Transport Studies of Doxorubicin in Model Membranes Indicate a Difference in Passive Diffusion across and Binding at the Outer and Inner Leaflets of the Plasma Membrane†

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ABSTRACT: The kinetics of passive transport of the anticancer drug doxorubicin were analyzed in relation to membrane composition in large unilamellar vesicles in which DNA was enclosed. Special attention was paid to lipids that are typical for the inner and outer leaflet of the plasma membrane of mammalian cells: Phosphatidylethanolamine and anionic phosphatidylserine versus phosphatidylcholine, sphingomyelin, and cholesterol, respectively. The presence of anionic phospholipids results in a highly efficient incorporation of the drug into biological and model membranes [de Wolf, F. A., et al. (1993) *Biochemistry 32,* 6688−6695]. Therefore, the effect of drug binding on the amount of free, transportable drug was explicitly taken into account. However, even after correction for binding the permeability coefficient was about 35% lower in membranes containing 50 mol % of the anionic phosphatidylserine than in membranes consisting only of zwitterionic phospholipids (0.71−0.79 versus 1.18−1.25 μm s^{-1}). This shows that drug binding and insertion also affect the intrinsic transport characteristics of the membranes. As compared to pure phosphatidylcholine, binding was not influenced by the incorporation of sphingomyelin or cholesterol, but equimolar amounts of sphingomyelin and cholesterol in phosphatidylcholine membranes decreased the rate of doxorubicin transport by 60% and 80%, respectively. The inhibitory effect of these two lipids is probably due to a closer packing of the membranes. In accordance, after the acyl chain order was decreased by adding the anaesthetic-like phenethyl alcohol (0.5% v/v), transport was stimulated more than 4-fold. The implications of our findings for the functioning and rate of drug pumping by the multidrug resistance-conferring P-glycoprotein in cancer cells are discussed.

Doxorubicin is a potent and widely applied anticancer agent exhibiting effects on DNA and DNA-associated enzymes (Chaires et al., 1985; Capranico et al., 1990) as well as on membranes [for a review, see Tritton (1991)]. In membranes, anionic phospholipids are important targets (Nicolay et al., 1984; Escriba et al., 1990; de Wolf et al., 1991a,b, 1993), and membrane binding involves electrostatic interaction as well as penetration of the electrostatically bound drug between the acyl chains (de Wolf et al., 1991b, 1993). The uncharged form of the drug (which is a weak base with a pK_a of 8.3) is transported via passive diffusion across the membrane (Mayer et al., 1986; Frézard & Garnier-Suillerot, 1991b).

Knowledge of parameters that govern the rate of this passive diffusion of doxorubicin across membranes is essential for several reasons. First, import of doxorubicin into the cell is accomplished by passive influx of which the driving force is a pH gradient (inside acid) (Harrigan et al., 1993) and the concentration gradient. The latter is initially maintained at a high level by the intracellular binding of the drug to DNA (Chaires et al., 1985; Capranico et al., 1990), RNA (Nicolay et al., 1984; Simpkins at al., 1984), and intracellular membranes (de Wolf et al., 1993) and by accumulation in acidic organelles (Willingham et al., 1986). Because of the large number of membranes present in a cell, both drug-binding and drug-transport properties of the membranes are essential determinants of the distribution of free and bound drug in the many intracellular compartments (de Wolf et al., 1993). Secondly, doxorubicin encapsulated in liposomes is at an increasing frequency used in chemotherapy (Leyland-Jones, 1993). A fast, stable, and efficient loading of the drug, facilitated for instance by the application of a pH gradient (inside acid), and a slow and/or selective release of the drug (Harrigan et al., 1993) once the liposomes are in the bloodstream is of clinical importance. Thirdly, in multidrug-resistant (mdr)[1] cells the distribution of doxorubicin is modulated by active, protein-mediated drug extrusion (Juliano & Ling, 1976; Horio et al., 1988; Frézard & Garnier-Suillerot, 1991c; Spoelstra et al., 1992). The ever increasing clinical importance of multidrug resistance calls for a detailed understanding of the parameters that determine passive as well as active drug transport and the distribution of free and bound drug in the cell.

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[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-morpholinoethanesulfonic acid; LUVET, large unilamellar vesicles prepared by extrusion; mdr, multidrug resistance; PEA, phenethyl alcohol; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; SM, egg yolk sphingomyelin.

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We have focused on model membranes composed of lipids that are considered to be typical for the plasma membrane. The plasma membrane is asymmetric with respect to its protein as well as its lipid distribution [for a review, see Op den Kamp (1981) and Devaux (1991)]. The erythrocyte membrane, which has been studied most extensively with respect to lipid asymmetry, contains 18% SM, 25% PC, 22% PE, 10% PS, and 25% cholesterol (Gennis, 1989). The outer leaflet mainly contains sphingomyelin (SM) and phosphatidylcholine (PC), whereas phosphatidylethanolamine (PE) and the anionic phosphatidylserine (PS) are preferentially present in the inner layer (Op den Kamp, 1979). Approximately 80% of the total SM and 75% of the PC is present in the outer leaflet, whereas 80% of total PE and 100% of PS is present in the inner leaflet. The distribution of cholesterol is controversial (Op den Kamp, 1979), but since cholesterol has a preference for SM (Demel et al., 1977), it may be preferentially present in the outer leaflet. Taken into account this distribution and the observation that the fatty acyl chains of PE and PS are more unsaturated than those of PC and SM (Devaux, 1991), the outer leaflet is more rigid compared to the inner leaflet.

Previous studies on the kinetics of doxorubicin transport largely disregarded the influence of the lipid composition (in particular the influence of drug binding to anionic phospholipids) and of membrane packing (Dalmark & Storm, 1981; Horio et al., 1988; Frézard & Garnier-Suillerot, 1991a,b; Spoelstra et al., 1992). Therefore, we investigated the effect of these parameters on passive transport of doxorubicin across model membranes which consist of lipids typical for the inner and outer leaflet of plasma membranes and which contain DNA. The enclosed DNA acts as a sink for the transported drug (Ramu et al., 1989; Frézard & Garnier-Suillerot, 1991a) and as a model for DNA in the cancer cell. In addition, we were interested in the possibility to modulate doxorubicin transport in cells and liposomes, and so we studied the effect of membrane fluidizers on doxorubicin transport.

MATERIALS AND METHODS

Preparation of Large Unilamellar Vesicles (LUVET) with or without Enclosed DNA. DNA was hydrated in 10 mM 1,4-piperazinediethanesulfonic acid (Pipes), 100 mM NaCl, and 1 mM EGTA, pH 7.4 (buffer A), sonicated (Bransonic B12 sonifier with a 3-mm microtip operated 10 times 30 s at 80 Js^{-1} with 90-s intervals, at 0 °C), and centrifuged for 20 min at 20000*g* to remove metal fragments. The pH of the sonicated DNA was adjusted if necessary and the DNA was extruded 10 times through 200-nm pore polycarbonate filters (Costar-Nucleopore, the Netherlands) and quantified by P_i content (around 40 mM P_i). The molecular size of such sonicated DNA is around 1000 base pairs (Wong et al., 1980).

Films of 60 μmol of lipid-P_i, obtained by evaporation of chloroform stock solutions, were dried overnight over P_2O_5 under vacuum and hydrated by gentle shaking in 2 mL of buffer A with 40 mM (P_i)-DNA or without DNA. After subjection to 10 freeze−thaw cycles using solid CO_2-cooled acetone, large unilamellar vesicles (LUVET) were prepared by subsequent extrusion through polycarbonate filters with 800-, 400-, and 200-nm pores, successively (10 times each). The P_i content of lipid and DNA in samples of the final extruded material were separately determined after an extraction according to Bligh and Dyer. External DNA was removed from the LUVET by application of sucrose gradient centrifugation and DNAse treatment. Thirty-five percent sucrose (w/v) in buffer A was added to the LUVET, to a final concentration of 29% (w/v). After layering 6 mL of the LUVET suspension in sucrose, 5 mL of buffer A with 15% (w/v) sucrose and 1−2 mL of normal buffer on top of each other, the gradient was centrifuged for at least 4 h (145000*g*; Beckmann SW41 Ti rotor), the vesicles were collected from the top 1−2 mL, and the density centrifugation was repeated. The vesicles were washed twice with 30 mL of a buffer in which the Pipes was replaced with Hepes (2 h at 160000*g*; Beckmann Ti60 rotor) and were resuspended in 1−2 mL of Hepes buffer. After degrading traces of nonenclosed DNA during 30 min at room temperature (10 mg of DNAse plus a final concentration of 10 mM $MgCl_2$) the vesicles were washed five times with 3 mL of buffer A (1 h at 540000*g*, TLA 100.3 rotor). Hepes was used because Pipes inhibited the DNAse. The DNAse/$MgCl_2$ treatment did not result in a significant loss of enclosed DNA from PS-containing and other LUVET, and doxorubicin permeabilities of similar magnitude were observed before and after DNAse treatment. "Control" LUVET without DNA were treated in the same way as DNA-LUVET. The ratio of enclosed DNA-P_i/total lipid-P_i was 0.025−0.030 mol/mol, the DNA being quantified by P_i as well as by its quenching effects on the fluorescence of doxorubicin (calibrated with DNA standards mixed with control LUVET and using 0.05 vol % Triton X-100 to permeabilize LUVET) (Frézard & Garnier-Suillerot, 1991a). Both methods yielded the same DNA/lipid ratio.

The particle size and distribution of LUVET with or without DNA was measured by dynamic light scattering. For LUVET composed of DOPC the average size was 147 and 166 nm, with a polydispersity of 0.35 and 0.30, in the absence and presence of enclosed DNA, respectively. For LUVET containing 50% negatively charged DOPS the size was 151 and 153 nm, with a polydispersity of 0.13 and 0.31, in the absence and presence of enclosed DNA, respectively.

Transport Measurements. Doxorubicin fluorescence was measured on an Aminco SPF 500 fluorimeter (excitation 490 nm; emission 594 nm). Data were corrected for light scattering, inner filtering, and drug dilution (de Wolf et al., 1991b), but these were usually negligible. The rate of doxorubicin import into LUVET was monitored at 25 °C by the rate of fluorescence quenching, resulting from interaction with enclosed DNA. The initial rate of doxorubicin transport was determined by on-line fluorometry after addition of DNA-LUVET to a doxorubicin solution. The amount of doxorubicin added was 1 μM, and the amount of vesicles added was between 100 and 200 μM lipid-P_i. DNA-binding data indicated that the enclosed DNA was an efficient sink for doxorubicin.

Calculation of Permeability Coefficients. The rate constant k was calculated as the ratio of the initial transport rate and the initial concentration of external free drug (initial driving force):

$$k = (\Delta F/\Delta t)/[(Q_{out} - Q_{in})F^0 f] \qquad (s^{-1})$$

where $\Delta F/\Delta t$ is the initial rate of fluorescence quenching, Q_{out} is the average quantum yield of the total drug (free plus

bound) outside the LUVET, Q_{in} is the average quantum yield of the total drug in the lumen of the DNA-LUVET, and F^0 is the original fluorescence of the drug before addition of the LUVET. So $Q_{out} - Q_{in}$ is the absolute change of quantum yield of each drug molecule transported from the outside to the inside of the vesicles (relative to F^0) and $(\Delta F/\Delta t)/(Q_{out} - Q_{in})$ is the rate of fluorescence quenching corrected for changes in quantum yield. f is the fraction of the external drug that is free shortly after addition of the LUVET, i.e., when the fraction of drug transported is still negligible.

Drug binding to the membranes is much faster than the rate of drug transport (see Results). This enabled us to determine Q_{out} directly from the fluorescence change upon addition of LUVET and also to determine f and Q_{in} in separate experiments under equilibrium conditions. Because of the small internal volume, a doxorubicin molecule in the vesicle lumen faces high lipid and DNA concentrations (at least 80 and 2 mM on P_i basis, respectively). Since these conditions cannot be mimicked in a cuvette due to high light absorbance and scattering, Q_{in} was determined at two different lipid concentrations in the range of 250−500 μM, after which an extrapolation to infinite high lipid and DNA concentrations was made, using the following formula:

$$(Q_H - (Q_H - Q_L))/(1 - R_{HL}Q_L/Q_H)$$

H and L refer to the highest and lowest lipid concentration, respectively. R_{HL} is the lipid concentration ratio (high/low). Q_H and Q_L are the average quantum yields in mixtures of DNA and control LUVET at the two different concentrations, both at the same DNA-P_i/lipid-P_i ratio. Since it is assumed that in ∅ 200-nm LUVET 47% of the lipids occupy the internal surface, Q_{in} determinations were performed in a mixture of control LUVET and DNA at a DNA/lipid ratio that was a factor 1/0.47 larger than that of the DNA containing LUVET used in the transport experiment. Addition of 0.05% Triton X-100 did not show any effect indicating that all potential binding sites were accessible under these equilibrium conditions.

For determination of the fraction of free drug f, equilibrium binding was assayed at room temperature after dialysis of 2 mL of control LUVET (0.5−1.0 mM P_i) in a Visking tube (size 2, 18/32", Medicell, U.K.) against stirred 200-mL doxorubicin baths, containing 0.3−1.5 μM doxorubicin, during 48 h in the dark under N_2 gas. Equilibrium was shown to be reached in less than 40 h. The bath and the tube were sampled for lipid-P_i (negligible outside of the tube) and doxorubicin. Doxorubicin was quantified by mixing 1 mL of sample with 4 mL of 7 mM SDS in buffer A and comparing the fluorescence to that of standard solutions in SDS (straight calibration curve). At each free drug concentration in the bath, the amount of bound drug per lipid-P_i in the tube was determined:

$$\text{drug}_{bound}/\text{lipid-P}_i\ (\text{mol/mol}) = \{[\text{drug}]_{total}(\text{in tube}) - [\text{drug}]_{free}(\text{in bath})\}/[\text{lipid-P}_i]$$

The dialysis assay has been tested at 10−20 μM free drug, where it yielded the same results as a previously described centrifugation assay (de Wolf et al., 1991a). Since we assumed that 53% of the lipid molecules in the LUVET are at the outer surface (L_{out} = 0.53 × lipid concentration in the cuvette), the equilibrium binding level to the transport experiment corresponding was calculated as

$$[\text{drug}]_{\text{bound in cuvette}} = L_{out}\{\text{drug}_{bound}/\text{lipid-P}_i\ (\text{mol/mol})\}$$

Using L_{out}, the total drug concentration (= $[\text{drug}]_{\text{bound in cuvette}} + [\text{drug}]_{free}$), was calculated and plotted as a function of the free drug concentration used in the dialysis assay. From this plot, the free drug concentration (D_f) that corresponds to the total drug concentration (D_t), used in the actual transport experiment, was determined. The fraction of free drug occurring just after addition of the LUVET to the cuvette, could then be calculated as $f = D_f/D_t$.

The clearance of the drug C_d [$L\ s^{-1}$ (mol lipid)$^{-1}$)] was calculated as k/L_{tot}, where k is the above defined rate constant and L_{tot} is the lipid concentration in the cuvette (on a [P_i] basis). Since only the initial transport rate was taken into account, the internal free drug concentration could be considered to remain negligible with respect to the external free drug concentration. Due to the efficient internal sink for the drug, the system behaved initially as if the internal volume was much larger than the external volume. Under those conditions the permeability coefficient can be approximated as

$$P = (kV_{ex})/A_{tot} = C_d/(aN1000)$$

V_{ex} is the external volume in the cuvette (≈ total volume) (m^3), A_{tot} is the total membrane area available in the cuvette during the transport assay (m^2), a is the area per lipid-P_i molecule (m^2), N is Avogadro's number (6.022×10^{23} P_i atoms per mol lipid), and P (in $\mu m\ s^{-1}$) equals [$C_d 1.66 \times 10^{-21}/a$]. Assuming that two opposing phospholipid molecules in the bilayer cover an area of 0.63 nm^2 (Demel et al., 1975), i.e., 0.315 nm^2 (3.15×10^{-19} m^2) per P_i, the permeability coefficient P ($\mu m\ s^{-1}$) becomes $5.271 \times 10^{-3} C_d$. The area of 0.315 nm^2 was used for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. For sphingomyelin an area of 0.24 nm^2 was assumed (Harmony et al., 1981). In the presence of cholesterol in the bilayer the effective area per lipid-P_i was increased by an amount of 0.18 nm^2 per cholesterol molecule (Demel et al., 1972).

The values of C_d and P are not calculated specifically for the uncharged form of the drug. The specific C_d^0 and P^0 for the uncharged drug are calculated to be 8.94-fold larger than C_d and P, the fraction of uncharged doxorubicin being $1/(1 + 10^{(pK_a - pH)})$, i.e., 11.2% of the free drug for a pK_a of 8.3 (Sturgeon & Schulman, 1977).

Other Methods. Lipids and DNA were separated by a Bligh−Dyer extraction (Bligh & Dyer, 1959). P_i was assayed by the method of Rouser et al. (1970). The amount of cholesterol incorporated in the LUVET after extrusion was determined as described (Huang et al., 1961) after a Bligh−Dyer extraction.

Materials. Doxorubicin (Pharmachemie, Haarlem, The Netherlands) was shown to be pure by high-performance thin-layer liquid chromatography (Nicolay et al., 1984) and was dissolved just before use and quantified as described (de Wolf et al., 1991a). DNA (type XIV, from herring testes, or type I from calf thymus) and DNAse I, type DN-25, from bovine pancreas, were obtained from Sigma, St. Louis, MO. Phospholipids were obtained from Avanti Polar-Lipids Inc., Birmingham, AL, and checked for purity by thin-layer chromatography. Cholesterol was from Merck, Darmstadt, Germany.

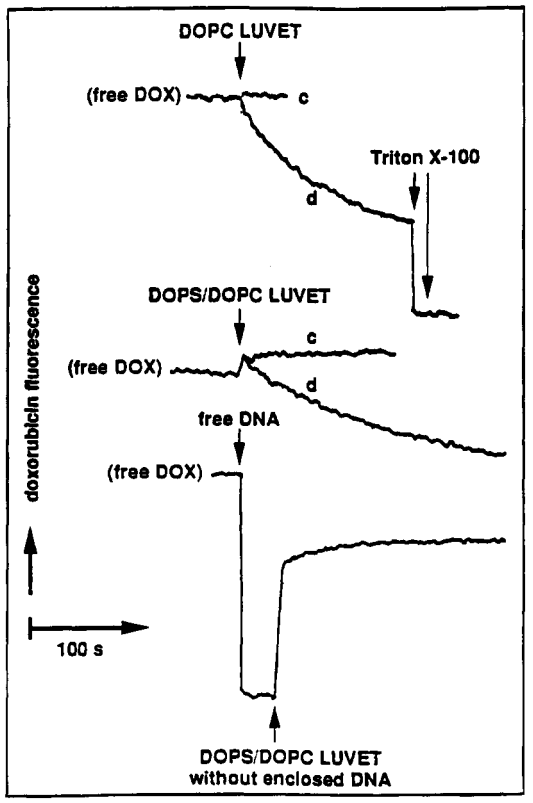

FIGURE 1: On-line recording of the quenching of doxorubicin fluorescence by DNA enclosed in LUVET (d) consisting of DOPC (top trace) or an equimolar DOPS/DOPC mixture (middle trace). (c) Control LUVET without enclosed DNA. Final concentrations are as follows: Doxorubicin, 1 μM, total lipid-P_i, 184 μM, Triton X-100, 0.05% (v/v). (Lower trace) Fast doxorubicin binding to DNA (4 μM Pi) and membranes (580 μM P_i) monitored by fluorescence. The arrow on the *Y*-axis corresponds to 14% of the total initial fluorescence. Transport measurements were performed at 25 °C.

RESULTS

Transport of Doxorubicin across Model Membranes. Method Establishment. Figure 1 shows examples of doxorubicin fluorescence changes that were elicited by addition of DNA-free (traces c) or DNA-containing LUVET (traces d). Only addition of DNA-containing LUVET induced a slow fluorescence quenching, due to interaction of translocated doxorubicin with enclosed DNA. Permeabilization with Triton X-100 resulted in a fast access to the DNA as indicated by the immediate fluorescence quenching. Triton X-100 itself did not induce quenching, since a second addition did not elicit further changes (Figure 1) and since 0.05−0.1% Triton X-100 had no significant effect on doxorubicin fluorescence in control LUVET without DNA (data not shown).

Depending on the experimental conditions, addition of DNA-containing or control LUVET to free doxorubicin also elicited a fast initial fluorescence change (for example, Figure 1, middle traces). Previous studies on doxorubicin−lipid fluorescence changes showed that such fluorescence changes are associated with an incorporation of the drug in the membrane and that the direction and amplitude of the changes depend on the concentrations of the drug and lipid (de Wolf et al., 1991a,b). For example, a fast quenching could be elicited with low amounts of control LUVET that consisted largely of anionic phospholipid (data not shown). The fast initial fluorescence change was identical for DNA-containing and control LUVET at equal lipid concentration (Figure 1, middle traces), showing that external DNA had been efficiently removed.

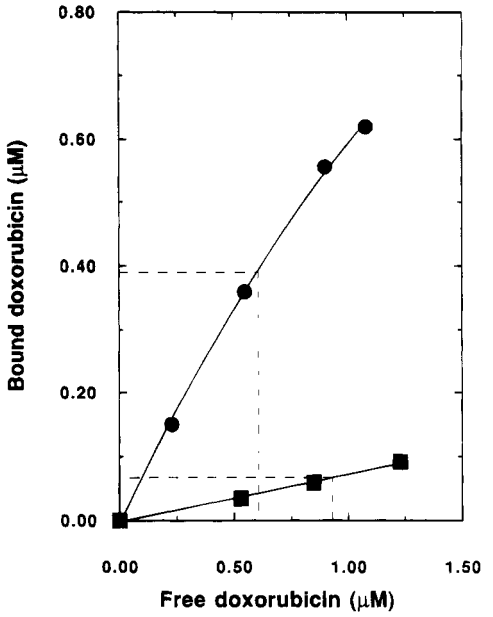

FIGURE 2: Binding to LUVET of DOPC (■) or equimolar DOPS/DOPC mixtures (●) without enclosed DNA, under the initial conditions of Figure 1. The binding levels (μM doxorubicin) were calculated from the experimentally determined equilibrium binding stoichiometries obtained with the dialysis assay (mol of doxorubicin bound per mol of lipid), for a total lipid concentration of 98 μM, i.e., 53% of the concentration in Figure 1. Assuming that the binding equilibrium was reached very soon after the addition of vesicles in Figure 1 (see text) and that 53% of the lipid molecules were facing the external compartment, and noting that the total drug concentration in Figure 1 was 1 μM, the initial binding of the drug to the outside of the DOPC and DOPC/DOPS vesicles in Figure 1 can be read from Figure 2.

After addition of control LUVET to a doxorubicin/DNA mixture (Figure 1, lower trace) a fast redistribution of doxorubicin between membranes and DNA occurred, demonstrating that the rate of binding to both lipid and DNA was high with respect to the transport rate. The high rate of binding (compared to the slow rate of transport) allowed us to directly measure the average quantum yield of the drug outside the LUVET and to estimate (from equilibrium data in separate experiments) the fraction of drug bound to the outer surface of the LUVET immediately after the addition of the vesicles (Figure 2) as well as the average quantum yield of the drug reaching the inside of the LUVET. Measurement of these parameters was required for the calculation of the rate constant of passive drug transport, the clearance of the drug, and the permeability coefficient (see Materials and Methods for more details). The addition of the DOPC LUVET resulted in the instantaneous binding of 8% of the external drug to the outer membrane surface (Figure 2 and Table 1). The Q_{in} for DNA-containing DOPC LUVET was 0.05, i.e., the fluorescence of doxorubicin in the vesicle lumen (in the presence of DNA and membranes) is 5% of the fluorescence of doxorubicin in buffer.

Preincubation of the LUVET with the protonophore FCCP or the Na^+/H^+ exchanger monensin did not have an effect on the initial transport rate of doxorubicin, indicating that transport was not inhibited by the immediate generation of a pH gradient (inside alkaline) due to protonation of doxorubicin inside the LUVET (data not shown).

Table 1: Different Parameters Determined To Calculate the Rate of Transport of Doxorubicin in DNA-Containing LUVET Composed of Various Phospholipids

lipid composition (mol/mol)	Q_{out}[a]	Q_{in}[a]	f[a,b]
DOPC	1.07	0.05	0.92
DOPS/DOPC (1/1)	1.15	0.19	0.65
DOPE/DOPC (1/1)	1.06	0.06	0.85
DOPS/DOPE (1/1)	1.05	0.13	0.62
SM/DOPC (1/1)	1.09	0.07	0.85
cholesterol/DOPC (1/3)	1.03	0.06	0.90
cholesterol/DOPC (2/3)	1.05	0.08	0.90
cholesterol/DOPC (3/2)	1.08	0.08	0.85
DOPC + 0.5% PEA	1.07	0.06	0.91

[a] The external quantum yield, Q_{out}, the internal quantum yield, Q_{in}, and the fraction of free drug, f, were determined as described under Materials and Methods at 25 °C. The standard deviation for Q_{out} determined was less than 2% ($n = 6-10$). The standard deviation in the determination of the fraction of free drug was 2−3%. For the determination of Q_{in} a standard deviation of 15−20% was obtained. The deviation between different liposome preparations ($n = 2-6$) of the same phospholipid composition was within 10% for Q_{out}, f, and P^0 and within 20% for Q_{in} determinations. [b] For the sake of comparison the f values in this table correspond to a fixed total lipid concentration of 200 μM P_i for each of the lipid mixture. In reality, the experiments were repeated at several values of lipid-P_i (and thus of f) for each of the lipid mixtures.

Transport of Doxorubicin across Model Membranes Composed of Inner Leaflet Phospholipids. Effect of Binding of Doxorubicin to Anionic Phospholipids. Two major phospholipid components of the inner leaflet of the plasma membrane are the zwitterionic phosphatidylethanolamine (PE) and anionic phosphatidylserine (PS). In view of the specific interaction of doxorubicin with anionic lipids and the resulting membrane incorporation of the drug, we first studied the possible effects of this binding on doxorubicin transport. As a reference membranes composed of (the outer lipid) DOPC were chosen, since it is known from previous studies that the interaction of doxorubicin with DOPC is weak. As compared to DOPC LUVET, drug binding to mixed DOPS/DOPC LUVET was more pronounced (Figure 2), and transport was decreased (Figure 1, middle traces). If we do not correct our data for drug binding, the calculated apparent permeability coefficient is nearly a factor 2.5 lower in membranes with 50% DOPS than in membranes of pure DOPC. This effect is partly due to the decreased availability of free drug due to binding. (Figure 2 and Table 1). Even after a correction for binding a significant difference in permeability of doxorubicin between DOPS/DOPC and pure DOPC membranes persists (see Figure 3), namely, 0.71 and 1.25 μm s^{-1}, respectively.

Q_{in} was higher in equimolar DOPS/DOPC (0.19) than in pure DOPC LUVET (0.05) (Table 1), because the anionic lipid apparently competed more effectively with the DNA for binding of the drug.

The permeability coefficient of doxorubicin across LUVET composed of 50% DOPC and 50% of the other typical inner leaflet lipid PE is shown in Figure 3. A minor, probably insignificant, effect on the rate of doxorubicin transport was observed as compared to pure DOPC LUVET. The fraction of free drug was somewhat lower in DOPE containing LUVET, whereas the Q_{out} and Q_{in} were comparable to the DOPC containing LUVET (Table 1). LUVET composed of 25% DOPE and 75% DOPC showed the same results as 50% DOPE/50% DOPC LUVET (data not shown). No attempts were made to prepare LUVET with a higher percentage DOPE, due to their tendency to form nonbilayer structures.

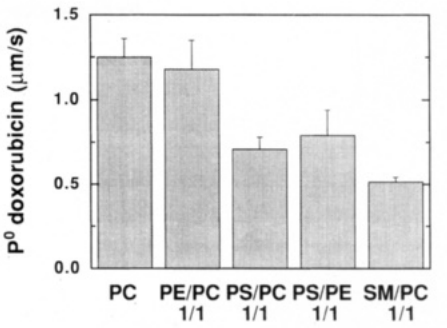

FIGURE 3: Permeability coefficients, P^0, for doxorubicin transport across model membranes composed of different phospholipids. The experiments were performed as shown in Figure 1, and P^0 was determined as described under Materials and Methods (see Table 1 for the corresponding values of Q_{in}, Q_{out}, and f). The average value of P^0 and standard deviation of six experiments is given.

Finally, LUVET composed of equimolar amounts of DOPS and DOPE, mimicking the inner leaflet of the plasma membrane, exhibited the same transport properties as compared to DOPS/DOPC LUVET, indicating again that the transport properties of DOPE and DOPC in the bilayer are very similar. Q_{in}, Q_{out}, and the fraction of free drug were comparable in both preparations (Table 1).

Transport of Doxorubicin across Model Membranes Composed of Outer Leaflet Lipids. Next to phosphatidylcholine, sphingomyelin (SM) and probably also cholesterol are lipids observed mainly in the outer leaflet of the plasma membrane. When 50 mol % sphingomyelin (SM) was incorporated in DOPC membranes, the rate of transport of doxorubicin strongly decreased from 1.25 to 0.51 μs s^{-1} (Figure 3). The effect of SM was not due to an altered Q_{out}, Q_{in}, or fraction of free drug (Table 1) and the zwitterionic SM exhibited the same binding affinities toward doxorubicin as DOPC.

Incorporation of an increasing amount of cholesterol into DOPC LUVET also elicited a strong decrease in the transport rate of doxorubicin. The permeability coefficient decreased from 1.25 to 0.20 μm s^{-1}, when the cholesterol concentration was increased (on a molar basis) from 0 to 60% (Figure 4). Again, Q_{out}, Q_{in}, and the fraction of free drug were not influenced (Table 1). So DOPC, SM, and cholesterol show little interaction (binding) with doxorubicin, and in the presence of the latter two lipids the rate of doxorubicin diffusion is decreased.

Effects of Membrane Fluidizers on Doxorubicin Transport. In order to study the effect of lipid packing on doxorubicin diffusion, the effect of membrane fluidization was examined. From previous work (Jordi et al., 1990) it is known that phenethyl alcohol (PEA) can severely decrease the average membrane packing (acyl chain order). At an increasing amount of PEA from 0 to 0.5% (v/v) the permeability coefficient in DOPC LUVET indeed strongly increased from 1.25 to as much as 5.2 μm s^{-1} (Figure 5). The barrier function of the membrane was still intact at the highest concentration of PEA used in our experiments (0.5%), since an addition of Triton X-100 still elicited an immediate decrease of the fluorescence to the equilibrium level (data not shown).

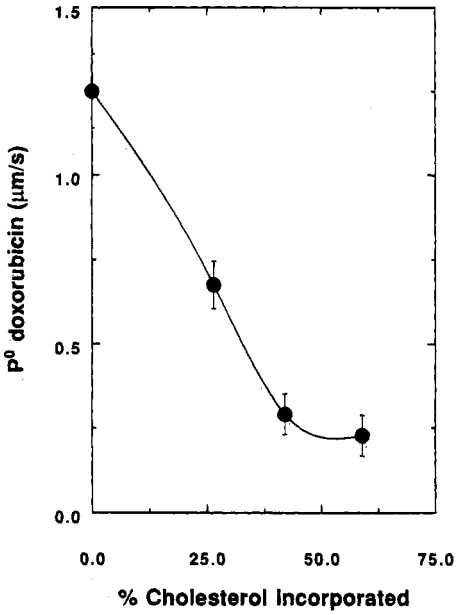

FIGURE 4: Effect of cholesterol on the transport rate of doxorubicin. LUVET consisting of DOPC and an increasing molar concentration of cholesterol were prepared as described under Materials and Methods. Conditions of the transport experiment were as described in the legend to Figure 1. The average value of P^0 and standard deviation of six experiments is given.

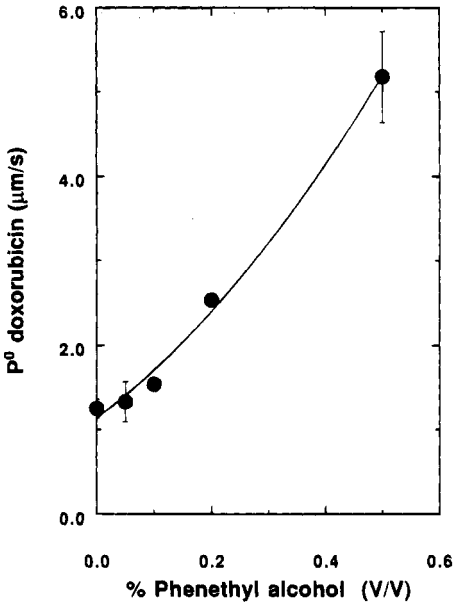

FIGURE 5: Effect of phenethyl alcohol on the transport rate of doxorubicin. LUVET composed of DOPC were prepared as described under Materials and Methods. Phenethyl alcohol was added from a 10% stock solution (v/v) to the cuvette and to the stock solution of LUVET to the final concentrations as shown on the *X*-axis. After a 5-min incubation, transport experiments were performed as described in the legend to Figure 1. The average value of P^0 and standard deviation of six experiments is given.

DISCUSSION

We have studied the effect of membrane composition and packing and the effect of binding of doxorubicin to membranes on the kinetics of passive diffusion of doxorubicin across membrane bilayers. The observed values of P^0 are in good agreement with the overall uptake rates (i.e., binding + transport) previously found in LUVET at high drug concentrations (Mayer et al., 1986). They are close to the *P* values estimated by model parameter fitting in a study on HL-60 human leukemia cells (Dordal et al., 1992), indicating the relevance of the LUVET system as a model for cellular membranes.

Biological membranes and closely related model membrane systems can bind significant amounts of doxorubicin in an anionic phospholipid dependent way, even at submicromolar concentrations of free drug, such that under pharmacologically relevant conditions the free cytosolic drug concentration is extremely low (de Wolf et al., 1993). Indeed, the extranuclear pool of the related drug daunorubicin was observed to reside mainly in intracellular membranes of cancer cells, besides in lysosomes (Rutherford & Willingham, 1993) rather than free in the cell. The importance of binding has been neglected in earlier transport studies. For example, the presently observed P^0 values are a factor of 50−100 higher than those determined by Dalmark and Storm (1981), most probably because drug binding to the plasma and internal membranes was disregarded in that study. In other studies (Frézard & Garnier-Suillerot, 1991a,b; Tarasiuk et al., 1993) binding was ignored, and the osmotically active intracellular drug concentration was determined by assuming that the fluorescent pool of anthracycline was equivalent to the pool of free (osmotically active) drug. Our work shows that even if membrane-induced fluorescence changes are small or nearly absent (Figure 1, middle traces), significant membrane binding can occur (Table 1). As a result, the rates of passive efflux reported in those studies on cancer cells were probably underestimated. The rate of active drug pumping by the P-glycoprotein was directly derived from the passive fluxes and was thus also underestimated. This shows that membrane binding is an important factor which should be thoroughly taken into account also in studies of passive and active drug transport in cells.

In order to compare lipids typical for the plasma membrane, we chose to use the zwitterionic bilayer-forming outer leaflet lipid PC as a reference lipid. In the presence of the anionic phospholipid DOPS more doxorubicin is bound, and this effects the transport rate of doxorubicin (1) by decreasing the concentration of free, transportable doxorubicin and (2) by an intrinsic decrease of the permeability coefficient, because even after correction for binding a significant decrease in the rate of transport in 50% DOPS-containing LUVET compared to LUVET containing zwitterionic phospholipids was observed. The latter point is a surprising result because it was previously demonstrated that drug binding to negatively charged lipids (including PS) resulted in a decreased acyl chain order (de Wolf et al., 1991a, 1992), which is expected to facilitate rather than to inhibit diffusion across the hydrophobic part of the membrane (see below). However, the decreased order is accompanied by drug insertion in between the headgroup region of the lipid molecules. This results in a tightening of the interfacial region (de Wolf et al., 1991a), which hinders the permeation of nonbound drug across the membrane. The previously observed inhibitory effect on the formation of type II nonbilayer structures points indeed to a preferential insertion of the drug into the interfacial region (de Wolf et al., 1992). Previous studies have also demonstrated the occurrence at the membrane surface of drug stacks that hinder penetration of water into the membrane (de Wolf et al., 1991a), but the contribution of this effect is probably small under the present conditions. The specific binding of doxorubicin to anionic phospholipids is mainly of an electrostatic nature (de Wolf

et al., 1991a, 1992) and results in a specific increase of the density of positively charged drug molecules in the membrane. Our data show that this does not stimulate drug transport; on the contrary, it is hindered by the presence of the charged form of the drug, clogging the membrane. Doxorubicin binding to anionic phospholipids can be reversed by adding agents like verapamil. In accordance, verapamil abolishes the difference between passive doxorubicin transport in zwitterionic and negatively charged membranes (G. Speelmans et al., manuscript submitted). An additional factor could be the special packing of mixed membranes (de Wolf et al., 1992). The negatively charged [11,11-$^2H_2$]oleoyl-labeled phospholipids appeared to have a higher order than the [11,11-$^2H_2$]oleoyl-labeled zwitterionic phospholipids, indicating that the headgroups of the former phospholipids are more exposed to the outside. This different membrane structure might hinder doxorubicin transport directly or might cause a special orientation of the bound doxorubicin molecules, which hinders doxorubicin diffusion. However, the effects of bound doxorubicin on membrane structure are very complex (de Wolf et al., 1992).

LUVET composed of PC/SM and PC/cholesterol showed an even stronger decrease in the permeability of doxorubicin with respect to pure DOPC. However, the binding to the membrane was not influenced. In case of SM a differentiation between (1) the specific chemical features of the polar region of sphingolipids and (2) the order of the aliphatic chains on transport rate cannot be easily made. At least from calorimetric studies it is known that different SM species (from bovine erythrocytes and synthetic palmitoyl SM) have an increased temperature at which the transition of the gel to liquid-crystalline state occurs compared to DOPC (Demel et al., 1977), so an increased membrane packing probably is involved. The effect of cholesterol on the influx of doxorubicin could be due to a direct interaction between doxorubicin and cholesterol, but the decreased transport is more likely due to the effect on membrane packing. In ^2H NMR experiments with lipids containing [11,11-$^2H_2$]oleoyl-labeled lipids, the deuterium quadrupolar splitting ($\Delta\nu_q$), which is a direct measure of the membrane order, increases when an increasing amount of cholesterol is incorporated in model membranes composed of DOPC, namely, from 6 to 13.3 (Gasset et al., 1988). On the other hand, addition of 0.5% phenethyl alcohol (PEA) to membranes is known to have a fluidizing effect on the membranes and to decrease the $\Delta\nu_q$ from 6 to 4.5 in LUVET (Jordi et al., 1990). In accordance, PEA and cholesterol appeared to have opposite effects on doxorubicin transport. The membrane order, expressed as $\Delta\nu_q$, in the presence of cholesterol or phenethyl alcohol, can be calculated from data provided by Gasset et al. (1988) and Jordi et al. (1990). Approximately the same inverse linear relationship between the doxorubicin permeability coefficient and the $\Delta\nu_q$ was found for cholesterol and PEA (Figure 6). This indicates that both cholesterol and PEA influence doxorubicin via the same mechanism, namely, indirectly via influencing the membrane order. On the basis of the increase of doxorubicin transport in the presence of PEA, it is expected that the use of membrane fluidizers such as anaesthetics will increase the rate of passive influx of doxorubicin into the cell and will thus counteract resistance of a cancer cell.

Due to the asymmetric distribution of lipids in the plasma membrane, a difference in the outer and inner leaflet with respect to binding and passive diffusion of doxorubicin is expected to occur. At the cytosolic site of the membrane the presence of anionic phospholipids (such as PS) affect passive transport by tightening of the interfacial region by formation of anionic phospholipid−doxorubicin complexes and by decreasing the free concentration of drug. An accumulation of doxorubicin at the interphase of the inner leaflet is expected to occur. At the outer leaflet due to the presence of SM, cholesterol, and relatively more saturated fatty acyl chains, (1) there is little interaction (binding) between doxorubicin and lipids of the outer leaflet, and (2) the rate of passive import of doxorubicin is relatively slow compared to the inner leaflet. If we assume (1) a lipid composition and distribution as mentioned in the introduction, (2) dioleoyl acyl chains for all phospholipids except SM, and (3) the same distribution for cholesterol and SM, we can calculate the P^0 relative to that of pure PC by interpolation of the results shown in Figures 3 and 4. These values are 0.188 and 0.614 for the outer and inner leaflet, respectively, i.e., a factor 3.3 lower in the outer leaflet than in the inner leaflet. Since phospholipids in the outer membrane are in general more saturated than PE and PS (Devaux, 1991), this difference is expected to be even larger *in vivo*.

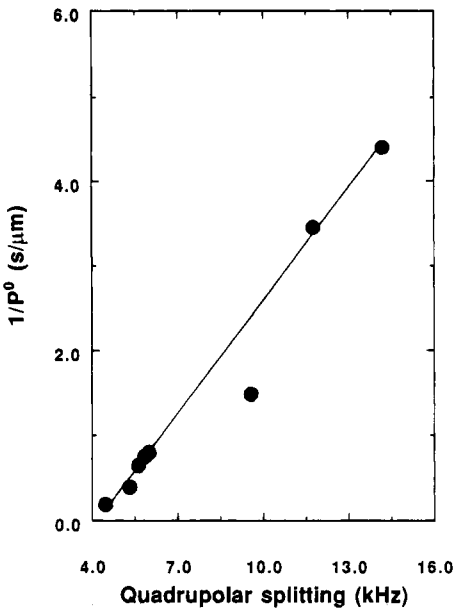

FIGURE 6: Inverse relationship between the membrane order (as determined by $\Delta\nu_q$ in kHz) and the permeability coefficient of doxorubicin. The permeability coefficients are taken from Figures 4 and 5. The $\Delta\nu_q$ of DOPC LUVET in the presence of varying amounts of cholesterol and phenethyl alcohol are calculated from Gasset et al. (1988) and Jordi et al. (1990), respectively. The line represents the best fit, with omission of the 29% cholesterol point.

Finally, besides the already mentioned underestimation of the rate of active drug pumping by disregarding membrane binding, we will discuss the consequences of our findings for the mechanism of multidrug resistance conferring enzymes. First, the highest concentration of its substrates (at least of doxorubicin) is at the interphase region of the inner leaflet of the plasma membrane, whereas the cytosolic concentration is very low (de Wolf et al., 1993). It can be expected that the ATP-dependent P-glycoprotein or other mdr-conferring enzymes could pump out doxorubicin more efficiently if they can obtain their substrate from the membrane, the compartment where the concentration is the

highest and in which the enzyme is embedded, instead of from the cytosol (Raviv et al., 1990). This compartment is most likely the interphase region of the inner leaflet. The active efflux of doxorubicin requires energy in the form of ATP. In case of pumping from the inner leaflet possible energy consuming steps are breaking of the electrostatic bonds between doxorubicin and anionic lipids or flipping of a phospholipid−doxorubicin complex. Secondly, once the drug is pumped out by an mdr-conferring transport protein, the rate of re-entry by passive diffusion across and the interaction with the outer leaflet lipids of the plasma membrane will be low.

Our present study demonstrates that knowledge of drug−membrane interactions and kinetics of passive transport in model membranes is essential to gain insight into the transport processes which occur in cancer cells and which determine the sensitivity or resistance of these cells to anticancer drugs.

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