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Synthesis and evaluation of water-soluble polyethylene glycol–paclitaxel conjugate as a paclitaxel prodrug

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Water-soluble paclitaxel may cause less side effects and be less costly to administer in comparison to a taxol formulation using a cremophor EL/alcohol vehicle. In this study, polyethylene glycol (PEG; MW 5000) was conjugated to the 2′ position of paclitaxel through a spacer succinyl group. PEG–paclitaxel as a non-ionic paclitaxel prodrug was highly water soluble (> 20 mg equiv. paclitaxel/ml). The release of paclitaxel from phosphate-buffered solution was pH dependent. The half-life of PEG–paclitaxel was 7.6, 54 and 311 min at pH 9.0, 7.4 and 6.0, respectively. PEG–paclitaxel inhibited the growth of B16 melanoma cells to an extent similar to that of paclitaxel. In MCA-4 mammary tumor-bearing mice, a single dose of PEG–paclitaxel (40 mg equiv. paclitaxel/kg body weight) significantly delayed tumor growth. The average number of days for the tumor to reach 12 from 8 mm in diameter increased from 6.5 days for control animals to 8.5 days for PEG–paclitaxel-treated animals and 9.4 days for paclitaxel-treated animals. These studies demonstrated that PEG may be used as an effective solubilizing carrier for paclitaxel.

Key words: Paclitaxel, polyethylene glycol, prodrug.

Introduction

Paclitaxel (taxol) has shown good antineoplastic activities against human cancer in clinical trials.[1] The initial phase II study of 25 women with metastatic breast cancer who had received only one previous chemotherapy regimen produced an objective response rate of 56%. Significant benefit has also been documented in patients receiving paclitaxel after failure of prior chemotherapy for advanced breast cancer.[2] Paclitaxel, which induces the formation of microtubule bundles within cells, represents a new class of drugs with unique mechanisms of action.

A major difficulty in the development of paclitaxel for clinical trial use, however, has been its poor solubility in water. Currently, paclitaxel is formulated as a concentrated solution containing 6 mg paclitaxel per milliliter of Cremophor EL (polyoxyethylated castor oil) and ethyl alcohol (50% v/v), and must be further diluted before administration.[3] Several toxic effects have been attributed to Cremophor, including vasodilatation, dyspnea and hypotension.[4] Cremophor has been shown to cause serious hypersensitivity in laboratory animals and humans.[5] In addition, Cremophor, as a surfactant, is known to leach phthalate plasticizers from polyvinylchloride bags and i.v. tubing.[3] Therefore, special costly provisions are necessary for the preparation and administration of paclitaxel solutions to ensure safe drug delivery to patients. A water-soluble paclitaxel prodrug would be safer, easier and less costly to administer. Previous attempts to prepare water-soluble prodrugs of paclitaxel involved placing solubilizing moieties at the C2′ or C7 position. Several paclitaxel derivatives including taxol succinate and glutarate, sulfonic acid derivatives, amino acid derivatives, and phosphate derivatives have been prepared and evaluated.[6–9]

Another approach to formulate water-soluble paclitaxel is to conjugate it to a water-soluble polymer. Many studies have established the advantages of macromolecular cytotoxic drug conjugates.[10] Both synthetic and natural polymers including *N*-(2-hydroxypropyl) methacrylamide copolymers, dextran and albumin have been used in these studies.[11–13] Polyethylene glycol (PEG) has been used in biological and biomedical applications due to its hydrophilicity, chain mobility and lack of ionic charge. These applications include protein modification to decrease antigenicity,[14] liposome

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modification to increase blood circulation time,[15] surface modification to produce biocompatible or protein-repelling activity[16] and polymer modification to control drug delivery.[17] Greenwald *et al.* recently published their work on the synthesis of 7-PEG carbamate and carbonate derivatives of taxol that exhibited high water solubility.[18] Here, we report the conjugation of PEG to paclitaxel at the C2′ position via ester linkage, and an *in vitro* and *in vivo* evaluation of the resulting paclitaxel prodrug.

Materials and methods

Materials

Methoxypolyoxyethylene amine (PEG-NH_2, MW 5000) and methoxypolyethylene glycol carboxylic acid (PEG-COOH, MW 5000) were obtained from Sigma (St Louis, MO). Paclitaxel (purity > 97%) was obtained from Hande Tech (Houston, TX). Silica gel (mesh 70-230) was purchased from EM Science (Gibbstown, NJ). Other chemicals of reagent grade were purchased from either Aldrich Chemical or Sigma. All solvents used were of HPLC grade.

Chemistry

Melting points were measured with a Mel-Temp II and were left uncorrected. UV spectra were obtained on a Beckman DU-70 spectrophotometer (Fullerton, CA). ^{1}H-NMR spectra were obtained on a GE-300 spectrometer (300 MHz). Samples were dissolved in appropriate solvents and chemical shifts (δ) were reported in p.p.m. relative to tetramethylsilane. Thin-layer chromatography (TLC) for in-process synthetic examination was performed on Whatman MK6F silica gel plates (thickness 0.25 mm, 60 Å).

The PEG–paclitaxel conjugate was synthesized in two steps (Figure 1). First, 2′-succinyl-paclitaxel was prepared according to a previously reported procedure.[6] Briefly, paclitaxel (200 mg, 0.23 mmol) and succinic anhydride (288 mg, 2.22 mmol) were allowed to react in anhydrous pyridine (6 ml) at room temperature for 3 h. The pyridine was then evaporated, and the residue was treated with water (10 ml), stirred for 20 min and filtered. The precipitate was recrystallized from acetone and water to yield 2′-succinyl-paclitaxel (180 mg, 81%). m.p.: 175–178°C (lit. m.p. 178–180°C). ^{1}H-NMR ($CDCl_3$) δ: 7.02 (N-H), 6.27 (s, C_{10}-H), 6.22 (t, C_{13}-H), 5.82 (dd, $C_{3'}$-H), 5.66 (d, C_2-H), 5.51 (d, $C_{2'}$-H), 4.93 (d, C_5-H), 4.40 (broad, C_7-H), 4.20 and 4.29 (d, C_{20}-H), 3.78 (d, C_3-H), 2.77 (m, $COCH_2CH_2CO_2$), 1.68 (C_{19}-H), 1.23 and 1.13 (s, $C_{16,17}$-H). The δ for C2′-H shifted from 4.79 to 5.51, indicating esterification at the C2′ position.

A *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)-mediated coupling reaction between the carboxyl group of 2′-succinyl-paclitaxel and the amino groups of methoxyl PEG-NH_2 yielded the targeted product. To a solution of 2′-succinyl-paclitaxel (400 mg, 0.45 mmol) and PEG-NH_2 (2.0 g, 0.40 mmol) in methylene chloride (50 ml) was added EEDQ (750 mg, 3.0 mmol). The reaction mixture was stirred at room temperature for 4 h. The course of reaction was followed by TLC (ethyl acetate mobile phase) which showed complete conversion of 2′-succinyl-paclitaxel ($R_f = 0.28$) to its corresponding polymer conjugate ($R_f = 0$). The crude product was chromatographed on silica gel with ethyl acetate to remove free paclitaxel followed by chloroform:methanol (10:1). This gave 1.42 g of amorphous solid (60%). ^{1}H-NMR ($CDCl_3$) δ: 6.27 (s, C_{10}-H), 6.20 (t, C_{13}-H), 5.79 (dd, $C_{3'}$-H), 5.66 (d, C_2-H), 5.51 (d, $C_{2'}$-H), 4.95 (d, C_5-H), 4.42 (broad, C_7-H), 3.61 (OCH_2CH_2O), 2.77 (m, $COCH_2CH_2CO_2$), 1.66 (s, C_{19}-H), 1.21 and 1.12 (s, $C_{16,17}$-H). The rest of the spectrum was consistent with the paclitaxel structure.

Physical properties

Water solubility was estimated by dissolving appropriate amounts of PEG–paclitaxel in 0.1 ml of water. The UV spectrum of PEG–paclitaxel in water was obtained and the absorbance at 288 nm was used to quantify the concentration of paclitaxel in PEG–paclitaxel. A paclitaxel standard series prepared in methanol was used to generate a calibration curve, assuming PEG–paclitaxel and paclitaxel have the same absorption coefficient.

Release studies

PEG–paclitaxel was dissolved in phosphate-buffered solutions (0.01 M) at various pHs at a concentration of 0.4 mM. The solutions were incubated at 37°C with gentle shaking. At selected time intervals, aliquots (200 μl) were removed and lyophilized. The resulting dry powders were redissolved in methylene chloride for gel permeation chromatography (GPC) analysis. The GPC system consisted of a Perkin-Elmer PL gel mixed bed column, a Perkin-Elmer isocratic LC pump, a PE Nelson 900 series

interface, a Spectra-Physics UV/Vis detector and a data station. The elutant (methylene chloride) was run at 1.0 ml/min with the UV detector set at 228 nm. The retention times of PEG–paclitaxel and paclitaxel were 6.1 and 8.2 min, respectively. Peak areas were quantified and the percentage of PEG–paclitaxel remaining and the percentage of paclitaxel released were calculated. The half-life was determined by a linear least-squares regression analysis.

Cytotoxic activity against B16 mouse melanoma cells

Cells were seeded onto 24-well plates at a concentration of 2.5×10^4 cells/ml and grown in a 1:1 Dulbecco's minimal essential medium and Ham's F12 medium containing 10% bovine calf serum at 37°C for 24 h in a 97% humidified atmosphere of 5.5% CO_2. The medium was then replaced with fresh medium containing paclitaxel or PEG–paclitaxel in concentrations ranging from 5×10^{-9} to 75×10^{-9} M. After 40 h, the cells were released by trypsinization and counted in a Coulter counter. DMSO was used to dissolve paclitaxel and the stock DMSO solution was diluted with a culture medium. The final concentrations of DMSO was less than 0.01%. This amount of solvent did not have any effect on cell proliferation as determined from the control experiments. Furthermore, PEG in the concentrations used to generate equivalent paclitaxel concentrations ranging from 5×10^{-9} to 75×10^{-9} M also had no effect on cell proliferation.

Antitumor effect of PEG–paclitaxel against MCA-4 tumor in mice

To evaluate the antitumor efficacy of PEG–paclitaxel against solid breast tumors, MCA-4 cells (5×10^5 cells) were injected into the right thigh muscle of female C3Hf/Kam mice. MCA-4 cells were originally derived from a non-immunogenic mammary carcinoma and are syngeneic to C3Hf/Kam mice. Previous studies have shown that MCA-4 tumors in mice respond well to treatment with paclitaxel.[19] The mice (20–25 g) were bred and maintained in our specific pathogen-free mouse colony in the Department of Experimental Radiotherapy. When the tumors had grown to 8 mm (after approximately 2 weeks), mice were divided into groups of five. A single dose of paclitaxel or PEG–paclitaxel was given i.v. in doses of 10, 20 and 40 mg equiv. paclitaxel/kg body weight. Paclitaxel was initially dissolved in absolute ethanol with an equal volume of Cremophor. This stock solution (30 mg/ml) was further diluted (1:4 by volume) with a sterile physiological solution within 15 min of injection. PEG–paclitaxel was dissolved in saline (6 mg equiv. paclitaxel/ml) and filtered through a sterile filter (Millipore, 4.5 μm). Saline, paclitaxel vehicle [absolute alcohol:Cremophor (1:1) diluted with saline (1:4)] and PEG solution in saline (600 mg/kg body weight) were used in control experiments.

The antitumor effect was measured in terms of tumor growth delay. Tumor growth was determined daily by measuring three orthogonal tumor diameters until the tumor reached 14 mm in diameter. The mice were weighed daily to estimate the toxicity of the injected drugs in terms of percent weight loss. The time (in days) for each tumor to reach 12 mm in diameter was calculated. In all experiments involving animals, the *Principles of Laboratory Animal Care* (NIH publication #85-23, revised 1985) was observed.

Statistics

The significance of differences in the tumor growth delay (number of days to reach 12 mm) was analyzed by an unpaired, two-tailed Student's *t*-test.

Results

Synthesis and characterization

In general, water-soluble paclitaxel prodrugs have been prepared by introducing ionic moieties to paclitaxel either by C2′ or C7 hydroxyl groups.[6–9] The C2′ substitution has been identified as a preferred position because C2′ ester hydrolyzes fairly rapidly back to taxol.[8] In those studies, water-soluble salts were prepared from paclitaxel derivatives that possess either a weak base, e.g. amine, or a weak acid, e.g. carboxylic acid. It is anticipated that the solubility of these paclitaxel prodrugs is a function of the pH of the aqueous media. Another approach to prepare water-soluble paclitaxel prodrugs is to use hydrophilic non-ionic moieties, such as sugar, polysaccharides or PEG as solubilizing agents and as carriers for paclitaxel.[18] Here, we report the synthesis and evaluation of a 2′-PEG–paclitaxel conjugate as a potential water-soluble paclitaxel prodrug.

Initial attempts to react the C2′ and/or C7 hydroxyl groups of paclitaxel with PEG-COOH in the

presence of *N,N'*-dicyclohexylcarbonyldiimide and dimethylaminopyridine failed to form ester bonds, probably because of the steric hindrance of paclitaxel's hydroxyl groups. Therefore, a spacer linkage to the C2′ hydroxyl group was introduced to facilitate conjugation with PEG. Reaction of paclitaxel with succinic anhydride in pyridine yielded C2′-succinyl-paclitaxel. C2′-succinyl-paclitaxel was then reacted with PEG-NH_2 in the presence of EEDQ to yield the desired PEG–paclitaxel conjugate in 60% yield (Figure 1).

Evidence for the site of esterification for succinyl-paclitaxel was obtained from ^{1}H-NMR, where the C2′ proton of paclitaxel at 4.78 p.p.m. appeared as a doublet at 5.51 p.p.m. The UV spectrum of PEG–paclitaxel in an aqueous solution was obtained; it showed characteristic paclitaxel absorption at 228 nm. Using UV assay, the concentration of paclitaxel in PEG–paclitaxel was found to be 11.6% (w/w). The calculated paclitaxel concentration in PEG–paclitaxel is 14.5% (w/w). Thus, a small amount of unreacted PEG (about 20%) was present in the final product. No further attempt was made to separate free PEG from the PEG–paclitaxel product. Both ^{1}H-NMR and UV data supported the formation of the PEG–paclitaxel ester bond. However, this data did not exclude the possibility of the final products containing unreacted succinyl-paclitaxel. To ascertain that the final product was not merely a mixture of PEG and succinyl-paclitaxel, GPC analyses were performed. In GPC chromatograms, PEG–paclitaxel appeared at 6.1 min, while paclitaxel had a retention time of 8.2 min. The data clearly demonstrated that the PEG–paclitaxel obtained was free of small molecular weight contaminants (Figure 2).

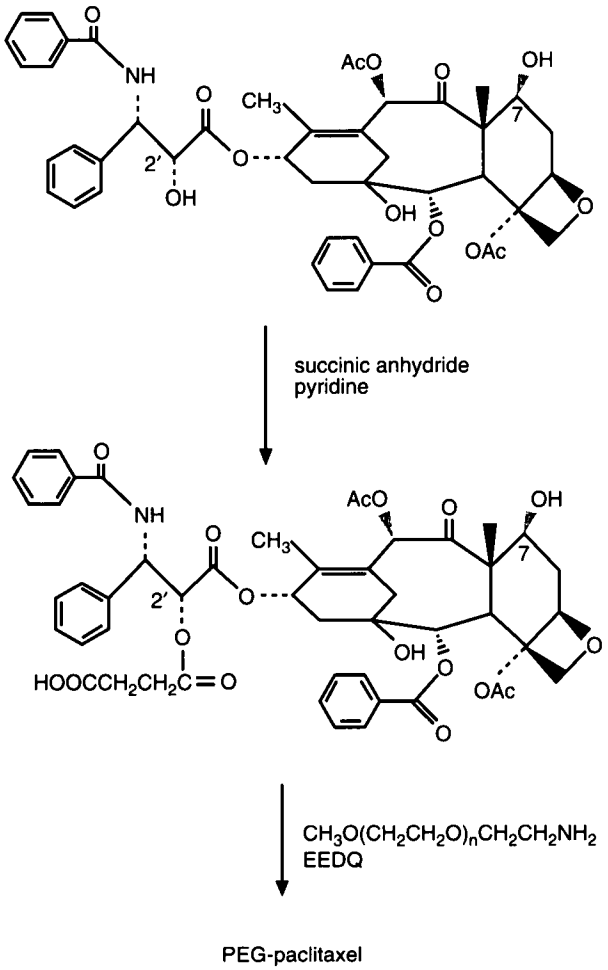

Figure 1. Synthesis of 2′-PEG–paclitaxel.

When 20 mg of conjugate was dissolved in 0.1 ml of water, a clear, viscous yet flowable liquid was obtained. An extremely viscous solution was obtained when the amount of PEG–paclitaxel was increased to 30 mg. Thus the solubility of PEG–paclitaxel was at least 200 mg/ml, which corresponded to an equivalent paclitaxel solubility of 20 mg/ml.

The stability of the PEG–paclitaxel conjugate was determined in a phosphate-buffered solution at various pHs at 37°C. The release of paclitaxel was quantified by GPC analysis. In the pH 9.0 and pH 7.4 media, paclitaxel was released rapidly (Figure 3). The half-life of PEG–paclitaxel in these media was only 7.6 and 54 min, respectively. In the

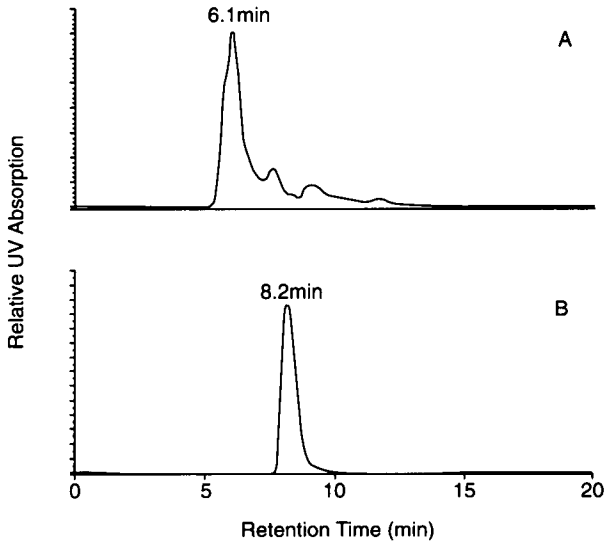

Figure 2. GPC chromatograms of 2′-PEG-paclitaxel (A) and paclitaxel (B). The samples were injected into a HPLC system consisting of a PL gel column. The eluent (methylene chloride) was run at 1.0 ml/min with a UV detector set at 228 nm.

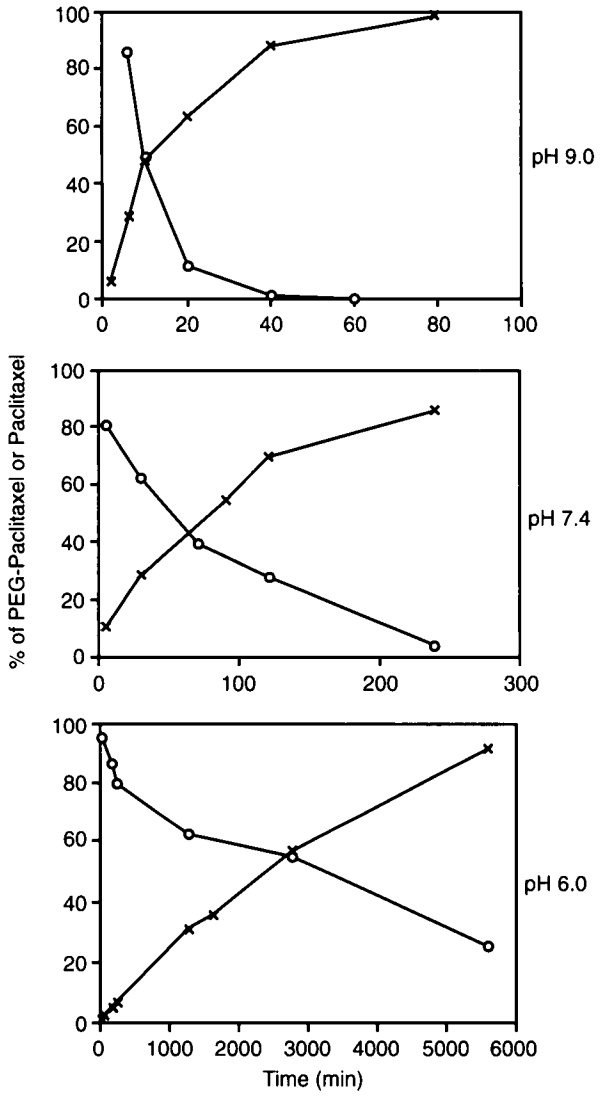

Figure 3. Release kinetics of paclitaxel from PEG–paclitaxel. 2'-PEG–paclitaxel was dissolved in phosphate buffered solutions at pH 9.0, 7.4 and 6.0. The concentrations of PEG–paclitaxel and paclitaxel were analyzed by GPC as described in the text. Paclitaxel, (×); PEG–paclitaxel, (○).

acidic medium, the conjugate was fairly stable, having $t_{1/2}$ of 311 min at pH 6.0 (Figure 3). Thus, PEG–paclitaxel may be formulated as an aqueous solution in acidic media (pH < 6.0) for practical reasons.

In vitro and *in vivo* studies

The minimal concentrations of paclitaxel and PEG–paclitaxel necessary to inhibit cell growth by 50% (IC_{50}) after incubation for 40 h at 37°C were determined. The IC_{50} for PEG–paclitaxel was 7.5 nM and 15 nM for paclitaxel. The results indicate that PEG–paclitaxel is as effective at inhibiting the growth of B16 melanoma cells as free paclitaxel.

To establish the antitumor effect of PEG–paclitaxel against breast tumor *in vivo*, C3Hf/Kam mice bearing MCA-4 tumor were treated with paclitaxel or PEG–paclitaxel. At doses of 10 or 20 mg equiv. paclitaxel/kg body weight, no significant tumor growth delay was observed as compared to controls. However, at a dose of 40 mg/kg, both PEG–paclitaxel and paclitaxel effectively delayed tumor growth. Paclitaxel was more effective than PEG–paclitaxel, although the difference was not statistically significant (Figure 4). Paclitaxel-treated tumors required 9.4 days to reach 12 mm in diameter whereas PEG–paclitaxel-treated tumors required 8.8 days. Statistically, these values were significant ($p > 0.05$) as compared to their corresponding controls, which were 5.9 days for the paclitaxel vehicle and 6.3 days for the saline solution of PEG.

Percentage weight changes for mice treated with PEG–paclitaxel and paclitaxel at the dose of 40 mg/kg were followed as a measure of drug toxicity (Figure 5). PEG–paclitaxel caused a decrease in weight which reached a nadir of 12% at 3 days post-injection. However, the weight recovered steadily over the next 10 days. Paclitaxel caused a maximal 6% decrease in weight at 2 days post-injection,

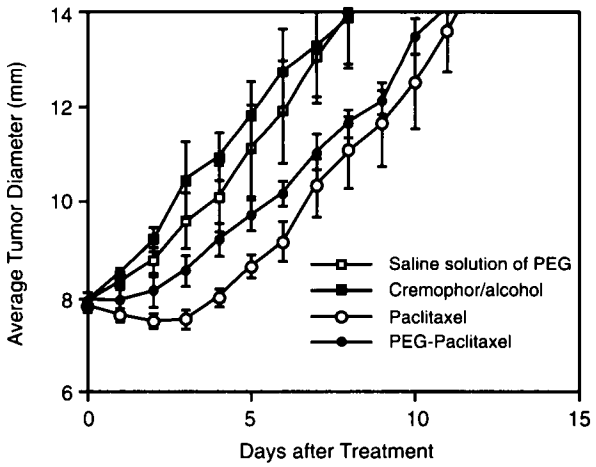

Figure 4. Antitumor effect of PEG–paclitaxel and paclitaxel against MCA-4 tumors in C2Hf/Kam mice. The drugs were injected i.v. in single doses of 40 mg equiv. paclitaxel/kg body weight. PEG–paclitaxel was dissolved in saline and paclitaxel was dissolved in a Cremophor/alcohol vehicle. The control animals were injected with either a PEG-containing saline solution (60 mg/ml) or a Cremophor/alcohol vehicle. Each piece of data represents mean ±SD ($n = 5$).

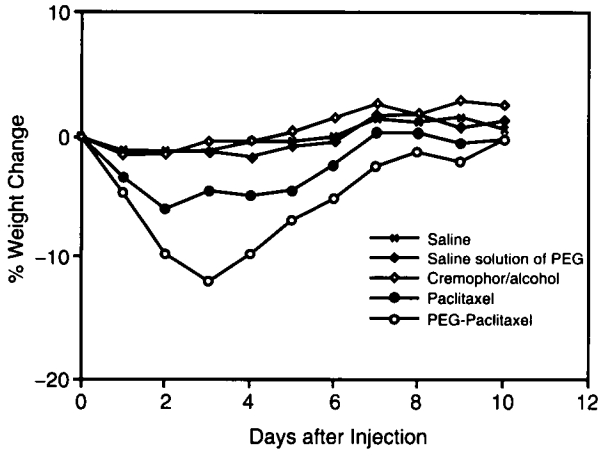

Figure 5. Weight changes in MCA-4 tumor-bearing mice after i.v. injection of PEG–paclitaxel and paclitaxel. The drugs were injected i.v. in single doses of 40 mg equiv. paclitaxel/kg body weight. Control groups included saline, a saline solution of PEG and a Cremophor/alcohol vehicle.

which was also recovered in the same time period. PEG and the Cremophor/alcohol vehicle did not cause significant weight loss as compared to saline.

Discussion

Development of water-soluble paclitaxel prodrugs with enhanced antitumor activity has been a subject of intense studies. Most efforts have been made by placing small molecular weight solubilizing moieties at the 2′ or 7 hydroxyl group.[6–9] However, with the possible exception of a cationic 2′ amino acid derivative,[7] those prodrugs are either chemically unstable for development or do not show a significantly improved therapeutic index as compared to free drug. The use of a polymeric drug carrier offers advantages in that improved therapeutic efficacy may be achieved by masking the toxicity of free drug[20] and by selective accumulation of polymer–drug conjugates in the tumors.[10] The purpose of this study was to examine whether conjugation of paclitaxel to PEG, a non-ionic water-soluble polymer, could yield polymer–paclitaxel with sufficient solubility, and more importantly, whether such a conjugate would maintain the antitumor activity of paclitaxel. A succinyl spacer was used to introduce a hydrolytically labile ester bond at the C2′ position of paclitaxel, and to facilitate coupling reaction between PEG and paclitaxel. As expected, the resulting conjugate is highly water-soluble. It behaved as a paclitaxel prodrug, as free paclitaxel was released from the conjugate upon exposure to aqueous environment. Both *in vitro* cytotoxicity and *in vivo* antitumor activity studies showed that PEG–paclitaxel had significant antitumor activity. A potential limiting factor in the use of PEG as a carrier for paclitaxel could be its toxicity. It is not clear at the present time why the PEG–paclitaxel conjugate was more toxic than free paclitaxel as judged by body weight lost. It is interesting to note that the molecular weight of PEG used in this study was only 5000 Da, which is far below the threshold molecular mass of 50 kDa considered necessary for polymer–drug conjugates to exhibit preferential uptake in tumors.[21] Low molecular weight of the conjugate could be one of the contributing factors responsible for increased toxicity. On the other hand, increasing the polymer molecular weight would effectively limit the amount of paclitaxel that PEG can carry. The calculated paclitaxel concentration in this PEG–paclitaxel is 14.5%. If the molecular weight of PEG was to be increased to 50 kDa, the paclitaxel concentration would be reduced to 1.7%. Our study on PEG–paclitaxel demonstrated the feasibility in the use of polymer-linked paclitaxel as an alternative strategy for the development of soluble paclitaxel prodrugs. These studies also suggest that favorable results will probably come from the selection of appropriate polymeric carriers (preferably polymers with side chain functional groups that allow attachment of multiple paclitaxel molecules per chain) and optimization of physicochemical properties of polymer–paclitaxel conjugates including their molecular weight.

Conclusion

Our current study demonstrated the feasibility of using a water-soluble polymer as a solubilizing carrier for the formulation of a paclitaxel prodrug. As exemplified in the use of PEG, both *in vitro* and *in vivo* studies showed that PEG–paclitaxel had significant antitumor activity. These findings will be useful for the future design of water-soluble prodrugs that will have improved antitumor efficacy.

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