Cancer Chemother Pharmacol (1989) 24: 349–353

Cancer Chemotherapy and Pharmacology

Modulation of the antineoplastic efficacy of mitomycin C by dicoumarol in vivo*

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Summary. Dicoumarol (DIC) modulates the intracellular metabolism of mitomycin C (MC) in vitro, increasing the toxicity of MC to hypoxic EMT6 cells and decreasing its toxicity to aerobic cells. The present experiments assessed whether DIC could be used to increase the therapeutic ratio attainable in vivo when MC was used as an adjunct to radiotherapy. Experiments with transplanted EMT6 tumors in mice showed that DIC increased the toxicity of MC to hypoxic tumor cells and increased the antineoplastic efficacy of regimens combining MC with radiation. DIC did not increase the hematologic toxicity of MC, and pretreatment with DIC plus MC did not augment radiation-induced skin reactions. The increase in antineoplastic effect was therefore obtained without a concomitant increase in normal tissue toxicities, and therapeutic gain was obtained.

Introduction

Mitomycin C (MC) has been used for many years in the chemotherapy of cancer, both as a single agent and as part of combination chemotherapy regimens [1]. Recently we explored the possibility of using MC as an adjunct to radiotherapy [5–8, 15–18, 20, 22, 23]. We hypothesized that regimens combining these two complimentary agents might be especially efficacious in treating solid tumors [5, 16, 22, 23], because solid tumors contain both hypoxic and aerobic cells [11] and radiation is preferentially toxic to well-oxygenated cells [11], whereas MC is selectively toxic to cells under hypoxic conditions [5, 6, 10, 18]. Such regimens have been tested in laboratory animals [5, 16, 17, 22, 23] and in a recent clinical trial [26], with very encouraging results.

Previous studies in our laboratories have shown that dicoumarol (DIC), an inhibitor of DT-diaphorase, can be used to modulate the metabolism of MC and the cytotoxicity of MC to EMT6 tumor cells in vitro [6–8, 20]. DIC increases the cytotoxicity of MC to hypoxic EMT6 cells but decreases the toxicity of the drug to aerobic cells [6, 8, 20]. We suggested [7] that DIC might be used to improve the therapeutic ratios attainable with MC in situations in which the toxicity of MC to hypoxic tumor cells was critical in determining its antineoplastic efficacy, as, for example, in regimens combining MC with radiation. Preliminary studies with mice [7] suggested that this approach might have merit. The experiments described in this report were carried out to examine in more detail the clinical potential of therapeutic regimens using DIC to modulate the metabolism of MC.

Materials and methods

Animals and tumors. BALB/c Rw mice, 2.5–3 months old, were bred and maintained at Yale University under specific-pathogen-free conditions [17]. EMT6 cells (subline EMT6-Rw) were maintained by alternate passage in mice and cell culture, as previously described [14]. Tumors were implanted by intradermally injecting mice in the flank with 2×10^5 cells harvested from exponentially growing cell cultures [14]. They were used for experiments at a volume of $\sim 100\ mm^3$, attained approximately 2 weeks after implantation. The viability of cells suspended from treated tumors was assayed by determining their ability to form colonies on cell culture dishes, as previously described [14, 17]. The survival of cells in treated tumors was calculated by comparing their clonogenicity with that of cells from untreated tumors assayed on the same day. The numbers of cells suspended from treated and control tumors were examined to ensure that there was no rapid loss of dead cells, which might compromise the cell-survival measurements [17]; no changes in cell yield were observed.

Analysis of the survival of marrow stem cells. Femurs were removed 1 h after MC treatment and the marrow was flushed from the femurs with a 25-gauge needle and supplemented McCoy's 5A medium [21]. The viability of the pluripotent progenitor cells (CFU-S) in the resulting single-cell suspension was assayed using the spleen colony technique of Till and McCulloch [25] exactly as previously done in our laboratory [21]. The viabilities of the granulocyte/monocyte and megakaryocyte progenitors (CFU-GM and CFU-MK) were assessed using the modification of the soft-agar assay of Williams and Jackson [27] described by Gamba-Vitalo et al. [3], again, exactly as previously done in our laboratory [21]. Surviving fractions were calculated using the cloning efficiencies of cells from untreated mice

* This research was supported by research grants PDT-145 from the American Cancer Society (SR) and CA-43659 from the National Cancer Institute (ACS, SRK)

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assayed the same day. The numbers of cells suspended from treated and control femurs were compared to detect any rapid cell death; no changes in yield were observed.

The toxicity of MC ± DIC was assessed by treating animals with graded doses of MC spanning the expected $LD_{50/30}$ for MC alone and examining the mice daily for 30 days to assess the resulting mortality [21]. Animals were euthanized when they showed physical or behavioral evidence of terminal toxicity. Data were analyzed using probit techniques [11, 21].

Skin reactions. The acute response of the skin to radiation was tested on the hind feet of BALB/c Rw mice. Mice (5–7/group) were lightly anesthetized with chloral hydrate; the left hind feet were immobilized and locally irradiated with 30 Gy 250 kV X-rays. Mice were examined three times per week. Changes in the irradiated skin were assessed using an arbitrary scale that grades the severity of the damage in terms of the degree of erythema, amount of dry desquamation, and area of moist desquamation [12]. Because of the subjectivity of this scoring process, one observer scored all reactions; scoring was blinded such that the observer did not know either the treatment or the previous reactions recorded for the animal.

Drugs. MC was a gift from Dr. T. W. Doyle of Bristol Laboratories (Wallingford, Conn). The drug was dissolved in warm physiologic saline, protected from light, and injected i.p. DIC (Sigma Chemical Co.; St. Louis, Mo) for i.p. injection was dissolved in sterile physiologic saline with stoichiometric amounts of NaOH. DIC for administration in the drinking water was dissolved in dionized water.

Irradiation. In tumor studies, unanesthetized mice were whole-body-irradiated with 250 kV X-rays at a dose rate of 1.6 Gy/min in a chamber designed to enable irradiation under controlled atmospheres [11, 14, 19]. During aerobic irradiation, mice were gassed with air. To induce hypoxia in the tumors, animals were asphyxiated with N_2 5 min before irradiation and were held under N_2 throughout the irradiation [14, 19]. Mice for marrow studies were irradiated similarly while breathing air [21]. In skin-reaction studies, the feet of mice were locally irradiated with the X-rays described above, at a dose rate of 6.4 Gy/min.

DT-diaphorase measurement. DT-diaphorase activity was assessed in sonicated preparations of bone-marrow cell suspensions by monitoring the reduction of dichlorophenolindophenol at 30° C and 600 nm, exactly as previously described [6].

Results

The experiments shown in Fig. 1 examined the effects of DIC on the antineoplastic effects of MC given alone or in combination with X-rays. In these studies, DIC was given by pretreating mice with two injections of DIC (34 mg/kg per injection) 24 and 2 h before sacrifice and supplying DIC (180 mg/l) in the drinking water throughout this 24-h period. DIC alone (without MC) was not toxic to the cells of EMT6 tumors (Fig. 1). The effect of DIC on the sensitivity to MC of the total tumor-cell population (aerobic plus hypoxic cells) was assessed in animals injected with MC 1 h after the second DIC injection, then assayed for tumor-cell viability 1 h after MC treatment. As we previ-

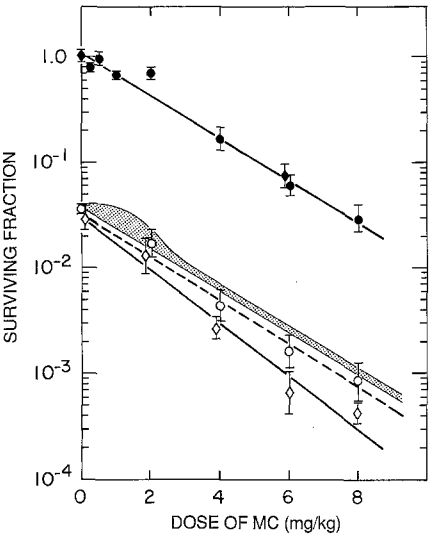

Fig. 1. Effect of DIC on the survival of EMT6 tumor cells treated with MC and/or X-rays in vivo: ●, tumors treated with MC as a single agent; ◆, tumors treated with DIC plus MC; ○, tumors treated with MC plus 15 Gy X-rays; ◇, tumors treated with DIC, MC, and 15 Gy. *Points:* means ± SEM of 3–14 independent determinations. The *stippled area* represents the area of additive cytotoxicity calculated for 15 Gy X-rays plus different doses of MC. The lower and upper limits of this area reflect the limits of additive survivals calculated from the full dose-response curves for X-rays alone and MC alone, assuming no interactions between the sublethal damage produced by the two agents (mode I) and maximal interaction between the sublethal damage produced by the two agents (mode II) [24], respectively

ously found that the cytotoxic effect of MC on EMT6 tumor cells is maximal by 30 min after MC injection [17], this interval is long enough to permit the complete expression of drug toxicity. DIC did not alter the survival of cells from tumors treated with MC in the absence of irradiation (Fig. 1).

In contrast, DIC decreased tumor-cell survival in animals treated with both MC and 15 Gy X-rays (Fig. 1). We have previously shown that DIC has no significant effect on the radiation responses of aerobic or hypoxic EMT6 cells in vitro [7]. In agreement with these findings, in the present study DIC did not alter the radiation response of EMT6 tumor cells in vivo (Fig. 1) on either the regimen described above or other regimens described below. Therefore, the increased cytotoxicity produced by regimens combining DIC, MC, and X-rays does not appear to reflect a potentiation of radiation cytotoxicity. The dose of radiation used in these studies (15 Gy) is sufficiently large that $< 0.1\%$ of the aerobic tumor cells survive irradiation, and the response of the tumors is dominated by the radiation-resistant hypoxic tumor cells [11, 16].

The dose-response curve for tumors treated with DIC, 15 Gy X-rays, and variable doses of MC (Fig. 1) was slightly steeper than those for tumors treated either with MC alone or with MC plus X-rays, suggesting that DIC increases the toxicity of MC to the hypoxic tumor cells. This finding is consistent with cell-culture data [7, 20], which show that DIC increases the toxicity of MC to hypoxic EMT6 cells in vitro. Regimens combining MC with 15 Gy X-rays produced cytotoxicities that fell just outside the area of additivity on Fig. 1, which was calculated from the complete dose-response curves for radiation alone and

MC alone. Similarly, isobologram analyses [24] showed that the cytotoxicities of regimens combining MC and X-rays fell just outside the areas of additivity calculated using the complete single-agent dose-response curves. Because the error limits on the survival determinations overlapped the area of additivity, the effects of these MC/X-ray combinations were statistically compatible with additive as well as supra-additive cytotoxicities. Regimens combining DIC, MC, and X-rays produced cytotoxicities that were higher than those produced by MC plus X-rays, which lay further outside the calculated areas of additivity and were compatible with supra-additive cytotoxicities.

The treatment regimen used in the studies shown in Fig. 1 appeared to be optimal. A more intensive DIC treatment (two injections of 68 mg/kg per injection plus 180 mg/l DIC in the drinking water) was no more efficacious than that used in the studies shown in Fig. 1. More intensive DIC treatments were toxic to the mice. Single injections of 34 or 68 mg/kg DIC given 1 h before MC were less efficacious and produced greater variability in tumor response, probably reflecting animal-to-animal variability in the biodistribution of DIC. Therefore, the 2-day regimen of 34 mg/kg DIC per injection plus 180 mg/l DIC in the water was used for subsequent studies in tumors and normal tissues.

The experiments shown in Fig. 2 also tested the hypothesis that DIC increases the effects of MC on the naturally hypoxic tumor-cell population. This figure shows dose-response curves for EMT6 tumors irradiated in unanesthetized, air-breathing mice and for tumors made uniformly hypoxic by N_2-asphyxiation of the host 5 min before irradiation. At radiation doses of ≥ 10 Gy, the survival curve for normally aerated tumors becomes parallel to that for tumors made uniformly hypoxic, because the aerobic cells have been killed and the curve reflects the survival of the radiation-resistant, hypoxic cells.

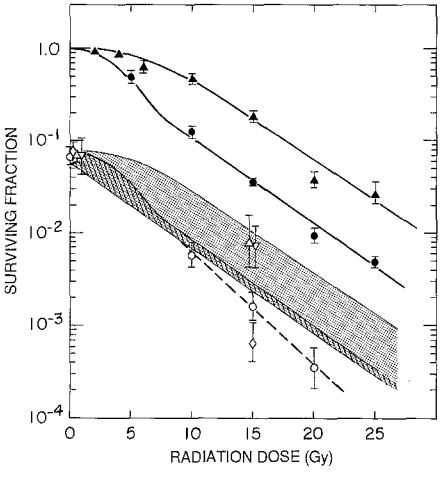

Fig. 2. Effect of tumor oxygenation on the survival of EMT6 tumor cells treated with DIC, MC, and/or X-rays: ●, tumors irradiated in normal, unanesthetized, air-breathing mice; ▲, tumors irradiated after the induction of uniform radiobiological hypoxia throughout the tumor; ○, tumors treated with 6 mg/kg MC, then irradiated (or sham-irradiated) in air-breathing mice; △, tumors treated with 6 mg/kg MC, then irradiated (or sham-irradiated) in hypoxia; ◇, tumors treated with DIC and 6 mg/kg MC, then irradiated (or sham-irradiated) in air-breathing mice; ▽, tumors treated with DIC and MC, then irradiated (or sham-irradiated) in hypoxia. *Points:* means ± SEM of 3–20 independent determinations. The *stippled area* defines the area of additivity for MC plus radiation given under hypoxic conditions, calculated from the full single-agent dose-response curves [24]. The *hatched area* represents the calculated area of additivity for MC plus radiation given to normally aerated tumors. The larger area of additivity for the hypoxic irradiation reflects the large shoulder on the hypoxic radiation dose-response curve

Cell survival in tumors treated with MC alone was not altered when mice were asphyxiated with N_2 55 min after MC; this finding was expected because the cytotoxic effects of MC are maximal by 30 min after injection of the drug [17] and are therefore insensitive to subsequent changes in tumor oxygenation. Cell survival in tumors treated with MC and then irradiated in hypoxia lay near the upper boundary of the area of additivity calculated from the dose-response curves for MC alone and hypoxic irradiation alone. DIC pretreatment did not alter the survival of cells in tumors irradiated under artificial hypoxia. N_2-asphyxiation renders all of the tumor cells uniformly hypoxic and therefore eliminates the differences in the radiation sensitivity of the naturally aerobic and naturally hypoxic tumor cells; as the aerobic cells comprises 80% of the population, they dominates the response of the tumor to this regimen. Therefore, MC and radiation produced additive toxicities to aerobic tumor cells; the cytotoxicity of these regimens was not increased by DIC.

In contrast, cell survival in tumors treated with MC and X-rays given under naturally aerated conditions lay outside the lower boundary of the calculated areas of additivity, in the area of supra-additivity (although some were statistically compatible with both additive or supra-additive cytotoxicities). DIC pretreatment decreased cell survival in normally aerated tumors treated with both MC and X-rays, and produced supra-additive cytotoxicity. These findings suggest that DIC increases the selective toxicity of MC to the hypoxic tumor cells.

As hematologic depression is one of the primary dose-limiting toxicities of MC, the effects of DIC on the viability and MC sensitivity of marrow CFU-GM, CFU-MK, and CFU-S were assessed (Fig. 3). The DIC treatment used in these experiments was the same as that used in the

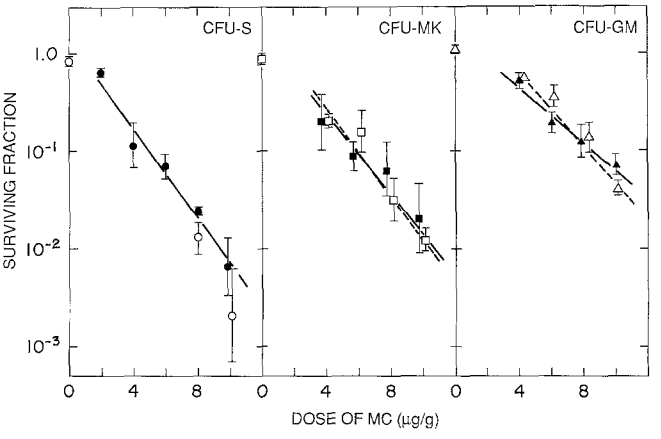

Fig. 3. Effect of DIC on the survival of CFU-S (*left panel*), CFU-MK (*middle panel*), and CFU-GM (*right panel*) in mice treated with different doses of MC: *closed symbols*, MC only; *open symbols*, DIC and MC; *lines*, least-squares regression lines fit through survival data. *Points:* means of 2–6 independent survival determinations (SEMs are shown for $n \geq 3$)

Table 1. Effect of DIC on the toxicity to BALB/c mice of a single MC treatment. The LD_{50} values calculated from these data were 9.0 mg/kg (95% confidence limits, 7.5–10.0) for MC and 9.0 mg/kg (95% confidence limits, 7.9–10.1) for DIC plus MC

Dose of MC (mg/kg)	Survivors/total mice	
	MC	DIC + MC
7	4/4	4/4
8	4/4	4/4
9	2/4	1/4
10	0/5	1/5

tumor studies shown in Figs. 1 and 2. DIC alone did not alter the viability of these stem cell lines. As expected, MC alone was toxic to the hematologic progenitors. The three stem cells had somewhat different sensitivities to MC; the hierarchy of sensitivities to MC was similar to those for radiation [4] and porfiromycin [21]. The survival curves for these three stem cells were not significantly altered by DIC treatment. Therefore, the enhanced antineoplastic effect obtained with the DIC/MC/radiation regimen described above was obtained without an increase in marrow toxicity.

It has been suggested that DIC might protect the marrow from MC damage, because it protects aerobic EMT6 cells in vitro [7, 20]. After obtaining the results shown in Fig. 3, we tested the effects of DIC (300 μM) on the toxicity of MC (6 μM, 1 h) to CFU-GM in suspension in vitro. This DIC treatment, which has been exceedingly effective in protecting EMT6 cells in vitro [20], did not alter the toxicity of MC to CFU-GM in vitro. Measurements of DT-diaphorase in suspensions of marrow cells revealed only low levels of enzyme ($\leq 13 \pm 5$ nmol/min per mg protein), at least 100-fold lower than those found in EMT6 cells [6]. The failure of DIC to protect the marrow against MC toxicity may therefore reflect the fact that hematopoietic cells lack DT-diaphorase. We propose that DT-diaphorase is one of the enzymes activating MC to a toxic species in aerobic EMT6 cells and that DIC may protect aerobic EMT6 cells from MC by inhibiting this enzyme, thereby preventing formation of this toxic species.

Treatment with DIC also failed to alter the host toxicity of MC (Table 1). The dose-response curve, the calculated LD_{50} (9.0 mg/kg in both cases), and the time of death were all similar in mice treated with MC and in those treated with DIC plus MC.

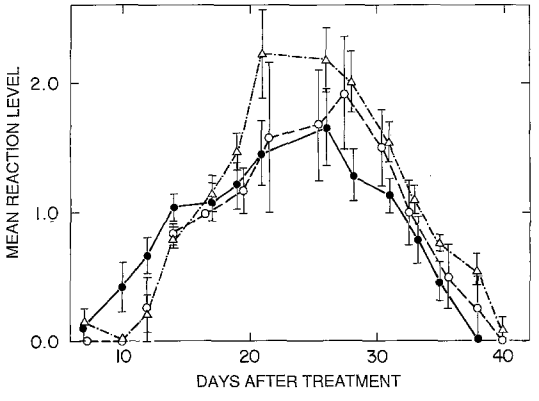

Fig. 4. Effect of treatment with either MC or DIC plus MC on the skin reactions induced by 30 Gy X-rays. *Points:* mean reaction levels ± SEMs. ●, radiation only; △, 6 mg/kg MC given 1 h before X-rays; ○, DIC plus MC plus X-rays

The effects of DIC plus MC on the radiation response of the skin was also examined (Fig. 4). Three experiments were carried out in which skin reactions were compared in mouse feet locally irradiated with 30 Gy X-rays, in mice treated with 6 mg/kg MC 1 h before X-rays, and in animals receiving DIC plus MC plus X-rays. A typical experiment is shown in Fig. 4. MC alone produced no change in the appearance of nonirradiated skin (data not shown). In all three experiments, skin reactions were slightly more severe in mice receiving MC plus X-rays than in those receiving radiation alone, but the differences were not statistically significant (Mann-Whitney U-test). Reactions in mice receiving DIC plus MC plus radiation were intermediate between those of animals receiving MC plus X-rays and those of mice receiving X-rays alone. In none of the three experiments did treatment with DIC plus MC significantly alter the time-course or severity of the skin reactions from those seen with radiation alone. As the dose of MC used in this study was two-thirds of the $LD_{50/30}$, one would not expect a significant augmentation of radiation-induced skin reactions by MC and DIC with clinically relevant drug treatment regimens.

Discussion

The experiments described in this report examined the clinical potential of DIC as an agent for modulating the selective toxicity of MC to hypoxic tumor cells and for increasing the therapeutic ratio attainable with MC as an adjunct to radiotherapy. Cell-culture studies have examined in detail the effects of DIC on the metabolism and cytotoxicity of MC [6–8, 20]. DIC was shown to act as a dose-modifying agent, altering the slopes of the MC survival curves [20]; hypoxic EMT6 cells were sensitized to the cytotoxic effects of MC, whereas aerobic EMT6 cells were protected. The change in the response of the cells to MC increased with increasing DIC dose [20]. DIC had to be present in the cultures during MC exposure to alter the sensitivity of cells; addition of DIC just before MC was as efficacious as its addition 30 min–2 h before MC. Sequential treatments (i.e., DIC followed by MC, with an intervening wash; MC followed by DIC) showed effects similar to those obtained with MC alone [20].

In vivo, treatment of air-breathing mice with the combination of DIC, MC, and radiation increased the tumor cell kill over that predicted from purely additive cytotoxicities and over that obtained with MC plus radiation, without DIC (Figs. 1, 2). As DIC did not alter the radiosensitivity of EMT6 cells in vitro [7] or in vivo ([7]; Fig. 1), this probably reflects the effects of DIC on the intracellular metabolism of MC and an increase in the toxicity of MC to hypoxic tumor cells in vivo, analogous to that observed in our cell-culture studies.

DIC did not increase the overall toxicity of MC to the tumor cells (Fig. 1). Thus, DIC would not be expected to improve the therapeutic ratio obtained with MC used as a single agent. The value of adjuvant DIC treatment would be limited to those situations in which the preferential toxicity of MC to hypoxic cells was being exploited (e.g., the use of MC as an adjunct to radiotherapy).

The DIC regimens used in this study were equivalent to the single doses of DIC in routine clinical use for anticoagulation [13]. Effective anticoagulation by DIC requires several days of treatment, as DT-diaphorase must be continuously inhibited for periods long enough to deplete stores of vitamin K [13]. In contrast, when DIC is used to modulate MC metabolism, the former must be present in the tumor cells only long enough to inhibit the enzymes involved in MC biotransformation during the short period of time [2] when high levels of active MC are present.

In the present experiments, pretreatment of mice with 6 mg/kg MC did not significantly increase the radiation-induced skin reactions. The skin reactions in BALB/c Rw mice treated with DIC and MC were intermediate between those of animals treated with X-rays only and those of mice treated with MC and X-rays; none of the reactions in these groups were significantly different from one another. Studies by van der Maase [9] have shown slight increases in skin reactions in mice pretreated with a maximum tolerated dose of MC, with dose-modifying factors of ~1.1 on regimens similar to those used here. MC did not augment skin reactions in patients receiving radiation therapy [26]. Therefore, treatment of patients with MC plus DIC would not be expected to produce significant augmentation of radiation reactions in skin or similar normal tissues within the treatment field.

DIC did not increase either the toxicity of MC to three different marrow stem cells (Fig. 2) or the $LD_{50/30}$ for MC (Table 1) and did not increase the MC-induced leukopenia [7]. Therefore, adjunctive treatment with DIC should not compromise hematologic tolerance in patients treated with MC. DIC did not have the protective effect on the hematopoietic progenitors that was anticipated from our studies on EMT6 tumor cells in vitro. These data may reflect the enzymatic phenotype of the mouse hematopoietic cells, which appear to have relatively low levels of DT-diaphorase and are not protected from MC by DIC in vitro. L1210 mouse leukemia cells, which also lack measurable DT-diaphorase, are likewise not protected from MC by DIC [8].

Because DIC increased the effects of MC as an agent toxic to hypoxic cells without increasing the host toxicities of either MC or radiation, therapeutic gain was obtained in this experimental system. However, the gain in these experiments was small. This is due partly to the small increase in the antineoplastic effect of MC and partly to the failure of DIC to protect the mice against the hematologic toxicity of MC. Although DIC is of value in studying the metabolism and cytotoxicity of MC in vitro, it is unclear whether this agent can be used to produce significant therapeutic benefit in the clinic.

Acknowledgements. The authors thank Marianne Kelley, Carolyn Irvin, and Jacqueline Mendes for their assistance with the experiments and Dr. C. Gamba-Vitalo for her helpful discussions of the CFU-GM and CFU-MK assays.

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