Drug Delivery Systems Employing 1,4- or 1,6-Elimination: Poly(ethylene glycol) Prodrugs of Amine-Containing Compounds

Richard B. Greenwald,* Annapurna Pendri, Charles D. Conover, Hong Zhao, Yun H. Choe, Anthony Martinez, Kwok Shum, and Shuiyun Guan

Enzon, Inc., 20 Kingsbridge Road, Piscataway, New Jersey 08854

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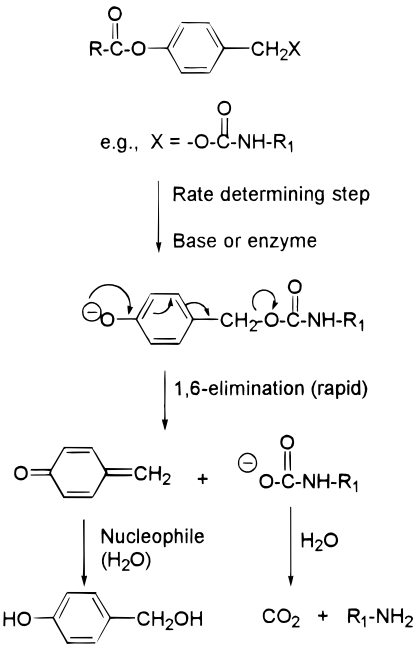
A general methodology for synthesizing poly(ethylene glycol) (PEG) prodrugs of amino-containing compounds has been developed and constitutes the basis for solubilization of insoluble drugs, extending plasma circulating half-lives and, in the case of anticancer agents, apparent tumor accumulation. Thus, we have successfully designed PEG conjugated specifiers or "triggers" as part of a double-prodrug strategy that relies, first, on enzymatic separation of PEG followed by the classical and rapid 1,4- or 1,6-benzyl elimination reaction releasing the amine (drug) bound in the form of a carbamate. The prodrug trigger was comprised of ester, carbonate, carbamate, or amide bonds in order to secure predictable rates of hydrolysis. Further refinement of the hydrolysis was accomplished by the introduction of steric hindrance through the use of *ortho* substituents on the benzyl component of the prodrug. This modification led to longer circulating plasma half-lives of the final tripartate form. The "*ortho*" effect also had the beneficial effect of directing nucleophilic attack almost exclusively to the activated benzyl 6-position of the heterobifunctional intermediates. In vivo testing of the PEG daunorubicin prodrugs (transport forms) prepared in the course of this study ultimately identified the type 1 carbamate (**34b**), with a circulating $t_{1/2}$ of 4 h, as the most effective derivative for solid tumor growth inhibition.

Introduction

Generally, 1,4- or 1,6-elimination of HX (where X is a good leaving group like halide, functionalized oxygen derivatives such as carboxylates, or a carbamic acid anion) from benzyl compounds bearing strong electron-releasing *o*- or *p*-hydroxy or -amino substituents is a fast reaction that occurs under mildly basic conditions. Concomitantly, quinone methides and quinonimine methides are produced (Scheme 1).[1] In fact, selective transformation of a precursor into a phenolic or anilinobenzyl derivative which subsequently fragments has been shown to be the basis for the mechanism of amino,[2a,b,3] hydroxy,[3] carboxy,[2c] and mercapto protecting groups,[4] active site-directed reagents, enzyme-activated irreversible inhibitors, bioreductive cleavable linkages, and prodrugs. While certain activated benzyl derivatives such as 4-(benzoyloxymethyl)-2-cyanophenol can be isolated under controlled conditions,[3] the reactivity of this class of compounds precludes their use as prodrug clinical candidates. A methodology which allows the use of these reactive compounds, especially for site-specific bioactivation, involves protecting the benzylic phenol (or aniline) derivative with a functionality that can be predictably hydrolyzed. This technology has been developed extensively and is generally referred to as the double-prodrug approach,[5] since in essence a pro-prodrug has been made. In such systems, the hydrolytic sequence involves a first step which usually is an enzymatic cleavage, followed by a second, faster step that is a molecular decomposition. One of the first applications of the 1,4- or 1,6-elimination (or BE) concept was used in designing model tripartate prodrugs[6] and involved the masking (or latentiation) of an aromatic NH_2 by forming an amide with lysine (Scheme 2). The amide derivative was stable in aqueous buffer, but subsequent treatment with trypsin initiated a reaction cascade regenerating the amine. A novel drug release strategy has also been designed which is based on the reductive fragmentation of a disulfide followed by an elimination reaction of a *p*-mercaptobenzyl carbamate derivative.[7] Nitro groups are precursors of amines, and so the (bio)reduction of *p*-nitrobenzyl esters or carbamates also leads to formation of an anilinoben-

Scheme 1



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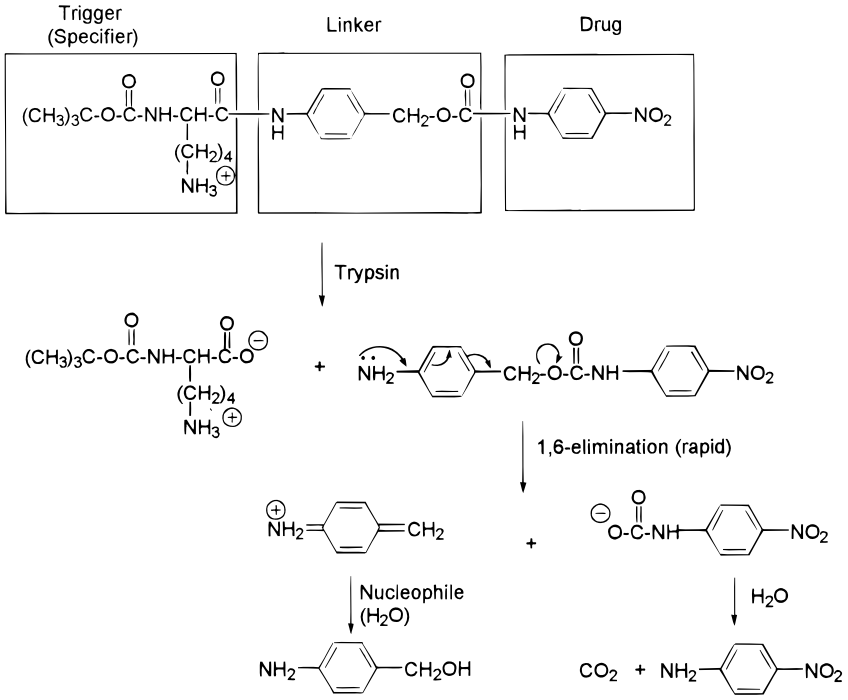
Scheme 2. Illustration of a Tripartate Prodrug



zyl derivative and rapid breakdown to either an acid or amine, respectively.[2c]

Several groups studying the application of antibody-directed enzyme prodrug therapy (ADEPT) with anticancer agents have used a reductive BE approach quite successfully. Mauger and co-workers[8] investigated *p*-nitro-substituted benzyl carbamates of mitomycin C, doxorubicin, and actinomycin D, an anilino mustard agent, and concluded that these types of prodrugs have potential application in the treatment of cancer. More recently a novel *p*-nitro-substituted benzyl carbamate enediyne prodrug activated by nitroreductase NR2 from *E. coli* in the presence of NADH has been demonstrated by Denny and co-workers[9] to possess in vitro activity and thus of potential utility in the treatment of cancer. A similar strategy using nitroreductase and NADH activation of a Tallmustine prodrug has also been reported.[10] Another very creative use of the BE system for use with anticancer drugs, as exemplified by doxorubicin or daunorubicin, has been pursued by two groups.[11,12] In this particular design, an amino group is latentiated by making it part of a benzyl carbamate linker with the *para* functionality (hydroxy or amino) bound to glucuronic acid or a galactopyranose derivative. Enzymatic cleavage of the protecting group by glucuronidase or galactosidase is a prerequisite for carbamate destruction and release of the amino drug. The application of 1,6-elimination for release of N-R as related peptides has recently been reported.[13] To demonstrate enzymatic cleavage and subsequent release of peptide from a water-insoluble phenolic acetyl derivative, solubilization with dimethyl-β-cyclodextrin was necessary.

Targeting of anticancer drugs is one of the primary goals of modern drug development.[14−18] If tumor selectivity can be achieved, then general toxicity is lowered and efficacy is enhanced thereby increasing the therapeutic index of the drug. ADEPT is one concept for accomplishing this objective, but other tumor properties can also be utilized. In particular, the passive accumulation of high molecular weight synthetic polymers in the interstitial tissue of tumors, known as the enhanced permeation and retention (EPR) effect, has been demonstrated to be a simple but useful property with which to target anticancer drugs.[16] Examples of various synthetic polymers conjugated to anticancer drugs and their applications have been reviewed recently.[19] However, no practical poly(ethylene glycol) (PEG) prodrugs conjugated through amino groups of anticancer agents have ever been synthesized. We felt that 1,6-elimination, one of the most versatile processes available for the design of prodrugs, would be an ideal structural platform from which to launch the synthesis of this novel class of compounds. Thus, the incorporation of PEG into the BE system as part of the specifier[6] or trigger[20] results in a neutral and highly water-soluble tripartate polymeric prodrug capable of passive tumor targeting. This PEG prodrug can be designed to attain predictable rates of hydrolysis by changing the nature of the trigger/linker bond and by adding steric hindrance using *ortho* substituents on the aromatic ring of the linker. This approach offers a great advantage in the final design of the prodrug since it enables a "mix and match" of triggers and linkers to be exploited and provides the means to achieve optimal pharmacokinetics for delivery of all types of drugs. Daunorubicin (DNR) is an anticancer agent that contains a primary amine function and readily lends itself as a model for the current PEG prodrug development strategy. Acylation of the amino group results in loss of activity of the anthracycline drug,[21] and therefore, antitumor activity can only result from cleavage of the conjugate.

Not only did the successful development of the desired water-soluble prodrugs in this program lead to possible anticancer prodrug clinical candidates, but also the PEG linkers synthesized were successfully applied to peptides

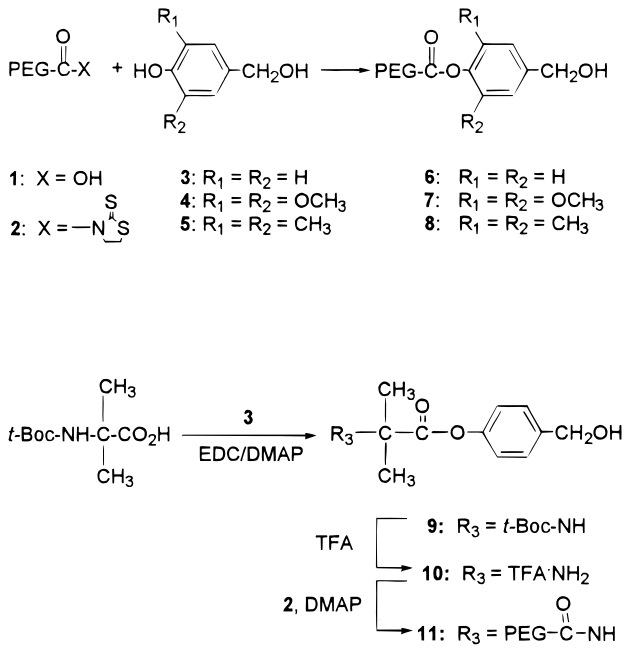
and proteins entirely under aqueous conditions and led to proforms of those more complex molecules with predictable plasma hydrolysis. These water-soluble proproteins can be stored for prolonged periods of time at refrigerated temperatures after lyophilization and are reconstituted prior to use. A description of this and other additional novel work on PEG amino prodrugs will be the subject of forthcoming reports.

Chemistry

To fully exploit the BE system for polymer-conjugated prodrugs, it was necessary to establish a consistent methodology for selective acylation of the phenolic hydroxyl group. This methodology must also be sufficiently versatile to allow the facile synthesis of different acyl-linking functionalities (triggers) such as esters, carbonates, carbamates, and amides in order to permit the deliberate adjustment of rates of hydrolysis in the first step of the cascade sequence (Scheme 1), culminating in the 1,4- or 1,6-elimination reaction of the benzyl derivative. Fortunately, in all the examples studied in the present work, exclusive acylation of the phenolic OH could be achieved by the judicious choice of reagents and reaction conditions. The greater availability of *p*-hydroxybenzyl alcohol derivatives focused most of our attention on 1,6-elimination. However, examples of PEG double prodrugs that depend on a 1,4-elimination have been synthesized using available *ortho* isomers (**36b** and **37b** in Table 1) and shown to behave in a fashion similar to the *para* isomers. It was anticipated that in the case of either *o*- or *p*-aminobenzyl alcohols functionalization as amide and carbamate derivatives would not be difficult. Our earlier work with paclitaxel[22] and camptothecin[23] demonstrated that in order to achieve useful levels of bioactivity with PEG prodrugs of low molecular weight (MW) organic compounds, 40 kDa was an optimal MW for the PEG conjugates. For disubstituted PEG 40 kDa, renal excretion is slower than hydrolysis, i.e., $t_{1/2}$ hydrolysis $< t_{1/2}$ elimination, while loading of native drug is sufficient to provide therapeutic dosages in most instances. Therefore, for practical purposes all PEG derivatives in this work will be limited to 40 kDa species.

PEG Ester Conjugates. Selective acylation of the phenolic hydroxyl group in earlier functionalization studies of hydroxybenzyl alcohols utilized acyl *N*-methylimidazolium derivatives to produce hydrolyzable ester triggers.[1,2] By utilizing PEG dithiazolidinethione (**2**, T-PEG) prepared according to published procedures,[24] acylation was also found to take place easily and selectively on the phenolic OH (Scheme 3). It is known that acylthiazolidinethione-transfer reagents demonstrate selectivity for amines in the presence of alcohols and also show a preference for primary over secondary alcohols.[25,26] Under basic conditions a phenolate anion will be more nucleophilic than the primary benzyl alcohol, and reaction with **3** should be favored to produce the desired product. In this reaction, use of excess **3** drove the reaction to completion; the unreacted excess substrate was easily separated from the PEG conjugate product by crystallization. In addition, selective coupling of PEG acid (**1**) with the phenolic moiety was achieved using large excesses of the phenolic component with an equivalent excess of diisopropylcarbodiimide (DIPC) in the presence of 4-(dimethylamino)-pyridine (DMAP): by contrast equimolar amounts of phenolic substrate, DIPC, and **1** often led to difunctionalization and a mixture of both types of esters. The first conjugation method is generally preferred over carbodiimide-mediated coupling.

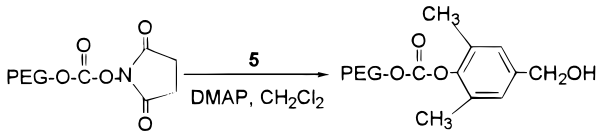
Scheme 3. Synthesis of PEG Esters and PEG Amino Acid Esters of 4-Hydroxylbenzyl Alcohols



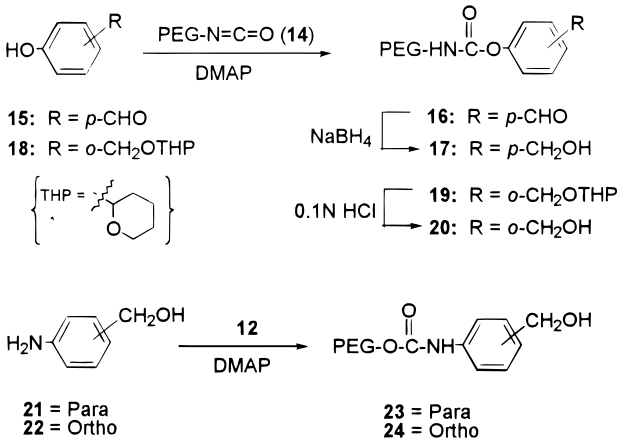
1. Amino Acid Ester Spacers. To introduce a great deal of steric hindrance in proximity to the ester bond and to lower the rate of hydrolysis of PEG-conjugated phenolic esters, α-aminoisobutyric acid (AIBA) was chosen as a representative spacer of this type. Condensation of **3** with *t*-Boc-AIBA in the presence of DIPC was difficult and often led to low yields of the hindered ester. Ultimately, **9** was obtained in 76% yield by using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) as condensing agent in the presence of pyridine (Scheme 3). Removal of the *t*-Boc protecting group of **9** by trifluoroacetic acid cleavage proceeded to give an 80% yield. At this point condensation of **10** with PEG diacid (**1**) in the presence of excess DIPC and DMAP (as was done in the case of the simple ester, *vide supra*) appeared to give the benzyl alcohol ester (O-PEG acylated product) in addition to the desired α-amino N-acylated product. This lack of discrimination probably can be attributed to the bulky dimethyl groups flanking the amino group which slows the desired amide formation and thus allows the less reactive benzyl OH group to compete for reagent. The solution to this problem was again found in the use of the more selective reagent T-PEG which resulted in a 95% yield of **11**. The overall method appears to be general for the use of other amino acid spacers.

PEG Carbonate Conjugates. Synthesis of the phenolic PEG carbonate derivative **13** utilizing PEG succinimidyl carbonate (**12**) in the presence of DMAP (Scheme 4) proved to be straightforward. Preparation of **12** was achieved by standard procedures used for lower MW PEG.[27]

Scheme 4. Synthesis of PEG Carbonates of 4-Hydroxybenzyl Alcohols



Scheme 5. Synthesis of PEG Carbamates of Hydroxy- and Aminobenzyl Alcohols

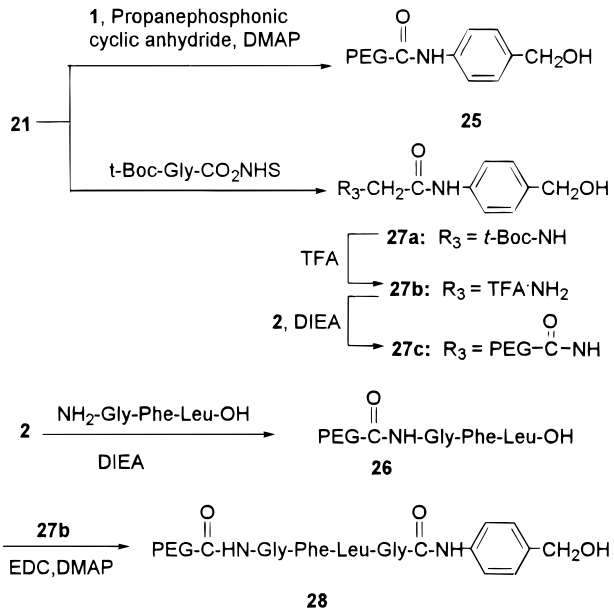


PEG Carbamate Conjugates. 1. Carbamates Based on a Phenolic OH (Type 1). A predictive model using octyl isocyanate and compound **3**, analyzed by ^{13}C NMR analysis, indicated that reaction was selective for the phenolic OH. PEG 40 diisocyanate (**14**) was prepared following the published procedure used for the lower MW, mPEG 5 kDa isocyanate[27] and condensed with **3**. Unexpectedly, selectivity was not observed in this case, and a mixture of both carbamates was detected. However, an alternate route for obtaining the desired product cleanly was devised and entailed the use of *p*-hydroxybenzaldehyde (**15**). Reaction with PEG-NCO (**14**) produced aldehyde **16** (80%), and this was subsequently reduced with $NaBH_4$ under mild conditions to yield the desired carbamate **17** in 78% yield (Scheme 5). *o*-Hydroxybenzaldehyde was similarly reacted with **14** to give an *ortho*-substituted carbamate, but the synthesis became problematic upon reduction of the aldehyde with $NaBH_4$. Another method was developed to synthesize type 1 *o*-carbamates that relied on blocking the benzyl alcohol and is also illustrated in Scheme 5. Thus, reaction of *o*-hydroxybenzyl alcohol with dihydropyran produced the tetrahydopyranyl-substituted derivative **18** which was reacted with PEG-NCO to give the blocked PEG carbamate. Dissolution of **19** in 0.1 N HCl for 3 h yielded the desired linker **20** by merely extracting the aqueous solution with dichloromethane. This path appears to be a general method and should also be applicable to the *para* isomer.

2. Carbamates Based on an Anilino NH_2 (Type 2). These were prepared without any difficulty in > 90% yield by the reaction of SC−PEG 40 with *p*- and *o*-aminobenzyl alcohols (**21, 22**) (Scheme 5).

PEG Amide Conjugates. The preparation of simple amides is illustrated by the reaction of PEG diacid and aminobenzyl alcohol (**21**) utilizing a condensing agent such as propanephosphonic cyclic anhydride (Scheme 6).

Scheme 6. Synthesis of PEG Amides of 4-Aminobenzyl Alcohols

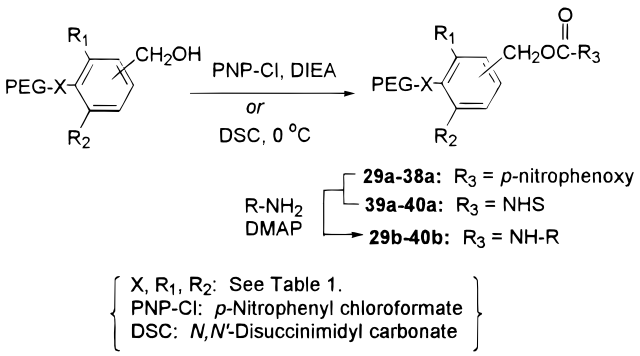


1. Amides Based on Amino Acids and Peptide Spacers. In the case of the tetrapeptide amide-linked compound **28**, *t*-Boc-glycine NHS ester was first reacted with **21** to give the glycinamide derivative **27a**, which after acid treatment yielded **27b**. Compound **27b** was in turn condensed with PEG−Gly-Phe-Leu-OH (**26**), prepared from T-PEG and the commercially available tripeptide Gly-Phe-Leu-OH as shown in Scheme 6. An example of an amide with an amino acid spacer (**27c**) was also synthesized using the intermediate **27b**.

Activation of PEG Linkers and Coupling to Amines. Activation of the benzylic alcohol moiety of the PEG-conjugated linker was done in several different ways. The choice of activating group used was often dictated by the reactivity of the amine present in the drug to be modified, e.g. primary or secondary amine, aromatic amine, or the presence of other functional groups such as hydroxyl which could compete for reagent. *p*-Nitrophenyl (PNP)-activated carbonates (**29a**−**38a**) serve well as acylating agents for small molecule substrates in organic solvents where crystallization readily removes the phenolic byproduct. This method also emerges as the simplest and most convenient method for activating the PEG linker and is shown in Scheme 7.

Using DNR as a model amine-containing drug, coupling reactions with PNP-activated PEG linkers (Scheme 7) were successfully carried out under mild reaction conditions: room temperature for several hours in methylene chloride or DMF as solvent and DMAP as base. When the drug doxorubicin, containing an additional reactive primary alcohol, was conjugated to PEG using PNP activation, lack of selectivity in obtaining a homogeneous product was encountered. The desired product resulted[28] using the more discriminating succinimidylcarbonate linker. Succinimidylcarbonate derivatives (**39a, 40a**) were best synthesized by reaction of PEG benzyl alcohols with disuccinimidylcarbonate[29] (DSC) in the presence of pyridine as base (Scheme 7).

Scheme 7. General Procedure for Activation of PEG Conjugates of Benzyl Alcohols and Coupling to Amines



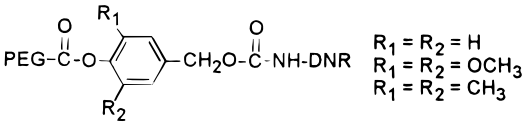
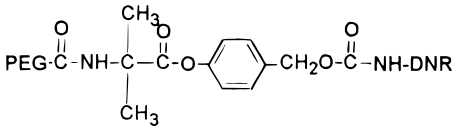
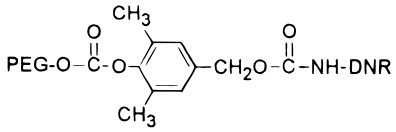
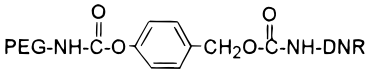
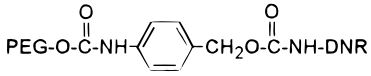
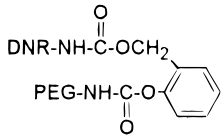
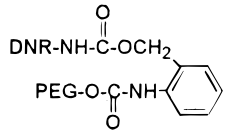
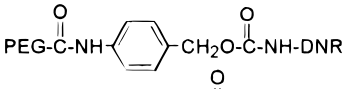
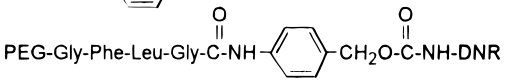
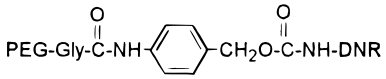
Results and Discussion

The synthetic methodology of the BE strategy accomplished in this work now allows the preparation of PEG amino prodrugs with many variable combinations of specifiers and linkers resulting in a broad, utilitarian PEG technology platform for designing prodrugs. The application of the PEG prodrug strategy produced compounds which had $t_{1/2}$ (hydrolysis) > 24 h in PBS buffer (pH 7.4) at 37 °C (Table 1). However, the rate at which DNR was released in fresh rat plasma (initiated by enzymatic action) provided information that clearly differentiated the various classes of prodrugs, as well as delineating the effects of specific substituent modifications carried out within a given series. The resulting PEG-linked prodrugs are listed in Table 1 with their rates of hydrolysis, cytotoxicities, and in vivo antitumor activities.

The efficacy of the PEG conjugates within the M109 model varied according to route of administration and their rate of in vitro dissociation. When the compounds were dosed intraperitoneally, the best activity was observed for native DNR followed by two carbamate derivatives (**34b** and **35b**). Similar activity for DNR in this ip model has been reported by others.[30] However, when the PEG prodrugs were administered by the more clinically relevant intravenous (iv) route, only those compounds with a $t_{1/2}$ rat plasma dissociation of 2−4 h (with the exception of **39b**) were effective in inhibiting solid tumor growth without causing toxicity and displayed a lower %T/C than an equivalent dose of DNR. Compounds **29b** and **30b** with $t_{1/2}$ of 1 h or less exhibited toxicity, while esters **31b** and **32b** with greater half-lives did not. Interestingly, a 2−6 h range for plasma hydrolytic half-life has also been demonstrated to be effective for antitumor activity of PEG camptothecin ester prodrugs (transport forms).[31] The reason behind this phenomenon probably lies in the biodistribution of the PEG−drug conjugates, especially with respect to their rates of drug elimination versus tumor uptake. To determine whether the intravenous results observed in this incipient fast growing M109 model were reproducible in more established slower growing solid tumor models, the type 1 (**34b**) and type 2 (**35b**) carbamates were further studied in an established (∼70 mm^3) SKOV3 ovarian cancer xenograft using nude mice (Table 2). Again, compound **34b** demonstrated a clear impact on tumor growth and was significantly more efficacious than native DNR. Surprisingly, the slow hydrolyzing type 2 carbamate also had an effect on these solid tumors with mean volumes less than those of DNR-treated animals. Therefore, while the slow release of DNR from **35b** was perhaps overwhelmed by the rapid division and necrosis of cells within fast growing tumors, its accumulation and slow release within slow growing tumors may have caused a more therapeutic effect. This is not to say that the attachment of 40 kDa PEG with its ability to accumulate in tumors[32] will automatically permit drugs to have greater antitumor activity. Obviously, for this technology to work, the compound must have an adequate circulatory retention to allow ample tumor accumulation (of either the free drug or the conjugate), and if the entire conjugate is taken up, it must contain a trigger which is cleaved within either the stromal environment or the neoplastic cells.

Fortunately, the 1,4- and 1,6-elimination strategy for PEG-conjugated amino prodrugs provides a flexible methodology for adjusting the rate of cleavage of the specifier to maximize its therapeutic impact. Starting with the ester series, the simplest compound (**29b**) resulted in a plasma $t_{1/2}$ that was only 0.4 h. However, introduction of steric hindrance in the vicinity of the trigger by introducing methoxy substituents in the 2,6-position of the ring (**30b**) resulted in an incremental increase in the half-life. A larger effect was obtained by the use of 2,6-methyl substituents (**31b**) which decelerated the rate to 2 h. Similarly, in the PEG carbonate series of specifiers, when 2,6-dimethyl substituents were introduced, they resulted in a $t_{1/2}$ of almost 3 h. Introduction of a AIBA spacer (**32b**) also had the desired effect of reducing hydrolytic rate, i.e., an increase of $t_{1/2}$ (1.3 h) compared to the unsubstituted ester (**29b**). The type 1 carbamate derivative **34b** had a $t_{1/2}$ of 4 h and, as noted earlier, produced the best solid tumor results in the study. In the *ortho* series, type 1 carbamate **36b** gave similar results (Table 1) and was found to have a $t_{1/2}$ of 3 h. In the case of amide triggers, compound **38b** was stable in plasma and did not appear to release DNR to the M109 model in a timely fashion: this was also reflected in the IC_{50} results (P388 leukemic model). We therefore turned to the use of an amide specifier based on the tetrapeptide sequence Gly-Phe-Leu-Gly. It has been adequately demonstrated that when *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer is conjugated to doxorubicin through a Gly-Phe-Leu-Gly spacer the conjugate is stable in rat plasma but passively accumulates in tumors. Release of anthracycline by degradation of the spacer in the lysosomal compartment by cathepsin B occurs following pinocytosis of the polymeric amide prodrug.[33] This peptide sequence was conjugated to PEG, and the resulting compound, **39b**, displayed the expected stability in PBS buffer. In rat plasma a half-life of 13 h for the disappearance of **39b** was measured (Table 1)—but without concomitant formation of free DNR (see Experimental Section). When compound **39b** was tested in the M109 syngeneic model, it proved to have a significant antitumor activity when administered by either ip or iv routes. PEG glycinamide (**40b)** was also synthesized and found to be stable at pH 7.4 (PBS) and in rat plasma ($t_{1/2}$ > 24 h). However, no significant tumor growth inhibition was observed in the M109 model for **40b.** Thus, as is the case for HPMA−Gly-Phe-Leu-Gly drug

Table 1. In Vitro and in Vivo Results of PEG−Daunorubicin Prodrugs

Compound	Compound #	$t_{1/2}$ (h)[a] buffer (pH 7.4)	$t_{1/2}$ (h)[a] rat plasma	IC $_{50}$ (nM) P388/0	M109 i.p. % T/C[b]	M109 i.v. % T/C[b]
Non-conjugated DNR				3	44.8	117.0
Esters						
 $R_1 = R_2 = H$; $R_1 = R_2 = OCH_3$; $R_1 = R_2 = CH_3$	**29b**	> 24	0.4	8	92.8	NA[c]
	30b	> 48	1.0	27	68.6	48.2
	31b	> 48	1.9	55	90.3	67.9
	**32b**	> 48	1.3	18	87.6	NA[c]
Carbonates						
	**33b**	> 48	2.9	179	90.3	74.4
Carbamates						
	**34b**	> 48	4	15	84.1	64.6
	**35b**	> 48	> 24	415	75.3	129.0
	**36b**	> 48	3	35	91.3	68.7
	**37b**	> 48	> 24	130	112.2	117.0
Amides						
	**38b**	> 48	> 24	457	122.7	NA[c]
	**39b**	> 24	13	160	87.6	82.6
	**40b**	> 24	> 24	825	91.1	204.3

[a] All experiments were done in duplicate. Standard deviation of measurements = ±10%. [b] 3 mg/kg/dose of active DNR administered to balb/c mice bearing subcutaneous Madison lung carcinoma on days 1 and 4 (intraperitoneal) or days 3 and 6 (intravenous) after inoculation. Percent treatment over control (%T/C) median tumor volumes was compared when control group's median tumor volume reached ~2000 mm^3. [c] Result not available.

conjugates, it appears that the analogous PEG derivatives are also capable of passively accumulating in solid tumors where pinocytosis and lysosomal cleavage of peptide spacers (but not simple amino acid spacers) take place.

The generation of quinone methides in vivo has been claimed to result in adduct formation with important biochemical and toxicological consequences.[34] To exclude the possibility that any quinone methide or quinone imide generated from the linker during the breakdown of the PEG−drug conjugates possessed toxic properties that would in any way affect the models, or was in part responsible for any of the tumor regression observed (especially those derived from compound **30b** which exhibited toxicity in the M109 model), we synthesized a conjugate of the dimethoxy ester BE system using a simple amino acid derivative of leucine (**41**), the 2-propylamine derivatives of dimethyl ester (**42**), and a type

Table 2. Efficacy of PEG−Daunorubicin Analogues against Established Subcutaneous Human Ovarian Tumors[a] (SKOV3) in Nude Mice

Treatment[b] (n = 5/treatment)	Mean Tumor Volume by day 30	% Tumor growth[c] (Δ from initial) by day 30	Mean Tumor Volume[d] (%T/C)	Median Tumor Volume[e] (%T/C)
Control (untreated)	1291	1588		
DNR	1154	1226	92	54
PEG-DNR (**34b**)	154*	86*	12	11
PEG-DNR (**35b**)	272	400	21	41

[a] Mean baseline (initial) tumor volume was 67 mm^3. [b] 3 mg/kg/dose (DNR content) given intravenously on q4d x 3. [c] Percent mean tumor volume change from initial based on individual tumors at day 30. [d] A comparison of treatment group's and control group's mean tumor volume on day 30. [e] The median tumor volume of treatment and control groups was measured and compared when the control group's median tumor volume reached approximately 1000 mm^3. *Significantly ($P < 0.05$) different from control (untreated).

2 carbamate (**43**) (Table 3). One problem encountered in using the activated ester PNP derivative **29a** (R_1,R_2 = H) was that nucleophilic attack at both sides of the molecule took place even when equimolar quantities of amine were used: this led to mixtures of the desired PEG prodrug products and simple PEG carbamates (Scheme 8). This underlines an important feature of the BE approach that must be considered in planning syntheses: namely, that when the trigger is an ester (Scheme 8) the activated linkers are, in fact, heterobifunctional reagents (phenoxide is a good leaving group) and the stoichiometry of the reaction, as well as the nature of the amine employed, must be taken into consideration. When PNP activation of the linker was employed in conjunction with *o,o*-dimethoxy flanking groups, attack at the carbonyl group of the trigger (path a) predominated with isopropylamine and the inert PEG carbamate was the sole product. However, use of leucine *tert*-butyl ester as nucleophile with the same linker proceeded exclusively via path b and produced only the desired prodrug **41**. Only path b product was identified when bulkier amines such as DNR were employed. *o,o*-Dimethyl substituents were the most effective in discouraging nucleophilic attack at the trigger, and a small excess of amine in this system produced only the desired benzyl carbamate (path b) product.

The three model compounds, which contained no anticancer agents, were tested in normal mice at 3 times the concentration of PEG linker used for the DNR-conjugated compounds **30b**, **31b**, and **35b**. No adverse effects or toxicity were noted at this dose. Since these simple amine prodrugs (**41**−**43**) demonstrated no IC_{50} activity, it appears that the anticancer agent and its delivery to the tumor target are entirely responsible for any inhibitions observed.

Conclusions

PEG conjugated to amino prodrugs that function via a 1,4- or 1,6-elimination has been demonstrated to be a feasible methodology to deliver drugs—especially anticancer agents—and offers several advantages over existing technologies. By using the double-prodrug strategy and changing promoieties (specifiers or triggers), alteration of PEG prodrug pharmacokinetics can easily be accomplished leading to greater drug efficacy. In a tripartate system this can be achieved not only by changing the PEG specifier but also by adding a spacer and/or introducing steric hindrance, both of which will affect the rate of cleavage of the specifier.

The exceptionally effective PEG delivery system for DNR, the type 1 carbamate, displayed a useful rat plasma half-life of 4 h: subsequent in vivo testing (M109, SKOV-3) demonstrated superior tumor growth inhibition compared to equimolar amounts of native drug. Although most amine drugs can be solubilized as acid salts, their rate of renal excretion is also high. When converted to neutral small prodrug species, the ability to form salts is lost, and solubility may again become problematic. Not so in the case of PEG−drug conjugates, where PEG confers water solubility to insoluble small organic compounds without the need for forming salts. PEG prodrug methodology can be accomplished in a rapid and facile manner. In practical terms, a PEG prodrug strategy will not result in any increase in the usual and customary costs associated with GMP manufacture of small organic molecules. Passive targeting of PEG to tumors has been demonstrated to be a real phenomenon, and the PEG prodrug approach applied to hydroxy-containing anticancer compounds has been shown to be an effective means for drug delivery of anticancer agents. The current work has now extended the usefulness of the PEG prodrug strategy to amino-containing anticancer compounds, and we believe these findings will also be applicable to other amino drugs which possess a diversity of activities.

Experimental Section

Chemistry. Reagents were obtained from Aldrich, Milwaukee, WI, Lancaster Synthesis, Lancaster, PA, and Advanced ChemTech, Louisville, KY, and were used without further purification. NMR spectra were obtained using a 270-MHz spectrometer and deuterated chloroform as the solvent unless otherwise specified. The percentage of daunorubicin in the PEG−daunorubicin conjugates was determined using UV assay techniques as previously described.[22] Rates of hydrolysis were obtained using an analytical HPLC employing a ZORBAX 300 SB C-8 column (150 × 4.6 mm) with a multiwavelength UV detector, using a gradient of 30−90% acetonitrile/0.1 M triethylammonium acetate (TEAA) at a flow rate of 1 mL/min. All PEG compounds were dried under vacuum or by azeotropic distillation from toluene prior to use.

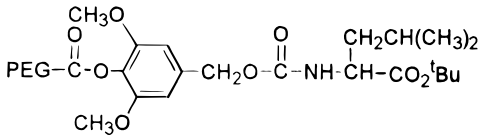
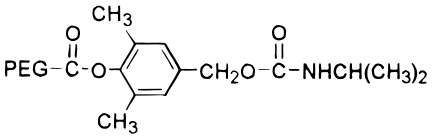
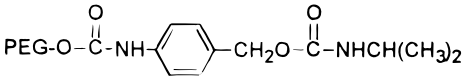
Compound 6. A solution of T-PEG (**2**; 4.0 g, 0.1 mmol), 4-hydroxybenzyl alcohol (**3**) (0.26 g, 2.1 mmol), and DMAP (0.25 g, 2.1 mmol) in anhydrous (anhyd) methylene chloride (40 mL) was refluxed overnight. The solvent was removed by distillation in vacuo, and the residue was crystallized from 2-propanol to yield 3.4 g (85%) of **6**: ^{13}C NMR δ 63.57, 68.36−71.86 (PEG), 120.69, 127.31, 139.15 149.25, 168.21.

Compound 7. Prepared in 80% yield by reacting **4** with **2** as described for **6**: ^{13}C NMR δ 55.93, 64.27, 67.97, 68.68−72.04 (PEG), 103.60, 140.09, 152.01, 167.61.

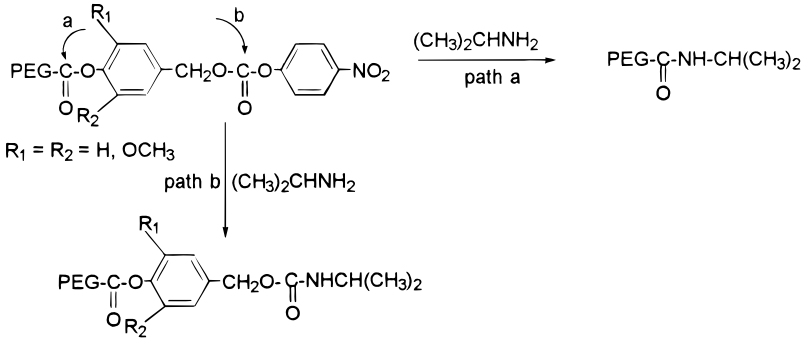
Compound 8. Prepared in 80% yield by reacting **5** with **2** as described for **6**: ^{13}C NMR δ 15.78, 63.86, −72.24 (PEG), 126.71, 129.58, 138.97, 146.69, 167.66.

Compound 9. A solution of *t*-Boc-α-aminoisobutyric acid (3.0 g, 0.015 mol), **3** (7.4 g 0.06 mol), and DMAP (7.3 g, 0.06 mol) in anhyd methylene chloride (100 mL) was cooled to 0 °C followed by the addition of EDC (11.52 g, 0.06 mol). The reaction mixture was allowed to slowly warm to room temperature and stirred for 18 h. The solvent was removed by distillation in vacuo, and the residue crystallized from 2-propanol to yield 500 mg (83%) of product: ^{13}C NMR δ 25.06, 28.01, 55.79, 63.73, 79.48, 120.93, 127.45, 138.76, 149.93, 154.54, 173.27; ES MS *m/z* 310.15 ($M^+ + H$, 80); HRMS calcd for $C_{16}H_{24}NO_5$ ($M^+ + H$) 310.1654, found 310.1660.

Table 3. In Vivo Acute Lethality of PEG Linkers

Compound	Compound #	Dose (mg/kg)	% Toxicity (Deaths)
	**41**	830	0
	**42**	830	0
	**43**	830	0

Scheme 8



Compound 10. To a solution of **9** (3.0 g) in methylene chloride (30 mL) was added 15 mL of trifluroacetic acid (TFA) followed by stirring for 30 min at room temperature. Ether was added until solid precipitated. The solid was filtered and thoroughly washed with ether to remove any residual TFA. After drying (2.5 g, 81% yield) the TFA salt **10** was used directly in the next step: ^{13}C NMR δ 23.40, 56.01, 62.25, 118.15(q), 120.90, 127.64, 140.97, 148.55, 158.70(q), 170.57; ES MS *m*/*z* 210.14 (M^+ + H, 100); HRMS calcd for $C_{11}H_{16}NO_3$ (M^+ + H) 210.1130, found 210.1132.

Compound 11. A solution of **2** (2.0 g, 0.05 mmol), **10** (0.07 g, 0.2 mmol), and DMAP (0.05 g, 0.4 mmol) in anhyd methylene chloride (25 mL) was refluxed overnight. The solvent was removed by distillation in vacuo, and the residue crystallized from 2-propanol to yield 1.9 g (95% yield): ^{13}C NMR δ 24.49, 55.24, 63.49, 68.65−71.26 (PEG), 120.85, 127.21, 138.79, 168.99.

Compound 13. A solution of **12** (6.0 g, 0.15 mmol), **5** (0.6 g, 4 mmol), and DMAP (0.6 g, 4 mmol) in anhyd methylene chloride (60 mL) was refluxed overnight. The solvent was removed by distillation in vacuo, and the residue crystallized from 2-propanol to give 5.4 g (90% yield) of **13**: ^{13}C NMR δ.15.49, 63.44, 67.06, 68.31, 68.58−70.90 (PEG), 126.53, 129.37, 138.79, 146.78, 152.36.

Compound 16. PEG diisocyanate (**14**)[27] was generated in situ as follows. A solution of PEG diamine hydrochloride[23] (3.0 g, 0.08 mmol) in 80 mL of toluene was azeotroped for 2 h. The solution was cooled to 30 °C followed by the addition of triphosgene (0.02 g, 0.06 mmol) and diisopropylethylamine (DIPEA; 0.07 g, 0.5 mmol). This mixture was stirred for 3 h at 70−80 °C followed by cooling to room temperature and addition of 60 mL of ethyl ether. **14** was collected by filtration under nitrogen and immediately added to a solution of 4-hydroxybenzaldehyde (**15)** (0.14 g, 1.1 mmol) and 4-(dimethylamino)pyridine (0.14 g, 1.1 mmol) in anhyd methylene chloride (30 mL), and the reaction refluxed overnight. The solvent was removed by distillation in vacuo, and the residue was crystallized twice from methylene chloride/ether to yield 2.4 g of **16** (80% yield): ^{13}C NMR δ 40.35, 121.26, 130.31, 132.58, 152.93, 156.01, 190.14.

Compound 17. To a solution of **16** (2.0 g, 0.05 mmol) in anhyd methanol (40 mL) at 0 °C was added sodium borohydride (6.0 mg, 0.15 mmol) followed by stirring for 2 h. The solvent was removed from the reaction mixture by distillation in vacuo and the residue dissolved in 30 mL of methylene chloride and washed with dilute aqueous HCl. The organic layer was separated and dried over anhyd sodium sulfate. The solvent was removed by distillation in vacuo, and the residue crystallized from 2-propanol to yield 1.5 g (75% yield) of **17**: ^{13}C NMR δ 40.35, 63.44, 67.97−71.45 (PEG), 120.82, 127.05, 137.99, 147.68, 154.13.

Compound 18. A solution of 6.0 g (0.05m) of 2-hydroxybenzyl alcohol, 2.1 g (0.025m) of dihydropyran, and 0.1 g (0.5 mmole) of *p*-toluenesulfonic acid monohydrate in 75 mL of methylene chloride was stirred for 1 h at room temperature, followed by the addition of 75 mL of saturated aqueous sodium bicarbonate. The mixture was stirred rapidly for 10 min and the methylene chloride layer separated, washed with water,

and dried over anhydrous sodium sulfate. Filtration and removal of the solvent by distillation in vacuo yielded 3.2 g of crude product. Purification of **18** was carried out by filtration through silica gel; elution using ethyl acetate/ethyl ether (7:3, v/v) yielded 2.5 g of pure product (49%): ^{13}C NMR δ 19.58, 24.99, 30.34, 63.19, 67.39, 98.44, 116.53, 119.80, 122.53, 129.16, 129.55 155.89; ES MS *m*/*z* 231.13 (M^+ + Na, 100); HRMS calcd for $C_{12}H_{16}O_3Na$ (M^+ + Na) 231.0997, found 231.0994.

Compound 19. A solution of 3.0 g (0.075 mmol) of PEG diamine hydrochloride in 80 mL of toluene was azeotroped for 2 h while removing 40 mL of toluene/water, followed by cooling to room temperature. Triphosgene (18 mg, 0.06 mmol) and 44 mg (0.34 mmol) of DIPEA were added, and the mixture stirred for 4 h at 50−60 °C, followed by removal of toluene by distillation in vacuo. The residue was dissolved in 40 mL of dry methylene chloride followed by the addition of 0.12 g (0.6 mmol) of the THP-protected alcohol **18**, 44 mg (0.34 mmol) of DIPEA, and 50 mg (0.08 mmol) of dibutyltin dilaurate catalyst. This mixture was refluxed for 18 h followed by removal of the solvent by distillation in vacuo and crystallization of the residue from 2-propanol to yield 2.5 g (83%) of protected carbamate **19**: ^{13}C NMR δ 18.85, 25.02, 30.06, 40.65, 61.48, 63.23, 67.80−71.07 (PEG), 97.28, 121.98, 124.98, 127.86, 128.60, 130.42, 148.46 154.08.

Compound 20. A solution of 2.0 g (0.05 mmol) of **19** in 80 mL of 0.6 N HCl was stirred at 37 °C for 3 h followed by extraction with methylene chloride (three times). The combined extracts were washed with water and dried over anhyd sodium sulfate. Filtration and removal of the solvent by distillation in vacuo yielded 1.8 g (90%) of PEG benzyl alcohol derivative **20**: ^{13}C NMR δ 40.31, 59.08, 67.70−70.80 (PEG), 121.40, 124.92, 127.59, 128.45, 133.17, 136.34, 154.21.

Compound 23. A solution of **12** (4.0 g, 0.1 mmol), **21** (0.1 g, 0.8 mmol), and DMAP (0.1 g, 0.8 mmol) in anhyd methylene chloride (30 mL) was stirred overnight at room temperature. The solvent was removed by distillation in vacuo, and the residue was crystallized from 2-propanol to yield 3.7 g (93%): ^{13}C NMR δ 63.64, 63.99, 68.91−71.32 (PEG), 118.57, 127.06, 136.13, 137.20, 153.08.

Compound 24. Prepared in 80% yield by reacting **22** with **12** as described for **23**: ^{13}C NMR δ 62.62, 63.13, 67.76−71.47 (PEG), 119.51, 122.07, 127.63, 136.96, 152.82.

Compound 25. A solution of **1** (5.0 g, 0.12 mmol), **21** (0.15 g, 1.2 mmol), 1-propanephosphonic acid cyclic anhydride (0.5 mL, 0.8 mmol, 50% solution in ethyl acetate), and DMAP (0.09 g, 0.8 mmol) in anhyd methylene chloride (100 mL) was stirred overnight at room temperature. The solvent was removed by distillation in vacuo, and the residue crystallized from 2-propanol to yield 2.54 g (56% yield) of **25**: ^{13}C NMR δ 63.98, 68.99−71.0 (PEG), 119.60, 127.01, 136.50, 137.35, 167.48.

Compound 26. A mixture of **2** (6 g, 0.15 mmol), tripeptide NH_2-Gly-Phe-Leu-OH (150.9 mg, 0.45 mmol), and DIPEA (76 mg, 0.6 mmol) in anhyd methylene chloride (60 mL) was stirred at room temperature for 18 h. The reaction mixture was washed with 0.1 N HCl (2 × 5 mL) followed by water (5 mL) and dried over anhyd magnesium sulfate. The solvent was removed under reduced pressure to yield a solid that was recrystallized from 2-propanol to give 4.9 g (80%) of **26**: ^{13}C NMR δ 21.41, 22.17, 24.21, 36.94, 40.66, 42.31, 50.42, 53.86, 70.64−72.22 (PEG), 126.14, 127.87, 128.73, 136.31, 168.42, 169.91, 170.28, 172.21.

Compound 27a. A solution of *t*-Boc-glycine NHS ester (1.1 g, 4.04 mmol) and **21** (1 g, 8.12 mmol) in anhyd methylene chloride (15 mL) was stirred at room temperature for 18 h. The reaction mixture was filtered to remove precipitated NHS and the filtrate washed with 0.1 N HCl (2 × 5 mL) followed by water (5 mL). The solvent was removed under reduced pressure to yield a residue that was triturated with ether to give 900 mg (75%) of **27a**: ^{13}C NMR δ 28.27, 44.87, 64.45, 80.50, 120.19, 127.66, 136.73, 137.01, 156.55, 168.24; ES MS *m*/*z* 319.14 (M^+ + K, 100); HRMS calcd for $C_{14}H_{20}N_2O_4K$ (M^+ + K) 319.1060, found 319.1062.

Compound 27b. A solution of **27a** (500 mg, 1.78 mmol) in methylene chloride (5 mL) and TFA (2.5 mL) was stirred for 30 min at room temperature. Anhydrous diethyl ether (50 mL) was added to precipitate crude **27b**. The solid was filtered, washed thoroughly with ether until all TFA was removed and dried in a vacuum oven to give 300 mg (60%) of the TFA salt **27b**: ^{13}C NMR δ 41.01, 62.58, 119.01, 127.22, 136.79, 138.13, 164.62; ES MS *m*/*z* 181.12 (M^+ + H, 100); HRMS calcd for $C_9H_{13}N_2O_2$ (M^+ + H) 181.0977, found 181.0968.

Compound 27c. A mixture of **2** (2 g, 0.05 mmol), **27b** (60 mg, 0.2 mmol), and DIPEA (39 mg, 0.3 mmol) in anhyd methylene chloride (20 mL) was stirred at room temperature for 18 h. The solvent was removed under reduced pressure to yield a solid that was recrystallized from 2-propanol twice to give 1.6 g (%) of **27c**: ^{13}C NMR δ 42.15, 63.74−72.17 (PEG), 118.84, 126.89, 136.39, 137.09, 166.88, 170.11.

Compound 28. To a solution of **26** (2 g, 0.05 mmol) and **27b** (60 mg, 0.2 mmol) in anhyd methylene chloride (20 mL) at 0 °C were added EDC (38.5 mg, 0.2 mmol) and DMAP (49 mg, 0.4 mmol), and the mixture stirred for 18 h at room temperature. The solvent was removed under reduced pressure, and the solid obtained crystallized from 2-propanol to give 1.5 g (75%) of **28**: ^{13}C NMR δ 20.42, 22.43, 24.00, 36.0, 39.0, 43.0, 52.50, 55.20, 63.5, 68.19−71.39 (PEG), 118.8, 126.1, 126.4, 127.8, 128.3, 136.0, 136.5, 137.0, 167.0, 170.5, 171.1, 172.0.

Compound 29a. A solution of **6** (3.0 g, 0.07 mmol) in 140 mL of toluene was azeotroped for 2 h. The solution was cooled to 30 °C followed by the addition of 4-nitrophenyl chloroformate (0.06 g, 0.3 mmol) and DIPEA (0.04 g, 0.3 mmol). The mixture was stirred for 18 h at 50−55 °C and then allowed to cool to ambient temperature and the solvent removed by distillation in vacuo. The residue crystallized from 20% methylene chloride in ethyl ether to yield 2.4 g (77%) of product **29a**: ^{13}C NMR δ 68.47−71.32 (PEG), 121.39, 121.47, 124.87, 129.45, 131.96, 145.54, 150.15, 155.01, 151.82, 168.19.

Compounds 30a−38a. Prepared in a manner similar to that of **29a** in 65−85% yields from the appropriate precursors.

Compound 30a. ^{13}C NMR δ 56.11, 67.98, 68.36−71.47 (PEG), 105.56, 105.62, 124.79, 132.56, 145.46, 151.93, 152.38, 155.32, 167.46, 167.49.

Compound 31a. ^{13}C NMR δ 15.78, 63.86, 68.10, 68.71−71.58 (PEG), 126.71, 129.58, 138.97, 148.39, 152.09, 167.66.

Compound 32a. ^{13}C NMR δ 24.50, 53.16, 68.76−70.89 (PEG), 121.37, 121.47, 124.73, 129.40, 131.31, 144.85, 151.05, 151.80, 154.98, 169.06, 172.21.

Compound 33a. ^{13}C NMR δ 15.44, 67.21, 68.23, 68.61−71.26 (PEG), 121.29, 124.65, 128.54, 130.19, 131.41, 144.79, 148.11, 151.73, 152.14, 154.91.

Compound 34a. ^{13}C NMR δ 40.72, 67.81−71.99 (PEG), 121.53, 121.66, 121.96, 124.68, 124.96, 125.20, 129.63, 130.73, 145.07, 151.37, 152.05, 154.12, 155.18.

Compound 35a. ^{13}C NMR δ 64.01, 68.60−71.45 (PEG), 118.78, 121.39, 124.86, 127.29, 128.85, 129.19, 139.13, 152.09, 153.19, 155.51.

Compound 36a. ^{13}C NMR δ 40.60, 65.54, 67.51−70.93 (PEG), 121.37, 122.36, 124.69, 125.13, 126.37, 129.68, 133.32, 144.86, 149.01, 151.72, 153.72, 155.05.

Compound 37a. ^{13}C NMR δ 63.86, 67.27, 68.03−70.82 (PEG), 121.37, 123.01, 124.05, 124.26, 129.54, 129.61, 130.13, 136.18, 144.90, 151.99, 153.55, 154.83.

Compound 38a. ^{13}C NMR δ 69.00−71.97 (PEG), 119.80, 121.32, 124.76, 128.99, 129.85, 138.19, 155.40, 167.79.

Compound 39a. A solution of **28** (1.45 g, 0.036 mmol) and disuccinimidylcarbonate (74 mg, 0.29 mmol) in anhyd methylene chloride (20 mL) was cooled to 0 °C followed by the addition of pyridine (12 mg, 0.15 mmol). The reaction mixture was stirred for 18 h at 0 °C followed by precipitation with ether. The residue was crystallized from 20% methylene chloride in ethyl ether to yield 1.2 g (85%) of **39a**: ^{13}C NMR δ 20.67, 22.58, 24.10, 35.81, 38.89, 43.03, 52.55, 55.12, 69.92−71.19 (PEG), 119.29, 126.40, 128.05, 128.49, 128.80, 135.78, 138.91, 151.21, 167.22, 168.17, 170.71, 171.5, 172.04.

Compound 40a. Prepared in 85% yield by reacting **27c** with disuccinimidylcarbonate as described for **39a**: ^{13}C NMR δ 24.76, 41.97, 69.88−71.87 (PEG), 118.84−128.98, 129.99, 138.97, 150.89, 167.06, 168.20, 169.96.

Compound 29b. A mixture of **29a** (2.3 g, 0.06 mmol), daunorubicin hydrochloride (115 mg, 0.20 mmol), and DMAP (83 mg, 0.68 mmol) in anhyd dimethylformamide (40 mL) was stirred at room temperature for 18 h. To this mixture was added 100 mL of ether until the precipitation was complete. The solid was collected by filtration, washed with ether, and then crystallized from 2-propanol to yield 2.2 g (88%) of final product. The amount of daunorubicin present in this compound as measured by UV assay was found to 2.3 wt %.

Compounds 30b−40b. Prepared in a manner similar to that of **29b** in 40−90% yields. The percent by weight of daunorubicin as determined by UV assay is given as follows: **30b**, 2.1; **31b**, 2.3; **32b**, 1.8; **33b**, 2.5; **34b**, 2.6; **35b**, 2.2; **36b**, 2.3; **37b**, 1.8; **38b**, 2.1; **39b**, 1.8; **40b**, 1.8%.

Compound 34b. 75% yield; ^{13}C NMR δ 16.83, 24.63, 29.94, 33.30, 34.99, 40.94, 47.23, 56.61, 65.74, 67.40, 67.80−71.34 (PEG), 102.15, 111.15, 111.31, 118.60, 119.63, 120.86, 121.44, 129.05, 133.41, 134.25, 134.39, 135.39, 135.65, 150.50, 154.45, 155.47, 155.73, 156.31, 161.00, 186.81, 187.04, 212.01.

Compound 35b. 71% yield; ^{13}C NMR δ 16.34, 24.18, 29.39, 32.71, 34.49, 46.64, 56.10, 66.90, 67.91, 68.11−70.87 (PEG), 100.22, 110.58, 110.74, 117.90, 118.06, 119.09, 120.23, 128.43, 130.40, 133.72, 133.85, 134.82, 135.17, 137.90, 152.92, 155.08, 155.18, 155.78, 160.44, 186.02, 186.29, 211.26.

Compound 36b. 78% yield; ^{13}C NMR δ 16.23, 24.06, 28.92, 32.60, 34.37, 40.44, 56.01, 69.83−70.93 (PEG), 101.48, 110.45, 110.52, 118.02, 119.00, 120.13, 122.12, 124.95, 128.89, 129.70, 133.66, 134.71, 135.11, 142.27, 148.37, 155.08, 155.69, 160.38, 186.06, 186.16.

Compound 37b. 38% yield; ^{13}C NMR δ 16.30, 24.07, 28.64, 32.66, 34.42, 46.81, 56.83, 62.81, 63.45, 67.10−71.74 (PEG), 101.05, 110.52, 110.71, 118.08, 119.02, 120.19, 123.31, 125.13, 129.67, 130.34, 133.78, 134.77, 135.17, 136.42, 142.24, 153.38, 155.10, 155.14, 155.73, 156.01, 160.42, 185.99, 186.23, 211.20.

Compound 38b. 92% yield; ^{13}C NMR δ 16.23, 23.98, 29.30, 32.63, 34.45, 46.62, 56.01, 65.30, 66.85, 68.28−70.84 (PEG), 100.13, 110.49, 110.66, 118.09, 118.99, 119.19, 120.16, 128.16, 131.81, 133.70, 133.76, 134.71, 135.09, 136.82, 155.02, 155.69, 160.39, 161.42, 167.50, 185.93, 186.14, 211.11.

Compound 39b. 86% yield; ^{13}C NMR δ 16.40, 20.74, 22.67, 24.23, 29.47, 32.90, 34.52, 35.91, 38.95, 43.06, 46.79, 52.69, 55.18, 56.17, 65.66, 66.96, 68.53, 69.0−72.0 (PEG), 100.22, 110.72, 110.95, 118.16, 119.17, 119.20, 120.45, 126.55, 128.09, 128.20, 128.58, 131.44, 133.82, 133.94, 134.97, 135.20, 135.78, 137.87, 155.13, 155.29, 155.87, 160.58, 167.21, 170.74, 171.69, 172.20, 186.16, 186.38, 211.0.

Compound 40b. 90% yield; ^{13}C NMR δ 16.45, 24.33, 29.23, 32.90, 34.71, 44.52, 56.24, 66.91, 70.92−72.37 (PEG), 100.42, 110.70, 110.87, 118.13, 119.27, 120.47, 133.76, 135.04, 135.27, 155.38, 155.96, 160.59, 168.70, 186.18, 186.50.

Compound 41. A mixture of 1.0 g (0.025 mmol) of **30a**, 22 mg (0.1 mmol) of leucine *tert*-butyl ester hydrochloride, and 14 mg (0.11 mmol) of DIPEA in 10 mL of anhyd methylene chloride was stirred at room temperature for 18 h followed by removal of the solvent by distillation in vacuo. The product was crystallized from 2-propanol to yield 0.7 g (70%): ^{13}C NMR δ 21.41, 22.28, 27.42, 41.24, 52.80, 55.56, 55.66, 67.58−71.26 (PEG), 80.11, 103.94, 127.16, 134.78, 151.47, 155.31, 167.59.

Compound 42. A mixture of 0.6 g (0.015 mmol) of **31a**, 100 mg (1.7 mmol) of isopropylamine, and 210 mg (1.7 mmol) of DMAP in 20 mL of dry methylene chloride was stirred at room temperature for 18 h followed by removal of the solvent by distillation in vacuo. The residue was crystallized from 2-propanol to yield 0.4 g (80%) of **42**: ^{13}C NMR δ 16.04, 22.70, 43.10, 66.41, 67.90−71.97 (PEG), 128.15, 129.85, 133.44, 143.77, 158.04, 167.92.

Compound 43. A mixture of 1.0 g (0.025 mmol) of **35a** and 30 mg (0.5 mmol) of isopropylamine in 10 mL of dry methylene chloride was stirred at room temperature for 18 h followed by removal of the solvent by distillation in vacuo. The residue was crystallized from 2-propanol to yield 0.8 g (80% yield) of **43**: ^{13}C NMR δ 22.36, 42.38, 60.93, 67.87, 68.21−72.00 (PEG), 117.93, 128.35, 130.05, 138.66, 153.25, 154.97.

Biological Results. 1. Materials. Daunorubicin HCl (Chem-Werth, Woodbridge, CT) and all PEG-conjugated daunorubicin compounds were dissolved in sterile saline (0.9%) for injection prior to in vivo drug treatments and were given as their daunorubicin equivalents (absolute amount of daunorubicin given).

2. Cell Lines and Cytotoxicity Assays. Studies using P388/0 cell lines for IC_{50} (drug concentration inhibiting 50% of cells) were maintained and conducted as previously reported.[22] Briefly, for IC_{50} determination cells were seeded into the plates at a density of 2×10^3 cells/50 μL/well. Plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 3 days. Cell growth was measured by the addition of 10 μL/well of Alamar Blue (Alamar Biosciences, Inc., Sacramento, CA), and the plates were incubated a further 4 h at 37 °C. The IC_{50} values for each compound were determined from absorbance-versus-dilution factor plots. All cell cultures for animal implantation were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2 and subcultured once a week. All cell lines were periodically tested for mycoplasma and were mycoplasma free. SKOV3 (human, ovarian adenocarcinoma, ATCC/HTB77) was raised in McCoy's 5a medium supplemented with 15% FBS (fetal bovine serum). M109 (Madison 109 murine lung carcinoma, NCI) was adapted to cell culture and grown in EMEM (Eagle's modified essential medium) with 10% FBS.

3. Hydrolysis Rates. Rates of hydrolysis of the PEG conjugates and the liberation of free drug were determined in phosphate-buffered saline (PBS) and fresh rat plasma as previously described.[31] In the case of **39b**, the disappearance of the PEG−peptide-linked drug was observed without the concomitant formation of native DNR. This result would appear to implicate binding of the PEG prodrug to a plasma component.

4. Antitumor Activity and PEG Linker Toxicity. Screening for antitumor activity against M109 tumors were conducted in 20−25-g, female balb/c mice (Hilltop Labs, Scottdale, PA). Mice were implanted (day 0) subcutaneously at the left flank with approximately 5×10^5 cells in 100 μL. Intraperitoneal treatments were administered on days 1 and 4, while intravenous dosages were given on days 3 and 6. Treatment groups (6/group) consisted of untreated controls, daunorubicin, and 12 PEG−daunorubicin derivatives. For solid tumor xenograft studies, female nu/nu mice (Harlan Sprague−Dawley, Madison, WI), 18−24 g, were inoculated subcutaneously at the left flank with tumor cells (1×10^6) in 0.1 mL of medium. Treatments began when the mean tumor volume reached aproximately 70 mm^3 (day 1). Experimental groups (5/group) consisted of daunorubicin, **34b**, and **35b** administered intravenously at 3 mg/kg/dose on days 1, 5, and 9 with their activity compared to control (untreated). Antitumor activity for both studies was calculated and determined as previously described.[35] To determine the toxicity of specific PEG linkers **41**−**43,** ICR mice (Harlan Sprague−Dawley, Madison, WI) were intravenously administered (25 mg/mouse) these simple PEG linker conjugates. Mice (4/group) were observed daily and weighed twice weekly for 2 weeks. 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 Institutes of Health. These experimental protocols were approved by the Institutional Animal Care and Use Committee of UMDNJ−Robert Wood Johnson Medical School.

5. Statistics. The differences between treatment groups were assessed by one-way ANOVA. Multiple comparisons, when significant differences existed, were determined by least significant differences techniques. Statistical analysis was conducted using the StatView software program (Abacus Concepts, Inc., Berkeley, CA).

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References

(1) Wakselman, M. 1,4- And 1,6-Eliminations From Hydroxy And Amino-Substituted Benzyl Systems: Chemical And Biochemical Applications. *Nouv. J. Chem*. **1983**, *7*, 439−447.

(2) (a) Wakselman, M.; Guibe-Jampel, E. J. An Alkali-labile Benyloxycarbonyl Amino-protecting Group. *J. Chem. Soc., Chem. Commun*. **1973**, 593−594. (b) Le Corre, G.; Guibe-Jampel, E.; Wakselman, M. Groupements Protecteurs Benzyloxycarbonyles Substitues Alcalinolabiles. *Tetrahedron* **1978**, *34*, 3105−3112. (c) Guibe-Jampel, E.; Wakselman, M. Selective Cleavage of *p*-Nitrobenzyl Esters With Sodium Dithionite. *Synth. Commun*. **1982**, *12*, 219−223.

(3) Kemp, D. S.; Hoyng, C. F. New Protecting Groups For Peptide Synthesis-I. The BIC Group Base And Solvent Lability of 5-Benzisoxylmethyleneoxycarbonylamino Function. *Tetrahedron Lett*. **1975**, 4625−4628.

(4) Taylor, L. D.; Grasshoff, M.; Pluhar, M. Use of *o*- and *p*-Hydroxybenzyl Functions as Blocking Groups Which Are Removable with Base. *J. Org. Chem*. **1978**, *43*, 1197−1200.

(5) (a) Cain, B. F. 2-Acyloxymethylbenzoic Acids. Novel Amine Protective Functions Providing Amides With the Lability of Esters. *J. Org. Chem*. **1976**, *41*, 2029−2031. (b) Bundgaard, H. The Double Prodrug Concept and Its Applications. *Adv. Drug Delivery Rev*. **1989**, *3*, 39−65.

(6) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. A Novel Connector Linkage Applicable in Prodrug Design. *J. Med. Chem*. **1981**, *24*, 479−480.

(7) Senter, P. D.; Pearce, W. E.; Greenfield, W. E. Development of a Drug-Release Strategy Based on the Reductive Fragmentation of Benzyl Carbamate Disulfides. *J. Org. Chem*. **1990**, *55*, 2975−2978.

(8) Mauger, A. B.; Burke, P. J.; Somani, H. H.; Friedlos, F.; Knox, R. J. Self-Immolative Prodrugs: Candidates for Antibody-Directed Enzyme Prodrug Therapy in Conjunction with a Nitroreductase Enzyme. *J. Med. Chem*. **1994**, *37*, 3452−3458.

(9) Hay, M. P.; Wilson, W. R.; Denny, W. A. A Novel Enediyne Prodrug For Antibody Directed Enzyme Prodrug Therapy (ADEPT) Using E. Coli B Nitroreductase. *Bioorg. Med. Chem. Lett*. **1995**, *5*, 2829−2834.

(10) Lee, M.; Simpson, J. E.; Woo, S.; Kaenzig, C.; Anlezark, G. M.; Eno-Amoquaye, E.; Burke, P. J. Synthesis of an Aminopropyl Analogue of the Experimental Anticancer Drug Tallmustine, and Activation of its 4-Nitrobenzylcarbamoyl Prodrug By Nitroreductase and NADH. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1065−1070.

(11) (a) Andrianomenjanahary, S.; Dong, X.; Florent, J.-C.; Gaudel, G.; Gesson, P.-P.; Jacquesy, J.-C.; Koch, M.; Mondon, M.; Pettit, P.; Renoux, B.; Tillequin, F. Synthesis of Novel Targeted Pro-Prodrugs of Anthracyclines Potentially Activated by a Monoclonal Antibody Galactosidase Conjugate. *Bioorg. Med. Chem. Lett*. **1992**, *2*, 1093. (b) Gesson, J.-P.; Jacquesy, J.-C.; Mondon, P.; Petit, P.; Renoux, S.; Andrianomenjanahary, H.; Dufat-Trinh, V.; Koch, M.; Bosslet, K.; Czech, J.; Hoffman, D. Prodrugs of Anthracyclines for Chemotherapy Via Enzyme-Monoclonal Antibody Conjugates. *Anti-Cancer Drug Des*. **1994**, *9*, 409−423. (c) Azoulay, M.; Florent, J.-C.; Moneret, C.; Gesson, J. P.; Jacquesy, J.-C.; Tillequin, F.; Koch, M.; Bosslet, K.; Czech, J.; Hoffman, D. Prodrugs of Anthracycline Antibiotics Suited for Tumor-Specific Activation. *Anti-Cancer Drug Des*. **1995**, *10*, 441−450. (d) Florent, J.-C.; et al. Prodrugs of Anthracyclines for Use in Antibody-Directed Enzyme Prodrug Therapy. *J. Med. Chem*. **1998**, *41*, 3572−3581.

(12) (a) Leenders, R. G. G.; Gerrits, K. A. A.; Ruijtenbeek, R.; Scheeren, H. W.; Haisma, H. J.; Boven, E. *β*-Glucuronyl Carbamate Based Pro-Moieties Designed for Prodrugs in ADEPT. *Tetrahedron Lett*. **1995**, *36*, 1701−1704. (b) Houba, P. H. J.; Leenders, R. G. G.; Boven, E.; Scheeren, J. W.; Pinedo, H. M.; Haisma, H. J. Characterization of Novel Anthracycline Prodrugs Activated by Human *β*-Glucuronidase for Use in Antibody-Directed Enzyme Prodrug Therapy. *Biochem. Pharmacol*. **1996**, *52*, 455−463.

(13) Nagele, E.; Schelhaas, M.; Kuder, N.; Waldmann, H. Chemoenzymatic Synthesis of N−Ras Lipopeptides. *J. Am. Chem. Soc*. **1998**, *120*, 6889−6902.

(14) Trouet, A. Increased Selectivity of Drugs by Linking to Carriers. *Eur. J. Cancer* **1978**, *14*, 105−111.

(15) Gardner, C. R. Potential and Limitations of Drug Targeting: An Overview. *Biomaterials* **1985**, *6*, 153−160.

(16) Maeda, H.; Seymour, L. W.; Miyamoto, Y. Conjugates of Anticancer Agents and Polymers: Advantages of Macromolecular Therapeutics in Vivo. *Bioconjugate Chem*. **1992**, *3*, 351−362.

(17) Jungheim, L. N.; Shepard, T. A. Design of Antitumor Prodrugs: Substrates for Antibody Targeted Enzymes. *Chem. Rev*. **1994**, *94*, 1553−1566.

(18) Kearny, A. S. Prodrugs and Targeted Drug Delivery. *Adv. Drug Delivery Rev*. **1996**, *19*, 225−239.

(19) Greenwald, R. B. Drug Delivery Systems: Anticancer Prodrugs and Their Polymeric Conjugates. *Exp. Opin. Ther. Patents* **1997**, *7*, 601−609.

(20) Denny, W. A.; Wilson, W. R. The Design of Selectively-Activated Anti-Cancer Prodrugs for Use in Antibody-Directed and Gene-Directed Enzyme-Prodrug Therapies. *J. Pharm. Pharmacol*. **1998**, *50*, 387−394.

(21) Chakravarty, P. K.; Carl, P. L.; Weber, M. J.; Katzenellenbogen, J. A. Plasmin-Activated Prodrugs for Cancer Chemotherapy. 2. Synthesis and Biological Activity of Peptidyl Derivatives of Doxorubicin. *J. Med. Chem*. **1983**, *26*, 638−644 and references cited.

(22) Greenwald, R. B.; Gilbert, C. W.; Pendri, A.; Conover, C. D.; Xia, J.; Martinez, A. Drug Delivery Systems: Water Soluble Taxol 2′-Poly(ethylene glycol) Ester Prodrugs-Design and In Vivo Effectiveness. *J. Med. Chem*. **1996**, *39*, 424−431.

(23) Greenwald, R. B.; Pendri, A.; Conover, C.; 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.

(24) Greenwald, R. B.; Pendri, A.; Martinez, A.; Gilbert, C.; Bradley, P. PEG Thiazolidine-2-thione, a Novel Reagent for Facile Protein Modification of Bovine Hemoglobin. *Bioconjugate Chem*. **1996**, *7*, 638−641.

(25) Plusquella, D.; Boczko, K. Sugar Chemistry Without Protecting Groups: A Novel Regioselective Synthesis of 6-O−Acyl-D-Glucopyranoses And Methyl-6-α-D-Glucopyranosides. *Tetrahedron Lett*. **1987**, *28*, 3809−3812.

(26) Yamada, S. Highly Selective Acylation of Di- and Polyhydroxyl Compounds by 3-Acylthiazolidine-2-thiones. *J. Org. Chem*. **1992**, *57*, 1591−1592.

(27) Greenwald, R. B.; Pendri, A.; Bolikal, D. Highly Water Soluble Taxol Derivatives: 7-Polyethylene Glycol Carbamates and Carbonates. *J. Org. Chem*. **1995**, *60*, 331−336.

(28) More detailed chemical and biological experimental results will be presented in a subsequent publication.

(29) Miron, T.; Wilchek, M. A. Simplified Method for the Preparation of Succinimidyl Carbonate Polyethylene Glycol for Coupling to Proteins. *Bioconjugate Chem*. **1993**, *4*, 568−569.

(30) Rose, W. C. Evaluation of Madison 109 Lung Carcinoma as a Model for Screening Antitumor Drugs. *Cancer Treat. Rep*. **1981**, *65*, 299−312.

(31) Greenwald, R. B.; Pendri, A.; Conover, C. D.; Lee, C.; Choe, Y. H.; Gilbert, C.; Martinez, A.; Xia, J.; Wu, D.; Hsue, M. Camptothecin-20-PEG Ester Transport Forms: The Effect of Spacer Groups on Antitumor Activity. *Bioorg. Med. Chem*. **1998**, *6*, 551−562.

(32) Murakami, Y.; Tabata, Y.; Ikada, Y. Tumor Accumulation of Poly (Ethylene Glycol) with Different Molecular Weights After Intravenous Injection. *Drug Delivery* **1997**, *4*, 23−31.

(33) Kopecek, J. Controlled Biodegradability of Polymers-a Key to Drug Delivery Systems. *Biomaterials* **1984**, *5*, 119−125 and references cited.

(34) Bolton, J. L.; Turnipseed, S. B.; Thompson, J. A. Influence of Quinone Methide Reactivity on the Alkylation of Thiol and Amino Groups in Proteins: Studies Utilizing Amino Acids and Peptide Models. *Chem.-Biol. Interact*. **1997**, *107*, 185−200.

(35) Corbett, T. T.; et al. Discovery of Solid Tumor Active Agents Using a Soft-Agar-Colony Formation Disk-Diffusion-Assay. In *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development*; Valeriote, F., Corbett, T., Baker, L., Eds.; Kluwer Academic: Boston, 1992; pp 35−87.

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