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Enhanced *in vivo* antitumor efficacy of fenretinide encapsulated in polymeric micelles

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ABSTRACT

Fenretinide (N-(4-hydroxyphenyl)retinamide, 4-HPR) is a synthetic retinoid with high antitumor activity against a variety of malignant cells *in vitro*, and is a promising candidate for cancer chemoprevention and chemotherapy. To enhance the antitumor efficacy of 4-HPR *in vivo*, 4-HPR were encapsulated into polymeric micelles for tumor targeting by enhanced permeability and retention effects. 4-HPR encapsulated in poly(ethylene glycol)–poly(benzyl aspartate) block copolymer micelles were prepared by the evaporation method. The mean particle size of 4-HPR encapsulated in polymeric micelles was about 173 nm. After intravenous injection into tumor-bearing mice, the delivery of 4-HPR by polymeric micelles increased the blood concentration and enhanced the tumor accumulation of 4-HPR over the injection of the 4-HPR encapsulated in oil-in-water (O/W) emulsions. Tumor growth was significantly delayed following treatment by 4-HPR encapsulated in polymeric micelles, which demonstrated the improved *in vivo* antitumor efficacy of 4-HPR. In addition, 4-HPR encapsulated in polymeric micelles did not cause any body weight loss. These results suggest that polymeric micelles are a promising and effective carrier of 4-HPR in order to enhance tumor delivery and have potential application in the treatment of solid tumor.

1. Introduction

Retinoids are a class of natural or synthetic derivatives of vitamin A that exert various biological actions on cellular growth and differentiation (Means and Gudas, 1995; Kagechika and Shudo, 2005). As a result of their unique characteristics, their application in novel cancer therapy has been progressing (Altucci and Gronemeyer, 2001; Clarke et al., 2004). In particular, fenretinide (N-(4-hydroxyphenyl)retinamide, 4-HPR), which was synthesized from all-trans retinoic acid (ATRA), is a promising candidate for cancer chemoprevention and chemotherapy, since it has higher antitumor activity and lower toxicity than other retinoids (Moon et al., 1979; Miller, 1998). It was reported that the antitumor activity of 4-HPR was through two main mechanisms: retinoic acid receptor-dependent cascade and retinoic acid receptor-independent cascade, including reactive oxygen species generation, ceramide synthesis, and proapoptotic bcl-2 family protein (Bax and Bak) induction (Hail et al., 2006; Corazzari et al., 2005). Clinical trials of 4-HPR have been progressing against breast cancer, prostate cancer, and neuroblastoma (Altucci and Gronemeyer, 2001; Clarke et al., 2004).

To date, 4-HPR has been clinically administrated using an oral gelatin capsule containing 4-HPR in corn oil and polysorbate 80 [available through the National Cancer Institute]; however, this formulation has poor bioavailability, since 4-HPR itself is too hydrophobic to pass through intestinal membrane easily (Kokate et al., 2007), and requires excessive or multiple administrations to achieve a higher blood concentration of 4-HPR (Villablanca et al., 2006). In addition, several animal studies have demonstrated that intravenously injected 4-HPR is rapidly eliminated from the body (Swanson et al., 1980; Hultin et al., 1986); therefore, the development of a targeted carrier of 4-HPR is needed to exert *in vivo* antitumor efficacy.

Polymeric micelles are a class of micelles that are formed from block copolymers typically consisting of hydrophilic and hydrophobic polymer chains (Kataoka et al., 2001; Torchilin, 2004). They are of particular interest because of their efficacy in entrapping a satisfactory amount of hydrophobic drugs within the inner core, their stability in the circulation and their ability to gradually release the drugs (Kwon, 2003). In addition, the highly hydrated outer

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shells of the micelles prevent reticuloendothelial system (RES) uptake and inhibit intermicellar aggregation of their hydrophobic inner cores. The characteristics of these polymeric micelles could be an advantage for passive delivery and to extravasate the drug at tumor sites by enhanced permeability and retention (EPR) effects (Maeda, 2001). In our previous report, we demonstrated that poly(ethylene glycol)–poly(aspartate) block copolymer micelles modified with benzyl groups could stably encapsulate 4-HPR and enhanced blood retention of 4-HPR after intravenous injection into mice (Okuda et al., 2008). This observation prompted us to investigate the potential use of polymeric micelles to enhance tumor retention and *in vivo* antitumor efficacy of 4-HPR in tumor-bearing mice.

In this study, we extended our previous study and tumor distribution and antitumor efficacy of 4-HPR encapsulated in poly(ethylene glycol)–poly(aspartate) block copolymer micelles modified with benzyl groups after intravenous injection were examined in mice bearing murine melanoma B16BL6 tumors. As a control pharmaceutical formulation of 4-HPR, oil-in-water (O/W) or PEGylated O/W emulsions were selected because of their preparation characteristics for delivery of highly lipophilic drugs (Tamilvanan, 2004).

2. Materials and methods

2.1. Materials

4-HPR was purchased from Tokyo Chemical Industry, Co. Ltd. (Tokyo, Japan). Egg yolk phosphatidylcholine (EggPC) and 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Soybean oil (SO) and ATRA were purchased from Wako Pure Chemicals Industry, Ltd. (Osaka, Japan). Acetonitrile (HPLC grade) and acetic acid (HPLC grade) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Distearoylphosphatidylethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-DSPE) was purchased from Nippon Oil and Fats Co. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Biowhittaker (Walkersville, MD). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Hank's balanced saline solution (HBSS) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

2.2. Synthesis of block copolymer

Poly(ethylene glycol)–poly(aspartic acid) (PEG–P(Asp)) block copolymer was obtained by alkaline hydrolysis of poly(ethylene glycol)–poly(β-benzyl-L-aspartate) (PEG–PBLA), as reported previously (Opanasopit et al., 2004). Briefly, the molecular weight of poly(ethylene glycol) (PEG) chain was 5000 and the average number of aspartic acid units was 27. Approximately 75% of the aspartic acid residues in poly(aspartic acid) chain were converted to the β-amide form by alkaline hydrolysis during the synthesis of this block copolymer. A hydrophobic benzyl group was bound to 77% of the poly(aspartic acid) residues by an ester-forming reaction between benzyl bromide and PEG–P(Asp), as reported previously (Yokoyama et al., 2004). Briefly, PEG–P(Asp) block copolymer was dissolved in N,N-dimethylformamide (DMF) and added to benzyl bromide along with a catalyst, 1,8-diazabicyclo[5,4,0]7-undecene (DBU). The reaction mixture was stirred at 50 °C for 15.5 h. Polymer was obtained by precipitation in excess of diethyl ether and collected by filtration. The dried polymer was dissolved in dimethyl sulfoxide (DMSO), and then 6N HCl was added, followed by dialysis against distilled water and finally, freeze-drying.

For determination of the polymer composition, such as the number of aspartic acid units and the benzyl ester content, ^{1}H NMR measurements were carried out on a 1% solution in 6D-DMSO containing 3% trifluoroacetic acid using a Varian Unity Inova NMR spectrometer at 400 MHz.

2.3. Preparation of 4-HPR encapsulated in polymeric micelles

4-HPR encapsulated in polymeric micelles was prepared by a conventional evaporation method (Kawakami et al., 2005; Chansri et al., 2008). Briefly, 4-HPR and polymer were dissolved in chloroform. After vacuum drying and desiccation, PBS (pH 7.4) was added for suspension in a bath sonicator for 3 min. The suspension was sonicated for 3 min (200 W) at 75 °C using a probe sonicator (US 300, Nissei, Inc., Tokyo, Japan). The preparation was centrifuged at 1400 × *g* for 10 min before the supernatant was passed through a 0.45 μm filter.

2.4. Preparation of 4-HPR encapsulated in O/W emulsions and PEGylated O/W emulsions

4-HPR encapsulated in O/W emulsions was prepared based on our previous reports (Takino et al., 1994; Chansri et al., 2006). Briefly, 4-HPR, EggPC, and SO (30:150:150, weight ratio) were dissolved in chloroform. After vacuum drying and desiccation, PBS (pH 7.4) was added for suspension in a bath sonicator for 3 min. The suspension was sonicated for 30 min (200 W) at 4 °C using a probe sonicator (US 300, Nissei, Inc., Tokyo, Japan). 4-HPR encapsulated in PEGylated O/W emulsions, composed of 4-HPR, EggPC, PEG-DSPE, and SO (30:105:45:150, weight ratio), was also prepared using this protocol.

2.5. Characterization of the formulations

The concentration of 4-HPR in the preparations was determined by UV absorption at 370 nm (UV–vis Spectrophotometer, Shimazu Co. Ltd., Kyoto, Japan) after dissolving in DMSO (the preparations: DMSO = 10:990, volume ratio). The recovery of 4-HPR was calculated from the concentration of 4-HPR in the preparations (C_P) as follows:

$$\text{Recovery}(\%) = \frac{C_P(\text{mg/mL}) \times \text{added PBS(mL)}}{\text{initial amount of 4-HPR(mg)}} \times 100$$

On the other hand, the recovery of polymer was defined as 100% because of high solubility of polymer in PBS (>50 g/L). The particle sizes and polydispersion indexes of the preparations were measured by Zetasizer Nano Series (Malvern Instruments Ltd., Worcestershire, UK).

2.6. Animals

Male C57BL/6 mice (4 weeks old, 14–19 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were carried out in accordance with the guidelines for Animal Experiments of Kyoto University.

2.7. Tumor cells

Murine melanoma B16BL6 cells were obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). They were grown in DMEM supplemented with 10% heat-inactivated FBS, 0.15% $NaHCO_3$, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in humidified air containing 5% CO_2.

2.8. MTT assay

MTT assay was performed by the method described previously (Kawakami et al., 2006). B16BL6 cells were placed on a 96-well cluster dish at a density of 3×10^3 cells/0.28 cm^2. Twenty-four hours later, medium containing various concentrations of 4-HPR, empty polymeric micelles, and 4-HPR encapsulated in polymeric micelles was added to the plates. At each exposure time point, the medium was removed and 5 mg/mL MTT solution was added to each well. Cells were incubated for 4 h at 37 °C in 5% CO_2 and then 10% sodium dodecyl sulfate (SDS) solution was added, followed by incubation overnight to dissolve formazan crystals. An absorbance was measured at wavelengths of 570 nm in a microplate photometer (Bio-Rad Model 550, Bio-Rad Laboratories, Inc., Hercules, CA, USA). IC_{50} values were determined from dose-response curves by a nonlinear regression analysis using MULTI program developed by Yamaoka et al. (Yamaoka et al., 1981) and represent the concentration required to inhibit cell viability by 50%.

2.9. Tumor-bearing mouse model

After harvesting by trypsin, B16BL6 cells were prepared at a concentration of 4×10^6 cells/mL by HBSS. Then, 0.05 mL of the cell suspension (2×10^5 cells) was inoculated subcutaneously in the lower back of each C57BL/6 mouse. A solid tumor was observed within 7 days after tumor inoculation.

2.10. In vivo distribution study

4-HPR encapsulated in polymeric micelles, O/W emulsions, and PEGylated O/W emulsions were adjusted to 7.5 mg/mL as 4-HPR. On 14 days after tumor inoculation, they were intravenously injected into the tail vein of B16BL6-bearing mice at a dose of 75 mg/kg as 4-HPR. At each collection time point, blood was collected from the vena cava under anesthesia, and mice were killed for tumor excision. Blood was centrifuged at 5000 × *g* for 5 min at 4 °C before 200 μL of plasma was collected. The plasma was added to 400 μL of acetonitrile and 4 μL of 2 mg/mL ATRA (dissolved in ethanol) as an internal standard and vortexed. Part 0.2 g of the excised tumor was collected and added to 500 μL of acetonitrile and 5 μL of 2 mg/mL ATRA, followed by sonication using a bath sonicator for 15 min. The extract from plasma and tumor was centrifuged at 13,000 × *g* for 3 min at 4 °C, and the supernatant was collected and passed through a 0.45 μm filter. The filtrated extract was analyzed by HPLC.

2.11. HPLC conditions

The extract was analyzed with a high-performance liquid chromatograph device, consisting of a system controller (SLC-10A VP, Shimazu Co. Ltd., Kyoto, Japan), a UV–vis detector (SPD-10A VP, Shimazu Co. Ltd., Kyoto, Japan), an auto injector (SIL-10A, Shimazu Co. Ltd., Kyoto, Japan), and a HPLC pump (LC-10AS, Shimazu Co. Ltd., Kyoto, Japan). The UV–vis detector was set at 350 nm. A C18 reverse-phase column (ODS-A 150 mm × 4.6 mm, YMC Co. Ltd., Kyoto, Japan) was used. The mobile phase consisted of acetonitrile:water:acetic acid (80:19:1, volume ratio) delivered at a flow rate of 1 mL/min. The injection volume was 50 μL. All samples were analyzed at room temperature.

2.12. Pharmacokinetic analysis

Pharmacokinetic parameters including the half-life ($t_{1/2}$), the area under the concentration versus time curve ($AUC_{t-\infty}$), the total body clearance (CL_{tot}), the volume of distribution at steady state (V_{dss}), and the mean residence time (MRT) were all calculated by moment analysis program developed by Yamaoka et al. (1978).

2.13. In vivo antitumor efficacy in tumor-bearing mice

On 8 days after tumor inoculation when the tumor volume reached approximately 100 mm^3, each treatment was started. 4-HPR encapsulated in polymeric micelles and O/W emulsions was intravenously injected into the tail vein of B16BL6-bearing mice at a dose of 75 mg/(kg day) as 4-HPR. The dose of empty polymeric micelles was 375 mg/(kg day) as polymer, which was an equivalent does to 4-HPR encapsulated in polymeric micelles. In the control groups, PBS was administrated. Each treatment was performed at 10 mL/(kg day) on 8, 10, and 12 days after tumor inoculation. Tumor diameter and body weight were measured for each mouse at 2-day intervals after the treatment started. Tumor volume was calculated as follows:

$$\text{Tumor volume} = \frac{\pi}{6} \times LW^2$$

where *L* is the long diameter and *W* is the short diameter.

2.14. Statistical analysis

Statistical comparison was performed by Student's *t*-test for two groups, and Dunnett's test for multiple groups. $P < 0.05$ was considered significant.

3. Results

3.1. Characteristics of 4-HPR encapsulated in polymeric micelles

Poly(ethylene glycol)–poly(benzyl aspartate) block copolymer was successfully synthesized from PEG–P(Asp), and about 77% of the aspartic residues were esterified with benzyl groups as reported previously (Opanasopit et al., 2004). The recovery of 4-HPR achieved approximately 70% in polymer/4-HPR weight ratio at 2.5 and 3.0 (Fig. 1). Therefore, polymer/4-HPR weight ratio was fixed at 2.5 in the following experiments. The mean particle size of polymeric micelles was about 173 nm (Fig. 2). On the other hand, the mean particle sizes of O/W emulsions and PEGylated O/W emulsions were about 176 and 178 nm, respectively, similar to polymeric micelles. The polydispersion indexes of polymeric micelles, O/W

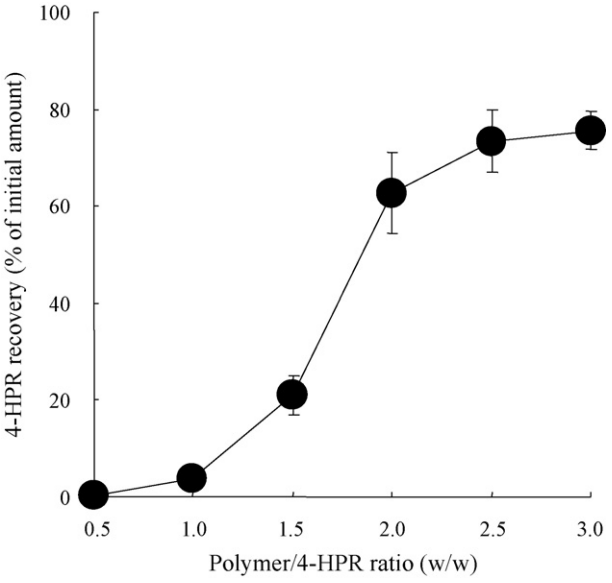


Fig. 1. Effect of polymer/4-HPR weight ratio on encapsulation of 4-HPR into prepared polymeric micelles. 4-HPR encapsulated in polymeric micelles was prepared at various weight ratio for 1 mg 4-HPR, and 4-HPR recovery was calculated. Each value represents the mean ± S.D. ($n = 4$).

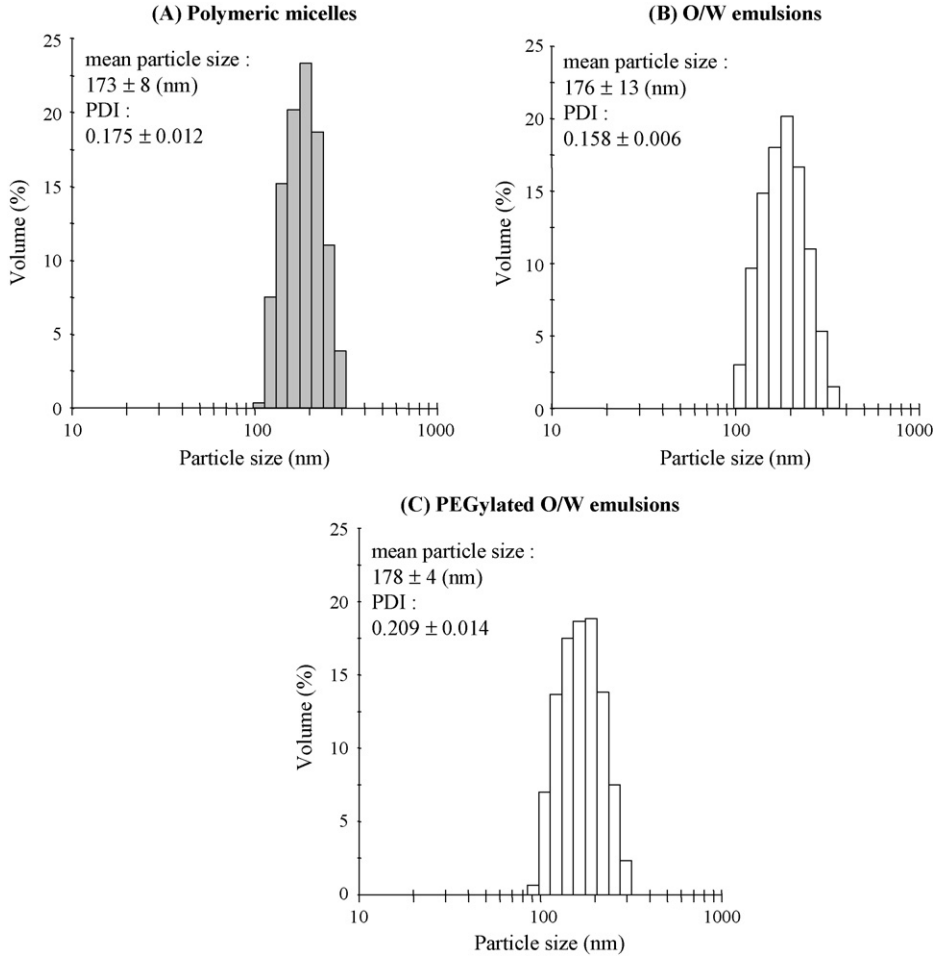


Fig. 2. Size distributions, mean particle sizes, and polydispersion indexes (PDI) of 4-HPR encapsulated in polymeric micelles (A), 4-HPR encapsulated in O/W emulsions (B), and 4-HPR encapsulated in PEGylated O/W emulsions (C). Each value of represents the mean ± S.D. ($n = 3$).

emulsions, and PEGylated O/W emulsions were about 0.175, 0.158, and 0.209, respectively, suggesting the narrow size distribution of these preparations. Furthermore, the particle size of 4-HPR encapsulated in polymeric micelles remained constant over 1 month at room temperature, 4, and −30 °C (data not shown).

3.2. *In vitro antitumor activity of 4-HPR and 4-HPR encapsulated in polymeric micelles*

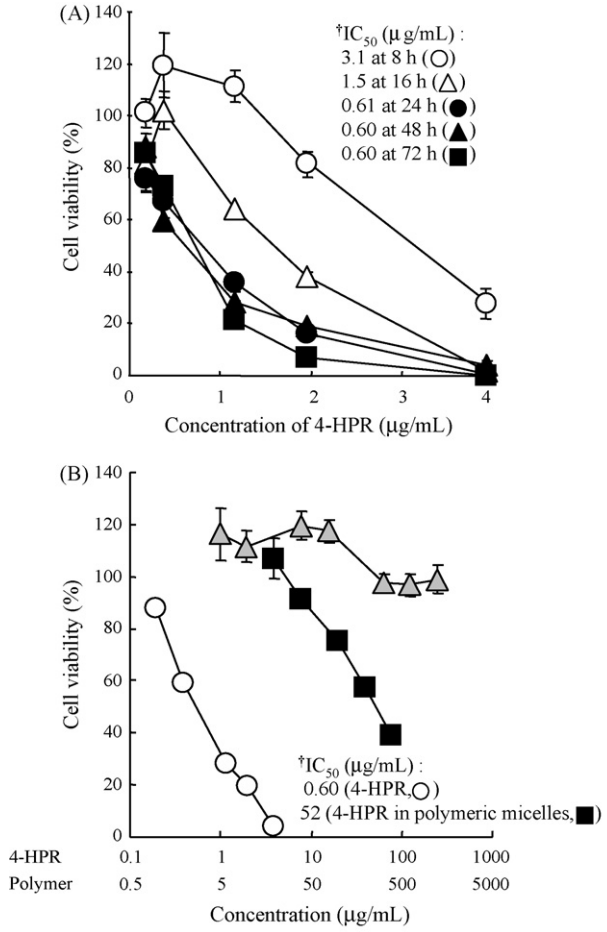
For evaluating *in vitro* antitumor activity of 4-HPR and 4-HPR encapsulated in polymeric micelles against B16BL6 cells, MTT assay was performed. The *in vitro* antitumor activity of 4-HPR was enhanced as the increase of dose and exposure time (Fig. 3(A)). IC_{50} value was settled on approximately 0.60 μg/mL at more than 24 h, suggesting full antitumor activity of 4-HPR could be obtained at 24 h. IC_{50} value of 4-HPR encapsulated in polymeric micelles (52 μg/mL at 48 h) was much larger than that of free form of 4-HPR (0.60 μg/mL at 48 h) (Fig. 3(B)). This result may indicate the stable encapsulation of 4-HPR into polymeric micelles in medium.

3.3. *Distribution of 4-HPR in tumor-bearing mice*

The blood concentration and tumor accumulation of 4-HPR after intravenous injection of 4-HPR encapsulated in polymeric micelles were evaluated in B16BL6-bearing mice (Fig. 4) and compared with those of 4-HPR encapsulated in O/W emulsions or PEGylated O/W emulsions as control. The blood concentration of 4-HPR encapsulated in polymeric micelles was significantly higher than those of 4-HPR encapsulated in O/W emulsions and PEGylated O/W emulsions, suggesting that polymeric micelles could enhance the blood retention of 4-HPR compared with O/W emulsions and PEGylated O/W emulsions. Moreover, the tumor accumulation of 4-HPR encapsulated in polymeric micelles was significantly higher and prolonged for 48 h compared with O/W emulsions and PEGylated O/W emulsions. Pharmacokinetic parameters of 4-HPR encapsulated in polymeric micelles, O/W emulsions, and PEGylated O/W emulsions were shown in Table 1. The area under the curve ($AUC_{0-\infty}$) in blood was approximately 23.9 times higher for 4-HPR encapsulated in polymeric micelles than for that in O/W emulsions. The $t_{1/2}$ in blood was approximately 5.02 times longer for 4-HPR encapsulated in polymeric micelles than for that in O/W emulsions. Furthermore, the maximum concentration (C_{max}), and the $AUC_{0-\infty}$ in tumors were approximately 3.03 and 16.9 times higher for 4-HPR encapsulated in polymeric micelles than for that in O/W emulsions, respectively. The $t_{1/2}$ in tumors was approximately 5.63 times longer for 4-HPR encapsulated in polymeric micelles than for that in O/W emulsions.

3.4. *In vivo antitumor efficacy of 4-HPR encapsulated in polymeric micelles in tumor-bearing mice*

In vivo antitumor efficacy of 4-HPR encapsulated in polymeric micelles was evaluated in B16BL6-bearing mice after intravenous injection of PBS (control), empty polymeric micelles, 4-HPR encapsulated in O/W emulsions, and 4-HPR encapsulated in polymeric micelles. Each treatment was performed on 8, 10, and 12 days (total



†IC_{50} values were determined from dose-response curves by a nonlinear regression analysis using MULTI program developed by Yamaoka et al. (Yamaoka et al., 1981) and represent the concentration required to inhibit cell viability by 50 %.

Fig. 3. *In vitro* antitumor activity of 4-HPR and 4-HPR encapsulated in polymeric micelles against B16BL6 cells. (A) Time and concentration dependence of *in vitro* antitumor activity of 4-HPR. Cell viability was measured by MTT method at 8 h (open circle), 16 h (open triangle), 24 h (filled circle), 48 h (filled triangle), and 72 h (filled square) after treatment. (B) *In vitro* antitumor activity of 4-HPR (open circle), empty polymeric micelles (shade triangle), and 4-HPR encapsulated in polymeric micelles (filled square) at 48 h after treatment. Each value represents the mean ± S.D. (n = 3–4).

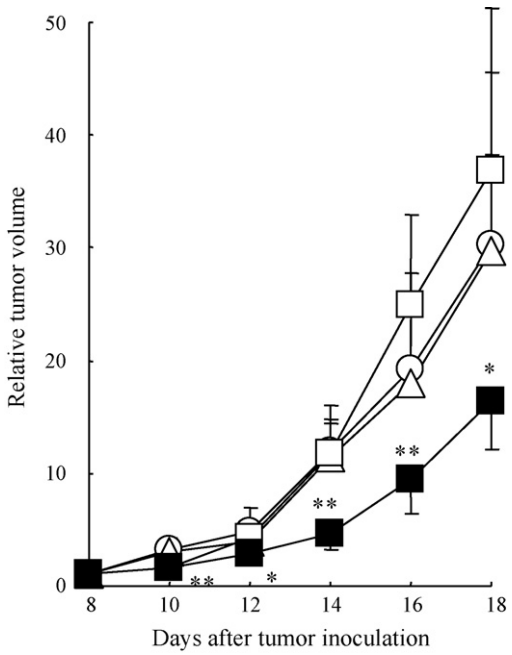


Fig. 5. Relative tumor volume of B16BL6-bearing mice after intravenous injection of PBS (open circle), empty polymeric micelles (open triangle), 4-HPR encapsulated in O/W emulsions (open square), and 4-HPR encapsulated in polymeric micelles (filled square). 4-HPR encapsulated in O/W emulsions and 4-HPR encapsulated in polymeric micelles were intravenously injected into mice at a dose of 75 mg/kg as 4-HPR on 8, 10, 12 days after tumor inoculation. The dose of empty polymeric micelles was 375 mg/kg as polymer, which was equivalent dose to 4-HPR encapsulated in polymeric micelles. Each value represents the mean ± S.D. (n = 6–12). Statistically significant differences compared with PBS-treated groups (**P < 0.01; *P < 0.05).

three times) after tumor inoculation. Only 4-HPR encapsulated in polymeric micelles significantly delayed tumor growth in B16BL6-bearing mice as compared with PBS (Fig. 5), while did not cause any significant weight loss (data not shown). On 18 days after tumor inoculation, approximately 55% of tumor growth inhibition was observed in mice treated with 4-HPR encapsulated in polymeric micelles. On the other hand, empty polymeric micelles and 4-HPR encapsulated in O/W emulsions did not have any effect on tumor growth (Fig. 5).

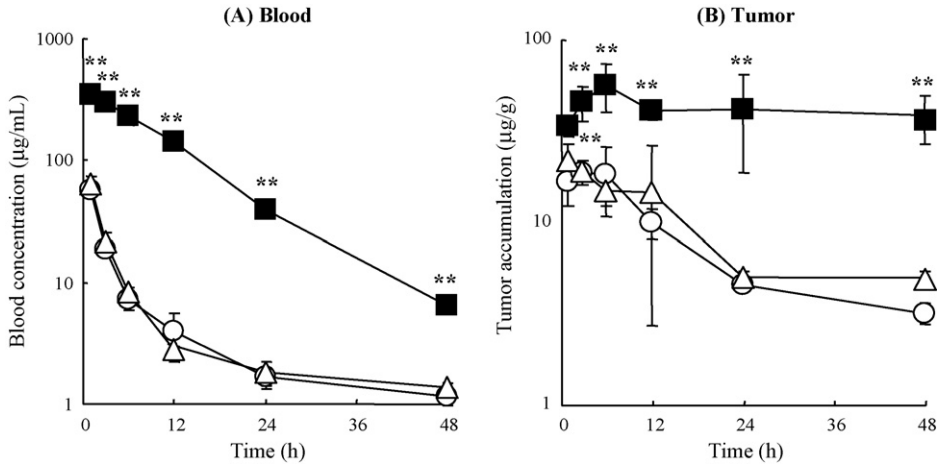


Fig. 4. Blood concentration (A) and tumor accumulation (B) of 4-HPR following intravenous injection of 4-HPR encapsulated in O/W emulsions (open circle), 4-HPR encapsulated in PEGylated O/W emulsions (open triangle), and 4-HPR encapsulated in polymeric micelles (filled square), into B16BL6-bearing mice. Each formulation was intravenously injected at 75 mg/kg dose of 4-HPR on 14 days after tumor inoculation. At indicated time point, blood and tumor were collected and amount of 4-HPR was measured by HPLC. Each value represents the mean ± S.D. (n = 3–6). Statistically significant differences compared with 4-HPR encapsulated in O/W emulsions (**P < 0.01).

Table 1
Pharmacokinetic parameters for blood and tumor concentration of 4-HPR after intravenous injection of 4-HPR encapsulated in polymeric micelles, 4-HPR encapsulated in O/W emulsions, and 4-HPR encapsulated in PEGylated O/W emulsions into B16BL6-bearing mice.

Formulation	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (μg h/mL)	CL_{tot} (mL/h kg)	V_{dss} (mL/kg)	MRT (h)
Blood[a]					
O/W emulsions	1.73	197	382	843	2.21
PEGylated O/W emulsions	1.73	225	333	737	2.21
Polymeric micelles	8.68	4717	15.9	199	12.5

Formulation	C_{max} (μg/g)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (μg h/g)	CL_{tot} (g/h kg)	V_{dss} (g/kg)	MRT (h)
Tumor							
O/W emulsions	18.7	3	17.7	430	175	4242	14.3
PEGylated O/W emulsions	21.7	1	20.1	509	147	4038	27.4
Polymeric micelles	56.6	6	99.8	7264	10.3	1505	146

$t_{1/2}$ = half-life; AUC = area under the curve; CL_{tot} = total body clearance; V_{dss} = volume of distribution at steady state; MRT = mean residence time; C_{max} = maximum concentration; T_{max} = time of maximum concentration; parameters were calculated from the mean value of three to six mice by moment analysis developed by Yamaoka et al. (1978).
[a] Parameters in blood were calculated for the initial phase of the experiment until 6 h after intravenous injection.

4. Discussion

Nano-particulate formulation is very attractive for intravenous injection of lipophilic drugs including 4-HPR. Previously, we have developed poly(ethylene glycol)–poly(aspartate) block copolymer micelles modified with benzyl groups and demonstrated their properties of stable encapsulation and enhanced blood circulation time of 4-HPR in mice (Okuda et al., 2008). In this study, we further investigated its disposition characteristics and pharmacological effects of 4-HPR encapsulated in polymeric micelles in tumor-bearing mice. We demonstrated here for the first time that *in vivo* antitumor effect was achieved by intravenous injection of 4-HPR encapsulated in polymeric micelles.

Spaces in the blood endothelium formed by solid tumors were reported to range between 300 and 4700 nm (Yuan et al., 1995; Hashizume et al., 2000). As shown in Fig. 2, the mean particle size of prepared polymeric micelles was approximately 173 nm. Furthermore, their mean particle size did not change for more than 1 month storage, even at room temperature (data not shown). These results suggested that they might be small enough to exert long circulating potential and pass through the endothelium of solid tumors.

As shown in Fig. 3(A), exposure to 4-HPR for more than 24 h was needed to exert high antitumor activity of 4-HPR against B16BL6 cells *in vitro*. This observation of *in vitro* antitumor characteristics of 4-HPR corresponds with a previous report (Wu et al., 2005) and in the other cancer cells (Holmes et al., 2003). Since EPR effects are known to enhance and prolong tumor distribution based on the characteristic vascular structure around tumor tissues, such passive targeting of 4-HPR to tumor tissues by EPR effects is expected to be an effective strategy for exerting *in vivo* pharmacological actions. After intravenous injection of 4-HPR encapsulated in polymeric micelles, the blood concentration of 4-HPR was extended (Fig. 4(A)). Investigating whether polymeric micelles can improve *in vivo* antitumor efficacy of 4-HPR, and the therapeutic efficacy of 4-HPR encapsulated in polymeric micelles was evaluated in B16BL6-bearing mice. As shown in Fig. 4(B), the tumor concentration of 4-HPR by 4-HPR encapsulated in polymeric micelles was from 33.9 to 56.6 μg/g of 4 HPR for 48 h and could significantly inhibit tumor growth (Fig. 5). The *in vivo* antitumor efficacy might be supported by the fact that *in vitro* IC_{50} value of 4-HPR against B16BL6 cells at more than 24 h was approximately 0.60 μg/mL. *In vitro* antitumor activity of 4-HPR encapsulated in polymeric micelles was much lower than that of 4-HPR in free form (Fig. 4(B)), but intravenously injected 4-HPR encapsulated in polymeric micelles showed the enhanced antitumor efficacy *in vivo* (Fig. 5). It might be partly explained that stably encapsulated 4-HPR was much slowly released in medium but was a little enhanced releasing by interaction with various biocomponents such as serum albumin and blood cells *in vivo*. With regard to the toxicity of 4-HPR encapsulated in polymeric micelles, no significant loss of body weight was observed in treated mice, suggesting little severe toxicity (data not shown). These results lead us to believe that enhanced *in vivo* antitumor efficacy of 4-HPR could be achieved by polymeric micelles without severe side-effects.

Lipid particles are known as conventional drug carriers, and have been already used clinically (Tamilvanan, 2004; Torchilin, 2005). In particular, O/W emulsions or PEGylated O/W emulsions can dissolve highly lipophilic drugs in the inner oil phase; however, few reports are available to compare the disposition of lipophilic drugs by encapsulation into polymeric micelles with that in these emulsions. After intravenous injection of 4-HPR encapsulated in O/W emulsions and PEGylated O/W emulsions, 4-HPR were rapidly eliminated from the blood and tumor (Fig. 4). Similarly, *in vivo* antitumor efficacy by 4-HPR encapsulated in O/W emulsion was not observed (Fig. 5). In our previous study, we systematically investigated the *in vivo* disposition of drugs with diverse lipophilicity, and concluded that the required lipophilicity of drugs for stable encapsulation into O/W emulsions was found to be 10^9 based on the partition coefficient between n-octanol and water (PC_{oct}) values (Takino et al., 1994). PC_{oct} of 4-HPR is $10^{8.03}$ (Kokate et al., 2007); therefore, 4-HPR might be rapidly released from O/W emulsions and PEGylated O/W emulsions to the blood stream by the intravenous injection.

Many factors can contribute to tumorigenesis, including inherited and acquired genetic changes, chromosomal rearrangements, epigenetic phenomena and chemical carcinogenesis. Retinoids can interfere with these events on several levels, their principal known actions being the induction of differentiation and apoptosis of tumor cells, and inhibition of tumor promotion in chemically induced cancers (Means and Gudas, 1995; Kagechika and Shudo, 2005). To date, many retinoids are candidates for the treatment of cancers (Altucci and Gronemeyer, 2001; Clarke et al., 2004); however, it is difficult to achieve therapeutic effect under *in vivo* conditions because their highly lipophilic nature and teratogenicity (Collins and Mao, 1999; Soprano and Soprano, 1995). In order to overcome these problems, we developed poly(ethylene glycol)–poly(benzyl aspartate) block copolymer micelles for 4-HPR delivery. In polymeric micelle delivery, drug encapsulation characteristics can be controlled by modification of the hydrophobic segments of block copolymers (Yokoyama et al., 2004; Watanabe et al., 2006; Okuda et al., 2008). Taking these into consideration, polymeric micelles would be effective carriers for various retinoids for cancer chemotherapy in the future.

In conclusion, we have examined the biodistribution characteristics of 4-HPR encapsulated in poly(ethylene glycol)–poly(benzyl

aspartate) block copolymer micelles after intravenous injection in tumor-bearing mice. We have demonstrated that 4-HPR encapsulated in polymeric micelles sustained blood retention of 4-HPR, passive accumulation at tumor sites, and led to superior therapeutic benefits of 4-HPR against solid tumor in tumor-bearing mice.

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