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# A comparative model membrane study on structural effects of membrane-active positively charged anti-tumor drugs

Klaas Nicolay [a], Anne-Marie Sautereau [b], Jean-Francois Tocanne [b], Robert Brasseur [c], Pascal Huart [c], Jean-Marie Ruysschaert [c] and Ben de Kruijff [a]

[a] *Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht (The Netherlands),* [b] *Centre de Recherche de Biochimie et de Génétique Cellulaires du CNRS, Toulouse (France) and* [c] *Laboratoire de Chimie-Physique des Macromolecules aux Interfaces, Université Libre de Bruxelles, Brussels (Belgium)*

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**The interaction of a number of positively charged anti-tumor drugs with cardiolipin-containing model membranes has been investigated using $^{31}P$ nuclear magnetic resonance ($^{31}P$-NMR), differential scanning calorimetry (DSC) and monolayer techniques. It appeared that the ellipticines used (i.e., celiptium and 2-*N*-methylellipticinium), and also ethidium bromide, completely blocked $Ca^{2+}$-induced $H_{II}$ phase formation in pure cardiolipin liposomes at molar ratios of drug-to-lipid of approx. 1:1. For the anthracyclines adriamycin and 4′-*epi*-adriamycin, a similar effect was observed, but now a 2:1 ratio was required. $^{31}P$-NMR experiments on dioleoylphosphatidylethanolamine/cardiolipin mixed liposomes indicated that the two anthracyclines, but not the other three drugs, were capable of inducing macroscopic phase separation into domains enriched in drug-cardiolipin complexes and domains enriched in the zwitterionic phospholipid species. DSC experiments on dipalmitoylphosphatidylcholine/cardiolipin mixtures led, with the exception of 2-*N*-methylellipticinium, to the same conclusion. Measurements of surface pressure and surface potential of cardiolipin monolayers at the air/water interface as well as conformational analysis of the various drug-cardiolipin recombinants showed that the ellipticines are deeply embedded in the acyl chain region of the bilayer, while the anthracyclines and ethidium bromide are preferentially localized in the interface. All drugs share an important electrostatic interaction with the negatively charged phosphates of cardiolipin.**

## Introduction

A number of positively charged anti-tumor drugs, including the anthracycline antibiotic adriamycin (and many of its derivatives) and the ellipticine family (for chemical structures, see Fig. 1), have recently been shown to have a strong interaction with the negatively charged phospholipid component of various cellular membranes. These membrane interactions can result in changes in lipid organization, and are believed to play an important role either in their anti-tumoral effect (ellipticines [1]) or in certain cytotoxic effects exerted by these drugs (e.g., the cardiotoxic action of adriamycin [2]). For a complete understanding of the way in which these cationic drugs affect membrane structure and function, informa-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Correspondence: K. Nicolay, Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, NL-3584 CH Utrecht, The Netherlands.

tion is required at the molecular level on the interaction of these drugs with their main potential membrane targets, i.e., the negatively charged phospholipids.

The objective of this study was to systematically compare the membrane structural effects of members of the anthracycline family of drugs (which has been studied most extensively (for review, see Ref. 2)) with those of the ellipticine class of drugs.

Cardiolipin, in pure form or in selected mixtures with other lipids, was chosen as model anionic lipid because of its well-characterized biophysical properties [3], the detailed knowledge of the adriamycin-cardiolipin complex [2,4,5], and its relation to the inhibitory action of the drug towards mitochondrial functions in which cardiolipin is involved [2,4,6]. The methods used in this comparative approach were $^{31}$P-NMR, differential scanning calorimetry (DSC) and monolayer techniques, while additional valuable information on the geometry of the drug-lipid recombinants was obtained from computer calculations using conformational analysis. The new data obtained will be integrated with literature data in models describing the different drug-lipid complexes.

## Materials and Methods

*Chemicals.* Adriamycin was obtained from either the National Cancer Institute, Aldrich (Brussels, Belgium) or Sigma (St. Louis, MO, U.S.A.), while 4′-*epi*-adriamycin was a gift from Dr. Giuliani (Farmitalia, Milan, Italy). L-α-Dipalmitoylphosphatidylcholine and ethidium bromide were purchased from Sigma. 2-*N*-Methylellipticinium and 2-*N*-methyl-9-hydroxyellipticinium (celiptium) were obtained from Sanofi Recherche (Toulouse, France). Cardiolipin (sodium salt) was isolated from bovine heart as described previously [7]. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (18 : $1_c$/18 : $1_c$-phosphatidylethanolamine) was synthesized as described before [8]. All other chemicals were of analytical grade and were obtained from regular commercial sources.

*Nuclear magnetic resonance (NMR).* $^{31}$P-NMR studies were carried out at 121.49 MHz on a Bruker MSL 300 spectrometer. Typically, 2500 scans were accumulated using 12 μs 90° radiofrequency pulses, a sweep width of 40 kHz and a recycle delay of 1 s. High-power $^{1}$H decoupling was employed during acquisition while probe temperature was maintained at 25°C. Chemical shift scales are drawn with the $^{31}$P-NMR peak of sonicated egg phosphatidylcholine vesicles at 0 ppm. Lipid dispersions for NMR were prepared at room temperature by vortex mixing of a dry film of the relevant phospholipid(s) (25–50 μmol of lipid phosphorus) in 0.8 ml 100 mM NaCl/25 mM Hepes/0.5 mM EDTA (pH 7.4). Drugs were added from 25–50 mM stock solutions. Subsequently, $Ca^{2+}$ was added in aliquots from a 0.25 M $CaCl_2$ solution. Where appropriate, the percentage bilayer in the $^{31}$P-NMR spectra was determined by computer substraction using reference spectra consisting of a single spectral component. The error in the percentages is estimated to be approx. 10%.

*Differential scanning calorimetry (DSC).* DSC measurements were performed on multilamellar liposomes as described elsewhere [4].

*Monolayer experiments.* Compression isotherms, surface pressure and surface potential were measured as described earlier [9], employing experimental procedures detailed elsewhere [10]. Lipids were spread as chloroform/methanol (5 : 1, v/v) solutions. The subphase consisted of 100 mM NaCl/25 mM Hepes buffer at pH 7.4. Ultrapure water from a Milli-Q apparatus (Millipore) was used to prepare buffers for the subphase. Throughout all experiments, reference surface potentials of aqueous subphases were around 20–30 mV. Film compressions were reproducible to within 1% ($\pm 5 \cdot 10^{-3}$ nm$^2$), whereas the reproducibility of surface potential determinations was ±5 mV. Monolayer data presented are averages of two or three experiments carried out at 21°C.

*Conformational analysis.* The total conformational energy is calculated as the sum of four terms: (i) the London-Van der Waals energy; (ii) the electrostatic interaction; (iii) the potential energy of rotation of torsional angles; (iv) the transfer energy of each part of the molecule from a hydrophobic to a hydrophilic medium [11]. The orientation of an isolated molecule at the lipid/water interface, the assemblage in monolayers and the interaction energy were calculated as described

earlier [11]. Calculations were made on an Olivetti M28 computer equipped with the 80287 arithmetic coprocessor using the PC-MSA (Molecular Structure Analysis) program, and the PC-TAMMO (Theoretical Analysis of Molecular Membrane Organization) procedure. Graphs were drawn with the PC-MGM (Molecular Graphics Manipulation) program.

## Results

### *$^{31}$P-NMR and DSC of drug-induced effects on the macroscopic organization of cardiolipin-containing model membranes*

First, the effects of adriamycin, 4′-*epi*-adriamycin, celiptium and 2-*N*-methylellipticinium (Fig. 1) on pure cardiolipin dispersions were studied by $^{31}$P-NMR. Ethidium bromide (Fig. 1) was included in these experiments because it had been shown earlier to bind to cardiolipin with an affinity comparable to that of adriamycin, while the two drug-lipid complexes formed have completely different properties [4]. All five cationic drugs tested caused an immediate precipitation upon addition to aqueous dispersions of bovine heart cardiolipin, as has been reported for adriamycin previously [12,13]. $^{31}$P-NMR (not shown) demonstrated that, with all drugs, cardiolipin retains its liquid-crystalline lamellar organization. However, the $^{31}$P-NMR spectra also revealed interesting differences in effect of the drugs on the residual chemical shift anisotropy, $\Delta\sigma$, measured as the distance between the high-field peak and the low-field shoulder in the asymmetric $^{31}$P-spectra [14]. This $\Delta\sigma$, which is a measure of the local order in the phosphate region of the phospholipid molecule [14], was found to be 27 ppm for the $Na^+$ salt of cardiolipin (not shown). The following values were measured in the presence of the various drugs (at drug-to-lipid molar ratios of 2 : 1): 27 ppm for adriamycin and 4′-*epi*-adriamycin and 33 ppm for celiptium, 2-*N*-methylellipticinium and ethidium bromide (data not shown). This implies that the two anthracyclines do not affect the local order in the phosphate region of cardiolipin, while the other three drugs do.

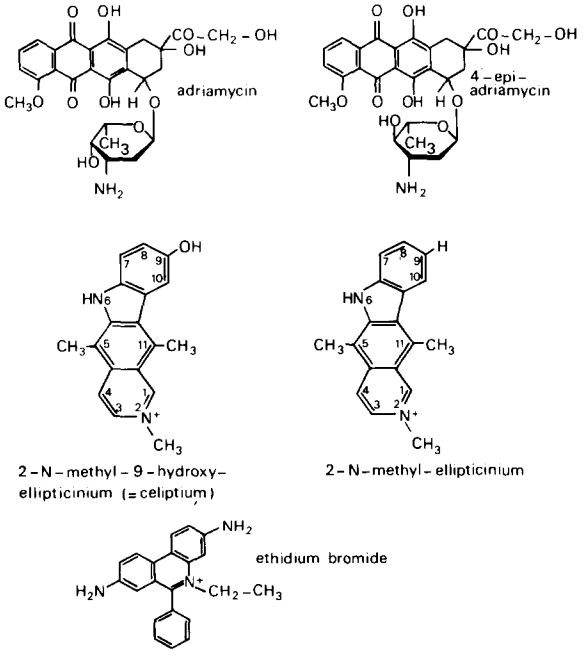


Fig. 1. Chemical structures of the antibiotics used in this study.

We then tested the various drugs with respect to their ability to interfere with the bilayer-to-hexagonal $H_{II}$ phase transition which occurs upon addition of stoichiometric amounts of $Ca^{2+}$ to the $Na^+$ salt of bovine heart cardiolipin [3]. These experiments, in which $^{31}$P-NMR was employed to assess the relative proportion of the cardiolipin molecules organized in a bilayer or a hexagonal $H_{II}$ organization, revealed considerable differences between the anthracyclines on one hand and the ellipticines and ethidium bromide on the other hand. As an example, Fig. 2 shows that, at a drug-to-cardiolipin molar ratio of 0.8, $Ca^{2+}$ addition to the sample containing 4′-*epi*-adriamycin (Fig. 2A and B) caused an almost complete bilayer-to-hexagonal $H_{II}$ phase transition, as evidenced by the reversal of the asymmetry and the reduction in width of the $^{31}$P-NMR spectra (for a description of the $^{31}$P-NMR line shapes of the various lipid structures, see Ref. 15). By contrast, an essentially complete bilayer configuration was retained for the celiptium/cardiolipin mixture (Fig. 2C and D).

A full evaluation of similar $^{31}$P-NMR experiments at various drug/lipid ratios is given in Fig. 3. In agreement with earlier findings for adriamycin [12], the two anthracyclines used here caused complete conservation of bilayer organization in

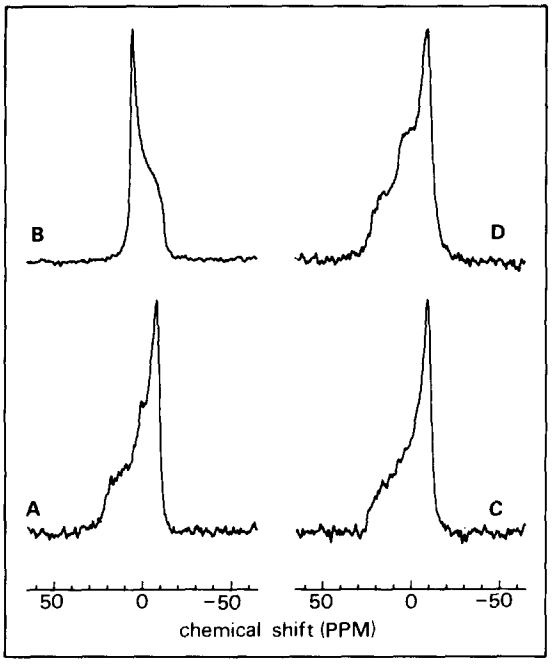


Fig. 2. 121.49 MHz $^{31}$P-NMR spectra of cardiolipin liposomes in the presence of 0.8 mol 4′-*epi*-adriamycin (A, B) or 0.8 mol celiptium (C, D) per mol cardiolipin and in the absence (A, C) of $Ca^{2+}$ or in the presence (B, D) of 0.4 mol $Ca^{2+}$ per mol cardiolipin.

the presence of $Ca^{2+}$ at drug/cardiolipin molar ratios of 2.0 (Fig. 3A). Interestingly, for celiptium, 2-*N*-methylellipticinium and ethidium bromide (Fig. 3B and C), a ratio of 1.0 was largely sufficient to reach the same bilayer-stabilizing effect.

It has been shown recently that adriamycin greatly affects the lateral distribution of cardiolipin in binary phospholipid mixtures in that it induces phase segregation [4,12]. In order to establish whether this property is shared by the ellipticines, we have employed $^{31}$P-NMR (Fig. 4) and DSC (Fig. 5) to compare the effects of the various drugs on binary mixtures of cardiolipin with a zwitterionic phospholipid species. $^{31}$P-NMR spectra of dioleoylphosphatidylethanolamine/cardiolipin (2:1) liposomes indicate that the two anthracyclines (Fig. 4B and C) induce structural phase separation as evidenced by the appearance of a prominent hexagonal $H_{II}$ component in the spectrum which was previously shown [12] to originate from dioleoylphosphatidylethanolamine. In isolated form, this lipid is organized in the hexagonal $H_{II}$ phase at 25 °C [16]. The $^{31}$P-NMR spectra obtained in the presence of ethidium bromide (Fig. 4D), 2-*N*-methylellipticinium (Fig. 4E) and celiptium (not shown) demonstrate that all phospholipids remained organized in a bilayer configuration, i.e., these three drugs do not induce macroscopic phase separation. Control experiments using $^{31}$P-NMR indicated that neither celiptium nor 2-*N*-methylellipticinium and ethidium bromide interfered with the ability of dioleoylphosphatidylethanolamine to adopt the hexagonal $H_{II}$ phase (not shown), while there was also no indication of binding of the drugs to this

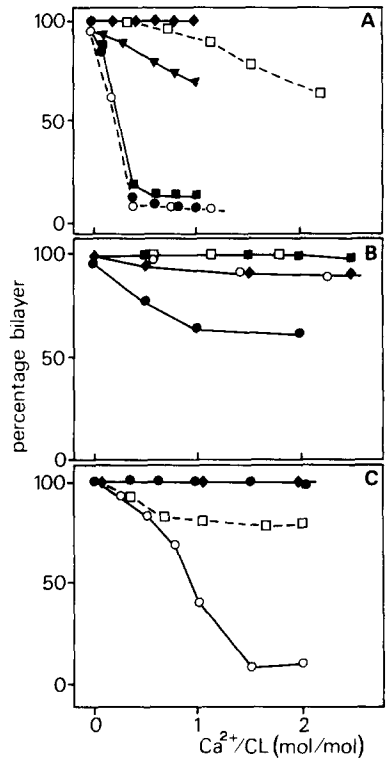


Fig. 3. Effects of (A) adriamycin (closed symbols) and 4′-*epi*-adriamycin (open symbols), (B) 2-*N*-methylellipticinium (open symbols) and celiptium (closed symbols), and (C) ethidium bromide on the $Ca^{2+}$-induced bilayer $H_{II}$ phase transition of cardiolipin (CL), as measured by $^{31}$P-NMR. Drug/cardiolipin molar ratios were: (A) 0.8 (○), 1.0 (●), 1.3 (■), 1.6 (▼), 1.8 (□), and 2.0 (◆); (B) 0.3 (●), 0.8 (○), 1.0 (◆), and 2.0 (■, □); (C) 0.2 (○), 0.5 (□), 1.0 (●), and 2.0 (◆). The ratio $Ca^{2+}$/CL refers to the molar ratio of $Ca^{2+}$ added to cardiolipin present. The data on adriamycin, which are displayed to allow a direct comparison, are taken from Ref. 8. For further details, see Materials and Methods and legend to Fig. 2.

lipid. This implies that the ability of the anthracyclines and the inability of the other three drugs to induce an isothermal phase separation is due to differences in the intrinsic properties of the drug-cardiolipin complexes.

Previous DSC data [4,17] on dipalmitoylphosphatidylcholine/cardiolipin mixtures indicated that adriamycin and closely related analogues, but not ethidium bromide, are capable of inducing cluster formation of drug-cardiolipin complexes, in agreement with the above $^{31}$P-NMR data. This was concluded from the fact that the lowering and broadening of the gel-to-liquid crystalline phase

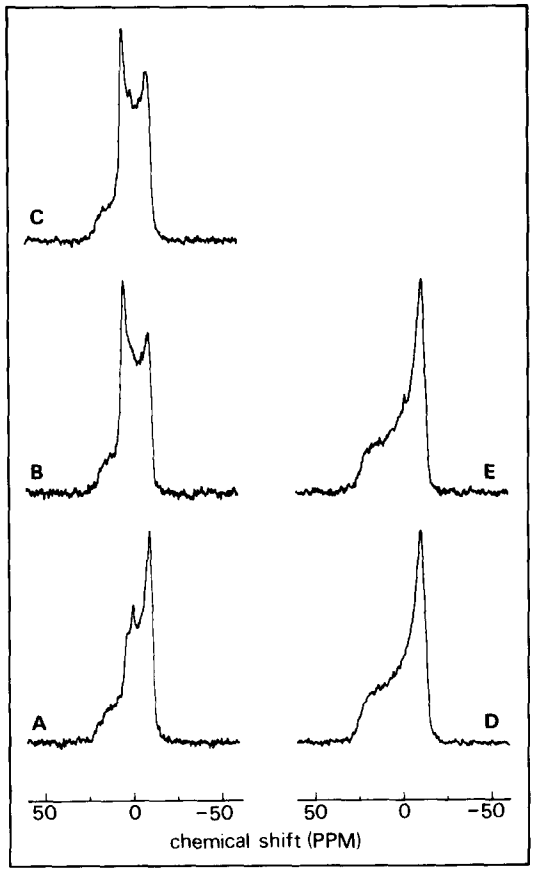


Fig. 4. 121.49 MHz $^{31}$P-NMR spectra of dioleoylphosphatidylethanolamine/cardiolipin (2:1) liposomes. Dispersions were made as described in Materials and Methods. Drugs were added from concentrated stock solutions, to a molar ratio of 2 per cardiolipin. The spectra displayed were obtained in the absence (A) and in the presence of adriamycin (B), 4′-*epi*-adriamycin (C), ethidium bromide (D) and 2-*N*-methylellipticinium (E).

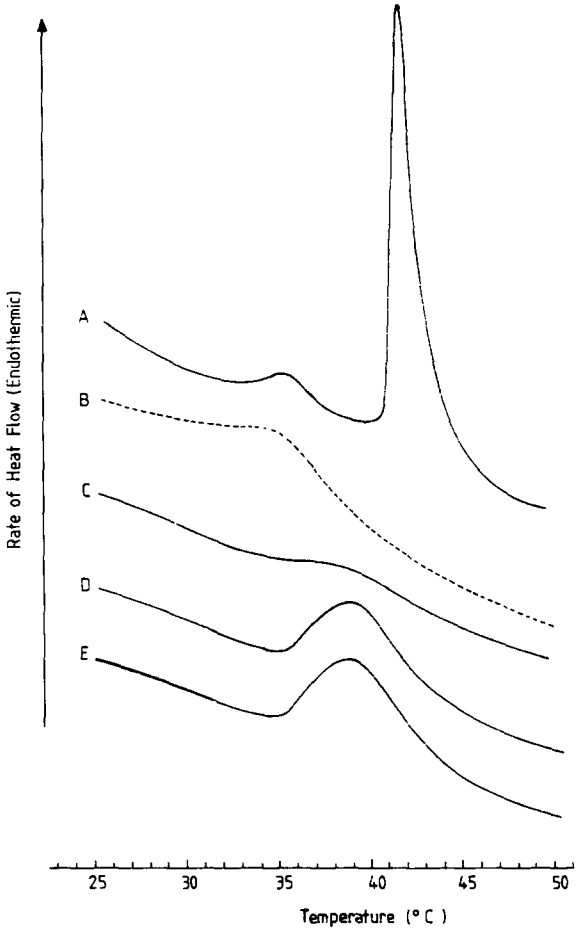


Fig. 5. DSC heating curves of dipalmitoylphosphatidylcholine multilamellar liposomes (A) and of dipalmitoylphosphatidylcholine/cardiolipin (6:1; molar ratio) liposomes (B–E). Curves C, D and E were obtained in the presence of celiptium, 2-*N*-methylellipticinium and adriamycin, respectively, at drug/cardiolipin molar ratios of 2:1. For further details, see Materials and Methods.

transition of dipalmitoylphosphatidylcholine by incorporation of low amounts of the unsaturated cardiolipin is largely reversed by the addition of adriamycin, and not by ethidium bromide [4,17]; compare also Figs. 5B and E). Fig. 5C and D represent DSC thermograms obtained after addition of celiptium and 2-*N*-methylellipticinium, respectively, to dipalmitoylphosphatidylcholine/cardiolipin liposomes. Only for the latter drug was a gel-to-liquid crystalline phase transition visible with characteristics comparable to that in the presence of adriamycin (Fig. 5E). The above ob-

servation seems to contradict the $^{31}$P-NMR results (Fig. 4), which demonstrated that in the dioleoylphosphatidylethanolamine-cardiolipin system, neither of the ellipticines could induce phase separation. However, besides the differences in the lipid system, the nature of the two phase separations is different. In the DSC experiment, phase separation is between gel and liquid-crystalline domains, whereas in the NMR experiment, phase separation is fully in the liquid-crystalline state.

*Effects on cardiolipin monolayers*

We then employed the monolayer technique to gain further insight into the nature of the interaction of the present drugs with cardiolipin. To this end, monolayers of pure bovine heart cardiolipin were spread on the air/water interface. Either in the absence or in the presence of drug in the subphase, the compression isotherms recorded for

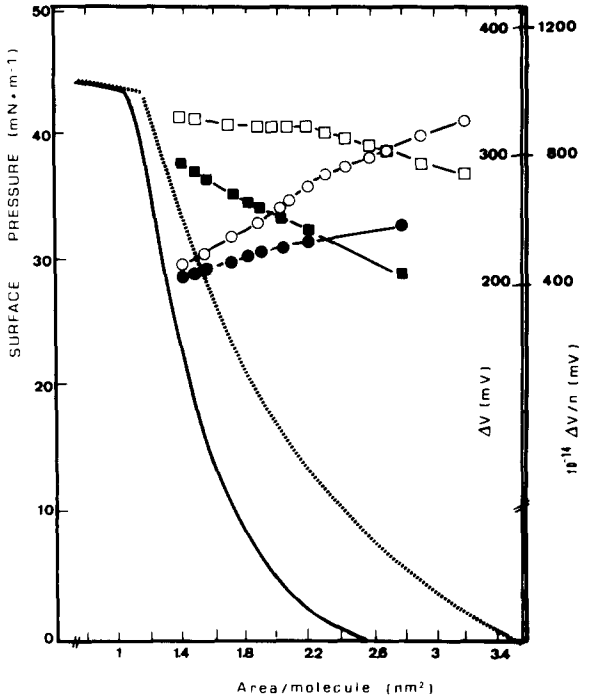


Fig. 6. Influence of adriamycin on the compression isotherms (curves) and surface potentials (symbols) of cardiolipin. Curve 1 and closed symbols: lipid alone; curve 2 and open symbols: lipid in the presence of $10^{-5}$ M drug in the subphase; (□, ■): surface potential, $\Delta V$; (○, ●): $\Delta V/n$, surface potential corrected for changes in lipid surface density which occur upon film compression. For further details, see text.

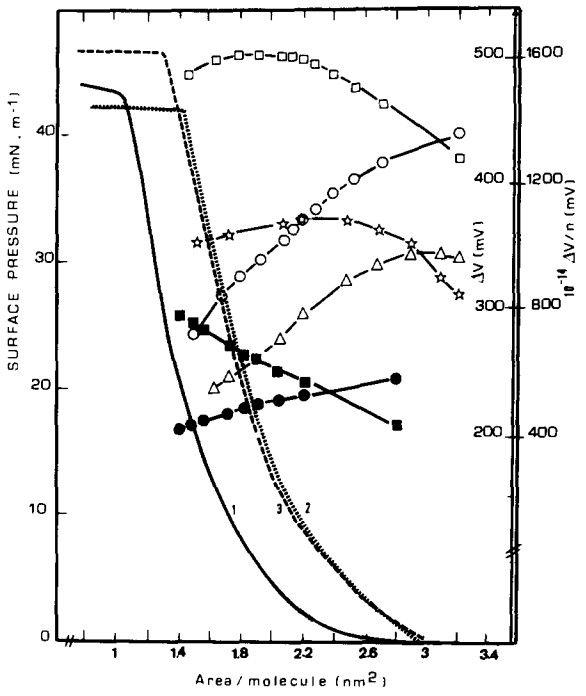


Fig. 7. Influence of celiptium and 2-*N*-methylellipticinium on the compression isotherms (curves) and surface potentials (symbols) of cardiolipin. Curve 1 and closed symbols (■, ●), lipid alone; curve 2 and open symbols (□, ○), lipid in the presence of $2 \cdot 10^{-5}$ M 2-*N*-methylellipticinium in the subphase; curve 3 and open symbols (☆, △), lipid in the presence of $2 \cdot 10^{-5}$ M celiptium. (□, ☆, ■): $\Delta V$; (○, △, ●): $\Delta V/n$.

cardiolipin were characteristic of a lipid in the liquid-expended state (Figs. 6 and 7). In the absence of drug (curve 1 in Figs. 6 and 7), a film collapse was observed at a surface pressure of about 43 mM · m$^{-1}$, corresponding to a limiting molecular area of 1.05 nm$^2$.

In the presence of adriamycin at a concentration of $10^{-5}$ M in the subphase, a large film expansion was observed at low and moderate surface pressure, which progressively reduced when increasing the packing density of the lipid (curve 2, Fig. 6). Nevertheless, the compression isotherm accounted for a film collapse at the same surface pressure, $\pi$, of 43 mM · m$^{-1}$ as above, with a slightly higher limiting molecular area of 1.15 nm$^2$.

Celiptium (curve 2, Fig. 7) and 2-*N*-methylellipticinium (curve 3, Fig. 7) at concentrations in the subphase of $2 \cdot 10^{-5}$ M and $10^{-5}$ M, respectively, also brought about large film expansions. These film expansions of about 0.50 nm$^2$ at low

surface pressure and 0.40 $nm^2$ at high surface pressure remained practically unaffected upon compressing the film. In both cases, a film collapse was detected at a surface pressure comparable to that found in the absence of drug in the subphase, with the same limiting area of 1.45 $nm^2$.

Surface potential data are reported on the same figures. They are plotted either as they were measured ($\Delta V$) or in terms of $\Delta V/n$, which corrects for the changes in lipid surface density which occur upon film compression [18]. As expected, owing to the respective cationic and anionic nature of the drug and lipid molecules, large increases in $\Delta V$ were observed when cardiolipin was spread in the presence of the three drugs, compared to the drug-free subphase. $\Delta V$ changed by about 80–90 mV in the presence of adriamycin at low and moderate surface pressure (Fig. 6), which is in good agreement with previous determinations [19]. Larger $\Delta V$ increases were measured upon addition of the ellipticine derivatives: about 80–140 mV with celiptium and 200–250 mV with 2-*N*-methylellipticinium (Fig. 7).

In terms of $\Delta V/n$, the surface potential measured for cardiolipin spread on drug-free subphases decreased only slightly (about 20–30 mV) upon compression of the film. This suggests that the orientation of the lipid molecules and their mutual interaction in the film were not strongly altered by the changes in lipid molecular packing. In contrast, $\Delta V/n$ decreased greatly when compressing cardiolipin in the presence of the three drugs in the subphase, suggesting a greater dependence of the orientation and interaction of the drug-lipid complexes on the lipid molecular packing. It should be noted that the plots of $\Delta V/n$ as a function of the lipid molecular area for the lipid alone or in the presence of adriamycin tend to converge precisely at the molecular area corresponding to the film collapse (Fig. 6). The same tendency was observed with celiptium (Fig. 7), but not with 2-*N*-methylellipticinium. For the latter drug, both $\Delta V$ and $\Delta V/n$ values remained considerably higher than those measured on a drug-free subphase, even in the region of the film collapse (Fig. 7).

In order to measure the association constant of the drug-cardiolipin complexes, experiments were carried out consisting of measuring the change in film surface pressure with increasing drug concentration in the subphase, at constant molecular area. The lipid was spread at an initial surface pressure, $\pi$, of 10 $mN \cdot m^{-1}$. Subsequently, the drug was added stepwise into the subphase, and the surface pressure was measured at each step. As an example, Fig. 8 shows that addition of adriamycin led to a progressive increase in surface pressure, up to a concentration of $3 \cdot 10^{-5}$ M. An apparent drug-to-lipid binding constant of about $3 \cdot 10^5$ $M^{-1}$ can be obtained from this curve as the concentration at which half-maximal film expansion occurs. This value is slightly lower than the $1.6 \cdot 10^6$ $M^{-1}$, as determined from surface potential and surface radioactivity measurements [19], and similar to that obtained by means of fluorescence experiments: $1.3 \cdot 10^5$ $M^{-1}$ [20].

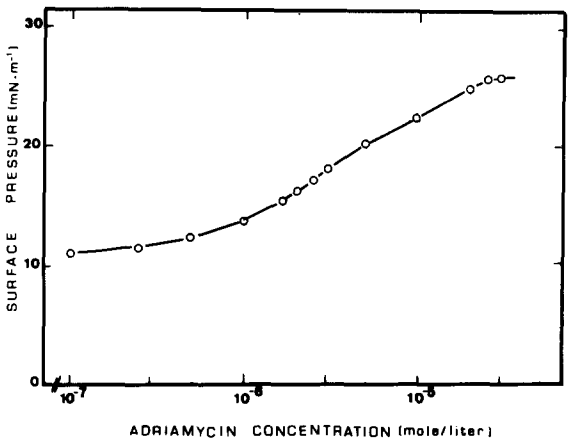


Fig. 8. Changes in the surface pressure of a film of cardiolipin with increasing adriamycin concentration in the subphase, at constant molecular area. The initial film pressure was 10 $mN \cdot m^{-1}$.

Similar experiments were performed with celiptium and 2-*N*-methylellipticinium. The estimated association constants of the complexes between cardiolipin and these two molecules amounted to $3 \cdot 10^4$ $M^{-1}$ and $2 \cdot 10^5$ $M^{-1}$, respectively (data not shown).

*Conformational analysis of drug-cardiolipin complexes*

It has been demonstrated recently that, in combination with experimental data, the conformational analysis procedure can contribute significantly to our understanding of the molecular de-

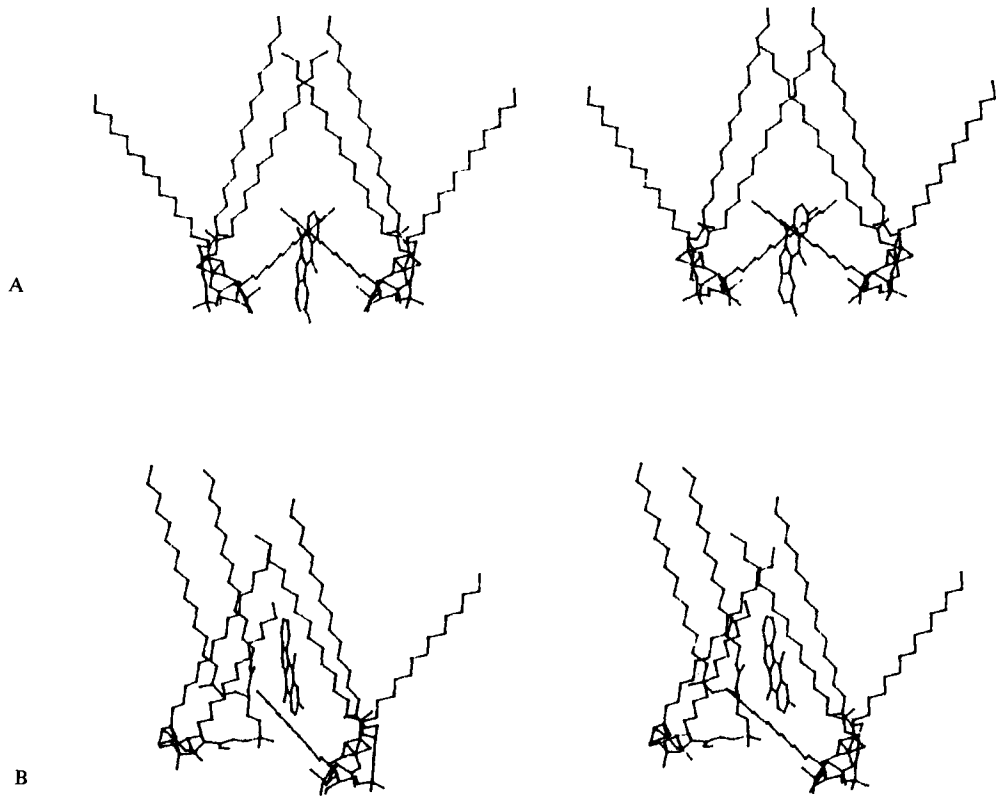


Fig. 9. Stereo views of the organization of mixed monolayers made of celiptium and cardiolipin (A), and of 2-*N*-methylellipticinium and cardiolipin (B), as calculated by conformational analysis. Note that the positively charged nitrogen-2 (Fig. 1) is pointing towards the aqueous phase in (B), while it is in contact with the phospholipid acyl chains in (A). For details, see Materials and Methods.

tails of the interaction between phospholipids and membrane-active drugs (for a review, see Ref. 11). Of the drugs used here, the above theoretical approach had been applied only to the adriamycin-cardiolipin and the ethidium bromide-cardiolipin systems [4,11]. For this reason, and in order to aid in interpreting the present experimental data, we have applied the conformational analysis procedure to the celiptium-cardiolipin and 2-*N*-methylellipticinium-cardiolipin systems and have concentrated on the orientation of the drugs with respect to the lipid/water interface and the interaction energy between the drug and lipid molecules.

Stereoviews of the complexes of cardiolipin with celiptium and 2-*N*-methylellipticinium as obtained by conformational analysis are shown in Figs. 9A and B, respectively. In both cases, the drug molecule is inserted into the hydrophobic region of the cardiolipin monolayer, although this is clearly more pronounced for 2-*N*-methylellipticinium (Fig. 9B). A more dramatic difference, however, lies in the fact that the positively charged nitrogen-2 (Fig. 1) is calculated to be in electrostatic interaction with the negatively charged phosphates of cardiolipin within its complex with 2-*N*-methylellipticinium, while in the complex with celiptium this positive charge is calculated to be situated rather deeply in the acyl chain region and the hydroxyl at position 9 is in the interface region. This difference in localization of the positive charge is also most probably mainly responsible for the difference in calculated interaction energy between the cardiolipin-2-*N*-methylellipticinium and the cardiolipin-celiptium complex ($-19.8$ and $-14.0$ kcal/mol, respectively).

## Discussion

Of the various cardiolipin-drug complexes studied here, the one with adriamycin is the best characterized. For that reason, this discussion will start out with an integration of the data currently available on adriamycin–cardiolipin interactions. Table I summarizes the most relevant properties and compares them with those of the other drugs.

Adriamycin is known from binding experiments [5,21] and monolayer studies [19] to form a 2 : 1 (i.e., charge neutral) complex with cardiolipin. This stoichiometry (at saturating drug concentrations) is observed with liposomes prepared from pure cardiolipin with liposomes prepared from mixtures of cardiolipin, and zwitterionic phospholipids as well as with mitochondrial membrane preparations [5,21]. Furthermore, this specific and high-affinity binding to cardiolipin has been shown to be primarily electrostatic in nature [19,22,23]. Therefore, it is generally accepted that complex formation occurs through coulombic interaction between the negatively charged phosphates in the cardiolipin headgroup and the positive charged amino group on the sugar moiety of the drug.

The precise orientation of the adriamycin molecule in its complex with cardiolipin appears to depend mainly on two factors, i.e., the drug-to-cardiolipin molar ratio and the surface pressure. Thus, it has been convincingly shown that in the

TABLE I

PROPERTIES OF THE COMPLEXES BETWEEN CARDIOLIPIN AND THE CATIONIC DRUGS USED IN THIS STUDY

| Drug | Properties of the complex | | | | | | |
|---|---|---|---|---|---|---|---|
| | Association constant ($M^{-1}$) [a] | Interfacial localization [b] | Monolayer penetration | Effect on chemical shift anisotropy [c] | Inhibition of $Ca^{2+}$-induced $H_{II}$ phase [d] | Induction of phase separation | |
| | | | | | | PE/CL [e] | DPPC/CL [f] |
| Adriamycin | $3 \cdot 10^5$ $1.6 \cdot 10^6$ [19] $1.3 \cdot 10^5$ [20] | membrane embedded [20, 24,25] membrane/water interface [20,24–26] | dependent upon surface pressure | – | 2:1 [12,13] | + [12] | + [4] |
| 4′-*epi*-Adriamycin | $1.3 \cdot 10^5$ [20] | membrane-embedded [20] membrane/water interface [20] | see adriamycin | – | 2:1 | + | + |
| Celiptium | $3 \cdot 10^4$ | membrane-embedded [33,34] | yes | + | 1:1 | – | – |
| 2-*N*-Methyl-elliptici-nium | $2 \cdot 10^5$ | membrane-embedded [33,34] | yes | + | 1:1 | – | ± |
| Ethidium bromide | $2 \cdot 10^6$ [17] | membrane/water interface [22] | n.d. | + | 1:1 | – | –[4] |

[a] Apparent association constants of drug-cardiolipin complexes.
[b] Special emphasis on localization of the drugs chromophore.
[c] Effects on the chemical shift anisotropy as measured by $^{31}$P-NMR from 2:1 drug/cardiolipin mixtures.
[d] Molar ratio of drug-to-cardiolipin at which a complete bilayer configuration is retained up to 1 $Ca^{2+}$/cardiolipin.
[e] Based on $^{31}$P-NMR data from dioleoylphosphatidylethanolamine (PE)/cardiolipin (CL) liposomes.
[f] Based on DSC data from dipalmitoylphosphatidylcholine (DPPC)/cardiolipin liposomes.
n.d., not determined.

2 : 1 complex the chromophore is localized in the interface between water and polar headgroups [20,24–27], as is also predicted by conformational analysis [4,11]. At low to intermediate drug-to-lipid molar ratios, however, the available experimental data [20,24,25] indicate that other orientations are accessible in which the chromophore is (partially) embedded in the hydrophobic region of the bilayer. It is important to stress that, in all above configurations, the drug is bound electrostatically to the cardiolipin phosphate via its α-amino group [25]. The effects of adriamycin on the surface pressure of cardiolipin monolayers (Fig. 6) are consistent with these two modes of organization of the drug in the complex. At low and medium surface pressures, adriamycin causes a large expansion of the monolayer, indicating that there is considerable penetration of the drug inbetween cardiolipin molecules. At higher surface pressures, including those between 31 and 34 mN/m suggested to hold in a bilayer [28,29], drug-induced film expansions are much smaller, indicating that the drug is progressively squeezed out of the monolayer upon compression.

An interesting point to discuss now is why, in a bilayer, the configuration of the adriamycin-cardiolipin complex appears to be dependent upon the drug/lipid molar ratio. The evidence available so far suggests that this is due to the ability of individual adriamycin molecules to undergo mutual plane–plane ring-stacking interactions in the membrane/water interface. Since this self-association of adriamycin is a second-order process, the drug at low concentration will exist mainly as monomers which penetrate the bilayer. At high drug concentrations, the ring-stacked configuration will prevail in which the drug has a complete interfacial localization. The self-association behaviour of adriamycin in the cardiolipin-bound state was proposed on the basis of visible absorption spectra [19], and by conformational analysis [4,11]. Interestingly, the latter theoretical approach also calculated that the geometry of the interfacial assembly of adriamycin is virtually identical to that in solution [4]. It is well known that self-association of adriamycin in solution occurs above approx. $10^{-5}$ M [30,31].

The above molecular details of the adriamycin-cardiolipin complex can now be used to interpret the effects of the drug on the macroscopic organization of cardiolipin-containing model membranes, as studied by $^{31}$P-NMR (Figs. 2–4). Thus, the 2 : 1 charge-neutral adriamycin-cardiolipin complex is required to block $H_{II}$ phase formation completely upon $Ca^{2+}$ addition (Fig. 3A). Furthermore, the capacity of adriamycin to induce phase separation in binary phospholipid mixtures containing cardiolipin, as demonstrated by $^{31}$P-NMR (Fig. 4, Ref. 12) and DSC [4,17], can be accounted for by ring-stacking interactions between adjacent membrane-bound drug molecules. This study clearly shows that 4′-*epi*-adriamycin behaves identically to adriamycin with respect to its effects on $H_{II}$ phase formation by $Ca^{2+}$ (Fig. 3A) and phase segregation (Fig. 4C).

Our comparative experiments on anthracycline– and ellipticine–cardiolipin interactions demonstrate that, although both classes of drugs show a strong interaction with cardiolipin, there are large differences in the resulting drug-lipid complexes (Table I). Although the ellipticines have a significant electrostatic contribution in the interaction with negatively charged lipids (see also Ref. 32), there appears to be also a prominent contribution of hydrophobic interactions to the stability of complexes between ellipticines and negatively charged phospholipids. First, the monolayer data (Fig. 7, see also Ref. 33) are indicative of considerable penetration between the acyl chains, irrespective of the lipid packing density. Secondly, fluorescence quenching data using membrane probes [34] show that there is insertion of ellipticines into the hydrophobic membrane interior. Interestingly, the latter data also show that 9-hydroxyellipticines penetrate less deeply into the lipid bilayer than nonhydroxylated compounds. This is in agreement with the present monolayer studies, since celiptium had smaller effects in cardiolipin monolayers than 2-*N*-methylellipticinium (Fig. 7). Thirdly, the conformational analysis approach (Fig. 9) as applied to the ellipticine-cardiolipin systems, yielded geometries of the complexes in which the drugs are in close contact with the acyl chains. It is interesting to note that conformational analysis predicts deeper penetration of 2-*N*-methylellipticinium than of celiptium, which is in agreement with the experimental data detailed above.

The prominent difference in mode of organization between the anthracycline-cardiolipin and the ellipticine-cardiolipin complexes leads to marked differences in the effects of the two classes of drugs on the modulation of the macroscopic organization of cardiolipin. First, we will compare the various drugs with respect to their ability to prevent the $Ca^{2+}$-induced bilayer to $H_{II}$ transition by cardiolipin (Fig. 3). Clearly, all five drugs block $H_{II}$ phase formation completely when present in charge-stoichiometric amounts relative to cardiolipin. Two factors which may be responsible for bilayer stabilization should be considered: (i) a general exclusion of $Ca^{2+}$ due to the presence of phosphate-bound drug, which seems especially relevant for the anthracyclines and ethidium bromide, since both have an interfacial localization in the charge-stoichiometric complex (see also below); (ii) the ability of the ellipticines to penetrate in between the headgroups will, within the terms of the shape-structure concept of lipid polymorphism [15], also mitigate against $H_{II}$ phase formation. Below charge stoichiometry, the various drug-cardiolipin complexes display remarkable differences in terms of bilayer stabilization towards $Ca^{2+}$. Thus, complete inhibition of $H_{II}$ phase formation by the anthracyclines occurred only at and above a drug-to-cardiolipin molar ratio of 2 (Fig. 3A), while for the ellipticines (Fig. 3B) (and ethidium bromide (Fig. 3C), a 1 : 1 molar ratio was sufficient to reach this effect. Although a satisfactory explanation in quantitative terms cannot be given as yet, it might well be that ring-stacking interaction in the adriamycin-cardiolipin system (below a drug/lipid molar ratio of 2 : 1) leads to the formation of patches enriched in drug-lipid complexes, leaving drug-free areas to which $Ca^{2+}$ has direct access. Since the ellipticines (and ethidium bromide) are not capable of phase separation (see below), the drug-cardiolipin complexes might be more randomly dispersed in the plane of the bilayer in these cases, thereby more efficiently preventing the $Ca^{2+}$-induced bilayer to $H_{II}$ transition through the two possible mechanisms detailed above.

The $^{31}$P-NMR studies on dioleoylphosphatidylethanolamine/cardiolipin (2 : 1) liposomes (Fig. 4) demonstrated that adriamycin and 4′-*epi*-adriamycin are capable of inducing phase separation under liquid-crystalline conditions, while celiptium, 2-*N*-methylellipticinium and ethidium bromide are not. DSC experiments on dipalmitoylphosphatidylcholine/cardiolipin liposomes (Fig. 5, Refs. 4 and 17) led to identical conclusions, except for 2-*N*-methylellipticinium. This drug partially restored the phase transition of the saturated phosphatidylcholine. These data, together with the calculated interfacial localization of the ellipticines in a cardiolipin monolayer, suggest that the lack of ring-stacking interactions between membrane-embedded drugs is the main reason for the reduced ability of these molecules to induce phase separation. In case of ethidium bromide, a preferential interfacial localization in the cardiolipin-bound state was suggested [22]. Therefore, interfacial ring-stacking interactions between nearby drug molecules could in principle occur. Conformational analysis [4,17] suggests that the reason for the absence of these interactions lies in the fact that the planar ring system of ethidium bromide cannot pack in regular parallel planes, either in solution or in the cardiolipin-bound state.

It remains to be established whether the remarkable differences in the interaction of anthracyclines and ellipticines with cardiolipin in model membranes as reported here also result in different effects on functions carried out by the mitochondrial inner membrane, i.e., the major natural source of cardiolipin. There is considerable evidence that adriamycin–cardiolipin interactions play an important role in the inhibition of mitochondrial function, both in vitro [4,6] and in vivo [35,36]. Moreover, the latter in vivo inactivation of mitochondrial function has been suggested to be responsible for development of anthracycline-induced cardiotoxicity (see Refs. 1, 36 and 37, and references cited therein). It would be interesting to establish the in vitro mitochondrial toxicity of ellipticines, e.g., by measuring their effects on the four complexes of the heart mitochondrial respiratory chain [6]. In this respect, it is of interest that Ruysschaert and co-workers have recently reported that ethidium bromide is several orders of magnitude less potent than adriamycin in inhibiting complex I–III [4] and complex IV activity [17] of bovine heart mitochondria, which was tentatively ascribed to the inability of the former

drug to induce lateral phase separation, as measured by DSC.

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