Synthesis and Evaluation of Water-Soluble Poly(vinyl alcohol)–paclitaxel Conjugate as a Macromolecular Prodrug

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Paclitaxel (PTX) is an antitumor agent for the treatment of various human cancers. Cremophor EL® and ethanol are used to formulate PTX in commercial injection solutions, because of its poor solubility in water. However, these agents cause severe allergic reaction upon intravenous administration. The aim of this study is to synthesize water-soluble macromolecular prodrugs of PTX for enhancing the therapeutic efficacy. Poly(vinyl alcohol) (PVA, 80 kDa), water-soluble synthetic polymer, was used as a drug carrier which is safe and stable in the body. The 2′-hydroxyl group of PTX was reacted with succinic anhydride and then carboxylic group of the succinyl spacer was coupled to PVA *via* ethylene diamine spacer, resulting the water-soluble prodrug of poly(vinyl alcohol)–paclitaxel conjugate (PVA–SPTX). The solubility of PTX was greatly enhanced by the conjugation to PVA. The release of PTX from the conjugate was accelerated at the neutral to basic conditions in *in vitro* release experiment. [^{125}I]-labeled PVA–SPTX was retained in the blood circulation for several days and was gradually distributed into the tumorous tissue after intravenous injection to the tumor-bearing mice. PVA–SPTX inhibited the growth of sarcoma 180 cells subcutaneously inoculated in mice. It was suggested that the water-solubility of PTX was markedly enhanced by the conjugation to PVA, and PVA–SPTX effectively delivered PTX to the tumorous tissue due to the enhanced permeability and retention (EPR) effect.

Key words poly(vinyl alcohol); paclitaxel; macromolecular prodrug; cytotoxicity; antitumor activity

Paclitaxel (PTX) is an anti-microtubule agent isolated from the trunk bark of the Pacific Yew tree, Taxus brevifolia.[1] It has been widely used as an anti-neoplastic agent for a variety of human cancers including breast, ovarian, non-small cell lung, head and neck cancers, leukemia, and melanoma.[2—6]

PTX is a highly hydrophobic drug and is hardly soluble in water (water solubility $<0.3\,\mu$g/ml).[7] Because of its poor solubility in water and many other acceptable pharmaceutical solvents, specific emulsionizers, such as Cremophor EL®, are used to formulate PTX in commercial injection solutions. However, serious hypersensitivity reactions have been reported in some individuals since the content of Cremophor EL® used in the PTX formulation is significantly higher than in any other marketed drug.[8,9] Side effects of PTX formulation include nausea and vomiting, diarrhea, mucositis, myelosuppression, cardiotoxicity and neurotoxicity.[10,11]

In addition, Cremophor EL® is known to leach phthalate plasticizers from polyvinylchloride bags and intravenous tubing.[12] Therefore, alternative dosage forms for the PTX administration need to be developed to reduce the undesirable side effects induced by using Cremophor EL®.

In recent years the use of water-soluble polymers as drug delivery systems has been received increasing attention. Li *et al.*[13] and Greenwald *et al.*[14] reported the conjugation of polyethylene glycol (PEG) to the 2′-position of PTX through a spacer succinyl group. They demonstrated that PEG may be used as an effective solubilizing carrier for PTX. Poly-L-glutamic acid was also used to make a water-soluble PTX conjugate.[15—17] It was demonstrated that the poly-(L-glutamic acid)–paclitaxel conjugate was more effective than standard PTX. On the other hand, Sugahara *et al.* constructed the PTX delivery system using amino acid linkers in the conjugation of PTX with carboxymethyldextran.[18]

The consequence of attachment of low molecular weight drugs to macromolecular carriers alters their rate of excretion from the body, changes their toxicity and immunogenicity, and limits their uptake by cells *via* endocytosis, thus providing the opportunity to direct the drug to the particular cell type where its activity is needed.[19] In addition, these macromolecular conjugates can accumulate in solid tumors due to the enhanced microvasculature of tumor tissue.[20,21] This phenomenon has been termed enhanced permeability and retention in relation to tumor targeting (EPR-phenomenon).[22—24]

Poly(vinyl alcohol) (PVA) is a polymer which is synthesized by polymerizing not a vinyl alcohol monomer but a vinyl acetate monomer. This monomer is polymerized in to poly(vinyl acetate) and then hydrolyzed to produce PVA. PVA's biocompatibility makes it an excellent material for use in medical applications such as soft contact lenses. Recently, PVA has been used for long-term implants, including a bioartificial pancreas, artificial cartilage, nonadhesive film, and esophagus or scleral buckling material.[25] Furthermore, PVA has numerous functional groups which are capable of covalently coupling drug molecules.

In the previous study, we first synthesized a PVA–doxorubicin conjugate and demonstrated that PVA provides a potential targetable drug delivery system.[26] In this paper we report on the synthesis of the poly(vinyl alcohol)–paclitaxel conjugate, its release experiment, cytotoxicity, tissue distribution, and antitumor activity.

MATERIALS AND METHODS

Animals Male ddY mice at 5 weeks of age were purchased from Shimizu laboratory supplies (Shizuoka, Japan) and housed under a standard condition of temperature and light. Mice were given free access to commercial food and tap water. All animal experiments were conducted in accordance with the institutional guideline for the care and use of laboratory animals for research, which conforms to the guideline of Science Council of Japan.

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Materials Paclitaxel (PTX) was obtained from Tokyo Chemical Industry Co., Ltd., Japan. PVA (80 K, MW=80520) was kindly supplied by Japan Vam & Poval Co., Ltd., Osaka, Japan. All other chemicals and reagents were of the highest grade commercially available. Purebright MB37-50T, a solubilizer of an amphiphilic polymer, was purchased from NOF Corporation, Tokyo.

Preparation of Poly(vinyl alcohol)–Paclitaxel Conjugate Ethylenediamine residues were introduced to the hydroxyl groups of PVA molecules by the 1,1′-carbonyldiimidazole (CDI) activation method.[26—29] Four milliliters of dimethyl sulfoxide (DMSO) containing CDI (74 mg) was added to 400 mg of PVA dissolved in 60 ml of DMSO, followed by stirring for 1 h at room temperature. After several precipitations in butanol to remove unreacted reagents, the fraction of CDI-activated PVA was dried *in vacuo*. Then, ethylenediamine (2 g) was added to the CDI-activated PVA (200 mg) dissolved in 50 ml of DMSO and stirred for 48 h at 50 °C. After several precipitations in butanol to remove unreacted reagents, the fraction of PVA–ethylenediamine was dried *in vacuo*. The free amino groups of ethylenediamine spacers introduced into the PVA molecule were measured by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method.[30—32]

2′-Succinyl-paclitaxel (SPTX) was synthesized by the modified method of Dosio *et al.*[33] Briefly, PTX (20 mg, 0.023 mmol) was added to succinic anhydride (14 mg, 0.14 mmol) in the presence of 4-dimethylamino-pyridine (0.88 mg, 0.0072 mmol). Then 0.4 ml of dry pyridine was added and the solution was stirred for 3 h at room temperature. The SPTX was purified by chromatography on a 30×2.5 cm SiO_2 column eluted with chloroform–methanol mixture (97 : 3 to 90 : 10) as determined by TLC (*Rf* 0.27 in chloroform–methanol 90 : 10). Its structure was confirmed by ^{1}H-NMR (500 MHz, $CDCl_3$) δ: S 1.13 (s, 17H), 1.22 (s, 16H), 1.67 (s, 19H), 1.90 (s, 18H), 2.17 (m, 14H), 2.20 (s, 4-OAc), 2.43 (s, 10-OAc), 2.57 (m, CH_2CH_2), 3.80 (d, 3H), 4.19 and 4.30 (d, 20H), 4.42 (m, 7H), 4.96 (d, 5H), 5.52 (d, 2′H), 5.68 (d, 2H), 5.98 (dd, 3′H), 6.23 (t, 13H), 6.29 (s, 10H), 7.10 (d, NH), 7.33 (m, 3′-Ph), 7.40 (m, 3′-NBz), 7.50 (m, 2-OBz), 7.75 (d, 3′-NBz), 8.13 (d, 2-OBz).

The SPTX (21.0 mg, 0.022 mmol), dissolved in DMSO–dimethylformamide (70 : 30) solution containing *N*-hydroxy-3-sulfo-succinimide (sulfo-NHS, Fluka Chimica, Milan, Italy) (9.7 mg, 0.044 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (8.6 mg, 0.044 mmol). After 14 h at room temperature the reaction was complete as determined by TLC (*Rf*=0.18 in chloroform–methanol, 90 : 10). Then, PVA–ethylenediamine (200 mg) dissolved in 20 ml DMSO was added to the activated-SPTX and stirred for 48 h at room temperature. After several precipitations in butanol to remove unreacted reagents, the fraction of PVA–SPTX conjugate was dried *in vacuo*. Characterization of the conjugate was carried out using a three dimensional high-performance size exclusion chromatography described later.

PTX Content of the Conjugate The conjugate was dissolved in distilled water, and the absorbance at 227 nm was measured. The PTX content of the conjugates was estimated using the calibration curve of PTX standard.

***In Vitro* Release Experiment** The release of PTX from the conjugate was determined in a 0.05 M phosphate buffer system (pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, μ=0.15) containing 1.5 M *N*,*N*-diethylnicotinamide (DENA) at 37 °C. The experiment was initiated by the addition of the stock solution to a preheated buffer solution to give a concentration of 0.25 mg/ml of PVA–SPTX conjugate, respectively. At a fixed time intervals, the amount of PTX released was determined by using a HPLC method described later.

***In Vitro* Cytotoxicity** Mouse leukemia L1210 was kindly provided by Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Cells were typically kept in continuous logarithmic growth at 37 °C in a humidified atmosphere in 5% CO_2–95% air in RPMI 1640 medium (Nacalai, Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Tokyo, Japan) and 50 U/ml penicillin and 50 μg/ml streptomycin (Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan). The number of viable cells was determined by the trypan blue exclusion method by using a Burker-Turk hematocytometer 3 d after incubation.

PVA–SPTX and PTX in Purebright MB37-50T were dissolved in RPMI1640 medium. PTX was dissolved in DMSO, then diluted by the tissue culture medium. The solutions of each sample were added to test wells to a final volume of 200 μl/well in 96-well plates. Cells were seeded into the plates at a density of 0.5×10^4 cells/200 μl/well. Plates were incubated at 37 °C for 3 d. Subsequently, cellular proliferation was examined by an additional incubation for 3 h with Alamar Blue (Kanto Reagents, Tokyo, Japan).[34] Cellular proliferation induces a chemical reduction of the Alamar Blue which results in a change in redox color from blue to red. The proliferation of cultures with Alamar Blue was determined by measuring absorbance at 570 nm and 600 nm in an immuno plate reader (NJ-2300, Inter Med Japan, Tokyo, Japan). Growth inhibition (%) was calculated as follows.

$$\text{growth inhibition (\%)}=(1-(T-B)/(C-B))\times100$$

where T, B, and C represent the scaled difference in absorbance at 570 nm and 600 nm of the test compound, the blank and the control, respectively.

Preparation of [^{125}I]-Labeled PVA–SPTX ([^{125}I]-PVA–SPTX) PVA–SPTX (200 μg) was labeled with 0.25 mCi of [^{125}I] iodine by using Bolton and Hunter reagent for protein iodination (GE Healthcare Bio-Sciences, Tokyo, Japan). Unreacted [^{125}I] was removed by chromatography on a PD-10 column (Amersham Pharmacia Biotech).

Tissue Distribution in Tumor-Bearing Mice S180 cells (1×10^6 cells/mouse) were inoculated into the subcutaneous tissue of the axillary region of ddY mice. Fourteen days after the inoculation, mice were injected with [^{125}I]-PVA–SPTX (6 mg/kg) in 0.2 ml of saline through the tail vein. Aliquots of the sample solution were stored for the calibration of the disintegration of radioiodine. At appropriate times after the administration, blood was collected from the *vena cava* under ether anesthesia and the tumor tissue, liver, spleen, kidney, lung, and heart were excised and weighed. The radioactivity was determined with a gamma counter (Aloka 301).

***In Vivo* Antitumor Activity** S180 cells (1×10^6 cells/mouse) were inoculated into the subcutaneous tissue of the axillary region of ddY mice. PVA–SPTX (25 mg/kg in PTX equivalents) or PTX (25 mg/kg) was given as intermittent

intravenous injections on days 7, 10, 14, 17 and 21 after tumor inoculation. Saline was used as a negative control. Tumor sizes were measured with calipers, and the tumor volumes (V) were calcualted as: $V=L\times W^2/2$, where L and W are length (mm) and width (mm), respectively.

Analytical Methods The amount of PTX was determined by HPLC. Chromatography was carried out using a Shimadzu liquid chromatographic system (LC-6A, Kyoto, Japan) with a variable-wavelength UV detector (SPD-6A, Shimadzu) operated at 227 nm. A 4.6×150 mm, 5-μm particle size, C_{18} reversed-phase column (TSK gel ODS-80TM, Tosho, Japan) was used at ambient temperature. The mobile phase was a mixture of acetonitrile and 2 mM phosphoric acid (55 : 45, v/v). The injection volume was 20 μl, and the flow rate was 1.0 ml/min. For every experimental sample the content of PTX was calculated by measuring the relevant peak area and calibrating against the corresponding peak area derived from the *n*-hexyl *p*-hydroxybenzoate internal standard.

Three dimensional high-performance size-exclusion chromatography was carried out using a Shimadzu liquid chromatographic system (LC-9A, Kyoto, Japan) equipped with a variable-wavelength UV detector (MCPD-3600, Otsuka, Osaka, Japan). A 7.8×300 mm, TSKge1 G4000PWXL column (Tosoh) was used at 40 °C. The mobile phase was 20% acetonitrile in 50 mM LiCl. The injection volume was 50 μl, and the flow rate was 1.0 ml/min.

RESULTS

Preparation of PVA–SPTX Conjugate PTX was bound to PVA according to the synthetic scheme shown in Fig. 1. The overall synthetic pathway involved three steps. The first was the preparation of PVA–ethylenediamine, the second the preparation of SPTX and the third the binding of SPTX and PVA–ethylenediamine.

Hydroxyl group of PVA was activated by CDI. Then, a 10-fold weight excess of ethylenediamine was reacted with CDI-activated PVA. An excess of the ethylenediamine was used in order to prevent crosslinking and cyclisation of the PVA chains. The number of free amino groups of the PVA–ethylenediamine was estimated by the TNBS method.[30—32] One mole of PVA showed the color intensity of 25 mol of free amino group.

SPTX was synthesized by the modified method of Dosio *et al.*[33] The crude SPTX was purified by chromatography on a SiO_2 column. It was confirmed by the ^{1}H-NMR analysis that the hydroxyl group of 2′ position of PTX was reacted with succinic anhydride. The yield of SPTX was 94.0%.

Free carboxylic group of SPTX molecule was activated with twice molar of EDC and sufo-NHS, and then reacted with PVA–ethylenediamine. The yield of PVA–SPTX was 91.4%. The PTX content of the conjugates estimated by the UV method was 6.2 w/w%, corresponding to 5.8 mol of PTX/mol of PVA.

Size-exclusion chromatography of the conjugate was performed by HPLC on an TSK gel G4000PWXL column. Elution peaks were detected by a photodiode array detector. The retention time of the peak top of PVA–SPTX was 8.4 min in the cross section at 227 nm (Fig. 2). PVA–SPTX eluted at the same retention time to the original PVA detected by a differential refractometer.

Kinetics of Regeneration of PTX from the Conjugate The degradation of PTX was investigated in citrate and phosphate buffer solutions containing 1.5 M DENA over the pH

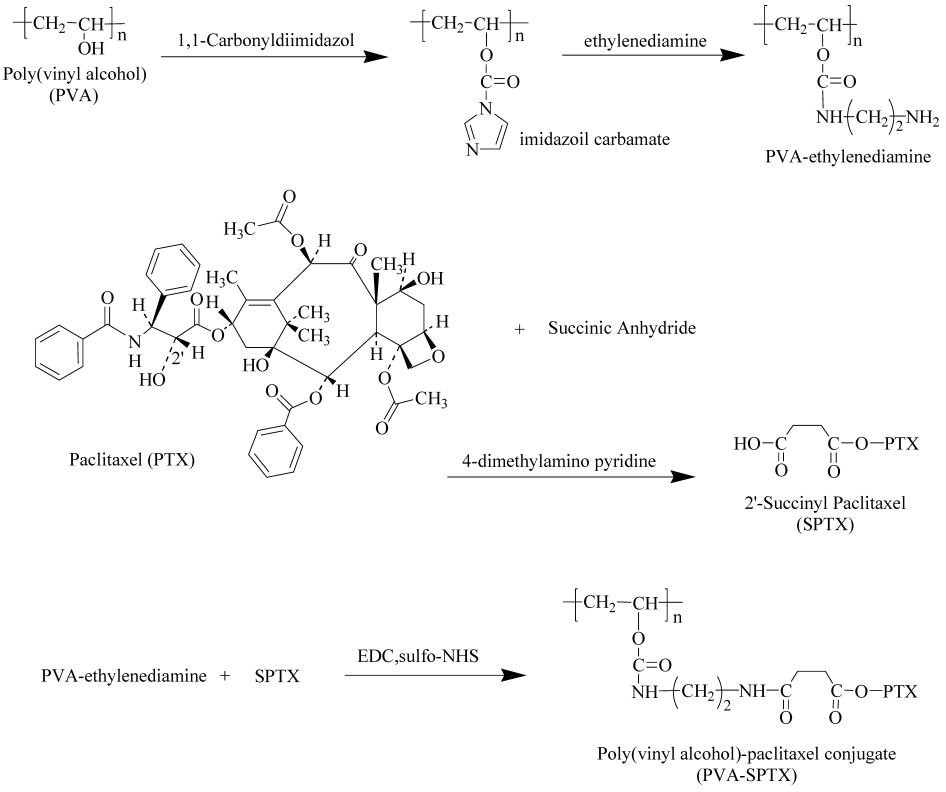

Fig. 1. Synthetic Pathway of PVA–SPTX Conjugate

EDC: 1-(3-dimethylaminopropyl)3-ethylcarbodiimide hydrochloride, sulfo-NHS: *N*-hydroxy-3-sulfo-succinimide.

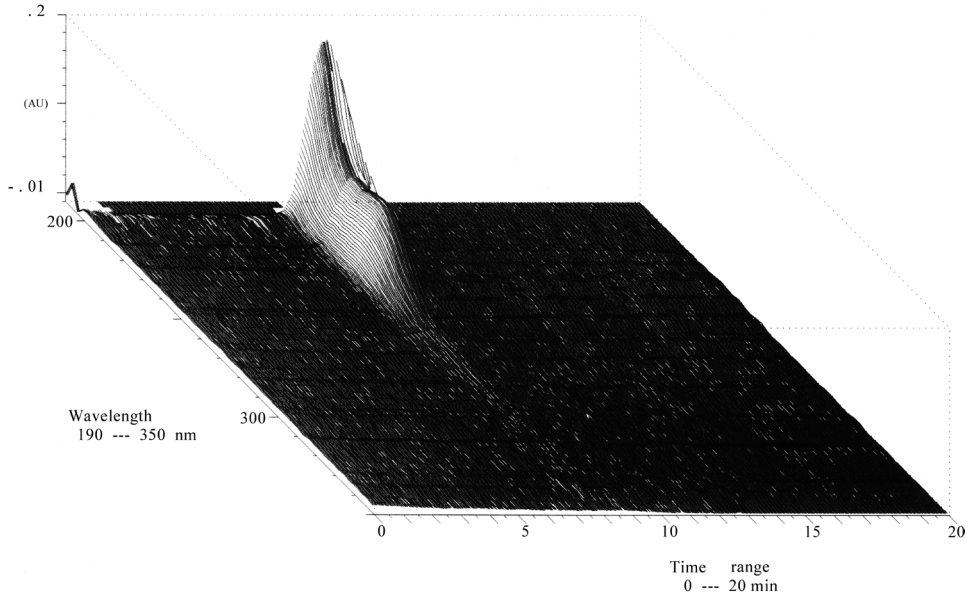

Fig. 2. Three Dimensional Chromatogram of PVA–SPTX

The retention time of the peak top of PVA–SPTX was 8.4 min in the cross section at 227 nm. High-performance size-exclusion chromatography was carried out using a HPLC system equipped with a photodiode array detector. A 7.8×300 mm, TSKge1 G4000PWXL column was used at 40 °C. The mobile phase was 20% acetonitrile in 50 mM LiCl and the flow rate was 1.0 ml/min.

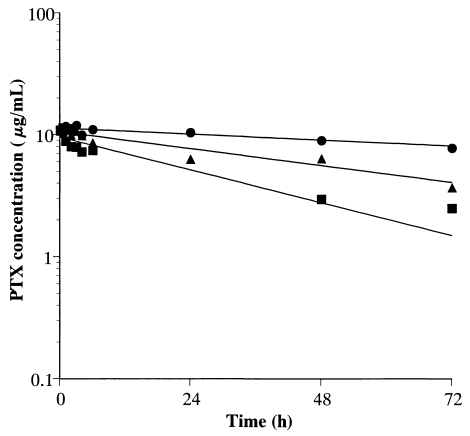

Fig. 3. Stability of PTX in 0.05 M Phosphate Buffer Solutions (μ=0.15) Containing 1.5 M DENA of pH 7.0 (●), pH 8.0 (▲) and pH 9.0 (■) at 37 °C

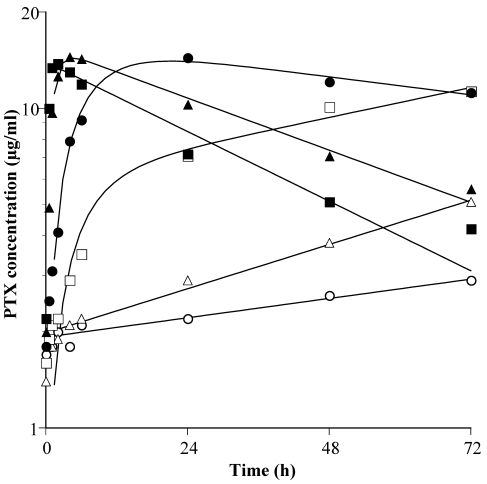

Fig. 4. Regeneration of PTX from PVA–SPTX in 0.05 M Citrate Buffer Solutions (μ=0.15) of pH 4.0 (○), 5.0 (△) or 6.0 (□) and in 0.05 M Phosphate Buffer Solutions (μ=0.15) of pH 7.0 (●), 8.0 (▲) or 9.0 (■) Containing 1.5 M DENA at 37 °C

range 4—9 at 37 °C. At pH 7—9 the degradation of PTX followed pseudo first-order kinetics as shown in Fig. 3, whereas PTX was quite stable at acidic conditions (pH 4—6). Time courses for PTX regenerated after incubation of PVA–SPTX in the buffer solutions are shown in Fig. 4. The apparent first-order rate constants for regeneration of PTX from PVA–SPTX (k_1) and for decomposition of PTX (k_2) were estimated by the curve fitting using a nonlinear least squares program (MULTI)[35] and are depicted in Fig. 5. The profiles of k_1 and k_2 showed pH-dependency in the investigated pH range.

***In Vitro* Cytotoxicity** *In vitro* biological efficacy of the soluble PVA–SPTX was tested using murine leukemia cell line L1210. The cytotoxicities of PVA–SPTX and PTX are shown in Fig. 6. As is evident, the cytotoxic effect of these compounds on L1210 cells was concentration dependent. PTX in DMSO showed the highest activity, whereas both PVA–SPTX and PTX in Purebright MB37-50T exhibited similar activity to that of the positive control of doxorubicin. The concentration of PVA–SPTX to produce a 50% inhibition of normal cell growth (IC_{50}) was 13.8 ng/ml (equivalent to PTX), whereas the IC_{50} of PTX in DMSO was 3.0 ng/ml.

Tissue Distribution in Tumor-Bearing Mice Mice were injected with [^{125}I]-PVA–SPTX (6 mg/kg). At appropriate times until 7 d, the radioactivities in a variety of organs were determined with a gamma counter. Figure 7 shows the time profile of the tissue distribution of [^{125}I]-PVA–SPTX after intravenous injection to mice. It was found that [^{125}I]-PVA–SPTX was retained in the blood circulation for several days and was gradually distributed into the tumorous tissue.

***In Vivo* Antitumor Activity** Figure 8 shows the growth inhibition curves against S180 subcutaneously inoculated in mice. To determine the effect of these drugs on S180 tumor growth, PVA–SPTX (25 mg/kg in PTX equivalents) or PTX (25 mg/kg) was given as intermittent intravenous injections

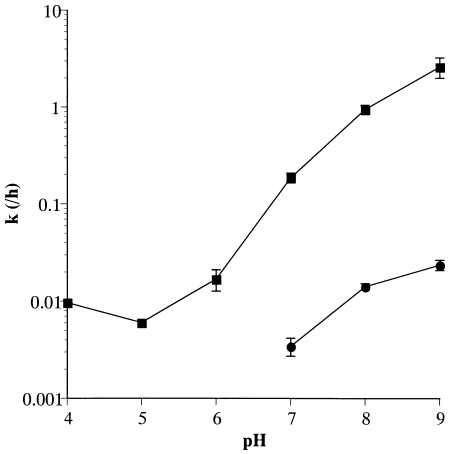

Fig. 5. pH Profile of Regeneration Rate Constant of PTX from PVA–SPTX (■) and Decomposition Rate Constant of PTX (●) in 0.05 M Citrate Buffer Solutions (μ=0.15) or Phosphate Buffer Solutions (μ=0.15) Containing 1.5 M DENA at 37 °C

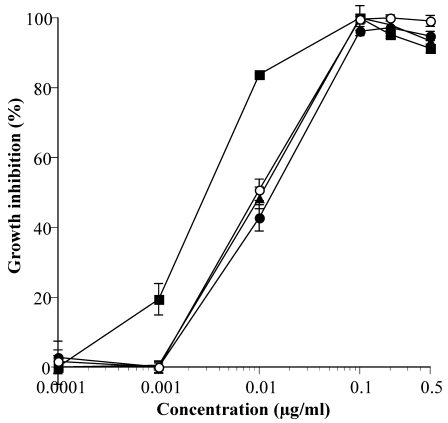

Fig. 6. Cytotoxicity of PVA–SPTX, PTX, and Doxorubicin against L1210 Cells

L1210 cells were cultivated in the medium containing each compound for 3 d, and the cells viability was determined by the alamar blue method. ●, PVA–SPTX; ▲, PTX (dissolved in Purebright MB37-50T solution); ■, PTX (dissolved in DMSO); ○, doxorubicin. Values are given as means±S.D. of three experiments.

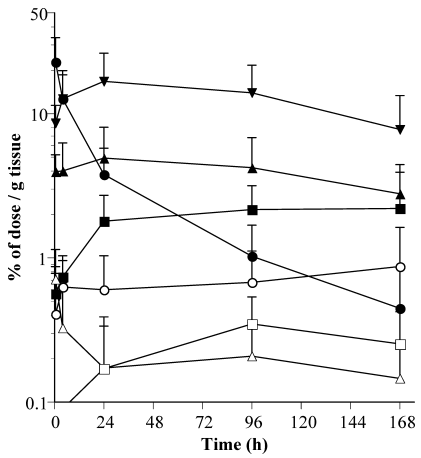

Fig. 7. Time Profile of Tissue Distribution of ^{125}I-PVA–SPTX after Intravenous Injection (6 mg/kg) to Mice

●, plasma; ▲, liver; ▼, kidney; ■, tumor; ○, spleen; △, lung; □, heart. Each point represents the mean±S.D. of three mice.

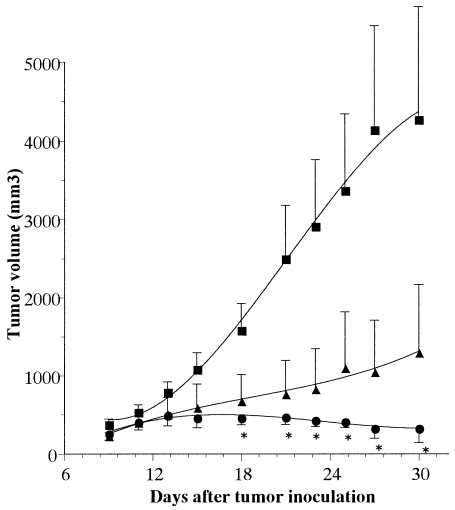

Fig. 8. Antitumor Effect of PVA–SPTX and PTX on Implanted S180 Tumor

Mice were inoculated with S180 cells (1×10^6 cells/mouse) subcutaneously. PVA–SPTX and free PTX were given as intermittent intravenous injections on days 7, 10, 14, 17 and 21 after the tumor inoculation. ●, PVA–SPTX (25 mg/kg in PTX equivalents); ▲, PTX (25 mg/kg); ■, control (5% glucose). Each point represents the mean±S.E. of six mice. Student's *t*-test was performed between control and PVA–SPTX, $*p<0.05$.

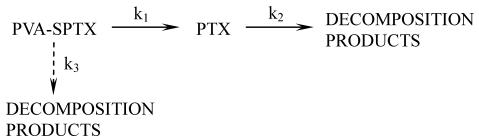

Chart 1

on days 7, 10, 14, 17 and 21 after tumor inoculation. PVA–SPTX caused significant tumor growth inhibition compared to the control. The control group of mice showed a progressive increase in tumor growth with the mean tumor volume increasing to 4278±1426 mm^3 on day 30 after tumor inoculation. The mice treated with PVA–SPTX showed significant tumor growth inhibition (mean tumor volume 329±187 mm^3) compared to the control group, which was almost similar to or less than the PTX-treated group (mean tumor volume 1304±864 mm^3). In this experiment, although both PTX and PVA–SPTX did not cause the side effect of body weight loss at 25 mg/kg in PTX equivalents, the macromolecular prodrug was expected to show the tolerability at the higher dose.

DISCUSSION

The kinetics of regeneration of PTX from the conjugate was examined in citrate and phosphate buffer solutions containing 1.5 M DENA over the pH range 4—9 at 37 °C. Since the degradation of PTX followed pseudo first-order kinetics, the overall reactions were described by Chart 1, where k_1, k_2 and k_3 are apparent first-order rate constants for the depicted reactions.

Then the concentration of PTX ([PTX]) has a time dependence given by the following equation:

$$[\mathrm{PTX}]=k_1[\mathrm{PTX}]^*/(k_2-(k_1+k_3))\times(\exp(-(k_1+k_3)t)-\exp(-k_2t)) \quad (1)$$

where [PTX]* is the initial concentration of PTX covalently

bound to PVA–SPTX. On the other hand, the stability of PTX is expressed by the following equation:

$$[PTX]=[PTX]_0 \exp(-k_2 t) \quad (2)$$

where $[PTX]_0$ is the initial concentration of PTX. At first the parameters of k_2 were calculated from the observed time courses shown in Fig. 3 using Eq. 2. Equation 1 was then fitted to the observed time courses shown in Fig. 4 using a nonlinear least squares program (MULTI).[35]

The model suggested in Chart 1 suffered the divergence in most of the computations; even if it was converged, the negative value of k_3 with an extraordinarily large standard deviation was obtained. When it was assumed that the degradationof PTX bound to PVA is negligible, however, the excellent convergence could be obtained in the curve fitting using the algorithm of Gauss–Newton method where the following equation was adopted instead of Eq. 1.

$$[PTX]=k_1[PTX]^*/(k_2-k_1)\times(\exp(-k_1 t)-\exp(-k_2 t)) \quad (3)$$

The apparent first-order rate constants for regeneration of PTX from PVA–SPTX (k_1) and for decomposition of PTX (k_2) are depicted in Fig. 5. The profiles of k_1 and k_2 showed pH-dependency in the investigated pH range. PTX was very stable at acidic conditions, but the decomposition was increased with the elevation of the pH. It was reported that PTX was susceptible to mild basic hydrolysis which resulted in the formation of baccatin III as the major product.[36] The regeneration rate of PTX was also enhanced with the rise in pH and reached a maximum at pH 9. At pH 7, the regeneration of PTX ($k_1=0.187\,h^{-1}$) was 54-times faster than the degradation of PTX ($k_2=0.00345\,h^{-1}$), showing half-lives of 3.7 and 201 h, respectively. These findings indicate that covalent binding to PVA stabilizes PTX ($k_3=0$) and the macromolecular prodrug, PVA–SPTX, releases PTX gradually at physiological conditions.

In addition, the amount of PTX covalently bound to PVA–SPTX was estimated by the value of [PTX]*, which was similar to that measured by the UV method. These findings indicated that approximately 100% of PTX was released from the conjugate.

In the *in vitro* release experiment, we observed that PTX released from the conjugate was precipitated as a white powder in buffer solutions. Consequently 1.5 M DENA was added to the release medium providing a chemical sink condition for the release of PTX from PVA–SPTX. Baek *et al.* reported that the PTX released from poly(lactic-co-glycol acid) (PLGA) matrices was much faster in DENA solution than in serum or phosphate buffer solution. They suggested that the presence of DENA in the release medium increased the hydrolysis rate of PLGA polymers.[37] However, in this study the regeneration rate of PTX from PVA–SPTX was not accelerated in 1.5 M DENA, being similar to those obtained in the ordinary buffer solutions, because PVA was stable in the 1.5 M DENA solution (data are not shown).

The *in vitro* cytotoxicity of PVA–SPTX was evaluated by using L1210 cells. PTX previously dissolved in a small amount of DMSO was a little more effective than the other compounds. Furthermore, one of the amphiphilic 2-methacryloxyethyl phosphorylcholine (MPC) polymers, Purebright MB37-50T, was also used as the solubilizer of PTX. It was reported that Purebright MB37-50T dissolved PTX as forming a polymeric lipid nanosphere.[38] As shown in Fig. 6, PVA–SPTX inhibited the proliferation of L1210 cells to the same extent as PTX dissolved in the Purebright MB37-50T. The cytotoxicity of PTX in the Purebright MB37-50T was similar to that reported by Wada *et al.*[39]

In the previous work, we examined the pharmacokinetics and biodisposition of PVA in experimental animals. [^{125}I] labeled PVA was retained in the blood circulation for several days after intravenous injection to mice. Although the tissue distribution of PVA was small, a significant accumulation in the liver, kidney and spleen was observed.[27] In this study, [^{125}I]-PVA–SPTX was also retained in the blood circulation very well and was gradually accumulated in the tumorous tissue as shown in Fig. 7. These findings indicated that PVA–SPTX was accumulated efficiently in the tumorous tissue by EPR effect.[22—24]

In conclusion, in this study the conjugate was generated from the combination of PVA and PTX for the first time. It was suggested that the water-solubility of PTX was markedly enhanced by the conjugation to PVA, and the conjugate, PVA–SPTX, effectively delivered PTX to the tumorous tissue due to the EPR effect. These studies demonstrated that PVA may be used as an effective solubilizing carrier for PTX.

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REFERENCES

1) Wani M. C., Taylor H. L., Wall M. E., Coggon P., McPhail A. T., *J. Am. Chem. Soc.*, **93**, 2325—2327 (1971).
2) Arbuck S. G., Christian M. C., Fisherman J. S., Cazenave L. A., Sarosy G., Suffness M., Adams J., Canetta R., Cole K. E., Friedman M. A., *J. Natl. Cancer Inst. Monogr.*, **15**, 11—24 (1993).
3) Cahan M. A., Walter K. A., Colvin O. M., Brem H., *Cancer Chemother. Pharmacol.*, **33**, 441—444 (1994).
4) Foa R., Norton L., Seidman A. D., *Int. J. Clin. Lab. Res.*, **24**, 6—14 (1994).
5) Long H. J., *Mayo Clin. Proc.*, **69**, 341—345 (1994).
6) Wiernik P. H., Einzig A. I., *J. Natl. Cancer Inst. Monogr.*, **15**, 185—187 (1993).
7) Lee J., Lee S. C., Acharya G., Chang C. J., Park K., *Pharm. Res.*, **20**, 1022—1030 (2003).
8) Fjallskog M. L., Frii L., Bergh J., *Lancet*, **342**, 8873—8875 (1993).
9) Panchagnula R., *Int. J. Pharm.*, **172**, 1—15 (1998).
10) Einzig A. I., Wiernik P. H., Sasloff J., Runowicz C. D., Goldberg G. L., *J. Clin. Oncol.*, **10**, 1748—1753 (1992).
11) Rogers B. B., *Oncol. Nurs. Forum*, **20**, 1483—1489 (1993).
12) Goldspiel B. R., *Ann. Pharmacother.*, **28**, S23—S26 (1994).
13) Li C., Yu D., Inoue T., Yang D. J., Milas L., Hunter N. R., Kim E. E., Wallace S., *Anticancer Drugs*, **7**, 642—648 (1996).
14) Greenwald R. B., Gilbert C. W., Pendri A., Conover C. D., Xia J., Martinez A., *J. Med. Chem.*, **39**, 424—431 (1996).
15) Li C., Price J. E., Milas L., Hunter N. R., Ke S., Yu D. F., Charnsangavej C., Wallace S., *Clin. Cancer Res.*, **5**, 891—897 (1999).
16) Li C., Yu D. F., Newman R. A., Cabral F., Stephens L. C., Hunter N., Milas L., Wallace S., *Cancer Res.*, **58**, 2404—2409 (1998).
17) Singer J. W., Baker B., De Vries P., Kumar A., Shaffer S., Vawter E., Bolton M., Garzone P., *Adv. Exp. Med. Biol.*, **519**, 81—99 (2003).

18) Sugahara S., Kajiki M., Kuriyama H., Kobayashi T. R., *Biol. Pharm. Bull.*, **25**, 632—641 (2002).
19) Ringsdorf H., *J. Polym. Sci. Polym. Symp.*, **51**, 135—153 (1975).
20) Seymour L. W., *Crit. Rev. Ther. Drug Carrier Syst.*, **9**, 135—187 (1992).
21) Yuan F., Dellian M., Fukumura D., Leunig M., Berk D. A., Torchilin V. P., Jain R. K., *Cancer Res.*, **55**, 3752—3756 (1995).
22) Maeda H., *Adv. Enzyme Regul.*, **41**, 189—207 (2001).
23) Maeda H., Wu J., Sawa T., Matsumura Y., Hori K., *J. Controlled Release*, **65**, 271—284 (2000).
24) Maeda H., Matsumura Y., *Crit. Rev. Ther. Drug Carrier Syst.*, **6**, 193—210 (1989).
25) Mallapragada S. K., McCarthy-Schroeder S., "Handbook of Pharmaceutical Controlled Release Technology," Marcel Dekker, New York, 2000, pp. 31—46.
26) Kakinoki A., Kaneo Y., Ikeda Y., Tanaka T., Fujita K., *Biol. Pharm. Bull.,* **31**, 103—110 (2008).
27) Kaneo Y., Hashihama S., Kakinoki A., Tanaka T., Nakano T., Ikeda Y., *Drug Metab. Pharmacokinet.*, **20**, 435—442 (2005).
28) Hearn M. T., *Methods Enzymol.*, **135**, 102—117 (1987).
29) Beauchamp C. O., Gonias S. L., Menapace D. P., Pizzo S. V., *Anal. Biochem.*, **131**, 25—33 (1983).
30) Habeeb A. F., *Anal. Biochem.*, **14**, 328—336 (1966).
31) Fields R., *Biochem. J.*, **124**, 581—590 (1971).
32) Fields R., *Methods Enzymol.*, **25**, 464—468 (1972).
33) Dosio F., Brusa P., Crosasso P., Arpicco S., Cattel L., *J. Controlled Release*, **47**, 293—304 (1997).
34) de Fries R., Mitsuhashi M., *J. Clin. Lab. Anal.*, **9**, 89—95 (1995).
35) Yamaoka K., Tanigawara Y., Nakagawa T., Uno T., *J. Pharmacobio-Dyn.*, **4**, 879—885 (1981).
36) Ringel I., Horwitz S. B., *J. Pharmacol. Exp. Ther.*, **242**, 692—698 (1987).
37) Baek N., Lee J., Park K., *J. Biomater. Sci. Polym. Ed.*, **15**, 527—542 (2004).
38) Konno T., Watanabe J., Ishihara K., *J. Biomed. Mater. Res. A*, **65**, 209—214 (2003).
39) Wada M., Jinno H., Ueda M., Ikeda T., Kitajima M., Konno T., Watanabe J., Ishihara K., *Anticancer Res.*, **27**, 1431—1435 (2007).