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ORIGINAL ARTICLE

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Fluorescence spectroscopy in a study of anticancer drugs: 7-tert-butyldimethylsilyl-camptothecin and 7-tert-butyldimethylsilyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin

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ABSTRACT

Background. Camptothecin (CPT), an alkaloid isolated from the Chinese tree *Camptotheca acuminata,* exhibits very high anticancer activity. 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. The cellular target of CPT is topoisomerase I. 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. This means that CPT is toxic to those 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.

Materials and methods. We used fluorescence anisotropy measurements to determine the properties of binding to membranes and proteins of two anticancer agents: 7-tert-butyldimethylsilyl-camptothecin and 7-tert-butyldimethylsilyl- 10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin. The samples of CPT, 1Si-CPT and 2Si-CPT were obtained from the laboratory of biotechnology, College of Pharmacy, University of Kentucky, Lexington, USA. 2 mM stock solutions of CPT, 1Si-CPT and 2Si-CPT were prepared in DMSO. As model membranes, small unilamelar liposomes formed by DMPC and DMPG lipids were used.

Results. Fluorescence anisotropy measurements prove that this new camptothecin analogue exhibits desirable properties: high affinity of lactone form to membranes and low affinity of carboxylate form to HSA. Such properties should ensure high stability of this drug in physiological fluids, including blood. The second considered compound, 7-tert-butyldimethylsilyl- camptothecin, exhibits very high affinity of its lactone form to membranes, but its carboxylate form exhibits also high affinity to HSA. The latter property excludes this compound as a candidate to be a good anticancer drug.

Conclusions. Fluorescence anisotropy measurements show that the lactone forms of 1Si-CPT and 2Si-CPT exhibit a high affinity to membranes but only the carboxylate form of 2Si-CPT behaves in a desirable way in HSA solution i.e. it exhibits a low affinity to this protein. This means that 2Si-CPT could be an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical trials in cancer chemotherapy.

Key words: camptothecin, silatecan, fluorescence anisotropy, membranes binding, HSA affinity

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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). The chemical structures of both forms of camptothecins are presented in Figure 1 and Table 1. Only the lactone form is biologically active. The cellular target of 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 those 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 two hours, equilibrium is achieved and both forms coexist. The concentration of the lactone form in equilibrium is much smaller than the carboxylate form, and depends on the environment in which the CPT is incubated. This 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 possible application of CPT in cancer chemotherapy. The presence of human serum albumin (HSA) has a critical influence on the activity of camptothecin. Two hours after introducing camptothecin to an HSA solution, it has totally lost its anticancer activity. 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].

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. Obtaining and studying camptothecin analogues has become a major challenge for many laboratories. Hundreds of analogues have been synthesised. Some selected camptothecin analogues are set out in Table 1 and Figure 1. Two of them, topotecan and irinotecan, have been approved by the FDA (US *Food and Drug Administration)* and are used in the chemotherapy of many tumours. These CPT analogues exhibit stability in blood that is higher than that of CPT. The equilibrium concentration of the active form in blood is equal to 12% for topotecan and 21% for irinotecan [7]. The increase in equilibrium concentration of these analogues is the result of weak binding of their carboxylate forms with HSA [7, 8]. Silatecans (chemical structures of some these agents are set out in Table 1) are a new class of promising camptothecin analogues. Three silatecans: DB-67 (7-*tert*-butyldimethylsil-10-hydroxy-camptothecin), DB-174 (7-trimethylsilyl-ethyl-10-hydroxy-camptothecin), and 7-TMSiE-10-A-CPT (7-trimethylsilyl-ethyl-10-amino-camptothecin) were the subjects of our previous studies [9–13]. Each of these three previously studied agents fulfills the criterion of desirable CPT analogue. Carboxylate forms of the above silatecans exhibit weak affinity to HSA, and their lactone forms exhibit a very high affinity to membranes.

Two other silatecans: 7-tert-butyldimethylsilyl-camptothecin and 7-tert-butyldimethylsilyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy- camptothecin, are the subject of this paper. We will call these CPT analogues '1Si-CPT' and '2Si-CPT', respectively. The chemical structures of these compounds are presented in Figure 2. The first of them (1Si-CPT), as can be seen in Table 1 and Figure 2, is obtained by substitution of hydrogen atom in position 7 of CPT molecule with the group $Si(CH_3)_2C(CH_3)_3$. This is the same position 7 as in DB-67, widely studied in previous papers. Some selected results of preliminary studies of this compound have been presented [5, 14, 15].

The second compound (2Si-CPT) is obtained by substitution of hydrogen atoms in position 7 by $Si(CH_3)_2C(CH_3)_3$ group (the same as in 1Si-CPT and DB-67) and in position 10 by $O_2CNC_5H_4NC_5H_5$. The latter is the same as 10 position group of irinotecan, the first CPT analogue approved by the FDA as an anticancer drug.

The results of the studies of the behaviour of these two agents, 1Si-CPT and 2Si-CPT, in the presence of model membranes — liposomes and in HSA solutions are presented in the next parts of this paper.

Materials and methods

Materials

The samples of camptothecin (CPT), 7-tert-butyldimethylsilyl-camptothecin (1Si-CPT) and 7-tert-butyldimethylsilyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin (2Si-CPT) were obtained from the

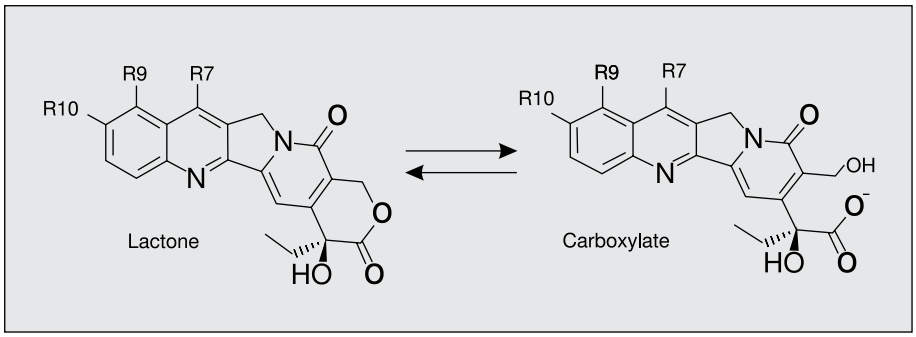

Figure 1. Chemical structure of camptothecin and its analogues

Table 1. Structure of camptothecin and its analogues

Compound	R_7	R_9	R_{10}
Camptothecin (CPT)	H	H	H
Topotecan (TPT)	H	$CH_2N(CH_3)_2$	OH
Irinotecan (CPT-11)	C_2H_5	H	$O_2CNC_5H_4NC_5H_5$
DB-67	$Si(CH_3)_2C(CH_3)_3$	H	OH
DB-174	$(CH_2)_2Si(CH_3)_3$	H	OH
7-TMSiE-10-A-CPT	$(CH_2)_2Si(CH_3)_3$	H	NH_2
1Si-CPT	$Si(CH_3)_2C(CH_3)_3$	H	H
2Si-CPT	$Si(CH_3)_2C(CH_3)_3$	H	$O_2CNC_5H_4NC_5H_5$

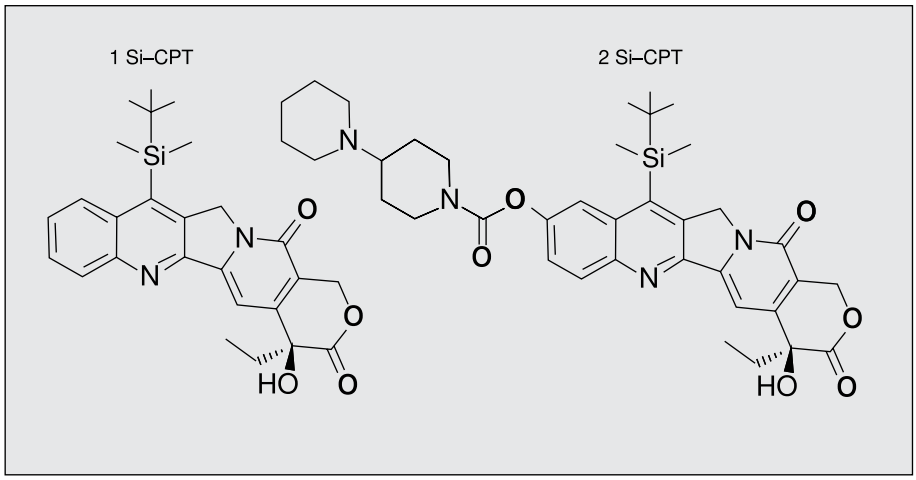

Figure 2. Chemical structure of 1Si-CPT (7-tert-butyldimethylsilyl-camptothecin) and 2Si-CPT (7-tert-butyldimethylsilyl--10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin)

laboratory of biotechnology, College of Pharmacy, University of Kentucky, Lexington, USA. 2 mM stock solutions of CPT, 1Si-CPT and 2Si-CPT were prepared in DMSO (dimethylsulfoxide, C_2H_6OS). Such stock solutions contain only a pure lactone form. 1 mM stock carboxylate solutions were obtained by diluting stock lactone solutions in phosphate buffered saline (PBS) at pH 12 in a volume ratio of 1:1.

As model membranes, small unilamelar liposomes formed by DMPC (dimyristoyl- phosphatidylcholine) and DMPG (dimyristoyl phosphatidylglycerol) lipids were used. DMPC and DMPG were 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 of 7.4 and temperature of 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 the addition of the stock lipid suspensions to PBS at pH 7.4.

Human serum albumin (95–97%) was purchased from Sigma-Aldrich (USA). 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 concentrations of CPT, 1Si-CPT and 2Si-CPT in the final samples were equal to 2 μM. The desired concentrations were obtained by the addition of the stock solutions of camptothecins under consideration to PBS at pH of 7.4 or to DMPC or DMPG liposomes suspensions at pH of 7.4, or to HSA solutions also at pH of 7.4.

Method of anisotropy measurement

Fluorescence light of solution is always depolarised [16]. For characterisation of depolarisation degree of fluorescence light, the fluorescence anisotropy r, defined as

$$r = \frac{I_V - GI_H}{I_V + 2GI_H}$$

[16], is most often used. I_V and I_H are the fluorescence intensities of the vertically and horizontally polarised emission, when the sample is excited by vertically polarised light.

$$G = \frac{S_V}{S_H}$$

is the ratio of the sensitivities of the detection system for vertically and horizontally polarised light. On the basis of fluorescence anisotropy measurements in dependence on concentration of lipids forming the model membranes, the association (binding) constants of drugs to membranes were determined. The association constant is determined by the formula: [17]

$$K = \frac{A_B}{A_F L} = \frac{F_B}{F_F L}, \quad (1)$$

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

The association constant K is a quantitative measure of affinity of drug to membranes. The procedure for determining the association constant on the basis of fluorescence anisotropy measurements has been described in detail in previous papers [10, 12].

Instrumentation

A PTI (Photon Technology International, Birmingham, NJ, USA) spectrofluorometer was used to record steady-state emission fluorescence spectra and to measure steady-state fluorescence anisotropy. To obtain the fluorescence spectra of CPT, 1Si-CPT and 2Si-CPT, lights at 374, 377 and 376 nm respectively were used for excitation. Measurements of fluorescence anisotropy were performed with the instrument in the 'L-format' using excitation at 375 nm and 420 nm long-pass filters on the emission channel. Using a long pass filter on the emission channel ensures the separation of fluorescence from scattered light. The temperature of the samples was kept constant (37°C) using the ultrathermostat TW2.03 (ELMI).

Results and discussion

Figure 3 presents the steady-state fluorescence spectra of lactone forms of 1Si-CPT, 2Si-CPT and comparable spectra of CPT. These compounds were diluted in PBS and in suspensions of liposome formed from DMPC and DMPG lipids. The concentration of DMPC lipids, as well as DMPG lipids used for the preparation of liposomes, was 1.5 mM. Spectra presented in Figure 3 are pure fluorescence spectra – the contribution of scattered light was removed by subtracting the scattering spectrum of PBS (or PBS and liposomes) from the recorded fluorescence spectrum of drug (1Si-CPT, 2Si-CPT and CPT). Emission fluorescence spectra of 1Si-CPT diluted in PBS, as shown in Figure 3A, exhibited maximum at 450 nm, while for 1Si-CPT diluted in suspension of DMPC liposomes, as well as DMPG liposomes, an increase in fluorescence intensity and a shifting toward shorter wavelengths (maximum at 439 nm for DMPC and at 437 for DMPG) were observed. Figure 3B presents analogical spectra of lactone form of 2Si-CPT diluted in PBS and in 1.5 mM suspensions of DMPC as well as DMPG liposomes. 2Si-CPT diluted in PBS exhibits fluorescence with a maximum at 457 nm. After incubation of this compound in liposomes suspension, as with 1Si-CPT, an increase in fluorescence intensity and a shifting toward shorter wavelengths (maximum at 443 nm for both DMPC and DMPG liposomes) were observed. The change in fluorescence spectra of 1Si-CPT and 2Si-CPT, after their incubation in liposomes suspensions, may prove strong binding of these agents to liposomes. It should be noted that, unlike 1Si-CPT and 2Si-CPT, the difference in fluorescence spectrum of the lactone form of CPT diluted in PBS and 1.5 mM DMPC liposomes suspension, as shown in Figure 3C, was not observed.

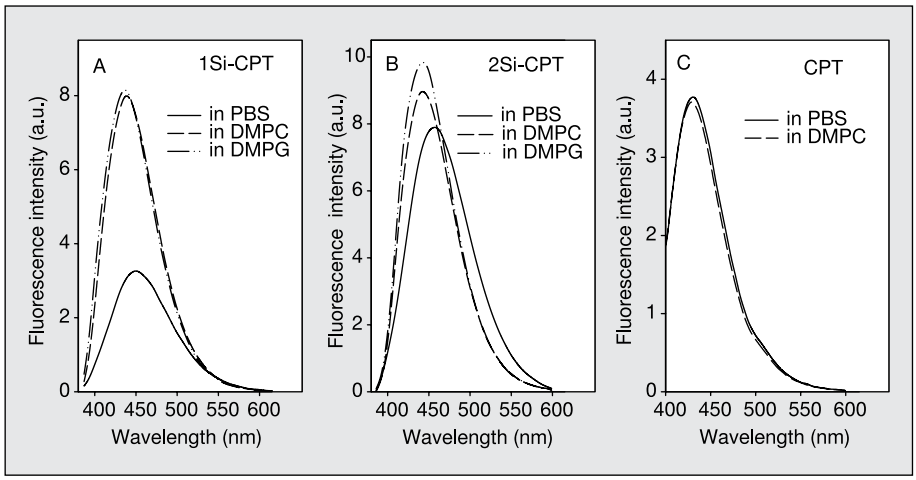

Figure 3. Steady-state fluorescence spectra of: (A) lactone form of 1Si-CPT diluted in PBS and in suspensions of DMPC and DMPG liposomes; (B) lactone form of 2Si-CPT diluted in PBS and in suspensions of DMPC and DMPG liposomes and; (C) lactone form of CPT diluted in PBS and in suspensions of DMPC liposomes. The concentration of liposomes was the same in each case (1.5 mM DMPC lipids and 1.5 mM DMPG lipids were used to prepare liposomes suspensions).

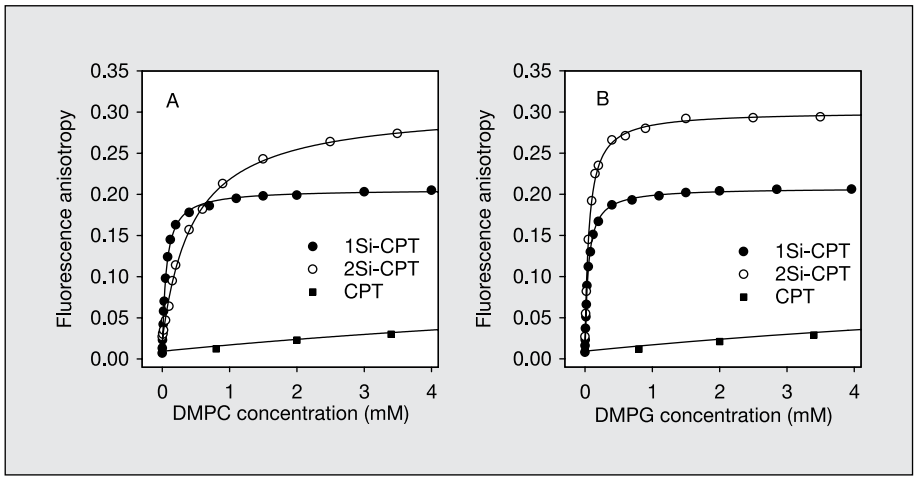

Figure 4. Steady-state fluorescence anisotropy of 1Si-CPT, 2Si-CPT and CPT depending on DMPC (A) and DMPG (B) liposomes concentration.

This proves that, unlike the two new camptothecin analogues considered here, CPT lactone exhibits poor binding to liposomes. Previous authors [17] have observed a 16 nm shifting of fluorescence spectrum of CPT after incubation of it in liposomes suspension, but they used very high concentrated liposomes (290 mM). For quantitative determination of 1Si-CPT and 2Si-CPT binding to liposomes, the fluorescence anisotropy as a function of lipids concentration was measured. The results of such measurements are presented in Figure 4. The fast increase of anisotropy with increasing lipids concentration was observed for 1Si-CPT and 2Si-CPT. This means that these two compounds bind very easily to membranes. The changes in fluorescence anisotropy of the parent drug CPT over lipid concentration are also for comparison depicted in Figure 4. The rate of fluorescence increase in this case is weak, which means that CPT binds poorly to DMPC and DMPG liposomes. However, the new CPT analogues under consideration here generally exhibit a high rate of anisotropy increase, although differences in behaviour of 1Si-CPT and 2Si-CPT in the presence of liposomes exist: (1) Anisotropy of free 1Si-CPT (in PBS) is smaller than anisotropy of free 2Si-CPT; observed differences in the value of anisotropies may be the result of differences in molecular weight of these two compounds (MW of 1Si-CPT = 472, while MW of 2Si-CPT = 673), and/or may be the result of a difference in fluorescence life time

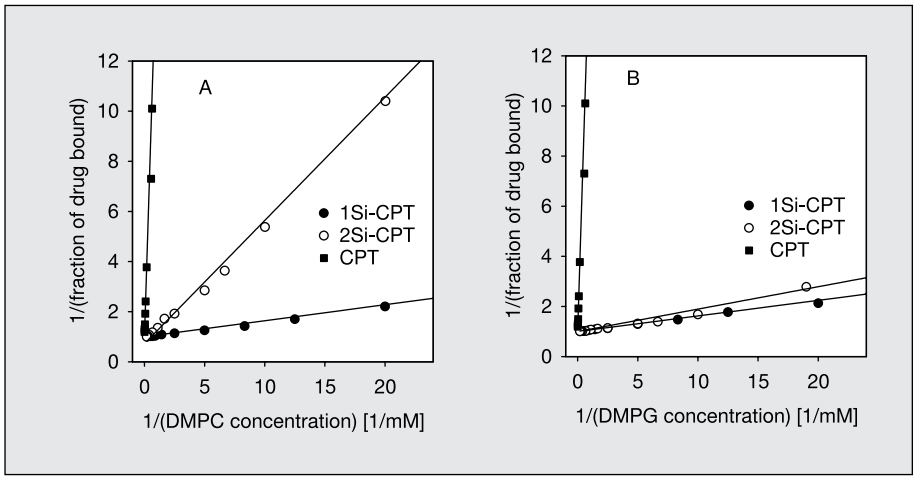

Figure 5. Double-reciprocal plots for the binding of lactone forms of 1Si-CPT, 2Si-CPT and CPT to the DMPC (A) and DMPG (B) liposomes

Table 2. Association constants of lactone forms of camptothecin, previously studied silatecans (DB-67, DB-174, 7-TMSiE-10-A-CPT) and new camptothecin analogues — silatecans 1Si-CPT and 2Si-CPT to DMPC and DMPG liposomes determined by fluorescence anisotropy method

Compound	K_{DMPC} (M^{-1})	K_{DMPG} (M^{-1})	References
CPT	70 ± 15	–	[10]
	100 ± 15	100 ± 15	[5, 12]
DB-67	4,500 ± 900	3,000± 600	[5, 12]
	6,600 ± 1,000	–	[10]
DB-174	9,000 ± 1,000	6,600 ± 800	[12]
7-TMSiE-10-A-CPT	5,700 ± 800	–	[13]
1Si-CPT	15,500 ± 800	16,000 ± 800	[5, 14, 15]
	15,000 ± 1,000	**16,000±1,000**	–
2Si-CPT	**2,500 ± 500**	**12,000 ± 1,000**	–

of these two compounds [16]; (2) The rate of anisotropy rise of 1Si-CPT in DMPC liposomes suspension is higher than the rate of anisotropy rise of 2Si-CPT; this indicates that 1Si-CPT exhibits a higher affinity to DMPC liposomes than 2Si-CPT does; (3) The rates of anisotropy rise of 1Si-CPT and 2Si-CPT in DMPG liposomes suspension are comparable; we may then suppose that the affinities of 1Si-CPT and 2Si-CPT to DMPG are comparable; (4) Saturation fluorescence anisotropy value in both DMPC and DMPG liposomes is smaller for 1Si-CPT (~ 0.20) than for 2Si-CPT (~ 0.30); this could be explained as a result of differences in the sizes of the complexes liposome – bound drug (complexes 'liposome - bound 1Si-CPT' most probably are smaller than complexes 'liposome — bound 2Si-CPT') and/or a difference in fluorescence life time of these two compounds [16].

On the basis of experimentally determined anisotropy using previously described methods, the concentrations of free and bound drugs in liposomes suspension were calculated and then the double reciprocal plots were drawn. They are shown in Figure 5. The slope of lines fitted to experimental values determined the inverse of association constants (1/K). The obtained parameters are summarized in Table 2. The value of association constants of CPT and previously studied silatecans are also set out in Table 2.

The results set out in Table 2 show that 1Si-CPT exhibits the highest affinity to liposomes, and values of this affinity (association constants) are almost the same for DMPC and DMPG liposomes. They are at least 150-fold higher than the association constants of camptothecin. 2Si-CPT exhibits also a high affinity to liposomes, but not as high as 1Si-CPT. Additionally, in the case of 2Si-CPT, a difference in affinity to DMPC and DMPG exists. The DMPC molecule is electrically neutral, while DMPG is negatively charged. Molecules of lactone forms of CPT, 1Si-CPT, DB-67, DB-174, and 7-TMSiE-10-A-CPT are electrically neutral, and therefore for each of these drugs affinity to DMPC liposomes is comparable with affinity to DMPG liposomes. Substituent group at position 10 in 2Si-CPT is positively charged and therefore this drug

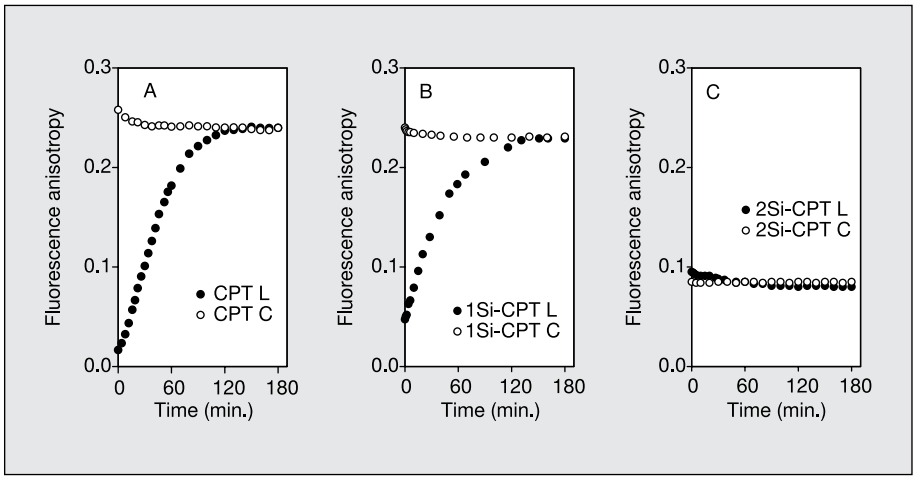

Figure 6. Time evolution of steady-state fluorescence anisotropy of lactone and carboxylate forms of CPT (A), 1Si-CPT (B) and 2Si-CPT (C) in 10 mM HSA solutions.

exhibits a higher affinity to negatively charged DMPG liposomes than to neutral DMPC liposomes.

The results of measurements of affinity of 1Si-CPT and 2Si-CPT to liposomes show that the two camptothecin analogues under consideration have promising properties. High lipophilicity of lactone forms of camptothecin analogues however, is not sufficient to assure high stability in blood and physiological fluids. The second desired property of camptothecin analogues is low affinity of their carboxylate forms to HSA. The results of studies by steady-state fluorescence anisotropy measurements of the behaviour of carboxylate and lactone forms of CPT, 1Si-CPT and 2Si-CPT in HSA solution, are presented in Figure 6. Figure 5A shows the steady-state fluorescence anisotropy of the carboxylate form of CPT in HSA solution is large and does not change over time, which proves that the molecules of CPT carboxylate are bound to big HSA molecules. CPT lactone just after incubation in HSA solution exhibits low steady-state anisotropy, which 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 two hours, as shown in Figure 6A, the anisotropy approaches that obtained for the pure carboxylate form. This means that after such time, the lactone form is converted almost totally to inactive carboxylate. Figure 6B shows that 1Si-CPT behaves in an HSA solution similar to CPT. Fluorescence anisotropy of the carboxylate form of 1Si-CPT is high, which means that this compound exhibits a high affinity to HSA. On the other hand, anisotropy of the lactone form is small, but it converts into carboxylate, which immediately binds to HSA, which results, as shown in Figure 6B, in an increase in fluorescence anisotropy. Figure 6C shows that 2Si-CPT, compared to CPT and 1Si-CPT, behaves totally differently in an HSA solution. Fluorescence anisotropy of both forms of this analogue is rather small, which means that both forms of this agent exhibit a low affinity to HSA. This is a desired property. Low affinity of the carboxylate form of 2Si-CPT to HSA, together with high affinity of its lactone form to membranes, ensures a sufficiently high stability of its lactone form in blood and in physiological fluids. Unlike 2Si-CPT, 1Si-CPT, despite its very high affinity to membranes, behaves undesirably in HSA solutions, and it may be supposed that this compound will behave badly in blood and in physiological fluids i.e. its lactone form will be transformed to inactive carboxylate form and this will be an irreversible transformation. It can therefore be supposed that after about 2–3 hours, the concentration in blood of the active lactone form of 1Si-CPT drops to zero. Because of such undesirable properties, 1Si-CPT will probably be excluded from further studies. The second silatecan studied here, 2Si-CPT, because of its desirable properties will be attached to a group of promising anticancer drugs such as DB-67, DB-174, and 7-TMSiE-10-A-CPT as a good candidate for further studies, including studies on animals.

Conclusion

Fluorescence anisotropy measurements show that the lactone forms of 1Si-CPT and 2Si-CPT exhibit a high affinity to membranes (a desirable property of camptothecin analogues), but only the carboxylate form of 2Si-CPT behaves in a desirable way in HSA solution i.e. it exhibits a low affinity to this protein. This means that 2Si-CPT could be an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical trials in cancer chemotherapy. Despite the very high

affinity of the lactone form of 1Si-CPT to membranes, the carboxylate form of this CPT analogue exhibits a high affinity to HSA, and therefore this compound will most probably be excluded from further studies. The high affinity of carboxylate form of 1Si-CPT to HSA proves that its stability in blood will be low.

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