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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 pathway 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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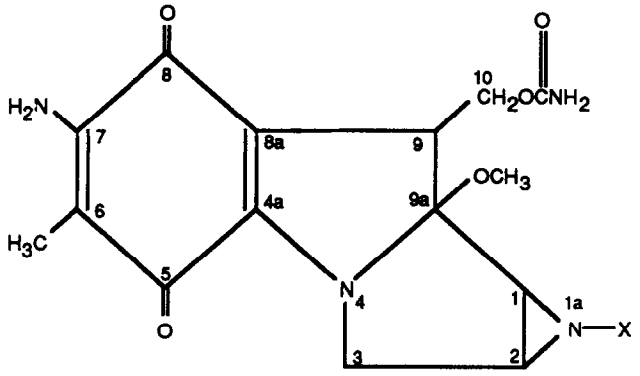

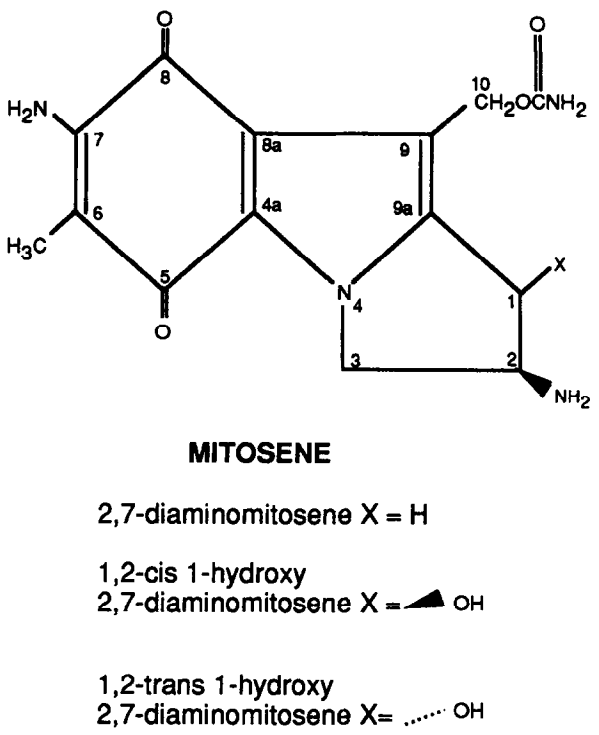

FIG. 1. Molecular structure of mitomycin C and its three principle mitosene metabolites.

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 N^2 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

	Cytosol			Microsomes			
	DT-diaphorase			DT-diaphorase		Cytochrome P450 reductase	Cytochrome b_5 reductase
Tumour	NADH	NADPH	'Others'	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 mobile 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 trochar 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
	1.04 ± 0.17	NADPH			
Microsomes	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			
Homogenate	0.14 ± 0.07		No effect		

The MAC 16 tumour was fractionated and all incubations were performed as described in Materials and Methods. Specific activity is expressed as 2,7-diaminomitosene 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 μM dicoumarol	Effect of antiserum
Cytosol	ND*	–	–	–	–
Microsomes	ND	–	–	–	–
Mitochondria	3.87 ± 0.71	NADPH	100% inhibition	No effect	No effect
Homogenate	1.45 ± 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-diaminomitosene formation in nmol/30 min/mg protein. * ND, no activity detected. Each value represents the mean ± 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 μg cytosolic or 17–33 μg microsomal protein, cytochrome *c* (77 μM), menadione (20 μM), 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 μM). DT-diaphorase activity was taken as the activity that could be inhibited by dicoumarol in the presence of NADPH or NADPH. All enzyme activities were calculated as nmol cytochrome *c* reduced/min/mg protein (*e* 21.1 × 10^3 M/cm).

Drug Incubations

In vitro drug incubations were performed at 37°C under aerobic and hypoxic conditions. Each incubation mixture contained 100 μg/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 incubations contained 100 μg/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 μM 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 μL aliquot was withdrawn from the incubation and 20 μL 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-diaminomitosene 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

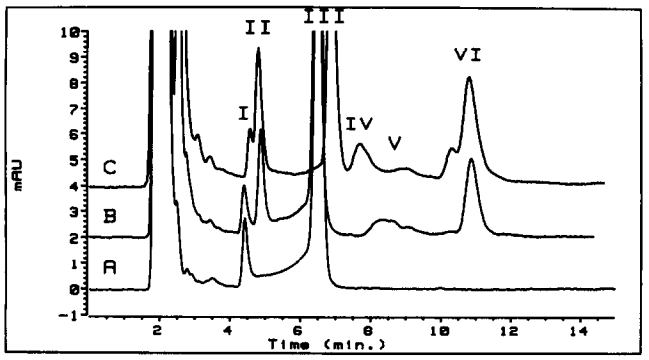

FIG. 2. *In vitro* pattern of mitomycin C metabolism in MAC 16 (C) and MAC 26 (B) mitochondrial fractions at 0 (A) and 60 (B, C) min as measured by HPLC. Incubations were performed using NADPH as cofactor under hypoxic conditions. Peaks: I, zwitterion form of mitomycin C; II, 1,2-trans-1-hydroxy-2,7-diaminomitosene; 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 μM 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 μM 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

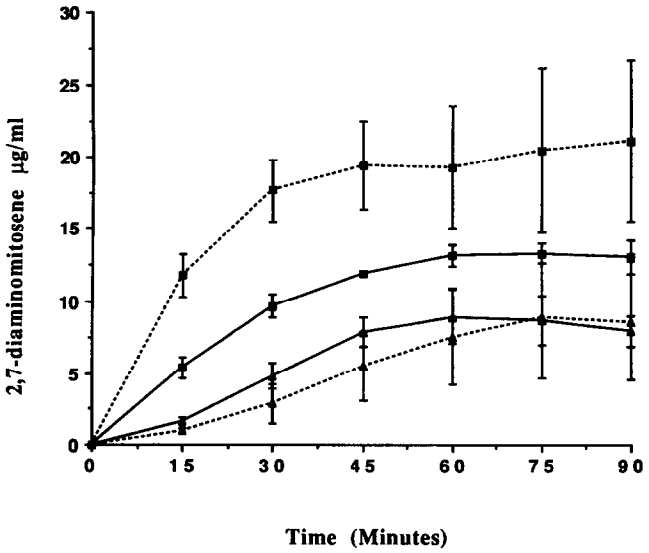

FIG. 3. *In vitro* production of the principle mitomycin C metabolite 2,7-diaminomitosene in MAC 16 (▲) and MAC 26 (■) tumour homogenates (33% w/v) under aerobic (—) and anaerobic (- - - -) conditions. Incubations were performed as described in Materials and Methods. Each point represents the mean ± 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-deoxyaglycone 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 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 [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 b_5/cytochrome b_5 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 predomination 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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