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# Role of NAD(P)H:(Quinone Acceptor) Oxidoreductase (DT-Diaphorase) in Activation of Mitomycin C under Acidic Conditions

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**SUMMARY**

The quinone antitumor agent mitomycin C is preferentially toxic to some cells under hypoxic and acidic conditions. The two-electron reducing enzyme DT-diaphorase may be a major contributor to mitomycin C activation under aerobic conditions, but its role in drug activation under hypoxic and acidic conditions is unclear. In this study, we observed that mitomycin C produced increased DNA cross-linking and cytotoxicity in Chinese hamster ovary cells at pH 6.6, compared with pH 7.2, under aerobic conditions, but drug activity was similar at these pH values under hypoxic conditions. The DT-diaphorase inhibitor dicoumarol completely inhibited the enhanced activity of mitomycin C at acidic pH under aerobic conditions but had no effect on DNA cross-linking or cytotoxicity under hypoxic conditions. These finding suggest that the enhanced activity of mitomycin C at acidic pH, in air, is due to increased drug activation by DT-diaphorase. However, the role of DT-diaphorase in activating mitomycin C under hypoxic conditions appears to be limited, even at acidic pH.

MMC is a quinone-containing antitumor antibiotic that has been widely used for the treatment of breast (1, 2) and head and neck (3, 4) tumors. It also has shown useful clinical activity in a number of other solid tumors including bladder, colorectal, lung, and gastric cancers (5, 6). The major toxicity associated with MMC treatment is a delayed myelotoxicity that is dose limiting (5).

The mechanism of action of MMC has been studied extensively, and two primary cytotoxic mechanisms have been identified for this agent. MMC can act as an alkylating agent and can produce a number of lesions in DNA, including monoadducts, intrastrand cross-links, and interstrand cross-links (7–11). Reduction of the quinone group is required to generate the active alkylating species, which may be either a semiquinone or a hydroquinone (12). In addition, redox reactions of the quinone group can generate superoxide radicals and other active oxygen species (13–17), which can produce DNA strand breaks (7). Several enzymes have been shown to activate MMC. NADPH-cytochrome P450 reductase (EC 1.6.2.4), xanthine oxidase (EC 1.1.3.22), and NADH-cytochrome $b_5$ reductase (EC 1.6.2.2) can activate MMC by one-electron reduction of the quinone group to a semiquinone (8–11, 18–20). In many cells cytochrome P450 reductase appears to be the major contributor to the activation of this agent (12, 18); however, the ability of this enzyme to activate MMC to an alkylating agent is decreased under aerobic conditions by the rapid reoxidation of the initially generated semiquinone. DT-diaphorase [NAD(P)H:(quinone acceptor) oxidoreductase, EC 1.6.99.2] and xanthine dehydrogenase (EC 1.1.1.204) can activate MMC by two-electron reduction to the hydroquinone, which can be converted to an active alkylating species (21–24). Considerable evidence has been presented that DT-diaphorase is an important contributor to the activation of MMC in many cell lines under aerobic conditions (22, 25–29).

The activity of MMC is highly dependent on its environment. MMC is preferentially toxic to hypoxic tumor cells both *in vitro* (13, 30) and *in vivo* (31). These findings have increased interest in the use of this agent to treat hypoxic cells in solid tumors, which do not respond well to conventional radiotherapy (32–34). The enhanced hypoxic activity of MMC likely results from the increased efficiency in generating active alkylating species via the one-electron reduction pathway in the absence of oxygen, because back-oxidation does not occur. Although DT-diaphorase can play an important role in the activation of MMC under aerobic conditions, we have recently shown that the contribution of this enzyme to MMC activity under hypoxic conditions may be more limited (12).

The activity of MMC is also strongly pH dependent. Studies have shown that MMC cross-linking (7, 35) and cytotoxicity (35) are increased under acidic conditions. In addition, the ability of DT-diaphorase to activate MMC has also been shown to be strongly pH dependent (22). The ability of DT-diaphorase

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**ABBREVIATIONS:** MMC, mitomycin C; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum; CHO, Chinese hamster ovary; DMO, 5,5-dimethyl-2,4-oxazolidinedione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

to reduce MMC and to generate alkylating metabolites that produce DNA cross-links increases significantly as the pH is lowered from 7.4 to 5.8 (22, 23). It has been suggested that the decreased activating efficiency of DT-diaphorase under less acidic conditions may result from inactivation of the enzyme by MMC (36). In contrast, there is some evidence that the activity of NADPH-cytochrome P450 reductase may be decreased under acidic conditions (37). It is not clear whether the increased activity of MMC under acidic conditions is due to a direct effect on the drug itself (7) or is mediated through an effect on the activating enzymes (22, 23). Furthermore, it is not known how changing pH affects the contribution of DT-diaphorase to the activation of MMC.

In the present study we examined the role of DT-diaphorase in the activation of MMC under acidic conditions. We have identified the mechanism responsible for the increased activity of MMC under acidic conditions and have determined the contribution of DT-diaphorase to drug activation under these conditions in the presence of oxygen and under hypoxia.

## Experimental Procedures

**Materials.** DMEM and HBSS were obtained from GIBCO Laboratories (Grand Island, NY) and FCS was from Hyclone Laboratories (Logan, UT). [$^3$H]Thymidine (specific activity, 60–90 Ci/mmol) and [$^{14}$C]thymidine (specific activity, 54 mCi/mmol) were obtained from ICN Radiochemicals (Irvine, CA). [$^3$H]Water (specific activity, 5 mCi/ml) was purchased from Amersham Canada Ltd. (Oakville, Canada), and [$^{14}$C]DMO (specific activity, 60 mCi/mmol) and [$^{14}$C]inulin (specific activity, 3 mCi/g) were obtained from New England Nuclear (DuPont Canada, Mississauga, Canada). Proteinase K was purchased from E. Merck (Darmstadt, Germany) and polycarbonate filters (0.8 μm) were obtained from Nucleopore Corp. (Pleasanton, CA). Dicoumarol, MMC, HEPES, mineral oil, and tetrapropylammonium hydroxide were obtained from Sigma Chemical Co. (St. Louis, MO), and Dow Corning 550 fluid was obtained from Dow Corning (Mississauga, Canada).

**Cells.** The CHO cell line was obtained from Dr. Gerald L. Forrest, Department of Biology, Beckman Research Institute/City of Hope (Duarte, CA). The cells were grown as a monolayer in DMEM containing 10% FCS, with a doubling time of 14–16 hr. For experiments, the cells were trypsinized and resuspended in DMEM/10% FCS at a concentration of $3.6 \times 10^4$/ml, and 5-ml aliquots were placed into 25-ml glass Erlenmeyer flasks and incubated for 2 days at 37° in 5% $CO_2$. Using 2,6-dichloroindophenol as electron acceptor (38), DT-diaphorase activity in these cells was 5.8 ± 0.83 nmol/min/mg of protein.

**Hypoxic and acidic conditions.** After the 2-day incubation in Erlenmeyer flasks, the DMEM/10% FCS was removed by aspiration and replaced with 5 ml of HBSS containing 10 mM HEPES. The flasks were sealed with rubber serum stoppers and hypoxia was produced by a modification of previously described methods (39, 40). The flasks were fitted with 21-gauge needles for inflow and outflow of gases. Flasks were gassed continuously for a total of 3 hr with a humidified mixture of 95% $N_2$/5% $CO_2$ in a shaking water bath at 37°. Dicoumarol, MMC, 1 N HCl, or 1 N NaOH was added to the cell cultures through the rubber stopper, using a Hamilton syringe. Aerobic cultures were incubated with 95% air/5% $CO_2$ as described above. After 2 hr of incubation the pH of the medium was adjusted by the addition of up to 25 μl of 1 N HCl or 1 N NaOH, to give extracellular pH values of 7.2, 6.8, or 6.4. The medium was allowed to equilibrate for 30 min before addition of MMC, and after drug addition cells were incubated for 30 min. To inhibit DT-diaphorase activity, dicoumarol was added to the incubation mixture 15 min before MMC.

**Determination of intracellular pH.** Intracellular pH was determined by the distribution of the weak acid DMO (41). Hypoxic and acidic conditions were produced as described above, except that cells were trypsinized and resuspended in HBSS, with 10 mM HEPES, at a concentration of $2 \times 10^6$ cells/ml. With 20 min left in the incubation period, 100 μl each of [$^3$H]water (50 μCi/ml) and [$^{14}$C]DMO (10 μCi/ml) or [$^{14}$C]inulin (10 μCi/ml) were added. Intracellular pH was determined using the protocol described by Chu and Dewey (42) and was plotted against extracellular pH. The intracellular pH values of experimental cells in the cross-linking and cytotoxicity studies were determined from the intracellular pH versus extracellular pH plot and were 7.2 at 7.2, 7.0 at 6.8, and 6.6 at 6.4, respectively. These values were similar to those observed previously in CHO cells (42, 43).

**Determination of DNA-DNA cross-links.** CHO cells were aliquoted into 25-ml Erlenmeyer flasks as described above, in DMEM/10% FCS containing 0.01 μCi/ml [$^{14}$C]thymidine, and were incubated for 24 hr. The medium was then replaced with unlabeled medium, and cells were incubated for an additional 24 hr. Hypoxic and acidic conditions were produced as described above. Final concentrations of 5 μM dicoumarol and 20 μM MMC were used in these experiments. After the 3-hr incubation, the cells were washed twice with citrate saline and gently scraped off the flask surface with a rubber policeman. Cells were irradiated (250 rad) on ice and were combined with [$^3$H]thymidine-labeled L5178Y cells (44). DNA interstrand cross-linking was measured using an elution assay and the level of cross-links was calculated as described previously (44, 45). Results were analyzed statistically by two-tailed *t* tests.

**Cytotoxicity assays.** Hypoxic and acidic conditions were produced as described above. Final concentrations of both dicoumarol and MMC were 5 μM. After the incubation, cells were washed twice with citrate saline (134 mM KCl, 15 mM sodium citrate), trypsinized, and resuspended in DMEM/10% FCS. The cells were counted and plated in triplicate at varying cell number. After 9 days of incubation at 37° in 5% $CO_2$, the plates were stained with methylene blue and colonies were counted. Average plating efficiencies for all treatments in the absence of MMC was 92.2 ± 1.8%, and this was not significantly different under aerobic or hypoxic conditions, under neutral or acidic conditions, or in the absence or presence of dicoumarol. Results were analyzed statistically by two-tailed *t* tests.

## Results

**Effect of pH on DNA cross-linking by MMC under aerobic conditions.** The effect of acidic pH on DNA cross-linking produced by MMC in CHO cells under aerobic conditions was assessed. Cells were incubated for 30 min at 37° with 20 μM MMC under aerobic conditions in medium whose pH had been adjusted to 7.2, 6.8, or 6.4. The levels of DNA cross-links produced by MMC were determined by alkaline elution assay and are shown at the corresponding intracellular pH in Fig. 1. Cross-linking increased from 118 ± 12 rad equivalents at an intracellular pH of 7.2 to 237 ± 20 rad equivalents at an intracellular pH of 6.6 ($p < 0.001$). However, when the cells were treated with 5 μM dicoumarol together with MMC, DNA cross-linking was significantly decreased to 32 ± 5 rad equivalents at pH 7.2 ($p < 0.001$) and to approximately the same level at the other pH values.

**Effect of pH on MMC cytotoxicity under aerobic conditions.** The effect of acidic pH on MMC cytotoxicity in CHO cells under aerobic conditions was assessed by clonogenic assays. Cells were incubated for 30 min at 37° with 5 μM MMC under aerobic conditions in medium whose pH had been adjusted to 7.2, 6.8, or 6.4. MMC cytotoxicity was similar in cells at intracellular pH values of 7.2 and 7.0; however, cell survival was significantly decreased ($p < 0.001$) for cells at an intracellular pH of 6.6 (Fig. 2). The addition of 5 μM dicoumarol with the MMC treatment inhibited drug toxicity ($p < 0.001$), with

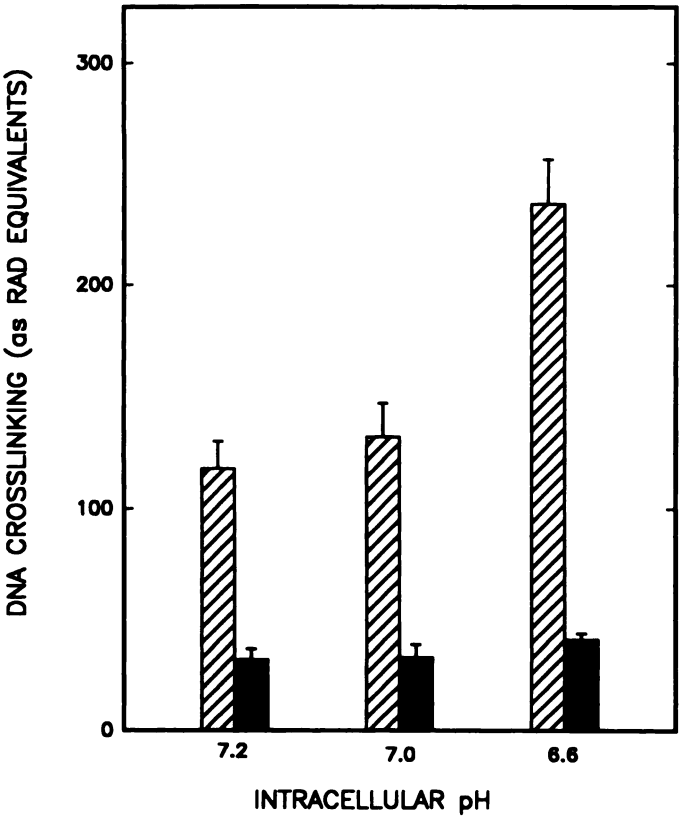


**Fig. 1.** Effect of pH on DNA cross-linking produced by MMC under aerobic conditions in the presence and absence of dicoumarol. CHO cells were incubated for 30 min at 37° with 20 μM MMC under aerobic conditions in the absence (▨) or presence (■) of 5 μM dicoumarol in medium at pH 7.2, 6.8, or 6.4. DNA cross-linking was determined by alkaline elution assay as described in the text. The level of cross-linking is presented as rad equivalents at the corresponding intracellular pH of the cells. Each *bar* represents the mean ± standard error of four to 10 determinations.

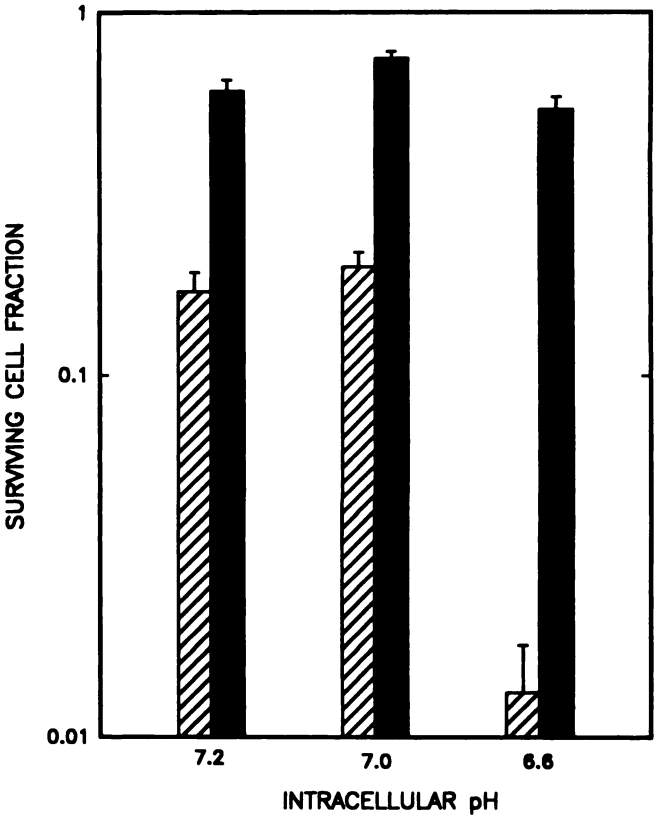


**Fig. 2.** Effect of pH on MMC cytotoxicity under aerobic conditions in the presence and absence of dicoumarol. CHO cells were incubated for 30 min at 37° with 5 μM MMC under aerobic conditions in the absence (▨) or presence (■) of 5 μM dicoumarol in medium at pH 7.2, 6.8, or 6.4. Cytotoxicity was determined by clonogenic assay as described in the text. Cytotoxicity is presented as the surviving cell fraction at the corresponding intracellular pH of the cells. Each *bar* represents the mean ± standard error of seven to 24 determinations.

the effect being greatest at pH 6.6, so that MMC cytotoxicity in the presence of dicoumarol was equal at all pH levels.

**Effect of pH on DNA cross-linking by MMC under hypoxic conditions.** The effect of acidic pH on DNA cross-linking produced by MMC in CHO cells under hypoxic conditions was measured. Cells were incubated for 30 min at 37° with 20 μM MMC under nitrogen in medium whose pH had been adjusted to 7.2, 6.8, or 6.4. The levels of DNA cross-links produced by MMC were determined by alkaline elution assay and are shown in Fig. 3. Levels of cross-linking at intracellular pH values of 7.2 and 7.0 were equal, but the level of cross-linking produced by MMC increased by approximately 35% at an intracellular pH of 6.6 ($p < 0.01$). MMC cross-linking was considerably higher at pH 7.2 and 7.0 under hypoxic conditions than in air, but this difference disappeared at pH 6.6. However, in contrast to the finding in air, when the cells were treated with 5 μM dicoumarol together with MMC under hypoxic conditions DNA cross-linking did not decrease.

**Effect of pH on MMC cytotoxicity under hypoxic conditions.** The effect of acidic pH on MMC cytotoxicity in CHO cells under hypoxic conditions was measured by clonogenic assays. Cells were incubated for 30 min at 37° with 5 μM MMC under hypoxic conditions in medium whose pH had been adjusted to 7.2, 6.8, or 6.4. MMC cytotoxicity did not change with changing pH, and 5 μM dicoumarol did not significantly affect drug toxicity at any of the pH levels (Fig. 4).

## Discussion

The activity of MMC is highly dependent on its environment. This agent is preferentially toxic to some tumor cells under hypoxic conditions both *in vitro* (12, 30) and *in vivo* (31), and its activity is also strongly pH dependent (7, 35). There is considerable evidence that DT-diaphorase can contribute significantly to the activation of MMC in many cell lines under aerobic conditions (22, 25–29). However, we have recently demonstrated that the role of DT-diaphorase in MMC activation under hypoxic conditions may be more limited (12). In addition, the ability of this enzyme to activate MMC has been shown to be strongly pH dependent, with enzyme activity increasing at lower pH (22, 23). Thus, we have examined the role of DT-diaphorase in the activation of MMC at acidic pH under both aerobic and hypoxic conditions. Furthermore, we have investigated whether the enhanced activity of MMC under acidic conditions is due to a direct effect on the drug itself or whether it is mediated through an effect on DT-diaphorase.

We observed that under aerobic conditions, at an intracellular pH of 7.2, approximately 75% of MMC cross-linking was inhibited by the DT-diaphorase inhibitor dicoumarol, and this increased to 85% at an intracellular pH of 6.6. A similar effect

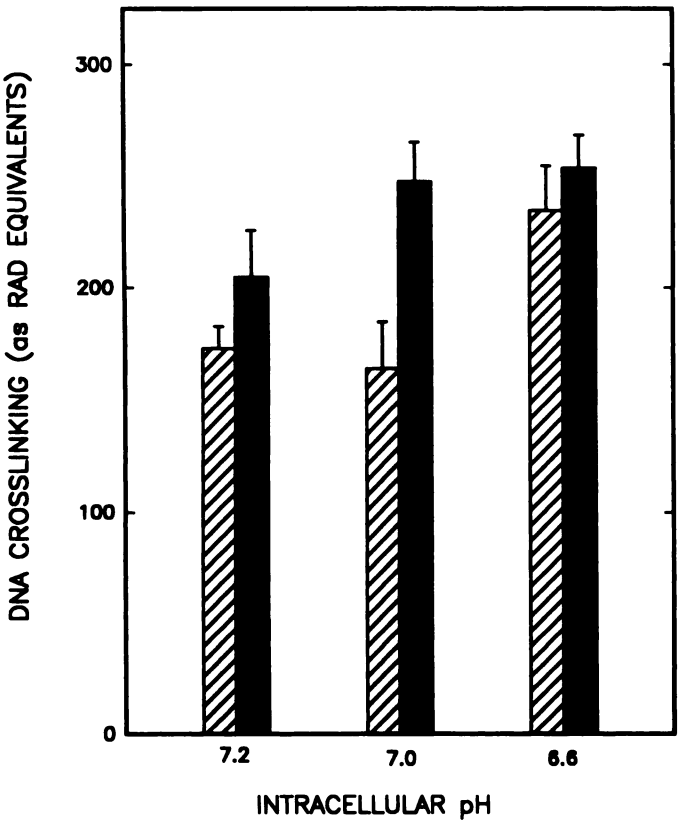


**Fig. 3.** Effect of pH on DNA cross-linking produced by MMC under hypoxic conditions in the presence and absence of dicoumarol. CHO cells were incubated for 30 min at 37° with 20 μM MMC under hypoxic conditions in the absence (▨) or presence (■) of 5 μM dicoumarol in medium at pH 7.2, 6.8, or 6.4. DNA cross-linking was determined by alkaline elution assay as described in the text. The level of cross-linking is presented as rad equivalents at the corresponding intracellular pH of the cells. Each *bar* represents the mean ± standard error of six to 15 determinations.

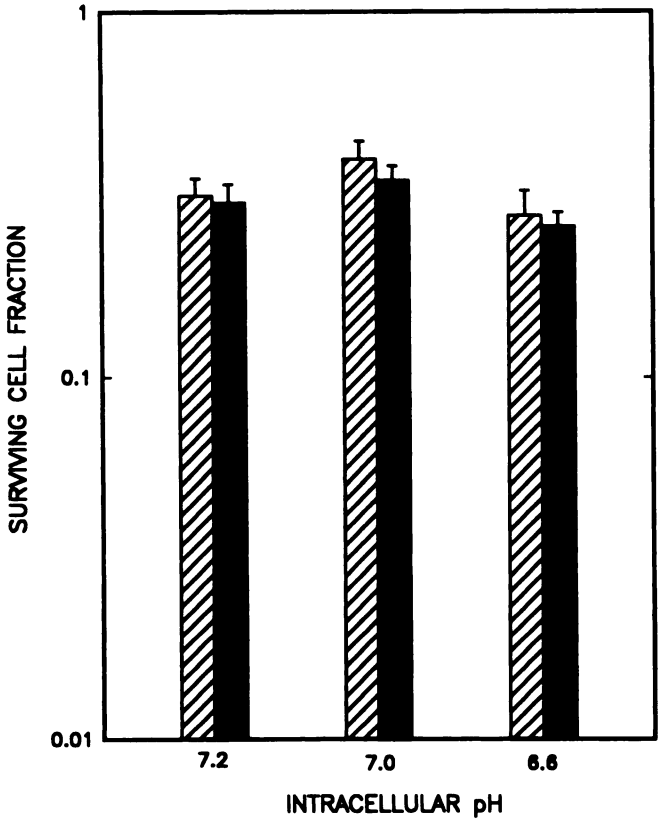


**Fig. 4.** Effect of pH on MMC cytotoxicity under hypoxic conditions in the presence and absence of dicoumarol. CHO cells were incubated for 30 min at 37° with 5 μM MMC under hypoxic conditions in the absence (▨) or presence (■) of 5 μM dicoumarol in medium at pH 7.2, 6.8, or 6.4. Cytotoxicity was determined by clonogenic assay as described in the text. Cytotoxicity is presented as the surviving cell fraction at the corresponding intracellular pH of the cells. Each *bar* represents the mean ± standard error of nine to 17 determinations.

on MMC cytotoxicity was also observed. These findings suggest that, in these CHO cells, DT-diaphorase is a major contributor to the activation of the alkylating metabolite of MMC under aerobic conditions and that its contribution increases at lower pH. In contrast, dicoumarol did not inhibit either MMC DNA cross-linking activity or cytotoxicity under hypoxic conditions at any pH, suggesting that DT-diaphorase does not contribute to MMC activation under hypoxia even under acidic conditions.

Although dicoumarol has been used as an inhibitor of DT-diaphorase activity in these studies, it can also inhibit other enzymes that may be involved in the activation of MMC, such as NADH-cytochrome $b_5$ reductase (19). Thus, the contribution of these enzymes to MMC activation under the conditions of these studies cannot be completely ruled out. However, there is little evidence that these enzymes contribute significantly to the activation of MMC in the cell line studied, and we have observed that the concentration of dicoumarol used in these studies does not significantly inhibit NADH-cytochrome $b_5$ reductase activity.[1]

Although DT-diaphorase does not appear to play a significant role in activating MMC under hypoxic conditions in the CHO cells, this is likely not due to a decreased ability of this enzyme to activate the drug under these conditions. Siegel *et al.* (22) showed that metabolism and bioactivation of MMC to produce DNA cross-links by purified DT-diaphorase were nearly identical under aerobic and hypoxic conditions. Thus, the absence of MMC activation by DT-diaphorase under hypoxia likely reflects the increased ability of one-electron reducing enzymes, such as NADPH-cytochrome P450 reductase, to generate an alkylating metabolite under conditions where the initially formed MMC semiquinone does not undergo redox cycling. Despite the lack of drug activation by DT-diaphorase, it is interesting to note that DNA cross-linking was generally higher under hypoxia than in air in the CHO cells. The decrease in MMC cytotoxicity in these cells under hypoxic, compared with aerobic, conditions likely represents the net effect of increased DNA cross-linking and decreased free radical-induced DNA damage resulting from redox cycling. These findings suggest that, unlike in other cell lines (12, 46), free radical-induced DNA damage resulting from redox cycling of MMC contributes significantly to MMC cytotoxicity in CHO cells. Our results are similar to those observed in previous studies with CHO (18, 47), Ehrlich (16), and MCF-7 breast cancer (17) cells. Thus, the net effect of hypoxia on the antitumor activity of MMC in different cells may depend on a number of factors, including the relative contribution of alkylation versus free radical damage to cytotoxicity and the relative levels and efficiencies of the activating enzymes in the cells.

Under aerobic conditions, MMC cross-linking increased from

[1] A. Begleiter and M. K. Leith, Unpublished observations.

118 rad equivalents at an intracellular pH of 7.2 to 237 rad equivalents at pH 6.6, and there was a corresponding increase in drug cytotoxicity. However, lowering the intracellular pH from 7.2 to 7.0 had only a small effect on DNA cross-linking and cytotoxicity, and this is consistent with the previously observed effect of pH on the ability of DT-diaphorase to activate MMC (22). Furthermore, all of the increased DNA cross-linking and cytotoxicity was inhibited by dicoumarol. These findings strongly support the suggestion that the enhanced antitumor activity of MMC at acidic pH is due to an increased ability of DT-diaphorase to activate the drug to its alkylating metabolite under these conditions.

In summary, we observed that MMC produced increased DNA cross-linking and cytotoxicity in CHO cells at an intracellular pH of 6.6, compared with pH 7.2, under aerobic but not hypoxic conditions. In addition, we demonstrated that the DT-diaphorase inhibitor dicoumarol completely inhibited the enhanced MMC activity at acidic pH under aerobic conditions but had no effect on DNA cross-linking or cytotoxicity under hypoxic conditions. These findings suggest that the enhanced activity of MMC, in air, at acidic pH is due to increased drug activation by DT-diaphorase. However, the contribution of DT-diaphorase to activation of MMC under hypoxic conditions appears to be limited, even at acidic pH.

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