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Mechanisms of Resistance to Ansamycin Antibiotics in Human Breast Cancer Cell Lines

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SUMMARY

We recently reported that multidrug-resistant, P-170 glycoprotein-positive, Adriamycin-selected, human breast tumor (MCF7/ADR^R) cells were resistant to the benzoquinonoid ansamycin antibiotics geldanamycin (GL) and herbimycin A (HA) and that significantly fewer hydroxyl radicals were formed in resistant cells. We have carried out additional studies to define the mechanisms of cytotoxicity of and resistance to GL and HA, by directly examining the interactions of these drugs with P-170 glycoprotein using photoaffinity labeling. We found that both GL and HA inhibited binding of azidopine to P-170 glycoprotein in a dose-dependent manner. We have developed a 10-fold GL-resistant cell line (MCF7/GL^R) by continuous drug exposure. Our studies indicated no significant differences in free radical formation between wild-type MCF7 cells and MCF7/GL^R cells. Uptake and efflux studies indicated a small decrease in the GL accumulation but no difference in the efflux of GL in these cells. Verapamil had no effect on cellular accumulation of GL in wild-type MCF7 cells or MCF7/GL^R cells. Verapamil significantly increased the accumulation of GL in MCF7/ADR^R cells and enhanced GL cytotoxicity 12-fold, suggesting that GL interacted with the P-170 glycoprotein. Using reverse transcription-polymerase chain reaction, we found no expression of the *mdr1* gene; however, expression of the multidrug resistance-associated protein was about 2-fold higher in MCF7/GL^R cells. Taken together, these studies indicate that the mechanisms of GL resistance are multifactorial. Although decreased free radical formation may not play a significant role in low levels of GL resistance, e.g., in MCF7/GL^R cells, both overexpression of *mdr1* and decreased free radical formation contribute to GL resistance in highly resistant cells such as MCF7/ADR^R cells.

The benzoquinone ansamycin antibiotics GL and HA have been reported to be cytotoxic to several tumor cell lines *in vitro* (1, 2). Although these antibiotics are potent inhibitors of *src* tyrosine kinases *in vitro* and have been shown to revert the phenotype of tyrosine kinase oncogene-transformed cell lines (2, 3), very little is known about their mechanisms of action and tumor cell kill. Recently, it was shown that the anticancer properties of GL did not appear to result from the inhibition of *src* kinase in certain tumor cells (4).

Because GL and HA contain quinone structures (Fig. 1) and because quinone-containing anticancer antibiotics, e.g., ADR, are enzymatically activated to reactive free radicals (5–10), we recently initiated studies to evaluate the roles of GL- and HA-induced free radical formation in cytotoxicity and resistance in human breast tumor MCF7 cells. We found that both GL and HA readily formed reactive oxygen radicals after reductive activation and, interestingly, significantly fewer of these radical intermediates were detected in the MDR breast tumor cell line MCF7/ADR^R (11). Furthermore, these cells were cross-resistant to GL and HA, suggesting a role for free radicals in the cytotoxicity of and resistance to GL and HA. We also found that verapamil, which binds to PgP (12, 13), partially reversed the resistance to GL and HA in MDR MCF7/ADR^R cells, suggesting that PgP may play a role in the mechanism of resistance to GL and HA in MCF7/ADR^R cells (11).

Because of these findings with GL, which is currently under consideration for clinical trails, we have carried out additional studies to define the mechanisms of cytotoxicity and resistance, by directly examining the interactions of GL and HA with PgP, using photoaffinity labeling. We have also developed a GL-resistant MCF7 cell line by continuous drug exposure, and we

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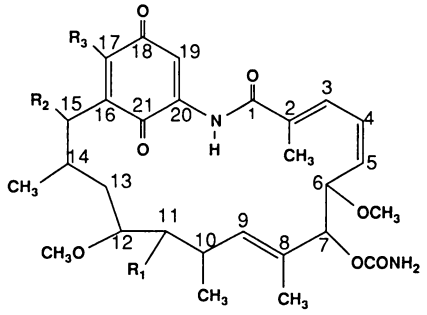
ABBREVIATIONS: GL, geldanamycin; HA, herbimycin A; ADR, Adriamycin; VCR, vincristine; MCF7/WT cells, sensitive wild-type MCF7 cells; MCF7/ADR^R cells, Adriamycin-selected resistant MCF7 cells; MCF7/GL^R cells, geldanamycin-selected resistant MCF7 cells; MDR, multidrug resistance or resistant; PgP, P-170 glycoprotein; MRP, multidrug resistance-associated protein; RT, reverse transcription; PCR, polymerase chain reaction; DMPO, 5,5′-dimethylpyrroline-*N*-oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; EGF, epidermal growth factor; GST, glutathione-*S*-transferase; GSH, glutathione; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; VP-16, etoposide.



	R_1	R_2	R_3
Geldanamycin (GL)	OH	H	OCH_3
Herbimycin A (HA)	OCH_3	OCH_3	H

Fig. 1. Chemical structures of GL and HA.

have used these cells to examine the roles of both free radical formation and PgP in GL resistance. Furthermore, because of recent implications of MRP in drug resistance (14, 15), we examined the expression of MRP in MCF7/GL^R cells using RT-PCR.

Materials and Methods

Chemicals. The chemicals used in this study, i.e., GL (NSC 122750), HA (NSC 305978), and ADR (NSC 123127), were provided by the Drug Synthesis and Chemistry Branch and the Drug Development Branch of the National Cancer Institute. GL and HA were dissolved in DMSO as 20 mM and 5 mM stock solutions, respectively, and were stored at −20°. Poly(Glu-Tyr) (4:1; M_r >50,000), MTT, superoxide dismutase, catalase, NADPH, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was obtained from DuPont. [^{3}H]GL, synthesized by Mr. E. G. Mimnaugh, was kindly provided by Dr. L. Whitesell (National Cancer Institute, National Institutes of Health, Bethesda, MD). DMPO was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was purified by passage over activated charcoal for spin-trapping studies.

Cell culture. MCF7 (WT and ADR-resistant) cells were provided by Dr. Ken Cowan (National Cancer Institute, National Institutes of Health). MCF7/GL^R cells were selected with stepwise increasing concentrations of GL (0.003–0.01 μM) over a period of 6 months. MCF7 cells were grown in improved minimum essential medium, supplemented with 2 mM L-glutamine, 2 mg/ml L-proline, 50 μg/ml gentamicin, and 5% fetal calf serum, under standard tissue culture conditions at 37° in a humidified 5% CO_2 atmosphere.

Cytotoxicity studies. For cytotoxicity studies, cells were harvested by trypsinization and plated at 2000 cells/well in 96-well microtiter plates (Costar, Cambridge, MA). Cells were allowed to reattach overnight, drugs were added, and cells were incubated for 5 days. The cells were then incubated with MTT for 3 hr and centrifuged at 2000 × *g*, and the medium was decanted. DMSO was then added, plates were gently shaken for 30 min, and the absorbance at 570 nm was read with a kinetic microplate reader (Molecular Devices, Menlo Park, CA), as described previously (11). For the clonogenic assay, 200 cells were plated and exposed to GL for 2 hr. The medium was replaced with fresh medium and cells were allowed to grow for 14 days, stained, and counted.

Free radical formation. The ESR studies were carried out as described previously (11, 16–19). Briefly, cells were harvested by trypsinization, washed twice with ice-cold PBS without calcium or magnesium, pH 7.4, and resuspended in ice-cold PBS at a cell density of 5 × 10^6/ml. The hydroxyl free radical formation was evaluated as DMPO-OH adduct formed. The ESR spectrum was recorded with a Varian E-109 spectrometer operating at 9.5 GHz.

GSH and GSH-related enzyme assays. GSH peroxidase activity was measured by the method of Paglia and Valentine (20), using hydrogen peroxide as the substrate; GST activity was assayed by monitoring the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (21), and GSH levels were measured by the kinetic assay of Tietze (22).

GST isozyme analysis. Cell lysates from MCF7/WT and MCF7/GL^R cells were prepared and 100 μg of cytosolic proteins from each cell line were loaded onto 14% polyacrylamide gels, with purified human GST standards, as described previously (23). The gel was electroblotted onto nitrocellulose (Schleicher & Schuell), blocked with 5% nonfat milk for 1 hr, and then incubated for 2 hr with affinity-purified rabbit anti-human polyclonal antibodies directed against each of the three (α, μ, and π) classes of GST (diluted 1/1000 in 5% nonfat milk). Blots were washed several times with PBS and then incubated for 2 hr with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies diluted 1/1000 in nonfat milk. After several washes with PBS, the blot was developed for 10 min in PBS containing 15% methanol, 0.5 mM 4-chloro-1-naphthol, and 0.15% hydrogen peroxide.

Uptake and efflux studies. Uptake and efflux studies were carried out as described previously (24, 25), using [^{3}H]GL (10 μM) and [^{3}H]VCR (2 μM). Effects of verapamil (50 μM) on the uptake of GL (10 μM) were similarly carried out in these cell lines.

Photoaffinity labeling with [^{3}H]azidopine. For photolabeling studies, 5 × 10^5 cells/assay in PBS containing 4% DMSO, in a final volume of 50 μl, were photolabeled with 0.5 μM [^{3}H]azidopine (specific activity, 44 Ci/mmol; Amersham Corp, Chicago, IL). The reaction mixture was preincubated for 30 min at 25°, in the presence or absence of the nonradioactive drugs (GL or HA), and was then irradiated for 20 min with a UV lamp equipped with two 15-W self-filtering 302-nm lamps, as described previously (26, 27). The photolabeled membranes were directly analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 5–15% gradient gel containing 4.5 M urea, followed by fluorography.

RT-PCR studies. One microgram of total RNA, isolated according to the method of Chomczynski and Sacchi (28), from MCF7/WT, MCF7/GL^R, MCF7/ADR^R, or MCF7/VP cells was reverse transcribed in 20 μl of RT buffer (10 mM Tris·HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM concentrations each of dATP, dGTP, dCTP, and dTTP) containing 2 units/μl RNase inhibitor, 0.003 A_{260} units of random hexanucleotides (both from Boehringer Mannheim, Indianapolis, IN), and 0.4 unit/μl avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). After incubation at 25° for 10 min and at 42° for 15 min, the reaction was terminated by incubation at 99° for 5 min. The resulting cDNA mixture was diluted 10- or 100-fold in RT buffer. The 10-fold dilutions were used to amplify MRP and *mdr1*, and the 100-fold dilutions were used to amplify the control gene *G3PDH* in the presence of 0.1 μl of DIG-dUTP (Boehringer Mannheim), using the hot-start modification (29). Amplification conditions included 25 cycles of 10 sec at 95° and 15 sec at 60°. The primers used were as follows: *G3PDH*, 5′ primer, nucleotides 75–100, 3′ primer, nucleotides 670–696 (30); MRP, 5′ primer, nucleotides 793–818, 3′ primer, nucleotides 1063–1088 (14); *mdr1*, 5′ primer, nucleotides 410–441, 3′ primer, nucleotides 664–695 (31). Fifteen microliters of the products were separated on a 2% agarose gel in TBE buffer. The gels were denatured, neutralized, and transferred to positively charged nylon membranes (Boehringer Mannheim) in 10× sodium saline citrate overnight. The PCR products were then detected by the Genius lumiphoschemiluminescence method, according to the manufacturer's instructions (Boehringer Mannheim). The amount of each band was quantitated by densitometry (Hoeffer densitometer program GS 370) and normalized to the amount of *G3PDH*. Under our experimental conditions, the amount of PCR products formed was directly proportional to the amount of MRP RNA present in the samples.

Tyrosine kinase activity assay. The tyrosine kinase activity in

cell lysates (prepared by brief sonication in 10 mM Tris·HCl, pH 7.4, at 4°) was measured by using poly(Glu-Tyr) (4:1) as a substrate. The reaction mixture contained 5 μl of poly(Glu-Tyr) (10 mg/ml), 5 μl of bovine serum albumin (1 mg/ml), cell lysate (50 μg), and 5 μl of EGF (100 μg/ml), in a total volume of 50 μl. The reaction was initiated by addition of radiolabeled ATP at 37° for 10 min. The reaction was stopped by addition of 200 μl of 10% trichloroacetic acid. The cell pellets were washed extensively with ice-cold trichloroacetic acid, dissolved in 1 N NaOH, neutralized with 1 N HCl, and counted for radioactivity. The effects of GL were measured on both total and EGF-dependent tyrosine kinase activity by including 20 μM GL in the incubation mixtures.

Results

Cytotoxicity studies. As depicted in Fig. 2 and reported previously, GL was significantly less cytotoxic to MCF7/ADR^R cells than to MCF7/WT cells, with a resistance factor of 100 (Table 1). These cells were also found to be cross-resistant to HA; however, HA was less active than GL in both MCF7/WT and MCF7/ADR^R cells (Table 1). As shown in Fig. 2, GL-selected MCF7 cells were 9-fold resistant to GL and 2-fold resistant to HA by the MTT assay (Table 1). The clonogenic assay also indicated that MCF7/GL^R and MCF7/ADR^R cells were 10-fold and 65-fold resistant to GL, respectively; IC_{50} values (GL, 2 hr) were 0.32 ± 0.008 μM for MCF7/WT cells, 3.0 ± 0.20 μM for MCF7/GL^R cells, and 21.0 ± 0.75 μM for MCF7/ADR^R cells. The respective IC_{50} values obtained in the clonogenic assay were significantly higher, due to a shorter drug exposure, than those obtained with the MTT assay, where drug exposure was for 5 days. Interestingly, further exposure of cells to higher concentrations of GL did not result in increased resistance to GL or HA. MCF7/GL^R cells were also cross-resistant (4-fold) to ADR, whereas no significant difference in cytotoxicity was observed between MCF7/WT and MCF7/GL^R cells with either VP-16 or camptothecin (Table 1), indicating no modifications in either topoisomerase activity or proteins. Furthermore, a small difference in VCR cytotoxicity was found between MCF7/WT and MCF7/GL^R cell lines (Table 1).

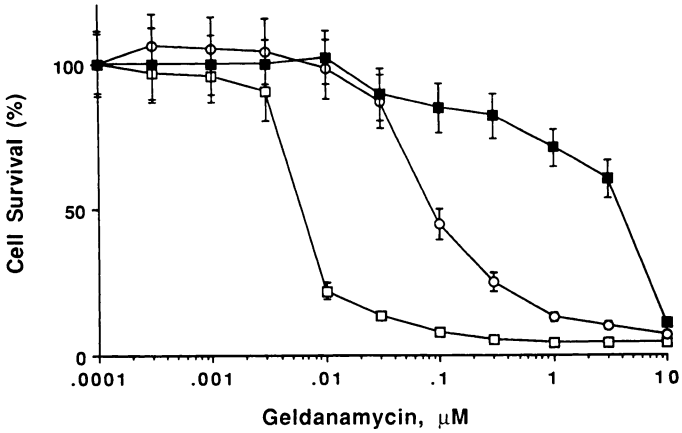


Fig. 2. Cytotoxicity profile of GL obtained in MCF7/WT (□), MCF7/GL^R (○), and MCF7/ADR^R (■) cells during 5-day continuous exposure, determined by the MTT assay. Data represent mean ± standard deviation of at least three different determinations carried out in triplicate.

TABLE 1

Cytotoxicity of anticancer drugs against human breast tumor MCF7 cell lines

The IC_{50} values were obtained from three to six independent experiments (mean ± standard deviation). The resistance factor (in parentheses) is the ratio of the IC_{50} value of the resistant cells to that of the MCF7/WT cells

	IC_{50}		
	MCF7/WT	MCF7/GL^R	MCF7/ADR^R
		μM	
GL	0.007 ± 0.001	0.06 ± 0.01 (9)	0.71 ± 0.09 (100)
HA	0.2 ± 0.032	0.43 ± 0.06 (2)	3.0 ± 0.48 (15)
ADR	0.004 ± 0.001	0.016 ± 0.003 (4)	2.5 ± 0.31 (600)
VP-16	0.56 ± 0.07	0.63 ± 0.05 (1)	
Camptothecin	0.018 ± 0.002	0.021 ± 0.002 (1)	
VCR	0.08 ± 0.02	0.18 ± 0.025 (2)	

Free radical formation. Because MCF7/GL^R cells were cross-resistant to ADR, we compared free radical formation from ADR and GL in MCF7 cells, using spin-trapping ESR techniques. As reported previously (11), both ADR and GL formed significantly less hydroxyl free radical, detected as DMPO-OH adducts, in MCF7/ADR^R cells, compared with MCF7/WT cells. As depicted in Fig. 3, GL formed less DMPO-OH in MCF7/GL^R cells than in MCF7/WT cells only in the presence of NADPH; this difference in DMPO-OH formation from GL in MCF7/WT and MCF7/GL^R cells was not significant in the absence of added NADPH. It is interesting to note that no significant difference in DMPO-OH formation was observed with ADR between MCF7/GL^R and MCF7/WT cells.

GSH and GSH-related enzymes. Because decreased free radical formation has been reported to result from increased detoxification (16, 32, 33), it was possible that GL treatment caused increased activities of GSH-dependent detoxification enzymes for the resistance observed with GL, HA, and ADR. Data in Table 2 show that GSH peroxidase activity was not significantly different between MCF7/WT and MCF7/GL^R cells. Moreover, both GST activity and GSH levels were also similar (Table 2). Western blot analysis was used to detect different classes of GST isozymes (π, α, and μ), and results shown in Fig. 4 indicated no overexpression of these isozymes in MCF7/GL^R cells, compared with MCF7/WT cells, suggesting that GSH-based detoxification enzymes were not altered and did not participate in GL resistance in MCF7/GL^R cells.

Photoaffinity labeling for PgP. Because our previous studies showed that the cytotoxicities of GL and HA can be increased by verapamil in resistant cells, resulting in decreased resistance in MCF7/ADR^R cells, we directly examined binding and inhibition of photoaffinity labeling of PgP by HA and GL. MCF7/ADR^R cells were photolabeled with azidopine and the interactions of GL and HA were examined. As shown in Fig. 5, both GL and HA inhibited labeling of PgP in a dose-dependent manner, indicating that both GL and HA interacted with PgP. GL was significantly more active in inhibiting this labeling than was HA, such that 10 μM GL completely inhibited the PgP labeling by azidopine. The specificity of PgP labeling was demonstrated by nonradioactive azidopine inhibition of labeling, in a dose-dependent manner.

Uptake and efflux studies. To gain some insight into the mechanisms and role of PgP in GL resistance, uptake and efflux studies were carried out in MCF7/WT, MCF7/GL^R, and MCF7/ADR^R cells. Results presented in Fig. 6 show that the uptake of GL was rapid in all three cell lines and about 30% less GL accumulated in MCF7/GL^R and MCF7/ADR^R cells, compared with MCF7/WT cells, between 5 and 30 min. Interestingly, the drug was removed rapidly from cells and >50% of

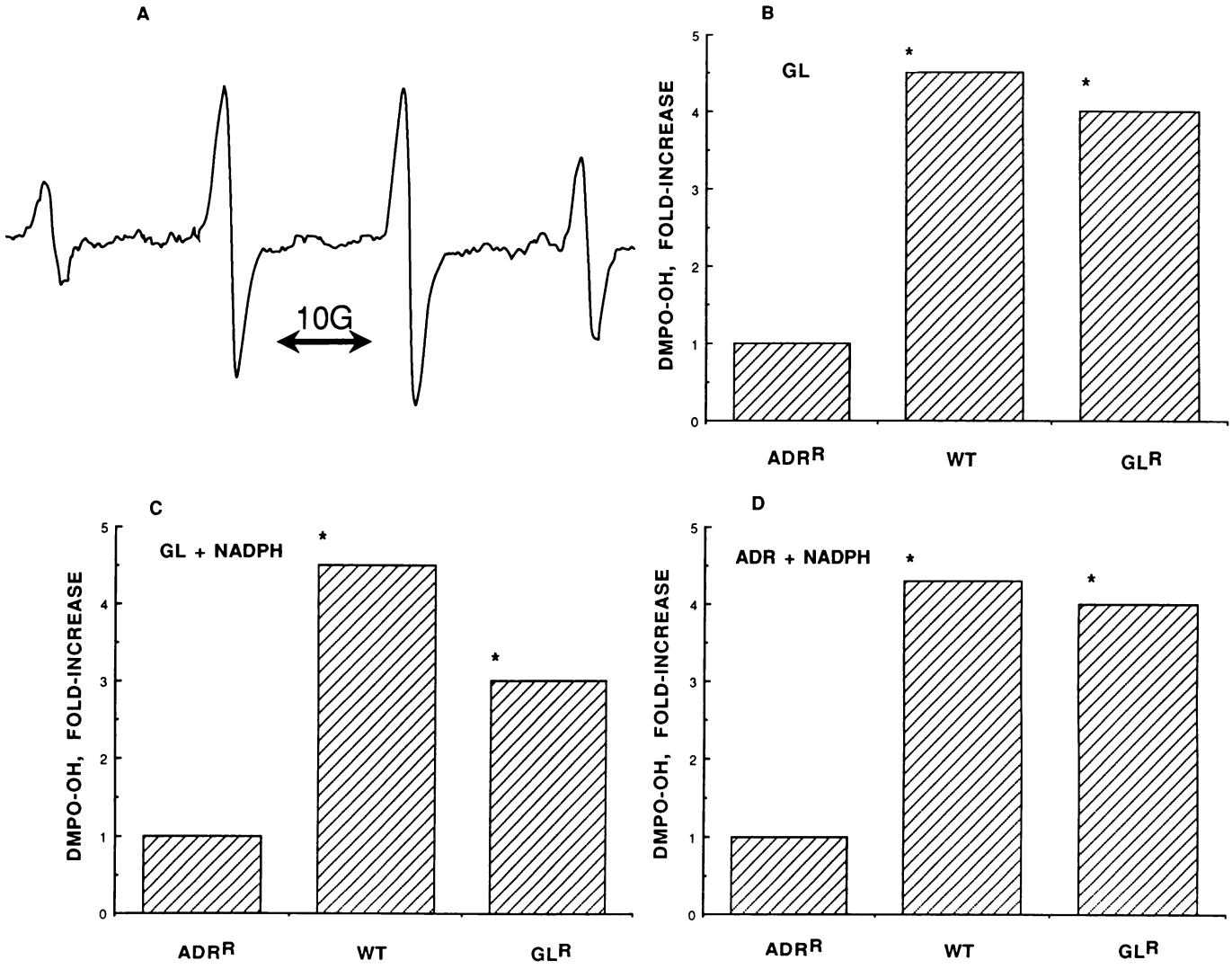


Fig. 3. Free radical formation from GL (100 μM) and ADR (200 μM) in the presence or in the absence of NADPH (1 mM) and DMPO (50 mM) in MCF7 cell lines. DMPO-OH adduct formed was detected by ESR spin-trapping techniques and was quantitated (arbitrary units) by measuring the peak height of the DMPO-OH adduct (1:2:2:1) signal (A) with hyperfine splitting constants of $a^N = a^H = 15.0$ G. The ESR settings were as follows: center field, 3364 G; microwave power, 20.0 G; modulation amplitude, 2.0 G; receiver gain, 8×10^4. *, Significantly different from MCF7/ADR^R cells ($p < 0.001$); values are normalized to DMPO-OH adduct values obtained in MCF7/ADR^R cells (standard deviation for DMPO-OH adduct formation was <15%).

TABLE 2

Relative activities of glutathione peroxidase and GST and GSH levels in MCF7/WT and MCF7/GL^R cells

The activies of GSH peroxidase and GST were determined as described in Materials and Methods

	MCF7/WT	MCF7/GL^R
GSH (nmol/mg of protein)	35.3 ± 6.40	39.4 ± 4.5
GSH peroxidase (nmol/mg of protein/min)	8.10 ± 0.76	7.42 ± 1.0
GST (nmol/mg of protein/min)	10.4 ± 2.0	10.0 ± 1.85

the drug effluxed out of the cells in 5 min; a small but significantly greater amount of GL was present in both MCF7/WT and MCF7/GL^R cells than in MCF7/ADR^R cells. However, after 15 min, the amounts of GL retained in these cells were similar.

Because GL cytotoxicity was increased in MCF7/ADR^R cells by verapamil (11) and because GL interacted with p170, we also measured uptake of GL in MCF7 cell lines in the presence of verapamil. Results presented in Fig. 7 show that verapamil had no significant effects on GL accumulation in MCF7/WT and MCF7/GL^R cell lines. As expected, verapamil significantly enhanced GL accumulation in the PgP-positive MCF7/ADR^R cells, confirming that GL interacted with PgP and likely effluxed via this protein in MCF7/ADR^R cells.

RT-PCR assay. Because the photoaffinity labeling and other studies indicated that both GL and HA interacted with PgP, expression of *mdr1* mRNA was examined in GL-selected (MCF7/GL^R) cells by RT-PCR. MCF7/WT and MCF7/ADR^R cells were used as the negative and positive controls, respectively, for the expression of the *mdr1* gene (34). Data presented in Fig. 8 show that selection of resistance to GL in MCF7/GL^R cells did not result in expression of the *mdr1* gene. However, MCF7/GL^R cells showed a slightly greater amount (2-fold) of MRP than did the parent cells (Fig. 7). For MRP expression, MCF7/VP cells were used as the positive control, because these cells have been shown to overexpress MRP (35). These cells were resistant to VP-16 (28-fold) and ADR (9-fold) (35) and

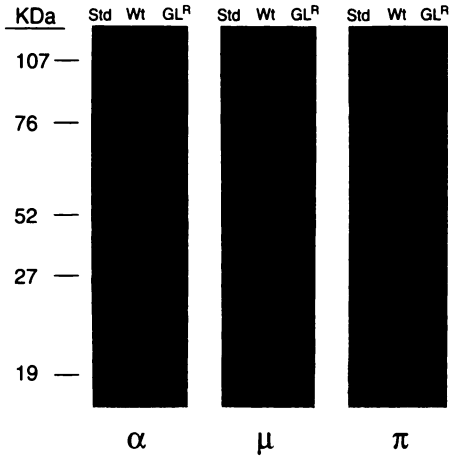


Fig. 4. Western blot analysis of GST isozymes in the human breast MCF7/WT and MCF7/GL^R cell lines. Rabbit antisera directed against human GST-α, -π, and -μ were used. Lanes were loaded with 100 μg of cell lysate and 0.25 μg of the standard (*Std*) and were probed as described in Materials and Methods.

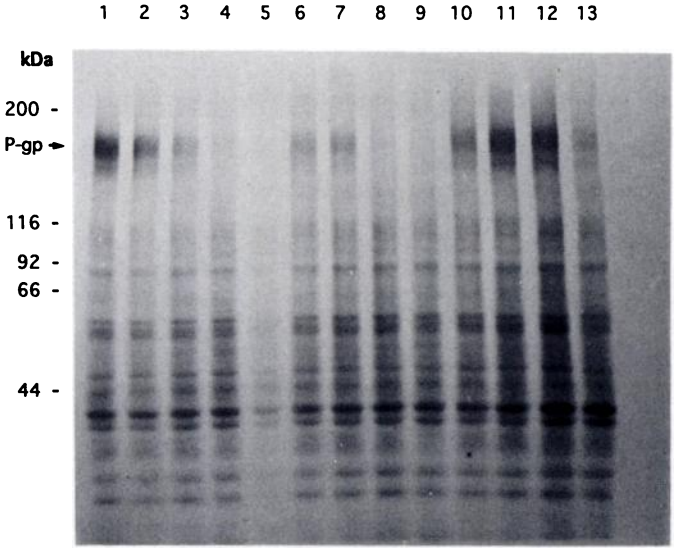


Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis fluorography of [^{3}H]azidopine (0.5 μM)-photolabeled MCF7/ADR^R cells (5 × 10^5 cells/lane), in the absence (*lane 1*) or in the presence of 0.1, 1, 10, and 100 μM azidopine (*lanes 2-5*), GL (*lanes 6-9*), or HA (*lanes 10-13*), respectively. *Numbers to the left*, molecular masses (in kDa); *arrow*, location of PgP.

were also found to be 3-fold cross-resistant to GL, suggesting a role for MRP in GL resistance in MCF7/GL^R cells.

Tyrosine kinase activity. Because the antitumor activity of GL may result from the inhibition of tyrosine kinase activity of tumor cells, it is possible that resistance to GL may be due to a decrease in tyrosine or tyrosine kinase activity. We have therefore evaluated the total and EGF-dependent tyrosine kinase activity in MCF7 cell lines. Our results presented in Table 3 show that, whereas the overall tyrosine kinase activity was similar in MCF7/WT and MCF7/GL^R cell lines, a small decrease (33%) was observed in MCF7/ADR^R cells, compared with MCF7/WT cells. However, GL (20 μM) had no effect on this tyrosine kinase activity. Furthermore, EGF receptor tyrosine kinase activity was similar in MCF7/WT and MCF7/GL^R cells and GL had no effect on EGF receptor-dependent tyrosine

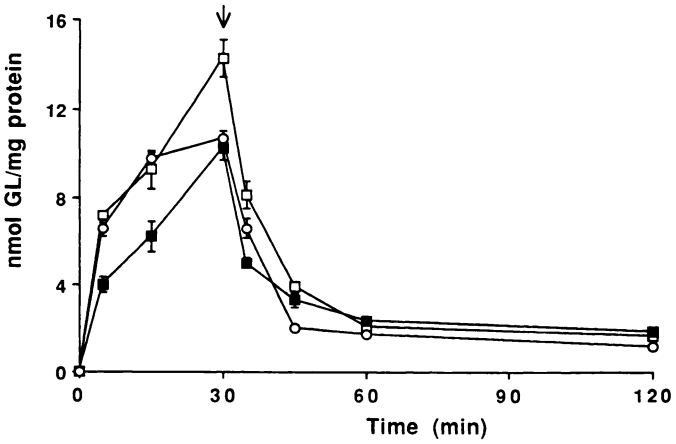


Fig. 6. Cellular accumulation and efflux of [^{3}H]GL (10 μM) in MCF7/WT (□), MCF7/GL^R (○), and MCF7/ADR^R (■) cells. Cells were exposed to the drug (10 μM) for the indicated times, washed three times with ice-cold PBS, and dissolved in 1 N NaOH, and the radioactivity was counted. For the efflux studies (*arrow*), cells were loaded with the drug for 30 min and then placed in fresh drug-free medium for the indicated times. The samples were then processed as described above. Data represent mean ± standard deviation of three separate experiments.

kinase activity. MCF7/ADR^R cells showed somewhat higher (30%) EGF receptor kinase activity, which was significantly inhibited by GL, consistent with the fact that MCF7/ADR^R cells overexpress a large number of EGF receptors.

Discussion

Several lines of evidence indicate that the benzoquinonoid ansamycin antibiotics GL and HA inhibit tyrosine kinase activities of certain oncogene products and are able to induce morphological changes in various *src*, *fbs*, and *abl* oncogene-transformed cells (2, 3). Recently, HA has been shown to inhibit tyrosine kinase activity of *ret* genes and to reverse the morphology of NIH(ret) cells (36). GL and HA have been reported to be potent antiproliferative agents; however, the antiproliferative activity of GL was not due to its effects on *src* tyrosine kinase activity (4). At present, little is known about the mechanisms of cytotoxicity of and resistance to these agents.

Our previous work (11) indicated that GL and HA are cytotoxic to human breast tumor cells and that ADR-selected cells are resistant to both GL and HA. Moreover, in human breast tumor cells GL and HA form toxic free radicals, which are known to induce lipid peroxidation and to cause DNA fragmentation, leading to cell death. The present work confirmed these findings with MCF7/WT and MCF7/ADR^R cells but showed no significant differences in free radical formation between MCF7/WT cells and GL-selected resistant (9–10-fold) MCF7 cells in the presence of GL. However, in the presence of NADPH and GL less DMPO-OH was trapped in MCF7/GL^R cells than in MCF7/WT cells. Although the reason for decreased DMPO-OH formation in the presence of NADPH in MCF7/GL^R cells is not clear, it may indicate that GL is more readily metabolized to an inactive and/or non-redox-cycling compound in MCF7/GL^R cells, compared with MCF7/WT cells. ADR also showed differential free radical formation in MCF7/WT and MCF7/ADR^R cells, as reported previously; however, no significant difference was observed between MCF7/WT and MCF7/GL^R cells, which were only 4-fold cross-resistant to ADR. These observations indicate that, whereas

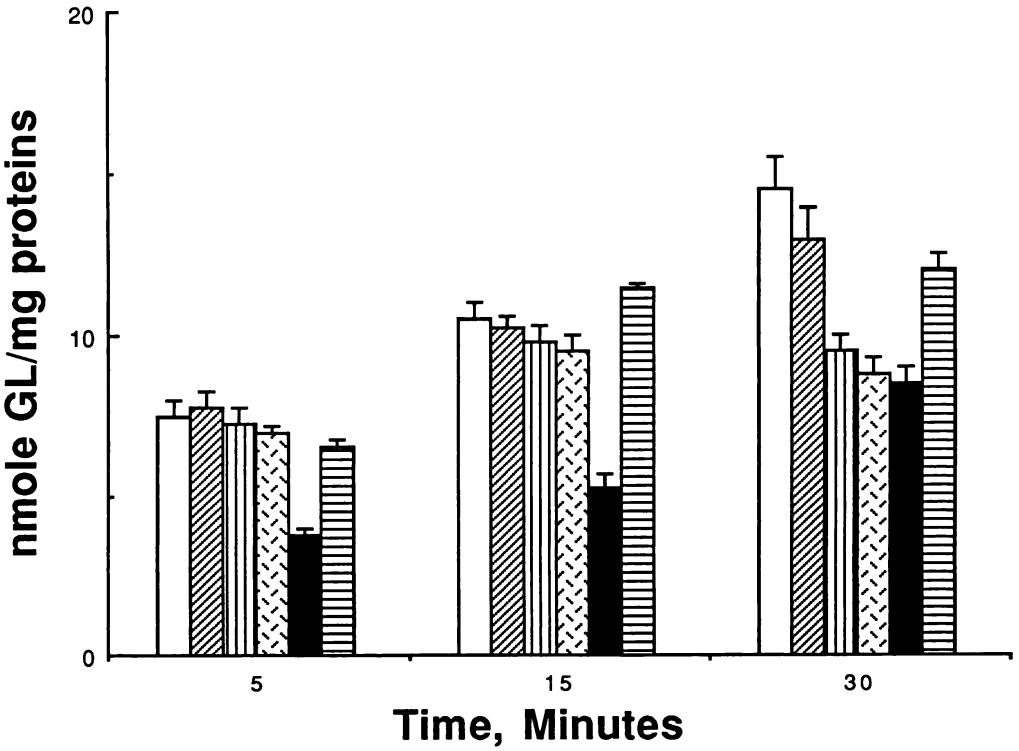


Fig. 7. Effects of verapamil (50 μM) on cellular accumulation of [^{3}H]GL in MCF7/WT (□, ▨), MCF7/GL^R (▥, ■), and MCF7/ADR^R (■, ■) cells. The uptake studies were carried out as described for Fig. 6.

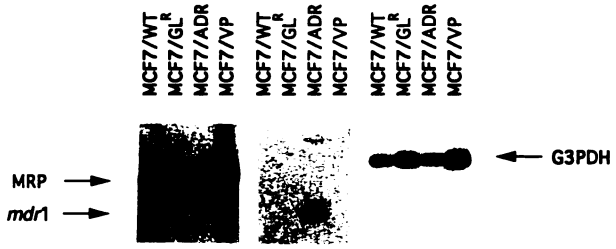


Fig. 8. RT-PCR studies for the detection of *mdr1* and MRP in MCF7/GL^R cells. MCF7/WT, MCF7/ADR^R, and MCF7/VP cells were used as controls for the detection of *mdr1* and MRP, respectively. The control *G3PDH* gene was used for quantitation of *mdr1* and MRP expression, using a Hoeffer densitometer with program GS 370. The experiment was repeated twice and the relative levels of MRP in MCF7/GL^R cells (normalized to that of *G3PDH*), compared with those in MCF7/WT cells, were 1.7 and 2.45, respectively.

TABLE 3

Relative tyrosine kinase activity in MCF7/WT, MCF7/GL^R, and MCF7/ADR^R cell lines and effects of GL on the tyrosine kinase activity

The tyrosine kinase activity was measured and quantitated using poly (Glu-Tyr) (4:1) as a substrate for specific tyrosine phosphorylation, in the presence or absence of EGF. The effects of GL (20 μM) on the inhibition of phosphorylation were determined by including GL in the incubation mixture before initiation of the reaction by the addition of ATP and MG^{2+}

	Tyrosine kinase activity					
	MCF7/WT		MCF7/GL^R		MCF7/ADR^R	
	−GL	+GL	−GL	+GL	−GL	+GL
	pmol of ^{32}P bound/mg of protein					
−EGF	105 ± 6	89 ± 20	76 ± 11	70 ± 10	63 ± 7	62 ± 12
+EGF	70 ± 10	79 ± 14	91 ± 14	73 ± 12	101 ± 13	43 ± 11

free radical formation is related to cell kill in MCF7/WT cells, the development of low levels of resistance to GL (10-fold) and to ADR (4-fold) in GL-selected MCF7 cells is not related to formation of reactive free radicals. However, when the drug selection pressure is long and the resulting resistance is high, as in MCF7/ADR^R cells, decreased free radical formation must play a role in the mechanisms of resistance to both GL and ADR.

Previous studies have indicated that GL and HA may interact with PgP, inasmuch as verapamil partially reversed GL and HA resistance in MCF7/ADR^R cells (11). The present study confirmed that GL and HA interacted with PgP, as shown by photoaffinity-labeling experiments in which GL and HA inhibited the specific labeling of PgP in a dose-dependent fashion. Our uptake and efflux studies with GL indicated a 30% decrease in cellular accumulation in MCF7/ADR^R and MCF7/GL^R cells and a 2-fold increase in efflux of GL in MCF7/ADR^R cells. No significant differences in GL efflux were found between MCF7/WT and MCF-/GL^R cells. Furthermore, we also found no significant difference in cellular accumulation of VCR, a substrate for PgP, between MCF7/WT and MCF7/GL^R cells (95 ± 0.8 and 88 ± 5.3 pmol of VCR/mg of protein at 60 min), whereas a significant decrease (7-fold, 13.0 ± 1.1 pmol of VCR/mg of protein at 60 min) was observed for MCF7/ADR^R cells. Verapamil neither affected the cellular accumulation of GL nor increased sensitivity to GL in MCF7/WT or MCF7/GL^R cells. However, verapamil caused a significant increase in GL accumulation in the PgP-positive MCF7/ADR^R cell line and significantly enhanced (10–15-fold) the cytotoxicity of GL in these cells (11). These results are consistent with our findings that the development of resistance to GL in the MCF7/GL^R cell line was not accompanied by overexpression of *mdr1*. Finally,

preliminary studies using MCF7/BC-19 cells showed that these cells were 15-fold resistant to GL and 50-fold resistant to ADR. MCF7/BC-19 cells were obtained from the MCF7/WT cell line after transfection with the *mdr1* gene (37). These observations, taken together, indicate that GL and HA, like ADR, also interact with PgP, and thus one of the mechanisms of resistance to GL and HA involves overexpression of the *mdr1* gene.

Although GL and HA are inhibitors of tyrosine kinases and this inhibition may play some role in the mechanism of GL- and HA-induced tumor cell death, our studies reported here indicate that inhibition of tyrosine kinases may not be related to the mechanism of GL and HA cytotoxicity or resistance in MCF7 cells, confirming recent observations of Whitesell *et al.* (4). We found that both EGF-dependent tyrosine phosphorylation and EGF-independent tyrosine phosphorylation were similar in MCF7/WT and MCF7/GL^R cells and that GL had no effect on this phosphorylation, indicating that tyrosine kinase activity did not play a major role in GL cytotoxicity and resistance in MCF7/GL^R cells. MCF7/ADR^R cells showed enhanced EGF-dependent phosphorylation, which was significantly inhibited by GL. Increased EGF-dependent tyrosine phosphorylation in MCF7/ADR^R cells is consistent with the fact that MCF7/ADR^R cells overexpress EGF receptors (38), which are receptor-dependent tyrosine kinases (39, 40). It is also noteworthy that we have found that MDA-231 human breast tumor cells are very sensitive to GL. MDA-231 tumor cells, like MCF7/ADR^R cells, express a large number of EGF receptors. In contrast to MDA-231 cells, however, MCF7/ADR^R cells are highly resistant to GL, indicating that EGF-dependent tyrosine kinase did not contribute to GL cytotoxicity or resistance in these cell lines.

In conclusion, GL and HA form free radicals in MCF7 cells upon reductive activation. Smaller amounts of free radicals were detected in highly resistant tumor cells, compared with drug-sensitive cells or cells that showed low levels of resistance to GL. GL and HA interact with PgP, which results in lower levels of drug accumulation and increased efflux. The mechanism of resistance to GL and HA in highly resistant cells appears to be due to overexpression of *mdr1* and decreased free radical formation, similar to that for ADR. Although the development of low levels of resistance to GL did not result in overexpression of the *mdr1* gene, a 2-fold increase in the expression of MRP was observed in MCF7/GL^R cells. The role of MRP in the mechanism of GL resistance is not known and is currently under investigation in our laboratory.

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