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Nucleoside Uptake and Membrane Fluidity Studies on *N*-Trifluoroacetyladriamycin-14-*O*-hemiadipate-treated Human Leukemia and Lymphoma Cells[1]

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ABSTRACT

N-Trifluoroacetyladriamycin-14-*O*-hemiadipate (AD 143), a DNA nonbinding derivative of Adriamycin, was studied for its effect on the uptake of labeled nucleosides into human leukemia (ML-1) and lymphoma (P3HR-1) cells in culture. After preincubation with AD 143 at concentrations as low as 5.2 μM (ML-1) or 13 μM (P3HR-1), the ability of the cells to take up extracellular labeled nucleosides was decreased by more than 50%. Similar experiments with *Escherichia coli* cells showed that AD 143 at the same concentrations did not have any effect. Influx of [^{3}H]thymidine or [^{3}H]uridine was studied by centrifugation of the cells through phthalate oil mixture, and it was found that the influx of the labeled nucleosides was decreased after treatment of the cells with AD 143. An increase in the membrane fluidity was observed after treatment of the cells with AD 143, as revealed by electron paramagnetic resonance spectroscopy studies. These observations suggest that the decreased incorporation of [^{3}H]thymidine and [^{3}H]uridine into acid-precipitable material that we observed earlier in the AD 143-treated cells may in part be the result of the AD 143-induced alteration of cell membrane activities, which in turn causes an inhibition of nucleoside uptake.

INTRODUCTION

Among many derivatives of ADR,[3] AD 143 has been demonstrated to have greater antitumor activity and lower cardiac toxicity than ADR (1). Hence, this new ADR analogue may be considered a potential cancer chemotherapeutic agent for clinical trials. In our previous *in vitro* studies, we showed that AD 143, unlike ADR, did not bind to DNA (2). Using terbium, a rare earth lanthanide which interacts preferentially with guanine bases in nonhelical regions of DNA, we have also found that AD 143 produced fewer effects than Adriamycin on terbium fluorescence with chromatin or nucleosomes (3). As a DNA-nonbinding agent, AD 143 had little or no effect on the enzymatic activities of leukemia DNA polymerase or the RNA and DNA polymerases of prokaryotic origin (2). Nevertheless, AD 143 was found to be a potent inhibitor of leukemia RNA polymerases I and II, and AD 143-induced inhibition of leukemia RNA synthesis was a result of inhibition of RNA chain initiation (2, 4). The susceptible step in the initiation process was found to be the formation of stable complexes between RNA polymerase and the DNA template. A preformed enzyme-DNA complex was less sensitive to AD 143 inhibition (4). While AD 143 caused no inhibition of *Escherichia coli* RNA polymerase activity, it was also found not to affect the *E. coli* RNA polymerase-template DNA complex formation (4). The inhibition of RNA synthesis by AD 143 in eukaryotic cells is, therefore, not an effect of AD 143-DNA template interaction, but a result of the formation of the AD 143-RNA polymerase association complex (5). This complex could be isolated through glycerol gradient sedimentation and subsequently dissociated upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5).

Although AD 143 was found not to inhibit DNA polymerase activity (2), it was found that AD 143 reduced the amount of incorporation of [^{3}H]dThd into the acid-precipitable materials of human leukemia ML-1 or human lymphoma P3HR-1 cells (6, 7). These observations prompted us to investigate whether the observed reduction of labeled nucleoside incorporation relates to an AD 143-induced alteration of cell membrane activity and hence an inhibition of cellular uptake of labeled nucleosides.

MATERIALS AND METHODS

[*methyl*-^{3}H]Thymidine ([^{3}H]dThd) and [5,6-^{3}H]uridine ([^{3}H]uridine) were purchased from New England Nuclear, Boston, MA. 5-Doxyl stearic acid was purchased from Sigma Chemical Co., St. Louis, MO. Di-*n*-butyl-phthalate (D = 1.043 g/ml) and di-*n*-octyl-phthalate (D = 0.981 g/ml), obtained from Aldrich Chemical Co., Milwaukee, WI, were blended 4:1 (v/v) yielding an oil mixture of D = 1.032 g/ml. Adriamycin was purchased from Adria Laboratories. AD 143 was synthesized in one of the authors' laboratories (M. I.). The human ML-1 leukemia cell line was originally isolated in 1978 from the peripheral blood of a 24-yr-old patient with acute myeloblastic leukemia (8). The human P3HR-1 cell line was originally derived from a patient with Burkitt's lymphoma (9). Both cell lines were maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum.

Thymidine and Uridine Uptake Studies. Cultures (6 ml) of human leukemia cells (ML-1), human lymphoma cells (P3HR-1), or *E. coli* cells in exponential growth were treated with various concentrations of AD 143, ADR, or buffer (as controls) for 2 h at 37°C and cooled to 4°C prior to the addition of [^{3}H]dThd or [^{3}H]uridine. The cells were then incubated with [^{3}H]dThd or [^{3}H]uridine at 4°C for 45 min to allow cellular accumulation, but not further utilization (or incorporation) into macromolecules, of labeled nucleosides (10). At 4°C, the cells were also unable to phosphorylate intracellular [^{3}H]dThd or [^{3}H]uridine, as determined by the DEAE-disc method of Cheng *et al.* (11, 12). After incubation, the cells were washed with cold medium, centrifuged at 1500 × *g* for 7 min, and then resuspended in ice-cold medium containing 0.12 mM thymidine or uridine. After a second centrifugation, the cells were lysed with 100 μl of 1% Triton X-100 and precipitated with 3 ml of 33% cold TCA. TCA-soluble (indicator of nucleoside uptake) and -precipitable (measure of nucleoside incorporation) materials were separated by filtration through GF/C filters.

Measurement of the Rapid Influx of Labeled Nucleosides. Cells (1.2 ml) in exponential growth phase (5 × 10^5 cells/ml) were treated with AD 143 or ADR for 2 h at room temperature. Labeled nucleoside ([^{3}H]dThd or [^{3}H]uridine) was added and the sample mixed gently. The influx of the nucleoside was terminated at specific times by centrifugation of 1 ml of the cells over 0.3 ml of phthalate oil mixture in an Eppendorf microcentrifuge tube (13). The supernatant fraction above the oil interface was sampled and counted in a scintillation counter to determine the amount of labeled nucleoside not taken in by the cells.

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[3] The abbreviations used are: ADR, Adriamycin; AD 143, *N*-trifluoroacetyladriamycin-14-*O*-hemiadipate; [^{3}H]dThd, [^{3}H]thymidine; AD 32, *N*-trifluoroacetyladriamycin-14-valerate; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; EPR, electron paramagnetic resonance.

The remainder of the supernatant fraction was aspirated and discarded, the walls of the microcentrifuge tube were washed 3 times with water, and most of the oil was removed with the final washing. Cell pellets were solubilized in 0.5 ml of 0.5 N NaOH or 1% Triton X-100. Scintillation fluid was added, and the sample was counted in a scintillation counter to determine the amount of labeled nucleoside taken up by the cells (13).

Electron Paramagnetic Resonance Studies. Human leukemic cells (ML-1) were grown to a cell density of 5×10^5 cells/ml. The cell suspension (20 ml) was centrifuged, and the cell pellet was washed with 20 ml of PBS solution at 37°C. The cells were collected by centrifugation and resuspended in 0.5 ml of PBS containing 20 mg/ml of 5-doxyl stearic acid. After a 15-min incubation at 37°C, the cells were again collected, washed once, and resuspended in 50 μl of PBS. At this point, 90 to 95% of the cells were viable as measured by the trypan blue exclusion method. ADR, AD 143, or buffer (as controls) was subsequently added; the cell suspension was drawn into a 50-μl capillary pipet; and the tip of the pipet was sealed. The capillary pipet was centrifuged for 1 min to sediment the cells gently and then placed in the cavity of a Varian E-3 spectrometer. Spectra were collected at a modulation amplitude of 10 G, microwave power of 5 milliwatts, modulation frequency of 100 kHz, scan time of 8 min, time constant of 1 s, and sweep width of 100 G (14, 15). The order parameter (S value), a measure of relative membrane fluidity, was determined from the separations of the outer ($2T_\parallel'$) and inner ($2T_\perp'$) hyperfine extrema (Fig. 1) according to the method of Gaffney *et al.* (16). Order parameters (S) were calculated according to the following equations (17)

$$S = \frac{T_\parallel' - T_\perp' - C}{T_\parallel' + 2\,T_\perp' + 2C} \times 1.723$$

where $C = 1.4 - 0.053\,(T_\parallel' - T_\perp')$.

In our experiment, the total time of exposure of the cells to the drugs did not exceed 45 min. Both drug binding to the plasma membrane and transport into the cells have been reported to have reached equilibrium by this time (14). We have observed, as it has also been reported (17), that after a 1-h period there was a considerable reduction in amplitude of the signal presumably due to chemical reduction of the paramagnetic nitroxide group by the cell components (17).

RESULTS

Effect of AD 143 on Nucleoside Uptake of Human Leukemia ML-1 and Lymphoma P3HR-1 Cells. AD 143, ADR, or buffer (as control)-treated ML-1 and P3HR-1 cells were incubated with [^{3}H]dThd or [^{3}H]uridine at 4°C for 45 min to allow cellular accumulation of labeled nucleosides as described in "Materials and Methods." The measurement of the amount of TCA-soluble radioactivity in the cell after 45-min incubation was an indication of the amount of uptake of the labeled nucleosides into the cells (10). Table 1 shows that, with increasing concentrations of AD 143, the uptake of the labeled nucleosides into ML-1 and P3HR-1 cells decreased. At AD 143 concentrations as low as 5.2 μM (ML-1) or 13 μM (P3HR-1), the ability of the cells to take up extracellular labeled nucleosides was decreased by more than 50%. The results also indicated that, at a similar concentration (13 μM), AD 143 had no effect on the uptake of labeled nucleosides into *E. coli* cells. Moreover, ADR, the parent compound of AD 143, at comparable concentrations caused less inhibition of the uptake of [^{3}H]uridine or [^{3}H]dThd into either ML-1 or P3HR-1 cells, and like AD 143, it had essentially no effect on the ability of *E. coli* cells to take up extracellular nucleosides (Table 1).

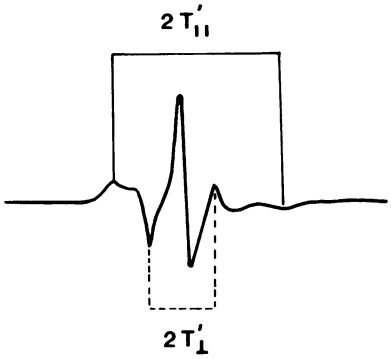

Fig. 1. Representative EPR spectrum of human leukemia ML-1 cells labeled with 5-doxyl stearic acid. Exponentially growing cells were labeled with the paramagnetic probe, 5-doxyl stearic acid, as described in "Materials and Methods." The approximate outer ($2T_\parallel'$) and inner ($2T_\perp'$) extrema used to determine the order parameters (S) are indicated.

Effect of AD 143 on Rapid Influx of Labeled Nucleosides. To demonstrate further that, after treatment with AD 143, the cultured human cancer cells took up less nucleosides from the medium, the rapid influx of labeled nucleosides into the drug-treated cells was determined. This was done by measuring, using phthalate oil mixture, the amount of labeled nucleoside that was taken up into the cells after only a short time (15 to 60 s) of exposure of the cells to [^{3}H]uridine or [^{3}H]dThd (see "Materials and Methods"). The results indicated that the influx of [^{3}H]dThd and [^{3}H]uridine into both ML-1 and P3HR-1 cells was inhibited after treatment of the cells with AD 143 for 2 h (Figs. 2 and 3). The inhibition of [^{3}H]uridine influx was as high as 70% for ML-1 cells pretreated with 52 μM AD 143. For the P3HR-1 cells, the influx of [^{3}H]uridine or [^{3}H]dThd was inhibited by 50% at AD 143 concentrations of 26 μM or higher. However, when the ML-1 cells were pretreated with ADR under similar conditions, the observed inhibition was not as significant (Fig. 4).

These data indicated that the inhibitory effect of AD 143 on the uptake of labeled nucleosides is observed even after exposure of the cells to the nucleoside for periods as short as 15 s.

EPR Studies. Using EPR spectroscopy, fatty acid spin labels were used to measure the inherent flexibility of lipid acyl chains in the intact cell membrane of ML-1 cells as affected by AD 143 or ADR treatment. The spin-labeled fatty acids provide a measure of the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chain in the lipid bilayer. The amplitude of motion of these labels is sensitive to the flexibility of membrane lipid chains.

A sample spectrum of 5-doxyl stearic acid-labeled cells is illustrated in Fig. 1. Spectral parameters are used to calculate the order parameter, S (16). The order parameter is related to the mean angular deviation of the labeled fatty acid chain from its average orientation in the lipid bilayer. High values of order parameters are characteristic of relatively solid lipid, and low values characterize very mobile lipids.

Table 2 shows the values obtained for the change in order parameter of ML-1 cells after treatment with AD 143 or ADR. Each value is the average of three or more experiments done in duplicate. A decrease in order parameter after the exposure to AD 143 was observed. ADR at the same concentrations, on the other hand, had much less effect on the S values of ML-1 cells (Table 2).

DISCUSSION

Adriamycin and its related anthracyclines inhibit cell growth by inhibiting cellular DNA and RNA syntheses (18, 19). The DNA-nonbinding Adriamycin derivatives AD 32 and AD 143, however, show a preferential inhibitory activity against cellular RNA *versus* DNA synthesis (2, 4, 5, 10, 20). The present studies demonstrated that, in addition to acting as a eukaryotic RNA polymerase inhibitor, AD 143 may affect other cellular functions by altering cell membrane activities.

Table 1 *Effect of AD 143 on [^{3}H]dThd and [^{3}H]uridine uptake*

Cultures (6 ml) of exponentially growing ML-1 or P3HR-1 cells (3 to 5 × 10^5 cells/ml) were treated with AD 143 at 37°C for 2 h and then labeled at 4°C with [^{3}H]dThd (4 μCi/ml) or [^{3}H]uridine (2 μCi/ml) for 45 min. TCA-soluble materials were counted as described in "Materials and Methods." For comparison purpose, [^{3}H]dThd or [^{3}H]uridine uptake of drug-treated *E. coli* cells and the effects of ADR on [^{3}H]dThd and [^{3}H]uridine were also studied.

Drug	Concentration (μM)	TCA-soluble radioactivity[a] (%)					
		ML-1		P3HR-1		*E. coli*	
		[^{3}H]dThd[b]	[^{3}H]Uridine[c]	[^{3}H]dThd[b]	[^{3}H]Uridine[c]	[^{3}H]dThd[b]	[^{3}H]Uridine[d]
AD 143	0	100	100	100	100	100	100
	5.2	39 ± 2.9[e]	48.7 ± 2.9	ND[f]	ND	ND	ND
	13	29 ± 2.9	33 ± 0.8	34 ± 3	ND	155	145 ± 5
	26	25 ± 1	27 ± 2.9	33.5 ± 1.5	38.5 ± 1.5	ND	ND
	52	22.5 ± 2.5	26 ± 2	30.5 ± 7.5	32 ± 4	129 ± 21	121 ± 17
	78	ND	ND	ND	27 ± 1	112	ND
ADR	0	100	100	100	100	100	100
	6.5	75.5 ± 0.5	80 ± 8	ND	87 ± 6	ND	ND
	13	75.5 ± 0.5	79 ± 3	60 ± 0.6	74.5 ± 2.5	112	103
	26	85 ± 5	74.5 ± 0.5	76 ± 1	70 ± 0.5	ND	ND
	52	ND	ND	61 ± 0.5	ND	94	95

[a] TCA-precipitable radioactivity was not detectable at 4°C.
[b] Range of counts, 9.8 × 10^4 to 1.5 × 10^6 cpm.
[c] Range of counts, 5.7 × 10^4 to 3.5 × 10^5 cpm.
[d] Range of counts, 2.9 to 5.3 × 10^4 cpm.
[e] Mean ± SD.
[f] ND, not determined.

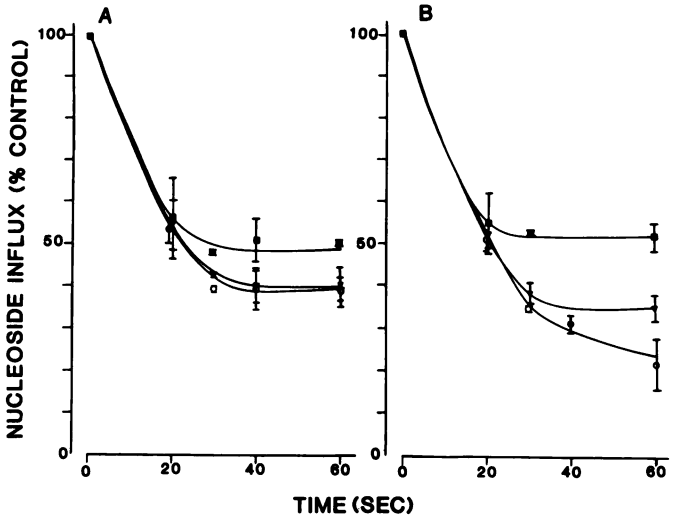

Fig. 2. Time course of nucleoside influx into human leukemia ML-1 cells after treatment with AD 143. [^{3}H]dThd (0.5 μCi/ml) (*A*) or [^{3}H]uridine (0.5 μCi/ml) (*B*) was added to a cell suspension (5 × 10^5 cells/ml) pretreated with AD 143. At the indicated time, the influx of nucleosides was terminated by centrifuging the cells over oil mixture, and the radioactivity inside the cells was determined as described in "Materials and Methods." The AD 143 concentration used was 13 μM (■), 26 μM (▼), or 52 μM (○). The control value (100%) for each *point* was the amount of nucleoside influx of the nontreated cells at the indicated time. The absolute values of radioactivity were in the following range of counts (cpm): [^{3}H]dThd, 1,309 to 4,295 (20 s) and 2,743 to 5,239 (60 s) for control values and 724 to 2,976 (20 s) and 1,396 to 2,800 (60 s) for drug-treated cells; [^{3}H]uridine, 3,997 to 7,805 (20 s) and 5,067 to 11,585 (60 s) for control values and 2,211 to 3,647 (20 s) and 2,154 to 4,394 (60 s) for drug-treated cells. *Bars*, SD.

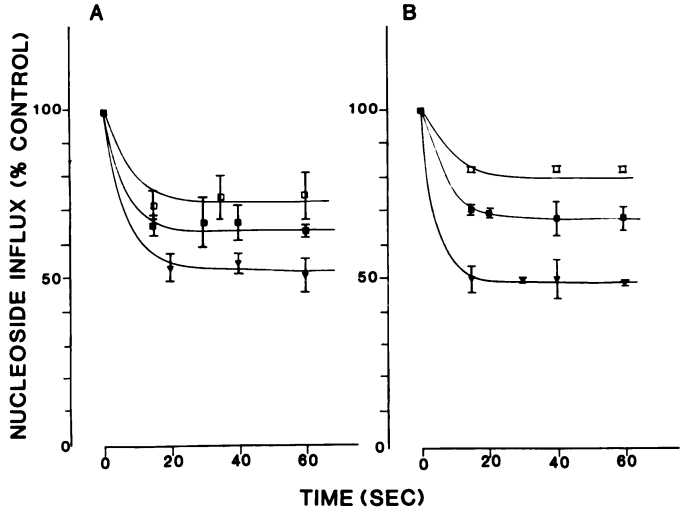

Fig. 3. Time course of nucleoside influx into human lymphoma P3HR-1 cells after treatment with AD 143. The cells were treated as described for Fig. 2, and the influx of [^{3}H]dThd (*A*) or [^{3}H]uridine (*B*) was measured after the time periods indicated. The AD 143 concentration used was 6.5 μM (□), 13 μM (■), or 26 μM (▼). The control value (100%) for each *point* was the amount of nucleoside influx of the nontreated cells at the indicated time. The absolute values of radioactivity were in the following range of counts (cpm): [^{3}H]dThd, 3049 to 3515 (15 s) and 5137 to 9181 (60 s) for control values and 2176 to 2662 (15 s) and 3401 to 6405 (60 s) for drug-treated cells; [^{3}H]uridine, 1279 to 1361 (15 s) and 2334 to 3384 (60 s) for control values and 654 to 1200 (15 s) and 1820 to 1870 (60 s) for drug-treated cells. *Bars*, SD.

AD 143 reduced the amount of intracellular [^{3}H]dThd and [^{3}H]uridine of human cancer cells as measured by TCA-soluble radioactivity (Table 1). Consequently, we observed that the amount of labeled nucleosides incorporated into TCA-precipitable material was also reduced after treatment of the cells with AD 143 (data not shown). Treatment of the cells with ADR, on the other hand, resulted in a much smaller change in the amount of TCA-soluble material in the cells (Table 1), although it was seen that the TCA-precipitable radioactivity decreased dramatically in the presence of ADR (data not shown). These results indicated that, although ADR exerts only a small effect on the uptake of [^{3}H]uridine and [^{3}H]dThd into ML-1 and P3HR-1 cells, it inhibited the nucleoside incorporation. These results are expected, since it is known that ADR can intercalate into DNA and inhibit its replication and transcription (18, 19).

The effect of AD 143 on the uptake of labeled nucleosides was further investigated by examining the influence of the drug on the fast influx of the nucleosides. It was again found that, after treatment of the cells with AD 143, the ability of the cells to take up [^{3}H]dThd and [^{3}H]uridine was decreased. This decrease was seen after the exposure of the cells to the labeled compounds for a time period as short as 15 to 20 s. These results also indicated that the cells started taking up the [^{3}H]-uridine and [^{3}H]dThd immediately after exposure to the nucleosides. As a comparison, the effect of ADR on the influx of the labeled nucleosides was also studied and was found to be minimal (Fig. 4).

To determine if the decreased uptake of the nucleosides after treatment of the cells with AD 143 resulted from a change in the membrane fluidity of these cells, EPR was used to study

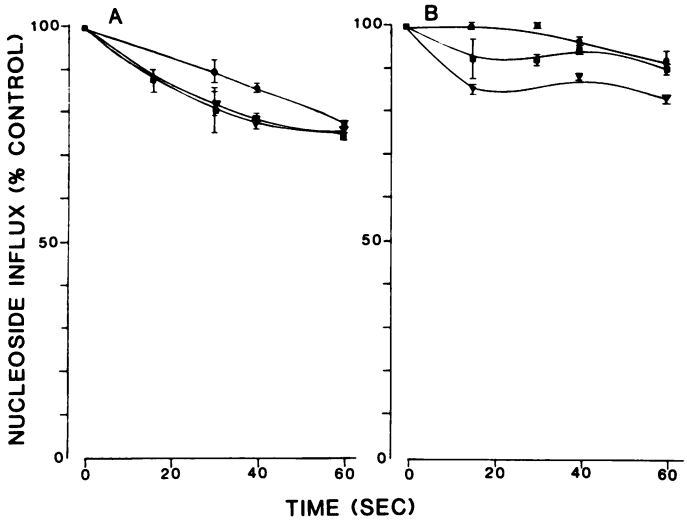

Fig. 4. Time course of nucleoside influx into human leukemia ML-1 cells after treatment with ADR. Experimental procedures were similar to those described in Fig. 2. The influx of [^{3}H]dThd (*A*) or [^{3}H]uridine (*B*) into the cells was measured after the cells were pretreated with ADR at a concentration of 5.2 μM (●), 13 μM (■), or 26 μM (▼). The control value (100%) for each *point* was the amount of nucleoside influx of the nontreated cells at the indicated time. The absolute values of radioactivity were in the following range of counts (cpm): [^{3}H]dThd, 1799 to 4125 (15 s) and 4754 to 5472 (60 s) for control values and 1511 to 3728 (15 s) and 3520 to 3976 (60 s) for drug-treated cells; [^{3}H]uridine, 1389 to 1543 (15 s) and 3522 to 4168 (60 s) for control values and 1223 to 1453 (15 s) and 3040 to 3888 (60 s) for drug-treated cells. *Bars*, SD.

Table 2 *Effect of ADR and AD 143 on the order parameter (S) of human leukemia ML-1 cells*

Each point is the average of three or more independent studies carried out in duplicate. Calculations of the *S* value were as described in "Materials and Methods."

Drug concentration (μM)	ADR		AD 143	
	Order parameter (*S*)	% of *S*	Order parameter (*S*)	% of *S*
0	0.5976 ± 0.0048[a]	100.0	0.6046 ± 0.0050	100.0
0.5	0.5976 ± 0.0040	100.0	0.6010 ± 0.0008	99.4
5	0.5957 ± 0.0046	99.7	0.5961 ± 0.0017	98.6
15	0.5953 ± 0.0017	99.6	0.5883 ± 0.0048	97.3
20	0.5922 ± 0.0050	99.1	0.5883 ± 0.0004	97.3

[a] Mean ± SD.

the membrane fluidity of the drug-treated and nontreated ML-1 cells. The results (Table 2) showed a 1.5 to 2.7% decrease in the order parameter, indicating an increase in membrane fluidity of the cells after exposure to AD 143 for a brief time (less than 45 min).

A variety of experiments have shown that changes in the order parameter of this magnitude can result in major alterations of cell functions (14, 21, 22). For example, comparable changes in the order parameter were shown to be coupled with the action of anesthetics (22). At general anesthetic concentrations, a 2.5% change in the order parameter would produce a 20 to 50% change in ion permeability (22). Thus, small changes of percentage in the order parameter can result in substantial modification of biological activity.

The above studies indicated that AD 143 inhibited the uptake of [^{3}H]dThd and [^{3}H]uridine into human cancer cells by affecting the membrane fluidity of these cells, whereas ADR at similar concentrations does not have the same effect. ADR has been shown to change membrane fluidity in mouse Sarcoma 180 cells (14); however, under our experimental conditions, AD 143 caused an even greater effect than ADR on the uptake of extracellular substrates and membrane fluidity of human cancerous cells. Therefore, the results of these studies may suggest that alteration in membrane functions be considered as one of the mechanisms of the cytotoxic action of the DNA-nonbinding anthracycline AD 143.

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