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ENCAPSULATION OF MITOMYCIN C IN ALBUMIN MICROSPHERES MARKEDLY ALTERS PHARMACOKINETICS, DRUG QUINONE REDUCTION IN TUMOUR TISSUE AND ANTITUMOUR ACTIVITY

IMPLICATIONS FOR THE DRUGS' *IN VIVO* MECHANISM OF ACTION

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Abstract—Pharmacokinetics and metabolism of mitomycin C (MMC) have been studied in NMRI mice bearing MAC 16 colon adenocarcinoma after direct intratumoural injection of either 500 μg free MMC or the same dose incorporated in albumin microspheres. Microspheres produced a tumour pharmacokinetic profile of steady state drug levels, avoiding the much higher early peak (20.5 μg/tumour vs 98.9 μg/tumour) and lower trough of free MMC, and reducing significantly the levels of drug reaching the systemic circulation (AUC 1.8 μg/mL × hr for microspheres vs 6.8 μg/mL × hr for free drug). 2,7-Diaminomitosene (2,7-DM), a key intermediate in MMC quinone bioreduction, was used as an indicator of drug metabolic activation in tumour tissue. Peak levels were 10-fold higher (11.2 μg/tumour vs 1.1 μg/tumour) and area under the curve 5-fold higher after free drug. Even taking into account differences in tumour pharmacokinetic profiles of the parent drug, microspheres actively inhibited 2,7-DM formation 3-fold. However, the microspheres generated a completely different pattern of drug metabolism where four previously uncharacterized mitosane metabolites and elevated levels of *cis* and *trans* 1-hydroxy 2,7-diaminomitosene were detected. Despite similar parent drug exposure in tumours, free drug was significantly more active ($P < 0.05$, Student's *t*-test) against MAC 16. These results suggest that formation of 2,7-DM correlates more closely with antitumour activity than sustained parent drug levels or appearance of other key metabolites. Potentially, they provide the first direct evidence for an *in vivo* mechanism of action dependent on bioreductive activation and formation of 2,7-DM.

Key words: drug delivery; HPLC; 2,7-diaminomitosene; mitosane metabolites; hypoxia; murine adenocarcinoma

MMC§ (Fig. 1) remains an important component in combination chemotherapy of breast, colorectal and prostate cancer and is probably the drug of first choice for intravesical administration in superficial bladder cancer [1]. Its clinical utility, however, is limited by severe bone marrow suppression and gastrointestinal damage [1]. Due to these dual properties of potent antitumour activity and high systemic toxicity, MMC has proved an ideal candidate drug for locoregional chemotherapy [2]. MMC has been subjected to a variety of drug delivery approaches including: microencapsulation using ethyl cellulose [3, 4] and polylactic acid [5]; incorporation in microspheres prepared from either human serum albumin (HSA) [6, 7] or poly(lactide-glycolide) co-polymers [8]; and covalent conjugation to biomacromolecules such as dextrans [9], albumin [10], asialofetuin [11] and monoclonal antibodies [12]. Unfortunately, these systems have met with limited therapeutic success when applied to the clinical context [13] and are often associated with decreased drug potency [14]. This may, in part, be due to the methods used in the preparation of these DDS which can actually significantly degrade the drug [10, 15]. In addition, very little is known about the effect (beneficial or otherwise) of incorporation in a DDS on the biochemical pharmacology of MMC *in vivo* [16].

Albumin microspheres have been shown to actively alter both drug disposition and the therapeutic properties of encapsulated doxorubicin (DOX) once they have reached the tumour [17–20]. As well as favourably altering tumour pharmacokinetics and increasing antitumour activity 3–5-fold, these microspheres (10–40 μm diameter) have the unique ability to stimulate drug AQR to 7-deoxyaglycone metabolites by a factor of up to 155-fold in tumour

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§ Abbreviations: MMC, mitomycin C; DDS, drug delivery systems; AQR, anaerobic quinone reduction; DOX, doxorubicin; *cis*-hydro, 1,2-*cis* 1-hydroxy 2,7-diaminomitosene; *trans*-hydro, 1,2-*trans* 1-hydroxy 2,7-diaminomitosene; 2,7-DM, 2,7-diaminomitosene; HSA, human serum albumin; PBS/Tween, phosphate-buffered saline and 0.5% poly(oxyethylene)sorbitan; AUC, area under the curve; R_d, regional drug delivery advantage; ROS, reactive oxygen species; and HCR, hypoxic cell kill ratio.

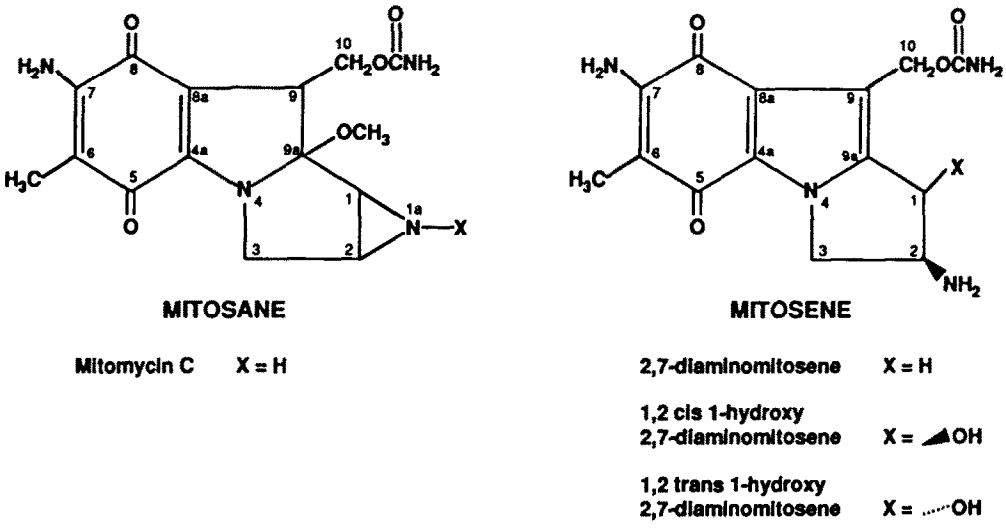

Fig. 1. Molecular structures of MMC and its three primary metabolites.

tissue [18]. This is achieved through a mechanism believed to involve the induction of hypoxia after a 16–24 hr latent period directing NADPH cytochrome P450 reductase to catalyse AQR [21]. In the case of DOX, it has now been demonstrated that AQR is a pathway which results in drug inactivation and that increased antitumour activity is due to the steady state drug levels produced in the tumour by the microspheres [20, 22]. Thus, the full potential of these microspheres to enhance antitumour activity is not realised with DOX due to the fact that a large fraction of active drug is lost through futile drug metabolism.

On the other hand, MMC is proposed to work through quinone reduction, operating preferentially under anaerobic conditions, resulting in bifunctional alkylation of DNA [23, 24]. Also, cytochrome P450 reductase is one of the main, if not the major enzyme, catalysing MMC metabolic activation [25]. Therefore, it was believed to be a rational decision to incorporate MMC in albumin microspheres and we have recently reported a new formulation which does not result in drug degradation during preparation [26]. In this work, the influence of microencapsulation on MMC antitumour activity, tumour pharmacokinetics and quinone reduction in tumour tissue is reported.

MATERIALS AND METHODS

Preparation of microspheres. The new method used in this work is essentially a modification of the original report on the preparation of DOX loaded albumin microspheres and is based on glutaraldehyde cross linking of protein during emulsification at room temperature [17, 26]. MMC (5 mg) with 125 mg sodium chloride excipient (Kyowa Hakko Kogyo Co., Tokyo, Japan) and 200 mg of HSA (Fraction V, Sigma Chemical Co. Ltd, Fancy Road, Poole, U.K.) were mixed in 400 μL of 5 mM sodium phosphate buffer pH 7.4 containing 0.1% sodium dodecyl sulphate (BDH, Merck Ltd, Merck House, Poole, U.K.). Distilled water (500 μL) was then added to make up the disperse phase. The continuous phase consisted of 60 mL cotton seed oil and 0.5 mL sorbitan monoleate (Span 80) (both Sigma). After addition of the disperse phase to the continuous phase, 100 μL of a 22% glutaraldehyde solution (Sigma, final concentration 2.2%, v/v) was immediately added. The emulsification process was carried out at 1250 rpm using a Silverson mixer emulsifier (Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, U.K.) for 1 hr at room temperature. The resulting microspheres were washed three times in petroleum ether, once in isopropanol (HPLC grade, Rathburn Chemicals, Walkerburn, U.K.) after which they were filtered. They were then stored dry at 4° and resuspended in PBS and 0.5% poly(oxyethylene)sorbitan (PBS/Tween, BDH) prior to use. A typical yield of microspheres by this procedure was 67% (dry weight); mean particle diameter was 16.9 μm (50% weight average) and mean content of MMC was 1.2% (w/w).

Drug analysis techniques. High performance liquid chromatography. All chromatographic analyses were carried out using a Hewlett–Packard Model 1090 liquid chromatograph equipped with a diode array detector. Standards of the major hydrolysis products of MMC, 1,2-*cis* 1-hydroxy 2,7-diaminomitosene (*cis*-hydro) and 1,2-*trans* 1-hydroxy 2,7-diaminomitosene (*trans*-hydro), were synthesised in house by treatment of MMC with 0.1 M hydrochloric acid for 25 min at room temperature. Authentic standards of the major product of MMC monofunctional drug activation, 2,7-diaminomitosene (2,7-DM, Fig. 1) and bifunctional drug activation, 10-decarbamoyl-2,7-diaminomitosene, were a kind gift from Professor Maria Tomasz, Department of Chemistry, Hunter College, New York, U.S.A. 10-Decarbamoyl-MMC was also a kind gift from Professor Tomasz.

Chromatographic conditions were adapted from those previously described to take into account the

greater number of metabolite standards [27]. The stationary phase was LiChrosorb RP-18 (7 μm particle size; column dimensions, 25 cm × 4 mm i.d.) (supplied by Crawford Scientific, 66A Kirk Street, Strathaven, U.K.). The mobile phase consisted of 10 mM sodium phosphate buffer pH 7.5 and methanol, 74:26 (HPLC grade, Rathburn Chemicals Ltd, Walkerburn, U.K.). Elution was isocratic at a flow rate of 1 mL/min and the column was maintained at 40°. Mobile phase components were filtered before use (0.2 μm filter, Waters-Millipore, Northwich, U.K.) and were continuously sparged with helium during chromatography.

Extraction of mitomycin C and its metabolites from tumour tissue. Tumours for drug analysis, which were stored at −40°, were only partially thawed in ice prior to homogenisation of the whole tumour in 154 mM potassium chloride (1:2 w/v). A portion of homogenate (1 mL) was then extracted twice with 5 mL chloroform–propan-2-ol–ethyl acetate (2:2:1) as described in detail previously [27]. The extracts were evaporated to dryness under a stream of nitrogen gas. Residues were reconstituted in 300 μL of HPLC mobile phase and 100 μL were then injected into the liquid chromatograph. Quantitation was by the external standard method where known quantities of MMC and its metabolites were added to control tumour homogenates and individual recoveries determined. This procedure was necessary since MMC and its metabolites exhibit different extraction efficiencies [27]. Mitosanes were quantitated at 360 nm and mitosenes at 310 nm.

Animal model and in vivo *drug treatments.* The animal model consisted of inbred mice of the NMRI strain and the subcutaneously growing MAC 16 murine adenocarcinoma tumour (breeding pairs and tumour very kindly supplied by Drs JA Double and MC Bibby, Clinical Oncology Unit, University of Bradford, U.K.). Animals were kept under standard laboratory conditions and fed on standard laboratory chow. The tumour was maintained by subcutaneous passage of 1–3 mg lumps of viable tissue via a trochar needle to male animals weighing between 25 and 30 g. When tumours had reached a size 0.15–0.3 g, animals were randomized in groups of five to 10 for experimental studies. Microspheres were injected intratumourally in a variable volume of between 0.25 and 0.5 mL PBS/Tween, in order that a dose of 500 μg was administered to each animal (actual dose, 468 μg ± 44 μg SE). The large injection volume was dictated by the relatively low drug loading achieved (1.2% w/w) and, thus, relatively large quantity of particles (approximately 50 mg) being made up in suspension. Free MMC was administered at the same dose level in the same volume of PBS. In antitumour experiments, tumour volumes were determined by measuring two diameters at right angles and calculating tumour weight from the following formula: 1/2 × shorter diameter × longer diameter. No treatment, PBS/Tween on its own and blank (non drug containing) microspheres served as controls. In pharmacokinetic experiments, animals were killed at the following times after drug administration: 5, 15, 30, 45, 60 min, 2, 4, 6 and 24 hr. Tumours were removed, washed, and then immediately placed in liquid nitrogen. Blood was also collected, from which plasma was harvested. All samples were stored at −40° prior to drug analysis.

Data analysis. Peak levels and time to peak levels of MMC and 2,7-DM were taken directly from concentration/time profiles. Area under concentration/time profiles (AUC) was determined by the trapezoidal rule. Clearance was calculated from the formula dose/AUC. Regional drug delivery advantage (R_d) was calculated from the following formula:

$$R_d = \frac{(AUC_{tumour}/AUC_{plasma})\ \text{microspheres}}{(AUC_{tumour}/AUC_{plasma})\ \text{free drug}}.$$

Antitumour activity for a treatment group was expressed in tumour weight as a percentage of the control group (T/C) on the final day of each experiment (normally day 11).

RESULTS

Effect of encapsulation of mitomycin C in protein microspheres on antitumour activity against MAC 16 *murine colon adenocarcinoma*

MAC 16 murine adenocarcinoma was chosen in this present study for its relative chemosensitivity to the closely related bioreductive alkylating agent, indoloquinone EO9 [28]. Chemosensitivity to MMC is unknown. In an initial study three different doses of MMC (250, 500 and 1000 μg) were administered intratumourally and a linear dose–response curve was established ($r = 0.992$). The intermediate dose of 500 μg was considered the most appropriate for studies with microspheres. Figure 2 shows the comparative antitumour activity (determined in separate studies from above) of 500 μg of free MMC versus the same dose incorporated in albumin microspheres together with various controls. In this study, free MMC was highly active recording a T/C of 8.3% ($P < 0.001$, Student's t-test), however, 11 days after commencement of treatment lethality occurred in 3/8 animals. In contrast, the microsphere formulation was significantly less active ($P < 0.05$) than free drug achieving a T/C of only 38.3% ($P < 0.05$ compared to no treatment control group) but no toxic deaths occurred. In fact, drug loaded microspheres were no more active (Student's t-test) than blank microspheres administered at the same dose (T/C 52.4%, $P < 0.05$ compared to no treatment control group). PBS/Tween injection vehicle was not significantly different (Student's t-test) from no treatment where 1/7 lethalites occurred by day 11. Antitumour studies were thus terminated after 11–12 days. In brief, microencapsulation has resulted in a large reduction in drug potency, despite the fact that the microsphere formulation was introduced directly into the tumour.

Effect of microencapsulation in albumin microspheres on the pharmacokinetics of MMC in MAC 16 *murine colon adenocarcinoma*

MMC concentration–time curves after intratumoural administration of 500 μg of free drug or the same dose incorporated in albumin microspheres are shown in Fig. 3 for the MAC 16 tumour and in

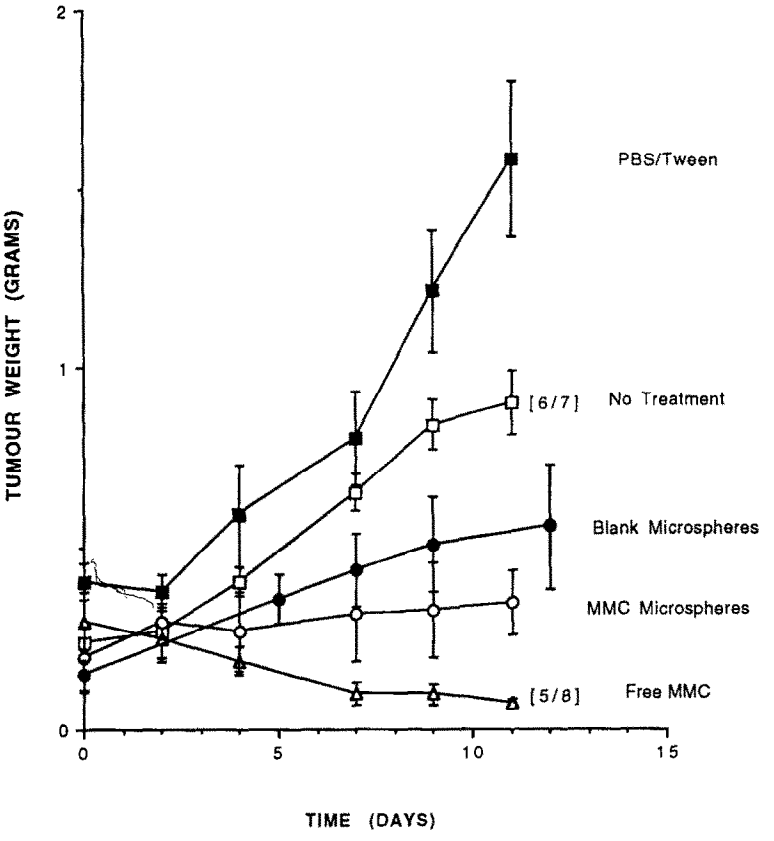

Fig. 2. Antitumour activity of free MMC (500 μg) versus the same dose of drug encapsulated in albumin microspheres (468 ± 44 μg). Treatments were administered in a volume of 0.25–0.5 mL PBS/Tween by direct intratumoural injection to MAC 16 murine adenocarcinoma. Control groups included: no treatment, 0.25–0.5 mL PBS/tween, and 0.25–0.5 mL of blank (non drug containing) microspheres. Each time point represents the mean ± SE of seven to 10 separate animals. For full details of experimental procedures see Materials and Methods and for levels of statistical significance see Results.

Fig. 4 for plasma. Pharmacokinetic parameters derived from these profiles are contained in Table 1. Although both treatments achieved a similar level of total drug exposure in the MAC 16 tumour (as determined by AUC: 46.9 μg/mL × hr for free drug, 30.0 μg/mL × hr for microspheres, Table 1), the microspheres generated a markedly different pharmacokinetic profile (Fig. 3). This was characterized by steady state drug levels, avoiding the much higher peak and lower trough of free drug. In this respect, the microspheres produced a similar pattern of pharmacokinetic modulation to that previously reported with DOX, though comparative steady state levels were much lower for MMC [18]. These data are also characteristic of controlled release of MMC from microparticles, which we have previously demonstrated *in vitro* is sustained over 20 hr with an initial burst effect (60% of total drug release) occurring during the first 3 hr [26]. Also consistent with slow drug release is the observation of reduced plasma drug levels after administration of microspheres (Fig. 4, Table 1). Overall, the microspheres achieved a regional drug delivery advantage (R_d, which is related to tumour drug exposure versus systemic drug exposure) 2.4 times better than free drug. However, this value compares poorly with an R_d of 109 previously demonstrated with DOX [29].

Effect of microencapsulation in albumin microspheres on drug metabolic activation of MMC in MAC 16 murine colon adenocarcinoma

2,7-DM was used as the principal indicator of metabolic activation in tumour tissue since this metabolite can only be formed after quinone reduction [30] and is not detected in plasma and, therefore, cannot be carried to the tumour from distal sites of production [31]. In addition, levels of 2,7-DM have been demonstrated to correlate with cytotoxicity in human colon cancer cell lines [32] and in a recent report is has even been shown that production of 2,7-DM is a prerequisite for N-7 alkylation of deoxyguanosines located in the major groove of DNA [33]. 2,7-DM concentration–time curves in MAC 16 after injection of free drug or microspheres are illustrated in Fig. 5. Neither 2,7-DM nor any other metabolite of MMC was identified in plasma, as expected. A high level of formation of 2,7-DM occurred after administration of free drug (AUC of 2,7-DM was 12.1% that of MMC).

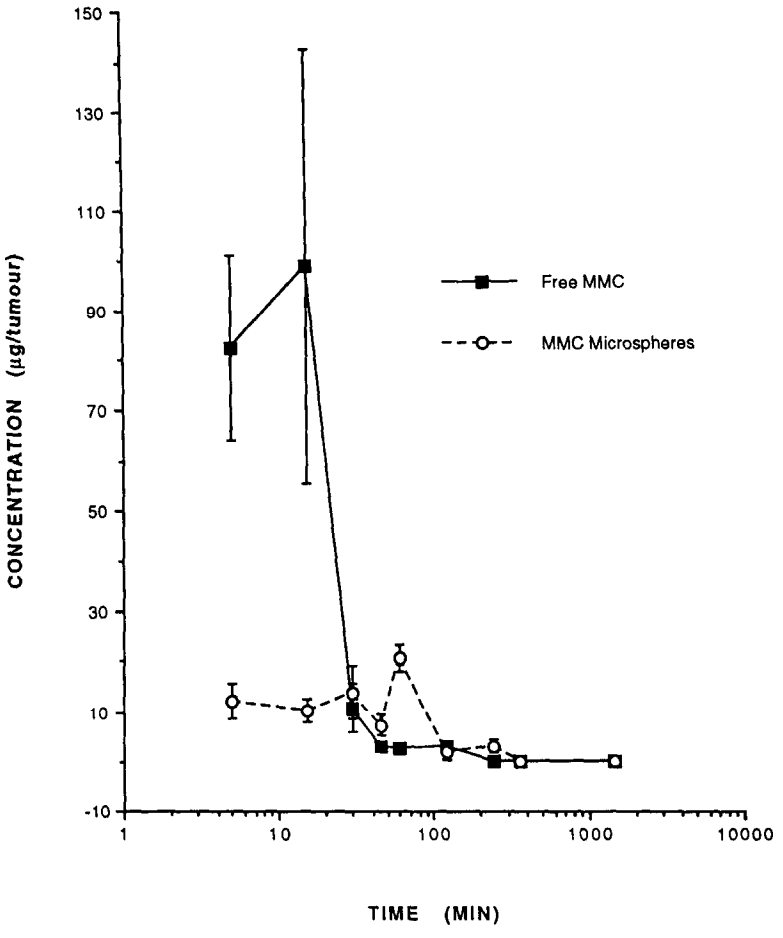

Fig. 3. Concentration–time profiles of MMC in the MAC 16 tumour after direct intratumoural injection of either 500 μg free drug or the same dose encapsulated in albumin microspheres (468 ± 44 μg). Results are expressed as the total tumour drug content in μg and represent the mean ± SE of five separate animals per time point.

Since the kinetics of MMC metabolite formation *in vivo* have never been reported previously, these data are clearly of significance [34–37]. Generation of 2,7-DM proceeded very rapidly in a burst of activity extending over the first 30 min after drug instillation and following closely the concentration–time profile of MMC (Fig. 5). Rapid decline in metabolite levels may reflect the fact that 2,7-DM is itself only an intermediate in a complex chain of metabolic events [40]. 2,7-DM was the *only* major metabolite detected after free drug administration (Fig. 6 chromatogram 1 and Fig. 7 chromatogram 2). In contrast, only trace levels of 2,7-DM were measured after injection of microspheres (Fig. 5, Table 1) but here again its production followed closely the pharmacokinetic behaviour of the parent drug. Taking into account the different levels of the parent drug, disproportionately less 2,7-DM was formed after the microspheres (3.6% of the AUC of MMC), indicating active inhibition of this pathway of metabolism. However, a completely different spectrum of metabolites emerged in preference to 2,7-DM, including elevated levels of *cis* and *trans*-hydro and at least four major mitosane products (Fig. 7; for UV-visible spectra of these peaks see Fig. 8). Assuming similar analytical behaviour to MMC (molar extinction coefficients and extraction efficiency), the mitosane peaks could account, together, for up to 50% of the concentration of parent drug, especially at earlier time points studied (5, 15 and 30 min).

DISCUSSION

Modulation of an anticancer drug's pharmacokinetic profile and pathways of metabolic activation in tumour tissue (by protein microspheres) can provide insights into the relative contribution of each of these processes to *in vivo* mechanism of action [20]. The data presented in this work show that while total exposure of the tumour with parent drug is not significantly compromised (although it is significantly altered) by inclusion in albumin microspheres, the large inhibition of quinone reduction to 2,7-DM has correlated with a significant loss in MMC activity. Thus, these results possibly

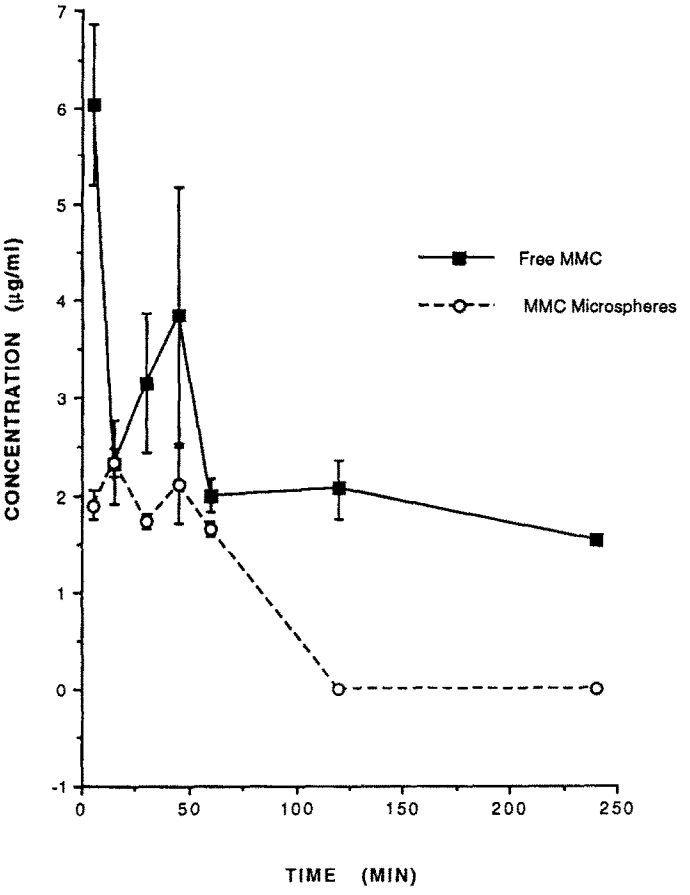

Fig. 4. Concentration–time profiles of MMC in plasma after direct intratumoural injection of either free drug or microspheres. Results are expressed in μg/mL and represent the means ± SE of five separate animals per time point.

Table 1. Pharmacokinetics, metabolic conversion to 2,7-diaminomitosene and antitumour activity of mitomycin C after direct intratumoural injection of 500 μg administered to MAC 16 murine colon adenocarcinoma either as free drug or incorporated in albumin microspheres

	Free mitomycin C		MMC/microspheres	
	Parent drug	2,7-DM	Parent drug	2,7-DM
Plasma				
Peak level (μg/mL or g)	6.03	ND	2.34	ND
Time to peak (min)	5.00		15.0	
$AUC_{0\text{-}24hr}$ (μg/mL or g × hr)	6.83		1.81	
Clearance (mL/hr)	73.2		233	
Tumour				
Peak level (μg/mL or g)	98.4	11.2	20.5	1.08
Time to peak (min)	15.0	15.0	60.0	60.0
$AUC_{0\text{-}24hr}$ (μg/mL or g × hr)	46.9	5.69	30.0	1.09
Regional drug delivery advantage (Therapeutic advantage, R_d)			×2.4	
Antitumour activity (% T/C)	8.3%		38.8%	

Pharmacokinetic terms and antitumour activity are defined in Materials and Methods.
ND, not detected.

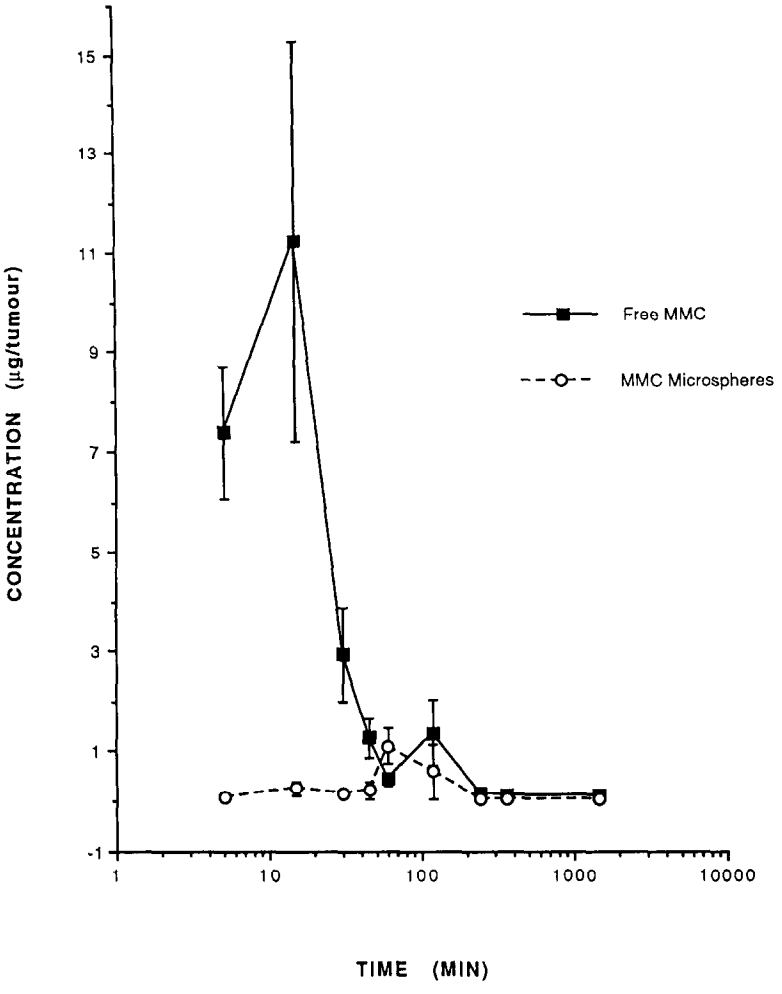

Fig. 5. Concentration–time profiles of 2,7-diaminomitosene in the MAC 16 tumour after free drug or microspheres. Results are expressed as the total tumour metabolite content in μg and represent the mean ± SE of five separate animals per time point.

provide the first ever direct *in vivo* confirmation, to back up the numerous *in vitro* studies which also support this conclusion [23, 24, 38–40], of a mechanism of action dependent on metabolic activation through quinone reduction. From this conclusion follows an interesting corollary: if levels of intact drug do not correlate with antitumour potency, then pharmacokinetic monitoring of the parent drug, especially in plasma, is unlikely to predict for patient response. Indeed, several clinical pharmacokinetic studies have shown this to be the case [34–37]. In addition, since stable active metabolites of MMC do not normally appear in plasma [31], the only reliable indicator of MMC activity would appear to be the concentration of metabolites in the target tissue itself.

Based on *in vitro* studies, several enzymes have been demonstrated to catalyse metabolic activation of MMC and these include: cytochrome P450 reductase [40, 41]; xanthine oxidase [40, 41] and cytochrome b5 reductase [42] acting as one electron reductases; and DT-diaphorase [32, 43] and xanthine dehydrogenase [44, 45] acting as two electron reductases. Cytochromes P450 do not directly transfer electrons to MMC but they may facilitate quinone reduction at least 2-fold through drug binding [46, 47]. The hierarchy of participation from each of these reductases to metabolic activation of MMC *in vivo* has still to be established [48], but it is emerging that under different physiologic conditions different enzymes may predominate. Cytochrome P450 reductase is believed to predominate in artificially hypoxic cells [46, 49, 50]. One electron reduction of MMC in the presence of molecular oxygen yields a drug semi-quinone free radical which will preferentially redox cycle to generate a series of ROS [51]. In comparison to DNA alkylation ROS production appears to be a relatively non-toxic event [43, 52, 53]. Generation of alkylating moieties by two electron reductases is unimpeded by molecular oxygen and numerous studies with cancer cell lines support a major role for DT-diaphorase in normally oxygenated cancer cells [32, 54–58]. However, these specialised roles in MMC metabolic activation (and a minor role for ROS) have been challenged for both

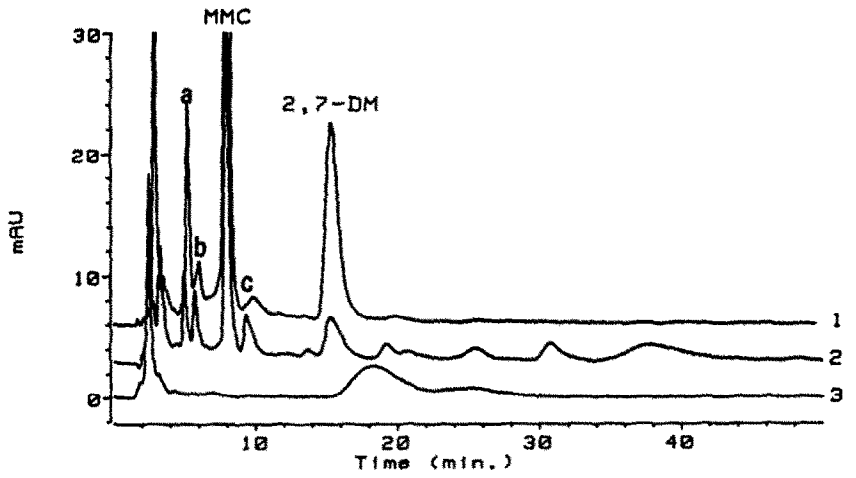

Fig. 6. Profile of metabolites produced in MAC 16 murine adenocarcinoma after direct injection of either 500 μg free drug or the same dose encapsulated in albumin microspheres (468 ± 44 μg). HPLC chromatograms were monitored at 310 nm (after extraction of tumour homogenates). Chromatogram 1 is a tumour collected 5 min after free drug administration and chromatographic peaks are identified as follows: MMC, mitomycin C (75.0 μg total tumour content); 2,7-DM, 2,7-diaminomitosene (6.5 μg); peak a, is an isomeric form of MMC; b, 1,2-*trans* 1-hydroxy 2,7-diaminomitosene (0.28 μg); peak c is not present. Chromatogram 2 is a tumour collected 15 min after microsphere administration and chromatographic peaks are identified as follows: MMC, mitomycin C (7.2 μg); 2,7-DM, 2,7-diaminomitosene (0.34 μg); peak b, 1,2-*trans* 1-hydroxy 2,7-diaminomitosene (0.35); peak c, 1,2-*cis* 1-hydroxy 2,7-diaminomitosene (0.23 μg). Chromatogram 3 is a control tumour extract.

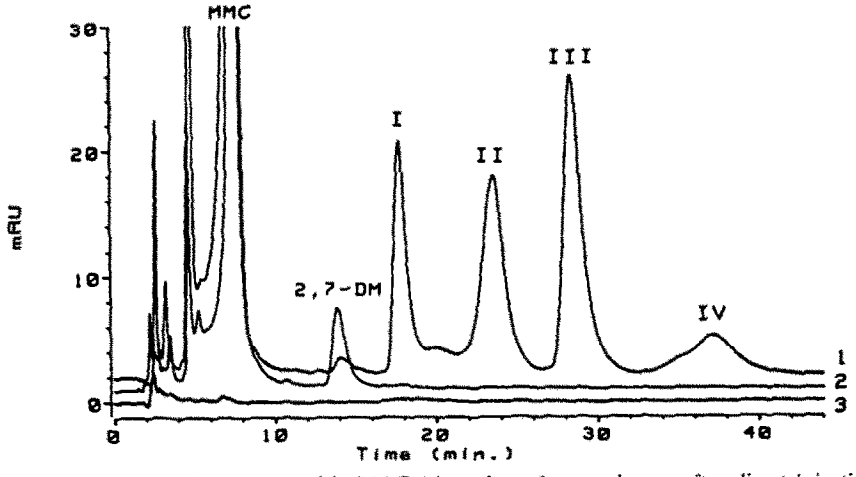

Fig. 7. Profile of metabolites produced in MAC 16 murine adenocarcinoma after direct injection of either free drug or microspheres. Samples (and concentrations) are identical to Fig. 6 except that HPLC chromatograms were monitored at 360 nm. Chromatogram 1 is microsphere administration; chromatogram 2 free drug; and chromatogram 3 blank tumour extract. Four mitosane metabolites (peaks I–IV, for UV-visible spectra see Fig. 8) appeared selectively in tumour only after injection of the microspheres.

cytochrome P450 reductase [25, 59] and DT-diaphorase [46, 60, 61].

MAC 16 tumour is comparatively rich in DT-diaphorase (98.3 nmol/min/mg protein) and cytochrome P450 reductase (10.7 nmol/min/mg protein) [28, 62, 63]. Biotransformation of MMC by cytochrome P450 reductase results in the formation of a large number of metabolites including *cis*- and *trans*-hydro and 2,7-DM [25, 40] whereas metabolism by DT-diaphorase yields exclusively 2,7-DM [32]. Part of the reason for this is undoubtedly the different pH dependency of these two enzymes where at more basic pH (7.8), which would favour the formation of *cis*- and *trans*-hydro [40, 64], DT-

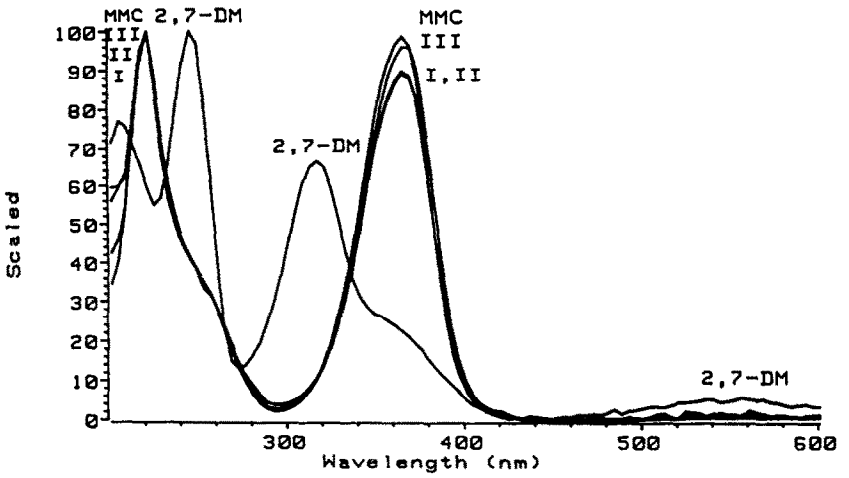

Fig. 8. UV-visible spectra of the major metabolite peaks detected in MAC 16 after free drug or microspheres. Spectra were recorded by a diode array detector during analysis of the chromatograms illustrated in Figs 6 and 7. The peak labelled 2,7-DM had a UV-visible spectrum identical to a standard of 2,7-diaminomitosene with maxima at 244, 313 and 558 nm. The peaks labelled I–III in Fig. 7 had spectra identical to that of MMC, which is also included for comparison, confirming their identity as mitosanes (see Fig. 1).

diaphorase is inhibited. However, it is significant that after administration of free MMC to MAC 16 only 2,7-DM was detected (characteristic of DT-diaphorase action) whereas immediately after administration of the microspheres 2,7-DM formation was decreased and a greater spectrum of metabolites were identified including *cis*- and *trans*-hydro (indicative of cytochrome P450 reductase). In previous studies, we have shown that intratumoural injection of microspheres to a rat mammary carcinoma (Sp 107) also produces hypoxia and apparent activation of cytochrome P450 reductase [21, 22]. Although in these studies there was a 16–24 hr delay before the onset of hypoxia, in the present study five times the dose of microspheres was administered to tumours which were on average 10 times smaller and this may have accelerated the establishment of an anaerobic environment.

Intratumoural administration of DOX/albumin microspheres to rat Sp 107 (DT-diaphorase activity, 94.2 nmol/min/mg; cytochrome P450 reductase activity, 14.4 nmol/min/mg) achieves a 155-fold stimulation in drug AQR [18, 21, 22]. A similar level of metabolic activation was anticipated with MMC and the MAC 16 tumour. However, a 3-fold inhibition of 2,7-DM formation was observed (taking into account the different tumour pharmacokinetic profiles between free drug and microspheres). This surprising difference may now be explained on the basis of the comparative enzymology of the two drugs. DOX is not a substrate for DT-diaphorase and does not undergo bioreductive transformation aerobically after one electron reduction but rather redox cycles [21, 65]. On the induction of hypoxia by microspheres, cytochrome P450 reductase catalysed AQR produces 7-deoxyaglycone metabolites. In contrast, the present data are consistent with MMC being a good substrate for DT-diaphorase with rapid and selective formation of 2,7-DM occurring under aerobic conditions. On the immediate induction of hypoxia by the microspheres metabolism by cytochrome P450 reductase is revealed, a greater spectrum of metabolites are evolved, and a concomittant reduction in 2,7-DM formation results.

Thus, whether the hypoxia created by the microspheres is likely to be beneficial or not *in vivo* would appear to depend on the balance of activities between one and two electron reductases, as well as other key factors such as tumour pH. Although MMC is considered to be the archetypal anaerobic bioreductive alkylating agent [49], its hypoxic cell cytotoxicity ratio (HCR, dose to kill 50% of cells growing under oxic conditions/dose to kill 50% of cells growing under hypoxia) is only 2 [66] and in some cell lines, such as CHO-K1, MMC-2 and MMC-3 no differential killing is observed [67, 68]. Initial observations on hypoxic cell selectivity were made in EMT6 cells where cytochrome P450 reductase was believed to be responsible for the majority of MMC activation [49, 69].

Four, more hydrophobic, mitosane metabolites of MMC appeared in the MAC 16 after administration of microspheres. All the major chemical degradation products of MMC have characteristic mitosene UV-visible spectra (70, see Fig. 1) with the exception of the zwitterion form of MMC (see peak a, Fig. 6) and decarbamoyl-MMC, both of which elute before MMC during reversed phase HPLC [26]. All major products of MMC quinone reduction either have regular mitosene spectra or dramatically different spectra [40]. Thus the basis for the formation of these products remains intriguing but unknown.

Finally, liver metastases of colorectal origin have been generally recognised as the optimal clinical condition to treat by locoregional perfusion with MMC microspheres [71]. MMC is one of only a few

drugs with even marginal activity in colon cancer. Nevertheless, human colon cancer is known to contain exceptionally high levels of DT-diaphorase [28, 61]. Results presented here, that the induction of hypoxia by microspheres, which can occur shortly after chemoembolisation [2, 72], may actually ablate the anticancer activity of MMC by transferring metabolism away from DT-diaphorase, indicate caution when following this approach. They do however suggest that drugs which are selectively toxic to anaerobic cells such as EO9 (HCR, 33); RSU 1069 (HCR, 30) and SR 4233 (HCR, 60) [66] may be ideal candidates to fully exploit the potential of microspheres to target to and induce local hypoxia in tumour tissue.

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