Brief Articles

Synthesis and in Vivo Antitumor Activity of Poly(L-glutamic acid) Conjugates of 20(*S*)-Camptothecin

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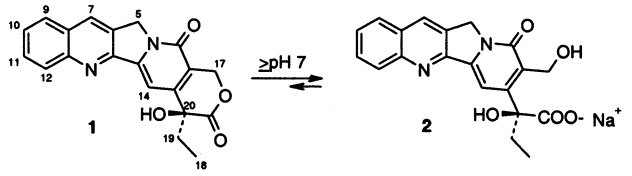
Poly-α-(L-glutamic acid) (PG) conjugates of 20(*S*)-camptothecin (**1**, CPT) displayed improved aqueous solubility compared to CPT, were stable in aqueous solution at neutral pH, and were potent antitumor agents in vivo. Evaluation of PG molecular weight, CPT loading, aqueous solubility, and CPT equivalent dosing with respect to in vivo antitumor potencies of various linked conjugates led to identification of a preferred conjugate composition.

Introduction

The water-soluble lactone hydrolyzed carboxylate form **2** of the water-insoluble antitumor agent 20(*S*)-camptothecin (**1**, CPT) was developed and evaluated in a clinical program[1] but was found to be both toxic and weakly active in patients. Results of these trials proved to be inconclusive in testing the value of CPT as a chemotherapeutic agent after it was found that the carboxylate was a relatively inactive antitumor agent in vitro compared to CPT[2] and that regeneration of the lactone did not occur in human blood because of preferential binding of the carboxylate to human albumin. At equilibrium in human plasma, less than 0.2% of CPT is in the lactone form with the remainder as the carboxylate.[3]



The 20(*S*)-hydroxyl group is believed to participate in the enhanced rate of lactone hydrolysis of CPT at neutral pH by shifting the lactone/carboxylate equilibrium in favor of the carboxylate form.[4] Acylation of the hydroxyl group blocks this participation. Although 20-O-acylated CPT derivatives possess no intrinsic topoisomerase I inhibitory activity,[5] they can function as prodrugs to release CPT in vivo and alter its pharmacokinetic, biodistribution, and toxicity profiles.[6−8]

Conjugation of water-insoluble camptothecins to water-soluble polymers has provided prodrugs with more favorable pharmacokinetic properties.[8−11] The ability of poly-α-(L-glutamic acid) (PG) to improve the pharmaceutical properties and therapeutic indices of oncologic agents including taxanes[12,13] and camptothecins[14,15] has been investigated. The potent in vivo antitumor efficacy of the PG conjugate of paclitaxel (Xyotax),[12,13] along with a 10-fold improvement in exposure of tumor tissue to Xyotax compared to paclitaxel,[16] presumably as the outcome of *e*nhanced *p*ermeability of tumor vessels and *r*etention of macromolecules (EPR),[17] validates the use of PG as a macromolecule for oncologic drug delivery. Useful attributes of PG include its ability as a polypeptide to be biodegraded and as a polyelectrolyte, with one free γ-carboxylate group per monomer unit, to "solubilize" hydrophobic molecules in aqueous media and to provide multiple available sites for drug conjugation.

Chemistry

Esterification of the 20(*S*)-hydroxyl group of CPT to γ-carboxyl sites on PG was accomplished using BOP-Cl and DMAP in DMF. The wt % CPT bound to PG was determined by analysis of integral ratios of signals in ^{1}H NMR of this conjugate (see Supporting Information). Attempts to increase the CPT loading on PG by altering reaction stoichiometry were unsuccessful because of steric constraints in acylation of the hindered 20(*S*)-hydroxyl group.

Limitations on CPT loading levels by direct conjugation methods were circumvented by use of short linkers (see Scheme 1). The 20-O-acylation of CPT with N-blocked amino acids or O-protected hydroxyl acids using DIPC-DMAP provided the corresponding protected aminoacyl- or hydroxyacyl-CPT (**4a**−**f**), which after deblocking yielded the corresponding aminoacyl- or hydroxyacyl-CPT (**5a**−**f**). N- or O-acylation with PG using DIPC-DMAP provided PG conjugates of CPT (**6a**−**f**) of significantly higher CPT loading and improved batch-to-batch reproducibility than was possible in the absence of linker. The improved control of CPT loading by stoichiometry exemplifies the greater reactivity of aminoacyl- and hydroxyacyl-CPT in the conjugation reaction compared to the hindered 20(*S*)-hydroxyl group of CPT.

Synthesis of PG−(γ-glu)−CPT (**7**) required that the α-carboxyl group of (γ-glu)−CPT be blocked during conjugation to PG. Conjugation of (γ-glu)−CPT α-*tert*-butyl ester (**5g**) with PG yielded PG−(γ-glu)−CPT

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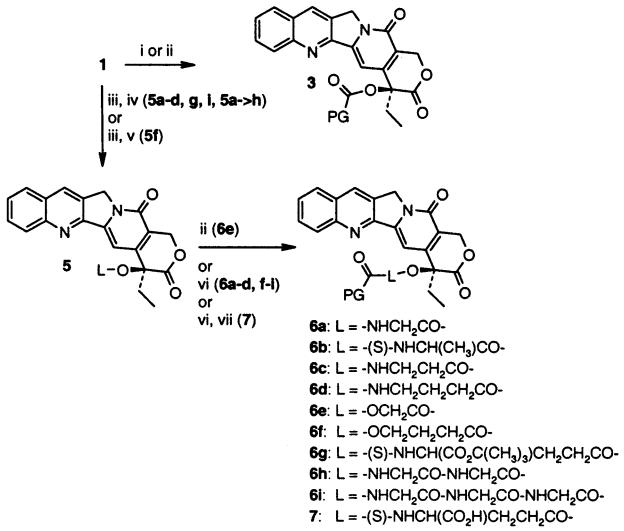
Scheme 1. Synthesis of PG Conjugates of CPT[a]



[a] Conditions: (i) PG, BOP-Cl, DMAP, DMF; (ii) PG, CMPI, DMAP, DMF; (iii) blocked linker, DIPC, DMAP, DMF; (iv) 50% TFA−CH_2Cl_2; (v) 10% Pd−C, cyclohexene, 25% dioxane−EtOH; (vi) PG, DIPC, DMAP, DMF; (vii) TFA.

α-*tert*-butyl ester (**6g**), which upon prolonged exposure to neat TFA afforded PG−(γ-glu)−CPT.

To evaluate the effects in our tumor models of lengthening the linker, we synthesized PG−$(gly)_2$−CPT (**6h**) and PG−$(gly)_3$−CPT (**6i**). Coupling of gly−CPT with *N*-*t*-BOC-glycine provided *N*-*t*-BOC−$(gly)_2$−CPT (**4h**), which after deblocking and conjugation of the resulting $(gly)_2$−CPT (**5h**) to PG yielded PG−$(gly)_2$−CPT. The tris-glycine linked conjugate was synthesized by O-acylation of CPT with *N*-*t*-BOC-$(gly)_3$ to yield *N*-*t*-BOC−$(gly)_3$−CPT (**4i**), which upon deblocking and conjugation with PG provided PG−$(gly)_3$−CPT.

Discussion

Our selection of a preferred chemical composition for PG−camptothecin was based on in vivo antitumor testing of conjugates in syngeneic mouse and human xenograft tumor models. C67BL/6 mice were injected with murine B-16 melanoma cells, or athymic nude mice were injected with either human HT-29 colon or NCI-H460 lung carcinoma cells. Tumor growth delays (TGD) were measured following single bolus intraperitoneal (ip) injection of conjugates (Tables 1 and 2). In most cases, the TGDs reported were obtained at or below the maximum tolerated dose (MTD). Other criteria considered in the selection process included aqueous solubility and chemical stability of conjugates, starting PG average MW, and wt % CPT loaded on PG.

The conjugates listed in Tables 1 and 2 were stable after overnight incubation in aqueous Na_2HPO_4 solution (0.1 M, 37 °C, pH 7.4). None released more than a trace of CPT as measured by TLC (data not shown).

Conjugates incorporating various linkers were compared for their effect on in vivo growth of B-16 melanomas (Table 1). On the basis of a comparison of CPT equivalent dosing levels, PG−gly−CPT and PG−(4-*O*-butyryl)−CPT appeared to have the highest antitumor activity.

The effect of polymer average MW was evaluated by comparison of in vivo efficacies of samples of PG−gly−CPT derived from PG with average MWs of 33 and 50 kDa. Both conjugates were 32 wt % CPT and dosed at the same CPT equivalent level (35 mg/kg). The TGD of B-16 melanomas was 2 days for the conjugate derived from 33 kDa PG and 4 days for the conjugate derived from 50 kDa PG. Further increase in polymer average MW to 74 kDa provided PG−gly−CPT with reduced aqueous solubility (data not shown).

We compared PG−gly−CPT, PG−$(gly)_2$−CPT, and PG−$(gly)_3$−CPT in the HT-29 colon carcinoma model (Table 2). When all three agents were dosed at comparable CPT equivalent levels (24−28 mg/kg) and at approximately 15 wt % CPT on PG, PG−$(gly)_3$−CPT gave the best response. Upon an increase in the CPT loading to 30% and in the CPT equivalent dose to 34 mg/kg, PG−$(gly)_3$−CPT displayed a TGD of 28 days in the HT-29 colon carcinoma model and 38 days in the NCI-H460 lung carcinoma model. However, this dosage of PG−$(gly)_3$−CPT was slightly above its MTD. In addition, PG−$(gly)_3$−CPT displayed diminished solubility compared to PG−gly−CPT. The aqueous solubility of PG−$(gly)_3$−CPT at 30 wt % CPT was 5 mg/mL. In

Table 1. Effects of PG Conjugates of CPT on in Vivo Growth of Subcutaneous B-16 Melanomas

conjugate[a]	wt % CPT[b]	dose[c]	B-16 TGD[e]	MTD[c]
PG−CPT[d]	9	48	4	≥48
PG−gly−CPT	32[d]	35	2[f]	~40
	32	35	4	
PG−ala−CPT	17	62	2[f]	≥62
PG−(β-ala)−CPT	31	67	1[g]	≥67
PG−(4-NH-butyryl)−CPT	24	60	1[g]	≥60
PG−(2-*O*-acetyl)−CPT	14	75	4	≥75
PG−(4-*O*-butyryl)−CPT	32	35	3[f]	~35
PG−(γ-glu)−CPT	16	41	1[g]	≥41

[a] MW of PG used in preparation of conjugates was 50 kDa (viscosity) unless specified otherwise. [b] The wt % CPT in conjugates was determined from ^1H NMR integral ratios as described in Supporting Information. [c] In units of mg of CPT/kg. [d] MW of PG used in preparation of this conjugate was 33 kDa (viscosity). [e] In units of days. TGD: difference of average times for tumors to reach a volume of 500 mm^3 between treatment and control groups. For Student's *t*-test, p vs vehicle, $p < 0.005$ unless specified otherwise. [f] $0.005 < p < 0.05$. [g] $p > 0.05$.

Table 2. Effects of PG Conjugates of CPT on in Vivo Growth of Human HT-29 Colon and NCI-H460 Lung Carcinomas

				TGD[e]		
conjugate[a]	wt % CPT[b]	solubility[c]	dose[d]	HT-29	NCI-H460	MTD[d]
CPT			20	17		~20
PG−gly−CPT	16		28	4[f]		~40
	23	35	33	7		
	29	34	33	20	16	
	36		39		30	
	38	15	39	26	38	
	47	7	40	6[f]	6	
PG−$(gly)_2$−CPT	16	34	28	4[f]		≥28
PG−$(gly)_3$−CPT	14	33	24	7		<34
	30	5	34	28	38	
PG−(4-*O*-butyryl)−CPT	22		33	13[f]		~35

[a] MW of PG used in preparation of conjugates was 50 kDa (viscosity). [b] The wt % CPT in conjugates was determined from ^1H NMR integral ratios as described in Supporting Information. [c] Aqueous solubility was measured in mg/mL of conjugate in aqueous Na_2HPO_4 solution (0.1 M, pH 7.4, and 37 °C). [d] In units of mg of CPT/kg. [e] In units of days. TGD: difference of average times for tumors to reach a volume of 500 mm^3 between treatment and control groups. For Student's *t*-test, p vs vehicle, $p < 0.005$ unless specified otherwise. [f] $0.005 < p < 0.05$.

Table 3. Plasma and Tumor Pharmacokinetics of PG−gly−[14-^{3}H]CPT and [14-^{3}H]CPT in Mice[a]

dosed drug	C_{max} (μg mL^{-1})	T_{max} (h)	AUC_{0-last} (μg h mL^{-1})	$T_{1/2}$ (h)	Vd (mL)	Cl (mL h^{-1})
			Plasma			
PG−gly−[^{3}H]CPT	179	0	240	97	166	1.2
[^{3}H]CPT	6	0	132	49	147	2.1
			Tumor			
PG−gly−[^{3}H]CPT	12	2	696	84[b]	*c*	*c*
[^{3}H]CPT	2	0	95	63[b]	*c*	*c*

[a] Female athymic nude mice were implanted with HT-29 carcinoma cells. When tumor volumes averaged 100−200 mm^3 (11 days), mice were injected iv with either PG−gly−[14-^{3}H]CPT (38 wt % CPT, 18 mg/kg CPT equivalent dose, 54 μCi/mg) in 0.1 M Na_2HPO_4 (4.8 mg/mL) or [14-^{3}H]CPT (15 mg/kg, 150 μCi/mg) in Liposyn III emulsion (0.75 mg/mL). All concentrations were expressed as microgram equivalents of CPT. Rsq > 0.90 for terminal elimination phase of each concentration vs time curve. For experimental details, see Supporting Information. [b] Mean residence time. [c] Not determined.

contrast, the aqueous solubility of PG−gly−CPT at comparable CPT loading (29%) was 34 mg/mL.

Improved efficacy of PG−gly−CPT in the HT-29 colon and NCI-H460 lung carcinoma models was observed with increased CPT loading. However, aqueous solubility was reduced with increased wt % CPT in the conjugate. Increasing the loading to 47 wt % CPT resulted in significantly diminished solubility, which was reflected in reduced efficacy. A preferred CPT loading for PG−gly−CPT was selected in the range 30−35%.

Studies in the HT-29 colon carcinoma model examining plasma and tumor pharmacokinetics of PG−gly-[14-^{3}H]CPT and [14-^{3}H]CPT indicated a 6-fold improvement in exposure of tumor tissue to CPT in the conjugate compared to free CPT (Table 3).

Considering its ease of synthesis, superior aqueous solubility and stability, and marked efficacy in various in vivo tumor models, we selected PG−gly−CPT with 30−35 wt % CPT for further study.

Experimental Section

Chemistry. CPT (>97% chemical purity) was purchased from Boehringer-Ingelheim Chemicals, Inc. (Petersburg, VA), and sodium PG was purchased from Sigma (St. Louis, MO). Average molecular weights of samples of PG were based on the viscosity of their sodium salts in aqueous solution. Other reagents were commercial grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 instrument. Mass spectra were recorded on a Micromass Quatro II electrospray mass spectrometer.

PG−CPT (3). To a solution of CPT (132 mg, 0.38 mmol), dry PG (33 kDa, 530 mg), and anhydrous DMF (20 mL), cooled in an ice bath, were added BOP-Cl (174 mg, 0.68 mmol), DMAP (167 mg, 1.37 mmol), and diisopropylethylamine (74 mg, 0.57 mmol). The mixture was warmed to room temperature over 4 h, stirred for 2 days, and cooled in an ice bath. A 10% aqueous NaCl solution (45 mL) was added over 25 min, and the mixture was acidified to pH 2.5 by addition of hydrochloric acid (0.5 M, 3.5 mL). After the mixture was stirred at room temperature for 1 h, the solid was filtered, washed with water (4 × 50 mL), dried under vacuum (<1 mm, 12 h), and ground to a powder. The solid was suspended in 2% MeOH−CH_2Cl_2 (10 mL), stirred for 3 h, and separated by centrifugation, and the supernatant was decanted. Extraction with 2% MeOH−CH_2Cl_2 was repeated 4 times to complete removal of unreacted CPT. The solid was dried under vacuum (<1 mm, 2 days) to yield **3** (521 mg, 87% mass balance, 15 wt % CPT).

PG−Linker−CPT Conjugates (6). To a solution of PG (1.24 g, 9.60 mmol of monomeric glu) in anhydrous DMF (31 mL) was added linker−CPT **5** (1 mol equiv based on targeted loading). Amino acid linked CPTs (**5a**−**d**,**g**−**i**) were added as TFA salts. After the mixture was cooled in an ice bath, DMAP (3 mol equiv) was added in portions followed by a solution of DIPC (1.2 mol equiv) in DMF (0.5 mL/mmol of DIPC) over 20 min. After being stirred at room temperature for 2 days, the mixture was cooled in an ice bath and 10% aqueous NaCl solution (75 mL) was added over 30 min. The mixture was acidified to pH 2.5 by addition of 1 M hydrochloric acid. After the mixture was stirred at room temperature for 1 h, the solid was filtered, washed with water (4 × 100 mL), dried under vacuum (<1 mm), stirred with 2% MeOH−CH_2Cl_2 (75 mL) for 1 h, and filtered. Extraction was repeated 3 times with 2% MeOH−CH_2Cl_2, once with CH_3CN (100 mL), and once with water (100 mL). The solid was dried under vacuum (<1 mm, 2 days) to yield **6**.

Supporting Information Available: Full experimental details along with spectral data, CPT loading analyses by NMR for compounds **3** and **6a**, and methods for in vivo tumor models and pharmacokinetic study. This material is available free of charge via the Internet at http://pubs.acs.org.

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