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

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SUMMARY

The role of the two-electron reducing enzyme DT-diaphorase in the activation of mitomycin C under hypoxic conditions was investigated. Mitomycin C activity was compared in L5178Y murine lymphoblasts, which have low levels of DT-diaphorase activity, and L5178Y/HBM10 cells, which have elevated levels of enzyme activity. The cytotoxic and DNA cross-linking activities of mitomycin C were greater in L5178Y/HBM10 cells than in L5178Y cells. In L5178Y/HBM10 cells, dicoumarol, an inhibitor of DT-diaphorase, decreased cell kill and DNA cross-linking by mitomycin C in air but had no significant effect on these activities under hypoxia. By comparison, in L5178Y cells, dicoumarol had no effect on drug activity under either aerobic or hypoxic conditions. A model for the activation of mitomycin C by both one-electron and two-electron reduction is proposed. Our findings suggest that two-electron reduction by DT-diaphorase has only a limited role in the activation of mitomycin C under hypoxic conditions, although this enzyme appears to be an important contributor to drug activation under aerobic conditions.

MMC is a quinone-containing antibiotic antitumor agent that is active in solid tumors (1). It is used clinically for the treatment of breast (2, 3) and head and neck tumors (4) and has shown useful activity in bladder, colorectal, lung, and gastric cancers (1, 5). MMC is preferentially toxic to hypoxic tumor cells both *in vitro* (6) and *in vivo* (7) and, thus, may be potentially valuable for the treatment of hypoxic cells in solid tumors, which do not respond well to conventional radiotherapy or other chemotherapeutic agents (8–10).

MMC is often considered the prototype bioreductive antitumor agent (11) and requires reduction of the quinone moiety for activation of its chemical and biological action (11, 12). Reduction of the quinone can proceed via one- or two-electron pathways, leading to the formation of reactive semiquinone or hydroquinone intermediates, respectively (13–17). Reaction of the semiquinone with molecular oxygen results in back-oxidation to the quinone moiety and the generation of superoxide radical and other active oxygen species (18–21). The active oxygen species can produce DNA strand breaks (13), and this activity has been implicated in the biological action of MMC (21–23). Reduced MMC intermediates form active alkylating species that can produce a number of lesions in DNA, including monoadducts, intrastrand cross-links, and DNA-DNA interstrand cross-links (13, 14, 16, 24, 25). The exact nature of the alkylating species, semiquinone or hydroquinone, remains unclear. In addition, the contribution of each of the mechanisms to the cytotoxic activity of MMC remains uncertain and likely depends upon the relative amounts of each type of damage produced within a cell and the ability of the cell to respond to the damage.

The reductive activation of MMC to alkylating species has been demonstrated chemically (13, 24), by liver microsomes (26, 27), and by nuclei (27). In addition, unequivocal evidence has been presented for the activation of MMC by the one-electron reducing enzymes NADPH:cytochrome P-450 reductase (EC 1.6.2.4) and xanthine oxidase (EC 1.2.3.2) (14–16, 22, 24, 25), although other one-electron reducing enzymes may also be capable of reducing MMC. However, the role of the two-electron reducing enzyme NAD(P)H:(quinone acceptor) oxidoreductase, or DT-diaphorase (EC 1.6.99.2), in the activation of MMC has been much more controversial (28). Dicoumarol, an inhibitor of DT-diaphorase (28), has been shown to inhibit MMC activity in EMT6 cells under aerobic conditions (29–31). We have observed that MMC produced increased cytotoxicity and DNA cross-linking in L5178Y cells selected for resistance to the model quinone antitumor agent hydrolyzed benzoquinone mustard (L5178Y/HBM10), compared with parental L5178Y cells (32). The L5178Y/HBM10 cells have 24-fold increased DT-diaphorase activity, compared with parental L5178Y cells (33), and dicoumarol inhibited both the cytotoxic and cross-linking activities of MMC in the L5178Y/HBM10 cells under aerobic conditions. Similarly, human fibroblast (34) and colon carcinoma cell lines (17) and Chinese hamster ovary cells (35) with elevated levels of DT-diaphorase were more

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ABBREVIATION: MMC, mitomycin C.

sensitive to MMC than were similar cell lines with low enzyme activity, and the increased sensitivity was inhibited by dicoumarol. Earlier attempts to demonstrate reduction of MMC by purified DT-diaphorase were unsuccessful and suggested that MMC was an inhibitor of the enzyme (36). However, Siegel *et al.* (17) have recently described the metabolism of MMC by purified rat hepatic DT-diaphorase and enzyme purified from human HT-29 colon carcinoma cells. These findings suggest that DT-diaphorase may play an important role in the activation of MMC.

The enhanced activity of MMC under hypoxic conditions has attracted considerable attention because of the potential for exploiting this property to produce tumor-selective cytotoxicity in radioresistant cells within poorly vascularized solid tumors. Cytochrome P-450 reductase may be important for this effect, because it has been suggested that there may be increased formation of an active alkylating species from MMC semiquinone under hypoxic conditions, possibly resulting from the absence of back-oxidation under these conditions (6). The role of DT-diaphorase in activating MMC under hypoxic conditions is still not known. Using isolated enzyme, Siegel *et al.* (17) demonstrated that DT-diaphorase activated MMC to the same extent under aerobic and hypoxic conditions. However, the enzyme inhibitor dicoumarol increased MMC activity in cells having either high or low levels of DT-diaphorase under hypoxia (31, 35).

In this study, we have examined the role of DT-diaphorase in the activation of MMC, with particular emphasis on its activity under hypoxic conditions. MMC cytotoxicity and DNA damage were compared in L5178Y murine lymphoblasts, which have low levels of DT-diaphorase, and L5178Y/HBM10 cells, which have elevated DT-diaphorase activity (33). Studies were carried out with these cell lines in the presence and absence of oxygen, with and without dicoumarol. Our findings suggest that DT-diaphorase can be an important activator of MMC under aerobic conditions but has only a limited role in modulating the activity of MMC under hypoxic conditions.

Experimental Procedures

Materials. MMC was obtained from Boehringer Mannheim (Mannheim, Germany). Dicoumarol and horse serum were obtained from Sigma Chemical Co. (St. Louis, MO), Fischer's medium was obtained from GIBCO Laboratories (Grand Island, NY), and [^{3}H]thymidine (specific activity, 50–80 Ci/mmol) and [^{14}C]thymidine (specific activity, 54 mCi/mmol) were obtained from ICN Radiochemicals (Irvine, CA). Proteinase K was from E. Merck (Darmstadt, Germany), tetrapropylammonium hydroxide was from Eastman Kodak Co. (Rochester, NY), and polycarbonate filters (0.8 μm and 2.0 μm) were from Nucleopore Corp. (Pleasanton, CA).

Cell lines. The L5178Y and L5178Y/HBM10 cell lines used in this study have been described previously (33). L5178Y cells were grown in Fischer's medium containing 12% horse serum, and L5178Y/HBM10 cells were grown in Fischer's medium containing 12% horse serum and 1.0 mM hydrolyzed benzoquinone mustard (37).

Hypoxic conditions. Cells were made hypoxic, in 25-cm^2 cell culture flasks sealed with rubber stoppers, by a modification of previously described methods (29, 38). The flasks were fitted with 21-gauge needles for inflow and outflow of gases and were gassed continuously for a total of 4 hr with a humidified mixture of 95% N_2/5% CO_2. Drugs or dicoumarol were added to the cell cultures through the rubber stopper, using a Hamilton syringe. Aerobic cultures were incubated as described above, using 95% air/5% CO_2. The pH of the medium remained constant at pH 7.4 during the 1-hr drug incubation, under both aerobic and hypoxic conditions.

Cytotoxicity assays. Cells were incubated under hypoxic or aerobic conditions at 37° for 3 hr in Fischer's medium containing horse serum. Various concentrations of MMC in dimethyl sulfoxide were added and cells were incubated at 37° for 1 hr. Cytotoxic activity was determined by a soft-agar cloning assay, as described previously (37, 39). Cloning efficiencies ranged from 22 to 67% for L5178Y cells and from 20 to 83% for L5178Y/HBM10 cells. A linear regression analysis for each concentration-survival curve was obtained, and the D_{10} (concentration of drug required to reduce the surviving cell fraction to 0.1) was derived from the negative reciprocal of the regression slope, as previously described (37). The degree of sensitivity was determined from the ratio of the D_{10} values in L5178Y and L5178Y/HBM10 cells. Cytotoxicities were compared statistically by *t* tests comparing the significance of the difference of the slopes of the concentration-survival curves.

For inhibition studies with dicoumarol, L5178Y cells were pretreated with or without 100 μM dicoumarol for 15 min before addition of drug. L5178Y/HBM10 cells were pretreated with or without 150 μM dicoumarol for 15 min. These concentrations of inhibitor were the highest nontoxic concentrations for each cell line. Results were analyzed statistically by a *t* test comparing the significance of the difference of the slopes of the concentration-survival curves in the absence or presence of dicoumarol. Control studies indicated that the concentrations of dimethyl sulfoxide and dicoumarol used in these studies were not toxic to the cells. In addition, all cytotoxic measurements were corrected for the cloning efficiency of cells treated under identical conditions in the absence of MMC.

Determination of DNA double-strand breaks. Cells labeled with [^{14}C]thymidine were treated at 37° for 1 hr with various concentrations of MMC, under air or hypoxic conditions, in medium containing horse serum. DNA double-strand breaks were measured using an elution assay, as described previously (40, 41). The level of DNA strand breaks was calculated from the elution profiles and was expressed as rad equivalents (dose of radiation inducing an equivalent number of breaks), as determined from calibration curves.

Determination of DNA-DNA cross-linking. Cells labeled with [^{14}C]thymidine were treated at 37° for 1 hr with various concentrations of MMC, under air or hypoxia, in medium containing horse serum. DNA-DNA cross-linking was measured using an elution assay, and the level of cross-linking was calculated as described by Kohn *et al.* (40, 41). A linear regression analysis of each concentration-activity curve was obtained, and cross-linking activity was compared statistically by *t* tests comparing the significance of the difference of the slopes of the concentration-activity curves.

For inhibition studies with dicoumarol, L5178Y cells were pretreated with or without 100 μM dicoumarol and L5178Y/HBM10 cells were pretreated with or without 150 μM dicoumarol, for 15 min. The results were analyzed statistically by *t* tests comparing the significance of the difference of the slopes of the concentration-activity curves. Control studies showed that dicoumarol alone did not induce DNA-DNA cross-links.

Results

Cytotoxicity of MMC in L5178Y and L5178Y/HBM10 cells under aerobic and hypoxic conditions. The cytotoxic activity of MMC was determined in L5178Y and L5178Y/HBM10 cells under aerobic and hypoxic conditions, using soft-agar clonogenic assays. Cells were treated for 1 hr at 37° with MMC, under air or nitrogen, and the results are shown in Table 1. The cytotoxic activity of MMC in air was approximately 4.5-fold greater in L5178Y/HBM10 cells than in L5178Y cells. Hypoxia increased the cytotoxicity of MMC in parental cells approximately 2-fold but had no effect on the cytocidal activity of MMC in L5178Y/HBM10 cells.

Effect of dicoumarol on cytotoxicity of MMC. The

TABLE 1

Cytotoxic activity of MMC in L5178Y and L5178Y/HBM10 cells

L5178Y or L5178Y/HBM10 cells were incubated with various concentrations of MMC for 1 hr at 37°, under air or nitrogen, in the absence or presence of 100 or 150 μM dicoumarol. The surviving cell fraction was determined by a clonogenic assay, as described in the text. A linear regression analysis of each concentration-survival curve was obtained, and the D_{10} (mean ± standard error) was derived from the negative reciprocal of the slope of the regression line. The cytotoxic activity of MMC in each cell line was compared under aerobic or hypoxic conditions and in the absence or presence of dicoumarol by two-tailed *t* tests comparing the significance of the difference in the slopes of the linear regression lines from the concentration-survival plots.

Treatment	D_{10}			
	L5178Y		L5178Y/HBM10	
	Aerobic	Hypoxic	Aerobic	Hypoxic
	μM			
MMC	1.27 ± 0.05[a]	0.73 ± 0.06[a]	0.28 ± 0.02[b]	0.25 ± 0.02
MMC + dicoumarol	1.26 ± 0.04[c]	0.69 ± 0.04[c]	0.36 ± 0.01[b]	0.29 ± 0.03

[a] Significantly different, by two-tailed *t* test ($p < 0.01$).
[b] Significantly different, by two-tailed *t* test ($p < 0.05$).
[c] Significantly different, by two-tailed *t* test ($p < 0.01$).

cytotoxicity of MMC was determined in L5178Y and L5178Y/HBM10 cells, in air or in nitrogen, in the presence of dicoumarol, and the results are shown in Table 1. The addition of dicoumarol decreased the cytotoxicity produced by MMC in L5178Y/HBM10 cells in air but had no significant effect on MMC cytotoxicity in these cells under hypoxia. In contrast, dicoumarol did not significantly affect the cell kill produced by MMC in L5178Y cells under either aerobic or hypoxic conditions.

DNA damage produced by MMC in L5178Y and L5178Y/HBM10 cells under aerobic and hypoxic conditions. The DNA damage produced by MMC in L5178Y and L5178Y/HBM10 cells under aerobic and hypoxic conditions was determined. Cells were treated with MMC at 37° for 1 hr, and DNA-DNA interstrand cross-links and DNA double-strand breaks were determined using elution assays. MMC produced significant levels of DNA-DNA cross-links in both cell lines, and the level of cross-linking was 10-fold greater in L5178Y/HBM10 cells than in parental cells ($p < 0.001$) (Fig. 1). Hypoxia increased cross-linking in L5178Y cells approximately 2.5-fold ($p < 0.05$) but had no effect on cross-linking in L5178Y/HBM10 cells. In contrast, MMC did not induce any measurable DNA double-strand breaks in L5178Y or L5178Y/HBM10 cells under either air or nitrogen.

Effect of dicoumarol on DNA cross-linking produced by MMC. DNA-DNA cross-links produced by MMC in L5178Y and L5178Y/HBM10 cells in the presence of dicoumarol, under air or nitrogen, were measured. The addition of dicoumarol produced a 2-fold decrease in cross-link formation in L5178Y/HBM10 cells in air ($p < 0.001$) but had a smaller effect on cross-linking in these cells under hypoxia (p = not significant) (Fig. 1). In contrast, dicoumarol did not significantly affect the production of DNA cross-links by MMC in L5178Y cells under either aerobic or hypoxic conditions.

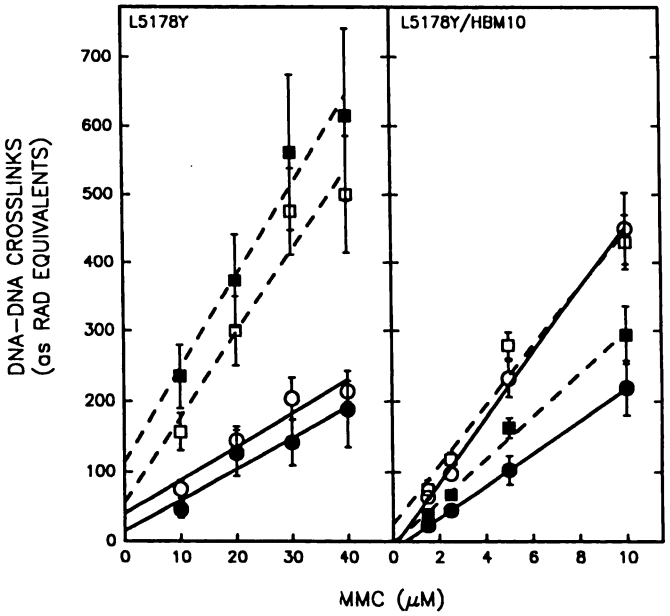

Fig. 1. DNA-DNA cross-linking by MMC under aerobic and hypoxic conditions, in the presence and absence of dicoumarol. L5178Y cells (*left*) or L5178Y/HBM10 cells (*right*) were incubated for 1 hr at 37° with various concentrations of MMC under aerobic (*circles*) or hypoxic (*squares*) conditions, in the presence (*closed symbols*) or absence (*open symbols*) of dicoumarol. DNA-DNA cross-linking was determined by alkaline elution assay, as described in the text. The level of cross-linking is presented as rad equivalents. *Points*, mean of three to seven determinations; *bars*, standard error; *lines*, linear regression lines. On occasion, the confidence intervals were too small to be shown.

Discussion

We have previously reported that L5178Y/HBM10 cells, which were derived from parental L5178Y cells and have a 24-fold elevated level of DT-diaphorase activity (33), were more sensitive than parental cells to MMC (32). In addition, MMC produced more DNA cross-links in the cells with elevated DT-diaphorase, and dicoumarol, an inhibitor of DT-diaphorase, decreased both the cytotoxic and cross-linking activities of MMC in L5178Y/HBM10 cells under aerobic conditions. These findings suggested an important role for DT-diaphorase in activating MMC in the L5178Y/HBM10 cells under aerobic conditions. We have now extended these studies to examine the role of DT-diaphorase in the activation of MMC under hypoxic conditions.

Evidence has been presented that both the induction of DNA strand breaks, resulting from the generation of active oxygen species by the MMC quinone (21, 23), and the formation of DNA alkylation products by activated MMC (13, 14, 25, 42) may contribute to the cytotoxic activity of MMC. However, we did not observe the formation of DNA strand breaks in either L5178Y or L5178Y/HBM10 cells treated with MMC, suggesting that DNA alkylation may be the most important cytotoxic

mechanism in these cells. MMC cytotoxicity and DNA cross-linking were increased in L5178Y cells under hypoxic conditions, compared with aerobic conditions, and these results are similar to previous findings (4, 6, 15, 34). In contrast, hypoxia appeared to have no effect on these activities in L5178Y/HBM10 cells. The DT-diaphorase inhibitor dicoumarol significantly inhibited the cytocidal and cross-linking activities of MMC in L5178Y/HBM10 cells under aerobic conditions, and these effects were similar to those reported by Keyes *et al.* (29–31), Marshall *et al.* (34), Siegel *et al.* (17), and Dulhanty and Whitmore (35). However, the inhibitor did not significantly affect MMC cytotoxicity and DNA cross-linking in these cells under hypoxic conditions. In addition, dicoumarol did not affect either MMC cytotoxicity or DNA cross-linking activity in parental L5178Y cells, which have low levels of activity of the enzyme. These observations differ from those of other studies. Keyes *et al.* (31) observed that dicoumarol increased MMC cytotoxicity and cross-linking under hypoxia in EMT6 murine mammary tumor cells, which have high levels of DT-diaphorase. Furthermore, these researchers found that dicoumarol increased MMC cytotoxicity in L1210 murine leukemia cells, which have no detectable levels of DT-diaphorase, under both aerobic and hypoxic conditions. Because the enhancement of MMC activity by dicoumarol in these latter studies appears to be independent of DT-diaphorase activity in the cells, these effects may result from other activities of the inhibitor, rather than from an effect on DT-diaphorase. Dicoumarol has been reported to produce changes in respiration (43) and calcium homeostasis (44) in cells. Similarly, Dulhanty and Whitmore (35) found that dicoumarol increased MMC cytotoxicity under hypoxic conditions in Chinese hamster ovary cell lines having either high or low levels of DT-diaphorase activity. Taken together, these findings strongly support an important role for DT-diaphorase in activating MMC under aerobic conditions; however, the present study suggests that this enzyme may make only a limited contribution to drug activation under hypoxia.

We believe that MMC activity observed in this and previous studies is consistent with a model in which MMC can be activated by both one-electron and two-electron reductions (Fig. 2). One-electron reduction by cytochrome P-450 reductase leads to a highly reactive semiquinone. The semiquinone can be oxidized by oxygen to the parent quinone, with the generation of oxygen radicals, or can undergo further chemical changes to produce highly active alkylating species. Reoxidation of the initially formed semiquinone intermediate is assumed to be more favorable than formation of the alkylating species in the presence of oxygen. In contrast, two-electron reduction of MMC by DT-diaphorase produces a more stable hydroquinone intermediate, which can also be reoxidized by oxygen or can be converted to an active alkylating species. Siegel *et al.* (17) have provided some evidence that the formation of alkylating species from the MMC hydroquinone is faster than reoxidation by oxygen. However, the exact nature of the alkylating species, semiquinone or hydroquinone, remains unknown, because the semiquinone intermediates may be further reduced by one-electron reduction or may undergo comproportionation to a quinone and a hydroquinone.

The model predicts that MMC alkylation should be increased under aerobic conditions in cells with elevated levels of DT-diaphorase activity, because of the formation of additional active alkylating species via the two-electron reduction pathway. Although oxygen radical formation may be decreased under these conditions, overall MMC cytotoxicity should increase because of increased alkylation. Dicoumarol would thus be expected to decrease significantly both MMC-induced DNA cross-linking and cytotoxicity in these cells under aerobic conditions. Under hypoxic conditions, the formation of reactive alkylating species via the one-electron pathway increases, because back-oxidation does not occur. Depending on such factors as the relative levels of DT-diaphorase and cytochrome P-450 reductase (17, 32, 34, 42), the relative efficiencies of the enzymes for reduction of MMC, the relative reactivities of the alkylating species produced by the two pathways, and the concentration of MMC available, DNA cross-linking by MMC may increase, decrease, or remain unchanged. Corresponding effects would be expected on MMC cytotoxicity. Dicoumarol would be expected to inhibit MMC activity, under these conditions, to the extent that reduction of MMC by DT-diaphorase contributes to drug activity.

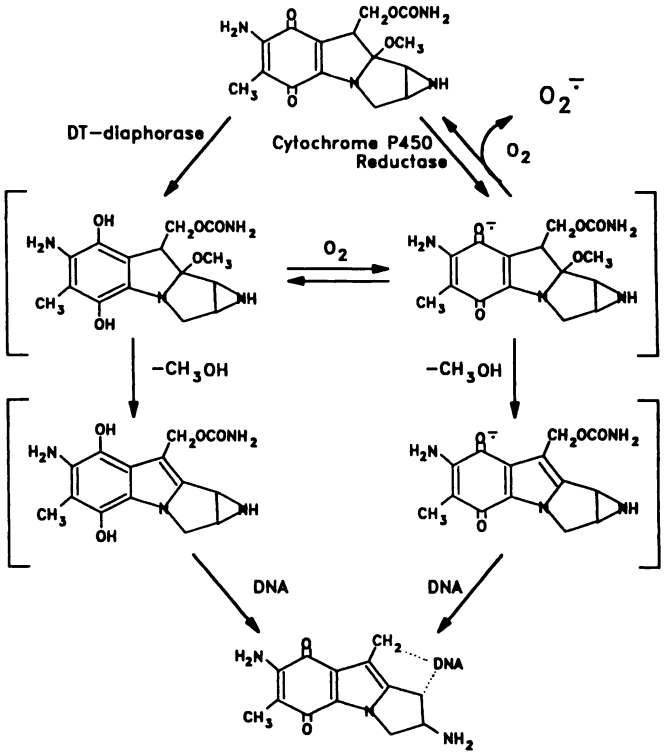

Fig. 2. Proposed model for activation of MMC.

The current study is also consistent with a model for the activation of MMC that was recently proposed by Dulhanty and Whitmore (35). These authors explained the apparent preferential activation of MMC via a one-electron pathway by postulating that the semiquinone is a more potent alkylating species than the hydroquinone and that cytochrome P-450 reductase has a higher affinity for MMC than does DT-diaphorase. To date, there have been no studies that have compared the alkylating activities of the MMC semiquinone and hydroquinone, nor are the affinities of the two activating enzymes for MMC known. We believe that the relative amounts of each enzyme present within cells may also contribute to this effect.

Although varying levels of MMC-induced DNA strand breaks have been observed in different cell lines (21, 23), we did not

observe the formation of DNA strand breaks by MMC in either the L5178Y or L5178Y/HBM10 cells. This may be due to the repair of such lesions in these cells, because we have shown previously that these cells repair radiation-induced DNA strand breaks very rapidly (45). In addition, dicoumarol did not completely eliminate the difference in MMC activity in L5178Y/HBM10 and L5178Y cells. This may have been due to incomplete inhibition of DT-diaphorase in the L5178Y/HBM10 cells. Higher concentrations of dicoumarol could not be used in these studies because the inhibitor produced significant toxicity at concentrations above 150 μM. Alternatively, the difference in MMC activity in the two cell lines might have resulted from other mechanisms, such as differences in DNA repair and drug inactivation by glutathione and glutathione *S*-transferase (33).

In summary, we have demonstrated that, under hypoxic conditions, the DT-diaphorase inhibitor dicoumarol has little effect on MMC DNA cross-linking and cytotoxicity in L5178Y/HBM10 cells, which have elevated DT-diaphorase activity. However, this enzyme inhibitor significantly decreases these activities under aerobic conditions. Furthermore, the inhibitor of DT-diaphorase has no effect on MMC activity in L5178Y cells, which have a low level of DT-diaphorase activity. These findings suggest that DT-diaphorase can play an important role in the activation of MMC to reactive alkylating species under aerobic conditions but that its role in MMC activation under hypoxic conditions may be more limited.

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