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INDUCTION OF DT-DIAPHORASE BY DOXORUBICIN AND COMBINATION THERAPY WITH MITOMYCIN C *IN VITRO*

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Abstract—Mitomycin C (MMC) is a bioreductive antitumor agent that is activated by NADPH:cytochrome P450 reductase (EC 1.6.2.4) and NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2) (DT-diaphorase). DT-diaphorase is a two-electron reducing enzyme that is induced by a variety of chemicals, including quinones. Doxorubicin (DOX) is an anthraquinone antitumor agent that has been used clinically with MMC for combination chemotherapy in breast cancer. In this study, we investigated whether DOX could selectively induce DT-diaphorase in tumor cells and whether combining this agent with MMC in an appropriate schedule could produce synergistic antitumor activity. Treatment of EMT6 murine mammary tumor cells with DOX resulted in a 40% increase in DT-diaphorase activity in these cells, but had no effect on this enzyme in murine bone marrow cells. Combination therapy with DOX and MMC produced a 1.4-fold level of synergistic cell kill in the tumor cells, but a similar level of synergy was also observed in normal bone marrow cells. Thus, DOX can selectively induce elevated levels of DT-diaphorase in tumor cells; however, the synergy observed by combining this agent with MMC appears to be unrelated to the induction of DT-diaphorase.

Key words: DT-diaphorase; doxorubicin; mitomycin C; combination therapy; EMT6 cells; mouse marrow cells

DOX[‖] is an anthracycline antitumor agent that is used extensively to treat acute leukemias, lymphomas and a wide variety of solid tumors, including breast cancer [1]. The use of this agent is limited by cardiotoxicity [2], myelosuppression [3] and inactivity against certain solid tumors [1]. DOX binds to DNA, RNA, chromatin and cell membranes; however, its antitumor activity likely results from the inhibition of topoisomerase II [4, 5]. The anthracycline can also undergo redox cycling to produce free radicals that cause DNA cleavage and membrane peroxidation [6]. Free radicals may contribute to the antitumor activity of anthracyclines, but are involved primarily in cardiotoxicity [6].

Bioreductive antitumor agents are an important new class of anticancer drugs [7]. The prototype agent in this class, MMC [8], is widely used for the treatment of breast [9], gastrointestinal [10], non-small cell lung [11], and head and neck [12] cancers. While MMC has been used in single agent therapy [9, 11–13], this antitumor agent generally produces higher response rates when combined with other antitumor agents [9–12], particularly DOX [9, 10, 14, 15]. The major toxicity associated with MMC treatment is a delayed myelotoxicity that is dose-limiting [13].

MMC is activated by reduction of the quinone group leading to the generation of alkylating species or to redox cycling that produces active oxygen species [8]. The alkylating intermediates produce DNA monoadducts, intrastrand cross-links and interstrand cross-links [16–18], while the active oxygen species can produce DNA strand breaks [16, 19]. Although both of these mechanisms may contribute to MMC cytotoxicity, DNA alkylation appears to be primarily responsible for MMC antitumor activity [19, 20]. MMC can be activated by the one-electron reducing enzymes, NADPH:cytochrome P450 reductase (EC 1.6.2.4) [8, 21, 22], xanthine oxidase (EC 1.1.3.22) [22] and NADH cytochrome b_5 reductase (EC 1.6.2.2) [23] and by the two-electron reducing enzymes, DT-diaphorase (EC 1.6.99.2) [24, 25] and xanthine dehydrogenase (EC 1.1.1.204) [26]. NADPH:cytochrome P450 reductase appears to be the major contributor to MMC activation in many cell lines [21, 24, 27, 28]; however, drug activation by this enzyme is decreased under aerobic conditions by rapid reoxidation of the initially formed semiquinone. Thus, DT-diaphorase may play an important role in MMC activation under aerobic conditions in many cells [8, 20, 21, 24, 25, 27, 29]. In contrast, the contribution of DT-diaphorase to MMC antitumor activity under hypoxic conditions, where reoxidation of MMC semiquinone does not occur, appears to be more limited [27, 29].

DT-diaphorase is a family of two-electron reducing enzymes that decrease the oxidative damage resulting from one-electron reduction of quinones [30, 31]. It is induced coordinately with other phase II detoxifying enzymes, such as the glutathione *S*-transferases [32], and may also play an important role in the detoxification of carcinogens [24, 33]. DT-diaphorase levels are relatively high in tissue from liver, stomach, bladder, intestine, colon and kidney [31, 34, 35], and the enzyme is inducible in liver, kidney and lung tissue [34]. In contrast, levels of DT-diaphorase are relatively low in spleen and

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‖ Abbreviations: DOX, doxorubicin; MMC, mitomycin C; DT-diaphorase, NAD(P)H:(quinone acceptor)oxidoreductase; IMDM, Iscove's modified Dulbecco's medium; CS, calf serum; HBSS, Hanks' balanced salt solution; FCS, FetalClone II; DMF, dimethyl formamide; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; CFU-G, colony forming unit-granulocyte; CFU-M, colony forming unit-monocyte; CFU-GM, colony forming unit-granulocyte monocyte; and CFU-GEMM, colony forming unit-granulocyte erythroid monocyte megakaryocyte.

thymus, and there is little induction of the enzyme in these tissues [34]. Elevated levels of expression of DT-diaphorase have been reported in breast, colon, liver and lung tumors compared with the corresponding normal tissues [24, 31, 35].

A wide variety of chemical compounds, including quinones, Michael reaction acceptors, polycyclic aromatic hydrocarbons, azo dyes, flavanoids, diphenols, 1,2-dithiol-3-thiones and isothiocyanates has been shown to induce DT-diaphorase in cells [32, 33]. The induction of this enzyme depends on the inducing agent providing an electrophilic chemical signal leading to increased transcription of the enzyme [32, 33]. Transcription appears to be regulated by an antioxidant response element in the 5′-upstream regulatory region of the DT-diaphorase gene [36, 37] that contains an AP1 binding site [31]. Increased levels of the enzyme are normally observed in cells 24–48 hr after exposure to the inducing agent [38, 39].

While DOX and MMC have been used extensively for combination chemotherapy in breast cancer [9, 14], the schedules for the administration of these two agents have been determined primarily by toxicity considerations [9]. Within a regimen the agents may be given simultaneously or several weeks apart. DOX contains a quinone group in its structure, and there is some evidence that anthracyclines can induce DT-diaphorase [40]. In this study, we examined whether DOX can induce DT-diaphorase in tumor and normal cells, and investigated if combining this agent with MMC in an appropriate schedule can produce synergistic antitumor activity in EMT6 murine mammary tumors.

MATERIALS AND METHODS

Materials

Waymouth's MB 752/1 medium, IMDM, CS, HBSS and trypsin were obtained from Gibco BRL (Grand Island, NY). Methocult M3430 and all related marrow culture supplies were from Stem Cell Technologies (Vancouver, Canada). FCS was from HyClone Laboratories (Logan, UT). All reagents for the DT-diaphorase assay were from the Sigma Chemical Co. (St. Louis, MO), as were DOX and MMC. Quickstain II and 100 μm mesh filters were from Baxter (Mississauga, Canada). Protein concentration was measured using the Bio-Rad DC Kit (Bio-Rad, Mississauga, Canada) with γ-globulin as standard. MMC was dissolved in PBS, while DOX was dissolved in DMF and then further diluted in PBS such that the final concentration of DMF did not exceed 0.04%. HBSS, which was used for all drug incubations, was supplemented with 5 mM HEPES, to maintain pH, and 2% FCS.

EMT6 cells were provided by Dr. C. Pritsos, University of Nevada, Reno, NV, and were grown as a monolayer in Waymouth's medium with 10% FCS and 5% CS. The cells had a doubling time of 14–16 hr and all experiments used cells in log phase growth. BALB/c mice were obtained from Charles River Canada (Montreal, Canada) and were used at 12–20 weeks of age.

Measurement of DT-diaphorase activity

EMT6 cells were plated in 60-mm plates and allowed to attach for 5 hr. The medium was removed and replaced with HBSS/2% FCS containing 0 or 2 μM DOX. Cells were incubated at 37° for 1 hr; medium was removed by aspiration; cells were washed twice with PBS, and then were incubated in Waymouth's/10% FCS/5% CS. At various times, the cells were washed twice with PBS, scraped into 2 mL PBS on ice, spun at 400 *g* for 10 min, resuspended in 100 μL of 0.25 M sucrose, sonicated three times for 10 sec on ice and stored at −80°.

BALB/c mice were asphyxiated with CO_2, and both femurs were removed. Marrow was obtained by flushing the femurs with HBSS/2% FCS using a 22-gauge needle. Marrow from the femurs of 2–3 BALB/c mice was pooled. A single cell suspension was ensured by passing the cells through a 25-gauge needle, and any particulate matter remaining was removed by passing through a 100-μm mesh nylon filter. Cells were treated with 0 or 1 μM DOX for 1 hr, washed twice with PBS, and resuspended in IMDM/20% FCS. At various times, cells were washed twice with PBS, resuspended in 25 μL of 0.25 M sucrose, sonicated three times for 10 sec on ice, and stored at −80°.

Protein concentration was determined in all samples with the Bio-Rad DC Protein Assay kit using bovine γ-globulin as standard. For EMT6 cells, 5 μg protein was assayed for DT-diaphorase activity, while for marrow 40–80 μg was assayed. DT-diaphorase activity was measured in the sucrose sonicates using a modification of the procedure of Prochaska and Santamaria [41] with a Cary 1 spectrophotometer (Varian). DT-diaphorase activity was expressed as nanomoles of MTT reduced per minute per milligram of protein.

Cytotoxicity assay for EMT6 cells

EMT6 cells were plated at 7.5×10^4 cells/well in 12-well plates and allowed to attach for 5 hr. Medium was removed by aspiration and replaced with HBSS/2% FCS containing 0 or 2 μM DOX; then the cells were incubated at 37° for 1 hr. Medium was removed, and the cells were washed twice with PBS and incubated for an additional 22 hr in Waymouth's/10% FCS/5% CS. Medium was removed, and the cells were incubated with 0–3 μM MMC in HBSS/2% FCS for 1 hr. For studies involving the simultaneous addition of DOX and MMC, cells were incubated for 23 hr in Waymouth's/10% FCS/5% CS, washed and then treated with 0 or 2 μM DOX and various concentrations of MMC in HBSS/2% FCS for 1 hr. Drug cytotoxicity was determined by clonogenic assay as described previously [29]. Cloning efficiencies averaged 59.7 ± 6.0%. The surviving cell fractions for cells treated with DOX alone, with various concentrations of MMC alone, and with DOX and various concentrations of MMC were determined from the clonogenic assays. The surviving cell fractions expected for an additive cytotoxic effect between DOX and MMC were calculated using the "effect multiplier principle" [42, 43]. For each MMC concentration, the expected surviving cell fraction was obtained from the product of: (surviving cell fraction for DOX alone) × (surviving cell fraction for MMC alone). The D_{10} (concentration of MMC required to reduce the surviving cell fraction by 90%) values for MMC alone, for DOX and MMC, and for an expected additive effect between DOX and MMC were obtained from linear regression lines of the corresponding surviving cell fraction versus MMC concentration curves.

Cytotoxicity for BALB/c mouse bone marrow cells

Marrow cells were obtained as described above. Cells were incubated in HBSS/2% FCS with 0 or 1 μM DOX

for 1 hr at 37°, washed twice with PBS, resuspended in IMDM/20% FCS and incubated for a further 22 hr at 37°. Medium was removed by centrifugation, and cells were resuspended in HBSS/2% FCS containing 0–10 μM MMC. After 1 hr of incubation, the cells were washed twice with PBS and resuspended in 200–500 μL IMDM/20% FCS. For control cultures, 2.5×10^4–5×10^4 cells were added to 1 mL Methocult M3430 following the protocol provided by Stem Cell Technologies. For drug-treated cells, increased cell numbers up to 7.5×10^5 were added to 1 mL Methocult M3430. Plates were counted after 14 days, and scored for CFU-G, CFU-M, CFU-GM and CFU-GEMM [44]. Total colony count was used to calculate the surviving cell fraction, and the D_{10} was determined as described for EMT6 cells. The colony forming efficiency for control cultures was within the expected range of 0.1 to 0.24% [45].

RESULTS

Induction of DT-diaphorase by DOX in EMT6 murine mammary tumor cells and normal murine bone marrow cells

EMT6 murine mammary tumor cells were incubated with 2 μM DOX at 37° for 1 hr *in vitro*. DT-diaphorase activity in the cells increased from a base level of 652 ± 21 nmol/min/mg protein, reaching a plateau level of 911 ± 101 nmol/min/mg protein after 24 hr (Fig. 1A), and this increase was statistically significant ($P < 0.02$; df, 9). In contrast, the base level of DT-diaphorase activity in normal bone marrow cells from BALB/c mice was 17.5 ± 1.4 nmol/min/mg protein. Treatment of the marrow cells with 1 μM DOX at 37° for 1 hr *in vitro* had no significant effect on DT-diaphorase activity in these cells (Fig. 1B).

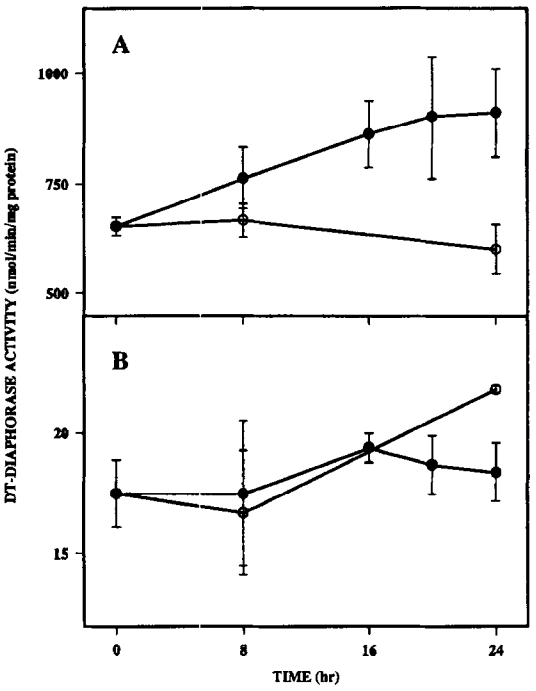

Fig. 1. Induction of DT-diaphorase by DOX in EMT6 cells and normal murine bone marrow cells. (A) EMT6 murine mammary tumor cells were treated *in vitro* at 37° without (○), or with (●), 2.0 μM DOX for 1 hr. (B) Normal murine bone marrow cells from BALB/c mice were treated *in vitro* at 37° without (○), or with (●), 1.0 μM DOX for 1 hr. The levels of DT-diaphorase activity in the cells were determined at various times as described in Materials and Methods. Values are the means ± SEM of 3–4 determinations.

Combination therapy with DOX and MMC in EMT6 cells and normal murine bone marrow cells

EMT6 cells were treated *in vitro* at 37° with, or without, 2 μM DOX for 1 hr. After incubation for 22 hr, the cells were treated with, or without, various concentrations of MMC for 1 hr. Cytotoxicity was determined by clonogenic assay (Fig. 2A). Treatment with DOX alone resulted in a surviving cell fraction of 0.20 ± 0.02. The D_{10} for treatment with MMC alone was 2.94 ± 0.29 μM, while that for MMC following DOX treatment was 2.14 ± 0.16 μM (Table 1). Combination therapy with DOX and MMC produced 1.4-fold greater cytotoxicity than was expected for an additive effect for these two agents and synergy increased with increasing MMC concentration (Fig. 2A). However, when EMT6 cells were treated simultaneously with 2 μM DOX and various concentrations of MMC for 1 hr, a similar 1.3-fold level of enhanced cell kill was observed (Fig. 2B and Table 1).

Bone marrow cells from BALB/c mice were treated *in vitro* at 37° with, or without, 1 μM DOX for 1 hr, followed 22 hr later by treatment with, or without, various concentrations of MMC for 1 hr. Cytotoxicity was determined by a methylcellulose clonogenic assay of marrow progenitor cells (Fig. 2C). Treatment with DOX alone produced a surviving cell fraction of 0.11 ± 0.02. The D_{10} for treatment with MMC alone was 17.64 ± 0.76 μM, and this decreased to 9.17 ± 1.24 μM with DOX pretreatment (Table 1) resulting in a 2.2-fold level of synergy (Fig. 2C and Table 1). Combination therapy with DOX and MMC did not change the relative survival of different progenitor cell populations compared with treatment with DOX or MMC alone.

DISCUSSION

In this study, we have demonstrated that DOX, an anthraquinone antitumor agent, can induce DT-diaphorase activity in EMT6 murine mammary tumor cells. Following treatment with DOX, the level of DT-diaphorase in these cells increased by approximately 40%. In contrast, similar treatment of murine bone marrow cells had no effect on the activity of this enzyme in these cells. It was necessary to use a lower concentration of DOX with the marrow cells compared with the tumor cells because the marrow cells were more sensitive to this agent. However, we did observe induction of DT-diaphorase in EMT6 cells treated with 1 μM DOX. These findings demonstrate that DOX can selectively induce the phase II detoxifying enzyme, DT-diaphorase, in tumor cells.

As elevated levels of DT-diaphorase activity have been shown to increase the cytotoxic activity of MMC [7, 8, 20, 21, 24, 25], we investigated whether pretreatment of EMT6 cells with DOX to increase the level of DT-diaphorase could potentiate the antitumor activity of MMC. We did observe increased tumor kill of EMT6 cells pretreated with DOX compared with cells treated with MMC alone, and the combination therapy resulted in 1.4-fold greater cell kill than was expected for an

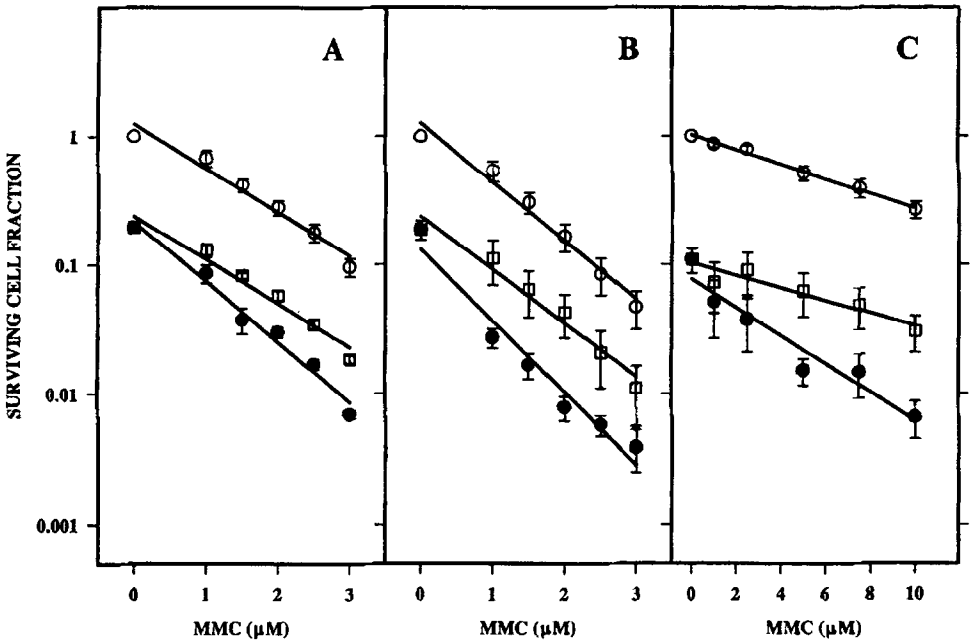

Fig. 2. Cytotoxicity of combination therapy with DOX and MMC in EMT6 mice cells and normal murine bone marrow cells. EMT6 murine mammary tumor cells were treated *in vitro* at 37° (A) with 0 or 2.0 μM DOX for 1 hr and then 22 hr later with various concentrations of MMC for 1 hr, or (B) together with 0 or 2.0 μM DOX and various concentrations of MMC for 1 hr. The surviving cell fraction was determined by clonogenic assay as described in Materials and Methods. (C) Normal murine bone marrow cells from BALB/c mice were treated *in vitro* at 37° with 0 or 1.0 μM DOX for 1 hr and then 22 hr later with various concentrations of MMC for 1 hr. The surviving cell fraction was determined by methylcellulose clonogenic assay as described in Materials and Methods. Symbols represent: surviving cell fraction of cells treated with MMC alone (○); surviving cell fraction of cells treated with DOX amd MMC (●), and surviving cell fraction expected for an additive cytotoxic effect between DOX and MMC (□). Values are the means ± SEM of 4–10 determinations. Lines are linear regression lines.

additive cytotoxic effect with these two agents. However, we observed a similar level of synergy for combination therapy with DOX and MMC in EMT6 cells when the cells were treated simultaneously with the two agents. Furthermore, we also obtained synergistic cell kill in normal murine bone marrow cells pretreated with DOX and then treated with MMC. These results demonstrate that combination therapy with DOX and MMC can produce synergistic cell kill; however, this effect does not appear to be related to an increase in the level of DT-diaphorase activity. In addition, this enhanced cell kill does not appear to be selective for tumor cells compared with normal marrow cells.

The mechanism responsible for the synergistic cell kill observed with DOX and MMC is unknown; however, both agents produce DNA damage [4–6, 16–18]. The combined accumulation of DNA damage in the cells may result in greater than expected cell kill. Alternatively, it has been shown that both agents can induce apoptosis in cells. It is possible that the induction of the cell death process by each agent via different mechanisms may result in enhanced cell kill.

Our observation that the cytotoxic activity of MMC in bone marrow cells was considerably lower than that in EMT6 cells is somewhat surprising in view of the clinical toxicity of MMC to marrow cells. However, this result may reflect the greater ability of MMC to reach the tumor cells in these *in vitro* studies compared with the clinical situation, where access of the agent to the tumor cells may be restricted by poor vascularization of the tumor.

In summary, we have shown that DOX, an anthracy-

Table 1. Combination therapy with DOX and MMC

Cells	Treatment schedule	DOX concentration* (μM)	D_{10}† (μM) MMC alone	D_{10}† (μM) DOX + MMC (Expected)	D_{10}† (μM) DOX + MMC (Actual)	Expected/ actual
EMT6	DOX followed by MMC	2.0	2.94 ± 0.29	2.93 ± 0.29	2.14 ± 0.16	1.4
EMT6	DOX and MMC together	2.0	2.19 ± 0.17	2.40 ± 0.23	1.81 ± 0.19	1.3
Bone marrow	DOX followed by MMC	1.0	17.64 ± 0.76	20.53 ± 3.35	9.17 ± 1.24	2.2

* Concentration of DOX used in combination therapy.

† Concentration of MMC that reduced the surviving cell fraction by 90%. The D_{10} values were obtained from the linear regression lines of the surviving cell fraction versus MMC concentration curves shown in Fig. 2. Values are means ± SEM (df = 5, N = 6).

cline antitumor agent, can selectively induce elevated levels of DT-diaphorase activity in murine tumor cells compared with normal murine bone marrow cells. Combination therapy with DOX and the bioreductive antitumor agent, MMC, resulted in synergistic cell kill, but the enhanced cell kill was not selective for tumor cells and appeared to be unrelated to the induction of DT-diaphorase by DOX. However, it is possible that combining MMC with more effective inducers of DT-diaphorase may lead to selective enhancement of antitumor activity.

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