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LIPOSOMAL FORMULATIONS OF ANTITUMOR DRUGS. I. CHOLESTEROL EFFECT ON MEMBRANE INTERACTIONS OF EUROPIUM COORDINATION COMPLEXES

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Among a wide variety of drug nanocarriers developed to date, liposome-based delivery systems are particularly attractive due to their advantageous features such as biocompatibility, complete biodegradability, low toxicity, ability to carry both hydrophilic and lipophilic payloads and protect them from chemical degradation and transformation, increased therapeutic index of a drug, improved pharmacokinetic and pharmacodynamic profiles compared to free drugs, reduced side effects, etc. The efficiency of drug encapsulation is largely determined by its membrane-partitioning properties as well as physicochemical characteristics of the lipid vesicles. In the present study we concentrated our efforts on the pre-formulation studies of the two synthesized Eu(III) coordination complexes, V3 and V4, the potential anticancer drugs. More specifically, our goal was twofold: i) to characterize the membrane partition properties of these complexes, and ii) to assess how the lipid-associating ability of V3 and V4 depends on membrane structural state being varied by introducing the different amounts of cholesterol (Chol) into phosphatidylcholine (PC) lipid vesicles. To achieve this goal, several fluorescent probes including pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), and 4-p-(dimethylaminostyryl)-1-dodecylpyridinium (DSP-12) have been employed. Partition coefficients of lanthanides determined using the equilibrium dialysis technique proved to depend on the amount of Chol content. Formation of drug-lipid complexes was found to affect pyrene excimerization and DSP-12 spectral properties but exerted no influence on pyrene vibronic structure and DPH anisotropy. Membrane composition was shown to have an impact on the spectral responses of the probes in drug-lipid systems. This finding was interpreted as arising from the sterol condensing effect on the structural state of the lipid bilayer.

KEY WORDS: europium complexes, liposome, cholesterol

Among a wide range of drug nanocarriers developed to date, liposome-based delivery systems are particularly attractive due to their following advantageous features. First, a variety of molecules could be encapsulated within the aqueous compartment of the liposomes as well as entrapped in the lipid bilayer [1]. Second, lipid vesicles can direct the drug to its specific target, protect the pharmacological compound against its degradation and chemical transformation, and prolong the duration of drug exposure. Finally, encapsulation of the compound into the lipid carriers allows reducing the adverse effects and controlling its movements in the human organism [1-3]. The efficiency of drug encapsulation is largely determined by its membrane-partitioning properties as well as physicochemical characteristics of the lipid vesicles. In the present study we concentrated our efforts on the pre-formulation studies of the two synthesized Eu(III) coordination complexes, V3 and V4, the potential anticancer drugs. More specifically, our goal was twofold: i) to characterize the membrane partition properties of these complexes, and ii) to assess how the lipid-associating ability of V3 and V4 depends on membrane structural state being varied by introducing the different amounts of cholesterol (Chol) into phosphatidylcholine (PC) lipid vesicles. To achieve this goal, several fluorescent probes including pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), and 4-p-(dimethylaminostyryl)-1-dodecylpyridinium (DSP-12) have been employed.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine was purchased from Biolek (Kharkov, Ukraine). DSP-12 was obtained from Zonde (Latvia), pyrene, DPH and cholesterol (Chol) were from Sigma (Germany). Eu(III) coordination complexes (Fig. 1) were synthesized as described previously [4]. Lipid vesicles composed of PC and its mixtures with 5 (Chol5), 15 (Chol15) or 30 (Chol30) mol% of Chol were prepared using the extrusion technique [5]. 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 suspension was extruded through a 100 nm pore size polycarbonate filter. Phospholipid concentration was determined according to the procedure of Bartlett [6]. Absorption measurements were conducted using SF-46 spectrophotometer. Fluorescence measurements were performed with CM 2203 spectrofluorimeter equipped with magnetically stirred, thermostated cuvette holder (SOLAR, Belarus).

RESULTS AND DISCUSSION

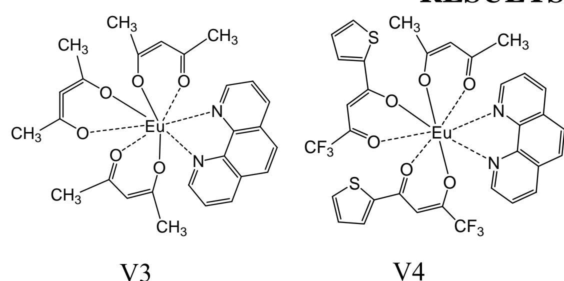

Fig. 1. Structure of europium coordination complexes.

At the first step of the study we evaluated the lipid association properties of compounds under study. Equilibrium dialysis was employed to quantify the drug partitioning into a lipid phase. In terms of this methodology the mole fraction partition coefficient is defined as [7]:

$$K_P = \frac{(A_0^c - A_{out}^{eq})[water]}{[lipid]A_{out}^{eq}} \quad (1)$$

where A_0^c and A_{out}^{eq} are the drug optical densities at 266 nm in liposome-free and liposome-containing systems, respectively, $[water]$ and $[lipid]$ represent water and lipid molar concentrations, respectively. Partition coefficients determined in such a way are presented in Table 1.

Table 1. Partition coefficients of V3 and V4 in different lipid systems

	PC	Chol5	Chol15	Chol30
V3	$2.7×10^3$	$1.6×10^3$	$2.1×10^4$	$1×10^4$
V4	$3.2×10^4$	n/d	n/d	n/d

* – not determined

As seen from this table, partitioning of coordination complexes into the lipid phase is characterized by high values of K_P suggesting the high efficiency of drug encapsulation into the liposomes. Notably, composition of the lipid vesicles strongly affects the partitioning of the lanthanides into the membranes. More specifically, Chol addition to PC bilayers resulted in the changing in K_P of V3 (Table 1). This finding can be interpreted as arising from the sterol condensing effect implicating the decreased free volume of lipid bilayer, augmented thickness of the membrane hydrocarbon region and increased number of PC molecules per unit area. Alterations in molecular volume occupied by PC are associated with the ordering of the lipid acyl chains and reduced rate of their *trans-gauche* isomerization due to specific orientation of the rigid sterol moiety. Within the framework of four-region model of lipid bilayer, Chol is suggested to localize in the Region 3 which involves polar/nonpolar interface

and initial parts of lipid tails. Since lanthanides are thought to reside at polar/nonpolar interface of the membrane [8-10], sterol-induced modifications in bilayer properties may result in the prevention of V3 partitioning into the liposomes.

Next, it seems of interest to assess whether the high lipid binding ability of V3 and V4 is coupled with their influence on lipid bilayer physicochemical properties. To this end, a series of fluorescent probes including pyrene, DPH and DSP-12 has been employed. As seen in Table 2, the intensity ratio of the third to the first pyrene vibronic bands ($R_{III} = I_{III} / I_I$, where I_I and I_{III} – pyrene emission intensities at 374 and 384 nm, respectively), which is reported to be related to the alterations in membrane polarity, remains virtually unchanged upon V3 and V4 association with all types of lipid vesicles under study. This finding suggests that the drugs do not affect the distribution of pyrene monomers and exert no influence on polarity of PC and PC:Chol lipid bilayers. In contrast, formation of drug-lipid complexes resulted in the changes in pyrene excimer-to-monomer intensity ratio (E/M), a parameter which reflects the rate of probe lateral diffusion within the membrane plane [11].

Table 2. Lanthanide effect on pyrene spectral parameters in PC and PC:Chol lipid vesicles

	Without V3		**+V3**		**Without V4**		**+V4**	
	E/M	R_{III}	E/M	R_{III}	E/M	R_{III}	E/M	R_{III}
PC	2.47	1.03	2.66	1.1	2.64	1.15	3.15	1.11
Chol5	3.46	1.07	3.26	1.12	1.99	1.14	2.06	1.13
Chol15	2.35	1.08	3.45	1.13	2.35	1.07	1.97	1.13
Chol30	2.65	1.07	1.89	1.06	2.07	1.11	2.43	1.1

Specifically, V3 and V4 encapsulation into PC liposomes led to the increase in E/M values (Table 2) being indicative of increased membrane free volume and decreased degree of lipid packing. Addition of Eu-complexes to PC:Chol vesicles resulted in ambiguous changes in E/M. V3 was found to reduce E/M in Chol5 and Chol30 membranes and increase E/M in Chol15 liposomes. V4 exhibited the opposite behavior – increased E/M ratio in the vesicles with low and high percentage of sterol, and decreased extent of pyrene excimerization in the vesicles containing 15 mol% of Chol. Apparently, the above effects may be explained by the balance of two competing processes – drug influence on bilayer structural state and modifying effect of Chol on membrane hydrophobic region [8].

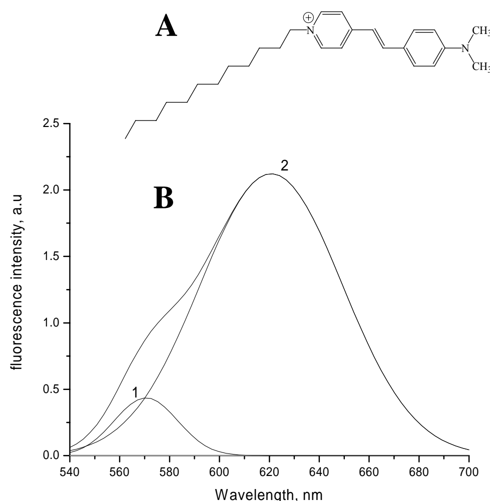

Fig. 2. Structure (A) and deconvoluted fluorescence spectrum (B) of DSP-12.

In contrast to pyrene, DPH was found to be virtually insensitive to the lanthanide binding to the model lipid membranes – changes in probe anisotropy due to the drug encapsulation into the liposomes did not exceed 7%. The observed discrepancy in pyrene and DPH data may be explained by the difference in the mechanisms by which these probes respond to the alterations in their microenvironment. While the changes in pyrene excimerization arise from the modifications in probe lateral movements, variations in DPH anisotropy mirrors the changes in probe rotational mobility.

In order to get additional information on the molecular details of lanthanide association with

the model membranes, we examined the spectral behavior of DSP-12 in the drug-lipid systems. DSP-12 is a fluorescent probe possessing a charged hydrophilic fluorophore moiety (Fig. 2) and a long hydrophobic tail. The fluorescence spectrum of DSP-12 is characterized by two spectral components with the maxima at 567 and 619 nm attributing to the different probe populations – the former locating in the nonpolar membrane region, and the latter residing at the lipid/water interface. Incorporation of V3 and V4 into the liposomes exerted no influence on the contribution of long-wavelength component to the overall DSP-12 emission. Meantime, the relative intensity of the short-wavelength constituent of DSP-12 fluorescence was found to decrease by 13% in Chol5 liposomes and increase by 8% at higher Chol content. The fact that only the short-wavelength spectral component undergoes drug-induced changes strongly suggests that hydrophobic interactions play predominant role in lanthanide-membrane association.

Ambiguous dependence of the data presented here on Chol amount may be a consequence of sterol ability to regulate order/disorder behavior of lipid bilayer. At low concentrations (5 mol%) Chol stabilizes the liquid-disordered phase of membrane, while high concentrations (30 mol%) favour lipid transition into the liquid-ordered phase. Critical point for this disorder→order phase transition is assumed to be 15 mol% Chol. This intermediate state of lipid bilayer may affect the binding of the employed probes to the lipid vesicles, thereby giving rise to nonmonotonous dependence of the recovered probe spectral parameters on sterol concentration.

CONCLUSIONS

The present study strongly suggests that newly synthesized anticancer drugs V3 and V4 can be efficiently entrapped by the lipid phase of the vesicles, thereby paving the way for the development of their liposomal formulations. The revealed ability of Chol to modify drug effect on lipid bilayer physicochemical properties may be of practical importance while optimizing the conditions for achieving maximal payload of the drug without compromising the liposome stability.

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