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Camptothecin-20-PEG Ester Transport Forms: the Effect of Spacer Groups on Antitumor Activity

Richard B. Greenwald,* Annapurna Pendri, Charles D. Conover, Chyi Lee, Yun H. Choe, Carl Gilbert, Anthony Martinez, Jing Xia, Dechun Wu and Mei-mann Hsue

Enzon, Inc., 20 Kingsbridge Road, Piscataway, New Jersey 08854-3969, USA

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Abstract—An improved synthesis of the hindered PEG-camptothecin diester transport form has been achieved using the Mukaiyama reagent. We have also assessed the effect of changing the electronic configuration of the (d-position of PEG-camptothecin transport forms on the rates of hydrolysis of the pro-moiety, and attempted to correlate these differences to efficacy in two animal models. In addition to the simple substitution of N for O, other synthetic modifications of these atoms were accomplished by employing heterobifunctional linker groups. The half lives by disappearance (rates of hydrolysis) of the transport forms in buffer and rat plasma were determined. It was established that anchimeric assistance to hydrolytic breakdown of the pro-moiety occurs in a predictable manner for some of these compounds. Results for the new derivatives in a P388 murine leukemic model and HT-29 human colorectal xenograft study are also presented. The use of a glycine linker group was found to provide similar efficacy in rodent models to that of simple camptothecin 20-PEG ester, and displayed enhanced pharmacokinetics.

Introduction

Synthetic polymers conjugated to cytotoxic drugs have long been postulated as novel chemical entities which should be capable of effective drug delivery.[1] Thus the greater solubility, enhanced tumor accumulation, and longer circulatory retention attributed to the polymeric ballast of various drug conjugates has been the focal point of research in this particular area of drug delivery for the past 20 years. Attesting to this fact is the litany of review articles[2] covering this essential approach to cancer chemotherapy, notwithstanding the reality that no clinically approved polymeric drug of this type has yet ensued in the U.S. Currently, Maeda reports[2g] that pilot clinical trials of poly(styrene-co-maleic acid *n*-butyl ester) conjugated neocarzinostatin (SMANCS) have demonstrated substantial efficacy in the treatment of hepatocarcinoma. Earlier studies employing daunorubicin and the polymers carboxymethyl dextran, alginic acid, polyglutamate, or carboxymethyl cellulose resulted in the conclusion that when daunorubicin was attached irreversibly to a macromolecule, its cytotoxic properties were lost; but attachments using a readily hydrolyzable bond (hydrazone) resulted in sustained in vivo activity.[3] This result has been interpreted to be due to hydrolysis in the acidic milieu of the tumor; possibly after cellular uptake. Further investigations by Ueda[4] utilizing doxorubicin Schiff base conjugates of oxidized dextran confirmed the utility of this approach, and led to a phase I clinical trial of the macromolecular prodrug.[5] Simultaneously, extensive work on the incorporation of doxorubicin into *N*-(2-hydroxypropyl)methylacrylamide (HPMA) copolymers appeared.[2,6] These conjugates employed tetrapeptide spacers which demonstrated controlled biodegradability (lysosomal degradation following endocytosis) and tumor accumulation while exhibiting lower toxicity than the unconjugated drug. A clinical trial of a HPMA-anthracycline conjugate has recently been initiated.[7]

*Corresponding author.

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Table 1. Rates of hydrolysis and IC_{50} values of various PEG-camptothecin derivatives

Compd	Linker	IC_{50} (nM)*	$t_{1/2}$ (h)[a]	
			PBS (pH 7.4)	Rat plasma
1	—	7	—	—
3	-O-CO-CH_2-O-PEG	15	27	2
10	-O-CO-CH_2-O-CH_2-CO-NH-PEG	16	5.5	0.8
11	-O-CO-CH_2-O-CH_2-CO-N(CH_3)-PEG	21	27	3
16	-O-CO-CH_2-O-CO-NH-PEG	7	0.2	ND
17	-O-CO-CH_2-O-CO-N(CH_3)-PEG	18	28	5
24	-O-CO-CH_2-NH-CO-CH_2-O-PEG	12	40	6
25	-O-CO-CH_2-N(CH_3)-CO-CH_2-O-PEG	15	97	10
28	-O-CO-CH_2-NH-PEG	24	12	3
29	-O-CO-CH_2-N(CH_3)-PEG	42	102	> 24

*All experiments were done in duplicate: standard deviation of measurements: ± 10%.
[a]These results more appropriately represent the half lives by disappearance of the transport form.
ND: Not determined.

The dramatic enhancement of circulating half life, solubility and in vivo activity of the potent anti-cancer drug camptothecin (**1**), which we recently reported,[8] was achieved utilizing a traditional type of prodrug strategy in conjunction with the non-immunogenic 40 kDa polymeric compound, polyethylene glycol (PEG) to transport the macro-molecular conjugate. Activation by the α-alkoxy substituent provided by PEG diacid[9] results in a scissile ester bond in the polymeric conjugates (**3**) which controllably releases **1** in its active lactone form. A distinction can now be made between polymer conjugated drugs which undergo relatively slow rates of internalization by endocytosis[2m] in order to function, and polymer conjugated prodrugs (transport forms) which are designed to hydrolyze in a predictable fashion within the tumor stroma. This latter type of easily prepared polymeric drug, with its controlled hydrolysis, should be extremely potent if passive tumor accumulation by the enhanced permeability and retention (EPR) effect postulated by Maeda[2g,2h,10] occurs in a relatively short time, that is, $t_{1/2}$ accumulation > $t_{1/2}$ hydrolysis: thus controlled release of the small hydrophobic active drug within the neoplasm, diffusion to tumorous parenchymal cells, followed by cellular uptake may now be predictable.* Presently though, the most serious drawback to the clinical consideration of the PEG-camptothecin conjugate **3** is that it consists of a mixture of mono (**3a**) and di-substituted ester (**3b**) prodrugs. Therefore our efforts were directed toward either improving the yield of **3b** or developing alternate synthetic strategies in order to obtain a similar PEG diester in a pure state. This latter approach would focus on designing transport forms by conjugating different linker groups to PEG while maintaining the integrity of the camptothecin 20(*S*)- ester functionality established for **3**. In this paper we report on an improved procedure for the preparation of **3b** as well as the synthesis of novel tripartate prodrugs[11], and the results of screening these new compounds using $t_{1/2}$ hydrolysis (Table 1), in vitro IC_{50} measurements (Table 1), and survival in a P388 murine leukemic model (Table 2). From the accumulated data, selections were made for more in-depth xenograft studies. The results of these studies were finally compared to those of **3** in order to find an analogous or improved clinical candidate. We achieved the goal of synthesizing an essentially single component camptothecin diester with efficacy comparable to **3** by synthesizing the glycine derivative **24**. Compound **24** also demonstrated enhanced circulatory retention in the mouse. More importantly, the hydrolytic profile of **24** in human plasma showed a significant increase in camptothecin release compared to **3** (Figure 1).

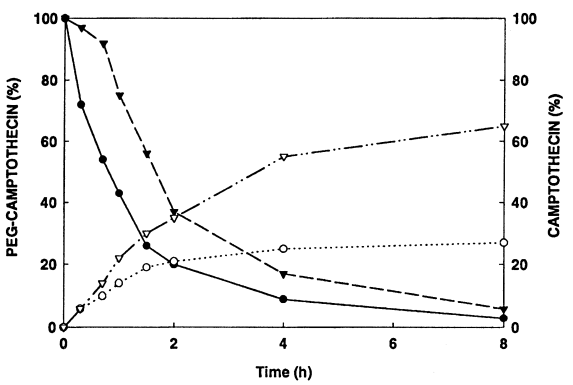


Figure 1. Kinetics of camptothecin (**1**) release from PEG-camptothecins **3** and **24** at 37 °C in human plasma: ●, rate of hydrolysis of **3**; ○, rate of formation of **1** from hydrolysis of **3**; ▼, rate of hydrolysis of **24**; △, rate of formation of **1** from hydrolysis of **24**.

*We have observed this phenomenon in a solid tumor model with compound **24**. The results of this investigation will be published at a later date.

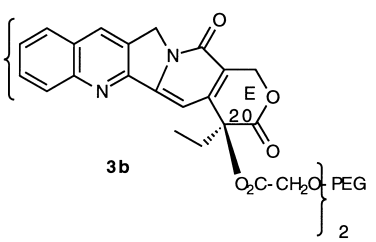
Table 2. Activity of PEG-camptothecin derivatives against P388 murine leukemia *in vivo*

Test compound	Linker	Total dose[a] (mg/kg)	Mean time to death (days)[b]	% ILS[c]	Survivors on day 40
Control	—	—	13.0	—	0/10
1	—	16	38.0*	192%	7/10
3	-O-CO-CH_2-O-PEG	16	38.0*	192%	9/10
10	-O-CO-CH_2-O-CH_2-CO-NH-PEG	16	17.4†	34%	4/10
11	-O-CO-CH_2-O-CH_2-CO-N(CH_3)-PEG	16	31.6*†	143%	6/10
17	-O-CO-CH_2-O-CO-N(CH_3)-PEG	16	23.4	80%	0/10
24	-O-CO-CH_2-NH-CO-PEG	16	35.0*	169%	8/10
25	-O-CO-CH_2-N(CH_3)-CO-PEG	16	19.3*†	48%	0/10
28	-O-CO-CH_2-NH-PEG	16	30.6*	135%	0/10
29	-O-CO-CH_2-N(CH_3)-PEG	16	21.4*†	65%	0/10

[a]Equivalent dose of camptothecin, mice dosed days 1–5.
[b]Kaplan–Meier estimates with survivors censored.
[c]Increased life span (ILS) is (T/C-1)×100.
*Significant ($P < 0.001$) compared to control (untreated).
†Significant ($P < 0.001$) compared to compound **1**.
In vivo efficacy study of the water soluble camptothecin derivatives using the P388/0 murine leukemia model. Compound **1** or prodrug derivatives were given daily [intraperitoneal(ip)×5], 24 h following an injection of P388/0 cells into the abdominal cavity with survival monitored for 40 days.

Chemistry

Generally, when working with PEG of molecular weight in excess of 1000 Da, it is necessary to develop functionalization reactions that proceed in high yield (> 90%) since resulting mixtures of PEG-OH and PEG-X (where X is any non OH moiety) are extremely difficult to separate. As previously reported,[8] condensation of PEG diacid **2** with camptothecin's hindered 3^0 alcohol moiety at position 20 in the presence of DIPC (EDC or DCC) led to multicomponent mixtures of mono and diester (35:65) under a variety of conditions (see experimental section for a typical procedure). However, employing the Mukaiyama reagent,[12] 2-chloro-1-methylpyridinium iodide, as condensing agent, the amount of diester **3b** was increased to approximately 90%.



3b

At this point, even though the product purity appeared adequate to proceed to pharmaceutical development, we elected to further explore other methods of high yield derivatization of camptothecin with PEG in order to determine if modification of the α position could provide alternative clinically relevant candidates. Using this approach we hoped to delineate the limits of the PEG prodrug strategy. For this reason we turned our attention to the alternative strategy outlined earlier, viz. first functionalizing the 20-OH group with a linker group capable of further reaction (i.e. a small heterobifunctional molecule which can maintain the desired placement of a heteroatom in the α position). Since the camptothecin prodrug is based on an ester, by necessity one of the functionalities of the heterobifunctional linker must be a carboxylic acid. The other moiety, located at the distal terminus can be designed to be situated on a 1° carbon and thus unhindered. At this stage, the camptothecin intermediate is usually obtained in high yield, and can be additionally purified without difficulty. Further reaction of the distal functionality with a complimentary functionalized PEG would then result in a stable polymeric conjugate. In this fashion, the synthesis of disubstituted camptothecin derivatives in high purity can be accomplished in a facile manner. For example, in the case of a carboxyl terminated linker one would employ an amino PEG to form an amide linked conjugate. By contrast, with an amino terminated linker a carboxy or carboxaldehydo PEG would be the appropriate choice leading to an amide and imine, respectively; the latter can easily be reduced to a stable 2° amine. One consequence of using linker groups is the introduction of rate accelerations due to neighboring group (anchimeric) participation. This enhancement to hydrolytic breakdown of the promoiety can be incorporated into the design of the transport form. The following examples of linkers illustrate the methodology:

Example 1: CO_2H-CO_2H

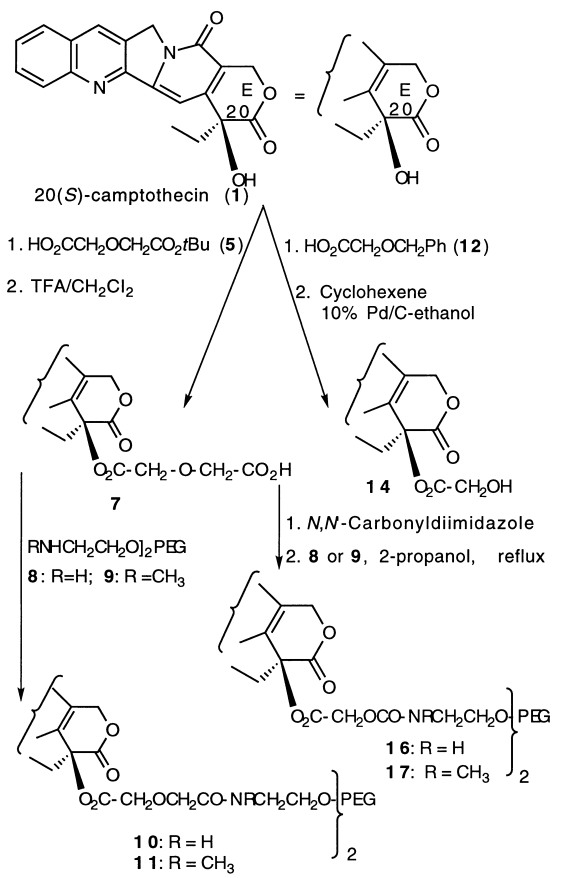
Conversion of camptothecin to the 20-acetate using acetic anhydride was reported by Wall, Wani and co-workers in 1966.[13] By substituting diglycolic anhydride (a bifunctional acylating agent)[14] the slightly water soluble camptothecin derivative **7**, containing an (α-alkoxy ester and a terminal carboxyl group, was obtained in moderate yield (Scheme 1). It was found more convenient to prepare **7** by first opening diglycolic anhydride with *t*-BuOH to give the protected ester acid **5**, followed by condensation with **1** in the presence of DIPC, and subsequent acid cleavage. The next step, as outlined in the strategy above, was to add PEG in a facile manner. This was accomplished by utilizing the diamine derivative **8** which was easily prepared from the chloride. Condensation of **7** in the presence of DIPC provided diamide **10** in high yield and purity. In a similar way the corresponding di-*N*-methyl amide **11** was also prepared from **9**. From the values observed in Table 1, this derivative gave the closest physical measurements to **3**, but far less in vivo activity as later experiments demonstrated.

Example 2: CO_2H-OH

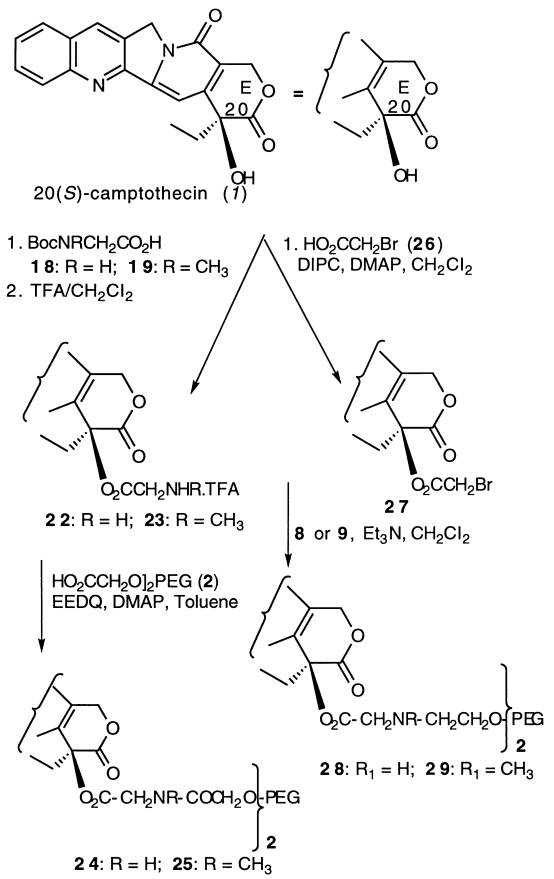
It was also of interest to synthesize an ester with an α-carbamate group, which provides increased electronegativity, in order to observe the effect of this substituent on the rate of hydrolysis. To achieve this objective we developed a simple route, as illustrated in Scheme 1, which utilizes the α-hydroxy ester **14**. This intermediate was obtained in modest yield, and was easily converted to the activated carbonyl imidazole derivative **15** (see experimental). Reaction with PEG amine **8** or **9** proceeded to give the dicarbamate derivatives (**16** or **17**) in high yield.

Example 3: CO_2H-NH_2

Many amino acid ester derivatives of water insoluble drugs have been prepared with the expressed purpose of enhancing the aqueous solubility of the resulting prodrugs as the salts of strong acids.[15] Anti-cancer drugs such as paclitaxel[16] and camptothecin[17] have already been modified in this fashion. Generally, the problem associated with this type of approach is the instability



Scheme 1.



Scheme 2.

of the charged α-ammonio substituted ester which undergoes rapid hydrolysis.[18] Bifunctional amino acid groups employed in a PEG prodrug strategy will, however, result in a water soluble nonionic α-amidoester prodrug that should be much less susceptible to hydrolysis. The route taken is shown in Scheme 2 where ester formation was easily accomplished leaving a reactive amino group, usually in a protected form, but easily unblocked. Attachment of PEG to the α-amino group of camptothecin 20-glycinate and sarcosinate was accomplished in standard fashion by utilizing PEG diacid **2** (Scheme 2). Although DIPC and the Mukaiyama reagent worked well in dichloromethane solvent to afford **24**, we preferred the use of EEDQ since it could be employed with the more environmentally acceptable solvent, toluene. Acylated amino acids appear to have a pKa in the range of about 3.0–3.6 (*N*-acetyl gly = 3.6, glygly = 3.12)[19] which is similar to that reported for α-alkoxy acids (3.48). We felt that since an α-amido substituent possesses similar electronegativity to α-alkoxy groups as evidenced by pKa, a similar hydrolytic profile to that of the effective α-alkoxy ester **3** should result for this novel series of esters. As Table 1 shows, the $t_{1/2}$ hydrolysis for **24** was only about 1.4 times greater than that of **3** in PBS buffer (pH 7.4). However, this was not the case for the *N*-substituted amide **25** derived from sarcosine which hydrolyzed at a rate of 3.5 times slower than **3** in buffer and 2.5 times slower in rat plasma. The rate of hydrolysis provided by the glycine linker, serendipitously, gave rise to a compound (**24**) with excellent efficacy in the biological models. For purposes of comparison, PEG was attached directly to the slightly less electronegative amino group (pK_a of neutral gly = 4.3)[19] by reaction of the bromo ester **27** with PEG amine **8** to give **28** (Scheme 2). The *N*-methyl derivative **29**, was synthesized in a similar way. In this case, anchimeric participation of the basic NH of amine **28**, probably via an α-lactam intermediate,[20] was clearly observed: compared to the *N*-methylamine derivative **29**, $t_{1/2}$ in PBS buffer was almost nine times faster and 16 times faster in plasma.

Results

In vitro cytotoxicity

The in vitro biological efficacy of the PEG-camptothecin transport forms were tested using the P388/0 murine leukemia cell lines. The cytotoxicities of **1**, **3** and the various PEG transport forms synthesized in this study are shown in Table 1.

In vivo murine leukemia screen

The relative in vivo equivalency of compound **1** to the new prodrugs synthesized in this study was assessed by monitoring survival in a P388/0 murine leukemia model. Mice were injected with P388/0 cells and then treated for five consecutive days. This was followed by daily survival monitoring for another 35 days. The results of this in vivo study are shown in Table 2. The mean time to death for native camptothecin (**1**, administrated within an intralipid suspension delivered ip) group at 16 mg/kg was 38 days which resulted in an increased life span (ILS) of 192% with a 70% cure rate. Similarly, the mean time to death for an equivalent dose of the prodrug, compound **3** (administered ip as an aqueous solution) was 38 days (ILS = 192%) with a cure rate of 90%. Both **1** and **3** showed no signs of overt acute toxicity. Thus, equivalency ($P = 0.66$) was demonstrated for compound **1** and its transport form, compound **3**, in this murine leukemia model. By comparison, compounds **11**, **24**, and **28** demonstrated an ILS similar to **3**, although compound **28**'s group was void of long term survivors.

In vivo colorectal xenograft

Prodrug congeners that exhibited a comparable ILS to **3** in the P388 model were subsequently evaluated in

Table 3. Antitumor activity against human colorectal xenografts (HT-29) in vivo

Test compound	Dose (mg/kg)[a]	Week 5 (end of treatment)		Week 7 (two weeks post)		Total mortality
		Efficacy (%)[b]	BW change (%)[c]	Efficacy (%)	BW change (%)	
Control	-	+727	+10	+1047	+18	0/10
Topotecan	2.5	+78	−8	+300	+6	0/10
1	2.5	−20	−3	+62	+9	5/10
3[d]	2.5	−89	−1	−98	+13	0/10
11[d]	2.5	−60	+1	−73	+13	4/10
24[d]	2.5	−80	0	−96	+25	0/10

[a]Mice dosed five days a week for five weeks.
[b]Efficacy was expressed as per cent tumor volume change from initial.
[c]BW, body weight.
[d]Equivalent dose of compound **1**.
In vivo efficacy study of the water soluble camptothecin derivatives using the HT-29 human colorectal xenograft. A subcutaneous injection of HT-29 cells was allowed to reach an average tumor volume of 250 mm^3, prior to treatments. Mouse weight and tumor size were measured at the beginning of study and weekly through week 7.

HT-29 human colorectal xenografts which were monitored for tumor growth kinetics after a five week dosing period. All treatment groups were monitored an additional two weeks in order to assess the short-term regrowth kinetics of the treated tumors. Compound **1** appeared more toxic (50% mortality) and less effective than equivalent doses of the prodrugs (Table 3). Both **3** and **24** showed considerable antitumor activity which resulted in a striking 95% or greater reduction in initial tumor burden by the end of the study without any significant weight loss. Topotecan treatments resulted only in tumor growth inhibition following the five weeks of treatment. Following drug treatment withdrawal, control, topotecan and compound **1** treated mice all displayed tumor regrowth (Table 3). In contrast, mice in all prodrug groups showed continued tumor regression, with all the groups displaying approximately a 10% further reduction in tumor volume during this period.

In vivo circulatory retention

Both compounds **3** and **24**, which showed the greatest efficacy in the solid tumor model, were tested for blood circulatory retention. The blood $t_{1/2\alpha}$ of compound **3** was estimated to be 5 min, and the $t_{1/2\beta}$ was 3.5 h with the area under the curve (AUC) of 17.7 mg/mL.h. Similarly, compound **24** displayed $t_{1/2\alpha} = 5$ min, and a $t_{1/2\beta} = 5.3$ h with an AUC of 15.9 mg/mL.h.

Discussion

It is evident from the IC_{50} values shown in Table 1 that all of the camptothecin transport forms prepared in this study demonstrate in vitro activity similar to native drug (**1**). Examination of the in vivo data presented in Table 2, clearly shows that significant differences in biological activity result from the structural changes made by the incorporation of a linker group. However, predictive changes, ultimately, could only be broadly characterized, and it appears that each linker must be evaluated on an individual basis.

Of the seven new compounds presented, three (**11**, **24**, and **28**) can be immediately distinguished as possessing an ILS greater than the other candidates in the P388 murine leukemic model. It is generally recognized that a per cent ILS > 120 is significant,[22] and **11**, **24**, and **28** all have values in excess of 130 albeit slightly less than that observed for compound **3**. Compound **28** was eliminated from further consideration since dosing with this compound provided no survivors on day 40 of the study compared to **11** and **24** where the survival rate was 60 and 80%, respectively. Some degree of correlation of physical parameters with P388 activity can be made from comparisons of $t_{1/2}$ hydrolysis in buffer (Table 1). Anchimeric assistance emerges as a primary mechanism in the hydrolytic process for those structures where an NH or NHC=O functionality is present, and a 3, 5, or 6 membered cyclic transition state can be formed with the terminal ester. Precedent for such rate enhancements are known for amides,[23] carbamates,[24] and amines.[25] Thus, **10** hydrolyzes at a rate five times faster than **11** in buffer, and three times faster in rat plasma. The P388 model reflects this in the mean time to death which is only minimally better than control, and would seem to indicate that a plasma circulating half life closer to that of **3** (2 h), is desirable. The rate enhancement is so pronounced for **16** ($t_{1/2} = 0.2$ h, pH 7.4) that no further experimentation could be justified for this compound since $t_{1/2}$ is less than that reported for camptothecin 20-glycinate TFA salt which releases **1** very rapidly, and has been shown to be less potent than **3**.[8] On the other hand, by replacing the N–H group of **16** by N–Me, the resulting derivative **17** has a rate of hydrolysis which is 140 times slower. Compound **17**, while exhibiting a similar hydrolytic profile to **3**, produces a result in the P388 model that is only half as efficacious. Similarly, the *N*-methyl derivative **25** with a $t_{1/2} = 97$ h at pH 7.4 is almost four times slower than **3**, and does not show a significant % ILS. We attribute this finding to the inability of **25** to release camptothecin in a timely fashion to the model. The *N*-unsubstituted analog **24** hydrolyzes 2.5 times faster than **25** at pH 7.4, and 1.5 times faster in rat plasma. Compared to **3**, compound **24** hydrolyzes at a rate ($t_{1/2} = 40$ h) that is only about 35% slower than **3** in buffer, but 3 times slower in plasma. Fortunately, in this case the difference in the timed release of **1** from **24** still results in a compound that demonstrates very similar P388 activity to **3**. Based on the data provided in Table 1, a circulating $t_{1/2}$ range of approximately 2–6 h in rat plasma is required to provide efficacy in the ascites P388 murine leukemic model. Rates either on the high or low side of this range results in a diminution of ILS.

On the basis of their performance in the simple P388 model, compounds **11** and **24** were chosen for more in-depth evaluation in a solid tumor (HT-29 colorectal xenograft) study which would compare them to **1**, **3**, and the water soluble camptothecin derivative, topotecan. The results of the study are presented in Table 3.[26] It is not clear at this time why a lesser tumor reduction and greater degree of toxicity exists for **11**: it may possibly be due to a more rapid release of **1** in vivo caused by an initial proteolytic attack on the 3^0 amide tripartate prodrug. Compounds **10**, **16**, **17**, **24** and **25** can also be classified as tripartate prodrugs[11] with two enzymatically mediated pathways available for hydrolysis. Kinetic differentiation between the two possibilities are beyond the scope of this investigation, but one would anticipate that biological results would be reflective of the pathways. In compound **3** (a bipartate prodrug)

only pH or esterase mediated release is possible. Therefore other tripartate prodrug candidates which mimic the overall biological activity of **3** might be considered to hydrolyze by a similar mechanism; those that manifest different biological parameters such as greater toxicity or lesser efficacy can be viewed as hydrolyzing by a different, or combination of, mechanisms including anchimeric assistance. While this interpretation is useful when explaining in vivo differences, the weakness in this simplistic view of the data is that alternate mechanisms can lead to the same result in a single model, and thus cannot be adequately distinguished from each other. Structure–activity correlations between di and tripartate prodrugs must therefore be regarded as only approximations of in vivo mechanisms. While the ester hydrolysis model for **24** in buffer and rat plasma is attractive to support its similarity to **3**, examination of rates of hydrolysis in human plasma clearly show that **24** can indeed behave quite differently when models are changed.[28] The $t_{1/2}$ hydrolysis of **24** in fresh human plasma is shown in Figure 2. The amount of **1** released from the transport form is approximately 65%. For comparison the curve for **3** is superimposed, where only about 25% of **1** becomes available. This decrease in recovery of **1** has been attributed to a species specific enzymatic interaction.[8] Additionally, a circulatory retention study for **24** in the mouse was carried out and yielded a $t_{1/2\beta}$ of 5.3 h; about 30% longer than that of **3**.[21] Overall, the ease of synthesis, long circulatory retention, and high efficacy makes **24** an ideal candidate for future development.

Conclusion

In the present study we have investigated various high yield synthetic approaches leading to PEG transport forms of camptothecin. For direct condensations the Mukaiyama reagent was found to produce quite acceptable yields of diester **3b**. Linker groups were also employed in order to examine the effect of modifying the electron withdrawing α substituent on the rates of hydrolysis of the promoiety (ester), as well as to permit high yield conversion to the final PEG transport form. Anchimeric assistance was observed for those systems that had NH or NHC=O moieties configured in such a way that a 3, 5, or 6 membered cyclic transition state could be formed, and increased the rate of hydrolysis of the drug-ester bond. We have endeavored to correlate structure and $t_{1/2}$ hydrolysis with activity in the P388 murine leukemic model. The results of this attempt has defined a range of about 2–6 h for $t_{1/2}$ circulation of the transport form in rat plasma which will result in a predictable per cent ILS. Additionally, HT-29 (human colorectal carcinoma) xenograft studies were used to refine the choice between compounds identified as possessing similar activities in the P388 model. It was found that a glycine linker group yielded a PEG transport form that was minimally as effective as the lead compound **3**, but provided enhanced pharmacokinetics, and demonstrated superior efficacy. It is noteworthy, that all the PEG-camptothecin transport forms tested appeared to demonstrate less toxicity and more efficacy than equal levels of free camptothecin. More detailed reports of this important finding will be published shortly. We are currently exploring the effect of substituting other amino acids for glycine in the PEG camptothecin tripartate delivery system, as well as examining different PEG-amino acid-drug transport forms in several in vivo systems.

Experimental

Biological assays

Materials. All PEG-camptothecin compounds were dissolved in sterile water for injection (WFI) prior to in vivo drug treatments. With the exception of the circulatory retention study, all PEG-camptothecin dosages were given as their camptothecin equivalents (absolute amount of camptothecin given). For in vivo administration, compound **1** was dispersed in intralipid (Liposyn® III 10%, Abbott Laboratories, North Chicago, IL) by sonication. Topotecan was synthesized according to published procedures.[29] All animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society of Medical Research and the 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health. These experimental protocols were approved by the Institutional Animal Care and Use Committee of UMDNJ-Robert Wood Johnson Medical School.

Cell lines and cytotoxicity assays. Studies using P388/0 cell lines for both IC_{50} and in vivo screens were maintained and conducted as previously reported.[9] The solid tumor HT-29 (human, colon adenocarcinoma) was obtained from the ATCC (HTB 38) and grown in DMEM supplemented with 10% FBS. Cells were subcultured once a week and for in vivo experiments viabilities were >90%. All cell lines were periodically tested for Mycoplasma and were Mycoplasma free.

In vivo murine leukemia model. Compound **1** and its prodrug forms were screened for in vivo activity against the murine leukemia cell line P388/0 (mouse, lymphoid neoplasm) as previously described.[9]

In vivo colorectal xenograft. Female nu/nu mice (Harlan Sprague–Dawley, Madison, WI), 18–24 g and 10–14 weeks old, at onset of treatment, were used to test the efficacy of camptothecin derivatives in a colorectal

xenograft model. When tumors reached the average volume of 250 mm^3, the mice were divided into their experimental groups ($n = 10$) which consisted of control, topotecan and compounds **1**, **3**, **11**, and **24**. This portion of the study was conducted and results analyzed as previously reported.[21]

In vivo circulatory retention. Circulatory retention studies were performed in 25 g, nontumor bearing CD1 female mice (Charles River Laboratories, Stone Ridge, NY). Mice received an i.v. bolus of 12 mg of test article (8 mg/kg CPT equiv) via the tail vein and were exsanguinated over a 48 h period (3 mice/time point). Exsanguination was conducted in unconscious (100% CO_2) animals via orbital bleeding into a sterile tube to remove a minimum of 1.0 mL whole blood/mouse. Blood samples were processed and assayed as previously described.[21] Half-life values of the $t_{1/2\alpha}$ (distribution) and $t_{1/2\beta}$ (elimination) phases for PEG-camptothecins were calculated using a two compartment, intravenous bolus, first order elimination model (WinNonlin, Scientific Consulting Inc., Apex, NC). The correlation between observed and predicted model time point values was greater than 97% for each test article examined.

Chemical methods. Unless stated otherwise, all reagents and solvents were used without further purification. NMR spectra were obtained using a 270 MHz spectrometer and deuterated chloroform as the solvent unless specified. PEG diols (40 kDa) were obtained from Serva (Crescent Chemical Company, NY). PBS buffer was purchased from Sigma Chemical Company. All PEG compounds were dried under vacuum or by azeotropic distillation from toluene prior to use. Elemental analysis were performed by Galbraith Laboratories, Knoxville, TN, and FAB-MS analyses were done at the mass spectrometry facility of Yale Medical School, New Haven, CT.

Abbreviations. DIPC (diisopropylcarbodiimide), DMAP (dimethylaminopyridine), TFA (trifluoroacetic acid), DMF (dimethylformamide), EEDQ (2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline).

Analysis of 20-camptothecin PEG 40 kDa esters. The percentage of camptothecin in the PEG-camptothecin esters was determined using an identical UV assay as previously published[9] for PEG-taxol.

Determination of rates of hydrolysis of camptothecin-PEG esters. The rates of hydrolysis were obtained by employing a C-8 reverse-phase column (Zorbax® SB 300 Å, 4.6 mm × 50 mm), using a gradient mobile phase consisting of (a) 0.05% TFA in water and (b) 0.05% TFA in acetonitrile.

Camptothecin-20-PEG 40 kDa ester. Method A: DIPC as the coupling agent (3). PEG 40 kDa diacid **2**, (11.5 g, 0.29 mmol,) was dissolved in 200 mL of anhydrous (anhyd.) methylene chloride at room temperature. The solution was chilled to 0 °C and DIPC (0.18 mL, 1.15 mmol), DMAP (140 mg, 1.15 mmol) and camptothecin (400 mg, 1.15 mmol) were added, in that order, and stirred for 2 h at 0 °C. The reaction mixture was allowed to warm to room temperature and left for 16 h. The solution was concentrated to about 100 mL and filtered through celite. The filtrate was washed with 0.1 N HCl, dried (anhyd $MgSO_4$), and evaporated under reduced pressure to yield **3** as a white solid which was recrystallized from DMF/ether. The solid was filtered and washed with 2-propanol. The product **3** (9.5 g, 82%) was found to be a mixture of monoester **3a** and diester **3b**. The structures of the compounds comprising the mixture were deduced by analysis of the NMR spectra and determination of per cent camptothecin in the product using the UV method outlined above: ^{1}H NMR: δ 1.01 (t, $J = 7.2$ Hz, 1H), 1.21 (s, 3H), 1.24 (s, 3H), 1.42 (s, 3H), 1.45 (s, 3H), 2.3 (s, 1H), 3.39 (PEG, t, $J = 5.2$ Hz), 3.49–3.71 (bs, PEG), 3.93 (PEG, t, $J = 5.2$ Hz), 4.1–4.32 (m, 6H), 5.44 (s, 1H), 5.5–5.8 (AB q, $J = 17.16$ Hz, 1H), 7.3 (t, $J = 7.92$ Hz, 1H), 7.6 (s, 1H), 7.7 (t, $J = 7.26$ Hz, 1H), 7.97 (d, $J = 8.5$ Hz, 1H), 8.23 (d, $J = 8.5$ Hz, 1H), 8.44 (s, 1H); ^{13}C NMR: δ 7.25, 19.6, 22.3, 31.4, 42.5, 46.38, 48.68, 53.48, 62.8, 66, 67–71 (PEG), 75, 94.21, 118.8, 127.2, 127.4, 127.5, 127.65, 128.5, 129.5, 130.5, 144.48, 145.67, 151.5, 152.7, 156.53, 166.2, 168.78.

Method B: 2-Chloro-1-methylpyridinium iodide (Mukaiyama reagent) (3b). A mixture of PEG 40 kDa diacid (**2**, 5 g, 0.125 mmol), and camptothecin (0.2 g, 0.57 mmol) in toluene (150 mL) was azeotroped for 2 h. The reaction mixture was cooled to room temperature and the solvent was completely removed by distillation in vacuo. Anhyd. dichloromethane (200 mL) was added to the reaction mixture and the solution was chilled to 0 °C for 15 min followed by the addition of 2-chloro-1-methylpyridinium chloride (0.25 g, 0.98 mmol) and DMAP (0.25 g, 2.05 mmol). The reaction mixture was cooled to room temperature and the solvent was completely removed by distillation in vacuo. Anhyd. dichloromethane (200 mL) was added to the reaction mixture and the solution was chilled to 0 °C for 15 min followed by the addition of 2-chloro-1-methylpyridinium chloride (0.25 g, 0.98 mmol) and DMAP (0.25 g, 2.05 mmol). The reaction mixture was allowed to warm to room temperature slowly and stirred for 48 h. The solution was washed with 0.5 N HCl (2 × 50 mL), dried (anhyd. $MgSO_4$), and evaporated under reduced pressure to yield a light-yellow solid that was recrystallized from 2-propanol (500 mL) to give a white solid, followed by a second recrystallization from DMF (60 mL).

The solid was filtered and washed with 25 mL of ice-cold DMF:ethyl ether (1:1, v/v) followed by ethyl ether (3×50 mL) to give **3b** (4.7 g, 92%) as a pale-yellow solid. HPLC analysis indicated that the product contained ~90% of diester: ^{1}H NMR: δ 1.01 (t, J=7.2 Hz, 1H), 2.3 (m, 1H), 2.80 (s, PEG), 3.49–3.71 (bs, PEG), 3.93 (t, J=5.2 Hz, PEG), 4.4 (s, 4H), 5.44 (s, 1H), 5.5–5.8 (AB q, J=17.16 Hz, 1H), 7.3 (s, 1H), 7.6 (m, 1H), 7.7 (t, J=7.26 Hz, 1H), 7.97 (d, J=8.5 Hz, 1H), 8.23 (d, J=8.5 Hz, 1H), 8.44 (s, 1H); ^{13}C NMR: δ 7.15, 31.28, 49.58, 66.70, 67.58–71.83 (PEG), 95.39, 119.78, 127.63, 127.79, 127.87, 128.10, 129.12, 130.26, 130.97, 144.95, 146.01, 148.37, 151.80, 156.84, 166.73, 169.21.

Mono *t*-butylester of diglycolic acid (5). A solution of diglycolic anhydride (10 g, 0.09 mol) and DMAP (10.5 g, 0.09 mol) in dry *t*-butanol (75 mL) was stirred at reflux temperature for 18 h. The solvent was removed under reduced pressure and the residue was dissolved in water (100 mL). The aq solution was acidified to pH 2.5–3.0 with 1 N HCl and extracted with dichloromethane. Removal of solvent from the dried extracts yielded 12.3 g (75%) of the mono *t*-butylester of diglycolic acid **5**: ^{13}C NMR: δ 27.84, 68.45, 68.78, 82.42, 169.54, 172.85.

Camptothecin-20-monoester of diglycolic acid (7). A mixture of **5** (4.2 g, 0.02 mol), camptothecin (4.0 g, 0.01 mol), DMAP (2.7 g, 0.02 mol), and DIPC (2.8 g, 0.02 mol) in anhyd. dichloromethane (40 mL) was stirred for 18 h at room temperature. The reaction mixture was washed with water, then saturated aq sodium bicarbonate, 0.1 N HCl and again with water. The organic layer was dried (anhyd $MgSO_4$) and the solvent was removed in vacuo. Recrystallization of the resultant solid from dichloromethane/ether gave **6** (3.1 g, 54%): ^{1}H NMR: δ 8.35(s), 8.15–8.18 (d), 7.89–7.92 (d), 7.80 (m), 7.63–7.66 (m), 7.20 (s), 5.35–5.72 (AB q,) 5.21 (s), 4.45–4.48 (d), 4.11–4.13 (d), 2.2–2.3 (m), 1.45 (s), 1.00 (t); ^{13}C NMR: δ 7.38, 27.86,31.53, 49.72, 66.96, 67.45, 68.36, 76.38, 81.75, 95.54, 119.94, 127.76, 127.99, 128.21, 129.32, 130.39, 130.97, 145.13, 146.25, 148.55, 151.94, 157, 166.98, 168.52, 168.97. MS (FAB) $(M+H)^+$ 521.4. A solution of compound **6** (0.8 g, 1.5 mmol) in dichloromethane (8 mL) and TFA (4 mL) was stirred at room temperature for 30 min. The solvent was removed under reduced pressure, and the resulting solid was recrystallized from dichloromethane/ether to yield **7** (0.6 g, 82%): ^{1}H NMR: δ 8.44 (s), 8.27–8.34 (m), 7.95 (d), 7.86 (m), 7.70 (m), 7.34 (s), 5.37–5.75 (AB q), 5.31 (s), 4.47–4.5 (d), 4.39–4.41 (d), 2.18–2.26 (m), 1.03 (t); ^{13}C NMR (DMSO-d_6): δ 171.15, 169.27, 167.41, 156.84, 152.63, 148.19, 146.41, 145.45, 131.88, 130.71, 130.11, 129.22, 128.83, 128.29, 128.02, 119.08, 95.24, 76.72, 67.57, 67.33, 66.62, 50.54, 30.4, 7.84. MS (FAB) $(M+H)^+$ 465.3.

PEG 40 kDa diamine dihydrochloride (8). Polyethylene glycol 40 kDa (500 g, 12.5 mmol) was placed in an 1 L round-bottomed flask and warmed to 70 °C under vacuum (0.1 Torr) for 16 h to remove traces of water. To the dried PEG-OH was added thionyl chloride (100 mL) and the reaction mixture was kept at 70 °C for 16 h. 2-Propanol (5 L) was added to the reaction mixture. The solution was cooled overnight at room temperature. The precipitated product was filtered and washed with 2-propanol followed by recrystallization from 2-propanol to yield 500 g (98%) of colorless crystalline PEG 40 kDa dichloride: ^{13}C NMR: δ 42.18, 69.94–71.76 (PEG). A solution of PEG 40 kDa dichloride (50 g, 1.25 mmol) in 30% ammonia solution (400 mL) was placed in a sealed polypropylene bottle and heated at 60 °C for 3 days. Subsequent removal of the solvent from the reaction mixture followed by recrystallization from 2-propanol (1.5 L) yielded **8** (44 g, 87%): ^{13}C NMR: δ 39.20, 66.04–71.49 (PEG).

PEG 40 kDa di-*N*-methylamine dihydrochloride (9). A solution of PEG 40 kDa dichloride (50 g, 1.25 mmol) in 40% methylamine (400 mL) was placed in a sealed polypropylene bottle and heated at 60 °C for 3 days. Subsequent removal of the solvent from the reaction mixture followed by recrystallization from 2-propanol (1.5 L) yielded **9** (44 g, 87%): ^{13}C NMR: δ 33.10, 48.38, 66.18–71.60 (PEG).

PEG 40 kDa diamide of acid 7 (10). A mixture of **7** (0.14 g, 0.3 mmol), PEG 40 kDa diamine hydrochloride **8** (3.0 g, 0.075 mmol), DMAP (55 mg, 0.45 mmol), and DIPC (38 mg, 0.3 mmol) in anhyd. dichloromethane (30 mL) was stirred for 18 h at room temperature. Removal of the solvent in vacuo and recrystallization from 2-propanol gave **10** (2.8 g, 90%). ^{13}C NMR: δ 168.39, 168.09, 166.51, 156.81, 151.80, 148.44, 146.22, 144.76, 131.04, 130.32, 129.17, 128.17, 127.91, 127.71, 119.77, 95.05, 76.72, 66.73–71.89 (PEG), 49.64, 38.24, 31.38, 7.0.

PEG 40 kDa di-*N*-methylamide of acid 7 (11). A mixture of **7** (0.14 g, 0.3 mmol), PEG 40 kDa di-*N*-methylamine hydrochloride **11**, (3.0 g, 0.075 mmol), DMAP (55 mg, 0.45 mmol), and DIPC (38 mg, 0.3 mmol) in anhyd dichloromethane (30 mL) was stirred for 18 h at room temperature. Removal of the solvent in vacuo and recrystallization from 2-propanol gave **11** (2.8 g, 90%): ^{13}C NMR: δ 168.73, 168.09, 166.51, 156.58, 151.80, 148.18, 145.91, 144.77, 130.84, 130.03, 128.95, 127.99, 127.71, 127.61, 127.40, 119.77, 95.05, 76.72, 66.46–71.65 (PEG), 49.41, 48.3, 47.5, 35.24, 33.3, 31.06, 6.97.

Camptothecin-20-ester of hydroxyacetic acid (14). To a suspension of camptothecin (1.4 g, 4.02 mmol) in CH_2Cl_2 (500 mL) was added benzyloxyacetic acid **12**

(2 g, 12.06 mmol, prepared by the aqueous hydrolysis of benzyloxyacetyl chloride), DIPC (1.9 mL, 12.06 mmol) and DMAP (982 mg, 8.04 mmol) at 0 °C and stirring was continued for 3 h. The resulting yellow solution was concentrated to about 100 mL and washed with 1 N HCl (2×10 mL), followed by 1% aq sodium bicarbonate solution (2×10 mL). The organic layer was dried (anhyd $MgSO_4$) and evaporated in vacuo to give a yellow solid which was recrystallized from ethyl acetate. The product was triturated with methanol (10 mL), and the slurry filtered to yield camptothecin-20-ester of benzyloxyacetic acid (**13**) (1.3 g, 65%). ^{1}H NMR: δ 1.0 (t), 1.84 (s), 2.1–2.3 (m), 4.31 (s), 4.59–4.69 (q), 5.28 (s), 5.4–5.8 (dd), 7.22 (s), 7.27 (s), 7.3–7.38 (m), 7.6–7.7 (m), 7.81–7.87 (m), 7.92–7.95 (d), 8.19–8.22 (d), 8.39 (s); ^{13}C NMR: δ 7.52, 31.74, 49.90, 66.59, 67.16, 73.27, 76.38, 95.80, 120.27, 127.97, 128.10, 128.43, 129.54, 130.63, 131.15, 136.81, 145.39, 146.36, 148.81, 152.22, 157.26, 167.19, 169.52. MS (FAB) $(M+H)^+$ 497.6. A suspension of **13** (1 g, 2.01 mmol) and 10% Pd/C (500 mg) in ethanol (100 mL) was degassed by sparging with nitrogen. Cyclohexene (5 mL) was added, and the mixture was refluxed for 20 h. The catalyst was filtered and the solvent was removed in vacuo followed by recrystallization of the solid product from acetonitrile to give **14** (500 mg, 50%): ^{1}H NMR (DMSO-d_6): δ 1.0 (t), 1.84 (s), 2.1–2.3 (m), 3.1 (s), 4.31 (s), 4.0–4.69 (m), 5.28 (s), 5.6 (m) 5.8 (m), 7.22 (s), 7.6–7.7 (m), 7.6–7.95 (m), 8.0–8.2 (m), 8.3 (s), 8.7 (s); ^{13}C NMR (DMSO-d_6) δ 7.5, 30.14, 50.20, 59.37, 66.21, 75.83, 79.14, 94.95, 118.84, 127.71, 127.97, 128.52, 128.87, 129.77, 130.42, 131.58, 145.35, 145.95, 147.85, 152.29, 156.53, 167.21,171.69. MS (FAB) $(M+H)^+$ 407.4.

PEG dicarbamate derivative of camptothecin-di-20-hydroxyacetate (16). A solution of **14** (240 mg, 0.59 mmol) and *N*,*N*′-carbonyldiimidazole (288 mg, 1.77 mmol) in chloroform (80 mL) was stirred at 50 °C for 18 h. Removal of solvent in vacuo followed by trituration of the residue with ethyl acetate gave the carbonylimidazole derivative of camptothecin **15** as a pale-yellow solid (170 mg, 58%). ^{1}H NMR δ 1.0 (t), 2.1–2.5 (m), 5.1 (d), 5.28 (s), 5.4–5.8 (dd), 7.0 (s), 7.5 (s), 7.7 (m), 7.9 (m), 8 (d), 8.1 (s), 8.3 (d), 8.4 (s). MS (FAB) $(M+H)^+$ 501.6. A solution of **15** (74 mg, 0.15 mmol) and PEG 40 kDa diamine **8** (2 g, 0.05 mmol) in 2-propanol (20 mL) was refluxed for 12 h. The solvent was removed under reduced pressure to yield **16** as a solid, which was further purified by recrystallization from 2-propanol: ^{13}C NMR δ 167.24, 166.44, 156.77, 154.83, 152.0, 148.0, 145.96, 144.35, 130.81, 130.10, 129.20, 128.09, 127.92, 127.89, 127.78, 127.60, 127.50, 119.72, 95.70, 76.72, 65.66–71.65 (PEG), 61.3, 49.51, 40.58, 31.30, 6.97.

PEG di-*N*-methylcarbamate derivative of camptothecin-di-20-hydroxyacetate (17). This was prepared in a similar manner as compound **16**, using PEG 40 kDa di-*N*-methyldiamine **9** as starting material. ^{13}C NMR δ 166.63, 166.08, 156.17, 151.39, 147.84, 145.41, 144.35, 130.84, 130.03, 128.95, 127.99, 127.71, 127.61, 127.40, 118.68, 94.30, 76.72, 65.66–71.65 (PEG), 61.3, 49.37, 48.52, 48.15, 47.82, 35.14, 34.54, 33.15, 30.97, 21.62, 6.97.

Camptothecin-20-glycinate TFA salt (22). *t*-Boc-glycine **18** (1.8 g, 9.39 mmol) was dissolved in 700 mL of anhyd methylene chloride at room temperature and to this solution were added DIPC (1.5 mL, 9.39 mmol), DMAP (765 mg, 6.26 mmol) and camptothecin (1.09 g, 3.13 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and left for 16 h. The solution was washed with 0.1 N HCl, dried and evaporated under reduced pressure to yield a white solid, which was recrystallized from methanol to give camptothecin-20-ester of *t*-Boc-glycine **20**: ^{1}H NMR(DMSO-d_6) δ 0.9 (t), 1.3 (d), 1.6 (s), 2.1 (m), 4 (m), 5.3 (s), 5.5 (s), 7.3 (s), 7.5–8.8 (m). Anal. ($C_{27}H_{27}N_3O_7$. 0.5 H_2O) C, H, N. Compound **20** (1.19 g, 2.12 mmol) was dissolved in a mixture of methylene chloride (15 mL) and TFA (15 mL) and stirred at room temperature for 1 h. Solvent was removed and the solid was recrystallized from methylene chloride and ether to give 1 g of product **22**. ^{1}H NMR (DMSO-d_6) δ 1.0 (t), 1.6 (d), 2.2 (m), 4.4 (m), 5.4 (s), 5.6 (s), 7.2 (s), 7.7–8.8 (m); ^{13}C NMR (DMSO-d_6) δ 7.5, 15.77, 30.09, 47.8, 50.27, 66.44, 77.5, 94.92, 119.10, 127.82, 128.03, 128.62, 128.84, 129.75, 130.55, 131.75, 144.27, 146.18, 147.90, 152.24, 156.45, 166.68, 168.69.

Camptothecin-20-sarcosinate TFA salt (23). A mixture of sarcosine (5 g, 56.1 mmol), *t*-Boc anhydride (14.7 g, 67.4 mmol) and sodium hydroxide (4.5 g, 112.3 mmol) in water (25 mL) was stirred at room temperature for 18 h. The reaction mixture was cooled to 0 °C and was acidified to pH 3 with 6 N HCl and extracted with ethyl acetate. Evaporation of the solvent gave *t*-Boc sarcosine **19** as a clear oil. ^{1}H NMR δ 4.58 (m), 3.0 (s), 4.0 (m). Compound **19** (1.63 g, 8.61 mmol) was dissolved in 100 mL of anhyd methylene chloride at room temperature and to this solution were added DIPC (1.3 mL, 8.61 mmol), DMAP (725 mg, 5.74 mmol) and camptothecin (1 g, 2.87 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and left for 2 h. The solution was washed with 0.1 N HCl, dried and evaporated under reduced pressure to yield a white solid which was recrystallized from 2-propanol to give camptothecin-20-*t*-Boc-sarcosinate (**21**, 750 mg, 50.3%). Compound **21** (750 mg) was dissolved in methylene chloride (4 mL) and TFA (4 mL) and the solution was stirred at room temperature for 1 h. Ether (10 mL) was added and the precipitated solid was filtered and dried to give **23** (550 mg, 85%) as a yellow

powder. ^{1}H NMR(DMSO-d_6) δ 1.0 (t), 2.2 (m), 2.7 (s), 2.84 (s), 4.4–4.6 (dd), 5.11 (brs), 5.34 (s), 5.65 (s), 7.36 (s), 7.6–8.3 (m), 8.74 (s), 9.46 (s); ^{13}C NMR (DMSO-d_6) δ 7.55, 30.21, 32.49, 47.87, 50.22, 66.40, 77.72, 95.34, 118.84, 127.74, 127.95, 128.76, 129.67, 130.51, 131.65, 144.64, 147.88, 152.24, 156.48, 158.23, 166.78. Anal. ($C_{25}H_{22}F_3N_3O_7$.0.5 H_2O) C, H, N.

Camptothecin-di-20-ester of PEG 40 kDa glycine (24). A solution of 1.0 g (0.025 mmol) of PEG 40 kDa dicarboxylic acid in toluene (35 mL) was azeotroped by distillation of 15 mL of toluene for 2 h. The mixture was cooled to room temperature and **22** (70 mg, 0.13 mmol), EEDQ (30 mg, 0.12 mmol), and DMAP (50 mg, 0.4 mmol) were added. The reaction mixture was stirred at 55–60 °C overnight and the solvent was removed by distillation in vacuo. The residue was crystallized from 200 mL of 2-propanol and washed with anhydrous ethyl ether to give the product as a white solid (0.8554 g, 86% yield). HPLC assay indicated that the product contained >90% of diester **24**. ^{1}H NMR δ 1.00 (t, J=7.2 Hz, 1H), 2.20 (m, 1H), 2.80 (s, PEG), 3.38–4.15 (m, PEG), 4.43 (s,4H), 5.30 (s, 1H), 5.43–5.64 (AB q, J=17.16 Hz, 1H), 7.32 (s, 1H), 7.70 (t, J=7.26 Hz,1 H), 7.84 (t, J=7.25 Hz, 1H), 7.95 (d, J=8.5 Hz, 1H), 8.26 (d, J=8.5 Hz, 1H), 8.46 (s, 1H); ^{13}C NMR δ 7.12, 31.30, 39.93, 49.53, 66.57, 68.32–71.62 (PEG), 95.63, 119.54, 127.57, 127.,75, 128.05, 129.20, 130.16, 130.89, 144.97, 145.97, 148.32, 151.78, 156.77, 166.57, 168.45, 170.27.

Camptothecin-20-ester of PEG 40 kDa sarcosinate (25). Diacid **2** (2 g, 0.05 mmol) was dissolved in 30 mL of anhyd methylene chloride at room temperature and to this solution were added DIPC (30 μL, 0.20 mmol), DMAP (24 mg, 0.20 mmol) and 20-camptothecin sarcosinate TFA salt **23** (112 mg, 0.21 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and left for 16 h. The solution was evaporated under reduced pressure to yield a white solid which was recrystallized from 2-propanol to give **25** (1.4 g, 69%). NMR data is in agreement with the structure.

Camptothecin-20-ester of bromoacetic acid (27). To a suspension of camptothecin (1 g, 2.87 mmol) in anhyd CH_2Cl_2 (700 mL) were added bromoacetic acid **26** (1.2 g, 8.61 mmol), DIPC (1.3 mL, 8.61 mmol) and DMAP (700 mg, 5.74 mmol) at 0 °C and stirring was continued for 4 h. The resulting yellow solution was concentrated to about 100 mL and washed with 1 N HCl (2×10 mL), followed by 1% aq sodium bicarbonate solution (2×10 mL). The organic layer was dried (anhyd $MgSO_4$) and evaporated in vacuo to give a yellow solid which was recrystallized from ethyl acetate. This crude product was triturated with methanol (10 mL), and the slurry was filtered to yield **27** (0.9 g, 67%). ^{1}H NMR δ 1.0 (t), 1.84 (s), 2.1–2.3 (m), 3.9–4.4 (q), 5.28 (s), 5.4–5.8 (dd), 7.2 (s), 7.27 (s), 7.6–7.7 (m), 7.81–7.87 (m), 7.92–7.95 (d), 8.19–8.22 (d), 8.39 (s); ^{13}C NMR δ 7.52, 24.97, 31.77, 49.97, 67.16, 76.53, 95.73, 120.29, 128.05, 128.17, 128.39, 129.64, 130.65, 131.17, 144.94, 146.48, 148.84, 152.18, 157.24, 165.97, 166.83. Anal. ($C_{22}H_{17}BrN_2O_5$.0.5 H_2O) C, H, N.

Reaction of 27 with PEG diamine hydrochloride 8 (28). A solution of PEG 40 kDa diamine hydrochloride **8** (5 g, 0.125 mmol), 20-bromoacetyl camptothecin **26** (322 mg, 0.63 mmol) and triethylamine (208 μL, 1.5 mmol) in anhyd methylene chloride (75 mL) was stirred at room temperature for 3 days. The solvent was evaporated under reduced pressure and the residual solid obtained was first recrystallized from DMF and then using 2-propanol to give **28** as a white solid (4.4 g, 87.4%). ^{13}C NMR δ 6,99, 32.16, 38.50, 47.97, 48.39, 66.17–70.84 (PEG), 75.96, 95.02, 119.26, 127.18, 128.10, 128.25, 129.22, 130.19, 131.20, 145.57, 146.64, 147.82, 152.27, 156.71, 167.25.

Reaction of 27 with PEG di-*N*-methyldiamine hydrochloride 9 (29). A solution of PEG 40 kDa di-*N*-methyldiamine hydrochloride **9** (5 g, 0.125 mmol), 20-bromoacetyl camptothecin **26** (322 mg, 0.63 mmol) and triethylamine (208 μL, 1.5 mmol) in anhyd. methylene chloride (75 mL) was stirred at room temperature for three days. The solvent was evaporated under reduced pressure and the solid obtained was first recrystallized from DMF and then using 2-propanol to give **29** as a white solid (4.5 g, 90%): ^{13}C NMR δ 6.84, 30.62, 41.53, 49.33, 53.96, 64.97, 66.12, 67–70.5 (PEG), 94.82,118.53, 127.21, 127.40, 127.55, 127.91, 128.59, 129.85, 130.81, 144.34, 145.84, 147.84, 151.23, 156.19, 165.77.

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