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Effects of tunicamycin on anthracycline resistance in P388 murine leukemia cells

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Studies of patterns and modes of resistance in animal tumor models have provided useful information concerning determinants of drug responsiveness. Several factors have been implicated in the development of resistance to natural products, an important class of anti-tumor agents. Cell lines selected for resistance to actinomycin D, Vinca alkaloids or anthracyclines tend to be cross-resistant to many other natural products and to certain synthetic agents [1–5]. Experimental evidence indicates that such drug-resistant cells have markedly altered properties when compared with parent cell lines. These include enhanced energy-dependent efflux of accumulated drug [6–10] and the appearance of new cell-surface glycoprotein [11–16]. The relation between these two phenomena is not known.

The present study was designed to determine whether inhibition of glycoprotein synthesis in an adriamycin-resistant subline of the P388 murine leukemia affects anthracycline responsiveness or drug efflux. Since P388/ADR is cross-resistant to daunorubicin *in vivo* [2], labeled daunorubicin was used in the present studies. The latter drug can be obtained labeled with ^{14}C, thereby minimizing ambiguities in the interpretation of radioactivity measurements.

The effect of tunicamycin on the cell-surface was independently monitored by a two-phase partitioning method [17–19] which can be used to assess cell-surface hydrophobicity [18, 20].

Materials and methods

[^{14}C]Daunorubicin was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD; [^{14}C]cycloleucine and [^{3}H]fucose were purchased from the New England Nuclear Corp., Boston, MA. Radioactive daunorubicin and cycloleucine were diluted with carrier so that 1 ml cell suspensions were exposed to 10 μM levels containing approximately 5×10^4 dpm. Tunicamycin was provided by Eli Lilly & Co., Indianapolis, IN, and by the Division of Cancer Treatment, National Cancer Institute.

P388 and P388/ADR cells were provided by Dr. R. K. Johnson, Arthur D. Little Corp., Cambridge MA. Both cell lines were grown in sealed flasks containing Fisher's medium supplemented with 10% horse serum. P388/ADR cells had an additional requirement for 10 μM mercaptoethanol. All media were obtained from GIBCO, Grand Island, NY.

The drug responsiveness patterns of these cell lines *in vivo* has been described [2]. In Ref. 10, sensitivity of these cell lines to daunorubicin and adriamycin *in vitro* was also reported. The LC_{99} levels (concentrations that kill 99 per cent of cells in 1 hr) were 0.11 μg/ml daunorubicin and 0.25 μg/ml adriamycin for P388, and 2.0 μg/ml daunorubicin and 200 μg/ml adriamycin for P388/ADR.

The effects of tunicamycin and daunorubicin on cell growth were determined by incubating cells with graded levels of drugs for 1 hr. The cells were then suspended in fresh growth medium and diluted to a density of 200 cells/ml. This suspension was supplemented with 0.3% agar and the tubes were incubated at 100% relative humidity in a 7% CO_2 atmosphere for 7 days. Cell viability was then measured by colony counting, with each experiment done in triplicate. The effect of drug on growth rate was monitored using a Coulter counter.

Glycoprotein synthesis was inhibited by a 24-hr exposure of exponentially growing cells to 3 μg/ml tunicamycin. The effect of overnight exposure of cells to tunicamyin on daunorubicin responsiveness was assessed by incubating control versus treated cells in medium containing the anthracycline for 1 hr. The resulting changes in cell viability were monitored as described above. Effects of tunicamycin on incorporation of labeled fucose into glycoprotein and of leucine into protein were measured [14]. This involved a 30-min exposure of 2×10^6 cells to 0.1 μCi of radioactive fucose or 0.01 μCi (0.5 μM) of radioactive leucine in 1 ml of growth medium. Radioactivity in the acid-insoluble fraction was then measured [14]. The effects of tunicamycin treatment on concentrative uptake of the non-metabolized amino acid cycloleucine were measured by incubation of control and treated cells with 10 μM [^{14}C]cycloleucine for 10 min at 37°. Concentrative uptake of cycloleucine was expressed in terms of the [intracellular]/[extracellular] distribution ratio.

Daunorubicin uptake studies were carried out as described [21]. This involved exposure of 1-ml aliquots of a suspension of 5×10^6 cells in HEPES-buffered growth medium to 1 μg/ml (1.8 nmoles/ml) of radioactive daunorubicin for 10 min at 37°.* The cells were then collected by centrifugation, washed once with cold isotonic NaCl, and the intracellular drug level was determined by liquid scintillation counting. Daunorubicin uptake was calculated in terms of pmoles per 10^6 cells.

Studies on daunorubicin efflux [6, 7] were carried out with cells previously loaded with drug during a 10-min incubation in glucose-free medium containing sodium azide, to inhibit exodus. These cells were suspended in fresh medium containing 1 mg/ml glucose, and then collected after 1, 3 or 5 min of incubation at 37° for measurement of intracellular radioactivity.

Partitioning studies were carried out using an aqueous two-phase system that contained 5% (w/v) Dextran T 500 (Pharmacia Fine Chemicals, Piscataway, NJ, Lot 78630), 4% (w/v) poly(ethylene glycol) (Pierce Chemical Co., Rockford, IL, mol. wt 6000), 10 mM sodium phosphate buffer at pH 7.0 and 140 mM NaCl. The partitioning system was supplemented with 0.0001% of 8%-esterified poly(ethylene glycol) palmitate [22]. The partitioning procedure is more fully described elsewhere [15, 17, 18]. Effects on partitioning of short-term treatment of cells with neuraminidase were determined [23]. All partitioning data are reported in terms of number of cells in the top phase, expressed as per cent total cells present, after a 20-min phase separation interval.

Results

Glycoprotein synthesis. Rates of glycoprotein synthesis were estimated from fucose incorporation studies. Treatment with tunicamycin resulted in a 60 per cent inhibition of such incorporation (Table 1). The uptake of leucine into the acid-insoluble fraction was not affected (data not shown).

Partitioning data. As previously described [14], the P388/ADR cell has an inherently more hydrophilic cell-surface than does the P388 cell. Treatment with tunicamycin

* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Table 1. Effects of tunicamycin on properties of murine leukemia cells*

	Partition coefficient†	Fucose‡	Cycloleucine§	ID_{50} DNR‖
P388				
Control	25	1510	3.5	0.042
Treated	83	558	3.7	0.037
P388/ADR				
Control	5.2	4210	3.7	0.61
Treated	81	1603	3.4	0.58

* Cells were exposed to 3 μg/ml tunicamycin for 24 hr. Values represent the average of three determinations with a range of <10 per cent of numbers shown.
† Per cent total cells found in upper phase after 20 min.
‡ Radioactive fucose incorporated as counts·min^{-1}·$(10^7$ cells$)^{-1}$.
§ Distribution ratio representing concentrative uptake of cycloleucine.
‖ Concentration of daunorubicin required to reduce cell viability by 50 per cent.

resulted in a substantial increase in the partition coefficient of both cell lines (Table 1). This same result was obtained after brief treatment of P388 or P388/ADR cells with neuraminidase. The resulting partition coefficients for P388 and P388/ADR were 85 and 83 respectively. This suggests that a major determinant of partitioning behavior was the degree of membrane sialation. Exposure of P388 or P388/ADR cells to 3 μg/ml of tunicamycin for 30 min did not affect partitioning behavior, indicating that the drug-induced modification of the partition coefficient was not related to tunicamycin binding to the cell surface.

Cycloleucine transport. The capacity of P388 and P388/ADR for concentrative uptake of this non-metabolized amino acid was not significantly different, nor did a 24-hr exposure to tunicamycin alter the capacity of these cells for such transport (Table 1).

Daunorubicin responsiveness. When the rate of division of P388 and P388/ADR cells was monitored, we observed a mean doubling time of 9–10 hr. When P388 cells were incubated for 1 hr in medium containing 0.03 μg/ml of daunorubicin, then washed and transferred to fresh medium, the soft-agar clonogenic assay indicated a decrease in cell viability by approximately 50 per cent. Measurement of growth rate with the Coulter counter gave a similar result. This same effect could be produced, in P388/ADR, by exposure of cells for 1 hr to medium containing 0.60 μg/ml of daunorubicin.

When cells of either line were exposed to 3 μg/ml of tunicamycin for 24 hr, transfer to fresh medium resulted in no apparent growth for 24 hr. After this interval, normal growth apparently resumed as shown by growth-curve studies. The soft-agar clonogenic assay indicated only a 5–10 percent loss in cell viability after exposure to tunicamycin. Exposure of tunicamycin-treated P388 cells to 0.04 μg/ml of daunorubicin, or of P388/ADR cells to 0.60 μg/ml of drug, for 1 hr after tunicamycin treatment did not thereby alter these results (Table 1).

Drug transport studies. Table 2 compares the net accumulation of daunorubicin in P388 and P388/ADR in growth medium. Impaired accumulation by the latter line was apparent. This difference could be abolished by the addition of 10 mM azide and the deletion of glucose from the medium.

Efflux was measured using cells pre-loaded with daunorubicin in the presence of azide. The rate of drug loss measured in fresh medium containing glucose, at 37°. Accumulation of daunorubicin was not affected by prior exposure of either cell line to tunicamycin, nor was efflux thereby affected.

Discussion

The finding of elevated levels of glycosylated membrane components in cell lines selected for anthracycline resistance [11–16] had prompted speculation that membrane lipid fluidity might thereby be reduced [24]. Decreased fluidity could impede uptake of agents that penetrate membranes by passive or facilitated diffusion processes, thereby decreasing the rate of drug accumulation.

But an energy-dependent drug exodus system appears to account for the decrease in net drug accumulation in

Table 2. Accumulation and loss of daunorubicin*

	Daunorubicin (pmoles/10^6 cells)				
	Conditions				
	10 min Uptake	10 min Uptake†	Washed (1 min)‡	Washed (3 min)‡	Washed (10 min)‡
P388					
Control	180	175	108	70	52
Treated	182	178	105	66	49
P388/ADR					
Control	68	130	88	59	47
Treated	65	133	71	42	39

* Treated cells were exposed to 3 μg/ml tunicamycin for 24 hr. These values represent the average of three determinations with a range of <10 per cent of the numbers shown.
† Uptake in the presence of 10 mM NaN_3 in glucose-free medium at 37°.
‡ Cells were loaded in medium containing NaN_3 and washed for 1, 3 or 10 min at 37°.

such cell lines [6, 7, 9]. The effect of enhanced efflux on net accumulation can be appreciated when studies are carried out in the presence versus the absence of the exodus inhibitor sodium azide (Table 2).

The present study was undertaken to determine whether interference with glycoprotein synthesis would affect either daunorubicin responsiveness, or the capacity for drug accumulation, of an adriamycin-resistant cell line. We had shown before [24] that the elevated level of membrane glycoprotein in P388/ADR was accompanied by a higher rate of incorporation of labeled fucose into such glycoprotein. This phenomenon is also illustrated by data shown in Table 1.

Suppression of glycoprotein synthesis by treatment with tunicamycin altered the cell-surface properties of both P388 and P388/ADR, as measured by two-phase aqueous partitioning. But neither daunorubicin transport characteristics nor drug responsiveness patterns were affected. These data indicate that the enhanced glycosylation of cell-surface components was associated with the development of drug resistance patterns in P388/ADR, but it was not a determinant of such resistance.

In a related study [25], Beck reported that treatment of a vinblastine-resistant subline of CEM leukemia cells with tunicamycin did not affect the degree of vinblastine resistance. Like P388/ADR, the vinblastine-resistant CEM cell line exhibited an elevated level of cell-surface glycoprotein [11] which was abolished by exposure to tunicamycin [25].

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