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An investigation of the antitumour activity and biodistribution of polymeric micellar paclitaxel

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Abstract *Purpose*: To evaluate in vitro cytotoxicity, in vivo antitumour activity and biodistribution of a novel polymeric (poly(DL-lactide)-block-methoxy polyethylene glycol) micellar paclitaxel. *Methods*: Hs578T breast, SKMES non-small-cell lung, and HT-29 colon human tumour cells were exposed, either for 1 h or continuously, to conventionally formulated paclitaxel (Cremophor paclitaxel) or polymeric micellar paclitaxel. After a period of incubation, cytotoxicity was measured using a radiometric system. In the in vivo antitumour study, B6D2F1 mice, bearing P388 leukaemia tumour intraperitoneally (i.p.), were treated with polymeric micellar paclitaxel or Cremophor paclitaxel by i.p. injection. The number of deaths and body weights were recorded. In the biodistribution study, CD-1 mice were given micellar paclitaxel i.p. at a dose of 100 mg/kg. The mice were sacrificed after a given time and the organs were harvested. Paclitaxel in the organs was extracted by acetonitrile and analysed using HPLC. *Results*: The polymeric micellar paclitaxel showed similar in vitro cytotoxicity to Cremophor paclitaxel against the tumour cell lines. The polymeric micellar formulation of paclitaxel produced a fivefold increase in the maximum tolerated dose (MTD) as compared with Cremophor paclitaxel when administered i.p. In addition, micellar paclitaxel was more efficacious in vivo when tested in the murine P388 leukaemia model of malignancy than Cremophor paclitaxel when both were administered i.p. at their MTDs. Micellar paclitaxel-treated animals had an increased survival time and, importantly, long-term survivors (20% of those tested) were obtained only in the polymeric paclitaxel formulation group. Biodistribution studies indicated that a significant amount of paclitaxel could be detected in blood, liver, kidney, spleen, lung and heart of mice after i.p. dosing of the polymeric micellar paclitaxel formulation. *Conclusion*: These preliminary results indicate that polymeric micellar paclitaxel could be a clinically useful chemotherapeutic formulation.

Key words Micellar paclitaxel · Antitumour activity · Biodistribution

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Introduction

Paclitaxel, a naturally occurring taxane which is extracted from *Taxus brevifolia*, has shown high activity against a wide range of tumours and has been clinically used in the treatment of metastatic breast cancer, refractory ovarian cancer and several other malignancies [1, 2]. Paclitaxel is a highly hydrophobic drug with very low water solubility in its native form. In order to enhance paclitaxel solubility, a mixture of 50:50 Cremophor EL (a polyoxyethylated castor oil) and ethanol is used in the current clinical formulation. The formulation is diluted 5–20-fold with saline or other aqueous intravenous (i.v.) solutions before i.v. infusion. This results in the administration of significant amounts of Cremophor and serious side effects (such as hypersensitivity and extraction of plasticizer from the i.v. infusion line) attributable to this agent have been observed [3, 4].

The duration of administration of paclitaxel has been shown to influence its clinical effectiveness. Sustained infusions of the drug have produced greater clinical

efficacy than bolus injections or more rapid infusion rates [1]. Administration of a paclitaxel formulation with an enhanced circulation time and sustained paclitaxel release could potentially increase efficacy without the need for prolonged costly continuous i.v. infusion dosing schedules.

To circumvent these problems, a great deal of effort has been given to developing new systemic formulations for paclitaxel which are Cremophor-free, highly soluble, and increase the circulation time of the drug. Cosolvents (ethanol/polysorbate-80, polyethylene glycol, or polyvinylpyrrolidone), oil-in-water emulsion, liposomes, cyclodextrins and surfactants (pluronic L64) have all been employed to enhance paclitaxel solubility [5]. Unfortunately, the problems of low paclitaxel solubility and drug precipitation upon dilution have as yet been incompletely resolved. We have developed polymeric micellar paclitaxel formulations using amphiphilic diblock copolymers of poly(DL-lactide)-block-methoxy polyethylene glycol (PDLLA-MePEG) [6]. The copolymers form micelles of hydrophobic PDLLA cores and hydrophilic MePEG shells in water. A solid paclitaxel/polymer matrix is prepared containing up to 25% paclitaxel (250 mg paclitaxel/750 mg polymer) and is solubilized to form a polymeric micellar paclitaxel system (maximum paclitaxel concentration 50 mg/ml) without precipitation upon dilution. Paclitaxel might be slowly released from the micelles due to the strong hydrophobic association between high molecular weight PDLLA and paclitaxel.

The physicochemical properties of the polymeric micellar formulation have been investigated and reported elsewhere [6]. In the study reported here, the antitumour activity and biodistribution of polymeric micellar paclitaxel were investigated.

Materials and methods

Chemicals

PDLLA-MePEG was synthesized from DL-lactide (Aldrich, Milwaukee, WI) and MePEG (Sigma, Mississauga, Ontario) through ring opening bulk polymerization [6]. Paclitaxel was obtained from Hauser Chemicals, Boulder, Colo. Paclitaxel formulation in Cremophor/alcohol (referred to as "Cremophor paclitaxel") was purchased from Bristol-Myers Squibb, Princeton, NY. Acetonitrile was obtained from Fisher Scientific (Vancouver, BC).

Preparation of micellar paclitaxel

Copolymers of PDLLA-MePEG 2000-40/60 (i.e. molecular weight of MePEG 2000, weight ratio of PDLLA to MePEG 40:60) or PDLLA-MePEG 2000-50/50 were used to formulate paclitaxel. Paclitaxel and the copolymer were dissolved in acetonitrile followed by evaporation of the solvent under a stream of nitrogen at 60 °C for about 2 h. The resulting paclitaxel/copolymer matrix was solubilized by adding hot (60 °C) water to the preheated matrix followed by vortex mixing.

In vitro cytotoxicity

Human tumour cell lines

Hs578T breast, SKMES non-small-cell lung, and HT-29 colon human tumour cell lines were obtained from the American Type Culture Collection. The HT-29 colon cell line was cultured in RPMI-1640 with 10% heat-inactivated fetal bovine serum (HIFBS), the Hs578T breast cell line in Iscove's modified Eagle's medium with 5% HIFBS plus 10^{-9} *M* insulin, and the SKMES lung cell line in Eagle's minimal essential medium with 10% non-heat-inactivated FBS. These cell lines have been shown to detect the activity of conventional antineoplastic agents [7–9].

Radiometric (Bactec) system

The Bactec system (Johnson Laboratories, Towson, Md.) is based on a clinical instrument which was developed to detect bacteria in blood cultures. The instrument has been used to screen for new antineoplastic agents [10]. This radiometric system is a rapid, semiautomated system which utilizes the inhibition of the conversion of ^{14}C-glucose to $^{14}CO_2$ as an index of cytotoxicity. The Bactec system automatically flushes out the $^{14}CO_2$ into an ion chamber where the signal of the radiolabeled CO_2 is changed into a proportional electrical signal or growth index value on a scale of 1–1000.

Percentage survival of the cells

The Hs578T breast, SKMES non-small-cell lung, and HT-29 colon human tumour cells were exposed, either for 1 h or continuously, to Cremophor paclitaxel, polymeric micellar paclitaxel (10% paclitaxel-loaded PDLLA-MePEG 2000-40/60 or 2000-50/50), copolymers alone, and control saline. After a period of incubation, cytotoxicity was measured using the radiometric (Bactec) system and the percent survival relative to saline controls was calculated. For the continuous exposure, tumour cells were added to 2 ml of the appropriate growth medium containing 2 μCi of ^{14}C-glucose plus Cremophor paclitaxel, polymeric micellar paclitaxel or copolymers alone and injected into 20-ml rubber-stoppered serum vials which contained a mixture of 5% CO_2 and air, and incubated at 37 °C for 12 days. For the 1 h exposure, cells and Cremophor paclitaxel, polymeric micellar paclitaxel or copolymers alone were incubated in 15-ml polypropylene conicals in a water bath at 37 °C for 1 h. The cells were then centrifuged and washed in medium, then resuspended in 2 ml of the appropriate growth medium containing 2 μCi ^{14}C-glucose and injected into 20 ml rubber-stoppered serum vials which contained a mixture of 5% CO_2 and air, and incubated at 37 °C for 12 days. At days 6, 9, and 12 the vials were removed and inserted into the Bactec instrument for determination of the amount of $^{14}CO_2$ produced by the cells upon metabolizing the ^{14}C-glucose. The growth index values of treated cells were compared with the growth index values of nontreated cells and the percentage survival compared with untreated controls was calculated.

In vivo efficacy

P388 leukaemia tumour inocula were prepared by removing ascites fluid containing P388 cells from tumoured B6D2F1 mice, centrifuging the cells, and then resuspending the cells in saline. B6D2F1 mice received tumour inocula, intraperitoneally (i.p.), containing 1×10^6 P388 cells on day 0. On day 1, tumoured mice were treated with polymeric micellar paclitaxel (10% paclitaxel-loaded PDLLA-MePEG 2000-40/60 in saline), Cremophor paclitaxel, polymeric

micelle (PDLLA-MePEG 2000-40/60) alone, or saline, by i.p. injection. Polymeric micellar paclitaxel was given at paclitaxel doses of 150, 100 or 50 mg/kg, on a daily × 5 schedule. Cremophor paclitaxel was given at 20 mg/kg (maximum tolerated dose, MTD), also on a daily × 5 schedule. Each group comprised ten mice. The number of deaths and body weight of the mice were recorded.

Biodistribution

Mature CD-1 mice (average weight 30 g) were injected i.p. with polymeric micellar paclitaxel (10% paclitaxel-loaded PDLLA-MePEG 2000-40/60 in saline) at a paclitaxel dose of 100 mg/kg. The mice were sacrificed by cervical dislocation under methoxyflurane anaesthesia. Samples of blood, liver, kidney, lung, heart and spleen were collected at 5 min, 0.5 h, 1.5 h, 3 h, 6 h and 12 h after injection. Paclitaxel was extracted from the tissue samples using acetonitrile [11, 12]. Typically, acetonitrile with a volume (millilitres) of no less than twice the tissue sample weight (grams) was added to the sample. This was followed by homogenization using a Polytron homogenizer. The supernatant was separated by centrifugation. More than 85% of the paclitaxel was extracted into the supernatant from tissue samples spiked with a known amount of paclitaxel.

The paclitaxel concentration in the supernatant was analysed directly using reverse phase high-performance liquid chromatography (HPLC) [11, 12]. HPLC analysis was performed using a 110A pump and C-18 ultrasphere column (Beckman), and a SPD-6A UV detector set at 232 nm, a SIL-9A autoinjector and a C-R3A integrator (Shimadzu). The injection volume was 40 µl and the flow rate was 1.8 ml/min. The mobile phase was 50:50 acetonitrile and water. The peak area was used to calculate paclitaxel concentrations in the supernatant, according to a standard calibration line obtained from pure paclitaxel 60:40 acetonitrile:water solutions. A new calibration was done for every set of sample measurements. The interday variability of the calibration coefficients was less than 15%. The detection limit was about 1 µg/ml. The assay was linear in the range of 1–100 µg/ml.

The area under the curve (AUC) was calculated by the trapezoidal rule. The maximum paclitaxel concentration (C_{max}) in the tissue and the time for reaching the C_{max} (t_{max}) were taken directly from the biodistribution data.

Results

In vitro cytotoxicity

The copolymers alone showed no toxicity in the experimental concentration range (below 90 µg/ml, data not shown). Both polymeric micellar paclitaxel (PDLLA-MePEG 2000-40/60 or 2000-50/50) and Cremophor paclitaxel showed the same efficacy in inhibiting the growth of Hs578T breast tumour cells, SKMES non-small-cell lung tumour cells, or HT-29 colon tumour (Fig. 1). The 1-h exposure resulted in the killing of more than 80% of the cells at a paclitaxel concentration of 0.25 µg/ml, while continuous exposure resulted in the same inhibition at paclitaxel concentrations as low as 0.025 µg/ml.

In vivo efficacy

The results are summarized in Table 1. The MTD was found to be 100 mg/kg and 20 mg/kg for the polymeric micellar paclitaxel and Cremophor paclitaxel given i.p., respectively. The change in mouse weight was measured on day 9, when nine or ten mice were still alive in each group (except in the group receiving 150 mg/kg polymeric micellar paclitaxel in which only five mice survived to this point). The weight increased in saline and polymer control groups, and decreased in all the paclitaxel-treated groups. The extent of the weight loss increased with increasing paclitaxel doses. No toxicity-related deaths occurred during the study (30 days) in the control groups, the Cremophor paclitaxel group, or the polymeric micellar paclitaxel 50 mg/kg group.

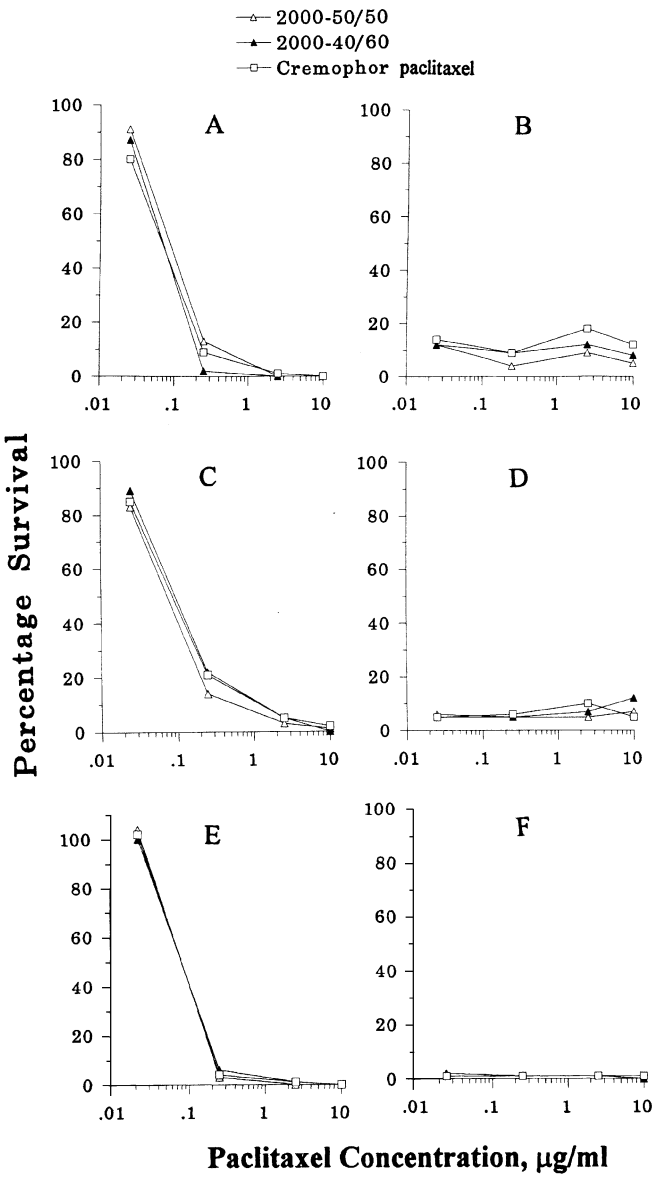

Fig. 1A–F The percentage survival of Hs578T breast tumour cells (**A**, **B**), SKMES non-small-cell lung tumour cells (**C**, **D**) and HT-29 colon tumour cells (**E**, **F**) after exposure to polymeric micellar paclitaxel or Cremophor paclitaxel at various concentrations (**A**, **C**, **E** 1 h exposure; **B**, **D**, **F** continuous exposure)

Table 1 Weight changes, toxic deaths, T/C values, and long-term survivors of i.p. P388 tumoured mice after i.p. administration of saline, copolymer alone, polymeric micellar paclitaxel and Cremophor paclitaxel ($n = 10$)

Group	Dosage/day (daily × 5)	Weight change, day 9 (%)	No. of toxic deaths	T/C (%)	No. of survivors (day 30)
Control	0.9% saline	+ 14.5	0	100	0
Control	Polymeric micelles	+ 10.9	0	121	0
Cremohor paclitaxel	20 mg/kg (MTD)	− 2.9	0	192	0
Polymeric micellar paclitaxel	150 mg/kg	− 22.4	9	212	0
Polymeric micellar paclitaxel	100 mg/kg (MTD)	− 20	1	212	2
Polymeric micellar paclitaxel	50 mg/kg	− 14.1	0	161	0

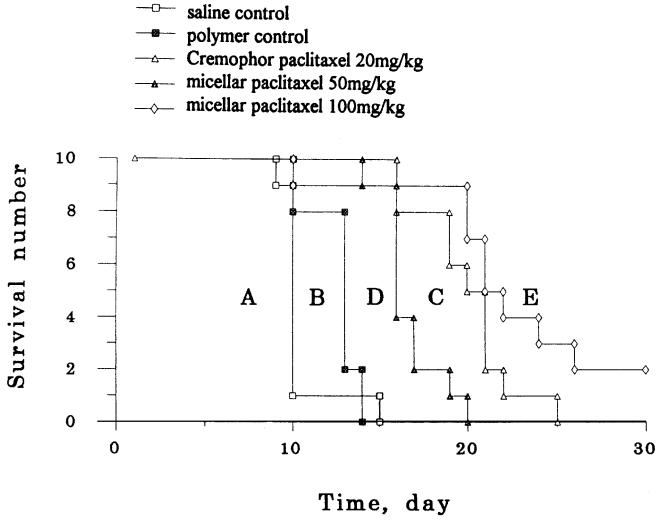

Fig. 2 The survival number of P388 tumoured (i.p.) mice versus time after i.p. injection of saline control (**A**), copolymer alone (**B**), Cremophor paclitaxel 20 mg/kg (**C**), polymeric micellar paclitaxel at dosages of 50 mg/kg (**D**), and 100 mg/kg (**E**)

There was one toxic death in the polymeric micellar paclitaxel 100 mg/kg group. Nine toxic deaths occurred within 11 days in the polymeric micellar paclitaxel 150 mg/kg group.

The survival data for mice in each group are presented in Fig. 2. Cremophor paclitaxel, and polymeric micellar paclitaxel at 100 mg/kg and 50 mg/kg significantly increased lifespan, with the first two groups demonstrating the most pronounced increase. The T/C value (i.e. mean survival of treated mice/mean survival of control mice × 100) was used to interpret the results (Table 1). In the calculation of the mean survival time, the toxic deaths in polymeric micellar paclitaxel groups (100 mg/kg and 150 mg/kg) and the two long-term survivors (survival beyond 30 days, Fig. 2) in the 100 mg/kg polymeric micellar paclitaxel group were not included. According to the NCI model, T/C values represent the following levels of activity: T/C < 125, no activity; T/C 125–150, weak activity; T/C 150–200, modest activity; T/C 200–300, high activity; T/C > 300 with long-term survivors, excellent, curative activity. Applying this index to the data obtained, the copolymer alone showed no antitumour activity. The 50 mg/kg polymeric micellar paclitaxel formulation and Cremophor paclitaxel displayed modest activity, while the 100 mg/kg polymeric micellar paclitaxel produced high antitumour activity with two long-term survivors.

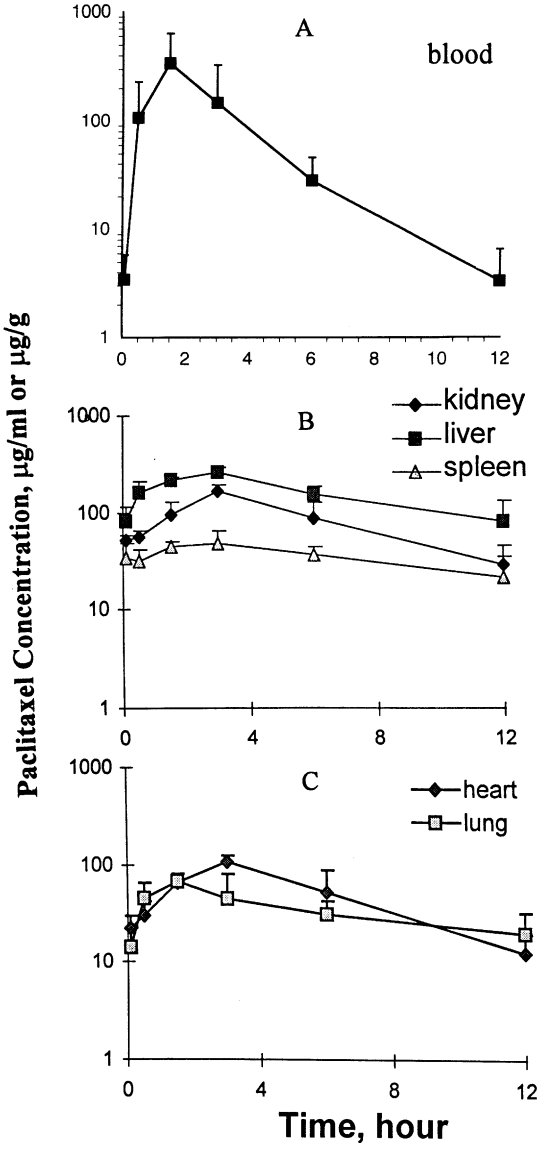

Fig. 3A–C Tissue distribution of paclitaxel in blood (**A**), liver, kidney, spleen (**B**), lung and heart (**C**) after i.p. injection of polymeric micellar paclitaxel (100 mg/kg) to mice ($n = 3$)

Table 2 The t_{max}, C_{max}, and AUC of paclitaxel in mice after i.p. injection of 100 mg/kg polymeric micellar paclitaxel ($n = 3$)

	Blood	Liver	Kidney	Heart	Lung	Spleen
t_{max} (h)	1.5	3	3	3	1.5	3
C_{max} (μg/g)	340	267	170	109	68	48
$AUC_{0\rightarrow 12\,h}$ (μg h/ml or μg h/g)	968	1962	1049	635	428	425

Biodistribution

Significant amounts of paclitaxel were detected in tissue samples of blood, liver, spleen, kidney, lung and heart following intraperitoneal administration of polymeric micellar paclitaxel (Fig. 3). Paclitaxel in each tissue examined reached a peak concentration followed by an exponential decrease. The time required to reach the peak concentration was approximately 1.5 h in blood and lung and 3 h in liver, kidney, spleen and heart. The highest peak concentration was found in blood, followed by (in decreasing order) liver, kidney, heart, lung and spleen. The AUC values from time 0 to 12 h decreased in the order liver > kidney > blood > heart > lung and spleen (Table 2).

Discussion

Amphiphilic diblock copolymers can form micelles which are effective carriers for hydrophobic drugs [13, 14]. The drugs can be either covalently coupled to diblock copolymers to form micellar structures or can be physically incorporated within the hydrophobic cores of polymeric micelles [15]. One type of diblock copolymer, poly(aspartic acid)-block-polyethylene glycol, has been evaluated as a micellar carrier of the anticancer drug adriamycin for i.v. administration [13–15]. A high drug payload, long circulation time in the blood, and good antitumor activity have been observed with this system [15–18]. Another type of diblock copolymer recently evaluated as a micellar drug carrier is poly(DL-lactide)-block-methoxy polyethylene glycol [6, 19–22].

In contrast to conventional surfactants (e.g. Cremophor), amphiphilic diblock copolymers such as PDLLA-MePEG possess unique properties. Owing to the high molecular weight of the copolymers, the association among the hydrophobic block (e.g. PDLLA) is very strong. Therefore the hydrophobic core of the micelle is highly viscous or effectively solid and the equilibration between single copolymer chains (or polymer molecules) and micelles is very slow [6, 23]. This results in very low critical micelle concentrations (CMC). The single copolymer chain even forms a core-shell structure itself [24] and the polymer molecules can solubilize paclitaxel through hydrophobic bonding between paclitaxel and PDLLA in the polymer molecules. The strong hydrophobicity of PDLLA allows for a large amount of paclitaxel to be loaded into the micelles and improves stability of the polymeric micellar paclitaxel system. The strong hydrophobic association between paclitaxel and the copolymer also results in different biodistribution, pharmacokinetics and in vivo efficacy. It may be speculated that the dissociation of free paclitaxel from the polymeric micelles or the polymer molecules to body components (such as plasma proteins and cells) is slow and provides a more sustained release of the drug.

The copolymer concentrations in the in vitro cytotoxicity studies (Fig. 1) were all less than 30 μ*M*, which is below the CMC [6]. The similarity between the activities of polymeric formulations and the Cremophor formulation (Fig. 1) may indicate that paclitaxel is readily available to the cells under these conditions or that the polymer molecule-bound paclitaxel is itself cytotoxic.

The higher MTD observed with polymeric micellar paclitaxel (Table 1) is probably attributable to the following circumstances. First, the dissociation rate of paclitaxel from the polymeric micelles is probably slower than from the Cremophor micelles which results in lower peak plasma levels. Second, the PDLLA-MePEG copolymers may not possess the toxicities associated with the Cremophor and ethanol vehicle and the amount of vehicle used in the polymer formulation is about 18 times lower than that required for the Cremophor formulation.

In the in vivo experiment, polymeric micellar paclitaxel produced greater efficacy in terms of extending the survival time of tumour-bearing mice and produced animals which were long-term survivors (Table 1). Although paclitaxel was administered to the tumour site directly (peritoneal cavity), the high antitumour activity obtained from the polymer formulation warrants further study in an animal model which requires the dissociation of paclitaxel from the administration site to distant disease sites in the body.

Paclitaxel peak concentrations in blood, liver, kidney, heart, lung and spleen represented 11%, 9%, 6%, 4%, 2% and 2% of the dose/g tissue, respectively. The $AUC_{0\rightarrow 12\,h}$ value in blood was 968 μg h/ml (Fig. 3, Table 2). Studies on biodistribution and pharmacokinetics of Cremophor paclitaxel in mice after i.p. injection have been reported [11, 12]. In one study, the bioavailability of i.p.-injected Cremophor paclitaxel in CD2F1 mice was found to be approximately 10% and

the $AUC_{0\rightarrow\infty}$ value in the plasma was about 6 μg h/ml (dose 22.5 mg/kg) [11]. In another study, $AUC_{0\rightarrow 30\,h}$ in plasma was found to be 113.2 μg h/ml (dose 18 mg/kg) or 141.9 μg h/ml (dose 36 mg/kg) after i.p. injection of Cremophor paclitaxel into Swiss female mice [12].

In summary, the polymeric micellar paclitaxel showed similar in vitro cytotoxicity to Cremophor paclitaxel against a variety of tumour cells. It also displayed a fivefold increase in the MTD as compared with conventionally formulated Cremophor paclitaxel when administered systemically (i.p.). In addition, micellar paclitaxel was more efficacious in vivo when tested in the murine P388 leukaemia model of malignancy than Cremophor paclitaxel when both were administered systemically at their MTDs. Biodistribution studies indicated that a significant amount of paclitaxel can be detected in blood, liver, kidney, spleen, lung and heart of mice after i.p. dosing of the polymeric micellar paclitaxel formulation. These preliminary results indicate that polymeric micellar paclitaxel could be a clinically useful chemotherapeutic formulation.

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