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INTERACTION OF CAMPTOTHECIN WITH HUMAN SERUM ALBUMIN DETERMINED BY FLUORESCENCE ANISOTROPY SPECTROSCOPY

TOMASZ WYBRANOWSKI*, BLANKA ZIOMKOWSKA, MICHAŁ CYRANKIEWICZ and STEFAN KRUSZEWSKI

Medical Physics Division, Biophysics Department, Collegium Medium of Nicolas Copernicus University, Jagiellońska 13, 85-067 Bydgoszcz, Poland

Abstract: The study can be useful for understanding the interaction of camptothecin with human serum albumin. There are two forms of camptothecin (the carboxylate form (CPT-C) and the lactone form (CPT-L)) but only the lactone one is pharmacologically active. It was reported earlier that in the presence of HSA, the active lactone form of camptothecin changes to inactive carboxylate form and it reduces the antitumor activities of camptothecin. However, those studies were performed at physiological pH (7.4) and with non-oxidized and non-glycosylated albumin. The aim of this study was to investigate the effect of oxidative stress, glycosylation, pH changes and competitor drugs on inactivation of lactone form of camptothecin in albumin solution using measurements of fluorescence anisotropy spectroscopy. It was tried to prove that *in vivo* camptothecin may be present in higher amount in lactone form than previously thought. Due to a reduction of pH value, a decreased rate of hydrolysis from CPT-L to CPT-C was observed. It was found *in vitro* a significant reduction in bound fraction of CPT-C to HSA oxidized by chloramine T or glycosylated by glucose. Moreover, as a result of blocking binding of CPT-C to HSA by competitive compound (flurbiprofen), a decrease in the fluorescence anisotropy of the HSA-CPT complex was found. This study opens the way to review an application of CPT and its derivatives in therapy.

Keywords: camptothecin, albumin, fluorescence, anisotropy

Camptothecin (CPT), an alkaloid isolated from the Chinese tree *Camptotheca acuminata*, exhibits very high anticancer activity. CPT exists in two forms: lactone and carboxylate, but only the lactone exhibits biological activity. Under physiological conditions (at pH 7.4), spontaneous hydrolysis occurs and the active lactone changes to inactive carboxylate form (1, 2). Currently, two CPT analogs are used in cancer therapy: topotecan and irinotecan. They exhibit relatively low affinity for plasma proteins. Some other derivatives are currently in the clinical trial phase. The new camptothecin derivatives are also the subject of research, and the goal is to obtain an analogue which has the greatest concentration of lactone form in the blood and moderately binds to albumin, thus extending the drug activity. Camptothecin is a fluorescent compound. The biophysical properties of CPT, determined by measurements of fluorescence anisotropy, help to predict the behavior of this compound under physiological conditions (3). The most important advantage of fluorescence methods is their high sensitivity, which enables to test drugs which have high affinity for albumin. Serum albumin is the basic protein of plasma (4). This protein serves a significant function in action of CPT – it binds inactive carboxylate form of camptothecin and accelerates the conversion of active lactone form into carboxylate form (2). The serious limitation of CPT and its analogs is achievement of high level of bound drug to HSA. Almost all studies were performed with commercially available human serum albumin. It should be taken into consideration that an excessive free radical formation and a reduced activity of antioxidant systems (oxidative stress) cause an oxidative damage to albumin, especially during cancer disease (5-7). This may result in a reduced CPT-HSA binding, and, consequently, in increased levels of free, pharmacologically active fraction of CPT (8, 9). In order to investigate the effects of oxidative stress on the binding of CPT to the albumin, human serum albumin was oxidized by chloramine T. The structure of albumin oxidized by chloramine T is similar to advanced oxidation protein products

* Corresponding author: e-mail: tomaszwybranowski@cm.umk.pl; phone: 52 585-34-05, fax 52 585-34-04

(AOPP) isolated from patients (10). In order to investigate the effects of glycosylation on the binding of CPT to the albumin, human serum albumin was glycosylated by glucose. Glycosylation of albumin may impair its transport functions (8). A significant amount of glycosylated albumin is observed in patients with cancer, as compared to healthy persons. Likewise, larger increase in level of glycosylated albumin is observed in diabetes with hyperglycemia (11). At pH 7.2, the CPT drug exists in an equilibrium of the active lactone and inactive carboxylate forms. Increase in pH value shifts the equilibrium towards an inactive form, while at low pH (< 5.5) only the lactone form of CPT is present (1, 2). It was demonstrated that inside and in the midst of the tumor pH is lower than physiological pH values, reaching even below 7 inside of the tumor (12-14). Moreover, in the course of cancer, a slightly decreased blood pH is observed. Therefore, one would expect a larger amount of lactone form of CPT in close proximity of the tumors and in the blood of cancer patients, compared to healthy persons. If both CPT and other drug pretend to take the same position in albumin, there may be an increase in free fraction of lactone form of CPT (15). Flurbiprofen was used as an active competitor, due to its strong binding to albumin.

MATERIALS AND METHODS

Flurbiprofen, chloramine T and human serum albumin (HSA) were received from Sigma-Aldrich. The samples of CPT were obtained from the laboratory of biotechnology, College of Pharmacy, University of Kentucky, Lexington (USA). A 2 mM stock lactone solution of CPT was prepared in DMSO (dimethyl sulfoxide C_2H_6OS). For fluorescence anisotropy measurements, concentration of lactone form of CPT in final samples was equal to 1 μM. The kinetic studies of the hydrolysis of lactone form to carboxylate form of CPT in albumin solution (40 μM) at different pH (from 7 to 8) consisted of a single series of measurements of fluorescence anisotropy in time function (up to 3 h). The PBS was adjusted to the desired value of pH using small quantities of 0.1 M KOH or HCl. For fluorescence anisotropy measurements, concentration of CPT in final samples was equal to 1 μM. A PTI (Photon Technology International, Birmingham, NJ, USA) spectrofluorometer was used for the measurement of steady-state fluorescence anisotropy. The effect of flurbiprofen on rate of hydrolysis of lactone form of CPT in HSA solution was studied using different concentration of flurbiprofen (from 10 μM to 2 mM). Stock solutions of flurbiprofen were prepared in ethanol. The oxidized form of HSA (40 μM) was obtained by an incubation of albumin in a solution of chloramine T (40 μM) for 60 min. The glycosylated form of HSA (40 μM) was obtained by an incubation of albumin in a solution of glucose (40 μM) for 60 min. The human blood from healthy people (n = 30) was received from the local blood-donation center (Bydgoszcz, Poland). A spectrophotometer UV-Vis JASCO V-550 was used to measure the absorbance. Concentration of AOPP was determined by measuring absorbance at 340 nm accord-

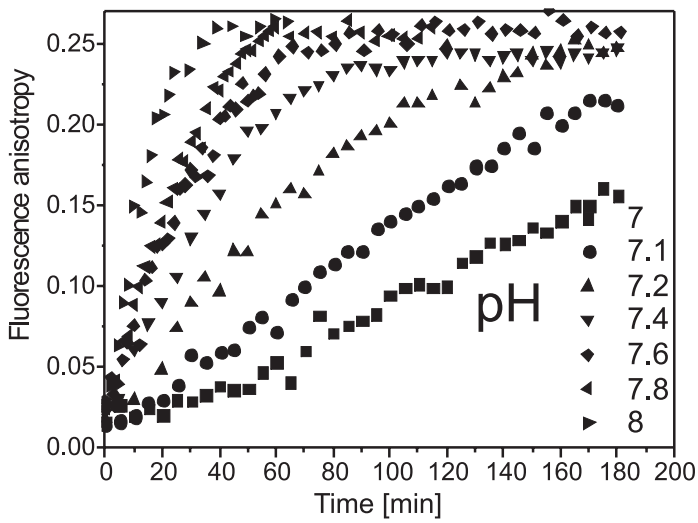

Figure 1. Fluorescence anisotropy of camptothecin in function of time at different pH

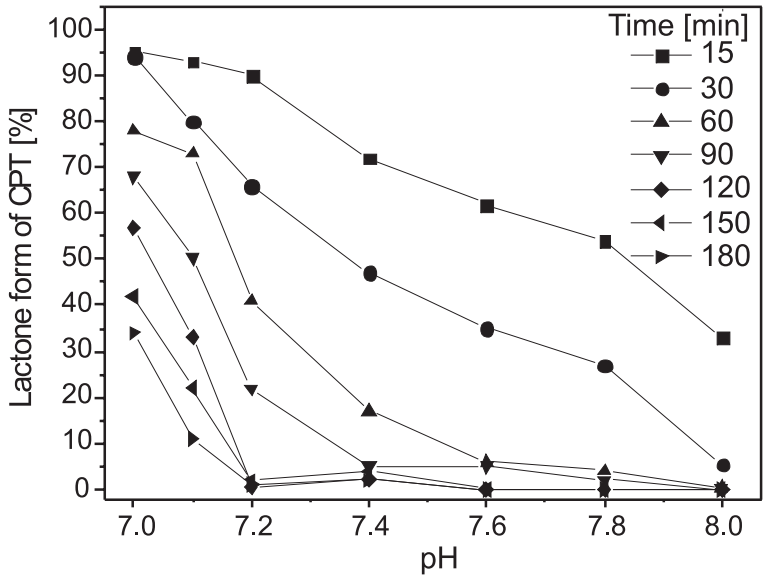

Figure 2. Fraction of lactone form of camptothecin in dependence on pH in different time

ing to the modified method described for the first time by Witko-Sarsat (16, 17). Briefly, the reactant mixture for AOPP assay contains 1.875 mL of 0.2 M citric acid and 25 µL of 1.16 M potassium iodide. Then, 1.9 mL of this mixture was added to 100 µL of the test sample and after 30 min the absorbance was recorded. In order to investigate the correlation between AOPP and free fraction of CPT in healthy subject, the single fluorescence anisotropy measurements of CPT-C for each albumin concentration (1, 2, 5, 10, 20 µM) were recorded. The method of determination of the affinity of drugs to the albumin and the free fraction of drugs in the plasma, based on measurements of the anisotropy, is described in our earlier publications (15). The temperature of the all sample was kept constant (37^{o}C) using the ultrathermostat TW2.03 (ELMI).

RESULTS AND DISCUSSION

Analysis of fluorescence anisotropy of CPT enables to follow the process of hydrolysis, i.e., the process of converting the biologically active lactone form into inactive carboxylate. Fluorescence anisotropy of bound CPT is high (close to 0.24) and of free (not bound) CPT – low (close to 0). The anisotropy measurements might be indicative of a degree of drug-albumin binding. As anisotropy reaches an upper limit (0.24), all drug molecules are linked to the macromolecules of albumin. Anisotropy equal to 0.12 indicates that about 50% of the drug is bound to albumin.

Figure 1 shows the time dependence of the fluorescence anisotropy of the lactone form of CPT introduced into an albumin solution at different pH (from 7 to 8). The value of the fluorescence anisotropy of the lactone form immediately after introduction to albumin solution is low (0.025). This results from the lack or weak binding to albumin. On the other hand, anisotropy increases over time. This is due to the gradual transition of the lactone form to the carboxylate form, which immediately binds with albumin. CPT molecules bound to albumin, do not re-enter an active form of lactone. Figure 1 shows that with increasing pH, the process of inactivation of CPT accelerates, and this is an undesirable effect. In turn, at pH lower than 7.4, the process of converting the lactone form of CPT into the inactive form is slowed down, and requires a much longer time. Considering that in the vicinity of tumors, the pH value is often less than 7.2, the results suggest that this will increase the therapeutic effect of CPT in patients with diagnosed cancer.

Figure 2 shows the content of free fraction of CPT-L in albumin solution, depending upon the pH of the solution and incubation time of CPT-L. After 3 h in albumin solution of pH 7.4, the amount of lactone form is only at the level of 4%. Of the solutions tested, the lactone form is most abundant at pH 7 (30% of the unbound form of lactone CPT after 3 h).

For given time, the amount of lactone form markedly lowers with an increase in pH of the solution. The lactone form almost disappears after only two hours of CPT-L incubation in pH 7.2. The concentration of the active form of topotecan and irinotecan in blood at pH 7.4 is equal to 12% and 21%, respectively (18). It is the result of weak binding of their carboxylate forms to albumin. The results shown in this study suggest that also in case of the derivatives of CPT, the concentration of the lactone form would increase with decreasing pH.

In order to check how the hydrolysis rate of CPT-L changes in the presence of competing compound, flurbiprofen in different concentrations (from 10 μM to 3 mM) was added to a solution of CPT-L-HSA (data not shown) and the fluorescence anisotropy was measured in time. It was observed that addition of flurbiprofen to the solution of CPT-L and HSA reduces the rate of an inactivation of lactone form of CPT in dependence on flurbiprofen concentration. It can be concluded that an addition of the competing drug to the solution of CPT-L-HSA would block the binding of CPT-C with albumin. Therefore, the solution may contain more CPT lactone form. However, only the concentrations of flurbiprofen above 200 μM may significantly contribute to the reduction of the growth rate of anisotropy. Such large concentrations, however, does not mimic the body condition, even in case of multidrug therapy.

Figure 3 compares the fluorescence anisotropy values of CPT obtained 3 h after the lactone form of CPT had been added to the 40 μM albumin solution prepared in diverse conditions. As it was previously shown in Figure 1, by lowering pH to 7, the anisotropy reaches the value of 0.16 within 3 h after the addition of CPT-L to HSA (the concentration of lactone form equals approximately 30%).

The addition of flurbiprofen at a concentration of 10 μM (at pH 7.4) results in a modest decline in the growth rate of anisotropy, by approximately 3%. Nonetheless, merely an addition of larger amounts of competing drug in non-physiological concentrations causes a significant decrease in the growth rate of anisotropy. Oxidative damage of albumin was induced *in vitro* by chloramine T. Glycosylation of albumin involves the non-enzymatic addition of glucose to albumin. An incubation of albumin in a chloramine T solution (10 μM) or a glucose solution (10 μM) at pH 7, followed by an addition of CPT-L,

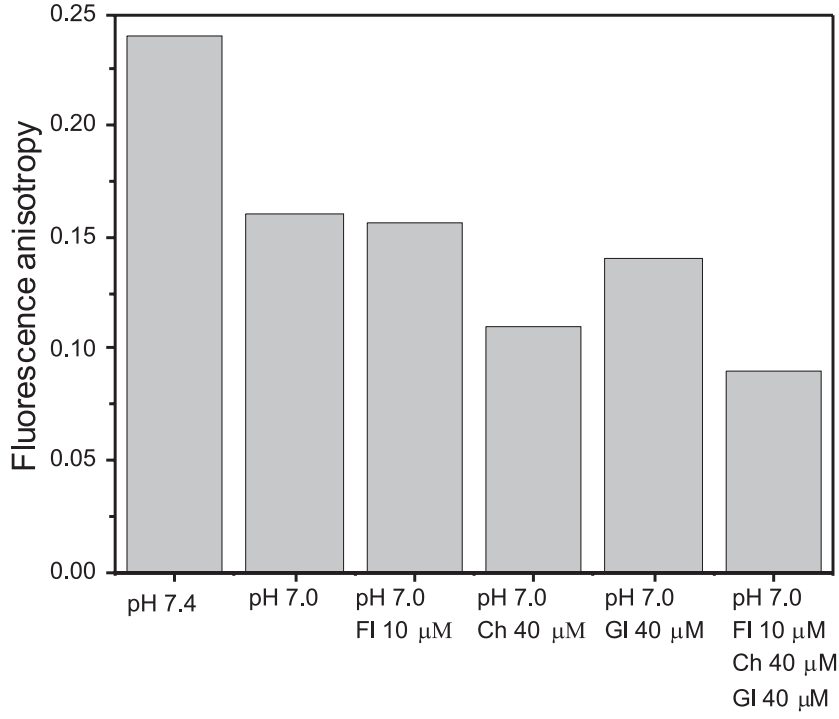

Figure 3. Fluorescence anisotropy of camptothecin in dependence on applied condition, 3 h after the lactone form of camptothecin had been added to the albumin solution after addition of flurbiprofen (Fl), after oxidation of albumin induced by chloramine T (Ch) and after glycosylation of albumin (Gl)

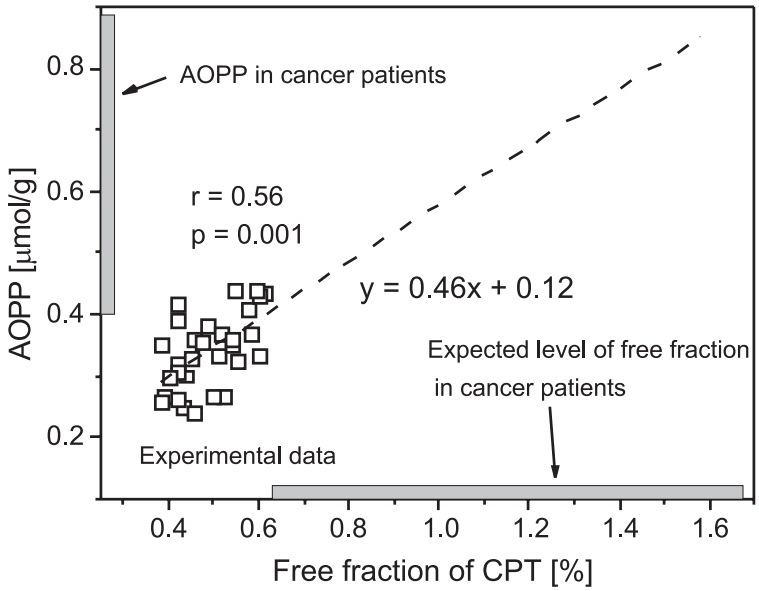

Figure 4. Correlation between advanced oxidation protein products (AOPP) and free fraction of camptothecin (CPT) in healthy subject and regression curve fitting

caused a decrease of the hydrolysis rate, after 3 h anisotropy was 0.11 or 0.14, respectively. These results show that due to an oxidation or glycosylation of albumin, an inactivation rate of lactone form is slower. Finally, it was examined how the fluorescence anisotropy of CPT in a solution of pH 7, containing all three study factors is reduced. Three hours following the introduction of CPT-L, the anisotropy value was only 0.09. After this time, the concentration of free CPT was about 63%. This clearly shows that in patients with cancer, the plasma concentration of free active lactone form of CPT is higher than it was previously believed. This will increase the therapeutic effect of the drug. However, this will depend on the patient condition and whether one uses the other drugs or is subjected to radiotherapy. Radiotherapy is associated with a strong oxidative stress, which also increases an oxidative damage of the proteins. It seems that the concomitant use of anticancer drugs and radiation therapy is beneficial from the viewpoint of the therapeutic effect of the drugs and may contribute to the improvement of the patient's condition.

Another observation is a decrease in the binding affinity of the carboxylate form to oxidized and glycosylated albumin by 22 and 10%, respectively, as compared to the affinity of CPT-C to unmodified albumin ($K = 290\ mM^{-1}$). For investigation of binding properties of glycosylated and oxidized albumin, 1 mol chloramine T or 1 mol glucose on 1 mol albumin were used. It demonstrates that the decrease in the rate of hydrolysis is associated rather with a reduction in binding of the carboxylate form to modified albumin.

The effects of oxidative stress on the binding of CPT to albumin are also confirmed by the study of CPT-C binding with plasma proteins, derived from 30 healthy subjects.

For each sample the level of oxidative stress was measured and in order to determine the affinity and free fraction of CPT, the fluorescence anisotropy of CPT-C was examined as a function of albumin concentration (from 1 to 40 μM) at pH 7.4. A significant positive correlation between the concentration of AOPP and free fractions of CPT in plasma at physiological concentration of albumin was observed ($r = 0.56$ $p = 0.001$) (Fig. 4). AOPP is a marker of oxidative stress detected first in the plasma of chronic uremic patients (16). In the course of cancer, the oxidative processes are more intense, resulting in an increase of the level of AOPP, ranging from a few to 250 percent, depending on the stage and type of the cancer (19-21). Linear regression between levels of AOPP and values of free fraction of CPT reveals a slope of 0.46 and an intercept of 0.12. The fitting trendline indicates that it may cause an increase of the free fraction of CPT by even 330% in some cancer patients. Undoubtedly, this will result in larger amounts of lactone form in the blood of these patients.

CONCLUSION

A reduced CPT drug binding to human serum albumin results in elevated active CPT lactone levels and thus improves the effectiveness of treatment in cancer therapy. This study showed that during cancer disease, level of active lactone form of CPT can be higher than previously thought. It is related to oxidative damages, glycosylation and decreased pH in tumor. Conclusions drawn from the research may also refer to the other derivatives of CPT. It seems that an impact of the examined factors on the affinity is important in the uses thereof as an anticancer agent. In addition, these studies show what can be done to increase the therapeutic effect of CPT and its derivatives. The induction of oxidative stress (e.g., by radiotherapy) may result in an improvement of the efficacy of an anticancer therapy with the use of CPT and its derivatives.

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