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PARTITIONING OF EUROPIUM CHELATE INTO LIPID BILAYER AS REVEALED BY *p*-TERPHENYL AND PYRENE QUENCHING

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Fluorescence quenching method is an effective tool for obtaining important information about different properties of biophysical and biochemical systems. In the present study quenching of fluorescent probes *p*-terphenyl and pyrene by europium chelate were observed in phosphatidylcholine liposomes. Europium chelates (EC) belong to a new class of potential antitumor drugs with high cytotoxic activity. These compounds are of particular interest for biomedical investigations and diagnostics, since their spectral characteristics are optimal for decrease of light scattering in biological patterns and background signal. However, the application of such drugs in a free form is limited by their high toxicity and metabolic instability. One efficient way to increase drug efficiency is based on using different drug delivery systems such as liposomes. Highly adaptable liposome-based nanocarriers currently attract increasing attention, because of their advantages, *viz*. complete biodegradability, ability to carry both hydrophilic and lipophilic payloads and protect them from chemical degradation and transformation, increased therapeutic index of drug, flexibility in coupling with targeting and imaging ligands, improved pharmacodynamic profiles compared to the free drugs, *etc*. The present study was focused on examination of lipid bilayer interactions of europium chelate (here referred to as V10). Fluorescence intensity of membrane-incorporated probes – pyrene and *p*-terphenyl – was found to decrease with increasing concentration of the drug, suggesting that V10 represents an effective quencher for these probes. This finding was explained by the drug penetration into hydrophobic membrane core, followed by the collision between V10 and probe molecules and subsequent fluorescence quenching. The acquired fluorescence quenching data were quantitatively interpreted in terms of the dynamic quenching model.

KEY WORDS: europium chelate, liposomes, fluorescent probes, fluorescence quenching

РАСПРЕДЕЛЕНИЕ ХЕЛАТА ЕВРОПИЯ В ЛИПИДНЫЙ БИСЛОЙ ПО ДАННЫМ ТУШЕНИЯ ФЛУОРЕСЦЕНЦИИ *n*-ТЕРФЕНИЛА И ПИРЕНА

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Метод тушения флуоресценции является эффективным средством получения важной информации о различных свойствах биофизических и биохимических систем. В данной работе тушение флуоресценции двух флуоресцентных зондов пирена и *n*-терфенила хелатом европия наблюдалось в фосфатидилхолиновых липосомах. Хелаты европия принадлежат к новому классу потенциальных противоопухолевых препаратов, обладающих высокой цитотоксичной активностью. Эти соединения представляют особый интерес для биомедицинских исследований и диагностики, поскольку их спектральные характеристики являются оптимальными для уменьшения вклада рассеяния биологических образцов и фоновой флуоресценции. Однако применение таких лекарственных препаратов в свободной форме ограничено их высокой токсичностью и метаболической нестабильностью. Один из методов увеличения эффективности подобных лекарств основан на использовании различных систем доставки, таких как липосомы. Применение липосомальных наноперенощиков фармакологических препаратов в настоящее время привлекает особое внимание благодаря их следующим преимуществам: полной биосовместимости, способности переносить как липофильные, так и гидрофильные соединения, уменьшению токсичности, увеличению терапевтического индекса и т. д. Данная работа была сфокусирована на исследовании взаимодействия хелата европия (обозначенного в работе как V10) с липидным бислоем. Было найдено, что интенсивность флуоресценции локализованных в

мембране зондов пирена и *n*-терфенила уменьшалась при увеличении концентрации препарата. Это свидетельствует о том, что V10 является эффективным тушителем для этих зондов. Наблюдаемое тушение флуоресценции является результатом столкновения данных флуорофоров с проникнувшими в бислой молекулами препарата. Полученные результаты были численно проанализированы в рамках модели динамического тушения.
КЛЮЧЕВЫЕ СЛОВА: хелат европия, липосомы, флуоресцентные зонды, тушение флуоресценции

РОЗПОДІЛ ХЕЛАТУ ЄВРОПІЮ В ЛІПІДНИЙ БІШАР ЗА ДАНИМИ ГАСІННЯ ФЛУОРЕСЦЕНЦІЇ *n*-ТЕРФЕНІЛУ Й ПІРЕНУ

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Метод гасіння флуоресценції є ефективним засобом одержання важливої інформації, стосовно різних властивостей біофізичних і біохімічних систем. У даній роботі гасіння флуоресценції двох флуоресцентних зондів пірену й *n*-терфенілу хелатом європію спостерігалося у фосфатидилхолінових ліпосомах. Хелати європію належать до нового класу потенційних протипухлинних препаратів з високою цитотоксичною активністю. Ці сполуки також привертають особливу увагу при використанні їх у біомедичних дослідженнях та діагностиці, оскільки їхні спектральні характеристики є оптимальними для зменшення внесків розсіювання біологічних зразків і фонової флуоресценції. Однак застосування таких лікарських препаратів у вільній формі обмежено їхньою високою токсичністю й метаболічною нестабільністю. Один з методів збільшення ефективності подібних ліків засновується на використанні різних систем постачання, наприклад – ліпосом. Застосування ліпосомальних нанопереносників фармакологічних препаратів має наступні переваги: повну біосумісність, здатність переносити як ліпофільні, так і гідрофільні з'єднання, зменшення токсичності, збільшення терапевтичного індексу й т. і. Дана робота була зосереджена на дослідженні взаємодії хелата європію (позначеного в роботі як V10) з ліпідним бішаром. Було знайдено, що інтенсивність флуоресценції локалізованих у мембрані зондів пірену й *n*-терфенілу зменшувалася при збільшенні концентрації препарату. Це свідчить про те, що V10 є ефективним гасником флуоресценції цих зондів. Спостережуване гасіння флуоресценції є результатом зіткнення даних флуорофорів із молекулами препарату, що проникли у бішар. Отримані результати були чисельно проаналізовані у рамках моделі динамічного гасіння.
КЛЮЧОВІ СЛОВА: хелат європію, ліпосоми, флуоресцентні зонди, гасіння флуоресценції

Interest in design of novel pharmaceutical formulations is currently focused on the achievement of correlation between the activity of a drug and its side effects. A lot of chemotherapeutic agents have a narrow therapeutic index. Increase of the drug doses to a concentration, required for therapy, is often causes various side effects [1]. Sometimes, drugs can't attain to invaded tissue because of their instability and destruction in an organism [2]. Thus, chemotherapeutic molecules either can affect the surrounding tissues or may be eliminated untimely. To improve drug pharmacokinetics and to achieve high therapeutic index it is necessary to use the targeted delivery of a drug close to its destination (invaded tissue). This problem has been discussed in recent reviews [3-5]. Modern drug delivery systems involve formulations based on polymer materials (polymer conjugates, microspheres and wafers) [6], osmotic pumps, microchips [5] and different lipid based carrier systems (lipid microbubbles, microspheres and microtubules, solid lipid nanoparticles, oily suspensions, lipid implants, submicron lipid emulsions, liposomes) [7]. Among these systems liposomes represent the most promising drug carrier due to the following advantages. First, liposomes are constructed from lipids which have biological origin [7]. This feature makes liposomes fully biocompatible and in the case of liposomes destruction, lipids can be used by organism as building material. Second, the amphiphilic structure of liposomal phospholipids allows loading these systems by both lipophilic and hydrophilic compounds [8]. The drugs, encapsulated into liposomes, become protected from the degradation and transformation in an organism. Variation of liposome size and composition allows to realize targeted delivery of a

drug, minimize side effects and enhance therapeutic index [9]. Recently, liposomes have been applied as delivery systems for proteins and peptides (interleukin-2, β-glucuronidase [7]), plasmid DNA [8,3] and local anesthetics (prilocaine, lidocaine and mepivacaine [10]). Utilization of delivery systems is especially important in cancer therapy. Liposome-based formulations of several antineoplastic drugs such as daunorubicin, amphotericin-B, doxorobicin [5], lurtotecan, nystatin, annamycin etc. [3] are currently available on the market. However, there are several problems limiting the application of liposomes *viz*. their stability, low drug entrapment, particle size control and short circulation half-life of vesicles [11]. Particularly, stability of liposomal formulations depends on a number of factors including liposomal size and composition as well as drug molecular weight and lipophilisity [12,13]. In view of this, prior to use the liposomes as nanocarriers it is necessary to know the character of interactions between compound in question and lipid membrane. One of the fundamental tasks related to the development of liposomal drug formulations is identification of drug-membrane partition coefficient. This parameter characterizes the ability of hydrophobic compound to incorporate into nonpolar membrane region. Lipid-to-water partition coefficient can be obtained using several physical techniques, *e.g.* dialysis [14,15], absorption and fluorescence spectroscopy [16], centrifugation [17], chromatography [16] *etc*. In spite of their advantages these methods have a number of shortcomings such as tediousness and requirement of relatively large quantities of the compounds under question [18]. Therefore, considerable efforts are currently devoted to elaboration of more accessible and convenient methods. Lakowicz et al. [19] have proposed a novel method for the assessment of drug-membrane partition coefficient. This approach is based on the dependence of probe fluorescence intensity on quencher concentration [20]. In the case of fluorescent probe incorporated into hydrophobic membrane region the more effective inclusion of investigated drug will result in more efficient quenching.

The present study was undertaken to evaluate lipid association ability of europium coordination complex, referred to here as V10. This compound belongs to a wide class of lanthanide chelates. Currently these substances became the subject of increasing interest in many areas, including biomedical analysis [21], NMR studies [22], laser techniques [23], and bioanalytical assays [24]. It is due to their unique photophysical features such as long fluorescent lifetime [25] and long Stokes' shift [24] allowing efficient temporal discrimination of background interferences. In addition, lanthanides and their complexes possess antibacterial and antitumor properties [26]**.**

In this study we use V10 as a collisional quencher for the fluorescent probes pyrene and *p*-terphenyl to obtain information on the binding of this potential drug to liposomal memranes consisting of phosphatidylcholine. The molar partition coefficient of V10 has been calculated by comparing the extent of probes fluorescence quenching obtained under different lipid molar concentrations.

MATERIALS AND METHODS

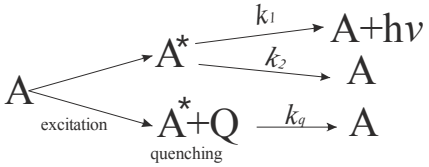
Egg yolk phosphatidylcholine (PC) was purchased from Biolek (Kharkov, Ukraine). Pyrene was obtained from Sigma (Germany), p-terphenyl was from Signe (Latvia). Eu(III) coordination complex V10 (Fig. 1) was synthesized as described previously [27]. Lipid vesicles composed of PC were prepared using the extrusion technique [28]. 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. Fluorescence measurements were performed with LS-55 spectrofluorimeter (Perkin Elmer, Great Britain) equipped with magnetically stirred, thermostated cuvette holder. The decrease in fluorescence intensity at 465 nm for pyrene and

at 358 nm for *p*-terphenyl (which were excited at 340 and 280 nm, correspondingly) was monitored as a function of increasing concentration of V10.

THEORY

Fluorescence lifetime and fluorescence intensity can be significantly changed by addition of different substances to the probe environment. Particularly, it can result to decrease of fluorescence intensity (fluorescence quenching). Quenching can be conditioned by various processes which occur upon interactions between fluorophore and surrounding molecules, *e.g.* collision (dynamic quenching) [29], ground-state complex formation (static quenching)[30], molecular rearrangements, energy transfer *etc* [20]. Fluorescence quenching is a source of valuable information in a study of drug-membrane interactions [18]. Particularly, this method was used to estimate the relative membrane location of ciprofloxacin [31], anti-inflammatory drug nimesulide [32] and oxicams [33]. Also it was applied to determine diffusion coefficients of quinone [34] and lipid/water partition coefficients of quinine [32], chlorpromazine [18] and nimesulide [34].

Since probe molecules emit from the lowest excited state, the measured fluorescence intensity is proportional to the concentration of excited probe molecules (A^*). The transition of probe from excited (A^*) to a ground state (A) may occur according to the following scheme:



(1)

where k_1 and k_2 and k_q are the rate constants, Q is a quencher. The first two processes in this scheme describe transition with emitting of light and without it [35]. The last process is a quenching.

At the absence of quencher the concentration of excited probe molecules is equal to:

$$[A^*] = [A^*]_0 e^{-(k_1+k_2)t} = [A^*]_0 e^{-t/\tau_0} \quad (2)$$

where $[A]_0$ – is initial concentration of excited probe molecules, τ_0 is a fluorescence lifetime in the absence of quencher:

$$\tau_0 = 1/(k_1 + k_2). \quad (3)$$

While probe molecules are accessible for quencher, the transition from excited to a ground state is described as:

$$[A^*] = [A^*]_0 e^{-(k_1+k_2+k_q[Q])t} = [A^*]_0 e^{-t/\tau} \quad (4)$$

where τ is a fluorescence lifetime in the presence of quencher:

$$\tau = 1/(k_1 + k_2 + k_q[Q]). \quad (5)$$

Since the decrease in fluorescence intensity caused by quenching is equal to the decrease in fluorescence lifetime, substituting eq. (3) into eq. (5) gives

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = \frac{k_1 + k_2 + k_q[Q]}{k_1 + k_2} = 1 + k_q\tau_0[Q] \quad (6)$$

where k_q is the bimolecular rate constant for the dynamic quenching, F_0 and F are fluorescence intensities in the absence and presence of quencher. The expression (6) is called Stern-Volmer equation and the product of $k_q\tau_0$ is referred to as the Stern-Volmer constant, K_{SV} [20,35].

RESULTS AND DISCUSSION

The structure of europium chelate (EC) under study is shown on Fig. 1, A. To gain insight into V10 influence on the structure of liposomal membranes the investigation of pyrene (Fig. 1, B) and *p*-terphenyl (Fig. 1, C) fluorescence quenching were carried out.

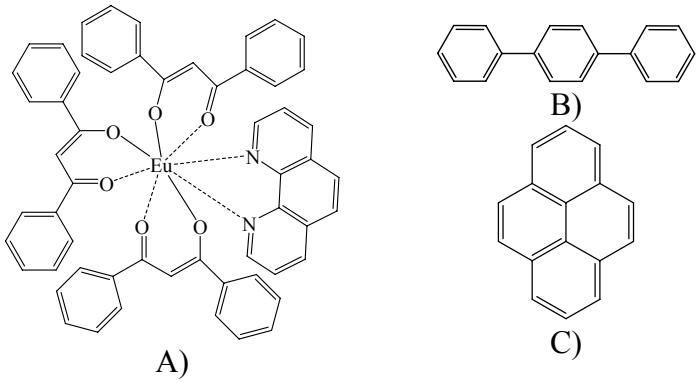


Fig. 1. Chemical structures of V10 (A), *p*-terphenyl (B) and pyrene (C)

Fluorescent probes employed in this work are highly lipophilic compounds, located in the hydrophobic membrane region. The changes in the fluorescence emission spectra of these probes have been monitored upon titrating PC liposomes with V10. It has been found that EC addition to the liposomes resulted in decrease of fluorescence intensity of pyrene and *p*-terphenyl without shift of maximum position (Fig. 2). This finding provides evidence that europium complex represents an effective quencher of pyrene and *p*-terphenyl fluorescence. The mechanism of quenching is most probably due to spin-orbit coupling and intersystem crossing to the triplet state [20].

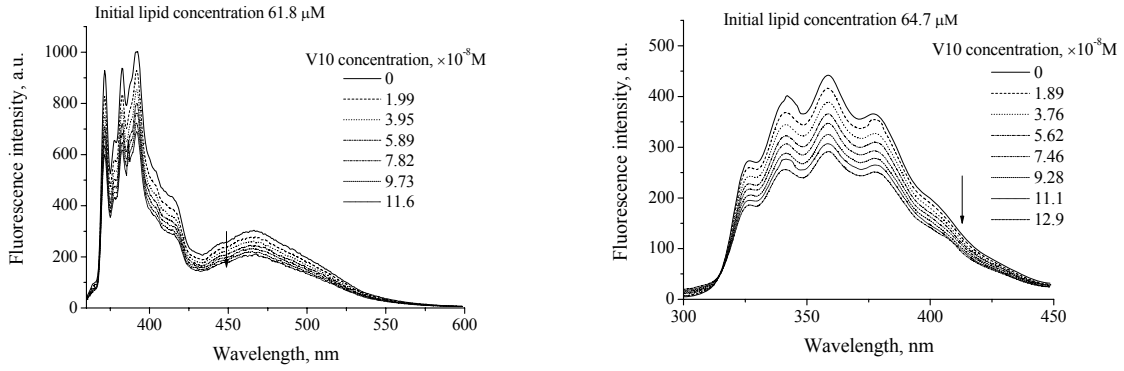


Fig. 2. Fluorescence spectra of pyrene (A) and *p*-terphenyl (B) in suspension of PC liposomes at varying EC concentrations. Initial pyrene and *p*-terphenyl concentrations were 1.4 and 1.1 μM, respectively.

V10 is a highly hydrophobic complex, tending to locate in the nonpolar membrane region. This process results in increase in frequency of collisions between the drug and probe molecules. EC molecules distribute between water and lipid phase:

$$[Q_T]V_T = [Q]_m V_m + [Q]_w V_w \quad (7)$$

where $[Q]_T$ – total quencher concentration, V_m and V_w are the volumes of membrane and water phases, respectively. Thereby, to give correct interpretation of the observed quenching of membrane-bound probes, it is necessary to evaluate the lipid–water partition coefficient of EC.

According to a commonly used methodology [16,20], partition coefficient is determined as a ratio of compound concentrations in lipid ($[Q]_m$) and water ($[Q]_w$) phases:

$$P = [Q]_m / [Q]_w \tag{8}$$

Quencher concentration in the membrane phase can be obtained using partition coefficient as follows:

$$[Q]_m = \frac{P[Q]_T}{P\alpha_m + (1-\alpha_m)} \tag{9}$$

where $\alpha_m = V_m / V_T$ is the volume fraction of membrane phase. Substitution of this expression into the Stern-Volmer equation (eq. (6)) gives:

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + \frac{\tau_0 k_m P[Q]_T}{P\alpha_m + 1 - \alpha_m} = 1 + \tau_0 k_{app}[Q]_T \tag{10}$$

where k_m is the bimolecular quenching constant for the membrane-bound fluorophore, $k_{app} = k_m P/(P\alpha_m + 1 - \alpha_m)$ is the apparent quenching constant. It is easy to notice that this parameter should depend on volume fraction of membrane phase α_m (and lipid concentration):

$$\frac{1}{k_{app}} = \alpha_m \left(\frac{1}{k_m} - \frac{1}{k_m P} \right) + \frac{1}{k_m P}. \tag{11}$$

According to this approach k_m and P can be obtained from the dependence of k_{app}^{-1} on α_m.

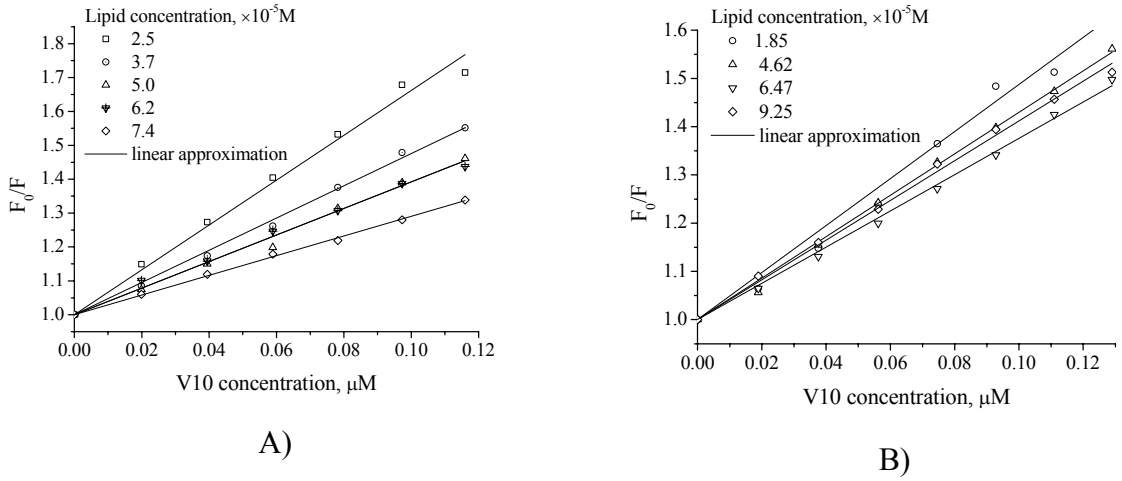


Fig. 3. Stern-Volmer plots of pyrene (A) and *p*-terphenyl (B) quenching by europium complex in PC liposomes.

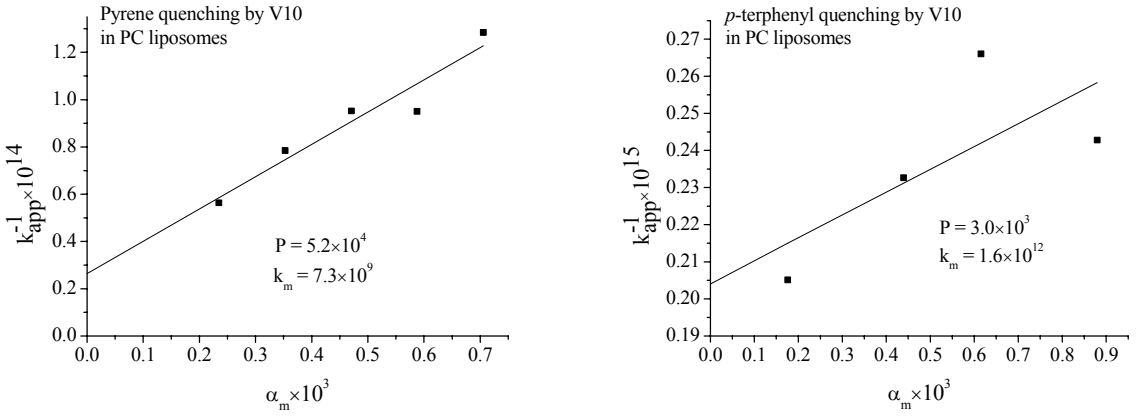
The dependences of relative fluorescence intensity (F_0/F) of pyrene and *p*-terphenyl on EC concentration were analyzed according to eq. (10). The obtained Stern-Volmer plots are shown on Fig. 3.

Table 1. Parameters of pyrene and *p*-terphenyl quenching by europium chelate

Pyrene quenching		***p*-terphenyl quenching**	
$[L]\times10^5$M	$k_{SV}^{app}\times10^{-6}$	$[L]\times10^5$M	$k_{SV}^{app}\times10^{-6}$
2.5	6.6 ± 0.15	1.9	4.9 ± 0.13
3.7	4.7 ± 0.07	4.6	4.3 ± 0.05
5.0	3.9 ± 0.08	6.5	3.8 ± 0.05
6.2	3.9 ± 0.07	9.3	4.1 ± 0.05
7.4	2.9 ± 0.03		

The linearity of these plots suggests that pyrene and *p*-terphenyl fluorescence quenching arises from collisions between probe and quencher molecules (dynamic quenching) [20].

Approximation of the experimental results by the eq. (10) allowed us to evaluate the apparent Stern-Volmer constants ($k_{SV}^{app} = \tau_0 k_{app}$) as a slope of linear curve. The obtained parameters are presented in Table 1.



A) B)

Fig. 4. Dependence of the apparent quenching constant of pyrene (A) and *p*-terphenyl (B) on volume fraction of lipid phase.

The values of k_{SV}^{app} were divided on the pyrene and *p*-terphenyl fluorescence lifetimes (37.3 [36] and 1 ns [20], respectively) to calculate k_{app}. As seen from Fig. 4, this parameter exhibits nearly linear dependence on lipid concentration. The results of $k_{app}(\alpha_m)$ approximation by equation (11) are presented in the Table 2.

Table 2. Parameters of V10 partitioning into PC bilayer

Fluorescent probe	**f_m***	**P**	**k_m**	**Correlation coefficient**
Pyrene	0.55-0.79	5.2×10^4	7.3×10^9	0.96
p-terphenyl	0.05-0.21	3.01×10^3	1.6×10^{12}	0.72

* f_m is a fraction of quencher in a lipid phase: $f_m = P\alpha_m/(P\alpha_m + 1 - \alpha_m)$.

The obtained high partition coefficients provide evidence that europium complex under study can by efficiently incorporated into the lipid phase of PC membrane. This finding is in a good agreement with our previous results, obtained for other europium complexes [37]. Smaller partition coefficient received using *p*-terphenyl probably is explained by different localization of probe and drug molecules. Apparently, *p*-terphenyl lies close to the membrane polar region, becoming more accessible to water quenching. This hypothesis is confirmed by astonishingly high value of k_m (1.6×10^{12}) exceeding diffusion limit ($\sim10^{10}$). On the other hand, lipid concentration used in experiment with *p*-terphenyl was small in order to obtain reasonable values of f_m. In this case EC was found to be less partitioned into the lipid phase.

CONCLUSIONS

The membrane interactions of europium chelate have been characterized using fluorescence quenching technique. A novel method for the quantitative analysis of the

membrane partition of this drug is proposed. The obtained results established that europium chelate under study represents an effective quencher for fluorescent probes *p*-terphenyl and pyrene. The recovered ability of V10 to quench these membrane probes provides direct evidence for incorporation of this drug into membrane interior. This assumption correlates with obtained high values of V10 partition coefficient (~10^4). This parameter was found to be only on order of magnitude smaller than the previously estimated by Mattila and co-workers for antipsychotics HPD and CPZ, and the antineoplastic compound DOX [38].

These results create the basis for the development of liposomal formulations of europium coordination complexes as highly promising potential antineoplastic drugs.

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