Determination of the Protein–Binding Properties of Camptothecins by Means of Optical Spectroscopy Methods

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Optical spectroscopy methods are widely used in studies of drugs. The affinity of camptothecins — anticancer agents — to human serum albumin (HSA) was determined in this work. Camptothecins (CPTs) exist in two forms: active lactone and open ring inactive carbonylate. In blood, the hydrolysis process of lactone form occurs which leads to deactivation of CPTs. Research is being done on biophysical properties of synthesized CPT compounds, in particular on binding to albumin. The affinity to plasma proteins is an important determinant of stability of CPTs in blood. The following analogues of CPT were tested in this paper: irinotecan, SN-38, topotecan, and 9-amino camptothecin. Using the method of fluorescence anisotropy measurement, the association constants of the studied compounds to HSA were determined. The authors attempted to determine the deactivation rate of topotecan in HSA solution using Principal Component Analysis and Factor Analysis of absorption spectra recorded during hydrolysis process of lactone form.

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1. Introduction

The methods of optical spectroscopy are useful tools in specifying biophysical properties of fluorescent compounds used in therapy. The analysis of shapes of absorption and fluorescence spectra and fluorescence anisotropy measurements of fluorophores both free and bound to proteins provide useful information about the binding and allow to determine quantitatively the degree of binding drugs to albumin [1]. The affinity of camptothecins to human serum albumin was determined in this work. Camptothecins (CPTs) are derivatives of a plant alkaloid which exhibit strong anticancer properties. CPT was isolated in the 1960s from a tree Camptotheca acuminata [2]. Interest in camptothecin increased in the 1980s and 90s, after the discovery of its mechanism of action based on inhibition of nuclear enzyme topoisomerase I which is involved in DNA replication [3]. This interest resulted in the synthesis of many analogues, two of which were introduced as a medicine in anticancer therapy (topotecan, irinotecan), while some other are at the stage of clinical trials [4]. Camptothecins exist in two forms: lactone, stable at pH<5.5, and open ring carbonylate, stable at pH>9 (Table I). Only the lactone form is active with respect to inhibition of topoisomerase I. In blood and fluids at physiological pH, the α-hydroxy β-lactone ring moiety hydrolyzes which leads to deactivation of CPTs [5]. The process of deactivation is accelerated in a solution containing albumin. It is caused by irreversible binding of carbonylate form to this protein and leads to rapid loss of anticancer properties of camptothecins. On the other hand, lactone form molecules of CPTs bound to membranes do not hydrolyze [6-8]. Taking into account these two points one can conclude that the best stability in blood have these analogues of CPT which exhibit high affinity of lactone form to cell membranes and low affinity of carbonylate form to human serum albumin. The aim of this study was to determine the affinity for albumin of the following camptothecin analogues: irinotecan (CPT-11), SN-38, topotecan (TPT), and 9-amino camptothecin (9-amino-CPT). Chemical structures of these compounds are presented in Table I. The 7-ethyl-10-hydroxy camptothecin (SN-38) is the active component of irinotecan.

The analysis of changes in the shapes of fluorescence and absorption spectra recorded during lactone ring opening allows to study the rate of hydrolysis. This approach is based on the differences in shapes of fluorescence or absorption spectra of the lactone and carbonylate forms of CPTs. Because of conversion of the lactone form into the carbonylate during the hydrolysis process, both fluorescence and absorption spectra evolve [9]. The set of evolving fluorescence/absorption spectra is analyzed using two methods of multivariate analysis, i.e. principal component analysis (PCA) and factor analysis (FA) [10, 11]. This paper presents the results of application of PCA and FA to sets of absorption spectra of camptothecin and topotecan recorded during hydrolysis in PBS and HSA solution.

The method of steady-state fluorescence anisotropy measurement enables to determine the quantitative measure of drug affinity to albumin, i.e. the association constant. The base for application of this method is that for a small, fast rotating fluorophore (e.g. free CPTs molecule), steady-state anisotropy is small, close to zero. In turn for a big, slow rotating fluorophore or a molecule

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of fluorphore small but bound to a big molecule of protein (e.g. CPTs molecule bound to albumin), the steady-state anisotropy reaches large value, close to the limiting anisotropy [1, 7, 12, 13]. The following chapters present the results of application of these methods to tested compounds. The values of association constants calculated for the studied compounds are compared with results obtained by the ultrafiltration method.

2. Experimental

2.1. Chemicals

Samples of camptothecin (CPT) were obtained from the laboratory of biotechnology, College of Pharmacy, University of Kentucky (Lexington, USA), and 7-ethyl-10-hydroxycamptothecin (SN-38) from Tigen Pharmaceuticals Inc. (USA). Samples of topotecan (TPT) and irinotecan (CPT-11) were purchased from Sigma-Aldrich (St. Louis, USA) and those of 9-amino camptothecin (9-amino-CPT) from Santa Cruz Biotechnology, Inc. (Dallas, USA). To prepare 2 mM stock lactone solutions, each camptothecin sample was dissolved in dimethylsulfoxide (DMSO). 1 mM stock solutions of the carboxylate forms were obtained by dilution of stock lactone solutions in phosphate buffered saline (PBS) at pH 10 in volume ratio of 1:1. Human serum albumin (97%) was purchased from Sigma-Aldrich (St. Louis, USA). The desired concentrations of HSA were obtained by adding the stock solution to PBS. The pH and temperature of these solutions were maintained at 7.4 and 37°C, respectively. In case of 9-amino-CPT for fluorescence measurements, the pH of solution was maintained at 3. For recordings of absorption spectra, the concentration of drug and HSA was 20 µM and 40 µM, respectively. For fluorescence anisotropy measurements, the concentration of camptothecins in the final samples was 2 µM. The desired concentration was obtained by adding the stock drug solutions to HSA solutions at varying concentration.

2.2 Principles of PCA and FA method

To determine the rate of camptothecins deactivation, the method of absorption spectra analysis was used. Conversion of the lactone to the carboxylate during hydrolysis caused changes in their absorption spectra. The set of absorption spectra recorded during opening of the lactone ring of CPT can be arranged as a data matrix X and analyzed using principal component analysis (PCA) [10]. The data matrix can always be written as a product of two other matrices X = CB, where C can be interpreted as a matrix of coefficients needed to reconstruct X from rows of B. Each row of B is termed a component (or a factor). PCA method allows to determine how many principal components (factors) are needed to reconstruct the data matrix X. There are many numerical criteria helpful in proper determination of the number of principal components. One of them is the inverse condition number (ICN) which compares the importance of given principal component with the importance of the first principal component. If the PCA method proves that only two principal components (factors) exist, they can be identified as the spectra of pure lactone and pure carboxylate forms of CPTs recorded just after introduction of the drug into solution. In such case, the factor analysis (FA) method allows to determine the contribution of each factor in each resultant spectrum [11]. The time dependence of the contributions obtained from the series of absorption spectra determines the deactivation rate of CPTs. The principles of PCA and FA methods implemented in MATLAB v.7.1.0.246 (The Math Works, Natick, MA, USA) for camptothecin studies were described previously [14].

2.3 Principle of fluorescence anisotropy method

The overall association constant is defined using formula $K = F_b/(F_L L)$, where $F_f$ and $F_b$ are the fractions of ligands free and bound to albumin, and $L$ represents the total concentration of HSA [7, 12, 13]. Because $F_f + F_b = 1$, one can obtain

$$1/F_b = 1 + 1/KL.$$  

The inverse of the fraction of bound ligands is a linear function of the inverse of HSA concentration. The $F_b$ fraction can be determined for all examined concentration L by fluorescence anisotropy measurements. The procedure was described previously [12]. Finally, by regression $1/F_b$ on $1/L$, the association constant can be calculated.

2.4 Instrumentation

A PTI (Photon Technology International, Birmingham, NJ, USA) spectrophotometer modified by the authors was used for the measurement of steady-state fluorescence anisotropy. Measurements of anisotropy were performed with the instrumentation in the "L-format" using excitation at 380 nm for all compounds, short-pass filter SPF 400 on the excitation channel and long-pass filters LPF 430 (for CPT, CPT-11, 9-aminoCPT) or LPF 490 (for TPT, SN-38) on the emission channel. The absorption spectra of camptothecin and topotecan were collected with a spectrophotometer Jasco V-550. The time needed for recording a single absorption spectrum was about 50 s. The spectra of the pure lactone and carboxylate forms were recorded immediately after mixing.
an adequate volume of the stock drugs solutions in PBS and HSA. For hydrolysis rate measurements, the single absorption spectrum sweeps were repeated every 3 minutes during the hydrolysis process. The temperature of the sample was kept constant using the ultrathermostat TW2.03 (ELMI). In the ultrafiltration method, the Cetrisfree UF Device (Merck Millipore, Billerica, MA USA) was used to separate free drug molecules in the albumin solution from the bound ones. The concentration of free molecules was determined by absorption measurements.

3. Results and discussion

3.1 Hydrolysis rate determination

Figure 1A presents absorption spectra of the lactone and carboxylate forms of camptothecin recorded in PBS buffer at pH 7.4. As one can see, the shapes of the spectra differ. During the hydrolysis process, the shape of lactone form spectrum changes over time and approaches the shape of the carboxylate form (Fig. 1B). The observed changes are the result of the conversion of the lactone form into the carboxylate one.

<table>
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<tr>
<th>PCs</th>
<th>ICN $\times 10^{-3}$</th>
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<tr>
<td></td>
<td>CPT in PBS</td>
</tr>
<tr>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>2.14</td>
</tr>
<tr>
<td>3</td>
<td>0.0122</td>
</tr>
<tr>
<td>4</td>
<td>0.0047</td>
</tr>
<tr>
<td>5</td>
<td>0.0017</td>
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</table>

The Principal Component Analysis of the set of the absorption spectra recorded during hydrolysis showed that only two principal components (factors) exist. The ICN numbers are collected in Table II. It is seen that the first two PCs are much more informative when compared to the third and subsequent ones. The set of absorption spectra was analyzed using FA, with the spectra of pure lactone and pure carboxylate forms as the factors. The obtained curve describing the rate of deactivation process is shown in Fig. 2.

![Fig. 2. Hydrolysis of lactones over time obtained by FA of series of absorption spectra of CPT in PBS and HSA.](image)

The analogous studies were carried out for camptothecin diluted in albumin solution. The Principal Component Analysis has revealed existence of two PCs and they were identified as the spectra of pure lactone and pure carboxylate forms of CPT recorded in HSA solution. The result of factor analysis is shown in Fig. 2. It is seen that about two hours after adding camptothecin into PBS or HSA solution, equilibrium is achieved and concentration of lactone form equals about 15% and 0% for PBS and HSA solution, respectively. The total decay of lactone form in albumin solution is caused by high affinity of carboxylate form of CPT to HSA. The lactone form, which exhibits low affinity for albumin, hydrolyzes and the carboxylate form binds immediately and irreversibly to albumin. Two kinds of fluorophores exist in the solution — free molecules of lactone form and bound molecules of carboxylate form.

Fig. 3 presents absorption spectra of topotecan recorded in PBS (A and B) and in HSA solution (C and D). It can be seen that both in PBS and albumin solution there are differences between the shapes of lactone and carboxylate form spectrum. Furthermore, the shapes of subsequent spectra recorded during hydrolysis process evolve. ICN values obtained from the Principal Component Analysis of the sets of absorption spectra (Table II) indicate that three PCs exist, both in PBS and HSA. The first three PCs are much more informative when
Fig. 3. (A,C) Absorption spectra of lactone and carboxylate form of TPT diluted in (A) PBS and in HSA (C). (B,D) Fragments of selected absorption spectra recorded during hydrolysis of CPT lactone in (B) PBS and in (D) HSA. The bottom spectra on panels B and D are of the pure lactone form, and the subsequent spectra were recorded 15, 90, 180, and 300 min after sample preparation. The top spectra are of the pure carboxylate form. Drug concentration was 20 \( \mu \text{M} \).

compared to the fourth and subsequent ones. Because the shapes of all three factors are unknown, the factor analysis cannot be applied and the deactivation curve cannot be determined.

3.2 Studies of CPTs binding with albumin

For quantitative determination of the studied drugs’ affinity to albumin, the steady-state fluorescence anisotropy over HSA concentration was measured. The results obtained for carboxylate and lactone forms of CPT, TPT, CPT-11, and SN-38 are shown in the Fig. 4 panels A and B. Using method described in [12], the double-reciprocal plots were drawn (C and D) and the association constants of drugs binding to albumin were calculated (Table III). The fluorescence anisotropy of 9-amino-CPT was measured at pH 3 because of low fluorescence of this compound at pH 7.4 [13]. Unfortunately, the addition of albumin causes a further fluorescence quenching which makes determination of affinity constant of 9-amino-CPT by the anisotropy measurements impossible.

The highest binding to albumin among the studied analogues is demonstrated by carboxylate form of CPT. The difference between the affinity of lactone and carboxylate form of CPT for albumin is so big that it is impossible to determine the affinity constant of CPT lactone to HSA using fluorescence anisotropy method. SN-38 binds to albumin to a moderate degree. Its association constant is large in comparison to TPT and CPT-11. However, carboxylate form of SN-38 binds HSA weaker than its lactone form. Its affinity constant is two times smaller. The difference between binding to albumin of lactone and carboxylate forms of TPT and CPT-11 is slight. One can notice a big difference between properties of CPT-11 and its metabolite SN-38. The association constants of TPT and CPT-11, summarized in Table III, are comparable with those obtained by ultrafiltration method, i.e. 3700 M\(^{-1}\) for TPT and 2500 M\(^{-1}\) for CPT-11. The value of binding constant determined for 9-amino-CPT by ultrafiltration method equals 1100 M\(^{-1}\), indicating that this analogue associates weakly with albumin.

4. Conclusions

PCA and FA analysis of set of absorption spectra recorded during CPT hydrolysis is a method competitive to the High Performance Liquid Chromatography

TABLE III

<table>
<thead>
<tr>
<th>Drug</th>
<th>( K [\text{M}^{-1}] )</th>
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<tbody>
<tr>
<td>lactone</td>
<td>carboxylate</td>
</tr>
<tr>
<td>CPT</td>
<td>290000 ± 40000</td>
</tr>
<tr>
<td>TPT</td>
<td>3000 ± 600</td>
</tr>
<tr>
<td>CPT-11</td>
<td>2700 ± 450</td>
</tr>
<tr>
<td>SN-38</td>
<td>80000 ± 12000</td>
</tr>
</tbody>
</table>
(HPLC) widely used in studies of camptothecins [9]. Limitation of the presented method is the required knowledge of the shape of spectra of all the factors. Difficulty in determining all three factors in the case of TPT preclude the use of the FA method to calculate the kinetics of hydrolysis in PBS and HSA solutions.

The fluorescence anisotropy method allows to determine the affinity of fluorescent camptothecin derivatives to albumin. Presented results confirm that the CPT carboxylate exhibits high affinity to HSA which leads to the total hydrolysis of the lactone form in albumin solution. The poor affinity of carboxylate forms of TPT, CPT-11, and 9-amino-CPT to albumin and weaker affinity of SN-38 carboxylate than SN-38 lactone are desirable features which improve stability of the studied compounds in blood.

References


