0026-895X/88/050574-06$02.00/0

MOLECULAR PHARMACOLOGY, 33:574-579

Structural Basis for the Binding of Antitumor Anthracycline Antibiotics to Model Membranes: Circular Dichroism Studies

NELLY HENRY-TOULME, BARBARA STEFANSKA, EDOUARD BOROWSKI, and JACQUES BOLARD

Laboratoire de Physique et Chimie Biomoléculaire (U.A. CNRS 198), Tour 22, Université Pierre et Marie Curie, 4 place Jussieu, 75252 Paris Cedex 05, France (N.H.-T., J.B.) and Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk, 80-952 Gdansk, Poland (B.S., E.B.)

Received August 11, 1987; Accepted February 10, 1988

SUMMARY

Circular dichroism was used to compare the binding of several anthracycline antitumor antibiotics to sonicated phosphatidylcholine vesicles. Daunorubicin analogues, differing from the parent by structural changes in the amino sugar moiety of the molecule, were tested both with vesicles that contained negatively charged phospholipids and with neutral vesicles. The self-association properties of the analogues were also investigated. Binding to negatively charged vesicles was not strictly dependent on electrostatic interactions, since the characteristics of daunorubicin binding were totally different from those of Adriamycin (doxorubicin). Furthermore, the cardiotoxicity of these molecules did not have its origin in their quantitatively preferential electrostatic binding to negatively charged cardiolipin-containing membranes: DR-19, a daunorubicin derivative having lower cardiotoxicity than the parent compound, which bound to negatively charged vesicles in a manner quite similar to that of Adriamycin, whereas DR-10, another daunorubicin derivative with higher cardiotoxicity, bound poorly to negatively charged vesicles.

The anthracycline antibiotics are effective against several types of cancer. Unfortunately, the therapeutic use of these drugs has been limited by their cardiotoxicity. Cumulative doses may cause irreversible cardiomyopathy. The cardiotoxicity of ADR (doxorubicin), the most widely used anthracycline, may be correlated with its interaction with cell membranes (for a review see Ref. 1). Furthermore, much evidence suggests that the mitochondrial membrane is the target for ADR cardiotoxicity and that the formation of a cardiolipin-ADR complex would be determinant (2). It has been suggested that such cardiolipin-anthracycline complexes are formed upon an electrostatic interaction between negatively charged phosphate groups of the phospholipids and positively charged nitrogen atoms of the sugar moiety of the antibiotic molecule.

In contrast, from a comparison of the biological data existing in the literature and the results of a study on the binding of several anthracyclines to phosphatidylcholine vesicles, Burke and Tritton (3, 4) on the one hand and Griffin *et al.* (5) on the other hand concluded that no significant relationship could be discerned between membrane binding and cardiac toxicity.

In the present study, the binding of several anthracyclines with charged and neutral phospholipid vesicles was compared in order to define the relationship between membrane electrostatic binding and cardiotoxicity.

The compounds selected for these studies (Table 1) differ in their ability to acquire a positive charge at the nitogen atom; furthermore, they are not cardiotoxic to the same extent. ADR, DR, and two novel alkyl (*N*-glycosyl) derivatives of the latter (DR-9, DR-10) are compounds in which the nitrogen atom has a comparable basicity. The nitrogen atoms of the two other derivatives of DR examined, an amidine (DR-2) and an enamine (DR-19) derivative, are much less basic. In contrast, ADR and DR-19 are less cardiotoxic than DR, whereas DR-10 and DR-2 are more so (6).

Here we report CD characteristics of DR, DR-2, DR-9, DR-10, and DR-19. We considered the influence of strongly hydrophilic glycoside substitutions on the self-association in water of the anthracycline derivatives DR-9 and DR-10. Then we determined the extent of anthracycline-vesicle binding, taking advantage of the high sensitivity of CD to detect changes in the conformation of the antibiotic molecule and the way in which it is embedded in the membrane bilayer. We have already used this method for measuring the binding of ADR to phospholipid-vesicles (7).

Materials and Methods

Chemicals. Purified ADR and DR were kindly provided by Roger Bellon (Neuilly-sur-Seine, France) and Rhône-Poulenc (Vitry, France),

This work was supported in part by a grant from Institut Curie and the Cellule des Relations Internationales de l'Université Pierre et Marie Curie.

ABBREVIATIONS: ADR, Adriamycin (doxorubicin); CD, circular dichroism; CF, carboxyfluorescein; DR, daunorubicin; EPA, egg yolk phosphatidic acid; EPC, egg yolk L-α-phosphatidylcholine; HEPES, *N*-(2-hydroxyethyl)piperazine-2-*N*′-2-ethanesulfonic acid; SUV, small unilamellar vesicles. DMPC, L-α-dimyristoyl phosphatidylcholine.

respectively. *N*-(*N'*,*N'*-dimethylaminomethinyl)daunorubicin (DR-2), *N*-(1-deoxy-fructos-1-yl)daunorubicin (DR-10), *N*-[1-deoxy-4 (α-D-glucopyranoside)-fructos-1-yl] daunorubicin (DR-9), and *N*-[1-carboethoxy-propen-1-yl]daunorubicin (DR-19) were synthesized as indicated in previous work (8–10). EPC and EPA were purchased from Sigma Chemical Co. (St. Louis, MO), as was HEPES. Cholesterol was from Carlo Erba (Italy). CF was obtained from Kodak Laboratory Chemicals (Rochester, NY) and purified according to the method of Ralston *et al.* (11). Potassium chloride was from Merck.

Unilamellar phospholipid vesicles. SUVs were prepared according to a method adapted from Newman and Huang (12). Sonication of the appropriate mixture of phospholipids was performed above their transition temperature, under nitrogen. The SUVs were prepared at a total lipid concentration of 10 mM. The electrically neutral vesicles contained EPC and cholesterol at a molar ratio of 8:2. Negatively charged vesicles contained EPC, EPA, and cholesterol at a molar ratio of 6.4:1.6:2.

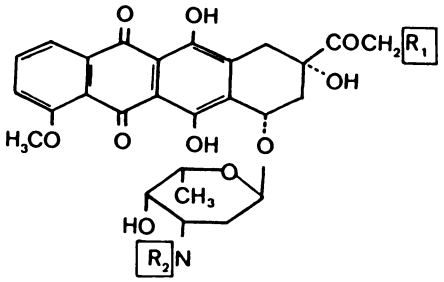
Methods. The experiments were performed in 0.01 M HEPES and 0.1 M KCl, pH 7.4, unless otherwise stated. Anthracycline stock solutions were prepared by directly dissolving the drug in the HEPES buffer except for DR-19, which we dissolved first in dimethyl sulfoxide before suspending the very concentrated aliquot in aqueous buffer (1 mg of DR-19 powder in 10 μl of dimethyl sulfoxide).

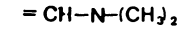
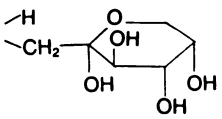
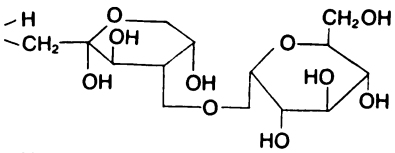
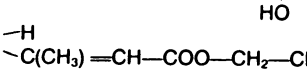
The CD studies were done using two different methods. (i) In the *direct method*, the CD of the anthracyclines in HEPES buffer was compared to those obtained in the presence of various concentrations of lipid vesicles having different compositions. The CD changes enabled us to monitor the anthracycline binding. (ii) The *indirect method* took advantage of the interactions of the anthracyclines with a dye molecule, CF. The association of CF with the plasma anthracyclic part of the drugs results in the appearance of a strong dichroic doublet. The intensity of this doublet has been used to trace the embedding of the anthracyclic part of ADR into lipid vesicles. Details concerning these methods are given in a previous paper (7), in which the interactions of ADR with model membranes were studied.

The CD spectra were recorded on a Jobin Yvon Mark IV dichrograph.

TABLE 1

Anthracycline derivative structures



Anthracycline	R_1	R_2
ADR	OH	
DR	H	
DR-2	H	
DR-10	H	
DR-9	H	
DR-19	H	

Results

CD of DR, DR-2, DR-9, DR-10, and DR-19. The self-association of ADR and DR in aqueous buffer has been shown in a number of cases, and its consequences on the spectroscopic characteristics of these molecules have been described (13–16). Some works have shown that an indefinite association model can describe experimental behavior of DR (17). Nevertheless, the monomer-dimer model correctly fits the results in a concentration range located between 10^{-6} M and 5.10^{-3} M, even if further aggregation may occur beyond these concentrations (16).

This association into dimer is accompanied by very characteristic spectral changes (13–16). A completely different CD spectrum is obtained.

The patterns of the spectra of the five derivatives that we studied were very sensitive to the aggregation state of the molecules, as it was with the parental compound ADR. Taking into account the anthracycline concentrations that were used, we will only consider the association into dimers. Fig. 1A shows the CD spectra recorded with solutions of monomeric and dimeric DR-2. The stacking of the anthracenic parts of the two molecules at higher concentrations induces the coupling of the dipole moments, which results in the appearance of a dichroic doublet around 500 nm. Very similar patterns were obtained with DR, DR-9, and DR-10. The main parameters for these compounds are recapitulated in Table 2. The derivative DR-19 displayed different CD characteristics (Fig. 1B). The general pattern of the monomeric form was comparable to those of the other derivatives. The main differences were observed for the dimeric form: the dichroic band, located around 356 nm for the other derivatives, had disappeared, and the position of the maximum of intensity for the negative part of the dichroic doublet had shifted from 522 nm for ADR, DR, DR-2, DR-9 and DR-10 to 560 nm for DR-19.

Fig. 2 shows the intensity of the negative band of the dichroic doublet as a function of the anthracycline concentration for the three derivatives DR, DR-10, DR-19, and ADR as a reference. This doublet, which has been interpreted as the signal of the stacking of the planar parts of the molecules, appeared at a concentration of DR-19 lower than that of the other derivatives. This result, together with the fact that DR-19 is less soluble in water and aqueous buffer than the other derivatives studied in this paper, suggests that its intermolecular forces are stronger than those of the other molecules.

Assuming the aggregation model of the dimer formation, we calculated the dimerization constant K_d for DR, DR-10, and DR-19, using the variation of the dichroic doublet as a function of concentration. The dimerization constants were found to be 1.6×10^4, 4×10^4, and 3×10^8 M^{-1} respectively. It should be pointed out that K_d is only an apparent constant, allowing the extent of self-association of the compounds to be compared to each other, as we have no experimental proof of their exact degree of association.

Interaction of the Anthracyclines with CF. Increasing amounts of DR-2, DR-9, DR-10, or DR-19 were added to a 4×10^{-4} M CF solution. We followed the intensity of the CD signal

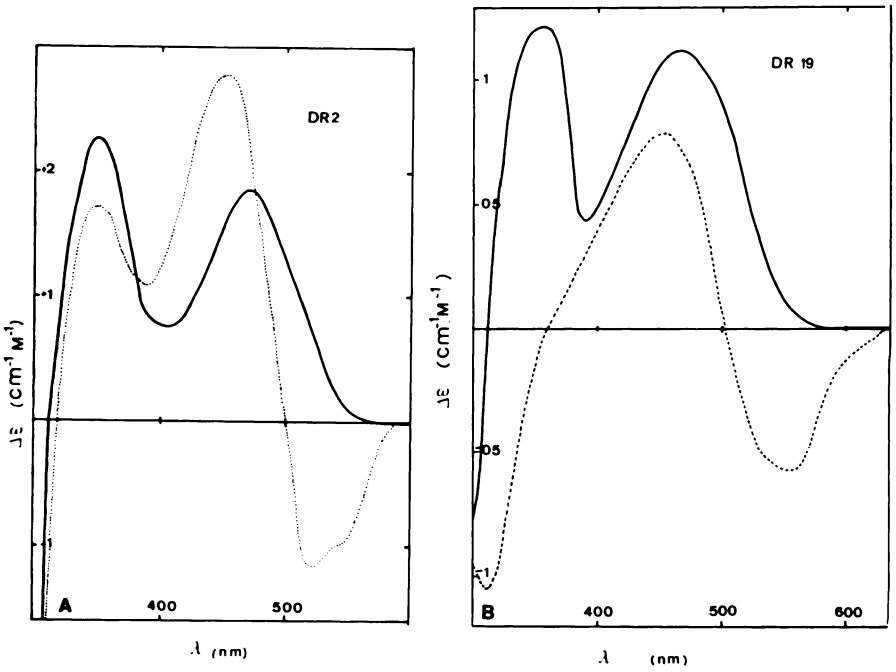


Fig. 1. CD spectra in HEPES buffer of DR-2 (A), 10^{-3} M (· · ·) and 5.65×10^{-6} M (—) and DR-19 (B), 5×10^{-4} M (· · ·) and 2×10^{-6} M (—). $\Delta\epsilon$ is the differential molar dichroic absorption coefficient.

at 518 nm resulting from the association of the anthracycline with CF and plotted it as a function of the anthracycline concentration. The contribution of the free compound, when it was significant, was subtracted (Fig. 3).

From these results, the stoichiometry of the complexes and the association constants were calculated as indicated in a previous paper (7). The values are given in Table 3. Comparable values were obtained for DR-2, DR-9, and DR-10 but no interaction was detected between CF and DR-19, which suggests a particular behavior for this last compound.

DR, DR-10, and DR-19 Interactions with SUVs. Direct method. Increasing amounts of vesicles were added to a DR solution. The CD spectra of a 2×10^{-4} M DR solution in the presence of various concentrations of vesicles consisting either of EPC/EPA/cholesterol or of EPC/cholesterol were recorded. We did not observe drastic modification of the CD profile following the addition of vesicles but only a slight reduction of the dichroic doublet located around 500 nm. The variation of $\Delta\epsilon$ at 520 nm, which corresponds to the maximum of intensity of the negative band of the doublet, is given in Fig. 4A. The similarity of the curves obtained for EPC/cholesterol vesicles and EPA/EPC/cholesterol vesicles was striking and will be discussed later on.

The same experiment was performed with DR-10 and DR-19. For DR-10, we obtained results very similar to those obtained with DR (Fig. 4B). For DR-19 rather different modifications occurred: the addition of EPC/cholesterol vesicles resulted in the appearance of a dichroic band at 356 nm. Fig. 5 reports the intensity of this band as a function of vesicle concentration: a plateau was reached for a lipid concentration around 5 mM, that is, in experimental conditions where the EPC/DR-19 ratio is 20. No significant variation of the intensity of the dichroic doublet located around 500 nm was observed. In contrast, the presence of EPA/EPC/cholesterol vesicles led to the appearance of a dichroic band located at 356 nm concomitant with the disappearance of the dichroic doublet located around 500 nm. These two variations reached a plateau for a lipid concentration around 2 mM, that is, in the conditions of the experiment, for an EPA/DR 19 ratio of 1.6.

With the two types of vesicles, the variation of the CD profiles was dependent on the incubation time; the kinetics are shown in Fig. 6 for a vesicle suspension of 1 mM. For other derivatives, the characteristic spectra were observed immediately after the addition of vesicles and showed no further evolution with time, which means that the interactions were established within the first 10 sec after mixing.

DR-2, DR-9, and DR-10 Interactions with SUVs. Indirect method. The complexation of DR-2, DR-9, or DR-10 with CF was studied in the presence of EPA/EPC/cholesterol vesicles. The variations of CD signals of the complexes (518 nm) of each derivative are presented in Fig. 7.

Discussion

Self-association of the anthracycline derivatives. The CD spectrum of the anthracycline derivatives that we have studied is largely dependent on the association state of the drug, as has been previously reported for ADR and DR (13–15). At pH 7.4 and 10^{-6} M, the anthracyclines were fully in the monomeric form and the CD spectrum consisted of two positive bands, one around 460 nm ($1L_b \leftarrow 1A$) and the other at 320 nm ($n \rightarrow \prod^*$). When the concentration was increased, the first band split into one positive band and one negative band. This doublet signal is an indication of excitonic coupling and self-association of the molecules.

The constants of self-association that we obtained with the model of the dimeric forms of the molecules were 1.6×10^4 for DR, 4.1×10^4 for DR-10, and 3×10^8 for DR-19 in 0.1 M KCl. The constant for DR self-association is in agreement with that found by Martin (15) and is close to what we found under the same conditions for ADR (1.1×10^4) (6). It appears that the adjunction of glycosyl groups in the amino sugar moiety of the anthracycline molecule promotes the self-association.

Binding of the anthracycline derivatives to phospholipid vesicles. CD took advantage of the self-association and

TABLE 2

Differential molar dichroïc absorption coefficients [$\Delta\epsilon(cm^{-1} \times M^{-1})$] of dimeric (d) and monomeric (m) forms of DR and DR derivatives. The positions of the bands are given with a precision of ±3 nm and the values of $\Delta\epsilon$ with a precision of ±10%. The absence of value denotes the absence of band.

λ_{max} (nm)	$\Delta\epsilon$	λ_{max} (nm)	$\Delta\epsilon$
DR			
d (5,10^{-4} M)		*m (5,10^{-6} M)*	
300	−3,75	293	−2,34
354	1,8	354	2
456	1,75	470	1,15
524	−0,95		
550	−0,9		
DR-2			
d (0,98 10^{-3} M)		*m (5,65 10^{-6} M)*	
296	−6,3	296	−3,63
348	1,73	348	+2,3
456	2,81	480	+1,91
522	−1,16		
550*	−0,96		
DR-9			
d (1,46 10^{-3} M)		*m (5,61 10^{-6} M)*	
296	−4,34	293	−4,91
358	1,64	348	2,53
455	2,77	480	1,89
524	−1,57		
550	−1,27		
DR-10			
d (1,27 10^{-3} M)		*m (5,87 10^{-6} M)*	
299	−3,58	293	−4,42
356	1,50	356	2,49
454	2,51	472	1,84
524	−1,42		
550	−1,12		
DR-19			
d 5 10^{-4} M		*m (2, 10^{-6} M)*	
310	−1,1	298	−0.75
356	1,2		
455	0,8	470	1,15
560	−0,55		

allowed us to work, using this technique, at high anthracycline to lipid ratios. We were able to show the existence of two classes of binding sites depending on this ratio (7) and the embedding of the anthracyclic part of the molecules. Fluorescence measurements can also be used to determine binding affinities and embedding of the molecules in the lipid bilayer (4), but the anthracycline to lipid ratio has to stay lower than 2×10^{-4} for anisotropy measurements and changes in the intensity and shape of the fluorescence spectra that occur upon anthracycline binding are very weak.

Anthracycline binding to neutral lipid vesicles. The characteristics of DR and DR-10 binding were similar to those we already observed with ADR (7); that is, a smooth simultaneous decrease of the amplitude of the bands at 528 and 460 nm in the presence of increasing concentrations of vesicles, corresponding to what we have called "type III" binding and which we have interpreted as resulting from a weak insertion of the planar anthracenic part of the anthracycline into the lipidic leaflet. The plots of $\Delta\epsilon$ as a function of lipid concentration drawn with ADR and DR were superimposable. It may be noted that this similarity in binding strength was not observed by Burke and Tritton (4) with DMPC vesicles at 25°, although DMPC is also a neutral phospholipid in the same physical state (liquid crystalline state) as EPC at 25°. Nevertheless, it is a determining factor that could explain the discrepancy of binding to EPC and DMPC in the degree of saturation of their fatty acyl chain. They obtained an apparent association constant of 410 M^{-1} with ADR and one of 2100 M^{-1} for DR.

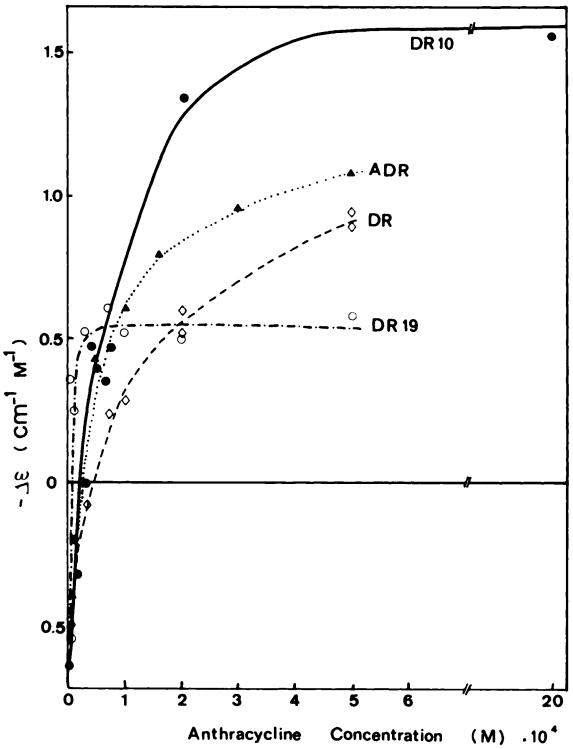


Fig. 2. Intensity of the negative band of the aggregated form of DR 10 (●), ADR (△), DR (◇), and DR-19 (○) as a function of the anthracycline concentration. The intensity has been measured at the maximum of $|-\Delta\epsilon|$, that is, $\lambda = 524$ nm for DR-10 and DR, $\lambda = 520$ nm for ADR, and $\lambda = 560$ nm for DR-19.

Surprisingly, the characteristics of DR-19 were totally different: the addition of vesicles did not affect the intensity of the dichroic doublet at 528–460 nm which testifies to the self-association of the drug. However, some interaction must have occurred since the CD dramatically changed around 350 nm. It would seem, therefore, that DR-19 does bind to neutral lipid vesicles but that the strength of self-association is such that the drug remains dimerized. Furthermore, the reaction is slower than that observed with the other derivatives that we examined.

Anthracycline binding to negatively charged vesicles. The data reported above indicate that the binding of DR-19 to negatively charged vesicles is of a different nature from that to neutral vesicles. The stoichiometry is different: 1 anthracycline molecule for 6.4 EPC molecules (or 1.6 EPA molecules) in the former case, and 1 anthracycline molecule for 20 EPC molecules in the second case. Furthermore, the disappearance of the dichroic doublet seems to indicate that DR-19 binds to negatively charged vesicles in a monomeric form, whereas it remains in a dimeric form in the presence of neutral molecules. The absence of a CF-DR-19 interaction prevented us from using the "indirect" method to measure the extent of DR-19 embedding in the membrane.

Surprisingly, the binding characteristics of DR and DR-10 to negatively charged vesicles were the same as those observed in the absence of charges. This means that the interaction of these drugs with negatively charged vesicles is significantly weaker than it is in the case of ADR. Indeed, ADR and DR bind similarly to neutral vesicles, but ADR binds more strongly

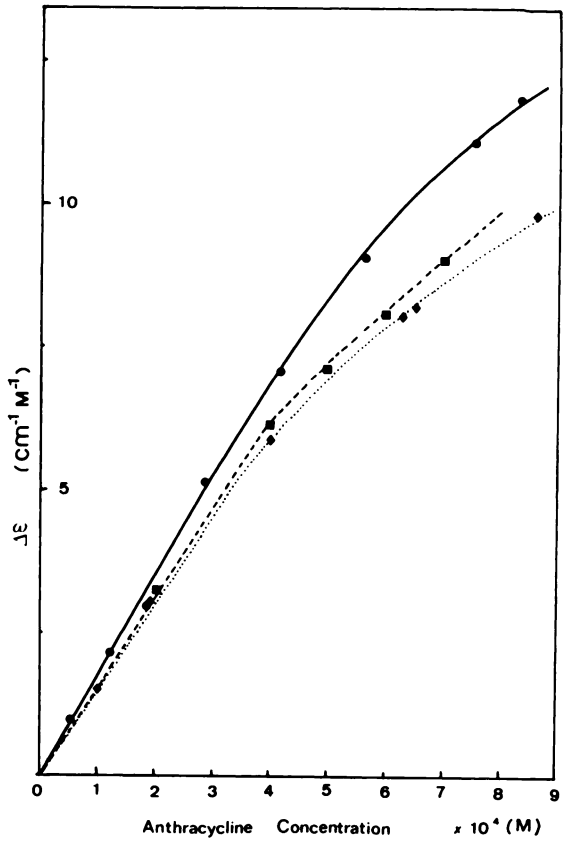


Fig. 3. Intensity of the dichroic signal at 518 nm of the complex anthracycline CF as a function of the anthracycline concentration. CF concentration was 4 × 10^{-4} M. The results are given for DR-9 (●), DR-2, (□), and DR-10 (◇).

TABLE 3
Association constants of ADR and DR derivatives with CF

Anthracycline	K_a
ADR	(2.0 ± 0.5) 10^3
DR-2	(1.2 ± 0.5) 10^3
DR-9	(1.7 ± 0.5) 10^3
DR-10	(1.1 ± 0.5) 10^3

to EPA-containing vesicles. This is in contrast to the results of Goldman *et al.* (18), whose fluorescence studies indicated that DR bound to dicetylphosphate-containing EPC vesicles more strongly than that of ADR.

We observed a decrease of the CD signal of the CF complex with DR-2, DR-9, and DR-10 in the presence of vesicles. As already discussed (7), this indicates the embedding of the drug into the lipid bilayer.

It appears, therefore, that the role of negative charges, in the strong interaction of ADR with cardiolipin-containing membranes and the consequent cardiotoxicity, is not straightforward, even though it has been considered as determinant. Indeed, while the interaction of ADR with membranes is strongly reinforced by the presence of negative charges, nothing similar is observed with DR. At the pH used in this study, however, both drugs bear a positive charge on the protonated amino group of the amino sugar, and the only difference between them is that the hydrogen atom of the *R*1 group of DR is replaced by an OH group in ADR (see Table 1). Furthermore, DR-19 strongly interacts with negatively charged vesicles,

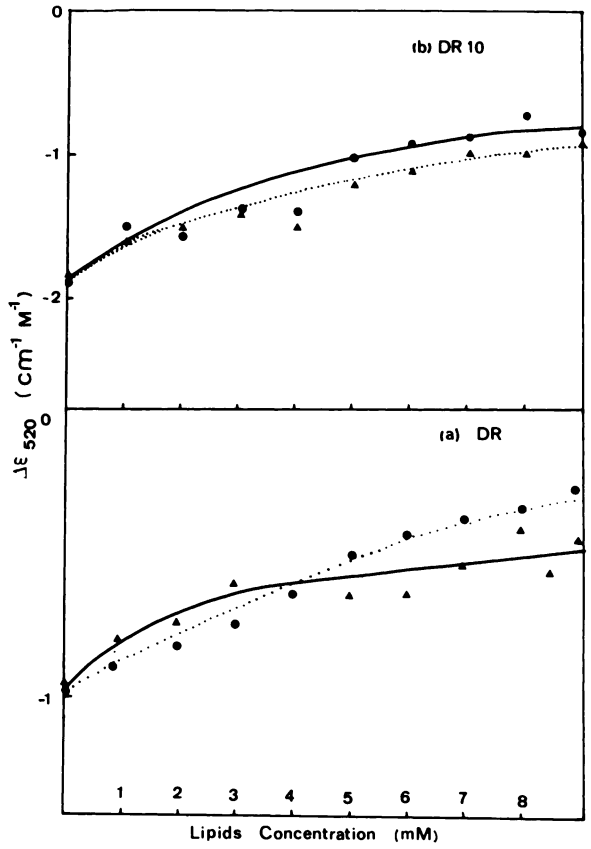


Fig. 4. Intensity of the negative band of the dichroic doublet of DR (a) and DR-10 (b) in the presence of various concentrations of EPC/EPA/cholesterol (△)- and EPC/cholesterol (●)-containing vesicles. Lipid molar ratios were 6.4:1.6:2 and 8:2, respectively. Anthracycline concentration was 2 × 10^{-4} M.

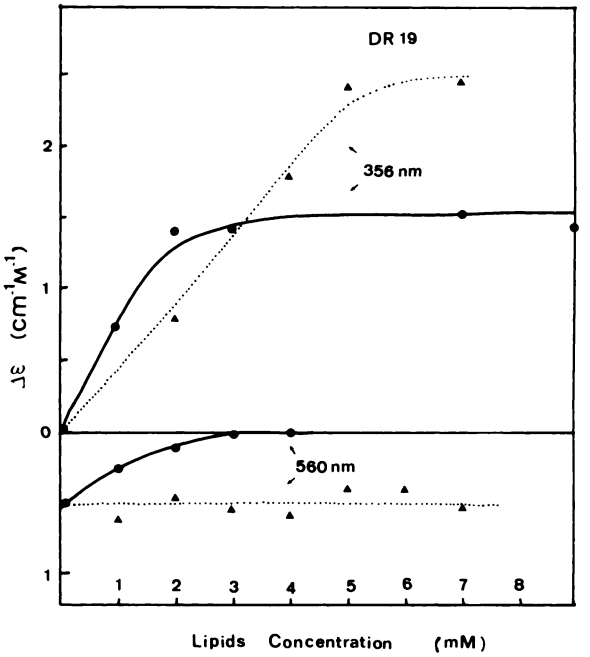


Fig. 5. CD of a 2 × 10^{-4} M DR-19 solution in the presence of increasing concentrations of EPC/EPA/cholesterol (●) and EPC/cholesterol (△). The same lipid molar ratios as in Fig. 4 were used. The dichroic intensity was measured at 560 nm and 356 nm, 60 min after the addition of the vesicles.

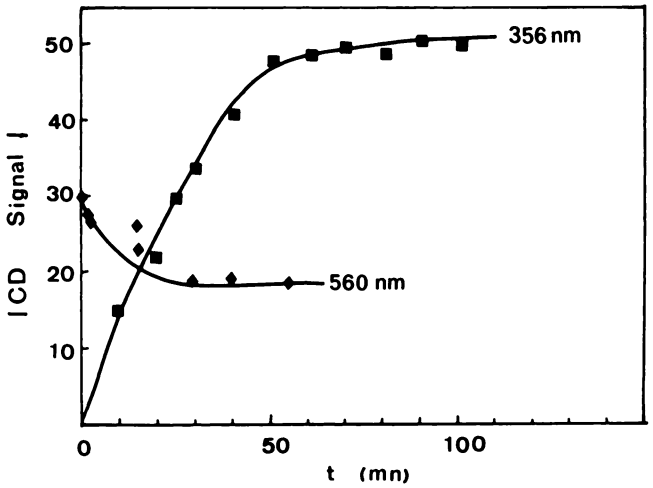


Fig. 6. Variation of the CD signals at 356 nm and 560 nm of a 2×10^{-4} M DR-19 solution as a function of the time elapsed after addition of EPC/EPA/cholesterol-containing vesicles. The final lipid concentration was equal to 1 mM.

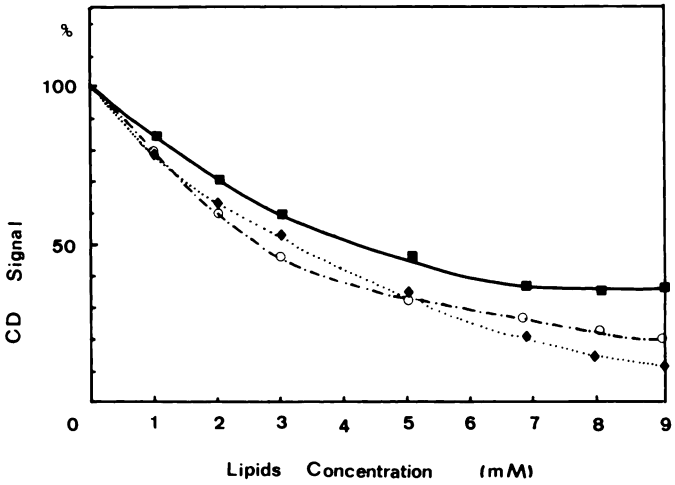


Fig. 7. Variation at 518 nm of the CD signal of the anthracycline-CF complex in the presence of increasing amounts of EPC/EPA/cholesterol-containing vesicles (molar ratio 6.4:1.6:2). ♦, DR-2; □, DR-9; ○, DR-10.

whereas it has the same substituent R_1 as DR but bears no charge at the amino group of the sugar; indeed, it is expected that at physiological pH, DR-19 bears no electric charge due to the poor susceptibility to protonation of the nitrogen atom. It has been demonstrated (19, 20) that enamines possessing a primary or secondary nitrogen atom are 2 or 3 orders of magnitude weaker bases than the corresponding saturated amines. The origin of the increased affinity of some anthracyclines for membranes containing negative charges must therefore lie in a molecular property other than the presence of electrostatic charge. We are currently investigating the role of strong hydrogen bonds between the anthracycline hydroxyl group and the phosphate group of phosphatidic acid or cardiolipin.

No significant relationship was discerned in the present study between membrane binding and cardiotoxicity. Although both ADR and DR are cardiotoxic (21), our results indicate that ADR binds weakly to neutral lipids and strongly to negatively charged lipids, whereas DR binds similarly to neutral and charged lipids. Morphological and ultrastructural studies on cardiac muscle in rats have led to the conclusion that DR-10 is much more cardiotoxic than DR-19 (6). Our results indicate that DR-19 binds strongly to negatively charged lipids, whereas DR-10 binds to neutral and charged lipids.

Acknowledgments

We are grateful for the excellent technical assistance of Liliane Leroy.

References

1. Goormaghtigh, E., and J. M. Ruysschaert. Anthracycline glycoside-membrane interactions. *Biochim. Biophys. Acta* **779**:271–288 (1984).
2. Goormaghtigh, E., P. Chatelain, J. Caspers, and J. M. Ruysschaert. Evidence of a specific complex between adriamycin and negatively-charged phospholipids. *Biochim. Biophys. Acta* **597**:1–14 (1980).
3. Burke, T. G., and T. R. Tritton. Ligand self-association at the surface of liposomes: a complication during equilibrium-binding studies. *Anal. Biochem.* **143**:135–140 (1984).
4. Burke, T. G., and T. R. Tritton. Structural basis of anthracycline selectivity for unilamellar phosphatidylcholine vesicles: an equilibrium binding study. *Biochemistry* **24**:1768–1776 (1985).
5. Griffin, E., J. Vanderkooi, G. Maniara, and M. Erecinska. Anthracycline binding to synthetic and natural membranes. A study using resonance energy transfer. *Biochemistry* **25**:7875–7880 (1986).
6. Chibowski, D., Z. Siezieniewska, Z. Kleinrok, and B. Chmielewska. Morfologiczne i ultrastrukturalne wykladniki kardiotoksycznosci wczesnej u szczurow po stosowaniu rubidomycyny i jej pochodnych. *Patol. Pol.* **35**:453–479 (1984).
7. Henry, N., E. O. Fantine, J. Bolard, and A. Garnier-Suillerot. Interaction of adriamycin with negatively charged model membranes: evidence of two types of binding sites. *Biochemistry* **24**:7085–7091 (1985).
8. Stefanska, B., E. Borowski, L. Falkowski, and S. Martelli. Synthesis and antileukemic activity of some novel *N*-substituted derivatives of daunorubicin. *Pol. J. Chem.*, in press.
9. Stefanska, B., P. Kolodziejczyk, L. Falkowski, and E. Borowski. Synthesis and antileukemic activity of some novel *N*-substituted derivatives of daunorubicin and adriamycin, in *Proceedings of the 14th IUPAC International Symposium on the Chemistry of Natural Products, Poznan, Poland, Abstracts Book* Vol 1, p. 387 (1984).
10. Stefanska, B., Z. Mazerska, L. Falkowski, and E. Borowski. The synthesis and antitumor activity of *N*-glucosyl derivatives of daunorubicin. *J. Antibiot.* **37**:1213–1216 (1984).
11. Ralston, E., L. M. Hjemeland, R. D. Klausner, J. N. Weinstein, and R. Blumenthal. Carboxyfluorescein as a probe for liposome-cell interactions. Effect of impurities and purification of the dye. *Biochim. Biophys. Acta* **649**:133–137 (1981).
12. Newman, C. G., and C. Huang. Structural studies on phosphatidylcholine-cholesterol mixed vesicles. *Biochemistry* **14**:3363–3368 (1975).
13. Schütz, F., A. Gollmick, and E. Stutter. Anthracycline self-association *Stud. Biophys.* **75**:147–150 (1979).
14. Barthalamey-Clavey, V., J. C. Maurizot, J. L. Dimicoli, and P. Sicard. Self-association of daunorubicin. *FEBS Lett.* **46**:5–10 (1974).
15. Martin, S. R. Absorption and circular dichroic spectral studies on self-association of daunorubicin. *Biopolymers* **19**:713–721 (1980).
16. Beraldo, H., A. Garnier-Suillerot, and L. Tosi. Cu(II)-adriamycin complexes. A circular dichroism and resonance Raman study. *Inorg. Chem.* **22**:4117–4124 (1983).
17. Chaires, J. B., N. Dattagupta, and D. M. Crothers. Studies on interaction of anthracycline antibiotics and desoxyribonucleic acid: equilibrium binding studies on interaction of daunomycin with desoxyribonucleic acid. *Biochemistry* **21**:3933–3940 (1982).
18. Goldman, R., T. Pacchinetti, D. Bach, A. Raz, and M. Shinitzky. A differential interaction of daunomycin, adriamycin and their derivatives with human erythrocytes and phospholipid bilayers. *Biochim. Biophys. Acta* **512**:254–269 (1978).
19. Starr, D. F., H. Bulbrook, and R. M. Hixon. Electron sharing ability of organic radicals. IV. Alpha substituted pyrolines and pyrolidines. *J. Am. Chem. Soc.* 3971–3976 (1932).
20. Stamhuis, E. J., W. Maas, and H. Wynberg. Mechanism of enamine reactions. III. The basicity of tertiary enamines. *J. Org. Chem.* **30**:2160–2162 (1965).
21. Ferrans, V. J. Overview of cardiac pathology in relation to anthracycline cardiotoxicity. *Cancer Treat. Rep.* **62**:955–961 (1978).

Send reprint requests to: J. Bolard, Laboratoire de Physique et Chimie Biomoléculaire (UA CNRS-198), Université Pierre et Marie Curie, Tour 22, 4 place Jussieu, 75252 Paris Cedex 05, France.