Biochemical Pharmacology, Vol. 44, No. 9, pp. 1869–1877, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

AN ENHANCED ABILITY FOR TRANSFORMING ADRIAMYCIN INTO A NONCYTOTOXIC FORM IN A MULTIDRUG-RESISTANT CELL LINE (LZ-8)

YIN ZHANG,* KEVIN M. SWEET, MARGUERITE A. SOGNIER and JAMES A. BELLI
Biology Division, The University of Texas Medical Branch, Department of Radiation Therapy, Galveston, TX 77555, U.S.A.

(*Received* 7 *April* 1992; *accepted* 24 *June* 1992)

Abstract—Multidrug-resistant LZ-8 cells are 9000-fold more resistant to Adriamycin® (ADRM) exposure than wild-type V79 cells. To understand more about the mechanisms producing such high level resistance, we tested whether LZ-8 cells inactivate ADRM toxicity to a greater extent than wild-type V79 cells. ADRM was recovered from (1) culture media of wild-type V79 and ADRM-resistant LZ-8 cells; (2) V79 and LZ-8 cells; and (3) LZ-8 cell plasma membrane, and the cytotoxicity was determined by treating V79 cells for 1 hr with a known concentration of the recovered ADRM. ADRM obtained from LZ-8 cells or its culture medium exhibited less cytotoxicity than that recovered from V79 cells or its culture medium. ADRM extracted from LZ-8 cell plasma membrane was noncytotoxic. HPLC analysis revealed that the extracted ADRM was structurally changed compared to stock ADRM. The retention time in the column was 7 min for stock ADRM, and 23 min for the recovered ADRM. Thus, LZ-8 cells have an increased ability to transform ADRM into a noncytotoxic form compared to wild-type V79 cells. This transformation involves structural conversion into a previously unidentified ADRM metabolite. The greatly increased survival of LZ-8 cells compared to V79 cells after ADRM treatment is due to at least two mechanisms: (1) an enhanced ability to inactivate the cytotoxicity of ADRM, and (2) increased drug efflux resulting from the amplification and overexpression of the *pgp* 1 gene in these cells. Our results suggest the possibility that P-glycoprotein participates in drug binding/inactivation in addition to serving as a drug efflux pump.

The most common, consistent alteration found in multidrug-resistant cell lines is an amplified and/or overexpressed gene for multidrug resistance that results in increased levels of P-glycoprotein (P-gp)† in the cell membrane [1–4]. P-gp is thought to function as an energy-dependent drug efflux pump, that removes drug from the intracellular environment [5]. Many other mechanisms have been described, including: alterations in the levels/activity of topoisomerase II [6]; altered redox enzyme systems, such as glutathione, glutathione *S*-transferase, cytochrome P450 and superoxide dismutase [7–9]; and altered intracellular drug distribution in which drug is sequestered away from target sites (i.e. DNA) [10, 11]. In some cells, two or more mechanisms operate simultaneously, suggesting that resistance may be multifactorial [12].

In our laboratory, a series of Adriamycin® (ADRM)-resistant cell lines have been selected and characterized. All cells from the LZ series exhibit greater ADRM resistance and higher levels of P-gp in the cell plasma membrane than wild-type V79 cells. We have shown previously that (1) different degrees of ADRM resistance are observed in LZ-8 and LZ-24 cells even though the amounts of P-gp in the plasma membrane are virtually identical (approximately 20% of the plasma membrane proteins) [13], and (2) a 1-hr, 100 μg/mL ADRM treatment of LZ-8 cells produces significant amounts of intracellular ADRM (detected by fluorescent microscopy and spectrophotometry), but does not result in enhanced cytotoxicity in LZ-8 cells compared to untreated LZ-8 cells; in addition, no DNA damage could be detected using alkaline and pH 9.6 filter elution techniques [14]. In contrast, wild-type V79 cells exhibit DNA strand breaks and DNA–protein cross-links, and the survival fraction is reduced to nondetectable levels after exposure to 100 μg/mL ADRM for 1 hr [14]. These observations suggest that another mechanism, in addition to the overexpression of P-gp, may contribute to ADRM resistance in LZ cells. One possible explanation is that LZ cells may have an enhanced ability to convert ADRM into a noncytotoxic form compared to V79 cells.

In these studies, this hypothesis was explored by testing the cytotoxicity of ADRM recovered from the following three sources on the colony-forming ability of V79 cells: (1) V79 and LZ-8 cells; (2) the growth medium from the two cell lines; and (3) LZ-8 cell plasma membrane. The results indicate that this ADRM was inactivated and structurally changed compared to stock ADRM as determined by HPLC, and that LZ-8 cells exhibited an enhanced ability to inactivate ADRM compared to V79 cells.

MATERIALS AND METHODS

Cell culture and ADRM survival studies. Our cell

* Corresponding author: Dr. Yin Zhang, The University of Texas Medical Branch, Department of Radiation Therapy, Mail Route F-56, Galveston, TX 77555. Tel. (409) 772-4242; FAX (409) 772-3387.

† Abbreviations: ADRM, Adriamycin; P-gp, P-glycoprotein; and PSA, Puck's Saline Solution A.

culture conditions have been described previously in detail [4, 14]. Chinese hamster V79 cells were maintained as monolayers in F-10 medium supplemented with 10% fetal bovine serum (ICN Flow Biomedicals Inc., Costa Mesa, CA) and penicillin/streptomycin. For LZ-8 cells, this medium also contained 8 μg/mL ADRM.

The ADRM survival response was determined by colony-forming ability using V79 cells exposed to various levels of stock or recovered ADRM for 1 hr at 37°. Following harvesting of exponentially growing V79 cells with 0.05% trypsin in Puck's Saline Solution A (PSA), cells were counted using a Coulter counter model ZM and subcultured into petri dishes in numbers sufficient to yield 100–200 colonies at each survival level. After exposure to stock or ADRM recovered from cells, growth medium, or cell membrane preparations, the dishes were rinsed once with PSA, filled with fresh medium, and incubated at 37° for colony formation.

ADRM recovery. To recover ADRM from the growth medium, V79 and LZ-8 cells were grown in 3 μg/mL ADRM. This concentration of ADRM was used since it is the highest concentration that V79 cells are able to tolerate and survive for 24 hr and also allows sufficient recovery of ADRM. After 3 days growth in ADRM, the medium was collected, filter sterilized using a 0.20 μm MFS 25 disposable syringe filter unit with a cellulose acetate membrane (Millipore Corp., Bedford, MA), and the concentration of ADRM present was determined using fluorescent spectrophotometry. This preparation was diluted with fresh medium to the desired final concentration and the cytotoxicity was assayed on virgin V79 cells (i.e. cells never before exposed to drug) by measuring colony-forming ability. It should be noted that some studies have reported that ADRM can absorb to some types of membranes during filtration. This is particularly true with dilute drug solutions and small volumes (a few mL). Under these conditions, more than 95% of ADRM is found to be adsorbed to cellulose ester membranes [15]. Under the conditions of our experiments, no detectable loss of ADRM or metabolites occurred with filtration based on comparisons of HPLC profiles of filtered versus centrifuged ADRM media collected from V79 or LZ-8 cells.

To recover ADRM from cells, V79 cells were exposed to 3 μg/mL ADRM and LZ-8 cells to 100 μg/mL for 2 hr at 37°. A higher concentration of ADRM was used in this experiment to treat LZ-8 cells in order to recover adequate amounts of intracellular ADRM (LZ-8 efflux is so rapid, little ADRM is retained intracellularly at lower concentrations). After ADRM treatment, culture dishes were rinsed three times with PSA. The cells were removed from the dishes using a rubber policeman, collected in cold water, and kept on ice for 20 min. Following cell lysis in cold water, cell suspensions were transferred into test tubes, an equal volume of *n*-butanol was added, and tubes were vortexed for 5 min at room temperature. The organic layer containing ADRM was separated by centrifugation at 2500 rpm for 1 hr, collected, and dried by lyophilization. The recovered ADRM was dissolved in distilled water, and the concentration was determined as above. Alternatively, after collection of cells, recovered ADRM was extracted using chloroform/methanol (2:1), and dried under N_2. HPLC analysis revealed that the two different extraction procedures did not affect the retention time in the column for ADRM or recovered ADRM.

To recover ADRM from the plasma cell membrane of LZ-8 cells, the cells were plated in roller bottles and grown in medium containing 8 μg/mL ADRM for 3 days. This concentration was used since it is noncytotoxic to LZ-8 and allows sufficient ADRM recovery from the membrane. After ADRM treatment, the roller bottles were rinsed three times with PSA to remove any free, unbound ADRM; the cells were removed using a rubber policeman, lysed in hypotonic solution, and homogenized. Unlysed cells and nuclei were removed by centrifugation (see details in Ref. 4). The cell membrane in the supernatant was precipitated and further centrifuged in a 16–60% sucrose gradient. The 16–31% interface containing the plasma cell membrane was collected and will be designated in this report as the ADRM–LZm complex. For some experiments, ADRM was extracted from this complex using *n*-butanol or chloroform/methanol (2:1). The same retention time in the column for extracted ADRM from the complex was observed using either *n*-butanol or chloroform/methanol (2:1).

HPLC analysis for ADRM and recovered ADRM. Stock ADRM and ADRM recovered from the culture media, cell membrane, or whole cells were analyzed by high performance liquid chromatography (Beckman 421A controller, 114 M solvent delivery module, San Ramon, CA) on an Ultrasphere C18 reverse phase column (25 cm × 4.6 mm diameter) with a linear gradient of 30% aqueous acetonitrile containing 0.1% trifluoroacetic acid to 90% over 30 min at room temperature. A flow rate of 1 mL/min was used. A fluorescent detector (spectroflow 980, programmable fluorescence detector, Kratos analytical, Ramsey, NJ) with an excitation wavelength of 480 nm and an emission filter of 580 nm was used to measure the amount of ADRM. Data were recorded on a curve integrator (Water 740 data module, Milford, MA). To protect against photolytic degradation, all procedures were protected from direct exposure to fluorescent light. The HPLC was protected from light by the use of steel tubing.

RESULTS

Cytotoxicity of intracellular ADRM. When cells are exposed to ADRM and the drug enters the cell, some drug may reach initial targets (i.e. DNA) while some may be metabolically inactivated and/or removed from the cell via P-gp. These experiments were designed to test whether V79 and LZ-8 cells inactivate ADRM to different degrees by assaying the cytotoxicity of recovered ADRM from the cells. To determine the cytotoxicity of ADRM recovered from V79 and LZ-8 cells, intracellular ADRM was extracted directly from the cells as described in Materials and Methods. This recovered ADRM was then used to treat virgin V79 cells at a concentration of 0.50 μg/mL for 1 hr at 37° in order to determine if the ADRM was cytotoxic. A survival fraction of

0.41 ± 0.02 for ADRM recovered from LZ-8 cells, and 0.14 ± 0.01 for ADRM recovered from V79 cells was observed. In addition, virgin V79 cells were exposed to stock ADRM at the same concentration (0.50 μg/mL) for 1 hr at 37° as a control. The cell survival fraction was 0.022 ± 0.005. These results suggest that both V79 and LZ-8 cells are able to alter ADRM into a less cytotoxic form. However, the efficiency of transforming ADRM into this less cytotoxic form was significantly different between the V79 and LZ-8 cells (relative survival 18.6 vs 6.4, respectively).

Our second approach was to determine relative cytotoxicity for intracellular ADRM released into the culture medium from lethally irradiated V79 or LZ-8. Cells were subcultured into dishes containing different numbers (2×10^2–2×10^5) of V79 or LZ-8 cells that had been pretreated with 8 μg/mL ADRM for 2 hr, and subsequently irradiated with 50 Gy. The culture dishes were rinsed three times with PSA to remove free ADRM and refilled with fresh medium. Over the next 24 hr, the cells lyse, and release intracellular ADRM into the medium. Virgin V79 cells were then innoculated into the same petri dishes containing the ADRM treated/irradiated cells. The survival fractions were determined by colony-forming ability. The amount of ADRM released into the culture medium from dead V79 or LZ-8 cells was proportional to the cell number seeded before treatment with radiation and ADRM as determined by fluorescent spectrophotometry. As a control, corresponding numbers of V79 or LZ-8 cells were subcultured into petri dishes and only exposed to irradiation with 50 Gy (no ADRM treatment). The top two curves in Fig. 1 represent the controls. The high survival fractions (95%) indicate that the presence of dead V79 or LZ-8 cells (lethal irradiation) did not affect the survival of virgin V79 cells plated in the same petri dish. In addition, HPLC analysis revealed that ADRM was not structurally modified by irradiation at 50 Gy (data not shown). Thus, under these experimental conditions, the altered ADRM was most likely formed by cellular metabolism. As shown in Fig. 1, when V79 cells were exposed continuously to 8.0×10^{-3} μg/mL ADRM released from lethally irradiated cells for 6 days to allow colony formation, the cell survival fraction was 0.88 for ADRM released from LZ-8 cells, and 0.66 for ADRM released from V79 cells. These results suggest that ADRM released from LZ-8 cells was approximately 33% less cytotoxic than that from V79 cells.

Cytotoxicity of ADRM recovered from growth medium of V79 or LZ-8 cells. If the ADRM recovered from V79 and LZ-8 cells in these first experiments represents metabolically altered drug, it would be expected that, with time, this ADRM might be effluxed out of the cell and into the culture medium. To test this possibility, the cytotoxicity of ADRM recovered from culture medium was determined as follows: 10^6 V79 or LZ-8 cells were plated into dishes containing medium with 3 μg/mL ADRM at 37° for 3 days. The medium was then collected, filter sterilized, and used to treat V79 cells. Since ADRM is sensitive to light and temperature, some degradation may occur in medium

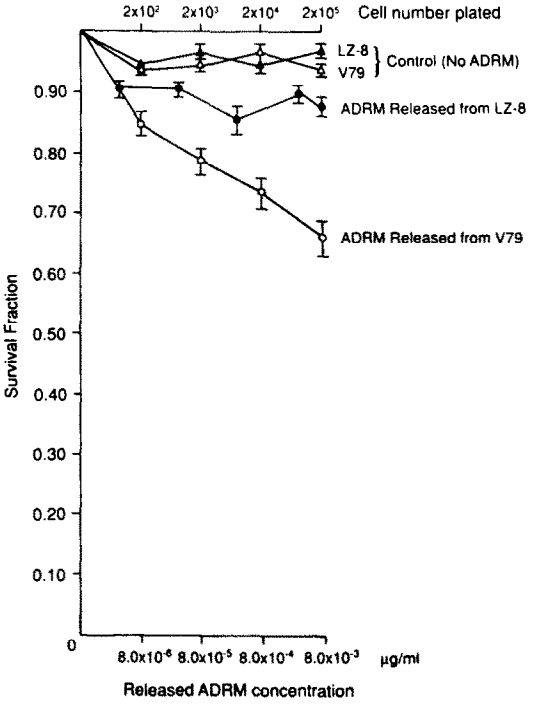


Fig. 1. Virgin V79 cell survival response to ADRM released from lethally irradiated V79 and LZ-8 cells. X-axis (bottom) represents the concentration of ADRM released from V79 or LZ-8 cells treated with radiation and ADRM. X-axis (top) represents cell number plated before treatment with radiation or radiation/ADRM. Data are means ± SD from three independent experiments in which all points represent the data from triplicate dishes.

at 37° [15]. Consequently, a dish containing medium and 3 μg/mL ADRM, but without cells, was incubated at 37° for 3 days as a control. After incubation for 3 days, the amount of ADRM recovered from either the culture medium with cells (V79 or LZ-8), or culture medium without cells exhibited no difference, with a value of 2.95 ± 0.05 μg/mL as determined by fluorescent spectrophotometry. To determine the survival response for ADRM recovered from growth medium, virgin V79 cells were exposed to various concentrations of recovered ADRM by dilution with fresh medium from 3 to 0.1 μg/mL, and colony-forming ability was assayed. As shown in Fig. 2, the results indicated that ADRM recovered from culture medium with cells exhibited less cytotoxicity than the control consisting of ADRM recovered from culture medium without cells. For example, the survival fraction for the 2 μg/mL (recovered) ADRM treatment was 0.18 for ADRM obtained from LZ-8 culture medium, 0.04 for ADRM from V79 medium, and 0.02 for ADRM from medium without cells. These results are consistent with those above and indicate that intracellularly inactivated ADRM can be transported out of cells.

Cytotoxicity of ADRM bound P-gp (ADRM–LZm complex) and ADRM recovered from the ADRM–

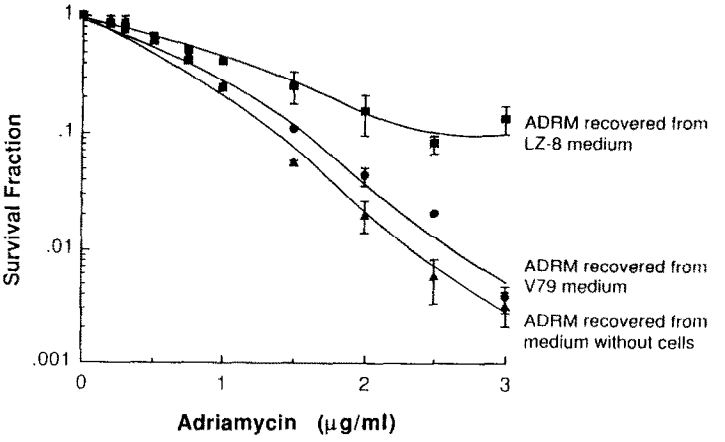


Fig. 2. V79 cell survival response to ADRM recovered from V79 or LZ-8 cell growth medium containing 3 μg/mL ADRM and ADRM incubated without cells. Data are means ± SD from three independent experiments in which all points represent the data from triplicate dishes (where not shown, the standard deviation was smaller than the size of symbol).

LZm complex. Several mechanisms for reducing ADRM cytotoxicity are known including detoxification by enzymes [7–9, 16, 17], exocytosis [18] and binding to P-gp [19]. Since P-gp binds ADRM and probably functions as an energy-dependent drug efflux pump, it is possible that P-gp (either alone or in conjunction with membrane-associated enzymes) may also inactivate ADRM. This can be tested by recovering ADRM bound to P-gp. If the ADRM bound to P-gp is always noncytotoxic, then either P-gp only transports inactivated ADRM or P-gp plays a role in the detoxification process.

To determine the cytotoxicity of the ADRM–LZm complex, the complex was isolated from LZ-8 cells grown in medium containing 8 μg/mL ADRM for 3 days at 37° as described in Materials and Methods. LZ-8 provides a suitable test system, since this cell line has such high levels of P-gp in the cell plasma membrane (approximately 20% of plasma membrane protein) that sufficient amounts of the ADRM–LZm complex can be recovered for analysis. The concentration of ADRM in the complex was determined using fluorescent spectrophotometry, and a concentration of 2 μg/mL ADRM present in the complex was used to treat V79 cells for 1 hr at 37°. The amount of membrane protein in the complex was determined using the Lowry method. To evaluate the cytotoxicity of the ADRM–LZm complex, monolayer V79 cells were also exposed to the following treatments for 1 hr at 37°: (1) 2 μg/mL of stock ADRM; (s) 2 μg/mL of stock ADRM plus LZ-8 cell membrane or V79 cell membrane; and (3) V79 or LZ-8 cell membrane alone (without ADRM). The amount of cell membrane used in the treatments was equivalent to the amount of cell membrane in the ADRM–LZm complex. The results, shown in Table 1, indicate that the ADRM–LZm complex exhibited much less cytotoxicity compared to stock ADRM with a relative survival of 2200. LZ-8 or V79 cell membrane alone was not cytotoxic (survival fraction 0.89). In addition, the cytotoxicity of stock

Table 1. Effect of ADRM and ADRM bound to LZ-8 membrane complex (ADRM–LZm) on survival of virgin V79 cells

Treatment	Survival fraction	Relative survival
2 μg/mL ADRM	0.00033	1.0
LZ-8 or V79 membrane	0.89	
ADRM–LZm complex	0.74	2200
ADRM + LZ-8 membrane	0.0033	10
ADRM + V79 membrane	0.0006	2

ADRM could be reduced two or ten times by mixing stock ADRM with V79 or LZ-8 cell membrane, respectively. Among the treated samples, the highest survival was exhibited by treatment with ADRM bound to LZ-8 membrane (ADRM–LZm complex). The lack of cytotoxicity of this ADRM could result from two possibilities: (1) ADRM could be in a noncytotoxic form in the complex; and (2) the immobilization of the ADRM by association with the membrane could render the drug incapable of penetrating the cell membrane by diffusion or transport, thereby reducing its cytotoxic effects.

To test these possibilities, ADRM was extracted from the ADRM–LZm complex using *n*-butanol or chloroform/methanol (2:1). Stock ADRM was extracted by *n*-butanol or chloroform/methanol (2:1) under the same conditions as a control. V79 cells were exposed to the extracted ADRM for 1 hr at 37°. The results are shown in Fig. 3 and indicate that the ADRM extracted from the ADRM–LZm complex exhibited significantly decreased cytotoxicity. For example, following a 2 μg/mL ADRM

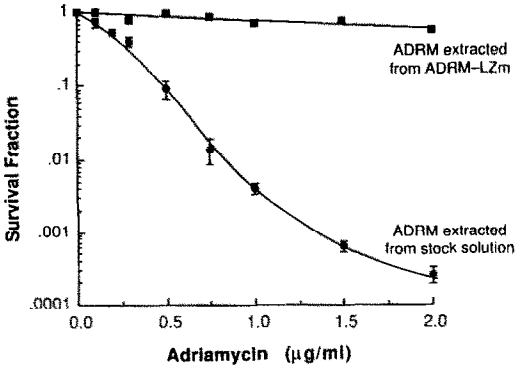


Fig. 3. V79 cell survival response to ADRM extracted from ADRM–LZm complex. Data are means ± SD from three independent experiments in which all points represent the data from triplicate dishes (where not shown, the standard deviation was smaller than the size of symbol). Survival response to ADRM extracted from stock solution served as a control.

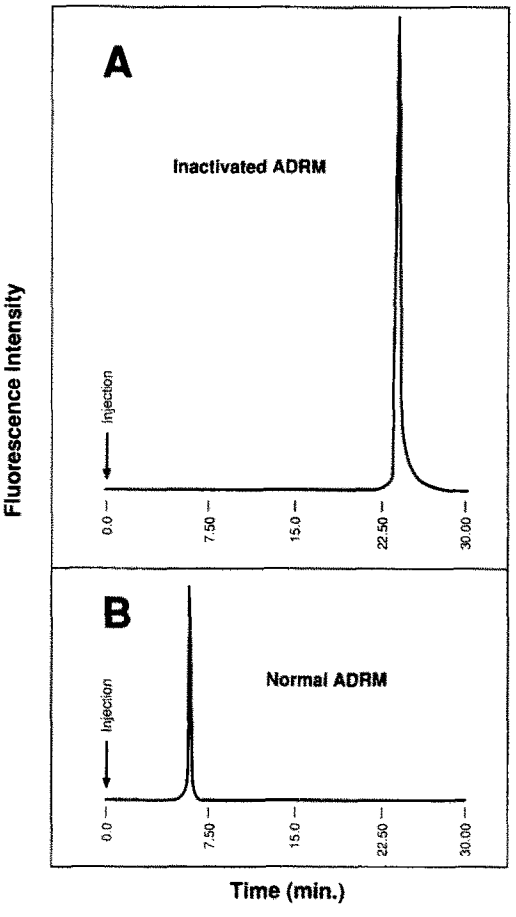


Fig. 4. HPLC profiles for normal ADRM and inactivated ADRM.

treatment, the survival fraction was 0.56 for ADRM extracted from ADRM–LZm complex, and 0.00026 for ADRM extracted from stock solution (ratio = 2138). The similar cell survival curves for ADRM with or without extraction suggest that this procedure did not affect the activity of ADRM. In addition, no structurally altered ADRM was formed using the extraction procedure as determined by HPLC.

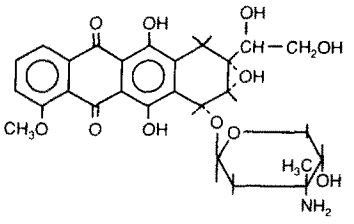
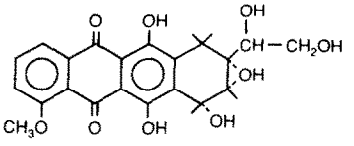
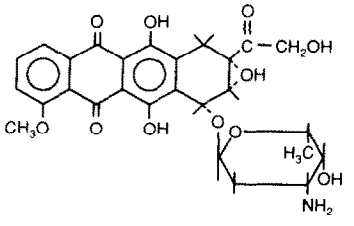
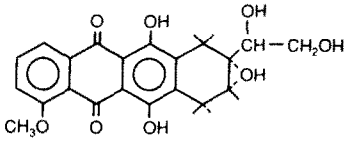
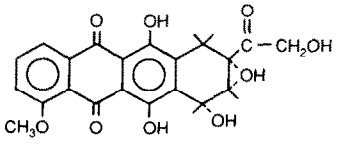
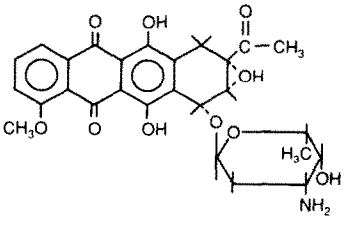
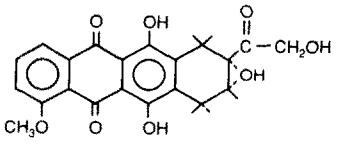
In the experiments reported above, ADRM was recovered from LZ plasma membrane preparations. Since ADRM is known to bind to many types of molecules, the ADRM extracted from LZ plasma membrane may not have been exclusively bound to P-gp. However, since P-gp has been shown to specifically bind ADRM and 20% of the plasma membrane protein in LZ cells is P-gp, we expect a significant fraction of the ADRM recovered to be bound to P-gp. In addition, if nonspecific binding of ADRM to non-P-gp molecules accounted for the majority of ADRM recovered, then V79 and 77A (which contain low levels of P-gp in the plasma membrane) would be expected to yield an equivalent amount of ADRM/mg plasma membrane protein. This was not observed. V79 and 77A yielded levels of ADRM consistent with the levels of P-gp in the cell membrane.

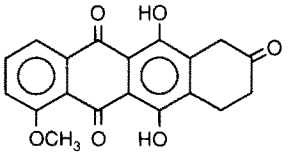
Taken together, these results indicate that the ADRM in the ADRM–LZm complex was significantly less cytotoxic. Thus, the decreased ADRM cytotoxicity observed in LZ-8 cells may be largely due to the ability of LZ-8 cells to convert ADRM to a noncytotoxic form which can be transported out of the cells.

HPLC analysis of ADRM and recovered ADRM. To determine whether ADRM extracted from the ADRM–LZm complex is structurally modified compared to stock ADRM, HPLC analysis was performed. The HPLC profiles for stock ADRM and recovered ADRM from the ADRM–LZm complex are shown in Fig. 4. The amount of ADRM injected into the column was approximately 0.1 μg for normal ADRM and 0.3 μg for the recovered ADRM. HPLC analysis revealed that the retention time in the column was 7 min for stock ADRM and 23 min for ADRM extracted from the ADRM–LZm complex. This suggests that the recovered ADRM (which was observed to be noncytotoxic) was structurally changed compared to stock ADRM, and exhibited higher hydrophobicity. Since only one peak with a retention time of 23 min was observed for ADRM recovered from the ADRM–LZm complex (see Fig. 4), it suggests that ADRM bound to P-gp may always be noncytotoxic. Furthermore, following incubation of stock ADRM and LZ-8 cell plasma membrane (which contains approximately 20% P-gp) at 37° and pH 7.5 for 4 hr, noncytotoxic ADRM (retention time of 23 min) was detected by HPLC. These results suggest that P-gp may play a role in ADRM inactivation.

The major metabolites of ADRM in humans and mammalian species, including cells in culture [20–28], are shown in Table 2. All known metabolites can be synthesized from ADRM by chemical reactions according to the methods described by

Table 2. Retention time for Adriamycin and its metabolites as determined by HPLC

Structure	Compound	Retention time (min)
	Doxorubicinol	2.2
	Doxorubicinol aglycone	3.7
	Doxorubicin	7.0
	7-Deoxydoxorubicinol aglycone	9.1
	Doxorubicin aglycone	10.1
	Daunorubicin	11.3
	7-Deoxydoxorubicin aglycone	12.0



Scheme 1. A speculative molecular structure for inactivated ADRM which was recovered from ADRM–LZm complex.

Takanashi and Bachur [23]. The retention time in the column for ADRM metabolites was determined under the same conditions we used for our analysis, and the results are shown in Table 2. None of the known metabolites of ADRM exhibited a retention time close to the ADRM recovered from the ADRM–LZm complex (23 min). This suggests that the ADRM recovered from the ADRM–LZm complex may be a previously unidentified metabolite of ADRM. To further clarify the nature of the metabolite, LZ-8 was treated with [^{14}C]ADRM (labeled at the keto C-14 position) and the [^{14}C]-ADRM–LZm complex was isolated. The ^{14}C-labeled ADRM was extracted from the complex by *n*-butanol and analyzed for fluorescence using HPLC and radioactivity using scintillation counting. The results indicate that the ADRM recovered from the ADRM–LZm complex had lost the radioactive label, suggesting that ADRM may undergo a two-carbon degradation of its α-ketol group. Based on these results, a putative chemical molecular structure for the recovered ADRM from ADRM–LZm complex (a noncytotoxic metabolite) may be a 9-keton-7-deoxy-adriamycin aglycone as shown in Scheme 1. Further refinement of the chemical structure of the ADRM metabolite will be determined by mass spectroscopy or nuclear magnetic resonance in future studies.

DISCUSSION

Our results indicate that intracellular ADRM obtained from LZ-8 cells exhibited less cytotoxicity than that from V79 cells. The inactivated ADRM can be effluxed via P-gp or transported (via exocytosis) out of the cells into the culture medium. The noncytotoxic metabolite extracted from the ADRM–LZm complex was found to be structurally changed compared to stock ADRM as determined by HPLC. These results suggest that an inactive metabolite of ADRM was produced in both ADRM-sensitive V79 cells and ADRM-resistant LZ-8 cells; however, ADRM-resistant LZ-8 cells have a greatly enhanced ability to transform ADRM into the noncytotoxic form. For LZ-8 cells, this represents a second mechanism of ADRM resistance in addition to enhanced efflux resulting from the amplification/overexpression of the *pgp* 1 gene. Metabolic inactivation of the drug before it reaches sensitive cellular targets is consistent with our previous studies in which no DNA damage could be detected in LZ-8 with filter elution methods after ADRM treatment [14]. A possible consequence of the simultaneous functioning of both enhanced drug inactivation and faster efflux in LZ-8 cells is that the rapid efflux of a noncytotoxic metabolite might establish a high concentration gradient of noncytotoxic ADRM around the perimeter of the LZ-8 cell. This gradient might serve as a protective barrier making it more difficult for cytotoxic ADRM to enter the cell due to competition with the noncytotoxic form. It is also possible that the noncytotoxic metabolite may diffuse back into the cell more easily (due to its nonpolarity) than unmodified ADRM. If true, this would also contribute to increased resistance of LZ-8 cells.

The mechanisms responsible for transforming ADRM into a nontoxic metabolite in ADRM-resistant cells are not yet well understood. It has been reported that the major enzymatic conversion of ADRM involves a carbonyl reduction by aldo-keto reductase [20, 23, 28]. In addition, there is reductive hydrolytic glycosidic cleavage [23]. The molecular structures of the known metabolites are shown in Table 2. The main metabolite Adriamycinol exhibits antitumor activity [29, 30], whereas the aglycones are no longer active [31]. Some investigations have demonstrated that 7-deoxy-adriamycin aglycone is a metabolite known to be formed from ADRM semiquinone radicals under anoxic conditions by NADPH-cytochrome P450 reductase. A free radical-dependent mechanism is thought to be involved in ADRM cytotoxicity [9, 16, 17].

The structures of ADRM and daunorubicin are very similar, differing only in the presence of a hydroxyl group on the alkyl side-chain (see Table 2); yet there are marked differences in their antitumor properties and in their cytotoxicity. ADRM has shown a much wider spectrum of activity in various solid tumors and leukemias, and greater cardiotoxicity than daunorubicin [31]. This implies that the alkyl side-chain may play an important role in ADRM cytotoxicity [32]. Our results show that ADRM extracted from the ADRM–LZm complex lost the ^{14}C label, suggesting that 9-ketone,7-deoxy-adriamycin aglycone may be formed through two-carbon degradation on the alkyl side-chain during cellular ADRM metabolism. Hasinoff [33] and Tong *et al.* [34] have reported that ADRM can undergo a mild two-carbon degradation of its α-ketol group by tautomerization of the α-ketol group to an α-hydroxyaldehyde. Stepwise oxidative degradation of ADRM has been shown to produce 9-COOH-adriamycin [35].

Based on our results and those of others, we speculate on a possible pathway for the production of 9-ketone,7-deoxy-adriamycin aglycone from ADRM in the two cell lines. When ADRM enters cells by passive diffusion or active uptake, following elimination of the daunosamine moiety by reductive glycosic and hydrolytic glycosidic cleavage, and reductive redox-cycling via NADPH-cytochrome P450 reductase, or detoxification enzymes and/or P-gp, 7-deoxy-adriamycin aglycone is formed. The 7-deoxy-adriamycin aglycone will subsequently undergo a two-carbon degradation on the alkyl side-chain through tautomerization or by stepwise oxidative degeneration to form 9-ketol-7-deoxy-adriamycin aglycone which is a noncytotoxic metabolite of ADRM.

Our data are also interesting in view of current models of the mechanism of P-gp drug efflux [2, 36]. The most common concept is that after the drug passes through the lipid bilayer into the cytoplasm, it becomes bound to P-gp and is effluxed out of the cell. With this model, at least some drug would have the opportunity to interact with target sites and/or other cellular components before efflux. Alternatively, it has been proposed that drug binds to P-gp and is effluxed from the cell while still in the lipid bilayer (i.e. without entering the cytoplasm of the cell) [2, 19]. With this mechanism, drug would not be expected to be able to interact with any internal cellular target sites. In these and other studies [14] we have shown that (1) ADRM enters LZ-8 cells with the majority localized to the cytoplasm; (2) ADRM recovered from LZ-8 membrane (which contains 20% P-gp) is in a noncytotoxic form; and (3) the noncytotoxic form of ADRM can exit LZ-8 cells. These results suggest that for LZ-8 cells, the first model may be the more likely mechanism but the second model cannot be excluded yet.

In summary, the results reported here suggest that the enhanced survival of LZ-8 cells after ADRM treatment is due to at least two mechanisms: (1) an enhanced ability to inactivate the cytotoxic properties of ADRM, and (2) increased drug efflux via P-gp. P-gp may not function exclusively as an energy-dependent pump but may also serve as a binding site for ADRM (and other cytotoxic agents). The resulting complex may induce unidentified enzymatic processes which cleave the ADRM molecule into noncytotoxic subunits. Thus, the presence of high levels of P-gp in multidrug-resistant cells may also serve to protect the cell membrane, which represents an important target for ADRM-induced toxicity as suggested by us and others [4, 37–39].

Acknowledgement—This research was supported by NIH grant CA 34269.

REFERENCES

1. van der Bliek AM and Borst P, Multidrug resistance. *Adv Cancer Res* **52**: 165–203, 1989.
2. Roninson IB, P-glycoprotein-mediated drug resistance: Puzzles and perspectives. In: *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells* (Ed. Roninson IB), pp. 395–402. Plenum Press, New York, 1991.
3. Bradley G, Juranaka PF and Ling V, Mechanisms of multidrug resistance. *Biochim Biophys Acta* **948**: 87–128, 1988.
4. Belli JA, Zhang Y and Fritz P, Transfer of Adriamycin resistance by fusion of *M*, 170,000 P-glycoprotein to the plasma membrane of sensitive cells. *Cancer Res* **50**: 2191–2197, 1990.
5. Beck WT, Drug accumulation and binding in P-glycoprotein-associated multidrug resistance. In: *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells* (Ed. Roninson IB), pp. 215–224. Plenum Press, New York, 1991.
6. Ross WE, Sullivan DM and Chow KC, Altered function of DNA topoisomerases as a basis for antineoplastic drug action. In: *Important Advances in Oncology* (Eds. De Vita VT Jr, Hellman S and Rosenberg SA), pp. 65–81. J. B. Lippincott, Philadelphia, 1988.
7. Russo A and Mitchell JB, Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione modulation. *Cancer Treat Rep* **69**: 1293–1296, 1985.
8. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE and Cowan KH, overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* **261**: 15544–15549, 1986.
9. Keizer HG, Pinedo HM, Schuurhuis GJ and Joenje H, Doxorubicin (Adriamycin): A critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmacol Ther* **47**: 219–231, 1990.
10. Gervasoni JE Jr, Fields SZ, Krishna S, Baker MA, Rosado M, Thuraisamy K, Hindenburg AA and Taub RN, Subcellular distribution of danorubicin in P-glycoprotein-positive and -negative drug resistant cell lines using laser-assisted confocal microscopy. *Cancer Res* **51**: 4955–4963, 1991.
11. Hindenburg AA, Gervasoni JE Jr, Krishna S, Stewart VJ, Rosado M, Lutzky J, Bhalla K, Baker MA and Taub RN, Intracellular distribution and pharmacokinetics of daunorubicin in anthryacycline-sensitive and -resistant HL-60 cells. *Cancer Res* **49**: 4607–4614, 1989.
12. Zijlstra JG, de Vries EGE and Mulder NH, Multifactorial drug resistance in an Adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* **47**: 1780–1784, 1987.
13. Sognier MA, Zhang Y, Eberle RL and Belli JA, Characterization of Adriamycin-resistant and radiation-sensitive Chinese hamster cell lines. *Biochem Pharmacol* **44**: 1859–1868, 1992.
14. Sognier MA, Eberle RL, Zhang Y and Belli JA, Interaction between radiation and drug damage in mammalian cells. V. DNA damage and repair induced in LZ cells by Adriamycin and/or radiation. *Radiat Res* **126**: 80–87, 1990.
15. Bosanguet AG, Stability of solutions of antineoplastic agents during preparation and storage for *in vitro* assay. *Cancer Chemother Pharmacol* **17**: 1–10, 1986.
16. Komiyama T, Kikuchi T and Sugiura Y, Interactions of anticancer quinone drugs, aclacinomycin A, Adriamycin, carbazilquinone, and mitomycin C, with NADPH-cytochrome P-450 reductase, xanthine oxidase and oxygen. *J Pharmacobiodyn* **9**: 651–664, 1986.
17. Gutierrez PL, Gee MV and Bachur NR, Kinetics of anthracycline antibiotic free radical formation and reductive glycosidase activity. *Arch Biochem Biophys* **223**: 68–75, 1983.
18. Steinman RM, Mellman IS, Muller WA and Cohn ZA, Endocytosis and the recycling of plasma membrane. *J Cell Biol* **96**: 1–27, 1983.
19. Gottesman MM and Pastan I, The multidrug transporter, a double-edged sword. *J Biol Chem* **263**: 12163–12166, 1988.
20. Huffman DH and Bachur NR, Daunorubicin metabolism by human hematological components. *Cancer Res* **32**: 600–605, 1972.
21. Bachur NR and Gee M, Daunorubicin metabolism by rat tissue preparations. *J Pharmacol Exp Ther* **177**: 567–578, 1971.
22. Bachur NR, Egorin MJ and Hildefrand RC, Daunorubicin and Adriamycin metabolism in the Golden Syrian hamster. *Biochem Med* **8**: 352–361, 1973.
23. Takanashi S and Bachur NR, Adriamycin metabolism in man. Evidence from urinary metabolites. *Drug Metab Dispos* **4**: 79–87, 1976.
24. Brown JR and Imam SH, Recent studies on doxorubicin and its analogues. *Prog Med Chem* **21**: 169–236, 1984.
25. Brenner DE, Galloway S, Cooper J, Noone R and Hande KR, Improved high-performance liquid chromatography assay of doxorubicin: detection of circulating aglycones in human plasma and comparison

with thin-layer chromatography. *Cancer Chemother Pharmacol* **14**: 139–145, 1985.
26. Mross K, Maessen P, van der Vijgh WJF, Gall H, Boven E and Pinedo HM, Pharmacokinetics and metabolism of epidoxorubicin and doxorubicin in humans. *J Clin Oncol* **6**: 517–526, 1988.
27. Pavlik EJ, Kenady DE, van Nagell JR Jr, Hanson MB, Donaldson ES, Casper S, Garrett D, Smith D, Keaton K and Flanigan RC, Stability of doxorubicin in relation to chemosensitivity determinations: loss of lethality and retention of antiproliferative activity. *Cancer Invest* **2**: 449–458, 1984.
28. Felsted RL, Gee M and Bachur NR, Rat liver daunorubicin reductase. An aldo-keto reductase. *J Biol Chem* **249**: 3672–3679, 1974.
29. Brenner DE, Grosh WW, Noone R, Stein R, Greco FA and Hande KR, Human plasma pharmacokinetics of doxorubicin: Comparison of bolus and infusional administration. *Cancer Treat Symp* **3**: 77–83, 1984.
30. Olson RD, Mushlin PS, Brenner DE, Fleischer S, Cusack BJ, Chang BK and Boucek RJ Jr, Doxorubicin cardiotoxicity may be caused by its metabolite, doxorubicinol. *Proc Natl Acad Sci USA* **85**: 3585–3589, 1988.
31. Bachur NR, Biochemical pharmacology of the anthracycline antibiotics. In: *Cancer Chemotherapy* (Ed. Sartorelli AC), pp. 58–70. American Chemical Society Symposium Series, No. 30, American Chemical Society, Washington, DC, 1976.
32. Zweier JL, Gianni L, Muindi J and Myers CE, Differences in O_2 reduction by the iron complexes of adriamycin and daunomycin: The importance of the sidechain hydroxyl group. *Biochim Biophys Acta* **884**: 326–336, 1986.
33. Hasinoff BB, Oxyradical production results from the Fe^{3+}–doxorubicin complex undergoing self-reduction by its α-ketol group. *Biochem Cell Biol* **68**: 1331–1336, 1990.
34. Tong GL, Wu HY, Smith TH and Henry DW, Adriamycin analogues. 3. Synthesis of N-alkylated anthracyclines with enhanced efficacy and reduced cardiotoxicity. *J Med Chem* **22**: 912–918, 1979.
35. Gianni L, Vigano L, Lanzi C, Niggeler M and Malatesta V, Role of daunosamine and hydroxyacetyl side chain in reaction with iron and lipid peroxidation by anthracyclines. *J Natl Cancer Inst* **80**: 1104–1111, 1988.
36. Gros P, Croop J and Housman D, Mammalian multidrug resistance gene: Complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell* **47**: 371–380, 1986.
37. Tritton TR and Yee G, The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science* **217**: 248–250, 1982.
38. Thierry AR, Jorgensen TJ, Forst D, Belli JA, Dritschilo A and Rahman A, Multidrug resistance in Chinese hamster cells: Effect of liposome-encapsulated doxorubicin. *Cancer Commun* **1**: 311–316, 1989.
39. Hasmann M, Valet GK, Tapiero H, Trevorrow K and Lampidis T, Membrane potential differences between Adriamycin-sensitive and -resistant cells as measured by flow cytometry. *Biochem Pharmacol* **38**: 305–312, 1989.