Optica Applicata, Vol. XXXVI, No. 2–3, 2006

Membranes affinity of hydroxycamptothecins, anticancer agents, determined by fluorescence spectra analysis

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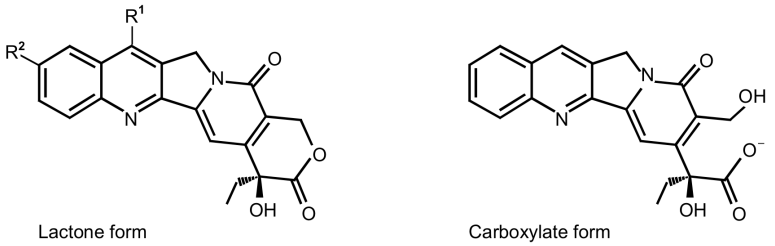
In this paper the method of determination of membranes affinity of hydroxycamptothecins is described. Under physiological conditions hydroxycamptothecins easily hydrolyze and convert into inactive carboxylate form. The process of deactivation is inhibited when the molecules of drug are bound to cell membranes so it is desirable that hydroxycamptothecins molecules bind easily to membranes. A quantitative measure of drugs affinity to membranes is the association constant. To determine this parameter the small unilamellar liposomes are used as model membranes. The affinities of 10-hydroxycamptothecin, SN-38 and DB-67 to membranes are determined. The association constants are calculated on the basis of changes of fluorescence spectra.

Keywords: 10-hydroxycamptothecin, SN-38, DB-67, fluorescence, liposomes.

1. Introduction

Camptotheca acuminata is a member of the family Nyssaceae and is native only to China and Tibet. Its primary anticancer ingredient is a quinoline alkaloid called camptothecin [1]. The latter and its close chemical relatives are the only known naturally-occurring topoisomerase inhibitors [2, 3]. Topoisomerases are the enzymes responsible for DNA replication. Replicating cells are destroyed under influence of camptothecin. The cancerous cells replicate much more rapidly, so they are much more susceptible to the action of these drugs than healthy ones.

Under physiological conditions active lactone form of camptothecin easily converts into inactive one. Hydrolysis process occurs. When the lactone ring opens, negatively charged and inactive carboxylate form arises. About 2 hours after dilution of pure lactone form in a buffer at pH 7.4 the equilibrium concentration is achieved. Both forms, lactone and carboxylate, coexist in the solution. The final concentration of lactone form is much less than the concentration of carboxylate form. For blood, its fraction is about 5% of the total concentration of drug [4]. Easiness of hydrolysis seriously limits the clinical applications of camptothecins.



	R^1	R^2
CPT	H	H
10 OH CPT	H	OH
SN-38	C_2H_5	OH
DB-67	$Si(CH_3)_2C(CH_3)_3$	OH

Fig. 1. Structural formulas of lactone and carboxylate forms of camptothecin and its analogues.

The process of deactivation is stopped when molecules of camptothecin are bound to cell membranes [5] so high affinity to membranes is a very precious property. Through a modification of camptothecin molecule one can obtain its analogues which are able to better fulfill this condition. Three of them are 10-hydroxycamptothecin, 7-ethyl-10-hydroxycamptothecin and 7-t-butyldimethylsilyl-10-hydroxycamptothecin (10-OH-CPT, SN-38 and DB-67, respectively). All mentioned agents contain a 10-hydroxy functionality. Their chemical structures are presented in Fig. 1.

The affinities of hydroxycamptothecins to cell membranes are determined in this work. Liposomes as the model cell membranes are used. Fluorescence spectra of these drugs change in presence of liposomes. On the basis of spectra changes over lipid concentration the association constants of hydroxycamptothecins to membranes are calculated.

2. Experimental

2.1. Materials

The samples of 10-OH-CPT and DB-67 were obtained from the laboratory of biotechnology, College of Pharmacy, University of Kentucky (Lexington, USA) while SN-38 from Tigen Pharmaceuticals Inc. (USA). 2 mM stock solutions of all examined hydroxycamptothecins were prepared in DMSO (dimethylsulfoxide C_2H_6OS). Such stock solutions contain only a pure lactone form. 1 mM stock solutions of hydroxy-camptothecins carboxylate forms were obtained by dilution of stock lactone solution in phosphate buffered saline (PBS) at pH 10 in volume ratio 1:1.

Dimyristoylphosphatidylcholine (DMPC) obtained from Avanti Polar Lipids (Alabaster, AL, USA) was used to form liposomes. Stock DMPC suspension in PBS at pH 7.4 and temperature 37ºC was prepared by Vortex mixing. Next, it was sonicated for about 2 hours using a bath sonicator (Sonic-5, POLSONIC, Poland) until optical

clarity was obtained. The desired concentrations of lipids were obtained by adding the stock lipid suspension to PBS at pH 7.4.

The concentration of hydroxycamtothecins in final samples was equal to 1 μM and it was obtained by adding the stock solution of lactone and carboxylate forms to liposomes suspension at pH 7.4.

2.2. Instrumentations and measurements

The following instrumentation was used for fluorescence excitation: 150 W xenon lamp, monochromator SPM2 (Carl Zeiss Jena) and a quartz lens focusing the excitation light on the sample. For fluorescence exciting of DB-67, 10-OH-CPT and SN-38 in DMPC suspension the radiation of wavelengths 390, 374 and 395 nm, respectively were applied. The fluorescence light was collected by photographic lens (SIGMA) of focal length 28–105 mm and F/2.8-4, onto the entrance slit of emission monochromator (SPM2 monochromator was also used) equipped with a photomultiplier M12 FQ51. A PC with measuring card (AMBEX, Poland) and software written in MATLAB environment [6] were used for monochromators control and manage with data acquisition. The time of single scan of fluorescence spectrum was about 50 seconds. The sample temperature during measurements was stabilized at 37°C with ultratermostat U7^c (Medengen, Germany).

3. Results and discussion

3.1. Experimental results

Due to fluorescence hydroxycamptothecins dissolved in water emit green light, while blue light appears, when they are diluted in a waterless solvent [4]. Figure 2**a** presents fluorescence spectra of 10-OH-CPT, dissolved in DMSO (waterless organic solvent),

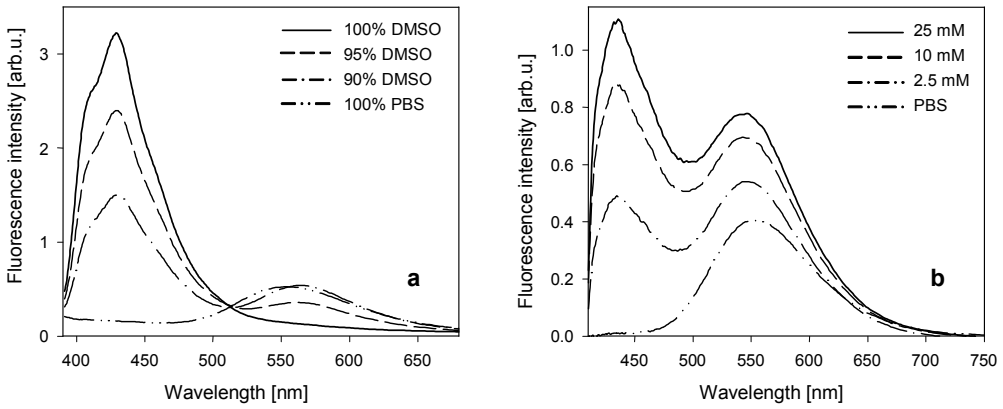


Fig. 2. Fluorescence spectra of lactone form of 10-OH-CPT diluted in: pure DMSO (100% DMSO), mixture of DMSO and PBS (95% DMSO, 90% DMSO) and in PBS (100% PBS) – **a**. Fluorescence spectra of lactone form of SN-38 diluted in liposomes suspensions of lipid concentrations of 25 mM, 10 mM, 2.5 mM and in pure PBS (lipids concentration equals 0) – **b**.

in DMSO/PBS mixture and in pure PBS. Analogical spectra were recorded for DB-67 and SN-38. The spectra of hydroxycamtothecins which are diluted in 100% DMSO contain blue band only. As the DMSO fraction diminishes, the blue band intensity decreases, and the green band emerges. The spectra of hydroxycamtothecins diluted in water consists only of green band.

Hydroxycamptothecins behave similarly when they are diluted in water suspension of liposomes. Figure 2**b** presents the fluorescence spectra of the lactone form of SN-38 diluted in liposomes suspensions. These spectra were recorded for various lipids concentration and in pure PBS. pH of each solution was kept at 7.4. All hydroxycamptothecins behave in the same manner.

The spectrum of SN-38 diluted in PBS exhibits green band with maximum at slightly more than 550 nm (555 nm for 10-OH-CPT and 560 nm for DB-67). After adding liposomes, the blue band with maximum at about 435 nm (440 nm and 450 nm for 10-OH-CPT and DB-67, respectively) arises. One can suppose that observed spectra result from existence of three kinds of fluorophores in the liposomes suspension: i) free molecules of hydroxycamptothecins surrounded by water molecules, ii) molecules of drug bound to liposomes but surrounded by water molecules – both represented by the green band, and iii) drug molecules which are built into lipid bilayers where water molecules do not occur – represented by the blue band. Using the principal components analysis (PCA) [7] one can find only two components existing in the spectra referred to free and bound molecules of drug. The detailed analysis of the changes in spectra over lipids concentrations, allow us to determine the affinity of hydroxycamptothecins to liposomes.

3.2. Association constant

The association constant K [M^{-1}] (where M = mol/dm^3) is a quantitative measure of drug affinity to membranes. It is determined by the following formula [4]:

$$K = \frac{A_B}{A_F L} = \frac{F_B}{F_F L} \tag{1}$$

where A_B [M] represents the concentration of drugs bound to liposomes, A_F [M] – the concentration of free drugs, L [M] represents the total concentration of lipids. $F_B = A_B/A$ is a fraction of bound drugs, $F_F = A_F/A$ is a fraction of free drugs and A [M] represents the total concentration of drugs (the same for all samples). Since $F_B + F_F = 1$, one can obtain from (1)

$$\frac{1}{F_B} = 1 + \frac{1}{K}\frac{1}{L} \tag{2}$$

Area under curves representing spectra of bound and free drug molecules are proportional to F_B and F_F, respectively: $S_B(L) = \alpha F_B(L)$ and $S_F(L) = \beta F_F(L)$, where α and β are the proportionality coefficients. Quantum yields of fluorescence

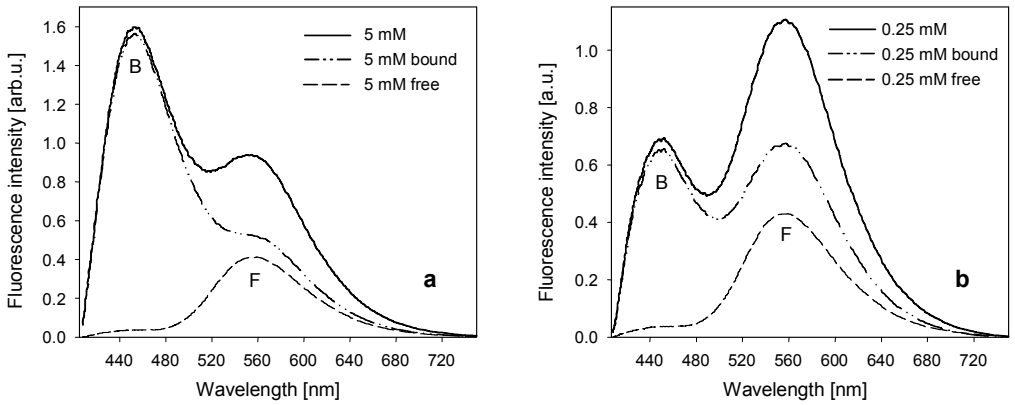


Fig. 3. Recorded fluorescence spectrum of carboxylate form of DB-67 diluted in 5 mM (**a**) and 0.25 mM (**b**) DMPC liposomes suspension (solid line), calculated spectrum of carboxylate form molecules bound to liposomes (dashed-dotted-dotted line) and spectrum of free carboxylate form molecules (dashed line). The shape of the spectrum of free carboxylate form is determined experimentally, while intensity is calculated in the case of both forms.

(defined as the ratio of the number of photons emitted to the number of photons absorbed) of both free and bound forms of hydroxycamptothecins can be different, therefore the coefficient α can also differ from β. The reason for this differences can be also in optical characteristics of a measurement equipment, *e.g.*, the efficiency of both the monochromator and the photomultiplier tube.

The first step is the decomposition of each spectra into their "free" $F(L)$ and "bound" $B(L)$ parts (Fig. 3). The spectrum of hydroxycamptothecins recorded in pure PBS (for L equal to 0) contains information on free drug only. On the basis of PCA one can assume that the shapes of $F(L)$ and $B(L)$ do not change with DMPC concentration. One can always find such set of component spectra to fulfill following linear dependence

$$\frac{S_B(L)}{S_F(L)} = K'L \tag{3}$$

for any value of K'. This condition and constancy of the shape of $F(L)$ and $B(L)$ allows to decompose the spectra unambiguously. Value of K' depends on the unknown coefficients α and β. To find the real association constant K one needs to solve the following equations system for each pair of concentrations L_i and L_j ($i \neq j$):

$$\begin{aligned} \frac{\alpha}{S_B(L_i)} &= 1 + \frac{1}{K_{ij}}\frac{1}{L_i} \\ \frac{\alpha}{S_B(L_j)} &= 1 + \frac{1}{K_{ij}}\frac{1}{L_j} \end{aligned} \tag{4}$$

T a b l e. Values of association constant of examined hydroxycamptothecins calculated using the method of fluorescence spectra analysis. The results obtained using fluorescence anisotropy method are included in the bracket. Results obtained for concentration of lipids equals 1 mM and less (*), 5 mM and more (**).

Compound	Lactone form	Carboxylate form
10-OH-CPT	140 ± 30 M^{-1}	—
SN-38	215 ± 40 (260) M^{-1}	—
DB-67	5300 ± 1200 (4500) M^{-1}	5800 ± 400 M^{-1} (*); 260 ± 70 M^{-1} (**)

The average of K_{ij} calculated in this way is taken as the association constant of drug to membranes. The standard deviation of K_{ij} is taken as the uncertainty of measurement of K.

3.3. Discussion

The method of analysis presented above was used to the set of fluorescence spectra of all examined hydroxycamptothecins. Obtained results are presented in the Table. This table contains also the results obtained by fluorescence anisotropy method [8]. It can be seen that the values of the association constants calculated for lactone forms of both DB-67 and SN-38, on the basis of this two methods, are very close to each other. The set of spectra of carboxylate form of DB-67 was split into two groups. The association constant obtained for small concentration of DMPC (1 mM and less) is very close to the one obtained for lactone form. One can suppose that in solution with low concentration of DMPC, the process of lactonization occurs and lactone form molecules bind easily with liposomes. So the association constant obtained in this case is in fact a constant calculated for lactone form of DB-67. This follows also from the comparison of spectra of bound drugs presented in Figs. 3**a** and 3**b**. They differ considerably.

The association constant of carboxylate form of 10-OH-CPT and SN-38 to membranes have not been yet determined. The value obtained for carboxylate form of DB-67 (calculated for DMPC concentrations between 5 and 50 mM) is about 20 times smaller than the one of lactone form. It is consistent with the results obtained for other camptothecin analogues on the basis of fluorescence anisotropy measurement. In each case association constants of carboxylate forms to membranes were many times smaller than those of lactone forms [9].

4. Conclusions

The presented method of analysis of fluorescence spectra seems to be competitive or supplementary to other ways of determining drugs affinity to membranes. Results obtained for lactone form of both SN-38 and DB-67 are consistent with those obtained by fluorescence anisotropy measurements. The experimental procedure of this methods is very simple and not time consuming. A simple spectrofluorometer which enables to record emission spectra appears to be good enough. The main limitation of

presented method lyes in necessity of the existence of difference in fluorescence spectra of free and bound drugs.

The high lipophilicity of the lactone form of DB-67 makes this drug the most promising among the analogues of camptothecin studied in this work.

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Received December 15, 2005
in revised form April 27, 2006