In vivo efficacy and toxicity of intratumorally delivered mitomycin C and its combination with doxorubicin using microsphere formulations

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The efficacy and toxicity of intratumorally (i.t.) administered anticancer drugs mitomycin C (MMC) and doxorubicin (Dox) incorporated in polymeric microspheres were investigated. Biodegradable sulfopropyl dextran microspheres and their oxidized products were used to load Dox and MMC, respectively. EMT6 mouse mammary cancer cells were injected into the hind leg of BALB/c mice. MMC microspheres, alone or combined with Dox microspheres, were injected i.t. once tumors had reached around 0.3 g. The tumor-plus-leg diameter was measured daily and the delay in time for the tumor to grow to 1.13 g relative to control (TGD) was employed as an indication of therapeutic effect. General toxicity was determined by monitoring weight, appearance and behavior of the mice. Morphology and histology of tumor and heart tissues were also examined. An average 79% TGD was observed after i.t. injection of MMC microspheres. The i.t. co-administration of MMC and Dox microspheres resulted in a 185% TGD. The i.t. injections of the microsphere formulations did not result in visible signs of toxicity in animals. In contrast, systemic (i.e. i.p.) injections of MMC solutions caused considerable general toxicity. This study suggests that i.t. delivery of anticancer drugs by polymeric microspheres is an effective way of improving the therapeutic index for cancer chemotherapy of selected solid tumors under special conditions. *Anti-Cancer Drugs* 16:423–433 © 2005 Lippincott Williams & Wilkins.

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Introduction

In the treatment of solid tumors with drugs, the efficacy of chemotherapy administered systemically is limited by the toxicity of chemotherapeutic agents to normal tissues, drug concentrations achievable in tumors and the development of drug resistance [1–3].

Traditional anticancer drugs (e.g. alkylating agents, antimetabolites) inevitably cause normal tissue toxicity because of their relatively poor specificity to target tissues and the premise that higher doses increase the likelihood of achieving a favorable therapeutic response [4]. The normal tissue toxicity of these drugs may limit the dose that can be given to patients, can lead to patient non-compliance and in some cases even cause death. Thus, decreasing the toxicity of these agents towards normal tissue, while maintaining efficacy towards tumor tissue, may further enhance the utility of these drugs.

The structure of solid tumors imparts a number of non-cellular resistance mechanisms that may prevent sufficient drug from reaching the tumor following systemic administration (e.g. i.v. or i.p.). The irregular vasculature of solid tumors can restrict drug access to poorly perfused areas—protecting some tumor cells from toxicity, while also resulting in hypoxic regions [1,5]. Tumor cells found in hypoxic regions are resistant to many anticancer agents [6] and have a propensity to drive malignant progression [7]. Solid tumors are also characterized by a malfunctioning lymphatic system, resulting in high interstitial fluid pressure that in turn makes it difficult for systemically delivered drugs to diffuse into the tumor interstitium from blood vessels [8].

Exposure of tumor cells to non-toxic anticancer drug doses, through the inability of systemically delivered drugs to access all tumor regions, may lead to the development of cellular-based multidrug resistance (MDR) [9]. MDR is often associated with the over-expression of transport proteins such as P-glycoprotein [10]. Once the cells have acquired resistance to one drug, they are simultaneously cross-resistant to drugs that are unrelated in terms of structure or cellular target [11].

In light of the aforementioned problems encountered in the chemotherapy of solid tumors, alternative anticancer

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drug delivery routes such as locoregional delivery have been explored in attempts to enhance the efficacy of chemotherapy and to reduce systemic toxicity. The intratumoral (i.t.) route has received increasing attention over the past 25 years because it establishes a much higher drug concentration in the tumor than in the general circulation, unlike systemic administration, which relies on the erratic tumor microvasculature and thus can only deliver a small fraction of the drug to a solid tumor [12]. This feature of i.t. delivery is important since most of the body is a non-target site. This feature restricts the use of systemic administration to tumors where local control of disease is a cause of failure and systemic metastases are not a concern [13] or to palliative approaches in situations where local tumors are not operable or recur after radiotherapy.

Previous studies have shown that prolonged residence time of anticancer drug in tumor tissues increases the probability of therapeutic cure [1,14]. In other words, a sustained supply of drug in the tumor mass is desirable. However, this condition cannot be met by i.t. injection of a drug solution since blood circulation and the elevated i.t. pressure will hasten clearance of the drug from tumor tissue. This has prompted the development of various drug delivery systems, such as microspheres (MS), for the i.t. delivery of anticancer drugs [15]. MS with suitable sizes and biodegradability are ideal as anticancer drug carriers since they can be administered to the tumor by direct injection and can remain anchored within the tumor mass. When properly formulated, MS can serve as a depot for the sustained release of tumoricidal agent [16–20], providing high local drug levels over an extended period of time, which can perceivably improve therapeutic efficacy and reduce the probability of developing acquired MDR [9].

The advantages of i.t. delivery of anticancer agents using MS have been demonstrated. Improved survival of animals bearing gliomas was found with i.t. administration of 5-fluorouracil-loaded poly(methylidene malonate 2.1.2) MS [17]. Mitoxantrone-loaded albumin MS [18], doxorubicin-loaded poly(L-lactide co-glycolide) MS [19] or dextran-based MS [20] also resulted in prolonged animal survival, with minimal systemic drug toxicity, when administered i.t. for the treatment of breast cancers. These positive results, coupled with the relatively low number of studies involving the i.t. route for cancer chemotherapy, suggest that this administration route has yet to be fully exploited and warrants further investigation.

Dextran-based MS for the locoregional delivery of anticancer drugs and chemosensitizing agents were extensively investigated *in vitro* and *in vivo* by Liu *et al.* [20–24] and Cheung *et al.* [25,26]. In these studies, sulfopropyl dextran MS and various modifications of them (e.g. oxidation) were found to have high loading efficiencies and extended drug release. Furthermore, the activity of the released drugs was also maintained against cancer cells *in vitro*. An *in vivo* study involving the i.t. injection of doxorubicin (Dox)-loaded MS into BALB/c mice bearing mammary tumors (EMT6) showed that, while no significant drug toxicity was observed, a smaller than expected delay (24–40%) in tumor growth was attained when compared with *in vitro* results [20]. Because of this, a dextran-based MS system with a fast degradation rate has been developed for i.t. delivery of mitomycin C (MMC) [25].

MMC is an ideal candidate for i.t. delivery because it is a potent anticancer agent that is limited by serious systemic toxicities (e.g. irreversible myelosuppression) [27]. Furthermore, its bioreductive activation mechanism and cell cycle-independent cytotoxicity also suggest that MMC is useful for targeting hypoxic cells in solid tumors [28,29]. The microsphere system, prepared by oxidation of sulfopropyl dextran MS (Ox-MS), released more than 30% of the loaded drug in a phosphate buffer solution over 48 h, accompanied by marked disintegration indicative of polymer degradation. As previously alluded to, the released drug was chemically unaltered and maintained its bioactivity against cancer cells *in vitro* [25]. Taken together, these findings warrant further *in vivo* studies involving the i.t. delivery of MMC using the Ox-MS system.

In clinical practice, combinations of two or more anticancer drugs are often administered to improve the therapeutic index (i.e. difference between the therapeutic drug dose and the dose of drug that elicits toxicity to normal tissues) of the combined drugs. Although Dox and MMC are not currently used together in the clinic, investigations of MMC/Dox combinations in tissue culture [30], animals [31] and humans [32,33] have demonstrated that the drug combination can produce synergistic effects on tumor cells. However, the therapeutic advantage of this combination, when the drugs are systemically administered, has been limited by significant toxicity.

Previous *in vitro* research conducted by Cheung *et al.* [30] has shown that Dox and MMC released from microspheres not only retain their activities, but exhibit synergistic cytotoxicity against EMT6 cells when the two drugs are applied simultaneously or MMC application follows Dox exposure. Given the apparent ability of i.t. administered anticancer drugs, using MS formulations, to minimize systemic toxicity, it may be possible to augment the benefit of MMC/Dox combinations by delivering both agents using MS via the i.t. route. The present work was therefore designed to investigate the *in vivo* efficacy

of i.t. administered MMC-Ox-MS, and to study whether the synergistic cytotoxicity observed from the previous *in vitro* study of MMC/Dox combinations can be realized *in vivo* by i.t. co-administration of Dox-MS and MMC-Ox-MS.

Materials and methods

Materials

Cross-linked dextran MS bearing sulfopropyl groups (Sephadex SP C-25; SP-MS) and Dox were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). MMC was acquired from Faulding (Kirkland, Quebec, Canada). All cell culture plasticware was purchased from Sarstedt (Montreal, Quebec, Canada). Cell culture medium, α-minimum essential medium (α-MEM), was obtained from the Ontario Cancer Institute (Toronto, Ontario, Canada) and fetal bovine serum (FBS) was purchased from Cansera International (Etobicoke, Ontario, Canada). The surfactant, Pluronic F-127, was generously provided by BASF (Mount Olive, NJ).

Preparation of oxidized MS

MS oxidation was carried out as previously described [25]. Briefly, 50 mg of SP-MS was exposed to 5 ml of 0.1 M sodium periodate for at least 15 h at 4°C. The oxidized SP-MS (Ox-MS) were washed successively with 5 ml of 0.2 M lead acetate aqueous solutions to remove unreacted periodate and 2 ml of 0.5 N HNO_3 to remove residual lead acetate, and then extensively rinsed with deionized/distilled (DDI) water to remove any residual chemicals. Rinsed Ox-MS were then lyophilized for storage and future use.

Drug loading into microspheres

Loading MMC and Dox into microspheres was conducted using an absorption method previously detailed [21,25]. Samples of 5 mg of Ox-MS were incubated with 1 ml of an aqueous MMC solution (300 μg/ml in DDI water) at 4°C or 1 mg of SP-MS were placed in a 1 ml of 250 μg/ml Dox solution at room temperature for at least 48 h. The resultant MS respectively contained 3.2% (w/w) MMC (MMC-Ox-MS) and 25% (w/w) Dox (Dox-MS). The drug-loaded MS were briefly rinsed with DDI water to remove unloaded drug, lyophilized and stored at room temperature for further studies.

In vitro drug release

A column system was used to determine *in vitro* drug release from MMC-Ox-MS and Dox-MS because a previous study showed that this method could provide good prediction of *in vivo* release kinetics from *in vitro* release profiles [26]. A hollow column (4.6 mm × 15 cm) was used to house 1 mg of MMC-Ox-MS or Dox-MS. Hank's balanced salts solution (HBSS; Sigma-Aldrich), the designated release medium, was injected from a 60-ml syringe into the column and then through a flow-through cell, by a syringe pump (Sage Model 355; Orion, Boston, MA) set at a flow rate of 0.6 ml/h. Drug concentration in the cell was monitored using the UV/vis spectrophotometer set at 364 nm for MMC and 234 nm for Dox.

Tissue cell culture

The EMT6 murine breast sarcoma cell line was generously provided by Dr I. F. Tannock (Ontario Cancer Institute). Cells cultures were grown as a monolayer on 175-cm^2 vented tissue culture flasks in α-MEM plus antibiotics (streptomycin and penicillin, 0.1 g/l each) supplemented with 10% FBS (designated the growth medium) at 37°C in a 5% CO_2/95% air humidified incubator.

In vivo treatment of mice bearing solid tumors

Nine-week-old female BALB/c mice, 1 week after being received from the Jackson Laboratory (Bar Harbor, ME), were inoculated intramuscularly in the hind leg with 5×10^5 EMT6 cells in 50 μl of the growth medium [20]. Treatments were initiated when the tumor-plus-leg (T + L) diameters reached approximately 8–9 mm (about 0.3 g), an average of 5–6 days following inoculation. All animal handling was carried out under an approved protocol from the Animal Care Committee at the Ontario Cancer Institute following Canadian Council on Animal Care (CCAC) guidelines.

The various treatment groups are listed in Table 1. On the day of treatment, animals were randomized into groups of five animals. Blank Ox-MS or MMC-Ox-MS were suspended in a 9% Pluronic F-127 (non-ionic surfactant) solution in DDI water to make 10 mg/ml suspensions. Mixtures containing equal amounts of MMC-Ox-MS and Dox-MS were suspended in the surfactant solution to make 20 mg/ml suspensions. The surfactant solution was selected to prevent microsphere clumping within the needle hub during injection. Aliquots of 100 μl of blank Ox-MS, MMC-Ox-MS or

Table 1 Overall summary of treatment groups, tumor growth times, TGD and toxicity assessment

Groups	*n*[a]	Average time (days) to 1.13 g tumor weight (mean ± SD)[b]	Overall TGD (%)[c]	Average toxicity score[d] (mean ± SD)
Control	25 (5)	5.6 ± 1.2	–	0.6 ± 1.3
Ox-MS	25 (5)	6.0 ± 1.9	+7	2.6 ± 4
MMC i.p.	25 (5)	10.0 ± 5.4	+79	10.2 ± 5[f]
MMC-Ox-MS	25 (5)	10.0 ± 4.1	+79	2 ± 4
MMC/Dox-MS	5 (1)	15.4 ± 4.9[f]	+185[e]	0

[a]Total number of animals used (number of trials).
[b]Determined by taking average of mean times for tumors to reach 1.13 g for each trial.
[c]Calculated using means determined by pooling all tumor growth data for each trial over all experiments.
[d]Determined by taking the average of the summed toxicity scores for each trial.
[e]Calculated using results from the control group of MMC/Dox-MS only.
[f]Statistical significance; analysis of variance with the Bonferroni *t* procedure, $p < 0.05$.

MMC-Ox-MS/Dox-MS (containing 1 mg of MS for the blank and drug-loaded systems, and 2 mg of MS for the combination regimen) suspensions were injected i.t. using a syringe fitted with a 21-gauge, 1-in. needle. This needle size was chosen because it was the smallest diameter that would allow MS (around 100 µm average diameter) to freely pass out of the syringe. The control group received a single i.t. injection of 100 µl of the surfactant solution.

For the animals receiving systemic MMC, a known concentration of MMC in sterile phosphate buffer solution was made up. Doses equivalent to the amount of MMC loaded onto the administered Ox-MS were injected i.p. (around 50 µl volumes) using a 26-gauge, 1-in. needle. For the animals receiving the microsphere combination, MMC-Ox-MS and Dox-MS were co-administered to minimize trauma to the animals and to make the results more directly comparable with other treatment groups.

Beginning on the day of treatment, the T + L diameter was measured daily for the initial 9 days and then at least every other day by passing the tumor-bearing leg through a plastic plate bearing a series of circular holes of increasing 0.5-mm diameters. When the T + L diameter reached 13 mm, the time was recorded and animals were sacrificed shortly thereafter. A diameter of 13 mm corresponded to an average tumor weight of 1.13 g. Once the animals were sacrificed, tumors were excised, weighed and stored in formalin for future examination.

The general toxicity of each treatment was evaluated in a semiquantitative manner using five parameters as previously described (i.e. weight loss, fur roughing, water and food intake, general activity, and tissue damage at the site of injection) [20]. Each parameter was evaluated on a scale of 0, 1 or 2, e.g. weight loss (0 = no weight loss; 1 = 1–2 g weight loss; 2 = > 2 g weight loss). Therefore, general toxicity scores could range from 0 (no observable toxicity) to 10 (maximum observable toxicity) for each animal. The sum of the general toxicity scores for each animal in a group was used as an indication of the general toxicity of the given treatment (maximum score of 50 per group). During the course of an experiment, any animal with a greater than 20% weight loss (measured from treatment initiation) was sacrificed.

Examination of tumor and heart tissue

Excised tumors were sectioned into halves (dissected along similar tumor planes) and subjected to gross examination, whereupon the tumor interiors were digitally photographed. Tumor tissue samples, randomly selected from each of the treatment groups, were also obtained for histological analysis. Samples were sent to the University Health Network Clinical Research Program (Toronto, Ontario) for hematoxylin & eosin (H & S) staining, and slide preparation.

Dox is known to cause potentially life-threatening, dose-related, cardiotoxicity [27,34]. Thus, for animals receiving Dox-MS, hearts were collected after the animals were sacrificed. Following organ collection, tissue slices were obtained and subjected to H & E staining, and then prepared slides were examined histologically as a component of toxicity assessment.

Statistics

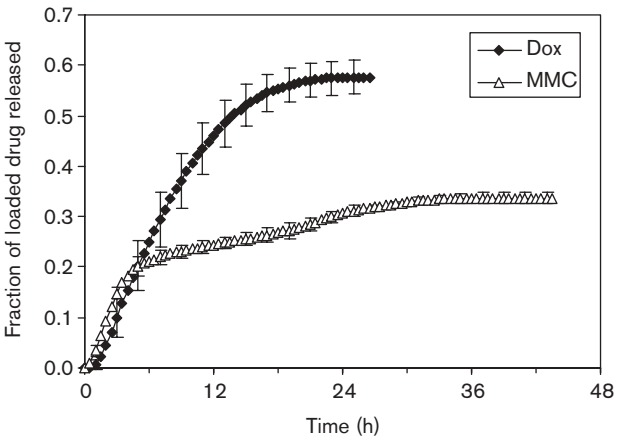
Time for the tumor weight to reach 1.13 g was expressed as the mean time ± SD (days), where mean values were determined from all animals in each group. The existence of differences in mean times for tumors to reach 1.13 g among the groups was determined using a two-way analysis of variance [35]. When differences were found, a Bonferroni t procedure was applied to identify the statistically different groups [35]. A value of $p \leq 0.05$ was considered statistically significant.

Results

Drug release from MS

Figure 1 shows the cumulative fractional release of MMC and Dox from 1 mg of Ox-MS (3.2% w/w MMC loading) or SP-MS (25% w/w Dox loading), respectively, over time. For both the MMC-Ox-MS and Dox-MS systems, the respective drug loading levels and the amount of MS placed within the column were equivalent to the drug loading levels and the amount of MS injected i.t. This allowed for the prediction of *in vivo* drug release from MS following administration.

Fig. 1



Cumulative fraction of loaded MMC or Dox released from 1 mg of MMC-Ox-MS (3.2% w/w loading) or Dox-MS (25% w/w loading), respectively, over time using the column system in HBSS delivered at a flow rate of 0.60 ml/h. Data points are means ± SD of measurements from independent experiments (n = 3).

MMC release appeared to occur in two phases: (i) a fast initial phase where an approximate 0.20 fraction of the loaded drug is released in the first 4 h, then gradually tapering off, and (ii) a slow extended phase lasting approximately 30 h, in which an approximate 0.10 fraction of additional loaded drug is released until equilibrium (total fractional release 0.34 ± 0.01). The first phase is likely the result of the release of drug molecules that are bound to MS by ionic interactions, which can be readily exchanged by counter-ions in the releasing medium. The second release phase may be due to drug molecules that are bound to MS by stronger covalent bonds and are released as the MS physically degrade [25]. The *in vitro* release of MMC from Ox-MS is extensively discussed elsewhere [25].

Dox release from SP-MS appeared to occur in a single phase and was more complete than that for MMC, as almost a 0.60 fraction of the loaded drug is released over 24 h. The interaction between Dox and SP-MS has previously been demonstrated to be entirely ionic [21]. Thus, drug release is once again due to the exchange of counter-ions between MS and the release medium. Based on previous investigations by Liu *et al.* [21], incomplete Dox release from SP-MS was expected based on the low ionic strength of the primary *in vitro* release medium (i.e. HBSS) and the tendency for Dox to self-associate at higher loading levels.

In comparing the release profiles of MMC and Dox from their respective systems, it can be seen that the rate of MMC release in the initial phase (i.e. first 4 h) closely follows that of Dox release over the same time period. This observation further supports the premise that MMC release during the first phase of release is due to ionic exchange.

Calibration curve for correlation of tumor size and weight

To convert T + L diameter to EMT6 tumor weight, a calibration curve is required. A calibration curve for the KHT mammary tumor spanning the T + L diameter range 7–17 mm was taken from Siemann *et al.* [36], while the data for the EMT6 tumor, over the range of 13–15 mm T + L diameters, was obtained in this study. The EMT6 data coincided with the calibration curve for KHT tumors (data not shown). Therefore, T + L diameters over the course of this study were converted to tumor weights using the KHT calibration curve.

Efficacy of various treatments

From the plots of tumor weight versus time, tumor growth delay (TGD) was estimated using the following equation, as described by Liu *et al.* [20] (previously termed prolonged survival): $\mathrm{TGD} = (T_{\mathrm{treat}} - T_{\mathrm{control}})/T_{\mathrm{control}} \times 100\%$, T_{treat} and T_{control} represent the mean number of days for the tumor weight to reach 1.13 g, beginning from the time of treatment initiation, for the various treatment and control groups.

The effect of the treatments (i.e. i.t. injection of MMC-Ox-MS and MMC-Ox-MS/Dox-MS combination) on EMT6 solid tumors was evaluated using tumor growth curves. Results from the efficacy investigations from the different treatment groups are presented in Table 1 where the averaged results are summarized. Figure 2 (A–D) shows representative tumor growth curves as a function of time following tumor cell inoculation for the control, blank Ox-MS, MMC-Ox-MS and MMC-Ox-MS/Dox-MS treatment groups (Table 1).

The average time for tumors to reach the tumor weight cut-off (1.13 g) for the untreated group was 5.6 ± 1.2 days post-treatment initiation (Table 1). Since it has previously been shown that SP-MS (i.e. unmodified MS) do not slow tumor growth [20], it was not surprising that there was no difference observed for tumor growth rates between the untreated group and the animals receiving i.t. blank Ox-MS (6.0 ± 1.9 days).

Efficacy of i.t. administered MMC-Ox-MS

MMC-Ox-MS were injected i.t. to investigate the effect of the device and administration route on tumor growth rate. The mean time for tumors to reach 1.13 g for animals receiving i.t. injections of MMC-Ox-MS was 10.0 ± 4 days post-treatment initiation, leading to an average TGD score of 79% when compared to untreated controls. However, the average time for the tumor to reach 1.13 g was not statistically different from the untreated control group (Table 1).

In comparison, for animals receiving aqueous solutions of MMC (equivalent in dose to the amount of MMC loaded onto Ox-MS) injected i.p. into mice, the overall average time for tumor weight to reach 1.13 g for MMC i.p. was 10.0 ± 5.4 days post-treatment initiation, leading to a TGD score of 79% when compared to the untreated control group. Surprisingly, this result was similar to that obtained by i.t. administered MMC-Ox-MS.

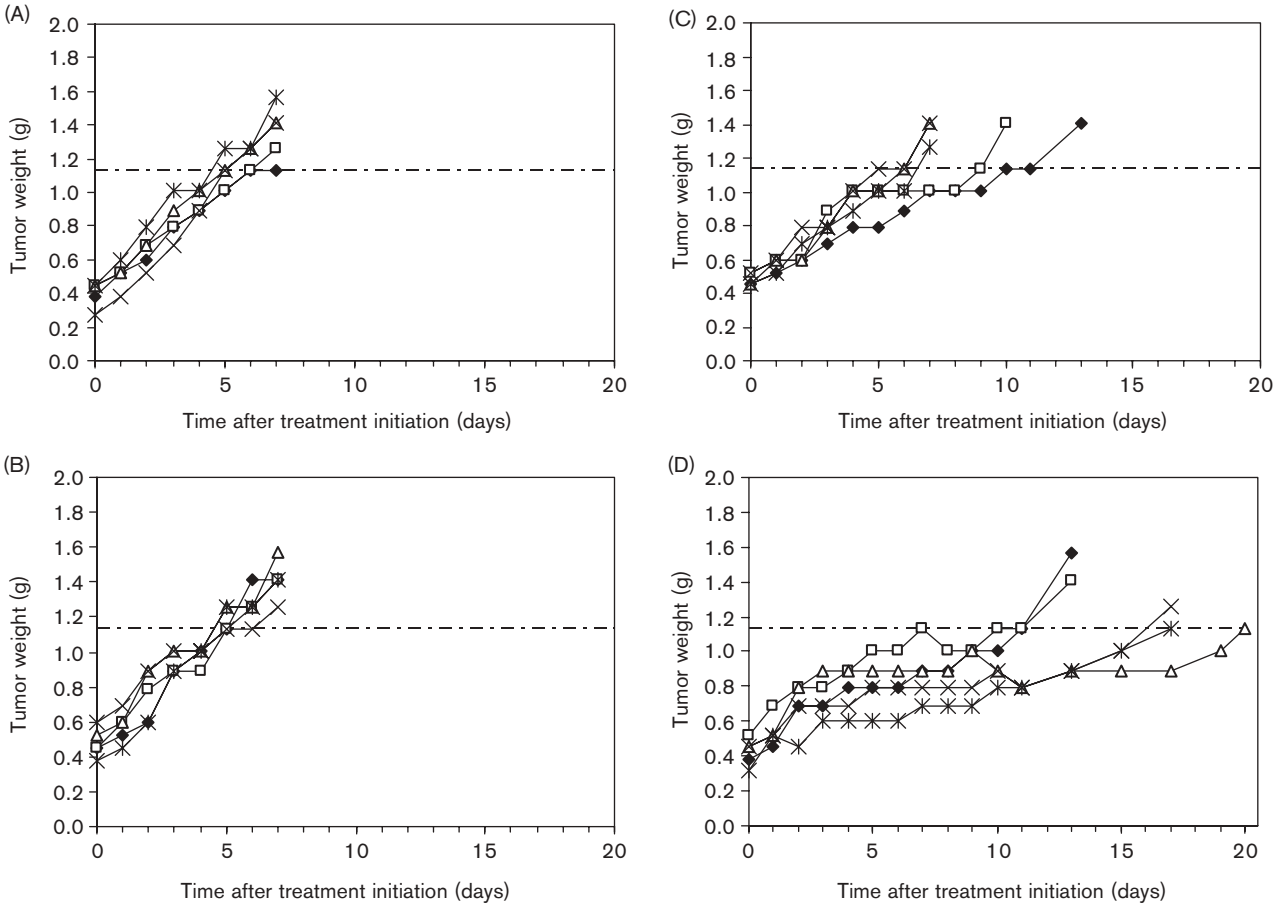
Efficacy of i.t. co-administered MMC-Ox-MS/Dox-MS combination

Figure 2(D) shows that the average time for tumors to reach 1.13 g following the co-administration of MMC-Ox-MS and Dox-MS was 15.4 ± 4.9 days, translating to a TGD score of 185% when compared to the mean time of the untreated control group for Trial 5 ($p < 0.0001$).

General toxicity of various treatments

The general toxicity to mice in the various groups was assessed using the previously mentioned parameters (i.e. weight loss, observation of fur roughing of the animal,

Fig. 2



Tumor weight as a function of time for individual mice from different treatment groups following treatment initiation (day 0) for Trial 5: (A) control (100 μl of surfactant given i.t.), (B) blank Ox-MS (1 mg of Ox-MS suspended in 100 μl of surfactant given i.t.), (C) i.t. MMC-Ox-MS [1 mg of MMC-Ox-MS (3.2% w/w loading) suspended in 100 μl of surfactant given i.t.] and (D) intratumoral co-administered MMC-Ox-MS + Dox-MS [1 mg of MMC-Ox-MS (3.2% w/w loading) and 1 mg of Dox-MS (25% w/w loading) suspended in 100 μl of surfactant given i.t.].

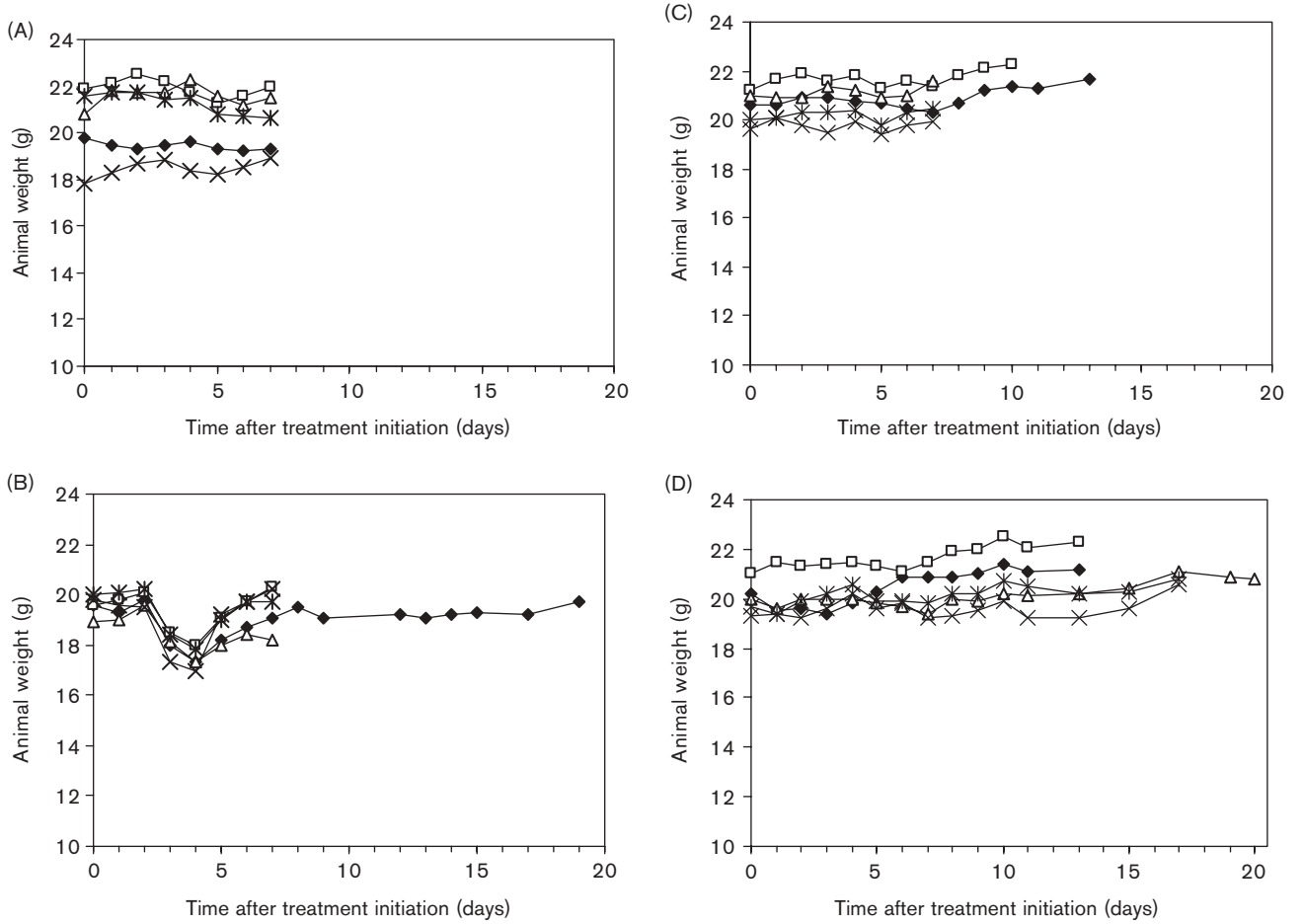
decreased water and food intake, general activity, and tissue damage at the site of injection). Figure 3 presents animal body weight versus time in a representative trial of the various treatments. It can be seen that the body weight is essentially unchanged over the course of the experiment for the control group (Fig. 3A), and the groups treated with i.t. injection of MMC-Ox-MS (Fig. 3C) and MMC-Ox-MS/Dox-MS (Fig. 3D). However, the group treated with i.p. injection of MMC solutions experienced a significant body weight loss at day 3 and day 4 (Fig. 3B), which is an indication of the toxicity of the systemic treatment.

As shown in Table 1, the general toxicity scores for the animals receiving i.t. injected blank Ox-MS, MMC-Ox-MS and MMC-Ox-MS/Dox-MS are virtually as low as the control group. The average toxicity scores for these three groups are 2.6 ± 4, 2 ± 4 and 0, respectively, which are not statistically different from the control group with a score of 0.6 ± 1.3. In contrast to the negligible toxicity observed in these groups, the systemically administered MMC solutions show significant toxicity with a mean toxicity score of 10.2 ± 5 ($p = 0.01$). This result suggests that the blank Ox-MS is a non-toxic carrier and that i.t. treatment using the MS systems causes no general toxicity. Despite similar TGD scores for systemic and i.t. administration of MMC, i.t. treatment is favored over systemic treatment due to its considerably lower toxicity.

Gross and histological examination of excised tumor tissue

Interiors of the excised tumors from representative sample mice from each treatment group are shown in Figure 4. Tumors from the untreated control and MMC i.p. groups (Fig. 4A and B), as well as those from the blank Ox-MS group, appear solid and consistent throughout the tumor mass. For the tumor excised from the mouse receiving i.t. administration of MMC-Ox-MS (Fig. 4C),

Fig. 3



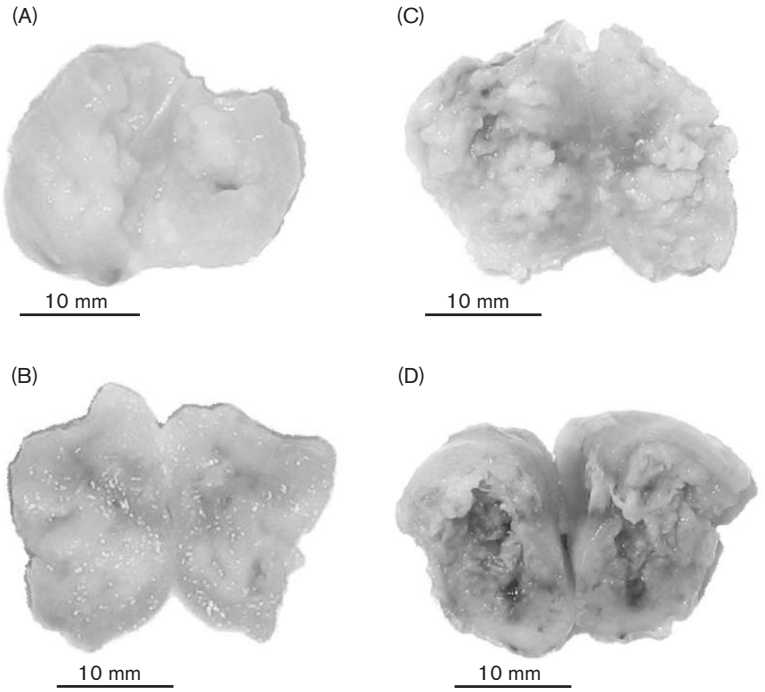
Animal body weight as a function of time for individual mice from different treatment groups following treatment initiation (day 0) for Trial 5: (A) control (100 μl of surfactant given i.t.), (B) MMC i.p. (32 μg of MMC dissolved in 50 μl of PBS given i.p.), (C) i.t. MMC-Ox-MS [1 mg of MMC-Ox-MS (3.2% w/w loading) suspended in 100 μl of surfactant given i.t.] and (D) i.t. co-administered MMC-Ox-MS/Dox-MS [1 mg of MMC-Ox-MS (3.2% w/w loading) and 1 mg of Dox-MS (25% w/w loading) suspended in 100 μl of surfactant given i.t.].

the tumor interior appears loose and irregular, suggesting that local drug administration may be exerting an effect within the tumor mass. The tumor interior of the mouse receiving i.t. injection of the MMC-Ox-MS/Dox-MS combination (Fig. 4D) appears to have hollow regions; a possible indication that the combination causes significant internal tissue necrosis resulting in delayed tumor growth.

The tumor tissue from a control mouse was viewed with optical microscopy. The EMT6 tumor was found to be a very aggressive tumor as there was extensive invasion of tumor cells into the surrounding muscle tissue only 11 days following tumor cell inoculation (data not shown). Histological examination of the excised tumor tissue from the i.t. treated groups (i.e. MMC-Ox-MS and MMC-Ox-MS/Dox-MS) did not reveal any cellular differences when compared to the control groups. A major difference between the tumors of i.t. treated animals and the control group was the observation of inflammation in the treated groups (data not shown), likely due to a combination of the high local drug concentration and the presence of a foreign substance (i.e. dextran). In addition, another notable difference occurred upon inspection of the tumor treated with the MMC-Ox-MS/Dox-MS combination. The necrotic region of the combination-treated tumor was greater than that in the control tumor, in agreement with the gross examination (Fig. 4D).

A section from the tumor treated with an i.t. injection of MMC-Ox-MS was of interest. It captured the i.t. injection tract produced by the needle during microsphere administration. Fragments of MMC-Ox-MS were visible within the passage, providing further evidence that Ox-MS biodegrade *in vivo* (data not shown).

Fig. 4



Gross histological examination of excised solid tumor interiors for individual mice from different treatment groups: (A) control, (B) i.p. MMC, (C) i.t. MMC-Ox-MS and (D) i.t. MMC-Ox-MS/Dox-MS.

Histological examination of excised heart tissue

No overt signs of Dox toxicity were observed in cardiac tissue samples obtained from mice receiving an i.t. injection of MMC-Ox-MS and Dox-MS (data not shown). This was anticipated, not only because the total Dox dose administered to the mice (250 μg or 12.5 mg/kg) was less than the median cumulative Dox dose known to cause cardiotoxicity in mice (36.4 mg/kg [37]), but also because general toxicity was absent in animals treated with Dox-MS and low levels of systemically circulating Dox were expected from i.t. administration [38].

Discussion

In the present work, a single i.t. administration of MMC-Ox-MS delayed EMT6 solid tumor growth by an average of 79% as compared to controls. This rate of reduction is more than 3-fold (24%) of that achieved by the single-dose i.t. injection of Dox-MS (250 μg Dox) into the same tumor model reported by Liu *et al.* [20], suggesting that MMC is a better therapeutic agent for EMT6 solid tumors. An equivalent delay in tumor growth was observed in the i.p. groups receiving an equal total dose of MMC in a solution. The group that received a combination treatment of MMC-Ox-MS and Dox-MS showed a 185% delay in tumor growth, double that of the groups treated by MMC alone. Previously published work of several groups has shown maximally tolerated single i.p. or i.v. injections of Dox into EMT6 tumor-bearing BALB/c mice cause only marginal cell killing [39,40] and little to no effect in terms of tumor regrowth delay [41,42].

Cheung *et al.* [30] investigated cytotoxicity of MMC and Dox combinations using EMT6 cells, and found that simultaneous administration of MMC and Dox or administration of Dox before MMC produced additive to more-than-additive toxic effects on EMT6 cells. As illustrated in Fig. 1, Dox and MMC are released from their respective microsphere systems at the same rate over the initial 4 h, after which the Dox release rate exceeds that of MMC for the ensuing 12 h. This release pattern indicates that the simultaneous delivery of both microsphere systems is warranted, as the regimen should optimize therapeutic effects by mirroring the results from the *in vitro* combination study. In other words, equivalent MMC and Dox initial release rates is similar to simultaneous drug administration *in vitro*, and increased Dox release accompanied by prolonged MMC release resembles the *in vitro* administration schedule employing Dox before MMC. As mentioned above, previous i.t. studies using Dox-MS containing 250 μg of Dox resulted in a TGD score of 24% [20], while the present study yielded a 79% TGD score for a single i.t. injection of MMC-Ox-MS containing 32 μg of MMC (Table 1).

Theoretically, based on the concept of simple additivity (sum of the TGD from a given dose of drug A and the TGD from a given dose of drug B), the effect of both agents with the same doses indicated above should lead to a 103% TGD score (i.e. 79 + 24%). Thus, the observed 185% TGD score can be considered as a greater-than-additive antitumor effect, which is consistent with the finding from previous *in vitro* studies of the Dox and MMC combination [30].

One of the well-documented toxicities associated with Dox solutions is local tissue necrosis which typically occurs as a result of extravasation (i.e. leakage of i.v. drugs from the vein into the surrounding tissue) [4]. The necrotic effect of Dox was evident from the results of the morphological and histological examination of tumors treated with Dox-MS and MMC-Ox-MS. Hollow regions (i.e. areas devoid of tumor tissue as a result of necrosis) are present in the tumors (Fig. 4D) treated with the combination, but are absent in the tumor with i.t. injection of MMC-Ox-MS alone, although the latter resulted in the development of loose and irregular tissue within the tumor interior. It is possible that the use of Dox-MS produces severe tumor tissue necrosis, contributing to the greater-than-additive therapeutic effect obtained with the combination therapy. Thus, while steps are normally taken to avoid local tissue necrosis during systemic drug administration, it may be an advantageous byproduct of i.t. Dox administration. The greater TGD achieved with the drug combination may also be attributed to the ability of Dox to slow the rate of cell division, which was observed by Cheung *et al.* during *in vitro* treatments of EMT6 cells with Dox [30]. Whether i.t. co-administration of Dox-MS and MMC-Ox-MS is of clinical significance requires more extensive investigation using either the same or alternate (e.g. human breast cancer cells) cell lines. Studies, *in vitro* and *in vivo*, employing similar MMC/Dox combinations are in progress in our laboratory.

The i.t. treatment using the present MS formulations not only improved the therapeutic efficacy of the loaded anticancer drugs, but also significantly reduced toxicity to normal tissues. Little general toxicity was observed in the groups treated with MMC-Ox-MS and the MMC-Ox-MS/Dox-MS combination. Furthermore, no Dox-induced cardiac toxicity from the combination treatment was found in the histological examination of heart tissue. In contrast, considerable toxicity occurred in the systemic i.p. treatment groups, which was not surprising given the dose administered, as discussed below. Moreover, previous investigations found that MMC/Dox combination therapy in animals [31] and humans [32,33] also resulted in considerable toxicity, compromising the beneficial synergistic activity of the two agents. The greatly reduced toxicity from the same anticancer drug combination in the present study can be credited to localized delivery of the drugs.

MMC is very toxic to animals, with a systemic LD_{50} of about 8 mg/kg in mice [43,44]. The typical dose of MMC administered i.v. in the clinic is 0.24–0.51 mg/kg, based on average human female body surface area and weight, which often results in some degree of toxicity to normal tissues [27]. The dose used in this study is 1.6 mg/kg, estimated from the *in vitro* results by Cheung *et al.* [30]. This dose, while lower than the LD_{50} for mice, is much higher than the clinical doses. However, the absence of normal tissue toxicity observed in i.t. treatment demonstrates the advantages of i.t. delivered MMC-Ox-MS. The lack of significant toxicity also suggests that higher doses of MMC, in the form of greater single doses or multiple injections, can be delivered via the i.t. route to further enhance therapeutic effects.

Recent studies on the effects of multi-site i.t. [18] and peritumoral [19] (surrounding the tumor) injections have demonstrated that these approaches may lead to additional therapeutic benefits. However, given the localization of the tumors to the hind leg and the small tumor dimensions at the time of treatment initiation, these methods were too technically difficult to perform and were not investigated in the present study. Nevertheless, these approaches are worthy of further investigation as they have the potential to eliminate cancer cells at tumor margins, which may be particularly important in aggressive tumors, potentially reducing the incidence of micrometastases.

As seen in Table 1, the SD of inter-trial variation is relatively large in the present study, making the results of some groups (e.g. MMC i.p. and MMC-Ox-MS) statistically insignificant. Although a strictly standardized administration method was developed during preliminary studies for i.t. injections, to ensure equal drug dose in MMC-Ox-MS delivered to each animal, some inter-trial deviation remained. The observed variability was not altogether unexpected as *in vivo* tumor models, like human clinical trials, often yield somewhat variable results compared to *in vitro* studies.

In a retrospective study, Revesz and Siracka [45] observed that following an identical radiotherapeutic treatment, half of a group of 45 cervical cancer patients survived for more than 5 years, while the other half succumbed to the disease within 5 years of treatment. Subsequent analysis of tumor tissue from the patients revealed that the difference in survival was likely due to differences in tumor vasculature, where the long-term survivors had highly vascularized tumors (i.e. low hypoxic fraction) and short-term survivors had tumors with low vascularization (i.e. high hypoxic fraction). This

variability in tumor response, following the same treatment, is further supported by the results of the MMC i.p. group in the present study. Although the i.p. group received equivalent doses of MMC in the form of a solution, where administered doses are precisely controlled, the mean time for tumors to reach 1.13 g (data not shown) varied as much as that for the other treatment groups (i.e. MMC-Ox-MS and MMC-Ox-MS/Dox-MS).

It should be noted that the actual available doses of MMC and Dox in the i.t. injected MS formulations may not be equal to the total theoretical doses because a fraction of the drugs may not be released from the carrier, as illustrated in Figure 1. Nonetheless, unlike Dox-MS that degrade slowly (i.e. in weeks or months), Ox-MS have a faster degradation rate (i.e. in a few days), which may lead to further MMC release beyond 40 h. The projected bioavailability of MMC from Ox-MS remains to be confirmed by further studies.

Conclusion

The i.t. injection of MMC-Ox-MS delayed growth of EMT6 solid tumors. The combination of MMC-Ox-MS with Dox-MS slowed tumor growth even more significantly. Although the TGD scores achieved by i.p. injected MMC solutions was equivalent to that of MMC-Ox-MS, noticeable general toxicity was evident in the animals with the systemic treatment. In contrast, general toxicity was not observed in the animals that received i.t. injection of blank Ox-MS and MMC- and Dox-loaded MS. The lack of observable toxicity, even following co-administration of high drug doses in the form of MMC-Ox-MS and Dox-MS, together with the inhibition of tumor growth, suggests that i.t. administration of anticancer drugs alone or in combination using MS carriers is an effective approach for the improvement of localized solid tumor chemotherapy and for the minimization of systemic toxicity associated with the anticancer drugs. An important limitation of this approach is that, in its current form, it does not deal with the major problem of metastatic disease.

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