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Affinity of new anticancer agent, 7-trimethylsilyl-ethyl-10-amino-camptothecin, to membranes and HSA determined by fluorescence spectroscopy methods

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The application of fluorescence anisotropy measurements in determining the properties of camptothecin analogue 7-trimethylsilylethyl-10-amino-camptothecin – a promising anticancer agent – is described in this paper. The fluorescence anisotropy measurements provide useful information about binding of camptothecins to membranes and proteins, including human serum albumin (HSA). Knowledge of these properties is important for potential clinical applications of these agents, and permits to select from camptothecin analogues only those which exhibit desirable properties. An active lactone form of camptothecin in fluids at pH 7.4 hydrolyses and converts into an inactive carboxylate form. The carboxylate form of camptothecin binds easily and irreversibly to HSA. Only free carboxylate form can transform back into lactone, then in the presence of HSA one direction transition occurs (from lactone to carboxylate); therefore in HSA solution, after about two hours, the lactone form almost totaly decays. On the other hand, the camptothecins bound to membranes do not hydrolyse. Fluorescence anisotropy measurements prove that 7-trimethylsilylethyl-10-amino-camptothecin exhibits desirable properties: high affinity of its lactone form to membranes and low affinity of its carboxylate form to HSA. Such properties should ensure high stability of this drug in physiological fluids, including blood.

Keywords: camptothecin, silatecan, fluorescence anisotropy, membranes binding, HSA affinity.

1. Introduction

Camptothecin (CPT) – an alkaloid isolated from the Chinese tree *Camptotheca acuminata* – exhibits very high anticancer activity [1]. CPT is a fluorescent compound and this is a very useful property. Using methods of fluorescence spectroscopy, one can determine or predict the biophysical properties of this promising compound. CPT can exist in two forms: lactone (stable at pH < 5.5) and carboxylate (stable at pH > 9). Chemical structures of both forms of camptothecin are presented in Fig. 1**a**. Only lac-

tone form is biologically active. The cellular target of the CPT is topoisomerase I, a nuclear enzyme responsible for DNA replication. CPT interacts only with those cells which are in the S-phase. CPT molecules bind to topoisomerase I – DNA complex and prevent the replication process [2, 3]. This means that CPT is toxic to the cells that are undergoing DNA synthesis. Cancerous cells are rapidly replicating and they spend more time in the S-phase in relation to healthy tissues, and therefore they are killed with much higher efficiency than the healthy host tissues. Such selectivity of cytotoxicity is a promising property of CPT. Due to the S-phase specificity of CPT, a continuous exposure to this drug must be maintained in order to achieve optimum therapeutic efficacy. Unfortunately, there is some difficulty in fulfilling this requirement. Under physiological conditions (pH = 7.4) CPT hydrolyses and converts to the "ring opened" inactive carboxylate form [3]. After about 2 hours an equilibrium is achieved and both forms coexist. The concentration of the lactone form in the equilibrium is much smaller than the carboxylate form and depends on the environment into which the CPT is introduced. It is about 15%, 5% and 0.2% for PBS (phosphate buffer saline), whole blood and blood plasma, respectively [4]. The low concentration of the lactone form under physiological conditions seriously limits the possibility of CPT application in cancer chemotherapy. The presence of HSA has a critical influence on the activity of camptothecin. 2 hours after introducing camptothecin to HSA solution, it loses totally its anticancer activity. An effect which destabilizes camptothecin in blood is the high affinity of its carboxylate form to HSA. Camptothecin carboxylate molecules bound to HSA do not convert into the lactone form. This leads to rapid decay of the active lactone form in blood [5]. However, a competing effect exists which improves the stability of camptothecin: CPT molecules bound to membranes do not hydrolyse [6].

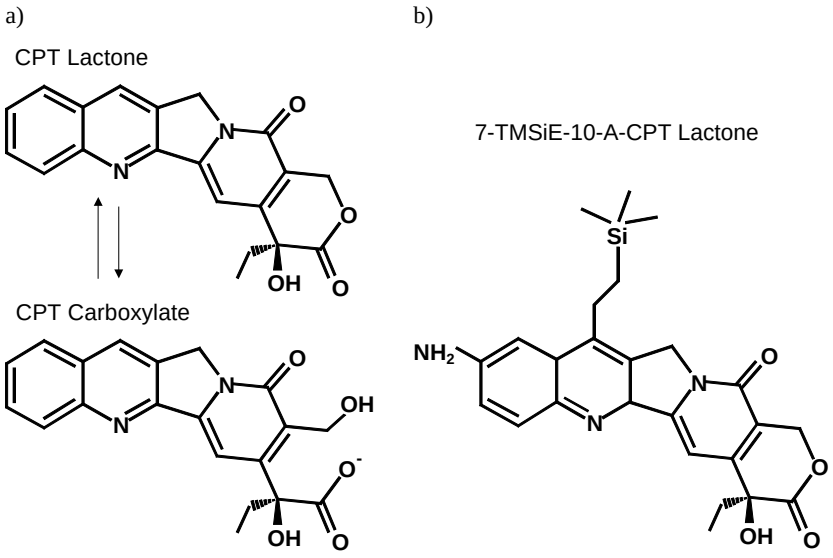

Fig. 1. Structures of the lactone and carboxylate forms of camptothecins (**a**) and structure of the lactone form of 7-TMSiE-10-A-CPT (**b**).

Much effort has been undertaken in order to discover analogues of camptothecin which exhibit improved blood stability. Such new compounds should retain anticancer properties and exhibit high affinity of the lactone forms to membranes and low affinity of the carboxylate forms to HSA. It is well known that among many new analogues, some of them, silatecans: DB-67 (7-tert-butyldimethylsilyl-10-hydroxy-campthothecin) [5, 7–9] and DB-174 (7-trimethylsilylethyl-10-hydroxy-camptothecin) [10], exhibit high affinity of lactone form to membranes and small affinity of its carboxylate form to HSA. Such properties ensure that the lactone form of this agent exhibits high stability in blood and therefore it seems to be the most promising camptothecin analogue and an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical trials in cancer chemotherapy. 7-trimethylsilylethyl-10-amino-camptothecin (7-TMSiE-10-A-CPT) is also a silatecan. Figure 1**b** presents the chemical formulae of this agent. It was obtained by camptothecin modification – at position 7, the hydrogen was replaced by a group $–CH_2CH_2\ Si(CH_3)_3$ and at position 10, by an amino-group. Properties of this compound, like these of DB-67 and DB-174, seem to be also very promising. Results of the studies of the behavior of this new agent in the presence of model membranes – liposomes and in HSA solutions are presented in the next part of this paper.

2. Materials and methods

2.1. Materials

The samples of camptothecin and 7-trimethylsilylethyl-10-amino-camptothecin were obtained from the laboratory of biotechnology, College of Pharmacy, University of Kentucky, Lexington, USA. 2 mM stock solutions of camptothecin and 7-trimethylsilyl-ethyl-10-amino-camptothecin were prepared in DMSO (dimethylsulfoxide C_2H_6OS). Such stock solution contains only a pure lactone form. 1 mM stock carboxylate solution was obtained by diluting a stock lactone solution in PBS at pH 12 in a volume ratio 1:1. The PBS was adjusted to the desired value of pH using small quantities of 0.1 M KOH or HCl.

As model membranes, small unilamelar liposomes formed by DMPC (1,2 dimyristoyl-sn-glycero-3-phosphatidylcholine) lipids were used. DMPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The small unilamelar liposomes were prepared in the following way. Stock lipid 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 (Ultrasonic Cleaner SONIC-5, POLSONIC, Poland) for a few hours until optical clarity was obtained. The desired concentration of lipids was obtained by adding the stock lipid suspensions to PBS at pH 7.4.

Human serum albumin (95–97%) was purchased from Sigma-Aldrich (USA–Poland). The solutions of HSA were prepared in PBS. The pH of these solutions was kept at 7.4 and their temperature at 37 °C.

For fluorescence spectra recording and fluorescence anisotropy measurements, the concentration of 7-trimethylsilylethyl-10-amino-camptothecin in final samples was equal to 2 μM. The desirable concentration was obtained by adding the stock solutions of 7-TMSiE-10-A-CPT to PBS at pH 7.4 or to DMPC liposomes suspensions at pH 7.4, or to HSA solutions also at pH 7.4.

2.2.Method of anisotropy measurement

Fluorescence light from a solution is always depolarized [11]. For characterization of a depolarization degree of fluorescence light, the fluorescence anisotropy *r* defined as $r = \frac{I_V - GI_H}{I_V + 2GI_H}$ [11] is most often used. 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. $G = \frac{S_V}{S_H}$ is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. On the basis of fluorescence anisotropy measurements depending on the concentration of lipids forming the model membranes, the association (binding) constants of drugs to the membranes were determined. The association constant is determined by the formulae [12]

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

where A_F represents the concentration of a free drug, A_B is the concentration of a drug bound to the membranes and L represents the total concentration of lipids forming the membranes. $F_F = A_F/A$ is the fraction of a free drug, $F_B = A_B/A$ is the fraction of a bound drug and $A = A_F + A_B$ represents the total concentration of a drug (the same for all samples).

The association constant K is a quantitative measure of the affinity of a drug to the membranes. The procedure of determining the association constant on the basis of fluorescence anisotropy measurements was described in detail in previous papers [8, 10].

2.3. Instrumentation

A PTI (Photon Technology International, Birmingham, NJ, USA) spectrofluorometer was used for recording steady-state emission fluorescence spectra and for measuring steady-state fluorescence anisotropy. To obtain the fluorescence spectra of 7-TMSiE-10-A-CPT, the light at 395 nm was used for excitation. Measurements of fluorescence anisotropy for 7-TMSiE-10-A-CPT were performed with the instrument in the "L-format" using excitation at 395 nm and 435 nm long-pass filters on the emission channel. Using the long-pass filter on the emission channel ensures the separation of fluorescence from scattering light. The temperature of the sample was kept constant (37 °C) using an ultrathermostat TW2.03 (ELMI).

3. Results and discussion

Figure 2 presents the steady-state fluorescence spectra of 7-TMSiE-10-A-CPT, and for comparison, the spectra of CPT diluted in PBS and in the suspension of liposomes formed from DMPC lipids. The concentration of DMPC lipids used for the preparation of liposomes was 1.5 mM. The spectra presented in Fig. 2 are pure fluorescence spectra – the contribution of scattering light was removed by subtractions of the scattering spectrum of PBS and liposomes from the recorded fluorescence spectrum of the drug (CPT and 7-TMSiE-10-A-CPT). The emission fluorescence spectra of 7-TMSiE-10-A-CPT diluted in PBS, as it is shown in Fig. 2**a**, exhibit maximum at 526 nm for both forms, while for 7-TMSiE-10-A-CPT diluted in the suspension of DMPC liposomes the increase fluorescence intensity, and shifting toward shorter wavelength (maximum at 503 nm) are observed. The change in the fluorescence spectrum of 7-TMSiE-10-A-CPT after introducing it into DMPC liposomes suspension may prove the strong binding of this agent to liposomes. Figure 2**b** presents analogical spectra of lactone and carboxylate forms of CPT diluted in PBS and of lactone form of CPT diluted in 1.5 mM of DMPC liposomes suspension. It shows differences in the fluorescence spectra of lactone and carboxylate forms of CPT. These differences were analysed in previous papers [13, 14]. It is necessary to notice that contrary to 7-TMSiE-10-A-CPT, the difference in the fluorescence spectrum of lactone form of CPT diluted in PBS and 1.5 mM of DMPC liposomes suspension is not observed. It proves that contrary to 7-TMSiE-10-A-CPT, the CPT lactone exhibits poor binding to liposomes. The authors of paper [12] observed 16 nm shifting of the fluorescence spectrum of CPT after introducing it into liposomes suspension, but they used very high concentrated liposomes (290 mM).

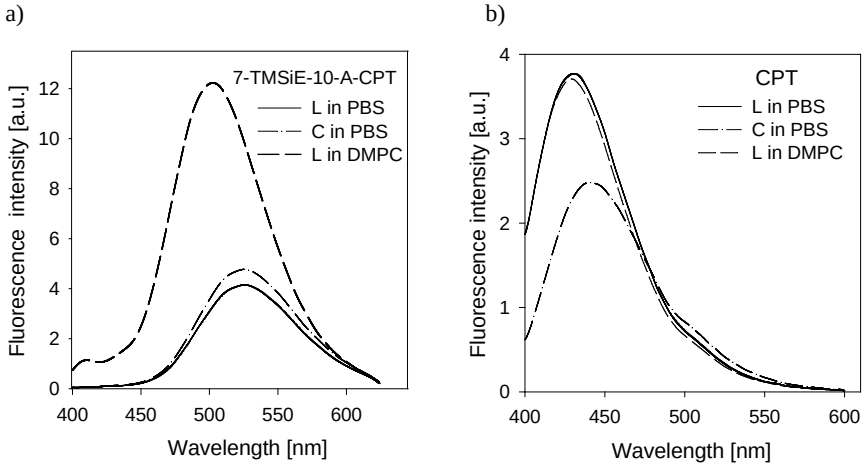

Fig. 2. Steady-state fluorescence spectra of 7-TMSiE-10-A-CPT lactone and carboxylate forms diluted in PBS and lactone form diluted in the suspension of DMPC liposomes (**a**); for comparison the analogical spectra of CPT (**b**). In both cases the concentration of liposomes was the same (1.5 mM of DMPC lipids was used while preparing liposomes suspension).

For quantitative determination of 7-TMSiE-10-A-CPT binding to liposomes, the fluorescence anisotropy as a function of lipids concentration was measured. Results of such measurements are presented in Fig. 3. This figure contains also the results of analogical measurements performed earlier for: camptothecin (poor binding with membranes), SN-38 (medium binding with membranes) and DB-67 (strong binding with membranes) [5, 7–9]. An extremely fast increase in anisotropy with increasing lipids concentration was observed for 7-TMSiE-10-A-CPT, as for DB-67 [5, 7–9]. This means that 7-TMSiE-10-A-CPT, like DB-67, bind very easily to membranes.

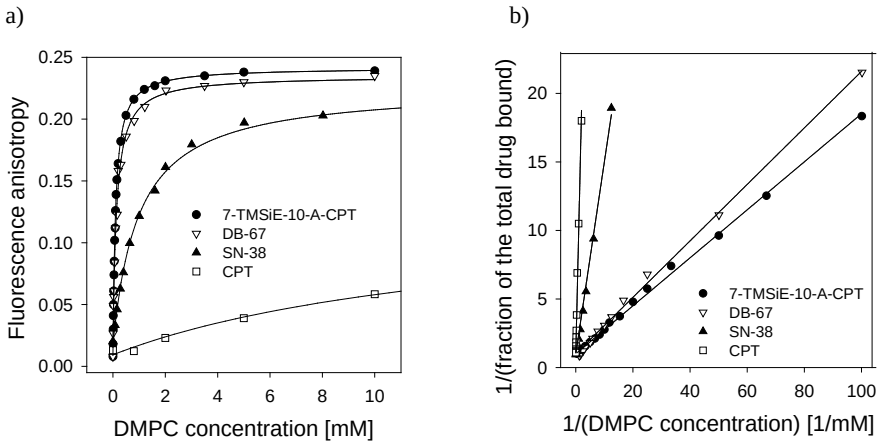

Fig. 3. Steady-state fluorescence anisotropy of 7-TMSiE-10A-CPT, DB-67, SN-38 and CPT depending on DMPC liposomes concentration (**a**) and double-reciprocal plots for the binding of 7-TMSiE-10A-CPT, DB-67, SN-38 and CPT to DMPC liposomes (**b**).

On the basis of experimentally determined anisotropy, using previously described methods, the concentration of free and bound drugs in liposomes suspension was determined and then the double-reciprocal plots were drawn. They are shown in Fig. 3**b**. The slope of lines fitted to experimental values determines the inverse of association constants (1/*K*). For studied here 7-TMSiE-10-A-CPT, the obtained value of *K* is equal to 5700 M^{-1}. Such big value of the association constant of 7-TMSiE-10-A-CPT confirms the earlier conclusion reached on the basis of changes in fluorescence spectrum or high rate of increasing of anisotropy with increasing liposome concentration. The obtained value of the association constant for 7-TMSiE-10-A-CPT is compared with the values of this parameter obtained earlier for CPT and studied CPT analogues [5, 8, 10]. These parameters are listed in the table. From the table it follows that 7-TMSiE-10-A-CPT, as DB-67 and DB-174, exhibit very high affinity to membranes. Then, if the carboxylate form of 7-TMSiE-10-A-CPT displays low affinity to HSA, this new agent will become a good candidate for a stable anticancer drug.

Steady-state anisotropy measurements can also provide useful information about the behaviour of lactone and carboxylate forms of camptothecins in HSA solution. From our previous results [8, 10, 15] it follows that lactone and carboxylate forms of

T a b l e . Summary of the association constants of camptothecins including 7-TMSiE-10-A-CPT to model membranes (DMPC liposomes) determined using the fluorescence anisotropy method.

Compound	K_{DMPC} [M^{-1}]	Reference
CPT	70 ± 15	[8]
	100 ± 15	[5, 10]
SN-38	800 ± 100	[8]
DB-67	4500 ± 900	[5, 10]
	6600 ± 1000	[8]
DB-174	9000 ± 1000	[10]
7-TMSiE-10-A-CPT	**5700 ± 800**	–

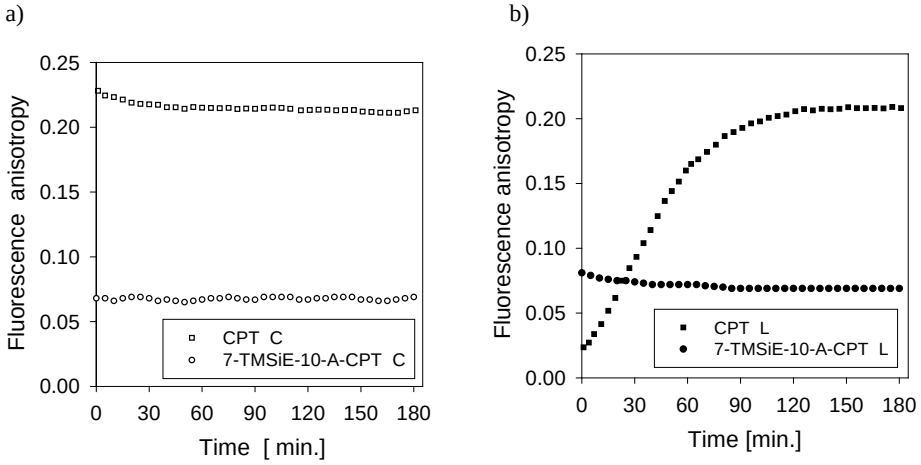

Fig. 4. Time evolution of steady-state fluorescence anisotropy of the carboxylate forms of 7-TMSiE-10-A-CPT and CPT (**a**) and lactone forms of these agents (**b**) in 10 μM HSA solutions.

camptothecins in HSA solution exhibit big differences in steady-state fluorescence anisotropy. The time dependences of steady-state anisotropy for both forms of CPT in HSA solution were determined. Results of such measurements are presented in Fig. 4. Figure 4**a** shows that the steady-state fluorescence anisotropy of the carboxylate form of CPT in HSA solution is high and does not change over time. The high value of the steady-state anisotropy proves that the molecules of CPT carboxylate are bound to big HSA molecules. Figure 4**b** shows that the CPT lactone just after the introduction into HSA solution exhibits low steady-state anisotropy (0.013). This means that CPT lactone 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 binds to HSA. After about 2 hours, as it is shown in Fig. 4**b**, the anisotropy approaches the value obtained for the pure carboxylate form. This means that after this period the lactone form is converted almost totally to inactive carboxylate. Figure 4 presents also the results of analogical measurement for 7-TMSiE-10-A-CPT. On the basis of obtained results, one can conclude that 7-TMSiE-10-A-CPT behave totally

different in HSA solution. The anisotropy of both forms of 7-TMSiE-10-A-CPT is equal to about 0.07 and is practically independent of the time. This means that both forms of 7-TMSiE-10-A-CPT exhibit rather poor affinity to HSA, even poorer than previously studied DB-67 and DB-174. Such results permit to conclude that 7-TMSiE-10-A-CPT will behave in a desirable way in blood, and therefore this agent can become a stable anticancer drug. It may be even more stable in blood than previously studied DB-67 and DB-174.

4. Conclusions

Fluorescence anisotropy measurements show that 7-trimethylsilylethyl-10-amino-camptothecin is one of the most promising camptothecin analogues. It exhibits very high affinity of the lactone form to membranes and poor affinity of its carboxylate form to HSA. It means that this agent can become an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical trials in cancer chemotherapy.

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