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Bioactivation of Mitomycin Antibiotics by Aerobic and Hypoxic Chinese Hamster Ovary Cells Overexpressing DT-Diaphorase

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ABSTRACT. DT-Diaphorase catalyzes a two-electron reduction of mitomycin C (MC) and porfiromycin (POR) to reactive species. Many cell lines that overexpress DT-diaphorase and are sensitive to the mitomycins are protected from the aerobic cytotoxicity of these drugs by the DT-diaphorase inhibitor dicumarol. The cytoprotective properties of this relatively non-specific inhibitor, however, vanish under hypoxic conditions. To ascertain the role of DT-diaphorase in mitomycin bioactivation and cytotoxicity in living cells, a rat liver DT-diaphorase cDNA was transfected into Chinese hamster ovary cells. MC was equitoxic to the parental cells under oxygenated and hypoxic conditions. In contrast, POR was less toxic than MC to these cells under aerobic conditions, but significantly more toxic than MC under hypoxia. Two DT-diaphorase-transfected clones displayed increases in DT-diaphorase activity of 126- and 133-fold over parental cells. The activities of other oxidoreductases implicated in mitomycin bioreduction were unchanged. MC was more toxic to both DT-diaphorase-transfected lines than to parental cells; the toxicity of MC to the transfected lines was similar in air and hypoxia. POR was also more toxic to the DT-diaphorase-elevated clones than to parental cells under oxygenated conditions. Under hypoxia, however, the toxicity of POR to the transfected clones was unchanged from that of parental cells. The findings implicate DT-diaphorase in mitomycin bioactivation in living cells, but suggest that this enzyme does not contribute to the differential toxicity of MC or POR in air and hypoxia. BIOCHEM PHARMACOL 51;12:1669–1678, 1996.

KEY WORDS. bioreductive activation; transfectants; differential toxicity; tumor hypoxia; mitomycin C; porfiromycin

MC§ and POR can preferentially kill hypoxic tumor cells *in vivo*, producing therapeutic gain when employed in combination with radiotherapy [1, 2]. Bioreductive alkylating agents such as MC and POR require enzymatic activation to exert their cytotoxic effects. Several enzymes can catalyze the bioreductive activation of the mitomycin antibiotics including DT-diaphorase (NAD(P)H:quinone reductase, EC 1.6.99.2) [3–8], NADPH:cytochrome c (P-450) reductase (EC 1.6.2.4) [9–17], NADH:cytochrome b_5 reductase (EC 1.6.2.2) [18, 19], xanthine dehydrogenase (EC 1.2.1.37) [20–22], and xanthine oxidase (EC 1.2.3.2) [11, 14, 23]. Xanthine oxidase is absent in most rapidly proliferating cell lines and consequently is unlikely to be a major contributor to the activation of the mitomycins in most tumor cell lines.

DT-Diaphorase is an obligate two-electron reductase that can utilize either NADH or NADPH as an electron donor and is inhibited by dicumarol [24]. This enzyme catalyzes the two-electron reduction of quinones such as the mitomycins. The reduction of MC by DT-diaphorase purified from rat liver or from HT-29 human colon carcinoma cells is pH dependent. While DT-diaphorase is susceptible to inhibition by MC at pH 7.8, this enzyme activates MC effectively at physiological pH, with greater activation occurring under increasingly acidic conditions [4, 5, 25]. The reduction of MC by DT-diaphorase produces 2,7-diaminomitosene as the predominant metabolite regardless of the degree of aeration; a small amount of oxygen consumption is observed under aerobic conditions [26].

Elevated DT-diaphorase activity, relative to normal tissues, has been reported in a number of different malignancies including primary human tumors from lung, liver, colon, and breast [27, 28]. A 20- to 50-fold increase in DT-diaphorase mRNA has been observed in human liver tumors [29]. Recent studies have examined the role of hypoxia in the regulation of DT-diaphorase gene expression. Interestingly, hypoxia markedly increases the transcription and stability of DT-diaphorase mRNA, as well as the specific activity of the enzyme, in HT-29 colon carcinoma cells [30]. The increased transcription of the DT-diaphorase gene in response to hypoxia and other stress factors has

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§ *Abbreviations:* MC, mitomycin C; and POR, porfiromycin.
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been correlated recently with the activity of the transcription factor NF-κB [31]. Moreover, DT-diaphorase expression increases as a function of depth within multicellular spheroids of HT-29 cells; interior regions of these spheroids are known to have lowered oxygen tensions and reduced pH [32, 33]. These findings suggest that hypoxic regions of tumors may be targeted by drugs amenable to reductive activation by DT-diaphorase.

A considerable body of evidence suggests that DT-diaphorase plays a major role in the bioreductive activation of the mitomycins under aerobic conditions in cell lines either selected for or possessing intrinsically different MC sensitivities; however, these same studies suggest a smaller role for this enzyme under hypoxic conditions (for references see [34] and Discussion). The contribution of other enzyme systems to the cytotoxic action of MC in the cell lines studied to date is not known, because complete profiles of the other enzymes known to activate MC were not measured. Moreover, a comparison of cell lines with distinct intrinsic or selected MC sensitivities, but of different origins, disregards potential heterogeneities in the many other genetic factors that can impact on drug resistance. In the present report, a Chinese hamster ovary (CHO) cell line was modified by transfection to overexpress a rat DT-diaphorase cDNA to gain information on the role of DT-diaphorase in the bioactivation of MC and POR in living cells under aerobic and hypoxic conditions. A preliminary report of these findings has been presented in abstract form [35].

MATERIALS AND METHODS

Materials

MC and POR were contributed by the Bristol-Myers Squibb Co. (Wallingford, CT). NADH, NADPH, NAD^+, chloroquine, and HEPES were purchased from the Sigma Chemical Co. (St. Louis, MO). Glutamine, hypoxanthine, thymidine, geneticin (G418), trypsin, penicillin, and streptomycin were purchased from GibcoBRL (Grand Island, NY). Tissue culture dishes (60 mm) were purchased from the Costar Corp. (Cambridge, MA). Tissue culture flasks and 100- and 150-mm tissue culture dishes were acquired from Becton Dickinson (Franklin Lakes, NJ). Dicumarol, potassium ferricyanide, and ethanol were obtained from the Aldrich Chemical Co. (Milwaukee, WI). 2-Mercaptoethanol was from Bio-Rad Laboratories, Inc. (Richmond, CA). $Na_2HPO_4 \cdot 2H_2O$, dextrose, $CaCl_2$, glycerol, Tris, EDTA, KH_2PO_4, KCl, and NaCl were obtained from J. T. Baker, Inc. (Phillipsburg, NJ).

Cell Culture and Medium

The cell line used in this study is a variant of the CHO-K1 cell line termed CHO-K1/dhfr$^-$ [36] and was obtained from the American Type Culture Collection (Rockville, MD). This cell line is deficient in dihydrofolate reductase. The cells were maintained in Iscove's Modified Dulbecco's medium (GibcoBRL) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), 2 mM glutamine, 0.1 mM hypoxanthine, 0.01 mM thymidine, and antibiotics (penicillin, 100 units/mL; streptomycin, 100 μg/mL). Transfected lines were maintained in the identical medium supplemented with 1 mg/mL of G418 to provide for selection of the expression vector. Cells were grown as monolayers in tissue culture flasks, petri dishes, or glass milk dilution bottles at 37° under an atmosphere of 95% air/5% CO_2 in a humidified incubator. The doubling time of CHO-K1/dhfr$^-$ cultures is 19 hr.

Plasmid Construction

The cDNA for rat DT-diaphorase was provided by Dr. Gerald L. Forrest of the Beckman Research Institute (Duarte, CA) [37, 38]. To allow for the subcloning of the 1500 bp cDNA into the eukaryotic expression vector pRC/CMV (Invitrogen Corp., San Diego, CA) and to correct sequences in the 5′ untranslated leader portion necessary for optimal translation of its mRNA, the cDNA was amplified by the polymerase chain reaction (PCR) utilizing the following oligonucleotides:

5′-CGCGGATCCAAGCTTGGTACCGCCAC-CATGGCGGTGAGAAGAGCCCTG-3′

and

5′-CGCGGATCCAAGCTTGCTAGCATAGTG-TACAAAAGACCTAGA-3′.

These oligonucleotides incorporate a *Hin*dIII restriction site (shown in italics) and four changes in the 5′ untranslated leader (shown underlined) upstream of the ATG translational initiation codon (shown in bold) [39]. The amplified PCR product was extracted with phenol:chloroform, precipitated with 2.5 vol. of ethanol, and resuspended in 50 μL of Tris:EDTA (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Following digestion of 5 μL of the amplified cDNA with *Hin*dIII (Boehringer Mannheim Corp., Indianapolis, IN), the fragment was subcloned into pRC/CMV, and recombinants were screened by restriction analysis to isolate the correct orientation. This plasmid, designated pRC/CMV-rDTD, contains the promoter sequences from the immediate early gene of the human cytomegalovirus and the appropriate sequences for polyadenylation and selection (neomycin resistance).

Transfections

Transfections were performed by the $Ca_3(PO_4)_2$–DNA coprecipitation method essentially as described by Sambrook *et al.* [40]. Briefly, CHO-K1/dhfr$^-$ cells were plated in 100-mm dishes at approximately 2×10^6 cells per dish and allowed to attach overnight. Three hours prior to transfection, the medium was changed. Plasmid DNA (30 μg) in 438 μL of 0.1× Tris–EDTA (1 mM Tris–HCl, pH 8.0, 0.1 mM EDTA) and 62 μL of 2 M $CaCl_2$ was added dropwise

to 500 μL of 2× HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, and 50 mM HEPES; the pH of the final solution was adjusted to 7.05) through which air was bubbled slowly. Following addition of the DNA solution, the mixture was vortexed gently (5 sec), and the precipitate was allowed to form at room temperature over 30 min. Then the medium was removed from the dishes, and the $Ca_3(PO_4)_2$–DNA coprecipitate mixture was added dropwise over the entire surface of the monolayer. After incubation for 20 min at room temperature, 10 mL of pre-warmed medium containing 100 μg/mL of chloroquine was added, and the cells were incubated at 37° under an atmosphere of 95% air/5% CO_2 for 4 hr. Then the medium was removed by aspiration, the monolayer was washed once with 5 mL of PBS (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), and 2 mL of glycerol shock solution (15% glycerol in 1× HEPES-buffered saline) was added. After a 3-min incubation, 5 mL of PBS was added and mixed, and the glycerol–PBS mixture was removed by aspiration. The monolayer was washed twice more with PBS to remove any remnants of the glycerol, and the cells were refed with 10 mL of pre-warmed culture medium. Following incubation for 18 hr at 37° under an atmosphere of 95% air/5% CO_2, the cells were removed from the dishes by trypsinization, diluted 500- to 1000-fold with culture medium containing 1 mg/mL of G418 (selection medium), and plated onto 150-mm dishes, where colonies were allowed to form over 10 days. Single colonies were introduced into wells of a 24-well plate using sterile cotton-tipped applicators (General Medical Corp., Richmond, VA). After expansion, the isolates were screened for expression of the DT-diaphorase cDNA gene. Isolates that had elevated enzyme activities were cloned by flow cytometry sorting, and the resulting clones were rescreened. Vector-transfected control clones were CHO-K1/dhfr$^-$ cells transfected with the plasmid without a DT-diaphorase cDNA insert.

Assays of Enzyme Activity

Exponentially growing cells (approximately 5×10^6 total cells) were harvested by trypsinization, washed in cell culture medium containing 10% fetal bovine serum to inactivate the trypsin, washed with PBS, and then resuspended in 2 mL of PBS. Cells were disrupted by sonication using a Branson sonicator (Branson Ultrasonics Corp., Danbury, CT) with three 10-sec bursts at a setting of 25 with 1 min cooling on ice between each sonication burst. Cell disruption was confirmed microscopically. DT-Diaphorase activity was measured as the dicumarol-inhibitable reduction of dichlorophenolindophenol, measured at 600 nm with a Beckman model 25 UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, CA) using an extinction coefficient of 21 mM^{-1} cm^{-1} at 30° [24]; the final concentration of dicumarol was 100 μM [16]. NADPH:cytochrome *c* (P-450) reductase activity in cell extracts was assayed by monitoring the rate of ferricytochrome *c* reduction at 550 nm (extinction coefficient of 27.7 mM^{-1} cm^{-1}) at 30° [41]. NADH:cytochrome b_5 reductase activity was measured as NADH:ferricyanide reductase at 420 nm (extinction coefficient of 1.02 mM^{-1} cm^{-1}) at 30° essentially as described [42] but using a final concentration of 0.34 mM NADH. Xanthine oxidase and xanthine dehydrogenase activities were assayed by monitoring the formation of uric acid at 293 nm (extinction coefficient of 12.2 mM^{-1} cm^{-1}) at 30° using oxygen and NAD^+ as the respective electron acceptors [43]. Glutathione *S*-transferase activity was determined at 340 nm and 25° utilizing 1-chloro-2,4-dinitrobenzene (extinction coefficient of 9.6 mM^{-1} cm^{-1}) as the electrophilic substrate [44]. Protein concentrations were assayed using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) [45].

Aerobic/Hypoxic Experiments

Exponentially growing monolayers of CHO-K1/dhfr$^-$, CHO-DTD-133, CHO-DTD-126, and CHO-VC (vector control) cells were seeded in glass milk dilution bottles at 2×10^5 cells per bottle and were used in mid-exponential phase (approximately 3–4 days of growth). 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 induction of hypoxia, cells were exposed to 1, 2.5, 5, 7.5, or 10 μM MC or POR for 1 hr; drugs were injected through the septum without compromising the hypoxia. Cells under aerobic conditions were treated with the mitomycins in an identical manner for 1 hr in a humidified atmosphere of 95% air/5% CO_2 at 37°. Treated cells were washed, harvested by trypsinization, and assayed for survival by measuring their ability to form macroscopic colonies [46]. Both aerobic and hypoxic vehicle controls (63 μL of 70% ethanol) were included in each experiment; the surviving fractions were normalized using these vehicle controls. The plating efficiencies (colonies/100 plated cells; means ± SD) for CHO-K1/dhfr$^-$, CHO-VC, CHO-DTD-133, and CHO-DTD-126 cells were 84 ± 7, 67 ± 3, 68 ± 7, and 64 ± 5, respectively. The surviving fractions for the aerobic vehicle controls (mean ± SD) were 1.01 ± 0.04, 1.01 ± 0.05, 1.00 ± 0.04, and 1.04 ± 0.04, while the surviving fractions for the hypoxic vehicle controls were somewhat lower, reflecting the toxic effects of the hypoxia: 0.67 ± 0.06, 0.72 ± 0.07, 0.65 ± 0.05, and 0.61 ± 0.04 for CHO-K1/dhfr$^-$, CHO-VC, CHO-DTD-133, and CHO-DTD-126 cells, respectively.

Subcellular Fractionation

Exponentially growing CHO-K1/dhfr$^-$ parental, and CHO-DTD-133 cells were collected and disrupted by sonication as described above, except that cells were resuspended in 20 mL of HEPES buffer (25 mM HEPES, 250 mM sucrose, pH 7.4) at a cell concentration of 2×10^7 cells/mL, and sonication was performed at a setting of 20. The cell sonicates

were centrifuged at 12,100 g for 10 min (mitochondrial/nuclear fraction), and then at 105,000 g for 1 hr (microsomal fraction). The resulting supernatant represented the cytosolic fraction. Each pellet was washed in HEPES buffer, recentrifuged, and then resuspended in 1 mL of the same buffer. Fractions were assayed for DT-diaphorase enzyme activity and protein concentration as described above.

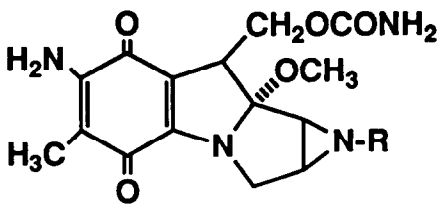
RESULTS

To assess the contribution of DT-diaphorase to the cellular activation of MC and POR (Fig. 1), the CHO-K1/dhfr$^-$ cell line was used to develop transfectants that overexpressed a rat DT-diaphorase cDNA [38], using the plasmid pRC/CMV-rDTD. Parental CHO-K1/dhfr$^-$ cells and vector-transfected control cells (CHO-VC) were equisensitive to the cytotoxic action of MC under oxygenated and hypoxic conditions at concentrations ranging from 2.5 to 10 μM (Fig. 2A). In contrast, POR was significantly less cytotoxic than MC to both cell lines in air, but was markedly more toxic than MC under hypoxia (Fig. 2B). In previous studies, POR was similarly more toxic than MC to CHO-AA8 cells under hypoxia and less toxic than MC in air [47]. These findings with CHO cells differ from those seen with EMT6 mouse mammary carcinoma cells for which the levels of hypoxic cell kill by MC and POR are similar [48]. Consistent with the findings in CHO cells, POR is considerably less cytotoxic than MC to EMT6 cells in air.

Two clones of CHO-K1/dhfr$^-$ transfected with pRC/CMV-rDTD were selected, which expressed 126-fold (CHO-DTD-126) and 133-fold (CHO-DTD-133) more DT-diaphorase activity than the parental cell line. Comparisons between the growth rates of the CHO-K1/dhfr$^-$ parental cells and the DT-diaphorase-transfected clones revealed no significant differences (data not shown). The levels of the other oxidoreductases implicated in the bioreductive activation of the mitomycin antibiotics were measured in the two transfectants, in a vector-transfected control clone, and in the parental cell line (Table 1). Except for the desired increase in DT-diaphorase activity in CHO-DTD-126 and CHO-DTD-133 cells, the levels of these oxidoreductases were not significantly different in the four cell lines. None of these CHO cell lines contained detectable levels of xanthine oxidase or xanthine dehydrogenase. Because increased glutathione S-transferase activity has been reported to correlate with increased resistance to MC in a human bladder cancer cell line [49], the total levels of these isoenzyme activities were measured in each cell line and were found to be essentially identical (Table 1). An analysis of the subcellular distribution of DT-diaphorase in the CHO-K1/dhfr$^-$ parental and CHO-DTD-133 cell lines demonstrated some possible differences, but over 80% of the DT-diaphorase enzyme activity was found in the cytosolic fraction in both lines (Table 2).

The effects of DT-diaphorase on the bioactivation of MC and POR were studied by comparing the cytotoxicities of these drugs under aerobic and hypoxic conditions in the parental and vector-transfected control cell lines and in the transfected clones with high DT-diaphorase levels. The vector-transfected control clone resembled the parental line in its sensitivities to MC and POR (Fig. 2). In both DT-diaphorase-rich transfected clones, sensitivity to MC was increased considerably under both oxygenated and hypoxic conditions (Fig. 3). The magnitude of the increase in sensitivity to MC was the same under aerobic and hypoxic conditions in both clones. As a result, like the parental line, both DT-diaphorase-transfected clones were equally sensitive to MC under aerobic and hypoxic conditions.

The results with POR were more complex. In air, both DT-diaphorase-transfected clones were significantly more sensitive to POR than was the parental line (Fig. 4). The increase in aerobic cell kill in the DT-diaphorase-rich transfectants, relative to parental control cells, was greater with MC than with POR (compare Fig. 3 with Fig. 4). However, under hypoxia, no difference was observed between the DT-diaphorase-transfected and parental cell lines in sensitivity to POR (Fig. 4). Thus, the aerobic/hypoxic differential for POR was slightly smaller for the DT-diaphorase-rich transfectants than for the control cells.



Mitomycin C: R= -H

Porfiromycin: R= -CH_3

FIG. 1. Structures of mitomycin C and porfiromycin.

DISCUSSION

Considerable evidence from our laboratories and others implicates DT-diaphorase in the aerobic, but not hypoxic, activation of the mitomycins. Several of these studies utilized the DT-diaphorase inhibitor dicumarol to demonstrate that under aerobic conditions, DT-diaphorase-rich cell lines were protected from the toxicity of MC and/or POR, whereas DT-diaphorase-deficient cell lines were not. For example, dicumarol protected oxygenated EMT6 mammary carcinoma cells from the toxicity of MC and POR [16, 50–53]; in contrast, the cytotoxicity of these drugs was increased by dicumarol under hypoxia. Similarly, L1210 leukemia cells, which have no measurable DT-diaphorase activity, were not protected from MC by dicumarol in air;

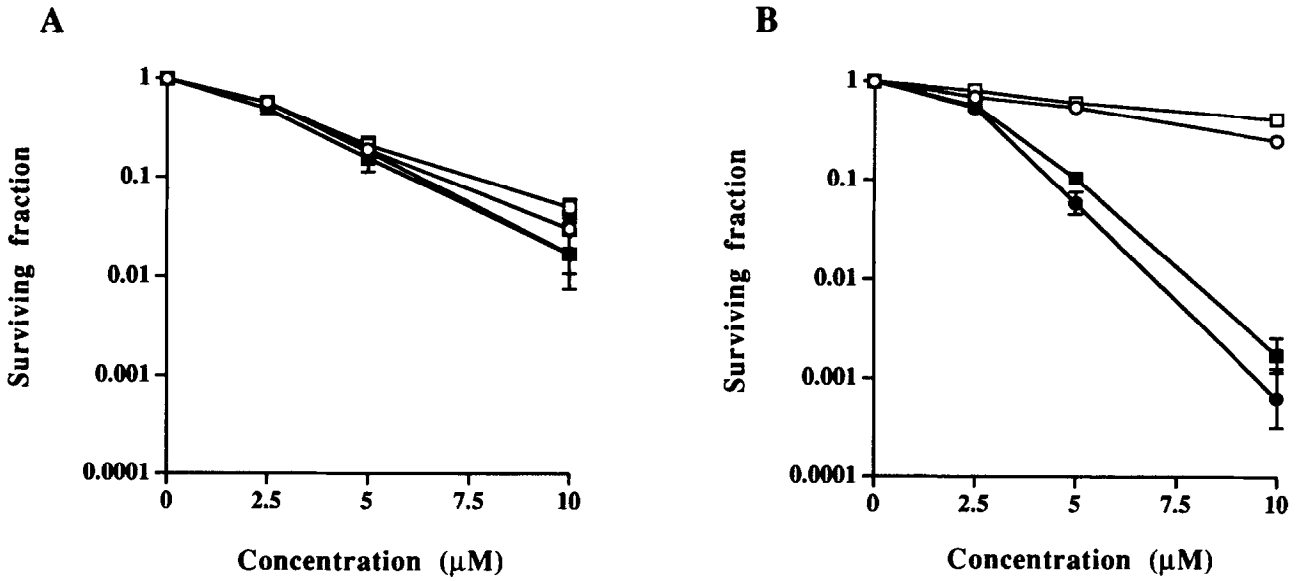


FIG. 2. Survival curves for CHO-K1/dhfr⁻ parental, and CHO-VC vector-transfected control cells treated with graded concentrations of MC or POR for 1 hr under aerobic (open symbols) and severely hypoxic (closed symbols) conditions. The surviving fractions were calculated using the plating efficiencies of the aerobic and hypoxic vehicle-treated controls. Points are geometric means of 3–8 determinations; SEM are shown where larger than the points. Key: (○, ●) CHO-K1/dhfr⁻ parental cells; and (□, ■) CHO-VC vector-transfected control cells. (A) MC survival curves. (B) POR survival curves.

dicumarol enhanced the cytotoxicity of MC to L1210 cells under hypoxia [50]. It should be noted that dicumarol is not a specific inhibitor of DT-diaphorase [54], but rather inhibits several other enzymes, including NADH:cytochrome b_5 reductase, an enzyme which has been shown recently to be involved in the reductive activation of the mitomycins *in vitro* [18]. Additionally, dicumarol appears to enhance the ability of xanthine dehydrogenase to metabolize MC [21].

Cell lines with different MC resistance profiles often have DT-diaphorase enzyme levels that correlate with their drug sensitivities. A hydrolyzed benzoquinone mustard resistant clone (designated L5178Y/HBM10), selected from the mouse lymphoblastic cell line L5178Y, and a non-transformed human skin fibroblast cell line, GM38, expressed 24- and 61-fold more DT-diaphorase, respectively, than their DT-diaphorase-deficient counterparts [7, 55–57]. These DT-diaphorase-rich cell lines were 4.5- and 7-fold more sensitive, respectively, to MC in air. Both DT-diaphorase-elevated lines were protected from MC by dicumarol in air, while the corresponding DT-diaphorase-deficient cells were not. Interestingly, under hypoxic conditions, the MC sensitivities of the DT-diaphorase-deficient cell lines increased to match those of their DT-diaphorase-rich counterparts. The drug sensitivities of the DT-diaphorase-rich cells were unaffected by hypoxia. Dicumarol had no effect on the MC sensitivity of L5178Y/HBM10 cells under hypoxia; GM38 cells were not tested. Similar findings were reported for the DT-diaphorase-rich, human colon carcinoma cell line, HT-29, and its DT-diaphorase-deficient counterpart, BE [4].

In related studies, derivatives of HCT 116 human colon carcinoma and CHO-AA8 cells, which were selected for MC resistance under aerobic conditions, expressed 20-fold and 2- to 5-fold less DT-diaphorase activity, respectively,

TABLE 1. Oxidoreductase and glutathione S-transferase activities of CHO-K1/dhfr⁻, vector-transfected control (CHO-VC), and rat DT-diaphorase cDNA transfected (CHO-DTD-126 and CHO-DTD-133) cell clones

Enzyme*	Activity† (nmol/min/mg protein)			
	CHO-K1/dhfr⁻	CHO-VC	CHO-DTD-126	CHO-DTD-133
DT-Diaphorase	10.1 ± 1.6‡	11.3 ± 0.7	1277 ± 137	1347 ± 58
NADH:Cytochrome b_5 reductase	2226 ± 151	2341 ± 162	2330 ± 594	2561 ± 190
NADPH:Cytochrome c (P-450) reductase	2.2 ± 0.9	2.8 ± 0.6	1.8 ± 1.4	1.0 ± 0.5
Glutathione S-transferase	151 ± 2	155 ± 14	182 ± 17	164 ± 10

* No detectable xanthine oxidase or xanthine dehydrogenase activity was observed in any cell line.

† All enzyme activities were determined by standard assays as desscribed in Materials and Methods.

‡ Values are means ± SEM of 3–6 determinations. There were no significant differences between the values for the parental and transfected cell lines (paired Student's *t*-test; $P > 0.05$), except for DT-diaphorase transfected clones.

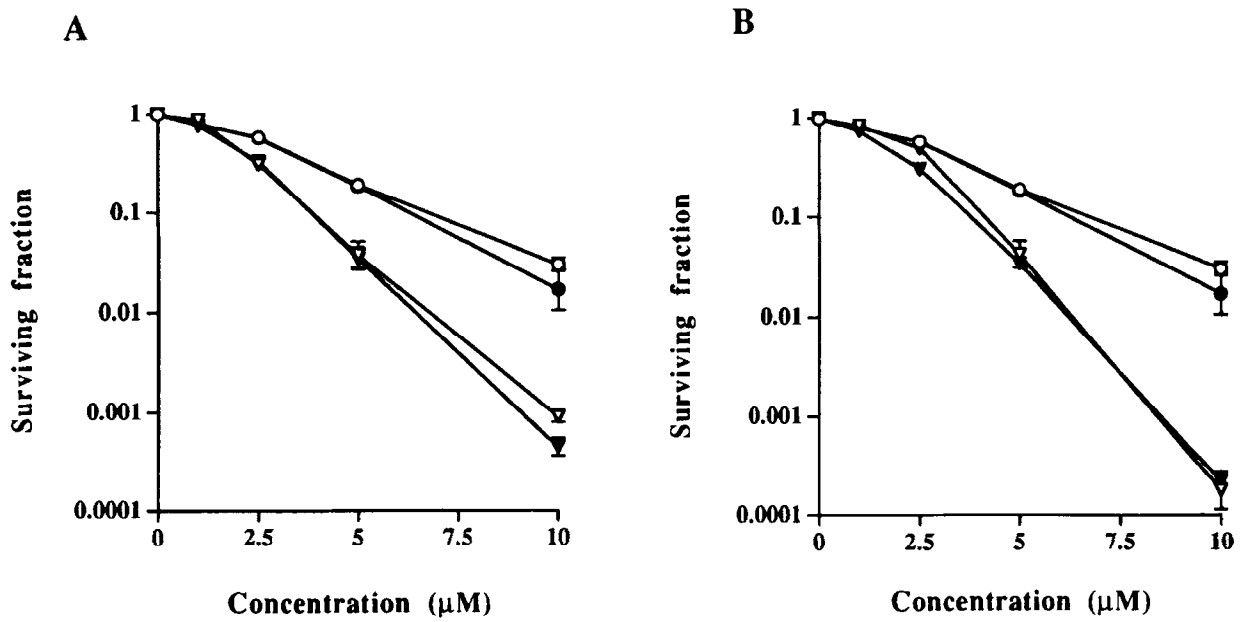


FIG. 3. MC survival curves for CHO-K1/dhfr⁻ parental (from Fig. 2), CHO-DTD-133 (A) and CHO-DTD-126 (B) cells. Cells were treated with graded concentrations of MC for 1 h under aerobic (open symbols) or severely hypoxic (closed symbols) conditions. The surviving fractions were calculated using the plating efficiencies of the aerobic and hypoxic vehicle-treated controls. Each point represents the geometric mean ± SEM of survivals determined in 3–8 independent experiments; SEM are shown where larger than the points. Key: (○, ●) CHO-K1/dhfr⁻ parental cells; and (▽, ▼) CHO-DTD-133 (A) or CHO-DTD-126 (B) cells.

than their parental cells [58, 59]. Parental and resistant CHO-AA8 cells had equivalent sensitivities to MC when treated under hypoxia. A comparison of two human bladder cancer cell lines demonstrated that SCaBER cells, which are resistant to MC, exhibited lower NADPH:cytochrome *c* (P-450) reductase and DT-diaphorase activities than MC-sensitive J82 cells [60]. When J82 cells were selected for resistance to MC, NADPH:cytochrome *c* (P-450) reductase and DT-diaphorase activities dropped 3- to 4-fold, while glutathione *S*-transferase activity increased marginally [49]. Collectively, this body of work strongly implicates DT-diaphorase as a major contributor to the activation of the mitomycins under aerobic conditions, and also suggests that the role of DT-diaphorase is less important under hypoxic conditions.

In this report, we describe an intact cell model in which the level of a single bioreductive enzyme was varied to assess directly the importance of that enzyme in the activation of the mitomycins in living cells. In CHO cells transfected with and overexpressing a rat DT-diaphorase cDNA, the cytotoxicity of MC was increased equally under aerobic and hypoxic conditions. This finding indicates that DT-diaphorase does not contribute to the aerobic/hypoxic differential in the toxicity of MC. Furthermore, these results demonstrated that DT-diaphorase can activate MC under hypoxic conditions. This finding is in contrast to those obtained using dicumarol; the non-specific nature of this inhibitor may explain this apparent contradiction. In support of the findings presented in this paper, Beall *et al.* [26] demonstrated that the cytotoxicity of MC was not altered by oxygenation in DT-diaphorase-rich HT-29 cells, but was potentiated markedly by hypoxia in DT-diaphorase-deficient BE cells. Likewise, Begleiter *et al.* [55] noted that the DT-diaphorase-rich cell line L5178Y/HBM10 was equally sensitive to MC in air and hypoxia, whereas MC was 2-fold more toxic to the DT-diaphorase-deficient parental line under hypoxia than in air. Collectively, these findings are consistent with the idea that the relative levels of enzyme activity in living cells are important in determining the extent to which any particular enzyme will reduce MC. Additionally, the results suggest that MC would be useful in the treatment of solid tumors that express elevated DT-diaphorase activities. Both aerobic and hypoxic cells in such tumors would be sensitive to the cytotoxic effects of MC. However, MC could be effec-

TABLE 2. Subcellular distribution of DT-diaphorase enzyme activity in CHO-K1/dhfr⁻ parental and CHO-DTD-133 transfected cells

Subcellular fraction*	% DT-Diaphorase activity†	
	CHO-K1/dhfr⁻	CHO-DTD-133
Mitochondrial/Nuclear	0	14
Microsomal	10	3
Cytoplasmic	90	82

* Subcellular fractions were prepared as described in Materials and Methods.

† DT-Diaphorase enzyme activity was determined as described in Materials and Methods. The percent DT-diaphorase activities were calculated as the activity in a particular fraction divided by the sum of the activities in all of the fractions.

tive in targeting hypoxic cells of solid tumors if DT-diaphorase enzyme expression were greater in hypoxic cells than in aerated cells; recent work demonstrating that prolonged hypoxic stress induces DT-diaphorase gene expression suggests that this may occur in some tumors [30–33].

Measurements of the effects of POR in CHO-DTD-133 and CHO-DTD-126 cells demonstrated that overexpression of DT-diaphorase activity increased the toxicity of this drug under aerobic conditions without increasing its toxicity in hypoxia, thus decreasing the aerobic/hypoxic differential in toxicity. This result, which differs significantly from that obtained with MC, is consistent with the idea that DT-diaphorase has a greater role in the activation of POR under aerobic conditions than in hypoxia and suggests that DT-diaphorase may have affinities for MC and POR that are different and are influenced by the degree of oxygenation. This latter conclusion is supported by the demonstration in the parental cell line that POR was more toxic than MC to hypoxic cells but less toxic than MC to oxygenated cells, producing an aerobic/hypoxic differential for POR that contrasted to the equal cytotoxicity of MC (Fig. 2). This difference occurred despite the fact that MC and POR have similar chemical structures and essentially identical redox potentials. These results differ somewhat from those seen with EMT6 cells, in which the level of hypoxic cell kill was similar for MC and POR [48], but is in agreement with findings in two other CHO sublines, CHO-HA-1 [61] and CHO-AA8 [47]. DT-Diaphorase activity is approximately 15-fold higher in EMT6 cells [16] than in CHO-K1/dhfr$^-$, CHO-HA-1 [16], or CHO-AA8 [59] cells. Interestingly, the cytotoxicities of MC and POR in the DT-diaphorase-transfected CHO-K1/dhfr$^-$ cell lines were similar under hypoxic conditions, suggesting that the variations between the CHO and EMT6 cell lines in their hypoxic sensitivities to MC and POR may reflect their relative levels of DT-diaphorase activities.

In an attempt to explain the different cytotoxicity profiles of MC and POR to aerobic and hypoxic CHO-K1/dhfr$^-$ parental cells (Fig. 2), we propose that MC is a better substrate than POR for the two-electron reductant, DT-diaphorase. We also suggest that MC and POR are similar as substrates for the one-electron reductants, and that the product of the one-electron reduction, the semiquinone anion radical, is a more potent alkylating species than is the product of the two-electron reduction, the hydroquinone. This latter assumption, also proposed by Dulhanty and Whitmore [59], is supported by Cera *et al.* [62] who found that the semiquinone of *N*-methylmitomycin A is a better cross-linking agent than its hydroquinone derivative. Under aerobic conditions, MC is more toxic than POR because it is primarily a substrate for the two-electron reductant DT-diaphorase, resulting in production of the oxygen-insensitive hydroquinone intermediate and in subsequent alkylations. The one-electron reduction of POR in air results in a futile cycle and little toxicity because the oxygen-sensitive semiquinone anion radical redox cycles with molecular oxygen, restoring the parent compound [63]. Marshall and Rauth [64] provide evidence suggesting that the

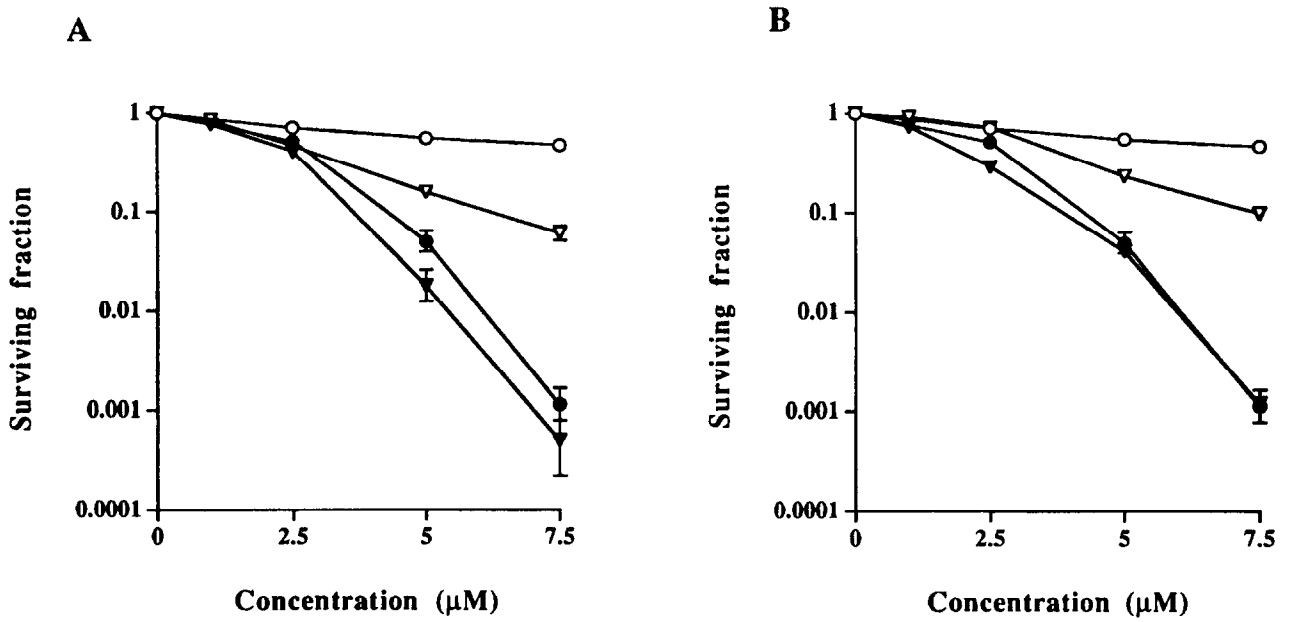


FIG. 4. POR survival curves for CHO-K1/dhfr$^-$ parental (from Fig. 2), CHO-DTD-133 (A) and CHO-DTD-126 (B) cells. Cells were treated with graded concentrations of POR for 1 hr under aerobic (open symbols) or severely hypoxic (closed symbols) conditions. The surviving fractions were calculated using the plating efficiencies of the aerobic and hypoxic vehicle-treated controls. Each point represents the geometric mean ± SEM of survivals determined in 3–8 independent experiments; SEM are shown where larger than the points. Key: (○, ●) CHO-K1/dhfr$^-$ parental cells; and (▽, ▼) CHO-DTD-133 (A) or CHO-DTD-126 (B) cells.

cytotoxicity of POR is considerably more sensitive to quenching by oxygen than is the cytotoxicity of MC; this implies that POR is reduced primarily through a one-electron pathway. Under hypoxic conditions, the one-electron reduction of POR results in the production of alkylating species and, concomitantly, a marked increase in cytotoxicity (Fig. 2). MC, being metabolized primarily by DT-diaphorase, shows little or no change in cytotoxicity with hypoxia in the parental cell line because the hydroquinone intermediate is oxygen insensitive. Under hypoxia, POR is more cytotoxic than MC because the semiquinone anion radical is a more potent alkylating species than the hydroquinone. Consistent with this model, elevating the levels of DT-diaphorase in CHO-K1/dhfr$^-$ cells increased the toxicity of MC equally under aerobic and hypoxic conditions (Fig. 3). However, in the DT-diaphorase-elevated cell lines, the increase in the aerobic toxicity of POR, relative to that of MC, was small, and no increase in POR cytotoxicity was observed under hypoxia (Fig. 4). In contrast, elevating the levels of NADPH:cytochrome *c* (P-450) reductase, which reduces the mitomycins through a one-electron mechanism, by transfection of CHO-K1/dhfr$^-$ cells results in increased cytotoxicity for both MC and POR regardless of the degree of oxygenation; a greater increase in toxicity occurs under hypoxia than in air [65]. This implicates NADPH:cytochrome *c* (P-450) reductase in the differential toxicity of the mitomycin antibiotics in air and hypoxia.

Begleiter *et al.* [7] suggested that the relative amounts of the various enzymes present within cells would determine the toxicities of the mitomycins under different conditions of aeration. Where reduction of MC by DT-diaphorase contributes to the toxicity of the drug, dicumarol would be expected to diminish the cytotoxicity. Additionally, the intracellular pools of reduced pyridine nucleotides would be expected to be influenced by the degree of oxygenation; these pools will partially determine which enzymes activate MC and POR in air and hypoxia (see [34] for references). Validation of the proposed mechanisms will require studies on the relative alkylating abilities of the semiquinone anion radical and the hydroquinone, a determination of the relative amounts of the activating oxidoreductases present in specific cell lines, a more detailed analysis of the interactions between the activating oxidoreductases and the mitomycins, and measurements of the intracellular concentrations of reduced pyridine nucleotides in air and hypoxia.

The reduction of MC by DT-diaphorase is virtually absent at pH 7.8 and increases progressively as the pH is reduced to 5.8 [4]. In contrast, the reduction of MC by NADPH:cytochrome *c* (P-450) reductase is optimal at pH 8.2 [15]. Reduction of MC by NADH:cytochrome b_5 reductase is greater at pH 6.6 than at pH 7.6 [18], and reduction of MC by xanthine dehydrogenase increases as the pH is lowered from 7.4 to 6.0 [20]. This suggests that different enzymes will predominate in the activation of the mitomycins at different pH values. As a result, there may be regional variations in the metabolism of the mitomycins within solid tumors, where pH is heterogeneous with some regions being acidic [66]. The predominant enzyme activities at a given pH will also dictate whether MC or POR will be more effective, as enzyme–substrate specificity will determine the relative activities of each drug at that pH. Studies in oxygenated EMT6 cells indicate that greater MC-induced cytotoxicity and more DNA cross-links occur as the pH is lowered from 7.5 to 5.7 [67], suggesting that the pH-dependent changes in enzyme activity observed *in vitro* are important in determining the cytotoxicities of the mitomycins in living cells.

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References

1. Rockwell S, Keyes SR and Sartorelli AC, Preclinical studies of porfiromycin as an adjunct to radiotherapy. *Radiat Res* **116:** 100–113, 1988.
2. Rockwell S and Sartorelli AC, Mitomycin C and radiation. In: *Antitumor Drug-Radiation Interactions* (Ed. Hill BT and Bellamy AS), pp. 126–139. CRC Press, Boca Raton, FL, 1990.
3. Ross D, Beall H, Traver SD, Siegel D, Phillips RM and Gibson NW, Bioactivation of quinones by DT-diaphorase, molecular, biochemical, and chemical studies. *Oncol Res* **6:** 493–500, 1994.
4. Siegel D, Gibson NW, Preusch PC and Ross D, Metabolism of mitomycin C by DT-diaphorase: Role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50:** 7483–7489, 1990.
5. Siegel D, Beall H, Senekowitsch C, Kasai M, Arai H, Gibson NW and Ross D, Bioreductive activation of mitomycin C by DT-diaphorase. *Biochemistry* **31:** 7879–7885, 1992.
6. Pritsos CA, Pardini LL, Elliot AJ and Pardini RS, Relationship between the antioxidant enzyme DT-diaphorase and tumor response to mitomycin C treatment. In: *Oxygen Radicals in Biology and Medicine* (Eds. Simic MG, Taylor KA, Ward JF and von Sonntag C), pp. 713–716. Plenum Press, New York, 1989.
7. Begleiter A, Robotham E and Leith MK, Role of NAD(P)H: (quinone acceptor) oxidoreductase (DT-diaphorase) in activation of mitomycin C under hypoxia. *Mol Pharmacol* **41:** 677–682, 1992.
8. Begleiter A, Robotham E, Lacey G and Leith MK, Increased sensitivity of quinone resistant cells to mitomycin C. *Cancer Lett* **45:** 173–176, 1989.
9. Kennedy KA, Sligar SG, Polomski L and Sartorelli AC, Metabolic activation of mitomycin C by liver microsomes and nuclei. *Biochem Pharmacol* **31:** 2011–2016, 1982.
10. Komiyama T, Oki T and Inui T, Activation of mitomycin C and quinone drug metabolism by NADPH-cytochrome P-450 reductase. *J Pharmacobio-Dyn* **2:** 407–410, 1979.
11. Bachur NR, Gordon SL, Gee MV and Kon H, NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc Natl Acad Sci USA* **76:** 954–957, 1979.
12. Pritsos CA and Sartorelli AC, Generation of reactive oxygen radicals through bioactivation of mitomycin antibiotics. *Cancer Res* **46:** 3528–3532, 1986.

13. Komiyama T, Kikuchi T and Sugiura Y, Generation of hydroxyl radical by anticancer quinone drugs, carbazilquinone, mitomycin C, aclacinomycin A and adriamycin, in the presence of NADPH-cytochrome P-450 reductase. *Biochem Pharmacol* **31:** 3651–3656, 1982.
14. Pan S-S, Andrews PA, Glover CJ and Bachur NR, Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. *J Biol Chem* **259:** 959–966, 1984.
15. Pan S-S, Iracki T and Bachur NR, DNA alkylation by enzyme-activated mitomycin C. *Mol Pharmacol* **29:** 622–628, 1986.
16. Keyes SR, Fracasso PM, Heimbrook DC, Rockwell S, Sligar SG and Sartorelli AC, Role of NADPH:cytochrome c reductase and DT-diaphorase in the biotransformation of mitomycin C. *Cancer Res* **44:** 5638–5643, 1984.
17. Bligh HFJ, Bartoszek A, Robson CN, Hickson ID, Kasper CB, Beggs JD and Wolf CR, Activation of mitomycin C by NADPH:cytochrome P-450 reductase. *Cancer Res* **50:** 7789–7792, 1990.
18. Hodnick WF and Sartorelli AC, Reductive activation of mitomycin C by NADH:cytochrome b_5 reductase. *Cancer Res* **53:** 4907–4912, 1993.
19. Fisher J and Olsen R, Mechanistic aspects of mitomycin C activation by flavoprotein transhydrogenases. *Dev Biochem* **21:** 240–243, 1982.
20. Gustafson DL and Pritsos CA, Bioactivation of mitomycin C by xanthine dehydrogenase from EMT6 mouse mammary carcinoma tumors. *J Natl Cancer Inst* **84:** 1180–1185, 1992.
21. Gustafson DL and Pritsos CA, Enhancement of xanthine dehydrogenase mediated mitomycin C metabolism by dicumarol. *Cancer Res* **52:** 6936–6939, 1992.
22. Pritsos CA and Gustafson DL, Xanthine dehydrogenase and its role in cancer chemotherapy. *Oncol Res* **6:** 477–481, 1994.
23. Kennedy KA, Mimnaugh EG, Trush MA and Sinha BK, Effects of glutathione and ethylxanthate on mitomycin C activation by isolated rat hepatic or EMT6 mouse mammary tumor nuclei. *Cancer Res* **45:** 4071–4076, 1985.
24. Ernster L, DT Diaphorase. *Methods Enzymol* **10:** 309–317, 1967.
25. Siegel D, Beall H, Kasai M, Arai H, Gibson NW and Ross D, pH-Dependent inactivation of DT-diaphorase by mitomycin C and porfiromycin. *Mol Pharmacol* **44:** 1128–1134, 1993.
26. Beall HD, Mulcahy RT, Siegel D, Traver RD, Gibson NW and Ross D, Metabolism of bioreductive antitumor compounds by purified rat and human DT-diaphorases. *Cancer Res* **54:** 3196–3201, 1994.
27. Schlager JJ and Powis G, Cytosolic NAD(P)H:quinone acceptor oxidoreductase in human normal and tumor tissue. Effects of cigarette smoking and alcohol. *Int J Cancer* **45:** 403–409, 1990.
28. Malkinson AM, Siegel D, Forrest GL, Gazdar AF, Oie HK, Chan DC, Bunn PA, Mabry M, Dykes DJ, Harrison SD and Ross D, Elevated DT-diaphorase activity and messenger RNA content in human non-small lung carcinoma: Relationship to the response of lung tumor xenografts to mitomycin C. *Cancer Res* **52:** 4752–4757, 1992.
29. Cresteil T and Jaiswal AK, High levels of expression of the NAD(P)H:quinone oxidoreductase (NQO_1) gene in tumor cells compared to normal cells of the same origin. *Biochem Pharmacol* **42:** 1021–1027, 1991.
30. O'Dwyer PJ, Yao K-S, Ford P, Godwin AK and Clayton M, Effects of hypoxia on detoxicating enzyme activity and expression in HT29 colon adenocarcinoma cells. *Cancer Res* **54:** 3082–3087, 1994.
31. Yao K-S and O'Dwyer PJ, Involvement of NF-κB in the induction of NAD(P)H:quinone oxidoreductase (DT-diaphorase) by hypoxia, oltipraz and mitomycin C. *Biochem Pharmacol* **49:** 275–282, 1995.
32. Phillips RM, de la Cruz A, Traver RD and Gibson NW, Increased activity and expression of NAD(P)H:quinone acceptor oxidoreductase in confluent cell cultures and within multicellular spheroids. *Cancer Res* **54:** 3766–3771, 1994.
33. Sutherland RM, Cell and environmental interactions in tumor microregions: The multicell spheroid model. *Science* **240:** 177–184, 1988.
34. Sartorelli AC, Hodnick WF, Belcourt MF, Tomasz M, Haffty B, Fischer JJ and Rockwell S, Mitomycin C: A prototype bioreductive agent. *Oncol Res* **6:** 501–508, 1994.
35. Hodnick WF, Belcourt MF, Kemple B, Rockwell S, and Sartorelli AC, Potentiation of mitomycin C and porfiromycin toxicity to Chinese hamster ovary (CHO) cells by overexpression of DT-diaphorase (DTD) cDNA. *Proc Am Assoc Cancer Res* **36:** 602, 1995.
36. Urlaub G and Chasin LA, Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci USA* **77:** 4216–4220, 1980.
37. Forrest GL, Qian J, Ma J-X, Kaplan WD, Akman S, Doroshow J and Chen S, Rat liver NAD(P)H:quinone oxidoreductase: cDNA expression and site-directed mutagenesis. *Biochem Biophys Res Commun* **169:** 1087–1093, 1990.
38. Robertson JA, Chen H-C and Nebert DW, NAD(P)H:menadione oxidoreductase. Novel purification of enzyme, cDNA and complete amino acid sequence, and gene regulation. *J Biol Chem* **261:** 15794–15799, 1986.
39. Kozak M, The scanning model for translation: An update. *J Cell Biol* **108:** 229–241, 1989.
40. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual* (Eds. Sambrook J, Fritsch EF and Maniatis T). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
41. Yasukochi Y and Masters BSS, Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J Biol Chem* **251:** 5337–5344, 1976.
42. Yubisui T and Takeshita M, Purification and properties of soluble NADH-cytochrome b_5 reductase of rabbit erythrocytes. *J Biochem (Tokyo)* **91:** 1467–1477, 1982.
43. Stirpe F and Della Corte E, The regulation of rat liver xanthine oxidase. *J Biol Chem* **244:** 3855–3863, 1969.
44. Habig WH, Pabst MJ and Jakoby WB, Glutathione S-transferases. *J Biol Chem* **249:** 7130–7139, 1974.
45. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
46. Rockwell S, *In vivo–in vitro* tumor systems: New models for studying the response of tumors to therapy. *Lab Anim Sci* **27:** 831–851, 1977.
47. Hughes CS, Irvin CG and Rockwell S, Effect of deficiencies in DNA repair on the toxicity of mitomycin C and porfiromycin to CHO cells under aerobic and hypoxic conditions. *Cancer Commun* **3:** 29–35, 1991.
48. Keyes SR, Loomis R, DiGiovanna MP, Pritsos CA, Rockwell S and Sartorelli AC, Cytotoxicity and DNA crosslinks produced by mitomycin analogs in aerobic and hypoxic EMT6 cells. *Cancer Commun* **3:** 351–356, 1991.
49. Xu BH, Gupta V and Singh SV, Characterization of a human bladder cancer cell line selected for resistance to mitomycin C. *Int J Cancer* **58:** 686–692, 1994.
50. Keyes SR, Rockwell S and Sartorelli AC, Modification of the metabolism and cytotoxicity of bioreductive alkylating agents by dicoumarol in aerobic and hypoxic murine tumor cells. *Cancer Res* **49:** 3310–3313, 1989.

51. Keyes SR, Rockwell S and Sartorelli AC, Enhancement of mitomycin C cytotoxicity to hypoxic tumor cells by dicoumarol *in vivo* and *in vitro*. *Cancer Res* **45:** 213–216, 1985.
52. Rockwell S, Keyes SR and Sartorelli AC, Modulation of the cytotoxicity of mitomycin C to EMT6 mouse mammary tumor cells by dicoumarol *in vitro*. *Cancer Res* **48:** 5471–5474, 1988.
53. Rockwell S, Keyes SR and Sartorelli AC, Modulation of the cytotoxicity of porfiromycin by dicoumarol *in vitro* and *in vivo*. Anticancer Res **9:** 817–820, 1989.
54. Murray RDH, Mendez J and Brown SA, Biological actions of 4-hydroxycoumarins. In: *The Natural Coumarins: Occurrence, Chemistry, and Biochemistry* (Eds. Murray RDH, Mendez J and Brown SA), Chap. 11. Wiley-Interscience, New York, 1982.
55. Begleiter A, Leith MK, McClarty G, Beenken S, Goldenberg GJ and Wright JA, Characterization of L5178Y murine lymphoblasts resistant to quinone antitumor agents. *Cancer Res* **48:** 1727–1735, 1988.
56. Marshall RS, Paterson MC and Rauth AM, Deficient activation by a human cell strain leads to mitomycin resistance under aerobic but not hypoxic conditions. *Br J Cancer* **59:** 341–346, 1989.
57. Marshall RS, Paterson MC and Rauth AM, Studies on the mechanism of resistance to mitomycin C and porfiromycin in a human cell strain derived from a cancer-prone individual. *Biochem Pharmacol* **41:** 1351–1360, 1991.
58. Pan S-S, Akman SA, Forrest GL, Hipsher C and Johnson R, The role of NAD(P)H:quinone oxidoreductase in mitomycin C- and porfiromycin-resistant HCT 116 human colon-cancer cells. *Cancer Chemother Pharmacol* **31:** 23–31, 1992.
59. Dulhanty AM and Whitmore GF, Chinese hamster ovary cell lines resistant to mitomycin C under aerobic but not hypoxic conditions are deficient in DT-diaphorase. *Cancer Res* **51:** 1860–1865, 1991.
60. Xu BH, Gupta V and Singh SV, Mechanism of differential sensitivity of human bladder cancer cells to mitomycin C and its analogue. *Br J Cancer* **69:** 242–246, 1994.
61. Fracasso PM and Sartorelli AC, Cytotoxicity and DNA lesions produced by mitomycin C and porfiromycin in hypoxic and aerobic EMT6 and Chinese hamster ovary cells. *Cancer Res* **46:** 3939–3944, 1986.
62. Cera C, Egbertson M, Teng SP, Crothers DM and Danishefski SJ, DNA cross-linking by intermediates in the mitomycin activation cascade. *Biochemistry* **28:** 5665–5669, 1989.
63. Sartorelli AC, Therapeutic attack of hypoxic cells of solid tumors: Presidential address. *Cancer Res* **48:** 775–778, 1988.
64. Marshall RS and Rauth AM, Oxygen and exposure kinetics as factors influencing the cytotoxicity of porfiromycin, a mitomycin C analogue, in Chinese hamster ovary cells. *Cancer Res* **48:** 5655–5659, 1988.
65. Belcourt MF, Hodnick WF, Rockwell S and Sartorelli AC, Differential toxicity of mitomycin C and porfiromycin to aerobic and hypoxic Chinese hamster ovary cells overexpressing human NADPH:cytochrome *c* (P-450) reductase. *Proc Natl Acad Sci USA* **93:** 456–460, 1996.
66. Wike-Hooley JL, Haveman J and Reinhold HS, The relevance of tumour pH to the treatment of malignant disease. *Radiother Oncol* **2:** 343–366, 1984.
67. Kennedy KA, McGurl JD, Leondaridis L and Alabaster O, pH Dependence of mitomycin C-induced cross-linking activity in EMT6 tumor cells. *Cancer Res* **45:** 3541–3547, 1985.