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EVIDENCE OF A COMPLEX BETWEEN ADRIAMYCIN DERIVATIVES AND CARDIOLIPIN: POSSIBLE ROLE IN CARDIOTOXICITY

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Abstract—Most of the mitochondrial damage induced by antimitotic drugs of the adriamycin family could be due to the high affinity of these drugs for the membrane. The prime interaction between the anthracycline drug and this membrane would explain specific alterations observed on mitochondria. Cardiolipin has been proposed as a privileged target. We have tested this hypothesis here. Model membranes (lipid monolayers, liposomes) were used to demonstrate the interaction between these anthracycline drugs and different phospholipids. A new surface potential technique showed the specificity of adriamycin derivatives for cardiolipin whereas no complexation was observed with neutral phospholipids (dipalmitoyl lecithin and egg lecithin). Association constants were evaluated and a good correlation was obtained between the mitochondrial toxicity of each drug and its affinity for cardiolipin. Fluorescence measurements were carried out in order to locate precisely the position of the drug in the lipid bilayer. Perturbations of the lipid organization after complex formation were analysed using phospholipase A_2 as an enzymic probe.

Adriamycin is one of the most promising new antineoplastic drugs [1, 2]. Most of its side-effects are reversible and are commonly seen with other antineoplastic drugs. Cardiac toxicity, however, is very specific and places a limit on the total dose of adriamycin that may be given, since the effect is cumulative [3]. The development of cardiac failure is characterized by a good correlation with the impairment of mitochondrial functions (O_2 consumption and proportional ATP synthesis) [4] without perturbation of the sliding of actin and myosin filaments across each other [5]. Histologically, mitochondria changes are characterized by formation of myelin-like figures [6, 7] generally related to the formation of a drug–lipid complex [8–10]. Other studies confirm that adriamycin can perturb the lipidic organization on model membranes [11–13]. It has been postulated that the formation of a strong complex between adriamycin and cardiolipin, a phospholipid of the inner mitochondria membrane, demonstrated on model membrane [14, 15], could be the prime cause of cell toxicity. Indeed, disorganization of the phospholipid fraction of the mitochondrial membrane could explain the decreasing activity of membrane enzymes [16]. It is striking that cardiolipin-dependent enzymes such as coenzyme Q [17] and cytochrome oxidase [5] seem to be particularly affected by adriamycin. On the other hand, peroxidation of lipids catalysed by anthraquinonic drugs has been demonstrated [18, 19] and could be another way of explaining the mitochondrial toxicity [20]. In this hypothesis, cardiolipin again could play a prominent role since it has been pointed out that peroxidized cardiolipin reacts covalently with lipids and proteins to form polymers [21].

The purpose of this paper is to demonstrate a correlation between the cardiac toxicity of the drug and its affinity for cardiolipin. A possible approach is the study of the behaviour of adriamycin derivatives having different mitochondrial toxic effects in the presence of model membranes (lipid monolayers and liposomes) containing cardiolipin. In this work, lipids were spread at the air–water interface and adriamycin derivatives were injected into the aqueous subphase. We developed a new method using surface potential and surface pressure measurements to determine the association constant. In order to evaluate the penetration of the drug into the lipophilic region of the bilayer, its fluorescence titration by liposomes was carried out. The fluorescence spectrum of each drug was correlated to the dielectric constant of the medium surrounding the chromophore. Finally, phospholipase A_2 (EC 3.1.1.4) was used as an enzymic probe to point out a change in the model membrane organization due to the lipid–adriamycin complex formation.

MATERIALS AND METHODS

DL-α-Dipalmitoyl phosphatidylcholine, egg phosphatidylcholine and cardiolipin were purchased from Sigma Chemical Co. Adriamycin, rubidazone and steffimycin (Figs. 1a, d, and f) were generously supplied by Dr. J. Hildebrand (Institut Jules Bordet) and by Dr. C. Deslover (Farmitalia). *N*-Acetyl-adriamycin (Fig. 1b) was a gift of Prof. A. Trouet and Dr. R. Baurin (Laboratoire de Chimie Physiologique, Université de Louvain). Cinerubin, rhodomycin and nogalamycin (Figs. 1c, e, and g) were obtained from the National Cancer Institute. Phos-

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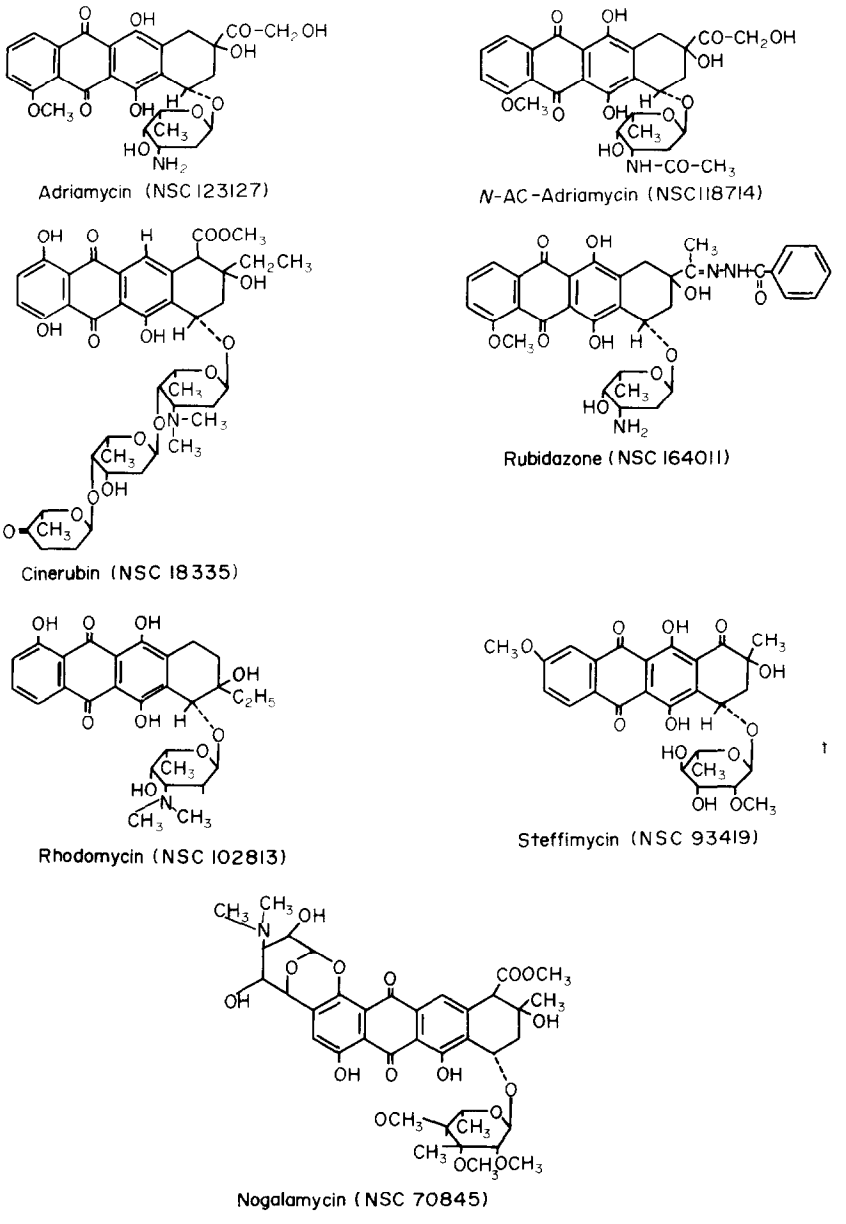

Fig. 1. Structure of the adriamycin analogs.

pholipase A_2 from porcine pancreas (EC 3.1.1.4), acitivity 800 U/mg, was supplied by Boehringer Mannheim. All chemicals were of analytical grade and water was tridistilled. Buffered solutions (Tris–HCl 10^{-4}M/l, pH 7.4, NaCl 10^{-3}M/L) were used to prepare the subphase. Phospholipids were spread at the air–water interface from a chloroform solution using an Agla Microlitre syringe. All experiments were carried out at 25°.

To prepare small unilamellar liposomes, lipids were dissolved in $CHCl_3$ in a spherical flask. The solution was evaporated to dryness and further dried under vacuum. Multilamellar liposomes were obtained by mechanical stirring (vortex mixer) of a lipid film in buffer. The temperature was maintained above the corresponding lipid phase transition temperature T_c [22]. Small unilamellar liposomes were obtained by sonication of the multilamellar liposome dipsersion (Branson Sonifier B12). The temperature was kept above T_c during sonication.

The vibration electrode technique was employed to measure the surface potential [23, 24]. Surface pressure measurements were made on a Cahn R.G. electrobalance using the Wilhelmy method [25]. A platimum plate was used. Fluorescence spectra were recorded with a differential spectrofluorimeter FOCCI ML 1.

RESULTS

Surface potential results. The knowledge of σ, the surface charge density after complexation, and of σ_0, the surface charge density before complexation, allows evaluation of the complexation constant.

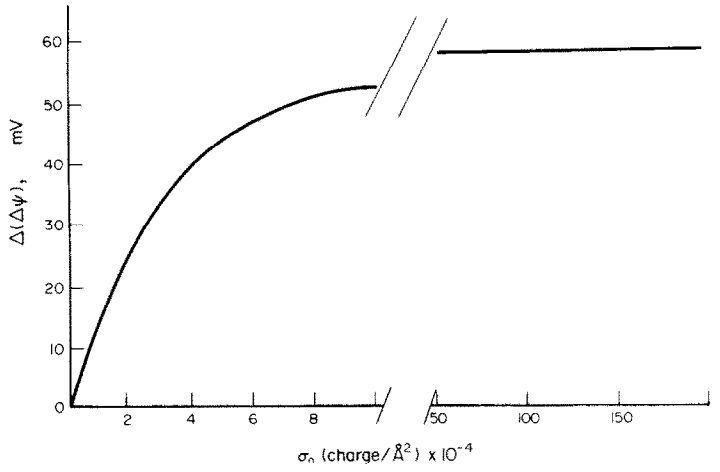

Fig. 2. Increase of the surface potential $\Delta(\Delta\psi)$ as a function of the monolayer charge density σ_0. The salt concentration was increased from 10^{-3} to 10^{-2} M by injection of $NaNO_3$ in the subphase. Buffered solution: Tris–HCl pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M, T =25°C. The surface pressure of the spread monolayer was 25 mN/m.

between a lipidic anionic site (P^-) and a positively ionized drug (D^+). The reaction can be written:

$$D^+ + P^- \rightleftharpoons DP \tag{1}$$

with an association constant

$$K = \frac{[D\,P]}{[D^+]_s\,[P^-]} = \frac{\sigma_0 - \sigma}{\sigma}\,\frac{1}{[D^+]_s}, \tag{2}$$

where σ and σ_0 are, respectively, the surface charge density after and before complexation and $[D]_s$ is the molar concentration of D^+ at the interface. $[D^+]_s$ is related to the bulk concentration through a Boltzmann distribution:

$$[D^+]_s = [D^+]_\infty \exp(-e\psi/kT), \tag{3}$$

where e is the electronic charge, k the Boltzmann constant and ψ the remaining negative surface potential after complexation. From a general point of view, the electrostatic potential ψ(mV) associated to the lipid monolayer is described by the Gouy–Chapman theory. At 25°,

$$\psi = 50.4\, sh^{-1}\,\frac{134\sigma}{\sqrt{C}}, \tag{4}$$

where σ is the surface charge density in charge/Å^2 and C is the molar salt concentration in the solution.

Classically, the lipid monolayer is spread at the air–water interface. After injection of the drug in the subphase, the evolution of the surface potential is followed until equilibrium is reached. The increase in surface potential ΔV (mV) can be described by:

$$\Delta V = \Delta\psi + \frac{12\pi\Delta\mu_z}{A}, \tag{5}$$

where $\Delta\psi$ is the change in the electrostratic potential expressed in mV, $\Delta\mu_z$ is the change of the vertical component of the total dipole moment expressed in mDebye and A is the area occupied per molecule

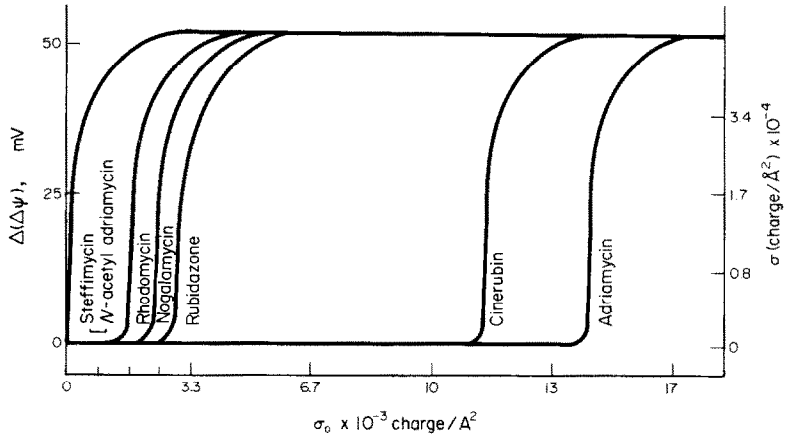

Fig. 3. Increase of the surface potential $\Delta(\Delta\psi)$ of the complexed monolayer after injection of $NaNO_3$ in the subphase as a function of the surface charge density before complexation. The $NaNO_3$ concentration was modified from 10^{-3} to 10^{-2} M. Right ordinate gives the surface charge density σ equivalent to the increase of the surface potential (equation 6). (Drug concentration: 2.10^{-5} M.) Buffered solution: Tris–HCl pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M, T = 25°C. The surface pressure was 25 mN/m.

Table 1. Association constants of the cardiolipin–adriamycin derivatives complex*

	$[D+]_\infty$ (moles/litre)	$[D+]_s$ (moles/litre)	σ_0 (charge/Å^2)	σ (charge/Å^2)	K (eqn 1) (litres/mole)
Adriamycin	2×10^{-5}	5.2×10^{-5}	1.5×10^{-2}	1.7×10^{-4}	1.8×10^{6}
N-Acetyladriamycin	2×10^{-5}	2.0×10^{-5}	1.7×10^{-4}	1.7×10^{-4}	No charge complexation
Cinerubin	2×10^{-5}	5.2×10^{-5}	1.1×10^{-2}	1.7×10^{-4}	1.2×10^{6}
Rubidazone	2×10^{-5}	5.2×10^{-5}	2.9×10^{-3}	1.7×10^{-4}	3.1×10^{5}
Nogalamycin	2×10^{-5}	5.2×10^{-5}	2.5×10^{-3}	1.7×10^{-4}	2.7×10^{5}
Rhodomycin	2×10^{-5}	5.2×10^{-5}	1.7×10^{-3}	1.7×10^{-4}	1.7×10^{5}
Steffimycin	2×10^{-5}	2.0×10^{-5}	1.7×10^{-4}	1.7×10^{-4}	No charge complexation

* Buffer Tris–HCl, pH = 7.4, 10^{-4}M; $NaNO_3$, 10^{-3} M. $T = 25°$. The surface pressure of the monolayer was 25 nM/m.

in Å^2/molecule. In order to avoid the problem of the evaluation of the change in the dipolar contribution after formation of the drug–lipid complex, we developed a new technique based on the Gouy–Chapman theory allowing the determination of σ. It consists of varying the salt concentration by injecting a saturated solution of $NaNO_3$ into the subphase. Since evidence has been presented to suggest that no modification of the dipolar orientation of the monolayer will occur [26], the increase of the surface potential $\Delta(\Delta\psi)$ after the salt injection can be described from the Gouy–Chapman theory. At 25°,

$$\Delta(\Delta\psi) = 50.4 \left(sh^{-1} \frac{134\sigma}{\sqrt{C_2}} - sh^{-1} \frac{134\sigma}{\sqrt{C_1}} \right), \quad (6)$$

where C_1 and C_2 are, respectively, the concentration in monovalent ions in the subphase (M/l) before and after $NaNO_3$ injection and σ is the surface charge density (charge/Å^2). C_1 was chosen equal to 10^{-3}M/l and C_2 equal to 10^{-2}M/l. Figure 2 has been drawn from equation 6. It appears that σ can be precisely extrapolated from $\Delta(\Delta\psi)$ in only a small range of $\Delta(\Delta\psi)$ values around 25 mV corresponding to a σ value of 1.7×10^{-4} charge/Å^2.

The following procedure was used to calculate the association constant K: monolayers of defined charge density σ_0 were obtained by spreading mixtures of a neutral lipid (DPPC) and of the negatively charged cardiolipin in different proportions to define precisely σ_0. Adsorption experiments on a pure DPPC monolayer have been carried out for each drug at the same concentration as in Fig. 3 using surface potential and surface pressure measurements. Both techniques demonstrated that no adsorption occurs ($\Delta\psi = 0 \pm 5$ mV; $\Delta\pi = 0 \pm 0.5$ mN/m). For this reason, DPPC was used as an inert lipid. For each σ_0 value, i.e. for each monolayer composition, a known amount of the drug was injected into the subphase. When equilibrium was reached, the salt concentration of the subphase was modified by injection of $NaNO_3$. The experimental data obtained by this procedure are shown in Fig. 3. For each drug, a σ_0 value was determined at $\Delta(\Delta\psi) = 25$ mV corresponding to $\sigma = 1.7\ 10^{-4}$ charge/Å^2. From σ and σ_0, ψ and $[D^+]_s$ the association constant K can be obtained (equations 2 and 3). The ψ value used in the evaluation of $[D^+]_s$ is the difference between the surface potential before complexation (equation 4 with $\sigma = \sigma_0$) and after complexation (equation 4 with $\sigma = \sigma$). K values are reported in Table 1.

Comparison between ΔV (equation 5) and $\Delta\psi$ allows an evaluation of the dipolar term change. $\Delta\psi$ is calculated from equation 7:

$$\Delta\psi = 50.4 \left(sh^{-1} \frac{134\sigma_0}{\sqrt{C_1}} - sh^{-1} \frac{134\sigma}{\sqrt{C_1}} \right). \quad (7)$$

ΔV and $\Delta\psi$ are reported in Table 2. Two kinds of behaviour are pointed out. For three drugs only, ΔV and $\Delta\psi$ exhibit compatible values. In these cases, we can assume that $\Delta\mu_z$ is roughly equal to 0. Greater deviations are observed for the other drugs. Low or near zero values of $\Delta\mu_z$ are generally associated with a low penetration of the drug into the lipophilic region of the model membrane. In the next part of

Table 2. Increase of surface potential of the cardiolipin–DPPC monolayer after injection of the adriamycin derivatives into the aqueous subphase. Comparison between experimental values and theoretical predictions calculated from eqn. 7*

	Experimental ΔV (mV)	Theoretical $\Delta\psi$ (mV)
Adriamycin	210 ± 20	225
N-Acetyladriamycin	0 ± 5	0
Cinerubin	180 ± 30	209
Rubidazone	105 ± 20	185
Nogalamycin	265 ± 30	135
Rhodomycin	150 ± 20	115
Steffimycin	60 ± 15	0

* Subphase was a buffered solution (Tris–HCl, pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M). The surface pressure of the monolayer was 25 mN/M. $T = 25°$. Each value represents the mean ± S.D. of seven experiments.

this work we will try to confirm this conclusion by fluorescence titration of each drug.

Fluorescence measurements. Since the maximum emission wavelength of the fluorescence λ_{max} is strongly dependent on the dielectric constant of the medium surrounding the dye, the fluorescence spectra can be used as a probe of the dielectric constant of the medium in which the dye is embedded [27]. In fact, the dielectric constant profile in a phospholipidic bilayer has been evaluated by Shinitzky [28]. Figure 4 shows the position of the maximum emission wavelength of each drug as a function of the dielectric constant. Media of different dielectric constants were

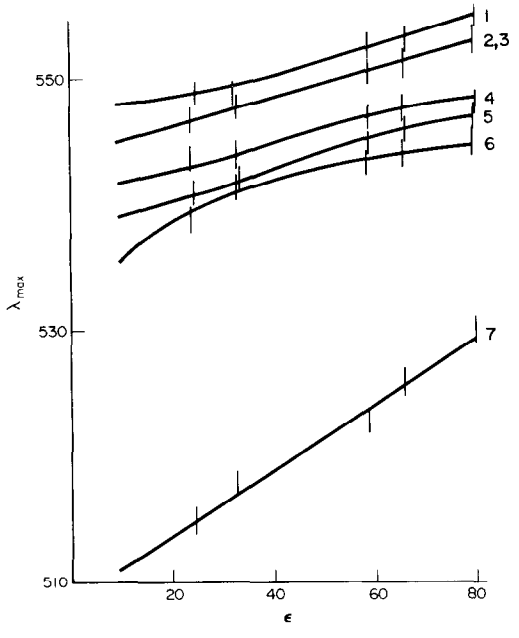

Fig. 4. λ_{max} (nm) of: (1) rubidazone, (2) *N*-acetyladriamycin, (3) adriamycin, (4) rhodomycin, (5) cinerubin, (6) nogalamycin, (7) steffimycin as a function of the dielectric constant of the solvent. The concentration of the drugs was 5.10^{-6} M. $T = 25°C$.

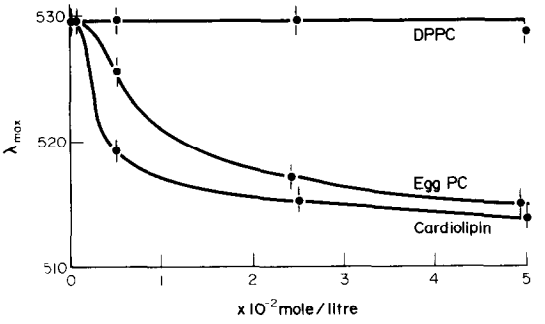

Fig. 5. Titration of steffimycin 5.10^{-6} M by unilamellar liposomes of DPPC, egg PC and cardiolipin in a buffered solution Tris–HCl pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M. T – 25°C. λ_{max} is plotted against the lipid concentration.

used: aqueous buffer ($\varepsilon = 80$), buffer–methanol 2/1 v/v ($\varepsilon = 66$), buffer–methanol 1/1 v/v ($\varepsilon = 58$), methanol ($\varepsilon = 33$), ethanol ($\varepsilon = 24$) [29]. For example, in Fig. 5, λ_{max} of steffimycin is plotted as a function of the liposome concentration. Cardiolipin, egg phosphatidylcholine and dipalmitoylphosphatidylcholine liposomes have been tested. At the end of the titration curve, it can be assumed that quantitatively no more free drug is present in the solution. At this point, the ε value is deduced from λ_{max} (Fig. 5). For each drug, λ_{max} and ε were reported in Table 3. A good correlation clearly appears between these results and that of surface potential.

Comparing Tables 2 and 3, one can see that drugs which did not change quantitatively the dipolar orientation of the cardiolipin model membrane were embedded in a medium of dielectric constant greater than 50 (adriamycin, *N*-acetyladriamycin, cinerubin), whereas the neighbourhood of all other drugs has a dielectric constant lower than 45 (rubidazone, nogalamycin, steffimycin, rhodomycin). In the latter case, the affinity and the penetration of the drug is no more dependent on the charge–charge interactions, but the physical state of the bilayer seems to be the driving factor. This kind of behaviour is well illustrated by the behaviour of steffimycin which penetrates into the fluid lipids (cardiolipin and egg phosphatidylcholine) but does not penetrate into DPPC which is in a gel state at the working temperature (Fig. 5).

It is of special interest to observe that the quantum yield of adriamycin in the presence of increasing amounts of cardiolipin liposomes presents a minimum at a defined liposome concentration (Fig. 6). At this concentration, one cardiolipin is associated to two adriamycin molecules. This must be correlated to the observation that in such conditions adriamycin forms a cardpack stacking complex as shown by absorbance measurements [15]. This organization disappears by surface dilution as a great excess of liposomes is added. In agreement with our previous studies [15], adriamycin is the only drug to display such a behaviour.

Enzymic hydrolysis. In many studies, enzymic hydrolysis of phospholipid membranes was used to study the phospholipid composition [30] and structure [31, 32]. It is an elegant method for describing the molecular organization of the lipid matrix. In

Table 3. Dielectric constants of the lipidic medium surrounding each drug in unilamellar liposomes*

	λ excitation (nm)	λ emission (nm)	Dielectric constant Cardiolipin ε	DPPC† ε	Egg PC‡ ε
Adriamycin	510	553.5	50	80	80
N-Acetyladriamycin	510	554.1	80	80	80
Cinerubin	503	547.1	58	80	80
Rubidazone	510	554.1	43	80	80
Nogalamycin	512	546.1	30	38	58
Rhodomycin	510	547.2	30	65	40
Steffimycin	493	530.5	21	80	35

* Buffer Tris–HCl, pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M. $T = 25°$.
† DPPD, dipalmitoylphosphatidylcholine.
‡ Egg PC, egg phosphatidylcholine.

order to study possible structural changes, cardiolipin monolayers were hydrolyzed by phospholipase A_2 from porcine pancreas. Kinetics were followed by surface pressure and the lag time (see Fig. 7) was used as the kinetic parameter [9, 10]. This lag time is found to be in good correlation with preceding results [10]. The drugs which do not modify the vertical projection of the monolayer dipole and which do not penetrate deeply into a liposomal bilayer do not modify or even reduce the lag time (Table 4). In contrast, the drugs which modify the dipolar term of the monolayer and penetrate deeply into the bilayer increase the induction time. This behaviour was not observed with rubidazone, probably because of its intermediate position between the two kinds of compounds (Tables 2–4). The inhibition found for the second group of drugs could be correlated with a perturbation affecting the β ester linkage conformation of the phospholipid. The decrease of the lag time observed with the first group of drugs is probably due to the decrease of the surface charge density subsequent to the complexation [33].

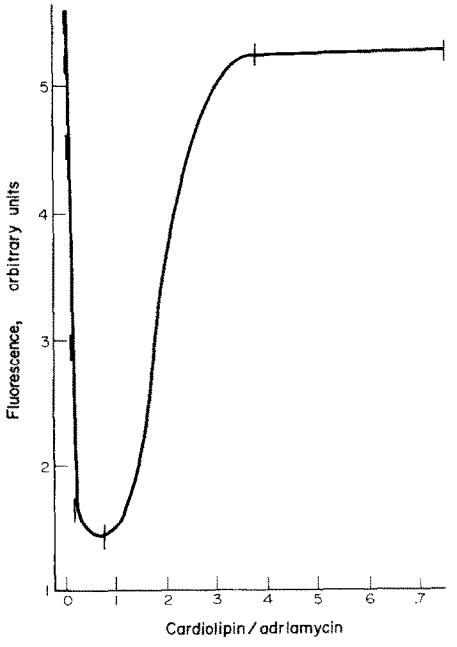

Fig. 6. Titration of adriamycin 5.10^{-7} M by cardiolipin unilamellar liposomes. Evolution of the fluorescence (arbitrary units) as a function of the molar ratio cardiolipin/adriamycin. Buffered solution: Tris–HCl pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M. $T = 25°C$.

DISCUSSION

Among the adriamycin derivatives, we have observed two different kinds of behaviour in the presence of model membranes. In the first class, we can collect drugs which display the highest association constant for cardiolipin and which do not penetrate deeply into the lipid bilayer. These drugs react specifically with cardiolipin. The second class would include the drugs weakly associated to cardiolipin and which penetrate without specificity into the lipid bilayer.

Concerning cardiac toxicity, cardiolipin seems to play a key role in the impairment of mitochondrial functions. A strong interaction of the antibiotic with this specific lipid of the internal mitochondrial membrane could be an important step in the toxicity mechanism. It is significant that adriamycin is the most toxic compound and forms the strongest complex with cardiolipin (Table 1). Rubidazone, which is less toxic than adriamycin at the mitochondrial level [4], binds less effectively to cardiolipin. Prob-

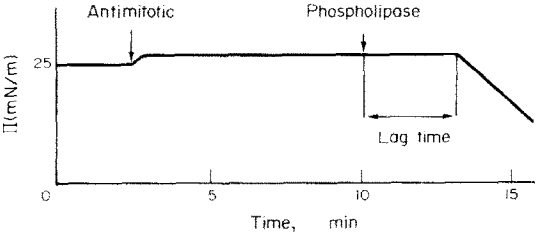

Fig. 7. Scheme of hydrolysis of a phospholipid monolayer by phospholipase A_2. The surface pressure Π (mN/m) of the monolayer is used as kinetic parameter. Buffered solution Tris–HCl pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M; $CaCl_2$, 10^{-3} M. $T = 25°C$. Phospholipase A_2 concentration: 0.1 μg/ml.

Table 4. Hydrolysis of a cardiolipin monolayer (surface pressure = 25 mN/m) by the pancreatic phospholipase A_2. Evolution of the lag time for different adriamycin derivatives*

Antimitotic 3×10^{-6} M	Lag time (min)
Control	4.4 ± 0.4
Adriamycin	2.9 ± 0.8
N-Acetyladriamycin	4.3 ± 0.4
Cinerubin	1.5 ± 0.3
Rubidazone	2.4 ± 0.4
Rhodomycin	5.5 ± 0.3
Nogalamycin	7.0 ± 0.5
Steffimycin	9.1 ± 0.5

* Buffered subphase: Tris–HCl, pH 7.4, 10^{-4} M; $NaNO_3$, 10^{-3} M; $CaCl_2$, 10^{-3} M. The phospholipase concentration was 0.1 μg/ml. $T = 25°$. Each value represents the mean ± S.D. of five experiments.

ably because of its highest hydrophobicity, rubidazone is also a weak uncoupling agent of mitochondrial respiration [4]. Nogalamycin has no effect on the pigeon heart and rat liver respiration [34, 35]. A weak toxicity at this level can again be correlated to a relatively weak affinity for cardiolipin. In contrast, *N*-acetyl-adriamycin, which does not bind to cardiolipin, does not perturb either rat electrocardiograms or mitochondrial respiration [35].

Moreover, the efficiency of adriamycin, cinerubin, rhodomycin and nogalamycin against L1210 leukemia cells has been reported by Goldin [36]:

adriamycin (ILS* = 68) > cinerubin (ILS = 37)
> rhodomycin (ILS = 0)
= nogalamycin (ILS = 0).

A surprising agreement appears between this efficiency sequence and our association constant. Linear regression analysis indicates a good correlation coefficient ($r = 1$, $P < 0.01$). A great specificity of the antimitotic drug for cardiolipin and a weak penetration into the lipid bilayer characterizes drugs which are the most active against different types of malignant cells. Less active agents are those which penetrate deeply into the lipid bilayer and which exhibit no specificity for any tested lipids. The good correlation between the susceptibility of neoplastic cells to adriamycin and derivatives and the affinity of the drug for cardiolipin could be explained by the fact that cardiolipin may be found in cellular membrane upon malignant transformation [37].

To conclude, the description of the complex cardiolipin–adriamycin derivatives has been compared with the mitochondrial toxicity side-effect of these drugs. The result supports the hypothesis that cardiolipin could play a prominent role in the first steps of the toxicity mechanism. Since the association constant measured here is 1.8×10^6 l/M for the complex adriamycin–cardiolipin and is 3.6×10^6 l/M for the complex adriamycin-DNA nucleotide [38], clearly cardiolipin could be a competitive target for adriamycin.

* ILS = per cent increase in survival time over controls.

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