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Membrane alterations associated with progressive adriamycin resistance

The anthracycline antibiotic adriamycin is effective in the
treatment of a broad spectrum of human tumors [1]. This
drug inhibits both DNA and RNA syntheses [2–5], pre-
sumably by intercalating between adjacent base pairs of
native DNA [6–8]. Other drug interactions may also con-
tribute to toxicity, e.g. with membrane components [9–11],
tubulin [12], and electron transport processes [13].

Several anthracycline-resistant variants have been
obtained *in vitro* and *in vivo* [14–16]. These resistant cells
exhibit a decreased ability to accumulate the anthracyclines
as compared with parental, drug-sensitive cells; this may
be related to an apparent enhanced energy-dependent drug
efflux [17–19] which could limit the cytoplasmic drug level
to sublethal concentrations.

Many studies on anthracycline resistance have employed
cell lines that are resistant to relatively high drug concen-
trations. Drug resistance is usually not characterized in cell
lines selected for resistance to clinically relevant drug levels.
A particular resistance mechanism may become more
effective with increasing drug resistance; alternatively, cells
may adapt to higher drug levels by multiple mechanisms.

To address this question we isolated, from a metastatic
murine tumor line, a series of variants which exhibit increas-

ing resistance to adriamycin. The parental tumor line
(MDAY-K2), originally described by Kerbel *et al.* [20–22],
rapidly metastasizes to most organs of the mouse following
intradermal or subcutaneous injections. The present study
describes isolation of adriamycin-resistant variants of
MDAY-K2 and effects of increasing drug resistance on
transport and other membrane-related cell properties.

[¹⁴C]Daunorubicin (30 mCi/mmole), obtained from the
Division of Cancer Treatment, National Cancer Institute,
Bethesda, MD, was used as a marker for anthracycline
transport. Growth media and newborn calf serum were
purchased from GIBCO, Grand Island, NY; fetal calf
serum was provided by Sterile Systems Inc., Logan, UT.
PEG* (mol. wt 6000) and Dextran T-500 (lot 7863) were
obtained from the Sigma Chemical Co., St. Louis, MO,
and Pharmacia Fine Chemicals, Piscataway, NJ,
respectively.

The MDAY-KD2 tumor cell line was provided by Dr.
Robert Kerbel, Queen's University, Kingston, Ontario,
Canada. Cultures were propagated in RPMI 1640 medium
supplemented with 5% fetal calf serum, 5% newborn calf
serum, 10 mM HEPES buffer, pH 7.2, 100 μg/ml penicillin
and 100 μg/ml streptomycin. Cultures of drug-resistant cell
lines were derived by exposing cells to a specific level of
adriamycin on a biweekly schedule.

The first adriamycin-resistant variants were selected in
the presence of 0.04 μg/ml adriamycin, until the generation

Table 1. Relative accumulation of daunorubicin

Selection level* (ADR) (μg/ml)	IC$_{50}$† (DNR) (μg/ml)	Uptake‡		
		Control	+ NaN$_3$	+ NEM
Control	0.02	200 ± 30	290 ± 4	220 ± 4
0.04	0.18	90 ± 2	280 ± 1	230 ± 8
0.08	0.40	66 ± 10	272 ± 2	220 ± 8
0.16	0.75	36 ± 8	242 ± 6	204 ± 2

* Concentration of adriamycin used for selection of drug resistant cell line.

† Concentration of daunorubicin which, during a 72–hr incubation, resulted in death of 50% of cells.

‡ Uptake of labeled daunorubicin (pmoles/10^6 cells) during 30-min incubations; extracellular drug concentration was 10 μg/ml. Data represent average ± S.D. of three determinations.

Table 2. Studies on daunorubicin net efflux

Selection level (ADR) (±g/ml)	DNR uptake*	Drug retention† (%)	
		No additions	+ NaN$_3$
Control	210	74 ± 10	96 ± 12
0.04	208	48 ± 10	91 ± 13
0.08	217	38 ± 15	89 ± 13
0.16	203	28 ± 3	88 ± 13

* Daunorubicin uptake (pmoles/10^6 cells) following 30 min of incubation at 37° in medium containing 10 μg/ml of labeled drug.

† Per cent retention of labeled drug during subsequent 10-min incubation of drug-loaded cells in fresh medium at 37° containing, where specified, 10 mM sodium azide with glucose omitted. Data represent average ± S.D. of three determinations.

time of the culture was approximately equal to that of the parental drug-sensitive line. Cells resistant to 0.08 and 0.16 μg/ml adriamycin were successively selected. The three variant lines were cloned by limiting dilution in microtiter plates. The fastest growing clones were isolated and grown in larger culture volumes. Clonal representatives of each of the adriamycin-resistant lines were used in the present experiments. Transport studies were carried out using labeled daunorubicin; IC$_{50}$ levels (Table 1) define the concentrations of daunorubicin which, during 72-hr incubations, decreased the number of viable cells by 50%. Several clonal variants at each resistance level were initially examined. Influx and efflux of daunorubicin were found to be essentially similar for a given adriamycin resistance level.

To measure net accumulation of drug, cells were incubated at a density of 5×10^6 cells/ml in MEM-Eagle's medium. In some studies, energy-dependent processes were inhibited by measuring uptake and exodus in the presence of 10 mM sodium azide using glucose-free medium. After 30-min loading incubations, cells were collected by centrifugation (200 g, 30 sec) and washed once with cold 0.9% NaCl, and drug uptake was assessed by liquid scintillation counting. To measure drug efflux, cells were loaded for 30 min at 37° with [^{14}C]daunorubicin in glucose-free medium containing 10 mM sodium azide. This procedure resulted in all cell lines accumulating approximately the same net amount of daunorubicin (Table 1). Drug-loaded cells were resuspended in fresh medium for 10 min at 37° and then collected for measurement of remaining intracellular radioactivity. The results (Table 2) are reported as percent drug retained. Distribution of drug between cytoplasm and nucleus was measured as described in Ref. 23.

An "uncharged" two-phase aqueous partitioning procedure has been used to delineate subtle alterations in properties of the cell membrane related to hydrophobic interactions with the cellular environment [24–27]. The partitioning mixture contained 5% (w/v) Dextran T-500, 4% (w/v) PEG (mol. wt 6000), 10 mM sodium phosphate

buffer at pH 7.0, and 140 mM NaCl, as described before [28]. The system was supplemented with 0.005% PEG-palmitate [29] in which 8% of the available OH groups were esterified. Partitioning data are expressed in terms of the percentage of the cells that remained in the top phase after a 20-min separation time. An estimate of cell-surface glycosylation was obtained by measuring sialic acid released by neuraminidase treatment as described in Ref. 30.

Membrane fluidity was estimated from fluorescence polarization studies [31] using DPH as a marker. Cells (7 mg) were incubated in 20 mM sodium phosphate buffer (pH 7.0), 130 mM NaCl, and 2 μm DPH for 30 min at 37°. Fluorescence polarization was measured at 37° using an Aminco-Bowman spectrophotofluorometer.

We found that both incubation of cells for 30 min with labeled daunorubicin and subsequent washing for 10 min produced steady-state intracellular drug levels. After the initial 30-min uptake, approximately 75% of the intracellular drug was associated with the nuclear fraction in the different cell lines examined.

The steady-state accumulation of [^{14}C]daunorubicin decreased as a function of adriamycin resistance (Table 1). Impaired drug accumulation in resistant cell lines was antagonized by metabolic inhibitors, e.g. sodium azide or N-ethylmaleimide (Table 1). The capacity of cells to retain accumulated daunorubicin also decreased with increasing drug resistance; this phenomena was antagonized by metabolic inhibitors (Table 2).

All of the radioactive drug retained by these cell lines, after a 30-min wash in medium containing glucose, was found associated with the nuclear fraction. Association of poorly diffusible intracellular daunorubicin with the nucleus has also been reported with Ehrlich tumor cells [32].

Partitioning data (Table 3) show that increased drug resistance was associated with a progressive decrease in the partition coefficient, i.e. with a less hydrophobic cell surface. This is presumably related to the increase in cell-surface glycoprotein generally associated with resistance to anthracyclines and other natural products [28, 33–35].

Table 3. Cell-surface properties

Selection level (ADR) ($\pm$g/ml)	Partition coefficient*	DPH polarization†	Cell-surface sialic acid‡
Control	78 $\pm$ 6	0.252 $\pm$ 0.008	105 $\pm$ 9
0.04	74 $\pm$ 2	0.243 $\pm$ 0.009	140 $\pm$ 11
0.08	56 $\pm$ 8	0.238 $\pm$ 0.008	192 $\pm$ 14
0.16	40 $\pm$ 10	0.231 $\pm$ 0.007	228 $\pm$ 17

* Expressed as per cent total cells found in upper phase after 20 min (mean $\pm$ S.D. for four trials).

† Polarization (P) value; mean $\pm$ S.D. of six trials.

‡ Neuraminidase-liberated sialic acid, μg/g wet cells; mean $\pm$ S.D. of three determinations.

In the present study, progressive resistance to adriamycin was also found associated with increased levels of cell-surface sialic acid (Table 3), an index of total cell-surface carbohydrate [28].

Ling [36] postulated that elevated levels of membrane glycoprotein might serve to reduce membrane fluidity and thereby impair drug influx. Our estimation of membrane fluidity via DPH polarization (P) measurements [37] indicates a *decrease* in P values with increasing drug resistance, hence *increased* membrane fluidity. The polarization data are subject to errors deriving from multiple DPH binding sites [31]. But the present data are not consistent with the supposition that drug resistance leads to decreased membrane fluidity.

As cited in the introduction, several investigators have suggested that anthracycline resistance is associated with energy-dependent enhanced drug efflux. The present investigation suggests that the efficiency of this exodus system increases with increasing drug resistance (Table 3). We also observed biological differences between anthracycline-resistant and parental cell lines studied here. The parental MDAY-KD2 cells, upon intraperitoneal implantation, often form a bloody ascites, while the drug resistant lines form a solid tumor. Subcutaneous inoculation of mice with any of these cell lines led to widespread metastases. Future studies will be necessary to determine if there are any differences in the *in vivo* growth rate of the drug sensitive and resistant cell lines and the site of metastases.

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