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Stability of the new prodrug 9-aminocamptothecin glucuronide (9ACG) in the presence of human serum albumin

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Abstract

9-Aminocamptothecin glucuronide (9ACG) is a new water-soluble prodrug of 9-aminocamptothecin (9AC) that is a substrate for β-glucuronidase and displays potent antitumor activity against human tumor xenografts. The lactone ring of camptothecins (CPTs) is required for antitumor activity but spontaneously opens under physiological conditions to an inactive carboxy form. The carboxy form of many CPTs, including 9AC, preferentially binds to human serum albumin (HSA), which further reduces the equilibrium amount of active lactone and greatly decreases antitumor efficacy. In this study, we examined the hypothesis that the unique structure of 9ACG might alter prodrug interaction with HSA and increase 9ACG lactone stability as compared with 9AC. HPLC analysis revealed that HSA did not affect the equilibrium level of 9ACG lactone whereas both CPT lactone and 9AC lactone were greatly reduced in the presence of HSA as compared to their equilibrium levels in PBS. Similar results were found in human serum and whole blood. The lactone ring of 9ACG also opened more slowly ($t_{1/2} = 50$ min) as compared with 9AC ($t_{1/2} = 20$ min) in the presence of HSA. Both 9ACG lactone and 9ACG carboxy bound HSA with similar affinities ($K_D \sim 4.5 \times 10^{-5}$ M^{-1}). Binding of 9ACG to HSA reduced prodrug toxicity to cancer cells by about 10-fold *in vitro*. Injection of HSA into nude mice prolonged the half-life of 9ACG by about 3-fold, indicating that albumin-bound 9ACG lactone may act as a depot of active prodrug *in vivo*. Our results suggests that in contrast to CPT and 9AC, HSA does not appear to adversely affect 9ACG and may enhance the selective antitumor activity of 9ACG in tumors that contain β-glucuronidase.

Keywords: 9-Aminocamptothecin; Glucuronide; Albumin; Lactone; Carboxy; Equilibrium

1. Introduction

CPT is an antitumor alkaloid that was isolated in 1966 from *Camptotheca acuminata* [1]. CPT exists in a pH-dependent equilibrium of closed-ring lactone and opened-ring carboxy forms. Early clinical trials employed a water-soluble sodium salt of CPT carboxy due to the poor water solubility of the lactone. The poor antitumor activity and frequent unpredictable toxicity of CPT carboxy [2–4], however, caused clinical investigation of CPT to be abandoned for almost 20 years until the molecular mechanism of CPT action was elucidated. The discovery that the lactone ring of CPT is necessary for specific interaction with topoisomerase I [5] and selective antitumor activity [6,7] generated renewed interest in CPT as an antitumor agent. Several water-soluble derivatives of CPT with improved lactone ring stability have been synthesized [8,9]. Two of these derivatives (irinotecan (CPT-11) and topotecan) have recently been approved for clinical use [10,11].

9AC is a potent derivative of CPT [12]. 9AC, however, suffers from two serious problems. First, 9AC is almost insoluble in water, causing problems in formulating an injection form of the drug. Second, although 9AC displays excellent antitumor activity in animal models [13], its potency was poor in phase I clinical trials [14–16]. We recently solved the first problem by synthesizing a 9AC glucuronide derivative, 9ACG (Fig. 1) [17]. 9ACG displays improved water solubility as compared to 9AC or CPT. 9ACG is a substrate for β-glucuronidase (E.C.3.2.1.31) and was designed as a prodrug for use in antibody-directed enzyme prodrug therapy [18]. Experiments in mice, moreover,

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Abbreviations: HSA, human serum albumin; CPT, camptothecin; 9AC, 9-aminocamptothecin; 9ACG, 9-aminocamptothecin glucuronide; CPT-11, irinotecan.

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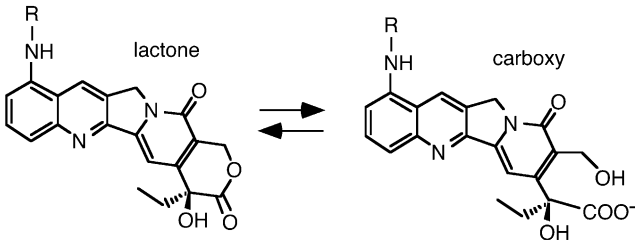


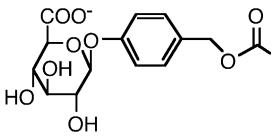
Compound	R
9-AC	H
9-ACG	

Fig. 1. 9AC and its glucuronide prodrug. Both 9AC lactone and 9ACG lactone exist in equilibrium with a corresponding open-ring carboxy form. Enzymatic hydrolysis by β-glucuronidase of the glucuronide group of 9ACG results in the spontaneous degradation of the carbamate linker and release of 9AC.

revealed that 9ACG possesses potent antitumor activity as a monotherapeutic against human tumor xenografts [19]. Solving the second problem, however, is more challenging. Experience with CPT-11 and topotecan suggests that the behavior of CPT derivatives in the presence of HSA is a determining factor of their clinical efficacy. The equilibrium concentration of 9AC lactone in blood is less than 0.5% due to preferential binding of 9AC carboxy to HSA which shifts the natural equilibrium between 9AC lactone and carboxy toward inactive 9AC carboxy [20–22]. CPT displays similar behavior [20,21,23]. In contrast, the clinically important CPT derivatives CPT-11 and topotecan display enhanced lactone stability in the presence of HSA [21]. Specific interaction of HSA with CPTs can also influence other pharmacologically important parameters such as elimination half-life and bioavailability [22].

9ACG possesses a bulky polar group that is negatively charged when dissociated. We therefore expected that it may interact differently with HSA as compared with CPT and 9AC. In the present study, we investigated the influence of HSA on 9ACG lactone ring stability. We also measured the binding affinity of 9ACG to HSA and examined the influence of HSA on 9ACG cytotoxicity against cancer cells and 9ACG circulating half-life in mice as critical parameters for predicting 9ACG utility in humans.

2. Material and methods

DMSO, lipid and IgG free HSA, PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma Chemical Co. (*S*)-(+)-CPT was purchased from Aldrich. CPT-11 was kindly provided by Dr. Mu-Hsien Yu, Department of Gynecology and Obstetrics, Tri-Service General Hospital, Taipei, Taiwan. 9AC and 9ACG were synthesized as described [17].

Human blood and serum was obtained from healthy normal male Caucasian volunteers by vein puncture. Blood samples were kept at 37° for 30 min and then centrifuged for 5 min at 3000 *g* to obtain serum. Ten millimolar stock solutions of drugs were made by dissolving the lactone form of drugs (CPT, 9AC and 9ACG) or diluting a commercially available lactone solution (CPT-11) in DMSO. All stock solutions were stored at −80°.

CL1-5 human lung adenocarcinoma cells [24] were cultured in DMEM supplemented with 10% bovine serum, 2.98 g/L HEPES, 2 g/L $NaHCO_3$, 100 U/mL penicillin and 100 μg/mL Streptomycin (complete medium) at 37° in an atmosphere of 5% CO_2 in air. The cells were free of mycoplasma as determined by a PCR-based detection kit (American Type Culture Collection).

Female BALB/c mice aged 10–12 weeks were obtained from the National Animal Center, Taipei, Taiwan. The animals were kept in cages with free access to food and water. The animal experiments were carried out with ethical committee approval. The ethical guidelines that were followed met the standards recommended by the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia [25].

2.1. Simultaneous determination of lactone and carboxy forms of drugs

Reversed phase HPLC was utilized for simultaneous determination of the lactone and carboxy concentrations of drugs. 9ACG and CPT were analyzed by a method developed for CPT [26] with minor modifications. The mobile phase consisted of a mixture of 5 mM potassium phosphate buffer pH 6.5 and methanol (1:1 v/v). The mobile phase was briefly degassed by ultrasound and the pH was re-adjusted to 6.5 with dilute phosphoric acid. Post-column acidification as described by Supko and Malspeis was adopted for simultaneous determination of 9AC lactone and 9AC carboxy [27]. A mobile phase containing 20:80 (v/v) acetonitrile–triethylamine acetate buffer (3% v/v, pH 5.5) was employed for simultaneous determination of CPT-11 lactone and CPT-11 carboxy [28]. All mobile phases were delivered at 1 mL/min. Post-column acidification used 0.2 mL/min of 1 M phosphoric acid.

The HPLC system consisted of two Waters 600E multisolvent delivery systems with He degassing, a Rheodyne 7725i manual injector with a 200 μL loop, a 250 mm × 4.6 mm column packed with Hypersil ODS (5 μm), a Gilson 121 fluorometer with 305–395 nm excitation and 430–470 nm emission and a Beckman Analog Interface Module 406 connected to a PC. Beckman Gold Version 6 software was used to control the system, record spectra and perform peak integration calculations.

2.2. Influence of HSA on the lactone–carboxy ratio of CPTs

The kinetics of conversion and the equilibrium concentrations of the lactone and carboxy forms of 9ACG, 9AC, CPT and CPT-11 were measured at pH 7.4 in PBS and DMEM with and without 40 mg/mL HSA. The behavior of 9ACG in human serum and whole blood was also examined. Stock solutions (10 mM) of the drugs in lactone form were diluted to 10 μM and incubated for various times. Samples were immediately treated with 4 vol. of ice-cold methanol followed by vortex mixing for 30 s to precipitate proteins and extract the total lactone and carboxy forms of the drugs. The recovery was essentially quantitative (data not shown). Upon centrifugation for 5 min at 10,000 *g* at 4° to remove precipitated proteins, the clear methanol extract was diluted 1:1 with 5 mM phosphate buffer to reconstitute the mobile phase. Five hundred microliter of sample was injected into the 200 μL HPLC loop. Three fully independent runs were performed for every drug and solvent combination. Human blood samples were kept under an atmosphere of 5% CO_2 in air to maintain constant pH.

2.3. Binding of 9ACG lactone and carboxy to HSA

A 2.5 μM equilibrium mixture of 9ACG lactone and 9ACG carboxy was incubated with graded concentrations of HSA from 2.5 mM to 250 nM for 12 hr at 37°. One half of each sample was then briefly spun in an Amicon microconcentrator unit (30 kDa cut off) to collect unbound drug. The resulting protein-free solution was diluted 1:1 with methanol and analyzed by HPLC for free 9ACG lactone and carboxy concentrations. The other half of each sample was precipitated with methanol and processed to measure total 9ACG lactone and carboxy concentrations. The K_D values were calculated using a one-site competition equation with Origin 6.0 (OriginLab).

2.4. Influence of HSA on in vitro growth inhibition

Exponentially growing CL1-5 cells were seeded overnight in 96-well plates at 5000 cells/well. Graded concentrations of 9AC lactone or 9ACG lactone were diluted in complete medium with or without 4 mg/mL HSA. This concentration of HSA produces kinetic and equilibrium values for the hydrolysis of CPT lactone that are similar to the values measured in physiological levels (30–50 mg/mL) of HSA [22]. The resulting solutions were immediately added to the cells or were allowed to equilibrate for 1 hr before addition to the cells. After 24 hr, the plates were washed twice with PBS and fresh medium was added for another 24 hr. Fresh medium containing 1 μCi/well [^{3}H]thymidine was then added for another 16 hr before the cells were harvested on glass-fiber filters and the radioactivity was measured on a Topcount scintillation counter. All experiments were performed in triplicate and mean values are shown. Corresponding controls for HSA and DMSO were performed at the same time. The DMSO concentration was less than 0.05% and did not cause any toxicity. The results are expressed as:

$$[^3\mathrm{H}]\text{Thymidine incorporation}\,(\%\,\text{control}) = 100\left(\frac{\text{cpm treated cells}}{\text{cpm untreated cells}}\right)$$

2.5. Influence of HSA on 9ACG pharmacokinetics in mice

Groups of three BALB/c mice were untreated or i.v. injected with HSA (2 g/kg) in PBS (total volume of 100–125 μL). After 30 min, 9ACG (50 mg/kg) in PBS was injected *via* the tail vein. Blood samples were periodically removed from the tail vein and the total concentration of 9ACG in serum samples was measured by HPLC.

3. Results

3.1. Influence of HSA on the lactone–carboxy ratio of 9ACG

The hydrolysis of 9ACG lactone to 9ACG carboxy was compared with three CPT drugs (CPT, 9AC and CPT-11) in PBS with and without 40 mg/mL HSA. The lactone form of all the derivatives in PBS was converted to a mixture containing both lactone and carboxy drugs (Fig. 2). Near equilibrium concentrations were reached within 1–2 hr. The percentages of drug remaining as lactone at equilibrium in

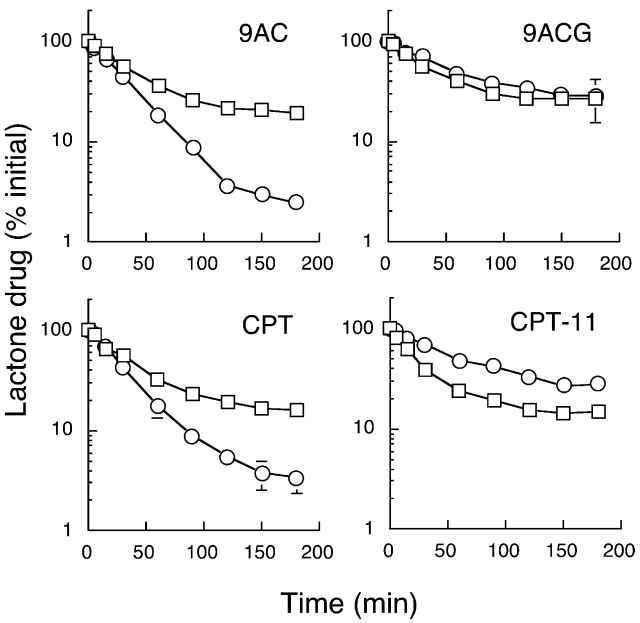


Fig. 2. Kinetics of drug lactone ring opening. The lactone form of 9AC, 9ACG, CPT and CPT-11 were incubated in PBS (□) or PBS containing HSA (○). Results show the mean percentage of the lactone form of drugs remaining at the indicated times. Bars, SD.

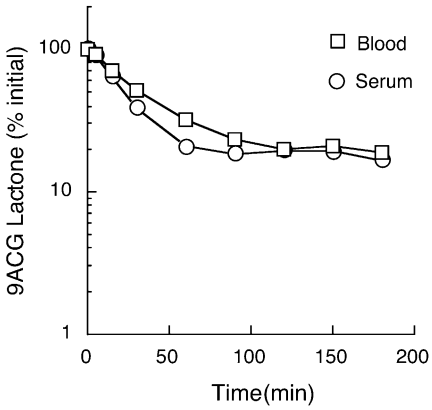


Fig. 3. Stability of 9ACG lactone in serum and blood. 9ACG lactone was incubated in whole blood (□) or serum (○). Results show the mean percentage of the lactone form of drugs remaining at the indicated times. Bars, SD.

PBS for CPT, CPT-11, 9AC and 9ACG were 15.7, 15.0, 19.1 and 27.1%, respectively. In the presence of 40 mg/mL HSA, 9ACG was hydrolyzed more slowly ($t_{1/2} = 50$ min) than 9AC ($t_{1/2} = 20$ min). In addition, only 3.4 and 2.5% of CPT lactone and 9AC lactone remained after 3 hr whereas HSA did not alter 9ACG lactone equilibrium (28% lactone) and the amount of CPT-11 lactone increased to 27%. Similarly, the percentage of 9ACG lactone remained above 20% in both human blood and serum (Fig. 3).

3.2. Binding of 9ACG to HSA

9ACG binding to HSA was measured by allowing an equilibrium mixture of 9ACG lactone and 9ACG carboxy to bind to graded concentrations of HSA. The unbound and total concentrations of 9ACG lactone and 9ACG carboxy were then measured. A single binding-site competitive model best fit the data with an estimated K_D value of $4.7 \times 10^{-5}\ M^{-1}$ for 9ACG lactone and $4.3 \times 10^{-5}\ M^{-1}$ for 9ACG carboxy (Fig. 4).

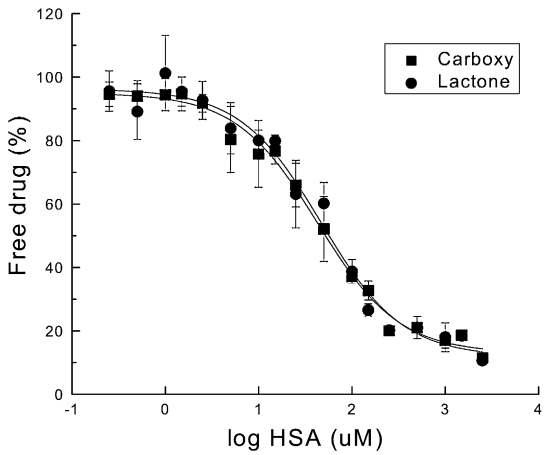


Fig. 4. Binding of 9ACG to HSA. An equilibrium mixture of 9ACG lactone and 9ACG carboxy were incubated with graded concentrations of HSA. Results show mean values of the percentage of total 9ACG carboxy (■) and 9ACG lactone (●) in solution at each HSA concentration. Bars, SD.

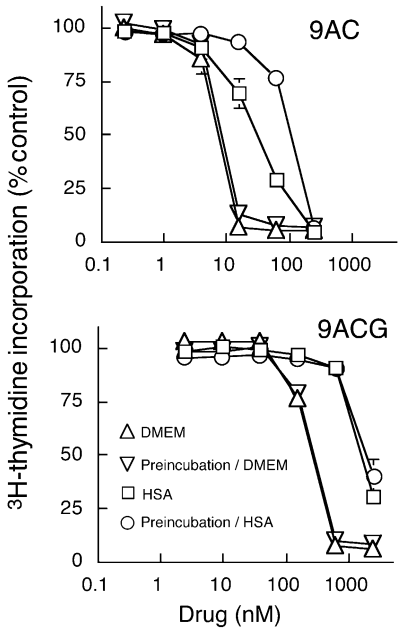


Fig. 5. Cytotoxicity of drugs in the presence of human albumin. 9AC and 9ACG were preincubated in complete medium (▽) or complete medium containing HSA (○) for 1 hr before addition to CL1-5 cells or directly added to CL1-5 cells in complete medium (△) or complete medium containing HSA (□). After 24 hr, the culture medium was changed to complete medium and the cells were cultured an additional 24 hr. Results show the mean incorporation of ^{3}H-thymidine into cellular DNA as a percentage of ^{3}H-thymidine incorporation in untreated control cells. Bars, SD.

3.3. Influence of HSA on the in vitro growth inhibition of 9ACG

The influence of HSA on the growth inhibition produced by 9ACG and 9AC against CL1-5 human lung cancer cells was investigated. The kinetics of 9AC and 9ACG hydrolysis were similar in culture medium containing HSA and in PBS with HSA, serum and whole blood. Figure 5 shows that addition of 9AC lactone in complete medium to CL1-5 cells for 24 hr inhibited cellular DNA synthesis with an IC_{50} value of 7 nM. 9AC lactone in complete medium containing 4 mg/mL HSA produced less toxicity with an IC_{50} value of 30 nM. Preincubation of 9AC lactone in complete medium containing HSA for 1 hr before addition to the cells resulted in further reduction in toxicity ($IC_{50} = 100$ nM), reflecting the effect of 9AC lactone conversion to 9AC carboxy on cytotoxicity. Figure 5 shows that similarly to 9AC, HSA reduced the toxicity of 9ACG lactone to CL1-5 cells ($IC_{50} = 200$ nM vs. 1800 nM). In contrast to 9AC, preincubation of 9ACG with medium containing HSA did not further reduce the cytotoxicity of 9ACG lactone to CL1-5 cells.

3.4. Influence of HSA on the pharmacokinetics of 9ACG

The binding affinity of 9ACG to HSA ($K_D = 4.5 \times 10^{-5}\ M^{-1}$) suggested that prodrug pharmacokinetics may be altered in the presence of HSA. To test this

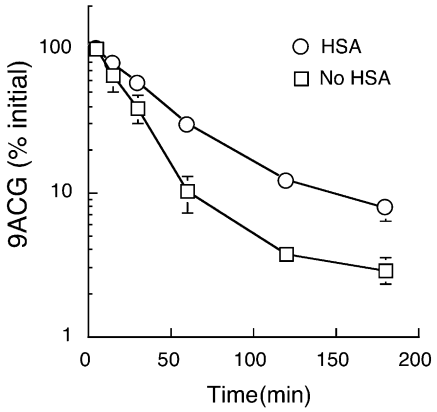


Fig. 6. HSA slows the elimination of 9ACG. BALB/c mice were untreated (□) or i.v. injected with 2 g/kg HSA (○) 30 min before i.v. injection of 50 mg/kg 9ACG. Results show mean 9ACG concentrations as a percentage of the initial 9ACG concentration in serum samples taken at the indicated times. Bars, SD.

hypothesis, the pharmacokinetics of 9ACG lactone were measured in mice that had previously been i.v. injected with HSA. Figure 6 shows that 1/10 of the physiological level of HSA in serum slowed the rate of 9ACG elimination, resulting in about three times higher concentrations of prodrug remaining in the circulation at 3 hr after administration.

4. Discussion

Although many CPTs display excellent antitumor activity against human tumor xenografts, the efficacy of CPTs can be dramatically different in humans. The most important effect is the preferential binding of the carboxy form of CPTs to HSA [21], thereby shifting the equilibrium to generate more carboxy drug with a corresponding reduction in the amount of active lactone. In addition, high affinity binding to HSA can reduce the availability of free CPT whereas moderate binding affinities may allow HSA to serve as a drug reservoir, thereby prolonging the half-life of drug in the circulation. We hypothesized that due to its structural differences with 9AC and CPT, 9ACG could differently interact with HSA and possess advantages for human use. Indeed, we found that 9ACG lactone displayed enhanced stability in serum as compared with 9AC. In addition, 9ACG lactone bound to HSA with moderate affinity, suggesting that HSA may act as a reservoir of active 9ACG lactone in humans.

Both CPT and 9AC lactone accounted for about 15–19% of total drug in PBS at equilibrium but just 2–3% of total drug in the presence of HSA. These results are in good concordance with previous investigations [22]. 9ACG lactone was more stable in both PBS and HSA; equilibrium concentrations of 9ACG lactone accounted for about 27% of total 9ACG in both cases. More importantly, 9ACG lactone was also relatively stable in serum and in whole blood. The equilibrium level of 9ACG lactone in blood (18%) was about 100 times greater than 9AC lactone (<0.2%) but similar to the clinically important analogs CPT-11 and topotecan lactone (∼20%) [22]. The small difference between 9ACG lactone in PBS with HSA (27%) and in blood (18%) could be due to the well known "salt effect" [22].

Even though HSA did not change the lactone-carboxy equilibrium of 9ACG, this prodrug did bind to HSA. The carboxy-lactone equilibrium was unaffected because both 9ACG lactone and 9ACG carboxy bound to HSA with similar affinities ($K_D = 4.7 \times 10^{-5}$ and 4.3×10^{-5} M^{-1}, respectively). 9ACG bound HSA with lower affinity than CPT-11 or SN-38 ($K_D \sim 1 \times 10^{-6}$ M^{-1}), allowing higher concentrations of free 9ACG lactone (about 10% of total 9ACG) to remain in solution. HSA possesses positively charged pockets that bind anions [29,30]. Both the carboxy group of the open lactone ring as well as the carboxy group of glucuronic acid may participate in such binding. The similar binding affinities of 9ACG lactone and carboxy for HSA suggests that the same structural motif, likely including the glucuronide group, binds to HSA. Crystallography, competitive binding and computer simulation may reveal the mechanism of binding.

We investigated how HSA binding affects 9ACG growth inhibition in a standard cytotoxic assay with CL1-5 lung carcinoma cells. HSA reduced the cytotoxicity of both 9AC and 9ACG to human lung cancer cells. The lower cytotoxicity of 9ACG in the presence of HSA is attributed to prodrug binding to HSA with a corresponding reduction of the amount of free 9ACG in solution. Preincubation of 9ACG lactone with HSA produced similar cytotoxicity as compared to direct addition of 9ACG lactone to cells containing HSA-spiked medium because HSA did not shift the lactone/carboxy equilibrium. In contrast, preincubation of 9AC lactone with HSA resulted in less cytotoxicity than when 9AC lactone was directly added with HSA to CL1-5 cells because 9AC lactone binds very poorly to HSA [21]. Preincubation of 9AC lactone with HSA therefore allowed generation of 9AC carboxy which could then bind to HSA with high affinity [21], resulting in low levels of free 9AC lactone. Direct addition of 9AC lactone to cells likely allowed some 9AC lactone to enter CL1-5 cells and produce cytotoxicity before 9AC carboxy was generated and bound by HSA. Indeed, CPT and 9AC pass across the lipid bilayer very rapidly [6,31]. These results emphasize that the differential interactions of 9AC and 9ACG with HSA result in distinct biological effects. Because HSA binds both 9ACG lactone and 9ACG carboxy with similar affinities, HSA can act as a depot for the slow release of 9ACG lactone without generation of 9ACG carboxy. For 9AC, in contrast, HSA can only act as a depot of 9AC carboxy, resulting in the prolonged exposure of patients to the inactive, but toxic open ring form of 9AC.

We addressed the question of whether HSA could act as a reservoir of 9ACG *in vivo* by injecting HSA into mice.

Due to physiological limitations, we could only attain in mice about 10% of the HSA concentration present in human serum. This level of HSA, however, resulted in slower elimination of 9ACG and about three times more 9ACG in circulation. 9ACG elimination may be even slower in humans since HSA concentrations are 10 times higher. About 90% of prodrug should be bound to albumin, serving as a pool of active 9ACG lactone.

Our results indicate that in contrast to CPT and 9AC, 9ACG is stable in HSA and in serum with properties similar to CPT-11. Binding of 9ACG lactone to HSA without conversion to 9ACG carboxy should slow the rate of prodrug elimination. This is advantageous for 9ACG since glucuronides are typically eliminated quite rapidly. Thus, by acting as a pool of 9ACG lactone, HSA may prolong the exposure of tumor cells to low concentrations of prodrug. Slow release of 9ACG lactone at tumors that contain β-glucuronidase should allow enzymatic generation of 9AC lactone for selective tumor therapy [19,32]. Overall, 9ACG appears to possess several advantages over 9AC for human cancer therapy.

Acknowledgments

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