Cancer Chemother Pharmacol (1995) 35: 377–386

ORIGINAL ARTICLE

Peter F. Lenehan · Peter L. Gutiérrez
John L. Wagner · Niteen Milak · Geoffrey R. Fisher
Douglas D. Ross

Resistance to oxidants associated with elevated catalase activity in HL-60 leukemia cells that overexpress multidrug-resistance protein does not contribute to the resistance to daunorubicin manifested by these cells

Received: 11 March 1994/Accepted: 15 July 1994

Abstract *Purpose*: It has been recognized that enhanced antioxidant defenses can contribute to the resistance of cancer cells displaying multidrug resistance (MDR) that arises in conjunction with the overexpression of P-glycoprotein (Pgp). The purpose of this study was to determine if the defenses against oxidant stress in MDR human leukemia cells (HL-60/AR) that overexpress multidrug-resistance-associated protein (MRP), but not Pgp, contribute to the mechanism of drug resistance in this cell line. *Methods*: HL-60/AR cells were evaluated in comparison with wild-type cells with respect to sensitivity to the oxidants hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (t-BuOOH), the activities and amounts of the antioxidant enzymes catalase and glutathione peroxidase (GSH-Px), and the effects that manipulation of the activities of these enzymes may have on cellular sensitivity to the oxidants and to daunorubicin. We also evaluated the ability of the cells to generate daunorubicin semiquinone free radical as measured by electron spin resonance (ESR) spectroscopy. *Results*: HL-60/AR cells were > 10-fold resistant to the cytotoxic effects of the H_2O_2 or t-BuOOH as compared with parental, drug-sensitive HL-60 cells. This phenomenon could be attributed largely to elevated activity and protein levels of catalase in HL-60/AR cells. Furthermore, inhibition of catalase by 3-amino-1,2,4-triazole (AT) diminished the resistance of HL-60/AR to these oxidants by > 80% or > 50%, respectively. Despite these findings, AT was incapable of causing sensitization of HL-60/AR cells to the cytotoxic effects of daunorubicin. We found that the activity and amount of selenium-dependent glutathione peroxidase (GSH-Px) was no greater in HL-60/AR cells than in HL-60 cells. Cultivation of cells in selenium-deficient medium caused a marked reduction in GSH-Px activity in HL-60/AR cells and a profound inhibition of GSH-redox cycling manifested by a decrease in baseline hexose monophosphate shunt activity (HMPS) and markedly blunted stimulation of the HMPS by the oxidant t-BuOOH in both wild-type and resistant cells. These variations in GSH-Px activity and GSH-redox cycling, however, were not associated with an alteration in cellular sensitivity to daunorubicin. The failure of catalase inhibition or selenium manipulation of GSH-Px activity to affect daunorubicin cytotoxicity was not due to the inability of these cells to produce free-radical species of daunorubicin, since ESR studies revealed that the generation of daunorubicin semiquinone free radical by HL-60/AR cells was equal to and, in fact, 3-fold that obtained with HL-60 cells. *Conclusions*: In comparison with parental HL-60 cells, MRP-overexpressing HL-60/AR cells have demonstrable alterations in

Supported in part by Special Research Initiative Support, University of Maryland at Baltimore Designated Research Initiative Fund (P.F.L., D.D.R.); National Research Service Award (1 T32 CA09633-01), Physician Scientist Training Program in Oncology, NCI, NIH (P.F.L.), and a Research Starter Grant, American Cancer Society, Maryland Division, Inc. (P.F.L.). This publication was also supported in part by grant RO-1-CA40188 from the NCI, NIH (D.D.R.), and grant RO-1-CA53491 (P.L.G.) from the NCI. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCI

P.F. Lenehan[1] · P.L.Gutiérrez · J.L. Wagner · N. Milak · G.R. Fisher · D.D. Ross (✉)
Division of Developmental Therapeutics, University of Maryland Cancer Center, Bressler Research Building, Room 9-015, 655 West Baltimore Street, Baltimore, MD 21201, USA

P.L. Gutiérrez
Department of Biochemistry, University of Maryland School of Medicine, Baltimore, MD 21201, USA

D.D. Ross · P.F. Lenehan
Division of Hematology and Oncology, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

[1] *Present address*: Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48105, USA

antioxidant defenses that are manifested by cellular resistance to the cytotoxic effects of H_2O_2 and t-BuOOH and by elevated protein levels and activity of catalase. Whether these alterations are epiphenomena or are related to overexpression of MRP remains to be determined. However, it does appear that the enhanced antioxidant defenses observed in HL-60/AR cells do not contribute to the resistance to daunorubicin manifested by this cell line. Although HL-60/AR cells generate daunorubicin semiquinone free radical to an extent equal to or greater than that observed in HL-60 cells, the failure of alterations in GSH-Px activity or inhibition of catalase to change the sensitivity of HL-60/AR cells to daunorubicin suggests that the cytotoxicity of daunorubicin in these cells in not mediated through H_2O_2 or other peroxide species detoxified by these enzymes.

Key words Antioxidant defenses · HL-60 · Leukemia · Catalase · Multidrug resistance

Abbreviations AML Acute myelogenous leukemia · MDR multidrug resistance · Pgp P-glycoprotein · MRP multidrug-resistance-associated protein · H_2O_2 hydrogen peroxide · GSH reduced glutathione · GSH-Px glutathione peroxidase · FDA/PI fluorescein diacetate/propidium iodide · LC_{50} concentration of agent lethal to 50% of the cell population studied · AT 3-amino-1,2,4-triazole · HMPS hexose monophosphate shunt · ESR electron spin resonance · PBS phosphate-buffered saline · SOD superoxide dismutase · t-BuOOH tert-butyl hydroperoxide · BSO buthionine sulfoximine · FBS fetal bovine serum

Introduction

The anthracycline daunorubicin is one of the most effective agents available for the treatment of AML [1]. It is known that daunorubicin undergoes metabolic reduction that results in the generation of daunorubicin semiquinone free-radical and reactive oxygen species [2–4]. The intracellular and extracellular oxidants such as H_2O_2 and hydroxyl free radicals produced by such a process may be a component of the cytotoxicity of daunorubicin [5,6]. That daunorubicin causes an oxidant stress to cells is further strengthened by observations that anthracycline exposure stimulates the activity of the HMPS [7]. The formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by the HMPS is important not only to the detoxification of reactive oxygen species but also to the generation of anthracycline free radicals [4,8,9]. Because of the tight regulation of NADPH formation by the HMPS, alteration in the activity of this pathway is commonly used as an index of cellular oxidant stress [9].

One of the most extensively studied mechanisms by which cells can become resistant to daunorubicin (and to a variety of other natural-product compounds) is that of MDR mediated by the membrane-resident efflux transporter Pgp [10]. Pgp expression in blast cells is being recognized as an important prognostic indicator in AML [11]. Most promising is the potential use of noncytotoxic substrates for Pgp, such as verapamil or cyclosporin A, to serve as inhibitors of the outward transport of cancer chemotherapeutic agents (such as daunorubicin) by Pgp [11]. Hence, theoretically these inhibitors should sensitize Pgp-overexpressing MDR cells to the cytotoxic effects of chemotherapeutic agents such as daunorubicin and the related anthracycline doxorubicin. However, Kramer et al. [7] found that verapamil was incapable of restoring full sensitivity to doxorubicin in the Pgp-overexpressing MDR cell lines MCF-7/Adr (human breast) and P388/Adr (murine lymphocytic leukemia) or in de novo MDR human colon-carcinoma cell lines, suggesting that the resistance of these lines cannot be explained solely by Pgp-mediated drug efflux. Since the combination of verapamil and buthionine sulfoximine (BSO, an inhibitor of GSH synthesis) did result in full sensitization of these cell lines to doxorubicin, these authors suggested that an increase in antioxidant metabolism in addition to drug efflux was responsible for the resistance manifested by these MDR cells. The resistance was uniformly associated with enhanced GSH-redox capacity and elevated activity of selenium-dependent GSH-Px in all MDR cell lines, and in MCF-7/Adr cells only, with higher activity of glutathione S-transferase, as compared with parental drug-sensitive cells. Although an anionic gluthathione S-transferase was observed to be overexpressed in MCF-7/Adr cells [12], Akman et al. [13] subsequently showed that the major alteration in antioxidant and xenobiotic-detoxifying enzyme expression in these cells was overexpression of GSH-Px mRNA, which contributed to the doxorubicin resistance of these cells. Furthermore, Mimnaugh et al. [14] have documented enhanced resistance of MDR MCF-7/Adr cells to the cytotoxic effects of exogenous superoxide free radical and H_2O_2 relative to that of drug-sensitive MCF-7 cells.

In addition to Pgp, a new 190-kDa membrane transporter named multidrug-resistance-associated protein (MRP) has been found to confer MDR to cells independent of Pgp [15]. The mRNA sequence of MRP has been determined [15]. Transfection of MRP cDNA into HeLa cells resulted in an MDR phenotype in these cells [16]. MRP is overexpressed in HL-60/AR cells [17], a subline of the human AML cell HL-60, which was selected for resistance with doxorubicin and does not overexpress Pgp [18]. HL-60/AR cells have a marked reduction in the ability to accumulate and retain daunorubicin as compared with HL-60 cells [18–20]. Despite their lack of measurable Pgp, the Pgp inhibitor cyclosporin A restores daunorubicin

accumulation in HL-60/AR cells to levels equal to those of HL-60 cells, suggesting that in addition to Pgp, these substances may also serve as inhibitors of MRP [20]. In contrast, cyclosporin A only partially sensitizes HL-60/AR cells to the cytotoxic effects of daunorubicin [20], making it possible that alterations in antioxidant metabolism such as those described by Kramer et al. [7] for Pgp-overexpressing cells may also be associated with MRP-overexpressing cells. Evidence in support of GSH and GSH-dependent enzymes being integrally associated with transport-mediated MDR in HL-60/AR cells was obtained by Lutzky et al. [21], who observed that inhibition of GSH synthesis by BSO partially reversed the daunorubicin resistance of HL-60/AR cells.

To test whether enhanced antioxidant metabolism is a component of the resistance to daunorubicin manifested by HL-60/AR cells, we compared HL-60/AR cells with wild-type cells with respect to their sensitivity to oxidants (H_2O_2 and t-BuOOH), the activities and amounts of the antioxidant enzymes catalase and GSH-Px, the effects of manipulating the activities of these enzymes on cellular sensitivity to the oxidants and daunorubicin, the activity of the HMPS, and the ability of the cells to generate daunorubicin semiquinone free radical. Selected studies were also performed in HL-60/Vinc cells [22], a Pgp-overexpressing MDR subline of HL-60 selected for resistance with vincristine. HL-60/Vinc does not overexpress MRP.

Materials and methods

Chemicals and pharmaceuticals

H_2O_2, t-BuOOH, and AT were purchased from Sigma Chemical Co. (St. Louis, Mo.). The concentration of H_2O_2 was standardized utilizing a molar extinction coefficient of 43.6 $M^{-1}\,cm^{-1}$ at 240 nm [23]. The exact concentration of t-BuOOH was determined by iodimetric titration with a standard solution of 0.1 *N* sodium thiosulfate (Aldrich Chemical Co., Milwaukee, Wis [24]. Daunorubicin was obtained from Wyeth Laboratories (Philadelphia, Pa.) Diaziquone was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda Md.) and was used without further purification.

Cell lines and culture methods

HL-60 cells were obtained from the original line described by Collins [25]. HL-60/AR and HL-60/Vinc cells were generous gifts from Drs. Alexander Hindenburg, Kapil Bhalla, and Steven Grant (respectively, at Winthrop University Hospital, Mineola, N.Y., Medical University of South Carolina, and Medical College of Virginia) and Dr. Melvin Center (Kansas State University, Manhattan, Kan.), respectively. Unless otherwise indicated, all cell lines were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56 °C for 30 min) FBS (JRH Biosciences, Lenexa, Kan.), 2 m*M* L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, penicillin (50 IU/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% air. The cells were passed in fresh media weekly at an initial density of 2.5×10^5 cells/ml and were split 1:1 on day 4 after every passage. Logarithmic growth was observed consistently from day 2 to day 6 of each passage cycle. Cells were routinely tested for mycoplasma to assure absence of contamination (Gen-Probe, Fisher Scientific, Pittsburgh, Pa.). "Serum-deficient" RPMI 1640 medium consisted of all of the above-mentioned constituents except that the FBS concentration was changed to $\leq 1\%$ and a combination of bovine insulin (5 μg/ml, Sigma) and iron-saturated transferrin (5 μg/ml, Sigma) was added [26]. Sodium selenate (Sigma) was added to serum-deficient medium to achieve the specific net selenium concentrations of "selenium-supplemented" (≥ 330 n*M*) and "selenium-deficient" (≤ 3 n*M*) media [27]. Cells cultured in serum-deficient media were also passed weekly but at an initial concentration of 5×10^5 cells/ml to permit satisfactory growth.

Cell-viability assays

The number of viable cells in culture was determined 72 h after the addition of peroxides or daunorubicin by the flow cytometric FDA/PI assay described by Ross et al. [28]. This cell-survival assay, previously developed in our laboratory, compares well with traditional clonogenic assays in the range of 1 log cell kill, the range of interest in our experiments. Assessment of cell viability prior to determination of HMPS activity or ESR spectra was done by trypan-blue dye exclusion [29].

HMPS activity

HMPS activity was assayed using a modification of the procedures described by Cohen et al. [30] and Rosenberg et al. [31]. Cells (2.5×10^6 viable cells/ml) were incubated for 60 min at 37 °C (pH 7.4) in 1 ml PBS containing 1 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, 2 m*M* D-glucose, and 0.2 μCi of either [1-^{14}C]-glucose or [6-^{14}C]-glucose (Du Pont Co., Wilmington, Del.) alone or in the presence of 500 μ*M* t-BuOOH. Incubations took place in septum-stoppered test tubes with a suspended central well (Kontes, Vineland, N.J.) containing 0.2 ml hyamine hydroxide (Sigma) as a CO_2-trapping agent. The incubation period was terminated by the addition of 1 ml 5 *N* sulfuric acid to the cell suspension. The trapped $^{14}CO_2$ was then quantified by liquid scintillation counting (model LS 5801, Beckman Instruments, Fullerton, Calif.). 6-$^{14}CO_2$ was subtracted from 1-$^{14}CO_2$ and the net activity, attributable to the HMPS, was expressed as the amount (in nanomoles) of glucose consumed per hour per 10^7 cells.

ESR spectroscopy

Quantification of daunorubicin and diaziquone semiquinone free-radical generation was done as previously described [3,32] by ESR spectroscopy with an X-band (9.3 GHz) Varian E-109 Century Series ESR spectrometer (Varian Analytical Instruments, Palo Alto, Calif.). A dual rectangular TE-104 cavity was used that contained strong pitch ($g = 2.0028$) in one section and the sample in an ESR flat cell in the other.

Preparation of cell lysates

Logarithmically growing cells were washed twice in PBS and then resuspended in 2 ml of β-mercaptoethanol/ethylenediaminetetraacetic acid (EDTA) stabilizing solution [33]. This cell suspension was subsequently sonicated at 35 W for 10 s with a model 250

Branson Sonifier (Branson Ultrasonics Corp., Danbury, Conn.). The sonicate was clarified by an initial low-speed spin (2,500 *g* for 5 min) and a subsequent high-speed (14,000 *g* for 15 min) spin. Aliquots of the clarified sonicate were kept frozen at − 70 °C until assayed for total protein (BCA Reagent, Pierce Chemical Co., Rockford, Ill.) or enzyme activity.

Enzyme-activity assays

Cell sonicates were assayed for catalase by spectrophotometric measurement of first-order kinetic loss of H_2O_2 (from 10.3 to 9.2 m*M*) at 240 nm (pH 7.0, 25 °C) [34,35]. GSH-Px was measured according to a modification of the spectrophotometric assays of Beutler [24] and Takahashi and Cohen [36], measuring the zero-order kinetic decrease in NADPH absorption at 340 nm with H_2O_2 or t-BuOOH as substrates (pH 7.0, 25 °C).

Western-immunoblot analysis

Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% resolving gel) of cell sonicates, protein was transferred to nitrocellulose membranes [37] and detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, Ill.) with rabbit anti-human erythrocyte catalase polyclonal antibodies (Calbiochem Corp., La Jolla, Calif.) or rabbit anti-human GSH-Px (selenium-dependent) generously provided by Dr. Harvey Cohen of the University of Rochester. Densitometric quantification of catalase or GSH-Px protein was accomplished by scanning the enhanced chemiluminescence photoautograph with a Pharmacia Gel Scan XL (version 2.0) laser scanner (Pharmacia Biotechnology, Inc., Piscataway, N.J.).

Statistical analysis

Statistical evaluation of the data employed the SYSTAT computer program (SYSTAT. Intelligent Software, Evanston, Ill.). The specific tests that were used are mentioned in the text. Differences were considered significant at $P < 0.05$.

Results

Cytotoxicity of H_2O_2, t-BuOOH, or daunorubicin

To assess whether MDR HL-60/AR or HL-60/Vinc cells exhibit enhanced antioxidant defenses as compared with parental, drug-sensitive HL-60 cells, all three cell lines were exposed to increasing concentrations of the known potent oxidants H_2O_2 and t-BuOOH. Cell survival was evaluated by the flow cytometric FDA/PI assay and reported in terms of the LC_{50} value. The mean LC_{50}s of these oxidants, shown in Table 1 (without AT), were calculated from duplicate experiments. As can be seen, relative to HL-60 cells, both HL-60/AR and HL-60/Vinc cells showed resistance to the cytotoxic effects of peroxides. Specifically, HL-60/AR was the more resistant cell line to these oxidants, with the LC_{50} of H_2O_2 obtained for these cells being 12-fold that recorded for HL-60 (321 vs 25 μM) and 6-fold that noted for HL-60/Vinc (321 vs 52 μM). Analogously, the LC_{50} of t-BuOOH recorded for HL-60/AR cells was 11-fold that noted for HL-60 (96 vs 8.5 μM) and more than 2-fold that obtained for HL-60/Vinc (96 vs 36 μM). Although HL-60/Vinc cells did not show significantly enhanced resistance to H_2O_2 ($P = 0.26$), they did prove to be 4 times more resistant than HL-60 cells to t-BuOOH (36 vs 8.5 μM; $P < 0.0001$).

Table 1 Cytotoxicity of peroxides and daunorubicin to HL-60 cells. Effects of inhibition of catalase

Cell line[b]	AT[c]	LC_{50}[a] (μM)		
		H_2O_2	*t*-BuOOH	Daunorubicin
HL-60	−	25 ± 4^{d}	$8.5 \pm 0.2^{f,h}$	$0.032 \pm 0.005^{i,j}$
HL-60	+	33 ± 6	9.1 ± 0.1	0.027 ± 0.001
HL-60/AR	−	$321 \pm 36^{d,e,k}$	$96 \pm 2^{f,g,l}$	1.5 ± 0.4^{i}
HL-60/AR	+	58 ± 1^{k}	45 ± 2^{i}	1.4 ± 0.3
HL-60/Vinc	−	52 ± 10^{e}	$36 \pm 8^{g,h}$	1.7 ± 0.01^{j}
HL-60/Vinc	+	49 ± 1	32 ± 5	1.6 ± 0.15

[a] Cell survival was assessed by the flow cytometric FDA/PI viability assay. Values represent means ± 1 SD from two experiments, each done in duplicate

[b] Cells were cultured under standard conditions in RPMI 1640 medium containing 10% FBS and were exposed to peroxides or daunorubicin during logarithmic growth

[c] ± Preincubation with 100 m*M* AT for 4 h. Cells were washed free of AT immediately prior to exposure to peroxides or daunorubicin

[d–j] Values with the same letter are significantly different from each other (analysis of variance and Scheffe's test of multiple comparisons; $P < 0.0001$)

[k,l] Values with the same letter are significantly different from each other (Mann-Whitney test; $P < 0.03$)

Although the HL-60/AR and HL-60/Vinc cell lines used were originally selected for resistance with doxorubicin and vincristine, respectively, both are known also to be resistant to the anthracycline daunorubicin by virtue of their MDR phenotype [18,20]. This is also documented in Table 1, where, relative to HL-60 cells, both HL-60/AR and HL-60/Vinc cells were 45–50 times more resistant to daunorubicin (LC_{50}, 1.5–1.7 vs. 0.032 μM). The degrees of resistance of HL-60/AR and HL-60/Vinc cells to daunorubicin do not differ significantly and compare favorably with the degrees of anthracycline resistance reported at the times of initial selection and isolation of these MDR cell lines [18,38].

Antioxidant enzyme quantification and activities

Constitutive activities of two major antioxidant enzymes, catalase (EC 1.11.1.6; H_2O_2:H_2O_2 oxidoreductase) and GSH-Px (EC 1.11.1.9; GSH:H_2O_2 oxidoreductase), were determined in the three cell lines. The constitutive level of catalase catalytic specific activity detected in HL-60/AR cells was 3-fold that found in either HL-60 or HL-60/Vinc cells (Table 2). Western-immunoblot analysis of catalase protein expression

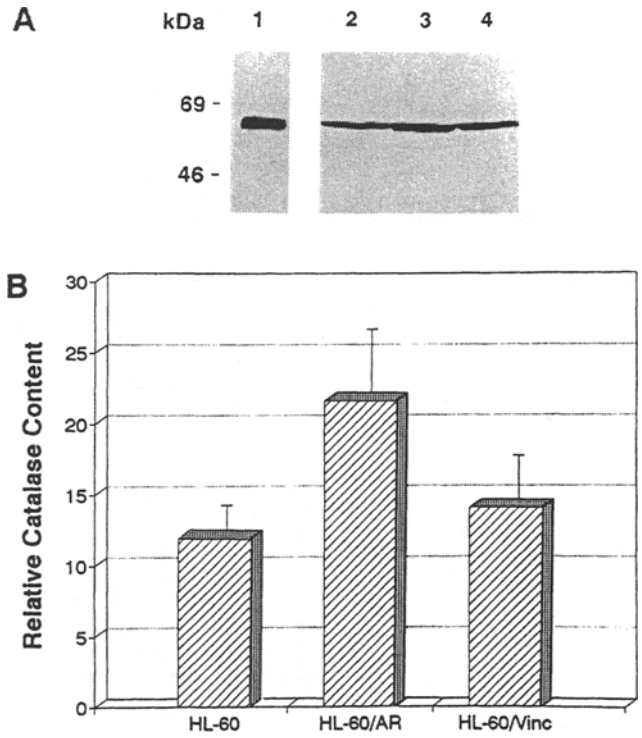

Fig. 1a,b Western-immunoblot analysis of the catalase protein content of cell lysates. **a** *Lane* 1, Purified bovine liver catalase (8 μg protein, Sigma); *lane* 2, HL-60; *lane* 3, HL-60/AR; *lane* 4, HL-60/Vinc. Lanes 2–4 contained 90 μg protein each. **b**Densitometric quantification of the catalase protein content relative to each other (arbitrary units). Values represent mean ± 1 SD of triplicate densitometric determinations. The value obtained for HL-60/AR was significantly greater than that recorded for HL-60 (analysis of variance and Scheffe's test of multiple comparisons; $P < 0.03$)

demonstrated a band with an approximate relative molecular weight of 60,000 Da that corresponded precisely to that obtained with denatured, partially purified bovine liver catalase (Sigma; Fig. 1A). Densitometric analysis of the immunoblot (Fig. 1B) showed nearly 2-fold greater catalase protein expression in HL-60/AR cells as compared with HL-60 cells, consistent with the corresponding enzyme specific activities.

The constitutive levels of selenium-dependent GSH-Px specific activity (assayed using H_2O_2 as the substrate) detected in drug-sensitive HL-60 cells were 1.4-fold those found in the MDR cells HL-60/AR or HL-60/Vinc (Table 2). This observation is in contrast to the reported findings in MDR P388/Adr or MCF-7/Adr cells [7], where GSH-Px activity was higher in the resistant cells than in the parental cell lines. Furthermore, using Western blots, we found that GSH-Px protein expression was comparable in HL-60 and HL-60/AR cells (data not shown). Because an anionic glutathione S-transferase exhibits "non-selenium-dependent" GSH-Px activity but can use only organic peroxides as hydrogen acceptors [12], total GSH-Px activity was determined by using t-BuOOH as the substrate in the enzyme assay. The specific activities

Table 2 Antioxidant enzyme specific activities[a]

Cell line[b]	Catalase[c]	GSH-Px[d]	
		H_2O_2	*t*-BuOOH
HL-60	25.9 ± 2.8[e]	41.6 ± 7.3[g,h]	31.3 ± 1.4[i,j]
HL-60/AR	87.5 ± 2.5[e,f]	30.4 ± 0.9[g]	26.2 ± 1.0[i]
HL-60/Vinc	24.4 ± 3.1[f]	29.7 ± 3.8[h]	23.2 ± 2.4[j]

[a] Activities are expressed as mean values ± 1 SD for 3–6 determinations. Statistical evaluation was done by analysis of variance and Scheffe's test of multiple comparisons

[b] Cells were cultured under standard conditions in RPMI 1640 medium containing 10% FBS and were lysed during logarithmic growth

[c] μmol H_2O_2 decomposed min^{-1} mg $protein^{-1}$ while the H_2O_2 concentration falls from 10.3 to 9.2 m*M* (pH 7.0, 25°C)

[d] nmol NADPH oxidized min^{-1} mg $protein^{-1}$ (pH 7.0, 25°C) using H_2O_2 or *t*-BuOOH as the substrate

[e,f,j] Values with the same letter are significantly different from each other ($P < 0.0001$)

[g–i] Values with the same letter are significantly different from each other ($P < 0.01$)

obtained with t-BuOOH as the substrate did not exceed those obtained using H_2O_2 (Table 2), implying that non-selenium-dependent GSH-Px activity was undetectable in the cells studied. Similar findings were reported for P388/Adr and MCF-7/Adr cells [7].

Effect of inhibition of catalase activity on cellular resistance to peroxides and daunorubicin

Cellular catalase was inhibited by incubating cells for 4 h at 37 °C with 100 m*M* AT, an irreversible inhibitor of catalase [39]. This resulted in 93%, 95%, and 87% inhibition of catalase activity in HL-60, HL-60/AR, and HL-60/Vinc cells, respectively, as compared with untreated control cells (Fig. 2). These incubation conditions were selected after preliminary studies in our laboratory had shown that in all three cell lines, such exposure to AT produced the maximal inhibition of catalase activity with maintenance of ≥ 95% cell viability, assessed by flow cytometry at 72 h after removal of the cells from AT. It was also confirmed that catalase activity remained inhibited by 82% and 71% in HL-60 and HL-60/AR cells, respectively, at 22 h following removal of the cells from AT.

The conditions under which significant inhibition of catalase activity could be achieved without cytotoxicity having thus been established, the effect of such inhibition on cellular resistance to peroxides and daunorubicin was examined. A 4-h preexposure of HL-60/AR cells to 100 m*M* AT immediately prior to exposure to peroxides results in marked reduction of the LC_{50}s for H_2O_2 and t-BuOOH by 82% and 53%, respectively (Table 1). In contrast, identical preexposure of HL-60 or HL-60/Vinc cells to AT resulted in no significant changes in the LC_{50}s for H_2O_2 or

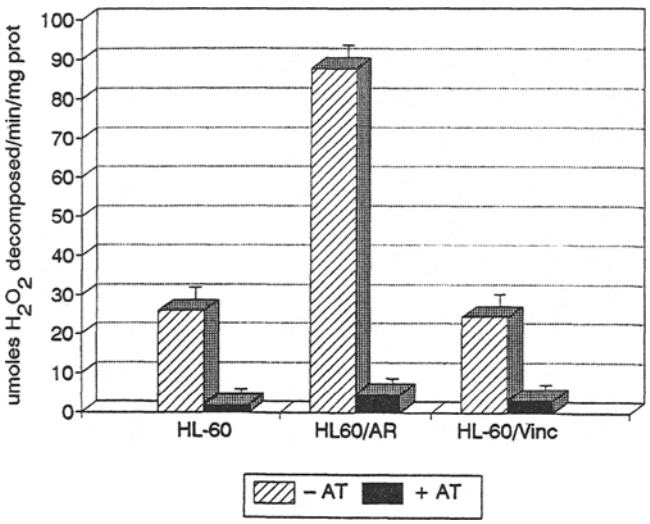

Fig. 2 Inhibition of catalase activity by AT. Cells were incubated for 4 h at 37°C in RPMI 1640 medium containing 10% FBS or 10% FBS + 100 m*M* AT. All cells were then washed 3 times in PBS and cell lysates were prepared. Values represent means ± 1 SD of triplicate determinations. In all 3 cells lines, activities obtained with AT preincubation were significantly lower than those obtained without AT (*t*-test for independent samples; $P < 0.01$)

t-BuOOH (Table 1), although catalase levels had been inhibited by $\geq 87\%$, as stated above. In no cell line was there seen a change in sensitivity to daunorubicin following preincubation with AT (Table 1).

Effect of selenium depletion on GSH-Px activity and cellular resistance to daunorubicin

Despite the absence of elevated GSH-Px activities in HL-60/AR and HL-60/Vinc cells relative to HL-60 cells (Table 2), the reported association between GSH and anthracycline resistance in HL-60/AR [21] prompted us to decipher further the role of the GSH-redox cycle in the resistance of HL-60/AR cells to daunorubicin. Significant modulation of selenium-dependent GSH-Px activity in HL-60 (11-fold range) and HL-60/AR (23-fold range) cells was achieved by culturing them for > 1 week in serum-deficient ($\leq 1\%$ FBS) RPMI 1640 medium with or without supplemental selenium. As compared with the selenium-supplemented controls, selenium-deficient conditions caused a 91% and 96% diminution in GSH-Px activity in HL-60 and HL-60/AR cells, respectively. That this modulation of GSH-Px activity was of a magnitude that could result in marked perturbation of the GSH-redox cycle was documented by a reduction in the baseline (unstimulated) activity of the HMPS under selenium-deficient conditions and by a marked reduction in the ability of the oxidant t-BuOOH to stimulate the HMPS (Fig. 3). Western-blot analysis of cells cultured in selenium-supplemented or selenium-deficient

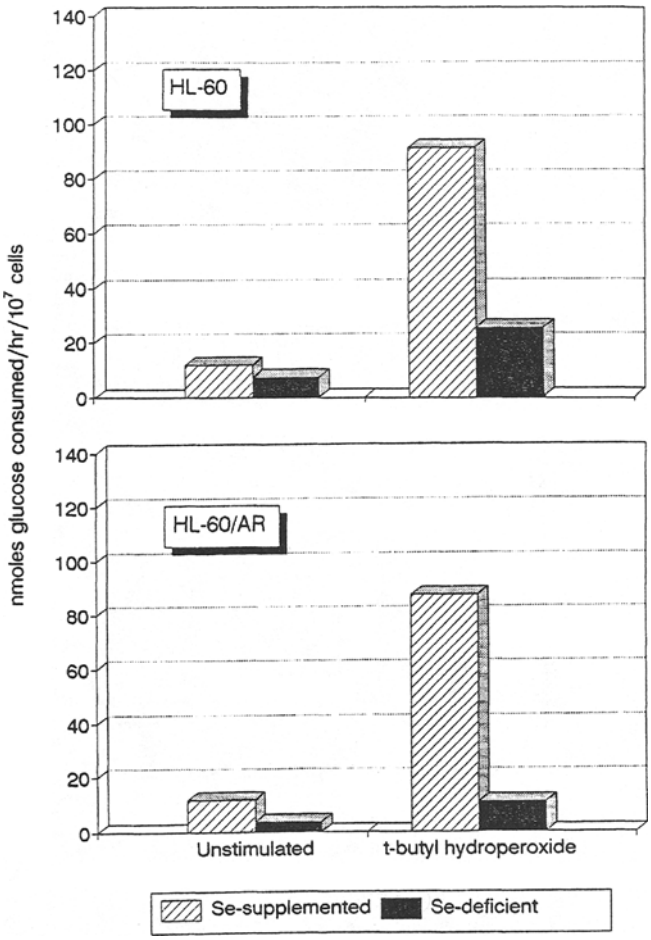

Fig. 3 Effect of selenium (*Se*) deficiency on baseline and inducible HMPS activity via the GSH-redox cycle. Following cell culture in Se-supplemented (≥ 330 n*M* Se) vs Se-deficient (≤ 3 n*M* Se) media for 1 week, stimulation of the GSH-redox cycle was achieved by incubation of equal numbers of viable cells (2.5×10^6/ml) with 500 μM *t*-BuOOH for 1 h. Values represent means of duplicate determinations

medium revealed diminution of GSH-Px protein to barely detectable levels in selenium-deficient cultures (data not shown). Although these culture conditions produced marked changes in the capacity of HL-60 and HL-60/AR cells to react to oxidative stress via the GSH-redox cycle, they did not translate into significant effects on resistance to daunorubicin in either cell line (Fig. 4).

One possible explanation for the inability of catalase inhibition or selenium depletion to sensitize HL-60/AR cells to the cytotoxic effects of daunorubicin could be that these cells are incapable of reducing daunorubicin to its semiquinone, consequently preventing the cascade of reactive oxygen species (superoxide free radical, H_2O_2, hydroxyl free radical) that could be generated by the semiquinone. To investigate this possibility, we studied the generation of daunorubicin semiquinone free radical in these cells using ESR.

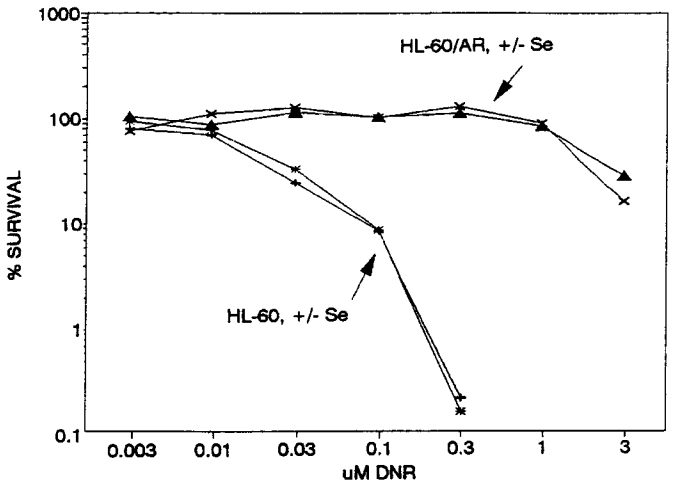

Fig. 4 Effect of inhibition of GSH-Px activity on resistance of HL-60 and HL-60/AR cells to daunorubicin (DNR). Representative survival curves of cells grown in serum-deficient media with or without supplemental selenium (Se; $\geq$ 330 vs $\leq$ 3 n*M*, respectively) for at least 1 week prior to exposure to daunorubicin. Cell viability was determined by the flow cytometric FDA/PI assay at 72 h after the addition of DNR. Points reflect individual experiments, with values representing means of duplicate determinations. The coefficient of variation for each point shown is < 15%

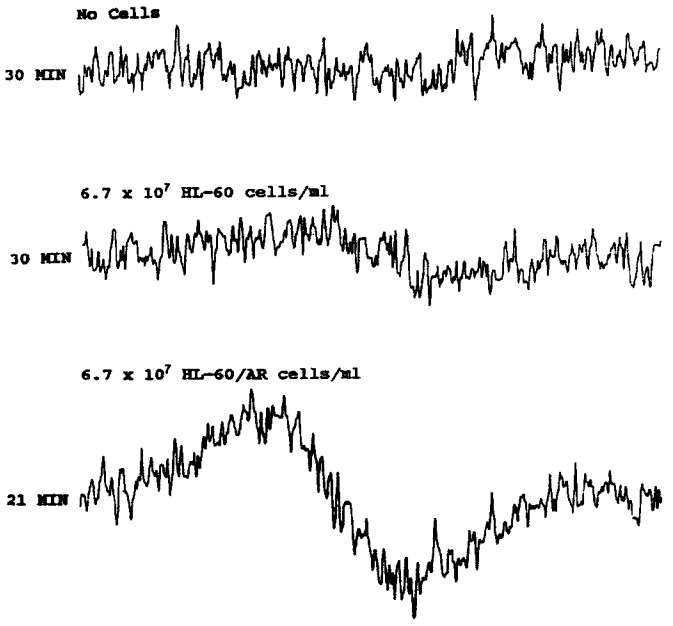

Fig. 5 ESR spectra of maximal daunorubicin semiquinone formation under anaerobic conditions. Washed whole cells suspended in RPMI 1640 medium without serum (6.7×10^7cells/ml) were exposed to 400 μM daunorubicin under anaerobic conditions (nitrogen gas bubbling for 15 min) at 25°C in the presence of 800 μM NADPH. ESR spectra showing the maximal intensity achieved following exposure of either HL-60 or HL-60/AR cells to daunorubicin are displayed. The times at which the maximal spectra were recorded are shown and are compared with a background spectrum for medium plus daunorubicin and NADPH only (*No Cells*). ESR parameters were: incident microwave power, 10 mW; modulation amplitude, 1.25 G; receiver gain, 20×10^4; time constant, 0.5 s; and scan rate, 2.5 G/min

ESR spectra of daunorubicin semiquinone formation

Because the concentration of the semiquinone is only a fraction of the concentration of parent compound in these systems [3], relatively high extracellular concentrations of daunorubicin are routinely required for the detection of daunorubicin semiquinone free-radical formation. Hence, intact cells were exposed to 400 μM daunorubicin under anaerobic conditions to allow accumulation of the semiquinone free radical by preventing its reoxidation back to the original stable daunorubicin quinone. Paradoxically, the generation of daunorubicin semiquinone detected in the resistant HL-60/AR cells was actually 3 times *greater* and occurred 30% *faster* (21 vs. 30 min) than that observed in an equal number of HL-60 cells (Fig. 5). To test the universality of this finding, ESR studies were carried out following exposure of cells to 400 μM diaziquone, an antitumor agent known to be activated to its semiquinone free-radical species in HL-60 and K562 human leukemia cell lines [40]. Even under aerobic conditions, diaziquone semiquinone was detectable in either HL-60/AR or HL-60 cells, but the semiquinone appeared nearly twice as fast (12 vs 22 min) in the relatively more diaziquone-resistant HL-60/AR cells (data not shown). This paradoxical phenomenon has also been reported to occur in MDR P388 cells selected for resistance with diaziquone [41].

Discussion

The "dual mechanism of drug resistance" proposed by Kramer et al. [7], based on observations of an increased GSH-redox cycling capacity in Pgp-overexpressing MDR cells, brought forth the intriguing suggestion that the definition of MDR be broadened to include enhanced antioxidant defenses in addition to the enhanced drug efflux mediated by Pgp. Our studies clearly show that MRP-overexpressing HL-60/AR cells have augmented antioxidant defenses, defined by their resistance to the oxidants H_2O_2 and t-BuOOH. However, unlike the observations in Pgp-overexpressing MDR P388 or MCF-7 cells [7], the antioxidant defenses seen in HL-60/AR cells were characterized not by an alteration in GSH-redox cycling but by increased catalase protein and activity. Pgp-overexpressing HL-60/Vinc were also found to be resistant to t-BuOOH, although this effect was significantly weaker than that displayed by HL-60/AR cells. GSH-Px activity was not elevated in HL-60/Vinc cells relative to wild-type HL-60 cells, unlike the Pgp-overexpressing MDR cells described previously [7].

The higher activity of catalase observed in HL-60/AR cells does not contribute to their resistance to daunorubicin, since treatment of cells with AT was capable of causing cellular sensitization only to the oxidants H_2O_2 or t-BuOOH but not to daunorubicin.

Inhibition of GSH-redox cycling by means of lowering GSH-Px activity under selenium-deficient culture conditions, similarly, did not sensitize the cells to daunorubicin, suggesting that GSH-redox cycling is not critical to the detoxification of daunorubicin in either HL-60 or HL-60/AR cells. These data suggest that H_2O_2 does not play a primary role in the mechanism of cytotoxicity of daunorubicin to HL-60 or HL-60/AR cells. Although we could demonstrate by ESR that the ability of HL-60/AR cells to generate daunorubicin semiquinone free radical is not reduced as compared with that of wild-type cells, the sensitivity of our ESR instrument may be such that at the LC_{50} concentrations of 32 n*M* and 1.5 μM obtained for HL-60 or HL-60/AR cells, respectively, the semiquinone may be difficult to detect. Hence, we cannot assess by ESR the amount of daunorubicin semiquinone free radical produced at physiologically relevant concentrations of daunorubicin. Taking this and the above-mentioned observations into consideration, it may be reasonable to conclude that the contribution to the overall cytotoxicity of daunorubicin caused by H_2O_2 generated from drug concentrations in the range of the LC_{50} for HL-60/AR is small and can be easily thwarted by endogenous catalase or peroxidases, even after partial (> 90%) inhibition of these enzymes, as was accomplished in our studies.

Although we found that inhibition of the GSH-redox cycle had no effect on the daunorubicin sensitivity of HL-60/AR or wild-type cells, Lutzky et al. [21] demonstrated that GSH depletion by BSO did partially sensitize HL-60/AR but not wild-type HL-60 cells to daunorubicin. This indicates that some GSH-dependent enzyme system appears to offer protection to HL-60/AR cells against the cytotoxic effects of daunorubicin. Though these authors found equivalent overall glutathione S-transferase activities in HL-60/AR and HL-60 cells, they discovered, using the fluorogenic dye monochlorobimane [42], that the intracellular distribution of glutathione S-transferase and GSH differed markedly, being highly localized to the Golgi apparatus in the resistant cells and having a diffuse cytoplasmic and nuclear distribution in wild-type cells. They implicated this specific localization of GSH/glutathione S-transferase as being a component of the resistance to daunorubicin manifested by HL-60/AR cells. Furthermore, these authors found equivalent GSH-Px activities in the sensitive and resistant cell lines. Hence, these findings complement the observations we present herein regarding the lack of involvement of GSH-redox cycling in curtailing the cytotoxic effects of daunorubicin and our conclusion that the cytotoxicity caused by reactive oxygen species (or at least H_2O_2 and related peroxides) generated from daunorubicin is probably nil at concentrations of drug that encompass the LC_{50} values for these cell lines.

Taking all these observations into consideration, it seems reasonable that the sensitizing effect of BSO may be mediated by limiting the supply of GSH available to cellular gluthathione S-transferases, thus impairing the ability of these diverse enzymes to conjugate and detoxify a xenobiotic such as daunorubicin. Although no one has yet reported a GSH conjugate of daunorubicin, if such a conjugate is formed, then under selenium-deficient conditions there must be enough intracellular reduced GSH present to meet the requirements of the glutathione S-transferases. Furthermore, if glutathione S-transferases are a component of the resistance of HL-60/AR cells, they represent a GSH-dependent enzyme system that does not detoxify reactive oxygen species (with the exception of the anionic glutathione S-transferases that have non-selenium-dependent GSH-Px activity [12], which we find to be virtually absent in HL-60 cells). This point lends support to our conclusion that cytotoxic oxidants generated from daunorubicin at physiologically relevant concentrations are minimal.

These studies are the first to demonstrate differential catalase activities among HL-60/AR and HL-60 cells as well as the dependent relationship between catalase and the enhanced peroxide resistance of HL-60/AR cells. In contrast to the murine cell lines of Nathan et al. [43] and doxorubicin-resistant MCF-7 cells [9], the resistance of HL-60/AR cells to H_2O_2 and t-BuOOH appears to be mediated at least in part by enhanced catalase activity. This finding complements the recent work of Kasuga and Yamada [44], who showed that of the antioxidant enzyme activities assayed (catalase, SOD, and GSH-Px) in peroxide-resistant HL-60 cells selected with different concentrations of H_2O_2, only catalase activity consistently increased in a dose-dependent fashion. They also demonstrated that myeloperoxidase in HL-60 cells becomes undetectable upon selection with H_2O_2, implying that it plays an insignificant role in H_2O_2 resistance in these cells. In our investigation, Western-immunoblot analysis confirmed that the enhanced catalase activity was a consequence of augmented catalase protein expression in HL-60/AR cells. Although catalase activities in HL-60, HL-60/AR, and HL-60/Vinc cells were significantly inhibited by AT, only in HL-60/AR cells did we see any decrease in resistance to peroxides. That HL-60/Vinc maintained its peroxide-resistance profile despite catalase inhibition implies that its mechanism of enhanced resistance to t-BuOOH is predominantly independent of catalase. Inhibition of catalase in HL-60/AR cells also decreased their resistance to t-BuOOH. Although t-BuOOH does not react directly with catalase [45], potentially toxic metabolites of t-BuOOH do. These metabolites may consist of a variety of alcohols (e.g., tert-butyl alcohol) that can be produced following reaction of t-BuOOH with cellular peroxidases (e.g., GSH-Px) under conditions of low intracellular concentrations of H_2O_2 [46].

These studies have helped to define the alterations in antioxidant metabolism that accompany drug

resistance associated with overexpression of MRP. Although antioxidant defenses were enhanced in these MDR cells, the changes do not support a "dual mechanism of resistance" in MRP-overexpressing cells, since the enhanced antioxidant defenses displayed did not appear to contribute to resistance to daunorubicin. In this sense, these studies may serve to expand the delineation of the "spectrum" of alterations in antioxidant defenses that accompany the development of transport-mediated MDR. Whether these enhanced antioxidant changes reflect cellular adaptations to a hostile environment that are linked to the overexpression of MRP or simply represent an epiphenomenon of the acquisition of drug resistance remains to be determined. These data from MRP-overexpressing HL-60/AR cells and Pgp-overexpressing HL-60/Vinc cells are consistent with the hypothesis that transport-mediated drug resistance is frequently accompanied by enhanced antioxidant defenses [7], but the specific defenses manifested can be diverse and may not necessarily be a component of the drug resistance manifested by the cell line.

Acknowledgements The authors wish to express their appreciation for the excellent technical assistance of Patricia Wooten of immunoblot analysis, Dennis Cuddy for tissue culture, and Natalie Wehman and Dr. José V. Ordóñez for flow cytometry. We thank Dr. Allen Eskenazi for his assistance in establishing the HMPS assay and Dr. Raji Sridhara for help with statistical analysis. We thank Dr. Harvey Cohen of the University of Rochester for providing us with the antibody to GSH-Px that was used in Western-blot studies. We are also grateful to Drs. Merrill Egorin and Nicholas Bachur for their suggestions and reviews of the manuscript.

References

1. Myers CE, Chabner BA (1990) Anthracyclines. In: Chabner BA, Collins JM (eds) Cancer chemotherapy, principles and practice. Lippincott, Philadelphia, p 356
2. Bachur NR, Gordon SL, Gee MV (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res 38:1745–1750
3. Gutierrez PL, Gee MV, Bachur NR (1983) Kinetics of anthracycline antibiotic free radical formation and reductive glycosidase activity. Arch Biochem Biophys 223:68–75
4. Powis G (1987) Anthracycline metabolism and free radical formation. In: Powis G, Prough RA (eds) Metabolism and action of anti-cancer drugs. Taylor and Francis, New York, pp 211–260
5. Doroshow JH (1986) Role of hydrogen peroxide and hydroxyl radical formation in the killing of Ehrlich tumor cells by anticancer quinones. Proc Natl Acad Sci USA 83:4514–4518
6. Sinha BK, Katki AG, Batist G, Cowan KH, Myers CE (1987) Differential formation of hydroxyl radicals by Adriamycin in sensitive and resistant MCF-7 human breast tumor cells: implications for the mechanism of action. Biochemistry 26:3776–3781
7. Kramer RA, Zakher J, Kim G (1988) Role of the glutathione redox cycle in acquired and de novo multidrug resistance. Science 241:694–697
8. Gessner T, Vaughan LA, Beehler BC, Bartels CJ, Baker RM (1990) Elevated pentose cycle and glucuronyltransferase in daunorubicin-resistant P388 cells. Cancer Res 50:3921–3927
9. Yeh GC, Occhipinti SJ, Cowan KH, Chabner BA, Myers CE (1987) Adriamycin resistance in human tumor cells associated with marked alterations in the regulation of the hexose monophosphate shunt and its response to oxidant stress. Cancer Res 47:5994–5999
10. Kartner N, Ling V (1988) Multidrug resistance in cancer. Sci Am 260:44–51
11. List AF (1993) Multidrug resistance: clinical relevance in acute leukemia. Onocology 7:23–38
12. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE, Cowan KH (1986) Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. J Biol Chem 261:15544–15549
13. Akman SA, Forrest G, Chu F, Esworthy RS, Doroshow JH (1990) Antioxidant and xenobiotic-metabolizing enzyme gene expression in doxorubicin-resistant MCF-7 breast cancer cells. Cancer Res 50:1397–1402
14. Mimnaugh EG, Dusre L, Atwell J, Myers CE (1989) Differential oxygen radical susceptibility of Adriamycin-sensitive and resistant MCF-7 human breast tumor cells. Cancer Res 49:8–15
15. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650–1654
16. Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley RG (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res 54:357–361
17. Krishnamachary N, Center MS (1993) The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. Cancer Res 53:3658–3661
18. Bhalla K, Hindenburg A, Taub RN, Grant S (1985) Isolation and characterization of an anthracycline-resistant human leukemic cell line. Cancer Res 45:3657–3662
19. Ross DD, Wooten PJ, Tong Y, Sridhara R, Ordonez JV, Lee EJ, Schiffer CA (1993) Enhancement of daunorubicin accumulation, retention, and cytotoxicity by verapamil or cyclosporin A in blast cells from patients with previously untreated acute myeloid leukemia. Blood 82:1288–1299
20. Ross DD, Wooten PJ, Tong Y, Cornblatt B, Levy C, Sridhara R, Lee EJ, Schiffer CA (1994) Synergistic reversal of multidrug resistance phenotype in acute myeloid leukemia cells by cyclosporin A and cremophor EL. Blood 83:1337–1347
21. Lutzky J, Astor MB, Taub RN, Baker MA, Bhalla K, Gervasoni JE Jr, Rosado M, Stewart V, Krishna S, Hindenburg AA (1989) Role of glutathione and dependent enzymes in anthracycline-resistant HL-60/AR cells. Cancer Res 49:4120–4125
22. Marquardt D, McCrone S, Center MS (1990) Mechanisms of multidrug resistance in HL-60 cells: detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. Cancer Res 50:1426–1430
23. Badwey JA, Barnovsky ML (1979) Production of superoxide and hydrogen peroxide by an NADH-oxidase in guinea pig polymorphonuclear leukocytes: modulation by nucleotides and divalent cations. J Biol Chem 254:11530–11537
24. Beutler E (1984) Red cell metabolism: a manual of biochemical methods, 3rd edn. Grune and Stratton, New York, pp 74–76
25. Collins SJ (1987) The HL-60 promyelocytic leukemia cell line: proliferation, Differentiation, and cellular oncogene expression. Blood 70:1233–1244
26. Breitman TR, Collins SJ, Keene BR (1980) Replacement of serum by insulin and transferrin supports growth and differentiation of the human promyelocytic cell line, HL-60. Exp Cell Res 126: 494–498
27. Speier C, Baker SS, Newburger PE (1985) Relationships between in vitro selenium supply, glutathione peroxidase activity, and phagocytic function in the HL-60 human myeloid cell line. J Biol Chem 260:8951–8955

28. Ross DD, Joneckis CC, Ordonez JV, Sisk AM, Wu RK, Hamburger AW, Nora RE (1989) Estimation of cell survival by flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number. Cancer Res 49:3776–3782
29. Sharpe PT (1988) Methods of cell separation. Elsevier, New York, pp 15–17
30. Cohen HJ, Tape EH, Novak J, Chovaniec ME, Liegey P, Whitin JC (1987) The role of glutathione reductase in maintaining human granulocyte function and sensitivity to exogenous H_2O_2. Blood 69:493–500
31. Rosenberg LE, Weinberg AN, Segal S (1961) The effect of high galactose diets on urinary excretion of amino acids in the rat. Biochim Biophys Acta 48:500–505
32. Fisher GR, Gutiérrez PL (1991) The reductive metabolism of diaziquone (AZQ) in the S9 fraction of MCF-7 cells: free radical formation and NAD(P)H: quinone-acceptor oxidoreductase (DT-diaphorase) activity. Free Rad Biol Med 10:359–370
33. Beutler E (1984) Red cell metabolism: a manual of biochemical methods, 3rd edn. Grune and Stratton, New York, p 11
34. Sigma Chemical Co. (1991) Catalase. In: Biochemicals, organic compounds for research, and diagnostic reagents. Sigma Chemical Co., St. Louis, p 218
35. Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195:133–140
36. Takahashi U, Cohen HJ (1986) Selenium-dependent glutathione peroxidase protein and activity: immunological investigations on cellular and plasma enzymes. Blood 68:640–645
37. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
38. McGrath T, Center MS (1987) Adriamycin resistance in HL-60 cells in the absence of detectable P-glycoprotein. Biochem Biophys Res Commun 145:1171–1176
39. Michiels C, Remacle J (1988) Use of inhibition of enzymatic antioxidant systems in order to evaluate their physiological importance. Eur J Biochem 177:435–441
40. Gutierrez PL, Egorin MJ, Fox BM, Friedman R, Bachur NR (1985) Cellular activation of diaziquone [2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone] to its free radical species. Biochem Pharmacol 34:1449–1455
41. Gutierrez PL, Wilder PJ, Biswal N (1989) In vitro multidrug resistance of P388 murine leukemia selected for resistance to diaziquone. Cancer Commun 1:181–190
42. Rice GC, Bump EA, Shrieve DC, Lee W, Kovacs M (1986) Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: some applications to radiation and drug resistance in vitro and in vivo. Caner Res 46:6105–6110
43. Nathan CF, Arrick BA, Murray HW, DeSantis KM, Cohn ZA (1980) Tumor cell anti-oxidant defenses: inhibition of the glutathione redox cycle enchances macrophage-mediated cytolysis. J Exp Med 153:766–782
44. Kasuga I, Yamada M (1992) High production of catalase in hydrogen peroxide-resistant human leukemia HL-60 cell lines. Leuk Res 16:173–179
45. Rush GF, Alberts D (1986) tert-Butyl hydroperoxide metabolism and stimulation of the pentose phosphate pathway in isolated rat hepatocytes. Toxicol Appl Pharmacol 85:324–331
46. Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527–605