Biochimica et Biophysica Acta, 512 (1978) 254—269

BBA 78135

A DIFFERENTIAL INTERACTION OF DAUNOMYCIN, ADRIAMYCIN AND THEIR DERIVATIVES WITH HUMAN ERYTHROCYTES AND PHOSPHOLIPID BILAYERS

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(Received February 22nd, 1978)

Summary

Drug-membrane association of daunomycin, adriamycin and three of its derivatives, adriamycin-14-octanoate (AD-14-OCTA), adriamycin-14-acetate (AD-14-ACE) and *N*-trifluoroacetyladriamycin-14-valerate (AD32), was studied using phospholipid bilayers and human erythrocytes. The various drugs exhibited a differential affinity to membrane-lipid domains.

Lipid-incorporated drugs exhibit a marked change in the shape of the emission spectrum which was utilized for the evaluation of the apparent dielectric constant, ϵ, of the environment surrounding the anthracycline moiety, as well as for the determination of the partitioning constant. By measuring the fluorescence polarization and the fluorescence lifetime of the incorporated drugs, rotational relaxation times of 4—8 ns were derived. These parameters provide a supportive evidence for the association of the fluorophore of the drugs with membrane-lipid domains.

The anthracycline derivatives interact to a different degree with dipalmitoyl phosphatidylcholine and phosphatidylserine as reflected by changes in their thermotropic properties assessed by differential scanning calorimetry. Daunomycin was the most effective in decreasing the temperature of the phase transition and brought about a comparable reduction in the enthalpy of melting as AD32 and AD-14-OCTA. Adriamycin was the least potent of the series.

AD-14-ACE and AD32 protected erythrocytes against hypotonic lysis, adriamycin and daunomycin had no significant effect on the susceptibility to hypotonic lysis, whereas AD-14-OCTA proved to be hemolytic even at low concentration (approx. 10^{-7} M).

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Abbreviations: AD-14-OCTA, adriamycin-14-octanoate; AD-14-ACE, adriamycin-14-acetate; AD32, *N*-trifluoroacetyladriamycin-14-valerate.

The interaction of erythrocytes with daunomycin, AD-14-ACE and AD-14-OCTA resulted in a shape change from biconcave discs to cups. Adriamycin and AD32 did not affect erythrocyte shape.

The differential drug-membrane interactions may be an important determinant in the antitumor differential efficiency of the drugs, especially in view of the fact that derivatives that do not intercalate into the DNA (AD32) are at least as potent as those that do.

Introduction

The antibiotics, daunomycin and adriamycin, are anthracycline aminoglycosides, isolated from cultures of *Streptomyces peucetius* possessing antitumor activity against certain animal and human neoplasms [1,2]. These two anthracyclines show a high affinity towards DNA, which is best accounted for by assuming an intercalative type of binding; non-polar insertion of the ligand between the stacked bases and charge interaction in the external anionic regions of the DNA helix [3]. Except for binding to the genome [4] the drugs have also been shown to perturb cellular RNA and DNA synthesis [4—6].

A mechanism for the antimitotic activity of these anthracyclines, based exclusively on the interaction with DNA and the inhibition of nucleic acid synthesis, has recently been questioned. New derivatives of the anthracycline aminoglycosides have been developed, with the hope of increasing the therapeutic value and overcoming major side effects, such as severe cumulative dose-dependent cardiotoxicity [7]. *N*-Acetyldaunomycin and *N*-trifluoroacetyladriamycin-14-valerate (AD32) have a very low affinity towards DNA and exert a weak effect on nucleic acid synthesis, yet they are capable of inhibiting cell mitosis [8]. Furthermore, AD32 exerts a therapeutic activity superior to that of adriamycin in a number of experimental rodent tumor systems [9].

A number of observations have implicated a direct interaction of anthracycline drugs with cell membranes [10] and the cytoskeleton [11]. Adriamycin has also been shown to partition into a lipophilic phase (chloroform/methanol/water; 86 : 14 : 1, v/v) of a Folch biphasic system, a partitioning much enhanced by the addition of acidic phospholipids [12].

In the present study we explore the interaction of daunomycin, adriamycin and three of their derivatives with phospholipid bilayers, human erythrocytes and erythrocyte membranes. Using the characteristic fluorescence spectra of the drugs in the membranes as a measure of the local dielectric constant and the fluorescence polarization as an indicator of the microviscosity prevailing in the milieu where the anthracycline fluorophore is located, it was possible to assess the different positions of the drugs within the phospholipid bilayer and to estimate their partitioning. The effect of the anthracycline drugs on the thermotropic behaviour of phospholipid bilayers and on the shape and osmotic fragility of human erythrocytes further suggested a differential interaction of the drugs with the phospholipid component of biological membranes.

Materials and Methods

Daunomycin, adriamycin, *N*-trifluoroacetyladriamycin-14-valerate (AD32), adriamycin-14-octanoate (AD-14-OCTA) and adriamycin-14-acetate (AD-14-

ACE) were kindly supplied by Farmitalia, Milano, Italy.

The partitioning of the various drugs to octanol was measured as follows. Drugs were dissolved or suspended (AD32, AD-14-OCTA, were dissolved in methanol and the solvent was evaporated from the tube) in 2 ml of Ca^{2+} + Mg^{2+}-free Dulbecco's phosphate-buffered saline, pH 7.2 (Gibco). Octanol (2 ml) was added and the tubes were vortexed vigorously for 1 min. After centrifugation (5 min, 200 × *g*) the aqueous layer was removed and its absorbance at 495 nm was determined. A range of drug concentrations (50—200 μg/ml, four concentrations) was tested in triplicate, and the partitioning was constant throughout the range.

Liposomes of egg phosphatidylcholine (Lipid Products, grade I, South Nutfield, England) and dicetylphosphate (Sigma Chem, Corp., St. Louis, Mo.) (9 : 1, w/w) were prepared by sonicating the lipid dispersion in Ca^{2+} + Mg^{2+}-free Dulbecco's phosphate-buffered saline at 100 W for 30 min at 4°C under argon atmosphere. These conditions lead to over 90% single-walled liposomes [13].

Erythrocyte membranes were prepared from human blood by the method of Dodge et al. [14].

Fluorescence spectra (uncorrected) were recorded in a Hitachi-Perkin-Elmer model MPF III. The degree of fluorescence polarization was determined as described [15] with an instrument constructed in our laboratory. Excited state lifetime, τ, was measured at 24°C by a sampling method and an instrument which was described elsewhere [16]. The fluorescence decay curves were evaluated by a least-square convolution method and in all cases were found to be close to pure single exponential decays.

The effect of adriamycin derivatives on the osmotic properties of heparinized human (adult male) venous blood was assessed essentially as described [17]. In brief, 20 μl of a 50% stock cell suspension was rapidly mixed with 1 ml of 2 mM sodium phosphate solution, pH 7.2, containing NaCl of the specifies concentration. The tested drugs were dissolved in water (daunomycin, adriamycin, and AD-14-ACE) or in methanol (AD32 and AD-14-OCTA) (final concentration of methanol, 0.5%) and mixed with the buffered NaCl solutions just prior to the addition of the erythrocytes. Controls of 0.5% methanol did not affect the osmotic fragility of the erythrocytes. The erythrocytes were incubated with the drugs for 10 min at room temperature (20—21°C) and subsequently centrifuged at 2000 × *g* for 5 min. Percentage hemolysis was determined by measuring the absorbance of hemoglobin in the supernatant at 540 nm. Each experiment was carried out in triplicates. A correction for drug absorbance at 540 nm was carried out at each point.

Scanning electron microscopy was carried out on erythrocyte suspensions that have been interacted with the specified drugs (25 μg/ml) for 10 min at 21°C, and fixed by mixing 1 : 1 with a 5% formaldehyde solution (in Dulbecco's phosphate-buffered saline, pH 7.2, 60 min, 21°C). After drying with a critical point drying apparatus (Polaron Equip. Ltd., Watford, Herts) with liquid CO_2, the dried specimens were coated with gold, using a rotating stage in a vacuum evaporator. A stereoscan Cambridge S-180 scanning electron microscope was used at an accelerating voltage of 30 kV and at a tilt angle of 30°.

For differential scanning calorimetry the drugs were dissolved in distilled

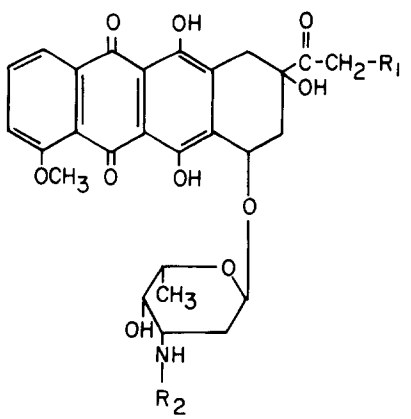
methanol (aprox. 5 mg/ml). Dipalmitoyl phosphatidylcholine, puriss grade, was purchased from Fluka-Buchs (Switzerland) and phosphatidylserine from bovine spinal cord, grade I, monosodium salt (in chloroform/methanol) was obtained from Lipid Products (South Nutfield, England).

Solutions of phospholipids dissolved in chloroform/methanol (2 : 1, v/v) were mixed with drugs dissolved in methanol and left for 60 min at room temperature. Subsequently, the solvents were evaporated by a stream of N_2 and high vacuum (3 h). The material was then transferred to aluminum pans, and a solution of 0.15 M NaCl in 10^{-3} M Tris · HCl buffer, pH 7.2, was added, giving a weight ratio of about 1 : 1 water to lipid. The pans were sealed and left overnight. The calorimetric measurements were performed on a DuPont 990 differential scanning calorimeter, equipped with cell base II. The calibrated mode was used, heating rate of 5°C/min.

Results

The octanol/buffer partition coefficients correlate with membrane/buffer partition coefficients for a wide range of anaesthetic compounds, both non-charged and positively charged (review ref. 18). The partitioning into octanol may thus serve as a first indication for possible membrane-drug interactions. The five anthracycline drugs used in this study differ to a variable degree in their chemical structure (Fig. 1) and accordingly, in their octanol/buffer partition coefficients, i.e. adriamycin, 1.1; daunomycin, 3.5; AD-14-ACE, 3.1; AD-14-OCTA, >99.9; AD32, >99.9.

The fluorescence spectra of the anthracycline drugs were of identical shape



	R_1	R_2
AM	OH	H
DM	H	H
AD32	$OCOC_4H_9$	$COCF_3$
AD-14 OCTA	$OCOC_7H_{15}$	H
AD-14 ACE	$OCOCH_3$	H

Fig. 1. Chemical structure of the anthracycline drugs used in this study. AM, adriamycin; DM, daunomycin.

when recorded in the same solvent. This is expected since the chemical variations in this series do not apply to the fluorophore moiety. The shape of the fluorescence spectrum of each of these drugs was found to change markedly with the hydrophobicity of the solvent. Fig. 2 presents fluorescence spectra of daunomycin in methanol/water mixtures. As can be clearly seen, the increase in methanol content is concomitant with an increase of emission quantum yield and an increase in the ratio of the emission peak intensities around 556 and 579 nm (peaks I and II, respectively). Similar changes were observed with all the other drugs, both in methanol/water and in ethanol/water mixtures. The dependence of the peak intensity ratio, I/II, on the dielectric constant of the mixture is shown in Fig. 3. The apparent linear relation, which is displayed in the figure, could serve as a convenient means for identifying the region of intercalation of the drug in membranes.

As a membrane model system for studying the interaction with the anthracycline drugs, we have used single-walled liposomes made of egg phosphatidylcholine and dicetylphosphate (9 : 1, w/w). The intercalation of these drugs into the lipid layer could be easily followed by changes in fluorescence spectrum, presented by I/II, upon increase in phospholipid concentration (see Fig. 4). Titrations of this sort are shown in Fig. 5 from which the dissociation constant could, in principle, be evaluated. However, the obtained titration curves are of a complex cooperativity nature which is presumably due to local changes in surface potential produced by the bound drug molecules. Changes in charge distribution upon binding of hydrophobic but charged molecules have been shown to result in a cooperative mode of binding [19] which could, at least partially, account for the binding profiles shown in Fig. 5. Since we were only interested in the average binding capacity of each of the drugs, we have

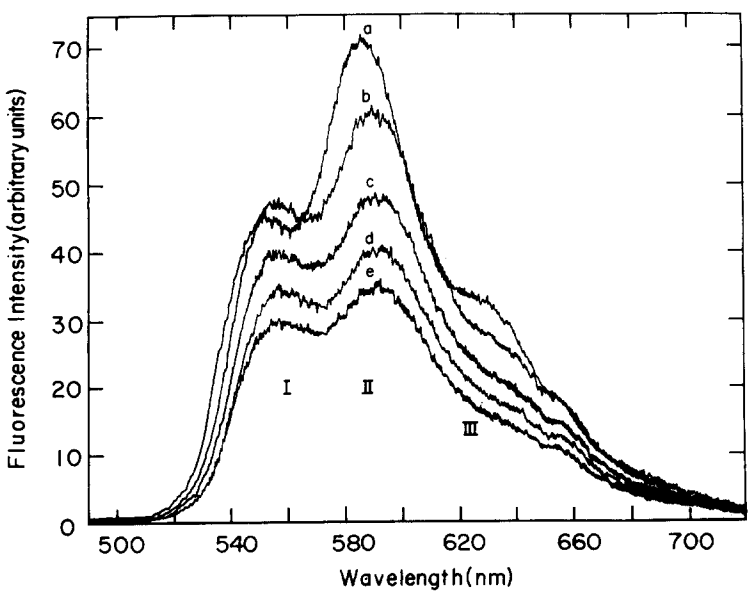


Fig. 2. Uncorrected fluorescence spectra of daunomycin ($5 \cdot 10^{-6}$ M) in different methanol/water mixtures. Methanol fraction: a, 100%; b, 70%; c, 40%; d, 20%; e, 0%. Excitation wavelength: 468 nm.

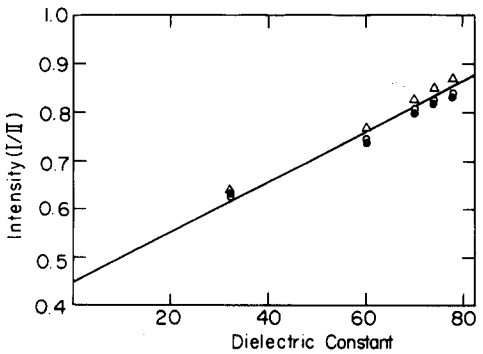


Fig. 3. The dependence of the ratio of fluorescence peak intensities (I/II) of daunomycin (△———△), adriamycin (●———●) and AD-14-ACE (○———○), on the dielectric constant of the solvent (methanol/water mixtures).

adopted a simplified procedure for the evaluation of the partitioning constant. This procedure, which is outlined below, ignores the cooperativity and essentially yields average values only. A partitioning constant, K_{part}, was defined by the non-cooperative expression:

$$K_{part} = \frac{(1-\alpha)\cdot L}{\alpha} \tag{1}$$

where α is the fraction of drug bound and L is the lipid concentration in mg/ml. Formally, K_{part} represents the lipid concentration at which 50% of the drug binding occurs. The factor α can be derived from the titration curve (Fig. 5),

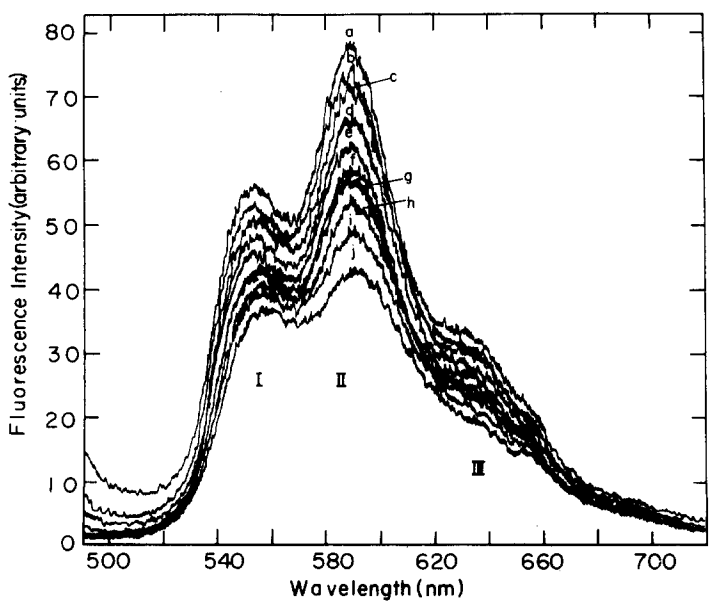


Fig. 4. Uncorrected fluorescence spectra of daunomycin ($5 \cdot 10^{-6}$ M) in liposome dispersions of different concentration in phosphate-buffered saline. Concentration of phospholipid in mg/ml: a, 5; b, 3; c, 2; d, 1; e, 0.5; f, 0.35; g, 0.25; h, 0.15; i, 0.05; j, 0. Excitation wavelength: 468 nm.

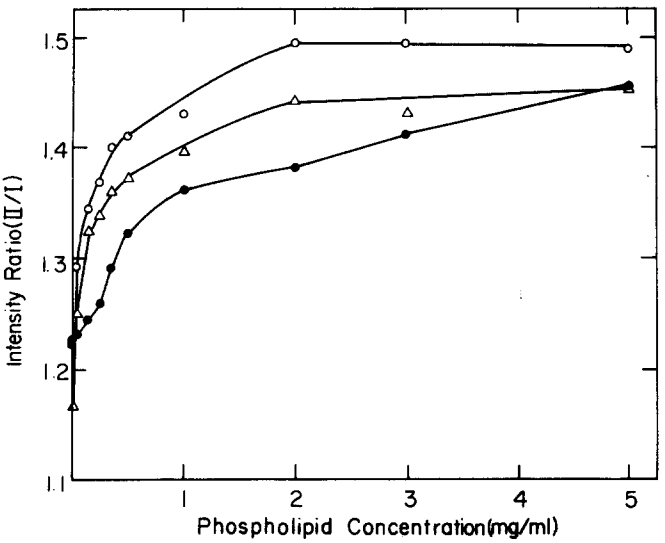


Fig. 5. Fluorescence titrations of drug binding to liposomes. △———△, daunomycin; ●———●, adriamycin; and ○———○, AD-14-ACE. Fluorescence intensities derived from spectra as the ones shown in Fig. 3.

using the expression:

$$\alpha = \frac{(I/II) - (I/II)_{min}}{(I/II)_{max} - (I/II)_{min}} \tag{2}$$

and the data in Fig. 5 can be replotted as $1/\alpha$ versus $1/L$. K_{part} is then obtained from the slope of the straight lines according to:

$$\frac{1}{\alpha} = 1 + K_{part} \cdot \frac{1}{L} \tag{3}$$

For the evaluation of K_{part} we selected the range of $\alpha = 0.2$—0.8. The apparent K_{part} values are given in Table I.

TABLE I

FLUORESCENCE AND PARTITIONING PROPERTIES OF ANTHRACYCLINE-LIPID INTERACTIONS

System	Anthracycline drugs	Apparent dielectric constant (ϵ)	K_{part} (mg/ml)	Fluorescence anisotropy (r)	Fluorescence lifetime at at 25°C (τ ns)	Rotational relaxation time at 25°C (ρ ns)
Liposomes	Daunomycin	11	0.16	0.222	1.1	4.8
	Adriamycin	20	0.73	0.195	1.1	3.6
	AD-14-ACE	6	0.27	0.237	1.2	6.2
	AD32	4	<0.05	0.249	1.2	7.1
	AD-14-OCTA	7	<0.05	0.241	1.2	6.4
Erythrocyte membranes	AD32	22	<0.05	0.191	1.1	3.5
	AD-14-OCTA	10	<0.05	0.230	1.1	5.2

The obtained values of K_{part} indicate that with the liposome system over 90% drug incorporation occurs at phospholipid concentration of 3 mg/ml. The fluorescence spectra at this concentration were used to identify the dielectric constant which surrounds the anthracycline moiety of the bound drug (see Table I). For all the five drugs the apparent dielectric constant, ϵ, falls in the range of 4—22 which suggests that the intercalation region of the anthracycline moiety is the upper hydrocarbon layer ($\epsilon \approx 4$—8) for AD32, AD-14-ACE and AD-14-OCTA and the hydrocarbon-water interface ($\epsilon \approx 10$—30) [15] for daunomycin and adriamycin.

Incorporation measurements were carried out analogously with erythrocyte membranes. However, only slight incorporation of daunomycin, adriamycin and AD-14-ACE could be detected at membrane concentrations of up to 1 mg/ml lipid. Beyond this concentration light scattering interfered with the fluorescence measurements and the titration curve could not be determined. It is thus apparent that for these drugs $K_{part} > 2$ mg/ml. On the other hand, AD32 and AD-14-OCTA, which exhibit a high partitioning into the liposomes (see Table I), partition very efficiently into the erythrocyte membrane as well.

The fluidity of the lipid region which accomodated the anthracycline group could be assessed by fluorescence depolarization measurements according to the well-known Perrin equation [20]:

$$\frac{r_0}{r} = 1 + \frac{3\tau}{\rho} \tag{4}$$

This equation expresses the relation between the measured and the limiting fluorescence anisotropy values, r and r_0, respectively, the excited-state lifetime, τ, and the rotational relaxation time, ρ. The latter factor is a combination of all hydrodynamic parameters which determine the various rotational modes of the fluorophore, and it is proportional to the effective viscosity and inversely proportional to the absolute temperature. It represents approximately the average time for a rotational displacement of about 60°.

The determined excited-state lifetime, τ, of the various anthracyclines in solution or in the lipid phase was in the range of 1 ns. Therefore, when dissolved in a highly viscous solvent like glycerol the rotations during τ can be considered as frozen, and the determined r value should approach r_0. Indeed, the r_0 value of the various anthracyclines obtained in glycerol solution at 25°C upon excitation at 475 nm was 0.375, which is very close to the upper theoretical limit value of $r = 0.4$. With this r_0 value and the measured r and τ values (see Table I) the rotational relaxation time, ρ, was evaluated. The obtained ρ values, given in Table I, are typical of molecules of the size of the anthracyclines which rotate freely in a medium of about 1 P. Since microviscosity of this range prevails in lipid layers [15] these results strongly indicate that the drug molecules, both in the liposomes and in the membranes, are dissolved in the lipid bilayer. The subtle differences of ρ obtained for the different drugs directly relate to the differences in microviscosity of the different intercalation regions, as is also reflected in the different dielectric constants (see Table I).

Since the data indicate that drug-membrane interactions are by and large limited to the lipid domain, we have studied the effect of the drugs on the thermotropic behaviour of dipalmitoyl phosphatidylcholine and of phos-

phatidylserine. Fig. 6 presents the thermograms and Table II presents the enthalpy of melting and melting temperature (taken at the middle of the transition peak) of the specified phospholipids and adriamycin derivatives. The thermogram of dipalmitoyl phosphatidylcholine (Aa) has the normal shape and

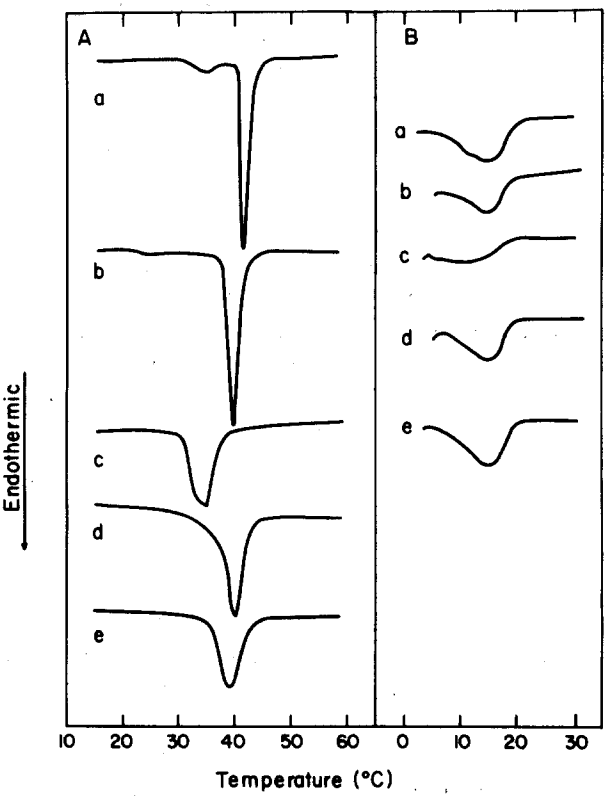


Fig. 6: The effect of adriamycin and its derivatives on the differential scanning calorimetry thermograms of dipalmitoyl phosphatidylcholine and of phosphatidylserine.

(A) Dipalmitoyl phosphatidylcholine	Molar ratio	Amount of lipid in the pan (mg)
a. Dipalmitoyl phosphatidylcholine alone		2.4
b. Dipalmitoyl phosphatidylcholine: adriamycin	6.6 : 1	1.5
c. Dipalmitoyl phosphatidylcholine: daunomycin	7.0 : 1	1.3
d. Dipalmitoyl phosphatidylcholine: AD-14-OCTA	8.9 : 1	1.7
e. Dipalmitoyl phosphatidylcholine: AD32	9.6 : 1	1.4

Sensitivity of a: 0.4 mcal/s inch. Others: 0.2 mcal/s inch. Heating rate in all samples: 5°C/min.

(B) Phosphatidylserine	Molar ratio	Amount of lipid in the pan (mg)
a. Phosphatidylserine alone		1.1
b. Phosphatidylserine: adriamycin	6.9 : 1	1.1
c. Phosphatidylserine: daunomycin	6.6 : 1	1.3
d. Phosphatidylserine: AD32	9.2 : 1	1.7
e. Phosphatidylserine: AD-14-OCTA	8.6 : 1	1.1

Sensitivity: 0.1 mcal/s inch.

TABLE II

THE EFFECT OF ADRIAMYCIN AND ITS DERIVATIVES ON THE THERMOTROPIC PROPERTIES OF DIPALMITOYL PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE

Phospholipid/drug (mol/mol)	Enthalpy of melting (ΔH) (kcal/mol phospholipid)	Temperature of the middle of the peak (T_m) (°C)
Dipalmitoyl phosphatidylcholine (alone)	9.8	42.0
Dipalmitoyl phosphatidylcholine/adriamycin	8.5	40.0 *
Dipalmitoyl phosphatidylcholine/daunomycin	7.4	35.0
Dipalmitoyl phosphatidylcholine/AD32	7.4	39.0
Dipalmitoyl phosphatidylcholine/AD-14-OCTA	7.3	39.5
Phosphatidylserine	4.7	15.0
Phosphatidylserine/adriamycin	3.4	14.5
Phosphatidylserine/daunomycin	1.8 **	12.5
Phosphatidylserine/AD32	2.5	15.0
Phosphatidylserine/AD-14-OCTA	3.2	15.0

* T_m variable from experiment to experiment. A maximum decrease of 7°C has been obtained.
** A minimum value disregarding possible heat absorption around 0°C (see Fig. 6).

melting parameters as described for this compound. Interaction with the four adriamycin derivatives influences the thermotropic behaviour to a different degree. The most significant change in the shape of the peak and in the value of the melting temperature is obtained with daunomycin. The enthalpy of melting decreases due to the interaction with the various derivatives. Interaction with daunomycin, AD32 and AD-14-OCTA resulted in a decrease of about 25%. Adriamycin had a smaller effect on the enthalpy of melting (≈15%). The melting temperature in the presence of this drug showed a variability not encountered with the other compounds, i.e. it fluctuated in six experiments from a decrease of 7°C to no detectable change.

Since anionic phospholipids are a common constituent of biological membranes and three of the compounds tested have a positive charge, it was of interest to study the interaction of the drugs with the negatively charged phosphatidylserine. Except for daunomycin that shifted significantly the melting temperature of phosphatidylserine to a lower value, the other drugs, both the positively charged and the non-charged, did not affect the melting temperature. AD-14-OCTA and adriamycin decreased the enthalpy of melting by about 30% and AD32 by about 47%. Daunomycin had the strongest effect on the enthalpy of melting, as compared to the other drugs, even taking into account that the calculated enthalpy is the lowest limit due to possible shifting of part of the peak into 0°C region (not detectable in the measurement).

The five anthracycline drugs were tested for their capacity to affect the osmotic fragility of fresh human erythrocytes. AD-14-ACE and AD32, at a concentration of 25 μg/ml, brough about a shift in the concentration of salt at which 50% hemolysis occurs, i.e. from 70 mM NaCl (control) to 68 and 66 mM NaCl, respectively. AD-14-OCTA brought about an 85% hemolysis even under iso-osmotic conditions. Adriamycin and daunomycin showed no effect on the osmotic fragility of erythrocytes under comparable conditions.

A more subtle assessment of potential drug-induced stabilization against

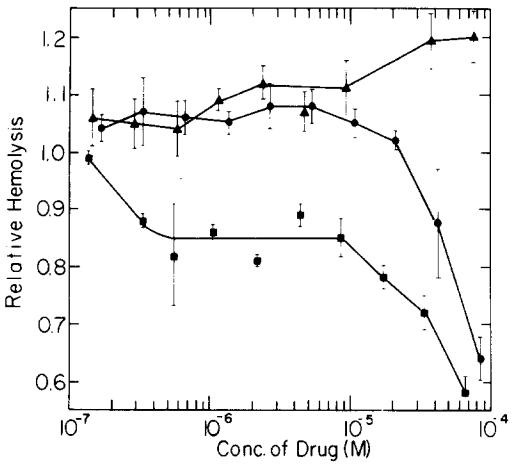


Fig. 7. Relative hemolysis of human erythrocytes as affected by anthracycline drugs. ●——●, AD-14-ACE; ▲——▲, AD-14-OCTA; ■——■, AD32.

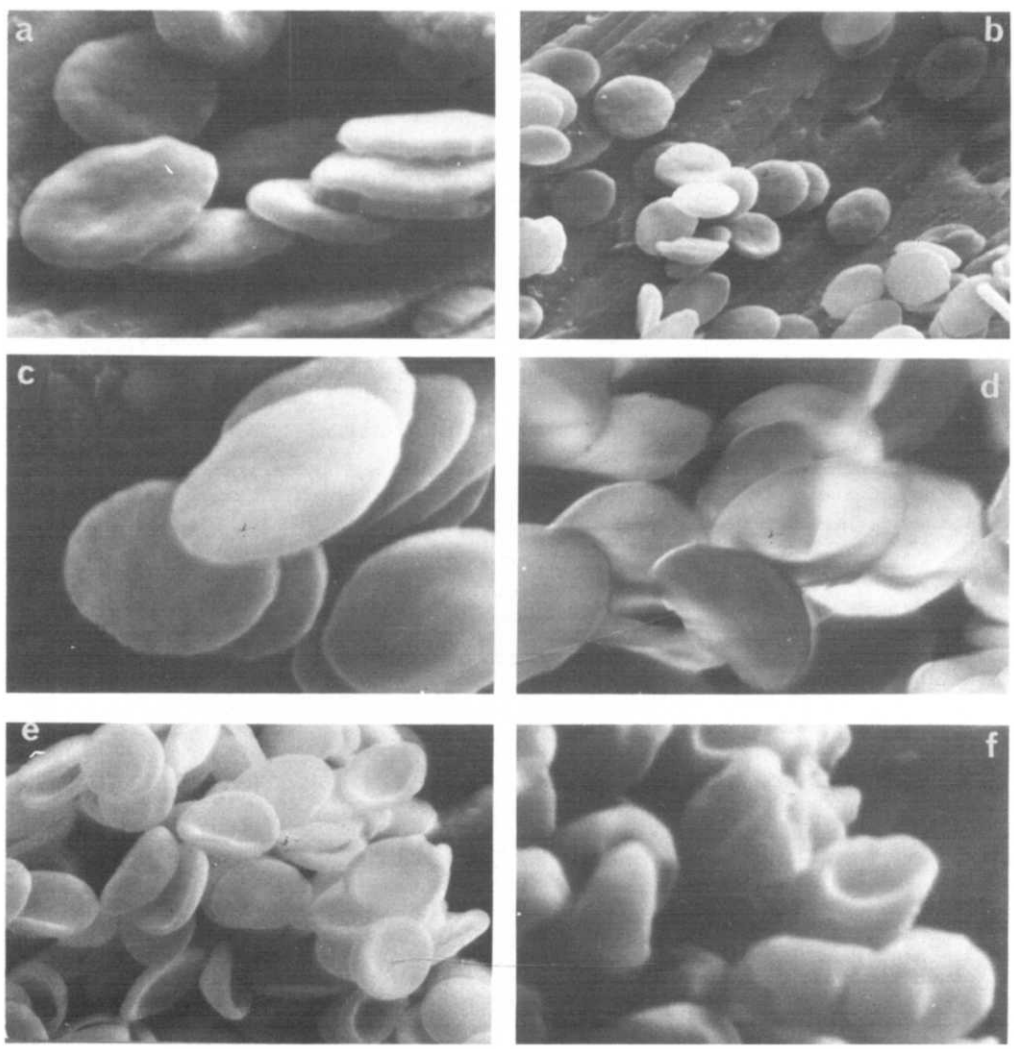


Fig. 8. Scanning electron micrographs of control erythrocytes and of erythrocytes treated with anthracycline drugs. a and b, controls X4000 and X1500, respectively; c, AD32 X4700; d, daunomycin X4700;

hemolysis is shown in Fig. 7. A relative hemolysis of 1 indicates the hemolysis of control erythrocytes at 68 mM NaCl. Both AD32 and AD-14-ACE were effective stabilizers against hypotonic lysis, AD-14-ACE exerted its effect at a higher concentration than AD32. AD-14-OCTA exerted lytic effects at concentrations above $4 \cdot 10^{-5}$ M. It is to be noted that AD32 and AD-14-OCTA, both strong lipophilic compounds (Table I), showed opposing effects at the concentration range tested (Fig. 7). The amphipathic charged drug (AD-14-OCTA) did not confer stability even at very low drug concentration (10^{-7} M), whereas the non-charged AD32 stabilized against hemolysis at relatively high concentrations ($\approx 5 \cdot 10^{-5}$ M).

The interaction of the various drugs with erythrocytes has also been probed using scanning electron microscopy. Figs. 8a and 8b show the well-documented biconcave disc of control erythrocytes. Fig. 8c shows that no dramatic shape changes occur upon interaction with 25 μg/ml of AD32 or adriamycin (not shown). Daunomycin and AD-14-ACE cause the cells to form cup-shapes (Figs. 8d and 8e) and AD-14-OCTA, which at 25 μg/ml causes 85% lysis, causes a dramatic swelling, as well as cup-shapes (Fig. 8f). It is of interest that daunomycin causes remarkable shape changes, whereas adriamycin does not.

Discussion

The physiological action of adriamycin and its derivatives is conferred upon intracellular organelles [21]. In order to cross the permeability barrier of the plasma membrane, the anthracycline drugs have either to utilize a specific carrier mechanism, for which there is no indication so far, or to be able to partition into lipid domains in the membrane.

There is a distinct correlation between the permeation of non-electrolytes through biological membranes and their lipid-water partition coefficients [22,23]. Non-polar non-electrolytes permeate the membrane forming a chain of interactions with the hydrocarbon tails of the lipids. Weak acids and bases can diffuse readily across biological membranes in their unionized form but not, or very slowly, when ionized. Unionized form of most drugs and antibiotics is the one which accounts for membrane crossing [24].

Adriamycin is a weak base ($pK_a = 8.22$ [25]), thus at pH 7.2 a significant fraction is still unprotonated and can partition into the non-polar lipid regions. The partitioning of adriamycin and its derivatives into octanol indicates that adriamycin is the least lipophilic derivative among the five drugs, that daunomycin and AD-14-ACE partition to the same extent into the lipid phase (more than three times the partitioning of adriamycin), and that AD32 and AD-14-OCTA are virtually taken totally (>99.9%) into the lipophilic phase.

Negatively charged liposomes are obviously better model for drug-membrane interactions. The apparent partition constants obtained (Table I) show essentially the same pattern obtained with octanol, i.e. about 4.5 more liposomes were needed for a 50% partitioning into the lipid phase in the case of adriamycin than in the case of daunomycin and AD-14-ACE was comparable in its partitioning to daunomycin. Both AD32 and AD-14-OCTA exhibit a high affinity to liposomes and to the erythrocyte membranes.

The apparent dielectric constant prevailing in the microenvironment of the

fluorophore (ϵ = 4—22) further reflects the association of the drugs with the phospholipid phase. Here, too, adriamycin appears to be the least intercalated into the hydrophobic core of the bilayer. The difference between the dielectric constant reported by the fluorophore of AD32 embedded in liposomes and in erythrocyte membranes suggests that the presence of proteins displaces the drug into a more hydrophilic environment.

The rotational relaxation times of the drugs within the membranes are an independent parameter relating to the microviscosities at which the fluorophore is embedded. The obtained ρ values are all in the range of 3.5—7.1 ns, which is expected for free rotation in a fluid domain. It is worth noting that those drugs that according to the calculated dielectric constant are embedded deeper in the phospholipid core are more restricted in their movement than those located at the hydrocarbon-water interface.

Again, we note the difference in the behaviour of AD32 and AD-14-OCTA in liposomes and in erythrocyte membranes. The rotational relaxation rates support the notion that the drugs rotate within a lipid phase and are not bound to a protein carrier. If the latter possibility was correct the expected ρ values would have been 10—100 timer greater.

Extensive studies of the interaction of various compounds with both model and biological membranes have shown that the interaction affects the thermotropic behaviour of both membranes [26—28]. In order to assess the extent of phospholipid bilayer perturbation, resulting from interactions with antracycline drugs, and the role that anionic head groups of phospholipids in biological membranes may play in this interaction, we have resorted to differential scanning calorimetry. The latter studies indicate that three drugs, daunomycin, AD32 and AD-14-OCTA, have the same perturbing effect on dipalmitoyl phosphatidylcholine as reflected by a change in enthalpy of melting, whereas adriamycin has a smaller effect. A decrease in the temperature of the middle of the peak (T_m) (Table II) was observed with all drugs, suggesting a fluidizing effect on membrane lipids [26,27]. The interaction of the drugs with phosphatidylserine resulted in a more pronounced decrease in the enthalpy of melting. A significant change in the T_m was observed only for daunomycin. It is of interest that the enhanced decrease in enthalpy of melting in phosphatidylserine as compared to that observed in dipalmitoyl phosphatidylcholine is not linked to the net charge of the drug, i.e. AD32 was more effective than the positively charged drugs (except for daunomycin). Phosphatidylserine, being charged and having unsaturated fatty acids, is structurally less rigid than dipalmitoyl phosphatidylcholine. It is thus possible that all drugs intercalate to a higher extent within its lipid domains, or that a comparable intercalation results in a higher perturbation. Tritton et al. [29] suggest on the basis of NMR studies that phospholipid bilayers become more fluid upon interaction with adriamycin and that the latter binds with the same affinity to liposomes of various compositions.

That the intercalation of the various anthracycline drugs into lipid domains of biological membranes may affect their properties is indicated from the influence of the drugs on the osmotic fragility (Fig. 7) and shape (Fig. 8) of fresh human erythrocytes. The highly lipophilic positively charged AD-14-OCTA exerted a lytic effect on erythrocytes even under iso-osmotic condi-

tions. AD32 and AD-14-ACE diminished the sensitivity of the cells to hypotonic lysis whereas daunomycin and adriamycin did not exert detectable changes in the susceptibility to hypotonic lysis. The differential effect of AD-14-ACE and daunomycin is not readily understood as they exhibit comparable partitioning into both octanol and liposomes. Schioppacassi and Schwartz [30] have recently shown that human erythrocytes show either increased or decreased susceptibility to hypotonic lysis upon interaction with daunomycin, and that the diverse reactions were proportional to the osmotic fragility of the non-drug-treated erythrocytes.

Sheetz and Singer [31] have proposed that membranes, in which the proteins and the polar lipids are distributed asymmetrically in the two halves of the membrane bilayer, can act as bilayer couples, i.e. the two halves can respond differently to a perturbation. When applied to the perturbation effected by amphipathic drugs on human erythrocytes, the theory explained the various patterns of shape-changes that the cells undergo. It was proposed that: (a) anionic drugs intercalate mainly in the external half of the bilayer, expand it in relation to the inner layer and induce cell crenation; (b) permeable cationic drugs intercalate preferentially in the inner half of the bilayer where phosphatidylserine is concentrated, expand this layer with respect to the exterior half and bring about cup-formation; (c) impermeable amphipathic drugs intercalate only in the exterior half of the bilayer and would therefore also be crenators.

In light of the above we have studied the effect of the various anthracycline drugs on the shape of human erythrocytes. The biconcave disc shape of the erythrocytes (Figs. 8a and 8b) was essentially unchanged after interaction with either adriamycin or AD32 (Fig. 8c). Daunomycin and AD-14-ACE were potent cup-formers (Figs. 8d and 8e). The latter two drugs are cationic amphipathic molecules and should, according to the theory, cause cup-formation. AD32, being non-charged, distributes apparently equally well in the two halves of the bilayer and therefore has no gross effect on the cell shape. The fact that adriamycin, a cationic amphipathic drug, does not lead to cup-formation is a further indication of a low partitioning into the membrane phase. AD-14-OCTA, a potent cationic lytic drug, has a dual effect, it causes extensive cell swelling while retaining its capacity to transform the biconcave shape into a cup (Fig. 8f). The shape change is thus a more sensitive parameter for assessing intercalation of cationic drugs into erythrocytes than protection against lysis, since in the latter both daunomycin and adriamycin did not have a detectable effect.

The protection against lysis is not related to the ability of the drugs to change the shape, since both AD32 and AD-14-ACE protect against hypotonic lysis while only AD-14-ACE is a cup-former. AD-14-OCTA is a cup-former and a potent lytic drug.

The various independent parameters tested indicate a differential drug-membrane interaction. Differences were observed in partitioning into the membrane, in location within the membrane and in perturbation of membrane organization.

The results predict differences in the rate of permeation of the various drugs, as well as in their intracellular distribution. Using fluorescence micro-

scopy we were able to show that adriamycin and daunomycin accumulate in the nuclei of macrophages in addition to some cytoplasmic inclusions (fluorescence microscopy could not identify the organelles), whereas AD32 accumulates rapidly in the cytoplasm and in cytoplasmic organelles, but is excluded from the nucleus. Macrophages exposed to either of the latter drugs lose their viability in a dose- and time-dependent process (Raz et al., unpublished). Since macrophages under normal conditions are non-proliferating, one cannot ascribe the cytotoxic effect to interference with DNA synthesis. It is of interest that adriamycin is significantly less toxic to marcrophages than daunomycin (ref. 32 and Raz et al., unpublished).

Experiments with various experimental tumor systems in vivo and in vitro have shown a differential growth inhibitory activity of the various drugs, even those that do not intercalate into DNA, and differential rates and extents of intracellular accumulation [33,34]. Various structures [11] and enzyme activities, such as those involved in the generation of free radicals and subsequent drug-dependent peroxidation of lipids [35], may contribute to the overall toxic effect exerted by the drugs.

The correlation between toxicity and membrane association, as indicated by the present study, should be kept in mind in further attempts to construct new anti-tumor drugs which are less harmful but more specific.

Acknowledgement

T.F. was supported by the Roth Fund for Development of Experimental Cancer Research, administered by the Mario Negri Institute Foundation of New York, N.Y., U.S.A.

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