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Fluorescence Spectra Analysis and Fluorescence Anisotropy Titration Methods in Determining the Hydroxycamptothecins Affinity to Membranes

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Abstract

Two methods of determining the affinity of hydroxycamptothecins – promising anticancer compounds - to membranes are described and applied in this work: fluorescence spectra analysis and fluorescence anisotropy titration. On the basis of changes of emission spectra as well as changes of steady-state anisotropy over lipids concentration, the fraction of free and bound drugs can be determined and next their association constants to membranes also calculated. One of the investigated agents, 7-t-butyldimethylsilyl-10-hydroxycamptothecin, exhibits a very high affinity to membranes, and therefore, one can expect that this agent will be very stable in blood and is worth further studies.

Keywords: hydroxycamptothecins, membranes, fluorescence anisotropy

Introduction

Camptothecin (CPT) - a pentacyclic alkaloid isolated and characterized by Wall et al. in 1966 [1] - is the prototype inhibitor of DNA topoisomerase I that is recognised as a useful target for antitumour therapy. CPT exists in two forms: lactone and carboxylate, but only the lactone exhibits biological activity. Under physiological conditions, spontaneous hydrolysis occurs and the instability of the lactone form seriously limits the clinical applications of CPT [2]. Fortunately, the process of deactivation is inhibited when molecules of CPT are bound to cell membranes [3]. This precious property of CPT has stimulated research activities to identify analogues of CPT which exhibit higher affinity to membranes. Some of the hydroxycamptothecins (CPTs with the hydrogen at 10-position replaced by a hydroxy group) exhibit this desirable property [4]. A study of the affinity of the lactone form of 10-hydroxycamptothecin (10-OH-CPT), 7-ethyl-10-hydroxycamptothecin (SN-38) and 7-t-butyldimethylsilyl-10-hydroxycamptothecin (DB-67) to membranes is the subject of this work. In order to determine the association constants to membranes of mentioned agents, fluorescence spectra analysis and fluorescence anisotropy titration methods are applied.

Experimental

Hydroxycamptothecins investigated in this work were obtained from the Laboratory of Biotechnology, College of Pharmacy, University of Kentucky, Lexington, USA. Liposomes used as biological membrane models were formed from dimyristoylphosphatidylcholine (DMPC) purchased from Avanti Polar Lipids (Alabaster, AL, USA). Stock DMPC suspension in phosphate buffer saline (PBS)

at pH=7.4 and temperature 37ºC was prepared by Vortex mixing and then sonicated until optical clarity was achieved. The desired concentrations of lipids were obtained by dilution of stock suspension in PBS. Finally, stock solutions of hydroxycamptothecins (initially preprared in dimethylsulfoxide) were added to the samples. The final concentration of drugs was 2 μM.

A PTI spectrofluorometer as modified by the authors was used for both the recording of steady-state emission fluorescence spectra and the measurement of steady-state anisotropy. To obtain the fluorescence spectra, DB-67, SN-38 and 10-OH-CPT in DMPC suspension were excited at 390 nm, 395 nm and 374 nm, respectively. Measurements of anisotropy were performed with the instrument in the "L-format". A wavelength of 370 nm for fluorescence excitation and a 400 nm long-pass filter on the emission channel were used.

Results

As one can see in Fig 1 (part A), the spectra of hydroxycampthothecins diluted in pure PBS hydroxycamtothecins have only a band in the green region and the intensity of the blue band increases with an increasing amount of DMPC. All examined hydroxy-camptothecins behaved in the same manner. The appearance of the band in the blue region is related to the binding of the drug to membranes. The steady-state fluorescence anisotropy of the drug also changes over the lipid concentrations (see part B). The higher value of anisotropy points out that more drug molecules were bound to liposomes.

On the basis of the analysis of these changes, the association constants K, defined as

$$K = \frac{F_B}{F_F L} \qquad (1)$$

were determined. F_F and F_B are the fractions of total concentration of free drugs and drugs bound to liposomes, respectively ($F_F + F_B = 1$). L represents the concentration of lipids.

The set of spectra recorded in digital form was arranged as a matrix. Rows of this matrix **X** consist of spectra (of length n) of drug solutions recorded at various lipid concentrations L_i (i = 1,…, m) without their scattering parts (this was achieved by subtraction of the spectra of drug-free DMPC suspensions). Any matrix of spectra can be decomposed into

$$\mathbf{X}_{m\times n} = \mathbf{F}_{m\times 2}\mathbf{S}_{2\times n} \qquad (2)$$

where columns of **F** represent fractions of the respective free and bound form of drug, and rows of the matrix **S**

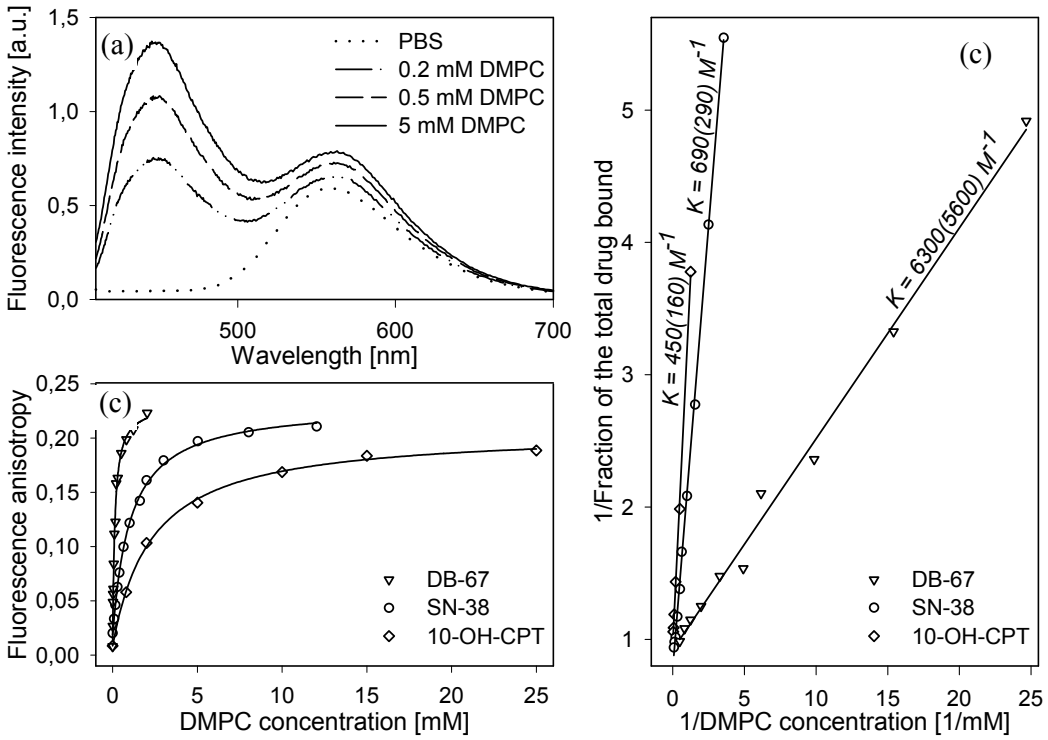

Fig. 1. (a) Fluorescence spectra of the lactone form of DB-67 diluted in various DMPC suspensions and in pure PBS. (b) Steady-state fluorescence anisotropy of hydroxycamptothecins depending on the DMPC liposome concentration. (c) Double-reciprocal plots for the association constant calculation. The slopes of regression lines are equal to 1/K. The values in the parenthesis refer to K as obtained from fluorescence spectra analysis.

contain the shapes of spectra of these forms. For any **F** modelled according to Eq. (1) (taking into account that $F_B = 1 - F_F$), with given K the unknown **S** can be calculated. The criterion for the choice of K is the similarity between the calculated shape of spectra of the free drug and a spectrum of the drug recorded in the absence of lipids. The value of K that maximises this criterion is taken as the association constant of the drug to the membranes. The association constants obtained for DB-67, SN-38 and 10-OH-CPT were $5600 \pm 1300\ M^{-1}$, $290 \pm 80\ M^{-1}$ and $160 \pm 50\ M^{-1}$, respectively.

The second method is based on the dependency:

$$r = r_F f_F + r_B f_B + r_S f_S \qquad (3)$$

where r is the fluorescence anisotropy of the mixture of free and bound drugs, r_F and r_B are the fluorescence anisotropies of the free and bound drugs, respectively, and f_F , f_B are the fractions of the total fluorescence provided by free and bound drugs, respectively. The ratio of f_F to f_B can differ from the ratio of F_F to F_B because of the differences in the quantum yields of fluorescence. The parameters r_S and f_S are related to background scatter. A detailed description of this method can be found in [5]. On the basis of experimentally determined values of r, r_F, r_S, f_S, estimated values of r_B (taking into consideration the changes in the fluorescence intensity of drug upon binding) the fractions of bound drugs F_B was calculated. Finally, by regression of $1/F_B$ on $1/L$ (see Fig. 1, part C) the association constants K were determined. As results, values of K of $6300 \pm 900\ M^{-1}$ for DB-67, $690 \pm 140\ M^{-1}$ for SN-38 and $450 \pm 100\ M^{-1}$ for 10-OH-CPT were obtained. These values are qualitatively consistent with these ones obtained by fluorescence spectra analysis. For the high lipophilic compound (DB-67) the both methods provide also quantitatively consistent results.

Conclusions

Results of both methods are comparable. Somewhat lower values of the association constants of the investigated agents to liposomes were obtained by fluorescence spectra analysis. This method is probably slightly less exact, but cheaper and requires only a simple spectrofluorometer. Despite these differences, the results obtained clearly prove that the biologically active lactone form of DB-67 is the most stable one in the vicinity of lipid membranes among the compounds presented in this work. It also allows to predict the stability of the DB-67 compound in the human blood environment.

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