

# The Anticancer Drug Adriamycin Interacts with the Human Erythrocyte Membrane

Mario Suwalsky<sup>a</sup>, Pedro Hernández<sup>a</sup>, Fernando Villena<sup>b</sup>, Felipe Aguilar<sup>c</sup> and Carlos P. Sotomayor<sup>c</sup>

<sup>a</sup> Faculty of Chemical Sciences, University of Concepción, Casilla 160-C, Concepción, Chile

<sup>b</sup> Faculty of Biological Sciences, University of Concepción, Chile

<sup>c</sup> Institute of Chemistry, Catholic University of Valparaíso, Valparaíso, Chile

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Adriamycin, Anticancer Drug, Erythrocyte Membrane, Phospholipid Bilayer

Adriamycin is an aminoglycosidic anthracycline antibiotic widely used in the treatment of cancer. Increasing reports point to the involvement of cell membranes in its mechanism of action. The interaction of adriamycin with human erythrocytes was investigated in order to determine the membrane binding sites and the resultant structural perturbation. Electron microscopy revealed that red cells incubated with the therapeutical concentration of the drug in human plasma changed their discoid shape to both stomatocytes and echinocytes. According to the bilayer couple hypothesis, this means that adriamycin was incorporated into either the inner or outer leaflets of the erythrocyte membrane. To explain this unusual result, the drug was incubated with molecular models. One of them consisted of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) multilayers, representative of phospholipid classes located in the outer and inner leaflets of the erythrocyte membrane, respectively. X-ray diffraction showed that adriamycin interaction perturbed the polar head and acyl chain regions of both lipids. Fluorescence spectroscopy on another model, consisting of DMPC large unilamellar vesicles (LUV), confirmed the X-ray results in that adriamycin fluidized its hydrophobic moiety. It is concluded that adriamycin incorporates into both erythrocyte leaflets affecting its membrane structure.

## Introduction

Adriamycin (doxorubicin) is an aminoglycosidic antibiotic of the anthracycline class widely used in the treatment of leukemias and solid tumors (Blum and Carter, 1979). The mechanism of action has been generally based on its interaction with nuclear DNA through the intercalation of the adriamycin planar ring between the base-pairs (Goormaghtigh *et al.*, 1990). However, a growing body of evidence has challenged the dogma that it exerts the cytotoxic effect solely through interaction with DNA. In fact, several reports increasingly point to cellular membranes, and in particular the plasma membrane, as important structures

involved in the mechanism of action of adriamycin (Tritton and Yee, 1982; Arancia *et al.*, 1988; Garnier-Suillerot and Gattegno, 1988; Dupou-Cezanne *et al.*, 1989; Marutaka *et al.*, 1994; Speelmans *et al.*, 1996). That the interactions with the cell surface are essential for the antitumor activity of adriamycin has been shown by demonstration of cytotoxicity using a non-penetrating polymer immobilized drug (Tritton and Yee, 1982). It has also been reported that no matter how much adriamycin is present inside the cell, there must also be extracellular drug available for membrane interaction in order to initiate nuclear DNA damage and the cytotoxic cascade (Vichi and Tritton, 1992). Its main route of entry into a cancer cell is passive diffusion across the plasma membrane of the uncharged form of the drug, which is moderately lipophilic (de Wolf *et al.*, 1993) and is a weak base with a pKa of 8.3 (Speelmans *et al.*, 1996). Several properties and functions of plasma membranes seem to be modulated by adriamycin, such as glycoprotein synthesis, phospholipid structure, transport, expression of hormone receptors (Arancia *et al.*, 1988), fluidity (Marutaka *et al.*, 1994), mor-

**Abbreviations:** DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; LUV, large unilamellar vesicles; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; r, fluorescence anisotropy; GP, general polarization; SEM, scanning electron microscopy.

Reprint requests to Prof. Suwalsky.

Fax: 56–41–245974

E-mail: msuwalsk@udec.cl.

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phology and ultrastructure (Arancia *et al.*, 1995). Adriamycin strongly interacts with lipids of biological membranes, especially cardiolipin, which is an anionic phospholipid present in cardiac mitochondria (Goormaghtigh *et al.*, 1990; Garnier-Suillerot and Gattegno, 1988; Dupou-Cezanne *et al.*, 1989; Speelmans *et al.*, 1996). Marked differences have been found in the interaction of adriamycin with acidic and neutral lipids. Thus, binding of adriamycin to cardiolipin should result in a complex including two stacked adriamycin molecules electrostatically bound on the two anionic phosphate groups of cardiolipin (Goormaghtigh *et al.*, 1990), decreasing the acyl chain order of the lipid (de Wolf *et al.*, 1993). In contrast, it should not affect the order and mobility in membranes consisting of zwitterionic phospholipids, even at drug concentrations exceeding 10 mM (de Wolf *et al.*, 1992). However, it has been reported that adriamycin interacts with phosphatidylcholine small unilamellar vesicles in which hydrophobic interactions predominate between the dihydroanthraquinone moiety of the drug with the hydrocarbon region of the lipid, and between the aminoglycosyl group with lipid phosphates (Dupou-Cezanne *et al.*, 1989). Therefore, the objective of this work was to examine the binding site of adriamycin in the membranes and the resulting structural perturbations induced by the drug. We have focused on the erythrocyte membrane and molecular models built up with phospholipid classes representative of those located in its outer and inner leaflets. These systems have been used in our laboratories to determine membrane interaction and perturbing effects of several agents (Suwalsky *et al.*, 1988; 1991; 1994; 1996; Suwalsky and Frías, 1993; Suwalsky and Villena, 1995). Scanning electron microscopy was used to detect human erythrocyte shape changes induced by adriamycin. The models consisted of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) multilayers, representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively (Devauux and Zachowsky, 1994), and of DMPC large unilamellar vesicles (LUV). Adriamycin perturbation of the DMPC and DMPE multilayer structure was determined by X-ray diffraction. Because of the amphipathic character of adriamycin and of both phospholipids, their interactions were as-

sayed in hydrophobic and aqueous media in a wide concentration range. The effect of adriamycin on the physical properties of the bilayer of DMPC large unilamellar vesicles was examined by evaluation of DPH steady state fluorescence anisotropy.

## Materials and Methods

### *Scanning electron microscope (SEM) studies on human erythrocytes*

Blood samples taken from clinically healthy male adult donors by puncture of the ear lobe disinfected with 70% ethanol were incubated with adriamycin. Two drops of blood were collected in a plastic tube containing 1 ml of saline (0.9% NaCl) at 5 °C. This solution was used to prepare the following samples: a) control, by mixing 0.1 ml with 0.9 ml of saline, b) 40 mM adriamycin, by mixing 0.1 ml with 0.9 ml of adriamycin in adequate concentration. This concentration was calculated to be about that present in plasma when it is therapeutically injected to humans (Goodman and Gilman, 1996). These samples were incubated at 37 °C for one h. They were then fixed with glutaraldehyde, adding one drop of each sample to a tube containing 1 ml of 2.5% glutaraldehyde in saline, reaching a final fixation concentration of about 2.4%. After resting overnight at 5 °C the fixed samples were directly placed on Al stubs, air dried at 37 °C for half to one h and gold coated for 3 min at  $10^{-1}$  Torr in a sputter device (Edwards S150). The observations and photographic records were performed in an Etec Autoscan SEM.

### *X-ray diffraction analysis of phospholipid multilayers*

Synthetic DMPC (lot 80H-8371 A grade MW 677.9), DMPE (lot 13H-83681 A grade MW 635.9) and adriamycin.HCl (lot 63H-0349 from Sigma and batch 6009B47F as a gift from Pharmacia & Upjohn, MW 580.0) were used without further purification. About 5 mg of each phospholipid were mixed with the corresponding weight of adriamycin in order to attain DMPC:adriamycin and DMPE:adriamycin powder mixtures in the molar ratios of 10:1, 5:1 and 1:1. Each mixture was dissolved in chloroform:methanol 3:1 v/v and left to dry. The recrystallized samples were placed in spe-

cial glass capillaries 0.7 mm dia. They were diffracted in Debye-Scherrer cameras of 114.6 mm dia and flat-plate cameras with 0.25 mm dia glass collimators provided with rotating devices. The same procedure was followed with samples of each phospholipid and adriamycin. The aqueous specimens were prepared in 1 mm dia glass capillaries, mixing each phospholipid and adriamycin in the proportions described above. Each capillary was then filled with about 100  $\mu$ l of distilled water. These specimens were X-ray diffracted 2 days after preparation in flat-plate cameras. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuK $\alpha$  radiation from a Philips PW1140 X-ray generator was used. The relative reflection intensities were obtained from films by peak-integration in the microdensitometers Joyce-Loebl MKIIICS and BIO-RAD using the Molecular Analyst/PC image software. No correction factors were applied. The experiments in water were performed at  $17 \pm 2^\circ\text{C}$ , which is below the main transition temperature of both DMPC and DMPE.

#### *Fluorescence measurements on large unilamellar vesicles (LUV)*

DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspension (final lipid concentration 0.5 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp.) under nitrogen pressure at  $10^\circ\text{C}$  over the lipid transition temperature. DPH and Laurdan were incorporated into LUV by addition of small aliquots of concentrated solutions of the probe in tetrahydrofuran and ethanol respectively to LUV suspensions and gently shaken for about 30 min. Fluorescence spectra and anisotropy measurements were respectively performed in a Spex Fluorolog and in a phase shift and modulation Greg-200 steady-state and time-resolved spectrofluorometer (I. S. S.), both interfaced to computers. Software from I. S. S. was used for data collection and analysis. Measurements of LUV suspensions were made at  $18^\circ\text{C}$  in 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circulator (Cole Parmer) and measured prior to and after each measurement using a digital thermometer (Omega). Anisotropy

measurements were made in the "L" configuration using prism polarizers (Glan Thompson) in both exciting and emitting beams. The emission was measured across a high pass filter (Schott WG-420) with negligible fluorescence. Laurdan fluorescence spectral shifts were quantified through the General Polarization (GP) concept which was evaluated by  $GP = (I_b - I_r) / (I_b + I_r)$ , where  $I_b$  and  $I_r$  are the intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of Laurdan in the gel and liquid crystalline phases, respectively (Parasassi and Gratton, 1995). Adriamycin was incorporated in LUV suspensions by addition of small aliquots of a concentrated ethanol solution and incubated at  $40^\circ\text{C}$  for ca. 15 min. Samples with probes, but without adriamycin showed no variation in the measured parameters during periods longer than those employed in the experiments. Blank subtraction was performed in all measurements using unlabelled samples without probes.

## **Results**

#### *Scanning electron microscopy (SEM) studies on human erythrocytes*

SEM of red cells incubated with 40 mM adriamycin, which is about the plasma concentration when therapeutically injected to humans (Goodman and Gilman, 1996), revealed abnormalities in their shapes. In contrast to the normal discoid erythrocyte profile (Fig. 1a), about half of the adriamycin treated cells showed stomatocytic shapes while the other half were crenocytic, i.e., evagination and invagination of their faces, respectively (Fig. 1b). This effect was observed in all the assayed samples and the extent of the shape alteration was always the same. Similar results were obtained with 0.8 mM adriamycin.

#### *X-ray studies on phospholipid multilayers*

On account of the amphipathic nature of adriamycin, DMPC, and DMPE, the molecular interactions of the drug with bilayers of both phospholipids were assayed in hydrophobic and aqueous media. Fig. 2a compares the diffraction patterns of DMPC, adriamycin and of their 10:1, 5:1 and 1:1

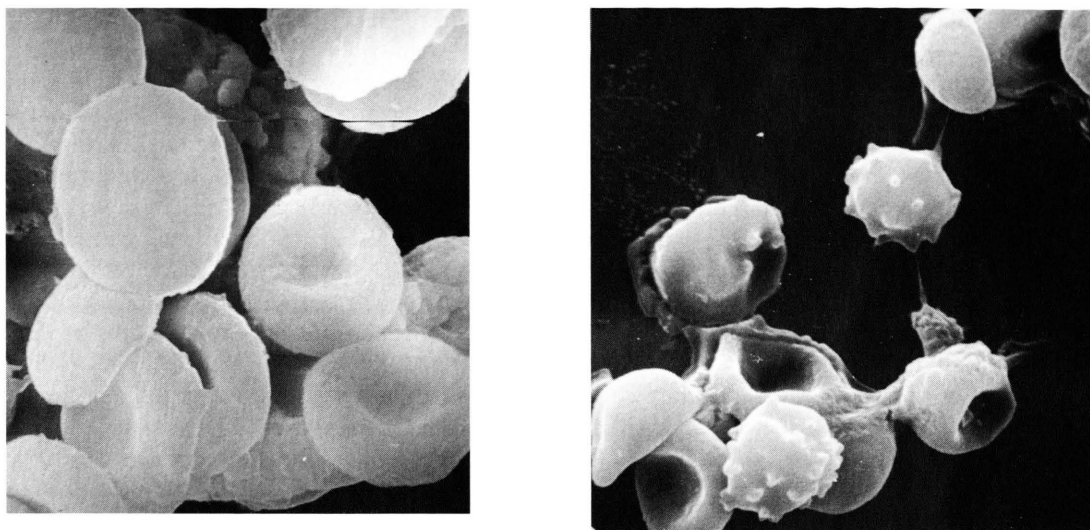


Fig. 1. Scanning electron microscope (SEM) images of human erythrocytes; (a) Control (3840x); (b) incubated with 40 mM adriamycin (2800x).

molar mixtures after interaction and recrystallization from chloroform:methanol 3:1 v/v solutions. Analysis of these results indicated that the X-ray pattern of DMPC was affected by adriamycin. At a lipid:drug molar ratio of 10:1 adriamycin decreased DMPC reflection intensities; several of them disappeared, in particular those of 4.30 Å, 4.13 Å and 3.88 Å, which were replaced by one of 4.2 Å. The appearance of this reflection was indicative of the fluid state reached by the lipid bilayer; in fact, it arises from the fully extended acyl chains organized with rotational disorder in a hexagonal lattice. This reflection is normally attained when DMPC is immersed in water below its main transition temperature (23 °C) as can be observed in Fig. 2b. What is surprising is that this reflection was also observed in dry specimens. On the other hand, the 54.5 Å bilayer width of DMPC gradually expanded with increasing proportions of adriamycin up to 56.6 Å in their 1:1 molar ratio. However, no reflections from the drug were observed in any of its mixtures. These results clearly indicated that adriamycin penetrated into the phospholipid bilayer structure, perturbing its molecular arrangement at the polar head and acyl chain regions.

Fig. 2b shows the results obtained after DMPC, adriamycin and their molar mixtures in the above mentioned ratios were immersed in an excess of

distilled water. As may be observed, water changed the X-ray pattern of DMPC; its bilayer width expanded from 54.5 Å when dry to 64.0 Å and its reflections were reduced to only the first three orders of the bilayer width and the relatively intense reflection of 4.2 Å. As before, increasing concentrations of adriamycin produced a gradual expansion of the bilayer width and a decrease of the phospholipid reflection intensities. These effects indicated that adriamycin in an aqueous medium was also able to penetrate and perturb the DMPC bilayer structure.

The results obtained after the interaction of DMPE with adriamycin in the hydrophobic medium are depicted in Fig. 2c. As reported elsewhere (Suwalsky *et al.*, 1988), DMPE exhibits two polymorphic forms; one ( $L_c1$ ) has extended hydrocarbon chains parallel to the bilayer normal, its bilayer width being about 52 Å. The other form ( $L_c2$ ) has hydrocarbon chains tilted about 30 °C and a bilayer width of nearly 44 Å. Adriamycin produced the following effects in DMPE: a) an  $L_c1$  to  $L_c2$  phase transition, and b) a marked decrease of the lipid reflection intensities. Fig. 2d shows the results of the adriamycin-DMPE interaction in water. The drug did not induce a phase transition in DMPE, which remained throughout in the  $L_c1$  form; however, it interacted with DMPE in such a way that at the 1:1 molar ratio only a scanty



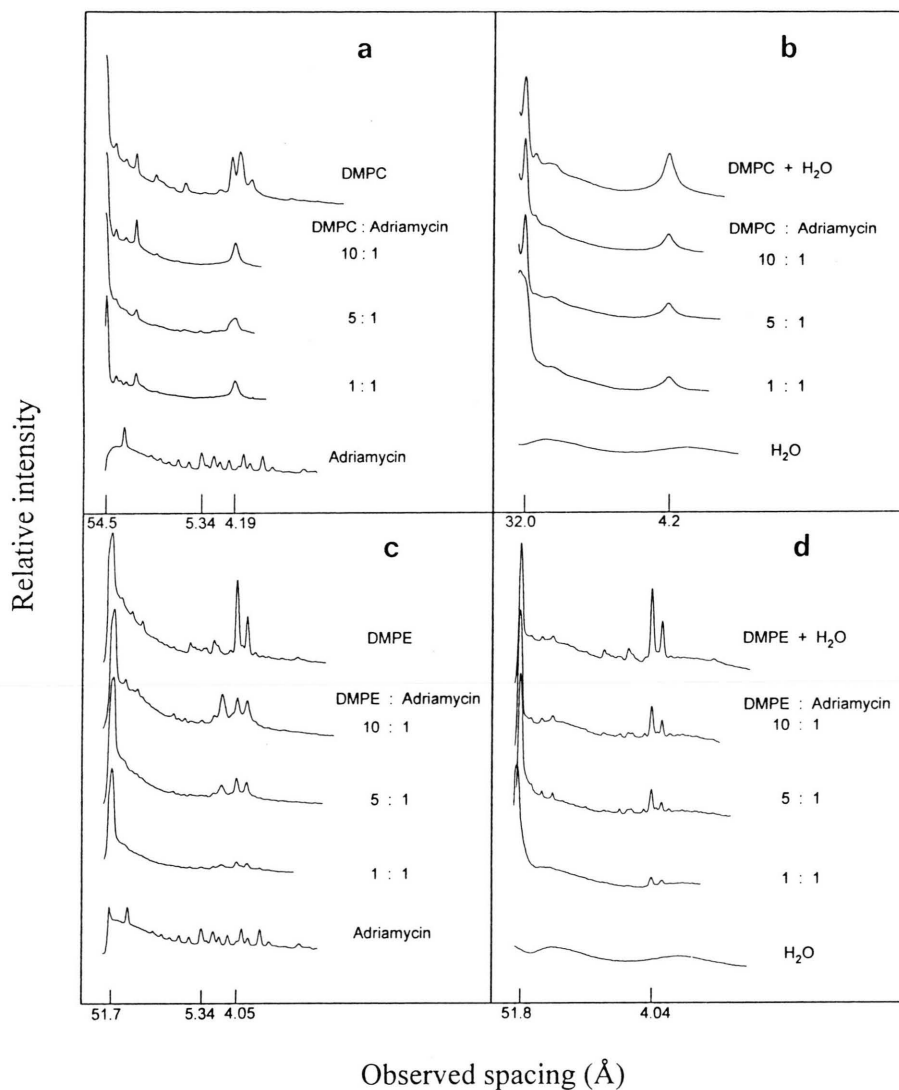


Fig. 2. Microdensitograms from X-ray diagrams of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), and of their 10:1, 5:1 and 1:1 molar mixtures with adriamycin. Flat-plate cameras; specimen-to-film distance 8 cm; (a) and (c): recrystallized from  $\text{CHCl}_3:\text{CH}_3\text{OH}$  3:1 (v/v); (b) and (d): immersed in water. The DMPE: adriamycin mixtures show the X-ray pattern of DMPE  $\text{Lc}_1$  form in (d) while that of its  $\text{Lc}_2$  form is present in (c).

number of considerably weakened reflections remained. These results implied a strong interaction of adriamycin with DMPE, equivalent to that observed with DMPC. This was rather surprising in view of the fact that DMPE bilayers pack closer and tighter than those of DMPC, therefore being more difficult to penetrate and perturb (Suwalsky, 1988).

#### *Fluorescence measurements on DMPC large unilamellar vesicles (LUV)*

The influence of adriamycin upon the structure of the phospholipid acyl chain hydrophobic core of DMPC LUV was determined by fluorescence spectroscopy. Increasing concentrations of the drug significantly decreased DPH fluorescence anisotropy ( $r$ ) (Table I). This effect would result

from the adriamycin-induced disorder of the acyl chain packing of DMPC, in agreement with the X-ray diffraction observations. The effects of the drug upon the hydrophilic/hydrophobic interface of the lipid through Laurdan general polarization could not be determined due to the absorption of adriamycin in the spectral region of the measurement.

Table I. Effect of adriamycin on the anisotropy ( $r$ ) of 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in large unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles (probe:lipid ratio 1:600).

Adriamycin conc. ( $\mu\text{M}$ )	$r$ DPH
0	0.319
8	0.308
80	0.268

## Discussion

Several reports increasingly point to the involvement of cell membranes in the anticancer mechanism of action of adriamycin, a drug which apparently modulates membrane properties and functions. Therefore, it was considered of interest to determine the membrane adriamycin-binding sites and the resulting structural perturbations. For these purposes we focused our research on the erythrocyte membrane, which has been extensively studied. This cell type lacks nuclear binding sites for anthracyclines and is, therefore, a useful model to investigate the interaction of adriamycin with cell membranes. Electron microscopy of human erythrocytes incubated with adriamycin revealed that they changed their normal discoid shape to both echinocytes and stomatocytes. According to the bilayer couple hypothesis (Sheetz and Singer, 1974), the shape changes induced in red cells by amphiphiles are due to differential expansion of the two monolayers. Thus, echinocytes are observed when the added molecules penetrate into the outer monolayer, whereas stomatocytes are induced when they enter into the inner monolayer. In order to explain this rather unusual result, i.e., the simultaneous presence of echinocytes and stomatocytes, molecular models of the erythrocyte membrane were exposed to adriamycin.

One model consisted of DMPC and DMPE multilayers, representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively (Devauux and Zachowsky, 1994). X-ray studies showed that adriamycin strongly interacted with both. This result, which implies that adriamycin is able to insert into the outer as well as in the inner lipid monolayers of the erythrocyte membrane, certainly might explain the erythrocytes shape changes. The membrane distribution of the drug is confirmed by kinetic studies, which have demonstrated the existence of two similar sized pools of adriamycin in the two leaflets of the erythrocyte membrane (Regev and Eytan, 1997). Although the interaction of the drug with membrane proteins can not be ruled out, our results indicate that adriamycin interacts with membranes by incorporation into lipid bilayers. Analysis of the X-ray diffraction experiments on DMPC and DMPE bilayers and those performed by fluorescence spectroscopy on DMPC LUV indicate that their polar head and the hydrophobic acyl chain regions were perturbed by adriamycin. This means that the drug interacts with the bilayers in such a way that part of the molecule incorporates into the hydrocarbon chains and another part is in contact with the polar groups. This conclusion agrees with experimental data reported in the literature (Garnier-Suillerot and Gattegno, 1988; Dupou-Cezanne *et al.*, 1989; Giuliani *et al.*, 1988). Thus, the dihydroanthraquinone residue of adriamycin could be bound through hydrophobic interactions with the lipid acyl chains, whereas the ammonium group of the drug could be electrostatically bound to the negatively charged phosphates.

To conclude, our observations indicate that adriamycin interacts with the cell membrane by its incorporation into both lipid monolayers. This action should modify cell membrane structure and physiological properties, such as fluidity, permeability, receptor and channel functions, and so on. Thus, the cytotoxic action of adriamycin might be mediated by its interaction with cell membranes. For instance, it has been reported that the ability of adriamycin to modulate the sensitivity of target cells for destruction by immune effectors is due to membrane alterations resulting from direct actions of the drug (Marutaka *et al.*, 1994).

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