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Synthesis of a Macromolecular Camptothecin Conjugate with Dual Phase Drug Release

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Abstract: A water soluble macromolecular conjugate of camptothecin (CPT) with a new, dual phase hydrolytic drug release mechanism was prepared on the basis of a 60 kDa biodegradable hydrophilic "stealth" polyacetal, poly(1-hydroxymethylethylene hydroxy-methyl formal). Succinamido-glycinate was used as a prodrug releasing group. A model preparation with 7.5% CPT content w/w was water soluble. The lipophilic camptothecin prodrug, camptothecin-(O20)-succinimidoglycinate, was released from the conjugate with $t_{1/2} = 2.2 \pm 0.1$ h in rodent plasma. The blood clearance in a rodent model as measured by CPT was release limited, $t_{1/2} = 2.1 \pm 0.2$ h, while the conjugate half-life was 14.2 ±1.7 h. In a xenograft tumor model, the conjugate demonstrated higher antineoplastic efficacy than CPT at a less than equitoxic dose. This improved therapeutic window is in line with the modified drug pharmacokinetics and with camptothecin release in a stabilized lipophilic prodrug form. Regulation of prodrug release and hydrolysis rates through linker structure modification will open the way to further improve both pharmacokinetics and pharmacodynamics.

Keywords: Drug release, drug delivery, cancer, camptothecin, polyals, polyacetals, biodegradable polymers

Introduction

Camptothecin[1] is a potent antineoplastic agent with topoisomerase I inhibiting activity. Therapeutic application of unmodified CPT is hindered by very low solubility in aqueous media, high toxicity, and rapid inactivation through lactone ring hydrolysis in vivo. Lactone hydrolysis, which is reversible in acidic media, leads to a water soluble carboxylate.[2] The latter is cleared by the kidneys and causes hemorrhagic cystitis, a severe adverse reaction to CPT administration. Acylation of the (O20) lactone ring hydroxyl significantly increases the stability.[3,4]

Hydrophilization of the CPT molecule results in water soluble forms, e.g., Irinotecan (CPT-11). The latter is the most widely used soluble prodrug, which (as well as other CPT prodrugs) requires endoplasmic activation, mainly in

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the liver, for conversion into the active form (SN38[5]). Such prodrugs, activated outside cancer tissue, are not feasible for tumor as well as cancer cell targeting.

Macromolecular and liposomal forms of CPT have shown improved efficacy, as compared to low molecular weight analogues.[6,7] However, bladder toxicity was still reported.[8] The dual phase drug release system described in this paper was intended to engineer soluble, potentially targetable macromolecular preparations with novel pharmacokinetics and reduced toxicity.

The dual phase strategy involves assembling of a hydrophilic conjugate that releases a lipophilic stabilized CPT prodrug, which, in turn, releases the active drug substance locally (intra- and extracellularly), without the requirement for prior metabolization by the hepatic microsomal P450 complex.

The model release system developed in this work is based on a known reaction, hydrolytic cyclization of succinamido esters. The reaction results in ester bond cleavage and simultaneous succinimide formation at the amide side. Attempts have been made to employ succinamido ester tethers with the amide group at the carrier side,[9] which does not result in dual phase drug release. In our system, the succinamido ester is oriented such that the ester is formed at the polymer side, while the opposite carboxyl forms an amide bond with an amine-containing drug or drug derivative (in this paper, CPT-(O20)-glycinate).

Hydrolysis of the succinamido ester tether leads to drug cleavage from the polymer in the form of a cyclic succinimidoglycyl-CPT (Figure 1). The reaction is base catalyzed, and in aqueous medium it goes to completion under mild conditions. The second stage is in vivo glycyl ester bond hydrolysis, which results in active drug release.

The potential advantages of this dual phase drug release system are the following. (1) The conjugate is water soluble and can be administered intravenously. (2) Unlike other CPT prodrugs, e.g., Irinotecan, the intermediate prodrug is activated "on site" rather than in the liver, so that local

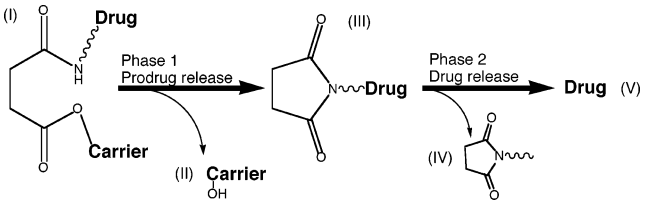

Figure 1. Two stages of drug release from a succinamido ester linked conjugate.

administration and targeting are possible. (3) CPT is released in a lipophilic, lactone-stabilized form, which ensures prodrug deposition in tissues and low rates of redistribution and carboxylate transfer to urine.

In this paper, a model fully biodegradable macromolecular CPT conjugate with dual phase release from an unsubstituted succinamido ester tether was synthesized and characterized in vitro. Initial results of ongoing in vivo characterization studies are also presented.

The conjugate was assembled using poly(1-hydroxymethylethylene hydroxy-methyl formal) (PHF) as a backbone. PHF is a highly hydrophilic, biodegradable "stealth" polymer developed in our laboratory.[10,11] Biodegradability of PHF reduces the potential risks associated with administration of large doses of nondegradable polymers, making the model PHF conjugate feasible for clinical development.

Experimental Section

Camptothecin was obtained from Hande Tech Development Co. (Houston, TX). Dextran (M_n 73000 Da) and *N*-BOC-glycine were obtained from Sigma Chemical Company (St. Louis, MO). Succinic anhydride (SA), sodium borohydride, sodium metaperiodate, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC), diisopropylcarbodiimide (DIPC), 4-(dimethylamino)pyridine (DMAP), trifluoroacetic acid, hydrochloric acid, and sodium hydroxide were purchased from Aldrich (St. Louis, MO). Other chemicals, of reagent or higher grade, were obtained from Acros Organics or Fisher Scientific and used as received. Anhydrous pyridine, methyl alcohol, ethyl alcohol, dimethylformamide, dimethyl sulfoxide, methylene chloride, diethyl ether and other solvents were obtained from Sigma-Aldrich and used without further purification. Deionized water (resistivity > 18 MΩ) was used for all synthetic and analytical procedures.

Size exclusion chromatography (SEC) in aqueous media and reversed phase (RP) chromatography were carried out using a Varian-Prostar HPLC system equipped with a BIO-RAD model 1755 refractive index detector and LDC/Milton Roy SpectoMonitor 3000 UV detector. HPSEC Biosil SEC-125 and Biosil SEC-400 (BIO-RAD) and low-pressure Superose-6 (Pharmacia) columns were used for size exclusion

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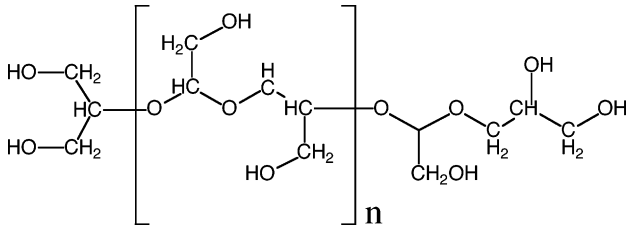

Figure 2. The structure of PHF, copolymer (copolyacetal) of glycerol and glycol aldehyde.

chromatography. SEC column calibration was performed on the basis of broad molecular weight dextran and protein standards. Unless otherwise stated, elution was performed isocratically with 50 mM phosphate buffered 0.9% NaCl, pH = 7.0.

An Altima C18 column (Alltech, 250 mm × 4.6 mm, 5 μm bead) was used for RP chromatographic determination of low molecular weight CPT derivatives and degradation products of polymer−CPT conjugates.

Preparative isolation and purification of polymers and polymer conjugates was carried on a G-25 gel SpectraChrom (60 cm × i.d. 10 cm) column equipped with a Milton-Roy liquid delivery system, MasterFlex CL peristaltic pump, Knauer-2401 RI detector, Foxy JR fraction collector, and Varian-Prostar data acquisition system. Alternatively, a QuixStend flow dialysis system (A/G Technology, Needham, MA) equipped with a UFP-10-C-4MA hollow fiber cartridge (cutoff 10 kDa) was used in high volume procedures. Photon correlation light scattering was carried out using a Brookhaven ZetaPlus analyzer.

Proton and ^{13}C NMR were carried out on Varian Mercury-300, Bruker DPX-300, and Bruker Aspect 3000 NMR spectrometers using solvent peaks as reference standards.

A Cary 300Bio UV−visible spectrophotometer equipped with a thermostated multicell Peltier block and a Molecular Devices Co. 96-well plate reader was used for spectroscopic measurements.

An Agilent 1100 series LC/MSD system was used for MS characterization of PHF−CPT hydrolysis products.

Male nu/nu mice, 18−24 g (8−10 weeks of age), were obtained from Charles River Laboratories, Boston, MA.

Human colorectal adenocarcinoma HT-29 cell culture was from ATCC (ATCC HTB-38).

Photoimaging was carried out using a Nikon Eclipse TE300 microscope with long working distance phase contrast optics, epifluorescence imaging setup, CCD camera, and MacOS based imaging workstation.

Carrier Polymer Synthesis. PHF is a semisynthetic acyclic polyacetal prepared by exhaustive lateral cleavage of Dextran B-512. Complete periodate cleavage of the (1→6)-polyglycoside sequence of Dextran B-512 results in poly(1-carbonylethylene carbonyl formal) (PCF). Borohydride reduction of the pendant aldehyde groups of PCF gives poly(1-hydroxymethylethylene hydroxy-methyl formal) (PHF), a copolymer (copolyacetal) of glycerol and glycol aldehyde (Figure 2).

The polymer was prepared using an accelerated modification of a previously described technique[10] allowing the formation of PHF with 5% 2,3-dihydroxyethylformal units originating from the C2−C3 of dextran. Dextran B-512, 73 kDa preparation (15.15 g, 93.4 mmol by glycopyranoside), was dissolved in 300 mL of deionized (DI) water at 0−5 °C and treated with 47.95 g (224.2 mmol) of sodium metaperiodate in a light protected reactor for 3 h. The crystalline sodium iodate was removed from the reaction mixture by filtration (1 μm glass filter). The pH of the filtrate was adjusted to 8.0 with 5 N NaOH, and the resultant solution was immediately treated with sodium borohydride (7.07 g, 187 mmol, dissolved in 70 mL of deionized water) for 2 h. The pH was then adjusted to 6.5 with 1 N HCl. The product was desalted on Sephadex G25 and lyophilized; yield: 80%. The results of SEC analysis were M_n = 60 kDa and polydispersity index (M_w/M_n) of 2.0. The proton NMR spectrum in DMF-d_6:D_2O (95:5 v/v) was found to be in agreement with the expected PHF structure (C1−H at δ 4.62 t, J = 5.2 Hz) with ca. 5% vicinal diol pendant groups originating from C2−C3 (δ 4.49 d, J = 5.2 Hz).

CPT-(O20)-glycinate Trifluoroacetate Salt (CPT-Gly·TFA). CPT-Gly·TFA was prepared in two steps according to the procedure reported by Greenwald[12] and modified by Minko.[13] Briefly, CPT was treated with BOC-glycine and DIPC in methylene chloride in the presence of DMAP. The *N*-BOC group was removed with trifluoroacetic acid, and the resultant CPT-Gly·TFA was crystallized from diethyl ether. Purity: >97% (HPLC, NMR).

PHF-Succinate (PHF-SA). PHF (10.00 g, 75.6 mmol), succinic anhydride (0.76 g, 7.6 mmol), and DMAP (1.2 mg, 0.01 mmol) were dissolved in 5 mL of anhydrous pyridine. After 18 h of agitation at 40 °C, pyridine was removed in a vacuum, the residue was suspended in deionized water, and the pH was adjusted to 7.0 with 1 N NaOH. The succinylated PHF was desalted on Sephadex G-25 and lyophilized with 86% yield. The succinic acid content, as determined by potentiometric titration, was 10.3% (mol/monomer). The ^{1}H NMR spectrum of the polymer (D_2O) contained signals characteristic for methylene protons of succinic acid ester at δ 2.66 and δ 2.57 (broad triplets) in addition to methylene and methine (δ 3.3−3.8), and acetal (δ4.4−4.7) protons of the PHF backbone.

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Table 1. PHF−CPT Conjugate Composition (by CPT, mol %)

	CPT derivatives	reaction mixture	isolated product
1	PHF−CPT	92.8	96.15
2	CPT-glycinate	1.9	0.32
3	CPT	2.3	0.34
4	CPT-CA (carboxylate)	0.3	0.44
5	CPT-Gly-SA	<0.05	0.53
6	CPT-Gly-SI	<0.05	0.66
7	other low MW	2.7	1.59

Camptothecin−PHF Conjugate (PHF−CPT). Conjugation of CPT-Gly·TFA with PHF−SA was conducted via (i) EDC mediated amidation of polymer-succinate with CPT-(O20)-glycinate trifluoroacetate salt in aqueous medium or (ii) DIPC mediated coupling under nonaqueous conditions (DMF). The first approach (described below) was found to be more efficient, on the basis of higher reaction rate, cleaner product, and simplicity of purification.

Prior to the preparative synthesis, conjugates with various CPT contents (ca. 5−15% w/w) were prepared on a lower scale to test solubility in aqueous media, which showed that conjugates with CPT content up to 10% w/w were readily soluble.

Preparative Synthesis. PHF-SA (15.0 g, 10.7 mmol of SA) was dissolved in 150 mL of deionized water and mixed with 30 mL of DMF, cooled to −2°C, and combined with CPT-Gly·TFA solution (2.0 g/3.85 mmol in 20 mL of 3:1 acetonitrile/water mixture). Under intense agitation, EDC (2.0 g) was added to the reaction mixture. The pH was adjusted to 5.9−6.0. After 30 min of agitation, the temperature of the reaction mixture was brought to ambient, and agitation was continued for another 3 h. The CPT conversion at this point was 93%, based on RP HPLC (UV at 360 nm). The pH was adjusted to 5.5 to prevent CPT release from the conjugate, and the reaction mixture was stored overnight at 8 °C. The mixture was then diluted with DMF and water to 600 mL (DMF content 10% v/v), and the conjugate was desalted on Sephadex G-25, lyophilized, and stored at −20 °C. The product was obtained as an off-white to pale-yellow solid with CPT content of 7.48% w/w (as determined spectrophotometrically at 360 nm). Yield based on CPT: 80%.

The proton NMR spectrum of PHF−CPT (DMSO-d_6/D_2O) contained the signals characteristic for the succinic acid modified PHF backbone: δ 3.3−3.8 (methylene and methine), δ 4.4−4.7 (acetal), δ 2.4−2.6 (−CH_2−, succinate); and signals corresponding to the pendant CPT structures: δ 0.95 (t), δ 2.21 (d), δ 5.26(m), δ 5.46(s), δ 7.20(s), δ 7.70-(t), δ 7.88(t), δ 8.09(d), δ 8.18(d), δ 8.45(s).

The reaction mixture and lyophilized product compositions are shown in Table 1.

Camptothecin-20-(*N*-succinimidoglycinate) (CPT-SI). CPT-SI is the lipophilic prodrug isolated from the products of PHF−CPT hydrolysis (see below). CPT-SI was synthesized as a control compound.

PHF−CPT (500 mg) was dissolved in 10 mL of 0.1 M phosphate pH 7.6 and incubated for 24 h at 37 °C. The resultant suspension was diluted to 150 mL and extracted with methylene chloride (3 × 150 mL). Methylene chloride layers were combined, washed with 0.01 N HCl, and dried over magnesium sulfate. Solvent was removed in a vacuum. The light yellow residue was redissolved in methylene chloride, filtered, and dried in a vacuum to yield 38 mg of a product containing, according to RP HPLC, >93% CPT-SI. The solubility of CPT-SI in water was found to be lower than that of unmodified CPT, <1.0 μg/mL, vs 2.5 μg/mL respectively.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.01(τ, 3H, J = 7.4 Hz, C19), δ 2.05−2.32 (m, 2H, C18), δ 2.66 (s, 4H, succinimide), δ 4.32−4.51 (AB, 2H, 17.2 Hz, C-α Gly), δ 5.32 (s, 2H, C-5), δ 5.29−5.65 (AB, 2H, 17.3 Hz, C-17), δ 7.20 (s, 1H, C-14), δ 7.60 (t, 1H, J = 7.5 Hz, C-11), δ 7.76 (t,-1H, J = 7.7 Hz), δ 7.86 (d, 1H, J = 8.3, C-12), δ 8.20 (d, 1H, J = 8.3, C-9), δ 8.32(s, 1H, C-7).

^{13}C NMR: 7.23, 28.36, 29.89, 32.04, 39.53, 50.17, 67.31, 77.45, 96.29, 120.54, 128.23, 128.33, 128.64, 130.00, 130.80, 131.35, 145.14, 146.70, 149.08, 152.46, 157.48, 166.27, 166.78, 175.95. MS: m/z 488.2 (M + H).

Camptothecin-20-(*N*-succinamidoglycinate) (CPT-SA, Control). CPT-Gly·TFA (50 mg, 0.096 mmol) and succinic anhydride (18 mg, 0.190 mmol) were dissolved in 2 mL of anhydrous pyridine. After an 18 h agitation at ambient temperature, pyridine was removed in a vacuum. The solid residue was suspended in deionized water and extracted with methylene chloride, washed with 0.01 N HCl and dried over magnesium sulfate. Solvent removal in a vacuum resulted in a light-yellow solid (41.4 mg, 85% yield) containing >90% CPT-SA (HPLC with 360 nm detection). LC-MS: m/z 506.2 (M + H). The product was used as HPLC standard for determination of PHF−CPT hydrolysis product composition.

PHF−CPT Hydrolysis. The hydrolytic stability of the PHF−CPT conjugate was tested in DI water and isotonic saline at ambient temperature and pH = 5.7, in 0.05 M phosphate buffered 0.9% saline (pH 7.4), and in freshly prepared rat plasma at 37 °C. PHF−CPT hydrolysis and accumulation of CPT derivatives was monitored by RP HPLC using a 20 min 10−70% acetonitrile/water gradient (both solvents with 0.1% TFA). Results were reproduced in two independent experiments.

The second stage hydrolysis of CPT-SI was investigated analogously.

The reaction of cyclization−elimination (Figure 1) requires folding of the succinamido ester into a cyclic intermediate structure, with subsequent intramolecular nucleophilic attack on the ester carbon. Thus, the reaction should be sensitive to the presence of (1) bulky substituents and (2) substituents altering the charge density on either of the carboxylic carbons of the tether. The second phase can also be affected by the substituents in the succinimide ring of the prodrug. Therefore, substitution in the succinate tether can be a powerful tool for regulation of the drug release profile. Furthermore, substitution in the succinate tether can open the way to the regulation of prodrug properties (hydrophobicity, transmem-

brane transfer, affinity to cell receptors, etc.), which can further enhance pharmacokinetics.

Four substituted analogues of PHF−CPT were synthesized (using methyl, 2,2-dimethyl, and 2-nonen-2-yl succinate). Using procedures analogous to those described above, CPT release from these conjugates was investigated in phosphate buffered saline as described above. The conjugates were also tested for cytotoxicity in HT29 cell culture.

Labeling. A dual labeled conjugate (^{3}H labeled CPT and ^{111}In labeled backbone) were used for parallel independent monitoring of the conjugate components.

A [^{3}H] labeled PHF−CPT conjugate with 0.210 mCi/g activity and 7.0% w/w CPT content was prepared using [5-^{3}H(N)]-camptothecin (Moravek Biochemicals, Inc.) as described for PHF−CPT. The polymer backbone of the conjugate was modified with DTPA and labeled with ^{111}In by transchelation from indium citrate at pH 5.5. Modification of PHF−CPT with DTPA was carried out in two steps. (1) Vicinal diols present in PHF structure (see above) were oxidized with sodium metaperiodate at a diol:periodate ratio of 1:1, pH 5.7, for 2 h at ambient temperature. The resultant pendant aldehyde groups were nonreductively aminated with DTPA amide of 1-amino-2-hydroxy-3-(aminooxy)propane. The latter "aminooxy-DTPA", which forms oxime bonds with aldehydes under mild conditions, was prepared in our laboratory (synthesis to be described elsewhere). In our opinion oximes, being significantly more stable under physiological conditions than hydrazides[15] and generally less toxic, are more suitable for carbonyl modification in modular conjugates.

Radiochemical purities of all labeled derivatives were >98% (HPLC).

Biokinetics. Biokinetics and biodistributions of PHF−CPT conjugates were studied in normal rats and in nude mice with HT29 and A2780 xenografts using conjugates containing double-labeled CPT conjugates. All animal studies were conducted in accordance with institutionally approved protocols.

Male nude/nu mice, average weight 28−32 g (Charles River Labs, Boston, MA), bearing 150−200 μL tumor xenografts (n = 6 per group), were injected iv with the double-labeled PHF−CPT in 0.9% saline at 20 mg/kg based on CPT. The injected activities were 1.25 μCi/animal for ^{3}H and 5 μCi/animal for ^{111}In.

Adult outbred 240 g male rats (Charles River Laboratories, Boston, MA), n = 6 per group, were injected iv with 800 μL of labeled PHF−CPT in 0.9% saline at 20 mg/ kg by CPT. The injected activities were 1.25 and 24 μCi/animal for ^{3}H and ^{111}In, respectively.

Blood samples were taken at 5, 15, and 30 min and 1, 2, 4, 8, and 24 h time points. At 24 h, the animals were euthanized; tumors and samples of major organs were harvested for counting. The total ^{3}H and ^{111}In activities in tissues were measured by scintillation (β) and γ counting, respectively, and expressed as % injected dose/g tissue to characterize the distributions of ^{3}H-CPT (total of all forms) and ^{111}In-PHF.

Antiproliferative Activity. Cytotoxicity of CPT derivatives was investigated in HT29 cell culture. Cells were grown in McCoy's 5a medium with 1.5 mM L-glutamine supplemented with 10% FBS. The (exponentially growing) cells were seeded in 24-well culture plates (~10000 cells/well), cultured for 24 h, and then treated with test compounds at various dilutions. Growth inhibition was assessed 72 h posttreatment (MTT assay).

In Vivo Antineoplastic Activity and Toxicity. The toxicity of CPT was evaluated in normal outbred mice, as well as in xenograft bearing nude athymic animals in the course of antineoplastic activity studies.

The antineoplastic activity of PHF−CPT was evaluated with a HT-29 xenograft model in athymic mice in accordance with institutionally approved protocols. Camptothecin and CPT-SI (the first phase release product) were used as controls.

The study was carried out using approximately equitoxic doses of CPT and PHF−CPT. Cells were injected subcutaneously into the left flank, 10^6 cells per animal in 50 μL. When tumor volume reached 100−150 mm^3, mice were randomly divided into four experimental groups: PHF−CPT, camptothecin, CPT-SI, and untreated control (n = 3 each). Animals of the first three groups received the respective experimental substance via the tail vein in five doses every 3 days (5×q3d). Each injection contained 22.5 mg of CPT equiv/kg of CPT and CPT-SI, and 45 mg of CPT equiv/kg for PHF−CPT. All formulations were prepared immediately prior to administration. PHF−CPT was administered as a solution in 0.9% saline. CPT and CPT-SI were administered as dispersions in Tween 80/water (9/1 v/v). Animal weight, tumor size, animal appearance, behavior, and survival rate were monitored for four weeks after administration. Weight loss over 20% and tumor growth over 1500 mm^3 were counted as lethalities (animals were euthanized).

Results

Synthesis and Properties of PHF−CPT. The synthesized PHF−CPT had CPT content of 7.5% (w/w) and was soluble in aqueous media. HPSEC showed Mn of ~65 kDa with essentially no aggregation (photon correlation light scattering). The viscosities of up to 20% solutions were feasible for injection through a high gauge needle used in the rodent studies; most injections were performed at 6% w/w (η = 4.05 cps).

The solutions (intrinsic pH = 5.5−5.7 with physiologically negligible buffer capacity) showed no significant decomposition after a week of storage at 8 °C or 24 h at ambient temperature. At neutral and slightly basic pH (7.0−7.4) and under mild conditions (8−37 °C), the conjugate did undergo slow hydrolysis yielding primarily CPT-(O20)-(*N*-succinimidoglycinate) (CPT-SI). For example, hydrolysis of the

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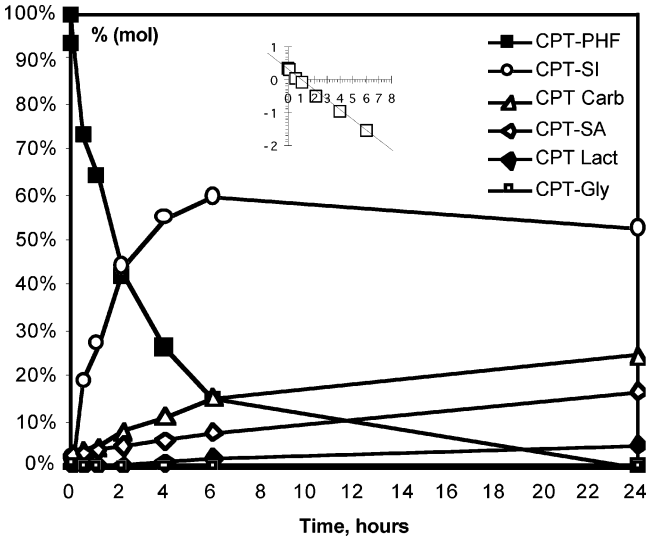

Figure 3. Prodrug release from PHF−CPT in rat plasma at 37 °C. Inset: log linearization of PHF−CPT kinetics. Mean values from two independent experiments, for all points SD < 10% of the mean, $p < 0.05$.

Table 2. Comparative Release Rates of Modified CPT−PHF Conjugates in PBS at pH 7.4/ 37 °C

compound	tether	CPT half-release time, h
PHF−CPT	Gly-succinate	2.1
PHF-MSA-CPT	Gly-(methyl succinate)	1.4
PHF-DMSA-CPT	Gly-(2,2-dimethyl succinate)	0.6
PHF-NSA-CPT	Gly-(2-nonen-2-yl succinate)	16.0

PHF−CPT conjugate (2 mg/mL in 0.05 M phosphate buffered 0.9% saline pH 7.4 for 24 h) resulted in the quantitative release of CPT from PHF−CPT, with CPT-SI lactone (87%), CPT carboxylate (8%), and CPT-SA lactone (5%) being the only detectable products. Notably, CPT released from the prodrug under these conditions was in the carboxylate but not lactone form, suggesting that the lactone ring, which was stable in CPT-SI and CPT-SA, was hydrolyzed during the second stage of CPT release.

A similar trend but slightly different composition of hydrolytic products were observed in freshly prepared rat plasma, as shown in Figure 3. This suggests the presence of additional CPT release mechanisms, possibly mediated by interactions with plasma proteins. Cleavage of CPT (all forms) from PHF−CPT was found to be monoexponential, with half-release time of 2.2± 0.1 h.

The half-time of the subsequent hydrolysis of CPT-SI was over 20 h, depending on the conditions (the exact pH dependence and enzyme sensitivity, if any, are to be determined in ongoing studies).

The three synthesized substituted analogues of PHF−CPT were also investigated to determine the first phase release rates. As expected, the bulky nonenyl group (which sterically hinders tether folding, which is necessary for hydrolytic cyclization) decreased the release rate, while methyl groups, which stabilize cyclic structures, increased it (Table 2; each

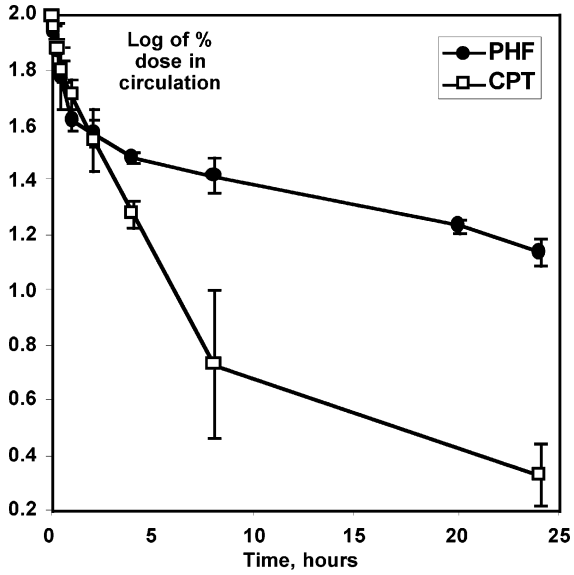

Figure 4. Biokinetics of PHF−CPT conjugate (^{111}In-DTPA labeled PHF backbone and ^{3}H labeled CPT).

result based on two independent experiments, $n = 4-6$ data points each; for all numbers SD < 10% of the mean, $p < 0.05$).

Biokinetics. The carrier polymer half-life in rat was found to be 14.2 ± 1.7 h, while the drug substance half-life was 2.1 ± 0.2 h, which corresponds well to the determined in vitro first phase release rate (Figure 4).

Both ^{3}H-CPT and ^{111}In-PHF showed substantial accumulation in the tumor tissue. At 24 h, CPT uptake in the tumor was 2.22% and 2.52% dose/g for A2780 and HT29, respectively, which is ca. 75-fold higher than for CPT ($p < 0.05$) and very similar to the results for PEG-CPT.[14] Mean tumor-to-muscle ratios were 2.4 and 1.5, respectively ($p < 0.2$ for the difference between two different xenografts).

Accumulation in other tissues (Figure 5) was also similar to that of PEG-CPT. However, 2−3-fold higher drug levels were detected in the reticuloendothelial system (RES) tissues. The latter could be either due to higher RES uptake of the CPT-SI than PEG-CPT or due to a blood volume dependent pharmacokinetics (see Discussion).

Photoimaging of unstained unfixed tumor tissue 24 h post administration showed relatively homogeneous CPT distribution with elevated drug accumulation in some areas adjacent to vascular beds (Figure 6). Diffuse intracellular distribution of CPT fluorescence indicated predominantly cytoplasmic (nonvesicular) drug localization.

Antiproliferative Activity. The ID_{50} of PHF−CPT in HT-29 cell culture was found to be 172 nM, which is 10-fold higher than that for CPT ID_{50} (17 nM) and 5-fold higher than that for CPT-SI ID50 (34 nM).

Efficacy and Toxicity in a Xenograft Model. The maximum tolerated dose (MTD) of PHF−CPT was found to be >24 mg/kg, which is at least 2-fold higher than for the low molecular weight CPT and Irinotecan (9−10 mg/kg for analogous schedules).

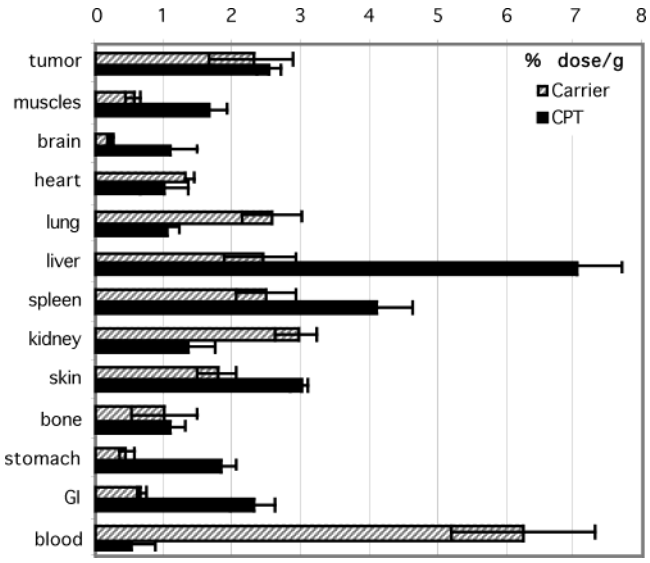

Figure 5. Biodistribution of the carrier polymer (^{111}In) and CPT (^{3}H) 24 h post iv administration of double-labeled PHF−CPT. Xenograft: HT29, 0.1−0.15 mL tumors; $n = 6$ per group.

Antineoplastic toxicity was tested at approximately equitoxic doses in the HT29 model (tumor size 100−150 μL). PHF−CPT administered at 45 mg/kg by CPT (5×q3d) was found to be both more effective and less toxic than unmodified CPT at 22.5 mg/kg (same schedule). The intermediate release product, CPT-SI, was found to have no significant effect on tumor dynamics, as determined by the time of tumor growth from 0.1−0.15 cm^3 to 1.5 cm^3 (27 days vs 24 days for untreated control and 40 days for CPT at 22.5 mg/kg 5×q4d). This is probably due to rapid hepatic clearance of CPT emulsion with no subsequent redistribution (to be determined in the future studies).

Discussion

A fully biodegradable soluble macromolecular conjugate with camptothecin content 7.5% and dual phase drug release was successfully synthesized and demonstrated the expected properties.

The lipophilic prodrug, CPT-SI, was released from the conjugate with $t_{1/2}$ of 2.1 ± 0.1 h. The release rate is faster than the blood half-life of the PHF carrier (14 h); thus no significant enhanced permeability and retention (EPR) effect[16] was expected. Considering the very low solubility and low blood protein binding of CPT-SI, the latter should be deposited in tissues immediately upon release. Therefore, prodrug deposition rate in any tissue site i should be proportional to the blood content in that tissue: $dD_i/dt = (B_i/T_i)C(t)$, where D is the amount of deposited drug, C is the concentration of carrier-bound drug in the blood, and B_i and T_i are blood and tissue weights in the site i, respectively.[17]

(16) Maeda, H.; Seymur, L. W.; Miyamoto, Y. Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjugate Chem.* **1992**, *3*, 351−362.

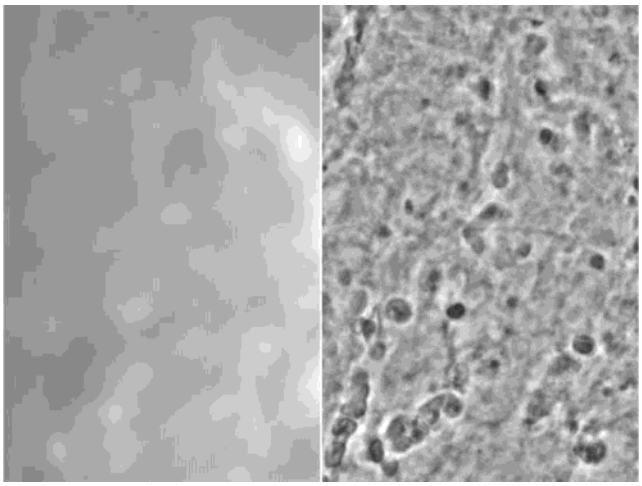

Figure 6. Microdistribution of CPT in tumor tissue 24 h post administration of PHF−CPT. CPT fluorescence (left) and phase contrast (right) images of the same region. Unstained unfixed 15 μm slice. Field: 80 × 130 μm.

This should result in a strong correlation of the initial drug disposition (before redistribution) with blood content per gram of tissue. The CPT biodistribution shown in Figure 5 is generally consistent with blood content in the tissues. The improved tumor-to-muscle ratio (2.4) may be due to the higher vascularization (thus higher blood content per gram of tissue) of the growing tumor. Alternatively (or in addition), there might be a modest influence of the EPR effect.

In view of the above, the observed RES uptake, which does not appear to result in significant toxicity, is likely to be a result of high blood content in RES tissue. Alternatively, this may be a result of conjugate uptake by the liver, which can be decreased via decreasing drug content in the conjugate (e.g., to ca. 5%) or using sterically protected branched rather than linear PHF.[10]

The influence of the EPR effect most likely can be significantly increased via prolongation of the first stage release, in which case the pharmacokinetics will be under tight control of the polymer carrier. Our current work shows that such prolongation can be achieved through substitution in the succinic acid tether with bulky groups that hinder tether folding, which is required for the hydrolytic cyclization (Figure 1). Substitution that favors folding would increase the rate of hydrolytic cyclization. Steric hindrance can also be introduced by a (bulky) drug molecule, as well as a bulky drug−tether bridge (in this study unhindered glycine).

The observed nonenzymatic prodrug hydrolysis time frame ($t_{1/2}$ > 20 h at pH = 7.4) appears to be nearly optimal. The possible role of lysosomal enzymes and pH in the second stage release, as well as regulation of release rate through

(17) Papisov, M. I. Modeling in vivo transfer of long-circulating polymers (two classes of long circulating polymers and factors affecting their transfer in vivo). *Adv. Drug Delivery Rev.* (Special issue on long circulating drugs and drug carriers) **1995**, *16*, 127−137.

alterations in the linker structure, is currently being investigated in our laboratory.

Drug concentration in tumor tissue was 50−70-fold higher than reported for unmodified CPT at the same time point.[16] The character of CPT microdistribution in the tumor tissue suggests that, by 24 h post administration, all tumor-localized drug is distributed relatively homogeneously. This can be explained by the lipophilic nature of the prodrug, which, being fully released from the carrier, is free to redistribute along cell membranes within the tumor via diffusion.

The biological activity test of PHF−CPT in a HT29 xenograft model demonstrated that the conjugate is more effective than CPT at an equitoxic dose. Data from a more extended study with other xenograft models, which is currently at its final stage, shows that PHF−CPT is actually both more effective *and* safer than soluble CPT analogues at equimolar dosing schedules (to be published elsewhere).

The relatively low toxicity of the conjugate at therapeutically effective doses can be at least in part explained by the slow free CPT release to plasma (and, therefore, urine) from the prodrug deposited in tissues. Intracellular compartmentalization of CPT-SI upon release from the carrier and intracellular release of CPT from CPT-SI might be contributing factors, and these topics are the subject of our future studies.

Conclusions

The dual phase drug release mechanism investigated in this study appears to be generally feasible for engineering of macromolecular drug conjugates with improved therapeutic windows. The mechanism can be used to simultaneously improve drug biokinetics and (for unstable substances such as CPT) drug stability in vivo.

On the basis of the most likely mechanism of action, the therapeutic window can be further widened by recruitment of the EPR effect. The latter is possible through engineering tethers with slower prodrug release rates, which will enable better carrier control over CPT circulation, and thus potentially much higher tumor to normal tissue ratios. Development of methods for regulation of the first and second stage release rates will also enable cell targeting.

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