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Research Paper

In vitro toxicity to breast cancer cells of microsphere-delivered mitomycin C and its combination with doxorubicin

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Abstract

To better understand and design microsphere systems for the locoregional delivery of anticancer drug combinations to solid tumors, (1) the cytotoxicity of microsphere-delivered mitomycin C (MMC) was evaluated and (2) various schedules of MMC and doxorubicin (Dox) were tested for their toxicity in vitro towards a murine breast cancer cell-line, EMT6. To accomplish the former MMC was loaded onto oxidized sulfopropyl dextran microspheres, released in a pH 7.4 buffer solution and tested for its potency against EMT6 cells versus a standard MMC solution. For the latter EMT6 cells were exposed to MMC or Dox as single agents or together using various drug concentrations and schedules. The efficacy of the treatments was measured using a clonogenic assay. MMC released from the microspheres showed similar activity against EMT6 cells to freshly prepared MMC solutions. Greater-than-additive toxicity was observed when MMC was given either simultaneously or after Dox exposure. In contrast, administration of MMC before Dox exposure resulted in toxicity that ranged from additive to sub-additive; this reduced toxicity was mainly due to increasing cell density arising from the design of the assay. These results help explain our previous in vivo investigations using microsphere-delivered combinations of the same agents in EMT6 solid tumors.

Keywords: Mitomycin C; Doxorubicin; Microspheres; Breast cancer cells; Anticancer drug combinations

1. Introduction

The efficacy of conventional chemotherapy in the treatment of solid tumors can be limited by several factors including poor drug penetration into the tumor, systemic toxicity of chemotherapeutic agents, and the development of multidrug resistance [1–4]. Although doxorubicin (Dox) is active against many solid tumors, and is one of the most widely used anticancer drugs in current clinical practice [5,6], it causes many acute toxicities (e.g. hair loss, nausea, and vomiting) as well as irreversible cardiac toxicity, which restricts the repeated administration of the drug. Mitomycin C (MMC) is also a potent anticancer agent but is currently used mainly as a second line or adjunctive agent [5]. Its bioreductive activation mechanism (i.e. activation by cellular enzymes) has suggested its usefulness in targeting cells in the hypoxic regions of solid tumors [7]. Like Dox, the use of MMC is associated with a number of acute toxicities; however, it is the chronic toxicities (e.g. irreversible myelosuppression and hemolytic-uremic syndrome) that limit the clinical applications of the drug [5,8].

In contrast to conventional systemic drug administration, locoregional delivery of anticancer agents to solid tumors by a slow-release system, such as microspheres (MS), does not rely on the tumor vasculature to deliver drug to target sites. As a result, it is possible to achieve both high local drug concentrations in tumor tissues for extended periods of time and reduced systemic circulation of the drug leading to higher therapeutic efficacy and lower systemic toxicity [9–13]. Locoregional delivery also has the potential to minimize the development of cellular drug resistance mechanisms (e.g. drug efflux transporters) that may be induced by continued exposure to non-lethal doses of anticancer drugs.

A MS system has previously been developed in our laboratory for the locoregional delivery of Dox to solid tumors [14–16]. Although the Dox-loaded MS (Dox-MS)

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demonstrated reduced systemic toxicity following intratumoral injection, the observed therapeutic efficacy was lower than expected based on the results of in vitro studies using cells in monolayer [14–16]. Solid tumors may contain hypoxic regions that can contribute to both chemotherapeutic and radiotherapeutic resistance [17]. It has been speculated that bioreductive agents like MMC may be more effective than Dox for locoregional delivery [17–19]. This speculation led us to develop a MS system using oxidized-dextran microspheres (Ox-MS) for the delivery of MMC [20]. This previous work has demonstrated that MMC-loaded Ox-MS (MMC-Ox-MS) is a better candidate for intratumoral administration to solid tumors than Dox-MS. Moreover, combining Dox-MS and MMC-Ox-MS for locoregional (intratumoral injection) yielded at least an additive toxicity in the EMT6 model tumor system without evidence of additional toxicity [21]. This previous in vivo work, which is summarized in Fig. 1, was only done with Dox-MS and MMC-Ox-MS being injected intratumorally at the same time. The effectiveness of drug combinations has been shown to be dependent on the timing of drug administration [22,23]. Therefore, it was of interest to determine if the scheduling of one drug relative to the other was important for their increased activity.

While in vivo preclinical tests are critical for determining the efficacy of microsphere-delivered drugs and drug combinations, the accuracy, simplicity and sensitivity of in vitro cell culture assays allows for more rapid evaluation of the developed microsphere systems and various drug combinations. Thus, the present work was designed to investigate—[1] the in vitro cytotoxicity of microsphere-delivered MMC and [2] the ability of MMC/Dox combinations to inhibit the clonal expansion of murine breast cancer cells. That is, to define effective schedules for possible future in vivo studies using the developed MMC-Ox-MS and Dox-MS systems. A monolayer-cell system was employed to test the nature of the interaction of MMC and Dox at various concentrations and schedules to eliminate the interference of Dox binding in a multilayer-cell model [24,25], which affects the available drug concentrations thus complicating the analysis of the concentration effect.

2. Materials and methods

2.1. Materials

Sulfopropyl dextran MS (SP-MS; Sephadex SP C-25) and Dox were purchased from Sigma Chemical Company (St Louis, MO, USA). MMC was obtained from Faulding (Canada), Inc. (Kirkland, QUE, Canada). All cell culture plastic-ware was purchased from Sarstedt (Montreal, QUE, Canada). Cell culture media, α-Minimum Essential Medium (α-MEM), was acquired from the Ontario Cancer Institute (Toronto, Ont., Canada) and fetal bovine serum (FBS) was purchased from Cansera International Inc. (Etobicoke, Ont., Canada).

2.2. Cell culture

Wild-type murine breast tumor cells (EMT6/WT) and its P-gp overexpressing multidrug resistant variant (EMT6/AR1.0) [26] were generously provided by Dr I. F. Tannock of the Ontario Cancer Institute, Toronto, Canada. The doubling time of the cell-line during the exponential growth phase was 14–16 h. Cells cultures were grown as a monolayer

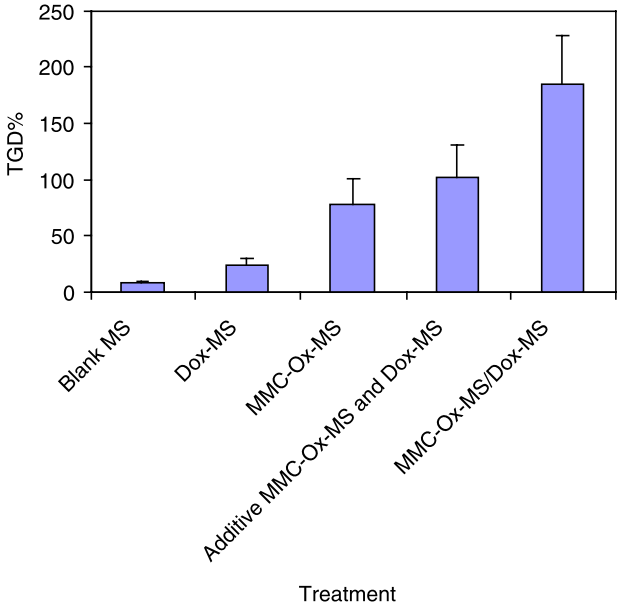

Fig. 1. Comparison of tumor growth delay (TGD%) in mice bearing EMT6 tumors treated by a single intratumoral injection of blank Ox-MS, Dox-MS, MMC-Ox-MS, calculated MMC-Ox-MS plus Dox-MS or measured Dox-MS with MMC-Ox-MS. The 'additive MMC-Ox-MS plus Dox-MS' was based on a calculation of simple additivity of the single agent experimental results. Data replotted from Ref. [21]. Percent growth delay is calculated relative to the time tumor bearing control mice intramuscular leg tumors take to grow from 300 to 1.1 gm. Bars are the mean values plus or minus the SEM except the experimental MMC-Ox-MS which shows the standard deviation from a single experiment.

on 175 cm^2 tissue culture flasks in α-MEM plus antibiotics (streptomycin and penicillin, 0.1 g/L each) supplemented with 10% FBS (growth medium), at 37 °C in a 5% CO_2/95% air humidified incubator.

2.3. Preparation of MMC loaded MS

The preparation of MMC-Ox-MS is described in detail elsewhere [20]. Briefly, to improve loading and release of MMC from the microsphere system, SP-MS was chemically modified. In a typical modification process, 50 mg of SP-MS were oxidized by incubation with 10 ml of an aqueous solution of 0.1 M sodium periodate for 15 h at 4 °C. Following the reaction, Ox-MS were washed successively with 5 ml of 0.2 M lead acetate and 2 ml 0.5 N HNO_3, extensively rinsed with deionized-distilled (DDI) water and dried by lyophilization.

In a typical loading process, 5 mg of dry Ox-MS were incubated in a 1 ml aqueous MMC solution (e.g. 100–300 μg/ml) for 48 h at 4 °C (to minimize drug degradation). MMC was loaded onto the microspheres by covalent bonding, ionic complexation, and hydrophobic interaction between the polymer and MMC as described previously [20]. The amount of drug in Ox-MS was estimated by determining the difference between the concentration of the MMC loading solution before and after incubation using an ultraviolet–visible (UV–vis) spectrophotometer (Hewlett Packard 8452, Palo Alto, CA, USA) at a wavelength of 364 nm ($\varepsilon_{364}=40\ mM^{-1}\ cm^{-1}$). MMC-Ox-MS were then rinsed with DDI water, lyophilized, and stored at room temperature for further studies. Stability of dry MMC-Ox-MS is maintained for at least 6 months (data not shown).

2.4. Evaluation of cytotoxicity for single agents and blank MS (Tests #1–#5, Table 1)

All in vitro toxicity tests were carried out using cultured cells grown as monolayers. Prior to drug or blank microsphere exposure, EMT6 cells (see Table 1) were seeded in 10 cm plastic Petri dishes containing 10 ml growth medium for 24 h. For the drug exposure experiments, aliquots of either Dox or MMC aqueous solutions were incubated with the cells at 37 °C for 1 h. Blank MS cytotoxicity was evaluated by incubating an excess (up to 10 mg/ml) of blank Ox-MS or SP-MS with the cells at 37 °C for 1 week. Following incubation, dishes were rinsed with phosphate buffer solution (PBS), cells trypsinized and cytotoxicity evaluated using a clonogenic assay (described below).

The toxicity of MMC delivered by Ox-MS (released MMC) was examined by incubating 5 mg of MMC-Ox-MS, in the absence of cells, in 3 ml of 0.15 M PBS at 37 °C for 48 h. The release medium was then collected and the MMC concentration (i.e. the amount of drug released from Ox-MS) was determined by spectrophotometry at 364 nm. Toxicities of increasing equivalent doses of aqueous solutions of either fresh MMC or released MMC were compared as described above. Specific details regarding the initial cell plating densities, drug doses employed, and the amount of blank MS used for the described exposure studies are summarized in Table 1.

Table 1
Evaluation of in vitro toxicity experiments (EMT6/WT)

Test	Experiment	Initial cell plating	Dose regimen
Single agent (drug solutions or MS)			
#1	Blank Ox-MS	2×10^5[a]	0–10 mg/ml
	Blank SP-MS		0–10 mg/ml
#2	MMC	5×10^5[b]	0–2 μg/ml
#3	Released MMC	5×10^5	0–1.5 μg/ml
#4	Dox	5×10^5	0–4 μg/ml
#5	Cell density	2×10^5	0.5 μg/ml (MMC and Dox)
MMC/Dox combinations			
#6	MMC pretreatment	5×10^5	0.17 μg/ml MMC 0–3 μg/ml Dox
#7	Exposure schedules	2×10^5	0.5 μg/ml (MMC and Dox)

[a] Initial plating was 2×10^5 but at time of actual drug exposure was 4×10^5.
[b] Initial plating was 5×10^5 but at time of actual drug exposure was 1×10^6.

2.5. Evaluation of non-toxic MMC pretreatment and subsequent Dox exposure (Test #6, Table 1)

The protocol for this set of experiments was adapted from that of Hamilton and coworkers [27] in which they sequentially incubated rat hepatoma cells (H4IIE) with the maximum non-toxic dose of MMC, which caused a drop in membrane P-glycoprotein (P-gp) levels, and resulted in a subsequent increase in sensitivity of the cells to a Dox exposure at later times. These workers measured cell sensitivity by a colorimetric assay. In the present study, a 1 h MMC or Dox incubation time was maintained to facilitate toxicity comparisons amongst the various treatment regimens. After initial plating, EMT6/WT cells were exposed to a maximally non-toxic dose (i.e. 0.17 μg/ml) of MMC (see Table 1). Following drug incubation, cells were washed with PBS and then re-immersed in 10 ml of drug-free growth medium. The culture dishes were incubated for 72 h at 37 °C. Following the incubation period, cells were exposed to increasing doses of Dox (0–3 μg/ml). The cytotoxicity of the combination was determined using a clonogenic assay, as described below, and compared to that of cells which were not pretreated with MMC prior to Dox exposure.

2.6. Evaluation of MMC and Dox exposure schedules and cell density effects (Test #7, Table 1)

As shown in Fig. 2a, at 0 h, aliquots of either single agent MMC or Dox were distributed amongst fourteen 10 cm diameter Petri dishes, with another dish receiving both drugs (Dox and MMC), and a final dish serving as an untreated control (for a total of 16 dishes). Following drug exposure, the cells were washed with PBS. Cells in four of the sixteen dishes (control dish containing no drug [A1], the Dox + MMC dish [D1], one MMC only dish [C1], and one Dox only dish [B1]) were trypsinized, counted, and plated in various dilutions in 6 cm Petri dishes containing growth medium. Twenty-four hours following the initial drug exposure, four dishes (two with initial MMC exposure [A2, B2] and two with initial Dox

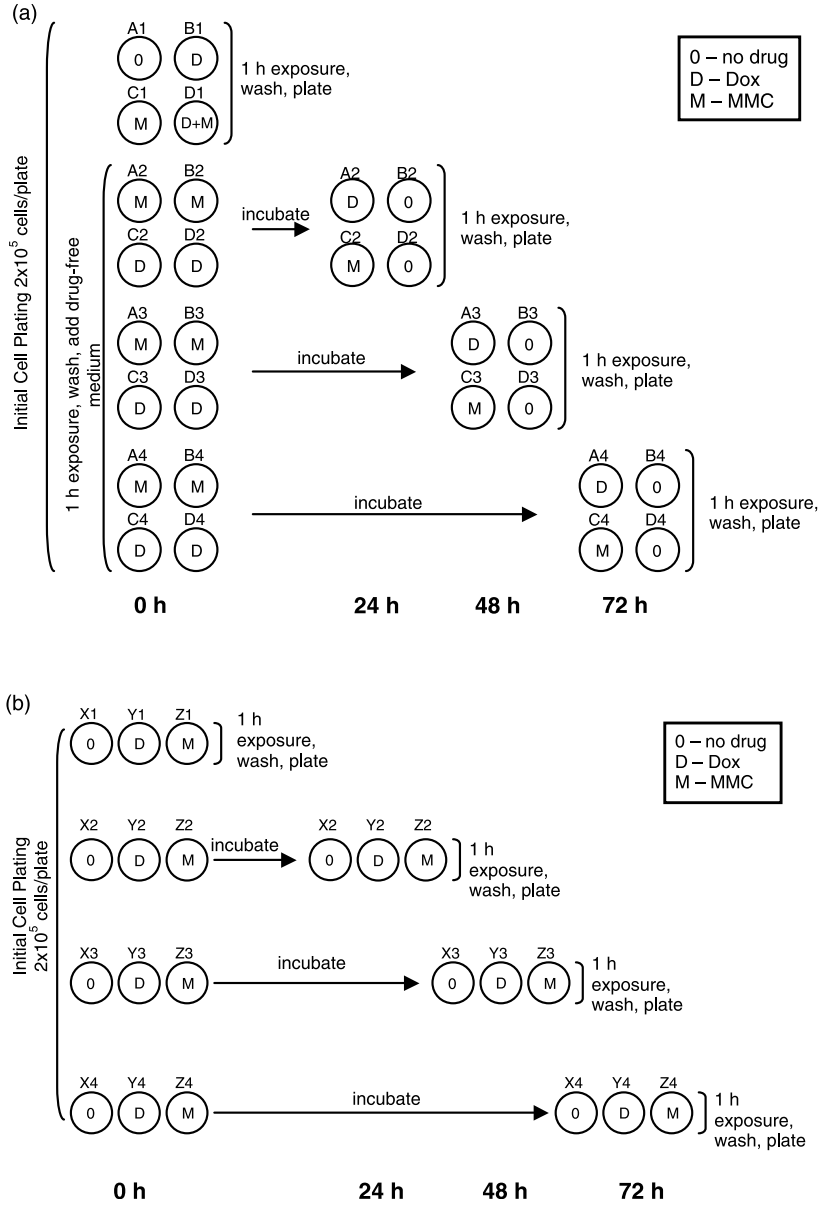

Fig. 2. (a) Experimental scheme of effect of Dox and MMC exposure schedules on the survival of EMT6/WT cells. 2×10^5 cells are plated 24 h prior to initial drug exposure (0 h). The following day, cells are exposed to no drug, MMC, Dox, or MMC + Dox for 1 h. After the drug exposure period, cells are either plated (i.e. A1–D1) or incubated in fresh medium (i.e. A2–D4). At 24 h (A2–D2), 48 h (A3–D3), and 72 h (A4–D4), selected dishes were exposed to no drug, MMC, or Dox for 1 h. After the drug exposure period, cells were plated. (b) Experimental scheme of effect of cell density on the toxicity of Dox and MMC to EMT6/WT cells. 2×10^5 cells are plated 24 h to prior to study initiation. The following day (0 h), cells on dishes X1–Z1 are exposed to no drug, MMC, or Dox for 1 h. After the drug exposure period, cells are plated. At 24 h (X2–Z2), 48 h (X3–Z3), and 72 h (X4–Z4), selected dishes were exposed to no drug, MMC, or Dox for 1 h. After the drug exposure period, cells were plated.

exposure [C2, D2]) were separated from the remaining 12 dishes. One of the two dishes that initially received MMC was subsequently exposed to Dox (MMC before Dox [A2]). Similarly, one of the two dishes initially receiving Dox was subsequently exposed to MMC (MMC after Dox [C2]). This drug exposure schedule continued at 48 h (dishes A3–D3) and at 72 h (dishes A4–D4).

In the preceding scheduling study, variability in cell number with time is inevitable. To assess the significance of this variability on cell sensitivity to the drugs, the following experiment was conducted. As illustrated in Fig. 2b (Test #5, Table 1), at different times following initial cell plating, dishes X1–Z1 (at 0 h), X2–Z2 (at 24 h), X3–Z3 (at 48 h), and X4–Z4 (at 72 h) were exposed to either Dox or MMC, with the final dish serving as an untreated control. The number of cells in each dish was determined by trypsinization and counting using a hemocytometer at the time of drug exposure.

2.7. Determination of cell survival by clonogenic assay

Ten centimeter diameter Petri dishes containing cells exposed to blank MS, or Dox and MMC, as single agents or

in combination, were washed with 5 ml of PBS and detached by treatment with 5 ml of PBS containing sodium citrate and supplemented with 0.25% (w/v) trypsin (VWR International, Mississauga, Ont., Canada) following treatment. The re-suspended cells were counted with a hemocytometer and plated at various dilutions in 6 cm Petri dishes containing 5 ml of growth medium. Cell cultures were then incubated for 1 week at 37 °C in a humidified incubator with a mixture of 95% air and 5% CO_2, allowing viable cells sufficient time to grow into macroscopic colonies. The medium was then removed and the cells were fixed and stained with a 0.5% solution of methylene blue (Sigma-Aldrich Canada, Oakville, Ont., Canada) in 70% ethanol. The number of colonies formed was counted on a light table, from which plating efficiencies were calculated based on the number of cells plated in the 6 cm culture dishes. Surviving fractions were determined by dividing the plating efficiency of the drug-treated cells by that of cells without exposure to the drug (i.e. the control) [28]. The control plating efficiency of EMT6/WT was 0.57 ± 0.09 ($n = 20$).

2.8. Western blotting

Crude membrane preparations from cultured EMT6/WT cells (untreated or collected at 0, 24, 48, and 72 h after MMC exposure) were prepared by a method described previously by Ronaldson et al. [29]. Protein concentrations of the crude membrane preparations were determined by the Bradford protein assay method.

For immunoblotting, 25 μg aliquots of crude proteins were mixed in Laemmli buffer and resolved on an 8.5% sodium dodecyl sulfate-polyacrylamide gel. The gel was then electrotransferred onto a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4 °C in Tris-buffered saline (15 mM Tris–HCl, 150 mM NaCl, pH 7.6) containing 0.05% (v/v) Tween-20 (TBS-T) and 5% (w/v) dry skim milk powder. Following six washes (5 min each) with TBS-T, the membrane was incubated with the monoclonal P-gp antibody (C219, ID Labs, London, ON, Canada; 1:500 dilution in 5% milk) for 4 h at room temperature for P-gp detection, or the monoclonal-actin antibody (AC-40, Sigma-Aldrich Canada, Oakville, Ont., Canada; 1:750 dilution in 5% milk) for 4 h at room temperature for β-actin detection. Following a second wash, the membranes were incubated for 2 h in the presence of anti-mouse [1:5000] horseradish peroxidase-linked secondary antibodies (Serotec Inc., Raleigh, NC, USA) in 5% milk at room temperature. Protein bands were detected by enhanced chemiluminescence and exposed to X-ray film for 1 min. The EMT6/AR1.0 P-gp over-expressing cell-line was used as a positive control.

2.9. Statistical analysis

All experiments were repeated 3–5 times. Data is presented as the mean value plus or minus the standard error of the mean (SEM). Students *t*-test was used to test the statistical significance of results ($P \leq 0.05$). Analysis for possible synergistic interactions of MMC and Dox was carried out by the isobologram technique of Steel and Peckham [30]

For each of the in vitro bioactivity tests, experiments were conducted at least in triplicate. Within each experiment, experimental data points were determined in quadruplicate. The results are expressed as means ± SEM. Data was entered into Microsoft Excel (Microsoft Corporation) and analyzed using the statistical package S-Plus 2000 (MathSoft, Inc.). For each of the drug combination studies, a linear regression analysis was used to assess the effect of dose and treatment on the log-transform of the measured outcome (i.e. fractional survival). An interaction term between dose and treatment was constructed in order to compare the dose-response relationship between the two treatment groups (i.e. single agent drug versus combined drugs). A statistical difference was declared if the interaction term was significant at a 0.05 level of significance.

3. Results

3.1. Cytotoxicity of blank Ox-MS or SP-MS

The surviving fraction of EMT6/WT cells directly incubated with up to 10 mg/ml of blank Ox-MS or SP-MS for 7 days was measured (Test #1, Table 1). Using a clonogenic assay method, no change in cell survival was observed indicating that the MS themselves, or any materials released from the blank MS, are not toxic to the cells under the conditions studied (data not shown).

3.2. In vitro activity of MMC released from Ox-MS

As shown in Fig. 3, the in vitro activity of released MMC in the EMT6/WT cell-line (Test #3, Table 1) is similar to that of a fresh MMC solution (Test #2). In general, EMT6/WT cells are sensitive to both released and fresh MMC, as both curves follow a similar trend of dose dependence, though released MMC appears to be slightly more effective than the fresh

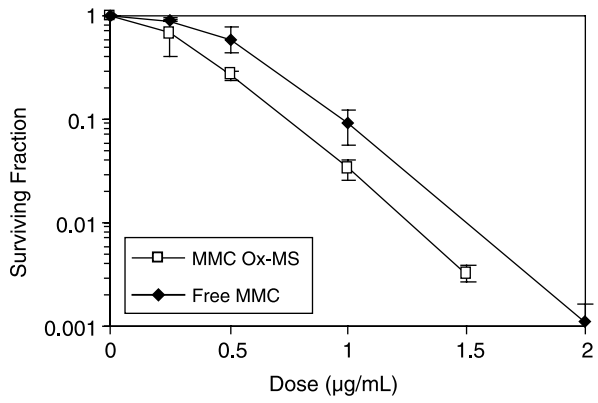

Fig. 3. Surviving fraction of EMT6/WT cells after 1 h exposure to increasing concentrations of either free or MMC released from Ox-MS (Tests #2 and #3). For MMC-Ox-MS loading, 5 mg of Ox-MS were loaded in a 300 μg/ml MMC solution. For MMC-Ox-MS release, the drug-loaded Ox-MS was incubated in 3 ml of a well-stirred 0.15 M PBS at 37 °C. *Data points,* means ± SE of measurements from independent experiments ($n = 3$).

MMC solution (dose resulting in 10% survival, $D_{10}=0.72$ μg/ml versus 0.93 μg/ml for fresh MMC; P-value $=0.007$). This result suggests that released MMC retains its activity against the clonogenic potential of EMT6 cells, and further confirms the usefulness of the MMC-Ox-MS system.

From the present study, released MMC was observed to have similar activity against EMT6/WT cells when compared to equal doses of a fresh MMC solution. Moreover, both blank Ox-MS and SP-MS were found to be non-toxic to EMT6/WT cells. Given that previous in vitro investigations of Dox released from SP-MS by Liu et al. [14] suggested that the activity of released Dox was unchanged from equivalent doses of a fresh Dox solution, all subsequent in vitro cell experiments were carried out using fresh drug solutions.

3.3. Effect of single agent Dox or MMC on the survival EMT6/WT cells

Prior to the investigation of the effects of various MMC/Dox combinations on the survival of EMT6/WT cells, the effect of single agent Dox on cell survival was also determined (Test #4, Table 1) to establish a baseline of cytotoxicity for the drug alone on the cell-line. As shown in Fig. 4, EMT6/WT cells are sensitive to Dox ($D_{10}=0.95$ μg/ml; 1.6 μM). Furthermore, Dox toxicity is similar, based on drug concentrations, to that observed with MMC ($D_{10}=0.93$ μg/ml; 2.8 μM).

3.4. Non-toxic MMC pretreatment and subsequent Dox exposure

Experiments conducted by Hamilton and coworkers showed that pretreatment of cancer cells with non-toxic doses of MMC was able to enhance the toxicity of Dox due to down regulation of P-gp [27]. A MMC pre-exposure time of 72 h was chosen to correspond with the time which was previously observed to yield minimum P-gp expression in rat hepatoma cells [27].

Over the concentrations investigated (i.e. 1 h pre-exposure of cells to 0.17 μg/ml MMC followed by a 1 h exposure to 0–3 μg/ml Dox 72 h post-MMC; Test #6, Table 1), no difference was observed between the surviving fraction of EMT6/WT cells exposed to the drug combination versus cells exposed to Dox alone (data not shown). Similarly, no difference in toxicity was observed between MMC pretreated cells and non-MMC treated cells when the combination was applied to the P-gp over-expressing AR1.0 cell-line (data not shown).

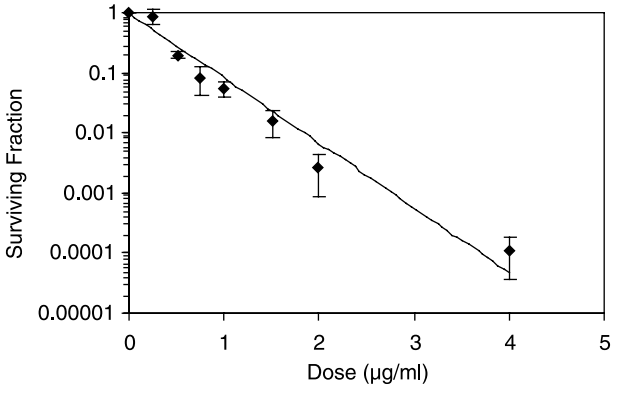

Fig. 4. Surviving fraction of EMT6/WT cells after exposure to Dox (Test #4). For all experiments, cells growing in a monolayer were exposed to varying concentrations of drug for 1 h. *Data points,* means ± SE of measurements from independent experiments ($n=5$).

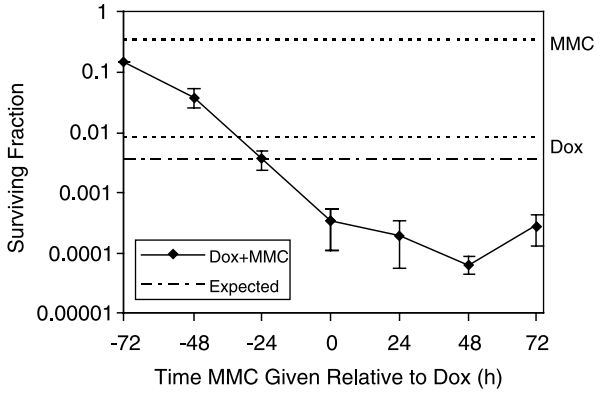

Fig. 5. Surviving fraction of EMT6/WT cells after treatment with MMC (0.5 μg/ml) and then Dox (0.5 μg/ml) at negative times (plates A2, A3, A4) or Dox and then MMC at positive times (plates C2, C3, C4) (Test #7 and Fig. 2a). Dotted lines represent the surviving fraction of cells from single doses of MMC and Dox and dashed line is the expected surviving fraction based on simple additivity of MMC and Dox. *Data points,* means ± range of measurements from independent experiments ($n=2$).

3.5. Effect of MMC and Dox exposure schedules on the survival of EMT6/WT cells

Given that non-toxic MMC pretreatment 72 h before Dox exposure had no effect on the sensitivity of EMT6 cells to Dox, the effect of applying toxic concentrations of MMC at various times before, or after Dox exposure using fixed toxic doses of each drug was investigated to determine if the sequence and interval of time between drug exposures would affect cell survival (Test #7, Table 1 and Fig. 2a). As shown in Fig. 5, when MMC and Dox are given together (time 0 h; dish D1), there is a significantly greater-than-additive toxicity observed as determined by a simple additivity model (depicted by dashed lines). Simple additivity is defined as the product of the surviving fraction from a given dose of drug A and the surviving fraction from a given dose of drug B. The regimen using MMC after Dox (positive times; dishes C2, C3, and C4 in Fig. 2a) seems to maintain the greater-than-additive effect, as indicated by the data points below the dashed line. However, when MMC is given before Dox (negative times; dishes A2, A3, and A4) the effect of the two agents varies from additive at −24 h (on the dashed line) to less-than-additive at −48 and −72 h (above the dashed line in Fig. 5). Experiments carried out using higher or lower doses (e.g. 0.5 μg/ml MMC, 0.25 μg/ml Dox) of Dox and MMC yielded curves similar in shape to the curve in Fig. 5 (data not shown). These results suggest that

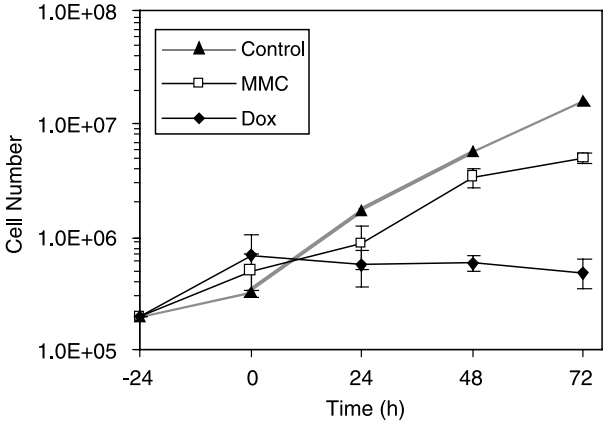

Fig. 6. Growth curves for EMT6/WT cells following exposure to either Dox (0.5 μg/ml) or MMC (0.5 μg/ml). *Data points,* means ± SE of measurements from independent experiments ($n = 4$).

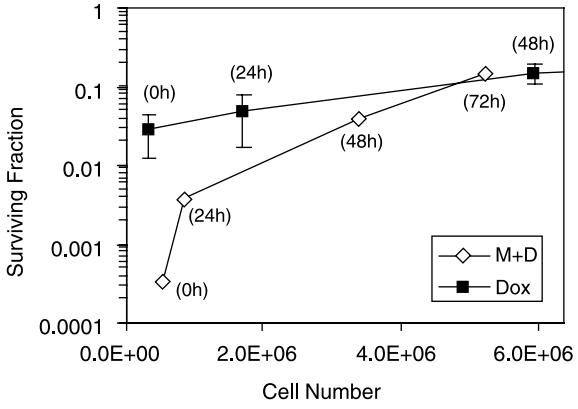

Fig. 8. Surviving fraction of EMT6/WT cells after treatment with Dox (0.5 μg/ml) at various times (0, 24, 48, or 72 h) following MMC (0.5 μg/ml) exposure (data re-plotted from Fig. 5) or single agent Dox (0.5 μg/ml) at various times following initial cell plating (data re-plotted from Fig. 7). The survival curves have been corrected for cell number.

the order and interval of MMC/Dox combinations are important to achieve maximum toxicity.

3.6. The effect of cell density on the sensitivity of EMT6/WT cells to Dox and MMC

The effect of EMT6/WT cell density on drug sensitivity was studied because as the time between the exposure of cells to MMC and Dox approached 72 h, the number of cells per dish increased. This can be seen in Fig. 6, where EMT6/WT cells grow substantially after treatment with 0.5 μg MMC alone. This growth is greater than for treatment with 0.5 μg Dox alone ($P < 0.0001$). Microscopic examination showed that at 72 h following MMC exposure (dish Z4), cells had overgrown to such an extent that cells were beginning to pile up on each other.

Fig. 7 presents the surviving fraction of cells exposed to Dox (0.5 μg/ml) or MMC (0.5 μg/ml) as a function of the interval between initial cell plating and drug exposure (Test #5, Table 1 and Fig. 2b). While the surviving fraction of cells exposed to Dox was observed to increase as the interval between initial cell plating and drug exposure lengthened, the surviving fraction of cells exposed to MMC remained constant over 24 h, with a marginal decrease in surviving fraction over 72 h. Since cell number increases with time after initial plating, this result suggests that the efficacy of Dox is influenced by the number of tumor cells present, whereas the efficacy of MMC is independent of cell density.

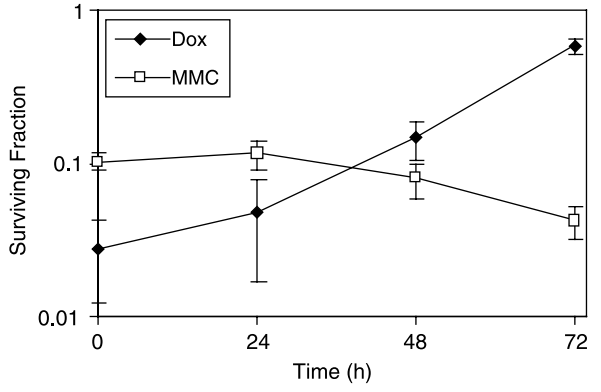

Fig. 7. Surviving fraction of EMT6/WT cells after treatment with either MMC (0.5 μg/ml) or Dox (0.5 μg/ml) at various times after initial cell plating (Test #5 and Fig. 1b). *Data points,* means ± SE of measurements from independent experiments ($n = 3$).

To examine the extent to which cell density contributes to the sensitivity of the cells to Dox following MMC (Fig. 5), the surviving fraction of cells is plotted against cell number in Fig. 8 for the cells exposed to Dox after MMC pretreatment (data from Fig. 5) or exposed to Dox alone at various times after cell plating (data from Fig. 7). This figure shows that the surviving fraction of cells increases with increasing cell number in both cases but the MMC pretreated cells lose their enhanced sensitivity rapidly between the 0 and 24 h point and enhanced sensitization is entirely lost by 48 h. After 48 h the loss of sensitivity in the combined drug treatment is primarily controlled by the increase in cell density.

3.7. Investigation of EMT6/WT cells for P-gp up-regulation

Western blot analysis was conducted to determine whether an up-regulation of P-gp by MMC treatment could be responsible for the observed reduction in Dox sensitivity. Investigation of EMT6/WT cells (both untreated and cells pretreated with MMC and allowed to grow for up to 72 h following drug exposure) using the monoclonal P-gp antibody, C219, did not detect a single band at approximately 170–180 kDa—a size previously reported for P-gp [31] (data not shown). The C219 antibody recognizes a conserved cytoplasmic epitope on all isoforms of murine P-gp [32]. The AR1.0 cell-line, a P-gp over-expressing variant of the EMT6/WT cell-line, served as a positive control and a single robust band was detected at the expected molecular weight (data not shown). A single band at approximately 43 kDa, which

corresponds to β-actin, was detected in each lane, suggesting that the appropriate quantity of protein was added for each sample. Taken together, these data imply that P-gp does not contribute to the observed reduction in Dox cytotoxicity in the EMT6/WT cell-line.

4. Discussion

We have previously studied the effectiveness of intratumorally injected microspheres containing Dox or MMC given alone or together in the in vivo EMT6 murine solid tumor system [13,21]. Results indicated a greater than simple addition of individual drug toxicities when the drugs were administered together when percent tumor growth delay was used as an endpoint (Fig. 1). The present in vitro studies using the same tumor cell line yielded additional information regarding [1] the biological effectiveness of the MMC released from MMC loaded microspheres and [2] the nature of the interaction between Dox and MMC. This information is useful for the interpretation of our previous in vivo results [21], suggesting that there is a true synergistic interaction between the two drugs and the planning of future in vivo experiments using this drug combination e.g. drug schedule testing.

4.1. Studies of MMC released from MMC-OX-MS loaded microspheres

In our previous in vivo studies, we found that MMC-Ox-MS injected intratumorally into EMT6 tumors had the same effectiveness in inhibiting tumor regrowth as an identical amount of free drug given by i.p. injection though the toxicity to the whole animal of intratumorally injected drug was less [21]. Two explanations for the equal effectiveness of the intratumour injected drug and the i.p. injected drug are possible. It was shown that only 35% of the microsphere loaded MMC was released in vitro [20], thus one explanation for the equal effectiveness was that the intratumorally injected animals practically received one-third the dose of active MMC as the i.p. injected animals. A second possibility was that the drug released from the MS was not as effective on a molar basis as freshly prepared drug. The results shown in Fig. 3 indicate the MS released drug is at least as effective as free drug on a molar basis arguing against this second possibility. Also unloaded MS were not toxic in vitro consistent with the lack of toxicity of unloaded microspheres in the in vivo system [21].

Several previous studies have shown that MMC can be incorporated into albumin microspheres [33–35], but no study has been carried out to evaluate the bioactivity of MMC released from the MS in cell culture. Cummings et al. investigated the anticancer activity of intratumorally injected MMC-albumin MS in mice bearing MAC16 tumor [35]. However the drug loaded MS were less active than free MMC and no more active than the blank MS. In contrast, MMC-Ox-MS developed in this work exhibited equivalent activity as free MMC and was over 70% more active than the blank Ox-MS [21]. This result and the data shown in Fig. 3 suggest that MMC released from the Ox-MS retains full anticancer activity and Ox-MS may be a more suitable delivery system for MMC than the albumin MS.

4.2. Studies of MMC and Dox toxicity alone and together

Exposing EMT6 cells to different MMC or Dox concentrations for 1 h yielded survival curves with a small shoulder followed by an exponential decline or a straight exponential survival curve respectively (Figs. 3 and 4). These curves were used to construct an envelope of additivity for isobologram analysis of the results for combinations of MMC and Dox. This decrease in cell survival, when Dox and MMC are given together (Fig. 5), is super additive (synergistic) when subjected to isobologram analysis. Isobologram analysis is an accepted means of establishing anticancer drug combination efficacy under the assumption that the effects of the drugs are mutually exclusive as discussed by Steel and Peckham [30]. Dox and MMC are two drugs that are rarely used in combination therapy clinically. However, the above in vitro results and our previous in vivo results [21] suggest that there is a potential benefit when these drugs are given together. We also studied the results of giving the same doses of MMC or Dox at different times relative to one another.

4.3. MMC/Dox interactions: MMC after Dox regimen

The apparent therapeutic advantage gained by using MMC/Dox combinations simultaneously is maintained if MMC follows Dox, as demonstrated by the greater-than-additive decrease in cell survival (Fig. 5). In addition, the increase in cytotoxicity does not appear to be significantly affected by the time interval between the applications of the two drugs for up to 72 h, as long as MMC follows Dox. This does not appear to involve the induction of the MMC activating enzyme DT-diaphaorase, which was suggested by Begleiter and Leith who investigated the DT-diaphorase inducing ability of Dox, and the in vitro cytotoxicity of a combination of the drugs against murine breast cancer cells (EMT6) and murine bone marrow cells [36]. Synergistic cell kill was found in both cell-lines when MMC was administered 22 h following Dox. However, this effect could not be directly related to elevated levels of DT-diaphorase. Dox pretreatment did lead to a 40% higher DT-diaphorase expression in EMT6 cells but it did not lead to an increase in the DT-diaphorase level in bone marrow cells, though it increased toxicity to both bone marrow cells and EMT6 cells [36]. Simultaneous treatment with Dox and MMC resulted in a similar level of synergy in EMT6 cells, which involved no change in DT-diaphorase.

The synergistic toxicity of simultaneously applied MMC and Dox or MMC following Dox is most likely due to differences in the mechanism of action of the two drugs. While various chemotherapeutic agents are able to produce a wide array of damage to cancer cells, DNA double-strand breaks are lesions that correlate closely with cell death [37]. Dox is known to produce DNA double-strand breaks via irreversible binding to topoisomerase II [5]. MMC is known to form interstrand crosslinks in DNA [5]. Perhaps the formation of these two

major types of lethal lesions in the same cell results in more efficient cell killing than either alone due to the involvement of multiple repair systems.

4.4. MMC/Dox interactions: MMC before Dox regimen

The combination of Dox and MMC has also been previously studied in vitro by Hamilton and coworkers using rat hepatoma H4IIE cells [27,38,39]. It was observed that the efficacy of Dox, a known P-gp substrate [40], was enhanced when applied to cells following a non-toxic dose of MMC, likely due to a functional decrease in P-gp expression. Results at the molecular level, using the monoclonal C219 antibody [41] and western blot analysis showed that P-gp protein is not detectable in cultured EMT6/WT cells consistent with previous results [42]. It was also demonstrated that P-gp could not be detected in EMT6/WT cells up to 72 h following exposure to MMC, suggesting that P-gp is not involved in reduced cellular Dox toxicity in EMT6/WT cells pretreated with MMC.

As illustrated in Fig. 5, at increasing times following MMC exposure (−24 to −72 h), the surviving fraction observed for cells receiving MMC before Dox becomes increasingly sub-additive (above the dashed line), implying that cellular resistance to Dox apparently increases. One marked difference between cells initially exposed to MMC versus Dox is that subsequent to MMC treatment, viable EMT6/WT cells continue the process of cellular division at a much higher rate than cells receiving Dox, as demonstrated by Fig. 6. Thus, cells exposed to Dox at subsequent times following MMC treatment were present at progressively higher cell densities as the time of Dox exposure following MMC treatment increased.

Dox toxicity is reduced at higher cell densities as previously shown by Tunggal et al. [24]. In these experiments, involving Chinese hamster ovary (AuxB1) cells growing in a multilayer model, it was observed that the efficacy of Dox decreased as the cell concentration plated increased from 10^5 to 10^7 cells/ml due to the inability of the drug to penetrate through successive cell layers. Furthermore, it was also shown that the penetration of Dox through multilayer EMT6 cells was reduced compared with cells growing in a monolayer [25]. This reduction in toxicity seemed to be primarily dependent on the ratio of drug concentration to cell number rather than a cell cycle dependent reduction in Dox toxicity [43]. As previously mentioned, due to the design of the drug scheduling experiment, increasing cell number was an uncontrollable and potentially confounding factor of the observed experimental results. Thus, the effects of cell density on the sensitivity of EMT6/WT cells to the drugs of interest were studied by treating cells at different times after initial plating.

The cell density experiments clearly show that cell number plays a role in the sensitivity of the cells to Dox (Fig. 7). For equivalent drug doses, the surviving fraction of cells is greater as cell number increases. These findings help to explain the in vivo results from the Dox-MS studies by Liu et al. [13], in which the effectiveness of the locally delivered Dox in dense solid tumors was less than that predicted by in vitro experiments using monolayer cells, and are consistent with the observation with multilayered cells reported by Tunggal et al. [25].

The reduction in Dox toxicity at high cell densities may be attributed to two mechanisms: (i) as a weak base, Dox may become sequestered in acidic endosomes within cells; and as an intercalating agent with high DNA affinity, Dox may accumulate in peripheral cell layers [25] and (ii) Dox toxicity is dependent on the ratio of Dox concentration to cell number [43]. As a result, sub-additive toxicity is observed when Dox was given post-MMC.

In contrast to Dox, the toxicity of MMC is not reduced when cells are plated at higher density. This observation is consistent with reports in the literature that both exponentially growing and plateau phase EMT6 cells have similar sensitivities to MMC [44–46], assuming that highly confluent cells are in the G_0 (i.e. non-proliferative or plateau) phase of the cell cycle. The independence of survival on cell density may be explained by the inability of EMT6 cells to repair potentially lethal damage caused by MMC, which was observed by Rockwell in both in vitro cell cultures and solid tumors [19,45,46]. After treatment with MMC, EMT6 cells are able to divide as shown by increased cell number, but they are unable to recover their clonogenic potential. Therefore, the cell survival fraction, as determined by the clonogenic assay, remains constant up to 72 h following MMC exposure.

The demonstrated efficacy of Dox [13,14] and MMC [20] microsphere systems when studied separately, in vitro and in vivo and together [21], coupled with the present in vitro results, suggest that Dox-MS and MMC-Ox-MS may offer a therapeutic advantage when administered simultaneously with no further advantage in scheduling one drug before the other.

Previous studies of Dox and MMC simultaneously administered intraperitoneally in C3H mice bearing 16/C mammary tumors demonstrated a superadditive efficacy [17]. But, significant toxicity was also found leading to the conclusion that this combination was not therapeutically beneficial. Combinations of Dox and MMC have also been investigated in the clinic for breast cancer treatment but results have been equivocal [47,48]. Since these clinical studies were limited by the toxicities of the respective drugs, it may be possible to enhance the benefit of MMC/Dox combinations by employing a MS locoregional administration approach to minimize systemic toxicity. The locoregional administration approach will of course limit the effectiveness of the drug combination to the primary tumor as opposed to systemic disease.

5. Conclusion

MMC released from Ox-MS retains its activity *in vitro* when compared to fresh drug solutions. An increasing level of resistance is observed when EMT6/WT cells are treated with a toxic dose of Dox up to 72 h after MMC. This is due to both a cell density effect, whereby cells initially exposed to MMC undergo increased proliferation and a rapid loss in toxicity of the drug combination over the first 24 h between MMC and Dox administration. Conversely, a toxic dose of MMC applied

simultaneously or up to 72 h after Dox, leads to a greater-than-additive decrease in surviving fraction of tumor cells, resulting from a synergistic combination of lethal effects. These results are consistent with in vivo investigations of locally administered combinations involving Dox-MS and MMC-Ox-MS [21]. Taken together, if Dox and MMC are administered to solid tumors via MS using an optimal schedule, not only would an advantage be gained from the drug combination itself, but the benefits associated with locally administered MS (e.g. significantly reduced systemic drug toxicity) could also be achieved.

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