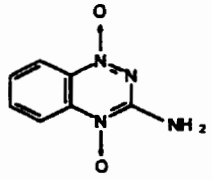
EO9



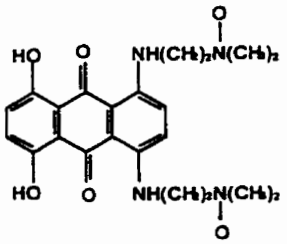
AZQ



MeDZQ



Tirapazamine



AQ4N

Figure 1: Bioreductive antitumour agents

DNA strand breaks and lipid peroxidation. Generally, this mechanism is not a major contributor to the antitumour activity of bioreductive agents (Rockwell et al, 1993). The reactive intermediates can undergo further one-electron reduction to form two-electron reduced intermediates, a process that is favored under hypoxia. This results in activation of a cytotoxic element like an alkylating group present in MMC, EO9 and MeDZQ, producing DNA damage by covalent reaction with DNA. The reduction of bioreductive agents by two-electron reducing enzymes can form two-electron reduced intermediates similar to those produced by sequential one-electron reduction. The end products can directly contribute to the cytotoxicity of a bioreductive agent by covalently binding proteins and/or DNA and by intercalating or cross-linking DNA.

New developments in bioreductive therapy

Interior regions of solid tumours are often poorly oxygenated, this is one mechanism responsible for the low response of some tumours to chemotherapy and radiation. Bioreductive agents are generally more toxic under hypoxic conditions because redox cycling does not occur in the absence of oxygen. This feature gives bioreductive agents potential value in the treatment of solid tumours that contain hypoxic regions compared to the normal tissues. Newer bioreductive agents, such as EO9 (Schellens, 1994), tirapazamine (Bedikian et al, 1997: Miller et al, 1997), AZQ, MeDZQ (Beall et al, 1995), RB 6145/RSU 1069, and streptonigrin (Beall et al, 1996), have been developed and extensively studied for use in treating solid tumours.

Interest has been raised by recent studies of combining bioreductive agents with radiation or other antitumour drugs to increase the anticancer effects. Radiation has been

tested in combination with tirapazamine, the nitroimidazoles RB 6145/RSU 1069 and various quinones (such as MMC and EO9) in many tumour models, such as head and neck tumours, NSCLC, and metastatic melanoma (Haffty et al, 1997; Adams et al, 1992). High doses of X-ray are most effective to kill the aerobic region of the tumour and less effective against hypoxic part. Immediately after irradiation, bioreductive agents are administered in an attempt to eliminate the residual hypoxic cell population and thereby increase the response of the tumours. Haffty et al (1997) have assessed the impact of the addition of mitomycin to radiotherapy for head and neck tumours and showed better response compared to irradiation alone. Phase I trials of combining tirapazamine with cytotoxic chemotherapy were first carried out in early 1990s. Several phase I and II studies have evaluated the efficacy of tirapazamine in combination with cisplatin in the treatment of non-small cell lung cancer (NSCLC) and showed better responses compared with the traditional treatment of this disease (Miller et al, 1997). Studies of combination of cisplatin and tirapazamine were also carried out in metastatic melanoma; a better response was also reported following this treatment strategy (Bedikian et al, 1997). Two large scaled phase II trials of tirapazamine and cisplatin, involving more than 70 centers, to confirm the synergy of this treatment strategy are in process. The cytotoxicity of tirapazamine was also increased when used in combination with melphalan, cyclophosphamide, etoposide, and bleomycin.

Mitomycin C

Structure and background

MMC is a quinone containing natural antibiotic that exhibits activity against a variety of human tumours, such as non-small cell lung, colorectal, and gastric cancer (Crooke and

Bradner, 1976; Gillis, 1996). It was first isolated from *Streptomyces Caespitosus* in 1958 by Wakaki and his colleagues (Cummings et al, 1995). MMC have been used in the treatment in various solid tumours. MMC contains an aziridine group, a quinone group, as well as a mitosane ring in its structure, and each of these can participate in covalent reactions with DNA (Structure of MMC is shown in Figure 1).

Clinical use of MMC

MMC has been used in the treatment of breast (Hortobagyi, 1993), non-small cell lung (Spain, 1993), head and neck (Coia, 1993), colorectal (Cummings et al, 1993), prostate cancer (Verweij) and gastric (Fujita et al, 1998) cancer. In general, MMC produces an overall response rate of 15-30% when used as a single agent. Toxicity of MMC highly reduces the response rate due to the limited dosage and treatment period that can be used. Higher response rates were achieved by combining MMC with other agents. Hortobagyi et al (1993) suggested that MMC along with doxorubicin are the two most potent single agents in breast cancer. MMC alone produced responses of 26-38% in previously untreated metastatic breast cancer and 15-25% in previously treated patients. Combination therapies with MMC and agents including doxorubicin, vincristine, vinblastine, mitoxantrone, methotrexate and fluorouracil produced responses of 15-77% in over 1200 patients with advanced or metastatic breast cancer (Jodrell et al, 1991). In the treatment of anal cancer, MMC is mostly combined with 5-fluorouracil in conjunction with radiotherapy. A study showed a significantly better rate of disease free survival of patients receiving combination therapy of MMC and fluorouracil compared with the patients receiving only fluorouracil (Flam et al, 1996). Similar results have been reported in combination therapy trials in head

and neck cancer (Haffty et al, 1997). Clinical trials have been testing the usefulness of the addition of MMC to radiation therapy for the treatment of solid tumours. Radiotherapy combined with MMC showed an improvement in disease-free survival compared to radiation or MMC alone in anal cancer and head and neck cancer treatments (Flam et al, 1996; Haffty et al, 1997). In these studies, MMC had a relatively large effect on the outcome of the cancer treatment.

Clinical toxicity of MMC

The major toxicity associated with the use of MMC is myelosuppression, which is dose related (Crooke and Brandner, 1976). The myelosuppression is characterized by marked leukopenia and thrombocytopenia. This toxicity may be delayed and cumulative, with recovery only after 6 to 8 weeks of pancytopenia. Nausea, vomiting, diarrhea, stomatitis, dermatitis, fever, and malaise are also observed after MMC administration. This drug may also potentiate the cardiotoxicity of doxorubicin when used in conjugation with this drug (Goodman, 1990).

Bioactivation and cytotoxicity of MMC

Several enzymes have been reported to be involved in the bioreductive activation of MMC. The two most important activating enzymes for catalyzing the activation of MMC are the one-electron reducing enzyme NADPH:cytochrome P450 reductase (Bligh et al, 1990), and the two-electron reducing enzyme DT-diaphorase (Keyes et al, 1984, Siegel et al, 1992). Figure 2 shows the bioreductive activation processes of MMC by NADPH:cytochrome P450 reductase and DT-diaphorase.

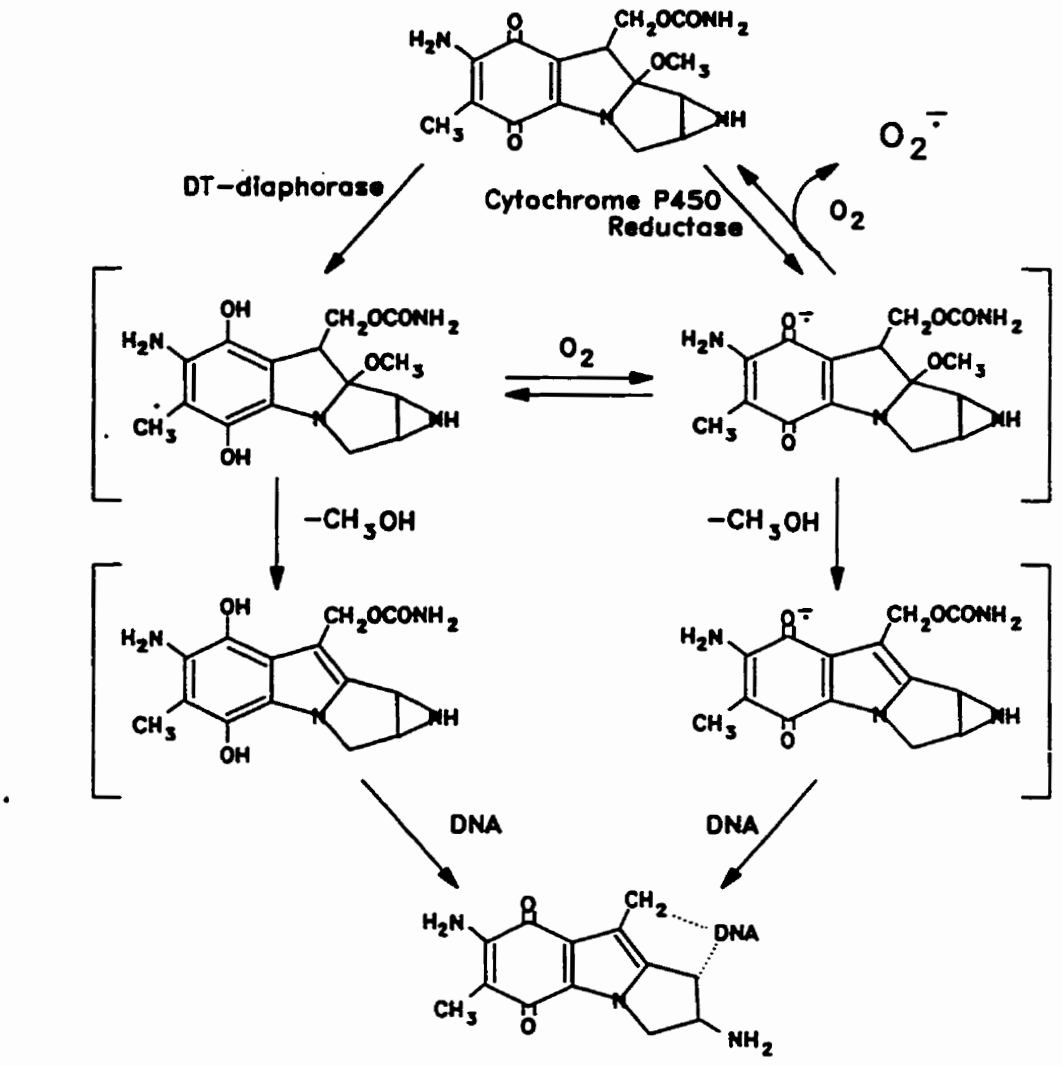


Figure 2: Bioreductive activation of MMC by NADPH:cytochrome P450 reductase and DT-diaphorase

Other enzymes, including NADH:cytochrome b5 reductase (Hodnick and Sartorelli, 1993), xanthine oxidase (Shao et al, 1995) and xanthine dehydrogenase (Gustafson and Pritsos, 1992; Pan et al, 1994), have also been reported to play a role in the activating process of the bioreductive agent, MMC.

Activation of MMC by either one-electron transfer enzymes or by the two-electron transfer enzymes generates reactive electrophilic species, which can alkylate cellular nucleophiles (Kennedy et al, 1980; Powis, 1987). The reactive species are capable of forming either DNA monoadducts or DNA crosslinks (Ross, 1993; Tomasz et al, 1987; Pritsos et al, 1986; Lown et al, 1976; Powis, 1987). However, crosslinking appears to be the most important mechanism for antitumour activity (Iyer and Szybalski, 1964; Powis, 1987; Rockwell et al, 1993).

Bligh has shown that expressing a rat liver NADPH:cytochrome P450 reductase in a *salmonella typhimurium* strain increased the sensitivity to MMC under aerobic conditions, suggesting that NADPH:cytochrome P450 reductase plays a role in the cytotoxic action of MMC (Bligh et al, 1990). NADPH:cytochrome P450 reductase transfers one electron from NADPH to MMC and forms a semiquinone anion radical. Under aerobic conditions, redox cycling can occur as the semiquinone reacts with the oxygen, and as a consequence, MMC is converted back to parent molecule. Reactive oxygen species (i.e., superoxide, hydrogen peroxide, and /or hydroxyl radical) formed through this redox cycle can trigger DNA strand breaks (Pan et al, 1984; Pritsos et al, 1986; Ross et al, 1994). But the toxicity generated by the reactive oxygen species is not considered as a major contributor to the antitumour activity of MMC. In fact, it may decrease the cytotoxicity of MMC by converting the cytotoxic reduced intermediates back to the parent form (Kappus, 1986: Butler et al, 1987;

Ross et al, 1994). Under hypoxic conditions, the semiquinone intermediates, or the hydroquinone intermediates, which are formed by further one electron reduction, can undergo structural rearrangement and lead to the formation of monofunctional or bifunctional alkylating species which then form DNA adducts.

Reduction of MMC through the two-electron reducing pathway by DT-diaphorase results in the formation of a hydroquinone. This pathway is not inhibited by aerobic conditions since the initially formed hydroquinone is not as susceptible to redox cycling as the semiquinone (Siegel et al, 1990; Workman and Stratford, 1993). After reduction of MMC, the methoxyl group is eliminated as methanol and the aziridine ring is activated to produce an alkylating site at the C1 position. The subsequent loss of the carbamoyl group generates a second alkylating site which can cross-link macromolecules through the C10 position of MMC (Powis, 1987). The activating process of MMC by DT-diaphorase is pH-dependent in cell-free systems and increases as the pH is decreased from 7.8 to 5.8 (Schlager and Powis, 1988; Siegel et al, 1993).

Activating enzymes for bioreductive antitumour agents

NADPH:cytochrome P450 reductase

NADPH:cytochrome P450 reductase is a membrane bound protein, which can be detected in liver, small intestine, pancreas, lung and kidney, and belongs to the cytochrome P450 monooxygenase system (Goldstein and Faletto, 1990). NADPH:cytochrome P450 reductase is responsible for oxidative metabolism of both exogenous compounds, including therapeutic drugs, toxins and carcinogens, and endogenous compounds including steroids and prostaglandins. NADPH:cytochrome P450 reductase is important in activation of

carcinogens by P450 enzymes and is a major contributor to the activation of bioreductive agents such as MMC (Pan et al, 1984; Bligh et al, 1990; Hoban et al, 1990) and tirapazamine (Riley, et al, 1993). NADPH:cytochrome P450 reductase has a molecular weight of 77.5 kDa and contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). It uses NADPH as the electron donor and transfers electrons to cytochrome P450 or bioreductive agents (McManus et al, 1989). The amino acid sequence of NADPH:cytochrome P450 reductase is highly homologous among species (Shen et al, 1993). This enzyme can be induced by dexamethasone, isosafrole vitamin E, phenobarbital, and some carcinogens, but most studies have been done in liver cells and it is not clear if NADPH:cytochrome P450 reductase can be induced in other tissues.

DT-diaphorase

Historical background

DT-diaphorase was first discovered by Ernster and Navazio in 1958 and described as a cytosolic-soluble diaphorase that utilized both the cofactors NADH and NADPH with equal facility (Ernster and Navazio, 1958). With further studies of this enzyme, a variety of names have been used including NAD(P)H:menadione reductase, NAD(P)H:(quinone acceptor) oxidoreductase, quinone reductase and azo-dye reductase.

During the purification and characterization of DT-diaphorase, Ernster and colleagues (1960) found that the anticoagulant dicoumarol was a highly efficient inhibitor of the enzyme. This property of dicoumarol helped the studies on the role of DT-diaphorase in the biosynthesis of quinone-containing vitamin K and coenzyme Q (also known as ubiquinone) (Ernster 1987). More recent work has indicated that DT-diaphorase may keep

these quinones in their reduced forms which facilitates the posttranslational modification of vitamin K (Wallin et al, 1987) and the function of coenzyme Q as an antioxidant (Beyer et al, 1996).

Huggins and coworkers found another physiological role for DT-diaphorase in the early 1960s (Ernster, 1987). Pretreating rats or mice with a small dose of polycyclic hydrocarbons or azo dyes prevented the toxicity of subsequent high doses. This prevention was correlated with increased DT-diaphorase activity suggesting that DT-diaphorase may detoxify a variety of xenobiotics including quinones, azo-dyes and nitro-compounds (Ernster et al, 1987).

Iyangi and Yamazaki (1970) discovered another novel feature of DT-diaphorase, This enzyme can catalyse obligatory two-electron reduction which is important for its role in cytoprotection against toxic chemicals. One of the mechanisms of how two-electron reducing enzymes detoxify toxins and prevent cancer formation is through detoxifying the toxic free radicals that can damage DNA and result in cancer formation. One-electron reducing enzymes, such as cytochrome b5 reductase and NADPH:cytochrome P450 reductase, reduce quinone-containing compounds to semiquinones, which are easily reoxidized to form reactive oxygen intermediates during this process. The reactive oxygen intermediates can attack cell macromolecules, including DNA to yield strand breaks and mutations. DT-diaphorase reduces the quinones to the relatively redox stable hydroquinone state. Conjugation of the hydroquinone by other phase II enzymes like the glutathione-S-transferase and UDP-glucuronosyltransferase leads to the elimination of the toxins (Cadenas et al, 1995).

Distribution and expression of DT-diaphorase in normal and tumour tissues

DT-diaphorase exists ubiquitously in eukaryotes. More than 90% of this enzyme is located in cytosol, the other 5-10% is in membrane bound form and is found in subcellular organelles, such as mitochondria, microsomes and Golgi apparatus (Edlund et al, 1982; Riley et al, 1992). The enzyme can be detected widely among organs with various levels of activity, and is particularly high in kidney, intestine and stomach, but generally very low in hemopoietic cells (Spencer et al, 1990; Schlager and Powis, 1990; Benson et al, 1980).

Many studies have shown that the expression of DT-diaphorase is upregulated in most tumour tissues and cells compared to normal cells of the same origin (Belinsky and Jaiswal, 1993; Smitskamp-Wilms et al, 1995). It has been observed that expression of DT-diaphorase is higher in human hepatomas, colonic carcinomas (Belinsky and Jaiswal, 1993), NSCLC (Schlager and Powis, 1990; Ross et al, 1994) and human liver tumours (Cresteil and Jaiswal, 1991). Schlager and Powis also reported increased levels of DT-diaphorase activity in lung, liver, colon and breast tumours (Schlager and Powis, 1990). The surrounding normal tissues of the hepatic tumours express higher level of DT-diaphorase activity compared to the normal tissue from the same region. It is assumed that soluble factor(s) which may exist in tumour tissues could diffuse into the surrounding tissue and upregulate the expression of DT-diaphorase (Cresteil and Jaiswal, 1991).

Smoking and alcohol can alter the expression of DT-diaphorase. Schlager and Powis (1990) reported that non-smokers appear to have higher DT-diaphorase activity in tumour tissues, whereas smokers have similar enzyme activity compared with the normal tissue from the same region. Consumption of alcohol seems to increase the enzyme activity in lung tumours with no effect on the normal tissues (Schlager and Powis, 1990).

Molecular biology

Gene family

Since DT-diaphorase plays an important role in activation of many bioreductive agents, there have been great efforts in trying to understand how this enzyme is regulated in cell systems. In humans, there are several forms of NAD(P)H:quinone oxidoreductases (NQOs) that are encoded by four gene loci. NQO1 gene is the most extensively studied and appears to be the most important form involved in chemoprevention and bioactivation of bioreductive agents (Edward et al, 1980). This gene is located on chromosome 16 and codes for DT-diaphorase or diaphorase 4. Jaiswal et al (1991) showed that the human gene is 20 kb in length and consists of six exons and 5 introns. DT-diaphorase mRNA has an extremely long 3' untranslated region and a coding region of 822 nucleotides. They identified four potential polyadenylation sites that are responsible for the formation of three mRNAs of 1.2, 1.7, and 2.7 kb. The possible roles of these mRNA isoforms in regulating DT-diaphorase activity are not clear.

Jaiswal and coworkers (1988, 1990) identified a second human gene isolated from human liver and named it NQO2 to distinguish it from NQO1. NQO2 gene is located on human chromosome 6 and was shown to be highly polymorphic. The polymorphism of chromosome 6 which contains NQO2 gene is often associated with the development of ovarian and colorectal cancer (Jaiswal, 1994). In contrast to NQO1 gene, which is expressed in all tissues, the NQO2 gene is expressed only in several human tissues including heart, lung, liver, skeletal muscle, kidney and pancreas (Jaiswal, 1994). The human NQO2 cDNA codes for a protein of 231 amino acid that is 43 amino acids shorter than the NQO1 protein. Comparison of the cDNA and the protein of human NQO2 and NQO1 showed 54%

and 49% similarity, respectively. The NQO2 protein is normally expressed at low levels, is 50-100 times less active in reducing menadione than DT-diaphorase (NQO1) and is not inducible by dioxin. (Joseph et al. 1994).

Gene structure and regulatory elements

The NQO1 gene contains several transcriptional activators regulating the expression of DT-diaphorase. The 5' upstream region of the DT-diaphorase coding sequence contains an antioxidant response element (ARE), a xenobiotic response element (XRE), a NF-kB binding site, an AP2 site, and a TATA box.

ARE, also known as electrophile response element (EpRE), contains three copies of AP1/AP1 like elements (TGACTCA) and a GCA box. ARE is the most important cis-element that is responsible for high basal expression of NQO1 gene in tumour tissues and for induced expression of this gene by xenobiotics and antioxidants. Similar ARE elements have been reported in the regulatory regions of the rat and mouse GST Ya genes. Mutation in the region of AP1 binding site of the NQO1 gene resulted in the loss of both the basal and the induced expression of the NQO1 gene. Band shift and supershift assays revealed that the transcription factors, Jun-D, Jun-B and C-Fos, and other unidentified regulatory proteins, bind to the AP1 sequences contained within the NQO1 gene ARE and mediate the regulation of NOQ1 gene expression (Li and Jaiswal, 1992; Favreau and Pickett, 1993). This is consistent with the increase in DT-diaphorase activity in small cell lung cancer (SCLC) cells when they are transfected with Ras gene, since Ras transfection is known to increase the levels of Jun and Fos (Malkinson et al, 1992; Sistonen et al, 1989). Studies have been done regarding the signal transduction mechanism of monofunctional (β-NF) and bifunctional

inducers (BHA) to increase the expression of DT-diaphorase. A model has been proposed to explain the mechanism that may account for the induction of NQO1 gene by mono and bifunctional inducers. Xenobiotics and antioxidants, after reductive metabolism, produce a "redox signal" (De Long et al, 1987) which may modulate Ref-1 or other unknown redox proteins that can increase the binding of Jun and Fos to the AP1 sites to activate the transcription of NQO1 gene (Jaiswal, 1994; Yao et al, 1994). Alternatively, the redox signal may activate protein kinase(s), such as PKC or others unknown kinase(s), which phosphorylate Jun and Fos proteins for increased binding to the ARE resulting in increased transcription and expression of the NQO1 gene. Further studies are needed to fully understand the mechanism of induction of the NQO1 gene.

The induction of the NQO1 gene expression by dioxin is mediated by the XRE sequence rather than the ARE sequence. It has been suggested that dioxin forms a complex with the Ah receptor (protein product of the aromatic hydrocarbon locus) and then binds to the XRE sequence in the 5' upstream region of the NQO1 gene (Jaiswal, 1991; Favreau and Pickett, 1991).

There is also evidence that the NF-kB response element in the 5' upstream region of NQO1 gene may be involved in the increased DT-diaphorase induced by hypoxia, MMC and oltipraz (Yao et al, 1995; Yao et al 1997). Xie and Jaiswal (1996) reported that the AP2 sequence might be involved in the basal and increased NQO1 gene expression induced by cAMP.

Induction of NQO1 gene expression

The expression of DT-diaphorase is highly inducible by a number of chemicals including

steroids, drugs, industrial chemicals and dietary constituents. Talalay (1989) has categorized enzyme inducing agents into two classes as monofunctional and bifunctional. Monofunctional inducers, including phenolic antioxidants, 1,2-dithiol-3-thiones, aromatic isothiocyanates, coumarins, thiocarbanates and, cinnamates, increase the activity of phase II detoxifying enzymes (such as glutathione S-transferases, DT-diaphorase, UDP-glucuronosyltransferases and epoxide hydrolase) in various tissues without significantly elevating phase I enzymes (e.g., cytochromes P450). Bifunctional inducers, such as 2,3,7,8-tetrachloradibenzo-p-dioxin (TCDD), polycyclic aromatic hydrocarbons, azo dyes and flavonoids, induces both phase I and phase II enzymes. Certain phase I enzymes are generally thought to be responsible for functionalizing hydrophobic, xenobiotic, endogenous compounds and some carcinogens by oxidation or reduction and converting them to more polar products which can interact with the nucleophilic centers of DNA. These toxic electrophilic metabolites activated by phase I enzymes can be conjugated with endogenous ligands by phase II enzymes to hydrophobic metabolites and excreted out of the body. This reduces the susceptibility of cells to the toxic electrophiles and decreased the incidence of tumour formation (Talalay, 1989). Prestera et al (1993) suggested this constellation of protective metabolic responses be designated as the electrophile counterattack.

Studies were carried out in a Hepa 1c1c7 murine hepatoma cell line to quantify the potency of inducers of DT-diaphorase (Talalay et al, 1989; Prochaska and Santamaria, 1988). The results revealed at least seven chemically distinct families of monofunctional inducers. These inducers are different in their structure but all share a common characteristic of being able to interact with sulfhydryl groups whether by nucleophilic substitution or oxidoreductions. It is postulated that the interaction between the inducer and the sulfhyldryl

groups of a target protein generates a signal for induction. This proposal is supported by a positive correlation between the inducing potency and the affinity for sulfhydryl groups (Putzer et al, 1995).

Structure of DT-diaphorase protein

The structure of rat DT-diaphorase has been revealed using X-ray diffraction studies by Li et al in 1995. Rat DT-diaphorase is a homodimer with each subunit of a molecular mass of 30,784 Da. Each subunit contains two separate domains, a major catalytic domain (residues 1-220) consisting of 7 α-helices and 7 β-sheets and a small C-terminal domain (residues 221-273) consisting of 1 α-helics, 2 β-sheets and several loops (Li et al, 1995). During dimerization of the two subunits, two identical catalytic domains for NAD(P)H, FAD, and the enzyme substrate are formed. The overlap of the binding sites for NAD(P)H and substrate is consistent with the ping-pong mechanism for DT-diaphorase (NAD(P)H on-off, and then substrate on-off). Dicoumarol, the DT-diaphorase inhibitor, has a binding site that overlaps with that of NAD(P)H but not substrate, which explains the competitive inhibitory effect with NAD(P)H over DT-diaphorase. Li et al (1995) hypothesized that NAD(P)H transfers two electrons to FAD to form FADH2, then FADH2 transfers the hydrogens to the quinone substrate after NAD(P)H leaves the binding site.

The structure of human DT-diaphorase has not been determined. Studies have shown that human DT-diaphorase is a dimer containing two identical subunits which have individual molecular weight of 32 kDa and two molecules of FAD (Lind et al, 1990; Riley and Workman 1992).

DT-diaphorase in different species

Human, mouse and rat DT-diaphorase cDNAs have been cloned and cDNAs and proteins have been sequenced (Jaiswal et al, 1988; Chen et al, 1994; Bayney et al, 1987). The human NOQ1 gene ARE contains a perfect AP1 consensus binding sequence while the rat NOQ1 gene ARE contains only imperfect AP1 like sequences. Supershift and band shift assays have shown that Jun and Fos bind to the human NOQ1 gene ARE but failed to bind to the rat NQO1 gene and this was later proven to be due to the lack of a perfect AP1 element (Joseph et al, 1994). Three RNA transcripts (2.7, 1.7 and 1.2 kb) are formed from the human NQO1 gene as compared with one 1.2 kb transcript from the rat gene. The amino acid sequence of mouse DT-diaphorase is more homologous to human DT-diaphorase compared to rat DT-diaphorase (Chen et al, 1994), and the catalytic capability of mouse DT-diaphorase is similar to the human enzyme in activating CB 1954 and EO9 (Chen et al, 1995). Chen and coworkers found that the residue 104 (Tyr in rat DT-diaphorase and Gln in human and mouse DT-diaphorase) within the carboxyl-terminal region is an important factor for the difference in catalytic activity between the rat, the human and mouse enzyme (Chen et al, 1997). It has been recognized that a major difference exists in the capability of human and rat DT-diaphorase to activate most quinone and nitrobenzenes. It has been shown that the rat enzyme reduces EO9 faster than human enzyme (Walton et al, 1991). It has also been showed that the rat enzyme is more efficient in reducing two clinically used bioreductive quinones, MMC and AZQ (Siegel et al, 1990 a, b). Beall et al (1994) compared the metabolism of a series of bioreductive antitumour compounds by purified rat and human DT-diaphorase and showed that the rat DT-diaphorase was 1.9-4.9 times faster in reducing bioreductive agents than the human DT-diaphorase.

Polymorphism of the NQO1 gene

When Traver et al (1992) were studying the sensitivity of MMC in two human colon cancer cell lines, HT-29 and BE, they found that the BE cells were resistant to MMC and lacked detectable DT-diaphorase activity. Further analysis using RNA single-strand conformational polymorphism analysis and subsequent sequencing of BE revealed a C to T mutation at nucleotide 609 in the NQO1 gene. This results in a proline to serine substitution in the amino acid sequence of the normal protein (Siegel et al, 1990; Marshall et al, 1991; Traver et al, 1992). Several other studies in E. coli showed that the mutant protein has little or no activity (Traver et al, 1997). Misra et al (1998) directly tested the effect of the 609 point mutation on DT-diaphorase activity by transfecting COS-1 cells with the cDNA with, or without, the altered nucleotide 609 base and showed a tenfold reduction in DT-diaphorase activity in cells containing the mutated gene.

Approximately one-half of healthy human (40% heterozygous and 9% homozygous) have been found to carry the C-to-T transition at base 609 (Kuehl et al, 1995). The occurrence of a homozygous base pair 609 polymorphism of DT-diaphorase in the human population has recently been studied by a group of researchers. It is reported that 4% among a large group of individuals in Britain showed no detectable enzyme activity due to the 609 point mutation (Edwards et al, 1980). Rosvold et al (1995) showed that 13% of individuals from a reference family panel carry only mutant allele. Recent studies by Traver et al (1997) showed that in 45 lung cancer patients, 7% had the gene mutation. Eickelman et al (1995) have shown that loss of NQO1 activity occurred in 3 of 23 patients with renal cancer .

The relationship between the mutation of the DT-diaphorase gene and the risk of developing cancer is still unclear. Mutation of DT-diaphorase has been reported to be associated with increased susceptibility to benzene poisoning caused hematological malignancy in human (Rothman et al, 1997) and menadione toxicity in mice (Radjendirane et al, 1998). In addition, Marshall et al (1991) have hypothesized that a genetic defect leading to a decrease in DT-diaphorase may have predisposed individuals in a cancer-prone family to the development of malignancies. Wiencke et al, (1997) have reported an interesting finding that wild type genotype of NQO1 gene is associated with increased risk of developing lung cancer among African-Americans in the study of ethnic and racial differences in lung cancer incidence. It is thought that DT-diaphorase may act as an activator for certain environment carcinogens such as nitroaromatic compounds and cigarette smoke condensate (Cenas et al, 1995).

More studies are required to determine the contribution of mutations of the detoxifying enzymes to cancer risk, and why there is a high incidence of the NQO1 mutation among human populations. The base 609 polymorphism leads to a lack of DT-diaphorase activity. Since DT-diaphorase acts as a protective factor against oxidative damage and as an activator for many bioreductive agents, the mutation in the NQO1 gene may impair both chemoprevention and response to chemotherapy. In addition, the presence of this polymorphism may present significant problems in the use of bioreductive antitumour agents, which are activated by DT-diaphorase, in the treatment of cancers.

An alternative spliced DT-diaphorase mRNA that lacks exon 4 has also been reported to be expressed in some human tumour cell lines. The mutant form mRNA was 114 bases smaller than the full length NQO1 mRNA (Gasdaska et al, 1995). The protein

encoded by this particular mRNA shows little catalytic activity due the lack of a putative quinone substrate binding site on DT-diaphorase coded by exon 4, and may be associated with resistance to MMC in human colon carcinoma HCT116-R30A (Hu et al, 1996). Yao et al (1996) found that the expression of this mRNA, lacking exon 4, was not increased following treatment with MMC while elevated full-length mRNA was detected.

Pan and the coworker (1995) identified another mutation in the NQO1 gene with a T to C substitution at base 464 that resulted in the replacement of an arginine 139 by tryptophan in the enzyme. The activity of the mutant protein for reducing MMC was 60% lower compared with the wild type DT-diaphorase.

DT-diaphorase and chemoprevention

Tumours can be initiated by damage to DNA by electrophilic carcinogen metabolites formed during phase I enzymes-mediated metabolism or by reactive oxygen species. These toxins can be detoxified through conjugation with endogenous substrates by Phase II enzymes, resulting in protection of cells from carcinogenesis. Induction of phase II enzymes, such as DT-diaphorase, glutathione S-transferase, UDP-glucoronosyltransferase, or epoxide hydrolase, is thought to be a major mechanism for prevention of carcinogenesis (Prestera et al, 1993). DT-diaphorase is considered a chemopreventive enzyme because it can reduce the formation of toxic free radicals when cells are exposed to endogenous or exogenous quinones. Joseph et al (1994) showed that DT-diaphorase also prevents the binding of semiquinones, activated by NADPH:cytochrome P450 reductase, to DNA, which may provide additional protection against the toxic effect of quinones. In addition, DT-diaphorase also reduces quinone and nitro groups to metabolites that can be conjugated with

glutathione and then be removed from cells.

Inducers of DT-diaphorase and other phase II enzymes are currently under investigation for their cancer prevention properties. Dithiolethiones, naturally occurring compounds in cruciferous vegetables, may play an important role in chemoprevention by inducing DT-diaphorase and other phase II detoxifying enzymes (Benson, 1993). Oltipraz, a dithiolethione analogue capable of inducing DT-diaphorase, has been extensively studied for its ability as a chemoprotective agent against a variety of chemical carcinogens in animal models systems. Tomasz et al (1987) reported that feeding rats with oltipraz, and related 1,2-dithiole-3-thiones, could significantly protect the animals against aflatoxin induced tomourigenicity. This result is further supported by the findings that oltipraz also inhibited the formation of intestinal carcinoma induced by azoxymethane (Rao et al, 1991) and hepatocellular carcinomas induced by aflatoxin B1 in F334 rats (Roebuck et al, 1991). Smith et al (1998) observed a dose-dependent decrease in the formation of dibenzpyrene adducts following exposure of human breast cancer cells to oltipraz. Phase I and II human trials of oltipraz as a chemopreventive agent have been carried out in the United States and China (Clapper, 1998). Pharmacokinetic studies of oltipraz following oral administration have been tested by Gupta et al (1995). O'Dwyer et al (1996) showed increased levels of phase II enzymes in lymphocytes and colon mucosa after treatment of oltipraz at low dose levels in high-risk population. A clinical trial of oltipraz showed decreased aflatoxin albumin adducts, a biomarker for development of hepatocellular carcinoma, in oltipraz treated groups with only minor adverse effects (Zhang et al, 1997; Kensler et al, 1998).

Phase II detoxifying enzymes can also be induced by isothiocyanates such as benzyl isothiocyanate and sulforaphane, a chemical isolated from broccoli and proved to been a

potent monofunctional inducer of phase II enzymes (Zhang et al, 1992). It has also been demonstrated that sulforaphane inhibited CYP2E, an isoenzyme of the cytochrome P450 enzymes which are responsible for activation of several carcinogens, suggesting that inhibition of CYP2E1 by sulforaphane may contribute to the chemoprotection against carcinogens activated by this enzyme (Barcelo et al, 1996). Administration of sulforaphane significantly decreased the incidence and multiplicity of tumours in rats treated with the 9,10-dimethyl-1,2-benzanthracene (Zhang et al, 1994). Sulforamate, a novel analogue of sulforaphane, has shown greater induction of DT-diaphorase and other phase II detoxifying enzymes with less toxicity compared with the parent compound and may be a promising new cancer chemopreventive agent (Gerhäuser et al, 1997). Based on these findings, a preclinic trial has been carried out to test the effectiveness of the broccoli supplements to induce phase II enzymes in blood lymphocytes and colon mucosa, however, no induction of GST activity has been observed. (Clapper et al, 1997).

Hypoxia and enzyme directed development of bioreductive agents

Background

Much attention has focused on improving cancer chemotherapy by increasing the selectivity and targeting of antitumour drugs to tumour cells (Workman and Stratford, 1993). The unique characteristics of bioreductive antitumour agents make them perfectly suited for improving tumour selectivity and targeting. First, bioreductive antitumour agents are generally more toxic towards hypoxic regions within tumours that are inherently resistant to radiation therapy (Workman and Stratford, 1993). This feature makes them the ideal agents to be used in combination with irradiation in the treatment of solid tumours. Second,

bioreductive antitumour agents are uniquely suited for an "enzyme-directed" approach to tumour targeting because of the requirement for intracellular activation by reductive enzymes. The rapidly growing knowledge of the enzymes involved in the activation of these drugs may make it possible to predict the responsiveness of individuals to a particular antitumour agent.

The concept of "enzyme-directed" bioreductive drug development was discussed in 1989 at an international conference held in Italy (Workman et al, 1990). Enzyme-directed bioreductive drug treatment comprises: (1) The use of tumour biopsy specimens to guide the selection of groups of patients most likely to respond to a given bioreductive agent. Since different enzymes participate to different extents in activating bioreductive prodrugs, the activity of a given drug may be enhanced for patients with a particular enzyme profile. For example, patients with high levels of DT-diaphorase may respond well to CB 1954, EO9, AZQ and MMC agents that are activated by this enzyme, but may be resistant to tirapazamine which is inactivated by DT-diaphorase. The concept could be expanded to enhance the cytotoxicity of the bioreductive antitumour agent by selectively increasing activating enzyme activity inside the tumour. (2) Enzyme-directed bioreductive drug discovery to optimize prodrug activation by particular reductases (Bailey et al, 1992). It is possible, by modifying the structure of a bioreductive agent, to increase the selectivity of the agent to be activated by a particular activating enzyme (Workman and Stratford, 1993).

Hypoxia directed activation of bioreductive agents

The conventional approach to bioreductive drug development has involved screening for selective activity in hypoxic versus oxygenated cells. Bioreductive agents have the

potential for achieving a high therapeutic index against solid tumours containing hypoxic malignant cell regions. This selectivity is thought to be due to the ability of the molecular oxygen to reverse the bioreductive activation process of bioreductive agents, particularly for agents that are activated by one electron reduction. Although this redox cycling will generate reactive oxygen species at presence of oxygen, these toxic species will likely be detoxified by protective enzymes such as catalase and superoxide dismutase.

Two bioreductive drugs discovered in mid-1980s, RSU1069/RB6145 and tirapazamine (formerly known as SR4233), were evaluated for their selective cytotoxicity to hypoxic cells. The typical hypoxic:oxic cytotoxicity ratios were in the range of 50 to 200 for murine and human cell lines for both compounds and was higher than the typical hypoxic:oxic cytotoxicity ratio of observed for MMC (Stratford and Stephens, 1989; Brown, 1993). Administration of RB 6145/RBSU 1069 one hour prior to irradiation caused a large increase in cell kill suggesting these drugs have radiosensitizing properties (Cole et al, 1990). Zeman et al (1990) showed that tirapazamine could act as a radiosensitizer *in vivo* and *in vitro*, and oxygenated tumour cells pretreated with tirapazamine under hypoxic condition were more sensitive to radiation. Phase I studies of the combination of tirapazamine and radiotherapy have been carried out by Shulman et al (1995).

An alternative way to increase the efficacy of the bioreductive agents against hypoxic cells is to selectively increase the tumour hypoxia. This has been tested by using vasoactive agents (such as hydralazine) to modify tumour blood supply and result in increased activity of RSU 1069 (Chaplin et al, 1989) and tirapazamine (Brown, 1987). Other agents, such as tumour vasculature damaging drugs (flavone acetic acid), immunomodulators (tumour necrosis factor) (Edwards et al, 1991a) and interleukin 1 (Braunschweiger et al, 1991) have

been tested to increase tumour hypoxia. The efficiencies of the three methods to increase tumour hypoxia were compared with mechanical occlusion of the tumour blood supply by clamping. Each of these methods have been shown to greatly increase the overall hypoxia (Bremner et al, 1990; Edwards et al, 1991b). Smith et al (1987) have reported that, flavone acetic acid, when given with RSU1069 or tirapazamine, substantially delayed tumour growth. It has also been reported that nude mice engrafted with both murine and human tumours, and treated with RSU 1069 following two hours of clamping to induce hypoxia, had significant inhibition of tumour growth compared to non-clamped controls (Cole et al, 1989; Bremner et al, 1990; Adams et al, 1991).

Enzyme directed bioreductive drug therapy

Enzyme profile-directed bioreductive drug therapy

Benzonidazole, the model drug in the nitroimidazoles family, has been shown to be activated mainly by microsomal NADPH:cytochrome P450 reductase, but the detailed mechanisms of the enzymatic activation of RSU 1069/RB 6145 are not clearly demonstrated. Microsomal NADPH:cytochrome P450 reductase catalyzed 70% of the reductive activation of tirapazamine, a heterocyclic N-oxide, suggesting that NADPH:cytochrome P450 reductase is a major activating enzyme for this drug. In addition, an anti-NADPH:cytochrome P450 reductase antibody was inhibitory and purified NADPH:cytochrome P450 reductase was able to produce cytotoxicity in the absence of the cytochrome (Walton and Workman 1987). It has also been shown that the cytotoxic metabolites of tirapazamine that induce single and double strand breaks in cells are formed mainly by NADPH:cytochrome P450 reductase (Lloyd et al, 1991). Other enzymes, such

as xanthine oxidase and aldehyde oxidase, may also be involved in the activation of tirapazamine (Walton and Workman, 1992). Since the one-electron reduced intermediate of tirapazamine is thought to be the major contributor to the cytotoxicity, it has been suggested that DT-diaphorase may inactivate tirapazamine. Thus, patients who have tumours that express high levels of DT-diaphorase activity may not be suitable for tirapazamine treatment.

DT-diaphorase is the best-studied enzyme in this enzyme-directed approach to chemotherapy. The major reasons for this are: (1) The levels of DT-diaphorase are often higher in tumour cells compared with the normal cells from the same region. (Cresteil and Jaiswal, 1991, Malkinson et al, 1992, Belinsky and Jaiswal, 1993, Schlager and Powis, 1990). In addition, DT-diaphorase activity in many tumour cells can be selectively induced by some inducers. (2) DT-diaphorase plays an important role in the activation of many bioreductive agents including clinically used drugs (MMC and AZQ).

Keyes et al (1984) first suggested the importance of DT-diaphorase in tumour sensitivity to MMC. HT-29 cells with higher levels of DT-diaphorase showed higher sensitivity to MMC than DT-diaphorase deficient BE cells (Siegel et al, 1990). A comparison of the activity of MMC in paired cell lines with different levels of DT-diaphorase activity further supported the importance of this enzyme to its antitumour effects (Begleiter and Leith, 1989; Begleiter and Leith, 1990a; Begleiter and Leith, 1990b). Further, transfection of the DT-diaphorase gene into small cell lung cancer cell line which had 80-fold less enzyme activity than a NSCLC cell line resulted in elevated MMC activity (Malkinson et al, 1992). Mikami et al (1996) reported that transfection of st-4 cells, a human gastric carcinoma cell line, with the NQO1 gene increased the DT-diaphorase activity

from 144 to 2085 nmol/min/protein, and increased sensitivity to MMC by 5-10 fold compared with parent st-4 cells that expressed no enzyme activity. Loss of DT-diaphorase activity has been reported associated with resistance to MMC treatment in tumour cells (Hu et al, 1996). However, Robertson et al (1992) failed to find a correlation between the MMC sensitivity and the level of DT-diaphorase in a panel of 15 human lung, breast and colon tumour cell lines. It is now apparent that although MMC activity is enhanced by elevated DT-diaphorase activity under aerobic conditions, MMC is only a poor substrate for DT-diaphorase at physiological pH.

EO9, a quinone-containing bioreductive antitumour agent, has also been tested for a correlation between antitumour and DT-diaphorase activity. EO9 is a better substrate than MMC for activation by DT-diaphorase at neutral pH (Walton et al, 1991). It was shown that cells with high levels of DT-diaphorase were up to 1000 times more sensitive to EO9 compared with cell lines with little DT-diaphorase activity (Robertson et al, 1992). Walton et al (1992) also reported an association of EO9 sensitivity with the level of DT-diaphorase. Transfection of the NOQ1 gene into CHO cells to express high levels of DT-diaphorase elevated the activity of EO9 by 3-fold (Gustafson et al, 1996). A study of the relationship between the enzyme activity and the sensitivity to EO9 was carried out in the National Cancer Institute Tumour Cell Line Panel. A good correlation was found between the level of DT-diaphorase activity and the tumour cells sensitivity to EO9 (Fitzsimmons et al, 1996). However, lack of correlation between enzyme activity and EO9 sensitivity has also been reported in two human colon cancer xenografts and two murine colon adenocarcinomas (Cummings et al, 1998).

Other bioreductive agents have also been studied as possible candidates for enzyme-directed drug therapy. Siegel et al (1990) showed that AZQ induced cytotoxicity in a DT-diaphorase rich cell line but not in enzyme deficient cell line suggesting the potential value of this agent in enzyme-directed tumour treatment. Two quinone derivatives, MeDZQ and RH1, are better substrates for DT-diaphorase and may have potential value in the treatment of tumours which are rich in the enzyme (Beall et al, 1994; Winski et al, 1998).

An alternative approach for enzyme-directed bioreductive agent therapy is to artificially increase the activating enzyme levels in tumours. DT-diaphorase is highly suitable for this purpose since it is readily inducible by many inducers. Studies have been done to determine whether 1,2-dithiole-3-thione (D3T) could selectively induce DT-diaphorase activity in tumour cells, and whether this induction would lead to the enhancement of antitumour activity of some bioreductive agents. Pretreatment of L5178Y murine lymphoma cells with D3T increased DT-diaphorase activity by 22-fold and enhanced MMC antitumour activity by 2-fold and EO9 antitumour activity by 7-fold with no increase in myelotoxicity (Begleiter et al, 1996). A similar approach was carried out in human leukemia cells. Pretreatment of HL60 cells with D3T enhanced the cytotoxic activity of EO9 by 2-fold with no increase in either enzyme activity or cytotoxicity of EO9 in normal kidney cells. All these studies suggest that it is possible to increase antitumour activities of some bioreductive agents by selectively increase the activating enzyme level.

Enzyme-directed bioreductive drug discovery

The controversial results in the enzyme profile-directed bioreductive therapy reflect the complexity of enzymology in the activation of these prodrugs. None of the bioreductive

agents developed to date are specifically activated by a single reductive enzyme. Thus, it has not been possible to specifically target one activating enzyme. A number of difficulties have limited the development of new bioreductive agents that could be used in enzyme directed targeting. Structure-activity relationships have been studied through altering the structure of a lead compound (such as AZQ, MMC, EO9 tirapazamine, and CB 1954) and determining the effect of such alterations on the kinetics of reduction by reductive enzymes. Substitution of AZQ by n-butyl and methyl groups resulted in good substrates for DT-diaphorase catalyzed activation (Gibson et al, 1992). In the case of analogues of EO9, replacement of the aziridine with methyl aziridine or methoxy slowed down the reduction (Jaffar et al, 1998; Naylor et al, 1997). The reductions of a series of analogues of EO9 by DT-diaphorase have also been carried out. Two analogues (EO4 and EO68) showed similar reduction rates to EO9 but with longer plasma half-lives and improved tissue penetration compared with the parent compound (Phillips, 1996). Beall studied the metabolism of a series of antitumour quinone compounds by purified DT-diaphorase, and MeDZQ and streptonigrin turned out to be excellent substrates for human purified DT-diaphorase (Beall et al, 1994). Recently, another new water soluble analogue of MeDZQ, RH1, was shown to be an excellent substrate for DT-diaphorase (Winski et al, 1998).

2-(Di(chloroethyl)amino-1,4-benzoquinone (BM), which contains a quinone bioreductive element and a nitrogen mustard cytotoxic element, was chosen to study the effect of different chemical functional groups on the ability of DT-diaphorase and NADPH:cytochrome P450 reductase to activate this model bioreductive antitumour agent. BM was previously studied in our laboratory as a model bioreductive agent to identify the role of the bioreductive and cytotoxic elements in the activity of quinone alkylating agent.

BM was more cytotoxic to tumour cells than the non-quinone alkylating agent, aniline mustard (Begleiter, 1983; Begleiter and Leith, 1990). BM produced both DNA crosslinks and strand breaks, but the crosslinks were the major contributor to the cytotoxic activity. A group of BM analogues with different functional groups in the quinone structure have been synthesized to investigate the role of functional groups in modifying the specificity for drug activation by reductive enzymes (Structures of BM analogues are shown in Figure 3).

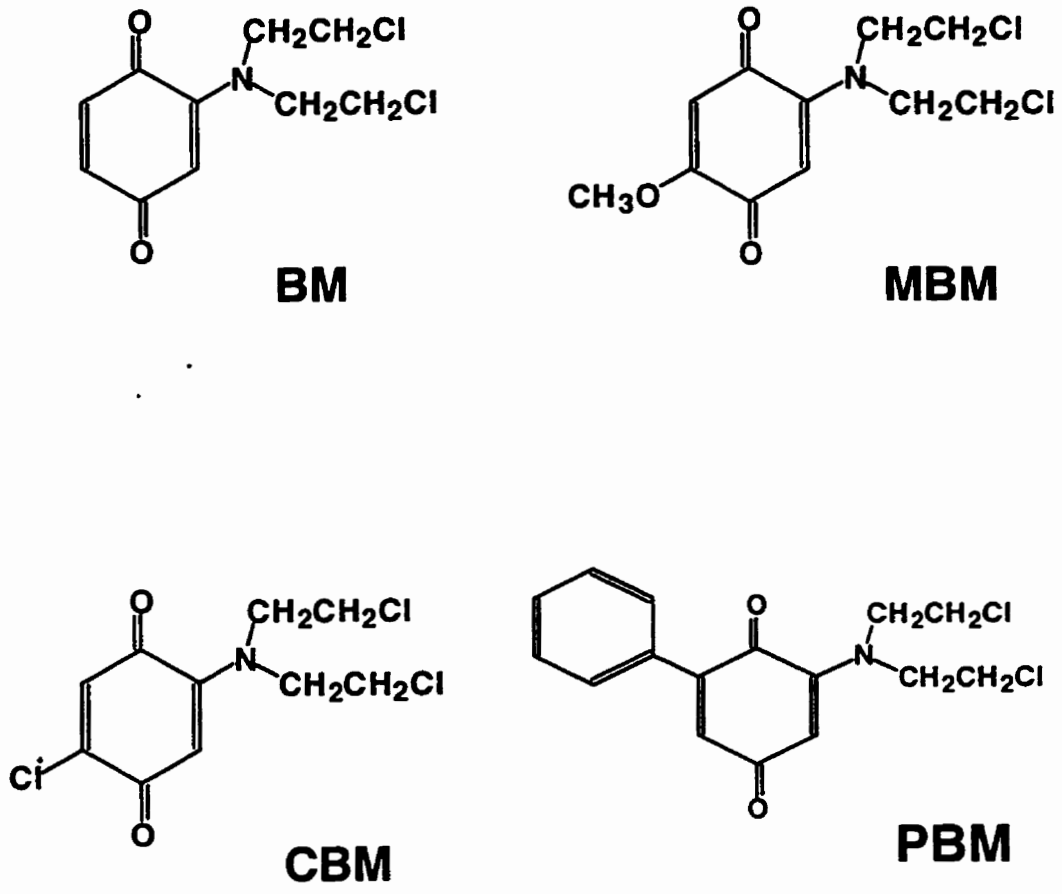


Figure 3: Structures of BM analogues

Specific objectives of the proposed research

1. To determine if inducers of DT-diaphorase can selectively enhance tumour cell kill by MMC in human tumour cell lines.

   We have previously shown that D3T could increase DT-diaphorase activity in most human tumour cell lines. In this study, D3T was tested for its ability to enhance the antitumour activity of MMC in human tumour cell lines. Dietary inducers of DT-diaphorase were screened for their ability to induce DT-diaphorase activity in human tumour cells. Inducers, which produce better induction of DT-diaphorase than D3T, were tested for the ability to increase the cytotoxicity of MMC in human tumour cells. Normal bone marrow cells were studied to ensure that the inducers do not increase MMC toxicity to these normal cells. Inducers that selectively increase the antitumour activity of MMC will go into *in vivo* study to test if these inducers can selectively enhance the antitumour activity of bioreductive agent in human tumour xenografts in nude mice. These studies will identify DT-diaphorase inducers that may be useful for enhancing the effectiveness of bioreductive agents in the clinic.

2. To study the effect of different functional groups on the activation and cytotoxicity of BM analogues.

   BM is a quinone-containing bioreductive antitumour agent that has been shown to be inactivated by DT-diaphorase. In analogues of BM, the presence of different functional groups, was shown to alter both antitumour activity of the analogues and the effect of DT-diaphorase on their activity. To understand the effects of the functional groups on the reduction of the quinone group by DT-diaphorase, reduction of BM and

its analogues by purified DT-diaphorase in a cell free system was studied using HPLC analysis. These studies are useful for identifying how functional groups can regulate the effect of DT-diaphorase on the activity of bioreductive agents.

MATERIALS AND METHODS

Materials

Media and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). All reagents for the DT-diaphorase assay, NADH, FAD, dicoumarol, potassium chloride, Tris HCl, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 13-cis-retinoic acid, genistein, ursolic acid, ibuprofen, caffeic acid, folic acid and MMC were obtained from Sigma (St Louis, MO, USA). Sulforaphane was purchased from LKT Labs, Inc. (St. Paul, MN, USA). Dimethyl sulfoxide (DMSO) and dimethyl formamide were obtained from BDH Laboratory Supplies (Darset, England). HPLC grade methanol was from Fisher Scientific (Nepean, ON, Canada). Protein concentration was measured using The Bio-Rad DC Kit for measuring protein concentration was obtained from Bio-Rad, Mississauga, ON, Canada. Vitamin K, dimethyl maleate (DMM), dimethyl fumarate (DMF), propyl gallate (PG), chalcone and aspirin were purchased from Aldrich Chem. Co. (Milwaukee WI, USA). Methocult GF H4434 was obtained from Stem Cell Technologies Inc (Vancouver, BC, Canada). MMC was dissolved in phosphate-buffered saline (PBS): DMSO (1:1, v/v). The final concentration of DMSO did not exceed 1%. BM, MBM, PBM and CBM were synthesized and purified by Curtis J. Oleschuk (1998).

Cell culture

The NCI-H661, human non-small cell lung carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and was grown in RPMI 1640 and 10% FBS. HCT116, a human colon carcinoma cell line, and SK-MEL-28, a human

malignant melanoma cell line, were from American Type Culture Collection and were grown in DMEM/F12 (1:1) and 10% FBS. AGS, a human gastric adenocarcinoma cell line, was obtained from Dr. J. A. Wright (Manitoba Institute of Cell Biology, Winnipeg, MB, Canada) and was grown in RPMI 1640 and 10% FBS. T47D, a human breast ductal carcinoma cell line, was obtained from Dr. S. Mai (Manitoba Institute of Cell Biology) and was grown in RPMI 1640 and 10% FBS. HS578T, a human breast ductal carcinoma cell line, was obtained from Dr. S. Pan (University of Maryland Cancer Center, Bethesda, MD, USA) and was grown in DMEM/F12 (1:1) and 10% FBS. Normal bone marrow was obtained from marrow donated for transplantation. Bone marrow cells were cultured in RPMI 1640 and 10% FBS.

Induction of DT-diaphorase activity

Cells were incubated in 10 µl of media and 10% FBS with inducers of DT-diaphorase at 37^0C in 5% CO_2 for 48 hrs. The concentrations of DT-diaphorase inducers employed were not toxic to the cells during the incubation time by measuring the cell number after incubation time. Following incubation, cells were washed with PBS, resuspended in 200 µl of 0.25 M sucrose, sonicated and stored at -80^0C. Protein concentration was measured using the Bio-Rad DC Kit with gamma globulin as standard. DT-diaphorase activity was measured spectrophotometrically by a modification of the procedure of Prochaska and Santamaria (Prochaska et al, 1988) using menadione as the electron acceptor. The DT-diaphorase cycling assay mixture was prepared immediately before the experiment, and contained 10 ml assay buffer (2.5×10^{-2} M Tris, 0.06% bovine serum albumin and 10 µl Tween-20), 5.0×10^{-6} M FAD, 1.0×10^{-3} M glucose

6-phosphate, 20 units glucose 6-phosphate hydrogenase, 3.0 X 10^{-5} M NADP, 7.2 X 10^{-6} M MTT in PBS and 5.0 X 10^{-5} M menadione in acetonitrile. The cycling assay mixture was warmed to 25 ^{0}C and kept out of light. Samples and 750 µl cycling assay mixture were added into a semimicro cuvette and the increase in absorbance was measured at 610 nm for 5-15 minutes using a Cary 1 Spectrophotometer (Varian, Mississauga, ON, Canada). A second measurement, in which an additional 10 µl of 1.0 X 10^{-5} M dicoumarol stock was added to the assay mixture, was carried out to determine non-DT-diaphorase related absorbance. DT-diaphorase activity was reported by the difference between the two measurements as the dicoumarol-inhibitable activity and expressed as nmol min^{-1} mg $protein^{-1}$.

Effect of DMM on NADPH: cytochrome P450 reductase activity

T47D cells (7.5 X 10^5) were incubated with 50 µM DMM in 10 µl of media and 10% FBS at 37^{0}C in 5% CO_2 for 48 hrs. Following incubation, cells were washed twice with PBS and resuspended in 100-500 µl of 20% glycerol, sonicated and stored at –80 ^{0}C. Protein concentration was measured in all samples using Bio-Rad DC Kit with gamma globulin as standard. The NADPH:cytochrome P450 reductase activity was measured by a previously described procedure using cytochrome C as the artificial electron receptor (Strobel and Digman, 1978). Phosphate buffer containing 1 mM KCN was prepared and warmed to 30^{0}C before the experiment. Immediately before the assay was run, 0.84 mM cytochrome C and 7.4 mM NADPH were prepared in phosphate buffer. Protein sample was mixed with 850 µl phosphate buffer and 50 µl cytochrome c solution in a semimicro

cuvette. 15 µl NADPH was added to the cuvette to start the reaction. The reaction was analyzed at 550 nm by a Cary 1 spectrophotometer for 5-10 minutes. NADPH:cytochrome P450 reductase activity was expressed as nmol min^{-1} mg $protein^{-1}$.

Effect of DMM on NADH:cytochrome b5 reductase activity

T47D cells (7.5 X 10^5) were treated with 50 µM DMM in 10 µl of media and 10% FBS at 37^0C in 5% CO_2 for 48 hrs. Following incubation, cells were: washed twice with PBS; resuspended in 500 µl of solution containing 10 mM Hepes/KOH (pH 7.4), 1.5 mM magnesium chloride and 0.05 mM dithiolthreitol; incubated 10 minutes on ice; sonicated; incubated another 10 minutes on ice, and spun at 11750 × g for 15 min at 4 ^{0}C. The supernatant was stored at -80 ^{0}C. Protein concentration was measured and the enzyme activity was determined using a previously described spectroscopic method (Barham et al, 1996). Assay buffer (0.05 M phosphate buffer pH 6.0 and 0.9 mM NADH) was prepared immediately prior to the assay was run. Protein sample was mixed with 880 µl assay buffer in a cuvette. Reaction was started by adding 20 µl cytochrome C to the cuvette to achieve a final concentration of 70 µM. The reaction mixture was analyzed at 550 nm by a Cary 1 spectrophotometer for 2 min. A second measurement was made in which p-hydroxymercuribenzoic acid (pHMB), at a final concentration of 0.2 mM, was added to the assay mixture to inhibit the enzyme activity. NADH:cytochrome b5 reductase activity was reported by the difference between the two measurements as nmol min^{-1} mg $protein^{-1}$.

Effect of DMM on Xanthine dehydrogenase activity

Xanthine dehydrogenase activity was measured by a spectrophotometric method. Xanthine dehydrogenase and xanthine oxygenase forms of the enzyme were distinguished by the formation of uric acid from xanthine in the presence and absence of NAD^+ (Gustafson et al, 1992). After 7.5 X 10^5 of T47D cells were incubated with DMM in 10 µl of media and 10% FBS for 48 hr at 37^0C in 5% CO_2. Cells were washed twice with 0.25 µM ice-cold sucrose, resuspended in homogenizing buffer consisted of 100 mM phosphate buffer (pH 7.8), 3 mM phenylmethyl sulfonylfluoride (PMSF) and 1 mM EDTA. The cells were then sonicated and spun at 13800 × g for 20 min at 4^0C. The supernatant was transferred to a fresh Eppendorf tubes. The protein concentration was determined in all samples. 2-Mercaptoethanol was then added to the supernatant at a concentration of 7 µl/ml supernatant. 100 µl of protein sample was mixed in a cuvette with 100 µl 12 mM NAD^+ and 2.8 ml xanthine dehydrogenase assay buffer consisting of 100 mM phosphate buffer (pH 7.8) and 0.2 mM xanthine. The cuvette was warmed to 25^0C and analyzed at 293 nm by a Cary 1 spectrophotometer for 7 min. Xanthine dehydrogenase activity was expressed as nmol min^{-1} mg $protein^{-1}$.

Effect of DMM on Glutathione S-transferase activity

T47D cells (7.5 X 10^5) were incubated in 10 µl of media and 10% FBS with 50 µM DMM at 37^0C in 5% CO_2 for 48 hrs. Following incubation, cells were washed twice with PBS and resuspended in 1 ml tissue culture quality H_2O, sonicated and supernatant was recovered for enzyme assay. Protein concentration was determined as previously

described for the DT-diaphorase assay. GST activity was measured by a procedure previously described (Habig et al, 1974). 1-Chloro-2,4-dinitrobenzene (CDNB) was used as the substrate and was previously prepared at 50 mM in ethanol. GSH was prepared fresh at 10 mM in phosphate buffer (pH 6.5). 100 µl GSH, 100 µl sample and H_2O were added into the semimicro cuvette for a the total volume of 880 µl, 20 µl CDNB was added to cuvette to start the reaction at 25 ^{0}C and the increase in absorbance was determined by a Cary 1 spctrophotometer at 340 mm for 5-10 minutes. GST activity was reported as nmol min^{-1} mg $protein^{-1}$.

Cytotoxicity studies with MMC

Cytotoxicity measured by MTT assay

Cells were incubated in 4 µl of media and 10% FBS with, or without, inducers for 48 hrs. Media was removed and cells were washed with citrate-saline solution and then were treated with various concentrations of MMC in fresh media and 10% FBS for 1 hr at 37^{0}C. Then, media was removed and cells were washed once with sterile citrate-saline. Cells were trypsinized and resuspended in fresh media and 10% FBS and plated in quadruplicate into 96-well microplates. The surviving cell fraction was determined by MTT assay (Johnston et al, 1994: Kirkpatrick et al, 1990) after 4-9 days. This length of time was sufficient to allow at least three cell doublings. Media was removed and 200 µl of fresh RPMI 1640 containing 10 µl of MTT stock was added to each well. Cells were incubated for 2-3 hours, then spun for 7 minutes at 480 X g. The medium was removed and 200 µl DMSO was added. The plate was shaken for 2-3 minutes to dissolve the

purple crystals of formazan and the absorbance was measured at 540 nm. The surviving cell fraction for each drug concentration and each cell line were the means of at least 3 experiments. The D_0 (concentration of drug required to reduce the surviving cell fraction to 0.37) was calculated from the linear regression line of the surviving cell fraction versus drug concentration curve. Data were analyzed using two-tailed t-tests and anova to compare the significance of the differences of the treatments.

Myelotoxicity measured by methylcellulose clonogenic assay

Marrow specimens were obtained from marrow donated for bone marrow transplantation. The mononuclear cells were isolated using a Ficoll-Hypaque gradient (Johnston et al, 1994). Normal human bone marrow cells were treated with, or without, D3T for 48 hrs, resuspended in RMPI 1640 media and 10% FBS and then incubated with various concentrations of MMC for 1 hr. Cells were washed and resuspended in methocult GF H4434 following the protocol provided by Stem Cell Technologies. Total colonies (CFU-G, CFU-M, CFU-GM, CFU-E and BFU-E) on the plates were counted after 7-10 days and the surviving cell fraction was determined by clonogenic assay. D_0 was calculated from the linear regression line of the surviving cell fraction versus drug concentration curve (Johnston et al, 1994: Petzer et al, 1991). Data were analyzed using two-tailed t-tests to compare the significance of the differences of the bone marrow toxicity with, or without, DT-diaphorase inducer.

Cytotoxicity of BM analogues in SK-MEL-28 cells

BM analogues were all prepared fresh in dimethyl formamide the day of the

experiment. SK-MEL-28 cells were first incubated with, or without, 100 μM dicoumarol for 20 minutes and then were incubated with, or without, 2 μM of BM analogues with the final concentration of dimethyl formamide not exceeding 1% in growth media. Surviving cell fraction was measured by MTT assay as described above.

Reduction of BM analogues by DT-diaphorase

Preparation of solutions of BM analogues

BM, MBM, PBM and CBM in dimethyl formamide were prepared fresh on the day of the experiment. In all cases, a final concentration of 50 μM of BM analogue in 25 mM Tris HCl (pH 7.4) containing 1% dimethyl formamide was used. Solutions of BM analogues were covered with aluminum foil paper to minimize photochemical decomposition.

Reductions of BM analogues under hypoxic and aerobic conditions

Reductions were carried out in 1.5 ml microcentrifuge tubes in 1 ml of a reaction buffer (50 mM Tris HCl, pH 7.4). DT-diaphorase, which was stored as -80°C frozen stock was prepared fresh in Tris HCl buffer on the day of the experiment. 0.01% Tween 20 was added to activate the enzyme. NADH and FAD were also prepared fresh on the day of the experiment. DT-diaphorase, NADH and FAD were added to a 1.5 ml Eppedorf microcentrifuge tube to give final concentrations of 0.1 μg/ml, 100 μM and 0.5 μM, respectively in a volume of 1 ml. The tube was sealed and the covering lid was perforated twice with a needle to create an inlet and outlet hole. A 1.5 inch stainless

steel needle was placed through the inlet into the reaction buffer. Through this needle, the reaction buffer was bubbled with nitrogen gas, or air, for at least 2 hours at 37°C. BM analogue was added through the outlet hole to the reaction buffer to give a concentration of 50 μM to start the reaction. Nitrogen, or air, was continuously bubbled into the reaction vessel throughout the entire reaction time. At various times, 10 μM dicoumarol was added through the outlet hole to stop the reaction, and the reaction solution was frozen quickly in a dry ice/ethanol bath and stored at -80°C for HPLC analysis. Samples were kept frozen for no longer than 5 days and only thawed immediately before the HPLC analysis.

HPLC analysis

Reduction of the BM analogues was determined by measuring the rate of consumption of NADH. The rate of consumption of NADH was quantified by HPLC, as described by Beall et al (1995). Reduction of NADH was analyzed with a Phenomenex Prodigy 5 μm ODS 150 X 4.6 mm analytical column with a Waters HPLC system (Waters WISP™ autoinjection system, Waters programmable multisolvent delivery system, Waters programmable multi-wavelength detector, and a Hewlett Packard integrator). A linear solvent gradient program of 10 mM potassium phosphate (pH 6.0) and HPLC grade methanol was used to isolate NADH. The solvent gradient program is described in Table 1. NADH was detected at 340 nm, at a retention time of 16 min, and was quantified by measuring the area under the curve.

Table 1: HPLC solvent gradient program

Time (min)	Flow rate (ml/min)	Phosphate buffer (%)	Methanol (%)
0	1.0	100	0
5	1.0	95	5
15	1.0	20	80
25	1.0	20	80
30	1.0	95	5
35	1.0	100	0
45	1.0	100	0

RESULTS

Effect of D3T on MMC cytotoxicity in human tumour cells

Combination treatment with D3T and MMC in NCI-H661 cells

NCI-H661 human non-small cell lung cancer cells were incubated with, or without, 50 μM D3T for 48 hr and then were treated with various concentrations of MMC for 1 hr. Cytotoxicity was determined by MTT assay. The concentration of D3T used was not toxic to the cells. DT-diaphorase activity was significantly increased from 117.2 ± 8.8 to 292.0 ± 14.0 nmol min^{-1} mg $protein^{-1}$ ($p < 0.001$) by D3T. D3T increased the cytotoxicity of MMC by 2.3-fold with the D_0 changing from 3.24 ± 0.19 μM without D3T to 1.43 ± 0.15 μM with D3T pretreatment ($p < 0.001$). The results are illustrated in Figure 4. The D_0 is listed in Table 2.

Combination treatment with D3T and MMC in T47D cells

T47D, human breast cancer cells, were incubated with, or without, 75 μM D3T for 48 hr and then were treated with various concentrations of MMC for 1 hr. The concentration of D3T used was not toxic to the cells. Cytotoxicity was determined by MTT assay. DT-diaphorase activity was significantly increased from 30.8 ± 0.8 to 100.0 ± 1.5 nmol min^{-1} mg $protein^{-1}$ ($p < 0.001$) by D3T. D3T increased the cytotoxicity of MMC by 2.4-fold with the D_0 changing from 3.27 ± 0.20 μM without D3T to 1.34 ± 0.13 μM with D3T pretreatment ($p < 0.001$). The results are illustrated in Figure 5. The D_0 is listed in Table 2.

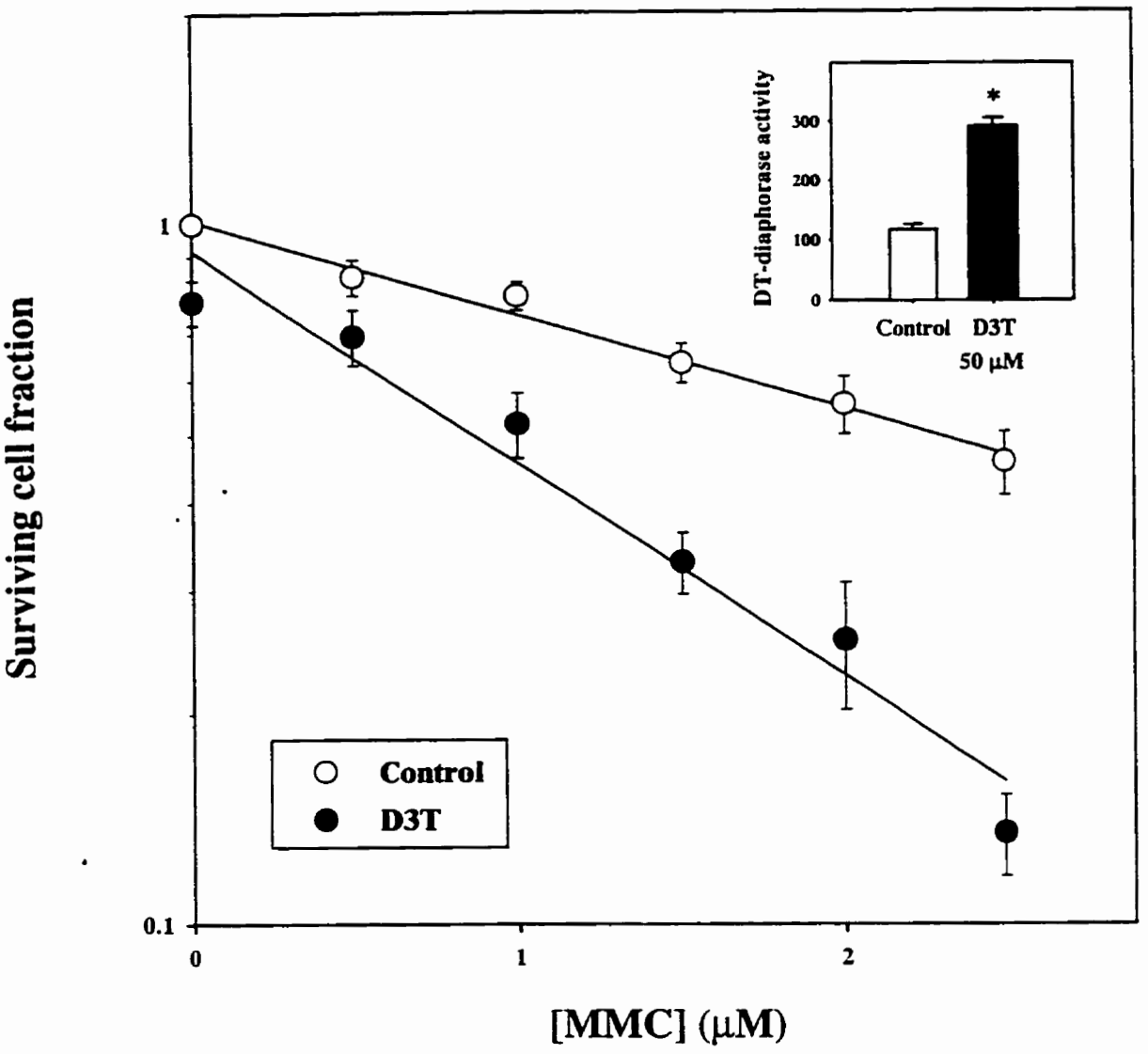


Figure 4: Combination treatment of NCI-H661 cells with D3T and MMC

Cells were incubated at 37^{0}C with, or without, 50 μM D3T for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of five determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.001$).

Table 2: Effect of inducers of DT-diaphorase on the cytotoxicity of MMC in human tumour cell lines.

Cells were treated with, or without, inducers at 37^{0}C for 48 hrs, then were incubated with various concentrations of MMC for 1 hr. Surviving cell fractions were measured by MTT assay for tumour cells or by methylcellulose clonogenic assay for normal human bone marrow cells. The antitumour activity of MMC is presented as the D_0, which was obtained from the linear regression line of the surviving cell fraction vs the drug concentration curve. The data represent the mean ± SE of three to eight determinations. A t-test comparing the significance of the difference of the slopes of the linear regression lines was used to compare the D_0s for cells treated with, or without, inducers.

Tumour Type	Cell Line	Inducer	D_0 (μM)		P Value
			Control	With Inducer	
Lung	NCI-H661	D3T	3.24 ± 0.19	1.43 ± 0.15	< 0.001
Breast	T47D	D3T	3.27 ± 0.20	1.34 ± 0.13	< 0.001
Breast	HS578T	D3T	3.72 ± 0.30	2.63 ± 0.10	< 0.01
Colon	HCT116	D3T	3.31 ± 0.25	1.67 ± 0.11	< 0.001
Skin	SK-MEL-28	D3T	4.40 ± 0.41	4.06 ± 0.34	NS
Stomach	AGS	D3T	0.78 ± 0.08	0.66 ± 0.05	NS
Breast	T47D	DMM	3.27 ± 0.20	1.13 ± 0.09	< 0.001
Breast	T47D	PG	3.27 ± 0.20	1.85 ± 0.14	< 0.001
Normal Human Bone Marrow		D3T	4.66 ± 0.41	2.95 ± 0.23	< 0.05

NS, not significant

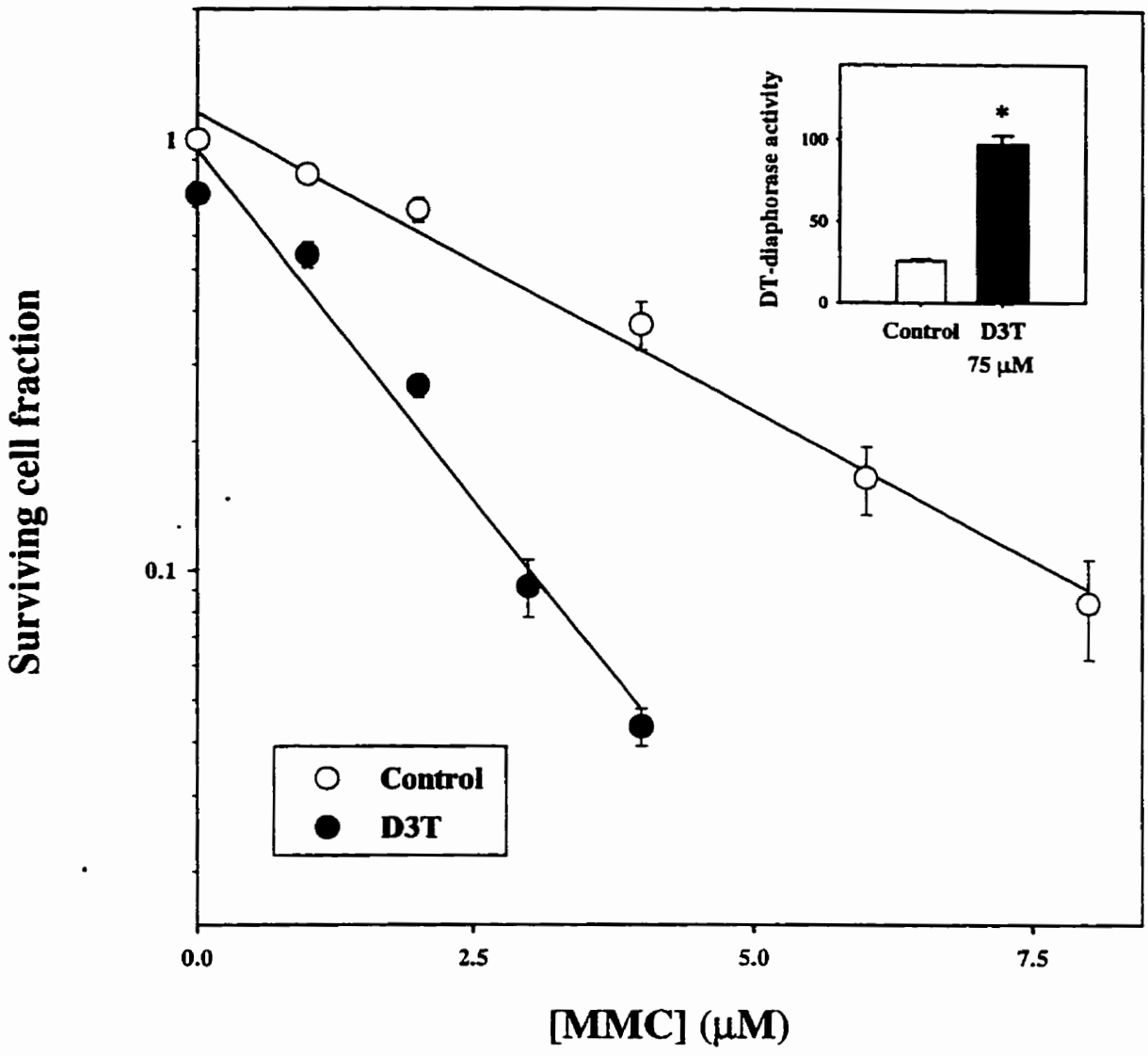


Figure 5: Combination treatment of T47D cells with D3T and MMC

Cells were incubated with, or without, 75 μM D3T for 48 hrs at 37^{0}C, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of three to eight determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.001$)

Combination treatment with D3T and MMC in HS578T cells

HS578T, human breast cancer cells, were incubated with, or without, 100 μM D3T for 48 hr and then treated with various concentrations of MMC for 1 hr. The concentration of D3T used was not toxic to the cells. Cytotoxicity was determined by MTT assay. DT-diaphorase activity was increased from 237.9 ± 13.9 to 420.8 ± 19.0 nmol min^{-1} mg $protein^{-1}$ ($p < 0.001$). Cytotoxicity of MMC was increased by 1.4-fold with the D_0 changing from 3.72 ± 0.30 μM without D3T to 2.63 ± 0.10 μM with D3T pretreatment ($p < 0.01$). The results are illustrated in Figure 6. The D_0 is listed in Table 2.

Combination treatment with D3T and MMC in HCT116 cells

HCT116, human colon cancer cells, were incubated with, or without, 50 μM D3T for 48 hr and then were treated with various concentrations of MMC for 1 hr. The concentration of D3T used was not toxic to the cells. Cytotoxicity was determined by MTT assay. DT-diaphorase activity was increased from 118.0 ± 17.9 to 267.0 ± 56.3 nmol min^{-1} mg $protein^{-1}$ ($p < 0.05$). D3T increased the cytotoxicity of MMC by 2-fold with the D_0 changing from 3.31 ± 0.25 μM without D3T to 1.67 ± 0.11 μM with D3T pretreatment ($p < 0.01$). The results are illustrated in Figure 7. The D_0 is listed in Table 2.

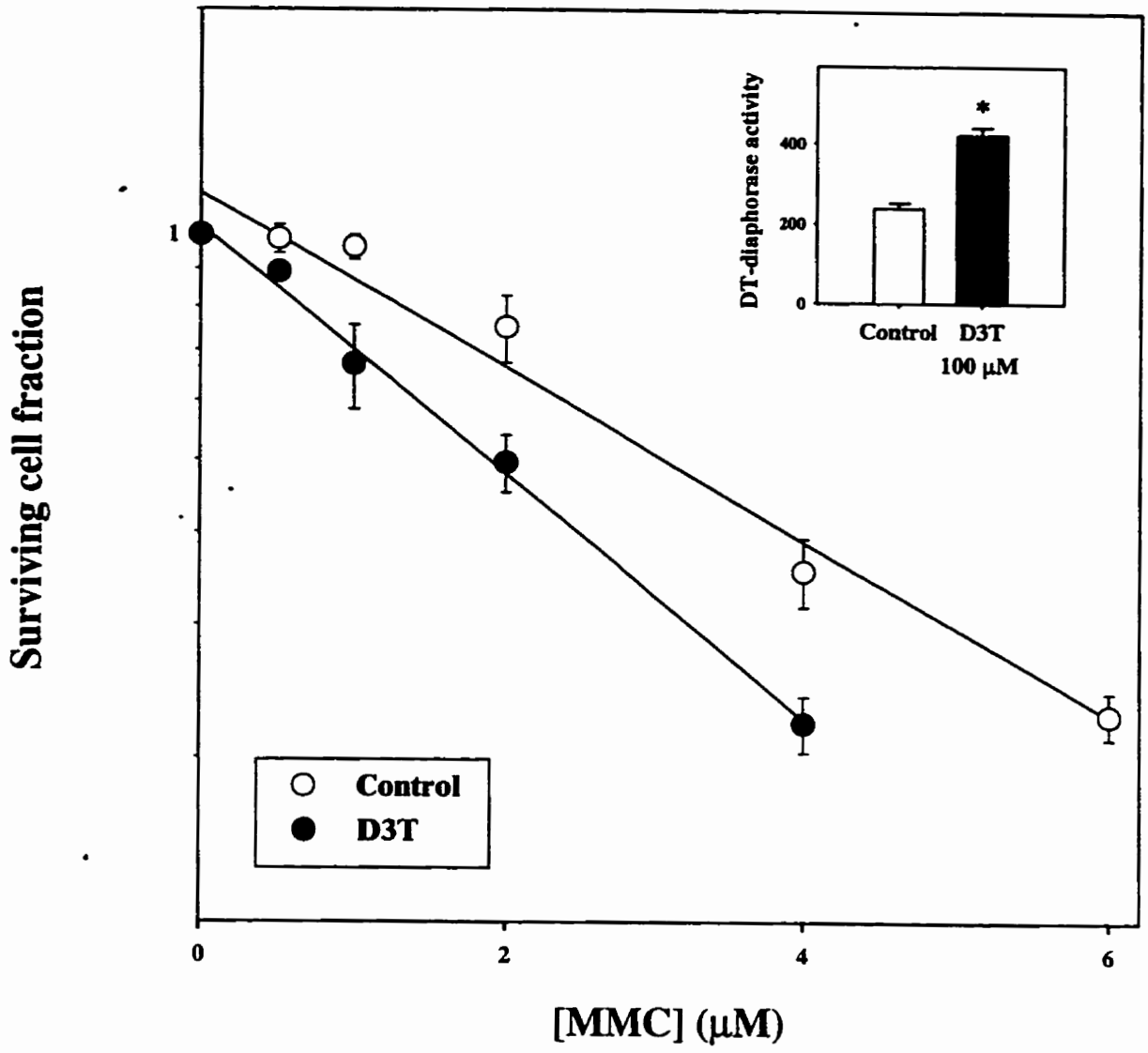


Figure 6: Combination treatment of HS578T cells with D3T and MMC

Cells were incubated at 37^{0}C with, or without, 100 μM D3T for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of three to six determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.001$)

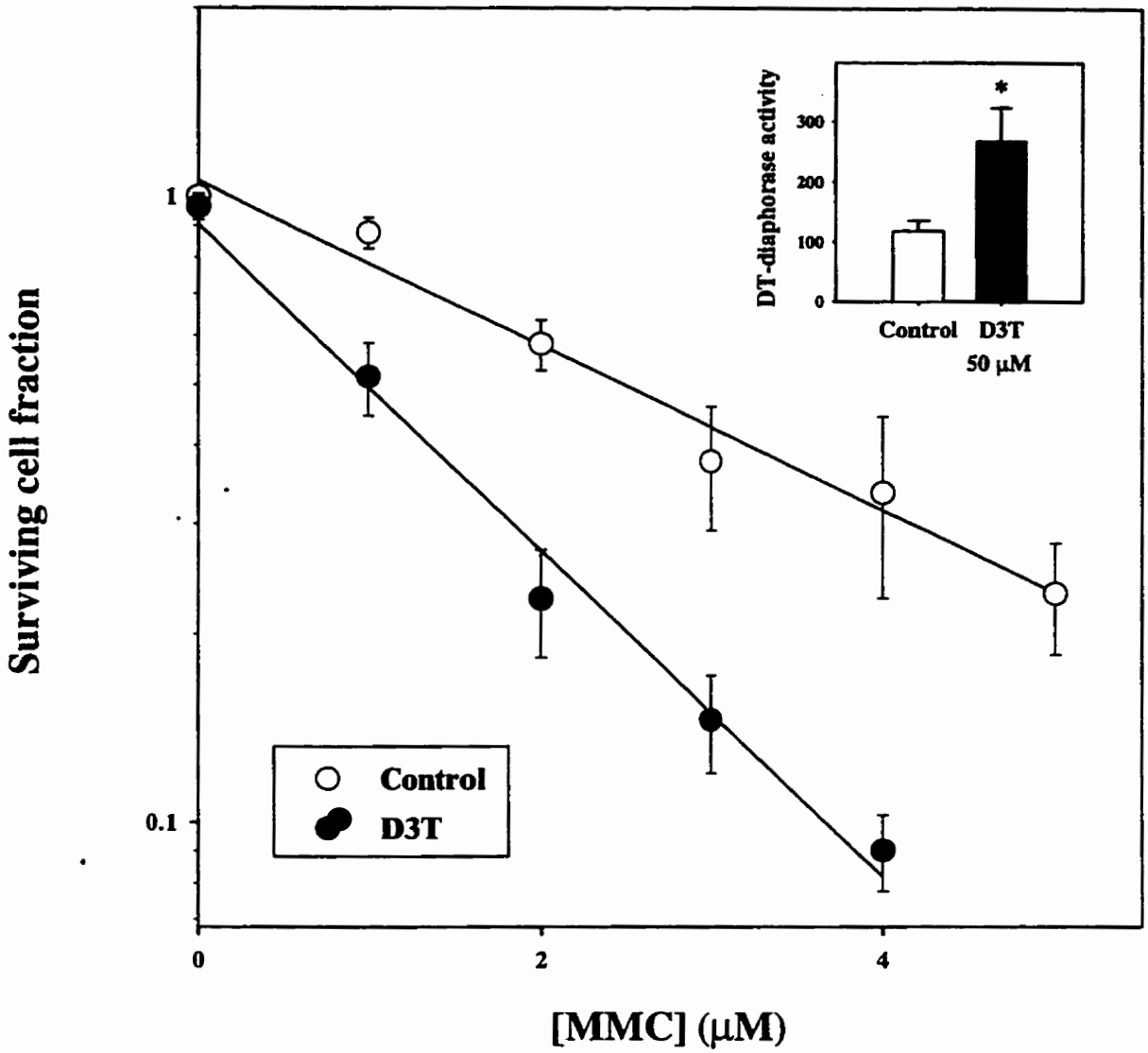


Figure 7: Combination treatment of HCT116 cells with D3T and MMC

Cells were incubated at 37°C with, or without, 50 μM D3T for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of four to six determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.05$)

Combination treatment with D3T and MMC in SK-MEL-28 cells

SK-MEL-28, human melanoma cells, were incubated with, or without, 50 μM D3T for 48 hr and then were treated with various concentrations of MMC for 1 hr. The concentration of D3T used was not toxic to the cells. Cytotoxicity was determined by MTT assay. DT-diaphorase activity was significantly increased from 270.0 ± 14.7 to 622.2 ± 22.9 nmol min^{-1} mg $protein^{-1}$ ($p < 0.001$) in SK-MEL-28 cells by D3T. D3T did not increase the cytotoxicity of MMC in SK-MEL-28 cells. The D_0 was 4.40 ± 0.41 μM without D3T and 4.06 ± 0.34 μM with D3T pretreatment. The results are illustrated in Figure 8. The D_0 is listed in Table 2.

Combination treatment with D3T and MMC in AGS cells

AGS, human gastric cancer cells, were incubated with, or without, 25 μM D3T for 48 hr and then were treated with various concentrations of MMC for 1 hr. The concentration of D3T used was not toxic to the cells. Cytotoxicity was determined by MTT assay. DT-diaphorase activity was increased from 216.5 ± 14.4 to 413.8 ± 13.4 nmol min^{-1} mg $protein^{-1}$ ($p < 0.001$). D3T did not significantly increase the cytotoxicity of MMC in AGS cells. The D_0 was 0.78 ± 0.08 μM without D3T and 0.66 ± 0.05 μM with D3T in AGS cells. The results are illustrated in Figure 9. The D_0 is listed in Table 2.

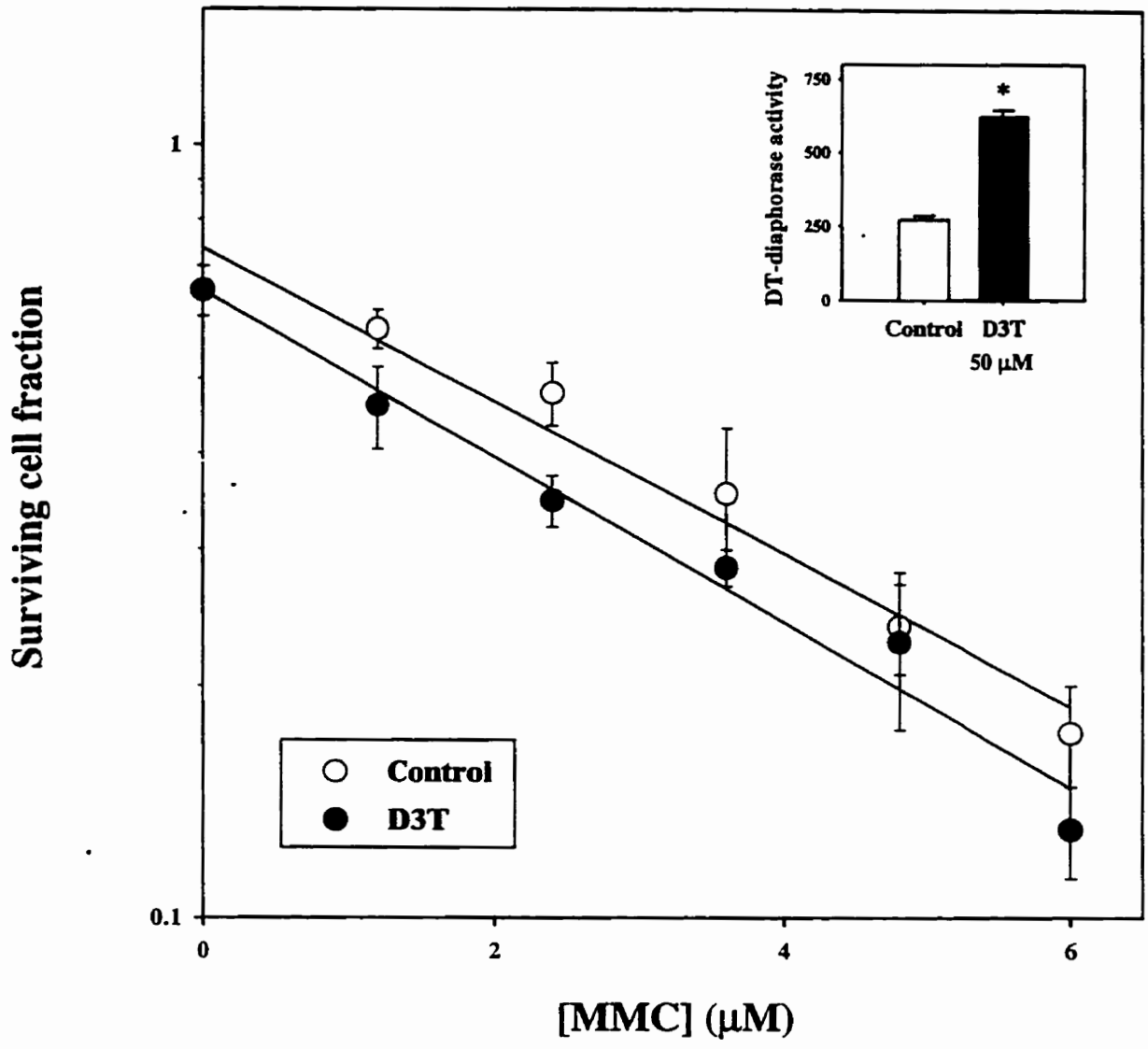


Figure 8: Combination treatment of SK-MEL-28 cells with D3T and MMC

Cells were incubated at 37^{0}C with, or without, 50 μM D3T for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of four determinations. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.001$)

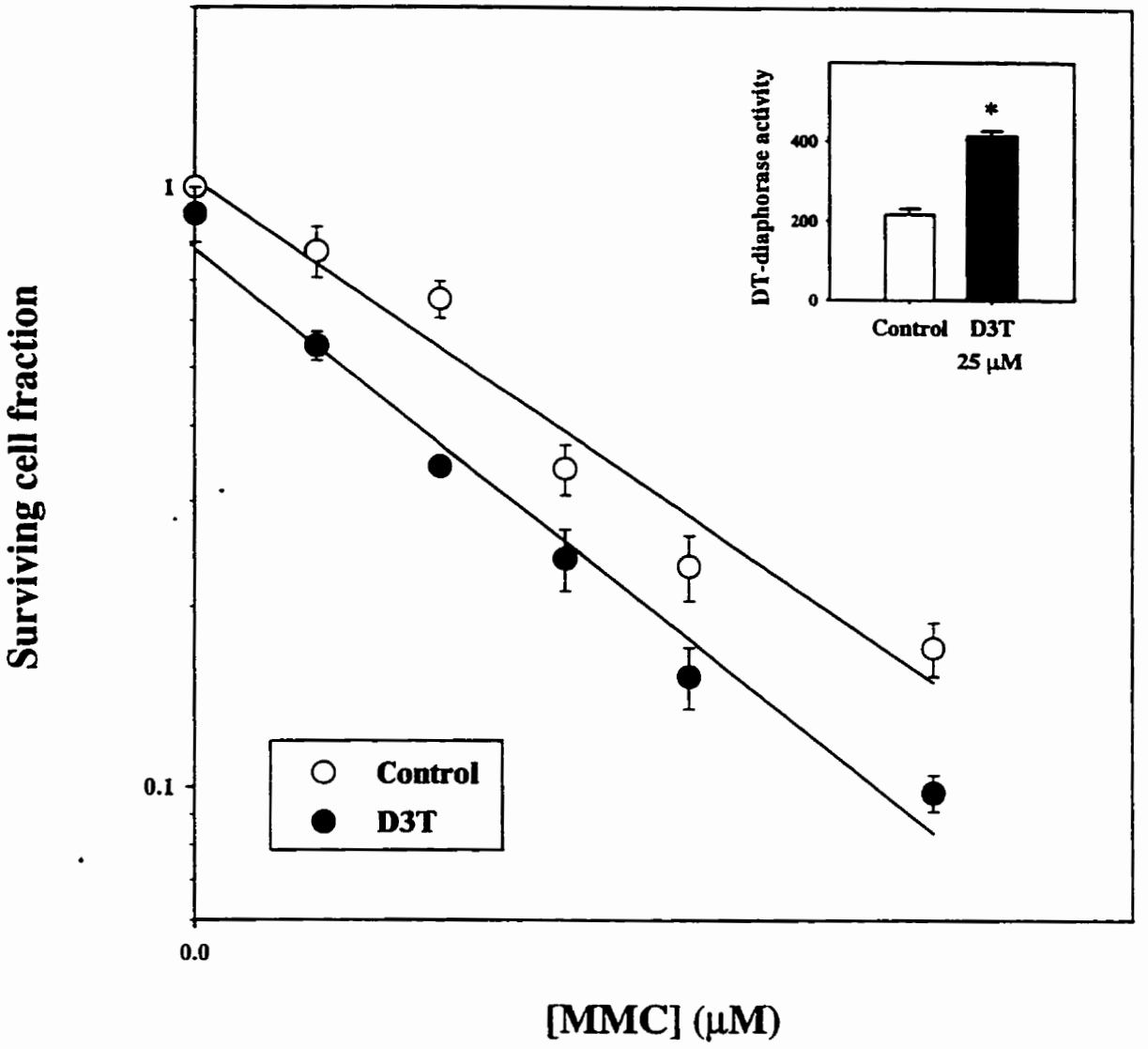


Figure 9: Combination treatment of AGS cells with D3T and MMC

Cells were incubated at 37^{0}C with, or without, 25 μM D3T for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of four determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.001$)

Induction of DT-diaphorase by dietary and pharmaceutical inducers in T47D cells

The ability of dietary components and pharmaceuticals to induce DT-diaphorase was examined in T47D, human breast cancer cells, which had a base level of DT-diaphorase of 27.8 ± 1.2 nmol min^{-1} mg $protein^{-1}$ and were readily inducible by D3T. Fourteen inducers were tested and eight inducers showed significant induction of enzyme activity. The induced enzyme levels ranged from 40.8 ± 1.2 to 128.5 ± 5.6 nmol min^{-1} mg $protein^{-1}$ (Table 3). D3T increased the DT-diaphorase level to 101.0 ± 2.8 nmol min^{-1} mg $protein^{-1}$. DMM, DMF and sulforaphane were good inducers of DT-diaphorase compared with D3T, with induced enzyme levels of 121.0 ± 3.9 with 50 μM DMM ($p < 0.001$), 128.5 ± 5.6 with 50 μM DMF ($p < 0.001$) and 102.3 ± 0.7 nmol min^{-1} mg $protein^{-1}$ with 10 μM sulforaphane ($p < 0.001$), respectively. The activity of DT-diaphorase was also significantly increased after treatment with propyl gallate, 13-cis-retinoic acid, chalcone and aspirin, although the enzyme activity following induction by these agents did not exceed the level of DT-diaphorase activity following induction by D3T in T47D cells. The enzyme levels after treatment with these inducers were: 80.5 ± 5.3 nmol min^{-1} mg $protein^{-1}$ with 10 μM propyl gallate ($p < 0.001$), 61.6 ± 4.9 nmol min^{-1} mg $protein^{-1}$ with 10 μM 13-cis-retinoic-acid ($p < 0.001$), 41.4 ± 3.4 nmol min^{-1} mg $protein^{-1}$ with 20 μM chalcone ($p < 0.001$) and 40.8 ± 1.2 nmol min^{-1} mg $protein^{-1}$ with 1 mM aspirin ($p < 0.001$). There was no significant induction of DT-diaphorase activity in T47D cells by treatment with genistein, ursolic acid, ibuprofen, caffeic acid, folic acid or vitamin K.

Table 3: Induction of DT-diaphorase in T47D human breast tumour cells by dietary and pharmaceutical inducers

Cells were incubated with inducers at the concentrations shown at 37^{0}C for 48 hrs. Cells were washed, pelleted, suspended in 200 µl of 0.25 M sucrose and sonicated. DT-diaphorase activity was measured using menadione as the electron acceptor. Data represent the mean ± SE of three to five determinations. Two-tailed t-tests were used to compare the significance of the difference of DT-diaphorase activity in control and inducer treated cells.

Inducers	Concentration	Enzyme Activity (nmol min^{-1} mg protein^{-1})	P Value
Control		27.8 ± 1.2	
DMF	50 µM	128.5 ± 5.6	< 0.001
DMM	50 µM	121.0 ± 3.9	< 0.001
* Sulforaphane	10 µM	102.3 ± 0.7	< 0.001
D3T	100 µM	101.0 ± 2.8	< 0.001
PG	10 µM	80.5 ± 5.3	< 0.001
13-cis-Retinoic Acid	10 µM	61.6 ± 4.9	< 0.001
Chalcone	20 µM	41.4 ± 3.4	< 0.001
Aspirin	1 mM	40.8 ± 1.2	< 0.001
Caffeic Acid	500 µM	33.4 ± 2.3	NS
Ursolic Acid	15 µM	29.6 ± 4.4	NS
Genistein	5 µM	23.8 ± 1.4	NS
Folic Acid	100 µM	17.3 ± 2.5	NS
Vitamin K	10 µM	17.3 ± 1.3	NS
Ibuprofen	200 µM	16.1 ± 1.1	NS

NS, not significant

Combination treatment of MMC with DT-diaphorase inducers

T47D cells were incubated with, or without, 10 μM PG, 75 μM D3T or 50 μM DMM for 48 hrs and then were treated with various concentrations of MMC for 1 hr. Cytotoxicity of MMC was determined by MTT assay. The concentrations of inducers used were not toxic to the cells. DT-diaphorase activity increased from 30.0 ± 0.8 nmol min^{-1} mg $protein^{-1}$ in control cells to 81.1 ± 2.8, 100.0 ± 1.5 and 121.0 ± 3.9 nmol min^{-1} mg $protein^{-1}$ in cells treated with PG, D3T and DMM, respectively ($p < 0.001$). The cytotoxicity of MMC was increased by 2-, 2.4- and 3-fold in cells pretreated with PG, D3T and DMM, respectively. The D_0 was 3.27 ± 0.20 μM with MMC alone, 1.85 ± 0.14 μM with PG, 1.34 ± 0.13 μM with D3T and 1.13 ± 0.09 μM with DMM ($p < 0.001$). The results are illustrated in Figure 10. The D_0s are summarized in Table 2.

Effect of DMM on NADPH:cytochrome P450 reductase

T47D cells were incubated with, or without 50 μM DMM at 37°C for 48 hrs. DMM did not have any significant effect on the level of NADPH:cytochrome P450 reductase activity. The activity of NADPH:cytochrome P450 reductase was 11.1 ± 1.0 nmol min^{-1} mg $protein^{-1}$ without 50 μM DMM and 10.4 ± 0.3 nmol min^{-1} mg $protein^{-1}$ with 50 μM DMM in T47D cells. Result is summarized in Table 4.

Effect of DMM on NADH:cytochrome b_5 reductase

T47D cells were incubated with, or without, 50 μM DMM at 37°C for 48 hrs. DMM had no significant effect on the level NADH:cytochrome b_5 reductase activity.

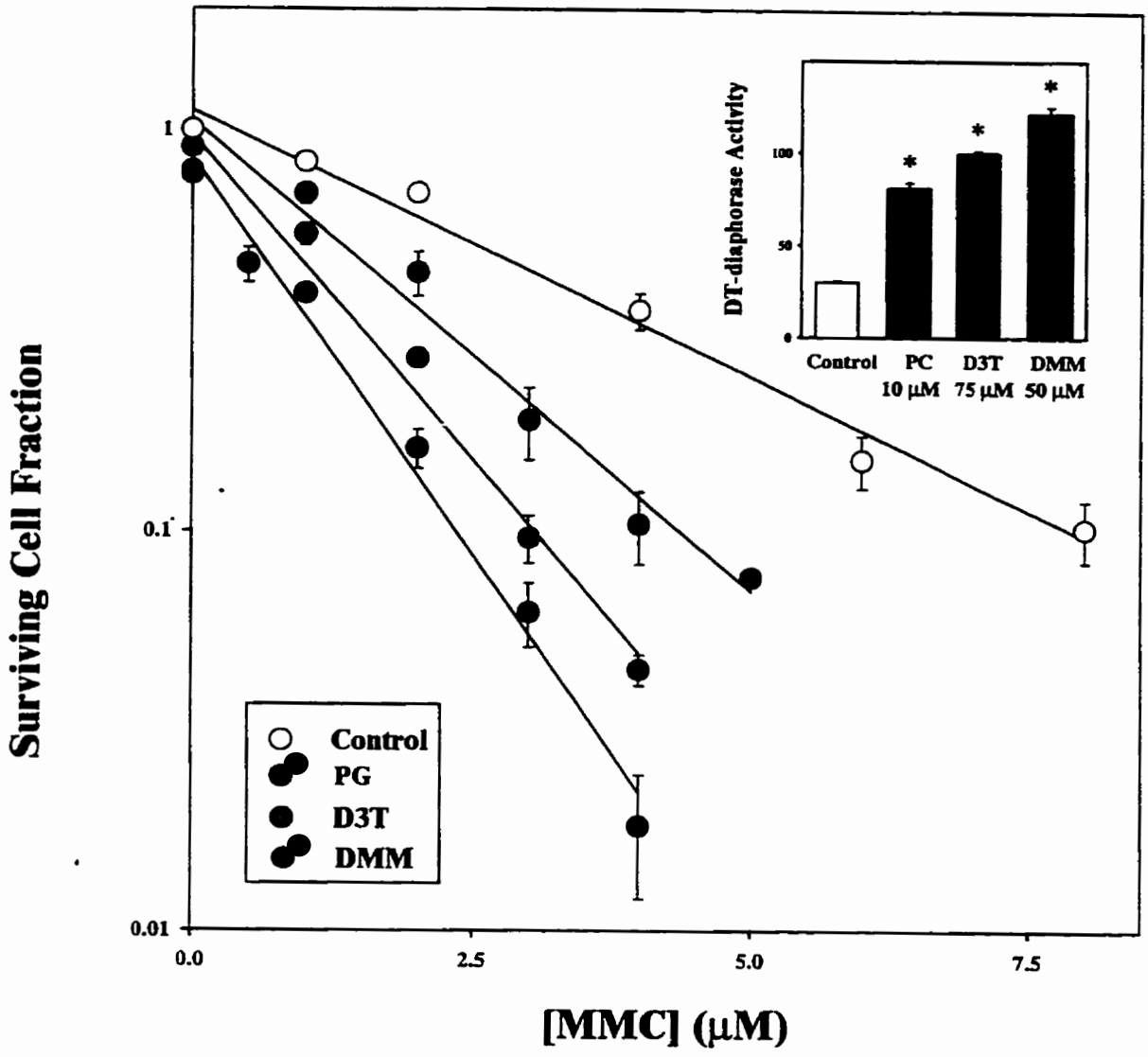


Figure 10: Combination treatment of T47D with PG, D3T or DMM and MMC

Cells were incubated at 37^{0}C with, or without, 10 μM PG, 75 μM D3T or 50 μM DMM for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by MTT assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of three to eight determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. ANOVA was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.001$)

Table 4: Induction of enzymes involved in activation of MMC and GST by DMM in T47D human breast cancer cells

T47D cells were incubated with DMM at 37^0C for 48 hrs. Cells were then washed, pelleted, suspended and sonicated. Enzyme activities were measured as described in "**Materials and Methods**". Data represent the mean ± SE of three to four determinations. Two-tailed t-tests were used to compare the significance of the difference of the enzyme activities in control and inducer treated cells

Enzyme	Enzyme activity (nmol min^{-1} mg $protein^{-1}$)		P Value
	Control	DMM induced	
NADPH:cytochrome P450 reductase	11.1 ± 1.0	10.4 ± 0.3	NS
NADH:cytochrome b5 reductase	36.4 ± 4.7	34.3 ± 4.2	NS
Xanthine dehydrogenase	ND	ND	
GST	20.2 ± 0.6	24.0 ± 1.1	< 0.05

NS, not significant

ND, not detected

The activity of NADH:cytochrome b_5 reductase was 36.4 ± 4.7 nmol min^{-1} mg $protein^{-1}$ without DMM and 34.3 ± 4.2 nmol min^{-1} mg $protein^{-1}$with 50 μM DMM in T47D cells (Table 4).

Effect of DMM on xanthine dehydrogenase

T47D cells were incubated with, or without, 50 μM DMM at 37^0C for 48 hrs. The activity of xanthine dehydrogenase/oxidase was too low to be detected in cells treated with, or without, DMM (Table 4).

Effect of DMM on GST activity

T47D cells were incubated with, or without 50 μM DMM at 37^0C for 48 hrs and the activity of GST was measured. There was a small increase in GST activity after incubation with DMM with GST levels increasing from 20.2 ± 0.6 nmol min^{-1} mg $protein^{-1}$ in control cells to 24.0 ± 1.1 nmol min^{-1} mg^{-1} protein in cells treated with DMM ($p < 0.05$) (Table 4).

Bone marrow toxicity

Normal human marrow cells were incubated with, or without, 100 μM D3T and then were treated with various concentrations of MMC. The concentration of D3T used was not toxic to the cells. DT-diaphorase activity increased from 1.9 ± 0.3 nmol min^{-1} mg $protein^{-1}$ in control cells to 12.7 ± 2.4 nmol min^{-1} mg $protein^{-1}$ in cells treated with 100 μM D3T ($p < 0.005$). The surviving cell fraction of human marrow progenitor cells

(CFU-G, CFU-M, CFU-GM, CFU-E and BFU-E) was determined by methylcellulose clonogenic assay. The D_0 for MMC cytotoxicity was 4.66 ± 0.41 μM without D3T and 2.95 ± 0.23 μM with D3T ($p < 0.05$). There was no obvious selective effect on any of the progenitor cell types. The results are illustrated in Figure 11. The D_0s are summarized in Table 2.

Cytotoxicity of BM analogues in SK-MEL-28 cells

SK-MEL-28 human melanoma cells were treated with, or without 100 μM dicoumarol for 20 min and then with, or without 2 μM of BM, MBM, PBM or CBM for 1 hr. The SK-MEL-28 cells had a DT-diaphorase level of 586.7 ± 19.6 nmol MTT min^{-1} mg $protein^{-1}$. The surviving cell fraction was determined by MTT assay (Figure 12). For BM, the surviving cell fraction was 0.64 ± 0.14 without dicoumarol and 0.27 ± 0.08 with dicoumarol ($p < 0.05$). For MBM, the surviving cell fraction was 0.10 ± 0.03 without dicoumarol and 0.28 ± 0.06 with dicoumarol ($p < 0.05$). For PBM, the surviving cell fraction was 0.73 ± 0.11 without dicoumarol and 0.70 ± 0.10 with dicoumarol. For CBM the surviving cell fraction was 0.62 ± 0.07 without dicoumarol and 0.38 ± 0.10 with dicoumarol.

HPLC analysis

Standard curve

The rate of reduction of BM analogues was measured as the rate of consumption of NADH, which is a cofactor of this enzymatic reaction. The HPLC analysis showed a

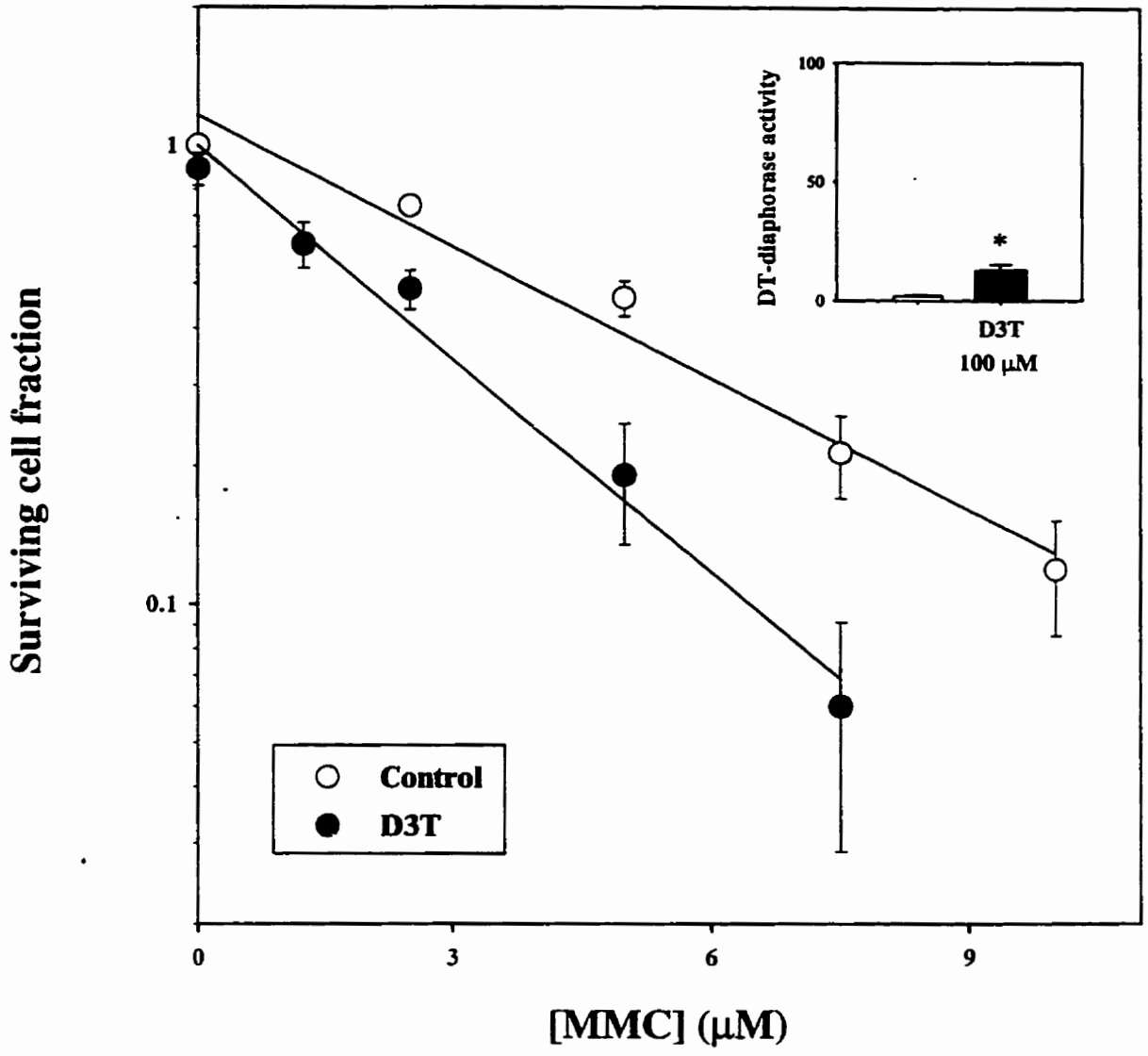


Figure 11: Combination treatment of normal human marrow cells with D3T and MMC

Cells were incubated at 37°C with, or without, 100 μM D3T for 48 hrs, then were treated with various concentrations of MMC for 1 hr. Surviving cell fraction was measured by methocellulose clonogenic assay. DT-diaphorase activity was measured as described in **Materials and Methods** and is shown in the inserted figure. Data represents the mean ± SE of four to five determinations. On occasion, the SE is too small to be shown. The lines are linear regression lines. A two-tailed t-test was used to determine the significance of the difference of the DT-diaphorase activities. (*, $p < 0.005$)

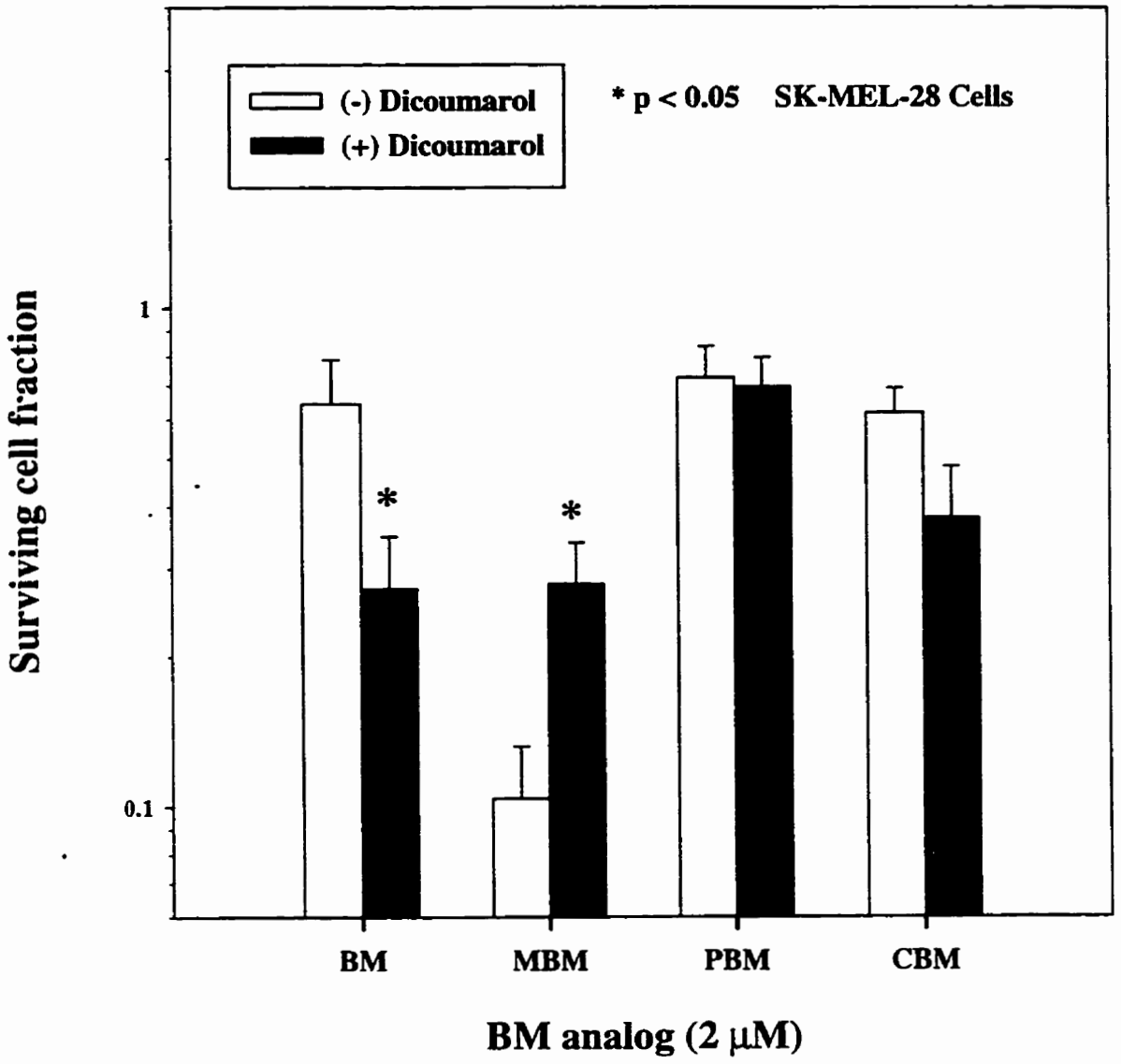


Figure 12: Cytotoxicity of BM analogues in SK-MEL-28 human melanoma cells

Cells were incubated with, or without, 100 μM dicoumarol for 20 min and then with 2 μM of BM analogues for 1 hr. Surviving cell fraction was determined by MTT assay. The results represent the mean ± SE of 6 to 10 determinations. Surviving cell fractions with, and without, dicoumarol were compared for each BM analogues by a two-tailed t-test comparing the significance of the difference of the means.

linear relationship between the absorbance of NADH at 340 nm and the concentration of NADH in the range from 0 to 100 μM (Figure 13).

Reduction of BM analogues

Reduction of the BM analogues by purified DT-diaphorase in cell free solution was carried out under both aerobic and hypoxic conditions. Two equivalents of NADH to one equivalent of BM analogues was used to ensure the availability of sufficient NADH in the reduction. 10 μM dicoumarol was used to stop the reaction at various time points. The reduction of the analogues was measured by detecting the loss of NADH, a cofactor of this enzymatic reaction, using HPLC analysis as described by Beall et al (1992).

The consumption of NADH in the reduction of BM by DT-diaphorase was measured at 0, 1, 2, 3, 5, 15, 20 and 30 min both under aerobic and hypoxic conditions. Under hypoxic conditions, one equivalent of NADH was consumed after approximately 10 min and there was no further decrease in the level of NADH. A similar result was obtained under aerobic condition. The time courses of BM reduction by purified human DT-diaphorase are illustrated in Figure 14.

The consumption of NADH in the reduction of MBM by purified DT-diaphorase was measured at 0, 5, 10, 15, 20, 40, and 60 minutes both under aerobic and hypoxic conditions. Under hypoxic condition, one equivalent of NADH was consumed after approximately 20 min and there was no further decrease in the level of NADH. In contrast, under aerobic conditions, there was greater consumption of NADH with two equivalents of NADH consumed by 60 min. The time courses of MBM reduction by

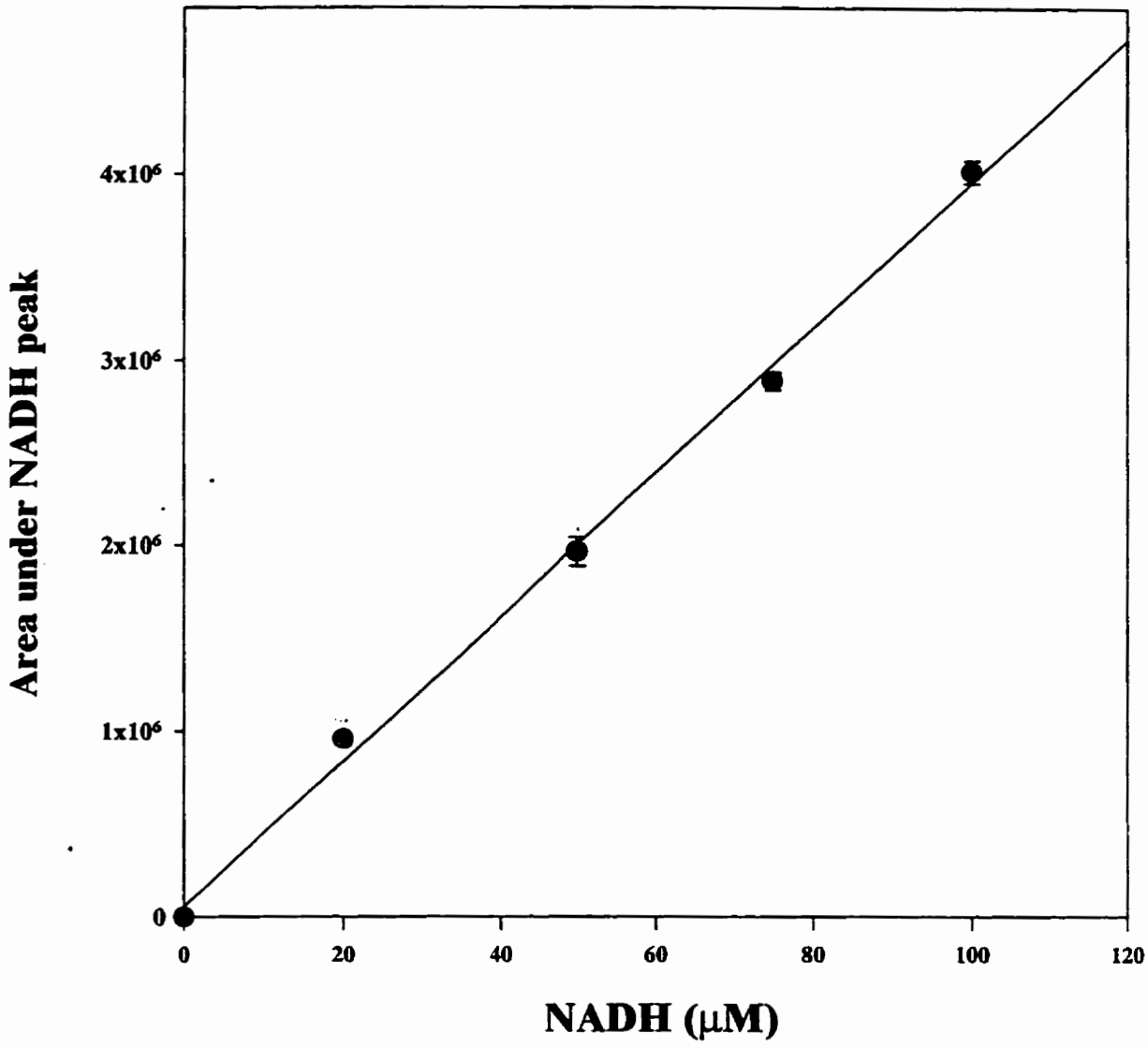


Figure 13: Calibration curve for measurement of NADH by HPLC

NADH was dissolved in 25 mM Tris HCl (pH 7.4) to make the final concentrations of 0, 25, 50, 75 and 100 μM. NADH was quantified by measuring the area under the NADH peak by HPLC analysis. The line is a linear regression line. The data represent the mean ± SE of 3 to 4 determinations.

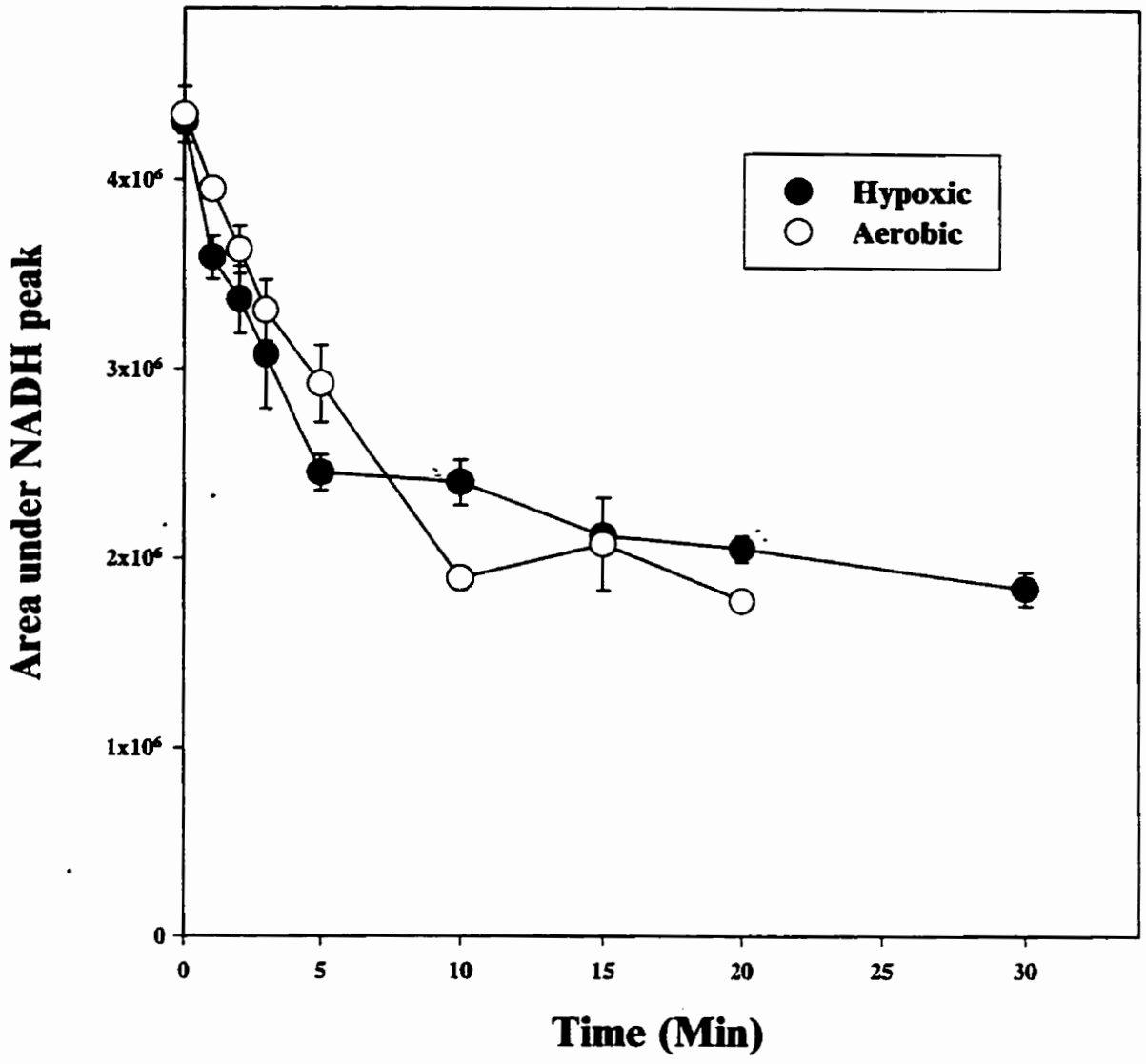


Figure 14: Reduction of BM by purified DT-diaphorase

The reaction buffer contained 100 μM NADH, 0.5 μM FAD and 0.1 μg/ml purified DT-diaphorase and 25 mM Tris HCl (pH 7.4). Air or nitrogen was continuously bubbled through the reaction buffer for 2 hrs prior to addition of BM and during the reduction reaction at 37 ^{0}C. 50 μM BM was added to start the reduction. At various time points, 10 μM dicoumarol was added to stop the reaction. Reduction of BM was measured by quantitating the loss of NADH by HPLC analysis. The data represent the mean ± SE of 5 determinations. On occasion, the SE was too small to be shown.

purified DT-diaphorase under both aerobic and hypoxic conditions are illustrated in Figure 15.

The consumption of NADH in the reduction of PBM by DT-diaphorase was measured at 0, 5, 15, 30 and 60 minutes under both aerobic and hypoxic conditions. Under hypoxic conditions, there was consumption of NADH for approximately 30 min after which the level of NADH was unchanged. Less than one equivalent of NADH was consumed. Under aerobic conditions, there was greater consumption of NADH, however, the loss of NADH also reached a plateau at approximately 30 min. The time courses of PBM reduction by purified DT-diaphorase are illustrated in Figure 16.

The consumption of NADH in the reduction of CBM by DT-diaphorase was measured at 0, 2, 5, 15, 30 and 60 minutes under both aerobic and hypoxic conditions. Under hypoxic conditions, there was little consumption of NADH with less than one-half equivalent of NADH consumed by 60 min. Under aerobic conditions, there was increased NADH consumption. The time courses of CBM reduction by purified DT-diaphorase are illustrated in Figure 17.

Under hypoxic conditions, the rate of reduction of the analogues was BM > MBM > PBM > CBM with $t_{1/2}$ of 3, 10, 31 and > 60 min, respectively. The reduction of BM by DT-diaphorase under aerobic condition was similar compared with under hypoxic conditions, while the rate of consumption of NADH was greater under aerobic conditions for MBM, PBM and CBM.

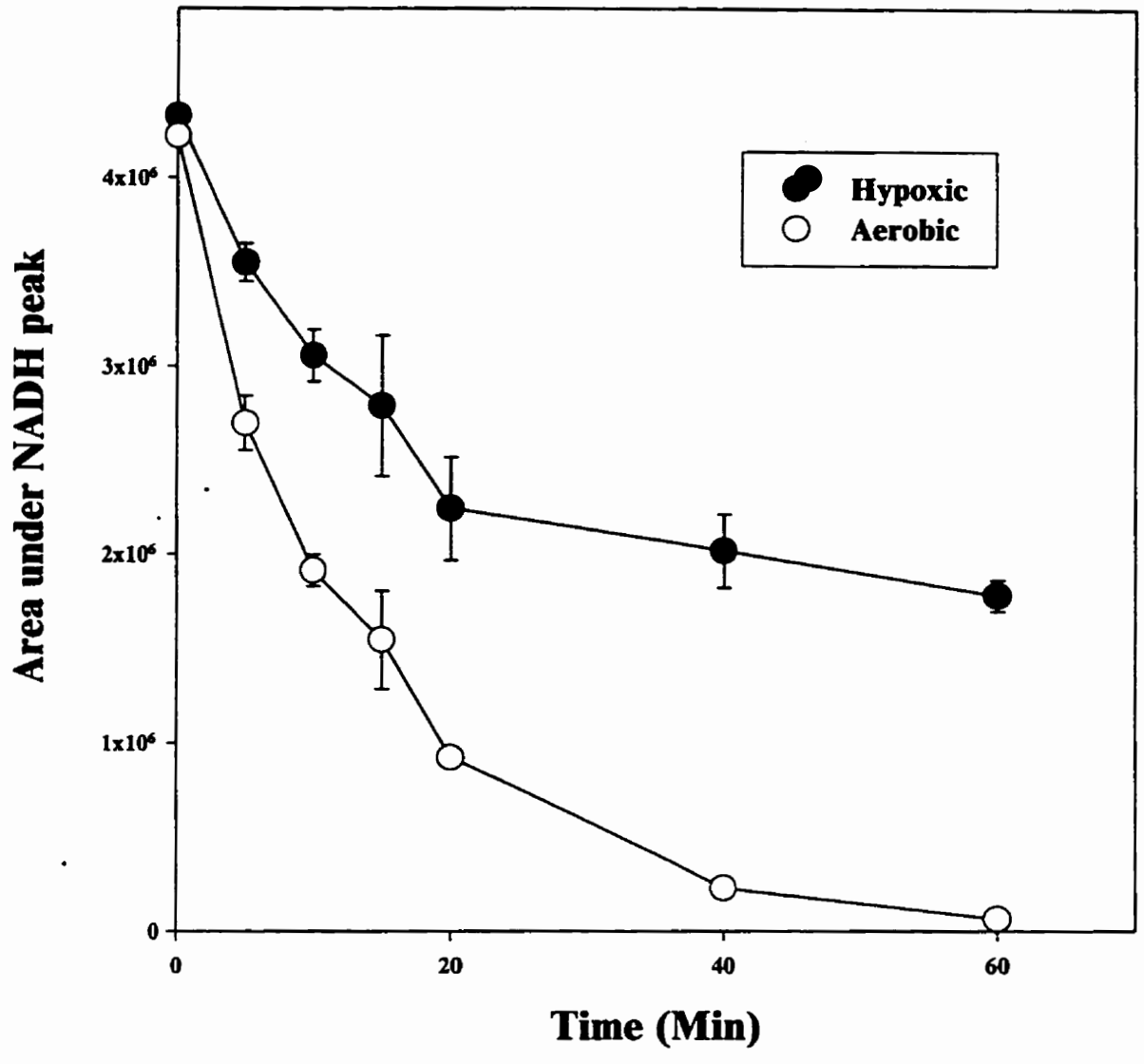


Figure 15: Reduction of MBM by purified DT-diaphorase

The reaction buffer contained 100 μM NADH, 0.5 μM FAD and 0.1 μg/ml purified DT-diaphorase and 25 mM Tris HCl (pH 7.4). Air or nitrogen was continuously bubbled through the reaction buffer for 2 hrs prior to addition of MBM and during the reduction reaction at 37 ^{0}C. 50 μM MBM was added to start the reduction. At various time points, 10 μM dicoumarol was added to stop the reaction. Reduction of MBM was measured by quantitating the loss of NADH by HPLC analysis. The data represent the mean ± SE of 4 determinations. On occasion, the SE was too small to be shown.

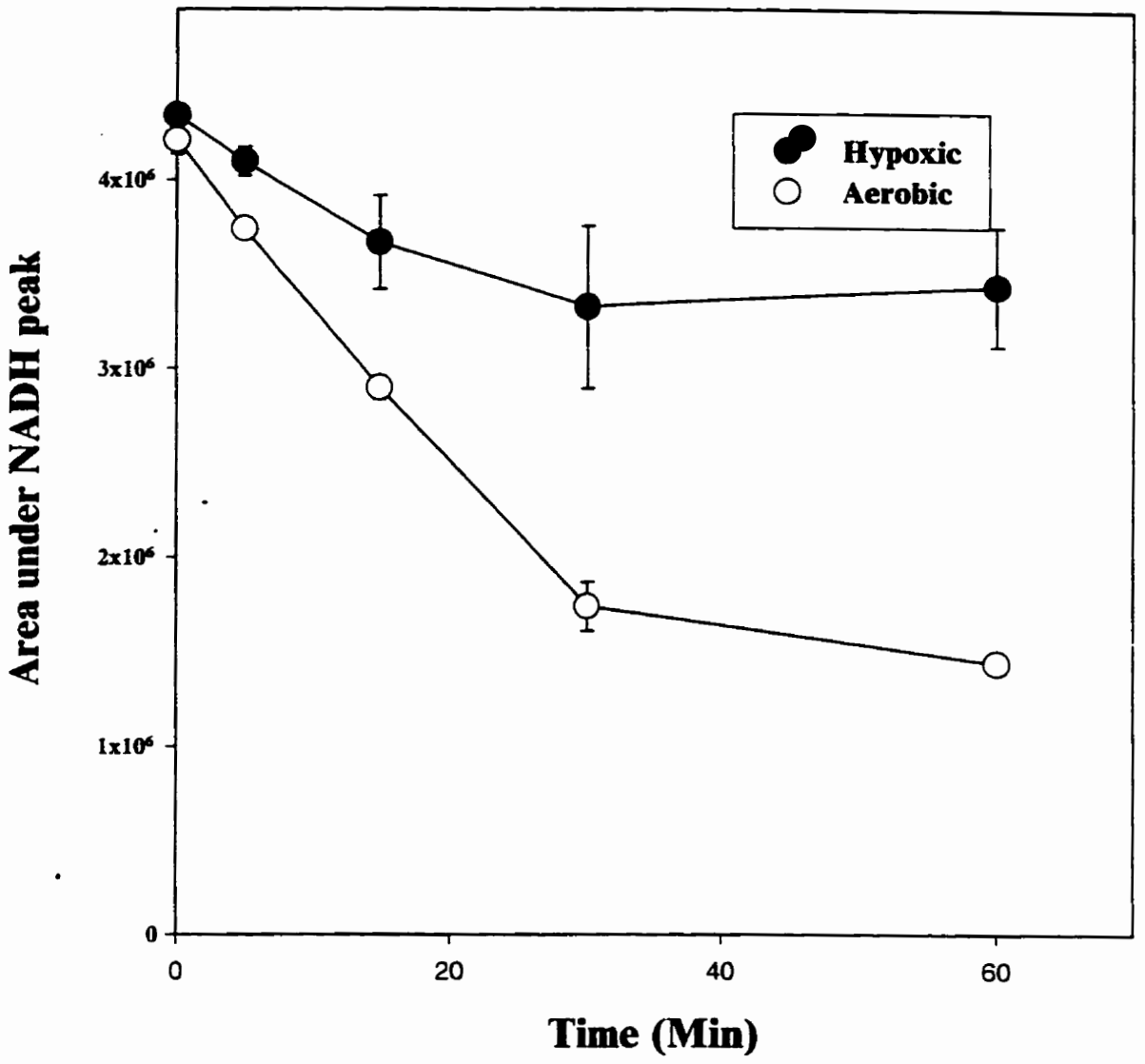


Figure 16: Reduction of PBM by purified DT-diaphorase

The reaction buffer contained 100 μM NADH, 0.5 μM FAD and 0.1 μg/ml purified DT-diaphorase and 25 mM Tris HCl (pH 7.4). Air or nitrogen was continuously bubbled through reaction buffer for 2 hrs prior to addition of PBM and during the reduction reaction at 37 ^{0}C. 50 μM PBM was added to start the reduction. At various time points, 10 μM dicoumarol was added to stop the reaction. Reduction of PBM was measured by quantitating the loss of NADH by HPLC analysis. The data represent the mean ± SE of 3 determinations. On occasion, the SE was too small to be shown.

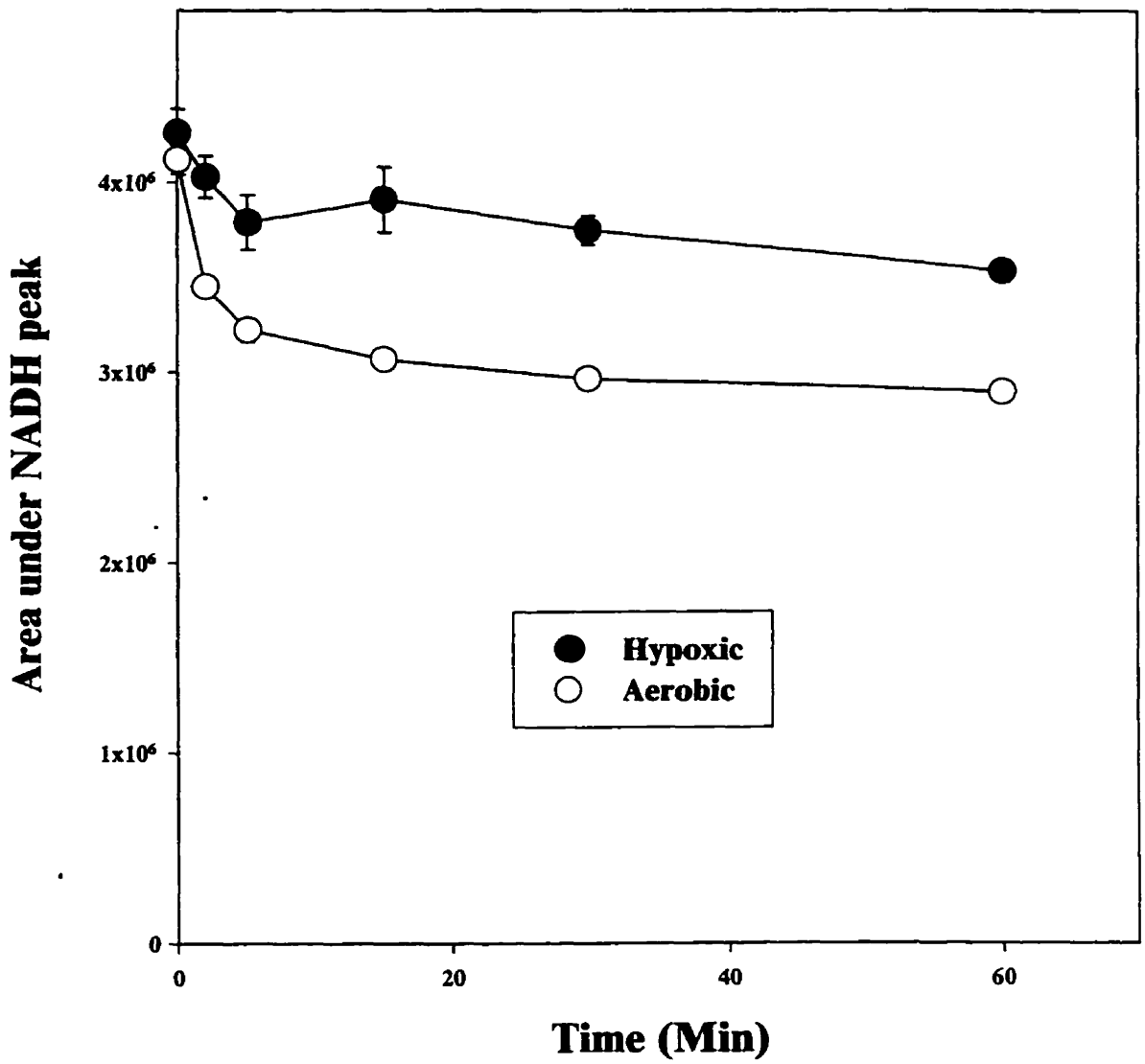


Figure 17: Reduction of CBM by purified DT-diaphorase

The reaction buffer contained 100 μM NADH, 0.5 μM FAD and 0.1 μg/ml purified DT-diaphorase and 25 mM Tris HCl (pH 7.4). Air or nitrogen was continuously bubbled through reaction buffer for 2 hrs prior to addition of CBM and during the reduction reaction at 37 ^{0}C. 50 μM CBM was added to start the reduction. At various time points, 10 μM dicoumarol was added to stop the reaction. Reduction of CBM was measured by quantitating the loss of NADH by HPLC analysis. The data represent the mean ± SE of 3 determinations. On occasion, the SE was too small to be shown.

DISCUSSION

Enhancement of bioreductive agents by induction of DT-diaphorase

Combination treatment of MMC and D3T in human tumour cells

DT-diaphorase is a two-electron reducing enzyme that plays an important role in activating many bioreductive antitumour agents including MMC, the prototype drug in this class of agents. It is also a phase II detoxifying enzyme that detoxifies xenobiotics and carcinogens and protects cells from tumourigenesis (Riley and Workman, 1992; Beyer el al, 1988). DT-diaphorase is highly inducible in many tissues by bifunctional and monofunctional inducers including 1,2-dithiole-3-thiones, quinones, isothiocyanates, diphenols and Michael reaction acceptors (Talalay, 1989; Prestera et al, 1993). We have shown previously that pretreatment with D3T, an inducer of DT-diaphorase, significantly increased the cytotoxic activity MMC and EO9 in mouse lymphoma LY5178 cells with no effect on normal mouse marrow cells (Begleiter et al, 1996). The major toxicity of MMC is a dose-limiting delayed bone marrow toxicity. Similar enhancement of EO9 activity was also observed in human tumour cells in combination with D3T with no effect on normal human kidney cell line (Doherty et al, 1998). Kidney is the major site of clinical toxicity for EO9. We have extended these studies to examine this combination therapy with MMC and D3T in six human tumour cell lines representing 5 human tumour types. MMC has been used clinically in the treatment of non-small cell lung cancer, advanced breast cancer, colon cancer, gastric and head and neck cancer. The six human tumour cell lines have varied DT-diaphorase activity from a low level of 30.8 ± 0.8 to a relatively high enzyme level of 270.0 ± 14.7 nmol min^{-1} mg $protein^{-1}$. We showed

previously that D3T could induce DT-diaphorase activity in 28 out of 37 human tumour cell lines by from 1.3- to 7.0-fold (Doherty et al, 1998). D3T significantly increased DT-diaphorase activity in all six cell lines used in this study. Pretreatment with the enzyme inducer significantly enhanced the cytotoxicity of MMC in four of the cell lines. Combination treatment with D3T and MMC increased the cytotoxic activity of MMC by 2.3-fold in NCI-H661, non-small cell lung cancer cells, by 2.4-fold in T47D breast cancer cells, by 1.4-fold in HS578T breast cancer cells and by 2-fold in HCT116 human colon cancer cells. .These data suggest that this combination strategy may have potential value for enhancing the effectiveness of MMC in the treatment of tumours.

These results support our previous findings that combination treatment with DT-diaphorase inducer and bioreductive agents produced a significant increase in the antitumour activity of bioreductive drugs in animal tumour cells (Begleiter et al, 1996). These results are also consistent with the findings by many researchers (Begleiter et al, 1989; Ross et al, 1993; Mikami et al, 1996; Fitzsimmons et al, 1996) that higher DT-diaphorase activity is associated with greater cytotoxicity for some bioreductive agents. Similarly, Malkinson et al (1992) found a relationship between the level of DT-diaphorase activity in a series of primary human lung xenografts and the sensitivity to MMC.

Although DT-diaphorase activity was significantly increased by pretreatment with D3T in AGS human stomach tumour cells and in SK-MEL-28 human melanoma cells, no increase in MMC antitumour activity was observed in these two cell lines. Both these cell lines have relatively high base levels of DT-diaphorase activity of 216.5 ± 14.4 and 270.0 ± 14.7 nmol min^{-1} mg $protein^{-1}$ for AGS cells and SK-MEL-28 cells, respectively,

compared with the other four cell lines. This suggests that there may be an upper threshold for the level of DT-diaphorase in this combination therapy approach. If the basal or induced level of DT-diaphorase is above this upper threshold, further induction may not lead to an increase in MMC antitumour activity. This hypothesis is further supported by a small 1.4-fold increase in MMC antitumour activity in the HS578T cells, which also have a relatively high base level of DT-diaphorase activity. Our results suggest that this upper threshold of DT-diaphorase is approximately 300 nmol min^{-1} mg $protein^{-1}$. This is supported by the work of Beall et al (1995) that correlated the cytotoxicity of MMC with DT-diaphorase activity in different tumour cells. They found that MMC cytotoxicity increased with increasing enzyme activity, until the DT-diaphorase activity reached 300 nmol min^{-1} mg $protein^{-1}$ (Beall et al, 1995). Mikami et al (1996) also observed a saturation of MMC activation by DT-diaphorase in st-4 cells that were transfected with the NQO1 gene and expressed different levels of DT-diaphorase activity. Although this may limit the use of DT-diaphorase inducers to increase the antitumour activity of bioreductive agents in some situations, it should not significantly impair the use of this therapeutic approach in the clinic, since primary tumours generally have base levels of DT-diaphorase that are less than 100 nmol min^{-1} mg $protein^{-1}$ (Belinsky and Jaiswal, 1993; Ross et al, 1994; Smitskamp-Wilms et al, 1995).

The lack of enhancement in MMC antitumour activity in the AGS and SK-MEL-28 cells these two cell lines may also be explained by other differences in these cells, such as higher levels of detoxifying and DNA repair enzymes, which help to detoxify or

remove MMC from tumour cells and repair the damage it produces. Further studies may be needed to clearly interperet the experimental results.

Induction of DT-diaphorase by dietary and pharmaceutical compounds in T47D cells

Inducers of phase II detoxifying enzymes have been categorized into two classes: monofunctional inducers that only induce phase II detoxifying enzymes, and bifunctional inducers that induce both phase I and phase II enzymes. The monofunctional inducers have general properties that (1) they are electrophiles like quinones and Michael reaction acceptors; (2) they can react with sulfhydryl groups; (3) they can coordinately induce DT-diaphorase and other phase II enzymes like GST, UGT, and epoxide hydrolase (Talalay, 1989; Prestera et al, 1993; Spencer et al, 1991).

To identify non-toxic enzyme inducers that produce better induction of DT-diaphorase than D3T in human tumours, we investigated the ability of some dietary components and pharmaceuticals as nontoxic inducers to increase DT-diaphorase activity in human tumour cells. We used T47D, human breast cancer cells, for these studies because we showed previously that these cells were readily inducible by D3T. In addition, they have a relatively low base level of DT-diaphorase activity that is similar to that found in many primary tumours. Thus, these cells were studied to identify enzyme inducers that were better than D3T.

Fourteen compounds were examined for their ability to induce DT-diaphorase in T47D cells and eight of the fourteen compounds significantly increased DT-diaphorase activity in this cell line. The base level of DT-diaphorase was 27.8 ± 1.2 nmol min^{-1} mg protein $^{-1}$and the induced level of enzyme activities ranged from 40.8 ± 1.2 to 128.5 ± 5.6

nmol min^{-1} mg protein $^{-1}$. Three compounds, DMM, DMF and sulforaphane, proved to be better inducers of DT-diaphorase activity than D3T at non-toxic concentrations in T47D cells. DMM and DMF are metabolites of fumaric and maleic acid that are commonly found in foods. Sulforaphane is extracted from broccoli and has been extensively studied as a potent chemopreventive agent (Zhang et al, 1992). Administration of sulforaphane to Sprague-Dawley rats showed a dose-dependent reduction in the incidence of mammary tumour development (Zhang et al, 1994). In addition to its ability to induce phase II enzymes, sulforaphane has also been observed to have some antitumour effects (Zhang and Talalay, 1994). This antitumour property of sulforaphane may be beneficial when combined with bioreductive antitumour drugs since the additional anticancer activity may further enhance the already increased cytotoxicity of bioreductive agents resulting from the increase in DT-diaphorase activity.

• Propyl gallate (PG), which is an antioxidant used in food and is reported to have chemoprotective activity (Williams et al, 1986; De Long et al, 1985), also showed moderate inducing activity producing a 2.5-fold increase in DT-diaphorase activity. Manson et al (1997) also observed similar induction of DT-diaphorase activity by PG in rat liver in a study of the mechanism of action of dietary chemopreventive agents.

There has been great interest in non-steroidal anti-inflammatory drugs (NSAIDs) for their ability to prevent cancer formation. Ibuprofen and aspirin have shown to be promising cancer chemopreventive agents in phase I and II/III clinical trials for preventing colon and bladder cancers (Kelloff et al, 1997). It has been suggested that the chemopreventive effects of NSAIDs might be due to their ability to restore immune response by inhibiting cyclooxygenase activity. However, the exact mechanism has not

been clearly established. Thus, we tested aspirin and ibuprofen in this study to see if these agents might produce their chemopreventive effects by induction of phase II detoxifying enzymes. Aspirin produced a small but significant induction of DT-diaphorase, whereas ibuprofen did not have any effect on enzyme activity. This suggests that induction of DT-diaphorase may not be responsible for the chemopreventive property of NSAIDs. In addition, it appears unlikely that these drugs would be useful in our approach to enhancing the antitumour activity of bioreductive agents.

Vitamin supplements and other antioxidants, such as caffeic acid and ursolic acid, have also been studied for their ability to prevent cancer formation. Retinoic acid has undergone large-scale clinical trial for chemoprevention. In our study, 13-cis-retinoic acid produced moderate induction of DT-diaphorase in T47D cells. No induction of DT-diaphorase was seen with folic acid, ursolic acid, caffeic acid and genistein in these cells. Manson et al (1997) reported that caffeic acid could induce the total GST activity, but not DT-diaphorase activity. This suggests that caffeic acid achieves chemoprevention by inducing GST but not DT-diaphorase. Although soy bean has been reported to increase the levels of phase II enzymes, including DT-diaphorase in rats and has proven to be a chemopreventive agent (Appelt and Reicks, 1997). However, genistein, the primary component of soy, did not increase DT-diaphorase activity in T47D cells.

In summary, we have tested a series of potential inducers of DT-diaphorase in human breast tumour cells and identified that DMM, DMF, and sulforaphane are better inducers of DT-diaphorase than D3T.

Cytotoxicity of MMC in T47D cells in combination with different DT-diaphorase inducers

It is difficult to directly compare the correlation between DT-diaphorase activity and cytotoxicity of bioreductive agents obtained in different cell lines, since other factors such as drug uptake, and levels of other activating, detoxifying and repair enzymes, can influence the antitumour activity of bioreductive drugs. To investigate if the level of enhancement of MMC activity is dependent on the level of DT-diaphorase induction, we used three DT-diaphorase inducers that produced different enzyme levels in combination with MMC in one tumour cell line. Pretreatment of T47D cells with PG, D3T or DMM increased DT-diaphorase activity from 30.0 ± 0.8 to 81.1 ± 2.8, 100.0 ± 1.5 or 121.0 ± 3.9 nmol min^{-1} mg $protein^{-1}$ ($p < 0.001$), respectively. The observed enhancement of MMC antitumour activity in these cells was 1.8-, 2.3- and 3.0-fold for PG, D3T and DMM, respectively. These results demonstrated that the higher the induction of DT-diaphorase, the greater enhancement of MMC antitumour activity, provided that the level of DT-diaphorase does not exceed the upper threshold level. Doherty et al (1998) reported that combination of the bioreductive agent EO9 with the DT-diaphorase inducers, D3T or oltipraz, in HL-60 cells produced a 1.4- or 1.8-fold enhancement in EO9 activity with a 1.7- or 4.7- fold induction in DT-diaphorase activity, respectively. Furthermore, pretreatment of NCI-H661 cells with D3T, or an analogue of D3T, increased DT-diaphorase activity by 1.3- or 1.6- fold, respectively and significantly increased the cell kill observed with EO9 by 1.6- or 2.2-fold, respectively. Thus, enhancement of EO9 toxicity by DT-diaphorase inducers also paralleled the effect on induction of DT-diaphorase. These results suggest that it may be possible to achieve

greater enhancement of the antitumour activity of bioreductive agents in the clinic by using more potent inducers of DT-diaphorase.

Studies of effect of the combination strategy on other enzymes

Many enzymes may be involved in the bioreductive activation of MMC inside cells including NADPH:cytochrome P450 reductase, NADH:cytochrome b5 reductase and xanthine dehydrogenase. NADPH:cytochrome P450 reductase has been reported by many laboratories to be involved in the activation of MMC (Pan et al. 1984; Rockwell et al, 1996). It has been reported that decreased NADPH:cytochrome P450 reductase activity is coupled with a lower rate of MMC metabolism. Incorporation of NADPH:cytochrome P450 reductase into drug resistant Chinese hamster ovary cells significantly sensitized cells to MMC (Bligh et al, 1990). Similarly, Hoban et al (1990) reported MMC resistance in a Chinese hamster ovary cell line due to decreased NADPH:cytochrome P450 reductase. In our study, NADPH:cytochrome P450 reductase activity in T47D cells was not changed by pretreatment with DMM, suggesting that this enzyme is not involved in the enhancement of MMC activity by DT-diaphorase inducers. This is consistent with our previous finding that there was no change in NADPH:cytochrome P450 reductase activity in HL-60, human promyelocytic leukemia cells, after pretreatment with D3T (Doherty et al, 1998). We also observed no increase in the activity of NADH:cytochrome b5 reductase after treatment of T47D cells with DMM, suggesting that this enzyme is not involved in the increased MMC cytotoxicity observed with combination treatment with DT-diaphorase inducers. Doherty et al (1998) also reported no increase in NADH:cytochrome b5 reductase activity following treatment

with D3T. The levels of xanthine dehydrogenase were too low to be detected either before or after DMM treatment in T47D cells. Since relatively high levels of this enzyme are needed to produce significant effects on the bioactivation of MMC (Gustafson and Pritsos, 1992), xanthine dehydrogenase is likely not involved in the increased MMC activity in T47D cells treated with DMM. Thus, DT-diaphorase appears to be the only bioactivating enzyme for MMC to be affected by the combination strategy and is likely responsible for the enhancement of MMC activity observed.

The GST's are a family of phase II detoxifying enzymes. The GST Ya gene contains a similar ARE sequence to the NQO1 gene in the 5' flanking region and can be coordinately induced with DT-diaphorase in some tissues (Talalay, 1989; Talalay et al, 1995). It has been recognized for many years that these enzymes may protect cells from toxic chemicals, including electrophiles and reactive oxygen species (Prestera et al, 1993). In addition, these enzymes may play a role in resistance to antitumour agents, including MMC, by helping to remove drugs from cells (Xu et al, 1994). If DT-diaphorase inducers increased the levels of GST in tumour cells, the elevated level of GST might remove more activated MMC from the tumour cells, resulting in no net increase in MMC antitumour activity. Although, in previous studies, we did not observe induction of GST with D3T in T47D and HL-60 cell lines, in this study, we saw a small, but significant, increase in GST activity following treatment of T47D cells with DMM. However, this did not appear to interfere with our combination treatment strategy as we did see a large enhancement of MMC cytotoxic activity in these cells following treatment with DMM. The difference in the induction of GST by D3T and DMM may suggest different induction mechanisms by these two inducers. It has been shown that induction

of DT-diaphorase can be mediated by NF-κb sequences instead of the ARE region. This suggests that selective inducers of DT-diaphorase should be identified to minimize the potential negative effect of GST induction on the drug enhancement strategy.

Effect of the combination treatment strategy on myelotoxicity

The major toxicity observed with MMC is a delayed dose limiting bone marrow toxicity. Pretreatment of D3T may also induce DT-diaphorase activity in normal bone marrow cells and increase MMC toxicity to these normal tissues. Begleiter et al (1996) showed that combination treatment of MMC combined with D3T had no effect on normal DBA/2 mouse marrow cells. In our study, D3T produced a significant increase in the levels of DT-diaphorase activity in normal human marrow cells. Although the absolute increase in enzyme activity was small, there was a 1.5-fold increase in MMC toxicity to the human marrow cells. However, there was no obvious selectivity for specific marrow progenitors (CFU-G, CFU-M, CFU-GM, CFU-E or BFU-E). The increase in bone marrow toxicity was small compared with the enhancement of MMC antitumour activity in T47D, NCI-H661 and HCT116 tumour cells. Therefore, the net result of the combination treatment was an increase in the therapeutic index for MMC.

Significance of the combination treatment strategy

Combination treatment of DT-diaphorase inducers and bioreductive agents may be able to improve the therapeutic index of bioreductive drugs by selectively increasing the level of activating enzyme in tumour cells. It may be possible to achieve higher antitumour activity with this strategy by combining the normal dose of the bioreductive

agent with an enzyme inducer. Alternatively, it may be possible to decrease the unwanted side effects of the bioreductive agent by using a lower dose of bioreductive agent in combination with a DT-diaphorase inducer.

MMC is a relatively poor substrate for DT-diaphorase (Walton et al, 1992) and this enzyme is generally thought to account for less than 30% of the cellular activation of the agent. Thus, the increase in DT-diaphorase activity produced by enzyme inducers may have only a limited impact on increasing the antitumour activity of MMC. In this study we observed a 3-fold enhancement of MMC cytotoxicity in T47D human breast cancer cells when this agent was combined with DMM. While such an increase in antitumour activity would be highly significant in the clinic, it may be possible to achieve greater enhancement of antitumour activity using bioreductive agents that have a greater specificity for activation by DT-diaphorase. There has been considerable effect to identify possible substrates for DT-diaphorase that may have clinical value. EO9 is an indoloquinone analogue of MMC that has been shown to be selectively activated by DT-diaphorase (Walton et al, 1991; Robertson et al, 1994). Metabolism of a series of quinones by purified DT-diaphorase was tested by Beall et al (1994), and showed that MeDZQ, diaziquone and streptonigrin are all better substrates for this enzyme than MMC. Gibson et al (1992) tested the rate of reduction by DT-diaphorase and the cytotoxicity of a group of aziridinylbenzoquinones, and again found that MeDZQ is a better substrate for DT-diaphorase than MMC. Recently, RH1, an analogue of MeDZQ, has been reported to have higher affinity for DT-diaphorase than the parent compound (Winski et al, 1998). Thus, with the continue development of new bioreductive agents that are mainly activated by DT-diaphorase, a greater increase in therapeutic index would

be expected by combining these agents with DT-diaphorase inducers. Indeed, we saw a 7-fold enhancement of the antitumour activity of EO9, a more potent substrate for DT-diaphorase than MMC, compared to a 2-fold increase with MMC in mouse lymphoma cells

In summary, this study has demonstrated that inducers of DT-diaphorase can selectively increase the antitumour activity of MMC in a variety human tumour cells of different tumour types. Enzyme inducers that produced more induction of DT-diaphorase also produced a greater enhancement of MMC antitumour activity. Thus, it may be possible to use non-toxic inducers of DT-diaphorase to enhance the efficacy of bioreductive antitumour agents. Additional studies with other bioreductive agents and enzyme inducers are required to optimize this new treatment strategy.

Structure-activity studies with BM analogues

Cytotoxicity of BM analogues

A main problem in cancer chemotherapy is that chemotherapeutic drugs lack selectivity between normal tissues and tumour tissues. Recently, an "enzyme-directed" approach to targeting of tumours has been studied to increase the effectiveness of cancer chemotherapy. Bioreductive antitumour agents are particularly suited for this approach since all drugs in this class need to be activated by reductive enzymes. Thus, tumours with high levels of a particular activating enzyme could be treated with bioreductive agents that are specifically activated by this enzyme. The antitumour agent would be selectively activated in tumour cells compared with normal tissues that do not have elevated level of this enzyme. Alternatively, using a bioreductive agent that is

specifically inactivated by an enzyme that is abundant in normal tissues compared with tumour cells could decrease toxicity to these tissues.

The enzymology for the activation of bioreductive drugs is very complicated. The bioreductive activation process of these drugs can involve many enzymes, as in the case with MMC that can be activated by many different enzymes like NADPH:cytochrome P450 reductase, DT-diaphorase, NADH:cytochrome b5 reductase and xanthine dehydrogenase. In addition, one enzyme can be an activating enzyme under one condition but an inactivating enzyme under another condition. For example, MMC can be activated by DT-diaphorase under acidic condition while inactivated when pH is beyond 7.4 (Siegel et al, 1992). Tirapazamine, a new bioreductive agent, is activated by NADPH:cytochrome P450 reductase but inactivated by DT-diaphorase (Walton and Workman, 1990). New bioreductive agents like EO9 and RH1, have demonstrated greater selectivity for activation by DT-diaphorase (Walton et al, 1991; Winski et al, 1998) and there has been considerable interest in developing new bioreductive molecules and methods for modifying enzyme selectivity (Denny et al, 1996). However, none of the bioreductive agents developed to date are activated by a single reductive enzyme. Thus, their application in enzyme directed targeting is limited. One major difficulty in developing agents that are specifically activated by one enzyme is a lack of knowledge of structural factors that influence the selectivity for activation of bioreductive drugs.

We have studied the mechanism of activation and cytotoxicity of the model bioreductive agent, BM, in comparison with a series of BM analogues having different function groups. BM contains a quinone bioreductive element and a nitrogen mustard-

alkylating element. It produces DNA strand breaks and DNA crosslinks, but the crosslinks appears to be the major contributor to the cytotoxicity (Begleiter, 1986; Begleiter and Blair, 1984). BM showed higher cytotoxicity and cross-linking activity in L5178Y lymphoblasts than aniline mustard, a compound that has the same alkylating group but no quinone function. This suggested that the quinone group plays a role in modulating the activity of the alkylating element (Begleiter, 1983; Begleiter and Blair, 1984). BM activity was lower in L5178Y/HBM2 and L5178Y/HBM10 cell lines that have elevated levels of DT-diaphorase, catalase, superoxide dismutase and GST. The lower BM activity was linked to DT-diaphorase, which reduces the quinone in BM directly to a hydroquinone form. This suggested that the semiquinone form of BM may be the most important cytotoxic form of BM, that NADPH:cytochrome P450 reductase might be the most important enzyme for the bioreductive activation of BM and that DT-diaphorase may act as an inactivating enzyme for BM (Begleiter and Leith, 1990).

Analogues of BM were chosen to study the effect of various functional moieties on the reduction of the quinone group by DT-diaphorase and NADPH:cytochrome P450 reductase. Three analogues of BM, which have different functional groups adjacent to the quinone structure, were synthesized, including 5-methoxy-BM (MBM), 6-phenyl-BM (PBM), and 5-chloro-BM (CBM). Comparison of the activities of MBM and BM illustrates the effect of an electron donating, methoxy group, while comparison of CBM and BM, illustrates the effect of an electron withdrawing, chloro group, on enzyme specificity. Both groups will change the electron density around the quinone group and may alter the ability of bioreductive enzymes to reduce these compounds. PBM was used to study the effect of the sterically bulky phenyl group on the reduction of the

quinone group. This bulky group might interfere with the binding of PBM to DT-diaphorase or NADPH:cytochrome P450 reductase.

The cytotoxic activities of BM analogues were studied in two human tumour cells, NCI-H661, human non-small-cell lung cancer and SK-MEL-28, human melanoma, with, or without the DT-diaphorase inhibitor, dicoumarol. MBM showed significantly higher tumour kill than BM in both cell lines, suggesting that the methoxy group affected the cytotoxicity of BM. PBM and CBM showed similar cytotoxic activity compared with BM, suggesting that the phenyl and chloro groups had little effect on the cytotoxicity of the parent drug. The DT-diaphorase inhibitor, dicoumarol, increased the cytotoxicity of BM but decreased the activity of MBM in both cell lines. These studies suggested that DT-diaphorase acts as a detoxifying enzyme for BM, and this is similar to our previous findings in mouse lymphoma cells (Begleiter and Leith, 1990). In contrast these results suggest that DT-diaphorase may serve as an activating enzyme for MBM. Dicoumarol had no effect on the cytotoxicity of PBM and CBM in these cell lines, suggesting that PBM and CBM are not dependent on activation by DT-diaphorase. This suggested that the methoxy, phenyl and chloro groups might have changed the role of DT-diaphorase in the activation of the BM analogues.

Reduction of BM analogues by purified DT-diaphorase

In order to directly examine the effect of the different functional groups on the ability of DT-diaphorase to reduce the quinone moiety, the reduction of the BM analogues by purified DT-diaphorase was studied in a cell free system by measuring the rate of consumption of NADH, the cofactor for DT-diaphorase. Reductions were carried

out under both hypoxic and aerobic conditions to provide information about the rate of reduction and redox cycling. Reduction of the BM analogues by DT-diaphorase *in vitro* under hypoxic conditions gives a true measure of the rate of reduction of the analogues since lack of oxygen prevents redox cycling. The whole reduction process of BM by purified DT-diaphorase takes 5 min to complete with a $t_{1/2}$ of 3 min under hypoxic conditions. The rate of reduction for MBM, PBM and CBM is slower than the parent compound with $t_{1/2}$ of 10, 31 and > 60 minutes, respectively. Thus, the methoxy, chloro and phenol groups appear to decrease the rate of reduction of the quinone by DT-diaphorase. We have shown previously that DT-diaphorase acts as a detoxifying enzyme for BM. The rapid reduction of BM by DT-diaphorase may in part explain the low cytotoxicity of BM in both NCI-H661 and SK-MEL-28 cells, which have relatively high levels of DT-diaphorase. Reduction of BM by DT-diaphorase to the hydroquinone form may produce an intermediate with low toxicity to cells. The observed slower reduction of MBM, PBM and CBM suggests that the methoxy, phenyl and chloro functional groups may interfere with the interaction of DT-diaphorase and the quinone group by altering the binding of the analogue with the active site of the enzyme, or by interfering with the electron transfer process. The slower reduction rate of MBM by DT-diaphorase is not consistent with the higher cytotoxicity of this agent observed in the tumour cells and with the effect of dicoumarol, which suggests that DT-diaphorase is an activating enzyme for this agent. This may suggest that other enzymes, such as NADPH:cytochrome P450 reductase or NADH:cytochrome b5 reductase may also activate MBM and play a role in determining its overall toxicity of MBM. For PBM and CBM, the slower reduction by

DT-diaphorase may explain why this enzyme appeared to have little effect on the cytotoxicity of these agents.

If the analogues have the ability to undergo redox cycling, under oxygenated conditions, the reduced form of the analogues may be converted back to the original drugs. For BM, reduction under aerobic conditions was similar to that under hypoxic condition with one equivalent of NADH being consumed under both conditions. More NADH was consumed under aerobic conditions compared with that under hypoxic condition for MBM, PBM, and CBM. These results suggest that, in the presence of oxygen, MBM, PBM, and CBM, once reduced, can undergo redox cycling back to the original compounds. In contrast, BM appears to be unable to re-oxidize back to its original form and stays in a reduced form. One possible reason is that, once BM is fully reduced to the hydroquinone form by DT-diaphorase, one of the two arms of the mustard group may react with one of the hydroxyl groups on the quinone ring to form a cyclized product. This product would not be able to undergo redox cycling, and, in addition, may no longer have the ability to cross-link DNA, leading to reduced cytotoxicity, and explaining why DT-diaphorase is an inactivating enzyme for BM.

In summary, the cytotoxicity studies of BM analogues in human tumour cell lines illustrated that the methoxy, chloro and phenyl groups changed the role of DT-diaphorase in the bioreductive activation of these analogues. DT-diaphorase appears to be an activating enzyme for MBM, an inactivating enzyme for BM, and has little effect on the activation of PBM and CBM. The reduction studies with these analogues in a cell free system showed that the functional groups decreased the rate of reduction of MBM, PBM and CBM by DT-diaphorase. These findings demonstrated that modifying the structure

of the quinone ring with functional groups could significantly effect the reduction and activation of bioreductive agents by DT-diaphorase. Further studies are required to identify other mechanisms through which these functional groups affect the activity of the quinone containing bioreductive agents.

FUTURE STUDIES

Combination treatment strategy with bioreductive agents and DT-diaphorase inducers

Combination therapy with DT-diaphorase inducers and MMC suggests a possible way to increase the therapeutic index of bioreductive antitumour drugs. We showed that pretreatment of tumour cells with DT-diaphorase inducers increased the cytotoxicity of MMC by up to 3-fold. We have tested a series of potential inducers of DT-diaphorase and identified several inducers that could dramatically increase the enzyme activity in tumour cells. Many dietary inducers of DT-diaphorase have been extensively studied for their ability to reduce the risk of cancer. These non-toxic dietary compounds should be examined for their potency of induction of DT-diaphorase and potential value in combination therapy. Bioreductive agents, like MeDZQ and EO9, have shown selectivity toward tumour cells that have high DT-diaphorase activity, compared to the normal tissues. RH1, a water-soluble analogue of MeDZQ, has been synthesized and also appears to be an excellent substrate for DT-diaphorase. These bioreductive models should be tested in the combination therapy strategy to evaluate the true potential of DT-diaphorase induction as an approach to enhancing the effectiveness of bioreductive drugs.

Finally, *in vivo* studies of the combination treatment of bioreductive agents and DT-diaphorase inducers should be carried out in a nude mice model implanted with a variety of human tumour types. *In vivo* studies should examine this combination strategy in a physiological environment to test the influence of a number of factors which may affect the DT-diaphorase inducers and the bioreductive agents, such as the absorption and

elimination of the inducers and bioreductive agents, influx and efflux of the inducers and bioreductive agents, microenvironment of tumours, repair of damaged DNA, and the levels of other activating enzymes. The results in the animal model may determine the clinical applicability of this combination therapy in the treatment of cancer.

Structure-activity studies of BM analogues

Cytotoxicity of BM analogues in enzyme transfected cell lines

Cytotoxicity of BM analogues should be studied in a series of tumour cells that have been transfected with NADPH:cytochrome P450 reductase or DT-diaphorase and express varying levels of enzyme activity, to demonstrate the function of these enzymes for the activation of these quinone containing agents. Patterson et al (1997) have transfected MDA-MB-231 cells with human NADPH:cytochrome P450 reductase to produce a cell line that had a 53-fold increase reductase activity. Mikami et al (1997) have also transfected the NQO1 gene into sk-4 cells to express a wide range of DT-diaphorase activity at a wide range. These transfected cell lines, different only in the levels of enzymes, should be used to test the cytotoxicity of the BM analogues. Comparison of the proportional cytotoxicity of BM analogues in MDA-MB-231 cells or sk-4 cells will identify the contribution of NADPH:cytochrome P450 reductase and DT-diaphorase in the activation of the BM analogues.

Kinetics of BM analogues reduced by purified DT-diaphorase and NADPH:cytochrome P450 reductase

A study of the kinetics of the reduction of the BM analogues by purified DT-

diaphorase should be investigated to reveal the precise mechanism underlying the differences in the variable rate of reduction among the analogues. A change of Vmax (the maximum velocity of the reduction) will suggest the differences are due to a change in the electron transfer rate from DT-diaphorase, while a change of Km (the affinity constant) will suggest that the differences are caused by altered binding of the BM analogues to the active site on the enzyme.

Kinetics of reduction of BM analogues by purified NADPH:cytochrome P450 reductase should also be studied to determine if the functional groups alter reduction of the quinone group by this enzyme and the mechanism(s) of these effects.

Crosslinking activity and DNA strand break activity

The cytotoxicity of the BM analogues is likely due to DNA crosslinking and DNA strand break activity. The measurement of DNA strand breaks and DNA crosslinks produced by the intermediates formed by reduction of the BM analogues by purified DT-diaphorase and NADPH:cytochrome P450 reductase in a cell free system will give a complete overview of the cytotoxic mechanism of the BM analogues, and the effect of the functional groups on each of these mechanisms.

The crosslinking activity of the BM analogues reduced by DT-diaphorase or NADPH:cytochrome P450 reductase could be measured by an agarose gel method developed by Hartley et al (1991). We have suggested that DT-diaphorase is an inactivating enzyme for BM, and an activating enzyme for MBM. We also suggested that the DT-diaphorase reduction product of BM could cyclize and be unable to crosslink DNA. Thus, we would expect that MBM, if reduced by DT-diaphorase, should produce

more crosslinks than BM reduced by DT-diaphorase. These studies should also clarify whether the phenyl and chloro groups altered the DNA crosslinking activity of the DT-diaphorase reduction products, or if any of the functional groups altered the DNA crosslinking activity of the NADPH:cytochrome P450 reductase reduction products.

The DNA strand break activity of the BM analogues, reduced by DT-diaphorase and NADPH:cytochrome P450 reductase, could be measured by the method developed by Chen et al (1995). The metabolites of BM analogues reduced by these enzymes can undergo redox cycling under oxygenated condition to generate reactive free radicals that can produce DNA strand breaks. DT-diaphorase should be tested for its ability to generate intermediates that can cause DNA strand breaks when reducing BM analogues. Since it is hypothesized that BM is unable undergo redox cycling once reduced by DT-diaphorase, we would expect MBM to produce more DNA strand breaks than BM following reduction by DT-diaphorase. These studies should also demonstrate whether the functional groups altered the DNA strand break activity of the NADPH:cytochrome P450 reductase products.

The kinetic, DNA damage and cytotoxic studies of BM analogues should give us a better understanding of the roles of electron donating, electron withdrawing, and bulky groups on the specificity of reduction by DT-diaphorase and NADPH:cytochrome P450 reductase. These structure-activity studies may make it possible to design new bioreductive agents that are more selectively activated by DT-diaphorase or NADPH:cytochrome P450 reductase. These new agents would be useful in enzyme directed targeting strategies to improve the effectiveness of bioreductive antitumour agents.

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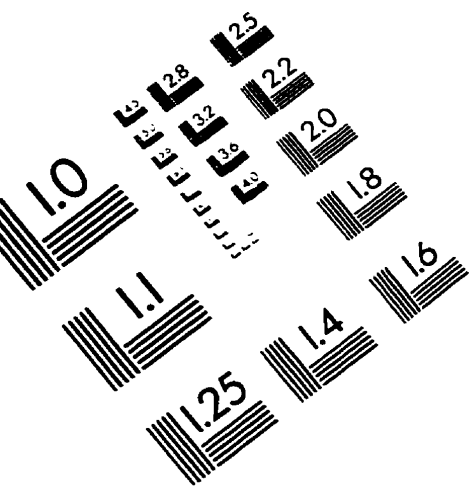
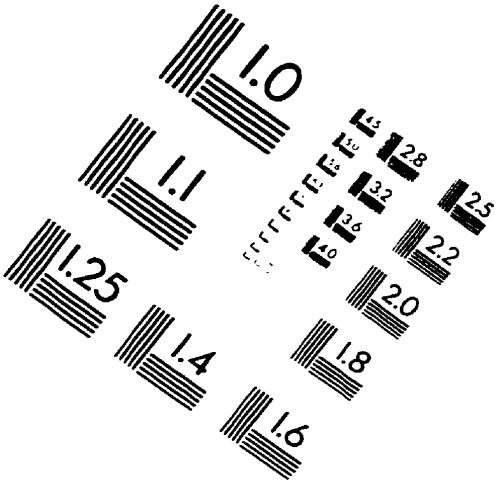
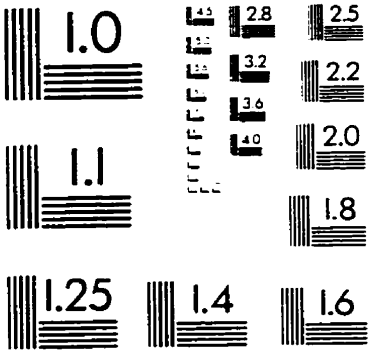
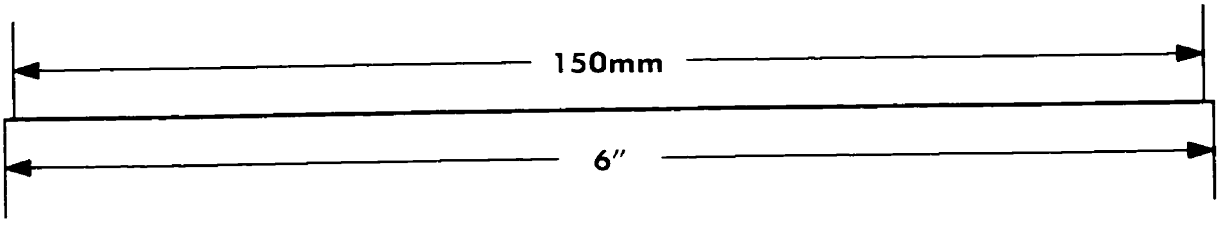
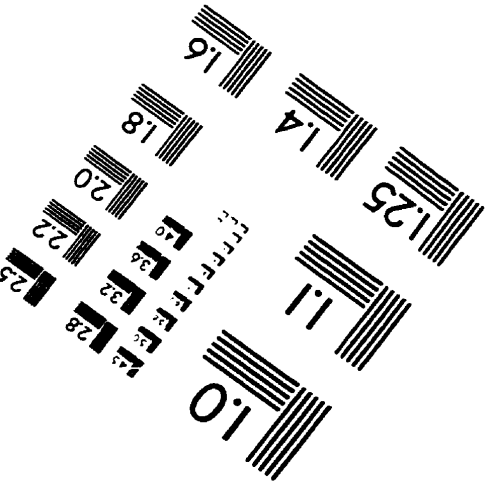
IMAGE EVALUATION
TEST TARGET (QA-3)

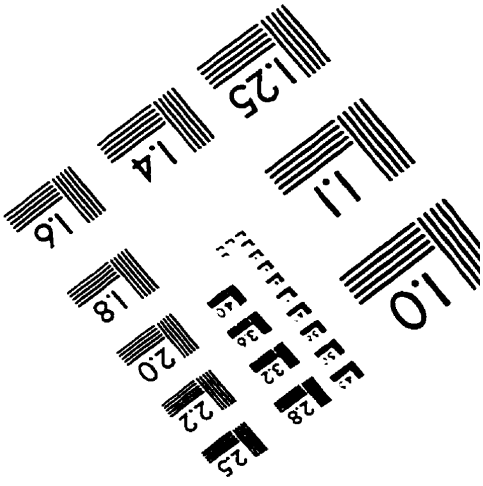












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