Synthesis, Characterization, and Preliminary in Vivo Tests of New Poly(ethylene glycol) Conjugates of the Antitumor Agent 10-Amino-7-ethylcamptothecin

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Despite the high antitumor activity of camptothecins, few derivatives have been developed and tested for human treatment of solid tumors, due to unpredictable toxicity mainly connected to their poor water solubility. We report the conjugation of the antitumor agent 10-amino-7-hydroxy camptothecin (SN-392) to linear or branched poly(ethylene glycol)s (PEGs) of different loading capacity through a tri- or tetrapeptide spacer selectively cleaved by lysosomal enzymes (cathepsins). A synthetic strategy based on the chemoselective acylation of the aromatic amino group in the presence of the unprotected C20 tertiary alcohol allowed high overall yields. Two conjugates demonstrated good stability at physiological pH and in mouse plasma (nonspecific proteases) but slowly released the drug payload in the presence of the lysosomal enzyme cathepsin B1. Compound **3**, selected for in vivo experiments, was very active against P388, P388/ADM leukaemia, and Meth A fibrosarcoma cell lines, scoring *T*/*C*% values comparable with the camptothecin derivative CPT-11. Pharmacokinetic studies indicated that **3** acts as a reservoir of 10-amino-7-ethylcamptothecin, as the mean residence time (MRT) is about 3-fold higher than that of the free drug.

Introduction

Camptothecin (CPT), an alkaloid which was isolated in the late 1950s from the Chinese tree "*Camptotheca acuminata*",[1] is the first member of a very promising class of anticancer agents. Despite the encouraging early trials, severe side effects (myelosuppression, severe diarrhea, and hemorrhagic cystitis), together with lack of sufficient information on the cellular target, eventually halted their clinical development in the 1970s. In the early 1980s a renewed interest in this class of agents was boosted from the understanding of their mechanism of action involving the nuclear enzyme topoisomerase I,[2,3] overexpressed in several types of solid tumors. In the same period, researchers discovered that the drug's poor water solubility was one possible cause for the clinically observed toxicity correlated to CPTs administration: the need to formulate water soluble salts of CPT (i.e. alkaline solutions for iv injections) led to chemical modifications of the molecule (**1**, Figure 1) with loss of antitumor activity and significant alterations in the toxicological profile of the drug.[4] Such findings encouraged the development of new, semisynthetic analogues of CPT with improved pharmacological and pharmaceutical properties while maintaining (or even increasing) the antitumor activity of the parent compound. Yaegashi and colleagues, among others, described the synthesis of 9-, 10-, and 11-amino-A-ring-substituted 7-ethylcamptothecin analogues, and those derivatives demonstrated some promising antitumor activity in preliminary in vitro experiments.[5] However, the administration of these compounds for in vivo assays and the efficient delivery to the tumor cells remain difficult to achieve, due to their very poor solubility in the physiological medium.

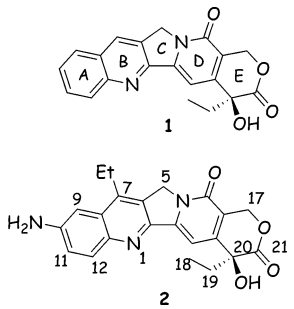

Figure 1. Structures of camptothecin (**1**) and 10-amino-7-ethylcamptothecin (SN-392) (**2**).

The conjugation of anticancer agents to water-soluble, biocompatible polymers is one of the most promising modifications in order to improve the pharmacokinetic and pharmacodynamic properties of the cytotoxic drug. The high molecular weight prodrug carriers can lead to higher water solubility, reduced systemic toxicity, increased half-life, improved biological distribution, and possibly higher therapeutic efficacy.

One of the cornerstone concepts of the polymeric prodrug approach is the so-called EPR (enhanced permeability and retention) effect, first postulated by

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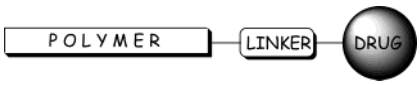


Figure 2. General scheme of polymeric prodrugs layout.

Maeda and co-workers.[6] This effect is due to the defective vascular architecture (hypervascularity and hyperpermeability of blood vessels) and poor drainage from an hypoplastic and inefficient lymphatic system inside the solid tumor. Polymers of appropriate size (ideally, between 5000 and 40 000 Da) are able to extravasate through the large fenestrations of the tumor vessels, concentrating in the tumor matrix, and the carried "payload" can be released either externally, in the extra cellular compartment, or inside the tumor cell, after uptake.[7,8]

Among the many biocompatible polymers that have been used for this purpose (HPMA,[9] dextrans,[10] poly(glutamic acids),[11] PEGs,[12−15] to name the most widely represented in the literature), we decided to use poly(ethylene glycol) (PEG) as carrier system, due to its well documented properties of water solubility, biocompatibility, and low immunogenicity, which enabled FDA approval for human usage.[15,16] The release of anticancer agents from polymeric carriers outside or inside the target cell is another issue that attracted many investigators in a research field closely related to the prodrug approach.[17] As in the classical produgs, the polymeric system must be sufficiently stable to survive storage and administration conditions, cross blood vessels unaltered, and release the active moiety selectively inside the target tissue by hydrolytic or enzymatic cleavage. In an idealized polymer−drug conjugate with general structure depicted in Figure 2, the linker should be a chemically cleavable system (carbamate or carbonate moieties, 1,6-elimination systems,[18] trimethyl-lock lactonization-based prodrugs[19]) or an enzymatically cleavable spacer, sensitive to specific enzymes that are present exclusively, or overexpressed, at the tumor site. Among such enzymes, lysosomal enzymes (cathepsins) have been selected by independent groups (Kopecek and Duncan,[20] Dubowchik,[21] Caiolfa[9]) as targets for their polymeric prodrug carriers of doxorubicin and camptothecin. A thorough investigation on the hydrolytic stability, lysosomal digestion, and antitumor activity was carried out by Coessen and co-workers[22] with polymer−drug conjugates using PEG (5 kDa), dextran, or poly(2-hydroxyethyl)-L-glutamine and different spacers. The tetrapeptide spacers H-Gly-Leu-Phe-Gly-OH and H-Gly-Phe-Leu-Gly-OH (and with minor efficiency the tripeptide H-Gly-Leu-Gly-OH) were effectively hydrolyzed by cathepsin B1, releasing the drug payload. On the basis of this experience and the experiments we carried out on PEGylated doxorubicins[23] and Ara-C,[24] we decided to prepare a series of polymeric conjugates of the anticancer agent 10-amino-7-ethylcamptothecin (SN-392, **2**, Figure 1), a CPT derivative that has been developed by some of us at Yakult Honsha Ltd., Co., Japan. SN-392 is an aminoCPT closely related to 9-aminocamptothecin (9-AC) and 9-nitrocamptothecin (9-NC), now under clinical Phase II trials. PEGs of different shape (linear or branched) and loading capacity (1 or 2 free hydroxyl terminal groups, respectively for mono-methoxy PEG and PEG diol) were conjugated to

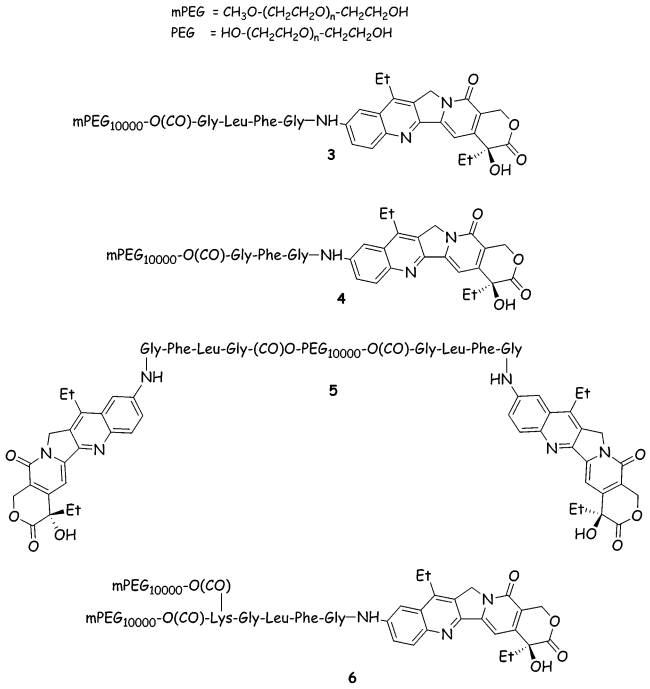


Figure 3. Synthesized PEG derivatives of 10-amino-7-ethylcamptothecin.

Table 1. Final Product Yield and Drug Loading

compd	yield,[a] %	drug loading,[b] %	loading efficiency (exptl/theoret), %	mass[c]
3	74	3.71 (3.72)	100	10765
4	96	3.21 (3.78)	85	10615
5	90	6.1 (6.78)	90	11500
6	76	1.82 (1.87)	97	20896

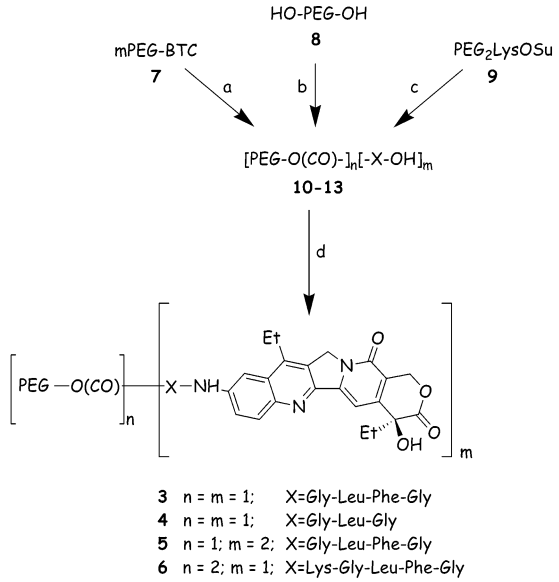
[a] Yield of final step. [b] Drug loading calculated as percentage of free drug weight on total conjugate weight (in parentheses the theoretical 100% w/w loading). [c] MALDI-TOF mass spectrometer, synapinic acid matrix; M^+ value is the center of the Gaussian distribution and is expressed in daltons.

SN-392 using the tetrapeptide H-Gly-Leu-Phe-Gly-OH and the tripeptide H-Gly-Leu-Gly-OH spacers. The synthesized compounds **3**−**6** are summarized in Figure 3. The overall yield, SN-392 loading (expressed as free drug % and calculated from UV absorption and amino acid analysis, as described in the Experimental section), and MALDI-TOF mass are reported in Table 1.

Chemistry

The synthetic strategy devised for the preparation of the conjugates **3**−**6** was designed with the purpose of reducing the chemical modifications that SN-392 might suffer during the reaction steps, maximizing the yield, and obtaining pure intermediates unambiguously by simple purification methods (Scheme 1). Linear monomethoxy PEG (mPEG) of 10 000 Da activated with benzotriazolyl carbonate (mPEG-BTC) was commercially available or prepared according to literature references.[25] The PEG diol was activated, after thorough removal of water by azeotropic distillation, with *p*-nitrophenyl carbonate, while the branched poly(ethylene glycol) $PEG_2LysOSu$ was used directly. The activated PEGs were added portionwise to a solution of the tri- or tetrapeptide in 1 M aqueous borate buffer (pH 8). During the reaction, the pH was carefully monitored, and 1 M NaOH was added to maintain pH 8. Reaction

Scheme 1[a]



3 n = m = 1; X=Gly-Leu-Phe-Gly
4 n = m = 1; X=Gly-Leu-Gly
5 n = 1; m = 2; X=Gly-Leu-Phe-Gly
6 n = 2; m = 1; X=Lys-Gly-Leu-Phe-Gly

[a] Reagents and conditions: (a) peptide (4 to 6 equiv), borate buffer 1 M, pH 8, rt, 24 h; (b) (1) *p*-nitrophenyl chloroformate, anhydrous CH_2Cl_2, Et_3N, rt, 12 h; (2) peptide (6 equiv), borate buffer 1 M, pH 8, rt, 24 h; (c) peptide (10 equiv), anhydrous CH_2Cl_2, Et_3N, rt, 24 h;(d) 10-amino-7-ethylcamptothecin, phenyl dichlorophosphate, pyridine, DPEA, anhydrous $CHCl_3$, rt, 12 h.

workup yielded a crude product that was purified by ion exchange chromatography. In this way, both purification and characterization of the reaction products was possible, as the only high molecular weight, negatively charged species that could be retained by the resin using water as eluent, and released by increasing the ionic strengths, were the PEG−peptide conjugates. Both starting PEGs (neutral) and low molecular weight reagents (peptide, activating agents, etc.) eluted separately through the resin using water as eluent. Titration of carboxylic groups and ^{1}H NMR confirmed the purity of the expected compounds.

The second step consisted in the conjugation of the PEG−peptide intermediate to the 10-amino group of SN-392. This reaction required chemoselectivity, as a mixture of 10-N and 20-O acylated products would not be easily separated. A first attempt was made using the classical EDCI/HOBt coupling method derived from peptide synthesis and employed by Greenwald and coworkers for the preparation of camptothecin derivatives PEGylated in position 20.[13] Several failures, using different bases (Et_3N, $^iPr_2NEt$, DMAP) and solvents (DMF, acetonitrile, chloroform) resulted in both low reactivity of the aromatic amino group of SN-392 and the concurrent formation of the 20-O-acylated species. A further explanation for such low reactivity was very recently proposed, for the 10-hydroxy camptothecin analogue (10-HCPT), by Greenwald:[26] 10-HCPT, according to the authors, would form strong intermolecular hydrogen bonding between the 10-OH and the 20-OH moieties (Figure 4a). In our case, the 10-NH_2 group is an aromatic amine para to a quinoline nitrogen; therefore, the free electron couple is highly delocalized onto the aromatic ring system, and the amine is a very poor nucleophile (Figure 4b).

Regioselective acylation of 20-hydroxycamptothecin with PEG carboxylic acids was achieved by Enzon researchers[27] using the coupling reagent phenyl dichlorophosphate in the presence of pyridine.[28]

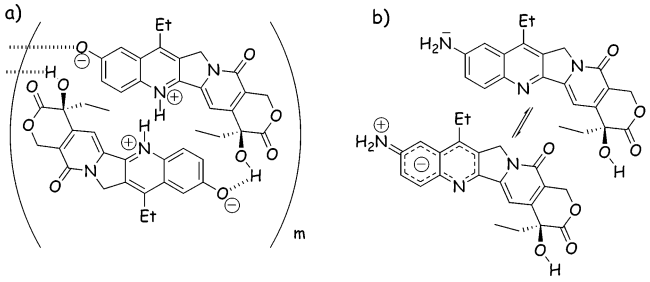


Figure 4. Proposed intramolecular hydrogen bonding for (a) 10-hydroxycamptothecin (from ref 22) and (b) 10-amino-7-ethylcamptothecin (SN-392).

Acylation of amino groups, either aliphatic or aromatic, with the same reagent, although not reported in the literature, seemed to us consistent with the use of diphenylphosphinic acids as coupling reagents in peptide synthesis. We were in fact able to apply the method devised for acylation of 10-HCPT to our compound, with careful adjustments in experimental conditions (pH, base used, reaction time)[29] that allowed full chemoselective control over the acylation site. No 20-*O*-acyl SN-392 could be detected (according to ^{1}H NMR single quartet around 1.9 ppm for the C-19 protons[26] and broad singlet at 6.5 ppm for 20-OH), and the only final product was the desired N-acylated species. Poor solubility of free SN-392 in the reaction mixture allowed a first purification of the PEG conjugate by filtration, followed by crystallization from 2-propanol. Final compounds were characterized by ^{1}H NMR, and drug loading was calculated by UV absorption.

In Vitro Hydrolysis Assays. Conjugates **3** and **4** were submitted to chemical and enzymatic hydrolysis to test the drug release at different pH values and in the presence of cathepsin B1, a lysosomal enzyme. Experiments in buffer at pH 5.5 and 7.4, in mouse plasma, and in buffer (pH 5.5) with cathepsin B1 are reported in Figure 5.

It can be appreciated from Figure 5a that **3** was progressively converted into SN-392 in the presence of cathepsin B1. After 24 h, about 25% of **3** was converted. Under the same conditions but without cathepsin B1 (Figure 5b) the compound is virtually unaffected, and after 24 h less than 5% was hydrolyzed with release of free SN-392. Compound **4** showed higher stability toward enzymatic degradation, as the tripeptide is a poorer substrate than the tetrapeptide spacer[17] (Figure 5e). Stability was observed also under physiological conditions (pH 7.44) with about 5% conversion after 5 h for **3** and 0.8% for **4** (Figure 5c and 5g). In mouse plasma **3** and **4** were slowly converted into SN-392, and after 24 h about 15% and 12% of starting product was hydrolyzed, respectively (Figure 5d and 5h). This suggests that, although the peptide spacer was selected among the sequences specifically targeted by lysosomal enzymes, still nonspecific degradation possibly involving plasma proteases can occur.

In Vivo Assays. Derivative **3** was tested against murine leukaemia P388 and adriamycin resistant P388 (P388/ADM) (Tables 2 and 3) and Meth A fibrosarcoma (Table 4) cell lines. Due to poor water solubility, the pharmacophore 10-amino-7-ethylcamptothecin was ad-

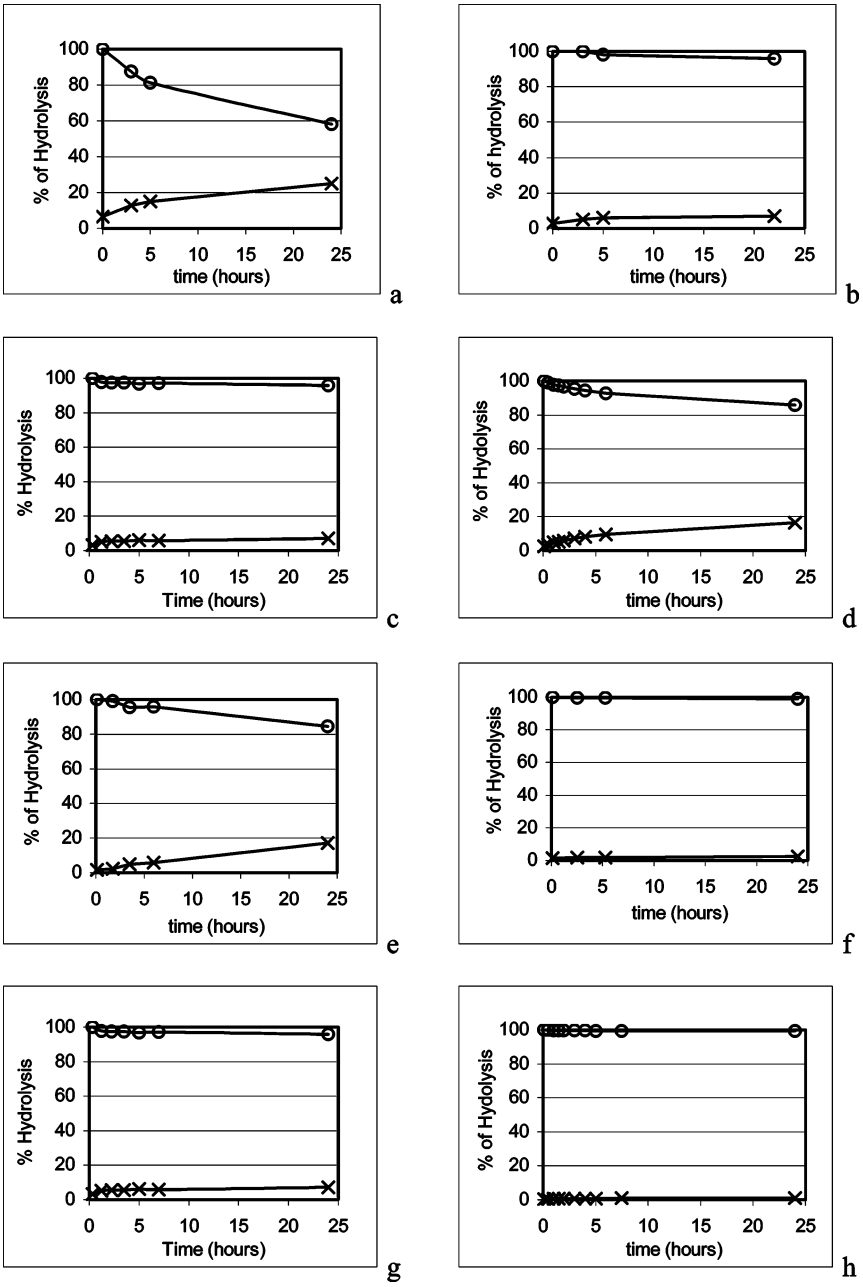


Figure 5. Hydrolysis experiments: (a) hydrolysis of **3** in the presence of cathepsin B_1 at pH 5.5; (b) hydrolysis of **3** at pH 5.5; (c) hydrolysis of **3** at pH 7.44; (d) hydrolysis of **3** in mouse plasma; (e) hydrolysis of **4** in the presence of cathepsin B_1 at pH5.5; (f) hydrolysis of **4** at pH 5.5; (g) hydrolysis of **4** at pH 7.44; (h) hydrolysis of **4** in mouse plasma. % Hydrolysis calculated from HPLC peak area at 368 nm (○ = PEG-bound camptothecin, X = free camptothecin). All experiments were conducted in thermostatic bath at 37 °C.

Table 2. Activity of CPT-11, Adriamycin (ADM), and Compound **3** against P388 Leukaemia Implanted on Female CDF1 Mice

sample	total dose (mg/kg)	survival time (days, mean ± SD)	*T/C* (%)[a]
3	5	11.8 ± 3.7	154
	10	10.8 ± 1.5	141
	20	13.0 ± 1.1	170
CPT-11	25	13.2 ± 1.7	172
	50	14.5 ± 1.5	189
	100	17.5 ± 0.8	228
ADM	25	20.0 ± 9.3	261
control	-	7.7 ± 0.5	100

[a] *T/C* (%) = survival rate calculated as ratio between mean survival days of treated group and control group.

Table 3. Activity of CPT-11, Adriamycin (ADM), and Compound **3** against P388/ADM Leukaemia Implanted on Female CDF1 Mice

sample	total dose (mg/kg)	survival time (days, mean ± SD)	*T/C* (%)[a]
3	5	11.2 ± 1.6	116
	10	13.2 ± 2.6	136
	20	13.2 ± 3.8	136
CPT-11	25	12.5 ± 0.8	129
	50	14.7 ± 2.3	152
	100	15.7 ± 1.6	162
ADM	25	9.8 ± 2.6	102
control	–	9.7 ± 1.2	100

[a] *T/C* (%) = survival rate calculated as ratio between mean survival days of treated group and control group.

ministered as sodium salt (SN-392Na, open lactone form). In addition, one of its water soluble analogues, namely CPT-11, was used for comparison. The survival rates (*T/C*%) against P388 and P388/ADM after admin-

Table 4. Activity of CPT-11, 10-Amino-7-ethylcamptothecin Sodium Salt (SN-392Na), and Compound **3** against Meth A Fibrosarcoma in BALB/c Mice[a]

sample	dose (mg/kg)	tumor weight (g) (mean ± SD)	inhibition rate (%)
control	–	2.45 ± 0.69	–
3	25	1.51 ± 0.23	38.4
	50	1.09 ± 0.29	55.5
	100	0.51 ± 0.28	79.1
SN-392Na	25	0.93 ± 0.30	62.3
	50	0.88 ± 0.21	64.0
	100	0.75 ± 0.09	69.3
CPT-11	25	1.24 ± 0.46	49.5
	50	1.31 ± 0.28	46.6
	100	1.16 ± 0.37	52.7

[a] Meth A cells (5×10^5/mouse) were inoculated subcutaneously into 7 week old male BALB/c mice (five/group) on day 0. Samples were injected intravenously on day 5, and the tumors were weighed on day 21. Control group was given with saline.

istration of **3** show that in this animal model the PEGylated compound is comparable with the clinically used CPT-11 and could be a good candidate for the development of a new water soluble, tumor-targeted camptothecin anticancer agent. Inhibition rate (%) of tumor growth at 50 and 100 mg/kg single iv administration (dose equivalent of free drug) against Meth A fibrosarcoma cells was higher than that of SN-392 sodium salt and CPT-11, again confirming the potency of this conjugate in a solid tumor model.

Pharmacokinetic Assays. The pharmacokinetic parameters of 10-amino-7-ethylcamptothecin after iv administration of compound **3**, 10-amino-7-ethylcamptothecin (dissolved in DMSO/10 mM H_3PO_4 10/1(v/v)) and its sodium salt (water soluble, opened lactone form of camptothecin) are listed in Table 5. 10-amino-7-ethylcamptothecin concentrations in plasma after iv administration of each compound to rats were measured over a 7 h period.

Results and Discussion

The poor solubility of camptothecins in general, and 10-amino-7-ethylcamptothecin (SN-392, developed by Yakult Honsha Inc., Japan) in particular, finds an ideal solution in the conjugation of this class of antitumor agents to water soluble polymers, acting as both reservoirs and carriers of the active moiety. The choice of carriers for this purpose is wide, but few polymers have the favorable characteristics of poly(ethylene glycol) (PEG): high biocompatibility, low toxicity, low immunogenicity, low polydispersivity, sound chemistry, and fixed number of anchoring sites (1 or 2) that avoid multicomponent mixtures of partially substituted molecules typical of other polymers, which are difficult to characterize and standardize and, most importantly, to gain FDA approval. One aspect of polymeric prodrug pharmacology that deserves a separate comment is the so-called EPR (enhanced permeability and retention) effect. First described by Maeda and colleagues, EPR allows the specific localization of a drug in the cancer tissue as a result of the higher permeability of blood capillaries in that area, accompanied by a reduced lymphatic drainage. Both phenomena permit the accumulation of the drug polymer within the tumor matrix.[6−8]

Besides the modification of the pharmacokinetic profiles, the macromolecular nature of the bioconjugates is per se responsible for the exploitation of a totally new cell penetration pathway other than adsorption- or receptor-mediated endocytosis. This new pathway has been used to advantage since new, specific linkages between polymer and drug have been designed. Such linkages ought to be stable in blood but cleavable intracellularly, by means of the acidic environment of endosomes or the rich enzymatic machinery of the lysosomes. For the second approach, specific amino acid sequences were designed to target lysosomal enzymes (like the cathepsin family) but survive from the attack of aspecific extracellular proteases.

The synthesis of compounds **3**−**6** was carried out in two steps (Scheme 1), optimizing the yield for each step by careful choice of reagents and reaction conditions. Table 1 summarizes the final step yields (between 74 and 96%) and the drug loading in the purified compounds (between 85 and 100% of maximun theoretical loading). Purification of the intermediates by ion exchange chromatography was also a key step, allowing both purification of the PEG−peptide intermediates **10**−**13** and their unequivocal characterization. In fact, the PEG−tetrapeptide intermediate bearing a carboxylic acid terminal group could be separated from the unreacted, neutral PEG and the coupling reagents and byproducts by fixation to an anion-exchange resin and two-step elution (milliQ water first, then 0.01 N aq NaCl). Final products were purified by crystallization with 2-propanol, a method that offered both high yields and low decomposition of the conjugate: attempts to purify the crude products by gel filtration using Pharmacia LH-20 resin in anhydrous conditions (eluent methanol) resulted in poor recovery and formation of uncharacterized byproducts during elution.

The determination of drug loading was a very critical issue that required an approach totally different from the reported methods,[27] as the acylation of the 10-amino

Table 5. Pharmacokinetic Parameters of 10-Amino-7-ethylcamptothecin in Rat Plasma after iv Administration of Compound **3**, 10-Amino-7-ethylcamptothecin, or Its Sodium Salt

	compound **3**		10-amino-7-ethylcamptothecin	10-amino-7-ethylcamptothecin sodium salt
parameter	100 mg/kg ($n = 2$)	50 mg/kg ($n = 2$)	4 mg/kg ($n = 2$)	4 mg/kg ($n = 1$)
AUC_{inf} (μg/h/mL)	1.89 (1.80, 1.98)[a]	0.531 (0.507, 0.554)	2.08 (2.34, 1.65)	1.71
C_{max}[b] (μg/mL)	1.35 (1.20, 1.51)	0.436 (0.439, 0.433)	10.53 (14.09, 6.99)	10.50
t_{max} (min)	2.00	2.00	2.00	2.00
MRT_{inf} (h)	3.56 (3.52, 3.61)	3.31 (3.31, 3.30)	1.16 (1.22, 1.09)	1.09
CL_{tot} (L/kg/h)	-	-	2.07 (1.71, 2.42)	2.42
V_{dss} (L/kg)	-	-	2.37 (2.09, 2.65)	2.65
$t_{1/2}$ (h)	2.82 (2.78, 2.84)	2.69 (2.71, 2.66)	2.25 (2.24, 2.26)	2.26

[a] Values in the parentheses are of the individual animals. [b] The value represents the maximum plasma concentration determined.

group significantly alters the UV spectra of the pharmacophore. The simple use of tabulated values for the molar extinction coefficient (ϵ) of free SN392 would therefore lead to misjudgments in the loading efficiency. As a result, we had to calculate the ϵ of PEG−Gly-Leu-Phe-Gly−SN392 product from a titration curve, starting from a solution at known concentration. As PEG is a highly hygroscopic compound, simple weight measurement cannot be entrusted. Quantitative amino acid analysis was instead the solution to the problem, as from the amino acid content we could calculate the exact molarity of the standard solution and therefore the concentration of the PEG derivative in the stock solution and in the dilutions used for the determination of ϵ.

Compounds **3** and **4** were selected for hydrolysis experiments in different conditions, varying the pH (7.44 and 5.5, respectively, physiological- and lysosomal-mimicking environments) and the presence of cathepsin B1. Mouse plasma was also used to check the stability of the compounds toward nonspecific proteases (Figure 5a−h). As expected from literature data[17] and our experience,[23,24] the tetrapeptide was a better substrate for cathepsin B1 than the tripeptide (25% of hydrolysis at 24 h vs 16%). Both derivatives were equally stable at pH 5.5, with negligible hydrolysis occurring. **3** and **4** were also stable at pH 7.44 (<5% hydrolysis at 24 h) but slowly released the drug in mouse plasma (around 15% and 12% hydrolysis at 24 h, respectively). The experiments clearly demonstrate that the PEG conjugates are able to release the drug payload in lysosomal environments but remain stable in plasma ($t_{1/2}$ hydrolysis $\gg$ $t_{1/2}$ elimination by renal ultrafiltration).

Caiolfa and co-workers[9] performed similar enzymatic hydrolysis experiments on their MAG−camptothecin compounds, finding that the release in the presence of cathepsin B was around 5% at 5 h, and background hydrolysis at pH 5.5 was negligible. Their results are comparable to our findings, although in our molecules initial release of SN-392 is higher (16% at 5 h for compound **3**). This could be explained with the higher accessibility of the peptidic spacer in our conjugates, where it is positioned at the end of the polymer. MAG polymers, on the contrary, have multiple binding sites distributed along the polymer chain, with different accessibility (and reactivity) toward the enzymatic degradation.

In vivo antitumor activity was tested in three separate experiments, using P388, P388/ADM leukaemia, and MethA fibrosarcoma cell lines in mice. The P388/ADM are tumor cells resistant to adriamycin expressing the MDR (multidrug resistance) protein. Against leukemia cell lines, compound **3** (at dose of 20 mg/kg expressed as free drug) was as active as the reference compound CPT-11 (Tables 2 and 3).

The same derivative, at doses of 50 and 100 mg/kg (expressed as free drug), was more potent than CPT-11 and SN-392 (sodium salt) against Meth A fibrosarcoma cells, with tumor growth inhibition rates of 55% and 79.1%, respectively (Table 4). Comparison of these data with the HPMA-bound camptothecin analogues MAG-CPT-1 and MAG-CPT-2 reported by Caiolfa[9] is difficult, because of the different experimental schedules, initial doses, and tumor cell lines, but substantially confirms the higher antitumor activity of the polymeric conjugates with respect to their free camptothecin analogues.

10-Amino-7-ethylcamptothecin concentrations in plasma were measured over 7 h after iv injection of compound **3** to rats at a dose of 100 or 50 mg/kg and compared to those after injection of 10-amino-7-ethylcamptothecin and 10-amino-7-ethylcamptothecin sodium salt (opened lactone form) at a dose of 4 mg/kg which was equivalent molecular numbers to compound **3** at a dose of 100 mg/kg (Table 5). It is immediately evident that (a) the maximal plasma concentration (C_{max}) after administration of both forms of 10-amino-7-ethylcamptothecin is 8-fold higher than that after compound **3** injection; (b) although AUC data are comparable, the mean resident time (MRT, a dose-independent measure of drug elimination expressing the average time a drug molecule remains in the body after rapid iv injection) after compound **3** injection is 3-fold higher than those after injection of both forms of 10-amino-7-ethylcamptothecin. Both data indicate that **3** acts as a circulating reservoir of the antitumor agents.

Conclusions

We prepared a series of PEGylated derivatives of 10-amino-7-ethylcamptothecin with PEGs of different shapes (linear and branched) and spacers (tri- and tetrapeptides). Compounds **3** and **4** were tested for selective enzymatic hydrolysis in the presence of cathepsin B1, a lysosomal enzyme overexpressed in tumors, and stability in physiological conditions. Compound **3** was the best candidate for in vivo evaluation, which was carried out against P388, P388/ADM, and MethA cells. The conjugate was compared to CPT-11 and 10-amino-7-ethylcamptothecin and was more active both on leukemia and MethA fibrosarcoma (solid tumor) models. Pharmacokinetic studies indicated that **3** acts as a reservoir of 10-amino-7-ethylcamptothecin, as the mean residence time (MRT) is about 3-fold higher than that of the free drug.

Experimental Section

Chemistry. General. All coupling reagents and solvents were purchased from Sigma-Aldrich Inc. or Fluka. Poly(ethylene glycols) were purchased from Shearwater Inc. (Huntsville, AL). NMR spectra were obtained using a 300 MHz spectrometer (Bruker Biospin) and deuterated chloroform as the solvent unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and coupling constants (J values) are given in hertz (Hz). Ion exchange chromatography was performed using a Pharmacia FPLC apparatus loaded with QAE Sephadex A-50 anion-exchange resin (10 × 2.5 cm bead). Gel filtration HPLC analyses were performed on a Jasco HPLC system (880-PU pump, 830-RI refractive index detector) coupled to a Shimadzu C−R4A Chromatopac integrator; stationary phase: BIOSEP SEC S3000 analytical gel filtration column; mobile phase 80% aq Na_2HPO_4 0.1 M, NaCl 0.3 M, pH 7, 20% acetonitrile. The column was calibrated with commercial standards of PEG 5, 10 and 20 KDa. RP-HPLC were run on a Gilson HPLC system equipped with a Vydac C18 analytical column (200 × 4.6 mm). Eluent A: milliQ grade water, 0.05% TFA; eluent B: acetonitrile, 0.05% TFA.

Final compounds weight was obtained by matrix-assisted laser desorbtion ionization time-of-flight (MALDI-TOF) analysis using a Hewlett-Packard G 2025 A LD-TOF mass spectrometer and synapinic acid as the matrix.

The percentage of 10-amino-7-ethylcamptothecin was determined using UV assay techniques as reported below. Rates

of hydrolysis were obtained using an analytical HPLC system equipped with an Alltima C-18 (Alltech Associates, Inc. Deerfield, IL) reversed-phase chromatography column (150 × 4.6 mm), a multiwavelength UV detector set up at 226 and 368 nm and a gradient of 10% to 80% acetonitrile/0.05% TFA in 30 min at a flow rate of 1 mL/min. All PEG compounds were dried under vacuum or by azeotropic distillation from toluene prior to use.

Analysis of 10-Amino-7-ethylcamptothecin (SN-392) Content in PEG Derivatives. Due to the absorbance change caused by acylation of the amino group of SN-392, for determination of drug content the following procedure was used: an exact amount of pure compound **3** (single peak from HPLC chromatogram) was weighed into a Pyrex tube. HCl (6 N, 1 mL) was added, and the tube was sealed under vacuum. After 22 h at 110 °C, the tube was opened and the content was injected into an amino acid analyzer Carlo Erba 3A30. Quantitative analysis of the amino acids (Gly, Leu, Phe) returned the exact title of the compound, which was used to determine the concentration of the solutions used for UV measurements.

UV absorption curves for different concentrations (obtained from progressive dilutions of a 0.06 mM mother solution) were recorded, and absorbance values at 368 nm were plotted against the concentrations. The resulting calibration curve was used to calculate the molar extinction coefficient of PEG-bound SN-392 ($\epsilon = 19325$).

m-PEG$_{(10kD)}$-O(C=O)-NH-Gly-Leu-Phe-Gly-OH (10). m-PEG$_{(10kD)}$-BTC (mw = 10 000) (1 g, 0.101 mmol) was added portionwise over 30 min to a solution of 0.24 g (0.606 mmol, 6 equiv) of tetrapeptide H-Gly-Leu-Phe-Gly-OH in 3 mL of 1 M borate buffer, pH 8. The resulting mixture was adjusted to pH 8 using 1 N NaOH and stirred at room temperature for 24 h. The reaction mixture was acidified with citric acid to pH 3 and extracted with chloroform (3 × 50 mL). The combined organic solutions were dried over sodium sulfate and concentrated to a small volume at reduced pressure. The resulting slurry was added dropwise to 200 mL of vigorously stirred diethyl ether. The white precipitate, which formed, was filtered and dried at reduced pressure, affording 0.96 g of crude product which was applied to a QAE Sephadex A-50 ion exchange column. Elution with MilliQ grade H_2O afforded 0.125 g of starting material (m-PEG-OH); with increase of ionic strength (0.01 N NaCl) the desired compound eluted together with NaCl (0.896 g combined). The combined fractions containing the m-PEG−tetrapeptide were freeze-dried, and the residue was suspended in chloroform to remove the salts. Recrystallization with diethyl ether afforded 0.77 g (77%) of the title compound. ^{1}H NMR ($CDCl_3$) ppm: 0.91 (t, J = 5.6 Hz, 6H, Hδ Leu); 1.24 (m, 1H, Hγ Leu); 1.60 (m, 2H, Hβ Leu); 3.01−3.20 (dd, J = 22.8 Hz; J = 6.6 Hz, 2H, Hβ Phe); 3.39 (s, 3H, PEG OCH_3); 3.40−3.88 (m, PEG + Hα Gly); 4.15 (m, 1H, Hα Leu); 4.19 (m, 1H, Hα Phe); 5.70 (m, 1H, O(CO)NH); 7.26−7.31 (m, 5H, arom Phe); 8.16 (s, 1H); 8.34 (s, 1H); 8.91 (s, 1H).

m-PEG$_{(10kD)}$-O(C=O)-NH-Gly-Leu-Phe-Gly-10-amino-7-ethylcamptothecin (3). Compound **10** (0.6 g, 0.06 equiv) and 45 mg (0.12 mmol, 2 equiv) of 10-amino-7-ethylcamptothecin were dissolved in 30 mL of toluene, and the mixture was azeotropically distilled with removal of 10 mL of toluene. The mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 30 mL of dry chloroform. Pyridine (0.24 mL, 3 mmol) and phenyl dichlorophosphate (0.35 mL, 2.28 mmol) were added, and the mixture was stirred at room temperature for 12 h, adjusting to pH 8 with DPEA. At the end, the yellow mixture was extracted with 1 N HCl (20 mL). The organic phase was concentrated at reduced pressure, and the residue, diluted with 20 mL of 2-propanol, was recrystallized. The pale yellow crystalline precipitate was washed with diethyl ether, affording 0.45 g of pure product. ^{1}H NMR ($CDCl_3$) ppm: 0.83−0.91 (m, 6H, Hδ Leu); 1.03 (t, 3H, CPT 18-H); 1.26 (m, 1H, Hγ Leu); 1.42 (t, 3H, CPT 7-CH_2CH_3); 1.61 (m, 2H, Hβ Leu); 1.89 (q, 2H, CPT H-19); 2.97 (m, 2H, CPT 7-CH_2CH_3); 3.05 (d, 2H, Hβ Phe); 3.38 (s, 3H, PEG OCH_3); 3.39−3.89 (m, PEG + Hα Gly); 4.17 (m, 1H, Hα Leu); 4.26 (t, 1H, Hα Phe); 5.24 (s, 2H, CPT H-5); 5.29 (d, 2H, CPT H-17); 5.72 (m, 1H, O(CO)NH); 6.5 (bs, 1H, 20-OH); 7.24−7.30 (m, 8H, arom); 7.7 (s, 1H, CPT H-14); 8.18 (s, 1H); 8.35 (s, 1H); 8.90 (bs, 1H); 9.50 (s, 1H).

RP-HPLC gradient time (% B): 0 (10%) 30′ (80%), 36′ (10%); single peak eluting at 18.2 min. MALDI-TOF mass spectroscopy (sinapinic acid matrix): M^+ centered at 10765 Da. Gel filtration HPLC: single peak eluting at 10.5 min. The amount of 10-amino-7-ethylcamptothecin (**2**) determined in the obtained derivative was 16.7 mg, corresponding to a w/w % of 3.71% (the theoretical 100% of loading is, for this conjugate, 3.72% according to UV absorption).

m-PEG$_{(10kD)}$-O(C=O)-NH-Gly-Leu-Gly-OH (11). Tripeptide H-Gly-Leu-Gly-OH·HCl (114.1 mg, 0.404 mmol) was solubilized in 7 mL of 1 M borate buffer, pH 8. One gram (0.101 mmol) of m-PEG-BTC (mw = 9876) was added portionwise over 60 min. The pH was maintained at pH 8 by 1 N NaOH and stirred at room temperature for 24 h.

The reaction mixture was acidified with 1 N HCl to pH 3 and extracted with chloroform (5 × 50 mL). The organic phases were dried over Na_2SO_4, filtered, and concentrated at reduced pressure. The remaining oil was dropped into 200 mL of diethyl ether. The mixture was maintained at 4 °C for 1 h and filtered, and the resulting white powder was dried at reduced pressure. The crude yield was 96%. The product was separated from the unreacted mPEG-OH by Sephadex QAE A-50 ion exchange column. Elution with MQ grade H_2O removed the undesired material (m-PEG-OH), while at increased ionic strength (0.01 N NaCl) the desired compound was recovered from the column. The solution was freeze-dried to obtain 603.7 mg, and the product was treated with chloroform to remove the salts and filtered. After concentration of the chloroform solution the remaining oil was added dropwise to diethyl ether. The final yield was 57% (w/w). ^{1}H NMR ($CDCl_3$) ppm: 0.88 (t, J = 5.8 Hz, 6H, Hδ Leu); 1.24 (m, 1H, Hγ Leu); 1.64 (m, 2H, Hβ Leu); 3.35 (s, 3H, PEG OCH_3); 3.42−3.90 (m, PEG); 4.17 (m, 1H, Hα Leu); 5.67 (m, 1H, O(CO)NH); 8.18 (bs, 1H); 8.35 (s, 1H).

m-PEG$_{(10kD)}$-O(C=O)-NH-Gly-Leu-Gly-10-amino-7-ethylcamptothecin (4). Compound **11** (0.1 g, 0.01 mmol, 1 equiv) and 7.8 mg (0.02 mmol, 2 equiv) of 10-amino-7-ethylcamptothecin were dissolved in 30 mL of toluene, and the mixture was azeotropically distilled with removal of 25 mL of toluene. The mixture was suspended in 30 mL of dry chloroform. Pyridine (0.040 mL, 0.5 mmol) and phenyl dichlorophosphate (0.06 mL, 0.4 mmol) were added, and the mixture was stirred at room temperature for 15 h, adjusting to pH 8 with DPEA (0.075 mL). At the end, the yellow mixture was washed with 1 N HCl (2 × 20 mL). The organic phase was concentrated at reduced pressure, and the residue, diluted with 20 mL of 2-propanol, was recrystallized at 4 °C. The pale yellow crystalline precipitate was washed with cold 2-propanol and then with diethyl ether, affording the pure product that was dried at reduced pressure. The yield was 96% (w/w). 10-amino-7-ethylcamptothecin loading (w/w) 3.21%, theoretical loading (W/W) 3.78%. ^{1}H NMR ($CDCl_3$) ppm: 0.86−0.95 (m, 6H, Hδ Leu); 1.10 (t, 3H, CPT 18-H); 1.28 (m, 1H, Hγ Leu); 1.38 (t, 3H, CPT 7-CH_2CH_3); 1.63 (m, 2H, Hβ Leu); 1.93 (q, 2H, CPT H-19); 3.20 (m, 2H, CPT 7-CH_2CH_3); 3.35 (s, 3H, PEG OCH_3); 3.40−3.92 (m, PEG + Hα Gly); 4.18 (m, 1H, Hα Leu); 5.25 (s, 2H, CPT H-5); 5.29 (d, 2H, CPT H-17); 5.69 (m, 1H, O(CO)NH); 6.30 (bs, 1H, 20-OH); 7.21−7.30 (m, 3H, arom); 7.65 (s, 1H, CPT H-14); 8.17 (s, 1H); 8.90 (bs, 1H); 9.25 (s, 1H).

RP-HPLC gradient time (% B): 0 (10%) 30′ (80%), 36′ (10%); single peak eluting at 18.7 min. MALDI-TOF mass spectroscopy (sinapinic acid matrix): M^+ centered at 10615 Da. Gel filtration HPLC: single peak eluting at 10.4 min.

PEG$_{(10kD)}$-[O−(C=O)-Gly-Leu-Phe-Gly-OH]$_2$ (12). Diol HO-PEG-OH (mw = 10000) (1 g, 0.101 mmol) was dissolved in 30 mL of toluene and refluxed in a Dean−Stark apparatus to azeotropically remove water. The solution was concentrated to 5−6 mL and then diluted with dry dichloromethane (5 mL). *p*-Nitrophenyl chloroformate (0.2 g, 10 equiv) and 0.14 mL (10 equiv) of triethylamine were added, and the resulting mixture

was stirred at room temperature for 12 h. At the end, the mixture was added dropwise to 200 mL of diethyl ether under vigorous stirring. The resulting white precipitate was filtered and dried, affording 1 g of PEG-di(*p*-nitrophenyl carbonate). The activated PEG diol was added portionwise over 30 min to a solution of 0.24 g (0.606 mmol, 6 equiv) of tetrapeptide H-Gly-Leu-Phe-Gly-OH in 3 mL of 1 M borate buffer, pH 8. The resulting mixture was adjusted to pH 8 using NaOH 1N and stirred at room temperature for 24 h.

The reaction mixture was then acidified with citric acid to pH 3 and extracted with chloroform (3 × 50 mL). The combined organic solutions were dried over sodium sulfate and concentrated to a small volume at reduced pressure. The resulting slurry was added dropwise to 200 mL of vigorously stirred diethyl ether. The white precipitate which formed was filtered and dried at reduced pressure, affording 0.96 g of crude product which was applied to a column packed with QAE Sephadex A-50 ion-exchange resin. Elution with MilliQ grade H_2O afforded 0.095 g of starting material (PEG-OH). The appropriate combined fractions were freeze-dried, and the residue was suspended in chloroform to remove the salts. Recrystallization afforded 0.86 g (79%) of title compound. Titration of COOH groups: 98%. ^{1}H NMR ($CDCl_3$) ppm: 0.87−0.95 (m, 12H, Hδ Leu); 1.28 (m, 2H, Hγ Leu); 1.56 (m, 4H, Hβ Leu); 3.06−3.25 (m, 4H, Hβ Phe); 3.40−3.87 (m, PEG + Hα Gly); 4.10 (m, 2H, Hα Leu); 4.15 (m, 2H, Hα Phe); 5.65 (m, 2H, O(CO)NH); 7.22−7.35 (m, 10H, arom Phe); 8.15 (s, 2H); 8.33 (s, 2H); 8.75 (s, 2H).

$PEG_{(10kD)}$-[O−(C=O)-Gly-Leu-Phe-Gly-10-amino-7-ethylcamptothecin]$_2$ (5). PEG_{10kD}-[O−(C=O)Gly-Leu-Phe-Gly-OH]$_2$ (**12**) (0.6 g, 0.06 mmol) and 90 mg (0.24 mmol, 4 equiv) of 10-amino-7-ethylcamptothecin were dissolved in 30 mL of toluene, and the mixture was azeotropically distilled with removal of 10 mL of toluene. The mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 30 mL of dry chloroform. Pyridine (0.48 mL, 6 mmol) and phenyl dichlorophosphate (0.7 mL, 4.56 mmol) were added, and the mixture was stirred at room temperature for 12 h, adjusting to pH 8 with DPEA. At the end the yellow mixture was extracted with 1 N HCl (20 mL). The organic phase was concentrated at reduced pressure, and the residue, diluted with 20 mL of 2-propanol, was recrystallized. The pale yellow crystalline precipitate was washed with diethyl ether affording 0.55 g of product (5). Loading (w/w % of aminocamptothecin) = 6.1% (theoretical = 6.78%) according to UV absorption. ^{1}H NMR ($CDCl_3$) ppm: 0.8−0.93 (m, 12H, Hδ Leu); 1.10 (t, 6H, CPT 18-H); 1.28 (m, 2H, Hγ Leu); 1.37 (m, 6H, CPT 7-CH_2CH_3); 1.64 (m, 4H, Hβ Leu); 1.95 (q, 4H, CPT H-19); 2.95−3.00 (m, 8H, CPT 7-CH_2CH_3 + Hβ Phe); 3.25−3.76 (m, PEG + Hα Gly); 4.14 (m, 2H, Hα Leu); 4.32 (t, 2H, Hα Phe); 5.27 (s, 4H, CPT H-5); 5.35 (d, 4H, CPT H-17); 5.74 (m, 2H, O(CO)NH); 6.70 (bs, 2H, 20-OH); 7.27−7.35 (m, 16H, arom); 7.65 (s, 2H, CPT H-14); 8.25 (s, 2H); 8.31 (s, 1H); 8.84 (bs, 2H); 9.26 (s, 2H). RP-HPLC gradient time (% B): 0 (10%) 30′ (80%), 36′ (10%); single peak eluting at 19.4 min. MALDI-TOF mass spectroscopy (sinapinic acid matrix): M^+ centered at 11500 Da. Gel Filtration HPLC: single peak eluting at 9.3 min.

[$mPEG_{(10kD)}$-O(C=O)]$_2$-Lys-Gly-Leu-Phe-Gly-OH (13). Two grams (0.1 mmol) of [$mPEG_{(10kD)}$-O(C=O)]$_2$-Lys-OSu (mw = 20 000) were added portionwise over 30 min to a stirred solution of H-Gly-Leu-Phe-Gly-OH (39 mg, 10 equiv) and Et_3N (0.14 mL, 10 equiv) in 20 mL of anhydrous dichloromethane. The resulting mixture was stirred at room temperature for 24 h and then was extracted with 1 N HCl (2 × 20 mL) to remove the excess of tetrapeptide. The combined organic solutions were dried over sodium sulfate and concentrated to a small volume at reduced pressure. The resulting slurry was added dropwise to 200 mL of vigorously stirred diethyl ether. The white precipitate which formed was filtered and dried at reduced pressure, affording 1.92 g (93%) of crude product which was used without further purification. ^{1}H NMR ($CDCl_3$) ppm: 0.91 (t, J = 5.6 Hz, 6H, Hδ Leu); 1.25 (m, 1H, Hγ Leu); 1.38 (m, 2H, Hγ Lys); 1.60 (m, 4H, Hβ Leu + Hδ Lys); 1.77 (m, 2H, Hβ Lys); 2.91 (m, 2H, Hϵ Lys); 3.00−3.15 (m, 2H, Hβ Phe); 3.36 (s, 6H, PEG OCH_3); 3.40−3.88 (m, PEG + Hα Gly); 4.17 (m, 1H, Hα Leu); 4.20 (m, 2H, Hα Phe + Hα Lys); 5.75 (m, 2H, O(CO)NH); 7.25−7.35 (m, 5H, arom Phe); 8.12 (s, 1H); 8.38 (s, 1H); 8.50 (s, 1H); 8.71 (s, 1H).

[$mPEG_{(10kD)}$-O(C=O)]$_2$-Lys-Gly-Leu-Phe-Gly-10-amino-7-ethylcamptothecin (6). [$mPEG_{(10kD)}$-O(C=O)]$_2$-Lys-Gly-Leu-Phe-Gly-OH (**13**) (1.23 g, 0.06 equiv) and 45 mg (0.12 mmol, 2 equiv) of 10-amino-7-ethylcamptothecin were dissolved in 30 mL of toluene, and the mixture was azeotropically distilled with removal of 10 mL of toluene. The mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 30 mL of dry chloroform. Pyridine (0.24 mL, 3 mmol) and phenyl dichlorophosphate (0.35 mL, 2.28 mmol) were added, and the mixture was stirred at room temperature for 12 h, adjusting to pH 8 with DPEA. At the end, the yellow mixture was extracted with 1 N HCl (20 mL). The organic phase was concentrated at reduced pressure, and the residue, diluted with 20 mL of 2-propanol, was recrystallized. The pale yellow crystalline precipitate was washed with diethyl ether, affording 0.95 g (76%) of title compound. Loading (w/w % of aminocamptothecin) = 1.82% (theoretical = 1.87%) according to UV absorption.

^{1}H NMR ($CDCl_3$) ppm: 0.91 (t, J = 5.6 Hz, 6H, Hδ Leu); 1.10 (t, 3H, CPT 18-H);1.25 (m, 1H, Hγ Leu); 1.37 (m, 5H, CPT 7-CH_2CH_3 + Hγ Lys); 1.60 (m, 4H, Hβ Leu + Hδ Lys); 1.77 (m, 2H, Hβ Lys); 1.93 (q, 2H, CPT H-19); 2.90−3.15 (m, 4H, CPT 7-CH_2CH_3 +Hϵ Lys + Hβ Phe); 3.32 (s, 6H, PEG OCH_3); 3.40−3.88 (m, PEG + Hα Gly); 4.17 (m, 1H, Hα Leu); 4.20 (m, 2H, Hα Phe + Hα Lys); 5.22 (s, 2H, CPT H-5); 5.28 (d, 2H, CPT H-17); 5.72 (m, 2H, O(CO)NH); 6.65 (bs, 1H, CPT 20-OH); 7.25−7.35 (m, 8H, arom Phe); 7.65 (s, 1H, CPT H-14); 8.12 (s, 1H); 8.38 (s, 1H); 8.50 (s, 1H); 8.71 (s, 1H); 9.25 (s, 1H).

RP-HPLC gradient time (% B): 0 (10%) 30′ (80%), 36′ (10%); single peak eluting at 18.4 min. MALDI-TOF mass spectroscopy (sinapinic acid matrix): M^+ centered at 20895 Da. Gel filtration HPLC: single peak eluting at 7.5 min.

UV Measures. Free SN-392 has two main UV absorption maxima at 275 and 390 nm, while in the N-acylated analogues **3**−**6** the 240−300 nm zone is shielded by the phenylalanine aromatic chromophore and the 390 nm peak is split into two peaks at 368 and 382 nm. For such reasons a measure of drug loading from the molar extinction coefficient (calculated either at 275 or 390 nm) of free SN-392 could not provide accurate results. We therefore had to devise a different method to determine the extinction coefficient of PEGylated SN-392. To obtain such a value, we measured the absorbance at 368 nm from solutions of PEG conjugate at different concentrations, obtained by progressive dilution of a stock solution at known concentration. As accurate weighing of PEG polymers is impossible, due to polydispersivity and the hygroscopic nature of the solid, we obtained the exact concentration of our stock solution from quantitative amino acid analysis. The mean value of the concentrations of each amino acid (Phe, Leu, Gly) returned the concentration of our PEG-(SN-392) conjugate. The molar extinction coefficient calculated from the absorbance/concentration curve was used to determine the drug loading (expressed as w/w percentage of free drug) for each final product.

Enzymatic Hydrolysis in the Presence of Cathepsin B1 at pH 5.5 and Chemical Hydrolysis of the Derivative at pH 5.5. Buffer solution A at pH 5.5 was prepared using KH_2PO_4·$2H_2O$ 0.15 M, 10^{-3} M EDTA. Solution B was prepared from solution A by addition of 5 μM of GSH. Solution C was prepared by addition of 850 μL of solution B to 50 μL of cathepsin B_1 solution, containing 0.285 mg/mL of enzyme (extracted from bovine spleen) in buffer solution A (1 mg of enzyme powder contains 11.36 units). The mixture C was incubated for 5 min at 37 °C. At the end 100 μL of derivative **2** in buffer solution A (580 μg/mL expressed as free drug) was added to solution C, and this mixture was incubated at 37 °C. At the same time derivatives **3** and **4** were solubilized in buffer solution at the same concentration. At fixed intervals a 50 μL sample of both drug mixtures was injected in an HPLC system

(RP-C18 column). The conversion of the derivatives and the release of 10-amino-7-ethylcamptothecin were monitored following the decreasing of peak area.

Chemical Hydrolysis at pH 7.44. A PBS buffer solution pH 7.44, containing 0.156 g/mL $NaH_2PO_4 \cdot H_2O$ and 0.88 g/mL of NaCl, was prepared. A 4.0 mg amount of derivatives **3** and **4** was dissolved in 3 mL of this solution in a volumetric flask. The mixture was incubated at 37 °C. At a fixed period of time, a 50 μL sample of the mixture was injected into an HPLC system (RP-C18 column).

Hydrolysis in Mouse Plasma. One milliliter of blood taken from female BALB mice was centrifuged at 12 000 rpm for 1 min, obtaining 600 μL of plasma. A 130 μg amount of derivatives **3** and **4** expressed as 10-amino-7-ethylcamptothecin was added to 75 μL of plasma containing 1/15 M of KH_2PO_4/Na_2HPO_4, pH 7.4. The plasma solution was incubated at 37 °C. At fixed periods of time, 50 μL samples of the mixture were added to 300 μL of acetonitrile and centrifuged for 3 min at 12 000 rpm. The supernatant liquid was dried in "speed-vac" concentrator. The residue was dissolved with 100 μL of deionized H_2O. The samples were analyzed by an HPLC system (RP-C18 column).

In Vivo Pharmacological Assay. (A) Derivative **3** was tested against murine leukaemia P388 and P388 resistant to adriamycin (P388/ADM). Female CDF1 mice were inoculated intraperitoneally with P388 or P388/ADM at a dose of 1×10^6 cells/mouse on day 0 and injected intravenously with the derivative on days 1, 5, and 9 at total doses of 5, 10, or 20 mg/kg, then monitored survival times for 40 days. The water-soluble analogue CPT-11 was used in comparison. The survival rate (T/C%) is calculated using the following formula:

T/C (%) = (mean survival days of treated group/mean survival days of control group) × 100.

(B) Derivative **3** was tested against Meth A fibrosarcoma cells. Seven week old male BALB/c mice were inoculated subcutaneously with Meth A cells at a dose of 5×10^5 cells/mouse on day 0 and injected intravenously with the derivative on day 5. The tumors were weighed on day 21 after animal sacrifice. Due to poor water solubility, a first reference group was given 10-amino-7-ethylcamptothecin as its open lactone sodium salt. A second reference group was given water soluble analogue CPT-11. Control group was treated with saline.

Pharmacokinetics. Male Sprague−Dawley rats were purchased from Japan Slc (Hamamatsu, Japan) and used for experiment after 1-week acclimatization with free access to water and commercial animal chow (F-2; Funabashi Farm, Funabashi, Japan). Rats weighing 316−402 g were used in the experiment. They were cannulated in the right femoral vain and the left femoral artery (Intramedic PE−50; Clay Adams, Parsippany, NJ) under light ether anesthesia. They were kept in Bollman cages after cannulation and free access to an ordinary diet and water.

Compound **3** at a dose of 100 or 50 mg/kg, 10-amino-7-ethylcamptothecin at a dose of 4 mg/kg, or its sodium salt at a dose of 4 mg/kg was administered via the right femoral vein after animals completely awoke from anesthesia, and the treatment was followed by flushing with physiological saline. Two hundred microliters of blood was collected from the left femoral artery cannula at 2, 5, 10, and 30 min and 1, 3, 5, and 7 h after administration.

The plasma was separated immediately after sampling, diluted 5-fold with 0.146 M H_3PO_4, and then added to an equal volume of the internal standard (IS) solution (0.146 M H_3PO_4 containing 1 μg/mL of CPT as IS). A high-performance liquid chromatographic method with a fully automated on-line solid-phase extraction system (PROSPEKT; Spark Holland, Emmen, The Netherlands) was used. One hundred microliters of the plasma sample was used for the solid-phase extraction with a Cartridge-C18 analytichem (Spark Holland). A C_{18} reversed-phase column (Symmetry Column C18, 150 mm × 4.6 mm I.D., 5 μm, Waters, Milford, MA) was used at 50 °C for chromatography. The fluorescence detector (470 scanning fluorescence detector; Waters) was set at 390 and 513 nm (excitation and emission wavelengths, respectively) for 0−3.5 min for detection of the peak of 10-amino-7-ethylcamptothecin, and at 373 and 428 nm for 3.5−6 min for IS. The mobile-phase consisted of 0.05 M KH_2PO_4/acetonitrile (70/30, v/v) containing 4 mM sodium 1-decanesulfonate (pH 3.5 with H_3PO_4), and the flow rate was 1.5 mL/min. The quantification limits of 10-amino-7-ethylcamptothecin was 2 ng/mL in plasma.

Plasma concentration−time curves were analyzed by noncompartmental models. The areas under the plasma concentration−time curves (AUC_{inf}) were calculated by the trapezoidal rule with estimation of AUC from the last sampling time to infinity by equation A.

$$\int_{\text{last}}^{\infty} C\,dt = C_{\text{last}}/\text{last log-linear phase slope} \qquad \text{(A)}$$

where C_{last} is the concentration at the last sampling time. Total clearance (CL_{tot}), mean resident time (MRT_{inf}), and distribution volume in steady state (V_{dss}) were calculated as follows.

$$CL_{tot} = \text{dose}/AUC_{inf}$$

$$MRT_{inf} = AUMC/AUC$$

$$\text{(AUMC: area under moment curve : } = \int_0^{\infty} tC\,dt)$$

$$V_{dss} = CL_{tot} \times MRT_{inf}$$

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References

(1) Wall, M. E. Camptothecin and taxol: discovery to clinic. *Med. Res. Rev.* **1998**, *18*, 299−314.
(2) Hsiang, Y. H.; Lihou, M. G.; Liu, L. F. Arrest of replication forks by drug-stabilized topoisomerase I − DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res.* **1989**, *49*, 5077−5082.
(3) Hsiang, Y. H.; Liu, L. F. Identification of mammalian DANN topoisomerase I as an intracellular target of the cancer drug camptothecin. *Cancer Res.* **1988**, *48*, 1722−1726.
(4) Potmesil, M. Camptothecins: from bench research to hospital wards. *Cancer Res.* **1994**, *54*, 1431−1439.
(5) Yaegashi, T.; Sawada, S.; Nagata, H.; Furuta, T.; Yokokura, T.; Miyasaka, T. Synthesis and antitumor activity of 20(S)-camptothecin derivatives. A-ring-substituted 7-ethylcamptothecins and their E-ring-modified water-soluble derivatives. *Chem. Pharm. Bull.* **1994**, *42*, 2518−2525.
(6) Maeda, H.; Matsumura, Y. Tumoritropic and lymphotropic principles of macromolecular drugs. *Crit. Rev. Ther. Drug Carrier Syst.* **1989**, *6*, 193−210.
(7) Matsumura, Y.; Maeda, H. A new concept for macromolecular therapies in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumour agent SMANCS. *Cancer Res.* **1996**, *6*, 6387−6392.
(8) Seymour, L. W.; Miyamoto, Y.; Brereton, M.; Styger, P. S.; Maeda, H.; Ulbrich, K.; Duncan, R. Influence of molecular size on passive tumour accumulation of soluble macromolecular drug carriers. *Eur. J. Cancer* **1995**, *5*, 766−770.
(9) Caiolfa, V. R.; Zamai, M.; Fiorino, A.; Frigerio, E.; Pellizzoni, C.; d'Argy, R.; Ghiglieri, A.; Castelli, M. G.; Farao, M.; Pesenti, E.; Gigli, M.; Angelucci, F.; Suarato, A. Polymer-bound camptothecin: initial biodistribution and antitumour activity studies. *J. Controlled Release* **2000**, *65*, 105−119.
(10) Harada, M.; Sakakibara, H.; Yano, T.; Suzuki, T.; Okuno, S. Determinants for the drug release from T-0128, camptothecin analogue − carboxymethyl dextran coniugate. *J. Controlled Release* **2000**, *69*, 399−412.
(11) Singer, J. W.; De Vries, P.; Bhatt, R.; Tulinsky, J.; Klein, P.; Li, C.; Milas, L.; Lewis, R. A.; Wallace, S. Conjugation of camptothecins to poly (L-glutamic acid). *Ann. N. Y. Acad. Sci.* **2000**, *922*, 136−150.
(12) Greenwald, R. B.; Pendri, A.; Conover, C. D.; Gilbert, C.; Yang, R.; Xia, J. Drug delivery systems. 2. Camptothecin 20-*O*-poly(ethylene glycol) ester transport forms. *J. Med. Chem.* **1996**, *39*, 1938−1940.
(13) Greenwald, R. B.; Pendri, A.; Conover, C. D.; Lee, C.; Choe, Y. H.; Gilbert, C.; Martinez, A.; Xia, J.; Wu, D.; Hsue, M. Camp-

tothecin-20-PEG ester transport forms: the effect of spacer groups on antitumor activity. *Bioorg. Med. Chem. Lett.* **1998**, *6*, 551−562.
(14) Zhao, H.; Lee, C.; Sai, P. K.; Choe, Y. H.; Boro, M.; Pendri, A.; Guan, S. Y.; Greenwald, R. B. 20-O-acylcamptothecin derivatives: Evidence for lactone stabilization. *J. Org. Chem.* **2000**, *65*, 4601−4606.
(15) Greenwald, R. B. Peg drugs: an overview. *J. Controlled Release* **2001**, *74*, 159−171.
(16) Greenwald, R. B.; Conover, C. D.; Choe, Y. H. Poly(ethylene glycol) conjugated drugs and prodrugs: a comprehensive review. *Crit. Rev. Ther. Drug Carrier Syst.* **2000**, *17*, 101−161.
(17) Soyez, H.; Schacht, E.; Vanderkerken, S. The crucial role of spacer groups in macromolecular prodrug design. *Adv. Drug Deliv. Rev.* **1996**, *21*, 81−106.
(18) Greenwald, R. B.; Pendri, A.; Conover, C. D.; Zhao, H.; Choe, Y. H.; Martinez, A.; Shum, K.; Guan, S. Drug delivery systems empoying 1,4- or 1,6-elimination: poly(ethylene glycol) prodrugs of amine-containing compounds. *J. Med. Chem.* **1999**, *42*, 3657−3667.
(19) Greenwald, R. B.; Choe, Y. H.; Conover, C. D.; Shum, K.; Wu, D.; Royzen, M. Drug delivery systems based on trimethyl lock lactonization: poly(ethylene glycol) prodrugs of amino-containing compounds. *J. Med. Chem.* **2000**, *43*, 475−487.
(20) Kopecek, J.; Duncan, R. Targetable polymeric prodrugs. *J. Controlled Release* **1987**, *6*, 315−327.
(21) Dubowchik, G. M.; Firestone, R. A.; Willner, D. A.; Hofstead, S. J.; Trail, P. A.; Larsh, S.; Henderson, A.; Jure, M.; Mosure, K.; Knipe, J. Peptide linkers for selective intralysosomal release of anticancer drugs from monoclonal antibody conjugates. In *Peptides 1996 − Proceedings of the 24th European Peptide Symposium*; Ramage, R., Epton, R., Eds.; Mayflower Scientific: Kingswinford, 1996; pp 349−350.
(22) Coessen, V.; Schacht, E. H.; Domurado, D. Synthesis and in vitro stability of macromolecular prodrugs of norfloxacin. *J. Controlled Release* **1997**, *47*, 283−291.
(23) Veronese, F. M.; Schiavon, O.; Pasut, G.; Duncan, R.; Ford, J.; Andersson, L.; Andersen, A. J.; Ferruti, P.; Vincenzi, V.; Cassidy, J.; Davies, J. W.; Orsolini, P.; Deuschel, C. Tumor targeting of doxorubicin bound to peg of different size and shape. Presented at the 27th International Symposium on Controlled Release of Bioactive Materials, Paris, France, July 7−13, 2000.
(24) Schiavon, O.; Pasut, G.; Orsolini, P.; Guiotto, A.; Veronese, F. M.; Unpublished results.
(25) Dolence, E. K., U.S. Patent 5,824,784, 1997.
(26) Greenwald, R. B.; Choe, Y. H.; Wu, D. Selective phenolic acylation of 10-hydroxycamptothecin using poly (ethylene glycol) carboxylic acid. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 577−580.
(27) Greenwald, R. B.; Pendri, A.; Choe, Y. H., U.S. Patent 6,011,-042, 1997.
(28) Liu, H. J.; Chan, W. H.; Lee, S. P. Convenient procedures for esterification of carboxylic acids. *Tetrahedron Lett.* **1978**, *46*, 4461−4464.
(29) Guiotto, A.; Canevari, M.; Orsolini, P.; Lavanchy, O.; Deuschel, C.; Kaneda, N.; Kurita, A.; Matsuzaki, T.; Yaegashi, T.; Sawada, S.; Veronese, F. M. *Bioorg. Med. Chem. Lett.*, in press.

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