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Hydroquinone Resistance in a Murine Myeloblastic Leukemia Cell Line

INVOLVEMENT OF QUINONE REDUCTASE AND GLUTATHIONE-DEPENDENT DETOXIFICATION IN NONCLASSICAL MULTIDRUG RESISTANCE

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ABSTRACT. A hydroquinone-resistant derivative of the M1 cell line, designated M1HQ, was generated and used to evaluate the biochemical mechanism responsible for resistance to oxidative stress-inducing agents. The hydroquinone concentrations that were cytotoxic to 50 and 90% of the parental M1 cell line in 48 hr were 25 and 90 μM, respectively, whereas exposure to 500 μM hydroquinone did not decrease M1HQ viability significantly. M1HQ cells grew slower than M1 cells and exhibited significantly higher resistance to colchicine, doxorubicin, hydrogen peroxide, 4-hydroperoxycyclophosphamide, and 1,3-bis(2-chloroethyl)-1-nitrosourea but not to benzoquinone, vinblastine, or γ-radiation. M1HQ cells possessed significantly higher levels of total thiols, glutathione, glutathione peroxidase, glutathione reductase, quinone reductase, and γ-glutamyl transpeptidase than the parental M1 cell line. Steady-state γ-glutamylcysteine synthetase mRNA expression also was 1.6-fold higher in M1HQ cells. P-glycoprotein transcripts were detectable in both M1 and M1HQ cells, but were 2-fold higher in M1HQ. Multidrug resistance-associated protein transcripts were not detectable in either M1 or M1HQ. Hydroquinone resistance in M1HQ cells was partially reversible with a combination of inhibitors of quinone reductase, γ-glutamylcysteine synthetase, glutathione peroxidase, and the multidrug resistance-associated protein, but not with inhibitors of P-glycoprotein, γ-glutamyl transpeptidase, or glutathione-*S*-transferase. When treated with [^{14}C]hydroquinone, M1HQ cells did not generate significant hydroquinone–protein adducts but did release an adduct similar to *N*-acetylcysteinyl-benzoquinone. In contrast, numerous [^{14}C]hydroquinone–protein adducts were produced in M1 cells, while the *N*-acetylcysteinyl-benzoquinone-like molecule was undetectable. Thus, hydroquinone resistance in M1HQ cells appeared to result from a glutathione-dependent detoxification and export mechanism. BIOCHEM PHARMACOL 52;6:945–956, 1996.

KEY WORDS. hematopoietic progenitor cells; hydroquinone; multidrug resistance; glutathione; quinone reductase; glutathione peroxidase

HPC† proliferation and differentiation depends upon signals transduced following interactions of cell surface receptors on HPCs with soluble, stromal cell-associated, and extracellular matrix-bound regulatory molecules [1]. Many cytokine receptors [2], protein kinases [3], and transcription factors [4] possess conserved cysteine residues essential to their function. Furthermore, several enzymes essential for cellular proliferation, such as ribonucleotide reductase, thioredoxin reductase, and DNA polymerases α and δ, have been shown to be sensitive to thiol-reactive agents [5, 6]. Thus, it is clear that many oxidation-sensitive cellular components exist in HPCs that are critical to viability and function.

The sensitivity of HPCs to oxidative stress is further supported by the fact that *in vitro* cytokine-induced colony formation is nearly 2-fold greater at physiological (2–5% O_2) than at near-ambient (19–20% O_2) pO_2 [7, 8]. In addition, many known hematotoxic compounds, such as the antitumor agents doxorubicin, cyclophosphamide, or BCNU and the benzene metabolites HQ and BQ are believed to induce hematotoxicity through the direct alkylation of nucleic acids and protein and nonprotein thiols or the generation of ROI [9]. Furthermore, ROI are produced endogenously by activated macrophages and neutrophils during inflammatory reactions or as a result of exposure to γ-radiation. Nevertheless, neither the mechanism respon-

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† *Abbreviations:* BQ, benzoquinone; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BSO, L-buthionine-(*S,R*)-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DDP, *cis*-dichlorodiammine platinum[II]; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); FBS, fetal bovine serum; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GLCLC*, γ-glutamylcysteine synthetase; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, GSH peroxidase; GST, GSH-*S*-transferases; 4HC, 4-hydroperoxycyclophosphamide; HPC, hematopoietic progenitor cell; HQ, hydroquinone; IMDM, Iscove's Modified Dulbecco's Medium; MDR, multidrug resistant; MRP, multidrug resistance-associated protein; NAC, *N*-acetylcysteine; pO_2, O_2 partial pressure; P-gp, P-glycoprotein; PI, propidium iodide; ROI, reactive oxygen intermediates; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; and TCA, trichloroacetic acid.

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sible for oxidative stress-induced hematotoxicity nor the HPC oxidation-sensitive cellular components has been defined clearly.

In addition to the need to clarify the mechanism of oxidative stress-induced hematopoietic injury, it is also important to define the cellular response capabilities following exposure to oxidative stress-inducing agents. This is especially true in the context of the development of an MDR phenotype in tumor cells. In many cases, MDR is the result, at least in part, of the overexpression of P-gp. P-gp is a member of the ATP-binding cassette superfamily of integral membrane glycoproteins and acts by decreasing intracellular drug concentrations through ATP-dependent, verapamil-sensitive efflux of unmodified drug from the cell [10]. The role of P-gp in the development of MDR is of particular importance since several normal cell types, including HPCs, have been shown to express substantial levels of P-gp [11]. Cross-resistance to multiple agents also correlates with the expression of the ATP-dependent, vanadate-sensitive MRP. MRP also belongs to the ATP-binding cassette superfamily and is capable of effluxing multiple anionic GSH conjugates from the cell [12]. An MDR phenotype is also associated with substantial increases in intracellular GSH concentrations and enhanced GSH-dependent detoxification capabilities [13].

Therefore, in order to study both the effects of oxidative stress on HPCs and the cellular response following long-term exposure to an oxidative stress-inducing compound, the M1 murine myeloid progenitor cell line was used to generate a cell line resistant to the oxidative stress-inducing agent HQ. HQ is one of the primary hepatic benzene metabolites that can be oxidized to the more reactive semiquinone and BQ species [14]. HQ was chosen as the model oxidative stress-inducing hematotoxic agent for two reasons. First, HQ is likely to be involved in the poorly understood hematotoxic and leukemogenic properties of benzene. Second, since no other HQ-resistant HPC lines have been described, it is possible that valuable information would be obtained regarding both the mechanism of HQ-induced hematotoxicity and the biochemical basis underlying the development of primary HQ resistance and subsequent cross-resistance to additional cytotoxic agents in HPCs. The results from this study show that this HQ-resistant cell line, designated M1HQ, does indeed exhibit cross-resistance to a number of oxidative stress-inducing agents. Furthermore, the experimental evidence suggests that the M1HQ-resistance mechanism is independent of P-gp and appears to be due to elevated GSH levels coupled with an increased ability to detoxify HQ and export it from the cell.

MATERIALS AND METHODS

Reagents

HQ and BQ were from the Fluka Chemical Co. (Ronkonkoma, NJ); doxorubicin was from the Cetus Corp. (Emeryville, CA); vinblastine was from Eli Lilly & Co. (Indianapolis, IN); BCNU was from the Bristol-Myers Squibb Co. (Evansville, IN); 4HC was from Scios Nova Inc. (Baltimore, MD); CDNB, colchicine, DDP, guanidine HCl, DTNB, GSH, H_2O_2, NAC, NADPH, PI, glutathione reductase, dicumarol, acivicin, ethacrynic acid, and aurothioglucose were from the Sigma Chemical Co. (St. Louis, MO); verapamil was from Abbott Laboratories (North Chicago, IL); BSO was from Schweizerhall (South Plainfield, NJ); formalin and sodium-*o*-vanadate was from the Fisher Scientific Corp. (Pittsburgh, PA); and [U-^{14}C]HQ (sp. act. 22 Ci/mmol) was obtained from Wizard Laboratories Inc. (Sacramento, CA).

Cell Culture

The M1 murine myeloblastic leukemia cell line [15] was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The M1HQ subline was derived from the M1 cell line over a period of 9 months by weekly passage in increasing HQ concentrations beginning with 25 μM. Cultures of each cell line were initiated at 2×10^5 cells/mL IMDM (BioWhittaker, Walkersville, MD) supplemented with 4 mM L-Gln, 20 μg/mL gentamicin sulfate (complete IMDM) and 20% FBS (Hyclone, Logan, UT). M1 and M1HQ cell culture was performed at 37° in a humidified atmosphere of 5% O_2, 7% CO_2 and 88% N_2. M1 cells were passed twice weekly and M1HQ cells were passed weekly. A stock 100X HQ solution was prepared weekly in sterile 0.9% NaCl and stored at 4°. Once HQ resistance was established, HQ was added to M1HQ cells every 3–4 days at a final concentration of 100 μM. The multidrug-sensitive cell line J774.2 and its P-gp expressing MDR derivatives, J7-V2-1 and J7-V3-1, were provided by Dr. Susan B. Horwitz and maintained as described [16]. All experiments were initiated using log phase cells that were ≥85% viable.

Cell Growth and Cell Cycle Analysis

M1 and M1HQ growth curves were generated by counting 0.3-mL aliquots of parallel 15-mL cultures in T-75 tissue culture flasks at the same time daily for 7 days using a Coulter ZM electronic cell counter. M1HQ cell growth experiments were performed in the presence or absence of 100 μM HQ. Cell cycle determinations were performed as described [17]. Briefly, cells were pelleted at 4°, washed three times with ice-cold PBS and resuspended in 0.5 mL PBS. The cells were fixed for 1 hr at 4° by adding an equal volume of 2% formaldehyde made from 10% formalin. The cells were pelleted, 5 mL of 0.5% Triton X-100 was added, and the cells were immediately pelletted again and resuspended at 1×10^6/mL in 50 μg/mL PI in PBS. RNase A was added to 10 μg/mL, and the cells were incubated at 37° for 45 min. Samples were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cytotoxicity, Growth Inhibition, and Resistance Reversal Assays

M1 and M1HQ cells, at 2×10^5 cells/mL, were exposed in duplicate 0.5-mL aliquots to 0–500 μM HQ, 0–100 μM BQ, 0–200 nM vinblastine, 0–400 nM colchicine, 0–500 μM BCNU, 0–360 μM 4HC or 0–400 μM H_2O_2 for 48 hr or to 0–25.6 μM DDP for 72 hr at 37° in complete IMDM/20% FBS. Viability was assessed by flow cytometry after staining the cells for 3 min with 10 μg/mL PI at 22°. Cytotoxicity was calculated as the percentage of the untreated controls. Inhibition of M1 and M1HQ growth by doxorubicin or γ-radiation was determined in duplicate using a clonogenic assay. M1 and M1HQ cells were diluted to 70 or 200 cells/mL and divided into aliquots at 2.5 mL/tube. Doxorubicin was added at 0–100 nM or the cells were exposed to 0–800 cGy γ-radiation using a ^{137}Cs source at 319 cGy/min. The cells were plated at 25 μL/well in Terasaki plates (Robbins Scientific Inc., Sunnyvale, CA) and incubated at 37° for 7 days. Wells with greater than the input cell number were considered positive, and the number of positive wells in each plate was calculated as the percent of the medium-treated controls. The mean concentrations that were cytotoxic (TC) to 50 and 90% of the cells in the cytotoxicity assay or inhibited cell growth (IC) in 50 or 90% of wells in the clonogenic assay were calculated for each agent from the concentration–response curves generated from 3–4 separate experiments. Experiments to reverse the HQ resistance in M1HQ cells were performed as follows. M1HQ cells were preincubated at 37° for 1 hr in complete IMDM without serum at 2.5×10^5 cells/mL with 40 μM dicumarol, 20 μM BSO, 100 μM aurothioglucose, 100 μM vanadate, 100 μM acivicin, 20 μM verapamil or 20 μM ethacrynic acid alone or in combination. These inhibitor concentrations were selected for maximal inhibition without significant cytotoxicity. FBS was then added to 20%, additional inhibitors were added to maintain their concentrations, the cells were divided into aliquots and 0–400 μM HQ was added in duplicate. The cultures were incubated for 24 hr at 37°, and viability was determined with PI by flow cytometry.

Total Thiol and GSH Determinations

Total thiols were determined using a modification of the procedure described by Ellman [18] with modifications [19]. Briefly, cells were washed in ice-cold Hanks' buffered salt solution and resuspended at 5×10^6 cells/mL (M1HQ) or 1×10^7 cells/mL (M1) in 10 mM Tris–HCl, pH 7.4, 0.9% NaCl. To 0.5 mL of cells, 1 mL of 6 M guanidine–HCl, 1 mM DTNB, 0.2 M Tris–HCl, pH 8.0, was added and mixed well, and the A_{412} was determined. The total thiol concentration was calculated using the DTNB molar extinction coefficient of 13.6 $mM^{-1}cm^{-1}$. Total GSH (GSH and GSSG) was assayed as described [20, 21] with modifications [22]. Briefly, cells were washed with ice-cold PBS, and 5×10^6 cells were pelleted at 600 × *g* and lysed in 125 μL H_2O. Sulfosalicylic acid was added to a final concentration of 5%, and the samples were mixed well and chilled on ice for 30 min. The precipitate was pelleted at 10,000 × *g* for 2 min at 4°, and the supernatant was frozen at −20°. GSH content in the supernatant was then assayed in a 96-well flat-bottom microtiter plate in triplicate wells containing 0.21 mM NADPH, 0.6 mM DTNB, and 0.1 unit glutathione reductase. The A_{405} was recorded at 2-min intervals for 10 min using a Bio-Tek plate reader (Bio-Tek Instruments, Winooski, VT). The GSH content of each sample was then determined by comparing the rate of A_{405} increase observed with that of a standard curve generated with known amounts of GSH in 5% sulfosalicylic acid.

Enzyme Assays

Cells were pelleted, washed in ice-cold PBS, and resuspended at 3×10^7/mL. Cell lysates were prepared and assayed following the published methods for GST [23], GPx [24], glutathione reductase [25], quinone reductase [26], γ-glutamyl transpeptidase [27], and *N*-acetyl transferase [28]. M1 and M1HQ cell protein content was determined by the bicinchoninic acid method (Pierce). Enzymatic specific activities in M1 and M1HQ cell lysates were calculated per mg protein for comparison. M1 and M1HQ cellular protein content was not significantly different (M1 = 85 ± 10 pg protein/cell; M1HQ = 100 ± 14 pg protein/cell).

Northern Blot Analyses

Total cellular RNAs from M1, M1HQ, J774.2, J7-V2-1, and J7-V3-1 cells were prepared using either the guanidine/phenol reagent (TriReagent; MRC Inc., Cincinnati, OH) [29] or by centrifugation of guanidine isothiocyanate lysates through CsCl [30]. RNA (20 or 30 μg) from each cell line was denatured with glyoxal/DMSO and fractionated in a 1% agarose gel [30], transferred to Hybond N (Amersham, Arlington Heights, IL), and fixed to the membrane by UV cross-linking in a Stratalinker (Stratagene, La Jolla, CA). Following transfer, gels were stained with ethidium bromide to verify that RNA transfer was uniform. The membranes were rinsed in 20 mM Tris–HCl, pH 8.0, for 15 min at 65° and then were prehybridized in 5× SSC, 5× Denhardt's solution, 0.1% SDS, 0.1% $NaPP_i$, and 100 μg/mL denatured salmon sperm DNA for ≥2 hr at 65°. Blotted RNAs were hybridized to [^{32}P]ATP-random prime-labeled probes to *MDR1a* and *b* [31] (ATCC), *GLCLC* [32], and *MRP* [33] (the *GLCLC* and *MRP* probes were provided by Drs. R. T. Mulcahy and S. P. C. Cole, respectively). The blots were washed at 65° in 2× SSC, 0.1% $NaPP_i$, 0.1% SDS and then in 1× SSC, 0.1% $NaPP_i$, 0.1% SDS. Hybridization signals were detected and quantitated using a Fujix BAS2000 phosphorimager. Blots were stripped by washing in 0.2% SDS, 10 mM Tris, pH 7.4, for 30 min to 1 hr at 85° and then rehybridized to a *GAPDH* probe (ATCC). *MDR1a* and *b*-, *MRP*- and *GLCLC*- specific hybridization signals [reported as background-subtracted, photostimulated-

luminescence units (PSL Units)] were compared in sensitive and resistant cell lines after being normalized first against *GAPDH* signals to account for RNA loading variations. The results shown are from 3 separate experiments.

[^{14}C]HQ Labeling Studies

Cells were pelleted and resuspended in ice-cold complete IMDM/20% FBS at 5 × 10^5 cells/mL. [U-^{14}C]HQ (22 Ci/mmol) was added to the cells at a final concentration of 25 μM, and the cells were divided into aliquots at 1 mL/well in 24-well plates and incubated at 37° (time zero samples were kept on ice and processed immediately). At various times, [^{14}C]HQ-treated M1 or M1HQ cells were harvested, immediately chilled on ice, and pelletted at 600 *g* at 4°. The medium was transferred to separate tubes and kept on ice; the pellets were resuspended in 100 μL PBS and disrupted by one freeze/thaw cycle. TCA was added to both the cells and the medium at a final concentration of 10%. Precipitable material was pelletted at 16,000 *g*, and the TCA supernatants were transferred to separate tubes and stored at −20°. In two [^{14}C]HQ experiments, TriReagent was used to isolate simultaneously M1 and M1HQ RNA, DNA, and protein. Contaminating protein was removed from isolated DNA by treatment with 100 μg/mL proteinase K, 1% SDS in 50 mM Tris–HCl, pH 8.0, for 4 hr at 56°. The DNA samples were phenol-extracted and ethanol-precipitated prior to analyses. Both RNA and DNA were quantified spectrophotometrically, and associated radioactivity was quantified by scintillation counting.

TLC and SDS–PAGE

TLC was performed using 20 × 20 cm cellulose-coated plastic sheets (Polygram CEL 300; Brinkmann Instruments, Westbury, NY). BQ adducts of GSH, Cys, and NAC were prepared by combining equimolar quantities of BQ and each thiol reagent in PBS and incubating at 22° for 30 min. Samples of cell and medium TCA supernatants or freshly prepared standards of 5–10 mM GSH, Cys, BQ, or adducts of GSH, Cys or NAC and BQ (GS–BQ, Cys–BQ or NAC–BQ, respectively) in PBS were spotted repetitively in 1-μL aliquots. TCA in the dry, spotted samples was removed by dipping the TLC sheet in ethyl ether several times. The desired chromatographic separation was achieved using 20:10:3:10 butan-1-ol:pyridine:acetic acid:water system [34]. Standards (except NAC–BQ which is yellowish-brown) were detected with ninhydrin, and [^{14}C]HQ and HQ adducts were detected and quantified using a Fujix BAS2000 phosphorimager. Prior to SDS–PAGE, TCA was removed from precipitated cellular proteins with ether; then the samples were dried and solubilized by boiling and sonication in SDS–PAGE sample buffer [35]. The proteins were electrophoresed through a 12.5% polyacrylamide gel as described [36]. The gels were stained with Coomassie blue and dried, and [^{14}C]HQ–protein adducts were detected and quantified by phosphorimagery.

RESULTS

HQ Sensitivity and Growth of M1 and M1HQ Cells

Experiments were conducted to evaluate the HQ concentration-response and growth characteristics of M1 and M1HQ cells. The 48 hr HQ TC_{50} and TC_{90} values for M1 cells were 25 ± 8 and 90 ± 8 μM, respectively (Fig. 1A). In contrast, 500 μM HQ did not affect M1HQ cell viability significantly. M1 cell growth lagged for 24 hr, then doubled every 16 hr during exponential growth, and plateaued at 5.4 × 10^6 cells/mL (Fig. 1B). M1HQ cells took nearly 48 hr to traverse the lag phase, exhibited a doubling time of 24 hr, and plateaued at 3.4 × 10^6/mL. Moreover, the M1HQ cell growth rate was the same whether HQ was present or not (data not shown). M1 and M1HQ cell cycle analysis was consistent with the slower M1HQ cell growth rate demonstrated by the growth curve analysis (data not shown). Using log phase cells, 36% of M1 cells were in S phase while only 20% of M1HQ cells were undergoing DNA replication. M1HQ cells exhibited corresponding increases in both the G_1 and G_2/M phases of the cell cycle. Thus, it is clear that M1HQ cell growth is substantially slower than that of M1 cells during all phases of the cell cycle, and M1HQ cells do not reach the same cell density as M1 cells.

Cross-Resistance of M1HQ Cells

Since many drug-resistant cell lines exhibit a broad pattern of cross-resistance to multiple agents, we were interested in determining whether M1HQ cells also were cross-resistant. Concentration-response curves for additional cytotoxic and growth inhibitory agents were generated for M1 and M1HQ cells. Compared with M1 cells, the M1HQ TC_{50} and TC_{90} were significantly higher for colchicine, BCNU, 4HC, H_2O_2 and DDP (Table 1). However, significant differences between M1 and M1HQ cell sensitivities to BQ or vinblastine were not observed. In addition, analysis of the inhibition of M1 and M1HQ cell growth shows that doxorubicin, but not γ-radiation, was significantly more inhibitory to M1 cell growth (Table 2). These results show that M1HQ cells are indeed resistant to multiple agents, but that the pattern of cross-resistance does not conform to a classical MDR phenotype.

Biochemical Analyses of HQ-Resistance in M1HQ Cells

Having established the M1HQ cell growth characteristics and the pattern of cross-resistance, the biochemical basis for HQ resistance was investigated. Analyses of the M1 and M1HQ total cellular thiol and GSH levels demonstrated that M1HQ cells possessed 1.6-fold higher total thiols and 6.8-fold higher total GSH than M1 cells (Fig. 2). In addition, M1HQ cells displayed significantly higher levels of several enzymatic activities: GPx was 4.3-fold higher using either H_2O_2 or cumene hydroperoxide as a substrate. Since GST-associated GPx is active against organic hydroperoxides but not H_2O_2, the increased GPx activity must be derived from selenium-dependent GPx. Glutathione reduc-

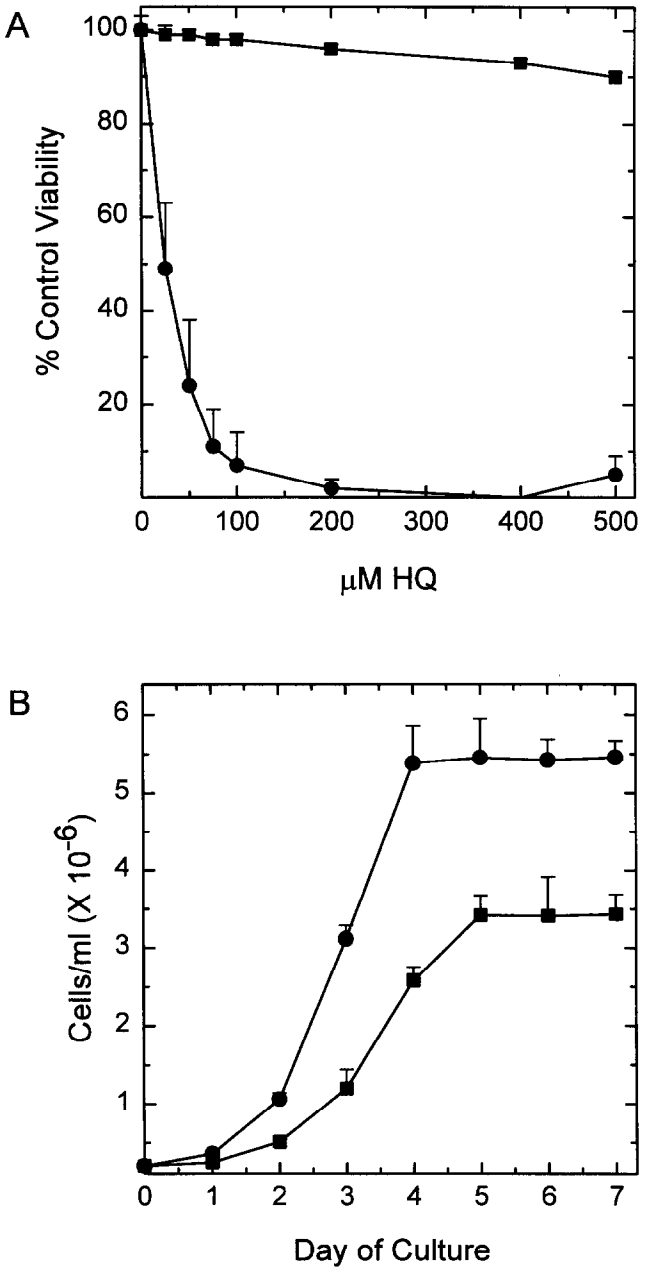

FIG. 1. M1 (●) and M1HQ (■) concentration–response to HQ and cell growth kinetics. (A) Cells (1×10^5) were incubated in 0.5 mL complete IMDM/20% FBS in 12 × 75 mm polystyrene tubes at 37° in a humidified atmosphere of 5% O_2, 7% CO_2, 88% N_2 in the presence of 0–500 μM HQ. After 48 hr, the fractions of viable (PI-negative) cells were determined by flow cytometry and converted to the percentages of the control samples that did not receive HQ. Control sample viability was ≥85%. Values are means ± SD from three separate experiments. (B) Cells (3×10^6) were cultured in 15 mL complete IMDM/20% FBS in T-75 flasks at 37° in a humidified atmosphere of 5% O_2, 7% CO_2, 88% N_2. Cell counts were obtained daily using an electronic cell counter. The mean cell count ± SD is presented from 3 separate experiments.

TABLE 1. Sensitivities of M1 and M1HQ cells to cytotoxic agents*

Cytotoxic agent†	M1		M1HQ	
	TC_{50}	TC_{90}	TC_{50}	TC_{90}
HQ	25 μM	90 μM	>500 μM‡	>500 μM‡
BQ	27 μM	42 μM	37 μM	48 μM
VB	19 nM	100 nM	37 nM	100 nM§
Colchicine	65 nM	163 nM	217 nM‡	>400 nM‡
BCNU	76 μM	269 μM	200 μM‡	392 μM‡
4HC	21 μM	32 μM	71 μM‡	164 μM‡
H_2O_2	188 μM	433 μM	>400 μM‡	≥400 μM
DDP	10 μM	23 μM	21 μM‡	>26 μM

* M1 and M1HQ cells were treated with various concentrations of the indicated agents in complete IMDM/20% FBS at 37° for 48 hr (except for DDP) or 72 hr (DDP). Cells were stained with propidium iodide for 3 min at 22°, and the fraction of viable cells was determined by flow cytometry. The mean concentrations that produced 50% (TC_{50}) and 90% (TC_{90}) cytotoxicity relative to the untreated controls are shown and were calculated from 3–4 concentration–response experiments.

† See text for abbreviations.

‡ Sensitivities of M1HQ cells were significantly less than those of M1 cells ($P < 0.02$), using the paired *t*-test.

§ Represents TC_{80}.

tase was 1.6-fold higher, quinone reductase was 143-fold higher, and γ-glutamyl transpeptidase was 17.7-fold higher (Table 3). Total GST and *N*-acetyl transferase activities of M1 and M1HQ cells were not significantly different. Northern blot hybridization analyses showed that, compared with M1 cells, *GLCLC* expression was elevated 1.8-fold in M1HQ cells (Table 4). Using a probe that detects both *MDR1a* and *MDR1b* transcripts, both M1 and M1HQ expressed *MDR1* levels higher than the multidrug-sensitive cell line J774.2 [37]. *MDR1* expression in M1HQ cells was 2-fold higher than that in M1 cells, but it was less than half that of the J774.2-derived MDR cell lines J7-V2-1 and J7-V3-1. More importantly, the P-gp inhibitor verapamil had no effect on M1, M1HQ, J7-V2-1, or J7-V3-1 cell sensitivity to HQ, but it did increase significantly the cytotoxicity of colchicine to these cells (data not shown). Nevertheless, even in the presence of verapamil, the colchicine TC_{50} for M1HQ cells was still 5-fold higher than that for M1 cells. *MRP* transcripts were not detectable in

TABLE 2. Inhibition of M1 and M1HQ growth by doxorubicin (DX) and γ-radiation (Rad)*

	M1		M1HQ	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
DX	28 nM†	58 nM	49 nM	90 nM
Rad	321 cGy	615 cGy	292 cGy	566 cGy

* Cells were diluted to 70 or 200 cells/mL in complete IMDM/20% FBS and divided into aliquots. DX was added or the cells were γ-irradiated. Samples were plated in Terasaki plates (1 plate/sample) at 2–5 cells/well. Wells with a greater than input cell number were scored on days 7 or 10. The mean DX concentrations or Rad doses that inhibited cell growth in 50% (IC_{50}) or 90% (IC_{90}) of wells relative to the controls are shown. The results represent the means of 3 separate experiments, each performed in duplicate.

† The M1 cell IC_{50} was significantly less than that for M1HQ cells ($P < 0.01$), as determined using the paired *t*-test.

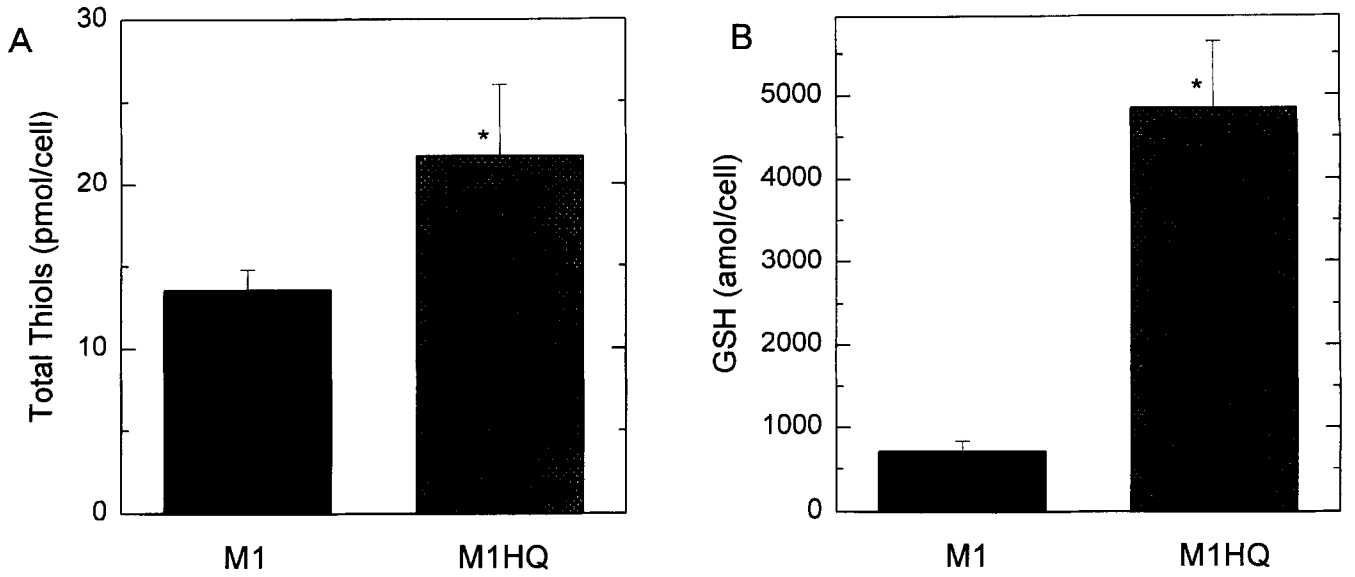

FIG. 2. M1 and M1HQ total thiols and GSH. (A) Total cellular thiols were calculated from the $O.D._{412\ nm}$ of cells disrupted in 4 M guanidine HCl, 0.667 mM DTNB, and 133 mM Tris–HCl, pH 8.0, using the DTNB molar extinction coefficient of 13.6 $mM^{-1}\ cm^{-1}$. (B) Total GSH (GSH and GSSG) was determined using sulfosalicylic acid supernatants of cell lysates and GSH standards from the increase in the $O.D._{405\ nm}$ for 10 min following the addition of 0.21 mM NADPH, 0.6 mM DTNB, and 0.1 unit glutathione reductase. The data shown in both graphs represent the means ± SD from 3 separate experiments. Key: (*) indicates statistical significance, $P \leq 0.01$, obtained using the paired *t*-test.

M1, M1HQ, J774.2, J7-V2-1, or J7-V3-1 cells by northern blot analysis, and the *MRP* genomic sequence was not amplified (data not shown). Thus, the development of HQ resistance by M1HQ cells does not appear to require amplification of *MDR1* or *MRP* steady-state transcript levels.

Partial Reversal of M1HQ Resistance

Additional biochemical assessment of the HQ-resistance mechanism was performed. M1HQ cells were pretreated for 1 hr with the quinone reductase inhibitor dicumarol, the γ-glutamylcysteine synthetase inhibitor BSO, the GPx inhibitor aurothioglucose, the MRP inhibitor vanadate, the γ-glutamyl transpeptidase inhibitor acivicin, the P-gp inhibitor verapamil, or the GST inhibitor ethacrynic acid, alone or in combination. The cells were then exposed to 0–400 μM HQ for 24 hr at which time HQ cytotoxicity was assessed. When used alone, none of these inhibitors produced statistically significant cytotoxicity or reversal of M1HQ HQ resistance (data not shown and Fig. 3). However, vanadate did increase slightly the HQ sensitivity of M1HQ cells (Fig. 3). The combination of dicumarol, BSO, and aurothioglucose significantly increased the sensitivity of M1HQ cells to HQ at each HQ concentration tested (Fig. 3). When vanadate was added to the combination of dicumarol, BSO, and aurothioglucose, an even greater reversal of HQ resistance was achieved (Fig. 3). However, this combination of inhibitors exhibited slight, but significant, cytotoxicity to M1HQ cells in the absence of HQ (data not shown). Thus, it is difficult to ascertain whether

TABLE 3. Enzymatic activities in M1 and M1HQ cells*

	Specific activities					
Cells	GST (nmol CDNB consumed/min/mg protein at 30°)	GPx† (nmol NADPH oxidized/min/mg protein at 25°)	GR (nmol NADPH oxidized/min/mg protein at 25°)	QR‡ (nmol MTT reduced/min/mg protein at 37°)	GT (fmol pNA liberated/min/mg protein at 37°)	NAT (nmol PABA metabolized/hr/mg protein at 25°)
M1	25.5 ± 2.5	27.3 ± 3.0	31.3 ± 1.2	0.2 ± 0	0.3 ± 0	567.5 ± 64.1
M1HQ	28.0 ± 3.6	117.0 ± 6.8§	50.7 ± 1.5§	28.6 ± 1.3§	5.3 ± 0.6§	497.0 ± 31.2

* M1 and M1HQ cells were pelleted and washed once with PBS. The cells were assayed for the indicated enzymatic activities as described in Materials and Methods. The mean specific activities (units/mg cellular protein) ± SD are presented from 2–3 separate experiments, each performed in duplicate or triplicate. Abbreviations: GST, GSH-*S*-transferase; GPx, GSH peroxidase; GR, GSSG reductase; QR, quinone reductase; GT, γ-glutamyl transpeptidase; NAT, *N*-acetyl transferase; pNA, *p*-nitroaniline; and PABA, *p*-aminobenzoic acid.

† GPx activities were identical using either H_2O_2 or cumene hydroperoxide as substrate.

‡ QR activities were dicumarol-inhibitable.

§ Activities were elevated significantly in M1HQ cell lysates ($P < 0.02$), using the paired *t*-test.

TABLE 4. Steady-state *GLCLC* and *MDR1a* and *b* mRNA-expression*

Cells	*GLCLC*	*MDR1*
M1	1	5.9
M1HQ	1.8	12.4
J774.2	ND†	1
J7-V2-1	ND	41.3
J7-V3-1	ND	24.9

* Northern blots of total cellular RNA were hybridized with radiolabeled probes to *GLCLC* or *MDR1a* and *b* and then rehybridized to a *GAPDH* probe to ensure that there were no loading differences. Hybridization signals were detected and quantitated using a Fujix BAS2000 phosphorimager, and relative levels of each transcript are shown.

† ND = not done.

vanadate actually contributes to the reversal of HQ resistance or whether its effect is the result of slight vanadate cytotoxicity. Furthermore, regardless of the inhibitor combination used, complete reversal of HQ resistance was not attainable.

Treatment of M1 and M1HQ with [^{14}C]HQ

Preliminary experiments indicated that [^{14}C]HQ underwent biotransformation when exposed to either M1 or M1HQ cells (data not shown). As a result, experiments were done to determine how M1 and M1HQ cells metabolize HQ to gain more insight into the mechanisms underlying the HQ toxicity to M1 cells and the HQ resistance exhibited by M1HQ cells. This was done by treating both cell lines with 25 μM [^{14}C]HQ in complete IMDM/20% FBS at 37° and analyzing medium and cellular TCA-soluble constituents using a TLC system capable of resolving GSH, Cys, NAC, and HQ/BQ from each other and their HQ/BQ adducts [34]. An adduct that exhibited an R_f equivalent to NAC–BQ was readily detected in TCA supernatants from M1HQ cell culture medium after 16 hr of [^{14}C]HQ exposure (Fig. 4A, lane 4). This adduct also was detected in the M1HQ cell-associated TCA-soluble fraction (data not shown). Adducts of GSH or Cys and [^{14}C]HQ were not detected in either M1HQ TCA-soluble fraction at any time. (The spot-like appearance at the GSH position in Fig. 4A, lane 4, is an artifact from either overloading or incomplete TCA removal and was not observed in other experiments.) In contrast, HQ adducts were not detected in medium (Fig. 4A, lane 2) or cellular (data not shown) TCA-soluble fractions from 16-hr M1 cultures. TCA-soluble HQ adducts were not detected at any time in the cell-free medium control (data not shown) or in the medium from M1 or M1HQ cells collected prior to 37° incubation (Fig. 4A, lanes 1 and 3). However, the NAC–BQ-like adduct was detectable in the M1HQ cellular TCA-soluble fraction in less than 3 min at 0° and was detected at all time points tested (data not shown). Analysis of M1 and M1HQ cellular proteins following 16-hr [^{14}C]HQ exposure showed that numerous M1 cellular HQ–protein adducts were generated (Fig. 4B, lane 2). Coomassie blue staining of the gel showed that while similar quantities of cellular proteins were present in each lane (data not shown), HQ adduction of cellular proteins from M1 cells at 0 hr (Fig. 4B, lane 1) or M1HQ cells at 0 or 16 hr was nearly undetectable (Fig. 4B, lanes 3 and 4, respectively). Adduction of either M1 or M1HQ cellular RNA or DNA following 16-hr exposure to [^{14}C]HQ was undetectable (data not shown). The kinetics of HQ adduct formation in the TCA-soluble and protein fractions from M1 and M1HQ cells also was examined. The presence of the NAC–BQ-like adduct was detectable in M1HQ cell culture medium within 2 hr and increased linearly before plateauing at 20–24 hr (Fig. 5). Production of this adduct was not detectable in M1 cell culture medium at any time point examined. Conversely, HQ adduction of M1, but not M1HQ, cellular proteins increased over an 8-hr period and then began to decline, probably due to HQ cytotoxicity since the 25 μM [^{14}C]HQ concentration used in these experiments was near the M1 cell TC_{50}.

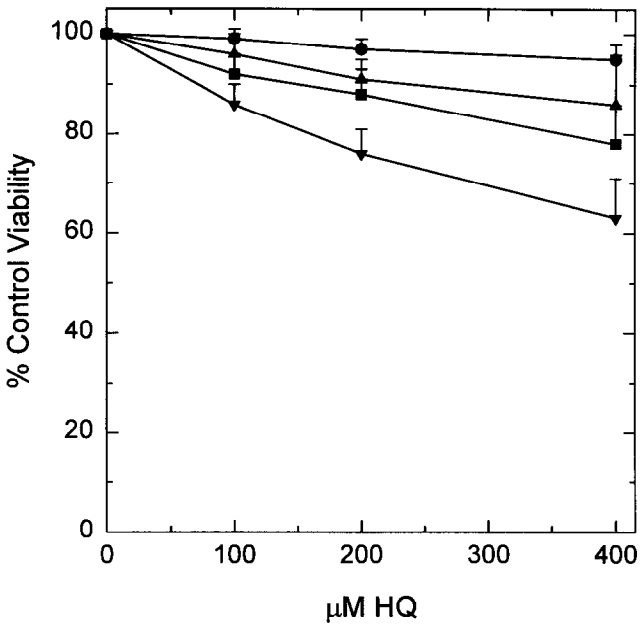

FIG. 3. Effects of inhibitors on M1HQ cell HQ resistance. Cells (1.25 × 10^6 in 5 mL) were pretreated in complete IMDM without FBS for 1 hr at 37° in the absence (●) or presence of 40 μM dicumarol, 20 μM BSO, and 100 μM aurothioglucose (■), 100 μM vanadate (▲) or dicumarol, BSO, aurothioglucose, and vanadate (▼). FBS was then added to each pretreatment group to 20%, additional inhibitors were added to maintain their concentrations, and 0–400 μM HQ was added to duplicate 0.5-mL aliquots. Samples were incubated at 37° for 24 hr, and cellular viabilities were determined as described in Fig. 1. Viabilities were converted to the percentages of the control samples in each group that did not receive HQ. The means ± SD are presented from ≥3 separate experiments. The mean viabilities from each group were as follows: no inhibitor: 89 ± 4%; dicumarol, BSO, and aurothioglucose: 88 ± 4%; vanadate: 83 ± 3%; and dicumarol, BSO, aurothioglucose, and vanadate: 83 ± 2%.

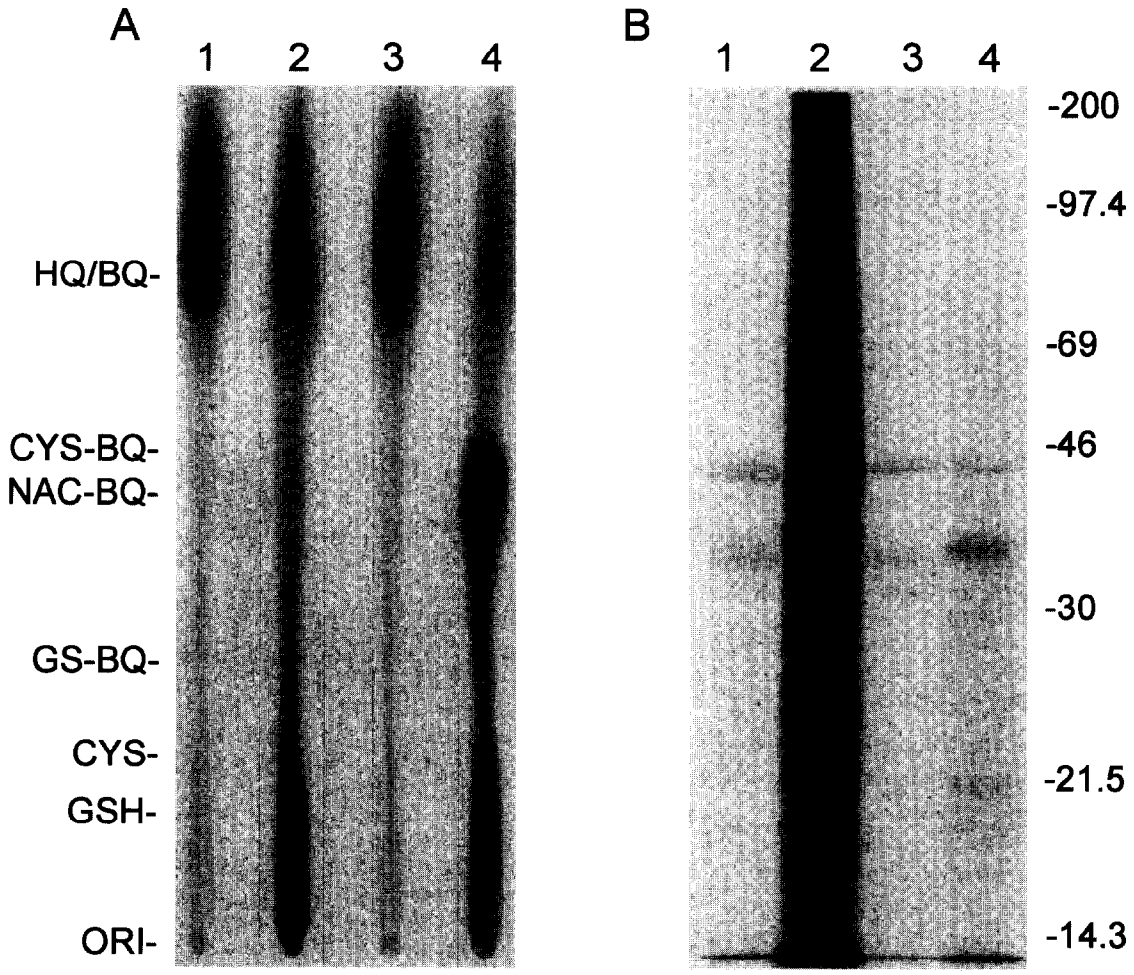

FIG. 4. Metabolism of [U-^{14}C]HQ by M1 and M1HQ cells. Cells (5 × 10^5 in 1 mL) were incubated with 25 μM [^{14}C]HQ (22 Ci/mmol) in complete IMDM/20% FBS at 37° for 0–16 hr in 24-well plates. Cells were pelletted, and the medium and cellular fractions were deproteinized by the addition of TCA to 10%. (A) Generation of nonprotein–[^{14}C]HQ adducts in the M1 (0 hr, lane 1; 16 hr, lane 2) and M1HQ (0 hr, lane 3; 16 hr, lane 4) medium fractions was monitored by TLC. (B) Formation of M1 (0 hr, lane 1; and 16 hr, lane 2) and M1HQ (0 hr, lane 3; 16 hr, lane 4) cellular [^{14}C]HQ–protein adducts was monitored by SDS–PAGE following solubilization of the TCA-precipitated cellular proteins by boiling in Laemmli SDS–PAGE sample buffer. The nonprotein- and protein–[^{14}C]HQ adducts were detected using a Fujix BAS2000 phosphorimager. The positions of the origin (ORI) and TLC nonprotein standards in (A) are indicated to the left and the M_r values in kDa of the protein standards are indicated in (B) to the right. The results shown are representative of 3 separate experiments.

DISCUSSION

The HQ-resistant derivative of the M1 murine myeloblastic leukemia cell line, M1HQ, was generated by continuous passage in increasing concentrations of HQ. Obvious HQ resistance began to develop after approximately 24 passages and became stable by passage 44. Interestingly, repeated attempts to derive HQ-resistant sublines of the KG-1 and KG-1a human HPC cell lines [38, 39] were unsuccessful. Thus, the possibility exists that human cells are incapable of developing HQ resistance.

The HQ TC_{50} for M1 and M1HQ cells demonstrated that M1HQ cells have greater than 20-fold higher resistance to HQ than the parental M1 cell line. Comparison of M1 and M1HQ cell growth curves revealed significant differences. The M1HQ cell lag phase was longer, exponential growth was slower, and M1HQ cells plateaued at a lower cell density than the M1 parental cell line. We also have observed that the growth of the J7-V2-1 and J7-V3-1 MDR cell lines [16] is slower than that of the J774.2 parental cell line (data not shown). Slower growth of resistant cell lines relative to their sensitive counterparts suggests that the resistant cell lines may require additional time to resynthesize cellular components damaged by the agent to which they are resistant or that drug resistance is maintained at the expense of cell growth. Since M1HQ cell growth remains slower than that of M1 cells regardless of whether HQ is present or not, it is likely that the maintenance of HQ resistance at the expense of faster cell growth is responsible.

The pattern of M1HQ cross-resistance shows that M1HQ cells are indeed resistant to many oxidative stress-inducing agents, including doxorubicin, BCNU, 4HC,

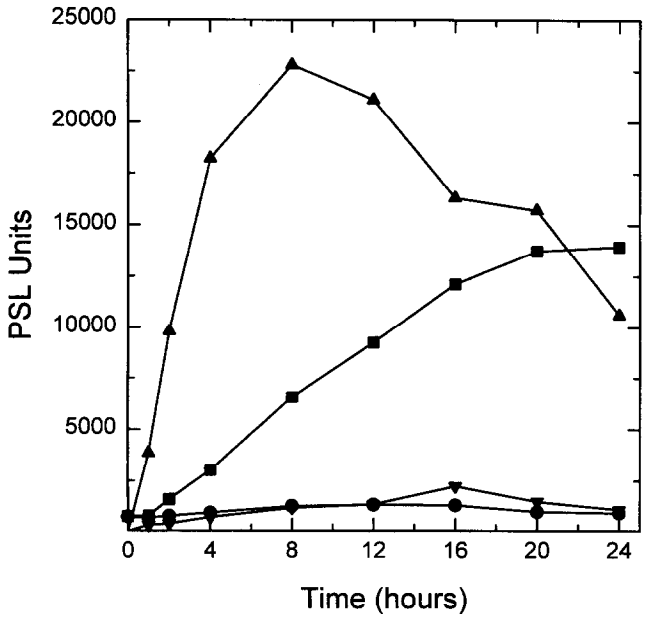

FIG. 5. Kinetics of [^{14}C]HQ adduct generation by M1 and M1HQ cells. Cells (5 × 10^5 in 1 mL) were incubated with 25 μM [^{14}C]HQ in complete IMDM/20% FBS at 37° for 24 hr in 24-well plates. The contents of wells were harvested at the indicated times, the cells were separated from the medium by centrifugation, and the fractions were deproteinized by the addition of 10% TCA. Generation of the NAC–BQ-like [^{14}C]HQ adduct in the M1 (●) and M1HQ (■) cell medium fractions was monitored by TLC. Formation of M1 (▲) or M1HQ (▼) cellular [^{14}C]HQ–protein adducts was monitored by SDS–PAGE following solubilization of the cellular proteins by boiling for 3 min in SDS–PAGE sample buffer. The nonprotein– and protein–[^{14}C]HQ adducts were quantified using a Fujix phosphorimager and are expressed in photostimulated luminescence (PSL) units. The results shown are from one representative experiment.

H_2O_2, and DDP. However, with the exception of colchicine and doxorubicin resistance, the pattern of cross-resistance exhibited by M1HQ cells is not consistent with a classical MDR phenotype involving P-gp overexpression. This conclusion also is supported by the fact that the P-gp inhibitor verapamil has no effect on the HQ resistance. The increased resistance to colchicine may have an explanation, however. Microtubule polymerization has been shown to involve thiols in tubulin monomers and thiol alkylation can interfere with colchicine binding to tubulin [40]. HQ is metabolized to thiol-reactive species within the cell and has been shown to interfere with tubulin assembly, probably via thiol alkylation [41]. Thus, it is possible that the observed colchicine resistance has resulted from changes that have taken place in M1HQ cell tubulin. Consistent with this hypothesis, others have demonstrated that mutations in α and β tubulin occur in colchicine-resistant cells, and these cells possess elevated levels of polymerized tubulin [42]. Interestingly, M1HQ cells do not exhibit significant resistance to vinblastine, another agent that induces microtubule depolymerization by binding to tubulin. The lack of M1HQ resistance to vinblastine may be due to the fact that vinblastine and colchicine bind to different sites on tubulin [43, 44]. Thus, it is conceivable that changes in tubulin which increase colchicine resistance may not increase vinblastine resistance. Additional studies are necessary to determine whether M1HQ cells express altered tubulin proteins.

Investigation of the M1HQ-resistance mechanism revealed that, compared with M1 cells, significant elevations had occurred in M1HQ total cellular thiols and GSH and in the activities of quinone reductase and the GSH-metabolizing enzymes GPx, glutathione reductase, and γ-glutamyl transpeptidase. These results share some similarities with those obtained comparing the human AML cell line ML-1 and the promyelocytic leukemia cell line HL-60 [45]. ML-1 cells had higher levels of GSH and quinone reductase than HL-60 cells, which correlated with increased resistance to HQ. Surprisingly, GST activity was not higher in M1HQ cells even though increased GST activity has been correlated with cellular resistance to alkylating agents [13].

Evidence for the HQ-resistance mechanism in M1HQ cells also was obtained from experiments performed with numerous enzyme inhibitors. None of the inhibitors alone had a significant effect on the HQ resistance of M1HQ cells, suggesting that the mechanism of HQ resistance is multifactorial. It also was observed that the combination of dicumarol, BSO, and aurothioglucose could partially reverse HQ resistance. Addition of ethacrynic acid, acivicin, or verapamil to this inhibitor combination did not increase the partial reversal of HQ resistance. However, vanadate combined with dicumarol, BSO, and aurothioglucose had a possible additive effect on the partial reversal of HQ resistance achieved with dicumarol, BSO, and aurothioglucose. The results with these inhibitors suggest two explanations for the HQ resistance. First, quinone reductase, GSH, and GPx are necessary for HQ resistance, and GST, γ-glutamyl transpeptidase, P-gp, MRP, and *N*-acetyl transferase activities may contribute to HQ resistance but are not absolutely required. Alternatively, since complete reversal of HQ resistance was not attainable, either additional as yet unidentified cellular activities are involved or one or more of the inhibitors did not inhibit its enzymatic target completely.

The results from the analysis of [^{14}C]HQ metabolism by M1 and M1HQ cells complemented those showing that M1HQ cells have increases in total cellular thiols, in total GSH, and in several enzymatic activities. These results clearly show that numerous HQ–protein adducts were generated with M1 cells while barely detectable HQ–protein adduct levels were produced by identically treated M1HQ cells. Conversely, M1HQ cells, but not M1 cells, produced a nonprotein–HQ adduct similar to NAC-BQ in the cell and exported it into the medium. Interestingly, significant levels of HQ–DNA or –RNA adducts were not detectable nor were HQ adducts of GSH or Cys.

These results have been used to construct a model that accounts for the HQ-induced cytotoxicity to M1 cells and the development of HQ resistance by M1HQ cells (Fig. 6). In M1 cells, a sufficiently high HQ concentration is achieved to cause cytotoxicity resulting primarily from thiol adduction of cellular proteins by HQ, or by its more reactive metabolite BQ, which affects one or more critical cellular proteins necessary for cell survival. The M1HQ cell resistance is posited to result from a combination of increased GSH levels and increased activities of quinone reductase, GPx, glutathione reductase, and γ-glutamyl transpeptidase. The increased GSH level and quinone reductase activity prevent excessive protein adduction and subsequent cytotoxicity by effectively increasing the intracellular concentration of expendable thiols while at the same time preventing the accumulation of the more reactive HQ-metabolite BQ. The increased GPx activity also participates in HQ resistance by eliminating ROI through GSH oxidation, and the increased glutathione reductase reduces the GSSG formed and contributes to the maintenance of the intracellular pool of reduced GSH. GS–BQ or Cys–BQ adducts (assuming they are formed) are expelled from the cells, possibly through the action of MRP. However, since *MRP* transcripts were not detectable in M1HQ cells and the effects of vanadate on reversing HQ resistance were marginal, it is questionable whether MRP is actually involved in the HQ-resistance mechanism. Thus, HQ resistance may be the result of a vanadate-insensitive efflux pathway for BQ adducts. In any case, GS–BQ is immediately metabolized through the actions of γ-glutamyl transpeptidase, dipeptidase, and *N*-acetyl transferase, yielding the mercapturate NAC–BQ. At this time, it is unclear whether GST activity is necessary for HQ resistance since GST activity is not higher in HQ-resistant cells and the GST inhibitor ethacrynic acid is unable to reverse the HQ resistance. GST may be required for HQ resistance, but if the parental M1 cells already possessed sufficiently high levels no further increases would be necessary. Alternatively, even though total GST activity is not increased, the relative proportions of the GST α, μ and π isozymes in M1HQ cells may be altered such that the GST with the highest affinity for HQ is overexpressed while the other GST classes are correspondingly down-regulated. Another possibility is that GST is simply not required for the development of primary HQ resistance or perhaps the generation of GS–BQ is, in fact, cytotoxic similar to other GS–quinone conjugates [46]. Studies are in progress to clarify what role, if any, GST has in the development of cellular HQ resistance. Additional questions concerning this model exist. The predicted GS–BQ and Cys–BQ adducts have not been detected, and the identity of the NAC–BQ-like adduct has not been confirmed. Efforts are presently underway to identify additional adducts, if they exist, and to confirm the identity of NAC–BQ. Furthermore, since this model was developed to explain the basis for HQ cytotoxicity and resistance, it is unclear whether the proposed model also accounts for the M1HQ cell resistance to the other agents. In any case, these well-characterized sensitive and resistant HPC lines are well suited for genetic approaches to further delineate the mechanisms of HQ cytotoxicity and nonclassical multidrug resistance.

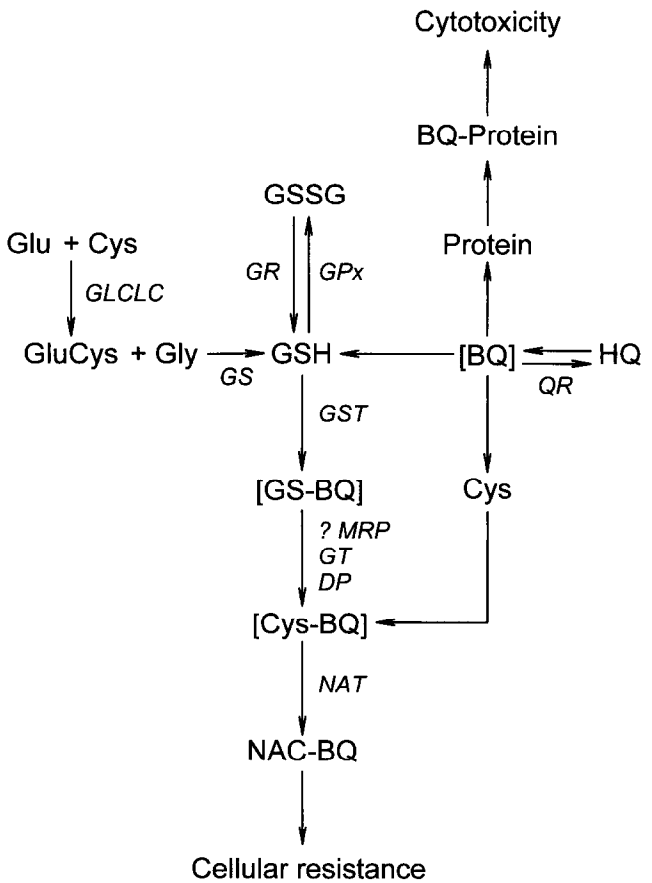

FIG. 6. Integrated model accounting for both HQ-induced cytotoxicity to M1 cells and the HQ resistance of M1HQ cells. HQ-induced cytotoxicity to M1 cells is proposed to result from entry of HQ into the cell where it is oxidized to BQ, which then covalently adducts one or more essential cellular proteins and leads to cell death. HQ resistance of M1HQ cells is posited to result from a combination of increases in quinone reductase and GSH-dependent detoxification activities that protect the cell from adduction of cellular proteins essential for survival. Enzymatic activities are italicized and proposed intermediates are bracketed. (Abbreviations not defined in the text: *GS*, glutathione synthetase; *GR*, glutathione reductase; *QR*, quinone reductase; *GT*, γ-glutamyl transpeptidase; *DP*, dipeptidase; and *NAT*, *N*-acetyl transferase.)

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