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Association of daunomycin to membrane domains studied by fluorescence resonance energy transfer

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1,6-Diphenyl-1,3,5-hexatriene and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene are fluorophores used to explore different hydrophobic domains of membrane bilayers (Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1265; Prendergast, F.G., Haugland, R.P. and Callahan, P.J. (1981) Biochemistry 20, 7333–7338). Fluorescence resonance energy transfer between these fluorophores, acting as energy donors, and the anthracycline, daunomycin, as the acceptor, was used to analyze the interaction of the drug with natural membranes, and its relative location within the membrane bilayer. The transfer process was demonstrated by: (1) emission fluorescence of the acceptor when the samples were excited at the excitation maximum of the donor (360 nm); and (2) progressive quenching of the energy donor (at 428 nm) when in the presence of increasing acceptor concentration. Also, the disruption of the energy transfer by solubilization of the membrane with Triton X-100 evidences a role for the membrane in providing the appropriate site(s) for energy transfer to occur. At moderately low daunomycin / membrane lipid ratios, the different efficiencies of resonance energy transfer between the two donors and daunomycin predicts a preferential, but not exclusive, location of the drug at membrane 'surface' domains, i.e., those regions of the bilayer explored by the 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene probe. In support of this observation, a large fraction (approx. 75%) of membrane-associated daunomycin was rapidly sequestered away from the membrane upon addition of excess DNA, which forms high-affinity complexes with daunomycin (Chaires, J.B., Dattagupta, n. and Crothers, D.M. (1982) Biochemistry 21, 3927–3932), thus acting as a drug 'sink'. Also, a large fraction of drug was accessible to fluorescence quenching by iodide, a collisional water-soluble quencher. On the other hand, a smaller population of the membrane-associated daunomycin was characterized by slow sequestering by the added DNA and inaccessibility to quenching by iodide. We conclude that the daunomycin, which is only slowly sequestered, is located deep within the hydrophobic domains of the bilayer, likely to be those probed by 1,6-diphenyl-1,3,5-hexatriene.

Introduction

Anthracycline antibiotics such as daunomycin, are potent cytotoxic drugs currently used as anti-tumour agents [1]. The classical model to explain anthracycline cytotoxicity has been based upon intercalation into nuclear DNA and interference with nucleic acid function [2].

Nevertheless, experiments using polymer-immobilized drug have shown that the drug is able to exert its cytotoxic activity without entering the cell, and is thus interacting solely with the plasma

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membrane [3–5]. Furthermore, several reports have appeared which demonstrate the interaction of anthracyclines with phospholipid vesicles [6,7], thus indicating that the lipid components might play a critical role in the incorporation of the drugs into cells.

In the present study we have exploited the intrinsic fluorescence of daunomycin by using the drug as an energy acceptor in fluorescence resonance energy transfer experiments. Two well-characterized membrane probes, which are able to explore different hydrophobic domains of the bilayer [8,9], have been used as energy donors. These were 1,6-diphenyl-1,3,5-hexatriene (diphenylhexatriene), which is assumed to partition deep within the membrane core [8], and its derivative, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, which partly interacts with the phospholipids' polar head groups [9]. The distances required between the energy donor and the acceptor for resonance energy transfer are comparable to the thickness of biological membranes [10] and, therefore, energy transfer between the membrane probes and the drug should occur primarily when the acceptor, daunomycin, is on or within the membrane bilayer and not free in solution. Our experiments were aimed at: (1) demonstrating the association of daunomycin with natural membranes, and (2) exploring the relative location of the drug within the lipid bilayer.

Materials and Methods

Daunomycin hydrochloride was purchased from Sigma. Stock solutions in bidistilled water were prepared at 300 μM and stored under nitrogen atmosphere at $-20\,^{\circ}$C. To determine daunomycin concentration in dilute solutions (below 10 μM), a molar absorption coefficient at 480 nm of 11 400 $M^{-1} \cdot cm^{-1}$ was used [11].

Sonicated stock solutions of calf thymus DNA (Sigma) at 3–4 mg/ml in 10 mM Tris buffer (pH 7.4), were used as described elsewhere [12].

Live *Torpedo marmorata* were obtained from local fishermen and electroplax membranes were obtained according to Ref. 13. Briefly, the electroplax was minced and homogenized 1 : 2 (w/v) in ice-cold 10 mM Tris buffer (pH 7.4)/5 mM EDTA/5 mM iodoacetamide/0.5 mM phenylmethylsulfonyl fluoride/0.02% sodium azide. The homogenate was centrifuged at 3500 rpm for 10 min in a GS-3 Sorvall rotor. The supernatant was filtered through cheese-cloth and centrifuged at 30 000 rpm for 30 min in a Beckman type 35 rotor. The resulting pellet was resuspended in 10 mM Hepes buffer (pH 7.4). Lipid phosphorus concentration in membranes was determined as in Ref. 14.

Diphenylhexatriene and its trimethylammonium derivative were obtained from Molecular Probes (Junction City, OR): fresh (1 mM) stock solutions were prepared in peroxide free tetrahydrofuran and *N,N'*-dimethylformamide, respectively. Incorporation of the probes into membranes was carried out at a molar ratio of 1 molecule per 500 phospholipid molecules, for 1 h at room temperature and in the dark.

Titration experiments to monitor fluorescence resonance energy transfer were done by incubating a fixed amount of donor-containing membranes with increasing daunomycin concentration (0.5–5 μM), for 2 h at 25 °C. Under these conditions self-association of daunomycin molecules is prevented [15]. Alternatively, aliquots from a 2 μM daunomycin solution were titrated with aliquots of donor-containing membranes up to 200 μM in terms of final phospholipid concentration. Incubation time and temperature were as described above.

Quenching of daunomycin by iodide was carried out by mixing aliquots of 1 M KI (2 mM $Na_2S_2O_3$ to prevent I_3^- formation [16]), stock solution in 10 mM Hepes (pH 7.4), with membranes in 10 mM Hepes (pH 7.4)/1.0 M NaCl, containing high levels of incorporated daunomycin (greater than 90% of the total 1.5 μM added drug), as estimated by fluorescence anisotropy [17]. Total emission (in the absence or in the presence of several KI concentrations) was measured by using a cut-off 3-67 Corning filter in a Perkin-Elmer LS-5 model fluorescence spectrofluorimeter, using 1 cm quartz cells.

Fluorescence intensity measurements were corrected for scattering contributions observed in membrane samples devoid of fluorophores. Scattering values were always less than 1% of the total fluorescence signal.

Results

Spectral overlap between the emission fluorescence spectrum of the energy donor-containing membranes and the excitation spectrum of the added daunomycin is shown in Fig. 1. Spectral overlapping constitutes an essential requisite for the fluorescence resonance energy transfer to take place. The transfer process will then occur when donor and acceptor (having the appropriate orientation) are within the Förster distance R_0 [10].

The occurrence of resonance energy transfer between daunomycin and diphenylhexatriene or its trimethylammonium derivative incorporated into membranes has been determined by exciting at 360 nm (excitation wavelength for the donor) and monitoring: (1) the increase in the fluorescence emission from a constant concentration of added daunomycin as the donor concentration increases (Fig. 2A); and (2) the decrease (quenching) of the emission fluorescence of the donor at increasing daunomycin concentrations (Fig. 2B). Control samples including free daunomycin in solution and membranes containing donors but no acceptor did not show significant emission at the characteristic daunomycin emission wavelength of 560 nm upon excitation at 360 nm.

The role of the membrane in providing the site(s) for the occurrence of energy transfer between donors and daunomycin was assessed by incubating a sample of membranes containing the acceptor/donor pairs ((b) in Fig. 3) with the non-ionic detergent Triton X-100. Disruption of the membrane by Triton X-100 abolishes energy transfer between either donor and daunomycin ((c) in Fig. 3), thus returning the emission fluorescence of the donor to its initial (control) value ((a) in Fig. 3). It should be noted, however, that, regardless of the presence of daunomycin, solubilization by Triton X-100 of membrane samples containing the donor probes produces a 10% enhancement of donor fluorescence emission.

The efficiency of energy transfer depends upon the distance between donor and acceptor [10], according to the equation:

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

where E is the efficiency of energy transfer, R_0 is

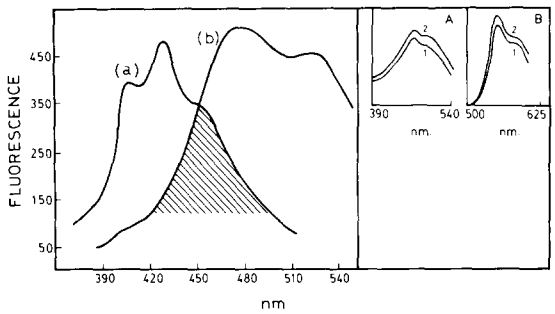

Fig. 1. (a) Fluorescence emission spectrum of diphenylhexatriene incorporated into *Torpedo* membranes (excitation at 360 nm). (b) Fluorescence excitation spectrum of 2 μM daunomycin upon incubation at equilibrium with membranes in 10 mM Hepes (pH 7.4). Shaded area represents overlapping between both spectra. Inset. (A) fluorescence excitation spectra of 4 μM daunomycin (1) and 4.5 μM adriamycin (2); (B) fluorescence emission spectra of 4 μM daunomycin (1) and 4.5 μM adriamycin (2). Excitation wavelength, 472 nm. Slits, 5 nm.

the Förster distance at which the efficiency of transfer is 50%, and R is the actual distance between the donor and the acceptor. Since, in our case, there is a random distribution of both donor and acceptor within their respective membrane domains, R_0 and R should represent an average of distances between the hydrophobic probes and daunomycin. Using diphenylhexatriene and adriamycin, an anthracycline analogue of daunomycin, and estimation of R_0 of 29 Å has been recently reported [17]. Because of the similarity in spectroscopic properties between diphenylhexatriene and its trimethylammonium derivative [9], and also between daunomycin and adriamycin (inset, Fig. 1), we have used the reported R_0 to calculate R when either of the donors and daunomycin are employed as donor/acceptor pairs.

Alternatively, since the quantum yield of a fluorophore is proportional to the fluorescence intensity at a given wavelength [10], the efficiency of the energy transfer can also be defined as:

$$E = 1 - (F/F_0) \quad (1)$$

where F and F_0 represent the fluorescence emission intensity of the donor in the presence and in the absence of the energy acceptor, respectively. Similarly to the situation found with other donor/acceptor pairs [18,19], the efficiency of energy

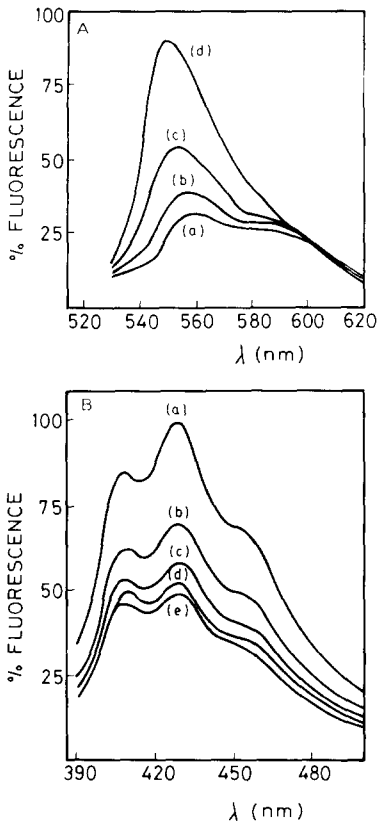

Fig. 2. (A) Fluorescence emission at 560 nm of 2 μM daunomycin-containing membranes at increasing donor concentrations (referred to as μM phospholipid concentration of membranes containing the donor): (a) 0 (control), (b) 50, (c) 100 and (d) 200 μM. Excitation wavelength, 360 nm. (B) Quenching at 428 nm of the energy donor incorporated into membranes (50 μM in phospholipid), at increasing daunomycin concentrations: (a) 0.0 (control), (b) 0.7, (c) 1.0, (d) 1.2 and (e) 2.0 μM. Excitation wavelength, 360 nm.

transfer between diphenylhexatriene (or its trimethylammonium derivative) and daunomycin is independent of the surface density of the energy donor, but has a dependence on the concentration of the energy acceptor (Fig. 4). A comparison between the two energy donors shows that, under conditions of low acceptor concentration, (1) the efficiency of the transfer is nearly proportional to the concentration of added daunomycin, and (2) the trimethylammonium derivative of diphenylhexatriene is a much more efficient energy donor than diphenylhexatriene. Because energy exchange from donor to daunomycin in solution has been

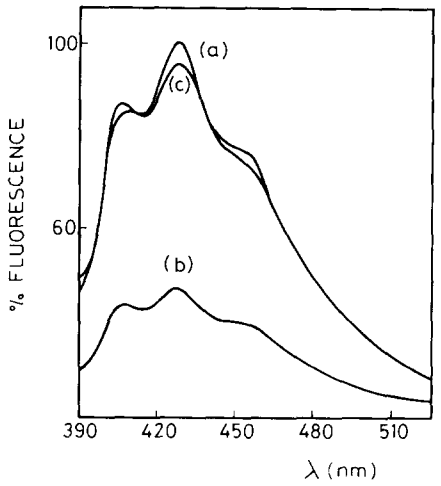

Fig. 3. Disruption of fluorescence resonance energy transfer from donors to daunomycin by Triton X-100. The process was followed as described in Figure 2B. (a) Control donor-containing membranes (50 μM in phospholipid); (b) control + 2.5 μM daunomycin; (c) sample (b) + 0.25% Triton X-100. Excitation wavelength was 360 nm.

shown not to occur (Fig. 3), the above suggests a preferential location of the acceptor daunomycin at hydrophobic membrane 'surface' domains, i.e., those where the trimethylammonium derivative partitions. It should be noticed however, that the abscissa in Fig. 4 indicates total concentration of daunomycin added into membrane suspensions,

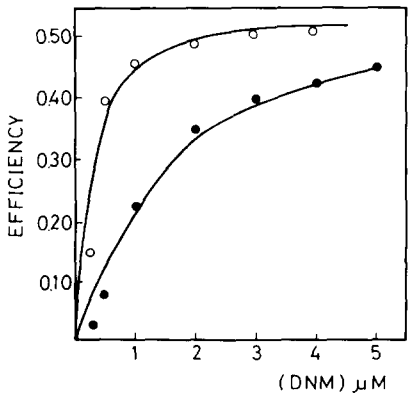

Fig. 4. Variation of the efficiency of transfer of energy from (●) diphenylhexatriene or (○) its trimethylammonium derivative to daunomycin as a function of acceptor concentration (up to 5 μM). The process was measured as described in Fig. 2B by using Eqn. 1 (see text).

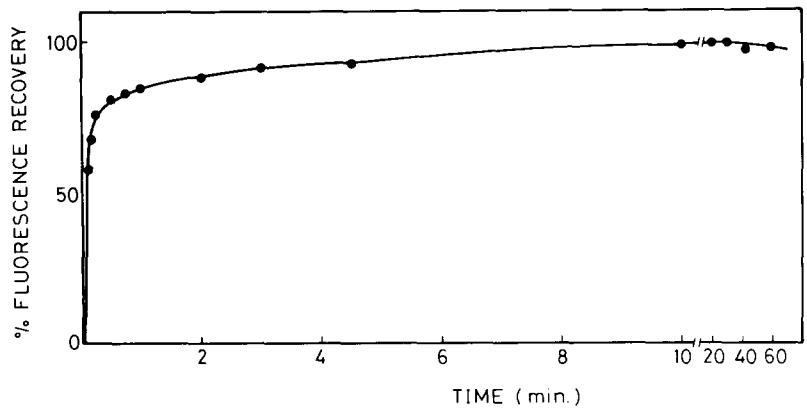

Fig. 5. Time-dependent recovery of the fluorescence emission of diphenylhexatriene into membranes (50 μM in phospholipid) in the presence of 2.6 μM daunomycin upon addition of 80-fold molar excess of DNA. Fluorescence emission intensity measurements were carried out at selected times indicated in the figure. Recovery was evaluated as % fluorescence recovery = $F - F_d/F_0 - F_d$, where F is fluorescence at indicated times; F_d is fluorescence before adding DNA and F_0 is initial fluorescence of donor-containing membranes.

and therefore does not provide a direct indication of the density of energy acceptor within the membrane. Nevertheless, a proportionality between added daunomycin (at least up to 8–10 μM), and the amount of drug incorporated into membranes has been observed through equilibrium binding experiments using *Torpedo* and other membrane systems (manuscript in preparation). At high concentrations of daunomycin, the transfer efficiencies corresponding to both donors tend to converge and, in fact, maximum efficiencies of 56–60% for either energy donor can be estimated by extrapolation to infinite daunomycin concentration from double-reciprocal plots of $1/E$ vs. $1/$[daunomycin]. When these maximum efficiency values are used in conjunction with the R_0 value reported in [17], an average distance (R) of 27 Å between either donor and daunomycin is obtained. This indicates that, differently from what is observed at low drug/lipid ratios, daunomycin partitioning into membranes under high drug/lipid conditions is less selective in distinguishing the different membrane domains explored by the two energy donor probes.

In order to confirm the existence of potentially different membrane domains for the accommodation of daunomycin, membrane samples containing donor-acceptor pairs under conditions of low or moderate daunomycin/membrane lipid ratios, were mixed with an excess (80-fold molar excess over daunomycin) of DNA, which has been demonstrated to form complexes with daunomycin and to be an effective static quencher of its fluorescence [12]. Under these conditions, free daunomycin in solution is almost instantaneously quenched (only measurable using rapid kinetic instruments [20]) by the added DNA. However, in the presence of membranes, a two-step, time-dependent recovery of the 428 nm emission of the initially quenched energy donor was observed (Fig. 5). As indicated, a large portion (approx. 75%) of

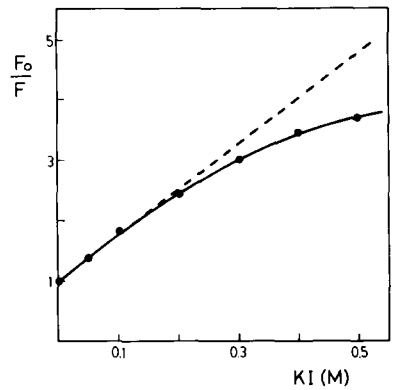

Fig. 6. Stern-Volmer plot corresponding to fluorescence quenching of a 1.5 μM daunomycin-containing membrane suspension (1 mM in phospholipid) by increasing KI concentrations (50–500 μM). Quenching of daunomycin in solution by KI yields a K_{SV} value of 10 M^{-1} (data not shown).

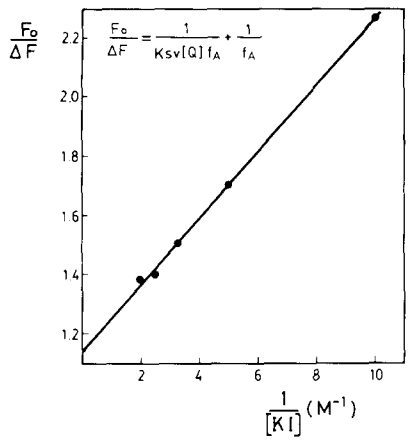

Fig. 7. Modified Stern-Volmer plot used to determine graphically the accessibility of daunomycin to iodide, according to the equation shown [21]. f_A represents fraction accessible to KI and is calculated from the y-intercept (equal to $1/f_A$). Experimental conditions used were as in Fig. 6.

the donor emission is recovered within 15 s upon addition of DNA, followed by a slower recovery step, lasting almost 60 min, which finally restores the donor's original emission value. These results suggest the sequestering of the membrane-bound daunomycin by DNA from at least two different membrane domains.

Complementary fluorescence quenching experiments of membrane-associated daunomycin by iodide, a water-soluble collisional quencher of daunomycin [6], also support the above suggestion. Fig. 6 shows a representative Stern-Volmer plot used to describe collisional quenching according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q]$$

where K_{SV} equals the Stern-Volmer quenching constant and [Q] refers to the concentration of the quencher. The downward curvature represented in Fig. 6 indicates heterogeneity of the membrane-bound daunomycin with regard to its accessibility to the collisional quencher [21]. The fraction of daunomycin which is accessible to quenching by iodide was determined according to the modified Stern-Volmer equation [16,21] inserted in Fig. 7. The variable f_A represents the fraction of daunomycin accessible to the quencher, and was estimated from the y-axis intercept in Fig. 7 as being approx. 85% of the total membrane-associated daunomycin.

Discussion

Our goal in this paper has been to demonstrate that hydrophobic domains of natural membranes constitute an appropriate environment for the accommodation of anthracyclines such as daunomycin. For these studies we chose postsynaptic membranes from *Torpedo* because (1) their protein and lipid components have been characterized in detail (see Refs. 25 and 26 for reviews) and (2) *Torpedo* membranes should be devoid of specific drug-binding proteins such as those associated with cellular drug resistance [24] in tumor cells.

Our rationale for demonstrating incorporation of daunomycin into membranes has been to use fluorescence resonance energy transfer between the fluorescence drug and two well-known extrinsic membrane probes, diphenylhexatriene and trimethylammonium diphenylhexatriene. The fluorescence of either probe is quenched by the energy acceptor, daunomycin, in a concentration-dependent manner via a Förster energy transfer mechanism. Since quenching of donor fluorescence is abolished upon disruption of the membrane with detergent, it is apparent that the energy transfer process requires the membrane to accommodate sufficient amounts of both the donor(s) and the acceptor within the required Förster distance.

Based on the dependence of the efficiency of energy transfer upon the concentration of the energy acceptor, it is suggested that, when low or moderate daunomycin/membrane lipid ratios are used, preferential incorporation of daunomycin ocurs at membrane 'surface' domains. Electrostatic forces between negatively charged phospholipid polar head-groups (phosphatidylserine is one of the major phospholipid components of *Torpedo* membranes [22]), and the ionized amino group at the daunosamine moiety of daunomycin, might be partly responsible for the stabilization of the drug within those regions of the bilayer.

Nevertheless, when high acceptor/lipid ratios are used in the experiments, the efficiency of the energy transfer to daunomycin from 'surface' and 'core' domains of the membranes is very similar,

thus suggesting that the drug may also traverse the membrane to reach internal regions of the bilayer. This is consistent with earlier reports [17] suggesting that hydrophobic interactions between the anthracycline ring and the hydrocarbon interior of membranes may be important in the binding of anthracyclines to synthetic phospholipid bilayers. Furthermore, we have observed that membrane-bound daunomycin can be partly displaced from the membrane by increasing the concentration of cholesterol within the bilayer (unpublished observation).

The presence of daunomycin in different membrane domains is also indicated by experiments of drug sequestering by externally added DNA. The recovery of energy donor emission upon removal of the quenching energy acceptor by DNA occurs in at least two steps, each characterized by a markedly different rate of daunomycin sequestering. This suggests different accessibility of drug pools within the membrane for binding to DNA and/or the existence of membrane sites with different affinities for binding of the drug. Heterogeneity in the accessibility of the membrane-bound drug from the aqueous environment can also be concluded from experiments of collisional quenching by iodide. In fact, two macroscopic membrane-associated drug populations were detected from iodide quenching experiments: a large fraction (approx. 85%) of membrane bound-daunomycin, which was accessible to the quencher and must therefore be located closer to the membrane surface, and a small non-accessible fraction, which is presumably buried within the bilayer core.

The observation reported here may offer some insight into the controversial subject of the possible mechanisms of transport of anthracyclines through membranes [25,26]. The ability of daunomycin to interact with both surface and core domains of membranes lends support to the hypothesis that anthracyclines may diffuse through the membranes passively.

Acknowledgements

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