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ORIGINAL ARTICLES

Liposomes as a Model for the Biological Membrane: Studies on Daunorubicin Bilayer Interaction

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Abstract In this study the interaction of the antitumoral drug daunorubicin with egg phosphatidylcholine (EPC) liposomes, used as a cell membrane model, was quantified by determination of the partition coefficient (K_p). The liposome/aqueous-phase K_p of daunorubicin was determined by derivative spectrophotometry and measurement of the zeta-potential. Mathematical models were used to fit the experimental data, enabling determination of K_p. In the partition of daunorubicin within the membrane both superficial electrostatic and inner hydrophobic interactions seem to be involved. The results are affected by the two types of interaction since spectrophotometry measures mainly hydrophobic interactions, while zeta-potential is affected by both interpenetration of amphiphilic charged molecules in the bilayer and superficial electrostatic interaction. Moreover, the degree of the partition of daunorubicin with the membrane changes with the drug concentration, due mainly to saturation factors. Derivative spectrophotometry and zeta-potential variation results, together with the broad range of concentrations studied, revealed the different types of interactions involved. The mathematical formalism applied also allowed quantification of the number of lipid molecules associated with one drug molecule.

Keywords Biomimetics · Membrane · Drug interaction · Liposome · Absorption spectroscopy · Zeta-potential

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Introduction

Biological membranes act as a physiological barrier for a drug in its path to reach the site of action. The diffusion (through the phospholipidic cell membrane) is a key step in the absorption and distribution of a drug as well as, ultimately, its action in the organism. The molecule must enter the membrane within the polar headgroup region, diffuse through the lipophilic hydrocarbon double layer and emerge throughout the headgroup region on the inner side. Even in cases where a specific transporter is involved, the drug's ability to interact with the membrane is often highly correlated with the velocity or extent of the transportation. This ability depends on the drug's hydrophilic/lipophilic equilibrium, which can be quantitatively expressed as a partition coefficient.

The *n*-octanol/water partition coefficient ($K_{O/W}$) has been correlated with the hydrophobicity of drugs since the studies of Hansch and coworkers in the 1970s (Leo et al. 1971; Hansch and Dunn 1972). The *n*-octanol/water system, however, is only an approximation of the actual environment found in the interface between the cellular membranes and extracellular/intracellular fluids since it is an isotropic environment.

Liposomes are self-closed structures composed of amphiphilic lipids that form a bilayer encompassing an

aqueous compartment and have been extensively used as cell membrane models (Peetla et al. 2009). Liposomes present a membrane structure similar to the cellular one, in which the lipophilic hydrocarbon region is sandwiched between two ordered polar headgroup regions, and a system more appropriate for determining partition coefficients than the traditional *n*-octane/water method.

We studied the interaction of the antitumoral drug daunorubicin with the membrane of unilamellar liposomes composed of phosphatidylcholine. Daunorubicin (Fig. 1) is a natural antitumoral drug classified as an anthracycline, widely used to treat acute lymphocytic and granulocytic leukemia; but its use is restricted by its toxicity and multidrug resistance (MDR) (Agrawal et al. 2005). MDR is greatly dependent on the overexpression of P-glycoprotein (P-gp) multidrug transporter, which acts by decreasing intracellular drug concentrations through an ATP-dependent efflux of drug throughout the lipid bilayer (Romsicki and Sharom 1999). Being an integral membrane protein, the partition of the substrate into the bilayer before it interacts with the transporter is probably a requirement. Therefore, the binding affinity of drugs to P-gp is highly correlated with their ability to partition into the lipid membrane (Romsicki and Sharom 1999). Moreover, this ability determines the amount of drug which is able to effectively cross the cellular and nuclear membranes and reach the DNA and topoisomerase II, which are the main sites of action of daunorubicin (Gallois et al. 1996).

As daunorubicin is a positively charged molecule at physiological pH, it can interact electrostatically with the biological membrane and will probably display a much higher affinity for the lipid bilayer than supposed from the $K_{O/W}$. Electrostatic interaction of the positively charged anthracycline antibiotics with the negatively charged cardiolipin, a lipid abundant in heart tissue, is thought to be involved in drug localization in heart tissue and in the high cardiotoxicity observed with these drugs (Ratna and Burke 1995). On the other hand, the incorporation of an ionized drug into a neutral membrane modifies the membrane's surface charge density, restraining the access of more drug molecules to be incorporated and thus decreasing the apparent partition coefficient.

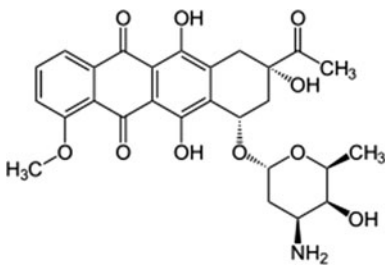

Fig. 1 Chemical structure of daunorubicin

In this context, the study of the mechanisms governing daunorubicin membrane interaction, either partitioning or electrostatic, is crucial.

Liposome/buffer partition coefficients (K_p) can be determined by different methods, including phase separation (for a comparative review on phase separation methods, consult Dipali et al. 1996). Since techniques used to separate the free drug from the liposome-encapsulated drug can potentially cause leakage of contents and, in some cases, uncertainty in the extent of separation, research using methods that do not rely on separation are of interest (Edwards and Baeumner 2006).

We used two different techniques to evaluate the K_p of daunorubicin without separation of phases: derivative spectrophotometry and zeta-potential evaluation.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC) and daunorubicin were from Sigma (St. Louis, MO) and used as received. All other chemicals were from Merck (Darmstadt, Germany). Solutions were prepared with HEPES buffer (10 mM, $I = 0.1$ M, pH 7.4). The ionic strength was adjusted with NaCl.

Vesicle Preparation

Liposomes were prepared by the thin film hydration method. In this method, a solution of lipid in chloroform was evaporated to dryness with a stream of nitrogen. The lipid film, in an amount that would provide a final lipid concentration of around 2 mM, was then left under vacuum overnight to remove all traces of the organic solvent. The resultant dried lipid film was dispersed using HEPES buffer and the mixture vortexed to yield multilamellar vesicles (MLVs).

Lipid suspensions were equilibrated at 25°C for 30 min and further extruded 10 times (10 ml Lipex^{TM} Thermobarrel Extruder; Northern Lipids, Burnaby, Canada) through polycarbonate filters (Nucleopore, Pleasanton, CA) with a diameter pore of 100 nm, to form large unilamellar vesicles (LUVs, stock solution). The EPC concentration in all vesicle suspensions was determined by phosphate analysis using the Fiske and Subbarow phosphomolybdate method (McClare 1971).

Derivative Spectrophotometry

The EPC stock suspension was diluted to prepare a set of suspensions with different EPC concentrations (range

0–1,400 μM), in which the drug concentration was kept constant in each assay but varied between assays (10–85 μM). A set of "blank" suspensions was prepared for each assay. After a period of 2 h in the dark, at 25°C, to allow drug partition equilibrium to be established, the spectra of all suspensions were collected by means of a double-beam spectrophotometer (V-650; Jasco, Easton, MD), at 25°C. The second derivative spectra were calculated after spectra smoothing, with Origin 8.5.1 software (OriginLab, Northampton, MA), using 13 points for the derivative calculus. Partition coefficients were determined by performing a nonlinear fitting of the proposed mathematical model (Eq. 2) to the experimental data using the same computer software.

Zeta-Potential and Size Determinations

For these studies, EPC concentration was kept constant (460 μM) in all the suspensions of the set and drug concentration ranged from 0 to approximately 200 μM across the set.

Size distribution of the extruded vesicles was determined by quasi-elastic light scattering analysis (Malvern ZetaSizer 5000; Malvern Instruments, Malvern, UK), using a helium-neon laser (633 nm) as a source of incident light, operating at a scattering angle of 90° and a temperature of 25.0°C, assuming a medium viscosity of 0.89 cP and a medium refractive index of 1.33.

Theory/Calculations

Determination of Partition Coefficients by Derivative Spectrophotometry

The theoretical background for the determination of partition coefficients by derivative spectrophotometry has been described previously (Castro et al. 2001; Marcelino et al. 2007). Briefly, the technique is based on the evaluation of the discrete spectral variations presented by the drug in the presence of increasing lipid concentrations. The main drawback is the high degree of scattering presented by the lipid solutions in the ultraviolet region, which can be attenuated by spectra derivation, since scattering has a negligible effect on derivatives. Moreover, this technique increases the sensitivity of spectroscopy since spectral details are enhanced and overlapping bands are separated.

The liposome/buffer partition coefficient is defined as the ratio between the number of moles of membrane-bound drug (n_m) per liter of phospholipid and the number of moles of free drug (n_w) per liter of buffer. This relation can be expressed as a function of bound drug ($[A_m]$), free drug ($[A_w]$) and lipid ($[L]$) concentrations and the lipid molar volume (V_Φ).

$$K_p = \frac{n_m/V_L}{n_w/V_w} = \frac{[A_m]}{[A_w]\,[L]V_\Phi} \tag{1}$$

The derivative intensities can be related with the partition coefficient by the following expression (Castro et al. 2001):

$$\mathrm{Abs_T} = \mathrm{Abs_w} + \frac{(\mathrm{Abs_m} - \mathrm{Abs_w})\,K_p[L]\,V_\Phi}{1 + K_p[L]V_\Phi} \tag{2}$$

where $\mathrm{Abs_T}$, $\mathrm{Abs_m}$ and $\mathrm{Abs_w}$ are the total (solution of the drug-containing lipid), lipid and aqueous derivative absorbance of the drug, respectively; K_p is the partition coefficient; $[L]$ is the lipid concentration; and V_Φ is the lipid molar volume. For EPC, the mean molecular weight was considered to be 770 and $V_\Phi = 0.688\ \mathrm{M}^{-1}$ (White et al. 1987).

Determination of Partition Coefficients by Zeta-Potential

The experimentally determined zeta-potential values can also be used to calculate partition coefficients, using a mathematical formalism fully explained in Matos et al. (2004) and used with other charged molecules previously (Ferreira et al. 2005a, b). Basically, the theory is based on the assumption that the membrane charging is a consequence of the stepwise incorporation of the charged drug. The use of Gouy-Chapman theory allows for the calculation of the amount of charged drug that has entered the membrane. Considering that zeta-potential is the potential measured at 2 Å from the particle surface (Winiski et al. 1988; Eisenberg et al. 1979), one can calculate the parameter α, the membrane potential at 0 nm (Ψ_0), and the surface charge density (σ), using the set of mathematical relations described in detail in Matos et al. (2004). The number of charged molecules per area unit (σ^*) can be calculated since $\sigma^* = \sigma.\mathrm{N/F}$. The σ^* value can be used to calculate the molar concentration of a positively charged drug in the membrane ($[A_m^+]$) as

$$[A_m^+] = \frac{\sigma^*\,[L]a_L}{1 - \sigma^*\,a_A} \tag{3}$$

where a_L is the molecular surface area of the lipid, a_A is the molecular surface area of the drug (daunorubicin), N is the Avogadro number and F is the Faraday constant. The value used for a_L was 60 $\mathrm{Å}^2$ (Rooney et al. 1983) and that for a_A was 75 $\mathrm{Å}^2$ (Heywang et al. 1998).

The calculation of the free drug concentration can now be achieved using a mass balance ($[A^T] = [A_m^+] + [A_w^+]$) since the total amount of drug added to the system, $[A^T]$, is known

and the drug is mainly (94%) in its ionized form. The value for the partition coefficient, K_p, can be obtained using Eq. 1.

The values of σ^*_{max} (maximum number of charged molecules per area unit) can be obtained from the plot of σ^* versus $[A^T]$, fitting the binding isotherm (Connors 1987):

$$\theta = \frac{K_b[A^T]}{1 + K_b[A^T]} \quad (4)$$

in which θ, the degree of saturation, is given by $\theta = \sigma^*/\sigma^*_{max}$.

Knowledge of σ^*_{max} allows calculation of $\bar{n}$, the mole ratio of lipid/drug (Matos et al. 2004):

$$\sigma^*_{max} = \frac{1}{(a_A + \bar{n}\, a_L)} \quad (5)$$

Results

Determination of Partition Coefficients by Derivative Spectrophotometry

Second derivative absorption spectra of daunorubicin in the presence of increasing lipid concentrations are shown in Fig. 2, together with the second derivative spectra of the "blank" lipid suspensions. As can be observed, the interference caused by the presence of liposomes was completely eliminated with the second derivative. Usually, LUV suspensions cause strong Rayleigh light scattering, especially in the UV region, hampering spectroscopic data collection and making necessary the use of high derivative orders (third or fourth), with consequent loss of signal to noise ratio. However, as daunorubicin absorbs in the visible region, where scattering is minimal, the second derivative provides an adequate elimination of lipid effect.

An increase in the derivative peaks is observed when the lipid concentration changes from 0 to ca. 1,400 μM (drug concentration kept constant). The derivative spectra show the existence of isosbestic points, which can indicate the existence of equilibrium between drug in polar aqueous and in nonpolar EPC bilayer phases (Castro et al. 2001).

Data points from the drug spectra (Abs_T in Eq. 2) were collected at 539 nm, a wavelength where scattering elimination was effective and the drug presented an intense peak, in order to achieve the best signal to noise ratio.

Equation 2 was fitted to the experimental second derivative spectrophotometric data, using a nonlinear least-squares regression method (Fig. 3), at wavelengths where the scattering is completely eliminated. The value of K_p obtained in HEPES buffer (pH 7.4) for daunorubicin was 1,084 ± 206 (mean and standard deviation of seven independent assays), but there appeared to be a decrease in partition as drug concentration increased, as can be observed in Fig. 4.

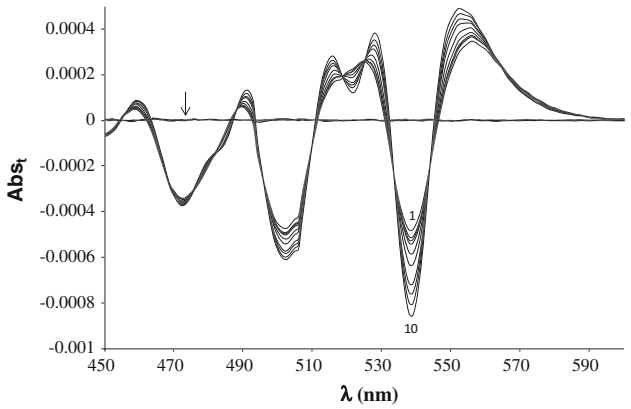

Fig. 2 Second derivative spectra of daunorubicin in different concentrations of EPC liposomes (from 0 [*1*] to 1,400 [*10*] μM), and of "blank" EPC liposomes (*arrow*)

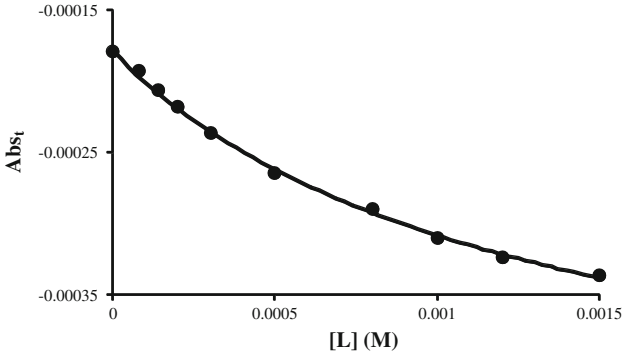

Fig. 3 Second-derivative spectrophotometric data at $\lambda = 539$ nm for daunorubicin in different concentrations of EPC liposomes. *Curve* represents best fit to Eq. 2

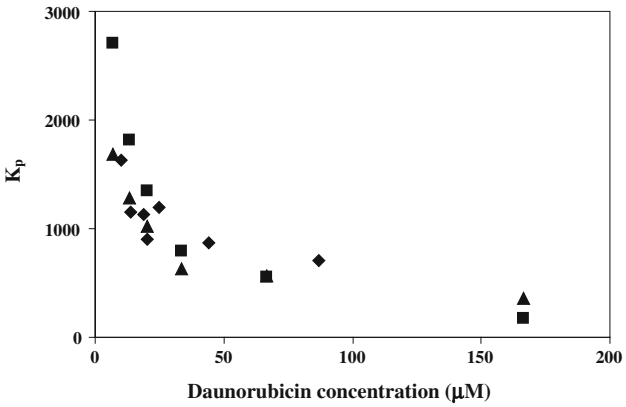

Fig. 4 Variation of partition coefficients on the concentration of daunorubicin obtained by derivative spectrophotometric (*diamonds*) and by zeta-potential measurements with (*triangles*) and without (*squares*) electrostatic effect corrections

Zeta-Potential and Size Determinations

The mean particle size of "blank" liposomes was 125 nm. Suspensions of liposomes with increasing concentrations of daunorubicin show a stepwise increase in vesicle diameter, reaching 305 nm for higher drug amounts. Similarly, results of zeta-potential for daunorubicin-incubated liposomes showed an increase from −7.2 to 1.3 mV (from 0 to 170 μM drug).

The variation of zeta-potential indicates that daunorubicin interacts with the membrane, causing a shift in its surface potential. The observed increase in size is too large to be considered only an expansion of the liposome due to drug incorporation and is probably affected by vesicle aggregation.

Comparison of Partition Coefficients Determined by Derivative Spectrophotometry and Zeta-Potential Variation

The results for partition coefficients determined by derivative spectrophotometry and zeta-potential variation are represented in Fig. 4 and Table 1, and two conclusions can be extracted:

1. For the lower drug concentrations (<20 μM), the K_p values obtained by zeta-potential evaluation (Fig. 4, squares) are around one-third higher than the ones obtained by derivative spectrophotometry (Fig. 5, diamonds). Since the liposome membrane is slightly negative in the absence of daunorubicin, the electrostatic superficial interaction (sensed mainly by zeta-potential) can be responsible for this difference. For higher drug concentrations, the partition of the positively charged daunorubicin, A^+, will lead to a neutralization of the membrane charge, this electrostatic attraction is diminished and the K_p values obtained by the two techniques are similar. Calculations of K_p were made (corrected) using the concentration of the charged form at the interface, $[A_i^+]$, instead of its bulk concentration, $[A_w^+]$. The value for $[A_i^+]$ can be obtained by the Boltzmann equation (McLaughlin and Harary 1976), and it is higher than $[A_w^+]$ due to the ionic attraction. Results for the corrected K_p (Fig. 4, triangles) show that the values obtained with this correction are not very different from the ones obtained with derivative spectrophotometry (diamonds).
2. A decrease in the partition coefficients can be observed as the drug concentration is increased. This decrease, although often described in the literature (Takegami et al. 2008), can be considered a deviation to the Nernst partition law since partition is an equilibrium state and the partition coefficient should be a constant. Such a decrease is probably due to a saturation effect: as the concentration of drug in the membrane increases, the maximum solubility of the drug in lipid is achieved and a minimum lipid/drug ratio is attained.

Table 1 Values for the liposome/buffer partition coefficients (K_p) obtained by two different methods: derivative spectrophotometry and zeta-potential determination

Derivative spectrophotometry		Zeta-potential determination		
Daunorubicin concentration (μM)	K_p	Daunorubicin concentration (μM)	K_p^a	K_p^b
10	1,631	7	2,702	1,690
14	1,150	13	1,820	1,284
19	1,131	20	1,349	1,024
25	1,198	33	796	628
44	872	67	551	568
87	703	167	174	356

In the case of results obtained with zeta-potential, K_p^a were calculated using the bulk drug concentration and K_p^b were calculated using the interfacial drug concentration, calculated by the Boltzmann equation

The values of σ^*_{max} (maximum number of charged molecules per area unit) can be obtained from the plot of σ^* versus $[A^T]$, fitting the binding isotherm (Eq. 4). Fitting is shown in Fig. 5. The value of σ^*_{max} obtained was 4.7×10^{-3} molecules/Å^2, while $\bar{n}$ was 29 molecules of lipid per molecules of drug.

Discussion

Daunorubicin is an amphoteric molecule, containing acidic functions (the ring phenolic groups) and a basic function (the sugar amino group). It is amphiphilic, the anthracycline ring being lipophilic and the sugar amino together with the hydroxyl groups being hydrophilic. The sugar amino group has a pK_a of 8.6 (Gallois et al. 1996), which, applying the

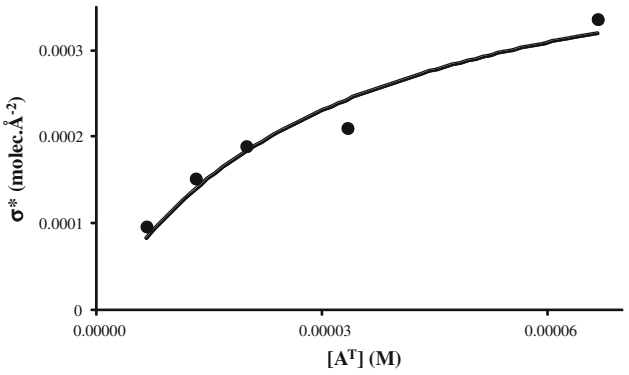

Fig. 5 Dependence of number of charged molecules per unit area (σ^*) on the concentration of daunorubicin at pH 7.4 in the presence of EPC. *Curve* represents best fit to Eq. 4

Henderson-Hasselbalch equation, gives a 94% positive ionization at the working pH value (7.4). Amphiphilic drugs that carry a positive charge can interact electrostatically with the negatively charged phosphate of the headgroup region of the bilayer, while the nonpolar portion inserts into the hydrophobic core. The overall result is an interfacial partitioning of the drug.

The daunorubicin partition in the membrane reflects the different types of interaction that an amphiphilic molecule can perform with a lipidic membrane, as a result of the structured bidimensional membrane, that holds a polarized superficial layer and a nonpolar inner core. This structure allows amphiphilic molecules to penetrate and orientate their polar part to the surface and nonpolar part to an inner position. Such an inner interaction can lead to a spectral modification as electronic distribution in the drug molecule is perturbed by the nonpolar environment, which can in extent lead to bathochromic effects, visible in the derivative spectra here presented.

On the other hand, a drug bearing a charged group can establish electrostatically driven interactions with positive or negative groups of the membrane, such as the negative phosphate or the positive amine groups. Such interactions appear, in the case of anthracyclines, to play a very important role in cardiotoxic effects (Ratna and Burke 1995).

Daunorubicin presents a spectral variation when it interacts with the membrane, which is an indication that the molecule establishes an inner partition. Nevertheless, an electrostatic interaction is also likely to occur, especially for lower drug concentrations, before membrane charging begins to cause electrostatic repulsion. Figure 4 shows that the results (squares) obtained by zeta-potential are higher than the results from derivative spectrophotometry (diamonds) for lower drug concentrations, possibly because of a superficial electrostatic attraction of the positive drug by the negative liposome. As drug concentration increases, this effect diminishes due to neutralization and then inversion of the liposome charge. When corrected for electrostatic effects, both techniques yield similar results. It appears that for lower drug concentrations (<20 μM), electrostatic interaction plays an important role in the interaction; for higher drug concentrations (>20 μM) the process is hydrophobically driven. Another finding was the decrease in K_p as drug concentration increases. That can be observed with the two techniques used and attributed to a saturation effect of the membrane, which is achieved when the lipid/drug ratio is around 30.

Gallois et al. (1996) determined the K_p for daunorubicin using negatively charged liposomes and found a value of 1,900 ± 540, which is higher than the values obtained in our study, probably due to some degree of electrostatic interaction as the positive drug can be attracted to the negative lipid. These authors also concluded that daunorubicin interaction does not depend on the drug's or liposome's charge, and in another study Gallois et al. (1998) detected only hydrophobic interactions by this molecule. These findings are probably due to the techniques used (fluorimetry and circular dichroism) or the drug concentration used (in fact, for higher drug concentrations there is only hydrophobic partition).

Conclusions

This article describes the interaction of daunorubicin with the membrane of EPC liposomes. In the interaction of daunorubicin with the membrane, both electrostatic and hydrophobic forces seem necessary for the stabilization of anthracyclines in the lipid bilayer. The determination of K_p by derivative spectrophotometry is based on the spectral changes occurring when drug and membrane interact and thus must reflect mostly the hydrophobic interaction. On the other hand, zeta-potential variations reflect a charge increase, caused by the partition of a charged molecule in the lipid bilayer.

Our results show that for lower drug concentrations (<20 μM) the electrostatic attraction to the membrane strongly accounts for the global partition coefficient, while for higher drug concentrations the hydrophobic interpenetration is the main single driven force for the interaction.

Another finding was the saturable nature of daunorubicin interaction within the membrane. In fact, a minimum of 30 lipid molecules seems to be necessary to dissolve each drug molecule in the bilayer environment.

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