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PEG liposomalization of paclitaxel improved its in vivo disposition and anti-tumor efficacy

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ABSTRACT

To find out potent paclitaxel (PTX) formulations for cancer chemotherapy, we formulated PTX in O/W emulsion and liposome selected as candidates of nanocarriers for PTX. Surface modification of these nanoparticles with polyethylene glycol (PEG) improved their in vivo behavior, but the effect of PEGylation on the pharmacokinetics of emulsion was not so remarkable and the release of PTX from emulsion was found to be very fast in blood circulation, indicating that emulsion would not be an adequate formulation for PTX. On the other hand, AUC of PEG liposome was 3.6 times higher than that of naked liposome after intravenous injection into normal rats due to the lower disposition into the reticuloendothelial system tissues such as liver and spleen. Since PEG liposome was able to stably encapsulate PTX in blood, AUC of PTX was also extensively enhanced after intravenous dosing of PTX-PEG liposome into normal rats. In the in vivo studies utilizing Colon-26 solid tumor-bearing mice, it was confirmed that PTX-PEG liposome delivered significantly larger amount of PTX to tumor tissue and provided more excellent anti-tumor effect than PTX-naked liposome. These results suggest that PEG liposome would serve as a potent PTX delivery vehicle for the future cancer chemotherapy.

1. Introduction

Paclitaxel (PTX) is one of the most useful anticancer agents which have been used for various cancers including ovarian, breast, head and neck, and non-small cell lung cancers (Rowinsky and Donehower, 1995). Due to its poor aqueous solubility, the commercially available product for intravenous infusion of PTX, known as Taxol®, formulates PTX in a 1:1 combination of Cremophor EL (polyethoxylated castor oil) and dehydrated ethanol. However, some drawbacks have been reported for its clinical applications of this formulation such as severe hypersensitivity reactions, neurotoxicity and neutropenia (Lorenz et al., 1977; Dye and Watkins, 1980). It was reported that these adverse effects associated with this formulation would be due to Cremophor EL rather than PTX itself (Weiss et al., 1990). In addition, upon contact of this formulation with polyvinyl chloride infusion sets, Cremophor EL causes leaching of plasticizer diethylhexylphtalate. Therefore, the use of non-plasticized containers is indispensable (Dye and Watkins, 1980).

To avoid these adverse effects and inconvenience of this formulation, alternative formulations have been exploited for both parenteral and oral delivery of PTX. Polymeric micro- and nanoparticles (Bilensoy et al., 2008), lipid nanoparticles (Arica Yegin et al., 2006), polymeric micelles (Kim et al., 2001; Hamaguchi et al., 2005; Hu et al., 2006), liposomes (Sharma and Straubinger, 1994; Torchilin, 2005; Yang et al., 2007a,b) and emulsions (Liu and Liu, 1995; Lundberg, 1997; Tamilvanan, 2004; Rossi et al., 2007) have been examined, and all of these formulations achieved the improvement of PTX solubility. Particulate preparations as described above are expected to be accumulated preferentially in many types of solid tumors due to the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Maeda et al., 2000), because generally most solid tumors possess unique pathophysiological characteristics that are not observed in normal tissues/organs, such as extensive angiogenesis, defective vascular architecture and impaired lymphatic drainage/recovery system. To date, various nanoparticles that exhibit the long blood-circulating property have been shown to preferentially accumulate in tumors due to the leakiness of microvasculature in solid tumors (Unezaki et al., 1996; Gabizon et al., 2006; Heyes et al., 2006). However, the uptake of nanoparticles by macrophages in the reticuloendothelial system (RES) should be avoided to achieve an efficient EPR effect-based passive targeting of drugs included (Poste et al., 1982; Moghimi and Patel, 1998; Ishida et al., 2002). Various strategies, therefore, have been developed including the modification of the surface of nanoparticles with ganglioside GM1, phosphatidylinositol or polyethylene glycol (PEG) (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen and Hansen, 1991; Allen et al.,

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1991; Maruyama et al., 1999). Among them, the surface modification with PEG, PEGylation, is known to be one of the most successful approaches to prolong blood circulating time of nanoparticles, which leads to better EPR effect-based tumor targeting of drugs encapsulated (Allen et al., 1991; Gabizon et al., 2003). Although some earlier studies have indicated that several nanoparticulate formulations of PTX provided favorable in vivo behavior of PTX (Crosasso et al., 2000; Yang et al., 2007b), there are few reports describing in vivo disposition characteristics of both PTX and nanoparticles.

In this study, we prepared PTX-nanoparticulate formulations, e.g. PEGylated emulsion and liposome, based on the previously reported studies (Kan et al., 1999; Yang et al., 2007b), and evaluated these formulations from the aspects of in vivo disposition characteristics of both nanoparticles labeled with ^{3}H-cholesteryl hexadecylether (^{3}H-CHE) and ^{14}C-PTX. Furthermore, PTX release properties for the formulations and their in vivo anti-tumor activity were also assessed and compared to find out a potent nanoparticulate formulation for PTX.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (EPC), Tween 80, Tricaprylin (C8), Cremophor EL, Cholesterol (Chol), 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT), RPMI-1640, fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Sigma (St. Louis, MO, USA). Gentamicin was purchased from Gibco BRL, Co. (Grand Island, NY, USA). Tricaproin (C6) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Distearoyl phosphatidylethanolamine-N-[methoxy poly (ethylene glycol)-2000] (PEG-DSPE) and hydrogenated soybean phosphatidylcholine (HSPC) were purchased from NOF Inc. (Tokyo). Paclitaxel (PTX) was kindly gifted from Sawai Pharmaceutical Co., Ltd. (Osaka, Japan). [^{3}H] Cholesteryl hexadecylether (^{3}H-CHE) and PTX [2-benzoyl ring(U)-^{14}C] (^{14}C-PTX) were purchased from Perkin Elmer Life Science Inc. (Boston, MA, USA) and Moravek Biochemicals Inc. (Brea, CA, USA), respectively. All other chemicals were commercial products of the finest grade.

2.2. Cells

Colon-26 carcinoma cells (C26) were kindly provided from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). C26 were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 20 μg/mL gentamicin at 37 °C under 5% CO_2/95% air.

2.3. Preparation of emulsions

O/W emulsion of PTX, "naked emulsion", was prepared based on the method reported previously with minor modifications (Kan et al., 1999). In brief, EPC, Tween 80, C6, C8 and PTX (160:120:300:100:1.2, weight ratio) were dissolved in chloroform with tracer amounts of ^{3}H-CHE and ^{14}C-PTX. For the preparation of PEGylated emulsion (PEG emulsion), PEG-DSPE was further added to the mixture (5 mol%). Then, the oil phase was dried under reduced pressure at 50 °C. Aqueous phase composed of 2.25% (w/v) glycerol was mixed with the oil phase, followed by the sonication for 30 min at 4 °C using a probe-type sonicator (50 W, Ohtake Works, Tokyo).

2.4. Preparation of liposomes

Both the conventional "naked" liposome (HSPC:Chol:PTX = 90:10:8, molar ratio) and PEG liposome (HSPC:Chol:PEG-DSPE:PTX = 90:10:5:8, molar ratio) were prepared by the thin-film hydration method (Yang et al., 2007a,b). In brief, PTX, HSPC, Chol and PEG-DSPE were dissolved in chloroform with tracer amounts of ^{3}H-CHE and ^{14}C-PTX. After evaporation of the solvent at 75 °C, the lipid mixture was dried in vacuo at room temperature over night. The resultant dried lipid film was hydrated with phosphate-buffered saline (PBS, pH 4.0) under mechanical agitation. The obtained multilamellar preparations were sized by repeated extrusion through polycarbonate membrane filters (Millipore, Bedford, MA) with the pore size of 200 nm followed by further extrusion through polycarbonate membrane filters with 100-nm pore size. The resulting liposomes were passed through a Sephadex G-25 column (Amersham Bioscience, Uppsala, Sweden) equilibrated with PBS (pH 7.4) to change the pH of the external phase and remove non-encapsulated PTX. Entrapment efficacies into both liposomes were determined to be approximately 34% reproducibly.

2.5. Size distribution and zeta potential of PTX-emulsions and PTX-liposomes

Particle sizes and zeta potentials of prepared emulsions and liposomes were determined by a dynamic light scattering spectrophotometer (Zetasizer Nano, Malvern Instruments Ltd., Worcestershire, UK).

2.6. Animals and preparation of tumor-bearing mice model

Male Wistar rats (7–8 weeks) and male BALB/c mice (6–7 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and from Charles River Laboratories Japan Inc. (Yokohama, Japan), respectively. Animals, maintained at 25 °C and 55% humidity, were allowed free access to standard chow and water. To prepare tumor-bearing mice, 10^{6} cells of C26 were subcutaneously inoculated into the back of mice. Our investigations were performed after approval by our local ethics committee at Okayama University and in accordance with the Principles of Laboratory Animal Care (NIH publication #85-23).

2.7. In vivo disposition experiments

After normal rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg), PTX-nanoparticles were injected into the femoral vein at a dose of 1 mg/kg as PTX. Blood samples were withdrawn from the jugular vein at fixed time periods, followed by immediate centrifugation at 4000 × *g*. The obtained plasma was collected (100 μL) and scintillation medium (Clear-sol II, Nacalai Tesque, Kyoto) was added. Then, the radioactivity derived from [^{3}H] and [^{14}C] was simultaneously, but separately measured in a liquid scintillation counter (TRI-CARB® 2260XL, Packard Instrument Inc., Meriden, CT, USA). In the case of the studies for tumor-bearing mice, PTX-nanoparticles were intravenously administered through the tail vein at a dose of 1 mg/kg as PTX. At each collection time point, blood was collected from the vena cava and the mice were then sacrificed at the end of experiment. The organs were excised, rinsed with saline and weighed. To solubilize the organs, Soluvable (PerkinElmer Inc., MA) was added to the minced organs and incubated for 2 h at 60 °C before the solubilized solution was neutralized by HCl. Then, scintillation medium was added to the samples, and the radioactivity was measured in the liquid scintillation counter as described above.

2.8. Pharmacokinetic analysis

Plasma concentrations of both nanoparticles themselves and PTX included (C_p) versus time curves were analyzed by Eq. (1) using the non-linear least-squares regression program MULTI (Yamaoka et al., 1981).

$$C_p = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad (1)$$

Area under plasma concentration–time curve (AUC) was calculated by the following equation:

$$AUC_0^t = \int_0^t C_p dt \quad (2)$$

Total body clearance (CL_{total}), distribution volume of central compartment (Vd_c), Vd at steady state (Vd_{ss}), volume of distribution β (Vd_β), and elimination rate constant (k_{el}) were calculated by the following equations:

$$CL_{total} = \frac{Dose}{AUC_0^\infty} \quad (3)$$

$$Vd_c = \frac{Dose}{A+B} \quad (4)$$

$$Vd_{ss} = \left(1 + \frac{k_{12}}{k_{21}}\right) \cdot Vd_c \quad (5)$$

$$Vd_\beta = \frac{Dose}{AUC_0^\infty \cdot \beta} \quad (6)$$

$$k_{el} = \frac{CL_{total}}{Vd_c} \quad (7)$$

where AUC_0^∞ means AUC value from 0 to infinity, and k_{12} and k_{21} represent the first-order transfer rate constants from the central to peripheral compartment and from the peripheral to central compartment, respectively.

In the cases of PEG liposome and PEG liposomal PTX in rats, one compartment model was more adequate for the analysis. Therefore, Eq. (8) was used to analyze the plasma concentration–time profile and to obtain the distribution volume (Vd) and k_{el}.

$$C_p = \frac{Dose}{Vd} \cdot e^{-k_{el}t} \quad (8)$$

Tissue uptake clearance (CL_{tissue}) was calculated by the following equation:

$$CL_{tissue} = \frac{X_{tissue}^t}{AUC_0^t} \quad (t = 24\,h) \quad (9)$$

where AUC_0^t means AUC value from 0 to time t and X_{tissue}^t represents the amount of emulsions or liposomes in a tissue at time t. In the case of tumor-bearing mice model, AUC values in the organs such as liver, spleen and tumor, were calculated by the trapezoidal rule.

2.9. Evaluation of PTX release profile based on MTT assay

Since cytotoxic effect of PTX included in nanoparticles is thought to be a release-limited, the cytotoxic effect of PTX-nanoparticles on C26 cells was evaluated using MTT assay to estimate release properties of nanoparticulate formulations (Ogawara et al., 2008). In brief, C26 cells were seeded (7000 cells/well) in a 96-well plate (Asahi Techno Glass, Chiba, Japan). After the plate was incubated at 37 °C for 12 h in a humidified 5% CO_2 atmosphere, the medium with various concentrations of PTX added as either solution, emulsions or liposomes was given to each well in the plate. After incubation for 48 h, each well was washed and rinsed with fresh medium. MTT solution (0.5 mg/mL) was then added to each well and the cultures were further incubated for 4 h. Formazan crystals were dissolved in 0.04 M HCl-isopropanol and subjected to sonication in a bath-type sonicator (ASONE Corporation, Osaka, Japan) for 15 min. Each plate was set into a microplate reader (Model 680, Bio-Rad, Hercules, CA) and absorbance values were measured at 570 nm (test wavelength) and 750 nm (reference wavelength). Results were expressed as cell viability (%) by the following formula:

$$\text{Cell viability (\%)} = \frac{OD_{570,sample} - OD_{750,sample}}{OD_{570,control} - OD_{750,control}} \times 100 \quad (10)$$

where sample and control mean the cells with and without PTX, respectively.

Cell viability (%) was plotted against PTX concentration and the plots were fitted with the Hill-type equation (Eq. (11)) (Eghbali et al., 2003; Lim et al., 2004) using the non-linear least-squares regression program MULTI (Yamaoka et al., 1981). The concentration at which 50% of cells survived corresponds to IC_{50}.

$$E = \frac{E_0 \times IC_{50}}{IC_{50} + C} \quad (11)$$

where E or E_0 is the percentage of cell survival with or without PTX treatment, respectively, and C is the final concentration of PTX in each well. Each experiment was performed using four replicated wells for each PTX concentration and carried out independently five times. In our preliminary experiments, any significant cytotoxicity was not observed for empty naked emulsions, empty PEG emulsions, empty naked liposomes or empty PEG liposomes at all.

2.10. Stability of PTX nanoparticles in serum

Stability of PTX-nanoparticles in serum was evaluated using the gel filtration method. In brief, ^{14}C-PTX-emulsions or liposomes labeled with ^{3}H-CHE incubated in rat serum for 30 min at 37 °C, were loaded onto the Sepharose CL-4B gel column (Amersham Bioscience, Uppsala) and eluted with PBS (pH 7.4) followed by the fractional collection. Then, the radioactivity of collected effluents was measured by the liquid scintillation counter as described above.

2.11. In vivo anti-tumor activity

When the tumor volume reached about 100 mm^3 after inoculation of C26 cells, PTX-emulsions or liposomes were intravenously administered through the tail vein at a dose of 0.5 mg/kg as PTX. Saline solution or PTX solution, which was prepared by dissolving PTX in a vehicle containing the mixture of equal volume of Cremophor EL and ethanol, was injected to mice of control or PTX solution-treated group, respectively. The size of tumor was measured every other day with a caliper in two dimensions, and the tumor volume was calculated using the following equation (Lee et al., 2005):

$$\text{Tumor volume (mm}^3\text{)} = \text{longer diameter} \times (\text{shorter one})^2 \times 0.52 \quad (12)$$

A slope of tumor volume–time curve, representing the growth rate of each tumor in the treatment group, was obtained from days 4 to 18 after injection. The T/C index of growth rate for the in vivo therapeutic effect of tumor-bearing mice model was obtained by dividing the growth rate of the treatment group (T) by that of the saline-treated control group (C). In addition, mean survival time (MST) after each treatment was measured. The T/C index of MST for tumor-bearing mice model was similarly obtained by dividing the MST of the treatment group (T) by that of the saline-treated control group (C).

2.12. Statistical analysis

Results are expressed as the mean ± S.D. of more than three experiments. Analysis of variance (ANOVA) was used to test the

Table 1
Physicochemical properties of PTX-nanoparticles.

Formulation	Composition	Ratio	Particle size (nm)	Zeta potential (mV)
Naked emulsion	EPC:Tween 80:C6:C8:PTX	160:120:300:100:1.2 (weight ratio)	98.2 ± 9.4	−1.88 ± 0.45
PEG emulsion	EPC:Tween 80:C6:C8:PTX:PEG-DSPE	160:120:300:100:1.2:29 (weight ratio)	102.9 ± 9.9	−2.18 ± 0.54
Naked liposome	HSPC:Chol:PTX	90:10:8 (molar ratio)	107.0 ± 5.3	−0.02 ± 1.71
PEG liposome	HSPC:Chol:PTX:PEG-DSPE	90:10:8:5 (molar ratio)	105.3 ± 3.5	−4.20 ± 1.18

Results are expressed as the mean ± S.D. ($n = 3$).

statistical significance of differences among groups. Statistical significance in the differences of the means was determined using Student's *t*-test or Dunnett's test for the single or multiple comparisons of experimental groups, respectively.

3. Results and discussion

Since several factors such as particle size, surface charge and composition of the formulation have been reported to influence the in vivo fate of nanoparticles after intravenous administrations (Ishida et al., 2002; Levchenko et al., 2002; Awasthi et al., 2003), the physicochemical properties of PTX-nanoparticles prepared were assessed. As shown in Table 1, particle sizes were around 100 nm for all the PTX-nanoparticles prepared in the present study. Zeta potential of them showed that surface charges of these PTX-nanoparticles were weakly negative or almost neutral. Earlier studies have demonstrated that nanoparticles with the size of around 100 nm and with weak negative charge would exhibit favorable in vivo behavior for the efficient EPR effect-based tumor disposition (Ishida et al., 1999; Gabizon and Papahadjopoulos, 1992). Therefore, the physicochemical properties of PTX formulations prepared in this study would be adequate.

Then, we have investigated the in vivo pharmacokinetics of PTX-nanoparticles after intravenous administration into normal rats. In this study, the in vivo disposition characteristics of nanoparticles themselves and of the drug included were separately evaluated using nanoparticles labeled with ^{3}H-cholesteryl hexadecylether (^{3}H-CHE) including ^{14}C-PTX. Fig. 1A shows plasma concentration–time profiles of naked and PEG emulsions and PTX included, and the pharmacokinetic parameters obtained are summarized in Table 2. PEGylation significantly increased AUC value of emulsion, but the extent of increase was not so remarkable. In addition, PEG emulsion provided a significantly smaller k_{el} than naked one, but Vd_c, Vd_{ss} and Vd_β values were similar for PEG and naked emulsions. On the other hand, although PEGylation was also found to significantly increase AUC of PTX and decrease Vd_c of PTX, plasma concentrations of PTX were much lower than those of corresponding emulsions just after dosing. The ratio of PTX to emulsion in AUC was calculated to be 0.26 for both naked and PEG emulsions. The difference in hepatic uptake clearance between the two emulsions was also quite small, although the clearance was reduced by PEGylation. These results suggest that PEGylation would not be so effective for PTX emulsion and the release of PTX from the two emulsions would be so rapid after intravenous administration.

Fig. 1B shows plasma concentration–time profiles of naked and PEG liposomes and PTX included after intravenous dosing. In contrast to the case of emulsions, PEGylation dramatically increased plasma concentrations of liposome and provided AUC that was about 3.6 times larger than that of naked one (Table 2). Plasma concentrations of PTX were also extensively increased for PEG liposome, providing the value of AUC that was around 3.6 times larger than that for naked liposome. The ratio of PTX to liposome in AUC was approximately 0.7 for both naked and PEG liposomes, indicating that PTX would be incorporated within liposomes much more stably than emulsions. Therefore, the pharmacokinetics of PTX was very similar to that of liposome for both liposomes. The value of β (0.06 ± 0.02) calculated for naked liposome was not so different from k_{el} value of PEG liposome (0.05 ± 0.01). However, a very large value of α (3.68 ± 2.19) was obtained for naked liposome and total body clearance (CL_{total}) of PEG liposome was, therefore, significantly smaller than that of naked one (Table 2). In addition, it was found that Vd of PEG liposome was significantly smaller than Vd_β and Vd_{ss} of naked liposome (Table 2). The decrease in distribution volume and disappearance of the initial distribution phase in plasma concentration profile by PEGylation of liposome would mean the decrease in rapid distribution to the liver just after dosing. Tissue uptake clearances calculated for RES tissues such as the liver

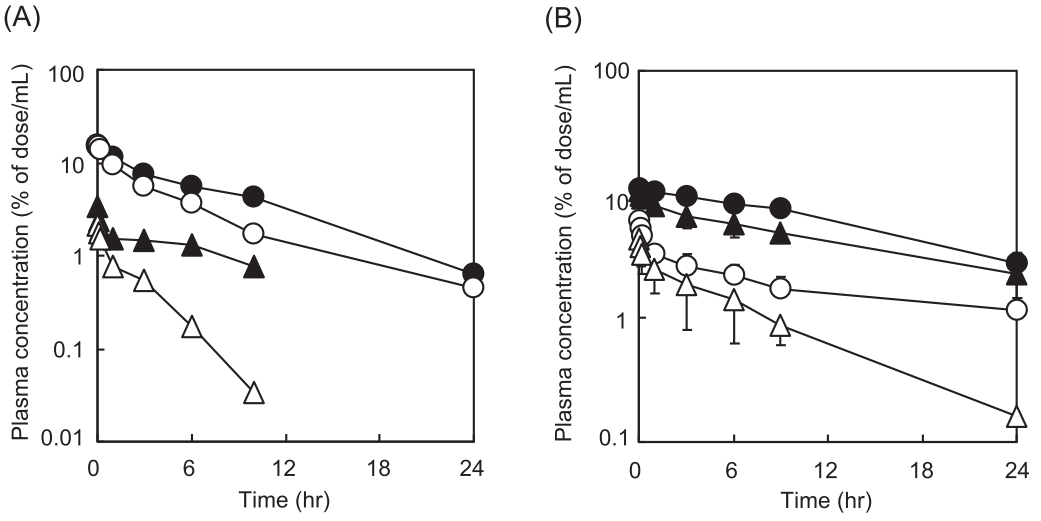

Fig. 1. Plasma concentration–time profiles of nanoparticles and PTX after intravenous injection of PTX-emulsions (A) and PTX-liposomes (B) into normal rats at a dose of 1 mg/kg as PTX. Keys: open circle, naked nanoparticles; closed circle, PEGylated nanoparticles; open triangle, PTX for naked nanoparticles; closed triangle, PTX for PEGylated nanoparticles. Results are expressed as the mean with the vertical bar showing S.D. ($n = 4$–6).

Table 2
Pharmacokinetic parameters of PTX-formulations after intravenous injection into normal rats.

Nanoparticles	Detected	AUC_0^∞ (% of dose/mL h)	CL_{tot} (mL/h)	Vd_c (mL)	Vd_{ss} (mL)	Vd_β (mL)	k_{el} (h^{-1})	α (h^{-1})	β (h^{-1})	CL_{liver} (mL/h)	CL_{spleen} (mL/h)
Naked emulsion	Emulsion	54.0 (4.67)	1.86 (0.16)	6.44 (0.40)	9.36 (1.06)	9.73 (1.18)	0.29 (0.01)	3.03 (1.36)	0.19 (1.18)	0.56 (0.04)	0.01 (0.002)
	PTX	14.0 (2.57)	7.33 (1.23)	39.2 (1.44)	77.3 (17.1)	80.2 (20.0)	0.19 (0.03)	4.73 (1.50)	0.10 (0.03)	–[a]	–[a]
PEG emulsion	Emulsion	102.1** (13.3)	0.99** (0.13)	6.75 (0.51)	8.69 (1.03)	8.99 (1.18)	0.15** (0.01)	1.23 (0.25)	0.11*** (0.004)	0.38* (0.06)	0.002** (0.001)
	PTX	26.4** (3.33)	3.83** (0.45)	23.5** (2.17)	58.3 (8.23)	58.6 (8.25)	0.17 (0.03)	17.9 (3.05)	0.07 (0.005)	–[a]	–[a]
Naked liposome	Liposome	57.6 (19.5)	1.93 (0.72)	14.5 (3.03)	32.0### (5.71)	32.3### (5.89)	0.13 (0.04)	3.68† (2.19)	0.06 (0.02)	1.23 (0.42)	0.35 (0.15)
	PTX	41.4 (20.6)	3.25 (2.18)	14.2 (2.65)	31.0## (9.68)	31.5## (9.75)	0.14 (0.03)	2.79† (0.85)	0.11 (0.08)	–[a]	–[a]
PEG liposome	Liposome	209.9*** (36.0)	0.49** (0.08)	8.88[b] (0.45)	–[c]	–[c]	0.05 (0.01)	–[c]	–[c]	0.14* (0.05)	0.05** (0.01)
	PTX	150.9*** (34.8)	0.80* (0.12)	10.9[b] (1.41)	–[c]	–[c]	0.07 (0.02)	–[c]	–[c]	–[a]	–[a]

Pharmacokinetic parameters were calculated by following the methods described in Section 2.8. Results are expressed as the mean with S.D. in parentheses ($n = 6$).
[a] Not calculated.
[b] Vd, obtained by analysis with one-compartment model.
[c] Not available because of analysis with one-compartment model.
* $p < 0.05$, compared with the corresponding naked nanoparticle.
** $p < 0.01$, compared with the corresponding naked nanoparticle.
*** $p < 0.001$, compared with the corresponding naked nanoparticle.
$p < 0.01$, compared with Vd of PEG liposome
$p < 0.001$, compared with Vd of PEG liposome.
† $p < 0.05$, compared with k_{el} of PEG liposome.

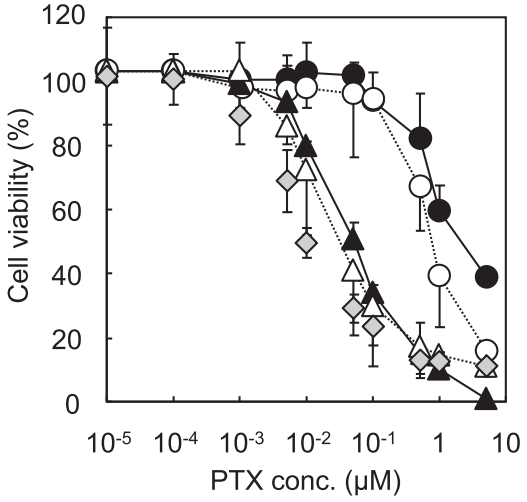

Fig. 2. MTT assay for evaluation of PTX release from nanoparticles. Keys: filled diamond, PTX solution; open triangle, naked emulsion; closed triangle, PEG emulsion; open circle, naked liposome; closed circle, PEG liposome. Results are expressed as the mean with the vertical bar showing S.D. ($n = 5$).

and spleen would support the above consideration. PEG liposome showed very small clearances for the liver and spleen, which were approximately one tenth of those for naked liposome (Table 2). These results clearly indicate that PEGylation markedly reduced the affinity of liposome for the liver and spleen, main organs for liposomes to be distributed to, resulting in the prolonged blood residence time of liposomes. It has been generally considered that PEGylation forms a fixed aqueous layer on the surface of nanoparticles via interaction between PEG and water molecule (Shimada et al., 1995), leading to reduced amounts of serum opsonins associated on the surface, thereby prolonging blood circulation time of nanoparticles (Sadzuka et al., 2002; Shehata et al., 2008; Yokoe et al., 2008). It would also be the case for PEG liposome containing PTX prepared in the present study. In contrast, the reason why PEGylation of emulsion was not so efficient would be speculated as follows. Since Tween 80 used as a surfactant to prepare emulsion has a polyoxyethylene chain in its structure, the molecules of Tween 80 locating at the interface between water and oil phase of emulsion may have exhibited the similar function as PEG molecules do. As a result, PEGylation would not have been able to show additional effect on the in vivo behavior of naked emulsion. However, the detailed mechanisms behind these observations for PEG emulsion including the conformational and functional properties of Tween 80 on the surface of emulsion remain to be elucidated and will be the subject of our further studies.

Since it was suggested that the release of PTX from emulsions would be much faster than that from liposomes (Fig. 1 and Table 2), we tried to evaluate release properties of PTX from these nanoparticles by applying MTT assay (Fig. 2). Because it is considered that endocytotic uptake of these nanoparticles by tumor cells would hardly take place, PTX included in the nanoparticles must be first released into the medium to be taken up by tumor cells via passive diffusion. Therefore, the release process should be the rate-limiting step in the uptake of PTX by tumor cells, and the concentration-dependent cytotoxic effect of PTX would reflect the PTX release profile from nanoparticles. IC_{50} values of naked and PEG emulsions (0.049 ± 0.028 μM ($n = 5$) and 0.11 ± 0.092 μM ($n = 5$), respectively) calculated based on cell viability–PTX concentration profiles, were found to be slightly higher than that of PTX-solution (0.009 ± 0.006 μM ($n = 5$)) and these differences were not statistically significant. On the other hand, IC_{50} values of naked and PEG liposomes (0.91 ± 0.11 μM ($n = 5$) and 1.8 ± 0.58 μM ($n = 5$),

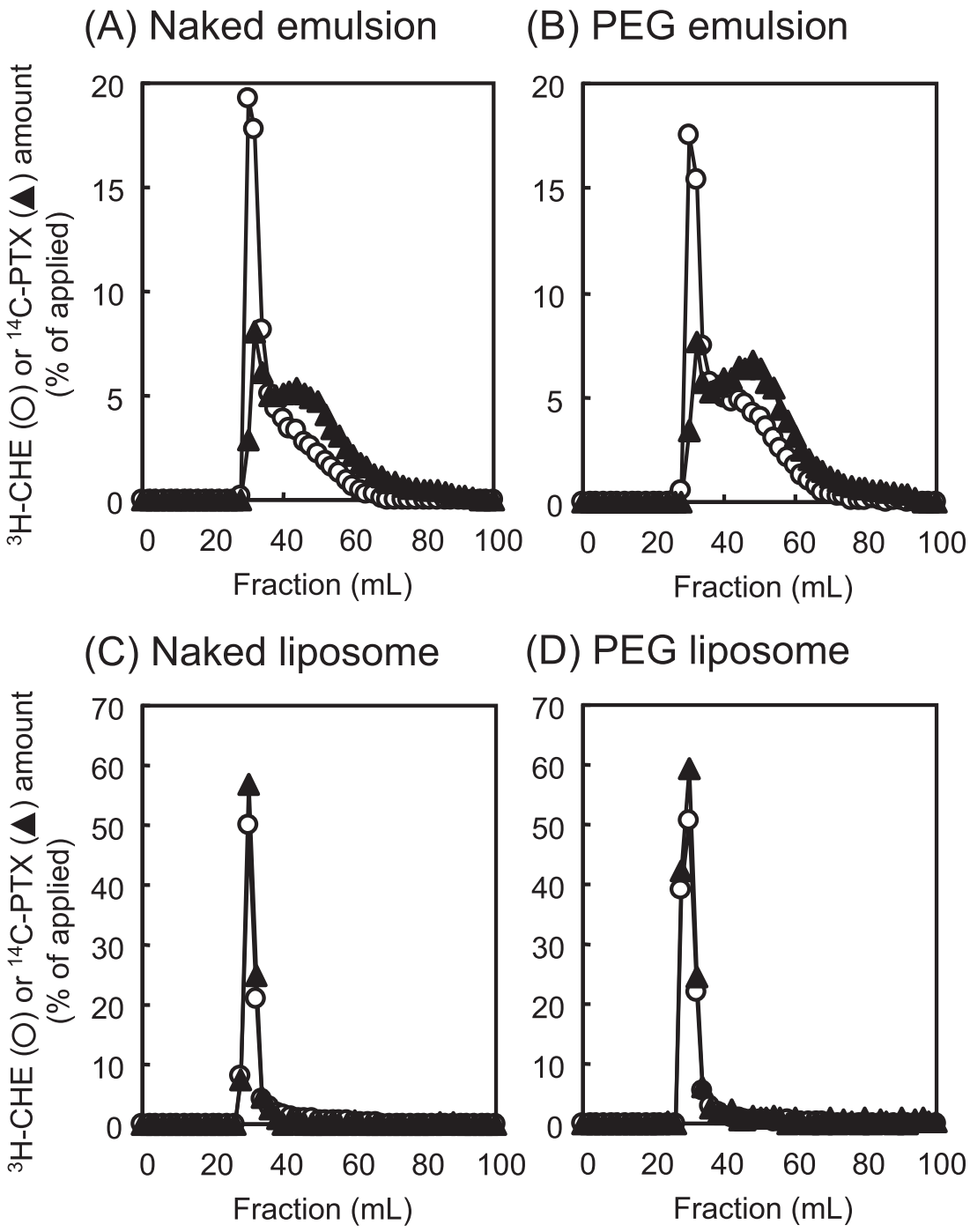

Fig. 3. Stability of PTX nanoparticles in serum. Gel filtration was performed after (A) naked emulsion, (B) PEG emulsion, (C) naked liposome or (D) PEG liposome nanoparticles were incubated in rat serum for 30 min at 37 °C. Keys: open circle, nanoparticle (^{3}H-CHE); closed triangle, PTX (^{14}C-PTX).

respectively) were dramatically and significantly higher than that of PTX-solution ($p < 0.001$). These results clearly demonstrate that PTX would be released from liposomes much more slowly than from emulsions in the culture medium containing FBS (10%, v/v).

Then, the stability of PTX-nanoparticles in rat serum was also evaluated by gel filtration (Fig. 3). In short, nanoparticles double-labeled with ^{3}H-CHE and ^{14}C-PTX were first incubated with serum, and the mixture was applied to gel filtration to separately trace the elution profiles of nanocarriers themselves and PTX. Elution pattern of naked or PEG liposome was almost the same with that of PTX for naked or PEG liposome, respectively (Fig. 3C and D). Since void volume of the column used was around 30 mL, this result indicates that PTX was stably incorporated within liposomes and was eluted as PTX-liposomes. On the other hand, elution profiles of naked and PEG emulsion showed that substantial amounts of ^{3}H-CHE were eluted at the fractions where smaller size molecules should be eluted, suggesting the possible degradation of emulsion in rat serum. Furthermore, the elution profile of PTX had a large second peak, indicating that PTX was eluted as small molecules (Fig. 3A and B). Our preliminary experiment showed that serum albumin was eluted from the column at the fractions of around 50 mL. Therefore, the second peak of the elution profiles of PTX would contain PTX bound to serum albumin after released from emulsions. The results shown in Figs. 2 and 3 suggest that PTX would be stably incorpo-

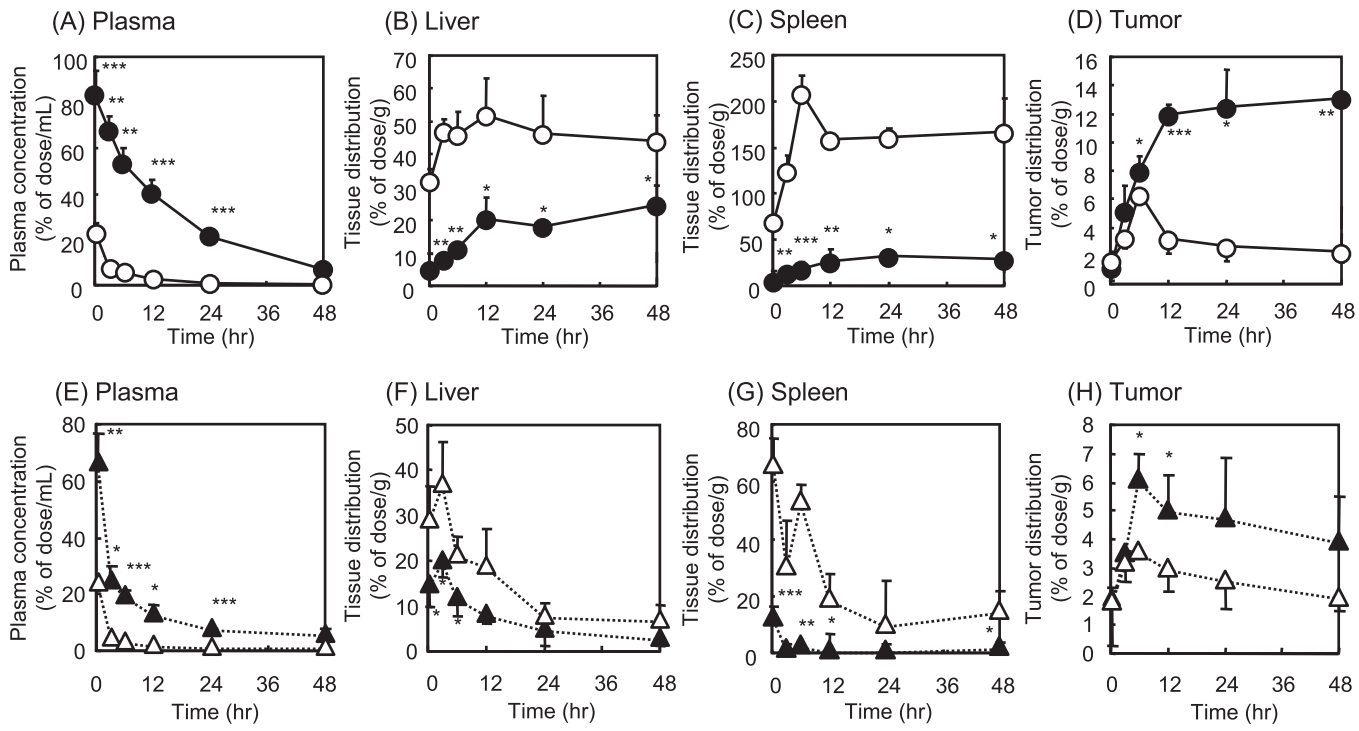

Fig. 4. Plasma concentration– and tissue distribution–time profiles of liposomes (upper panel) and PTX (lower panel) after intravenous injection of PTX-liposomes into C26-bearing mice. Each formulation was intravenously injected at dose of 1 mg/kg as PTX. Keys: (A)–(D) liposome; open circle, naked liposome; closed circle, PEG liposome; (E)–(H) PTX; open triangle, naked liposome; closed triangle, PEG liposome. Results are expressed as the mean with the vertical bar showing S.D. ($n = 3$). $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$, compared with the corresponding naked liposome.

rated within liposomes and that PTX included in emulsions would be rapidly released in blood, which would strongly support the results of in vivo experiments shown in Fig. 1 and Table 2. One of the possible reasons for the structural instability of emulsions would be that C6 and C8 used as oil components of emulsion are soluble in aqueous phase to some extent. Utilization of more hydrophobic triglyceride with longer alkyl chain to might lead to the formation of more stable emulsion, which will be the subject of our further study. In addition, metabolism of emulsions in rat is known to be comparable to that of chylomicrons (Gotto et al., 1986) and the hydrolysis of some surface and core lipids via the actions of lipoprotein lipase (LPL) is the first step in chylomicron metabolism (Miller and Small, 1983; Redgrave and Maranhao, 1985; Handa et al., 1994). Therefore, it would be speculated that the instability of emulsions and rapid release of PTX from them in blood are partly ascribed to LPL-mediated hydrolysis of C6 or C8 as well. However, the detailed mechanisms behind the rapid release of PTX from emulsions just after intravenous administration remain to be elucidated.

Since it was shown that liposomal preparations would give more favorable EPR effect than emulsion as a candidate for PTX formulation, we tried to elucidate the in vivo disposition characteristics including the tumor disposition of liposomes themselves and PTX included after intravenous administration into tumor-bearing mice. As mentioned in Section 1, the main objective of the present study is to achieve the efficient passive targeting of PTX into solid tumor by utilizing EPR effect. In our previous study, we found that C26 tumor-bearing mice showed the most efficient EPR effect-driven tumor disposition of doxorubicin-encapsulating PEG liposome, leading to the most prominent in vivo anti-tumor effect of doxorubicin among several types of solid tumor-bearing mice models (Ogawara et al., 2008), and we confirmed that C26 was sensitive enough to PTX as shown in Fig. 2. Since these findings clearly demonstrate that C26 tumor-bearing mice should be a good model to evaluate both EPR effect-driven tumor disposition of nanoparticles and in vivo anti-tumor effect of PTX, C26 tumor was selected as a model tumor in this study. Fig. 4 shows plasma concentration– and tissue distribution–time profiles of liposomes and PTX, and the pharmacokinetic parameters obtained are summarized in Table 3. Similar to the results obtained in rats (Fig. 1 and Table 2), it was clearly demonstrated that PEGylation of lipo-

Table 3
Pharmacokinetic parameters of PTX-formulations after intravenous injection into C26-bearing mice.

Pharmacokinetic parameters	Liposomes (^{3}H-CHE)		Paclitaxel (^{14}C-PTX)	
	Naked	PEG	Naked	PEG
$AUC^{0-48\,h}_{plasma}$ (% of dose/mL h)	119	1375	69	630
CL_{tot} (mL/h)	0.83	0.07	1.44	0.15
Vd_c (mL)	3.66	1.04	1.89	1.36
Vd_{ss} (mL)	8.58	1.32	15.9	4.08
Vd_β (mL)	9.90	1.32	19.6	4.67
k_{el} (h^{-1})	0.23	0.07	0.77	0.11
$AUC^{0-48\,h}_{liver}$ (% of dose/g h)	2220	888	623	311
$AUC^{0-48\,h}_{spleen}$ (% of dose/g h)	7739	1313	908	39.9
$AUC^{0-48\,h}_{tumor}$ (% of dose/g h)	144	542	122	215

Pharmacokinetic parameters were calculated utilizing the mean values of plasma or tissue concentrations by following the methods described in Section 2.8.

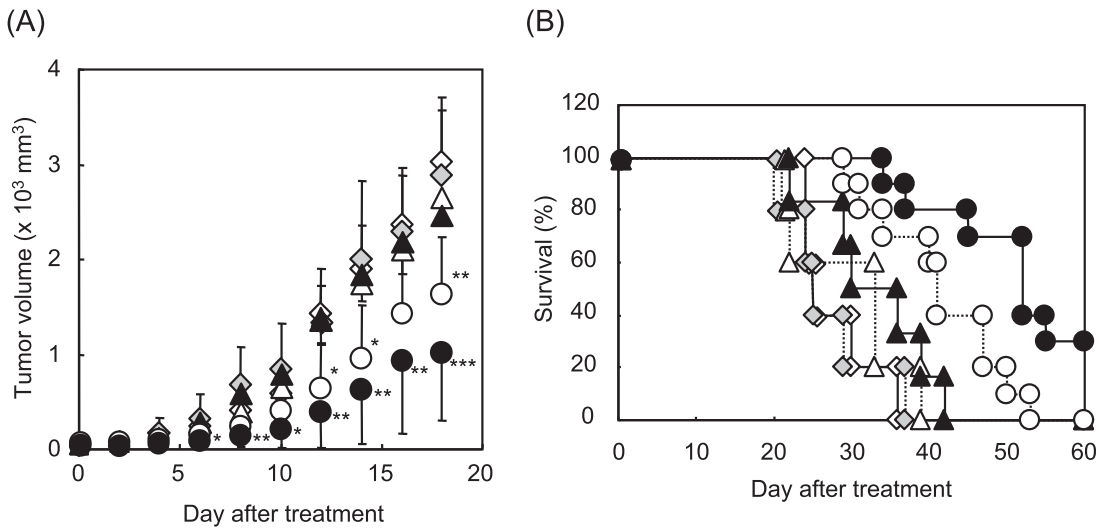

Fig. 5. In vivo anti-tumor activity of PTX-nanoparticles after intravenous injection into C26-bearing mice. Each formulation was intravenously injected at a dose of 0.5 mg/kg as PTX one week after the tumor inoculation. Keys: open diamond, saline; filled diamond, PTX solution; open triangle, naked emulsion; closed triangle, PEG emulsion; open circle, naked liposome; closed circle, PEG liposome. (A) Tumor volume and (B) survival time after treatment. Results are expressed as the mean with the vertical bar showing S.D. ($n = 5$). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, compared with saline-treated groups.

some remarkably reduced the amount of liposome distributed to the liver and spleen and markedly increased the blood circulation time of liposome (Fig. 4A–C and Table 3). It was also the case for PTX, where PTX incorporated in PEG liposome was distributed to the liver and spleen much less and was retained in the systemic circulation for a much longer time than PTX incorporated in naked liposome (Fig. 4E–G and Table 3). Comparing with the pharmacokinetics of PEG liposomal PTX in mice reported by Crosasso et al. (2000), the values of both CL_{total} (0.15 mL/h) and Vd_{ss} (4.09 mL) were much smaller for our PEG liposomal PTX than those for Crosasso's (CL_{total} = 0.35 mL/h, Vd_{ss} = 83.53 mL), clearly indicating that PTX-PEG liposome prepared in the present study provided more favorable kinetics of PTX for passive targeting based on EPR effect. One of the reasons for the difference between the two PTX-PEG liposomes would be attributed to the difference in the main lipid component of liposomes. It is well known that release of drugs from liposomes prepared with EPC, which was used for Crosasso's liposomes, is more rapid than that from liposomes prepared with HSPC, which was used for our liposomes (Charrois and Allen, 2004; Garbuzenko et al., 2005). Therefore, the slow release of PTX from our PEG liposome would result in smaller values of CL_{total} and Vd_{ss} of PTX.

On the other hand, Fig. 4B, C, F and G indicates that distributed amount–time profiles of PTX for the liver and spleen were quite different from those of liposomes, which would be explained by the following reasons. ^{3}H-CHE, used as a tracer for liposome, is a non-exchangeable lipid marker and is known to continuously accumulate in the tissue over the entire period of the study (Goren et al., 1996). On the other hand, PTX would be released from liposomes in the liver and spleen after the liposomes are taken up by these tissues, and would be eliminated from these tissues by following its own pharmacokinetic characteristics.

More importantly, it is worth to note that distribution of both liposome and PTX to the tumor tissue was enhanced by PEGylation of liposome (Fig. 4D and H). AUC_{tumor} values of liposome and PTX for PEG liposome were both larger than those for naked liposome, and PEGylation of liposome increased AUC_{tumor} values of liposome 3.8 times and those of PTX 1.8 times (Table 3). These results clearly indicate that PEG liposome gives an EPR effect-based tumor disposition of PTX more efficiently than naked liposome.

Finally, the anti-tumor activity of PTX-liposomes was evaluated in C26-bearing mice (Fig. 5 and Table 4). Treatment with PTX solution, PTX-naked or PTX-PEG emulsion was also examined, but any of them did not significantly inhibit tumor growth. On the other hand, PTX-liposomes significantly inhibited tumor growth compared with control group. Furthermore, PTX-PEG liposome exhibited more prominent inhibition of tumor growth than PTX-naked liposome and T/C value for PTX-PEG liposome was the smallest among all the treatments examined. Survival time of tumor-bearing mice after the treatment was also evaluated (Fig. 5B and Table 4). Significant extension of mean survival time (MST) compared with control group was observed in the tumor-bearing mice treated with PTX-liposome. Moreover, PTX-PEG liposome exhibited a statistically significant survival benefit compared with

Table 4
Tumor growth rates and mean survival time (MST) of C26-bearing mice treated with PTX formulated in various nanoparticles.

Formulation	Growth rate (mm^3/day)	T/C (%)	MST (days)	T/C (%)
Saline	216.6 ± 48.2	–	27.8 ± 5.2	–
PTX solution	197.9 ± 43.9	91	27.4 ± 5.9	99
Naked emulsion	190.3 ± 22.1	88	29.6 ± 7.8	106
PEG emulsion	207.1 ± 35.3	96	33.0 ± 7.4	119
Naked liposome	113.2 ± 41.7**	52	41.3 ± 8.1**	149
PEG liposome	67.1 ± 54.1***	31	50.7 ± 9.3***,†	182

Results of growth rate and MST are expressed as the mean ± S.D. (n = 5–10). T/C values were calculated according to the method described in Section 2.11.
** $p < 0.01$, compared with saline-treated control group.
*** $p < 0.001$, compared with saline-treated control group.
† $p < 0.05$, compared with naked liposome.

PTX-naked liposome. These results obtained in the in vivo anti-tumor studies are reasonably explained by the pharmacokinetics of liposomes and PTX shown in Fig. 4 and Table 3, clearly indicating that PEG liposome would be the promising formulation for PTX based on its efficient EPR effect.

In conclusion, PEG liposome was able to encapsulate PTX stably in blood and provided longer residence time in blood circulation than naked liposome because of its less affinity to RES tissues. These properties of PTX-PEG liposome made it possible for PTX to be distributed to tumor tissues more efficiently based on EPR effect, which led to more effective in vivo anti-tumor effect. Our present study clearly indicated that PEG liposome would serve as a potent PTX delivery vehicle for the future cancer chemotherapy.

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