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Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism

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Abstract

The objectives of the present work are to characterize the transport of mitoxantrone and three anthracyclines in terms of binding to the membrane surface, flip-flop across the lipid core of the membrane, and release into the medium. Mitoxantrone and anthracyclines are positively charged amphipathic molecules, and as such are located at the surface of membranes among the headgroups of the phospholipids. Therefore, their transport across membranes occurs by a flip-flop mechanism, rather than by diffusion down a continuous concentration gradient located in the lipid core of the membrane. Flip-flop rates have been estimated with liposomes labeled at their surface with 7-nitrobenzo-2-oxa-1,3-diazol-4-yl (NBD) moiety attached to the headgroup of phosphatidylethanolamine. Flip-flop of mitoxantrone, doxorubicin, daunorubicin, and idarubicin occurred with half-lives of 6, 0.7, 0.15, and 0.1 min, respectively. Partition of the drugs into the membrane occurred with lipid phase/aqueous medium coefficients of 230,000, 8600, 23,000, and 40,000 for mitoxantrone, doxorubicin, daunorubicin, and idarubicin, respectively, which are much higher than their corresponding octanol/aqueous medium values. There was no direct correlation between the lipophilicity of the drugs and their lipid phase/aqueous medium partition coefficient or their flip-flop rate. Mitoxantrone exhibited the highest affinity toward liposome membranes, but the slowest flip-flop across the lipid core of the membranes. Simulation of drug uptake into liposomes revealed that transmembrane movement of the mitoxantrone and anthracyclines is determined by their flip-flop rate and affinity toward membranes.

Keywords: Membrane transport; Anthracyclines; Doxorubicin; Mitoxantrone; Multidrug resistance; P-glycoprotein

1. Introduction

The mechanism of passive transport of solutes across membranes was formulated by the Finnish botanist, Collander [1]. He measured the permeability of membranes of plant alga to various organic non-electrolytes and observed that the best correlation occurred with their hydrophobicity, which was estimated as olive-oil/water partition coefficient. He deduced that the barrier to solute diffusion is the hydrophobic lipid core of the membranes. Stein [2] elaborated this model further by assuming that solutes partition into the lipid core according to their hydrophobicity and size, and diffuse down a concentration gradient located in the lipid core of the membrane. The model proved useful in describing the passive diffusion of small organic solutes and gases. However, anthracyclines and other drugs are complex molecules and exhibit at least partial amphipathic characteristics. As such, they are preferentially located at the surface of the membrane among the phospholipid headgroups, rather than inside the lipid core. Thus, the transport of anthracyclines is best described in terms of insertion into the outer leaflet of the membrane and subsequent flip-flop across the lipid core.

In cells lacking drug pumps, drug concentration in the cytoplasm will eventually reach a level similar to that outside the cells. In contradistinction, in resistant cells, the rate of drug uptake plays a crucial role in the determination of the steady state drug concentration in the cytoplasm. In these cells, drug concentration is the outcome of competition between the passive uptake of drugs and their extrusion by pumps, such as P-glycoprotein. It has been suggested that the successful prevention of drug uptake depends on active

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleyl-*sn*-glycero-3-phosphoglycerol; MDR, multidrug resistance; NBD-PE, 7-nitrobenzo-2-oxa-1,3-diazol-4-yl-phosphatidylethanolamine

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transporters overcoming the passive drug leak across the membranes [3]. Thus, passive drug transport plays a crucial role in the success of phenomena such as multidrug resistance [4–6] and prevention of drug transport across the blood-brain barrier [7,8]. Measurements of drug pumping and leak in MDR cells confirm the crucial role of drug leak rates in cellular drug resistance [9–11].

It has been suggested that P-glycoprotein pumps its substrates directly from the lipid bilayer [12] and that it functions as a flippase [13], i.e. it catalyzes the translocation of drugs from the inner membrane leaflet of the plasma membrane to the outer leaflet (from which they can diffuse into the medium) or directly to the extracellular medium [13,14]. Such a flippase mechanism is consistent with substrates of P-glycoprotein crossing the membrane by means of a flip-flop mechanism. Doxorubicin has been shown to traverse lipid and erythrocyte membranes by a flip-flop exhibiting a half-life of about a minute [15]. This has been achieved by taking advantage of the quenching of doxorubicin fluorescence by DNA.

The aim of the present work is to study the transbilayer movement of mitoxantrone and anthracyclines in terms of binding to the membrane, flip-flop across the lipid core of the membrane, and desorption from the opposing membrane leaflet.

2. Materials and methods

Routinely used chemicals were obtained from Sigma, Israel. Lipids were purchased from Avanti Polar Lipids, AL, USA; daunorubicin, doxorubicin, mitoxantrone, and NBD-PE were purchased from Sigma, Israel.

2.1. Liposome preparation

Liposomes were prepared essentially according to Szoka et al. [16]. Films of lipids (10 μmol of lipid-Pi/ml liposomes) were obtained by evaporation of appropriate stock solutions under vacuum. Chloroform (1 ml) and 0.3 ml buffer A (10 mM Hepes/Tris, 100 mM NaCl, and 1 mM EGTA, pH 7.4), respectively were added per 1 ml final volume of liposomes. Emulsion was formed by sonication in a round bath-type sonicator (Laboratory Supplies Co., Hicksville, NY, USA). The emulsion was dried in an evaporator until gel was formed. A further 0.7 ml buffer A was added per milliliter liposomes. The gel was dispersed and a uniform liposome suspension was obtained by repeated cycles of vigorous shaking and evaporation under vacuum. The liposome suspension was diluted five-fold with medium A, incubated under vacuum for an hour, and downsized by successive extrusions through polycarbonate filters with 400 and 200 nm pores (10 times each). The volume trapped within the liposomes was determined by inclusion of 0.001% trypan blue in buffer A. The medium outside the liposomes was exchanged with buffer A by gel filtration.

Liposomes symmetrically labeled with NBD were produced by including NBD-PE (1% of total phospholipids) in their lipid composition. The fraction of fluorescent lipids present in the inner versus the outer leaflet of these vesicles is proportional to the total amount of lipid present in these leaflets [17]. Liposomes labeled exclusively on their inner leaflet were produced essentially as described by McIntyre and Sleight [18]. NBD label exposed at the surface of symmetrically labeled liposomes (1 mM Pi in medium A) was chemically eliminated by the addition of sodium dithionite (20 μl/ml of a 1 M solution in 1 M Tris pH 10 buffer) and further incubation for 15 min at 24 °C. Excess dithionite was removed by chromatography through a Sephadex-G 50 spin column equilibrated with medium A.

2.2. Transport measurements

Drug flip-flop rates across membranes of liposomes were measured as the reduction in fluorescence of NBD. Fluorescence of NBD-phosphatidylethanolamine (NBD-PE) containing liposomes was monitored continuously after temperature equilibration at 24 °C (except when otherwise noted) in glass cuvettes on a thermostated Cary Eclipse fluorescence spectrometer (excitation 450 nm; emission 530 nm). The addition of drugs resulted in rapid partial quenching of NBD fluorescence. Subsequently, fluorescence was further quenched, due to the flip-flop of drugs from the outer membrane leaflet to the inner leaflet of the liposomes. This phase of fluorescence quenching was fitted by a non-linear regression analysis to a single exponential that is described by the following first-order kinetic system:

$$Fl_{(t)} = Fl_{(\infty)} + [Fl_{(0)} + Fl_{(\infty)}] \exp^{-kt}$$

where t is the time, $Fl_{(t)}$ the NBD fluorescence at time t, $Fl_{(0)}$ the NBD fluorescence at time 0, $Fl_{(\infty)}$ the NBD fluorescence at time infinity, k the rate constant.

The half-life (the time taken for the fluorescence to fall to half its value) was calculated as $0.693/k$.

2.3. Mitoxantrone and anthracyclines binding to liposomes

The binding of drugs to liposomes was determined after equilibrium dialysis. Binding was assayed at room temperature after dialysis of 0.5 ml liposomes (1 mM Pi suspension) in a 10 mm dialysis tubing (purchased from Spectrum Laboratories, CA, USA) against stirred 100 ml 5 μM drug in medium A supplemented with 1 mM NaN_3 for 4 days in the dark under nitrogen gas. Equilibrium was reached in less than 3 days. Drugs were quantified by mixing 1 ml of sample with 4 ml of 7 mM SDS in medium A and comparing the fluorescence to that of standard solutions in SDS. Samples were diluted to a concentration included in the linear part of a calibration curve. The drug

amount bound per phospholipids was determined as the drug concentration inside the dialysis tube minus the drug concentration in the dialysis outer medium divided by the volume occupied by the phospholipids inside the dialysis tube. The drug partition coefficient (lipid phase/aqueous medium) was defined here as the ratio of drug concentrations in the lipid phase to that in the membrane phase, somewhat similar to the way in which a partition coefficient (octanol/water) used to express the hydrophobicity of molecules.

2.4. Modeling of transmembrane drug transport

Mitoxantrone and anthracycline transport in lipid vesicles was simulated essentially as previously described for cellular pharmacokinetics [19]. The considerations leading to the formulation of the model and the principles of its simulation by the use of a *Mathematica* program have been detailed previously [19]. The model is based on a four-compartment system in which the compartments represent the extra vesicular medium (m), the outer leaflet of the vesicle membrane (ol), the inner leaflet of the membrane (il), and the vesicle lumen (l). The transport of drugs between the compartments is described by rate constants. The flip-flop rate constants (k_2 and k_{2n}) were estimated experimentally. Since drug binding to the membrane is much faster than drug flip-flop across membranes, the rate constants, k_1 and K_{3n} were set arbitrarily at 10,000 times the flip-flop rate constant (k_2 and k_{2n}). The drug desorption rates were expressed as the corresponding binding rate divided by the drug partition coefficient. The code of the *Mathematica* simulation program is presented in Appendix A.

3. Results

3.1. Mitoxantrone and anthracyclines binding to liposomes

Mitoxantrone and anthracyclines penetrate liposomes by (a) rapid binding to the outer surface of the liposomes, (b) transit across the lipid core, presumably by a flip-flop mechanism, and (c) release into the inner volume of the liposomes. The binding of the drugs was assessed by equilibrium dialysis of liposomes with doxorubicin, daunorubicin, mitoxantrone, or idarubicin. Under the experimental conditions described in Table 1, acidic liposomes bound large amounts of drugs that are equivalent to 1 drug molecule bound per 45, 21, 4.5, and 13.3 phopholipid molecules, respectively. Thus, immediately upon the addition of drugs, a large fraction of the drugs became associated with the liposomes, which resulted in high local concentrations at the liposome surface. The binding affinity, expressed as the partition between the lipid phase and an aqueous medium, far exceeded the partition coefficient of the drugs between octanol and an aqueous medium. Since no clear correlation was observed between the binding affinities of the drugs to liposomes and their partition coefficient (octanol/aqueous medium), it would seem that other factors besides drug hydrophobicity determine the drug binding affinity. The reduced affinity of the drugs to liposomes that lack an acidic phopholipid indicates that this affinity is at least partially due to electrostatic attraction between the partially basic drugs and acidic groups in the membrane. In the case of daunorubicin, electrostatic attraction and streo arrangements seem to be the determining factors, since its binding to neutral liposomes was negligible, relative to that of the other drugs.

3.2. Assay methodology of drugs flip-flop in liposomes

Flip-flop across artificial lipid membranes was measured in liposomes labeled, either at both surfaces or only at the inner surface, with the highly fluorescent moiety, NBD. NBD was included in the lipid mixture used to prepare the liposomes as a moiety attached to the headgroup of the phospholipid, phosphatidylethanolamine. NBD is hydrophilic and as such is located among the headgroups of the phospholipids. Liposomes were prepared as large vesicles and downsized to a diameter of 0.2 μm by repeated extrusion through appropriate filters. The NBD moieties exposed at the surface of the liposomes could be reduced with dithionite, leading to the loss of their fluorescence. In experiments similar to that shown in Fig. 1, it has been shown that the reduction of liposomes prepared with NBD-PE resulted in elimination of 54 ± 2% of the fluorescence. Dissolving the liposomes with Triton-X-100 exposed the internal NBD groups to reduction by dithionite. These results are consistent with the liposomes being unilamellar

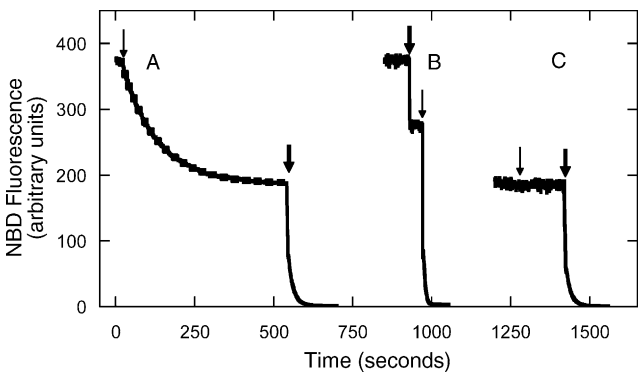

Fig. 1. Preparation of symmetrically and asymmetrically labeled liposomes. Liposomes containing DMPC:DMPG:cholesterol:NBD-PE (5:5:2:0.1, weight ratios) were prepared as described under "Section 2". Symmetrically (Traces A and B) or asymmetrically (trace C) labeled liposomes (1 mM Pi) were incubated in 2 ml medium A and the NBD-fluorescence was continuously monitored at 30 °C. At the time points marked by thin arrows, dithionite (20 μl of 1 M dithionite in 1 M Tris pH 10.0) was added. At the time points marked by thick arrows, Triton-X-100 (0.1%) was added. The reduction in fluorescence induced by dithionite (Trace A) was fitted by a non-linear regression (dashed thick line).

Table 1
Binding of mitoxantrone and anthracyclines to liposomes

Anthracycline	DOPC:DOPG:cholesterol		DOPC:cholesterol		Partition coefficient (octanol/medium)
	Binding ratio	Partition coefficient	Binding ratio	Partition coefficient	
Doxorubicin	6.3 ± 1.2	10200	0.7 ± 0.3	1150	0.8 [9], 1.1 [3]
Daunorubicin	14.3 ± 2.1	23000	0.08 ± 0.02	130	3.5 [9], 7.4 [3]
Mitoxantrone	144 ± 43	230000	6.6 ± 0.6	10800	7.2 [3]
Idarubicin	24.5 ± 3.2	40000	1.0 ± 0.2	1600	15 [9]

Liposomes containing either DOPC:DOPG:cholesterol (5:5:2, weight ratios) or DOPC:cholesterol (10:2, weight ratios) were prepared as described under "Section 2". The binding ratio (ratio of concentrations liposome bound drug to drug concentration in the medium) of the drugs was measured using 5 μM drug solutions and liposome (0.1 mM Pi) suspensions. Partition coefficients (ratio of drug concentrations in the lipid phase and in the medium) were calculated assuming a specific gravity of 1.03 for the lipids.

vesicles with no significant presence of multilamellar vesicles. Liposomes labeled only at their inner surface were prepared by reduction of the externally exposed fluorescent groups. During a 2 h incubation period, NBD moieties located at the inner surface of the liposomes did not become available to reduction by externally added dithionite (Fig. 1C). Thus, liposomes with the specific composition detailed in Fig. 1 exhibited no significant flip-flop of the fluorescent phospholipid.

Transport of drugs across membranes occurs by initial binding to the outer surface of the liposomes and subsequent transport across their membrane by a flip-flop mechanism. Since the inner volume of the liposomes is small compared to the outer medium, the drug amount released into the internal volume of the liposomes is negligible compared to the amount bound to the liposomes and free in the outer medium. Flip-flop of drugs was followed by continuous monitoring of NBD fluorescence associated with liposomes. As shown in Fig. 2A, the addition of mitoxantrone to liposomes resulted in an immediate, extensive decrease in NBD fluorescence followed by a relatively slow further decrease exhibiting first-order kinetics. The initial decrease in fluorescence is due to filter effects of drug dissolved in the medium and to localized interactions of the high drug concentrations bound to the liposome surface with the NBD groups present in their proximity. The contribution of the drug dissolved in the medium was assessed by the addition of same drug concentrations to liposomes dissolved with a detergent (such as Triton-X-100). Under these conditions, no localized interaction of the drugs with NBD occurs. As shown in Fig. 2C, under these conditions the decrease in fluorescence is small, which indicates that most of the initial decrease in fluorescence observed upon the addition of mitoxantrone to liposomes is due to localized effects at the liposome surface. The spectrum of mitoxantrone and NBD-labeled liposomes (Fig. 3), apart or together, show that the decrease in NBD fluorescence (emission at

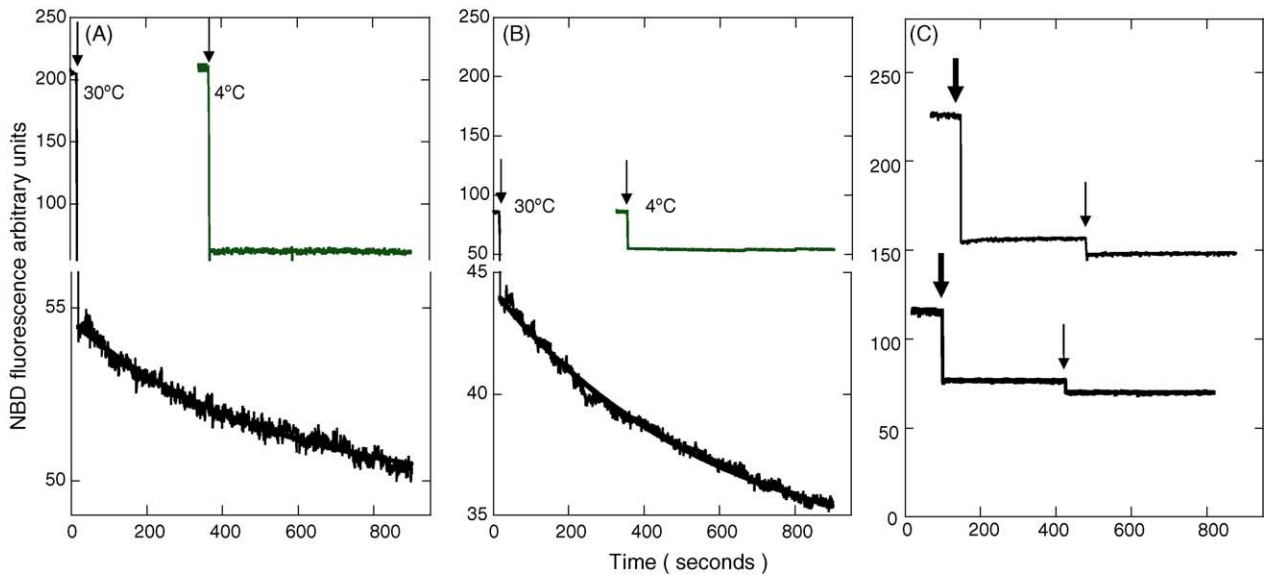

Fig. 2. Assay of mitoxantrone flip-flop. Liposomes containing DMPC:DMPG:cholesterol:NBD-PE (5:5:2:0.1, weight ratios) were prepared as described under "Section 2". Symmetrically (Trace A and upper C trace) or asymmetrically (Trace B and lower C trace) NBD-labeled liposomes (1 mM Pi) were incubated at 30 or 4 °C. NBD-fluorescence was monitored continuously. Mitoxantrone (10 μM) was added at the time points marked by thin arrows. Triton-X-100 (0.1%) was added at the time point marked by thick arrows. The reduction in fluorescence induced by mitoxantrone (Traces A and B) was fitted by a non-linear regression as described under "Section 2" (dashed thick line).

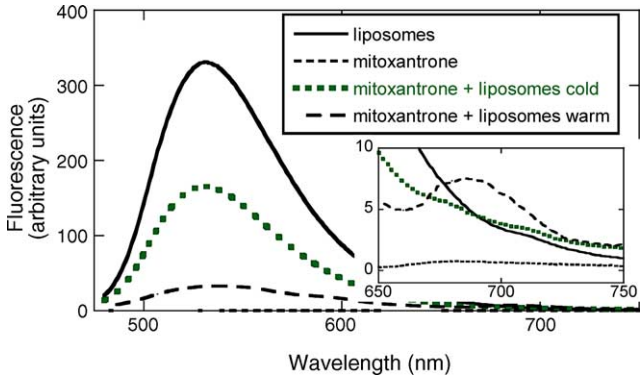

Fig. 3. Effect of mitoxantrone on fluorescence spectra of NBD-labeled liposomes. Liposomes containing DMPC and NBD-PE (10 and 0.02 mg/ml, respectively) were prepared as described under "Section 2". Liposomes (0.1 mg/ml in PBS) were excited with 490 nm light and the fluorescence spectra determined in the absence or presence of 50 μM mitoxantrone after 30 min incubation either at 4 °C (cold) or 37 °C (warm). The insert contains the same spectra measured over a more limited wavelength range.

540 nm) is accompanied by an increase in mitoxantrone fluorescence (emission 680–700 nm), which indicates that fluorescence energy was transferred from NBD to mitoxantrone. Incubation of mitoxantrone with liposomes below their melting point did not affect drug binding to the liposomes (data not shown). Under these conditions, the drug is prevented from penetrating the solid core of the membranes. As shown in Fig. 3, drug bound to the liposomes under these conditions quenched the NBD fluorescence of the liposomes with little increased mitoxantrone fluorescence. Presumably, the bound drug quenched the fluorescence mainly by localized filter effects.

The initial rapid fluorescence quenching is followed by a slow fluorescence decrease that can be described as a first-order reaction. This slow fluorescence decrease, caused by drug flip-flop across the membrane, does not occur at temperatures below the melting point of the liposomes (Fig. 2). Further insight into the assay mechanism can be gained from studies of drug interactions with liposomes labeled with NBD only at their inner surface (Fig. 2B). In this case, the initial reduction in fluorescence due to drug binding to the outer surface is diminished; presumably there is little optical interaction between drug bound at the outer surface and NBD located at the inner surface of the liposomes. As expected, the relative decrease in NBD fluorescence due to drug flip-flop was large in these liposomes, compared to the corresponding decrease in liposomes labeled at both surfaces.

Results similar to those reported above for mitoxantrone were observed with idarubicin, daunorubicin, and doxorubicin except that due to the overlapping of fluorescence emission peaks, energy transfer from NBD to the drugs could not be demonstrated (data not shown). In any case, the fluorescence quantum yield of anthracyclines, and especially mitoxantrone, is much lower than that of NBD. As a result, energy transferred from NBD to the drugs is emitted mainly as heat and not as fluorescence.

In order to measure the rate of drug flip-flop by the method described above, two potential problems must be solved: (1) the measured flip-flop rate reflects the transfer of drug from the outer membrane leaflet to the inner. However, the resulting reduction in drug concentration in the outer leaflet allows for further insertion of drug from the medium into the liposomes. Thus, the measured flip-flop rate reflects both the intrinsic flip-flop and uptake of further drug amounts into the outer liposome leaflet. This results in an apparent flip-flop rate slower than the actual flip-flop rate. (2) The estimate of the flip-flop rate could be in error, due to modulation of membrane characteristics by the inserted drugs. The high affinity of the agents to membranes results in high local drug concentrations in the membranes. Such concentrations can modulate the flip-flop rate of drugs. To solve both these problems, the rate of the drug flip-flop was measured at various liposome concentrations and the intrinsic flip-flop rate was deduced by extrapolation to high liposome concentration. As shown in Fig. 4, whereas high membrane concentrations of mitoxantrone seemed to interfere with its own flip-flop, idarubicin accelerated its own flip-flop, and doxorubicin and daunorubicin had little effect. Thus, apparent flip-flop rates measured at high liposome concentrations are an adequate approximation of intrinsic flip-flop values.

3.3. Passive transport of drugs by a flip-flop mechanism

Mitoxantrone traversed the lipid core of the liposomes membrane by a flip-flop mechanism with a rate constant of

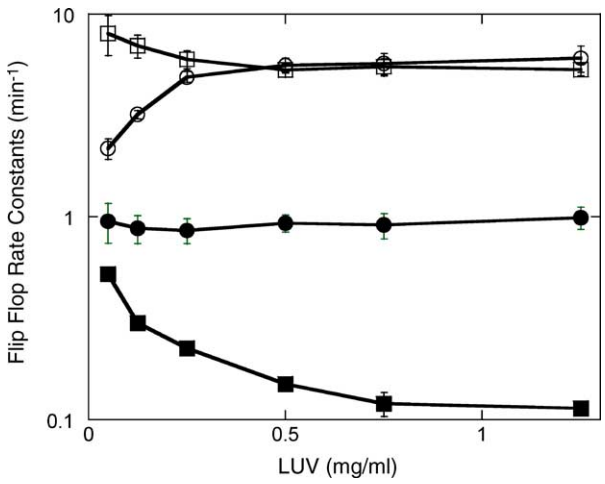

Fig. 4. Effect of liposome concentrations on apparent flip-flop rate of anthracyclines and mitoxantrone. Liposomes containing DOPC:DOPG:-cholesterol:NBD-PE (5:5:2:0.1, weight ratios) were prepared as described under "Section 2". Various concentrations of liposomes were incubated in medium A at 24 °C and l0 μM of idarubicin (empty circles), daunorubicin (empty squares), doxorubicin (full circles), or mitoxantrone (full squares) were added. The fluorescence of the NBD moiety was monitored continuously. The rate constants of the slow phases of the fluorescence quenching induced by the drugs were estimated by non-linear regression to exponentials that describe a first-order system. All values are means ±S.D. of four independent measurements.

$0.114\ min^{-1}$. This means that a mitoxantrone molecule inserted into the outer leaflet of the membrane dwelt in that leaflet for an average of 6 min before flipping to the inner leaflet of the liposome. This phenomenon was not dependent on the liposome composition and neither cholesterol nor acidic charge of the membrane had a significant effect on it. Although doxorubicin and daunorubicin are less hydrophobic than mitoxantrone, they exhibited faster flip-flop rates with average dwelling times of 0.7 and 0.1 min, respectively, while mitoxantrone exhibited the slowest flip-flop rate. Thus, the flip-flop rate of these drugs is not determined solely by their hydrophobicity. In contradistinction to the case with mitoxantrone, the presence of cholesterol or an acidic phospholipid in the liposomes slowed down flip-flop of doxorubicin, daunorubicin, and idarubicin. Arrhenius plots revealed that flip-flop of anthracyclines was uniformly accelerated by temperature rise (Fig. 5). As expected, membrane fluidization by benzyl alcohol accelerated the flip-flop rate of anthracyclines (Fig. 6). The local anesthetic, benzyl alcohol, has been previously shown to increase the fluidity of model bilayers [20] and biological membranes [21,22].

Drug transport from the outer medium into the lumen of the liposomes can be simulated once the extent of drug binding and the flip-flop rate are known. Uptake of drugs into liposomes involves insertion of the drug into the outer leaflet of the liposome membrane, flipping across the lipid core of the membrane, and release into the lumen of the liposome. The insertion of the drug into the membrane and its release are much faster compared to the flip-flop rate (see kinetics shown in Fig. 2 and [15,23]). Thus, in our simulations of drug uptake, we have assumed for these steps rate constants much larger than the rate constant that describes

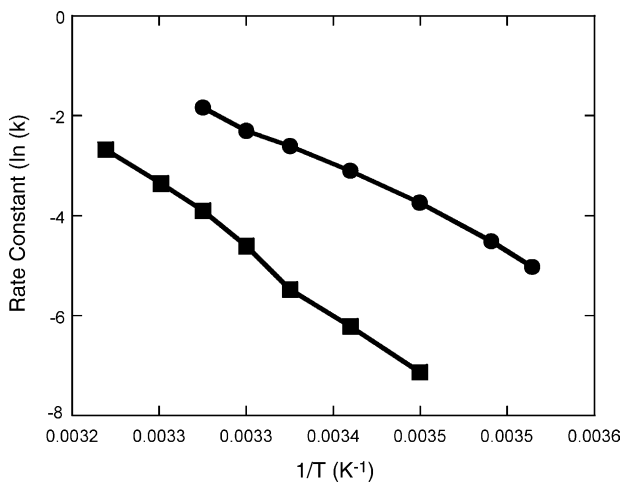

Fig. 5. Arrhenius plot of flip-flop rate. Liposomes containing DOPC:NBD-PE (10:0.1, weight ratios) were prepared as described under "Section 2". Liposomes (0.1 mM Pi) were incubated in medium A at various temperatures and 10 μM of either mitoxantrone (circles) or doxorubicin (squares) were added. The rate constants (expressed in terms of seconds) of the slow phases of the anthracyclines-induced fluorescence quenching were estimated by non-linear regression to exponentials that describe a first-order system.

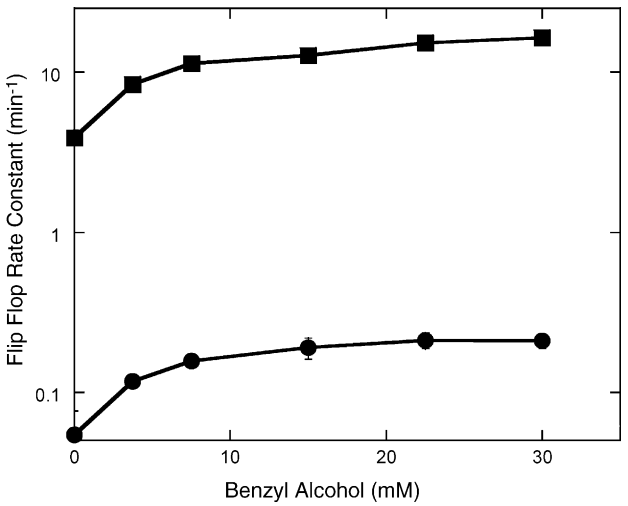

Fig. 6. Effect of the fluidizer, benzyl alcohol, on the apparent flip-flop rate of anthracyclines. Liposomes containing DOPC:DOPG:NBD-PE (5:5:0.1, weight ratios) were prepared as described under "Section 2". Liposomes (0.1 mM Pi) were incubated in medium A at 24 °C for 5 min before 10 μM of either doxorubicin (squares), or mitoxantrone (circles) were added. The fluorescence of the NBD moiety was monitored continuously. The rate constants of the slow phases of the anthracyclines-induced fluorescence quenching were estimated.

drug flip-flop. A simulation of drug uptake into liposomes under conditions similar to those employed in actual experiments, namely 1 μM drug and 0.5 mg/ml liposomes, is shown in Fig. 7. As expected, at equilibrium, drug concentration in the liposome lumen was equivalent to that in the outer medium and was determined by the partition coefficient of the drug. At equilibrium, the vast majority of the drugs were associated with the liposome membranes and not free in the lumen and the outer medium. Less than 0.5% of mitoxantrone was free in solution. Even doxorubicin, which exhibits the lowest partition coefficient, was more than 90% bound to the liposome membranes. The rate of drug uptake seemed correlated with the flip-flop rates of the various drugs. To further investigate the effect of flip-flop rates on drug uptake into liposomes, drug uptake was simulated as a function of its flip-flop rate (Fig. 8). As expected, drug concentration in the liposome at equilibrium was not dependent on the flip-flop rate. The course of drug uptake into liposomes could not be fitted to first-order kinetics and the rate was expressed as the time period required for the drug concentration within the liposome to reach half its concentration at equilibrium. As shown in Fig. 8, whereas flip-flop rates determine the transport rate, partition coefficients had little effect. Thus, simulated uptake rate of a drug with a high partition coefficient, such as mitoxantrone, was almost identical to that of a drug with a low partition coefficient, such as doxorubicin. Simulation of drug uptake assuming a wide range of drug partition coefficients revealed that, indeed, partition coefficients with values less than 10,000 determined the uptake rate and drug concentration at equilibrium (Fig. 9). At higher values, partition coefficients had little additional effect on the

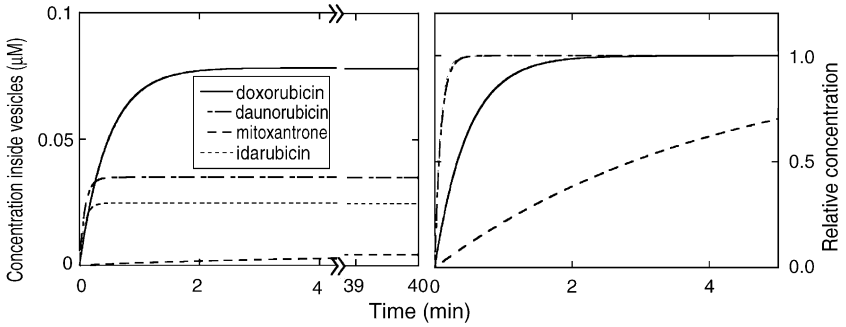

Fig. 7. Simulation of drug uptake kinetics. Uptake of mitoxantrone, doxorubicin, daunorubicin, and idarubicin from an initial 1 μM drug solution into 1 mg/ml of DOPC:DOPG:cholesterol (5:5:2, weight ratios) liposomes was simulated using the experimentally obtained partition coefficients (Table 1) and flip-flop rates (Table 2). The rate constant of drug insertion into the outer leaflet was assumed to be 10^{-7} s. The rate constant describing the release of drug from the membrane was equivalent to the adsorption rate constant divided by the partition coefficient. The drug concentrations within the liposomes were simulated as they are (left panel) or relative to the drug concentration achieved at equilibrium (right panel).

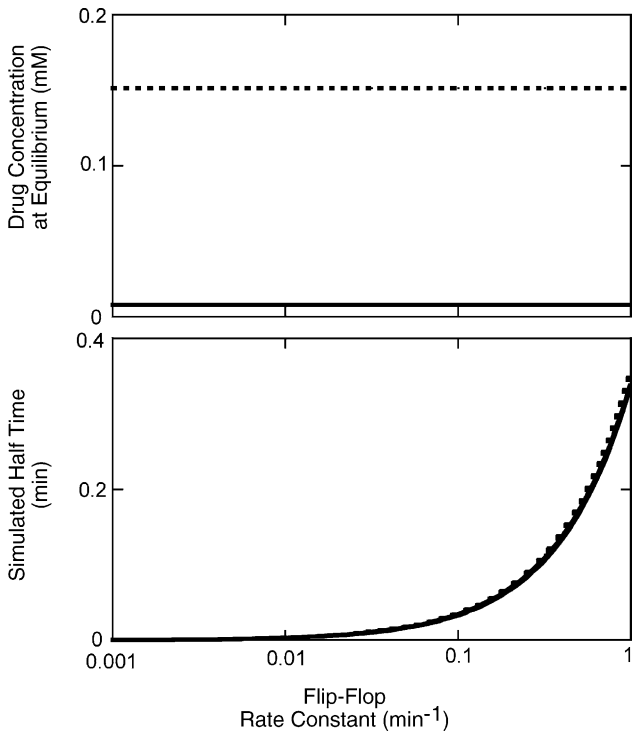

Fig. 8. Simulation of the effect of flip-flop rate on drug uptake into liposomes. Uptake of mitoxantrone (solid lines) and doxorubicin (dashed lines) from an initial 1 μM drug solution into 1 mg/ml of DOPC:DOPG:-cholesterol (5:5:2, weight ratios) liposomes were simulated using the experimentally obtained partition coefficients (Table 1) and various theoretical flip-flop rate constants.

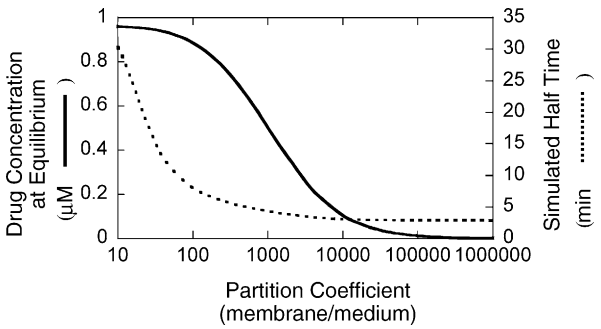

Fig. 9. Simulation of the effect of drug partition coefficient into membranes on drug uptake into liposomes. Uptake of a putative drug with a flip-flop rate of mitoxantrone from an initial 1 μM drug solution into 1 mg/ml of DOPC:DOPG:cholesterol (5:5:2, weight ratios) liposomes was simulated using various theoretical drug partition coefficients (lipid phase/aqueous medium).

uptake rate (which was fast) and the drug concentration at equilibrium (which was low).

4. Discussion

Flip-flop rates of mitoxantrone, doxorubicin, daunorubicin, and idarubicin occurred with a half-life equivalent to 6, 0.7, 0.15, and 0.1 min, respectively when measured with liposomes containing an acidic phospholipid, phophatidylcholine and cholesterol (Table 2). The presence of either cholesterol or an acidic phospholipid in the liposomes slowed down the flip-flop of doxorubicin, daunorubicin, and idarubicin, but had little effect on mitoxantrone flip-flop.

Flip-flop rates of doxorubicin measured with NBD-labeled lipids confirm previously reported rates obtained by DNA quenching of drug fluorescence [15]. Flip-flop rates with a half-life of minutes are consistent with a transport mechanism whereby the amphipathic anthracyclines and mitoxantrone are inserted into the outer lipid leaflet of the vesicles. Subsequently, drugs traverse the lipid core of the membrane by distinct events of flip-flop occurring after an average dwell equal to the half-life, which in mitoxantrone is 6 min. Complex amphipathic drugs, such as anthracyclines, are not soluble in the hydrophobic core of the membrane, but are oriented at the surface of the membranes [24,25]. Thus, their transmembrane transport is consistent with relatively rare events of flip-flop occurring after a long dwelling time at the origin leaflet, rather than diffusion down a putative gradient located in the lipid core of the membrane.

Transport of drugs, such as anthracyclines, involves partition of the drugs into the outer surface of the membrane, flip-flop across the lipid core of the membrane, and

Table 2
Flip-flop rate constants: liposomes were prepared with NBD-PE (1% of total lipids) and DOPC:DOPG:cholesterol (5:5:2, weight ratios), DOPC:cholesterol (10:2), DOPC:cholesterol (5:5), or DOPC

	DOPC:DOPG:cholesterol (min^{-1})	DOPG:cholesterol (min^{-1})	DOPC:DOPG (min^{-1})	DOPC (min^{-1})
Mitoxantrone	0.114 ± 0.008	0.15 ± 0.09	0.157 ± 0.01	0.15 ± 0.015
Doxorubicin	1.0 ± 0.045	2.9 ± 0.11	3.48 ± 0.24	4.4 ± 0.48
Daunorubicin	5.3 ± 0.35	11.5 ± 0.6	12.8 ± 1.2	na
Idarubicin	6.1 ± 0.45	14.7 ± 0.8	14.2 ± 2.3	na

Flip-flop rate constants were determined at liposome and drug concentrations equivalent to 1.5 mM Pi and 10 μM, respectively. The numbers in square parentheses denote reference numbers. na: not available (too fast to be measured).

release of the drugs from the membrane into the medium. Partition of the drugs into the membrane is extremely fast compared to the flip-flop (results presented here and [15]). Since drug binding to membranes does not involve interaction with specific sites, it is quite possible that drug partition is limited by drug diffusion toward the membrane. Thus, the transport rate across membranes is a function of drug partition into the membrane and the rate of its flip-flop across the lipid core.

The partition of the drugs tested here between the lipid phase of negatively charged membranes and aqueous medium ranged between 10,200 and 230,000. There was no clear correlation between these partition coefficients and drug hydrophobicity. Similar high values of partition coefficients have been previously reported for doxorubicin [24,26] and other anthracyclines [27]. The outcome of such high partition coefficients is that upon the addition of drugs to liposomes, most of the drug becomes immediately associated with the outer leaflet of the membrane. In the case of mitoxantrone, upon its addition to liposomes, less than 1% remains free in solution. Since, in the human body membranes and lipid globules are abundant, there too the vast majority of anthracyclines, and especially, mitoxantrone, become associated with lipid matrix and are not free in solution. Simulation of drug transport in liposomes indicated that variation in the extent of drug binding to membranes had a significant effect only at partition coefficients lower than 10,000. It is only under these conditions that the amount of drug available for flip-flop across the membrane is limiting. Expressing the distribution of drugs as a lipid phase/aqueous medium partition coefficient is somewhat misleading in the sense that the drugs are not distributed uniformly throughout the membrane, but are localized at the lipid/medium interface [28].

The rate limiting step in transmembrane movement of anthracyclines, and especially mitoxantrone, is their flip-flop rate across the lipid core of the membrane. There was no correlation between flip-flop rate and hydrophobicity of the drugs (as previously reported [3,23]). Thus, mitoxantrone is more lipophilic compared to doxorubicin and daunorubicin and still exhibits a slower flip-flop rate compared to them. The fast flip-flop rate exhibited by idarubicin is consistent with the limited success of P-glycoprotein in excluding this drug from cells [29]. P-glycoprotein catalyzes the translocation of drugs from the inner leaflet of the plasma membrane to the outer or to the external medium [13]. Fast flip-flop, such as is exhibited by idarubicin, will overcome P-glycoprotein-mediated drug pumping. On the other hand, slow flip-flop, as exhibited by mitoxantrone and doxorubicin, is consistent with their high resistance levels, since drugs evicted from the inner leaflet will be slow to return.

Appendix A. Mathematica program used for simulation of drug transport

```
(*The names of the reactants are: Dm = drug outside liposome; Dol =
drug in the outer leaflet of the liposome membrane; Dil = drug in
the inner leaflet of the liposome membrane; Dl = drug in the
liposome lumen.*)
Clear[Dm, Dol, Dil, Dl, t, Fliposome, Bov, Kbinding, Biv, k1, kn1,
k2, kn2, k3, kn3];
Module [
{maxT = 3000., (*This is the maximum time of the simulated time-course,
in seconds.*)
Fliposome = 0.022, (*Fliposome = liposome volume as a fraction of
total volume
Bov = fraction of liposome volume in the outer leaflet of the membrane
Biv = fraction of liposome volume in the inner leaflet of the membrane*)
    Bov = 0.044 0.53
    Biv = 0.044 0.47
    k1 = 10000
    k2 = 0.0019
    k3 = 10000,
    Kbinding = 2460}
    kn1 = (k1/Kbinding) (1-Fliposome)/(BovFliposome)
    kn2 = k2 Bov/Biv;
    kn3 = (k3 Kbinding) (Biv Fliposome)/(Fliposome)
    vel1[t_] := k1Dm[t];
    vel2[t_] := kn1Dol[t];
    vel3[t_] := k2Dol[t];
    vel4[t_] := kn2Dil[t];
    vel5[t_] := k3Dil[t];
    vel6[t_] :=  kn3 Dl[t];
P1 := Dm'[t] == −vel1[t] + vel2[t] (Bov Fliposome)/(1-Fliposome)
P2 := Dol'[t] == vel1[t]                    ((1 − Fliposome)/(Fliposome-
Bov)) − vel2[t] − vel3[t] + vel4[t] (Biv/Bov);
P3 := Dil'[t] == vel3[t](Bov/Biv) − vel4[t] − vel5[t] + vel6[t]    ((Flipo-
some)/(Fliposome Biv));
P4 := Dl'[t] == vel5[t] ((Biv Fliposome)/(Fliposome)) − vel6[t]; sol = ND-
Solve[{P1,P2,P3,P4,  Dm[0.] == 1.0,  Dol[0.] == 0.0,  Dil[0.] == 0.0,
Dl[0.] == 0.0},{Dm[t],Dol[t],Dil[t],Dl[t]},  {t,  0,  maxT},  WorkingPreci-
sion->15,AccuracyGoal->10,  PrecisionGoal->10,  StartingStepSize->1.0
10^-12,  MaxSteps->10000]
graph1 = Plot[Dl[t]/.sol, {t,0.,maxT}, PlotRange->Automatic, AxesLabel-
>{"'Time (s)", "[Dm] (μmoles L^−1)"}]
```

```
graph2 = Plot[Dl[t]/.sol, {t,0.,maxT}, PlotRange->Automatic, AxesLabel->{"'Time (s)", "[Dm] (μmoles L^−1)"}]
graph3 = Plot[Dl[t]/.sol, {t,0.,maxT}, PlotRange->Automatic, AxesLabel->{"'Time (s)", "[Dm] (μmoles L^−1)"}]
graph4 = Plot[Dl[t]/.sol, {t,0.,maxT}, PlotRange->Automatic, AxesLabel->{"'Time (s)", "[Dm] (μmoles L^−1)"}]
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