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The role of NAD(P)H:quinone oxidoreductase in mitomycin C- and porfiromycin-resistant HCT 116 human colon-cancer cells*

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Summary. A mitomycin C (MMC)- and porfiromycin (PFM)-resistant subline of the HCT 116 human colon-cancer cell line was isolated after repeated exposure of HCT 116 cells to increasing concentrations of MMC under aerobic conditions. The MMC-resistant subline (designated HCT 116-R30A) was 5 times more resistant than the parent cells to MMC and PFM under aerobic conditions. Both the MMC-resistant cells and the parent HCT 116 cells accumulated similar amounts of PFM by passive diffusion, but levels of macromolecule-bound PFM were about 50% lower in the resistant cell line, implying a decrease in PFM reductive activation in the resistant cells. The finding that microsomes from either sensitive or resistant cells showed an equal ability to reduce MMC and PFM indicated that the activity of NADPH cytochrome P-450 reductase (EC 1.6.2.4) was not changed in the resistant subline. Soluble extracts of HCT 116 cells reduced MMC and PFM more effectively at pH 6.1, and NADH and NADPH were utilized equally well as electron donors under both aerobic and anaerobic conditions. These data suggest that quinone reductase (EC 1.6.99.2; DT-diaphorase) in soluble extracts is responsible for the reduction of MMC. Quinone reductase activities in soluble extracts of HCT 116-R30A cells for the reduction of dichlorophenol indophenol (DCPIP) and menadione-cytochrome c at optimal pHs were decreased by 95% as compared with those obtained in parent cells. However, the MMC-reducing activity of HCT 116-R30A soluble extracts was only 50% lower than that of the parent cell extracts. The kinetic constants (K_m, V_{max}) found for quinone reductase in the two cell lines with respect to the substrates DCPIP and menadione differed. Two species of mRNA for quinone reductase (2.7 and 1.2 kb) were detected in both cell lines, and there was no detectable difference between parent and resistant cells in the steady-state level of either of these mRNA species. Furthermore, incubation with the quinone reductase inhibitor dicoumarol rendered HCT 116 cells more resistant to MMC. Alteration of the quinone reductase activity in HCT 116-R30A cells appears to be the mechanism responsible for their resistance to MMC and PFM.

Introduction

Mitomycin C (MMC) and porfiromycin (PFM), two bioreductive alkylating agents, are effective antitumor drugs. MMC is widely used in the treatment of various solid tumors [5]. Unfortunately, clinical resistance to MMC has been reported in patients with colon cancer [23], gastric cancer [21], and breast cancer [18]. In vitro studies have also shown that human colon-cancer cells isolated from patients who had never undergone chemotherapy were resistant to MMC [40]. Several laboratories have developed MMC-resistant sublines of various cancer lines by repeated drug exposure, and different mechanisms have been suggested to explain the development of resistance against MMC by these tumor-cell lines.

One type of MMC resistance, which has been observed in an L1210 leukemia subline by Dorr et al. [7] and in a P388 leukemia subline by Rose et al. [30], has the characteristics of multiple drug resistance (MDR). These cells exhibited cross-resistance to anthracyclines and vinca alkaloids, and expression of membrane p-glycoprotein was seen in the L1210 cells. Another type of resistance to MMC involves reductive enzymes that activate the drug.

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Abbreviations: NAD(P)H, reduced nicotinamide adenosine dinucleotide (phosphate); MMC, mitomycin C; PFM, porfiromycin; PBS, phosphate-buffered saline; DCPIP, 2,6-dichlorophenol indophenol; DIC, dicoumarol; c- and t-AHME, *cis*- and *trans*-7-amino-1-hydroxyl-2-methoxyaminomitosene; EDTA, ethylenediaminetetraacetic acid; AME, 7-amino-2-methoxyaminomitosene; DMSO, dimethylsulfoxide; SSC: 20 × SSC contains 3 M NaCl and 0.3 M sodium citrate; SSPE: 20 × SSPE contains 3 M NaCl, 0.2 M monosodium phosphate, and 20 mM EDTA; SDS, sodium dodecyl sulfate

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Cells exhibiting this mechanism of resistance include (1) the MMC-resistant sublines of HCT 116 human colon carcinoma that produce less DNA cross-linking [39] and show less MMC-activating ability [3], (2) an MMC-resistant Chinese hamster ovary subline (CHO-K1) that shows decreased activity of NADPH cytochrome P-450 reductase [12], and (3) a MMC-resistant human skin fibroblast strain (3437T) that is deficient in the activation of MMC under aerobic conditions due to reduced DT-diaphorase activity [22]. Conversely, Begleiter et al. [2] have developed a L5178Y subline that shows greater sensitivity to MMC due to an increase in DT-diaphorase. In terms of MMC activation by reductive enzymes, it has been shown that microsomal NADPH cytochrome P450 reductase is a major enzyme that activates MMC under anaerobic conditions [26, 36]. Indirect evidence indicates that DT-diaphorase also plays a role in the cytotoxicity of MMC [2, 16, 29] and in the resistance of cells to this alkylating agent [22]. However, until recently, the capability of DT-diaphorase to activate MMC in vivo was uncertain because purified quinone reductase (DT-diaphorase) from human kidney had shown no ability to activate MMC [32, 41]. Recent reports by Siegel et al. [33, 34] have clarified some of the uncertainty and demonstrated that at low pH, DT-diaphorases from various sources are capable of reducing MMC.

We developed a group of MMC-resistant sublines of the HCT 116 line by repeatedly exposing the cells to progressively increasing concentrations of MMC under aerobic conditions. One subline, HCT 116-R30A, was subcloned. This report presents (1) a description of the enzymatic differences between the parental HCT 116 cells and this subline, (2) an elucidation of the possible mechanisms underlying the resistance of this subline to MMC, and (3) an explanation of the involvement of reductive enzymes in the cytotoxicity of MMC.

Materials and methods

Reagents. PFM was kindly supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.) and by Dr. J. P. McGovren, Upjohn Co. (Kalamazoo, Mich.). [^{14}C]-PFM was synthesized from $^{14}CH_3I$ and MMC according to Steven et al. [35], with modifications [25]. The MMC and PFM metabolites *trans-* and *cis-*1-hydroxyl-2,7-diaminomitosene (t- and c-AHME), and 2,7-diaminomitosene (AME) were generated by enzymatic reduction of MMC and PFM and were purified by high-performance liquid chromatography (HPLC) [25]. SF-1250 silicone fluid was generously donated by General Electric Co., Silicone Products Division (Waterford, N.Y.). Other biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cell lines. HCT 116 human colon-carcinoma cells were obtained from the American Type Culture Collection (CCL247; Rockville, Md.). Cells were maintained and subcultured as described by Long et al. [19]. MMC-resistant sublines were developed by 1 h exposure of monolayer HCT 116 cells to progressively increasing concentrations of MMC ranging from 4 to 32 μM under aerobic conditions. Following drug exposure, cells were washed with phosphate-buffered saline (PBS) and subcultured in fresh, drug-free medium until the growth of surviving cells had reached confluence, after which the drug-treatment cycle was resumed. Sublines were subcultured and kept frozen in liquid nitrogen. HCT 116-R30A, the subline used in the present study, was cloned from one colony that survived exposure to 32 μM MMC.

Cell volume and cell water. Cell volume was determined using a Coulter Channelyzer (Coulter Electronics, Hialeah, Fla.) calibrated with 10.0- and 14.48-μm-diameter latex microspheres (volume, 524 and 1,590 μm^3, respectively). Intracellular water and extracellular space were measured as described by Hissin and Hilf [11].

Cell survival. The cytotoxicity of MMC and PFM to HCT 116 and HCT 116-R30A cells was assessed using the clonogenic assay described by Rockwell and Kallman [28], with modifications. Cells were plated in triplicate in 1.5 ml drug-free medium at three cell concentrations ranging between 300 and 900 cells/35-mm dish for HCT 116 cells and between 600 and 1,800 cells/35-mm dish for HCT 116-R30A cells. The cultures were incubated overnight at 37° C in an atmosphere containing 5% CO_2 and at 95% relative humidity to allow cells to adhere. Then, dicoumarol (DIC, 100 μM) was added to the cultures. Following 15 min preincubation, MMC (or PFM) was added to the cultures to yield the desired drug concentrations. Control cultures were treated appropriately. Incubation was resumed under the same conditions for 60 min. Next, the medium in each plate was removed, and the plates were rinsed twice with PBS. Fresh medium was added to the plates for further incubation. After 10 days, the plates were rinsed with cold saline, and the colonies were fixed and stained with 0.25% crystal violet in 90% methanol and 10% formalin for 20 min. The stain was then removed and the plates were rinsed with deionized water. Colonies measuring >60 μm in diameter (>50 cells) were counted. The drug concentration producing 50% inhibition of cell growth (IC_{50}) was calculated for each cell line by median-effect analysis using a program developed by Chou and Chou [4].

Initial drug uptake. The uptake of [^{14}C]-PFM by HCT 116 and HCT 116-R30A cells was measured according to the method previously described by Pan et al. [27].

Distribution of radioactivity in cells. Confluent cells were trypsinized, recovered by centrifugation, washed with PBS, and resuspended in fresh medium at 4×10^6 cells/ml. Next, 5-ml aliquots of cells were distributed in 25-cm^2 culture flasks and preincubated for 15 min. Radioactive drug or the vehicle was then added to each flask, and incubation was resumed for the designated period time. During incubation, the flasks were periodically agitated to prevent adherence of cells to the flask walls. At the end of incubation, cells were immediately chilled to 4° C and recovered by centrifugation through silicone fluid SF-1250 for analysis [27]. For studies on the effect of DIC on PFM metabolism, either DIC at 100 μM or vehicle was included during the 15-min preincubation interval and the drug-treatment period. Cells recovered following drug treatment were fractionated and analyzed for the distribution of radioactivity in various cellular components. The procedures used have been described elsewhere [27].

HPLC analysis. Soluble cellular radioactive material and metabolites of MMC produced by enzymatic reduction were analyzed using a reversed-phase HPLC method developed in this laboratory [25–27]. For the analysis of radioactivity, pure PFM as well as c- and t-AHME and AME were used as internal standards, and [^{14}C]-PFM was used as an external standard. Fractions of 0.5 ml were collected, and the radioactivity in each fraction was measured by liquid scintillation counting. Peaks of radioactivity were integrated and converted into picomoles per microliter of cells using the specific activity of [^{14}C]-PFM and the intracellular water content of each cell line. For the measurement of the rate of reduction of MMC by enzymes, the same HPLC system was used. Metabolites were detected at 312 nm. The identity of each metabolite was confirmed by thermospray mass spectrometry [24]. Peak areas were used to calculate the quantities of metabolites by comparison with external standards of authentic metabolites (molar absorptivities for t-AHME and AME are 11,400 and 10,800, respectively).

Enzyme preparations. Cell homogenates were prepared from confluent HCT 116 and HCT 116-R30A cells. Cells were harvested, washed with cold PBS, and lyzed by two methods. To obtain soluble enzymes, cells were resuspended at 2×10^7 cells/ml in cold 50 mM potassium phosphate (various pHs) and lyzed by three cycles of freezing and thawing. To

isolate microsomes, cells were suspended in 0.25 M sucrose, 0.5 mM TRIS (pH 7.4), and 0.2 mM $MgSO_4$ and then disrupted by intracytoplasmic cavitation with nitrogen gas [37]. Complete lysis was verified by microscopic observation. The crude extracts of both preparations were centrifuged at 10,000 *g* for 20 min to remove large debris, mitochondria, and nuclei. The supernatant was further centrifuged at 144,000 *g* for 1 h to separate microsomes and soluble enzymes. Microsomal pellets were dissolved in 50 mM potassium phosphate buffer (at various pHs) in volumes equivalent to 2×10^7 cells/ml and centrifuged again. Washed microsomes were redissolved in the same manner and used for assays. Possible contamination of mitochondrial enzymes in the soluble or microsomal preparation was checked by measuring glutamate dehydrogenase levels (see below). The activity of xanthine oxidase in all preparations was checked. Rat-liver microsomes were prepared according to the method of Yasukochi and Masters [42].

Enzyme assays. Reduction of MMC by cellular fractions was performed aerobically and anaerobically by a previously published method, with some modifications [26]. Reaction mixtures contained 0.1 mM MMC, 0.5 mM NADH (or NADPH), and 0.25 ml cell fraction in a total volume of 0.5 ml. Anaerobic conditions were obtained by closing the test tubes with rubber stoppers and flushing them with nitrogen for 2 min. All reactions were initiated by the addition (or injection) of NADH (or NADPH), were performed at 37° C and were stopped by the addition of 0.25 ml methanol. The reaction time was 15 min, which lay within the linear portion of the reaction as determined in preliminary experiments conducted for both microsomes and soluble extracts under each condition in all cases. The reaction mixtures were centrifuged at 10,000 *g* for 10 min, and the resulting supernatant was analyzed by HPLC for the production of metabolites.

Quinone reductase was measured by two methods previously described by Ernster [9]. The reduction of DCPIP and menadione-cytochrome c was followed at 600 and 550 nm, respectively, using a Beckman DU-70 spectrophotometer (Beckman Instrument Co., Fullerton, Calif.). The 1.0-ml reaction mixture for DCPIP reduction contained potassium phosphate buffers (50 mM, various pHs), 40 μM DCPIP, 0.07% bovine serum albumin, 0.3 mM NADH (or NADPH), and enzyme preparation equivalent to 2×10^5 HCT 116 cells (or 2×10^6 HCT 116-R30A cells). DCPIP was replaced with 10 μM menadione and 40 μM cytochrome c for the menadione-cytochrome c assay. For kinetic analyses, DCPIP at 5–200 μM, menadione at 0.1–80 μM, and cytochrome c at 30–80 μM were used. The concentration of DIC used to study the inhibition of the reduction of DCPIP and menadione was 10 μM and the MMC concentration used was 100 μM.

Glutamate dehydrogenase was assayed according to Lowry [20]. Xanthine oxidase was assayed by the oxidation of xanthine as detected at 295 nm. Protein content was determined with a Pierce protein-assay kit using Coomassie brilliant blue G-250 (Pierce Chemical Co., Rockford, Ill.).

Quinone reductase cDNA-probe preparation. Quinone reductase cDNA was cloned from a λ-gt11 rat-liver cDNA library [10]. A 1.2-kb EcoRI fragment of the cDNA was isolated and labeled with [^{32}P]-deoxycytidine triphosphate to a specific activity of $0.8-1.0 \times 10^9$ cpm/ μg by random priming with an Amersham kit (Amersham Co., Arlington Heights, Ill.) according to the supplier's instructions. Prior to their use, labeled probes were purified by chromatography through Sephadex G-50.

RNA isolation, gel electrophoresis, and nucleic acid transfer. Total cellular RNA from confluent cells was isolated with RNAzol B (Cinna Biotech, Friendswood, Tex.) according to the supplier's instructions. Denaturing glyoxal/formaldehyde gels were prepared and run according to the method of Sambrook et al. [31], with 20 μg total RNA being loaded per lane. After electrophoresis, gels were soaked for 5 min in distilled water followed by 1 h in two changes of 10 × SSC. The RNA was then transferred to Genetrans nylon filter paper (Plasco Inc., Woburn, Mass.) by capillary transfer with 10 × SSC. Following RNA transfer, the filters were baked under vacuum for 2 h at 80° C.

Hybridization. Filters were prehybridized for 4 h at 42° C in 5 × Denhardt's solution [31], 2 × SSPE, 1% sodium dodecyl sulfate (SDS), 0.5 mg denatured herring-sperm DNA/ml, 20 μg yeast RNA/ml, and 50% formamide. They were then hybridized for 15 h at 42° C in the above solution containing 10% dextran sulfate plus probes labeled with ^{32}P at $1-2 \times 10^6$ cpm/ml. After hybridization, the filters were washed according to a previously published protocol [1], after which autoradiograms were made on Kodak XAR-5 film (Kodak Co., Rochester, N.Y.) using two Dupont Cronex Lithning Plus intensifying screens (Dupont, Wilmington, Del.) at –70° C. Following autoradiography, the filters were stripped and reprobed with a 2.0-kb human β-actin cDNA fragment labeled with ^{32}P to account for variability in gel loading and transfer efficiency. Autoradiographic signal intensities were compared by densitometric scanning on a Hoefer GS 300 scanning densitometer (Hoefer Scientific, San Francisco, Calif.) using GS 360 software.

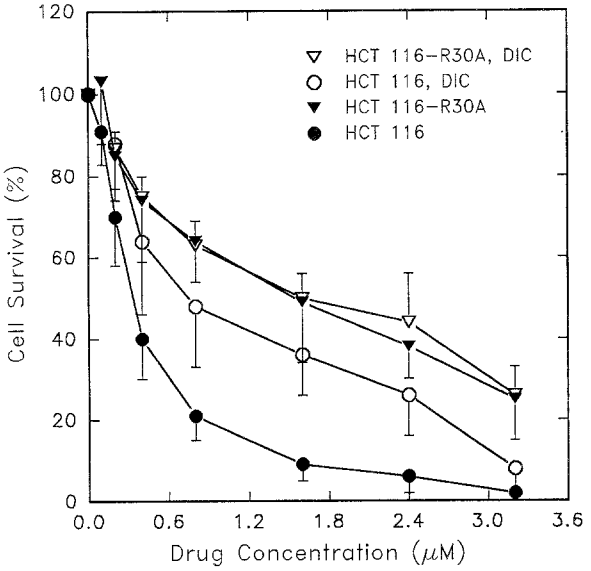

Fig. 1. Survival of HCT 116 and HCT 116-R30A cells following exposure to various concentrations of MMC and modulation of MMC cytotoxicity by DIC. Cell plating and drug exposure were carried out as described in Materials and methods. The cloning efficiency of HCT 116 cells treated with MMC alone (●) and with MMC plus DIC (○) and HCT 116-R30A cells treated with MMC alone (▲) and with MMC plus DIC (△) was determined and expressed as a percentage of control values. *Points* represent mean values ± SE for at least four experiments

Results

Cell survival

Parental (HCT 116) cells and the MMC-resistant subline (HCT 116-R30A) cloned with an efficiency of 25% and 15%, respectively. Their responses to MMC were determined by comparing the percentage of surviving colonies relative to control values as a function of drug concentration (Fig. 1). The IC_{50} values for MMC in HCT 116 and HCT 116-R30A cells under aerobic conditions were calculated as 0.3 and 1.6 μM, respectively, indicating that the resistance of HCT 116-R30A cells to MMC was about 5-fold that of the parent line. Inclusion of DIC (100 μM) during MMC exposure increased the resistance of HCT 116 cells to MMC but had no effect on the HCT 116-R30A subline (Fig. 1). Under anaerobic conditions, the two cell lines were equally sensitive to MMC (IC_{50}, 0.03 μM). The degree of resistance remained unchanged through 20 passages in MMC-free media over a period of 4 months. How-

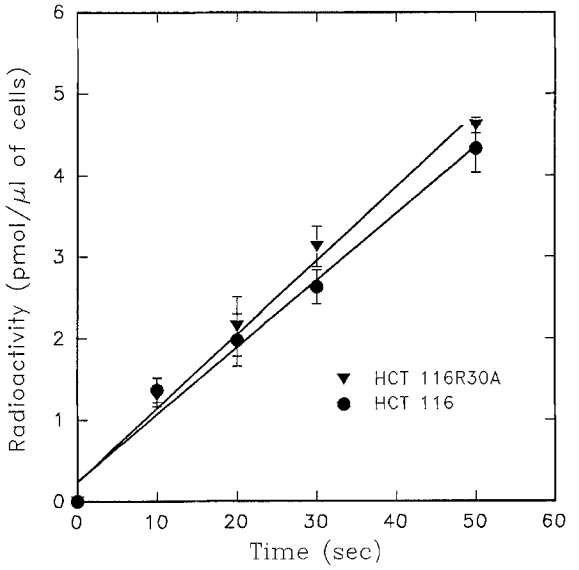

Fig. 2. Initial uptake of PFM by HCT 116 (●) and HCT 116-R30A (▲) cells. Cell suspensions (2×10^6 cells/ml) were exposed to 5 μM [^{14}C]-PFM at 37° C for 10–50 s and analyzed for radioactivity. Details of the experiments have been described elsewhere [27]. Each point derives from three experiments performed in triplicate. The values shown indicate the intracellular (i. e., total) radioactivity associated with cells minus the drug nonspecifically associated with the cell surface. Lines were plotted by linear regression. *Points*, Mean values; *bars*, SE

Table 1. Cellular disposition of radioactivity in HCT 116 and HCT 116-R30A cells

Cells	Exposure period	Radioactivity (pmol/μl cells)		
		PFM	Metabolites	Macromolecule--bound
HCT 116	80 s	2.58±0.50	0.99±0.06	0.41±0.07
	5 min	4.32±0.67	1.76±0.02	0.58±0.03
	15 min	4.58±0.48	2.02±0.35	0.99±0.12
	30 min	4.98±0.74	2.12±0.21	1.59±0.29
	60 min	4.70±0.75	2.85±0.51	2.64±0.26
HCT-116 R30A	80 s	2.38±0.65	0.65±0.15	0.26
	5 min	4.84±0.72	0.77±0.13	0.49±0.15
	15 min	4.91±0.70	0.89±0.07	0.69±0.17
	30 min	4.75±0.64	1.09±0.18	0.99±0.20
	60 min	4.58±0.48	1.08±0.19	1.38±0.10

Cells exposed to 5 μM [^{14}C]-PFM for periods ranging from 80 s to 60 min at 37° C were harvested, lyzed, separated into soluble and insoluble fractions, and analyzed as described in Materials and methods. Aqueous extracts were fractionated with internal standards by HPLC. Radioactive peaks were identified and were grouped as PFM and metabolites. Calculations were based on the specific activity of [^{14}C]-PFM. The insoluble fraction that contained macromolecule-bound radioactivity was calculated accordingly. Data were derived from duplicates of three experiments as corrected for the nonspecifically associated radioactivity measured in cells following 10 s exposure at 4° C

ever, revertants did occur after further passages. HCT 116-R30A cells did not show cross-resistance against melphalan, cisplatin, etoposide, or cytosine arabinoside. The effect of PFM on HCT 116 and HCT 116-R30A cells was similar to that of MMC, indicating that HCT 116-R30A cells were equally resistant to PFM and MMC (data not shown).

Cell water and cell volume

The intracellular water content of HCT 116 and HCT 116-R30A cells was 0.82±0.05 and 0.95±0.06 μl/10^6 cells, respectively, and the cell volume amounted to 0.85±0.033 and 0.98±0.017 μl/10^6 cells, respectively. These values, obtained in five experiments performed in triplicate, were used in calculations of the uptake and distribution of [^{14}C]-PFM-related radioactivity.

Initial uptake of PFM radioactivity

The accumulation of radioactivity by HCT 116-R30A cells exposed to 5 μM [^{14}C]-PFM at 37° C for 10–50 s was similar to that of parental cells (Fig. 2). The linear accumulation rate in the initial 50 s was similar for both cell lines. Statistical analysis (Student's *t*-test) of two sets of the linear regression data showed no significant difference ($t = -0.992$, $df = 6$).

Disposition of PFM

Studies on the distribution of radioactivity following the exposure of MMC-sensitive and -resistant cells to 5 μM PFM for 80 s and for 5, 15, 30, and 60 min indicated that the intracellular pools of free [^{14}C]-PFM were similar in the resistant subline and the sensitive parental cells (Table 1). Both cell types maintained a PFM pool that was in equilibrium with the media after 5 min. However, decreased amounts of macromolecule-bound ^{14}C were found in the resistant cells as compared with the sensitive parental cells. This difference between the two cell lines in macromolecule-bound ^{14}C increased progressively with prolonged incubation time.

The accumulation of macromolecule-bound ^{14}C during drug exposure decreased in both cell lines in the presence of DIC (Table 2). HCT 116 cells responded to DIC more strongly than did HCT 116-R30A cells. Analysis of cellular soluble radioactivity showed that [^{14}C]-PFM reached the same intracellular concentration at the same rate in both cell types, indicating that PFM uptake was not affected by DIC.

Reductive activity of the soluble extracts. Assays of the soluble preparations for xanthine oxidase and contamination by mitochondrial enzymes produced negative results.

Reduction of DCPIP. The reduction of DCPIP by soluble extracts of sensitive cells was affected slightly by pH (Table 3). The soluble fraction of the resistant cells showed <10% of the reductive activity of the soluble extract of sensitive cells. The kinetics of DCPIP reduction observed for soluble extracts of the two cell types differed greatly. Substrate saturation of the enzyme was not obtained for soluble extracts of resistant cells at DCPIP concentrations of up to 200 μM (Fig. 3B). However, soluble extracts of sensitive cells followed typical second-order kinetics (Fig. 3A) and yielded a K_m value of 31.5±4.5 μM for DCPIP as analyzed by Lineweaver-Burk plot (Fig. 3A, inset). Parallel experiments using either NADH or

Table 2. Effect of DIC on the cellular distribution of radioactivity in HCT 116 and HCT 116-R30A cells

Cells	Exposure period (min)	Radioactivity (pmol/μl cells)			
		Without DIC		With DIC	
		Total soluble[a]	Macromolecule-bound	Total soluble[a]	Macromolecule-bound
HCT 116	15	6.80±0.55	0.87±0.07	6.25±0.90	0.46±0.0
	30	7.36±0.90	1.35±0.25	7.27±0.22	0.57±0.0
	60	7.12±0.30	2.05±0.45	7.05±1.02	0.75±0.1
HCT 116-R30A	15	6.36±0.17	0.72±0.04	6.29±0.46	0.33±0.09
	30	5.80±0.57	0.94±0.10	6.13±0.58	0.35±0.06
	60	6.90±0.65	1.23±0.20	6.81±0.58	0.67±0.06

Cells were treated as described in Materials and methods
[a] Includes PFM and metabolites

Table 3. Effect of DIC on the reductive activity of soluble extracts of HCT 116 and HCT 116-R30A cells

Substrate	pH	HCT 116		Inhibition (%)	HCT 116-R30A		Inhibition (%)
		–DIC	+DIC		–DIC	+DIC	
DCPIP[a]	6.5	730 ± 25	105 ±33	86	45 ±5	30 ±7	33
	7.3	640 ±123	48 ± 9	93	36 ±5	27 ±5	25
	8.2	530 ± 74	23 ± 9	96	29 ±3	19 ±5	34
Menadione-cyto c[a]	6.5	37 ± 7	30 ±10	20	11 ±2	5 ±5	55
	7.3	566 ± 60	30 ± 7	95	39 ±6	5 ±5	87
	8.2	1,052 ± 81	18 ± 5	98	55 ±9	9 ±4	84
MMC (aerobic)[b]	6.1	17.5± 1.8	9.3± 1.0	47	7.8±1.9	7.3±1.5	NS
	6.5	8.3± 1.0	5.6± 0.4	33	3.9±0.9	5.2±0.8	NS
	6.9	3.8± 1.6	2.8± 0.9	27	2.2±0.4	2.7±0.7	NS
	7.3	1.6± 0.3	1.1± 0.4	31	1.2±0.3	1.1±0.3	NS
	7.8	T	T		T	T	
	8.2	T	T		T	T	

Cells were treated as described in Materials and methods. T, Trace of activity too low for calculation; NS, not significant
[a] Reduction of DCPIP and menadione as expressed in nmol min^{-1} 2×10^{-7} cells. The data represent mean values ± SE for at least three experiments involving triplicate assays
[b] MMC reduction rates were obtained during the linear portion of the reaction (15 min). The data are expressed in nmol h^{-1} 2×10^{-7} cells and represent mean values ± SE for three experiments

NADPH as electron donors were conducted. Similar results were obtained (data for NADPH not shown).

Reduction of menadione. The reduction of menadione by soluble extracts of sensitive cells (HCT 116) was highly pH-dependent (Table 3). The activity was 25 times greater at pH 8.2 than at pH 6.5. In comparison, soluble extracts of resistant cells showed low reductive activity that was about 20 times lower than that of sensitive cells when assayed at high pH. However, the difference between the two cell types was much less marked at lower pH. The K_m values for menadione in the menadione-cytochrome c assay were 5.8±0.72 and 1.5±0.23 μM for the sensitive and resistant cell extracts, respectively. The V_{max} values for this reaction amounted to 90±15 and 2.4±0.4 nmol min^{-1} 10^{-6} cells for the extracts of sensitive and resistant cells, respectively. As in the studies on DCPIP reduction, parallel experiments were conducted using NADH or NADPH as electron donors, and similar results were obtained (data for NADPH not shown).

Reduction of MMC. Under aerobic conditions, MMC reduction by soluble extracts of both cell types was pH-dependent when NADH served as the electron donor (Table 3). The rate of reduction increased with decreasing pH. The MMC-reductive activity exhibited by the cytoplasm of resistant cells was about half of that exerted by the sensitive cells throughout the pH range tested. The rate of MMC reduction by soluble extracts of both cell types at pH 6.1 depended on the drug concentration used. Substrate saturation of the enzyme was not obtained at MMC concentrations of up to 200 μM, and the K_m value for this reaction could not be determined. Inhibition of the soluble enzyme by MMC at pH 6.1 was not observed. When the reactions were conducted anaerobically or when NADPH was used as the electron donor, similar results were obtained (data not shown). Only one major metabolite (peak c in Fig. 4A), which co-chromatographed with 2,7-diaminomitosene, was separated from this reaction (Fig. 4A). The structural identification of this product was confirmed by thermospray mass spectrometry.

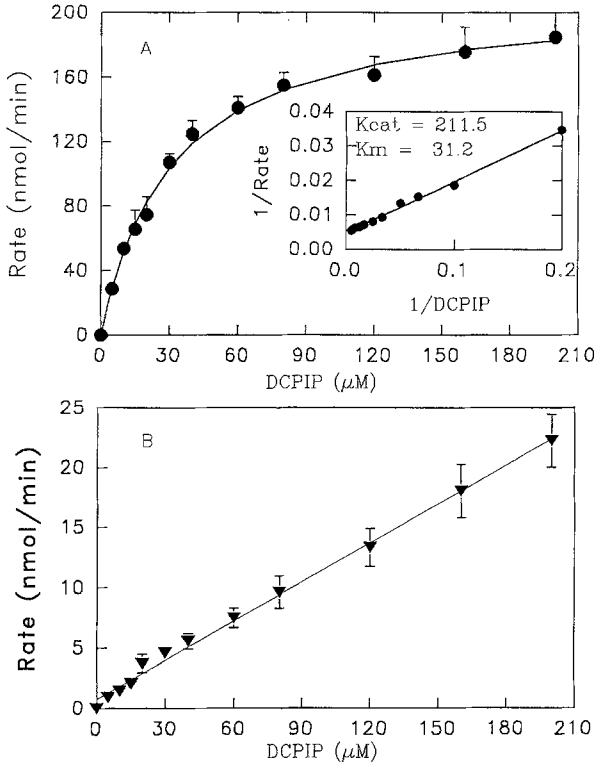

Fig. 3 A, B. Kinetic analysis of the reduction of DCPIP by soluble extracts of **A** HCT 116 and **B** HCT 116-R30A cells. Details of the assay are described in Materials and methods. NADH (0.3 mM) served as the electron donor. *Inset*: linear Lineweaver-Burk plot of 1/v versus 1/s

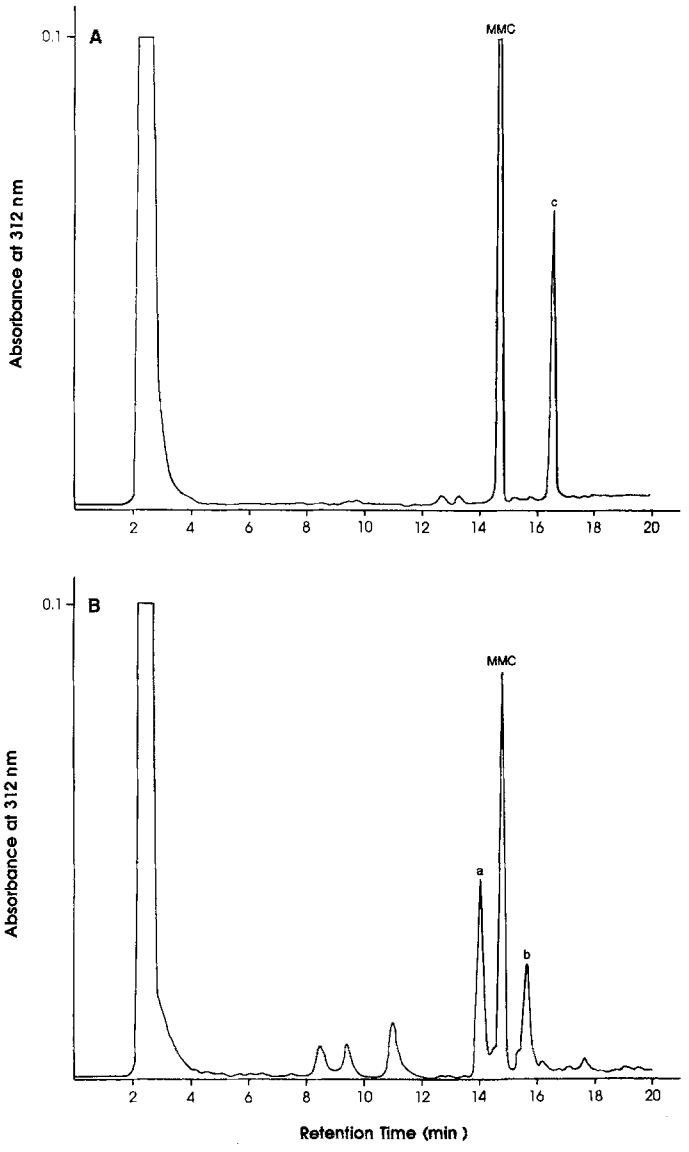

Fig. 4. HPLC separation of MMC and metabolic products in reaction mixtures containing cellular fractions. Experimental details on the preparation of cell extracts, the reduction of MMC, and the HPLC assay are described in Materials and methods. **A** Profile of the reaction products produced by the soluble extract of HCT 116 cells at pH 6.2. **B** Profile of the reaction products produced by the microsomal fraction of HCT 116 cells at pH 7.8. Absorbance at 312 nm was used to detect MMC and metabolites, and the full scale was set at 0.1 absorbance units

Effects of DIC on activity of soluble extracts

The concentration of DIC required to achieve maximal inhibition of the cytoplasmic reductive activity for DCPIP, menadione, and MMC was determined. Total inhibition of DCPIP and menadione reduction occurred at 1.0 μM DIC. The inhibition of MMC reduction was linear at DIC concentrations of up to 100 μM. Total inhibition of MMC reduction could not be achieved. Therefore, 100 μM DIC was used in all MMC studies and 10 μM DIC was used in the other investigations.

DIC effectively inhibited the reduction of DCPIP by sensitive cells at pH 7.3–8.2 (>93%) and was somewhat less effective at pH 6.5 (86%, Table 3). It inhibited only one-third of the DCPIP-reductive activity in soluble extracts of resistant cells; however, the reliability of this estimate was limited by the small amount of activity found in the absence of the inhibitor. The inhibition of menadione reduction by DIC was more effective at higher pH in the sensitive cell line. DIC inhibition of MMC reduction by the cytoplasm of sensitive cells reached about 50% at pH 6.1 and 30% at higher pH. Cytoplasmic reduction of MMC in resistant cells was not greatly affected by DIC.

Microsomal activities

Reduction of DCPIP and menadione. The DCPIP- or menadione-reducing activity of the microsomal fraction of sensitive cells was <10% of that shown by the soluble extract of that cell type. However, the reducing activity of the microsomal fraction of resistant cells was about equal to that found in the corresponding soluble extract (data not shown). The microsomes from the two cell sources showed limited differences between each other in their ability to reduce both DCPIP and menadione, and the effect of pH on the reduction of both substrates by microsomes was similar to the influence of pH on soluble fractions.

Reduction of MMC. Under anaerobic conditions, MMC reduction by microsomes was also pH-dependent when NADPH served as the electron donor. However, this pH dependency was the opposite of that observed for the soluble extracts, with the reduction rate increasing at higher pH. The generation of major metabolites by the microsomal reaction was also pH-dependent. NADPH was

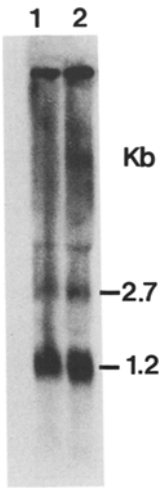

Fig. 5. Northern blot of total cellular RNA from HCT 116 and HCT 116-R30A cells probed with a cDNA fragment of rat-liver quinone reductase. RNA was electrophoresed under denaturing conditions, blotted, and hybridized as described in Materials and methods. In all, 20 μg RNA was loaded per lane. *Lane 1*, HCT 116-R30A cells; *lane 2*, HCT 116 cells. Following autoradiography, the blots were stripped by heating to 85° C in 0.1 × SSC, then reprobed with a 2.0-kb human β-actin cDNA probe. No difference was observed in the signal intensity of the β-actin mRNA bands (data not shown).

the preferred electron donor (Table 4), and NADH was poorly utilized for anaerobic reduction of MMC by the microsomes of either source. Under aerobic conditions, some reduction of MMC by the microsomal fractions of both cell lines was detected at lower pH (Table 4). NADH and NADPH were utilized equally well as electron donors under these conditions (data of NADPH not shown). Some differences were observed in the aerobic reduction of MMC by the two microsomal fractions.

Two major metabolites (Fig. 4B, peaks a and b) that co-chromatographed with t- and c-1-hydroxyl-2,7-diaminomitosene were detected in the higher-pH reaction mixture, whereas one metabolite that co-chromatographed with 2,7-diaminomitosene was detected in the lower-pH reaction mixture (data not shown). The product of microsomal metabolism at low pH exhibited the same HPLC profile as did the reaction mixture produced by the soluble extract at a similar pH (Fig. 4A). The anaerobic reduction of MMC by the microsomes of the two cell sources produced the same types of metabolites at each pH and at similar rates.

mRNA of quinone reductase

Transcripts of the quinone reductase gene in human cells may include up to three different species (1.2, 1.7, and 2.7 kb) due to alternative polyadenylation [14]. Two species of quinone reductase mRNA (2.7 and 1.2 kb) were detected in the MMC-resistant HCT 116-R30A cells as well as in the parent MMC-sensitive HCT 116 cells (Fig. 5). The steady-state levels of both species of mRNA in the two cell lines were similar. Hybridization to the high-molecular-weight species is probably nonspecific, since the probe is of mouse origin.

Discussion

In our attempts to explain the mechanisms underlying the MMC resistance of HCT 116 cells, we examined several critical properties of the parental wild-type cell line and the MMC-resistant subline. In addition, we compared the enzyme systems that are capable of activating MMC in both cell lines.

The MMC-resistant subline HCT 116-R30A and the parent HCT 116 cells have identical uptake kinetics describing free-drug accumulation in the cells (Fig. 2, Table 1). More specifically, both MMC-sensitive and MMC-resistant cells accumulate PFM by passive diffusion. However, the reduced accumulation of macromolecule-bound drug by the HCT 116-R30A cell line suggests that the reductive mechanisms of the MMC-resistant subline are different from those of the parent cells. Further evidence supporting this premise is the differential effect

Table 4. Effect of DIC on the reductive activity of microsomal fractions of HCT 116 and HCT 116-R30A cells

Substrate	pH	HCT 116		Inhibition (%)	HCT 116-R30A		Inhibition (%)
		–DIC	+DIC		–DIC	+DIC	
MMC, NADPH (anaerobic)[a]	6.1	7.7±0.5	7.5±1.8	NS	8.8± 1.8	10.1 ±0.5	NS
	6.5	9.1±3.2	11.0±4.3	NS	11.6± 0.8	10.5 ±2.8	NS
	6.8	10.5±3.9	12.3±4.6	NS	30.4± 5.6	24.9 ±2.7	NS
	7.4	45.8±1.0	51.8±2.4	NS	56.0± 3.0	63.3 ±3.1	NS
	7.8	62.4±4.8	65.7±4.9	NS	62.7± 7.7	77.3 ±4.0	NS
	8.2	76.3±4.6	84.6±1.8	NS	71.7±11.4	81.2 ±5.0	NS
MMC, NADH (aerobic)[a]	6.1	4.4±0.8	3.2±1.0	NS	1.8± 0.2	1.26±0.1	NS
	6.5	2.4±0.8	2.0±0.7	NS	1.1± 0.1	1.26±0.5	NS
	6.8	1.4±0.8	1.2±0.5	NS	0.5± 0.2	0.5 ±0.2	NS
	7.4	0.9±0.4	T	NS	T	T	NS
	7.8	T	T	NS	T	T	NS
	8.2	T	T	NS	T	T	NS

Cells were treated as described in Materials and methods. T, Trace of activity too low for calculation; NS, not significant

[a] MMC reduction rates were obtained during the linear portion of the reaction (15 min). The data are expressed in nmol h^{-1} 2×10^{-7} cells and represent mean values ± SE for three experiment

of DIC on the accumulation of macromolecularly bound radioactivity by the two cell lines. Due to a lack of radioactive MMC, these studies and the resultant conclusions were based on investigations using radioactive PFM. However, we have established that MMC and PFM share the same uptake mechanism and metabolic pathways [25, 27].

MMC and PFM were reduced by isolated microsomal fractions of the parent and resistant cell sources, with no apparent differences being observed under anaerobic conditions. The anaerobic reduction of MMC was most likely catatyzed by NADPH cytochrome P-450 reductase [26, 36]. Therefore, it is reasonable to assume that NADPH cytochrome P-450 reductase was not altered in the MMC-resistant subline and was thus not responsible for the difference observed in the accumulation of macromolecule-bound radioactivity by the sensitive and the resistant cells. The aerobic reduction of MMC by microsomes was probably attributable to microsomal quinone reductase because NADH and NADPH were utilized equally well as electron donors. Since our microsomes were washed, the possibility of contamination by soluble quinone reductase is quite unlikely. The difference in the aerobic activity exerted by the two cell sources was not significant. Therefore, the difference in the accumulation of bound radioactivity by the two sublines was not likely to have resulted from microsomal enzyme reduction and must have been due to other reductive enzymes present in other cellular components.

We found that the soluble cellular extract of HCT 116 cells was capable of reducing at pH 6.1–6.8. This reduction produced one major metabolite that was identified as 2,7-diaminomitosene. Siegel et al. [33, 34] have obtained similar results using quinone reductase from HT-29 cells and rat liver. Quinone reductase is the only reductive enzyme that is capable of utilizing NADH and NADPH with equal efficiency. Because of these facts quinone reductase is the probable soluble enzyme in HCT 116 cells responsible for reducing MMC. Furthermore, contamination by mitochondrial reductive enzymes was negligible, and these enzymes required a higher pH for optimal reactions. Other soluble enzymes that reportedly have the ability to reduce quinones include carbonyl reductase and xanthine oxidase, but the former utilizes MMC poorly as a substrate [38] and the latter occurred in negligible amounts in both types of cells investigated in the present study.

Our initial evidence for the involvement of soluble quinone reductase in MMC resistance was the ability of DIC, a potent inhibitor of quinone reductase, to make HCT 116 cells more resistant to MMC. Subsequent studies on soluble extracts containing quinone reductase revealed the dramatic difference between the parent cell line and the MMC-resistant subline in their ability to reduce DCPIP and menadione. Our initial impression was that the amount of quinone reductase in the MMC-resistant subline was reduced. However, our failure to find a difference in the amount of quinone reductase mRNA in the two cell lines suggested otherwise. As additional evidence, the kinetics of quinone reductase in the two cell lines for the reduction of DCPIP and menadione were very different. One possible explanation for the observed difference in quinone reductase activity in the MMC-resistant subline would involve a change in the catalytic activity of the enzyme. This premise is supported by a study conducted by Forrest et al. [10], who used site-directed mutagenesis of rat quinone reductase cDNA in the region of NAD(P)H binding sites and found that single base substitution can dramatically alter the activity of the enzyme. Another explanation such as posttranslational modification is also possible. Dulhanty and Whitmore [8] have recently reported evidence for molecular defects in the DT-diaphorase (quinone reductase) gene or gene transcript for an MMC-resistant Chinese hamster ovary cell line.

It is possible that another as yet unidentified enzyme may have been involved in the observed reduction of MMC. This hypothesis is supported by the inability of DIC to inhibit completely the reduction of MMC by the soluble extracts. If this were indeed the case, the observed decrease in quinone reductase in the HCT 116 MMC-resistant subline would have been only coincidental. Purification of MMC-reductive enzyme(s) from these cells is currently in progress, and their kinetics will be further investigated. In addition, other factors besides an alteration of the ability to reduce MMC may have contributed to the MMC resistance of our cell line. Studies of factors such as increased levels of intracellular protective agents and increased efficiency of DNA-repair systems are under way.

In that enzymes are essential for the reduction of MMC to alkylating cytotoxic intermediates, several factors such as oxygen and pH play modulating roles in the cytotoxic activity of MMC. In the presence of oxygen, active intermediates of MMC produced by NADPH cytochrome P-450 reductase undergo redox cycling, thereby decreasing the alkylating activity of MMC but increasing the levels of active oxygen products such as superoxide and hydroxyl radical. The generation of active oxygen products by MMC has been demonstrated in vitro by a number of laboratories [6, 15]. In the absence of oxygen, active intermediates of MMC produced by NADPH cytochrome P-450 reductase result in the alkylation of DNA and other molecules. The alkylating ability of hydroquinone, the intermediate of MMC produced by quinone reductase, is not affected by oxygen. The pH dependency of these two enzymes for the reduction of MMC corresponds to opposite ends of the intracellular pH range. NADPH cytochrome P-450 reductase is more active at higher pH, and quinone reductase is more active at lower pH. In the case of HCT 116 cells, microsomes containing NADPH cytochrome P-450 reductase at its optimal pH reduced MMC at least 4 times more efficiently than did soluble extracts containing quinone reductase. On the other hand, quinone reductase is more active than NADPH cytochrome P-450 reductase in generating MMC alkylation under aerobic conditions at lower pH. Therefore, the intracellular pH and oxygen tension of tumor cells determine the ability of these two enzymes to produce alkylating intermediates of MMC. Furthermore, other reductive enzymes such as NADH cytochrome b reductase and xanthine oxidase also reduce MMC [13, 17, 26].

Quinone reductase has been suggested as a target enzyme for the modulation of bioreductive drug activity [2, 22, 41]. In view of the complexity of reductive enzymes in various cell systems as indicated by the present study,

clinical studies attempting to modulate of the activity of bioreductive alkylating agents should be designed and carried out with caution.

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