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Camptothecins affinity to HSA and membranes determined by fluorescence anisotropy measurements

STEFAN KRUSZEWSKI

Department of Medical Physics, Ludwik Rydygier Medical University, ul. Jagiellońska 13, 85–067 Bydgoszcz, Poland.

Institute of Mathematics and Physics, University of Technology and Agriculture, ul. Kaliskiego 7, 85–796 Bydgoszcz, Poland.

THOMAS G. BURKE

Division of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY, USA.

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

1. Introduction

Camptothecin (CPT) as 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). Only the lactone form is biologically active.

The cellular target of the CPT is topoisomerase I, a nuclear enzyme responsible for DNA replication. CPT interacts only with 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 cells that are undergoing DNA synthesis. Rapidly replicating cells, such as a cancerous cell, spend more time in the S-phase relative to healthy tissues and therefore cancerous cells 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, continuous exposure to this drug must be maintained in order to achieve optimum therapeutic efficacy. Unfortunately, there is some difficulty for CPT to fulfil this requirement. Under physiological conditions (pH 7.4) CPT hydrolyses and converts to the "ring opened" inactive carboxylate form [2]. After about 2 hours an equilibrium is achieved and both forms coexist. The concentration of the lactone form is much smaller than the carboxylate form and it 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. The low concentration of the lactone form under physiological conditions seriously limits the possibility of CPT application in cancer chemotherapy. Efforts to find more stable CPT analogues have been undertaken. The new analogues were obtained by modifying the camptothecin molecule. The fluorescence spectroscopy methods were applied to determine the biophysical properties of these new camptothecin analogues. Among many new analogues, one of them, silatecan DB-67 (10-hydroxy-7-tert-butyldimethylsilylcampthothecin) exhibits impressively high blood stability. It is now the most promising camptothecin analogue. It is an excellent candidate for further *in vivo* pharmacological studies, and most probably for clinical trials in cancer chemotherapy.

2. Materials and methods

The purity of samples of camptothecin and topotecan (obtained from the National Cancer Institute, Betheseda, MD, USA), SN-38 (Yakult Honsha Tokio, Japan), and Silatecan (laboratories of Prof. Dennis Curran, University of Pittsburgh, USA) was greater than 98%. 2 mM stock solutions of the drugs were prepared in dimethylsulfoxide. For chromatographic and fluorescence measurements the stock solution was added to various physiological fluids. The final drug concentration was 1 μM. The red blood cells and human plasma were obtained from the Kentucky Red Cross (Lexington, KY, USA), HSA from Sigma Chemical Co. (St. Louis, MO, USA) and lipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) from Avanti Polar Lipids (Alabaster, AL, USA).

The hydrolysis kinetics of camptothecin and that of its analogues in various physiological fluids were determined by high-performance liquid chromatography (HPLC) using the Waters Alliance 2690 Separations Module with a fluorescence detector. Separation of the lactone and carboxylate forms was achieved using an isocratic mobile phase consisting of varying mixtures of acetonitrile and a 2% triethylamine acetate buffer (pH 5.5).

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 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 was used [4]. As model membranes, small unilamelar liposomes were used. 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

Fluorescence light emitted by a solution is always depolarized. For the characterization of depolarization of fluorescence light the anisotropy r defined as $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ is most often used. $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the vertically ($\parallel$) and horizontally ($\perp$) polarized emission, when the sample is excited with vertically polarized light. Maximal possible values of anisotropy r_0 of fluorescence light emitted by a solution is always smaller than 0.4 [5]. This is a result of photoselection and internal conversion [5]. Rotation of fluorescent molecules during the life of an excited state causes the further decrease in anisotropy according to the relation $r(t) = r_0 \exp(-t/\Theta)$, where Θ is a rotational correlation time [5]. Anisotropy decays provide information about the rate of rotation of fluorescent molecules. For small molecules value of Θ is small, *i.e.*, fast anisotropy decay occurs. On the other hand, if fluorophore is bound to big molecules (*e.g.*, HSA) or membranes, value of Θ is large and slow anisotropy decay occurs.

Anisotropy decay can only be recorded using pulse excitation. By continuous excitation one can determine the steady-state anisotropy. It depends on the fluorescence lifetime t and rotational correlation time as follows [5]:

$$r = \frac{r_0}{1 + \frac{\tau}{\Theta}}.$$

From this formulae it follows that for small, fast rotating fluorofores ($\Theta < \tau$) r is small, close to zero, while for big, slow rotating fluorofores ($\Theta > \tau$) r is large, close to r_0.

Free and bound drugs exist in the suspension of liposomes or membranes. The association constant [6]

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

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

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

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, one can determine using Eq. (2) the association constant K. The fluorescence anisotropy methods can be used for determining the concentration of bound drugs in relation to the lipid concentration. The total fluorescence anisotropy r observed for a mixture of fluorophores [5] 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 Eq. (3) 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 anisotropies r_F and r, estimated values of r_B and Eq. (5) the fraction of the bound drug F_B can be calculated. By making the graph $1/F_F$ in dependence on $1/L$, *i.e.*, double-reciprocal plot, the association constant K according to Eq. (2) can be determined.

4. Results and discussion

4.1. Kinetics of camptothecin hydrolysis

Figure 1 presents the structure of the lactone and carboxylate form of camptothecin. The lactone form is electroneutral and is stable in acid environment at pH < 5.5. In a base environment the lactone ring (ring E) opens and CPT transforms into a negatively charged carboxylate form which is stable at pH > 9. For therapeutic applications it is important to know how camptothecin behaves under physiological conditions, *i.e.*, at pH 7.4.

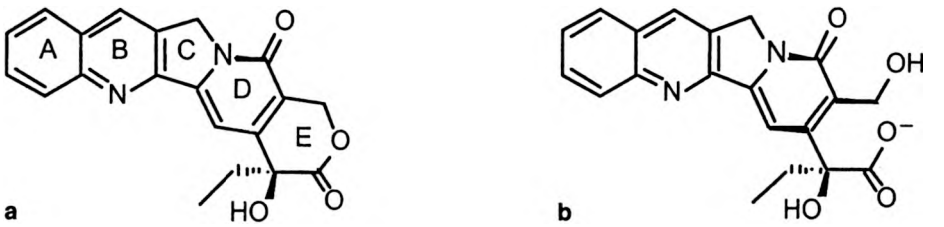


Fig. 1. Structures of the lactone (**a**) and carboxylate (**b**) forms of camptothecin.

Figure 2**a** presents the stability of CPT in physiological fluids obtained by HPLC separation of the lactone and carboxylate forms. It follows from this figure that in each fluid the concentration of the lactone form decreases over time. This decrease is extremely fast in the human plasma and in the HSA (plasma protein) solution. The slowest decrease in the lactone form is observed in the red blood cells (RBC) suspension. This is caused by the reversible binding of CPT to the RBC membrane. The CPT bound to membranes does not hydrolyse. On the other hand, the carboxylate form binds irreversibly to HSA, and therefore in plasma or in HSA solutions the continuous hydrolysis leads to the almost total disappearance of the biologically active lactone form [7].

Time resolved fluorescence anisotropy decays of lactone and carboxylate forms in HSA solutions proved that big differences exist in the behaviour of these forms [8]. Anisotropy of the lactone form decays very fast (rotational correlation time $\Theta_1 = 90$ ps). This fast anisotropy decay informs us that fluorophores are free and rotate

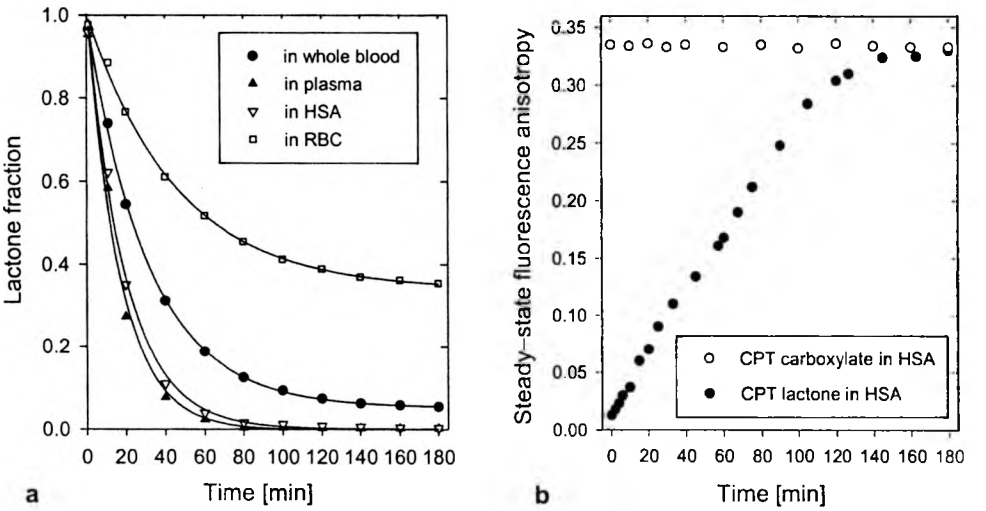


Fig. 2. Kinetic evaluation of the rate of lactone ring opening for camptothecin in whole human blood, human plasma, 10 μM solution of HSA and plasma-free red blood cells (**a**); time evolution of steady-state fluorescence anisotropy of lactone and carboxylate forms of CPT in 10 μM HSA solution (**b**).

very fast. It is proof of the poor affinity of the lactone form to HSA. The carboxylate form behave totally differently in the presence of HSA. A slow anisotropy decay is observed. The rotational correlation time Θ_2 = 16 ns is 180 times higher than the analogical time recorded for the lactone form. This slow anisotropy decay informs us that the fluorescent molecules of the carboxylate form are bound to the big slow rotating molecules of HSA.

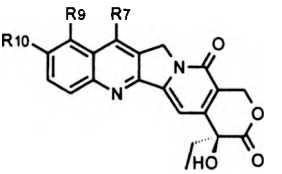
Steady-state anisotropy measurements also provide useful information about differences in the behaviour of lactone and carboxylate forms in the presence of HSA. Figure 2**b** presents the time dependence of steady-state anisotropy of lactone and carboxylate forms of CPT in HSA solution. It shows that the steady-state fluorescence anisotropy of the carboxylate form in HSA solution is large (0.33) and does not change over time. The large value of the steady-state anisotropy proves that the molecules of CPT carboxylate are bound to big HSA molecules. CPT lactone just after 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 bind to HSA. After about 2 hours, as is shown in Fig. 2**b**, the anisotropy approaches to that obtained for the pure carboxylate form. This means that after such a time the lactone form is converted almost totally to inactive carboxylate.

On the basis of the results presented in Fig. 2**a** and **b** one can conclude that two opposing processes occur in the blood: reversible binding of the lactone form to RBCs improves CPT stability, and irreversible binding of the carboxylate form to HSA decreases substantially the concentration of the lactone form. The strong affinity of the carboxylate form to HSA is disadvantageous. Carboxylate CPT molecules which are bound to HSA cannot convert back into lactone. Only free carboxylate in acid and neutral environments convert back into lactone. At pH 7.4 in fluids free of HSA after about 2–3 hours an equilibrium is achieved and both forms coexist, however the concentration of the lactone form is about 6 times less than that of carboxylate. In such conditions, permanent and reversible processes of hydrolysis and lactonization occur and the active lactone form can exist for a long period of time. This is impossible in the presence of HSA. Because the carboxylate form binds irreversibly to HSA, lactonization becomes impossible and after a few hours the active lactone form disapears totally. The goal of many research groups was to discover camptothecin analogues that display improved human blood stability. The new analogues should: retain anticancer properties, exhibit the high affinity of the lactone form to cell membranes, and exhibit a poor affinity of the carboxylate form to HSA.

4.2. Properties of some new camptothecin analogues

It has been proved that camptothecin derivatives obtained by modification of CPT molecules at positions 7, 9 or 10 retain the anticancer activity [2]. Some camptothecins obtained by such modifications are presented in Tab. 1. Topotecan (TPT) and SN-38 (7-ethyl-10-hydroxycamptothecin) are approved as anticancer drugs and are

T a b l e 1. Structure of camptothecin and its analogues.



Compound	R7	R9	R10
Camptothecin	H	H	H
Topotecan	H	$CH_2N(CH_3)_2$	OH
SN-38	C_2H_5	H	OH
DB-202	$Si(CH_3)_2C(CH_3)_3$	H	H
DB-67	$Si(CH_3)_2C(CH_3)_3$	H	OH

used in chemotherapy of many human cancers [7]. These compounds exhibit substantially higher stability in the human blood in comparison to camptothecin. The concentrations of the lactone form of TPT, and SN-38 in whole blood in equilibrium are 12 and 20%, respectively [5]. The improved blood stability of these congeners is a result of the poor affinity of their carboxylate form to HSA. Stability in blood is not only determined by binding to HSA. Affinity to membranes also strongly influences the stability of the lactone form. The association constant K [6] is a quantitative measure of drug affinity to membranes. Small unilamellar DMPC and DMPG liposomes were used as model membranes for determining K.

The fluorescence light emitted by free drugs is characterized by small anisotropy (small fluorophores rotate fast and the fluorescence light is almost totally depolarized).

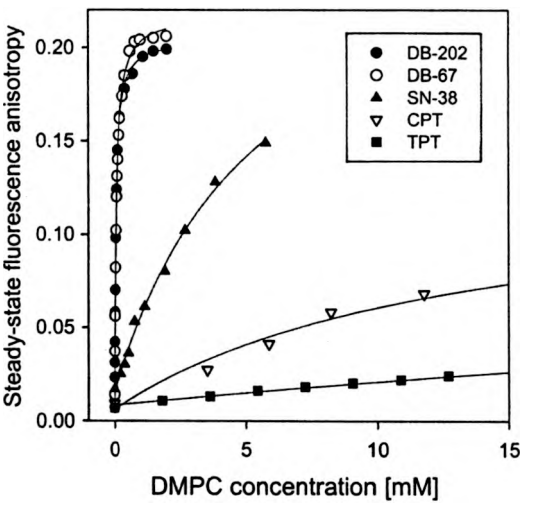


Fig. 3. Steady-state fluorescence anisotropy of camptothecin and its analogues depending on DMPC liposomes concentration.

On the other hand, the light emitted by the drug bound to liposomes (membranes) has a substantially higher anisotropy, because of slower rotation of the bound fluorophores.

Figure 3 presents the results of steady-state anisotropy measurements of camptothecin and some of its analogues as a function of lipids concentration. 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 analogues. The slowest increase is observed for topotecan. This means that this compound exhibits the poorest affinity to membranes. An extremely fast increase in anisotropy is observed for silatecans: DB-67 (10-hydroxy-7-tert-butyldimethylsilylcampthothecin) and DB-202 (7-tert-butyl-dimethylsilylcampthothecin). They are the new promising camptothecin derivatives. The extremely fast increase in anisotropy with increasing lipids concentration means that these compounds bind very easily to membranes.

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 association constants K were determined from the double reciprocal plots. They are presented in Tab. 2. These values of K explain why topotecan, despite the small affinity of its carboxylate form to HSA [8], exhibits relatively low blood stability. This is a result of the poor affinity of topotecan to RBC membranes. Silatecans – DB-67 and DB-202 manifest very high affinity to membranes, about 45 and 150 times higher,

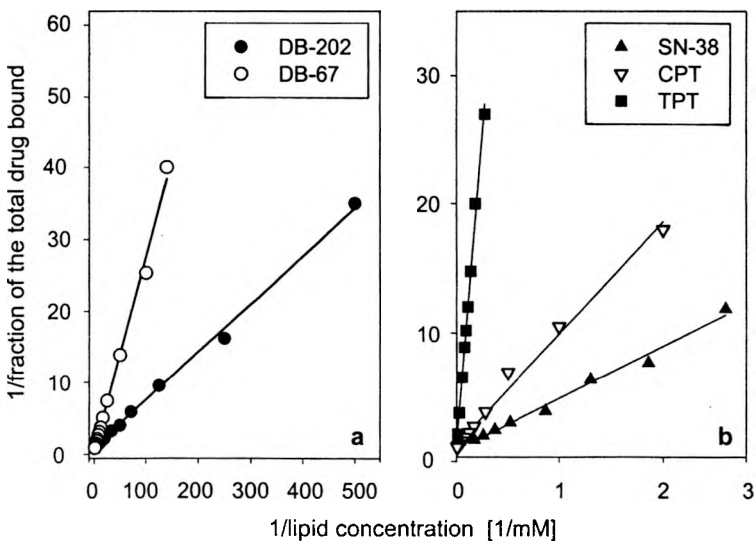


Fig. 4. Double-reciprocal plots for the binding of DB-202 and DB-67 (**a**) and the less lipophilic SN-38, CPT, TPT (**b**) to the DMPC small unilamellar liposomes.

T a b l e 2. 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	100
Topotecan	10	50
SN-38	260	160
DB-67	4 500	3 000
DB-202	15 500	16 000

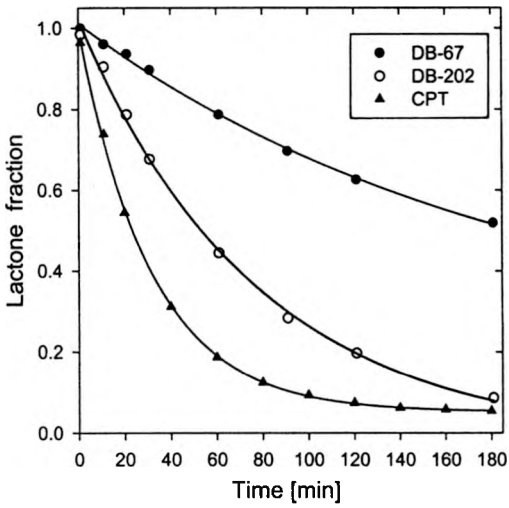


Fig. 5. Kinetic evaluation of the rate of lactone ring opening for silatecans (DB-67 and DB-202) and camptothecin in whole human blood determined using HPLC methods.

respectively, than camptothecin. If their carboxylate forms display low affinity to HSA, they have the promise of becoming very stable anticancer drugs.

Figure 5 shows that DB-67 is indeed very stable in human blood. Unfortunately, DB-202, although it shows 3 times as high affinity to membranes as DB-67, exhibits rather poor blood stability. It is comparable with the stability of camptothecin. The poor blood stability of DB-202 is most probably the result of the high affinity of its carboxylate form to HSA. Because of its low blood stability, DB-202 was excluded from further studies. Efforts were concentrated on the study of DB-67 [9]–[11].

The high lipophilicity of the lactone form of DB-67 and the poor affinity of its carboxylate form to HSA make this drug very promising as a topoisomerase I inhibitor. The poor water solubility of this drug is a disadvantage of this agent. Delivering it as a liposomal formulation is the best resolution of its water insolubility [7]. Additionally, the stability of its lactone form is improved in this way.

5. Conclusions

Fluorescence anisotropy measurements and HPLC study show that silatecan DB-67 is one the most promising camptothecin analogue. It exhibits impressive blood stability that is a result of the high affinity of the lactone form to membranes and the poor affinity of its carboxylate form to HSA. It is an excellent candidate for further in vivo pharmacological studies, and most probably for clinical trials in cancer chemotherapy.

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