The Anionic Phospholipid-Mediated Membrane Interaction of the Anti-Cancer Drug Doxorubicin Is Enhanced by Phosphatidylethanolamine Compared to Other Zwitterionic Phospholipids†

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ABSTRACT: The interaction of doxorubicin and lipids has been studied using large unilamellar vesicles (LUVET) composed of mixtures of anionic phospholipids and various zwitterionic phospholipids. Dilution of anionic lipids with zwitterionic lipids leads to decreased membrane association of the drug because electrostatic forces are very important in doxorubicin−membrane interaction. However, binding of doxorubicin to LUVET composed of anionic phospholipids combined with phosphatidylethanolamine (PE) is much higher than binding to LUVET made of anionic lipids plus a range of other zwitterionic lipids such as phosphatidylcholine (PC) and the *N*-methylethanolamine and *N*,*N*-dimethylethanolamine derivatives of PE. This preferential interaction is observed with all negatively charged phospholipids tested and is, in the case of phosphatidylserine (PS), confirmed in monolayer experiments. The increase in surface area observed in a monolayer composed of PS and PE (1/3) was 3 times higher than in a monolayer of PS/PC (1/3). The preferential interaction appears not to be due to the ability of PE to adopt inverted nonbilayer structures, but probably involves a combination of the ability of PE to form additional hydrogen bonds and of the intrinsic curvature of a bilayer containing PE because of its small headgroup. Implications of our finding for the *in vivo* membrane interaction and transport of the drug will be discussed.

The anthracycline antibiotic doxorubicin is a potent and widely applied anti-cancer agent. Its activity is attributed to the interaction with nucleic acids and/or with nuclear components, such as DNA topoisomerase II (Chaires et al., 1985; Capranico et al., 1990; D'Arpa et al., 1989). In addition, this drug binds to various cellular membranes (De Wolf et al., 1993; Escriba et al., 1990; Nicolay et al., 1984), where it can disturb vital cellular processes, such as signal transduction (Thompson et al., 1987; Posada et al., 1989), electron transfer (Nicolay et al., 1984; Thompson et al., 1987), or translocation of proteins and lipids (Eilers et al., 1989; Phoenix et al., 1993; Voelker, 1991). Doxorubicin−membrane interaction also has important consequences for the availability of the drug to DNA (Speelmans et al., 1995) and passive and active transport of the drug (Gottesman & Pastan, 1993; Henry et al., 1985; Speelmans et al., 1994, 1995). At physiological pH, almost 90% of the doxorubicin molecules are positively charged (Figure 1). Therefore, in membranes, anionic phospholipids are important targets (De Wolf et al., 1991a,b, 1993; Escriba et al., 1990; Nicolay et al., 1984). Much insight into the interaction of doxorubicin and lipids has been obtained using model membrane systems. A strong interaction of the drug with anionic phospholipids was observed (Constantinides et al., 1986, 1988; De Wolf et al., 1991a,b; Goormaghtigh et al., 1980a,b, 1983), which underscores the electrostatic nature of the interaction. In addition, also hydrophobic interactions play a role. The drug partially penetrates between the lipids, resulting in disordering of the acyl chains (De Wolf et al., 1991b, 1992).

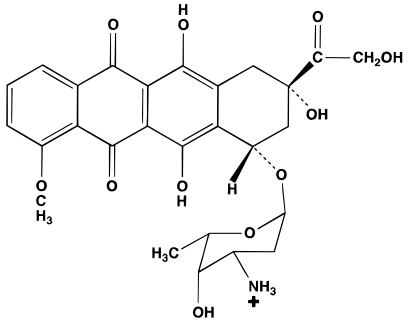

FIGURE 1: Molecular structure of doxorubicin. The p*K* of the charged group is 8.3.

Most studies of the interaction of doxorubicin with negatively charged phospholipids have been performed in model membranes composed of negatively charged phospholipids only. Few studies have been performed with mixtures of anionic and zwitterionic phospholipids, invariably using phosphatidylcholine (PC)[1] as the model zwitterionic

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[1] Abbreviations: CF, carboxyfluorescein; CL, cardiolipin from bovine heart; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPE-NMe, 1,2-dioleoyl-*sn*-glycero-3-phospho-*N*-methylethanolamine; DOPE-N(Me)$_2$, 1,2-dioleoyl-*sn*-glycero-3-phospho-*N*,*N*-dimethylethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-N,*N*,*N*′,*N*′-tetraacetic acid; LUVET, large unilamellar vesicles prepared by extrusion; PI, phosphatidylinositol from soybean; Pipes, piperazine-1,4-diethanesulfonic acid.

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phospholipid (Burke & Tritton, 1985a,b; De Wolf et al., 1991a,b). However, in biological membranes, anionic phospholipids often occur in the presence of the zwitterionic phospholipid phosphatidylethanolamine (PE). For instance, the major negatively charged phospholipid of the plasma membrane of erythrocytes, phosphatidylserine (PS), is exclusively located in the inner leaflet, where it comprises 20% of the phospholipid fraction. PE and PC comprise 35.2 and 12.5% of the phospholipids in this leaflet, respectively (Gennis, 1989; Op den Kamp, 1979). In the inner mitochondrial membrane, the major negatively charged phospholipid is cardiolipin (CL), which is a target for doxorubicin (Nicolay et al., 1984). For rat liver mitochondrial inner membranes, the ratio of PC/PE/CL as percentage of the total phospholipids is 39.2/43.4/13.7 (Hovius et al., 1990).

Whether the interaction of doxorubicin with anionic phospholipids is different in the presence of PE instead of PC is not known, but previous studies with model membranes composed of phospholipids isolated from *Escherichia coli* composed of 71% PE and 29% anionic phospholipids show an increased binding of doxorubicin compared to model membranes composed of 75% PC and 25% PG (De Wolf et al., 1993), suggesting an interaction-enhancing effect of PE over PC. Therefore, the interaction of doxorubicin with model membranes composed of anionic phospholipids and various zwitterionic phospholipids was studied in detail. The striking observation was made that interaction of doxorubicin to anionic phospholipids was greatly enhanced in the presence of PE compared to other zwitterionic lipids for all negatively charged phospholipids tested.

MATERIALS AND METHODS

Materials. Doxorubicin (Pharmachemie, Haarlem, The Netherlands) was shown to be pure by high-performance thin-layer chromatography (Nicolay et al., 1984) and dissolved just before use. Phospholipids were obtained from Avanti Polar-Lipids Inc., USA, and checked for purity by thin-layer chromatography. Cardiolipin was from bovine heart, and phosphatidylinositol from soybean. 6-Carboxyfluorescein was obtained from Kodak, USA, and purified according to Smaal et al. (1986).

Preparation of Large Unilamellar Vesicles (LUVET). LUVET used in the binding experiments and carboxyfluorescein efflux measurements were prepared in Pipes buffer (10 mM Pipes, 100 mM NaCl, 1 mM EGTA, pH 7.4) by 10 times extrusion through polycarbonate filters with 400 nm pores (Costar-Nucleopore Europe, Badhoevedorp, The Netherlands) as described previously (Speelmans et al., 1994). Extrusion was performed at room temperature for all LUVET except for LUVET containing DEPE and DEPC. In this case, the temperature was kept at 40 °C.

Binding Assays. Doxorubicin binding to LUVET was assayed as described by De Wolf et al. (1991b) and Speelmans et al. (1995) by mixing 100 μL of vesicle suspensions (10 mM phospholipid) with 900 μL of a doxorubicin solution in the range of 25−2000 μM (final concentration) (both in Pipes buffer). After incubation of duplicate samples in the dark at room temperature for 1−2 h, the LUVET and bound doxorubicin were pelleted (60 min at 435000*g* and at 20 °C). DEPE and DEPC containing LUVET were incubated at 40 °C. Taking into account the rate of passive diffusion of doxorubicin across the membrane, an incubation period of 1−2 h is sufficient to have both leaflets of the membrane exposed to the drug (Speelmans et al., 1994). The top 800 μL was collected for determination of free drug. The amount of phospholipid in this supernatant fraction was negligible. Blanks without lipids were used to determine the amount of total drug. The concentration of doxorubicin was determined after dilution to 5−10 μM drug, by light absorption at 480 nm using an extinction coefficient of 1.06×10^4 M^{-1} cm^{-1}. Binding experiments were performed at room temperature and at pH 7.4, a value at which doxorubicin hydrolysis is negligible.

Monolayer Experiments. Monolayer experiments were performed as described by De Wolf et al. (1991a). Pipes buffer (pH 7.4) was used in the subphase, and the lipids were spread from solutions in CH_3Cl. Surface area increases were measured at room temperature and at a constant surface pressure of 30 $mN \cdot m^{-1}$. Equilibration at a given doxorubicin concentration was allowed to take place for at least 20 min.

Other Methods. Leakage of 6-carboxyfluorescein was determined as described previously (De Wolf et al., 1991b) in Pipes buffer, pH 7.4. Phospholipids were quantified based on the amount of phosphate according to Rouser et al. (1970).

RESULTS

Doxorubicin Binding to Large Unilamellar Vesicles (LUVET) Composed of Phosphatidylserine and/or Zwitterionic Phospholipids. Binding was studied of doxorubicin to large unilamellar vesicles prepared by extrusion (LUVET) containing the negatively charged phospholipid phosphatidylserine (DOPS) and/or the zwitterionic phospholipids phosphatidylcholine (DOPC) or phosphatidylethanolamine (DOPE). LUVET composed of pure PS bind doxorubicin efficiently (Figure 2) with a saturation level of about 1.6 mol of doxorubicin bound per mole of phospholipid (Figure 2A) and an apparent affinity, expressed as the concentration of free doxorubicin at which 50% of the total lipid binding sites are occupied, of 35 μM (De Wolf et al., 1991b; Figure 2C). Interaction of doxorubicin with LUVET composed of 100% DOPC or mixtures of DOPC/DOPE (1/1) is low (Figure 2). In these model membrane systems, an apparent affinity, saturation binding level, or existence of positive interaction cannot be deduced because of this low interaction. Membranes composed of pure DOPE were not employed because it is not possible to obtain stable LUVET from DOPE due to the tendency of DOPE to adopt nonbilayer structures. Subsequently, LUVET composed of mixtures of DOPS/DOPC or DOPS/DOPE (1/3) were examined. The latter vesicles can be formed because DOPS stabilizes the bilayer organization of DOPE. A comparable saturation level of doxorubicin binding was observed in these model systems. About 0.4 mol of doxorubicin was bound per mole of phospholipid in the case of DOPS/DOPE and about 0.3 mol of doxorubicin was bound per mole of phospholipid in the case of DOPS/DOPC (Figure 2A). Expressed as moles of doxorubicin bound per mole of negatively charged phospholipid, these values are 1.6 and 1.2, respectively, which is comparable to the value observed for LUVET composed of pure DOPS. However, a dramatic difference in apparent affinity was demonstrated. Assuming the saturation levels as mentioned above, the apparent binding affinity was 500 μM for DOPS/DOPC LUVET and 40 μM for DOPS/DOPE LUVET (Figure 2C). From Figure 2B, it is clear that

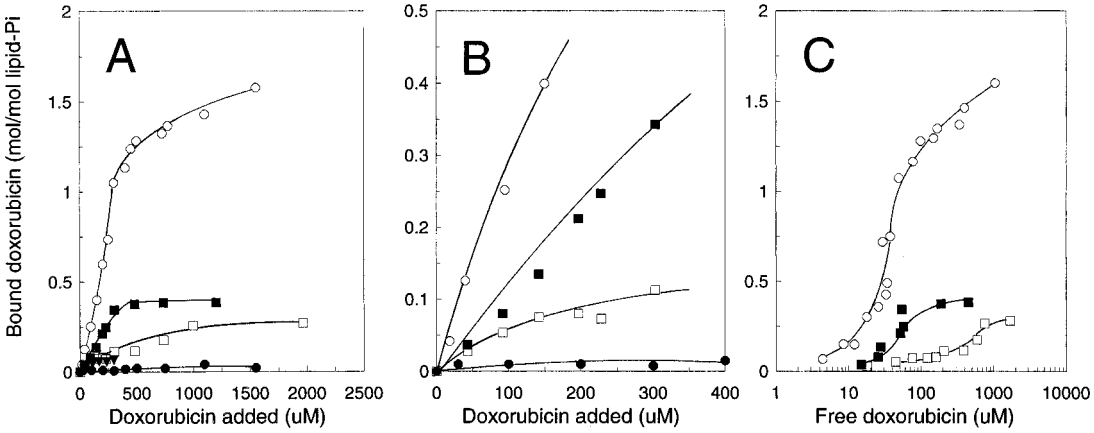

FIGURE 2: Binding of doxorubicin to LUVET composed of various phospholipids. (A) Saturating stoichiometries. (B) Binding at low and intermediated concentrations of doxorubicin. (C) Binding affinities. LUVET were composed of DOPS (○), DOPC (●), DOPE/DOPC 1/1 (▼), DOPS/DOPC (1/3) (□), and DOPS/DOPE (1/3) (■). Binding studies were performed as described under Materials and Methods. The results show mean values of 2−5 experiments. The standard deviation is below 10%.

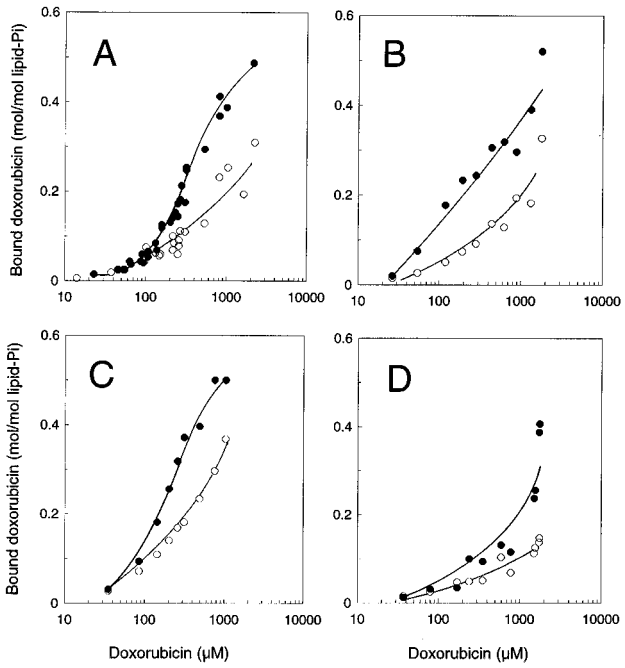

FIGURE 3: Binding of doxorubicin to LUVET containing various anionic phospholipids and either DOPE or DOPC. LUVET contained 75% DOPE (●) or 75% DOPC (○), and (A) 25% DOPG, (B) 25% CL, (C) 25% DOPA, and (D) 25% PI. Binding studies were performed as described under Materials and Methods. The results show mean values of 2−5 experiments. The standard deviation is below 10%. The *x*-axis represents the amount of total doxorubicin added.

especially at low and intermediate drug concentrations, in the range from 0.1 to 1 mM, an enhanced interaction of doxorubicin to DOPS/DOPE is observed compared to DOPS/DOPC.

Doxorubicin Binding to LUVET Composed of Various Anionic Phospholipids and Zwitterionic Phospholipids. It was examined whether the increased interaction of doxorubicin to DOPS in the presence of DOPE instead of DOPC is also observed for other negatively charged phospholipids. We therefore tested the negatively charged phospholipids phosphatidylglycerol (DOPG) (Figure 3A), cardiolipin (CL) (Figure 3B), phosphatidic acid (DOPA) (Figure 3C), and phosphatidylinositol (PI) (Figure 3D). For all negatively charged phospholipids tested, binding was higher in the presence of DOPE than in the presence of DOPC. The

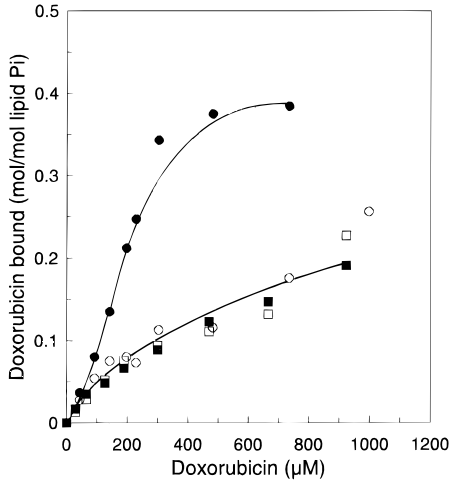

FIGURE 4: Binding of doxorubicin to LUVET containing DOPS and zwitterionic phospholipids. LUVET were composed of 25% DOPS and 75% DOPC (○), DOPE (●), DOPE-NMe (■), and DOPE-N(Me)$_2$ (□). Binding studies were performed as described under Materials and Methods. The results show mean values of 2−5 experiments. The standard deviation is below 10%. The *x*-axis represents the amount of total doxorubicin added.

saturation level could not be achieved. Therefore, an apparent affinity cannot be deduced. The difference in interaction is especially observed for doxorubicin concentrations from 0.1 to 1 mM, except for PI where a difference is observed at high drug concentration only.

Subsequently, binding of doxorubicin to LUVET composed of 25% PS and 75% of various zwitterionic phospholipids was investigated. DOPE, DOPC, and the intermediate phospholipids monomethylphosphatidylethanolamine (DOPE-NMe) and dimethylphosphatidylethanolamine [DOPE-N(Me)$_2$] were employed. Figure 4 shows that the highest binding is observed to LUVET containing DOPE. LUVET containing DOPE-NMe and DOPE-N(Me)$_2$, however, do not show a binding behavior intermediate between DOPE and PC, but show exactly the same interaction as LUVET containing DOPC (Figure 4).

Monolayer Experiments. Monolayer experiments can give insight into the mode of interaction of the drug, in particular the ability to insert in between the acyl chains. Therefore, interaction of doxorubicin with negatively charged phospholipids in the presence of DOPE or DOPC was also

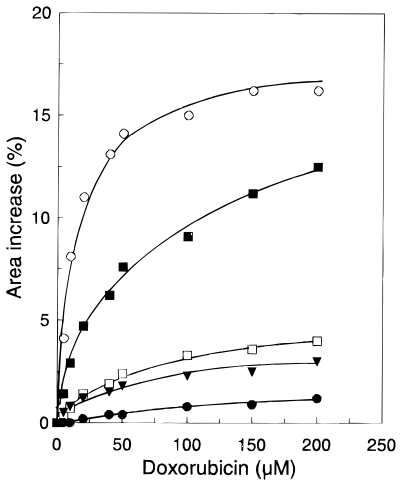

FIGURE 5: Doxorubicin-induced area increase in monolayers at a constant surface pressure of 30 mN·m^{-1}. Monolayers were composed of DOPS (○), DOPE (▼), DOPC (●), DOPS/DOPC (1/3) (□), and DOPS/DOPE (1/3) (■).

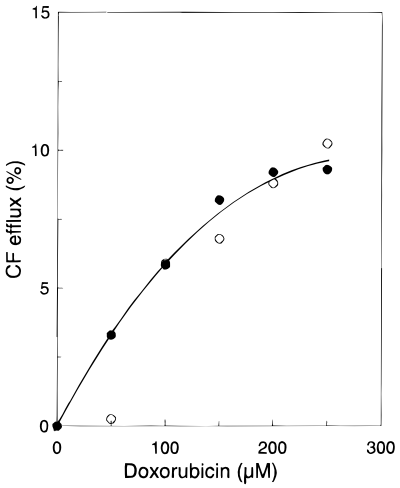

FIGURE 6: Doxorubicin-induced carboxyfluorescein efflux of LUVET. LUVET were composed of DOPG/DOPC (1/3) (○) or DOPG/DOPE (1/3) (●).

examined using the monolayer technique. In the experiments shown in Figure 5, drug penetration was monitored by the drug-induced increase of the monolayer area at constant surface pressure (30 mN·m^{-1}) in analogy to De Wolf et al. (1991a). The area increase is highest when pure DOPS monolayers are employed and lowest in monolayers of pure DOPC and DOPE, although DOPE shows a slightly higher interaction than DOPC. In mixtures of DOPS/DOPC (1/3), the area increase is 24% of that of pure DOPS at a doxorubicin concentration of 200 μM, and this can be entirely explained by the interaction with DOPS. Interestingly, the area increase in monolayers of a DOPS/DOPE mixture (1/3) is much higher, namely, 76% of that of pure DOPS at a drug concentration of 200 μM, than expected based on the behavior of the monolayers composed of the pure lipids. This indicates either that more molecules of doxorubicin are inserted into the membrane to the same extent or that the same amount of doxorubicin molecules insert to a greater extent into monolayers composed of DOPS/DOPE than of DOPS/DOPC. Since under these conditions more moles of doxorubicin are bound per mole of phospholipids (Figure 2), the former explanation is the most likely.

Enhanced Interaction of Doxorubicin with Anionic Lipids in the Presence of PE Compared to PC Is Not Due to the Formation of Nonbilayer Structures by PE. In the experiments described so far, DOPE was used as a representative of naturally occurring phosphatidylethanolamines. These, like DOPE, have a strong tendency to form inverted nonbilayer phases. It can therefore be questioned whether the strong interaction between mixed PE/anionic lipids systems is related to the introduction of nonbilayer structures in the system. To test this hypothesis, we studied the barrier properties of the vesicles using a carboxyfluorescein (CF) leakage assay. If, during the doxorubicin vesicle interaction, nonbilayer structures would be formed, this should cause a loss of barrier function and result in an immediate efflux of CF. Therefore, LUVET were enclosed with CF, and the efflux of CF was measured upon addition of doxorubicin, using the same conditions as during the binding experiment, i.e., with the same incubation period, lipid concentration, temperature, and centrifugation step. In the presence of increasing amounts of doxorubicin, an increased efflux of CF is observed from LUVET composed of DOPG/DOPC (Figure 6). This is due to the interaction of doxorubicin with the negatively charged phospholipids (De Wolf et al., 1991b) and not to the formation of nonbilayer strucures, since this lipid mixture does not show an ability to adopt nonbilayer structures. In LUVET composed of DOPG/DOPE, the same extent of carboxyfluorescein efflux was observed as in LUVET composed of PG/PC (Figure 6), demonstrating that only the interaction of doxorubicin with negatively charged phospholipids is involved in carboxyfluorescein efflux and that additional mechanisms, such as the occurrence of nonbilayer structures, are not involved.

Another approach to establish whether the increased interaction of doxorubicin to LUVET containing DOPE instead of DOPC was due to the presence or ability to form inverted nonbilayer phases in the case of DOPE was the use of dielaidoylphospholipids. The transition temperature between the liquid-crystalline bilayer phase and the inverted hexagonal phase is 55 °C for DEPE, instead of 8 °C for DOPE. Therefore, membranes containing this lipid have a much lower tendency to form nonbilayer structures at room temperature than membranes containing DOPE. When binding of doxorubicin to LUVET composed of 25% DOPG and 75% of either DEPE or DEPC was determined, it appeared that again binding to LUVET containing DEPE was higher than to LUVET containing DEPC (Figure 7).

DISCUSSION

The key result of this study is the finding that the interaction of the anti-cancer drug doxorubicin with model membranes containing mixtures of various anionic and various zwitterionic phospholipids is highest when phosphatidylethanolamine is employed as zwitterionic phospholipid. This enhanced interaction was observed in binding as well as in monolayer studies and was observed when DOPE was combined with all anionic phospholipid species tested.

Our data strongly suggest that the mechanism of this enhanced interaction in the presence of PE over PC does not involve the formation of nonbilayer structures in the case of PE. The tendency of PE to form nonbilayer structures is also not involved. This conclusion can be drawn from the experiments with the dieilaidoyl phospholipids and by comparing the results with DOPC and DOPE-NMe. Both

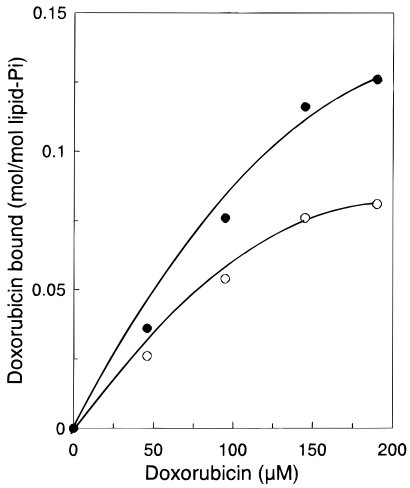

FIGURE 7: Binding of doxorubicin to LUVET containing phospholipids with dielaidoyl fatty acyl chains. LUVET were composed of DOPG/DEPE (1/3) (●) or DOPG/DEPC (1/3) (○). Binding studies were performed as described under Materials and Methods. The results show mean values of 2−5 experiments. The standard deviation is below 10%. The *x*-axis represents the amount of total doxorubicin added.

lipids show the same interaction with doxorubicin, whereas DOPE-NMe is able to form nonbilayer structures at 73 °C (Siegel et al., 1989) and DOPC is not. Several other hypotheses can be conceived. For instance, the enhanced interaction of doxorubicin with anionic phospholipids in the presence of PE compared to other zwitterionic phospholipids might involve the formation of hydrogen bonds. The ethanolamine part of the headgroup of PE contains a primary amine which can in potential form hydrogen bonds, whereas the headgroup of PC, choline, contains a quaternary amine, which does not have this property. In this hypothesis, the formation of hydrogen bonds between PE and doxorubicin is involved in the increased binding at nonsaturating levels of the drug. However, this cannot be the entire explanation, since DOPE-NMe and DOPE-$N(Me)_2$ do not show an intermediate effect. An alternative or additional explanation involves the effect of the size of the headgroup. The small headgroup of PE and the effect of this on the intrinsic curvature of the membrane might favor binding of more molecules of doxorubicin into the membrane. Apparently, in both hypotheses, the presence of one or two methyl groups in DOPE-NMe and DOPE-$N(Me)_2$, respectively, sterically hinders this enhanced interaction.

Interestingly, an enhanced interaction of membrane-associated molecules with anionic phospholipids present in membranes together with PE compared to PC is also observed in other studies. This observation is made for the signal sequence of prePhoE of *E. coli* (Van Raalte et al., 1996), the precursor of the chloroplast protein ferredoxin (Van't Hof & De Kruijff, 1995), and for the periplasmic domain of the leader peptidase of *E. coli* (van Klompenburg, submitted for publication). Interestingly, PE acts as a chaperone for the lactose permease of *E. coli* in enabling this protein to obtain its correct conformation in the membrane, whereas PC is unable to do this (Bogdanov et al., 1996). Apparently PE plays a more general role in the interaction between membranes and membrane-associated proteins and drugs.

Our data indicate that studies obtained with model membranes containing mixtures of anionic lipids and PC instead of PE underestimate the effect of doxorubicin with biological membranes. For *E. coli* cytoplasmic membranes and membranes prepared from lipids extracted from *E. coli*, indeed a higher interaction with doxorubicin is observed compared to model membranes composed of PC (De Wolf et al., 1993). This higher interaction can now be explained, at least partially, because of the presence of PE in the membranes. This observation may also have implications for two target membranes of doxorubicin, the inner mitochondrial membrane and the plasma membrane. The inner mitochondrial membrane contains as major negatively charged phospholipid cardiolipin (CL). Interaction of doxorubicin with CL disturbs the process of oxidative phosphorylation and is a possible cause of the most severe side-effect of doxorubicin, namely, cardiotoxicity (Nicolay et al., 1984). In this membrane, PE is present in equal or even slightly higher amounts than PC (Hovius et al., 1990). Therefore, interaction of doxorubicin with cardiolipin is probably higher than predicted from studies with model membranes containing PC (Nicolay et al., 1984; Goormaghtigh et al., 1980a,b, 1982). The plasma membrane determines the access of the drug to the internal target, the DNA. Anionic phospholipids, the major being phosphatidylserine (PS), are exclusively located in the inner leaflet. Interestingly, PE is also preferentially located in the inner leaflet (Op den Kamp, 1979). A high interaction of doxorubicin with headgroups of phospholipids decreases its passive diffusion (Speelmans et al., 1994, 1995). Studies with model membranes predict that the location of doxorubicin in the membrane is at the headgroup region of the inner leaflet. Our results demonstrate that the concentration at this location is even higher than anticipated from studies using model membranes. This is an interesting finding since strong indications are present that multidrug resistance conferring enzymes, such as the P-glycoprotein, extrude drugs from this compartment (Homoloya et al., 1993; Raviv et al., 1990; Shapiro & Ling, 1995).

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