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Design, synthesis, and evaluation of water-soluble morpholino-decorated paclitaxel prodrugs with remarkably decreased toxicity

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ABSTRACT

Novel water-soluble paclitaxel prodrugs were designed and synthesized by introducing morpholino groups through different linkers. These derivatives showed 400–20,000-times greater water solubility than paclitaxel as well as comparable activity in MCF-7 and HeLa cell lines. The prodrug PM4 was tested in the S-180 tumor mouse model, with paclitaxel as the positive control. The results showed that PM4 had comparable antitumor activity as paclitaxel, with tumor inhibition of 54% versus 56%, and remarkably decreased toxicity. The survival rate of treated mice was 8/8 in the PM4 group, compared to 3/8 in the paclitaxel group.

Paclitaxel has been widely used in the clinic as an antitumor drug for lung, breast, and ovarian cancers.[1] It is a natural antimicrotubule diterpenoid isolated from the bark of the pacific yew tree (*Taxus brevifolia*).[2] However, due to its poor water solubility, paclitaxel is dissolved in dehydrated ethanol and Cremophor EL for clinical use, which causes serious side effects associated with hypersensitivity.[3,4] Numerous attempts to improve the water solubility of paclitaxel have been done by conjugating paclitaxel to some hydrophilic molecules, such as amino acids,[5,6] sugars,[7–9] malic acid,[10] polyethylene glycol,[11–13] dextran,[14] heparin,[15] and sulfonate.[16] Although most of these derivatives have much better water solubility than paclitaxel, some problems still remain, such as low stability, limited improvement in solubility, decreased activity, and high toxicity.

Morpholine is a hydrophilic molecule that could possibly improve the water solubility when introduced into paclitaxel, especially when the amino group is salified. Moreover, upon entering tumor tissues, the morpholino group would be protonated at the slightly acidic extracellular pH of tumors (6.5–7.2).[17–19] This would promote the interactions of prodrugs containing morpholino groups with negatively charged cell membranes and accelerate their endocytosis by tumor cells. Our previous work found that morpholino-decorated polymeric micelles exhibit higher cellular uptake at lower pH values (6.5–7.0).[20] Therefore, the toxicity of morpholino compounds to normal tissues, where the extracellular pH is 7.4, might be decreased.

Here, we report the design, synthesis, and evaluation of a series of new paclitaxel prodrugs by introducing morpholino groups through different linkers (Fig. 1). The primary aim of this work was to study the influence of morpholino groups on improving the water solubility of insoluble drugs like paclitaxel. Second, we wanted to examine whether the administration of morpholino derivatives of paclitaxel would prolong the survival time of tumor-bearing mice by decreasing the drug toxicity to normal tissues. In addition, the influence of different linkers on the stability and activity of the derivatives was studied. The linker of the ester bond in PM1 and the carbamate bond in PM2 may lead to different release rates of paclitaxel, possibly resulting in activity variation. In PM3, paclitaxel was conjugated to 4-(2-aminoethyl)morpholine (AEM) through a succinyl group; while in PM4, two AEM groups were introduced to reinforce the influence of the morpholino group. In PM4, a disulfide linker was incorporated because it has been reported that cleavage would occur only after cellular entry of the conjugate after encountering a high glutathione concentration (typically 15 mM intracellular compared to 15 μM extracellular).[21] The thiol resulting from glutathione cleavage has been reported to cyclize into the proximate carbonyl group of the linker, subsequently leading to the release of free paclitaxel.

Synthesis: The synthetic routes of the designed conjugates are given in Schemes 1–4. PM1 was obtained by directly reacting 2-morpholineacetic acid with paclitaxel through an ester bond in

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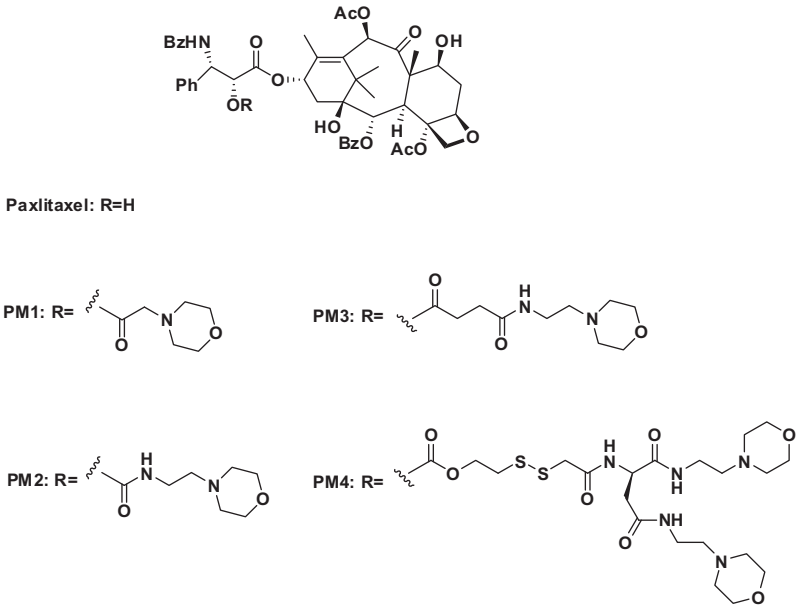
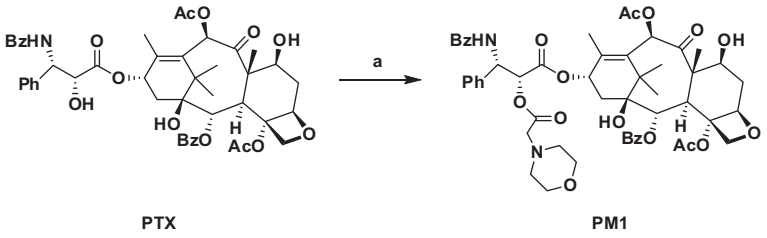
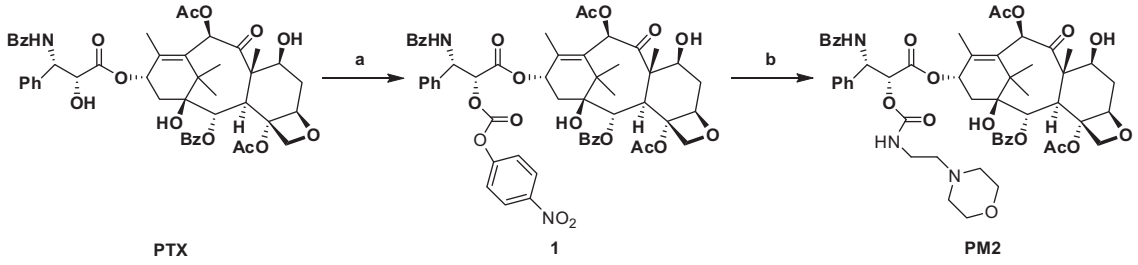


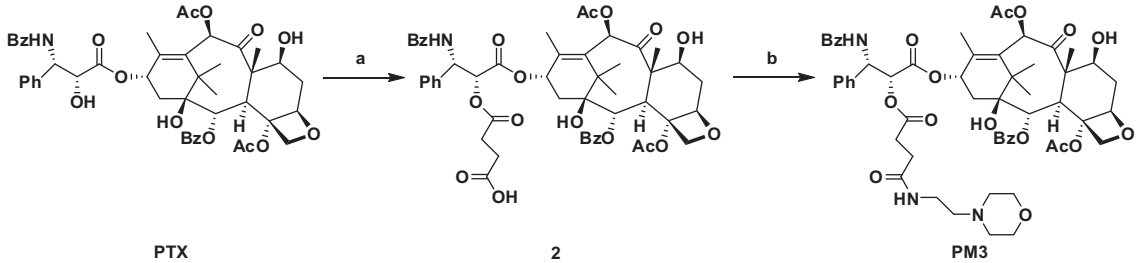
Figure 1. Structures of paclitaxel and its four prodrugs modified by morpholino groups.



Scheme 1. Synthesis of PM1. Reagents and conditions: (a) 2-morpholineacetic acid, EDCI, DMAP, DCM, rt.

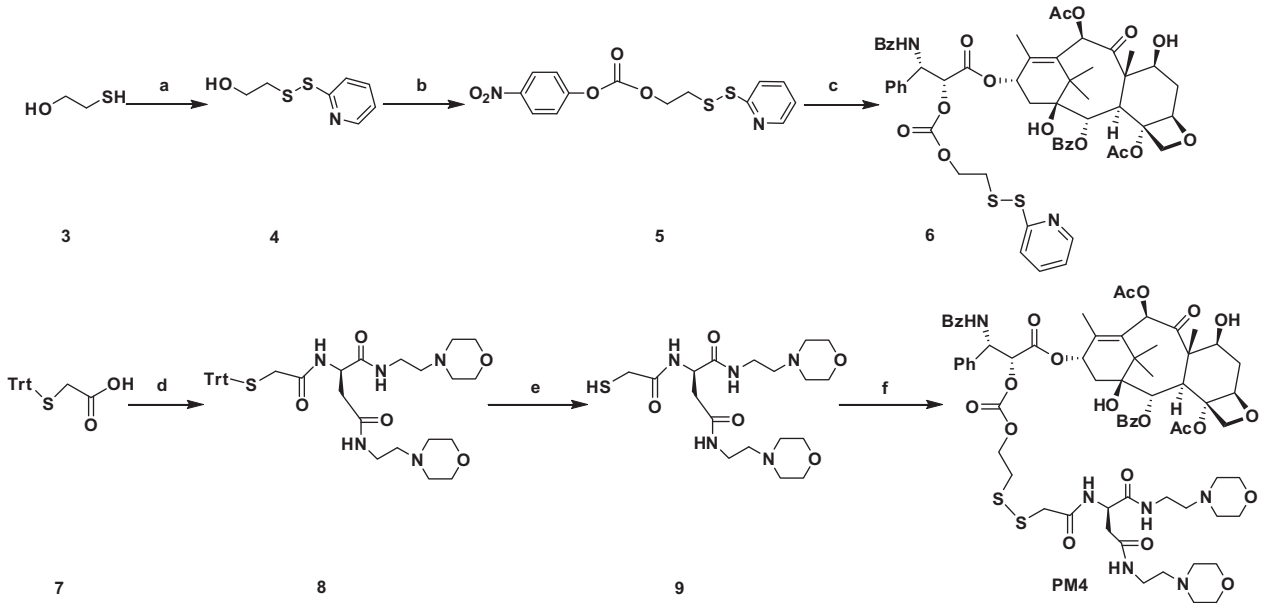


Scheme 2. Synthesis of PM2. Reagents and conditions: (a) 4-nitrophenyl chloroformate, pyridine, DCM, rt; (b) 4-(2-aminoethyl)morpholine, DMAP, DCM, rt.



Scheme 3. Synthesis of PM3. Reagents and conditions: (a) succinic anhydride, pyridine, rt; (b) 4-(2-aminoethyl)morpholine, EDCI, HOBt, DMF, rt.

the presence of 1-(3-dimethylaminopropyl)-3-ethylcardodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) in dichloromethane (DCM) at room temperature (rt). The reaction was completed in approximately 10 h. Next, DCM was evaporated,



Scheme 4. Synthesis of PM4. Reagents and conditions: (a) 2-2′-dithiodipyridine, DMAP, MeOH, rt; (b) 4-nitrophenyl chloroformate, pyridine, DCM, rt; (c) PTX, DMAP, DCM, rt; (d) AEM, EDCI, HOBt, DCM, rt; (e) 5% EDT/TFA, rt; (f) **6**, DMF, rt.

and the mixture was dissolved in a 50% acetonitrile/water solution before it was purified to give a white powdery solid. To obtain PM2, paclitaxel was first reacted with 4-nitrophenyl chloroformate in the presence of pyridine in DCM to get compound **1**. Then compound **1** was reacted with AEM in the presence of DMAP in DCM to obtain PM2. PM3 was prepared in two steps. First, paclitaxel was reacted with succinic anhydride in dry pyridine to obtain compound **2**, which was then reacted with AEM in the presence of EDCI and HOBt in DCM to obtain PM3. PM4 was synthesized in six steps. First, compound **6** was prepared as described previously.[22] Compound **9** was then prepared by first coupling AEM with compound **7**, followed by removal of the triphenylmethyl group. Compound **6** was then reacted with compound **9** in dimethylformamide (DMF) to obtain PM4. All four prodrugs were purified by reverse-phase C4 silica gel column chromatography and lyophilized in the form of acetic acid salts.

With the purified prodrugs in hand, their water solubility was measured. The results showed that their solubility in water was remarkably improved to at least 400 times greater than that of paclitaxel (Table 1). Among them, PM4, with two morpholino groups, showed the highest water solubility of up to 5 mg/mL, which is 20,000 times greater than that of paclitaxel. The solubilities of PM2 and PM3 were both around 0.5 mg/mL. Although PM1 had the lowest water solubility of 0.1 mg/mL, it was still much higher than that of paclitaxel.

The chemical stability of these four derivatives was then evaluated.[22] Briefly, solutions of the derivatives in DMSO (0.5 mM,

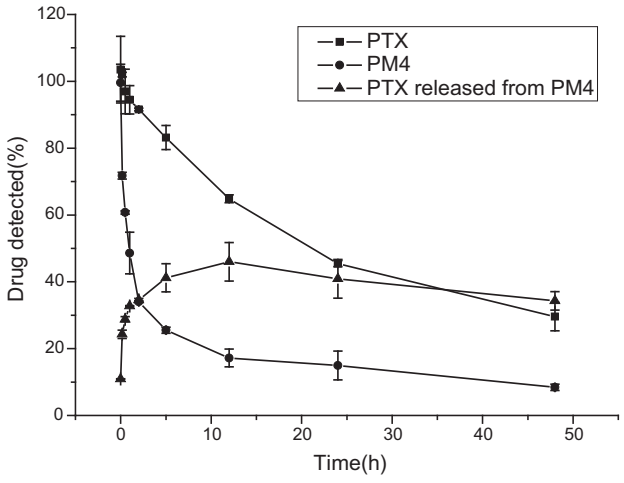


Figure 2. Stability of PTX and PM4 in mouse plasma (*Detected drug concentration divided by total concentration).

400 μl) were added to fetal bovine serum (FBS, 3600 μl) and incubated at 37 °C. At the desired time points 100 μl aliquots were removed and quenched with 300 μl acetonitrile. The mixture was centrifuged for 5 min at 10,000 rounds per minute. The supernatant was analyzed by HPLC. The results showed that paclitaxel

Table 1
The water solubility and cytotoxicity of paclitaxel conjugates in two tumor cell lines

Compd	Water solubility* (mg/mL)	IC_{50} (nM)			
		MCF-7 (nM) 48 h	MCF-7 (nM) 72 h	HeLa (nM) 48 h	HeLa (nM) 72 h
Paclitaxel	0.00025[24]	3.10	1.25	23.29	12.62
PM1	0.1	11.96	3.49	49.20	22.95
PM2	0.5	>1000	>1000	>1000	>1000
PM3	0.5	4.23	0.74	19.82	14.55
PM4	5	7.45	4.07	20.65	14.01

* Equivalent dose of paclitaxel.

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Table 2
In vivo activity of paclitaxel and its prodrugs against an S-180 ascites tumor[a]

Group	Dose[b] (mg/kg)	Average tumor weight (g)	Tumor inhibition[c] (%)	Body weight changes[d] (%)	No. of deaths
Control	—	7.35	—	−2.8	1/8
Paclitaxel	25	3.34	56	−28.1	5/8
PM4	25	3.43	54	−16.1	0/8

[a] Prodrugs and paclitaxel were given every other day (iv × 4). Treatment was initiated 24 h after implantation.
[b] Equivalent dose of paclitaxel.
[c] Tumor inhibition = (1 − average tumor weight of treated group/average tumor weight of control group) × 100.
[d] Body weight changes = (average body weight of mice at the tenth day/average body weight of mice at the first day − 1) × 100.

could be released from PM1, PM3 and PM4 while no paclitaxel released from PM2 was detected (Fig. S1). In PM1 and PM4, about 40% paclitaxel was released in 15 h while in PM3, paclitaxel was completely released in about 5 h. PM2 degraded nearly completely in 4 h. However, no paclitaxel was detected, probably, due to the high stability of the carbamate bond (Fig. S2).

Biological evaluation: The cytotoxicity of paclitaxel and the prodrugs was detected in two tumor cell lines, including a human breast cancer (MCF-7) cell line and a human cervical cancer (HeLa) cell line. Cells were seeded at a density of 4×10^3 cells/well in 96-well plates 24 h before treatment. Paclitaxel and the prodrugs were then added to the cells and incubated for 48 h or 72 h, respectively. The cell viability was determined by an MTS assay. The results showed that all derivatives except for PM2 had comparable cytotoxicity to paclitaxel, with IC_{50} values ranging from 0.6- to 3.9-fold of that of paclitaxel (Table 1). As predicted, the IC_{50} values decreased as the incubation time proceeded from 48 h to 72 h, indicating that an adequate exposure time is necessary for the drugs to kill the tumor cells effectively. Thus, the parent paclitaxel could be released effectively from these prodrugs. In contrast, PM2 did not kill either cell line, even at a concentration of 1 μM. This result may be due to the carbamate bond, which made the prodrug too stable to release paclitaxel effectively. This was in accordance with the result of the chemical stability evaluation.

Water-soluble PM4 was given intravenously to mice at different doses to evaluate its acute toxicity. Mice administered PM4 at a dose of 90 mg/kg (equivalent dose of paclitaxel) were still alive after 1 week. It has been reported that the maximum tolerated dose of paclitaxel is approximately 30 mg/kg,[23] and we found that when paclitaxel was given at a dose of 60 mg/kg, the mice died within 24 h. Therefore, PM4 appears to be less toxic than the parent paclitaxel and was chosen to carry out further activity evaluation in vivo.

The in vivo activity evaluation was performed on mice bearing an ascites tumor. S-180 cells were implanted subcutaneously in mice, and 24 h later, PM4 dissolved in 5% glucose solution was administered intravenously to the mice. Paclitaxel dissolved in cremophor/ethanol (50%/50%) diluted 10 times by 5% glucose solution was used as the positive control, and 5% glucose solution was used as the negative control. The prodrug was given every other day for a total of four times, and the mice alive at the tenth day were weighed and sacrificed. The tumors were retrieved and weighed, and the tumor inhibition was calculated. A summary of the preliminary results is given in Table 2.

As shown in Table 2, at a dose of 25 mg/kg, the tumor inhibition of paclitaxel was 56%, which is very close to that of PM4, which was 54%, meaning that the in vivo activity of the prodrug was equivalent to that of the parent paclitaxel. And as expected, judging from the body weight changes and the number of deaths, the toxicity of PM4 seemed to be much lower than that of the parent paclitaxel. In the paclitaxel group, the average body weight of the mice decreased by as much as 28.1%; while in the PM4 group, it was only 16.1%. Moreover, at the end of the experiment, only three out of eight mice survived in the paclitaxel group; while in the PM4 group, all eight mice were alive. In the 5% glucose aqueous solution group (control), only one of the eight mice died, possibly due to the fast proliferation of the tumor cells.

To further investigate the reason for the greatly reduced toxicity of PM4, the stability of PM4 in plasma was studied. PM4 was incubated in mouse plasma at 37 °C for 48 h, and the concentrations of PM4 and paclitaxel released at different times were detected. The results showed that PM4 degraded almost completely in first 10 h and only around 50% paclitaxel was released (Fig. 2). However, when incubated with 10 mM dithiothreitol, paclitaxel was completely released from PM4 in 10 min (Fig. S3). These results demonstrate that paclitaxel could be sufficiently released from PM4 after entering cells with high concentration of glutathione. Different from paclitaxel, PM4 may be taken up faster in the slightly acidic tumor surroundings than in normal tissues for the introduction of morpholino groups, leading to reduced toxicity to normal tissues.

In summary, we report the design, synthesis, and evaluation of a series of novel water-soluble paclitaxel prodrugs that contain morpholino groups. All the derivatives possessed much better water solubility and all but PM2 exhibited an equivalent in vitro activity compared to the parent paclitaxel in MCF-7 and HeLa cells. The linkers that conjugated the morpholino groups to the parent paclitaxel had an important influence on the solubility and stability of the derivatives, which may have affected their activity and the release of paclitaxel. The optimal prodrug PM4 was administered intravenously to mice bearing ascites tumors and showed equivalent tumor inhibition compared to the parent paclitaxel, with remarkably decreased toxicity. These morpholino-decorated paclitaxel prodrugs may have great potential for further development. This strategy and methodology may also be applied to the design of water-soluble prodrugs of other anticancer drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.012.

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