Biophysical Chemistry, 35 (1990) 259–264
Elsevier

BIOCHE 01428

Transverse location of anthracyclines in lipid bilayers

Paramagnetic quenching studies

Panayiotis P. Constantinides [a], Yan Yan Wang [b], Thomas G. Burke [c] and Thomas R. Tritton [d]

[a] *Lipogen, Inc., Knoxville, TN 37932 and Department of Biochemistry, University of Tennessee, Knoxville, TN 37996,* [b] *Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510,* [c] *Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010 and* [d] *Department of Pharmacology and Vermont Regional Cancer Center, University of Vermont College of Medicine, Burlington, VT 05405, U.S.A.*

Received May 1989
Accepted 19 September 1989

Fluorescence quenching; Anthracycline fluorescence; Fluorophore-membrane binding; Spin label

Quenching of anthracycline fluorescence by a series of spin-labeled fatty acids was used to probe the transverse location of the drug in phosphatidylcholine bilayers in the form of small unilamellar vesicles. Stern-Volmer plots of the quenching data indicate that the fluorophore moiety of the anthracycline is intercalated into the hydrocarbon region of the bilayer, with deeper penetration observed in fluid-phase than in solid-phase vesicles. ^{31}P-NMR parameters (T_1 and nuclear Overhauser enhancement (NOE)) are unaffected by the presence of drug, consistent with a binding site removed from the interfacial region. Comparison of intensity (F_0/F) plots with lifetime (τ_0/τ) data shows that the predominant mechanism of anthracycline quenching by membrane-bound nitroxides is static. Since the membrane-bound drug is also accessible to quenching by I^-, the binding site in the membrane must create a channel which is accessible to solvent. Two other fluorescent probes, 12-(9-anthroyloxy)stearate (12-AS) and diphenylhexatriene (DPH), were employed to confirm the results obtained with the anthracyclines, giving quenching data representative of their location in the bilayer.

1. Introduction

Anthracycline antibiotics, particularly adriamycin, are among the most active anticancer drugs known and are widely used in the treatment of a variety of human tumors. One primary target for anthracyclines has been considered to be DNA, and DNA-processing enzymes like topoisomerase, within the cell nucleus. There is also considerable evidence implicating membranes as an alternative target for many chemotherapeutic agents, including anthracyclines [1,2].

Correspondence address: T.R. Tritton, Department of Pharmacology and Vermont Regional Cancer Center, University of Vermont College of Medicine, Burlington, VT 05405, U.S.A.

High-affinity binding of anthracyclines to small unilamellar vesicles has been observed in our laboratory using fluorescence methods [3]. We have also shown by magnetic resonance spectroscopy (NMR and EPR) that adriamycin induces changes in the physical properties of liposomal membranes such as fusion and permeability [4], lipid order and fluidity [5]; these effects depend on the phospholipid composition and are particularly sensitive to the presence of cardiolipin. Strong interaction of anthracyclines with membranes, especially those rich in cardiolipin, has also been observed by other investigators [2,6]. Finally, we have recently developed methodology to measure the angular distribution of fluorophores in an ordered bilayer, and have shown that adriamycin takes on

a particularly disruptive angle of approx. 55° when bound to phosphatidylcholine membranes [7]. The current status is that although a considerable amount of understanding of the membrane interactions of anthracyclines is in hand, detailed information on the drug location and dynamics in the membrane is still lacking. In an effort to obtain some insight into the relative location of the anthracyclines in a membrane we have begun to develop the paramagnetic quenching experiments reported in this communication.

A recently developed method of fluorescence quenching by paramagnetic fatty acid and lipid analogues has been employed to gather information on the transverse location of fluorophores in lipid bilayers [8–11], micelles [11,12], and biological membranes [13,14]. Although the mechanisms of this kind of quenching as well as the critical distance between the fluorophore and quencher have not been completely established so far, electron exchange and energy transfer are probably the main contributors to quenching. Thus, information can be extracted about the proximity of the fluorophore and the quencher and their relative location in the bilayer. Taking advantage of the intrinsic fluorescence of anthracyclines, a series of spin-labeled fatty acids having the doxyl group attached at different positions along the fatty acyl chain, and thus probing different depths in the bilayer, have been tested in their efficiency to quench adriamycin fluorescence in small unilamellar phosphatidylcholine vesicles. We employed Stern-Volmer plots to analyze the data which show that the drug is more accessible for quenching to deeper probes, suggesting a drug location in the bilayer removed from the polar interface. The validity of the method has been tested with other widely used and extensively studied fluorescent probes.

2. Materials and methods

Adriamycin and carminomycin were generously provided by Adria Laboratories (Columbus, OH) and Bristol Laboratories (Syracuse, NY), respectively. Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma (St. Louis, MO). All lipids were stored in a desiccator at −20°C. No impurities were detected in any of the samples used as judged by thin-layer chromatography on a silica gel plate using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25 : 15 : 4 : 2, v/v). Spin-labeled stearic acids with the 4,4-dimethyl-3-oxazolidyloxy group (doxyl group) attached to either carbon atom 5 (5-DS), or 12 (12-DS) or 16 (16-DS) and their methylated esters, were obtained from Aldrich (Milwaukee, WI). Stock solutions of spin-labeled fatty acids (1 mg/ml) were made in absolute ethanol and stored at 4°C in the dark. The fluorescent probes 12-(9-anthroyloxy)stearate (12-AS) and diphenylhexatriene (DPH) were purchased from Molecular Probes (Junction City, OR). Stock solutions of 12-AS and DPH were made in ethanol and benzene, respectively, and stored at 4°C.

Liposome solutions were made in phosphate-buffered saline (PBS), pH 7.4. Small unilamellar vesicles were prepared as described elsewhere [5], by sonicating multilamellar vesicles until optical clarity (2–3 h) in a bath sonicator. Following sonication, the vesicles were allowed to anneal for 45 min above the transition temperature [15] and used subsequently for the fluorescence experiments. Anthracyclines were added to the annealed vesicles, either from a concentrated stock solution or by mixing and briefly sonicating the vesicles with the dry film of the drug. The latter method was also employed to introduce the fluorescent probes 12-AS and DPH into the liposomes. Drugs were equilibrated with the vesicles above the T_m for about 2 h. Spin-labeled fatty acids were introduced into the vesicles by using the same procedure as for introducing the fluorophores, followed by a 30 min incubation above the phase transition temperature to allow complete partitioning in the bilayer. EPR spectra of the spin-labeled vesicles confirmed that all spin-labeled fatty acids employed were completely bound and that no signal from any remaining free label was present under the conditions of our experiments. This is an important consideration, since Blatt and Sawyer [16] have reported that partitioning of the quencher between aqueous and membrane phases, if it occurs, can complicate the fluorescence analysis.

An SLM model 4800 LPS instrument interfaced to a Hewlett-Packard 9825 data system was employed for the fluorescence measurements. For temperature control the instrument was connected to a thermostatted cuvette compartment. Fluorescence lifetimes were obtained by the phase-shift method [17]. A 25 mg/ml glycogen solution was used as a phase reference and neutral density filters were employed to adjust the amount of scattered light. The samples were placed in a 1 cm quartz cuvette and excited with light modulated at 30 MHz. Between temperature changes the samples were equilibrated at the desired temperature for at least 15 min. No time dependence of the fluorescence intensity was observed, suggesting that equilibration of both the quenchers and fluorophores was completed before the fluorescence was recorded.

^{31}P-NMR spectra were recorded on a Bruker CXP-200 instrument operating at 80.9 MHz with proton noise decoupling. The samples of approx. 1 ml were contained in 10-mm NMR tubes at 25 mg/ml lipid. A deuterium field-frequency lock was used with $^{2}H_2O$ present in either the sample or a concentric capillary. Spin-lattice relaxation times (T_1) were measured by progressive saturation [18], and nuclear Overhauser enhancements (NOE) by comparing the integrated peak areas of fully decoupled spectra with those where the decoupling frequency was gated [19].

3. Results and discussion

Stern-Volmer plots for the quenching of carminomycin fluorescence by doxylstearates are shown in fig. 1. The following points can be noted. Firstly, fluorescence lifetimes varied linearly over the quencher concentration employed representative of dynamic (collisional) quenching, whereas for the fluorescence intensity measurements an upward curvature is evident, due to the simultaneous occurrence of both dynamic and static quenching [9,10]. The anomalous slightly downward curvature of 5-DS below the phase transition (fig. 1A) may be explained in terms of co-clustering of fluorophore and quencher molecules in the solid bilayer as originally suggested by Bieri and Wallach [8]. Secondly, since the dynamic quenching of a membrane-bound fluorophore is reflected by the fluorescence lifetime [9,11], comparison of the F_0/F and τ_0/τ plots shows that the predominant quenching component is static, particularly below the gel ↔ liquid-crystal phase transition. This is likely due to the fact that in the gel phase the resident molecules are more immobile and

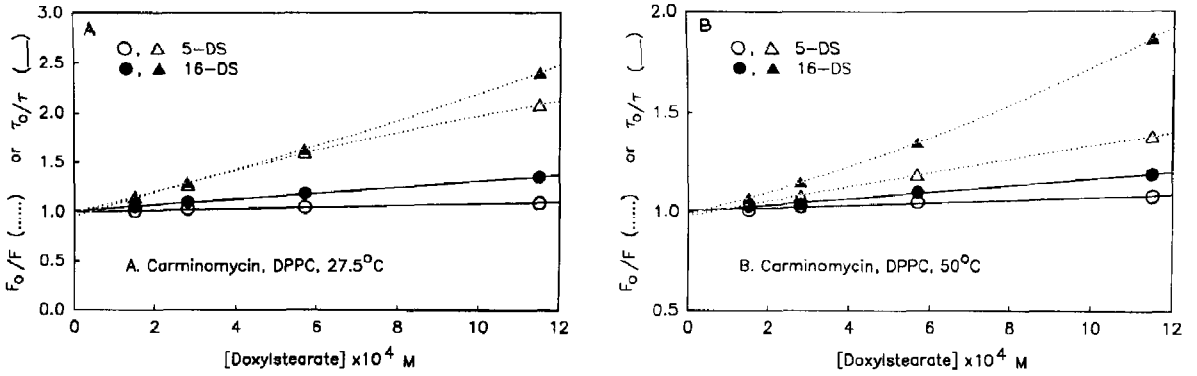

Fig. 1. Stern-Volmer plots for paramagnetic quenching of carminomycin bound to (A) solid-phase and (B) fluid-phase DPPC vesicles, in PBS at pH 7.4. F_0/F represents the static and dynamic quenching whereas τ_0/τ reflects only the dynamic component. The lipid concentration was at 10 mg/ml or approx. 1.4×10^{-2} M with the drug at 5×10^{-6} M, a condition which ensures that more than 97% of carminomycin is bound [3]. An excitation wavelength of 470 nm was used and fluorescence intensities and lifetimes were determined as described in section 2.

Table 1

Quenching of adriamycin and carminomycin fluorescence in dipalmitoylphosphatidylcholine vesicles [a] by spin-labeled fatty acids

Quencher [b]	Drug [c]	Quenching (%)	
		Below T_m (27.5 °C)	Above T_m (50 °C)
5-DS	adriamycin	8.1	1.9
	carminomycin	13.5	4.8
12-DS	adriamycin	10.6	2.6
	carminomycin	21.3	8.8
16-DS	adriamycin	6.6	2.6
	carminomycin	9.6	5.9

[a] The DPPC concentration was 25 mg/ml when adriamycin was used and 10 mg/ml with carminomycin. In both cases, more than 97% of the drug at a final concentration of 5×10^{-6} M was bound to the vesicles [3].
[b] At 1 mol% relative to the lipid, or 1.4×10^{-4} M.
[c] Both drugs at a final concentration of 5×10^{-6} M.

thus engage less readily in collisional interactions. A static quenching by paramagnetic spin labels has also been suggested by others [9,10,12]. Finally, although in both solid (fig. 1A) and fluid-phase bilayers (fig. 1B) 16-DS seems to be the most efficient quencher, in fluid-phase bilayers the drug appears to be located deeper in the bilayer. This last observation is consistent with the data in table 1 using a constant quencher concentration, where the order of efficiency in quenching carminomycin fluorescence was 12-DS > 5-DS > 16-DS for solid and 12-DS > 16-DS > 5-DS for fluid bilayers. The same order holds also for adriamycin but the effects are smaller compared to carminomycin, possibly due to adriamycin's shorter lifetime [28]. Using a formalism developed by Chattopadhyay and London [20], we estimate from the quenching data that the average carminomycin distance from the center of the bilayer is 6.2 Å in the solid phase and 3.8 Å in the fluid phase. These results are consistent with I^- quenching studies conducted previously [28] which revealed a more accessible binding site for anthracyclines in solid-phase bilayers.

Interestingly, when TEMPO-stearamide, a spin label which probes changes on or near the liposomal surface, was employed to quench adriamycin fluorescence almost negligible quenching was observed (data not shown). Smaller quenching by TEMPO-stearamide as compared to the methyl esters of 5-, 12- and 16-DS has also been observed in a fluorescence study on the location of the ionophore A23187 in DPPC and egg yolk PC bilayers, suggesting deeper location of the fluorophore in the bilayer [22].

The results shown in fig. 1 and table 1 together with the TEMPO-stearamide effect are consistent with our previous observations on the interaction of adriamycin with small unilamellar vesicles of phosphatidylcholine using paramagnetic resonance methods [5]. EPR spin labeling provided evidence for drug effects on the lipid chain order, and fluidity detected by 16-DS and TEMPO partitioning [5]. Furthermore, table 3 shows ^{31}P-NMR results indicating that neither the spin-lattice relaxation time (T_1) nor the nuclear Overhauser effect (NOE) of the DPPC vesicles was significantly affected by the drug, suggesting that although drug interaction can modulate the hydrocarbon chain organization, the head-group region of the lipid bilayer is not dramatically altered by the drug.

In order to confirm the results obtained with the anthracyclines and to gain more insight into the mechanism of quenching, the widely used fluorescent probes 12-AS and DPH were employed. Fluorescent derivatives of fatty acids having the

Table 2

Fluorescence quenching of 12-(9-anthroyloxy)stearate in phosphatidylcholine vesicles by spin-labeled fatty acids [a] and their methyl esters

System	Quenching (%)
DMPC [b] + 12-AS [c]	control
+ 5-DS	19.8
+ methyl 5-DS	9.9
+ 12-DS	36.5
+ methyl 12-DS	22.6
+ 16-DS	28.9
+ methyl 16-DS	19.1

[a] At 1 mol% relative to the lipid, or 1.5×10^{-5} M.
[b] DMPC at 1 mg/ml and 27.5 °C.
[c] The final concentration of 12-AS was 5×10^{-6} M. The excitation and emission wavelengths were 365 and 446 nm, respectively [17].

Table 3

Effect of adriamycin on the ^{31}P spin-lattice relaxation time T_1, and the nuclear Overhauser enhancement (NOE) of phosphatidylcholine vesicles

	Below T_m (25°C)		Above T_m (53°C)	
	NOE [b]	T_1 (s)	NOE	T_1 (s)
DPPC	1.54	1.35±0.04	1.31	1.30±0.04
DPPC + adriamycin [a]	1.47	1.41±0.04	1.29	1.28±0.04

[a] The drug concentration was 2.4×10^{-4} M with the lipid at 25 mg/ml.

[b] Intensity ratios of the fully decoupled spectra and spectra with the decoupling frequency gated.

anthroyloxy group at specific carbon atoms of the hydrocarbon chain have been used extensively to provide information on the transverse fluidity and polarity gradients in the bilayer and on the molecular order of the lipids [21]. In addition, these probes have been used to establish the position and orientation of other ligands in lipid bilayers such as gramicidin A [23] and local anesthetics [24]. A combination of a given fluorescent fatty acid derivative with a series of spin-labeled fatty acids is used to determine its transverse location in the membrane [11]. Using 12-AS and 5-, 12- and 16-DS in our system, we obtained the data shown in table 2. It is clear from table 2 that the order of quenching, 12-DS > 16-DS > 5-DS, is what is expected on the basis of the presumed transverse locations of fluorophore and quenchers. Moreover, the same order was observed when the spin-labeled fatty acids were replaced by their methyl esters, but the magnitude of quenching was smaller, probably because of differences in partitioning or electrostatic interactions in the vesicles [22,27].

DPH, a highly lipophilic probe, has spectral characteristics which make it a useful fluorescence probe [17]. Its excitation and emission wavelengths are well separated and their appropriate choice can minimize problems due to depolarization by energy transfer and Rayleigh or Raman scattering [17]. Because of its high lipophilicity, DPH is exclusively associated with the hydrocarbon phase of lipid bilayers, a property which can be used to sense the solid-to-fluid phase-transition temperature of the lipid and to estimate the membrane microviscosity [25]. Taking into account the hydrocarbon phase association of DPH, paramagnetic quenching was employed to verify its location in phosphatidylcholine bilayers and to confirm further the results obtained with the anthracyclines. Again, in both solid (fig. 2A) and fluid liposomes (fig. 2B), 12-DS was a more efficient quencher than 5-DS, although the amount of dynamic quenching was increased relative to that observed with carminomycin (fig. 1). Static quenching of DPH fluorescence by a spin-labeled phospholipid in phosphatidylcholine vesicles has

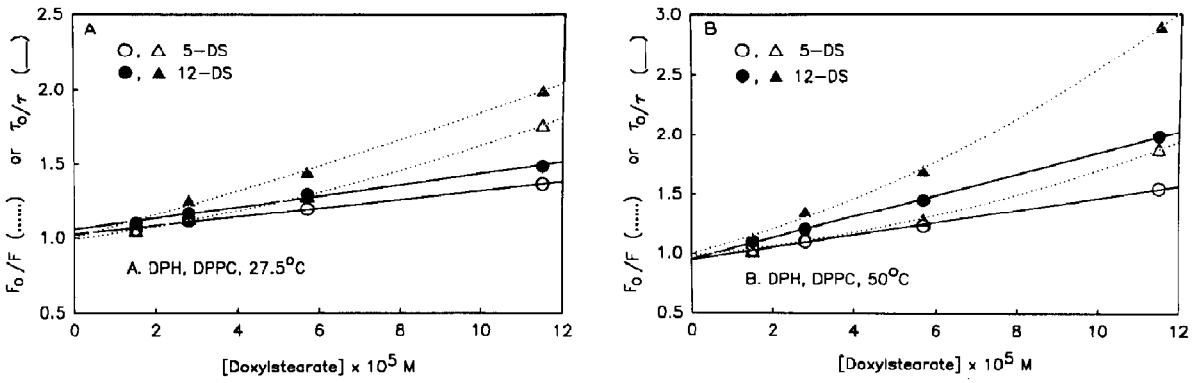

Fig. 2. Stern-Volmer plots for the quenching of diphenylhexatriene by 5- and 12-DS bound to (A) solid-phase and (B) fluid-phase DPPC vesicles in PBS at pH 7.4. DPH was introduced into the vesicles as described by Lakowicz et al. [17]. Its final concentration was 1×10^{-6} M with the lipid at 1.4×10^{-3} M. An excitation wavelength of 360 nm was employed and fluorescence intensity and lifetime measurements were performed as described elsewhere [3,17].

also been reported recently with a critical distance in the neighborhood of 10–12 Å [10]. Although fluorescence quenching by nitroxides free in solution resulted in the determination of a smaller critical distance, 4–7 Å [12], quenching by membrane-bound nitroxides is sensitive to larger fluorophore-quencher separations [10,11,22]. It has been suggested that the higher viscosity of the hydrocarbon phase, as well as lateral diffusion during a fluorescence lifetime of 1–10 ns, would increase the effective critical distance between fluorophore and quencher calculated through a Perrin equation using a hard-sphere approximation model [10].

The experiments reported here indicate that the technique of paramagnetic quenching can be used to determine the transverse location of anthracyclines in small unilamellar vesicle lipid bilayers. The data are consistent with a rather deep location of the fluorophore moiety of the drug within the bilayer, and the static mechanism is predominantly present during the quenching process. It should be noted that quenching experiments with I^-, which is large and polar, reveal that bilayer-bound adriamycin is accessible to quenching by this species as well [26]. Thus, although the drug is relatively deeply buried in the bilayer, there is still an accessible pathway to the aqueous solution, probably because the polar amino sugar forces the lipid chains apart. However, knowledge of the contribution of relevant factors to the observed quenching, such as the structure and orientation of the drug in the membrane, the curvature of the bilayers [5], as well as the lipid composition and presence of proteins, is necessary as the next step before the conclusions from these studies can be extended to biological membranes and correlated with drug action.

Acknowledgements

We thank the NIH for supporting this work (CA-44729). P.P.C. was the recipient of a Brown-Coxe Fellowship.

References

1 T.R. Tritton and J.A. Hickman, in: Experimental and clinical progress in cancer chemotherapy, ed. F. Muggia (Martinus Nijhoff, The Hague, 1985) p. 81.
2 E. Goormaghtigh and J.M. Ruysschaert, Biochim. Biophys. Acta 779 (1984) 271.
3 T.G. Burke and T.R. Tritton, Biochemistry 24 (1985) 1768.
4 S.A. Murphree, D. Murphy, A.C. Sartorelli and T.R. Tritton, Biochim. Biophys. Acta 691 (1982) 97.
5 P.P. Constantinides, T.R. Tritton and A.C. Sartorelli, J. Liposome Res. 1 (1988) 35.
6 K. Nicolay, R.J.M. Timmers, E. Spoelstra, R. van der Neut, J.J. Fok, Y.M. Huigen, A.J. Verkleij and B. de Kruijff, Biochim. Biophys. Acta 778 (1984) 359.
7 M. Adler and T.R. Tritton, Biophys. J. 53 (1988) 989.
8 V.G. Bieri and D.F.H. Wallach, Biochim. Biophys. Acta 389 (1975) 413.
9 K.A. Sikaris, K.R. Thulborn and W.H. Sawyer, Chem. Phys. Lipids 29 (1981) 23.
10 E. London and G.W. Feigenson, Biochemistry 20 (1981) 1932.
11 E. Blatt, R.C. Chatelier and W.H. Sawyer, Photochem. Photobiol. 39 (1984) 477.
12 S.S. Atik and L.A. Singer, J. Am. Chem. Soc. 100 (1978) 3234.
13 D.F.H. Wallach, S.P. Verma, E. Weidekamm and V. Bieri, Biochim. Biophys. Acta 356 (1974) 68.
14 R.C. Chatelier, P.J. Rogers, K.P. Chiggino and W.H. Sawyer, Biochim. Biophys. Acta 776 (1984) 75.
15 R. Lawaczek, M. Kainosho and S.I. Chan, Biochim. Biophys. Acta 443 (1976) 313.
16 E. Blatt and W.H. Sawyer, Biochim. Biophys. Acta 822 (1985) 43.
17 J.R. Lakowicz, F.G. Prendergast and D. Hogen, Biochemistry 18 (1979) 508.
18 R. Freeman, H.D.W. Hill and R. Kaptein, J. Magn. Reson. 7 (1972) 82.
19 K.F. Kuhlmann, D.M. Grant and R.K. Harris, J. Chem. Phys. 52 (1970) 3429.
20 A. Chattopadhyay and E. London, Biochemistry 26 (1987) 39.
21 K.R. Thulborn and W.H. Sawyer, Biochim. Biophys. Acta 511 (1978) 125.
22 J.S. Puskin, A.I. Vistnes and M.T. Coene, Arch. Biochem. Biophys. 206 (1981) 164.
23 E.A. Haigh, K.R. Thulborn and W.H. Sawyer, Biochemistry 18 (1979) 3525.
24 K.A. Sikaris and W.H. Sawyer, Biochem. Pharmacol. 31 (1982) 2625.
25 M. Shinitzsky and Y. Barenholz, J. Biol. Chem. 249 (1974) 2652.
26 G. Karczmar and T.R. Tritton, Biochim. Biophys. Acta 557 (1979) 306.
27 E. London, Mol. Cell Biochem. 45 (1982) 181.
28 T.G. Burke and T.R. Tritton, Biochemistry 24 (1985) 5972.