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Poly(*N*-vinylpyrrolidone)-block-poly(D,L-lactide) as a new polymeric solubilizer for hydrophobic anticancer drugs: in vitro and in vivo evaluation

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Abstract

The majority of novel anticancer drugs developed to date are intended for parenteral administration. Paradoxically, most of these drugs are water-insoluble, delaying their clinical development. A common approach to confering water solubility to drugs is to use amphiphilic, solubilizing agents, such as polyethoxylated castor oil (e.g., Cremophor® EL, CrmEL). However, these vehicles are themselves associated with a number of pharmacokinetic and pharmaceutical concerns. The present work is aimed at evaluating a novel polymeric solubilizer for anticancer drugs, i.e., poly(*N*-vinylpyrrolidone)-block-poly(D,L-lactide) (PVP-b-PDLLA). This copolymer self-assembles in water to yield polymeric micelles (PM) that efficiently solubilize anticancer drugs, such as paclitaxel (PTX), docetaxel (DCTX), teniposide (TEN) and etoposide (ETO). A PM-PTX formulation was evaluated, both, in vitro on three different cancer cell lines and in vivo for its safety, pharmacokinetics, biodistribution and antitumor activity. In vitro, cytotoxicity studies revealed that the drug-loaded PM formulation was equipotent to the commercial PTX formulation (Taxol®). In the absence of drug, PVP-b-PDLLA with 37% DLLA content was less cytotoxic than CrmEL. In vivo, acute toxicity was assessed in mice after a single injection of escalating dose levels of formulated PTX. PM-PTX was well tolerated and the maximum tolerated dose (MTD) was not reached even at 100 mg/kg, whereas the MTD of Taxol® was

Abbreviations: AUC, area under the plasma concentration–time curve; C_0, extrapolated peak plasma concentration; CAC, critical association concentration; CrmEL, Cremophor® EL; DCTX, docetaxel; DMAc, *N*,*N*-dimethylacetamide; DSC, differential scanning calorimetry; ETO, etoposide; FBS, fetal bovine serum; H&E, hematoxylin and eosin; ^{1}H-NMR, proton nuclear magnetic resonance; IC_{50}, inhibitory concentration of drug producing 50% cell growth inhibition or death; i.v., intravenous; M_n, number-average molecular weight; MTD, maximum tolerated dose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMP, *N*-methyl-2-pyrrolidinone; PBS, phosphate-buffered saline; PDLLA, poly(D,L-lactide); PEG, poly(ethylene glycol); PG, propylene glycol; PM, polymeric micelles; PTX, paclitaxel; PVP-*b*-PDLLA, poly(*N*-vinylpyrrolidone)-*block*-poly(D,L-lactide); RBC, red blood cells; s.c., subcutaneous; TBA, *tert*-butanol; TEN, teniposide; TNF-α, tumor necrosis factor-alpha; Vd_{ss}, volume of distribution at steady state; $t_{1/2}$, elimination half-life.

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established at 20 mg/kg. At 60 mg/kg, PM-PTX demonstrated greater in vivo antitumor activity than Taxol® injected at its MTD. Finally, it was shown in mice and rabbits that the areas under the plasma concentration–time curves were inversely related to PM drug loading.

Keywords: Polymeric micelles; Taxanes; Maximum tolerated dose; Pharmacokinetics; In vivo antitumor activity

1. Introduction

Nonionic, low-molecular weight surfactants are widely used as parenteral formulation vehicles to solubilize hydrophobic anticancer drugs. For example, taxane and podophyllotoxin derivative solutions for intravenous (i.v.) administration are formulated with polyethoxylated castor oil (Cremophor® EL, CrmEL) or polysorbate 80 (Tween® 80). Most low-molecular weight surfactants are biologically and pharmacologically active, and their use has been associated with clinically significant side effects, including acute hypersensitivity reactions and peripheral neurotoxicity [1]. CrmEL and polysorbate 80 have also been shown to alter the pharmacodynamic characteristics of the solubilized drugs [1,2]. Thus, much research is being carried out to identify alternative strategies for in vivo delivery of hydrophobic anticancer compounds. Among the wide panel of novel formulations under investigation, some turned out to be promising and are in a relatively advanced stage of development. For example, a cholesterol-rich emulsion of paclitaxel (PTX) was shown to be remarkably well tolerated in rats with a 50% lethal dose 10-fold greater than the commercial formulation [3]. Liposome-encapsulated PTX has entered clinical phase I for the treatment of advanced malignancies [4]. More recently, a CrmEL-free protein-stabilized nanoparticle formulation of PTX permitted drug administration without routine premedication for the prevention of hypersensitivity reactions, and exhibited a higher maximum tolerated dose (MTD) than PTX formulated in CrmEL. These results provided support for phase II trials to profile the antitumor activity of the drug [5].

Over the last two decades, polymeric micelles (PM) have received increasing interest as a means to solubilizing poorly water-soluble drugs [6–10]. Exhibiting remarkable stability in solution, PM can achieve high drug loading compared to low-molecular weight surfactants. Owing to their core-shell architecture, they can protect drugs against premature degradation in vivo. Moreover, their small size (5–100 nm) may potentially increase the drug circulation time after i.v. administration. Several amphiphilic polymer structures have been described in the literature [11–19]. Among these, diblock copolymers are particularly well suited for the preparation of PM. They consist of a shell-forming hydrophilic block linked to a core-forming hydrophobic segment. Due to its biodegradability and excellent biocompatibility, poly(D,L-lactide) (PDLLA) is widely used as a core-forming block. In particular, PM obtained from the self-assembly of poly(ethylene glycol)-block-PDLLA (PEG-b-PDLLA) have been investigated extensively [12,20]. PEG-b-PDLLA micelles can accommodate high levels of PTX, and substantially increase the drug's MTD. At their respective MTD, PTX-loaded PEG-b-PDLLA micelles exhibited greater activity than Taxol® against human ovarian adenocarcinoma tumor xenografts (OVCAR-3) [12].

Recently, poly(*N*-vinylpyrrolidone) (PVP) was proposed as an alternative to PEG as the shell-forming block [21]. PVP is a biocompatible, water-soluble polymer that can be found in various drug delivery systems, including microspheres, nanoparticles, liposomes, and polymer conjugates [22–28]. For instance, Kamada et al. [27] showed that PVP conjugated to tumor necrosis factor-alpha (TNF-α) was a more potent antitumor therapeutic agent than PEGylated TNF-α. It was also reported that PVP-coated liposomes displayed prolonged in vivo circulation time after i.v. administration [26]. Due to its cryo/lyoprotectant properties, PVP can overcome resuspension problems encountered after freeze-drying [29].

Given the unique properties of PVP and PDLLA, it was hypothesized that poly(*N*-vinylpyrrolidone)-block-poly(D,L-lactide) (PVP-b-PDLLA) PM could

prove to be useful carriers to solubilize hydrophobic drugs. In this work, four anticancer drugs, namely PTX, docetaxel (DCTX), teniposide (TEN) and etoposide (ETO), were formulated with PVP-b-PDLLA. The PM were loaded with the different drugs either by direct dissolution or by a novel one-step, freeze-drying process and assessed for their stability upon dilution with isotonic fluids [30]. A PTX-loaded formulation was selected as a model for further investigation and was characterized both in vitro and in vivo with respect to its toxicity, pharmacokinetics, biodistribution, and antitumor activity.

2. Materials and methods

2.1. Materials

PTX, DCTX, and TEN were kindly provided by the Shanghai Fudan Taxusal New Technology (Shanghai, China). ETO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from the Sigma-Aldrich (Oakville, ON, Canada). Taxol® (Bristol-Myers Squibb, New York, NY) was purchased in a retail pharmacy. Cremophor® EL was a generous gift from Cercan-BASF (Montreal, QC, Canada). The internal standard *N*-heptylbenzamide was synthesized according to a simple and convenient Schotten–Baumann method using benzoyl chloride and heptylamine, as described elsewhere [31,32]. All other chemicals and solvents were of reagent grade and were processed as received. Aqueous solutions were prepared in deionized distilled water from a Milli-Q water system (Millipore, Fisher Scientific, Nepean, ON, Canada).

2.2. Synthesis and characterization of PVP-b-PDLLA

PVP-b-PDLLA was synthesized, purified, and characterized as described previously [21,33]. Briefly, hydroxy-terminated PVP was prepared by free radical polymerization of *N*-vinyl-pyrrolidinone with 2-mercaptoethanol as a chain transfer agent and 2,2′ azobis(2-hydroxyethyl)propionamide (Wako Chemicals, Richmond, VA) as hydroxyl-bearing azo initiator. Polymerization was carried out in isopropyl alcohol at 85 °C in an argon atmosphere. PVP homopolymer was recovered by precipitation in diethyl ether. Ring-opening polymerization of D,L-lactide was initiated by the terminal hydroxyl groups of PVP in the presence of potassium hydride. PVP-b-PDLLA was purified by dialysis against water (molecular weight cutoff: 3500, Spectrum Laboratories, Laguna Hills, CA), centrifuged to remove any precipitated PDLLA homopolymer, freeze-dried, and stored at −20 °C (yield ~65%). The polymers were characterized by size exclusion chromatography with a refractometer and light scattering detector, and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The critical association concentrations (CACs) of the copolymers were determined in water at 25 °C by a steady-state pyrene fluorescence method, as described elsewhere [34].

2.3. Preparation of drug-loaded PVP-b-PDLLA micelles

2.3.1. Method A: preparation of powdered formulations

PTX and DCTX-loaded PM (2.5%, 4% or 5% w/w) were prepared by first dissolving the drug in *tert*-butanol (TBA), then mixing the organic solution with an aqueous PVP-b-PDLLA solution to obtain a water/TBA ratio of 70/30 (v/v). The solutions were gently stirred for 3 h at 4 °C, sterilized by passage through a 0.2-μm filter and freeze-dried. Drug-loaded PM were obtained after adding dextrose 5% (w/v) to the lyophile (Fig. 1) [30]. Stability testing of these solid-state formulations at 48 h, 2 weeks and 1 month was undertaken by differential scanning calorimetry (DSC) and X-ray diffraction, as described earlier [33,35].

2.3.2. Method B: preparation of liquid formulations

Stock solutions of drug and PVP-b-PDLLA were processed separately in injectable organic solvents [i.e., *N*-methyl-2-pyrrolidinone (NMP), *N*,*N*-dimethylacetamide (DMAc), or ethanol] at concentrations of 40 and 200 mg/ml, respectively. The solutions were mixed at different polymer-to-drug ratios to obtain final drug loadings ranging from 5% to 20% (w/w). The mixtures were then diluted in dextrose 5% (w/v), to produce clear solutions containing less than 3% (v/v) organic solvent.

The stability of the drug-loaded PM obtained by either Method A or B was monitored over time by dynamic light scattering (Malvern Autosizer 4800,

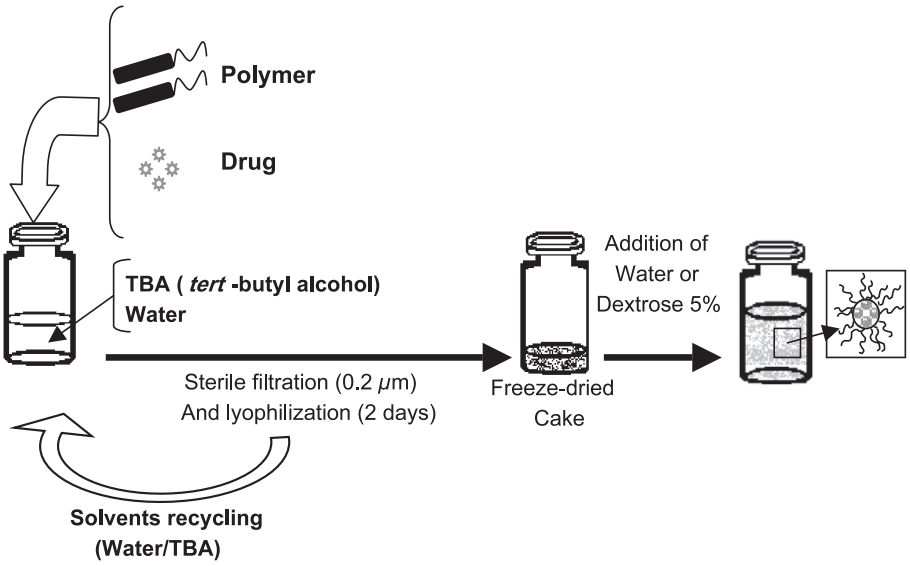

Fig. 1. Scheme of the freeze-drying, drug-loading procedure (Method A).

Malvern Instruments, Worchestershire, UK) and visually for signs of opalescence and precipitation. All PTX-loaded PM for the in vitro cytotoxicity assays and in vivo experiments were prepared by Method A.

2.4. Cell lines and culture conditions

Murine colon adenocarcinoma tumor C26 and mammary carcinoma tumor EMT-6 cells were kindly donated by Prof. J.E. van Lier of the University Hospital of Sherbrooke (QC, Canada). Human OVCAR-3 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture medium (RPMI 1640, Waymouth) and heat-inactivated fetal bovine serum (FBS) were obtained from Invitrogen Canada (Burlington, ON, Canada). C26 and OVCAR-3 cells were maintained in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin G, and 100 μg/ml streptomycin. EMT-6 cells were maintained in Waymouth's medium supplemented with 15% FBS, 1% L-glutamin, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

2.5. Cytotoxicity assays

In vitro cytotoxicity of the vehicles, PTX-loaded PM and Taxol® was determined by MTT assay [36]. The cells (5×10^3 C26, 5×10^3 EMT-6 cells/ml or 5×10^4 OVCAR-3 cells/ml) were seeded in 96-well tissue culture plates, and allowed to adhere for 24 h at 37 °C in a humid atmosphere containing 5% CO_2. Ten microliters of various concentrations of vehicles (2.1×10^{-3} to 5.2×10^3 μg/ml) or formulated PTX (1×10^{-4} to 25.6 μg/ml) was then added to the wells in triplicate. The cells were incubated for 96 h, and 10 μL MTT [5 mg/ml in phosphate-buffered saline (PBS); 53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl, pH 7.4] was added to each well. After another 3-h incubation, sodium dodecyl sulfate (100 μl of a 10% w/v solution containing 0.01 N hydrochloric acid) was added to the wells to dissolve reduced MTT. Absorbance was measured 24 h later at 570 nm with a PowerWave microplate reader (Biotek Instruments, Winooski, VT). The toxicity of the vehicles and PTX formulations was expressed as the inhibitory concentration at which 50% of cell growth inhibition was obtained (IC_{50}). Each experiment was performed three times.

2.6. In vitro hemolysis assay

This procedure was described previously by Murthy et al. [37]. Human red blood cells (RBC) were collected from a healthy donor in vacutainer tubes (Fisher Scientific, Montreal, QC, Canada)

containing 7.5% (w/v) K_3EDTA. The tubes were centrifuged at 4 °C (200×*g*, 5 min), and the cells were washed three times (centrifugation followed by redispersion) with PBS. Hemolysis assay was performed by adding RBC to the vehicles dissolved in PBS (final cell concentration 1×10^8 RBC/ml). The samples were incubated for 30 min at 37 °C under stirring, cooled on ice, and centrifuged at 4 °C (5300×*g*, 5 min) to determine the extent of membrane disruption. Hemoglobin absorbance in the supernatant was measured at 541 nm, using a PowerWave microplate reader. To obtain 100% hemolysis, cells (1×10^8 RBC/mL) were lysed by dispersion in water. Controls were prepared by mixing the RBC with buffer. Each concentration was evaluated in triplicate.

2.7. Animals

Female Balb/C mice weighing 16–20 g were obtained from Charles River Breeding Laboratories (Montreal, QC, Canada) and acclimatized for 7 days after arrival. Female New Zealand White rabbits weighing 3–4 kg were purchased from the same supplier and acclimatized for 2 days after arrival. The animals were provided with food and water ad libitum, and were exposed to alternating 12-h periods of light and darkness. All experiments followed a protocol approved by an in-house ethics committee in accordance with Canadian Council on Animal Care guidelines.

2.8. Evaluation of toxicity and determination of the MTD

The in vivo toxicity of PM-PTX and Taxol® after i.v. injection was investigated in healthy female Balb/C mice. Eight groups of mice (*n*=4–8/group) received a single dose of Taxol® at 20 or 30 mg/kg, PVP-b-$PDLLA_{37\%}$–PTX (5% w/w) at 20, 40, 60, 80 or 100 mg/kg, or dextrose 5% as a control. The formulations were administered via the subclaviar vein (0.2 ml/20 g). Mouse survival and body weight variations were monitored daily for 13 days in all groups. On day 8, two mice per group were sacrificed by cardiac exsanguination for blood cell count and histological tissue analysis (liver). The MTD was defined as the highest dose that induced no more than 15% weight loss vs. control, caused no toxic death, and was not associated with remarkable changes in vital signs within a week after administration. Hematotoxicity was evaluated by RBC and hemoglobin analysis of blood samples (Biovet, St-Hyacinthe, QC, Canada). For histological analysis, liver was fixed in neutral-buffered 10% formalin, processed by standard methods in paraffin, sectioned at 5–10 μm, and stained with hematoxylin and eosin (H&E).

2.9. Inoculation of tumor cells

One day before tumor implantation, hair on the hind legs and back of the mice was removed by shaving and chemical depilation. C26 cells were harvested by trypsinization and resuspended in growth medium. Approximately 2×10^6 cells in 0.05 ml growth medium were injected subcutaneously (s.c.) into the back of the mice and allowed to grow to a mean volume of 20 mm^3. Tumor volume was calculated as $1/2(4\pi/3)(L/2)(W/2)H$, where L is the length, W is the width and H is the height of the tumor.

2.10. Pharmacokinetics and biodistribution

Three C26 tumors were injected s.c. into the back of the mice, and drug injection was started 10 days after cell inoculation when tumor volume reached approximately 20 mm^3. Tumor-bearing mice were anesthetized with isoflurane, and injected i.v. via the subclaviar vein with Taxol® at 20 mg/kg or PVP-b-$PDLLA_{37\%}$–PTX (5% w/w) at 20 or 60 mg/kg for a total volume of 0.2 ml/20 g body weight. Blood was sampled by heart puncture in heparinized syringes at different time intervals (3, 15, 30 min; 1, 2, 4, 8, 12 and 24 h) after drug administration. The animals (*n*=4/group) were sacrificed at all time intervals. Blood was centrifuged in Eppendorf tubes at 4 °C (1200×*g*, 5 min), and plasma was collected. Organs and tissues of interest were harvested, washed with 0.9% saline (w/v), blotted dry and stored at −80 °C until analysis for PTX.

The effect of PTX loading on plasma pharmacokinetics was evaluated in mice and rabbits. Healthy female Balb/C mice were injected with PM loaded with 1%, 2%, 3.6%, or 5% (w/w) PTX, as described above. Healthy female New Zealand rabbits (*n*=4/group) were injected i.v. via an indwelling catheter in

the marginal ear vein with Taxol® or PM containing 2.5 or 5% (w/w) PTX at 5 mg/kg in a volume ranging from 1.3 to 1.95 ml/kg body weight. Blood was sampled via the marginal ear vein in heparinized syringes at different time intervals (3, 15, 30 min; 1, 2, 4, 8, 12, and 24 h) after drug administration, and plasma was collected as described above. After the last blood sample (24 h), the rabbits were sedated with a mixture of xylazine (0.25 mg/kg) and ketamine hydrochloride (0.35 mg/kg), then euthanized with an overdose of i.v.-injected sodium pentobarbital followed by exsanguination.

2.11. HPLC method

Plasma and tissue concentrations of PTX were determined by reverse-phase HPLC coupled with a UV detector [32,38]. Briefly, 100-μl plasma samples were mixed with 1200 μl of 0.05% (w/v) trichloroacetic acid and 50 μl of *N*-heptylbenzamide (20 mg/l in methanol) as the internal standard. The mixture was extracted with 7.2 ml of ethyl acetate by vortexing for 30 s, stirring for 10 min at room temperature, and centrifugating at 4 °C (1290×*g*, 20 min). Five milliliters of the organic phase was transferred into a clean tube and evaporated to dryness in a SpeedVac concentrator (Model SC210A, Savant Instruments, Farmingdale, NY, USA) at 40 °C (1730×*g*, 2 h). The extraction residue was redispersed in 200 μl of the mobile phase (acetonitrile/water/methanol, 48:41:11 v/v/v). To minimize contamination from the protein pellets, the 200-μl aliquot was transferred to a 1.5-ml centrifuge tube and centrifuged at 4 °C (2300×*g*, 5 min). The supernatant was collected and injected for analysis. Whole tumors and aliquots of harvested organs (80–150 mg) were homogenized in 12-fold volume of 0.05% (w/v) trichloroacetic acid with 50 μl of *N*-heptylbenzamide (20 mg/l in methanol) using a PowerGen homogenizer (Model 125, Fisher Scientific, Montreal, QC, Canada). The probe was rinsed with 4000 μl ethyl acetate that was transferred to the homogenates. The homogenates were then mixed with 72-fold volume minus the 4000 μl of ethyl acetate used for rinsing, and extracted as described above. Chromatographic separation was performed with a Waters HPLC system equipped with a 1525 Binary pump, a Waters Nova-Pack C18, 60 Å, 4 μm (3.9×300 mm) column, a 2487 Dual Wavelength Absorbance Detector, and Breeze Chromatography Software (Waters, Milford, MA). The flow rate, detection wavelength, and injection volume were set at 1.0 mL/min, 232 nm and 55 μL, respectively. The assay was linear over a concentration range of 0.025–20 mg/L, with a standard correlation coefficient >0.995. The pharmacokinetic profiles underwent noncompartmental routine analysis with WinNonlin software (WinNonlin, Professional Edition, version 1.5, Pharsight, Mountain View, CA). The area under the plasma concentration–time curve (AUC) in tissues was calculated according to the linear trapezoidal method. Standard deviations of AUC in the pharmacokinetic and biodistribution experiments were calculated by the bootstrap test method with S-PLUS 2000 software (S-Plus 2000 Professional, Cambridge, MA) [39].

2.12. Study of the influence of drug loading on in vitro blood:plasma ratios

To ascertain the extent of drug partitioning into blood cells, in vitro blood:plasma concentration ratios were assessed at different PTX loadings in mouse blood. Aliquots of heparinized blood (3 ml) were placed in a 37 °C shaking bath for 5 min, then mixed with the PTX formulations (Taxol®, PM-PTX at 1%, 2%, and 5% w/w drug loading) to a final concentration of 0.3 mg/ml. After 60-min incubation, three blood aliquots (100 μl) were withdrawn from the incubation tubes and kept at −80 °C for at least 5 min to achieve complete hemolysis. The remaining blood was centrifuged at 4 °C (1200×*g*, 5 min) for plasma collection. Blood and plasma samples were assayed by HPLC as described above.

2.13. In vivo antitumor activity assay

The antitumor activity of PM-PTX against solid tumors was evaluated on murine C26 colon adenocarcinoma cells. A single C26 tumor was injected s.c. into the back of female Balb/C mice, which were randomly assigned to one of five treatment groups. Treatment was initiated on day 0 when the tumor reached approximately 20 mm^3. Taxol® and PM-PTX (5% w/w PTX) were injected i.v. at doses corresponding to 20 and 60 mg/kg PTX, respectively, on days 0, 1, 2, 7, 8, and 9 (n=15 mice/group). Control groups (n=12/group) received dextrose 5%, Taxol® vehicle

(CrmEL:dehydrated alcohol 1:1 v/v) or PVP-b-PDLLA$_{37\%}$ at a dose corresponding to the amount of vehicle injected for the drug-loaded formulations. Tumor size was measured every day from day 0 to day 12, then every 2 days until day 18. Body weight change was also monitored. In a separate and parallel study, hematotoxicity and histology evaluations of the PTX formulations were conducted on healthy and C26 tumor-bearing mice (n=8) after a block of three successive daily injections.

2.14. Statistical analysis

Differences between the AUC in pharmacokinetics and biodistribution were analyzed for statistical significance by the Kruskal–Wallis test followed by Nemenyi's post hoc test for multiple comparisons. Differences in mean tumor volumes at each time point of the antitumor activity experiment were analyzed by one-way analysis of variance followed by Scheffé's post hoc test, or the Kruskal–Wallis test followed by the Dunn's post hoc test for multiple comparisons. p-Values lower than 0.05 were considered to be significant.

3. Results

3.1. Characterization of PVP-b-PDLLA-based formulations

Two copolymers with different DLLA content, i.e., PVP-b-PDLLA$_{27\%}$ and PVP-b-PDLLA$_{37\%}$ (Table 1), were synthesized using PVP-OH with number-average molecular weights (M_n) of 2300 and 2480, respectively. CAC was low and inversely related to DLLA content. Drug-loaded micelles of PVP-b-PDLLA$_{37\%}$ were associated with the best results in terms of particle size, visual aspect and stability of the aqueous dispersions (data not shown). Incorporation of either ETO, TEN, DCTX or PTX yielded drug-loaded PVP-b-PDLLA$_{37\%}$ micelles with sizes ranging between 15 and 60 nm, regardless of the drug-loading method used (Table 2). The two loading procedures, A and B, resulted in at least 95% entrapment efficiency since less than 10% of drug was lost in the filtration step. PTX and DCTX incorporation by the freeze-drying procedure (Method A) required extensive optimization. Parameters such as water/TBA ratio, order of addition of solvents, mixing temperature (4 vs. 20 °C), stirring time and freezing temperature (−50 vs. −80 °C) were investigated. The best conditions in terms of micelle size and stability of the reconstituted formulations were a water/TBA ratio of 70/30 (v/v), a mixing time of 3 h at 4 °C, and a freezing temperature of −50 °C. Under these conditions, the freeze-dried cake obtained was highly porous and gave a transparent micelle solution (~30 nm) after redispersion in dextrose 5%. DSC and X-ray diffraction analyses demonstrated remarkable stability of PM-PTX in the solid state over a 1-month period at 4 °C, with no sign of drug crystallization (data not shown). The reconstituted PTX and DCTX formulations were stable at least 6 and 24 h at room temperature, respectively.

ETO and TEN could not be incorporated in PM with Method A due to their lack of solubility in TBA. Drug loadings reaching 10% and 20% (w/w) could be achieved with TEN and ETO, respectively, when the drug and polymer were initially dissolved in an organic solvent approved for parenteral use (Method B). Dilution of the solvent with dextrose 5% yielded clear solutions with near-monodispersed micelles (Table 2). The TEN formulation was stable for at least 1 week after dilution, which is far superior to the stability of diluted commercial formulations of most anticancer drugs. PTX, DCTX, and ETO formulations were stable for at least 24 h, more than 24 h, and 48 h at room temperature, respectively.

Table 1
Characterization of PVP-b-PDLLA copolymers

Polymer	PVPOH M_n[a] (g mol^{-1})	M_n[a] (g mol^{-1})	M_w/M_n[a]	PDLLA content (mol%)[b]	CAC (mg/l)
PVP-b-PDLLA$_{27\%}$	2300	3800	1.3	27	10
PVP-b-PDLLA$_{37\%}$	2480	4300	1.2	37	6

[a] Determined by size exclusion chromatography.
[b] Determined by ^{1}H-NMR spectroscopy.

Table 2
Characterization of different formulations of anticancer drugs incorporated in PVP-b-PDLLA$_{37\%}$ micelles

Drug	Drug-loading (% w/w)	Drug-loading method	Organic solvent (% after final dilution)	Diameters (nm)[a]	Stability after reconstitution[a]
∅	0	–	–	33.7±7.8	–
DCTX	4	A	TBA <20 ppm[b]	21.8±5.1	48 h
PTX	5	A	TBA <20 ppm[b]	56.0±9.6	48 h
ETO	20	B	DMAc (2.8)	30.7±5.8	48 h
TEN	10	B	NMP (2.8)	30.0±6.6	1 week
TEN	10	B	DMAc (2.8)	29.8±4.1	1 week
DCTX	4	B	DMAc (1.8)	25.6±5.5	48 h
DCTX	4	B	ethanol (1.8)	20.6±5.5	48 h
PTX	5	B	DMAc (1.8)	17.1±4.2	24 h
PTX	5	B	ethanol (1.8)	15.9±5.0	24 h

[a] Redispersed in dextrose 5% (w/v), 25 °C.
[b] Residual TBA in micelles was assayed by head space gas chromatography [53].

3.2. In vitro cytotoxicity experiments

PVP-b-PDLLA$_{37\%}$–PTX, PVP-b-PDLLA$_{27\%}$-PTX (2.5 or 5%), Taxol®, and the drug-free vehicles were tested in vitro for their cytotoxicity against murine C26, EMT-6 and human OVCAR-3 cell lines (Table 3). The IC_{50} of PVP-b-PDLLA alone was two orders of magnitude higher than that of the CrmEL/ethanol vehicle used in the Taxol® formulation. As reported in Table 3, PTX-loaded PM and Taxol® showed similar concentration-dependent growth inhibition for all cell lines. However, each cell line exhibited different sensitivities to the drug. OVCAR-3 cells were the most sensitive to PTX, with an IC_{50} value of 0.004 μg/ml. In contrast, C26 cells were relatively resistant to this drug, with an IC_{50} value 10-fold higher than that of OVCAR-3 cells. Toxicity of the vehicle was also investigated by hemolysis assay. The block copolymers in this study were amphiphilic and could solubilize lipids or be inserted into phospholipid membranes to destabilize them [40–42]. The hemolytic activity of PVP-b-PDLLA diblock copolymers and CrmEL was negligible (less than 2%), even at concentrations up to 10 mg/ml (data not shown).

Table 3
Comparison of in vitro cytotoxicity of PM-PTX vs. Taxol®

Formulation	Tumor cell lines		
	C26	EMT-6	OVCAR-3
	IC_{50} (μg/ml)		
PVP-b-PDLLA$_{27\%}$-PTX (2.5% w/w)	0.039	0.017	0.004
PVP-b-PDLLA$_{27\%}$-PTX (5% w/w)	0.039	0.017	0.004
PVP-b-PDLLA$_{37\%}$-PTX (2.5% w/w)	0.041	0.017	0.004
PVP-b-PDLLA$_{37\%}$-PTX (5% w/w)	0.039	0.017	0.004
Taxol®	0.029	0.016	0.003
PVP-b-PDLLA$_{27\%}$	1890	1041	2929
PVP-b-PDLLA$_{37\%}$	1193	1250	4405
CrmEL	745	772	865

IC_{50}, inhibitory concentration of paclitaxel or vehicles producing 50% cell growth inhibition or death.

3.3. Evaluation of in vivo toxicity

To determine the MTD of PTX formulations, increasing amounts of PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) and Taxol® were injected i.v. into healthy Balb/C female mice. Representative data are shown in Table 4. PVP-b-PDLLA$_{37\%}$–PTX was very well tolerated and exhibited no apparent side effects (quick wake-up, no signs of respiratory distress). The MTD after single dosing was not even reached at 100 mg/kg, whereas the MTD of Taxol® was established at 20 mg/kg. At this dose levels, Taxol® induced severe prostration, apathy, respiratory distress, and catatonia. At 30 mg/kg, toxic deaths were observed. As seen in Table 4, weight loss was less than 5% for PM formulations at all dose levels, indicating that the MTD was not reached. PM-PTX at 80 and 100 mg/kg, seemed to cause less weight variation than at lower

Table 4
Determination of MTD at day 8 after i.v. injection of a single dose

Formulations	Taxol®		Micelle formulations PVP-b-PDLLA$_{37\%}$-PTX (5% w/w)				
Doses (mg/kg)	20	30	20	40	60	80	100
Deaths	0/8	2/4	0/4	0/4	0/8	0/8	0/8
Observation postinjection	apathy, catatonia, prostration	apathy, catatonia, prostration	no apathy	no apathy	no apathy	no apathy	no apathy
Body weight loss	<5%	<10%	<5%	<5%	<5%	<5%	<5%
Liver toxicity							
Hepatocellular regeneration	+++	ND	+	+	+	+	+
Microvacuolar steatosis	+++		+	+	−	−	−
Vascular congestion	++		−	−	−	−	−
Anemia	+	+++	+	+	+	+	+

Not determined (ND).

dose levels (20–60 mg/kg). This might be related to a change in pharmacokinetic parameters when higher amounts of polymer are administered (see Discussion section). Histological studies of the liver revealed the absence of toxicity with PM-PTX at high doses (Fig. 2A). However, slight hepatic inflammation and steatosis were induced by Taxol® (20 and 30 mg/kg) and PM-PTX (20 mg/kg) (Fig. 2B). No significant anemia was observed 24, 48 h, and a week after the injection.

3.4. Pharmacokinetic profiles

Plasma concentrations of PTX after i.v. injection of Taxol® (20 mg/kg) and PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) at 20 and 60 mg/kg are depicted in Fig. 3. The pharmacokinetic profiles were best described by a noncompartmental open model. The extrapolated peak plasma concentrations (C_0) after the administration of 20 mg/kg PM-PTX and Taxol® were 45.4 and 199.2 μg/g, respectively. It can be seen in Table 5 that at 20 mg/kg, Taxol® produced significantly higher plasma $AUC_{0\rightarrow\infty}$ ($p<0.0001$) compared to the PM-PTX formulation (91.8±10.9 vs. 18.6±3.7 μg h/g). The elimination half-life ($t_{1/2}$) was also longer (1.05 vs. 0.56 h). In addition, PM-PTX demonstrated higher total body clearance and volume of distribution. However, due to their better safety profile, PM-PTX could be administered at a higher dose. At 60 mg/kg, the C_0 and plasma $AUC_{0\rightarrow\infty}$ were 213.2 μg/g and 139.7±12.6 μg h/g, respectively.

Interestingly, the pharmacokinetic parameters of PM were found to be influenced by the drug-to-polymer ratio (Fig. 4 and Table 5). In mice, at 20 mg/kg PTX, the plasma $AUC_{0\rightarrow\infty}$ as well as the elimination half-life ($t_{1/2}$) were inversely related to

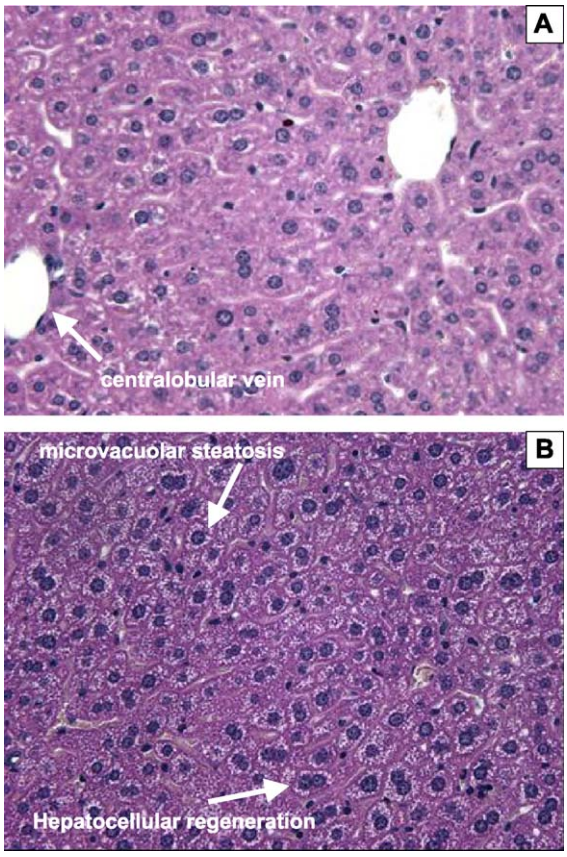

Fig. 2. Micrographs of H&E-stained histological sections showing the effect of PTX on the Balb/c mice liver 8 days after intravenous injection of (A) PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) at 60 mg/kg, or (B) Taxol® at 20 mg/kg.

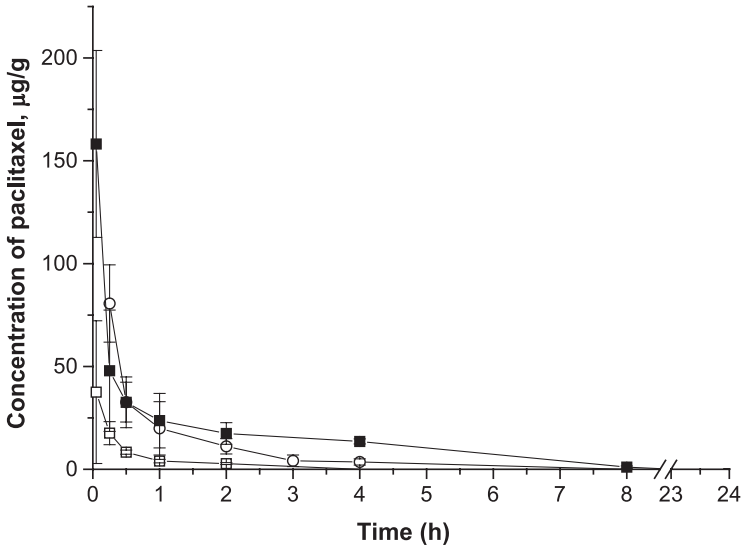

Fig. 3. PTX levels in mice plasma after intravenous administration of Taxol® at 20 mg/kg (○), or PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) at 20 mg/kg (□) and 60 mg/kg (■). Each point represents the mean±S.D. of four mice per time point.

drug loading. The plasma $AUC_{0\rightarrow\infty}$ reached 52.0±7.3 μg h/g at a PTX content of 1% (2.7-fold increase vs. PM-PTX 5%) ($p<0.001$). Total body clearance decreased as drug loading declined, whereas the volume of distribution at steady state (Vd_{ss}) was not affected. It is noteworthy that at similar drug loadings (1.13% vs. 1% w/w for Taxol® and PM-PTX, respectively), the Taxol® plasma $AUC_{0\rightarrow\infty}$ was higher than that of PM-PTX, but the difference was non significant. With the polymer-based formulations, a similar trend was observed in rabbits, where the plasma $AUC_{0\rightarrow\infty}$ rose from 2.2±0.3 to 4.3±0.8 μg h/g as drug-loading decreased from 5% to 2.5% (w/w), although the difference was not statistically significant (Fig. 4 and Table 6). To determine whether this drug-loading dependence of pharmacokinetics could be attributed to PTX release from the micelles and its redistribution to other hydrophobic compartments, including uptake by RBC, the in vitro blood:plasma ratio of PTX was evaluated in mouse blood. This ratio provides information on drug transfer from the PM (in plasma) to blood cells. The blood:plasma ratios of Taxol® and PVP-b-PDLLA$_{37\%}$–PTX at 1%, 2%, and 5% (w/w) drug content were 0.76±0.21, 0.63±0.05, 0.75±0.03, and 0.80±0.06, respectively. These results showed that increasing the polymer concentration decreased drug partition to blood cells.

Table 5
Pharmacokinetic parameters after i.v. administration of Taxol® and PVP-b-PDLLA$_{37\%}$-PTX (1%, 2%, 3.6% and 5% w/w) in mice

Formulation	Dose (mg/kg)	$t_{1/2}$ (h)	C_0 (μg/g)	$AUC_{0\rightarrow\infty}$ [a] (μg h/g)	CL (l/h/kg)	Vd_{ss} (l/kg)
Taxol®	20	1.05	199.2	91.8±10.9	0.21	0.22
PVP-b-PDLLA$_{37\%}$-PTX (5% w/w)	20	0.56	45.4	18.6±3.7	1.02	0.78
PVP-b-PDLLA$_{37\%}$-PTX (3.6% w/w)	20	1.27	62.4	25.0±4.4	0.78	1.12
PVP-b-PDLLA$_{37\%}$-PTX (2% w/w)	20	1.45	54.4	34.5±6.5	0.62	1.01
PVP-b-PDLLA$_{37\%}$-PTX (1% w/w)	20	2.08	123.9	52.0±7.3	0.37	0.83
PVP-b-PDLLA$_{37\%}$-PTX (5% w/w)	60	2.60	213.2	139.7±12.6	0.43	1.61

$t_{1/2}$, elimination half-life; C_0, extrapolated peak plasma concentration; AUC, area under the plasma concentration–time curve; CL, total body clearance; Vd_{ss}, volume of distribution at steady state.

[a] Values are mean±S.D.

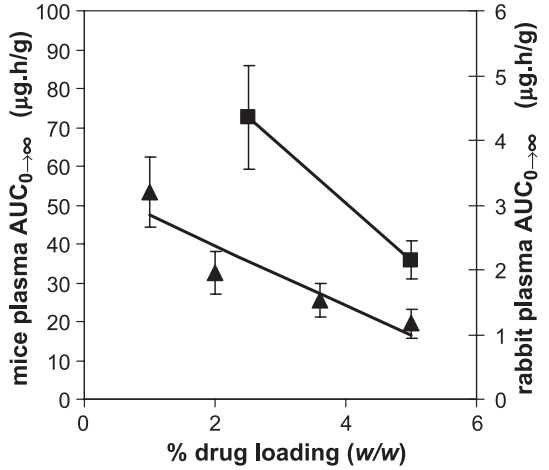

Fig. 4. Effect of % drug loading (w/w) on the plasma $AUC_{0\rightarrow\infty}$ in mice (▲) and rabbits (■) after intravenous injection of PVP-b-$PDLLA_{37\%}$–PTX. Each point represents the mean±S.D. (n=4).

3.5. Biodistribution

Fig. 5 reveals the PTX concentrations in tumors after i.v. administration of 20 mg/kg PTX formulated in PM or CrmEL, and 60 mg/kg PTX formulated in PM, in C26 tumor-bearing mice. At 20 mg/kg, PTX incorporation into PM did not improve its tumoral uptake since the tumor $AUC_{0.25-24h}$ of PM-PTX (5%) (45.7±3.8 µg h/g) was not significantly different from that of Taxol® (48.0±6.3 µg h/g) (p>0.05). However, when considering the tumor/muscle $AUC_{0.25-24h}$ ratio, PTX seemed to have slightly more affinity for the tumor compared to the muscle when loaded in PM rather than in CrmEL micelles (2 vs. 1.4, respectively), although this was not demonstrated statistically. For both formulations at 20 mg/kg, the highest PTX concentrations and $AUC_{0.25-24h}$ values were found in the liver, kidneys, and lungs (Tables 7 and 8). In all tissues except for the tumor and liver, PTX concentrations were considerably lower when the drug was incorporated in PM. For example, the Taxol® $AUC_{0.25-24h}$ values for the spleen, kidneys and heart were 1.8 (p<0.01), 2.6 (p<0.001) and 3.1 (p<0.001) times higher than the values obtained with the PM-PTX formulation. At 60 mg/kg, the maximal PTX tumor concentration reached 11.9 µg/g (t=4 h), with a tumor $AUC_{0.25-24\ h}$ value of 93.7±9.0 µg h/g.

3.6. In vivo antitumor activity

To evaluate antitumor activity, PTX was administered once daily for three consecutive days, and this dosing schedule was repeated after 7 days. Taxol® and PM-PTX were injected at a dose corresponding to 20 and 60 mg/kg PTX, respectively. In the case of the PM formulation, a dose lower than the MTD established after a single injection was chosen to avoid excessive toxicity that might occur with repeated injections. During this study, body weight change was monitored, and the results are shown in Fig. 6. It can be seen that the vehicles (CrmEL and PVP-b-PDLLA) and Taxol® at 20 mg/kg did not cause significant weight loss. Some weight loss occurred with PM-PTX, reaching 7% after 12 days. On day 4, both PTX formulations caused a similar extent of slight anemia (120±6 vs. 117±12 g/l), but no liver toxicity was observed (data not presented). Fig. 7 dicloses that drug-free vehicles and Taxol® were inefficient in preventing tumor growth, while PM-PTX exhibited significant antitumor activity (p<0.01 at days 3 and 4, p<0.05 at days 6 and 10). However, tumor regression was not achieved,

Table 6
Pharmacokinetic parameters after i.v. administration of Taxol® and PVP-b-$PDLLA_{37\%}$-PTX (2.5% and 5% w/w) in rabbit

Formulation	Dose (mg/kg)	$t_{1/2}$ (h)	C_0 (µg/g)	$AUC_{0\rightarrow\infty}$ [a] (µg h/g)	CL (l/h/kg)	Vd_{ss} (l/kg)
Taxol®	5	0.9±0.1	29.4±3.9	8.8±1.7	0.6±0.1	0.32±0.03
PVP-b-$PDLLA_{37\%}$-PTX (5% w/w)	5	1.1±0.1	9.3±1.2	2.2±0.3	2.4±0.4	1.72±0.27
PVP-b-$PDLLA_{37\%}$-PTX (2.5% w/w)	5	0.9±0.1	21.0±3.0	4.3±0.8	1.2±0.2	0.55±0.05

$t_{1/2}$, elimination half-life; C_0, extrapolated peak plasma concentration; AUC, area under the plasma concentration–time curve; CL, total body clearance; Vd_{ss}, volume of distribution at steady state.

[a] Values are mean±S.D.

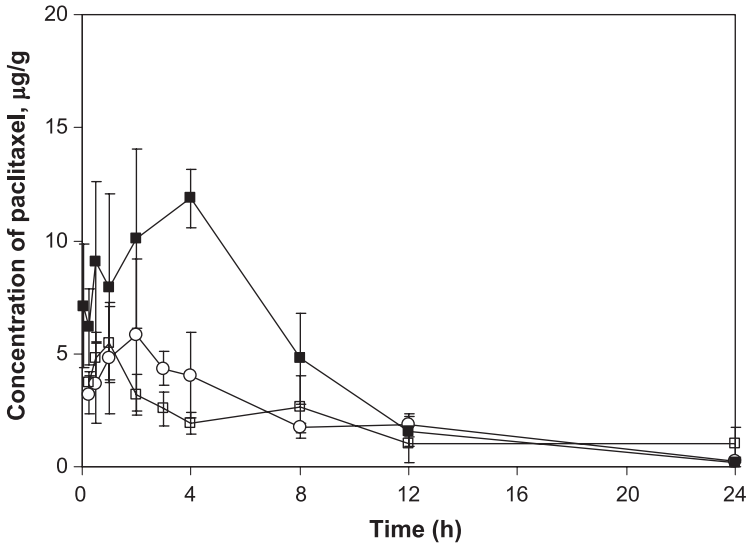

Fig. 5. PTX tumoral levels in C26 tumor-bearing mice after intravenous administration of Taxol® at 20 mg/kg (○), or PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) at 20 mg/kg (□) and 60 mg/kg (■). Each point represents the mean±S.D. of four mice per time point.

and this may have been due to the intrinsic resistance of murine C26 cells to taxane derivatives.

4. Discussion

Several vehicles have been investigated for the administration of poorly water-soluble anticancer drugs such as PTX, DCTX, ETO and TEN. These include hydroxypropylcellulose, carboxymethylcellulose, PEG 300, 400 and 600, poloxamer 184, polysorbate 20 and 80, bile salts, dimethylsulfoxide, DMAc, and CrmEL plus ethanol [43–48]. Despite its known serious and potentially life-threatening adverse effects, current commercial formulations of PTX and TEN contain CrmEL/ethanol. In contrast to conventional surfactant micelles, amphiphilic block copolymer micelles are generally more stable, and can solubilize substantial amounts of hydrophobic compounds [9,49,50]. Many studies have reported that the most important factor influencing the drug-loading capacity of PM is the compatibility between the guest molecule and the core-forming block [51,52]. In the present work, it was demonstrated that PVP-b-PDLLA copolymers possess good loading capacity for all evaluated anticancer drugs. For instance, tenoposide and etoposide were loaded at 10% and 20% (w/w), respectively. The loading capacity of PTX and DCTX was thoroughly investigated and described in a previous study [35]. It was found that at a 10%

Table 7
PTX biodistribution in mice after i.v. administration of Taxol® 20 mg/kg

Time (h)	Liver (µg/g)	Spleen (µg/g)	Kidney (µg/g)	Lung (µg/g)	Heart (µg/g)	Muscle (µg/g)
0.25	93.2±11.1	25.0±6.1	87.0±10.7	76.7±13.7	55.8±3.2	4.8±1.5
0.5	ND	ND	ND	ND	ND	4.2±0.8
1	84.9±15.8	10.6±3.0	31.1±11.0	19.7±8.1	23.6±11.5	4.4±2.3
2	47.9±9.3	13.4±5.5	24.3±2.7	19.8±1.4	12.8±3.9	4.3±0.8
4	35.0±10.9	11.1±4.3	16.8±3.6	8.0±5.7	8.6±1.9	3.2±1.1
8	2.2±0.8	2.4±0.3	1.6±1.1	–	0.8±0.3	1.2±0.3
12	ND	ND	ND	ND	ND	0.8±0.1
24	–	0.4±0.1	–	–	0.0±0.1	0.5±0.1
$AUC_{0.25–24}$ (µg h/g)	301.9±35.8	83.8±12.2	162.3±14.8	99.8±14.7	94.8±9.8	35.4±3.4

Values are mean±S.D. of four mice. ND: not determined. (–) Values below the detection limit.

Table 8
PTX biodistribution in mice after i.v. administration of PVP-b-PDLLA$_{37\%}$-PTX (5% w/w) 20 mg/kg

Time (h)	Liver (μg/g)	Spleen (μg/g)	Kidney (μg/g)	Lung (μg/g)	Heart (μg/g)	Muscle (μg/g)
0.25	125.6±14.2	13.6±1.9	45.7±8.0	28.8±5.4	18.9±1.5	3.3±0.5
0.5	ND	ND	ND	ND	ND	5.4±2.8
1	77.1±17.1	7.6±2.5	15.1±3.2	14.7±3.7	8.7±2.4	2.8±0.8
2	58.7±9.2	9.0±1.2	11. 5±4.0	10.2±1.6	5.0±1.2	2.0±0.4
4	15.5±7.2	3.9±1.0	4.6±2.1	6.4±1.7	2.5±0.6	1.3±0.2
8	0.6±0.9	1.3±0.3	–	–	0.1±0.2	0.8±0.1
12	ND	ND	ND	ND	ND	0.54±0.02
24	–	0.7±0.1	–	–	–	0.4±0.1
AUC$_{0.25–24}$ (μg h/g)	260.1±25.0	46.3±55.4	61.5±8.6	58.1±5.2	30.3±3.3	20.7±1.4

Values are mean±S.D. of four mice. ND: not determined. (–) Values below the detection limit.

(w/w) initial feed ratio, drug precipitation occurred after 24 h for both drugs. Drug-loaded polymeric micelles prepared at 5% and 7.5% (w/w) initial feed ratios were more stable and exhibited no signs of drug precipitation over 48 h [35]. In the present work, paclitaxel-loaded micelles were prepared at a drug loading of 5% (w/w) to avoid any risk of precipitation before injection in mice. This good loading capacity might be attributed to the fact that the drug has good affinity for the PDLLA hydrophobic core. It has to be mentioned that PVP-b-PDLLA alone does not demonstrate superior properties vs. PEG-b-PDLLA with respect to PTX solubilization. The uniqueness of the system lies in the combination of the use of PVP-b-PDLLA with the one-step freeze-drying process involving TBA. Indeed, PEG is practically insoluble in TBA and drugs are therefore generally solubilized by more complex loading procedures (e.g., dialysis). In addition, the PVP corona can help to solubilize some drugs (i.e., indomethacin), leading to higher loading than PEG-b-PDLLA [21].

The drug-loaded micelles were prepared either by lyophilizing water/TBA mixtures of polymer and drug (Method A), or by dissolving both components in an

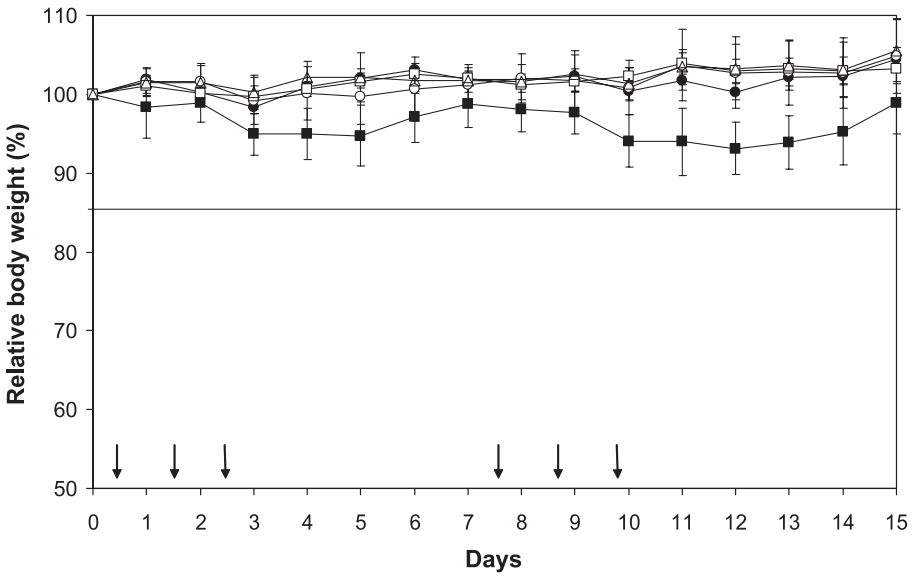

Fig. 6. Body weight change of C26 tumor-bearing mice after intravenous injection according to a dose schedule regimen of six injections (arrows). Untreated controls received injections of either dextrose 5% (△), CrmEL (○), or PVP-b-PDLLA$_{37\%}$ (□) without PTX (n=12). The treatment groups consisted of 20 mg/kg Taxol® (●) and 60 mg/kg PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) (■) (n=15). Each point represents the mean±S.D.

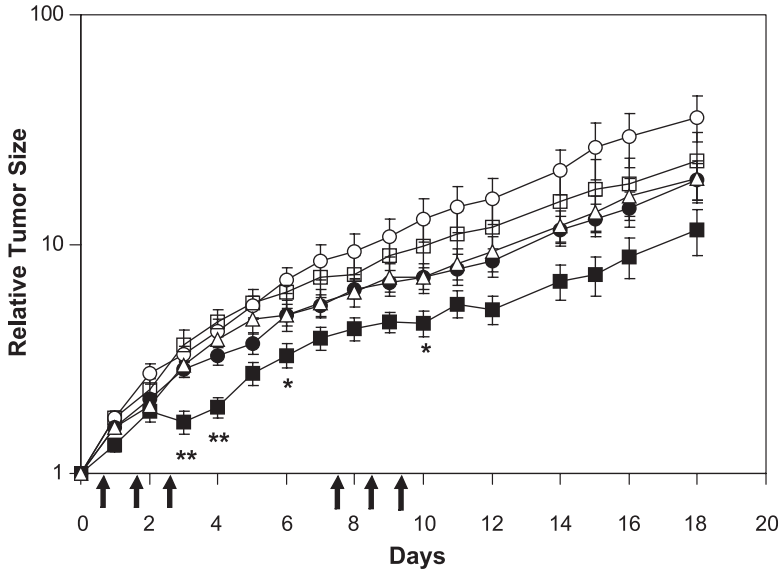

Fig. 7. Antitumor effect of micellar PTX formulations on C26 tumor-bearing mice. Subcutaneous C26 murine colon adenocarcinoma cells were inoculated as described in Materials and methods. Ten days after tumor implantation, intravenous treatment was initiated on day 0, and was repeated on days 1, 2, 7, 8 and 9 (arrows). Untreated controls received injections of either dextrose 5% (△), CrmEL (○) or PVP-b-PDLLA$_{37\%}$ (□) without PTX (n=12). The treatment groups consisted of 20 mg/kg Taxol® (●) and 60 mg/kg PVP-b-PDLLA$_{37\%}$–PTX (5% w/w) (■) (n=15). Each point represents the mean±S.E.M. *p<0.05 and **p<0.01.

injectable organic solvent that was then diluted with an isotonic aqueous solution (Method B). Both methods are simple and easily scalable. Incorporating PTX, DCTX, ETO, and TEN in PVP-b-PDLLA micelles resulted in clear solutions that contained as much as 5–20% (w/w) drug. Near-monodisperse, nanosized PM were obtained with both incorporation methods. The freeze-drying procedure might be advantageous, since the final product can be supplied as a dry powder with increased shelf life. The water/TBA mixture was selected for this method as it induces the self-assembly of PVP-b-PDLLA into swollen micelles where the drug can easily partition. The amount of residual TBA in the lyophile was found to be less than 20 ppm, which is far less than the authorized limit for most Class 3 solvents [53]. However, this method is not suited for drugs such as TEN and ETO as they are not soluble in water/TBA mixture.

Zhang et al. [20] formulated PTX in PEG-*b*-PDLLA micelles by dissolving the drug and copolymer in acetonitrile, followed by heating to remove the solvent. The resulting waxy solid film was reconstituted in water by heating to 60 °C and vortexing to dissolve the matrix. A similar process was used by Kim et al. [12], with an added final step of lyophilization. Although the resulting micelle solutions were stable up to 24 h, this incorporation method could be deleterious to heat-labile drugs [50].

PTX was selected as a prototype anticancer drug to evaluate PM formulations both in vitro and in vivo. The cytotoxicity results indicated that PTX entrapped in PVP-b-PDLLA micelles was readily available to interact with cancer cells and retained its antimitotic potency. In the absence of the drug, CrmEL demonstrated higher cell toxicity than PVP-b-PDLLA at high concentrations. Similar results have been reported in the literature for other amphipathic block copolymers [11,12,14,54]. PTX incorporated into PEG-b-PDLLA or poly(2-ethyl-2-oxazoline)-block-poly(ε-caprolactone) [P(EtOz)-b-PCL] was as potent as Taxol® when tested on different cancer cell lines, affirming that the micelles did not generally impair the drug's efficacy in vitro. Both PVP-b-PDLLA and CrmEL were nonhemolytic in vitro towards human RBC, even at relatively high concentrations (<2% hemolysis at 10 mg/ml), suggesting that these vehicles

would be nontoxic towards erythrocytes after i.v. injection. Similar results have been obtained by Lee et al. [14] with P(EtOz)-b-PCL on rat RBC, whereas Miwa et al. [15] found that *N*-lauryl-carboxy-methyl-chitosan, a polymer used for PTX solubilization, induced 50% hemolysis of dog RBC at a concentration of 5 mg/ml.

Previous in vivo toxicity studies carried out with PEG-b-PDLLA demonstrated that the MTD of PTX loaded into PM (60 mg/kg i.v., 100 mg/kg i.p.) was generally higher than that of CrmEL-based formulations [11,12,55]. Similarly, the data presented here showed that PVP-b-PDLLA–PTX was considerably less toxic than Taxol®, with a MTD exceeding that of the commercial formulation by at least fivefold. In vitro testing diclosed that Taxol® and PVP-b-PDLLA–PTX were equipotent, arguing against a loss of potency for the PM formulation. The gain in MTD can thus be explained by the better tolerability of PVP-b-PDLLA vs. CrmEL. The latter surfactant is known to exert a range of biological effects, such as severe anaphylactoid hypersensitivity reactions, hyperlipidemia, erythrocyte aggregation, and peripheral neuropathy. It is also known to cause nonlinear pharmacokinetics of various drugs. Indeed, CrmEL may affect drug elimination through different mechanisms, including modulated protein binding characteristics, altered hepatobiliary secretion, and inhibition of endogenous P-glycoprotein-mediated biliary secretion [56]. Furthermore, one may hypothesize that CrmEL-based micelles are more toxic than PVP-b-PDLLA-based micelles because they interact more favorably with systemic mediators that are involved in the classical complement activation pathway. Szebeni et al. [57] have suggested that recognition of CrmEL micelles could happen after binding constitutive anticholesterol antibodies to their hydroxyl-rich surface. These antibodies are abundant in most human plasma, and the epitopes that they recognize contain an –OH group. Additionally, the mechanism of CrmEL-induced complement activation could be similar to the one described for nonionic block copolymers, such as poloxamer 331, i.e., binding of the C3 complement fragment by polyoxyethylene chains present in the micelle shell [57].

After bolus injection, PTX seemed to be rapidly released from the PM, as evidenced by the rapid decline of PTX plasma levels and a lower AUC vs. Taxol® (Fig. 3). CrmEL is known to alter PTX pharmacokinetics in part by sequestering the drug in the plasma compartment [58]. Indeed, the $t_{1/2}$ of CrmEL is long, and its volume of distribution is not much higher than the volume of the central blood compartment. All these results indicate that PTX probably displays greater affinity for CrmEL than for PVP-b-PDLLA. As shown in Fig. 4, the plasma AUC of the PM formulation could be increased by reducing the drug payload. One possible explanation is that partition into the micelle core is favored at high polymer concentrations (i.e., low drug loading). This hypothesis was partially confirmed by measuring the blood:plasma PTX concentration ratios in vitro. At low drug loadings, PTX redistribution to the other hydrophobic sites, such as blood cell membranes, was minimized. In vivo, higher polymer concentrations could also help in maintaining levels above the CAC, thus slowing down micelle dissociation and PTX redistribution to other sites. Finally, it can be hypothesized that saturation of the mononuclear phagocyte system may also be involved in AUC dependence on drug loading. These results corroborate those of Zhang et al. [55], who clearly showed that PEG-b-PDLLA micelles, despite their strong solubilizing capacity, could not retain PTX in their hydrophobic core upon injection. In this study, the diblock copolymer was also rapidly eliminated in urine (within 15 h). On the opposite, Yamamoto et al. [59] demonstrated that drug-free PEG-*b*-PDLLA micelles with a modulated surface charge could exhibit long circulation times in the blood compartment, with 25% of the injected polymer dose persisting 24 h after injection. Strategies based on slowing down PTX diffusion from the PDLLA core and PM dissociation kinetics may be successful in increasing PTX's half-life [16]. Recently, Lee et al. [60], reported that this could be achieved by ionically cross-linking PDLLA. Alternatively, the length of the core-forming block could be augmented to strengthen the core, minimize surface area, and prolong the diffusion path of the drug [51]. It is notable that despite its lower plasma AUC, the tumoral accumulation of PTX was similar for PM and Taxol® formulations, with only a slight difference in the t_{max} values (Fig. 5). However, because of its higher MTD, PM-PTX could be injected at higher

doses. At 60 mg/kg, plasma and tumor AUCs increased three- and twofold, respectively, vs. Taxol® at 20 mg/kg.

Many studies have reported that PTX was more effective in inhibiting tumor growth when formulated in PM than in CrmEL micelles [11,12]. This generally results from the better tolerability of PM formulations, which allows increased doses to be administered. A study established that PTX incorporated at similar doses in PEG-b-PDLLA and CrmEL micelles (25 and 20 mg/kg, respectively) was equally efficacious against human lung tumors in nude mice after i.v. injection [55]. In this study, PVP-b-$PDLLA_{37\%}$–PTX at 60 mg/kg exhibited greater antitumor activity on C26 colon adenocarcinoma tumor cells than the commercial formulation at its MTD (20 mg/kg). Only slight weight loss was observed during treatment, and the mice rapidly recovered after each PTX injection session. The PM system induced a significant delay in tumor growth, but was unable to provide tumor regression due to the intrinsic resistance of the C26 model to PTX. It is generally acknowledged that murine tumors are somewhat resistant to PTX [61]. Still, these results provide evidence for the clinical superiority of this PM formulation, which has demonstrated a better therapeutic index than Taxol®. In future studies, it is planned to use tumor models that are more sensitive to PTX, such as human ovarian tumors (OVCAR-3, A121), in athymic nude mice.

In summary, it was shown that PVP-b-PDLLA can be used to solubilize a variety of hydrophobic anticancer drugs and form near-monodisperse micelles. PM had mean diameters ranging from 15 to 60 nm, depending on the drug and the incorporation method. These formulations are intended to be extemporaneously diluted in an aqueous solvent prior to their i.v. administration. Such a system proved to be safe after bolus injection, allowing the delivery of PTX doses exceeding those of the commercial Taxol® formulation. In turn, higher tumoral accumulation of the drug and greater antineoplastic activity could be achieved in vivo. However, the PM could still be improved, especially with respect to their pharmacokinetic parameters. Better drug retention in the micelle core is a key condition to ensure prolonged circulation times and eventually maximize drug accumulation at the tumoral site via the enhanced permeation and retention effect. This could be achieved by modifying the properties of the core either chemically or physically [62,63]. Taxane-loaded micelles with various PVP/DLLA ratios are currently being tested for their ability to retain the drug in their core. Some of these new formulations demonstrated up to 2.5-fold increase in drug retention compared to the current micelle preparation (unpublished results). It is expected that these formulations will exhibit better pharmacokinetics profiles. In addition, attaching a targeting ligand at the extremity of the micelle corona may further augment drug localization in the tumor [64]. Recently, in a very thorough study, Torchilin et al. [65] evaluated the biodistribution of PTX loaded into tumor-specific PEG-DSPE-2C5 (PEG-phosphatidylethanolamine attached to the monoclonal antibody mAb 2C5) immunomicelles. Their i.v. administration into mice bearing Lewis lung carcinomas enhanced PTX accumulation at the tumoral site vs. CrmEL-PTX or PTX loaded in nontargeted micelles [66].

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