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Flip-Flop of Doxorubicin across Erythrocyte and Lipid Membranes

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ABSTRACT. Doxorubicin, an anticancer drug, is extruded from multidrug resistant (MDR) cells and from the brain by P-glycoprotein located in the plasma membrane and the blood–brain barrier, respectively. MDR-type drugs are hydrophobic and, as such, enter cells by diffusion through the membrane without the requirement for a specific transporter. The apparent contradiction between the presumably free influx of MDR-type drugs into MDR cells and the efficient removal of the drugs by P-glycoprotein, an enzyme with a limited ATPase activity, prompted us to examine the mechanism of passive transport within the membrane. The kinetics of doxorubicin transport demonstrated the presence of two similar sized drug pools located in the two leaflets of the membrane. The transbilayer movement of doxorubicin occurred by a flip-flop mechanism of the drug between the two membrane leaflets. At 37°, the flip-flop exhibited a half-life of 0.7 min, in both erythrocyte membranes and cholesterol-containing lipid membranes. The flip-flop was inhibited by cholesterol and accelerated by high temperatures and the fluidizer benzyl alcohol. The rate of doxorubicin flux across membranes is determined by both the massive binding to the membranes and the slow flip-flop across the membrane. The long residence-time of the drug in the inner leaflet of the plasma membrane allows P-glycoprotein a better opportunity to remove it from the cell. BIOCHEM PHARMACOL **54**;10:1151–1158, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. drug resistance/multiple; antineoplastic drugs; P-glycoprotein; doxorubicin; anthracyclines; biological transport

Doxorubicin is a potent and widely applied anticancer drug that exhibits effects on DNA and DNA-associated enzymes [1], as well as on membranes [2]. Understanding the parameters that govern the rate of passive diffusion of doxorubicin across membranes is essential for the following reasons: (i) Import of doxorubicin into cells occurs by passive diffusion down a concentration gradient maintained by the intracellular binding of the drug to DNA [1], RNA [3], and intracellular membranes [4]. (ii) Doxorubicin is excluded from MDR† cells by active Pgp-mediated drug extrusion [5]. (iii) Another role of Pgp is to exclude MDR-type drugs, including doxorubicin, from the brain by their active efflux across the blood–brain barrier. Pgp is highly expressed in brain capillary endothelial cells and prevents MDR-type drugs from penetrating the brain [6]. In concord with this notion is the finding that mice in which the *mdr1a* gene is disrupted display a new sensitivity to a centrally neurotoxic pesticide [7]. Thus, Pgp, whose physiological role is to protect the brain, interferes with anticancer chemotherapy of brain tumors. MDR-type drugs are hydrophobic and, as such, enter cells by diffusion through the membrane without the requirement for a specific transporter. Transport studies, performed in erythrocytes [8] and in the alga *Chara ceratophylla* [9], formulated the generally accepted dogma that the transbilayer diffusion of solutes is determined by their hydrophobicity, measured as the organic solvent:water partition coefficient. Yet, Pgp efficiently excludes these drugs from MDR cells and from the brain. This apparent contradiction between the presumably free influx of MDR-type drugs into MDR cells and the efficient removal of the drugs by Pgp, an enzyme with a limited ATPase activity (turnover = 30 sec^{-1} [10]), prompted us to examine the passive transport mechanism of doxorubicin.

We recently estimated the transbilayer movement rate of numerous MDR drugs and modulators [11] and observed widely varying rates of transbilayer movement rates, with the modulators crossing the membranes much faster than the MDR drugs. The transbilayer movement rates were not correlated with drug hydrophobicity. The flux of doxorubicin across membranes has been characterized, using DNA to bind the drug and quench its fluorescence [11]. Alternatively, a pH gradient (inside acidic) was used to trap doxorubicin within liposomes [12]. We concentrated on characterizing the transport within the membrane of both artificial lipid membranes and erythrocyte membranes, instead of measuring the flux across the membranes.

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† *Abbreviations:* IOV, inside-out vesicles; LUV, large unilamellar vesicles; MDR, multidrug resistant; MLV, multilamellar vesicles; Pgp, P-glycoprotein; PC, phosphatidylcholine; and PS, phosphatidylserine.

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MATERIALS AND METHODS

Materials

Doxorubicin, herring testes DNA, cardiolipin, and phosphatidylglycerol were purchased from Sigma (St. Louis, MO, U.S.A.). PC, PS, and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). [^{14}C]Doxorubicin hydrochloride was purchased from Amersham (Buckinghamshire, England).

Preparation of Lipid Vesicles

LUV were prepared essentially as described [13]. Films of 10 mg lipids were dried for 1 hr under vacuum and dissolved in 1.0 mL ether. Then 0.3 mL of 25 mM HEPES–Tris (pH 7.5), 200 mM NaCl, and 1 mM NaN_3 (medium A) were added and sonicated to homogeneity in a round bath-type sonicator (Laboratory Supplies Co., Hicksville, NY, U.S.A.). The suspension was dried under vacuum to a gel form. Medium A (0.7 mL) was added, and the gel was broken by repeated cycles of vigorous shaking and incubation under vacuum. The concentration of the vesicles was estimated by determinations of lipid P_i. The vesicles were incubated under vacuum for 30 min, and then were downsized by three extrusions through polycarbonate filters with 200 nm pores. MLV were prepared by drying 10 mg lipids as described above followed by suspension into 1 mL medium A. The vesicles were centrifuged for 2 min at 2500 × *g* in an Eppendorf 5415C centrifuge.

The encapsulated volume of the lipid vesicles was determined by preparation of the vesicles in medium A containing 1 mM calcein, separation on Sephadex G-50, and determination of fluorescence in medium A containing 0.1% Triton-X-100. [^{14}C]Doxorubicin binding to lipid vesicles was determined as the amount of radioactivity associated with the vesicles after separation from the medium by centrifugation at 100,000 × *g* for 30 min.

Preparation of Sealed Ghosts and IOV

Blood was withdrawn from healthy volunteers, heparinized, and centrifuged (1300 *g*, 10 min, 4°). The erythrocytes were washed three times in phosphate-buffered saline. IOV and sealed ghosts were prepared essentially as described by Steck and Kant [14]. The packed cells were lysed by dilution into 40 vol. of 5 mM HEPES–Tris buffer (pH 8.0), incubation for 10 min, and centrifugation (27,500 × *g*, 15 min), and then were washed in the same buffer. The white ghosts, but not the red tight pellets, were suspended and incubated for 1 hr at 37° in a medium containing 5 mM HEPES–Tris buffer (pH 7.5) and either 0.1 mM EGTA to produce IOV or 5 mM $MgSO_4$ to seal the ghosts. The membranes were centrifuged at 100,000 × *g* for 30 min and suspended in medium B containing 5 mM HEPES–Tris (pH 7.5) and 1 mM NaN_3. The closed IOV were separated from open vesicles by centrifugation into 4.3% dextran in a medium B cushion (35,000 × *g* 30 min in a Beckman SW28 rotor).

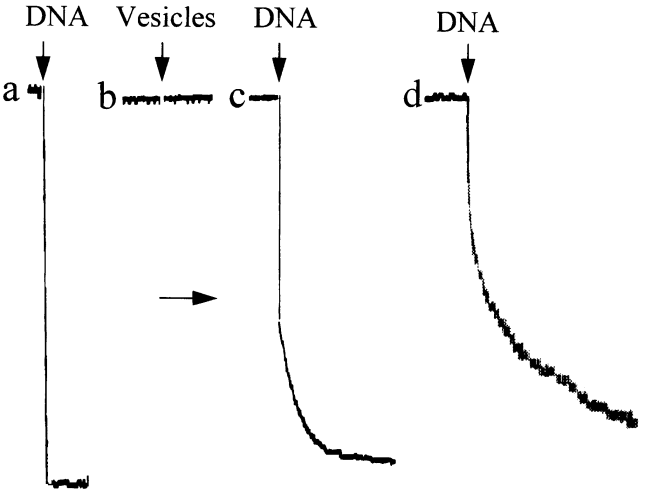


FIG. 1. Outward transbilayer movement of doxorubicin in PS vesicles. Doxorubicin (10 μM) was incubated at 23° and its fluorescence was monitored continuously. After a brief incubation, either DNA (0.5 mg/mL) or PS LUV (trace b, 0.5 mg/mL) was added. Similar concentrations of doxorubicin and either PS LUV (trace c) or PS MLV (trace d) were equilibrated by overnight incubation at 23° prior to the addition of DNA (0.5 mg/mL). The horizontal arrow corresponds to either 5 min (traces a–c) or 30 min (trace d).

Membrane sidedness and the fraction of sealed vesicles were determined by measuring the latency of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase [14].

Transbilayer Movement Assays

Transbilayer movement assays in lipid vesicles and erythrocyte membranes were performed in medium A and medium B, respectively. DNA was prepared as described by Speelmans *et al.* [15]. Doxorubicin was monitored continuously in a thermostatically controlled Perkin–Elmer LS-5 spectrofluorimeter (excitation 490 nm; emission 550 nm). Data were corrected for light scattering, inner filtering, and drug dilution [4].

RESULTS

Transbilayer Movement of Doxorubicin in Liposomes

As a first step toward characterization of doxorubicin transbilayer movement across biological membranes, doxorubicin movement within the membrane was studied in liposomes. DNA served as an agent capable of rapidly and efficiently quenching the fluorescence of doxorubicin. As shown in Fig. 1, doxorubicin fluorescence was quenched upon mixing with DNA (trace a), but it was not changed upon mixing with liposomes (trace b) even after complete equilibration (data not shown). Binding studies, using [^{14}C]doxorubicin, showed that after equilibration 90% of the drug was bound to PS vesicles. The bound drug was

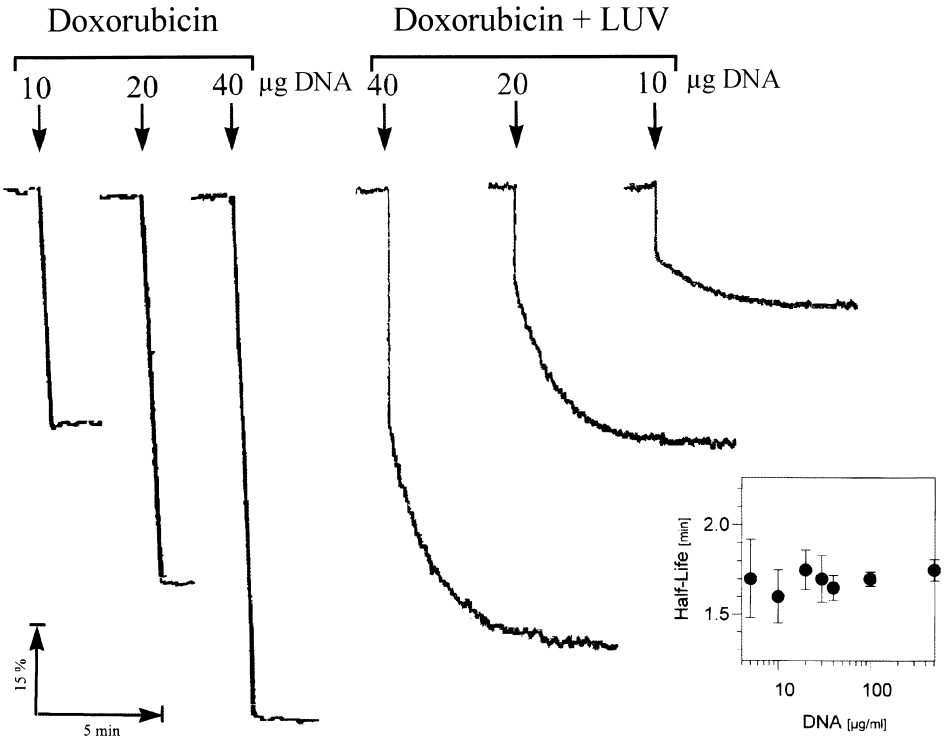


FIG. 2. Effect of DNA concentration on the transbilayer movement of doxorubicin. At the points marked by the arrows, DNA (concentrations as noted in the figure, μg/mL) was added to either free doxorubicin (10 μM) or doxorubicin (10 μM) pre-equilibrated overnight with PS LUV (1 mg/mL) Doxorubicin fluorescence was monitored continuously. The slow phases of fluorescence quenching were fitted to first-order reaction curves, and the resulting half-lives were plotted in the inset as a function of DNA concentration. Values in the inset are means ± SD, N = 4.

distributed equally between the two phospholipid leaflets in association with the acidic headgroups of the phospholipid [3, 15]. The volume encapsulated in the vesicles comprised less than 0.2% of the total volume, rendering negligible the contribution of the doxorubicin free within the lumen of the vesicles compared with the drug amount bound to the membranes. This was confirmed by repeating the transport studies described below in the presence of 2 M sucrose, which had no effect on the results (data not shown).

Due to the high affinity of doxorubicin for DNA, addition of DNA to doxorubicin, pre-equilibrated with vesicles, induced desorption of drug from the vesicles and binding to the DNA, leading to fluorescence quenching. The fluorescence quenching occurred in two steps (Fig. 1, trace c). About 50–60% of the total fluorescence decrease was too fast to be recorded under our experimental conditions. The second phase of fluorescence decrease occurred slowly, with a time–course that could be fitted to an exponential decay with a half-life of 1.1 to 1.3 min. The initial rapid fluorescence decrease observed in the first phase represents quenching of doxorubicin, both free in the medium and bound to the outer leaflet of the vesicles. The slow phase of fluorescence decrease is the outcome of the outward transbilayer movement of drug originally bound to the inner leaflet. When MLV (traced) were used instead of LUV, the fast phase of fluorescence quenching was equivalent to a loss of 20% of the total drug fluorescence, corresponding to the drug, both free in the medium and bound to the outer leaflet of the outer membrane. Compared with LUV, the second phase of fluorescence decrease in MLV was much slower, could not be curve-fitted to a single first-order reaction, and presumably is the result of the outward movement of drug across multiple membranes.

Repeating the experiment with various concentrations of DNA and vesicles resulted in varying extents of fluorescence quenching, but the life-time of the slow phase of fluorescence quenching remained 1.2 min (Fig. 2). Varying the concentrations of both the LUV and the drug had no effect on the rate of the slow phase of fluorescence quenching (Fig. 3). The amount of drug participating in the transbilayer movement, measured as the extent of the slow phase of drug quenching, remained equivalent to half the total amount of drug bound to the LUV.

Thus, factors expected to modulate the interaction rate of the drug with the vesicles, such as the concentrations of DNA, drug, and LUV, had no effect on the slow phase of fluorescence quenching. In contradistinction, parameters affecting the fluidity of the membranes and expected to affect the movement of drugs within the membrane did modulate the rate of the slow phase of fluorescence quenching. As shown in Fig. 4, cholesterol, known to limit membrane fluidity [16], inhibited the transbilayer movement rate of doxorubicin. Inclusion of 30% cholesterol in the LUV increased the doxorubicin half-life 7-fold. On the other hand, the membrane fluidizer benzyl alcohol [17] accelerated the transbilayer movement. Furthermore, fluidizing the membrane by raising the temperature led to shorter doxorubicin transbilayer movement lifetimes in both the presence and absence of cholesterol. The half-lives could be plotted in an Arrhenius-type plot yielding straight lines corresponding to an increase by a factor of 4–5 for each 10° rise in temperature (Fig. 5). Presumably, this temperature dependence reflects both a fluidization of the

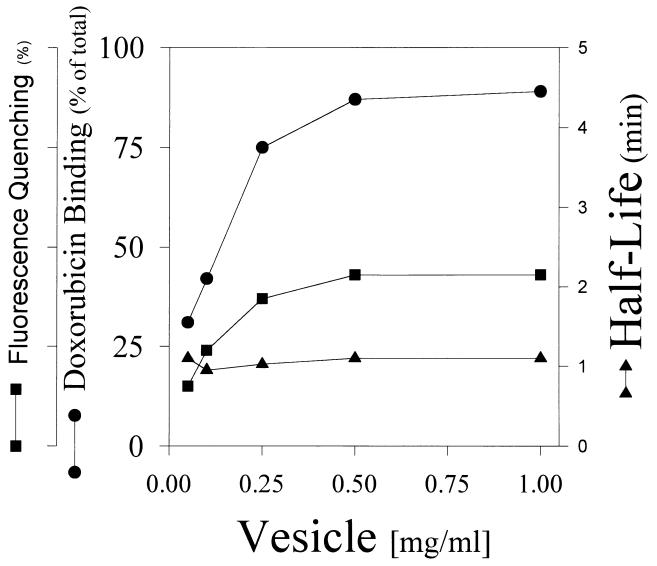


FIG. 3. Effect of vesicle and drug concentrations on the transbilayer movement of doxorubicin. Various concentrations of PS LUV were equilibrated overnight with doxorubicin (PS/drug molar ratio of 25). Subsequently, DNA (0.5 mg/mL) was added, and the rates and extents of the slow phases of fluorescence quenching were determined by curve fitting. These extents of fluorescence quenching are expressed as a percentage of initial doxorubicin fluorescence. Doxorubicin binding to the vesicles was determined with [^{14}C]doxorubicin as described under Materials and Methods. This experiment was performed four times; the data shown are those from a typical experiment.

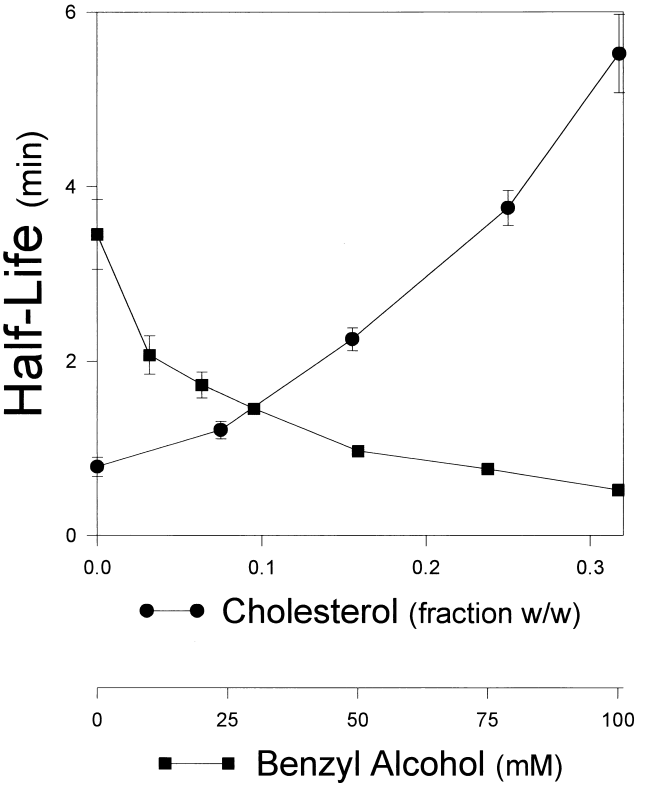


FIG. 4. Effect of cholesterol and benzyl alcohol on doxorubicin transbilayer movement rate. The half-life of doxorubicin transbilayer movement was determined as described in the legend of Fig. 1 (trace c) except that the LUV were of various compositions of lipids. To demonstrate the effect of cholesterol on doxorubicin transbilayer movement, the vesicles contained soybean phospholipids and various concentrations of cholesterol. Vesicles containing soybean phospholipids and cholesterol [0.7: 0.3 (w/w), respectively] were used to assess the fluidizing effect of benzyl alcohol on the rate of doxorubicin transbilayer movement. Each value is the mean ± SD of triplicate experimental points.

membranes and a direct acceleration of the transbilayer movement rate.

The nature of both the acidic and the neutral phospholipid present in the LUV had little effect on the transbilayer movement rate of doxorubicin. Thus, substitution of the PS with either phosphatidylglycerol or cardiolipin did not alter the transbilayer movement rate (data not shown). Likewise, partial substitution of PC with phosphatidylethanolamine had little effect on the transbilayer movement rate of the drug. As expected, total elimination of the acidic phospholipid results in reduced binding of doxorubicin [3]. However, the transbilayer movement rate observed in neutral liposomes was only twice as fast as that observed in acidic liposomes (Fig. 6). A similar effect could be observed at pH 9.7, which is above the pK of doxorubicin (pH 8.6). At this pH, most of the drug was unchanged, the binding to the liposomes was reduced, and the transbilayer movement rate of doxorubicin was twice as fast.

Variation of the strategy employed to measure drug transport from the inner to the outer leaflet was used to measure the drug transport rate from the outer to the inner leaflet. In the latter case, we monitored drug transfer from the DNA to the membranes. For this purpose, doxorubicin was equilibrated with small amounts of DNA, resulting in intercalation of drug into the DNA and a corresponding fluorescence quenching (Fig. 7). Upon subsequent addition of an excess of LUV, the lipid vesicles competed for the drug and partially relieved the fluorescence quenching. The fluorescence relief, due to transfer of drug from the DNA to the membranes, occurred in two phases. The first phase was fast and represents the transfer of doxorubicin from DNA to the outer leaflet of the LUV. The second phase, due to the inward movement of drug from the outer to the inner leaflet, could be fitted to an exponential function with a half-life of 1.1 min, similar to the outward movement. As for the outward transbilayer movement, the relative concentrations of DNA and vesicles determined the extent of quenching relief, but had no effect on the half-life of the slow phase of fluorescence increase (Fig. 7).

Transbilayer Movement of Doxorubicin across Erythrocyte Membranes

Upon equilibration of [^{14}C]doxorubicin under the experimental conditions described in the legend of Fig. 8 with either IOV or sealed ghosts, about 90% became membrane bound. Upon subsequent addition of DNA to IOV, the pattern of fluorescence decrease was similar to that ob-

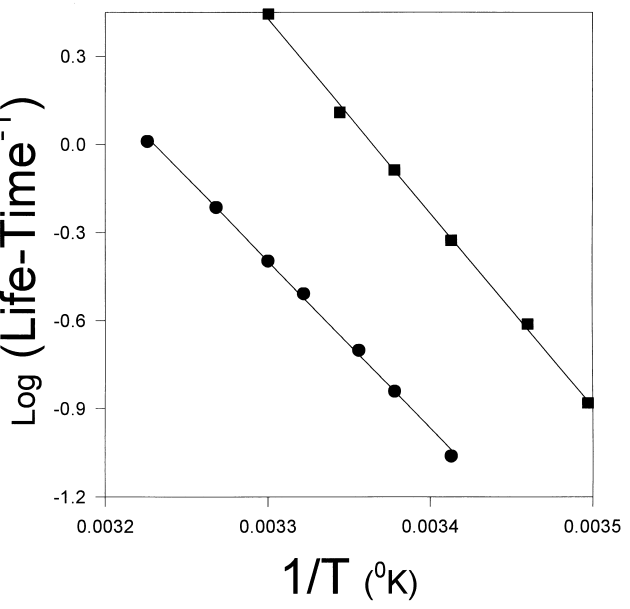


FIG. 5. Effect of temperature on doxorubicin transbilayer movement rate. The half-life of doxorubicin outward transbilayer movement was determined at various temperatures as described in the legend of Fig. 1, with vesicles containing soybean phospholipids and either 0 (●) or 30% (w/w, ■) cholesterol. This experiment was performed four times; the data shown are those from a typical experiment.

served with LUV, except that the half-life of the drug transbilayer movement was 0.7 min at 37° compared with 9 sec for PS LUV. The extent of the slow phase of fluorescence decrease was equivalent to close to 50% of the drug bound to IOV. On the other hand, the corresponding extent in sealed ghosts was lower, presumably due to an unsealed fraction of the ghosts. The results described in Fig. 8 were reproduced after osmotic collapse of the vesicles in the presence of 2 M sucrose (data not shown). Thus, in both IOV and sealed ghosts, the contribution of drug entrapped within the vesicles to the measurements of doxorubicin transbilayer movement was negligible. As with lipid vesicles, the half-life of transbilayer movement in erythrocyte membranes was not affected by concentrations of DNA, drug, or vesicles. On the other hand, both higher temperatures and membrane fluidization by benzyl alcohol accelerated the drug transbilayer movement (Figs. 9 and 10, respectively).

DISCUSSION

The mechanism by which doxorubicin traverses membranes might be clarified by analogy to the well-understood slow transbilayer movement of gramicidin A [17]. This channel-forming ionophore is an amphipathic peptide anchored by a hydroxyl group at the membrane surface and traverses the membrane slowly by rare flip-flop events. Doxorubicin is likewise an amphipathic molecule with a hydrophilic amine group present in the daunosamine moiety and hydroxyl groups limited to the same moiety of the

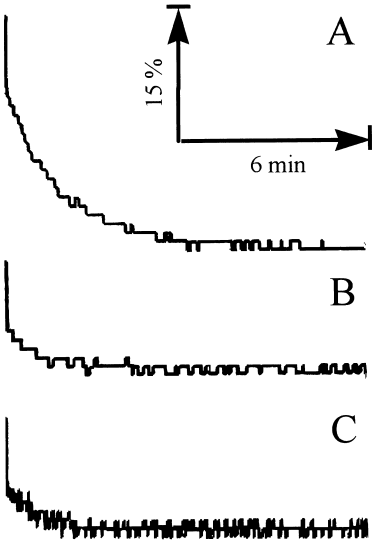


FIG. 6. Effect of pH and phospholipid charge on transbilayer movement of doxorubicin. The outward transbilayer movement rate of doxorubicin (trace A) was determined as described in the legend of Fig. 1 (trace c) except that the doxorubicin concentration was 25 μM and the LUV contained PC:PS and cholesterol (0.35:0.35:0.3 weight ratio). The half-life of the first-order process describing doxorubicin influx was determined, by curve-fitting, to be 1.25 min. The experiment was repeated (trace B) except that the pH medium was 9.7 (Na^+-glycine buffer). Under these conditions, the half-life of doxorubicin transbilayer movement was 0.6 min. The experiment described in trace A was repeated (trace C) except that the vesicles contained only PC and cholesterol [0.7:0.3 (w/w), respectively]. The half-life was determined to be 0.7 min.

drug (Scheme 1). Thus, for the following reasons the transbilayer movement of doxorubicin is best described in terms of distinct events of flip-flop across the membrane and not as diffusion down a continuous gradient located in the membrane core: (i) Transbilayer movement occurred

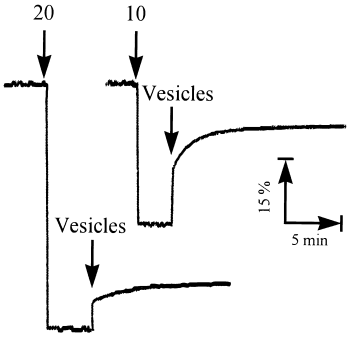


FIG. 7. Inward transbilayer movement of doxorubicin. The fluorescence of doxorubicin (10 μg/mL) was monitored continuously. The fluorescence was partially quenched by the addition of either 10 or 20 μg/mL DNA (marked by arrows). Subsequently, LUV (1 mg/mL containing PC:PS and cholesterol at 0.35:0.35:0.3 weight ratio) were added. The slow phase of the relief of fluorescence quenching was curve-fitted to a first-order reaction. The half-life of this relief was equivalent to the half-life of the outward movement of doxorubicin measured with LUV of similar lipid composition under conditions similar to those described in Fig. 1.

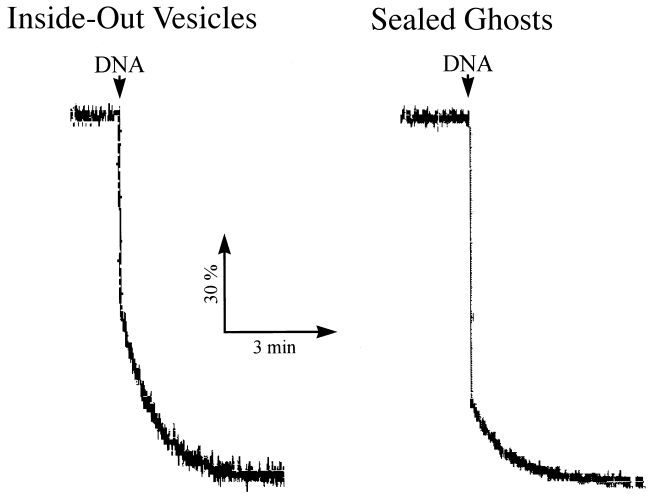


FIG. 8. Transbilayer movement of doxorubicin in erythrocyte membranes. Doxorubicin (10 μM) was incubated at 37° with either IOV (0.5 mg/mL) or sealed ghosts (1 mg/mL) prepared from fresh blood. Doxorubicin fluorescence was monitored continuously, and DNA (1 mg/mL) was added. The vertical arrow represents 30% fluorescence of the total doxorubicin fluorescence.

with a half-life close to a minute, which is hard to reconcile with free diffusion across the short width of membranes. (ii) The measurements of both the inward and the outward transbilayer movements of doxorubicin indicated that the amount of doxorubicin located in the membrane is divided into two similar-sized drug pools. Evidence of two similar-sized drug pools in the membrane is consistent with two similar compartments, presumably identical with the two

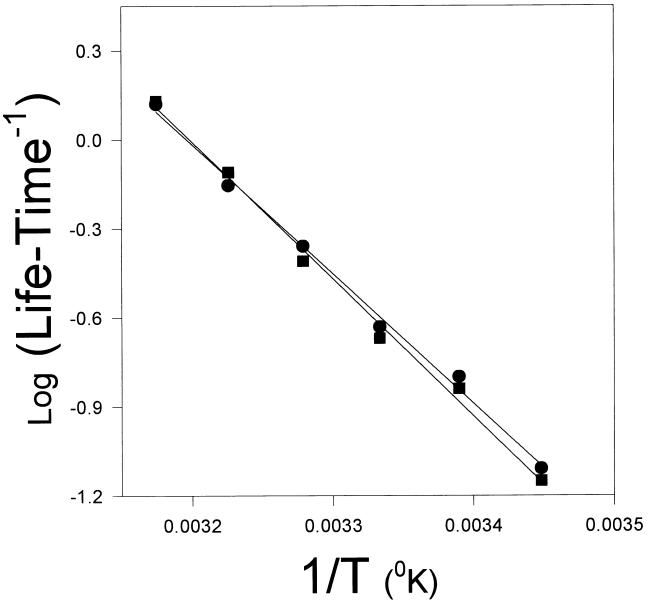


FIG. 9. Effect of temperature on doxorubicin transbilayer movement in erythrocyte membranes. The half-life of doxorubicin transbilayer movement was determined at various temperatures as described in the legend of Fig. 8 with either IOV (■) or sealed ghosts (●). This experiment was performed four times; the data shown are those from a typical experiment.

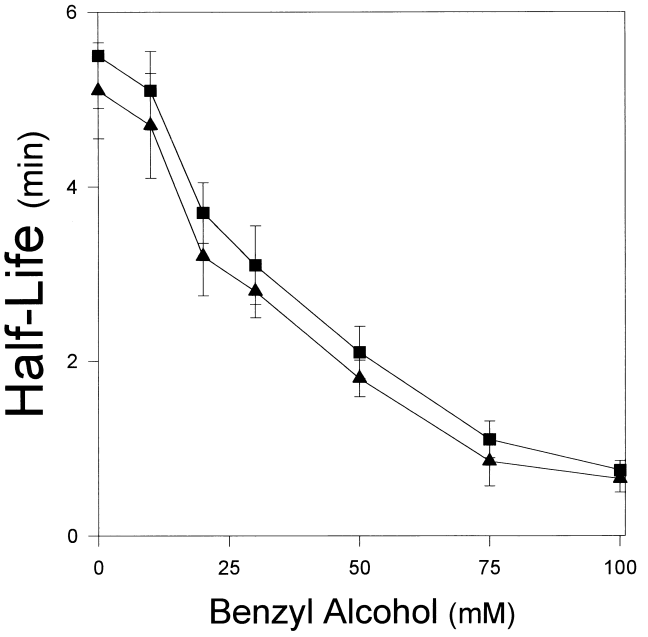
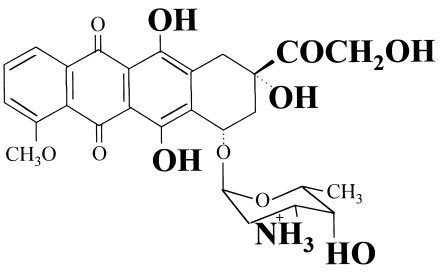


FIG. 10. Effect of benzyl alcohol on doxorubicin transbilayer movement in erythrocyte membranes. The half-life of doxorubicin transbilayer movement was determined at 15° in the presence of various benzyl alcohol concentrations as described in the legend of Fig. 8 with either IOV (■) or sealed ghosts (▲). Each value is the mean ± SD of triplicate experimental points.

membrane leaflets. (iii) Doxorubicin was soluble in polar solvents such as chloroform and octanol, but not in apolar solvents, such as benzene and CCl_4 (Table 1). Thus, doxorubicin is not soluble in the hydrophobic core of the membrane, but rather is located mainly at the membrane surfaces. (iv) Doxorubicin bound to lipid membranes is associated primarily with the acidic headgroups of the phospholipids, presumably due to its amphipathic nature and the electrostatic attraction of the basic amine group of doxorubicin to the acidic headgroups of the phospholipids in both liposomes and erythrocyte membranes [3, 4]. (v) The transbilayer movement of doxorubicin was not modulated by varying the concentrations of drug, vesicle, or DNA, but was correlated with membrane fluidity, indicating that the step limiting the movement rate is located in the membrane matrix. (vi) Osmotic collapse of both LUV and erythrocyte vesicles did not alter the time–course of fluorescence decrease, indicating that drug efflux from the



SCHEME 1. Doxorubicin. Polar and charged groups are designated by large, bold letters.

TABLE 1. Partition coefficients of doxorubicin into various organic solvents

	Octanol	Chloroform	Benzene	CCl_4
	Dielectric constants			
	10.3	4.8	2.28	2.23
	Partition coefficients*			
pH 7.5†	1	9	<0.05	<0.05
pH 8.6‡	8	17	0.2	<0.05
pH 9.7§	>20	>20	0.4	<0.05

* The partition coefficients into the organic solvents were determined after overnight mixing at room temperature.

† The aqueous phase contained HEPES–Tris buffer (100 mM).

‡ The aqueous phase contained Tris–HCl buffer (100 mM).

§ The aqueous phase contained Na^+-glycine buffer (100 mM).

medium encapsulated in the vesicles did not contribute to the transport measurements presented here. (vii) The decrease in doxorubicin fluorescence observed upon addition of DNA to MLV, pre-equilibrated with drug, exhibited prolonged and complex kinetics, consistent with the drug crossing multiple membrane barriers. This prolonged and complex kinetics excludes the possibility that the two phases of fluorescence reduction observed with LUV are due to two modes of drug interaction with the vesicles or to phase separation in the liposomes.

Thus, the transbilayer movement of doxorubicin is best described as a fast and massive binding to the membrane, a relatively long residence at the surface associated with the headgroups of the phospholipid, distinct flip-flop events across the membrane to the other leaflet, and fast equilibration with the aqueous medium across the membrane.

The barrier to doxorubicin transport posed by the hydrophobic core of the plasma membrane divides the cellular environment into two topological domains: (i) the intracellular domain, which is close to equilibrium with the inner plasma membrane leaflet, and (ii) the extracellular domain, which is close to equilibrium with the outer plasma membrane domain. It has been suggested that the Pgp function may involve substrate transport from the lipid bilayer itself to the external medium ("vacuum cleaner" hypothesis [18]). Within this hypothesis, Pgp could be a flippase, i.e. it could catalyze the translocation of hydrophobic substrates inserted in the cell membrane from the inner to the outer leaflet [19]. The near equilibration of doxorubicin between the inner leaflet of the plasma membrane and the intracellular milieu makes it extremely difficult to prove whether Pgp pumps its substrates from the aqueous phase or directly from within the plasma membrane [20]. Pgp-mediated drug pumping directly from the cytoplasm will result in almost concomitant reduction in drug levels in the inner plasma membrane leaflet. On the other hand, reduction in drug levels in the inner plasma membrane leaflet will lead to a concomitant decrease in intracellular drug levels.

The slow flip-flop of doxorubicin across biological membranes fits the hypothesis we recently suggested [21], that slow passive transbilayer movement rate allows the efficient Pgp removal of MDR drugs, while rapid transbilayer movement allows MDR-modulators to inhibit cellular Pgp. Clearly, a close to a minute residence of doxorubicin in one membrane leaflet before flip-flopping allows pumps, such as Pgp, an ample opportunity to efficiently remove drug molecules from the cells, practically without competition from passive drug influx. Moreover, doxorubicin present in the extracellular medium cannot efficiently compete with intracellular drugs on the Pgp pharmacophore. Only molecules with a rapid transbilayer movement can efficiently inhibit cellular Pgp.

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