Drug Delivery Systems: Water Soluble Taxol 2′-Poly(ethylene glycol) Ester Prodrugs—Design and *in Vivo* Effectiveness

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Water soluble 2′-taxol poly(ethylene glycol) (PEG) esters have been synthesized and shown to function *in vitro* as prodrugs. However, *in vivo* experiments clearly establish that in order for these prodrugs to behave in a predictable fashion, the molecular weight of PEG must be of such magnitude so as to maintain a $t_{1/2}$(circulation) > $t_{1/2}$(hydrolysis). When PEG derivatives of molecular weight ∼40 kDa were employed with paclitaxel, ca. 4% by weight of paclitaxel was carried by the water soluble prodrug form, and equivalent *in vivo* toxicity and increased life expectancy in the P388-treated mouse was observed. An effective method for prescreening prodrugs was found to be the acute murine lethality, which reflects the equivalency of the solubilized transport form and the native drug.

Introduction

The insoluble nature of paclitaxel, one of the most potent chemotherapeutic agents used in the treatment of breast and ovarian cancers, has led to the formulation of this drug as a 1:1 ethanol:cremophore concentrate which is diluted prior to lengthy infusion. Unfortunately, various hypersensitive reactions have been found in patients undergoing paclitaxel treatment. It is unclear at this time as to what extent cremophore is responsible for these side effects, but similar hypersensitivity reactions in dogs have been attributed to histamine release by cremophore EL.[1] These side effects can be ameliorated by employing antihistaminic drugs such as dexamethasone and diphenhydramine, but the inescapable fact is that this protocol results in additional medication, discomfort, and cost to the patient. Taxotere, reportedly a highly potent semisynthetic modified taxane, has only marginally increased aqueous solubility[2d] but can be formulated with the more innocuous excipient Tween 80. This formulated drug has not yet been approved by the FDA for use in the United States.

To date a great deal of chemical research has gone into the modification of paclitaxel in order to create a more soluble and, therefore, a more easily formulated and delivered drug. Numerous approaches have been considered, but prodrug synthesis[2] seems to be the most studied and probably represents the molecular design closest to providing a utilitarian solution to the aqueous solubilization and reformulation of paclitaxel.

Prodrug strategies consist of transient modification of the physicochemical properties of a given compound through chemical derivatization. Such temporary chemical modification is usually designed to alter aqueous solubility and biodistribution while the inherent pharmacological properties of the parent drug remain intact.[3] Prodrugs, or transport forms[4] as they are sometimes referred to, can be designed to reliably function, i.e., to self-destruct in a predictable fashion, *in vivo*, to the active drug by either an enzymatic mechanism or simple hydrolysis initiated under physiological pH conditions, once the barrier to delivery has been circumvented. In his initial investigation of paclitaxel prodrugs, Nicolaou et al.[5] embraced a hydrolytic approach and prepared a series of 2′-taxol esters with electron-withdrawing substituents in the α-position which were reported to have half-lives of >8 h at approximately physiological pH. These prodrugs also demonstrated cellular toxicity similar to paclitaxel but had only modestly increased aqueous solubility. Subsequently, Nicolaou et al. developed a new type of prodrug based on an enzymatic catalyzed hydrolysis of an onium salt (taxol 2′-methylpyridinium acetate, taxol 2′-MPA) which was elegant in its simplicity.[6] Taxol 2′-MPA demonstrated a meaningful water solubility of 1.5 mM, and *in vivo* testing, in a mouse model using a human prostate carcinoma xenograft, produced equivalent results to paclitaxel. The most unusual finding in this report was the apparent lack of toxicity of the prodrug when administered at the maximum tolerated dose (MTD) for paclitaxel.

A general approach to the controlled release of drugs based on enzymatic cleavage has been explored extensively by Kopecek et al.,[7] utilizes *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers containing oligopeptide side chains terminated in anticancer drugs,[8] and has recently been extended to paclitaxel.[9] *In vivo* data for this paclitaxel−copolymer employing a solid tumor model (murine melanoma B16F10) was also reported to show greater efficacy than that of a paclitaxel control.

Thus, two different water soluble prodrugs of paclitaxel, both employing enzymatic cleavage as their mode of activation, have been synthesized and tested *in vivo* in two different murine models. Both appear to possess at least equivalent activity to paclitaxel. We have approached the problem of paclitaxel transport forms (prodrugs) primarily from a hydrolytic perspective, i.e., a breakdown based on pH and not enzymatic participation. Since the linking moiety that we employed is an ester, it was anticipated that substantial rate enhancement due to nonspecific esterases would nonetheless occur *in vivo* and add to the effective use of the prodrug. We wish to report on the development of an alternate technology, poly(ethylene glycol) (PEG) conjugation, for solubilizing and delivering paclitaxel. This technology can also be applied to other highly insoluble oncolytic agents, and we are currently pursuing this course of

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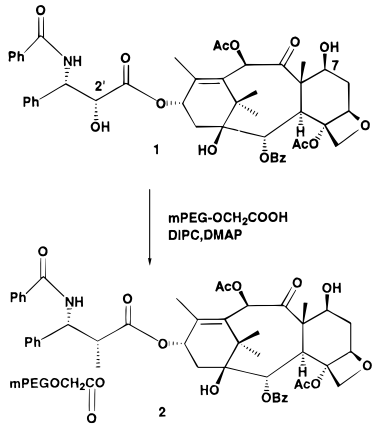
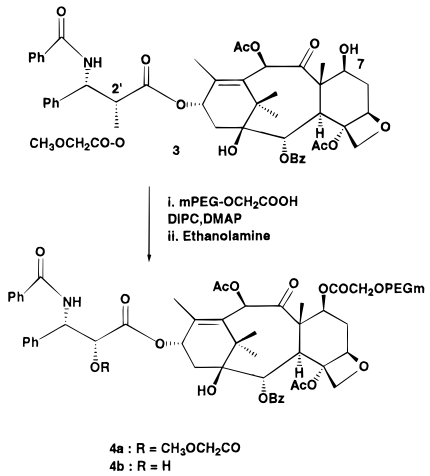
Scheme 1



Scheme 2



research in order to further demonstrate the utility of PEG in prodrug strategies.

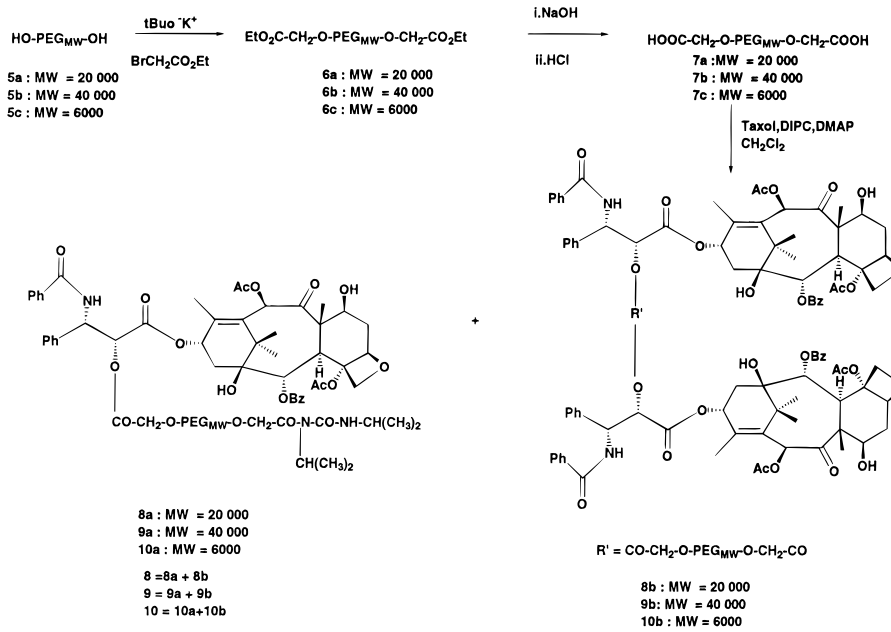
Chemistry

The use of esters as prodrugs has been extensively employed for modifying biologically active molecules containing either hydroxyl or carboxyl functionalities.[3,4] The simplicity of synthesis coupled with facile enzymatic hydrolysis dictates esters as a first choice when considering prodrug strategies. Earlier studies of 2′-taxol esters as prodrugs demonstrated that esters without α-substituents hydrolyzed only to the extent of 10% over a 24 h period,[10] while those with electron-withdrawing substituents in the α-position showed remarkable enhancements in their rates of hydrolysis.[2c,5,11] We recently utilized the features of methoxyacetate (MAc) esters[12] (stable in water and dilute acid, unstable to base) to block the 2′-OH group of paclitaxel and rationalized that replacement of methoxy by the amphiphilic macromolecule PEG would lead to a water soluble paclitaxel ester that would be useful as a prodrug by virtue of its facile cleavage. We described the results of this substitution in an earlier communication[11] and now wish to report on these findings in more detail.

The esters used in this study (**2**, **4b**, **8**, and **9**) were prepared by the condensation of the appropriate acid with paclitaxel (or 2′-MAc taxol, **3**) in methylene chloride employing diisopropylcarbodiimide (DIPC) as condensing agent and (dimethylamino)pyridine (DMAP) as base (Schemes 1 and 2). The synthesis of taxol 7-PEG ester (**4b**) was accomplished employing the MAc protecting group at the 2′-position and illustrates the diminished reactivity of the more hindered taxol 7-ester since treatment of **4a** with ethanolamine removed only the MAc group. In PBS buffer (pH 7.4) and rat plasma, the 7-PEG ester **4b** had $t_{1/2}$(hydrolysis) > 400 h and therefore was not considered to be a practical prodrug candidate.

When the molecular weight of the polymer was ≥20 kDa, bifunctional PEG was employed in order to increase the loading of paclitaxel in the prodrug. The diacids were conveniently prepared from the diols and ethyl bromoacetate in the presence of base according to published procedures.[13] Condensation of PEG dicarboxylic acids **7a**,**b** with paclitaxel in the presence of DIPC and DMAP led to the diesters **8b** and **9b** in addition to small amounts (usually 10−15%) of the monoesters **8a** and **9a** terminated on one end with the ureido moiety (Scheme 3). The structural assignment of the ureido group was arrived at by using lower molecular weight models in order to obtain more clearly defined NMR peaks to which assignments could be made.[14a] As a model, we chose molecular weight 6 kDa PEG diacid **7c** in the condensation reaction with paclitaxel to give compound **10** (Scheme 3). In addition to all the predicted peaks, the presence of an *N*,*N*′-diisopropylureido moiety could clearly be observed in the ^{13}C NMR spectrum of compound **10**: 169.8 (*CO*-N-CH(CH$_3$)$_2$), 152.7 (CO-N-*CO*-NH-CH(CH$_3$)$_2$, 47.0 (CO-N-*CH*(CH$_3$)$_2$), 42.0 (CO-N-CO-NH-*CH*(CH$_3$)$_2$), 22.0 (CO-N-CH(*CH*$_3$)$_2$), 20.0 (CO-NH-CH(*CH*$_3$)$_2$) ppm. The same peaks are present but cannot be distinguished as easily in the higher molecular weight compounds **8** and **9**. Similarly the ^{1}H NMR of **10** displays resonances at δ 1.19, 1.21 (2s, CO-N-CO-NH-CH(CH$_3$)$_2$), 1.39, 1.42 (CO-N-CH(CH$_3$)$_2$, 3.92−4.18 (N-*CH*(CH$_3$)$_2$). In the case of compounds **8** and **9**, resonances at δ 1.19, 1.21, 1.39, and 1.42 can also be observed but not as major peaks. Finally, to confirm the structure of the ureido moiety, we prepared the 6 kDa PEG diureido compound **11** by treating **7c** at room temperature with DIPC/DMAP in methylene chloride in the absence of paclitaxel (Scheme 4). Under these conditions all of the diacid **7c** was completely converted to the rearranged product **11** with ureido moieties on both ends. Resonances in both the ^{13}C and ^{1}H NMR spectra of **11** matched the minor peaks observed in the spectra of **8**−**10**. Apparently intramolecular rearrangement of the intermediate PEG-*O*-acylisourea[14b] derivative occurred at a sufficiently rapid rate to compete with the desired esterification of the paclitaxel and produced the mono-PEG-*N*-acylureas **8a**, **9a**, and **10a** with PEG diacids but, interestingly, only to a very minor extent during the synthesis of **2** which employs a monoacid (mPEG 5 kDa acid). The determination of percent paclitaxel contained in the product was based on UV absorbance values as described in the Experimental Section. For **9**, a value of 3.8 ± 0.1% was obtained. Theoretically a value of 4% is expected for a diester. Therefore **9** must consist of a mixture of monoester **9a** and diester **9b** in an approximate ratio of 1:9. Separation of **9a** from the diester **9b** proved to be difficult as HPLC could not differentiate the two compounds. Therefore for purposes of determining rates of hydrolysis, solubility, and *in vitro* and *in vivo* testing, the mixture was used as such and the ureido moiety was simply ignored since it is biologically inert.

Scheme 3



Scheme 4

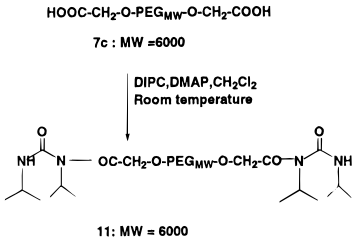


Table 1. Aqueous Solubility of Taxol 2′-PEG Esters

compd	MW of PEG	solubility[a] (mg/mL)	wt % of paclitaxel calcd	wt % of paclitaxel obsd
2	5000	660	14.5	14.5
8	20 000	200	7.8	7.4
9	40 000	125	4	3.8
10	6000	ND	22	21

[a] Solubility was determined as previously reported.[12] ND = not determined.

Table 2. Rates of Hydrolysis[a] of Taxol-2′-PEG Esters **2**, **8**, and **9** in Various Media at 37 °C

media	pH	$T_{1/2}$ (h)
distilled water	5.7	>72
PBS buffer	5.8	>50
	7.0	15
	7.4	5.5
rat plasma		0.5
human plasma		1
human whole blood		2.0

[a] All runs done in duplicate. Limit error is ±10%.

Taxol 2′-PEG esters **2**, **8**, and **9**, regardless of molecular weight, exhibited the same half-life (Table 2). Although it has been shown that faster rates of hydrolysis in buffer could be obtained by varying the α-substituent,[11] no significant advantage could be justified by employing them for the present *in vivo* study. In fact, $t_{1/2}$(hydrolysis) for **9** was found to be 2.0 h in whole human blood, while in deionized water it was >72 h (Figure 2). These results clearly demonstrate that **9** can be formulated prior to injection by dissolution in sterile water without concern for any appreciable degree of hydrolysis occurring.

Table 3. *In Vitro* Cytotoxicities of Paclitaxel and PEG-taxols against P388/O and L1210/O Leukemias

compd	IC_{50} (nM) P388/O	IC_{50} (nM) L1210/O
paclitaxel	6	6
2	15	17
8	17	14
9	10	16
12	3100	ND

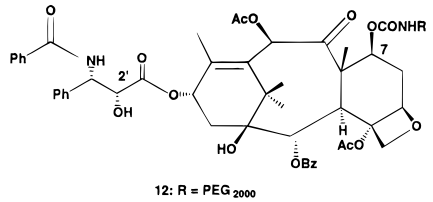


Figure 1.

Results

***In Vitro* Cytotoxicity of PEG-taxols.** The *in vitro* biological efficacy of the soluble PEG-taxols was tested using murine leukemia cell lines P388/O and L1210/O. The cytotoxicities of paclitaxel and the prodrug PEG-taxols are shown in Table 3. The IC_{50} for paclitaxel was 6 nM in both cell lines (P388/O and L1210) which is within the reported range for paclitaxel,[2c,15] while the IC_{50}'s for the prodrugs (compounds **2**, **8**, and **9**) were between 10 and 17 nM. These values are virtually equivalent to that for the native drug, indicating that paclitaxel is being released into the medium. For PEG-carbamate **12**[12] (Figure 1) (a stable derivative of paclitaxel), the IC_{50} was found to be about 3100 nM.

***In Vivo* P388/O Leukemia Screen.** Since all of the paclitaxel 2′-PEG esters exhibit *in vitro* cytotoxicity activities very similar to paclitaxel, excellent solubility properties (≥125 mg/mL), and hydrolysis rates of ca. 0.5 hours in rat plasma, we selected a candidate (compound **2**) to test in an *in vivo* efficacy screen. The unexpected

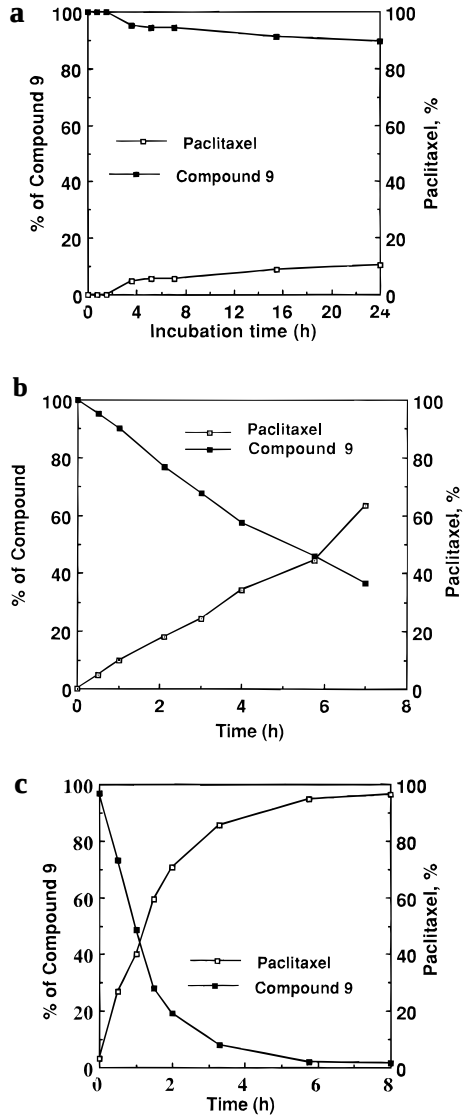


Figure 2. (a) Kinetics of hydrolysis of compound **9** in sterile water, 37 °C (pH 5.7). Compound **9** (□) was incubated in the presence of sterile water, and the concentration of compound **9** and paclitaxel (■) was determined at the time points indicated using HPLC. (b) Kinetics of hydrolysis of compound **9** in PBS buffer, 37 °C, pH 7.4. Compound **9** (□) was incubated in the presence of PBS buffer, and the concentration of compound **9** and paclitaxel (■) was determined at the time points indicated using HPLC. (c) Kinetics of hydrolysis of compound **9** in fresh human plasma, 37 °C. Compound **9** (□) was incubated in the presence of fresh human plasma, and the concentration of compound **9** and paclitaxel (■) was determined at the time points indicated using HPLC.

results of this experiment are shown in Table 4. The mean time to death for the paclitaxel group at 1.75 μmol was 18.7 days, while for an equivalent dose of prodrug **2** it was 14.1 days (T/C ratio = 1.13). A 3 × dose (5.25 μmol) of prodrug **2** only increased the mean time to death to 15.7 days, while an equivalent dose of paclitaxel was toxic (mean time to death = 6.7 days). A second experiment was performed using prodrug **9** as the test substance. The dosing range results of the second experiment are also shown in Table 4. The mean time to death for paclitaxel in this experiment was 17.4 days at the 1.75 μmol dose, while the mean time to death for compound **9** was 18.3 days at the same dose. At a dose of 2.63 μmol of compound **9**, the mean time to death was extended to 20.2 days. At the 3 × dose (5.25 μmol/mouse), compound **9** was as toxic as paclitaxel with the mean time to death being 6.3 and 6.7 days, respectively. The T/C ratio for compound **9** is essentially the same as that for paclitaxel for the 1.75 μmol dose of drug, making this prodrug form of paclitaxel (compound **9**) equivalent to the native drug.

Murine Acute Lethality Studies. To investigate the role that PEG molecular weight and circulating half-life play, as it effects drug delivery and efficacy, a murine acute lethality experiment was performed. A single dose of either paclitaxel or the molar equivalent of the prodrugs (compounds **2**, **8**, and **9**) was administered to mice in order to determine the lethal dose. The results are presented in Table 5. Compound **2** (5 kDa) is not lethal at 10 μmol/mouse, while compound **9** (40 kDa) is lethal at 10 μmol/mouse, as is native paclitaxel. At intermediate PEG molecular weights (20 kDa), prodrug **8** shows an intermediate acute lethality profile (50% lethality at 10 μmol/mouse but 0% lethality at 5 μmol/mouse).

Discussion

It is generally accepted that the greatest nonimmunogenicity, solubility, and circulating half-life of PEG-conjugated proteins is achieved by employing polymers in the molecular weight range of 2−12 kDa.[16] In fact, two PEG-conjugated enzymes which have been commercialized, Adagen and Oncaspar, both employ PEG 5 kDa. Essentially complete solubilization of paclitaxel has also been accomplished by using PEG 2−5 kDa to form stable carbamates in the 7-position.[12] Thus in our earlier communication on PEG prodrugs,[11] all of the examples described utilized PEG of molecular weight 5 kDa and the *in vitro* cytotoxicity of PEG prodrug **2** was shown to be essentially equivalent to paclitaxel. We were therefore surprised to find no acute toxicity exhibited in the mouse when treated with **2** at a dose of 5.25 μmol since paclitaxel at this dose was profoundly toxic. Subsequently, carbamate **12**[12] (which exhibited an IC_{50} of 3.1 μM)[17] was examined at a level of 10 μmol/mouse and also found to be nontoxic. The half-life of hydrolysis for prodrug **2**, at physiological pH 7.2−7.4, has been determined to be 5−6 h, while in rat plasma it was 0.5 h. In order for **2** not to exhibit paclitaxel toxicity, it stands to reason that the prodrug must have been excreted prior to its timed breakdown. Similar findings for other paclitaxel prodrugs have also been reported[6] and thus led to the realization that a dichotomy exists when considering this class of water soluble macromolecular prodrug.

Generally, the two opposing elements of the hydrophilicity of the prodrug and the hydrophobicity of the parent drug are addressed through a linking moiety, which is usually designed in such a way as to enhance *in vivo* efficacy by accelerating the rate of conversion of one form to the other. This is expressed by the following relationship:

$$ED_{50}(\text{prodrug}) = ED_{50}(\text{drug}), \quad \text{when } t_{1/2}(\text{hydrolysis}) > t_{1/2}(\text{elimination}) \quad (1)$$

However, in addition to this approach, an alternative solution to the problem of prodrug efficacy would be to extend the circulating lifetime of the water soluble modification. *By increasing the circulating life of the prodrug in plasma relative to its rate of hydrolysis,*

Table 4. Activity of Paclitaxel and Compounds **2** and **9** against P388 Leukemia *in vivo*

group	dose/day (μmol)	total dose (μmol)	mean time to death (days ± SD)	T/C[d]	ILS (%)[e]	p values[d] compared to control (untreated)	paclitaxel (1.75 μmol)
			experiment 1[b]				
control			12.5 ± 0.8				
paclitaxel	0.35	1.75	18.7 ± 1.3	1.50	50	$p < 0.001$	
	1.05	5.25	6.7 ± 1.4	0.54	−46	$p < 0.001$	$p < 0.001$
compound **2**	0.35	1.75	14.1 ± 2.3	1.13	13	$p < 0.06$	$p < 0.001$
	1.05	5.25	15.7 ± 2.1	1.26	26	$p < 0.001$	$p < 0.001$
			experiment 2[c]				
control			14.5 ± 1.8				
paclitaxel	0.35	1.75	17.4 ± 3.3	1.20	20	$p < 0.001$	
compound **9**	0.09	0.44	16.4 ± 1.3	1.13	13	$p < 0.001$	$p = 0.001$
	0.18	0.88	16.6 ± 1.1	1.13	13	$p < 0.001$	$p = 0.002$
	0.35	1.75	18.3 ± 3.6	1.26	26	$p < 0.001$	$p = 0.02$
	0.53	2.63	20.2 ± 4.8	1.39	39	$p < 0.001$	$p < 0.001$
	0.70	3.50	11.3 ± 7.9	0.78	−22	$p < 0.001$	$p < 0.001$
	1.05	5.25	6.3 ± 0.5	0.43	−57	$p < 0.001$	$p < 0.001$

[a] *t*-Test: two sample assuming equal variances. [b] $N = 10$ mice/group. [c] $N = 20$ mice/group. [d] T/C is mean survival time of treated group/mean survival time of the control group. [e] ILS (%) = (T/C − 1) × 100.

Table 5. *In Vivo* Acute Lethality of Paclitaxel and PEG-taxols as Expressed by Percent Toxicity (Deaths)

	dose (μmol/mouse)	
compd	5	10
paclitaxel	25%	100%
2	0%	0%
8	0%	50%
9	50%	100%
10	0%	0%

equivalent potency should result. This statement can be summarized as:

$$ED_{50}(\text{prodrug}) = ED_{50}(\text{drug}), \quad \text{when } t_{1/2}\,(\text{circulation}) > t_{1/2}(\text{hydrolysis}) \quad (2)$$

Consequently, eq 2 strongly suggests that if a means of prolonging the circulating half-life of the prodrug can be accomplished, then linking moieties of greater stability, which have previously not been considered useful, can be employed in constructing prodrugs. One way to accomplish this objective is to prevent rapid excretion of the hydrophilic form of the drug through the kidneys by increasing the molecular weight of the solubilizing agent. It has long been recognized that for dendritic polymer−low molecular weight drug conjugates the biodistribution of the polymer alone will determine the fate of the conjugate.[18] An informative report[19] on the effect of molecular weight of HPMA copolymers on body distribution and rate of excretion identified a molecular weight threshold-limiting glomular filtration to 45 kDa; below this limit the half-life of the polymer was quite short, e.g., $t_{1/2}$(circulation) for a 12 kDa copolymer was reported to be only 3 min. To date, few investigations have reported on the biodistribution of PEG in spite of its extensive use as a polymeric modifier of protein drugs. Recently, however, a detailed study by Ikada[20] on the distribution and tissue uptake of PEG of different molecular weights after intravenous administration to mice provided the necessary information to determine how to achieve the conditions stated in eq 2. In the mouse, Ikada found that the renal clearance of PEG decreased with an increase in molecular weight, with the most dramatic change occurring at 30 kDa. The half-life of PEG circulating in blood also showed a concomitant and dramatic increase. For instance, $t_{1/2}$(circulation) for PEG went from ca. 18 min to 16.5 h as the molecular weight increased from 6 to 50 kDa. In fact, a recent report[20b] on the PEGylation of dipeptide thrombin inhibitors has not only demonstrated that conjugation with PEG extends the $t_{1/2}$ of circulation but that $t_{1/2}$ increases as the molecular weight of PEG is increased. We tested the validity of relationship 2 by preparing a prodrug of paclitaxel and PEG of molecular weight 40 kDa which has an estimated $t_{1/2}$(circulation) = 8−9 h. Since the rate of hydrolysis of the ester linkage is the same for all the prodrugs, a clear distinction can now be made as to whether or not relationship 2 is accurate using as the criterion the acute murine lethality for **2** and **9** as compared to paclitaxel. The results, presented in Table 5, clearly demonstrate an apparent greater lethality of the PEG 40 kDa prodrug and thus confirms eq 2. It can also be appreciated that for PEG 20 kDa with an estimated $t_{1/2}$(circulation) of 2.8 h, the acute lethality will probably be borderline. Experimental evidence produced a murine lethality for **8** which was one-half that of **9**, the 40 kDa prodrug, at 10 μmol, and at a dose of 5 μmol no deaths were observed. On the basis of these findings, the results obtained[6] for taxol 2-MPA appear to be in question. This prodrug was reported to completely revert to taxol in <10 min when incubated at 37 °C with human plasma but purportedly showed no visible murine toxicity at paclitaxel's MTD. One possible explanation for this observation is that some of the water soluble paclitaxel 2-MPA was excreted prior to its breakdown and thus resulted in less than the MTD of the prodrug being present: i.e., relationship (1) has not been met.

Extrapolation of results obtained *in vitro* to events expected to occur *in vivo* can be misleading since mechanisms for distribution and metabolism of polymeric drugs are generally missing from most *in vitro* tests. It is therefore not surprising that a poor correlation often exists between *in vitro* and *in vivo* results.[22] The fate of water soluble polymers in the body, a key aspect of drug delivery, must be addressed if the design of PEG drugs is to be successful. By choosing the appropriate molecular weight for PEG, a prodrug of paclitaxel was produced that is as efficacious as paclitaxel/cremophor EL/ethanol in an *in vivo* model. In fact **9** may possibly be more potent than the native drug (Table 4).

Another interesting and useful ramification of our observations is that if PEG is employed simply as a

substituent with a low molecular weight drug, i.e., in a permanently bonded fashion, then the PEG−drug species, even if it manifests *in vitro* activity, will not show *in vivo* activity unless the molecular weight of the PEG is sufficiently high enough to prevent the rapid excretion associated with lower molecular weight polymers (*supra vide*, carbamate **12**). Earlier PEG−organic drug disclosures[23] did not recognize this important feature of drug design, and therefore PEG−organic drugs, where there is usually only one site considered for PEGylation, generally show no efficacy.[24] Ostensibly, employing polymer of molecular weight 5 kDa produces a rapidly excreted species which can be erroneously interpreted as inactive. On the other hand, development of PEG−protein drugs has benefited from conjugation with PEG 5 kDa since multiple attachment sites increase the effective molecular weight and elimination of the PEGylated protein is virtually halted, thus leading to a long circulating life and enhanced pharmacokinetics.[16] It has been claimed[25] that conjugation of superoxide dismutase with only one to four strands of higher molecular weight PEG leads to a product with longer circulating life, less immunogenicity, and 90−100% of the native enzyme's activity. Similar findings for PEG interleukin-2[26a] also underline the importance of considering PEG molecular weight in rational drug design.

Conclusion

A prodrug strategy employing PEG as a solubilizing agent has been successfully demonstrated in the case of paclitaxel. Thus, prodrug **9** simply dissolved in water delivers paclitaxel as effectively *in vivo* as the current cremophor EL formulation. Esters with PEG as an electron-withdrawing group in the α-position are especially effective linking groups to use in the design of the prodrug. *In vivo* testing clearly illustrates the importance of the molecular weight of the solubilizing group being employed and underlines the necessity of animal testing to verify *in vitro* cytotoxicity results as has been previously suggested.[3,22] This can be seen in the case of prodrug **2** (5 kDa) where, *in vivo*, a 3 × dose of prodrug still lacks the toxicity of a 1 × dose of cremophor EL-formulated paclitaxel, although both compounds are equally toxic *in vitro*. Application of PEG to either drugs or prodrugs mandates the use of polymer with a molecular weight of ≥30 kDa in order to prevent rapid elimination of the PEGylated species. PEG properly employed, i.e., as a transport form conjugated to insoluble organic drugs, appears to offer an effective methodology for drug delivery.

Experimental Section

General Methods. Unless stated otherwise, all reagents and solvents were used without further purification. Analytical HPLC's were performed using a C_8 reverse phase column (Beckman, ultrasphere) under isocratic conditions with a 75:25 mixture (v/v) of methanol−water as the mobile phase. Peak elutions were monitored at 227 nm using a UV detector.[27] To detect the presence of any free PEG acid and also to confirm the presence of PEGylated product, an evaporative light-scattering detector (ELSD), Model PL-EMD 950 (Polymer Laboratories), was employed.[28] Based on ELSD analysis, products 8 and 9 did not contain any free PEG acid as contaminants. NMR spectra were obtained using a 270 MHz spectrometer. Deuterated chloroform was used as the solvent unless otherwise specified. Paclitaxel was supplied by PhytoPharmaceuticals, Inc. mPEG (α-hydroxy-ω-methypoly(oxy-1,2-ethanediyl); 5 kDa) was obtained from NOF America (New York, NY). PEG diols (α-hydro-ω-hydroxypoly(oxy-1,2-ethanediyl); 20 and 40 kDa were obtained from Serva (Crescent Chemical Co., NY), and PEG diol 6 kDa was obtained from Union Carbide Corp. PBS buffer was purchased from Sigma Chemical Co. All PEG compounds were dried under vacuum or by azeotropic distillation from toluene prior to use.

Abbreviations: MTD, maximum tolerated dose; PEG, poly(ethylene glycol); mPEG, monomethyl ether of PEG; MAc, methoxyacetate; DIPC, diisopropylcarbodiimide; DMAP, dimethylamino)pyridine; WFI, water for injection; ELSD, evaporative light-scattering detector.

Cell Lines and Cytotoxicity Assays. P388/O and L1210/O cell lines were obtained from Southern Research Institute (Birmingham, AL). These lines were grown in RPMI 1640 supplemented with 10% FBS and subcultured 2−3 times/week. All lines were tested for *Mycoplasma* periodically and were *Mycoplasma* free. Assays to determine the cytotoxicity of paclitaxel derivatives were performed using growth medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), fungizone (0.25 μg/mL), and gentamicin (50 μg/mL). Samples of the PEG prodrugs were dissolved in WFI quality water (Baxter Healthcare Corp., Deerfield, IL). Control paclitaxel was dissolved in DMSO and diluted into tissue culture medium before assay. Sequential serial 1:2 dilutions of each sample were prepared to a final volume of 50 μL/well in 96-well plates. 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.[29] The IC_{50} values for each compound were determined from absorbance versus dilution factor plots.

Murine Acute Lethality Studies. Acute lethality studies (single-dose intraperitoneal (ip) administration) were performed in groups of non-tumor-bearing CD2F1 female mice (Taconic, Germantown, NY) 10−12 weeks old. Mice were monitored for survival up to 7 days. Paclitaxel was suspended in ethanol:cremophor EL:water,[30] and PEG prodrugs were solubilized in sterile water immediately before injection. The doses of PEG-taxols were chosen to be in the range of published paclitaxel lethalities[1,31] (5−10 μmol).

***In Vivo* P388/O Leukemia Screen.** Paclitaxel and prodrug forms of PEG-taxols were screened for *in vivo* activity against the murine leukemia cell line P388/O.[32] All paclitaxel derivatives were administered ip as aqueous solutions. The protocol was as follows: Female CD2F1 mice (10 or 20/group) were implanted ip with P388/O cells (5×10^5 cells/mouse) on day 0. The mice were then treated with paclitaxel (0.35 μmol/mouse/day) or the PEG prodrug for five consecutive days (days 1−5). Control groups received no treatment or vehicle (ethanol:cremophore EL:water). The mice were monitored daily for survival which was expressed as ILS (increased life span). Both the murine acute lethality study and the *in vivo* P388/O leukemia screen animal protocols were approved by IACUC boards.

Taxol 2′-mPEG Ester (2). mPEG acid (5 kDa)[13] (625 mg, 0.125 mmol) was dissolved in 20 mL of anhydrous methylene chloride, and to this solution at 0 °C were added DIPC (26 μL, 0.17 mmol), paclitaxel (146 mg, 0.17 mmol), and DMAP (32 mg, 0.26 mmol). The resulting solution was allowed to warm to room temperature and left for 16 h. The reaction mixture was washed with 0.1 N HCl, dried, and evaporated *in vacuo* to yield the product as a white solid which was crystallized from 2-propanol (585 mg, 80%). ^{13}C and ^1H NMR confirmed the structure.

Taxol 7-PEG Ester (4b). mPEG (5 kDa) acid (625 mg, 0.125 mmol) was dissolved in 20 mL of anhydrous methylene chloride, and to this solution at 0 °C were added DIPC (26 μL, 0.17 mmol), 2′MAc taxol (**3**)[12] (156 mg, 0.17 mmol), and DMAP (32 mg, 0.26 mmol). The reaction was carried out as described for **2**. The product obtained was dissolved in 2-propanol (10 mL) followed by the addition of 20 μL (0.32 mmol) of ethanolamine. The resulting solution was refluxed for 16 h and cooled to room temperature. The solid separated was filtered, washed

with ether, and recrystallized from 2-propanol to give 500 mg (73%) of 7-ester **4b** whose structure was confirmed by the NMR spectra.

PEG (40 kDa) Dicarboxylic Acid 7b. A solution of PEG diol **5b** (50 g, 1.3 mmol) in toluene (750 mL) was azeotroped with the removal of 150 mL of distillate. The reaction mixture was cooled to 30 °C followed by the addition of 1 M potassium *tert*-butoxide in *tert-butyl* alcohol (4 mL, 4.0 mmol). The resulting mixture was stirred for 1 h at room temperature followed by the addition of ethyl bromoacetate (1.6 mL, 14 mmol). The solution was heated to reflux and then stirred at room temperature for 18 h. The reaction mixture was filtered through Celite, and the solvent was removed *in vacuo*. The residue was recrystallized from methylene chloride/ether to give the PEG (40 kDa) diethyl ester **6b** (45.2 g, 86%). ^{13}C NMR: C═O, 170.9 ppm.

A solution of **6b** (20 g, 0.5 mmol), in 1 N sodium hydroxide (100 mL) was stirred at room temperature for 4 h. The basic solution was cooled in an ice bath, adjusted to pH 3.0 with 2 N HCl, and extracted three times with methylene chloride. The pooled extracts were washed with water and concentrated to 15 mL, and the solution was added to ethyl ether (200 mL) with stirring. The precipitate was filtered, washed with ether, dried, and crystallized from 2-propanol to yield 16.9 g (84%) of **7b**.

PEG (20 kDa) Dicarboxylic Acid 7a and PEG (6 kDa) Dicarboxylic Acid 7c: prepared according to the above procedure in 84% yield starting from the PEG (20 or 6 kDa) diol **5a** or **5c**.

General Procedure for the Preparation 2′-Taxol PEG Esters 8−10. PEG diacid (0.125 mmol) was dissolved in 20 mL of anhydrous methylene chloride at room temperature, and to this solution at 0 °C were added DIPC (52.2 μL, 0.34 mmol), DMAP (64 mg, 0.523 mmol) and paclitaxel (292 mg, 0.34 mmol). 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 2-propanol. The product (80−90% yield) was diester **8b**, **9b**, or **10b** which contained about 10−15% of monoester **8a**, **9a**, or **10a**. Structures of these compounds were confirmed by ^{13}C and ^{1}H NMR analysis. The ratio of diester to monoester was determined by the UV assay as described below. Separation of the ester mixture was not attempted, as it is difficult to differentiate between PEG-derived compounds of similar molecular weights using standard chromatographic and crystallization techniques.[33] For the purposes of *in vitro* and *in vivo* testing, the mixture was used as such.

Spectral Data for Compound 10: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.1 (s), 1.19 (s), 1.21 (s), 1.26 (s), 1.39 (s), 1.42 (s), 1.75 (s), 1.8 (s), 1.9 (s), 2.2 (s), 2.24 (s), 2.51 (s), 2.54 (s), 3.39 (m), 3.59−3.71 (br s, PEG), 3.91 (s), 4.2−4.4 (m), 4.5−4.6 (m), 5.0 (d, J = 8 Hz), 5.6 (m), 5.8 (d, J = 5.4 Hz), 6 (m), 6.2−6.4 (m), 7.3 (s), 7.4−7.7 (m), 7.8 (d, J = 8.1 Hz), 8.2 (d, J = 8.1 Hz); ^{13}C NMR (270 MHz, $CDCl_3$) δ 9.40, 14.61, 20.0, 20.64, 21.92, 22.0, 22.54, 26.63, 35.37, 42.0, 42.99, 45.39, 47.0, 52.71, 58.30, 68.0, 70.38, 71.91, 74.19, 74.91, 75.39, 78.91, 80.86, 84.23, 126.72, 127.10, 128.38, 128.56, 128.88, 129.04, 130.05, 131.67, 132.61, 133.58, 136.73, 142.54, 152.7, 166.80, 167.17, 167.66, 169.65, 169.82, 170.98, 203.61.

PEG (6 kDa) Diureido Compound 11. PEG diacid **7c** (765 mg, 0.125 mmol) was dissolved in 20 mL of anhydrous methylene chloride at room temperature, and to this solution were added DIPC (52.2 μL, 0.34 mmol) and DMAP (64 mg, 0.523 mmol). The reaction mixture was stirred at room temperature 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 2-propanol. The product (**12**, 756 mg, 95% yield) was characterized by NMR analysis: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.19 (s), 1.21 (s), 1.39 (s), 1.42 (s), 2.61 (s), 3.38−4.1 (br s, m, PEG), 4.3 (s); ^{13}C NMR (270 MHz, $CDCl_3$) δ 20.0, 22.0, 42.0, 47.0, 69.8, 70.6, 152.7, 169.8.

Analysis of Taxol PEG Esters. The UV absorbance of native paclitaxel in methylene chloride was determined at 227 nm[27] for five different concentrations ranging from 4 to 21 μM. From the standard plot of absorbance vs concentration, the absorption coefficient, ϵ, for paclitaxel was calculated to be 2.96 $\times$ 10^4 mol^{-1} cm^{-1}. Taxol PEG esters were dissolved in methylene chloride at an approximate concentration of 4 μM (based on MW of 40 or 20 kDa), and the UV absorbance of these compounds at 227 nm was determined. Using this value, and employing the absorption coefficient ϵ obtained from above, the concentration of paclitaxel in the sample was determined. Thus, dividing this value by the taxol PEG ester concentration provided the percentage of paclitaxel in the esters.

Determination of Rates of Hydrolysis of Taxol PEG Esters. The rates of hydrolysis were obtained by employing a Hydropore-5-HIC column (Rainin; 4.6 $\times$ 100 mm, 5 μm particle size, 300 Å pore size, spherical silica gel, chemically bonded to poly(ethylene glycol)), using a gradient mobile phase consisting of (a) hexane/2-propanol (80/20, v/v) and (b) acetonitrile/2-propanol (60/40, v/v). A flow rate of 1 mL/min was used, and chromatograms were monitored using a UV detector at 254 nm. Taxol PEG esters were dissolved in methylene chloride at a concentration of 10 mg/mL. From this stock solution 50 μL was transferred into a 1.5 mL poly(propylene) centrifuge tube and lyophilized. To this lyophilized powder was added 100 μL of 0.1 M, pH 7.4, PBS (or rat plasma or human plasma), and the mixture was incubated at 37 °C for various periods of time. 2-Propanol (300 μL) was added at the proper intervals to precipitate the proteins, and the resulting solution was vortexed and centrifuged for 3 min. Supernatant solution (350 μL) was transferred into another vial and lyophilized to a powder. Methylene chloride (100 μL) was added to dissolve the solid, and 25 μL of this solution was injected into the HPLC. On the basis of the peak area, the amount of paclitaxel/PEG taxol was estimated, and the half-life of each compound in different media was calculated.

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Supporting Information Available: Copies of selected ^{1}H and ^{13}C NMR spectra for compounds **2**, **4b**, **7a**,**b**, and **8−11** (14 pages). Ordering information is given on any current masthead page.

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