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ADRIAMYCIN ACCUMULATION AND METABOLISM IN ADRIAMYCIN-SENSITIVE AND -RESISTANT HUMAN OVARIAN CANCER CELL LINES

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Abstract—Adriamycin accumulation and metabolism were studied in three distinct groups of human ovarian cancer cell lines: those derived from previously untreated patients, those from clinically refractory (relapsed) patients, and those with induced resistance to adriamycin *in vitro*. The 2-hr [^{14}C] adriamycin accumulation in cell lines from previously untreated patients (A2780 and A1847 [Eva *et al.*, *Nature, Lond.* **295**, 116 (1982)] and OVCAR-5 [National Institutes of Health human OVarian CARcinoma cell line no. 5]) was 11–14 ng/10^6 cells. 2780^{AD} and 1847^{AD} (variants with *in vitro* induced resistance to adriamycin) accumulated one-third as much adriamycin after 2 hr (4 ng/10^6 cells). However, three cell lines derived from clinically refractory patients accumulated the same amount of adriamycin as cell lines from untreated patients (8–13 ng/10^6 cells). A high-performance liquid chromatography (HPLC) assay for adriamycin and its analogs confirmed these results and demonstrated only parent drug (no metabolites) in any of the cell lines tested. These results demonstrate that the primary mechanism of adriamycin resistance in some ovarian cancer cells from clinically refractory patients is not enhanced metabolism of drug or a transport defect leading to a decreased net accumulation such as has been described for cells with *in vitro* induced resistance to adriamycin.

The usefulness of adriamycin in the treatment of ovarian cancer has been limited by the frequent development of drug resistance, and much attention has been directed toward identifying possible mechanisms of adriamycin resistance. Most studies have been performed using animal tumor cell systems in which resistance to adriamycin has been developed *in vitro* by incubating cells with gradually increasing doses of drug. In Ehrlich ascites tumor and P388 murine leukemia, anthracycline resistant cells have been shown to have impaired uptake and retention of drug [1–5]. Adriamycin influx is thought to be via a carrier mediated mechanism [3, 5, 6]. In contrast, efflux is felt to be via an active transport system since metabolic inhibitors can virtually abolish adriamycin efflux [1–3, 5, 6]. Both anthracycline resistant P388 and Ehrlich ascites cells demonstrate an enhanced outward transport of adriamycin [1–3, 5]. Other reports have suggested that pleiotropic drug resistance is based on changes in the cell membrane, either the presence of a P170 glycoprotein or alterations in the lipid structural order of the membrane [7–9]. In contrast, transport studies in human red blood cells, which lack the metabolic apparatus and DNA present in nucleated cells, indicate that adriamycin is transported by simple Fickian diffusion [10, 11].

We have reported recently several human ovarian cancer cell lines established from previously untreated patients as well as from relapsed patients [12–17]. In addition, adriamycin-resistant variant cell lines have been developed from the untreated cell lines by stepwise incubation with drugs [13, 14, 17]. Using A1847 (from a previously untreated patient) as well as its adriamycin-resistant variant (1847^{AD}), we have demonstrated that 1847^{AD} has diminished adriamycin accumulation which is secondary to increased drug effux [13]. Further experimental studies on these cells with *in vitro* adriamycin resistance as well as on cells from clinically refractory patients may provide a better understanding of the mechanism(s) involved in clinical adriamycin resistance. The purpose of this study was to investigate adriamycin accumulation in human ovarian cancer cell lines established from previously untreated patients and from relapsed patients, as well as in cell lines with adriamycin resistance developed *in vitro*, in an effort to determine the importance of drug accumulation as a mechanism of clinical adriamycin resistance.

METHODS

Chemicals and reagents. RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin, and glutamine were obtained from the Grand Island Biological Co., Chagrin Falls, OH. Type VII agarose was obtained from the Sigma Chemical Co., St. Louis, MO. Insulin (Iletin U-100) was from Eli Lilly & Co., Indianapolis, IN. Aquassure scintillation fluid (New England Nuclear, Boston, MA) was used. Adriamycin hydrochloride[14-^{14}C] (sp. act.

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30.0 mCi/mmole) was from the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute.

Tissue culture medium. Cells were passaged and maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% (v/v) FBS, 0.25 units/ml insulin, 100 μg/ml streptomycin, 100 units/ml penicillin, and 0.3 mg/ml glutamine as previously described [18]. Cells were incubated at 37° in a humidified atmosphere of 5% (v/v) CO_2.

Clonogenic assay. Drug sensitivity curves and cross-resistance patterns were determined using clonogenicity in soft agarose as previously described [18]. Briefly, cells were harvested with a trypsin (0.05%, w/v)/EDTA (0.02%, w/v) solution and counted on a Coulter Counter (Coulter Electronics model ZBI). Cells in single cell suspension were plated in a mixture of 0.3% (w/v) agarose and RPMI 1640 (including the ingredients listed above) over a layer of solidified 0.6% (w/v) agarose in 10 sq cm dishes. Colonies measuring greater than 60 μm and containing greater than 50 cells were counted on a Bausch & Lomb Omnicon FAS II Image Analysis System.

Cell sizing. Cells were harvested with trypsin as previously described and resuspended at a concentration of 20,000 cells/ml of Isoton. A Coulter Counter model ZBI with a channelizer was used to determine the mean cell volume for each cell line (using the formula V = KAIT, where K = 1). A2780 was arbitrarily assigned a value of 1.00 and all other lines were assigned a relative volume compared to A2780.

Adriamycin accumulation. Cells were harvested with trypsin as previously described and plated as a monolayer in 30 sq cm dishes at a concentration of approximately 0.8×10^6 cells/dish. Forty-eight hours later, 0.15 μM [^{14}C]adriamycin was added to the medium and dishes were incubated at 37° in an atmosphere of 5% (v/v) CO_2. At 15 min, 30 min, 1 hr, 2 hr, 3 hr and 4 hr after addition of drug, dishes were washed with iced phosphate-buffered saline (PBS) five times (washes were discarded). Trypsin/EDTA (0.5 ml) was used to remove cells from the dish. Each dish was then washed twice with 0.75 ml of cold PBS and cells, as well as both washes, were placed in a scintillation vial. Aquassure scintillation fluid (20 ml) was added and mixed. Samples were counted in a Beckman LS2800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

In those experiments in which [^{14}C]adriamycin accumulation was performed simultaneously in all cell lines, the same procedure as that described above was used except that dishes were harvested at a single 2-hr time point.

Different numbers of cells (range 0.3 to 5.0×10^6) were plated and exposed to 0.15 μM [^{14}C]adriamycin for 2 hr. Duplicate dishes were harvested and counted as described above. As can be seen from Fig. 1, 2-hr [^{14}C]adriamycin accumulation varied linearly with cell number from approximately 0.5 to 4.0×10^6 cells/dish. Outside this range, uptake was either elevated (with $<0.5 \times 10^6$ cells/dish) or depressed ($>4 \times 10^6$ cells/dish) by the nonlinear kinetics of cell number variation. Thus, all experiments described in this report were performed with cell counts between 0.5 and 4.0×10^6 cells/dish.

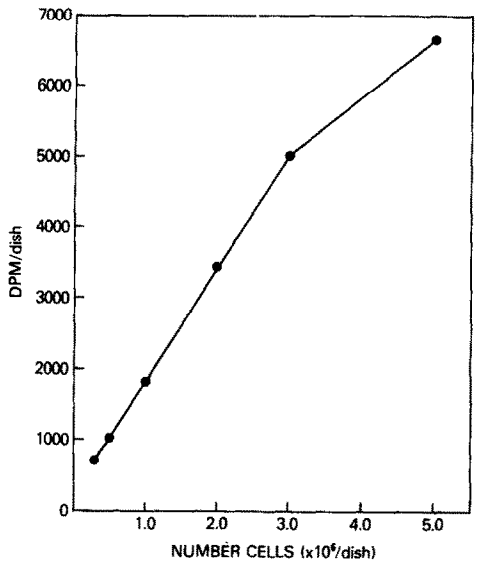

Fig. 1. Adriamycin accumulation versus cell number. Two-hour [^{14}C]adriamycin accumulation was performed using the A2780 cell line with cell numbers varying from 0.3 to 5.0×10^6 per dish. These results represent the mean of duplicate cultures. For each point 1 S.D. is ≤ 50 dpm.

This method determines the total amount of intracellular, as well as membrane associated, adriamycin in the cell lines studied. Since the cells vary considerably in size, the total adriamycin accumulation for each cell line has been divided by the relative cell volume of those cells to yield a corrected adriamycin accumulation based on a normalization of cell size.

High-performance liquid chromatography (HPLC) assay. Cells were plated and incubated with 0.15 μM nonradioactive adriamycin for 2 hr as described above. At 2 hr each dish was washed five times with cold PBS and harvested with 1 ml trypsin/EDTA followed by two 2-ml washes with cold PBS. Cells and washes were placed in polypropylene tubes. Samples were analyzed as previously described [13]. Briefly, 40 ng of daunomycin was added to each sample as internal standard. The samples were frozen and lyophilized. Methanol (3.0 ml) and 0.01 M EDTA (100 μl) were added to the dried pellet. The samples were mixed vigorously and centrifuged at 2000 rpm for 5 min. The methanol was transferred to another polypropylene tube and dried under a gentle stream of nitrogen. The residue was redissolved in 300 μl methanol, and 50 μl was injected into an HPLC system consisting of a reverse phase column with detection by fluorescence at an excitation wavelength of 229 nm [19]. The detection limit of the assay was 2 ng/sample for adriamycin, adriamycinol, and 7-deoxyaglycone.

RESULTS

Characteristics of human ovarian cancer cell lines. By stepwise incubation with drug, A2780 and A1847 were made resistant to adriamycin as previously

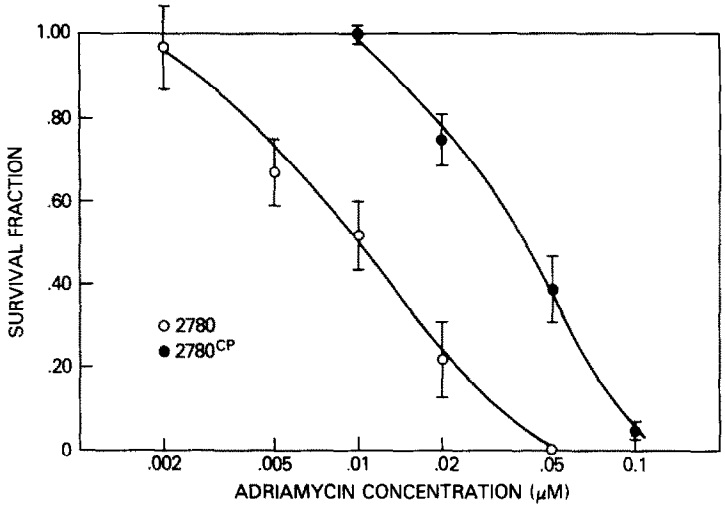

Fig. 2. Cross-resistance to adriamycin in the cisplatin resistant cell line, 2780^{CP}. Clonogenic dose–response curves for A2780 and 2780^{CP} versus continuous exposure adriamycin were performed. Each point represents the mean ± 1 S.D. of three experiments each with triplicate dishes.

described. NIH:OVCAR-3* and NIH:OVCAR-4 were derived from patients refractory to cyclophosphamide, adriamycin, and cisplatin. NIH:OVCAR-2 was established from a patient who had been treated with cyclophosphamide and cisplatin, but not with adriamycin. NIH:OVCAR-5 was from an untreated patient. A1847 and NIH:OVCAR-5, though from untreated patients, are inherently more resistant to adriamycin than A2780. The IC_{50} (drug concentration which inhibits colony growth by 50%) for A1847 is 0.03 μM [17] and for OVCAR-5 0.09 μM as compared to 0.012 μM for A2780 (Table 1). 2780^{AD}† and 2780^{ME} were approximately 100-fold and 2-fold, respectively, more resistant to adriamycin than A2780 (Table 1) [20]. 2780^{CP} was 4-fold cross-resistant to adriamycin (Fig. 2). 1847^{AD} is 5-fold more resistant to adriamycin than A1847 [13]. OVCAR-2, OVCAR-3, and OVCAR-4 inherently showed some resistance to adriamycin with IC_{50} values of 0.10, 0.03 and 0.07 μM compared to A2780 with 0.012 μM. Thus, OVCAR-2 cells, derived from a patient who was not treated with adriamycin, are 3- and 8-fold more resistant to adriamycin than A1847 or A2780 respectively.

Table 1. Cell volume and sensitivity to adriamycin in ovarian cancer cell lines

Cell line	Relative cell volume	Adriamycin (IC_{50} (μM + 1 S.D.)
A2780	1.00	0.012 ± 0.006
2780^{AD}	1.10	1.13 ± 0.54
2780^{ME}	0.94	0.025 ± 0.003
2780^{CP}	1.08	0.042 ± 0.014
A1847	2.22	0.03 [17]
1847^{AD}	3.94	0.15 [17]
OVCAR-2	3.50	0.10 [17]
OVCAR-3	2.44	0.03 ± 0.003
OVCAR-4	1.94	0.07 ± 0.004
OVCAR-5	1.72	0.09 ± 0.01

Cell volumes were generated with a Coulter channelizer. Adriamycin IC_{50} results were determined by continuous exposure clonogenic assay from at least two experiments, each with triplicate dishes (except for A1847, 1847^{AD}, and OVCAR-2 which are cited from Hamilton *et al.* [17].

* OVCAR-2, 3, 4, and 5, National Institutes of Health human OVarian CARcinoma cell lines no. 2, 3, 4, and 5.

† 2780^{AD}, 2780^{ME}, and 2780^{CP}, variants of A2780 selected for resistance to adriamycin, melphalan, and cisplatin respectively.

Relative cell volumes. Relative cell volumes were determined using a Coulter Counter chanelizer. A2780 was assigned a value of 1.00 and all other cell lines were compared to A2780. 2780^{AD}, 2780^{ME}, and 2780^{CP} varied by less than 10% from A2780 with sizes of 1.10, 0.94, and 1.08 respectively (Table 1). A1847 was 2-fold and 1847^{AD} 4-fold larger than A2780, with volumes of 2.22 and 3.94 respectively. The relative volumes of OVCAR 2–5 were 3.50, 2.44, 1.94, and 1.72 respectively.

Adriamycin accumulation. A 4-hr adriamycin study was performed with A2780 (an untreated cell line), 2780^{AD} (with *in vitro* induced resistance to adriamycin), and OVCAR-3 (derived from a clinically refractory patient). From Fig. 3 it can be seen that corrected adriamycin accumulation achieved a steady state for 2780^{AD} after 2 hr at 5 ng/10^6 cells. For OVCAR-3, the rate of accumulation also decreased after 2–3 hr of incubation (with a 2-hr accumulation of approximately 10 ng adriamycin/10^6 cells). However, in A2780 adriamycin accumulation continued to rise after 2 hr. The failure of A2780 to demonstrate a plateau of adriamycin accumulation may be attributed to cell death and possible loss of membrane integrity after approximately 2 hr. Due to sensitivity limitations of the assay, the dose used in these experiments (0.15 μM) was 12-fold higher than the IC_{50} for A2780 (0.012 μM). Figure 3 also depicts the importance of cell volume upon transport studies since the 2-hr uncorrected OVCAR-3 accumulation was roughly twice that of A2780,

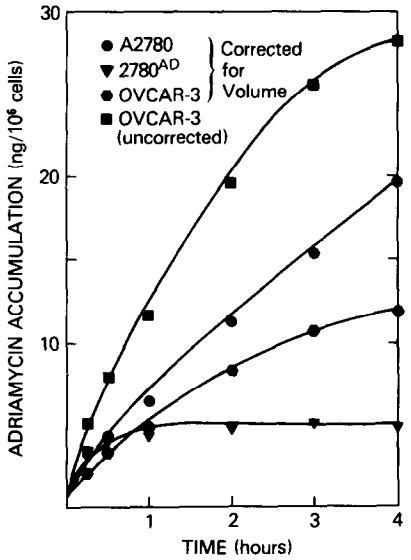

Fig. 3. Adriamycin accumulation in ovarian cancer cell lines. [^{14}C]Adriamycin accumulation (15 min to 4 hr) normalized for cell size was performed on lines A2780, 2780^{AD}, and OVCAR-3. These results represent a single experiment with triplicate dishes. For each point 1 S.D. is <0.8 ng/10^6 cells except for the 4-hr accumulation of OVCAR-3 where 1 S.D. = 1.05 ng/10^6 cells. For OVCAR-3, a curve demonstrating adriamycin accumulation which was not normalized for cell size is also included.

whereas the corrected accumulation was the same as A2780. Table 2 lists the 2-hr adriamycin accumulation of all lines tested. The cell lines A2780, A1847, and OVCAR-5 from untreated patients had total cell-associated (membrane bound and intracellular) adriamycin levels of 11.57, 11.68, and 14.13 ng/10^6 cells after the 2-hr incubation with [^{14}C]adriamycin. These values have all been divided by relative cell volume to correct for variation in cell size. The cell lines with *in vitro* induced resistance to single agent adriamycin, 2780^{AD} and 1847^{AD}, accumulated approximately one-third as much adriamycin as the untreated cells (4.32 and 3.97 ng/10^6 cells respectively). These results are statistically different when compared to each untreated parent line. However, those lines which were established from relapsed patients (OVCAR-2, OVCAR-3, and OVCAR-4) did not demonstrate a diminished accumulation of adriamycin after a 2-hr exposure (with 8.39, 10.70, and 12.50 ng adriamycin/10^6 cells respectively). These results are not statistically different from the accumulation of the three cell lines from untreated patients. In addition, 2780^{ME} and 2780^{CP}, which are cross-resistant to adriamycin (Table 1 and Fig. 2), took up as much adriamycin (18.80 and 10.14 ng/10^6 cells) as lines from untreated patients (Table 2).

HPLC assay. The HPLC assay with fluorescence detection was performed on each cell line after a 2-hr incubation with nonradioactive adriamycin in exactly the same manner as for adriamycin transport above. The results confirm those of the [^{14}C]adriamycin accumulation study. However, the absolute 2-hr adriamycin uptake as determined by HPLC cannot be given since the assay was performed as a qualitative run. Duplicate determinations were obtained on all cell lines simultaneously; therefore, results are expressed as ratios with the 2-hr adriamycin accumulation of A2780 assigned a value of 1.00. From Table 2 it can be seen that the uptake of untreated cell lines was 1.00, 1.52, and 2.07 for A2780, A1847, and OVCAR-5 respectively. Cell lines from clinically refractory patients had 2-hr adriamycin accumulations of 0.99 for OVCAR-2, 0.86 for OVCAR-3, and 1.23 for OVCAR-4, results which are not statistically different from those of the untreated cell lines. However, 2780^{AD} and 1847^{AD} accumulated 1/3–1/4 as much adriamycin at 2 hr as their parent lines. No fluorescent metabolites were detected in any of the cell lines tested (Table 2).

DISCUSSION

The mechanism for adriamycin cytotoxicity in human tumors remains to be established. The lack of

Table 2. Adriamycin accumulation and metabolism in ovarian cancer cell lines

Cell line	Corrected 2-hr [^{14}C]adriamycin accumulation (ng/10^6 cells ± 1 S.D.)	P	Relative 2-hr adriamycin accumulation by HPLC*	Relative 2-hr adriamycin accumulation by ^{14}C-uptake*	Presence of metabolites
A2780	11.57 ± 3.32		1.00	1.00	—
2780^{AD}	4.32 ± 1.13	<0.001	0.24	0.37	—
A1847	11.68 ± 1.67		1.52	1.01	—
1847^{AD}	3.97 ± 0.78	<0.001	0.32	0.34	—
OVCAR-2	8.39 ± 1.37	NS	0.99	0.73	—
OVCAR-3	10.70 ± 2.95	NS	0.86	0.92	—
OVCAR-4	12.50 ± 2.90	NS	1.23	1.08	—
OVCAR-5	14.13 ± 3.18		2.07	1.22	—
2780^{ME}	18.80 ± 1.44	NS	ND	1.62	—
2780^{CP}	10.14 ± 2.85	NS	ND	0.88	—

[^{14}C]Adriamycin accumulation results were derived from at least four experiments performed simultaneously on all lines. The HPLC adriamycin accumulation was obtained from two determinations taken simultaneously on all cell lines. Statistical significance was determined by a two-tailed *t*-test. (NS, not significant, $P > 0.05$; ND, not determined; —, absence of metabolites by HPLC).

* Results are expressed as ratios with the 2-hr adriamycin accumulation of A2780 assigned a value of 1.00.

defined pathway(s) by which adriamycin kills tumor cells has somewhat hampered studies on how tumor cells become resistant to adriamycin. It is known that adriamycin can intercalate within DNA itself; however, it may not be necessary for adriamycin to enter the cell in order to be cytotoxic [21–23]. The cardiac toxicity of adriamycin is thought to be caused by a free radical mechanism, but it is unclear whether antineoplastic activity is similarly mediated [21].

Most previous studies on mechanisms of adriamycin resistance have been in murine systems. Adriamycin resistant Ehrlich ascites and P388 leukemia cells have been shown to have a transport defect which results in decreased anthracycline accumulation secondary to an enhanced outward transport of drug [1–3, 5, 24]. We have demonstrated recently a similar mechanism in human ovarian cancer cells with *in vitro* induced resistance to adriamycin [13]. Other studies have suggested altered membrane glycoproteins or an altered membrane lipid structural order as potential mechanisms to account for drug resistance [7–9]. Human ovarian cancer cell lines derived from clinically refractory patients with ovarian cancer have not been studied in detail previously.

From the results in Table 1, it is apparent that cell lines derived from previously untreated patients (A2780, A1847, and OVCAR-5) have different levels of innate resistance to adriamycin. This is not surprising considering the variable clinical response of patients to chemotherapeutic drugs, including adriamycin which has a 40% response rate as a single agent in untreated patients [25]. OVCAR-2 cells from a patient treated with cyclophosphamide and cisplatin, but not adriamycin, demonstrate cross-resistance to adriamycin, with the IC_{50} actually being higher than for OVCAR-3 (0.10 vs 0.03 μM, respectively) (Table 1). This finding agrees with *in vitro* results showing that human ovarian cancer cells made resistant to either melphalan or cisplatin are cross-resistant to adriamycin (Fig. 2) [20]. In addition, patients treated with a non-adriamycin combination chemotherapy are usually refractory to adriamycin as a second line therapy [26].

Adriamycin accumulation has been studied by [^{14}C]adriamycin uptake and by HPLC assay both in cells with *in vitro* induced resistance to single agent drugs (adriamycin, melphalan, and cisplatin), as well as in cells from previously untreated and clinically refractory (relapsed) patients. From Table 2, it can be seen that the cell lines from previously untreated patients all accumulated the same amount of [^{14}C] adriamycin over 2 hr (approximately 11–14 ng/10^6 cells). The cells with *in vitro* induced resistance to adriamycin, 2780^{AD} and 1874^{AD}, accumulated only one-third to one-fourth as much adriamycin as the cells from untreated patients, thus manifesting the transport defect previously described [2, 3, 13, 24]. These results were confirmed by the HPLC assay. Since 1847^{AD} cells are only 5-fold more resistant to adriamycin than A1847, the mechanism of anthracycline resistance in these cells can be explained by a transport defect which results in 3- to 4-fold less adriamycin uptake. However, since 2780^{AD} is 100-fold more resistant to adriamycin than A2780, transport cannot be the only mechanism involved. Cells with induced resistance to melphalan or cisplatin, while cross-resistant to adriamycin, also did not demonstrate decreased accumulation. All of the cell lines from clinically refractory patients accumulated as much adriamycin as those cells from previously untreated patients (Table 2) (both by ^{14}C-uptake as well as by HPLC assay), suggesting that some mechanism other than transport is primarily responsible for their anthracycline resistance as well.

It should be noted that studies on tumor cell lines *in vitro* may not be truly representative of the tumor *in vivo*. There has been much discussion in the literature recently about tumor cell heterogeneity. Culture and continuous passage *in vitro* may exert a selective effect on cells, causing some subsets to die off while others continue to grow. Therefore, results obtained from *in vitro* systems must be looked at with caution when attempting to draw conclusions regarding the chemosensitivity or resistance of the tumor *in vivo*. Nevertheless, these studies on clinically refractory cell lines provide evidence that at least some cells from clinically refractory patients lack the transport defect described for cells with *in vitro* induced resistance to adriamycin.

After an intravenous bolus of adriamycin in patients, metabolites have been detected in plasma. Adriamycinol, produced by aldoketoreductases, and 7-deoxyaglycone, produced by the P-450 reductase system, are the major metabolites identified [21, 27]. Minimal metabolism has been detected in Chinese hamster ovary cells and Ehrlich ascites cells *in vitro* [28, 29]. However, daunomycinol has been detected in human myeloblastic leukemia cells exposed to daunorubicin *in vitro* [30]. In an effort to determine whether significant metabolism occurs in anthracycline resistant and clinically refractory cell lines, an HPLC assay was performed on cells from each line after a 2-hr incubation with adriamycin (Table 2). No metabolites were identified in any of the cell lines tested. The presence of nonfluorescent metabolites cannot be completely ruled out; however, the absence of any other metabolites makes this possibility less likely.

These results indicate that resistance to adriamycin in cell lines from refractory ovarian cancer patients is a complex multifactorial process and that neither altered drug accumulation nor metabolism to a less active drug is the primary mechanism involved in the expression of drug resistance.

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