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**MOLECULAR MECHANISMS OF BIOREDUCTIVE DRUG ACTIVATION
IN SOLID TUMOUR TISSUE**

VICTORIA JANE SPANSWICK

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1996



DECLARATION

In accordance with the regulations of the University of Edinburgh, I declare that this thesis has been composed by myself, and that the work presented is my own, except where acknowledgement has been indicated.

Victoria J Spanswick

ACKNOWLEDGEMENTS

I would like to thank everyone at the Imperial Cancer Research Fund Medical Oncology Unit, Western General Hospital, Edinburgh, for their support and friendship over the past three years. In particular, I would like to thank my supervisor Dr. Jeffrey Cummings and the pharmacology group for their help. Thanks are also due to Alison Ritchie for her assistance with the *in vivo* studies.

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ABSTRACT

Bioreductive agents are a class of anticancer drug primarily designed to exploit to their advantage the hypoxic areas and cellular enzymology present within solid tumours, ultimately via their metabolic activation leading to the generation of toxic species. Such a therapy provides an encouraging approach to treat solid tumours, in particular hypoxic cells which often remain resistant to conventional therapy such as irradiation. The quinone-containing antitumour antibiotic mitomycin C, often regarded as the prototype bioreductive, requires enzymatic metabolic activation which is known to be enhanced under hypoxia. The enzymes or quinone reductases involved in this process are numerous and their exact individual roles, in particular that of the two-electron reductase DT-diaphorase, remains controversial. Ultimately, the identification of a single or predominant reductase responsible for this bioactivation may lead to the realisation of enzyme-directed bioreductive drug therapy, a treatment programme tailoring a specific drug to an individuals' tumour enzyme profile.

The enzymology of mitomycin C bioactivation was studied *in vitro* in two solid mouse adenocarcinomas of the colon MAC 16 (high DT-diaphorase) and MAC 26 (low DT-diaphorase). Metabolism of mitomycin C by tumour subcellular fractions revealed a novel mitochondrial reductase active under hypoxia in both tumours. DT-diaphorase and

NADPH:cytochrome P-450 reductase activity was confirmed in MAC 16 but not MAC 26. The role of DT-diaphorase was examined in a tumour homogenate system and was found to play a protective role under hypoxia in MAC 16, predominating over the other enzymes present. MAC 26 showed enhanced hypoxic metabolism due to the presence of the mitochondrial reductase. The studies were extended to a human perspective, using the human colon xenografts HT-29 (high DT-diaphorase) and BE (low DT-diaphorase). *In vivo* studies, again using the MAC tumours, revealed comparable mitomycin C metabolism. Marked antitumour activity was also evident in both MAC 16 and MAC 26. Such a result supports the use of DT-diaphorase as a target for enzyme-directed bioreductive drug therapy with mitomycin C.

The mitomycin C analogue indoloquinone EO9 is a promising new bioreductive drug, although little is known about its metabolism and mechanism of action. The chemical properties of the reactive intermediates of EO9 were studied under controlled conditions via pulse radiolysis with the aim of proposing a mechanism for its cytotoxicity. Results indicated that whether EO9 undergoes reduction via one- or two-electron reduction the hydroquinone intermediate, product of two-electron reduction via enzymes such as DT-diaphorase, will predominate and dictate the pattern of cytotoxicity. *In vitro* metabolism of EO9 by the tumours described above produced a number of metabolites which were characterised by high-performance liquid chromatography. Unlike

ABBREVIATIONS

1,2- <i>cis</i> -1-hydroxy-2,7-diaminomitosen	cis-hydro
1,2- <i>trans</i> -1-hydroxy-2,7-diaminomitosen	trans-hydro
2,7-diaminomitosen	2,7-DM
Adenosine triphosphate	ATP
Area under curve	AUC
β -nicotinamide adenine dinucleotide phosphate (reduced)	NADPH
β -nicotinamide adenine dinucleotide (reduced)	NADH
Bovine serum albumin	BSA
Dihydrofolate reductase	DHFR
DT-diaphorase	DTD
Dulbecco's modified eagle's medium	DMEM
High-performance liquid chromatography	HPLC
Hypoxic cell cytotoxicity ratio	HCR
Imperial Cancer Research Fund	ICRF
Intratumoural	i.t.
Leucoaziridinomitosen	LAZM
Leucomitomycin C	LeucoMMC
Methotrexate	MTX
Mitomycin C	MMC
Mouse adenocarcinoma of the colon	MAC
Multidrug resistance	MDR

ABBREVIATIONS

1,2- <i>cis</i> -1-hydroxy-2,7-diaminomitosen	cis-hydro
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Bovine serum albumin	BSA
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DT-diaphorase	DTD
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High-performance liquid chromatography	HPLC
Hypoxic cell cytotoxicity ratio	HCR
Imperial Cancer Research Fund	ICRF
Intratumoural	i.t.
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Methotrexate	MTX
Mitomycin C	MMC
Mouse adenocarcinoma of the colon	MAC
Multidrug resistance	MDR

**NADPH-linked aquacobalamin-
reductase**

National Cancer Institute

Photodynamic therapy

Phosphate buffered saline

Ultraviolet

Visible

**NADPH-linked AqCbl-
reductase**

NCI

PDT

PBS

UV

Vis

INDEX OF FIGURES

CHAPTER 1 INTRODUCTION

Figure		page
1.1	Summary of the main categories of anticancer agents and their cellular effects.	3
1.2	The molecular structure of mitomycin C showing its three unique structural features.	16
1.3	The molecular structure of the three principle mitosene metabolites.	18
1.4	Difference in absorption spectrum of mitomycin C and the principle metabolite 2,7-diaminomitosene.	19
1.5	Proposed pathways of metabolic activation of mitomycin C after one- and two-electron quinone reduction.	28
1.6	Structures of mitomycin C-DNA adducts formed via mono- and bifunctional activation.	33
1.7	Comparative molecular structures of analogue indoloquinone EO9 and the parent drug mitomycin C.	37

CHAPTER 3 RESULTS AND DISCUSSION

Figure		page
3.1	<i>In vitro</i> pattern of mitomycin C metabolism in MAC 16 cytosolic fractions under aerobic and hypoxic conditions using NADH as cofactor.	82
3.2	<i>In vitro</i> pattern of mitomycin C metabolism in MAC 16 cytosolic fractions under aerobic and hypoxic conditions using NADPH as cofactor.	83
3.3	<i>In vitro</i> pattern of mitomycin C metabolism in MAC 16 microsomal fractions under hypoxia using NADPH as cofactor in the absence and presence of cytochrome P-450 reductase antiserum.	85

3.4	<i>In vitro</i> pattern of mitomycin C metabolism in MAC 16 and MAC 26 mitochondrial fractions under hypoxic conditions using NADPH as cofactor.	89
3.5	Electron micrograph of mitochondria isolated from MAC 16 murine adenocarcinoma of the colon.	91
3.6	Cross-sectional electron micrograph of a mitochondrion isolated from the tumour mitochondrial fraction.	92
3.7	Electron micrograph of microsomes isolated from MAC 26 murine adenocarcinoma of the colon.	93
3.8	<i>In vitro</i> production of the principle mitomycin C metabolite 2,7-diaminomitosene in MAC 16 and MAC 26 tumour homogenates under aerobic and hypoxic conditions.	100
3.9	<i>In vitro</i> pattern of mitomycin C metabolism in MAC 16 and MAC 26 tumour homogenates under aerobic conditions.	103
3.10	<i>In vitro</i> pattern of mitomycin C metabolism in MAC 16 and MAC 26 tumour homogenates under hypoxic conditions.	104
3.11	Reaction sequence of DT-diaphorase via the 'ping-pong' mechanism.	112
3.12	Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism.	113
3.13	Concentration-time profiles of mitomycin C in MAC 16 and MAC 26 murine adenocarcinomas of the colon after a direct i.t. injection of 500 µg mitomycin C.	122
3.14	Concentration-time profiles of mitomycin C in plasma from MAC 16 and MAC 26 bearing animals after a direct i.t. injection of 500 µg mitomycin C.	123

3.15	Histological appearance of MAC 16 murine adenocarcinoma of the colon.	126
3.16	Histological appearance of MAC 26 murine adenocarcinoma of the colon.	127
3.17	Concentration-time profiles of 2,7-diaminomitosenone in MAC 16 and MAC 26 murine adenocarcinomas of the colon after a direct i.t. injection of 500 µg mitomycin C.	129
3.18	<i>In vivo</i> metabolite profile of MAC 16 and MAC 26 murine adenocarcinomas of the colon 15 minutes after a direct i.t. injection of 500 µg mitomycin C.	130
3.19	Antitumour activity of mitomycin C in MAC 16 murine adenocarcinoma of the colon at doses of 50, 75, 125 and 250 µg.	133
3.20	Antitumour activity of mitomycin C in MAC 26 murine adenocarcinoma of the colon at doses of 50, 75, 125 and 250 µg.	134
3.21	<i>In vitro</i> production of the principle mitomycin C metabolite 2,7-diaminomitosenone in HT-29 and BE human colon xenograft homogenates under aerobic and hypoxic conditions.	141
3.22	<i>In vitro</i> pattern of mitomycin C metabolism in HT-29 and BE human colon xenograft homogenates under hypoxic conditions.	144
3.23	<i>In vitro</i> pattern of Indoloquinone EO9 related chromatographic peaks generated by a single 500 Gy irradiation dose.	148
3.24	UV/Visible absorption spectra of Indoloquinone EO9 and related products of irradiation.	150
3.25	Effect of time on the <i>in vitro</i> pattern of Indoloquinone EO9 related products generated by a single 500 Gy irradiation dose.	152

3.26	Effect of time on the stability of Indoloquinone EO9 and the major hydrolysis product, EO5A.	153
3.27	Effect of time on the stability of Indoloquinone EO9 related products of irradiation.	154
3.28	UV/Visible absorption spectra of Indoloquinone EO9 related products of irradiation.	156
3.29	<i>In vitro</i> pattern of EO9 metabolism in MAC 16 and MAC 26 tumour homogenates under aerobic and hypoxic conditions.	161
3.30	<i>In vitro</i> pattern of EO9 metabolism in HT-29 and BE tumour homogenates under aerobic and hypoxic conditions.	163
3.31	UV/Visible absorption spectra of Indoloquinone EO9 metabolites produced by HT-29 tumour homogenate.	165
3.32	<i>In vitro</i> formation of Indoloquinone EO9 metabolites I, II, III and EO5A in MAC 16 tumour homogenate under aerobic and hypoxic conditions during the 90 minute incubation.	168
3.33	<i>In vitro</i> formation of Indoloquinone EO9 metabolites I, II, III and EO5A in MAC 26 tumour homogenate under aerobic and hypoxic conditions during the 90 minute incubation.	169
3.34	<i>In vitro</i> formation of Indoloquinone EO9 metabolites I, II, III and EO5A in HT-29 tumour homogenate under aerobic and hypoxic conditions during the 90 minute incubation.	171
3.35	<i>In vitro</i> formation of Indoloquinone EO9 metabolites I, II, III and EO5A in BE tumour homogenate under aerobic and hypoxic conditions during the 90 minute incubation.	172
3.36	The proposed degradation pathway of Indoloquinone EO9 to the hydroxy metabolite under alkaline conditions.	175

3.37	<i>In vitro</i> degradation profile of Indoloquinone EO9 to the hydroxy metabolite under alkaline conditions.	176
3.38	<i>In vitro</i> formation of potential glutathione-aziridinyl EO9 adducts.	178
3.39	UV/Visible absorption spectra of potential glutathione-aziridinyl EO9 adducts.	179
3.40	<i>In vitro</i> pattern of Indoloquinone EO9 related products generated by a single 500 Gy irradiation dose.	185
3.41	Mass spectral analysis of unirradiated Indoloquinone EO9.	188
3.42	Mass spectral analysis of irradiated Indoloquinone EO9.	189
3.43	The difference spectrum of Indoloquinone EO9 semiquinone recorded at 20 μ s after pulse irradiation.	195
3.44	The variation of the absorbance of the semiquinone of Indoloquinone EO9 in relation to pH.	197
3.45	The dependence of the rate of decay of Indoloquinone EO9 semiquinone free radicals on oxygen concentration.	200
3.46	The autoxidation of the Indoloquinone EO9 hydroquinone.	205

INDEX OF TABLES

CHAPTER 3 RESULTS AND DISCUSSION

Table		page
3.1	Quinone reductase activity in cytosolic and microsomal subcellular fractions isolated from MAC 16 and MAC 26 murine adenocarcinomas of the colon.	78
3.2	Characterisation of mitomycin C bio-reduction in MAC 16 murine adenocarcinoma of the colon.	80
3.3	Characterisation of mitomycin C bio-reduction in MAC 26 murine adenocarcinoma of the colon.	81
3.4	Specific activities of mitomycin C metabolism by MAC 16 and MAC 26 tumour homogenates under aerobic and hypoxic conditions.	101
3.5	Pharmacokinetics and metabolic conversion of mitomycin C to the principle metabolite 2,7-diaminomitosenone in MAC 16 and MAC 26 murine adenocarcinomas of the colon.	124
3.6	Antitumour activity of i.t. mitomycin C in MAC 16 and MAC 26 murine adenocarcinomas of the colon at doses 50, 75, 125 and 250 μg on day 7 after treatment.	136
3.7	Quinone reductase activity in cytosolic and microsomal subcellular fractions isolated from HT-29 and BE human colon xenografts.	139
3.8	Specific activities of mitomycin C metabolism by HT-29 and BE human colon xenograft homogenates under aerobic and hypoxic conditions.	142
3.9	Characterisation of Indoloquinone EO9 related products generated via irradiation.	157

3.10	Antitumour activity of Indoloquinone EO9 in MAC 16 and MAC 26 murine adenocarcinomas of the colon and HT-29 and BE human colon xenografts.	159
3.11	Overall characterisation of products/metabolites generated by irradiation and enzymatic reduction of the parent drug Indoloquinone EO9.	183
3.12	Specific activities of Indoloquinone EO9 metabolism by MAC 16, MAC 26, HT-29 and BE tumour homoegnates under aerobic and hypoxic conditions and correlation with antitumour activity.	192
3.13	Absorbance of Indoloquinone EO9 semiquinone free radicals at equilibrium, $A_e \times 10^{-3}$, for a given concentration of EO9 at 415 and 490 nm and corresponding equilibrium constants, K.	203

CONTENTS

	page
Declaration	i
Acknowledgements	ii
Abstract	iii
Abbreviations	vi
Index of Figures	viii
Index of Tables	xiii
Contents	xv

CHAPTER 1 INTRODUCTION

1.1	Cancer Chemotherapy	1
1.2	Limitations of Cancer Chemotherapy	2
1.2.1.	Drug Toxicity and Therapeutic Index	4
1.2.2	Drug Resistance	6
1.2.3	Treatment of Solid Tumours	7
1.3	Rationale for the Use of Bioreductive Drug Therapy	9
1.3.1	Tumour Hypoxia	10
1.3.2	Tumour Enzymology and pH	13
1.4	Mitomycin C - The Prototype Bioreductive Drug	15
1.4.1	Chemistry of Mitomycin C	15

1.4.2	Clinical Properties of Mitomycin C	20
1.4.2.1	Antitumour Activity of Mitomycin C	20
1.4.2.2	Pharmacokinetics and Metabolism of Mitomycin C	21
1.4.2.3	Toxicity of Mitomycin C	23
1.4.2.4	Mechanisms of Mitomycin C Resistance	23
1.4.3	Mechanism of Action of Mitomycin C	24
1.4.3.1	Bioreductive Activation	24
1.4.3.2	Enzymology of Mitomycin C Activation	29
1.4.3.2	Modification of DNA by Mitomycin C	30
1.4.3.3	Redox Cycling and Oxygen Free Radical Formation	34
1.5	New Approaches to Bioreductive Therapy	36
1.5.1	Indoloquinone EO9	36
1.5.2	Benzotriazine-di-N-oxide SR 4233	42
1.5.3	Nitroimidazoles	44
1.5.4	Micellaneous Agents	46
1.5.5	Enzyme-Directed Bioreductive Drug Development	48
1.6	Scope of the Thesis	50

CHAPTER 2 MATERIALS AND METHODS

2.1	Materials	52
2.2	Apparatus	52
2.2.1	High-Performance Liquid Chromatography	52
2.2.2	Linear Accelerator	53
2.2.3	High-Performance Liquid Chromatography-Mass Spectroscopy	53
2.2.4	Human Colon Adenocarcinoma Cell Line BE and Establishment of the BE Xenograft	54
2.2.5	Animal Models	56
2.3	Methods	58
2.3.1	Isolation of Mitochondrial, Microsomal and Cytosolic Subcellular Fractions from MAC 16 & MAC 26 Murine Adenocarcinomas and HT-29 & BE Human Adenocarcinomas of the Colon	58
2.3.2	Protein Determination of Tumour Homogenates and Subcellular Fractions	59
2.3.3	Quinone Reductase Assessment of Tumour Subcellular Fractions	60
2.3.4	Electron Microscopy of MAC 16 and MAC 26 Mitochondrial and Microsomal Subcellular Fractions	61
2.3.5	<i>In Vitro</i> Metabolism of Mitomycin C by Tumour Subcellular Fractions	63

2.3.6	<i>In Vitro</i> Metabolism of Mitomycin C by Tumour Homogenates	64
2.3.7	High-Performance Liquid Chromatography Analysis of Mitomycin C and its Metabolites	65
2.3.8	Synthesis and Purification of Mitomycin C Primary Metabolites	66
2.3.9	Extraction of Mitomycin C and its Metabolites from Tumour Tissue and Plasma	67
2.3.10	Pharmacokinetics of Mitomycin C in MAC 16 and MAC 26 Murine Adenocarcinomas of the Colon	68
2.3.11	Antitumour Activity of Mitomycin C in MAC 16 and MAC 26 Murine Adenocarcinomas of the Colon	70
2.3.12	Histological Preparation of MAC 16 and MAC 26 Murine Adenocarcinomas of the Colon	70
2.3.13	High-Performance Liquid Chromatography of Indoloquinone EO9	72
2.3.14	<i>In Vitro</i> Metabolism of Indoloquinone EO9 by Tumour Homogenates	72
2.3.15	Degradation of Indoloquinone EO9 under Alkaline Conditions	73
2.3.16	Reaction of Indoloquinone EO9 with Reduced Glutathione	73
2.3.17	High-Performance Liquid Chromatography-Mass Spectroscopy of Indoloquinone EO9 and Intermediates Generated by Irradiation	73
2.3.18	Generation of Indoloquinone EO9 Reactive Intermediates by Pulse Radiolysis	75

CHAPTER 3 RESULTS AND DISCUSSION

3.1	Characterisation of a Novel Mitochondrial Reductase	77
3.2	Role of DT-diaphorase in the Metabolic Activation of Mitomycin C	99
3.3	Pharmacokinetics and Metabolism of Mitomycin C <i>In Vivo</i> : Implications for <i>In Vivo</i> Drug Treatment	120
3.4	Properties of Indoloquinone EO9 Intermediates Generated by Irradiation	147
3.5	Characterisation of the Metabolites Produced by Enzymatic Reductive Activation of Indoloquinone EO9	158
3.6	Identification of Indoloquinone EO9 Metabolites by High-Performance Liquid Chromatography-Mass Spectroscopy	184
3.7	Properties of Indoloquinone EO9 Reactive Intermediates: A Pulse Radiolysis Study	194
3.8	Summary	211

213

CHAPTER 4 REFERENCES

APPENDIX I

Reagent Suppliers	238
Apparatus Suppliers	241
Supplier Addresses	242
Collaborator Addresses	244

APPENDIX II

Publications

245

CHAPTER 1

1. INTRODUCTION

1.1 Cancer Chemotherapy

Cancer chemotherapy is one of three main approaches to cancer treatment. Unlike surgery and radiation which provide local treatment of the disease, systemic drug therapy is still the principle way of treating metastatic tumour growth. The use of cytotoxic drugs ie: drugs that can damage or kill growing cells, was first introduced in 1942 with the treatment of Hodgkins disease with the alkylating agent nitrogen mustard (Gilman, 1963). In the next decade, a wide variety of malignancies were treated with the newly discovered alkylating agents and antimetabolites.

With this success it became apparent that tests were required to allow successful introduction of new compounds into the clinic - clinical trials. Essentially, phase I trials establish the dose, route of administration, excretion pattern and prominent toxicities. Once this information is available, phase II trials are carried out in patients with varying malignancies so that the spectrum of tumours sensitive to the agent can be defined. Finally, phase III trials allow for in depth evaluation of patients usually being treated for one form of malignancy in which the new agent is compared to the best established method of treatment currently in use.

Cancer chemotherapeutic agents can be classified in a number of ways. Broadly, they can be divided into alkylating agents, antimetabolites, antitumour antibiotics, plant alkaloids and miscellaneous agents. The latter group would include agents such as enzymes for example L-asparaginase, the platinum coordination complexes cis- and carboplatin and steroid hormones. Alternatively, it is possible to divide agents into groups depending on what stage of the cell cycle they show selective lethal toxicity. Non-phase specific agents are effective throughout the cell cycle, whilst those only effective in a particular phase are referred to as phase specific. The primary mechanisms of action of the main groups of anticancer agents are summarised in figure 1.1.

1.2 Limitations of Cancer Chemotherapy

Cancer chemotherapy is used in a number of ways: (1) as the major curative modality for rare malignancies such as Hodgkins disease and other lymphomas, childhood acute leukaemias and testicular cancer; (2) adjuvant therapy before or after local treatment for primary disease with the aim of treating micrometastases; (3) as palliative treatment for advanced cancers; and (4) in combination with other modalities to improve therapeutic effects. Despite these regimes, limitations to their use have arisen of which normal tissue toxicity and drug resistance are the commonest.

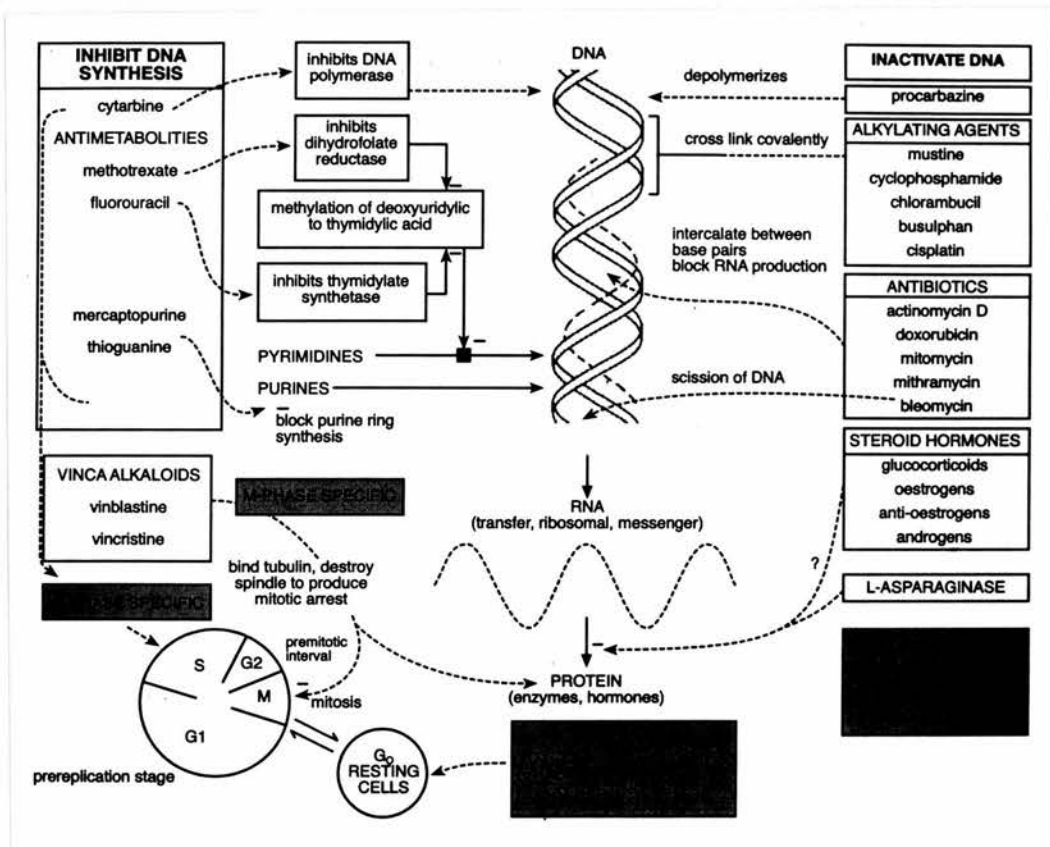


Figure 1.1. Summary of the main categories of anticancer agents and their cellular effects.

1.2.1 Drug Toxicity and Therapeutic Index

Although cancer chemotherapy is targeted at dividing malignant cells, normal tissues are exposed to such agents. Normal tissues with a high rate of cellular proliferation are at risk from the effects of cytotoxic drugs. The major tissues affected are the bone marrow, hair follicles and intestinal mucosa and are the commonest dose-limiting toxicities associated with most anticancer agents. Other less common toxicities are linked to specific agents, for example, cardiotoxicity is the main toxicity limiting the use of doxorubicin (Bristow, 1982).

Toxicity can be divided into 4 main categories depending on time of onset:- immediate, early, delayed and late. Immediate toxicity usually occurs within hours ranging from nausea/vomiting to phlebitis. Early toxicity is associated with myelosuppression, more commonly leucopenia and thrombocytopenia, alopecia and diarrhoea and usually presents within days to weeks. Anaemia and liver damage are considered to be delayed toxicities presenting weeks or months later. Finally sterility and second malignancies arise years later and are classed as late toxicities.

The clinical evaluation of any potential anticancer agent is a long and complex process. The key to evaluating any new agent is to compare the benefits achieved by the therapy with treatment related toxicity. This is known as the therapeutic index. Therapeutic index was originally defined

in mice from the following formula.

$$\text{Therapeutic Index} = \frac{\text{maximum non-toxic dose}}{\text{minimum effective dose}}$$

However this formula does not take into account individual variability therefore the following more widely-used definition has been introduced.

$$\text{Therapeutic Index} = \frac{\text{LD}_{50}}{\text{ED}_{50}}$$

where LD_{50} and ED_{50} are the lethal and effective dose for 50% of the group respectively. This gives an indication of the safety of the drug by drawing attention to the relative toxicity and effective dose of a drug.

A high therapeutic index is generally considered to be of benefit with toxic side effects at a minimum for a given dose. However, a low therapeutic index may be accepted for treatment in patients with advanced malignancy who have a significant change of meaningful palliation or survival prolongation as a result of that therapy. This would however be considered inappropriate for use in surgical adjuvant therapy in a patient population with a significant chance of cure.

One method of improving the therapeutic index is by combining one anticancer agent and/or radiotherapy with another. Two agents may often be combined with only a small reduction in doses compared to those used

when the agents are given alone but achieve less additive toxicity towards normal tissues and increased antitumour activity.

1.2.2 Drug Resistance

Resistance to chemotherapeutic agents has been observed for many years with many solid tumours showing infrequent response to treatment. The mechanisms underlying drug resistance are numerous, spanning from morphological factors such as poorly organised tumour vasculature leading to limited drug penetration, stage of cell proliferation to altered biochemical pathways and genetic factors. Broadly, drug resistance can be divided into two categories: (1) intrinsic resistance observed before the cancer has been exposed to treatment and (2) acquired resistance which manifests itself after prior exposure.

Many agents enter cells by facilitated membrane transport involving binding to a receptor protein located on the cell membrane followed by internalisation. Alteration or reduction of these receptor proteins may subsequently lead to impaired membrane transport, lower intracellular drug concentrations and reduced toxicity. Well documented examples include methotrexate (MTX) (Goldman, 1971) and nitrogen mustard (Goldenberg & Begleiter, 1980). A similar type of transport alteration is seen in cells possessing the so-called multidrug resistance (MDR) phenotype (Endicott & Ling, 1989). Characteristically, cells exhibiting this

phenotype are selected for resistance to one single agent. However, with further investigation it emerges that cross-resistance has developed to a variety of unrelated cytotoxic agents. These drugs were often observed to be of plant or fungal origin such as the vinca alkaloids and antitumour antibiotics respectively. Juliano and Ling (1976) linked this phenomenon to the presence of a 170 kD cell surface membrane glycoprotein, now commonly known as P-glycoprotein. Tumours high in P-glycoprotein tend to develop from normal tissue high in the protein such as kidney, colon and liver. Various methods have been used in an attempt to circumvent MDR and in particular the use of calcium channel blocking agents such as verapamil have potential (Tsuru, 1983).

Other forms of drug resistance involve altered intracellular targets. A good example is MTX resistance and its target dihydrofolate reductase (DHFR). Cells resistant to MTX may show increased amounts of DHFR due to amplified gene expression (Schimke, 1984) or alternatively produce variant forms of the enzyme disrupting DHFR inhibition (Goldie *et al.*, 1980). Other examples include alterations in type II topoisomerase expression (Morrow & Cowan, 1990) and glutathione metabolism.

1.2.3 Treatment of Solid Tumours

Despite the success of chemotherapy in treating blood borne diseases such as the leukaemias, treatment of solid tumours remains a challenge. The

highly disorganised vascular system, slow blood flow and high interstitial pressure of solid tumours hinder efficient drug perfusion leaving some areas unexposed (Jain, 1987a), whilst the presence of necrotic and hypoxic areas consequently lead to resistance to radiotherapy and treatment by conventional anticancer agents. The use of alternative routes of drug administration known as locoregional chemotherapy have achieved higher drug concentrations in the bulk of the tumour. Such methods include regional vascular perfusion, intraperitoneal, intrapleural and intrathecal chemotherapy (Kerr & Los, 1993). An alternative approach is to attack the endothelial cells lining the tumour vasculature rather than the tumour cells themselves. Such a treatment would result in an occlusive thrombosis halting blood flow throughout the tumour. This can be achieved using a variety of immunotoxins directed at such endothelial cells (Burrows & Thorpe, 1994).

1.3 Rationale for the Use of Bioreductive Drug Therapy

Solid tumours generally develop areas of reduced blood flow, decreasing pH and ultimately regional hypoxia. It has long been recognised that such hypoxia is a major obstacle in the successful treatment of solid tumours with particular reference to radiotherapy (Bush *et al.*, 1978; Moulder, 1984; Overgaard, 1992). However, despite its negative impact on cancer treatment, such a problem can be exploited as a potential difference between malignant and normal tissues. One strategy is the development of bioreductive therapy in which hypoxic cells are eliminated through the use of cytotoxic drugs selectively toxic in the absence of oxygen. Such hypoxia-activated prodrugs or so-called bioreductive drugs require metabolic activation via a number of enzyme-dependent pathways consequently resulting in highly cytotoxic species. Under aerobic conditions cells are placed under oxidative stress due to a process known as redox cycling. Essentially the free radical intermediate generated via a reduction step is back oxidised to the parent molecule. Although superoxide and the highly damaging hydroxyl radicals are produced via this futile cycling, this pathway is often less damaging than the toxic species predominating in hypoxic cells and aerobic toxicity is at a minimum (Powis, 1987).

The quinone-containing antitumour antibiotic mitomycin C is considered to be the prototype bioreductive alkylating agent and its

bio-reductive properties have led to the generation of a number of related quinone analogues (Oostveen & Speckamp, 1987). Other agents toxic to hypoxic cells include the nitroheterocycles such as the nitroimidazoles (Adams & Stratford, 1994) and the benzotriazines, the lead compound being SR 4233 (Brown, 1993). Bio-reductive drugs and their mechanism of action will be discussed in more detail throughout.

The ability of bio-reductive drugs to eradicate hypoxic cells can be expressed as the hypoxic cell cytotoxicity ratio or HCR. This is obtained by dividing the dose required to kill a proportion of oxygenated cells with that required to destroy an equal fraction of the same cells under hypoxia. For example, the HCR for mitomycin C is approximately 2 (Stratford & Stephens, 1989) although this may vary with the cell type used. In comparison, an improved HCR is observed with the nitroimidazole RSU 1069 which exhibits a HCR of 69 (Horwich *et al.*, 1986).

The next section briefly deals with the rationale behind the use of bio-reductive anticancer drugs in preference to existing treatments.

1.3.1 Tumour Hypoxia

Differences in tumour blood vessels and blood flow in comparison to their normal tissue counterparts are predominately responsible for tumour hypoxia (Jain, 1994). Lack of smooth muscle, abnormal vessel

branching, lack of innervation and often no endothelial lining results in an inadequate tumour blood vessel network depleting areas of essential nutrients particularly oxygen (Jain, 1994). In addition, the absence of a lymphatic drainage system often contributes to high interstitial pressures and consequently sub-optimal blood flow within solid tumours (Jain, 1987b).

Essentially as the distance from a microcapillary increases, tissue oxygenation decreases falling to a level insufficient to support cell division. Ultimately oxygen-deprived or anoxic cells die resulting in regional necrosis. However, areas of slowly proliferating cells do survive.

Hypoxic tissues can be divided into 2 groups. Firstly, Thomlinson-Gray or irreversible 'chronic' hypoxia results as a consequence of oxygen diffusion limitations leading to zones of chronically hypoxic cells (Thomlinson & Gray, 1955). These are often observed between the interface of well-oxygenated and necrotic regions at distances 150-200 μm from capillaries (Gray *et al.*, 1953). This is often described as the diffusion limited model.

Secondly, reversible 'acute' hypoxia arises through transient blood flow changes ie: varying oxygen delivery, often due to perfusion defects such as intermittent vascular occlusion (Brown, 1979; Chaplin *et al.*, 1986; Chaplin *et al.*, 1987).

It is possible to exploit tumour hypoxia in a number of ways using bioreductive therapy. As single antitumour agents, the benefit of bioreductive drugs could potentially be minimal as hypoxic cells may only constitute a minor proportion of the cells within the tumour. However, used in conjunction with other treatments active against well-oxygenated cells, for example irradiation, they may prove beneficial. In this proposal, the administration of bioreductive drugs after irradiation will decrease the number of hypoxic cells. This in combination with irradiation induced cell death will improve tumour response.

Alternatively, using bioreductive drugs specifically designed to kill surrounding oxygenated as well as hypoxic cells is a promising option. Such bioreductive 'prodrugs' would produce stable cytotoxins capable of diffusing out of hypoxic regions eradicating oxygenated cells or continuing to act after hypoxic areas have reoxygenated. One example of such a prodrug is the cobalt (III)-nitrogen mustard complex, SN 24771 (Wilson *et al.*, 1994).

A third option is to increase the already existing hypoxic fraction which in turn increases tumour cell sensitivity to that bioreductive drug. This can be achieved by a wide variety of agents capable of modulating tumour blood flow. These range from vasoactive drugs eg: hydralazine, anaesthetics, cytokines eg: tumour necrosis factor and interleukin-1 and cytokine inducers such as flavone acetic acid. More recently the treatment

of tumours with the nitric oxide synthase inhibitor, nitro-L-arginine has proved successful in hypoxia induction (Adams & Stratford, 1994). Other methods include photodynamic therapy (PDT) and hyperthermia, both of which result in increased ischaemic cell death.

1.3.2 Tumour Enzymology and pH

Bioreductive drugs require metabolic activation to generate toxic species. A number of enzymes are able to catalyse this process. This can occur via a one- or two-electron reduction step. Such enzymes include the one-electron reductases NADPH:cytochrome P-450 reductase, xanthine oxidase and NADH: cytochrome b_5 reductase, and the two-electron reductases DT-diaphorase [NAD(P)H:(quinone acceptor) oxidoreductase, EC 1.6.99.2] and xanthine dehydrogenase. With the quinone bioreductive drug mitomycin C, all of the above enzymes have been implicated in the drug's mechanism of action (Pan *et al.*, 1984; Siegel *et al.*, 1990a; Gustafson & Pritsos, 1993; Hodnick & Sartorelli, 1993). In comparison NADPH:cytochrome P-450 reductase and cytochrome P-450 itself have been demonstrated to be important in the catalysis of benzotriazine di-N-oxide SR 4233 reduction (Walton & Workman, 1990). However, the exact role of each of the above reductases remains complex and multiple enzymes may be involved in one or more parts of a given activation pathway (Workman & Walton, 1990).

The presence of bioreductive enzymes as well as hypoxia provides an attractive target for potential cancer therapy as many human tumours are known to have increased levels of these enzymes in comparison to normal tissues (Schlager & Powis, 1990; Fitzsimmons *et al.*, 1996). However different enzymes participate to different extents in the activation of the various bioreductive drugs. Therefore patients may benefit from tumour enzyme profiling so that the activity of a given drug may be enhanced by the presence of a particular enzyme. For example, a tumour rich in the enzyme DT-diaphorase may be an excellent target for mitomycin C and its analogue indoloquinone EO9 but may prove resistant to SR 4233.

Other exploitable areas of solid tumours include tumour pH. As hypoxic cells undergo anaerobic metabolism, it follows that such cells will produce greater than normal levels of acid creating a low pH environment. Potentiating this low pH throughout the tumour can be of advantage in making the tumour more sensitive to treatments such as hyperthermia (Hiraoka & Hahn, 1989) or increasing pH gradients between intra- and extracellular environments (Gerweck *et al.*, 1991). Using such strategies, low tumour pH can be exploited by aiding in the activation of cytotoxic drugs (Tannock & Rotin, 1989). Low extracellular pH, in addition to low oxygen levels have proved an ideal environment for increasing mitomycin C cytotoxicity *in vitro* (Newell *et al.*, 1992; Parkins *et al.*, 1993)

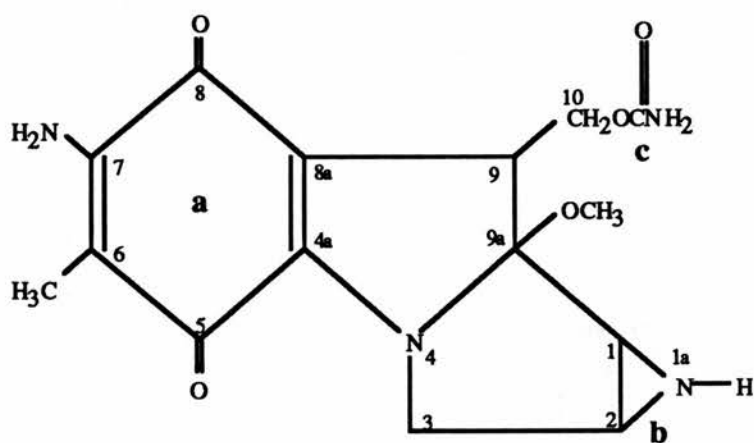
1.4 Mitomycin C - The Prototype Bioreductive Drug

Mitomycin C is a naturally occurring antitumour antibiotic originally isolated from *Streptomyces caespitosus* in 1958 (Wakaki *et al.*, 1958). As previously mentioned mitomycin C is often described as the prototype bioreductive drug. By studying its properties and behaviour at clinical, chemical and biological levels, it provides a firm basis on which to design novel highly specific bioreductive drugs. The following sections detail those properties of mitomycin C.

1.4.1 Chemistry of Mitomycin C

The structure of mitomycin C was first defined by Webb *et al.* (1962a, 1962b) and has a molecular weight of 334.13. The structural features of mitomycin C are illustrated in figure 1.2. Essentially, the unique features involve a quinone group, fused aziridine ring and a carbamate function arranged around a pyrrolo[1,2-*a*]indole nucleus and is commonly known as a mitosane structure.

Mitomycin C is unstable in aqueous solution and decomposition is strongly pH dependent. Mild acid hydrolysis of mitomycin C results in the cleavage of the C-9a O-methyl group subsequently followed by the loss of methanol, formation of a C-9-C-9a double bond and the opening of the aziridine ring (Beijnen & Underberg, 1985) and it is the protonation of



a = quinone group

b = aziridine ring

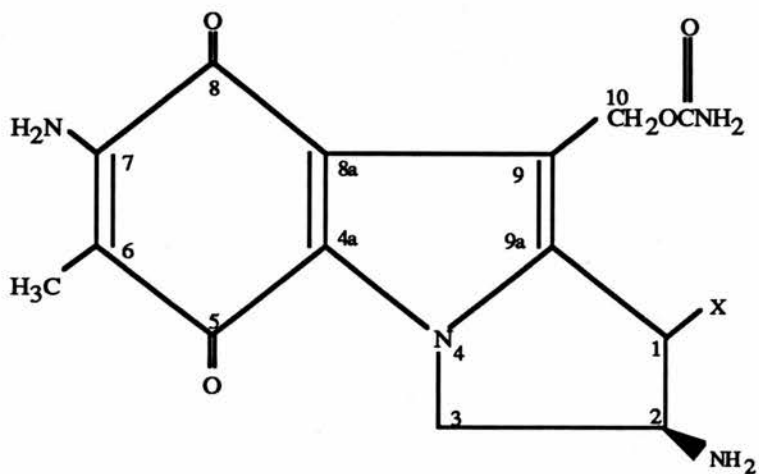
c = carbamate function

Figure 1.2. The molecular structure of mitomycin C showing its three unique structural features (**a**, **b**, **c**).

this methyl group that is thought to be the triggering factor in acid hydrolysis (Underberg & Lingeman, 1983). The overall result is the formation of 1,2-disubstituted indoloquinone or mitosene structure of which 1,2-*cis*- (cis-hydro) and 1,2-*trans*-1-hydroxy-2,7-diaminomitosenes (trans-hydro) are the primary products, figure 1.3. Extended acid hydrolysis also causes the replacement of the C-7-amino function by a hydroxyl group and cleavage of the C-10-carbamate function. Destruction of the quinone chromophore also occurs under alkaline hydrolysis whilst the aziridine ring remains unaffected and hence no additional products can be detected (Beijnen *et al.*, 1985).


Mitomycin C and its mitosene metabolite/degradation products exhibit different UV/Visible (UV/Vis) absorption spectra. Mitomycin C is characterised by λ maxima at 216, 360 and 555 nm. The mitosene structures exhibit altered absorption spectra with λ maxima ranging from 244-250, 308-313, 343 and 550-558 nm depending on the product formed. The change in absorption spectra is accompanied by a visible colour change from the mauve mitosane to the violet mitosene.

Figure 1.4 illustrates the difference in the absorption spectrum of mitomycin C and the primary metabolite 2,7-diaminomitosenes (2,7-DM). Similar spectra apply to *trans*- and *cis*-hydro metabolites.



2,7-diaminomitosene X = H

1,2-cis 1-hydroxy

2,7-diaminomitosene X =  OH

1,2-trans 1-hydroxy


2,7-diaminomitosene X =  OH

Figure 1.3. The molecular structure of the three principle mitosene metabolites.

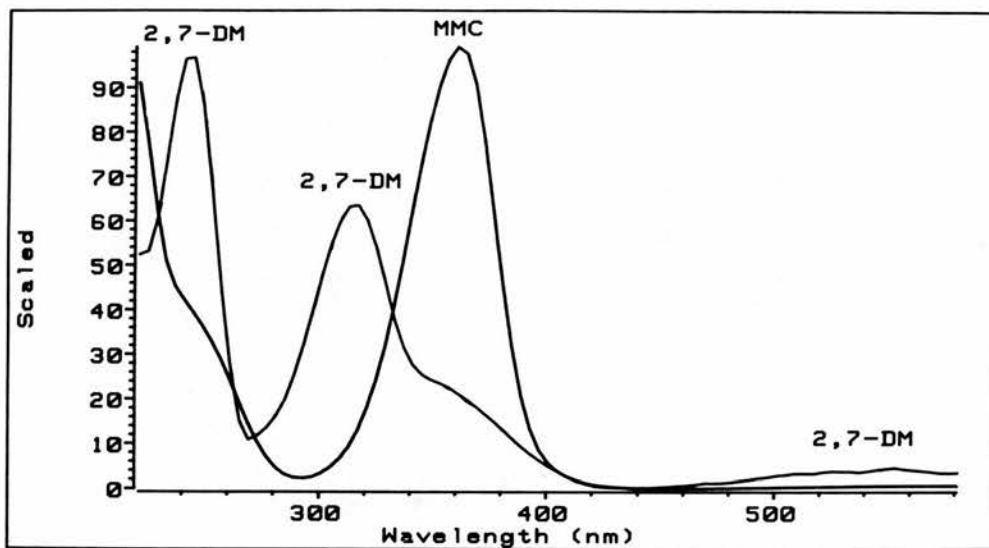


Figure 1.4. Difference in absorption spectrum of mitomycin C (MMC) and the principle metabolite 2,7-diaminomitosenone (2,7-DM).

1.4.2 Clinical Properties of Mitomycin C

1.4.2.1 Antitumour Activity of Mitomycin C

Initially clinical studies employed daily low dose schedules which unfortunately resulted in severe dose-limiting toxicities, namely cumulative myelosuppression (Crooke, 1979). This was followed by the introduction of an intermittent dosing schedule using bolus injections every four to eight weeks (Baker & Vaitkevicius, 1979). The net result was reduced and often more manageable haematologic toxicity. Using such a schedule mitomycin C was found to be active against a wide variety of solid tumours including breast, lung, gastrointestinal and superficial bladder cancers (Carter & Crooke, 1979).

Mitomycin C is now predominantly used in combination and intravesical therapy. It has proved curative in the treatment of squamous cell carcinoma of the anus when administered intravenously in combination with continuous infusion of 5-fluorouracil and radiation therapy (Nigro *et al.*, 1983). Its use prophylactically and therapeutically in the treatment of superficial bladder carcinoma when administered intravesically has also proved successful (Zincke *et al.*, 1985). Palliatively mitomycin C as a single agent has produced remission in a number of cancers including pleural mesothelioma (Bajorin *et al.*, 1987) and recurrent colorectal, bladder and breast cancers (Doll *et al.*, 1985). However, its use in combination

regimens still remains an integral component for the treatment of recurrent or metastatic stomach, pancreatic, oesophageal and lung cancers (Doll *et al.*, 1985).

In an attempt to increase local exposure to mitomycin C, incorporation into a variety of drug delivery systems has been employed, for example ethylcellulose microparticles (Kato *et al.*, 1980), polylactic acid microcapsules (Tsai *et al.*, 1986) and biodegradable albumin protein microspheres (Fujimoto *et al.*, 1985). Infused via the hepatic artery the net result is reduced systemic toxicity and maintenance of high, local drug concentrations.

1.4.2.2 Pharmacokinetics and Metabolism of Mitomycin C

Early pharmacokinetic data were obtained by the use of insensitive microbiologic assays possessing sensitivity in the range of 0.1 µg/mL (Fujita, 1971). However, with the introduction of high-performance liquid chromatography (HPLC), the detection limit was improved to approximately 1 ng/mL (Dorr, 1988). Using such methods the pharmacokinetic disposition of mitomycin C in blood has been shown to follow a first-order elimination process best described by a two-compartment model (Dorr, 1988). After a rapid initial half-life of distribution, $t_{1/2 \alpha}$, of 2-10 minutes, there is an elimination half-life, $t_{1/2 \beta}$, of 25-90 minutes (mean 54 minutes). Very little mitomycin C is

excreted intact in urine, approximately 1-20% (Verweij *et al.*, 1990).

Despite overwhelming evidence for the major role of drug metabolism in the mechanism of action of mitomycin C and its clearance from the body, no preclinical or clinical pharmacokinetic study has reported detection of mitomycin C metabolites. Recently an HPLC analytical method and liquid-liquid extraction sample preparation have been reported and used to determine the levels of mitomycin C and its three primary metabolites 2,7-DM, trans- and cis-hydro in transplantable rodent tissue after *in vivo* drug administration by intratumoural (i.t.) injection (Cummings *et al.*, 1993). *In vitro* 2,7-DM has a half-life of 13 minutes when incubated with either NADPH:cytochrome P-450 reductase or xanthine oxidase (Pan *et al.*, 1984). In a separate study, 2,7-DM was shown to have a half-life of 30 minutes after i.t. injection of mitomycin C (Chirrey *et al.*, 1995). The secondary metabolite 10-decarbamoyl 2,7-diaminomitosene was also detected in this study exhibiting a half-life of 130 minutes.

The primary metabolites have been shown to be formed after initial enzymatic metabolic activation of mitomycin C (Tomasz & Lipman, 1981; Pan *et al.*, 1984; Siegel *et al.*, 1992). Subsequent secondary metabolites, 10-decarbamoyl 2,7-diaminomitosene, are produced as a result of further enzymatic metabolism of the primary metabolites (Pan *et al.*, 1984; Siegel *et al.*, 1992).

1.4.2.3 Toxicity of Mitomycin C

The toxicities of mitomycin C are substantial, unpredictable and always considered to be life-threatening. The most significant and frequent side effect is delayed myelosuppression and is directly related to schedule and total dose (Crooke & Bradner, 1976). More common toxicities such as anorexia, nausea, vomiting and diarrhoea also occur. Hair loss is rare and infrequent and extravasation injury and necrosis result due to mitomycin C's vesicant properties. Potentially lethal chronic side effects include microangiopathic haemolytic anaemia and congestive cardiomyopathy and the latter has been shown to occur with greater frequency in patients receiving doxorubicin and mitomycin C in combination (Buzdar *et al.*, 1978).

Mitomycin C is highly clastogenic, particularly to bone marrow cells resulting in a number of chromosomal abnormalities including sister chromatid exchanges and micronuclei (Perry & Evans, 1975). More recent studies have shown that mitomycin C is however only a weak inducer of gene mutations (Hayashi *et al.*, 1992), despite being demonstrated to be carcinogenic in rats (Crooke & Bradner, 1976).

1.4.2.4 Mechanisms of Mitomycin C Resistance

The mechanisms of mitomycin C resistance result from changes in drug

accumulation, bioactivation, inactivation of alkylating species and DNA excision repair. In some cell lines expression of the MDR phenotype corresponded to mitomycin C resistance and cross-resistance with other natural-product anticancer agents including the anthracycline antibiotics, vinca alkaloids and MTX (Dorr *et al.*, 1987). Increased intracellular sulphhydryl levels correlate with resistance to certain alkylating agents leading to enhanced DNA repair in the form of cross-link removal (Lindahl *et al.*, 1982). In addition, as metabolic bioactivation is a prerequisite for mitomycin C activity, reduced expression of the enzymes involved such as DT-diaphorase and NADPH:cytochrome P-450 reductase have frequently been reported as causing resistance (Hoban *et al.*, 1990; Marshall *et al.*, 1991).

1.4.3 Mechanism of Action of Mitomycin C

1.4.3.1 Bioreductive Activation

Iyer & Szabalski (1964) initially postulated mitomycin C's dependence for enzymatic metabolic activation. This was subsequently adapted by Moore (1977). This led to the introduction of the term 'bioreductive alkylation' to describe the proposed mechanism of drug action (Lin *et al.*, 1972).

The first stage in the activation of mitomycin C is quinone reduction. For experimental purposes, reduction can be achieved chemically, for

example sodium dithionite (Schiltz & Kohn, 1993), catalytically using hydrogenation over platinum oxide (Kohn *et al.*, 1987) and finally electrochemically by reduction on a platinum or mercury electrode (Andrews *et al.*, 1986). However, the more biologically relevant enzymatic activation is usually employed since it may correspond to possible mechanisms occurring *in vivo*. The enzymes involved in this activation process are discussed in more detail in section 1.4.3.2.

Quinone reduction can occur through a one-electron pathway producing the semiquinone free radical intermediate or a two-electron pathway yielding the hydroquinone intermediate, figure 1.5. In the presence of oxygen, the semiquinone will enter into a redox cycle generating reactive oxygen species (section 1.4.3.4), pathway 2, figure 1.5. In contrast, the generation of alkylating moieties by two-electron reduction is unimpeded by molecular oxygen (Powis, 1987). Upon bioreduction, the C-9a O-methyl group leaves the parent mitosane nucleus as methanol resulting in a C-9-C-9a double bond producing the mitosene nucleus. This promotes proton assisted aziridine ring opening exposing an electrophilic carbon centre at C-1 which subsequently alkylates DNA (Moore, 1977).

The critical intermediate produced in this metabolic cascade that leads to DNA cross-linking is thought to be the quinone methide (Tomasz & Lipman, 1981; Peterson & Fisher, 1986). It is the binding of the quinone methide to DNA at C-1 that promotes a second DNA attachment point

producing DNA cross-linking via the production of a C-10 reactive centre (Tomasz & Lipman, 1981), after the loss of the carbamate group. While mitomycin C possesses no DNA binding activity, the quinone methide is believed to bind non-covalently to DNA with high affinity by non-specific intercalation through a mechanism which is dependent on the ionisation of its 2-amino group and is enhanced under acidic conditions (Kumar *et al.*, 1995). Reversible non-covalent binding can possibly be viewed as a precursor event to irreversible covalent bonding.

The quinone methide is believed to be ambivalent in nature ie: it has both electrophilic and nucleophilic properties. However, it has been demonstrated more recently by purely chemical means that rather than acting as an electrophile (figure 1.5, pathway 3) at neutral pH resulting in DNA binding and a nucleophile (figure 1.5, pathway 1) at acidic pH as originally hypothesised (Peterson & Fisher, 1986), the quinone methide is predominantly nucleophilic in character at all functional pHs between 5.5 and 8.5 (Schiltz & Kohn, 1993). This theory explains why 2,7-DM, the product of the quinone methide acting as a nucleophile, is the major metabolite of mitomycin C seen *in vivo*. The electrophilic metabolites of mitomycin C, *cis*- and *trans*-hydro are believed to be derived from a 7-aminoaziridinomitosenone intermediate, the oxidised form of leucoaziridinomitosenone (LAZM), figure 1.5, pathway 4. The formation of 7-aminoaziridinomitosenone predominantly under alkaline conditions explains why these metabolites are observed at more alkaline pH (Hoey *et*

al., 1988; Schiltz & Kohn, 1993).

Using the two-electron reductase DT-diaphorase to activate mitomycin C, two other key intermediates have been proposed to be precursors to DNA covalent bonding (Ross *et al.*, 1993), figure 1.5, pathway 5. These are the quinone-reduced intact aziridino products leucomitomycin C (leucoMMC) or fully reduced mitomycin C and LAZM, fully reduced mitomycin C minus the C-9a O-methyl group. Their dependence on proton-assisted aziridine ring opening to yield electrophilic intermediates may explain the pH dependence of mitomycin C-DNA cross-links.

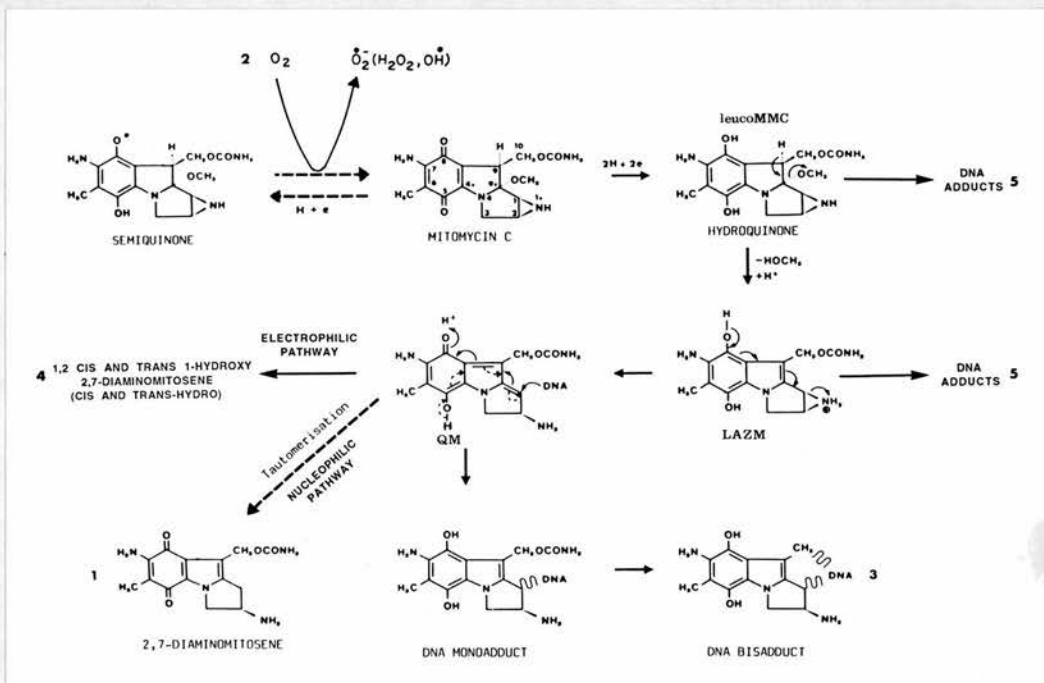


Figure 1.5. Proposed pathways of metabolic activation of mitomycin C after one- and two-electron quinone reduction. Pathways 1-5 are described within section 1.4.3.1.

1.4.3.2 Enzymology of Mitomycin C Activation

The enzymology of mitomycin C metabolic activation has been studied in a number of systems ranging from bacteria and liver to neoplastic cells and purified enzymes. Several enzymes have been implicated in the bioactivation of mitomycin C. These include one-electron reductases such as NADPH: cytochrome P-450 reductase (Kennedy *et al.*, 1982; Pan *et al.*, 1984), xanthine oxidase (Pan *et al.*, 1984) and NADH: cytochrome *b*₅ reductase (Hodnick & Sartorelli, 1993), and the two-electron reductases DT-diaphorase [NAD(P)H:(quinone acceptor) oxidoreductase, EC 1.6.99.2] (Siegel *et al.*, 1992) and xanthine dehydrogenase (Gustafson & Pritsos, 1993). In addition, cytochromes P-450, although unable to transfer electrons directly to mitomycin C, may in fact facilitate quinone reduction through drug binding (Keyes *et al.*, 1984).

The role of each of the reductases in the metabolic activation of mitomycin C *in vivo* has yet to be clarified, but it is emerging that different reductases may prevail under different physiological conditions (Cummings *et al.*, 1994). For example, NADPH:cytochrome P-450 reductase is thought to predominate in artificially hypoxic cells (Kennedy *et al.*, 1980; Krishna *et al.*, 1991). In contrast, numerous studies support a major role for DT-diaphorase in mitomycin C metabolic activation in normally oxygenated but not hypoxic cells (Siegel *et al.*, 1990a; Dulhanty & Whitmore, 1991).

1.4.3.3 Modification of DNA by Mitomycin C

Early studies on the mechanism of action of mitomycin C indicated that the principle cellular target was DNA (Iyer & Szybalski, 1963, 1964). Mitomycin C itself does not react with DNA. Only upon enzymatic or chemical activation does mitomycin C have the capability to interact with DNA. A variety of mitomycin C-DNA adducts have been reported. It was initially claimed that the major sites of covalent bonding are at O-6 guanine, followed by N-6 adenine and finally N-2 guanine (Hashimoto & Shudo, 1984).

Tomasz and colleagues (1986) demonstrated that monofunctional alkylation by the quinone methide led to the formation of a covalent guanine N²-linked mitosene monoadduct, Figure 1.6, adduct 2. This adduct accounted for 90 % of the mitomycin C bound to DNA after reductive activation with NADPH cytochrome P-450 reductase, xanthine oxidase and H₂/platinum oxide. Two minor guanine-N² adducts were also characterised, one a secondary degradation product of the major adduct and the second a 1- β stereoisomer arising from attack of the β side of the guanine amino group. Both accounted for 2-5 % of the total adduct pool. In the same study, model-building studies indicated that the guanine-N²-linked drug molecule fitted excellently inside the minor groove of the β DNA exhibiting no obvious distortion of the structure of the double helix.

In addition, a number of bisadducts have subsequently been formed, characterised and identified. The major product isolated under reductive activation with sodium dithionite was an interstrand cross-linked adduct bonded to the position of N-2 guanine on complementary strands of DNA (Tomasz *et al.*, 1987), figure 1.6, adduct 4. The drug-DNA complex produced minimal DNA distortion. The same bisadduct was also isolated from DNA from the livers of rats that had been injected with mitomycin C (Tomasz *et al.*, 1987). This study provided the first direct chemical proof for formation of covalent cross-links between mitomycin C and DNA and also postulated that oxygen and its possible role as an inhibitor of bifunctional activation, may explain the enhanced mitomycin C toxicity seen under hypoxia. The latter hypothesis was later demonstrated under a purely chemical system (Tomasz *et al.*, 1988a).

A second less frequently observed bisadduct, a cross-link within one strand of DNA or intrastrand cross-link, was isolated exhibiting a single 2,7-diaminomitosene residue linked bifunctionally to guanines at their N² atoms to the C-1 and C-10 positions of the mitosene (Bizanek *et al.*, 1992), figure 1.6, adduct 5. In contrast to the monoadduct and interstrand bisadduct, observed bending of DNA near the cross-link site was a notable effect of the intrastrand cross-link (Rink *et al.*, 1996).

Finally, nucleophilic attack by water at the C-10 position subsequently resulted in the formation of the 10-decarbamoyleated monoadduct, figure

1.6, adduct 3, and is also a major product of bifunctional activation (Tomasz *et al.*, 1988b).

All four major adducts previously isolated and characterised as described above have been detected *in vivo* (Bizanek *et al.*, 1993). The major species observed under aerobic conditions were the interstrand cross-linked adduct, followed by the 10-decarbamoyleated monoadduct, then the intrastrand adduct and finally the monofunctional monoadduct. A fifth adduct, adduct Y, was also seen but not identified chemically. This product may represent the N⁷ guanine adduct that appears to account for greater than 90 % of all mitomycin C covalently bonded to DNA after metabolic activation with purified rat DT-diaphorase (Prakash *et al.*, 1993).

The structures of all the adducts indicated that the reaction of mitomycin C with DNA was base specific: both the C-1 aziridine and C-10 carbamate alkylating functions of the drug react exclusively with 2-amino groups of guanines in the minor groove of DNA. In addition, the reaction with DNA is sequence specific. Mitomycin C alkylates guanines in the sequence CpG.CpG on average five to ten-fold faster than guanines in other sequence contexts (Li & Kohn, 1991; Kumar *et al.*, 1992). However, only 5 % of all guanines in the mammalian genome are present in the CpG context compared to 70 % for *Micrococcus luteus* DNA which is used throughout a number of the described studies. Therefore overall, mammalian DNA is cross-linked poorly by mitomycin C and may be seen

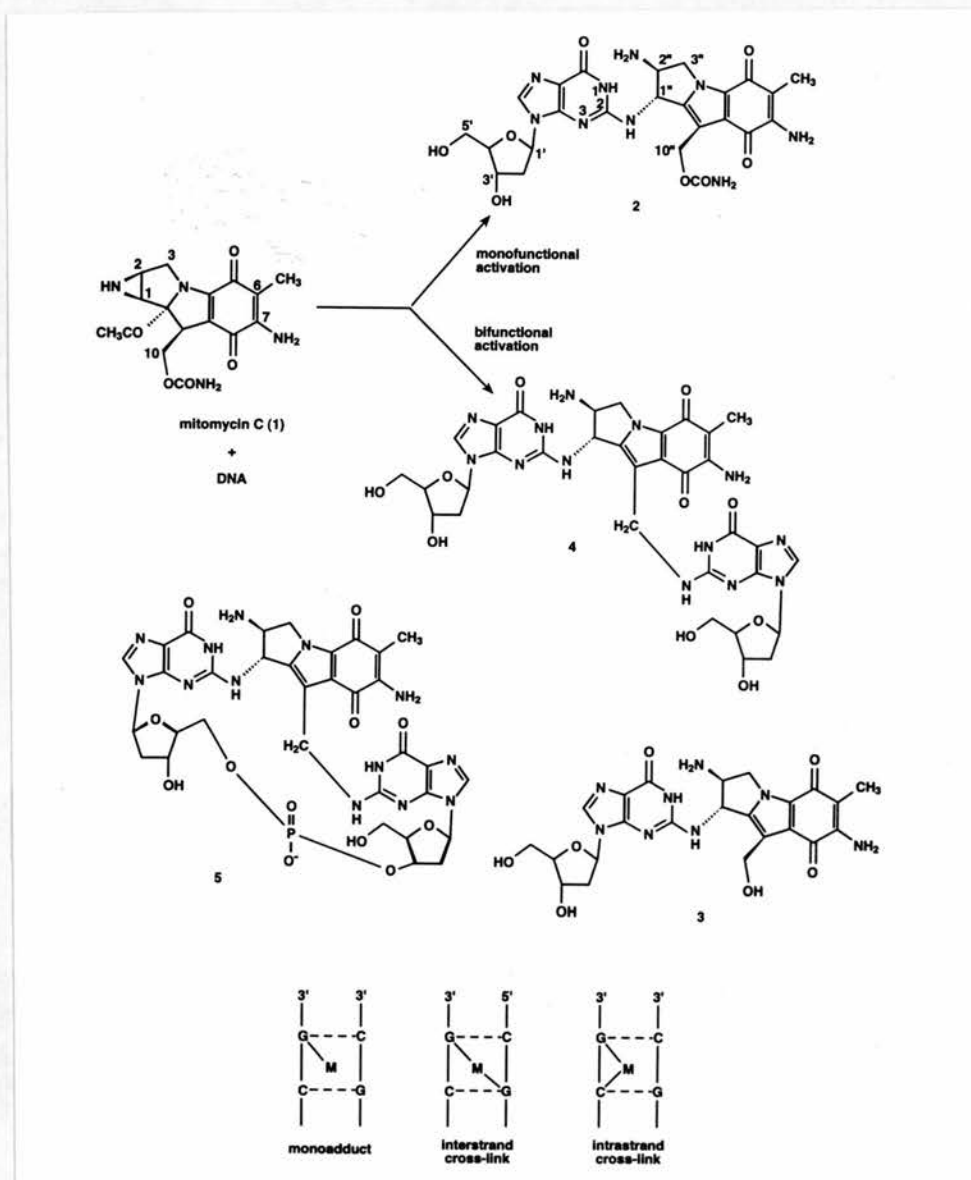


Figure 1.6. Structures of the mitomycin C-DNA adducts formed via mono- and bifunctional activation. 1, mitomycin C; 2, N2-linked mitosene monoadduct; 3, 10-decarbamoylated monoadduct; 4, interstrand cross-link adduct; 5, intrastrand cross-link adduct. Adapted from Rink *et al.*, 1996.

as a disadvantage in the treatment of tumour cells. However, such a problem may prove vital in designing cross-linking agents that are more efficient in killing tumour cells by altering their sequence specificity.

The non-covalent binding of mitomycin C directly to DNA has proved difficult to examine. Unreduced mitomycin C does not bind to DNA and the reductively activated form is too short lived to perform direct studies. Teng *et al.* (1989) however, detected binding of reduced species to synthetic oligonucleotides observing competitive inhibition of cross-linking of GC containing oligonucleotides by those containing AT base pairs and therefore no covalent drug binding sites. Kumar *et al.* (1995) have subsequently used a stable tautomeric form of the quinone methide, namely the metabolite 2,7-DM, and found that the positively charged molecule intercalated non-specifically into DNA. This intercalation has been demonstrated to hold DNA together under denaturing conditions (Peterson *et al.*, 1995). All of the above studies may represent a new mechanism of DNA damage induced by mitomycin C.

1.4.3.4 Redox Cycling and Oxygen Free Radical Formation

In the presence of oxygen a second mechanism occurs through which mitomycin C may develop its cytotoxic effect. The semiquinone free radical intermediate of mitomycin C is capable of transferring an electron to molecular oxygen. This leads to the formation of the superoxide anion

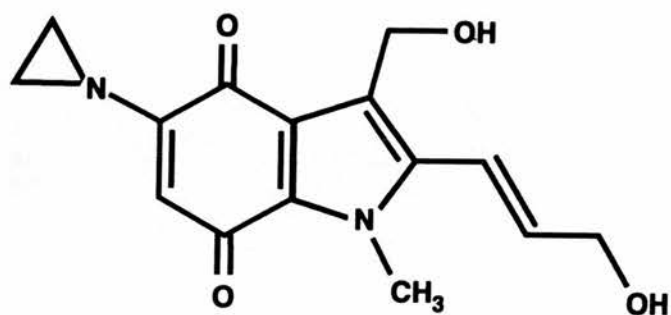
radical and regeneration of the parent molecule, mitomycin C. The repetitive reduction and reoxidation of mitomycin C under aerobic conditions is known as redox cycling. The superoxide radical is not a particularly reactive species but can give rise to the hydroxyl radical. The powerful oxidising capabilities of this radical can cause oxidation of lipids, degradation of DNA and protein damage (Halliwell & Gutteridge, 1984). The formation of the hydroxyl radical during redox cycling of mitomycin C has been demonstrated (Doroshov, 1981; Pritsos *et al.*, 1985) and subsequent lipid peroxidation has been observed with the co-incubation of mitomycin C, pulmonary microsomes and NADPH (Trush, 1982). However, although reactive oxygen species are evolved from this process, it is generally accepted as not being critical for mitomycin C antitumour activity (Powis, 1987; Butler & Hoey, 1987).

1.5 New Approaches to Bioreductive Therapy

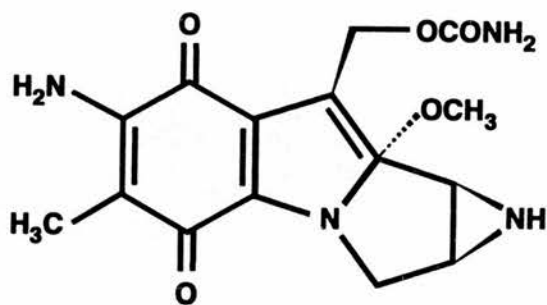
The ultimate goal in the use of any anticancer agent is to exploit a potential difference between normal and tumour tissue. Physiological differences such as those described in section 1.3 combined with the knowledge of existing bioreductive drugs can potentially lead to the rational design of highly specific anticancer agents.

1.5.1 Indoloquinone EO9

EO9 or (*E*)-5-(1-aziriny)-3-(hydroxymethyl)-2-(3-hydroxy-1-propenyl)-1-methyl-1*H*-indole-4,7-dione, figure 1.7, is the lead compound of a series of novel and fully synthetic indoloquinone cytotoxic agents structurally related to mitomycin C (Oostveen & Speckamp, 1987). However, despite its structural similarities to mitomycin C, in preclinical models EO9 exhibits a different antitumour profile. It was found to be very active *in vitro* exhibiting IC₅₀ values ranging from 1-100 ng/mL when tested against R-1 rat rhabdomyosarcoma, the human tumour cell lines A204 carcinoma, IgR-37 melanoma, human small cell lung cancer cell lines Oc-NyH/Oc-Tol (Roed *et al.*, 1989) and the National Cancer Institute (NCI) disease-orientated *in vitro* screen (Hendriks *et al.*, 1993). In the majority of cases EO9 was more potent than mitomycin C. In addition, bone marrow toxicity, a major dose limiting toxicity associated with mitomycin C was not seen in animal toxicology studies (Hendriks *et al.*, 1993).



EO 9



mitomycin C

Figure 1.7. Comparative molecular structures of analogue indoloquinone EO9 and the parent drug mitomycin C.

EO9 has since passed through Phase I and has now entered Phase II clinical trials. Initially EO9 was administered as a 5 minute intravenous infusion once every 3 weeks at a starting dose of 2.7 mg/m² (Schellens *et al.*, 1994). Results indicated that EO9 could be administered according to this schedule with tolerable and predictable toxicity. The dose-limiting toxic effect was proteinuria in coincidence with oedema development. The maximum tolerated dose was reached at 27 mg/m². Bone marrow toxicity was absent in all patients in agreement with the animal toxicity studies.

With the partial response of a number of patients with lung and liver metastases and its distinct preclinical antitumour profile, EO9 entered Phase II clinical trials (Wanders *et al.*, 1995). Patients with breast, colorectal, gastric and pancreatic cancer received a recommended weekly dose of 12 mg/m². Additional non-small cell lung cancer patients received 22 mg/m² once every three weeks. Once again proteinuria was the main toxicity recorded in 39% of patients. This was more frequent in the 3 weekly schedule (52%). Nausea and vomiting were also noted. As yet no antitumour responses have been observed.

As with mitomycin C, EO9 requires bioreductive activation via a one- or two-electron reduction pathway (Smitskamp-Wilms *et al.*, 1996). EO9 can undergo one- and two-electron reduction resulting in the formation of the semiquinone and hydroquinone respectively. Redox cycling of the

semiquinone radical under aerobic conditions yielding potential toxic superoxide radicals also occurs (Smitskamp-Wilms *et al.*, 1996). Unlike mitomycin C, no data has been published on the structure of DNA adducts, if any, formed by EO9. However, there is an indication that EO9 is capable of interacting with DNA. Walton *et al.* (1991) demonstrated that EO9 was reduced to species capable of causing single strand breaks in pBR322 plasmid DNA. This was later supported by the use of the alkaline elution technique (Bailey *et al.*, 1992a). Both techniques required DT-diaphorase. Recently, the pH and hypoxic dependence of EO9 interstrand cross-link formation for both DT-diaphorase and xanthine oxidase has been shown (Maliepaard *et al.*, 1995).

The enzymology of EO9 bioreduction is much clearer than mitomycin C with respect to the role of DT-diaphorase. Initially based on the use of DT-diaphorase preparations from rodent and human tumour cell lines (Walton *et al.*, 1991), a good correlation between DT-diaphorase expression and *in vitro* EO9 sensitivity in the presence of oxygen has been established (Phillips *et al.*, 1992; Plumb & Workman, 1994; Robertson *et al.*, 1994; Smitskamp-Wilms *et al.*, 1994; Collard *et al.*, 1995; Fitzsimmons *et al.*, 1996). The role of DT-diaphorase may explain the dose-limiting toxicity proteinuria observed during the clinical trials due to high expression of the enzyme in human kidney (Schlager & Powis, 1990).

In comparison there is little evidence, as yet, to support the role of DT-diaphorase with the *in vivo* solid tumour activity of EO9. Walton *et al.* (1992a) however, have demonstrated such a correlation using the transplantable mouse adenocarcinoma of the colon (MAC) tumours MAC 16 and MAC 26 and intraperitoneal administration of EO9. MAC 16 which exhibits a 15-fold higher level of DT-diaphorase than MAC 26 was found to be more responsive to EO9 than MAC 26 suggesting an important role for DT-diaphorase *in vivo*.

Under hypoxia the exact role of DT-diaphorase is unclear. Using the human colon cell lines HT-29 and BE, high and deficient in DT-diaphorase respectively, Plumb & Workman (1994) demonstrated a 1000-fold enhancement in EO9 cytotoxicity under hypoxia in the BE cell line compared to aerobic conditions. HT-29 only showed a 2 to 5-fold shift in hypoxic sensitivity. This was later supported by data obtained with breast, non- and small-cell lung cancer and additional colon cell lines (Robertson *et al.*, 1994). Such an observation may implicate one-electron reductases in EO9 cytotoxicity (Bailey *et al.*, 1993).

There are few studies detailing the metabolism of EO9. Acid-catalysed degradation may be a possible activation step of EO9 in the process of binding to DNA, in a manner analogous to mitomycin C (Beijnen, 1986a). Acid-catalysed degradation was shown to result in a number of products (de Vries *et al.*, 1993). Essentially, protonation of the trivalent nitrogen in

the aziridine ring results in the formation of an aziridinium ion. This electrophilic centre is then open to attack by nucleophilic species ie: water leading to the production of a degradation product possessing an ethanolamine group at C-5. This process is accompanied by a number of spectral changes.

This breakdown product has been identified as the aziridine ring open product EO5A (Workman *et al.*, 1992) and has been observed in patient samples (Schellens *et al.*, 1993; Verweij *et al.*, 1994). However, EO5A is very much less cytotoxic than EO9 and a poor substrate for DT-diaphorase (Bailey *et al.*, 1992b). Other metabolites have been detected by HPLC but as yet have not been further characterised (Maliepaard *et al.*, 1995).

Clinically EO9 has a number of possible uses. The use of EO9 as a post-irradiation sensitiser has proved beneficial with enhanced hypoxic cytotoxicity observed with EO9 administered immediately after a 10 Gy dose of x-rays (Adams *et al.*, 1992). In addition, when used in combination with the vasoactive agent hydralazine, the previously observed EO9 inactivity in the well vascularised MAC 26 murine adenocarcinoma is rendered active (Bibby *et al.*, 1993). Other uses may include the use of EO9 with interstitial photodynamic therapy, although only a minor improvement in tumour regression was seen when compared to mitomycin C (Baas *et al.*, 1994).

The high specificity of EO9 for DT-diaphorase-dependent bioactivation and an HCR of 33 (Adams *et al.*, 1992) makes it an ideal candidate for enzyme-directed bioreductive drug therapy. Its unique structure may aid in the development of structurally modified third generation analogues (Bailey *et al.*, 1992b; Philips, 1996 unpublished).

1.5.2 Benzotriazine-di-N-oxide SR 4233

SR 4233 (3-amino-1,2,4-benzotriazine 1,4-dioxide, WIN 59075) or tirapazamine is the lead compound of the benzotriazine di-N-oxide bioreductive drugs and is currently undergoing Phase I clinical testing. First reported by Zeman and colleagues (1986), it was found to exhibit differential hypoxic cytotoxicity against a number of mammalian cell lines with HCR varying from 25 to 500. As with other bioreductive drugs SR 4233 exhibits a complex mechanism of metabolic activation. Essentially, SR 4233 is metabolised via a one-electron reduction step to the radical anion SR 4233^{•-}. At this point the reaction can proceed in a number of directions. In the presence of oxygen the radical is back oxidised to the parent molecule in conjunction with the production of superoxide radicals. Under hypoxia the radical can either disproportionate resulting in SR 4233 and the stable two-electron reduction product SR 4317 or abstract hydrogen from cellular molecules producing an oxidised target and SR 4317. The damaging species of SR 4233 has been shown to be the radical anion. This is supported by evidence that SR 4317 is non-toxic

to both aerobic and hypoxic cells (Baker *et al.*, 1988; Costa *et al.*, 1989) and a decrease in hypoxic cytotoxicity is observed with the addition of DMSO, a known radical scavenger (Brown, 1990a).

The enzymes responsible for SR 4233 metabolic activation remains unclear. Initially, studies utilising mouse liver microsomes identified cytochrome P-450 as the major reductase involved based on the inhibitory effects of carbon monoxide (Walton & Workman, 1990; Walton *et al.*, 1992b). This was consequently confirmed in tumour/cell line homogenates (Wang *et al.*, 1993). However, Cahill & White (1990) and Lloyd *et al.* (1991) found no carbon monoxide-dependent inhibition using rat liver microsomes as the enzyme source and subsequently identified NADPH:cytochrome P-450 reductase as the major enzyme involved. Similar activity has also been demonstrated in a number of human breast cell lines under hypoxia and chronic exposure to air (Patterson *et al.*, 1995).

The role of other enzymes in particular DT-diaphorase remains unclear and may only play a minor part in SR 4233 bioactivation (Cahill *et al.*, 1993).

DNA is thought to be the major site of SR 4233 toxicity as a result of hydrogen atom abstraction (Baker *et al.*, 1988) and production of single strand breaks in DNA co-incubated with SR 4233 in the presence of

NADPH:cytochrome P-450 reductase have been demonstrated (Fitzsimmons *et al.*, 1994).

The use of SR 4233 has numerous potential clinical uses. Combination with vasoactive drugs such as flavone acetic acid, its analogue 5,6-dimethylxanthenone acetic acid, and hydralazine have all potentiated SR 4233 cytotoxicity in preclinical models (Sun & Brown, 1989; Cliffe *et al.*, 1994; Brown, 1987). Alternatives include combining SR 4233 with radiation (Brown & Lemmon, 1990b) and conventional chemotherapy. The latter has been found to markedly potentiate the tumouricidal effect of a number of alkylating agents with an overall therapeutic gain with minimal toxicity (Holden *et al.*, 1992). Although none of the above techniques have yet to be demonstrated in human tumours, all remain viable strategies for improving cancer treatment.

1.5.3 Nitroimidazoles

The concept of radiosensitisation using the 'oxygen mimetic' 5-nitroimidazole, metronidazole, was discovered in 1974 (Sutherland, 1974) and subsequently entered clinical trials. However, such agents displayed weak potency compared to the 2-nitroimidazoles. The initial 2-nitroimidazole, misonidazole, went through a number of clinical trials in combination with irradiation (Dische *et al.*, 1976; Thomlinson *et al.*, 1976) but dose-limiting toxicities, mainly neurotoxicity, prevented its use at

doses high enough to produce a beneficial effect. This disappointing result led to the development of etanidazole (SR 2508) (Brown & Lee, 1980) and with its lack of neurotoxicity higher doses were achieved *in vivo* (Coleman *et al.*, 1986). Pimonidazole, a more potent agent, entered Phase III clinical trials for cervical cancer but the results were once again disappointing.

A promising approach stemming from the above compounds has been the development of RSU 1069 (α -(1-aziridinylmethyl)-2-nitro-1H-imidazole-1-ethanol) (Stratford *et al.*, 1986). This compound acts as a very potent 'mixed function' sensitiser due to the presence of the sensitising nitro-group and an alkylating aziridine moiety and has demonstrated *in vitro* and *in vivo* radiosensitiser and bioreductive drug characteristics (Hill *et al.*, 1986; Stratford *et al.*, 1989). However, subsequent clinical trials showed dose-limiting gastrointestinal toxicity (Horwich *et al.*, 1986). In order to maintain the advantageous antitumour activity but reduce dose-limiting toxicities of RSU 1069, the analogue RB 6145 (α [2-bromoethyl)amino]) containing a masked aziridine moiety was developed (Jenkins *et al.*, 1990). The compound, a prodrug for RSU 1069, displayed the required radiosensitiser/bioreductive drug properties with reduced dose-limiting toxicity (Jenkins *et al.*, 1990; Sebolt-Leopold *et al.*, 1992).

Preclinical investigations have demonstrated that RB 6145's radiosensitiser/bioreductive properties can be potentiated in a number of

ways. These include x-rays (Cole *et al.*, 1990), physical clamping of the tumour, use of agents affecting oxyhaemoglobin dissociation and vasoactive agents such as hydralazine (Bremner, 1993). However, the use of RB 6145 in combination with PDT induced the largest increase in antitumour activity (Bremner *et al.*, 1994).

1.5.4 Miscellaneous Agents

Often structurally based on existing bioreductive agents, numerous new agents are being developed, each with their own specific mechanism of action. The mitomycin C analogues BMS-181174, previously designated BMY 25067 and KW-2149, have both entered clinical trial exhibiting a greater spectrum of activity including activity against mitomycin C resistant tumours in addition to reduced myelosuppression (Verweij *et al.*, 1993; Talbot *et al.*, 1994).

Other agents include the 4-alkylamino-5-nitroquinolines which have demonstrated varying degrees of hypoxic-selective toxicity (Denny *et al.*, 1992). The various chemical substitutions within the series have provided the opportunity to study the determinants of hypoxia-selectivity (Siim *et al.*, 1994). Also the novel fused pyrazine mono-N-oxides, the lead compound of which is RB90740, has shown a 20-fold enhancement in cytotoxicity under hypoxia and show great potential (Naylor *et al.*, 1993, 1995).

Other interesting compounds such as dimethyl-diaziridinylbenzoquinone, MeDZQ, were designed specifically with DT-diaphorase activation in mind. Unlike other bioreductive agents it shows little hypoxic enhancement *in vitro* but exhibits very potent *in vivo* antitumour activity against non-small cell lung cancer xenografts known to be highly refractory to most cytotoxic drugs (Cummings *et al.*, 1996).

The DNA intercalation and structural properties of the anthraquinones has led to the development of the alkylaminoanthraquinone N-oxide A4QN, a prodrug analogue of mitoxantrone (Patterson, 1989). This agent, upon hypoxia-dependent reduction to A4Q, in common with other bioreductive drugs, mediates its cytotoxic effects through high affinity drug-DNA binding but additionally possesses topoisomerase II inhibitory effects (Patterson, 1993). A4QN itself has no intrinsic binding affinity for DNA and low toxicity. A4QN may have potential when used in combination with radiation therapy (McKeown *et al.*, 1995).

Another recent approach to potentiate bioreductive therapy has been in the development of hypoxia-selective prodrugs of diffusible cytotoxins which are capable of back diffusing to kill surrounding oxygenated cells in addition to hypoxic regions (Denny & Wilson, 1993). The nitobenzyl mustard quaternary salt SN 25246 and the cobalt (III)-nitrogen mustard complex SN 42771 both show potential (Denny *et al.*, 1994; Wilson *et al.*, 1994).

1.5.5 Enzyme-Directed Bioreductive Drug Development

The general concept of enzyme-directed bioreductive drug development was first expressed in 1989 and subsequently reported a year later (Workman & Walton, 1990) and consists of two major elements. Firstly, it can be used to facilitate medicinal chemists in tailoring drugs and hence optimise prodrug activation by a particular reductase. This may be aided by the use of three-dimensional crystal structures of the reductases in association with advanced computational chemistry techniques (Li *et al.*, 1995). Secondly, so-called enzyme profiling of tumour specimens used in association with other parameters such as tumour hypoxia and pH measurement may aid in the selection of patients most likely to respond to a specific bioreductive drug. The net result is therefore to promote rational drug design/development whilst individualising patient treatment.

The concept can be approached by a variety of directions. The use of a drug against a specific tumour type known to express high levels of a particular reductase is the simplest approach. Alternatively, one could base the choice of drug on the analysis of a tumour biopsy specimen.

Which ever approach is used, a number of criteria must be fulfilled:-

a. Knowledge of a particular reductase predominating in the

activation of the drug concerned.

- b. Knowledge of the expression and activity of the reductase in tumour cell lines and biopsy specimens from patients with different tumour types.
- c. Correlation between enzyme activity and responsiveness in *in vitro* cell lines and *in vivo* human tumour xenografts.
- d. Possibilities of clinical trials to establish if the relationship is observed in humans and under what circumstances.
- e. Individual patient profiling using methods that can be easily incorporated and used in routine clinical practice.

The role of a specific reductase in the reductive activation of certain bioreductive drugs has been discussed throughout. In particular DT-diaphorase remains a very attractive target for such an approach and elevated levels of the enzyme have been reported in tumour material obtained from patients with lung, breast and colon cancers (Koudstaal *et al.*, 1975; Schlager & Powis, 1990). NADPH:cytochrome P-450 reductase and NADH:cytochrome b₅ reductase also play important roles and with the profiling of the NCI's human tumour cell line panel using techniques that could possibly be used in a clinical situation (cytochrome *c* reduction

assay, western blot analysis and messenger RNA expression) (Fitzsimmons *et al.*, 1996), it will in no doubt be a powerful asset in the future directions of enzyme-directed bio-reductive drug therapy.

1.6 Scope of the Thesis

The enzymology of mitomycin C metabolic activation has been extensively studied with the aid of purified enzymes and cell lines. The role of specific enzymes, in particular the two-electron reductase DT-diaphorase, have been clarified. However, although such studies have in no doubt provided considerable impact of the area of mitomycin metabolic activation, little evidence exists on the role of such enzymes in solid tumour tissue. This formed the basis of the thesis. The following questions were addressed:-

- (1). What enzymes are responsible for mitomycin C metabolic activation in tumour tissue *in vitro* and *in vivo*?
- (2). Is there a role for the two-electron reductase DT-diaphorase?
- (3). Can one predict which tumour types are optimum for mitomycin C treatment with regards to quinone reductase activity?

These studies were carried out using two murine adenocarcinomas of the

colon, MAC 16 and MAC 26, known to exhibit high and low levels of DT-diaphorase respectively.

EO9 is currently undergoing microsphere encapsulation for potential use as a locoregional drug formulation for the treatment of colorectal liver metastases (Gardiner *et al.*, 1996). Preliminary results indicate good antitumour activity particularly in those tumours high in DT-diaphorase.

In contrast to mitomycin C, the metabolites of EO9 have not been well characterised. Therefore, it was of interest to determine whether the metabolites of EO9 could be detected and quantified following EO9 reduction and if so, if any one metabolite correlated to the observed antitumour activity. Also could levels of any metabolite give an indication of EO9 metabolic activation with regards to specific quinone reductase expression. Tumour models used were once again MAC 16 and MAC 26 in addition to the human colon xenografts, HT-29 and BE. The latter tumours expressed high and low levels of DT-diaphorase respectively.

Finally from a biophysical perspective, the properties of the short lived reactive intermediates of EO9 were examined in order to determine the cytotoxic form(s) of EO9 produced after bioreduction.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 MATERIALS

All reagents used were of the highest grade commercially available from suppliers listed in Appendix 1 eg: Aristar grade from BDH Merck Ltd. and HPLC grade from Rathburn Chemicals Ltd. Reagents were made up in distilled water purified to Grade II (ISO 3696) equivalent to double distilled water obtained from a Milli-U10 water purification system.

2.2 APPARATUS

All suppliers of apparatus used throughout are listed in Appendix 1.

2.2.1 High-Performance Liquid Chromatography

The liquid chromatograph used throughout was a Hewlett-Packard Model 1090 equipped with the following features; a PV5 ternary low-pressure mixing solvent delivery system; a variable volume automatic injector and autosampler, 10-250 μ l injection volume; a heated column compartment and multi-diode rapid scanning UV/Vis spectrophotometric detector. System control and data evaluation were performed by a Hewlett-Packard Series 9000 300 "Chemstation" computer.

2.2.2 Linear Accelerator

The linear accelerator facility was based at the Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester, U.K. The system consisted of a "L" band linear accelerator capable of producing a beam of pulsed electrons with an energy between 8 and 12 MeV and variable pulse widths from 5 ns to 5 μ s. The optical detection system consisted of a high stability xenon arc or tungsten halogen lamp, Kratos GM 252 high intensity quarter meter monochromator and an EMI 9558QA photomultiplier. Flow-through capillary cells were composed of high purity fused silica cylindrical microbore tubing, internal diameter 3 mm, with Spectrosil quartz windows. Optical path lengths between 0.4 and 2.5 cm were used.

Signals from the photomultiplier were recorded on a dual channel Tektronix 7612AD programmable digitiser and processed using a Hewlett Packard Series 200 model 9836S computer. Stopped flow measurements used an Applied Photophysics RX1000 stopped-flow apparatus coupled to HP 8452A diode array spectrophotometer.

2.2.3 High-Performance Liquid Chromatography-Mass Spectroscopy

The high-performance liquid chromatography-mass spectroscopy facility was based at the Paterson Institute for Cancer Research, Christie Hospital

and Holt Radium Institute, Manchester, U.K. The system used consisted of a Trio 2 mass spectrometer (VG MicroMass) in conjunction with a Gilson 307 manually operated HPLC pump and Waters 484 MS UV/Vis detector. VG Masslynx and MaxEnt were used for software purposes.

2.2.4 Human Colon Adenocarcinoma Cell Line BE and Establishment of BE Xenograft

The human colon carcinoma cell line BE was kindly supplied by Dr. Neil W. Gibson, Pfizer Inc. Groton, U.S.A. via Dr. Jane Plumb, CRC Department of Medical Oncology, Glasgow, U.K. and was grown as a monolayer culture in a 50:50 mixture of Ham's F10 and Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine and 10% foetal calf serum. Penicillin-streptomycin solution was added to a final concentration of 100 IU to prevent bacterial contamination. Cells were grown in 175 cm² tissue culture flasks in a humidified atmosphere of 95 % oxygen and 5 % carbon dioxide at 37°C (Scotlab VSL incubator). Once the cells had reached late log phase, cells were trypsinised with 3 mL trypsin (0.25 %):versene (1 mM EDTA, 0.5 % phenol red) (1:1) until the cells had rounded up and detached. Trypsinisation was neutralised by the addition of 7 mL 50:50 Ham's F10:DMEM to each flask. Samples were transferred into 20 mL sterile universal containers and centrifuged at 1000 g for 5 minutes to pellet down the cells. The media were discarded and each pellet was resuspended in 5 mL sterile phosphate buffered saline

(PBS) then all transferred into one universal container which was centrifuged once again. The supernatant was discarded and the cell pellet resuspended in 20 mL PBS and syringed using a 21 gauge needle and 20 mL syringe to produce a single cell suspension.

0.5 mL of the single cell suspension was added to 5 mL PBS (dilution factor $\times 11$), mixed thoroughly and cell number counted using an Improved Neubauer haemocytometer. The original cell suspension was centrifuged once again, supernatant discarded and the cell pellet was left undisturbed covered by a few drops of PBS ready for implantation into nu/nu nude mice. Approximately 1×10^8 cells were available for implantation.

All of the above procedures were carried out under sterile conditions in M.D.H. Interlab Class II tissue culture hoods.

3 nu/nu mice in total were implanted with BE cells subcutaneously into the flank of the animal. Once the "tumour" material became palpable, the animals were sacrificed, "tumours" removed and samples were sent to Department of Pathology, Western General Hospital, Edinburgh, to confirm that the tissue was of tumour origin and not due to an inflammatory response. When establishment of the tumour was confirmed, routine passaging of the tumour was carried out as detailed in section 2.2.5b. At every third passage all tumours were examined

histologically to confirm tumour status.

2.2.5 Animal Models

a. Murine Tumour Models

The murine model used was the inbred NMRI mouse and the subcutaneously murine adenocarcinoma of the colon, MAC 16 or MAC 26, (original tumours and breeding pairs kindly supplied by Professor J.A. Double and Dr. M.C. Bibby, Clinical Oncology Unit, University of Bradford, Bradford U.K). All mice were fed Teklad mouse diet and water was available freely. Tumours were maintained by passaging 1-3 mg lumps via trochar needle to male animals weighing between 25 and 30 g. When tumours reached 0.15-0.2 g, animals were either randomised for experimental studies or sacrificed for tumour removal. All tumours were stored at -80°C prior to use.

b. Human Xenograft Tumour Models

The animal model used was the nu/nu nude mouse (originally bred at Imperial Cancer Research Fund (ICRF), London) supplied by Harlan U.K. Ltd. and the subcutaneously human adenocarcinomas, HT-29 or BE. Mice were housed in Morden isolators and fed on Teklad mouse diet. Water was available freely. Tumours were maintained by passaging 0.5-1 mg

lumps via trochar needle to male animals weighing between 20 and 25 g. When tumours reached 0.1-0.2 g, animals were sacrificed for tumour removal. Again all tumours were stored at -80°C prior to use.

2.3 METHODS

2.3.1 Isolation of Mitochondrial, Microsomal and Cytosolic Subcellular Fractions from MAC 16 & MAC 26 Murine Adenocarcinomas and HT-29 & BE Human Adenocarcinomas of the Colon

Isolation of tumour subcellular fractions was carried out using a standard differential ultracentrifugation method in 0.25 M sucrose using a modification of the previously described method (Cummings *et al*, 1992). Whole tumours, 0.5-1.5 g, were first washed in saline then transferred to ice cold 0.25 M sucrose, 5 mM Tris-buffer, 0.5 mM EDTA (STE), pH 7.4 in which a homogenate was produced (33% w/v). Portions of the homogenate were either used directly for drug incubation (sections 2.3.6 & 2.3.14) or processed to isolate mitochondrial, microsomal and cytosolic fractions. Homogenates were first centrifuged at 600 g (Beckman GPR centrifuge) for 10 minutes to remove cellular debris and the resulting supernatant subjected to a high speed spin of 24,000 g (Beckman L7 ultracentrifuge) for 10 minutes to isolate the mitochondria. The supernatant was retained for microsomal isolation.

The mitochondrial pellet was resuspended in STE solution and centrifuged once again at high speed. The final pellet was resuspended in 5 mL STE solution. Microsomes were isolated from the post-mitochondrial supernatant by centrifugation at 80,000 g (Beckman L7

Ultracentrifuge) for 1 hour. The supernatant was retained for isolation of the cytosolic fraction.

The resulting microsomal pellet was resuspended in 0.1 M sodium phosphate pH 7.4 and centrifuged once again at 80,000 g. The final pellet was resuspended in 5 mL 10 mM Tris-HCl buffer pH 7.4 containing 20% glycerol and 0.1 mM EDTA. The post-microsomal supernatant was centrifuged at 80,000 g for 4 hours. The resulting supernatant, cytosol, was made up to 10 mL with STE solution.

All centrifugation steps were carried out at 4°C and samples kept on ice at all times to prevent enzyme degradation. Subcellular pellets were resuspended using hand-held glass homogenisers. Samples were stored in 1 mL aliquots at -80°C for no more than one month and freeze-thaw repetitions avoided.

HT-29 and BE subcellular fractions were prepared purely for determination of tumour quinone reductase levels and not for drug incubations.

2.3.2 Protein Determination of Tumour Homogenates and Subcellular Fractions

Protein concentrations of all homogenates and subcellular fractions were

determined using the Biuret method (Layne, 1957) using bovine serum albumin (BSA) as a standard. Standards containing 0-10 mg protein were prepared from a 10 mg/mL stock solution and test samples diluted 1 in 10 with distilled water before analysis. 4 mL Biuret reagent was then added to 1 mL standard or diluted subcellular fraction/homogenate, vortexed and incubated at room temperature for 30 minutes. Each sample and standard was carried out in duplicate. The absorbance of each sample at 550 nm against distilled water was determined spectrophotometrically using a Unicam UV/Vis spectrophotometer. The extinction ΔE 550 was then calculated for all standards and samples. Sample protein concentrations were calculated from the standard curve (ΔE 550 versus mg protein) and expressed as mg protein.

2.3.3 Quinone Reductase Assessment of Tumour Subcellular Fractions

Quinone reductase activity was assessed in MAC 16 /MAC 26 and HT-29/BE subcellular fractions following the spectrophotometric reduction of cytochrome *c* at 550 nm (Unicam UV/Vis spectrophotometer) using a modification of the method of Ernster, 1967, as described elsewhere (Riley & Workman, 1992). The reaction mixture contained 65-100 μ g cytosolic or 17-33 μ g microsomal protein, 77 μ M cytochrome *c*, 20 μ M menadione as an intermediate electron acceptor, 2 mM NADH or NADPH as cofactor and 0.14% w/v BSA. Reactions were performed at 37°C in a total volume of 1 mL 50 mM Tris-HCl buffer, pH 7.4 in 1 mL plastic cuvettes in the

presence or absence of 10 μ M dicoumarol. Control cuvettes contained all reagents with the exception of the cytosolic/microsomal protein. Cuvettes were maintained at 37°C using a water circulating Unicam Cell Temperature Controller and Unicam UV5-220 Thermostatted cell holders. Each reaction was carried out in triplicate.

Cytosolic and microsomal DT-diaphorase activity was taken as the activity that could be inhibited by dicoumarol in the presence of either cofactor. Other cytosolic activity may include xanthine dehydrogenase/oxidase and aldoketo reductase. NADPH:cytochrome P-450 reductase was calculated as NADPH-dependent microsomal activity. NADH:cytochrome b_5 reductase was calculated as the residual NADH-dependent microsomal activity unaffected by dicoumarol inhibition. Activities were expressed as nmol cytochrome c reduced/min/mg protein using the extinction coefficient ϵ 21.1×10^3 M/cm. Mitochondrial quinone reductase levels were not assessed due to the presence of cytochrome c oxidase.

2.3.4 Electron Microscopy of MAC 16 and MAC 26 Mitochondrial and Microsomal Subcellular Fractions

Subcellular fractions were prepared as described in section 2.3.1 with the exception of the following. After the initial spin at 24,000 g for isolation of the mitochondria, the supernatant was retained for isolation of

tumour microsomes. The mitochondria were resuspended in 10 mL fixation buffer, 3% glutaraldehyde in 0.1 M sodium cacodylate/HCl pH 7.4. Samples were incubated in this solution for 30 minutes, mixing occasionally, before subjecting to another high speed spin at 24,000 g. The same procedure was used for isolation of the microsomes with fixative added after the initial 80,000 g centrifugation step. Once fixation was complete samples were stored in fixative at 4°C for a minimum of 2 hours.

Fixed samples were first washed with distilled water for 3 x 20 minutes to remove primary fixative. This was followed by a secondary fixation step using 1% osmium tetroxide incubated at room temperature for 45 minutes and subsequent dehydration in increasing methanol concentrations (50, 75, 100%) and absolute ethanol. Formation of the sections were carried out by 30 minutes propylene oxide linking, impregnation overnight with Emix resin and polymerisation at 70°C for 18-24 hours.

90 nm sections were cut using a glass knife microtome and mounted on copper grids. Section staining was carried out using a standard uranyl acetate/lead citrate method.

Sections were viewed by a Jeol 100CXII transmission electron microscope and photographed using Kodak 4489 film.

2.3.5 *In Vitro* Metabolism of Mitomycin C by Tumour Subcellular Fractions

Incubations were carried out in 50 mL tapered glass tubes at 37°C under aerobic and hypoxic conditions. Each incubation mixture contained approximately 1 mg MAC 16 or MAC 26 derived mitochondrial, microsomal or cytosolic protein, 3.3 mM NADH, NADPH or hypoxanthine acting as cofactors and 100 µg/mL mitomycin C. Both cofactors and drug were made up in 0.1 M sodium phosphate buffer, pH 7.4. Protein and cofactor mixtures were allowed to equilibrate to 37°C for 10 minutes prior to the addition of mitomycin C. For hypoxic incubations, mixtures were sparged with helium for 5 minutes prior to drug addition. Hypoxic environments were maintained with a flow of helium gas delivered via a rubber stopper and glass pastette and allowed to vent off through an outlet hole.

In the case of inhibitor incubations, the enzyme inhibitors dicoumarol and the polyclonal cytochrome P-450 reductase antiserum CH59 (kindly supplied by Professor C.R. Wolf, ICRF Molecular Pharmacology Unit, Dundee) were added. 10 µM dicoumarol, made up in 0.1 M sodium phosphate pH 7.4: 0.1 M sodium hydroxide 50: 50 and antiserum, 1 in 100 dilution, were added 30 minutes before the start of the reaction. The inhibitory properties of cytochrome P-450 reductase antiserum on quinone reductase activity were assessed using the quinone reductase

assay detailed in section 2.3.3. The concentration of antiserum giving 50 % inhibition of NADPH-microsomal dependent cytochrome *c* reduction was used (1 in 100 dilution).

After the addition of mitomycin C, a 100 μ L aliquot was withdrawn from the incubation at 15 minute intervals (0-90 minutes) and 20 μ L subjected immediately to HPLC to determine the concentration of mitomycin C and its metabolites at 360 and 310 nm respectively. Each incubation was carried out in triplicate. Reaction rates were calculated from the linear portions of the reaction curves and expressed as 2,7-DM formation in nmol/30min/mg protein.

Control incubations containing all components of the incubation mixture, with the exception of subcellular protein, were carried out under aerobic and hypoxic conditions in duplicate.

2.3.6 *In Vitro* Metabolism of Mitomycin C by Tumour Homogenates

MAC 16/MAC 26 and HT-29/BE homogenates were prepared as detailed in section 2.3.1. Drug incubations consisted of 100 μ g/mL mitomycin C made up in 0.1 M sodium phosphate buffer, pH 7.4 and 0.9 mL tumour homogenate. The incubation procedure follows that detailed in section 2.3.5 with the exception of the addition of enzyme inhibitors. At 15 minute intervals 100 μ L samples were withdrawn, mixed with 100 μ L

methanol and centrifuged in an Eppendorf microcentrifuge at 15,000 g for 2 minutes to remove tumour protein. 20 μ L of the supernatant was then subjected to HPLC analysis monitoring at 360 and 310 nm. Each incubation was carried out in triplicate. Reaction rates were calculated from the linear portions of the reaction curves and expressed as 2,7-DM formation in nmol/30min/mg protein.

Again, control incubations containing all components of the incubation mixture with the exception of tumour homogenate, were carried out under aerobic and hypoxic conditions.

2.3.7 High-Performance Liquid Chromatography Analysis of Mitomycin C and its Metabolites

Chromatographic conditions were modified from those previously described (Cummings *et al*, 1993). The stationary phase was a LiChrosorb RP-18 HPLC cartridge, 7 μ m particle size, column dimensions 25 cm x 4 mm and a LiChrospher 100 RP-18 precolumn, 5 μ m particle size, column dimensions 4 x 4 mm enclosed in a LiChrocart Manu-fix cartridge holder. The mobile phase consisted of 10 mM sodium phosphate buffer, pH 7.5 and methanol at a ratio of 76:24. Elution was isocratic at a flow rate of 1 mL per minute with the oven temperature maintained at 40°C. The mobile phase was filtered before use through 0.2 μ m filters and sparged continuously with helium throughout chromatographic analyses. Results

were recorded using the diode array detector at 360 nm for the parent drug and 310 nm for its metabolites.

The assay was standardised using mitomycin C and its primary metabolites 2,7-DM, trans- and cis-hydro as synthesised and purified in section 2.3.8.

2.3.8 Synthesis And Purification of Mitomycin C Primary Metabolites

Production of the major metabolite 2,7-DM was achieved by irradiation of mitomycin C. This was kindly carried out by Dr. John Butler, Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester, U.K. using the linear accelerator facility. The mitomycin C/metabolite mixture was initially concentrated using solid phase extraction. Essentially, a 5 g C2 Bondesil column was prepared and activated using 1 column volume of methanol. The column was then washed with distilled water immediately followed by the mitomycin C/metabolite mixture. The column was washed once again with distilled water to remove any unretained products, dried under vacuum and the sample eluted with approximately 15 mL methanol. The sample was concentrated to 3-5 mL under a stream of nitrogen. 2,7-DM was isolated from this concentrated mixture by preparative HPLC using the conditions described in section 2.3.7. Each distinct peak was collected using an online

Gilson fraction collector. The peak corresponding to 2,7-DM, retention time approximately 12 minutes, was purified from the HPLC mobile phase by solid extraction as above. The final methanol eluant was dried under nitrogen, checked for purity by HPLC and stored at 4°C.

The hydrolysis products trans- and cis-hydro were synthesised in house. 10 mg mitomycin C was subjected to acid hydrolysis by addition of 2 mL 0.1 M hydrochloric acid. The sample was maintained at room temperature for 25-35 minutes before stopping the reaction by placing the sample on ice. Retrieval of the sample was by the solid phase extraction method described above. Fractionation was essentially the same with the peaks of interest eluting at approximately 5 minutes for trans-hydro and 7-8 minutes for cis-hydro. Samples were purified once again using solid phase extraction, dried and stored at 4°C.

2.3.9 Extraction of Mitomycin C and its Metabolites from Tumour Tissue and Plasma

a. Tumour Tissue

MAC 16 and MAC 26 tumours were partially thawed on ice, weighed and to a known tumour weight, 154 mM potassium chloride solution added in proportions 1:2 w/v. Tumours were homogenised using a tissue homogeniser rinsing the cutting device between each tumour. 1 mL of

the resulting homogenate was extracted with 5 mL chloroform:propan-2-ol:ethyl acetate (2:2:1) in 50 mL tapered glass centrifuge tubes by vortexing constantly for 15 minutes at room temperature. Samples were centrifuged at 1000 g (Beckman GPR centrifuge) for 15 minutes at 4°C prior to removal and retention of the drug containing solvent layer. The remaining aqueous layer was extracted once again with a further 5 mL of solvent. The combined solvent layers were then evaporated to dryness under a stream of nitrogen and the resulting residues reconstituted with 300 µl 10 mM sodium phosphate pH 7.5:methanol, 74:26. Finally, samples were vortexed for 2 minutes, centrifuged at 15000 g in an Eppendorf microcentrifuge and filtered before HPLC analysis of 100 µL.

b. Plasma

Plasma samples were totally thawed prior to extraction of mitomycin C/metabolites. The procedure was essentially the same with the total volume of plasma obtained extracted twice with 2 mL chloroform:propan-2-ol:ethyl acetate (2:2:1) rather than 5 mL.

2.3.10 Pharmacokinetics of Mitomycin C in MAC 16 and MAC 26 Murine Adenocarcinomas of the Colon

MAC 16 or MAC 26 bearing NMRI mice were maintained as described in section 2.2.5a. Animals were randomized into groups of 3 and injected i.t.

with 500 µg free mitomycin C in a final volume of 0.25 mL distilled water. Mitomycin C consisted of preweighed vials containing 2 or 10 mg as a sterile powder. The animals were then sacrificed at the following times after drug administration:- 0, 1, 5, 15, 30, 45, 60, 120 and 360 minutes for both MAC 16 and MAC 26, tumours removed and immediately placed in liquid nitrogen.

Blood samples collected were centrifuged at 15000 g for 5 minutes in an Eppendorf microcentrifuge to obtain plasma. Plasma was removed, transferred to a cryotube and placed in liquid nitrogen. Plasma samples were not collected for the following time points:- 0 and 1 minutes since the time taken to sacrifice the animal and remove the blood sample exceeded 5 minutes. All samples were stored at -80°C for a maximum of 2 weeks prior to parent drug and metabolite extraction.

Peak levels and time to peak levels of mitomycin C and its metabolites were taken directly from concentration-time profiles. Area under concentration/time curves ($AUC_{0-360 \text{ min}}$) was calculated by the trapezoidal rule. Clearance was calculated as dose/AUC. The *in vivo* metabolic conversion of mitomycin C to 2,7-DM in both tumour types was calculated as the AUC ratio MMC:2,7-DM.

2.3.11 Antitumour Activity of Mitomycin C in MAC 16 and MAC 26 Murine Adenocarcinomas of the Colon

Tumour bearing animals were randomised into 5 groups of 8 consisting of the following dosage/control groups:- 50, 75, 125, 250, 500 and 1000 µg mitomycin C, vehicle control (distilled water) and untreated control. Mitomycin C or distilled water were injected i.t. as a single dose in a total volume of 0.25 mL. Approximately every two days, the mice were weighed and tumour volume (V) determined by measuring two diameters at right angles and calculated in cm³ using the formula $V = \pi/6 \times \text{length} \times \text{width}^2$. Once the study was complete, the animals were sacrificed, tumours removed and stored at -80°C.

Antitumour activity for a treatment group was expressed as percentage increase/decrease in tumour volume compared to day 0 (100%). The antitumour activity of mitomycin C compared to control, tumour/control (T/C) values, were calculated for each dose at day 7.

2.3.12 Histological Preparation of MAC 16 and MAC 26 Murine Adenocarcinomas of the Colon

Tumours no less than 0.5 cm diameter were initially fixed in 4% paraformaldehyde solution for a minimum of 24 hours and subsequently embedded in paraffin wax ready for sectioning. 12 sections of each tumour

type (3 per slide), approximately 3 μm thickness were prepared, mounted on non-coated glass slides and dried at 60°C overnight. Sections were then ready for staining with haematoxylin and eosin.

Sections were de-waxed in xylene for 2 x 4 minutes followed by rinses in decreasing alcohol concentrations (absolute, 74%, 64%) and water. The rehydrated sections were first stained with Harris's haematoxylin for 3-4 minutes or until the nuclei were stained and then rinsed once again in water. Excess haematoxylin was removed by rinsing in acid alcohol for 10-15 seconds followed by a water wash until all alcohol had been removed. Sections were placed in lithium carbonate until a colour change from red-brown to blue was observed, then washed in water for 2-3 minutes. Counterstaining was achieved by staining with alcoholic Y eosin solution for 1 minute. Slides were rinsed in water, dehydrated in increasing concentrations of alcohol (64%-absolute), rinsed in xylene, air dried and mounted using DPX mounting media. Sections were then ready for morphological assessment. Sections were examined using a Leitz Dialux 20 microscope equipped with Leitz 10, 40 and 100 objectives and light box. Sections were optimally seen using a blue filter to reduce the redness of the section. Photographs were taken with a Wild 51 S camera and MPS 45 photoautomatic controller using Kodak Ektar 25 tungsten balanced film at a magnification of 12.5.

2.3.13 High-Performance Liquid Chromatography Analysis of Indoloquinone EO9

Chromatographic conditions were essentially the same as those used for mitomycin C analysis as described in section 2.3.7 with the exception of the following. Results were recorded using the diode array detector at 280 nm for both EO9 and its metabolites. The assay was standardised with EO9 only.

2.3.14 *In Vitro* Metabolism of Indoloquinone EO9 by Tumour Homogenates

MAC 16/MAC 26 and HT-29/BE homogenates were prepared as detailed in section 2.3.1. Drug incubations consisted of 100 µg/mL EO9 made up in 0.1 M sodium phosphate buffer, pH 7.4 and 0.9 mL tumour homogenate. Incubations were carried out under both aerobic and hypoxic conditions as described in section 2.3.6. At 45 minute intervals, 100 µL samples were withdrawn, mixed with 100 µL methanol and centrifuged in an Eppendorf microcentrifuge at 15,000 g for 2 minutes to remove tumour protein. 20 µL of the supernatant was then subjected to HLPC analysis. Each incubation was carried out in triplicate.

Control incubations containing all components of the incubation mixture, with the exception of tumour homogenate, were carried out

under aerobic and hypoxic conditions in duplicate.

2.3.15 Degradation of Indoloquinone EO9 under Alkaline Conditions

Reactions were carried out using an adaptation of the method of de Vries *et al.* (1993). Essentially, a 1 mM solution of sodium hydroxide, pH 12, containing 100 µg EO9 was incubated at room temperature. At 30 minute intervals, a 100 µL aliquot was withdrawn from the reaction mixture and 20 µL analysed by HPLC for the presence of any degradation products.

2.3.16 Reaction of Indoloquinone EO9 with Reduced Glutathione

PBS containing 2 mM reduced glutathione was sparged with helium for 10 minutes. The PBS was previously filtered and degassed using 0.2 µM filters. 100 µg EO9 was subsequently added. At 30 minute intervals a 100 µL aliquot was withdrawn from the reaction and 20 µL analysed by HPLC. All reactions were carried out at room temperature and solutions continuously sparged with helium throughout.

2.3.17 High-Performance Liquid Chromatography-Mass Spectroscopy of Indoloquinone EO9 and Intermediates Generated by Irradiation

High-performance liquid chromatography-mass spectroscopy was carried out at the Paterson Institute as described in section 2.2.3. A sub-saturated

solution of EO9 in 10 mM phosphate buffer pH 7 containing 0.1M isopropanol was sparged with argon and subsequently irradiated with 500 Gy using the linear accelerator described in section 2.2.2. The irradiated sample was mixed with air and 100 μ L injected into the mass spectrophotometer. Samples were initially analysed by the mass spectrophotometer via a LiChrosorb RP-18 HPLC cartridge, 7 μ m particle size, column dimensions 25 cm x 4 mm and LiChrospher 100 RP-18 precolumn, 5 μ m particle size, column dimensions 4 x 4 mm and Waters 484 MS UV/Vis detector. The mobile phase consisted of water in 25% acetonitrile. Analyses were carried out at room temperature at a flow rate of 1 mL per minute. Additional samples were injected directly into the mass spectrophotometer. For both analyses, the following positive atmospheric pressure chemical ionisation (APCI +ve) parameters were used:-

Probe voltage	3.48 KV
Counter voltage	0.43 KV
Cone voltage	32 V
Lens 2	40 V
Lens 3	12 V
Source temperature	120°C
Probe temperature	460°C

2.3.18 Generation of Indoloquinone EO9 Reactive Intermediates by Pulse Radiolysis

Pulse radiolysis experiments were conducted at the Paterson Institute linear accelerator facility as described in section 2.2.2. All pulse radiolysis solutions contained 0.1 M sodium formate buffered using 10 mM phosphate, pH 4.5 - 9.0 or 10 mM borate/sodium hydroxide, pH 8.5-10.0. Pulse lengths were less than 0.1 μ s. Kinetic analysis was carried out using the Fig P program assuming second order kinetics. All experiments were performed at $21 \pm 2^\circ\text{C}$.

Generation of free radical intermediates was initially carried out in argon saturated solutions containing 20 μ M - 2 mM EO9 in 100 mM sodium formate solution pH 4.5 - 10.0. Pulses of 20 μ s were used.

The reduction potential of EO9 was assessed at pH 7.4 and was determined by setting up an equilibrium between the semiquinone free radicals of EO9 and mitomycin C monitoring at 415 and 490 nm. 100 μ M mitomycin C was used in conjunction with varying concentrations of EO9, 10-40 μ M. Autoxidation of EO9 was studied using 20-50 μ M EO9 initially reduced in one half of a stopped flow mixing chamber with 0.6 U xanthine oxidase, 100 μ M hypoxanthine in 0.1 M phosphate buffer, pH 7.4, until the solution became colourless. All solutions were argon saturated. The reduced solution was then rapidly mixed with an air saturated solution

containing 200 μM allopurinol and 2 μM EDTA in 0.1 M phosphate buffer, pH 7.4. Total time for the initial reduction and subsequent exposure to air was less than 10 minutes.

CHAPTER 3

3. RESULTS AND DISCUSSION

3.1 Characterisation of a Novel Mitochondrial Reductase

The aim of this section of the thesis was to characterise the enzyme(s) responsible for mitomycin C bioreduction in MAC 16 and MAC 26 murine adenocarcinomas to provide an insight into the drug's *in vivo* mechanism of action. MAC 16 and MAC 26 subcellular fractions were prepared as detailed in section 2.3.1 and subsequently used for quinone reductase determination and mitomycin C bioreduction studies.

The quinone reductase profile of the cytosolic and microsomal fractions isolated from both tumours are shown in table 3.1. MAC 16 exhibited a 22-fold higher level of cytosolic DT-diaphorase but similar levels of microsomal NADPH:cytochrome P-450 reductase to those of MAC 26, consistent with previously published data (Walton *et al.*, 1992a). 'Other' cytosolic enzymes, which may include xanthine oxidase and aldoketo reductase were also calculated, with MAC 16 possessing approximately 8-fold greater levels of these enzymes compared to MAC 26. In addition, both tumour types exhibited similar levels of microsomal NADH:cytochrome *b*₅ reductase. No microsomal DT-diaphorase was present in MAC 26. Mitochondrial quinone reductase activity could not be assessed due to the presence of cytochrome *c* oxidase. As the assay relies upon the reduction of cytochrome *c*, the reduced form will be re-oxidised.

TUMOUR	CYTOSOL		MICROSOMES			
	DT-diaphorase NADH	'Others' NADPH	DT-diaphorase NADH	Cytochrome P450 reductase NADPH	Cytochrome <i>b</i> ₅ reductase NADH	
MAC 16	447.13±19.2	328.46±20.5	5.12±0.9	9.69±3.1	17.21±2.1	18.41±0.9
MAC 26	19.33±5.9	15.67±3.6	ND	ND	14.82±1.4	16.89±4.9

Table 3.1. Quinone reductase activity in cytosolic and microsomal subcellular fractions isolated from MAC 16 and MAC 26 murine adenocarcinomas of the colon. All enzyme activities are expressed as nmol cytochrome *c* reduced/min/mg protein using the extinction coefficient, ϵ 21.1 × 10³M/cm. Each value represents the mean±SE from three separate experiments. ND = not detected.

Mitomycin C bioreduction in MAC 16 and MAC 26 subcellular fractions was measured by monitoring the formation of the principle metabolite 2,7-DM. This metabolite was used as an indicator of the rate of metabolic activation of mitomycin C as it can only be formed after quinone reduction rather than acid catalysed degradation (Beijnen *et al.*, 1986b). In addition, levels of 2,7-DM have been demonstrated to correlate with cytotoxicity in human colon carcinoma cell lines (Siegel *et al.*, 1990a) and antitumour activity in MAC 16 (Cummings *et al.*, 1994) and Sp 107 rat mammary carcinoma (Chirrey *et al.*, 1995). Table 3.2 and 3.3 illustrate mitomycin C bioreduction in MAC 16 and MAC 26 subcellular fractions and revealed a number of enzyme activities present within all three fractions of MAC 16 compared to one in MAC 26.

MAC 16 cytosolic activity utilised both NADH and NADPH as cofactors, showing similar activity under aerobic and hypoxic conditions. The chromatographic profiles of mitomycin C metabolism by MAC 16 cytosol under aerobic and hypoxic conditions in the presence of NADH or NADPH are illustrated in figure 3.1 and 3.2 respectively. 2,7-DM could be clearly seen at approximately 10.2 minutes. Significant levels of other metabolites were not seen. The addition of 10 μ M dicoumarol, a known inhibitor of the two-electron reductase DT-diaphorase (Lind *et al.*, 1982a), resulted in 100 % inhibition of 2,7-DM formation. Addition of cytochrome P-450 reductase antiserum had no inhibitory effect. No cytosolic activity was observed in MAC 26.

TUMOUR FRACTION	2,7-DM FORMATION (nmol/30min/mg protein)	COFACTOR REQUIREMENT	EFFECT OF OXYGEN	EFFECT OF 10µM DICOUMAROL	EFFECT OF ANTISERUM
CYTOSOL	0.76 ± 0.10	NADH	No effect	100% inhibition	No effect
	1.04 ± 0.17	NADPH	No effect	100% inhibition	No effect
	0.00	Hypoxanthine	-	-	-
MICROSOMES	0.00	NADH	-	-	-
	2.04 ± 1.09	NADPH	100% inhibition	No effect	100% inhibition
MITOCHONDRIA	0.93 ± 0.09	NADH	100% inhibition	No effect	No effect
	3.67 ± 0.58	NADPH	100% inhibition	No effect	No effect

Table 3.2. Characterisation of mitomycin C bioreduction in MAC 16 murine adenocarcinoma of the colon. Specific activity is expressed as 2,7-DM formation in nmol/30min/mg protein. Each value represents the mean±SE from three separate experiments.

TUMOUR FRACTION	2,7-DM FORMATION (nmol/30min/mg protein)	COFACTOR REQUIREMENT	EFFECT OF OXYGEN	EFFECT OF 10 μ M DICOUUMAROL	EFFECT OF ANTISERUM
CYTOSOL	0.00	NADH	-	-	-
	0.00	NADPH	-	-	-
	0.00	Hypoxanthine	-	-	-
MICROSOMES	0.00	NADH	-	-	-
	0.00	NADPH	-	-	-
MITOCHONDRIA	0.00	NADH	-	-	-
	3.87 \pm 0.71	NADPH	100% inhibition	No effect	No effect

Table 3.3. Characterisation of mitomycin C bio-reduction in MAC 26 murine adenocarcinoma of the colon. Specific activity is expressed as 2,7-DM formation in nmol/30min/mg protein. Each value represents the mean \pm SE from three separate experiments.

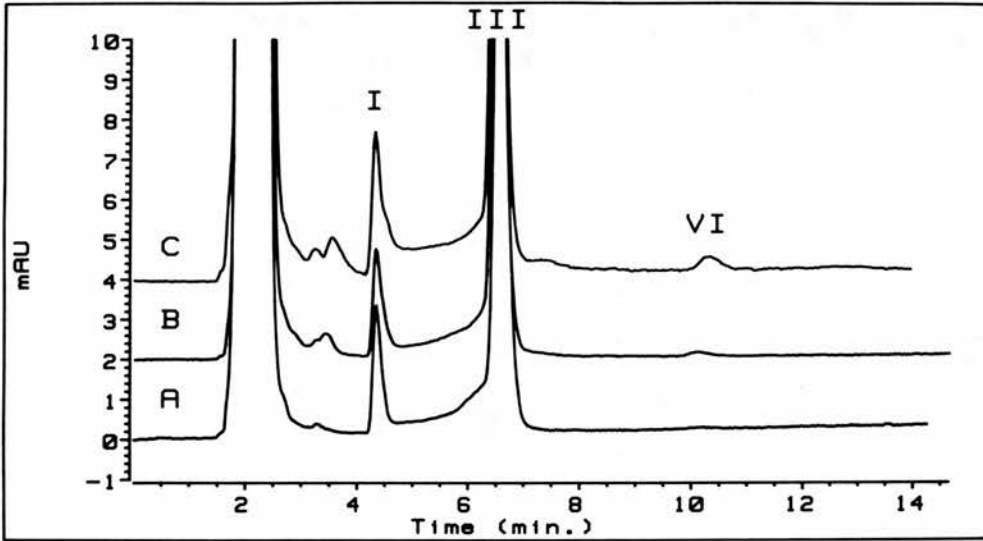


Figure 3.1. *In vitro* pattern of mitomycin C metabolism in MAC 16 cytosolic fractions under aerobic (B) and hypoxic (C) conditions using NADH as cofactor. Samples were analysed at 0 (A) and 60 (B, C) minutes and metabolite levels measured by HPLC. Chromatographic peaks were plotted as retention time (min) against UV milliabsorbance units (mAU) and applies to all subsequent chromatographic figures throughout.

Peaks: I, zwitterion form of mitomycin C; III, mitomycin C; VI, 2,7-diaminomitosenone.

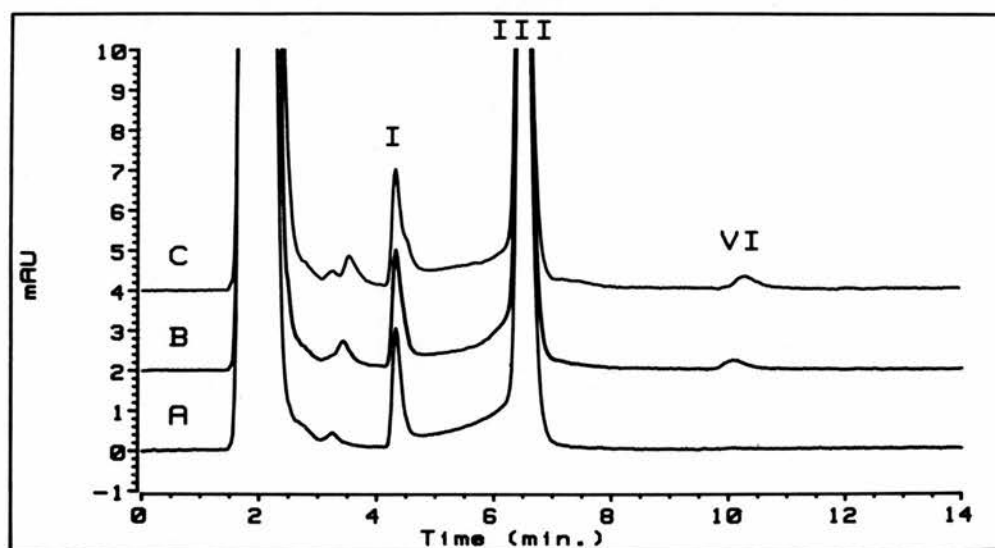


Figure 3.2. *In vitro* pattern of mitomycin C metabolism in MAC 16 cytosolic fractions under aerobic (B) and hypoxic (C) conditions using NADPH as cofactor. Samples were analysed at 0 (A) and 60 (B, C) minutes and metabolite levels measured by HPLC.

Peaks: I, zwitterion form of mitomycin C; III, mitomycin C; VI, 2,7-diaminomitosenone.

No cytosolic activity was recorded in either tumour when hypoxanthine was utilised as cofactor under both aerobic and hypoxic conditions.

MAC 16 microsomal activity had an absolute requirement for NADPH and hypoxia and 100 % inhibition was achieved with the addition of antiserum. Figure 3.3 illustrates the chromatographic profile of mitomycin C metabolism by MAC 16 microsomes and the inhibitory effect of cytochrome P-450 reductase antiserum. In addition to 2,7-DM, small amounts of cis-hydro and the secondary metabolite 10-decarbamoyl 2,7-diaminomitosene could be seen at 8 and 9.5 minutes respectively. The co-elution of trans-hydro with the zwitterion form of mitomycin C may be responsible for the increase in peak height observed in the 60 minute uninhibited sample. Co-incubation with dicoumarol had no effect on the rate of mitomycin C metabolism. Again as with the cytosol, no microsomal activity was present in MAC 26 despite cytosolic and microsomal activity being demonstrated using the cytochrome *c* reduction assay, table 3.1.

DT-diaphorase and NADPH:cytochrome P-450 reductase have both been shown to metabolise mitomycin C in a number of cellular systems (Pan *et al.*, 1984; Ross *et al.*, 1993; Fitzsimmons *et al.*, 1996). Their identification in many cases has relied upon comparison of their characteristics with those of purified enzymes in conjunction with specific enzyme inhibitors.

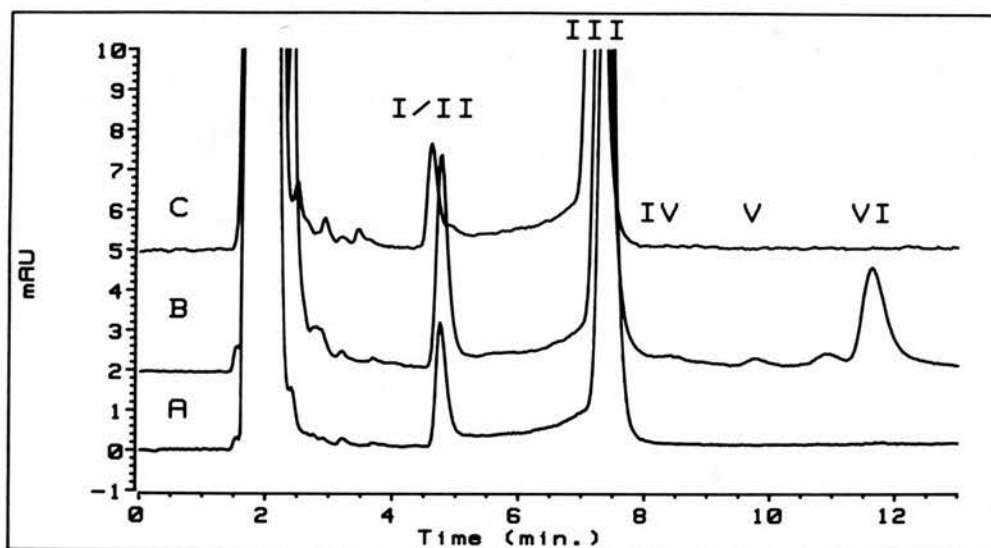


Figure 3.3. *In vitro* pattern of mitomycin C metabolism in MAC 16 microsomal fractions under hypoxia using NADPH as cofactor in the absence (B) and presence (C) of cytochrome P-450 reductase antiserum. Samples were analysed at 0 (A) and 60 (B, C) minutes and metabolite levels measured by HPLC.

Peaks: I, zwitterion form of mitomycin C; II, 1,2-*trans*-1-hydroxy-2,7-diaminomitosene; III, mitomycin C; IV, 1,2-*cis*-1-hydroxy-2,7-diaminomitosene; V, 10-decarbamoyl 2,7-diaminomitosene VI, 2,7-diaminomitosene.

Probably the most widely studied enzyme, DT-diaphorase, characteristically utilises both NADH and NADPH as cofactor and with certain substrates can show equivalent activity under aerobic and hypoxic conditions. This latter characteristic is associated with its ability to act as an obligate two-electron reductase. In addition, its activity can be inhibited by relatively low concentrations of the coumarin derivative dicoumarol (Lind *et al.*, 1982a). Using these characteristics, the results presented in table 3.2 have identified an enzyme present within the cytosol of MAC 16 exhibiting these properties. This, along with the high level of DT-diaphorase observed in the cytosolic quinone reductase assay, table 3.1, leads one to the reasonable conclusion that the cytosolic enzyme in MAC 16 is possibly the two-electron reductase DT-diaphorase.

The cytosolic enzyme is unlikely to be xanthine dehydrogenase. Although this enzyme is predominantly located within the cytosol and activates mitomycin C in a similar manner to DT-diaphorase, it requires a different cofactor (NAD⁺) and readily converts to xanthine oxidase during the process of tissue homogenisation (Gustafson & Pritsos, 1992a, 1993). In addition, mitomycin C activation by xanthine oxidase is solely by one-electron reduction pathway and is inhibited by oxygen (Pan *et al.*, 1984; Gustafson & Pritsos, 1992a). Other cytosolic enzymes such as carbonyl reductase, a member of the aldoketo reductase family, have been demonstrated to play a role in quinone-mediated oxidation (Forrest *et al.*, 1991,1994; Jarabak, 1991). However, carbonyl reductase has been reported

not to reduce mitomycin C and therefore it is an unlikely candidate (Wermuch *et al.*, 1986).

Early studies using rat liver preparations have implicated a microsomal reductase utilising NADPH as cofactor in mitomycin C metabolism under hypoxic conditions (Schwartz, 1962). Similar experiments later confirmed the possible role of the one-electron reductase NADPH:cytochrome P-450 reductase (initially referred to as NADPH:cytochrome *c* reductase) in liver (Kennedy *et al.*, 1982), EMT6 mouse mammary tumour cells (Keyes *et al.*, 1984) and purified enzyme preparations (Bachur *et al.*, 1979; Pan *et al.*, 1984). A rat liver microsomal system was also used to generate mitomycin C metabolites, particularly with reference 2,7-DM (Tomasz & Lipman 1981). More recently, Chinese hamster ovary cells transfected with the gene for this enzyme and hence overexpressing NADPH:cytochrome P-450 reductase have demonstrated differential mitomycin C toxicity under hypoxia (Belcourt *et al.*, 1996).

Activity characteristic of the one-electron reductase NADPH:cytochrome P-450 reductase was observed in MAC 16. Localised within the microsomal fraction, it had an absolute requirement for NADPH and hypoxia and it is this inhibitory effect of oxygen which is considered to be indicative of one-electron reduction. Activity was completely inhibited by the addition of cytochrome P-450 reductase antiserum, which has been previously shown to inhibit the biotransformation of doxorubicin to

doxorubicin 7-deoxyglycone by purified rat liver NADPH:cytochrome P-450 reductase (Cummings *et al.*, 1992). This, in conjunction with the results presented in table 3.1 demonstrating NADPH:cytochrome P-450 reductase activity in MAC 16, suggests that microsomal activity is due to this enzyme. Despite DT-diaphorase and NADPH:cytochrome P-450 reductase activities being confirmed in MAC 26 by cytochrome *c* reduction assays, table 3.1, there was no indication of any involvement of these enzymes in cytosolic and microsomal mitomycin C metabolism respectively. Reasons for this remain unclear.

The majority of mitomycin C metabolism on a per mg protein basis was associated with the mitochondrial fraction from both MAC 16 and MAC 26, table 3.2 and 3.3 respectively. NADPH combined with hypoxia produced the greatest formation of 2,7-DM with specific activities being similar in both tumour types. NADH-dependent mitomycin C metabolism was only seen in MAC 16 and activity was approximately 4-fold less than that seen with NADPH. The presence of oxygen resulted in complete inhibition of metabolism in both tumour types. In contrast to the other enzyme activities detected, the addition of 10 μ M dicoumarol and cytochrome P-450 reductase antiserum failed to produce any inhibitory effect. The chromatographic profiles of mitomycin C metabolism by MAC 16 and MAC 26 mitochondria in the presence of NADPH and hypoxia are shown in figure 3.4.

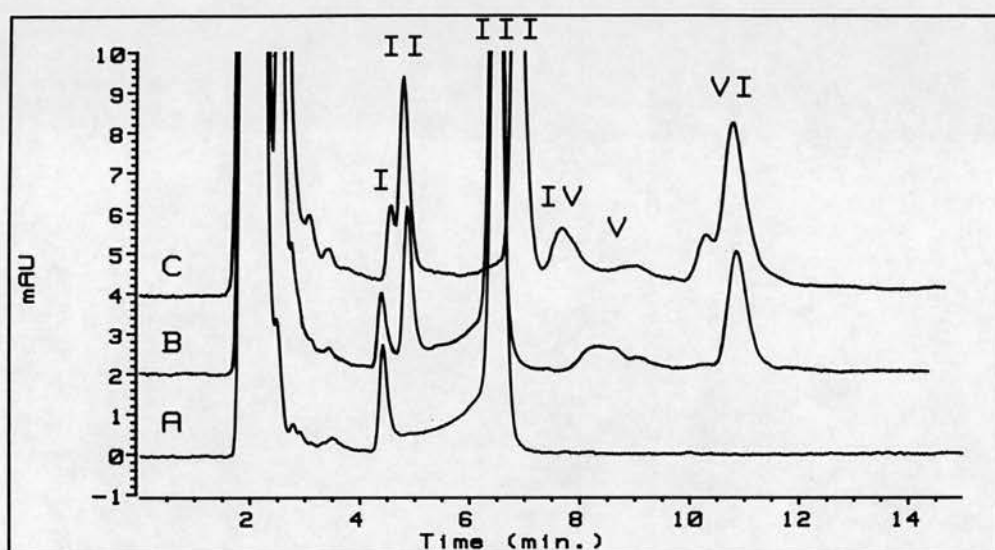


Figure 3.4. *In vitro* pattern of mitomycin C metabolism in MAC 16 (C) and MAC 26 (B) mitochondrial fractions under hypoxic conditions using NADPH as cofactor. Samples were analysed at 0 (A) and 60 (B, C) minutes and metabolite levels measured by HPLC.

Peaks: I, zwitterion form of mitomycin C; II, 1,2-*trans*-1-hydroxy-2,7-diaminomitosenone; III, mitomycin C; IV, 1,2-*cis*-1-hydroxy-2,7-diaminomitosenone; V, 10-decarbamoyle 2,7-diaminomitosenone; VI, 2,7-diaminomitosenone.

Again 2,7-DM can be clearly seen at 10.8 minutes as well as small amounts of cis- and trans-hydro and the secondary metabolite 10-decarbamoyl 2,7-diaminomitosenone at approximately 8, 5 and 9 minutes respectively.

The inability of dicoumarol and cytochrome P-450 reductase antiserum to inhibit NADPH-dependent activity suggests that the enzyme is neither DT-diaphorase or NADPH:cytochrome P-450 reductase respectively. In addition the enzyme's dependency for hypoxia suggests it is a one-electron reductase rather than a two-electron reductase. Similar inhibitory patterns were observed with the NADH-dependent enzyme present in MAC 16 only.

Although quinone reductase assessment of the mitochondria was not performed due to the presence of cytochrome *c* oxidase, the presence of mitochondria was confirmed by electron microscopy, figure 3.5 and 3.6. Mitochondria can be clearly seen exhibiting the classic double outer membrane and cristae, but due to the nature of the fractionation process, the method did not prevent mitochondrial membranes from a degree of disruption (Wehrle & Pedersen, 1981; Cummings *et al.*, 1984). This has the advantage of allowing the inner mitochondrial membranes access to exogenously added nicotinamide cofactors. For comparison, the microsomal fraction is shown in figure 3.7. The microsomes appeared as dark bodies. No microsomal contamination could be seen within the mitochondrial fractions.

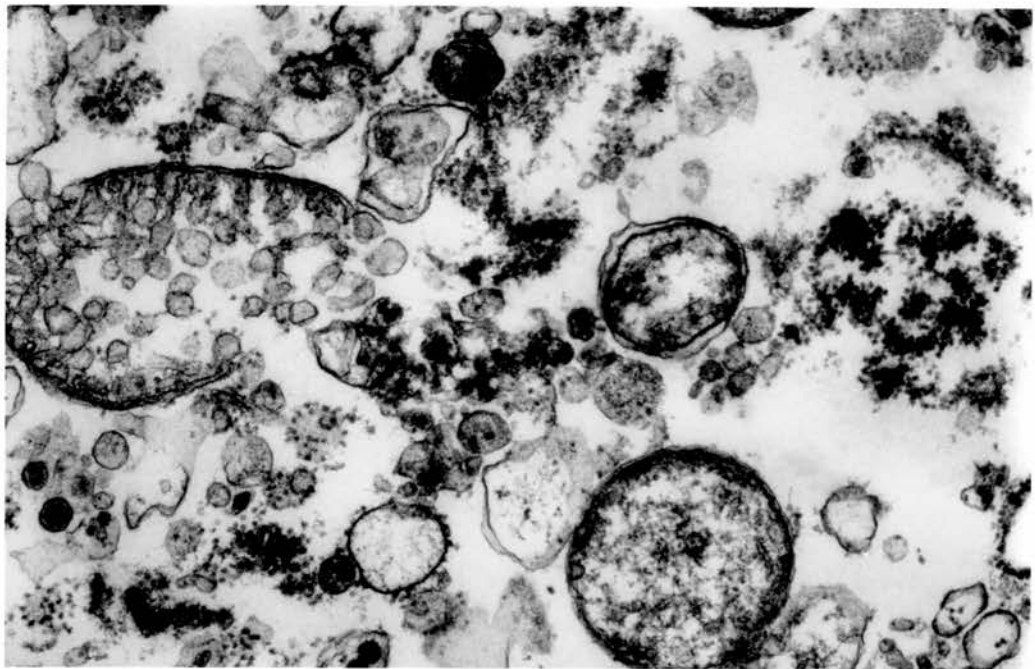


Figure 3.5. Electron micrograph of mitochondria isolated from MAC 16 murine adenocarcinoma of the colon. Magnification $\times 28080$.

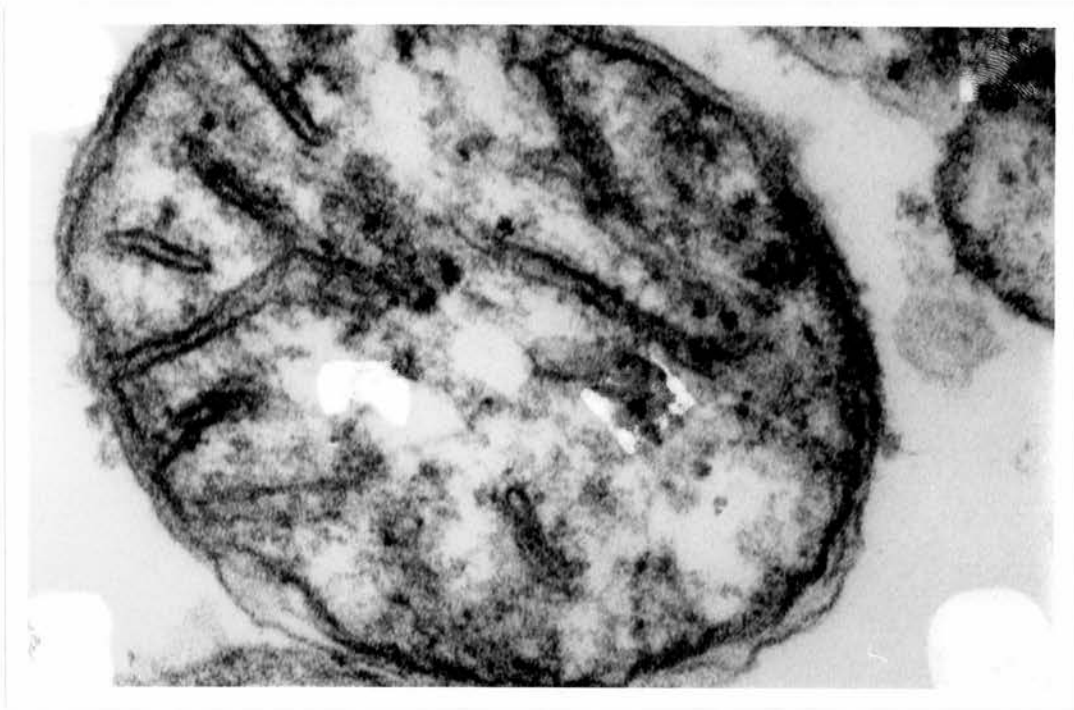


Figure 3.6. Cross-sectional electron micrograph of a mitochondrion isolated from the tumour mitochondrial fraction. Magnification $\times 138240$.

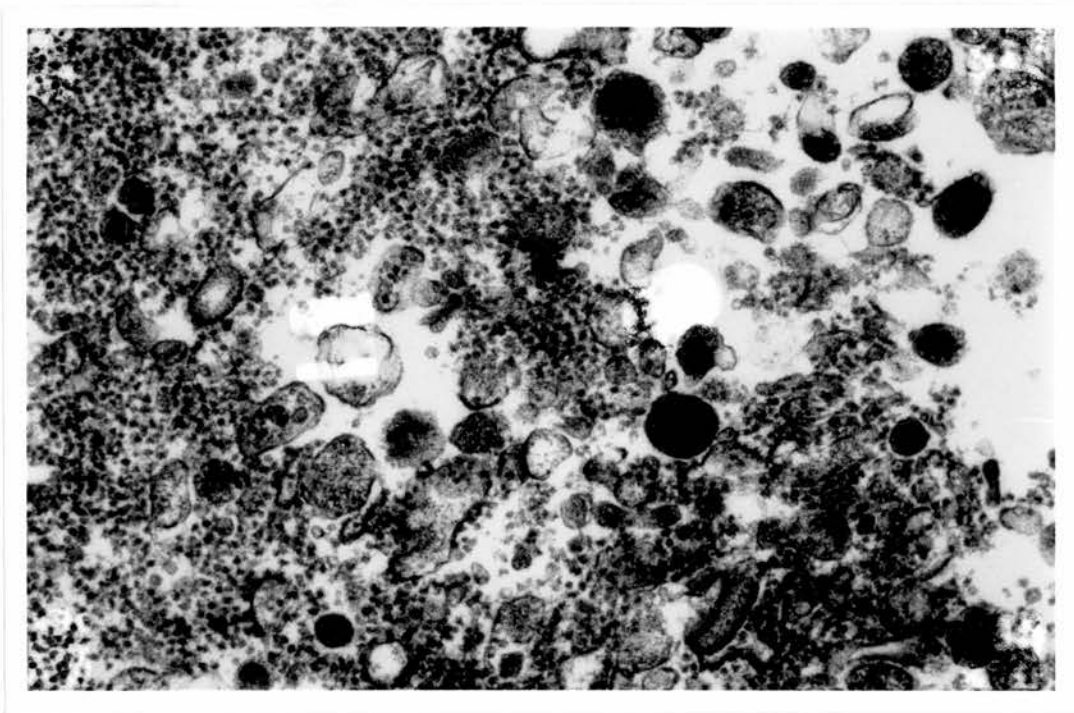


Figure 3.7. Electron micrograph of microsomes isolated from MAC 26 murine adenocarcinoma of the colon. Magnification x 41040.

It has previously been suggested that mitochondria (Pritsos & Sartorelli, 1986) and mitochondrial reductases (Butler & Hoey, 1987; Workman *et al.*, 1989) are capable of metabolising mitomycin C and other quinones. However, no experimental data, as yet, has been published to support this statement. In addition, recent *in vitro* and *in vivo* data studying mitochondrial function after mitomycin C treatment has shown that considerable mitochondrial damage occurred after drug administration (Pritsos & Vimalachandra, 1995). This implies for the first time that this mitomycin C-mitochondrial interaction may contribute to mitomycin C's mechanism of toxicity.

Also, quinone metabolites derived from the environmental pollutant benzo[*a*]pyrene have also been shown to exhibit the capacity to deplete cellular ATP and induce morphological changes within mitochondria in treated stromal cells, suggesting that benzo[*a*]pyrene quinones may elicit their toxicity through directly disrupting mitochondrial energy metabolism (Zhu *et al.*, 1995).

One enzyme that exhibits similar properties to the NADPH-dependent activity identified in MAC 16 and MAC 26 is NADPH-linked aquacobalamin reductase [EC 1.6.99.8] (NADPH-linked AqCbl reductase) which catalyses the reduction of aquacobalamin to cob(III)alamin in the synthesis of cobalamin enzymes (Watanabe *et al.*, 1990). First studied in bacterial systems (Walker *et al.*, 1969), this enzyme has now been

identified in mammalian tissues (Watanabe *et al.*, 1990). After further characterisation of this enzyme from rat liver microsomes, results indicated that it was NADPH:cytochrome *c* reductase, more commonly known as NADPH:cytochrome P-450 reductase (Watanabe *et al.*, 1992a). At this time a NADPH-linked AqCbl reductase had been reported to occur in mitochondrial membranes although its purification and characterisation were yet to be carried out (Watanabe *et al.*, 1989).

Subsequent isolation from rat liver mitochondrial membranes revealed similarities, but also distinct differences to its microsomal counterpart, namely molecular weight, 65 kDa compared to 79 kDa for the microsomal enzyme, and peptide elution profile (Saido *et al.*, 1993). However, similar to the microsomal enzyme, the mitochondrial enzyme was specific for NADPH and had the ability to reduce artificial electron acceptors such as cytochrome *c* and 2,6-dichlorophenolindophenol (Saido *et al.*, 1993). In addition, the mitochondrial enzyme immunoreacted with an antibody against microsomal NADPH-linked AqCbl reductase (NADPH:cytochrome P-450 reductase) indicating that the two enzymes have common antigenic determinants. This, in association with evidence showing the same high specific activity and identical submitochondrial location, the outer membrane, led the authors to conclude that the purified rat liver mitochondrial NADPH-linked AqCbl reductase may be NADPH:cytochrome *c* reductase (NADPH:cytochrome P-450 reductase). This hypothesis was supported by an early observation that NADPH:

cytochrome *c* reductase had been reported to occur, not only in microsomes, but also the mitochondrial outer membranes (Brunner & Bygrave, 1969).

This enzyme may be capable of metabolising mitomycin C in MAC 16 and MAC 26 tumour tissue and may be a good candidate for the NADPH-dependent enzyme identified in this study although identification and localisation in tumours has not yet been addressed. The lack of immunoreactivity of the cytochrome P-450 reductase antiserum (CH59) may be explained by the different antigenic determinants found between the mitochondrial enzyme and the enzyme to which the antibody has been raised.

Rat liver NADH-linked AqCbl reductase activity derived from the cytochrome *b*₅/cytochrome *b*₅ reductase complex has also been identified (Watanabe *et al.*, 1992b) and subsequently isolated in rat liver mitochondria within the outer membrane (Saido *et al.*, 1994). This enzyme may represent the NADH-dependent mitochondrial activity observed in MAC 16. Other possibilities include mitochondrial NADH-dehydrogenase which has been linked to the metabolism of the anthracycline doxorubicin (Davies & Doroshow, 1986; Thornally *et al.*, 1986) and mitochondrial ubiquinol-cytochrome *c* reductase (Hatefi, 1985). No published evidence, as yet, exists to support the role of these enzymes in mitomycin C metabolic activation.

Both NADPH- and NADH-linked AqCbl reductase activities have been located in a variety of human tissues ranging from liver and kidneys to brain and bone marrow (Watanabe *et al.*, 1991). Interestingly, the colon expresses both enzymes with the NADH-linked enzyme displaying the highest specific activity, although exact cellular localisation was not identified. Human liver NADPH- and NADH-linked AqCbl activity was observed in both microsomes and mitochondria. This, in association with its localisation within the colon, suggests that these enzymes may exist within tumours derived from this tissue.

It is interesting to note the different pattern of metabolites produced by the different fractions. MAC 16 cytosolic activity produced predominantly 2,7-DM. This had been previously demonstrated to occur *in vivo* (Cummings *et al.*, 1994) and *in vitro* (Siegel *et al.*, 1990a, 1992), with the latter study demonstrating that 2,7-DM was the major metabolite formed as pH decreased from pH 7 to 5.8. This is thought to be characteristic of DT-diaphorase and two-electron reduction. In contrast, MAC 16 microsomal activity produced a spectrum of metabolites, namely 2,7-DM, cis-hydro and 10-decarbamoyl 2,7-diaminomitosene. The trans-hydro metabolite is possibly co-eluting with the zwitterion form of mitomycin C, peaks I/II, figure 3.3. A similar pattern of metabolism was observed using rat liver microsomes in the presence of NADPH and hypoxia to activate mitomycin C (Tomasz & Lipman, 1981). This pattern of metabolism was replicated using purified rat liver NADPH:cytochrome P-

450 reductase showing increasing levels of cis- and trans-hydro and decreasing 2,7-DM levels with increasing pH (Pan *et al.*, 1984).

The mitochondrial fractions isolated from MAC 16 and MAC 26 both showed a similar spectrum of metabolites to those seen with MAC 16 microsomes. This supports the theory that the mitochondrial enzyme is possibly a form of NADPH:cytochrome P-450 reductase as discussed above.

In summary, the implications of the presence of a new enzyme capable of metabolising mitomycin C are important. With its high level of specific activity and activation under hypoxia, it provides an attractive target for enzyme-directed bioreductive drug therapy utilising drugs that are selectively toxic under hypoxic conditions and indeed provides a new insight into the metabolic activation of mitomycin C itself.

3.2 Role of DT-diaphorase in the Metabolic Activation of Mitomycin C

In the light of the three enzyme activities present in MAC 16 versus one in MAC 26, the role of these enzymes was investigated in whole tumour homogenates. This was considered to be a more representative tumour system where all enzyme activities were present together. Figure 3.8 shows the formation of the principle metabolite 2,7-DM in MAC 16 and MAC 26 homogenates under aerobic and hypoxic conditions. No added cofactors were required. In agreement with the subcellular incubations, MAC 26 homogenates exhibited an increase in metabolic activity under hypoxia similar to that seen with the mitochondrial fraction from this tumour. In contrast, MAC 16 homogenates did not show hypoxic enhancement displaying similar activity under both aerobic and hypoxic conditions. Table 3.4 details the specific activities produced by the tumour homogenates under both conditions clearly showing hypoxic enhancement in MAC 26.

The presence of the mitochondrial enzyme(s) may explain this hypoxic enhancement of mitomycin C metabolism in MAC 26 homogenate incubations. Although no aerobic metabolic activation was observed in the purified mitochondrial fraction of MAC 26, aerobic homogenate metabolism may rely upon the presence of these enzymes in the whole tumour environment that may, in turn, occur *in vivo*. The inability of hypoxia to potentiate mitomycin C metabolism in MAC 16 despite the

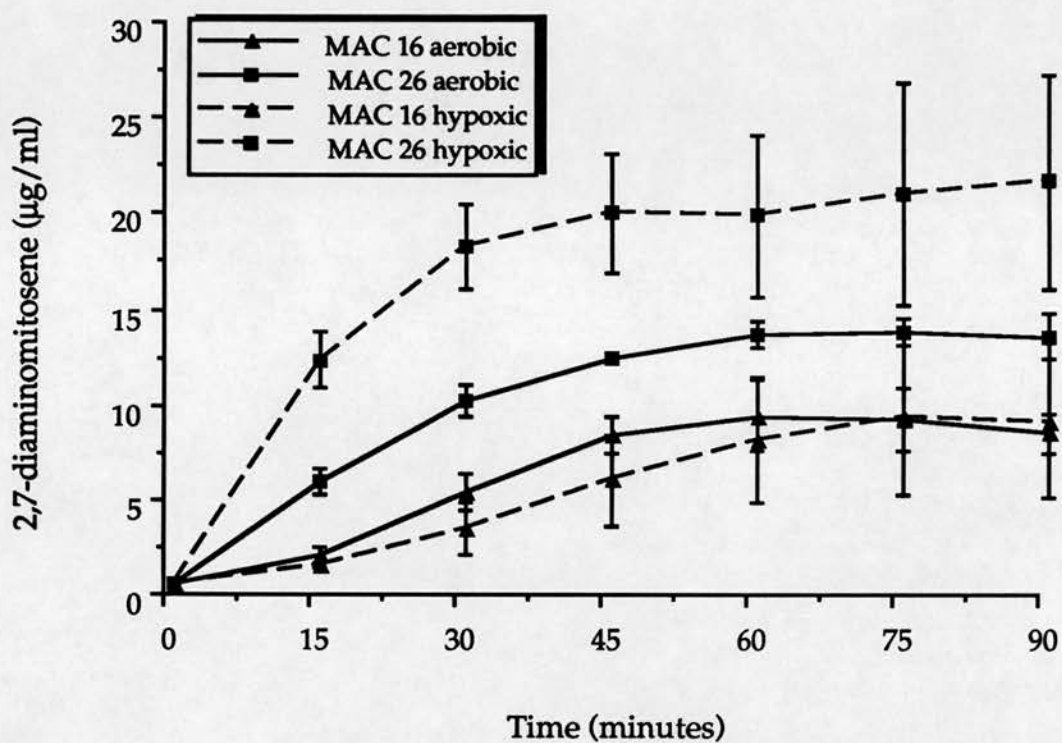


Figure 3.8. *In vitro* production of the principle mitomycin C metabolite 2,7-diaminomitosene in MAC 16 and MAC 26 tumour homogenates under aerobic (—) and hypoxic (---) conditions. Each point represents the mean \pm SE from three separate experiments.

TUMOUR HOMOGENATE	2,7-DM FORMATION (nmol/30min/mg protein)		EFFECT OF OXYGEN
	AEROBIC	ANAEROBIC	
MAC 16	0.22±0.04	0.14±0.07	No inhibition
MAC 26	0.80±0.06	1.45±0.18	45% inhibition

Table 3.4. Specific activities of mitomycin C metabolism by MAC 16 and MAC 26 tumour homogenates under aerobic and hypoxic conditions. Specific activity is expressed as 2,7-diaminomitosenone formation in nmol/30min/mg protein. Each value represents the mean±SE from three separate experiments.

presence of the NADPH-dependent mitochondrial enzyme, suggests preferential metabolism by other enzyme(s).

The metabolite profiles seen in these incubations were similar to those observed with the subcellular fractions. Figures 3.9 and 3.10 illustrate the HPLC profiles produced by MAC 16 and MAC 26 homogenates incubated under aerobic and hypoxic conditions respectively.

Again the principle metabolite formed under both aerobic and hypoxic conditions was 2,7-DM with clear hypoxic enhancement observed in MAC 26. This hypoxic enhancement was accompanied by a clear decrease in mitomycin C when compared to oxic conditions where levels of parent drug were similar at 60 minutes in both tumour types. MAC 26 also showed significant levels of additional metabolites suggesting the role of a one-electron reductase as detailed in section 3.1. MAC 16 homogenate however produced different metabolite levels. Similar levels of 2,7-DM but lower levels of additional metabolites were seen under both conditions similar to the cytosolic-dependent metabolism in figures 3.1 and 3.2. Metabolite profiles were monitored at 310 nm and hence the mitomycin C peak appears reduced compared to 360 nm. The retention time of 2,7-DM in figures 3.9 and 3.10 was approximately 13.5 minutes, slightly prolonged compared to previous figures. This was due to column ageing and should be taken into account throughout.

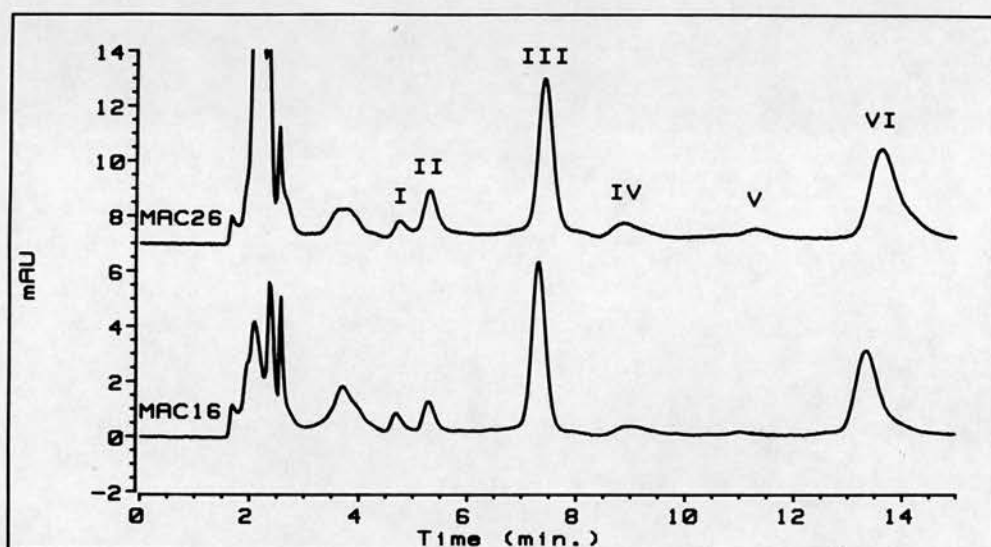


Figure 3.9. *In vitro* pattern of mitomycin C metabolism in MAC 16 and MAC 26 tumour homogenates under aerobic conditions. Samples were analysed at 60 minutes and metabolite levels measured by HPLC.

Peaks: I, zwitterion form of mitomycin C; II, 1,2-*trans*-1-hydroxy-2,7-diaminomitosenes; III, mitomycin C; IV, 1,2-*cis*-1-hydroxy-2,7-diaminomitosenes; V, 10-decarbamoyl 2,7-diaminomitosenes, VI; 2,7-diaminomitosenes.

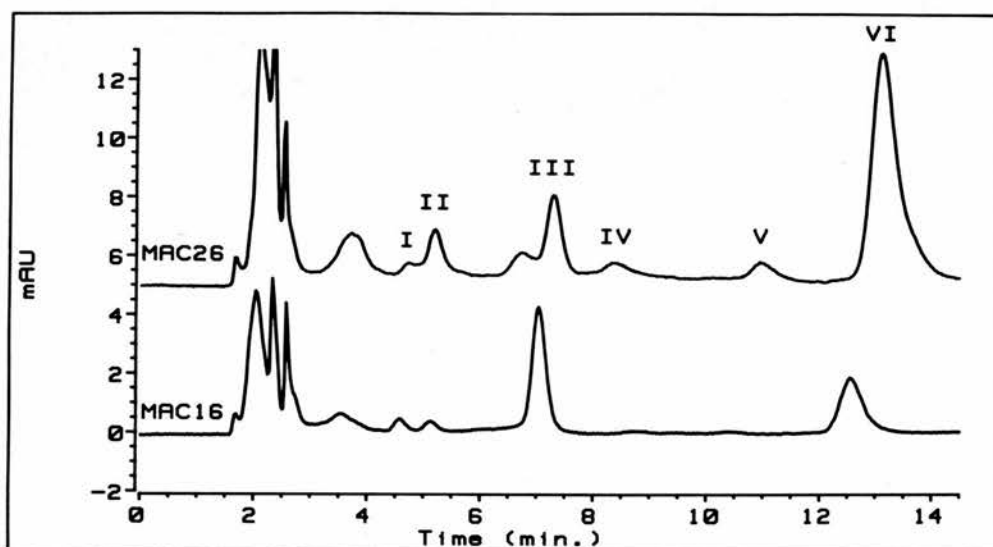


Figure 3.10. *In vitro* pattern of mitomycin C metabolism in MAC 16 and MAC 26 tumour homogenates under hypoxic conditions. Samples were analysed at 60 minutes and metabolite levels measured by HPLC.

Peaks: I, zwitterion form of mitomycin C; II, 1,2-*trans*-1-hydroxy-2,7-diaminomitosenes; III, mitomycin C; IV, 1,2-*cis*-1-hydroxy-2,7-diaminomitosenes; V, 10-decarbamoyle 2,7-diaminomitosenes, VI; 2,7-diaminomitosenes.

The phenomenon of hypoxic enhancement has been previously observed in the human colon adenocarcinoma cell line BE, a cell line that lacks DT-diaphorase activity due to mutation in the DT-diaphorase gene NQO1 (Traver *et al.*, 1992). BE displayed approximately a 10-fold increase in sensitisation to mitomycin C under hypoxia compared to that in the presence of air (Plumb & Workman, 1994). In addition, Beall *et al.* (1994) demonstrated potentiation of mitomycin C toxicity under hypoxia in the same cell line. This result was in contrast to that seen with another human colon adenocarcinoma cell line, HT-29, expressing a DT-diaphorase specific activity of 60 nmol/min/10⁶ cells at the time of drug exposure. HT-29 did not show any increase in sensitivity when exposed to mitomycin C under hypoxic conditions, a result similar to that observed above in MAC 16 (high in DT-diaphorase, table 3.1). In the same study, similar results were obtained using the mitomycin C analogue indoloquinone EO9 with BE showing a 1000-fold increase in sensitisation to the drug. HT-29 in combination with EO9 and hypoxia produced no enhancement.

Plumb and colleagues (1994) proposed that one-electron reduction was responsible for the hypoxic activity seen in BE. Such activity may be due to the presence of the mitochondrial reductase characterised and discussed in section 3.1.

The use of dicoumarol (200 μ M) in conjunction with HT-29 cells under

hypoxia markedly increased their sensitivity to EO9, suggesting that due to possible enzyme inhibition, a one-electron reductase may now prevail over other enzymes present (Plumb & Workman, 1994). Similar hypoxic enhancement in the presence of dicoumarol (100 μM) has also been observed in EMT6 murine mammary tumour cells (Keyes *et al.*, 1985) and L1210 murine leukaemia cells (Keyes *et al.*, 1989), again suggestive of one-electron reductase activity. Such activity may again be due to the mitochondrial enzyme identified. It should be noted that neither HT-29 nor BE cell lines were shown to have detectable levels of the one-electron reductase xanthine oxidase (Siegel *et al.*, 1990b).

Bizanek *et al.* (1993) extended the studies of Keyes and colleagues (1985, 1989) examining the effect of dicoumarol on adduct patterns generated by mitomycin C in EMT6 murine mammary tumour cells. Mitomycin C-DNA adduct frequencies were increased 1.5 fold under hypoxia in the presence of dicoumarol (300 μM). A decrease of 1.6 fold was observed under aerobic conditions. Such a result has a number of implications. Firstly, the enhancement of DNA adduct formation under hypoxia may be due to mitochondrial-dependent metabolism as seen in the MAC tumours. Secondly, the decrease under aerobic conditions may be due to DT-diaphorase inhibition and thirdly that this enzyme might be predominating under aerobic conditions.

Although the above results present some intriguing findings concerning

the role of quinone reductase in mitomycin C metabolic activation, they were generated using extremely high concentrations of dicoumarol (100-300 μ M). These should be viewed with caution as dicoumarol itself can also inhibit NADH:cytochrome *b*₅ reductase (Ross *et al.*, 1993), and NADPH:cytochrome P-450 reductase (Cummings *et al.*, 1992), stimulate xanthine dehydrogenase (Pan *et al.*, 1984; Gustafson & Pritsos, 1992a,b) and induce the formation of unique DNA covalent adducts (Bizanek *et al.*, 1993). Additional effects include inhibition of xanthine oxidase (Hajos & Winston, 1991), glucuronyl transferases (Segura-Aguilar *et al.*, 1986) and uncoupling of mitochondrial oxidative phosphorylation (Ernster, 1987).

However, the following questions arise from these observations. What enzyme is predominating over the identified mitochondrial enzyme in MAC 16 and thus preventing increased metabolism under hypoxia and does this apply to the observations above? With this lack of metabolic enhancement under hypoxia and the identification of an enzyme exhibiting similar properties in the cytosol, it appears that the enzyme responsible for mitomycin C metabolism in MAC 16 predominating under all conditions, is DT-diaphorase. Plumb and colleagues (1994) also suggested a dominant role for DT-diaphorase in HT-29.

If DT-diaphorase is predominating over other enzyme(s) present within tumour, why? And why is less mitomycin C metabolism seen with DT-

diaphorase when compared to the mitochondrial one-electron reductase? The first documented report of DT-diaphorase [NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2)] was by Ernster and Navazio, who in 1958 described activity of a highly active diaphorase in the soluble fraction of rat liver microsomes. DT-diaphorase is a unique flavoenzyme in a number of ways. Firstly, it exhibits nonspecific reactivity to NADH and NADPH reacting with equal but low maximal velocities (Ernster *et al.*, 1962), although the K_m value for NADH is a little higher than that for NADPH, 45 and 85 μM respectively. The very low affinity constant, K_m , also applies to mitomycin C bio-reduction via DT-diaphorase and the other major enzymes involved. Xanthine oxidase and NADPH:cytochrome P-450 reductase both have a K_m of 2 mM (Pan *et al.*, 1984, 1986), whilst xanthine dehydrogenase has a K_m of 299 μM (Gustafson & Pritsos, 1993). DT-diaphorase also has a low K_m , 1 mM (Walton *et al.*, 1992c). This suggests that neither one will show preference for mitomycin C as a substrate at concentrations used in the homogenates and hence the enzyme present in the largest quantity is likely to carry out the majority of mitomycin C bio-reduction irrespective of the enzymes intrinsic catalytic activity. The pattern of metabolic activation produced will therefore parallel the biochemical properties of that particular enzyme.

In the case of MAC 16, DT-diaphorase accounts for 66 % of the cytosolic and microsomal combined quinone reductase activity, table 3.1. Although the contribution of mitochondrial DT-diaphorase and the additional

mitochondrial enzyme(s) identified in section 3.1 are not accounted for in this calculation, it is likely that DT-diaphorase is present in the largest quantity since the pattern of metabolism is consistent with DT-diaphorase activity. It is therefore possibly responsible for the majority of mitomycin C metabolic activation in MAC 16 under both aerobic and hypoxic conditions, hence its predomination over other enzyme(s) present and the characteristic pattern of metabolism.

DT-diaphorase accounts for considerably less of total quinone reductase activity in MAC 26 (30 %). This may contribute towards the aerobic metabolism seen in this tumour, figure 3.8 and table 3.4. Under hypoxia, the identified one-electron mitochondrial reductase, perhaps present in the greatest quantity when compared to DT-diaphorase, predominates.

A second feature of DT-diaphorase is that it is extremely sensitive to dicoumarol (Ernster *et al.*, 1960), a property which initiated research to ascertain whether the enzyme was involved in the metabolism of vitamin K (Wallin *et al.*, 1978). Third, and perhaps the most important property is the ability of DT-diaphorase to catalyse obligatory two-electron reduction, a feature which is crucial to the enzymes' cytoprotective properties (Lind *et al.*, 1982b; Preusch *et al.*, 1991).

DT-diaphorase is a cytosolic enzyme, although activity has been isolated to the endoplasmic reticulum (Danielson *et al.*, 1960), mitochondria

(Conover & Ernster, 1963) and golgi apparatus (Edlund *et al.*, 1982). The mitochondrial enzyme reacts only with intramitochondrial NADH and NADPH, therefore it is located within the mitochondrial inner matrix or the inner surface of the inner membrane (Conover & Ernster, 1960, 1962). It is widely distributed throughout the organs of the body with liver, kidney and gastrointestinal tract expressing high levels (Schlager & Powis, 1990). Similar patterns of expression are also observed in tumour material taken from patients (Koudstaal *et al.*, 1975; Schor & Cornelisse, 1983) with the exception of kidney and stomach tumours which show the reverse trend (Schlager & Powis, 1990).

The dimeric enzyme, molecular weight approximately 55,000, exists as a number of isoforms with mouse and rat livers possessing two and three variants respectively (Prochaska & Talalay, 1986; Segura-Aguilar & Lind, 1987). Genetic investigations in humans indicated that the various forms of DT-diaphorase appeared to be encoded by four gene loci, *DIA* 1 to 4 (Edwards *et al.*, 1980). Diaphorase 4, now known as NQO₁, has recently been characterised (Shaw *et al.*, 1991). With gene localisation on chromosome 16, this form of DT-diaphorase accounts for the majority of the enzyme expressed and shows 84% homology compared to the rat NQO₁ protein (Jaiswal *et al.*, 1988). The NQO₂ gene located on chromosome 6 shares 54 % homology to NQO₁ and in contrast to NQO₁, is only expressed in heart, lung liver, brain and skeletal muscle (Jaiswal *et al.*, 1990; Jaiswal, 1994). Its exact function has yet to be determined.

Like many flavoproteins, DT-diaphorase reacts with its electron donor and acceptor, in this case quinones, according to a 'ping-pong' mechanism (Hosoda *et al.*, 1974), figure 3.11. This is also known as the binary-complex mechanism and the quinone acts as the second substrate. It is interesting to note that both substrates, cofactor and quinone, display competitive inhibition of the enzyme with respect to each other. This may suggest a cooperative relationship between the donor and acceptor binding sites of the enzyme.

Perhaps DT-diaphorase's most important function is its capability to function as a two-electron transfer enzyme. This was first demonstrated by Iyanagi and Yamazaki (1970). In the case of mitomycin C and other quinone-containing compounds, this leads to the production of the hydroquinone. The fate of the hydroquinone determines whether DT-diaphorase acts in an antioxidant/protective or prooxidant capacity, the latter leading to toxicity (Cadenas, 1995). Three types of hydroquinone can theoretically exist, redox-stable and redox-labile hydroquinone and hydroquinones that finally participate in bioalkylation, figure 3.12. Although all share common production by DT-diaphorase, the inherent chemical reactivity of a particular hydroquinone is determined by the substitution pattern of the quinone. The formation of redox-stable hydroquinones (figure 3.12, pathway I) and subsequent manifestation of a protective role for DT-diaphorase, has been examined with simple quinones.

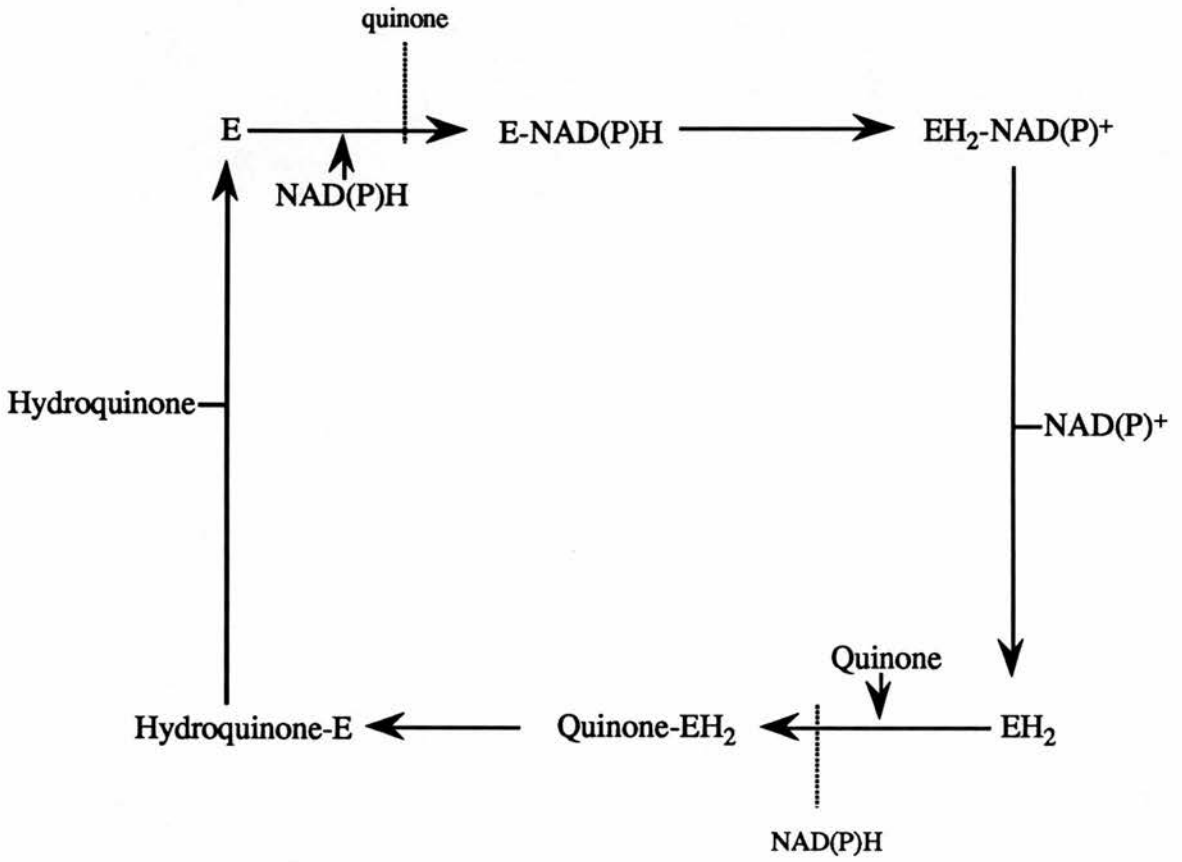


Figure 3.11. Reaction sequence of DT-diaphorase (E) via the 'ping-pong' mechanism. Dotted lines represent sites of substrate inhibition. Adapted from Ernster, 1987.

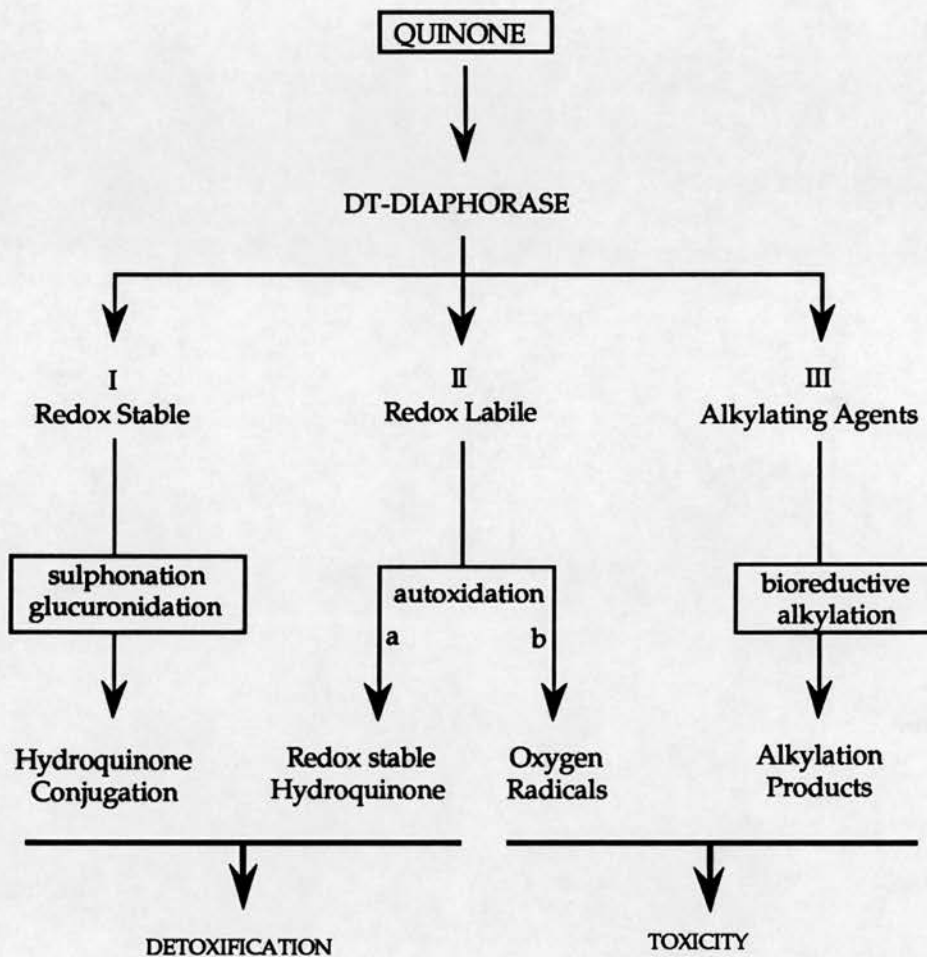


Figure 3.12. Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. (a) Autoxidation inhibited by superoxide dismutase; (b) Autoxidation stimulated by superoxide dismutase. Adapted from Cadenas, 1995.

Prevention of quinone toxicity occurs via aromatisation of the quinone ring by DT-diaphorase thus decreasing its electrophilic character. This prevents its participation in certain arylation reactions. Therefore, DT-diaphorase indirectly protects against cytotoxicity arising from such reactions. Conjugation of hydroquinones with glucuronide or sulphide groups may also facilitate metabolic inactivation. The use of dicoumarol to inhibit glucuronidation of benzo[*a*]pyrene-2,6-quinone suggests a possible role for DT-diaphorase in this process (Lind, 1985). Another body of evidence supporting an antioxidant role for DT-diaphorase has been derived from studies utilising the metabolism of benzene in a number of bone marrow stroma cells, macrophages and fibroblastoid cells (Ross, 1989; Thomas *et al.*, 1990).

In common with other detoxification or so-called phase II enzymes, DT-diaphorase is induced by a number of xenobiotics, due to up regulation of at a transcriptional level (Bayney *et al.*, 1989; Spencer *et al.*, 1990). This leads to additional antioxidant activity.

It is unlikely that the above formation of redox-stable hydroquinones applies to DT-diaphorase dependent protection from mitomycin C cytotoxicity.

A second type of hydroquinone formed during DT-diaphorase dependent catalysis is the redox-labile hydroquinone (figure 3.12, pathway II). Potent

antioxidant properties of this hydroquinone can be achieved in association with superoxide dismutase. Autoxidation of the hydroquinone via superoxide free radicals is inhibited by this enzyme. This results in the inhibition of semiquinone and hydrogen peroxide formation leading to subsequent enhancement of redox-stable hydroquinone concentrations (figure 3.12, pathway IIa). Such antioxidant activity has been demonstrated using a dopamine-derived *o*-quinone resulting in the suppression of oxygen radical formation (Segura-Aguilar & Lind, 1989).

In contrast to the above inhibitory effects the converse applies. Superoxide dismutase also has the capacity to elicit toxic effects through the formation of oxygen free radicals via enhanced hydroquinone autoxidation (figure 3.12, pathway IIb). Such toxicity has proved important in the mechanism of action of the diaziridinylbenzoquinones (O'Brien *et al.*, 1990) and anthraquinone-based antitumour agents (Fisher *et al.*, 1992).

However, in the context of mitomycin C, perhaps the most important hydroquinones formed are those that readily rearrange to potent electrophiles which subsequently participate in bioalkylation reactions (figure 3.12, pathway III). The protective/predominant role of DT-diaphorase observed by Plumb and colleagues (1994) gave rise to two valid propositions concerning the role of such hydroquinones: (i) one-electron

reductases are more efficient at metabolising mitomycin C than two-electron reductases such as DT-diaphorase, although DT-diaphorase prevails under aerobic conditions; and (ii) the semiquinone form of mitomycin C is more toxic than the hydroquinone, product of DT-diaphorase metabolism, under hypoxic conditions.

The latter proposition can be discounted as under hypoxic conditions the hydroquinone form of mitomycin C is considered to be a common intermediate after both one- and two-electron reduction due to the rapid disproportionation of the semiquinone radical (Hoey *et al.*, 1988; Ross *et al.*, 1993). Regarding the former proposition, this can be supported by an emerging body of evidence indicating that DT-diaphorase is less effective at metabolising mitomycin C than one-electron reductases. Firstly, the pH dependency of DT-diaphorase is more pronounced than one-electron reductases. Siegel and colleagues (1992, 1993) demonstrated that while mitomycin C was reduced effectively at pH 5.8, total enzyme inhibition was observed at pH 7.8. This has been explained by the ambivalent nature of the quinone methide where it acts as an electrophile at alkaline pH (pH 8.0), while at more acidic pH (pH 6.5) it can act as a nucleophile (Petersen & Fisher, 1986). The latter results in the production of 2,7-DM. Ross and colleagues (1993) subsequently demonstrated alkylation of DT-diaphorase by mitomycin C at pH 7.8 but not pH 5.8 resulting in enzyme inhibition. It was then suggested that the 7-NH₂ group of the enzyme may play an essential role in this inhibitory process. However, it should be noted that

bioactivation of mitomycin C by DT-diaphorase at physiological pH, pH 7 to 7.4, can occur leading to DNA cross-linking (Siegel *et al.*, 1992).

Secondly, DT-diaphorase produces predominantly monofunctional N-7 guanine DNA adducts which are believed to be less cytotoxic than the bifunctional adducts formed by one-electron reductases (Bizanek *et al.*, 1993). The formation of such adducts also follows the pH dependency of DT-diaphorase bioactivation of mitomycin C suggesting that a fewer number of cross-links will be formed at physiological pH compared to acidic pH (Prakash *et al.*, 1993). The reduced toxicity of mitomycin C monoadducts however has recently been challenged by Rockwell & Kim (1995) based on evidence that decarbamoyl mitomycin C, which is incapable of forming bisadducts, was at least as toxic as mitomycin C to EMT6 mouse mammary cells and DNA repair-deficient Chinese hamster ovary cells.

Therefore in conclusion, although DT-diaphorase is present in MAC 16 in the greatest quantity it is less efficient at metabolising mitomycin C. Hence, this results in less metabolism compared to the mitochondrial one-electron reductase responsible for metabolism in MAC 26.

The precise role of DT-diaphorase in mitomycin C metabolic activation has been somewhat controversial and unresolved for a number of years (Workman *et al.*, 1989; Doroshow, 1992). The use of tumour cell lines

with contrasting high and low DT-diaphorase levels have provided evidence that this enzyme may be responsible for aerobic mitomycin C bioactivation (Begleiter *et al.*, 1988; Siegel *et al.*, 1990a; Marshall *et al.*, 1991). In contrast, mitomycin C was shown not to be activated by purified kidney or rat liver DT-diaphorase but was in fact acting as a powerful inhibitor of the enzyme (Schlager & Powis, 1988). Incubation with a DT-diaphorase rich preparation isolated from the Walker 256 tumour also led to a negative result (Workman *et al.*, 1989). Both studies were carried out at the non-optimum pH for mitomycin C, pH 7.8, and shed doubt on the tissue culture studies which indirectly proposed a significant role for this enzyme in aerobic mitomycin C bioactivation. pH was in fact finally found to be the crucial determinant of purified DT-diaphorase-dependent mitomycin C metabolism (Siegel *et al.*, 1990a), with metabolism nevertheless occurring at pH 7.0, albeit at reduced levels.

The effect of hypoxia on the DT-diaphorase catalysed metabolism of mitomycin C remained unknown. Until recently studies using the murine lymphoblasts L5178Y and L5178Y/HBM10, having low and high DT-diaphorase levels respectively, established a limited role for DT-diaphorase in mitomycin C bioactivation under hypoxia (Begleiter *et al.*, 1992). In contrast, using the DT-diaphorase rich and deficient cell lines, HT-29 and BE, results concluded that bioactivation of mitomycin C by DT-diaphorase was similar under both aerobic and hypoxic conditions (Beall *et al.*, 1994), as one would expect.

The results discussed within this section have shed some light on the above uncertainties of DT-diaphorase. DT-diaphorase remains a very important enzyme. With its predomination over other quinone reductases and equivalent activity under both aerobic and hypoxic conditions, DT-diaphorase probably plays a unique and central role in the metabolic activation of mitomycin C in tumour tissue.

3.3 Pharmacokinetics and Metabolism of Mitomycin C *In Vivo*: Implications for *In Vivo* Drug Treatment

The next question that arises is what implications do the observations in section 3.2 have for the metabolism of mitomycin C *in vivo* and can one predict what tumour types are optimum for mitomycin C drug treatment with regards to the levels of quinone reductases? In order to assess the above question, the pharmacokinetics, *in vivo* metabolism and antitumour activities of mitomycin C in MAC 16 and MAC 26 were studied.

In both pharmacokinetic and antitumour studies, mitomycin C was given as a direct i.t. injection. Although i.t. drug treatment is not the normal route of drug administration, it has previously been employed in the treatment of human pancreatic cancer with mitomycin C (Moriai *et al.*, 1989) and has been the chosen route for a number of additional anticancer agents and biological response modifiers (Brincker, 1993; Dubinett *et al.*, 1993). For the purpose of these experiments, it was necessary to achieve the highest concentration of mitomycin C within the tumour in order to study the formation of metabolites. This method has previously been employed successfully in MAC 16 and the rat Sp 107 mammary carcinoma (Cummings *et al.*, 1994; Chirrey *et al.*, 1995).

The dose of mitomycin C, 500 µg, was chosen as it has previously been

used in such studies both as free drug and encapsulated into human albumin microspheres (Allan *et al.*, 1993; Cummings *et al.*, 1994). The primary metabolite 2,7-DM was once again used as the principle indicator of mitomycin C metabolic activation.

The pharmacokinetics of mitomycin C in tumour tissue and plasma are illustrated in figures 3.13 and 3.14 respectively. Pharmacokinetic parameters are summarised in table 3.5. Both MAC 16 and MAC 26 showed similar patterns of drug distribution, peak levels at 0 minutes with levels steadily decreasing with time. However, the initial peak level of mitomycin C seen at 0 minutes was approximately 2-fold lower in MAC 26 when compared to MAC 16. Indeed on calculation of the area under the curve (AUC), drug exposure levels were considerably less in MAC 26, 195.60 compared to 318.79 $\mu\text{g}/\text{total tumour content} \times \text{hour}$ for MAC 16. This may suggest a number of possibilities. Firstly, that mitomycin C in MAC 26 is rapidly cleared from the tumour or secondly, metabolism is occurring at such a high rate that levels of parent drug are immediately reduced.

The first statement can be supported by the pharmacokinetics of mitomycin C in plasma, figure 3.14 and table 3.5. The levels of mitomycin C in plasma derived from MAC 26 bearing mice followed a similar profile to that seen in tumour tissue, ie. steady decreasing levels over time. In comparison, plasma derived from MAC 16 bearing mice displayed a peak

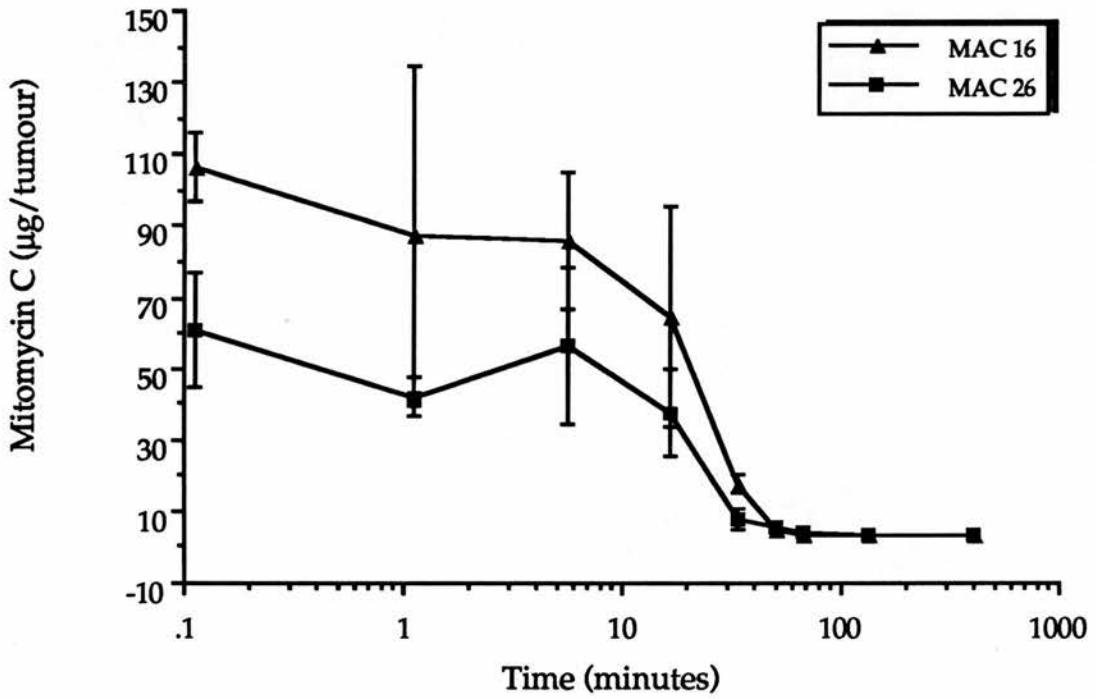


Figure 3.13. Concentration-time profiles of mitomycin C in MAC 16 and MAC 26 murine adenocarcinomas of the colon after a direct i.t. injection of 500 µg mitomycin C. Results are expressed as µg/total tumour content and represent the mean±SE of three separate animals per time point.

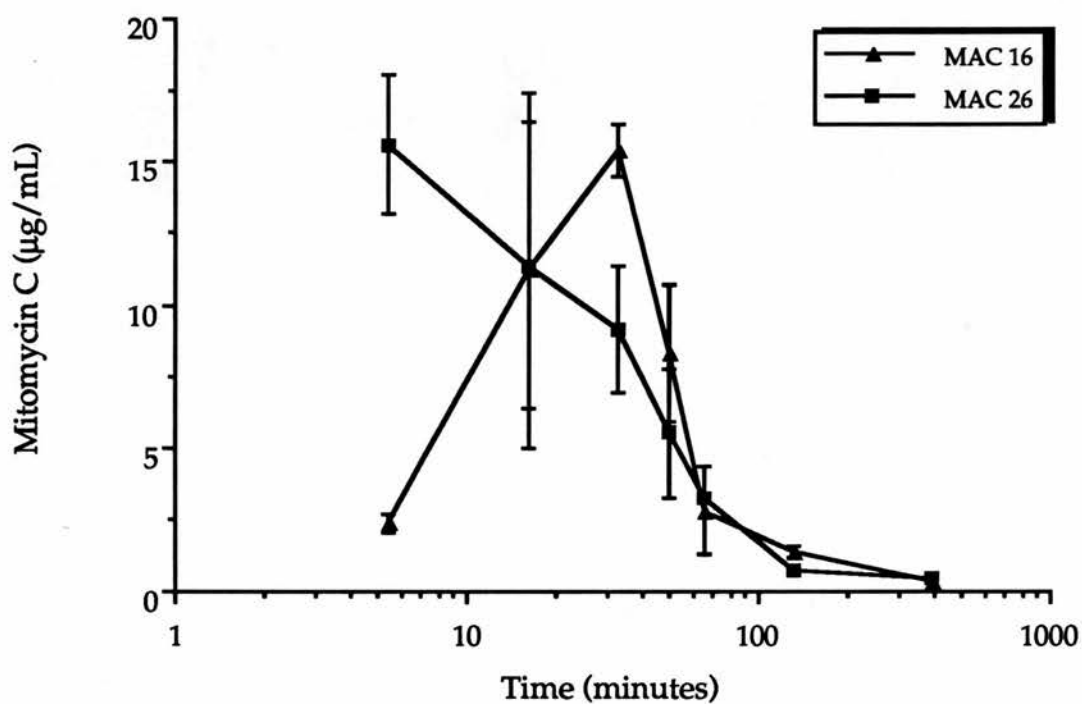


Figure 3.14. Concentration-time profiles of mitomycin C in plasma form MAC 16 and MAC 26 bearing animals after a direct i.t. injection of 500 µg mitomycin C. Results are expressed as µg/mL and represent the mean ± SE of three separate animals per time point.

	MAC 16		MAC 26	
	MMC	2,7-DM	MMC	2,7-DM
TUMOUR				
Peak level ($\mu\text{g}/\text{TTC}$)	103.42	5.85	57.77	5.12
Time to peak (min)	0	0	0	5
$\text{AUC}_{0-360\text{min}}$ ($\mu\text{g}/\text{TTC}\times\text{hr}$)	318.79	19.57	195.60	49.92
2,7-DM : MMC ratio	0.06		0.26	
PLASMA				
Peak level ($\mu\text{g}/\text{mL}$)	15.03	ND	15.23	ND
Time to peak (min)	30		5	
$\text{AUC}_{0-360\text{min}}$ ($\mu\text{g}/\text{mL}\times\text{hr}$)	129.50		88.09	
Clearance ($\mu\text{g}/\text{mL}$)	3.86		5.68	

Table 3.5. Pharmacokinetics and metabolic conversion of mitomycin C (MMC) to the principle metabolite 2,7-diaminomitosenone (2,7-DM) in MAC 16 and MAC 26 murine adenocarcinomas of the colon. TTC = total tumour content; ND = not detected.

level of mitomycin C at 30 minutes. Both observations support greater immediate tumour clearance of mitomycin C in MAC 26 compared to MAC 16.

The underlying morphology of the tumours may explain this 'clearance' effect. The MAC tumours are similar to human colon tumours in that they display varying histological characteristics and are active mucin producers (Cowen *et al.*, 1980). However, significant histological differences exist between MAC 16 and MAC 26. MAC 16 is a highly necrotic chemoresistant tumour which causes severe cachexia in its host (Double & Bibby, 1989). MAC 16 shows little differentiation, figure 3.15. In contrast, MAC 26 is a very well differentiated but slow growing adenocarcinoma with an extensive blood supply (Double & Bibby, 1989), figure 3.16.

It is this blood supply which may contribute to the clearance of mitomycin C from the tumour. As mitomycin C was injected i.t., a significant amount will immediately enter directly into the tumour capillary system. In comparison, MAC 16 may display slower tumour clearance due to its necrotic nature. This is supported by the different plasma AUCs. As it was difficult to obtain a 0 minute plasma sample, it can be proposed that a large amount of mitomycin C was removed from the plasma via metabolism and excretion in MAC 26 bearing mice in the first 5 minutes, shown by the high clearance and low AUC, table 3.5.

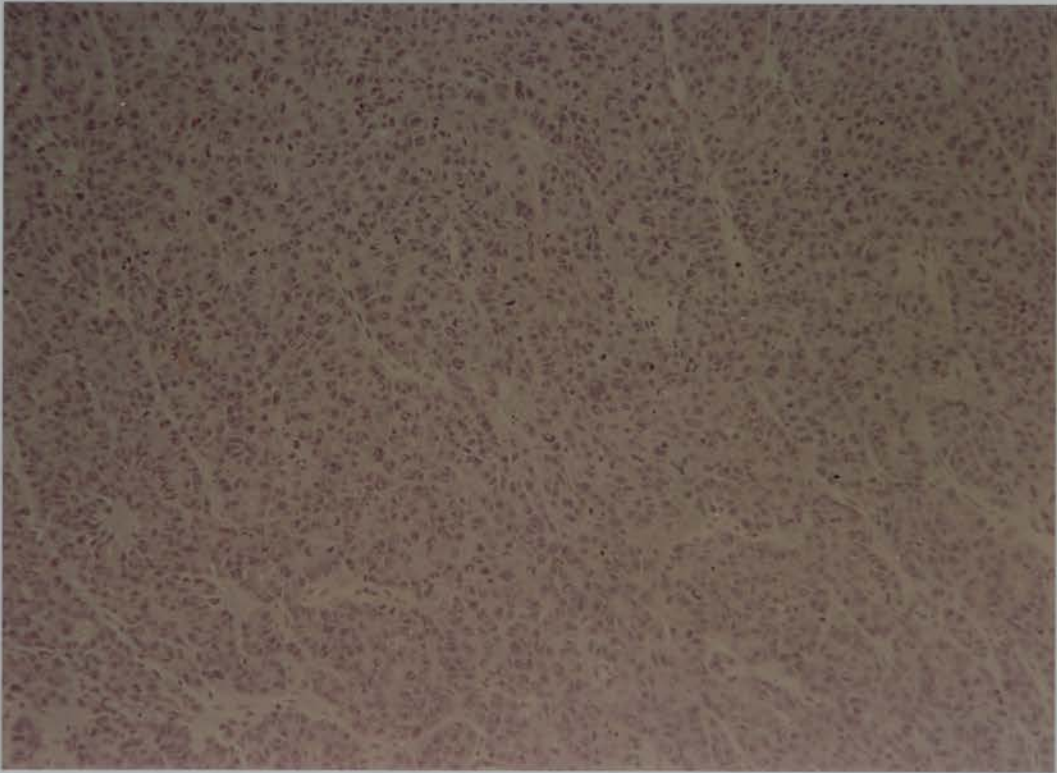


Figure 3.15. Histological appearance of MAC 16 murine adenocarcinoma of the colon. Haematoxylin and eosin preparation. Magnification $\times 12.5$.

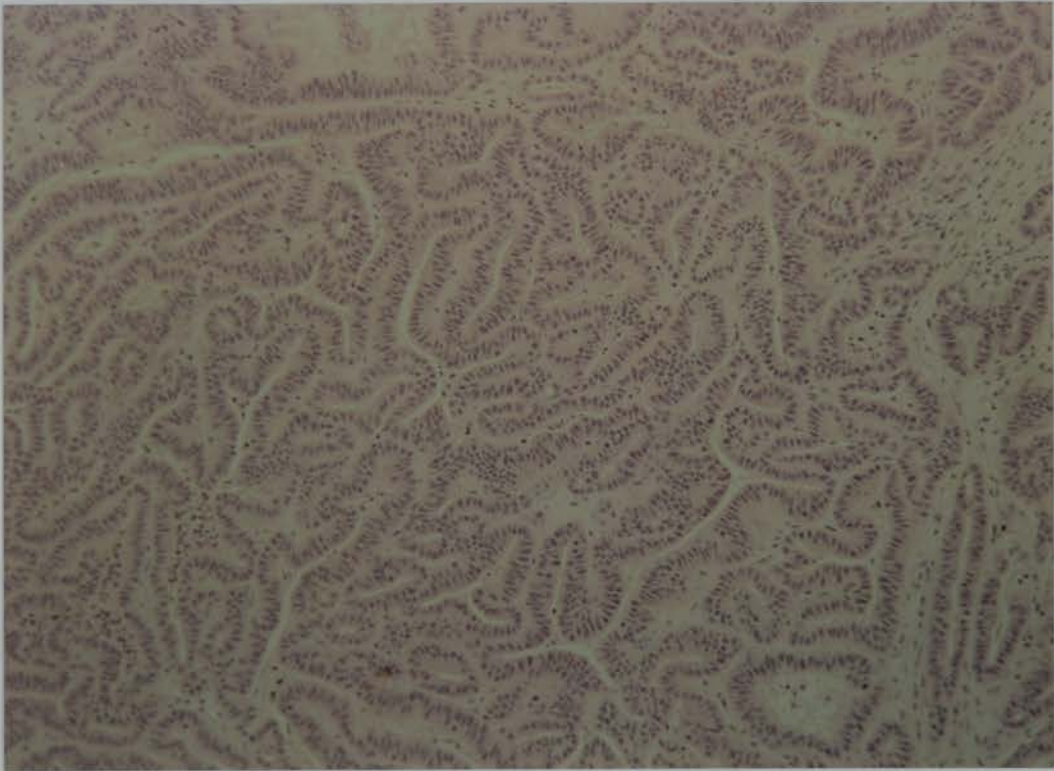


Figure 3.16. Histological appearance of MAC 26 murine adenocarcinoma of the colon. Haematoxylin and eosin preparation. Magnification $\times 12.5$.

MAC 16 however had a lower clearance and higher AUC indicating a slower rate of clearance and overall increased plasma concentrations.

The second possibility for low parent drug levels in MAC 26, is significant immediate metabolism. Figure 3.17 illustrates the concentration-time profiles of 2,7-DM in both tumour types. The formation of 2,7-DM differed significantly in both tumour types. Peak levels were achieved within the first minutes in MAC 16 then followed by a steady decline. In contrast, MAC 26, again showing early metabolism albeit lower than MAC 16, displayed a steady increase reaching peak levels over the first 5 to 10 minutes greater than those seen in MAC 16 at the same time points. Calculation of AUCs revealed greater tumour exposure to 2,7-DM in MAC 26 than MAC 16 suggesting that a significantly greater conversion to 2,7-DM was occurring in MAC 26. This is supported by the 4-fold increase in the 2,7-DM:MMC ratio in MAC 26 when compared to MAC 16. Overall, the well vascularisation of MAC 26 and rapid metabolism to 2,7-DM may both contribute to the reduced levels of parent drug.

However, more important is the elevated levels of metabolism seen in MAC 26 with respect to MAC 16 and what implications this may propose. Figure 3.18 illustrates the metabolite profile of MAC 16 and MAC 26, 15 minutes after mitomycin C administration. These metabolite profile can be compared to those obtained by the subcellular fractions from both

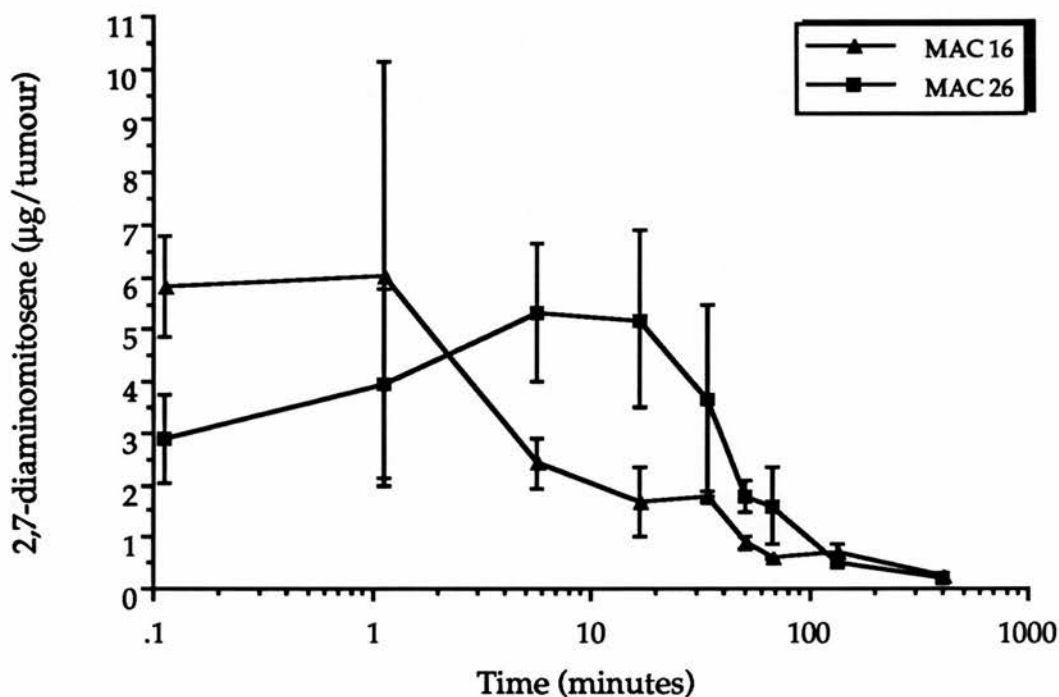


Figure 3.17. Concentration-time profiles of 2,7-diaminomitosenone in MAC 16 and MAC 26 murine adenocarcinomas of the colon after a direct i.t. injection of 500 µg mitomycin C. Results are expressed as µg/total tumour content and represent the mean±SE of three separate animals per time point.

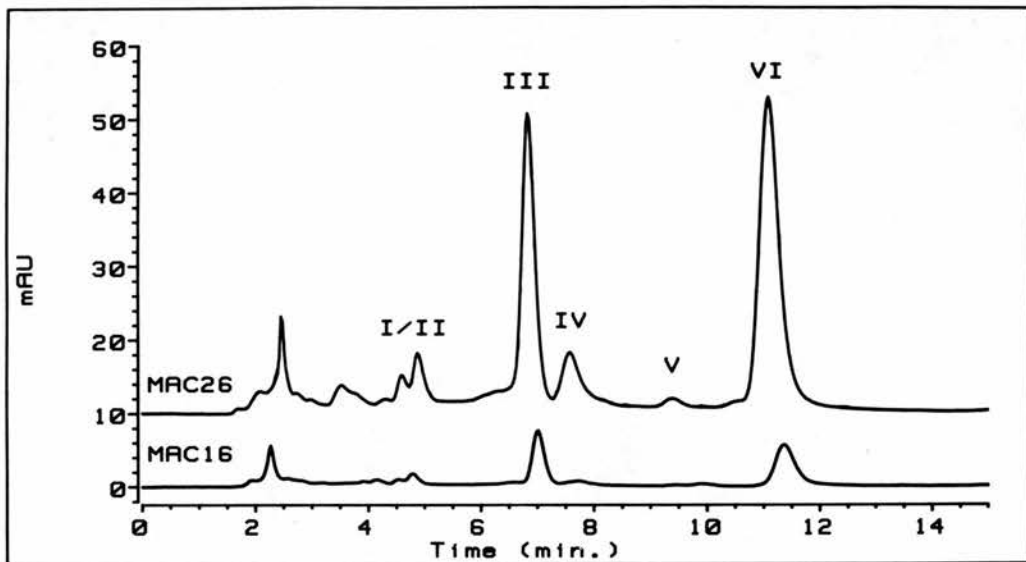


Figure 3.18. *In vivo* metabolite profile of MAC 16 and MAC 26 murine adenocarcinomas of the colon 15 minutes after a direct i.t. injection of 500 μg mitomycin C.

Peaks: I, zwitterion form of mitomycin C; II, 1,2-*trans*-1-hydroxy-2,7-diaminomitosenes; III, mitomycin C; IV, 1,2-*cis*-1-hydroxy-2,7-diaminomitosenes; V, 10-decarbamoyle 2,7-diaminomitosenes; VI, 2,7-diaminomitosenes.

tumours. MAC 16 exhibits predominantly 2,7-DM with only trace levels of trans- and cis-hydro. This has previously been seen in MAC 16 after a similar dose of mitomycin C (Cummings *et al.*, 1994). Such a profile, in particular to the predomination of 2,7-DM, is similar to that observed in the cytosolic fractions, figures 3.1 and 3.2 and tumour homogenates, figures 3.9 and 3.10, suggesting DT-diaphorase-dependent metabolism.

In comparison, MAC 26 showed significant levels of all metabolites, an identical profile to that observed in the mitochondrial fraction, the only enzyme activity detected in MAC 26.

From these metabolite profiles, it can be proposed that in MAC 16 DT-diaphorase is once again predominating over other enzymes present, resulting in significant but low levels of mitomycin C metabolism. MAC 26 metabolism however, is likely to be due to the mitochondrial enzyme identified in section 3.1. Such mitochondrial-dependent metabolism results in significant and comparable levels of 2,7-DM to MAC 16. This casts yet another doubt on the efficiency of DT-diaphorase to metabolise mitomycin C, this time *in vivo* and raises the possibility of a role for mitomycin C drug treatment in tumours low in DT-diaphorase and high in one-electron reductases.

With such observations, studies were extended to assess the antitumour activity of mitomycin C to ascertain if a corresponding increase in

antitumour activity can be achieved in tumours low in DT-diaphorase compared to those high in the enzyme.

The antitumour activity of mitomycin C in both MAC 16 and MAC 26 was assessed as described in section 2.3.11. Figures 3.19 and 3.20 illustrate the antitumour activity of the lower doses of mitomycin C against untreated and vehicle control groups. The data represent a number of combined investigations hence the differing measurement days. Tumour volumes at the time of injection ranged from 0.005-0.114 cm³ (median = 0.022 cm³) and 0.090-0.190 cm³ (median = 0.075 cm³) for MAC 16 control and drug treated groups respectively. Similarly, values for MAC 26 control and drug treated groups tumours ranged from 0.010-0.305 cm³ (median = 0.082 cm³) and 0.021-0.388 cm³ (median = 0.098 cm³) respectively. A clear antitumour response could be seen in both tumour types at all doses, with MAC 16 exhibiting end point tumour sizes of 0.058-0.720 cm³ (median = 0.225 cm³) for controls and 0.003- 1.267 cm³ (median = 0.083 cm³) for drug treated groups. MAC 26 controls ranged from 0.188-3.897 cm³ (median = 2.630 cm³) and drug treated from 0.017-1.675 cm³ (median = 0.444 cm³). It is interesting to note that MAC 26 has responded significantly to mitomycin C treatment. This is in contrast to the data of Double & Bibby (1989) where no significant growth inhibition was observed. The drug was however administered systemically suggesting that high local tumour levels of mitomycin C are required to elicit an antitumour response.

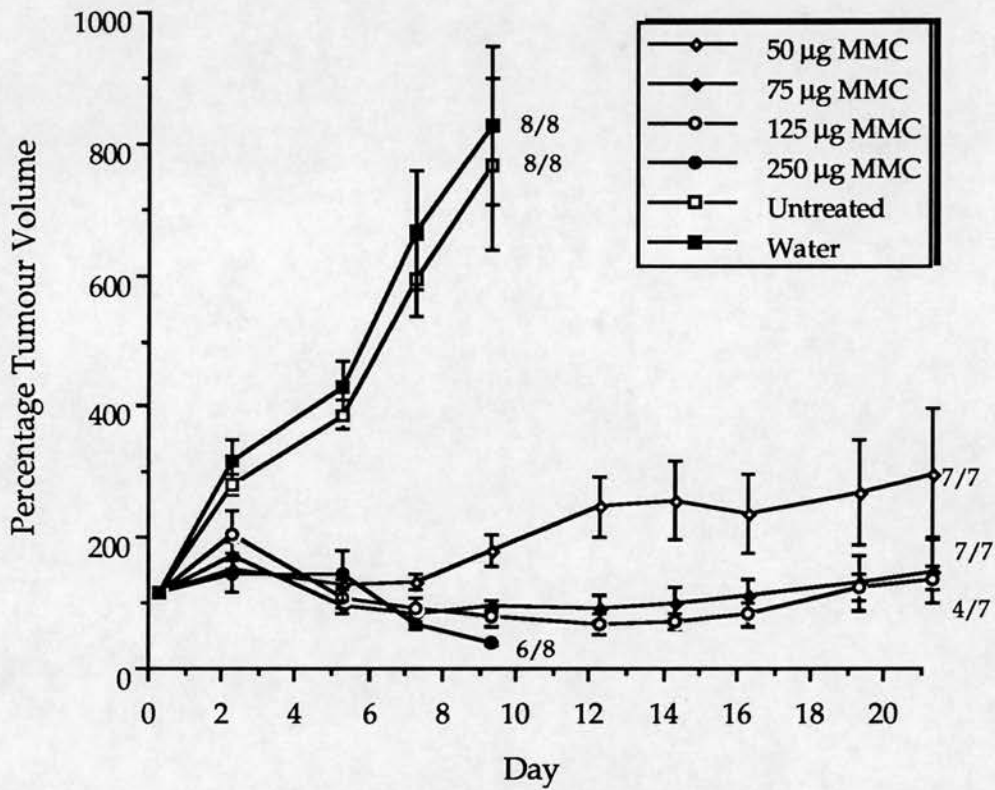


Figure 3.19. Antitumour activity of mitomycin C in MAC 16 murine adenocarcinoma of the colon at doses of 50, 75, 125 and 250 µg. Treatments were administered in a volume of 250 µL sterile water by direct i.t. injection. Controls consisted of untreated and vehicle (water) treatment groups. Each point represents the mean±SE of seven/eight separate animals.

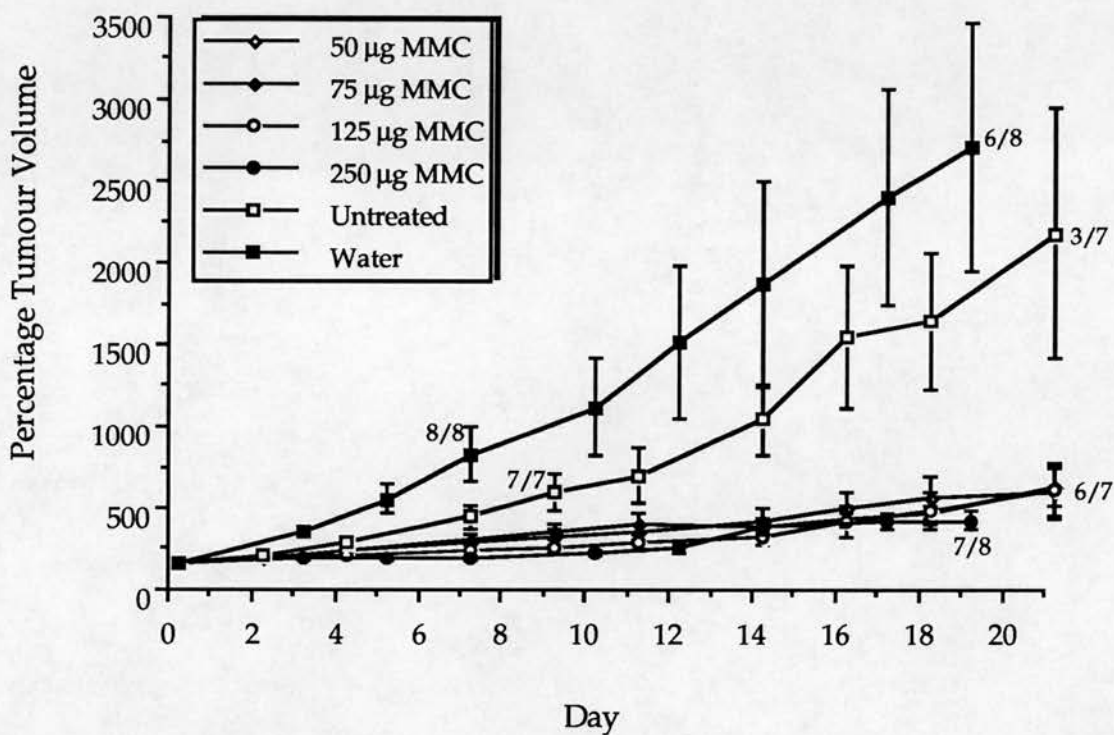


Figure 3.20. Antitumour activity of mitomycin C in MAC 26 murine adenocarcinoma of the colon at doses of 50, 75, 125 and 250 µg. Treatments were administered in a volume of 250 µL sterile water by direct i.t. injection. Controls consisted of untreated and vehicle (water) treatment groups. Each point represents the mean \pm SE of seven/eight separate animals.

However, both tumour types showed no significant dose response. This suggests that the maximum effect has been achieved at 50 µg mitomycin C. It would therefore be appropriate to extend studies using lower doses. Toxicity was noted in both MAC 16 and MAC 26 bearing mice and was particularly evident in MAC 16 at 500 and 1000 µg (data not shown). This may possibly be related to the greater plasma AUC seen in MAC 16 (129.50 µg/mLxhr) compared to MAC 26 (88.09 µg/mLxhr), table 3.5. The cachexic nature of MAC 16 may also have contributed (Double & Bibby, 1989).

T/C values for a treatment group compared to vehicle control calculated at day 7 after treatment are shown in table 3.6. The vehicle control group was used as it takes into account the effect of the i.t. injection such as cellular destruction and general tumour distention.

On assessing this data, results showed that there was no significant difference between the antitumour effect of mitomycin C in tumours high and low in DT-diaphorase. Therefore no firm conclusion can be drawn from this study concerning the role of DT-diaphorase in *in vivo* mitomycin C metabolism and antitumour activity. Using lower doses of mitomycin C and taking into account the doubling time of each tumour, calculating specific antitumour activity may be a more appropriate measurement.

Mitomycin C µg	MAC 16 T/C %	MAC 26 T/C %
50	18	29
75	11	31
125	12	23
250	8	17

Table 3.6. Antitumour activity of i.t. mitomycin C in MAC 16 and MAC 26 murine adenocarcinomas of the colon at doses 50, 75, 125 and 250 µg. Results are expressed in tumour volume as a percentage of the vehicle (sterile water) control group, T/C value. Values were calculated at day 7 after treatment.

In conclusion, despite the presence of the highly active mitochondrial reductase and the greater metabolising capacity of MAC 26, this tumour failed to produce a greater antitumour response compared to MAC 16. The rapid metabolism and significant tumour clearance of parent drug may have contributed to this reduced antitumour activity.

Therefore, it appears from this study that tumours high and low in DT-diaphorase (the latter being correspondingly high in one-electron reductases) are equally responsive to mitomycin C *in vivo* if given by the i.t. route of administration. This provides an unclear conclusion to the role of solid tumour DT-diaphorase levels and activity in enzyme directed bioreductive drug therapy and which tumours are optimum for mitomycin C drug treatment. Taking into account the limitations and reservations of the above study and making the suggested modifications may provide significant and interesting advances in this area of research.

What conclusions concerning the role of DT-diaphorase can be drawn from a human perspective? This was examined by assessing the metabolism of mitomycin C in two human colon xenografts, HT-29 and BE.

Both tumours have previously been employed in a number of studies using mitomycin C and indoloquinone EO9 due to their differing levels of DT-diaphorase (Siegel *et al.*, 1990a; Traver *et al.*, 1992; Beall *et al.*, 1994;

Plumb & Workman, 1994; Plumb *et al.*, 1994). However, as the majority of studies used cell lines, no evidence exists on metabolic studies carried out using tumour tissue.

The quinone reductase profiles of HT-29 and BE are shown in table 3.7. Consistent with the above studies, HT-29 possessed significant levels of DT-diaphorase which was the main enzyme found in the tumour. In contrast, BE possessed no DT-diaphorase activity, both cytosolic and microsomal, due to the point mutation in the NQO₁ gene (Traver *et al.*, 1992). 'Other' enzymes represented a lower proportion of the total enzyme activities of HT-29, approximately 49-fold less than cytosolic DT-diaphorase.

In comparison, such enzymes accounted for the only cytosolic activity seen in BE. Both tumour types have previously shown undetectable levels of the one-electron reductase xanthine oxidase, although this was assessed in cell lines not tumour tissue (Siegel *et al.*, 1990b). NADPH:cytochrome P-450 reductase and NADH:cytochrome *b*₅ reductase activities in HT-29 were approximately 44- and 50-fold less than that of total DT-diaphorase respectively. Low activities of both these enzymes were observed in BE.

TUMOUR	CYTOSOL		MICROSOMES			
	DT-diaphorase NADH	'Others' NADPH	DT-diaphorase NADH	Cytochrome P450 reductase NADPH	Cytochrome <i>b</i> ₅ reductase NADH	
HT-29	2776.80±205	2103.3±371	98.04±7.1	50.63±10.5	115.42±9.4	100.92±14.1
BE	ND	ND	ND	ND	15.55±1.57	29.57±3.09

Table 3.7. Quinone reductase activity in cytosolic and microsomal subcellular fractions isolated from HT-29 and BE human colon xenografts. All enzyme activities are expressed as nmol cytochrome *c* reduced/min/mg protein using the extinction coefficient, ϵ 21.1 x 10³M/cm. Each value represents the mean±SE from three separate experiments. ND = not detected.

Mitomycin C metabolism by HT-29 and BE tumour homogenates was assessed under both aerobic and hypoxic conditions to give an indication of the role of DT-diaphorase. Figure 3.21 and table 3.8 illustrate the formation of 2,7-DM under these conditions. In many respects, these tumours exhibited similar metabolism characteristics to those seen in MAC 16 and MAC 26 homogenates.

Firstly, HT-29, high in DT-diaphorase, showed no significant hypoxic enhancement similar to MAC 16. Secondly, BE, with no detectable DT-diaphorase activity, in common with MAC 26, did show hypoxic enhancement suggestive of one-electron reduction. However, the absolute amount of change in 2,7-diaminomitosene formation under hypoxia was greater in HT-29 (0.18 $\mu\text{g}/\text{mL}$) compared to BE (0.11 $\mu\text{g}/\text{mL}$). This suggests a possible limited role for one-electron reductases, such as the NADPH-dependent mitochondrial enzyme, in the hypoxic enhancement of mitomycin C metabolic activation observed BE.

It is interesting to note the elevated level of metabolism in HT-29 compared to BE both in the absence and presence of oxygen in contrast to MAC 16 and MAC 26. This is undoubtedly due to the very high activity of DT-diaphorase in HT-29 and is of benefit to this tumour type over BE, low in DT-diaphorase.

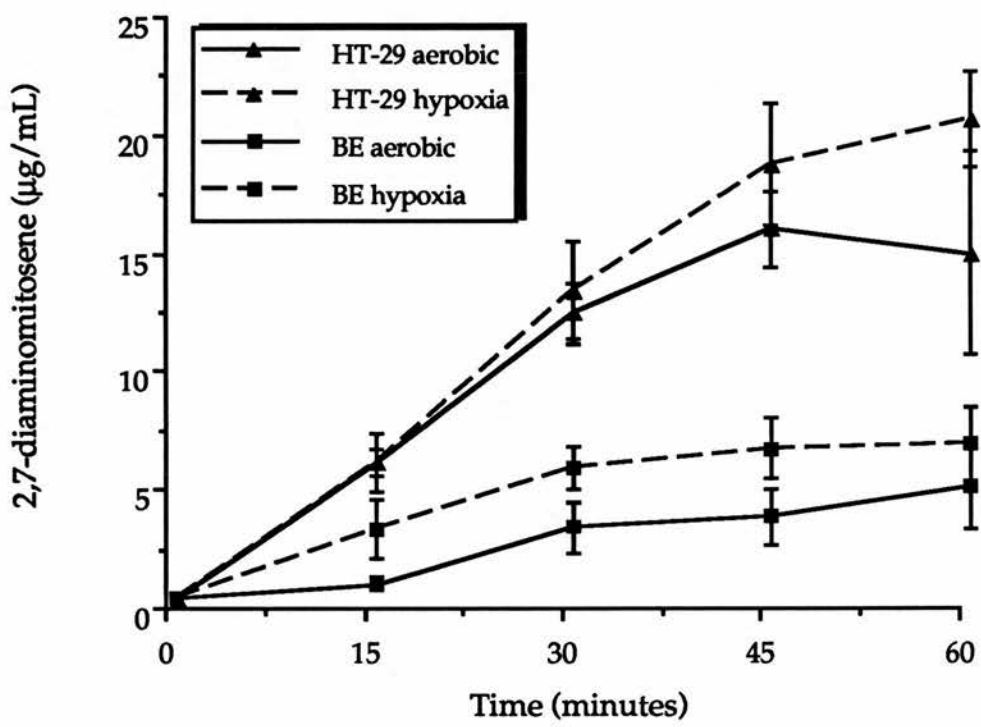


Figure 3.21. *In vitro* production of the principle mitomycin C metabolite 2,7-diaminomitosenone in HT-29 and BE human colon xenograft homogenates under aerobic (—) and hypoxic (---) conditions. Each point represents the mean±SE from three separate experiments.

TUMOUR HOMOGENATE	2,7-DM FORMATION (nmol/30min/mg protein)		EFFECT OF OXYGEN
	AEROBIC	ANAEROBIC	
HT-29	0.99±0.09	1.17±0.19	15% inhibition
BE	0.13±0.05	0.24±0.03	46% inhibition

Table 3.8. Specific activities of mitomycin C metabolism by HT-29 and BE human colon xenograft homogenates under aerobic and hypoxic conditions. Specific activity is expressed as 2,7-diaminomitose formation in nmol/30min/mg protein. Each value represents the mean±SE from three separate experiments.

The metabolite profiles of mitomycin C metabolism under hypoxia by HT-29 and BE are illustrated in figure 3.22. Both tumours showed significant levels of all metabolites, both primary and secondary, although 2,7-DM once again was the major metabolite, particularly in HT-29. In contrast to MAC 16 cytosolic fractions, homogenates and *in vivo* metabolism which yield predominantly 2,7-DM (with trace levels of other metabolites) suggesting DT-diaphorase activity, HT-29, possessing approximately 7-fold greater DT-diaphorase activity, yielded significant levels of all metabolites. This may be due to additional metabolism by one-electron reductases (Tomasz & Lipman, 1981; Pan *et al.*, 1984) or metabolism of 2,7-DM itself to further metabolites (Pan *et al.*, 1984; Siegel *et al.*, 1992). BE metabolism appeared to be characteristic of one-electron reduction similar to that seen in MAC 26.

What relevance do such results have in a clinical situation? In the first instance, tumours low in DT-diaphorase and high in one-electron reductases appear to be an attractive target for mitomycin C over those with high DT-diaphorase activities if a significant degree of hypoxia can be achieved. In fact, the specific activity of MAC 26 under hypoxia, table 3.4, was greater than that seen with HT-29 under similar conditions, table 3.8. Indeed, it would be appropriate to examine the expression and activity of the one-electron mitochondrial reductase in a wider spectrum of tumours and normal tissues, particularly those of human derivation, and to assess whether its activity can be exploited for therapeutic benefit.

This is currently being examined with respect to the mitomycin C analogue indoloquinone EO9 in a panel of cell lines transfected with NADPH:cytochrome P-450 reductase where varying levels of expression have been observed (Saunders *et al.*, 1996).

Two major obstacles hinder this notion. Firstly, such enzymes require hypoxia for activation. Although solid human tumours possess significant areas of central hypoxia, peripheral well oxygenated cells may remain less responsive or perhaps resistant to mitomycin C treatment. This may however be circumvented to a certain extent by the use of mitomycin C in conjunction with hypoxia-potentiating agents as discussed in section 1.3.1. Secondly, very few tumours have very low or lack DT-diaphorase activity. This has been demonstrated using the NCI tumour cell line panel which revealed that the majority possessed significant levels of the enzyme in association with reduced levels of NADPH:cytochrome P-450 reductase and NADH:cytochrome *b*₅ reductase (Fitzsimmons *et al.*, 1996). In addition, extensive data obtained directly from human tumour samples now indicate predominantly high levels of DT-diaphorase compared to normal tissue and is particularly applicable to colon cancer (reviewed in Smitskamp-Wilms *et al.*, 1996).

Therefore, are there any benefits of treating high DT-diaphorase tumours with mitomycin C? Mitomycin C has been shown to be metabolised by DT-diaphorase under both aerobic and hypoxic conditions. The presence

of this enzyme can lead to metabolism in areas of varying oxygen tensions within a tumour unlike the hypoxia-dependent one-electron reductases. However, if hypoxia is a prerequisite for a particular drug's mechanism of activation, then clearly such tumours may not be ideal since DT-diaphorase can successfully compete against one-electron reductases for substrates and resulting in a lower rate of metabolic activation as seen in MAC 16.

The ideal scenario would be in some way to prevent predomination of DT-diaphorase under hypoxia allowing one-electron reductase-dependent metabolism to prevail in conjunction with aerobic metabolism via DT-diaphorase. This may prove a somewhat difficult goal to achieve.

As the majority of human tumours possess considerable levels of DT-diaphorase, one can conclude that in reality tumours high in the enzyme are optimum for mitomycin C drug therapy.

The role of one-electron reductases and hypoxic enhancement should not be discounted however. With the use of patient tumour enzyme profiling, the measurement of tumour hypoxia and a greater understanding of the interaction between all quinone reductases within themselves and with hypoxia, this observation may prove a successful exploitable parameter.

3.4 Properties of Indoloquinone EO9 Intermediates Generated by Irradiation

Although significant progress has been made in defining the enzymology of EO9 bioreductive activation (reviewed in Bailey *et al.*, 1996; Smitskamp-Wilms *et al.*, 1996), little data exists on the following key areas (a) generation of reactive intermediates, (b) formation of drug metabolites and (c) structure of DNA adducts. Pulse radiolysis of EO9 provides a controlled *in vitro* reduction of the drug without the need of reductive enzymes and cofactors, and also allows reductive processes to be studied as well as the formation of potential metabolites. Chemical properties of reductive products can then be characterised by HPLC and UV/Vis spectrophotometry (Salmon & Sykes, 1993).

EO9 was subjected to irradiation and subsequent HPLC analysis as described in sections 2.3.17 and 2.3.13 respectively. Reduction occurs via a series of free radical reactions, ultimately resulting in the formation of an isopropanol ion which reduces EO9. Figure 3.23 illustrates the pattern of EO9 related chromatographic peaks generated by a single dose of irradiation compared to an unirradiated sample. Both samples were analysed approximately 18 hours after irradiation due to sample transportation. The parent drug, EO9, could be clearly seen with a retention time of approximately 14-16 minutes and was the predominant peak in both samples.

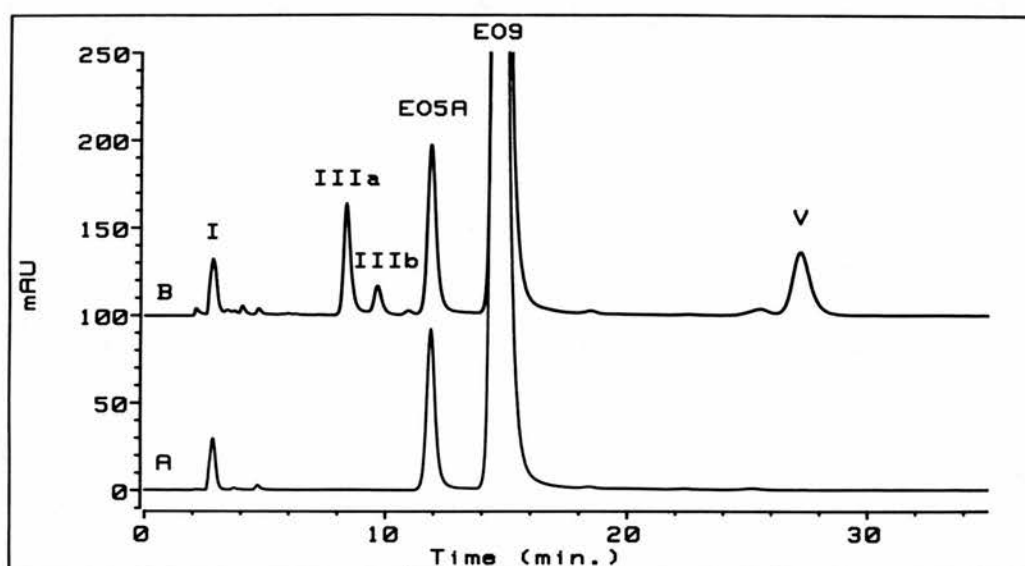


Figure 3.23. *In vitro* pattern of Indoloquinone EO9 related chromatographic peaks generated by a single 500 Gy irradiation dose (A). Results were compared to the unirradiated control (B). Samples were analysed by HPLC.

Peaks I, IIIa, IIIb and V correspond to previously unidentified products.

The open aziridine ring hydrolysis product EO5A was observed in both irradiated and unirradiated samples and eluted before EO9 in a similar manner to previous HPLC protocols (Binger & Workman, 1990; Schellens *et al.*, 1993). The presence of both parent drug and EO5A was confirmed by their different UV/Vis absorption spectra, figure 3.24. Consistent with previous spectral studies (Binger & Workman, 1990), EO9 showed characteristic UV absorption at 268 nm with a shoulder at 313 nm and visible absorption at 505 nm. In contrast, the spectra of EO5A exhibited a bathochromic shift resulting in UV absorption at 280 and 321 nm and visible absorption at 550 nm. This shift in spectral characteristics has been proposed to be a result of hydrogen bonding and conjugation of the quinone moiety by a lone pair of electrons (Griffiths, 1967; Binger & Workman, 1990).

It is interesting to note the presence of EO5A in the unirradiated and sample. This is most likely to be a result of the general instability of EO9 in aqueous solution < pH 7 (de Vries *et al.*, 1993). Also, as the samples were not analysed immediately, this may have enhanced this effect resulting in the hydrolysis of EO9 and hence the formation of EO5A. The similar levels of EO5A seen in the control may suggest that this product is a result of acid catalysed degradation rather than irradiation.

In addition to EO5A, an additional peak, peak I in figure 3.23, arose in both samples. With spectral characteristics similar to EO5A, figure 2.34,

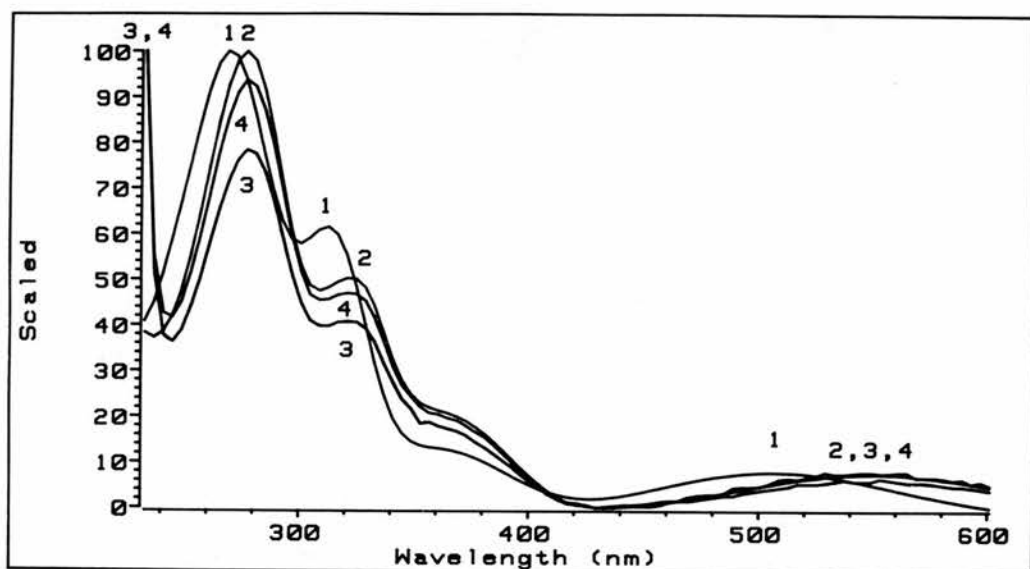


Figure 3.24. UV/Visible absorption spectra of Indoloquinone EO9 and related products of irradiation. Products were identified during HPLC analysis of irradiated and unirradiated samples as shown in figure 3.23. Spectrum 1, EO9; Spectrum 2, EO5A; Spectrum 3, peak I unirradiated sample; Spectrum 4, peak I irradiated sample.

and elution at the solvent front, retention time 3 minutes, such peaks are possibly aziridinyl adducts formed with phosphate buffer constituents. No further peaks were observed in the unirradiated sample. However, together with EO9, EO5A and peak I, three additional intermediates were identified in the irradiated sample, peaks IIIa, IIIb and V. With retention times 8.2, 9.4 and 27 minutes respectively, these peaks are likely to be a direct result of EO9 irradiation due to their absence in the control sample.

The effect of time on the levels of these identified products was also studied to assess their stability. Figure 3.25 illustrates the effect of time on the pattern of EO9 related products generated by irradiation as measured by HPLC. Samples were analysed at 18 hours and 20 days. The variation in retention times between samples was due to the use of a new HPLC column.

The effect of time had dramatic results. The loss of EO9 was accompanied by a corresponding increase in EO5A, possibly again due to significant hydrolysis. However, what is interesting was the reduction in peaks IIIa and IIIb, increase in peak I and V and appearance of peak VI, retention time 4 minutes. The effect of time on peak area can be seen more clearly in figures 3.26 and 3.27, with additional samples taken on days 2 and 5. The steady reduction in peaks IIIa and IIIb suggest that such products are relatively unstable and possible breakdown into additional peaks may occur. This may result in the formation of peak VI which appears

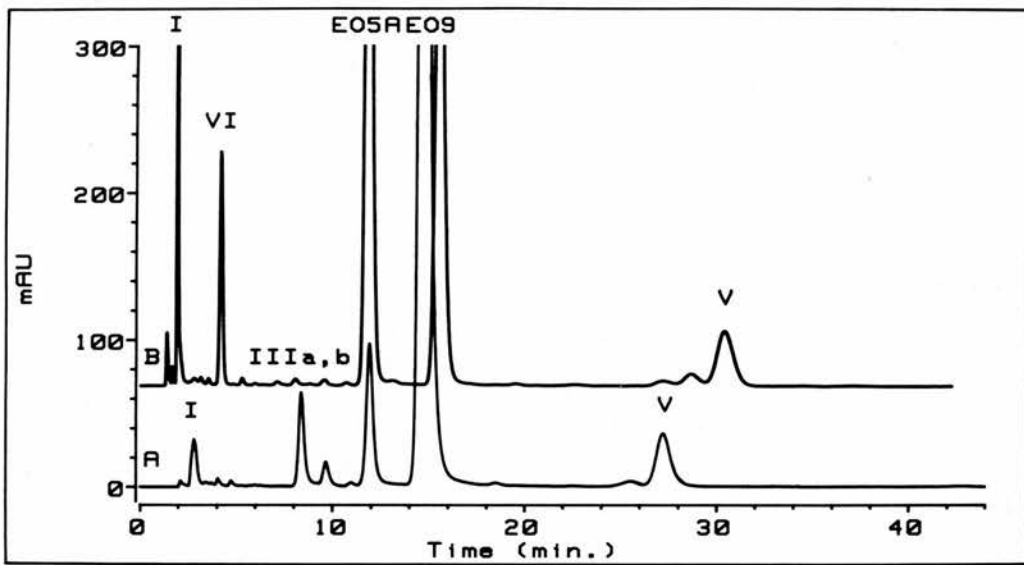


Figure 3.25. Effect of time on the *in vitro* pattern of Indoloquinone EO9 related products generated by a single 500 Gy irradiation dose. Samples were analysed at 18 hours (A) and 20 days (B) and product levels measured by HPLC.

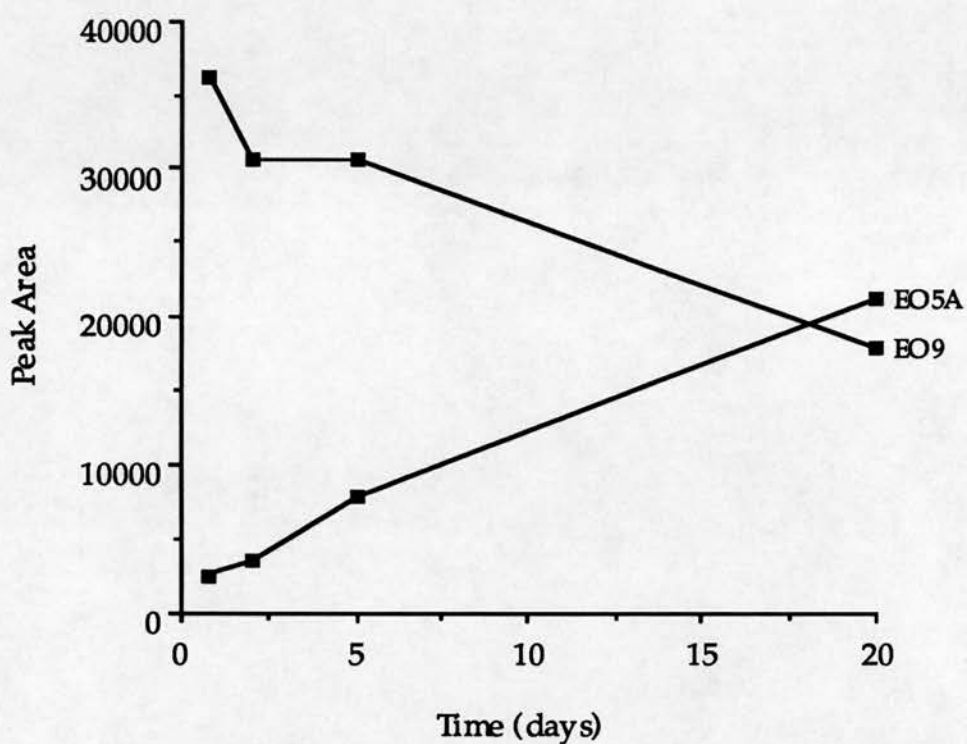


Figure 3.26. Effect of time on the stability of Indoloquinone EO9 and the major hydrolysis product, EO5A. Results are expressed as increase or decrease in peak area at 18 hours, 2, 5 and 20 days after irradiation.

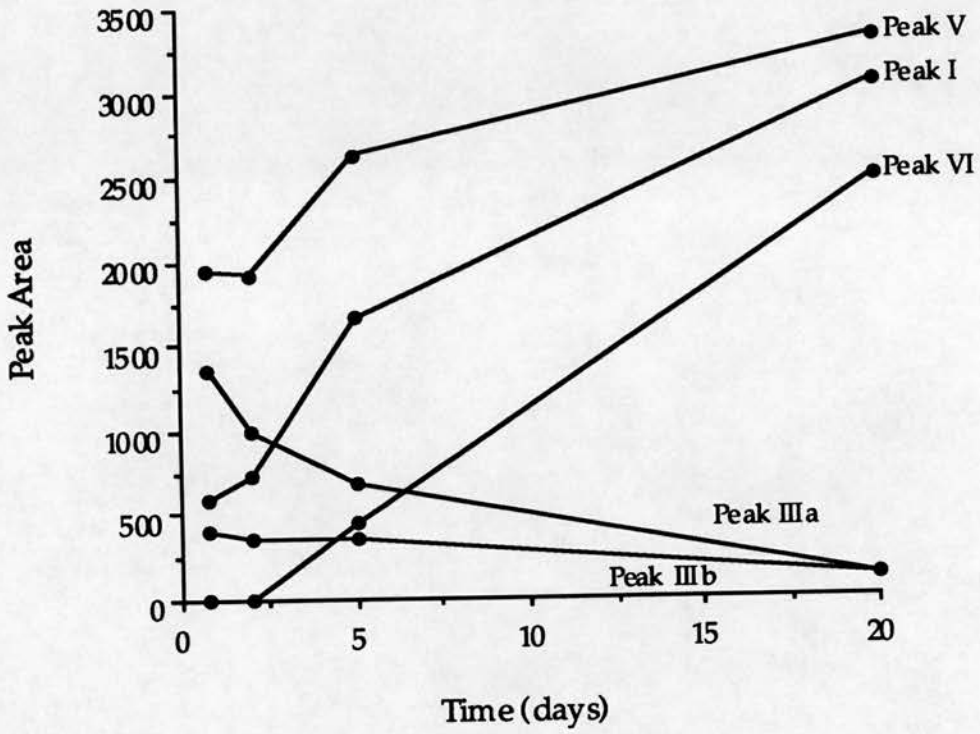


Figure 3.27. Effect of time on the stability of Indoloquinone EO9 related products of irradiation. Results are expressed as increase or decrease in peak area at 18 hours, 2, 5 and 20 days after irradiation.

between day 2 and 5 and increases sharply with time. This peak was not observed in the control and previous EO9 stability studies (data not shown) suggesting its formation results from degradation of products generated by irradiation. Little increase in peak V suggests it is relatively stable. The rise in peak I is consistent with increased formation of phosphate adducts over time.

The spectral characteristics of peaks I, IIIa, IIIb, V and VI are illustrated in figure 3.28. Peak I spectrum, obtained from the day 20 sample, was identical to that seen in figure 3.24 suggesting an increase in the adduct(s) not coelution of peaks bearing a different chromophore. Both peaks V and VI had similar spectra to EO9 and EO5A respectively. However, peaks IIIa and IIIb, which exhibited identical spectra, possess significant differences to those spectra observed above. Firstly, UV absorption was seen at 240 and 340 nm, wavelengths far removed from EO9 and the other products. Secondly, the characteristic shoulders seen in previous spectra were not present. And thirdly, perhaps the most important difference was the lack of visible absorption.

In conclusion, EO9 can be converted to several distinct chemical entities via irradiation. Such intermediates display both similarities and differences compared to the parent drug. The characteristics of EO9 and all these products produced as a result of irradiation and time are summarised in table 3.9.

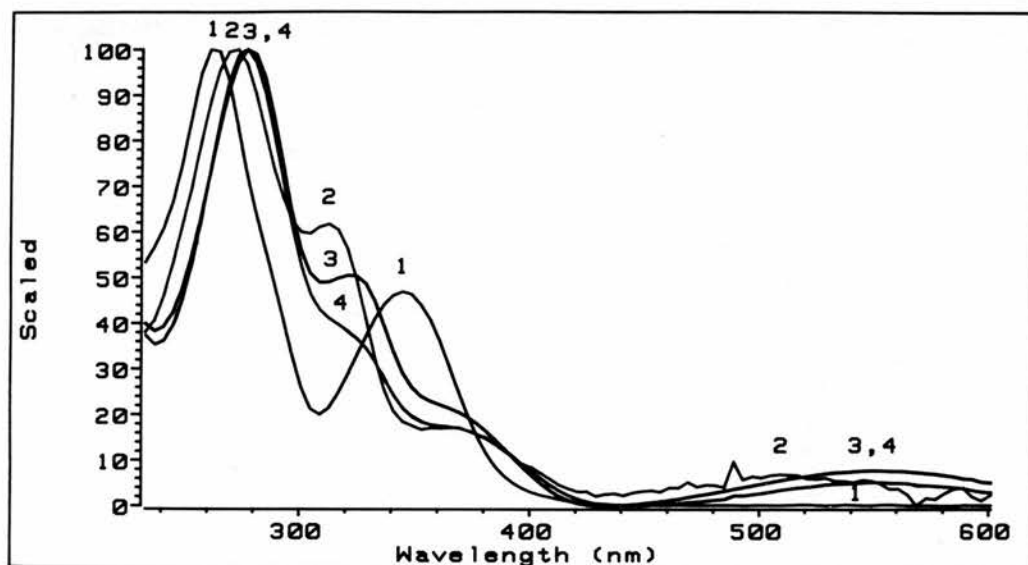


Figure 3.28. UV/Visible absorption spectra of Indoloquinone EO9 related products of irradiation. Products were identified during HPLC analysis as shown in figure 3.25.

Spectrum 1, peak IIIa/b; Spectrum 2, peak V; Spectrum 3, peak I; Spectrum 4, peak VI.

Peak	Retention Time (min)	UV Absorption (nm)	Visible Absorption (nm)	Effect of Time on Concentration
EO9	14-16	268, 313	505	Decrease
EO5A	12	280, 321	550	Increase
I	3	280, 321	550	Increase
IIIa	8.2	240, 340	nil	Decrease
IIIb	9.4	240, 340,	nil	Decrease
V	27	268, 313	505	Increase
VI	4	280, 321	550	Increase

Table 3.9. Characterisation of Indoloquinone EO9 related products generated via irradiation.

3.5 Characterisation of the Metabolites Produced by Enzymatic Reductive Activation of Indoloquinone EO9

The antitumour activity of EO9 given i.t. has been studied in both MAC 16 and MAC 26 murine adenocarcinomas of the colon and HT-29 and BE human colon xenografts (Gardiner *et al.*, unpublished). Table 3.10 shows the antitumour activity of EO9 in each tumour type. Clearly it can be seen that HT-29 was the most responsive to EO9 followed by BE, MAC 16 and MAC 26. Relating such results to the quinone reductase profiles of these tumour types, tables 3.1 and 3.7, it is likely that DT-diaphorase is responsible for EO9 metabolism in HT-29 and MAC 16. The latter tumour has previously been shown to be more responsive to EO9 than MAC 26 when given intraperitoneally and the levels of DT-diaphorase hypothesised to be responsible for this differential sensitivity (Walton *et al.*, 1992a). The low levels of DT-diaphorase in BE and MAC 26 resulted in decreased antitumour activity.

However, EO9 did show significant antitumour activity in BE with comparable T/C to MAC 16. This may be due to the different reductive capacities of human and murine quinone reductases. Murine DT-diaphorase has previously been shown to be less effective at reducing EO9 than the human variety (Lewis *et al.*, 1994). This may apply to other quinone reductases such as one-electron reductases which may be responsible for EO9 metabolism in BE.

TUMOUR	ANTITUMOUR ACTIVITY T/C%
MAC 16	33
MAC 26	60
HT-29	15
BE	27

Table 3.10. Antitumour activity of Indoloquinone EO9 in MAC 16 and MAC 26 murine adenocarcinomas of the colon and HT-29 and BE human colon xenografts. Measurements were taken on day 14 for the MAC tumours and day 21 for HT-29 and BE. EO9 was given as a single 250 µg i.t. dose. Results are expressed as tumour volume as a percentage of the vehicle control group, T/C value. Consent has been given by Dr. Gardiner to use these results.

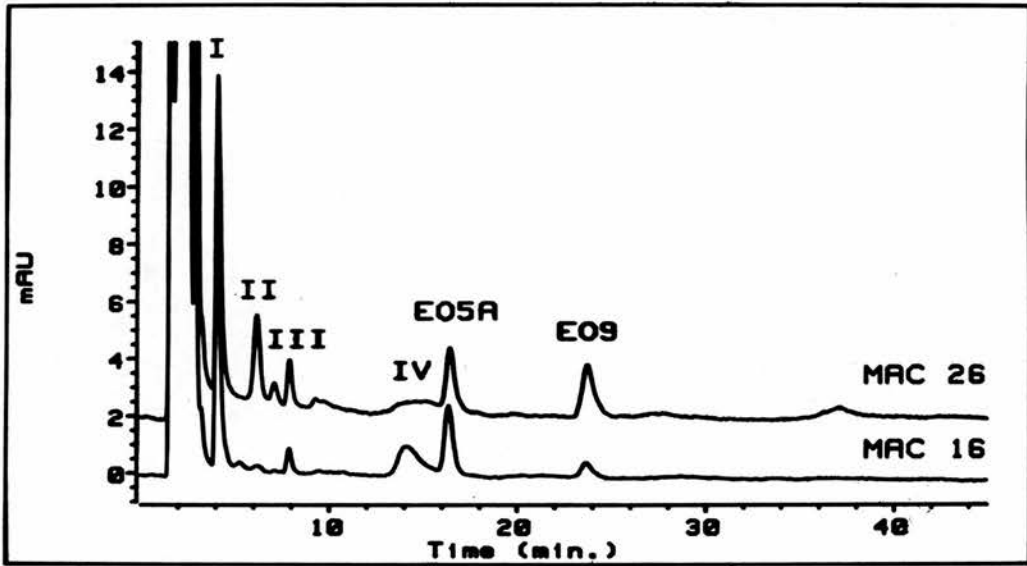
With these antitumour results and the results presented in section 3.4 regarding the generation of EO9 intermediates, can such tumours generate similar EO9 intermediates, now termed metabolites, and is it possible to relate the production such metabolites to DT-diaphorase activity and EO9 chemosensitivity?

This was studied using the tumour homogenate system previously used for mitomycin C as discussed in sections 3.2 and 3.3. Metabolism of EO9 was carried out under both aerobic and hypoxic conditions for all tumour types as described in section 2.3.14.

Figure 3.29 illustrates the *in vitro* metabolite profile generated by MAC 16 and MAC 26 tumour homogenates under aerobic and hypoxic conditions. It should be noted that the retention times of both the parent drug and EO5A vary to those in section 3.4. This is due to further optimisation of the HPLC technique.

A number of metabolites could be detected in both tumour types, although there was little difference in peak levels between incubations carried out under aerobic and hypoxic conditions. Once again EO5A could be seen eluting approximately 7 minutes before EO9. Four additional metabolites were also detected. Metabolites I and III were the predominant peaks seen in MAC 16 with trace levels of metabolite II. Metabolite IV was only observed under aerobic conditions.

A



B

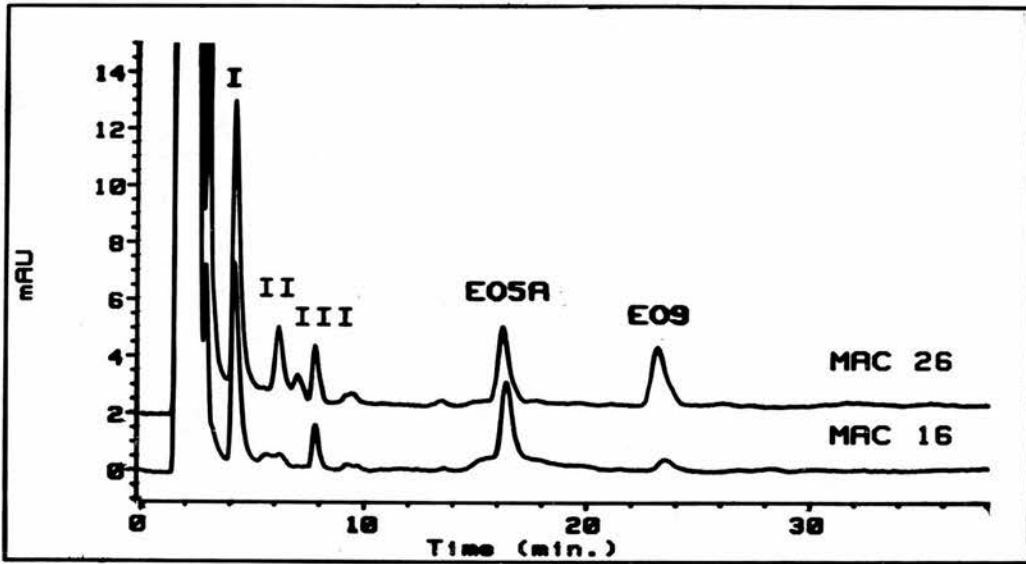


Figure 3.29. *In vitro* pattern of EO9 metabolism in MAC 16 and MAC 26 tumour homogenates under aerobic (A) and hypoxic (B) conditions. Samples were analysed at 45 minutes and metabolite levels measured by HPLC.

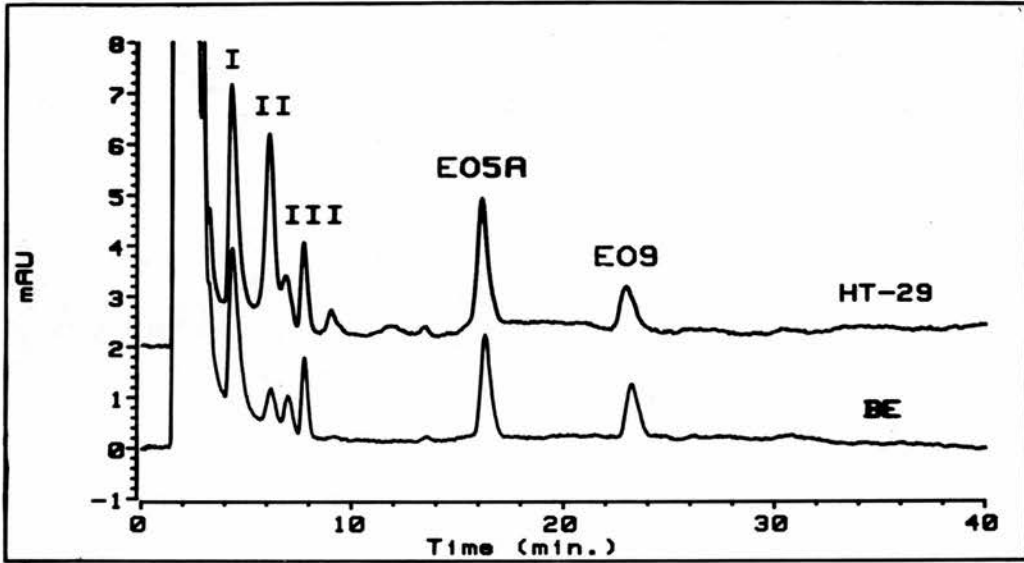
Peaks I, II, III and IV represent previously unidentified metabolites.

In contrast, MAC 26 produced higher levels of metabolites I, II and III under all conditions. Metabolite IV was present in aerobic incubations only.

A similar metabolite profile was observed in HT-29 and BE homogenates under aerobic and hypoxic conditions, figure 3.30. Once again metabolites I, II and III in addition to EO9 and EO5A were observed. Similar profiles were seen under aerobic and hypoxic conditions for each tumour type. In contrast to MAC 16, HT-29 displayed significant levels of metabolite II under all conditions. Metabolism in the BE homogenate resulted in considerably lower levels of this metabolite similar to MAC 16. Metabolite IV was not observed in either tumour type.

Metabolites of EO9 generated via purified DT-diaphorase have previously been detected. Maliepaard *et al.* (1995) observed three possible metabolites under hypoxic conditions similar to those seen in HT-29, figure 3.30. The high level of DT-diaphorase in HT-29, table 3.7, may be responsible for these metabolites and due to the lower DT-diaphorase activity in MAC 16, table 3.1, this may lead to reduced levels of metabolites. However, these metabolite profiles are also similar to MAC 26 and BE respectively, indicating that it may be difficult to associate metabolite levels to DT-diaphorase activity.

A



B

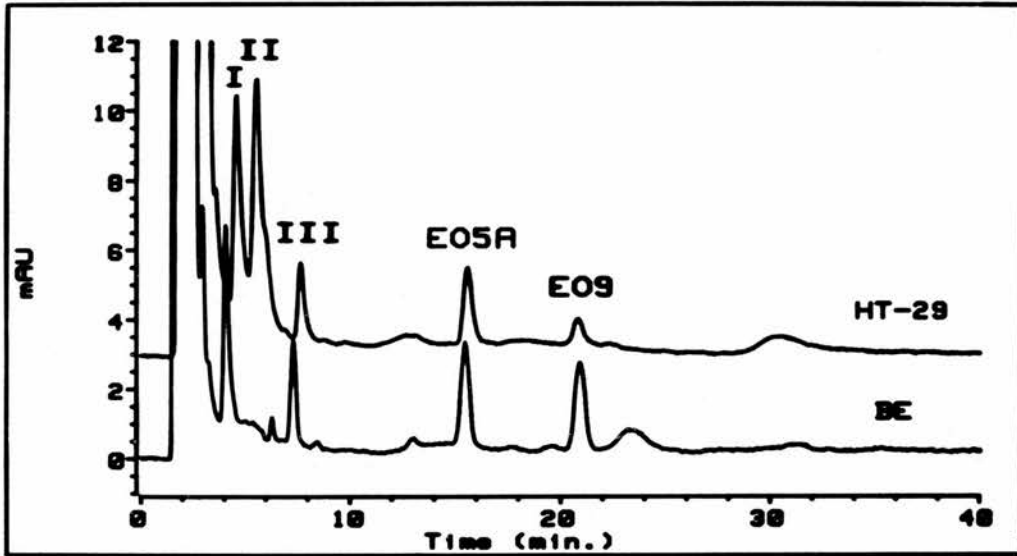


Figure 3.30. *In vitro* pattern of EO9 metabolism in HT-29 and BE tumour homogenates under aerobic (A) and hypoxic (B) conditions. Samples were analysed at 45 minutes and metabolite levels measured by HPLC. Peaks I, II and III represent previously unidentified metabolites.

With the presence of such metabolites after enzymatic reduction of EO9, do any correspond to those generated via irradiation and do levels change with time? Figure 3.31 illustrates the absorption spectra of EO5A and metabolites I, II and III. These spectra, although obtained from metabolites generated in HT-29 homogenate incubations due to their high levels, are the same as those spectra from MAC 16, MAC 26 and BE derived metabolites.

Metabolite I showed UV absorption at 280 nm (spectrum 2). No visible absorption was seen. This differed slightly to peak I observed after irradiation which had additional absorption at 321 and 550 nm. Therefore metabolite I and peak I are not the same product. However, as suggested in section 3.4, peak I is possibly an adduct formed with buffer constituents due to its presence in the control sample. A similar adduct, but of protein origin, may result in the drug-homogenate incubations. Retention times for both metabolite and peak I were 4 and 3 minutes respectively.

Metabolite II (spectrum 4) possessed a spectra similar to EO5A (spectrum 3) UV absorption 280 and 321 nm, visible absorption 550 nm. This metabolite, retention time approximately 6 minutes for all tumour incubations, was not present after irradiation indicating that it is generated by the bioreductive activation of EO9.

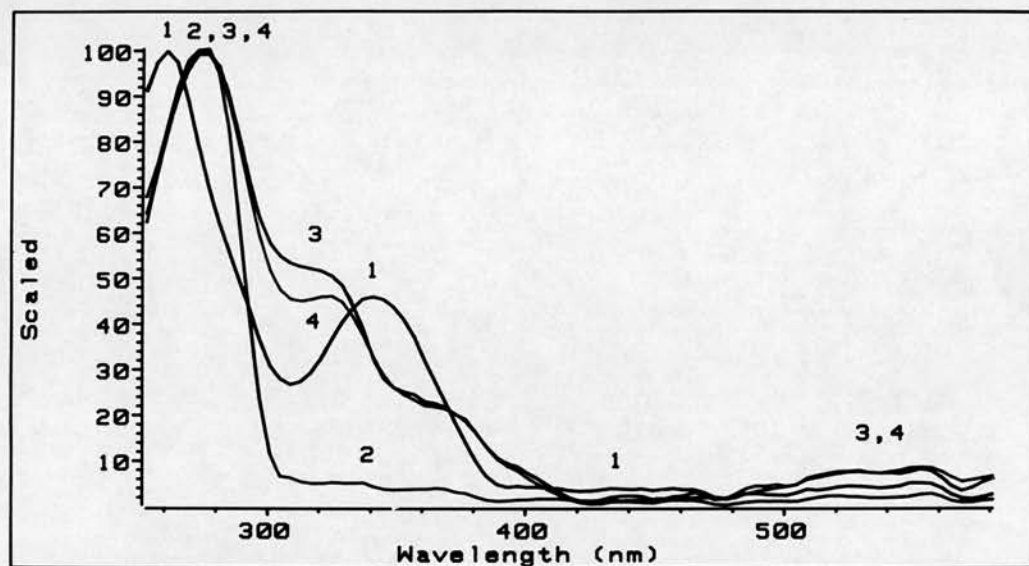


Figure 3.31. UV/Visible absorption spectra of Indoloquinone EO9 metabolites produced by HT-29 tumour homogenate. Metabolites were identified during HPLC as shown in figure 3.30.

Spectrum 1, metabolite III; Spectrum 2, metabolite I; Spectrum 3, EO5A; Spectrum 4, metabolite II.

Metabolite III (spectrum 1) had the most interesting spectrum. Identical to peaks IIIa and IIIb generated via irradiation, figure 3.28, this metabolite exhibited UV absorption at 240 and 340 nm only, distinct from any other metabolite. Therefore, metabolite III, retention time approximately 8 minutes, is possibly related chemically to peaks IIIa and IIIb from the irradiation studies. Metabolite IV, only observed in MAC 16 under aerobic conditions, figure 3.29, appeared in two out of the three repeat experiments. No further characterisation of this metabolite was carried out. Peak V produced via irradiation, retention time 27 minutes, could not be detected in any of the homogenate incubations.

The increase or decrease in the formation of EO9 metabolites was investigated in all tumour types. This was carried out to assess whether it was possible to correlate metabolite formation to the antitumour profile in observed in table 3.10 and hence identify a marker for EO9 metabolic activation. Figures 3.32 and 3.33 illustrate the changing levels of metabolites in MAC 16 and MAC 26 respectively.

Clearly it can be seen that there was little difference in the levels of all metabolites produced under aerobic and hypoxic conditions in both MAC 16 and MAC 26. Metabolite I in both tumours was present at 0 minutes, supporting the theory that this is a chemical adduct not requiring metabolic activation and not a direct result of EO9 metabolism. Levels of metabolite II were higher in MAC 26 than MAC 16 under both conditions.

Increasing in the first 45 minutes, little increase or decrease in levels of this metabolite were seen once standard errors were taken into account. A similar scenario applies to metabolite III, although levels did genuinely decrease with time implying possible instability.

EO5A formation was consistent throughout all experiments increasing steadily with time. Maximal levels were once again similar for both MAC 16 and MAC 26 under aerobic and hypoxic conditions.

From these results, no single metabolite could be identified to indicate if any difference in EO9 metabolism occurred under either aerobic or hypoxic conditions. This was particularly evident in MAC 26, low in DT-diaphorase, which exhibited enhancement in mitomycin C metabolic activation under hypoxia, sections 3.1 and 3.2.

In the context of quinone reductase levels, particularly DT-diaphorase, no correlation could be found between the formation of the above metabolites and quinone reductase activity, table 3.1. No hypoxic enhancement, analogous to that seen with mitomycin C, was observed in MAC 26 possibly suggesting a limited role for the mitochondrial one-electron reductase identified in section 3.1. However in contrast, levels of metabolites I, II and III did increase under hypoxia in MAC 16 incubations which is unexpected if DT-diaphorase-dependent metabolism is thought to be occurring.

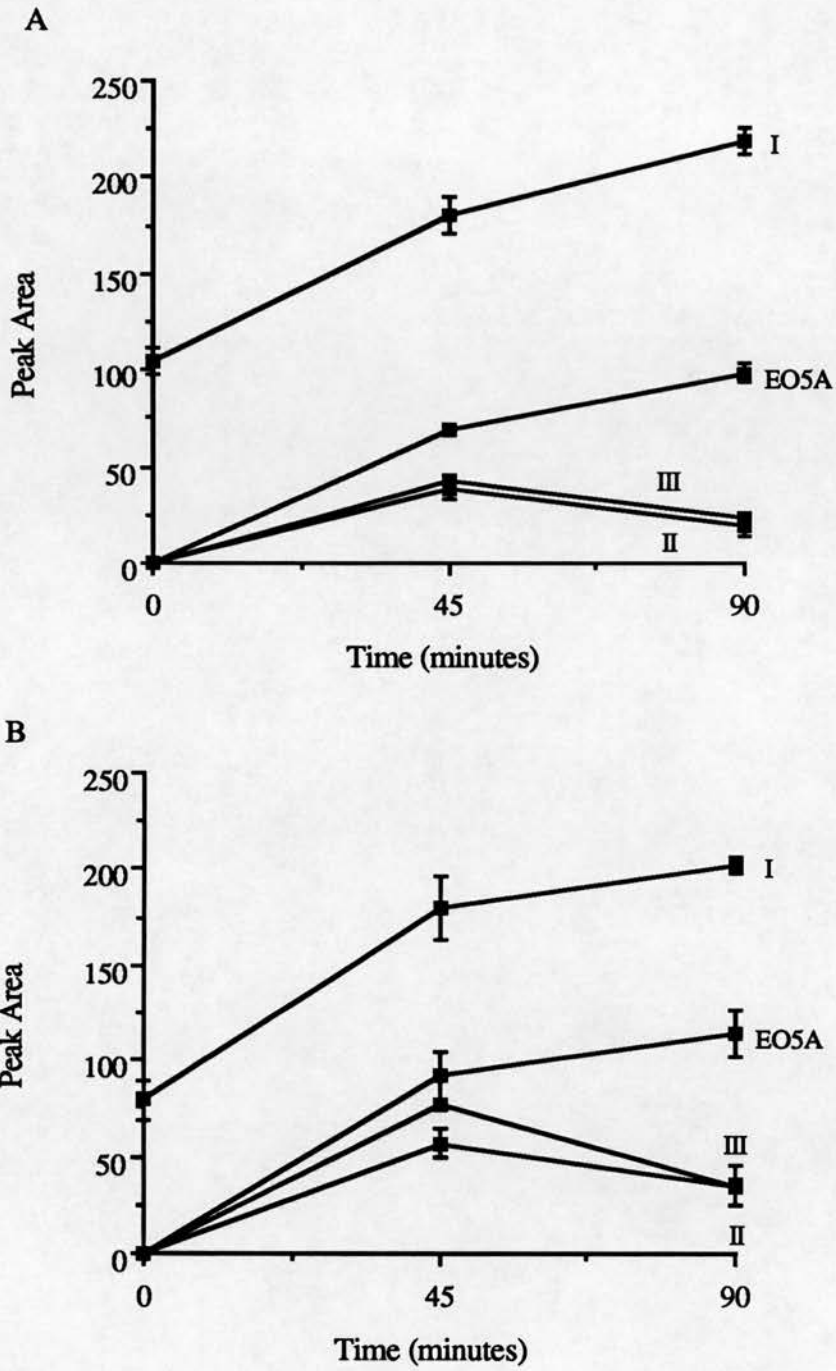


Figure 3.32. *In vitro* formation of Indoloquinone EO9 metabolites I, II, III and EO5A in MAC 16 tumour homogenate under aerobic (A) and hypoxic (B) conditions during the 90 incubation. Results are expressed as mean peak area \pm SE for three separate experiments.

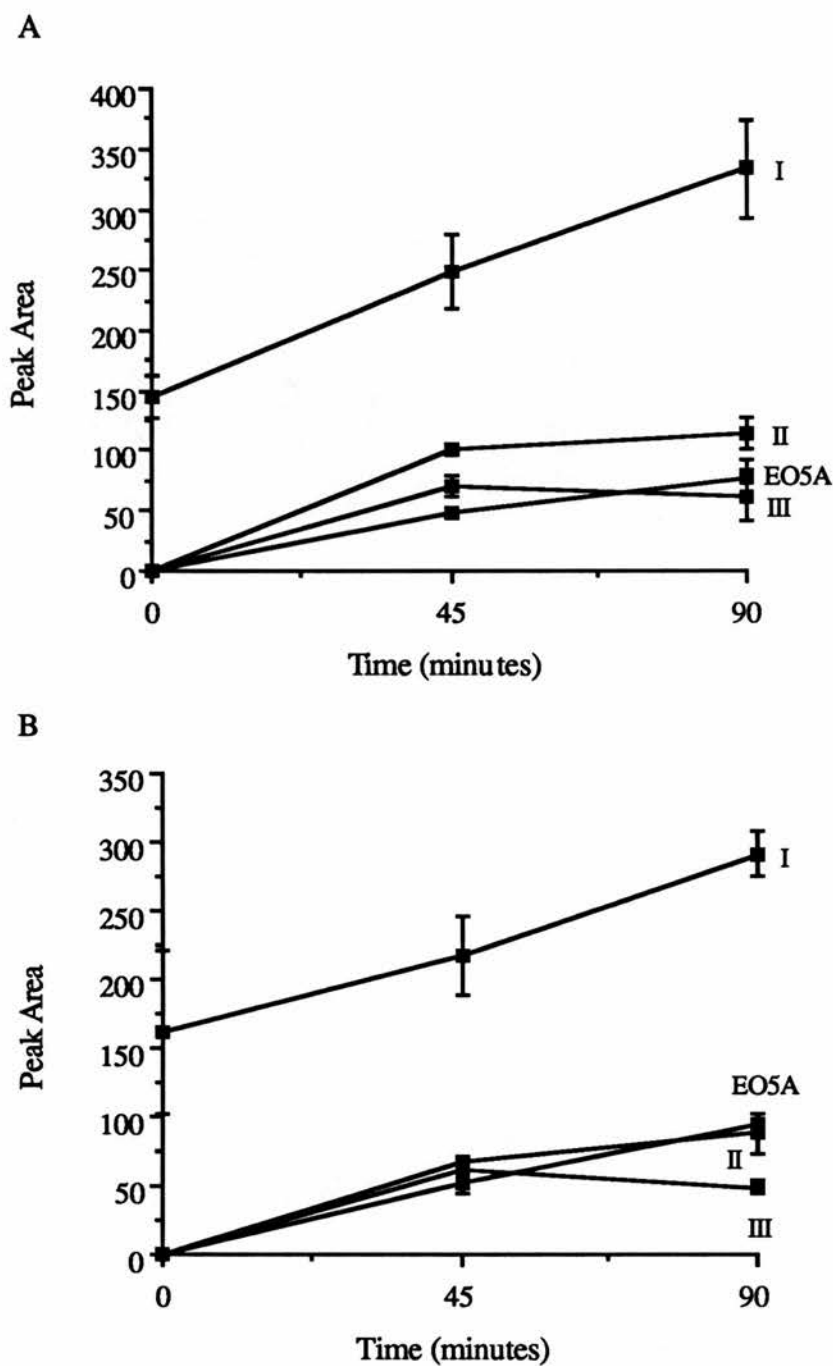


Figure 3.33. *In vitro* formation of Indoloquinone EO9 metabolites I, II, III and EO5A in MAC 26 tumour homogenate under aerobic (A) and hypoxic (B) conditions during the 90 incubation. Results are expressed as mean peak area \pm SE for three separate experiments.

Do such observations apply to the human colon xenografts HT-29 and BE? Figures 3.34 and 3.35 illustrate the formation of EO9 metabolites during the 90 minute incubation period. Once again metabolite I was present at 0 minutes in both HT-29 and BE incubations. Levels of metabolite III and EO5A followed patterns similar to those seen in MAC 16 and MAC 26, although EO5A did decrease sharply in HT-29 possibly due to further metabolism. However, in contrast to the MAC tumours and BE, levels of metabolite II increased in HT-29 over the first 45 minutes to levels greater than other metabolites produced. This was particularly enhanced under hypoxia. Taking into account standard errors however, this metabolite failed to increase significantly over the last 45 minutes of the incubation period and in fact decreased slightly.

Therefore, in parallel with MAC 16 and MAC 26, no single metabolite can be identified to represent chemosensitivity and therefore the extent of EO9 metabolic bioactivation. Although levels of metabolite II were higher in HT-29 than BE, possibly indicating DT-diaphorase dependent metabolism, this did not extend to MAC 16. In fact, MAC 26 exhibited greater levels of this metabolite under aerobic conditions compared to MAC 16. Therefore in conclusion, no correlation between human tumour xenografts high and low in DT-diaphorase levels and the production any of the EO9 metabolites observed could be established.

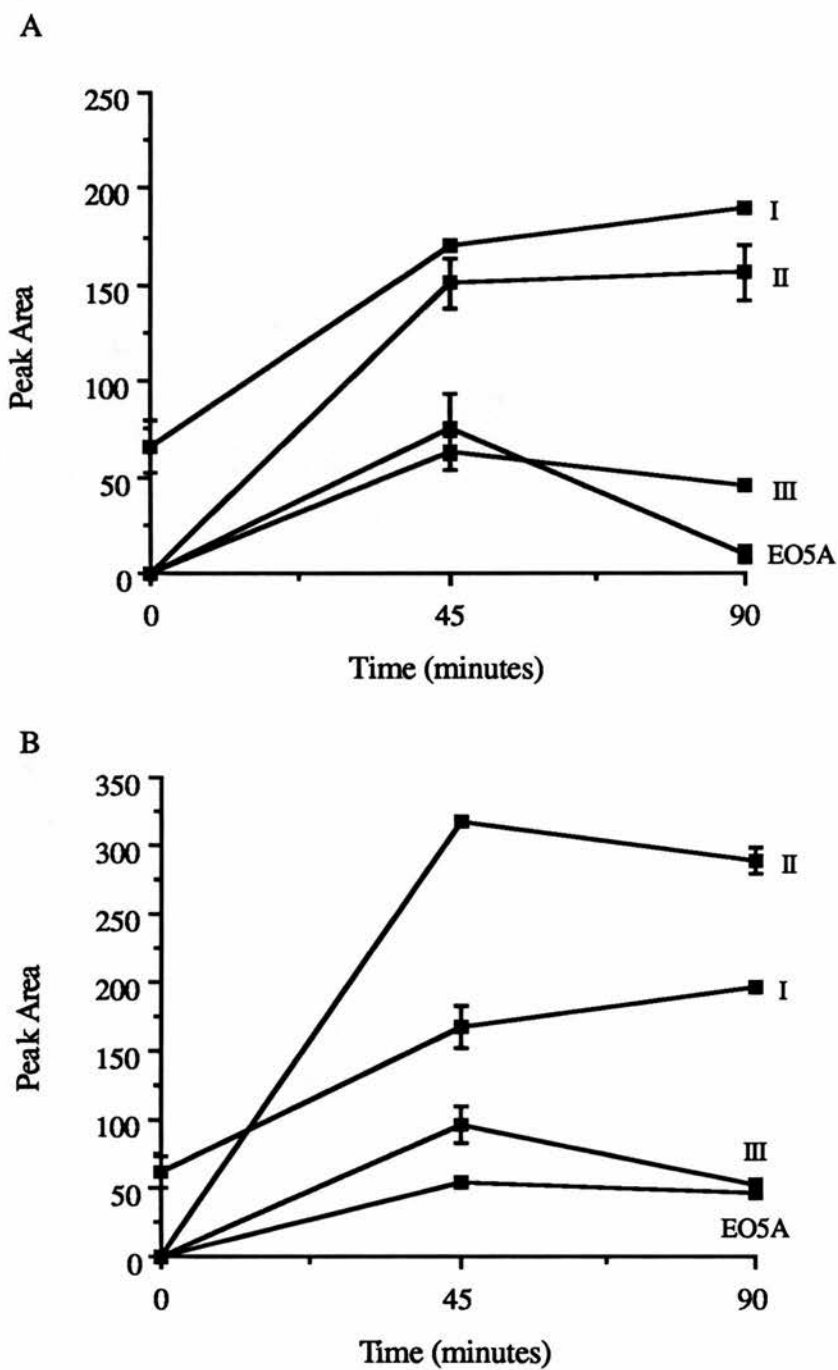


Figure 3.34. *In vitro* formation of Indoloquinone EO9 metabolites I, II, III and EO5A in HT-29 tumour homogenate under aerobic (A) and hypoxic (B) conditions during the 90 incubation. Results are expressed as mean peak area \pm SE for three separate experiments.

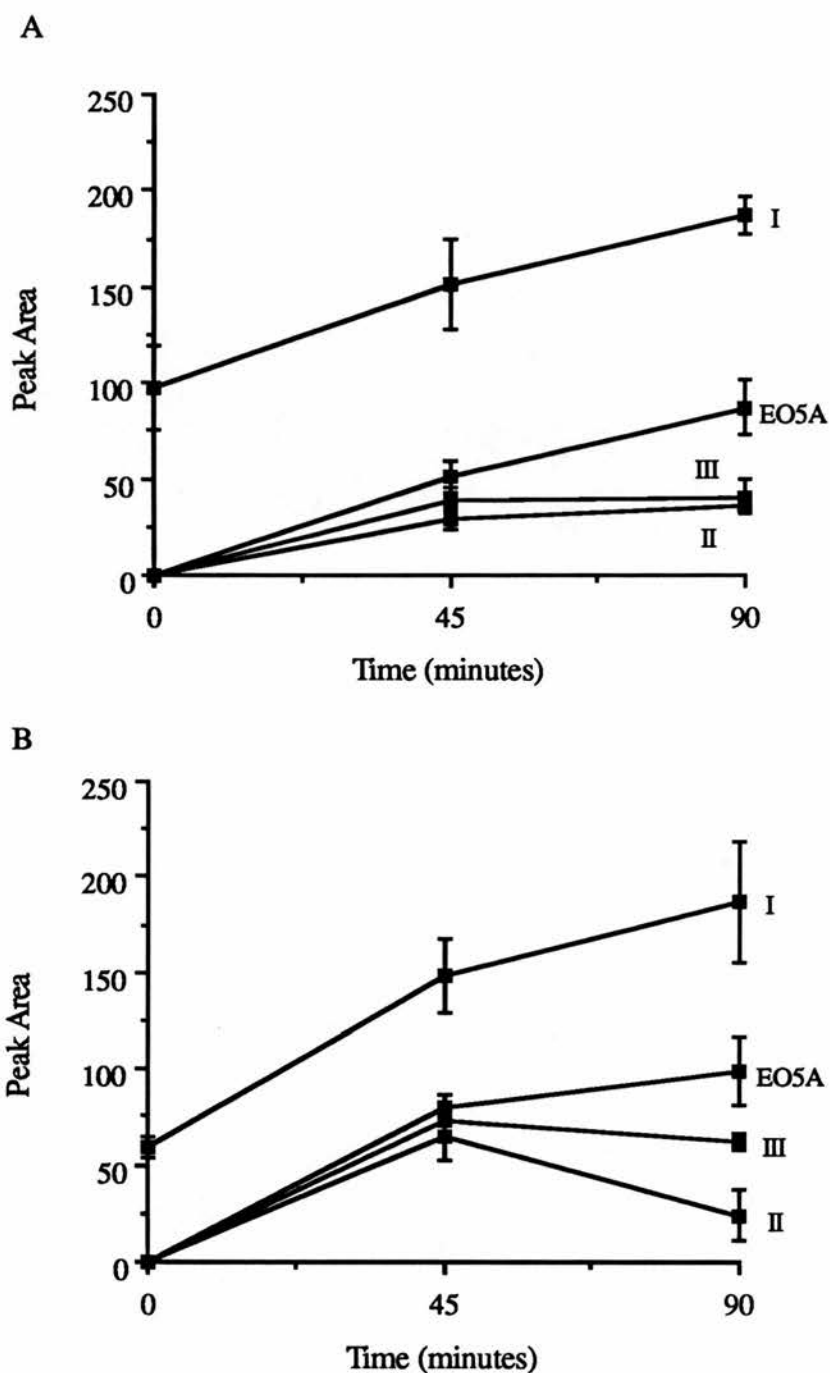


Figure 3.35. *In vitro* formation of Indoloquinone EO9 metabolites I, II, III and EO5A in BE tumour homogenate under aerobic (A) and hypoxic (B) conditions during the 90 incubation. Results are expressed as mean peak area \pm SE for three separate experiments.

Although it is disappointing that no one metabolite was found to be indicative of EO9 metabolic activation in tumour tissue, it is nonetheless important to propose their possible identities. Previous to the work detailed within this thesis, Binger & Workman (1990) detected four urine and nine plasma EO9 metabolites and were characterised on the basis of their spectral qualities. No further studies as yet have been carried out. Similarly, Maliepaard *et al.* (1995), although detecting three possible metabolites in addition to EO5A after EO9 reduction by DT-diaphorase, did not investigate these metabolites further. It is therefore of potential interest to speculate on the possible identities of the metabolites.

Metabolite I, as discussed throughout, is possibly a drug-protein adduct. A similar adduct, possibly derived from phosphate buffer constituents, could be seen after irradiation. However, due to the presence of both possible adducts in the control sample and 0 minute time point of the irradiation and homogenate studies respectively, such adducts are not a result of EO9 reductive activation. It is interesting to note that the spectrum of the adduct in the irradiation studies was identical to that of EO5A suggesting the presence of an open aziridine ring (Binger & Workman, 1990). The loss of visible absorption of metabolite I may in some way be a consequence of the absorption spectrum of the protein masking that of the adducted EO9 metabolite.

Metabolite II again exhibited an absorption spectrum identical to that of

EO5A indicating the opening of the aziridine ring. The metabolite could potentially be a number of possibilities. The first is a hydroxy metabolite. de Vries *et al.* (1993) demonstrated the formation of such a metabolite after EO9 degradation in alkaline solution, pH>10. In common with metabolite II, this hydroxy metabolite eluted close to the solvent front demonstrating increase absorption at 280-290 nm. Formation of this metabolite may follow the pathway outlined in figure 3.36. Essentially, the aziridine ring is substituted by a hydroxyl ion at the C5 position of the molecule. This results in the loss of ethanolamine. Similar degradation pathways have been observed with mitomycin C (Beijnen *et al.*, 1985) and aziridinybenzoquinone (Kusai *et al.*, 1981, 1982).

To assess whether metabolite II is potentially a hydroxy metabolite, EO9 was incubated with sodium hydroxide, pH 12 (section 2.3.15) and any metabolite production assessed by HPLC. The use of contrasting HPLC conditions to those use by de Vries *et al.* (1993) may reveal different retention times and confirm or refute the hypothesis of such metabolite formation.

Figure 3.37 illustrates the profile of metabolites formed via alkaline degradation of EO9. In agreement with de Vries and colleagues (1993), the hydroxy metabolite eluted at 3 minutes and exhibited a spectrum similar to EO5A. In contrast, metabolite II possessed a retention time of 6 minutes although it did share similar spectral qualities.

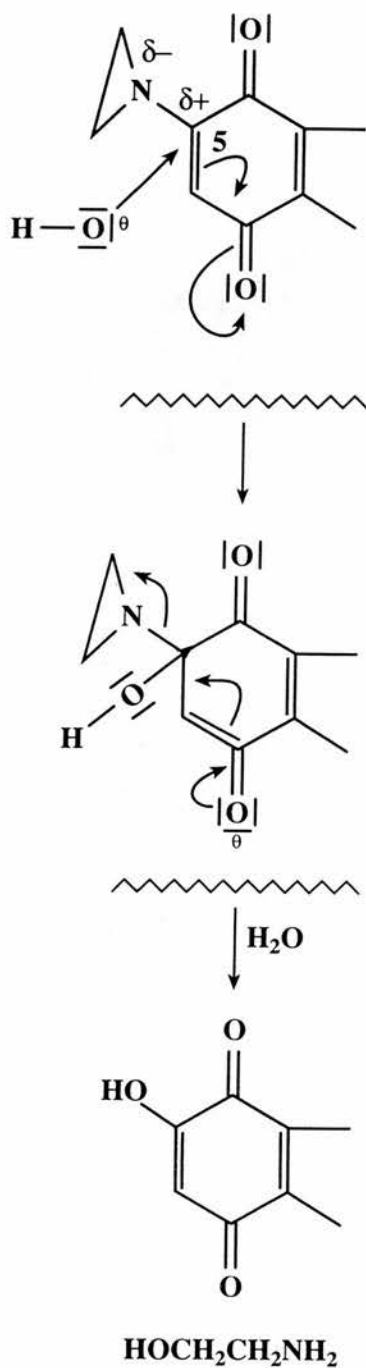


Figure 3.36. The proposed degradation pathway of Indoloquinone EO9 to the hydroxy metabolite under alkaline conditions. Adapted from de Vries *et al.*, 1993.

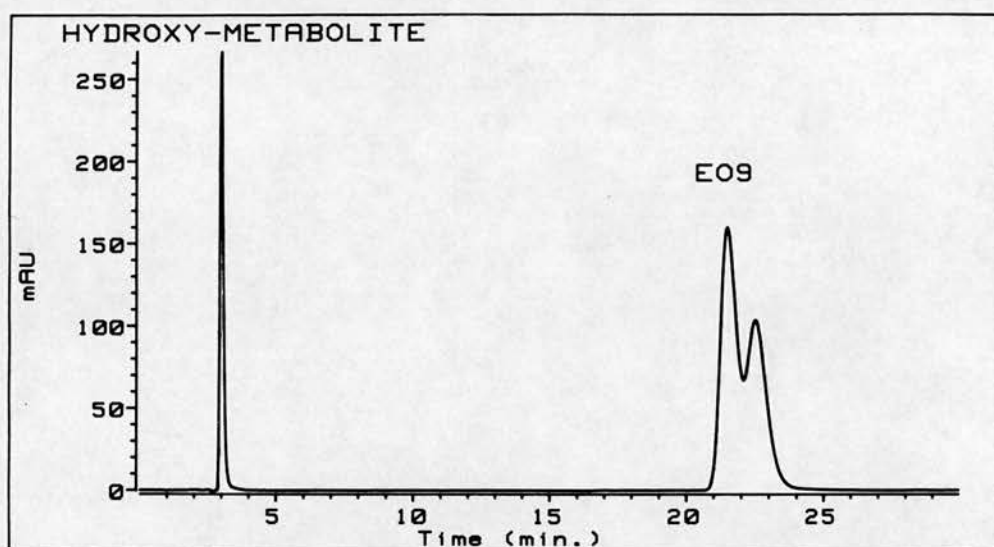


Figure 3.37. *In vitro* degradation profile of Indoloquinone EO9 to the hydroxy metabolite under alkaline conditions. The sample was analysed at 60 minutes and metabolite levels measured by HPLC.

Metabolite II is therefore not a hydroxy metabolite. If a hydroxy metabolite was formed during the homogenate incubations, it would coelute with the solvent front and not be seen. The abnormal EO9 peak was due to the effects of sodium hydroxide on the HPLC column packing material.

A second possibility is the formation of a glutathione-aziridinyl EO9 adduct. Such adducts have been previously shown to form with mitomycin C (Sharma *et al.*, 1994) and benzoquinones (Butler & Hoey, 1992).

Figure 3.38 illustrates the metabolite profile generated during co-incubation of EO9 with reduced glutathione. Reactions were carried out as described in section 2.3.16. Three possible 'adducts' were formed:- **b**, **c** and **d**. Adduct **a** appeared in both time 0 and 120 minutes samples and is likely to be once again a EO9-phosphate adduct analogous to that seen in the irradiation studies, figure 3.23. Of all the possible adducts, adduct **d**, was a likely candidate with a retention time similar to metabolite II, approximately 5.75 minutes.

Further similarities or differences were examined spectrally as shown in figure 3.39.

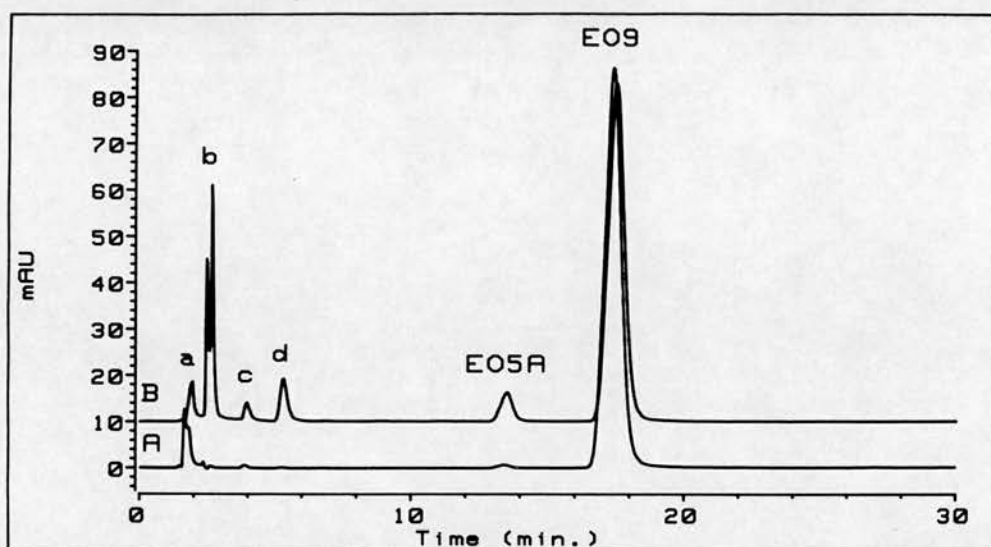


Figure 3.38. *In vitro* formation of potential glutathione-aziridinyI EO9 adducts. Samples were analysed at 0 (A) and 120 (B) minutes and measured by HPLC.

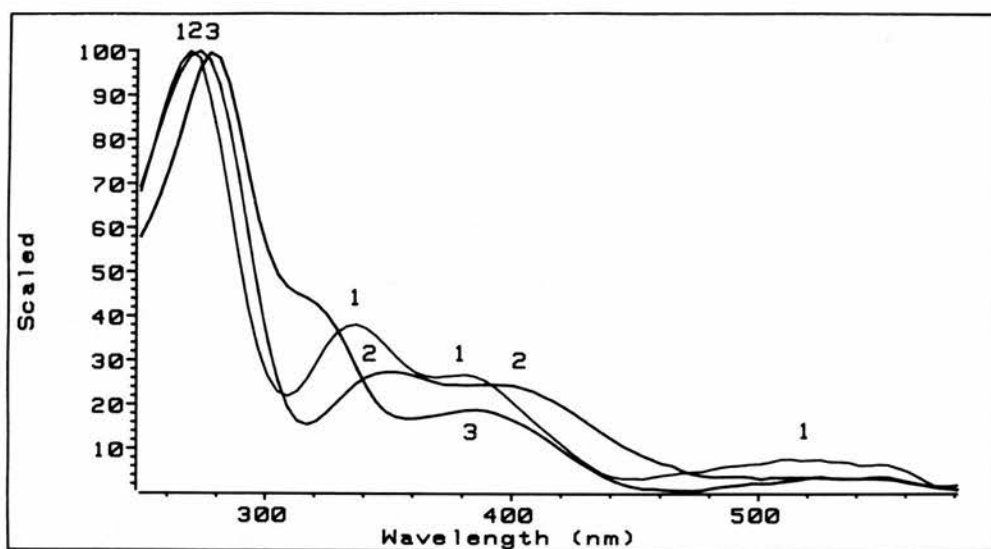


Figure 3.39. UV/Visible absorption spectra of potential glutathione-aziridinyl EO9 adducts. Potential adducts were identified during HPLC analysis as shown in figure 3.38.

Spectrum 1, adduct c; Spectrum 2, adduct b; Spectrum 3, adduct d.

No adduct showed spectra identical to that of metabolite II (280, 321, 550 nm). Although the likely candidate, adduct **d**, exhibited spectra at 280 and 321 nm corresponding to that of metabolite II, additional absorption was seen at 390 nm. This was not observed with metabolite II. Therefore, despite the formation of three potential glutathione-aziridinyl EO9 adducts, none corresponded to metabolite II.

Metabolite II is therefore speculated to be an aziridinyl adduct of unknown identity formed during the reduction process. This is supported by its spectrum characteristic of an open aziridine ring product and its significant water solubility resulting in earlier retention time to EO5A.

However, it is metabolite III which proposes the most interest. This metabolite appeared to decrease over time in both the irradiation and metabolite studies. This suggests that it is a possible unstable intermediate. Its absorption spectrum, distinct from any other metabolite, gives an indication of its identity. The lack of visible absorption observed has been demonstrated to be characteristic of certain hydroquinone intermediates (Land *et al.*, 1985; Cummings & Morrison, 1986) which can be detected using HPLC in association with a diode array detector (Cummings & Morrison, 1986). Although in theory, continuous generation of the hydroquinone of EO9 could be occurring throughout the 90 minute incubation period whilst parent drug is available for reduction, it is unlikely that the native hydroquinone of EO9 would

survive intact during HPLC, since it only has a half life of 1.5 ± 0.3 seconds (refer to section 3.7).

Metabolite III also corresponds to peaks IIIa and IIIb seen in the irradiation studies (section 3.4). Such peaks, although decreasing, were present for a significant period of time after irradiation and could still be detected at 20 days. It is more plausible that metabolite III (peaks IIIa and IIIb) is a hydroquinone adduct.

The additional intermediates observed in the irradiated sample, peak V and VI also display distinct properties. Peak V, retention time 27 minutes, has a spectrum identical to that of EO9, table 3.9. In contrast to metabolites II and III, this suggests the aziridine ring has remained intact. It is possible that it is a dimeric form of EO9. In contrast, peak VI, possessing an open aziridine ring, is undoubtedly a product formed via the degradation of EO9 and other intermediates over a significant period of time and is possibly another aziridinyl adduct.

Although the characterisation of EO5A structure and formation has been carried out, its formation within the different tumour types does differ. In HT-29 tumour homogenate incubations under aerobic conditions increased levels of EO5A occurred within the first 45 minutes. This was then followed by a sharp decline. Further metabolism was proposed as the potential degradation pathway. This is supported by work carried out by

Binger & Workman (1990), in which EO5A derived metabolites were detected in both plasma and urine from animals after a intravenous dose of EO5A. Such an observation in addition to its formation as a result of EO9 hydrolysis, indicates that EO5A is not a suitable marker for EO9 metabolic activation. It is unlikely however that such degradation is mediated via DT-diaphorase as EO5A has been demonstrated to be a very poor substrate for the enzyme (Bailey *et al.*, 1992b).

The characteristics and proposed identities of the EO9 metabolites are summarised in table 3.11.

In conclusion, the metabolism of EO9 in tumour tissue results in the formation of a number of complex and somewhat unstable metabolites. No one metabolite was found to be significantly affected by the presence and absence of oxygen. In addition, no correlation with EO9 *in vivo* chemosensitivity was observed.

Metabolite	Retention time (min)	UV Absorption (nm)	Visible Absorption (nm)	Present After Irradiation	Present After Metabolism	Effect of Time on Concentration	Proposed Identity
EO9	21-23	268, 313	505	Yes	Yes	Decrease	Parent Drug
EO5A	14-16	280, 321	550	Yes	Yes	Increase	Aziridine Ring Opened Product
I	4	280	nil	Yes	Yes	Increase	Drug/Protein Adduct
II	6	280, 321	550	No	Yes	Increase	Aziridinyll Adduct
III	8	240, 340	nil	Yes	Yes	Decrease	Hydroquinone Adduct
V	27	268, 313	505	Yes	No	Increase	Dimer
VI	4	280, 321	550	Yes	No	Increase	Aziridinyll Adduct

Table 3.11. Overall characterisation of products/metabolites generated by irradiation and enzymatic metabolism of the parent drug Indoloquinone EO9. Metabolites I, II, III, V and VI correspond those discussed throughout sections 3.4 and

3.5.

3.6 Identification of Indoloquinone EO9 Metabolites by High-Performance Liquid Chromatography-Mass Spectroscopy.

In this section, an attempt was made to characterise the chemical identity of the putative metabolites of EO9. This was carried using high-performance liquid chromatography in conjunction with mass spectroscopy. The facility was based at the Paterson Institute for Cancer Research as detailed in section 2.2.3. EO9 was irradiated as described in section 2.3.17 to generate a spectrum of metabolites similar to those in figure 3.23.

Figure 3.40 illustrates the HPLC profile of EO9 related products generated via irradiation. EO9 and EO5A exhibited retention times of 28.25 and 35.13 minutes and were the predominant peaks. These were extended compared to previous figures due to the differing mobile phase constituents, water in 25 % acetonitrile and temperature. Analyses were carried out at room temperature. The use of phosphate and formate containing mobile phases was avoided as the subsequent formation of phosphate and formate ions has been demonstrated to interfere with the mass spectroscopy.

A number of potential metabolite peaks could be seen. Without the aid of a diode array detector however, it was difficult to assign a particular peak to a specific metabolite.

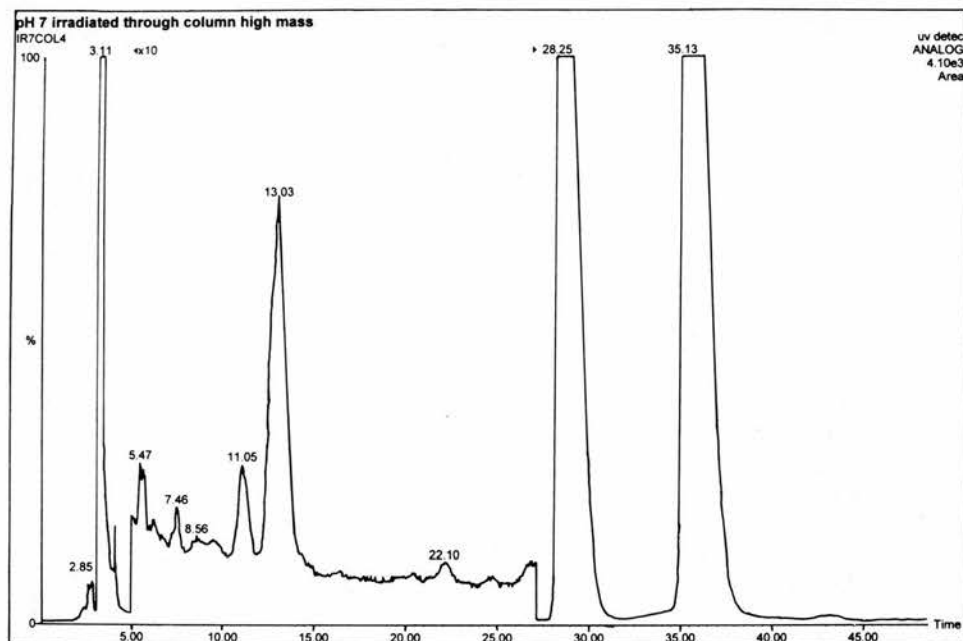


Figure 3.40. *In vitro* pattern of Indoloquinone EO9 related products generated by a single 500 Gy irradiation dose. Results as measured using the modified HPLC method.

Peaks at 3.11, 11.05 and 13.03 minutes correspond to previously characterised intermediates. Peaks at 28.25 and 35.13 minutes correspond to EO5A and EO9 respectively.

However, taking into consideration the extended retention times, it is likely that the major peaks seen at 3.11, 11.05 and 13.03 minutes, correspond respectively to peaks I, IIIa and IIIb seen in figure 3.23. Peaks IIIa and IIIb subsequently correspond to metabolite III observed after EO9 metabolism. Additional peaks at 5.47, 7.46, 8.56 and 22.10 minutes were extremely small and therefore were considered not to be major products of EO9 reduction via irradiation.

Subsequent mass spectroscopy produced disappointing results. Only EO9 and EO5A could be detected. EO9, molecular weight 288.30, produced a protonated molecular ion ($M + H^+$) of 289. Similarly, the open aziridine ring hydrolysis product EO5A, molecular weight 306, possessed a protonated molecular ion of 307, ie. $(M + H^+) + H_2O$. Similar mass spectral properties have been observed (Phillips *et al.*, 1992). The extremely low levels of the additional peaks in the sample may have rendered them undetectable by mass spectroscopy giving no clear signal. If peaks IIIa (11.05 minutes) and IIIb (13.03 minutes) are the native hydroquinones of EO9 and EO5A, then they should possess a molecular ion of 291, $(M + H^+) + 2H$ and 308, $(M + H^+) + 2H + H_2O$, respectively. No such molecular ion was observed.

In order to increase the sensitivity of the procedure, a similar highly concentrated/sub-saturated solution of EO9 was injected directly into the mass spectrometer. A control unirradiated sample was investigated.

Figure 3.41 illustrates the mass spectral analysis of the control unirradiated sample. The molecular ion of EO9 could be clearly seen. EO5A could not be detected showing that hydrolysis had not occurred.

In conjunction with EO9, several major additional ions were generated. Firstly, the loss of water resulted in the formation of molecular ion 271, $(M + H^+) - H_2O$. A dimeric form of this ion could also be seen with a nominal molecular weight of 559. Secondly, similar dimeric forms of EO9 were also formed, molecular ion 577. The formation of dimers during the ionisation process has been associated with the use of highly concentrated solutions. The presence of dehydration products may be a result of the extremely high temperatures used throughout.

The mass spectral analysis of irradiated EO9 is illustrated in figure 3.42. A molecular ion of EO9, $M^+ = 288$, was observed, in addition to a single dehydration product, $(M + H^+) - H_2O = 269$. No dimeric forms were formed. The inconsistency between the molecular ions of these products in the unirradiated and irradiated samples, may be an anomaly of the ionisation process.

Although additional ions were formed, no possible metabolite could be derived from these results. Such ions may result from fragmentation of parent ions to form so-called daughter ions.

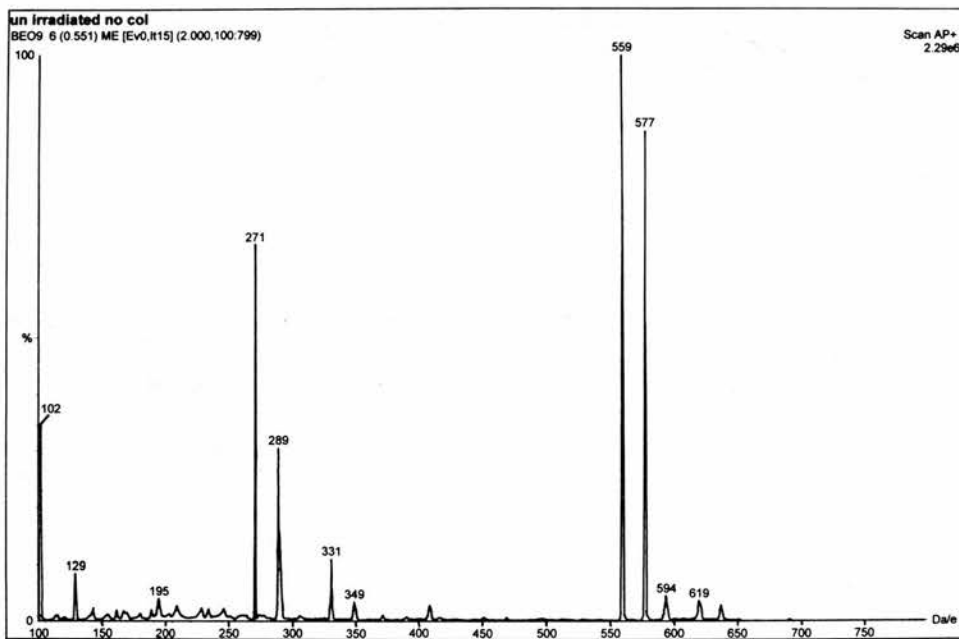


Figure 3.41. Mass spectral analysis of unirradiated Indoloquinone EO9. The sample was analysed by-passing the high-performance liquid chromatography column.

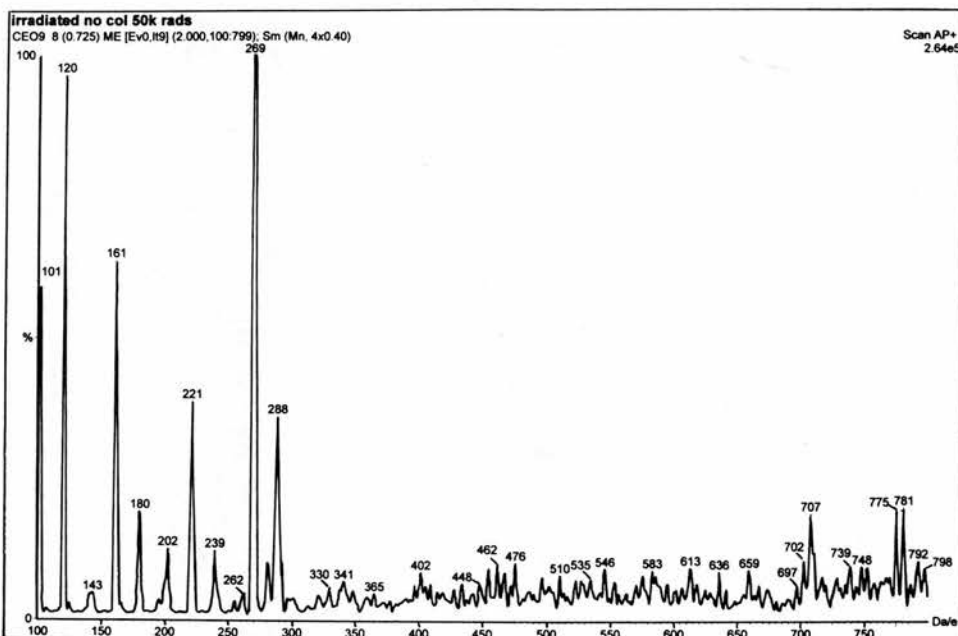


Figure 3.42. Mass spectral analysis of irradiated Indoloquinone EO9. The sample was analysed by-passing the high-performance liquid chromatography column.

In summary, identification has proved unsuccessful. The purification of substantial quantities of these metabolites from both irradiated and metabolised EO9 and subsequent mass spectroscopy as single agents rather than as a combined entity, may circumvent such problems.

Although the formation and preliminary characterisation of EO9 metabolites is in no doubt of interest, the fact remains that no appropriate parameter has been found to correlate to chemosensitivity of EO9 observed in table 3.10. In association with the formation of these metabolites which only represent a small fraction of the drug, the overwhelming majority of EO9 disappears into products which are not detected by HPLC. This process may be more indicative of EO9 bioactivation and may therefore correlate to chemosensitivity.

The ability of the MAC 16, MAC 26, HT-29 and BE tumour homogenates to metabolise EO9 is shown in table 3.12. Metabolism under both aerobic and hypoxic conditions has been calculated.

A number of observations can be drawn from these results. Firstly, in contrast to the metabolites detected in section 3.5, the loss of EO9 corresponds to the antitumour activity of the drug shown in table 3.10. Loss of EO9 was greatest in HT-29 followed by BE, MAC 16 and MAC 26. In addition, the same order applies for both aerobic and hypoxic conditions. From this it can be seen that the tumours most sensitive to EO9 are those

that are high in DT-diaphorase, confirming numerous studies showing such a correlation (Walton *et al.*, 1992a; Robertson *et al.*, 1994; Smitskamp-Wilms *et al.*, 1994; Fitzsimmons *et al.*, 1996) . This applies to both murine and human tumour types.

Secondly, once standard error was taken into account, no enhancement under hypoxia was seen in those tumours low in DT-diaphorase, BE and MAC 26. Particularly in the case of HT-29 and BE, this does not follow the observations of Plumb and colleagues (1994) in which BE exhibited significant hypoxic enhancement. Similar hypoxic enhancement has been seen in a number of cell lines including T47D breast cells (Adams *et al.*, 1994; Robertson *et al.*, 1994) and the mitomycin C resistant non-small cell lung cancer cell line PC-9/MC4 (Bando *et al.*, 1995). Both have low levels of DT-diaphorase.

Also, *in vivo* EO9 chemosensitivity has been enhanced in MAC 26 using the vasoactive agent, hydralazine (Bibby *et al.*, 1993). Such treatment resulted in 63% vascular shutdown (Quinn *et al.*, 1992), increasing tumour hypoxia and potentiating EO9 cytotoxicity.

This suggests that measuring the loss of EO9 may not be the best indicator of metabolic activation under aerobic and hypoxic conditions *in vitro*. A technique measuring the direct consequence of EO9 metabolic activation, such as DNA adducts levels would be much more appropriate,

TUMOUR/ HOMOGENATE	EO9 METABOLISED (nmol/45min/mg protein)		ANTITUMOUR ACTIVITY (T/C%)
	AEROBIC	HYPOXIC	
MAC 16	4.10±0.15	3.91±0.95	33
MAC 26	2.41±0.86	2.09±0.75	60
HT-29	13.76±3.22	8.95±0.13	15
BE	5.66±2.93	7.13±2.24	27

Table 3.12. Specific activities of Indoloquinone EO9 metabolism by MAC 16, MAC 26, HT-29 and BE tumour homogenates under aerobic and hypoxic conditions and correlation with antitumour activity. Specific activity is expressed as nmol EO9 metabolised/45 min/mg protein. Each value represents the mean±SE from three separate experiments. Antitumour activity was expressed as tumour volume as a percentage of the vehicle control, T/C value.

as measuring the rate of disappearance of EO9 takes into account the sum of all the processes under consideration ie: metabolism and DNA, protein, protein and glutathione adduct formation etc.

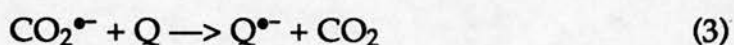
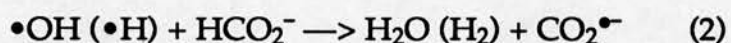
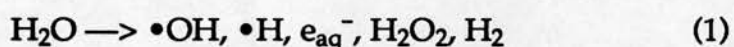
The use of *in vitro* chemosensitivity assays may still provide a better indication of EO9 antitumour activity *in vivo*. However, caution should be taken when examining *in vitro* chemosensitivity data. Although such studies are ideal for examining preliminary chemosensitivity, such 'clean' systems do not take into account the complex tumour environment. In addition, although good correlation was achieved between DT-diaphorase levels and *in vitro* chemosensitivity, no correlation was seen *in vivo* (Collard *et al.*, 1995).

In conclusion, although no metabolite produced after EO9 metabolic activation correlated to EO9 antitumour activity, the loss of EO9 via such metabolism was found to give a better correlation. However, such parameters failed to indicate the extent of hypoxic enhancement, if any, in those cell lines low in DT-diaphorase.

3.7 Properties of Indoloquinone EO9 Reactive Intermediates: A Pulse Radiolysis Study

Since it now appears that EO9 and not a drug metabolite is possibly the active form of the drug, it became important to understand which reactive intermediates of EO9 are responsible for cytotoxicity, how they were formed, their properties and the optimal conditions for their formation. This was carried out using the linear accelerator facility at the Paterson Institute for Cancer Research as detailed in section 2.3.18.

The pulse irradiation of quinone (Q) in an argon saturated buffered solution leads to the following reactions.



Essentially, the primary water free radicals reduce the quinone to produce the semiquinone, $\text{Q}^{\bullet-}$. Spectral properties of the semiquinone of EO9 are illustrated in figure 3.43. The so-called difference spectrum was calculated as the absorbance of the semiquinone minus that of EO9. The spectrum of the radical was essentially the same at both pH 7.4 and 10.

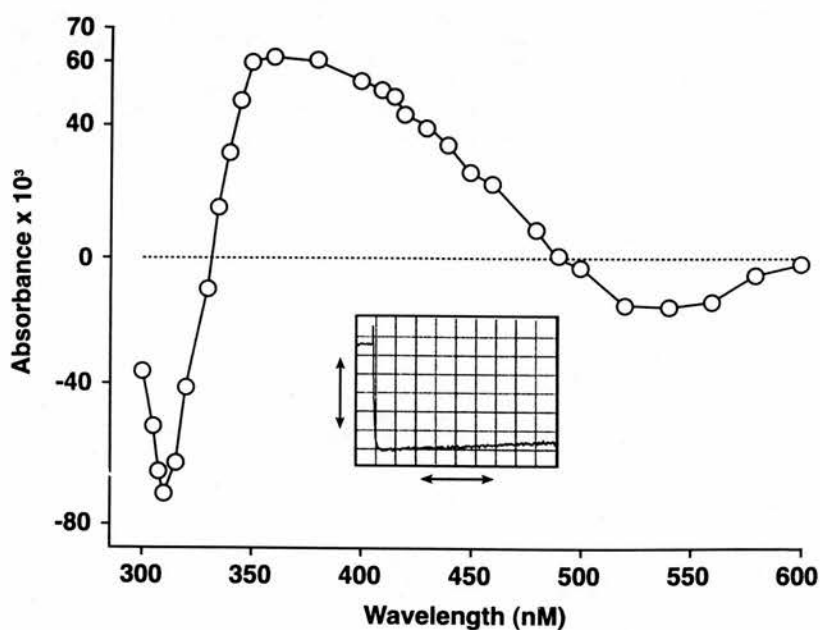


Figure 3.43. The difference spectrum of Indoloquinone EO9 semiquinone recorded at 20 μ s after pulse irradiation. All absorbances were normalised to a radical concentration of 6.5 μ M in a 2.5 cm optical cell.

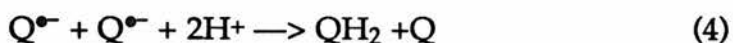
Insert: Typical trace produced by pulsing 150 μ M EO9 in 0.1 M formate, 10 mM phosphate buffer, pH 7.4 monitored at 400 nm. Radical concentration = 5.7 μ M, 20 μ s/division, 1.8% transmission/division.

Figure 3.43 (insert) depicts a typical trace produced during the reduction process.

However, the absorbance was found to decrease in the range of 350 and 450 nm at less than pH 6. This pH dependency of the semiquinone free radical can be seen more clearly in figure 3.44. pH values through 6, 5.5, 5 and 4.5 led to a corresponding decrease in absorbance. Due to the instability of EO9 in both acidic and alkaline solutions (de Vries *et al.*, 1993), it was not possible to examine the effect of pH outside the pH 4.5-10.

The inability of pH values 6.5 - 10 to change the spectrum of the semiquinone has a number of implications. Firstly it indicates that the aziridine group on the semiquinone free radical does not have a dissociation constant, pK, in this region. Secondly, the decrease in absorbance between pH 4.5 and 6.5 probably results from the protonation of the aziridine moiety on the semiquinone free radical.

Figure 3.44, insert A, shows the decay of the semiquinone radical in the absence of oxygen. This occurred over a period of milliseconds and occurs via the following reaction.



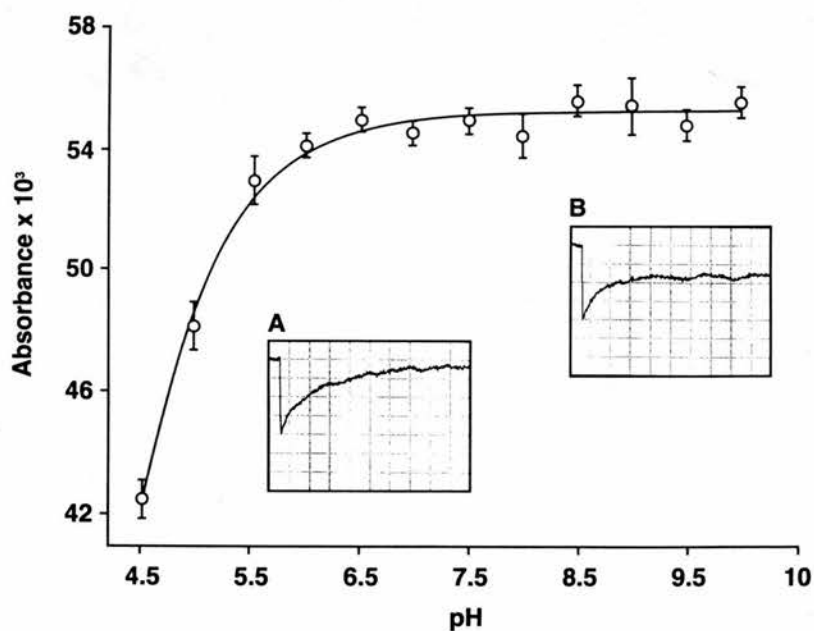


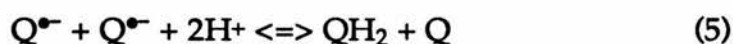
Figure 3.44. The variation of the absorbance of the semiquinone of Indoloquinone EO9 in relation to pH. All values were normalised to a radical concentration of $6.5 \mu\text{M}$ in a 2.5 cm optical cell.

Insert A: The decay of the semiquinone of Indoloquinone EO9 in the absence of oxygen at pH 7.4.

Insert B: The formation of an equilibrium at pH 9.

This forms the hydroquinone, QH₂, and the parent quinone, EO₉. The rate constant for this disproportion reaction was calculated to be 5.2±1.6 x10⁷ M⁻¹ s⁻¹. At alkaline pH, pH 8 - 10, the reaction was found to be at equilibrium. This is illustrated in figure 3.44, insert B, reaction 5.

Equilibrium follows the following reactions.



The equilibrium constant, K₄, was calculated as 84±12 at pH 9 and 8±2 at pH 10. Using a higher concentration of EO₉, the equilibrium constant at pH 7.4 was greater than 4000, in favour of the hydroquinone. Although this could not be accurately calculated, there is no reason to suppose that this does not occur.

The possibility of such an equilibria at pH 7.4 is supported by the detection of semiquinone radicals via electron spin resonance spectroscopy (ESR), formed from DT-diaphorase dependent reduction (Bailey *et al.*, 1993).

Similar equilibria have been observed with other quinones such as simple benzoquinones (Baxendale & Hardy, 1953), naphthoquinones (Mukherjee *et al.*, 1988) and adriamycin (Mukherjee *et al.*, 1989).

The semiquinone radicals formed at this pH (pH 7.4) were unstable in the presence of oxygen as follows:-



The variation in rate with oxygen concentration leading to decay of EO9 semiquinone radicals is shown in figure 3.45. This was determined by adding small concentrations of oxygen and observing subsequent decay at 400 nm. A clear relationship between rate of decay and oxygen concentration could be seen with greatest decay achieved at levels of oxygen greater than 200×10^{-6} M. The rate constant for this reaction was calculated to be $1.3 \pm 0.15 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. This suggests that the semiquinone free radical is expected to be removed in well oxygenated cells.

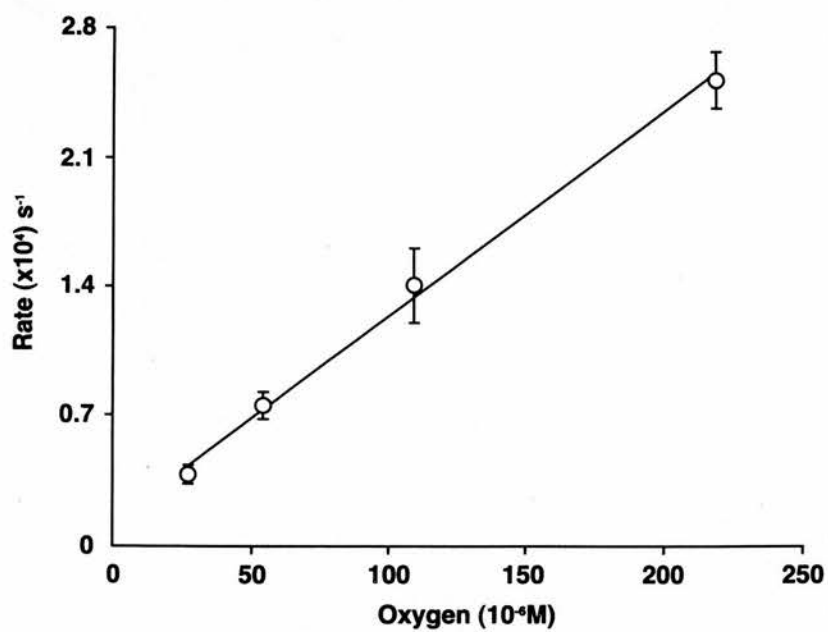


Figure 3.45. The dependence of the rate of decay of Indoloquinone EO9 semiquinone free radicals on oxygen concentration.

The reduction potential of the quinone/semiquinone couple, E(Q/Q^{•-}), was determined by setting up an equilibrium between EO9 semiquinone free radicals and mitomycin C (MMC) at pH 7.4 as follows:-



This reaction was investigated at 415 nm, where EO9^{•-} radicals have a higher absorbance than that of MMC^{•-}. This was extended to 490 nm where the MMC^{•-} have a much higher absorbance than EO9^{•-} radicals (Hoey *et al.*, 1988).

The equilibrium constant, K, can be shown to equal the following formula (Swallow, 1982):-

$$K = (A_e - A_{\text{MMC}}) / (A_{\text{EO9}} - A_e) \times [\text{MMC}] / [\text{EO9}] \quad (8)$$

where A_e is the absorbance value at equilibrium, A_{MMC} is the absorbance of mitomycin C radicals in the absence of EO9 and A_{EO9} is the absorbance of EO9 radicals in the absence of mitomycin C. The equilibrium constant is subsequently related to reduction potential as follows (Swallow, 1982; Wardman, 1989):-

$$E(\text{MMC}/\text{MMC}^{\bullet-}) = E(\text{EO9}/\text{EO9}^{\bullet-}) - 59 \log K \quad (9)$$

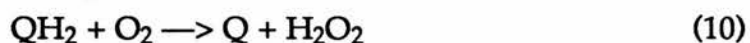
The absorbance values at equilibrium (A_e) for a given wavelength and concentration of EO9 and subsequent K values are summarised in table 3.13. Each value was normalised to a radical concentration of 6.5 μM . The absorption of EO9 radicals at 415 and 490 nm were 49.5 and 0.7×10^{-3} per 6.5 μM radicals respectively in the absence of mitomycin C. In addition, the absorption of mitomycin C radicals were 0 and 70.4×10^{-3} per 6.5 μM radicals at the same wavelengths respectively in the absence of EO9.

Using the average value of K, 9.33, derived from table 3.13, and assuming $E(\text{MMC}/\text{MMC}^{\bullet-}) = -310 \pm 6$ mV (Wardman, 1989), it can be shown that the reduction potential, $E(\text{EO9}/\text{EO9}^{\bullet-}) = -253 \pm 6$ mV.

EO9 Concentration (μM)	Wavelength (nm)	Absorbance at Equilibrium ($A_e \times 10^{-3}$)	K
10	490	37.4	9.0
	415	24.2	9.6
20	490	25.0	9.3
	415	31.8	9.0
40	490	15.1	9.6
	415	39.2	9.5

Table 3.13. Absorbance of Indoloquinone EO9 semiquinone free radicals at equilibrium, $A_e \times 10^{-3}$, for a given concentration of EO9 at 415 and 490 nm and corresponding equilibrium constants, K.

The effect of oxygen on the product of two-electron reduction, the hydroquinone, was investigated by rapidly mixing the hydroquinone (produced by xanthine oxidase) with air. Results indicated that the hydroquinone was unstable in air leading to autoxidation and regeneration of the parent drug.



The autoxidation of the EO9 hydroquinone is illustrated in figure 3.46. Clear differences could be seen in the spectrum of the hydroquinone with increasing exposure to oxygen. Particularly absorbance within the wavelength range 440 - 600 nm which increases steadily with time. Loss of visible absorbance has previously been associated with hydroquinones (Land et al., 1985; Cummings & Morrison, 1986). Appearance of absorbance here suggests the production of the parent drug EO9, which has been shown throughout this thesis to absorb in the visible end of the spectrum. The loss of visible absorbance also supports the hypothesis proposed in section 3.5, that metabolite III is a form of the hydroquinone, possibly an adduct.

The rate constant for this reaction was calculated to be $2.1 \pm 0.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The hydroquinone at low concentration subsequently exhibited a half life of 1.5 ± 0.3 seconds in air.

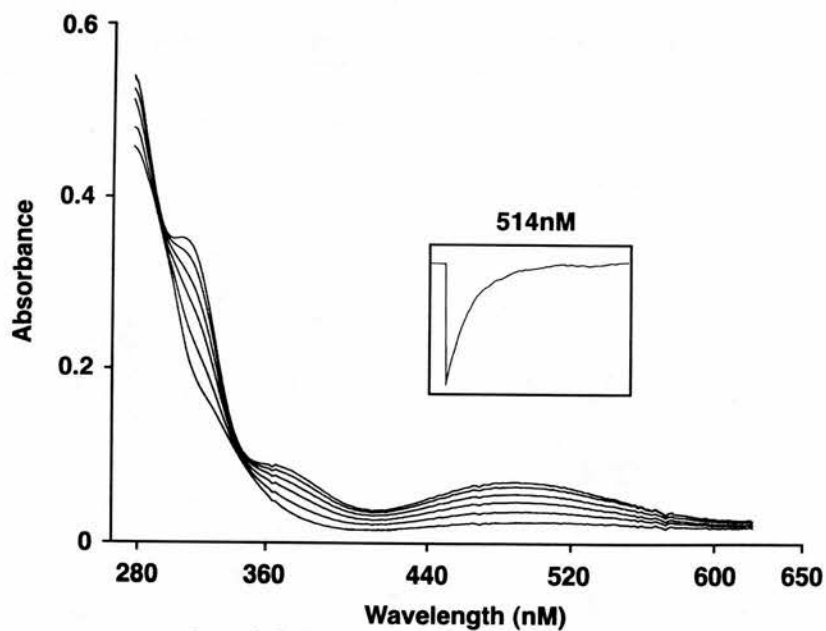


Figure 3.46. The autoxidation of the Indoloquinone EO9 hydroquinone. Spectra was measured at 2 second intervals.

Insert: Decay of Indoloquinone EO9 hydroquinone at 514 nm in the presence of 110 μM oxygen.

The semiquinone reduction potential was -253 ± 6 mV. Therefore, according to previous studies on the dependence of enzyme rates and reduction potentials (Clarke *et al.*, 1980, 1982; Butler & Hoey, 1993), EO9 should undergo enzymatic reduction via one-electron reductases such as NADPH:cytochrome P-450 reductase and xanthine oxidase more readily than mitomycin C. This has been demonstrated with xanthine oxidase (Maliepaard *et al.*, 1995) and NADPH:cytochrome P-450 reductase (Bailey *et al.*, 1993).

Therefore, although EO9 is an excellent substrate for DT-diaphorase, selective toxicity may also be mediated through one-electron reductase enzymes within tumour cells both *in vitro* and *in vivo*. This may be significant in those cell lines that possess low or deficient levels of DT-diaphorase which show significant sensitivity to EO9 (Walton *et al.*, 1992a; Plumb & Workman, Plumb *et al.*, 1994; Robertson *et al.*, 1994).

A number of studies have demonstrated the formation of DNA strand breaks as a result of DT-diaphorase-dependent EO9 metabolism in the presence of oxygen (Walton *et al.*, 1991; Bailey *et al.*, 1994). Walton *et al.* 1991, proposed that the strand breaks were a result of direct DNA alkylation by EO9. This was supported by the failure of superoxide dismutase, a enzyme known to inhibit hydroquinone oxidation hence leading to increase concentrations of such hydroquinone species and inhibition of hydrogen peroxide formation (Cadenas, 1995), reaction 10, to

affect this cross-linking process. However, the effect of catalase was not investigated and therefore such strand breaks may have been a direct result of hydrogen peroxide formation.

In addition, the formation of EO9-DNA cross-links produced via DT-diaphorase under hypoxia has been demonstrated (Maliepaard *et al.*, 1995). This may explain the enhanced cytotoxicity of EO9 seen in a number of cell lines under hypoxia (Plumb & Workman, 1994; Robertson *et al.*, 1994). If it is assumed that aerobic toxicity is predominantly due to hydrogen peroxide and superoxide radicals, then such products are expected to be less damaging to the cell than the formation of DNA cross-links and strand breaks.

Plumb *et al.*, (1994) and Workman (1994) proposed that the semiquinone free radical of EO9 may be the more toxic reactive species, both predicting that the formation of the semiquinone free radical would activate the aziridine group more effectively than the hydroquinone. This is not the case as shown by figure 3.44. The pK of the aziridine group on the semiquinone free radical must be less than 5.5. The pK of the aziridine of EO9, although not yet reported, is expected to be similar to mitomycin C, 2.8 (McClelland & Lam, 1985), and the diaziridinybenzoquinones, between 2.5 and 4.0 (Butler *et al.*, unpublished). The predicted order in pK's of the aziridines is quinone<hydroquinone<semiquinone, therefore it is unlikely that the difference in pK between hydroquinone and

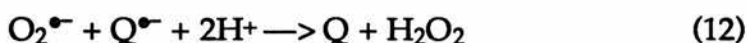
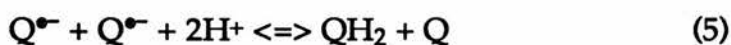
semiquinone is responsible for the different reactivities. However, the semiquinone may be more reactive than the hydroquinone towards DNA alkylation due to factors such as different hydrogen bonding between DNA bases which may favour the formation of cross-links (Hartley *et al.*, 1991; Mayalarp *et al.*, 1996). In addition, selective activation of the vinylogous side chains may occur. This could be assessed with the aid of the DNA cross-links structures generated from reduced EO9. These, as yet, are not known.

The role of the EO9 semiquinone in relation to DT-diaphorase levels was also assessed (Workman, 1994).

Examining the first scenario, a cell line expressing one-electron reductases in association with low levels of DT-diaphorase, the predominant species was predicted to be the semiquinone. In the presence of oxygen its formation will be reversed leading to the generation of the parent molecule and superoxide anion both of which will enter futile redox cycling. This has been demonstrated in reaction 6 and figure 3.45. In the absence of oxygen, enhanced toxicity in such cell lines was therefore predicted to be due to the semiquinone free radical. However, this is not clear cut as the semiquinone has been demonstrated to be unstable under hypoxic conditions resulting in the formation of the hydroquinone and EO9, reaction 4, figure 3.44, insert A. Indeed the equilibrium at pH 7.4 is in favour of the hydroquinone, $K_4 > 4000$, reaction 5. However, at alkaline pH

the equilibrium favours the semiquinone. For simple quinones this is often associated with the deprotonation of the hydroquinone (Baxendale & Hardy, 1953). Thus, in association with the increase in DNA cross-links between pH 5 and 7 (favouring a greater presence of semiquinone) observed after DT-diaphorase and xanthine oxidase dependent EO9 reduction (Maliepaard *et al.*, 1995), suggests that the semiquinone may be the active form of the drug.

The alternative scenario is a cell line high in DT-diaphorase in addition to one-electron reductase expression. Under aerobic conditions Workman (1994) proposed that the hydroquinone will initially predominate and then generate semiquinone free radicals as a result of autoxidation. This does occur with the hydroquinone rapidly autoxidising possibly via the following semiquinone mediated reactions:-



Therefore, toxicity under aerobic conditions could potentially be mediated

through oxygen free radical and hydrogen peroxide formation in association with the transient appearance of the semiquinone (Plumb & Workman, 1994).

Under hypoxic conditions, Workman (1994) proposed that the balance of the ratio of semiquinone and hydroquinone was in favour of the hydroquinone resulting in protection under hypoxia. This can be supported once again by the equilibrium constant $K_4 > 4000$, reaction 5.

In summary, the following conclusions can be drawn. Firstly, under aerobic conditions, whether after one- or two-electron reduction, EO9 is most likely to redox cycle and generate reactive oxygen species. Secondly, under hypoxic conditions, again after one- or two-electron reduction, the hydroquinone free radical intermediate could predominate. The hydroquinone can potentially be detoxified and excreted following glucuronidation and sulphonation (Cadenas, 1995). However, although the hydroquinone ultimately predominates under hypoxia after one-electron reduction, the initial formation of the semiquinone on balance possibly produces a more toxic effect than the hydroquinone, hence hypoxic enhancement. In addition, at high alkaline pH, the equilibrium shifts in favour of the semiquinone. This, in association with increased DNA cross-links with corresponding increasing pH, pH 5 to 7 (Maliepaard *et al.*, 1995), suggests that the semiquinone is the active form of EO9.

3.8 Summary

The studies detailed in this thesis have led to the following conclusions. Firstly, the enzymology of mitomycin C metabolic activation in solid tumour tissue involves a complex interplay between several quinone reductases. The identification of a novel one-electron mitochondrial reductase exhibiting activity under hypoxia has provided a new insight into mitomycin C metabolism. Such enzymes have the potential to be exploited in the activation of hypoxia dependent bioreductive drugs. Full identification, expression, cloning, transfection and metabolism studies will in no doubt be of benefit. The two-electron reductase DT-diaphorase occupies a unique and central role in mitomycin C metabolic activation in tumour tissue both *in vitro* and *in vivo*. Its apparent predomination over one-electron reductases *in vitro*, equal antitumour activity of mitomycin C observed in tumours both high and low in the enzyme and its increased expression in numerous tumour types, still make DT-diaphorase a very attractive target for enzyme directed bioreductive drug therapy using mitomycin C.

Secondly, the mitomycin C analogue Indoloquinone EO9 has a complex but distinct pattern metabolism giving rise to a number of metabolites. However, the appearance of no single metabolite correlated to tumour chemosensitivity. Rate of metabolism of the parent drug rather than specific metabolite formation was found to correlate to antitumour

activity. However, purification and identification of metabolites would indeed provide important information regarding the drug's activation and mechanism of action. A study of free radical processes identified the reactive intermediates of Indoloquinone EO9 most likely to be responsible for its therapeutic efficacy.

The role for specific quinone reductases present within tumour tissue remains unclear in the context of Indoloquinone EO9 metabolic activation. The use of additional techniques such as DNA adduct formation and characterisation will facilitate and perhaps provide a good basis from which to start such correlations.

CHAPTER 4

4. REFERENCES

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APPENDIX I

REAGENT SUPPLIERS

Reagent	Supplier
Absolute Alcohol	Hayman Ltd.
Acetonitrile	Rathburn Chemicals Ltd.
Allopurinol	Sigma Chemical Co. Ltd.
Argon	BOC
β -nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)	Sigma Chemical Co. Ltd.
β -nicotinamide adenine dinucleotide (reduced form) (NADH)	Sigma Chemical Co. Ltd.
Bondesil	Anachem Ltd.
Bovine serum albumin Fraction V	Sigma Chemical Co. Ltd.
Chloroform	Rathburn Chemicals Ltd.
Cytochrome <i>c</i>	Sigma Chemical Co. Ltd.
Cytochrome P-450 reductase antiserum	Professor C.R. Wolf
Dicoumarol	Sigma Chemical Co. Ltd.
Disodium EDTA	Sigma Chemical Co. Ltd.
Disodium Hydrogen Orthophosphate	BDH Merck Ltd.
DPX mounting media	Fisons Chemicals Ltd.
Dubecco's Modified Eagle's Medium	Gibco Life Technologies Ltd.
Emix resin	Fisons Chemicals Ltd.
EO9	Kyowa Hakko Kogyo Co. Under the auspices of Mr John Kelly.
Eosin Y solution (alcoholic)	Sigma Chemical Co. Ltd.

Ethyl Acetate	Rathburn Chemicals Ltd.
Foetal Calf Serum	Gibco Life Technologies Ltd.
Glutaraldehyde	Sigma Chemical Co. Ltd.
Glycerol	Fisons Chemicals Ltd.
Ham's F10	Gibco Life Technologies Ltd.
Harris Haematoxylin (Modified Solution)	Sigma Chemical Co. Ltd.
Helium	BOC
Hypoxanthine	Sigma Chemical Co. Ltd.
Lithium Carbonate	BDH Merck Ltd.
Menadione	Sigma Chemical Co. Ltd.
Methanol	Rathburn Chemicals Ltd.
Mitomycin C	Kyowa Hakko Kogyo Co.
Mitomycin C Standards	
2,7-DM	In house
Cis-hydro	In house
Trans-hydro	In house
Nitrogen	BOC
Osmium tetroxide	Sigma Chemical Co. Ltd.
Paraformaldehyde	Sigma Chemical Co. Ltd.
Penicillin-Streptomycin	Gibco Life Technologies Ltd.
Phosphate Buffered Saline	In House
Potassium Chloride	Sigma Chemical Co. Ltd.
Propan-2-ol (Isopropanol)	Rathburn Chemicals Ltd.
Propylene oxide	Sigma Chemical Co. Ltd.
Sodium borate	BDH Merck Ltd.

Sodium cadodylate	BDH Merck Ltd.
Sodium Dihydrogen Orthophosphate	BDH Merck Ltd.
Sodium Dithionite	Sigma Chemical Co. Ltd.
Sodium formate	BDH Merck Ltd.
Sodium hydroxide	BDH Merck Ltd.
Sucrose	Sigma Chemical Co. Ltd.
Tris Base	Sigma Chemical Co. Ltd.
Tris - HCl	Sigma Chemical Co. Ltd.
Trypsin	Gibco Life Technologies Ltd.
Xanthine oxidase	Sigma Chemical Ltd.
Xylene	Fisons Chemicals Ltd.

APPARATUS SUPPLIERS

Apparatus	Supplier
Beckman GPR centrifuge	Beckman Instruments Ltd.
Beckman L7 ultracentrifuge	Beckman Instruments Ltd.
Eppendorf microcentrifuge	Eppendorf Ltd.
Gilson 307 HPLC pump	Gilson Medical Electronics
Leitz Dialux 20 microscope	E. Leitz (Instruments) Ltd.
LiChrosorb RP-18 HPLC Cartridge	BDH Merck Ltd.
LiChrospher 100 RP-18 precolumn	BDH Merck Ltd.
LiChrocart Manu-fix holder	BDH Merck Ltd.
Liquid Chromatograph Model 1090	Hewlett Packard Ltd.
M.D.H Interlab tissue culture hood	M.D.H. Ltd.
Milli-U10 water purification system	Millipore (U.K.) Ltd.
Morden Isolators	Morden Scientific Ltd.
Scotlab VSL incubator	Scotlab Ltd.
Unicam UV/Vis spectrophotometer	ATI Unicam Ltd.
Unicam cell temperature controller	ATI Unicam Ltd.
Unicam UV5-220 thermostatted cell holders	ATI Unicam Ltd.
VG MicroMass Spectrophotometer	Fisons Scientific Equipment
Waters 484 MS UV/Vis detector	Waters Ltd.

SUPPLIER ADDRESSES

Supplier	Address
Anachem Ltd.	Charles Street, Luton, U.K.
ATI Unicam Ltd.	York Street, Cambridge, U.K.
BDH Merck Ltd.	Merck House, Poole, Dorset, U.K.
Beckman Instruments Ltd.	Progress Road, High Wycombe, Bucks,U.K.
BOC	Dear Park Road, London, U.K.
Fisons Chemicals Fisons Scientific Equipment	Bishop Medow Road, Loughborough, U.K.
E. Leitz (Instruments) Ltd.	Luton, U.K.
Eppendorf Ltd.	Merck House, Poole, Dorset, U.K.
Gibco Life Technologies Ltd.	Renfrew Road, Paisley, Scotland.
Gilson Medical Electronics	Charles Street, Luton, U.K.
Harlan U.K. Ltd.	Shaw's Farm, Blackthorn, Bicester, U.K.
Hewlett Packard Ltd.	Wokingham, Berkshire, U.K.
Kyowa Hakko Kogyo Co.	Martindale Pharmaceuticals, Romford, U.K.
M.D.H Ltd.	Walwarth Road, Andover, U.K.
Millipore (U.K.) Ltd	Blackmoor Lane, Watford, U.K.

Morden Scientific Ltd.

Rathburn Chemicals Ltd.

Scotlab Ltd.

Sigma Chemical Co. Ltd.

Waters Ltd.

Penicuik, Scotland.

Walkerburn, Scotland.

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APPENDIX II

PUBLICATIONS

Abstracts

Spanswick V.J., Cummings J., Ritchie A.A., Smyth J.F. (1994). Evidence that neither DT-diaphorase nor cytochrome P-450 reductase are the major enzymes involved in the metabolic activation of mitomycin C *in vivo* in tumour tissue. *International Conference on Bioreductive Drug Activation*, Lake Tahoe, U.S.A.

Spanswick, V.J., Cummings, J., Ritchie, A.A., Smyth, J.F. (1995). Identification of new mitomycin C metabolising enzymes in tumour tissue. *British Journal of Cancer*, **71**, 39.

Spanswick V.J., Cummings J., Smyth J.F. (1995). The enzymology of mitomycin C bioactivation in tumour tissue - Is there role for DT-diaphorase? *Ninth International Conference on Chemical Modifiers of Cancer Treatment*, Oxford, U.K.

Spanswick, V.J., Butler, J., Cummings, J., Smyth, J.F. (1996). The molecular pharmacology of indoloquinone EO9. *British Journal of Cancer*, **73**, 28.

Refereed Papers

Spanswick, V.J., Cummings, J., Smyth J.F. (1996). Enzymology of mitomycin C metabolic activation in tumour tissue: Characterization of a novel mitochondrial reductase. *Biochemical Pharmacology*, **51**, 1623-1630.

Butler, J., Spanswick, V.J., Cummings, J. (1996). The autoxidation of the reduced forms of EO9. *Free Radical Research*, **25**, 141-148.

Spanswick, V.J., Cummings, J., Ritchie, A.A., Smyth J.F. (1996). Role of DT-diaphorase in the metabolism and antitumour activity of mitomycin C. *Cancer Chemotherapy and Pharmacology*, (In Preparation).

Review Articles

Cummings, J., Spanswick, V.J., Smyth, J.F. (1995). Re-evaluation of the molecular pharmacology of mitomycin C. *European Journal of Cancer*, **31A**, 1928-1933.

Invited Articles

Spanswick V.J. (1996). Enzymology of mitomycin C metabolic activation in tumour tissue. *Biochemical Pharmacology*.

Spanswick V.J., Cummings J., Smyth J.F. (1996). Enzymology of mitomycin C. *General Pharmacology*.

Evidence That Neither DT-Diaphorase Nor Cytochrome P-450 Reductase Are The Major Enzymes Involved In The Metabolic Activation Of Mitomycin C *In Vivo* In Tumour Tissue.

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There is controversy as to the role of DT-diaphorase in the metabolic activation of mitomycin C (MMC). Metabolism of MMC has been studied in NMRI mice bearing one of 2 adenocarcinoma tumour models, one having a 15-fold higher DT-diaphorase (DTD) activity (MAC 16) than the other (MAC 26). Cytochrome P-450 reductase activity was similar in both. MMC (500 μ g), was administered by direct intratumoural injection and bio-reductive metabolites were measured by high performance liquid chromatography in tumour and plasma after extraction.

Peak level of MMC was approximately 19-fold lower in MAC 26 (5.1 μ g/TTC MAC 26, 98.4 μ g/TTC MAC 16) and plasma levels were higher indicating faster tumour clearance. A greater degree of conversion to 2,7-diaminomitosenone was observed in MAC 26, (AUC 11.4 μ g/TTC x hr in contrast to MAC 16, AUC 5.69 μ g/TTC x hr), despite the low level of parent drug. High levels of 1,2-*trans*- and 1,2-*cis*-1-hydroxy-diaminomitosenone were also detected.

Results indicate that MAC 26, despite its lower DTD activity, has a greater capacity to metabolise MMC than MAC 16. Preliminary results obtained from *in vitro* enzymology studies also suggest that MAC 26 has a greater capacity to metabolise MMC particularly under anaerobic conditions, when cytochrome P450 reductase might be expected to predominate.

It is concluded that neither DTD nor Cytochrome P-450 reductase are principally responsible for MMC metabolism in the MAC tumours. The identification of the enzyme(s) involved is currently underway.

*Presented at the International Conference on Bioreductive Drug Activation, Lake Tahoe, U.S.A., August 1994.

Identification Of New Mitomycin C Metabolising Enzymes In Tumour Tissue.

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Mitomycin C (MMC) requires bioreductive activation in order to exert its cytotoxic effects. Numerous enzymes have been implicated in the activation pathway including DT-diaphorase (DTD) and cytochrome P450 reductase, although their exact role is under much debate. To establish their importance, the metabolism of MMC has been studied in NMRI mice bearing one of 2 adenocarcinoma tumour models, one having a 15-fold higher DTD activity (MAC 16) than the other (MAC 26). Cytochrome P450 reductase activity was similar in both. MMC (500 μ g) was administered by direct intratumoural injection and bioreductive metabolites were measured by high performance liquid chromatography in tumour and plasma after extraction.

Peak level of MMC was 19-fold lower in MAC 26 (5.1 μ g/total tumour content (TTC) MAC 26, 98.4 μ g/TTC MAC 16) and plasma levels were higher indicating faster tumour clearance. A greater degree of conversion to the principle metabolite 2,7-diaminomitosenone was observed in MAC 26 (AUC 11.80 μ g/TTC x hr in contrast to MAC 16, AUC 25.39 μ g/TTC x hr), despite the low level of parent drug.

Results indicate that MAC 26, despite its lower DTD activity, has a greater capacity to metabolise MMC than MAC 16. Preliminary results obtained from MAC 16 and MAC 26 *in vitro* enzymology studies also suggest that DTD and cytochrome P-450 reductase are not the major enzymes involved in the metabolic activation of MMC. Although low levels DTD

and cytochrome P-450 reductase dependent activity were seen in MAC 16, the majority of metabolism was associated with the mitochondrial fraction utilising both NADH and NADPH optimally under anaerobic conditions. Only mitochondrial activity was seen in MAC 26.

The above findings may give an insight to MMC activation in human tumours and have important implications in the development of enzyme directed drug therapy.

*Presented at the 36th Annual Meeting of the British Association for Cancer Research, Nottingham, U.K., April 1995.

The Enzymology of Mitomycin C Bioactivation in Tumour Tissue - Is There a Role for DT-diaphorase?

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AIM

The role of DT-diaphorase (DTD) in the bioactivation of mitomycin C (MMC) has been the subject of intensive research and is under much debate. We have studied the *in vitro* biotransformation of MMC in order to establish the enzymology of MMC bioactivation in tumour tissue and explore the nature of the role of DTD. This information may in turn have important *in vivo* implications in the development of enzyme directed drug therapy.

BACKGROUND

The *in vivo* mechanisms of MMC have previously been studied in 2 murine adenocarcinomas of the colon, MAC 16 & MAC 26, chosen for their contrasting enzyme profiles (Spanswick *et al.*, 1995, British Journal of Cancer, 71, suppl XXIV, 39). MAC 16 contains 15-fold higher levels of DTD compared to MAC 26. Cytochrome P-450 reductase activity was similar in both. Results showed that MAC 26, despite its lower DTD activity and equivalent cytochrome P450 reductase activity, had a greater capacity to metabolise MMC than MAC 16. These results indicated the presence of different metabolising enzyme(s) apart from DTD and cytochrome P-450 reductase and the possibility of a minor role for DTD.

METHODS

The animal model consisted of inbred NMRI mice and the subcutaneously growing MAC 16 and MAC 26 murine adenocarcinomas.

Once tumours became palpable, they were removed and immediately placed in liquid nitrogen. All tumours were stored at -80°C prior to use. Homogenates and subcellular fractions incubations with MMC were prepared as described in detail, Cummings *et al.*, 1992, *Biochemical Pharmacology*, 44, 2175-2183, after which quinone reductase activity was assessed. *In vitro* incubations were performed at 37°C under aerobic and anaerobic conditions. Each incubation contained 100 µg/mL MMC, approximately 1 mg subcellular protein and 3.3 mM cofactor (NADH, NADPH & Hypoxanthine) in 0.1 M sodium phosphate buffer pH 7.4. Tumour homogenate incubations contained 100 µg/mL MMC only. At 15 minute intervals, an aliquot was withdrawn and analysed via HPLC to determine the presence of MMC and its metabolites (Cummings *et al.*, 1993, *Journal of Chromatography*, 612, 105-113).

RESULTS

Tumour	Condition	Cofactor	Homogenate	Fraction		
				Cytosol	Microsomes	Mitochondria
MAC 16	Aerobic	NADH	0.23±0.04	0.64±0.06	ND	ND
		NADPH		0.66±0.02	ND	ND
	Anaerobic	NADH	0.14±0.07	0.76±0.10	ND	0.93±0.09
		NADPH		1.04±0.17	2.09±1.09	3.67±0.58
MAC 26	Aerobic	NADH	0.80±0.06	ND	ND	ND
		NADPH		ND	ND	ND
	Anaerobic	NADH	1.45±0.18	ND	ND	ND
		NADPH		ND	ND	3.87±0.71

Table 1 Characterisation of MMC bioreduction in MAC 16 & MAC 26 Adenocarcinomas. Results are expressed as mean specific activity of 2,7-diaminomitosenone formation in nmol/30min/mg protein±SE.

Subcellular fraction incubations revealed high activity associated with the mitochondrial fraction in both MAC 16 & MAC 26, Table 1 (ND = no activity detected). NADPH combined with hypoxia produced the greatest level of 2,7-diaminomitosenone (2,7-DM) formation, the principle metabolite of MMC metabolism. In MAC 16 only, no mitochondrial activity was observed until 30 minutes into the reaction. In both MAC 16 & MAC 26 activity was not inhibited by co-incubation with 10 μ M dicoumarol or cytochrome P450 reductase antiserum indicating that the mitochondrial enzyme(s) are probably not DTD or cytochrome P-450 reductase.

In the cytosolic fractions of MAC 16 no significant increase in metabolism occurred under hypoxia and activity was inhibited by 10 μ M dicoumarol suggesting activity characteristic of DTD. Enzyme activity characteristic of cytochrome P450 reductase was detected in microsomes exhibiting activity under hypoxia in the presence of NADPH. Here the addition of cytochrome P-450 reductase antiserum resulted in complete loss of MMC metabolism.

CONCLUSION

A new MMC metabolising enzyme located within the mitochondria has been identified in MAC 16 & MAC 26 and is likely to be responsible for MMC metabolism in MAC 26 *in vitro* and *in vivo*. The 30 minute latency period seen in MAC 16 may suggest the enzyme is located within the mitochondrial inner membrane resulting in slower drug uptake and hence delayed metabolism.

The inability of hypoxia to enhance MMC metabolism and the inhibitory effects of dicoumarol in the MAC 16 homogenate appears to be characteristic of DTD. It suggests that if DTD is in high quantities it will predominate over other enzyme systems present within the tumour and effectively partially inhibit bioreduction since DTD is not as efficient as

one-electron reductases in MMC metabolic activation (Cummings *et al.*, In Press). The opposite applies to MAC 26. When DTD is absent other enzymes will predominate.

IMPLICATIONS

The implication of this data is that tumours high in DTD should be protected from MMC cytotoxicity, particularly under hypoxia in agreement with results observed by Plumb *et al.*, 1994, *British Journal of Cancer*, 70, 1136-1143, in which cell lines high in DTD resulted in little increase in EO9 cytotoxicity under hypoxia, whilst those low in DTD showed a marked increase. Together this suggests that tumours low in DTD are optimal for treatment with bioreductive drugs such as MMC and its analogue EO9 if hypoxia can be achieved.

However, total tumour hypoxia may be difficult to achieve clinically, therefore tumours that are high in DTD, such as colon tumours, may nevertheless still be optimal for MMC treatment.

*Presented at the 9th International Conference on Chemical Modifiers of Cancer Treatment, Oxford, U.K., August 1995.

The Molecular Pharmacology of Indoloquinone EO9.

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EO9 is a novel bioreductive compound currently undergoing phase II clinical trials as an anticancer drug. While considerable progress has been made in understanding its mechanism of action, it is still unclear which molecular forms of the drug are responsible for the induction of cytotoxicity and how these may behave under different conditions of oxygenation. In the present studies, pulse radiolysis was employed to generate reactive intermediates of EO9 and characterize their properties.

The one electron reduction potential of EO9 was determined to be -253 ± 6 mV, which is less negative than other quinone containing anticancer drugs and thus, EO9 should undergo enzyme catalyzed bioreduction more readily. The semiquinone free radical of EO9, the product of one electron reduction, rapidly reacted with oxygen (rate constant, $1.3 \pm 0.15 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and its fate under aerobic conditions is that it will preferentially redox cycle with oxygen. Unusually, the hydroquinone form, the product of two electron reduction, was unstable in the presence of oxygen and underwent rapid autoxidation back to the parent compound (half life 1.5 ± 0.3 seconds; rate constant $2.1 \pm 0.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). Hence, under aerobic conditions, whether the drug undergoes one or two electron enzyme catalyzed reduction the principal species formed will be reactive oxygen species rather than drug metabolites or DNA adducts. Under anaerobic conditions, the semiquinone free radical rapidly disproportionated into the hydroquinone (equilibrium constant > 4000 in favour of hydroquinone).

Therefore, whether the drug undergoes one or two electron reduction under hypoxia, the hydroquinone intermediate will predominate. These data provide new insights into the molecular pharmacology of EO9 and will aid in the design of second generation analogues.

*Presented at the 37th Annual Meeting of the British Association for Cancer Research, Edinburgh, U.K., March 1996.



Enzymology of Mitomycin C Metabolic Activation in Tumour Tissue

CHARACTERIZATION OF A NOVEL MITOCHONDRIAL REDUCTASE

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ABSTRACT. In this study, the enzymology of mitomycin C (MMC) bioactivation in two murine colon adenocarcinomas, MAC 16 and MAC 26, was examined. Subcellular quinone reductase assessment *via* cytochrome *c* reduction confirmed a number of active enzymes. MAC 16 exhibited 22-fold greater levels of cytosolic DT-diaphorase than MAC 26, while microsomal NADPH:cytochrome P-450 reductase levels were similar in both tumour types. Metabolism of MMC by subcellular fractions isolated from both MAC 16 and MAC 26 was quantitated by monitoring the formation of the principle metabolite 2,7-diaminomitosene (2,7-DM) *via* high-performance liquid chromatography (HPLC). In MAC 16 only, activity displaying the properties of cytosolic DT-diaphorase and microsomal NADPH:cytochrome P-450 reductase was detected and confirmed, using the enzyme inhibitors dicoumarol and cytochrome P-450 reductase antiserum, respectively. The highest level of MMC metabolism was associated with the mitochondrial fraction from both tumours and was the sole enzyme activity detected in MAC 26. The greatest mitochondrial drug metabolism was achieved in the presence of NADPH as cofactor and hypoxia (MAC 16-specific activity, 3.67 ± 0.58 nmol/30 min/mg; MAC 26 specific-activity, 3.87 ± 0.71 nmol/30 min/mg) and was unaffected by the addition of the inhibitors dicoumarol and cytochrome P-450 reductase antiserum. NADH-dependent mitochondrial activity was only observed in MAC 16 at approximately 4-fold less than that seen with NADPH. MAC 26 homogenate incubations displayed enhanced metabolism under hypoxia, presumably due to the presence of the identified mitochondrial enzyme. MAC 16 homogenates showed no increase in metabolism under hypoxia, suggesting that other enzyme(s) may be predominant. These data indicate the presence of a novel mitochondrial one-electron reductase capable of metabolising MMC in MAC 16 and MAC 26. *BIOCHEM PHARMACOL* 51;12:1623-1630, 1996.

KEY WORDS. mitomycin C; metabolism; mitochondria; DT-diaphorase; NADPH:cytochrome P-450 reductase; tumour tissue

The quinone-containing antitumour antibiotic MMC,† Fig. 1, remains an important component of cancer chemotherapy, exhibiting activity against a variety of solid tumours, such as breast, lung, and gastrointestinal cancers [1]. Considered to be the archetypical bioreductive alkylating agent, MMC undergoes reduction of its quinone moiety before it is able to covalently bond to DNA and elicit its cytotoxic effects [2]. This process can occur through two enzyme-catalyzed activation pathways. The first pathway, a one-electron reduction step, results in the formation of a semiquinone free radical intermediate which, in the presence of oxygen, is capable of redox cycling where it consequently reacts with molecular oxygen leading to the regeneration of the quinone molecule [3]. The second path-

way occurs *via* a two-electron reduction step resulting in the formation of the hydroquinone intermediate and, unlike one-electron reduction, alkylating species generated by this pathway are generally thought to be unimpeded by molecular oxygen. Under hypoxic conditions, rapid disproportionation of the semiquinone free radical suggests that the hydroquinone probably acts as the common intermediate in both one- and two-electron pathways [4, 5].

Upon reduction, the MMC C-9a methoxy group is eliminated from the parent mitosane molecule, resulting in the formation of reduced mitosene intermediates displaying the characteristic double bond between the C-9a and C-9 positions of the molecule. This promotes aziridine ring opening, to expose an electrophilic carbon centre at C-1 that is capable of DNA alkylation [6]. The critical intermediate produced in this metabolic cascade that leads to DNA crosslinking is thought to be a quinone methide [7, 8] and its binding to DNA at C-1 promotes a second DNA attachment point producing DNA crosslinks *via* the production of a C-10 reactive centre [8]. The quinone methide is believed to behave differently at different pH. At low pH,

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† Abbreviations: MMC, mitomycin C; 2,7-DM, 2,7-diaminomitosene; cis-hydro, 1,2-cis-1-hydroxy 2,7-diaminomitosene; trans-hydro, 1,2-trans-1-hydroxy 2,7-diaminomitosene; NADPH-linked AqCbl reductase, NADPH-linked aquacobalamin reductase.

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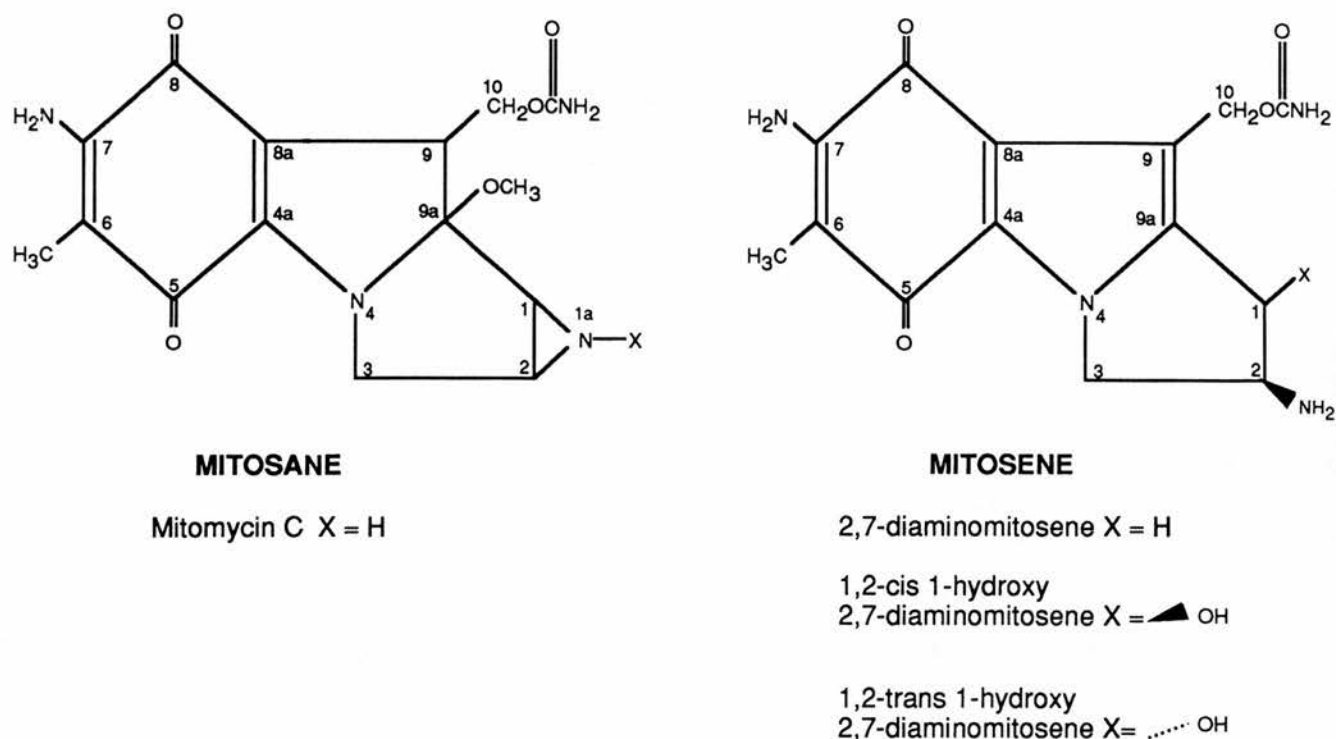


FIG. 1. Molecular structure of mitomycin C and its three principle mitosene metabolites.

the principle metabolite observed *in vivo*, 2,7-DM, is formed *via* protonation of the quinone methide. At higher pH, the quinone methide acts as an electrophile leading to the formation of cis-hydro and trans-hydro [7].

Using cell-free activation systems, both enzymatic and chemical, a number of MMC-DNA adducts have been identified. An interstrand crosslink [9], intrastrand crosslink [10], and three monoadducts [11, 12] have all been isolated using HPLC, and these studies indicate that the major alkylation site detectable is the N² position of guanine. Four of these DNA adducts, two monoadducts and two bisadducts, have now been detected in EMT6 mouse mammary tumour cells following MMC treatment [13], suggesting that they may occur *in vivo*.

Several enzymes have been shown to catalyse the *in vitro* bioreduction of MMC, ultimately resulting in the formation of MMC metabolites. These included one-electron reductases, such as NADPH:cytochrome P-450 reductase [14], xanthine oxidase [14], and NADPH:cytochrome *b*₅ reductase [15], and the two-electron reductases DT-diaphorase [NAD(P)H:(quinone acceptor) oxidoreductase, EC 1.6.99.2] and xanthine dehydrogenase [16]. The role of these enzymes in the bioactivation of MMC under aerobic and hypoxic conditions, particularly DT-diaphorase, has been the subject of intense research and controversy [17, 18]. Numerous studies using cancer cell lines with high and low DT-diaphorase activities have provided evidence that this enzyme may be responsible for aerobic MMC bioactivation [19–21], although these results were not in agreement with those using purified enzyme preparations, in which MMC was actually found to be an inhibitor of DT-

diaphorase [17, 22]. The effect of hypoxia on DT diaphorase-dependent MMC metabolism remained unknown until studies using the DT-diaphorase-rich and -deficient cell lines, HT-29 and BE, revealed that bioactivation of MMC by DT-diaphorase was similar under both aerobic and hypoxic conditions [23].

The *in vivo* metabolism of MMC has previously been studied in two murine adenocarcinomas of the colon, MAC 16 and MAC 26 [24]. The tumours were chosen for two reasons. First, their differing levels of quinone reductases: MAC 16 exhibiting a 16-fold higher activity of DT-diaphorase than MAC 26 and NADPH:cytochrome P-450 reductase being similar in both [25], and second, their histological similarity to human tumours of that origin [26]. Despite MAC 26 having reduced levels of DT-diaphorase, metabolism of MMC to its metabolites was comparable to that of MAC 16. This result suggested that an enzyme(s) other than DT-diaphorase may be catalysing MMC metabolism *in vivo*.

In this paper, we have studied the enzymology of MMC bioactivation in MAC 16 and MAC 26. The following approaches were used to identify and characterise the reductase(s) involved; (1) measure levels of quinone reductases in both tumour types; (2) incubate MMC with subcellular fractions isolated from MAC 16 and MAC 26 and characterise their cofactor requirements, the effect of oxygen, and response to specific enzyme inhibitors, and (3) incubate MMC with whole tumour homogenates to assess how the identified enzyme(s), if any, will behave in the tumour environment. This may, in turn, provide important information on the drug's *in vivo* mechanism of action and

TABLE 1. Quinone reductase activity in cytosolic and microsomal subcellular fractions isolated from MAC 16 and MAC 26 murine adenocarcinomas

Tumour	Cytosol			Microsomes			
	DT-diaphorase		'Others'	DT-diaphorase		Cytochrome P450 reductase	Cytochrome b ₅ reductase
	NADH	NADPH		NADH	NADPH	NADPH	NADH
MAC 16	447.13	328.46	378.81	5.12	9.69	17.21	18.41
MAC 26	19.33	15.67	49.78	1.22	0.24	14.82	16.89

Preparation of cytosolic and microsomal fractions and enzyme activity measurements were carried out in both tumour types as described in Materials and Methods. Each value represents the mean from 3 separate experiments and all enzyme activities are expressed as nmol cytochrome c reduced/min/mg protein. Enzymes grouped into the 'other' category would include xanthine oxidase/dehydrogenase and aldoketo reductase.

have implications for the use of bioreductive alkylating agents in enzyme-directed drug therapy.

MATERIALS AND METHODS

Chemicals

MMC was obtained from Kyowa Hakko Kogyo Co., Tokyo, Japan. HPLC-grade methanol was from Rathburn Chemicals Ltd., Walkerburn, U.K. Aristar grade sodium dihydrogen phosphate and disodium hydrogen phosphate were from BDH, Merck Ltd., Merck House, Poole, U.K. All other chemicals were of the highest grade available from Sigma Chemical Co. Ltd., Fancy Road, Poole, U.K. Cytochrome P-450 reductase antiserum (CH59) was a kind gift from Professor C. Roland Wolf, Imperial Cancer Research Fund, Molecular Pharmacology Unit, Dundee, U.K.

High-Performance Liquid Chromatography

All chromatographic analyses were carried out using a Hewlett-Packard Model 1090 liquid chromatograph equipped with a diode array detector. Chromatographic conditions were modified from those previously described [27]. Essentially, the stationary phase consisted of LiChrosorb RP-18 (7 µm particle size, column 25 cm, 4 mm internal diameter) (supplied by BDH, Merck Ltd.). The mo-

bile phase consisted of 10 mM sodium phosphate buffer, pH 7.5 and methanol, 74:26. Elution was isocratic at a flow rate of 1 mL/min and the column was maintained at 40°C. Mobile phase was filtered before use (0.2 µm filter, Waters-Millipore, Northwich, U.K.) and continuously sparged with helium throughout chromatography.

Animal Models and Tumours

The animal model consisted of inbred NMRI mice and the subcutaneously growing MAC 16 or MAC 26 murine adenocarcinoma of the colon (breeding pairs and tumours kindly supplied by Professor J. A. Double and Dr. M. C. Bibby, Clinical Oncology Unit, University of Bradford, U.K.). Tumours were maintained by subcutaneous passage of 1–3 mg lumps of viable tissue into the flank of the animal via a trocar needle. After tumours became palpable, they were removed and immediately placed in liquid nitrogen. All tumours were stored at -80°C prior to use.

Subcellular Fractionation and Quinone Reductase Assessment

MAC 16 and MAC 26 homogenates and subcellular fractions were prepared following a modification of the previously described method [28]. Whole tumours, 0.5–1.5 g,

TABLE 2. Characterisation of mitomycin C bioreduction in MAC 16 mouse adenocarcinoma of the colon

Tumour fraction	2,7-DM formation (nmol/30 min/mg protein)	Cofactor requirement	Effect of oxygen	Effect of 10 µM dicoumarol	Effect of antiserum
Cytosol	0.76 ± 0.10	NADH	No effect	100% inhibition	No effect
Microsomes	1.04 ± 0.17	NADPH	100% inhibition	No effect	100% inhibition
Mitochondria	2.04 ± 1.09	NADPH	100% inhibition	No effect	100% inhibition
	0.93 ± 0.09	NADH	100% inhibition	No effect	No effect
Homogenate	3.67 ± 0.58	NADPH	No effect		
	0.14 ± 0.07				

The MAC 16 tumour was fractionated and all incubations were performed as described in Materials and Methods. Specific activity is expressed as 2,7-diaminomitosenone formation in nmol/30 min/mg protein. Each value represents the mean ± SE from 3 separate experiments.

TABLE 3. Characterisation of mitomycin C bioreduction in MAC 26 mouse adenocarcinoma of the colon

Tumour fraction	2,7-DM formation (nmol/30 min/mg protein)	Cofactor requirement	Effect of oxygen	Effect of 10 μ M dicoumarol	Effect of antiserum
Cytosol	ND*	—	—	—	—
Microsomes	ND	—	—	—	—
Mitochondria	3.87 \pm 0.71	NADPH	100% inhibition	No effect	No effect
Homogenate	1.45 \pm 0.18	—	45% inhibition	—	—

The MAC 26 tumour was fractionated and all drug incubations were performed as described in Material and Methods. Specific activity is expressed as 2,7-diaminomitosen formation in nmol/30 min/mg protein. * ND, no activity detected. Each value represents the mean \pm SE from 3 separate experiments.

were first washed in saline then transferred to 0.25 M sucrose, 5 mM Tris-buffer, 0.5 mM EDTA, pH 7.4 in which a homogenate was produced (33% w/v). Portions of the homogenate were either used directly in drug incubations or processed to isolate the mitochondrial, microsomal, and cytosolic fractions. Homogenates were first centrifuged at 600 g for 10 min to remove cellular debris, and the resulting supernatant was subjected to a high-speed spin (24,000 g for 10 min) to isolate the mitochondria. The supernatant was retained for microsomal isolation. The mitochondrial pellet was resuspended in STE and centrifuged once again at high speed. The final pellet was resuspended in 5 mL STE. Microsomes were isolated from the postmitochondrial supernatant by centrifugation at 80,000 g for 1 hr. Again, the supernatant was retained as the cytosolic fraction. The pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.4, and centrifuged again at 80,000 g. The final pellet was resuspended in 5 mL 10 mM Tris-HCl buffer, pH 7.4 containing 20% glycerol and 0.1 mM EDTA. The cytosolic fraction was centrifuged at 80,000 g for 4 hr to remove remaining cellular debris. The resulting supernatant was made up to 10 mL with STE. All centrifugation steps were carried out at 4°C and fractions stored at -80°C in 1 mL aliquots. The protein concentration of all homogenate and fractions was determined using the Biuret method [29] with BSA as the standard.

Cytosolic and microsomal NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2, DT-diaphorase) activity was determined by following the spectrophotometric reduction of cytochrome *c* using a modification of the method of Ernster [30], as reported in detail elsewhere [31]. In brief, the reaction mixture contained 65–100 μ g cytosolic or 17–33 μ g microsomal protein, cytochrome *c* (77 μ M), menadione (20 μ M), NADH, or NADPH (2 mM) as cofactor and BSA (0.14% w/v). Reactions were performed at 37°C in a total volume of 1 mL 50 mM Tris-HCl, pH 7.4, in the presence or absence of the inhibitor dicoumarol (10 μ M). DT-diaphorase activity was taken as the activity that could be inhibited by dicoumarol in the presence of NADPH or NADH. All enzyme activities were calculated as nmol cytochrome *c* reduced/min/mg protein (e 21.1 \times 10³ M/cm).

Drug Incubations

In vitro drug incubations were performed at 37°C under aerobic and hypoxic conditions. Each incubation mixture

contained 100 μ g/ml MMC, approximately 1 mg subcellular protein, and 3.3 mM exogenously added cofactor (NADH, NADPH, or hypoxanthine) in 0.1 M sodium phosphate buffer, pH 7.4. Tumour homogenate incubation: contained 100 μ g/mL MMC in a total volume of 1 mL. In the case of hypoxic incubations, mixtures were sparged with helium for 5 min prior to the addition of MMC, which started the reaction. The enzyme inhibitor dicoumarol (10 μ M made up in 0.1 M sodium phosphate, pH 7.4:0.1 M sodium hydroxide) and cytochrome P-450 reductase antiserum (1 in 100 dilution) were added 30 min before the start of the reaction. At 15-min intervals (0–90 min), a 100 μ L aliquot was withdrawn from the incubation and 20 μ L subjected to HPLC to determine the concentration of MMC and its metabolites. Reaction rates were calculated from linear portions of the reaction curves and expressed as nmol/30 min/mg protein.

RESULTS

Quinone Reductase Activity in MAC 16 and MAC 26 Murine Adenocarcinomas

The quinone reductase activity of the cytosolic and microsomal fraction isolated from MAC 16 and MAC 26 are shown in Table 1. MAC 16 exhibited a 22-fold higher level of cytosolic DT-diaphorase but similar levels of microsomal NADPH:cytochrome P-450 reductase to those of MAC 26, which is consistent with previously published data [25]. Other cytosolic enzyme activities (Table 1) were also calculated and may include xanthine oxidase and aldoketo reductase. MAC 16 activity possessed approximately 8-fold greater levels of these enzymes compared to MAC 26. Both tumours exhibited similar levels of microsomal NADH:cytochrome *b*₅ reductase.

Mitomycin C Bioreduction to the Metabolite 2,7-diaminomitosen by MAC 16 and MAC 26 Subcellular Fractions and Whole Tumour Homogenates

Tables 2 and 3 illustrate MMC bioreduction in MAC 16 and MAC 26 subcellular fractions and tumour homogenates under conditions described in Materials and Methods. Subcellular data revealed a number of enzyme activities present within all 3 fractions in MAC 16 compared to one in MAC 26. MAC 16 cytosolic activity utilised both

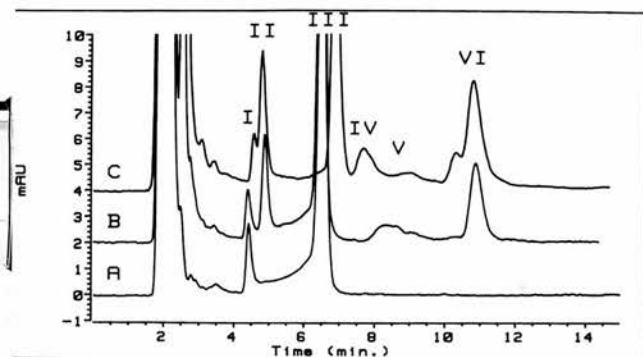


FIG. 2. *In vitro* pattern of mitomycin C metabolism in MAC 16 (C) and MAC 26 (B) mitochondrial fractions at 0 (A) and 30 (B, C) min as measured by HPLC. Incubations were performed using NADPH as cofactor under hypoxic conditions. Peaks: I, zwitterion form of mitomycin C; II, 1,2-trans-1-hydroxy-2,7-diaminomitosene; III, mitomycin C; IV, 1,2-cis-1-hydroxy-2,7-diaminomitosene; V, 10-decarbamoyl 2,7-diaminomitosene; VI, 2,7-diaminomitosene.

NADH and NADPH, showing similar activity under aerobic and hypoxic conditions. The addition of dicoumarol, a known inhibitor of the two-electron reductase DT-diaphorase [32], resulted in 100% inhibition of 2,7-DM formation. Addition of cytochrome P-450 reductase antiserum had no inhibitory effect. No cytosolic activity was observed in MAC 26. MAC 16 microsomal activity had an absolute requirement for NADPH and hypoxia and 100% inhibition was achieved with the addition of antiserum. Coincubation with 10 μ M dicoumarol had no effect on metabolism. Again, no microsomal activity was present in MAC 26.

The majority of MMC metabolism was associated with the mitochondrial fraction from both tumour types. NADPH combined with hypoxia produced the greatest formation of 2,7-DM with specific activities being similar in both tumour types. NADH-dependent MMC metabolism was only seen in MAC 16 and activity was approximately 4-fold less than that seen with NADPH. The presence of oxygen resulted in complete inhibition of metabolism in both tumour types. The addition of 10 μ M dicoumarol and cytochrome P-450 reductase antiserum to MAC 16 and MAC 26 mitochondria failed to produce any inhibitory effect. The chromatographic profiles of MMC metabolism by MAC 16 and MAC 26 mitochondria in the presence of NADPH and hypoxia are shown in Fig. 2. The principle metabolite, 2,7-DM, can be clearly seen at 10.8 min as well as a small amount of cis-/trans-hydro and the secondary metabolite 10-decarbamoyl 2,7-diaminomitosene.

In the light of three different enzyme activities being present in MAC 16 vs one in MAC 26, the role of these enzymes were investigated in whole tumour homogenates. Figure 3 shows the formation of 2,7-DM in MAC 16 and MAC 26 homogenates under aerobic and hypoxic conditions. In agreement with the subcellular incubations, MAC 26 homogenates exhibited an increase in metabolic activity

under hypoxia (Table 3), similar to that seen with the mitochondrial fraction from this tumour. In contrast, MAC 16 homogenates did not show hypoxic enhancement, despite the presence of the identified mitochondrial enzyme, and activity was similar under aerobic and hypoxic conditions.

DISCUSSION

The aim of the present study was to identify the enzyme(s) responsible for MMC bioreduction in the MAC 16 and MAC 26 murine adenocarcinomas, to provide an insight into the drug's mechanism of action *in vivo*. The results have demonstrated a number of previously characterized enzyme activities, in addition to a novel mitochondrial reductase(s) capable of reductive metabolism of MMC in both MAC 16 and MAC 26.

DT-diaphorase and NADPH:cytochrome P-450 reductase have both been shown to metabolise MMC in a number of cellular systems [5, 14]. Their identification, in many cases, has relied upon comparison of their characteristics with those of purified enzymes in conjunction with specific enzyme inhibitors. Probably the most widely studied enzyme, DT-diaphorase, characteristically utilises both NADH and NADPH as cofactors, showing equivalent activity under aerobic and hypoxic conditions. In addition, its activity can be inhibited by relatively low concentrations of the coumarin derivative dicoumarol. Using these characteristics, the results presented in Table 2 have identified an enzyme present within the cytosol of MAC 16 exhibiting these properties. This result, along with the high level of DT-diaphorase observed in the cytosolic quinone reductase assay (Table 1) leads to the conclusion that this enzyme is

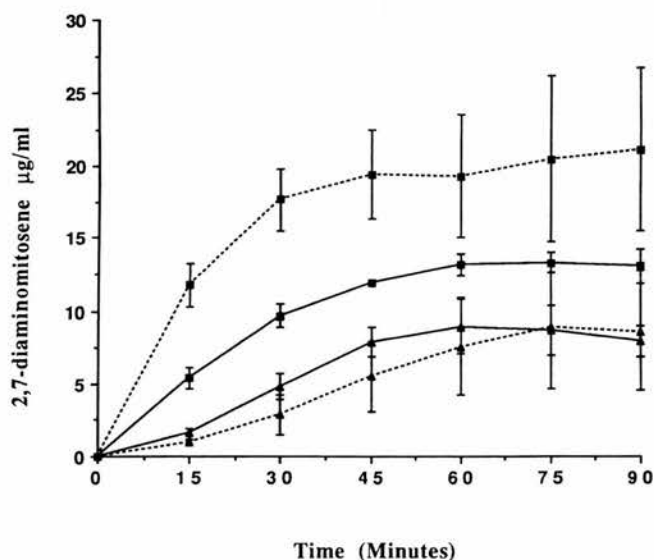


FIG. 3. *In vitro* production of the principle mitomycin C metabolite 2,7-diaminomitosene in MAC 16 (▲) and MAC 26 (■) tumour homogenates (33% w/v) under aerobic (—) and anaerobic (-----) conditions. Incubations were performed as described in Materials and Methods. Each point represents the mean \pm SE from 3 separate incubations.

possibly DT-diaphorase, an enzyme that has long been associated with metabolism of both MMC and its analogue EO9 in MAC 16 [25, 33, 34]. Therefore, perhaps it is not surprising that this enzyme activity was observed, although the specific activity was considerably less than with other enzymes identified.

Activity characteristic of the one-electron reductase NADPH:cytochrome P-450 reductase was also observed in MAC 16. Localised within the microsomal fraction, it had an absolute requirement for NADPH and hypoxia. Activity was completely inhibited by the addition of cytochrome P-450 reductase antiserum, which has been previously shown to be capable of inhibiting the biotransformation of doxorubicin to doxorubicin 7-deoxyglycone by purified rat liver NADPH:cytochrome P-450 reductase [28]. Despite NADPH:cytochrome P-450 reductase and DT-diaphorase activities being confirmed in MAC 26 by cytochrome *c* reduction assays, there was no indication of any involvement in microsomal and cytosolic MMC metabolism, respectively.

The majority of subcellular MMC metabolism in MAC 16 and MAC 26 was associated with the mitochondria. Although quinone reductase assessment of the mitochondria was impossible due to the presence of cytochrome *c* oxidase, the presence of mitochondria was confirmed by electron microscopy (data not shown). Due to the nature of the fractionation process, the method will not prevent mitochondrial membranes from disruption and, therefore, will allow the inner mitochondrial membranes access to exogenously added nicotinamide nucleotides [35, 36]. The greatest metabolism was observed under hypoxia using NADPH as the cofactor. The inability of dicoumarol and cytochrome P-450 reductase antiserum to inhibit activity suggests that the enzyme is neither DT-diaphorase or NADPH:cytochrome P-450 reductase, respectively. The dependency of this enzyme for hypoxia suggests it is a one-electron reductase rather than a two-electron reductase, such as DT-diaphorase, which is equally active under aerobic and hypoxic conditions. MAC 16 mitochondria also showed a small amount of activity using NADH as the cofactor; again, only under hypoxia, which may indicate the presence of more than one enzyme. MAC 26 showed no NADH-dependent activity. Again, the inhibitors failed to produce any inhibitory effect, implying that the enzyme is once again not DT-diaphorase or NADPH:cytochrome P-450 reductase.

It has previously been suggested that mitochondrial reductases are capable of metabolising MMC and other quinones [17, 37], although no experimental data has been published to support this statement. In addition, recent *in vitro* and *in vivo* data studying mitochondrial function after MMC treatment have shown that considerable mitochondrial DNA damage occurred after drug administration, implying, for the first time, that this MMC-mitochondrial interaction may contribute to MMC's mechanism of toxicity [38]. Quinone metabolites derived from the environ-

mental pollutant benzo[*a*]pyrene have also been shown to exhibit the capacity to deplete cellular ATP and induce morphological changes within mitochondria in stromal cells, suggesting that benzo[*a*]pyrene quinones may elicit their toxicity through directly disrupting mitochondrial energy metabolism [39].

One enzyme that exhibits similar properties to the NADPH-dependent mitochondrial enzyme identified in this study is NADPH-linked AqCbl reductase. First studied in bacterial systems [40], this enzyme has now been identified in a number of mammalian tissues [41]. After characterisation of this enzyme from rat liver microsomes, results indicated that it was NADPH:cytochrome *c* reductase now more commonly known as NADPH:cytochrome P-450 reductase [42]. At this time, a NADPH-linked AqCbl reductase had been reported to occur in mitochondrial membranes [43], although its purification and characterization were yet to be carried out. Subsequent isolation from rat liver mitochondrial membranes revealed similarities, but also distinct differences to its microsomal counterpart namely molecular weight and peptide elution profile [44]. However, despite these differences, the identified mitochondrial enzyme exhibited the same high specific activity and identical submitochondrial location (outer membrane) of the cytochrome *c* reductase, and the authors concluded that the purified NADPH-linked AqCbl reductase may be the NADPH-cytochrome *c* reductase in rat liver mitochondria. This was supported by an early observation that NADPH-cytochrome *c* reductase had been reported to occur, not only in microsomes, but also the mitochondrial outer membranes [45]. This enzyme may be capable of metabolising MMC in tumour tissue and may be a good candidate, although identification and localisation in tumours has never been addressed.

Rat liver microsomal NADH-linked AqCbl reductase activity derived from cytochrome *b₅*/cytochrome *b₅* reductase complex has also been identified [46], although whether or not a similar enzyme exists within the mitochondria remains unknown and, therefore, it is not yet a likely candidate for the NADH-dependent enzyme identified in MAC 16. More likely, NADH-dependent enzymes included mitochondrial NADH dehydrogenase, which has been linked to the metabolism of the anthracycline doxorubicin (adriamycin) [47, 48] and NADH-ubiquinone oxidoreductase.

The presence of the mitochondrial enzyme(s) may explain the hypoxic enhancement of MMC metabolism observed in MAC 26 homogenate incubations. Although no aerobic metabolism was observed in the purified mitochondrial fractions, aerobic homogenate metabolism may rely upon the presence of these enzymes in the whole tumour environment that may, in turn, occur *in vivo*. In contrast, MAC 16 homogenates showed no hypoxic enhancement, despite the presence of mitochondrial activity, suggesting the predominance of other enzyme(s). This phenomenon has been previously observed in a number of cell lines containing high and low DT-diaphorase activities [49]. Because

MAC 16 homogenates showed no increase in MMC metabolism under hypoxia, this suggests that an enzyme(s) other than the mitochondrial enzyme(s) was predominating. With the lack of metabolic enhancement under hypoxia and the identification of an enzyme exhibiting similar properties in the cytosol, it appears that the enzyme responsible for MMC metabolism in the MAC 16 homogenates under all conditions is DT-diaphorase. However, it seems that DT-diaphorase is less effective at metabolising MMC than the mitochondrial reductase(s) [50].

The conclusions drawn from these observations have clear clinical implications. Many tumours are known to have high levels of DT-diaphorase and are an attractive target for antitumour agents such as MMC and EO9 [51]. Data presented in this paper suggest that tumours low in DT-diaphorase are optimal for treatment with such agents, if hypoxia can be achieved, and tumours high in the enzyme may not benefit. However, total tumour hypoxia may be difficult to achieve *in vivo* and, therefore, on balance, tumours high in DT-diaphorase may, nevertheless, prove optimal for treatment with MMC and related drugs.

In conclusion, we now report the presence of a novel mitochondrial one-electron reductase(s) capable of metabolising MMC in MAC 16 and MAC 26 (i.e. tumours both high and low in DT-diaphorase, respectively). This enzyme(s) is possibly responsible for MMC metabolism in MAC 26 where DT-diaphorase levels are reduced *in vitro* and, possibly *in vivo*.

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The Autoxidation of the Reduced Forms of EO9

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The properties of the semiquinone radical from [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-indione)-prop-β-en-α-ol], EO9, have been studied using pulse-radiolysis techniques. The reduction potential of the semiquinone of EO9 at pH 7.4, $E(\text{EO9}/\text{EO9}^{\cdot-})$, is -253 ± 6 mV and hence this quinone can be readily reduced by one-electron reducing enzymes such as cytochrome P450 reductase and xanthine oxidase. However, the radical is unstable in the presence of oxygen ($k = 1.3 \pm 0.15 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$). The semiquinone radicals and the hydroquinone are in equilibrium, although the formation of the semiquinone is favoured at physiologically relevant pH. The hydroquinone of EO9 is also unstable in the presence of oxygen and it is predicted that in fully aerated solutions, its half life is 1.5 ± 0.3 seconds. These results are discussed in view of the selective cytotoxicity of EO9 and its ability to undergo bioreductive activation by one-electron reducing enzymes and DT-diaphorase.

Key words: EO9, semiquinone, hydroquinone, DT-diaphorase, autoxidation, pulseradiolysis

INTRODUCTION

EO9 [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-indione)-prop-β-en-α-ol] is a bioreductive alkylating indoloquinone (Figure 1) with a distinct activity towards solid tumours and is cur-

rently undergoing phase I/II clinical trials.¹ Several *in vitro* studies have shown that this compound is up to 1000 times more toxic towards hypoxic cells compared to cells which are fully oxygenated.^{2,3}

It has been proposed that EO9 undergoes bioreductive activation and alkylates DNA. The reduction can be produced by several one-electron reducing enzymes including cytochrome P450 reductase, xanthine oxidase and cytochrome b₅ reductase.^{4,5} However, the quinone is an excellent substrate for the obligatory two-electron reducing enzyme, DT-diaphorase [NAD(P)H quinone oxidoreductase, EC 1.6.99.2] and there is a good correlation between the activity of DT-diaphorase present in a tumour cell and the cytotoxicity of the drug.⁶ However, recent work has

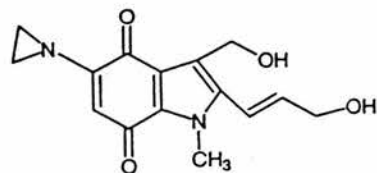


FIGURE 1 The structure of EO9

shown that this correlation is only true under oxic conditions and indeed, it appears that under hypoxic conditions, where EO9 is most toxic, DT-diaphorase may be protecting the cells against damage by the drug.⁵

In view of the conflicting mechanisms proposed for the cytotoxicity of EO9, we have investigated the chemical properties of the semiquinone radical and the reactivities of the radical and the hydroquinone with oxygen.

MATERIALS AND METHODS

EO9 was obtained from the EORTC under the auspices of Kyowa Hakko, UK and was found to be >95% pure, based on determination by HPLC. Bovine xanthine oxidase, allopurinol and hypoxanthine were from Sigma (St Louis, MO, USA). All of the pulse radiolysis solutions contained 0.1 M sodium formate and were buffered using 10mM phosphate (pH 4.5–9.0) or 10 mM borate/NaOH (pH 8.5–10.0). All other reagents were of the highest grade commercially available.

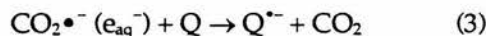
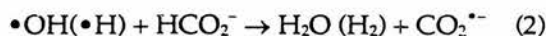
The pulse radiolysis experiments were con-

ducted at the Paterson Institute linear accelerator facility.⁷ The pulse lengths were less than 0.1 μ s. The optical detection system consisted of a xenon or tungsten lamp, a Kratos monochromator and an EMI 9558QA photomultiplier. The optical cells were capillary cells (3 mm internal diameter) with path lengths of 0.4 and 2.5 cm. The signals from the photomultiplier were recorded on a Tektronix 7612AD programmable digitizer.

The stopped flow measurements used an Applied Photophysics RX1000 stopped-flow apparatus coupled to HP8452A diode array spectrophotometer. The kinetic analysis were carried out using the Fig P (registered trademark, Biosoft, Cambridge, UK) program assuming second order kinetics. All experiments were performed at $21 \pm 2^\circ\text{C}$.

RESULTS

When an argon saturated buffered solution of sodium formate (100 mM, pH 4.5–9) and quinone (20 μ M–2mM) is irradiated, the following reactions are initiated:



Hence within a few microseconds, all of the primary radicals from water produce semiquinone radicals. The difference spectrum (absorbance of semiquinone minus absorbance of parent quinone) produced at 20 μ s after the pulse for the solutions at pH 7.4 is shown in Figure 2. The spectrum of the radical was the same at pH 7.4 and 10 although at pH values below about 6, the absorbances were found to decrease in the range 350–450 nm. The pH dependence of the absorbances at 400 nm are shown in Figure 3. Unfortunately, due to the instability of EO9 in acid and

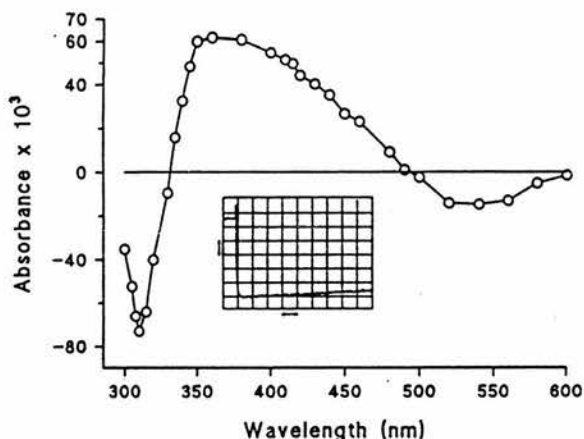
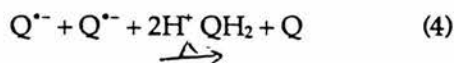


FIGURE 2 The difference spectrum of the EO9 semiquinone recorded at 20 μ s after the pulse. All absorbances were normalised to a radical concentration of 6.5 μ M in a 2.5 cm optical cell. Insert: A typical trace produced by pulsing an argon saturated solution containing 150 μ M EO9 in 0.1M formate, 10mM phosphate (pH 7.4) at 400nm. Radical concentration = 5.7 μ M, 20 μ s/div, 1.8% transmission/div.

alkaline pH,^{4,8,9} it was not possible to do the experiments outside the pH range 4.5–10.0.

In the absence of oxygen, the semiquinone radicals were found to decay at pH 7.4 over a period of milliseconds in a dose dependent manner. A typical trace is included in Figure 3.

This is consistent with the well established reaction for semiquinone radicals:



Where QH_2 is the hydroquinone of EO9.

This reaction was investigated at 400 nm where the rate constant was calculated to be $5.2 \pm 1.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4. This value was independent of the radical concentration in the range 1.0–5.2 μM and quinone concentrations up to 2.0 mM. However, within the pH range ca. 8–10 the apparent rate constant for reaction 4 was found to significantly increase and the absorptions due to the radicals did not disappear completely, but relaxed to a value which remained stable for at least several tens of milliseconds. The apparent rates of the reaction and the absorbance values at the end of the reaction were found to be dependent on the

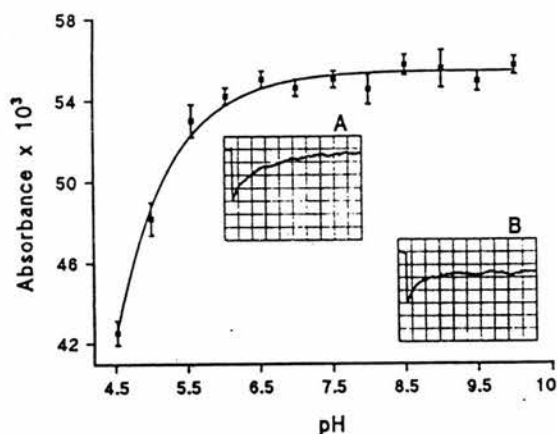
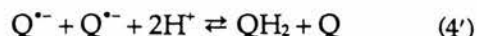


FIGURE 3 The variation of the absorbance of EO9 semiquinone radicals as a function of pH. All values were normalised to a radical concentration of 6.5 μM in a 2.5 cm optical cell. Inset A: The decay of EO9 semiquinone radicals at pH 7.4 ($\lambda = 400 \text{ nm}$: radical concentration = 2.65 μM , 10 ms/division and 1.2% transmission/division). Inset B: The formation of an equilibrium with 100 μM , EO9 at pH 9.0 ($\lambda = 400 \text{ nm}$: radical concentration = 2.6 μM , 4 ms/div and 1.2% transmission/division).

initial quinone concentration. A typical trace at pH 9.0 is included in Figure 3. This is consistent with reaction 4 being an equilibrium in alkaline pH:

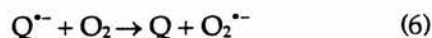


Measurements of the final absorbance at 400 nm, where only the semiquinone have appreciable absorptions, enabled the concentration, R_e , of the semiquinone at equilibrium to be calculated. The equilibrium constant K_4 was then calculated from the formula:

$$K_4 = \frac{\left(A_0 - \frac{(R_0 + R_e)}{2} \right) \left(\frac{(R_0 - R_e)}{2} \right)}{R_e^2} \quad (5)$$

where A_0 is the initial quinone concentration (50–200 μM) and R_0 is the concentration of semiquinone radicals produced immediately after the pulse (1.7–5.6 μM). From these calculations it was found that K_4 was 8 ± 2 at pH 10.0 and 84 ± 12 at pH 9.0. By using high concentrations of quinone (100–500 μM) and lower concentrations of radicals (0.4–1.7 μM), it was observed that the equilibrium occurred at pH 8. However, due to the relatively small absorbance values at equilibrium, only a lower limit of $K_4 > 700$ could be determined. Similarly, from using a much higher concentration of quinone (2.0 mM in a 0.4 cm optical cell), the limit on the value at pH 7.4 was found to be >4000 .

The semiquinone radicals formed at pH 7.4, were unstable in the presence of oxygen. This is consistent with the reaction.²⁷



The rate of this reaction was determined by adding small concentrations of oxygen to the argon saturated solutions and observing the decay of the semiquinone radicals at 400 nm. The variation of rate with oxygen concentration is shown in Figure 4. From the slope of the line, the rate constant for reaction 6 was calculated as $1.3 \pm 0.15 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$

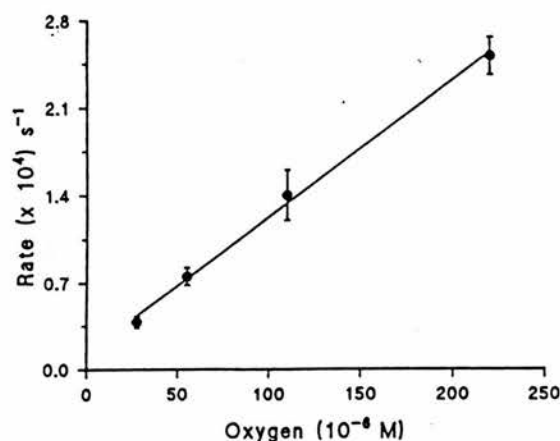


FIGURE 4 The dependence of the rate of decay of semiquinone radicals on oxygen concentration.

The reduction potential of the quinone/semi-quinone couple at pH 7.4 was determined by setting up an equilibrium between the semiquinone radicals of EO9 and mitomycin C (MMC):



The equilibria were investigated at 415nm, where the $\text{EO9}^{\cdot-}$ radicals have a much higher absorbance than that of $\text{MMC}^{\cdot-}$ radicals and at 490nm, where the $\text{MMC}^{\cdot-}$ radicals have much higher absorbance than $\text{EO9}^{\cdot-}$ radicals¹⁰. The concentration of mitomycin C was kept at 100 μM while the EO9 concentration was varied between 10–40 μM .

It can easily be shown¹¹ that at equilibrium:

$$K = \frac{(A_e - A_{\text{MMC}})/(A_{\text{EO9}} - A_e) \times [\text{MMC}]/[\text{EO9}]}{\quad} \quad (8)$$

where, A_e is the absorbance value at equilibrium and A_{MMC} is the absorbance of Mitomycin C radicals in the absence of EO9 and A_{EO9} is the absorbance of the EO9 radicals in the absence of Mitomycin C. The equilibrium constant, K is related to the reduction potentials^{11,12}:

$$E(\text{MMC}/\text{MMC}^{\cdot-}) = E(\text{EO9}/\text{EO9}^{\cdot-}) - 59 \log K \quad (9)$$

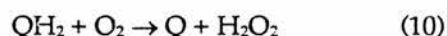
TABLE 1

Concentration of EO9 (μM)	λ (nm)	Absorbance at equilibrium (A_e) $\times 10^{-3}$	K
10	490	37.4	9.0
	415	24.2	9.6
20	490	25	9.3
	415	31.8	9.0
40	490	15.1	9.6
	415	39.2	9.5

The absorptions of EO9 radicals at 415 and 490 nm are 49.5 and $0.7 \times 10^{-3}/6.5 \mu\text{M}$ radicals in the absence of MMC. The absorption of MMC radicals are 0 and $70.4 \times 10^{-3}/6.5 \mu\text{M}$ radicals at the same wavelengths respectively.

The absorbance values, normalised to a radical concentration of 6.5 μM , are given in Table 1. From using the derived average value of K in Table 1 and assuming $E(\text{MMC}/\text{MMC}^{\cdot-}) = -310 \pm 6 \text{ mV}$ ¹⁰, it can be shown that $E(\text{EO9}/\text{EO9}^{\cdot-}) = -253 \pm 6 \text{ mV}$.

It has recently been shown that the reduction of EO9 by the obligatory two electron reducing enzyme, DT-diaphorase is influenced by the presence of oxygen.¹³ Several preliminary experiments involving rapidly mixing the hydroquinone produced in the pulse radiolysis experiments with air indicated that the hydroquinone is unstable with respect to autoxidation:



This reaction was studied by initially reducing the quinone (20–50 μM) in one half of a stopped flow mixing chamber with xanthine oxidase/hypoxanthine (100 μM hypoxanthine, 0.1 M phosphate buffer (pH 7.4), 0.6U xanthine oxidase, argon saturated) until the solution became colourless. This was then rapidly mixed with an air saturated solution (200 μM allopurinol and 2 μM EDTA in 0.1 M phosphate buffer, pH 7.4). The total time for the initial reduction of the quinone and the subsequent mixing with air was less than 10 min and it was determined by HPLC analysis, consistent with recent studies,⁴ that the reactions did not produce any changes in the

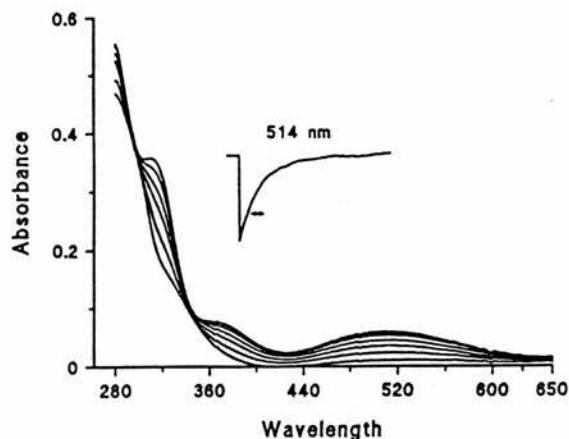


FIGURE 5 The autoxidation of the hydroquinone of EO9. Spectra measured at 2 sec intervals. Insert: Decay of EO9 hydroquinone at 514nm in the presence of 110 μ M oxygen. The arrow refers to a time period of 2 sec.

structure of the quinone during this period. A typical stopped-flow trace is shown in Figure 5 and the insert shows the kinetics at 314 nm. If it is assumed that the oxygen concentration in a fully air-saturated solution is 220 μ M (ie. 110 μ M in the stopped flow mix), then the rate constant for reaction 10 is $2.1 \pm 0.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. Thus the half-life of a low concentration of the hydroquinone of EO9 in air is 1.5 ± 0.3 seconds.

DISCUSSION

The semiquinone radicals of EO9 have a reduction potential, $E(Q/Q^{\cdot-})$, of $-253 \pm 6 \text{ mV}$ at pH 7.4. Hence, according to previous studies on the dependence of enzyme rates and reduction potentials,^{14,15,16} EO9 should be readily reduced by the one electron reducing enzymes such as cytochrome P450 reductase and xanthine oxidase. This has recently been demonstrated and indeed EO9 can be reduced by xanthine oxidase in the presence of DNA to form crosslinks.⁴ Therefore, although EO9 may be an excellent substrate for diaphorase, the selectivity cytotoxicity of the quinone should also be mediated by the levels of

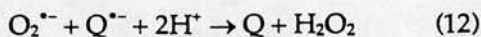
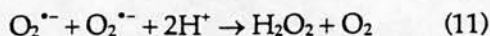
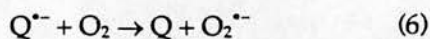
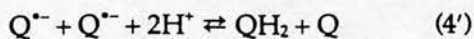
the one-electron reducing enzymes within the cells.

The spectrum of the radical did not change between pH 6.5 and 10. (Figure 3). This implies that the aziridine on the semiquinone radical does not have a pK in this region. However, the absorbances at 400 nm were found to decrease in the pH range of 4.5–6.5. This decrease is probably due to both the protonation the aziridine and the $-O^{\cdot-}$ on the semiquinone radical (ie. $Q^{\cdot-}$ in neutral protonating to QH^{\cdot} in acid). Most semiquinone radicals have a pK of around 4.0 to 6.0 (11). Unfortunately, due to the instability of EO9 in acid solutions,^{4,8} it was not possible to accurately determine either the pK of the aziridine or of the semiquinone.

The semiquinone radicals were found to be in equilibrium with the hydroquinone and parent quinone. The equilibrium constant, K_4 was 84 ± 12 at pH 9.0 and 8 ± 2 at pH 10.0. Similar equilibria have been observed for several quinones including simple benzoquinones, naphthoquinones and adriamycin.^{17,18,19} For simple quinones, the decrease in the equilibrium constant in going from neutral to alkaline pH is normally due to the involvement of protons in the equilibrium reaction and the deprotonation of the resulting hydroquinone in alkaline pH.¹⁷ Hence, although the equilibrium constant could not be accurately measured at pH 7.4, there is no reason to suppose that an equilibrium does not occur at this pH and indeed it has been reported that when EO9 is reduced by DT-diaphorase, semiquinone e.s.r signals can be detected.²⁶

The semiquinone of EO9 reacts with oxygen (reaction 6) with a rate constant of $1.3 \pm 0.15 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and thus is expected to be removed in well oxygenated cells. Similarly, the hydroquinone of EO9 also reacts relatively rapidly with oxygen to form hydrogen peroxide (reaction 10).

The autoxidation of hydroquinones has been extensively studied^{17,24} and is believed to be mediated by the semiquinone radicals involving reactions of the type:



Interestingly, the one-electron potentials for duroquinone and EO9 are similar¹² and yet the rate of autoxidation of durohydroquinone is several orders of magnitude slower.¹⁷ However, in the case of duroquinone, the equilibrium is strongly in favour of hydroquinone formation¹¹ and hence the amount of available semiquinone that can react with oxygen at any one time, is expected to be much less than that of EO9.

In a previous study, it was shown that DNA strand breaks are formed when DNA is incubated with EO9 and DT-diaphorase in the presence of air.²⁰ The extent of strand breaks was unaffected by the presence of superoxide dismutase and so it was proposed that the strand breaks were as a consequence of DNA alkylation by EO9. Unfortunately, the effects of catalase or metal chelators were not reported in this study. Hence, an alternative explanation could be that the strand breaks were produced simply as a consequence of the formation of hydrogen peroxide. It is significant that a recent study on the formation of EO9-DNA cross links produced by DT-diaphorase under nitrogen reported that strand breaks were not detected.⁴ These simple reactions could, at least in part, explain the enhanced cytotoxicity of EO9 in cells under hypoxia.²³ If it is assumed that the toxicity in air is primarily due to hydrogen peroxide and superoxide anions, then these products are expected to be much less damaging than the formation of DNA cross links and strand breaks formed from the direct interactions of the reduced form(s) of EO9 with DNA.

However, it has recently been shown that although DT-diaphorase contributes to the cyto-

toxicity under aerobic conditions, it appears to protect cells under hypoxia.⁵ The conclusion from this study would be that, under hypoxia, the hydroquinone reactions are not damaging. It was suggested that as a protonated aziridine is necessary for reactions with DNA bases, the pK of the aziridine on the semiquinone may be much higher than that of the hydroquinone and hence at physiologically relevant pH the semiquinones could be more reactive. However, the present studies show (Figure 3) that the pK of the aziridine on the semiquinone must be less than about 5.5. Surprisingly, the pK of the aziridine on EO9 has not been reported but it is expected to be similar to that of other aziridinylquinones which are 2.8 for mitomycin C²¹ and typically between 2.5 and 4.0 for diaziridinylbenzoquinones (Butler *et al.*, unpublished). As the predicted order in the pK's of the aziridines should be quinone < hydroquinone < semiquinone, it is unlikely that the pK's of the semiquinone and the hydroquinone will be sufficiently different to account for the difference in reactivities. However, the semiquinones could be more reactive than the hydroquinones towards DNA alkylation due to other factors such as different hydrogen bonding between the DNA bases (which does, in some instances, favour the formation of crosslinks as opposed to single alkylations),^{22,23} or selective activation of the vinyl group. Unfortunately, the structures of the toxic DNA crosslinks produced from reduced EO9 are not known.

The present results clearly show that the semiquinones and hydroquinones can be in equilibrium although it is extensively in the direction of hydroquinone formation at physiologically relevant pH. Nonetheless, the DNA crosslinks formed by the reduction of relatively high concentrations of EO9 with purified DT-diaphorase⁴ could be explained by a direct reaction of a more reactive semiquinone which is produced from the equilibrium in a similar manner as the hydroquinone apparently reacts with oxygen. It is also significant that in this study, the DNA crosslinking from both the DT-diaphorase and the

xanthine oxidase reductions was found to increase as the pH was changed from 5.5 to 7.0. This is contrary to what would be expected from the activation of the aziridines^{10,22} but it is consistent with the equilibrium being more in favour of semiquinone formation.

The question arises as to whether this equilibrium will be of significance in cellular systems under hypoxic conditions. The concentrations of EO9 necessary to kill cells are typically in the nanomolar range.^{2,3,5} Hence although it may be possible that the semiquinones could, in principle, react together and form hydroquinones is unlikely that the back reaction of the equilibrium will be important at these very low concentrations of drug. Thus, within cellular systems, the reduction of EO9 by DT-diaphorase should lead simply to the formation of the hydroquinone. Within the cell, the hydroquinone may be less reactive than the semiquinone, as discussed above, or the hydroquinone could be detoxified and excreted following the formation of glucuronide-, sulphate- or glutathione-adducts. Consequently, hypoxic cells which have high levels of DT-diaphorase could be protected against damage by EO9⁵ by these mechanisms.

Acknowledgements

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Review

Re-evaluation of the Molecular Pharmacology of Mitomycin C

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INTRODUCTION

Mitomycin C (MMC) is a naturally occurring antibiotic that was isolated in 1958 from the fermentation broth of *Streptomyces caespitosus*. It was shown to exhibit a broad spectrum of anti-tumour activity in preclinical animal screens and less toxicity compared to other mitomycins, and was introduced into clinical trials in Japan where anticancer activity was confirmed in humans [1]. MMC, which remains an important component in combination chemotherapy of breast, lung and prostate cancer, is among the few drugs to possess even marginal activity against colorectal cancer, and is probably the drug of choice for intravesical administration in superficial bladder cancer [2].

A number of recently published studies have extended and challenged many of our longstanding views on the molecular pharmacology of MMC, particularly in the areas of mechanism of action, DNA adduct profiles, enzymology of metabolic activation and drug metabolism. Therefore, it was considered timely to re-evaluate these areas and attempt to present a picture which is both cohesive and consistent.

MECHANISM OF ACTION

In 1964, it was first proposed that MMC required biotransformation, preferentially under anaerobic conditions, before drug activation occurred resulting in crosslinking of DNA [3]. To this day, this mechanism, termed anaerobic bioreductive alkylation [4], is generally accepted as holding true, even though considerably more details are now available on the type of metabolism, the chemical intermediates involved, the types of DNA adducts formed and the sequence specificity of alkylation.

The first stage in activation of MMC is quinone reduction, which can be catalysed by several different enzymes, either through a one electron pathway producing a semiquinone free radical intermediate or a two electron pathway producing a hydroquinone intermediate (Figure 1). In the presence of oxygen, the semiquinone will enter into a redox cycle, which although evolving reactive oxygen species, is generally accepted as not being critical for antitumour activity (pathway 2, Figure 1) [5, 6]. Generation of alkylating moieties by two electron reductases is unimpeded by molecular oxygen. After bioreduc-

tion, the C9a *O*-methyl group leaves the mitosane nucleus as methanol, a C9, 9a double bond forms producing the mitosene nucleus followed by proton assisted aziridine ring opening to generate an electrophilic carbon centre at C1 which alkylates DNA [4]. In this scheme, the key intermediate formed is a quinone methide (QM) [7]. Once the QM bonds to DNA, this promotes the carbamate group to leave yielding a C10 reactive centre which acts as the second point of attachment to produce DNA crosslinks [8, 9]. While MMC possesses no DNA binding activity, the QM is believed to bind non-covalently to DNA with high affinity by non-specific intercalation through a mechanism which is dependent on the ionisation of its 2-amino group and is enhanced under acidic conditions [10]. Reversible non-covalent binding can be viewed as a precursor event to irreversible covalent bonding. The QM reaction scheme is the pathway of MMC metabolic activation which is accepted by the majority of workers but at least three different alternative reaction schemes have been proposed [11-13].

It has been demonstrated recently by purely chemical means that, rather than acting as an electrophile at neutral pH (resulting in DNA bonding) (pathway 3, Figure 1) and a nucleophile at acidic pH as originally hypothesised (pathway 1, Figure 1) [7], the QM is predominantly nucleophilic in character at all functional pHs between 5.5 and 8.5 [14]. This theory does not challenge the concept of the QM, but explains why 2,7-diaminomitosene (2,7-DM, the product of the QM acting as a nucleophile) is the major metabolite of MMC seen *in vivo* (see below). It also suggests that DNA bonding is precluded unless the QM is in close proximity to a nucleophilic centre on DNA, otherwise it will preferentially react with a solvent proton to yield 2,7-DM. The electrophilic metabolites of MMC—1,2 *cis* and 1,2 *trans* 1-hydroxy 2,7-diaminomitosene (*cis* and *trans*-hydro)—are now proposed to be derived from a 7-aminoaziridinomitosene intermediate. This, in turn, is the oxidised form of the MMC quinone-reduced intact aziridino product, leucoaziridinomitosene (LAZM) (Figure 1) [14]. Since 7-aminoaziridinomitosene is only likely to form under more alkaline conditions, this explains why the *cis*- and *trans*-hydro metabolites are also only observed at more alkaline pH [11, 14].

Based on an extensive series of biological studies employing the enzyme DT-diaphorase to activate MMC, two other key

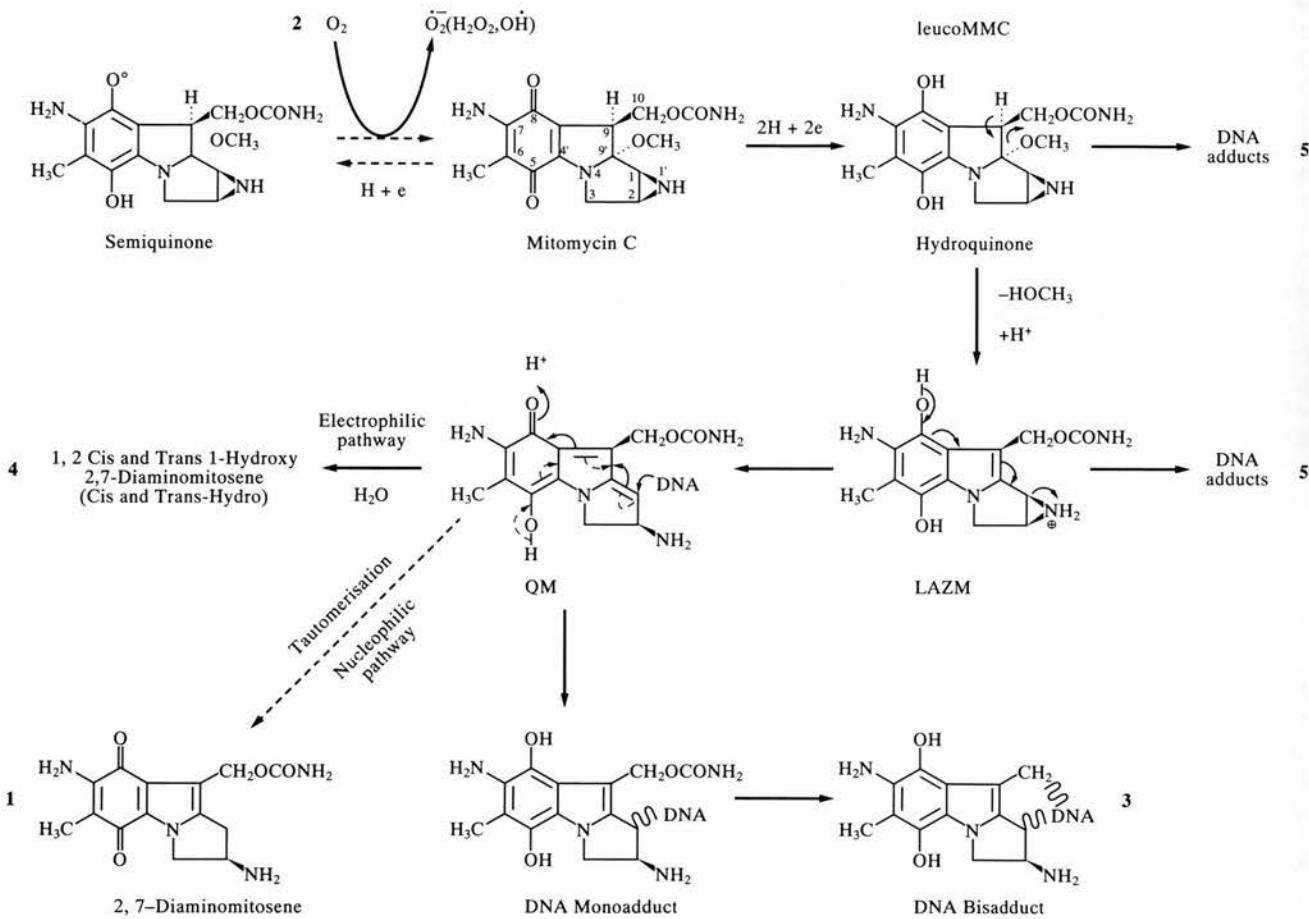


Figure 1. Proposed pathways of metabolic activation of mitomycin C (MMC) after one or two electron quinone reduction. Pathways are explained in more detail throughout the text.

intermediates have been proposed together with the QM as the precursors to DNA covalent bonding (pathway 5, Figure 1) [15]. These are the quinone-reduced intact aziridino products, leucoMMC (fully reduced MMC) and LAZM (fully reduced MMC minus the C9a O-methyl group), and their dependence on proton-assisted aziridine ring opening to yield electrophilic intermediates has been cited to explain the pH dependence of MMC DNA interstrand crosslinks (ISC).

COVALENT MODIFICATION OF DNA

A variety of MMC DNA adducts have been reported. It has been claimed that the major sites of covalent modification are at O-6 guanine followed by N-6 adenine and then N-2 guanine [16]. In a series of elegant studies, Tomasz's group has demonstrated that N-2 guanine is the preferred site of monoalkylation (90% of all DNA bonding) after bioreduction and chemical reduction, and identified the first bifunctional alkylation product as the N-2 guanine/N-2 guanine crosslinked adduct [9, 17]. The second site of attachment on MMC was the C-10 position, as originally hypothesised by Iyer and Szybalski in 1964. Computer models have indicated that this bisadduct fits snugly into the minor groove of DNA with minimal distortion to the structure of B-DNA, and have provided a rationale for the base specificity of MMC alkylation for guanine [17]. Using molecular biology techniques, an absolute requirement for guanine has been confirmed, and a sequence selectivity of ISC for 5'-CpG repeats has been demonstrated [18]. Studies by several independent

laboratories have confirmed N-2 guanine as the principal site of covalent attachment to DNA [19, 20]. As well as ISC, MMC forms a dGpdG intrastrand crosslinked adduct [21]. An orientation model has been proposed to explain the sequence selectivity of MMC crosslinks [22, 23]. The C-1 monofunctional adduct complexed at N-2 guanine (which always forms first) can only point in one direction where the C-10 carbamate group of MMC is facing the 3' direction of the minor groove. Therefore, only when a G is situated 3' of the monofunctional adduct in the complementary strand of DNA can a crosslink possibly form. This case is only satisfied with CG.CG or less frequently with GG.GG.

After metabolic activation with DT-diaphorase, an N-7 guanine adduct appears to account for greater than 90% of all MMC covalently bonded to DNA [24], and this probably reflects the unique manner in which the enzyme metabolises MMC. At pH 7.8, where predominately electrophilic intermediates are evolved, enzyme inhibition ensues as a result of protein alkylation and crosslinking [25]. At more acidic pH (5.8), nucleophilic intermediates are evolved resulting in the efficient formation of 2,7-DM. This metabolite can then act as a substrate for further bioreduction by DT-diaphorase, yielding the N-7 guanine monofunctional adduct complexed to C-10 of MMC. Evidence in favour of this proposal was presented with the finding that, when 2,7-DM was used as a substrate for DT-diaphorase instead of MMC, similar patterns of DNA alkylations to MMC were observed. Thus, DT-diaphorase-mediated DNA

monoalkylation by MMC may have an absolute requirement for 2,7-DM metabolite formation. Other enzymes involved in MMC metabolic activation, such as cytochrome P450 reductase, xanthine oxidase and xanthine dehydrogenase, are not inhibited at physiological pH, although their activity can fall significantly from pH 6.0 to 7.4 [26, 27]. The mechanism of DT-diaphorase inhibition has been speculated to be due to the orientation of MMC in the active site of the enzyme, bringing it in close proximity to specific nucleophilic sites on the protein [15]. *N*-2 guanine alkylation and minor groove 5'-CG ISC can also be detected after DT-diaphorase-mediated metabolism, but these were believed to be derived from the leucoMMC and LAZM intermediate forms of MMC and were only minor products [24].

The chemical structure of all the major *in vivo* DNA adducts formed in EMT6 mouse mammary tumour cells after exposure to MMC has recently been reported [28]. The major species detected under aerobic conditions were the *N*-2 guanine-*N*-2 guanine crosslinked adduct, followed by the decarbamoyl *N*-2 guanine monofunctional adduct, then the dGpdG intrastrand crosslink and finally the *N*-2 guanine monoadduct. These four adducts correspond to the same species that are formed *in vitro* after either chemical reduction or enzyme catalysed metabolism [9, 17, 21, 29]. A fifth dG adduct, codenamed Y, was detected but not identified chemically, and this product may represent the *N*-7 guanine adduct produced by DT-diaphorase. Under hypoxic conditions, levels of crosslinked adducts increased significantly, along with adduct Y. Treatment with the DT-diaphorase inhibitor, dicoumarol (DIC), resulted in elimination of the *N*-2 monofunctional adduct and a significant reduction in Y under hypoxic conditions, but produced large increases in both the inter- and intrastrand crosslinks. These results add weight to the view that the DNA modifications induced by DT-diaphorase are principally monoalkylations, and suggest that bifunctional alkylations are generated predominately under hypoxia by one electron reductases, such as cytochrome P450 reductase, cytochrome b5 reductase and xanthine oxidase, all of which have been shown to be present in this cell line [30–32]. This conclusion, if true, has major significance since it is generally recognised that crosslinks are more lethal to cells than monofunctional adducts [28, 33, 34]. In aerobic conditions, DIC still eliminated the *N*-2 monofunctional adduct, reduced Y but also reduced the number of crosslinks. Interestingly, DIC enhanced the formation of a sixth DNA adduct, codenamed X, particularly under hypoxia, and this has been attributed to the action of xanthine dehydrogenase, which has been previously isolated from EMT6 cells and shown to be stimulated 6–7-fold by this agent [31].

ENZYMOLGY OF METABOLIC ACTIVATION

Several enzymes can catalyse the *in vitro* metabolic activation of MMC, and these include cytochrome P450 reductase [26, 29], xanthine oxidase [26, 29] and cytochrome b5 reductase [30] acting as one electron reductases; and DT-diaphorase [20] and xanthine dehydrogenase [31] acting as two electron reductases. Cytochromes P450 are believed not to transfer electrons directly to MMC, but may facilitate quinone reduction at least 2-fold through drug binding [32]. This view has been recently challenged where cytochromes P450 have been shown to reduce MMC to a semiquinone free radical, which caused toxicity to hepatocytes by alkylating reduced glutathione [35]. The hierarchy of participation from each of these reductases to metabolic activation of MMC *in vivo* has still to be established, but it is emerging that under different physiological conditions

different enzymes may prevail [36]. Cytochrome P450 reductase is believed to predominate in artificially hypoxic cells [37, 38], although there have been reports that reduced expression of this enzyme leads to resistance only in normo-oxic cells [39]. Numerous studies with cancer cell lines support a major role for DT-diaphorase in MMC metabolic activation in normally oxygenated cancer cells but not in hypoxic cells [40–44]. However, a recent study has shown that DT-diaphorase can reduce MMC with equal facility under aerobic and anaerobic conditions in HT-29 human colon cancer cells [45].

A new role has been suggested for DT-diaphorase in the metabolic activation of MMC and its closely related analogue indoloquinone, EO9 [46, 47], namely, that DT-diaphorase protects cells from the hypoxic cytotoxicity of these drugs. According to this hypothesis, cell lines which express high levels of DT-diaphorase are more resistant to MMC than cell lines which express low levels of the enzyme, but only under hypoxic conditions. Under normal oxidative conditions, the converse applied where a direct correlation was observed between DT-diaphorase expression and cytotoxicity, confirming many previous studies which have also shown this to be the case [40–44]. A major cornerstone in the hypothesis was the finding that DIC increased hypoxic cytotoxicity of MMC and EO9 preferentially in cell lines rich in DT-diaphorase (5–10-fold increase versus a 1–3-fold). However, results generated using extremely high concentrations (200 μ M) of DIC should be viewed with great caution since this agent can also inhibit cytochrome b5 reductase [15] and cytochrome P450 reductase [48], stimulate xanthine dehydrogenase [26] and induce the formation of unique DNA covalent adducts [28].

While it is unclear what the biochemical basis is behind these results, similar observations have been made by other groups in different cell lines [28, 32, 45], indicating that this is probably a real phenomenon. Two valid propositions have been put forward to explain their results: (i) that one electron reductases are more effective at metabolising MMC than DT-diaphorase (but that DT-diaphorase prevails under hypoxic conditions); and (ii) that the semiquinone form of MMC (the product of one electron reduction) is more toxic than the hydroquinone form of MMC (the product of DT-diaphorase metabolism) under anaerobic conditions [46]. The second explanation can be discounted more easily since, under hypoxic conditions, due to rapid disproportionation of the semiquinone free radical, the hydroquinone form of MMC is believed to act as a common intermediate after both one and two electron reduction [11, 15]. The first explanation is probably more valid since it can be supported by an emerging body of results which indicate that DT-diaphorase is less effective than one electron reductases in MMC metabolic activation. Firstly, pH dependent alkylation of DT-diaphorase results in enzyme inhibition at physiological pH, but this effect is less pronounced with one electron reductases. Secondly, DT-diaphorase produces predominately monofunctional *N*-7 guanine DNA adducts which are believed to be less cytotoxic than the bifunctional crosslinks formed by one electron reductases [28]. Thirdly, all the major enzymes involved in MMC bioreduction exhibit very low affinity constants (K_m) for the drug, thus, neither one should show a preference for MMC as a substrate. Xanthine oxidase and cytochrome P450 reductase have a K_m of 2 mM [26, 49], xanthine dehydrogenase a K_m of 299 μ M [27] whilst DT-diaphorase also has a low K_m [50]. Therefore, the enzyme which is present in largest amount is likely to carry out the majority of MMC bioreduction regardless of intrinsic catalytic activity, and the pattern of metabolic

activation produced will parallel the biochemical properties of that particular enzyme.

Recent results from our laboratory lend support to the above conclusion [51]. When MMC was incubated with whole homogenates of the mouse colon adenocarcinoma MAC 16, which is rich in DT-diaphorase, the pattern of metabolism recorded was characteristic of DT-diaphorase, that is no increase in activity under hypoxic conditions and inhibition by DIC at a lower concentration (10 μ M). However, when the tumour was subjected to subcellular fractionation, three fractions were isolated with the ability to convert MMC into 2,7-DM (mitochondria, microsomes and cytoplasm) and the fraction containing DT-diaphorase exhibited the lowest intrinsic activity. In contrast, the MAC 26 tumour, which is low in DT-diaphorase, but contained the same highly active mitochondrial enzyme, produced a pattern of metabolism consistent with one electron reduction: stimulation of metabolism by hypoxia and higher levels of activity under hypoxia compared to MAC 16 [51].

Many human tumours, such as liver, colon, breast and non small cell lung cancer, are rich in DT-diaphorase, making this enzyme a good candidate for the enzyme-directed approach to cancer chemotherapy [52–54]. However, recent data with DT-diaphorase and MMC suggest caution when applying that approach to this drug and related compounds, such as EO9. In tumours that are high in DT-diaphorase, induction of hypoxia will only increase antitumour activity by a small margin, if at all, but this should be from a level which is much higher than in tumours low in DT-diaphorase. Tumours that are low in DT-diaphorase may be less responsive, but could become substantially more sensitive by the induction of hypoxia, if such modulation was achievable *in vivo*. Thus, on balance, tumours high in DT-diaphorase would appear to be optimal for treatment with MMC. The one caveat to this conclusion is that it is based on data generated *in vitro* with cell lines, and these studies do not take into account the obvious biological and physiological differences that exist in solid tumours and their cellular heterogeneity. With this in mind, it has been demonstrated in a panel of four human xenografts that an inverse relationship exists *in vivo* between DT-diaphorase expression and antitumour activity, and that DIC increased antitumour activity to a greater extent in the tumours expressing the highest levels of DT-diaphorase [53]. These data parallel more closely the *in vitro* scenario of hypoxic cancer cells treated with MMC [46, 47].

DRUG METABOLISM

Despite overwhelming evidence for the major role of drug metabolism in the mechanism of action of MMC and in its clearance from the body, no previously published preclinical (or clinical) pharmacokinetic study has reported detection of MMC metabolites in either plasma or tissues [55–58]. Recently, analytical techniques have been published which can detect *in vivo* MMC and its three primary bioreductive metabolites 2,7-DM, *cis*- and *trans*-hydro in rodent transplantable tumour tissue (the Sp 107 rat mammary carcinoma and MAC 16 and 26 murine colon cancer) [59]. Five MMC metabolites were detected in Sp 107 10 min after drug administration, including the three primary metabolites. These techniques have been applied to characterise further MMC metabolism *in vivo* in tumour tissue [36, 60]. In an initial study, metabolism was investigated in the MAC 16 tumour which is rich in DT-diaphorase. One single metabolite was detected, 2,7-DM, which was generated in a burst of activity that extended over 30 min after drug instillation [36]. The rapid clearance of 2,7-DM may reflect the fact that

this metabolite is only an intermediate in a complex chain of metabolic events [8, 11]. *In vitro*, 2,7-DM only has a half life of 13 min when incubated with either cytochrome P450 reductase or xanthine oxidase [26]. Induction of hypoxia by administration of albumin microspheres resulted in a reduction in 2,7-DM formation, confirming that DT-diaphorase protects MMC from metabolic activation under anaerobic conditions [36]. In a separate study, 2,7-DM was shown to have a half life of 30 min after intratumoural (i.t.) injection of 1 mg MMC in the Sp 107 tumour and 5 min after i.t. injection of 100 μ g. In that study, decarbamoyl 2,7-DM (DC 2,7-DM, a secondary metabolite) was also detected [60]. This metabolite may reflect bifunctional metabolic activation of MMC at C1 and C10, whereas 2,7-DM may reflect monofunctional metabolic activation [16, 17]. Levels of DC 2,7-DM were 5–10-fold lower than 2,7-DM. The formation of DC 2,7-DM was also much slower than 2,7-DM and its half life was 130 min. These *in vivo* data parallel closely the kinetics of DNA covalent bonding after treatment of cells with MMC, or incubation of the drug with purified cytochrome P450 reductase and xanthine oxidase in a cell free system [49, 61]. DNA bonding occurred in a rapid burst of activity during the first 6 min of drug incubation: by 20 min the levels of monofunctional DNA adducts appeared to plateau and thereafter there was a steady rise in the formation of crosslinks for a further 70 min [49]. The rapid appearance and disappearance of the major metabolite (2,7-DM) may explain why all previous preclinical pharmacokinetic studies have failed to detect this product in either plasma or tissues.

In those *in vivo* studies, tumour levels of 2,7-DM correlated better than MMC to antitumour activity [36]. *In vitro* studies have also identified a correlation between 2,7-DM levels and cytotoxicity in human colon cancer cell lines [40]. Together, these data may shed light on the nature of the ultimate cytotoxic species after MMC treatment in view of the fact that generation of 2,7-DM appears to be an obligatory step in *N*-7 monoalkylation of guanine [24].

CONCLUSIONS

The molecular pharmacology of MMC remains a very complex subject. Over the past five years, a number of seminal papers have expanded our understanding, clarified certain areas of controversy and at the same time challenged some of our longstanding opinions. The most significant development has been in defining the role of the obligate two electron reductase DT-diaphorase. While it was originally believed by some that this enzyme did not accept MMC as a substrate, it has now emerged that it occupies a unique and central role in the molecular pharmacology of the drug. Since this enzyme is expressed at very high levels in a number of human tumours, this role clearly has clinical significance. If DT-diaphorase is present at high levels, it will act as the main MMC metabolising enzyme, resulting in metabolic activation both under aerobic and anaerobic conditions. However, because the enzyme is not as efficient as one electron reductases in activating the drug, the enzyme effectively protects cells from the maximum degree of drug-induced damage possible under hypoxic conditions. When DT-diaphorase is present at lower levels, it may still predominate in aerobic cells because one electron reductases are effectively inhibited by oxygen. Under anaerobic conditions, one electron reductases will participate significantly and the drug may have greatly increased activity. Ultimately, the pattern of MMC metabolic activation, and hence its antitumour activity, will

depend on the balance of enzyme activities present in a tumour and on its oxidative state.

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