ORIGINAL PAPER

Europium Coordination Complexes as Potential Anticancer Drugs: Their Partitioning and Permeation Into Lipid Bilayers as Revealed by Pyrene Fluorescence Quenching

Valeriya Trusova · Andrey Yudintsev ·
Ludmila Limanskaya · Galyna Gorbenko ·
Todor Deligeorgiev

Abstract The present study was undertaken to evaluate the membrane-associating properties of a series of novel antitumor agents, Eu(III) coordination complexes (EC), using the pyrene fluorescence quenching as an analytical instrument. Analysis of EC-induced decrease in pyrene fluorescence intensity in terms of partition and solubility-diffusion models allowed us to evaluate the partition and permeation coefficients of the examined compounds into the lipid vesicles prepared from zwitterionic lipid phosphatidylcholine (PC) and its mixtures with cholesterol (Chol) and anionic lipid cardiolipin (CL). The drug-lipid interactions were found to have the complex nature determined by both EC structure and lipid bilayer composition. High values of the obtained partition and permeation coefficients create the background for the development of EC liposomal formulations.

Keywords Europium complexes · Lipid membranes · Liposomal formulations · Partitioning · Permeability

V. Trusova · A. Yudintsev · L. Limanskaya · G. Gorbenko
Department of Nuclear and Medical Physics, V.N. Karazin
Kharkov National University,
4 Svobody Sq.,
Kharkov 61077, Ukraine

T. Deligeorgiev
Department of Applied Organic Chemistry,
Faculty of Chemistry and Pharmacy, University of Sofia,
Sofia 1164, Bulgaria

V. Trusova (✉)
19-32 Geroyev Truda Str.,
Kharkov 61144, Ukraine
e-mail: valtrusova@yahoo.com

Introduction

During the past decades lanthanide complexes attract ever growing interest due to their favorable photophysical properties, such as exceptionally long lifetime, large Stokes' shifts, and line-like emission [1] arising from Laporte-forbidden f-f transitions in lanthanide ions [2]. These unique spectral characteristics resulted in the intensive utilization of lanthanide chelates in a wide variety of scientific and technological areas as luminescent materials [3], chemosensors [4], fluorescent labels [5], photoluminescence devises [6], etc. In addition, lanthanides are successfully used in medicine as effective MRI contrast agents, hypophosphatemic agents for hemodialysis and palliative pharmaceuticals for osteosarcoma patients [7–10]. Recently medical applications of lanthanide complexes were expanded by introducing the newly synthesized europium compounds (EC) as novel anticancer drugs [9, 11]. More specifically, europium (III) tris-β-diketonates were shown to exhibit profound cytotoxic effect presumably arising from abundance of DNA-intercalating pharmacophore in their structure [11]. However, clinical application of these drugs in the free form may not provide complete benefit to the patient unless supported by the adequate drug delivery system. These limitations are connected with high toxicity, metabolic instability and low therapeutic index of the antineoplastic compounds. Therefore, it seems of importance to evaluate the possibility for development of the effective delivery systems for EC, capable of not only acting as delivery vehicle, but also altering the biodistribution of the drugs and increasing their therapeutic potential. Among available micro- and nanosized drug carriers, liposomes represent the most promising systems due to their versatility, biocompatibility, biodegradability and lack of immunogenicity [12–14]. Encapsulation of drugs in liposomes provides a way to reduce the distribution volume

and decrease toxic side effects in healthy tissues. Furthermore, amphiphilic nature of the lipid molecules allows effective encapsulation of both hydrophilic and hydrophobic compounds. Finally, liposome-entrapped drugs become protected from the degradation and transformation in an organism. One of the fundamental tasks related to the development of drug liposomal formulations is evaluation of the drug-membrane partition coefficient (K_P) and estimation of lipid bilayer permeability (P_e), the parameters characterizing the ability of a certain compound to accumulate within the membrane. Analytical methods currently used for assessment of the lipid-to-water partition coefficient include equilibrium dialysis [15, 16], absorption and fluorescence spectroscopy [17], centrifugation [18], chromatography [17]. Of these, fluorescence spectroscopy seems to be one of the most effective tools for accurate determination of the above parameters due to its high sensitivity, specificity, informativity, noninvasive nature, and immense range of measured parameters [19]. In the present work we evaluated the lipid associating ability of ten newly synthesized EC using the methodological approach developed by Lakowicz et al. [19, 20]. This approach is based on the dependence of quenching efficacy of membrane-bound probe on the quencher partitioning into lipid bilayer. To derive EC partition and permeation coefficients, these compounds were recruited as collisional quenchers for the hydrophobic membrane probe pyrene.

Experimental

Materials

Egg yolk phosphatidylcholine (PC) and beef heart cardiolipin (CL) were purchased from Biolek (Kharkov, Ukraine). Pyrene and cholesterol (Chol) were obtained from Sigma (Germany). Eu(III) coordination complexes defined here as V3 – V12 (Fig. 1) were synthesized at the Faculty of Applied Organic Chemistry, University of Sofia, as described previously [11]. All other chemicals were of analytical grade and used without further purification.

Preparation of Lipid Vesicles

Large unilamellar lipid vesicles composed of PC or PC mixtures with 30 mol% of Chol or 5 and 10 mol% of CL were prepared using the extrusion technique [21]. The thin lipid film was obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4). Lipid suspensions were extruded through a 100 nm pore size polycarbonate filter. The size of lipid vesicles was controlled by dynamic light scattering. The phospholipid concentration was determined according to the procedure of Bartlett [22]. Hereafter, liposomes

containing 30 mol% Chol or 5 or 10 mol% CL will be referred to as Chol30, CL5 and CL10, respectively.

Fluorescence Measurements

Fluorescence measurements were performed with LS-55 spectrofluorimeter (Perkin Elmer, Great Britain) equipped with magnetically stirred, thermostated cuvette holder. The decrease in pyrene fluorescence intensity at 465 nm (excitation wavelength was 340 nm) was monitored as a function of increasing concentration of EC.

Theoretical Background

Partition Model

If the quencher distributes between the lipid and aqueous phases, its partition coefficient is given by:

$$K_P = [Q]_m/[Q]_w \tag{1}$$

where $[Q]_{m,w}$ are the concentration of quencher in a membrane and water, respectively.

Total concentration of the quencher $[Q_T]$ is:

$$[Q_T]V_T = [Q]_m V_m + [Q]_w V_w \tag{2}$$

where $V_{m,w}$ are the volumes of lipid and aqueous phases, respectively, V_T is the total volume of the system.

Defining $\alpha_m = V_m/V_T$ as the volume fraction of membrane phase one obtains [19, 20]:

$$[Q]_m = \frac{K_P[Q]_T}{K_P\alpha_m + (1 - \alpha_m)} \tag{3}$$

Substitution of this expression into the Stern-Volmer equation ($F_0/F = 1 + k_q\tau_0[Q]$, k_q– bimolecular rate constant for the dynamic quenching) gives:
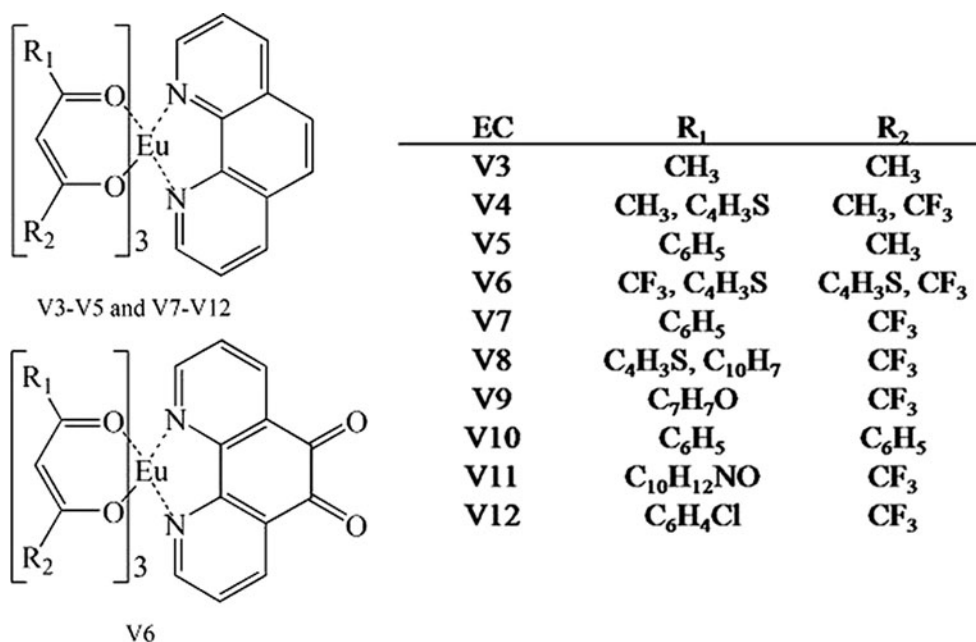
$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + \frac{\tau_0 k_m K_P[Q]_T}{K_P\alpha_m + 1 - \alpha_m} = 1 + \tau_0 k_{app}[Q]_T \tag{4}$$

where F_0 and F are fluorescence intensities of the probe in the absence and presence of quencher, respectively, k_m is the bimolecular quenching constant for the membrane-bound fluorophore, $k_{app} = k_m K_P/(K_P\alpha_m + 1 - \alpha_m)$ is the apparent quenching constant. It is easy to notice that this parameter depends on volume fraction of membrane phase α_m, which, in turn, is a function of lipid concentration:

$$\frac{1}{k_{app}} = \alpha_m \left(\frac{1}{k_m} - \frac{1}{k_m K_P}\right) + \frac{1}{k_m K_P} \tag{5}$$

According to this approach, k_m and K_P can be obtained from the dependence of k_{app}^{-1} on α_m. Successful determination of the partition coefficients allows evaluation of the

Fig. 1 Chemical structures of europium complexes

V3-V5 and V7-V12

V6

EC	R₁	R₂
V3	CH_3	CH_3
V4	CH_3, C_4H_3S	CH_3, CF_3
V5	C_6H_5	CH_3
V6	CF_3, C_4H_3S	C_4H_3S, CF_3
V7	C_6H_5	CF_3
V8	$C_4H_3S, C_{10}H_7$	CF_3
V9	C_7H_7O	CF_3
V10	C_6H_5	C_6H_5
V11	$C_{10}H_{12}NO$	CF_3
V12	C_6H_4Cl	CF_3

fraction of quencher in a lipid phase as:

$$f_m = K_P \alpha_m / (K_P \alpha_m + 1 - \alpha_m) \qquad (6)$$

Solubility-Diffusion Model

According to the solubility-diffusion model, the transmembrane permeability of a certain compound, in our case europium chelate, P_e is proportional to its lipid/water partition coefficient [23]:

$$P_e = \frac{K_P D_{EC}}{d} \qquad (7)$$

where D_{EC} is EC diffusion coefficient inside the membrane, d is the lipid bilayer thickness. The diffusion coefficients of the examined EC were determined using the Smoluchowski equation:

$$k_m = \frac{4\pi N}{1000} (R_{pyr} + R_{EC})(D_{pyr} + D_{EC}) \qquad (8)$$

where N is Avogadro's number, $R_{pyr,EC}$ are the molecular radii of pyrene, taken as 4 Å, and EC, respectively. R_{EC} were calculated from EC molecular volumes assuming the spherical shape of the drug molecule and were found to range from 8 to 11 Å depending on EC structure. D_{pyr} is pyrene diffusion coefficient in the membrane. Calculation of D_{pyr} was made on the basis of thorough analysis of pyrene excimerization in lipid bilayers (data not shown) and included the following steps [24]:

a) determination of pyrene excimerization constant:

$$K_E = \frac{\xi E/M}{\tau_M [Pyr]} \qquad (9)$$

where $\xi = 0.43$ is a proportionality coefficient, $\tau_M = 160$ ns is fluorescence lifetime of pyrene monomers, E/M—pyrene excimer-to-monomer intensity ratio, $[Pyr]$ is pyrene concentration in the lipid phase,

b) calculation of pyrene diffusion coefficient:

$$D_{pyr} = \frac{K_E}{N \cdot 10^3 \cdot 8\pi \cdot 2R_{pyr}} \qquad (10)$$

Calculated in such a way, pyrene diffusion coefficients were shown to be 0.95×10^{-11} m²s⁻¹, 0.92×10^{-11} m²s⁻¹, 0.88×10^{-11} m²s⁻¹ and 0.85×10^{-11} m²s⁻¹ for PC, Chol30, CL5 and CL10 vesicles. Importantly, calculations of D_{pyr} were made both in the neat lipid bilayers, as well as in EC-containing vesicles. It was found that incorporation of the drugs into the membranes results in statistically insignificant changes (<4 %) in D_{pyr}. Therefore, for the sake of simplicity, the values of pyrene diffusion coefficients in the neat lipid bilayers were taken. The derived D_{EC} values were further substituted into Eq. 7 to evaluate the permeability coefficients.

Results

Fluorescence quenching of aromatic hydrocarbons, such as pyrene, naphthalene, anthracene by metal ions is a well-known phenomenon encountered while investigating electron transfer reactions [25], intersystem crossing processes [26], exciplex formation [27], evaluating critical micellar concentration [28], etc. In the present contribution we utilized the quenching of pyrene fluorescence by a range of Eu

complexes, the novel potential antineoplastic agents, to characterize their incorporation into the lipid bilayers of different composition.

As seen in Fig. 1, the examined compounds are asymmetric Eu(III) coordination complexes with diverse O-containing chelate ligands, which, apparently, serve at least two main functions: i) bind tightly Eu(III) atom, providing the rigidity of the whole structure, and ii) shield lanthanide ion from the quenching and destabilizing effects of water. EC contain also organic chromophores which are responsible for absorbance of excitation light and energy transfer to lanthanide ion. Since the examined compounds are uncharged and possess relatively high hydrophobicity, it was assumed that EC-lipid association is controlled mainly by hydrophobic interactions. Therefore, in our study we focused mainly on PC and Chol30 membranes. However, to check the validity of the above assumption, for some EC we conducted analogous quenching experiments with anionic CL-containing lipid bilayers.
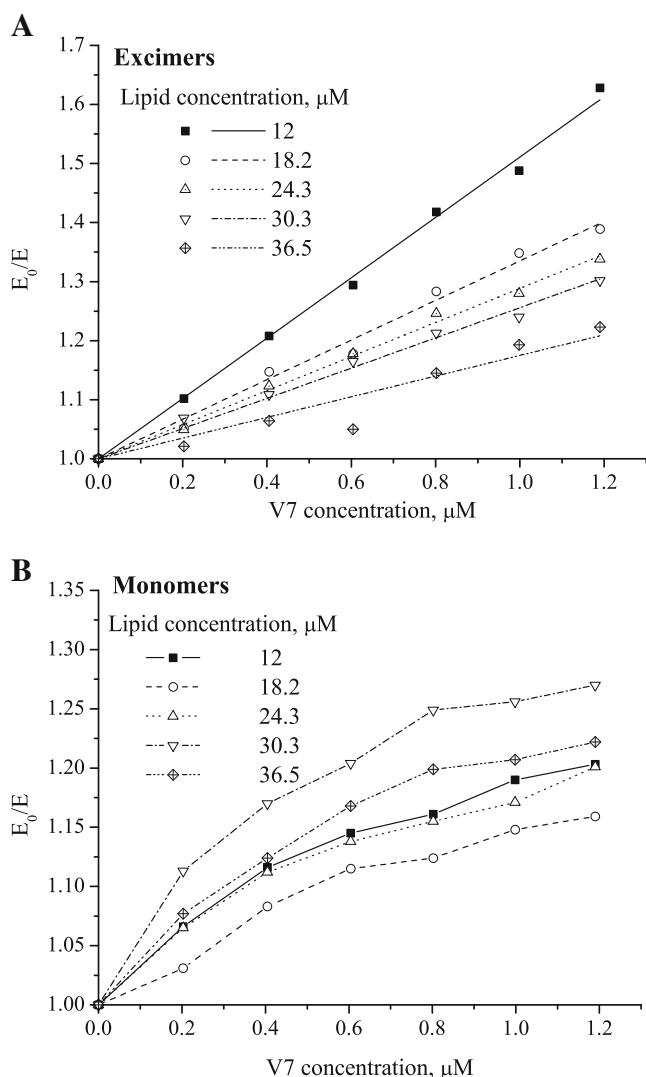
Association of EC with pyrene-doped lipid vesicles resulted in the decrease of probe fluorescence intensity, suggesting that europium complexes are effective quenchers of pyrene fluorescence (representative spectra are given in Fig. 2). Notably, membrane incorporation of EC brought about the decrease in fluorescence intensity of both pyrene monomers and excimers. The representative Stern-Volmer plots are shown in Fig. 3a. To extract quantitative information concerning EC affinity to the lipid bilayers, we applied the methodological approach developed by Lakowicz et al. [19, 20]. This approach is based on the idea that bimolecular quenching constant for the collisional quenching is proportional to the local quencher concentration which makes it possible to extract not only the quenching parameters but also lipid/water partition coefficient of the quencher. The data treatment algorithm included the following main steps: 1) determination of k_{app} from Stern-Volmer plots according to Eq. 4, 2) estimation of k_m and K_P values from k_{app}^{-1} (α_m) dependencies according to Eq. 5, 3) calculation of f_m parameter based on known K_P values (Eq. 6). Next, the recovered k_m values were used for evaluating the diffusion coefficients of europium complexes (D_{EC}) according to Smoluchowski equation (Eq. 8). The obtained D_{EC} were further substituted into the solubility-diffusion model (Eq. 7) to yield the permeability coefficients (P_e) of the examined drugs.

First, we tried to analyze the results of pyrene monomer fluorescence quenching by europium chelates. Since the basic Stern-Volmer plots (Fig. 3b) displayed downward curvature, we made an attempt to analyze the quenching data in terms of modified Stern-Volmer equation. However, the obtained K_P values were unrealistic, even attaining in some cases negative values. The most probable reason for such a behavior is the observation that formation of drug-
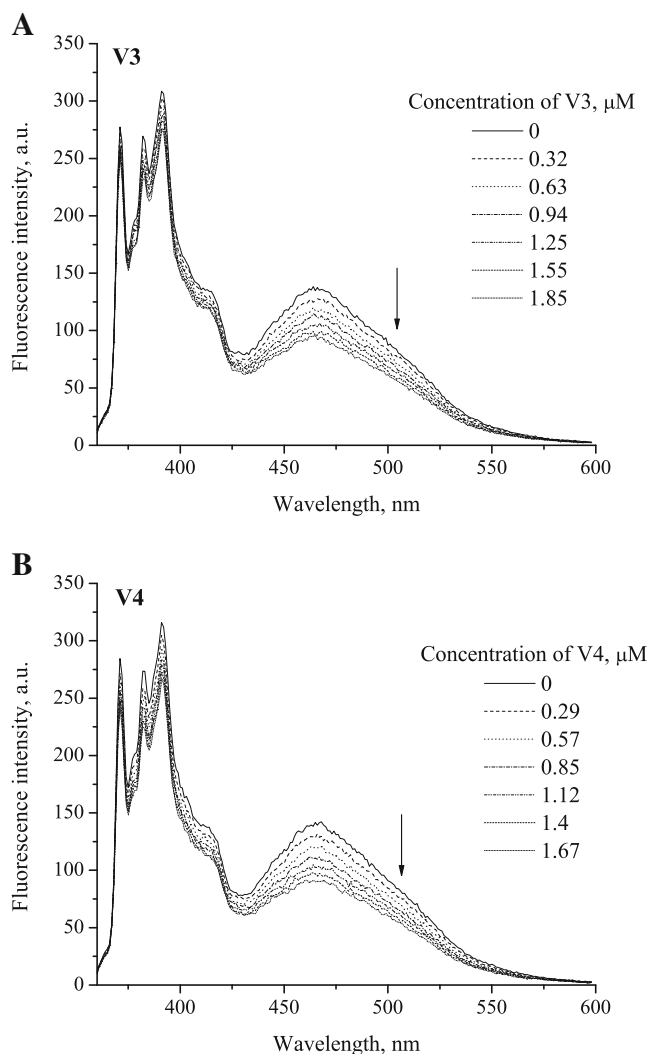
Fig. 2 Pyrene fluorescence spectra in PC lipid vesicles at varying concentrations of V3 (**a**) and V4 (**b**). Lipid and pyrene concentrations were 12 and 0.6 μM, respectively

membrane complexes induces the changes in the intensity ratio of the first to third vibronic bands of pyrene monomers (data not shown). This means that EC induce the changes in emission spectra of pyrene monomers not only by fluorescence quenching, but also through modifying the lipid bilayer polarity profile. In other words, fluorescence quenching of monomers seem to interfere with the changes in membrane polarity and thus cannot adequately reflect the EC partitioning. Therefore, EC partition coefficients were derived from the quenching data obtained for pyrene excimers. Analysis of the experimental results in terms of the partition and solubility-diffusion models allowed us to recover the set of parameters characterizing the drug binding and accumulation in the membrane (Table 1). In this context it should be stressed that the employed model is based on two main statements: i) the quencher is distributed between the bulk and lipid bilayer, and ii) the quenching may occur

Fig. 3 Stern-Volmer plots for the quenching of pyrene excimers (**a**) and monomers (**b**) in PC liposomes by V7

$(10^{-6}–10^{-4}\,cm/s)$ also indicate that membrane is highly permeable for europium chelates as compared to other pharmaceuticals ($4\times10^{-6}\,cm/s$ for camptothecin [34], $6.1\times10^{-6}\,cm/s$ for carbamazepine, $1.3\times10^{-6}\,cm/s$ for serratiopeptidase [36], $2\times10^{-5}\,cm/s$ for chlorpromazine [34], $1.9\times10^{-4}\,cm/s$ for ibuprofen [34], etc.). High values of permeability coefficients suggest that due to their lipophilic nature EC readily penetrate the membrane and accumulate therein without thermodynamically unfavourable exiting the lipid phase. Finally, it is noteworthy that the trends in K_P and P_e behavior correlate with the changes in f_m values, and that high partition and permeability coefficients, recovered, for instance, for V7 in PC liposomes or V8 in Chol30 vesicles correspond to ~90 % of the drug incorporated in the membrane.

Discussion

Successful development of liposomal formulations of pharmaceuticals and their therapeutic action in an organism are determined basically by the drug ability to accumulate within the lipid bilayer. The process of drug bioaccumulation involves three main steps: i) uptake of the drug molecule by membrane lipids, ii) diffusion of the compound through the lipid bilayer, iii) drug accumulation in the membrane without exiting the lipid phase. Therefore, drug bioaccumulation should be considered from the viewpoint of both its kinetics (permeation or passive diffusion) and partition in the membrane. Furthermore, passive permeation of the drugs into/across the membrane requires a delicate balance between liposolubility (solubility in lipid phase) and hydrosolubility (solubility in blood and cytosol). This balance is generally described in terms of lipophilicity parameters the main of which is drug partition coefficient. These considerations also necessitate the importance of mutual analysis of partition-permeation processes. In addition, one of the necessary prerequisites for the drug partitioning into the lipid bilayer and diffusion through it, is the existence of membrane free volume characterized by the difference between the effective and van der Vaals lipid volumes. Free volume reflects the degree of lipid packing. Lateral movements of lipid molecules as well as the existence of their different conformers disturb the homogeneous packing and induce the formation of the structural defects in the lipid bilayer [37]. The most stable is *trans*-conformation, in which the acyl chain is fully prolate and does not change its direction. In *gauche*-conformation the direction of the lipid chain is varied resulting in the formation of packing defect. The sequence of *gauche – trans – gauche*-conformations for three adjacent –C–C–bonds results into creation of the kinks and defects along the acyl chain, and consequently, free volume.

Fluorescence quenching technique provides a unique analytical tool for simultaneous characterization of diffusional

only in the lipid phase. The correctness of applying this model to our systems may be roughly confirmed by the observation that the values of bimolecular quenching constant appeared to be the same orders of magnitude as those obtained by Lemmetyinen et al. [29]. While examining pyrene fluorescence quenching by membrane-incorporated tetracyanoquinodimethane (TCNQ) these authors proved the validity of the employed methodological approach by comparing the steady-state and time-resolved fluorescence quenching data. In the present study, the values of EC partition coefficients were found to be $10^3–10^6$ orders of magnitude indicating rather high efficiency of chelate incorporation into lipid bilayers. For the sake of comparison, K_P for other drugs are 2.2×10^3 for dibucaine [30], 1.2×10^4 for tetracaine [31], 1.4×10^3 for indomethacine [32], 2.2×10^3 for acemetacin [33], 4.2×10^5 for trifluoperazine [34], 2.8×10^5 for chlorpromazine [35], etc. The magnitudes of the recovered permeability coefficients

Table 1 Parameters of EC—lipid complexation

| EC	Lipid system	K_P	k_m, $M^{-1}s^{-1}$	f_m range	P_e, cm s^{-1}
V3	PC	1.1×10^5	1.3×10^8	0.55–0.79	3.9×10^{-4}
	Chol30	1.5×10^5	9.8×10^7	0.59–0.82	7.5×10^{-4}
V4	PC	6.7×10^4	1.9×10^8	0.43–0.69	1.1×10^{-4}
	Chol30	2.7×10^4	2.5×10^8	0.21–0.44	8.1×10^{-5}
V5	PC	1.3×10^5	1.7×10^8	0.6–0.82	4.7×10^{-4}
	Chol30	4.9×10^4	2.4×10^8	0.32–0.59	1.1×10^{-4}
V6	PC	4.9×10^4	4.5×10^8	0.36–0.63	8.3×10^{-5}
	Chol30	1.0×10^5	2.4×10^8	0.51–0.76	5.3×10^{-4}
	CL5	9.8×10^3	1.3×10^9	0.11–0.26	4.6×10^{-6}
	CL10	2.9×10^4	7.9×10^8	0.28–0.53	9.7×10^{-6}
V7	PC	5.7×10^5	1.9×10^8	0.87–0.95	10.1×10^{-4}
	Chol30	3.5×10^4	3.1×10^8	0.25–0.51	9.8×10^{-5}
V8	PC	1.9×10^4	8.3×10^8	0.18–0.39	2.6×10^{-4}
	Chol30	1.4×10^6	3.5×10^8	0.91–0.96	9.1×10^{-4}
V9	PC	1.7×10^5	2.5×10^8	0.66–0.86	7.8×10^{-4}
	Chol30	6.0×10^4	3.6×10^8	0.37–0.64	3.2×10^{-4}
	CL5	3.9×10^4	2.4×10^8	0.32–0.58	2.8×10^{-5}
	CL10	4.2×10^4	6.6×10^9	0.44–0.68	3.5×10^{-5}
V10	PC	4.7×10^4	7.5×10^9	0.53–077	6.7×10^{-5}
	Chol30	1.3×10^5	3.5×10^9	0.61–0.87	6.7×10^{-4}
	CL5	2.6×10^4	7.8×10^9	0.24–0.49	9.3×10^{-6}
	CL10	4.6×10^4	2.5×10^{10}	0.37–0.64	5.5×10^{-5}
V11	PC	2.6×10^5	3.0×10^8	0.74–0.9	9.9×10^{-4}
	Chol30	2.9×10^4	8.2×10^8	0.22–0.46	9.0×10^{-5}
	CL5	8.6×10^3	1.4×10^8	0.11–0.24	2.4×10^{-6}
	CL10	3.5×10^4	4.3×10^8	0.29–0.51	2.7×10^{-5}
V12	PC	1.6×10^5	3.2×10^8	0.62–0.85	6.7×10^{-4}
	Chol30	7.3×10^4	4.4×10^8	0.42–0.68	4.1×10^{-4}
	CL5	1.4×10^4	7.4×10^8	0.15–0.34	7.1×10^{-6}
	CL10	2.6×10^4	8.4×10^8	0.25–0.51	8.9×10^{-6}

and binding phenomena occurring in a membrane [19]. In the present contribution dynamic quenching of pyrene excimer fluorescence by a series of europium compounds, newly synthesized potential anticancer agents, was utilized to analyze the distribution of the drugs in the membrane phase. Analysis of the obtained results within the framework of partition and solubility-diffusion models yielded quantitative information on drug-lipid complexation. As seen from the Table 1, the values of K_P, f_m and P_e strongly depend on both the drug structure and lipid bilayer composition. Due to nonpolar nature of the examined agents, we attributed the predominant role in EC—lipid association to hydrophobic interactions. This assumption is corroborated by the finding that CL decreases the above partition and permeability parameters (Table 1). In the present context it seems of importance to note that according to modern concepts, lipid bilayer is considered not as homogeneous phase, but as lamellar anisotropic environment consisting of 4 main regions: 1) the layer of bound water, 2) the region of polar headgroups, 3) the region of ordered segments of lipid acyl chains, 4) the region of unordered segments of lipid acyl chains [38]. This lamellar structure of lipid bilayer underlies the existence of sharp gradient of dielectric constant, electrostatic potential, lipid order parameter etc. Following these gradients, nonpolar compounds such as EC tend to distribute in the hydrophobic core of the membrane. Along with this, the observations that: i) Chol exerted ambiguous influence on K_P, f_m and P_e, ii) for all EC increasing the content of CL from 5 to 10 mol% brought about the increase in the derived parameters indicate that EC encapsulation into the vesicles cannot be considered exclusively in terms of electrostatic or hydrophobic interactions. Instead, complex superposition of the factors, such as drug structure from one hand, and lipid bilayer physicochemical properties from the other hand, seem to play essential role in the drug membrane incorporation. Additional argument in favor of this statement comes from the fact that in PC

liposomes the dye row showing the increase in K_P is V8 – V10 – V6 – V4 – V3 – V5 – V12 – V9 – V11 – V7. In turn, in Chol-containing vesicles this row appeared to be V4 – V11 – V7 – V5 – V9 – V12 – V6 – V10 – V3 – V8. In other words, inclusion of Chol modifies the properties of lipid bilayer, and these changes have different consequences for different drugs – while Chol embedment significantly enhances the partition of V8 (as can be judged from K_P and f_m values), V7, for example, shows much lower affinity for Chol-containing liposomes compared to neat PC bilayers. All these considerations highlight the idea that the process of EC binding to the lipid membranes should be discussed within the framework of universal and specific interactions occurring in the membrane. The interactions of the first kind (the long range dipole-dipole interactions) act at long distances and are classified into orientation, induction, polarization and dispersive types. Specific interactions (hydrogen bonding and different complexes formed by charge transfer), in turn, operate locally on the molecules which are in quasi-chemical coupling. Apparently, inclusion of Chol into the lipid bilayer changes the physico-chemical properties of the latter, controlling in such a way the mode of subsequent drug-lipid binding. According to the modern theories, the effects of Chol incorporation on the membrane nonpolar region (where EC are expected to localize) include (i) reduced content of the acyl chain gauche conformations, (ii) decreased hydration degree, and (iii) tighter lateral packing of hydrocarbon chains (condensing effect) [36, 39–43]. From the other hand, the hydroxyl group in Chol molecule has a hydrogen bond capability both as donor and acceptor. Apparently, for some EC such Chol-induced rigidization of the membrane hydrophobic region results in the decreased drug affinity for the lipids. Along with this, for those EC which possess high potential to the formation of H-bonds, specific interactions between the drug and Chol prevail over sterol bilayer-condensing effect, favoring thereby drug partitioning and accumulation in the membrane. In analogous manner one can explain the influence of CL on the EC—lipid complexation. Though the overall lipid binding ability of the drugs tends to reduce in the presence of anionic lipid, increasing the K_P and P_e values with CL content may reflect the enhancement of specific interactions between CL and the examined agents.

Additional arguments in favor of significant role of specific interactions in drug-lipid association come from the following rationales. The term "drug lipophilicity" is traditionally described by n-octanol/water partition coefficient expressed as log$P$ parameter [34]. However, despite its successful application in drug design studies, this parameter received a lot of criticism during the last decades due to isotropic nature of organic solvents with only superficial similarity with biological membranes. In such isotropic systems different solutes do not show distinct topographical modes of interactions with the liquid phase. To circumvent this problem, the growing attention in lipophilicity studies is currently given to anisotropic liposome/water systems where different solutes exhibit different topographical relations with the lipid phase [44]. Furthermore, when partition coefficient is expressed by octanol/water log$P$ parameter the lipophilicity is given as the outcome of hydrophobic and polar solvent—solute interactions (lipophilicity = hydrophobicity–polarity). The polar interactions encoded in log$P$ involve H-bonds, orientation (Keesom) and induction (Debye) forces, while nonpolar interactions are dispersion (London) and hydrophobic contacts [44, 45]. When the drug partitions into lipid bilayer, the existence of the dipole field of phospholipids highlights the necessity of electrostatic interactions to be allowed for as an additional intermolecular recognition force. The equation for lipophilicity is thus replenished by additional term—ionic bonds and could be written as lipophilicity = hydrophobicity–polarity + ionic bonds [44, 45]. Using the resources of Virtual Computational Chemistry Laboratory (http://www.vcclab.org) [46, 47], we estimated the log$P$ values for europium chelates, and found that there exists a good correlation between log$P$ and K_P in the case of PC vesicles (Fig. 4). However, log$P$ and K_P obtained for Chol- or CL-containing systems turned out to have different values (data not shown). In view of the above discussion, this finding can be explained assuming that ionic bonds between EC and PC molecules are absent or insignificant, and partitioning of the drugs into PC membranes is similar to their distribution into octanol/water systems. In turn, in PC/Chol or PC/CL vesicles, ionic EC-lipid interactions gain their strength contributing either positively (increase in K_P, and consequently, in P_e) or negatively (decrease in K_P and P_e) to the lipophilicity. Although EC are thought to be mostly hydrophobic in nature, the formation of ionic bonds between charged functional groups of EC and lipid headgroups cannot be ruled out.
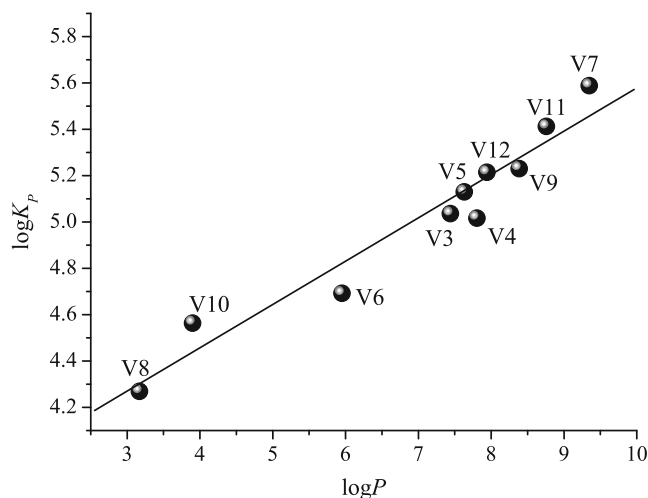
Fig. 4 Correlation between octanol/water and liposome/water partition coefficients of the examined drugs
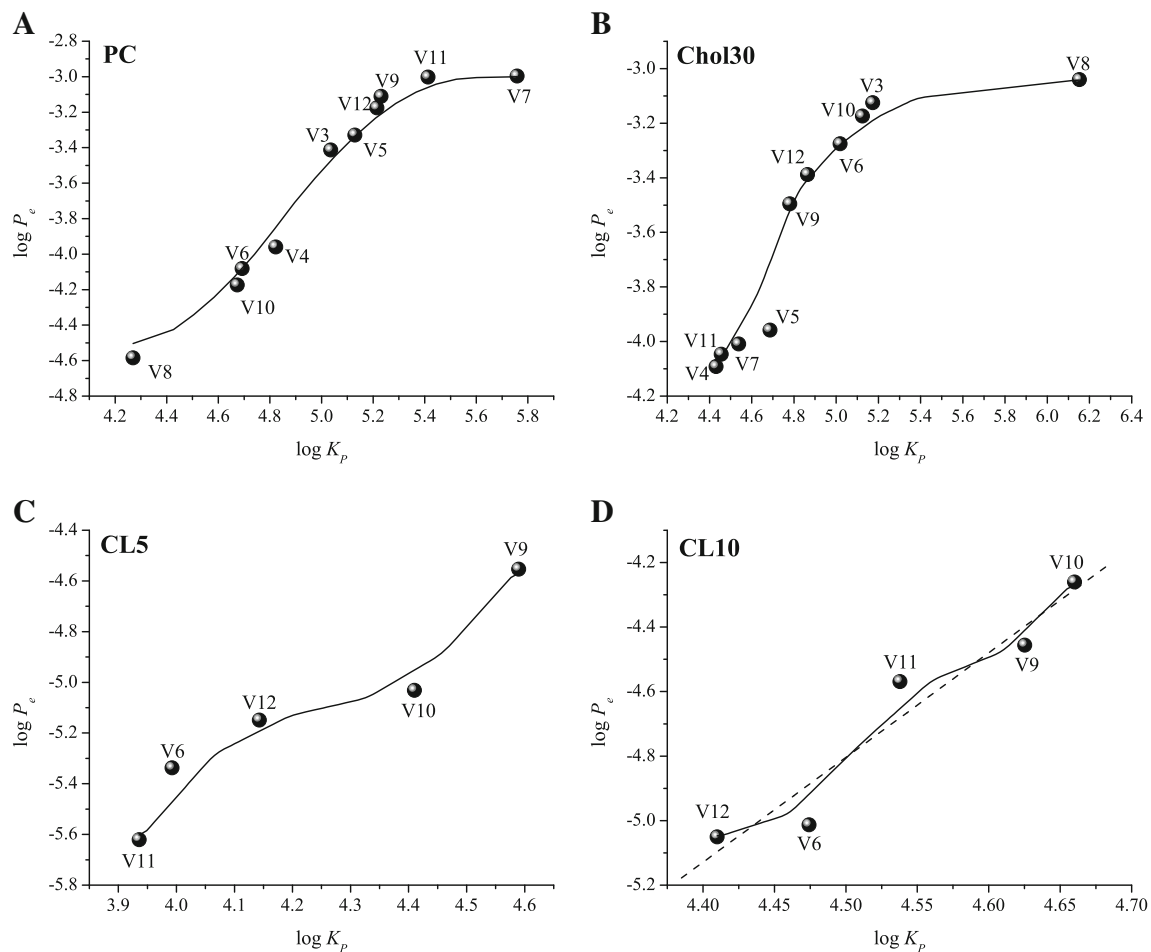
Fig. 5 Permeability-lipophilicity curves for europium complexes in PC (a), Chol30 (b), CL5 (c) and CL10 (d) lipid membranes

In the following, it seems of interest to draw attention to the shapes of the permeability-lipophilicity curves (PLC). As shown in Fig. 5, for PC membranes this plot is highly sigmoidal, for Chol30 bilayers the curve is almost hyperbolic, and for CL-containing systems the permeability-lipophilicity relations exhibit mirror-reflected sigmoidal behavior with the steepness decreasing with increasing CL content and approaching linear dependence in CL10 vesicles. In fact, one may suppose that due to small number of the drugs examined in PC/CL bilayers, the PLC resemble sigmoidal dependencies, but if the number of experimental points increases, the curves will be linear with the data points fluctuating around the linearity and the slope of the curve being dependent on CL content. Thus, PLC in CL5 and CL10 vesicles can be considered to exhibit the "apparent" linear behavior.

The shapes of PLC were in the focus of a good deal of works [34, 48, 49]. Specifically, it was demonstrated that linear region in PLC (region *b* in Fig. 6) corresponds to the case of transcellular lipid diffusion of the drugs, i.e. diffusion through the lipoid structures of the membrane, permeability plateau at high lipophilicities (region *c* in Fig. 6) is due to diffusion limitation through the stagnant water layers present

at the membrane interface, and the "tailing" effect at low lipophilicities (region *a* in Fig. 6) is due to the paracellular diffusion contribution, i.e. diffusion through membrane water-
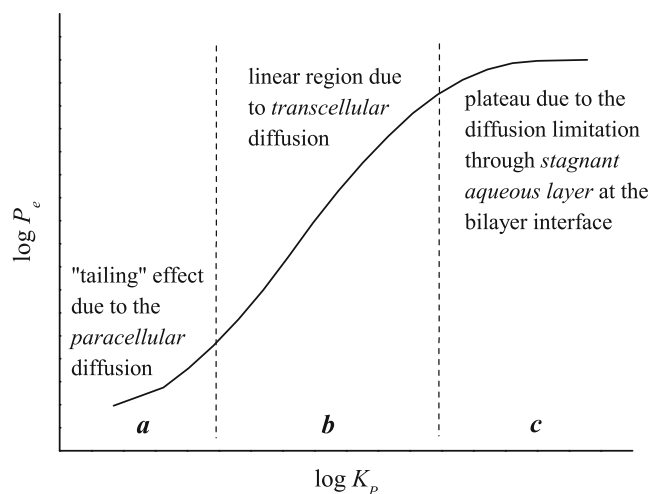
Fig. 6 Theoretical sigmoidal relationship between the permeability and lipophilicity according to aqueous pore membrane permeation model

filled pores (or defects). Transferring this model to our systems, it appears that for:

- PC bilayers—complex paracellular + transcellular permeation of EC with some extent of diffusion barrier through the standing water layers at the bilayer interface,
- Chol30 vesicles—the contribution of paracellular diffusion is insignificant and only transcellular diffusion accounting for stagnant aqueous layers is predominant,
- CL—containing membranes—"apparent" linearity of the plots allows suggesting the occurrence of only transcellular EC diffusion across the membrane without the limitation through the stagnant aqueous layer adjacent to the bilayer surface.

The above findings may indicate that the packing of lipid molecules is more rigid in PC/CL and PC/Chol systems as compared with neat PC bilayers, corroborating the well-known condensing effects of sterol and anionic lipid on the membrane structure [43, 50]. Apparently, lipid ordering results in the narrowing of water-filled voids between the lipid molecules, thereby excluding the possibility of paracellular permeation in these types of liposomes. In turn, in PC bilayers less tight lipid packing creates the bilayer defects of a size comparable with EC dimensions, favoring the pore diffusion. One more interesting moment is the observation that in CL-containing membranes diffusion through the aqueous layer adjacent to the membrane surface is not hampered. This finding can be a consequence of CL effect on electrostatic properties of lipid bilayer. Inclusion of CL changes the membrane zeta potential which may modify lipid polar head group conformation [51]. Negative charge of CL moves the N^+-end of P-N dipole parallel to the membrane surface, thereby causing the rearrangement of interfacial water bridges and stabilization of the intramolecular hydrogen bonds including the water molecules of hydration layer. All these processes may influence the drug transport into/through the lipid bilayer. Of course, one should bear in mind that translation of *in vivo* mechanisms of drug permeation into *in vitro* systems is far from being straightforward, however, we believe that our observations may shed light on the molecular level details of EC accumulation in the membrane.

In conclusion, by recruiting europium chelates as the quenchers of pyrene fluorescence, we found that the examined agents, potential anticancer drugs, readily partition and accumulate in the lipid bilayers, thereby paving the way for the development of their liposomal formulations. Both the drug chemical structure and lipid bilayer composition proved to control the extent of EC entrapment by the lipid phase and the mode of drug-lipid complexation. The revealed ability of Chol and CL to modify drug partitioning may be of practical importance while optimizing the conditions for achieving maximal payload of the drug without compromising the liposome stability.

References

1. Bünzli JG, Piguet C (2005) Taking advantage of luminescent lanthanide ions. Chem Soc Rev 34:1048–1077
2. Vogler A, Kunkely H (2006) Excited state properties of lanthanide complexes: beyond ff states. Inorg Chim Acta 359:4130–4138
3. Maas H, Currao A, Calzaferri G (2002) Encapsulated lanthanides as luminescent materials. Angew Chem Int Ed 41:2495–2497
4. Orcutt KM, Jones WC, McDonald A, Schrock D, Wallace KJ (2010) A lanthanide-based chemosensor for bioavailable Fe^{3+} using a fluorescent siderophore: an assay displacement approach. Sensors 10:1326–1337
5. Yuan J, Wang G (2005) Lanthanide complex-based fluorescence label for time-resolved fluorescence bioassay. J Fluoresc 15:559–568
6. Bakker BH, Goes M, Hoebe N, van Ramesdonk HJ, Verhoeven JW, Werts MHV, Hofstraat JW (2000) Luminescent materials and devices: lanthanide azatriphenylene complexes and electroluminescent charge transfer systems. Coord Chem Rev 208:3–16
7. Dean Sherry A (1989) Lanthanide chelates as magnetic resonance imaging contrast agents. J Less Common Met 149:133–141
8. Thompson K, Orvig C (2006) Lanthanide compounds for therapeutic and diagnostic applications. Chem Soc Rev 35:499–499
9. Kostova I (2005) Lanthanides as anticancer drugs. Curr Med Chem Anticancer Agents 5:591–602
10. Evans CH (1983) Interesting and useful biochemical properties of lanthanides. Trends Biochem Sci 8:445–449
11. Momekov G, Deligeorgiev T, Vasilev A, Peneva K, Konstantinov S, Karaivanova M (2006) Evaluation of the cytotoxic and pro-apoptotic activities of Eu(III) complexes with appended DNA intercalators in a panel of human malignant cell lines. Med Chem 2:439–445
12. Chonn A, Cullis P (1995) Recent advances in liposomal drug-delivery systems. Curr Opin Biotechnol 6:698–708
13. Maurer N, Fenske D, Cullis P (2001) Developments in liposomal drug delivery systems. Expert Opin Biol Ther 1:1–25
14. Mickova A, Buzgo M, Benada O, Rampichova M, Fisar Z, Filova E, Tesarova M, Lukas D, Amler E (2012) Core/shell nanofibers with embedded liposomes as a drug delivery system. Biomacromolecules 13:952–962
15. Ladokhin AS, Selsted ME, White SH (1997) Bilayer interactions of indolicidin, a small antimicrobial peptide rich in tryptophan, proline, and basic amino acids. Biophys J 72:794–805
16. Word RC, Smejtek P (2005) Partitioning of tetrachlorophenol into lipid bilayers and sarcoplasmic reticulum: effect of length of acyl chains, carbonyl group of lipids and biomembrane structure. J Membrane Biol 203:127–142
17. Santos NC, Prieto M, Castanho M (2003) Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. Biochim Biophys Acta 1612:123–135
18. Wang J, Gambhir A, Hangyas-Mihalyne G, Murray D, Golebiewska U, McLaughlin S (2002) Lateral sequestration of phosphatidylinositol 4,5-bisphosphate by the basic effector domain of myristoylated alanine-rich C kinase substrate is due to nonspecific electrostatic interactions. J Biol Chem 277:34401–34412
19. Lakowicz JR (2006) Principles of fuorescence spectroscopy. Plenum Press, New York

20. Lakowicz JR, Hogen D, Omann G (1977) Diffusion and partitioning of a pesticide, lindane, into phosphatidylcholine bilayers: a new fluorescence quenching method to study chlorinated hydrocarbo membrane interactions. Biochim Biophys Acta 471:401–411

21. Mui B, Chow L, Hope MJ (2003) Extrusion technique to generate liposomes of defined size. Meth Enzymol 367:3–14

22. Bartlett G (1959) Phosphorus assay in column chromatography. J Biol Chem 234:466–468

23. Mathai J, Tristam-Nagle S, Nagle J, Zeidel M (2008) Structural determinants of water permeability through the lipid membrane. J Gen Physiol 131:69–76

24. Dobretsov GE (1989) Fluorescent probes for the cell, membrane and lipoprotein studies. Nauka, Moscow

25. Inada T, Funasaka Y, Kikuchi K, Takahashi Y, Ikeda H (2006) Electron-transfer fluorescence quenching of aromatic hydrocarbons by europium and ytterbium ions in acetonotrile. J Phys Chem A 110:2595–2600

26. Nakamura T, Kira A, Imamura M (1982) Enhancement of the intersystem crossing of pyrene by metal ions in sodium dodecyl sulfate micelle solutions. J Phys Chem 86:3359–3363

27. Lee J, Carraway E, Schlautman M, Yim S, Herbert B (2004) Chaarcterizing pyrene-Ag$^+$ exciplex formation in aqueous and ethanolic solutions. J Photochem Photobiol A 167:141–148

28. Dederen J, Auweraer M, Schryver F (1981) Fluorescence quecnhing of solubilized pyrene and pyrene derivatives by metal ions in SDS micelles. J Phys Chem 85:1198–1202

29. Lemmetyinen H, Yliperttula M, Mikkola J, Kinnunen P (1989) Quenching of fluorescence of pyrene-substituted lecithin by tetra-cyanoquinodimethane in liposomes. Biophys J 55:885–895

30. de Paula E, Schreier S (1995) Use of a novel method for determination of partition coefficients to compare the effect of local anesthetics on membrane structure. Biochim Biophys Acta 1240:25–33

31. Zhang J, Hadlock T, Gent A, Strichartz G (2007) Tetracaine-membrane interactions: effects of lipid composition and phase on drug partitioning, location, and ionization. Biophys J 92:3988–4001

32. De Castro B, Gameiro P, Lima J, Matos C, Reis S (2001) Location and partition coefficients of anti-inflammatory drugs in EPC liposomes. A fluorescence quenching study using n-(9-anthroyloxy)-stearic probes. Colloids Surf A 190:205–212

33. Takegami S, Kitamura K, Kitade T, Takashima M, Ito M, Nakagawa E, Sone M, Sumitani R, Yasuda Y (2005) Effects of phosphatidyl-serine and phosphatidylethanolamine content on partitioning of tri-flupromazine and chlorpromazine between phosphatidylcholine-aminophospholipid bilayer vesicles and water studied by second-derivative spectrophotometry. Chem Pharm Bull 53:147–150

34. Avdeef A (2003) Absorption and drug development. Wiley, New Jersey

35. Sandhya KV, Devi G, Mathew S (2008) Liposomal formulations of serratiopeptidase: in vitro studies using PAMPA and Caco-2 models. Mol Pharm 5:92–97

36. Pasenkiewicz-Gierula M, Rog T, Kitamura K, Kusumi A (2000) Cholesterol effects on the phosphatidylcholine bilayer polar region: a molecular simulation study. Biophys J 78:1376–1389

37. Kinnunen PKJ, Koiv A, Lehtonen JYA, Mustonen P (1994) Lipid dynamics and peripheral interactions of proteins with membrane surfaces. Chem Phys Lett 73:181–207

38. Marrink SJ, Sok RM, Berendsen HJC (1996) Free volume properties of a simulated lipid membrane. J Chem Phys 104:9090–9099

39. Merkle H, Subczynski WK, Kusumi A (1987) Dynamic fluorescence quenching studies on lipid mobilities in phosphotidylcholine-cholesterol membranes. Biochim Biophys Acta 897:238–348

40. Bittman R, Blau L (1972) The phospholipid-cholesterol interaction. Kinetics of water permeability in liposomes. Biochemistry 11:4831–4839

41. Bach D, Miller IR (1998) Hydration of phospholipid bilayers in the presence and absence of cholesterol. Biochim Biophys Acta 1368:216–224

42. Rog T, Pasenkiewicz-Gierula M (2001) Cholesterol effects on the phosphatidylcholine bilayer nonpolar region: a molecular simulation study. Biophys J 81:2190–2202

43. Demel RA, de Kruijff B (1976) The function of sterols in membranes. Biochim Biophys Acta 457:109–132

44. van Balen G, Martinet C, Caron G, Bouchard G, Reist M, Carrupt PA, Fruttero R, Gasco A, Testa B (2004) Liposome/water lipophilicity: methods, information content, and pharmaceutical applications. Med Res Rev 3:299–324

45. Giaginis C, Tsantili-Kakoulidou A (2008) Alternative measure of lipophilicity: from octanol-water partitioning to IAM retention. J Pharm Sci 97:2984–3004

46. Tetko IV, Gasteiger J, Todeschini R, Mauri A, Livingstone D, Ertl P, Palyulin VA, Radchenko EV, Zefirov NS, Makarenko AS, Tanchuk VY, Prokopenko VV (2005) Virtual computational chemistry laboratory—design and description. J Comput Aid Mol Des 19:453–463

47. Tetko IV (2005) Computing chemistry on the web DOI:10.1016/S1359-6446(05)03584-1. Drug Discov Today 10:1497–1500

48. Camenisch G, Folkers G, van de Waterbeemd H (1998) Shapes of membrane permeability-lipophilicity curves: extention of theoretical models with an aqueous pore pathway. Eur J Pharm Sci 6:325–329

49. Camenisch G, Folkers G, van de Waterbeemd H (1996) Review of theoretical passive drug absorption models: historical background, recent developments and limitations. Pharm Acta Helv 71:309–327

50. Ioffe VM, Gorbenko GP (2005) Lysozyme effect on structural state of model membranes as revealed by pyrene excimerization studies. Biophys Chem 114:199–204

51. Shibata A, Ikawa K, Shimooka T, Terada H (1994) Significant stabilization of the phosphatidylcholine bilayer structure by incorporation of small amounts of cardiolipin. Biochim Biophys Acta 1192:71–78