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ORIGINAL ARTICLE

Effect of pH and human serum albumin on the cytotoxicity of a glucuronide prodrug of 9-aminocamptothecin

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Abstract
Purpose 9-aminocamptothecin glucuronide (9ACG) is a prodrug of 9-aminocamptothecin (9AC) that displays potent antitumor activity against human tumor xenografts in nude mice. Camptothecins exist in a pH dependent equilibrium between active lactone and inactive carboxy forms that can be altered by binding to human serum albumin (HSA). Here we investigated the influence of pH and HSA on the lactone-carboxy equilibrium, HSA binding, and cytotoxicity of 9ACG.
Methods Microfiltration and HPLC were used to measure the influence of pH on lactone to carboxy conversion and HSA binding of 9ACG as compared to other camptothecins. In vitro cytotoxicity of drugs was determined against EJ human bladder carcinoma cells and CL1-5 human lung cancer cells.
Results The rate of lactone to carboxy conversion was similar for 9ACG and 9AC. Decreasing the pH from 7.6 to 6.0 increased the equilibrium levels of the lactone forms of the drugs from 20 to almost 95% of total drug. HSA moderately diminished the amount of free 9ACG lactone but did not change the ratio of 9ACG lactone to 9ACG carboxy. Consistent with the effect of pH on lactone levels, lowering the pH of EJ human bladder carcinoma cells from 7.6 to 6.8 decreased the IC_{50} of 9ACG from 480 to 98 nM and 9AC from 33 to 12 nM. Activation of 9ACG by human β-glucuronidase anchored on the surface of EJ cells further decreased its IC_{50} value to 26 nM. Although HSA significantly decreased the cytotoxicity of 9AC and 9ACG, activation of 9ACG at cancer cells with an antibody-β-glucuronidase immunoconjugate produced greater cytotoxicity than 9AC.
Conclusions Acidification and targeted delivery of β-glucuronidase can enhance 9ACG cytotoxicity even in the presence of HSA.

Keywords 9-Aminocamptothecin · Human serum albumin · β-Glucuronidase · pH · Prodrug · Cancer therapy

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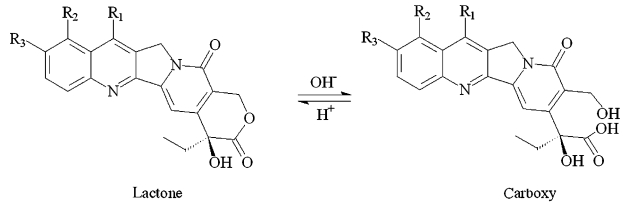
Introduction

The bark of the South Asian tree Camptotheca Acuminata has been used in traditional Chinese medicine to treat colon cancer. The active natural product camptothecin was isolated in the 1960s [1]. Camptothecin exists in an equilibrium of lactone and carboxy forms (Fig. 1) [2, 3]. The lactone form is favored under acidic conditions (pH < 4) whereas, the carboxy form is favored at neutral and alkaline pH values (pH > 7).

The behavior of camptothecins in vivo is complex (Fig. 2). Human serum albumin (HSA) but not albumins from other species has strong affinity for the carboxy form of most camptothecins [4–8], shifting the



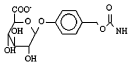
Compound	R1	R2	R3
camptothecin	H	H	H
9AC	H	NH_2	H
9ACG	H		H
10-hydroxy CPT	H	H	OH
SN-38	C_2H_5	H	OH
topotecan	H	$(CH_3)_2NCH_2$	H
irinotecan	C_2H_5	H	

Fig. 1 pH dependent equilibrium and chemical structures of camptothecin derivatives

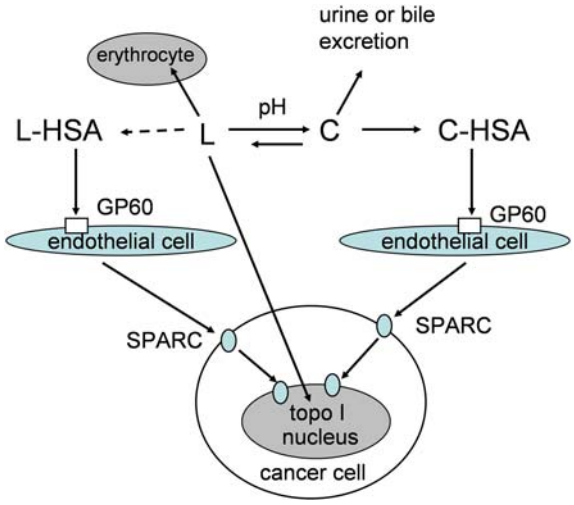


Fig. 2 Role of pH and HSA in camptothecin lactone and carboxy disposition. The active lactone form of camptothecins (*L*) can diffuse across the plasma membrane of cells, enter the nucleus and inhibit topoisomerase I activity. The lactone (*L*) is in a pH dependent equilibrium with the inactive carboxy form (*C*). Preferential binding of the carboxy form to HSA (*C-HSA*) drives the equilibrium to the *right*. Albumin-bound drugs may enter cancer cells by GP60-mediated transport across endothelial cells followed by interaction with SPARC on cancer cells

equilibrium toward the inactive carboxy form of the drug. Binding to other blood proteins also influences the equilibrium [9]. The hydrophobic lactone form of camptothecins also readily dissolves into the membrane of erythrocytes [10], thereby preserving the lactone ring. Binding to HSA may also affect cell uptake via GP60 and SPARC proteins [11–13].

Due to low solubility in water and difficulties in formulating the lactone form, early clinical trials utilized the water-soluble sodium salt of camptothecin, which produced high toxicity and poor antitumor effects [14–16]. The investigation of camptothecin was abandoned for many years until the mechanism of action was discovered. The lactone ring of camptothecin is essential for S-phase dependent inhibition of topoisomerase I activity [17, 18] and consequent antitumor activity [3, 19, 20].

Recently, several approaches have been used for generation of camptothecin analogues with better pharmacological properties including synthesis of derivatives that are more water-soluble, possess more stable (six-member) lactone ring moieties or display reduced binding to HSA [21–24]. Karenitecin, a lipophilic CPT derivative, has shown activity against malignant melanoma in phase II clinical trials [25].The synthesis of camptothecin derivatives that are water-soluble and display enhanced lactone stability has resulted in the clinical use of two derivatives [irinotecan (CPT-11) and topotecan] [26–28].

9-aminocamptothecin (9AC) is a camptothecin derivative [29, 30] that possesses remarkable activity against several cancer cell lines in vitro and in animal tumor models [31, 32]. Clinical trials, however, revealed poor activity in humans [33–35]. One important reason for poor in vivo efficacy is the interaction of 9AC with HSA [10]. The carboxy form of 9AC, like many other camptothecins, binds more strongly to HSA than does the lactone form [9]. As a result, the equilibrium is shifted to the carboxy-form, lowering the lactone form to less than 0.5% of the total dose, an amount insufficient for antitumor activity [4].

We previously synthesized a glucuronide prodrug of 9AC, named 9-aminocamptothecin glucuronide (9ACG) [36], that is composed of glucuronic acid linked by a self-immolative spacer to the amino-group of 9AC (Fig. 1). This compound is a substrate for β-glucuronidase (E.C. 3.2.1.31) and is designed for antibody-directed enzyme prodrug therapy of tumors [37, 38]. Upon enzymatic hydrolysis of the glucuronic acid moiety, self-re-arrangement of the spacer occurs leading to the release of active 9AC. 9ACG exhibits less toxicity and much higher solubility in water than 9AC. Moreover, we showed that if applied as a monotherapeutic agent, 9ACG displays potent antitumor activity against human tumor xenografts [39]. Later investigations revealed that, unlike 9AC, 9ACG binds to HSA in both lactone and carboxy forms equally well and with lower affinity ($K_d \sim 4 \times 10^{-5}$/M) [8], suggesting that 9ACG may exhibit greater antitumor activity than 9AC in humans.

The pH in the interstitial space of many solid tumors is lower (~6.8) as compared with normal tissue (~7.4) [40–42]. Intratumoral pH can be further lowered to 6.0–6.5 by pre-treatment with glucose [40] or glucose

combined with bicarbonate [43] or the mitochondrial inhibitor m-iodobenzylguanidine [44, 45]. Lowering the pH can increase the in vitro cytotoxicity of camptothecin and topotecan by two to threefold [46] and up to 27-fold for some analogues [47]. This behavior has not been found for other classes of drugs investigated [47]. The presumed mechanism includes favoring the ratio of lactone to carboxy forms of the drugs as well as influencing drug uptake [46, 48]. Acidification could also influence the efficacy of 9ACG by increasing the rate of 9ACG enzymatic hydrolysis since the pH optimum of human glucuronidase is 4.5–5.0 [49]. Here we investigated how moderate acidification, interaction with HSA and prodrug activation by β-glucuronidase influence 9ACG cytotoxicity.

Materials and methods

All cell culture media, HSA and buffers were obtained from Sigma Chemical Co. (St Louis, MO, USA). 9AC and 9ACG were synthesized as previously described [36]. Stock solutions were made by dissolving the pure drugs in DMSO to 10 mM. Working solutions were made just before use by diluting the stock solutions in PBS or cell culture medium.

Cell culture

EJ human bladder carcinoma cells and hybridomas producing mAb L6, with specificity for tumor-associated antigen L6 and mAb $H_{25}B_{10}$, with specificity for hepatitis B surface antigen, were obtained from the American Type Culture Collection (Manassas, VA, USA). CL1-5 human lung adenocarcinoma cells [50] were kindly provided by Dr. P. C. Yang, Department of Internal Medicine, National Taiwan University Hospital (Taipei, Taiwan). The cells were routinely propagated in RPMI medium supplemented with 2.98 g/l HEPES, 2 g/l bicarbonate, 10% bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 in air. All cells were free of mycoplasma as determined by PCR.

Enzyme-immunoconjugates

Immunoconjugates (L6-eβG-PEG and $H_{25}B_{10}$-eβG-PEG) were formed by covalently linking tumor specific monoclonal antibody L6 or control monoclonal antibody $H_{25}B_{10}$ to polyethylene glycol-modified β-glucuronidase as previously described [51]. Briefly, L6 and $H_{25}B_{10}$ antibodies were purified from ascites fluid by affinity chromatography on Protein A gel and F(ab')$_2$ fragments were generated by bromelain digestion as described [52]. Recombinant β-glucuronidase derived from *Escherichia coli* (eβG) containing an N-terminal his-tag was purified by Ni-chelate affinity chromatography and polyethylene glycol was covalently attached to the enzyme as described [51]. The L6 or $H_{25}B_{10}$ F(ab')$_2$ fragments were covalently attached to eβG-PEG using the bifunctional reagent SMCC as described [53]. The antigen-binding and enzyme activities of the conjugates were measured as described [53] and found to be similar to the original activities of the antibodies and eβG (data not shown).

Construction and transduction of membrane-anchored hβG

A human βG cDNA was fused to the B7 extracellular and transmembrane domain present in p2C11-eB7 [54] and then inserted into the retroviral vector pLNCX (BD Biosciences, San Diego, CA, USA) to generate pLNCX-hβG-eB7. Recombinant retroviral particles were packaged by co-transfection of pVSVG (Clontech, BD Biosciences, Taipei, Taiwan) with pLNCX-hβG-eB7 into GP2-293 cells (Clontech). After 48 h, the culture medium was filtered, mixed with 8 µg/ml polybrene and added to EJ human bladder carcinoma cells. The cells were selected in G418 and sorted on a flow cytometer to generate EJ-hβG cells.

HPLC

For determination of the lactone forms of drugs, samples were diluted 1:4 in ice-cold methanol. For determination of total drug lactone concentrations in samples containing HSA, proteins were precipitated with ice-cold methanol and the samples were then centrifuged for 5 min at 10,000*g* at 4°C. The recoveries of CPT, CPT-11, 9AC, and 9ACG were 103, 103, 95, and 108%, respectively. Samples containing CPT, 9AC or 9ACG were then diluted 1:1 with 10 mM phosphate buffer pH 6.5 whereas samples containing CPT-11, 10-hydroxy CPT or topotecan were diluted 1:3 with an 80:20 (vol : vol) mixture of acetonitrile and 3% triethanol amine, adjusted to pH 5.5 with acetic acid. For determination of free drugs in samples containing HSA, protein-bound drug was separated from free drug by fast filtration through 30 kDa cut-off spin concentrators (Millipore, Bedford, MA, USA) before the samples were treated as described above. Reversed-phase HPLC for determination of CPT,

9AC, and 9ACG was performed as previously described [8]. Briefly, the mobile phase of 50% methanol in 5 mM phosphate buffer pH 6.5 was run at 1 ml/min. For detection of 9AC, post-column acidification was performed by addition of 0.5 M phosphoric acid at a flowrate of 0.2 ml/min. The mobile phase for analysis of samples containing CPT-11, 10-hydroxy CPT, and topotecan was an 80:20 (vol : vol) mixture of acetonitrile and 3% triethanol amine, adjusted to pH 5.5 with acetic acid and delivered at a flowrate of 1.5 ml/min. The HPLC system consisted of two Waters 600E multi-solvent delivery systems, a Rheodyne 7725i manual injector with a 200 µl loop, a 250×4.6 mm^2 column packed with Hypersil ODS (5 µm), a Gilson 121 fluorometer with 305–395 nm excitation and 430–470 or 500–700 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.

^{3}H-thymidine incorporation assay

RPMI medium containing 10% bovine serum was supplemented with 20 mM HEPES, 20 mM MOPS, and 20 mM bicarbonate for pH 7.6 or 20 mM HEPES, 20 mM MOPS, and 5 mM bicarbonate for pH 6.8 [55]. The pH of the medium was adjusted by addition of NaOH and pre-equilibrated for 12 h at 37°C in an atmosphere of 5% CO_2 in air. The pH of the culture medium was checked both before and after the experiment to assure that cell growth did not significantly affect the pH. Cell lines were seeded overnight in 96-well microplates in pH 7.6 or 6.8 medium. In some experiments, L6-eβG-PEG, and $H_{25}B_{10}$-eβG-PEG conjugates (2.5 µg/ml) were added to CL1-5 cells for 1 h before the cells were washed twice with PBS. Graded concentrations of 9AC or 9ACG were added to the cells in triplicate for 16 h at 37°C. In some experiments, HSA was also added to the culture medium at a final concentration of 40 mg/ml. The cells were subsequently washed and cultured in complete medium without drugs for 24 h before they were pulsed for 16 h with [^{3}H]-thymidine (1 μCi/well) in fresh medium. Radioactivity was measured in a Topcount scintillation counter. Results are expressed as percent inhibition of [^{3}H]-thymidine incorporation compared with untreated control cells by the following formula:

$$\text{Inhibition}(\%) = 100 \times \frac{\text{cpm}_{\text{sample}} - \text{cpm}_{\text{background}}}{\text{cpm}_{\text{control}} - \text{cpm}_{\text{background}}}.$$

Results

Influence of pH on the rate of 9AC and 9ACG lactone to carboxy conversion

The conversion of the lactone to the carboxy forms of 9AC and 9ACG was investigated by incubating the lactone form of the drugs at defined pH values ranging from 7.4 to 6.0 and monitoring the lactone concentrations by HPLC (Fig. 3). The rate of conversion as well as the equilibrium concentrations of the lactone forms of 9AC and 9ACG were similar and strongly pH dependent. Equilibrium conditions were reached within 1–3 h. Even a small decrease in pH resulted in a relatively large decrease in the rate of hydrolysis resulting in a much higher level of lactone remaining after equilibrium was reached (about 20% at pH 7.4, but as high as 95% at pH 6.0).

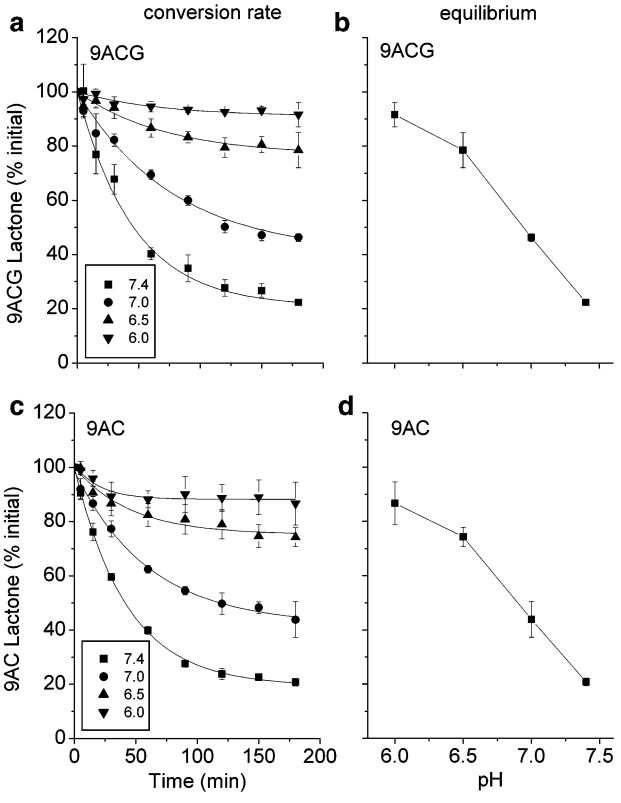


Fig. 3 Kinetics and equilibrium of the conversion of the lactone to carboxy forms of 9ACG and 9AC. The lactone form of 9ACG (**a, b**) or 9AC (**c, d**) were exposed to different pH values and the percentage of lactone present at the indicated times was determined by HPLC. The equilibrium percentage of lactone at various pH values is also shown (**b, d**). *Error bars* indicate the standard deviation of triplicate determinations

pH and HSA dependent lactone-carboxy equilibrium

The influence of HSA on the total and free (non-protein bound) lactone equilibrium levels of 9ACG, 9AC, and four other camptothecin drugs (irinotecan, topotecan, camptothecin, and 10-hydroxy camptothecin) was investigated (Fig. 4). At pH 7.4, HSA greatly reduced the equilibrium concentrations of the lactone forms of 9AC, camptothecin and 10-hydroxy camptothecin from about 20 to 0.5–3%. In contrast, the lactone forms of 9ACG, irinotecan, and topotecan remained at about 20%. At lower pH values, the trend was the same but both initial and final lactone levels were increased. Addition of HSA reduced the amount of free (unbound) lactone at all pH values examined (7.4–6.0) except for topotecan, which does not bind to HSA. HSA had the greatest effect on camptothecin, reducing the amount of free lactone to almost undetectable levels at pH 7.0. HSA had less effect on 9ACG, reducing the amount of free lactone from 80 to about 40% at pH 6.0. It is interesting that the level of free 9AC lactone at pH 7.4 was almost as low as camptothecin (Fig. 4), but was greatly increased (~55%) at pH 6.0, reaching a level that was comparable to that observed with Irinotecan (CPT-11).

Influence of pH, HSA, and β-glucuronidase on 9ACG cytotoxicity

The effect of 9AC and 9ACG on EJ human bladder carcinoma cells was investigated by exposing the cells to graded concentrations of the drugs for 16 h and then measuring the incorporation of ^{3}H-thymidine into cellular DNA 24 h later. The cells were cultured in drug-free medium for 24 h to minimize measurement of the cytostatic effects of the drugs. At pH 7.6, the IC_{50} of 9AC to EJ cells was 33 nM whereas, as expected, 9ACG displayed reduced toxicity with an IC_{50} value of 480 nM (Fig. 5a). Acidification of the cells to pH 6.8 resulted in about a threefold decrease in the IC_{50} of 9AC to EJ cells (IC_{50} = 12 nM) but about a fivefold decrease in the IC_{50} of 9ACG (IC_{50} = 98 nM) (Table 1). When HSA was present at pH 7.6, neither 9AC nor 9ACG displayed measurable cytotoxicity at the highest concentrations examined (1,000 nM for 9AC and 10,000 nM for 9ACG) (Fig. 5b). Acidification to pH 6.8 lowered the IC_{50} of 9AC to 580 nM.

Since 9ACG is a glucuronide prodrug, we wished to investigate the effect of the presence of local β-glucuronidase on the cytotoxicity of the drugs. We therefore generated a membrane-anchored form of β-glucuronidase by fusing the cDNA for hβG to a DNA fragment encompassing the Ig-like C2-type and Ig-hinge like domains, transmembrane domain, and cytoplasmic tail of murine B7-1 (CD80). Stable EJ-hβG cells were then generated by retroviral transduction of EJ bladder carcinoma cells. As expected, expression of hβG on EJ cells had essentially no effect on the toxicity of 9AC (Fig. 5c). In contrast, the IC_{50} of 9ACG was decreased from 480 nM for EJ cells to 215 nM for EJ-hβG cells at pH 7.6 and from 98 to 26 nM at pH 6.8. The greater cytotoxicity of 9ACG to EJ-hβG cells at pH 6.8 can be attributed to the greater activity of hβG at acidic pH

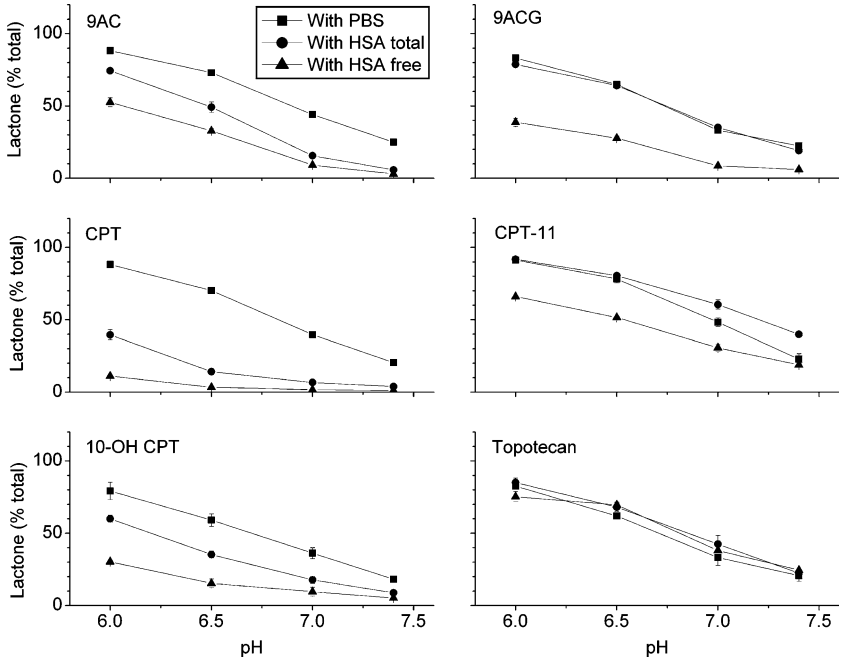


Fig. 4 Influence of pH and HSA on the equilibrium levels of total lactone and free lactone levels of six camptothecin drugs. The lactone forms of the drugs were incubated at defined pH values in PBS or in PBS with 40 mg/ml HSA for 4 h. The percentages of each drug that was in the lactone form in PBS (*filled square*) or in the lactone form in PBS containing HSA (*filled circle*) as well as the amount of unbound (''free'') lactone in PBS containing HSA (*filled triangle*) are indicated. *Error bars* show the standard deviation of triplicate determinations

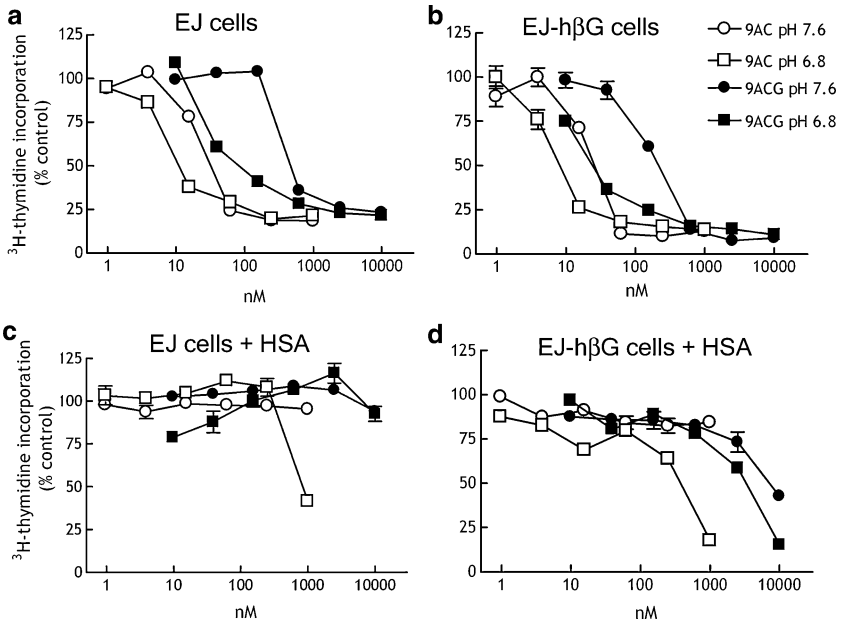


Fig. 5 Influence of pH, HSA, and activation by human *β*-glucuronidase on 9ACG and 9AC cytotoxicity. EJ cells (**a, b**) and EJ-h*β*G cells (**c, d**) were exposed for 16 h to graded concentration of 9AC or 9ACG in PBS (*top panels*) or 40 mg/ml HSA (*bottom panels*) at pH 7.6 or 6.8. The incorporation of ^{3}H-thymidine into cellular DNA was measured after the cells were cultured in fresh medium for an additional 24 h. Results represent the mean values of triplicate determinations. *Bars*, SD

Table 1 Summary of the effect of pH, HSA and human *β*-glucuronidase (as model of monotherapy with acidification in humans) on 9AC and 9ACG cytotoxicity

Cell type	Drug:	9AC (nM)				9ACG (nM)			
	HSA:	No HSA		HSA		No HSA		HSA	
	pH:	7.6	6.8	7.6	6.8	7.6	6.8	7.6	6.8
EJ		33 ± 0.3	12 ± 0.9	>1,000	580 ± 22	480 ± 12	98 ± 12	≫10,000	≫10,000
EJ-h*β*G		25 ± 1.4	8 ± 0.6	>1,000	390 ± 21	215 ± 17	26 ± 1.6	7,900 ± 500	3,400 ± 230

Results show the mean IC_{50} values of triplicate determinations ± standard deviation

(optimum pH = 4.5). In the presence of HSA, the IC_{50} of 9ACG decreased from >10,000 nM for EJ cells to 7,900 nM for EJ-h*β*G cells at pH 7.6 and to 3,400 nM at pH 6.8 (Fig. 5d).

Immunoconjugate activation of 9ACG

The cytotoxicity displayed by 9ACG in the presence of HSA at EJ-h*β*G cells suggested that local generation of 9AC at tumors might help overcome the neutralizing effects of HSA. To investigate this hypothesis further, CL1-5 human cancer cells were incubated with the L6-e*β*G-PEG immunoconjugate containing *β*-glucuronidase derived from *E. coli*, which displays high activity at neutral pH values. The L6 antibody portion of the immunoconjugate can bind to tumor-associated L6 antigen present on the surface of CL1-5 cells, thereby coating the surface of the cells with e*β*G. Table 2 and Fig. 6a shows that 9AC displayed an IC_{50} value of 28 nM to CL1-5 cells whereas 9ACG was less toxic with an IC_{50} value of 240 nM. Incubation of the cells with L6-e*β*G-PEG allowed effective activation of 9ACG, producing similar cytotoxicity as 9AC. This result shows that the enzymatic activity of L6-e*β*G-PEG was greater than the enzymatic activity of h*β*G present on EJ-h*β*G cells since 9ACG cytotoxicity was substantially less than 9AC to EJ-h*β*G cells (Fig. 5c). Incubation of the cells with a control antibody immunoconjugate, in contrast, did not increase the cytotoxicity of 9ACG, showing that the effect of L6-e*β*G-PEG required antigen-specific binding to CL1-5 cells. Remarkably, in the presence of HSA, CL1-5 cells coated with L6-e*β*G-PEG were more sensitive to 9ACG than to 9AC (Fig. 6b), indicating that local generation of 9AC at the tumor cell surface can partially prevent neutralization of the drug by HSA.

Discussion

Maintaining camptothecins in the active lactone form during therapy is crucial for antitumor activity [56]. The pH in the interstitial space of many tumors is lower (pH ≈ 6.8) than that in the corresponding normal tissue [40–42] and can be decreased to 6.5 or in some cases as low as 6.0 by pharmacological manipu-

Table 2 Summary of the effect of HSA and activation by immunoconjugate-delivered *Escherichia coli* β-glucuronidase (model for ADEPT) on 9AC and 9ACG cytotoxicity

Cell type	Drug:	9AC (nM)		9ACG (nM)	
	HSA:	No HSA	HSA	No HSA	HSA
CL1-5		28 ± 1.5	≫1,000	240 ± 25	≫10,000
CL1-5 + H25-eβG-PEG		–	–	420 ± 35	≫10,000
CL1-5 + L6-eβG-PEG		–	–	24 ± 0.3	970 ± 42

Results show the mean IC_{50} values of triplicate determinations ± standard deviation

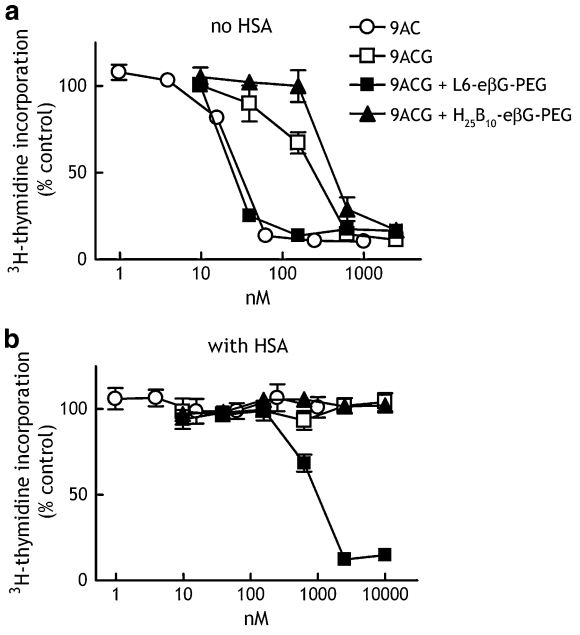


Fig. 6 Influence of HSA of the cytotoxicity generated by immunoconjugate activation of 9ACG. CL1-5 cells were incubated with PBS (*open symbols*), 5 μg/ml L6-eβG-PEG (*filled square*) or 5 μg/ml control immunoconjugate $H_{25}B_{10}$-eβG-PEG (*filled triangle*), washed and then exposed for 16 h to graded concentrations of 9AC or 9ACG in medium (*top panel*) or medium containing 40 mg/ml HSA (*bottom panel*). The incorporation of ^{3}H-thymidine into cellular DNA was measured after the cells were cultured in fresh medium for 24 h. Results represent the mean values of triplicate determinations. *Bars*, SD

lation [40, 43–45].We therefore investigated whether these relatively moderate differences in pH could influence the conversion of 9ACG lactone to 9ACG carboxy. Decreasing the pH dramatically influenced the rate of conversion as well as the equilibrium levels of the lactone form of 9ACG. The equilibrium level of 9ACG lactone increased from about 20% at pH 7.4 to 70% at pH 6.5 and to as high as 95% at pH 6. These differences in the equilibrium levels of 9ACG lactone could be even more emphasized in vivo due to slower elimination of 9ACG from the circulation, because the lactone form of camptothecins can incorporate into the lipid membranes of erythrocytes, thereby slowing elimination [5, 10]. The kinetics and equilibrium of the lactone to carboxy conversion for 9ACG and the parent drug 9AC (Fig. 3) were similar and agreed well with data published for other camptothecins [2, 7, 9, 57]. Differences in the structures of these drugs apparently do not influence the lactone equilibrium probably because the modifications are located far from the lactone ring. Similarly, the presence of a bulky spacer and polar sugar moiety did not influence the lactone ring stability of 9ACG.

Human serum albumin binds to most camptothecins with the expected behavior in which the negatively charged carboxy form binds more strongly to HSA (K_d ~10^{-6}/M) than does the corresponding lactone form (K_d~10^{-4}/M) [4–8]. Some camptothecins such as SN-38, however, do not follow this pattern, with the lactone form binding more strongly than the carboxy form [5]. An extreme case is topotecan, which does not bind to HSA in either form [58] and is in human use already. Binding to other blood proteins [9] and erythrocyte membranes [10] also influences the equilibrium. The hydrophobic lactone form of camptothecins readily dissolves into the lipid bilayer of erythrocytes, thereby preserving the lactone ring. This may be a reason why the lactone to carboxy ratio is more favorable in full blood than in plasma [59]. It is generally believed that interactions that maintain the lactone form of the drug are critical for good antitumor activity [5, 9, 56, 58].

Because HSA interactions involve charged groups on both HSA and the ligand, it is reasonable to expect that it could be sensitive to pH changes. In addition, 9ACG could interact with HSA differently than other camptothecins due to the presence of additional polar and charged groups. We therefore compared the effect of pH and HSA on the level of the lactone form of several camptothecins. HSA dramatically reduced the amount of CPT, 9AC, 10-hydroxy CPT, and CPT-11 lactone at pH 7.4. In contrast, HSA did not affect the equilibrium amounts of 9ACG and topotecan lactone. The stability of 9ACG lactone in the presence of HSA may be beneficial for its use in humans. Lowering the pH to 6.0 increased the total lactone levels of all of the

camptothecin derivatives to 80–90%, again emphasizing the potential benefits of tumor acidification.

The amount of free drug (unbound to HSA) was also measured since only the free form is primarily responsible for antitumor activity. The level of free CPT and 9AC lactone were extremely low at neutral pH (<1%), much lower than for irinotecan and topotecan (>20%). The 9ACG and 10-hydroxy CPT displayed intermediate levels of free lactone (about 8%). Acidification to pH 6.0 increased free lactone levels for all drugs investigated to 30–80% except for CPT, which remained at about 10%. Interestingly, 9AC displayed the greatest sensitivity to pH with about a 50-fold increase in free lactone levels when the pH was lowered from 7.4 to 6.0. Acidic pH may promote the uptake and retention of weak bases such as 9AC and 10-aminocamptothecin [47, 60]. These results suggest that the antitumor activity of 9AC may benefit from tumor acidification.

9-aminocamptothecin glucuronide lactone and carboxy forms bind to HSA equally well but with less affinity than does 9AC and CPT [8]. It results in higher levels of free 9ACG lactone in circulation at neutral pH as compared to camptothecin and 9AC. Furthermore, 9ACG lactone bound to HSA could act as a pool of active drug to prolong the circulation of 9ACG lactone. Exposure of the tumor to lower but prolonged levels of the lactone may enhance antitumor activity [31, 59]. Moreover, the HSA-bound lactone may be transported into tumors by a mechanism involving the SPARC protein [13]. On the contrary, the carboxy forms of 9AC and CPT bind more strongly to HSA than do the lactone forms, resulting in a large pool of the carboxy forms of these drugs bound to HSA. This could lead to a short and ineffective exposure of the tumor to the lactone forms of 9AC and CPT, but long and possibly toxic exposure of organs to the carboxy form as it is released from HSA or transported into tumor cells as an HSA complex.

Human serum albumin, and to a lesser degree pH, profoundly influenced the cytotoxicity of 9AC and 9ACG. Acidification from pH 7.6 to 6.8 increased the cytotoxic effect of both 9AC and 9ACG to EJ cells by three to fivefold. The increased cytotoxicity of 9AC and 9ACG at pH 6.8 can be attributed to increased amounts of the lactone forms of the drugs at decreased pH (Fig. 4). Furthermore, β-glucuronidase may be released from dying cells at low pH values, allowing increased enzymatic conversion of 9ACG to 9AC. The addition of physiological concentrations of HSA (40 mg/ml) to the cells at pH 7.6 totally blocked the cytotoxicity of 9AC and 9ACG over the range of the concentrations examined (1 μM for 9AC and 10 μM for 9ACG), consistent with the reduction of free lactone drug measured at neutral pH values (Fig. 4). Modest acidification of the cells to pH 6.8 partially restored the cytotoxic effects of 9AC, also consistent with the pH dependence of 9AC lactone binding to HSA (Fig. 4).

9-aminocamptothecin glucuronide is a prodrug that was designed to be enzymatically activated by β-glucuronidase. Glucuronide prodrugs are sequestered from endogenous lysosomal β-glucuronidase because the charged carboxylic acid group of the glucuronic acid moiety largely prevents diffusion of glucuronide prodrugs across the lipid bilayer of the plasma membrane of cells [61]. Glucuronides can be excreted by cMOAT into the intestine and be activated by bacterial β-glucuronidase. Thus, diarrhea is often a serious side-effect in patients treated with CPT-11 because the active metabolite SN-38 is readily converted to a glucuronide that is excreted into the intestine [59]. However, we did not detect any signs of diarrhea in nude mice after treatment with 9ACG [39].

Glucuronide prodrugs can be selectively hydrolyzed in tumors due to accumulation of β-glucuronidase in the interstitial tumor space [62] or by targeting β-glucuronidase to tumors as an antibody-enzyme immunoconjugate [53, 63–66]. We therefore investigated the effect of β-glucuronidase on the cytotoxicity of 9ACG by anchoring human β-glucuronidase on the membrane of EJ cancer cells to model local activation of 9ACG in the interstitial space of tumors. The presence of β-glucuronidase on EJ cells increased the cytotoxicity of 9ACG, especially at pH 6.8 where human β-glucuronidase is more active due to its acidic pH optimum [49]. In the absence of HSA, acidification to pH 6.8 and expression of human β-glucuronidase increased the cytotoxicity of 9ACG about 18-fold. In the presence of HSA, expression of human β-glucuronidase also increased the cytotoxicity of 9ACG from undetectable levels to the low-micromolar level. It is important to note that human β-glucuronidase expressed on the plasma membrane of EJ cells activates 9ACG inefficiently, resulting in only slow and partial hydrolysis of 9ACG to 9AC [67].

The effect of local prodrug activation in the presence of HSA was further investigated by targeting a L6 immunoconjugate containing β-glucuronidase derived from *E. coli* to CL1-5 tumor cells. In contrast to human β-glucuronidase on EJ cells, the bacterial enzyme displays optimal activity at neutral pH values. CL1-5 cells express high levels of the L6 antigen on their surface [5], allowing accumulation of high β-glucuronidase activities on the tumor cells. The L6-eβG-PEG immunoconjugate effectively activated 9ACG at CL1-5

cells and generated cytotoxic effects that were similar to direct treatment of the cells with 9AC. Remarkably, in the presence of HSA, the L6 immunoconjugate-treated cells were even more sensitive to 9ACG than to 9AC. This result shows that local generation of 9AC from 9ACG at the tumor cell membrane can produce greater cytotoxicity than systemic 9AC. This suggests that some enzymatically generated 9AC may enter the cells before it is converted to 9AC carboxy or is bound by HSA. This could potentially improve the therapeutic index of 9ACG since intratumorally generated 9AC may enter tumor cells and exert cytotoxicity whereas 9AC that leaked from the tumor into the circulation could be bound and effectively neutralized by HSA, thereby reducing systemic cytotoxicity. The rate and extent of 9ACG hydrolysis appears to be important for improved antitumor activity in the presence of HSA, since human β-glucuronidase on EJ cells was less effective at increasing 9ACG potency.

The data presented highlight the benefits of lowering intratumoral pH for 9AC and 9ACG due to stabilization of the lactone ring and reducing binding to HSA. However, HSA binding remains a serious obstacle to 9AC, emphasizing that the development of low HSA-binding drugs is highly desirable. HSA may be less of an obstacle for 9ACG if the prodrug can be enzymatically activated at the tumor surface since active drug can apparently enter tumor cells before it is sequestered by HSA. Binding of active 9ACG lactone by HSA may also create a pool of prodrug to allow sustained exposure of the tumor to low concentrations of active 9AC lactone, which can be effective for cancer therapy [31]. The in vivo behavior of 9ACG is likely more complicated and the net effect of HSA on 9ACG antitumor activity will require further in vivo studies against immunoconjugate-treated tumors.

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