ENZYMOLOGY AND STRUCTURE-ACTIVITY RELATIONSHIPS OF QUINOXALINE BIOREDUCTIVE CYTOTOXINS

By

Sara Ann Fitzsimmons, BSc.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

Tumour hypoxia, a phenomenon described within many solid tumours, arises primarily from the development of a deficient vasculative system. The failure of conventional radiotherapy and chemotherapy in the treatment of solid tumours has been related to the presence of hypoxia within such tumours. For this reason, several methods have been introduced in an attempt to overcome these problems, and hence, lead to effective killing of the whole tumour. The development of bioreductive anticancer agents as a means of exploiting hypoxic cells within solid tumours has been based upon their ability to be preferentially cytotoxic towards hypoxic cells. Furthermore, these agents express a requirement for enzyme-mediated reductive activation in order to exert their cytotoxic effects. The implementation of an enzyme-directed bioreductive drug development programme, therefore, has been based not only upon understanding the mechanism of action of particular bioreductive anticancer agents, but also upon determining the level of expression of reductases within both normal and tumour tissues.

The National Cancer Institute (NCI) possesses a panel of over 60 human tumour cell lines from at least nine different tumour origins. This cell line panel was used, therefore, as a means of determining the expression of several important reductase enzymes (DT-diaphorase; NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase) within cell lines from different tumour origins. Importantly, patterns of expression were found for each of the reductase enzymes studied. Subsequent incorporation of this data into the NCI database demonstrated the potential benefits of such a system by indicating possible mechanisms of action of various anticancer agents. For example, a large number of quinone compounds were among those showing positive correlations between DT-diaphorase activity and cell sensitivity under oxic conditions. Of particular importance, were the correlations between DT-diaphorase activity and cell sensitivity towards both the prototype bioreductive, mitomycin C and the investigative indoloquinone agent EO9.

A group of novel ZENECA pharmaceutical compounds were selected for study as potential bioreductive agents due to their structural similarities to some of the more active compounds already under investigation. These compounds consisted of a nitroacridine, a large nitroheterocycline and a series of quinoxaline di-*N*-oxides. The ability of these compounds to act as hypoxia-selective bioreductives was investigated using a small panel of human tumour cell lines selected from the NCI panel and hence, known to possess different enzyme activity levels. Of the three structurally distinct compounds, the quinoxaline di-*N*-oxide ZM 81853 showed the best potential as a bioreductive agent. Furthermore, ZM 81853 was the only compound from a series of structurally-related quinoxaline di-*N*-oxides to exhibit hypoxia-selective cytotoxicity. This finding indicated that small structural changes greatly influenced the ability of the compounds to act as a

hypoxia-selective cytotoxins. Interestingly, ZM 81853 demonstrated similar hypoxic : oxic differentials as the benzotriazine di-*N*-oxide currently in Phase II clinical trial, tirapazamine. Importantly, this differential was greatest in T47D cells which were shown to possess the highest NADPH : cytochrome P450 reductase activity. *In vitro* metabolism studies in the presence of various chemical inhibitors, in combination with high performance liquid chromatography (HPLC), were used to confirm the predominant role of NADPH : cytochrome P450 reductase in the metabolism of ZM 81853 under hypoxic conditions. Furthermore, these studies were used in conjunction with the technique of mass spectroscopy, to identify a four-electron reduced product as the predominant metabolite. Collectively, therefore, these studies suggest that ZM 81853 undergoes a series of one-electron reductions by NADPH : cytochrome P450 reductase to form the four-electron reduced product. Kinetic analysis of ZM 81853 metabolism in the presence of several enzyme preparations, including a purified, recombinant human form of NADPH : cytochrome P450 reductase, suggested that ZM 81853 was reduced under hypoxic conditions with reasonable efficiency. The ability of NADPH : cytochrome P450 reductase to activate ZM 81853 to a cytotoxic species was further supported by the demonstration of DNA damage in the presence of the purified, recombinant human form of the enzyme. Interestingly, the lack of inhibitory effect of the oxygen radical scavengers, superoxide dismutase and catalase, suggested a possible role for a free radical, such as a nitroxide based species, in causing DNA damage under hypoxic conditions.

In conclusion, this thesis has determined the expression and activity of several important reductases within human tumour cells. The potential use of this information in determining the mechanism of action of anticancer agents has been demonstrated with respect to both the NCI database and also to the novel compounds investigated within this thesis. In this regard, the quinoxaline di-*N*-oxide ZM 81853 was shown to possess the ability to act as a hypoxia-selective bioreductive agent. Importantly, ZM 81853 exhibited similar cytotoxic and metabolic characteristics as the benzotriazine di-*N*-oxide, tirapazamine. The work of this thesis, therefore, will aid selection of potential bioreductive anticancer agents for further *in vivo* studies within ZENECA Pharmaceuticals.

DEDICATION

To my brother, Mark

DECLARATION

The work described in this thesis was performed personally unless otherwise acknowledged.

Sara A. Fitzsimmons,
October, 1995.

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Finally, special thanks must go to Alan and the rest of my family for their support and encouragement over years. I am especially grateful to Alan for his patience and understanding (a great deal of which was needed) during the course of my PhD. Here's looking forward to a stress-free time!!

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ABBREVIATIONS

CHO	chinese hamster ovary
CNS	central nervous system
DEX	dexamethasone
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ESR	electron spin resonance
FAB	fast atom bombardment
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HCR	hypoxic cytotoxicity ratio
HPLC	high performance liquid chromatography
IC_{50}	concentration of drug required to kill 50% of cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWt.	molecular weight
NADH	ß-nicotinamide adenine dinucleotide (reduced)
NADPH	ß-nicotinamide adenine dinucleotide phosphate (reduced)
NCI	National Cancer Institute
PETG	polyethylene terephthalate glycol
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase Polymerase chain reaction
SOD	superoxide dismutase
SRB	sulforhodamine B

CHAPTER 1

GENERAL INTRODUCTION

More than 300,000 people develop cancer each year in the U.K (Cancer Research Campaign [CRC] Scientific Yearbook 1994 - 1995). Furthermore, this condition accounts for 20% of annual deaths in the U. K.. At present, the most common form of the disease in men is lung cancer (22% of all male cancers) whilst in women, it is cancer of the breast (approximately 30,000 new cases annually) (CRC Scientific Yearbook 1994 - 1995). Survival is dependent upon the type and stage of the disease, with the majority of deaths arising from metastatic spread (CRC Scientific Yearbook 1994 - 1995).

Conventional treatments of cancer include surgery, radiotherapy and chemotherapy. The former two regimes are used in the treatment of localised disease but neither is suitable on their own for the treatment of metastatic disease. In addition, the dose of radiation which can be delivered to the tumour site is often limited by damage to surrounding normal tissues. This is particularly applicable to tumours located deep inside the body. Chemotherapy plays an important role in cancer treatment, particularly in cases of metastatic disease. It can be used as adjuvant treatment before and after surgery or in combination with other treatments such as radiotherapy. Chemotherapeutic treatments have exhibited major curative effects for rare types of malignancy such as Hodgkin's lymphoma and testicular cancer in men. Unfortunately, however, successful drug treatment of common solid tumours such as lung, colon and breast has been limited. As with radiotherapeutic treatments, problems of toxicity towards normal cells occurs with conventional chemotherapeutic agents. Selection of drugs is generally based upon their ability to inhibit DNA synthesis and hence, interfere with the uncontrolled proliferation that is associated with tumour cells (Pratt *et al.* , 1994; Schwartsmann & Workman, 1992). Normal cell toxicity arises, however, as many of these cells are also undergoing rapid proliferation, for example those within the bone marrow, hair follicles and gastrointestinal tract. In addition to toxicity, a further problem with current

chemotherapeutic treatment is resistance of the tumour towards the cytotoxic agents used. This resistance can either be intrinsic, i.e. be present before treatment begins, as for many colon tumours, or it can be acquired. In this latter case, a tumour which has previously been treated with chemotherapeutic agents becomes resistant to those agents when the patient relapses and fails to respond to therapy (Kaye, 1988). To further complicate matters, in certain cases, the tumour frequently becomes cross resistant towards agents other than those already used (Kaye, 1988). Clearly there is a need, therefore, for more effective radiotherapeutic treatments and chemotherapeutic agents to be developed, with particular emphasis on the treatment of the major common solid tumours. In the case of chemotherapeutic treatment, for example, the selectivity of agents towards tumour cells could be improved as this would increase the effectiveness of the agents.

1.1 Hypoxia as a Target for Cancer Therapy

1.1.1. Existence of Hypoxic Cells within Solid Tumours

The histological structure of solid tumours, for example those of the lung, indicated that such tumours possessed a central necrotic region surrounded by intact tumour cells (Gray *et al.* , 1953). This is caused by the existence of a deficient vasculature, and a decreasing gradient of oxygen tension between the periphery and the central region of the tumour, so much so that those cells in the centre of the tumour are deprived of both oxygen and nutrients and as a result are necrotic. Cells at the periphery of the tumour, and at sites close to blood vessels are known as the oxic cell population as oxygen and nutrients are in abundant supply. Hypoxic cells are those cells which exist at an intermediate oxygen tension sufficient to maintain viability but only a low level of growth and metabolism. Two types of hypoxia exist within the solid tumours (Figure 1.1) (Workman, 1993). The first, chronic hypoxia, is caused by the limited diffusion distance of molecular oxygen (150 - 200 microns), in which cells at this distance from the blood capillary become hypoxic. This is often referred to as diffusion limited hypoxia. The second form of hypoxia, acute hypoxia, results from the intermittent opening and closing

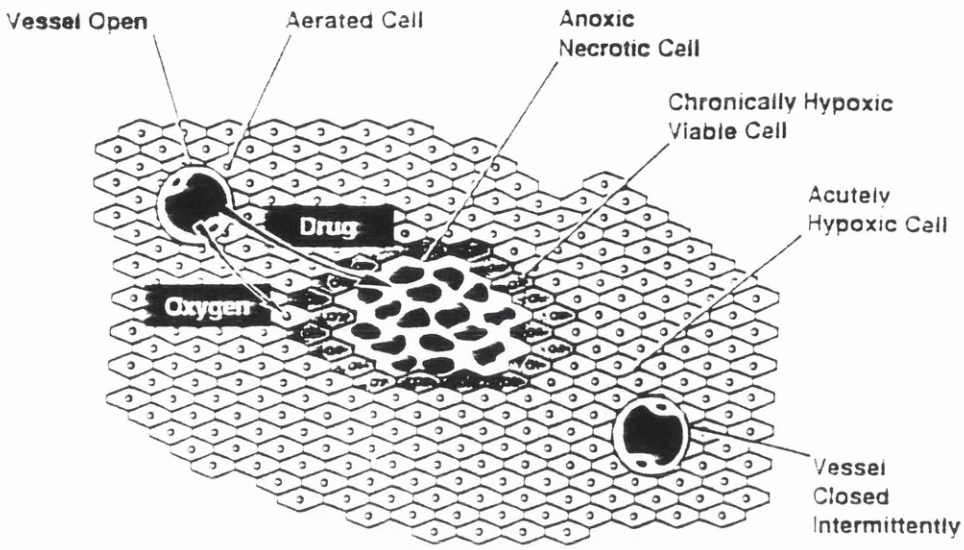


Figure 1.1 Hypoxia within solid tumours.

Two types of hypoxia are described within solid tumours.*Chronic hypoxia* is frequently described as the diffusion limited form due to a lack of diffusion of oxygen beyond 200 μM from the blood vessels. *Acute hypoxia* is the more transient form of hypoxia and is described as perfusion limited.

[taken from Workman, 1993]

of blood vessels and hence is perfusion limited. Closure of a capillary, therefore, results in rapid consumption of available oxygen by the surrounding cells and subsequent hypoxia. Cells then become reoxygenated upon re-opening of the capillary. Importantly, hypoxic regions have been identified in various solid tumours including head and neck (Gatenby *et al.*, 1988), breast (Vaupel *et al.*, 1991), cervix (Hockel *et al.*, 1993) and brain (Rampling *et al.*, 1994) using a computer-linked needle electrode (Eppindorf) to measure tumour partial oxygen pressure (pO_2).

1.1.2. Hypoxia and Resistance

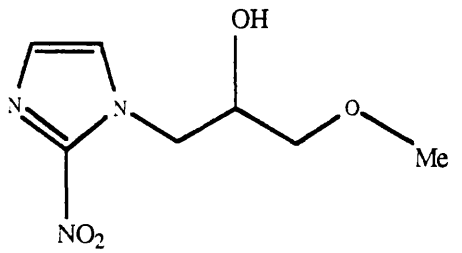
Advances in the treatment of solid tumours have been limited due to the resistance of many of these tumours to conventional chemotherapy and radiotherapy (Coleman, 1988). In the case of chemotherapeutic agents, resistance is believed to be due, in part, to the presence of deficient vasculature such that movement of the agents within the tumours is restricted. Resistance may also arise from a lack of oxygen-mediated activation or because of hypoxia-induced cell cycle effects (Workman, 1993). In addition, the presence of hypoxic cells within solid tumours are thought to be responsible for resistance towards radiotherapy, as these cells have been shown to be 2 - 3 fold more resistant than oxic cells towards the effects of radiation (Gray *et al.*, 1953; Hall, 1988). This phenomenon is frequently referred to as the oxygen effect. Under normal circumstances, i.e. in well-oxygenated areas, molecular oxygen would interact with short-lived radicals in DNA to cause irreversible damage to the cells (Coleman, 1988). In the case of hypoxic cells, however, such a reaction cannot occur, thereby rendering the cells more resistant to the action of radiation. As a consequence, the presence of hypoxic cells, and the subsequent marked resistance towards conventional treatments, has been viewed as a major clinical problem in the treatment of solid tumours.

1.1.3. Mechanisms of Overcoming Resistance

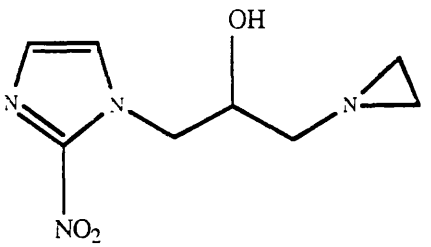
Various clinical strategies have been designed in an attempt to overcome the resistance of hypoxic cells to treatment. Solid tumours are known to undergo a

reoxygenation step following radiation treatment (Hall, 1988) such that the number of hypoxic cells within the tumour remains relatively constant. As a result, fractionated radiation dosing schedules were implemented to exploit this reoxygenation step and thereby, to improve the treatment of solid tumours by radiotherapy. In addition, the administration of hyperbaric oxygen to patients was proposed as a means of reoxygenating the tumour before subsequent radiotherapy treatment (Coleman, 1988; Henk, 1981). Although positive results have been obtained in head and neck and cervical cancers (Henk, 1981), results on the whole have been inconsistent. This may, however, be partly explained by the logistic difficulty of administration of such therapy to patients. More recent studies, however, have focused on the combined use of nicotinamide and carbogen (95% oxygen, 5% carbon dioxide) to improve the oxygen status of tumours (Laurence *et al.*, 1995). These studies have shown that in patients with advanced disease, significant increases in pO_2 values were obtained with only low levels of toxicity. As a result, the combined use of nicotinamide and carbogen breathing has been proposed as a possible means of tumour radiosensitisation.

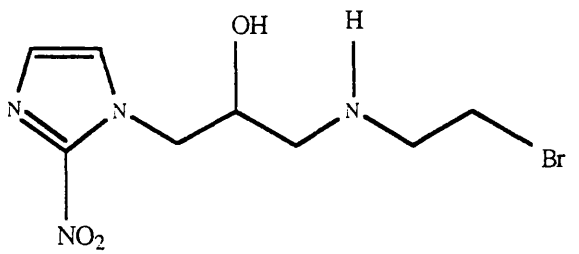
Hypoxic cell radiosensitisers have also been introduced with a view to specifically increasing the radiation sensitivity of hypoxic cells (Adams *et al.*, 1979) by mimicing oxygen and causing irreversible damage following interaction with the target DNA radical (Coleman, 1988). It was predicted that these agents would be more beneficial over the use of hyperbaric oxygen as the diffusion distance of the agents would be greater (Adams *et al.*, 1979). The earliest hypoxic cell radiosensitisers to be developed were the nitroimidazoles, such as the 5-nitroimidazole, metronidazole, and the 2-nitroimidazole, misonidazole (Figure 1.2). More recently, new developments have included the 'dual-functioning' agents such as RSU 1069 (Stratford *et al.*, 1986) and its derivative RB 6145 (Jenkins *et al.*, 1990) which contain not only the sensitising nitro group but also an alkylating aziridine group or a prodrug. A common problem with these agents has been the severe dose-limiting toxicities observed during clinical trials (Overgaard, 1994). For example, progress of misonidazole was marred by the dose-limiting peripheral and central neurotoxicity, while for RSU 1069, the dose-limiting toxicity observed was in the



MISONIDAZOLE



RSU 1069



RB 6145

Figure 1.2 Chemical structures of 2-nitroimidazole radiosensitisers.

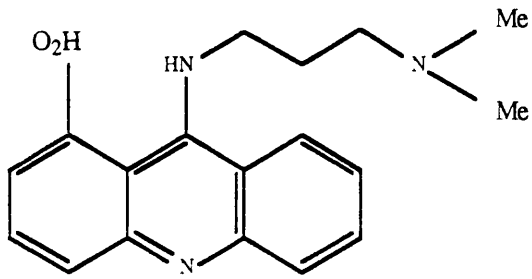
gastrointestinal tract. Attempts to overcome the neurotoxicity of nitroimidazoles have included the discovery of the hydrophilic analogue etanidazole (Brown & Workman, 1980) and the more potent, basic analogue pimonidazole (Smithen *et al.*, 1980). RB 6145 and one of its enantiomers have also shown less gastrointestinal toxicity than RSU 1069 (Jenkins *et al.*, 1990). Encouragingly, a meta-analysis of all clinical trials with nitroimidazole radiosensitisers has shown small but significant advantages in terms of local control and survival (Overgaard, 1992). This provides added support for the view that hypoxia is indeed a limiting feature in radiation therapy.

Sartorelli and co-workers (Lin *et al.* , 1972) hypothesised that hypoxic cells within solid tumours would possess a greater ability to cause reductive reactions than the surrounding well-oxygenated cells. As a consequence, the presence of a hypoxic environment could be exploited by developing chemotherapeutic prodrugs with the potential to be reduced to more cytotoxic species under these conditions. During the development of the hypoxic cell radiosensitisers, it was noted that certain agents such as misonidazole were preferentially cytotoxic under hypoxic conditions (Stratford *et al.*, 1980). The selective cytotoxicity was believed to be due to the production of toxic metabolites following specific reduction under hypoxic conditions. These agents, therefore, formed a new class of anticancer agent, namely the bioreductive hypoxic cell cytotoxins (Workman & Stratford, 1993).

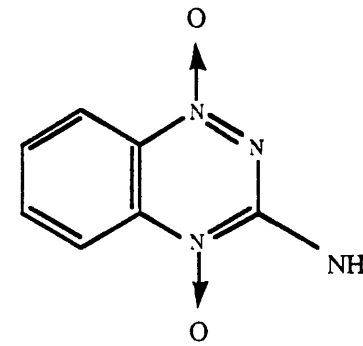
1.2 Bioreductive Hypoxic Cell Cytotoxins

1.2.1. Selectivity of Bioreductive Hypoxic Cell Cytotoxins

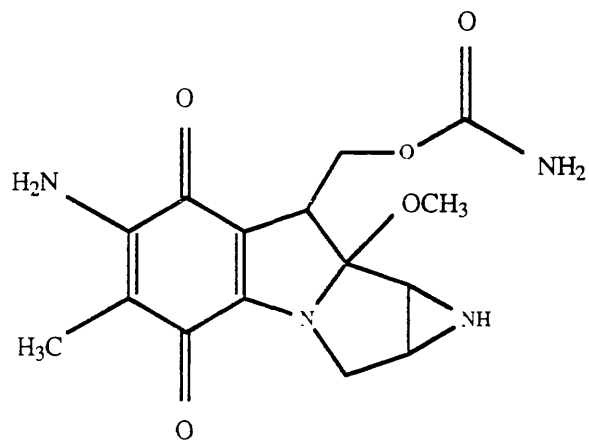
The bioreductive cytotoxins exist as inactive prodrugs which are able to undergo selective reductive metabolism under hypoxic conditions to form species capable of causing cellular damage (Workman & Stratford, 1993). Several different classes of these agents have been studied. Examples are shown in Figures 1.2 and 1.3, and include the nitro compounds, misonidazole, RSU 1069, nitracrine and CB 1954, and the quinone



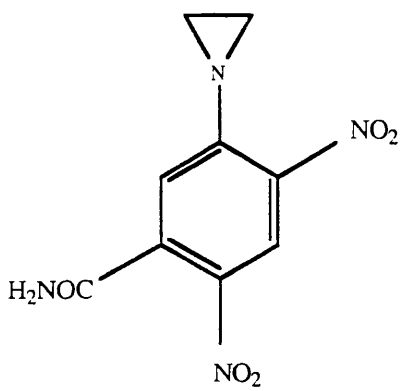
NITRACRINE



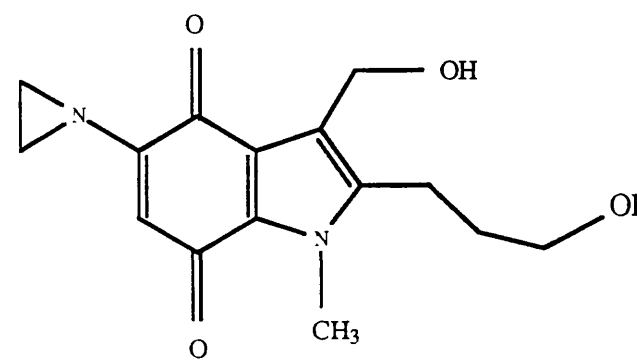
TIRAPAZAMINE



MITOMYCIN C



CB 1954



EO9

Figure 1.3 Structures of various bioreductive hypoxic cell cytotoxins.

alkylating agents such as mitomycin C and EO9. More recent developments have seen the introduction of the *N*-oxides, of which tirapazamine is the lead compound (see Figure 1.3 for structure). Many of these agents possess the ability to exhibit selective toxicity towards hypoxic versus oxic cells (Plumb *et al.* , 1994b; Stratford & Stephens, 1989; Wilson *et al.* , 1984) by virtue of their preferential reduction to cytotoxic metabolites under hypoxic conditions (Workman, 1992). For example, tirapazamine has been shown by Plumb and Workman (Plumb & Workman, 1994) to exhibit an oxic : hypoxic differential of approximately 95 against the BE human colon tumour cell line, while for mitomycin C, the hypoxic differential was shown to be significantly lower with a value of only 9. These results were consistent with reported studies by Stratford and Stephens (Stratford & Stephens, 1989) in which mitomycin C exhibited a much lower oxic : hypoxic differential than tirapazamine (2 compared to 50). Although these HCR results do not directly relate to the *in vivo* activity of the compounds, they are important for determining the ability of such compounds to be selectively active under hypoxic conditions. *In vitro* screening has therefore demonstrated that bioreductive cytotoxins exhibit quite variable degrees of selectivity under hypoxic conditions. In addition, the variability in selectivity is also seen for the same agents in different human tumour cell lines. For example, the indoloquinone EO9 has been shown to exhibit oxic : hypoxic differentials of approximately 1700 and 3 in BE and HT-29 human colon tumour cell lines, respectively (Plumb & Workman, 1994). These differences between bioreductive anticancer agents and also between different cell lines, result from the varying pathways of metabolic reduction of the individual agents (see Section 1.2.2. below) and those enzymes involved in these pathways (see Section 1.2.3).

1.2.2. Metabolic Reduction of Bioreductive Cytotoxins

The reductive metabolism of several bioreductive agents has been widely studied but while reduction products have been identified for many of these agents, the precise pathway of metabolism is often complex and as yet not completely understood.

The reductive pathway proposed for quinones such as menadione, EO9 and mitomycin C is shown in Figure 1.4. Under hypoxic conditions, quinones can undergo one-electron reduction to form the semiquinone free radical product. This product is relatively unstable, and in the presence of oxygen back-oxidises to the parent product with the subsequent production of toxic oxygen species (Workman & Walton, 1990). These radicals, for example superoxide and hydroxyl radicals, are capable of causing oxidative stress and cell death. In the case of simple quinones such as menadione, toxicity under oxic conditions is caused by oxidative stress alone (Workman & Walton, 1990). In contrast, however, the situation is more complicated in the case of bioreductive alkylating agents such as mitomycin C and EO9 since toxicity arises from both oxidative stress and activation to DNA-alkylating species (Workman & Walton, 1990). For example, electron spin resonance (ESR) (Bailey *et al.* , 1993) and DNA damage (Bailey *et al.* , 1994b; Walton *et al.* , 1991) studies involving EO9 have indicated that aerobic toxicity, involving DNA strand breaks, resulted from the action of the semiquinone radical product and also the oxygen radicals. Further one-electron reduction under hypoxic conditions would lead to the production of the stable product, the hydroquinone. In addition, obligate two-electron reduction of quinones can also occur (Iyanagi & Yamazaki, 1970), under both oxic and hypoxic conditions, to yield the more stable hydroquinone product (Pan *et al.* , 1984). In the case of EO9, for example, it has been proposed that the hydroquinone possessed the ability to alkylate DNA and cause both DNA strand breaks and DNA adduct formation in the form of cross-links (Bailey *et al.*, 1994b; Walton *et al.*, 1991).

Nitro compounds, for example the 2-nitroimidazoles misonidazole and RSU 1069, are similar to the quinone compounds in their complexity of metabolism. The reduction products which appear to be important are the nitroradical anion (1-electron), the nitroso (2-electron) and the hydroxylamine (4-electron) as these are believed to be the toxic species, unlike the amine (6-electron) (Workman, 1992). Whilst the predominant reaction under aerobic conditions is thought to be redox cycling via the radical anion, less is known about those reactions occurring under hypoxic conditions. Toxicity, however, is

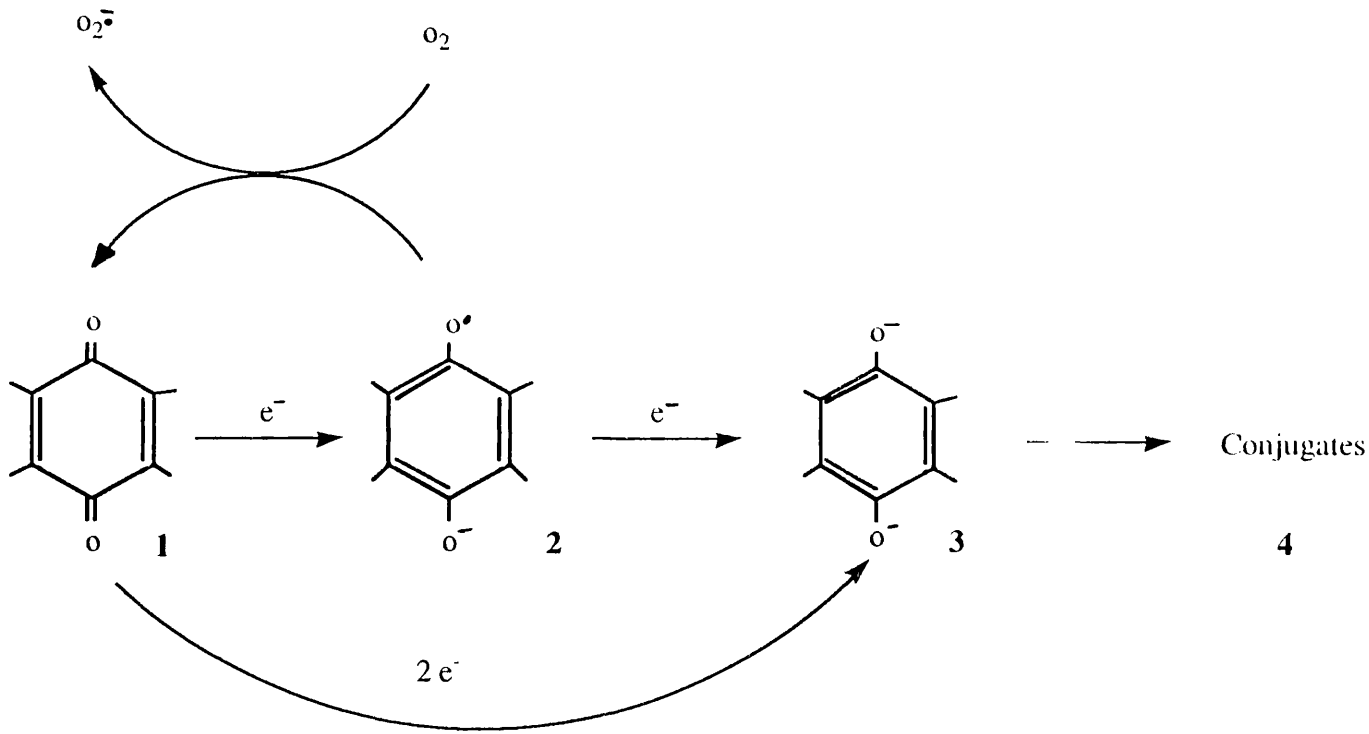


Figure 1.4 Predominant roles of enzymatic 1-electron versus 2-electron transfer in the toxification versus detoxification reactions of simple quinones.

(1) Quinone ; (2) semiquinone ; (3) hydroquinone ; (4) quinone conjugates.

thought to be caused by the actions of the nitroso and the hydroxylamine reduction products (Workman, 1992). In addition, fragmentation products such as glyoxal and species obtained from the reaction of the hydroxylamine intermediate with water are believed to be responsible, in part at least, for toxicity of the 2-nitroimidazoles (Franko, 1986; Raleigh & Liu, 1984; Varghese & Whitmore, 1984a; Varghese & Whitmore, 1984b). In contrast, the 'dual-function' nitroimidazoles such as RSU 1069 are believed to exert their toxic effects via DNA alkylation involving the aziridine moiety (Silver & O'Neill, 1986).

The nitroacridines such as nitracrine (1-NC) and 5-nitraquine have been studied for their selective toxicity towards hypoxic cells (Wilson *et al.*, 1984; Wilson *et al.* , 1992), however, only preliminary reports have been made in the literature concerning their reductive metabolism. Collectively, these studies suggest that cytotoxicity of 1-NC arose from nitroreduction of the compound (Wilson *et al.* , 1986) and subsequent production of a reactive metabolite capable of forming DNA adducts (Konopa *et al.* , 1983; Pawlak *et al.* , 1983). Problems of a lack of inhibition of the reaction by oxygen have, however, complicated the issue and as a result, more oxygen-sensitive nitroacridines are being sought whose reductive metabolism would be more restricted to the hypoxic environment.

In contrast to both the quinones and the nitro agents, the reduction pathway of *N*-oxides such as tirapazamine is relatively more straight forward. Therapeutic toxicity is believed to be caused by one-electron reduction of the agent to produce a toxic free radical species, capable of causing DNA damage (Figure 1.5) (Laderoute *et al.* , 1988). A nitroxide radical has since been identified following reduction of tirapazamine under hypoxic conditions (Lloyd *et al.* , 1991). Furthermore, the ability of tirapazamine to be reduced by one-electron reducing enzymes under hypoxic conditions to a DNA damaging species has been previously demonstrated (Fitzsimmons *et al.* , 1994; Walker *et al.* , 1994). Such damage is believed to arise by hydrogen abstraction from sugar residues in the DNA (Laderoute *et al.*, 1988).

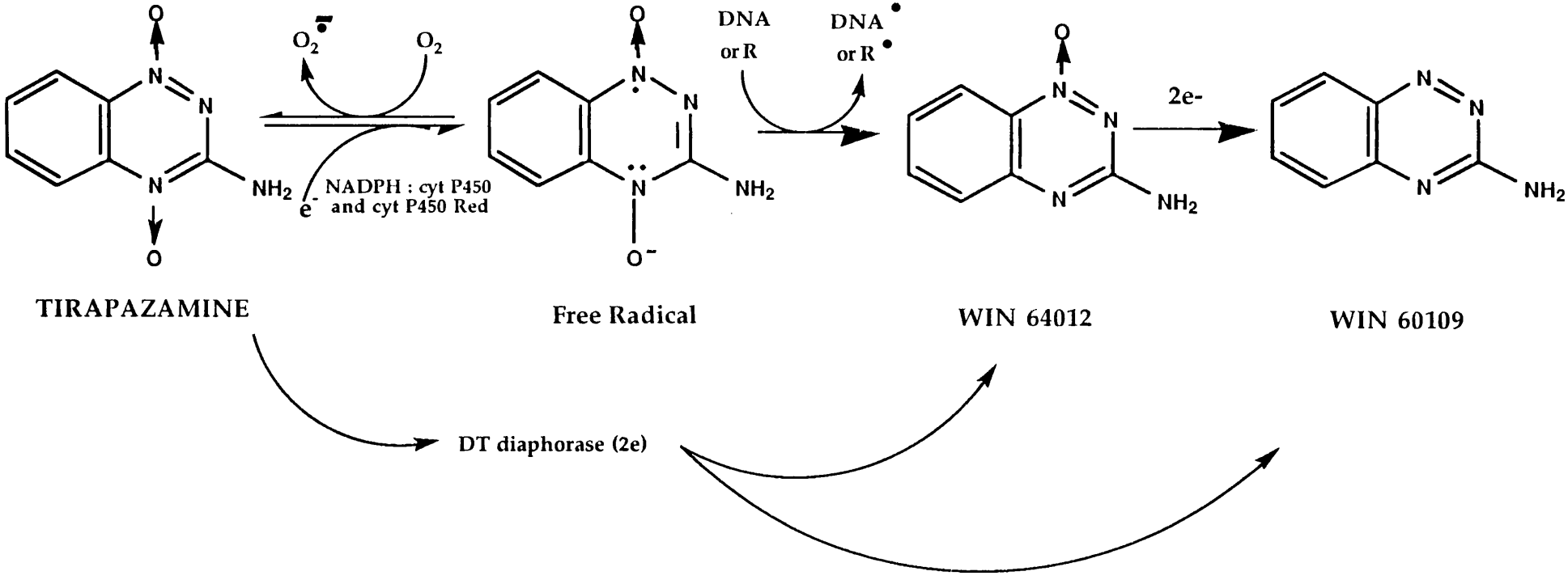


Figure 1.5 Putative reductive metabolism scheme for tirapazamine.

Direct 2-electron reduction of the parent compound results in the production of the 2-electron reduced product, WIN 64012 and, more predominantly, the 4-electron reduced product, WIN 60901. In contrast, 1-electron reducing enzymes are believed to reduce the drug to its 2-electron reduced product via production of an oxidising free radical , capable of exerting DNA damage.

As mentioned in the previous section (1.2.1.), variations between the activities of different bioreductive agents are not only dependent upon the metabolic pathway of the agents, but also on those enzymes involved in such a pathway. For this reason, investigations to establish those enzymes of importance in the metabolic pathway of bioreductive agents are important.

1.2.3. Enzymology of the Metabolic Reduction of Bioreductive Cytotoxins

As described in the previous section, both one- and two-electron reducing enzymes are involved in the reduction of bioreductive agents. This area of research has, therefore, attracted a great deal of attention in recent years (Workman, 1992; Workman, 1994 and references therein). Examples of those enzymes involved in the reduction of bioreductive agents include the one-electron reducing enzymes NADPH : cytochrome P450s (Lewis *et al.* , 1995; Riley *et al.* , 1993), NADPH : cytochrome P450 reductase (Fitzsimmons *et al.* , 1994; Walton *et al.* , 1992b), xanthine oxidase (Gustafson & Pritsos, 1992; Gustafson & Pritsos, 1993), and the two-electron reducing enzyme DT-diaphorase (Bailey *et al.* , 1994b; Knox *et al.* , 1988; Walton *et al.*, 1991). Depending on the chemistry of the individual agent and the particular enzyme involved, reduction can be either bioactivating or bioprotective (Workman, 1992). Examples of the enzymology of known reduction pathways are described below.

The benzotriazine di-*N*-oxide, tirapazamine, is perhaps the best understood of all the bioreductive agents developed so far, in terms of enzymes responsible for either bioactivation and bioprotection. For example, the obligate two-electron reducing enzyme DT-diaphorase has been shown to directly reduce tirapazamine (Riley & Workman, 1992b) to the inactive two-electron and more predominantly the four-electron reduced products (Baker *et al.* , 1988; Zeman *et al.* , 1986) (Figure 1.5). Reduction in this case, therefore, may cause a bioprotective effect. Interestingly, however, more recent studies have indicated that DNA damage may occur following metabolism of tirapazamine by DT-diaphorase (Walker *et al.*, 1994). In contrast, the one-electron reducing enzymes

NADPH : cytochrome P450 reductase and NADPH : cytochrome P450 2B6 are believed to cause bioactivation of tirapazamine, through the production of a nitroxide radical capable of exerting DNA damage (Fitzsimmons *et al.*, 1994; Laderoute *et al.*, 1988; Lewis *et al.*, 1995; Riley *et al.*, 1993; Walker *et al.*, 1994).

In contrast to tirapazamine, the nitro compounds have a more complex reduction pathway in that different enzymes have been shown to reduce the compounds at different stages of the reaction. For example, NADPH : cytochrome P450 reductase has been shown to be responsible for reduction of the misonidazole analogue, benznidazole, during the early stages, i.e. formation of the one- and two-electron reduced products. In contrast, NADPH : cytochrome P450s are responsible for production of the amine product in the latter stages of the reduction pathway (Walton & Workman, 1987). Furthermore, the cytosolic enzyme aldehyde oxidase has also been shown to play a minor role in the reduction of this particular agent. Similar studies using the 2-nitroimidazole hypoxic probe SR 4554 (Aboagye *et al.*, 1995) have also shown that NADPH : cytochrome P450 reductase is involved in the early stages of reduction of this agent.

Perhaps the most studied reduction pathway of a potential bioreductive agent to date is that of the prototype quinone compound mitomycin C (Sartorelli *et al.*, 1994). Xanthine oxidase, xanthine dehydrogenase (Gustafson & Pritsos, 1992; Gustafson & Pritsos, 1993), NADPH : cytochrome P450 reductase (Hoban *et al.*, 1990; Keyes *et al.*, 1984), NADH : cytochrome b_5 reductase (Hodnick & Sartorelli, 1993) and DT-diaphorase (Beall *et al.*, 1994a; Ross *et al.*, 1994; Siegel *et al.*, 1990a) have all been associated with the reductive metabolism of this bioreductive compound. However, the precise role of these enzymes in the reduction of mitomycin C has still to be determined, particularly in the case of DT-diaphorase. A great deal of controversy has surrounded the role of DT-diaphorase in the activation of mitomycin C (Ross *et al.*, 1994; Schlager & Powis, 1988; Workman, 1994; Workman & Walton, 1989). It is now known, however, that the ability of DT-diaphorase to metabolise mitomycin C is very much dependent upon pH conditions (Siegel *et al.*, 1990a). In addition, hypoxic conditions appear to cause only a small increase in the cytotoxic effects of mitomycin C (Plumb & Workman,

1994; Stratford & Stephens, 1989). Although this is perhaps the most studied reduction pathway, it has thus far proved to be extremely complex.

Although enzymology of reduction is certainly important, recent studies (Plumb & Workman, 1994) have shown the importance of considering both enzymological effects and environmental effects together. For example, although several studies have found a direct relationship between DT-diaphorase activity and EO9 sensitivity (Robertson *et al.* , 1992; Smitskamp-Wilms *et al.* , 1994; Walton *et al.* , 1992a), these studies have been performed only under oxic conditions. Plumb and colleagues (Plumb & Workman, 1994; Plumb *et al.* , 1994a; Plumb *et al.*, 1994b) have found that while high levels of DT-diaphorase, for example in HT-29 cells, related to sensitivity towards EO9 under oxic conditions, little increase in sensitivity occurred under hypoxic conditions. In contrast, those cells possessing low levels of DT-diaphorase activity, for example BE cells, exhibited increased sensitivity to EO9 under hypoxic conditions. It is now believed that this is due to the activating effects of one-electron reducing enzymes such as NADPH : cytochrome P450 reductase under these hypoxic conditions. Similar findings were reported by Robertson and colleagues (Robertson *et al.* , 1994). Collectively, they show the importance of considering all possible influential factors when studying the enzymology of bioreductive reduction.

It is clear that those enzymes demonstrated above are extremely important for bioreductive drug activation. They are of interest, therefore, in this thesis and as a result, are discussed below.

1.2.4. DT-diaphorase

1.2.4.1. Background and Nomenclature

DT-diaphorase (NAD(P)H : quinone oxidoreductase, NQO_1, E.C. 1.6.99.2) was discovered in 1958 as a highly active enzyme form in the soluble fraction of rat liver homogenates (Ernster & Navazio, 1958). Interestingly, early studies indicated the ability of this enzyme to utilise either NADH and NADPH with equal efficiency (Ernster &

Navazio, 1958). Subsequent purification and characterisation of DT-diaphorase revealed a high degree of sensitivity towards dicoumarol (Ernster *et al.* , 1962), a property which has led to the increased use of dicoumarol as a diagnostic inhibitor of this enzyme (Dulhanty *et al.* , 1989; Keyes *et al.* , 1985). A further characteristic of DT-diaphorase was its ability to catalyse obligatory two-electron reduction reactions (Lind *et al.* , 1982).

1.2.4.2. Purification, Protein and Gene Sequence

DT-diaphorase has been purified from several sources, including rat liver (Ernster *et al.*, 1962; Segura-Aguilar *et al.* , 1992), mouse liver (Prochaska & Talalay, 1986) and human Hep G2 cells (Boland *et al.* , 1991). The enzyme is known to exist as a dimer with two subunits of equal size (molecular weight approximately 27,500 each) and two molecules of flavin adenine dinucleotide (FAD) (Lind *et al.* , 1990; Lind *et al.*, 1982). Currently, two isoforms of DT-diaphorase are known to exist within the mouse (Prochaska & Talalay, 1986) while in the rat, at least three isoforms have been identified (Hojeberg *et al.* , 1981; Segura-Aguilar *et al.*, 1992). The presence of four genetically distinct isoforms of DT-diaphorase has been suggested in the case of humans, with each isoform encoded by a separate gene locus (Edwards *et al.* , 1980). Two such isoforms have been identified. The first is a dioxin-inducible form of DT-diaphorase, known as NQO_1 (Shaw *et al.* , 1991), which is believed to account for the majority of DT-diaphorase activity within tissues. The gene for NQO_1 has been identified on human chromosome 16 and codes for a protein similar in size to that of rat liver NQO_1, i.e. 274 residues (Jaiswal *et al.* , 1988). Further sequence analysis has revealed that 83% and 85% homology exists between human NQO_1 and rat NQO_1 cDNA and protein, respectively (Jaiswal *et al.*, 1988) and therefore it is not surprising to find that antisera raised towards the rat enzyme cross-react with the human form of NQO_1 (Shaw *et al.*, 1991).

The second form of human DT-diaphorase to be identified is that of NQO_2 which differs from NQO_1 in several ways : (a) it is not dioxin-inducible, (b) the gene is present on chromosome 6, and (c) the protein is 231 residues in size (Jaiswal *et al.* , 1990). Importantly, however, NQO_2 cDNA and protein are 54% and 49% homologous to the

NQO_1 form previously identified (Jaiswal *et al.*, 1990). In addition, antisera raised against the rat form of NQO_1 cross-reacted with human NQO_2 suggesting that both NQO_1 and NQO_2 are immunologically related (Jaiswal *et al.*, 1990).

Expression of DT-diaphorase (NQO_1) in human tissues is variable and has been shown to change when tissues convert to the tumourogenic phenotype (Schlager & Powis, 1990). These differences between normal and tumour tissues will be discussed in more detail later (see Chapter 2). Deletion and mutagenesis studies in the DT-diaphorase promoter region have indicated that the presence of an antioxidant response element (ARE), a xenobiotic response element (XRE) and an AP2 element are important for the regulation of NQO_1 expression in human tissues (Joseph *et al.*, 1994). The human ARE (hARE) is believed to be the most important region for basal expression of DT-diaphorase and for induction in response to xenobiotics and antioxidants (Belinsky & Jaiswal, 1993) as it contains an AP1 binding site (important for transcriptional regulation), and a GCA box (important for optimal expression and induction of DT-diaphorase gene expression) (Joseph *et al.*, 1994). Analysis of protein binding to the hARE element and subsequent induction of DT-diaphorase expression have suggested a role for Jun and Fos proteins and indeed other nuclear proteins (Joseph *et al.*, 1994; Li & Jaiswal, 1992a; Li & Jaiswal, 1992b), however, this remains a complex area requiring further study.

1.2.4.3. Role of DT-diaphorase in Cancer Chemotherapy

An important feature of DT-diaphorase is its ability to catalyse obligatory two-electron reductions of cytotoxic quinones and their derivatives (Lind *et al.*, 1982). Such a reaction by-passes the production of free radicals and toxic oxygen metabolites which would form during one-electron reductions of the compounds (Riley & Workman, 1992a) (see Figure 1.4). DT-diaphorase, therefore, acts in a protective manner in the case of simple quinones such as menadione. Although protection from naturally occurring toxic agents is the first and foremost role of DT-diaphorase, it is by no means its only role. DT-diaphorase also has the potential to cause bioprotection and / or bioactivation of a

number of anticancer agents, for example, the bioreductive alkylating aziridinyl benzoquinones mitomycin C, EO9 and diaziquone (AZQ) (Riley & Workman, 1992a; Ross *et al.* , 1994; Workman, 1994). The controversial role of this enzyme in the metabolism of both mitomycin C and EO9 has been mentioned previously in Section 1.2.3. The fact that DT-diaphorase levels are frequently raised in human tumours (Schlager & Powis, 1990) compared to normal tissue suggests that this enzyme could be important for successful treatment of tumours with anticancer agents as these agents may be designed to be activated by the enzyme and targeted towards the tumour (Riley & Workman, 1992a).

1.2.5 NADPH : Cytochrome P450 Reductase

1.2.5.1. Background and Nomenclature

NADPH : cytochrome P450 reductase (NADPH : cytochrome P450 oxidoreductase, NADPH : cytochrome *c* reductase, E.C. 1.6.2.4.) was first isolated in 1950 (Horecker, 1950). Along with NADPH : cytochrome P450, this enzyme forms an essential component of the mixed function oxidase system in the endoplasmic reticulum (Iyanagi & Mason, 1973). The role of NADPH : cytochrome P450 reductase is to transfer reducing equivalents from NADPH and H^+ to NADPH : cytochrome P450 (Vermillion *et al.* , 1981). NADPH : cytochrome P450 reductase acts by accepting two-electrons donated by NADPH and H^+ and subsequently passing them to the electron acceptor, NADPH : cytochrome P450, as single electrons. In addition, enzymes can also be transferred to other microsomal enzyme systems, such as haem oxygenase (Schacter *et al.*, 1972) and NADH : cytochrome b_5 (Enoch & Strittmatter, 1979). This system is important for the metabolism of lipids, drugs and other foreign compounds including anticancer agents (Conney, 1967; Gillette *et al.* , 1972).

1.2.5.2. Purification, Protein and Gene Structure

NADPH : cytochrome P450 reductase has been purified from the microsomal component of many species of liver including rat, pig, rabbit and human (Guengerich *et al.*, 1981; McManus *et al.*, 1987; Yasukochi & Masters, 1976). The enzyme exists as a single polypeptide (molecular weight approximately 78,000), and contains equal amounts of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Iyanagi & Mason, 1973). A high level of sequence homology has been shown between NADPH : cytochrome P450 reductase and ferredoxin : $NADP^+$ reductase (Porter & Kasper, 1986). Furthermore, homology between the FMN-binding domain of NADPH : cytochrome P450 reductase and the bacterial flavodoxins has been noted (Porter & Kasper, 1986). These studies have suggested, therefore, that NADPH : cytochrome P450 reductase arose from the fusion of the two ancestral genes of these flavoproteins. More recent studies by Smith *et al* (Smith *et al.*, 1994) have supported this finding. In addition, they have also reported the potential role of the functional domains (Figure 1.6). For example, the FAD/NADPH domain alone was able to catalyse reduction of certain compounds suggesting that it is important for the one-electron reduction of many compounds. In contrast, the FMN binding domain appeared to be important for binding NADPH : cytochrome P450 and subsequent transfer of electrons. The passage of electrons, therefore, is believed to proceed as follows (Vermillion *et al.*, 1981) :

NADPH -> FAD [NADPH : cytochrome P450 reductase] FMN ->

NADPH : cytochrome P450

1.2.5.3. Role of NADPH : Cytochrome P450 Reductase in Cancer Chemotherapy

As mentioned in 1.2.5.1., NADPH : cytochrome P450 reductase is important in the monooxygenase system for successful metabolism of lipids and other foreign compounds (Conney, 1967; Gillette *et al.*, 1972). In addition, however, NADPH : cytochrome P450 reductase also possesses the ability to reduce a number of bioreductive anticancer agents (Section 1.2.3.). The enzyme operates by causing one-electron reductions of the compounds to form toxic metabolites. For example, in the case of the benzotriazine di-*N*-oxide, tirapazamine, both NADPH : cytochrome P450 and NADPH :

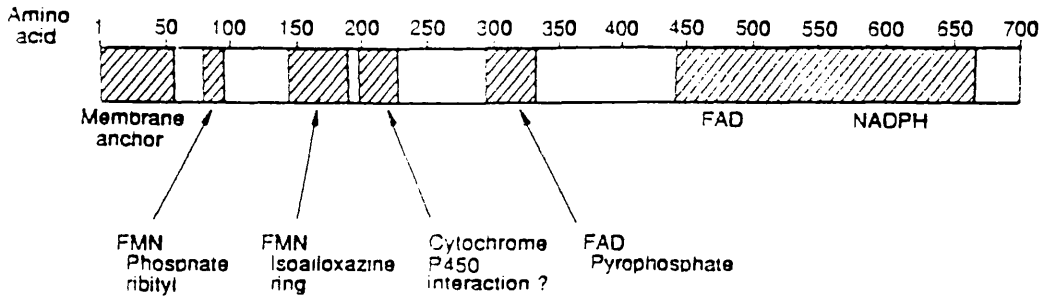


Figure 1.6 **Functional segments of the NADPH : cytochrome P450 reductase molecule.**

[taken from Gibson and Skett. 1994]

cytochrome P450 reductase have been shown to activate the compound following one-electron reduction (Riley *et al.*, 1993; Walton *et al.*, 1992b). Unlike NADPH : cytochrome P450 enzymes, however, NADPH : cytochrome P450 reductase has been shown to directly metabolise tirapazamine to a DNA damaging species (Fitzsimmons *et al.*, 1994; Walker *et al.*, 1994). The role for NADPH : cytochrome P450 reductase in the activation of the bioreductive EO9 has also been reported recently (Bailey *et al.*, 1994a; Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b). From these studies, therefore, it is clear that the level of expression of NADPH : cytochrome P450 and NADPH : cytochrome P450 reductase within human tissues, in particular tumour tissues, is an important factor in the ability of these enzymes to cause selective activation of anticancer agents. To date, very little is known regarding expression within human tissues. However, Rampling *et al* (Rampling *et al.*, 1994) have reported the presence of the enzyme within a series of human glioma biopsy material. If anything though, the levels of NADPH : cytochrome P450 reductase and NADPH : cytochrome P450 appear to be decreased within tumour tissues (de Waziers *et al.* , 1991; Massaad *et al.* , 1992; Philip *et al.* , 1994). Further studies are therefore required to fully investigate NADPH : cytochrome P450 reductase enzyme expression in human tumour cell lines and biopsies.

1.2.6 NADH : Cytochrome b5 Reductase

1.2.6.1. Background and Nomenclature

A second haem protein involved in the mixed function oxidase system is cytochrome b5 (Chance & Williams, 1954; Noshiro & Omura, 1978). This haem protein can be reduced by the FAD containing flavoprotein NADH : cytochrome b5 reductase (NADH : ferricytochrome b5 oxidoreductase, E.C. 1.6.2.2.) (Strittmatter & Velick, 1957) as well as NADPH : cytochrome P450 reductase (Enoch & Strittmatter, 1979). Interestingly, these latter two enzymes have been shown to express a high degree of similarity in their amino acid sequences (Porter & Kasper, 1986) suggesting common ancestry. NADH : cytochrome b5 reductase has been purified from both microsomal and

mitochondrial membranes of cells and was genetically similar in both cases (Kuwahara *et al.* , 1978). As well as a role in methemoglobin reduction in erythrocytes (Kuma & Inomata, 1972), evidence of a role for NADH : cytochrome b5 reductase in the transfer of electrons from cytochrome b5 to NADPH : cytochrome P450 has been provided by Noshiro and Omura (Noshiro & Omura, 1978). The importance of NADH : cytochrome b5 reductase in the mixed oxidase system was noted by the suggestion that NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase were involved in the transfer of the first and second electrons, respectively, to the terminal electron acceptor NADPH : cytochrome P450 (Peterson & Prough, 1986). As a result, NADH : cytochrome b5 reductase may also be important for the metabolism of lipids, drugs and other foreign compounds (Conney, 1967; Gillette *et al.*, 1972).

1.2.6.2. Role of NADH : Cytochrome b5 Reductase in Cancer Chemotherapy

NADH : cytochrome b5 reductase is perhaps one of the less studied enzymes both in terms of its levels of expression in human tissues, and also its role in the metabolic reduction of therapeutic agents. Due to its participation in the mixed oxidase system, however, it is clearly an important enzyme. Fisher and Olsen (Fisher & Olsen, 1982) first reported the role of human NADH : cytochrome b5 reductase in the activation of the prototype quinone bioreductive agent mitomycin C. Subsequent reports by Hodnick and Sartorelli (Hodnick & Sartorelli, 1993) have shown that the enzyme can, in fact, reduce mitomycin C to an alkylating species under both oxic and hypoxic conditions. These studies indicate that this enzyme may be important for activation of other potential bioreductive anticancer agents and as a consequence merits further study.

1.2.7 Enzyme-directed Bioreductive Drug Development

Bioreductive cytotoxins have been developed with a view to exploiting the presence of hypoxic cells within solid tumours. It is clear from the prior discussion, however, that both the level of hypoxia and the role of specific enzymes are important in

determining the effects of bioreductive cytotoxin treatment, i.e. bioprotection or bioactivation. This has led to the proposal of an 'enzyme-directed' approach to bioreductive drug development (Workman, 1994; Workman & Walton, 1990) as a means of enhancing the antitumour selectivity of these agents. Such an approach would involve gaining a better understanding of those enzymes involved in activation of different bioreductive classes. This would give a clearer picture as to the mode of action of bioreductive anticancer agents. Based on this knowledge, improved prodrugs could be designed to suit the catalytic preferences of particular reductases. From studies mentioned in section 1.2.3., it is clear that information is accumulating in this area. In addition, the determination of expression levels of different enzymes within human tumours would be extremely important as patient selection based on 'enzyme profiling' would then be possible. To date, only a limited amount of information is known regarding enzyme expression within both normal and tumour tissues (discussed in more detail in Chapters 2 and 3). The ultimate goal would be to use this information as a basis for future 'enzyme-directed' bioreductive drug development by designing agents which would be selectively activated by those enzymes present at high levels within human tumours (Workman, 1992).

1.2.8 Bioreductive Anticancer Agents in Clinical Trial

Progression of bioreductive anticancer agents towards routine clinical use has not been straight forward. Frequent problems of toxicity have been noted during clinical trials. For example, misonidazole and RSU 1069, exhibited peripheral neuropathy and gastro-intestinal dose-limiting toxicities, respectively (Overgaard, 1994). These studies, however, indicated a possible role for these agents in head and neck, and bladder cancers if the problems of toxicity could be overcome (Overgaard, 1994). Interestingly, the RB 6145 enantiomer (Jenkins *et al.*, 1990) will enter clinical trial shortly. In a similar manner to those mentioned above, Phase I clinical trials with the indoloquinone agent EO9 revealed problems of reversible renal toxicity (Schellens *et al.* , 1994); however, unlike

the prototype quinone compound mitomycin C (Sartorelli *et al.*, 1994), no evidence of myelosuppression was noted. Interestingly, however, some tumour responses were observed in the case of EO9 (Schellens *et al.*, 1994). The benzotriazine di-*N*-oxide, tirapazamine, is another bioreductive anticancer agent which has undergone Phase I and Phase II clinical trial. These trials have involved treatment with tirapazamine as a chemotherapeutic agent alone (Senan *et al.* , 1994), or in combination with either cisplatin (O'Dwyer *et al.* , 1995) or radiation (Doherty *et al.* , 1994). Muscle toxicity, in the form of muscle cramping was the major form of toxicity in Phase I studies (Doherty *et al.*, 1994). Although this was a problem, however, it was not shown to be dose-limiting. It is clear that toxicity is a major obstacle to be overcome in the treatment of solid tumours with bioreductive anticancer agents.

1.3 Aims of this Thesis

The main aims of these studies can be summarised as follows :

(i) To measure the levels of expression and activity of three important reductase enzymes within the NCI panel of human tumour cell lines.

(ii) To determine whether this information can be used within the National Cancer Institute database to establish correlations between different enzyme activities and cell sensitivities towards both existing and novel anticancer agents.

(iii) To determine the potential of several novel, structurally distinct and also a small series of structurally-related compounds to act as hypoxia-selective bioreductive anticancer agents.

(iv) To extend the above study to determine structure-activity effects of a small series of quinoxaline *N*-oxides.

(v) To investigate the enzymology and DNA damaging capacity of any of the above compounds shown to act as hypoxia-selective bioreductive anticancer agents.

These questions were addressed within the overall context of improving our knowledge of the reductase enzymology of human tumours and the relationships between

enzyme expression and bioreductive sensitivity, as well as identifying novel structures which possess the potential to act as bioreductive anticancer agents.

1.4 Lay-out of Thesis

To address the existing problem of limited availability of information regarding enzyme expression within human tumours, Chapter 2 describes the expression and activity of three important reductase enzymes within the National Cancer Institute (NCI) human tumour cell line panel. Analysis in over 60 cell lines from at least nine different tumour origins provides a detailed picture of possible enzyme expression patterns within human tumours. The potential use of such information in determining mechanisms of action of established and novel anticancer agents is described in Chapter 3, with particular reference to the prototype quinone alkylating agent mitomycin C and the indoloquinone agent EO9.

A small series of human tumour cell lines were selected from the NCI panel, based upon enzyme expression levels, for subsequent analysis of a group of structurally novel compounds. Chapter 4 describes the ability of these compounds to act as potential bioreductive anticancer agents, with particular reference to enzyme levels within each cell line. To establish possible reasons for differences in the activity of a small series of novel quinoxaline di-*N*-oxide compounds under oxic and hypoxic conditions, information regarding the metabolic properties of their reduction were obtained.

Chapter 5 describes the development of sensitive high performance liquid chromatography assays for detection of each of the five quinoxaline di-*N*-oxide compounds. The ability of these compounds to be metabolised in the presence of mouse liver enzymes is described in Chapter 6. In addition, chemical inhibition studies to establish those enzymes responsible for metabolism of the compound ZM 81853 are also discussed within this chapter. Finally, Chapter 7 compares the kinetics of ZM 81853 reduction in the presence of several enzyme preparations with those of previously

published anticancer agents. Furthermore, the ability of ZM 81853 to be metabolised to a DNA damaging species and hence cause strand breaks within DNA was investigated.

The final chapter, Chapter 8, summarises the work of this thesis and states the conclusions from the work. Suggestions for further investigations are also put forward in this chapter.

CHAPTER 2

DIFFERENTIAL EXPRESSION OF REDUCTASE ENZYMES IN THE NATIONAL CANCER INSTITUTE (NCI) HUMAN TUMOUR CELL LINE PANEL

2.1 Introduction

In many cases, antitumour agents require to be metabolised in order to exert their therapeutic activity. A good example of this is the so-called bioreductive drugs which require enzyme-catalysed reduction (Workman & Stratford, 1993). Preferential bioactivation in tumour versus normal tissue may provide a means of achieving improved therapeutic selectivity. The level of expression of these enzymes within human tumours, therefore, is thought to be very important in determining the outcome of tumour treatment with these particular agents. Depending on the chemistry of the individual agent concerned and the enzyme(s) involved, reduction can either be bioactivating or bioprotecting (Workman, 1992). The distribution of these drug-metabolising enzymes is, therefore, important in determining the outcome of specific drug treatments. To date, only a limited knowledge has been gained concerning the distribution of important drug-metabolising enzymes within human tumour tissues or cell lines. This chapter describes a detailed study to determine the differential expression of three important bioreductive reductase enzymes within a large panel of human tumour cell lines. This may help to establish a better understanding of the distribution of enzymes within human tumours, and hence, support the proposal that 'enzyme-profiling' of tumour biopsies would enable selection of antitumour agents for particular tumour treatments based upon their enzymology (Workman, 1994).

A wide variety of enzymes are involved in the metabolism of anticancer agents and could therefore be analysed for expression within human tumours and cell lines. Examples include the one-electron reducing enzymes such as NADPH : cytochrome

P450s, NADPH : cytochrome P450 reductase and xanthine oxidase, and the two-electron reducing enzymes such as DT-diaphorase and carbonyl reductase.

The presence of NADPH : cytochrome P450s and NADPH : cytochrome P450 reductase proteins within normal skin, breast and prostate tissues have been shown using immunohistochemical staining (Baron *et al.* , 1983). Furthermore, NADPH : cytochrome P450 reductase protein has been demonstrated in human liver, gastrointestinal tract, pancreas, lung and kidney by Western blot procedure (de la Hall *et al.* , 1989; Stralka & Strobel, 1991). While these studies are important, possible differences between normal and tumour tissues need to be identified. In this regard, the level of DT-diaphorase RNA within tumours and surrounding tissues of patients with hepatocarcinoma has been measured to be 20 to 50-fold higher than in the livers of normal individuals (Cresteil & Jaiswal, 1991). Similarly, Schlager and Powis (Schlager & Powis, 1990) have reported increased levels of DT-diaphorase activity in primary tumours of liver, lung, colon and breast compared to surrounding normal tissue. In contrast to these findings, however, some other studies by de Wazier *et al* (de Waziers *et al.* , 1991) have shown similar levels of DT-diaphorase activity to be present in peritumoural and tumour tissues from patients with colorectal adenocarcinoma when compared to normal tissue. These discrepancies may be real or may be explained by the possibility that in the latter case, normal tissue was present within the tumour biopsy sample. Other enzyme activities have been measured including the NADPH : cytochrome P450s. With the exception of the CYP3A isoform, NADPH : cytochrome P450s have been found to be lower in colorectal tumours compared to normal tissues (de Waziers *et al.*, 1991; Massaad *et al.* , 1992). These findings have been supported by more recent studies in which lower levels of expression of several NADPH : cytochrome P450 isoforms were found using immunohistochemical staining in tumour tissues (Philip *et al.* , 1994).

All of the aforementioned studies have increased existing knowledge as regards to expression of reductase enzymes within human normal and tumour tissues. One of the main problems in these studies, however, has been the limited quantity and range of biopsy material that has been available. Interestingly, several groups have recently

reported the activity of enzymes within different tumour histiotypes using panels of human tumour cell lines (Patterson *et al.*, 1994; Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; Robertson *et al.*, 1992; Smitskamp-Wilms *et al.*, 1994). While these particular studies have generally involved only small numbers of human tumour cell lines, they have indicated that the use of cell lines may be extremely important in overcoming the current problem of limited tumour biopsy material.

The US National Cancer Institute (NCI) annually screens more than 20,000 compounds of defined chemical structure against an *in vitro* panel of over 60 different human cell lines derived from tumours with a spectrum of histologies and organs of origin (Boyd & Paull, 1995). At least 42,000 compounds have been evaluated against the cell line panel thus far. The use of computer methods such as the COMPARE programme (Paull *et al.*, 1992; Paull *et al.*, 1989), neural networks (Weinstein *et al.*, 1992) and Kohonen self-organising mapping (van Osdol *et al.*, 1994) has provided a unique means by which to identify the potential mode of action of established agents and novel chemical entities. The inclusion of relevant biochemical and molecular parameters, such as enzyme activity measurements, within the same relational data base would provide an additional method of identifying critical factors influencing *in vitro* antitumour activity. For example the anthrapyrazoles DuP 937 and DuP 941, currently in clinical trial, have been shown to act as potent topoisomerase II inhibitors in a similar manner as that displayed by mitoxantrone (Leteurtre *et al.*, 1994). In addition, incorporation into the data base of *mdr*-1/P-glycoprotein expression levels within the cell line panel produced good correlations between expression and cell resistance to a number of compounds (Alvarez *et al.*, 1995). These compounds were previously found to be substrates for P-glycoprotein (Lee *et al.*, 1994). This demonstrates the usefulness of the inclusion of biochemical parameters along side the more usual cytotoxicity measurements.

For this reason, an extensive collaboration between the NCI, European Organisation for Research and Treatment of Cancer (EORTC) and the U.K Cancer Research Campaign was established to study bioreductive enzyme expression in over 60 human tumour cell lines originating from at least nine different tumour histiotypes. DT-

diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase were chosen to be studied. DT-diaphorase was selected because of the current degree of interest in its potential involvement in the activation of various bioreductive agents, particularly quinones (Riley & Workman, 1992a; Workman, 1994). Importantly, some of these quinone agents are in clinical trial, for example EO9 (Schellens *et al.* , 1994) (see section 1.2.8.). NADPH : cytochrome P450 reductase has also been extensively investigated for its role in the metabolism of a wide range of bioreductive agents (Fitzsimmons *et al.* , 1994; Rampling *et al.* , 1994). NADH : cytochrome b5 reductase has been less extensively studied but has been shown to have the ability to bioactivate the clinically used agent mitomycin C (Hodnick & Sartorelli, 1993). A further, practical advantage of selecting these particular reductases was the similarity in the analytical methodology used to measure their activities, which facilitated the efficient processing of the limited sample size available. Enzyme activity was measured by spectrophotometric analysis using a modified version of the cytochrome *c* reduction assay described by Segura-Aguilar (Segura-Aguilar *et al.* , 1990). To help interpret the enzyme activity information, the amount of protein and messenger RNA (for both DT-diaphorase and NADPH : cytochrome P450 reductase) were also analysed by Western immunoblotting and reverse-transcriptase polymerase chain reaction (RT-PCR), respectively. This chapter, therefore, details the expression and activity of the three selected bioreductive enzymes within a large panel of human tumour cell lines. It was hoped that enzyme activities within human tumours could be predicted from known activities within the human tumour cell line panel. In addition, the data may be useful in predicting the sensitivity of known and novel compounds towards tumours with known enzyme activities. Furthermore, an understanding of enzyme expression within the human tumour cell line panel would enable the selection of cell lines for cytotoxicity studies later in this thesis.

2.2 Materials and Methods

2.2.1. Chemicals and Reagents

All general chemicals were of Analar grade and supplied by Merck (Thornliebank, Glasgow, U.K.) or Fisons (Loughborough, U.K.) unless otherwise stated. NADH, NADPH, cytochrome *c*, bovine albumin, menadione, dicoumarol, aprotinin, ß-mercaptoethanol, bromophenol blue, mineral oil and ethidium bromide were all purchased from Sigma Chemical Company (Poole, Dorset, U.K.). The Pierce Bicinchoninic Acid (BCA) Protein Assay was supplied as a kit from Pierce and Warriner (Chester, U.K.). TEMED, Tween 20 and ammonium persulphate were obtained from BIORAD Laboratories (Bromley, Kent, U.K.) while the acrylamide / bis acrylamide (30% w/v) was purchased from Severn Biotech Ltd. (Kidderminster, U.K.). RNAzol™B was from Biogenesis Ltd. (Bournemouth, U.K.) while the reverse-transcriptase reagents were all obtained from Gibco BRL (Paisley, U.K.). Hexamer primers used in the reverse transcriptase reaction were from Pharmacia (Milton Keynes, U.K.) and the enzyme primers used in the PCR reaction were made by Oswel DNA Service (University of Edinburgh, Edinburgh, U.K.). All other reagents used in the polymerase chain reaction were supplied in a kit by Boehringer Mannheim (East Sussex, U.K.) which contained *Taq* DNA polymerase.

2.2.2. Enzyme Preparations and Specific Antibodies

Highly purified preparations of rat DT-diaphorase and NADPH : cytochrome P450 reductase were provided by Dr. R. Knox (ICR Department of Molecular Pharmacology, Sutton, U.K.) and Prof. C. R. Wolf (ICRF Laboratory of Molecular Pharmacology, Dundee, U.K.), respectively. The enzyme preparations had previously been purified from rat Walker tumour cells in the case of DT-diaphorase (> 95 % pure, Dr. R. Knox, personal communication), and from the liver microsomal fraction of male

Wistar rats for NADPH : cytochrome P450 reductase (> 90 % pure, Prof. C. R. Wolf, personal communication). In both cases, a specific polyclonal antibody raised in rabbits against the purified rat enzyme was provided.

2.2.3. Preparation of Human Glioma Tumour Tissue Homogenates

A series of human malignant brain tumours were provided by Dr. G. Cruikshank (Department of Neurosurgery, Southern General Hospital Trust, Glasgow, U.K.) for analysis of bioreductive enzyme activity. Resected tissue was homogenised in two volumes of buffer (10 mM potassium phosphate, pH 7.7; 1.15 % (w/v) potassium chloride; 0.1 mM diaminoethanetetra-acetic acid disodium salt (EDTA)) using a Citenco varicontrol homogeniser (Citenco Ltd., Hertfordshire, U.K.). Following ultracentrifugation at 18,000 rpm (4°C, 20 mins.) using a Beckman Optima TLX ultracentrifuge (Beckman Ltd., High Wycombe, Bucks., U.K.), the S9 supernatant was aliquoted into smaller volumes (40 µl) and stored at -70°C until use.

2.2.4. Preparation of Human Tumour Cell Line Sonicates

A total of 69 tissue-specific human tumour cell lines were kindly provided by the NCI (Division of Cancer Treatment, Bethesda, MD, U.S.A.) for bioreductive enzyme profiling. The cells had been grown to confluence at the NCI laboratories, and either harvested using trypsin (0.25 %) / EDTA (1 mM) solution or, for suspension cell lines, by centrifugation. Cell pellets were then prepared and forwarded to our laboratories as frozen cell pellets. When required, pellets were thawed and resuspended in 0.45 ml of phosphate buffered saline (PBS) and 0.05 ml aprotinin (10 %). Cell suspensions were sonicated at 22 kHz with an amplitude of 8 µm peak for 3 x 10 s. (MSE probe sonicator, Fisons Instruments, Loughborough, U.K.), ensuring that vials were kept on ice as often as possible. Samples were then spun down using a bench top centrifuge, at 15,000 rpm for

20 mins. at 4°C (Heraeus Equipment Ltd., Brentwood, Essex, U.K.). Aliquots (40 µl) of supernatant were removed and stored at -70°C until use.

2.2.5. Measurement of Protein Content

The Pierce BCA Protein Assay (Smith *et al.*, 1985) was used to determine protein content in each tumour cell line sonicate or tumour tissue homogenate. This assay allowed for the accurate measurement of protein concentration ranges of 10 - 2000 µg/ml in small (10 µl) sample sizes. Essentially, a series of known albumin standards were prepared (0 - 2 mg/ml) and 10 µl of each added to a microtiter plate in quadruplicate wells. Test samples were diluted as appropriate and 10 µl of each applied to the wells in duplicate. BCA protein reagent was prepared by mixing Reagent A (sodium carbonate; BCA detection reagent; sodium tartate in 0.1 M sodium hydroxide) with Reagent B (4 % copper sulphate solution) in a 50:1 ratio. 200 µl of working reagent was added to all wells. The plate was then placed on a microtiter plate shaker (BIORAD microplate reader, BIORAD laboratories, Bromley, Kent, U.K.) for 30 s before being covered and placed at 37°C for 30 mins. Absorbence in each well at 570 nm was determined using Softmax Version 2.3, installed on an Emax Precision microplate reader (Molecular Devices Corporation, Menlo Park, CA, U.S.A.). Protein concentration was determined from a plot of the standard curve and expressed as mg/ml.

2.2.6. Measurement of DT-diaphorase, NADPH : Cytochrome P450 Reductase and NADH : Cytochrome b_5 Reductase Activity

(a) Assay for DT-diaphorase

An indirect, coupled assay system was used to determine DT-diaphorase activity within the samples. Activity was assayed at 550 nm on a Lambda 2 UV/VIS spectrophotometer (Perkin Elmer Ltd., Beaconsfield, Bucks., U.K.) using a modification of the cytochrome *c* reduction method (Segura-Aguilar *et al.*, 1990). A mixture of

cytochrome *c* (77 μM) and bovine albumin (0.14 %, w/v) was monitored for its reduction in the presence of NADH (200 μM) as cofactor and menadione (20 μM) which acts as an intermediate electron acceptor. The reaction was initiated by addition of a sample of supernatant, the volume of which was dependent upon an amount known to be in the linear portion of the reaction. The activity attributable to DT-diaphorase was the portion which was inhibited by dicoumarol (10 μM), a known inhibitor of DT-diaphorase (Ernster, 1967). As a positive and standardised control for the reaction conditions, a 1:600 dilution of purified rat DT-diaphorase was assayed both in the absence, and the presence of dicoumarol. Inhibition of DT-diaphorase activity within the purified preparation was found to be between 90 - 95 %, on repeated occasions, thereby showing that dicoumarol was an efficient inhibitor of DT-diaphorase. Cytochrome *c*, bovine albumin and NADH were all dissolved in Tris buffer (Tris 50 mM, adjusted to pH 7.4 with HCl) while menadione and dicoumarol were dissolved in a mixture of dimethyl sulphoxide (DMSO)/bovine albumin or 0.1 M sodium hydroxide/bovine albumin, respectively.

(b) Assay for NADPH: Cytochrome P450 Reductase and NADH : Cytochrome b5 Reductase

NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase activity were measured using a direct assay system as both enzymes were able to reduce cytochrome *c* in the presence of cofactor alone (NADPH [200 μM] and NADH [200 μM], respectively). Appropriate controls were performed to determine background rates of reduction within all of the reactions.

All reactions were carried out at 37°C and initiated by the addition of the cell sonicate / tissue homogenate. Rates of reduction were calculated from the initial linear part of the reaction progress curve and were expressed as nmoles of cytochrome *c* reduced/min/mg of protein. An extinction coefficient for cytochrome *c* of 21.1 $mMcm^{-1}$ was used in the calculation.

2.2.7. Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The levels of DT-diaphorase and NADPH : cytochrome P450 reductase protein within each cell sonicate and tissue homogenate was determined by gel electrophoresis and Western blot analysis. For detection of DT-diaphorase, samples were run on a 12 % separating gel (1.5 M Tris base; 0.5 % sodium dodecyl sulphate (SDS) pH 8.8) while for NADPH : cytochrome P450 reductase, a 9 % separating gel was used. The percentage of separating gel used in the experiment was based upon the known molecular weights (MWt) of the human form of each enzyme. The stacking gel buffer was the same in all cases (0.5 M Tris base; 0.5 % SDS, pH 6.6). Samples were diluted to a 1 mg/ml protein concentration in boiling mix (10 % stacking gel buffer; 2 % SDS; 5 % *b*-mercaptoethanol; 10 % glycerol; 0.005 % bromophenol blue) and boiled for 5 mins. In the case of DT-diaphorase, 50 µg of protein was loaded for each sample, while for NADPH : cytochrome P450 reductase, 100 µg of protein was loaded. In addition, purified preparations of either rat DT-diaphorase or NADPH : cytochrome P450 reductase were used as a positive controls (0.1 µg loaded). Samples were subjected to electrophoresis (Pharmacia LKB-EPS 500/400 lab pack, Pharmacia, Milton Keynes, U.K.) at 50 mA for 2 hrs., then 6 mA overnight in electrode buffer (0.05 M Tris base; 0.05 M glycine; 0.1 % SDS, pH 8.3). A semi-dry graphite electroblotter (Millipore Corporation, U.S.A.) was used to transfer proteins from the gel onto an Immobilon-P transfer membrane (Millipore Corporation, U.S.A.). The transfer buffer consisted of 48 mM Tris, 39 mM glycine and 0.037 % (v/v) SDS. Following blocking with 5 % non-fat milk (Marvel®) for 1 hr., a primary antibody was applied for 1 hr. (1:500 dilution for DT-diaphorase; 1:250 dilution for NADPH : cytochrome P450 reductase). The primary antibodies had been raised against the purified rat proteins and were known to crossreact with the human forms of the enzyme. The membrane was washed (0.05 M Tris base; 0.15 M sodium chloride, pH 7.9; 0.01 % Tween 20) and then the membrane was washed with a 1:5000 dilution of Protein A linked to Horseradish Peroxidase (Amersham International plc, Amersham,

U.K.) for 18 mins. Following further washing, the membrane was processed using an Enhanced Chemiluminescence kit (Amersham International plc, Amersham, U.K.) and exposed to X-ray film (XR Fuji Medical film, Fuji, London, U.K.). The film was processed using a KODAK X-OMAT 480 RA Processor (KODAK Ltd., Hemel Hempstead, U.K.). The level of protein present within each cell sonicate was quantified by densitometric analysis using a Molecular Dynamics Laser Densitometer with Image Analysis Software (Protein Databases Incorporated, New York, U.S.A.).

2.2.8. Detection of DT-diaphorase and NADPH : Cytochrome P450 Reductase Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

(a) RNA Extractions

The expression of DT-diaphorase (NQO1) and NADPH : cytochrome P450 reductase was investigated using tumour cell lines originating from the breast and the CNS. RNA from individual cell pellets was isolated using the RNAzol™ B method described by Chomczynski & Sacchi (Chomczynski & Sacchi, 1987). This method was carried out as described in the manufacture's protocol and involved several steps. Firstly, cells were lysed and RNA solubilised by the addition of 1 - 2 ml RNAzol™ B (0.2 ml per 10^6 cells). RNA was extracted by the addition of chloroform (0.2 ml per 2 ml of homogenate) and subsequent placement of the samples on ice for 15 mins., followed by centrifugation (12,000 rpm, 4°C, 15 mins.). The aqueous phase was transferred to a clean eppendorf tube for the next step, RNA precipitation. An equal volume of isopropanol was added to the eppendorf and the sample stored at 4°C for 15 mins. before centrifugation (12,000 rpm, 4°C, 15 mins.). The RNA precipitate which formed was then washed by firstly removing the supernatant, adding 1 ml of 75 % ethanol, vortexing the sample and finally performing centrifugation (7,500 rpm, 4°C, 8 mins.). Ethanol was then removed and the pellets left to dry for 1 - 2 hrs. Pellets were then resuspended in distilled water (30 µl) and the optical density (OD) at 260 nm and 280 nm determined. An OD_{260}/OD_{280}

ratio higher than 1.9 was indicative of a pure RNA preparation with the sample substantially free from contaminating DNA and protein.

(b) Reverse Transcriptase Reaction

The reverse transcriptase reaction was performed in duplicate for each cell line. A reaction mixture consisting of hexamer primers (0.1 μg), Molony-murine leukaemia virus (MMLV; 10 U), nucleotides (dATP, dTTP, dCTP, dGTP; 2 mM), 5x hexamer reverse transcriptase (H-RT) buffer (0.25 M Tris HCl pH 8.3; 0.375 M potassium chloride; 15 mM magnesium chloride), dithiothreitol (DTT; 10 mM) and distilled water was made. This was then added to RNA (1 μg/μl) in a ratio of 9 μl reaction mix : 1 μl RNA. Reverse transcriptase was carried out at 37°C for 1 hr. Following this procedure, the cDNA was diluted 1:5 with distilled water and stored at -70°C until the polymerase chain reaction was carried out.

(c) Polymerase Chain Reaction

For each cell line, several dilutions of the cDNA were used in the polymerase chain reaction starting at 100 ng cDNA. These dilutions had previously been found to be in the linear portion of the reaction for each enzyme concerned. A separate master reaction mix was made for each enzyme under investigation, i.e. DT-diaphorase, NADPH : cytochrome P450 reductase, the human ribosomal 28S protein (used as a positive control) and an internal standard (no cDNA) in order to ensure no cross-contamination. This reaction mix consisted of the appropriate primers (see below), 10 x PCR buffer (100 mM Tris HCl, pH 8.3; 500 mM potassium chloride; 15 mM magnesium chloride), nucleotides (as above; 0.9 mM), *Taq* DNA polymerase and distilled water. DT-diaphorase PCR was performed using two different primers as detailed in Horikoshi *et al* (Horikoshi *et al.*, 1992) and consisting of (a) bases 392 - 413 of the DT-diaphorase coding sequence (Jaiswal *et al.*, 1988) and (b) bases 640 - 661 of the complimentary DT-diaphorase coding sequence (Jaiswal *et al.*, 1988). These primers were DT-diaphorase 1 (5'-ATTTGAATTCGGGCGTCTGCTG-3') and DT-diaphorase 2 (5'-AGGCTGGTTTGAGCGAGTGTTC-3'). For NADPH : cytochrome P450 reductase, the

PCR primers were selected from the amino acid sequence derived from the human cDNA (Shephard *et al.* , 1992) and were NADPH : cytochrome P450 reductase 1 (5'-GACGCCCACCGCTACGGAAT-3') and NADPH : cytochrome P450 reductase 2 (5'-CTGCATCCAGTCGTAGAAGT-3'). A positive control was performed alongside DT-diaphorase and NADPH : cytochrome P450 reductase involving the use of primers against the ribosomal 28S gene selected from the nucleotide sequence detailed in Gonzalez *et al* (Gonzalez *et al.* , 1985). Ribosomal 28S 1 consisted of (5'-TTACCAAAAGTGGCCCACTA-3') while ribosomal 28S 2 consisted of (5'-GAAAGATGGTGAACTATGCC-3'). For each reaction, 20 µl of reaction mix was added to 5 µl of cDNA and then overlaid with 50 µl mineral oil. A negative control was performed in which no cDNA template was present. All reaction tubes were then spun for a few seconds and placed in the thermal cycler (PREM™III, LEP Scientific, Milton Keynes, U.K.). The programme consisted of the following steps ; denature (95°C for 45 s), anneal (60°C for 45 s), extension (72°C for 2 mins. 15 s). This was repeated for 35 cycles and then a final extension at 72°C for 7 mins. was performed before holding reactions at 4°C. Samples were mixed with DNA loading buffer (300 mM sodium hydroxide; 6 mM EDTA; 18 % Ficoll in water; 0.15 % bromocresol green; 0.25 % Xylene cyanol FF) in a ratio of 5:1 and then loaded (10 µl) into the wells of a 2 % agarose gel prepared in 0.5 % TBE buffer (0.89 M Tris base; 0.89 M boric acid; 0.02 M EDTA). A 123 bp DNA ladder (Gibco BRL, Paisley, U.K.) was run alongside the test samples. The gel was electrophoresed at 180 V for 4 hrs. using a Hybaid Electro-4 Gel Tank and then stained with ethidium bromide (1 µg/µl) before being photographed under UV transillumination.

2.2.9. Statistical Analysis

The sensitivity data of anticancer drugs tested against the NCI cell line panel are routinely and conveniently displayed as COMPARE analysis plots (Paull *et al.*, 1992; Paull *et al.*, 1989). For a given drug, these COMPARE plots are illustrated on a log10 scale with the deviation from the mean sensitivity calculated across all cell lines in the

panel. This is known as the mean graph. The same method of analysis was used to display the enzyme activity data for the NCI panel.

The student's unpaired t-test was used to compare results for enzyme activity in groups of human tumour cell lines from different origins, and in addition, to compare those results for the human glioma (glioblastoma) biopsy panel with the CNS (mainly glioblastoma) human tumour cell lines.

2.3 Results

2.3.1 Enzyme Activity Measurements within the NCI Human Tumour Cell Line Panel

A total of 69 human tumour cell lines from nine different tissue origins were analysed for DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase enzyme activities using a cytochrome *c* reduction assay.

(a) DT-diaphorase

Figure 2.1 represents a mean graph of log DT-diaphorase activity within the cell line panel. This type of analysis enables cell lines with either higher or lower than average activities to be clearly displayed. Enzyme activity across the cell line panel varied approximately 2000-fold (2.6 - 5123.1 nmol/min/mg of protein ; five cell lines possessed activity below that which was detectable, i.e. below 2 nmol/min/mg). Considerable variation existed, however, patterns of high and low enzyme activity expressing groups were clearly seen. For example, those cell lines originating from tumours of the colon, CNS, non-small cell lung and melanoma histiotypes showed higher than average activities while the leukaemic/lymphoma, breast and, to some extent, the ovarian lines showed lower than average activities. Nevertheless heterogeneity of expression was seen within as well as between particular tumour types. Of the two prostate cell lines assayed, for example, one was found to express relatively high DT-diaphorase activity while the other expressed a much lower level.

To illustrate intra-tumour heterogeneity of expression more precisely, the enzyme activity measurements for each group of cell lines is shown in Table 2.1. The variation in DT-diaphorase activity within each group was as follows : CNS, 12-fold (Table 2.1a); melanoma, 19-fold (Table 2.1b); colon, 25-fold (Table 2.1c); ovarian, 10-fold (Table 2.1d); breast, 115-fold (Table 2.1e); leukaemia/lymphoma, 1.2-fold (Table 2.1f); NSCLC, 20-fold and SCLC, 6-fold (Table 2.1g); prostate, 11-fold (Table 2.1h) and finally, kidney,

Figure 2.1 Mean graph of log DT-diaphorase activity within the NCI human tumour cell line panel.

The mean activity for all of the cell lines is represented by a vertical line through individual plots. Horizontal bars show, on a logarithmic scale, the individual enzyme activities for each cell line relative to the mean value. Those cell lines with activity below or above the mean lie to the left and right of the vertical line, respectively. MG-MID, Delta and Range are defined as the calculated mean value of the panel; the number of $\log_{10}$ units by which the values for the individual value of the panel differs (+ or -) from the MG-MID and the number of $\log_{10}$ units by which the value for the most sensitive line differs from the least sensitve line, respectively.

A) MEAN GRAPH OF LOG10 (DT DIAPHORASE)

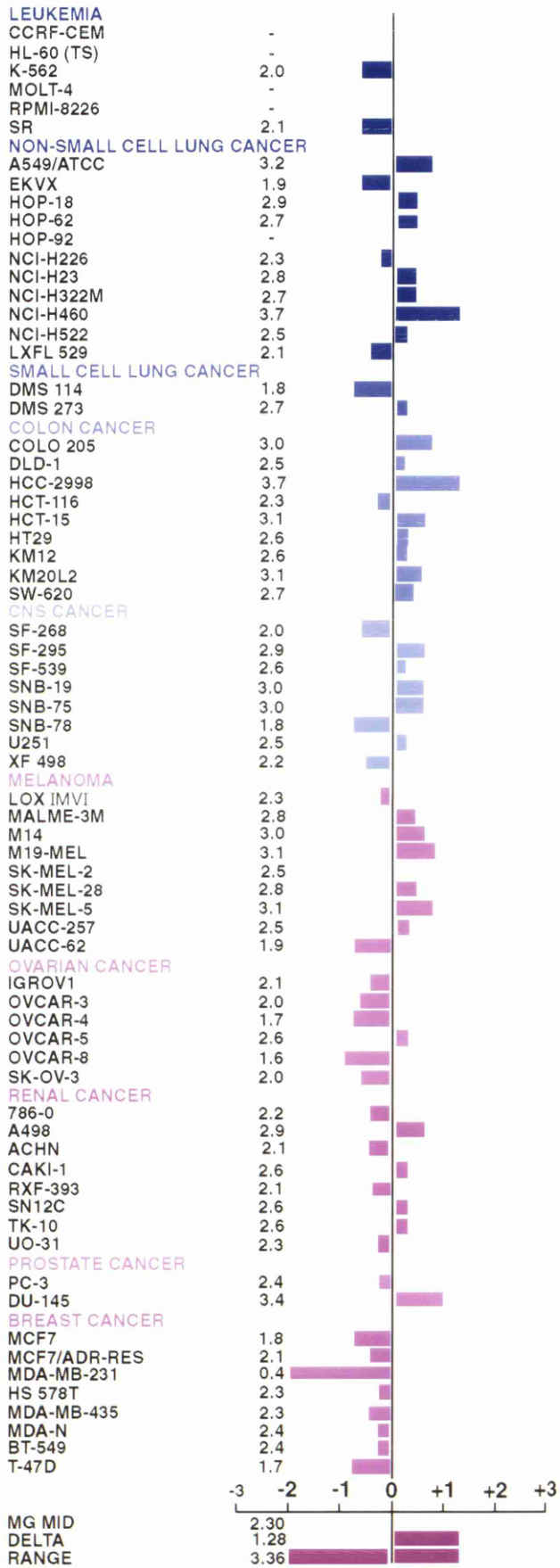


Table 2.1 Bioreductive enzyme activity measurements for the NCI human tumour cell line panel.

Units are nmol of cytochrome c reduced/min/mg of protein. Data represent a mean $\pm$ SE (n=3), except when denoted by * (two independent values). ND represents a level of activity (2 nmol/min/mg of protein or less) which is undetectable in this assay system.

a) CNS

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
SF 268	108.3 ± 13.9	20.9 ± 2.8	63.4 ± 6.8
SF 295	799.1 ± 27.1	15.2 ± 1.3	36.4 ± 0.5
SF 539	421.9 ± 18.8	19.9 ± 0.4	52.8 ± 2.4
SNB 19	1065.8 ± 68.2	9.8 ± 2.6	36.4 ± 3.9
SNB 75	1030.2 ± 6.9	16.7 ± 1.6	92.8 ± 5.3
SNB 78	76.2 / 101.1 *	14.7 / 30.8 *	72.8 ± 4.3
U 251	446.8 ± 11.6	13.6 ± 2.1	49.6 ± 3.3
XF 498	157.1 ± 2.8	9.7 / 12.9*	60.3 ± 6.6

b) Melanoma

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
UACC 62	81.0 / 78.1 *	13.2 / 18.8*	63.0 ± 2.7
UACC 257	355.5 ± 30.1	17.5 ± 1.3	40.6 ± 0.3
MALME	740.9 ± 13.1	37.7 ± 2.4	121.9 ± 12.9
M14	1038.6 ± 53.9	40.6 ± 1.1	87.7 ± 7.7
SK MEL 2	316.5 ± 39.0	6.6 ± 0.8	113.9 ± 8.2
SK MEL 5	1515.9 ± 71.7	36.7 ± 1.0	83.8 / 103.0 *
SK MEL 28	692.0 ± 45.0	25.4 ± 2.3	77.3 ± 0.5
M19 MEL	1497.1 ± 30.8	20.5 ± 0.9	52.7 ± 1.9

c) Colon

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
HT-29	412.3 / 488.9 *	20.2 / 21.7 *	34.6 / 33.9 *
HCT-116	199.4 / 210.7 *	21.0 / 21.0 *	53.3 / 57.7 *
HCT-15	1396.9 / 1660.2 *	21.4 / 41.0 *	64.1 / 65.9 *
DLD-1	345.6 / 361.4 *	15.8 / 21.6 *	15.8 / 40.3 *
COLO-205	948.6 / 1106.7 *	28.3 / 33.3 *	33.3 / 43.3 *
SW 620	460.9 / 586.1 *	22.4 / 44.7 *	35.8 / 53.7 *
KM20L2	1247.5 ± 35.7	13.1 ± 1.6	31.0 ± 3.2
KM12	490.8 ± 18.4	16.4 ± 1.0	29.2 ± 2.8
HCC 2998	5123.1 ± 263.4	21.4 ± 1.6	104.0 ± 10.8

d) Ovarian

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
SKOV-3	105.0 / 112.8 *	10.0 / 10.0 *	22.1 / 24.3 *
OVCAR 3	99.4 / 105.3 *	18.9 / 18.9 *	28.8 / 30.8 *
OVCAR 4	44.4 / 61.9 *	11.9 / 13.5 *	36.5 / 42.1 *
OVCAR 5	439.0 / 453.7 *	19.5 / 21.5 *	27.3 / 31.2 *
OVCAR 8	37.1 / 52.1 *	18.4 / 23.0 *	20.7 / 29.9 *
IGROV 1	123.1 / 145.9 *	16.9 / 16.9*	49.9 / 53.3 *

e) Breast

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
LOX IMVI	247.9 ± 16.0	16.4 ± 0.8	71.6 ± 3.6
MDA-N	299.6 ± 10.6	9.0 ± 1.3	17.1 ± 0.8
MDA-MB 231	2.6	13.7 ± 4.0	31.9 ± 5.1
MDA-MB 435S	198.1 ± 3.6	6.3 ± 1.2	34.2 ± 4.8
MCF-7	65.6 ± 2.4	11.8 ± 1.2	23.0 ± 1.7
MCF-7 / ADR	127.8 ± 9.2	13.4 ± 2.7	43.3 ± 11.0
HS 578T	213.1 ± 5.1	17.3 ± 1.5	81.1 ± 4.0
BT 549	271.9 ± 17.3	5.5 ± 0.4	36.2 ± 3.2
T 47 D	61.6 ± 8.9	6.7 / 8.2 *	53.2 ± 1.9

f) Leukaemia / Lymphoma

Tumour Cell Lines	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
K562	106.1 ± 7.3	29.1 ± 3.2	35.3 / 27.0 *
HL-60	*ND*	21.4 / 17.1 *	21.4 / 17.1 *
SR (TB)	111.8 / 140.6 *	25.8 ± 5.9	61.7 ± 2.3
MOLT 4	*ND*	19.9 / 27.1 *	34.3 / 37.9 *
RPMI 8226	*ND*	11.4 / 15.3 *	41.9 ± 5.8
CCRF CEM	*ND*	7.3 / 7.3 *	13.9 ± 1.7

g) Lung

Tumour Cell Lines	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochome b_5 Reductase
NSCLC			
LXFL 529	135.3 ± 6.6	12.5 ± 0.5	26.7 ± 0.7
A549 / ATCC	1952.1 ± 84.6	*ND*	9.2 / 11.4 *
NCI H522M	352.3 ± 11.4	9.3 ± 1.2	19.4 ± 2.1
NCI H23	666.9 ± 28.2	6.8 ± 0.8	15.2 ± 2.0
NCI H322M	568.0 / 594.7 *	4.9 ± 0.9	4.4 / 6.3 *
HOP 92 / 02	*ND*	2.4 / 4.8 *	22.8 ± 1.4
NCI H226	212.3 / 200.4 *	5.7 ± 0	13.7 ± 1.0
HOP 62	528.2 ± 13.2	2.9 / 2.9 *	19.5 ± 0.6
HOP 18	795.0 ± 24.0	8.1 ± 0.6	8.8 / 8.8 *
EKVX	93.5 ± 2.4	7.2 / 6.2 *	12.4 ± 2.1
SCLC			
DMS 114	73.8 ± 3.8	4.4 ± 0.3	7.9 / 6.8 *
DMS 273	495.5 ± 25.4	7.1 ± 0.9	13.9 ± 0.9
LARGE CELL			
NCI H460	6018.4 ± 213.7	12.8 / 12.8 *	34.3 ± 5.2

h) Prostate

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b_5 Reductase
DV 145	2834.4 ± 24.2	20.3 ± 2.0	75.2 ± 4.0
PC 3	251.2 ± 6.4	11.9 / 14.4 *	50.6 / 61.9 *

i) Kidney

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-Diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b_5 Reductase
CAKI 1	492.9 ± 36.3	18.4 ± 0.7	69.8 ± 3.7
SN 12C	455.5 ± 38.9	24.9 ± 0	48.2 ± 3.9
TK 10	466.4 ± 39.0	14.3 ± 0.8	48.0 ± 6.1
RXF 393	132.5 ± 11.8	19.6 / 24.2 *	50.9 ± 3.1
UO 31	216.5 ± 20.3	18.5 ± 0.6	35.5 ± 0
786.0	193.0 ± 10.9	26.8 / 33.0 *	57.0 ± 6.5
A498	827.5 ± 44.7	66.9 / 69.0 *	43.9 ± 1.7
ACHN	128.8 ± 10.6	14.0 ± 1.7	127.4 ± 12.3

6-fold (Table 2.1i). Interestingly, only two out of six leukaemia and lymphoma lines (Table 2.1f) exhibited detectable levels of DT-diaphorase activity.

The inter-tumour heterogeneity in DT-diaphorase activity across the cell line panel can be viewed graphically in Figure 2.2. Statistical analysis revealed interesting differences between the groups. Clearly, statistical analysis on all nine groups of cell lines could be included, however, as this would involve a total of 72 separate analysis for each enzyme, only selected examples are shown. As expected from the figure, the mean DT-diaphorase activity within the leukaemia/lymphoma group was significantly lower than, for example, the melanoma group (20.1-fold, $p<0.001$) or the CNS group (13.3-fold, $p<0.001$). Perhaps more surprising from the diagram, was the significant difference between the leukaemia/lymphoma group and the ovarian group (3.8-fold, $0.002<p<0.01$). Examples of other analysis reveal significant differences between colon / CNS cell lines (2.4-fold, $0.002<p<0.01$) and, colon / NSCLC (2.3-fold, $0.001<p<0.002$).

(b) NADPH : Cytochrome P450 Reductase

The activity of NADPH : cytochrome P450 reductase within the panel of cell lines is represented in Figure 2.3 in mean graph format. Although differences in enzyme activities existed both between and within the different groups of cell lines, the variation was less pronounced than that for DT-diaphorase (15-fold compared to 2000-fold). In addition, the overall level of NADPH : cytochrome P450 reductase activity was lower than for DT-diaphorase. Despite the lower overall range, patterns of high and low expressing groups did emerge. Cell lines originating from the colon, melanoma, kidney and, to some extent, the ovarian and leukaemic/lymphoma lines were found to have enzyme activities above the average level. In contrast, cell lines from the lung (both non-small cell and small cell) and breast possessed activities which were below average.

The range of NADPH : cytochrome P450 reductase activities within individual selected histiotypic groups of cell lines can be seen more clearly in Table 2.1 and in Figure 2.4. For example in the CNS group (Table 2.1a) the level of activity varied only 2-3-fold, while in the melanoma group (Table 2.1b) the variation was approximately 6-fold.

Figure 2.2 DT-diaphorase activity in the NCI human tumour cell line panel.

Graph shows the actual enzyme activities for all 69 cell lines within the panel. The mean value within each group is indicated by the bar.

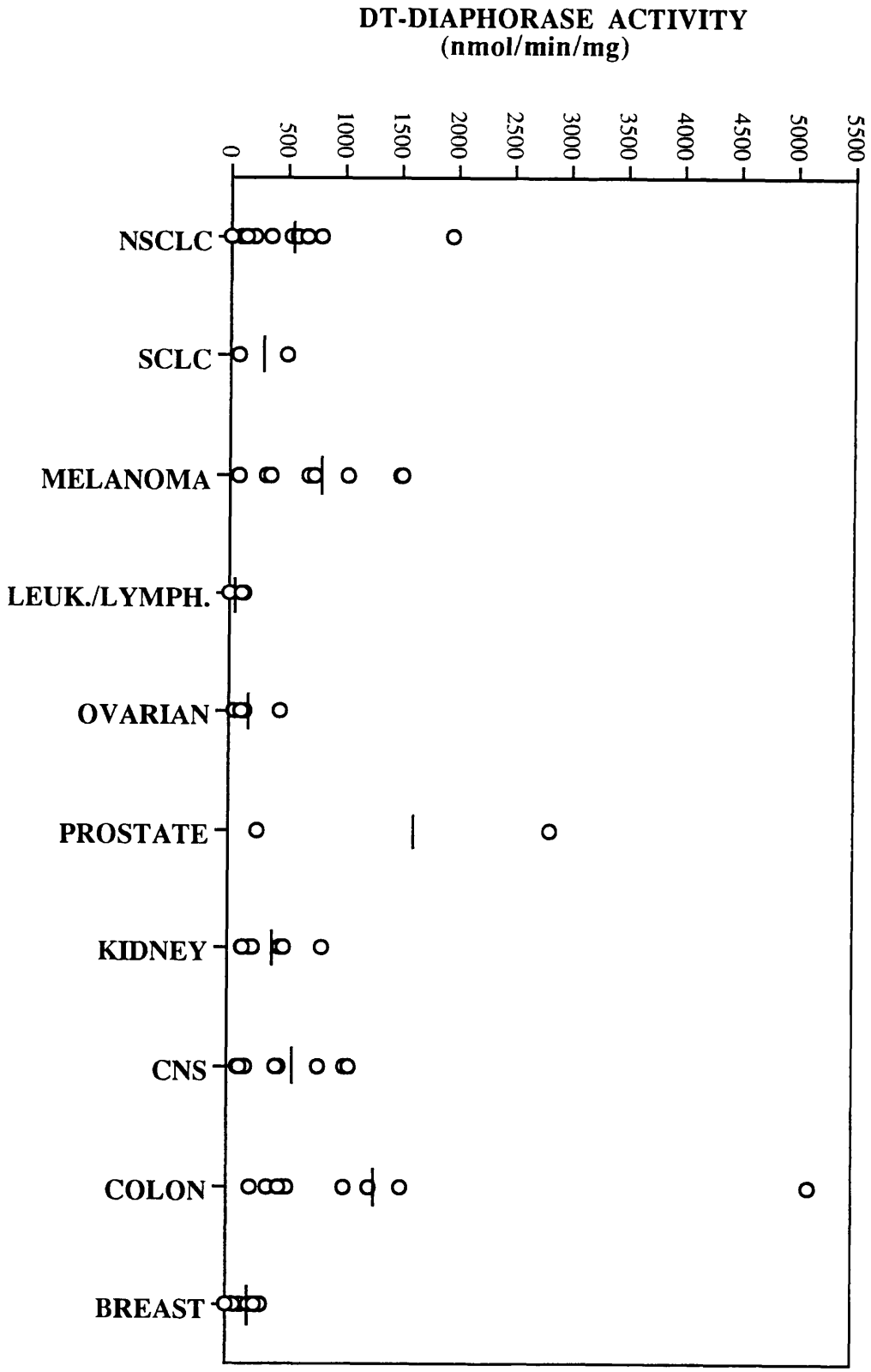

DT-DIAPHORASE ACTIVITY
(nmol/min/mg)
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
NSCLC
SCLC
MELANOMA
LEUK./LYMPH.
OVARIAN
PROSTATE
KIDNEY
CNS
COLON
BREAST

Figure 2.3 Mean graph of log NADPH : cytochrome P450 reductase activity within the NCI human tumour cell line panel.

The mean activity for all of the cell lines is represented by a vertical line through individual plots. Horizontal bars show, on a logarithmic scale, the individual enzyme activities for each cell line relative to the mean value. Those cell lines with activity below or above the mean lie to the left and right of the vertical line, respectively. For definition of MG-MID, Delta and Range see Figure 2.1 legend.

B) MEAN GRAPH OF LOG 10 (CYTOCHROME P450 REDUCTASE)

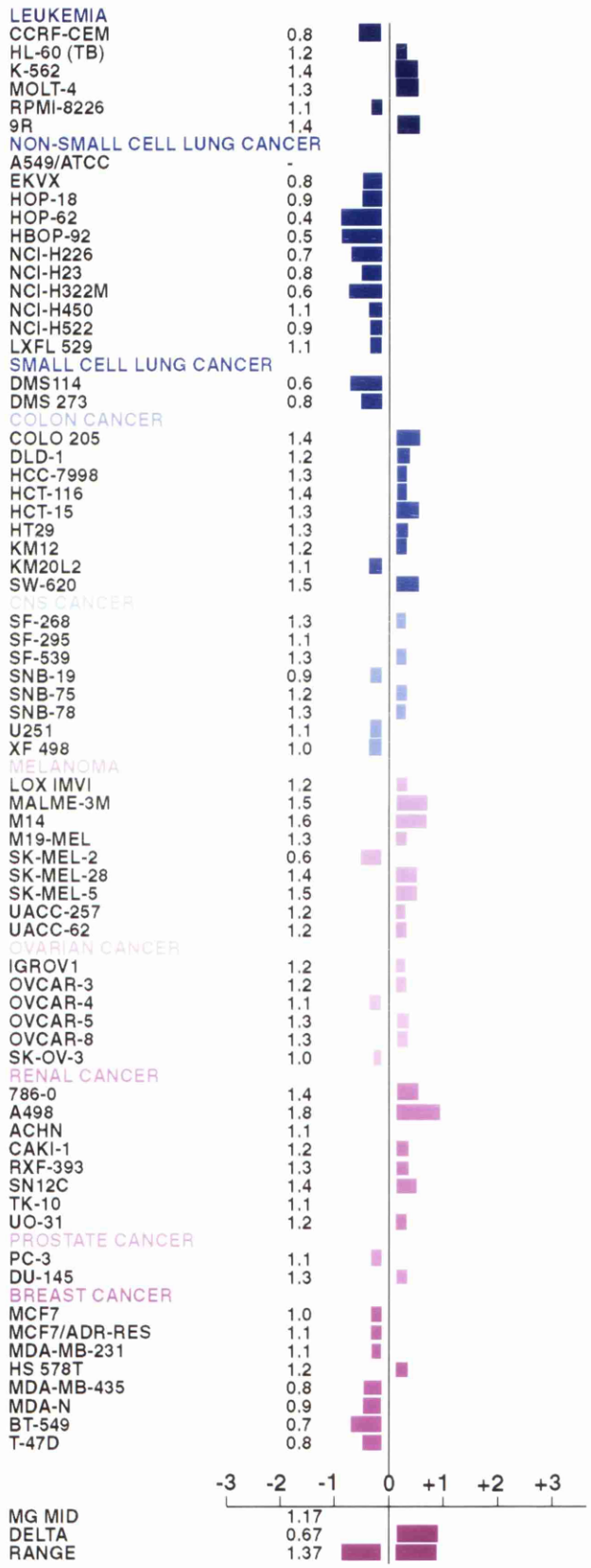
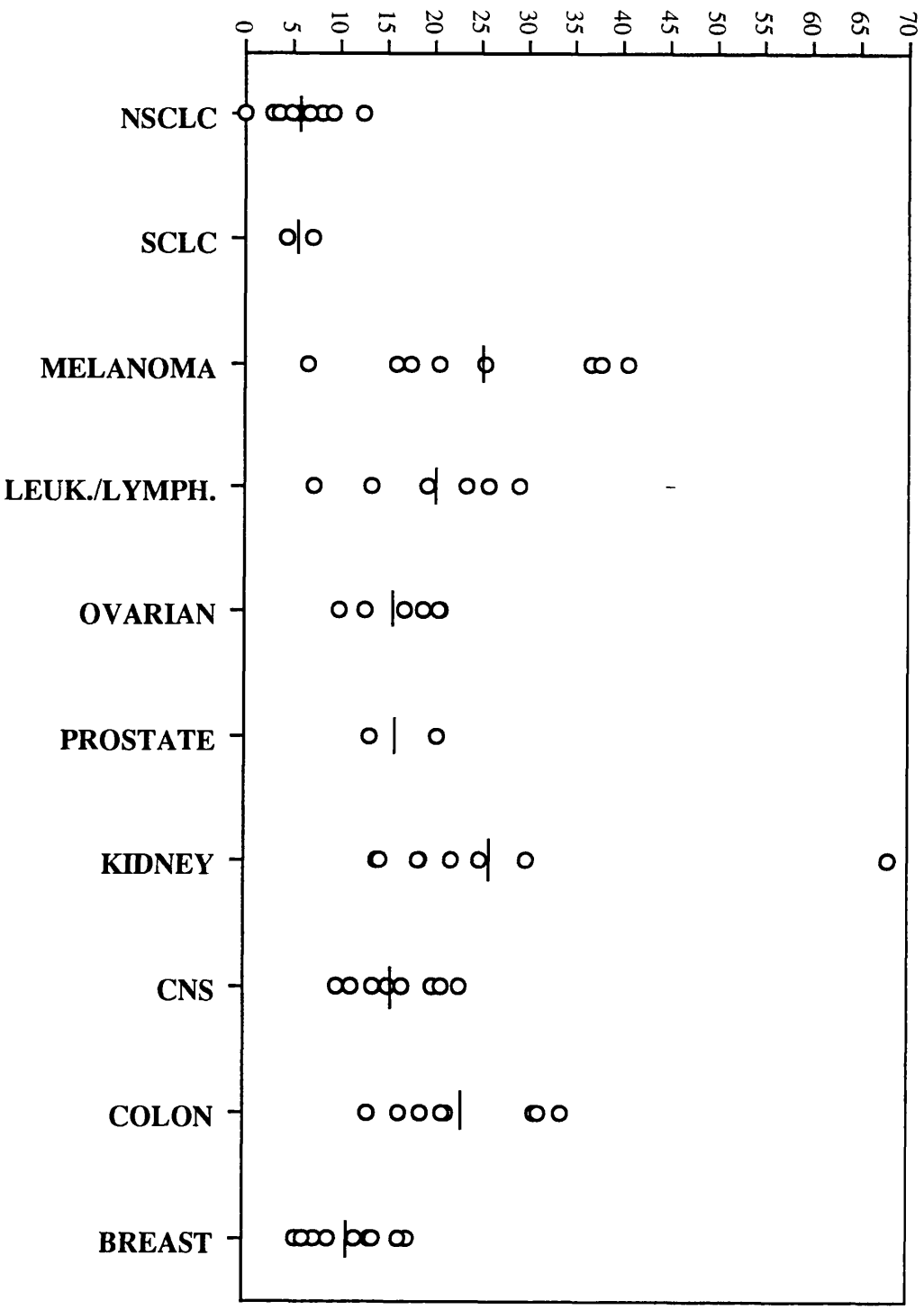


Figure 2.4 NADPH : cytochrome P450 reductase activity in the NCI human tumour cell line panel.

Graph shows the actual enzyme activities for all 69 cell lines within the panel. The mean value within each group is indicated by the bar.

NADPH : CYTOCHROME P450 REDUCTASE
ACTIVITY (nmol/min/mg)



The pattern of lower variation for NADPH : cytochrome P450 reductase compared to DT-diaphorase was true for all of the groups of cell lines studied. Inter-tumour heterogeneity can be viewed in Figure 2.4. In contrast to DT-diaphorase activity, the differences in mean NADPH : cytochrome P450 reductase activity between leukaemia/lymphoma and several other groups of cell lines, for example, melanoma ($0.02<p<0.05$) and CNS ($0.02<p<0.05$) were non-significant. Interestingly, however, the mean activity of the breast group differed significantly from those of the colon, ovarian, NSCLC and melanoma ($p<0.001$ in all cases).

(c) NADH : Cytochrome b5 Reductase

A mean graph of log NADH : cytochrome b5 reductase activity within the cell line panel is shown in Figure 2.5. Similar to NADPH : cytochrome P450 reductase, the overall level of NADH : cytochrome b5 reductase was lower than that for DT-diaphorase (24-fold compared to 2000-fold). As with the other two enzymes under study, patterns of high and low enzyme expressing groups were found. For example, cell lines from the CNS, melanoma and kidney expressed activities which were higher than average. In contrast, cell lines from the lung (both non-small cell and small cell), ovary and, to some extent the breast, possessed activities which, with the exception of one ovarian cell line, were below average.

Although the activity of NADH : cytochrome b5 reductase was generally higher than that of NADPH : cytochrome P450 reductase in all of the groups of cell lines studied, the range between the highest and lowest activity within the groups was similar. For example, the range in both NADH : cytochrome b5 reductase and NADPH : cytochrome P450 reductase activities within the CNS cell lines (Table 2.1a) was 2-3-fold. As with the previous two enzymes, the inter-tumour heterogeneity of NADH : cytochrome b5 reductase activity across the groups of cell lines is shown graphically in Figure 2.6. Both significant and non-significant differences were again clear from the graph. For example, while NADH : cytochrome b5 reductase activity was shown to be significantly different between melanoma / CNS (1.4-fold, $p<0.001$) and melanoma /

Figure 2.5 Mean graph of log NADH : cytochrome b_5 reductase activity within the NCI human tumour cell line panel.

The mean activity for all of the cell lines is represented by a vertical line through individual plots. Horizontal bars show, on a logarithmic scale, the individual enzyme activities for each cell line relative to the mean value. Those cell lines with activity below or above the mean lie to the left and right of the vertical line, respectively. For definition of MG-MID, Delta and Range see Figure 2.1 legend.

C) MEAN GRAPH OF LOG10 (CYTOCHROME b5 REDUCTASE)

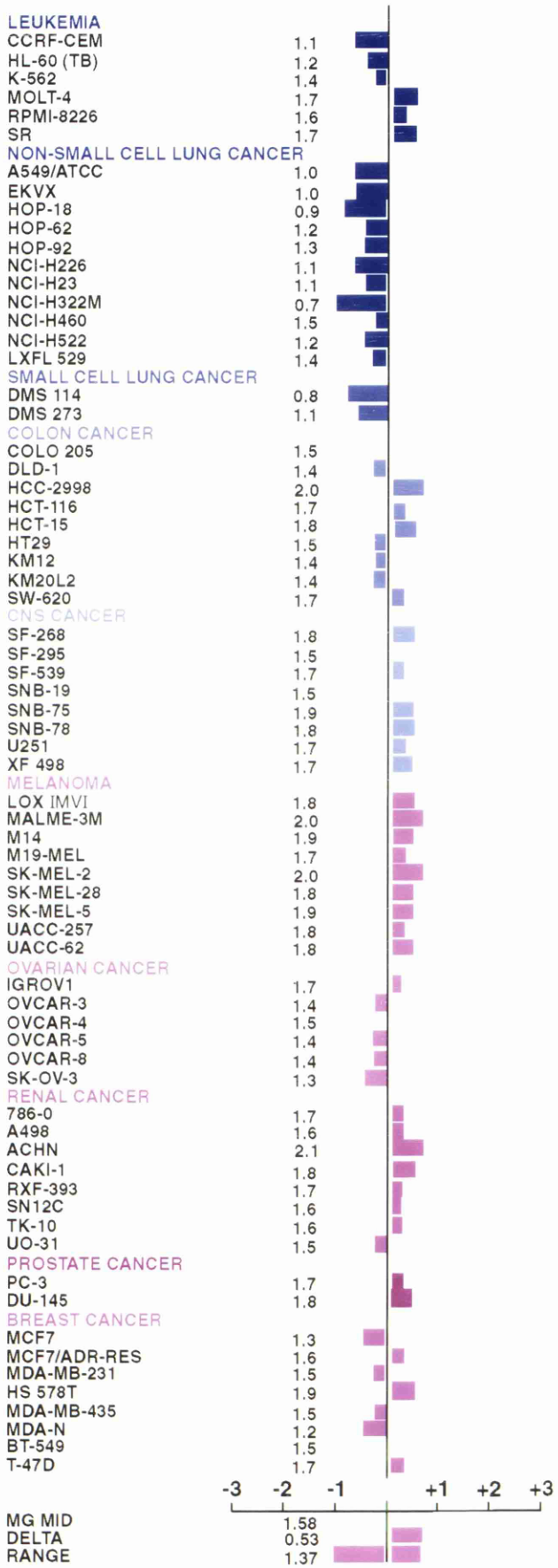
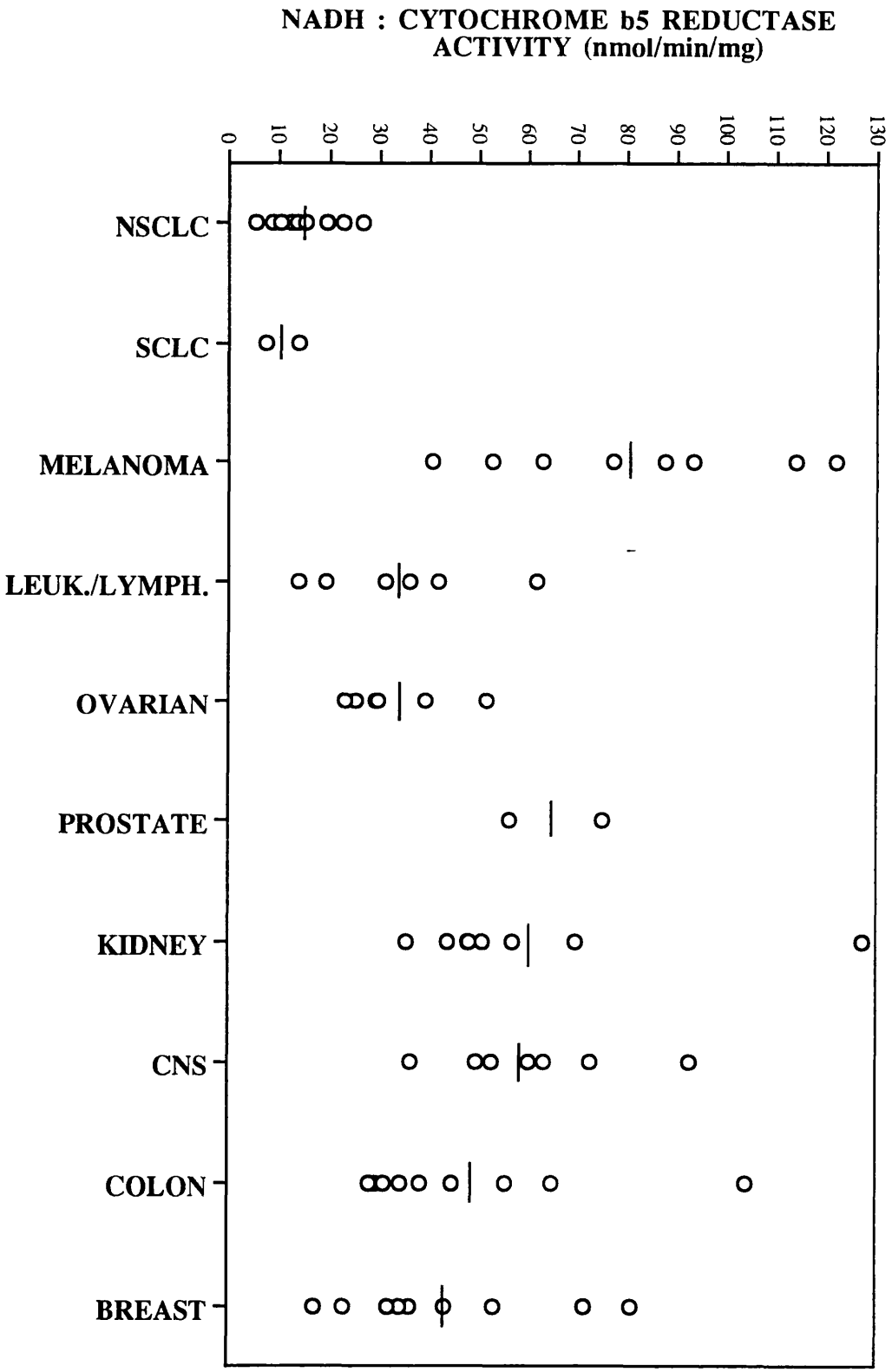


Figure 2.6 NADH : cytochrome b_5 reductase activity in the NCI human tumour cell line panel.

Graph shows the actual enzyme activities for all 69 cell lines within the panel. The mean value within each group is indicated by the bar.


NADH : CYTOCHROME b5 REDUCTASE
ACTIVITY (nmol/min/mg)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
NSCLC
SCLC
MELANOMA
LEUK./LYMPH.
OVARIAN
PROSTATE
KIDNEY
CNS
COLON
BREAST

leukaemia/lymphoma cell lines (2.4-fold, $p<0.001$), it was non-significant between melanoma / prostate cell lines ($p>0.1$) and leukaemia/lymphoma / ovarian ($p>0.1$).

Collectively, therefore, DT-diaphorase was found to be the enzyme exhibiting highest activity within the cell line panel. In addition, the range in activity was also greatest for this enzyme (approximately 2000-fold). In contrast, the variability in NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase activities was much lower (15- and 24-fold, respectively). Importantly for each enzyme, significant differences were observed between the mean enzyme activities of different groups of cell lines. An interesting point to note, however, was that the differences between NSCLC and SCLC cell lines were non-significant (DT-diaphorase - $p>0.1$; NADPH : cytochrome P450 reductase - $p>0.1$; NADH : cytochrome b_5 reductase - $0.02<p<0.05$) for each of the enzymes.

2.3.2 Protein Expression within the NCI Human Tumour Cell Line Panel

To complement the enzyme activity measurements, all human tumour cell lines within the panel were analysed for both DT-diaphorase and NADPH : cytochrome P450 reductase protein expression. NADH : cytochrome b_5 reductase was not studied as no antibody against the enzyme was available.

(a) DT-diaphorase

DT-diaphorase protein expression was detected in 65 out of a total of 69 cell lines. As with the enzyme activities, a wide range of protein expression was observed both within and between the different tumour types. The Western blots for each group of cell lines are shown in Figure 2.7. For ease of discussion, however, melanoma, CNS and breast groups will be taken as examples. All of the melanoma cell lines studied were shown to express DT-diaphorase protein with the range of levels clearly seen (Figure 2.7b). In contrast, only 6 out of 8 CNS cell lines (Figure 2.7a) exhibited DT-diaphorase

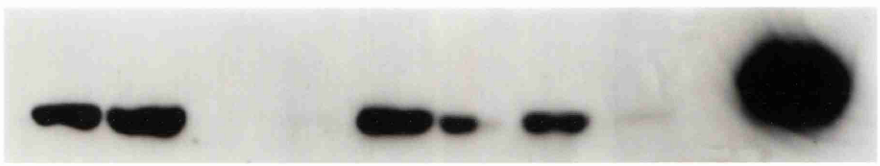
Figure 2.7 Western blot analysis of DT-diaphorase expression within human tumour cell lines.

(a) **CNS** : lanes 1 - 8 represent SNB 19, SNB 75, SNB 78, SF 268, SF 295, SF 539, U251 and XF 498 cell lines, respectively. A purified preparation of the rat form of DT-diaphorase (33kD) was also run as a standard.

(b) **Melanoma** : lanes 1 - 8 represent SK MEL 2, SK MEL 5, SK MEL 28, M14, M19 MEL, MALME, UACC 62 and UACC 257 cell lines, respectively. A purified preparation of the rat form of DT-diaphorase (33kD) was also run as a standard.

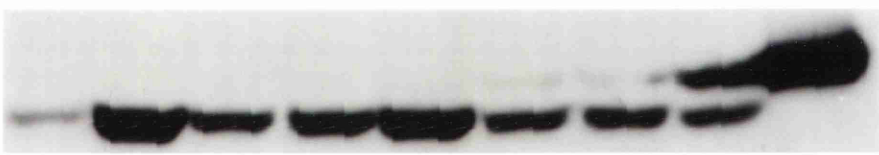
(c) **Colon** : lanes 1 - 9 represent KM 12, KM 20L2, HCC 2998, HCT-116, COLO-205, SW 620, DLD-1, HCT-15 and HT-29 cell lines, respectively. Note that the samples were run on two separate gels, and hence, no DT-diaphorase standard is shown in this case.

A)



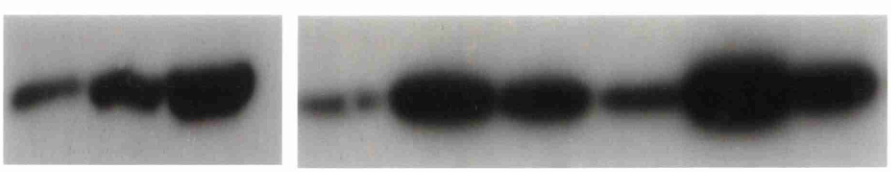
1 2 3 4 5 6 7 8 RAT P450R STD RAT DT-D STD

B)



1 2 3 4 5 6 7 8 RAT DT-D STD

C)



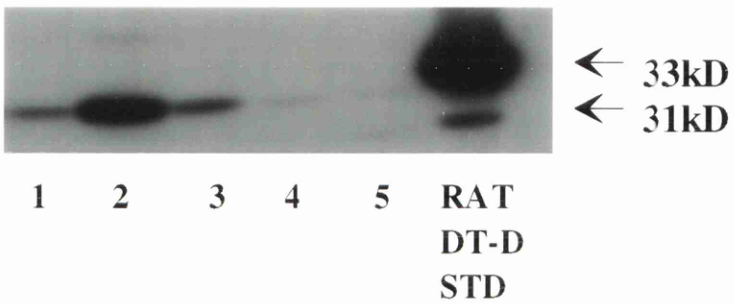
1 2 3 4 5 6 7 8 9

(d) **Ovarian** : lanes 1 - 6 represent OVCAR 8, OVCAR 5, SKOV-3, OVCAR 3 and OVCAR 4 cell lines, respectively. A purified preparation of the rat form of DT-diaphorase (33kD) was also run as a standard.

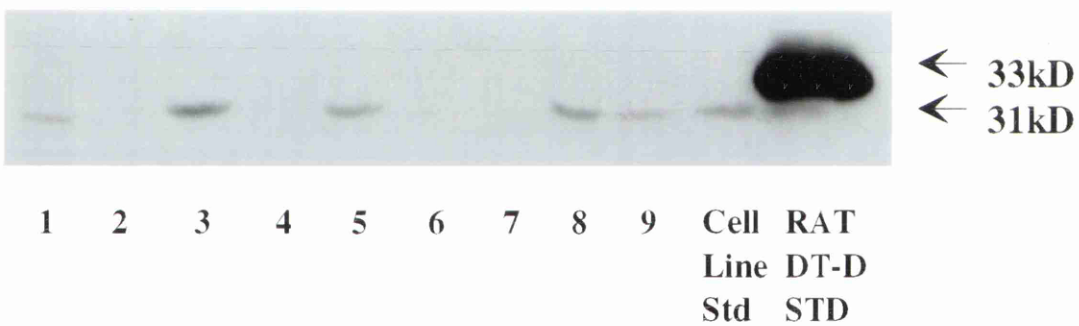
(e) **Breast** : lanes 1 - 9 represent MCF-7, MCF-7/Adr, MDA-N, MDA-MB 231, MDA-MB 435S, LOX IMVI, T47D, BT 549 and HS 578T cell lines, respectively. An HT-29 tumour cell line sample and a purified preparation of the rat form of DT-diaphorase (33kD) were ran as a standards.

(f) **Leukaemia/Lymphoma** : lanes 1 - 6 represent SR (TB), HL-60, K562, CCRF CEM, RPMI 8226 and MOLT 4 cell lines, respectively. A purified preparation of the rat form of DT-diaphorase (33kD) was also run as a standard.

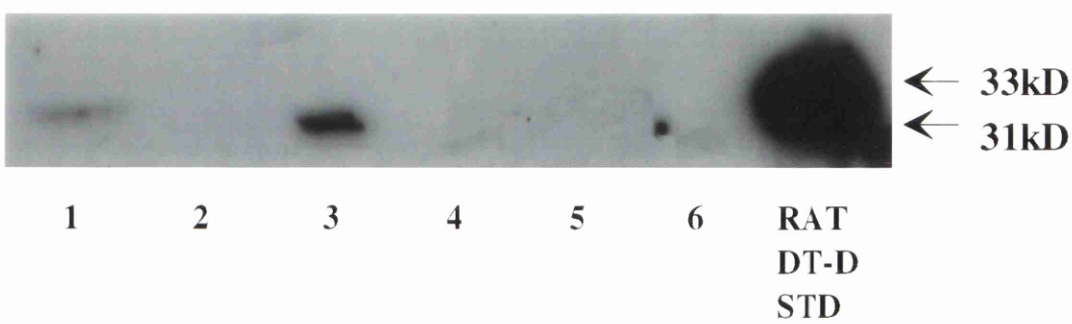
D)


33kD
31kD
1
2
3
4
5
RAT
DT-D
STD

E)


33kD
31kD
1
2
3
4
5
6
7
8
9
Cell
Line
Std
RAT
DT-D
STD

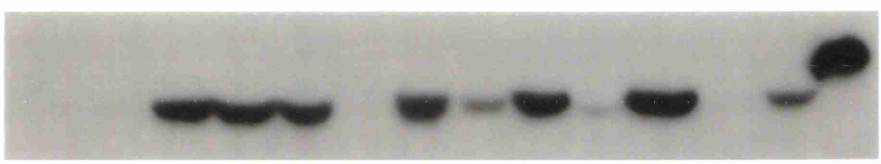
F)


33kD
31kD
1
2
3
4
5
6
RAT
DT-D
STD

(g) **Lung** : lanes 1 - 10 represent NSCLC cell lines EKVX, LXFL 529, A549/ATCC, HOP 18, HOP 62, HOP 92/02, NCI H23, NCI H226, NCI H322M and NCI H522M, respectively. Lane 11 contains the large cell line NCI H460 and lanes 12 - 13 contains the SCLC lines DMS 114 and DMS 273, respectively. A purified preparation of the rat form of DT-diaphorase (33kD) was also run as a standard.

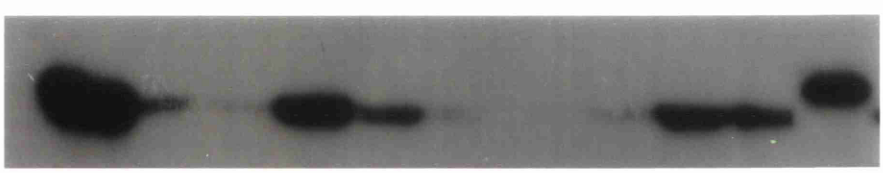
(h) **Prostate and kidney** : lanes 1 - 2 represent prostate cell lines DV 145 and PC 3, respectively. Lanes 3 - 10 represent kidney cell lines ACHN, A598, CAKI 1, 786.0, RXF 393, UO 31, TK 10 and SN 12C, respectively. A purified preparation of the rat form of DT-diaphorase (33kD) was also run as a standard.

G)



1 2 3 4 5 6 7 8 9 10 11 12 13 RAT DT-D STD

H)



1 2 3 4 5 6 7 8 9 10 RAT DT-D STD

protein, although the range in the level of expression was more notable, with some having relatively high levels of expression (Lanes 1, 2, 5 and 7) and others showing little or no detectable protein (Lanes 3, 4 and 8). In the case of the breast cell lines (Figure 2.7e), the overall protein expression was less than for the other two groups of cell lines. As with the CNS cell lines however, not all cell lines showed protein expression.

To determine whether the observed wide range of enzyme activities could be explained by a correlation with the DT-diaphorase protein content of the cell line, the results obtained with the two different endpoints were compared. Enzyme activity and protein expression (quantified by densitometry) were found to correlate well in the vast majority of cell lines studied. The comparison between DT-diaphorase activity and protein expression for each of the cell line groups (except the prostate group which contained only two cell lines) are illustrated in Figure 2.8. Correlation coefficients are shown in Table 2.2 and range from 0.394 - 0.938. A small number of discrepancies between DT-diaphorase protein expression and enzyme activity were found. These were mainly confined to several of the breast or ovarian cell lines which had very low levels.

(b) NADPH : Cytochrome P450 Reductase

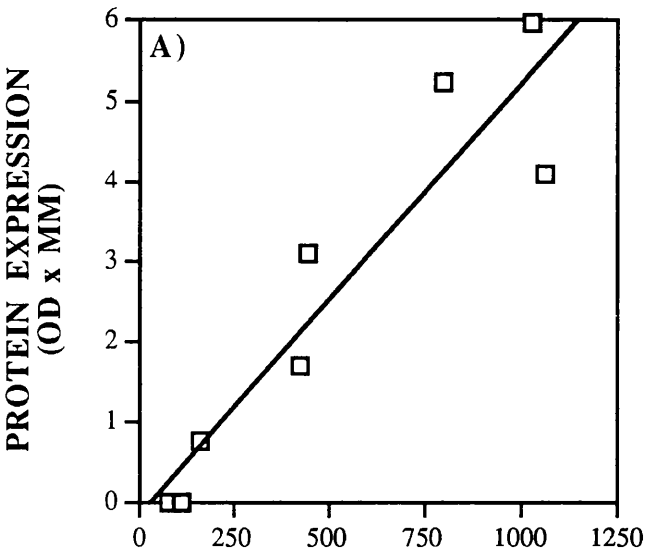
As far as NADPH : cytochrome P450 reductase expression was concerned, no detectable protein was found in any of the cell lines studied (data not shown). The antibody used to detect the enzyme was, however, functional as the purified rat NADPH : cytochrome P450 reductase standard was detected on each occasion. Therefore, the lack of NADPH : cytochrome P450 reductase protein expression within the cell lines was thought to be due to protein levels being too low for detection using the antibody against the rat protein.

2.3.3 Detection of DT-diaphorase and NADPH : cytochrome P450 reductase by RT-PCR

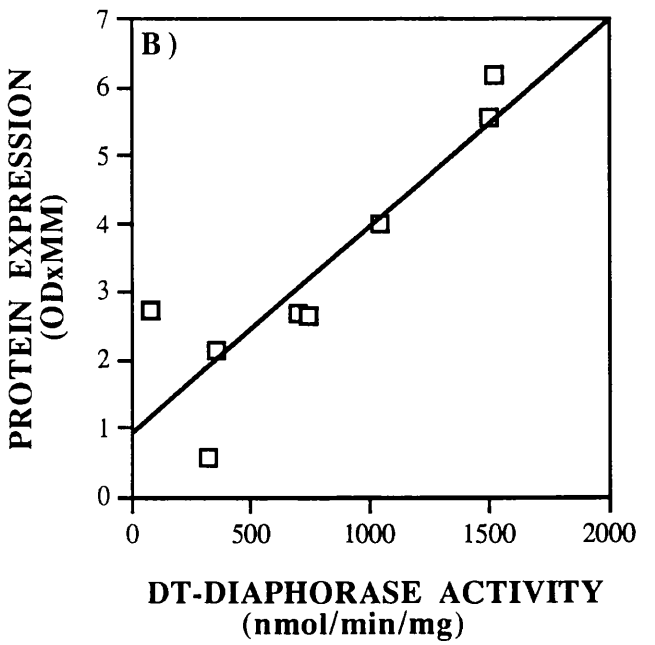
To explore the reasons underlying those few instances in which weak correlations were seen between DT-diaphorase protein expression and enzyme activity within the

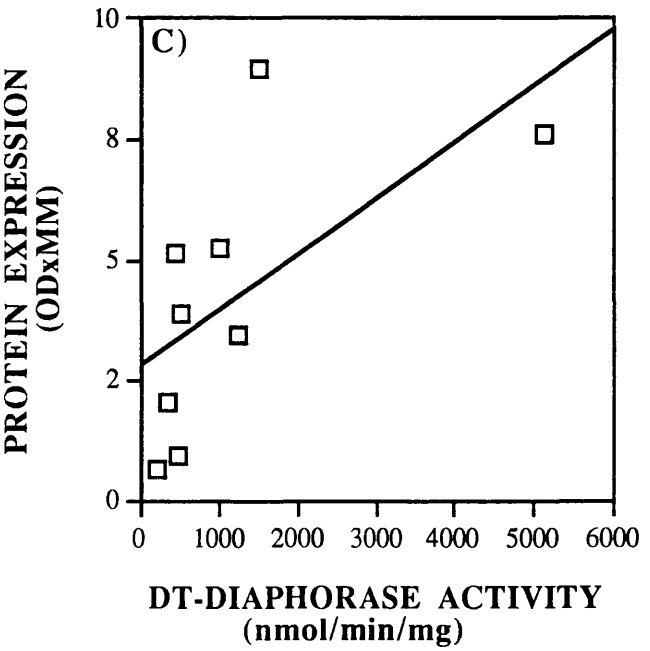
Figure 2.8 Correlation between DT-diaphorase protein expression and enzyme activity in individual groups of human tumour cell lines.

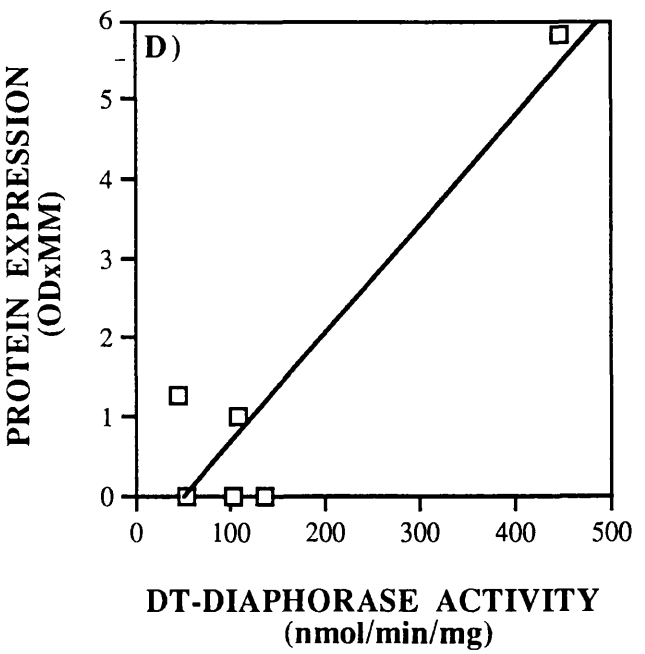
(A) CNS ; (B) melanoma ; (C) colon : (D) ovarian ; (E) breast ; (F) leukaemia / lymphoma ; (G) lung and (H) kidney.

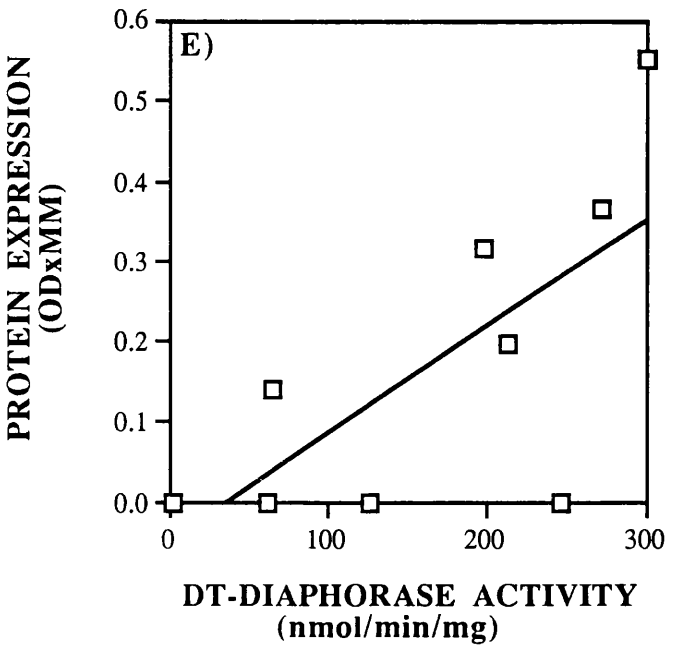


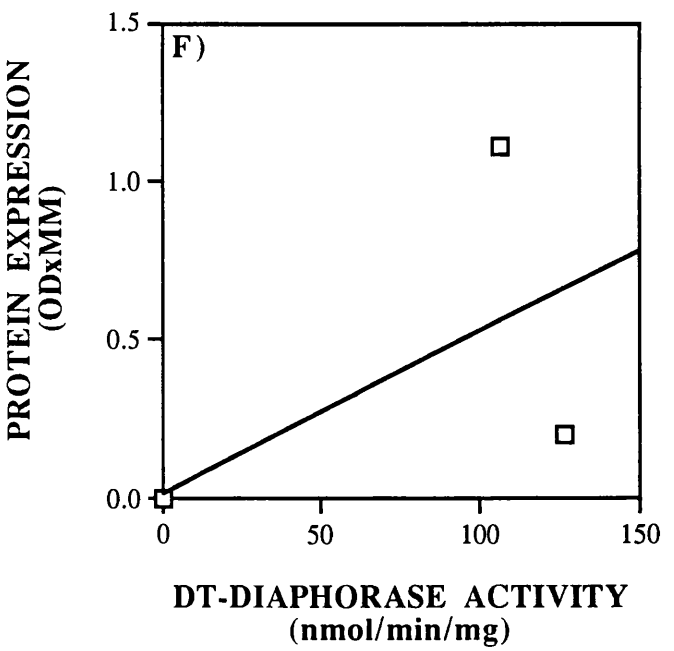
$y = 0.005x - 0.167 \quad r^2 = 0.872$

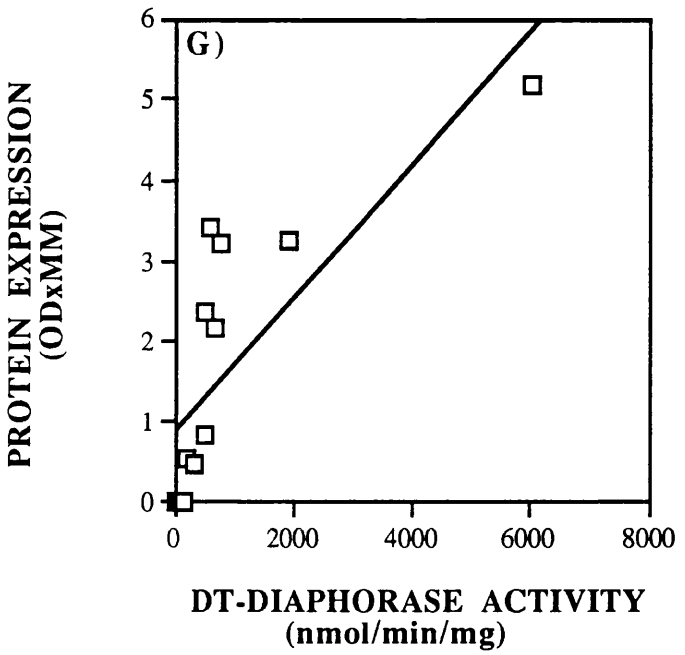


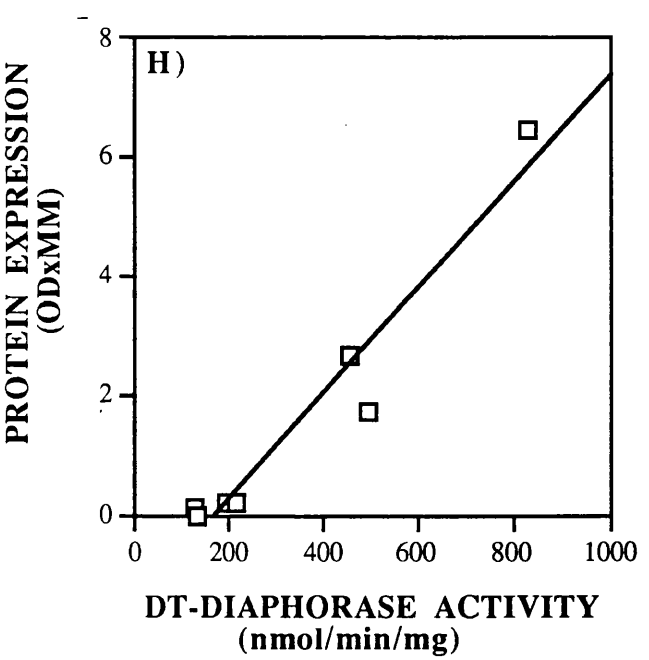

C)
PROTEIN EXPRESSION
(ODxMM)
10
8
5
2
0
0
1000
2000
3000
4000
5000
6000
DT-DIAPHORASE ACTIVITY
(nmol/min/mg)


D)
PROTEIN EXPRESSION
(ODxMM)
6
5
4
3
2
1
0
0
100
200
300
400
500
DT-DIAPHORASE ACTIVITY
(nmol/min/mg)


E)
PROTEIN EXPRESSION
(ODxMM)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
DT-DIAPHORASE ACTIVITY
(nmol/min/mg)


F)
PROTEIN EXPRESSION
(ODxMM)
1.5
1.0
0.5
0.0
0
50
100
150
DT-DIAPHORASE ACTIVITY
(nmol/min/mg)


G)
PROTEIN EXPRESSION
(ODxMM)
0
1
2
3
4
5
6
0
2000
4000
6000
8000
DT-DIAPHORASE ACTIVITY
(nmol/min/mg)


H)
PROTEIN EXPRESSION
(ODxMM)
0
2
4
6
8
0
200
400
600
800
1000
DT-DIAPHORASE ACTIVITY
(nmol/min/mg)

ORIGIN OF CELL LINE	CORRELATION COEFFICIENT
MELANOMA	0.788 (n=8)
OVARY	0.847 (n=6)
CNS	0.872 (n=8)
KIDNEY	0.938 (n=8)
BREAST	0.498 (n=9) **
LUNG	0.595 (n=13) **
COLON	0.394 (n=9)
PROSTATE	- *
LEUKAEMIA / LYMPHOMA	0.475 (n=5) **

Table 2.2 Level of correlation between two end-points, DT-diaphorase enzyme activity and protein expression, within each group of human tumour cell lines.

Values represent the correlation coefficient (r^2) obtained when two different methods of assaying DT-diaphorase levels within the cell line panel were compared. * denotes insufficient numbers available; ** denotes those groups of cell lines which contained obvious outliers resulting in lower correlation coefficients (see Figure 2.8).

breast and ovarian cell lines, the more sensitive RT-PCR assay for DT-diaphorase was performed. All human tumour cell lines originating from the breast, and as a control four cell lines originating from the CNS, were assayed for both DT-diaphorase and NADPH : cytochrome P450 reductase RNA levels using RT-PCR. The breast tumour cell lines were selected for study in preference to the ovarian lines due to the higher number within the group.

In the case of the human breast tumour cell lines, all nine were found to express DT-diaphorase RNA although the level of expression varied. For illustration, Figure 2.9 shows the results for two breast cell lines, MCF-7 and MCF-7/Adr. The level of NADPH : cytochrome P450 reductase RNA in the breast cell lines was low, although it was detectable in seven out of nine of the cell lines.

As a control, four CNS human tumour cell lines were analysed. The results for two of these cell lines, XF 498 and SF 539, are shown in Figure 2.10. Once again, DT-diaphorase RNA was detectable in all four cell lines studied, although the levels varied. NADPH : cytochrome P450 reductase RNA was detected only at very low levels in the CNS cell lines.

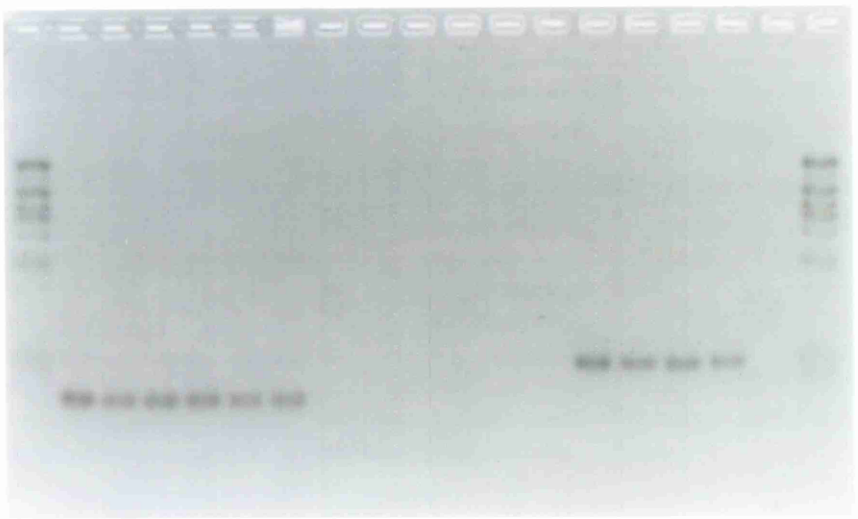
2.3.4 Enzyme Activity Measurements within Human Malignant Brain Tumours

A series of human malignant brain tumours were available to study the levels of bioreductive enzymes such as DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase. Table 2.3 shows the enzyme activity measurements obtained for eight tumour biopsies using a cytochrome *c* reduction assay (section 2.2.6.). As with the cell lines, heterogeneity of expression was seen within the tumour biopsies for each enzyme studied. The variability in activity for DT-diaphorase, NADH : cytochrome b_5 reductase and NADPH : cytochrome P450 reductase was approximately 17-fold, 11-fold and 8-fold, respectively. Interestingly, DT-diaphorase activity exhibited within the human tumour biopsies was significantly lower than those measured in the cell lines, and more significantly CNS cell lines (8.9-fold, $p<0.001$) (Table 2.1a). The

Figure 2.9 Expression of DT-diaphorase and NADPH : cytochrome P450 reductase RNA in the human breast tumour cell lines MCF-7 (A), and MCF-7/Adr (B).

Lanes 2 - 7 represent DT-diaphorase RNA, lanes 8 - 13 represent NADPH : cytochrome P450 reductase RNA and lanes 14 - 17 represent ribosomal 28S RNA as a positive control. Lanes 1 and 19 contain DNA ladders while lane 18 contains a minus RNA control.

A)


492 bp
369 bp
DT-D
246 bp
Cyt P450 Red
123 bp
28S Ribosomal
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

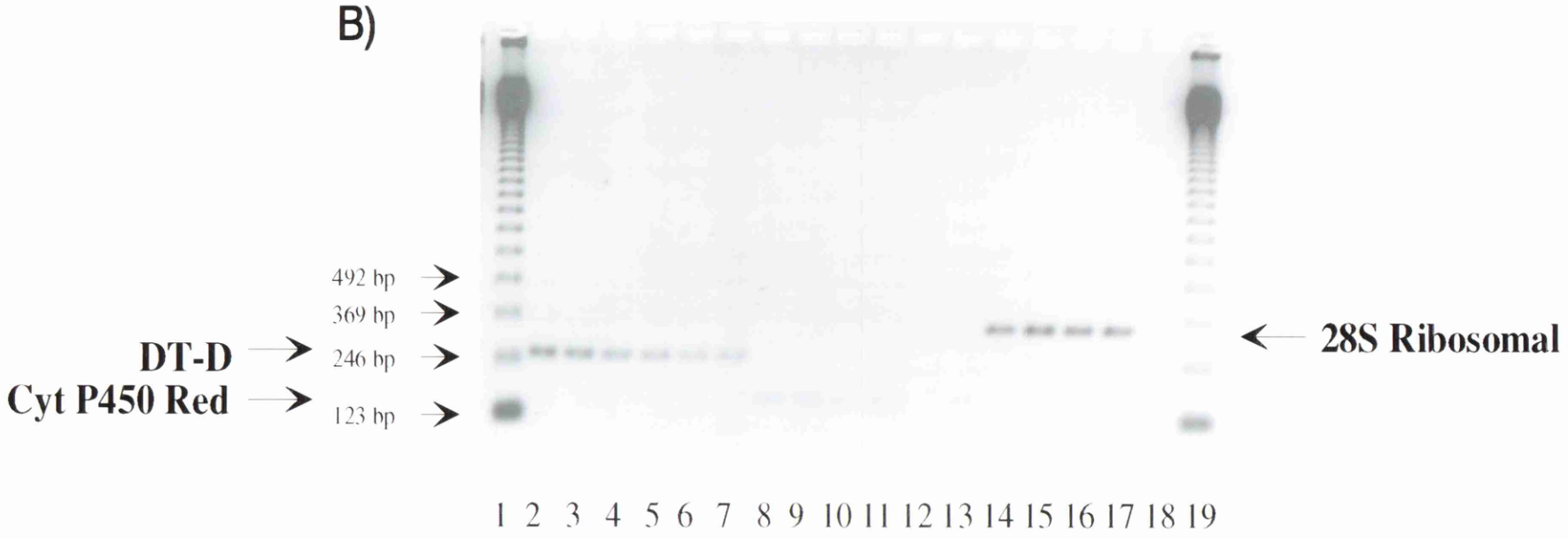
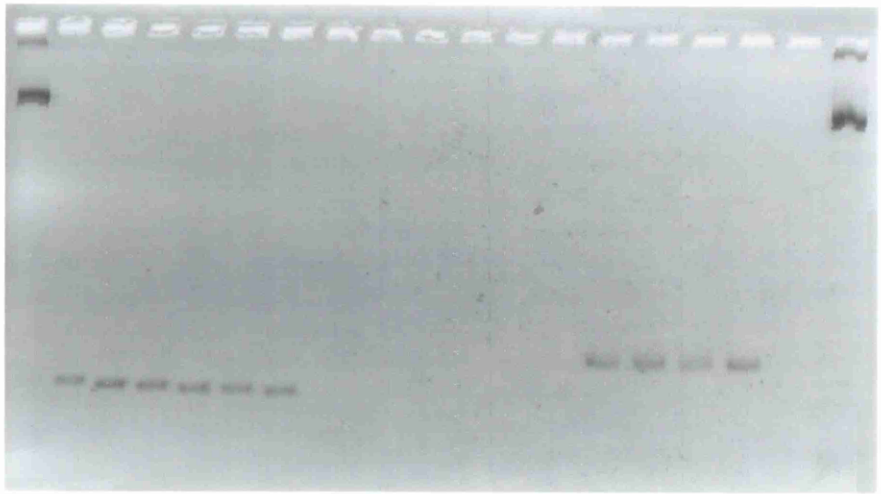

B)
492 bp
369 bp
DT-D
246 bp
Cyt P450 Red
123 bp
28S Ribosomal
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

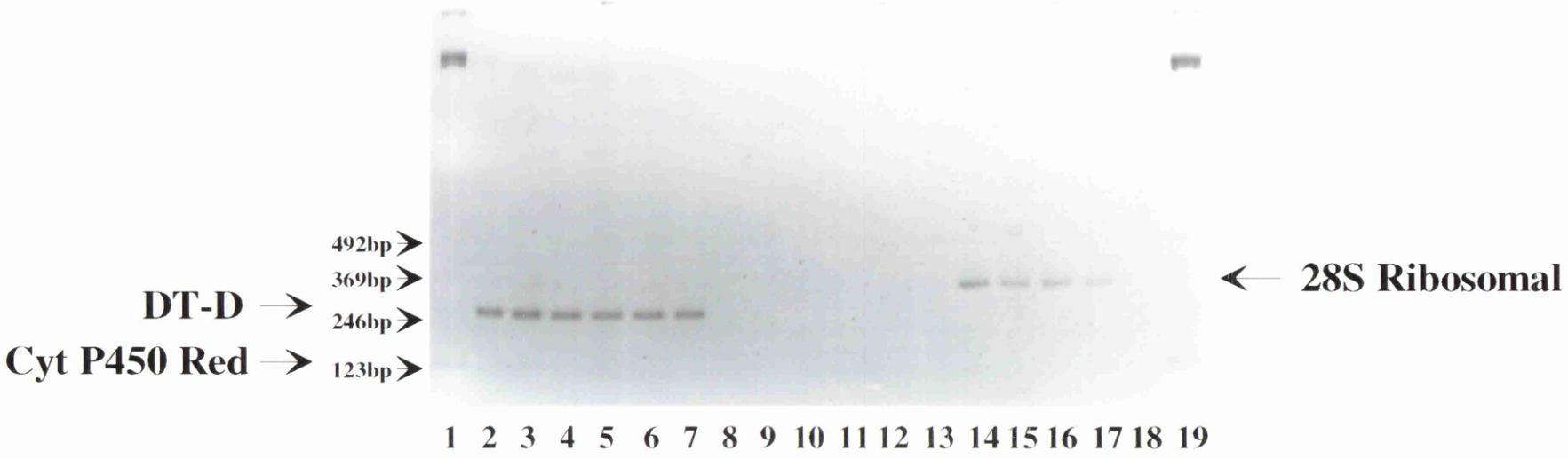
Figure 2.10 Expression of DT-diaphorase and NADPH : cytochrome P450 reductase RNA in the human CNS tumour cell lines XF 498 (A), and SF 539 (B).

Lanes 2 - 7 represent DT-diaphorase RNA, lanes 8 - 13 represent NADPH : cytochrome P450 reductase RNA and lanes 14 - 17 represent ribosomal 28S RNA as a positive control. Lanes 1 and 19 contain DNA ladders while lane 18 contains a minus RNA control.

A)


492bp
369bp
246bp
123bp
DT-D
Cyt P450 Red
28S Ribosomal
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

B)


492bp
369bp
246bp
123bp
DT-D
Cyt P450 Red
28S Ribosomal
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Tumour Pathology	Enzyme Activity (nmol/min/mg)		
	DT-diaphorase	NADH : Cytochrome b5 Reductase	NADPH : Cytochrome P450 Reductase
Glioblastoma	24.1 / 18.2	20.8 / 21.4	10.2 / 9.6
Sarcoma	67.5 / 46.7	85.0 / 90.5	22.3 / 16.4
Low grade glioma	189.8 / 176.1	180.4 / 179.7	41.1 / 38.2
Glioblastoma	25.5 / 25.0	16.2 / 16.0	7.8 / 7.5
Glioblastoma	11.3 / 10.3	25.0 / 22.1	11.8 / 13.8
Glioblastoma	28.9 / 25.1	31.2 / 30.2	5.8 / 4.8
Glioblastoma	106.6 / 121.5	33.6 / 30.6	41.8 / 44.0
Glioblastoma	24.5 / 24.1	21.0 / 19.9	17.0 / 13.5

Table 2.3 Bioreductive enzyme activity measurements in human malignant brain tumours.

Units are nmol of cytochrome c reduced/min/mg of protein. Data represent two independent readings.

NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase activities however, were not significantly different ($0.05<p<0.1$ and $p>0.1$, respectively) between the tumor biopsies and CNS cell lines.

2.3.5 Protein Expression within Human Malignant Brain Tumours

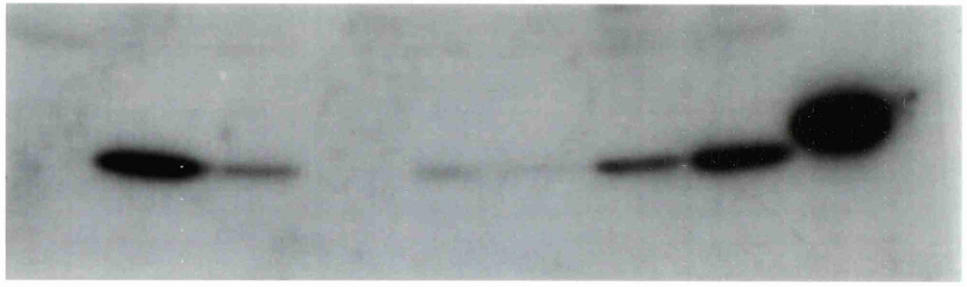
As was the case with the tumour cell lines, human tumour biopsies were analysed for both DT-diaphorase and NADPH : cytochrome P450 reductase protein expression, the results of which are shown in Figure 2.11. Note that at time of determining protein expression levels, only six samples were available. Of the six brain tumour biopsies studied, 4 were shown to express DT-diaphorase protein. The level of expression was variable, but correlated with the enzyme activity ($r^2=0.876$) (Figure 2.12). As previously demonstrated for the human tumour cell lines, NADPH : cytochrome P450 reductase protein was not detected in the tumour biopsies, most probably due to the low levels of protein present within the samples and the sensitivity of the antibody.

Figure 2.11 Western blot analysis of DT-diaphorase expression (A) and NADPH : cytochrome P450 reductase (B) within human malignant brain tumours.

A) Lanes 1 - 6 represent individual brain biopsy samples. In addition, lanes 7 and 8 show two human melanoma tumour biopsies. A purified preparation of the rat form of DT-diaphorase (33kD) was run as a standard.

B) As in A) but with a purified preparation of the rat form of NADPH : cytochrome P450 reductase ran as a standard.

A)



RAT DT-D
HUMAN DT-D

1 2 3 4 5 6 7 8 DT-D STD

B)

RAT P450 REDUCTASE

1 2 3 4 5 6 7 8 P450R STD

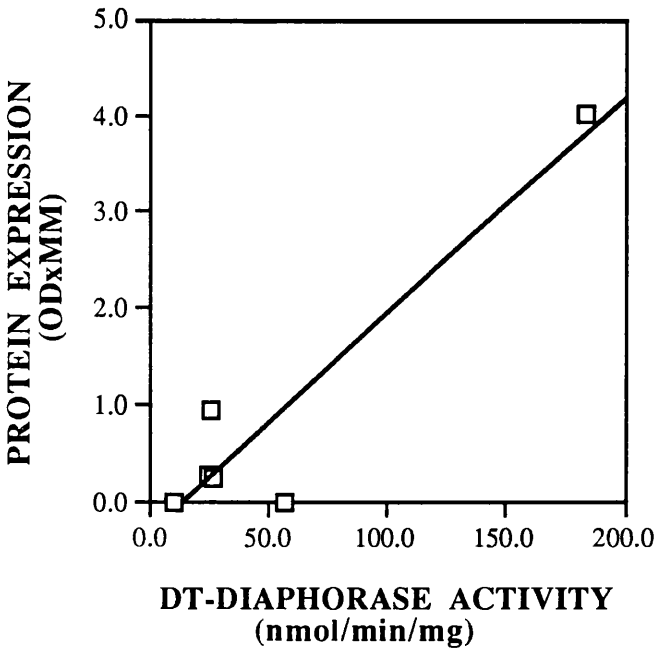


Figure 2.12 **Correlation between DT-diaphorase protein expression and enzyme activity in human malignant brain tumours (n=6) (r^2=0.876)**

2.4 Discussion

Several reductase enzymes have been studied for their levels of expression within the NCI human tumour cell line panel. Notable differences were observed within and in particular, between cell lines from different tissues of origins. DT-diaphorase enzyme activity and protein expression were generally found to be higher than those of NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase, although in all cases heterogeneity in the expression patterns was observed.

The results described in this chapter represent one of the first in a series of ongoing studies to characterise selected important biochemical and molecular parameters across the entire NCI panel of more than 60 human tumour cell lines. The power of such analysis has already been demonstrated by the incorporation of topoisomerase II inhibition levels (Leteurtre *et al.*, 1994) and *mdr-1*/P-glycoprotein expression levels (Alvarez *et al.*, 1995) and subsequent elucidation of mechanism of action of various compounds. The existing relational data base on the *in vitro* antitumour activity of at least 42,000 discrete chemical entities permits the assignment of a putative mechanism of action to both established and novel compounds (Boyd, 1993; Paull *et al.*, 1992; Paull *et al.*, 1989; Weinstein *et al.*, 1992). The addition of biochemical and molecular parameters, such as enzyme levels within the cell lines, allows the growing data base to be interrogated to determine the likely involvement of these parameters in the antitumour mechanism.

The selected biochemical parameters in this particular study were the activities of the enzymes DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase. These are among the reductases known to be involved in activation of bioreductive anticancer agents, including quinones, nitro compounds and *N*-oxides (Workman, 1993). Additional enzymes are also involved in bioreductive activation, including for example NADPH : cytochrome P450s, xanthine oxidase and xanthine dehydrogenase and, as a consequence, these could also be examined to characterise expression across the cell lines. It was felt appropriate, however, to restrict

the initial analysis to just three enzymes. The three enzymes studied were selected because of the extensive investigations currently underway into their role in metabolism of a wide range of bioreductive agents (Fitzsimmons *et al.*, 1994; Hodnick & Sartorelli, 1993; Riley & Workman, 1992b; Workman, 1994). A further, practical advantage of selecting these particular reductases is the similarity in the analytical methodology, which facilitated the efficient processing of the samples. To help interpret the enzyme activity information and also to assess technical feasibility for future clinical studies, the amount of protein and messenger RNA (DT-diaphorase and NADPH : cytochrome P450 reductase) were also analysed by Western immunoblotting and RT-PCR.

In addition to providing a valuable resource for the future analysis of mechanism of action of potential anticancer compounds tested in the NCI screen, the present study represents the most comprehensive characterisation to date of the bioreductive enzymes examined against human tumour cell lines *in vitro* (for previous studies see for example, Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; Robertson *et al.*, 1992; Robertson *et al.* , 1994).

In the present study a total of 69 cell lines were examined. It is clear that the expression of bioreductive enzyme activities showed a considerable degree of heterogeneity of expression both within and between tumour cell lines of different origins. Moreover, quite distinct patterns of activity emerged for particular tumour types. Overall the level of DT-diaphorase activity (Figure 2.1) in the cell lines was higher than that of NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase. This finding was in agreement with other studies using smaller groups of human tumour cell lines (Patterson *et al.*, 1994; Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b). In the case of DT-diaphorase, a relatively high degree of activity was seen in non-small cell lung cancer, colon, CNS and melanoma cell lines. Relatively low activities were noted in leukaemic/lymphoma, ovarian and breast cell lines. Other studies using smaller panels of cell lines have also shown similar patterns of DT-diaphorase activity. For example, high levels of DT-diaphorase activity have been observed within human tumour cell lines originating from the lung and colon (Patterson *et al.*, 1994; Plumb &

Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; Robertson *et al.*, 1992; Robertson *et al.*, 1994; Smitskamp-Wilms *et al.*, 1994) while very low levels in leukaemias have also been noted (Smitskamp-Wilms *et al*, 1994). In addition, enzyme activity measurements have also been previously carried out using human tumour samples. In this regard, Schlager and Powis (Schlager & Powis, 1990) showed that DT-diaphorase was enhanced (compared to paired normal tissue) in tumours of the lung, breast and colon. The results of this current analysis also showed higher than average activities in the lung and colon lines. Although tumour cell lines originating from the breast were shown to possess lower than average DT-diaphorase activities, the average activity within the group was, in fact, higher than that for the breast tumour tissue reported by Schlager and Powis (Schlager & Powis, 1990). It is notable that within the current study, the level of DT-diaphorase activity was in fact significantly higher within the CNS human tumour cell lines when compared to a human glioma biopsy panel. These discrepancies are hard to explain. Certainly the exact site of origin of the CNS cell lines is important when directly comparing them to a specific glioma panel. Differences were noted within the glioma panel itself (Table 2.3) dependent upon the nature of the tumour. A further possible explanation is that depending upon the means of biopsy sampling, some tumour biopsies may contain what is considered to be normal tissue, and thus the enzyme activity measurements are influenced. Although the level of DT-diaphorase activity is known to increase with cell density (Plumb & Workman, 1994), this possible influence was ruled out by the use of cell lines grown to the same density. Differences can clearly exist in the patterns of enzyme activities noted between human tumour cell lines and human tumour biopsies and care must therefore be taken when extrapolating between the two.

In comparison with DT-diaphorase, the activities observed for NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase were in general much lower. With respect to NADPH : cytochrome P450 reductase (Figure 2.3) the tumour types showing the highest activities were leukaemic/lymphoma, colon, melanoma and renal. Low levels were more characteristic of non-small and small cell lung cancer and

breast cancer, while both high and low levels were seen in CNS, ovarian and the two prostate cell lines. In the case of NADH : cytochrome b5 reductase (Figure 2.5), the two lung cancer histiotypes were again low in activity, as were the ovarian lines. Relatively high activities were associated with CNS, melanoma, renal and prostate lines, while a mixed pattern was observed in leukaemic/lymphoma, colon and breast cell lines. In contrast to DT-diaphorase, the mean activity of NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase across the human glioma biopsy panel was not significantly different to those in the CNS cell lines, although the range in activities was greater.

Enzyme activity was determined using a modified version of the cytochrome *c* reduction assay detailed by Segura-Aguilar *et al* (Segura-Aguilar *et al.*, 1990). In many studies (for example, Schlager & Powis, 1990; Traver *et al.* , 1992) 2, 6-dichlorophenol-indophenol (DCPIP) is used as the electron acceptor when measuring DT-diaphorase activity. In this current study, however, menadione (2-methyl-1, 4-napthoquinone; vitamin K_3 menadione) was used as it was previously found to be more active than DCPIP (Ernster, 1987). According to Segura-Aguilar (Segura-Aguilar *et al.*, 1990), DT-diaphorase activity is the activity which is dicoumarol-sensitive and NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase activities are those that are dicoumarol-insensitive in the presence of NADPH and NADH as electron donors, respectively. A great deal of emphasis has been placed on dicoumarol being a potent, specific inhibitor of DT-diaphorase (Dulhanty *et al.* , 1989; Keyes *et al.* , 1985). Controversially, however, it is now known that dicoumarol lacks specificity and that it can inhibit other enzymes such as NADH : cytochrome b5 reductase (Preusch *et al.* , 1991) and some carbonyl reductases (Maser & Netter, 1989). In an attempt to overcome the problems associated with dicoumarol, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase activity measurements were made using a direct assay system, omitting the electron acceptor menadione and therefore in the absence of dicoumarol. In the case of DT-diaphorase, enzyme activity was determined by the activity

which was inhibited by a low concentration of dicoumarol (10μM) in the presence of NADH as electron donor.

The Western immunoblotting studies for DT-diaphorase were useful in showing that protein levels associated with DT-diaphorase were generally high in those cell lines rich in enzyme activities and lower in those with poor enzyme activities. This suggests that it is the amount of protein expressed that governs the level of enzyme activity in the various cell lines. In breast cell lines which have very low levels of DT-diaphorase activity, no protein could be detected at the limits of detection of Western immunoblotting. For this reason the more sensitive technique of RT-PCR was carried out on these breast cell lines. As a control, several CNS cell lines which possessed high activity were also included. DT-diaphorase RNA was detected in all of the cell lines studied, including those with very low levels of DT-diaphorase activity. The extent of RNA expression was variable in a heterogeneous fashion with respect to the enzyme activities. It was important to note that NADPH : cytochrome P450 reductase RNA was detectable by RT-PCR in the cell lines. Although activity had been detectable throughout the cell line panel, no protein had been detected by Western immunoblotting. The reason for this may be due to the latter technique not being sensitive enough to detect the overall lower levels of enzyme activity (in comparison to DT-diaphorase).

Overall only a low number of cell lines within the NCI panel were found to lack DT-diaphorase activity completely. Interestingly, however, one out of ten (i.e. 10 %) non-small cell lung cancer lines studied was found to lack DT-diaphorase activity. This finding was in agreement with the frequency of a point mutation in the DT-diaphorase gene reported by Rosvold *et al* (Rosvold *et al.* , 1993) and more recently by Ross *et al* (Ross *et al.* , 1994) in lung cancers. Thus it may be an indication of the presence of this point mutation in those cell lines lacking DT-diaphorase activity. Clearly, the presence of this point mutation has important implications in terms of the ability to target DT-diaphorase within human tumours with specific chemotherapy treatments.

In summary, the expression and more importantly, the functional activity of three reductase enzymes has been determined across the NCI human tumour cell line panel.

The results reveal interesting differences within and in particular between cell lines from different tissues of origin. Heterogeneity in the expression patterns of DT-diaphorase were especially noteworthy. This information could be important in the targeting of particular agents to certain tumour types, depending on the levels of activating and deactivating enzymes, as suggested by the 'enzyme-directed' approach to bioreductive drug development (Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; Robertson *et al.*, 1994; Workman, 1994; Workman & Walton, 1990). For example, agents which are activated by DT-diaphorase such as EO9 (Walton *et al.* , 1991) and mitomycin C (Ross *et al.*, 1994) would be best used in the treatment of colon, NSCLC and melanoma tumours. In contrast, agents such as tirapazamine which is activated by NADPH : cytochrome P450 reductase (Fitzsimmons *et al.*, 1994) would be best used for the treatment of melanoma and kidney tumours. With regard to this thesis, enzyme activity data within the human tumour cell lines was used to determine possible correlations between enzyme activities and cell sensitivities towards the many compounds previously screened through the NCI *in vitro* screening programme (Chapter 3). The potential use of such studies has been demonstrated (Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; Robertson *et al.*, 1992; Robertson *et al.*, 1994; Smitskamp-Wilms *et al.*, 1994), although on a much smaller scale. Furthermore, the accumulation of enzyme activity data within human tumour cell lines enabled the selection of specific cell lines for cytotoxicity studies based upon enzyme activities to be made (Chapter 4).

CHAPTER 3

RELATIONSHIP BETWEEN ENZYME ACTIVITY AND CELL SENSITIVITY TOWARDS COMPOUNDS WITHIN THE NCI SCREENING PANEL

3.1 Introduction

Screening of novel compounds as potential anticancer agents has been ongoing for many years. These screens have involved a diverse array of experimental systems. The NCI has, for a long time, relied upon the use of the *in vivo* L1210 and P388 murine leukaemia models for their primary screening programme (Boyd, 1993). However, more recently they have employed an *in vitro* primary screening programme which involves the use of a diverse, disease-orientated panel of approximately 60 human tumour cell lines which can be arranged into distinct disease-specific subpanels (Stinson *et al.*, 1992). This new screening method allowed for the study of a greater number of different tumour types, the overall goal being to identify compounds with selective activity against particular tumour histiotypes as opposed to the more familiar broad spectrum cytotoxins.

Having fully established the new screening system, the NCI now annually screens approximately 20,000 novel compounds of defined chemical structure against the panel of *in vitro* human tumour cell lines (Boyd & Paull, 1995). Use of computer methods such as the COMPARE program (Paull *et al.*, 1989; Paull *et al.*, 1992), neural networks (Weinstein *et al.*, 1992) and Kohonen self-organising mapping (van Osdol *et al.*, 1994) to search the data base of at least 42,000 compounds so far evaluated against the panel of human tumour cell lines, provides a unique means by which to identify the potential mode of action of established agents and novel chemical entities. The inclusion of relevant biochemical and molecular parameters into the data base is now being sought as an additional means of identifying mechanisms of action of particular anticancer agents (Boyd & Paull, 1995). An example of such a study was the correlation of the level of topoisomerase II inhibition and cytotoxicity of the anthrapyrazoles DuP 937 and DuP 941

(Leteurtre *et al.* , 1994). Subsequent studies have determined the expression levels of *mdr-1*/P-glycoprotein within each of the cell lines and correlated this to the screening profiles of a series of compounds (Alvarez *et al.*, 1995).

A number of reports correlating enzyme activity with cell sensitivity towards various compounds have been published recently. These studies, while producing valuable data, are usually performed on a much smaller scale than those involving the NCI panel of cell lines. The novel indoloquinone anticancer agent EO9 is known to be metabolised under aerobic conditions by DT-diaphorase (Walton *et al.* , 1991). Cell sensitivity towards EO9 has now been correlated with DT-diaphorase activity in both human tumours and tumour cell line panels (Phillips *et al.* , 1992; Plumb & Workman, 1994; Plumb *et al.* , 1994b; Robertson *et al.* , 1994; Smitskamp-Wilms *et al.*, 1994; Walton *et al.* , 1992a). However, the maximum number of cell lines used in any of these studies was only 23. As expected, cell sensitivity towards the benzotriazine di-*N*-oxide, tirapazamine, was shown not to correlate with DT-diaphorase activity in a panel of 18 human tumour cell lines originating from the breast, small cell and non-small cell lung cancers (Patterson *et al.* , 1994). This is not surprising as DT-diaphorase is known, if anything, to detoxify tirapazamine (Workman & Stratford, 1993). Some compounds such as the prototype alkylating agent mitomycin C have proved to be more controversial. Several studies have reported the correlation of DT-diaphorase activity and cell sensitivity towards mitomycin C under aerobic conditions (Dulhanty & Whitmore, 1991; Marshall *et al.* , 1991; Siegel *et al.* , 1990a), whereas a contrasting report by Robertson *et al* (Robertson *et al.* , 1992) showed a lack of correlation. The role of DT-diaphorase in the activation of mitomycin C is much disputed (Schlager & Powis, 1988; Workman, 1994; Workman & Walton, 1989). However biochemical evidence suggest that under physiological pH (7.0 - 7.4), bioactivation can occur under aerobic conditions (Ross *et al.* , 1994).

It was felt that the availability of a larger number of human tumour cell lines may provide more conclusive evidence of a role for reductase enzymes in drug sensitivity. In a collaborative project, therefore, between the NCI, the European Organisation for

Research and Treatment of Cancer (EORTC) and the UK Cancer Research Campaign (CRC), the analysis of expression of three selected bioreductive enzymes, namely DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase, within the human tumour cell line panel has been undertaken. In addition to gaining an understanding of the pattern of expression of these enzymes both within and between the different categories of human tumour cell lines (Chapter 2), this information was generated in order to create a data base with which to seek correlations between the different enzyme activities and sensitivities to both existing and novel antitumour agents which had been screened through the NCI panel. The potential use of this new data base is illustrated in this chapter by the correlation and COMPARE analysis of enzyme activity and cell sensitivity towards the compounds already screened and stored within the NCI data base. A total of approximately 24,000 compounds were included in this particular analysis. In view of the information presented earlier in this introduction, possible correlations between enzyme expression and antitumour activity of EO9, an agent in Phase I / II clinical trial, and mitomycin C, a currently used clinical agent, were also investigated.

3.2 Materials and Methods

3.2.1. Human Tumour Cell Lines

The NCI panel of tissue-specific human tumour cell lines (Stinson *et al.*, 1992) were used in this analysis. Cell lines are in routine use at the NCI (Bethesda, MD, U.S.A.) as part of their drug screening programme. Details of the preparation of cell pellets and subsequent storage of cell sonicates for analysis of enzyme activity levels are described previously in Chapter 2 (Section 2.2.4.). DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase activities were determined as described in Chapter 2 (Section 2.2.6.) and are expressed in nmol of cytochrome *c* reduced/min/mg of protein.

3.2.2. Cytotoxicity Testing

Sensitivity of the human tumour cell lines towards approximately 24,000 compounds within the NCI database were determined previously at the US National Cancer Institute using standard procedures. Adherent cell lines were detached from routine culture flasks using 0.05 % trypsin-EDTA. Trypsin was then inactivated by adding 5 % serum-containing RPMI-1640 medium and the cells counted, along with suspended cell lines. Cells were plated out in a volume of 100 µl per well at densities between 5000 - 40,000 cells per well (see Monks *et al*, (Monks *et al.* , 1991) for details of cell lines and their plating densities). 100 µl of complete medium was then added to cell-free wells as controls. Microtitre plates were then pre-incubated for 24 hrs. at 37°C before addition of drug. Each compound was tested at five 10-fold dilutions by adding 100 µl of each to the appropriate plate wells. Cells were exposed to drug under aerobic conditions for 48 hrs. at 37°C (5 % CO_2 and 100 % relative humidity).

The end-point of the assay was determined by the Sulforhodamine B (SRB) assay (Skehan *et al.* , 1990). In the case of the adherent cells, 50 µl cold 50 % (w/v)

trichloroacetic acid (TCA) (final concentration 10 %) was added in order to fix the cells in situ for 60 mins. at 4°C. Supernatant was discarded and plates rinsed five times with deionised water before being dried. Fixed cells were stained at room temperature for 10 mins. with 0.4 % (w/v) SRB dissolved in 1 % acetic acid. SRB was poured from the plates and 1 % acetic acid used to rinse the plates five times. Once plates were dry, the dye was solubilised using 10 mM unbuffered Tris base (pH 10 - 10.5) for 2 - 5 mins. The method was essentially the same for suspension cell lines (e.g. leukaemia/lymphoma lines), except that at the end of the drug incubation period the settled cells were fixed to the bottom of the plates by adding 50 µl of 80 % cold TCA (final concentration 16 %). Optical densities were read on an automated spectrophotometric plate reader at 515 nm. Note that a measure of cell population density at time 0 (the time at which drugs were added) was also made by fixing two reference plates of inoculated cells prior to drug addition to the test plates.

Data was analysed by computer to produce three measurements ; (1) control optical density, (2) test optical density, and (3) optical density at time zero. These measurements were then used to calculate growth inhibition of 50 % (GI_{50}) [drug concentration causing 50 % reduction in protein relative to control cells during the drug incubation], total growth inhibition (TGI) [drug concentration resulting in total inhibition], and the LC_{50}, [concentration of drug causing 50 % reduction in measured protein at end of the drug exposure compared to at the start] (Monks *et al.*, 1991). Values were calculated for each of the above parameters providing the required effects were seen, otherwise they were expressed as greater or less than the maximum or minimum drug concentration tested.

3.2.3. Statistical Analysis

All of the statistical analysis was performed by Dr. K. Paull at the NCI (Information Technology Branch, Division of Cancer Treatment).

Statistical analysis in the form of the Pearson Correlation Coefficient was used to determine the degree of similarity and significance between cell sensitivity towards compounds within the NCI database and enzyme activities. For this analysis, GI_{50} values were used as an indication of cell sensitivity as this is the parameter generally employed by the NCI for sensitivity analysis.

The sensitivity data of anticancer drugs tested against the NCI screening panel are routinely and conveniently displayed as COMPARE analysis plots (Paull *et al.*, 1989; Paull *et al.*, 1992). For a given drug, these COMPARE plots illustrate on a log10 scale the deviation from the mean sensitivity calculated across all cell lines in the panel. This is known as the mean graph. The relationship between DT-diaphorase enzyme activity and cell sensitivity within the human tumour cell line panel towards both mitomycin C and EO9 was determined in more detail using the COMPARE programme as these agents are known to be promising bioreductive anticancer agents.

3.3 Results

3.3.1. Relationship between enzyme activity and cell sensitivity towards compounds within the NCI screening panel

DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase enzyme activities were determined within a large panel of human tumour cell lines, as reported in Chapter 2. These data were then entered into the NCI database as $\log_{10}$ values, and statistical analysis in the form of the Pearson Correlation was used to determine any possible relationship between enzyme activity and cell sensitivity (expressed as $\log_{10}$ GI_{50} values) under aerobic conditions within the NCI screening panel. A total of approximately 24,000 compounds were included in the analysis.

Examples of those compounds demonstrating a good correlation between DT-diaphorase enzyme activity and cell sensitivity are listed in Table 3.1. The highest correlation coefficient was obtained for the complex quinone streptonigrin. Indeed, the majority of compounds exhibiting the highest correlation coefficients were quinone-containing compounds. For example, cell sensitivity towards the indoloquinone compound EO9 and DT-diaphorase enzyme activity correlated significantly across the cell line panel (Pearson Correlation Coefficient of 0.446; $p<0.0013$; $n=49$). A graph showing this correlation can be seen in Figure 3.1. It should be noted that five of the cell lines used in the analysis exhibited extremely low levels of DT-diaphorase activity and as such, lie apart from the others in Figure 3.1. In addition, a significant correlation was detected with the prototype bioreductive agent mitomycin C (Pearson Correlation Coefficient of 0.420; $p<0.0005$; $n=64$). Figure 3.2 shows the correlation between cell sensitivity towards mitomycin C and DT-diaphorase activity. Once again, five cell lines lie apart from the others due to the their extremely low DT-diaphorase activity.

Perhaps more intriguing in the analysis, was the presence of several 5-fluorouracil-related compounds among those compounds showing the highest correlation coefficients.

CHEMICAL NAME	PEARSON CORRELATION COEFFICIENT
streptonigrin	0.563 (n=42)
5-fluorouracil prodrug	0.550 (n=54)
napthoquinone D	0.550 (n=54)
n-heterocycle D	0.484 (n=54)
EO9	0.446 (n=49)
mitomycin C	0.420 (n=64)
5-fluorouracil	0.401 (n=55)
aziridinylimidazoloquinone	0.399 (n=54)
porfiromycin	0.390 (n=50)

Table 3.1 Level of correlation between DT-diaphorase activity and sensitivity of the NCI human tumour cell line panel towards selected compounds within the screening program.

The degree of correlation was determined using statistical analysis in the form of the Pearson Correlation Coefficient (r^2). n represents the number of cell lines in which sensitivity data towards each compound was available and hence, the number of comparisons made.

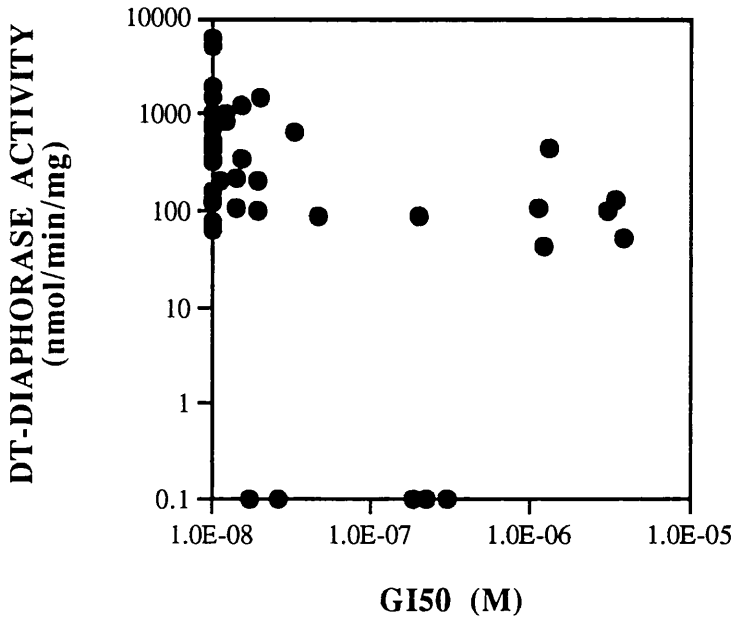


Figure 3.1 **Correlation of cell sensitivity towards the indoloquinone EO9 and DT-diaphorase enzyme activity within the NCI human tumour cell line panel.**

Sensitivity of the cell line panel towards EO9 was determined using the SRB assay and is represented by GI_{50} values, while DT-diaphorase enzyme activity was determined by the cytochrome *c* reduction assay and values are in nmol of cytochrome *c* reduced/min/mg of protein. Statistical analysis was performed in the form of the Pearson Correlation Coefficient (r^2=0.446).

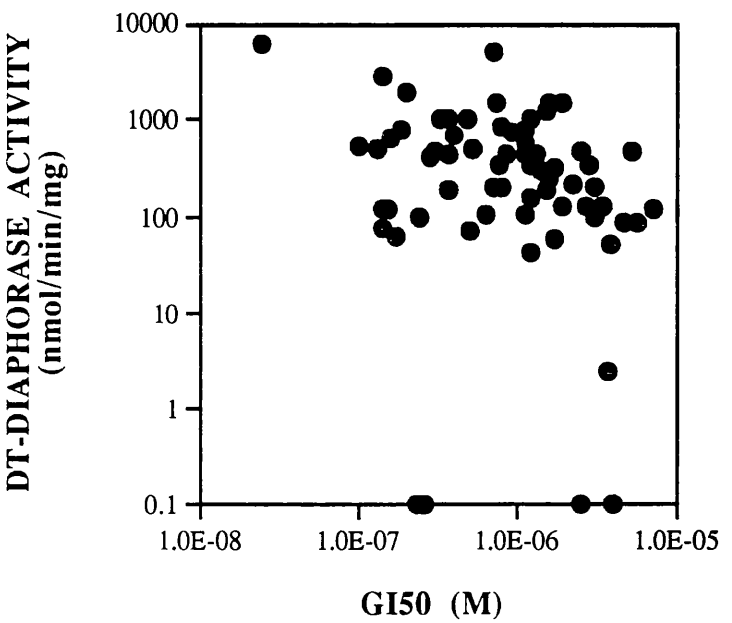


Figure 3.2 Correlation of cell sensitivity towards the prototype alkylating agent mitomycin C and DT-diaphorase enzyme activity within the NCI human tumour cell line panel.

Sensitivity of the cell line panel towards mitomycin C was determined using the SRB assay and is represented by GI_{50} values, while DT-diaphorase enzyme activity was determined by the cytochrome *c* reduction assay and values are in nmol of cytochrome c reduced/min/mg of protein. Statistical analysis was performed in the form of the Pearson Correlation Coefficient (r^2=0.420).

In contrast to DT-diaphorase, no significant correlations were detected across the cell line panel for either NADPH : cytochrome P450 reductase or NADH : cytochrome b_5 reductase.

3.3.2. Cell sensitivity towards the indoloquinone EO9 and DT-diaphorase enzyme activity : correlations within individual groups of cell lines

A COMPARE analysis was carried out to investigate the correlation between DT-diaphorase enzyme activity and cell sensitivity towards the indoloquinone compound EO9 under aerobic conditions. Figure 3.3 shows the pattern of differential cytotoxicity [GI_{50} (A); TGI (B) and LC_{50} (C)] in the form of a mean graph for the compound. Each of the individual parameters show that on the whole, leukaemic/lymphoma and ovarian cell lines were most resistant, while those originating from the melanoma, colon, renal, CNS, small cell and non-small cell lung cancer appear to be more sensitive towards EO9. The exact number of cell lines following this pattern within each group appears to depend to some extent on the parameter (i.e. GI_{50}) used to express cell sensitivity.

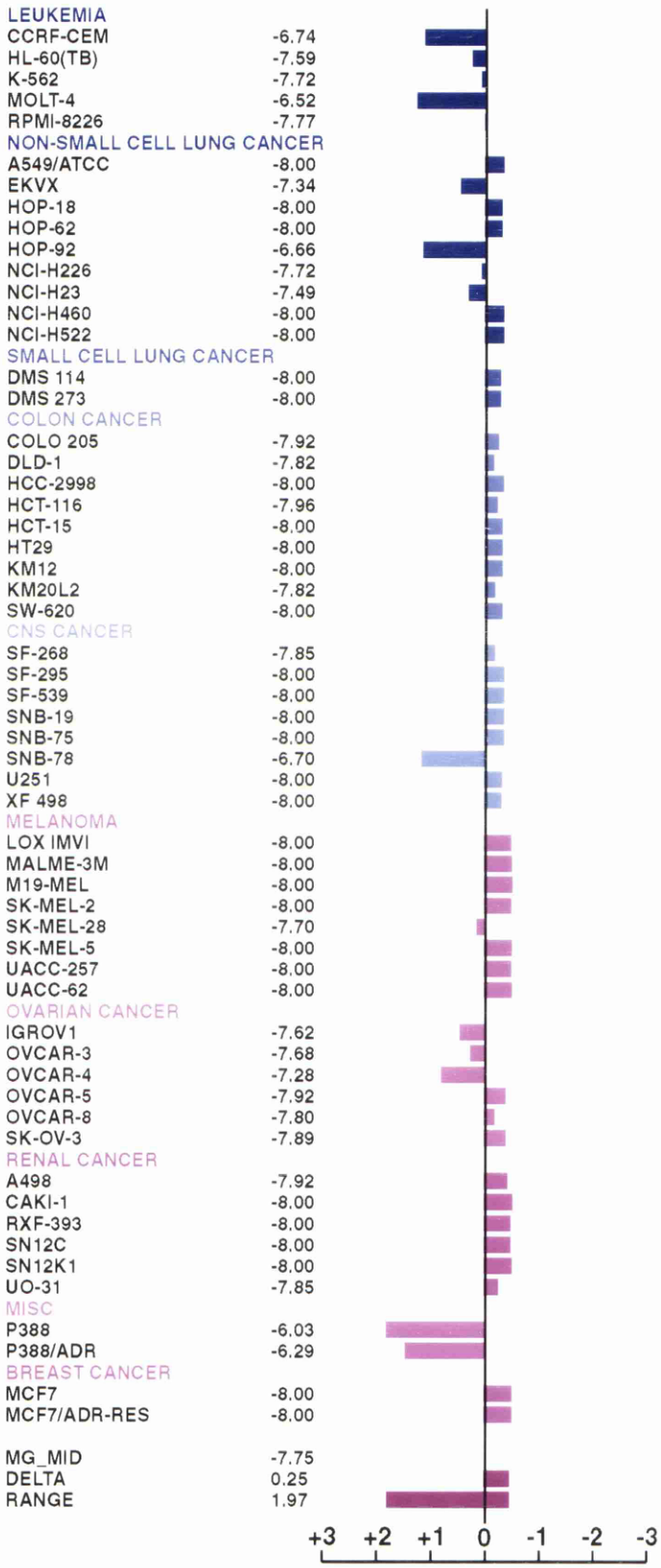
3.3.3. Cell sensitivity towards the prototype alkylating agent mitomycin C and DT-diaphorase enzyme activity : correlations within individual groups of cell lines

As with EO9, a COMPARE analysis was carried out with mitomycin C to investigate any correlations between DT-diaphorase activity and cell sensitivity within individual groups of cell lines. Figure 3.4 shows the patterns of differential cytotoxicity in the form of a mean graph for mitomycin C. Three different cytotoxicity parameters were again used in the analysis, GI_{50} (A), TGI (B) and LC_{50} (C). The results were found to be somewhat different to those with EO9, with less clear-cut patterns arising. Even so, cell lines originating from the breast, ovaries and colon appeared to be relatively resistant while those from the CNS and melanoma were more sensitive towards mitomycin C.

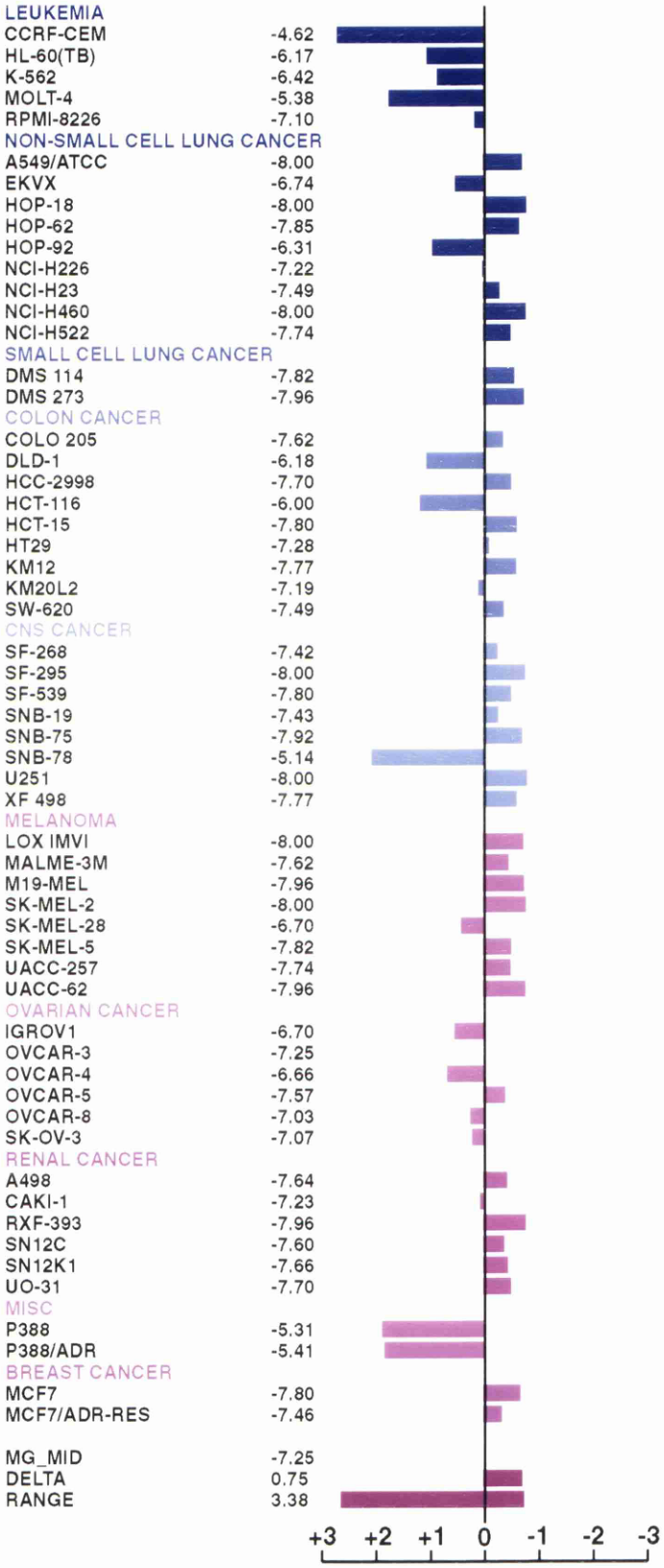
Figure 3.3 Patterns of differential cytotoxicity of the indoloquinone EO9 towards human tumour cell lines.

For each cell line, the GI_{50} (A), TGI (B) and the LC_{50} (C) are obtained. For each parameter, a mean log is determined and is defined as the mean of the $\log_{10}$ of the individual values for that parameter. The difference between the $\log_{10}$ of each cell line and the mean log is then determined, to yield positive values for cell lines more sensitive than average (bars projecting to the right) and negative values for cell lines less sensitive than average (bars projecting to the left). MG-MID, Delta and Range are defined as the calculated mean value of the panel; the number of $\log_{10}$ units by which the values for the individual value of the panel differs (+ or -) from the MG-MID and the number of $\log_{10}$ units by which the value for the most sensitive line differs from the least sensitve line, respectively.

A)


LEUKEMIA
CCRF-CEM -6.74
HL-60(TB) -7.59
K-562 -7.72
MOLT-4 -6.52
RPMI-8226 -7.77
NON-SMALL CELL LUNG CANCER
A549/ATCC -8.00
EKVX -7.34
HOP-18 -8.00
HOP-62 -8.00
HOP-92 -6.66
NCI-H226 -7.72
NCI-H23 -7.49
NCI-H460 -8.00
NCI-H522 -8.00
SMALL CELL LUNG CANCER
DMS 114 -8.00
DMS 273 -8.00
COLON CANCER
COLO 205 -7.92
DLD-1 -7.82
HCC-2998 -8.00
HCT-116 -7.96
HCT-15 -8.00
HT29 -8.00
KM12 -8.00
KM20L2 -7.82
SW-620 -8.00
CNS CANCER
SF-268 -7.85
SF-295 -8.00
SF-539 -8.00
SNB-19 -8.00
SNB-75 -8.00
SNB-78 -6.70
U251 -8.00
XF 498 -8.00
MELANOMA
LOX IMVI -8.00
MALME-3M -8.00
M19-MEL -8.00
SK-MEL-2 -8.00
SK-MEL-28 -7.70
SK-MEL-5 -8.00
UACC-257 -8.00
UACC-62 -8.00
OVARIAN CANCER
IGROV1 -7.62
OVCAR-3 -7.68
OVCAR-4 -7.28
OVCAR-5 -7.92
OVCAR-8 -7.80
SK-OV-3 -7.89
RENAL CANCER
A498 -7.92
CAKI-1 -8.00
RXF-393 -8.00
SN12C -8.00
SN12K1 -8.00
UO-31 -7.85
MISC
P388 -6.03
P388/ADR -6.29
BREAST CANCER
MCF7 -8.00
MCF7/ADR-RES -8.00
MG_MID -7.75
DELTA 0.25
RANGE 1.97
+3 +2 +1 0 -1 -2 -3

B)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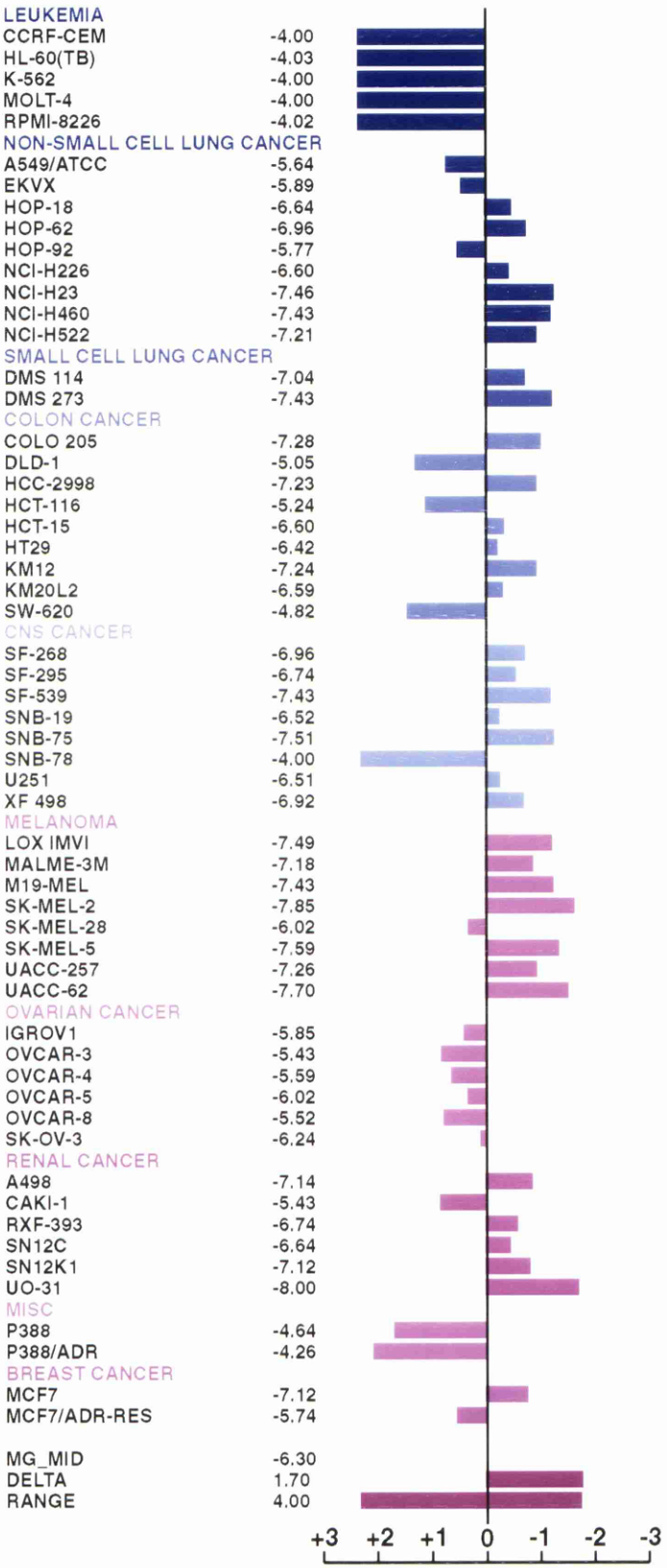
RPMI-8226 -7.10
NON-SMALL CELL LUNG CANCER
A549/ATCC -8.00
EKVX -6.74
HOP-18 -8.00
HOP-62 -7.85
HOP-92 -6.31
NCI-H226 -7.22
NCI-H23 -7.49
NCI-H460 -8.00
NCI-H522 -7.74
SMALL CELL LUNG CANCER
DMS 114 -7.82
DMS 273 -7.96
COLON CANCER
COLO 205 -7.62
DLD-1 -6.18
HCC-2998 -7.70
HCT-116 -6.00
HCT-15 -7.80
HT29 -7.28
KM12 -7.77
KM20L2 -7.19
SW-620 -7.49
CNS CANCER
SF-268 -7.42
SF-295 -8.00
SF-539 -7.80
SNB-19 -7.43
SNB-75 -7.92
SNB-78 -5.14
U251 -8.00
XF 498 -7.77
MELANOMA
LOX IMVI -8.00
MALME-3M -7.62
M19-MEL -7.96
SK-MEL-2 -8.00
SK-MEL-28 -6.70
SK-MEL-5 -7.82
UACC-257 -7.74
UACC-62 -7.96
OVARIAN CANCER
IGROV1 -6.70
OVCAR-3 -7.25
OVCAR-4 -6.66
OVCAR-5 -7.57
OVCAR-8 -7.03
SK-OV-3 -7.07
RENAL CANCER
A498 -7.64
CAKI-1 -7.23
RXF-393 -7.96
SN12C -7.60
SN12K1 -7.66
UO-31 -7.70
MISC
P388 -5.31
P388/ADR -5.41
BREAST CANCER
MCF7 -7.80
MCF7/ADR-RES -7.46
MG_MID -7.25
DELTA 0.75
RANGE 3.38
+3 +2 +1 0 -1 -2 -3

C)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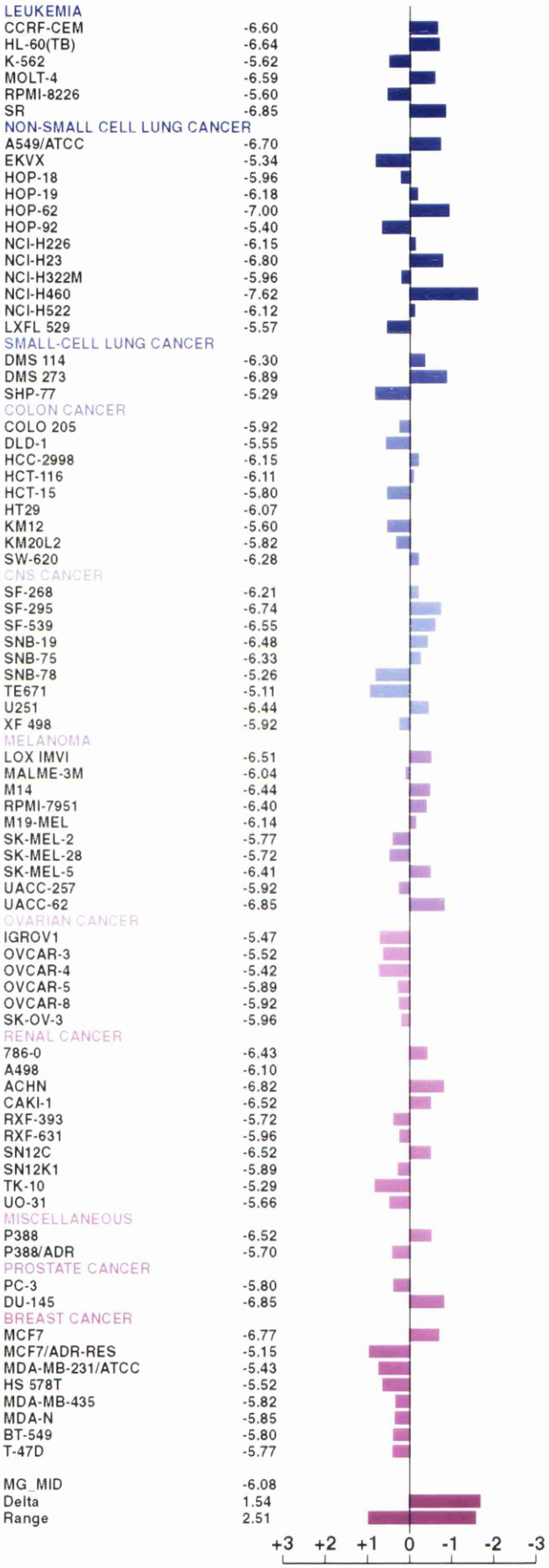
HL-60(TB) -4.03
K-562 -4.00
MOLT-4 -4.00
RPMI-8226 -4.02
NON-SMALL CELL LUNG CANCER
A549/ATCC -5.64
EKVX -5.89
HOP-18 -6.64
HOP-62 -6.96
HOP-92 -5.77
NCI-H226 -6.60
NCI-H23 -7.46
NCI-H460 -7.43
NCI-H522 -7.21
SMALL CELL LUNG CANCER
DMS 114 -7.04
DMS 273 -7.43
COLON CANCER
COLO 205 -7.28
DLD-1 -5.05
HCC-2998 -7.23
HCT-116 -5.24
HCT-15 -6.60
HT29 -6.42
KM12 -7.24
KM20L2 -6.59
SW-620 -4.82
CNS CANCER
SF-268 -6.96
SF-295 -6.74
SF-539 -7.43
SNB-19 -6.52
SNB-75 -7.51
SNB-78 -4.00
U251 -6.51
XF 498 -6.92
MELANOMA
LOX IMVI -7.49
MALME-3M -7.18
M19-MEL -7.43
SK-MEL-2 -7.85
SK-MEL-28 -6.02
SK-MEL-5 -7.59
UACC-257 -7.26
UACC-62 -7.70
OVARIAN CANCER
IGROV1 -5.85
OVCAR-3 -5.43
OVCAR-4 -5.59
OVCAR-5 -6.02
OVCAR-8 -5.52
SK-OV-3 -6.24
RENAL CANCER
A498 -7.14
CAKI-1 -5.43
RXF-393 -6.74
SN12C -6.64
SN12K1 -7.12
UO-31 -8.00
MISC
P388 -4.64
P388/ADR -4.26
BREAST CANCER
MCF7 -7.12
MCF7/ADR-RES -5.74
MG_MID -6.30
DELTA 1.70
RANGE 4.00
+3 +2 +1 0 -1 -2 -3

Figure 3.4 Patterns of differential cytotoxicity of quinone alkylating agent mitomycin C towards human tumour cell lines.

For each cell line, the GI_{50} (A), TGI (B) and the LC_{50} (C) are obtained. For each parameter, a mean log is determined and is defined as the mean of the $\log_{10}$ of the individual values for that parameter. The difference between the $\log_{10}$ of each cell line and the mean log is then determined, to yield positive values for cell lines more sensitive than average (bars projecting to the right) and negative values for cell lines less sensitive than average (bars projecting to the left). See Figure 3.3 for definition of MG-MID, Delta and Range.

A)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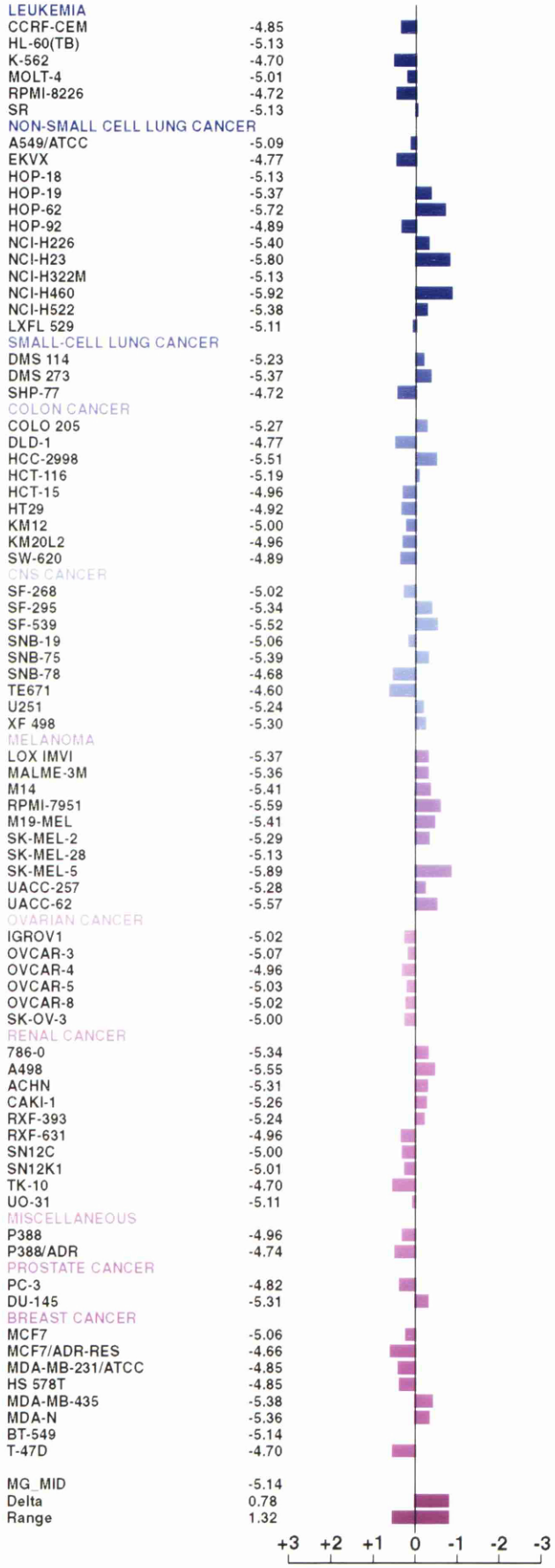
HL-60(TB) -6.64
K-562 -5.62
MOLT-4 -6.59
RPMI-8226 -5.60
SR -6.85
NON-SMALL CELL LUNG CANCER
A549/ATCC -6.70
EKVX -5.34
HOP-18 -5.96
HOP-19 -6.18
HOP-62 -7.00
HOP-92 -5.40
NCI-H226 -6.15
NCI-H23 -6.80
NCI-H322M -5.96
NCI-H460 -7.62
NCI-H522 -6.12
LXFL 529 -5.57
SMALL-CELL LUNG CANCER
DMS 114 -6.30
DMS 273 -6.89
SHP-77 -5.29
COLON CANCER
COLO 205 -5.92
DLD-1 -5.55
HCC-2998 -6.15
HCT-116 -6.11
HCT-15 -5.80
HT29 -6.07
KM12 -5.60
KM20L2 -5.82
SW-620 -6.28
CNS CANCER
SF-268 -6.21
SF-295 -6.74
SF-539 -6.55
SNB-19 -6.48
SNB-75 -6.33
SNB-78 -5.26
TE671 -5.11
U251 -6.44
XF 498 -5.92
MELANOMA
LOX IMVI -6.51
MALME-3M -6.04
M14 -6.44
RPMI-7951 -6.40
M19-MEL -6.14
SK-MEL-2 -5.77
SK-MEL-28 -5.72
SK-MEL-5 -6.41
UACC-257 -5.92
UACC-62 -6.85
OVARIAN CANCER
IGROV1 -5.47
OVCAR-3 -5.52
OVCAR-4 -5.42
OVCAR-5 -5.89
OVCAR-8 -5.92
SK-OV-3 -5.96
RENAL CANCER
786-0 -6.43
A498 -6.10
ACHN -6.82
CAKI-1 -6.52
RXF-393 -5.72
RXF-631 -5.96
SN12C -6.52
SN12K1 -5.89
TK-10 -5.29
UO-31 -5.66
MISCELLANEOUS
P388 -6.52
P388/ADR -5.70
PROSTATE CANCER
PC-3 -5.80
DU-145 -6.85
BREAST CANCER
MCF7 -6.77
MCF7/ADR-RES -5.15
MDA-MB-231/ATCC -5.43
HS 578T -5.52
MDA-MB-435 -5.82
MDA-N -5.85
BT-549 -5.80
T-47D -5.77
MG_MID -6.08
Delta 1.54
Range 2.51
+3 +2 +1 0 -1 -2 -3

B)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K-562 -4.70
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RPMI-8226 -4.72
SR -5.13
NON-SMALL CELL LUNG CANCER
A549/ATCC -5.09
EKVX -4.77
HOP-18 -5.13
HOP-19 -5.37
HOP-62 -5.72
HOP-92 -4.89
NCI-H226 -5.40
NCI-H23 -5.80
NCI-H322M -5.13
NCI-H460 -5.92
NCI-H522 -5.38
LXFL 529 -5.11
SMALL-CELL LUNG CANCER
DMS 114 -5.23
DMS 273 -5.37
SHP-77 -4.72
COLON CANCER
COLO 205 -5.27
DLD-1 -4.77
HCC-2998 -5.51
HCT-116 -5.19
HCT-15 -4.96
HT29 -4.92
KM12 -5.00
KM20L2 -4.96
SW-620 -4.89
CNS CANCER
SF-268 -5.02
SF-295 -5.34
SF-539 -5.52
SNB-19 -5.06
SNB-75 -5.39
SNB-78 -4.68
TE671 -4.60
U251 -5.24
XF 498 -5.30
MELANOMA
LOX IMVI -5.37
MALME-3M -5.36
M14 -5.41
RPMI-7951 -5.59
M19-MEL -5.41
SK-MEL-2 -5.29
SK-MEL-28 -5.13
SK-MEL-5 -5.89
UACC-257 -5.28
UACC-62 -5.57
OVARIAN CANCER
IGROV1 -5.02
OVCAR-3 -5.07
OVCAR-4 -4.96
OVCAR-5 -5.03
OVCAR-8 -5.02
SK-OV-3 -5.00
RENAL CANCER
786-0 -5.34
A498 -5.55
ACHN -5.31
CAKI-1 -5.26
RXF-393 -5.24
RXF-631 -4.96
SN12C -5.00
SN12K1 -5.01
TK-10 -4.70
UO-31 -5.11
MISCELLANEOUS
P388 -4.96
P388/ADR -4.74
PROSTATE CANCER
PC-3 -4.82
DU-145 -5.31
BREAST CANCER
MCF7 -5.06
MCF7/ADR-RES -4.66
MDA-MB-231/ATCC -4.85
HS 578T -4.85
MDA-MB-435 -5.38
MDA-N -5.36
BT-549 -5.14
T-47D -4.70
MG_MID -5.14
Delta 0.78
Range 1.32
+3 +2 +1 0 -1 -2 -3

C)


LEUKEMIA
CCRF-CEM -4.60
HL-60(TB) -4.64
K-562 -4.62
MOLT-4 -4.62
RPMI-8226 -4.62
SR -4.62
NON-SMALL CELL LUNG CANCER
A549/ATCC -4.62
EKVX -4.64
HOP-18 -4.60
HOP-19 -4.64
HOP-62 -4.74
HOP-92 -4.60
NCI-H226 -4.80
NCI-H23 -4.96
NCI-H322M -4.62
NCI-H460 -4.96
NCI-H522 -4.89
LXFL 529 -4.74
SMALL-CELL LUNG CANCER
DMS 114 -4.74
DMS 273 -4.82
SHP-77 -4.60
COLON CANCER
COLO 205 -4.85
DLD-1 -4.60
HCC-2998 -5.02
HCT-116 -4.74
HCT-15 -4.66
HT29 -4.66
KM12 -4.68
KM20L2 -4.62
SW-620 -4.62
CNS CANCER
SF-268 -4.62
SF-295 -4.66
SF-539 -4.82
SNB-19 -4.62
SNB-75 -4.70
SNB-78 -4.60
TE671 -4.60
U251 -4.72
XF 498 -4.80
MELANOMA
LOX IMVI -4.77
MALME-3M -4.89
M14 -4.92
RPMI-7951 -4.77
M19-MEL -4.89
SK-MEL-2 -4.85
SK-MEL-28 -4.77
SK-MEL-5 -5.34
UACC-257 -4.85
UACC-62 -4.96
OVARIAN CANCER
IGROV1 -4.72
OVCAR-3 -4.68
OVCAR-4 -4.68
OVCAR-5 -4.64
OVCAR-8 -4.77
SK-OV-3 -4.62
RENAL CANCER
786-0 -4.80
A498 -4.96
ACHN -4.60
CAKI-1 -4.64
RXF-393 -4.85
RXF-631 -4.64
SN12C -4.62
SN12K1 -4.68
TK-10 -4.60
UO-31 -4.74
MISCELLANEOUS
P388 -4.64
P388/ADR -4.66
PROSTATE CANCER
PC-3 -4.60
DU-145 -4.60
BREAST CANCER
MCF7 -4.60
MCF7/ADR-RES -4.62
MDA-MB-231/ATCC -4.60
HS 578T -4.60
MDA-MB-435 -4.85
MDA-N -4.82
BT-549 -4.77
T-47D -4.62
MG_MID -4.72
Delta 0.62
Range 0.74
+3 +2 +1 0 -1 -2 -3

3.4 Discussion

The potential benefits of incorporating biochemical and molecular parameters into the existing relational data base on the *in vitro* antitumour activity of approximately 42,000 chemical entities are considerable (Boyd, 1993; Paull *et al.*, 1989; Paull *et al.*, 1992; Weinstein *et al.*, 1992). Clearly, it would increase the valuable information which can be obtained by facilitating the determination of the possible involvement of these parameters in the antitumour mechanism. In this chapter, the effectiveness of this type of analysis has been put to the test by searching the data base for correlations between bioreductive enzyme activity (detailed in Chapter 2) and cell sensitivity within the large panel of approximately 60 human tumour cell lines. The normal procedure at the NCI is to use GI_{50} values in the correlation analysis as an indication of cell sensitivity (i.e. the concentration of drug that caused 50% inhibition in protein increase in drug-treated cells compared to control cells). This is in fact equivalent to the IC_{50} value more frequently used within our laboratory.

As an initial test of the ability to use enzyme activity measurements to investigate involvement in *in vitro* antitumour activity, the data base was searched for any correlations between DT-diaphorase activity and cell sensitivity towards compounds within the NCI screen. Certain quinones are well documented as being bioactivated by DT-diaphorase (Cadenas & Hochstein, 1992; Ross *et al.*, 1994 and references therein) and therefore it was not surprising to find that several quinone-containing compounds, such as the indoloquinone EO9 and the prototype alkylating agent mitomycin C, were common among the top 40 compounds (based on the Pearson Correlation Coefficient). Interestingly, the quinone-containing compound streptonigrin was found to exhibit the highest correlation between cell sensitivity and DT-diaphorase activity. Streptonigrin has been reported to be an excellent substrate for both rat and human recombinant DT-diaphorase (Beall *et al.* , 1994a). Additional studies by this group (Ross *et al.*, 1994) have shown that compared to other compounds, streptonigrin displayed the greatest selective cytotoxicity to cell lines containing high DT-diaphorase activity. These latter findings,

although based on a smaller study, are in direct agreement with those in the current study and provide an explanation for the finding that cell sensitivity towards streptonigrin showed the best correlation with DT-diaphorase activity out of a total of approximately 24,000 compounds included in this analysis. Perhaps more surprising was the fact that 10 fluorouracil derivatives, including 5-fluorouracil (5-FU) itself, were present within the top 40 compounds. 5-FU is the most effective single agent used in the treatment of colorectal carcinoma (Pinedo & Peters, 1988). It was interesting to note therefore that human tumour cell lines originating from colon cancers appeared to be more sensitive to 5-FU (data not shown). These particular cell lines were shown in Chapter 2 (2.3.1.) to have higher than average DT-diaphorase enzyme activities. Although there have been no previous reports available concerning a link between 5-FU and DT-diaphorase, it may be that the high levels of DT-diaphorase present in colon cancers (Schlager & Powis, 1990) play a role in the antitumour activity of this agent. An alternative, and perhaps more likely, explanation is that co-ordinate regulation of enzymes such as DT-diaphorase and dihydropyrimidine dehydrogenase (known to regulate 5-FU metabolism and hence, sensitivity (Pinedo & Peters, 1988)) occurs, such that in colon tumour cell lines several enzymes are in fact co-ordinately up-regulated. This unexpected finding may therefore have important implications in understanding 5-FU activity and also for future therapeutic applications.

Although positive and highly significant Pearson Coefficient Correlations were obtained in this study, the degree of correlation could be described as only moderate (see correlation coefficients in Table 3.1). A possible explanation for this is that enzyme activity measurements were performed in the laboratories in Glasgow while the cell sensitivity data was carried out at the NCI laboratories. Perhaps greater correlation coefficients would have been obtained had both studies taken place within the same laboratory environments. For example, a recent study by Plumb *et al* (Plumb *et al.*, 1994a) produced a much higher correlation coefficient of 0.87 when comparing DT-diaphorase activity and cell sensitivity towards EO9, both of which were measured in the same laboratory (Plumb *et al.* , 1989). This collaboration was established in this manner,

however, as the NCI possessed a large panel of human tumour cell lines in which the aerobic cell sensitivity towards compounds within their data base had been determined. This would therefore be the first study of its kind to use such a large panel of human tumour cell lines. A great deal of variability exists across the panel of cell lines (i.e. origins of the cell lines) and this may contribute to lower correlations being obtained.

A protein-binding dye, SRB is used as an end-point to the cell sensitivity assay at the NCI laboratories (see Section 3.2.2.). In contrast, other studies (Plumb & Workman, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; ; Robertson *et al.*, 1992) have used MTT-dye reduction as the end-point to the assay. The NCI elected to use the SRB method following a pilot study (Monks *et al.*, 1991) in which two different types of tetrazolium assays (one of which was MTT) and an assay using the protein-binding dye SRB were compared for their ability to measure cell viability and cell growth. Comparisons of the MTT and SRB assays suggested that the results were similar in each case (Rubinstein *et al.* , 1990). However, the MTT assay was found to be both time-consuming and also dictated by time restrictions (i.e. critical time period at the plate reading stage). As the NCI required an extremely efficient and least-time consuming assay for their future annual screening of approximately 20,000 agents in potentially 100 different cell lines, they concluded that the SRB assay presented the most efficient assaying system for their purpose.

In addition to DT-diaphorase activity, the data base was searched for any correlations between either NADPH : cytochrome P450 reductase or NADH : cytochrome b_5 reductase activity and cell sensitivity towards compounds within the NCI screen. In both cases, no significant correlations were detected between enzyme activity and cell sensitivity. It is important to remember, however, that cell sensitivity studies have been performed under aerobic conditions and that the results may be quite different under hypoxic conditions.

As a further test of the ability to use enzyme activity measurements to investigate involvement in *in vitro* antitumour activity, the data base was interrogated in more detail with respect to two selected compounds, namely the clinically used mitomycin C and the

structurally-related compound EO9 which has been undergoing recent Phase I clinical trial (Verweij *et al.* , 1994). The former was selected as an established agent for which a number of enzymes are known biochemically to activate the drug (Gustafson & Pritsos, 1992; Gustafson & Pritsos, 1993; Hoban *et al.* , 1990; Hodnick & Sartorelli, 1993; Siegel *et al.* , 1990a). Moreover, whereas DT-diaphorase is thought to be one of these, a previous study in a panel of 15 human tumour cell lines found no correlation between DT-diaphorase expression and cytotoxicity (Robertson *et al.*, 1992). EO9 was selected as a developmental drug in which a strong correlation between DT-diaphorase expression and cytotoxicity has been seen in several smaller studies but for which information on the role of additional reductases is lacking (for review see Workman, 1994). The results of the current study show that in this much larger cell line panel covering a wider range of tumour histiotypes, a highly significant correlation was seen between DT-diaphorase expression and sensitivity to both EO9 (Figure 3.1) and mitomycin C (Figure 3.2) under aerobic conditions. These results are in direct agreement with several other reports suggesting a role for DT-diaphorase in the activation of these two compounds (Plumb & Workman, 1994; Robertson *et al.*, 1994; Ross *et al.*, 1994; Walton *et al.*, 1992a and references therein). As mentioned above, Robertson and colleagues (Robertson *et al.*, 1992) found contrasting results in that no correlation between mitomycin C cell sensitivity and DT-diaphorase activity. The reasons for this finding are unclear. A very small number of human tumour cell lines were used in the study (Robertson *et al.*, 1992) and the analysis may have been influenced by outliers thereby giving no correlation. Mitomycin C is known to be activated by DT-diaphorase at physiological pH, i.e. in the range of 7 - 7.4 (Siegel *et al.* , 1992), but at pH 7.8 it is an effective inhibitor of the enzyme (Siegel *et al.* , 1993). It may be possible that the culture conditions could affect the interaction of mitomycin C and DT-diaphorase.

When looking at the sensitivity of individual tumour types to EO9 (Figure 3.3), leukaemic/lymphoma and ovarian lines tended to be more resistant whereas small-cell and non-small cell lung cancer, colon, CNS and melanoma were generally more sensitive. The results corresponded with those tumour types expressing above average DT-

diaphorase activity. In contrast, there was no significant relationship between cell sensitivity and activities of either NADPH : cytochrome P450 reductase or NADH : cytochrome b_5 reductase. These results would suggest therefore that DT-diaphorase is an important enzyme in the activation of the bioreductive quinone EO9 in intact cells under aerobic conditions whereas the other enzymes are much less important or not involved at all.

The correlation between mitomycin C cell sensitivity and DT-diaphorase activity, although still highly significant, was lower than with EO9. Hence, it was not surprising that the situation was less clear cut as far as sensitivity of individual tumour types to mitomycin C was concerned. Patterns of resistance and sensitivity, however, were still detectable across the panel of cell lines. For example, breast and ovarian lines tended to be more resistant whereas melanoma, CNS and non-small cell lung cancer lines were more sensitive. As mentioned previously, no relationship between the other two enzyme activities and cell sensitivity towards mitomycin C were detected. As is the case with EO9, the results suggested that DT-diaphorase is an important enzyme in the activation of mitomycin C in intact cells under aerobic.

The correlations shown in Figure 3.1 and 3.2, which are supported by the statistical analysis, nevertheless demonstrate that although DT-diaphorase is a major determinant of cellular sensitivity to EO9 and mitomycin C it is clearly not the only factor. This is not surprising. Not only may other activating and detoxifying systems play a role, but factors such as intrinsic sensitivity to DNA alkylation and DNA repair capacity will also play a role.

It should be emphasised that the correlations reported above concern the cytotoxicity of EO9 and mitomycin C under aerobic conditions. The sensitivity of the NCI human tumour cell line panel to these drugs has not been determined under hypoxic conditions, and the present configuration of the screen would not permit this. Very recent studies have shown that different results can be obtained under aerobic versus hypoxic environments (Plumb & Workman, 1994; Plumb *et al.*, 1994a; ; Robertson *et al.*, 1994) but the significance of these differences for the *in vivo* situation remains unclear.

In summary, the ability to use enzyme activity measurements as an additional parameter in establishing possible correlations with *in vitro* antitumour activity has been investigated. The correlations observed between enzyme activity and tumour cell line sensitivity to various quinones including the indoloquinone EO9 and mitomycin C, suggests that the inclusion of the enzyme activity measurements within the relational data base should allow similar questions to be asked across the 42,000 or more discrete chemical entities that have been screened to date. It should be possible to determine not only where compounds are activated by the reductases concerned, but also where reductive metabolism leads to deactivation in intact cells. Moreover, new agents under study in the panel can also be analysed in this way. This could lead to the targeting of particular agents to certain tumour types, depending on the levels of activating and deactivating enzymes, as suggested by the 'enzyme-directed' approach to bioreductive drug development (Plumb & Workman, 1994; Plumb *et al.*, 1994a; Robertson *et al.*, 1994; Workman, 1992; Workman, 1994; Workman & Walton, 1990). This study has therefore confirmed the feasibility and value of measuring biochemical and molecular parameters as potential aids to the diagnostic power of the NCI human tumour panel to investigate potential mechanism of action.

CHAPTER 4

CYTOTOXICITY OF POTENTIAL BIOREDUCTIVE ANTICANCER AGENTS IN A PANEL OF HUMAN BREAST TUMOUR CELL LINES

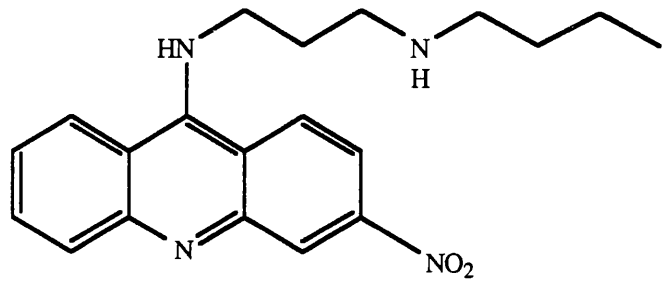
4.1 Introduction

Early studies of potential bioreductive anticancer agents involved the electron-affinic radiosensitisers which, during the course of study, were shown to demonstrate preferential toxicity towards hypoxic cells *in vitro* (Stratford *et al.* , 1980) and *in vivo* (Rauth *et al.* , 1978). Perhaps the most promising compounds of this group were the nitroimidazole class which included the 2-nitroimidazole misonidazole and, at a later stage, the 'dual-function' radiosensitiser RSU 1069 (see Chapter 1, Figure 1.2). During clinical trials, however, several of the compounds were shown to possess dose-limiting toxicities such as neurotoxicity and gastrointestinal toxicity (Workman, 1993) and as such proved unsuccessful. Furthermore, the level of potency of the compounds under hypoxic conditions was reported to be low (Wilson *et al.* , 1984). In an attempt to develop more potent radiosensitisers, Denny and colleagues have studied the potential of a second group of nitro-containing compounds to act as bioreductive anticancer agents. The nitroacridines, as they are known, are grouped within the acridine class and are large heterocyclic compounds (Ferguson & Denny, 1991). In addition to their intercalative DNA binding ability (Roberts *et al.* , 1990), it was hoped that they would act as more potent agents than the nitroimidazoles. The antitumour agent nitracrine (1-NC; 1-nitro-9-(dimethylaminoporopylamino)-acridine) has, in fact, been shown to be approximately 100,000 times more potent than misonidazole under hypoxic conditions (Wilson *et al.*, 1984). This is thought to be due in part to the ability of the agent to intercalate with DNA and cause DNA alkylation, but also due to reductive metabolism of the compound under hypoxic conditions (Wilson *et al.*, 1984). Subsequent studies, however, have shown that

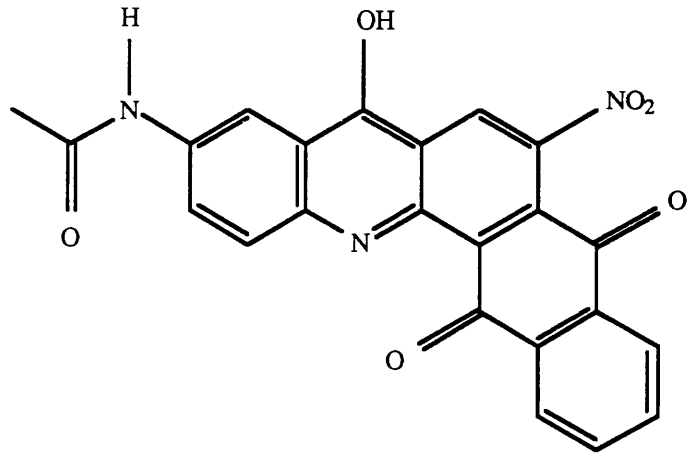
while this is the case, the cytotoxic activity was not completely inactivated by the presence of oxygen (Wilson *et al.* , 1986). Cytotoxicity in this situation was believed to arise from DNA adduct formation (Konopa *et al.* , 1983; Pawlak *et al.* , 1983). As a consequence, further studies are under way to further develop new nitroacridines as potentially useful clinical bioreductive agents (Roberts *et al.*, 1990). In addition, the same group have also been investigating compounds such as the quinoline analog of 1-NC, 5-nitraquine (Wilson *et al.* , 1992) and the 4-alklyamino-5-nitroquinoline derivative (8Me-5NQ) (Siim *et al.* , 1994) as potential bioreductive anticancer agents. To date, however, these studies have revealed problems of lower potency (Wilson *et al.*, 1992) and oxygen sensitivity (Siim *et al.*, 1994).

One of the most promising areas of bioreductive anticancer agents has been the recent development of a series of benzotriazine di-*N*-oxides, the lead compound being tirapazamine (SR 4233; WIN 59075). Although tirapazamine has now progressed to Phase II clinical trial, minor problems such as muscle cramping and solubility (Doherty *et al.* , 1994) were observed during the Phase I clinical trial. As discussed previously for the nitroheterocyclic compounds, further developmental studies are underway to produce a potentially better compound than tirapazamine for use in the clinic. For example, structure-activity relationship studies revealed that the analogue SR 4482 was more toxic to hypoxic cells *in vitro* and less toxic *in vivo* than tirapazamine (Zeman *et al.* , 1989). In addition, the analogue SR 4895 has been shown to be as effective as tirapazamine itself in causing DNA damage *in vitro* (Morecroft *et al.* , 1994). Further studies will determine whether results such as these lead to better agent for use in the clinic.

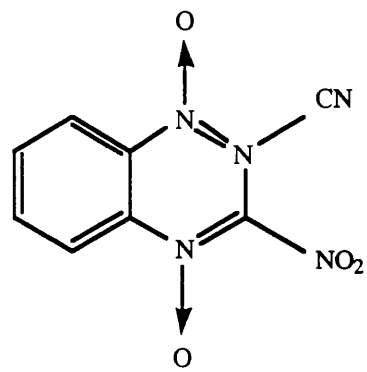
In an attempt to discover more potent and selective bioreductive anticancer agents, three novel compounds were selected for study as potential bioreductive anticancer agents based upon their structural similarities with some of the more active agents currently studied. These compounds, provided by ZENECA Pharmaceuticals, consisted of a nitroacridine compound (ZM 33191), a large nitroheterocyclic compound (ZM 29362) and a quinoxaline (ZM 81853) (Figure 4.1a). The former was selected due to its structural similarity to nitracrine (1-NC) (Wilson *et al.*, 1984; Wilson *et al.*, 1986).



ZM 33191



ZM 29362



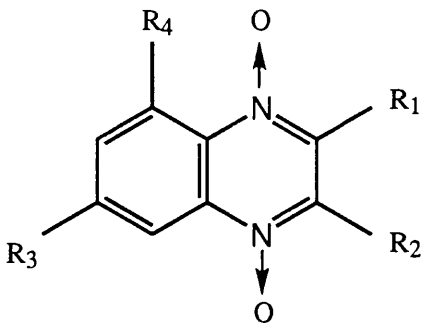
ZM 81853

Figure 4.1a Structures of novel compounds from ZENECA Pharmaceuticals.

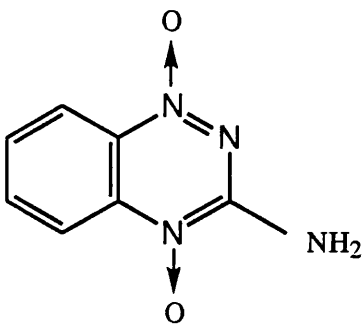
However, it was hoped that it would prove to be less toxic under oxic conditions. Although both ZM 33191 and ZM 29362 would be classed as nitroheteocyclic compounds, an additional interesting feature of ZM 29362 was the presence of a quinone moiety in the structure. This would create two possible reduction sites within the compound and as such make it an intriguing compound to study. The final compound, ZM 81853, was selected due to its structural similarity with tirapazamine and could be directly compared to that compound. The main aim of the study was to determine the ability of each of the compounds to be preferentially activated under hypoxic conditions and to compare this ability to known bioreductive anticancer agents.

Previous studies, for example with tirapazamine (Minchinton *et al.* , 1992; Morecroft *et al.*, 1994) and with the indoloquinone EO9 (Bailey *et al.* , 1992), have shown that small structural changes can alter the activity of compounds considerably. The structure-activity relationship of the quinoxaline ZM 81853 was therefore investigated by comparing the ability of a small series of the quinoxalines to act as bioreductive anticancer agents. The specific structures chosen are displayed in Figure 4.1b.

An *in vitro* cell cytotoxicity assay was used to establish the ability of these novel compounds to act as bioreductive anticancer agents and to determine the potential one- and / or two-electron reducing enzymes involved in their activation. A small panel of human breast tumour cell lines was selected for the study, based upon the varying levels of enzyme activity displayed previously in the NCI cell line panel (Chapter 2). Furthermore, as hypoxic regions have previously been demonstrated within breast tumours (Vaupel *et al.* , 1991), it was of interest to study the effects of these agents in cell lines originating from such tumours. In addition, two Chinese Hamster Ovary cell lines were used in the study, one of which had been transfected with the active gene for NADPH : cytochrome P450 reductase and as such should possess significantly higher enzyme levels compared to other cell lines. A modified version of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay (Plumb *et al.* , 1989) was used as a measure of the potential ability of the novel compounds to act as bioreductive anticancer agents. This assay measures the ability of live cells to reduce a



QUINOXALINE SERIES



TIRAPAZAMINE
(SR 4233; WIN 59075)

Analogue	R_1	R_2	R_3	R_4
ZM 8173	-	-	-	-
ZM 10309	-	-	NH_2	-
ZM 65086	-	-	-	NH_2
ZM 81853	CN	NH_2	-	-
ZM 12557	CH_3	CONHOH	-	-

Figure 4.1b Structural differences between the quinoxaline series and tirapazamine.

tetrazolium dye, and hence produces an estimate of cell numbers in a simple chemosensitivity assay. Such an assay system has been shown previously to produce similar results to those determined by clonogenic assay (Plumb *et al.*, 1989). The MTT assay system was selected for use in preference to the more widely used clonogenic assay for several reasons. First and foremost, not all of the human breast tumour cell lines selected for study were colony-forming cells, for example, T47D cells. Secondly, the clonogenic assay procedure is considerably more time-consuming (only 2 - 3 can be performed at the one time) and is unsuitable for comparing larger numbers of cell lines and drugs as more variables are introduced into the system. In addition, the possibility that the drugs interfere with the ability of the cells to reduce MTT is overcome by removal of drugs after a 3.5 hr. exposure, followed by a 3 day drug-free period. Perhaps the main advantage of the clonogenic assay system is that both cytotoxic (colony number) and cytostatic (colony size) effects can be studied. Early results, however, indicated that any effects were most likely cytotoxic as all cells in the top drug concentration and some in the other concentrations are dead within 24 hr. after drug treatment. A final factor was the limited availability of compounds.

Thus the present chapter describes the ability of structurally distinct compounds to act as bioreductive anticancer agents, and discusses possible differences between the current results and those obtained with previously studied bioreductive compounds.

4.2 Materials and Methods

4.2.1. Chemicals and Reagents

Seven novel compounds including quinoxalines, a nitroacridine and a heterocyclic compound were kindly provided by ZENECA Pharmaceuticals (Macclesfield, Cheshire, U.K.). Generous donations of tirapazamine (SR 4233; WIN 59075) and EO9 were received from Drs. M. Tracy and W. W. Lee of SRI International (Menlo Park, CA, U.S.A.) and by the New Drug Development Office of the EORTC, respectively. All tissue culture media components were purchased from Life Technologies (Paisley, Scotland). Tissue culture flasks and 96-well microtitration plates were purchased from Bibby Sterilin (Stone, Staffordshire, U.K.) and the PETG Tulip 96-well plastic plates were from Alpha Laboratories (Eastleigh, Hampshire, U.K.). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company (Poole, Dorset, U.K.).

4.2.2. Cell Lines

The source and doubling time of the cell lines used in this study are shown in Table 4.1. T47D and MDA-MB 231 cell lines were from the American Type Tissue Collection (ATCC). MCF-7 cells were originally from Dr. K. Cowan (NCI, Bethesda, MD, U.S.A.). Chinese Hamster Ovary cells transfected with either vector and human NADPH : cytochrome P450 reductase gene (CHO-P450R) or with vector and gene in opposite orientation (control cell line) (CHO-K1-WT) were kindly provided by Prof. C.R. Wolf (ICRF Laboratory of Molecular Pharmacology, Dundee, U.K.).

4.2.3. Routine Cell Maintenance

All epithelial cell lines were maintained in RPMI 1640 supplemented by sodium bicarbonate (0.075 %), L-glutamine (2 mM), foetal calf serum (10 %, v/v), Penicillin (50

Cell Line	Doubling Time (days)	Characteristics	Source	Reference
MCF-7	1.2	Human Breast Adenocarcinoma	Dr. K. Cowan, NCI, Bethesda, U.S.A.	(Soule *et al.*, 1973)
T47D	2.1	Human Breast Carcinoma	ATCC HTB 133	(Sher *et al.*, 1981)
MDA-MB 231	1.5	Human Breast Adenocarcinoma	ATCC HTB 26	(Cailleau *et al.*, 1974)
CHO-K1-WT	0.6	Chinese Hamster Ovary (transfected with non-functional NADPH : cytochrome P450 reductase gene)	Prof. C. R. Wolf	-
CHO-P450R	0.8	Chinese Hamster Ovary (transfected with functional NADPH : cytochrome P450 reductase gene)	Prof. C. R. Wolf	-

Table 4.1 Source, characteristics and cell doubling times of the cell lines used in these studies.

U/ml) and Streptomycin (50 μg/ml). In the case of T47D and MDA-MB 231, cells were passaged at weekly intervals at a 1:10 dilution. MCF-7 cells were passaged at weekly intervals but at a 1:25 dilution. The CHO-K1-WT and CHO-P450R cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) containing L-glutamine (2 mM), sodium bicarbonate (0.075 %), foetal calf serum (10 %, v/v), Penicillin (50 U/ml) and Streptomycin (50 μg/ml). CHO-K1-WT cells were passaged twice weekly at a dilution of 1:25. In contrast, the CHO-P450R cell line was passaged at weekly intervals at a 1:10 dilution. Following passaging of this latter cell line, cells were maintained in media as detailed above for 2 - 3 dys. before being replaced by selective media containing puromycine (2.5 μg/ml). All cell lines were incubated at 37°C in an atmosphere of 2 % CO_2 in air.

Cells were passaged by removal of all medium from the flask then adding 2.5 mls of phosphate buffered saline (PBS) containing EDTA (1 mM) and trypsin (0.25 %) to the flask. This solution was left on the cells for 5 - 15 mins., depending on the cell line, to allow the cells to detach from the surface of the flask. Cells were resuspended by the addition of 7.5 mls of the relevant medium and diluted into fresh medium in sterile tissue culture flasks.

4.2.4. Mycoplasma Testing

All cell lines were free of mycoplasma as confirmed by monthly screening. Cells were fixed with ice-cold glacial acetic acid (25 %, v/v) in methanol and then stained with the fluorescent DNA stain Hoescht 3328 (Sigma Chemical Company, Poole, Dorset, U.K.) at a concentration of 100 ng/ml for 15 mins. at room temperature (Chen, 1977). Plates were then examined under a fluorescent microscope (Polyvar Microscope, Reichert, Leica Ltd., Milton Keynes, U.K.) for visible evidence of infection, i.e. characterised by non-nuclear staining.

4.2.5. Enzyme Activities within Cell Lines

DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase enzyme activities were determined in each of the five cell lines using the method described in Chapter 2 (Section 2.2.6.). At the time of passaging cells, three additional flasks were set up for each cell line. Having reached confluence, the cells from each flask were harvested separately as described in Section 4.2.3., pelleted by centrifugation at 200 g for 5 mins. at 4°C (Centra-7R centrifuge, International Equipment Company, U.S.A.) and the cells washed twice with PBS. The final cell pellet was resuspended in 0.5 mls PBS containing 10 % aprotinin. Cells were then sonicated, centrifuged in an Eppendorf centrifuge at 4°C to remove debris, and aliquoted as described previously (Section 2.2.4.). Protein content of each set of aliquots was determined using the Pierce BCA Protein Assay (detailed in Section 2.2.5.).

4.2.6. Drugs

ZM 33191 and EO9 were prepared as 5 mM stock solutions in dimethylsulphoxide (DMSO), while tirapazamine was made as a 200 mM stock solution. All other compounds were prepared as 50 mM stock solutions. In the case of the novel quinoxaline compounds, the range of concentration used for each drug was based on preliminary studies performed at ZENECA Pharmaceuticals. Dilutions were made from the stock solution into culture medium. The range of concentrations used for ZM 81853, ZM 10309, ZM 12557, ZM 65086 and ZM 8173 under oxic conditions was 19.5 - 2500 μM. For all of these compounds except ZM 81853, a range of 7.8 - 1000 μM was used under hypoxic conditions. In the case of ZM 81853, the range was 2.0 - 250 μM. Both the nitroacridine ZM 33191 and the heterocyclic compound ZM 29362 were used at the wider concentration ranges of 6.4×10^{-4} - 50 μM and 0.032 - 2500 μM under both oxic and hypoxic conditions as neither compound had been used before. Several of the compounds required high concentrations of stock solution to be made and as a result, the

level of DMSO on the cells was as high as 5 %. For this reason, solvent controls (2 % and 5 %) were used in the assays.

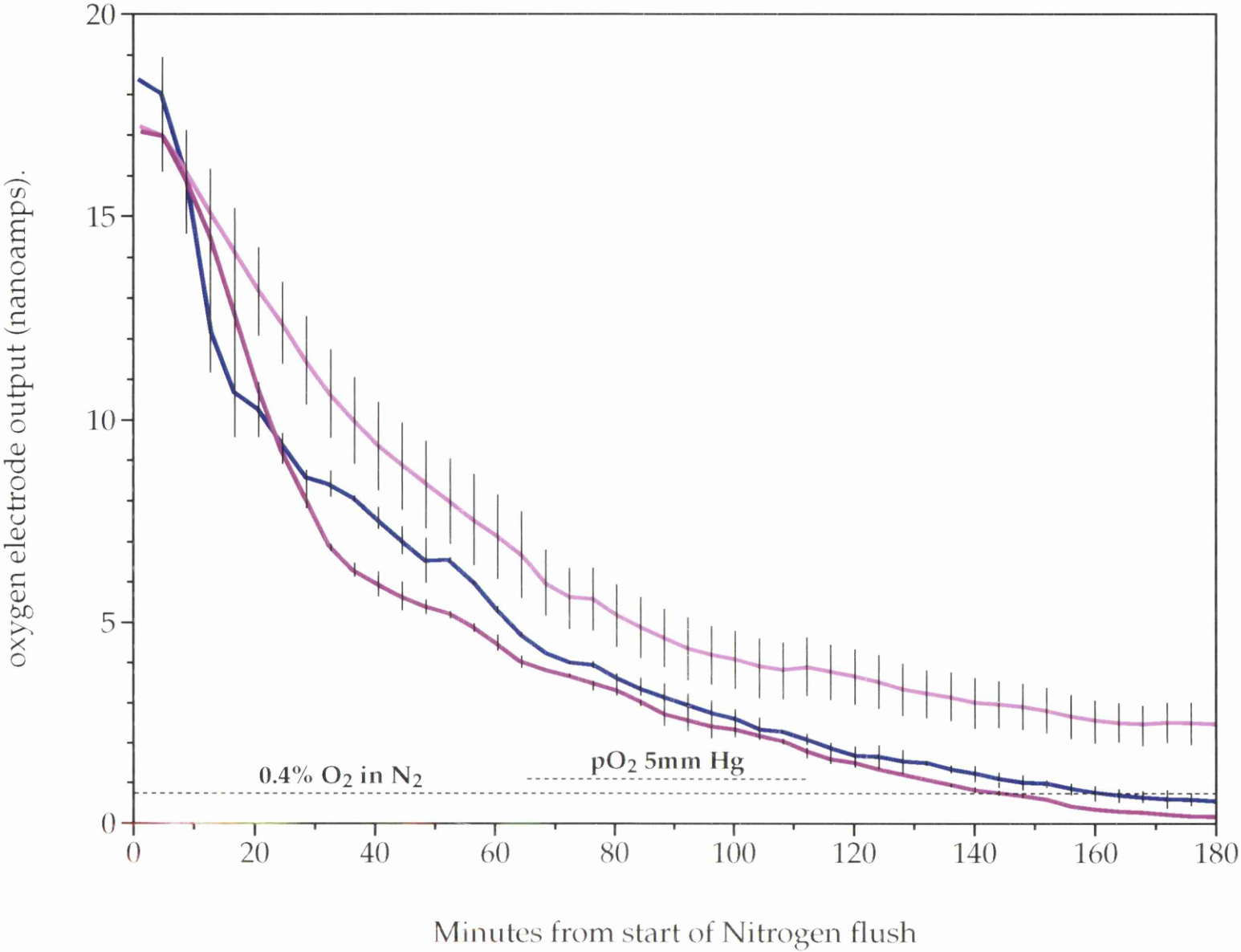
4.2.7. Cytotoxicity Assays

Cytotoxicity was determined using an adaptation of the tetrazolium-based microtitration assay described previously (Plumb *et al.*, 1989). When measuring cell cytotoxicity under oxic conditions, this assay is normally performed using 96-well plastic microtitre plates (for example, Costar, High Wycombe, Bucks., U.K.). However, general tissue culture plastic is known to be soluble to oxygen (Chapman *et al.* , 1970), and as such is unsuitable for studies involving hypoxic conditions. This problem is avoided by the use of glass dishes for drug exposure and cell survival determined by colony formation. Glass 96-well plates are not available, however. In an attempt to overcome this problem, Mr. M. Fennell (ZENECA Pharmaceuticals, Macclesfield, Cheshire) tested the ability of PETG (polyethylene terephthalate glycol) 'Tulip' 12-well plastic plates to absorb oxygen, and compared the findings to those of general tissue culture plastic 12-well plates (Costar, High Wycombe, Bucks., U.K.) and 12-well glass plates. The procedure employed was to measure oxygen levels using an oxygen electrode immersed in approximately 8 mm of saline at the bottom of the well over a period of time (3 hrs.) during which a constant flow of nitrogen (0.6 l/min) was maintained. The electrode was calibrated at the start in fully aerated saline (the top value) and with saline gassed for 18 hrs. with 0.4 % oxygen in nitrogen (horizontal line across graph). The electrode current was measured on a nanometer at regular intervals. Results of the study are shown in Figure 4.2, and clearly show that the level of oxygen in the PETG plates decreases over time in a similar manner to that in the glass wells. In contrast, the level of oxygen within the Costar plastic plates, whilst decreasing with time, is consistently higher than the other two. It is important to note that the level of oxygen falls below that generally considered to be present in hypoxic tumour regions (pO_2 5 mm mercury) following equilibration for 2 hrs. with nitrogen. Dr J. Plumb (Plumb & Workman, 1994) has also performed a small

Figure 4.2 Level of oxygen present within several different types of tissue culture apparatus.

Oxygen electrode measurements were made within 12-well plates made of either glass (deep purple), PETG plastic (blue) or Costar plastic (lilac). Each well contained 8mm of saline and readings were made at regular intervals using an oxygen electrode. Each line on the graph represents the mean $\pm$ SE of triplicate sets of data. Explanations of the oxygen electrode output level represented by the two horizontal dotted lines are made in the text.

[Reproduced by kind permission of Mr. M. Fennell, ZENECA Pharmaceuticals.]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5
0
oxygen electrode output (nanoamps).
0.4% O_2 in N_2
pO_2 5mm Hg
0
20
40
60
80
100
120
140
160
180
Minutes from start of Nitrogen flush

scale validation study comparing the differential cytotoxicity of tirapazamine in HT29 cells under oxic and hypoxic conditions. This was determined using either the clonogenic assay or the MTT assay (with PETG plates). Results (Table 4.2) indicated that cells are less sensitive to tirapazamine under both oxic and hypoxic conditions when exposed to drug in PETG plates (MTT) compared to glass (clonogenic). However, the hypoxic cytotoxicity ratio (HCR) values obtained by the two methods were comparable. As a consequence, the MTT cytotoxicity assay was adapted for use with the PETG Tulip plastic 96-well plates.

For the human breast lines, cells were seeded (750 cells/well) into 96-well PETG plates in 150 µl of medium. In contrast, studies involving the CHO-K1-WT and CHO-P450R cells required seeding levels of 525 and 775 cells/well respectively. The first and last row (8 wells in each) of each plate contained medium only. Plates were incubated for 72 hrs. at 37°C in an atmosphere of 2 % CO_2 in air to allow the cells to attach and grow. Medium from the wells was then replaced with medium containing a range of drug concentrations. A total of eight concentrations were used for each drug and four wells per concentration. In addition, medium containing 2 % DMSO or 5 % DMSO was added to two rows of wells as a solvent control. Plates were then exposed to the drugs for 3.5 hrs. in an atmosphere of 2 % CO_2 in either air (oxic) or nitrogen (hypoxic). Following drug exposure, the drug-containing medium was removed and replaced by fresh medium. Plates were then left under normal incubation conditions for a further 3 dys. At the end of the growth period, 30 µl of medium was carefully removed from the wells and replaced by MTT (30 µl; 5 mg/ml PBS). Plates were then wrapped in aluminium foil and incubated for a further 4 hrs. Medium and MTT were removed from the wells and the formazan crystals dissolved in 150 µl DMSO which was added via a dispenser (Welltech Wellfill 3; Denley, Sussex, U.K.). The dissolved crystals were immediately transferred to a rigid plastic 96-well plate, and glycine buffer added (16.7 µl; glycine (0.1 M); sodium chloride (0.1 M), adjusted to pH 10.5 by sodium hydroxide (1 M)) to adjust the final pH. The absorbance was then recorded at a wavelength of 570 nm using Softmax Version 2.3 installed on an Emax Precision microplate reader (details in Section 2.2.5.). The first and

Assay	Conditions	IC_{50} (μM)	HCR
Clonogenic	Oxic	577 ± 88	74
	Hypoxic	7.8 ± 0.9	
MTT (PETG)	Oxic	3550 ± 275	50.0
	Hypoxic	71 ± 19	

Table 4.2 **Comparison of two assay techniques for determining cytotoxicity of tirapazamine in HT-29 cells.**

[Reproduced by permission of Dr J. Plumb (Plumb & Workman, 1994)]

last rows which contained only medium were used to blank the plate reader. Results were expressed as the IC_{50} concentration which was determined as the drug concentration required to reduce the absorbance to half that in the control untreated cells.

4.2.8. Statistical Analysis

The student's unpaired t-test was used to compare results for enzyme activity in the different cell lines.

4.3 Results

4.3.1. Enzyme Activity Measurements within the Panel of Cell Lines

The human breast tumour cell lines were selected for use in the cytotoxicity assay based upon enzyme activity measurements across the NCI cell line panel. However, as differences do exist between batches of the same cell line, the activities of DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase were determined using the cytochrome *c* reduction assay described previously (Chapter 2). The levels of enzyme activities are shown in Table 4.3. MCF-7 cells were found to possess the highest DT-diaphorase and NADH : cytochrome b5 reductase activities amongst the breast lines. In contrast, the highest NADPH : cytochrome P450 reductase activity was found within T47D cells. These results were different from those determined for the same cell lines within the NCI panel (Chapter 2) as the highest NADH : cytochrome b5 reductase and NADPH : cytochrome P450 reductase were obtained within T47D cells and MDA-MB 231 cells, respectively. This result is not surprising, however, as the cells used in this current study were from a different source. Importantly, clear differences were still present between the three cell lines.

In an attempt to obtain cell lines expressing greater differences in one-electron reducing enzymes, two Chinese Hamster Ovary cell lines transfected with either the active or inactive gene for human NADPH : cytochrome P450 reductase, were studied. As the table shows, however, little variation in NADPH : cytochrome P450 reductase was observed. In fact, the activity was not significantly different ($p>0.1$). In addition NADH : cytochrome b5 reductase, although showing 3.6-fold higher activity in the CHO-P450R cells, was not significantly different ($0.05>p>0.02$). The level of DT-diaphorase was, however, 2.9-fold higher in the CHO-P450R cell line (significant; $0.01>p>0.002$).

4.3.2. Cytotoxicity of the Compounds in the Human Breast Tumour Cell Lines

The cytotoxicity of the compounds, determined under both oxic and hypoxic conditions for the three human breast tumour cell lines is shown in Tables 4.4 - 4.6. Also

Tumour Cell Line	Enzyme Activities (nmol/min/mg)		
	DT-diaphorase	NADPH : Cytochrome P450 Reductase	NADH : Cytochrome b5 Reductase
MCF-7	568.4 ± 55.6	19.0 ± 4.3	85.4 ± 13.4
T47D	108.5 ± 8.0	82.8 ± 17.6	24.5 ± 3.7
MDA-MB 231	24.7 ± 13.7	14.8 ± 1.0	55.1 ± 13.1
CHO-K1-WT	113.3 ± 32.0	13.1 ± 1.2	276.7 ± 45.3
CHO-P450R	325.4 ± 39.2	15.8 ± 4.8	993.8 ± 277.1

Table 4.3 Activities of DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase within the panel of cell lines used in the cytotoxicity studies.

Units are nmol of cytochrome *c* reduced/min/mg of protein. Data represent a mean ± SE (n=3). ND represents a level of activity (2 nmol/min/mg of protein or less) which is undetectable in this assay system.

	IC_{50} (μM)		
Compound	**Air**	**N_2**	**HCR**
EO9	**0.67, 0.79**	**0.29, 0.23**	**2.31, 3.43**
Tirapazamine	**1150, 1300**	**40, 20**	**28.8, 65.0**
ZM 81853	**1700 ± 190**	**40 ± 10**	**42.5**
ZM 10309	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 8173	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 65086	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 12557	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 33191	**1.8 ± 0.12**	**2.1 ± 0.99**	**0.9**
ZM 29362	**520 ± 160**	**1690, 1250**	**0.4**

Table 4.4 Sensitivity of the human breast tumour cell line, MCF-7, to EO9, tirapazamine and seven novel compounds.

Sensitivity was determined using the MTT-based cytotoxicity assay. Cells were exposed to drug for 3.5 hr. and sensitivity was expressed as IC_{50} values. Results represent the mean ± SE of triplicate estimations, and are one of three repeated experiments.

	IC_{50} (μM)		
Compound	**Air**	**N_2**	**HCR**
EO9	**2.11, 2.05**	**0.39, 0.12**	**5.41, 17.1**
Tirapazamine	**600, 450**	**10, 10**	**60, 45**
ZM 81853	**970 ± 40**	**20 ± 3**	**48.5**
ZM 10309	**740 ± 150**	**840 ± 440**	**0.9**
ZM 8173	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 65086	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 12557	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 33191	**1.06 ± 0.21**	**0.36 ± 0.17**	**2.9**
ZM 29362	**> 2500 ± 0**	**560 ± 420**	**> 4.5**

Table 4.5 Sensitivity of the human breast tumour cell line, T47D, to EO9, tirapazamine and seven novel compounds.

Sensitivity was determined using the MTT-based cytotoxicity assay. Cells were exposed to drug for 3.5 hr. and sensitivity was expressed as IC_{50} values. Results represent the mean ± SE of triplicate estimations, and are one of three repeated experiments.

	IC_{50} (μM)		
Compound	**Air**	**N_2**	**HCR**
EO9	**6.22, 6.56**	**1.55, 3.22**	**4.01, 2.04**
Tirapazamine	**1300, 1940**	**40, 60**	**32.5, 32.3**
ZM 81853	**1520 ± 30**	**100 ± 20**	**15.2**
ZM 10309	**910 ± 70**	**580 ± 40**	**1.6**
ZM 8173	**2300 ± 40**	**> 1000 ± 0**	**-**
ZM 65086	**1800 ± 250**	**> 1000 ± 0**	**-**
ZM 12557	**> 2500 ± 0**	**> 1000 ± 0**	**-**
ZM 33191	**0.55 ± 0.04**	**0.22 ± 0.04**	**2.5**
ZM 29362	**420 ± 60**	**540 ± 90**	**0.8**

Table 4.6 Sensitivity of the human breast tumour cell line, MDA-MB 231, to EO9, tirapazamine and seven novel compounds.

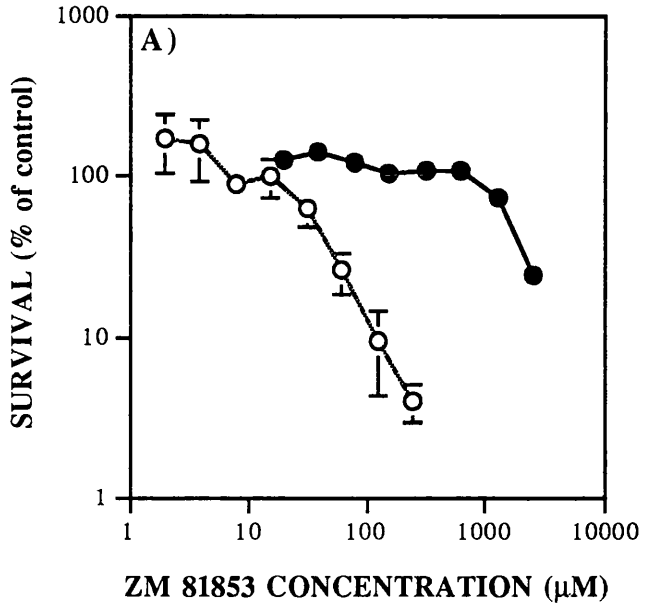
Sensitivity was determined using the MTT-based cytotoxicity assay. Cells were exposed to drug for 3.5 hr. and sensitivity was expressed as IC_{50} values. Results represent the mean ± SE of triplicate estimations, and are one of three repeated experiments.

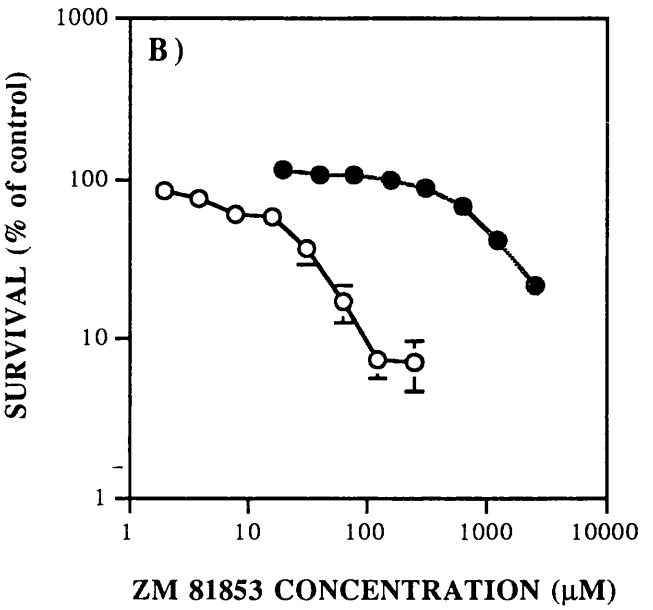
shown is the hypoxic cytotoxicity ratio (HCR) which is the ratio of the sensitivity under oxic conditions to that under hypoxic conditions. Considering first the quinoxaline series. None of the cell lines were sensitive to ZM 12557, ZM 65086 or the unsubstituted parent compound ZM 8173, suggesting that all of these compounds are inactive under both oxic (air) and hypoxic (N_2) conditions. ZM 10309 showed little cytotoxic effects under either oxic or hypoxic conditions in the high DT-diaphorase containing cell line MCF-7 (Table 4.4). Furthermore, although both T47D and MDA-MB 231 cells (Tables 4.5 and 4.6, respectively) were more sensitive to ZM 10309 under oxic conditions, no significant enhancement of this sensitivity occured under hypoxic conditions. In contrast, all three cell lines exhibited enhanced sensitivity to the quinoxaline ZM 81853 under hypoxic conditions (Tables 4.4 - 4.6 and Figure 4.3). Interestingly, ZM 81853 showed similar characteristics to those of tirapazamine in each of the three cell lines (Table 4.4 - 4.6) both in terms of HCR and IC_{50} values. In particular, T47D cells (Table 4.5) which contained the highest level of NADPH : cytochrome P450 reductase activity were approximately 48-fold (ZM 81853) and 52-fold (tirapazamine) more sensitive towards these agents under hypoxic conditions. The enhanced cytotoxic effects of tirapazamine can be viewed graphically in Figure 4.4. As expected, MCF-7 cells showed only a small shift (2 to 3-fold) in sensitivity towards the control compound EO9 under hypoxic conditions (Table 4.3) while in the case of T47D and MDA-MB 231 cells (Tables 4.5 and 4.6) this shift was greater (5 to 17-fold and 2 to 4-fold, respectively). Typical cytotoxicity curves for each of the cell lines are shown in Figure 4.4.

In addition to the quinoxaline series, two further novel compounds were studied for their selectivity towards hypoxic conditions. The results for both compounds are shown in Tables 4.4 - 4.6. The nitroheterocyclic compound, ZM 29362, exhibited a very low level of cytotoxicity towards the cell lines under oxic conditions. Incubation under hypoxic conditions resulted in no significant enhancement of this cytotoxicity, suggesting that this compound did not possess the ability to act as a particularly good hypoxia-selective anticancer agent. A similar low level of enhancement in cytotoxicity occured with ZM 33191 under hypoxic conditions. However, it was interesting to note that the

Figure 4.3 Sensitivity of Human Breast Tumour Cell Lines MCF-7 (A), T47D (B) and MDA-MB 231 (C) to ZM 81853.

Cells were exposed to drug for 3.5 hr. under oxic (2 % CO_2 in air; closed circle) or hypoxic (2 % CO_2 in N_2; open circle) conditions, and cell survival determined by an MTT-based assay. Points on the graphs represent a mean $\pm$ SE of triplicate readings and are from one of three repeat experiments.


A)
1000
100
10
1
SURVIVAL (% of control)
1
10
100
1000
10000
ZM 81853 CONCENTRATION (μM)


B)
1000
100
10
1
SURVIVAL (% of control)
1
10
100
1000
10000
ZM 81853 CONCENTRATION (μM)

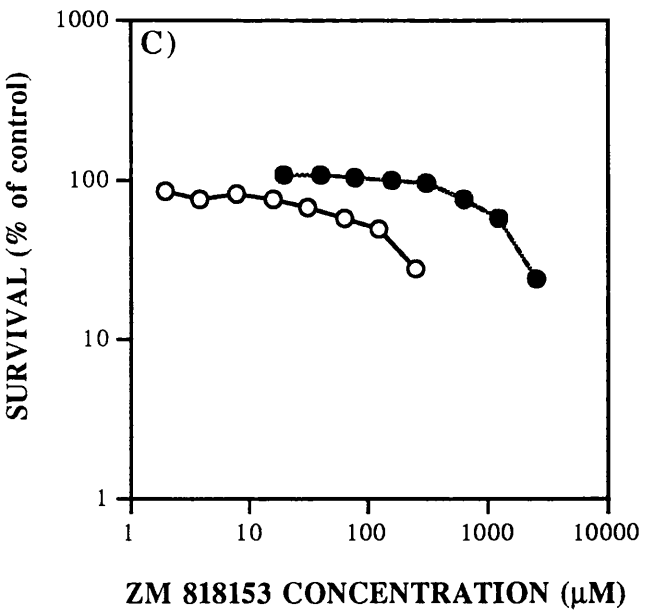
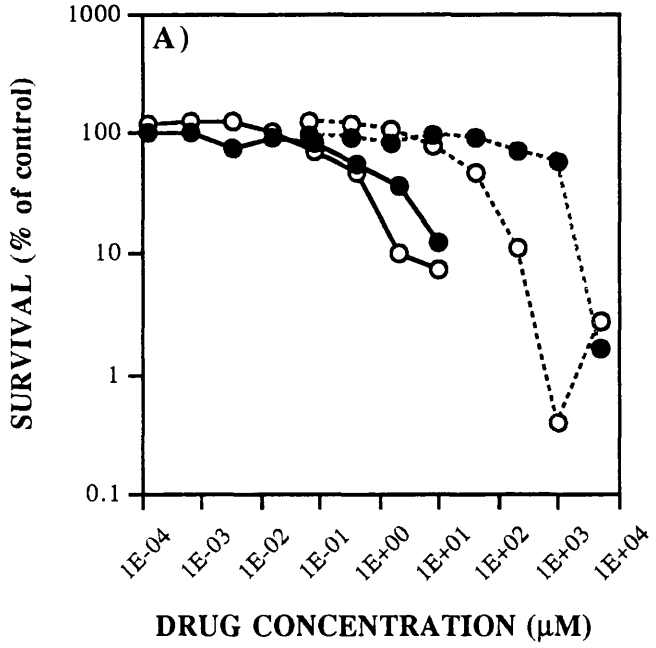
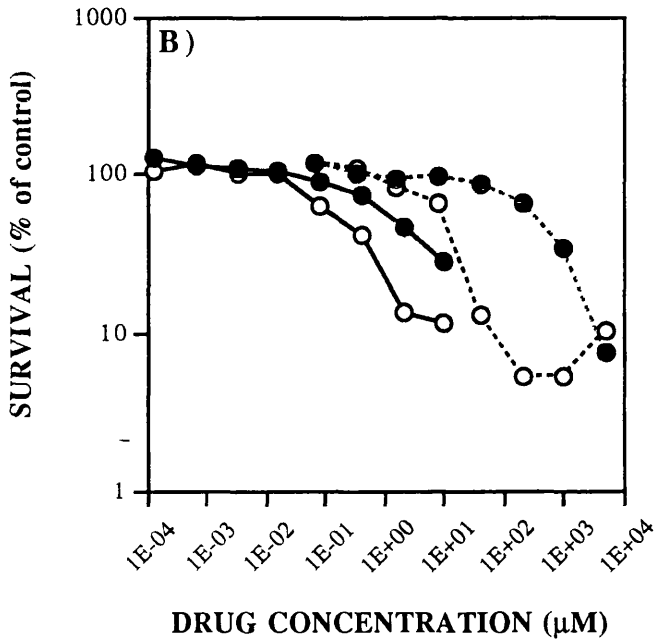
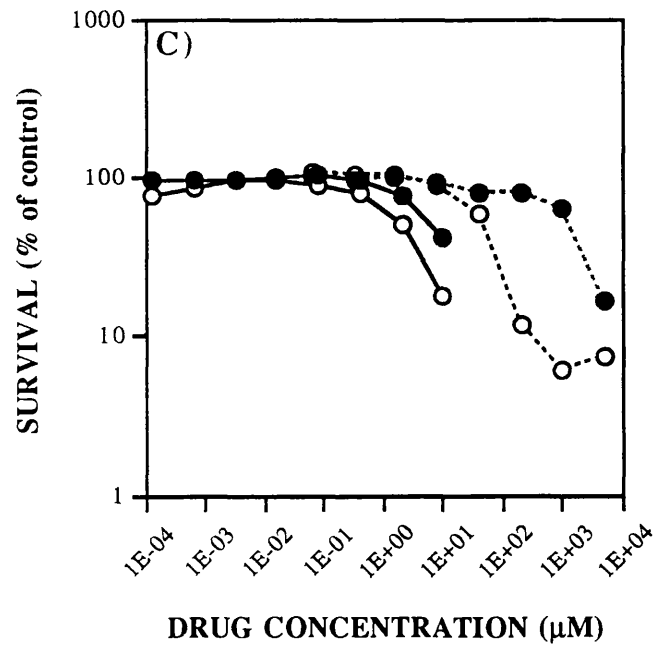

C)
1000
100
10
1
SURVIVAL (% of control)
1
10
100
1000
10000
ZM 818153 CONCENTRATION (μM)

Figure 4.4 Sensitivity of Human Breast Tumour Cell Lines MCF-7 (A), T47D (B) and MDA-MB 231 (C) to EO9 and Tirapazamine.

Cells were exposed to drug (EO9 solid line; Tirapazamine dashed line) for 3.5 hr. under oxic (2 % CO_2 in air; closed circle) or hypoxic (2 % CO_2 in N_2; open circle) conditions, and cell survival determined by an MTT-based assay. Points on the graphs represent a mean of two estimations. This experiment was repeated three times.


A)
1000
100
10
1
0.1
SURVIVAL (% of control)
1E-04
1E-03
1E-02
1E-01
1E+00
1E+01
1E+02
1E+03
1E+04
DRUG CONCENTRATION (μM)


B)
1000
100
10
1
SURVIVAL (% of control)
1E-04
1E-03
1E-02
1E-01
1E+00
1E+01
1E+02
1E+03
1E+04
DRUG CONCENTRATION (μM)


C)
1000
100
10
1
SURVIVAL (% of control)
1E-04
1E-03
1E-02
1E-01
1E+00
1E+01
1E+02
1E+03
1E+04
DRUG CONCENTRATION (μM)

IC_{50} under oxic conditions for ZM 33191 was, in fact, similar to that for EO9 (Tables 4.4 - 4.5).

4.3.3. Cytotoxicity of the Compounds in the CHO-K1-WT and CHO-P450R Cell Lines

Due to the similarities in cell cytotoxicity data between ZM 81853 and tirapazamine, and the known involvement of one-electron reducing enzymes such as NADPH : cytochrome P450 reductase in the activation of tirapazamine under hypoxic conditions, further cytotoxicity studies were undertaken using two CHO cell lines, one of which had been transfected with the active gene for human NADPH : cytochrome P450 reductase and the other with the inactive gene (control cell line). However, as the results show in Table 4.3, the level of activity of the enzymes was not as expected.

Table 4.7 shows the cytotoxicity of compounds, determined under oxic and hypoxic conditions, for both of the CHO-transfected cell lines. In addition, the HCR is also shown. As in the human breast tumour cell lines, quinoxalines ZM 10309, ZM 8173, ZM 65086 and ZM 12557, along with the nitroheterocyclic compound ZM 29362 showed little, if any, increased sensitivity under hypoxic conditions in either of the cell lines. Although IC_{50} values for the nitroacridine, ZM 33191, were similar to those of EO9 under oxic conditions, they were not altered significantly under hypoxic conditions. In contrast to the other quinoxalines, ZM 81853 exhibited increased cytotoxic effects under hypoxic conditions (Table 4.7, Figure 4.5B). Interestingly, however, the level of enhanced sensitivity under hypoxic conditions was similar in each cell line (75-fold, CHO-K1-WT; 73-fold, CHO-P450R). Whilst both cell lines experienced increased sensitivity towards EO9 and tirapazamine (Table 4.7) under hypoxic conditions, neither drug was as selective as ZM 81853 (with the exception of those results marked by *, which appear to be ambiguous results). Interestingly, in the case for ZM 81853, the degree of enhancement was similar for each of the two cell lines.

	IC_{50} (μM)					
	CHO-K1-WT			CHO-P450R		
Compound	Air	N_2	HCR	Air	N_2	HCR
EO9	0.85, 1.3	0.11, 0.14	7.7, 9.3	1.3, 1.2	0.18, 29.0 *	7.2, 0.04 *
Tirapazamine	410, 540	20, 20	20.5, 27.0	470, 550	20, 4 *	23.5, 137.5 *
ZM 81853	1500 ± 60	20 ± 1	75.0	730 ± 30	10 ± 1	73.0
ZM 10309	2000 ± 180	> 1000 ± 0	-	1200 ± 80	> 1000 ± 0	-
ZM 8173	> 2500 ± 0	990 ± 130	> 2.5	> 2500 ± 0	> 1000 ± 0	-
ZM 65086	> 2500 ± 0	> 1000 ± 0	-	> 2500 ± 0	> 1000 ± 0	-
ZM 12557	> 2500 ± 0	> 1000 ± 0	-	> 2500 ± 0	> 1000 ± 0	-
ZM 33191	2.4 ± 0.15	1.5 ± 0.32	1.6	2.5 ± 0.03	1.4 ± 0.03	1.8
ZM 29362	1100 ± 50	1100 ± 110	1.0	> 2500 ± 0	> 1000 ± 0	-

Table 4.7 Sensitivities of CHO-K1-WT and CHO-P450R cells to EO9, tirapazamine and seven novel compounds.

Sensitivity was determined using the MTT-based cytotoxicity assay. Cells were exposed to drug for 3.5 hr and sensitivity was expressed as IC50 values. Results represent the mean ± SE of triplicate estimations, except in the case of EO9 and tirapazamine for which duplicate estimations are shown. (see text for explanation of *, Section 4.3.3.).

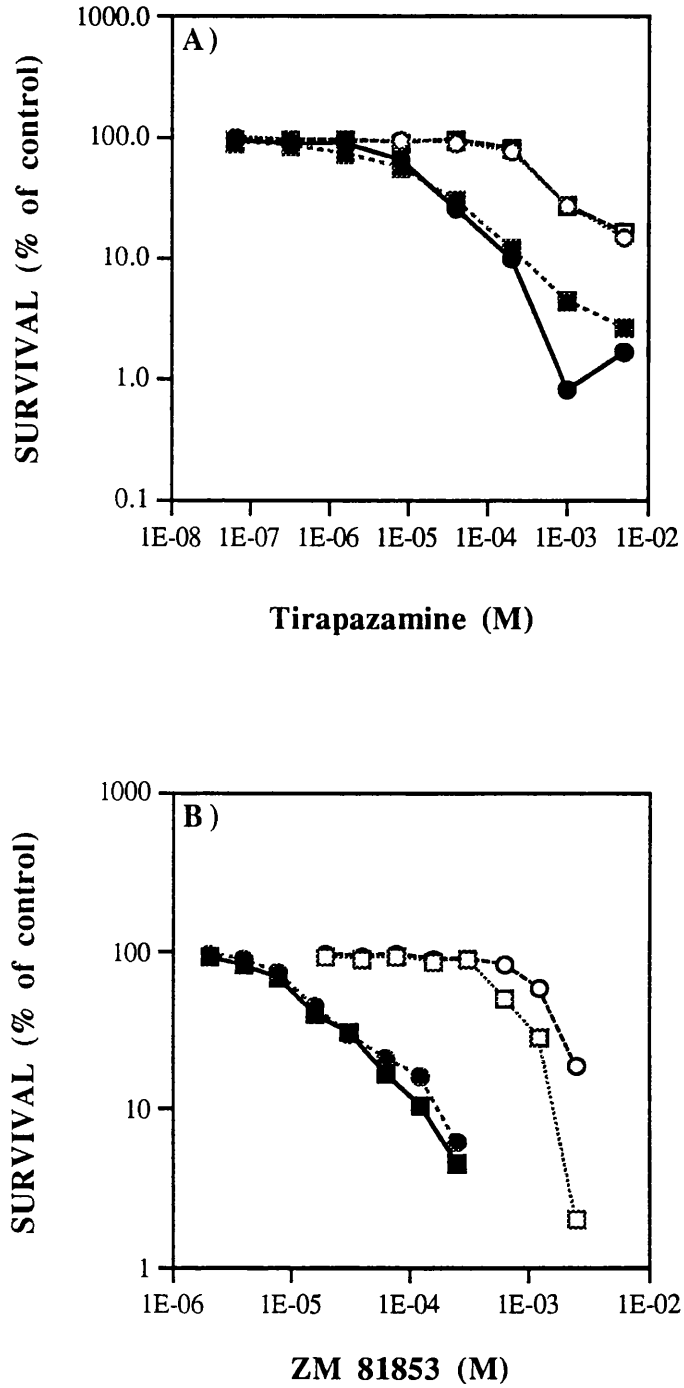


Figure 4.5 Sensitivity of CHO-K1-WT (circles) and CHO-P450R (squares) towards Tirapazamine (A) and ZM 81853 (B).

Cells were exposed to drug for 3.5hrs under oxic (2% CO_2 in air; open symbols) or hypoxic (2% CO_2 in N_2; closed symbols) conditions, and cell survival determined by an MTT assay. Points on the graph represent a mean of duplicate (tirapazamine) and triplicate (ZM 81853) estimations and are from one of two separate experiments.

4.4 Discussion

Based upon enzyme activity measurements within the NCI cell line panel (Chapter 2), three human breast tumour cell lines were selected to study the cytotoxic effects of seven novel compounds under both oxic and hypoxic conditions. Enzyme activities were shown to be slightly higher than those in the NCI panel. However, similar differences in each of the three enzymes were noted within each cell line. Sensitivity to the reference compounds EO9 and tirapazamine were demonstrated. Of the seven novel compounds investigated, only the quinoxaline ZM 81853 showed significant enhancement under hypoxic conditions. This compound was, in fact, structurally similar to tirapazamine.

The potency of potential anticancer agents has previously been measured using either the clonagenic assay or the MTT cell growth assay. More recent studies have involved large numbers of both cell lines, and potential anticancer agents (for example, the NCI screening programme (Boyd, 1993; Boyd & Paull, 1995)). As a result, the MTT-based microtitration assay described by Plumb *et al,* (Plumb *et al.*, 1989) has proved to be a valuable assay system for rapidly and efficiently measuring the cell cytotoxicity of large numbers of compounds and thereby determining their potential as anticancer agents. Problems arise, however, when investigating the cytotoxic potential of anticancer agents under hypoxic conditions, since normal tissue culture plastics absorb oxygen (Chapman *et al.*, 1970) and hence prevent the attainment of a hypoxic environment. Previous studies investigating the differential cytotoxicity of bioreductive compounds under oxic and hypoxic conditions have exposed cells to the gassing conditions in glass dishes and determined cell survival by colony formation (Plumb & Workman, 1994; Plumb *et al.* , 1994a). The MTT assay has been used successfully when modified for 24-well plates with glass inserts (Stratford & Stephens, 1989). However, neither of these assays would enable a large scale study to be carried out rapidly. As a result, the MTT assay (Plumb *et al.*, 1989) has been adapted for oxic and hypoxic conditions by the use of Tulip PETG-plastic 96-well tissue culture plates which act in a similar manner as glass as far as

absorption of oxygen is concerned. Although the assay system appeared to be slightly less sensitive than the conventional clonogenic assay, the HCR values were comparable (Table 4.2, Section 4.2.7. and also Plumb and Workman, (Plumb & Workman, 1994)), suggesting that this system was efficient for use in such a study. Furthermore, the HCR results obtained in this thesis for the reference compounds, tirapazamine and EO9, were comparable with those reported in other human tumour cell lines (for example MCF-7 and HT-29 cells, (Plumb & Workman, 1994); T47D cells, (Adams & Stratford, 1994)). One exception, however, was the low HCR values obtained in low DT-diaphorase containing cell lines such as MDA-MB 231 (Table 4.6). This result was in sharp contrast to published results in similar cell lines (Plumb *et al.*, 1994a). However, more recent studies by Dr. Plumb have also shown significant HCR values for EO9 in low DT-diaphorase containing cell lines using the PETG MTT assay (personal communication). The assay system therefore appeared to be working efficiently. An interesting point, however, is that the activity of NADPH : cytochrome P450 reductase in MDA-MB 231 cells appeared to be lower in this current study compared to that reported by Plumb *et al* (Plumb *et al.*, 1994a) and as such may account for the lack of enhancement under hypoxic conditions.

In the first instance, human breast tumour cell lines were used to assess the cellular potency of the novel compounds under study. The selection of human breast tumour cell lines was based upon two factors. Firstly, the prior analysis of the NCI human tumour cell line panel (Chapter 2) had indicated that several enzymes expressed variable activity between different cell lines originating from breast tumours. This factor in itself however did not separate breast cell lines from any other group. The second influential factor was that hypoxic regions have been measured previously within human breast tumours (Vaupel *et al.*, 1991) and this particular tumour type would therefore have the potential to act as a good target for bioreductive therapy. The highest average HCR (hypoxic cytotoxicity ratio) was obtained for tirapazamine within the cell line T47D. This observation was consistent with the fact that NADPH : cytochrome P450 reductase is known to be the enzyme responsible for activation of the drug to a DNA damaging

species under hypoxic conditions (Fitzsimmons *et al.* , 1994). Interestingly, the novel quinoxaline compound ZM 81853 showed similar results, with T47D cells proving to be the most sensitive under hypoxic conditions. Collectively, these results suggest therefore that ZM 81853 possesses the ability to be selectively activated under hypoxic conditions, and that under these conditions the drug may in fact be activated within the cells in a similar manner to tirapazamine. As a means of addressing this possibility, further cytotoxicity studies were performed in the presence of two CHO cell lines. One of the cell lines (CHO-P450R) had been transfected with the active gene for human NADPH : cytochrome P450 reductase while the other (CHO-K1-WT) was transfected with the gene in the reverse orientation, and hence, the enzyme was inactive. Unfortunately the expected increase in activity of human NADPH : cytochrome P450 reductase within the CHO-P450R cell line was not present and as a result, although HCR values were obtained for both ZM 81853 and tirapazamine, no difference in sensitivity of the two cell lines was detected. Original Western immunoblot data provided by colleagues at the ICRF in Dundee showed that the human NADPH : cytochrome P450 reductase protein was present within the CHO-P450R cell line; however, no comparison to the CHO-K1-WT cell line was provided. It may be possible that although the protein was detectable, the vector may not be expressing the enzyme sufficiently within the cell line to result in increased activity. As successful transfection of NADPH : cytochrome P450 reductase into human tumour cells is known to be difficult, the results of this particular study suggest that the selection of human tumour cells with defined enzyme activities may in fact be more reliable as an *in vitro* screen of novel compounds.

A further four novel quinoxaline compounds were studied for cellular potency towards the human breast tumour cell lines and the two CHO cell lines. ZM 10309, although more cytotoxic than ZM 81853 in two out of three breast cell lines under oxic conditions, showed only a small increase in cytotoxicity under hypoxic conditions. This suggested therefore that ZM 10309 may not act as an efficient hypoxia-selective bioreductive anticancer agent. All three other quinoxaline compounds, namely ZM 12557, ZM 65086 and the unsubstituted parent compound ZM 8173, showed no cytotoxic

effects towards any of the cell lines. In addition, ZM 10309 was the only one of the four compounds to exhibit any degree of cytotoxicity towards the CHO cell lines, and this was observed under oxic conditions.

These studies therefore show that small structural changes within a series of novel quinoxaline compounds greatly influence the cytotoxic nature of the compounds. Similar studies by Naylor (Naylor, 1994) demonstrated that structural changes within a series of new imidazo [1,2-*a*] quinoxaline mono-*N*-oxides influenced the ability of the compounds to act as bioreductive cytotoxins. There are several possible explanations for the current finding including lack of solubility of the compounds, instability of the parent compound and/or metabolites, inability of reductase enzymes to interact with the active site of the compound, lack of appropriate enzymes in the cells, inability of the compounds to penetrate into the cell and also inappropriate reduction potentials to allow efficient reduction of the compounds under hypoxic conditions. In an extended study of the quinoxaline series (Monge *et al.* , 1995), electrochemical studies suggested that the cytotoxic potential of these compounds, and hence the HCR, could be influenced by changes in the one-electron reduction potential. Such changes were brought about by the addition of either electron donating (CH_3) or electron withdrawing (Cl, F, CF_3) groups into the compounds. Links between hypoxic cytotoxicity / DNA damaging capacity and reduction potentials of nitro-containing compounds have been reported previously (Adams *et al.* , 1980; Morecroft *et al.*, 1994). The reduction potentials of quinoxalines used in this thesis are not available, however, and therefore the role of this factor in determining cytotoxic effects of the compounds cannot be determined. In addition to the possible influence of reduction potentials, Monge *et al* (Monge *et al.*, 1995) also showed that substitution of an amine in the R_3 position of the 7-chloroquinoxalines led to decreased potency, but a 7-fold increase in HCR. Interestingly, ZM 81853 possesses an amine in this position (see Figure 4.1b). A further explanation of lack of cytotoxicity was that those enzymes possibly required for activation of the quinoxalines (with the exception of ZM 81853) were not present at all, or at high enough activities, to cause

activation. Clearly, therefore, several factors were involved in influencing the potency and HCR of the larger quinoxaline series.

In addition to a small series of quinoxaline di-*N*-oxides, two further structurally independent compounds were studied. The nitroacridine compound, ZM 33191, was selected for study due to the presence of the potentially active nitro group and its structural similarity to the nitroacridine, nitracrine (1-NC), extensively by Wilson and colleagues (Wilson *et al.*, 1984; Wilson *et al.*, 1986; Wilson *et al.*, 1992). As with 1-NC, ZM 33191 exhibited the ability to be cytotoxic under oxic conditions suggesting that it may act in a similar manner to that of 1-NC, i.e. by intercalation with DNA and subsequent DNA alkylation. In contrast, however, further enhanced cytotoxicity was not observed with ZM 33191 under hypoxic conditions. As with the quinoxaline series, the lack of hypoxic cytotoxicity may well be due to a lack of appropriate activating enzymes within the cells and / or the reduction potential of ZM 33191, however, in the latter case, no conclusions can be made. These results certainly suggest that the potential of ZM 33191 as a selective bioreductive anticancer agent is limited. Interestingly, however, cytotoxicity under oxic conditions was similar to that of EO9. ZM 33191 may be useful, therefore, as an anticancer agent in oxic tumour cells.

ZM 29362, a large nitroheterocyclic compound, was also investigated for its potential as a selective bioreductive agent. This compound was interesting due to the presence of both a quinone moiety and a nitro group within the structure. As with the nitroacridine ZM 33191, however, it displayed only very small HCR in each of the cell lines. Unlike ZM 33191, however, the compound was inactive under oxic conditions. A lack of activity may result from an inability of the compound to penetrate into the cells due to its large size. As previously mentioned, however, it may be due to a lack of activating enzyme, or perhaps more likely, the reduction potential of the compound. The size of the compound does not usually restrict access of the reductases to the active site, therefore, this was probably not a reason for the lack of cytotoxicity.

In summary, a group of novel compounds has been investigated for their ability to act as hypoxia-selective anticancer agents. Three structurally distinct compounds were

analysed, based upon their structural similarities to known active compounds. The results suggested that quinoxaline compounds such as ZM 81853, in contrast to the nitroacridine and the nitroheterocyclic compounds, can be preferentially activated under hypoxic conditions to form potent cytotoxins. Activation of ZM 81853 appeared to take place in a similar manner to that of the benzotriazine di-*N*-oxide, tirapazamine. Further studies involving a small series of quinoxaline di-*N*-oxides suggested that small structural differences were important for determining the cytotoxic activity of the quinoxaline compounds. Results from a more detailed study by Monge *et al* (Monge *et al.*, 1995) suggested that activity of the 2-cyano-3-aminoquinoxaline di-*N*-oxides was enhanced by the presence of an electron withdrawing group (such as Cl, CF_3 or F) in the R7 position. Of interest, substitution of the amino by a dialkylaminoalkylamino group further enhanced potency and the HCR of the quinoxalines, especially in the presence of a CF_3 group at R7.

CHAPTER 5

DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETECTION OF NOVEL QUINOXALINE COMPOUNDS

5.1 Introduction

In the previous chapter, a small series of structurally-related quinoxaline di-*N*-oxide compounds was studied for the ability to act as bioreductive cytotoxins in a panel of human breast tumour cell lines. It was evident that even small changes in their structure resulted in vast differences in the ability of the compounds to be cytotoxic under hypoxic conditions. This may have been a reflection on the actual reduction potentials of the compounds not being appropriate for reduction under hypoxic conditions (as discussed in Section 4.4). A further requirement for successful reduction is the presence of reductase enzymes within the human tumour cell lines which are involved in metabolism of the compounds. For example, the reduction potentials of the quinoxaline compounds may have been suitable for reduction, however, enzymes involved in their reduction may not have been sufficiently active within the cell lines to cause metabolism. To study further the potential of these compounds to be reduced under hypoxic conditions, *in vitro* metabolism studies were performed. Carried out under both oxic and hypoxic conditions in the presence of drug, cofactor (for example, NADH and / or NADPH) and various enzyme sources, these studies were designed to indicate whether the compounds possessed the ability to be metabolised. In order to undertake such studies, however, a sensitive and reproducible assay was required for the quantification of parent compound loss and possible metabolite compound formation.

Several methods are currently available to study the metabolism of anticancer agents to possible metabolites. One such method is that of high performance liquid chromatography (HPLC). This method allows for the quantification of both parent loss

and metabolite formation following *in vitro* metabolism in the presence of various enzyme preparations. To date, extensive studies have been performed to investigate the reductive metabolism of other bioreductive agents such as tirapazamine using isocratic reverse-phase chromatography and ultra-violet (UV) detection (Fitzsimmons *et al.*, 1994 and references therein). As this benzotriazine di-*N*-oxide compound is structurally similar to the quinoxaline compounds used in this current study, the development of an HPLC assay for the latter compounds will be based initially on that which has been used successfully for tirapazamine.

This chapter, therefore, describes the development of an HPLC assay for the detection of each of the five novel quinoxaline compounds used in this study. In addition, the reproducibility of the assays was tested by performing a small validation study using the lead compound ZM 81853.

5.2 Materials and Methods

5.2.1. Chemicals and Reagents

All general chemicals and solvents (of the highest grade commercially available) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.) or Fisons plc. (Loughborough, U.K.) unless otherwise stated. Zero grade nitrogen (< 5 vpm oxygen) was obtained from the British Oxygen Company (London, U.K.)

5.2.2. Development of HPLC Conditions for the Quinoxaline Compounds

Due to the similarities in structure, conditions for separating and subsequently quantifying the quinoxaline compounds by HPLC were based upon those previously used for tirapazamine (Fitzsimmons *et al.*, 1994). In order to achieve optimal conditions for each compound, however, changes were made to the original protocol used for tirapazamine separation.

Chromatography was carried out using modular HPLC equipment and columns obtained from Waters Chromatography (Millipore U.K. Ltd., Watford, U.K.). Injections were made using a 712 WISP injector, controlled by a 600E System Controller. Separations were performed using a μBondapak Phenyl steel column (3.9 x 300 mm), protected by a Phenyl pre-column cartridge. Detection was made by ultra-violet (UV) using a 991 Photodiode Array Detector. All quinoxalines were analysed by gradient reverse-phase HPLC. The details of mobile phases, flow rate and run time for each individual compound are noted in Table 5.1. All mobile phases were degassed with helium (Air products plc., Walton on Thames, U.K.) before use.

5.2.3. Validation of HPLC Assay for ZM 81853

In order to validate the HPLC assay for its reproducibility in measuring drug levels, a range of different drug concentrations were tested. Stocks (15 mM) of ZM 81853

Quinoxaline	Mobile Phase A	Mobile Phase B	Flow Rate (ml/min)	Nature of Run		
				Run Time (mins)	Mobile Phase	Gradient
ZM 81853	18% MeOH	45% MeOH	1.5	0 - 15.0	A	-
				15.01 - 30	A -> B	Linear
ZM 10309	15% MeOH	45% MeOH	1.5	0 - 7.0	A	-
				7.01 - 14	A -> B	Linear
ZM 12557	15% MeOH	45% MeOH	1.5	0 - 7.0	A	-
				7.01 - 14	A -> B	Linear
ZM 8173	15% MeOH	45% MeOH	1.5	0 - 7.0	A	-
				7.01 - 14	A -> B	Linear
ZM 65086	15% MeOH	45% MeOH	1.5	0 - 7.0	A	-
				7.01 - 14	A -> B	Linear

Table 5.1 Optimal conditions for analysis of each compound by HPLC.

MeOH = methanol in water; each mobile phase was adjusted to pH 3.2 using concentrated HCl.

were made up in dimethyl sulphoxide (DMSO) and diluted to the appropriate concentration (15 - 1920 μM) in a total volume of 1 ml buffer (50 mM Tris HCl, pH 7.4). In addition to the drug, the sample mixture also contained cofactor NADPH (1 mM final concentration) as this would be present within metabolism reactions. Three separate 100 μl aliquots of each concentration were set up for injection onto HPLC and the amount of drug that was injected in each case, determined by the area of the peak.

5.2.4. Stability of Quinoxaline ZM 81853 under Varying Incubation Conditions

The stability of ZM 81853 under several different conditions was investigated. Two different concentrations (100 and 300 μM) of ZM 81853 were analysed for their stability at 37°C in both light and dark conditions. These concentrations were selected based upon those conditions used for reductive metabolism studies of tirapazamine (Riley & Workman, 1992b; Walton & Workman, 1990). A 5 mM stock concentration of ZM 81853 was made in dimethyl sulphoxide (DMSO) and diluted to 100 μM or 300 μM in 50 mM Tris HCl, pH 7.4 (buffer to be used at a later stage in metabolism studies). Samples were kept under the appropriate incubation conditions and aliquots removed at 0, 2.5, 5, 7.5, 10 & 20 mins. In the case of the 300 μM sample, a 1:3 dilution of the aliquot was made into 100 % methanol, samples were spun down and then loaded onto HPLC (details in Section 5.2.2.). To determine whether the drug was unstable when diluted into the methanol, aliquots from the 100 μM samples were removed and injected directly onto HPLC. The level of ZM 81853 present at each time point was determined by the area of the drug peak.

5.3 Results

5.3.1. Development of HPLC conditions for Quinoxaline Compounds

General conditions for detection of each of the quinoxaline compounds are shown in Table 5.1. Having established these conditions, compounds (100 μM) were then injected onto HPLC and the retention time and optimal absorption wavelength determined. Table 5.2 shows these parameters for each quinoxaline compound. The chromatograms obtained in these studies are shown in Figure 5.1 and illustrate that the assays allow for sensitive detection of each of the compounds.

5.3.2. Validation of the HPLC Assay for ZM 81853 Quantification Purposes

To ensure the reproducibility of the HPLC assay for future quantification studies, a validation study was undertaken with ZM 81853. The results are shown in Table 5.3 and Figure 5.2. The amount of drug present within a sample is quantified using the peak area, therefore, this parameter was used in the validation study. Importantly, the peak area was shown correlate well with increasing drug concentration (r^2 = 1.0) (Figure 5.2). In addition, repeated injections of the drug were shown to produce little variability in the peak area i.e. the largest SE observed was 1.4 % of the mean peak area (240 μM; Table 5.3).

5.3.3. Stability of ZM 81853

Two different concentrations (100 μM and 300 μM) of ZM 81853 were analysed for stability at 37°C under light and dark conditions. These particular conditions were selected as it was felt that they were the most likely to be used in subsequent metabolism studies. In addition the stability of a concentrated stock (15 mM) of ZM 81853 was tested at -20°C as this would be the storage conditions for the drug. In all cases, the drug was

Quinoxaline	Retention Time (mins.)	Optimum Absorbence (nm)
ZM 81853	11.0	265
ZM 10309	5.5	260
ZM 12557	4.7	260
ZM 8173	6.8	240
ZM 65086	8.3	250

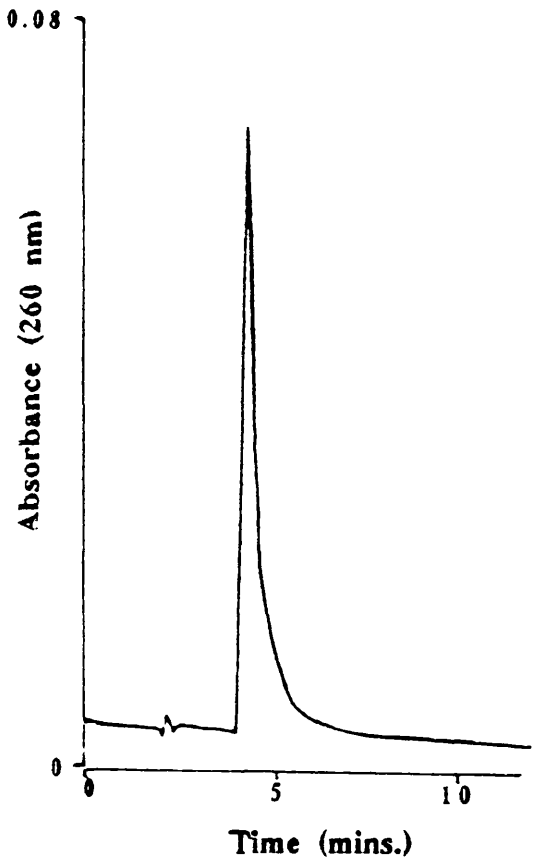
Table 5.2 Detection conditions for each quinoxaline compound.

Compounds were run under the conditions detailed in Table 5.1 and their retention times determined. Optimum absorbence of each compound was determined by performing chromatogram analysis to indicate the wavelength at which absorbence was greatest.

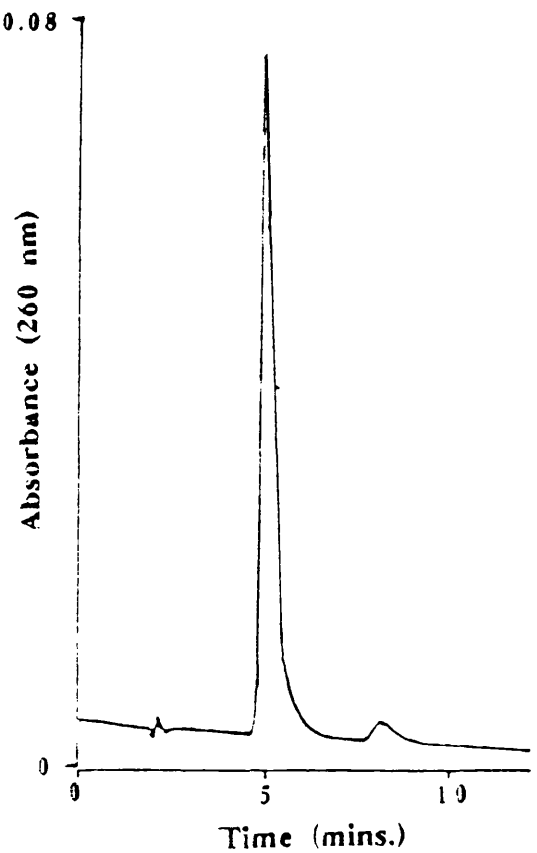
Figure 5.1 Representative HPLC chromatograms of ZM 81853 (A), ZM 8173 (B), ZM 12557 (C), ZM 10309 (D) and ZM 65086 (E).

Compounds (100 μM) were injected onto HPLC and analysed for retention time and optimum absorbence (as displayed in Table 5.2).

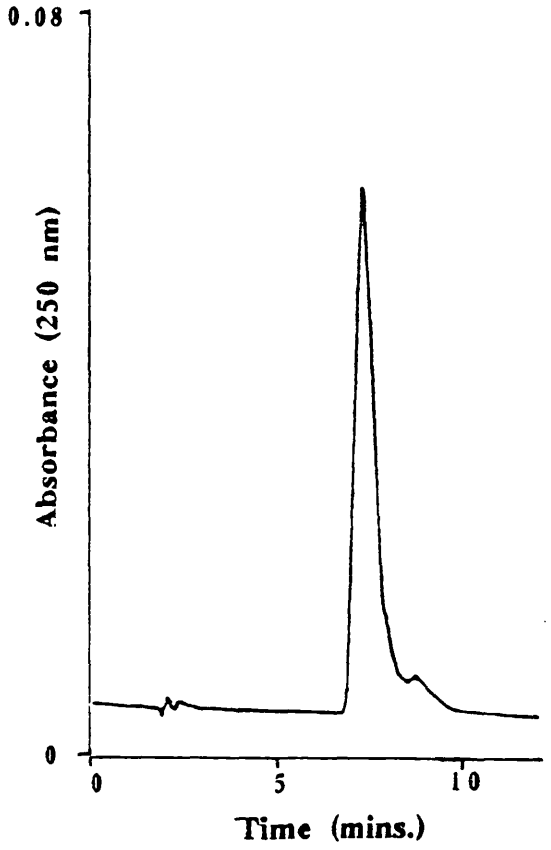
A)


0.08
Absorbance (260 nm)
0
0
5
10
Time (mins.)

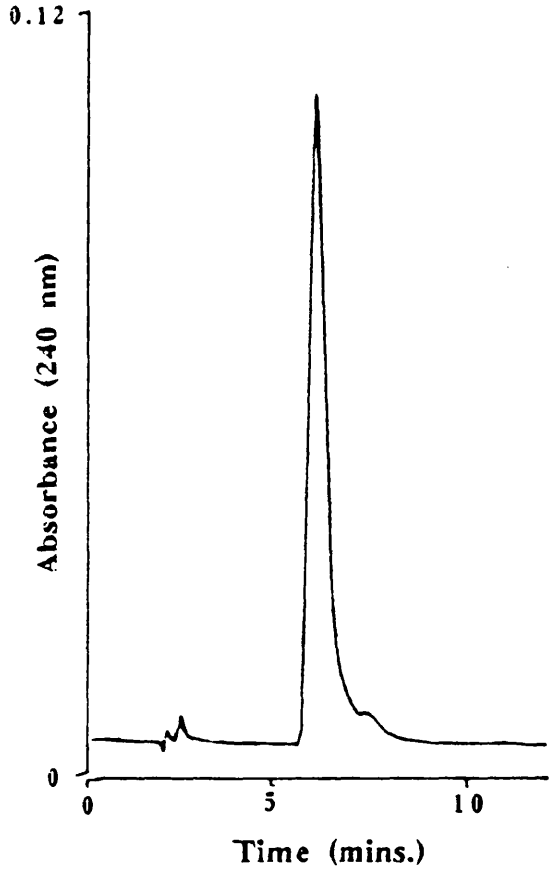
B)


0.08
Absorbance (260 nm)
0
0
5
10
Time (mins.)

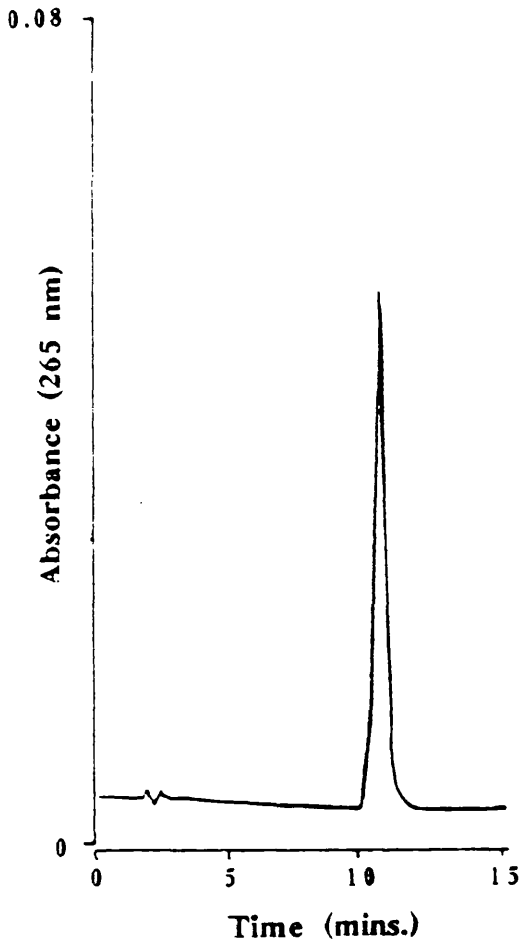
C)


0.08
Absorbance (250 nm)
0
0
5
10
Time (mins.)

D)


0.12
Absorbance (240 nm)
0
0
5
10
Time (mins.)

E)



ZM 81853 Concentration (μM)	Mean Peak Area (Peak Height x time)	Standard Error
15	8.1×10^{-3}	4.8×10^{-5}
30	1.4×10^{-2}	7.5×10^{-5}
60	2.6×10^{-2}	2.8×10^{-4}
120	5.4×10^{-2}	3.3×10^{-4}
240	9.9×10^{-2}	1.4×10^{-3}
480	18.8×10^{-2}	9.2×10^{-5}
960	37.5×10^{-2}	3.1×10^{-4}
1920	76.2×10^{-2}	5.4×10^{-3}

Table 5.3 Validation of HPLC assay for detection of ZM 81853.

Triplicate samples for each drug concentration were injected onto HPLC and a mean peak area determined. Table shows the mean peak area $\pm$ SE, n=3.

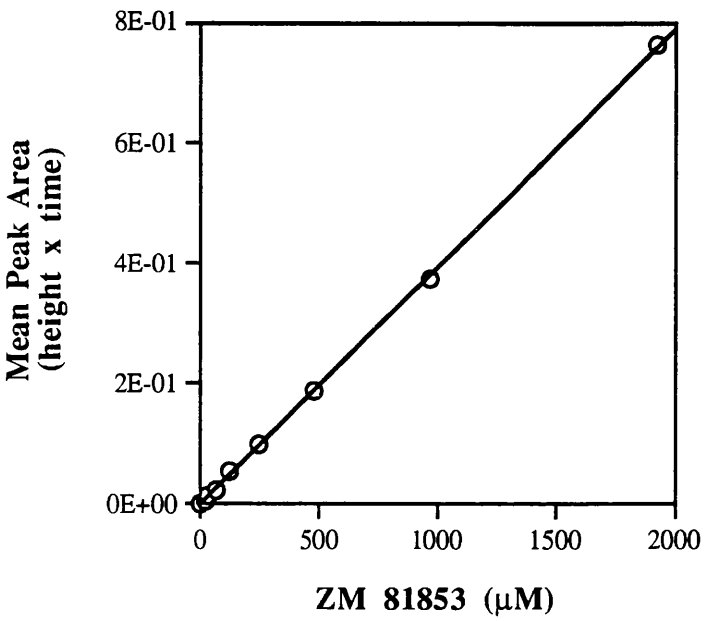


Figure 5.2 Validation of HPLC assay for detection of ZM 81853.

Triplicate samples for each drug concentration were injected onto HPLC and a mean peak area determined. Graph represents the mean ± SE (n=3) for each drug concentration.

found to be stable and hence exhibit no notable loss when analysed by HPLC (data not shown).

5.4 Discussion

In order to study the reductive metabolism of a small series of structurally-related quinoxaline compounds, a sensitive and reproducible HPLC assay technique is required. This chapter described the development of such an assay for the quinoxaline ZM 81853, and the subsequent minor alterations that were required for the successful detection of four structurally-related quinoxalines.

Several methods of investigating the metabolism of anticancer agents are available. These include, among others, spectrophotometric studies which directly monitor the loss of parent compound (change in absorbence) in the presence of enzyme and cofactor. Perhaps more useful and informative, however, is the technique of HPLC analysis involving reverse phase chromatography and either ultra-violet (UV) or radioisotope detection. The latter form of detection involves the use of radiolabelled compounds (for example ^{14}C labelled) to monitor both parent loss and metabolite formation. In this particular study, however, UV detection was selected for use due to the fact that, in the first place, no radiolabelled compounds were available for use, and secondly, an HPLC method involving UV detection had already been developed for the structurally similar compound, tirapazamine (Walton & Workman, 1988). Initial analysis of the lead compound ZM 81853 showed that although detection could be made under those conditions used for tirapazamine, a more sensitive assay was produced following a number of adjustments to the protocol. These changes included the use of steel μBondapak phenyl column, the use of a gradient mobile phase system, and adjustments to the MeOH : water ratios of the mobile phase. These changes improved the separation of the quinoxaline compounds by HPLC. Importantly, the new assay conditions were shown to be reproducible for ZM 81853. Having established the assay conditions for the lead compound ZM 81853, further minor adjustments were made to the mobile phase constituents and the gradient flow system to enable the successful detection of the four structurally-related quinoxalines.

Following the successful development of both sensitive and reproducible HPLC assays for the detection of quinoxaline compounds, the stability of the lead compound ZM 81853 was investigated under conditions which would be used in subsequent metabolism studies. ZM 81853 was selected as this compound has been shown to be the most cytotoxic compound (Chapter 4) of the group studied, and as such would be used for more detailed metabolic analysis (see Chapter 6 and 7). In addition, due to a limited supply of the four other quinoxaline compounds, care had to be taken in the use of each compound. ZM 81853 was shown to be stable as a diluted sample at 37°C for at least 20 mins. and also as a concentrated sample at - 20°C over a number of weeks. Unfortunately only ZM 81853 could be studied due to only a small quantity of the other quinoxalines being made available.

In summary, therefore, a sensitive and reproducible HPLC assay has been developed for the lead quinoxaline ZM 81853. This has been used to show that the compound is stable under the conditions required for future metabolism studies. In addition, the assay has been adapted for the detection of four structurally-related quinoxaline compounds.

CHAPTER 6

METABOLISM OF A SERIES OF QUINOXALINE DI-*N*-OXIDES BY MOUSE LIVER ENZYMES

6.1 Introduction

Cytotoxicity studies using a panel of human breast tumour cell lines indicated that the quinoxaline di-*N*-oxide ZM 81853 exhibited the potential to act as an effective hypoxia-selective bioreductive cytotoxin (Chapter 4). In addition, results indicated that one-electron reducing enzymes such as NADPH : cytochrome P450 reductase may play a role in this action. Further investigations using a small series of quinoxaline di-*N*-oxide compounds (shown in Chapter 4, Figure 4.1b) indicated that structural changes altered the ability of the compounds to act as hypoxia-selective cytotoxins. To further investigate the activation of the quinoxaline di-*N*-oxide ZM 81853 under hypoxic conditions, and to establish those enzymes responsible for its activation, metabolism studies using various enzyme preparations were performed. In addition, the lack of hypoxia-selective cytotoxicity displayed by the structurally-related quinoxaline series was investigated by determining their ability to be metabolised under hypoxic conditions.

The importance of reductase enzymes in the metabolism of bioreductive anticancer agents, and hence determination of their expression in human tissues, has been demonstrated and described in detail earlier in this thesis (Chapters 1 and 2). An understanding of the both the metabolic pathway and those enzymes involved for different bioreductive agents may allow the targeting of particular agents to a specific tumour type depending upon the levels of activating and deactivating enzymes present within that tumour. This concept has been suggested by the 'enzyme-directed' bioreductive drug development approach described by Workman and colleagues (Workman & Walton, 1990; Workman, 1994). In this regard, the enzymology of many bioreductive agents has been studied in detail (for examples, see reviews by Workman

(Workman, 1992; Workman, 1993)). Of particular interest to this thesis, however, is the benzotriazine di-*N*-oxide bioreductive agent tirapazamine as it is structurally similar to the quinoxaline di-*N*-oxides (Chapter 4, Figure 4.1b). This bioreductive agent is thought to be metabolised by one-electron reducing enzymes to a nitroxide radical (Fitzsimmons *et al.*, 1994; Lloyd *et al.*, 1991; Riley *et al.*, 1993) while two-electron reducing enzymes metabolise the drug to the inactive products, SR 4317 and SR 4330 (Cahill *et al.*, 1993; Riley & Workman, 1992b) (see Chapter 1, Section 1.2.3.). Data such as this has been gained by performing metabolism studies in the presence of an enzyme source such as crude tissue extracts, cell lysates, subcellular fractions or even purified enzymes. The use of crude tissue extracts or cell lysates provides a means of studying enzymes in an environment similar to the cell as many enzymes are present. The use of subcellular fractions such as liver microsomal or cytosolic fractions in combination with chemical and immunoinhibition, however, gives a cleaner enzyme system for studying specific roles of individual enzymes in metabolism of a bioreductive agent. This is further enhanced by the use of purified enzymes although such enzymes are always hard to obtain and are in short supply. It is important to remember, however, that purified enzymes act in complete isolation of other enzymes and may therefore produce different results than in a true cell system.

The liver is frequently used as an enzyme source as its morphological integrity can be disrupted by physical means to yield subcellular fractions following differential centrifugation. The two most important subcellular fractions for drug metabolism studies are the endoplasmic reticulum (or microsomal fraction) and the cytosolic fraction. In general, Phase I metabolising enzymes are present within the microsomal fraction (e.g. NADPH : cytochrome P450s and NADPH : cytochrome P450 reductase) while Phase II enzymes are present within the cytosolic fraction (e.g. DT-diaphorase). Although cytotoxicity studies indicated a similar activation of ZM 81853 as tirapazamine, and hence, a possible involvement of one-electron reducing enzymes, initial metabolism studies were performed in the presence of both microsomal and cytosolic subcellular enzyme preparations. As mentioned above, chemical inhibitors and epitope-specific

inhibitory antibodies have been used in combination with subcellular enzyme fractions to determine the role of different enzymes in metabolism (for example Riley *et al.*, 1993; Walton *et al.* , 1992b). In this thesis, therefore, chemical inhibitors were used to investigate those enzymes responsible for activation of ZM 81853. The chemical inhibitors used are shown in Table 6.1. Hepatic enzyme induction can also be used as a further means of addressing the possible involvement of NADPH : cytochrome P450 enzymes in metabolism (Meehan *et al.* , 1988; Riley *et al.*, 1993). For this reason, both control (mice administered with the carrier solution only) and dexamethasone (DEX) induced subcellular fractions were prepared. DEX had been previously shown to induce NADPH : cytochrome P450 Cyp2b, Cyp2c and Cyp 3a isoenzymes (Meehan *et al.*, 1988; Riley *et al.*, 1993).

Collectively, therefore, it was hoped that these investigations would indicate which enzymes were involved in the activation of ZM 81853 under hypoxic conditions. In addition, investigations into the ability of the structurally-related quinoxalines to be metabolised by the subcellular fractions were performed in an attempt to determine the reasons behind the lack of cytotoxicity shown by these compounds (Chapter 4).

Inhibitor / Substrate	Enzymes	Reference
Carbon Monoxide (CO)	NADPH : cytochrome P450s	(Omura & Sato, 1964)
SKF 525A	NADPH : cytochrome P450s	(Schenkman *et al.*, 1972)
Erythromycin	NADPH : cytochrome P450 CYP 3A *	(Wrighton *et al.*, 1985)
Metyrapone	NADPH : cytochrome P450 Cyp 2a, Cyp 2b, Cyp 2c and Cyp 3a *	(Riley *et al.*, 1993)
Thallium Chloride	NADPH : cytochrome P450 reductase	(Woods & Fowler, 1984)
Diphenyliodonium Chloride (DIPC)	NADPH : cytochrome P450 reductase	(Tew, 1993)
Dicoumarol	DT-diaphorase, NADH : cytochrome b_5 reductase, carbonyl reductase	(Maser & Netter, 1989; Preusch *et al.*, 1991; Segura-Aguilar *et al.*, 1990)
Rutin	Carbonyl reductase	(Wermuth, 1981)

Table 6.1 **Inhibitors / substrates of reductase enzymes known to be involved in bioreductive drug metabolism.**

[* Note that NADPH : Cytochrome P450 nomenclature recommended by Nebert *et al* (Nebert *et al.*, 1991) was used throughout the thesis.]

6.2 Materials and Methods

6.2.1. Chemicals and Reagents

All general chemicals and solvents (of the highest grade commercially available) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.) or Fisons plc. (Loughborough, U.K.). SKF 525A (proadifen hydrochloride) was provided by SmithKline and French Research Ltd. (Welyn, Hertfordshire, U.K.) while diphenyliodonium chloride (DIPC) and thallium (III) chloride tetrahydrate were from Aldrich Chemical Company (Gillingham, U.K.). Zero grade nitrogen (< 5 vpm oxygen) was obtained from the British Oxygen Company (London, U.K.) and research grade carbon monoxide (CO) from BDH Ltd. (Poole, Dorset, U.K.). Sodium dithionate was also purchased from BDH Ltd. (Poole, Dorset, U.K.).

6.2.2. Isolation of Hepatic Microsomes

Male BALB/c mice (18 - 21 g) were purchased from (Harlam Olac, Glasgow, U.K.) and were administered with dexamethasone (DEX) (100 mg/kg) by intraperitoneal injection in corn oil daily for 3 dys. A control group of animals received the equivalent dose volume of vehicle (corn oil; 10 ml/kg) once a day for 3 dys. Following the final injection, mice were fasted for 24 hrs. The animals were killed by cervical dislocation and their livers excised, washed in ice-cold buffer (buffer A : 50 mM Tris HCl, pH 7.4; 150 mM potassium chloride), weighed and an equivalent volume of buffer A was added. Livers were then homogenised (Citenco Varilab, Citenco Ltd., Borhamwood, Herts., U.K.) with a loose fitting pestle. The homogenate was spun down in a high speed centrifuge (Beckman U2-21 centrifuge with JA 20 fixed-angle rotor, Beckman Ltd., High Wycombe, Bucks., U.K.) at 10,000 g for 30 mins. at 4°C. Cell pellets were discarded and the supernatant respun (10,000 g; 1hr; 4°C) in an ultracentrifuge (Beckman L60 Ultracentrifuge with SW 40 swing out rotor, Beckman Ltd., High Wycombe, Bucks.,

U.K.). Following completion of the spin, the supernatant (cytosolic fraction) was removed and stored at -70°C until use. The remaining pellet was resuspended in buffer A and respun (as indicated above). The resulting supernatant was discarded and the microsomal pellets were stored either as intact pellets or concentrated suspensions (in buffer B : 0.1 M Tris/HCl, pH 7.4) at -70°C until required.

Protein content of both the microsomal and cytosolic fractions were determined using the Pierce BCA protein assay previously described (Chapter 2, Section 2.2.5.). Total NADPH : cytochrome P450 content was measured by the standard method of Omura and Sato (Omura & Sato, 1964). Briefly, 1 ml of microsomal or cytosolic protein was placed in each of two cuvettes. A few grains of sodium dithionate were added to both cuvettes, and the contents mixed. Samples were then scanned for absorbance (400 - 500 nm) using a Lambda 2 UV/VIS spectrophotometer (Perkin Elmer Ltd., Beaconsfield, Bucks., U.K.). The sample cuvette was then removed and the contents bubbled with CO for 30 s. A second scan was then performed. Total NADPH : cytochrome P450 content (nmol/mg) was determined by using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ and the absorbance change at 450 nm).

6.2.3. Establishment of Optimal Conditions for Metabolism of ZM 81853 in the Presence of Control or DEX-induced Mouse Liver Microsomes or Cytosol

Reductive metabolism of ZM 81853 was carried out at 37°C in specially modified Erhlenmeyer flasks, the contents of which were shaken throughout the reaction. The incubation mixtures were pregassed for 7 mins. with zero grade N_2, which was further deoxygenated by passage through an oxytrap (Alltech, Camforth, U.K.). Oxic incubations were performed in flasks open to the air. Incubations contained 1 mM NADPH, 1 mM NADH, 0.1 - 1.0 mg/ml microsomal protein or 0.5 - 15 mg/ml cytosolic protein in a total volume of 1 ml 50 mM Tris HCl, pH 7.4. Reactions were initiated by the addition of the substrate in DMSO (ZM 81853; 75 - 600 μM final concentration) through air-tight seals. Aliquots (100 μl) of the reaction mixture were removed at 4 - 5 consecutive time-points

and added to 2 volumes of ice-cold methanol. Samples were then centrifuged at 4°C (15,000 g for 15 mins.) and the supernatants analysed by HPLC (as described in Chapter 5; Section 5.2.2.). Having established the optimal conditions for metabolism of ZM 81853 by microsomal or cytosolic protein preparations, several control incubations were performed including, among others, cofactor dependence reactions.

6.2.4. Metabolism of ZM 81853 and Four Structurally-related Quinoxalines by DEX-induced Mouse Liver Microsomes and Cytosol

The ability of the four structurally-related quinoxalines to be metabolised by either cytosolic or microsomal protein preparations was investigated, and the rate of metabolism for each compound determined. The incubation conditions used were those which had previously been optimised for ZM 81853 (Section 6.2.3.), i.e. a set protein (0.3 mg/ml microsomal or 1 mg/ml cytosolic protein) and drug (300 μM) concentration. Unfortunately, limited availability of each structurally-related quinoxaline compound prevented a more detailed study being performed to establish optimal metabolism conditions. Incubations were carried out as detailed above (Section 6.2.3.) following which, samples were analysed by HPLC under the conditions detailed previously in Chapter 5 (Section 5.2.2.).

6.2.5. Chemical Inhibition of ZM 81853 Metabolism by DEX-induced Mouse Liver Microsomes and Cytosol

Incubations and analysis of samples were performed as detailed above except that various diagnostic substrates / inhibitors (Table 6.1) were added at concentrations of 20 - 2500 μM. In each case control experiments were performed with the same amount of buffer or vehicle (DMSO or methanol) required for solubilisation of the inhibitor. As far as possible, the concentrations used were based upon previous studies performed in this laboratory.

6.2.6. Identification by Mass Spectroscopy of Predominant Product Following Metabolism of ZM 81853 by DEX-induced Mouse Liver Microsomes

Metabolism of ZM 81853 by DEX-induced mouse liver microsomes was performed essentially as described in Section 6.2.3.. To ensure a large enough concentration of the predominant product for subsequent mass spectroscopy (approximately 100 µg), reaction constituents were used at a higher concentration : ZM 81853 (495 µM), NADPH (5 mM) and protein (2.5 mg/ml). Furthermore, the incubation was performed for 45 mins. before removing all of the sample and centrifuging for 15 mins. (15,000 rpm, 4°C). The sample was then injected (50 µl at a time) onto HPLC (as detailed in Chapter 5, Section 5.2.2.) and the predominant peak collected and stored on ice. The final fraction was dried down using an Alpha freeze drier (Christ, V. A. Howe Ltd., Banbury, U.K.).

Mass spectroscopy was performed in collaboration with Dr. D. G. Watson at the Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow. The dried sample was dissolved in 100 µl of glycerol as fast atom bombardment (FAB) matrix. An FAB spectrum was obtained using a JEOL JMS-AX505HA double focusing mass spectrometer (JEOL Ltd., Tokyo, Japan) with xenon gas as the source of fast atoms. Spectra were obtained with a resolution of 3000. Calibration for accurate mass measurement was carried out against the peaks (MWt. 185, 277 and 369) of glycerol matrix.

6.2.7. Statistics

The Student's unpaired t-test was used to compare the rates of metabolism for each compound and also the significance of the level of inhibition observed with various inhibitors.

6.3 Results

6.3.1. Establishment of Optimal Conditions for Metabolism of ZM 81853 by Mouse Liver Microsomes and Cytosol

To determine whether DEX-induction of the mouse liver microsomes had been successful, the total NADPH : cytochrome P450 content was measured in both control and DEX-induced microsomal preparations. DEX-induction resulted in approximately 2-fold higher levels of NADPH : cytochrome P450 content within the microsomes (data not shown).

The ability of ZM 81853 to be metabolised, under hypoxic conditions, in the presence of either control or DEX-induced mouse liver microsomes and cytosol, was investigated. Metabolism was measured as the loss of ZM 81853 over time. In the case of the mouse liver microsomes, DEX-induction appeared to cause increased loss of ZM 81853 by 10 mins. when compared to control microsomes (Figure 6.1). The level of loss was shown to become non-linear between 0.25 and 0.5 mg/ml of protein (Figure 6.1A) and above 300 μM drug concentration (Figure 6.1B).

A similar situation was observed in the presence of mouse liver cytosol, with ZM 81853 loss shown to be greater in the presence of the DEX-induced cytosol. In this case, however, protein concentrations above 1 mg/ml and drug concentrations between 200 and 400 μM resulted in non-linear loss of ZM 81853 (Figure 6.2A and 6.2B, respectively).

Collectively these results suggest that optimal metabolism of ZM 81853 would occur in the presence of DEX-induced microsomes and cytosol, and that the ideal protein and drug concentrations were 0.3 mg/ml and 300 μM, and 1.0 mg/ml and 300μM, respectively.

6.3.2. Metabolism of ZM 81853 by DEX-induced Mouse Liver Microsomes

Having established optimal conditions for the metabolism of ZM 81853 in the presence of DEX-induced mouse liver microsomes, a series of control incubations were

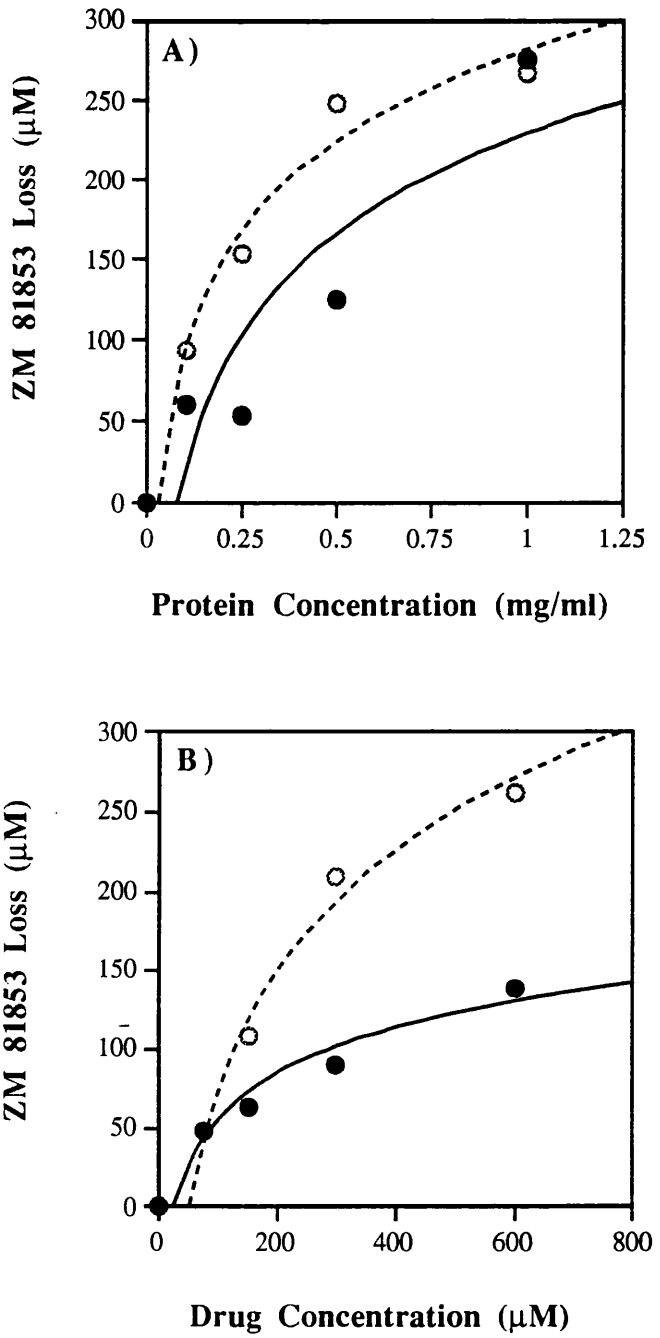


Figure 6.1 **ZM 81853 metabolism in the presence of control (closed circles) or DEX-induced (open circles) mouse liver microsomes. Effect of increasing protein (A) and drug (B) concentration on ZM 81853 metabolism.**

Incubations contained 0.1 - 1.0 mg/ml microsomal protein, 1 mM NADH and NADPH and 75 - 600 μM ZM 81853 in a total volume of 1 ml 50 mM Tris HCl, pH 7.4. Results represent one of two repeat experiments. Graphs show amount of ZM 81853 lost after a 10 mins. incubation. In (A), 300μM of ZM 81853 was used, while in (B), 0.3 mg/ml of protein was used.

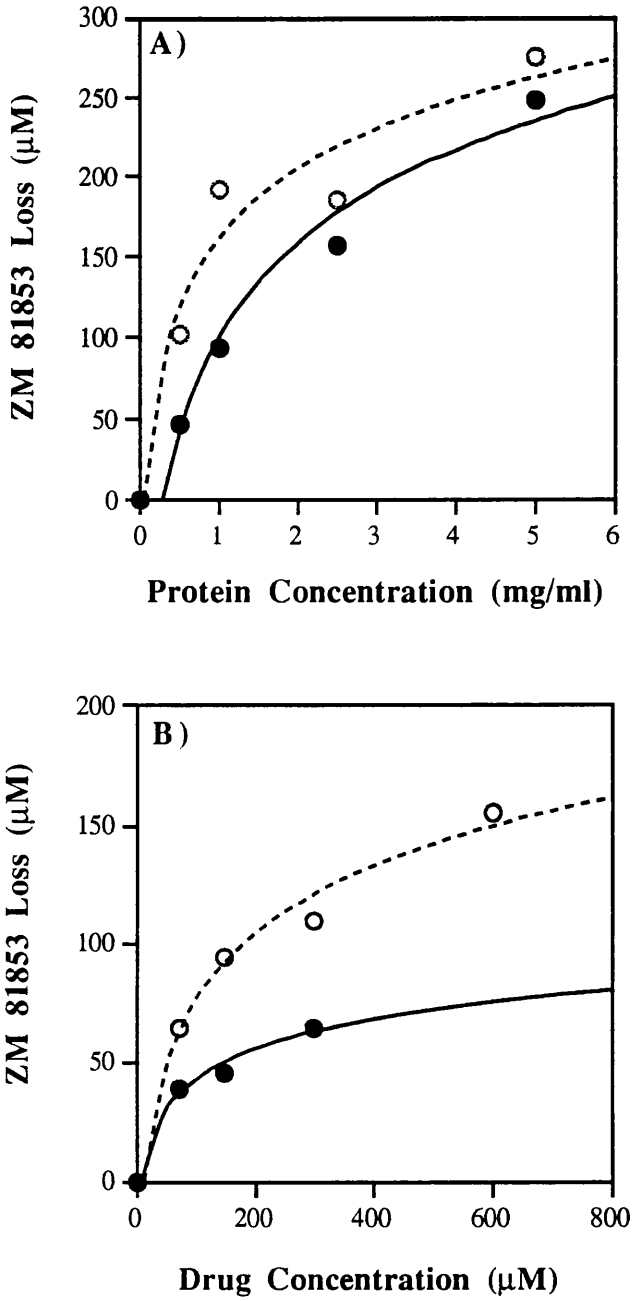


Figure 6.2 ZM 81853 metabolism in the presence of control (closed circles) or DEX-induced (open circles) mouse liver cytosol. Effect of increasing protein (A) and drug (B) concentration on ZM 81853 metabolism.

Incubations contained 0.5 - 5.0 mg/ml cytosolic protein, 1 mM NADH and NADPH and 75 - 600 μM ZM 81853 in a total volume of 1 ml 50 mM Tris HCl, pH 7.4. Graphs show the amount of ZM 81853 lost after a 10 mins. incubation. In (A), 300 μM of ZM 81853 was used while in (B), 1 mg/ml of protein was used.

performed. The results in Table 6.2 show that metabolism of ZM 81853 was predominantly dependent upon the presence of NADPH as cofactor, and to a lesser extent on NADH. In addition the reaction was completely inhibited in the presence of air and boiled microsomes. Importantly, metabolism of ZM 81853 in the presence of 300 μM drug and 0.3 mg/ml protein can be seen to be linear with time up to at least 15 mins. (Figure 6.3).

A representative HPLC chromatogram of ZM 81853 metabolism under control (N_2) conditions is shown in Figure 6.4. At 0 mins., ZM 81853 formed the predominant peak (no. 1) with a retention time of approximately 11 mins. Following a 15 mins. incubation under hypoxic conditions, ZM 81853 was significantly reduced in size (no. 1) and several potential metabolites had formed. The predominant new peak eluted at 16 mins. (no. 2).

6.3.3. Metabolism of ZM 81853 by DEX-induced Mouse Liver Cytosol

As with the mouse liver microsomes, a series of control incubations were performed in the presence of DEX-induced cytosolic protein. The results in Table 6.3 show that metabolism of ZM 81853 by cytosolic protein was fully dependent upon hypoxic conditions, active enzyme and the presence of NADPH as a cofactor. The rate of loss of ZM 81853 was significantly lower (3.5-fold; $p<0.001$) than in the presence of microsomal protein. Metabolism was shown to be linear with time up to at least 15 mins. (Figure 6.5). A similar HPLC chromatogram was obtained to that which was seen in the presence of microsomal protein (see Figure 6.4). However as previously noted, the level of ZM 81853 loss was lower (data not shown).

6.3.4. Metabolism of Four Structurally-related Quinoxaline Compounds

Using the conditions found to be optimal for ZM 81853, metabolism of the structurally-related quinoxaline compounds in the presence of both microsomal and

Incubation	Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein) *	Range in Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein)	% Inhibition in Metabolism (compared to control incubation)
Control (N_2) **	59.5 ± 17.9 (n=11)	33.8 - 91.7	-
Air	0 (n=3)	0	100
Boiled Microsomes	0 (n=3)	0	100
minus NADH	46.4 ± 6.4 (n=4)	38.0 - 53.7	22
minus NADPH	0 (n=3)	0	100

Table 6.2 Effect on metabolism of ZM 81853 by DEX-induced mouse liver microsomes in the presence of various controls.

* Figures represent the mean ± SD; n value is noted in brackets

** Control incubations were carried out under N_2 and contained 0.3 mg/ml microsomal protein, 1 mM NADH and NADPH and 300 μM ZM 81853.

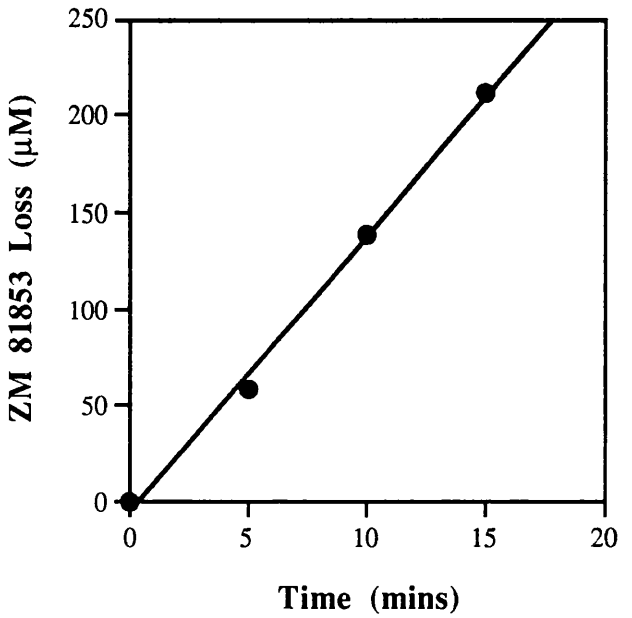
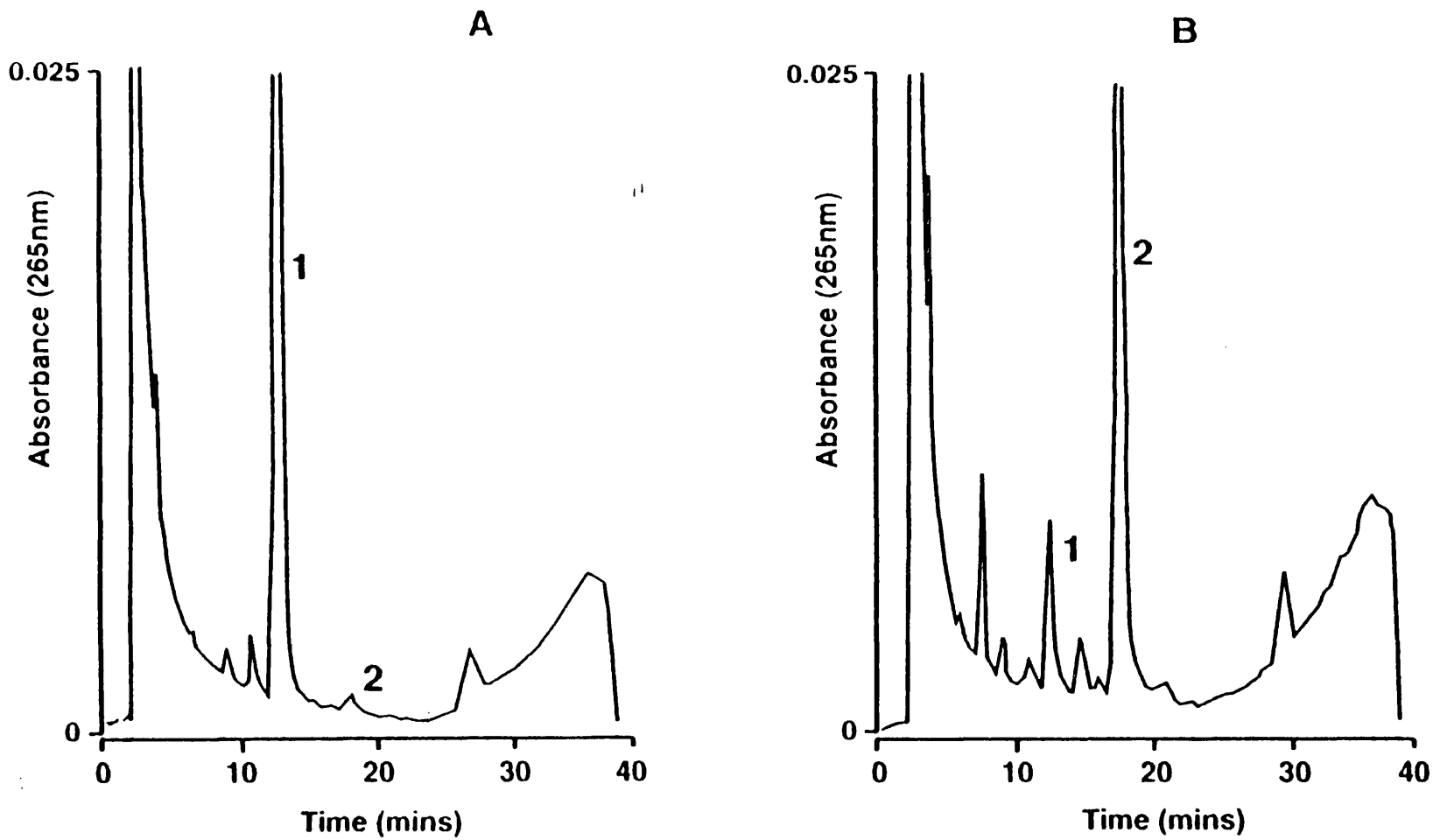


Figure 6.3 Typical progress curve for the metabolism of ZM 81853 by DEX-induced mouse liver microsomes under nitrogen.

Incubations contained 0.3 mg/ml microsomal protein, 1 mM NADH and NADPH and 300 μM ZM 81853 in a final volume of 1 ml 50 mM Tris HCl, pH 7.4.

Figure 6.4 A representative HPLC chromatogram of ZM 81853 metabolism in the presence of DEX-induced mouse liver microsomes.

Incubations were conducted under N_2 for 15 mins. in the presence of 300 μM ZM 81853, 1 mM NADH and NADPH and 0.3 mg/ml protein. (A) represents a sample removed at 0 mins. while (B) represents a sample removed at 15 mins. Peak 1 = ZM 81853, while peak 2 = predominant product.


A
0.025
Absorbance (265nm)
1
2
0
0
10
20
30
40
Time (mins)
B
0.025
Absorbance (265nm)
2
1
0
0
10
20
30
40
Time (mins)

Incubation	Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein) *	Range in Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein)	% Inhibition in Metabolism (compared to control incubation)
Control (N_2) **	17.2 ± 9.3 (n=6)	10.7 - 36.2	-
Air	0 (n=2)	0	100
Boiled Cytosol	0 (n=2)	0	100
minus NADH	20.0 ± 13.7 (n=3)	11.4 - 35.8	0
minus NADPH	0 (n=2)	0	100

Table 6.3 Effect on metabolism of ZM 81853 by DEX-induced mouse liver cytosol in the presence of various controls.

* Figures represent the mean ± SD; n value is noted in brackets

** Control incubations were carried out under N_2 and contained 1.0 mg/ml cytosolic protein, 1 mM NADH and NADPH and 300 μM ZM 81853.

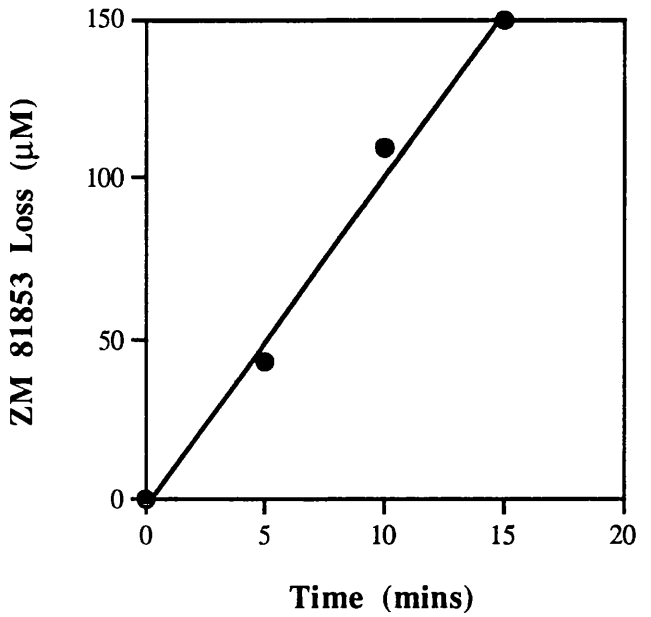


Figure 6.5 Typical progress curve for metabolism of ZM 81853 by DEX-induced mouse liver cytosol.

Incubations contained 1.0 mg/ml cytosolic protein , 1 mM NADH and NADPH and 300 μM ZM 81853 in a final volume 1 ml 50 mM Tris HCl, pH 7.4.

cytosolic protein was performed. Only ZM 10309 was shown to be metabolised by both microsomal and cytosolic enzyme preparations (Table 6.4). The rates of loss in the presence of both cytosolic and microsomal protein, however, were significantly lower than that of ZM 81853 (2.7-fold; $0.002>p>0.001$, and 3.6-fold; $p<0.001$, respectively). The resulting chromatograms were similar to ZM 81853 (Figure 6.4) and indicated that in both cases, the ZM 10309 peak had reduced in size, following a 15 mins. incubation, and a new peak had formed with a retention time of approximately 9 mins. With the exception of one particular experiment (ZM 8173 incubated with cytosolic protein), all the three other quinoxalines were found not to act as substrates for either enzyme preparation. The very low level loss of ZM 8173 detected in the presence of cytosolic protein was believed to a spurious result as loss was non-linear over the incubation period and was not reproduced in a subsequent experiment.

6.3.5. Chemical Inhibition of ZM 81853 Metabolism by DEX-induced Mouse Liver Microsomes

The effects of various chemical inhibitors on the metabolism of ZM 81853 by DEX-induced mouse liver microsomes are shown in Table 6.5. In addition, the effect of CO on the reaction is shown. Metabolism of ZM 81853 was significantly reduced (26.6 %) in the presence of CO ($0.01>p>0.002$). Subsequent reactions in the presence of SKF 525A, an established cytochrome P450 inhibitor, showed an even higher level of inhibition (43.7 %; $p<0.001$). Collectively these results, along with those which show an increased level of metabolism in the presence of DEX-induced enzyme preparations, suggest a possible role for NADPH : cytochrome P450 enzymes in the metabolism of ZM 81853 under hypoxic conditions. This is supported further by the inhibition, although at a lower rate, caused by metyrapone (an inhibitor of NADPH : cytochrome P450 Cyp 2b, Cyp 2c and Cyp 3a in mice). Interestingly, no inhibition was observed in the presence of erythromycin (an inhibitor of NADPH : cytochrome P450 CYP3A in the rat) suggesting no role for the Cyp 3a isoenzyme of NADPH : cytochrome P450. Furthermore, no

	Rate of loss (nmol of compound lost/min/mg of protein)	
Drug	**Microsomes**	**Cytosol**
ZM 81853	**59.5 ± 17.9 ***	**17.4 ± 10.6 ***
ZM 10309	**19.1, 13.9**	**7.5, 5.3**
ZM 8173	**0, 0**	**1.0, 0**
ZM 12557	**0, 0**	**0, 0**
ZM 65086	**0, 0**	**0, 0**

Table 6.4 Rate of loss of compound in the presence of either DEX-induced mouse liver microsomes or cytosol.

Figures represent two independent experiments except where denoted by *, in which case figures are the mean ± SD (n=11 and 5, repectively). Incubations contained 300 μM drug, 0.3 mg/ml microsomal or 1.0 mg/ml cytosolic protein, 1 mM NADH and 1 mM NADPH.

Conditions	Substrate / Inhibitor Concentration (μM)	Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein) *	Number of Repetitions (n)	Range in Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein)	% Inhibition of Metabolism (compared to control incubation)
Control (N_2) **	-	59.5 ± 17.9	11	33.8 - 91.7	-
+ Carbon Monoxide	-	43.7 ± 18.2 †	4	20.9 - 65.3	26.6
+ SKF 525A	2500	33.5 ± 6.8 ¶	3	28.8 - 41.3	43.7
+ Metyrapone	1000	49.8 ± 1.7	3	48.0 - 51.3	16.3
+ Erythromycin	1000	60.8 ± 2.7	3	58.7 - 63.7	0
+ Rutin	20	63.6 ± 27.6	3	37.2 - 92.3	0
+ Thallium Chloride	500	3.2 ± 5.6 ¶	3	0 - 9.7 [0, 0, 9.7]	94.6
+ DIPC	250	4.1 ± 4.1 ¶	4	0 - 9.0 [0, 1.5, 6, 9]	93.1

Table 6.5 Effect of various chemical inhibitors on the metabolism of ZM 81853 by DEX-induced mouse liver microsomes.

* Figures represent mean ± SD where appropriate; ** Control reactions were carried out under N_2 and contained 0.3 mg/mL microsomal protein, 1mM NADH and NADPH and 300 μM ZM 81853; † significantly different from control value, $0.01>p>0.002$; ¶ significantly different from control value, $p<0.001$.

inhibition was observed in the presence of rutin (an inhibitor of carbonyl reductase). The highest, and most significant level of inhibition, however, was obtained in the presence of either thallium chloride or DIPC (94.6 % and 93.1 %, respectively), both of which are known to inhibit NADPH : cytochrome P450 reductase.

6.3.6. Chemical Inhibition of ZM 81853 Metabolism by DEX-induced Mouse Liver Cytosol

Metabolism of ZM 81853 by cytosolic proteins has been demonstrated earlier, although at a lower level than in the presence of microsomal protein. The effects of two inhibitors on this metabolism are shown in Table 6.6. Only in the presence of rutin was the level of inhibition seen to be significant (58 %; $0.01>p>0.002$). This chemical is known to be an inhibitor of carbonyl reductase; therefore this enzyme may be involved in the metabolism of ZM 81853 by cytosolic protein under hypoxic conditions. Although the level of inhibition by dicoumarol (primarily used as a DT-diaphorase inhibitor) appeared to be similar, this was shown to be non-significant ($0.05>p>0.02$). Interestingly, in the presence of both inhibitors the level of inhibition was reduced to only 27 %.

6.3.7. Identification by Mass Spectroscopy of Predominant Product Following ZM 81853 Metabolism by DEX-induced Mouse Liver Microsomes

As shown in Figure 6.4, metabolism of ZM 81853 by DEX-induced mouse liver microsomes resulted in one predominant peak. To identify this product, mass spectroscopy was performed. The resulting FAB spectrum is shown in Figure 6.6, and indicates a major ion in the spectrum at 207 with an elemental formula of $C_9H_{11}O_2N_4$. As FAB spectra usually indicate the MWt. of the compound (+ H), the result indicated that a four-electron reduction product with a MWt. of 206 may be produced following reduction of ZM 81853 in the presence of DEX-induced mouse liver microsomes.

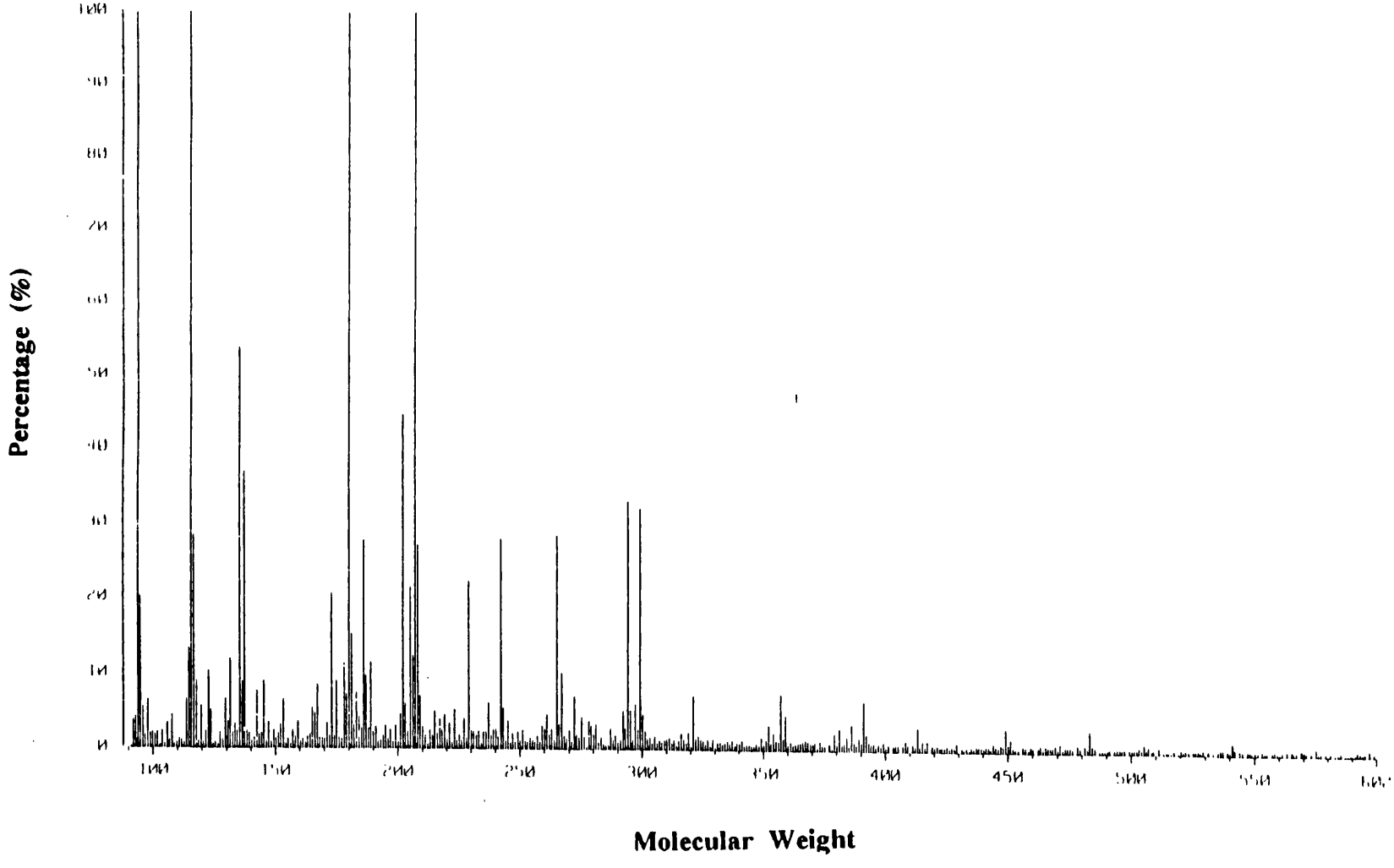
Conditions	Substrate / Inhibitor Concentration (μM)	Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein) *	Number of repetitions (n)	Range in Rate of Metabolism (nmol of ZM 81853 lost/min/mg of protein)	% Inhibition of Metabolism (compared to control incubation)
Control (N_2) **	-	17.4 ± 10.6	5	10.7 - 36.2	-
+ dicoumarol	100	7.4 ± 6.5	3	0 - 11.9	57.5
+ rutin	20	7.3 ± 1.3 †	3	6.3 - 8.8	58.0
+ dicoumarol / rutin	100 / 20	12.7	2	11.9, 13.4	27.0

Table 6.6 Effect of various chemical inhibitors on the metabolism of ZM 81853 by DEX-induced mouse liver cytosol.

* Figures represent the mean ± SD, except in the final row in which the average of duplicate values is quoted; ** Control reactions were carried out under N_2 and contained 1.0 mg/mL cytosolic protein, 1 mM NADH and NADPH and 300 μM ZM 81853; † significantly different from control value, $0.01>p>0.002$.

Figure 6.6 FAB Spectra resulting from Mass Spectroscopy of the Predominant Product of ZM 81853 Metabolism by DEX-induced Mouse Liver Microsomes.

Analysis was performed by Dr. D. G. Watson at the Department of Pharmaceutical Sciences, University of Strathclyde using a double focussing mass spectrometer.


Percentage (%)
Molecular Weight

6.4 Discussion

The novel quinoxaline di-*N*-oxide ZM 81853 was shown in earlier studies in this thesis (Chapter 4) to exhibit similar hypoxia-selective cytotoxicity to that of the benzotriazine di-*N*-oxide, tirapazamine and as such, may undergo similar metabolic activation as tirapazamine. Further analysis of a series of structurally-related quinoxalines suggested that small changes in the structure of ZM 81853 resulted in loss of the observed hypoxia-selective cytotoxicity. It was of interest, therefore, to investigate this further by studying the metabolic properties of all of the quinoxaline compounds under hypoxic conditions. Furthermore, the role of particular reductase enzymes in metabolism was investigated as it is known that the action of particular enzymes can influence the selective toxicity of bioreductive cytotoxins (discussed in Chapter 1). ZM 81853 was metabolised by enzymes within the cytosolic and the microsomal fractions, although the rate of ZM 81853 loss was greater in the latter case. Interestingly, chemical inhibition studies suggested that NADPH : cytochrome P450 reductase was the enzyme predominantly responsible for metabolism in the presence of microsomes. ZM 10309 was the only other quinoxaline from the series to undergo metabolism. However, the rate of loss of the compound was significantly lower in the presence of both cytosolic and microsomal fractions than was the case for ZM 81853.

Enzymes such as the NADPH : cytochrome P450 family, DT-diaphorase and xanthine oxidase have been implicated in the reductive metabolism of many drugs, including various nitro and *N*-oxide compounds (McLane *et al.* , 1983; Workman & Walton, 1990). Their role can either be one of activation or detoxification of the drug (Workman & Walton, 1990). For example in the case of tirapazamine, the one-electron reducing enzymes NADPH : cytochrome P450 (CYP 2B6) and NADPH : cytochrome P450 reductase (Fitzsimmons *et al.*, 1994; Lewis *et al.* , 1995; Riley *et al.*, 1993) are known to be involved in activation whilst the two-electron reducing enzyme DT-diaphorase was shown to be involved in detoxification (Riley & Workman, 1992b). Information of this nature has generally been accumulated by performing metabolism

studies in the presence of subcellular fractions from mouse livers. The role of reductases can be investigated with the use of various chemical enzyme inhibitors and, more specifically, confirmed with the use of highly purified enzyme preparations. In addition, hepatic enzyme induction has also been incorporated to investigate the role of different enzymes. For example, significant induction by DEX of total NADPH : cytochrome P450 Cyp 2b, Cyp 2c and Cyp 3a isoenzyme content and of DT-diaphorase activity in the microsomal fraction of mouse livers have been demonstrated (Meehan *et al.*, 1988; Riley *et al.*, 1993). Such techniques have been used to predict those reductase enzymes involved in metabolism of a large number of bioreductive compounds (reviewed in Workman, 1993).

Due to the similarities between ZM 81853 and tirapazamine, in both structure and hypoxic-selectivity in human breast tumour cell lines (Chapter 4), it was felt that an appropriate starting point for determination of enzymes involved in reductive metabolism of ZM 81853, and indeed the other quinoxalines, would be to use subcellular fractions from mouse liver (both control and DEX-induced preparations). To further address the role of specific enzymes in metabolism, various chemical inhibitors (Table 6.1) were included in the analysis. Whilst it was realised that a more detailed investigation could be performed by combining the use of chemical inhibitors and antibodies, the latter were in limited supply and therefore were not included at this stage. Investigations involving diagnostic inducers, inhibitors and / or antibodies are extremely complex, not least because of inherent problems of species variation (Boobis *et al.* , 1990). Nevertheless, it was believed that this initial study would provide valuable information regarding the potential role of different enzymes in ZM 81853 metabolism.

ZM 81853 was shown to be metabolised by microsomal protein, but only in the presence of active enzyme and cofactors. Interestingly, although both NADH and NADPH played a role as cofactors in the metabolism of the compound, the latter cofactor was shown to be the most important. Similar results were reported by Sugiura and colleagues (Sugiura *et al.* , 1976) using the tertiary amine *N*-oxides of imipramine, tiarimide and *N, N*-dimethylanaline; however, in contrast to these findings, reduction of

tirapazamine showed a complete dependence upon NADPH (Walton & Workman, 1990). Interestingly, however, the rate of loss of ZM 81853 was found to be approximately 4-fold lower than that for tirapazamine under similar conditions (Walton & Workman, 1990). Metabolism of ZM 81853 was shown to be completely inhibited by the presence of oxygen, a finding which is in agreement with previous studies involving tirapazamine (Riley *et al.*, 1993) and the tertiary amine *N*-oxides (Sugiura *et al.*, 1976). Therefore, metabolism of ZM 81853 was shown to occur preferentially under hypoxic conditions, as indicated by the cytotoxicity studies (Chapter 4).

As mentioned above, DEX is known to induce total NADPH : cytochrome P450 content within mouse liver microsomes approximately 2-fold, with the emphasis on the Cyp 2b, Cyp 2c and Cyp 3a isoenzymes (Meehan *et al.*, 1988; Riley *et al.*, 1993). Metabolism of ZM 81853 was shown to be higher in the presence of DEX-induced mouse liver microsomes, thereby suggesting a possible involvement of NADPH : cytochrome P450 enzymes in the reaction. This possibility was further supported by the observation that metabolism of ZM 81853 in the presence of the established NADPH : cytochrome P450 inhibitors, CO and SKF 525A, was reduced by approximately 26 % and 44 %, respectively. Similar roles for NADPH : cytochrome P450 enzymes have been reported for other *N*-oxides such as tirapazamine (Riley *et al.*, 1993), and the tertiary amine *N*-oxides (Sugiura *et al.*, 1976). In contrast, however, some studies (Walton *et al.*, 1992b) have previously reported an increase in the level of metabolism in the presence of the type I ligand, SKF 525A. Proposed mechanisms for this effect, have suggested that an increase in NADPH : cytochrome P450 reduction occurs in the presence of the ligand (Gigon *et al.* , 1969), or that the presence of the ligand causes shunting of electrons towards a more efficient NADPH : cytochrome P450 enzyme and hence causes an increase in metabolism (Walton *et al.*, 1992b). It is important to note, however, that an aerobic preincubation step has been reported as a requirement for inhibition of drug metabolism by SKF 525A (Gillette & Sasame, 1964) and those studies performed previously by Walton and colleagues, did not involve such a step. Subsequent studies involving tirapazamine and SKF 525A inhibition have shown that in the presence of an

aerobic preincubation step, approximately 58 % inhibition of metabolism occurs (Riley *et al.*, 1993). Further analysis using chemical inhibitors suggested that the NADPH : cytochrome P450 Cyp 3a isoenzyme did not play a role in metabolism of ZM 81853, as no inhibition occurred in the presence of the macrolide antibiotic erythromycin (a substrate and competitive inhibitor of rat CYP3A subfamily (Wrighton *et al.*, 1985)). In contrast, a low level of inhibition occurred in the presence of metyrapone (a type II ligand and inhibitor of NADPH : cytochrome P450 Cyp 2a, Cyp 2b, Cyp 2c and Cyp 3a isoenzymes (Riley *et al.*, 1993 and references therein) suggesting the possible involvement of one or more of the aforementioned NADPH : cytochrome P450 isoenzymes, other than Cyp 3a, in the metabolism of ZM 81853.

Previous studies have demonstrated an important role for rodent liver NADPH : cytochrome P450 reductase in the metabolism of tirapazamine (Fitzsimmons *et al.*, 1994; Riley *et al.*, 1993). In contrast, no direct role for NADPH : cytochrome P450 reductase has been reported for the tertiary amine *N*-oxides (Sugiura *et al.*, 1976), although these studies were with rat rather than mouse liver microsomes. As for total NADPH : cytochrome P450 content, NADPH : cytochrome P450 reductase activity has been shown to be increased in DEX-induced mouse liver microsomes (Riley *et al.*, 1993). In this present study, metabolism of ZM 81853 by DEX-induced microsomes was inhibited by approximately 94 % in the presence of thallium chloride, a known inhibitor of NADPH : cytochrome P450 reductase (Woods & Fowler, 1984). This result was further supported by the extensive inhibition which occurred in the presence of DIPC, an alternative inhibitor of NADPH : cytochrome P450 reductase (Tew, 1993). These results strongly suggest that NADPH : cytochrome P450 reductase was the enzyme predominantly responsible for metabolism of ZM 81853 in the presence of DEX-induced mouse liver microsomes. This is in agreement with the possible role of NADPH : cytochrome P450 reductase in the hypoxic-selective cytotoxicity of ZM 81853, as demonstrated in Chapter 4 of this thesis. Therefore, the results of the metabolism studies further support the view that NADPH : cytochrome P450 reductase may play an important role in the hypoxia-selective activity of ZM 81853. A secondary role for NADPH : cytochrome P450

enzymes was also suggested by the inhibition of metabolism in the presence of CO or SKF 525A. Whilst the specificity of CO for haem-containing enzymes such as NADPH : cytochrome P450 enzymes is known to be due to the irreversible binding to the haem group within the enzyme structure, the specifity of SKF 525A is less clearly understood. In the presence of SKF 525A, inhibition of metabolism was approximately 17% greater than CO suggesting that inhibition of enzymes other than the NADPH : cytochrome P450 family may have occurred. SKF 525A was in fact shown to cause almost complete inhibition of ZM 81853 metabolism in the presence of a purified, recombinant form of human NADPH : cytochrome P450 reductase (data not shown), thereby helping to explain the higher level of inhibition obtained in the presence of the ligand.

Metabolism of ZM 81853 in the presence of cytosolic protein was also studied. The rate was again shown to be greater in the presence of the DEX-induced enzyme preparation. However, the actual rate of metabolism was significantly lower (3.5-fold) than that in the presence of microsomal proteins. Interestingly, the observed rate of ZM 81853 loss (17.4 nmol ZM 81853 lost/min/mg of protein) in the presence of mouse liver cytosol was approximately 3-fold greater than that of the di-*N*-oxide, tirapazamine (5 nmol/min/mg) (Walton & Workman, 1990). However, in the latter case metabolism was performed in the presence of a non-induced cytosolic preparation. The fact that metabolism of ZM 81853 was higher in the presence of the DEX-induced cytosol was interesting as DEX treatment has been previously shown by Riley *et al* (Riley *et al.*, 1993) to significantly increase the activity of the 2-electron reducing enzyme DT-diaphorase within the microsomal fraction of mouse livers. It may therefore be possible that induction within the mouse liver cytosol also occurs. Importantly, however, metabolism was shown to be fully dependent upon NADPH as a cofactor indicating that the enzyme(s) involved in metabolism would utilise only NADPH. This finding, therefore, ruled out a role for DT-diaphorase in the metabolism of ZM 81853 as this enzyme has the ability to utilise both NADH and NADPH equally (Ernster *et al.*, 1962). These results are in sharp contrast to those of tirapazamine for which reduction displayed an equal dependence upon both NADH and NADPH for metabolism in the presence of

cytosolic protein, and for which metabolism was shown to involve DT-diaphorase (Walton & Workman, 1990). These results with tirapazamine were confirmed by the use of a purified human preparation of DT-diaphorase in which equal dependence for NADH and NADPH was displayed (Walker *et al.* , 1994). Further analysis using either dicoumarol, an known inhibitor of DT-diaphorase (Segura-Aguilar *et al.*, 1990), or rutin, a potent inhibitor of carbonyl reductase (Wermuth, 1981), revealed that both inhibitors caused approximately 58 % inhibition of ZM 81853 metabolism. Inhibition in the presence of dicoumarol alone was shown to be statistically non-significant. However, it was clear that a reasonable level of inhibition could be obtained. Dicoumarol, once considered to be a very specific inhibitor of DT-diaphorase (Dulhanty *et al.* , 1989; Keyes *et al.* , 1985), is now known to inhibit a large number of enzymes including NADH : cytochrome b_5 reductase (Hodnick & Sartorelli, 1993; Preusch *et al.* , 1991) and some forms of carbonyl reductase (Maser & Netter, 1989). Therefore, due to the similar levels of inhibition and the possible cross reactivity for carbonyl reductase, it was felt that metabolism of ZM 81853 in the presence of DEX-induced mouse liver cytosol may be due to a NADPH-utilising carbonyl reductase enzyme, most likely via 2-electron reduction of the compound. A possible complication in this hypothesis is that in the presence of both inhibitors, the percentage inhibition is reduced to only 27 %. However, the reasons for this observation are unclear at this stage.

The novel quinoxaline di-*N*-oxide ZM 81853 appears, therefore, to be reduced by carbonyl reductase and, more predominantly, by NADPH : cytochrome P450 reductase from mouse liver under hypoxic conditions. Mass spectroscopy following reduction of ZM 81853 by DEX-induced mouse liver microsomes suggested that the predominant reduction product may have a MWt of 206 (see Section 6.3.7 for explanation). This information indicated, therefore, that the reduction product may result from four-electron reduction of ZM 81853 (MWt. 202). Although these studies were not performed following DEX-induced cytosolic metabolism, results from HPLC analysis suggested that the predominant product was, in fact, the same species, as retention time and wavelength spectra were identical (data not shown). Collectively, therefore, these results suggest that

ZM 81853 can undergo a series of one-electron reductions by NADPH : cytochrome P450 reductase to yield a four electron reduced product. In addition, they suggest that ZM 81853 may also undergo a two-electron reduction pathway by carbonyl reductase to form the same four-electron reduced product. Certainly in the case of NADPH : cytochrome P450 reductase these results are in agreement with the proposed role of this enzyme in the metabolism of ZM 81853 to a cytotoxic species under hypoxic conditions.

Metabolism of the structurally-related quinoxalines to cytotoxic species was investigated to determine possible reasons as to why these compounds were not active in the human tumour cell lines (Chapter 4). The parent unsubstituted quinoxaline ZM 8173, and two other substituted quinoxalines ZM 12557 and ZM 65086 were found to be inactive as substrates for both microsomal and cytosolic enzyme preparations under hypoxic conditions. Although these latter studies were performed in the presence of mouse enzymes (as opposed to human), the results do support earlier suggestions that the compounds lacked the ability to be metabolised to active species under hypoxic conditions. As discussed previously in Chapter 4 (Section 4.4), this may be due to the fact that the compounds possess inappropriate reduction potentials for reduction by enzymes present within the human cells or the mouse liver fractions. The influence of reduction potentials (one-electron) of a range of nitro-compounds on cytotoxicity towards Chinese hamster V79 cells was clearly demonstrated by Adams and colleagues (Adams *et al.* , 1980). More recently, a direct correlation between reduction potential and rate of reduction by NADPH : cytochrome P450 reductase has been demonstrated (Butler & Hoey, 1993). Morecroft and colleagues (Morecroft *et al.* , 1994) also demonstrated a correlation between reduction potentials of a series of tirapazamine analogues and their ability to be metabolised by NADPH : cytochrome P450 reductase. Therefore this aspect is clearly important when analysing the ability of compounds to act as bioreductive cytotoxins.

In contrast to the other quinoxalines, ZM 10309 exhibited some degree of reduction by both mouse liver microsomes and cytosol, although the rate of loss of the compound was significantly lower than that of ZM 81853. The cytotoxicity studies had

previously revealed that although ZM 10309 showed no notable HCR, the IC_{50} values for this compound under both oxic and hypoxic conditions were the lowest, and therefore most potent, for all of the four structurally-related compounds in question. It appears, therefore, that ZM 10309 was metabolised but at only a low level under both oxic and hypoxic conditions and hence exhibited no hypoxic-selectivity.

In summary, the preferential metabolic activation of ZM 81853 under hypoxic conditions was confirmed with the use of mouse liver enzyme preparations. In addition, chemical inhibition studies revealed that the one-electron reducing enzyme NADPH : cytochrome P450 reductase may be the principal enzyme responsible for activation of ZM 81853 under hypoxic conditions. This was certainly suggested in the cytotoxicity studies, as the highest HCR value was obtained in the human tumour cell line with the greatest NADPH : cytochrome P450 reductase activity (Chapter 4). Roles for one or more of the NADPH : cytochrome P450 isoenzymes Cyp 2a, Cyp 2b or Cyp 2c, and also carbonyl reductase were also indicated in this study. Furthermore, mass spectroscopy suggested that the predominant product was a four-electron reduced product. Interestingly, three out of four structurally-related quinoxaline compounds did not act as substrates for mouse liver enzymes, thereby suggesting that the lack of cytotoxicity observed (Chapter 4) was due to a lack of metabolic activation of the compounds. Although ZM 10309 did undergo metabolism under hypoxic conditions, it was to a much lesser degree than for ZM 81853. Collectively this study supports the cytotoxicity data by indicating that small changes in the quinoxaline structure clearly affect the ability of the compound to be metabolised, and hence exert its activity under hypoxic conditions. Although strongly implicated, the present results do not provide direct evidence for a role of NADPH : cytochrome P450 reductase in the cytotoxic effects of ZM 81853. Purified enzyme preparations can be extremely valuable in establishing such roles. Chapter 7 describes an investigation to establish the ability of a purified, recombinant preparation of human NADPH : cytochrome P450 reductase to reduce ZM 81853 to a DNA damaging species.

CHAPTER 7

ZM 81853 METABOLISM : KINETIC ANALYSIS AND ABILITY TO CAUSE DNA DAMAGE

7.1 Introduction

ZM 81853 has been shown to be metabolised under hypoxic conditions by both DEX-induced mouse liver microsomes and cytosol (Chapter 6). Furthermore, chemical inhibition studies in the presence of DEX-induced mouse liver microsomes and cytosol indicated a predominant role for NADPH : cytochrome P450 reductase in the metabolism of ZM 81853 under hypoxic conditions. In this current chapter, the kinetics of ZM 81853 metabolism in the presence of DEX-induced mouse liver microsomes and cytosol were investigated as a means of comparing ZM 81853 to other established bioreductive agents. The potential role of NADPH : cytochrome P450 reductase was examined more specifically by studying the kinetics of ZM 81853 metabolism in the presence of a purified, recombinant human form of the enzyme. In order to establish a direct role for the NADPH : cytochrome P450 reductase in the selective toxicity observed within tumour cell lines, the ability of this enzyme to metabolise ZM 81853 to a DNA damaging species under hypoxic conditions was also examined. Although chemical inhibition studies indicated a role for carbonyl reductase in the metabolism of ZM 81853 by cytosolic proteins, no purified enzyme preparation was available to study such a role. Since dicoumarol had been shown to inhibit metabolism and a purified, recombinant form of human DT-diaphorase was available, this was used to determine the possible role of this enzyme in ZM 81853 metabolism .

The enzymology of bioreductive anticancer agents has been proposed as an important factor in future drug development programmes (Workman & Walton, 1990). To date, a number of potential bioreductive anticancer agents have been studied in detail to determine which enzymes are involved in metabolism. For example, the benzotriazine

di-*N*-oxide tirapazamine is known to be metabolised by one-electron reducing enzymes such as NADPH : cytochrome P450s and NADPH : cytochrome P450 reductase (Riley *et al.* , 1993; Walton *et al.* , 1992b), and by two-electron reducing enzymes such as DT-diaphorase (Riley & Workman, 1992b). Furthermore, DT-diaphorase has been shown to be involved in metabolism of the novel indoloquinone, EO9 (Walton *et al.* , 1991) and also the dinitrobenzamide, CB 1954 (Boland *et al.* , 1991). It would be reasonable, therefore, to predict that tumours rich in enzymes such as NADPH : cytochrome P450 reductase and DT-diaphorase would be susceptible to the actions of these bioreductive agents.

In addition to determining those enzymes involved in the metabolism of bioreductive agents, it is also important to establish the level of efficiency with which metabolism occurs. This can be achieved by performing kinetic analysis to determine the V*max* (maximum rate of reaction) and K*m* (substrate concentration at which V*max* is half its maximum rate) of the enzyme-catalysed reaction (Henderson, 1978). By establishing such parameters for individual bioreductive agents, comparisons between the efficiency of metabolism of such agents could then be made. In the previous chapter, mouse liver enzymes were shown to metabolise ZM 81853 under hypoxic conditions. Here kinetic analysis of these reactions were performed in the presence of both DEX-induced mouse liver microsomes and cytosol in order to allow comparisons between ZM 81853 and other *N*-oxides such as tirapazamine and the tertiary *N*-oxides (Sugiura *et al.* , 1976; Walton & Workman, 1990). Studies of this nature can also be performed in the presence of purified enzyme preparations to establish more conclusively a direct role for specific enzymes in the metabolism of bioreductive agents. Chemical inhibition studies (Chapter 6) previously indicated that NADPH : cytochrome P450 reductase played a predominant role in metabolism of ZM 81853 by DEX-induced microsomal proteins under hypoxic conditions. For this reason, the ability of a purified, recombinant human form of the enzyme to metabolise ZM 81853 was investigated and kinetic parameters of metabolism determined. Similar studies were undertaken in the presence of a purified, recombinant

form of human DT-diaphorase since no such preparations were available for carbonyl reductase. The results of the kinetic analysis are described in this chapter.

Both the cytotoxicity (Chapter 4) and chemical inhibition (Chapter 6) studies indicated that ZM 81853 may undergo a similar pathway of activation to that of tirapazamine. Tirapazamine has previously been shown to be metabolised under hypoxic conditions by purified rat (Fitzsimmons *et al.* , 1994) and human (Walker *et al.*, 1994) NADPH : cytochrome P450 reductase to a DNA damaging species, believed to be a nitroxide radical (Lloyd *et al.* , 1991). Similar studies were performed, therefore, to investigate the ability of ZM 81853 to be metabolised to a DNA damaging species by a purified, recombinant form of human NADPH : cytochrome P450 reductase. The results of this investigation are also described here.

7.2 Materials and Methods

7.2.1. Chemicals and Reagents

All general chemicals and solvents (of the highest grade commercially available) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.) or Fisons plc. (Loughborough, U.K.) unless otherwise stated. Zero grade nitrogen (< 5 vpm O_2) was obtained from the British Oxygen Company (London, U.K.).

7.2.2. Enzyme Sources

DEX-induced microsomal and cytosolic fractions were prepared as previously detailed in Section 6.2.2. A highly purified, recombinant form of human NADPH : cytochrome P450 reductase was kindly provided by Prof. C. R. Wolf (ICRF Department of Molecular Pharmacology, Dundee, U.K.). Purified, recombinant human DT-diaphorase was also kindly provided by Dr. S. Chen (City of Hope, Duarte, CA, U.S.A.) (Chen *et al.* , 1995). In all four cases, total protein content was determined using the Pierce Microtiter Plate BCA assay (detailed in Section 2.2.5.). Activity of the purified, recombinant forms of the human enzymes were determined spectrophotometrically using the cytochrome *c* reduction assay detailed in Section 2.2.6.

7.2.3. ZM 81853 Metabolism Under Hypoxic Conditions

Reductive metabolism of ZM 81853 and analysis of samples were carried out essentially as detailed in previous chapters (Sections 5.2.2. 6.2.3. and 6.2.4.). All reactions were performed under conditions in which the rate of loss of ZM 81853 was shown to be linear with respect to protein / activity level and time. A control reaction in the absence of cofactor was performed during each experiment. Reactions were pre-incubated for 7 mins. under the flow of N_2 before initiation. In the case of DEX-induced

mouse liver cytosol and microsomes, standard incubations contained 1.0 mg/ml or 0.3 mg/ml protein, respectively, and 1 mM NADPH in a total volume of 1 ml 50 mM Tris HCl buffer, pH 7.4. Reactions were initiated by the addition of ZM 81853 in DMSO to give final substrate concentrations of 15 - 540 μM. Aliquots (100 μl) were removed at 0 and 7.5 mins. and added to 200 μl ice-cold methanol. Samples were then processed as described before (Section 6.2.3.).

For analysis in the presence of purified recombinant human enzyme, standard incubations contained 40 mU of NADPH : cytochrome P450 reductase activity (where 1U = 1μmol cytochrome *c* reduced/min/mg protein measured at 37°C) and 1 mM NADPH in a total volume of 1 ml 50 mM Tris HCl buffer, pH 7.4. This level of activity had been shown to metabolise ZM 81853 successfully (data not shown). Reactions were initiated by the addition of ZM 81853 in DMSO to give final substrate concentrations of 30 - 800 μM. Aliquots (100 μl) were removed at 0 and 10 mins. in this case, and were added to 200 μl ice-cold methanol. Samples were processed as described before (Section 6.2.3.). Similar studies were performed in the presence of the structurally-related quinoxaline ZM 10309 as it was the only other compound to be metabolised in the presence of mouse liver enzymes (Chapter 6).

Reductive metabolism of ZM 81853 in the presence of a purified, recombinant form of human DT-diaphorase was performed using a range of enzyme activities (up to 1400 mU (1U = 1μmol cytochrome *c* reduced/min/mg protein measured at 37°C)). In this case, incubations contained 1 mM NADH as cofactor. Aliquots of the reaction mixture were processed as above.

7.2.4. Kinetic Analysis

Michaelis-Menten enzyme kinetics were defined under optimal conditions for ZM 81853 using the criteria described by Henderson (Henderson, 1978). Apparent K*m*, V*max* and K*cat* were determined by computer graphics analysis (Enzyme Kinetics, Trinity Software, Campton, NH, U.S.A.) using a total of nine different substrate concentrations.

K*cat* is defined as the catalytic constant of the enzyme and describes the capacity of the enzyme-substrate complex to form the product under saturating substrate conditions. As these conditions are unlikely to be achieved intracellularly, however, K*cat* / K*m* can be a more useful parameter as this takes into consideration non-saturable conditions. The ratio V*max* / K*m* was also determined to enable comparisons of overall activity of different substrates.

7.2.5. DNA Damage Assays

The ability of purified, recombinant human NADPH : cytochrome P450 reductase to activate ZM 81853 to a DNA damaging species under both oxic and hypoxic conditions was investigated *in vitro* by measuring the level of conversion of a supercoiled preparation (form I) of plasmid pBR322 DNA (Boehringer Mannheim, East Sussex, U.K.) to the relaxed, circular configuration (form II) or the linearised conformation (form III). Incubation mixtures contained ZM 81853 (9.4 - 600 μM) with NADPH (1 mM), pBR322 DNA (1000 ng) and purified, recombinant NADPH : cytochrome P450 reductase (0.039 - 60 mŪ) in a total volume of 60 μl of 100 mM sodium phosphate buffer, pH 7.4. Superoxide dismutase (SOD) (150 - 600 μg/ml) and catalase (150 - 600 μg/ml) were also added to some of the reactions. NADPH : cytochrome P450 reductase (40 mU) was added to all reactions (except those investigating effect of enzyme concentration) at the equivalent activity to that used in the previous metabolism studies (Section 7.2.3.). Control incubations omitted various reagents, while boiled enzyme preparations were used as an inactive enzyme control. Hypoxic reaction mixtures were pre-incubated in sealed vials under N_2 at 37°C for 2 mins. before the addition of ZM 81853 through air-tight seals to initiate the reaction. The reactions were then incubated for a further 30 mins. before being stopped by the addition of 15 μl of stop buffer (5 mM EDTA, 0.5 % SDS, 60 % (v/v) glycerol and 0.1 % (v/v) bromophenol blue). Oxic reaction mixtures were incubated in vials open to the air for 30 mins. at 37°C following

initiation of the reaction by the addition of ZM 81853. Reactions were stopped as described above.

Aliquots (25 µl) of stopped reaction mixtures were added to a horizontal 1 % (w/v) agarose gel (20 x 24 cm) and electrophoresed at 40 Volts for 15 - 20 hrs. at room temperature. DNA in gels was stained with ethidium bromide (0.5 µg/ml) and photographed under UV transillumination (TF 35M UV box, Vilber Lourmat, Marne La Vallee, France). Densitometric analysis was performed using a Molecular Dynamics Laser Densitometer with Image Analysis Software (Protein Databases Incorporated, NY, U.S.A.). Correction factors were employed to account for differential staining of DNA forms by ethidium bromide.

7.3 Results

7.3.1 Kinetics of ZM 81853 Metabolism by Mouse Liver Enzymes

ZM 81853 has previously been shown to be metabolised in the presence of both DEX-induced mouse liver microsomes and cytosol, although in the latter case, metabolism was demonstrated to be significantly lower (Chapter 6).

Metabolism of ZM 81853 by cytosolic protein was shown to conform to Michaelis-Menten kinetics. This was indicated by the plots of *V* versus *S* (Figure 7.1A), which gave rectangular hyperbolae, and by the linear nature of the Hanes-Woolf plots (Figures 7.1B). Values of apparent K*m* and V*max* (mean ± SD, n=3) were obtained using five different kinetics plots and were in the range 56 - 198 μM for K*m* and 11 - 21 nmol of ZM 81853 lost/min/mg protein for V*max*. The kinetic parameters for non-linear regression and Hanes-Woolf analysis are shown in Table 7.1. In addition V*max* / K*m* ratios were calculated and found to be 0.13 and 0.12, depending upon the type of analysis employed.

Similar studies to investigate the kinetics of ZM 81853 metabolism by microsomal protein also indicated that metabolism conformed to Michaelis-Menten kinetics. Plots of *V* versus *S* (Figure 7.2A) and Hanes-Woolf plots (Figure 7.2B) are shown. As before, values of apparent K*m* and V*max* (mean ± SD, n=3) were obtained using five different kinetics plots and were in the range 208 - 518 μM for K*m* and 131 - 238 nmol of ZM 81853 lost/min/mg protein for V*max*. The kinetic parameters for non-linear regression and Hanes-Woolf analysis are shown in Table 7.2, including ratios for V*max* / K*m* of 0.51 and 0.46.

7.3.2. Kinetics of ZM 81853 and ZM 10309 Metabolism by Purified, Recombinant Human Enzymes

Purified, recombinant human NADPH : cytochrome P450 reductase was found to catalyse the reduction of ZM 81853. Chromatogram analysis indicated that one

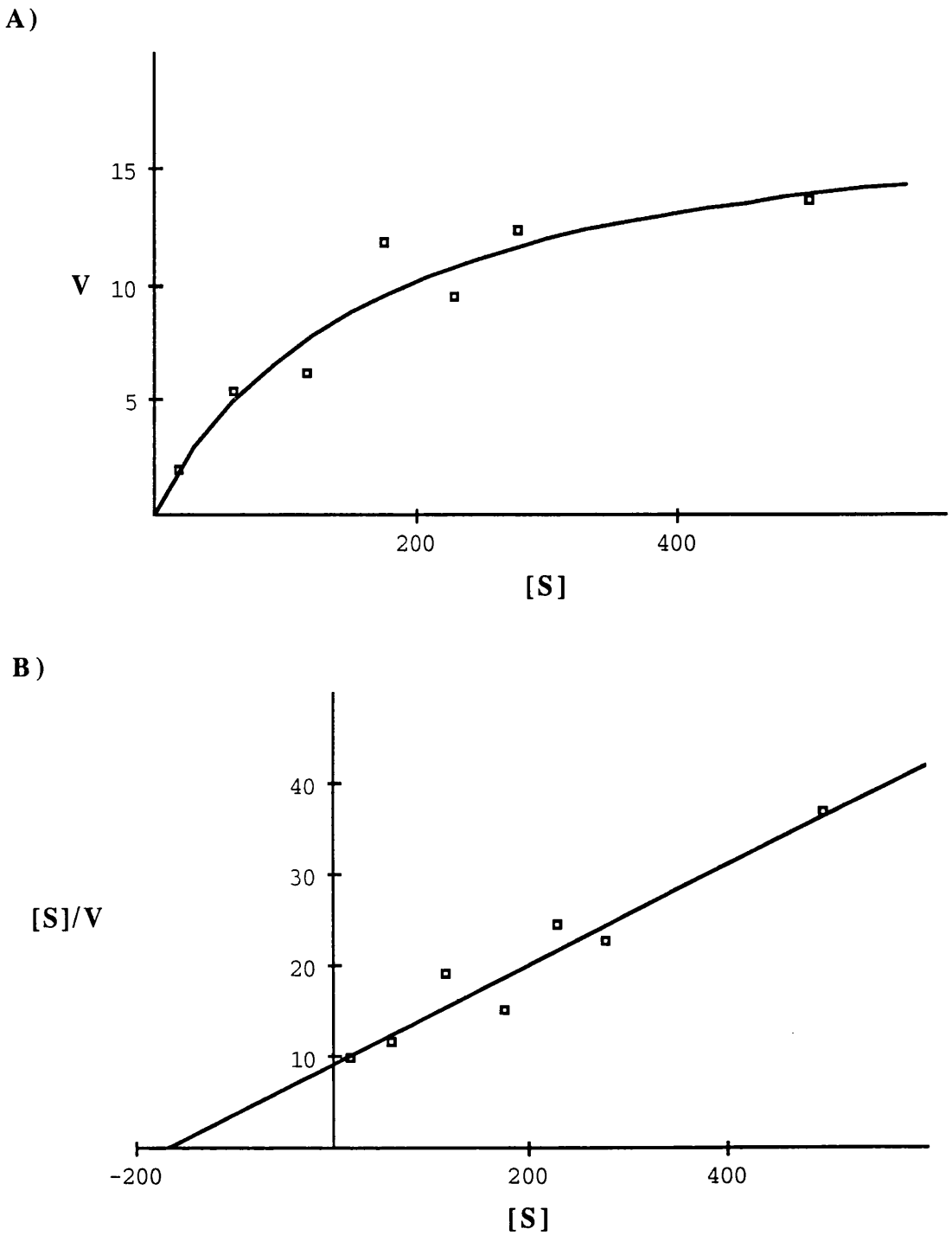


Figure 7.1 **Typical plot of *V* versus *S* (A) and corresponding Hanes-Woolf linear transformation plot (B) for ZM 81853 metabolism by DEX-induced mouse liver cytosol.**

Units of *S* and *V* are μM and nmol of ZM 81853 lost/min/mg protein, respectively.

Parameter	Non-linear regression	Hanes-Woolf
K*m* (μM)	121.3 ± 35.9	130.4 ± 30.7
V*max* (nmol/min/mg)	15.6 ± 3.0	15.2 ± 3.0
V*max* / K*m* ratio	0.13	0.12

Table 7.1 Michaelis-Menten parameters for ZM 81853 loss following metabolism by DEX-induced mouse liver cytosol *in vitro*.

Values were obtained from three independent experiments, each with nine substrate concentrations. K*m* and V*max* values represent a mean ± SD, n=3.

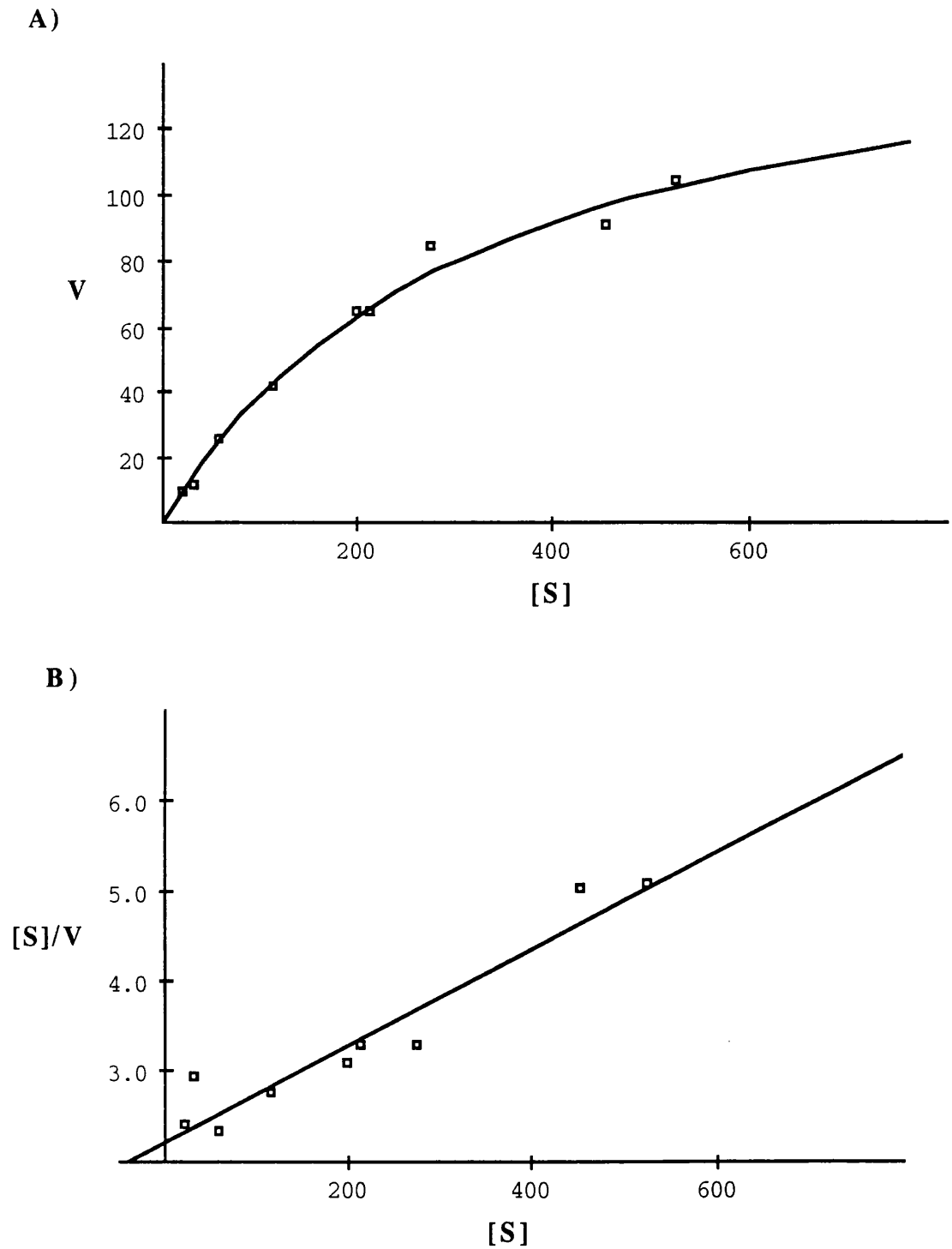


Figure 7.2 Typical plot of *V* versus *S* (A) and corresponding Hanes-Woolf linear transformation plot (B) for ZM 81853 metabolism by DEX-induced mouse liver microsomes.

Units of *S* and *V* are μM and nmol of ZM 81853 lost/min/mg protein respectively.

Parameter	Non-linear regression	Hanes-Woolf
K*m* (μM)	319.2 ± 82.9	374.3 ± 51.1
V*max* (nmol/min/mg)	162.5 ± 27.6	170.9 ± 22.2
V*max* / K*m* ratio	0.51	0.46

Table 7.2 Michaelis-Menten parameters for ZM 81853 loss following metabolism by DEX-induced mouse liver microsomes *in vitro*.

Values were obtained from three independent experiments, each with nine substrate concentrations. K*m* and V*max* values represent a mean ± SD, n=3.

predominant new peak eluted at approximately 16 mins., closely similar to previous studies involving DEX-induced mouse liver microsomes (Chapter 6, Figure 6.4). Reduction was shown to be dependent upon NADPH as cofactor. Furthermore, progress curves for ZM 81853 loss were linear with time up to at least 10 mins. at all substrate concentrations used.

Loss of ZM 81853 in the presence of NADPH : cytochrome P450 reductase was shown to conform to Michaelis-Menten kinetics, with plots of *V* versus *S* (Figure 7.3A) showing the expected rectangular hyperbolae, and Hanes-Woolf plots being linear in nature (Figure 7.3B). As with those studies detailed above, kinetic parameters were determined using five different plots and were in the range of 59 - 327 μM for K*m* and 3075 - 6800 nmol of ZM 81853 lost/min/mg protein for V*max*. The parameters for non-linear regression and Hanes-Woolf analysis are shown in Table 7.3. The additional parameters of K*cat* and K*cat*/K*m* were also determined, as was the ratio V*max* / K*m*. Interestingly, studies with purified, recombinant human DT-diaphorase produced no metabolism of ZM 81853, even at enzyme concentrations as high as 1400 mU.

The structurally-related quinoxaline ZM 10309 had been shown to be metabolised by DEX-induced mouse liver enzymes, although at a lower rate than ZM 81853 (Chapter 6). Kinetic analysis of ZM 10309 metabolism by NADPH : cytochrome P450 reductase was performed in order to compare the two quinoxaline compounds. Although metabolism of ZM 10309 by purified, recombinant NADPH : cytochrome P450 reductase was detected (data not shown), loss of ZM 10309 was not linear and did not conform to Michaelis-Menten kinetics.

7.3.3. DNA damage in the presence of ZM 81853 and NADPH : cytochrome P450 reductase

From chemical inhibition studies (Chapter 6) and those detailed above, ZM 81853 appears to be metabolised predominantly by the one-electron reducing enzyme NADPH : cytochrome P450 reductase under hypoxic conditions. In addition, ZM 81853 was shown

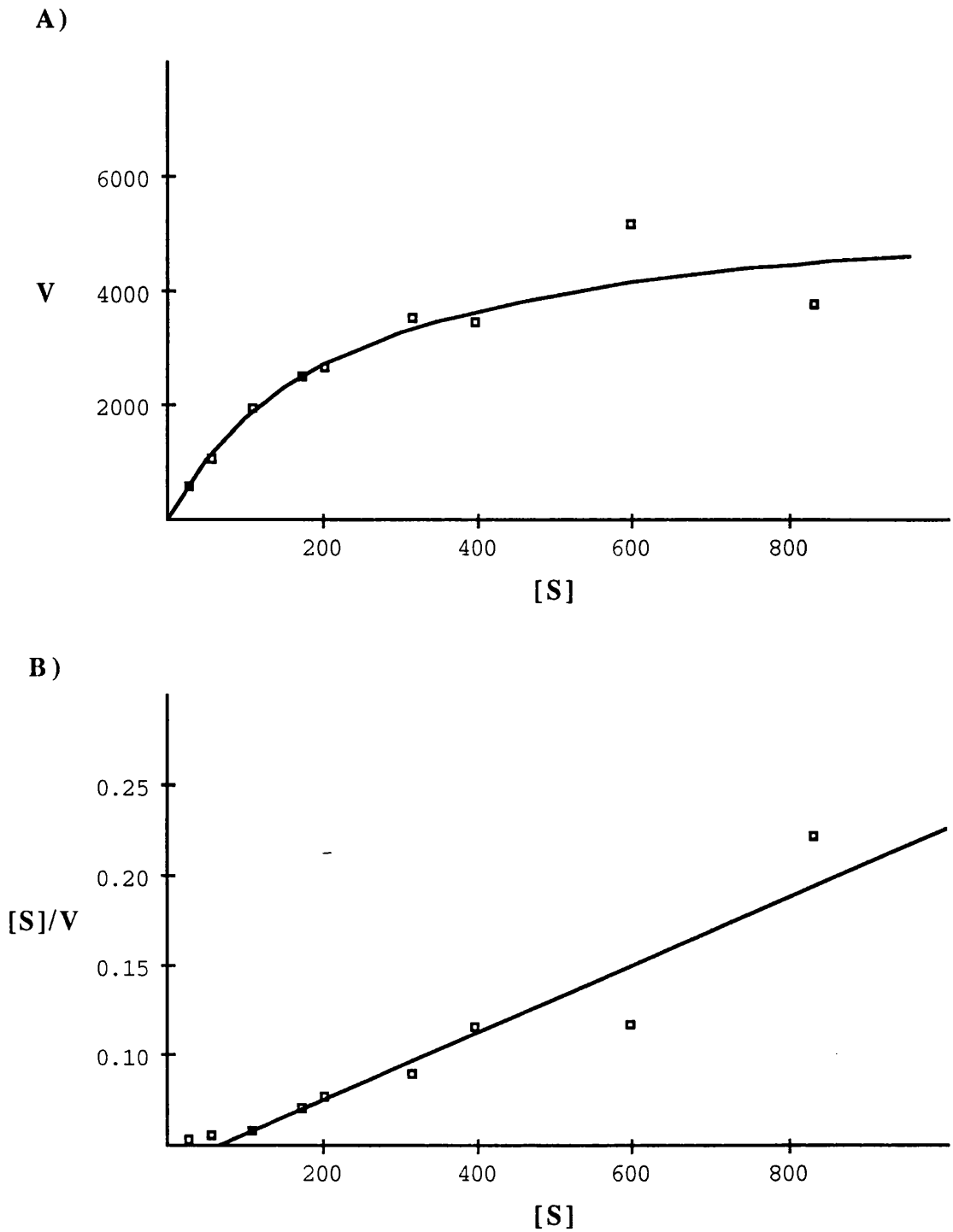


Figure 7.3 Typical plot of *V* versus *S* (A) and corresponding Hanes-Woolf linear transformation plot (B) for ZM 81853 metabolism by purified, recombinant human NADPH : cytochrome P450 reductase.

Units of *S* and *V* are μM and nmol of ZM 81853 lost/min/mg protein, respectively.

Parameter	Non-linear regression	Hanes-Woolf
K*m* (μM)	289.8 ± 61.8	241.8 ± 42.8
V*max* (nmol/min/mg)	5825.4 ± 397.0	5264.4 ± 708.7
K*cat* (/s)	7.6 ± 0.5	6.8 ± 0.9
K*cat* / K*m* (/μM s)	0.03 ± 0.01	0.03 ± 0
V*max* / K*m* ratio	20.1	21.8

Table 7.3 Michaelis-Menten parameters for ZM 81853 loss following metabolism by purified, recombinant human NADPH : cytochrome P450 reductase *in vitro*.

Values were obtained from three independent experiments, each with nine substrate concentrations. K*m* and V*max* values represent a mean ± SD, n=3.

to exhibit similar cytotoxic characteristics as tirapazamine in each of the cell lines studied (Chapter 4). In particular, this was the case in the cell line possessing the highest NADPH : cytochrome P450 reductase activity. Tirapazamine has been shown previously to cause DNA damage when activated by purified rat and human preparations of NADPH : cytochrome P450 reductase (Fitzsimmons *et al.*, 1994; Walker *et al.*, 1994). Similar studies were undertaken with ZM 81853 to determine whether this compound could also be activated by human NADPH : cytochrome P450 reductase to a DNA damaging species under hypoxic conditions.

Initially, the extent of DNA damage in the presence of increasing concentrations of ZM 81853 (0 - 600 μM) was investigated. These concentrations were selected as they were in the range used for kinetic analysis. Figure 7.4 demonstrates the results of such an experiment. With the exception of one result (Figure 7.4, lane 10), DNA damage was seen to become more extensive with increasing ZM 81853 concentration (Figure 7.4, lanes 4 - 11). No sample was detected in the case of 300 μM ZM 81853 (Figure 7.4, lane 10), possibly due to lack of movement from the loading well. DNA damage was seen, however, in a repeat experiment. Interestingly, DNA damage was not complete even at the highest concentration of ZM 81853 (600 μM).

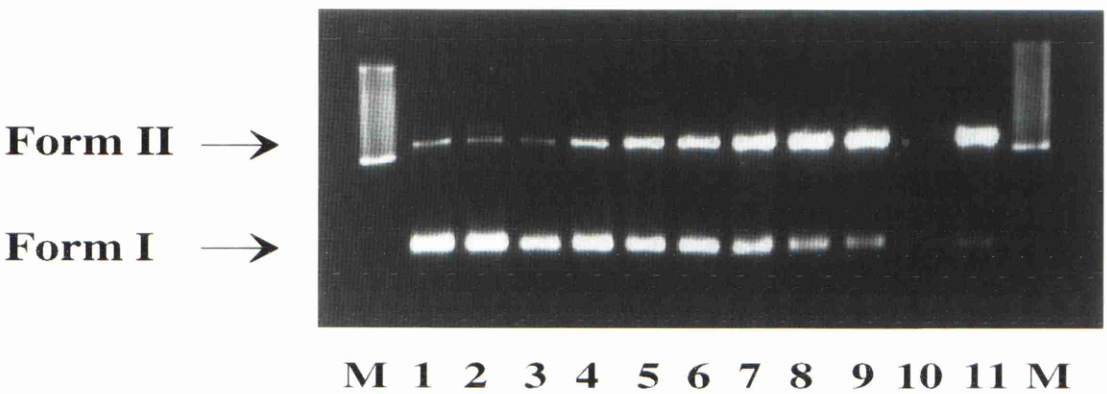
Although NADPH : cytochrome P450 reductase had been used above at an activity of 40 mU (the concentration used in kinetic analysis), the ability of ZM 81853 to be metabolised to a DNA damaging species in the presence of a range of enzyme concentrations was investigated. Figure 7.5 shows the ability of NADPH : cytochrome P450 reductase to cause conversion of ZM 81853 to a DNA damaging species at a variety of different concentrations (0.039 - 2.5 mU per assay). Considerable damage was observed at enzyme concentrations as low as 0.3 mU. A repeat experiment which further extended the range of enzyme concentrations used to 60 mU (not shown) confirmed that at concentrations of 1.25 mU and above, DNA damage was considerable, i.e. approximately 80% conversion.

Finally, the possible influence of oxygen on the ability of ZM 81853 to be metabolised to a DNA damaging species was investigated. Conditions used for the assay,

Figure 7.4 **(A) Dose-dependent effect of increasing ZM 81853 concentration on DNA damage induced in plasmid pBR322 DNA by purified, recombinant human NADPH : cytochrome P450 reductase and (B) the corresponding densitometric analysis [open bar, supercoiled plasmid DNA (form I); hatched bar, relaxed, circular plasmid DNA (form II)].**

Lanes 1 - 3 are controls: (1) plasmid DNA (1000 ng) alone, (2) DNA with enzyme (40 mU) and 600 μM ZM 81853 (no cofactor), (3) DNA with 600 μM ZM 81853 and NADPH (no enzyme). Lanes 4 - 11 show the effect of increasing concentrations of ZM 81853 (0, 9.4, 18.8, 37.5, 75, 150, 300 and 600 μM) in the complete system. Molecular weight markers are present on the extreme left and right of the gel. Results represent one of two repeat experiments.

A)


Form II
Form I
M 1 2 3 4 5 6 7 8 9 10 11 M

B)

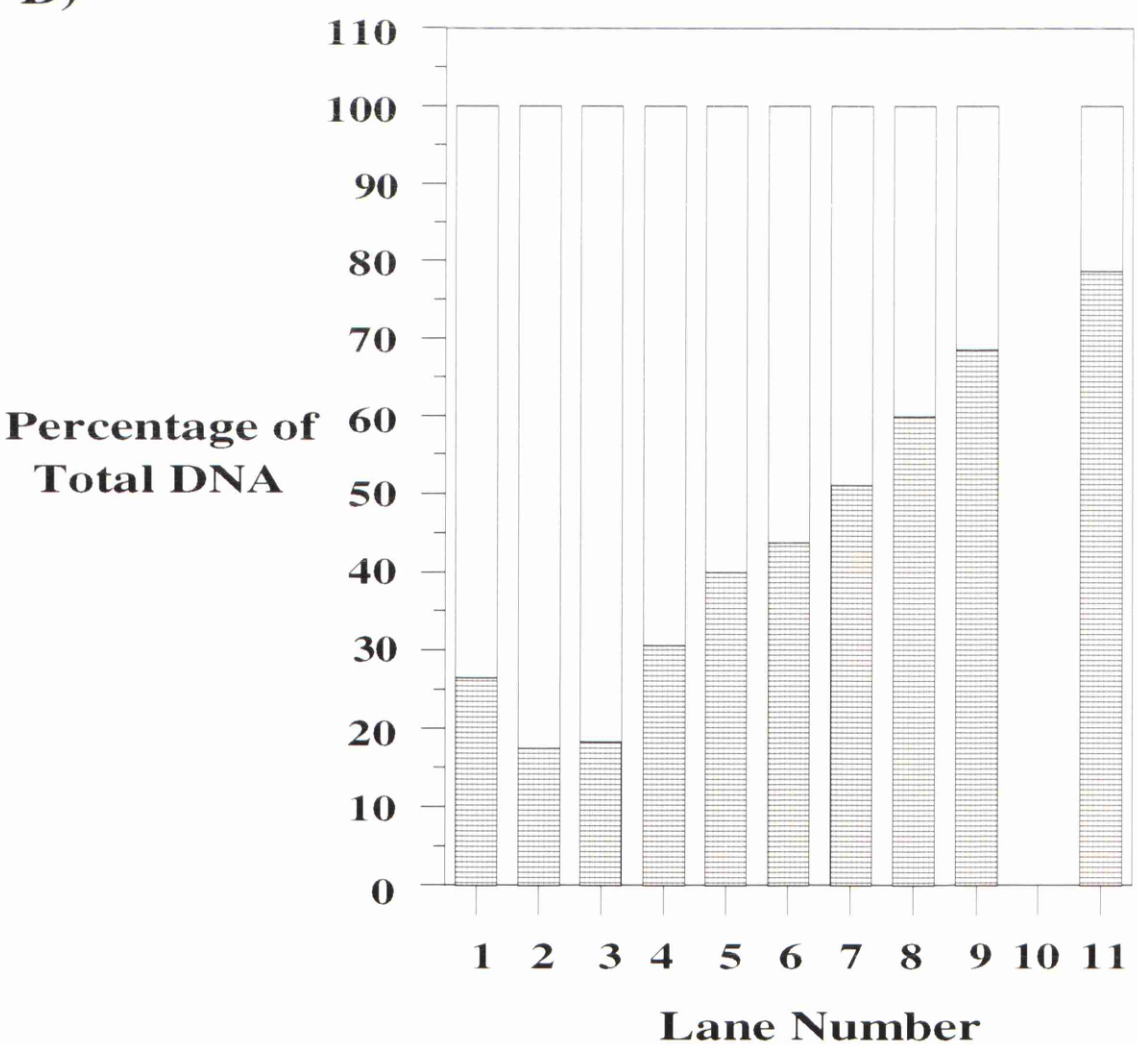
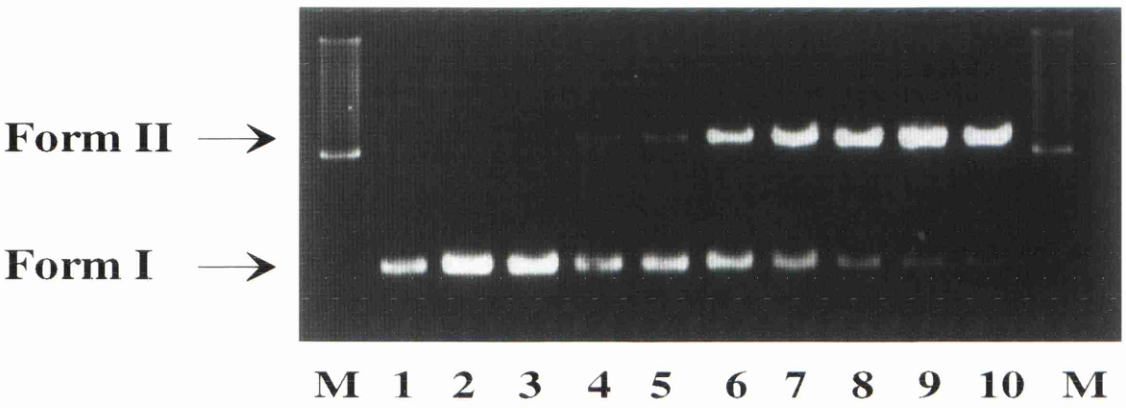

Percentage of Total DNA
110
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11
Lane Number

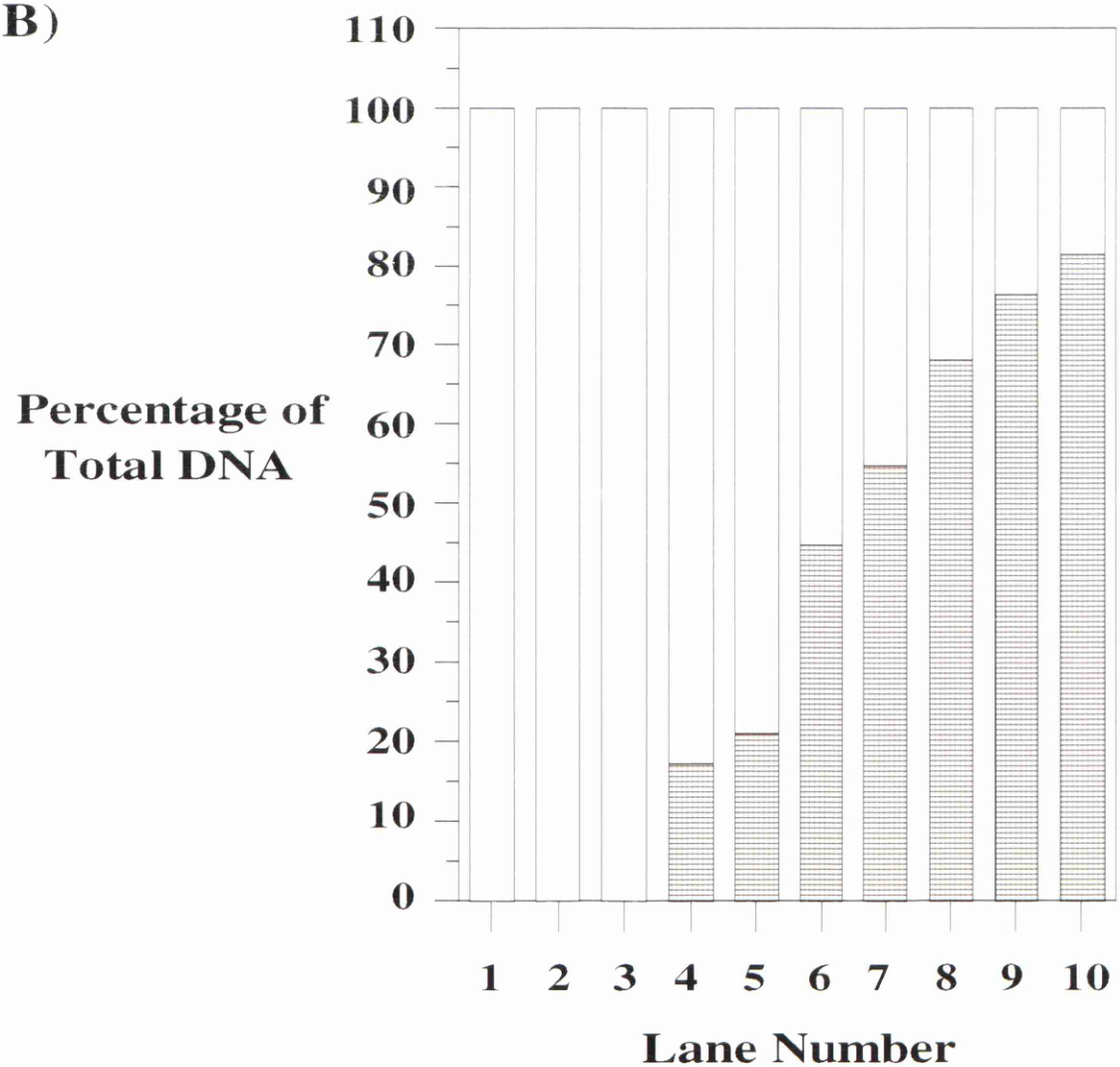
Figure 7.5 **(A) Effect of increasing enzyme concentration on DNA damage induced in plasmid pBR322 DNA by purified, recombinant human NADPH : cytochrome P450 reductase and (B) the corresponding densitometric analysis [open bar, supercoiled plasmid DNA (form I); hatched bar, relaxed, circular plasmid DNA (form II)].**

Lanes 1 - 3 are controls: (1) plasmid DNA (1000 ng) alone, (2) DNA with enzyme (2.5 mU) and 300 μM ZM 81853 (no cofactor), (3) DNA with ZM 81853 and NADPH (no enzyme). Lanes 4 - 11 show the effect of increasing concentrations of NADPH : cytochrome P450 reductase (0.039, 0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 mU) in the complete system. Molecular weight markers are present on the extreme left and right of the gel. Results represent one of two repeat experiments.

A)


Form II
Form I
M 1 2 3 4 5 6 7 8 9 10 M

B)


Percentage of Total DNA
110
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10
Lane Number

i.e. ZM 81853 concentration and NADPH : cytochrome P450 reductase concentration, were those used previously in the kinetic studies, and shown to produce DNA damage (experiments above). Figure 7.6 shows the effect of hypoxic and oxic conditions, respectively, on the damage induced in plasmid DNA following metabolism of ZM 81853. Various control incubations were performed under hypoxic (Figure 7.6, Lanes 1 - 8, see figure legend) and oxic conditions (not shown). The effect of increasing concentrations of SOD and catalase on the level of DNA damage under hypoxic conditions is shown in lanes 9 - 11, while damage in the absence of SOD and catalase is shown in lane 12. The same incubations performed under oxic conditions are shown in lanes 13 - 16. Both the gel (Figure 7.6A) and the densitometry data (Figure 7.6B) indicated that the background level of relaxed, circular DNA (Form II) was not significantly altered under different control conditions (lanes 1 - 8). This background level did not rise above approximately 35% of total DNA. Similar observations were made under oxic conditions. Clearly, this basal level of relaxed, circular DNA was present in the original preparation of plasmid DNA.

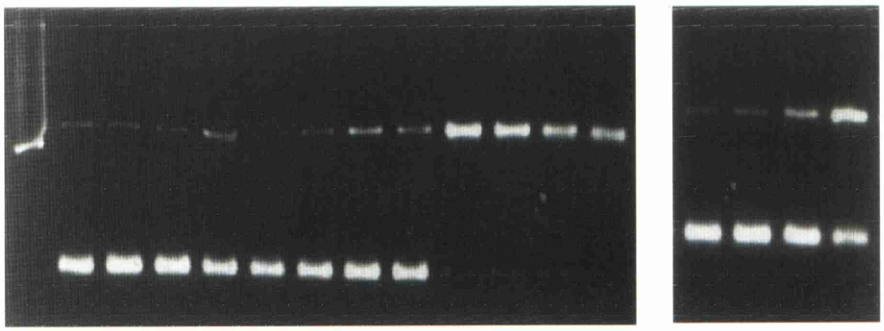
DNA damage occurred under both hypoxic and oxic conditions (lanes 12 and 16, respectively). In both cases, metabolism of ZM 81853 by NADPH : cytochrome P450 reductase resulted in conversion of the supercoiled plasmid DNA (Form I) to the relaxed, circular form (Form II), indicative of single strand breaks (Figure 7.6, lanes 12 and 16). No evidence for the occurrence of double strand breaks was seen. Importantly, however, the level of conversion under hypoxic conditions was approximately 60% higher than under oxic conditions (Figure 7.6B, lanes 12 and 16). DNA damage in the presence of SOD and catalase was reduced by approximately 20 - 30% under hypoxic conditions (Figure 7.6, lanes 9 -11). In contrast, however, the presence of SOD and catalase under oxic conditions reduced the level of DNA damage observed by approximately 50% (Figure 7.6A and B, lanes 13 - 16).

Although ZM 10309 had been demonstrated to be metabolised by mouse liver enzymes (Chapter 6), the level of metabolism was significantly lower than for ZM 81853.

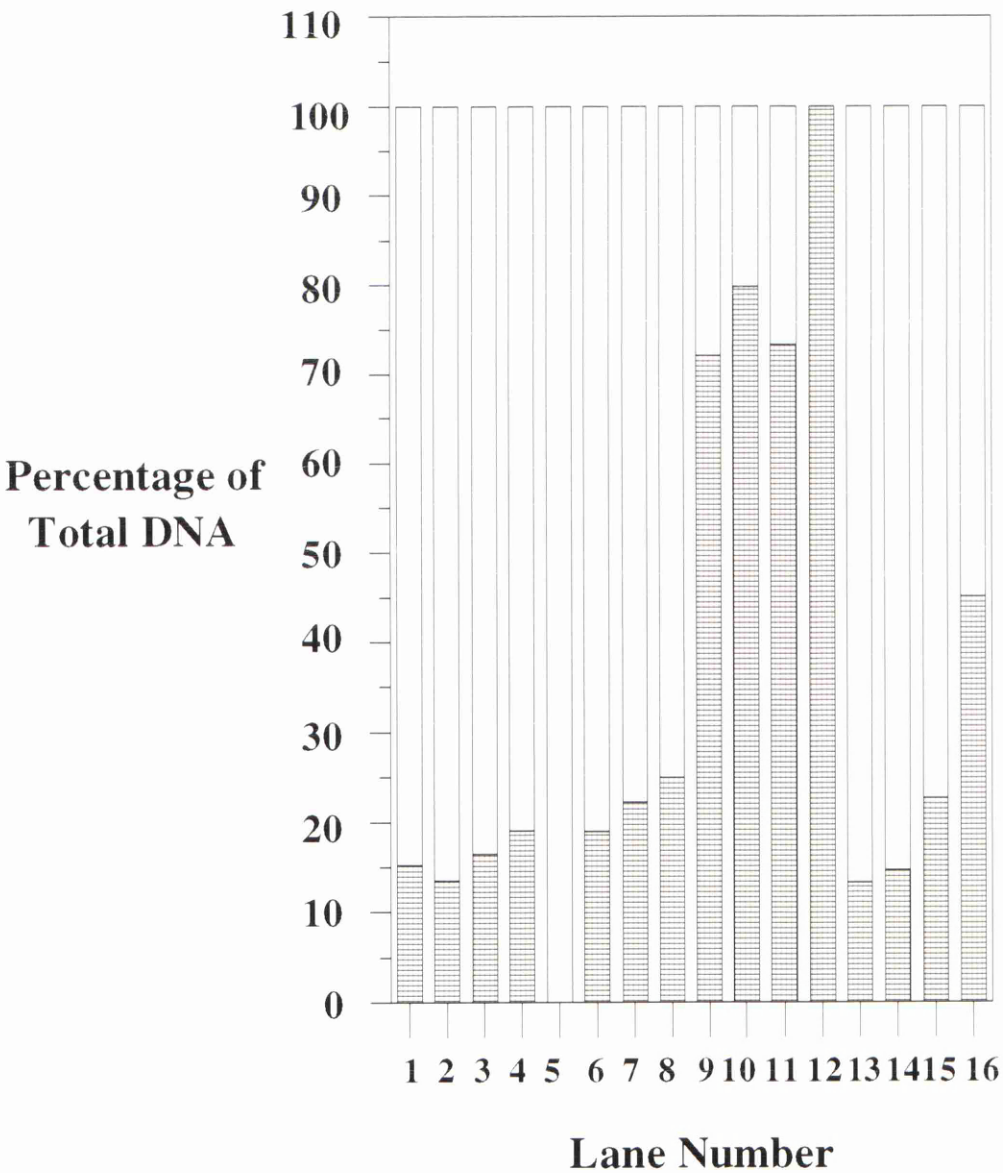
Figure 7.6 **(A) Effect of hypoxic versus oxic conditions on the damage induced in plasmid pBR322 DNA by the reduction of ZM 81853 by purified, recombinant human NADPH : cytochrome P450 reductase and (B) the corresponding densitometric analysis [open bar, supercoiled plasmid DNA (form I); hatched bar, relaxed, circular plasmid DNA (form II)].**

Lanes 1 - 12 represent hypoxic incubations. Lanes 1 - 8 are controls: (1) plasmid DNA (1000 ng) alone, (2) DNA with enzyme (40 mU), (3) DNA with ZM 81853 (300 μM), (4) DNA with NADPH (1 mM), (5) DNA with enzyme and ZM 81853 (no cofactor), (6) DNA with ZM 81853 and NADPH (no enzyme), (7) DNA with enzyme and NADPH (no ZM 81853) and (8) DNA with boiled enzyme, ZM 81853 and cofactor. Lanes 9 - 11 show the effect of increasing concentrations of SOD and catalase (150 - 600 μg/ml) on the DNA damage in the complete system. Lane 12 shows the complete incubation reaction in he absence of SOD and catalase. Lanes 13 - 16 represent oxic incubations performed within the same experiment but samples were run on a separate gel. The effect of increasing SOD and catalase concentrations on DNA damage in the complete system are shown in lanes 13 - 15. Lane 16 shows the complete incubation reaction in the absence of SOD and catalase. Molecular weight markers are present on the extreme left of the gel. Results represent one of two repeat experiments.

A)


Form II
Form I
M 1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16

B)


Percentage of Total DNA
110
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Lane Number

For this reason, and also due to a limited availability of ZM 10309, DNA damage assays were not performed using this compound.

7.4 Discussion

ZM 81853 metabolism under hypoxic conditions has been demonstrated previously in the presence of mouse liver enzymes (Chapter 6). In order to establish the efficiency of bioreductive metabolism by particular enzyme preparations, kinetic analysis can be performed to determine the apparent affinity (K*m*) and also the rate at which metabolism occurs (V*max*). Several studies have previously used enzyme kinetics as a means of investigating the association between metabolism of bioreductive agents and particular enzymes ; CB 1954 (Boland *et al.*, 1991); EO9 (Walton *et al.*, 1991); Tirapazamine (Fitzsimmons *et al.*, 1994; Walton & Workman, 1990); mitomycin C (Beall *et al.* , 1994a). In this current chapter, the kinetics of ZM 81853 metabolism by both DEX-induced mouse liver microsomes and cytosol have also been studied. The results suggest that although ZM 81853 may have a lower K*m* for cytosolic compared to microsomal enzymes (K*m* 130.4 μM and 374.3 μM, respectively), the speed of the reaction (V*max*) was, in fact, considerably slower (15.2 compared with 170.9 nmol/min/mg protein). The ratio V*max* / K*m* also indicated that ZM 81853 may be a better substrate for microsomal enzymes. To further investigate the possible role of NADPH : cytochrome P450 reductase in metabolism, and indeed, activation of ZM 81853 under hypoxic conditions, kinetic analysis and DNA damage assays in the presence of a purified, recombinant human form of the enzyme were performed. ZM 81853 was shown to be a good substrate for the enzyme (K*m* 241.8 μM and V*max* 5264.4 nmol/min/mg protein) and, perhaps even more importantly, was also shown to be metabolised to a DNA damaging species under hypoxic conditions.

In this present study, the kinetics of ZM 81853 loss in the presence of DEX-induced mouse liver enzymes was investigated. ZM 81853 had previously been shown to be metabolised to a greater extent by microsomal as opposed to cytosolic enzymes (Chapter 6). This finding was further supported by the fact that the rate of reaction (V*max*) in the presence of microsomal enzymes was significantly higher than with cytosolic enzymes (approximately 11-fold, $p<0.001$). Interestingly, the K*m* of ZM 81853

for microsomal enzymes was also significantly higher (approximately 3-fold, $p<0.001$). This may indicate a weaker interaction between the enzyme(s) and the substrate. The ratio V*max* / K*m* also indicated that ZM 81853 may act as a better substrate for microsomal enzymes compared to cytosolic enzymes. Previous studies (Sugiura *et al.*, 1976) involving several *N*-oxides have shown that compounds such as imipramine *N*-oxide, tiaramide *N*-oxide and *N*, *N*-dimethylaniline *N*-oxide undergo reduction by rat liver microsomes. K*m* and V*max* values for these compounds were shown to be in the range 0.2 - 0.4 mM and 2.5 - 3.2 nmol/min/mg protein. It is of interest, therefore, that although K*m* of the novel *N*-oxide ZM 81853 for mouse microsomal enzymes was higher than that obtained for cytosolic enzymes, it was in fact comparable with earlier studies of *N*-oxides (Sugiura *et al.*, 1976). In addition, the rate of reduction was approximately 60-fold higher in the case of ZM 81853 (V*max* 170 compared to 2.9 nmol/min./mg protein), suggesting that this compound acts as a better substrate for reduction by microsomal enzymes. It is important to note, however, that the experiments by Sugiura *et al* (Sugiura *et al.* , 1976) were performed using microsomes isolated from different rodent species. It has been noted previously that differences in enzyme levels may be present as a result of species variation (Boobis *et al.* , 1990). More recently, Walton and colleagues (Walton & Workman, 1990) have reported K*m* and V*max* values in the range of 1.1 - 1.7 mM and 790 - 1100 nmol SR 4233 lost/min/mg protein, respectively for metabolism of tirapazamine by mouse liver microsomes. Clearly, the rate of reduction of tirapazamine is much greater (approximately 11-fold) than for ZM 81853, although the K*m* value suggested that the interaction with microsomal enzymes may be higher in the latter case. Overall, however, tirapazamine appeared to be the better substrate for microsomal enzymes when compared to ZM 81853 as shown by the significantly higher ratio V*max* / K*m* (675 compared to 0.49).

From the present studies, therefore, ZM 81853 appears to be a better substrate for microsomal enzymes than the tertiary N-oxides, imipramine *N*-oxide, tiaramide *N*-oxide and *N*, *N*-dimethylaniline *N*-oxide, but, tirapazamine appeared to be significantly better again as a substrate for these enzymes.

A role for both NADPH : cytochrome P450 reductase and NADPH : cytochrome P450s has been postulated for tirapazamine (Walton & Workman, 1990). Current studies suggest that a similar situation occurs in the case of ZM 81853 (Chapter 6). In contrast, however, only NADPH : cytochrome P450 enzymes have been shown to be responsible for metabolism of the tertiary *N*-oxides in the presence of rat microsomal protein (Sugiura *et al.*, 1976). NADPH : cytochrome P450 enzymes are known to be dependent upon the presence of NADPH : cytochrome P450 reductase for their metabolic activity (Peterson & Prough, 1986). The enhanced reduction efficiency observed for ZM 81853, and indeed for tirapazamine, may be a result of both a direct and an indirect role for NADPH : cytochrome P450 reductase in the metabolism of these compounds. The ability of NADPH : cytochrome P450 reductase to act directly on ZM 81853 was investigated with the use of a purified enzyme preparation. Similar studies were not performed with NADPH : cytochrome P450s or indeed, for carbonyl reductase, however, for two reasons ; firstly, no purified enzyme preparations were available for use, and secondly, the role of these enzymes in the metabolism of ZM 81853 was shown to be only minimal (Chapter 6).

To further investigate the direct involvement of NADPH : cytochrome P450 reductase in the reductive metabolism of ZM 81853, kinetic analysis in the presence of purified, human NADPH : cytochrome P450 reductase was performed. Studies using enzymes purified from both rat and human livers have been previously reported for tirapazamine, and have confirmed a direct role for the involvement of NADPH : cytochrome P450 reductase in its reductive metabolism (Fitzsimmons *et al.*, 1994; Walker *et al.*, 1994). In this current study, metabolism of ZM 81853 was investigated in the presence of a purified, recombinant form of human NADPH : cytochrome P450 reductase. Comparisons between different purified enzyme forms have been made and show that they act in a similar manner (Lewis, A.D., personal communication). In this particular study, ZM 81853 was shown to be a good substrate for purified NADPH : cytochrome P450 reductase with an apparent K*m* and V*max* of 241.8 μM and 5264.4 nmol of ZM 81853 lost/min/mg protein, respectively. The predominant involvement of

NADPH : cytochrome P450 reductase, as indicated by chemical inhibition studies (Chapter 6), was supported further by the fact that the K*m* values of 374.3 and 241.8 μM for microsomes and purified enzyme, respectively, were similar. As expected, the rate of reduction of ZM 81853 by purified NADPH : cytochrome P450 reductase was at least 30-fold higher than that found in the presence of microsomal enzymes (5264.4 and 170.9 nmol/min/mg protein respectively), once again emphasising an important and direct role for this enzyme in reduction. The V*max* / K*m* ratios, in this case, were 20.1 and 21.8 dependent upon which kinetic plot was used, suggesting, as expected, that ZM 81853 was a better substrate for the purified enzyme preparation. K*cat* (the enzyme turnover number reflecting the absolute rate of ZM 81853 loss to the enzyme / substrate complex) was determined and shown to be in the range 6.8 - 7.6/s, dependent upon the analysis used. In addition, the parameter K*cat*/K*m*, which takes into consideration non-saturable conditions, was also determined and shown to be 0.03/μM/s. Previous studies with tirapazamine (Fitzsimmons *et al.*, 1994) reported a K*cat* /K*m* value in the range of 5.56 - 6.54/mM/s for WIN 64012 formation following tirapazamine reduction by purified rat NADPH : cytochrome P450 reductase. It is important to remember, however, that the latter studies were performed in the presence of purified rat enzyme, and that K*cat*/K*m* was in fact representative of metabolite formation, not compound loss. These two differences may account for the fact that ZM 81853 appeared to be the better substrate for NADPH : cytochrome P450 reductase.

Whilst a direct role for human NADPH : cytochrome P450 reductase in the metabolism of ZM 81853 has now been demonstrated in this chapter, the precise effect of reduction by this enzyme or indeed other one-electron-reducing enzymes within the cell remains unknown. Earlier cytotoxicity studies using a panel of human breast tumour cell lines revealed that ZM 81853 was capable of causing a high degree of selective toxicity under hypoxic conditions (Chapter 4). The selectivity of ZM 81853 was comparable with that observed for tirapazamine. The mechanism of action and hence, the observed cytotoxicity, for tirapazamine has been postulated to be through one-electron reduction to form a nitroxide free radical (Lloyd *et al.*, 1991). This radical is believed to exert its

effect by DNA damage via hydrogen abstraction from sugar residues. DNA damage arising from the reduction of tirapazamine in the presence of several one-electron-reducing enzymes has been demonstrated (Fitzsimmons *et al.*, 1994; Laderoute *et al.* , 1988; Walker *et al.*, 1994). Due to the similarities between tirapazamine and ZM 81853 in both cytotoxic effects (Chapter 4) and reduction in the presence of purified NADPH : cytochrome P450 reductase (this Chapter) it was thought likely that the novel compound, ZM 81853, would undergo a similar mechanism of action as tirapazamine. To investigate this further, DNA damage assays were performed. Under hypoxic conditions, ZM 81853 was metabolised by purified human NADPH : cytochrome P450 reductase to a DNA damaging species capable of causing strand breaks in plasmid DNA. Complete conversion from the supercoiled plasmid DNA (Form I) to the relaxed, circular conformation (Form II) occurred, a result which was indicative of single strand breaks. DNA damage, however, was reduced by 20 - 30% in the presence of SOD and catalase. As SOD and catalase are known to interfere with the action of oxygen radicals (Fisher & Gutierrez, 1991), this result suggested that incomplete expulsion of air had occurred during the experiment. If this had been the case, back-oxidation to ZM 81853 from the reduction product could have occurred with the production of oxygen radicals as a by-product. This is similar to the explanation postulated for a similar effect shown with tirapazamine (Fitzsimmons *et al.*, 1994). The remaining damage (70 - 80%), however, was not affected by the presence of SOD and catalase and therefore may have been caused by the formation of radicals such as nitroxides, as shown for tirapazamine (Fitzsimmons *et al.*, 1994; Lloyd *et al.*, 1991). Further studies involving, for example, electron spin resonance (ESR), would be required in order to identify the exact nature of any such radical. In contrast to the damage seen under hypoxic conditions, the degree of damage under oxic conditions was much lower (approximately 40% conversion to the relaxed, circular configuration). The presence of SOD and catalase, however, reduced the level of damage still further, resulting in only 10 - 20% conversion of the DNA to the relaxed, circular configuration (Form II). This finding suggested that oxygen radicals such as superoxide are involved in the DNA damage observed under oxic conditions. The

oxygen radicals would form by back-oxidation from the reduction product. Importantly, however, complete inhibition by SOD and catalase was not achieved, thereby implying that other radicals such as nitroxides play a role in DNA damage even under oxic conditions. Similar conclusions were made from the studies on tirapazamine in the presence of purified rat and human NADPH : cytochrome P450 reductase (Fitzsimmons *et al.*, 1994; Walker *et al.*, 1994). Importantly, ZM 81853 did not produce double strand breaks in the DNA even at high enzyme and drug concentrations, unlike tirapazamine (Fitzsimmons *et al.*, 1994), suggesting that overall, the DNA damage observed in the presence of ZM 81853 was less extensive.

The results of this present chapter support the earlier finding that NADPH : cytochrome P450 reductase played a role in the reductive metabolism of ZM 81853. Furthermore, reduction in the presence of this enzyme was shown to result in single strand breaks in plasmid DNA. Important to the therapeutic action of these compounds, NADPH : cytochrome P450 reductase has been identified in many human tumour cell lines (Chapter 2) and normal cells from breast, skin and prostate tissues (Baron *et al.* , 1983). In addition, NADPH : cytochrome P450 reductase activity within human glioma biopsies (Rampling *et al.*, 1994 and within this thesis) has been demonstrated. Also of potential importance is the fact that nuclear localisation of NADPH : cytochrome P450 reductase has been demonstrated in rat liver (Cahill & White, 1990), thereby enabling activation of ZM 81853 even closer to the DNA than would be the case for cytosol or microsomal reduction.

In summary, reduction of ZM 81853 by purified, recombinant human NADPH : cytochrome P450 reductase was demonstrated, although it was found to be less efficient than that of tirapazamine. Importantly, however, reduction of ZM 81853 resulted in the production of a DNA damaging species. A possible mechanism of action for ZM 81853 may, therefore, be via one-electron reduction to form a nitroxide radical product which is capable of causing DNA damage. Such a pathway has already been proposed for tirapazamine (see Chapter 1, Figure 1.5). Further studies would be required to determine

whether a radical species is formed on ZM 81853 one-electron reduction, and whether this is the species responsible for causing the DNA damage observed.

CHAPTER 8

GENERAL DISCUSSION

The aims of this thesis which were initially set out at the beginning of the project have been achieved. The aims were :

(i) To measure the level of expression and activity of reductase enzymes within a panel of human tumour cell lines derived from various tumour tissue origins.

(ii) To determine whether this information could be used within the National Cancer Institute database to establish correlations between different enzyme activities and sensitivities towards both existing and novel anticancer agents.

(iii) To establish the potential of three structurally distinct and novel compounds to act as hypoxia-selective bioreductive anticancer agents.

(iv) To extend the above study to determine structure-activity effects of a small series of quinoxaline di-*N*-oxides.

(v) To investigate the enzymology and DNA damaging capacity of any of the above compounds that were shown to act as possible hypoxia-selective bioreductive anticancer agents.

The results of this thesis contribute towards a better understanding of the enzymology of human tumour cells and also towards the future application of an enzyme-directed approach to bioreductive drug development.

The attainment of information regarding enzyme expression within human tumours has been proposed as an important factor for the future success of an enzyme-directed approach to anticancer drug development (Workman, 1994; Workman & Walton, 1990). While expression of a number of enzymes have been reported (see for example, Baron *et al.* , 1983; Cresteil & Jaiswal, 1991; Schlager & Powis, 1990), these studies have involved only limited numbers of tumour types. In an attempt to improve the current situation, this thesis has investigated enzyme expression in over 60 human

tumour cell lines in a collaborative project between the NCI, EORTC and the U.K. CRC (Chapter 2). Heterogeneity of expression was demonstrated both within and between cell lines from different origins. Importantly, however, clear patterns of expression within different tumour cell origins emerged. This was particularly pronounced in the case of DT-diaphorase (see Chapter 2, Section 2.3.1 for more details). The results are generally consistent with previous findings using smaller numbers of human tumour cell lines. For example high DT-diaphorase activity was seen in lung and colon cell lines (Patterson *et al.*, 1994; Plumb *et al.*, 1994a; Plumb *et al.*, 1994b; Robertson *et al.*, 1992; Robertson *et al.*, 1994; Smitskamp-Wilms *et al.*, 1994). The establishment of such patterns from the analysis of human tumour cell lines could prove to be extremely valuable in forming the platform for successful targeting of anticancer agents to specific tumour types based upon the enzymology of the tumour (and, of course, nature of the drug).

A possible problem, however, is how applicable such measurements are to the *in vivo* situation. This issue was addressed, to a limited degree in this thesis, by comparing enzyme activities in human tumour cell lines to previously published data involving tumour biopsies (for a more detailed discussion see Chapter 2, Section 2.4). Enzyme activities such as DT-diaphorase were observed to be lower in tumour samples (Rampling *et al.*, 1994; Schlager & Powis, 1990) compared to cell lines. Interestingly, and importantly for therapeutic action, normal tissues have been reported to have lower enzyme activities than tumour tissues (Schlager & Powis, 1990). A plausible explanation, therefore, is that contamination of tumour biopsies by normal tissue, i.e. stroma, can occur upon surgical removal, thereby resulting in underestimation of enzyme activity within the biopsy sample. This may also explain the findings of de Wazier and colleagues (de Waziers *et al.*, 1991) who reported no change in DT-diaphorase activity between tumoural and normal tissues. The use of human tumour cell lines essentially rules out this problem. As a result, they provide an invaluable tool with which to study the enzymology of many different tumour histiotypes.

The NCI annually screens approximately 20,000 compounds of distinct chemical structure in the aforementioned panel of *in vitro* human tumour cell lines (Boyd & Paull,

1995). It has been proposed that incorporation of relevant biochemical and molecular parameters into the database would provide additional means of identifying mechanisms of action of potential anticancer agents (Boyd & Paull, 1995). Such a proposal was tested in this thesis by the incorporation of enzyme activities into the database, and subsequent statistical analysis (in collaboration with Dr. K. Paull, NCI) to search for correlations between enzyme activity and sensitivity towards the many anticancer agents already screened through the NCI programme. Several interesting correlations between DT-diaphorase activity and cell sensitivity were discovered in the analysis (see Chapter 3, Sections 3.3 and 3.4 for more details), including those of the quinone-containing compound streptonigrin, and the clinical agent 5-FU. Perhaps the most significant finding of this study with regards to the controversy in the current literature was the clear correlation shown between sensitivity towards mitomycin C and DT-diaphorase activity across the human tumour cell line panel. A great deal of controversy has surrounded the role of this enzyme in mitomycin C activation (ERoss *et al.* , 1994 and references therein), in particular concerning a lack of correlation between the aforementioned parameters (Robertson *et al.*, 1992). This current study strongly supports a role for DT-diaphorase in the activation of mitomycin C under aerobic conditions. Furthermore it also provided a means of establishing in more detail those individual tumour types which were either sensitive or resistant to different bioreductive agents. In the case of EO9, for example, cell lines high in DT-diaphorase activity such as lung and colon were more sensitive towards EO9 than those low in activity such as leukaemia and lymphoma cell lines (Chapter 3; Figure 3.3). Whilst this result was not surprising considering the known involvement of DT-diaphorase in the activation of EO9 under oxic conditions (Plumb *et al.*, 1994a), it demonstrated that the incorporation of enzyme activities into the NCI database as an additional biochemical parameter proved to be an invaluable resource with which to study mechanisms of action of potential new and existing anticancer agents. Interestingly, this study current study revealed no significant correlations involving either NADPH : cytochrome P450 reductase or NADH : cytochrome b5 reductase. Whilst this thesis has focused on only

three enzymes, further studies to determine the activities of other important bioreductive enzymes, for example, carbonyl reductase and xanthine oxidase / dehydrogenase, would significantly increase the potential of such an application in the NCI database. In light of recent reports concerning the role of one-electron reducing enzymes such as NADPH : cytochrome P450 reductase in the activation of EO9 under hypoxic conditions (Plumb & Workman, 1994; Plumb *et al.*, 1994b), the incorporation into the NCI database of cytotoxicity data under hypoxic conditions would also prove to be extremely valuable. This would of course apply to all other potential bioreductive anticancer agents.

As discussed in the introduction to this thesis, a number of bioreductive anticancer agents have been, or are currently, undergoing clinical trial. However, muscle cramping and dose-limiting toxicity have proved problematic during their progress (Doherty *et al.*, 1994; Schellens *et al.*, 1994). In an attempt to identify more potent and hypoxia-selective agents for use in an 'enzyme-directed' bioreductive programme (Workman, 1994; Workman & Walton, 1990), the potential of three groups of novel compounds, with diverse molecular structures, to act as hypoxia-selective anticancer agents was investigated in this thesis (Chapter 4). These particular compounds were selected in conjunction with ZENECA Pharmaceuticals, due to their structural similarities towards some of the more active agents (see Chapter 4, Figure 4.1 for structures) currently in development. The NCI routinely use a large panel of *in vitro* human tumour cell lines as an initial screen for potential anticancer agents. The knowledge gained by determining enzyme activity levels within the NCI panel was put to further use by selecting a small panel of cell lines, based upon their enzyme activity levels, with which to screen the novel compounds under investigation. As both one- and two-electron reducing enzymes are known to be important in the metabolism of bioreductive anticancer agents (Chapter 1), this was seen as a means of predicting possible mechanisms of action of the compounds. Neither the nitroheterocyclic agent, ZM 29362, nor the nitroacridine, ZM 33191, exhibited the potential to act as selective bioreductive agents. Interestingly, however, ZM 33191 was at least as potent as the indoloquinone bioreductive agent EO9 under oxic conditions. Activation of EO9 by DT-

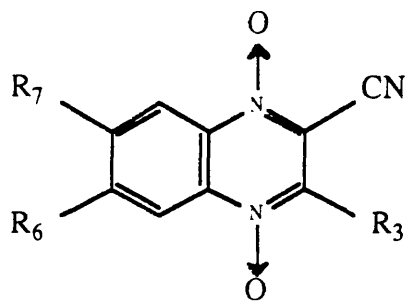
diaphorase under oxic conditions is known to result in DNA alkylation (Bailey *et al.*, 1994b; Maliepaard *et al.*, 1995); therefore, it is possible that ZM 33191 acted in a similar manner. Such a mechanism of action was further supported by the fact that DNA adduct formation has also been proposed as the mechanism of action, under oxic conditions, of the nitroacridine, nitracrine (1-NC) which is similar in structure to ZM 33191 (Konopa *et al.*, 1983; Pawlak *et al.*, 1983). While ZM 33191 may still have a future as an anticancer agent under oxic conditions, Denny and colleagues (Denny *et al.*, 1992; Siim *et al.*, 1994; Wilson *et al.*, 1992) are currently developing new analogs of 1-NC in an attempt to identify structures which display more hypoxic-selectivity.

A more detailed study involving a small series of structurally-related quinoxaline di-*N*-oxides revealed that ZM 81853 was the only compound with the potential to act as a bioreductive anticancer agent, as it exhibited similar hypoxic-selectivity to the benzotriazine di-*N*-oxide, tirapazamine, a bioreductive compound currently in clinical trial. To further investigate the metabolic properties of these quinoxaline compounds, sensitive HPLC methods were developed to allow detection of each compound (Chapter 5). Successful assays were established for all of the compounds concerned. Further similarities between ZM-81853 and tirapazamine were revealed in subsequent studies (Chapters 6 and 7) as ZM 81853 was metabolised by the one-electron reducing enzyme NADPH : cytochrome P450 reductase to a species capable of causing single strand DNA damage, under hypoxic conditions. Although a four-electron reduced product (identified in collaboration with Dr. D. Watson, University of Strathclyde, U.K.) was detected by HPLC analysis as the predominant metabolite following incubation in the presence of mouse liver enzymes, it is unclear at this stage whether this was, in fact, the DNA damaging, and hence, cytotoxic species. Further studies involving isolation of this product, and subsequent cytotoxicity and DNA damage experiments would determine whether this was the case. As ZM 81853 is likely to undergo an one-electron reduction under hypoxic conditions, the most likely metabolic pathway for the compound would involve four sequential one-electron reductions resulting, ultimately, in production of the four-electron reduced product. Due to the similarities in metabolic properties of

tirapazamine and ZM 81853, it is possible that the mechanism of action of the latter compound under hypoxic conditions would be similar to that of tirapazamine (Lloyd *et al.* , 1991). Hence, DNA damage would arise from the action of a toxic radical species. Certainly, DNA damage experiments ruled out a possible influence of oxygen radicals (Chapter 7, Section 7.3.3.). Importantly, studies such as those previously performed for tirapazamine (Lloyd *et al.*, 1991) would identify whether a radical species is involved, and if so, determine the nature of that radical.

As mentioned previously, ZM 81853 was the only compound from a small series of quinoxaline di-*N*-oxides to demonstrate the potential to act as a bioreductive anticancer agent. Unfortunately, however, this compound did not appear to be any more potent or hypoxia-selective than the benzotriazine di-*N*-oxide, tirapazamine, which is currently in Phase II clinical trial in combination with cisplatin and shortly seeking approval for Phase III clinical trial. These findings were consistent with a more detailed study, at ZENECA Pharmaceuticals, involving 15 structurally-related 2-cyano-1, 4-quinoxaline di-*N*-oxides including ZM 81853 (Monge *et al.* , 1995). Importantly, however, this latter study revealed that substitution of a strong electron withdrawing group (Cl, F, CF_3) in the R7 position (Figure 8.1) increased the overall activity of the compounds e.g. IC_{90} 0.3 - 15 μM; HCR up to 340 compared to tirapazamine. In addition, preliminary *in vivo* data suggested that these compounds may have greater therapeutic benefit over tirapazamine. Clearly, therefore, further studies involving selected compounds from this quinoxaline di-*N*-oxide series are warranted and may provide a potential bioreductive agent with greater potency and hypoxic-selectivity over tirapazamine.

The development of effective bioreductive anticancer agents is an important concept with regards to overcoming the problem of resistant hypoxic cells within solid tumours. One of the principle problems arising during clinical trials, however, has been the dose-limiting toxicity caused by these agents (see Chapter 1 for more details). This frequently arises from a lack of specificity towards hypoxic cells. In the case of EO9, for example, the reversible kidney toxicity observed during Phase I clinical trial (Schellens



R3	R6	R7	P (μM)	HCR	MTD (mmol/kg)
NH_2	H	H	30	80	-
$NH(CH_2)_3N(CH_3)_2$	H	Cl	0.4	250	0.055
$NH(CH_2)_3N(CH_3)_2$	H	F	0.6	170	0.07
$NH(CH_2)_3N(CH_3)_2$	H	CH_3	0.3	340	<0.05
$NHCOCH_3$	H	Cl	15	50	-
tirapazamine	-	-	30	75	0.3

Figure 8.1 Characteristics of a series of novel quinoxaline di-*N*-oxides compared to tirapazamine.

P=dose for 1% V-79 survival in N_2; HCR=ratio of equitoxic doses in air/nitrogen; MTD=maximum tolerated single dose (mice).

[taken from Monge *et al*, 1995]

et al., 1994) maybe attributed to the aerobic metabolism of the drug by DT-diaphorase, which is known to be present at high levels within normal kidney tissue (Schlager & Powis, 1990). In order to improve the long-term prospects of bioreductive anticancer agents, specificity of action must therefore be improved. The aforementioned study involving quinoxaline di-*N*-oxides may prove to be important, therefore, in the future development of more specific agents.

Denny (Denny, 1995) recently proposed that the design of agents with narrower ranges of reduction potentials would improve the specificity of activation of bioreductive anticancer agents. It was also proposed that the development of enzyme-specific bioreductive agents, based upon current knowledge regarding enzyme expression within both normal and tumour tissues would be important. An exciting new concept introduced quite recently, is that of antibody-directed enzyme prodrug therapy (ADEPT) (Anlezark *et al.*, 1992; Bagshawe, 1987; Bagshawe *et al.*, 1994; Knox *et al.*, 1993). This approach involves the conjugation of drug reducing enzymes to a tumour-selective monoclonal antibody and subsequent specific localisation of this complex to the tumour site. Local activation of an inactive prodrug would then occur at the tumour site. This process is particularly promising with regard to the use of non endogenous enzymes, as the whole process would then be extremely specific towards the tumour of interest. One promising example is that of the monofunctional alkylating agent CB 1954, which has previously been shown to be a much better substrate for rat DT-diaphorase (isolated from rat Walker tumour cells) than for human DT-diaphorase (Boland *et al.*, 1991). Conjugation of the rat form of DT-diaphorase, or indeed the more active nitroreductase purified from Escherichia coli (Anlezark *et al.*, 1992), to a tumour-selective antibody could improve the potential of CB 1954 to act as an anticancer agent, by creating an extremely tumour specific reaction. Studies are currently underway involving the tumour selective antibody A5B7 (Knox *et al.*, 1993). Furthermore, this technique also has the potential for improving the bioreductive activity of different agents by conjugating enzymes of interest to hypoxic regions of tumours. Although promising, however, potential problems may arise with ensuring localisation of the

enzyme to the tumour site in sufficient quantities, and also location of the inactive prodrug to the tumour site. Similar proposals have also been introduced involving gene-directed enzyme prodrug therapy (GDEPT), involving the specific insertion of a gene of a particular enzyme into the tumour site.

To date, bioreductive anticancer agents have been developed with the view to overcoming the problem of hypoxic cells within solid tumours via production of toxic species. This treatment usually takes place alongside that of chemotherapy or radiotherapy in order to kill both the oxic and hypoxic fractions of the tumour. A further exciting area in bioreductive drug development, however, has been the introduction of hypoxia-selective prodrugs of diffusible cytotoxins (HPDCs) (Denny & Wilson, 1993). In addition to killing hypoxic cells upon activation, these agents also release cytotoxins into the surrounding oxic cells and cause cell death. Two such agents, namely SN 25246, a nitrobenzyl mustard quaternary salt, and SN 24771, a cobalt-nitrogen mustard complex have both been shown, *in vitro*, to exhibit some potential as HPDCs (Denny *et al.*, 1994; Wilson *et al.*, 1994).

In summary, this thesis has increased awareness as regards to the importance of understanding enzyme expression within human tumour cell lines and perhaps, therefore, more importantly, within human tumours. In addition, a small series of structurally distinct compounds has been evaluated for their potential to act as bioreductive anticancer agents. Unfortunately, although the quinoxaline di-*N*-oxide ZM 81853 showed similar *in vitro* characteristics (HCR's in cytotoxic studies, and metabolic activity in the presence of different enzyme preparations) as the benzotriazine di-*N*-oxide tirapazamine, it did not appear to have the potential to be any better than tirapazamine as a bioreductive agents. More detailed studies within ZENECA Pharmaceuticals, however, indicated that several compounds within a larger series of the quinoxaline di-*N*-oxides (Monge *et al.*, 1995) could prove to be better bioreductive anticancer agents than tirapazamine (Figure 8.1). Overall, however, it is believed that the future for bioreductive anticancer agents must be dependent upon the ability to improve

both selectivity and potency of the agents, using mechanisms such as ADEPT and GDEPT, for example .

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APPENDIX

LIST OF PUBLICATIONS ARISING FROM THIS THESIS

Full Papers

(1). Rampling, R., Cruickshank, G., Lewis, A. D., **Hemingway, S. A.** and Workman, P. Direct Measurement of pO_2 distribution and bioreductive enzymes in human malignant brain tumours. *International Journal of Radiation, Oncology, Biology and Physics,* **29**, 427-431, 1994.

(2). **Fitzsimmons, S. A.**, Workman, P., Grever, M., Paull, K., Camalier, R. and Lewis, A. D. Differential reductase enzyme expression across the NCI tumor cell line panel : Correlation with sensitivity to mitomycin C and EO9. *Journal of the National Cancer Institute,* (in press), 1995.

Published Abstracts

(1). **Fitzsimmons, S. A.,** Workman, P., Grever, M., Paull, K., and Lewis, A. D. Differential expression of DT-diaphorase, cytochrome P450 reductase and cytochrome b_5 reductase in the NCI human tumour cell line panel. *Proceedings of the American Association of Cancer Research,* **35**, 369, 1994.

(2). Paull, K., Camalier, R., **Fitzsimmons, S. A.,** Lewis, A. D., Workman, P., Grever, M. Correlations of DT-diaphorase expression with cell sensitivity data obtained from the NCI human tumour cell line panel. *Proceedings of the American Association of Cancer Research,* **35**, 369, 1994.

(3). **Fitzsimmons, S. A.**, Paull, K., Workman, P., Camalier, R. and Lewis, A. D. Relationship between DT-diaphorase, cytochrome P450 reductase and cytochrome b_5 reductase enzyme activities and cell sensitivity towards various compounds within the NCI drug screening panel. *British Journal of Cancer*, **71**, 42, 1995.

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● *Manipulation of Tumor Oxygen and Physiology*

DIRECT MEASUREMENT OF pO_2 DISTRIBUTION AND BIOREDUCTIVE ENZYMES IN HUMAN MALIGNANT BRAIN TUMORS

ROY RAMPLING, PH.D., MBBS,* GARTH CRUICKSHANK, PH.D., M.D.,† ALEXANDER D. LEWIS, PH.D.,‡ SARA A. FITZSIMMONS, BSC.‡ AND PAUL WORKMAN, PH.D.‡

*Beatson Oncology Centre, Western Infirmary, Glasgow G11 6NT; †Institute of Neurological Science, Southern General Hospital, Glasgow; and ‡CRC Dept. of Medical and Radiation Oncology, Glasgow, Scotland, UK

Purpose: To measure the oxygen status of human malignant brain tumors *in vivo* and to determine the activities and expression of bioreductive enzymes in these same human brain tumor samples, as a means of assessing their suitability as targets for bioreductive drug therapy.

Methods and Materials: A polarographic oxygen electrode was used to measure the intratumoral oxygen tension in twenty patients with malignant brain tumors during open brain surgery, performed under standard anaesthetic conditions. Six different tracks, each with a path length of 22 mm, were recorded per patient representing 192 readings. Following pO_2 measurements the tumors were resected and stored in liquid N_2 for subsequent bioreductive enzyme analysis. Eight human malignant brain tumors were assessed, by enzyme activity and western blot expression, for the presence of various bioreductive enzymes. These enzymes included DT-diaphorase, NADH cytochrome b5 reductase, and NADPH cytochrome P-450 reductase. Of these eight gliomas analyzed six samples were incubated with the bioreductive drug tirapazamine, in the presence of cofactor(s), to establish whether human brain tumors could metabolize this compound.

Results: Both the high grade intrinsic and metastatic brain tumors showed significant regions of hypoxia. All the tumors subjected to *enzyme profiling* contained the bioreductive enzymes, DT-diaphorase, NADH cytochrome b5 reductase and NADPH cytochrome P-450 reductase. Also all six of the brain tumors investigated could metabolize tirapazamine to the two-electron reduction product.

Conclusion: These findings would favor primary brain tumors as suitable targets for bioreductive therapy.

Human brain tumors, Glioma, Tirapazamine (WIN 59075, SR 4233), Enzyme profiling, Bioreductive enzymes, DT-diaphorase, NADPH cytochrome P-450 reductase, NADH cytochrome b5, Reduction of tirapazamine, Bioreductive therapy, pO_2 distribution, Hypoxia.

INTRODUCTION

Bioreductive drug activation depends on the oxygen status and enzyme concentration-activity profile of the target tissue. In selecting tumors as potential targets for such drugs it is important to know that these parameters exist within a particular tumor to determine whether the drug is likely to be metabolized to its active form.

Direct measurements of partial oxygen pressure (pO_2) using a tube type sensor have suggested that hypoxia exists within brain tumors but the spatial resolution of the technique is limited (6). Indirect measurements of oxygen status have yielded conflicting results with the presence of hypoxic areas being questioned (3). No direct measurements of intratumoral pO_2 have been reported using micro electrode techniques. Our aim was to use such methods to determine the profile of pO_2 in the micro environment of brain tumors. The demonstration of significant regions of hypoxia inside these tumors would recommend brain tumors as potential targets for bioreductive drugs provided that the enzymes involved in the bioreductive drug activation were present.

Tirapazamine (SR 4233; WIN 59075 or 1,2,4-benzotriazine 1,4-di-*N*-oxide) is a lead bioreductive compound which is currently undergoing Phase I clinical trial in Stanford, Harvard and Glasgow Universities. Interestingly, this drug has been shown to be metabolized by a variety of enzymes as indicated in Figure 1. These enzymes include the one-electron reducing enzymes NADH cytochrome b5 reductase, xanthine oxidase, and NADPH

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Reprint requests to: Roy Rampling.

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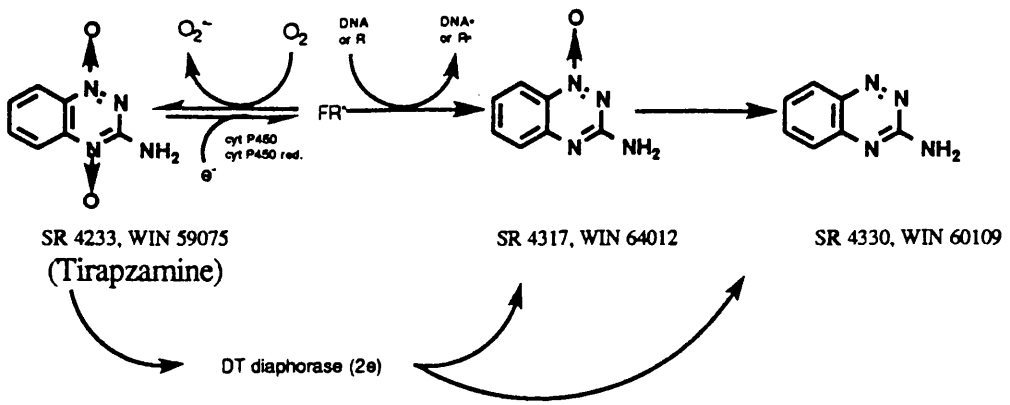

Fig. 1. Scheme for tirapazamine (WIN 59075; SR4233) reduction.

cytochrome P-450 reductase, and also the two-electron reducing enzyme DT-diaphorase which bypasses the reactive drug radical intermediate (15, 17). In addition to hypoxia, therefore, these enzymes must be present within the tumor to activate the drug to the therapeutic species.

As a consequence, the purpose of this study was to investigate both the degree of hypoxia which exists within human brain malignancies and also, on resection of these tumors, the presence of enzymes which can reduce bioreductive agents—a concept we describe as enzyme profiling (17). The ability of these human brain tumors directly to metabolize the bioreductive drug tirapazamine was also studied.

METHODS AND MATERIALS

Oxygen measurements

The entire study was approved by the ethical committee of the Institute of Neurological Sciences, Glasgow, UK. All patients were given verbal and written information concerning the study and gave their written consent prior to participation. Twenty patients with a clinical and radiological diagnosis of malignant brain tumor (primary or secondary) were entered into the study. All patients were receiving corticosteroids, two had undergone previous craniotomy and one had also received prior radiotherapy. All patients underwent anaesthetic with preoxygenation and alfentanil combined with atricurium bolus injection. Subsequent maintenance was with isofluorane (0.5–1.0%) or propofol infusion (0.5–1.0 mg/Kg/h). Halothane was specifically avoided. Controlled ventilation was used with a fractional inspired oxygen maintained between 0.4–0.5 with the arterial pCO_2 kept between 3.5–4.5 KPa.

The patients head was held in a fixator[1] to reduce movement. A self-adhesive silver chloride anode was positioned on cleaned skin, usually over the mastoid process. The oxygen probe[2] was then carefully calibrated. During this period a craniotomy was performed and the dura opened. A 10-min equilibration period was allowed after which samples were removed for blood gas analysis and the pO_2 readings were begun.

Tissue oxygen tension (pO_2) was measured using a 300 μm needle electrode[2] containing a 12 μm concentric O_2-sensitive, membrane covered, gold wire cathode held at −700 mV relative to an indifferent Ag/AgCl anode. Oxygen reduced at the cathode resulted in a current directly proportional to the pO_2. Using a microprocessor controlled micromanipulator, multiple spatially separate measures of pO_2 were collected automatically over a step length of 700 μm (1.0 mm advance, 0.3 mm backstep). The electrode was inserted through the open craniotomy under direct vision. Normally six tracks, each with a total path length of 22 mm, were recorded per patient representing 192 readings. After each track the probe returned automatically to the starting position. It was then reangled a few degrees and a new track begun. A 6 MHz, 16 mm ultrasound probe was used to locate the margins of the tumor and confirm the needle trajectory. The pO_2 values were plotted automatically as frequency histograms with a intervals of 2.5 mmHg. Following the procedure the probe was recalibrated.

Following all the pO_2 measurements, the tumor including all areas sampled by the oxygen probe was resected. In some instances pathologic samples were preserved in liquid nitrogen for subsequent bioreductive enzyme analysis. Postmeasurement intraoperative and postoperative management proceeded as normal and no complications relating to the pO_2 measurements were encountered.

[1] Patients heads were restrained in a Mayfield fixator during pO_2 measurements.

[2] The polarographic oxygen electrode micromanipulator and microprocessor were supplied by Eppendorf Ltd., Hamburg, Germany.

Bioreductive enzyme measurements and metabolism of tirapazamine

Resected tissue was homogenized as described by Adams *et al.* (1) with the S9 fraction prepared by differential centrifugation. Levels of bioreductive enzymes such as DT-diaphorase, NADH cytochrome b5 reductase, and NADPH cytochrome P-450 reductase were determined using a modification of the cytochrome *c* reduction method (11). Confirmation of the expression of the DT-diaphorase and NADPH cytochrome P-450 reductase enzymes in the human malignant glioma tumor samples was carried out by western blot analysis as described by Lewis *et al.* (7). The DT-diaphorase and NADPH cytochrome P-450 reductase antibodies used in the western blot analysis were kindly provided by Dr. R. Knox, ICR, Sutton, UK and Dr. C. R. Wolf, ICRF, Dundee, UK, respectively. Selected tumor homogenates (3 mg/ml) including the murine EMT 6 tumor were incubated with 1.5 mM tirapazamine (WIN 59075; SR 4233) and 10 mM NAD(P)H in 50 mM Tris HCl pH 7.5 for 0–30 min at 37°C. Reduction of the drug to metabolite(s) were quantified by HPLC analysis (10).

RESULTS

Oxygen measurements in human brain malignancies

The pO_2 values determined in 20 human brain tumors were presented as frequency histograms. This form of presentation is illustrated in Figure 2 for the pooled data from the ten patients with glioblastomas. The mean, median, and 90th percentiles from the individual histograms were used to describe the distribution. The results for all 20 patients are shown in Table 1 together with the tumor histology and an approximate measure of their sizes,

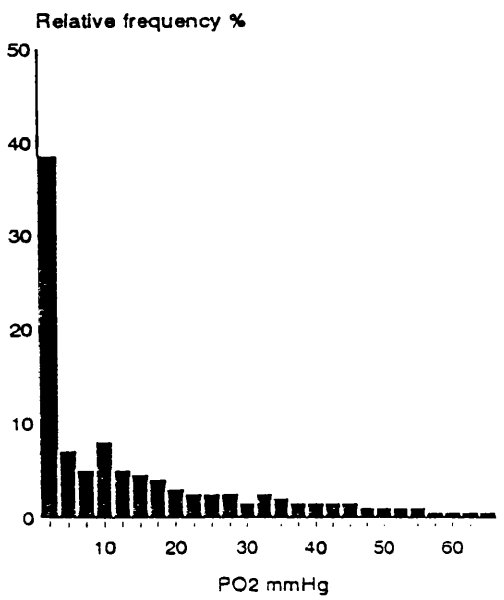

Fig. 2. Histogram of pO_2 incidence in ten patients with glioblastoma. Pooled data; N = 10, n = 2486, median value 7.4 mmHg.

Table 1. Intratumoral tissue oxygen tension results for human malignant brain tumors

Patient no.	Tumor pathology	Max dim* (mm)	Median pO_2† (mmHg)	% pO_2 values < 2.5† mmHg
1	Glioblastoma	36	24.3	9.5
2	Glioblastoma	46	3.2	43.7
3	Glioblastoma	32	19.3	22.5
4	Glioblastoma	34	5.4	35.5
5	Glioblastoma	64	2.5	56.7
6	Glioblastoma	37	4.5	47.0
7	Glioblastoma	45	0.1	67.2
8	Glioblastoma	63	5.4	39.5
9	Glioblastoma	38	7.7	30.5
10	Glioblastoma	37	0.1	68.5
11	Anaplastic	30	10.0	19.5
12	Anaplastic	45	42.3	9.0
13	Anaplastic	38	5.0	41.5
14	Intermediate	39	3.7	22.5
15	Low grade	41	6.4	0
16	Bronchus	43	14.1	1.5
17	Bronchus	46	3.0	29.5
18	Breast	36	4.3	46.5
19	Breast	35	4.5	32.5
20	Melanoma	30	23.7	20.0

* Max Dim (mm) is an approximate measure of the tumor size estimated by CT scan.

† Measurement of pO_2 distribution was determined as described in the Methods and Materials section. The median pO_2 values and the number of pO_2 values less than 2.5 mmHg were calculated from histogram plots. A typical example of a plot is shown in Figure 2.

which were estimated from CT scan images. Presented also in this Table is a measure of the number of pO_2 readings for each tumor recorded to be less than 2.5 mmHg, a level of pO_2 which is thought to be radiobiologically important.

The pooled median pO_2 value for the ten patients with glioblastoma was determined to be 7.4 mmHg. In this tumor type, a high percentage (28%) of low pO_2 values were recorded indicating a radiobiologically significant hypoxic component in all glioblastomas studied. By definition anaplastic (WHO Grade 3) astrocytomas do not contain regions of necrosis. Interestingly, however, in our study these also show hypoxic regions, with up to 41.5% of recorded values in individual tumors being under 2.5 mmHg. Noticably, the median pO_2 values were generally higher in this small series than for the glioblastomas. Of obvious interest was the single low grade tumor which, when measured by this method, recorded no pO_2 values < 2.5 mmHg. This is in contrast to the mixed group of metastatic tumors which demonstrated marked hypoxia. Taken together, these results confirm the expected pattern of increasing hypoxia with increasing tumor aggressiveness and rapidity of growth. Other interesting clinical information obtained from this study included the lack of correlation between the percentage of low pO_2 values and the patient age, sex, tumor position, size, the arterial pO_2, or pCO_2.

Bioreductive enzyme expression in human brain malignancies

Eight human malignant brain tumors were assessed for bioreductive enzyme activity. The results of this study are shown in Table 2. All the tumors investigated expressed DT-diaphorase, NADH cytochrome b5 reductase and NADPH cytochrome P-450 reductase activity. The levels between the tumor samples varied, however. An 18-fold, 10-fold, and 8-fold range in DT-diaphorase, NADH cytochrome b5 reductase, and NADPH cytochrome P-450 reductase activity, respectively, was measured between the tumor samples. Interestingly, the low grade glioma had the highest overall bioreductive enzyme content. This high level of expression of bioreductive enzymes, in particular DT-diaphorase and NADPH cytochrome P-450 reductase, was confirmed in the brain tumor samples by western blot analysis (data not shown). To investigate whether the human malignant brain tumors could metabolize bioreductive drugs, six of the brain tumors were incubated with the bioreductive agent tirapazamine (WIN 59075; SR 4233) in the presence of cofactor. The results of this experiment are shown in Table 3. All the brain tumors investigated could metabolize tirapazamine to the 2-electron reduced product (WIN 64012; SR 4317). The murine tumor EMT 6, which was used as a control in this experiment, gave a value for the rate of formation of WIN 64012 of 5.56 nmol/min/mg protein.

DISCUSSION

It has long been recognized that the presence of hypoxia in tumors can modify their response to therapy. Traditionally, hypoxia has been considered a disadvantage to be overcome. However if agents are used that are selectively cytotoxic to hypoxic cells, then their presence in a tumor may be seen as an advantage to be exploited. Tirapazamine is such an agent which, by undergoing a one-electron reduction in a hypoxic environment, forms cytotoxic free radicals (17). These radicals, if formed close enough to the cellular DNA, can cause strand breaks and potentially cell death. The early clinical development of this drug is now almost complete. Further development, will require the identification of suitable targets, that is, tumors in which there is a sizeable hypoxic fraction and an appropriate enzyme complement. This is known as enzyme profiling of tumors (17).

Since Thomlinson and Gray (12) postulated the presence of hypoxic cells in human tumors many attempts have been made to measure the pO_2 directly. The use of direct electrode methods began with animal tumor model work in the 1970s (2, 13) but it was not until the development of the polarographic micro-electrode that any sustained program of work was undertaken to determine, by direct measurement, the pO_2 distribution in human tumors (14). This work has demonstrated the existence of hypoxic regions in cancers of the breast and cervix. Furthermore, there have been suggestions that the hypoxia correlates with response to treatment with radiation (9).

There has been one previous attempt directly to measure the pO_2 in human brain tumors (6). In this study, a single measurement was made using a 700 μm Clarke electrode. In 12 malignant gliomas the average pO_2 was found to be 15.3 ± 2.3 mmHg (range, 5–38). This figure was much lower than that measured in normal brain and demonstrated relative hypoxia in the tumor tissue. However, this method had a slow response time (90 s) and did not provide any spatial information. Our results give a similar average figure, but show that at a microscopic level the pO_2 varies dramatically within the tumor with many values falling below 2.5 mmHg. This is a level of hypoxia which is significant for the activation of bioreductive agents, and also, at which the tissue is likely to demonstrate hypoxic radio-resistance. Heterogeneity of pO_2 levels was also a feature of measurements reported by Kallinowski in both animal model systems and in human breast and

Table 2. Bioreductive enzyme activities measured in human malignant brain tumors

Tumor pathology	Enzyme activity (nmol/min/mg) DT-diaphorase	Cytochrome b5 reductase	Cytochrome P-45 reductase
Glioblastoma	21.1	21.1	9.9
Sarcoma	57.1	87.8	19.3
Low grade glioma	182.9	180.0	39.7
Glioblastoma	25.3	16.1	7.7
Glioblastoma	10.1	23.6	12.8
Glioblastoma	27.0	30.7	5.3
Glioblastoma	114.1	32.1	42.9
Glioblastoma	24.5	20.4	15.3

Note: The enzyme activity measurements for DT-diaphorase, NADH cytochrome b5 reductase and NADPH cytochrome P-450 reductase were carried out as described in Methods and Materials. The results presented are the average of at least two measurements.

Table 3. Rate of metabolism of Tirapazamine (WIN 59075; SR 4233) by human malignant brain tumors

Tumor pathology	Tumor number	Rate of WIN 64012 formation (nmol/min/mg)
Sarcoma	T3	2.56
Low grade glioma	T5	2.09
Glioblastoma	T7	1.06
Glioblastoma	T8	0.84
Glioblastoma	T9	1.21
Glioblastoma	T14	1.10

Note: The human brain tumor samples were incubated with tirapazamine as described in Methods and Materials. The rate of formation of WIN 64012 (SR 4317) was found to be linear with time and quantified by HPLC as described in Methods and Materials.

cervix tumors *in situ* (5). This heterogeneity is likely to comprise both spatial and temporal components, as has been discussed by Chaplin (4).

Hypoxia arising within tumors can offer the potential of a novel approach to therapy, using bioreductive drug therapy (4, 17). The levels of hypoxia required for drug activation are similar to those at which cells develop hypoxic radioresistance, that is, ~ 0–10 mmHg. Hypoxia of this order has been demonstrated in all but one of the intracranial tumors investigated in this study. In particular, the high grade gliomas show particularly large hypoxic components which may well contribute to their known radioresistance. Therapeutic strategies directed specifically at viable and possibly clonogenic hypoxic cells using bioreductive therapy may well prove beneficial.

Tirapazamine has been shown to have considerable anti-tumor activity under hypoxic conditions in animal model and *in vitro* systems (8). While the oxygen profiles measured in this study suggest that high grade gliomas may be a good target for bioreductive therapy, the appropriate enzyme profile must also exist within the tumor. In this study, we have demonstrated that bioreductive enzymes are present within the human brain tumor malignancies and these tumors can also reduce the drug presumably via the therapeutic drug radical. Interestingly, this reduction was calculated to be at ~ 50% of that of the murine EMT 6 tumor, a tumor which is sensitive to tirapazamine.

Data in mice have shown that tirapazamine can cross the blood–brain barrier to produce high levels within the brain (16). As a consequence, all the conditions appear to be met which allow access and appropriate metabolism of the drug. All patients with high grade gliomas will die from their disease. Median survival without treatment is less than 6 months. Radiation alone may double this survival, but the prognosis is still short and patients are never cured. If the hypoxia demonstrated in this study contributes to the radioresistance of these tumors then a combination of tirapazamine with radiation may improve the survival prospects for this difficult group of patients.

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Differential reductase enzyme expression across the NCI tumor cell line panel: Correlation with sensitivity to mitomycin C and EO9.

Sara A. Fitzsimmons[1], Paul Workman[1,3], Michael Grever[2,4], Kenneth Paull[2], Richard Camalier[2], Alexander D. Lewis[1]

[1] Cancer Research Campaign Beatson Laboratories, CRC Department of Medical Oncology, University of Glasgow, Alexander Stone Building, Garscube Estate, Switchback Road, Bearsden, Glasgow, G61 1BD, Scotland, U.K.

[2] Information Technology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892, U.S.A.

[3] Present Address, proofs and correspondence to : Dr Paul Workman,
ZENECA Pharmaceuticals,
Cancer Research Department,
Mereside, Alderley Park,
Macclesfield, Cheshire, U. K.
SK10 4JG.
Telephone : 44-1625-513938
Fax : 44-1625-513624

[4] Present Address : Oncolgy Center, No. 3-127
John Hopkins University,
Hematologic malignancies,
600 N. Wolfe Street,
Baltimore
MD 2187-8985

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ABSTRACT

Background: Numerous enzymes metabolize anticancer agents, including bioreductive drugs. Tumor enzyme expression may affect treatment outcome aswell as influence new drug devlopment strategies but we have only limited information on drug metabolizing enzyme levels in human tumors and cell lines.

Purpose: 1) To characterize systematically the expression of three drug metabolizing reductase enzymes across the different histiotypes in the NCI human tumor cell line panel. 2) To correlate the results with sensitvity to the established bioreductive agent mitomycin C and the related indoloquinione EO9, currently in phase II clinical trial with the EORTC.

Methods: 69 human tumor cell lines from 9 histiotypes were assayed for DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase using cytochrome c reduction methodology. Where appropriate, protein expression was determined by Western blot and mRNA by reverse-transcriptase polymerase chain reaction. *In vitro* antitumor activity was determined under aerobic conditions using sulforhodamine B to measure cell mass. The relationship between enzyme activities and drug sensitivity (GI_{50}) was assessed by the COMPARE analysis, with significance tested by the Pearson Correlation Coefficient.

Results : Compared to DT-diaphorase activities which were generally high, cytochrome P450 and b5 reductase were both relatively low across the panel. Levels

of all three varied both between and within histiotypes, but clear patterns did emerge. For example, DT-diaphorase was relatively low in leukemic, ovarian and breast lines but high in non-small cell lung, colon, CNS and melanoma. In several instances expression patterns agreed with data reported for clinical tumor material. Activities of cytochrome P450 and b5 reductase did not correlate with chemosensitvity, whereas a clear relationship was seen between DT-diaphorase levels and response to both EO9 and mitomycin C (Pearson Correlation Coefficient = 0.446, $p \leq 0.0013$ for EO9, and 0.424, $p \leq 0.0005$ respectively).

Conclusions: Bioreductive enzyme expression is heterogeneous across human tumor cell lines and tissue-specific patterns are apparent. DT-diaphorase expression is predictive of sensitivity to mitomycin C and EO9, supporting a role for DT-diaphorase in the mechanism of action of these bioreductive quinone alkylators.

Implications: 1) The results may be used to support the enzyme-directed approach to bioreductive drug development. 2) Incoporation of these and other biochemical/molecular parameters into the relational data base should enhance the capability of the NCI human tumor panel to predict the mode of action and activity of existing and novel agents.

INTRODUCTION

In many cases, antitumor agents require metabolism in order to exert their antitumor activity. A good example of these are the so-called bioreductive drugs which require enzyme-catalyzed reduction (*1*). Preferential bioactivation in tumour versus normal tissue may provide a means of achieving improved therapeutic selectivity. One approach to establish the importance of a given reductase in the activation of an individual bioreductive agent within intact cells is to determine the correlation between the activity of that particular reductase enzyme and drug sensitivity across a panel of appropriate cell lines. For example, this approach has been applied to demonstrate the clear involvement of DT-diaphorase (NAD(P)H:-quinone oxidoreductase; E.C. 1.6.99.2) in the activation of the investigational indoloquinone drug EO9 under aerobic conditions (*2-7* and reviewed in reference *8*). EO9 is currently undergoing Phase II clinical trial with the EORTC (European Organisation for Research and Treatment of Cancer) (*9,10*) and may have a particular value against tumors rich in the DT-diaphorase enzyme. Although mitomycin C is also reduced by DT-diaphorase, and there is evidence that this enzyme may be involved in the drug's activation within intact cells (reviewed in references *11,12*), the results obtained in at least one cell line panel have challenged this view (*3*).

The US National Cancer Institute (NCI) annually screens more than 10,000 compounds of defined chemical structure against an *in vitro* panel of over 60 different human cell lines derived from tumors with a spectrum of histologies and organs of origin (*13*). Use of computer methods such as the COMPARE program (*13,14*), neural networks (*15*) and Kohonen self-organizing mapping (*16*) to mine the data base of at least 42,000 compounds so far evaluated against the cell line panel, provides a unique means by which to identify the potential mode of action of established agents and novel chemical entities. The inclusion of relevant biochemical and molecular parameters within the same relational data base would provide an additional method of identifying critical factors influencing *in vitro* antitumor activity.

In a collaborative activity between the NCI, the EORTC and the UK Cancer Research Campaign, we have piloted this approach by determining the expression of three selected bioreductive enzymes, namely DT-diaphorase, NADPH : cytochrome P450 reductase (E.C. 1.6.2.4) and NADH : cytochrome b5 reductase (E.C. 1.6.2.2) The

pattern of expression both within and between cell lines from different tissue origins is described and the potential utility of this new resource is illustrated by the correlation observed between enzyme expression and antitumor activity of the established anticancer agent mitomycin C and the investigational indoloquinone drug EO9.

MATERIALS AND METHODS

Chemicals and Reagents

NADH, NADPH, cytochrome *c*, bovine albumin, menadione, dicumarol and aprotinin were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). TEMED and ammonium persulphate were obtained from BIORAD Laboratories (Richmond, U.S.A.) while the acrylamide / bis acrylamide (30% w/v) was purchased from Severn Biotech Ltd. (Kidderminster, U.K.). RNAzol™B was from Biogenesis Ltd. (Bournemouth, U.K.), while purified MMLV-RT, DTT and 5x First strand Buffer were all obtained from Gibco BRL (Paisley, U.K.). The hexamer primers used in the reverse transcriptase reaction were from Pharmacia (Milton Keynes, U.K.) while all other reagents used in the Polymerase Chain Reaction were in a kit supplied by Perkin Elmer Cetus (Beaconsfield, U.K.).

Enzyme Preparations and Specific Antibodies

Highly purified preparations of rat Walker tumor cell DT-diaphorase and rat liver NADPH : cytochrome P450 reductase were provided by Dr. R. Knox (ICRF Department of Molecular Pharmacology, Sutton, U.K.) and Prof. C. R. Wolf (ICRF Laboratory of Molecular Pharmacology, Dundee, U.K.), respectively. In each case, a specific polyclonal antibody raised against the purified rat enzyme was provided.

Cell Lines

A range of tissue-specific human tumor cell lines were grown to confluence in RPMI 1640 containing 5% serum, and cells were either harvested using trypsin (0.25%) / EDTA (1mM) solution or, for suspension cell lines, by centrifugation. These were susequently frozen at -70°C until use. Pellets were thawed and resuspended in 0.45ml of PBS and 0.05ml aprotinin (10%). Cell suspensions were sonicated for 3x10s, ensuring that vials were kept on ice as often as possible, and then spun down (15,000rpm) using a Biofuge 15 microfuge (Heraeus, Germany) for 20 minutes at 4°C to obtain an S9 supernatant. This S9 supernatant containing a mixture of cytosolic and microsomal proteins. Aliquots (40µl) of S9 supernatant were removed and stored at -70°C until use. The Pierce Microtiter Plate BCA Assay (*17*) was used to measure total protein content of each cell sonicate.

Measurement of DT-diaphorase, NADPH : Cytochrome P450 Reductase and NADH : Cytochrome b5 Reductase Activity

An indirect, coupled assay system was used to determine DT-diaphorase activity within the S9 cell sonicates. Activity was assayed by measuring cytochrome *c* reduction at 550nm on a spectrophotometer (Perkin Elmer) in the presence of NADH. Standard reaction conditions contained cytochrome *c* (77 µM), NADH (200µM), as cofactor, menadione (10µM) as an intermediate electron acceptor, and 0.14% bovine serum albumin in a final volume of 1 ml 50mM Tris HCl buffer (pH 7.5). The activity attributable to DT-diaphorase was that which was inhibited by dicumarol (10µM), a known inhibitor of DT-diaphorase (*18*).

NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase activity were measured using a direct assay system as both enzymes were able to reduce cytochrome *c* in the presence of cofactor alone (NADPH and NADH, respectively). Conditions used, therefore, were as described above but in the absence of menadione. A range of volumes of S9 supernatants (2.5µ to 25µl) were added to the reactions to

ensure that the amount of protein added, gave a linear rate of reaction. Appropriate controls were performed to determine background rates of reduction within the reactions. In addition, a 1:600 dilution of purified rat DT-diaphorase and an HT-29 cell sonicate were used in the assay as positive and standardized controls.

All reactions were carried out at 37°C and initiated by the addition of the cell sonicate. Rates of reduction were calculated from the initial linear part of the reaction progress curve and units of enzyme activity were expressed as nmoles of cytochrome *c* reduced/min/mg of protein. An extinction coefficient for cytochrome c of 21.1 $mMcm^{-1}$ was used in the calculation.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The level of DT-diaphorase protein within each cell sonicate was determined by gel electrophoresis and Western blot analysis. For detection of DT-diaphorase, samples were run on a 12% separating gel. Samples were diluted to a 1mg/ml protein concentration in boiling mix (10% stacking gel buffer, 2% SDS, 5% *b*-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue) and boiled for 5 minutes. 50µg of protein was loaded into each lane. A purified preparation of rat DT-diaphorase were used as a positive control (0.1µg loaded). Samples were subjected to electrophoresis at 50mA for 2 hours and subsequently at 6mA overnight. A semi-dry graphite electroblotter (Millipore Corporation, U.S.A.) was used to transfer proteins from the gel onto an Immobilon-P transfer membrane (Millipore Corporation, U.S.A.). Following blocking for 1 hour with 5% non-fat milk in a buffer containing Tris (50mM), Saline (0.9% NaCl) and Tween 20 (0.05%), pH7.9 (TBST pH7.9), a primary antibody was applied for 1 hour (1:500 dilution in TBST pH7.9 for DT-diaphorase) at room temperature. The primary antibody had been raised against the rat Walker tumor protein, and was known to crossreact with the human form of the enzyme. The membrane was washed (in TBST pH7.9) and a 1:5000 dilution of Protein A linked to Horseradish Peroxidase (Amersham International plc, Amersham, U.K.) was applied for 15 minutes at room temperature. Following further washing (in TBST pH7.9), the membrane was processed using an Enhanced Chemiluminescence kit (Amersham International plc, Amersham, U.K.). The level of protein present within each cell sonicate was analysed by densitometric methods using a Molecular Dynamics Laser Densitometer with Image Analysis Software (Protein Databases Incorporated, New York, U.S.A.).

Detection of DT-diaphorase and NADPH : Cytochrome P450 Reductase Expression by RT-PCR

RNA from individual cell pellets was isolated using the RNAzol™ B method described by Chomczynski & Sacchi (*19*). This method was carried out as described in the manufacture's protocol and involved several steps including homogenization, RNA extraction, RNA precipitation and an RNA wash. The final preparation of RNA was diluted down to a 1µg/µl concentration. Reverse transcriptase was performed at 37°C for 1 hour in the presence of RNA (1µl of a 1µg/µl stock), Hexamer primers (0.1µg), MMLV-RT (10U), dNTP's (2mM), 5X First strand buffer and DTT (10mM). Following this procedure, the cDNA was diluted 1:5 with distilled water and stored at 70°C until the polymerase chain reaction was carried out. For each cell line, several dilutions of the cDNA were used in the polymerase chain reaction starting at 100ng cDNA. These dilutions had previously been found to be in the linear portion of the reaction for each enzyme concerned. A separate master reaction mix (primers, Perkin Elmer PCR buffer, dNTP's, Amplitaq, distilled water) was made up for each enzyme under investigation i.e. DT-diaphorase, NADPH : cytochrome P450 reductase, the human ribosomal 28S protein (used as a positive control) and an internal standard (no cDNA). The DT-diaphorase primers used in this study are those detailed by Horikoshi *et al* (*20*) and consist of (a) bases 392 - 413 of the DT-diaphorase coding sequence (*21*) and (b) bases 640 - 661 of the DT-diaphorase coding sequence (*21*). For NADPH : cytochrome P450 reductase, the primers were selected from the

sequence of the human cDNA (*22*). A positive control was performed alongside DT-diaphorase and NADPH : cytochrome P450 reductase involving the use of primers against the ribosomal 28S gene selected from the sequence detailed in Gonzalez *et al* (*23*). For each reaction, 20µl of reaction mix was added to 5µl of cDNA and then overlaid with 50µl mineral oil. A negative control was performed in which no cDNA template was present. All reaction tubes were then spun for a few seconds and placed in the thermal cycler (PREM™III, LEP Scientific, Milton Keynes, U.K.). The program consisted of the following steps ; denature (95°C for 45secs.), anneal (60°C for 45secs.), extension (72°C for 2mins.15secs.). This was repeated for 35 cycles and then a final extension at 72°C for 7mins. was performed before holding reactions at 4°C. Products were run on a 2% agarose gel in 0.5% TBE, and then stained with ethidium bromide (1µg/µl) before being photographed under UV transillumination.

Cytotoxicity Testing

Sensitivity of the human tumor cell lines to mitomycin C and EO9 were determined using standard procedures at the US National Cancer Institute. Results were expressed using the standard calculated response parameters GI_{50} (drug concentration causing a 50% reduction in the protein increase within the test wells compared to the control wells i.e. 50% growth inhibition); TGI (drug concentration resulting in total growth inhibition) and LC_{50} (drug concentration causing a 50% reduction in protein present in the test well at the end of drug treatment, compared to that at the start i.e. 50% cell kill). For a more full description see (*24*). Cultures, in the logarithmic phase of growth, were incubated with test agents for 48 hours, and the end point of the assay the amount of protein present was determined by the Sulforhodamine B (SRB) method (*25*). Briefly, adherent cells were fixed in situ for 60 minutes at 4°C using 50µL cold 50% (wt/vol) trichloroacetic acid (TCA) (final concentration 10%). Supernatant was then discarded and 96 well plates rinsed five times with deionized water and then dried. Fixed cells were stained at room temperature for 10 minutes with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. SRB was poured from the 96 well plates and 1% acetic acid used to rinse the plates five times. Once the plates were dry, the dye was solubilized using 10mM unbuffered tris base (pH 10-10.5) for 2-5 minutes. Optical densities were read on an automated spectrophotometric plate reader at 515nm. In the case of suspension cells, the method is essentially the same, except that at the end of the drug incubation period the settled cells are fixed to the bottom of the plates by adding 50µL of 80% cold TCA (final concentration 16%).

Statistical Analysis

The sensitivity data of anticancer drugs tested against the NCI cell line panel are routinely and conveniently displayed as COMPARE analysis plots (*13,14*). For a given drug, these COMPARE plots illustrate on a $\log_{10}$ scale the deviation from the mean sensitivity calculated across all cell lines in the panel. This is known as the mean graph. We have used the same method to display our enzyme activity data for the NCI panel.

The relationship between enzyme activity and cell sensitivity (using the parameter generally employed by the NCI for sensitivity analysis; GI_{50} values) within the human tumor cell line panel towards both mitomycin C and EO9 was determined using the COMPARE program (*13,14*). Statistical anaylsis in the form of the Pearson Correlation Coefficient was used to determine the degree of similarity between cytotoxicity towards EO9 and mitomycin C, and enzyme activity.

RESULTS

Enzyme Activity Measurements

A total of 69 human tumor cell lines from 9 different tissue origins were analysed for DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase enzyme activities using standard methodolgy based on reduction of cytochrome *c* .

a) DT-diaphorase

Figure 1A represents a mean graph of log DT-diaphorase activity within the cell line panel. It is clear that although there is considerable variation in levels of enzyme activity across the panel, patterns of high and low enzyme activity expressing groups do occur. For example, those cell lines originating from tumors of the colon, CNS, non-small cell lung and melanoma histiotypes show higher than average activities while the leukemic, breast and, to some extent, ovary lines show lower than average activities. Nevertheless heterogeneity of expression can be seen within as well as between particular tumor types. For example, it is interesting to note that of the two prostate cell lines assayed, one was found to express relatively high DT-diaphorase activity while the other expressed a much lower level.

For clarity and as an example of the intra-tumor heterogeneity of expression, Tables 1 and 2 show more details of the enzyme activity measurements for eight CNS and melanoma cell lines. In the case of the CNS group, DT-diaphorase activity was shown to vary approximately 12-fold between the cell lines. The range of DT-diaphorase activity within the melanoma group was slightly higher at 19-fold. Similar results were obtained for the other tissue-specific groups of cell lines, although it is interesting to note that measurable levels of DT-diaphorase activity could be detected in only two out of five leukemia and lymphoma lines.

b) NADPH : Cytochrome P450 Reductase

The activity of NADPH : cytochrome 450 reductase within the panel of cell lines is represented in Figure 1B in mean graph format. Although differences in enzyme activities existed both between and within the different groups of cell lines, the variation was less pronounced than that for DT-diaphorase. In addition, the overall level of NADPH : cytochrome P450 reductase activity was lower than that for DT-diaphorase. Despite the lower overall range, patterns of high and low enzyme activity expressing groups did emerge. Cell lines originating from the colon, melanoma, kidney and to some extent the ovarian and leukemic lines were found to have enzyme activities above the average level. In contrast, cell lines from the lung (both non-small cell and small cell) and breast possessed activities which were below average. The range of NADPH : cytochrome P450 reductase activities within individual selected histiotypic groups of cell lines is illustrated in more detail in Tables 1 and 2. In the case of the CNS cell lines (Table 1), NADPH : cytochrome P450 reductase activity was lower than that of DT-diaphorase and varied only 2-3-fold between cell lines. This pattern was true for all of the groups of cell lines studied.

c) NADH : Cytochrome b_5 Reductase

A mean graph of log NADH : cytochrome b_5 reductase activity within the cell line panel is shown in Figure 1C. Similar to NADPH : cytochrome P450 reductase, the overall level of NADH : cytochrome b_5 reductase activity was lower than that for DT-diaphorase. As with the other two enzymes under study, patterns of high and low enzyme expressing groups were found. For example, cell lines from the CNS, melanoma and kidney expressed activities which were higher than average. In contrast, cell lines from the lung (both non-small cell and small cell), ovary and, to some extent the breast, possessed activities which, with the exception of one ovarian cell line, were below average. Although the activity of NADH : cytochrome b_5 reductase was higher than that of NADPH : cytochrome P450 reductase in the CNS group (Table 1), and indeed in many of the other groups studied, the range between the highest and lowest cell lines was similar at 2-3-fold.

DT-diaphorase Protein Expression

To complement the enzyme activity measurements, all human tumor cell lines within the panel were analysed for DT-diaphorase protein expression. DT-diaphorase protein was detected in 65 out of a total of 69 cell lines. As for the enzyme activities, a wide range of protein expression was found both within and between different tumor types. Examples of the results obtained in melanoma and CNS cell lines are shown in Figure 2. All of the melanoma lines studied were found to express DT-diaphorase with the range of levels clearly seen in Figure 2A. Figure 2B clearly shows the results of Western blot analysis for detection of DT-diaphorase protein in cell lines originating from the CNS. Here the range of DT-diaphorase expression was even greater, with some cell lines having relatively high levels of expression (Lanes 1, 2, 5 and 7) and others showing little or no detectable protein (Lanes 3, 4 and 8).

To determine whether the observed wide range of enzyme activities could be explained by a correlation with the DT-diaphorase protein content of the cell line, the results obtained with two different endpoints were compared. Enzyme activity and protein expression were found to correlate extremely well in the vast majority of cell lines studied. For illustration, the comparison between DT-diaphorase activity and protein expression in the eight CNS cell lines is shown in Figure 3 (correlation coefficient = 0.872). Correlation coefficients for all of the other tissue groups investigated were in the range 0.394 - 0.938. The small number of discrepancies found between DT-diaphorase protein expression and enzyme activity were mainly confined to a few of the breast or ovarian cell lines which had very low levels (data not shown).

Detection of DT-diaphorase and NADPH : Cytochrome P450 Reductase by RT-PCR

To explore the reasons underlying those few instances in which weak correlations were seen between DT-diaphorase protein expression and enzyme activity within the breast and ovarian cell lines, the more sensitive RT-PCR assay for DT-diaphorase RNA was performed. All human tumor cell lines originating from the breast, and as a control four cell lines originating from the CNS, were assayed for both DT-diaphorase and NADPH : cytochrome P450 reductase RNA levels using RT-PCR.

In the case of the human breast tumor cell lines, all nine were found to express DT-diaphorase RNA although a wide range of levels were observed (data not shown). The level of NADPH : cytochrome P450 reductase RNA in breast lines was extremely low in most of the lines investigated and was undetectable in others. In the case of CNS, DT-diaphorase RNA was detectable in all four cell lines investigated, with a range in values once again observed. NADPH : cytochrome P450 reductase RNA was detected only at very low levels in some of the CNS cell lines.

Correlation between cell sensitivity towards anticancer agents and enzyme activity

A COMPARE analysis was carried out to investigate the correlation between DT-diaphorase activity and cell sensitivity to the bioreductive quinones EO9 and mitomycin C under aerobic conditions. Figure 4 shows the patterns of differential cytotoxicty (GI_{50} (A), TGI (B) and LC_{50} (C)) in the form of the mean graph for EO9. Each of the individual parameters show that leukemic and ovarian cell lines were the most resistant, while those originating from the melanoma, colon, renal, CNS, small cell and non-small cell lung cancer appear to be more sensitive towards EO9. The results for mitomycin C (figure 5) were found to be somewhat different with cell lines originating from breast, ovaries and colon being relatively resistant and those from the CNS and melanoma being more sensitive towards mitomycin C.

A statisical analysis of the data was performed using the Pearson Correlation Coefficient. The results of the 42,000 compounds screened against the NCI panel

indicated that quinone compounds such as EO9 and mitomycin C were among those compounds exhibiting the best correlation between the GI_{50} and DT-diaphorase activity under investigation. For example, a Pearson Correlation Coefficient of 0.446 ($p \leq 0.0013$) was obtained for EO9 (Figure 6), whilst for mitomycin C (not shown) the coefficient was 0.424 ($p \leq 0.0005$). A similar trend for correlations with the other parameters TGI and LC_{50} were also observed. In contrast, no correlation was found between NADPH : cytochrome P450 reductase or NADH : cytochrome b_5 reductase activities, and sensitivity towards either mitomycin C or EO9. It should be noted that five of the cell lines used in the analysis exhibited extremely low levels (or none) of DT-diaphorase activity and as such, lie apart from the others in figure 6.

DISCUSSION

The results described in this paper represent one of the first in a series of ongoing studies to characterize selected important biochemical and molecular parameters across the entire NCI panel of more than 60 human tumor cell lines. The existing relational data base on the *in vitro* antitumor activity of at least 42,000 discrete chemical entities permits the assignment of a putative mechanism of action to both established and novel compounds (*13-15,26*). The addition of biochemical and molecular parameters allows the growing data base to be interrogated to determine the likely involvement of these parameters in the antitumor mechanism.

The selected biochemical parameters, in this study, were the activities of the enzymes DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b_5 reductase. These are among the reductases known to be involved in activation of bioreductive anticancer agents, including quinones, nitro compounds and *N*-oxides (*1*). Additional enzymes are also involved in bioreductive activation, including for example cytochrome P450s, xanthine oxidase and xanthine dehydrogenase. DT-diaphorase was selected because of the current degree of interest in its potential involvement in the activation of various bioreductive agents, particularly quinones (*8,27*). NADPH : cytochrome P450 reductase has also been extensively investigated for its role in the metabolism of a wide range of bioreductive agents (*28,29*). NADH : cytochrome b_5 reductase has been less extensively studied but has been shown to have the ability to bioactivate mitomycin C (*30*). A further, practical advantage of selecting these particular reductases is the similarity in the analytical methodology, which facilitated the efficient processing of the samples. To help interpret the enzyme activity information and also to assess technical feasibility for future clinical studies, the amount of protein (DT-diaphorase) and messenger RNA (DT-diaphorase and NADPH : cytochrome P450 reductase) were also analyzed by Western immunoblotting and RT-PCR, respectively. Additional reductases could also be examined in a similar fashion but given the extensive nature of the work involved to characterize expression across close to 70 cell lines, it was felt appropriate to restrict the initial analysis to three drug metabolizing reductase enzymes.

In addition to providing a valuable resource for the future analysis of mechanism of action of potential anticancer compounds tested in the NCI screen, the present study represents the most comprehensive characterization to date of the bioreductive enzymes examined against human tumor cell lines *in vitro* (for previous studies see for example, references *3,4,31*).

It is clear that the expression of bioreductive enzyme activities showed a considerable degree of heterogeneity of expression both within a particular histotype and between the 69 tumor cell lines of different origins. In the case of DT-diaphorase, for example, a relatively high degree of expression was seen in non-small cell lung cancer, colon, CNS and melanoma cell lines. Relatively low activities were noted in leukemic, ovarian and breast cell lines. Other studies using smaller panels of cell

lines have also shown similar patterns of DT-diaphorase expression. For example, high levels of DT-diaphorase activity have been observed within human tumor cell lines originating from the lung and colon (*3,4,7,31,32*) while very low levels in leukemias have also been noted (*7*). In addition, enzyme activity measurements have been carried out using human tumor samples. Schlager and Powis (*33*) showed that DT-diaphorase was enhanced (compared to paired normal tissue) in tumors of the lung and colon, results very much in line with the tumor cell lines in this study. Similar to our study using human tumor cell lines, Schlager and Powis (*33*) found that DT-diaphorase was also enhanced in breast tumors but interestingly our breast cell lines contained higher levels of DT-diaphorase activity compared to the tumor data. This is not unusual, since we ourselves noted that the level of DT-diaphorase activity was also found to be higher within the CNS human tumor cell lines when compared to a human glioma biopsy panel (*28*). These differences, however, may be partly explained by problems concerning tissue aquisition, such as the amount of contaminating normal tissue present (generally normal tissue has lower enzyme activity (*33*)) within the tumor. In order to answer this question more fully, therefore, more work is required in the investigation of reductase levels within human tumors. Interestingly, in comparison with DT-diaphorase, the activities in the panel (and tumors) observed for NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase were in general much lower.

Overall only a low number of cell lines within the NCI panel were found to lack DT-diaphorase enzyme activity completely. Interestingly however, one out of ten (i.e.10%) non-small cell lung cancer lines studied was found to lack DT-diaphorase activity. This finding was in agreement with the frequency of a point mutation in the DT-diaphorase gene reported by Rosvold *et al* (*34*) and more recently by Ross *et al* (*12*) in lung cancers and may be an indication of the presence of this point mutation in our cell lines lacking DT-diaphorase activity. Further studies would be required to establish this.

As an initial test of the ability to use the enzyme activity measurements to investigate involvement in *in vitro* antitumor activity, we interrogated the data base with respect to two selected compounds, namely the clinically used, prototype bioreductive agent mitomycin C and the structurally related, novel indoloquinone agent EO9 which is currently undergoing Phase II clinical trial (*9*). The former was selected as an established agent for which a number of enzymes are known to be capable of bioactivation (*30,35-38*). Moreover, whereas DT-diaphorase is thought to be one of these, a previous study in a panel of 15 human tumor cell lines found no correlation between DT-diaphorase expression and cytotoxicity (*3*). EO9 was selected as a developmental drug in which a strong correlation between DT-diaphorase expression and cytotoxicity has been seen in several smaller studies but for which information on the role of additional reductases is lacking (for review see reference *8*). Our results showed that in this much larger cell line panel, covering a wider range of tumor histiotypes, a highly significant correlation was seen between DT-diaphorase expression and sensitivity to both EO9 and mitomycin C. In contrast, there was no significant relationship between sensitivity to either drug and activities of either NADPH : cytochrome P450 reductase or NADH : cytochrome b5 reductase. These results would suggest that DT-diaphorase is an important enzyme in the activation of these bioreductive quinones in intact cells whereas the other enzymes are much less important or not involved at all.

The correlation shown in Figure 6 for EO9, and similar data for mitomycin C, suggest that although DT-diaphorase is a major determinant of cellular sensitivity to EO9 and mitomycin C it is clearly not the only one. This is not suprising. Not only may other activiating and detoxifying systems be involved, but factors such as intrinsic sensitivity to DNA alkylation and DNA repair capacity will also play a role. It is a

feature of the large NCI panel that individual parameters can be identified from a multivariant data base.

It should be emphasized that the correlations reported above concern the cytotoxicity of EO9 and mitomycin C under aerobic exposure conditions. The sensitivity of the NCI human tumor cell line panel to these drugs has not been determined under hypoxic conditions and the present configuration of the screen would not permit this. Very recent studies have shown that different results can be obtained under aerobic versus hypoxic environments (*4,31,39*) but the significance of these differences for the *in vivo* situation remains, at present, unclear.

In summary, the expression and, importantly, the functional activity of three reductase enzymes has been determined across the NCI human tumor cell line panel. The results reveal interesting differences within and, in particular, between cell lines from different tissues of origin. Heterogeneity in the expression patterns of DT-diaphorase were especially noteworthy. The correlations observed between enzyme activity and tumor cell line sensitivity to the indoloquinone EO9 and to mitomycin C suggest that the inclusion of the enzyme activity measurements within the relational data base should allow similar questions to be asked across the 42,000 or more discrete chemical entities that have been screened to date. It should be possible to determine not only whether particular compounds are activated by the reductases concerned, but also where reductive metabolism leads to deactivation in intact cells. This suggests a possible role for the cell line panel in predicting metabolic detoxification of xenobiotics. Moreover, new agents under study in the panel can also be analysed in a similar way. A potential practical application of this knowledge is the targeting of particular agents to certain tumor types, depending on the levels of activating and deactivating enzymes, as suggested by the 'enzyme-directed' approach to bioreductive drug development (*4,8,31,39-41*). A caveat is that whereas the patterns of expression displayed in the cell lines are in many cases consistent with expression in fresh clinical tumor specimens, more information on the latter is required to underpin the enzyme-directed approach. In addition, prospective clinical studies would be needed to determine clinical utility. Finally, this study has illustrated the feasibility and value of measuring biochemical and molecular parameters as potential aids to the diagnostic power of the NCI human tumor panel to investigate the potential mechanism of action of existing agents and new chemical entities.

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FOOTNOTES

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FIGURE LEGENDS

Fig 1 : Mean graphs of log DT-diaphorase (A), NADPH : cytochrome P450 reductase (B), and NADH : cytochrome b5 reductase (C) activities within the NCI cell line panel. For each enzyme, the mean activity for all of the cell lines is represented by a

vertical line through individual plots (these mean activities being 199.5, 14.8 and 38.0 nmoles cytochrome *c* reduced/min/mg protein for DT-diaphorase, NADPH : cytochrome P450 reductase and NADH : cytochrome b5 reductase respectively). Horizontal bars show, on a logarithmic scale, the individual enzyme activities for each cell line relative to the mean value. Those cell lines with activity below the mean lie to the left of the vertical line. Cell lines which contain no enzyme activity are indicated with (-). MG_MID, Delta and Range are defined as the calculated mean value of the panel; the number of $\log_{10}$ units by which the values for the individual lines of the panel differs (+ or -) from the MG_MID value and the number of $\log_{10}$ units by which the value for the most sensitive line differs from the least sensitive line, respectively.

Fig 2 : DT-diaphorase protein expression within melanoma (A) and CNS (B) tumor cell lines. In the melanoma panel, lanes 1 - 8 represents S9 supernatant fractions prepared from SKMEL2; SKMEL5; SKMEL28; M14; M19MEL; MALME; UACC62 and UACC257 cell lines respectively. In the CNS panel, lanes 1 - 8 represents S9 supernatant fractions prepared from SNB19; SNB75; SNB78; SF268; SF295; SF539; U251 and XF498 cell lines respectively. A purified preparation of the rat Walker form of DT-diaphorase (33kD) was run as a standard in each case.

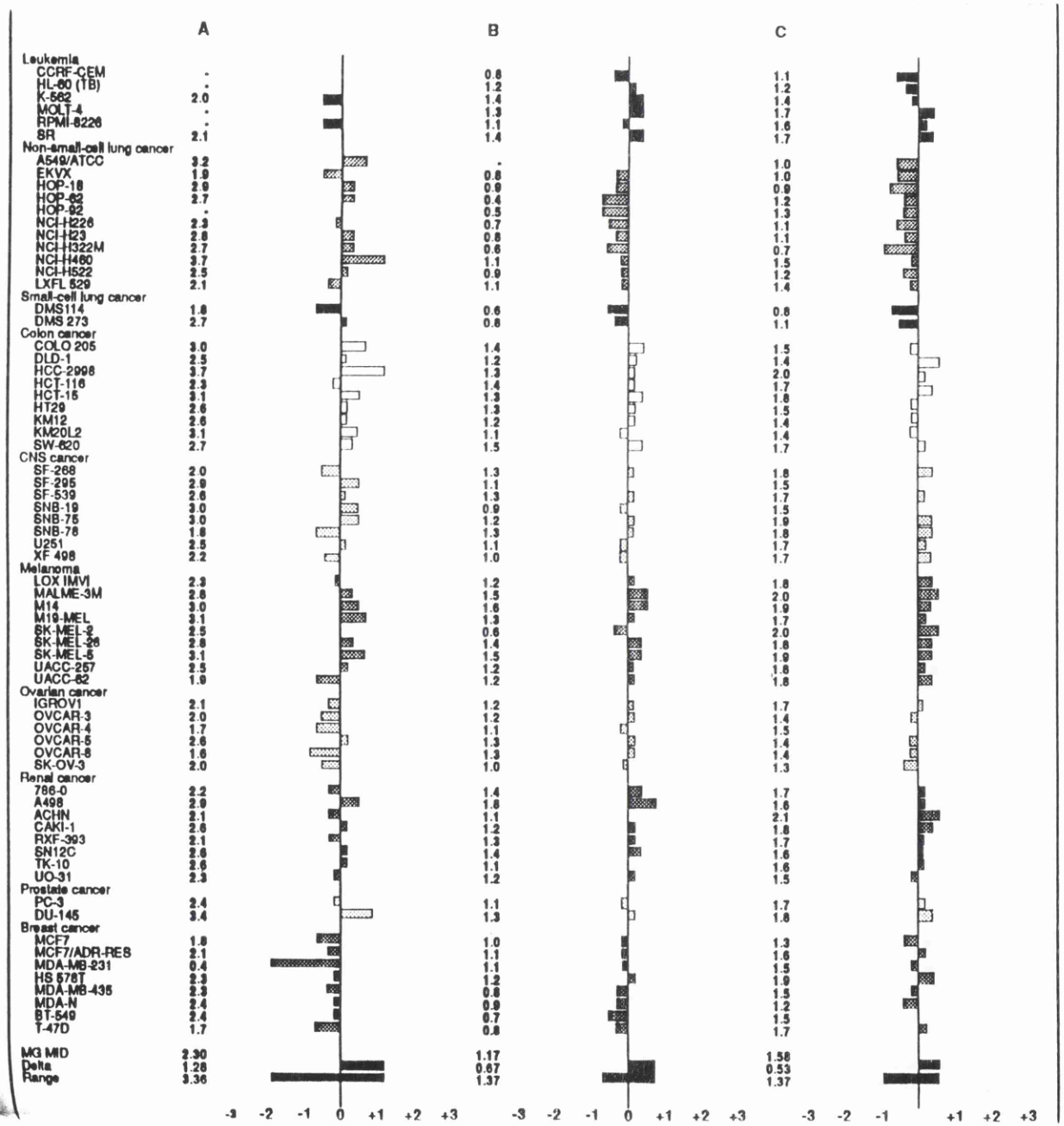
Fig 3 : Correlation between DT-diaphorase protein expression and enzyme activity within eight CNS tumor cell lines. OD x MM is defined as an integrated area under the curve over a fixed width of band.

Fig 4 : Patterns of differential cytotoxicity of the indoloquinone EO9 towards human tumor cell lines. For each cell line, the GI_{50} (A), TGI (B) and the LC_{50} (C) are obtained. These different measures of *in vitro* antitumor activity are described in Materials and Methods and in reference 24. For each parameter, a mean log is determined and is defined as the mean of the $\log_{10}$ of the individual values for that parameter. The difference between the $\log_{10}$ of each cell line and the mean log is then determined , to yield positive values for cell lines more sensitive than average (bars projecting to the right) and negative values for cell lines less sensitive than average (bars projecting to the left). For definition of MG_MID, Delta and Range see legend of figure 1.

Fig 5 : Patterns of differential cytotoxicity of mitomycin C towards human tumor cell lines. For each cell line, the GI_{50} (A), TGI (B) and the LC_{50} (C) are obtained. These different measures of *in vitro* antitumor activity are described in Materials and Methods and in reference 24. For each parameter, a mean log is determined and is defined as the mean of the $\log_{10}$ of the individual values for that parameter. The difference between the $\log_{10}$ of each cell line and the mean log is then determined , to yield positive values for cell lines more sensitive than average (bars projecting to the right) and negative values for cell lines less sensitive than average (bars projecting to the left). For definition of MG_MID, Delta and Range see legend of figure 1.

Fig 6 : Correlation between EO9 sensitivity and DT-diaphorase activity. The parameter used in the analysis shown is that of GI_{50}. The statistical analysis used is the Pearson Correlation.

Fig 1


A
B
C
Leukemia
CCRF-CEM
HL-60 (TB)
K-562
MOLT-4
RPMI-8226
SR
Non-small-cell lung cancer
A549/ATCC
EKVX
HOP-18
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522
LXFL 529
Small-cell lung cancer
DMS114
DMS 273
Colon cancer
COLO 205
DLD-1
HCC-2998
HCT-116
HCT-15
HT29
KM12
KM20L2
SW-620
CNS cancer
SF-268
SF-295
SF-539
SNB-19
SNB-75
SNB-78
U251
XF 498
Melanoma
LOX IMVI
MALME-3M
M14
M19-MEL
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62
Ovarian cancer
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3
Renal cancer
786-0
A498
ACHN
CAKI-1
RXF-393
SN12C
TK-10
UO-31
Prostate cancer
PC-3
DU-145
Breast cancer
MCF7
MCF7/ADR-RES
MDA-MB-231
HS 578T
MDA-MB-435
MDA-N
BT-549
T-47D
MG MID
Delta
Range
2.0
2.1
2.2
2.4
2.4
1.8
2.1
0.4
2.3
2.3
2.4
2.4
1.7
2.30
1.28
3.36
0.8
1.2
1.4
1.3
1.1
1.4
0.8
0.9
0.4
0.5
0.7
0.8
0.6
1.1
0.9
1.1
0.6
0.8
1.4
1.2
1.3
1.4
1.3
1.3
1.2
1.1
1.5
1.3
1.1
1.3
0.9
1.2
1.3
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1.3
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1.2
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1.8
1.7
1.6
1.6
1.5
1.7
1.8
1.3
1.6
1.5
1.9
1.5
1.2
1.5
1.7
1.58
0.53
1.37
-3
-2
-1
0
+1
+2
+3

Fig 2

A.

B.

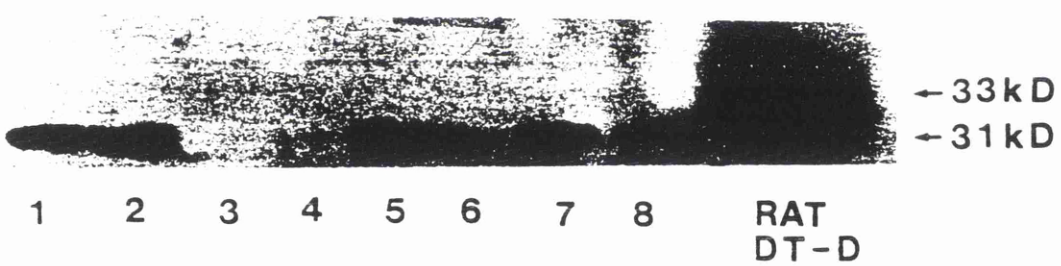


Figure 3

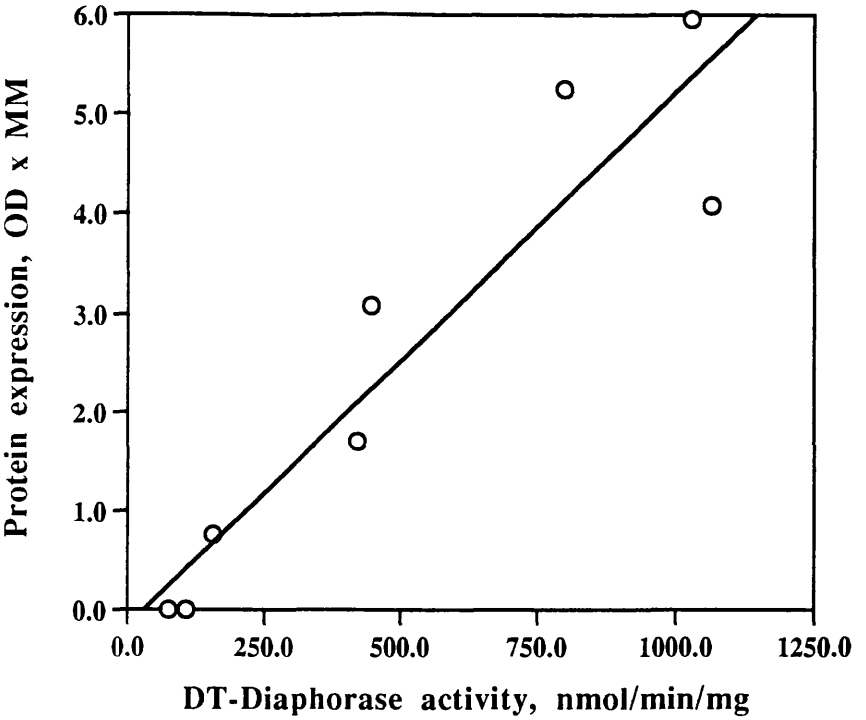


Figure 6

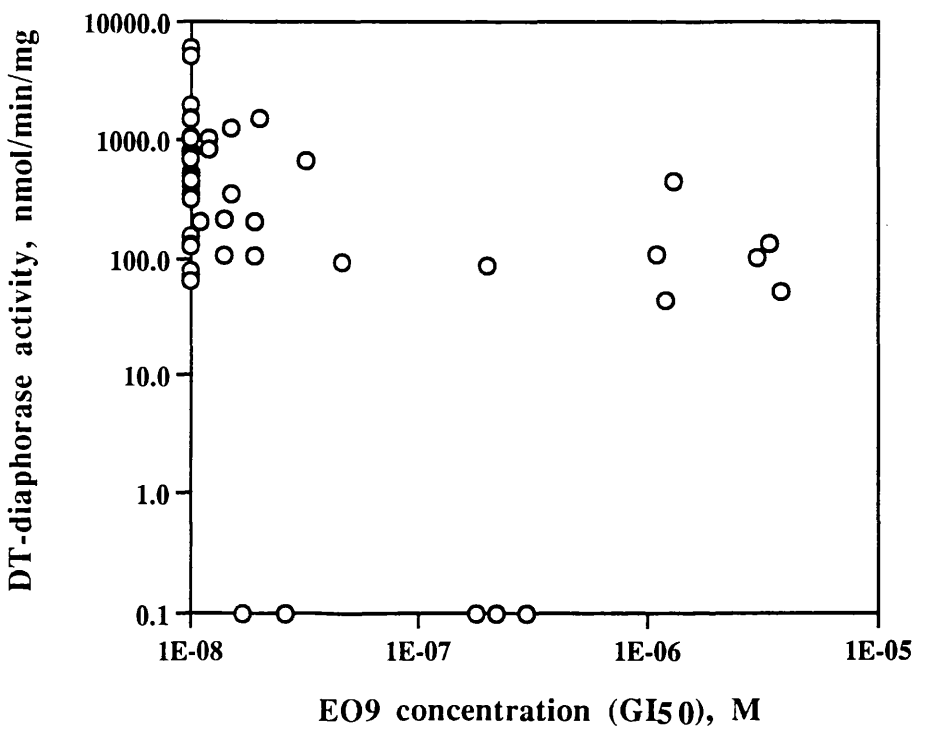
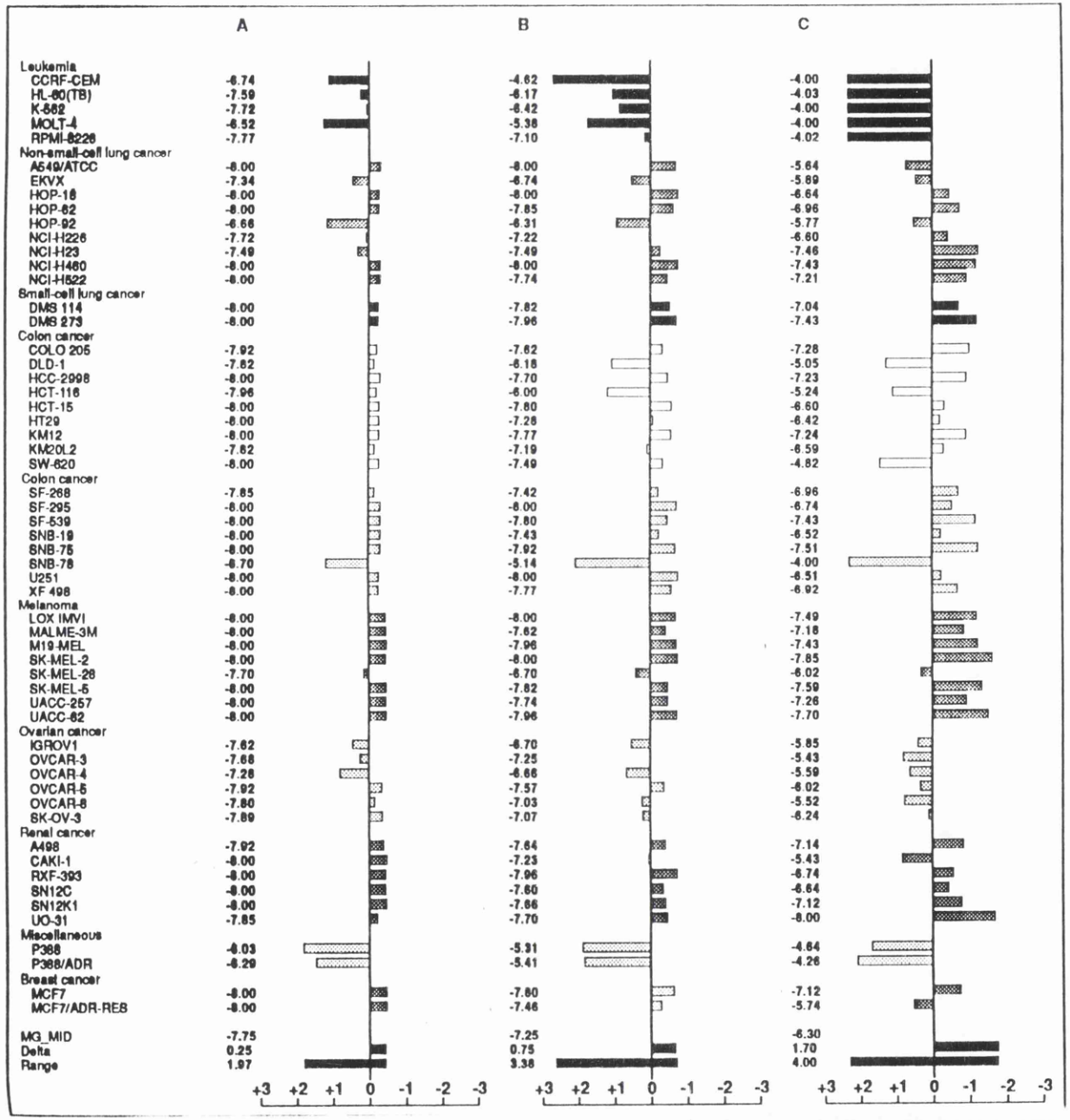


Fig 4


A
B
C
Leukemia
CCRF-CEM -6.74 -4.62 -4.00
HL-60(TB) -7.59 -6.17 -4.03
K-562 -7.72 -6.42 -4.00
MOLT-4 -6.52 -5.38 -4.00
RPMI-8226 -7.77 -7.10 -4.02
Non-small-cell lung cancer
A549/ATCC -8.00 -8.00 -5.64
EKVX -7.34 -6.74 -5.89
HOP-18 -8.00 -8.00 -6.64
HOP-62 -8.00 -7.85 -6.96
HOP-92 -6.66 -6.31 -5.77
NCI-H226 -7.72 -7.22 -6.60
NCI-H23 -7.49 -7.49 -7.46
NCI-H460 -8.00 -8.00 -7.43
NCI-H522 -8.00 -7.74 -7.21
Small-cell lung cancer
DMS 114 -8.00 -7.82 -7.04
DMS 273 -8.00 -7.96 -7.43
Colon cancer
COLO 205 -7.92 -7.62 -7.28
DLD-1 -7.82 -6.18 -5.05
HCC-2998 -8.00 -7.70 -7.23
HCT-116 -7.96 -6.00 -5.24
HCT-15 -8.00 -7.80 -6.60
HT29 -8.00 -7.26 -6.42
KM12 -8.00 -7.77 -7.24
KM20L2 -7.82 -7.19 -6.59
SW-620 -8.00 -7.49 -4.82
Colon cancer
SF-268 -7.85 -7.42 -6.96
SF-295 -8.00 -8.00 -6.74
SF-539 -8.00 -7.80 -7.43
SNB-19 -8.00 -7.43 -6.52
SNB-75 -8.00 -7.92 -7.51
SNB-78 -6.70 -5.14 -4.00
U251 -8.00 -8.00 -6.51
XF 498 -8.00 -7.77 -6.92
Melanoma
LOX IMVI -8.00 -8.00 -7.49
MALME-3M -8.00 -7.62 -7.18
M19-MEL -8.00 -7.96 -7.43
SK-MEL-2 -8.00 -8.00 -7.85
SK-MEL-28 -7.70 -6.70 -6.02
SK-MEL-5 -8.00 -7.82 -7.59
UACC-257 -8.00 -7.74 -7.26
UACC-62 -8.00 -7.96 -7.70
Ovarian cancer
IGROV1 -7.62 -6.70 -5.85
OVCAR-3 -7.68 -7.25 -5.43
OVCAR-4 -7.28 -6.66 -5.59
OVCAR-5 -7.92 -7.57 -6.02
OVCAR-8 -7.80 -7.03 -5.52
SK-OV-3 -7.89 -7.07 -6.24
Renal cancer
A498 -7.92 -7.64 -7.14
CAKI-1 -8.00 -7.23 -5.43
RXF-393 -8.00 -7.96 -6.74
SN12C -8.00 -7.60 -6.64
SN12K1 -8.00 -7.66 -7.12
UO-31 -7.85 -7.70 -8.00
Miscellaneous
P388 -6.03 -5.31 -4.64
P388/ADR -6.29 -5.41 -4.26
Breast cancer
MCF7 -8.00 -7.80 -7.12
MCF7/ADR-RES -8.00 -7.46 -5.74
MG_MID -7.75 -7.25 -6.30
Delta 0.25 0.75 1.70
Range 1.97 3.38 4.00
+3 +2 +1 0 -1 -2 -3
+3 +2 +1 0 -1 -2 -3
+3 +2 +1 0 -1 -2 -3

~~Fig 5~~

FIGURE 5

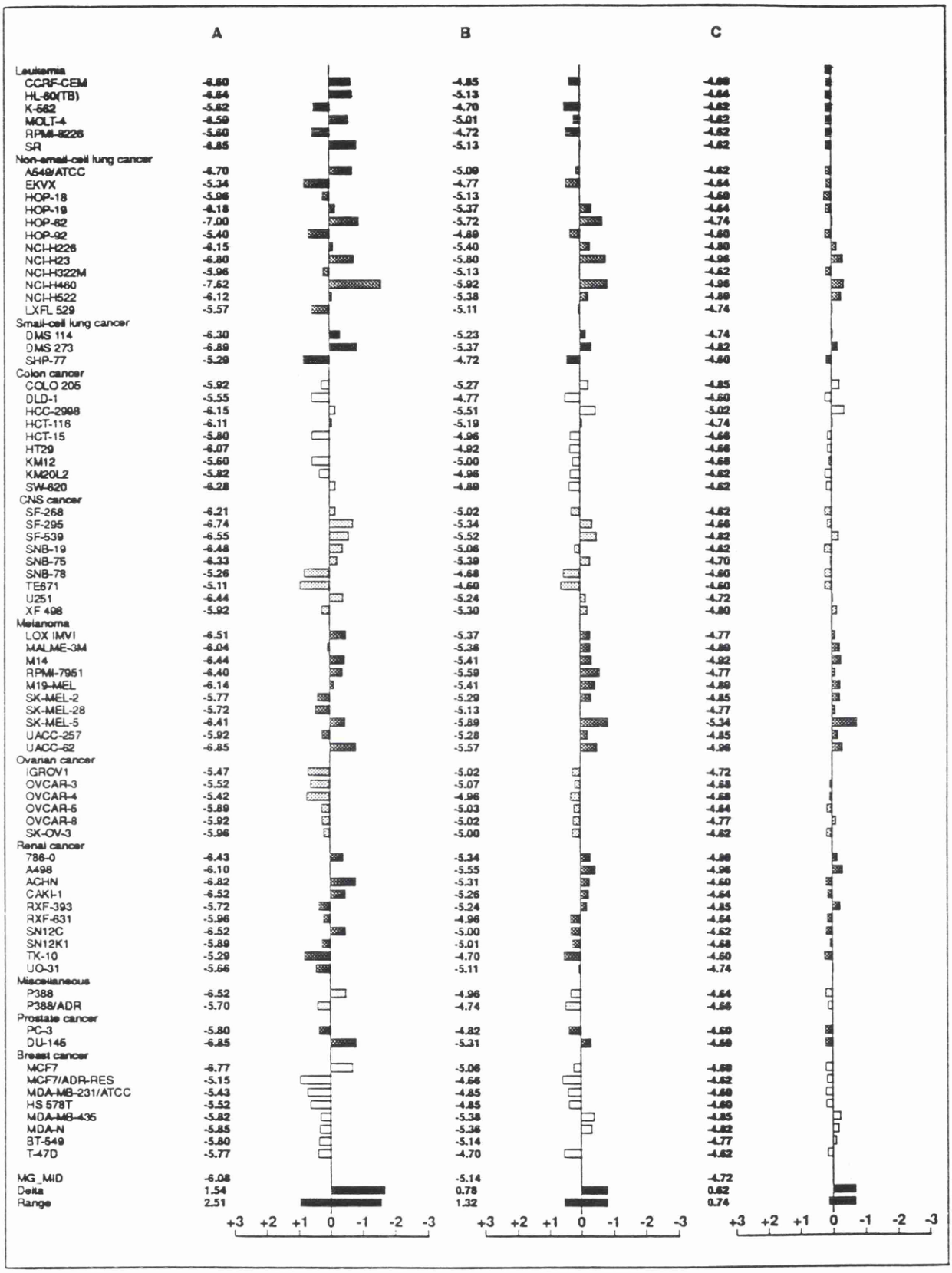


Table 1: Enzyme activity measurements for eight CNS tumor cell lines.

Tumor cell lines	Enzyme Activities, nmol/min/mg		
	DT-diaphorase	NADPH: cytochrome P-450 reductase	NADH: cytochrome b5 reductase
SF 268	108.3 ± 24.0	20.9 ± 4.8	63.4 ± 11.8
SF 295	799.1 ± 46.9	15.2 ± 2.2	36.4 ± 0.8
SF 539	421.9 ± 32.5	19.9 ± 0.7	52.8 ± 4.1
SNB 19	1065.8 ± 118.2	9.8 ± 2.8	36.4 ± 6.7
SNB 75	1030.2 ± 12.0	16.7 ± 2.8	92.8 ± 9.1
SNB 78	76.2 / 101.1 *	14.7 / 30.8 *	72.8 ± 7.4
U 251	446.8 ± 20.1	13.6 ± 1.3	49.6 ± 5.7
XF 498	157.1 ± 4.9	9.7 / 12.9 *	60.3 ± 11.4

Units are nmol of cytochrome *c* reduced/min/mg of protein. Data represent a mean ± SD (n=3) except where indicated by * where duplicate values are given.

Table 2: Enzyme activity measurements for eight melanoma tumor cell lines.

Tumor cell lines	Enzyme Activities, nmol/min/mg		
	DT-diaphorase	NADPH: cytochrome P-450 reductase	NADH: cytochrome b5 reductase
UACC 62	81.0/78.1*	13.8/18.8*	63.0 ± 4.6
UACC 257	355.5 ± 52.2	17.5 ± 2.3	40.6 ± 0.5
MALME	740.9 ± 22.7	37.7 ± 4.1	121.9 ± 22.4
M14	1038.6 ± 93.4	40.6 ± 1.9	87.7 ± 13.3
SK MEL 2	316.5 ± 67.6	6.6 ± 1.4	113.9 ± 14.2
SK MEL 5	1515.9 ± 124.2	36.7 ± 1.8	83.8/103.0*
SK MEL 28	692.0 ± 77.9	25.4 ± 4.0	77.3 ± 0.9
M19 MEL	1497.1 ± 53.3	20.5 ± 1.5	52.7 ± 3.3

Units are nmol of cytochrome *c* reduced/min/mg of protein. Data represent a mean ± SD (n=3) except where indicated by * where duplicate values are given.