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

Affinity of new anticancer agent, DB-174, to membranes and HSA determined by fluorescence spectroscopy methods

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The application of fluorescence spectroscopy methods to determining the properties of camptothecins – promising anticancer agents – are described in this paper. The fluorescence anisotropy measurements provide useful information about the binding of camptothecin and its analogues to cell membranes and human serum albumin (HSA) that is important for potential clinical applications of these agents, and permits the selection from many camptothecin analogues those ones exhibiting desirable biomedical properties. Binding properties of 7-trimethylsilyl-ethyl--10 hydroxy-camptothecin are the subject of this paper.

Keywords: camptothecin, fluorescence anisotropy, membranes, human serum albumin (HSA).

1. Introduction

The fluorescence spectroscopy methods are very useful in determining of biophysical properties of fluorescent drugs. Camptothecin (CPT) and its analogues are fluorescent compounds exhibiting strong anticancer properties [1–3]. A disadvantage which seriously limits application of camptothecins in antitumor chemotherapy is the hydrolysis of these compounds in neutral and base solutions. Under this condition they convert from biologically active lactone form into inactive carboxylate [2]. Knowledge of the kinetics of camptothecin hydrolysis, which cause its biological deactivation, is important in the search of camptothecin derivatives with desirable stability in human blood and human tissues or the methods of improving stability of these compounds.

Interaction of these compounds with membranes and proteins has the crucial meaning for stability of its active forms. Methods of fluorescence spectroscopy play special role in determining of behaviour of camptothecin in presence of blood components. Such techniques as fluorescence lifetime measurements, fluorescence

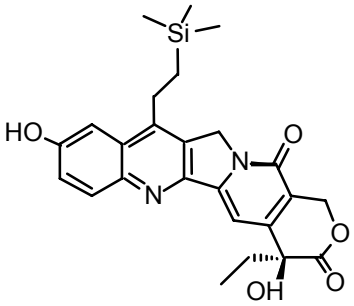

Fig. 1. Structure of 7-trimethylsilylethyl-10 hydroxy--camptothecin (DB-174).

anisotropy decay, steady-state fluorescence anisotropy measurements, steady-state fluorescence spectra analysis, fluorescence microscopy, two-photon fluorescence spectroscopy and fluorescence microscopy [4], permit to determine many important biophysical properties of camptothecins. By measurements of fluorescence lifetime one can determine the affinity of camptothecin and its analogues to human serum albumin (HSA) [5, 6]. For potential clinical applications of camptothecin analogues the low affinity of their carboxylate form to HSA is desirable. In other hand the high affinity of active lactone form to membranes is wanted. Principal criterion in search of new camptothecin analogues is low affinity of carboxylate form to HSA and high affinity of lactone form to membranes. The fluorescence lifetime measurements and fluorescence anisotropy measurements are very useful in determining the above affinities. Results obtained on the basis of such measurement are decisive for further testing of these drugs in biological systems including those of animals.

Camptothecins which bind to cell membranes or liposomes do not hydrolyse [7]. So, the high affinity of camptothecins to membranes is also a wanted property. It is well known, that among many new analogues, one of them, silatecan DB-67 (7-tert-butyldimethylsilyl-10-hydroxy-campthothecin) exhibits the high affinity of lactone form to membranes and small affinity of its carboxylate form to HSA [8–10]. Such properties ensure, that lactone form of this agent exhibits the high stability in blood and therefore it seems to be now both one of the most promising camptothecin analogue and an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical trials in cancer chemotherapy [11]. 7-trimethylsilylethyl-10--hydroxy-camptothecin, called DB-174 is also silatecan. Figure 1 presents the chemical formulae of this agent. It was obtained by camptothecin modification – at position 7 the hydrogen was replaced by group $-CH_2CH_2Si(CH_3)_3$ and at position 10 by hydroxygroup. Properties of DB-174, like that of DB-67, seem to be also very promising. Results of studies of behaviour of this agent in the presence of model membranes – liposomes and in HSA solution are presented in the next part of this paper.

2. Materials and methods

DB-174 was obtained from laboratories of Prof. Dennis Curran, University of Pittsburgh, USA. Its purity was greater than 98%. 2 mM stock solution of this drug

was prepared in dimethylsulfoxide. For fluorescence measurements the stock solution was added to various fluids (PBS, liposomes suspension, HSA solution). The final drug concentration for fluorescence measurements was 1 μM. The lipids (dimyristoyl-phosphatidycholine (DMPC) and dimyristoylphosphatidyl-glycerol (DMPG)) used for liposomes preparation, were from Avanti Polar Lipids (Alabaster, AL, USA). The HSA was purchased from Sigma-Aldrich.

A SLM model 8100 spectrofluorometer was used for fluorescence measurements. It permitted the recording of steady-state excitation and emission fluorescence spectra as well as the determination of the steady-state anisotropy of emission. Measurements of fluorescence anisotropy were performed with the above instrument in the "T-format". 370 nm exciting light and 400 nm long-pass filters on each emission channel were used. For determining camptothecins affinities to membranes the method of fluorescence anisotropy titration [12], was used like that in previous paper [10]. Small unilamelar liposomes were used as model membranes. They were prepared in the following way. Stock lipid (DMPC or DMPG) suspensions in a phosphate buffered saline at pH = 7.4 and temperature 37°C were prepared by Vortex mixing for 5–10 minutes and then sonicated using a bath sonicator (Laboratory Supplies Co., Hicksville, NY, USA) for a few hours until optical clarity was obtained.

3. Principle of fluorescence anisotropy methods

The fluorescence light emitted by a solution is always depolarized. The anisotropy r defined as $r = (I_V - GI_H)/(I_V + 2GI_H)$ [4] is most often used for characterization of depolarization of fluorescence light. I_V and I_H are the fluorescence intensities of the vertically and horizontally polarized emission, when the sample is excited by vertically polarized light. $G = S_V/S_H$ is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. Maximal possible values of anisotropy r_o of fluorescence light emitted by a solution is always smaller than 0.4 [4]. This is a result of photoselection and internal conversion [4]. Rotation of fluorescent molecules during the life of an excited state cause the further decrease of anisotropy according to the relation $r(t) = r_o \exp(-t/\Theta)$, where Θ is a rotational correlation time [4]. By continuous excitation one can determine the steady-state anisotropy. It depends on the fluorescence lifetime τ and rotational correlation time as follows [4]:

$$r = \frac{r_o}{1 + \frac{\tau}{\Theta}}$$

From this formulae it follows that for small, fast rotating fluorofores ($\Theta < \tau$) r is small, being close to zero, while for big, slow rotating fluorofores ($\Theta > \tau$) r it is great, being close to r_o.

Free and bound drugs exist in suspension of liposomes or membranes. The association constant [7]

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

is a quantitative measure of a drugs affinity to membranes. A_B represents the concentration of bound drugs, A_F represents the concentration of free drugs, $F_B = A_B/A$ is a fraction of bound drugs, $F_F = A_F/A$ is a fraction of free drugs, A represents total concentration of drugs and L represents total concentration of lipids – molecules forming the membranes. Because $F_B + F_F = 1$, from (1) we obtain:

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

The inverse of the fraction of bound drugs is a linear function of the inverse of lipid concentration. The slope of this function $1/K$ represents the inverse of association constant. On the basis of experimentally determined fractions of bound drugs in dependence on lipid concentration, the association constant K can be determined using Eq. (2). The fluorescence anisotropy methods can be used for determining the concentration of bounds drugs in relation to the lipid concentration. The total fluorescence anisotropy r observed for a mixture of fluorophores [4] is given by

$$r = \sum_i r_i f_i \tag{3}$$

where r_i is the anisotropy of i-th individual fluorophore and f_i is the fractional fluorescence intensity of i-th fluorophore. In the suspension of liposomes, two kinds of fluorophores exist: free drugs and drugs bound to liposomes. According to (3) the anisotropy of such a mixture is given by

$$r = r_F f_F + r_B f_B \tag{4}$$

where r_F and r_B are the anisotropies of the free and bound drugs, respectively, f_F and f_B are the fractional fluorescence intensity of the free and bound drugs, respectively. Of course $f_F + f_B = 1$. If we assume that the quantum yield of the fluorophores is not altered by binding then $f_F = F_F$ and $f_B = F_B$. Equation (4) can be easily rearranged into

$$F_B = f_B = \frac{r - r_F}{r_B - r_F} \tag{5}$$

On the basis of experimentally determined r_F and r, estimated values of r_B and Eq. (5) the fraction of bound drug F_B can be calculated. By making the graph of $1/F_F$ versus $1/L$, *i.e.*, double-reciprocal plot, the association constant K according to Eq. (2) can be determined.

4. Results and discussion

Figure 2 presents the steady-state fluorescence spectra of DB-174 in PBS and in suspension of liposomes formed from DMPC and DMPG lipids. This figure shows the big difference in spectra of the free DB-174 and that bound to liposomes. The free DB-174 in water exhibits green fluorescence, while if this agent is diluted in liposomes suspension a strong blue fluorescence band appears beside the green band.

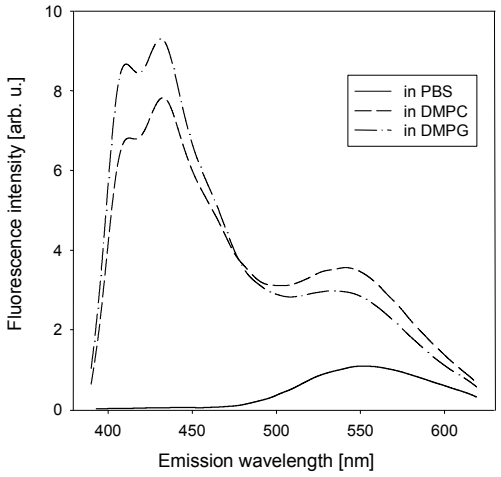

Fig. 2. Steady-state fluorescence spectra of DB-174 in PBS and in suspension of liposomes formed from both DMPC and DMPG lipids.

The liposomes formed from DMPC and DMPG lipids are used to determine the affinity of DB-174 to membranes. The fluorescence anisotropy of DB-174 diluted in suspensions of DMPC and DMPG liposomes was measured as a function of lipids concentration. Results of such measurements for DMPC liposomes are presented in Fig. 3. This figure contains also results of analogical measurements performed for camptothecin and some camptothecin analogues previously studied (topotecan, SN-38, DB-67) [10]. In solutions free of liposomes the steady-state anisotropy of camptothecins is small (about 0.01). A rise in lipids concentration causes an increase in fluorescence anisotropy. The rate of this increase depends on the kind of camptothecin analogue. The slowest increase is observed for topotecan. This means that this compound exhibits the poorest affinity to the membranes. For camptothecin a faster increase of anisotropy is observed. The anisotropy of SN-38 rises more fast than for camptothecin. An extremely fast increase of anisotropy with increasing lipids concentration is observed for DB-67 and DB-174. This means that these last two compounds bind very easily to membranes.

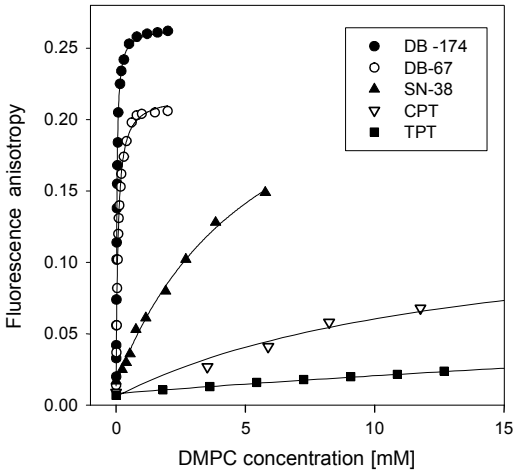

Fig. 3. Steady-state fluorescence anisotropy of DB-174, camptothecin and some other camptothecin analogues depending on DMPC liposomes concentration.

On the basis of experimentally determined anisotropies and formulae (5), the concentrations of free and bound drugs in a liposome suspension were determined and then the double-reciprocal plots were drawn. They are shown in Fig. 4. Values of the association constants *K* were determined from the double reciprocal plots. Average values of association constants together with standard deviation are summarized in the Table. Each value of association constant is the mean value of at least 3 values obtained by independent measurements. From the results summarized in the Table it

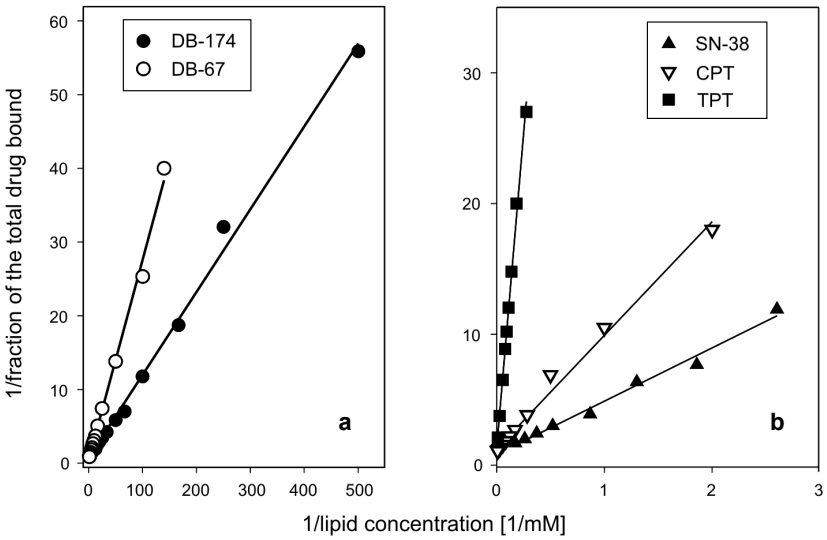

Fig. 4. Double-reciprocal plots for the binding of DB-174 and DB-67 (**a**) and the less lipophilic SN-38, CPT, TPT (**b**) to the DMPC small unilamellar liposomes.

T a b l e. Association constants for camptothecin analogues interacting with unilamellar liposomes of DMPC and DMPG in PBS buffer at pH 7.4 and 37°C.

Compound	K_{DMPC} [M^{-1}]	K_{DMPG} [M^{-1}]
Camptothecin	100±15	100±15
Topotecan	10±3	50±10
SN-38	260±80	160±50
DB-67	4500±900	3000±600
DB-174	9000±1000	6600±800

follows that among the camptothecins considered here, DB-174 exhibits the highest affinity to membranes. Then, if their carboxylate form displays low affinity to HSA, they offer the promise of becoming very stable anticancer drugs.

Steady-state anisotropy measurements can also provide useful information about the behaviour of lactone and carboxylate form of camptothecins in HSA solution. From our previous results [10] it follows that the lactone and carboxylate forms of camptothecin in HSA solution exhibit the big differences in steady-state fluorescence anisotropy. The time dependences of steady-state anisotropy for both forms of CPT in HSA solution were determined. The results of such measurements are presented in Fig. 5. They show that the steady-state fluorescence anisotropy of the carboxylate form of CPT in HSA solution is large (0.33) and it does not change in time. The great value of the steady-state anisotropy proves that the molecules of CPT carboxylate are bound to big HSA molecules. CPT lactone just after their introduction into HSA solution exhibits a low steady-state anisotropy (0.013). This means that CPT lactone

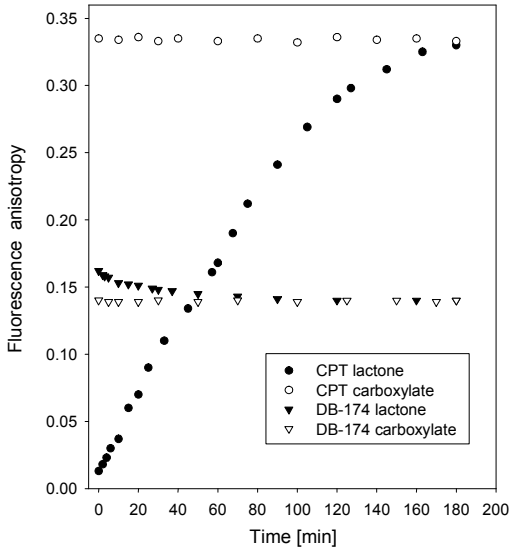

Fig. 5. Time evolution of steady-state fluorescence anisotropy of lactone and carboxylate forms of CPT and DB-174 in 10 μM HSA solution.

does not bind or binds poorly to HSA. However, the anisotropy rises over time. This increase is caused by the hydrolysis process. The free CPT lactone molecules convert into carboxylate, which immediately bind to HSA. After about 2 hours, as is shown in Fig. 4, the anisotropy approaches that obtained for the pure carboxylate form. This means that after such a time the lactone form is converted almost totally to inactive carboxylate. This figure presents also the results of analogical measurement for DB-174. On the basis of obtained results one can conclude that DB-174 behave totally different in HSA solution. The anisotropy of both forms of DB-174 is equal to about 0.15 and is practically independent of the time. This means, that both forms of DB-174 exhibit rather poor affinity to HSA. Such results permit to conclude that DB-174 will behave in desirable way in blood, and then this agent can become stable anticancer drug.

5. Conclusions

Fluorescence anisotropy measurements show that DB-174 is one of the most promising camptothecin analogue. It exhibits a very high affinity of the lactone form to membranes and rather a poor affinity of its carboxylate form to HSA. This means that this agent can become an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical attempting applications to cancer chemotherapy.

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Received December 15, 2005
in revised form March 15, 2006