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# Modulation of adriamycin transport by hyperthermia as measured by fluorescence-activated cell sorting

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**Summary.** Heat-induced (45.5° C) modification of adriamycin uptake and efflux were measured by flow cytometry in CHO cells in vitro. Administration of adriamycin with simultaneous 15-min or 30-min heat treatment increased drug uptake in a dose-dependent manner. Fluorescence-activated cell sorting showed that cytotoxicity to adriamycin was correlated with relative cellular concentration (fluorescence) for both unheated cells and those heated and simultaneously treated with adriamycin. However, if adriamycin administration followed the heat treatment, accumulation was significantly reduced, primarily as a result of decreased passive drug diffusion (rather than increased efflux) in the heated cells. Cells made heat-tolerant by prior heating also exhibited reduced adriamycin uptake 12 h later, and further heating did not increase uptake. Cell sorting experiments indicated that cytotoxicity of adriamycin was not necessarily correlated with intracellular drug levels when drug administration followed the heat treatment. Also, heat-sterilized cells exhibited a two-fold increase in adriamycin uptake over surviving cells, as assessed by simultaneous measurement of dansyl lysine and adriamycin content. These results indicate that sensitization to adriamycin by simultaneous heat treatment is probably due to increased drug uptake. The decreased sensitization observed when drug administration is followed by heating is probably the result of both decreased uptake and decreased drug DNA accessibility.

## Introduction

Hyperthermia has been shown to increase the cytotoxicity of a variety of chemotherapeutic agents, both in vitro and in vivo [4]. Such a combined regimen may allow for localized tumor chemosensitization of specific, localized tumors at drug levels that are compatible with critical normal tissue' tolerance. One possible exception has been adriamycin, whose cytotoxicity has been demonstrated to exhibit a marked schedule dependency with hyperthermia [6]. The present study was designed to examine in more detail the transport-related mechanisms involved with interactions of adriamycin and heat, using the techniques of flow cytometry and cell sorting.

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Heat has been shown to exert pleiotropic effects on numerous membrane end points examined, including differential ion permeability [22, 25], decreases in membrane potential [14], decrease in number and/or affinity of certain membrane receptors [1, 11, 13, 18] and decreases in membrane capping [21]. Thus, it is not surprising that it should have such a dramatic effect on adriamycin permeability. We have previously shown that cell killing by heat may primarily be a result of membrane protein denaturation and aggregation and lateral phospholipid domain segregation [17]. Heat-sensitization studies employing a variety of membrane-active agents [5, 10, 24] and heat-tolerant cells indicate that the ability to resist these structural alterations correlates with a cell's ability to survive a given heat treatment.

This report examines the multiple effects of hyperthermia on adriamycin transport. We then attempt to correlate these changes to previously published cytotoxicity data [6] as well as relate them to our present understanding of the effects of heat on mammalian cells.

## Materials and methods

*Cell Culture.* The cell line employed was the HA-1 line of Chinese hamster fibroblasts (CHO-HA-1) cultured in alpha-MEM medium supplemented with 10% fetal calf serum. Monolayer stock cultures were trypsinized at least 24 h prior to uptake or heating experiments and seeded into suspension in Corning 15-ml plastic centrifuge tubes and placed on a rotating roller drum at 37° C. Cells were cultured at all times at $10^5$ to $5 \times 10^5$ cells/ml. pH values were strictly maintained between 7.2 and 7.4 during the course of the experiment. All experiments were performed at least in duplicate.

*Hyperthermia.* Cells were centrifuged and resuspended in 1 ml of the appropriate medium and submerged in Haake controlled (±0.05° C) Plexiglas water baths for the prescribed duration. Immediately after heating, 9 ml of the prewarmed medium was rapidly added to each test tube and returned to 37° C. All thermometers were calibrated by standardization against a National Bureau of Standards Certified Thermometer, with corrections made for emergent stem effects.

*Flow cytometry, cell sorting and cytochemistry.* Flow cytometry measurements were made on the Livermore flow

cytometer [15]. Adriamycin-treated cells ($1\text{-}5 \times 10^5$ cells/ml) were analyzed at 500-mW 488-nm excitation and fluorescence was collected with a Corning 520-nm wavelength pass filter. Adriamycin was added to the cell suspensions in full medium at 37° C and the test tube was rapidly inverted and transferred to the flow cytometer, which was modified with a continuous flow 37° C water jacket. Efflux was also measured at 37° C after rinsing stained cells 3 times with 5 ml medium. Uptake (or efflux) was measured by noting the change in the peak mean fluorescence intensity (PMFI) as a function of time after addition (or removal) of adriamycin. The PMFI was calculated numerically using all the cells within the distribution. A Coulter Epics V was used to sort HA-1 cells directly into Petri dishes for survival assay. The cells were exposed for 1 h to 2 μg/ml adriamycin in full medium. The laser power was decreased to 200 mW for these studies. Survival was measured by colony formation.

Simultaneous multiparameter analysis of staining by adriamycin and by the structure-sensitive dye dansyl lysine (DL) was undertaken on the Livermore dual beam cytometer. Briefly, cells were incubated with 10 μg/ml adriamycin for 1 h and stained with 500 μ*M* DL immediately prior to analysis. Adriamycin was excited with 1-W 488-nm UV and DL with 500-mW UV, and fluorescence was collected for both employing a Corning 3–69 pass filter, with removal of any signal overlap electronically.

*Drugs.* Adriamycin was obtained from Adria Labs and a stock solution of 1 mg/ml in phosphate-buffered saline (PBS) was made up fresh for each experiment. DL was obtained from Sigma Chemicals and dissolved to $10^{-3}$ *M* in PBS.

## Results

Since cytotoxic sensitization to adriamycin was previously demonstrated when HA-1 cells were treated for relatively short periods with heat in the presence of adriamycin [6], we examined adriamycin uptake during simultaneous heat exposure in this cell line. Cultures were treated with various concentrations of adriamycin for 1 h and either incubated at 37° C for the whole hour or heated for 15 or 30 min at 45.5° C and then returned to 37° C for the remainder of the hour. The data in Fig. 1 show that a dose-dependent increase in mean fluorescence intensity was observed for the heated cultures as compared to the nonheated controls. Heating increased the mean cellular fluorescence by approximately 20% for each 15-min heat interval.

To establish if the fluorescence observed was correlated to adriamycin cytotoxicity, cells were sorted from five arbitrary collection windows across the adriamycin flow cytometry distribution as shown in Fig. 2. These cells were stained for 1 h with 2 μg/ml adriamycin with or without noncytotoxic heat treatment (10 min, 45.5° C) simultaneous with drug exposure. As expected (from Fig. 1) the mean fluorescence intensity of the heated population (panel A) was greater than that of the nonheated population (panel C), by approximately 20%. Figure 2 (right) also shows that survival was highest in the sort window of lowest fluorescence, and decreased with increasing fluorescence. This relationship was observed both for the control (i.e., nonheated) cells and for the cells treated simultaneously with adriamycin and heat (panel A).

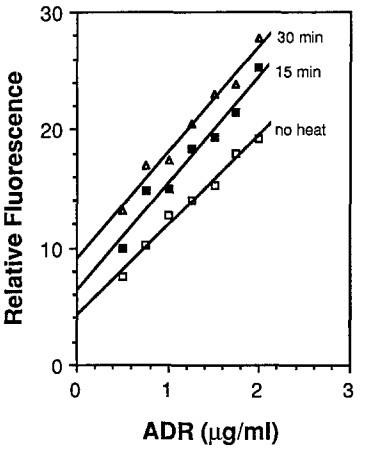


**Fig. 1.** The increase in adriamycin permeability when heat and drug are applied simultaneously. HA-1 cells were heated for 0, 15 or 30 min at 45.5° C with simultaneous addition of various concentrations of adriamycin. Cells were stained for a total of 1 h before analysis of mean cellular fluorescence by flow cytometry

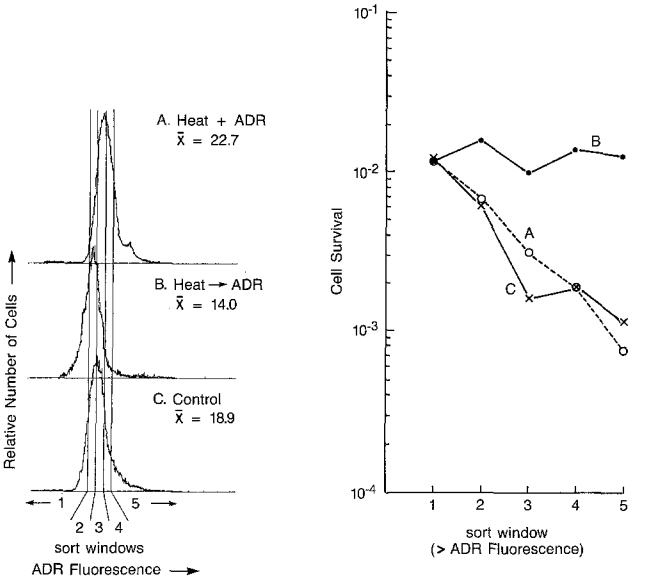


**Fig. 2.** The relative amount of adriamycin fluorescence correlates with the amount of drug-induced cytotoxicity for control and heated (drug added simultaneously) cells, but not when the heat treatment precedes drug exposure. Panel *C* shows a flow cytometry histogram of HA-1 cells that were treated with 2 μg/ml adriamycin for 1 h. Panel *A* shows a flow cytometry histogram similarly stained except with a noncytotoxic 10 min/45.5° C heat treatment applied within the hour. These two histograms are directly comparable, and show that this mild heat treatment increases mean fluorescence by approximately 20% (as expected; Fig. 1). Arbitrary windows were selected and cells were sorted for survival assay within these windows. The survival curve on the *right* shows cell survival levels within the five sort windows from both panel *C* (control) and panel *A* (simultaneous heat). These data show enhanced cytotoxicity with increasing fluorescence. Also, since the sort windows encompassed the same relative fluorescence levels (and since the heat treatment was not cytotoxic), both survival levels are similar for the five sort windows. Panel *B* shows the FCM distribution for cells heated (10 min/45.5° C) *before* addition of adriamycin. Mean fluorescence levels were decreased by approximately 25% from control (panel *C*). Also, there was no correlation between fluorescence and cytotoxicity within any of the five sorted windows. (Again, these sort windows are directly comparable to panels *A* and *C*.) This figure shows the results of one of four experiments in which similar data were observed

HA-1 cells heated for 10 min and then returned to 37° C exhibited marked inhibition in uptake when the drug was added following the heat treatment (Fig. 3 A). For instance, by 240 min, the cells heated for 10 min showed nearly a twofold reduction in adriamycin fluorescence over controls. Heating for 40 min (survival $< 10^{-4}$, see insert in Fig. 3 A) decreased net adriamycin accumulation by a lesser amount than did the 10-min treatment. This is probably due to inclusion of a highly permeable subset of the dead cells within the distribution (see below) used to calculate the PMFI.

When HA-1 cells were exposed to a brief heat treatment and returned to 37° C for a period of time, the cells display thermal tolerance, i.e., a marked resistance to a second heat treatment. In Fig. 3 B, tolerance was induced in HA-1 cells by a 10-min 45.5° C heat treatment followed by a 12-h incubation at 37° C. As shown in the figure uptake was still inhibited 12 h later and could not be increased with further heating of 10 or 40 min. Efflux measurements of adriamycin are shown in Fig. 3 C. Cells were stained and rinsed as described in "Material and methods" and the decrease in fluorescence was measured as a function of time at 37° C. The control cells lost fluorescence in a biphasic manner; an initial, rapid decrease for approximately 20 min was followed by a slower rate of loss thereafter up to 140 min. The efflux measurements of cells heated for 10 min showed very little difference from control; however, those cells heated for 40 min initially exhibited marked depression in efflux capacity. This effect was transient, since by 100 min adriamycin fluorescence had dropped to control levels.

The relationship between cellular adriamycin fluorescence and cytotoxicity when adriamycin administration followed the heat treatment is shown in Fig. 2 where panel B shows a population of cells preheated for a non-cytotoxic treatment of 10 min at 45.5° C before addition of the adriamycin for 1 h. In this case, cellular fluorescence was decreased by approximately 25% from control (panel C) and there was no correlation between adriamycin content and cytotoxicity within the fluorescence distribution (Fig. 2, right). Even though mean cellular fluorescence was reduced from control levels, the high fluorescence sort windows of the preheated population overlapped the low fluorescence windows of the control population. A comparison of survival values within these windows of similar adriamycin fluorescence indicated significant resistance of the preheated population as compared to the control, given equal fluorescence.

The results for the cytotoxic heat-treatments might be affected by heat-killed cells. We therefore examind whether such cells exhibited adriamycin uptake significantly different from that of those cells surviving the heat treatment. For these studies we stained cells both with adriamycin and with DL. The fluorescence of these dyes was examined simultaneously by dual laser flow cytometry (Fig. 4). DL is a membrane-specific probe that binds to cholesterol-depleted domains on the plasma membrane [7]. We have shown that cells staining with DL are clonogenically dead. The insert in Fig. 3 A shows that the fraction of DL-excluding cells shows a good, though not absolute, correlation with cell survival 24 h after the heat treatment. (By 72 h after heating, this correlation is nearly 1:1 [17].) Further, freeze fracture electron microscopy indicated that the lipid domain segregation was due to a heat-induced membrane

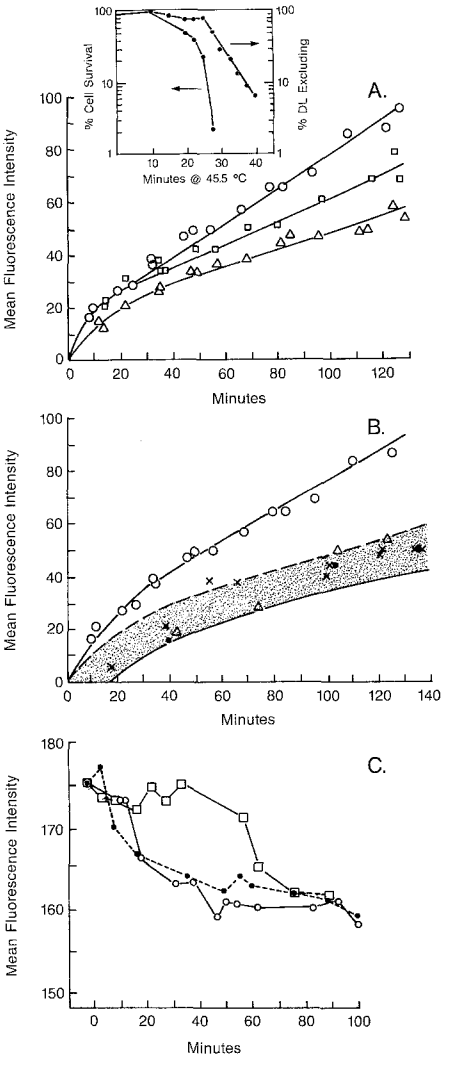


**Fig. 3 A–C.** Adriamycin uptake and efflux measurements for heated and control HA-1 cells. The insert shows both cell survival and percentage of DL-excluding cells as a function of heat treatment. (DL stains heat-killed cells). **A** Adriamycin uptake (2 μg/ml immediately after 0 min (○), 10 min (△) or 40 (□) min at 45.5° C. **B** CHO cells made tolerant by a heat treatment 12 h previously. These tolerant cells were then heated again for 0 min (X), 10 min (△) or 40 min (◆). Also shown (*dashed line*) is the data from **A** for cells heated for 10 min and assayed immediately. **C** Efflux measurements for HA-1 cells first treated for 1 h with 2 μg/ml adriamycin, then heated for either 0 min (●), 10 min (○) or 40 min (□), rinsed 3 times in medium before returning to 37° C for flow cytometric assay

protein aggregation [17]. Cells killed by adriamycin (not heat) are not stained by DL [17]. Figure 4 shows that those cells stained by DL were highly permeable to adriamycin as compared to non-DL staining cells within the population. Both nonheated (Fig. 4 A) and heated (Fig. 4 B) populations had DL staining fractions (in an untreated cell population typically 2%–5% of the cells stain DL). Both DL staining fractions exhibited an approximately twofold increase in average adriamycin content per cell.

## Discussion

Several investigators have presented data showing that a good correlation exists between intracellular adriamycin

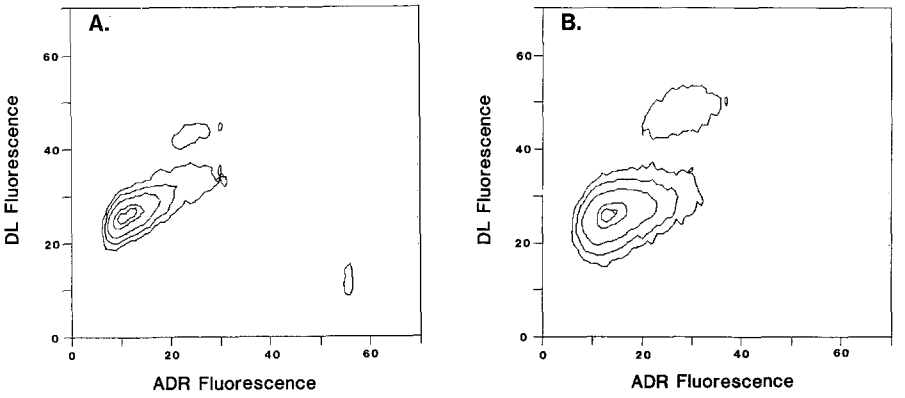


**Fig. 4 A, B.** Simultaneous bivariate analysis of adriamycin and dansyl lysine (DL) staining. Cells were heated, treated with adriamycin for 1 h and DL was added immediately prior to flow analysis. The two populations depicted are DL staining (*upper* subpopulation) and nonstaining (*lower* subpopulation) cells. **A** nonheated cells; **B** cells heated for 40 min, 45.5° C

content (and that of another anthracycline, daunorubicin) as measured either in intact cells by flow cytometry or fluorimetrically by organic extraction techniques [2, 12]. The mechanism of adriamycin cytotoxicity is thought to be dependent on high-affinity binding of the drug to DNA [3] leading to decreased template initiation, although other mechanisms involving the plasma membrane have been postulated [23]. Although the present study does not permit any conclusions regarding the precise mode of adriamycin uptake and efflux in HA-1 cells, the kinetic data are compatible with the "leak and pump" system observed in other mammalian cell lines [9]. Uptake is via passive diffusion and the "pump" is responsible for active efflux. Uptake kinetics showed little evidence of a saturable response, thus suggesting a passive uptake mechanism. The biphasic efflux kinetics are similar to those observed for the energy-dependent efflux of adriamycin seen in other cell lines [9, 20].

We observed an increase in fluorescence in HA-1 cells treated simultaneously with adriamycin and heat over that seen in nonpreheated controls. This correlates well with earlier cytotoxicity data [6], indicating that the hyperthermic potentiation under these conditions was probably due to a direct and immediate effect of heat to increase adriamycin uptake. Our cell sorting data show that a good correlation exists between adriamycin fluorescence and adriamycin-associated cytotoxicity in unheated cells or when cells are heated in the presence of the drug (Fig. 2, panels A, C). This suggests that the mechanism of increased cytotoxicity observed with simultaneous exposure is simply one of increased drug permeability.

In the case of administration of adriamycin after heating, however, our data suggest two mechanisms for decreased sensitization. First, as shown in Fig. 3, membrane permeability is decreased. This effect is due primarily to decreased uptake rather than increased efflux. Second, comparison of survival in control and heated cells given equal doses of adriamycin (Fig. 2) indicates decreased effectiveness (in terms of cytotoxicity) of adriamycin when heat precedes drug administration. Primary or secondary heat effects may mask target sites for adriamycin. For instance, it is known that a large increase in nuclear nonhistone chromosomal proteins is induced following heating [19] and could compete with or mask DNA-binding sites.

Dansyl lysine was used in this study to distinguish heat-sterilized cells from viable cells [16]. Simultaneous measurement of DL staining and adriamycin content, undertaken by dual laser flow cytometry analysis, indicated that heat-sterilized cells (i.e., staining cells) accumulated approximately twofold more adriamycin than did cells not stained by DL. Previously, lateral phospholipid domains have by themselves been shown to impart altered membrane permeability to glucose, fluorescein and other species [8].

In conclusion, our results indicate that heat decreases the net accumulation and cytotoxicity of adriamycin unless treatments are applied simultaneously. Decreased accumulation is a result of an inhibition of inward passive drug diffusion (rather than an increase in efflux). This is in addition to, and independent from, a decrease in the cytotoxic efficiency of intracellular adriamycin following heating. Cells made heat-tolerant remain refractive to drug accumulation. These transport data correlate with earlier cytotoxicity data [6] and point to precautions that must be taken if hyperthermia and adriamycin are to be used within the clinic.

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