Tumor-targeted and activated bioconjugates for improved camptothecin delivery

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Earlier reports from our laboratory described bioconjugates of camptothecin (CPT) for tumor targeting. In the current work, the rate and site of CPT release from the bioconjugates were modulated using increasingly sterically hindered amino acids and cysteine proteinase-sensitive peptide linkers, respectively. Polyethylene glycol served as a spacer/scaffold between CPT and folic acid. The folic acid receptor, overexpressed on many cancer cells, was targeted using folate. The delivery system was tested *in vitro* for hydrolytic stability, enzyme-mediated cleavage, cytotoxicity and targeting potential. The linkers successfully modulated the hydrolysis rate (around 1–100 h) and potential site (tumor microenvironment) of CPT release. Preliminary molecular modeling approaches were utilized to assess the influence of molecular volume on hydrolysis half-life (i.e. CPT release). There was a clear, but non-linear, relationship between *in vitro* CPT release and increasing steric hindrance offered by the peptide linker. The efficacy of four conjugates was studied in a syngeneic rat breast cancer model. Histopathological analysis on treated tumors was performed to evaluate disease prognosis. The results demonstrate that programmed bioconjugates may provide superior efficacy and greater control over the rate and site of CPT release, resulting in higher anti-tumor efficacy and lower toxicity. *Anti-Cancer Drugs* 16:763–775 © 2005 Lippincott Williams & Wilkins.

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Introduction

Camptothecin (CPT) is a water-insoluble, naturally occurring alkaloid isolated from *Camptotheca acuminata*, a tree native to China, and from *Mappia foetida*, a tree abundant in Western India [1]. As a class of anti-cancer compounds, the CPTs pose a variety of challenges in terms of optimal delivery to the target tissue [2]. Our laboratory has previously reported bioconjugates of CPT with improved targeting potential to cancer cells [3,4]. The aim of the present study was to improve upon the earlier design by modulating the *rate* and *site* of CPT release using novel linkers. It is hypothesized that, by designing a targeted bioconjugate with selective and programmed release properties, dose-limiting side-effects associated with CPT can be minimized while the anti-tumor activity can be preserved and focused in diseased tissues.

The CPT bioconjugate described earlier [3,4] used polyethylene glycol (PEG) and glycine as the spacer and linker, respectively, to covalently attach CPT to a targeting moiety. Upon internalization into cells by active transport/receptor-mediated endocytosis, the bioconjugate enters endosomes and lysosomes that have a mildly acidic (pH 5) environment. This pathway offers a selective mechanism of drug release if the drug-carrier linkers possess adequate differences in their rates of hydrolysis. Anti-tumor conjugates that are internalized and processed within lysosomes or tumor cells are also exposed to a multitude of proteolytic enzymes that serve a catabolic role in cells [5]. Most of these proteases are not found extracellularly. This presents an opportunity to use peptide linkers that are enzymatically cleaved only upon internalization [6,7]. One way to enhance the plasma stability of bioconjugates is to use steric hindrance to reduce access to cleavable bonds [8]. Simple carboxylic acid esters of amino acids are not very stable in plasma; however, by virtue of increased steric hindrance around the ester bond, it is hypothesized that reasonable control over the rate of hydrolysis can be achieved. The ultimate aim of achieving control over the rate of drug release is to increase the half-life of hydrolysis in plasma and control the half-life of hydrolysis at the target tissue. Thus, the bioconjugate should be inactive in circulation, only releasing active free drug in the target tissue. Greater control over the pharmacodynamics of the drug delivery system can be attained by incorporating the features of enzyme-sensitive linkers for site-specific cleavage and steric hindrance for stability in plasma.

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When investigating targeted drug delivery systems that are dependent on the tumor microenvironment for the targeting effect (cell surface receptors, tumoral drug metabolism, lysosomal enzymes, etc.), *in vivo* studies become very important. Since the *in vivo* milieu of the tumor is very different from *in vitro* cell culture models, the targeting mechanism may not be fully functional in the *in vitro* environment. The *in vivo* study becomes more important in the present research since the *in vitro* systems used thus far do not test the folate competition for limited folate receptors posed in an *in vivo* situation.

In our continuing efforts to devise a tumor-selective targeted delivery system for CPT, CPT–amino-acid–PEG–folate conjugates have been synthesized. The present studies are aimed at achieving greater control over the site and rate of CPT release. In this study, tumor-selective bioconjugates were synthesized by introducing increasingly sterically hindered amino acids as linkers to modulate the release properties of cleavable bonds and cysteine proteinase-sensitive peptide linkers to control the site of release. Cancer cell specificity was achieved using folic acid, which has been extensively studied for increasing the delivery of pro-drugs to various tumors by virtue of overexpression of folate receptors in various cancer cells [9]. A macromolecular conjugate approach was taken using PEG because of the enhancement of aqueous solubility and increase in circulation half-life that can be achieved [10]. The bioconjugates were tested *in vitro* and in *in vivo* syngeneic breast cancer models. By using this multifaceted strategy, the goal is to achieve targeted delivery and selective release of CPT. It is hypothesized that these conjugates will improve delivery of CPT to cancer cells leading to better and enhanced tumor regression.

Materials and methods

Materials

CPT, folic acid and cathepsin B from human liver were obtained from Sigma (St Louis, Missouri, USA). t-boc protected amino acids and short peptides were obtained from Bachem (King of Prussia, Pennsylvania, USA). t-boc–PEG–NHS (molecular weight around 3400 kDa) was obtained from Shearwater Corporation (Huntsville, Alabama, USA). *N*,*N*′-Dicyclohexylcarbodimide (DCC), 4-dimethylamino-pyridine (DMAP) and *N*-hydroxysuccimide (NHS) were obtained from Nova Biochem (San Diego, California, USA). All CPT bioconjugates and CPT were dissolved in intralipid vehicle prior to *in vivo* drug treatments and doses were based on their CPT equivalents (absolute amount of CPT given). Intralipid (20% emulsion) was obtained from Sigma and used as received. Digital Vernier calipers and 1-cm^3 tuberculin syringes were obtained from VWR Scientific (West Chester, Pennsylvania, USA). All other chemicals were purchased from Sigma or Fisher Scientific (Fair Lawn, New Jersey, USA) and used as received.

Cell culture

KB, a human nasopharyngeal carcinoma cell line known to overexpress folate receptor, was obtained from ATCC (Rockville, Maryland, USA) and frozen at −80°C. Prior to the experiment cells were cultured at 37°C/5% CO_2 in folate-free RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids and 2% penicillin/streptomycin. The heat-inactivated serum contained its normal complement of endogenous folates which enabled the cells to sustain growth in this more physiologically relevant medium [11]. The cells were seeded on a Corning Costar polycarbonate 96-well plate for MTT assay or on 12-well polycarbonate plates for uptake and inhibition studies. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. All experiments were performed on cells in the exponential growth phase. MAT III-B rat breast carcinoma cell line was obtained from Dr Michael Liebowitz at the UMDNJ (Piscataway, New Jersey, USA) and frozen at −80°C. Prior to the experiment cells were cultured at 37°C/5% CO_2 in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids and 2% penicillin/streptomycin.

Design and synthesis of CPT bioconjugates

The design of the bioconjugates remained essentially similar as described earlier [3,4]. CPT, the anti-cancer compound, was linked to a 3.4-kDa PEG via amino acid/peptide linkers. The other end of PEG is linked to folic acid, the targeting moiety. Two series of bioconjugates were synthesized with (i) linear, increasingly sterically hindered amino acid linkers (Ala, Pro, Pro–Gly, Pro–Ala), and (ii) tri- and tetrapeptide linkers sensitive to cysteine proteinases (Gly–Gly–Gly and Gly–Gly–Phe–Gly).

A similar procedure was used to synthesize all the bioconjugates. The results are tabulated in Table 1. Synthesis of CPT–Ala–PEG–folate is described below as an example. The synthesis of CPT–Gly–PEG–folate has been published previously [4].

The general synthetic route is shown in Figure 1. The final bioconjugate was synthesized in three steps—Step 1: Synthesis of CPT–Alanine ester. Step 2: Synthesis of CPT–Ala–PEG conjugate and Step 3: Synthesis of CPT–Ala–PEG–folate.

The alanine ester of CPT was synthesized by a modified procedure as described earlier. Briefly, t-boc-L-alanine (163 mg, 0.87 mmol) was dissolved in 100 ml of anhydrous dichloromethane (DCM) at room temperature. To this was added CPT (100 mg, 0.29 mmol), DCC (177 mg, 0.87 mmol) and DMAP (70 mg, 0.58 mmol) at 4°C. The

Table 1 Synthesis of CPT bioconjugates: NMR signals, mass and amino acid analysis

Bioconjugate	Amino acid analysis
CPT–Ala–PEG–folate	alanine/glutamic acid
CPT–Pro–PEG–folate	proline/glutamic acid
CPT–Gly3–PEG–folate	glycine/glutamic acid
CPT–Gly2PheGly–PEG–folate	glycine/phenylalanine/glutamic acid
CPT–Pro–Gly–PEG–folate	proline/glycine/glutamic acid
CPT–Pro–Ala–PEG–folate	proline/alanine/glutamic acid

^{1}H-NMR (DMSO-d_6):CPT–Ala–PEG–folate: δ 4.3 CH (m), δ 4.4 CH_2 (s), δ 8.7 CH (s), δ 6.6 CH_2 (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m), δ 7.6 *o*-CH, *o*-CH (s), δ 0.9 CH_3 (t), δ 1.9 CH_2 (m), δ 5.3 CH_2 (s), δ 5.5 CH_2 (s), δ 6.7 CH (s), δ 7.4 *p*-CH (s), δ 8.2 *o*-CH, *o*-CH (d), δ 7.6-8.2 2H *m*-CH (d), δ 1.4 CH_3 (d), δ 3.7 CH (t). Mass: 4384. CPT–Pro–PEG–folate: δ 4.3 CH (m), δ 4.4 CH_2 (s), δ 8.7 CH (s), δ 6.6 CH_2 (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m), δ 7.6 *o*-CH, *o*-CH (s), δ 0.9 CH_3 (t), δ 1.9 CH_2 (m), δ 5.3 CH_2 (s), δ 5.5 CH_2 (s), δ 6.7 CH (s), δ 7.4 *p*-CH (s), δ 8.2 *o*-CH (d), δ 7.6-8.0 2H *m*-CH (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m). Mass: 4410. CPT–Gly3–PEG–folate: δ 7.6 *o*-CH, *o*-CH (s), δ 4.3 CH (m), δ 4.4 CH_2 (s), δ 8.7 CH (s), δ 6.6 CH_2 (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m), δ 0.9 CH_3 (t), δ 1.9 CH_2 (m), δ 5.3 CH_2 (s), δ 5.5 CH_2 (s), δ 6.7 CH (s), δ 7.4 *p*-CH (s), δ 8.2 *o*-CH, *o*-CH (d), δ 7.6-8.0 2H *m*-CH (d), δ 3.7 CH_2 6H (s). Mass: 4484.84. CPT–Gly2PheGly–PEG–folate: δ 7.6 *o*-CH, *o*-CH (s), δ 4.3 CH (m), δ 4.4 CH_2 (s), δ 8.7 CH (s), δ 6.6 CH_2 (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m), δ 0.9 CH_3 (t), δ 1.9 CH_2 (m), δ 5.3 CH_2 (s), δ 5.5 CH_2 (s), δ 6.7 CH (s), δ 7.4 *p*-CH (s), δ 8.2 *o*-CH, *o*-CH (d), δ 7.6-8.0 2H *m*-CH (d), δ 3.6 CH_2 7H (s), δ 3.0 CH_2 (d), δ 7.2 5H (benzene ring of Phe). Mass: 4631.4. CPT–Pro–Gly–PEG–folate: δ 4.3 CH (m), δ 4.4 CH_2 (s), δ 8.7 CH (s), δ 6.6 CH_2 (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m), δ 7.6 *o*-CH, *o*-CH (s), δ 0.9 CH_3 (t), δ 1.9 CH_2 (m), δ 5.3 CH_2 (s), δ 5.5 CH_2 (s), δ 6.7 CH (s), δ 7.4 *p*-CH (s), δ 8.2 *o*-CH, *o*-CH (d), δ 7.6 -8.0 2H *m*-CH (d), δ 3.7 CH_2 (t), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m). Mass: 4467. CPT–Pro–Ala–PEG–folate: δ 4.3 CH (m), δ 4.4 CH_2 (s), δ 8.7 CH (s), δ 6.6 CH_2 (d), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m), δ 7.6 *o*-CH, *o*-CH (s), δ 0.9 CH_3 (t), δ 1.9 CH_2 (m), δ 5.3 CH_2 (s), δ 5.5 CH_2 (s), δ 6.7 CH (s), δ 7.4 *p*-CH (s), δ 8.2 *o*-CH, *o*-CH (d), δ 7.6-8.0 2H *m*-CH (d), δ 1.4 CH_3 (d), δ 3.7 CH (t), δ 2.0 CH_2 (m), δ 2.3 CH_2 (m). Mass: 4481.

reaction was stirred for 1 h, allowed to come to room temperature and left overnight. After removing the dicyclohexylurea (DCU) precipitate the filtrate was washed with 5% aqueous sodium bicarbonate ($NaHCO_3$) and dried over magnesium sulfate ($MgSO_4$). The solvent was evaporated under reduced pressure. The t-boc protection group was removed by dissolving the precipitate in 100 ml of 1:1 trifluoroacetic acid (TFA): methylene chloride mixture and stirring for 1 h. The solvent was removed under reduced pressure and the product subsequently dissolved in 50 ml DCM; to it was added excess methanolic HCl and this was allowed to stand at room temperature for 30 min. The solvent was removed under reduced pressure and the product was precipitated using dry ether. The product was recrystallized from a 1:1 mixture of methanol:ethanol to give CPT–Ala. MS (ESI) m/z (M + 1) = 419.9. Retention time (R_t) on analytical high-performance liquid chromatography (HPLC; μBondapak C_{18} RP): 5.9 min.

Conjugation of CPT–Ala to PEG (3.4 kDa) was achieved using activated derivative of PEG. Briefly, t-boc-PEG–NHS (100 mg, 0.03 mmol) was dissolved in 10 ml dimethylformamide (DMF) and to it were added CPT–Ala (36 mg, 0.09 mmol) and 1% diisopropylethylamine (DIEA, 100 μl). The reaction was stirred for 3 h at room temperature. The solvent was removed under reduced pressure and the t-boc protection was removed by stirring with 10 ml of 1:1 mixture of TFA:DCM for 3 h. The solvent was evaporated under reduced pressure. The product CPT–Ala–PEG–NH_2 was precipitated in dry ether and washed 3 times with cold ether. The precipitate was recrystallized from 2-propanol, washed with ether and vacuum dried. The product was purified using size-exclusion chromatography (SEC; LH-20 Sephadex). Briefly, a Sephadex LH-20 column (40 ml) was packed and eluted with DMF using gravity flow. The fractions were monitored for fluorescence at excitation/emission λ 370/420 (CPT) using a Tecan multiwell plate reader. Unreacted CPT–Ala eluted in the column volume while the pure CPT–Ala–PEG–NH_2 eluted in the void volume. The relevant fractions were pooled and DMF was removed under reduced pressure. The purified product was precipitated using ether, washed 3 times and dried under vacuum overnight.

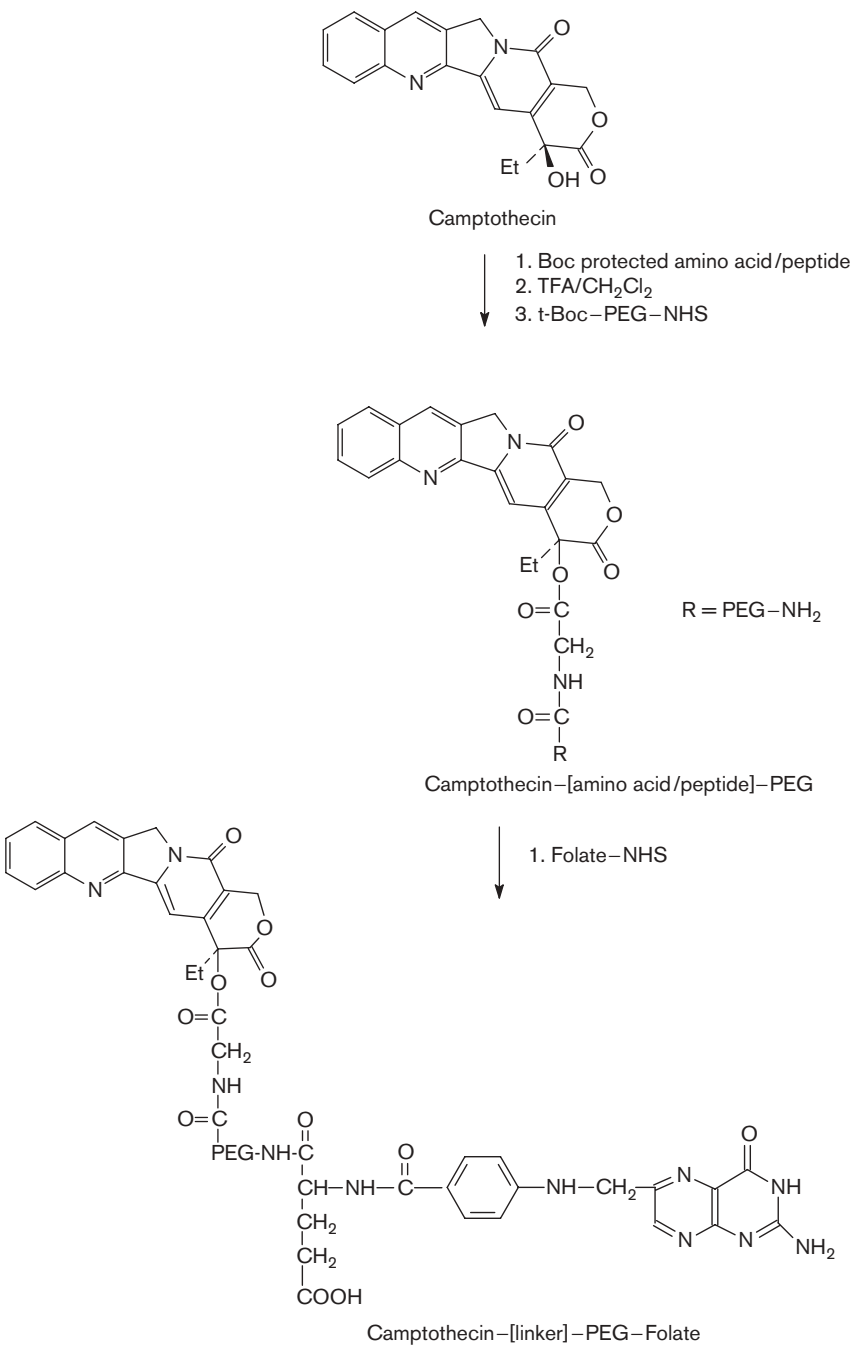
In the third step, pre-activated folic acid was reacted with CPT–Ala–PEG–NH_2. Folate–NHS was synthesized as follows. Folic acid (56 mg, 0.19 mmol), NHS (16 mg, 0.14 mmol), DCC (28.8 mg, 0.14 mmol) and DMAP (17 mg, 0.14 mmol) were dissolved in 2 ml anhydrous DMSO, and stirred at room temperature overnight. The reaction was filtered to remove precipitated DCU and directly reacted with CPT–Ala–PEG–NH_2. CPT–Ala–PEG (100 mg, 0.03 mmol) and folate–NHS (0.13 mmol) were dissolved in 2 ml anhydrous DMSO. Then 1% DIEA (20 μl) was added to the reaction. After stirring for 3 h at room temperature the solution was passed directly through a LH-20 Sephadex SEC column as described above to obtain the purified bioconjugate.

The conjugate was characterized using ^{1}H-NMR (Varian, 600 MHz). Fourier transform (FT)-IR (Mattson Cygnus 100; KBr pellet) analysis: 1500–1650 cm^{-1} (amide) and 1727 cm^{-1} (ester). Mass spectrometry was performed as described previously using Applied Biosystems Voyager DE Pro matrix-assisted laser desorption time of flight mass spectrometer (MALDI-TOF). The difference in the molecular weight of the final conjugate and unreacted PEG corresponds to the molecular weight additions on the PEG skeleton [3,4]. Amino acid analysis was performed on the bioconjugate to confirm the presence of alanine (from the linker) and glutamic acid (from folic acid) as described previously [4].

Stability of CPT amino acid esters in phosphate-buffered saline (PBS)

The stability of the amino acid/peptide esters of CPT was tested in PBS, pH 7.4 at 37°C. An approximately 10 μg/ml solution of various esters was prepared in PBS (pH 7.4). Aliquots of this solution were incubated at 37°C for various periods of time. After the specified incubation, the aliquots were diluted with 0.1 N HCl (1:1), vortexed and analyzed by reverse-phase HPLC to quantitate CPT

Fig. 1



General synthetic scheme for CPT bioconjugates.

esters. The column was C_{18} (μBondpack, 10 μm, 3.9 × 300 mm; Waters Associate, Milford, Massachusetts, USA) preceded by a C_{18} μBondpack guard column. The mobile phase was a mixture of 35% acetonitrile with 65% aqueous buffer containing 100 mM potassium dihydrogen phosphate and 1 mM sodium heptane sulfonate (pH 4.0). The flow rate was maintained at 1 ml/min. CPT esters were detected by a Shimadzu RF-551 fluorescence detector (Shimadzu Scientific Instruments, Princeton, New Jersey, USA) with λ_{ex} at 370 nm and λ_{em} at 420 nm. The concentration of CPT ester remaining at various time points was determined by using a standard curve generated for each ester under similar conditions. The slope of the semi-log plot of concentration of CPT ester

remaining versus time gave the rate constant (first-order reaction). Hydrolysis half-life was calculated using this rate constant. The CPT–Gly2PheGly–PEG–folate conjugate was additionally tested for stability in rat plasma using a similar assay. Rat blood was collected after cardiac puncture and centrifuged at 14 000 r.p.m. for 10 min. The supernatant was collected and pooled to give rat plasma. The stock solution of the bioconjugate was diluted appropriately with the rat plasma and incubated at 37°C. Aliquots of samples were drawn at different time points. The proteins were precipitated using acetonitrile followed by vortexing for 1 min. The supernatant was diluted 1 : 1 with 0.1 N HCl and directly injected onto the HPLC.

Stability of CPT amino acid esters in presence of cathepsin B

To test the hypothesis of site-specific cleavage inside the cancer cells, stability of CPT–Gly–Gly–Phe–Gly and CPT–Gly–Gly–Gly esters was additionally determined in PBS in the presence of human cathepsin B enzyme, known to be present in lysosomes and in tumors. The two CPT esters used in this study were designed to be cleaved by cysteine proteinases based on literature reports [12]. A 10 µg/ml solution of the esters was incubated with 0.002 U of human cathepsin B (diluted from the stock solution of 110 U) in 1 ml pH 6.0 (88 mM KH_2PO_4, 12 mM Na_2HPO_4 and 1.33 mM EDTA) buffer containing 3 mM cysteine. The concentration of enzyme was selected on the basis of *in vivo* concentrations encountered in humans [7]. The mixture was incubated for 6 h. Periodically, samples from this mixture were spotted on a MALDI plate. Subsequently, MALDI analysis was performed on an Applied Biosystems Voyager DE Pro MALDI-TOF spectrometer as described previously (Fig. 2). The mass spectra were then interpreted for the presence of any fragmentation products; parent CPT, CPT–Gly–Gly–Phe, CPT–Gly–Gly, CPT–Gly for CPT–Gly–Gly–Phe–Gly ester or CPT, CPT–Gly–Gly, CPT–Gly for CPT–Gly–Gly–Gly ester.

Cytotoxicity assay

The cytotoxicity of bioconjugates was assessed using a MTT assay as described previously [3].

Uptake inhibition studies in KB cells

The involvement of folate receptor in the KB cell monolayer transport of CPT–[amino acid]–PEG–folic acid was investigated through inhibition studies by co-incubating the bioconjugates with competitive substrate of folate receptor in the donor compartment of 12-well polycarbonate plates. [3′,5′,7,9-^{3}H]Folic acid was co-incubated with 10 µM of CPT–Gly–Gly–Phe–Gly–PEG–folate, 10 µM of CPT–Ala–PEG–folate, 10 µM of untargeted CPT–Gly for 30 min at either 37 or 4°C. Uptake of [3′,5′,7,9-^{3}H]folic acid (10 nM) alone was determined in the respective control study. Briefly, KB cells were seeded on 12-well polycarbonate plates at the density of 1×10^5 cells/ml 4 days before the uptake studies to confirm cell attachment. The cells were washed twice with uptake buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM MES. The pH was adjusted to 6.0 with 3 M Tris. Subsequently the cells were incubated with folic acid and respective competitive substrate for 30 min (time determined in a preliminary folic acid uptake study). At the end of incubation period the cells were washed with ice-cold buffer to stop the uptake. The cells were solubilized by incubating with 0.1% v/v Triton X-100 for 30 min and then repeat pipetting to break the cell membrane. From this solution, 600 µl was used for scintillation counting. From the remaining volume, 10 µl was used for protein concentration determination using the Bio-Rad (Hercules, California, USA) reagent according to the Bradford assay with bovine serum albumin as the standard. Absorbance was read at 595 nm and the amount of protein per well was determined based on the standard curve.

Molecular modeling

Molecular modeling was performed on an SGI Octane workstation (Silicon Graphics, Mountain View, California, USA). Structural manipulations were performed with the molecular modeling package SYBYL 6.9 (Tripos, St Louis, Missouri, USA) using the standard TRIPOS force field. Partial atomic charges of the ligands were calculated with Gasteiger–Marsili formalism implemented in SYBYL. Structures of seven different peptide/amino acid linkers were built using the standard biopolymer fragments. The drug (CPT) and the targeting moiety (folic acid) were constant across all the bioconjugates, and hence were not used for these studies. The structures were then optimized by energy minimization using the conjugate gradient method. Alignment of all seven linkers was performed using the backbone heavy atoms of the linkers.

Molar volume, the volume occupied by 1 mol of ideal gas at standard temperature and pressure (STP), was used as the steric descriptor and it was correlated to experimentally calculated hydrolysis $t_{1/2}$ values. The structures built in SYBYL were imported into a molecular software package SPARTAN (Wavefunction, Irvine, California, USA), and the molar volume and molar surface area were calculated.

Animal protocol and model development

All the animal protocols were reviewed and approved by the Animal Care and Facilities Committee at Rutgers, State University of New Jersey. Inbred female Fischer 344 rats weighing 190–224 g (around 16 weeks) were obtained from Hilltop Laboratory Animals (Scottdale, Pennsylvania, USA). The animals were acclimatized for 1 week. MAT III-B cells were grown in RPMI 1640 media,

washed with Hank's buffer and trypsinized for 5 min. Cells were then collected in PBS and centrifuged at 1500 r.p.m. for 5 min. Cell pellets (1×10^6 cells) were re-suspended in 0.2 ml of PBS and injected s.c. using 1-ml insulin syringes into the back of the rats [13]. To design the treatment protocol, maximum tolerated dose was determined in preliminary experiments by the body weight loss method [14]. Healthy female Fisher 344 rats ($n = 2$/group) were injected i.p. with CPT–Gly–PEG–folate at different dose levels (2.5, 5 and 7.5 mg/kg, CPT content). Body weights were measured daily for 4 weeks. The highest dose to cause a loss of less than 20% of initial weight was considered the MTD.

Anti-tumor efficacy and safety screens

CPT and amino acid–PEG–folate or amino acid–PEG–biotin [3] conjugated forms were tested i.p. in a syngeneic rat MAT III-B breast carcinoma model. The drugs were administered after palpable tumor growth was detected (13th day after tumor injections). All drug solutions were made in Intralipid. The groups included control ($n = 3$), CPT and series of CPT bioconjugates ($n = 5$). The rats were then dosed with 0.5 mg/kg/day (total dose = MTD, CPT equivalents) for 5 consecutive days. Control groups received vehicle (Intralipid). The rats were monitored for 33 days, and the treatment was evaluated as percentage decrease in the tumor volume that was determined by measuring two dimensions with digital calipers and calculated using the formula: tumor volume = (length × width^2)/2.

Treatment groups were evaluated on the basis of percentage tumor growth, tumor growth delay and complete regression of tumors. Percentage tumor growth was calculated as the mean of the tumor volume at the end of the treatment divided by the initial tumor volume (volume at the beginning of the treatment, 13th day). Tumor growth delay (%T/C) represents the percentage by which the treated groups mean tumor volume is delayed in reaching 400 mm^3 as compared to the control group. The tumors were designated as completely regressed when no palpable mass was detected on the back of the rats.

Histopathology of tumors

At the end of study protocol, the rats were sacrificed by CO_2 asphyxiation. The tumors were surgically removed from six rats (one from each treatment group and control), and stored in 10% buffered formalin (10% formaldehyde in PBS + 1 ml methanol) and blinded before sending to the veterinary pathologist. The tumors were sent for histopathology to Taconic Anmed (Rockville, Maryland, USA). Each tumor was trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological evaluation. The histopathological evaluation was performed by a veterinary pathologist.

Statistical analysis

All statistical tests were performed using Jandel SigmaStat version 2.03 (Jandel Scientific, San Raphael, California, USA). A minimal P value of 0.01 was used as the significance level for all tests. One-way analysis of variance and Tukey's test was performed on the uptake data. All data are reported as the mean ± SD unless otherwise noted. The cytotoxicity data were fitted to a sigmoidal curve using Sigma Plot 2001 version 7.0. All other graphs were constructed using GraphPad 4.0.

Results

Table 2 outlines the compounds used in various assays and the rationale for selecting them.

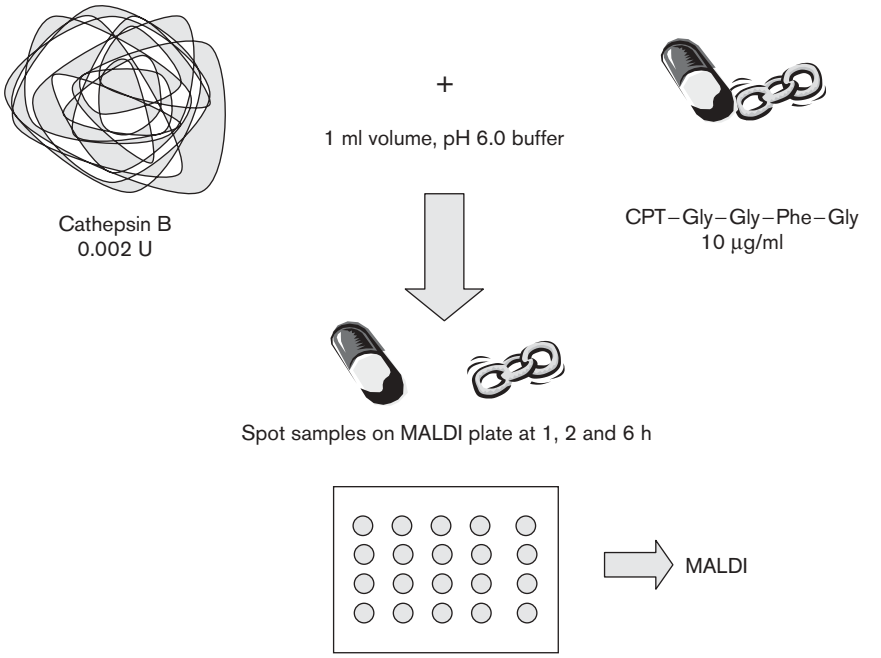
Synthesis and characterization of CPT bioconjugates

The CPT bioconjugates CPT–Ala–PEG–folate, CPT–Pro–PEG–folate, CPT–Pro–Ala–PEG–folate, CPT–Pro–Gly–PEG–folate, CPT–Gly3–PEG–folate and CPT–Gly2PheGly–PEG–folate were successfully synthesized using a simplified three-step procedure (Fig. 2). In the first step, the preparation of an ester of CPT was achieved using a well-established DCC coupling procedure in the presence of DMAP. The ester formation was confirmed by MS (ESI), ^{1}H-NMR, FT-IR and reverse-phase HPLC. In the second step, the CPT–ester was coupled with t-boc–PEG–NHS to form a stable amide bond. The product was purified using SEC (LH-20 Sephadex) and the process was monitored using the fluorescence of CPT. In the third step, the folate was conjugated to the rest of the molecule using an activated form of folic acid, folate–NHS. The pre-activation was achieved by coupling NHS to folic acid in the presence of DCC. The final product was purified using SEC as described in methods. The bioconjugate was characterized using ^{1}H-NMR, FT-IR and mass spectrometry. A comparison of MALDI-TOF spectra of unreacted PEG (molecular weight 3560) with those of the final bioconjugate revealed a shift of around 786 Da in the single mass peaks, indicating that the polymer conjugate carried exactly one CPT and one folic acid molecule. Additionally, no subdistribution of unreacted PEG was observed in the mass spectra of the CPT bioconjugate confirming that the conjugate solutions contained no free amino–PEG.

Stability of CPT amino acid esters in PBS

The stability of all CPT amino acid/peptide esters were tested in PBS (pH 7.4) at 37°C (Table 3). In accordance with the steric hindrance hypothesis, the stability increased as the amino acid was changed from glycine (0.9 h) to alanine (1.0 h) to proline (12 h). In the second series of bioconjugates short peptides were used as linkers. CPT esters increased in stability as the linker

Fig. 2

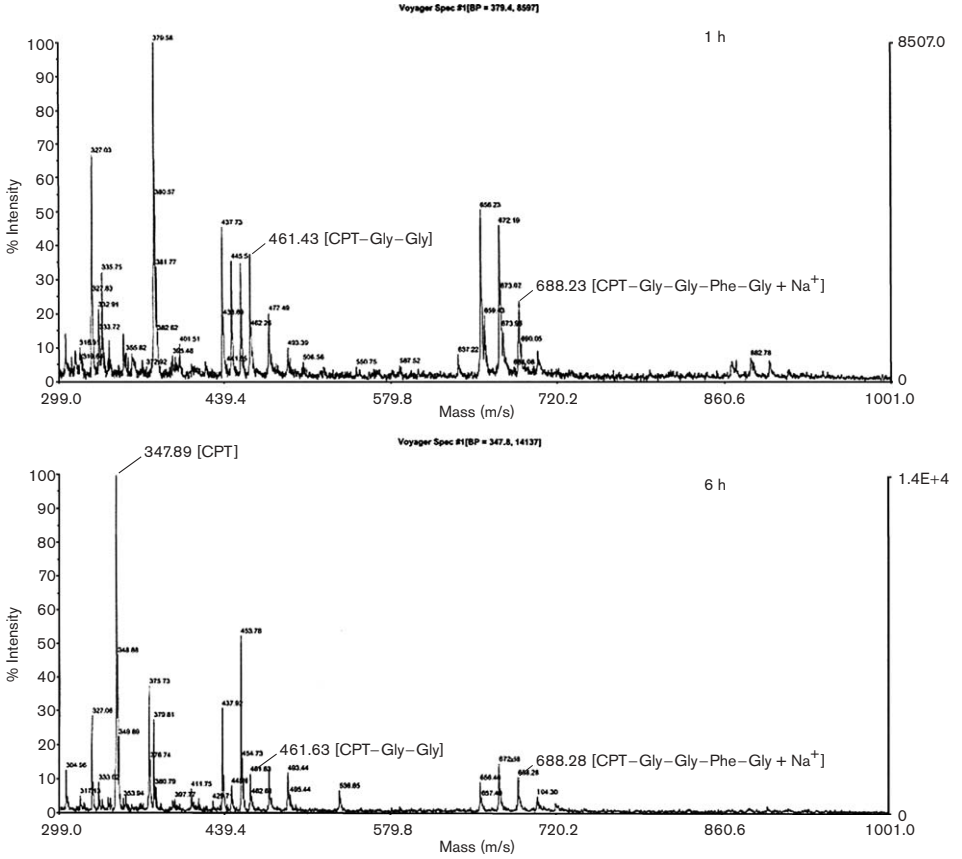


Experimental scheme for Cathepsin B-mediated cleavage.

Table 2 Compounds used for each assay and rationale for selection (a sequential selection procedure was applied and compounds were dropped based on results of each assay)

No.	Test	Compound	Selection rationale
1	Synthesis (seven bioconjugates)	CPT–Gly–PEG–folate CPT–Ala–PEG–folate CPT–Pro–PEG–folate CPT–Pro–Gly–PEG–folate CPT–Pro–Ala–PEG–folate CPT–Gly–Gly–Gly–PEG–folate CPT–Gly–Gly–Phe–Gly–PEG–folate	Two linker series: (i) linear, increasingly sterically hindered amino acids/peptides and (ii) cathepsin B-sensitive peptides. Synthesis of CPT–Gly–PEG–folate has been published previously.
2	Hydrolysis in PBS, pH 7.4, 37°C (seven CPT esters)	CPT–Gly CPT–Ala CPT–Pro CPT–Pro–Gly CPT–Pro–Ala CPT–Gly–Gly–Gly CPT–Gly–Gly–Phe–Gly	Esters of CPT (without PEG–folate part). CPT–Gly–Gly–Phe–Gly was additionally tested in rat plasma.
3	Hydrolysis in presence of cathepsin B (two bioconjugates)	CPT–Gly–Gly–Gly CPT–Gly–Gly–Phe–Gly	Series (ii) linkers, known to be sensitive to cathepsin B.
4	Cytotoxicity, MTT assay (five bioconjugates)	CPT CPT–Gly–PEG CPT–Gly–PEG–folate CPT–Ala–PEG–folate CPT–Pro–PEG–folate CPT–Gly–Gly–Gly–PEG–folate	Cytotoxicity of CPT–Gly–PEG–folate has been published previously. CPT–Pro–Gly–PEG–folate and CPT–Pro–Ala–PEG–folate were not tested due to long half-lives.
5	Targeting Potential, uptake inhibition (three bioconjugates)	CPT–Gly–PEG CPT–Ala–PEG–folate CPT–Gly–Gly–Phe–Gly–PEG–folate CPT–Gly–PEG–biotin	Representative compounds to show targeting, one each from series (i) and (ii), unrelated but targeted CPT–Gly–PEG–biotin and untargeted, CPT–Gly–PEG.
6	*In vivo* (four bioconjugates)	CPT CPT–Gly–PEG–biotin CPT–Gly–PEG–folate CPT–Ala–PEG–folate CPT–Gly–Gly–Phe–Gly–PEG–folate	Selected based on results of 1–4.

Fig. 3



MALDI spectra of CPT–Gly2PheGly–PEG–folate after 1 and 6 h incubation with cathepsin B.

was changed from Gly3 (4.8 h) to Gly2PheGly (6.4 h) to Pro–Gly (29 h) to Pro–Ala (116 h). Additionally, the CPT–Gly2PheGly–PEG ester was found to be stable in rat plasma.

Stability of CPT amino acid esters in presence of cathepsin B

The series of bioconjugates with short peptide linkers were designed to be sensitive to lysosomal enzymes, especially cathepsin B. The stability of CPT–Gly2PheGly and CPT–Gly3 was tested in the presence of this enzyme to test this hypothesis. After just 1 h of incubation with cathepsin B the CPT–Gly2PheGly was degraded to CPT–Gly2 (M^+ 461.43) and CPT–Gly2PheGly ($M^+ + Na$ 688.23). After 6 h the original CPT was visible in the MALDI spectra. CPT (M^+ 347.89) as well as residual CPT–Gly2 and CPT–Gly2PheGly (Fig. 3). A similar assay using CPT–Gly3 did not show appreciable acceleration in hydrolysis over PBS alone (data not shown).

Cytotoxicity assay

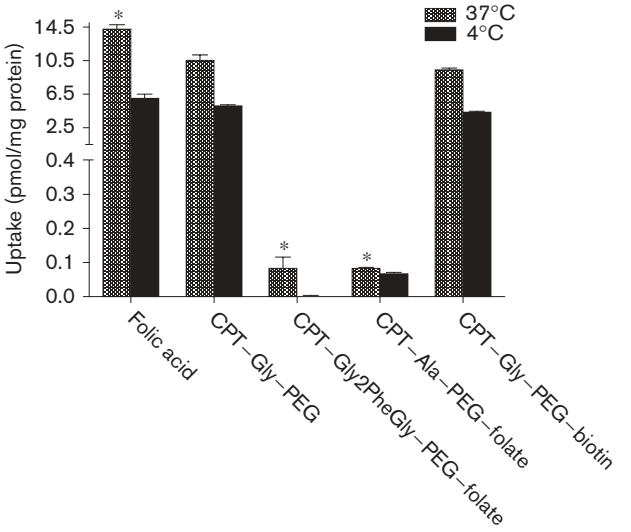
The cytotoxicity of CPT bioconjugates was evaluated using a 72-h MTT assay (Table 3). In KB cells, the IC_{50} values obtained for CPT–Ala–PEG–folate, CPT–Gly3–PEG, CPT–Gly2PheGly–PEG–folate were 6.27 ± 0.43, 6.45 ± 0.38 and 10.45 ± 0.62 nM, respectively. The bioconjugates with alanine and Gly3 were similar in cytotoxicity, about 10 times more toxic than untargeted CPT, while that with Gly2PheGly was much less

Table 3 Hydrolysis of CPT amino acid esters in PBS, pH 7.4 and 37°C, and cytotoxicity of targeted bioconjugates (means ± SD for at least three observations are shown)

Bioconjugate	Half-life (h)*	IC_{50}(nM)
CPT–Gly–PEG–folate	0.9	3.75 ± 0.22
CPT–Ala–PEG–folate	1.0	6.27 ± 0.43
CPT–Pro–PEG–folate	12	non-toxic
CPT–Gly3–PEG–folate	4.8	6.45 ± 0.38
CPT–Gly2PheGly–PEG–folate	6.4	10.45 ± 0.62
CPT–Pro–Gly–PEG–folate	29	not reported
CPT–Pro–Ala–PEG–folate	116	not reported

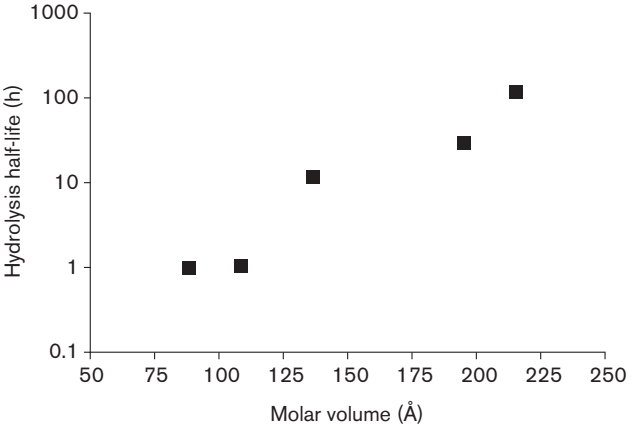
IC_{50} of CPT = 65 ± 3.75 nM. *hydrolysis was determined using CPT-amino acid/peptide esters

Fig. 4



Uptake inhibition of folic acid in KB cells. Plot of [^{1}H]folic acid (10 nM) uptake in the presence of various inhibitors (10 μM). Means ± SD for at least three observations are shown. *Statistically significant difference between the treatments and control (folic acid, 37°C) at $p < 0.01$.

Fig. 5



Dependence of hydrolysis half-life on molar volume.

cytotoxic (around 6 times) in this *in vitro* assay of cytotoxicity. The CPT–Pro–PEG–folate conjugate was not cytotoxic at the concentrations used.

Uptake inhibition studies in KB cells

The uptake of 10 nM folic acid in KB cells at 37°C was nearly completely inhibited by CPT–Gly2PheGly–PEG–folate (around 99%). CPT–Ala–PEG–folate at 10 μM also inhibited uptake by 99%. The untargeted CPT–Gly–PEG and unrelated, but targeted bioconjugate, CPT–Gly–PEG–biotin did not inhibit the uptake appreciably (26 and 33%, respectively). Uptake at 4°C was much lower than that at 37°C, but followed a similar trend in percent inhibition (Fig. 4).

We have reported the specificity of targeting using CHO cell uptake inhibition in an earlier publication [4].

Molecular modeling

The molar volume was plotted versus the hydrolysis half-lives as shown in Figure 5. The Gly3 and Gly2PheGly linkers are considered outliers since the bond available for ester cleavage is still with the sterically unhindered glycine, but the molar volumes are large due to the presence of longer chains and relatively sterically hindered phenylalanine. The molar volume calculations substantiate our hypothesis. Although limited in scope, these trends suggest that novel linkers can be designed that will give greater control over the release rates of the active CPT can be designed.

Tumor model development

The rats developed palpable tumors within around 10–12 days after injecting MAT III-B cells. The animals were monitored for tumor size, skin lesions, distress or ascites. Some of the animals developed skin lesions, but most were in good condition. The rat body weight was monitored continuously during the first 10 days of tumor development. The body weights did not go below 20% of the original weight, suggesting a lack of toxicity. Histopathological studies (described later) revealed angiocentric, moderately to poorly differentiated, multifocal carcinoma. Healthy rats ($n = 2$) after injecting i.p. with three dose levels (2.5–7.5 mg/kg) were monitored for body weight changes. The animals receiving the highest dose level showed indications of toxicity early on. Animals that survived post-injection generally regained their lost body weight quickly and survived for the duration of the study. The highest dose (5 mg/kg) was selected as the maximum tolerated dose based on these results (data not shown).

Anti-tumor efficacy

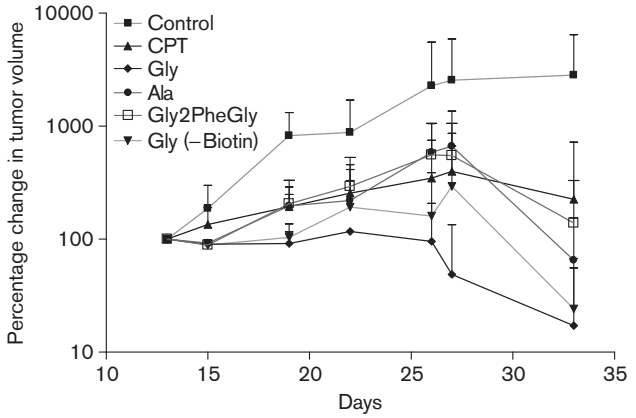
The relative *in vivo* anti-cancer efficacy of the synthesized CPT bioconjugates was assessed by monitoring tumor volume in a syngeneic rat MAT III-B breast cancer model. Rats were injected with MAT III-B cells and then treated i.p. for 5 consecutive days. This was followed by daily survival monitoring for 33 days, and measurement of tumor volume and body weight twice a week. The results of this *in vivo* screen are shown in Table 4 and Figure 6. The rank order of effectiveness was CPT–Gly–PEG–folate > CPT–Gly–PEG–biotin > CPT–Ala–PEG–folate > CPT–Gly2PheGly–PEG–folate > CPT > control.

Table 4 Summary of *in vivo* efficacy results

Treatment	%T/C	Degenerating tumor foci (histological ranking[a])
Control	–	–
CPT	100	*****
CPT–Gly–PEG–biotin	115	***
CPT–Gly–PEG–folate	146	****
CPT–Ala–PEG–folate	100	**
CPT–Gly2PheGly–PEG–folate	115	*

[a]Ranking:* = most foci observed; ***** = least foci observed.

Fig. 6



Percent change in tumor volume. The tumor volume was monitored twice a week after treatment onset (day 13). Means ± SD for at least five observations are shown (except control where $n = 3$).

The only treatments that demonstrated signs of toxicity, as evaluated by the toxicity indicator body weight change, were CPT–Gly–PEG–biotin and CPT alone. Animals receiving all other treatments lost weight immediately after dosing, but regained it quickly. All CPT bioconjugates resulted in tumor regression, while the control treated animals showed a steady increase in tumor volume. The mean tumor volumes at the end of the study were observed to be greater in control and CPT (i.e. drug only)-treated rats than in CPT bioconjugate-treated animals. All of the bioconjugates except CPT–Ala–PEG–folate demonstrated a favorable T/C ratio.

Histopathology of tumors

The six tumors evaluated histologically (Fig. 7) are ranked in the Table 4 according to the number of foci of viable tumor cell angiocentric centers within the capsular lumen. They are also ranked according to the number of degenerating angiocentric tumor cell foci present within the section evaluated. This evaluation was entirely subjective in nature and was based on '*' representing the greatest number of foci and '*****' representing the least number of foci per section.

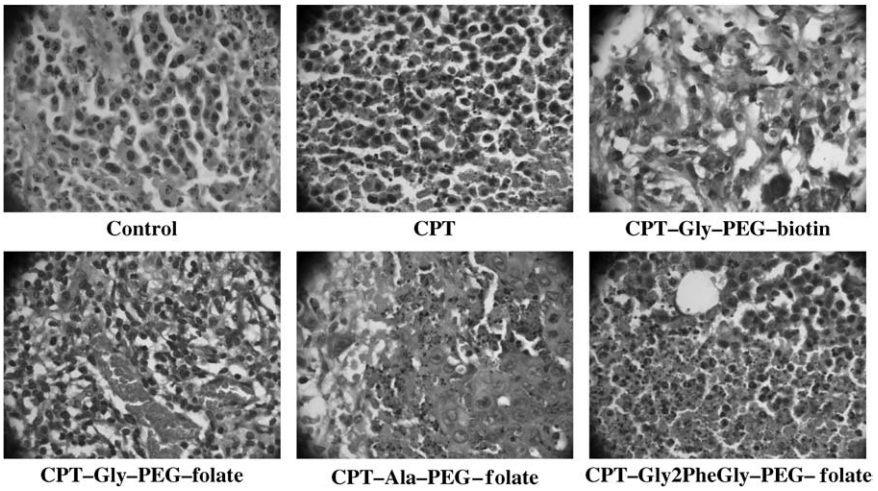
Discussion

In an earlier report from our laboratory, we presented tumor-targeted CPT bioconjugates utilizing folic acid as a targeting moiety in order to get the conjugate to interact selectively with cancer cells [4]. A second critical requirement for the targeted delivery of anti-cancer compounds is the site-specific release of the drug within the target cell. Failure to release the drug from the polymer carrier has been shown to result in a therapeutically inactive conjugate [15]. However, fast release of active drug in plasma would result in a broad distribution of drug, possibly increasing toxic side-effects. A rational approach to solving this problem is the use of peptide spacers to link CPT to the polymeric scaffold and targeting moiety. Ideally, the peptides should be selected to be stable in the blood circulation and release active drug inside the tumors. The current paper describes such a dual targeting approach to improve CPT delivery with a greater control on rate and site of active CPT release.

The results of the stability of various CPT amino acid esters in PBS are shown in Table 3. Two series of bioconjugates were synthesized—one increasing in steric hindrance using single amino acids and the other series comprised of short peptides synthesized to test the site-specific release via cathepsin hydrolysis. The hydrolysis half-life ($t_{1/2}$) increases (i.e. has greater stability in PBS) as the steric hindrance increases from glycine to alanine to proline. The observed variations in $t_{1/2}$ are likely due to a combination of steric factors and the hydrophobicity of the amino acid. There are three bonds in the current CPT bioconjugates that might be hydrolyzed depending upon which mechanism takes preference. The bond between CPT and the amino acid is an ester, while the bond between the amino acid and PEG and that between PEG and folic acid are amide bonds. The hydrolysis of the amide bond might result in a CPT–amino acid ester that would then undergo slow hydrolysis of the ester to release CPT. Alternatively, it is more likely for glycine and alanine esters that the ester bond might be hydrolyzed before the amide bonds leading to a faster release of active CPT. This series of esters release free CPT slowly enough for the folate receptor mediated endocytosis to occur (time course of folic acid uptake across KB cells shows maximal uptake at 30 min [4]). Additionally, theory supports the hypothesis that this release will be even slower for the complete bioconjugates due to the steric hindrance attributed by PEG and folate. These bioconjugates are transported across the cell membrane more efficiently than free CPT. The results from this study demonstrate the importance of carefully modulating the rate of release of free CPT once inside the cancer cell.

The second set of bioconjugates was designed to take advantage of their susceptibility to cysteine proteinases. Several classes of proteinases are known to be over

Fig. 7



Histopathology of the tumors. The tumors were trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological evaluation. These are the light microscopy pictures of the six tumors showing the number of degenerating tumor foci.

expressed by many tumor cells [16,17]. In particular, cathepsin B has been the most widely investigated and is shown to play a critical role in tumor progression [5]. The two linkers Gly–Gly–Gly and Gly–Gly–Phe–Gly are known to be cleaved by cathepsin B and other lysosomal enzymes [12]. The results of incubation with human cathepsin B confirmed these reports for the Gly–Gly–Phe–Gly ester. Within 6 h most of the ester was converted to parent CPT as seen in the MALDI spectra. The same conjugate had a $t_{1/2}$ of 6.4 h in PBS (pH 7.4). This result suggests that CPT–Gly–Gly–Phe–Gly–PEG–folate will be preferentially cleaved inside cancer cells where it will likely release the active form of CPT. This conjugate also exhibited fair stability in rat plasma. The last two esters in this series, Pro–Gly and Pro–Ala, were unusually stable in PBS. The observed hydrolysis half-life ranged from 1 to 5 days. This stability is due to the dual stabilizing effect of steric hindrance imparted by the second amino acid added to the already significant steric hindrance presented by proline. Given the extreme stability of these two conjugates in PBS, they were not tested any further since non-releasable CPT is not active. In the future, the linker design can be modulated to incorporate solution stability of the ester bond at 20-OH position of CPT, and site-specific cleavage and release of active CPT by using a cathepsin B labile linker. While these two aspects were studied individually in the present studies in order to obtain a clear mechanistic interpretation, it is envisioned that incorporating these elements into a single linker would be optimal.

A molecular modeling approach was used to gain insight into the linkers and dependence of hydrolysis on the steric factors. A good review of the various attempts to quantitatively describe the dependence of reaction rates/hydrolysis on steric effects is available [18–20]. In order to validate the hypothesis, the steric descriptors, molar volume and molar surface area were calculated. The experimentally obtained hydrolysis rates correlated well but in a nonlinear manner with the hydrolysis half-life (i.e. release rate) of CPT. Although the data set is limited in size, this correlation shows useful trends for designing conjugates with enhanced plasma stability.

The cytotoxicity results show increased efficacy for all targeted bioconjugated in comparison to free CPT. Additionally the cytotoxicity data matches the rank order of ester stability. Although the ester hydrolysis rates were not explored beyond $t_{1/2}$ of around 1 day, the results demonstrate the utility of this controlled release approach. In future more sterically hindered linkers based on molecular modeling results can be designed. We have shown the targeting potential and specificity of targeting using an indirect method earlier [4]. Uptake inhibition studies were done on representative bioconjugates in this study to confirm the targeting potential.

Over the past several years, a considerable number of studies of tumor-specific, carrier-conjugated macromolecules have been reported. There have been numerous studies showing the utility of the folate receptor pathway for targeting cancer cells. Most of these studies have proved the significance of folate receptor mediated endocytosis in increasing cytotoxicity, delivery, uptake in *in vitro* cell culture models like KB cells [11,21–24]. However, very few studies have shown the targeting

effect of the folate receptor pathway in *in vivo* models [25,26]. Among other factors, the *in vivo* fate of the folate-conjugated delivery system and folate concentration in blood available for competition for the limited number of folate receptors are important considerations [27]. Given the limitations of *in vitro* models and the importance of the two previously mentioned factors, CPT bioconjugates with folate were studied in an *in vivo* model of cancer.

We have used a syngeneic breast carcinoma model in rat for the reasons of convenience and have ample evidence of its validity (versus KB xenografts in nude mice). There have been studies published that validate several important aspects of the current studies. CPT has been used in the mitigation of breast cancer. The syngeneic rat breast carcinoma model using MAT III-B cells has been previously used to test the prevention of breast cancer growth, invasion and metastasis by tamoxifen alone or in combination with a urokinase inhibitor [13,28]. It has also been used in the development of autologous tumor vaccination to safely generate tumor-specific immunity to breast cancer cells in patients bearing those cancers [29]. A CPT analog has been tested as a dextran conjugated form for the treatment of mammary carcinoma in rats [30]. A breast cancer xenograft, MX-1, has also been used as a model to evaluate the efficacy of glycoconjugates of CPT [31]. Although most folate-targeting studies have used ovarian and/or nasopharyngeal carcinoma models, there are a few reports of overexpression of folate receptor in breast cancer [32–34]. We have previously reported the expression of folate receptor in MAT III-B cells [4]. Therefore, the use of the rat syngeneic breast carcinoma model is appropriate for testing the CPT bioconjugates *in vivo*.

The selection of bioconjugates for the *in vivo* study was based on the *in vitro* results. Three conjugates with varying degrees of *in vitro* stability and steric/chemical/enzymatic stability (CPT–Gly–PEG–folate, CPT–Ala–PEG–folate and CPT–Gly2PheGly–PEG–folate) were selected to test the hypothesis. The CPT–Gly–PEG–biotin bioconjugate [3] was included in this study to compare the targeting effect of biotin with folic acid. The results obtained in this study show a rank order similar to the results obtained *in vitro* with the CPT–Gly–PEG–folate conjugate being the most active of the series. The CPT–Gly2PheGly–PEG–folate conjugate, as per the hypothesis, should have been the most active by virtue of its 'dual' targeting effect—via the folate receptor and selective enzymatic cleavage once inside the tumors. However, this conjugate was not more efficacious than the simpler CPT–Gly–PEG–folate conjugate with no tumor specific cleavage mechanism. Several factors, such as an alteration in pharmacokinetics, tumor and tissue distribution, stage of tumor growth, site and sequence of pro-drug activation, and rate of tumor uptake [14], may have an impact on the *in vivo* activity of the bioconjugates. The glycine derivative, as seen from its PBS hydrolysis rate, is rapidly converted to active CPT. In comparison, Gly2PheGly, a considerably sterically hindered conjugate, has a much longer half-life. Since these conjugates were not designed to promote retention in tumors, faster drug release resulted in enhanced efficacy, explaining the reversal of the trend observed *in vitro*. It is expected that, if the conjugates had prolonged residence in tumor, the slower release rate would result in prolonged exposure and better efficacy. However, given these limitations, when evaluated on the basis of toxicity and histopathology of the tumors, the CPT–Gly2PheGly–PEG–folate conjugate fares much better than any other conjugate. The body weight steadily increased after treatment with this conjugate without much fluctuation. The numbers of degenerating tumor foci were at a maximum in the tumors treated with this particular bioconjugate as shown by the histopathological analysis. Reduced toxicity and better prognosis are important considerations when evaluating a targeted treatment modality such as this. These results also underscore the importance of *in vivo* studies in interpreting encouraging *in vitro* targeting results.

Another interesting result obtained in these studies was the activity of CPT–Gly–PEG–biotin conjugates. This conjugate was more efficacious than the conjugates with alanine or Gly2PheGly linkers (with folate). There are a few reports of using the biotin transporter as a targeted delivery route for cancers [3,35,36]. However, the similarity between the CPT–Gly–PEG–folate and CPT–Gly–PEG–biotin is probably attributable more to the nature of the linker rather than to the targeting moiety used. The simple glycine linker, as described previously, has a much faster release rate for the active form of CPT and the kinetics of delivery to the tumors versus release of active CPT might be playing a more important role in defining the final efficacy of these bioconjugates [37].

The presence of PEG in each of these conjugates also plays an important role in defining the *in vivo* fate of conjugates. We have reported the importance of using spacer with optimal length between targeting moiety and drug for activity [4]. Using PEG results not only in increased total CPT delivered to tumors, but also the conjugates provide higher tumor to normal tissue ratios of CPT over a longer period of time, as compared with the administration of free CPT [38]. These results need to be further confirmed and optimized using pharmacokinetic data in suitable healthy and tumor models.

In conclusion, tumor-selective CPT bioconjugates were designed, synthesized, and tested *in vitro* and *in vivo*. The folate targeting moiety led to *in vivo* tumor volume reduction and appears to be important in attaining an

overall level of cancer control. The current studies demonstrated the possibility that rate of active CPT delivery could be adequately controlled using increasingly sterically hindered amino acids, while the site-specific release can be incorporated using short peptide linkers sensitive to enzymatic milieu present in *in vivo* conditions. Finally, while this study establishes the feasibility of this multifaceted tumor selective bioconjugate approach, further mechanistic studies are required to optimize CPT delivery.

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