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Bioreductive metabolism of mitomycin C in EMT6 mouse mammary tumor cells: cytotoxic and non-cytotoxic pathways, leading to different types of DNA adducts. The effect of dicumarol

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Abstract

The six DNA adducts formed in EMT6 mouse mammary tumor cells upon treatment with mitomycin C (MC) fall into two groups: (1) four guanine adducts of MC and (2) two guanine adducts derived from 2,7-diaminomitosene (2,7-DAM), the major reductive metabolite of MC. The two groups of adducts were proposed to originate from two pathways arising from reductive activation of MC: (a) direct alkylation of DNA and (b) formation of 2,7-DAM, which then alkylates DNA. The aim of this study was to test the validity of this proposal and to evaluate the significance of alkylation of DNA by 2,7-DAM. Treatment of the cells with 2,7-DAM itself yielded the same 2,7-DAM-guanine adducts as treatment with MC; however, 2,7-DAM was approximately 100-fold less cytotoxic than MC. The uptake and efflux of 2,7-DAM by EMT6 cells was comparable to that of MC, but 2,7-DAM alkylated DNA with higher efficiency than MC. These results validate the two proposed pathways and show that formation of 2,7-DAM–DNA adducts in MC-treated cells represents a relatively non-toxic pathway of reductive metabolism of MC. A selective stimulatory effect of dicumarol (DIC) on 2,7-DAM–DNA adduct formation in EMT6 cells treated with MC was also investigated. DIC had no effect on alkylation by MC in cell-free systems, nor did it have significant effects on adduct formation or cell survival for cells treated with 2,7-DAM. It is proposed that in the cell DIC stimulates a reductase enzyme located at subcellular sites where the activated MC species has no direct access to DNA and therefore is diverted into the non-cytotoxic pathway, which leads to the formation of 2,7-DAM and its adducts.

Keywords: Mitomycin C; DNA adducts; Bioreductive; Metabolism; Tumor cells; 2,7-Diaminomitosene; Dicumarol

1. Introduction

The antitumor antibiotic MC is used in the treatment of several human malignancies [1]. MC is the prototype of the bioreductive anticancer drugs, a class of agents whose antitumor activity is dependent upon the reductive activation of their prodrug form. Antitumor selectivity of bioreductive drugs may be achieved through two possible mechanisms: targeting of the hypoxic regions of the tumor [2] or targeting of tumor cells in which reductase enzymatic activity is elevated compared to that in normal tissue [3]. MC is known to induce DNA damage in cells in the form of DNA cross-links and monofunctional DNA alkylation products [4]. The precise molecular structures of the cross-links and the monofunctional adducts have been elucidated previously [4–6]. In cell-free systems, both types of DNA damage are dependent on the reduction of MC. In living cells, DNA adduct and cross-link levels produced by MC have been shown to be higher under hypoxia than under aerobic conditions, indicating that O_2 can decrease the reduction-dependent DNA damage produced by this drug in intact cells. Since MC is more cytotoxic to cells under hypoxic conditions than under aerobic conditions, these findings demonstrate that the cytotoxic action of MC is correlated with the

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Abbreviations: MC, mitomycin C; DIC, dicumarol; 2,7-DAM, 2,7-diaminomitosene; and XDH, xanthine dehydrogenase.

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generation of reduction-dependent DNA damage by the drug. Furthermore, the decreased damage that occurs in the presence of O_2 *in vivo* is likely to be the basis for the preferential activity of MC against the hypoxic cells of solid tumors [7].

An understanding of the relationship between the metabolic activation of bioreductive drugs and the death of cancer cells is clearly desirable to optimally exploit the dependence of the cytotoxic activity of MC and other bioreductive drugs on enzymatic reduction for more effective chemotherapy, as well as to develop new bioreductive agents. This understanding has been intensively pursued in a number of laboratories, particularly in the case of MC, leading to the recognition that reductive activation of MC in tumor cells can be catalyzed by several different enzymes, none of which shows any particular specificity for MC. This fact explains why no consistent correlations have been observed between the expression of individual bioreductive enzymes and total antitumor activity, either in tumor cell lines or *in vivo*, and also explains why assaying the activity of an individual reductase will not lead to a straightforward prediction of chemosensitivity to MC (for a review, see Ref. 8). An alternative assay, which is more specific to the metabolic reduction of MC, was attempted by Spanswick and co-workers [9]. These authors determined the levels of 2,7-DAM, the major reductive metabolite of MC, in MC-treated murine adenocarcinomas, by HPLC. Again, no correlation was found between the concentration of 2,7-DAM and the chemosensitivity of the tumors. It was concluded from all of these results taken together that no simple relationship existed *in vivo* between reductase expression, reductive metabolism of MC, and chemosensitivity.

In contrast, analysis of DNA adducts of MC in EMT6 tumor cells showed a positive correlation between MC adduct levels and cytotoxicity when the effects of treatment were modulated by the oxygen tension or by co-treatment of the cells with dicumarol [10]. Similarly, analysis of DNA cross-linking by MC [11,12] as well as of the DNA adducts of porfiromycin formed in EMT6 cells [13] and in P388 murine leukemia cells [14] under conditions of oxygen modulation, all demonstrated a positive correlation between levels of DNA adducts and cytotoxicity. In these early studies, the MC– and porfiromycin–DNA adducts had not yet been completely resolved and identified.

Elucidation of the six major DNA adducts formed from MC in EMT6 cells (Fig. 1) was recently completed [6]. The structures (Fig. 2) revealed that four are adducts of MC and two are derived from 2,7-DAM. The 2,7-DAM adducts were thought to originate from reductive metabolism of MC to give 2,7-DAM, followed by alkylation of DNA by this metabolite. Since 2,7-DAM was much less cytotoxic than MC [15], it was proposed that reductive conversion of MC to 2,7-DAM within the cell represents a relatively non-cytotoxic pathway that competes with the cytotoxic pathway of DNA cross-linking by MC and MC–DNA monoadducts (Fig. 3) [6,15]. This pathway could thus be considered a pathway for the inactivation of MC. However, alternative

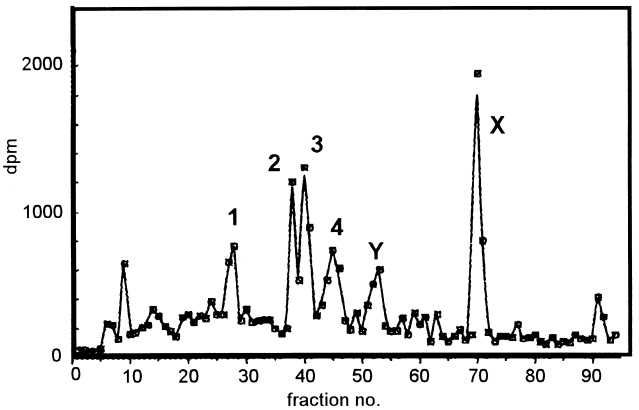


Fig. 1. HPLC of DNA adducts formed in EMT6 cells upon treatment with [^{3}H]MC. Digest of DNA from cells treated with 20 μM [^{3}H]MC (specific activity 1.8 mCi/μmol), under hypoxia. The numbers or letters above the peaks refer to the adducts in Fig. 2. (Reproduced with permission from Ref. 15.)

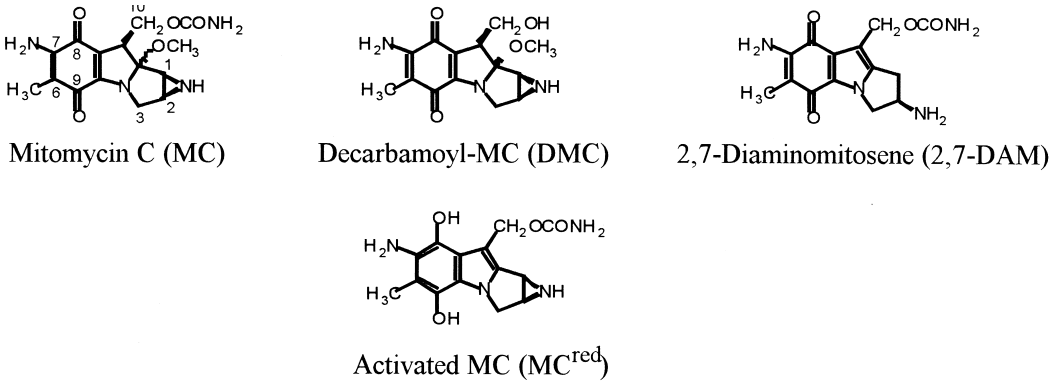
explanations for the observed low cytotoxicity of 2,7-DAM remained possible: (1) 2,7-DAM was not taken up by cells; (2) 2,7-DAM entered cells, but did not alkylate DNA, only 2,7-DAM formed intracellularly from MC being capable of this action; or (3) the 2,7-DAM–DNA adducts formed in MC-treated cells originated directly from MC, rather than from 2,7-DAM.

The goal of the studies reported here was to characterize the reductive metabolism of MC to 2,7-DAM and to 2,7-DAM–DNA adducts and to clarify the role of 2,7-DAM and its DNA adducts with respect to the cytotoxicity of MC in tumor cells. To this end, cells were treated with exogenous 2,7-DAM. It was found that exogenous 2,7-DAM was rapidly taken up by cells, and that it alkylated nuclear DNA, forming the same 2,7-DAM–DNA adducts as those formed in MC-treated cells. These results verified that although a major path of the reductive activation of MC leads to the formation of 2,7-DAM and 2,7-DAM–DNA adducts, this path is relatively non-cytotoxic. We also observed a selective effect of DIC on the non-cytotoxic 2,7-DAM pathway. Experimental evidence is presented that this is a cellular phenomenon and that the cytotoxic and non-cytotoxic pathways of MC metabolism may occur at separate subcellular sites.

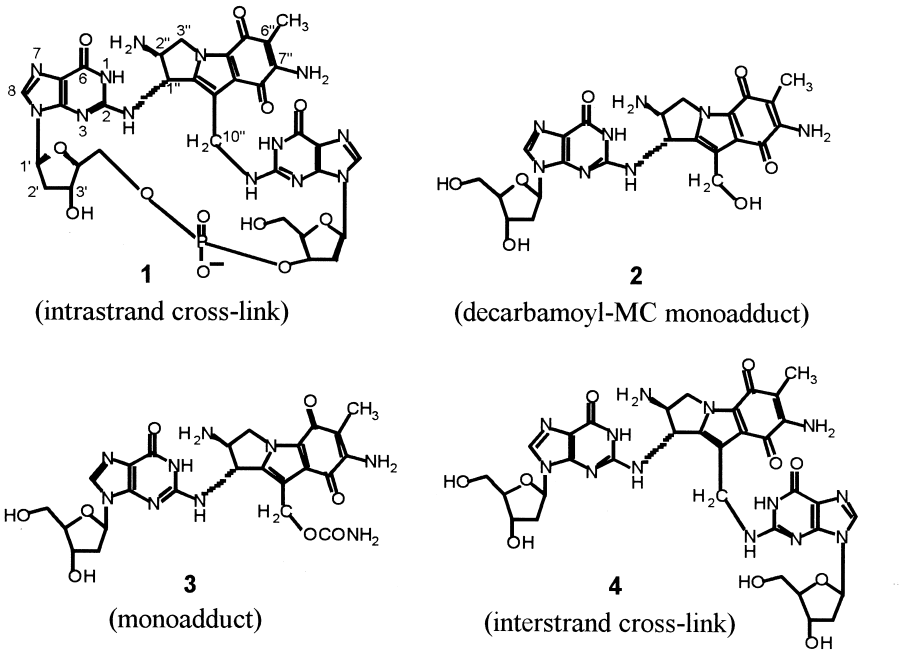
2. Materials and methods

2.1. Chemicals and enzymes

Calf thymus DNA was purchased from Sigma Chemical Co. and was sonicated before use. NADH-cytochrome *c* reductase (NADH-FMN oxidoreductase; E. C. 1.6.99.3), NADPH, NADH, and dicumarol were obtained from Sigma. Nuclease P1 (*Penicillium citrinum*; E. C. 3.1.30.1) was purchased from Amersham Pharmacia Biotech. Phosphodiesterase I (snake venom diesterase; *Crotalus adamanteus venom*; E. C. 3.1.4.1) and alkaline phosphatase (*Esch-*



DNA adducts of MC:



DNA adducts of 2,7-DAM:

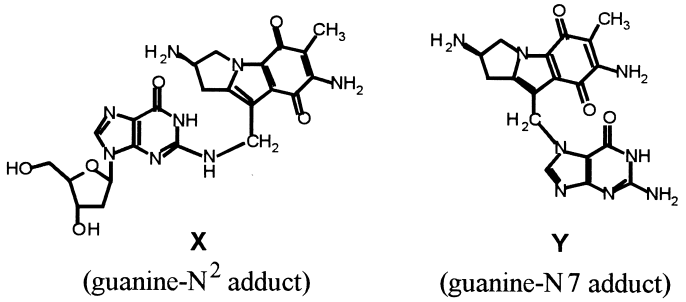


Fig. 2. Structures of mitomycins and their DNA adducts.

erichia coli; E.C. 3.1.3.1) were obtained from Worthington Biochemical Corporation.

NADPH-cytochrome P450 reductase (same as NADPH-cytochrome *c* reductase) (E.C. 1.6.2.4) (purified, recombinant human) was purchased from Panvera Corp. Xanthine dehydrogenase (E.C. 1.2.1.37) was purified from EMT6 tumors grown in athymic nude mice according to the method of Suleiman and Stevens [16] as modified for EMT6 tissue by Gustafson and Pritsos [17]. Xanthine oxidase (E.C. 1.1.3.22) ("From Microorganism") was purchased from Sigma.

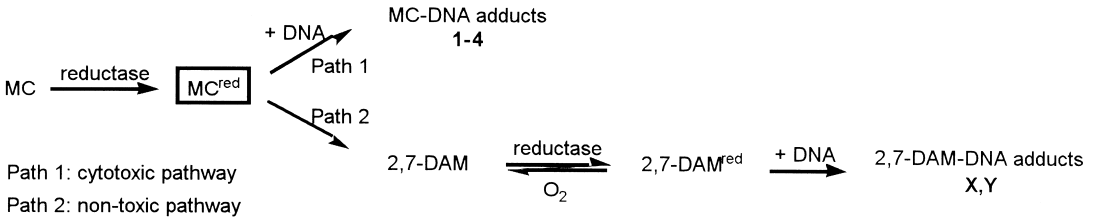


Fig. 3. Reductive metabolism of MC in tumor cells: MC–DNA adduct pathway and 2,7-DAM–DNA adduct pathway.

2.2. Drugs

MC was supplied by D. M. Vyas (Bristol-Myers Squibb Co.). 2,7-DAM was synthesized from MC as previously described [18]. [6-CH_3-^{3}H]MC (36 Ci/mmol), synthesized by the method of Arai and Kasai [19], was donated by Kyowa Hakko Kogyo Co. (Tokyo, Japan). The material was diluted to a desired lower specific activity by mixing 3.6 μmol unlabeled MC with 49 μCi of ^{3}H-labeled MC, followed by purification by chromatography on Sephadex G-25 (fine) column (2.5 $\times$ 56 cm), eluted with 0.02 M NH_4HCO_3. The UV-absorbing fraction, corresponding to MC, was found to have 7.73 μCi/μmol specific activity by determining its absorbance at 360 nm and radioactivity by liquid scintillation spectrometry.

[6-CH_3-^{3}H]2,7-DAM was synthesized from [6-CH_3-^{3}H]MC. Briefly, 8 μmol non-radiolabeled MC was mixed with 54 μCi of [^{3}H]MC (36 Ci/mmol), and the mixture was reduced by H_2/PtO_2 to give [^{3}H]2,7-DAM as described for the synthesis of non-radiolabeled 2,7-DAM [18]. The specific activity of the purified material was 6.70 μCi/μmol.

2.3. Cells

EMT6 mouse mammary carcinoma cells were cultured and maintained as exponentially growing monolayers in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics (all from Life Technologies, Inc.) as described previously [10,20,21].

2.4. Treatment of cells in adduct isolation studies

Cells were grown in T75 flasks for 3 days, collected by trypsinization, then washed and pooled to form a single cell suspension. The suspension was seeded in 100 mL of Waymouth's medium in 250-mL side-arm-equipped spinner flasks at a cell density of 10^6 cells/mL and slowly stirred at 37° to prevent cell attachment. Cells were gassed with a humidified mixture of 95% air/5% CO_2 or 95% N_2/5% CO_2 ($<$10 ppm O_2; hypoxic conditions) at 37° through a rubber septum that was fitted with 18-gauge inflow and outflow needles placed in the side arms of the spinner flasks for 1 or 2 hr before the start of the drug treatment. Following these incubations, cultures were treated with drug or drug + dicumarol for various time periods and at concentrations specified in the text.

2.5. Cytotoxicity studies

For experiments examining the cytotoxic effects of 2,7-DAM and MC, exponentially growing EMT6 cells were seeded in glass cell culture flasks at 2×10^5 cells per bottle (2×10^4 cells per mL) and were used in mid-exponential phase (approximately 3–4 days of growth). At the beginning of the experiment, the medium on these monolayer cultures was removed and the cells were recovered with 5 mL of Waymouth's medium, at pH 7.2. Hypoxia was induced by gassing the cultures with a humidified mixture of 95% N_2/5% CO_2 ($<$10 ppm O_2) at 37° for 2 hr through a rubber septum fitted with 13-gauge (inflow) and 18-gauge (outflow) needles. Following the induction of hypoxia, drugs (dissolved in ethanol) were added to the cultures by injecting them through the rubber septum in a small volume of solvent, without breaking the hypoxia, and the culture flask was swirled gently to ensure uniform distribution of the drug in the culture medium. Aerobic cells were treated in an identical manner, under a humidified atmosphere of 95% air/5% CO_2 at 37°. Treated cells were then washed, harvested by trypsinization, and assayed for survival by measuring their ability to form macroscopic colonies in Petri dishes as described in detail in our past publications [20,22,23]. Both aerobic and hypoxic vehicle-treated controls were included in each experiment, along with untreated controls.

In the experiments comparing the cytotoxicities of MC and 2,7-DAM, graded doses of these drugs were added to the cultures 2 hrs after the induction of hypoxia, and the cells were exposed to the drugs for 2 hr under hypoxia. The doses used for each drug in these experiments were chosen to span a range of cell survivals expected, ranging from a surviving fraction of about 1.0 (i.e. no measurable toxicity) to a surviving fraction of $\sim$0.05 –1.0, so that the resulting survival data could be analyzed to calculate and compare IC_{50}s for the two drugs under these conditions of treatment. These IC_{50}s were calculated as the concentrations needed to reduce the surviving reactions to 0.5 (50% survival). For the experiments examining the effect of the oxygenation of the cultures on the cytotoxicity of 2,7-DAM and studying the effect of dicumarol on the cytotoxicity of 2,7-DAM in air and hypoxia, cultures were treated with 40 μM 2,7-DAM for periods of 2–8 hr. This concentration was chosen because it was the highest concentration of 2,7-DAM that would reliably remain in solution in the culture medium.

Under aerobic conditions, this dose produced only minimal cytotoxicity, even with a treatment time of 8 hr (with a relative survival of 0.57 when normalized to the vehicle-treated controls incubated for the same long duration). The same dose of drug was used in hypoxia, so that hypoxic and aerobic cells could be studied at equal concentrations; however, the longest incubation time examined was 6 hr, because the greater toxicity of this drug in hypoxia made higher doses uninteresting. DIC was added to some cultures by injecting a small volume of concentrated stock solution into the culture medium, exactly as described above for MC and 2,7-DAM. DIC was added approximately 1 min before the MC or 2,7-DAM, to produce a final concentration of 300 μM DIC in the culture medium. This concentration of DIC was chosen because it produced no discernible toxicity to EMT6 cells and produced very significant changes in the survival of EMT6 cells treated with MC [23].

These studies use high doses of 2,7-DAM and long treatment times. They should, however, be considered in the context of the clinical use of MC. In contrast to many anticancer drugs, the best therapeutic ratios for MC in the clinic are obtained using regimens that incorporate very large doses of MC, given infrequently (e.g. 15 mg/m^2, given every 6 weeks). Data on blood counts in patients receiving MC in typical clinical regimens suggest that each treatment with MC probably reduces cell survival by 1–2 logs in marrow, an aerobic tissue. Under aerobic conditions, the maximal dose of 2,7-DAM used in these studies therefore produced cytotoxicities that are well within the range anticipated from the clinical administration of a single, standard treatment with MC. This treatment therefore represents a clinically relevant treatment intensity, and an appropriate *in vitro* model system in which to study the modulation of the cytotoxicity and metabolism of 2,7-DAM by environmental factors (e.g. hypoxia) and by the enzyme modulator dicumarol. The doses of MC used in these studies likewise produce cytotoxic effects in the range anticipated from the administration of single, clinically used doses of MC in patients.

2.6. Isolation of nuclear DNA from 2,7-DAM-treated cells

Cells treated with 2,7-DAM under hypoxia or aerobic conditions, as described above, were washed twice with PBS (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and resuspended in the same buffer at a cell density of 1×10^7 cells/mL. Nuclear DNA was isolated using a Qiagen blood and cell culture DNA maxi kit (Qiagen), exactly following the manufacturer's instructions. DNA was resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), precipitated using ice-cold ethanol, and resuspended in the same buffer. The yield of DNA was 15 to 20 A_{260} units/10^8 cells.

2.7. Treatment of calf thymus DNA with MC +/− DIC using various reductases as activators

Xanthine dehydrogenase (XDH). A solution of 6 mM calf thymus DNA, 0.25 mM MC, 1.5 mM NADH, and 0.73 units of XDH per μmol MC in 0.1 M potassium phosphate, pH 5.8 or pH 7.4, was incubated at 37° for 1 hr, either under aerobic or anaerobic conditions. The latter was maintained by bubbling argon gas through the solution. The reaction mixture was extracted with phenol/$CHCl_3$ (1:1, v/v), and the drug–DNA complex was isolated by ethanol precipitation. In "+DIC" experiments, 300 μM DIC was added to the solution before addition of XDH. *Xanthine oxidase* was used instead of XDH in an otherwise identical procedure.

NADH-cytochrome c *reductase.* A solution of 12 mM calf thymus DNA, 1 mM MC, and 2 mM NADH in 15 mM Tris–HCl buffer, pH 7.4, was deaerated by bubbling argon gas through the solution. NADH-cytochrome *c* reductase in a stock solution of 20 U/mL was added to give a concentration of 0.58 U/mL in the reaction mixture. Incubation under positive pressure of argon was continued for 1 hr at 37°. The MC–DNA complex was isolated as in XDH.

NADPH-cytochrome c *reductase.* A solution of 12 mM calf thymus DNA, 1 mM MC, 2 mM NADPH, and 0.6 units/mL of NADPH-cytochrome *c* reductase was incubated under anaerobic conditions, and the drug–DNA complex was isolated, as in XDH.

2.8. Treatment of calf thymus DNA with 2,7-DAM +/− DIC using NADH-cytochrome c *reductase as activator*

This procedure was analogous to that described above for the treatment of calf thymus DNA with MC +/− DIC, using NADH-cytochrome *c* reductase as activator.

2.9. Enzymatic digestion of the drug–DNA complex to nucleosides and MC–nucleoside adducts

The lyophilized drug–DNA complex was digested to the nucleoside level by the following protocol. Nuclease P1 (1.0 unit/A_{260} unit of complex) was added to the solution of the complex in dilute aqueous acetic acid, pH 5.0 (2.5 A_{260} units/mL), followed by incubation for 4 hr at 37°. The pH was adjusted to 8.2 by addition of 0.5 M Tris, and $MgCl_2$ was added to a concentration of 1.0 mM. Addition of snake venom diesterase (2.25 units/A_{260} unit of complex) and a 2-hr incubation at 37° were followed by the addition of alkaline phosphatase (1.6 units/A_{260} unit of complex) and incubation overnight at 37°. The digest was then heated at 90° for 1 hr to hydrolyze guanine–N7 adducts, lyophilized, and redissolved in varying volumes of water for HPLC analysis.

2.10. HPLC analysis of adducts in digests

For HPLC analysis, a Beckman model 366 HPLC system, including a model 168 diode array detector module,

was used throughout. A Rainin Microsorb MV C18 column (4.6 × 250 mm) was employed for elution with a 6–18% acetonitrile gradient in 30 mM KH_2PO_4 at pH 5.5 for 60 min at a flow rate of 1 mL/min. Digests originating from experiments using cell-free systems were injected and the UV absorbance of the eluate was recorded continuously. Digests of DNA from EMT6 cells were analyzed by a two-stage procedure, employing an additional Sep-Pak C-18 column (Waters Associates) fractionation step as follows. The digested DNA (15–20 A_{260} units) was applied on a 1 × 1-cm Sep-Pak column. The unmodified nucleoside fraction was eluted with water, and the adducted nucleoside fraction, together with unmodified deoxyadenosine, left over from the first fraction was subsequently eluted with 60% methanol (approx. 5 mL). Both fractions were then chromatographed by HPLC as described above. The identity of the adducts was established by comparison of HPLC elution times and UV spectra with those of authentic standards [6,10,15]. The frequency of adducts (mol adduct/mol DNA nucleotide) was calculated from areas of corresponding peaks in the HPLC chromatogram of the digest, as previously described. In this procedure, the area of the deoxythymidine peak of the digest serves as internal reference, in order to control for run-to-run differences in the amount of DNA digest loaded on to the column [6].

2.11. Uptake/efflux studies

EMT6 cells were harvested and suspended as described above and incubated as stirred suspension cultures in glass vials at 37°, exactly as described previously [20]. Cultures contained 7 mL of medium with 5×10^6 cells/mL. Cultures were gassed with 95% air/5% CO_2 or 95% N_2/5% CO_2 through 18-gauge inflow and outflow needles for 2 hr, and radiolabeled drugs were then injected into the cultures as described above. Previous studies [20] showed that cultures treated with porfiromycin under these conditions had survivals indistinguishable from those of cells treated in the monolayer cultures described above. Cells were collected at various times from 2 min to 2 hr after the initiation of drug treatment, centrifuged through oil, and lysed in acid exactly as described previously [20]. After 2 hr of incubation, cells were collected by centrifugation, washed twice, resuspended in fresh medium, and incubated under aerobic conditions to assess the efflux of the radiolabel. Cell-associated radioactivity was measured with a scintillation spectrometer and cellular drug concentrations were estimated using the specific activity of the drugs and an estimated cell volume of 2.2 pL.

3. Results

3.1. Isolation of 2,7-DAM–DNA adducts from EMT6 cells treated with 2,7-DAM

Cells were treated with 40 μM non-radiolabeled 2,7-DAM for 4 hr under various conditions. Under aerobic conditions, both 2,7-DAM adducts *Y* and *X* were detected at 9.6×10^{-6} and 7.9×10^{-6} adduct/nucleotide frequencies, respectively (Fig. 4a). Under hypoxia, adduct *X* was the single product, formed at a 3.8×10^{-5} adduct frequency (Fig. 4b). The adducts were also detectable when a 10-fold lower dose of 4 μM 2,7-DAM was used for treatment; the adduct frequencies were $\leq 10^{-6}$ (*X* + *Y*, aerobic treatment) and 3.4×10^{-6} (*X*, hypoxia) (data not shown). These results indicate that adduct frequencies are directly proportional to drug treatment concentrations in the 4–40 μM range. Curiously, adduct *Y* was formed only with aerobic cell treatments. Thus far, we have found no adequate explanation for this difference.

3.2. Effects of DIC co-treatment on the DNA adduct profile in 2,7-DAM-treated cells and cell-free systems

Treatment of cells with 40 μM 2,7-DAM under aerobic conditions in the absence or presence of 300 μM DIC resulted in the formation of the 2,7-DAM adducts *X* and *Y*, in very similar distribution and frequencies (1.7×10^{-5} total adduct without DIC vs 2.3×10^{-5} total adduct in the presence of DIC; Fig. 4, a and b). Treatment under hypoxia gave higher frequencies of adducts than treatment under aerobic conditions, both in the absence or presence of DIC (3.8×10^{-5} and 5.7×10^{-5} total adduct, respectively) (Fig. 4, c and d). However, the presence of 300 μM DIC itself did not significantly alter the DNA adduct profiles under either aerobic or hypoxic conditions. In the cell-free system, employing NADH-cytochrome *c* reductase as the activator of 2,7-DAM, no significant difference in (*Y* + *X*) adduct frequencies was observed (2.8×10^{-3} in the absence of DIC and 2.3×10^{-3} in the presence of DIC; Fig. 4, e and f).

3.3. Lack of effect of DIC on 2,7-DAM adduct formation in cell-free MC/DNA systems with XDH as activator

The formation of the reductive metabolite 2,7-DAM from MC, catalyzed by purified XDH, is accelerated in the presence of DIC [21]. We tested whether this effect of DIC led to an enhanced formation of 2,7-DAM–DNA adducts when DNA was present in the MC activation system. A mixture of calf thymus DNA, MC, NADH, and XDH was incubated with or without DIC. Additional variables were pH 7.4 or 5.8 and aerobic or anaerobic conditions, as described in the Methods section. DNA was isolated from the incubation mixtures and adducts were analyzed by the digestion–HPLC method. The results of anaerobic (no DIC) and anaerobic (+ DIC) incubations at pH 5.8 are shown in Fig. 5a, and the results of analogous aerobic incubations are displayed in Fig. 5b. DIC had very little influence on the adduct frequencies: only a 1.1-fold increase of adduct *X* under anaerobic conditions (Fig. 5a) and a 2.0-fold and 1.1-fold increase of adduct *X* and adduct *Y*, respectively, under aerobic conditions (Fig. 5b). Similar results were observed in

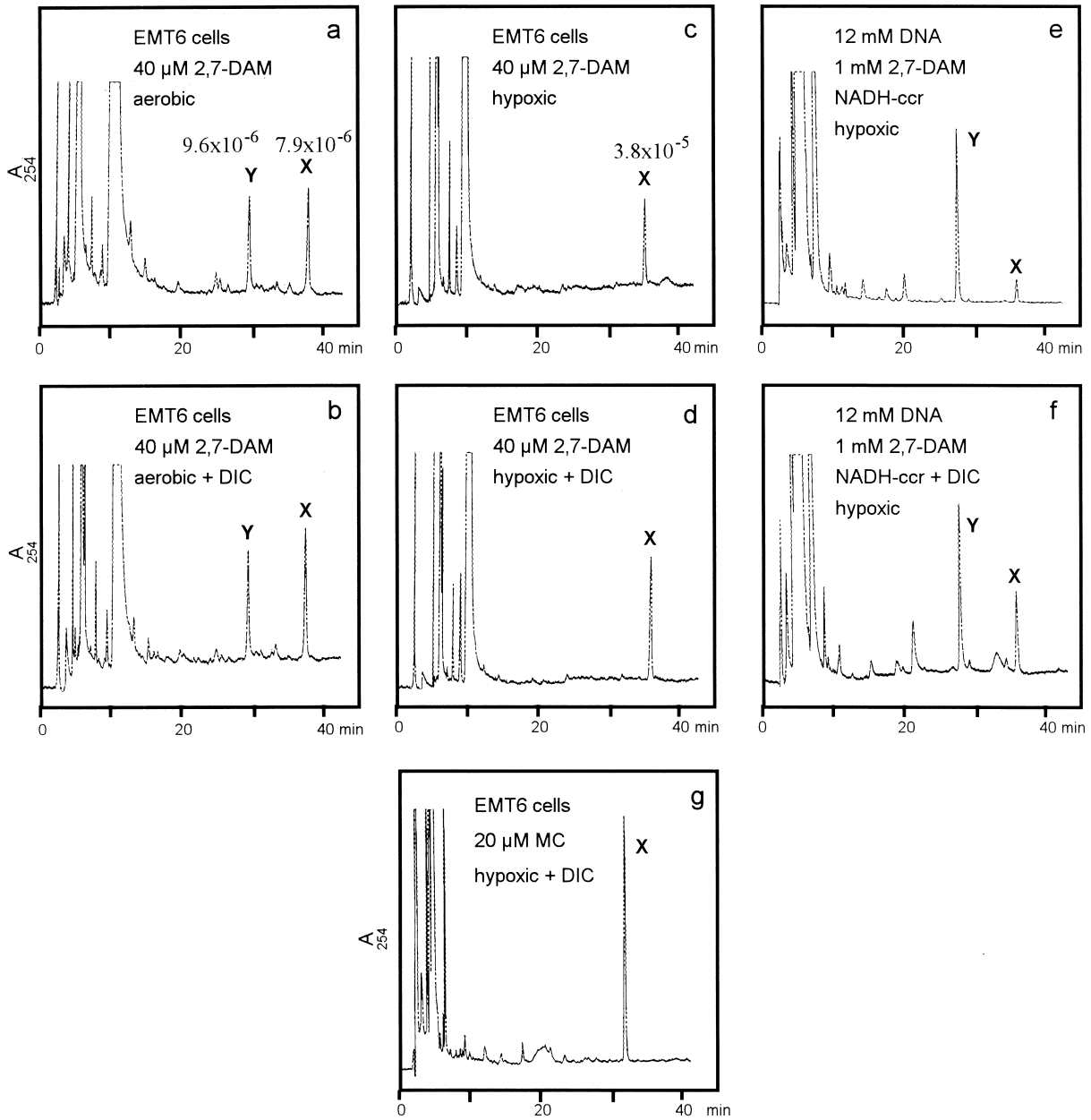


Fig. 4. HPLC of DNA adducts of 2,7-DAM formed in EMT6 cells and in a cell-free system upon treatment with 2,7-DAM or 2,7-DAM plus dicumarol. (a)–(d) DNA was isolated from cells after drug treatment and digested to nucleosides. Treatment: (a) 40 μM 2,7-DAM, 4 hr, aerobic conditions; (b) 40 μM 2,7-DAM + 300 μM DIC, 4 hr, aerobic conditions; (c) 40 μM 2,7-DAM, 4 hr, hypoxia; (d) 40 μM 2,7-DAM + 300 μM DIC, 4 hr, hypoxia (e) and (f) Calf thymus DNA adducts of 2,7-DAM were formed in a cell-free system using NADH-cytochrome *c* reductase as activator in the absence (e) or presence (f) of DIC. (g) DNA adducts resulting from treatment of EMT6 cells with 20 μM MC + 300 μM DIC, 4 hr, under hypoxia. Panel g is reproduced from Ref. 6 with permission. In (a) and (c), the frequencies of adduct *X* and *Y* are indicated above the corresponding peaks. A_{254}, absorbance at 254 nm.

the case of the pH 7.4 incubations (data not shown). It may be noted that most adduct frequencies were approximately 3-fold higher at pH 5.8 than at pH 7.4, with the exception of adduct X, the frequency of which was approximately 16-fold higher at pH 5.8 than pH 7.4, both in the absence or presence of DIC (data not shown). The increased adduct frequencies at the lower pH parallel the greater efficiency of the reduction of MC by XDH at pH 6.0 than at pH 7.4 [17]. However, a *selective* effect of DIC on the frequency of adduct *X* was clearly absent in the XDH-activated cell-free systems. Xanthine oxidase gave identical results (data not shown).

3.4. NADH-cytochrome c *reductase and NADPH-cytochrome* c *reductase as activators*

Anaerobic conditions were used, as described in the Materials and Methods section. The DNA patterns and frequencies are shown in Fig. 5, c and d, respectively. In the

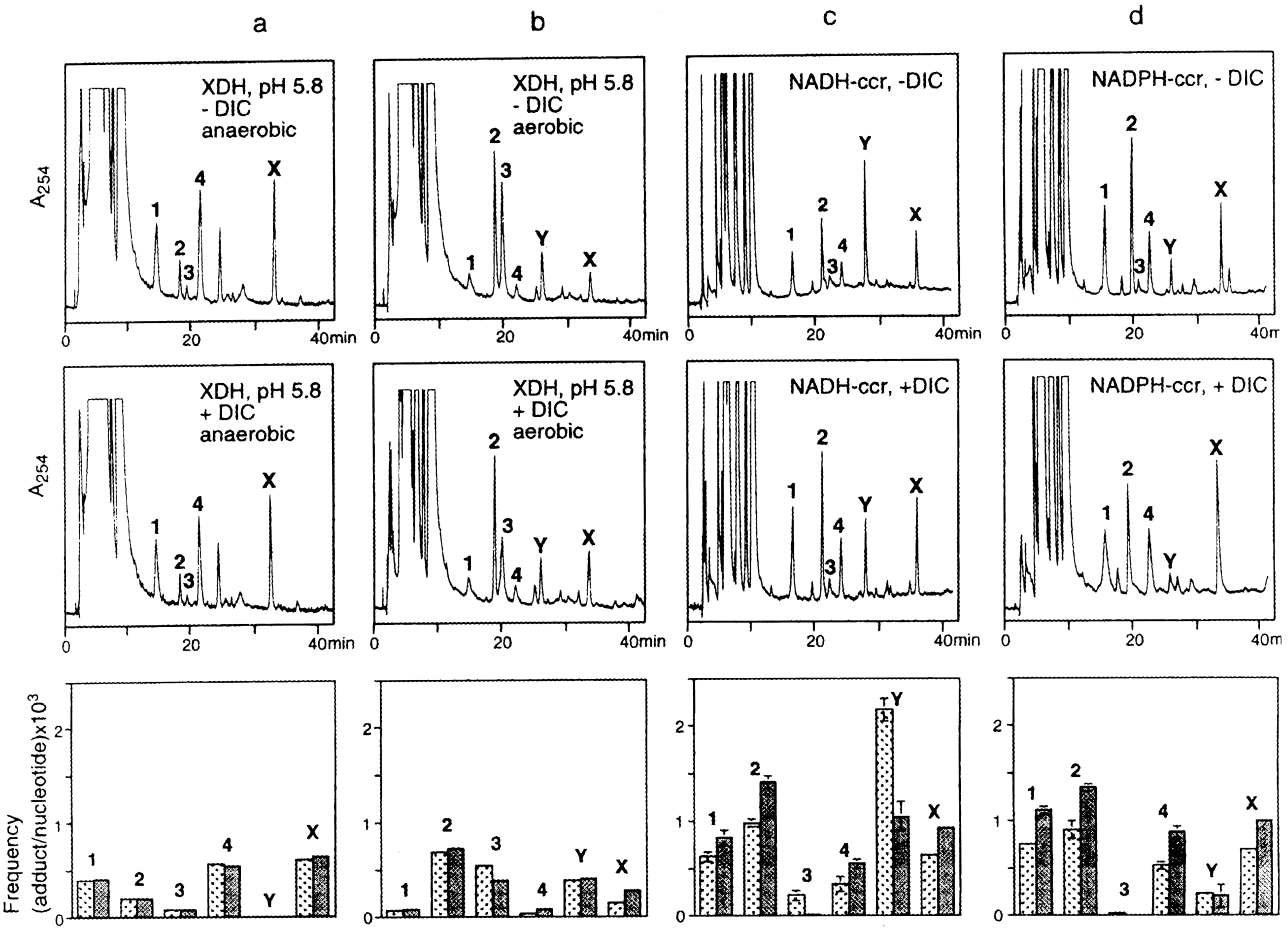


Fig. 5. HPLC patterns and frequencies of DNA adducts of MC formed in enzymatic cell-free systems in the absence or presence of DIC. Calf thymus DNA was used as substrate. The enzymatic activities were: (a) xanthine dehydrogenase, anaerobic conditions; (b) xanthine dehydrogenase, aerobic conditions; (c) NADH-cytochrome *c* reductase, anaerobic conditions; (d) NADPH-cytochrome *c* reductase, anaerobic conditions. The frequencies of individual adducts are shown in the bar graph form; ▨ −DIC; ▩, +DIC. Numbers or letters above peaks in the HPLC tracings refer to the adducts in Fig. 2. A_{254}, absorbance at 254 nm.

presence of DIC, the frequencies of both the MC adducts and adduct *X* increased in the 1.3- to 1.6-fold range. Again, no *selective* effect of DIC on adduct *X* was discernible. NADH-cytochrome *c* reductase produced a 2-fold *decrease* of *Y* in the presence of DIC, in a reproducible manner. The reason for this is not clear.

3.5. Cytotoxicity of 2,7-DAM

To assess the biological implications of the adduct studies described above, the cytotoxicities of MC and 2,7-DAM were compared under the conditions used in the adduct comparisons, and the effects of oxygenation and of DIC on the cytotoxicity of 2,7-DAM were examined. A direct comparison of the cytotoxicity (clonogenicity) of 2,7-DAM and MC was performed using a 2-hr treatment with graded doses of each drug in hypoxia. The IC_{50} for 2,7-DAM under these conditions was 15 μM, while the IC_{50} for MC was 0.15 μM (Fig. 6). 2,7-DAM was therefore approximately 100-fold less toxic than MC under hypoxic conditions. The cytotoxic effects of 2,7-DAM were examined further in the experiments shown in Fig. 7, which tested the effect of oxygenation and of DIC on the cytotoxicity of 2,7-DAM. Under aerobic conditions, 2,7-DAM produced minimal cytotoxicity even after prolonged treatment with 40 μM drug: the surviving fraction after 8 hr of treatment, calculated

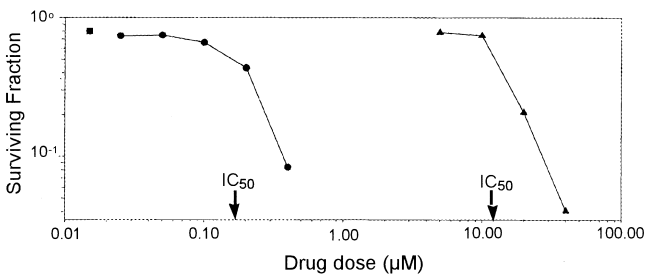


Fig. 6. Dose–response curves for EMT6 cells treated with MC or 2,7-DAM in hypoxia. EMT6 cells were treated with MC, 2,7-DAM, or vehicle for 2 hr in hypoxia at the doses indicated. Points are means from two experiments. ●, MC; ▲, 2,7-DAM; ■, vehicle (no drug).

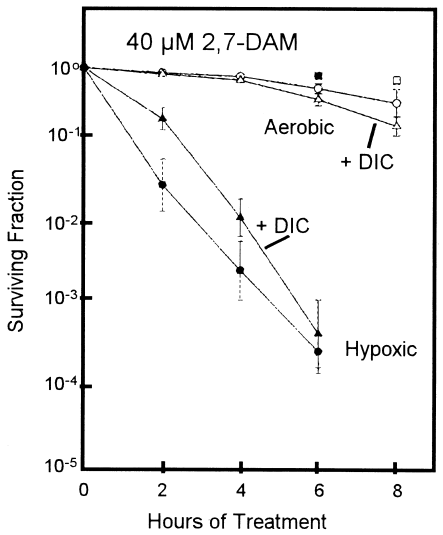


Fig. 7. Effect of hypoxia and DIC on the cytotoxicity of 2,7-DAM. EMT6 cells were treated with 40 μM 2,7-DAM under aerobic or hypoxic conditions, either with or without co-treatment with 300 μM DIC. Treatment with DIC + vehicle (■) had minimal toxicity to the cells under either aerobic or hypoxic conditions. Points are means ± SEM of 4 to 8 independent experiments.

relative to the vehicle-treated control, is 0.57. Greater cytotoxicity was observed in hypoxia (Fig. 7). In the same experiments, the cytotoxicity of 2,7-DAM was determined in the presence of co-treatment with 300 μM DIC, under both aerobic and hypoxic conditions. DIC did not produce statistically significant changes in the cytotoxicity of 2,7-DAM to EMT6 cells under either aerobic or hypoxic conditions; this is in contrast to the moderate, but statistically significant, effects of DIC on the cytotoxicity of MC [23], which are also qualitatively opposite from the effects of DIC on 2,7-DAM.

3.6. Cellular uptake and efflux of [^{3}H]MC and [^{3}H]2,7-DAM by EMT6 cells

The rates of uptake of 7.5 μM [^{3}H]MC and 7.5 μM [^{3}H]2,7-DAM (Fig. 8) were determined by measuring cell-associated radioactivity, using a previously established methodology [20,23]. Both MC and 2,7-DAM were taken up at a higher rate under hypoxia than in air, in agreement with previous reports on the uptake of MC and porfiromycin [20,24]. There was no evidence for a plateau in the uptake of either drug with the dose (7.5 μM) and maximum treatment time (2 hr) used in these studies. For both MC and 2,7-DAM, the activity retained by hypoxic cells was greater than that retained by aerobic cells; this higher level of retained activity could reflect a higher level of tightly bound drug, and could be related to the increased adduct formation and increased cytotoxicity seen with both of these drugs under hypoxic conditions. The uptake and efflux of 2,7-DAM were indistinguishable from those of MC.

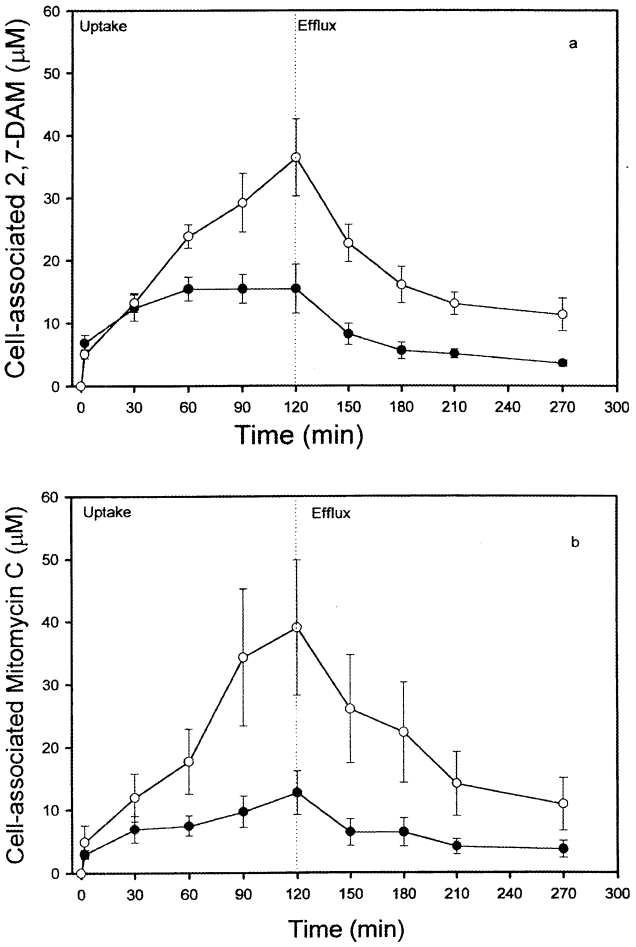


Fig. 8. Uptake and efflux of [^{3}H]MC and [^{3}H]2,7-DAM by EMT6 cells in air and in hypoxia. Cells were treated in suspension cultures under aerobic (●) and hypoxic (○) conditions with 7.5 μM MC (7.73 μCi/μmol) or 7.5 μM 2,7-DAM (6.70 μCi/μmol). Uptake was followed for times of 2–120 min, after which cells were collected by centrifugation, washed twice, and resuspended in drug-free medium to allow assessment of the efflux of the radiolabel. (a) Cell-associated 2,7-DAM. (b) Cell-associated MC. Points are means ± SEM of 3 to 4 independent experiments.

4. Discussion

Elucidation of the structure of all six major DNA adducts formed in EMT6 mouse mammary tumor cells following exposure to MC (Fig. 2) revealed that the adducts fall into two groups: adducts of activated MC and adducts of the MC metabolite, 2,7-DAM [6]. A mechanism has been proposed to explain the simultaneous formation of adducts of MC and 2,7-DAM observed in cell-free systems [25]: Reduction of MC yields a reactive intermediate (MC^{red}; Fig. 2), which either alkylates DNA to form MC adducts or rearranges to produce the reduced stable metabolite, 2,7-DAM. The latter can be reductively activated in a secondary reduction cycle to monofunctionally alkylate DNA, forming a 2,7-DAM–DNA adduct. This mechanism has been based on extensive experimental evidence obtained using cell-free systems [5,26]. Formation of the 2,7-DAM adducts *X* and *Y* upon treatment of EMT6 cells directly with 2,7-DAM (Fig. 4,

Table 1
Lack of significant cytotoxicity of 2,7-DAM–DNA adducts

Cell treatment	Cell survival	MC adduct (1–4) frequency[a]	2,7-DAM adduct (*X*) frequency[a]
2 μM MC, 1 hr, hypoxia	$\sim$.01[b]	$\sim 10^{-7}$[c]	$\sim 10^{-7}$[c]
40 μM 2,7-DAM, 4 hr, hypoxia	.01[d]	0[d]	3.8×10^{-5}[d]

[a] Adduct/nucleotide.
[b] Surviving fraction from data in [22].
[c] [15].
[d] This work.

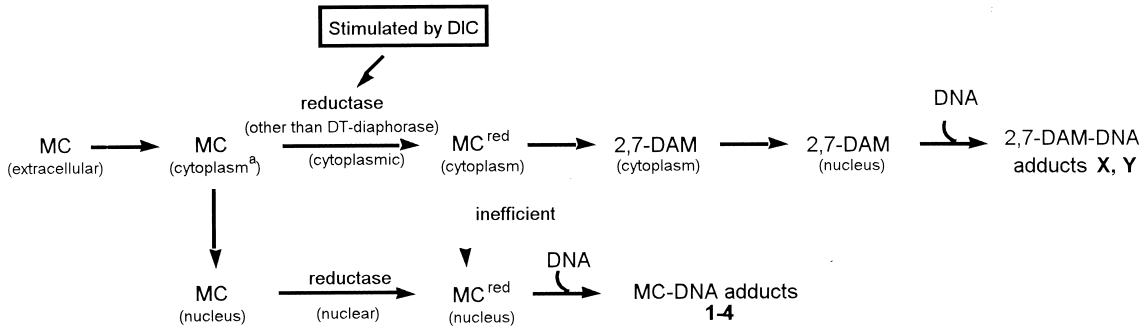
a–d) now provides critical confirmatory evidence that the adducts *X* and *Y* produced in the cell do indeed originate from 2,7-DAM.

Since a large proportion of the DNA alkylation damage in MC-treated cells is caused by the MC metabolite 2,7-DAM, rather than by MC itself [6,15], the question arose as to whether the formation of 2,7-DAM–DNA adducts played any major role in the cytotoxicity of MC. It was noted previously [15], and confirmed here, that 2,7-DAM itself was approximately 100-fold less toxic than MC to the EMT6 cells under hypoxic conditions. Since 2,7-DAM was taken up by and effluxed from EMT6 cells at the same rates as MC (Fig. 8), a slower uptake or faster efflux of 2,7-DAM by the cells was ruled out as the reason for its lesser cytotoxicity. Furthermore, formation of 2,7-DAM–DNA adducts in the 2,7-DAM-treated cells demonstrated that 2,7-DAM does indeed alkylate DNA after entering the cell. Thus, it is concluded that the 2,7-DAM–DNA adducts themselves are relatively non-cytotoxic and that their contribution to the cytotoxicity of MC is negligible. A quantitative illustration of this conclusion is provided by data summarized in Table 1: 40 μM 2,7-DAM treatment for 4 hr and 2 μM MC treatment for 1 hr, both under hypoxia, resulted in equivalent cytotoxicities (a surviving fraction of $\sim$0.01). The frequency of 2,7-DAM–DNA adducts (*X*) was 3.8×10^{-5}, i.e. 380-fold higher in the 2,7-DAM-treated cells than in the cells treated with MC (10^{-7}). Clearly, the major part of the cytotoxicity resulting from 2 μM MC treatment is due to the MC–DNA adducts, which are formed at a frequency of only 10^{-7}, yet result in the same level of cytotoxicity as the 2,7-DAM adducts, which are formed at 380-fold higher frequency in the 2,7-DAM-treated cells. That only the MC adducts, and not adduct *X*, showed a correlation with the cytotoxicity of MC as modulated by dicumarol co-treatment of EMT6 cells [10] is consistent with this conclusion.

The basis of the low cytotoxicity of the 2,7-DAM adducts *X* and *Y* is not obvious. Because of their monofunctionality, these adducts would be expected to be less cytotoxic than the MC cross-link adduct 4 [27]. Nevertheless, the very low cytotoxicity (Figs. 6 and 7; [15]) of such bulky adducts as *X* and *Y* is puzzling, especially since decarbamoyl MC (DMC; Fig. 2), another monofunctional MC derivative, is nearly as cytotoxic as MC [22]. DMC is known to monoalkylate DNA to give adduct 2 [28]. There are several possible reasons for the higher cytotoxicity of DMC compared with that of 2,7-DAM. Alkylation by DMC may occur at higher frequency or at more sensitive sites than alkylation by 2,7-DAM. C-1 adducts of mitosenes may be more toxic than C-10 adducts in general: the 2,7-DAM adducts may be more easily repaired or bypassed by polymerases than the DMC monoadducts, because of the structural differences between the C-1 adduct of DMC and C-10 adduct of 2,7-DAM (Fig. 2). Studies with DMC are underway to clarify the mechanism underlying the differences between the cytotoxicities of DMC and 2,7-DAM.

DIC, a potent inhibitor of DT-diaphorase [29], has been widely used as a tool to study the reductive enzymology of MC and other bioreductive agents, both in cell-free systems and in intact cells. Determination of the effects of simultaneous treatment of cells with MC and DIC on the cytotoxicity of MC may provide some estimate of the importance of DT-diaphorase in the cytotoxic process [30], although the validity of such estimates has been questioned [31]. DIC is not fully specific as an inhibitor of DTD and also inhibits activation of MC by NADH-cytochrome b_5 reductase [33]. We reported recently that the frequency of adduct *X* showed a disproportionately large increase with MC–DIC co-treatment, as compared with MC treatment alone, under both hypoxic and aerobic conditions [10]. Specifically, treatment of EMT6 cells with 2.0 μM [^{3}H]MC for 1 hr under hypoxia led only to a 2.0-fold increase in the frequency of total MC adducts, but to a much larger (12-fold) increase in the frequency of adduct *X* when the treatment included 300 μM DIC. Under aerobic conditions, although total MC adduct frequency decreased 1.5-fold, the frequency of adduct *X* increased 5.5-fold in the presence of 300 μM DIC. As a qualitative illustration of more recent results of MC–DIC co-treatment of EMT6 cells, Fig. 4g is reproduced here from Ref. 6 to show the 23-fold predominance of adduct *X* (1.5×10^{-5} frequency) over MC adducts in cells treated under hypoxia.

What is the mechanism for the enhancement of the 2,7-DAM adduct *X* under the influence of DIC? A possibility was that DIC enhanced the chemical reactivity between 2,7-DAM and DNA. This was ruled out by showing that in cell-free 2,7-DAM activation systems, DIC did not influence the frequency of the 2,7-DAM–DNA adducts (Fig. 6, e and f). This lack of a direct effect proved to be true in intact cells as well: co-treatment with 2,7-DAM plus DIC did not result in an increased frequency of adduct *X*, compared to 2,7-DAM treatment alone (Fig. 4, a–d). Thus, DIC does not affect either the reactivity of 2,7-DAM with DNA or the reductive activation of 2,7-DAM, which is necessary for its reactivity. Another alternative is that DIC stimulates the activity of a specific reductase that reduces MC to an active form producing predominantly (or exclusively) 2,7-DAM–DNA adducts. We tested four reductases, known to reduce MC, as candidates for this mechanism: xanthine



[a] The term "cytoplasm" is used here to designate "other than nucleus".

Fig. 9. Proposed scheme to explain the effect of DIC treatment on the DNA adduct profile in MC-treated EMT6 cells.

dehydrogenase, xanthine oxidase, NADH-cytochrome *c* reductase, and NADPH-cytochrome *c* reductase. DT-diaphorase and NADH-cytochrome b_5 reductase were not tested, because DIC inhibits rather than stimulates their activities [29,33]. All four reductases activated MC in the DNA-containing cell-free systems to generate, side-by-side, the set of four MC–DNA adducts and the two 2,7-DAM–DNA adducts, with similar patterns of distribution (Fig. 5). Furthermore, no selective increase in adduct *X* was observed when 300 μM DIC was included in any of the reaction mixtures. These results indicate that all four enzymes reduce MC to the same intermediate, MC^{red}, which then partitions into paths 1 and 2 (Fig. 3) in a proportion which is independent of DIC. Although these negative results do not rule out the above hypothesis with absolute certainty, the possibility that an unknown, significant MC reductase exists in EMT6 cells and would have tested positively in our cell-free system containing DIC is unlikely.

It appears, then, that the preferential increase in the non-cytotoxic pathway of MC in the presence of DIC is a phenomenon occurring only in intact cells. We propose a mechanism by which this can occur, based upon the known chemistry of MC activation, as follows. In the absence of DNA, enzymatic reduction of MC by either one- or two-electron reductases in cell-free systems leads predominantly to path 2, i.e. rearrangement to 2,7-DAM (Fig. 3; [21,34, 35]). By extension to intact cells (Fig. 9), reduction of MC to MC^{red} in the cytoplasm,[1] where nuclear DNA is not directly accessible for alkylation, is expected to generate predominantly 2,7-DAM. However, since 2,7-DAM is stable, it can diffuse into the nucleus to alkylate DNA, yielding 2,7-DAM–DNA adducts. In contrast, when reduction of MC occurs in the nucleus or otherwise in the vicinity of DNA, path 1, i.e. the direct reactions of MC^{red} with DNA, can compete more effectively with path 2, i.e. with the rearrangement of MC^{red} to 2,7-DAM. It follows that stimulation of MC reduction by DIC in the cytoplasm would lead only to increased 2,7-DAM production and therefore to a selective increase in 2,7-DAM–DNA adducts. Both XDH and NADPH-cytochrome *c* reductase are known to be stimulated by DIC to reduce MC [21,36] and thus could be involved in this process.[2] We propose that the observed preferential increase in 2,7-DAM–DNA adducts in the presence of DIC is an indication that reductive metabolism of MC occurs in distinct locations within EMT6 cells; the locations differ in their efficiency in mediating the DNA alkylation/cross-linking path, 1. In the cytoplasm[3] the detoxification path, 2, may prevail, while in the nucleus the cytotoxic path, 1, proceeds more efficiently (Fig. 9).

In support of this concept, a dependence of the cytotoxicity of MC on the subcellular location of MC reductases was recently demonstrated in several cell lines. Overexpression of NADPH-cytochrome *c* reductase, which is located in the endoplasmic reticulum, led to increased sensitivity to MC; however, overexpression of this reductase resulted in an even greater increase in sensitivity when the enzyme was fused with a nuclear localization signal peptide, which concentrated it in the nucleus [37]. Similar results were obtained upon comparison of the effects of DT-diaphorase overexpressed in the cytoplasm or in the nucleus [38]. In this context, it is notable that natural MC reductase activity was detected in four distinct subcellular fractions of mouse liver [39], and high tirapazamine reductase activity was observed in the nucleus [40].

In summary, bioreduction of MC leads to both a cytotoxic pathway and a non-cytotoxic pathway, which generate cytotoxic and non-cytotoxic DNA adducts, respectively. Although parallel reductive activation and inactivation pathways exist with other bioreductive therapeutic agents, such as tirapazamine [41] and metronidazole [42], formation of relatively non-toxic DNA adducts as part of an inactivation pathway has not been documented previously. The effects

[1] "Cytoplasm" is understood here to encompass distinct fractions such as cytosol, mitochondria, microsomes, etc.

[2] DIC may also stimulate the *expression* of certain reductase genes, in analogy to coumarin [43].

[3] "Cytoplasm" is understood here to encompass distinct fractions such as cytosol, mitochondria, microsomes, etc.

of DIC on the relative distribution of the two types of DNA adducts suggest that the two pathways function independently of one another; it is proposed that the basis of this independence is the different intracellular location of the reductases involved in the initiation of the reductive pathways. The cytotoxic pathway, leading to DNA cross-linking and MC–DNA monoadducts, is efficient when reduction of MC occurs in the vicinity of DNA, while the non-cytotoxic pathway results from reduction of MC in the cytoplasm, where the target nuclear DNA is not accessible. Thus, the overall cellular level of a reductase, capable of reducing MC, may not be a reliable predictor of the sensitivity of the cells to MC. Since 2,7-DAM is the major product of the non-cytotoxic pathway, intracellular levels of 2,7-DAM should be similarly non-predictive in this regard, as indeed was found to be the case [9]. Furthermore, the 2,7-DAM–DNA adducts *X* and *Y* also lack a relationship to the sensitivity of the EMT6 cells to MC. The levels of DNA–MC cross-links and/or MC monoadducts formed in proportion to the cross-links appear to be the most accurate indicators of the cytotoxic activity of MC currently available.

Acknowledgments

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