INDUCTION OF DT-DIAPHORASE BY 1,2-DITHIOLE-3-THIONES AND THE ENHANCEMENT OF ANTITUMOUR ACTIVITY OF BIOREDUCTIVE AGENTS

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
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MASTER OF SCIENCE

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University of Manitoba
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Abstract

DT-diaphorase is a two-electron reducing enzyme that is an important activator of bioreductive antitumour agents, such as mitomycin C and EO9. DT-diaphorase is highly inducible by a wide variety of compounds, including 1,2-dithiole-3-thiones (D3T's), and these compounds could be used to enhance the antitumour activity of bioreductive agents. We investigated the ability of D3T to increase the level of DT-diaphorase activity in human tumour cells lines and normal human cells. D3T significantly increased DT-diaphorase activity in 11 of 18 human tumour cell lines representing 6 tissue types. There were no obvious relationships between the tumour type, or the basal level of DT-diaphorase activity in the cells, and the ability of D3T to increase the enzyme activity. Tumour cells of all tissue types were induced except head and neck tumour cells and hepatoma cells. DT-diaphorase was induced by D3T in normal human bone marrow, kidney, and lung cells, but the increases were small in the marrow and kidney cells. We examined the ability of 13 D3T analogs to induce DT-diaphorase activity in 8 human tumour cell lines from 5 different tissues. The parent D3T appeared to be the best overall inducer of DT-diaphorase in the cell lines studied, but induction of enzyme activity varied markedly with the D3T structure. D3T increased tumour cell kill by EO9 in HL-60 human leukemia cells and this was inhibited by an inhibitor of DT-diaphorase, dicoumarol. In contrast, D3T had no effect on EO9 cell kill in normal human kidney cells, the site of dose-limiting toxicity for EO9 therapy. Pretreatment of HL-60 cells and H661 cells with D3T analogs also enhanced the antitumour efficacy of EO9 with the increases in cytotoxicity parallelling the increases in DT-diaphorase activity. The combination of MMC and D3T increased MMC cytotoxicity in HCT116 human colon carcinoma cells. These studies demonstrate that D3T analogs can increase DT-diaphorase activity in human tumour cells and that this can enhance the antitumour activity of the bioreductive agents, MMC and EO9.

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Abbreviations

αMEM	Alpha minimum essential medium
ADT	Anethole 1,2-dithiole-3-thione
AFB_1	Aflatoxin-B_1
ARE	Antioxidant response element
AZQ	2,5-Diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone
BHA	2(3)-*tert*-Butyl-4-hydroxyanisole
BSA	Bovine serum albumin
CB 1954	5-Aziridin-1-yl-2,4-dinitrobenzamide
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
EORTC	European organization for research and treatment of cancer
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
GST	Glutathione *S*-transferase
IMDM	Iscove's modified Dulbecco's medium

ITS	Insulin transferrin selenium
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)
nmol MTT min^{-1} mg $protein^{-1}$	Nanomole of MTT reduced per minute per milligram protein
NSCLC	Non small-cell lung cancer
Oltipraz	5-(2-Pyrazinyl)-4-methyl-1,2-dithiole-3-thione
PBS	Phosphate buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TPA	12-*O*-Tetradecanoyl-phorbol-13-acetate
XRE	Xenobiotic response element

BACKGROUND AND REVIEW OF THE LITERATURE

CANCER AND CANCER CHEMOTHERAPY

Cancer can be defined as a disease characterized by a shift in the control mechanisms that govern cell proliferation and differentiation (Katzung, 1995; Weinberg, 1996) that results in the eventual inhibition of various organ systems and death (Hardman and Limbird, 1996). Cancer is the second leading cause of deaths in the United States and Canada after heart disease (Katzung, 1995). It causes approximately 560,000 deaths per year in the United States (Katzung, 1995; Hardman and Limbird, 1996; Rennie and Rusting, 1996), 60,000 deaths per year in Canada (Parker et al, 1997), and 2,700 deaths per year in Manitoba (Manitoba Cancer Treatment and Research Foundation, 1995). During the period from 1973-1992, overall cancer mortality in North America increased by 6.3% (Rennie and Rusting, 1996). Mortality has decreased during this period in such cancers as leukemia, Hodgkin's disease, and testicular, cervical, stomach and colon carcinomas; however, the incidence of mortality from lung and prostate cancer, melanomas, and multiple myeloma, for example, have increased (Rennie and Rusting, 1996).

Cancer can be currently treated with a combination of three modalities: surgery, radiation and chemotherapy. Local control measures, surgery and radiation, used alone or, more often, in combination can cure 33% of all cancer cases (Katzung, 1995; Hardman and Limbird, 1996). Dissemination of the tumour at diagnosis greatly diminishes the chances of curing the patient by any of the treatment modalities. Advances in diagnosis have allowed much earlier detection and, as a result, some patients have benefited from better control of the tumour. Chemotherapy contributes to the cure of 17% of cancer in patients (Katzung,

1995).

Cancer chemotherapeutic agents attempt to cause cellular differentiation, cell cycle arrest, or cell death by necrosis or programmed cell death (Katzung, 1995). Cancer chemotherapeutic agents are numerous and constitute 5 different classes (Katzung, 1995; Hardman and Limbird, 1996). The first class is alkylating agents and includes cyclophosphamide, chlorambucil, melphalan, and cisplatin. The second class is antitumour antibiotics, such as the antracyclines and bioreductive agents. The third class is antimetabolites which are represented by methotrexate, fluorouracil, fludarabine, and hydroxyurea. Vincristine, etoposide, and taxol are natural products that constitute the fourth class of chemotherapeutic agents. Finally, hormones and other biological agents, such as antisteroids, interferons, interleukins, and growth factors, are also used in the treatment of cancer. Further development is underway in the field of anticancer drugs in the attempt to aid in the therapy of the other 50% of cancer cases that result in patient mortality.

BIOREDUCTIVE ANTITUMOUR AGENTS

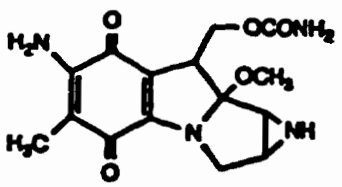
Background

Bioreductive agents are a relatively new class of anticancer drugs that have varied chemical structures but are characterized by a common requirement for intracellular reductive activation (Workman and Stratford, 1993; Adams and Stratford, 1994). The class of bioreductive agents includes mitomycin C (MMC), porfiromycin, 3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol (EO9), tirapazamine, streptonigrin, 5-aziridin-1-yl-2,4-dinitrobenzamide (CB 1954), 2,5-dimethyl-3,6-diaziridinyl-

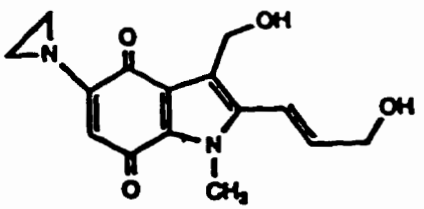
1,4-benzoquinone (MeDZQ), and diaziridinylbenzoquinone (AZQ) (Sunters et al, 1991; Hendriks et al, 1993; Siegel et al, 1993; Beall et al, 1996). The chemical structures of a number of bioreductive agents are depicted in Figure 1. Bioreductive antitumour agents are an important class of drugs because hypoxic conditions may enhance their cytotoxicity (Workman and Stratford, 1993) and such regions of tumours are inherently resistant to radiation and typically more resistant to chemotherapy. These drugs are effective in treating solid tumours because these tumours contain regions of hypoxic cells and the agents may be able to diffuse to cells in the interior of the tumour without being inactivated. There has been major interest in using bioreductive agents in combination with radiation and drugs that kill oxygenated cells in order to enhance solid tumour treatment.

Bioreductive antitumour agents can be activated by a number of intracellular enzymes and the mechanisms involved have been extensively studied (Rockwell et al, 1993; Ross et al, 1993). 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), xanthine oxidase (EC 1.1.3.22) (Pan et al, 1994) and NADH:cytochrome b_5 reductase (EC 1.6.2.2) (Hodnick and Sartorelli, 1993), and by the two-electron reducing enzymes, NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase) (EC 1.6.99.2) (Ross et al, 1993; Plumb et al, 1994) and xanthine dehydrogenase (EC 1.1.1.204) (Gustafson and Pritsos, 1992).

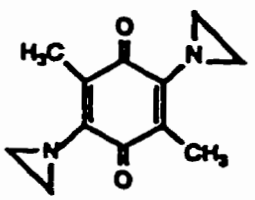
The three principal types of bioreductive agents, the mitomycins, the nitroimidazoles, and the benzotriazine di-N-oxides (Rauth et al, 1993; Workman and Stratford, 1993) undergo metabolic reduction to generate cytotoxic species. The metabolic reduction by one- or two-



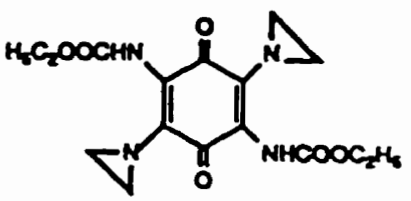
Mitomycin C



EO9



MeDZQ



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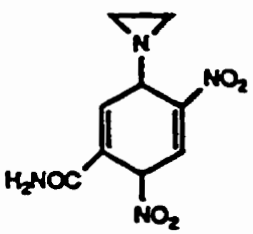
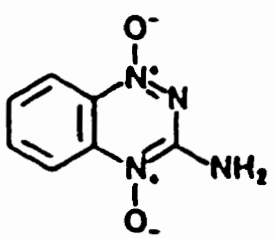
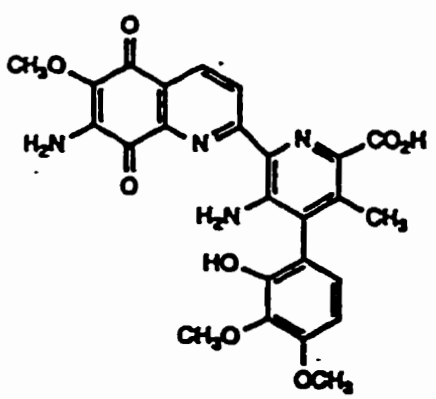


CB 1954



Tirapazamine



Streptonigrin

Figure 1: Chemical Structures of Bioreductive Antitumour Agents

electron reducing enzymes of the agent's bioreductive element, such as a quinone, a nitro, or an N-oxide group (Rauth et al, 1993), is dependent on a number of factors. The structure and the redox potential of the bioreductive element affects the compound's interaction with and reduction by the reducing enzymes (Workman and Stratford, 1993). For all three types of compounds, pH and tumour oxygenation has the ability to modulate the concentration of reduced toxic species occurring primarily at the one-electron reduction step (Rockwell et al, 1993; Smitskamp-Wilms et al, 1996). Following one-electron reduction under oxygenated conditions, the reactive radical intermediate can transfer an electron to molecular oxygen resulting in the formation of a superoxide anion radical (Kappus, 1986; Butler et al, 1987; Ross et al, 1994) and regeneration of the oxidized form of the agent. The oxidized bioreductive agent can again undergo one-electron reduction and this cycle is referred to as "redox cycling" (Ross et al, 1994). The redox cycling may contribute to the cytotoxicity of the bioreductive agent by generating superoxide radical and other reactive oxygen species which can produce DNA strand breaks and lipid peroxidation (Ross et al, 1994). One- and two-electron reduction of bioreductive agents may also result in further reactions or molecular rearrangement to activate a cytotoxic element, often an alkylating group, which may then covalently bind proteins and/or DNA or may intercalate or cross-link DNA.

Other bioreductive agents such as porfiromycin (Rockwell et al, 1988) and diaziquone (Chamberlain et al, 1988) have been used in the clinic, and interest has been raised with new agents in clinical trials, such as EO9 (McLeod et al, 1996; Smitskamp-Wilms et al, 1996) and tirapazamine (Senan et al, 1997), and with combination therapy of MMC and radiation (Fischer, 1994).

Mitomycin C

Chemistry

MMC is an antitumour antibiotic isolated from *Strep. caespitosus* in 1958 by Wakaki and associates (Cummings et al, 1995; Hardman and Limbird, 1996). MMC is the prototype bioreductive antitumour drug (Rockwell et al, 1993) and has been used clinically for over 20 years in treatment of various solid tumours (Gillis, 1996). MMC contains an aziridine group and a quinone group in its structure, as well as a mitosane ring, and each of these participates in the alkylation reactions with DNA upon reduction by intracellular enzymes (Gillis, 1996).

Antitumour Activity

The principal intracellular reductive enzymes involved in the activation of MMC are NADPH:cytochrome P450 reductase and DT-diaphorase, but the contribution of each enzyme to MMC activation varies with cell type, oxygen level, and pH (Keyes et al, 1984; Riley and Workman, 1992; Cummings et al, 1995). It has been reported that NADH:cytochrome b_5 reductase (Hodnick and Sartorelli, 1993), xanthine oxidase (Shao et al, 1995), and xanthine dehydrogenase (Gustafson and Pritsos, 1992; Pan et al, 1994) may also be involved with the reduction and activation of MMC. Reduction occurs preferentially in hypoxic cells (Keyes et al, 1985; Begleiter et al, 1992; Walton et al, 1992b; Nishiyama et al, 1993) and under acidic conditions (Siegel et al, 1990; Begleiter and Leith, 1993) in some experimental systems.

The one-electron reduction of MMC results in the formation of a semiquinone. Under oxygenated conditions, redox cycling can occur as the semiquinone group is oxidized

by molecular oxygen to the quinone resulting in the formation of superoxide radicals (Kappus, 1986; Butler et al, 1987; Ross et al, 1994). It has not been clarified if redox cycling is a component of the antitumour activity of MMC (Butler et al, 1987) or may principally explain some of the undesired toxicity (Cummings et al, 1995). Briggs *et al* (1997) have proposed that MMC can induce mitochondrial DNA damage by free radical generation. The MMC semiquinones can be further reduced by one-electron reduction to the hydroquinone or undergo chemical modification to activate two alkylating sites that can bind proteins or form monoadducts with DNA (Butler et al, 1987; Workman and Stratford, 1993,Rockwell et al, 1993). The two-electron reduction of MMC results in the formation of a hydroquinone which reacts with molecular oxygen at a much slower rate than the semiquinone radical (Workman and Stratford, 1993). This process bypasses the redox cycling of the bioreductive agent that occurs under aerobic conditions and allows more direct DNA damage by covalent reaction of bioreductive metabolites with DNA (Workman and Stratford, 1993). After hydroquinone formation, the mitosane nucleus demethylates and a double bond is created producing a mitosene nucleus. This is followed by proton-assisted aziridine ring opening to generate an electrophilic carbon center which can alkylate DNA. This alkylation promotes the carbamate group to leave creating another reactive carbon center which acts as the second point of attachment to produce DNA cross-links. The drug inhibits DNA synthesis and cross-links DNA at the N^6 position of adenine and at the O^6 and N^7 positions of guanine (Cummings et al, 1995; Hardman and Limbird, 1996). Sun and Ross (1996) have suggested that DT-diaphorase activation of antitumour quinones induces apoptosis via a p53-independent pathway.

Pharmacokinetics

The usual dose of MMC (6-20 mg/m^2) may be administered I.V. as a single bolus infusion every 6 weeks (Gillis, 1996; Hardman and Limbird, 1996) or as 2 mg/m^2/day I.V. for 5 days followed by 2 drug-free days then 2 mg/m^2/day for an additional 5 days (Gillis, 1996). Dosage is modified based on hematologic recovery. Peak concentrations of 0.5-1.7 μg/ml after doses of 20 mg/m^2 are observed within minutes (Gillis, 1996; Hardman and Limbird, 1996). Plasma half-life is 25-90 minutes. The drug is widely distributed throughout the body but is not detected in the brain (Hardman and Limbird, 1996). Inactivation occurs by metabolism or chemical conjugation. Clearance is primarily effected by the liver but other tissues can be involved (Gillis, 1996). Less than 10% is excreted in the urine or bile in its active form (Gillis, 1996; Hardman and Limbird, 1996). Since some metabolic pathways are saturated at relatively low doses, the percent of excretion in urine increases with increasing dose.

Clinical Uses

MMC is used as a single agent solely in the treatment of superficial bladder carcinomas (Gillis, 1996; Hardman and Limbird, 1996). MMC is more commonly used in a combination protocol with 5-fluorouracil, cisplatin, doxorubicin, or radiation in the treatment of carcinomas of the cervix (Hardman and Limbird, 1996), gastrointestinal system (Schnall and Macdonald, 1993; Gillis, 1996; Hardman and Limbird, 1996), breast (Hortobagyi, 1993; Hardman and Limbird, 1996), bladder (Hardman and Limbird, 1996), head and neck (Coia, 1993; Hardman and Limbird, 1996), and lung (Folman, 1993; Hardman

and Limbird, 1996).

MMC use is contraindicated in patients with a known hypersensitivity to mitomycin and in patients with thrombocytopenia, leukopenia, coagulation disorder, or an increased bleeding tendency due to other causes (Gillis, 1996). Mitomycin should not be administered to a patient with a white blood cell count below 4,000 mm^3 and a platelet count below 150,000 mm^3, or those with potentially serious infections (Gillis, 1996).

Adverse Reactions

The major dose-limiting toxicity observed with MMC treatment is delayed bone marrow suppression (Walters et al, 1992; Gillis, 1996; Hardman and Limbird, 1996). This myelosuppression is characterized by marked leukopenia and thrombocytopenia (Hardman and Limbird, 1996). Nausea, vomiting, diarrhea, stomatitis, alopecia, dermatitis, anorexia, hypoglycemia, fever, and malaise may also occur (Gillis, 1996; Hardman and Limbird, 1996). A hemolytic-uremic syndrome is the most dangerous adverse effect and is believed to result from drug-induced endothelial damage (Hardman and Limbird, 1996). In approximately 5% of patients (Klein and Wilds, 1983), MMC can also cause potentially fatal interstitial pulmonary fibrosis characterized initially by dyspnea and a non-productive cough (Gillis, 1996; Hardman and Limbird, 1996). MMC may also potentiate the cardiotoxicity observed with doxorubicin when used in conjunction with this drug (Hardman and Limbird, 1996).

EO9

Chemistry

Oostveen and Speckamp (1987) synthesized a number of MMC analogues in 1987. EO9, an indoloquinone analog of MMC with an aziridine ring substitution, was selected for clinical evaluation as the leading compound of a series of bioreductive agents (Workman et al, 1992; Hendriks et al, 1993).

Antitumour Activity

The indoloquinone analogues were designed to undergo redox cycling and formation of alkylating intermediates under bioreductive conditions (Hendriks et al, 1993). Bioreductive activation of EO9 induces the release of two hydroxyl groups and the opening of the aziridine group which can generate three possible reactive carbon centers (Smitskamp-Wilms et al, 1996). EO9 activity appears to be particularly sensitive to activation by DT-diaphorase under aerobic conditions (Workman et al, 1992; Plumb et al, 1994). EO9 exhibits superior hypoxic cell cytotoxicity ratio and activation by DT-diaphorase at physiological pH when compared to MMC (Workman et al, 1992). EO9 activation in the absence of DT-diaphorase only generated monofunctional DNA adducts (Bailey et al, 1994). Its intracellular reduction by DT-diaphorase has been shown to generate DNA damaging species *in vitro*, with the development of DNA single strand breaks and cross-links (Maliepaard et al, 1995; Smitskamp-Wilms et al, 1996). EO9 was shown to be more efficient at DNA cross-linking than MMC under identical conditions (Maliepaard et al, 1995).

Animal Studies

Preliminary *in vitro* screens of murine and human tumour cell lines (Phillips et al, 1992) and the NCI tumour cell line panel (Hendriks et al, 1993) revealed preferential activity against a broad spectrum of solid tumour cell lines and a more potent antiproliferative effect than MMC. Hendriks *et al* (1993) used murine tumours and human tumour xenografts to study the sensitivity to EO9 *in vivo*. Human gastric, ovarian and breast tumour xenografts were sensitive to EO9 and this showed the broad spectrum of activity of this new antitumour agent. The preliminary screen for EO9 cytotoxicity in mice showed no bone marrow depression in the mice but did find dose-limiting gastrointestinal tract toxicity (Hendriks et al, 1993). EO9 was very quickly metabolized in mice and rats with extensive biotransformation of EO9 including the aziridine ring-opened hydrolysis product and no parent drug was detectable in the urine (Workman et al, 1992).

Clinical Phase I and II Studies

The increased efficacy under hypoxic conditions and the lack of haematological toxicity in animals were reasons to select this agent for phase I studies within the framework of the Early Clinical Studies Group of the European Organization for Research and Treatment of Cancer (EORTC). Schellens *et al* (1995) reported the results of the phase I trial. The peak plasma concentration was 1-2 μg/ml after 22-27 mg/m^2 and the clearance was 8.9 ± 7.7 l/min. The dose-limiting toxicity is reversible renal toxicity, presented by proteinuria, which coincided with renal sodium and water retention, and development of edema (Schellens et al, 1995; Smitskamp-Wilms et al, 1996). Renal toxicity did not appear

in the animal preclinical toxicity studies (Schellens et al, 1995). Maximum tolerated dose was 27 mg/m^2. These pharmacokinetic parameters for EO9 were confirmed by a second group (McLeod et al, 1996). Its distinct antitumour profile and lack of myelosuppression indicated further support of a phase II trial by the EORTC (Riley and Workman, 1992; Smitskamp-Wilms et al, 1996).

The phase II trial was carried out for the EORTC by Dirix *et al* (1996). Ninety human patients with breast, pancreatic, gastric and colon cancer were treated with 12 mg/m^2 EO9 as a weekly bolus injection. Main toxicities were nausea and vomiting which were controlled pharmacologically. Moderate rises in creatine levels were observed, as a sign of some renal toxicity, but this was always reversible and mild urinary protein loss, proteinuria, was noticed in 45% of the drug courses. Despite reports of partial remissions in 2 patients with adenocarcinomas of unknown origin and in one patient with bile duct cancer (Schellens et al, 1995), no antitumour activity was found (Wanders et al, 1995; Dirix et al, 1996). The lack of clinical effectiveness of EO9 may be related to poor delivery to tumours due to a very short plasma half-life in patients (Workman et al, 1992; Schellens et al, 1995; McLeod et al, 1996) and insufficient drug penetration of multicell layers (Phillips et al, 1997). Although the reasons for the lack of activity of EO9 in the human clinical trials are not known, there are some concerns about the design of the clinical trials. In particular, DT-diaphorase activity in patients' tumours was not measured routinely and many of these patients may have had tumours with inherently low DT-diaphorase activity or with NQO_1 polymorphisms. In addition, the scheduling of EO9 for treatment as a single agent may not have allowed for it to be properly evaluated. There is the belief that bioreductive drugs should be administered

as part of a combination therapy regimen with either radiation or chemotherapy in order to observe a significant clinical response (Workman and Stratford, 1993).

Other Indoloquinone Analogs

Phillips (1996) used comparative chemosensitivity assays to demonstrate that the indoloquinone analogs, EO4, EO9, and EO68, are preferentially toxic to H460 human non-small cell lung carcinoma (NSCLC) cells (a cell line expressing high levels of DT-diaphorase activity) compared to H596 human NSCLC cells (a cell line expressing non-detectable levels of DT-diaphorase activity) with the ratio of IC_{50} values for H596 to H460 being 113.8, 92.2, and 103.9, respectively. The IC_{50} values for EO4, EO9, and EO68 in the H460 cells were 23.9, 34.5, and 37.8 nM, respectively. The potency of such compounds indicates the potential for further indoloquinone drug development. Although EO9 may not be used clinically in the future, EO9 was used in this study as a model for a bioreductive agent that is activated preferentially by DT-diaphorase.

Activation of MMC and EO9 by DT-diaphorase

Various intracellular reductive enzymes can activate both MMC and EO9. A number of investigators have found that cell lines expressing higher levels of DT-diaphorase activity show greater sensitivity to the bioreductive antitumour agents, MMC (Begleiter et al, 1989; Malkinson et al, 1992; Ross et al, 1993) and EO9 (Plumb et al, 1994), than cells with lower levels of enzyme activity. EO9 has been shown to be selectively activated by DT-diaphorase (Walton et al, 1991; Bailey et al, 1992; Walton et al, 1992a; Robertson et al,

1994). Stable expression of cloned, rat liver DT-diaphorase in Chinese hamster ovary cells and exposure to MMC resulted in decreased free radical production and an increase in DNA cross-links (Doroshow et al, 1992; Belcourt et al, 1996). MMC caused DNA interstrand cross-links in a human cell line expressing high levels of DT-diaphorase, but this was not observed in a human cell line with no detectable levels of DT-diaphorase (Siegel et al, 1990). Finally, targeted disruption of the DT-diaphorase gene, NQO_1, in mouse embryonic stem cells resulted in a significant decrease in the MMC sensitivity of these cells (Yoshida and Tsuda, 1995).

In contrast, other groups have questioned the role of DT-diaphorase in MMC activation based on observations in their experimental models (Keyes et al, 1985; Nishiyama et al, 1993; O'Dwyer et al, 1996a). Stable expression of cloned, human liver DT-diaphorase in Chinese hamster ovary cells resulted in increased sensitivity to EO9, streptonigrin, and MeDZQ, but the sensitivity to MMC remained unchanged (Gustafson et al, 1996). The transfection of murine NIH 3T3 cells and stable expression of human DT-diaphorase in these cells did not change MMC cytotoxicity (Powis et al, 1995). The use of human DT-diaphorase in animal cell lines may have modified the normal metabolism of MMC by the enzyme in this model. Expression of rat NADPH:cytochrome P450 reductase in *Salmonella typhimurium* (Bligh et al, 1990) and expression of human NADPH:cytochrome P450 reductase in Chinese hamster ovary cells (Sawamura et al, 1996) both significantly increased the cytotoxicity of MMC.

An analysis of data from the National Cancer Institute tumour cell line panel, consisting of 60 human tumour cell lines from a wide range of tissues, showed a highly

significant correlation between DT-diaphorase expression levels and sensitivity to both EO9 and MMC (Fitzsimmons et al, 1996). In contrast, no correlations were found between NADPH:cytochrome P450 reductase activity or NADH:cytochrome b_5 reductase activity and sensitivity to either MMC or EO9. A smaller study by Mikami *et al* (1996) found that DT-diaphorase activity was required for effective cytotoxicity of MMC in 13 human colon and gastric carcinoma cell lines. These findings suggest that the activation of MMC is complex and the importance of either one- and two-electron reducing enzymes varies by cell type and by environmental conditions.

A potential practical application of this knowledge is the targeting of particular antitumour agents to specific tumour types depending on the levels of activating and deactivating enzymes, as suggested by the "enzyme-directed" approach to bioreductive drug development (Robertson et al, 1994; Beall et al, 1995; Fitzsimmons et al, 1996). Developing a strategy to detect and target certain tumours that have high levels of DT-diaphorase with bioreductive antitumour agents may provide a novel approach to enhancing the effectiveness of these agents in cancer treatment.

DT-DIAPHORASE

The Enzyme

DT-diaphorase was first described by Ernster *et al* (1958) and partially purified in 1960 (Ernster et al, 1960). DT-diaphorase is primarily located in the cytosol with 5-10% being membrane bound to mitochondria, microsomes and Golgi apparatus (Riley and Workman, 1992). The enzyme requires reduced β-nicotinamide adenine dinucleotide

(NADH) or reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor for enzymatic activity, has two identical subunits with individual molecular weights of 32 kDa, and contains two molecules of flavin adenine dinucleotide (FAD) (Riley and Workman, 1992). The three-dimensional structure of rat liver DT-diaphorase was determined by Li *et al* (1995). They isolated the 2.1 Å crystal structure of DT-diaphorase from the rat liver. The three-dimensional structure localized the binding sites for FAD and NAD(P)H in the dimeric protein and it clarified the simple rationale for the "ping-pong" mechanism of two-electron reduction of DT-diaphorase substrates.

Role of DT-diaphorase

Vitamin K (Preusch and Smalley, 1990), coenzyme Q_{10} (Beyer et al, 1996), and vitamin E (Siegel et al, 1997) have been hypothesized to be the endogenous substrates for DT-diaphorase and its proposed roles in blood clotting and antioxidant protection. It is known that DT-diaphorase is involved in the detoxification of xenobiotics and carcinogens that contain quinones, quinone imines, epoxides, azo dyes, arylamines, and other nitrogen oxides by two-electron reduction (Riley and Workman, 1992,37).

The fate of chemical carcinogens is controlled by the balance between phase I and phase II detoxifying enzymes (Gordon et al, 1991). Phase I enzymes, such as NADPH:cytochrome P450 isozymes, convert chemical xenobiotics to highly reactive electrophilic forms that may undergo further conjugation for elimination or that may damage DNA (Miller and Miller, 1980). Phase II enzymes, which include DT-diaphorase, glutathione *S*-transferases (GST's), UDP-glucuronosyltransferases, and epoxide hydrolase,

promote the conjugation of the reactive electrophile metabolites of phase I enzymes with endogenous ligands, such as glutathione and glucuronic acid, to less toxic products for elimination (Talalay et al, 1987; Gordon et al, 1991).

DT-diaphorase is classified as a phase II enzyme since it does not introduce new functional groups, is often induced coordinately with other phase II detoxifying enzymes, and exerts protective functions for the cell (Beyer et al, 1988; Gordon et al, 1991; Prestera et al, 1993). DT-diaphorase may play an important role in detoxifying chemically reactive metabolites, thereby protecting the cell from their toxic and mutagenic effects (Beyer et al, 1988; Riley and Workman, 1992). DT-diaphorase can be induced by monofunctional inducers, those compounds which induce phase II detoxifying enzymes selectively, or by bifunctional inducers, which induce both phase I and II detoxifying enzymes (Gordon et al, 1991).

DT-diaphorase Expression in Normal and Tumour Tissues

DT-diaphorase is ubiquitous in eukaryotes and is expressed at varying levels in most tissues (Benson et al, 1980; Riley and Workman, 1992; Belinsky and Jaiswal). Relatively high levels of enzyme activity have been found in liver, stomach, bladder, intestine, colon and kidney, but the levels are consistently low in hematopoietic cells (Benson et al, 1980; Schlager and Powis, 1990). DT-diaphorase activity is generally higher in tumour cells compared with normal cells from the same tissue, and high levels of enzyme activity have been found in some human hepatoma and colon, breast and lung carcinoma cell lines (Schlager and Powis, 1990; Cresteil and Jaiswal, 1991; Malkinson et al, 1992; Belinsky and

Jaiswal, 1993). Some studies have found DT-diaphorase activity to be unchanged or lower in other tumours (Schlager and Powis, 1990; De Waziers et al, 1991). DT-diaphorase activity may be higher in tumour cells *in vitro* than in similar cells *in vivo* (Collard et al, 1995). There are species differences in DT-diaphorase activity, with the rat enzyme being significantly more effective in activating MMC than the human and mouse enzymes (Chen et al, 1997). DT-diaphorase is induced in many tissues by a wide variety of chemicals including 1,2-dithiole-3-thione (D3T), quinones, 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), polycyclic aromatics, diphenols, vitamins A, D, and E, isothiocyanates, hydroperoxides, mercaptans, and heavy metals (Talalay, 1989; Prestera et al, 1993; Wang and Higuchi, 1995).

Molecular Biology of DT-diaphorase

DT-diaphorase Human Gene Family

NQO_1 is located on chromosome 16 (Jaiswal et al, 1988; Gasdaska et al, 1995), is 20 kb in length, contains 5 introns and 6 exons, and encodes a protein of 273 amino acids (Belinsky and Jaiswal, 1993; Gasdaska et al, 1995). The gene product is termed DT-diaphorase (or NAD(P)H:quinone oxidoreductase or quinone reductase). The NQO_1 gene is highly conserved in human and rat (Jaiswal, 1991; Belinsky and Jaiswal, 1993) but there is one principal exception. The length of the sixth exon in the human and rat NQO_1 is 1807 and 907 base pairs, respectively (Belinsky and Jaiswal, 1993). This difference results in an Alu repetitive sequence and 4 different polyadenylation sites in the human gene (Belinsky and Jaiswal, 1993). This modification results in 3 human NQO_1 mRNA transcripts (2.7, 1.7, and 1.2 kb), whereas only one rat mRNA can be detected (1.2 kb). It is assumed that all three

human NQO_1 transcripts are translated into functional protein with similar catalytic activities. Though several different DT-diaphorase genes have been identified in humans (Jaiswal et al, 1990; Jaiswal, 1991), the NQO_1 gene has been the most extensively studied and appears to be the most important DT-diaphorase for activation of bioreductive agents (Jaiswal, 1991; Riley and Workman, 1992; Belinsky and Jaiswal, 1993).

NQO_2 is found on chromosome 6, has only low levels of expression, is not induced by dioxin unlike NQO_1, encodes for a protein 231 amino acids in length, and has 49% homology to NQO_1 (Jaiswal et al, 1990; Zhao et al, 1997). The NQO_2 has a low activity with the conventional substrates used in assays for NQO_1 activity, such as menadione, and the difference between the activities was 210-fold (Jaiswal et al, 1990; Jaiswal, 1994).

NQO_1 Gene Regulation

As depicted in Figure 2, the 5' regulatory region of NQO_1 contains a number of promoter elements. The 5' region contains an Antioxidant Response Element (ARE) (Egner et al, 1994), a Xenobiotic Response Element (XRE) (Jaiswal, 1991), two AP-1 like sites (Li and Jaiswal, 1992), an AP-2 like site (Li and Jaiswal, 1992), and a NF-κB binding site (Yao and O'Dwyer, 1995).

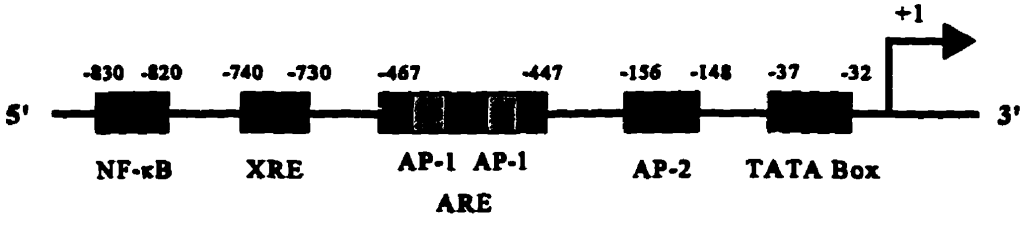


Figure 2: The Regulatory Sequences of the Human NQO_1 Gene

The ARE is responsible for maximal basal transcription (Favreau and Pickett, 1995), and induction by a wide range of compounds, such as Michael reaction acceptors, diphenols, quinones, isothiocyanates, D3T's, peroxides, vicinal dimercaptans, arsenicals, heavy metals, estrogen, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), β-naphthoflavone, and phenolic antioxidants (Favreau and Pickett, 1993; Prestera et al, 1993; Egner et al, 1994; Favreau and Pickett, 1995; Wang and Williamson, 1996). The XRE is responsible for induction by bifunctional inducers such as the dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Belinsky and Jaiswal, 1993).

The two AP-1 like sites are contained within the ARE of the human NQO_1 (Li and Jaiswal, 1992). The AP-1 sites have been observed to regulate NQO_1 induction by hypoxia (O'Dwyer et al, 1994), phenobarbital (Pinkus et al, 1993; Bergelson et al, 1994), MMC (Yao et al, 1997), t-butylhydroquinone, and β-naphthoflavone (Bergelson et al, 1994). Yao *et al* (1994) found that hypoxia induced NQO_1 expression and they observed that this increase was preceded by induction of jun, fos, and Ref-1. Specifically, c-jun and junD were involved in complex formation with c-fos at the AP-1 site and it was speculated that this may result in a series of AP-1 complexes, further regulating NQO_1 expression. Yao *et al* suspected that Ref-1 induction may be in response to possible O_2 damage since Ref-1 is a endonuclease or it may be used for its redox activity to help maintain reduced state of cysteine residues of fos-jun complexes for optimal DNA binding.

The NF-κB binding site is involved in the induction of NQO_1 by hypoxia, the D3T analog, 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (Oltipraz), and MMC (Yao and O'Dwyer, 1995; Yao et al, 1997). NF-κB consists of two proteins (p50 and p65) which in

unstimulated cells reside in the cytoplasm bound to a specific inhibitor, I-κB (Baeuerle and Baltimore, 1988). Upon activation, p50 and p65 dissociate from I-κB, translocate to the nucleus, and bind DNA as a p50 homodimer or p50/p65 heterodimer (Baeuerle and Baltimore, 1989).

Other researchers have found evidence that NQO_1 gene regulation is further regulated by a number of elements. Yao *et al* (1997) have shown that a percentage of the inducibility of NQO_1 by MMC depends on a novel factor binding element in a 38-base-pair region (-40 to -78) that is devoid of known transcription binding elements. Wang *et al* (1996) found a TPA responsive element that overlapped with the ARE. This element was involved in basal expression only. Montano and Katzenellenbogen (1997) found that antiestrogens, such as tamoxifen, can increase NQO_1 expression which implies that estrogen receptor complexes are negative regulators of NQO_1 gene expression. O'Dwyer *et al* (1994) have also reported that there was an increase in NQO_1 message stability after exposure to hypoxia. The only evidence of posttranslational modification of the DT-diaphorase protein is a report of glycosylation for the rat isoform (Segura-Aguilar et al, 1992). A combination of complex transcriptional activation and enhanced message stability may contribute to the mechanism of induction of NQO_1 expression.

The full picture of NQO_1 gene regulation is very complicated and is far from being clarified. The gene product of NQO_1 is used for many stress responses and it is reasonable to expect it to be regulated and induced by a variety of DNA:protein interactions.

The Mechanism of NQO_1 Induction

Although the regulation of NQO_1 expression is slowly being elucidated, the mechanism by which the inducers of DT-diaphorase send their signal to the gene is still unknown. Although there are many regulatory regions through which the inducers may work, the range of DT-diaphorase inducers is much greater. A large number of inducers possibly share a common pathway of induction of NQO_1.

Talalay (1989) reviewed the structural requirements for the induction of DT-diaphorase by monofunctional inducers and postulated that the inducers are all Michael reaction acceptors characterized by olefinic or acetylenic linkages that are rendered electrophilic by conjugation with electron-withdrawing groups. A universal property of most NQO_1 inducers may be their capacity for reaction with sulfhydryls by oxidoreduction or alkylation (Egner et al, 1994). Prestera *et al* (1993) also surveyed a list of DT-diaphorase inducers, including D3T, and the only apparent universal property was their capacity for reaction with sulfhydryls by either oxidoreduction or alkylation. Structural modifications of the inducer, such as D3T, may, therefore, alter the inducer's electrophilic character or its ability to react with sulfhydryls. Indeed, Egner *et al* (1994) looked at the induction of NQO_1 by the D3T analog, Oltipraz, and found that it was extensively metabolized in rodents, primates, and humans. In Hepa1c1c7 cells, a complete rearrangement was observed with the principal detected metabolite being 7-methyl-6,8-bis(methylthio)pyrrolo-[1,2-a]-pyrazine. This metabolite was glucuronidated and was further oxidized. The exogenous addition of 7-methyl-6,8-bis(methylthio)pyrrolo-[1,2-a]-pyrazine did not cause NQO_1 induction. The metabolism of Oltipraz was critical to NQO_1 induction.

A number of research groups have postulated how the metabolism of such inducers could induce NQO_1. Fleury *et al* (1991) made the claim that mixed disulfides, such as D3T's, can form bonds with glutathione. The metabolism of D3T to an opened ring may also cause anionic species to form. Egner *et al* (1994) pointed out that D3T's do not readily form disulfides with thiols but more probably act as oxidizing agents. Gates *et al* (1997) determined that D3T's, in the presence of thiols, mediated the conversion of molecular oxygen to reactive oxygen radicals. It was speculated that molecular oxygen was converted to a peroxide species that underwent a trace-metal catalyzed, Fenton-type reaction to generate oxygen radicals. The oxygen radical production by D3T's may, in addition to their electrophilicity, have played a role in the induction of protective phase II detoxifying enzymes. Induction may occur because of a decrease in thiol levels created by generation of intracellular oxidants since induction can be prevented by increasing intracellular thiol levels (Bergelson et al, 1994). Free radicals and sulfhydryl groups serve as molecular recognition sites of sensor proteins regulating gene expression in response to a variety of stresses (Abate et al, 1990; Storz et al, 1990). The outline of NQO_1 gene regulation indicates that redox processes and many sensor mechanisms are involved with NQO_1 enhancement.

Mutations in NQO_1 Expression

Traver *et al* (1992) compared a human colon carcinoma cell line, HT29, to another human colon tumour cell line, BE, that was found to be highly resistant to MMC. The BE cell line had a 90% reduction in NQO_1 levels and no detectable DT-diaphorase activity. This cell line was homozygous for a point mutation (C→T) at position 609 in NQO_1 cDNA and

this caused an amino acid change from proline 187 to serine 187. This residue was determined to be only 6 amino acids from an important cysteine residue in DT-diaphorase. The loss of a rigid proline amino acid residue in this region may result in a critical conformational change that caused a loss of DT activity. Eickelmann *et al* (1994) developed a RT112 human bladder carcinoma cell line variant that was found to be highly resistant to MMC. This cell line, RT112MMC, had its NQO_1 gene sequenced and the same homozygous point mutation was observed. The H596 NSCLC cell line was found to be homozygous for the same point mutation at position 609 in NQO_1 (Traver et al, 1997). In all these cases, the mutant human NQO_1 had moderate mRNA levels but no detectable protein or enzymatic activity levels. Traver *et al* (1997) have expressed and detected the mutant protein in *E. coli* and Misra *et al* (1997) have been able to achieve similar but transient transfection in the COS-1 monkey kidney cell line. The possible role of the mutant protein expression and the reason for the lack of stable mutant detection in human cells are being investigated.

Another mutant form of NQO_1 was found in human tumour cell lines that appeared to be an alternatively spliced version of the full-length NQO_1 mRNA lacking exon 4 (Gasdaska et al, 1995; Yao et al, 1996). It was also present in paired human normal and primary tumour tissue (Gasdaska et al, 1995). This mutant form of NQO_1 mRNA was 114 bases smaller than the full length NQO_1 mRNA. The loss of these bases resulted in a loss of amino acid residues 102 to 139, which included the putative quinone-binding domain and residues important for NAD(P)H cofactor binding (Gasdaska et al, 1995). This mutant product had no enzymatic activity towards various compounds that are typically reduced by normal full length NQO_1 product (naphthoquinone, benzoquinone, diaziquone, epoxide) .

However, it was not known if the alternatively spliced form of the protein would be active with other substrates. Pan *et al* (1995) found that a mutant human colon carcinoma cell line, HCT116R, had a splice deletion variant at exon 4 that resulted in MMC resistance, a decrease of 20% in mRNA levels for this mutant, and a decrease of 95% in DT-diaphorase activity. The reason for alternative splicing in normal tissue is not known but, in the latter case, overexpression of the exon 4 splice variant conferred MMC resistance.

Yao *et al* (1996) carried out an interesting study where they examined the mRNA from peripheral mononuclear cells of 16 patients with malignant solid tumours and found substantial interindividual variability in the patterns of NQO_1 transcript expression. After receiving a course of MMC treatment, the patients' transcript profiles were determined. In most patients, exon 4 deletion mutant expression remained constant while the expression of the full-length transcript was elevated. The extent of induction of full length NQO_1 mRNA varied. The study also examined two human colon carcinoma cell lines, HT29 and BE, and similar patterns of induction were observed after MMC treatment. These data demonstrate that NQO_1 expression in human cells is polymorphic, and that the levels of individual transcripts can be regulated by exogenous factors. Selective alternative splicing of NQO_1 RNA could provide another important mechanism for the regulation of NQO_1 gene expression.

The findings of these mutations is interesting but the relevance for the normal population should be considered. DT-diaphorase activity was lacking in 4% of a British population (Edwards et al, 1980) but the reason for this lack of activity in these patients has not been clarified at the molecular level. Nine and 40% of the population have been found

to be homozygous and heterozygous for the NQO_1 point mutation, respectively (Kuehl et al, 1995). Another study (Rosvold et al, 1995) used single-strand conformation polymorphism (SSCP) analysis to show that the point mutation allele occurred with a frequency of 0.13 in a reference population (a Centre d'Étude Polymorphisme Humain reference panel). The group tested the association of this mutation with lung cancer and their preliminary evidence suggested that the mutation was over-represented in lung cancer cases. An additional research group examined lung cancer patients and the incidence of the point mutation was analyzed from paired normal and tumour tissue (Traver et al, 1997). The mutation did not appear to be tumour, tissue, sex, or race specific. A total of 51% of the lung cancer samples were homozygous for the wild-type protein, 42% were heterozygous for the mutation and 7% were homozygous for the mutation. 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.

DT-diaphorase is a critical phase II detoxifying enzyme and an important activator of bioreductive antitumour agents. The existence of NQO_1 polymorphisms in the normal population has significant implications in the fields of chemoprotection, chemoprevention, cancer susceptibility, and cancer therapy.

DT-diaphorase and Chemoprevention

Chemoprevention, a term coined in 1976 (Sporn, 1976), is an approach which recognizes that cancer is not caused by a simple threshold event, but rather a multistep molecular and cellular process which is characterized by a period of time between the initiation

of carcinogenesis and the clinical presentation of invasive and metastatic disease (Greenwald et al, 1995). Due to the multistep progression of carcinogenesis, opportunities exist for intervention at early as well as later stages of the process. The goal of chemoprevention is to develop and administer compounds to prevent the formation or absorption of carcinogens, prevent carcinogens from reaching or reacting with cellular targets, or suppress the expression of cancerous cells (Greenwald et al, 1995). The study of epidemiological data and the understanding of molecular processes of cellular damage and tumour progression has lead to the clinical study and evaluation of a number of chemopreventative agents, such as retinoic acid, vitamin E, carotenoids, D3T's, and finasteride (Greenwald et al, 1995).

Recent studies have investigated using inducers of DT-diaphorase and other phase II detoxifying enzymes in cancer prevention or chemoprevention (Benson et al, 1980; Kelloff et al, 1990; Kensler and Helzlsouer, 1995; Wang and Higuchi, 1995). The induction of such enzymes could protect cells against the toxicity, mutagenicity, and carcinogeneicity of various chemical agents (Prestera et al, 1993). Benson *et al* (1980) used dietary BHA to show that the activity of DT-diaphorase and other detoxifying enzymes was significantly induced in murine liver, kidney, lung, and small intestine mucosa. The enhanced levels of the phase II detoxifying enzymes was proposed to increase the conjugation and elimination reactions of toxic substances and their metabolites. DT-diaphorase induction has been shown to be a critical component in the field of chemoprevention and this knowledge is being used in further clinical trials. The key point of these studies is that the phase II detoxifying enzyme, DT-diaphorase, can be induced by chemical compounds which have inherently very low toxicity to the organism.

1,2-DITHIOLE-3-THIONES (D3T's)

Chemistry

D3T is a five-membered disulfide-containing ring compound depicted in Figure 3 (Davidson et al, 1990). Substitutions to the D3T ring can be made at the 4- and 5- positions. D3T analogs are found in nature in cruciferous vegetables, such as cabbage, cauliflower, and Brussels sprouts (Jirousek, 1958; Jirousek and Starka, 1959).

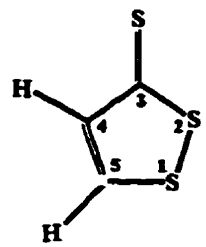


Figure 3: Chemical Structure of D3T

Biochemical Effects of D3T's

Ansher *et al* (1986) carried out a study to examine the administration of a number of D3T analogs to the diets of CD-1 and C3H mice and F344 rats. Their results found that D3T's were monofunctional inducers which enhanced the activity of a number of phase II detoxifying enzymes involved, directly or indirectly, in the detoxification of carcinogenic compounds. The enzyme activities of GST's, DT-diaphorase, glutathione reductase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were increased in a number of tissues, including lung, liver, and upper jejunal mucosa. Glutathione levels were also increased. Induction of phase II detoxifying enzymes, such as DT-diaphorase, epoxide hydrolase, GST, and UDP-glucuronosyl transferase, in animal and human cell lines and primary tissues by D3T analogs has been reported by a number of research groups (Kensler et al, 1987; Davidson et al, 1990; Gordon et al, 1991; Kensler et al, 1992; Twerdok et al,

1992; Li et al, 1994; Kensler et al, 1995).

Uses of D3T's

A number of synthetic D3T compounds have antioxidant, chemotherapeutic, and chemoprotective properties (Davidson et al, 1990). D3T analogs have been used as an antioxidant in the commercial preparation of rubber, metals, oils, and greases. The D3T analog, Oltipraz, has been used as an antischistosomal agent (Bueding et al, 1982; Davidson et al, 1990; Fleury et al, 1991). Another D3T analog, anethole 1,2-dithiole-3-thione (ADT), has been used as a therapeutic choleretic drug (Warnet et al, 1989). The study of D3T's as chemoprotective agents have only recently begun (Warnet et al, 1989; Kelloff et al, 1990; Kensler et al, 1992; Egner et al, 1994; Clapper et al, 1995; Kensler and Helzlsouer, 1995; Maxuitenko et al, 1996; Arif et al, 1997; Maxuitenko et al, 1997).

D3T and Chemoprevention

Epidemiological and experimental evidence indicates that consumption of diets high in certain vegetables reduces the incidence of various cancers (Colditz et al, 1985). Analogs of D3T are being investigated as cancer preventative agents due to their presence in the relevant cruciferous vegetables, their ability to induce phase II detoxifying enzymes and their relatively low toxicity (Egner et al, 1994). Oltipraz induces DT-diaphorase and other phase II detoxifying enzymes (Kensler et al, 1992; Egner et al, 1994; Kensler and Helzlsouer, 1995) and has been shown to significantly inhibit tumourigenesis in various animal models (Kelloff et al, 1990; Kensler et al, 1992; Clapper et al, 1995; Kensler and Helzlsouer, 1995; Arif et

al, 1997). Another D3T analog, ADT, reduced the hepatotoxicity of acetaminophen in mice (Warnet et al, 1989). In one study, Maxuitenko *et al* (1997) have shown that D3T can be given orally to rats and can significantly prevent aflatoxin-B_1 (AFB_1)-induced hepatotoxicity in rats. The rats were fed low doses of D3T (0.3 mmol/kg body weight, 3 times per week) and phase II detoxifying enzymes were found to be induced in a number of tissues. Oltipraz is currently in a phase II clinical trial as a chemoprotective agent in a region of China that has a high incidence of AFB_1-induced tumours (Kensler et al, 1992; Maxuitenko et al, 1996). O'Dwyer *et al* (1996b) have recently shown that a single oral dose of Oltipraz increased the levels of DT-diaphorase mRNA in colon mucosa and peripheral lymphocytes in humans. These studies indicate that D3T analogs are bioavailable in oral doses, are chemoprotective, and induce phase II detoxifying enzymes in a number of tissues in the whole organism.

D3T and Cancer Chemotherapy

D3T has been extensively examined in its ability to induce phase II detoxifying enzymes for the role of chemoprotection and cancer prevention. Recent studies have begun to examine the potential use of induction of these same enzymes in cancer chemotherapy. Our laboratory has previously reported that D3T selectively induces DT-diaphorase in murine L5178Y lymphoma cells compared with normal murine bone marrow cells (Begleiter et al, 1996). 100 μM D3T increased DT-diaphorase levels by 22-fold. Combination treatment with D3T and the bioreductive antitumour agents, MMC and EO9, produced increases in tumour cell kill of 2- and 7-fold, respectively. Similar treatment of normal mouse bone marrow cells with D3T and the bioreductive agent produced only a small

increase in cytotoxicity in these cells.

Shao *et al* (1995) found that high levels of dietary fish oil lowered the growth rate of MX-1, a human mammary carcinoma cell line, in athymic mice and increased the response of the explanted tumour to MMC used as a single therapy. The high levels of fish oil were shown to increase xanthine oxidase and DT-diaphorase activity in the tumour. They did not determine if this combination treatment increased the toxicity of MMC in normal tissues. This was the first study to show *in vivo* that a non-toxic dietary component could be given prior to administration of a bioreductive antitumour agent, MMC, and this resulted in an increase in reductive enzyme activity and a corresponding enhancement of MMC cytotoxicity towards a human tumour.

Induction of DT-diaphorase by D3T may allow for similar responses to human tumours to bioreductive agents. Egner *et al* (1994) determined that induction of DT-diaphorase by 25 D3T analogs in Hepa 1c1c7, mouse hepatoma cells, was dependent on the chemical structure of the D3T compound. These studies suggest that D3T analogs should be screened for selective induction of DT-diaphorase in human tumour cells compared to normal tissues and then possibly used for selectively enhancing the antitumour efficacy of bioreductive antitumour agents.

The ability of D3T to induce DT-diaphorase activity has not been evaluated in human normal cells and human tumour cell lines. Tissue and basal enzyme level specificities may exist for DT-diaphorase induction by D3T in human cells. The study of a number of structural D3T analogs may display possible structure-activity relationships that, in turn, could be used to develop D3T analogs that maximize DT-diaphorase induction selectively

in human tumour cells. The combination of significant induction of DT-diaphorase in tumour cells with bioreductive agents, such as MMC or EO9, may increase the cytotoxic activity of these agents *in vitro*. D3T may also increase the cytotoxicity of bioreductive agents in normal tissues and this issue should be evaluated. If it can be shown that the induction of DT-diaphorase by D3T analogs can selectively increase the antitumour activity of bioreductive agents *in vitro*, then this would lend support to an *in vivo* evaluation of this potential regimen in cancer treatment.

MATERIALS AND METHODS

Materials

All media and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). All reagents for the DT-diaphorase assay, hydrocortisone, Ficoll 400, lactalbumin hydrosylate, and MMC were from Sigma (St. Louis, MO, USA). Protein concentration was measured using the Bio-Rad DC Kit (Bio-Rad, Mississauga, ON, Canada) with gamma globulin as standard. EO9 was kindly supplied by Dr. H.R. Hendriks, New Drug Development Office, European Organization for Research and Treatment of Cancer, Amsterdam, The Netherlands, dissolved in dimethyl sulfoxide (DMSO):ethanol (2:3, v:v), and stored at -20°C. MMC was dissolved in phosphate buffered saline (PBS) and stored at -20°C. Appropriate media and FBS at pH 7.2 were used for all DT-diaphorase studies and incubations with MMC or EO9. The D3T analogs were synthesized by Dr. T.J. Curphey, Dartmouth College, Hanover, NH, USA. D3T analogs were prepared in ethanol at a concentration of $2x10^{-2}$ M with the exceptions of ADT, which was prepared at $1x10^{-2}$ M in ethanol, and Oltipraz, which was prepared at $2x10^{-2}$ M in DMSO or at $5x10^{-3}$ M in acetone. All the D3T analog solutions were stored at -20°C. The final concentration of acetone did not exceed 2% and that of DMSO or ethanol did not exceed 1% in the cell incubation media.

Cells

HL-60, promyelocytic leukemia cells, were obtained from Dr. A.H. Greenberg, Manitoba Institute of Cell Biology, Winnipeg, MB, Canada, and were grown in RPMI 1640 medium and 10% FBS. THP-1, monocytic leukemia cells, were obtained from Dr. D.

Houston, Manitoba Institute of Cell Biology, and were grown in RPMI 1640 and 10% FBS. WIL-2, B lymphoblastoid leukemia cells, were purchased from the American Type Culture Collection, Rockville, MD, USA, and were grown in RPMI 1640 and 10% FBS. SSC-25, tongue squamous carcinoma cells, were from American Type Culture Collection and were grown in DMEM/F-12(1:1) medium with 0.4 µg/ml of hydrocortisone and 10% FBS. Detroit 562, pharynx carcinoma cells, were from American Type Culture Collection and were grown in DMEM/F-12(1:1) with 0.1% lactalbumin hydrosylate and 10% FBS. FaDu, pharynx squamous carcinoma cells, were from American Type Culture Collection and were grown in DMEM/F-12(1:1) and 10% FBS. NCI-H596, lung adenosquamous carcinoma cells, NCI-H209, lung small cell carcinoma cells, NCI-H661, lung large cell carcinoma cells, and NCI-H520, lung squamous carcinoma cells, were obtained from American Type Culture Collection and were grown in RPMI 1640 and 10% FBS. NCI-H125, lung non-small cell adenocarcinoma cells, were obtained from Dr. S.S. Pan, University of Maryland Cancer Center, Baltimore, MD, USA, and were grown in RPMI 1640 and 10% FBS. HCT116 and LS174T, colon carcinoma cells, were from American Type Culture Collection and were grown in DMEM/F-12(1:1) and 10% FBS. Colo205, colon adenocarcinoma cells, were obtained from American Type Culture Collection and were grown in RPMI 1640 and 10% FBS, while HT29, colon adenocarcinoma cells, were obtained from Dr. J.B. Johnston, Manitoba Cancer Treatment and Research Foundation, Winnipeg, MB, Canada, and were grown in RPMI 1640 and 10% FBS. RF-1 and RF-48, gastric adenocarcinoma cells, were from Dr. J.A. Wright, Manitoba Institute of Cell Biology, and were grown in Alpha Minimum Essential Medium (αMEM) and 10% FBS. AGS, gastric adenocarcinoma cells,

and Kato III, gastric carcinoma cells, were obtained from Dr. J.A. Wright and were grown in RPMI 1640 and 10% FBS. MDA-MB-231, breast adenocarcinoma cells, were from American Type Culture Collection and were grown in Iscove's Modified Dulbecco's Medium (IMDM) and 10% FBS. T47D, breast ductal carcinoma cells, were from Dr. S. Mai, Manitoba Institute of Cell Biology, and were grown in RPMI 1640 with 1% insulin transferrin selenium (ITS) and 10% FBS. MDA-MB-468, breast adenocarcinoma cells, were obtained from Dr. A.H. Greenberg and were grown in Dulbecco's Modified Eagle Medium: Nutrient Powder F-12 (Ham) (1:1) (DMEM/F-12(1:1)) with 1% ITS and 10% FBS. BT474, breast ductal carcinoma cells, were from Dr. S.S. Pan and were grown in DMEM/F-12(1:1) with 1% ITS and 10% FBS. SK-Br-3, breast adenocarcinoma cells, were obtained from American Type Culture Collection and were grown in McCoy's medium and 10% FBS. MDA-MB-435, breast ductal carcinoma cells, were from Dr. E.A. Turley, Hospital for Sick Children, Toronto, ON, Canada, and were grown in DMEM/F-12(1:1) and 10% FBS. HS578T, breast ductal carcinoma cells, and ZR-75-1, breast carcinoma cells, were obtained from Dr. S.S. Pan and were grown in DMEM/F-12(1:1) and 10% FBS. MCF-7, breast adenocarcinoma cells, were from Dr. A.H. Greenberg, and were grown in RPMI 1640 and 10% FBS. OVCAR-3, ovarian adenocarcinoma cells, were from American Type Culture Collection and were grown in RPMI 1640 with 1% ITS and 20% FBS. SK-OV-3, ovarian adenocarcinoma cells, were from American Type Culture Collection and were grown in McCoy's and 10% FBS. PC-3, prostate adenocarcinoma cells, and DU145, prostate carcinoma cells, were obtained from Dr. J. Dodd, University of Manitoba, Winnipeg, MB, Canada, and were grown in DMEM/F-12(1:1) and 10% FBS. LnCAP, prostate

adenocarcinoma cells, were from Dr. J. Dodd, and were grown in RPMI 1640 and 10% FBS. SK-MEL-28, SK-MEL-2 and SK-MEL-5, malignant melanoma cells, were obtained from American Type Culture Collection and were grown in DMEM/F-12(1:1) and 10% FBS. HepG2, hepatocellular carcinoma cells, were from American Type Culture Collection and were grown in αMEM and 15% FBS.

Normal marrow specimens were obtained from marrow donated for transplantation and mononuclear cells were isolated using a Ficoll-Hypaque gradient (Johnston et al, 1994). Marrow samples were diluted 1:1 (v/v) with PBS and gently layered over 3-4 ml Ficoll 400. The sample was spun for 30-35 min at 1400 rpm in a IEC Centra GP8R centrifuge (Fisher Scientific, Nepean, ON, Canada). The top and interphase layers, which contain the white blood cells and serum, were removed and spun again for 10 min at 1500 rpm. The pellet was then resuspended in 2 ml RPMI 10% FCS. A small sample, 100 µl, was counted using a Coulter Counter® and approximately $5.0x10^6$ cells were used in each incubation with D3T for DT-diaphorase activity. WI-38, human embryonic lung cells, were obtained from Dr. J.A. Wright and were grown in αMEM and 10% FBS, while 293, human embryonic kidney cells, were from Dr. M. Mowat, Manitoba Institute of Cell Biology, and were grown in DMEM/F-12(1:1) and 10% FBS.

Incubation Conditions for DT-diaphorase Induction by D3T

To determine the proper incubation conditions for DT-diaphorase induction by D3T and its analogs, dose response and time course experiments were carried out. HL-60 cells were incubated in 5% CO_2 at 37°C for 48 hr with various concentrations of D3T. NCI-H661

cells were incubated in 5% CO_2 at 37°C for various time periods with 100 µM D3T.

Measurement of DT-diaphorase Activity

DT-diaphorase activity was measured in the sucrose sonicates by a modification of the protocol developed by Prochaska and Santamaria (1988). After the cells were incubated with the D3T analog for the required period of time, aliquots of cells were washed twice with PBS, resuspended in 200-300 µl of 0.25M sucrose, sonicated on ice and stored at -80°C. Protein concentration was determined in all samples. The assay consisted of adding a determined protein concentration to the DT-diaphorase cycling assay solution. The cycling solution consisted of 10 ml cycling buffer ($2.5x10^{-2}$ M Tris, 0.06% bovine serum albumin (BSA) w/v, and 10 µl Tween 20) and $5.1x10^{-6}$ M FAD, $1.0x10^{-3}$ M glucose 6-phosphate, 20 units glucose 6-phosphate hydrogenase, $7.2x10^{-6}$ M 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (stock stored in PBS), and $5.0x10^{-5}$ M menadione (stock stored in acetonitrile). The buffer was kept out of direct light and at room temperature. Immediately before the assay was run, 400 µl of the cycling buffer was added to 1 mg β-nicotinamide adenine dinucleotide phosphate (NADP). The sample was vortexed and 100 µl of the sample was added back to the DT-diaphorase cycling solution. The protein samples and 750 µl cycling assay solution were placed in a semimicro cuvette and analyzed at 610 nm by a Cary 1 spectrophotometer (Varian, Mississauga, ON, Canada) for 4-8 minutes. Ten µl of $1.0x10^{-5}$ M dicoumarol stock (in 5% $5x10^{-2}$ N NaOH) was added to a second reading of the protein sample to determine the non-DT-diaphorase related reduction observed in the sample. The second assay reading was subtracted from the first assay reading

to calculate the DT-diaphorase activity. This value was divided by the mg of protein used for the assay to calculate DT-diaphorase activity. DT-diaphorase activity was expressed as nanomole of MTT reduced per minute per milligram protein (nmol MTT min^{-1} mg $protein^{-1}$).

Measurement of Glutathione *S*-Transferase Activity

GST activity was measured in the supernatant of cell sonicates by a previously described procedure (Habig et al, 1974) using 1-chloro-2,4-dinitrobenzene as substrate. After the cells were incubated with D3T for 48 hr, aliquots of cells ($5x10^6$ cells) were washed twice with PBS, resuspended in 1 ml of water, sonicated on ice, and spun at 12,500 rpm in an Eppendorf 5415 C centrifuge for 20 min. The supernatant was transferred to a fresh Eppendorf tube and the assay was run immediately. The protein concentration was determined in all samples. The GST assay phosphate buffer consisted of 80 ml 1 M K_2HPO_4 added to 100 ml 1 M KH_2PO_4 and this solution was verified to have pH 6.5. Before the assay was run, 1-chloro-3,4-dinitrobenzene (CDNB) was made at 50 mM in ethanol and glutathione in its reduced form was prepared at 10 mM in phosphate buffer. In a semimicro cuvette, 100 µl glutathione solution was added to 100 µg protein sample and water for a total volume of 880 µl. A blank consisting of 880 µl water was also measured. The cuvettes were let to warm to 25°C in the Cary 1 spectrophotometer. To start the reaction, 20 µl CDNB was added and mixed into each cuvette. The reaction was analyzed at 340 nm by a Cary 1 spectrophotometer for 5-10 minutes. GST activity was expressed as nmol CDNB min^{-1} mg $protein^{-1}$.

Measurement of NADPH:Cytochrome P450 Reductase Activity

NADPH:cytochrome P450 reductase activity was determined in supernatant from cell sonicates using cytochrome c as the artificial electron acceptor (Strobel and Digman, 1978). After the cells were incubated with D3T for 48 hr, aliquots of cells ($1x10^7$ cells) were washed twice with PBS, resuspended in 500 µl 20% glycerol (v/v), sonicated on ice and stored at -80°C. Protein concentration was determined in all samples. Phosphate buffer was prepared for the assay and consisted of 100 ml 0.3 M K_2HPO_4, pH solution to 7.7, and then 6.5 mg KCN (1 mM). The buffer was prewarmed to 30°C. Before the assay was run, cytochrome c and NADPH were prepared at 0.84 mM and 7.4 mM, respectively, in phosphate buffer. In a semimicro cuvette, a determined protein concentration (to a maximum 100 µl volume) was added to 850 µl phosphate buffer and 50 µl cytochrome c solution. To start the reaction, 15 µl NADPH was added and mixed into each cuvette. 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 cytochrome c min^{-1} mg $protein^{-1}$.

Cytotoxicity Assays

Cytotoxicity Assay for HL-60 Cells

HL-60 cells were incubated at 37°C for 48 hr with, or without, 50 µM D3T or Oltipraz. The differentiation of HL-60 cells can be initiated by prolonged exposure to such polar solvents as DMSO (Collins et al, 1978). Thus, Oltipraz at $5x10^{-3}$ M in acetone was used for the clonogenic assay in HL-60 cells. Medium was removed, cells were washed, and cells were incubated with various concentrations of EO9 for 1 hr in RPMI and 10% FBS.

For studies with dicoumarol, 50 μM dicoumarol was added 15 minutes prior to treatment with EO9. Drug cytotoxicity was determined by clonogenic assay as described previously (Begleiter et al, 1989). Cells were diluted to 10,000, 2,500, and 625 cells/ml in RPMI 1640 and 15% FBS. Two ml melted agar mixture was added to 20 ml of prewarmed RPMI 1640 and 15% FBS (44°C). 400 μl of each cell dilution was placed in the wells of a Falcon® 12-well plate (Becton Dickinson Labware, Lincoln Park, NJ, USA) and then 600 μl of the RPMI 1640 and agar solution was added to each well. The dilutions for the control points were seeded in duplicate. The cells were placed on ice for a short period of time to solidify the mixture and then placed in an incubator with humidified atmosphere containing 5% CO_2. After 14 days, the colonies of viable cells were counted and surviving cell fractions were calculated. The doses of D3T, Oltipraz, and dicoumarol used for the clonogenic studies were not toxic to the cells.

Cytotoxicity Assay for 293 Human Normal Kidney Cells

The 293 human normal kidney cell line was incubated at 37°C for 48 hr with, or without, 50 μM D3T. Medium was removed, cells were washed, and cells were incubated with various concentrations of EO9 for 1 hr in RPMI 1640 and 10% FBS. The surviving cell fraction was determined by MTT assay (Johnston et al, 1994). For each drug concentration, treated cells were diluted and then seeded at 1,000, 800, 600, 400, and 200 cells/well in 250 μl total volume of DMEM/F12(1:1) and 10% FBS in a Falcon® 96-well plate (Becton Dickinson Labware). The experimental points and dilutions were all seeded in quadruplicate. The cells were then placed in an incubator with humidified atmosphere

containing 5% CO_2. After 8 days, or a minimum of 4-5 cell cycles, the cells were spun at 1,200 rpm for 10 minutes in a IEC Centra GP8R centrifuge. At this time, 2.5 ml MTT stock (12 mM in PBS) was added to 50 ml RPMI 1640 and 10% FBS. After the plates had been spun, the medium was removed and replaced with 200 µl of the RPMI/MTT solution. The cells were placed in the incubator for 4 hr to allow the metabolism of MTT by viable cells. The cells were then spun again at 1,500 rpm for 15 minutes. The medium was removed and the MTT crystals were dissolved in 300 µl DMSO. The plates were vortexed for 10 seconds, read at 540 nm with a Titertek Multiskan® MCC/340 plate reader (Flow Laboratories, Woodcock Hill, Great Britain), and the surviving cell fractions were calculated. The dose of D3T used for the cytotoxicity studies was not toxic to the cells.

Cytotoxicity Assay for NCI-H661 Cells

NCI-H661 cells were incubated at 37°C for 48 hr with, or without, 50 µM D3T or Analog 8. Medium was removed, cells were washed, and cells were incubated with various concentrations of EO9 for 1 hr in RPMI 1640 and 10% FBS. The surviving cell fraction was determined by MTT assay (Johnston et al, 1994). For each drug concentration, treated cells were diluted and then seeded at 1,000, 800, 600, 400, and 200 cells/well in 250 µl total volume of RPMI 1640 and 10% FBS in a 96-well plate. The experimental points and dilutions were all seeded in quadruplicate. The cells were then placed in an incubator with humidified atmosphere containing 5% CO_2. After 7 days, 10 µl MTT stock was added to each well, and the plates were placed in the incubator for 3.5 hr to allow the metabolism of MTT by viable cells. The cells were then spun for 10 minutes at 1,500 rpm. The medium

was removed and the MTT crystals were dissolved in 300 µl DMSO. The plates were vortexed for 10 seconds, read at 540 nm with the plate reader, and the surviving cell fractions were calculated. The doses of D3T and Analog 8 used for the cytotoxicity studies were not toxic to the cells.

Cytotoxicity Assay for HCT116 Cells

HCT116 cells were incubated at 37°C for 48 hr with, or without, 50 µM D3T. Medium was removed, cells were washed, and cells were incubated with various concentrations of MMC for 1 hr in RPMI 1640 and 10% FBS. The surviving cell fraction was determined by MTT assay (Johnston et al, 1994). Treated cells were diluted and then seeded at 1,200, 1,000, 800, 600, and 400 cells/well in 250 µl total volume of DMEM/F12(1:1) and 10% FBS in a 96-well plate. The experimental points and dilutions were all seeded in quadruplicate. The cells were then placed in an incubator with humidified atmosphere containing 5% CO_2. After 5 days the cells were spun at 1,500 rpm for 7 minutes. At this time, 2.5 ml MTT stock was added to 50 ml RPMI 1640 and 10% FBS. After the plates had been spun, the medium was removed and replaced with 200 µl of the RPMI/MTT solution. The cells were placed in the incubator for 3.5 hr to allow the MTT metabolism by viable cells. The cells were then spun again at 1,500 rpm for 10 minutes. The medium was removed and the MTT crystals were dissolved in 300 µl DMSO. The plates were vortexed for 10 seconds, read at 540 nm with the plate reader, and the surviving cell fractions were calculated. The dose of D3T used for the cytotoxicity studies was not toxic to the cells.

Calculation of Surviving Cell Fraction

The surviving cell fractions for each drug concentration and each cell line were the means of at least 4 experiments. The D_{10} values, the concentration of drug reducing the surviving cell fraction to 0.1, were determined from the linear regression lines of the surviving cell fraction versus drug concentration curves and were calculated from the neagtive recriprocal of the slopes of the regression lines.

Statistical Analysis

In the experiments measuring the induction of DT-diaphorase activity, the control and D3T analog treated cells were compared by a t-test evaluating the significance of the difference of the mean enzyme activities in the control and D3T analog treated cells. For the cytotoxicity experiments, the D_{10} values were compared by a t-test comparing the significance of the differences of the slopes of the linear regression lines.

RESULTS

Induction of DT-diaphorase in Human Tumour Cells

Incubation of HL-60, promyelocytic leukemia cells, with increasing concentrations of D3T for 48 hr resulted in increasing levels of DT-diaphorase activity that reached a maximum at 100 μM D3T and decreased at higher concentrations (Fig. 4). When NCI-H661, large cell lung carcinoma cells, were incubated with 100 μM for up to 72 hr, the level of DT-diaphorase activity increased with time but reached a maximum at 48 hr. These results indicated that for optimal induction of DT-diaphorase activity the incubation of each cell line with any of the D3T analogs should be for 48 hr at 100 μM.

The level of DT-diaphorase activity was measured in 37 human tumour cell lines after incubation with, or without, 100 μM D3T for 48 hr (Table 1). Tumour cell lines from 10 different tumour types were studied including leukemia, head and neck, lung, colon, stomach, breast, ovary, prostate, melanoma, and liver. The basal level of DT-diaphorase activity in the cells ranged from 0.9 to 2120 nmol MTT min^{-1} mg $protein^{-1}$ in absolute terms. Enzyme activity was significantly increased with D3T incubation in 28 of the cell lines with the increase ranging from 1.3- to 7.0-fold, or from 2.5 to 590 nmol MTT min^{-1} mg $protein^{-1}$ in absolute terms. Cells from all tumour types appeared to be inducible with the exception of head and neck and liver tumours.

All 3 leukemia cell lines were significantly induced by D3T (Fig. 5). Four of 5 lung tumour cell lines showed increased DT-diaphorase activity following incubation with D3T and this included both small cell and non-small cell tumours (Fig. 6). All 4 colon tumour cell lines (Fig. 7) and each of the 3 gastric tumour cell lines were inducible by 100 μM D3T.

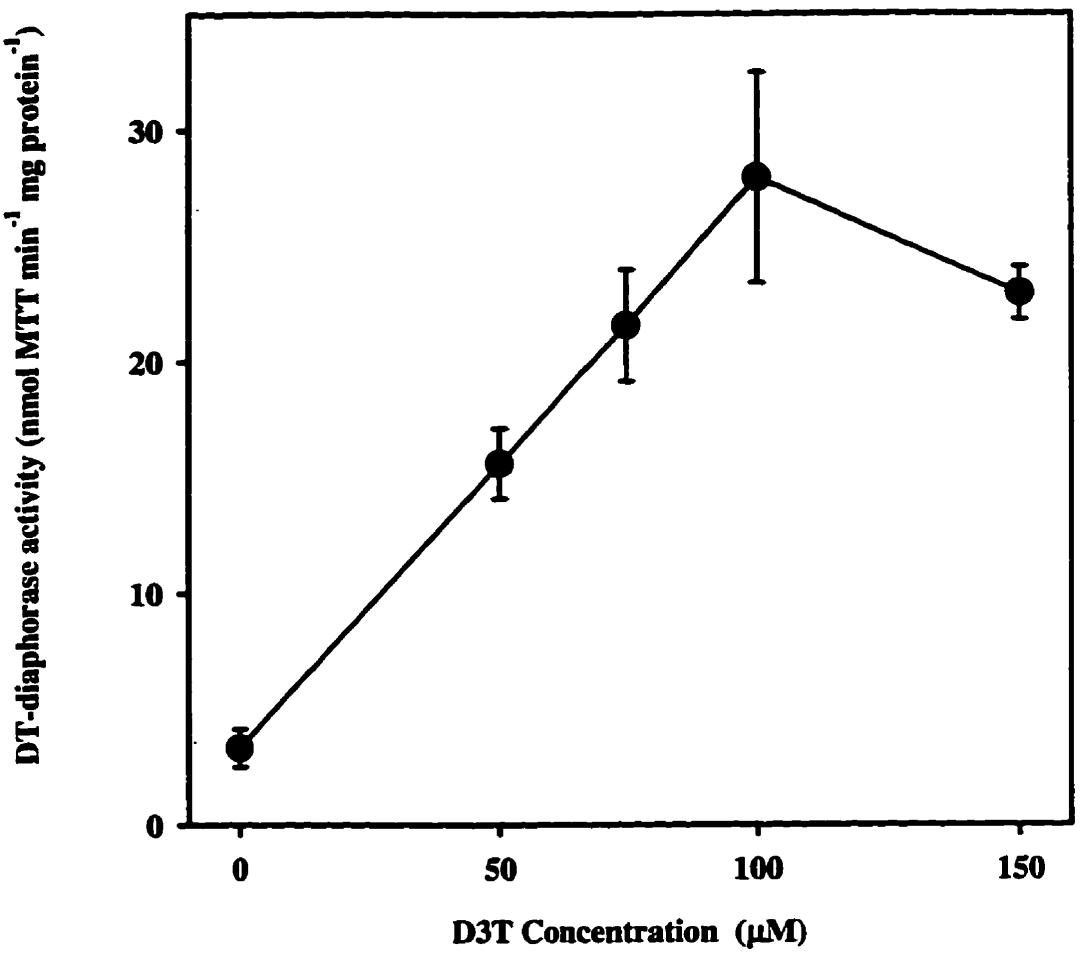


Figure 4: Dose Response of Induction of DT-diaphorase by D3T in HL-60 Cells

HL-60 cells were incubated with various concentrations of D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 4-5 determinations; bars, standard error.

Table 1: Induction of DT-diaphorase in Human Tumour Cell Lines by D3T

Cells were incubated with, or without, 100 μM D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated and stored at -80°C. DT-diaphorase activity was measured (Prochaska and Santamaria, 1988) using menadione as the electron acceptor. The data represent the mean ± standard error of 3-15 determinations. Statistical significance was determined by a 2-tailed t-test comparing the significance of the difference of the mean DT-diaphorase activity in control and D3T treated cells.

Tumour Type	Cell Line	DT-diaphorase Activity Mean ± S.E. (nmol MTT min^{-1} mg $protein^{-1}$)		p
		Control	D3T Induced	
Leukemia	HL-60	4.5 ± 0.5	31.6 ± 3.6	<0.001
	THP-1	26.1 ± 2.3	102.8 ± 5.4	<0.001
	WIL-2	58.8 ± 4.4	139.2 ± 8.9	<0.001
Head and Neck	SSC-25	39.7 ± 7.8	66.1 ± 16.7	NS
	Detroit 562	167.7 ± 27.9	199.7 ± 39.3	NS
	FaDu	463.2 ± 29.9	444.1 ± 45.2	NS
Lung	NCI-H596	1.0 ± 0.2	3.5 ± 0.6	<0.005
	NCI-H209	8.3 ± 0.8	40.2 ± 3.1	<0.001
	NCI-H661	112.7 ± 12.4	284.8 ± 27.3	<0.001
	NCI-H520	230.1 ± 31.3	267.0 ± 36.5	NS
	NCI-H125	689.5 ± 39.0	1136.0 ± 52.1	<0.001
Colon	HCT116	91.5 ± 15.2	224.4 ± 38.1	<0.02
	LS174T	314.6 ± 39.0	776.0 ± 97.4	<0.005
	Colo205	671.2 ± 64.5	1117.3 ± 111.5	<0.005
	HT29	713.1 ± 47.4	944.6 ± 69.1	<0.02
Stomach	RF-48 *	6.9 ± 0.3	22.7 ± 1.0	<0.002
	RF-1 *	7.7 ± 0.5	22.7 ± 0.4	<0.001
	AGS *	113.0 ± 1.6	266.0 ± 15.3	<0.001
	Kato III *	161.0 ± 7.3	278.0 ± 10.6	<0.001
Breast	MDA-MB-231	0.9 ± 0.3	1.9 ± 0.5	NS
	T47D *	25.8 ± 1.0	92.2 ± 5.5	<0.001
	MDA-MB-468 *	90.5 ± 4.8	87.1 ± 4.9	NS
	BT474 *	191.5 ± 10.0	430.8 ± 36.1	<0.001
	SK-Br-3 *	213.0 ± 1.5	331.0 ± 13.7	<0.002
	MDA-MB-435 *	232.2 ± 16.2	291.7 ± 16.0	<0.02
	HS578T *	237.9 ± 13.9	420.8 ± 19.0	<0.001
	ZR-75-1 *	355.5 ± 43.0	592.5 ± 71.7	<0.02
	MCF-7	939.1 ± 88.3	981.2 ± 96.5	NS
Ovary	OVCAR-3 *	47.1 ± 5.9	129.3 ± 10.9	<0.001
	SK-OV-3 *	177.8 ± 8.6	259.0 ± 15.9	<0.002
Prostate	PC-3 *	149.3 ± 5.0	183.0 ± 1.7	<0.005
	LnCAP *	166.0 ± 15.1	266.2 ± 34.5	<0.02
	DU145 *	652.3 ± 29.9	632.0 ± 20.1	NS
Skin	SK-MEL-28 *	586.7 ± 19.6	828.7 ± 38.4	<0.01
	SK-MEL-2 *	666.5 ± 30.6	796.3 ± 36.5	<0.05
	SK-MEL-5 *	2120.0 ± 51.3	2710.0 ± 60.8	<0.005
Liver	HepG2	1292.5 ± 162.0	1356.3 ± 141.3	NS

*, refer to Begleiter and Leith, 1997.
NS, not significant.

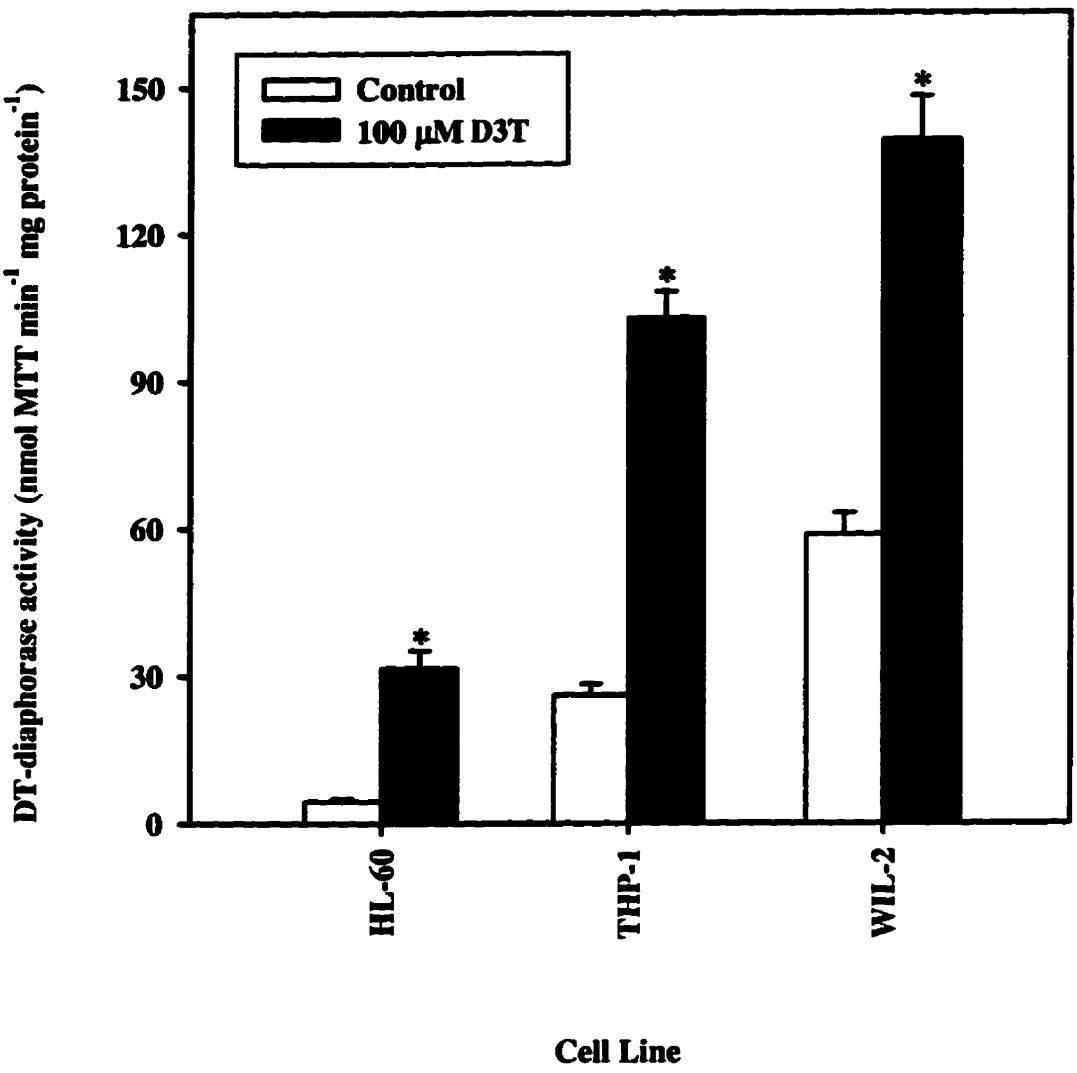


Figure 5: Induction of DT-diaphorase in Human Leukemia Cells by D3T

Human leukemia cell lines were incubated with, or without, 100 µM D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 µl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 6-10 determinations; bars, standard error. *, $p < 0.05$.

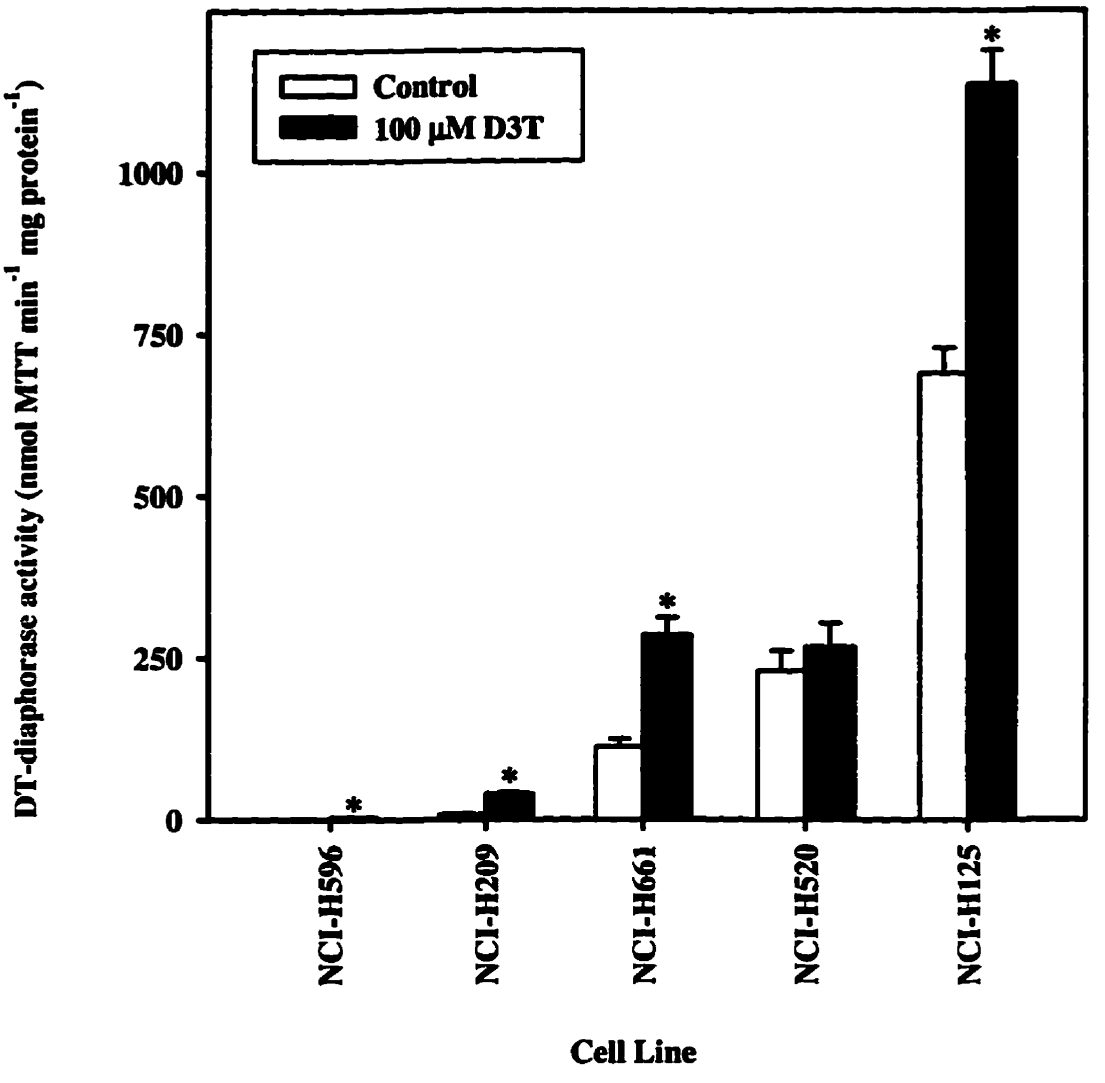


Figure 6: Induction of DT-diaphorase in Human Lung Tumour Cells by D3T

Human lung tumour cell lines were incubated with, or without, 100 µM D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 µl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 5-13 determinations; bars, standard error. On occasion the error bars were too small to be shown. *, $p < 0.05$.

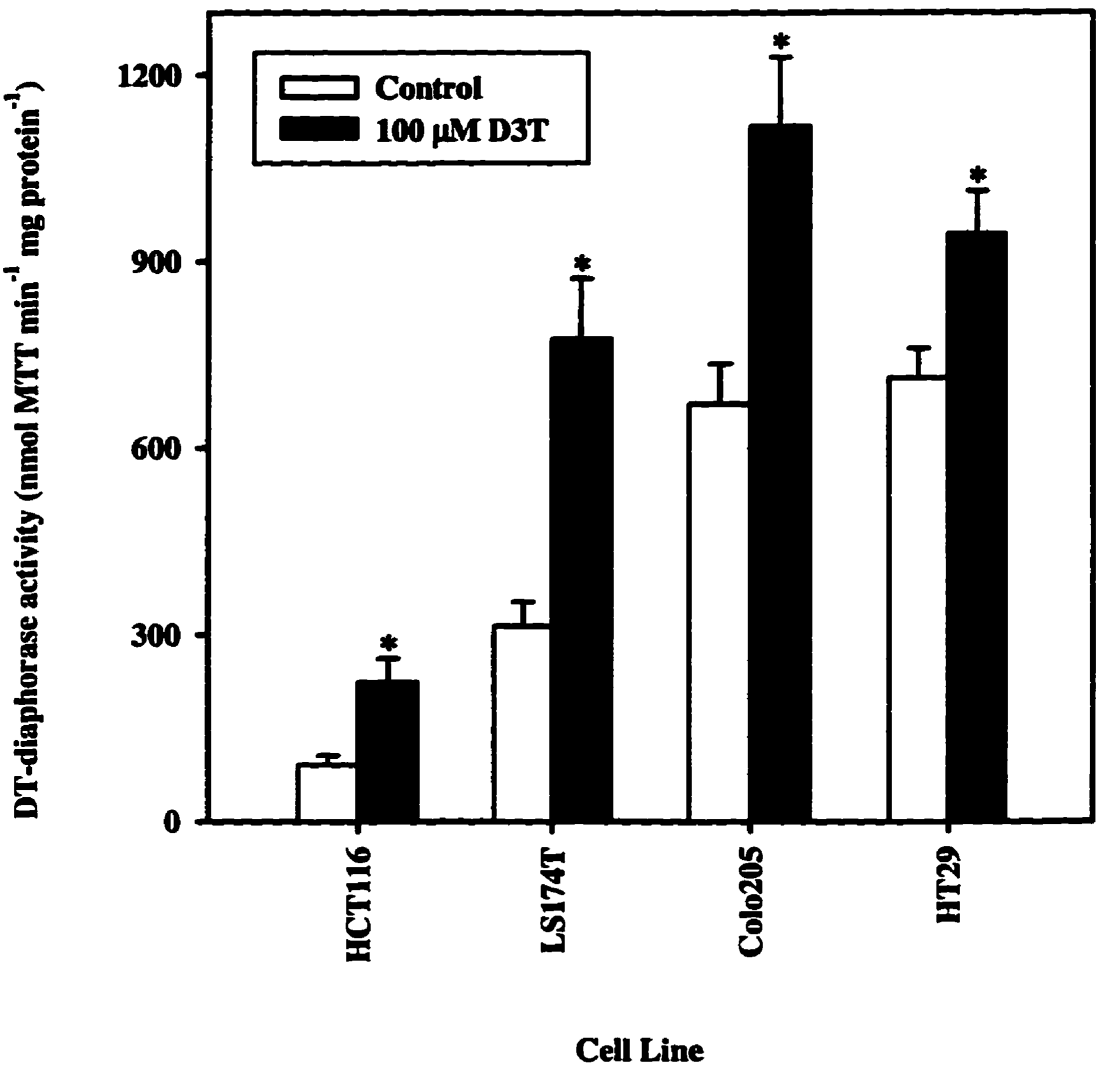


Figure 7: Induction of DT-diaphorase in Human Colon Tumour Cells by D3T

Human colon tumour cell lines were incubated with, or without, 100 µM D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 µl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 4-11 determinations; bars, standard error. *, $p < 0.05$.

Six of 9 breast carcinoma cell lines were inducible by D3T, with most of the cell lines having intermediate basal levels of DT-diaphorase being induced (Fig. 8). The hormone receptor status of the cell lines did not appear to affect the induction or lack of induction by D3T (Table 2). Both estrogen receptor (ER) positive and ER negative breast tumour cell lines were induced by D3T. Of the cell lines that were not inducible, 2 of 3 were ER negative. Both ovary tumour cell lines were induced by D3T. Two of three prostate tumour cell lines showed significant induction of DT-diaphorase activity by D3T. The hormone receptor status of the cell lines did not appear to affect the induction by D3T (Table 2). One androgen receptor (AR) positive and one AR negative prostate tumour cell line were induced by D3T. All three melanoma cell lines were inducible. All the colon and malignant melanoma cell lines showed significantly increased levels of DT-diaphorase activity after treatment with D3T despite the fact that many of these cells had very high basal levels of enzyme activity. In contrast, incubation of HL-60 cells with 100 μM D3T for 48 hr did not increase the levels of GST activity or NADPH:cytochrome P450 reductase activity (Table 3).

Induction of DT-diaphorase in Human Normal Cells

The basal level of DT-diaphorase activity in human normal bone marrow cells was very low, 1.9 ± 0.3 nmol MTT min^{-1} mg $protein^{-1}$; however, incubation with D3T resulted in a small, but significant increase in this activity to 12.7 ± 2.4 nmol MTT min^{-1} mg $protein^{-1}$ ($p < 0.005$) (Table 4). The basal level of enzyme activity in 293 human normal kidney cells was also very low, 2.8 ± 0.2 nmol MTT min^{-1} mg $protein^{-1}$, and D3T also increased DT-diaphorase activity in these cells to 7.7 ± 1.3 nmol MTT min^{-1} mg $protein^{-1}$ ($p < 0.001$). In

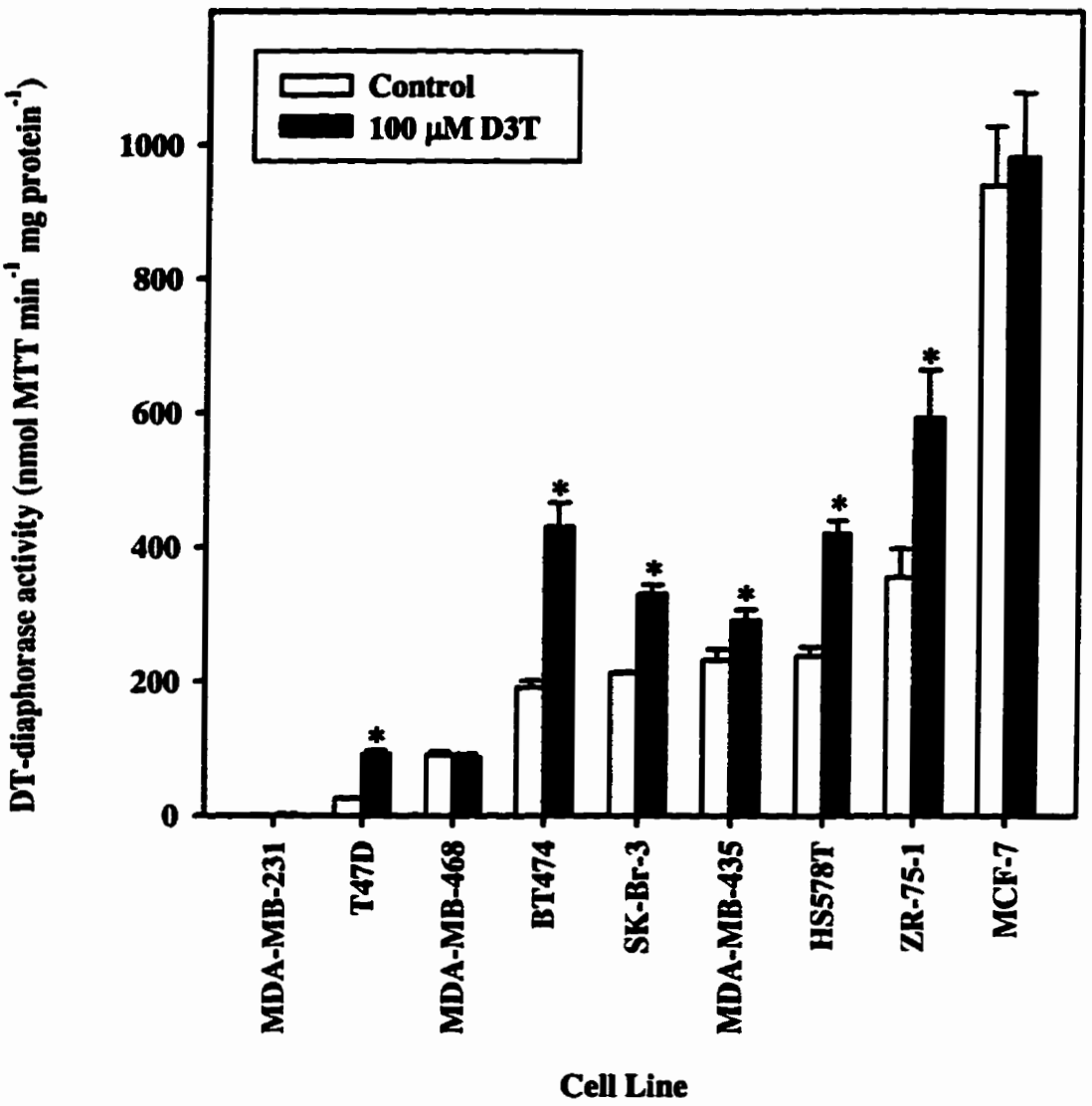


Figure 8: Induction of DT-diaphorase in Human Breast Tumour Cells by D3T

Human breast tumour cell lines were incubated with, or without, 100 µM D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 µl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 3-8 determinations; bars, standard error. On occasion the error bars were too small to be shown. *, $p < 0.05$.

Table 2: Hormone Receptor Status in Human Breast and Prostate Cell Lines

Tissue	Cell Line	Description	Hormone Receptor
Breast	MDA-MB-231	Breast Adenocarcinoma	ER Negative
	T47D	Breast Ductal Carcinoma	ER Positive
	MDA-MB-468	Breast Adenocarcinoma	ER Negative
	BT474	Breast Ductal Carcinoma	ER Positive
	SK-Br-3	Breast Adenocarcinoma	ER Negative
	MDA-MB-435	Breast Ductal Carcinoma	ER Negative
	HS578T	Breast Ductal Carcinoma	ER Negative
	ZR-75-1	Breast Carcinoma	ER Positive
	MCF-7	Breast Adenocarcinoma	ER Positive
Prostate	PC-3	Prostate Adenocarcinoma	AR Negative
	LnCAP	Prostate Adenocarcinoma	AR Positive
	DU145	Prostate Adenocarcinoma	AR Negative

Table 3:

Induction of GST and NADPH:Cytochrome P450 Reductase by D3T in HL-60 Cells

Cell Line	Enzyme	Enzyme Activity Mean ± S.E. (nmol min^{-1} mg $protein^{-1}$)		p
		Control	D3T Induced	
HL-60	GST	112.4 ± 9.5	114.5 ± 9.3	NS
	NADPH:Cytochrome P450 Reductase	7.2 ± 0.6	7.4 ± 0.5	NS

Cells were incubated with, or without, 100 µM D3T at 37°C for 48 hr. Cells were washed with PBS, pelleted, and prepared for enzyme assays using the protocols in "Materials and Methods". GST activity was measured in the supernatant of cell sonicates by a previously described procedure (Habig et al, 1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GST activity was expressed as nmol CDNB min^{-1} mg $protein^{-1}$. NADPH:cytochrome P450 reductase activity was determined in supernatants from cell sonicates using cytochrome c as the artificial electron acceptor (Strobel and Digman, 1978). NADPH:cytochrome P450 reductase activity was expressed as nmol cytochrome c min^{-1} mg $protein^{-1}$. The data represent the mean ± standard error of 4 determinations. Statistical significance was determined by a 2-tailed t-test comparing the significance of the difference of the mean enzyme activity in control and D3T treated cells. NS, not significant.

Table 4: Induction of DT-diaphorase in Human Normal Cells by D3T

Cells	DT-diaphorase Activity Mean ± S.E. (nmol MTT min^{-1} mg $protein^{-1}$)		p
	Control	D3T Induced	
Bone Marrow	1.9 ± 0.3	12.7 ± 2.4	<0.005
293 Kidney	2.8 ± 0.2	7.7 ± 0.1	<0.001
WI-38 Lung	76.5 ± 6.5	182.0 ± 12.2	<0.002

Cells were incubated with, or without, 100 µM D3T at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 µl of 0.25M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured (Prochaska and Santamaria, 1988) using menadione as the electron acceptor. The data represent the mean ± standard error of 3 or 4 determinations. Statistical significance was determined by a 2-tailed t-test comparing the significance of the difference of the mean DT-diaphorase activity in control and D3T treated cells.

contrast, WI-38 lung cells had an intermediate basal level of DT-diaphorase activity, 76.5 ± 6.5 nmol MTT min^{-1} mg $protein^{-1}$, and this was increased to 182.0 ± 12.2 nmol MTT min^{-1} mg $protein^{-1}$ ($p < 0.002$) by treatment with D3T.

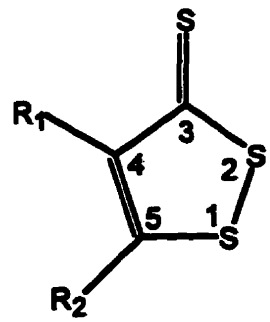
1,2-Dithiole-3-thione Analogs

The structures of the D3T analogs used in this study are shown in Figure 9. The analogs had substituents at either or both the 4- and 5- positions of the disulfide ring structure. Some of the analogs had unique features to their structure. Analog 8 had a S-oxide on the thione group. Analog 9 had a structure that corresponded to a possible opened-ring metabolite of D3T. Analog 10 was the only compound that had a salt structure.

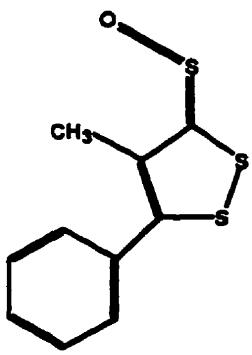
DT-diaphorase Induction by D3T Analogs

The ability of the D3T analogs to induce DT-diaphorase activity was examined in 8 cell lines from 5 tissue types: two leukemia cell lines, the HL-60 and THP-1; two lung carcinoma cell lines, NCI-H661 and NCI-H209; two colon carcinoma cell lines, LS174T and HT29; one breast cancer cell line, MCF-7, and one liver tumour cell line, HepG2. Cells were incubated with 100 μM of each D3T analog for 48 hr. The control and induced DT-diaphorase enzyme activity levels for these cell lines are summarized in Table 5.

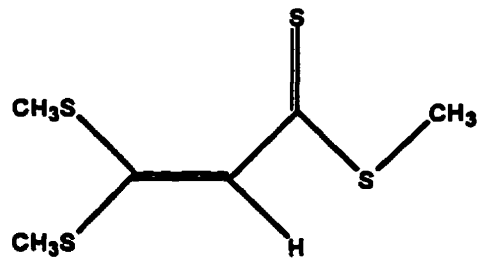
The basal levels of DT-diaphorase activity in HL-60 and THP-1 were 4.5 ± 0.5 and 26.1 ± 2.3 nmol MTT min^{-1} mg $protein^{-1}$, respectively. For the HL-60 cell line, 11 of the 13 D3T analogs significantly induced DT-diaphorase activity (Fig. 10), with the induced levels of DT-diaphorase activity ranging from 7.4 ± 0.8 to 38.4 ± 5.8 nmol MTT min^{-1} mg $protein^{-1}$.



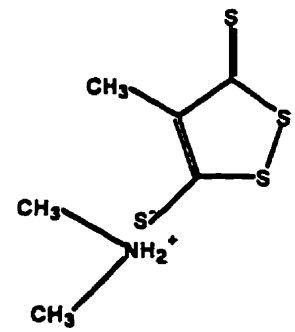
Analog	R_1	R_2
D3T	-H	-H
Oltipraz	$-CH_3$	-2-pyrazinyl
ADT	-H	-methoxyphenyl
1	$-CH_3$	-H
2	-H	$-CH_3$
3	$-CH_2CH_3$	-H
4	-H	$-CH_2CH_3$
5	-H	$-C(CH_3)_3$
6	-phenyl	-H
7	-H	$-CONH_2$



Analog 8



Analog 9



Analog 10

Figure 9: Structure of D3T Analogs

Table 5: Induction of DT-diaphorase in human tumour cell lines by D3T analogs

Cells were incubated with, or without, 100 µM of each D3T analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 200-300 µl of 0.25M sucrose, sonicated and DT-diaphorase activity was measured (Prochaska and Santamaria, 1988). The data represent the mean ± standard error of 3-13 determinations. Statistical significance was determined by a 2-tailed t-test comparing the significance of the difference of the mean DT-diaphorase activity in control and D3T analog treated cells.

INDUCER	DT-diaphorase Activity Mean ± S.E. (nmol MTT min^{-1} mg $protein^{-1}$)							
	HL-60	THP-1	NCI-H209	NCI-H661	LS174T	HT29	MCF-7	HepG2
Control	4.5 ± 0.5	26.1 ± 2.3	8.3 ± 0.8	112.7 ± 12.4	314.6 ± 39.0	713.1 ± 47.4	939.1 ± 88.3	1292.5 ± 162.0
D3T	31.6 ± 3.6*	102.8 ± 5.4*	40.2 ± 3.1*	284.8 ± 27.3*	776.0 ± 97.4*	944.6 ± 69.1*	981.3 ± 96.5	1356.3 ± 141.3
Oltipraz	17.8 ± 3.3*	57.8 ± 0.8*	10.7 ± 3.5	132.0 ± 24.9	421.7 ± 43.2	813.1 ± 82.2	N.D.	N.D.
ADT	7.6 ± 1.9*	22.3 ± 0.6	10.4 ± 4.3	158.8 ± 25.2	524.7 ± 73.2*	718.0 ± 31.6	N.D.	N.D.
Analog 1	7.7 ± 0.8*	45.3 ± 2.8*	6.2 ± 1.7	203.2 ± 35.6*	658.4 ± 48.7*	793.3 ± 72.9	904.0 ± 122.6	1174.9 ± 143.2
Analog 2	9.3 ± 0.6*	52.1 ± 4.5*	10.0 ± 0.8	171.7 ± 39.5	573.7 ± 72.4*	766.1 ± 64.9	920.7 ± 252.3	1123.2 ± 94.2
Analog 3	6.4 ± 0.6	41.6 ± 3.2*	7.3 ± 0.5	180.1 ± 41.0*	672.8 ± 55.9*	833.0 ± 69.4	657.2 ± 153.7	1069.2 ± 144.2
Analog 4	12.2 ± 1.0*	59.4 ± 5.0*	13.6 ± 2.4*	187.7 ± 33.1*	666.5 ± 49.9*	892.2 ± 80.0	1041.5 ± 154.3	1175.3 ± 156.6
Analog 5	7.5 ± 1.4*	53.9 ± 5.6*	7.7 ± 1.7	168.0 ± 26.8*	482.7 ± 29.6*	907.7 ± 78.5*	672.3 ± 219.1	1011.4 ± 76.2
Analog 6	9.9 ± 1.1*	48.7 ± 4.4*	6.9 ± 2.3	190.0 ± 35.3*	652.5 ± 90.2*	682.0 ± 91.4	638.9 ± 130.0	1272.2 ± 102.1
Analog 7	38.4 ± 5.8*	124.2 ± 11.6*	26.4 ± 2.7*	309.9 ± 58.9*	596.9 ± 40.9*	752.7 ± 98.2	805.1 ± 120.4	1048.4 ± 143.7
Analog 8	7.4 ± 0.8*	76.1 ± 2.8*	8.0 ± 3.2	205.9 ± 34.2*	640.5 ± 67.0*	1110.5 ± 87.4*	604.4 ± 217.5	1031.4 ± 107.1
Analog 9	24.2 ± 2.5*	38.4 ± 6.3*	24.3 ± 3.0*	289.9 ± 37.9*	678.8 ± 79.1*	842.4 ± 134.0	691.7 ± 225.8	1232.6 ± 95.7
Analog 10	3.9 ± 0.5	44.5 ± 3.7*	8.1 ± 1.0	129.2 ± 18.3	336.4 ± 46.6	672.9 ± 58.0	N.D.	993.7 ± 116.9

*, $p < 0.05$ compared with control N.D., Not Determined

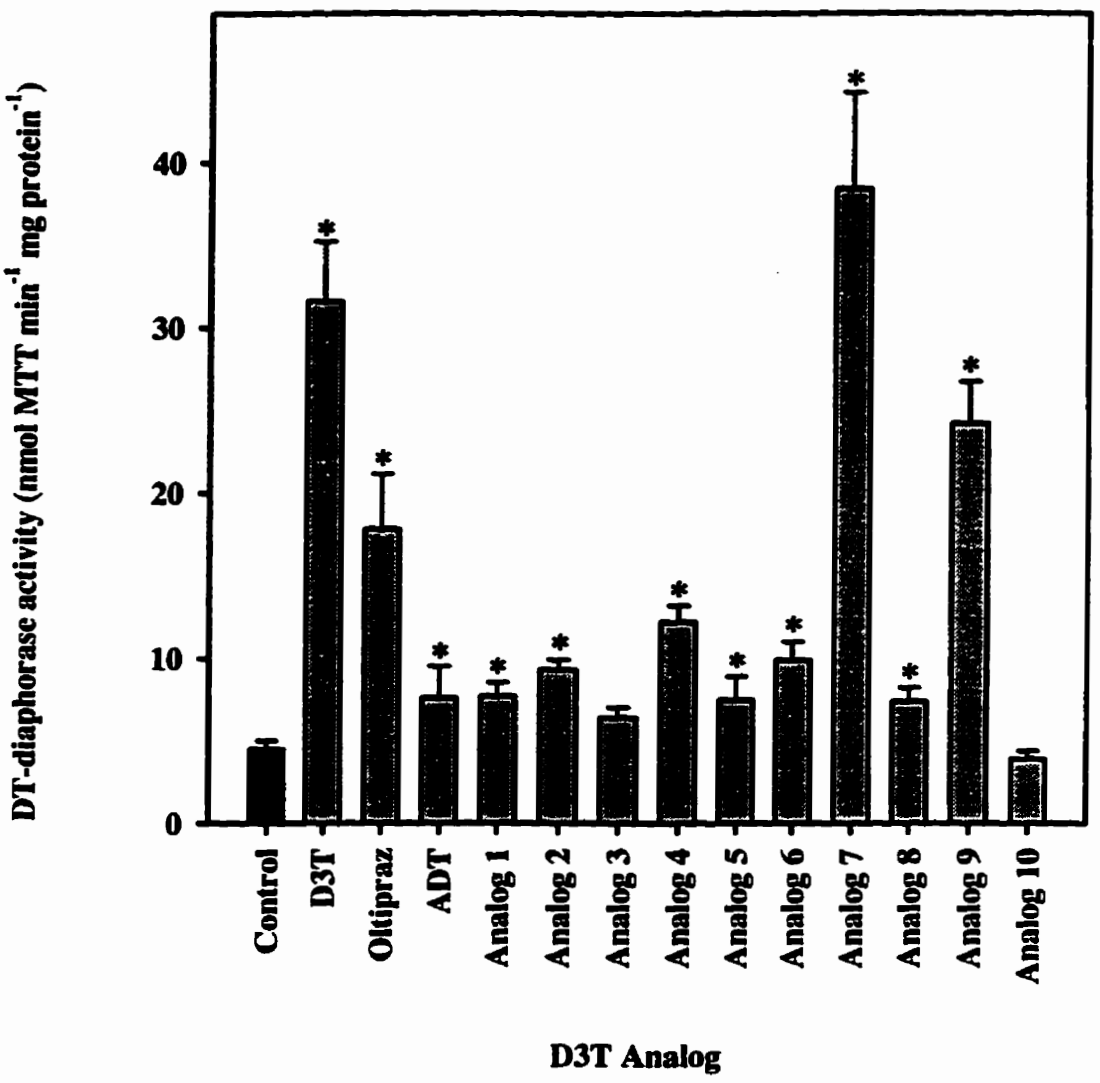


Figure 10: Induction of DT-diaphorase by D3T Analogs in HL-60 Cells

HL-60 cells were incubated with, or without, 100 μM D3T Analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 4-10 determinations; bars, standard error. *, $p < 0.05$.

The best inducers of DT-diaphorase in the HL-60 cells were Analog 7, D3T, Analog 9 and Oltipraz. In the THP-1 cell line, 12 of the 13 analogs significantly induced DT-diaphorase activity (Fig. 11). DT-diaphorase activity following induction ranged from 38.4 ± 6.3 to 124.2 ± 11.6 nmol MTT min^{-1} mg $protein^{-1}$. The best inducers in the THP-1 cells were Analog 7, D3T, and Analog 8.

The NCI-H209 and NCI-H661 cell lines had basal DT-diaphorase activities of 8.3 ± 0.8 and 112.7 ± 12.4 nmol MTT min^{-1} mg $protein^{-1}$, respectively. In the NCI-H209 cells, significant induction occurred with only 4 of the 13 analogs, D3T, Analog 7, Analog 9, and Analog 4, and the induced activity ranged from 13.6 ± 2.4 to 40.2 ± 3.1 nmol MTT min^{-1} mg $protein^{-1}$ (Fig. 12). In the NCI-H661 cell line, 9 of the 13 analogs significantly induced DT-diaphorase activity (Fig. 13). The induced enzyme levels varied from 168.0 ± 26.8 to 309.9 ± 58.9 nmol MTT min^{-1} mg $protein^{-1}$. The best DT-diaphorase inducers were Analog 7, Analog 9, and D3T.

LS174T and HT29 had control DT-diaphorase activities of 314.6 ± 39.0 and 713.1 ± 47.4 nmol MTT min^{-1} mg $protein^{-1}$, respectively. In the LS174T cell line, 11 of the 13 analogs increased DT-diaphorase activity levels significantly (Fig. 14). Induced levels varied from 482.7 ± 29.6 to 678.8 ± 79.1 nmol MTT min^{-1} mg $protein^{-1}$. The best inducers were D3T, Analog 9, Analog 3, and Analog 4. Only 3 analogs significantly induced DT-diaphorase activity in the HT29 cell line (Fig. 15). The induced enzyme levels ranged from 907.7 ± 78.5 to 1110.5 ± 87.4 nmol MTT min^{-1} mg $protein^{-1}$ for Analog 5, D3T, and Analog 8.

The basal level of enzyme activity in the MCF-7 breast carcinoma cells was 939.1

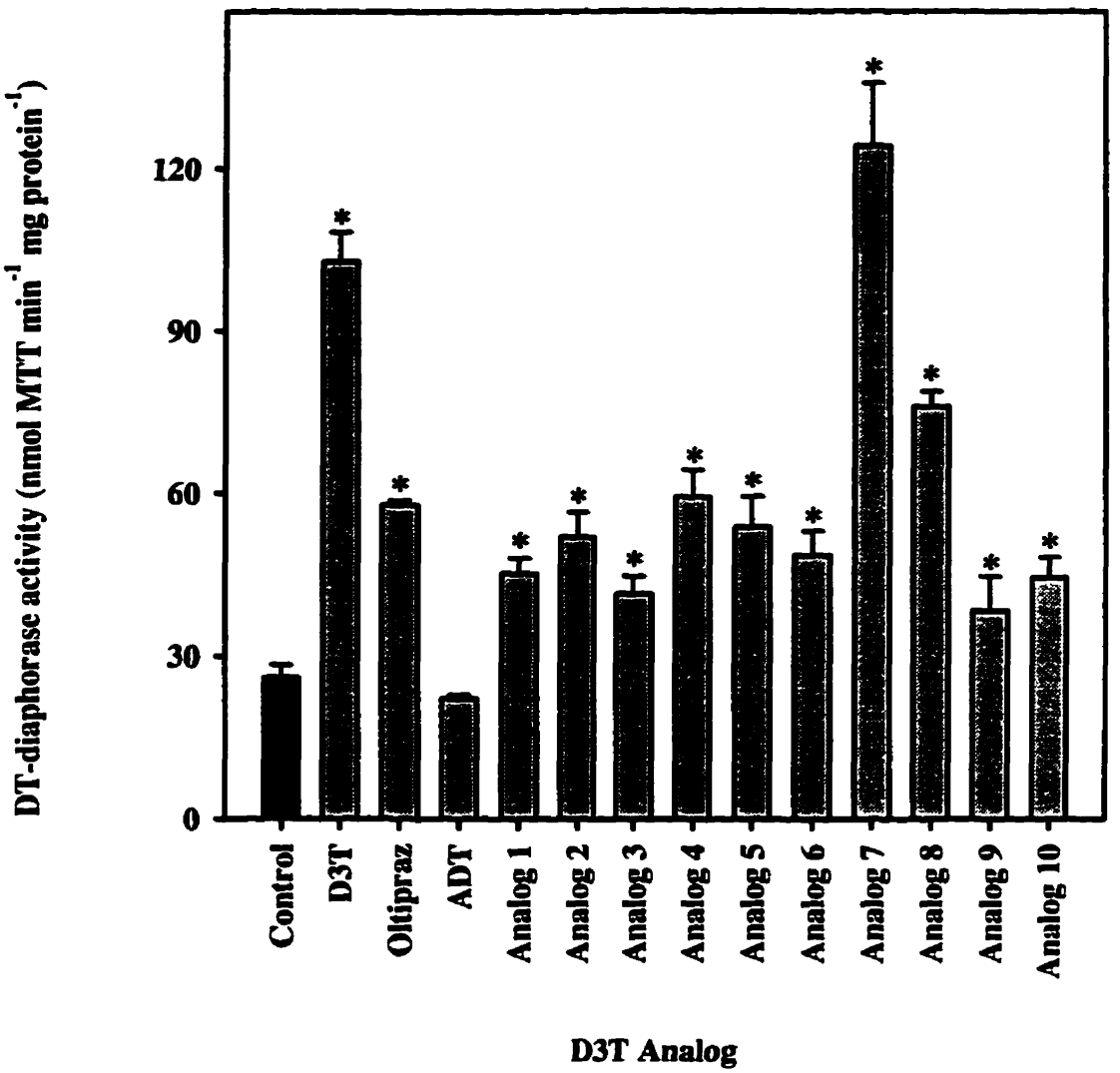


Figure 11: Induction of DT-diaphorase by D3T Analogs in THP-1 Cells

THP-1 cells were incubated with, or without, 100 μM D3T Analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 3-9 determinations; bars, standard error. *, $p < 0.05$.

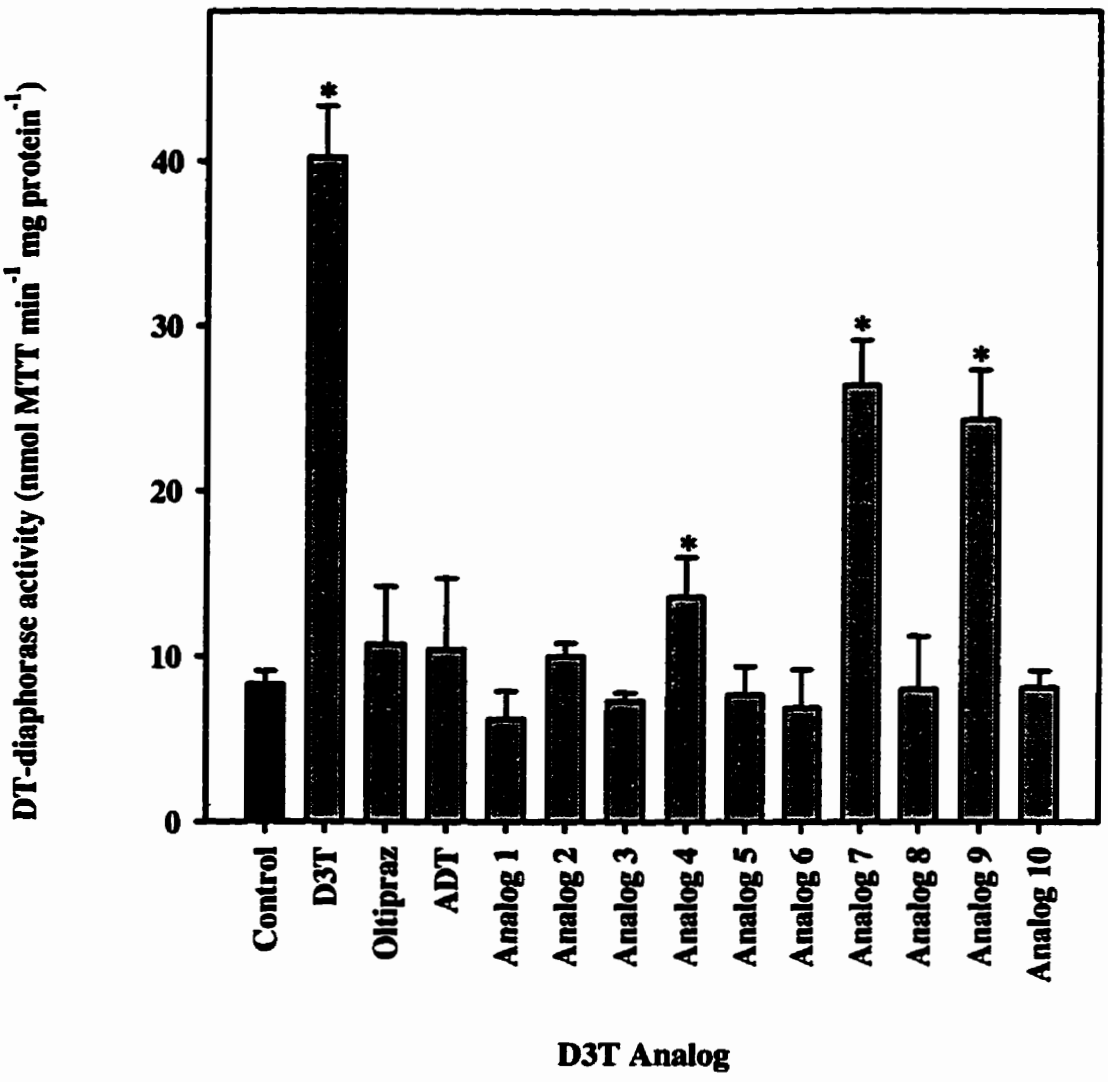


Figure 12: Induction of DT-diaphorase by D3T Analogs in NCI-H209 Cells

NCI-H209 cells were incubated with, or without, 100 μM D3T Analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 4-8 determinations; bars, standard error.
*, $p < 0.05$.

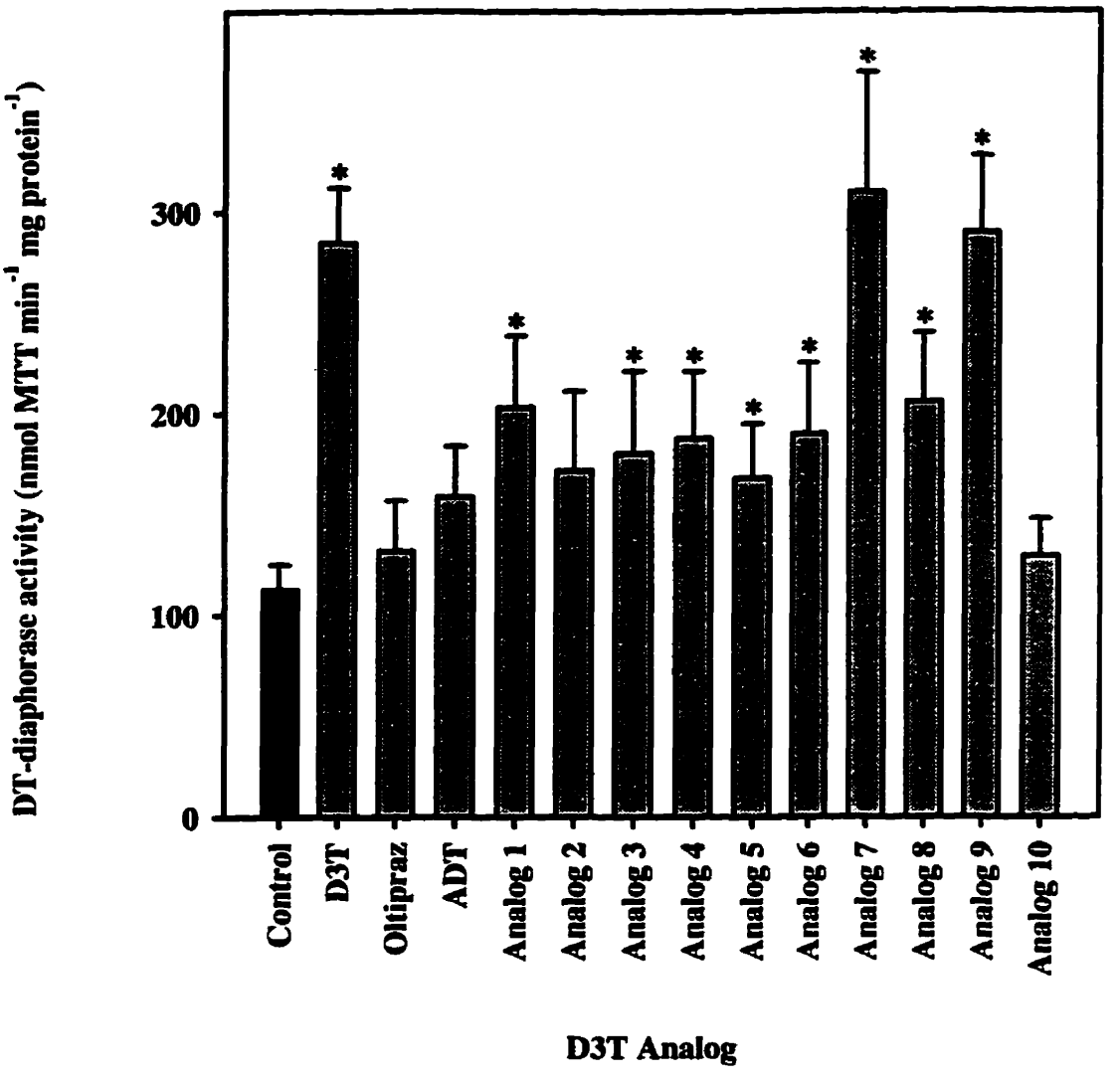


Figure 13: Induction of DT-diaphorase by D3T Analogs in NCI-H661 Cells

NCI-H661 cells were incubated with, or without, 100 μM D3T Analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 5-13 determinations; bars, standard error. *, $p < 0.05$.

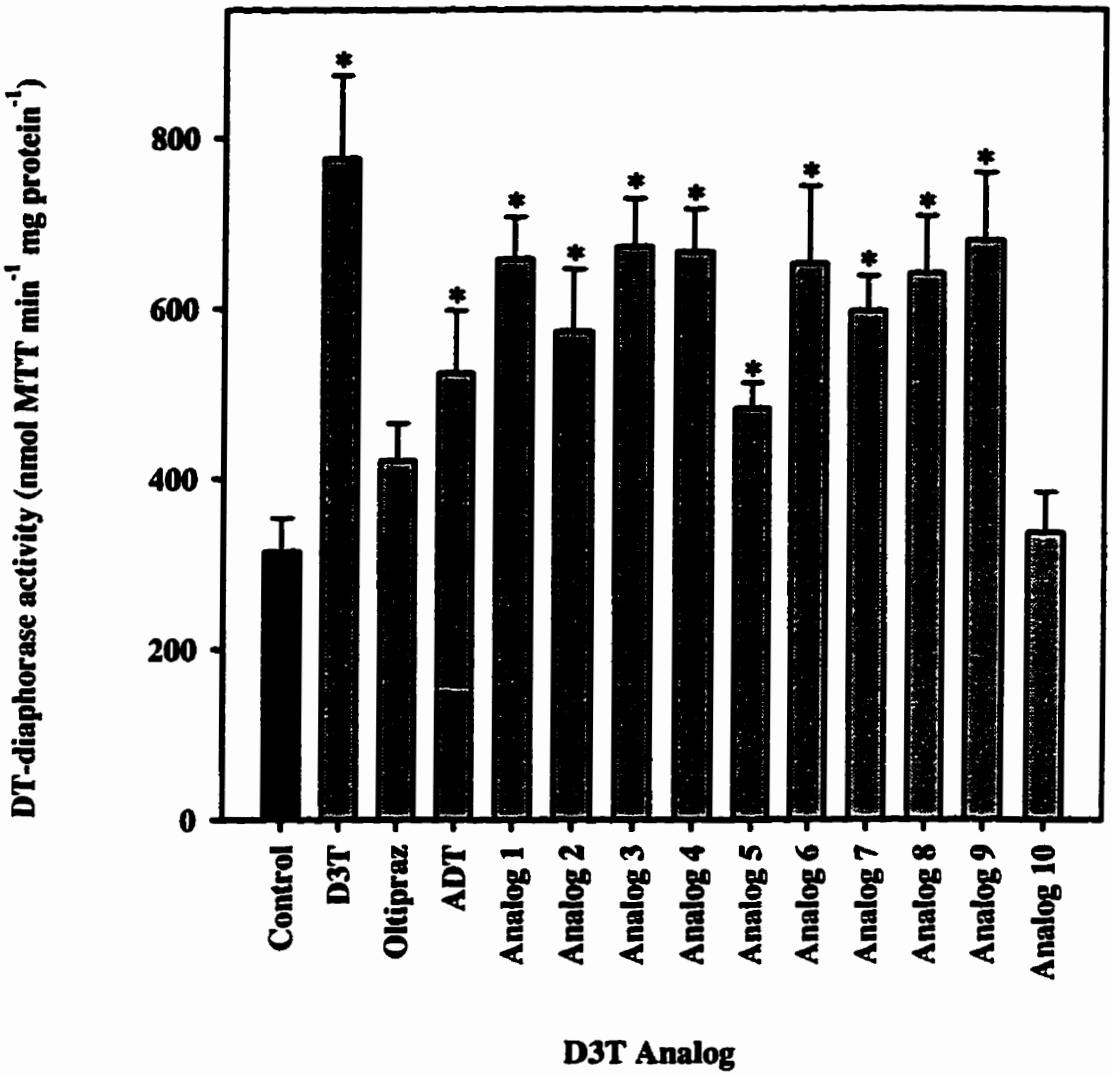


Figure 14: Induction of DT-diaphorase by D3T Analogs in LS174T Cells

LS174T cells were incubated with, or without, 100 μM D3T Analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 4 determinations; bars, standard error.
*, $p < 0.05$.

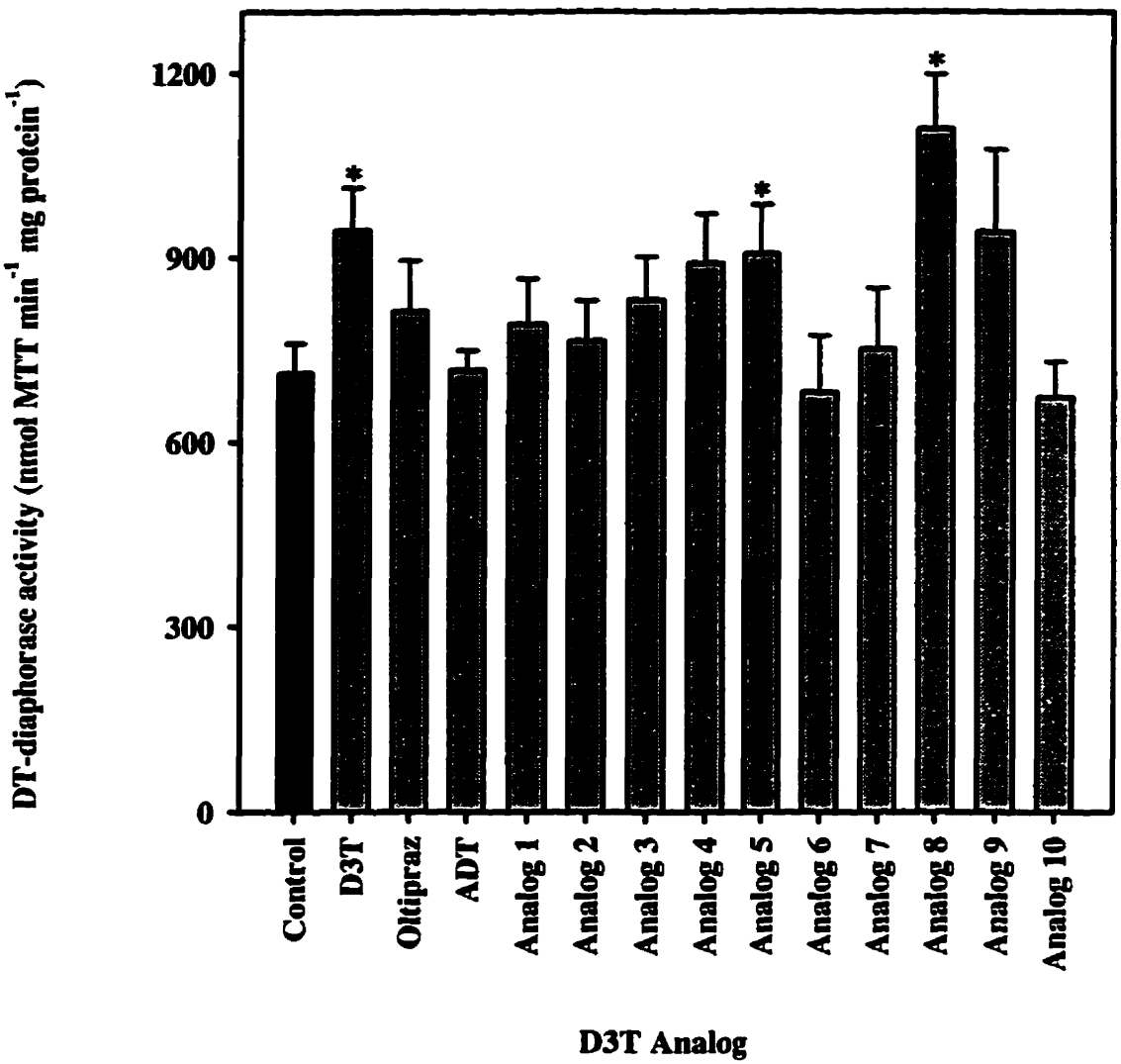


Figure 15: Induction of DT-diaphorase by D3T Analogs in HT29 Cells

HT29 cells were incubated with, or without, 100 μM D3T Analog at 37°C for 48 hr. Cells were washed, pelleted, suspended in 100-200 μl of 0.25 M sucrose, sonicated, and stored at -80°C. DT-diaphorase activity was measured using the protocol (Prochaska and Santamaria, 1988) described in "Materials and Methods". Points, mean of 5-11 determinations; bars, standard error. *, $p < 0.05$.

± 88.3 nmol MTT min^{-1} mg $protein^{-1}$ and none of the D3T analogs increased DT-diaphorase activity significantly. The control level of DT-diaphorase activity in the hepatoma cell line, HepG2, was also high with a level of 1292.5 ± 162.0 nmol MTT min^{-1} mg $protein^{-1}$, and no significant induction of enzyme activity was observed with exposure to any of the D3T analogs.

Combination Treatment with D3T Analogs and Bioreductive Antitumour Agents

Combination Treatment with EO9 and D3T in HL-60 Cells

HL-60 cells were incubated with, or without, 50 μM D3T for 48 hr and then were treated with various concentrations of EO9 for 1 hr. Cytotoxicity was determined by clonogenic assay (Fig. 16). Pretreatment with D3T increased the level of DT-diaphorase activity from 3.3 ± 0.4 to 15.6 ± 1.5 nmol MTT min^{-1} mg $protein^{-1}$ ($p < 0.05$), and also significantly increased the cell kill produced by EO9 with the D_{10} decreasing from 15.1 ± 1.7 μM to 7.7 ± 0.5 μM ($p < 0.001$). The addition of the DT-diaphorase inhibitor, dicoumarol, prior to treatment with EO9 reversed this effect ($p < 0.005$).

Combination Treatment with EO9 and D3T in 293 Human Normal Kidney Cells

The 293 human normal kidney cell line was incubated with, or without, 50 μM D3T for 48 hr and then were treated with various concentrations of EO9 for 1 hr. Cytotoxicity was determined by MTT assay (Fig. 17). The basal level of DT-diaphorase activity in these cells was 1.5 ± 0.4 nmol MTT min^{-1} mg $protein^{-1}$ and the treatment of 293 cells with 50 μM D3T did not change this activity in a statistically significant manner. The D_{10} for EO9 in the

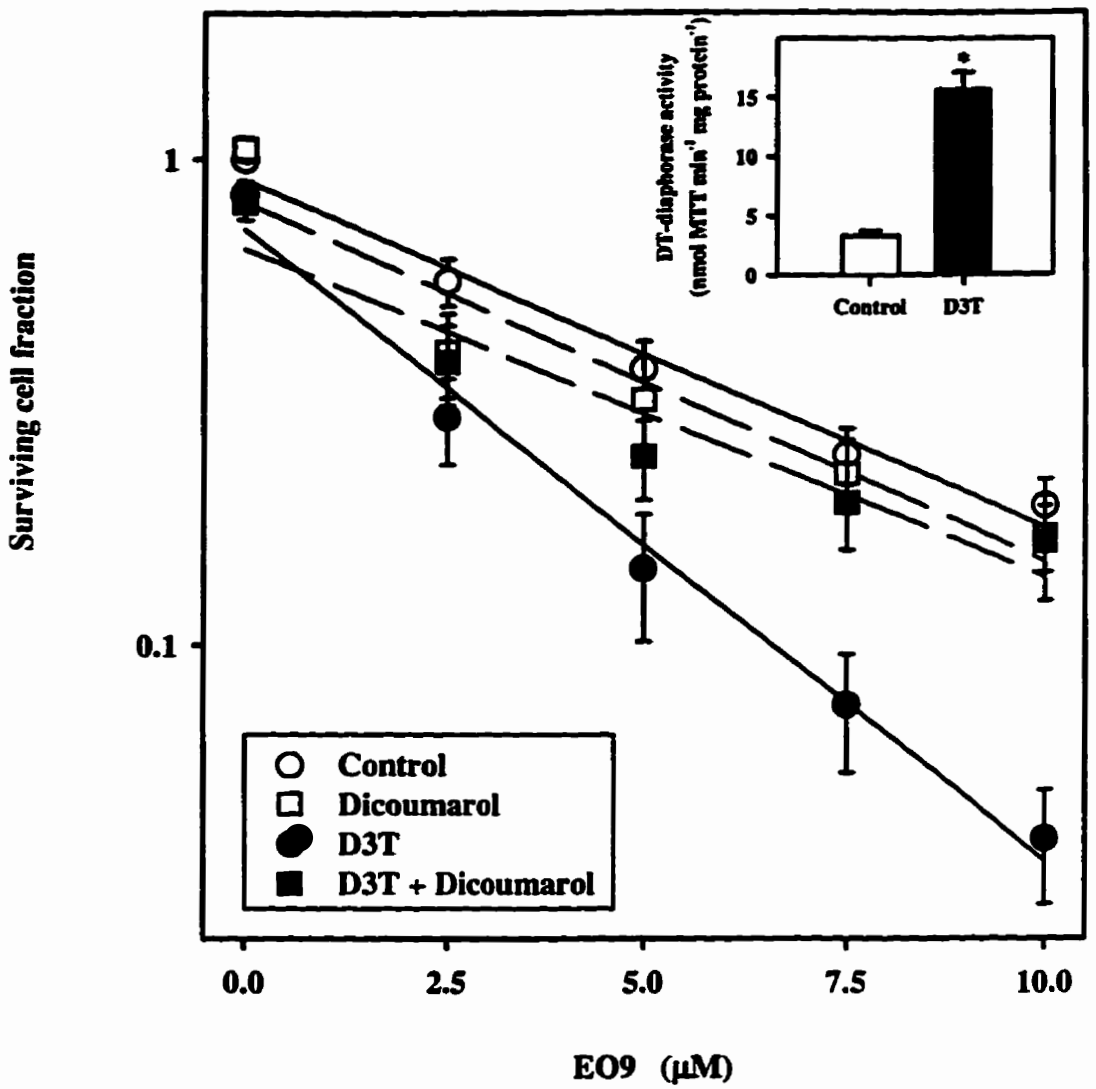


Figure 16: Combination Treatment with EO9 and D3T in HL-60 Cells

HL-60 cells were incubated at 37°C with, or without, 50 μM D3T for 48 hr. Cells were then treated with various concentrations of EO9 for 1 hr. For the experiments with dicoumarol, 50 μM dicoumarol was added 15 min before the addition of EO9. Surviving cell fraction was determined by a clonogenic assay (Begleiter et al, 1989) as described in "Materials and Methods". Points, mean of 7-13 determinations; bars, standard error; lines, linear regression lines.

Inset: Level of DT-diaphorase activity in control cells and cells treated with D3T alone. Points, means of 4-10 determinations; bars, standard error; *, $p < 0.05$.

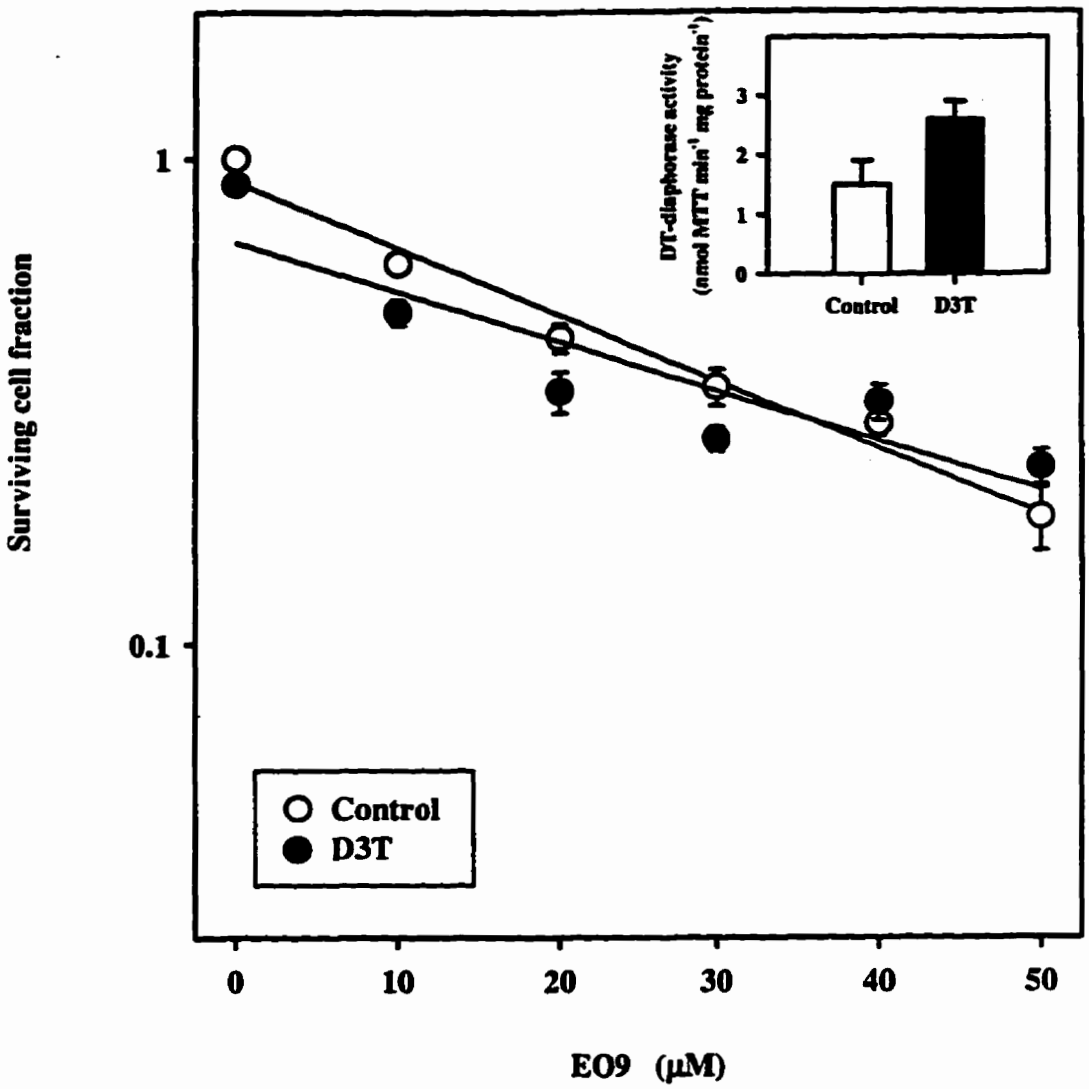


Figure 17: Combination Treatment with EO9 and D3T in 293 Cells

293 human normal kidney cells were incubated at 37°C with, or without, 50 μM D3T for 48 hr. Cells were then treated with various concentrations of EO9 for 1 hr. Surviving cell fraction was determined by MTT assay (Johnston et al, 1994) as described in "Materials and Methods". Points, mean of 5 determinations; bars, standard error; lines, linear regression lines.

Inset: Level of DT-diaphorase activity in control cells and cells treated with D3T. Points, means of 4 determinations; bars, standard error.

293 cells was 73.0 ± 5.7 μM and pretreatment with D3T had no significant effect on this activity.

Combination Treatment with EO9 and D3T Analogs in HL-60 Cells

HL-60 cells were incubated with, or without, 50 μM Oltipraz or 50 μM D3T for 48 hr and then were treated with various concentrations of EO9 for 1 hr. Cytotoxicity was determined by clonogenic assay (Fig. 18). Treatment of HL-60 cells with 50 μM Oltipraz or 50 μM D3T increased DT-diaphorase activity from 3.3 ± 0.4 to 5.8 ± 0.4 or 15.6 ± 1.5 nmol MTT min^{-1} mg $protein^{-1}$, respectively ($p < 0.05$, compared with control). Pretreatment with Oltipraz increased the cytotoxicity of EO9 in HL-60 cells with the D_{10} decreasing from 14.0 ± 1.1 μM to 10.0 ± 0.7 μM ($p < 0.02$). Pretreatment with D3T also increased the cytotoxicity of EO9 with the D_{10} decreasing from 14.0 ± 1.13 μM to 7.7 ± 0.5 μM ($p < 0.002$), and this effect was significantly greater than the increase observed with Oltipraz ($p < 0.05$).

Combination Treatment with EO9 and D3T Analogs in NCI-H661 Cells

NCI-H661 cells were incubated with, or without, 50 μM Analog 8 or 50 μM D3T for 48 hr and then were treated with various concentrations of EO9 for 1 hr. Cytotoxicity was determined by MTT assay (Fig. 19). Treatment of NCI-H661 cells with 50 μM Analog 8 or 50 μM D3T increased DT-diaphorase activity from 168.4 ± 15.6 to 226.8 ± 17.9 or 266.7 ± 43.6 nmol MTT min^{-1} mg $protein^{-1}$, respectively ($p < 0.05$, compared with control). Pretreatment with Analog 8 increased the cytotoxicity of EO9 in NCI-H661 cells with the

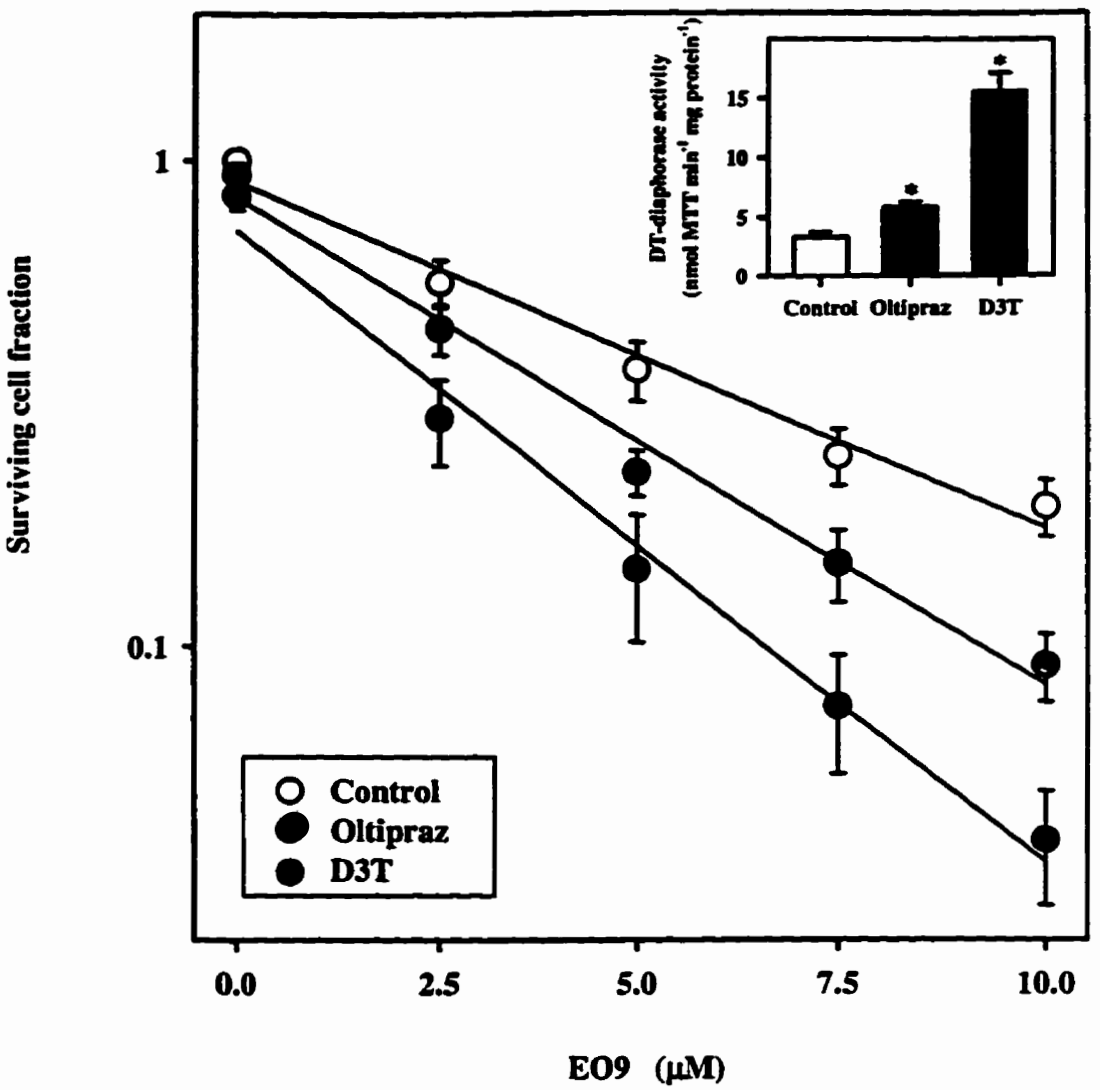


Figure 18: Combination Treatment with EO9 and D3T or Oltipraz in HL-60 Cells

HL-60 cells were incubated at 37°C with, or without, 50 μM Oltipraz or 50 μM D3T for 48 hr. Cells were then treated with various concentrations of EO9 for 1 hr. Surviving cell fraction was determined by a clonogenic assay (Begleiter et al, 1989) as described in "Materials and Methods". Points, mean of 7-13 determinations; bars, standard error; lines, linear regression lines.

Inset: Level of DT-diaphorase activity in control cells and cells treated with Oltipraz or D3T. Points, means of 4-10 determinations; bars, standard error; *, $p < 0.05$.

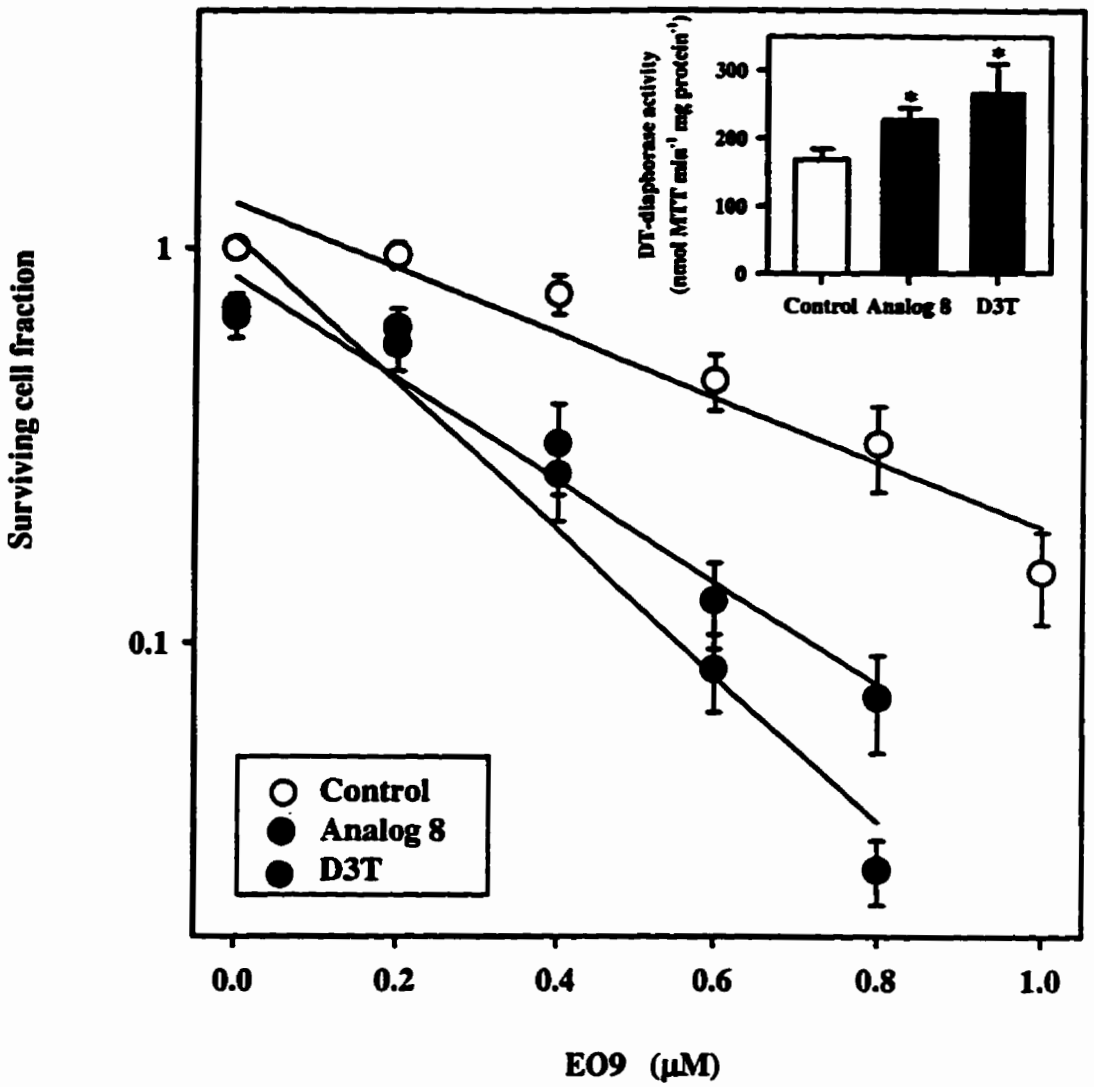


Figure 19: Combination Treatment with EO9 and D3T or Analog 8 in NCI-H661 Cells

NCI-H661 cells were incubated at 37°C with, or without, 50 μM Analog 8 or 50 μM D3T for 48 hr. Cells were then treated with various concentrations of EO9 for 1 hr. Surviving cell fraction was determined by MTT assay (Johnston et al, 1994) as described in "Materials and Methods". Points, mean of 5-7 determinations; bars, standard error; lines, linear regression lines.

Inset: Level of DT-diaphorase activity in control cells and cells treated with Analog 8 or D3T. Points, means of 4 determinations; bars, standard error; *, $p < 0.05$.

D_{10} decreasing from 1.21 ± 0.18 μM to 0.77 ± 0.09 μM ($p < 0.05$). Pretreatment with D3T also increased the cytotoxicity of EO9 with the D_{10} decreasing from 1.21 ± 0.18 μM to 0.54 ± 0.08 μM ($p < 0.005$), but this effect was not significantly greater than the increase observed with Analog 8.

Combination Treatment with MMC and D3T in HCT116 Cells

HCT116 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 (Fig. 20). Treatment of HCT116 cells with 50 μM D3T increased DT-diaphorase activity from 75.6 ± 10.1 to 174.1 ± 28.8 nmol MTT min^{-1} mg $protein^{-1}$ ($p < 0.02$). Pretreatment with D3T increased the cytotoxicity of MMC in HCT116 cells with the D_{10} decreasing from 7.6 ± 0.6 μM to 3.8 ± 0.3 μM ($p < 0.002$).

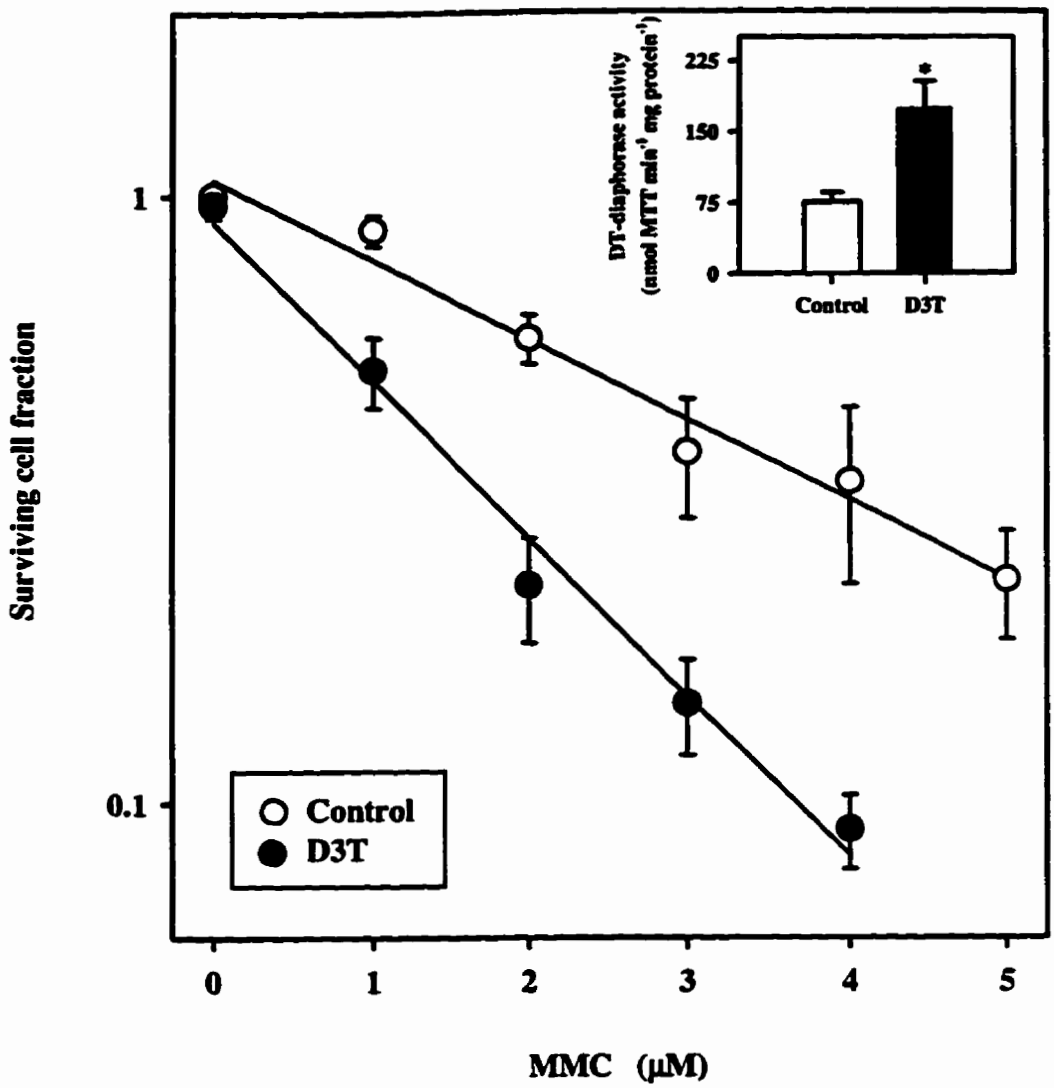


Figure 20: Combination Treatment with MMC and D3T in HCT116 Cells

HCT116 cells were incubated at 37°C with, or without, 50 μM D3T for 48 hr. Cells were then treated with various concentrations of MMC for 1 hr. Surviving cell fraction was determined by MTT assay (Johnston et al, 1994) as described in "Materials and Methods". Points, mean of 4 determinations; bars, standard error; lines, linear regression lines.

Inset: Level of DT-diaphorase activity in control cells and cells treated with D3T. Points, means of 4 determinations; bars, standard error; *, $p < 0.05$.

DISCUSSION

DT-diaphorase has been shown to play an important role in detoxifying chemically reactive metabolites in cells, thus protecting the cell from their toxic and mutagenic effects (Beyer et al, 1988; Riley and Workman, 1992). The enzyme is highly inducible by a wide variety of compounds and recent studies have investigated the use of inducers of DT-diaphorase in cancer prevention (Kelloff et al, 1990; Kensler and Helzlsouer, 1995; O'Dwyer et al, 1996b). DT-diaphorase can reduce quinone moieties and, as a result, has been shown to be an important activator of bioreductive antitumour agents like MMC and EO9 which have this functional group. MMC has limited clinical uses due to a small therapeutic index and severe side effects of which delayed bone marrow depression is dose-limiting. We have previously shown that D3T, an inducer of DT-diaphorase, can increase the level of DT-diaphorase activity in murine lymphoma cells without altering the level of enzyme activity in normal murine marrow cells (Begleiter et al, 1996). When D3T was used with MMC or EO9, an increase in the antitumour activity of the bioreductive agents was observed. This suggested that inducers of DT-diaphorase could be used to enhance the antitumour activity of bioreductive agents. The evaluation of selective inducers of DT-diaphorase in human tumours could lead to their use in improving bioreductive agent cancer chemotherapy.

In this study we found that D3T can increase the level of DT-diaphorase activity in most human tumour cell types. Enzyme induction with D3T in NCI-H661 human lung tumour cells reached a maximum at 48 hr. Induction of DT-diaphorase activity in HL-60 human leukemia cells increased with increasing D3T concentrations reaching a maximum at 100 μM of D3T (Fig. 4). The induction of DT-diaphorase by D3T in leukemia cell lines

and 5 different solid tumour tissue types was examined in these studies. In addition, Begleiter and Leith (1997) examined DT-diaphorase induction by D3T in 5 additional solid tumour tissue types. Overall, 28 of 37 tumour cell lines showed significant increases in enzyme activity following treatment with D3T that ranged from 1.3- to 7.0-fold or from 2.5 to 590 nmol MTT min^{-1} mg $protein^{-1}$ in absolute terms (Table 1). Leukemia, lung, colon, stomach, breast, ovary, prostate and melanoma tumour cell lines were all inducible. In contrast, 3 head and neck tumour and a liver tumour cell line were not induced by D3T. In addition, some tumour types appeared to be more readily induced. All the leukemia cell lines were induced (Fig. 5), as were all the colon tumour (Fig. 7), gastric tumour, ovarian tumour and malignant melanoma cell lines. However, the differences in inducibility of DT-diaphorase activity in the different cell types may simply reflect the particular cell lines of the tumour types examined in this study.

D3T did not increase the level of enzyme activity in 3 head and neck tumour cell lines, each with different basal levels of DT-diaphorase activity. This could be significant and representative for the tumour tissue but may simply reflect the particular cell lines sampled. Further head and neck tumour cell lines should be studied to determine if this lack of induction is truly tissue specific. In the liver cell line, HepG2, DT-diaphorase was not induced by D3T but its high basal level of DT-diaphorase activity may indicate that a threshold level was reached for gene expression in this particular cell line. Studies with other liver tumour cell lines may show that this tissue can have increased activity of DT-diaphorase after exposure to D3T. Indeed, numerous studies have demonstrated induction of DT-diaphorase activity in murine liver (Kensler et al, 1987; Maxuitenko et al, 1996) and other

tissues (Li et al, 1994; Maxuitenko et al, 1997) following treatment with D3T's.

There was no obvious relationship between the basal level of DT-diaphorase activity in the tumour cells and the ability of D3T to increase enzyme activity. Although 4 of the 9 tumour cell lines that were not induced by D3T had high basal levels of DT-diaphorase activity, all the melanoma tumours, and other cell lines, that had high basal levels of enzyme activity were induced. While the increase in enzyme activity relative to the basal level was low in these cells, the absolute increase in enzyme activity was very high.

The basal levels of DT-diaphorase activity were very low in the normal marrow and kidney cells we examined (Table 4). While D3T produced statistically significant increases in enzyme activity in these cells, the actual increases were only 10.8 and 4.9 nmol MTT min^{-1} mg $protein^{-1}$, respectively, which were less than half the increase observed in HL-60 leukemia cells. The basal levels of DT-diaphorase activity in the normal lung cells, 76.5 ± 6.5 nmol MTT min^{-1} mg $protein^{-1}$, was in the intermediate range of DT-diaphorase activity measured in the tumour cell lines. In contrast to the other normal cells, D3T increased the level of DT-diaphorase activity in normal human lung cells by 2.4-fold and by 105.5 nmol MTT min^{-1} mg $protein^{-1}$ in absolute terms.

The mechanisms responsible for the different basal levels of DT-diaphorase activity and different levels of induction are unknown. The lack of significant induction by D3T in some of the tumour cell lines may be due to the cells having reached an upper threshold level of NQO_1 gene expression. This might occur in cell lines like the HepG2 and MCF-7 cell lines which express high basal levels of DT-diaphorase activity. In contrast, other factors involved in NQO_1 expression may explain the lack of enzyme induction in cell lines, such

as MDA-MB-231 and FaDu, which have low or intermediate basal levels of DT-diaphorase activity. It has been shown that NQO_1 can have a critical point mutation (C→T) at position 609 in NQO_1 cDNA resulting in an amino acid change at position 187 from proline to serine (Traver et al, 1992; Eickelmann et al, 1994; Traver et al, 1997). Indeed, one of the non-inducible cell lines, NCI-H596, has been found to be homozygous for the point mutation that results in decreased levels of mRNA, no measurable protein, and almost no detectable enzyme activity (Traver et al, 1997). Traver *et al* (1997) expressed the mutated protein in *E. coli* and determined that the point mutation results in only 2% of the enzyme activity obtained with the wild-type gene product. This may explain why we did see a very slight, but significant, increase in DT-diaphorase activity in the NCI-H596. MDA-MB-231, the breast tumour cell line with very low levels of DT-diaphorase and no significant induction by D3T, may also be homozygous for the point mutation. Cell lines with no induction by D3T may have other gene expression anomalies, such as transcription factor or NQO_1 promoter region mutations or deletions. Alternatively, an exon 4 deleted splice variant mRNA that results in no protein activity has been reported (Gasdaska et al, 1995; Pan et al, 1995; Yao et al, 1996). Other possible explanations for the lack of induction of DT-diaphorase by D3T may be differences in micro-envioronment conditions, such as intracellular pH or oxygen tension, that may affect the response of DT-diaphorase activity to an exposure to D3T. Additional studies are underway in our laboratory to study these possible mechanisms for the lack of induction of DT-diaphorase activity by D3T.

Eight human tumour cell lines from 5 different tissues were examined to compare the ability of D3T and 12 of its analogs to induce DT-diaphorase and to identify possible

structure-activity relationships (Table 5). D3T was used in the induction of DT-diaphorase because it has been shown to be a good inducer of this enzyme in other systems (Ansher et al, 1986; Kensler et al, 1987; Twerdok et al, 1992; Begleiter et al, 1996). The analogs were synthesized by Tom Curphey and were selected on the basis of induction of DT-diaphorase activity in Hepa1c1c7 cells *in vitro* and enzyme induction in normal mouse liver *in vivo*. Oltipraz and ADT were selected due to their clinical use and their extensive study in the field of chemoprevention (Warnet et al, 1989; Kelloff et al, 1990; Clapper et al, 1995; Arif et al, 1997). We initially found that a leukemia cell line, HL-60, a lung tumour cell line, NCI-H661, and a colon tumour cell line, HT29, were induced by the D3T analogs. Two cell lines, the MCF-7 breast tumour and the HepG2 hepatoma, were not induced by D3T analogs. An additional cell line for each of the leukemia, lung tumour, and colon tumour tissue types was examined for induction of DT-diaphorase activity by the D3T analogs to determine any tissue specificity for enzyme induction by the D3T analogs and to study structure-activity relationships for the D3T analogs. The results of these experiments demonstrate that D3T analogs are generally good inducers of DT-diaphorase enzyme activity in human tumour cells.

D3T and Analog 7 were the best inducers in the two leukemia cell lines, while D3T, Analog 7 and Analog 9 were the best inducers in the lung tumour cell lines. The only common inducers in the two colon carcinoma cell lines were D3T, Analog 5, and Analog 8. None of the D3T analogs induced DT-diaphorase activity in the breast or liver tumour cell lines. The lack of induction by D3T analogs in the MCF-7 breast tumour cell line was not representative of the tumour tissue type. Other breast tumour cell lines were induced by D3T

and the study of induction by the D3T analogs in an inducible breast cancer cell line could possibly show tissue specific induction. Thus, in the examined cell lines, the D3T analogs did not appear to show any obvious tissue specificity for enzyme induction.

Some of the D3T analogs were better inducers of DT-diaphorase than others in the human tumour cell lines examined, and this may indicate possible structure-activity relationships. The parent compound, D3T, was the most consistent inducer, producing significant increases in enzyme activity in 6 of the 8 cell lines. Analogs 4, 5, 7, 8, and 9 were also consistent inducers of DT-diaphorase activity, producing significant increases in enzyme activity in 5 cell lines. Of these, D3T and Analogs 7, 8, and 9 generally produced the largest increases in DT-diaphorase activity. In contrast, Analog 10 was the poorest inducer in the cell lines studied.

These results indicate that the parent D3T may be the best inducer of DT-diaphorase activity of the analogs studied. However, the carbamoyl group in Analog 7, the S-oxide in Analog 8, and the ring-opened structure in Analog 9, in some instances, may produce greater increases in DT-diaphorase activity than D3T. These studies do not indicate any obvious difference in induction capacity between analogs with substituents at the 4' or 5' position of the D3T ring. However, the salt structure in Analog 10 may reduce induction capacity, possibly as a result of decreased cellular uptake. In some of the examined cell lines, other D3T analogs seemed to be better inducers of DT-diaphorase activity than D3T, such as Analog 7 in the THP-1 cells and Analog 8 in the HT29 cells, although these observations were not statistically significant. This indicates that certain chemical features must be shared by inducers of DT-diaphorase and that some substitutions to the D3T ring may increase D3T

induction capacity.

Talalay (1989) reviewed the structural requirements for induction of DT-diaphorase by monofunctional inducers and postulated that the inducers are all Michael reaction acceptors characterized by olefinic or acetylenic linkages that are rendered electrophilic by conjugation with electron-withdrawing groups. Prestera *et al* (1993) also surveyed a list of DT-diaphorase inducers, including D3T, and the only apparent universal property was their capacity for reaction with sulfhydryls by either oxidoreduction or alkylation. Structural modifications of D3T may, therefore, alter the electrophilic character of the analogs or their ability to react with sulfhydryls.

Egner *et al* (1994) compared induction of DT-diaphorase in Hepa1c1c7, mouse hepatoma cells, by 25 D3T analogs. This group of analogs included D3T, Oltipraz, Analogs 1-6, and Analog 8, which we also examined in our study. Consistent with our findings, D3T was one of the more potent DT-diaphorase inducers in the Hepa 1c1c7 cells. In addition, the 5-ethyl analog (Analog 4) and the S-oxide analog (Analog 8) were relatively potent inducers in both studies. In contrast, the 4-ethyl analog (Analog 3), the 4-phenyl analog (Analog 6) and the 5-*t*-butyl analog (Analog 5) were good inducers in Hepa 1c1c7 cells but poor inducers in the human tumour cell lines. Furthermore, the ring-opened analog (Analog 9) was a good inducer of DT-diaphorase in human tumours, but a ring-opened analog of Oltipraz was inactive in the mouse hepatoma cells. Comparison of the results of these two studies indicate both similarities and differences in induction of DT-diaphorase by D3T analogs in human and mouse tumour cells. Whether these relate to species differences or differences in tumour type is unknown.

In a recent abstract publication by Maxuitenko *et al* (1997), rats were treated with a number of the D3T analogs synthesized by Tom Curphey to measure the chemoprotective effects of these compounds. Rats were fed 0.3 mmol/kg body weight, 3 times per week, and compounds were selected based on their ability to induce phase II detoxifying enzymes, including DT-diaphorase, in a number of tissues *in vivo*. Of 17 compounds tested, D3T was the best analog for the prevention of AFB_1-induced hepatotoxicity in rats, followed by 4-ethyl D3T (Analog 3), 4-carbamoyl D3T, and cyclopentano D3T.

These studies showed that D3T analogs are potent inducers of DT-diaphorase activity in normal and tumour tissues. The structure of the analog is important for good induction of the enzyme. Modifications to the basic D3T structure, such as the 5-carbamoyl substituent, the S-oxide function, or opening of the dithiolethione ring results in compounds that have high enzyme inductive capacity. However, other modifications, such as the salt form, can severely decrease inductive capacity. Additional structure-activity studies are required in normal human tissues to identify D3T analogs that produce the optimum selective increase in DT-diaphorase activity in tumour cells compared with normal cells. Combining one or more of the structural features of Analogs 7, 8, and 9 may allow for increased induction of DT-diaphorase activity. In addition, further studies of D3T analogs may determine if other substitutions at the 4' or 5' position of the D3T ring selectively affect the capacity of the analog to induce DT-diaphorase activity.

The mechanism by which D3T causes an increase in DT-diaphorase activity is still unknown. In addition, the process by which D3T enters the cell is also unknown. There has been evidence of metabolism of Oltipraz in cells and the metabolism itself may mediate the

signal to induce DT-diaphorase activity (Egner et al, 1994). Due to the fact that D3T was the best overall inducer and that the next best analogs, Analog 7, Analog 8, and Analog 9, had very dissimilar structures, we can conclude that metabolism or interaction of the D3T analogs with various cellular components is critical to the induction of DT-diaphorase activity. The regulatory sequences of the NQO_1 gene are being clarified and NF-κB (Yao et al, 1995; Yao et al, 1997) and AP-1 (Bergelson et al, 1994; Yao et al, 1994; Yao et al, 1997) binding sites seem to be critical for induction of DT-diaphorase.

O'Dwyer *et al* (1994) reported that there was an increase in NQO_1 message stability after exposure of HT29, human colon carcinoma cells, to hypoxia. The 1.2 kb mRNA had a control half-life of 1 hr and after exposure to hypoxia they observed increased transcription and an increase of the message half-life. We attempted to verify these results in the HT29 cells. Begleiter *et al* (1997) used similar experimental conditions with induction by D3T. Several repeats of these experiments yielded confusing results. We could identify both the 1.2 and 2.7 kb transcripts of NQO_1 and each mRNA appeared to undergo increased transcription. However, the control message half-life for each transcript was approximately 24 hr and this fact did not allow us to observe any changes in message stability. The very long half-life raised questions on how to control for sample quantity loading and other experimental variables. These results and the difficulties in methodology caused us to delay further experiments into the mechanism of induction by D3T.

The selective induction of DT-diaphorase in tumour cells by D3T analogs offers an opportunity to increase the effectiveness of bioreductive antitumour agents without increasing the toxicity of these agents. MMC is used clinically in the treatment of

carcinomas of the breast (Hortobagyi, 1993; Hardman and Limbird, 1996), head and neck (Coia, 1993; Hardman and Limbird, 1996), gastrointestinal system (Schnall and Macdonald, 1993; Gillis, 1996; Hardman and Limbird, 1996), cervix (Hardman and Limbird, 1996), bladder (Hardman and Limbird, 1996), and lung (Folman, 1993; Hardman and Limbird, 1996), but its severe toxicities greatly limit its use and other drugs are typically used as primary treatment in these diseases (Gillis, 1996; Hardman and Limbird, 1996). However, the preferential toxicity of MMC towards hypoxic cells is a feature possessed by no other class of antitumour drugs and this has maintained clinical interest in its possible applications in combination with radiation (Fischer, 1994). MMC, along with doxorubicin, is one of the best single agents in the treatment for breast cancer (Hortobagyi, 1993). A protocol that increases the therapeutic index of MMC or other bioreductive agents would offer another potential regimen in the treatment of cancer. The increased therapeutic index of such agents could increase the possible applications and clinical situations in which these agents could be used. The determination of the structural requirements for DT-diaphorase induction by D3T analogs could lead to the development of more potent and selective D3T compounds that would be useful as enhancers of chemotherapeutic agents in the clinic.

In order to determine if induction of DT-diaphorase activity by D3T analogs in human tumours would enhance antitumour activity of bioreductive agents, a number of human tumour cell lines were evaluated *in vitro*. Combination treatment with D3T and EO9 increased the antitumour activity of EO9 in HL-60, human promyelocytic leukemia cells (Fig. 16). D3T increased the level of DT-diaphorase activity in these cells by 5-fold ($p < 0.05$) and also increased the cytotoxic activity of EO9 by 2-fold in these cells ($p < 0.001$).

The increased cytotoxic activity was due to the elevated DT-diaphorase activity, as dicoumarol, an inhibitor of the enzyme, reversed the effect of D3T ($p< 0.005$). Dicoumarol is the strongest known inhibitor of DT-diaphorase and the inhibition is competitive with respect to the electron donors NADH or NADPH with the K_i values in the range of 10^{-8} to 10^{-10} M (Lind et al, 1990). Treatment of HL-60 cells with dicoumarol alone did not decrease the cytotoxicity of EO9 to these cells and this may be due to the low basal levels of DT-diaphorase activity in the these cells.

This study also showed that pretreatment of HL-60 cells with Oltipraz significantly increased DT-diaphorase activity by 1.8-fold ($p < 0.05$) and the cytotoxicity produced by the bioreductive agent, EO9, increased by 1.4-fold in these cells ($p < 0.02$) (Fig. 18). Similarly, the pretreatment of a solid tumour cell line, NCI-H661, human large cell lung carcinoma cells, with D3T or Analog 8 increased DT-diaphorase activity in these cells by 1.6- and 1.3-fold, respectively ($p < 0.05$), and this resulted in corresponding increases in EO9 cell kill of 2.2- ($p < 0.005$) and 1.6-fold ($p < 0.05$), respectively (Fig. 19). In both cell lines, the enhancement of EO9 cytotoxicity by the D3T analogs parallelled their effect on induction of DT-diaphorase. Thus, other D3T analogs that produce greater induction of DT-diaphorase activity may further enhance the cytotoxic activity of bioreductive agents.

Although the phase II clinical trials with EO9 have proven to be very disappointing and will potentially prevent further testing of EO9 as a clinical agent, the combination treatments with EO9 and D3T described in these studies are clinically relevant. Other groups have proposed the targeting of particular antitumour agents to specific tumour types depending on the levels of activating and deactivating enzymes, as suggested by the

"enzyme-directed" approach to bioreductive drug development (Robertson et al, 1994; Beall et al, 1995; Fitzsimmons et al, 1996). Agents that were activated preferentially by DT-diaphorase, such as MeDZQ, would benefit clinically by a protocol that selectively enhanced DT-diaphorase activity in tumours.

Similar studies were carried out with D3T and MMC to determine if D3T could enhance the tumour cell kill by this clinically used bioreductive antitumour drug. D3T increased the level of DT-diaphorase activity in HCT116, human colon carcinoma cells, by 2.3-fold ($p < 0.02$) and also increased the cytotoxic activity of MMC by 2-fold in these cells ($p < 0.002$) (Fig. 20). Similarly, Begleiter and Leith (1997) have shown that D3T pretreatment could also increase MMC cytotoxicity in two human breast tumour cell lines. D3T increased the level of DT-diaphorase activity in T47D, human breast tumour cells, by 4-fold ($p < 0.001$) and also increased the cytotoxic activity of MMC by 3-fold in these cells ($p < 0.001$). D3T increased the level of DT-diaphorase activity in HS578T, human breast carcinoma cells, by 1.8-fold ($p < 0.001$) and increased MMC cell kill by 1.4-fold in these cells ($p < 0.02$).

It is difficult to directly compare the results of the cytotoxicity assays obtained in these studies in different cell lines. Each cell line had different culture conditions and properties that resulted in the use of both clonogenic and MTT cytotoxicity evaluation assays. These studies were intended to examine any changes in cytotoxicity with, or without, D3T pretreatment and found that D3T pretreatment increased the cytotoxicity of bioreductive agents, MMC or EO9, in HL-60, NCI-H661, HCT116, T47D, and HS578T cell lines. However, it is not possible to directly compare the D_{10} values for the same bioreductive

agents between cell lines due to variations in the cells' cloning efficiency, other drug-metabolizing enzyme activities, state of DNA repair, and cell cycling times. Similarly, the comparison of different drugs in the same cell line is difficult to control for the complex variations in the mechanism of activation and detoxification for each bioreductive agent. A correlation between the increases in DT-diaphorase activity and bioreductive agent cytotoxicity could not be observed with the data collected in this study due to the small number of completed experiments, but with further observations in different cell lines and tumour types such a correlation may be calculated.

D3T produced significant increases in the levels of DT-diaphorase activity in normal human marrow and kidney cells. These tissues represent the major toxicities observed with MMC and EO9, respectively (Hortobagyi, 1993; Schellens et al, 1995). Although the increased levels of DT-diaphorase activity by D3T were small in the normal human bone marrow and 293 human normal kidney cells, combination treatment of bioreductive agents and D3T may increase the toxicity observed in these normal tissues. Although we were not able to evaluate the effect of combination treatment on MMC cytotoxicity in human bone marrow cells, Begleiter *et al* (1996) have previously shown that D3T pretreatment increased MMC cytotoxicity by 2-fold towards L5178Y murine lymphoma cells but had very little effect on normal DBA/2 mouse bone marrow cells. Sufficient quantities of human bone marrow were difficult to acquire on a regular basis for clonogenic assays in order to evaluate any enhanced MMC cytotoxicity. For this reason, the 293 normal kidney cell line was used for MTT assay to evaluate EO9 cytotoxicity. The DT-diaphorase activity was not significantly increased by 50 μM D3T and there was correspondingly no change in EO9 cell

kill (Fig. 17). This contrasts with the 1.8- and 2.2- fold increases in EO9 cytotoxicity produced by 50 μM D3T in HL-60, human promyelocytic leukemia cells, and NCI-H661, human large cell lung carcinoma cells, respectively. These results indicate that the combination treatment increased the therapeutic index of EO9 by 2-3 fold in this *in vitro* model.

However, such conclusions made from the use of the 293 cell line as normal kidney cells are not immune from criticism. The 293 cell line is a permanent line of primary human embryonal kidney cells transformed with sheared human adenovirus type 5, or Ad5, DNA (Aiello et al, 1979). The 293 cells express Ad5 early region 1 functions that confer the adenovirus' ability to transform cells and this region of adenoviral DNA was used with human embryonic kidney cells to establish the 293 cell line. This early region 1 contains the transcriptional units for the E1A and E1B proteins (Schmitz et al, 1995). The E1A genes produce two major spliced mRNAs 13S and 12S. The E1B transcriptional unit encodes two major proteins, 19 kDa and 55 kDa or 19K and 55K.

NF-κB activity is activated by E1A 13S but E1B 19K can prevent this activation and inhibit NF-κB activity (Schmitz et al, 1995). However, it has been found that NF-κB is only transiently inhibited by E1B 19K because the continuous stimulatory action of E1A 13S can finally override the antagonistic effects of 19K on NF-κB activity (Limbourg et al, 1996). The permanent integration of the Ad5 early region 1 into the 293 genome may allow the transcriptional activity of NF-κB to recover from E1B 19K inhibition; therefore, the induction of DT-diaphorase by D3T, which can be mediated by NF-κB binding to the promoter region of NQO_1, observed in the 293 cells may still be indicative of normal kidney

cells.

E1A 13S also transforms cells and is a potent initiator of apoptosis and this cell death is inhibited by both the E1B 19K (Boulakia et al, 1996; Chen et al, 1996; Han et al, 1996) and E1B 55K proteins (Schmitz et al, 1995; Grand et al, 1996). These anti-apoptotic activities of the E1B gene products may have directly affected the observations made in the combination treatment of EO9 and D3T in the 293 cells. The presence of the early region 1 adenovirus proteins likely affected the toxicity of EO9 which is mediated by DNA damage triggering apoptosis. However, the 293 cells in this study were sensitive to a 50 μM dose of EO9 and it can be concluded that the blockage of apoptosis was not complete. But it is possible that the E1B gene products may have affected the cytotoxicity resulting from combination treatment with EO9 and D3T. The 293 cell line was the only normal kidney line available for our experiments and, ultimately, the ability of D3T to enhance cancer chemotherapy with bioreductive agents will have to be further studied *in vivo* in a relevant mouse model.

In contrast to the normal marrow and kidney cells, the increase in enzyme activity in the normal human lung cells was considerably larger. MMC has been shown to produce pulmonary fibrosis in approximately 5% of patients (Klein and Wilds, 1983) by a mechanism that likely involves redox cycling and the formation of reactive oxygen species. The increase in DT-diaphorase activity with D3T treatment may serve to decrease this lung toxicity as two-electron reduction of MMC to its hydroquinone form by DT-diaphorase would decrease reactive oxygen species formation. Thus, the clinical use of combination treatment with MMC and D3T may actually decrease the incidence of pulmonary toxicity in patients.

Increasing the cell kill obtained with MMC may be of use in the increasing treatment of cancer by high dose chemotherapy. In laboratory models of cancer chemotherapy, there is a direct relationship between drug dose and cytotoxicity, suggesting that some component of tumour drug resistance must be relative rather than absolute (Vahdat et al, 1995). Clinically, dose intensification therapy appears to offer a progression-free survival advantage over standard therapy in breast cancer patients (Fields et al, 1995) and other tumour types (Vose et al, 1993; Fields et al, 1995). Although MMC is not presently one of the drugs is these clinical regimens, an improved therapeutic index for MMC achieved with D3T pretreatment may allow this bioreductive agent to be combined with other drugs in dose intensification strategies for more effective cancer chemotherapy.

DT-diaphorase was shown in this study to be the most important bioreductive activating enzyme increased by D3T pretreatment. Two pieces of experimental evidence support this conclusion. Firstly, dicoumarol reversed the effect of D3T pretreatment on EO9 cytotoxicity observed in the HL-60 cells. Secondly, the increases in EO9 cell kill in the HL-60 and NCI-H661 cells parallelled the increases in DT-diaphorase activity by the D3T analogs. However, other activating enzymes could possibly be involved in the activation of bioreductive agents and affected by D3T pretreatment, such as NADPH:cytochrome P450 reductase, xanthine dehydrogenase and NADH:cytochrome b_5 reductase. We measured no changes in NADPH cytochrome P450 reductase activity in the HL-60 cells with, or without, D3T. Begleiter and Leith (1997) also found no increase in NADPH:cytochrome P450 reductase activity in T47D breast tumour cells treated with D3T. The level of activity of other activating enzymes should be determined with all such experiments to eliminate other

possible mechanisms. The same should be done with detoxifying and DNA repair enzymes in order to more confidently correlate experimental results. The evaluation of such enzymatic approaches could explain why there are distinct differences in cytotoxicity between various cell types and tissues.

The GST's are a family of phase II detoxifying enzymes that have been shown to be coordinately induced with DT-diaphorase in some tissues (Talalay, 1989). These enzymes may also protect cells from the toxic and mutagenic effects of foreign chemicals (Prestera et al, 1993). In addition, GST's have been shown to play an important role in resistance to a variety of antitumour agents, including MMC, by aiding in the removal of the drugs from cells (Waxman, 1990; Xu et al, 1994). Thus, if D3T were to increase the levels of both DT-diaphorase and GST activity in tumour cells, this might not result in a net increase in antitumour activity. This did not occur in our studies as we did observe an increase in antitumour activity with both EO9 and MMC in a number of tumour cell lines. Furthermore, we did not find any increase in GST activity in a leukemia cell line, HL-60, or in a solid tumour cell line, T47D (Begleiter and Leith, 1997), treated with D3T, as has been observed previously (Li et al, 1994). Thus, it appears that it is possible to increase DT-diaphorase activity in tumour cells without increasing GST. Additional studies to determine if this effect is observed in other tumour cells, in normal tissues or with other inducers are required.

In summary, we have shown that D3T significantly increased the level of DT-diaphorase activity in 28 of 37 human tumour cell lines representing a wide variety of tumour types. DT-diaphorase activity was also increased in normal human bone marrow and kidney cells but the increases were small in these cases. D3T produced a significant increase in

enzyme activity in normal human lung cells. Combination treatment of human tumour cells with D3T and the bioreductive antitumour agents, MMC or EO9, did produce significant increases in cytotoxic activity. The combination treatment also enhanced the *in vitro* therapeutic index of EO9 by 2-3 fold in the HL-60, human promyeolcytic leukemia cells, and H661, human large cell lung carcinoma cells, due to the observation that D3T did not effect the toxicity of EO9 in normal kidney cells.

These results demonstrate that it is possible to enhance the antitumour activity of bioreductive agents in human tumour cells with inducers of DT-diaphorase. Such increases in cytotoxic activity may play an important role in dose intensification strategies. In addition, this approach appears to be applicable to different agents and in different tumour cells. Additional studies with other inducers, antitumour agents and cells are required to identify the optimum uses for this new treatment strategy.

FUTURE STUDIES

There are many questions that remain to be answered with respect to bioreductive agents, DT-diaphorase, and D3T analogs. Newer bioreductive agents, such as tirapazamine, MeDZQ, and EO9, are in or beginning clinical trials. Tirapazamine and MeDZQ show promise in the treatment of hypoxic tumour regions (Patterson et al, 1994) and tumours presenting higher DT-diaphorase activity levels than found in the surrounding normal tissue (Ross et al, 1994), respectively. EO9 has been disappointing in phase II clinical trials (Dirix et al, 1996) and it may not reach the clinic as a therapeutic bioreductive agent. Other indoloquinone analogs have been synthesized and may overcome some of the potential problems encountered with EO9 (Schellens et al, 1995; McLeod et al, 1996; Phillips et al, 1997). In addition, newer experimental models should be developed to clearly evaluate the contribution and importance of DT-diaphorase in the activation of present and future bioreductive agents.

It has been proposed that any cytotoxicity studies with bioreductive agents be carried out in the closest possible physiological conditions to that existing in a solid tumour, such as low pH and low oxygen tension (Pritsos et al, 1996). In reality, differences in the *in vivo* sensitivity to bioreductive agents may be related to a large number of factors, including differences in tumour microenvironment conditions, NQO_1 expression, relative uptake and efflux of bioreductive drug, levels of enzymes involved in DNA repair, and levels of other bioreductive enzymes. The study of the connections between these factors would aid in the design of new *in vitro* methodologies to use in bioreductive agent development and the evaluation of potential clinical relevance.

The finding of DT-diaphorase mutations in the population (Edwards et al, 1980; Traver et al, 1992; Rosvold et al, 1995; Traver et al, 1997) raises the question whether the point mutation is of any clinical relevance in a homozygous, or even heterozygous, individual. The heterozygous genotype may result in a dominant negative phenotype because the active DT-diaphorase protein is a homodimer (Riley and Workman, 1992). An NQO_1 knock-out mouse is under development (Yoshida and Tsuda, 1995) and it could clarify many issues related to the importance of DT-diaphorase in chemoprevention and chemotherapy. In addition, our study of DT-diaphorase induction by D3T in human tumour cell lines indicates that there may be tissue specificity in DT-diaphorase induction. Further studies of induction in other head and neck tumour cell lines should be carried out to determine if combination treatment with D3T could be considered for this type of tumour that is presently treated with MMC. The role of high basal DT-diaphorase activity levels on the induction by D3T and the reasons for differences in induction in different tumour cell lines also must be clarified.

Possible structure-activity relationships for DT-diaphorase induction by D3T analogs have been found in our studies. Additional D3T analogs will be synthesized by Tom Curphey (Dartmouth College, Hanover, NH, USA) that will combine some of the structural features of the inducers that had similar activity to the parent D3T to see if the induction can be further increased. Other non-toxic compounds, such as various dietary components, should be examined for possible greater induction of DT-diaphorase. The molecular mechanism of induction by D3T and other dietary compounds in normal and tumour tissue should be given much attention and this could maximize the induction capacity of the

compound and selective induction in tumours.

Finally, the clinical potential of combination treatment of bioreductive agents and D3T or other inducers should be examined in an *in vivo* nude mouse xenograft model with a range of human tumours. Successful results in such a model with a number of tumour tissue types will determine the scope and applicability of this approach and validate the clinical testing of this protocol in the therapy of cancer. I am hopeful that the correct D3T analog will prove to be as encouraging as the study by Shao *et al* (1995) in which they found that menhaden oil enhanced the cytotoxic activity of MMC towards MX-1 human breast tumour cells in an athymic mouse. The enhanced efficacy of bioreductive agents resulting from this strategy may then benefit the large number of people who don't respond to conventional therapies and the 50% of cancer patients that eventually die from their disease (Katzung, 1995).

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