European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 741–748

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Anti-tumour and immuno-modulation effects of triptolide-loaded polymeric micelles

Lingyun Xu, Huabing Chen, Huibi Xu, Xiangliang Yang *

College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China

ARTICLE INFO

Article history:
Received 14 May 2008
Accepted in revised form 27 July 2008
Available online 13 August 2008

Keywords:
Polymeric micelles
Triptolide
Anti-tumour
Immuno-modulation
Sarcoma 180-bearing mice
A2780 cells

ABSTRACT

Triptolide (TP) possesses both anti-tumour and immuno-suppressive activities. Its immuno-suppressive activity may be disadvantageous for the therapy of cancers. A novel polymeric micelle system containing TP (TP-PM) was constructed by the solvent evaporation method using methoxypolyethylene glycol–poly(D,L-lactic acid)-block copolymer as the carrier, and was characterised using photon correlation spectroscopy, transmission electron microscopy and high performance liquid chromatography. The anti-tumour and immuno-modulation effects of TP-PM were evaluated in sarcoma 180-bearing mice and A2780 cells. Results demonstrated that TP-PM had an average diameter of 78.9 nm, encapsulation efficiency of 66.7%, core–shell morphology and a long-term stability. TP-PM could significantly inhibit tumour growth via intravenous injections at the dose levels of 0.0375, 0.075 and 0.15 mg/kg, and their inhibition rates were 42.5%, 46.0% and 49.9%, respectively; they showed similar cytotoxicity against A2780 cells compared to that of TP. Simultaneously, TP-PM had no effect on the thymus index, spleen index, spleen lymphocyte proliferation and the TNF-α and IL-2 levels in serum as compared with TP. Therefore, TP encapsulated in polymeric micelles does not demonstrate immuno-suppressive activity but does not lose its anti-tumour effect. These results show that polymeric micelles are a promising carrier for cancer therapy using TP.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Triptolide (TP), a diterpenoid triepoxide, isolated from the Chinese medicinal herb *Tripterygium wilfordii* Hook F (TWHF), shows both anti-tumour and immuno-suppressive activities [1]. Recently, there has been a growing interest in anti-tumour researches and applications of TP due to its broad anti-tumour spectrum and potent anti-tumour activity. TP has been reported to inhibit the proliferation of many different types of cancer cells *in vitro* and to reduce the growth and metastasis of some solid tumours *in vivo* [2–6]. However, the clinical use of TP is restricted due to its poor water solubility and some toxic effects. On the other hand, TP has stronger immuno-suppressive effects than the conventional immuno-suppressant cyclosporine [7], and the strong immuno-suppressive activity of TP may become a side effect when it acts as an anti-tumour agent, and thus may be disadvantageous for the therapy of cancers. This is because there are immuno-suppressive networks in human tumours; recent literatures have highlighted the potential immuno-suppressive mechanisms in tumours which block effective anti-tumour immune responses in cancer patients [8]. As evidence, the cancer morbidity and mortality of transplant recipients was greatly increased when long-term immuno-suppressive drug therapy was used [9]. Therefore, it is against the therapeutic interests of the patient if the immune function of the body is suppressed.

In the recent years, controlled release delivery systems and targeted drug delivery systems have been used to deliver TP to reduce its toxicities [10,11]. Among the new delivery systems, polymeric micelles have been used as a promising nanocarrier for anti-tumour drugs with several advantages including prolonged blood circulation, reduced cumulative toxicity, deeper tumour penetration and improved endocytosis. In addition, their use can increase anti-tumour activities and reduce the toxic side effects of anti-tumour drugs [12,13]. Generally, diblock copolymers with a block of polyethylene glycol such as polyethylene glycol–poly(D,L-lactide) (PEG-PDLLA) are preferable as carriers for anti-tumour drugs [14,15]. The polymeric micelles with a high density of PEG shells can avoid reticuloendothelial system (RES) recognition and preferentially accumulate in solid tumours due to the enhanced permeability and retention (EPR) effect [12]. Because RES is a part of the immune system, it is possible that polymeric micelles play an important role in decreasing the immuno-suppressive side effects of anti-tumour agents.

In order to explore a novel formulation containing TP with high anti-tumour activity and low immuno-suppressive activity, a poly-

* Corresponding author. College of Life Science and Technology, Huazhong University of Science and Technology, Luoyu Road 1037, Wuhan 430074, China. Tel.: +86 27 87792147; fax: +86 27 87794517.
E-mail address: yangxl@mail.hust.edu.cn (X. Yang).

0939-6411/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2008.07.017

meric micelle system containing TP (TP-PM) was constructed by the solvent evaporation method. The physicochemical characteristics of TP-PM were investigated. The anti-tumour activities of TP-PM on human ovarian cancer cell line A2780 *in vitro* and on sarcoma 180 (S-180)-bearing mice *in vivo* were evaluated. Simultaneously, the influence of TP-PM on the immunity of tumour-bearing mice was also evaluated. The long-term goal of this research is to develop an intravenous formulation for clinical use to treat solid tumours.

2. Materials and methods

2.1. Drugs and reagents

Triptolide (purity > 99%) was supplied by Fujian Chinese Medical Research Institute (Fuzhou, China). Cyclophosphamide (CTX) was purchased from Jiangsu Hengrui Pharmaceutical Co. Ltd. (Lianyungang, China). D,L-Lactide (DLLA) was purchased from Tianyuan Biomaterials Co. Ltd. (Fushun, China). Monomethoxypolyethylene glycol (MePEG, Mn = 5000) and stannous octoate were obtained from Aldrich (Milwaukee, WI, USA). Purified deionised water was prepared by the Milli-Q plus system from Millipore Co. (MA, USA). RPMI 1640, MTT, DMSO, ConA and penicillin–streptomycin sulphate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Iodine [^{125}I] Tumour Necrosis Factor Radioimmunoassay kit and Iodine [^{125}I] Interleukin-2 Radioimmunoassay kit were purchased from Beijing East Asian Institute of Immunology (Beijing, China). All other chemicals were obtained from Aldrich.

2.2. Cells and animals

Human ovarian carcinoma A2780 cell lines were kindly supplied by Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China). Murine sarcoma 180 (S-180) cells were kindly supplied by the Hubei Cancer Institute (Wuhan, China). Kunming mice (male and female, 20 ± 2 g) were obtained from the Laboratory Animal Center, Hubei Academy of Preventive Medicine (Wuhan, China). The animals were housed in a well-ventilated room with a constant temperature of 23 ± 2 °C, relative humidity of 40–70%, and illumination (12 light/dark cycles). The protocol of the study was approved by the Ethical Committee of Huazhong University of Science and Technology.

2.3. Synthesis and characterization of MePEG-PLA

MePEG-PLA was synthesised and purified as described previously [14]. Briefly, 4 g DLLA, 1 g MePEG and 20 mg stannous octoate were mixed in a bottle-neck flask, and polymerization was carried out at 150 °C for 8 h. The synthesised copolymers were characterised by the gel permeation chromatography system (GPC, Agilent 1100 Chromatographic Instrument, USA), the Fourier transform infrared spectrum (FTIR, Perkin Elmer 1700 spectrometer, USA) and nuclear magnetic resonance (NMR, Bruker AV 400 spectrometer, Switzerland). The critical micelle concentrations (CMCs) of the copolymers were determined in water at 25 °C by the steady-state pyrene fluorescence method as described elsewhere by using a spectrofluorimeter (Hitachi F-4500, Japan) [16]. Pyrene concentration in each sample was 6×10^{-7} M, whereas polymer concentrations ranged between 5×10^{-6} and 1 g/L. The excitation spectra were recorded at from 300 to 360 nm with the emission wavelength at 393 nm.

2.4. Preparation and characteristics of TP-PM

TP-PM was prepared by the solvent evaporation method [17]. Briefly, MePEG-PLA (100 mg) and TP (1.5 mg) were co-dissolved in 5 ml mixture organic solvent consisting of acetone/ethanol (9/6, v/v). The solution obtained was then added dropwise into 20 ml deionised water in a 100-ml flask with stirring at 300 rpm. Polymeric micelles were formed immediately and the organic solvents were removed from the dispersion through overnight evaporation under magnetic stirring at room temperature. The obtained transparent solution was filtered through a 0.45-μm membrane filter (Whatman) and then ultra-centrifuged for 1 h at 36,000 rpm (4 °C) using a super-speed centrifuge (Hitachi Co., Japan). The supernatant was discarded and the pellet was resuspended in a purified deionised water. The obtained transparent solution was then used for analysis or freeze-dried by using a freeze-dryer (Labconco Co., USA) to obtain the white powder. The mean size and zeta potential of TP-PM were measured by photon correlation spectroscopy (PCS) with a Nano-ZS90 laser particle analyzer (Malvern Instruments Corp., UK). Fresh solution of TP-PM was dropped on copper grids, stained with phosphotungstic acid solution (1%, w/v, pH = 6.0), and was observed under a transmission electron microscope (TEM, Tecnai G220, FEI Co., Netherlands). Drug-loaded micelles were stored in a dark place at 4 °C for 4 months. The stability was monitored by changes in particle size, zeta potential and drug encapsulation efficiency (EE) during the storage period. EE was determined by using HPLC as follows.

2.5. Encapsulation efficiency

The amount of TP incorporated in the polymeric micelles was determined by high performance liquid chromatography (HPLC) (Agilent 1100, USA), using a reverse phase Lichrospher ODS C_{18} column. Then, 5 mg of TP-PM was dissolved in 1 ml acetone, evaporated until dry by using the rotor evaporation technique under reduced pressure at 35 °C, and followed by the addition of 2 ml ethanol. The clear solution obtained after membrane filtration was put into the vial for HPLC to detect the TP concentration. The mobile phase consisted of a mixture of acetonitrile–water (30/70, v/v), and the flow rate was 1.0 ml/min. The column effluent was detected at 224 nm by a variable wavelength detector and the column temperature was 30 °C. The calibration curve for the quantification of TP was linear as given by $A = 15.347C - 7.229$ (A = peak area; C = TP concentration) over the range of standard concentration of TP from 1.88 to 56.28 μg/ml ($n = 7$) with a correlation coefficient of $R = 0.9999$. The drug encapsulation efficiency (EE) was calculated by the following equation:

$$\text{EE}\ (\%) = M_1/M_2 \times 100 \quad (1)$$

where M_1 is the mass of TP in the polymeric micelles and M_2 is the mass of TP used in the formation.

2.6. In vitro release

The release experiment of TP from polymeric micelles was performed by the dialysis bag diffusion technique [16]. Ten milligrams of TP-PM were suspended in 2 ml phosphate buffer solution (PBS, pH = 7.4) and placed in a dialysis bag (cut-off, 8000; Millipore Co., MA, USA). Then, it was hermetically sealed and immersed into 28 ml PBS containing 10% (v/v) ethanol with continuous and gentle magnetic stirring at 60 rpm at 37 °C. At predetermined time intervals, 0.5 ml aliquots was taken from the receptor compartment and contemporarily restored with the same volume of fresh buffer. All samples were taken for the analysis of TP concentration, and HPLC analysis was carried out as described in Section 2.5. A profile showing the cumulative amount of drug release as a function of time was plotted.

2.7. In vitro cytotoxicity of TP-PM against A2780 cells

A2780 cells were seeded at 10^4 cells/well in a 96-well plate and cultured in RPMI 1640 medium (pH = 7.4, with 10% calf serum and

1% penicillin–streptomycin) in a humidified 37 °C environment with 5% CO_2. After 24 h incubation, the medium was replaced with a medium containing different concentrations of TP-PM or TP formulations. After additional 24, 48 or 72 h incubation, the media were replaced with PBS containing 0.1 mg/ml MTT, and the cells were incubated for 4 h at 37 °C with 5% CO_2. The cytotoxicity was measured following the absorbance of the degraded MTT (formazan) with 150 μl DMSO at 490 nm using a microplate reader (EX800, Bio-Tec Co., USA). The cell viability (CV) was expressed as a percentage compared to a control that had not been treated with drugs using the following equation:

$$\text{CV}\ (\%) = A_t/A_c \times 100 \quad (2)$$

where A_t and A_c are the average absorbance values at 490 nm in the drug-treated cell groups and in the untreated cell group, respectively.

2.8. In vivo anti-tumour effects of TP-PM in S-180-bearing mice

Under sterile conditions, 0.2 ml of S-180 cell suspension (approximately 1×10^7 cells/ml) was subcutaneously inoculated into mice in the axillary region. The mice inoculated were divided into eight groups: S-180-bearing control group, 20 mg/kg CTX, 0.0375, 0.075, 0.15 mg/kg TP-PM and free TP-treated groups, with five males and five females in each group. A normal control group was also used in this experiment. After the day of inoculation (day 1), CTX, TP-PM and TP were administrated by intravenous injection every second day (six times) with a volume of 10 ml/kg for 12 days. Normal control and S-180-bearing control groups received the same volume of sodium chloride 0.9% injection. On day 13, all animals were executed. The excised tumours were weighed, and the inhibition rate (TIR) was calculated by Eq. (3) [18]. Then, the tumours were fixed in 10% neutral buffered formalin solution, trimmed, embedded in paraffin, sectioned at 4–6 μm, stained with *haematoxylin* and *eosin* and examined by light microscopy (Olympus BH-2, Japan)

$$\text{TIR}\ (\%) = \frac{W_c - W_t}{W_c} \times 100 \quad (3)$$

where W_c is the average tumour weight in the S-180-bearing control group and W_t is the average tumour weight in the drug-treated groups.

2.9. Effects of TP-PM on the immune function of S-180-bearing mice

Serum was collected by retro-orbital venous puncture from the above-mentioned S-180-bearing mice 24 h after the last administration, and the IL-2 and TNF-α levels in the serum were determined according to the radioimmunoassay kit instructions. After the mice were executed, the excised thymuses and spleens were weighed under sterile conditions. Thymus and spleen indices were expressed as the thymus (or spleen) weight (mg) over the body weight (g). Then, the spleens were aseptically removed in cold PBS, gently homogenised with a lose Teflon pestle and passed through a sterilised mesh to obtain single cell suspensions, which were used to assess the lymphocyte proliferation according to the method of Yuan et al. [19]. Finally, the cells were suspended to a final density of 3×10^6 cells/ml in RPMI 1640 medium supplemented with 10% foetal calf serum. Spleen cells (100 μl/well) were seeded into a 96-well plate in the presence of ConA (7.5 mg/ml) and cultured at 37 °C with 5% CO_2. After incubation for 72 h, 10 μl MTT (5 mg/ml) were added to each well and the plate was incubated for another 4 h. Then, a total of 200 μl DMSO was added to each well and shaken till the crystals were dissolved. The absorbance at 570 nm (A_{570}) was detected on a microplate reader (EX800, Bio-Tec Co., USA). The lymphocyte proliferation rate (LPR) was evaluated as follows:

$$\text{LPR}\ (\%) = \frac{A_t - A_c}{A_c} \times 100 \quad (4)$$

where A_c is the absorbance in the control group and A_t is the absorbance in the drug-treated groups.

2.10. Statistical analysis

Results were expressed as mean ± deviation (SD). The statistical significance of the differences between groups was evaluated by variance analysis, followed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characteristics of MePEG-PLA

The NMR and FT-IR spectrum of the synthesised MePEG-PLA showed that the reaction between lactide and MePEG had occurred, and successful synthesis of the MePEG-PLA copolymer with high purity was demonstrated (data not shown). From GPC analysis, the weight–average molecular weight (M_W) of the copolymer was 2.8×10^4. The HLB (hydrophile–lipophile balance) value and CMC value were the two important factors influencing MePEG-PLA in assembling micelles in water [20]. According to the Griffin formula [21], the HLB value of the micelles was 7.1 and could form o/w emulsification. CMC was determined by using pyrene as a hydrophobic fluorescence probe [16]. I_{338} and I_{333} represent the fluorescence intensities of the third peak and the first peak in the pyrene excitation spectra, respectively, and their peak height-intensity ratio (I_{338}/I_{333}) can be used as a sensitive parameter to represent the polarity of the microenvironment. The shift of pyrene from water to the micelle core occurred at the onset of micelle formation. As expected, micelles were not formed at a low polymer concentration, and the I_{338}/I_{333} ratio was almost constant, close to the characteristic value of pyrene in water, while pyrene partitioned into the hydrophobic core, the I_{338}/I_{333} ratio varied dramatically due to the formation of micelles. The intensity ratio of I_{338}/I_{333} was plotted against the logarithm of polymer concentration, and two tangents were drawn, one to the curve at high concentrations and another through the points at low concentrations. The CMC value was taken from the intersection of the two tangents. The result is shown in Fig. 1. The CMC of the polymer MePEG-PLA was determined to be 8.9×10^{-7} mol/L, which was much lower than that of common low-molecular weight surfactants; further, its dissociation was kinetically slow, and this property may allow the micelles to circulate in the bloodstream until accumulation at target tissues [12].

3.2. Preparation, characteristics and stability of TP-PM

MePEG-PLA polymeric micelles containing TP were conveniently obtained by using the solvent evaporation method. The mean particle size of TP-PM was 78.9 ± 5.0 nm ($n = 4$) with a narrow polydispersity index (PDI = 0.18 ± 0.02), and its zeta potential was −6.1 ± 2.8 mV ($n = 4$). Compared to the previously prepared TP-loaded poly(D,L-lactide) nanoparticles by the solvent evaporation method [23], TP-PM has smaller size and less negative electric charge and is suitable for intravenous administration, which is promising as an injectable sustained-release delivery system [22]. TP-PM was in fine spherical shape with the core–shell structure determined by TEM, in which MePEG groups were located outside and PLA groups were assembled in the core part (Fig. 2a and b). The drug TP encapsulation efficiency into polymeric mi-

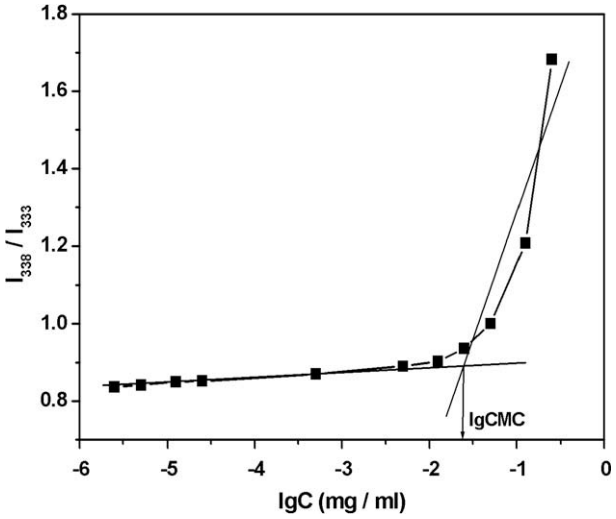

Fig. 1. Plot of the intensity ratio of I_{338}/I_{333} from pyrene excitation spectra vs. log*C* for the MePEG-PLA diblock copolymer.

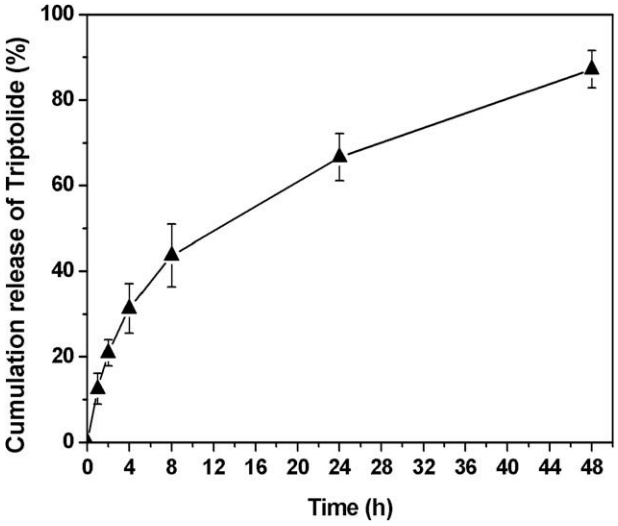

Fig. 3. *In vitro* release profile of TP from polymeric micelles in pH 7.4–PBS at 37 °C by the dialysis bag method. The results are expressed as mean ± SD (n = 3).

celles was 66.7 ± 2.3% (n = 3). After storing TP-PM for 4 months at 4 °C in the dark, it exhibited a size of 85.5 ± 8.4 nm, a zeta potential of −7.1 ± 3.0 mV and an encapsulation efficiency of 65.6 ± 3.1%, which were all similar to the original pre-storage measurements with no significant differences. This implied a long-term stability of TP-PM.

3.3. In vitro release

The *in vitro* release profile of TP from the polymeric micelles in PBS (pH = 7.4) is presented in Fig. 3, which shows that after the incubation for 24 h at 37 °C, the release of TP was 66.6 ± 5.6%. When kept for 48 h at 37 °C, the TP release was 87.2 ± 4.4%. In contrast to a significant burst release at the initial stage that was reported by Liu et al. in their study concerning the TP-loaded PLA nanoparticle system [23], the release profile obtained here exhibited a very steady sustained-release pattern with negligible initial burst release.

3.4. In vitro cytotoxicity against A2780 cells

The A2780 cell line is a type of solid tumour cell line, and the results of our pre-experimental studies suggested that in *in vitro* studies, A2780 cells are more sensitive to triptolide therapy than are other solid tumour cell lines, such as the MCF-7 cell line and the H_{22} cell line. The anti-proliferation activities of free TP or TP-PM formulation against A2780 cells after 24, 48 and 72 h incubation were assessed by MTT assay (Fig. 4). Treatment of the A2780 cells with the increasing concentrations of free TP or TP-PM resulted in a significant decrease of cell viability for the incubation period of 48 and 72 h, while A2780 cells incubation with free TP or TP-PM for 24 h did not result in significant loss of cell viability. Recent studies have demonstrated that TP induces apoptosis of a broad range of human cancer cells by inhibiting or activating different molecular targets, such as NF-KB, p53, c-Jun NH_2-terminal kinase, and 5-lipoxygenase gene [6,24,25]. In this study, TP-PM exhibited the same anti-tumour potential as free TP against

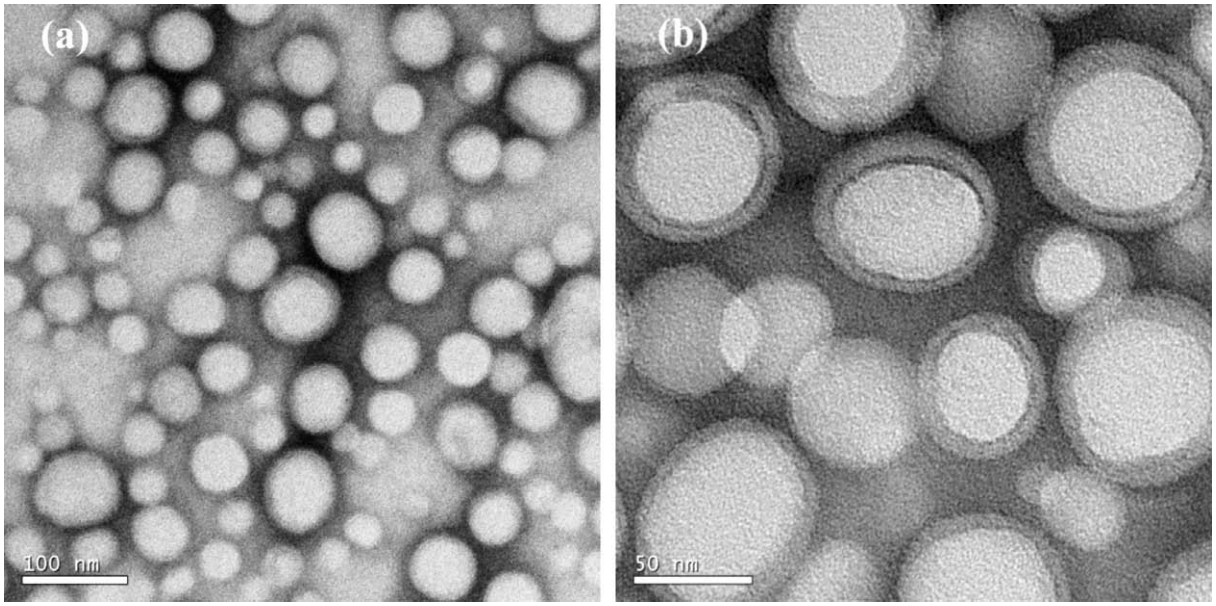

Fig. 2. Transmission electron micrographs of TP-PM (a and b).

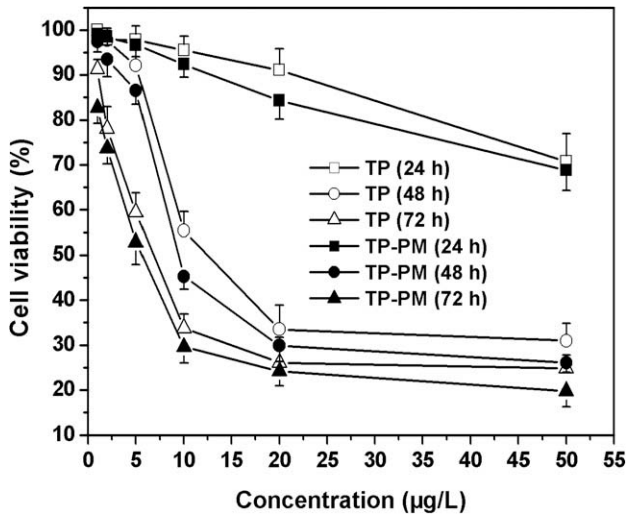

Fig. 4. Cytotoxicity of TP delivered as a free drug or in polymeric micelles against A2780 cells at different concentrations at different time points by the MTT assay. Data are represented as mean ± SD ($n = 3$).

A2780 cells in a time- and dose-dependent manner. This implies that TP is released from MePEG-PLA polymer micelles carrier without losing its cytotoxicity and that TP-PM may induce tumour-cells apoptosis in the same manner as that of free TP.

3.5. In vivo anti-tumour activities of TP-PM

Table 1 lists the tumour inhibition rates of all the tested groups. The groups treated with TP-PM and TP showed a significant tumour inhibition (inhibition rate more than 30%, $P < 0.01$) compared to the negative control group. TP-PM inhibited tumour growth in a dose-dependent manner, whose inhibition rates were 42.5%, 46.0% and 49.9%, respectively. The inhibition rates of TP at the equal dose levels were 29.6%, 35.7% and 35.7%, respectively. Meanwhile, the inhibition rate of CTX was 52.5%. The inhibition rates in TP-PM groups were 10% higher than those in the TP groups at equal dose levels. The anti-tumour efficacy of TP-PM at a low dose level was comparable with that of TP at a high dose level. These results showed that TP-PM had a stronger anti-tumour effect than TP.

The representative microphotographs are shown in Fig. 5. There are two colours on microphotographs: blue stands for tumour cells with good growth and red stands for tumour necrosis. Fig. 5A revealed the presence of typical tumour cell groups with typical nuclear changes in the S-180 control group with little necrosis in the central area of the tumour. A few residual neoplastic cells inserted into a large central area of necrosis were observed in the tumour tissue of animals treated by CTX at the dose level of 20 mg/kg and free TP at the dose level of 0.15 mg/kg (Fig. 5B and D). However, more extensive necrotic areas were observed in the tumour tissue of animals treated by TP-PM at the dose level of 0.15 mg/kg (Fig. 5C).

Table 1
Effects of TP-PM on tumour growth in S-180-bearing mice

Group	Dose (mg/kg)	Tumour weight (g)	Inhibitory rate (%)
S-180 control	–	2.46 ± 0.61	–
CTX	20	1.17 ± 0.25**	52.5
TP	0.0375	1.73 ± 0.50**	29.6
	0.075	1.58 ± 0.54**	35.7
	0.15	1.58 ± 0.50**	35.7
TP-PM	0.0375	1.41 ± 0.31**	42.5
	0.075	1.33 ± 0.34**	46.0
	0.15	1.23 ± 0.32**	49.9

Results are expressed as mean ± SD of 10 mice for each group, *$P < 0.05$, **$P < 0.01$ compared to S-180 control (Dunnett's t-test).

S-180 cell is one of the classical solid tumour models since a very long time. TP-PM possessed better anti-tumour activity than TP and induced tumour necrosis more significantly than free TP. This might be related to the increase in the circulating time of TP in blood and drug accumulation in tumours due to its characteristics of small particle size, high structure stability and extended drug release, as well as the above-mentioned EPR effect of polymeric micelles [12,26]. Particularly, polymeric micelles, as an ideal application for EPR-based selective anti-cancer drug delivery, may extravasate into the tumour vasculature and become trapped in the tumour vicinity; further, with time, due to lack of efficient lymphatic drainage in the solid tumour, the drug concentration of the tumour would increase to a level that is several times higher than that in the plasma [27]. TP-PM showed a stronger anti-tumour effect *in vivo* than *in vitro* when compared to TP, and it may be far more complex involving multiple factors *in vivo*, such as vascularization and the immune response.

3.6. Effects of TP-PM on the immune function of S-180-bearing mice

TP is a molecule with both anti-tumour and immuno-suppressive activities. The effects of TP-PM on the immune function of S-180-bearing mice are shown in Table 2. Compared to the normal control, the spleen index was significantly increased ($P < 0.05$) and the spleen lymphocyte proliferation was significantly decreased ($P < 0.05$) in the S-180-bearing control group. Compared to the S-180-bearing control group, TP significantly decreased ($P < 0.05$) the thymus index and very significantly increased ($P < 0.01$) the spleen index only at the high dose level of 0.15 mg/kg, and significantly decreased the spleen lymphocyte proliferation, IL-2 and TNF-α levels in the serum in a dose-dependent manner ($P < 0.05$). At the same time, the positive control CTX group also significantly decreased the lymphocyte proliferation and the IL-2 and TNF-α levels in serum ($P < 0.05$). However, CTX had no effect on the thymus index and spleen index in S-180-bearing mice, and TP-PM had no effect on any of the above parameters in S-180-bearing mice ($P > 0.05$).

The occurrence, development and prognosis of cancer are closely related to the immune status of the cancer patient, and cellular immunity has been proved to play a particularly important role in the anti-tumour immunity of the host [28]. The thymus and the spleen are two important immune organs. The T lymphocytes in the peripheral blood originate from the thymus and the B lymphocytes originate from the spleen. IL-2 and TNF-α are two important cytokines mediating the immune response. IL-2 produces a marked effect through the receptor system with distribution in the T, B, NK, and K cells; TNF-α is able to increase the immunogenicity of tumour cells due to the enhanced expression of MHC antigens and thus can be used to identify the anti-tumour immune response [19]. ConA-induced proliferation of spleen-derived T lymphocytes is a typical cell-mediated immune response. The changes of these detected parameters could reflect the immune status of the host.

TP has been reported to have strong immuno-suppressive effects on both humoral immunity and cellular immunity, and the effective dose level of TP as an immuno-suppressive agent (9.31 μg/kg for rat) is also smaller than that of TP as an anti-tumour agent [29,30]. In this study too, TP exhibited obvious immuno-suppressive activity. Immuno-suppressive status increases the risk of suffering from cancers and harms to the effectiveness of cancer therapy [31]. One of the side effects of many chemotherapy agents, such as CTX, is the immuno-suppressive effect. In this study, the immuno-suppressive activity of CTX was also demonstrated, and the immuno-suppressive activity of TP was equal to

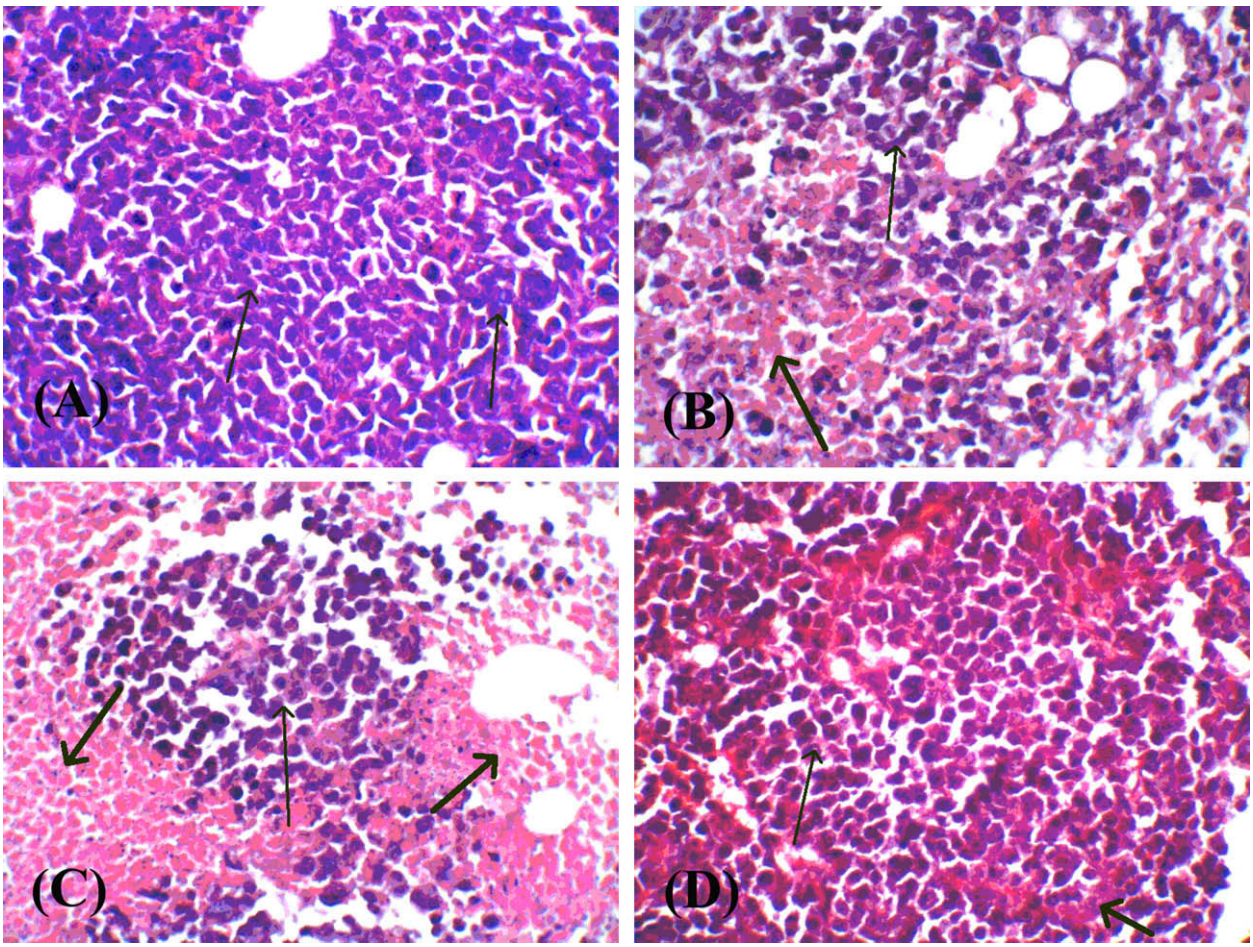

Fig. 5. Histopathological analyses of tumours in sarcoma 180-bearing mice (H & E, 40 × 17): blank control (A), CTX (B), TP-PM at the dose level of 0.15 mg/kg (C) and free TP at the dose level of 0.15 mg/kg (D). Thin arrows indicate tumour cells with good growth and thick arrows show tumour necrosis.

Table 2
Effects of TP-PM on immune function of S-180-bearing mice

Group	Dose (mg/kg)	Thymus index (mg/g)	Spleen index (mg/g)	TNF-α (ng/ml)	IL-2 (ng/ml)	Lymphocyte proliferation rate(%)
Normal control	–	3.05 ± 0.58	7.97 ± 1.21	3.1 ± 0.2	3.1 ± 1.6	46.9 ± 12.4
S-180 control	–	3.17 ± 0.70	10.12 ± 2.35$^{\triangle}$	3.0 ± 0.7	2.8 ± 1.8	28.3 ± 11.8$^{\triangle}$
CTX	20	2.66 ± 0.54	9.34 ± 2.63	2.5 ± 0.4*	1.2 ± 1.0*	12.1 ± 9.5*
TP	0.0375	3.04 ± 1.51	10.26 ± 2.21	2.6 ± 0.4	2.1 ± 0.4	24.2 ± 18.8
	0.075	3.33 ± 0.65	12.23 ± 3.56	2.5 ± 0.6	1.6 ± 0.6*	11.9 ± 11.0*
	0.15	2.09 ± 0.88*	15.56 ± 1.17**	2.4 ± 0.6*	1.1 ± 1.1*	9.4 ± 7.7**
TP-PM	0.0375	3.46 ± 0.79	10.89 ± 1.61	2.8 ± 0.5	2.3 ± 1.1	31.8 ± 21.7
	0.075	2.84 ± 0.65	11.67 ± 2.46	2.7 ± 0.9	1.8 ± 0.4	19.7 ± 23.6
	0.15	2.89 ± 0.80	13.46 ± 5.96	2.9 ± 0.4	1.6 ± 0.8	16.5 ± 15.7

Results are expressed as mean ± SD of 10 mice for each group, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to S-180 control (Dunnett's *t*-test). $^{\triangle}P < 0.05$ compared to normal control (Dunnett's *t*-test).

or even stronger than that of the conventional chemotherapy agent CTX. Therefore, the immuno-suppressive activity of TP may become a side effect when it is used as an anti-tumour agent.

Spleen is an organ that creates lymphocytes for the destruction and recycling of old red blood cells along with being a blood reservoir. In this study, the spleen index in S-180-bearing mice was significantly greater than that in normal mice ($P < 0.05$). Pathological splenomegaly was demonstrated under a microscope, where the red pulp was observed to be enlarged and the white pulp was shrunken (data not shown). This suggested an immuno-suppressive status in the tumour-bearing mice. The spleen index in S-180-bearing mice by the intravenous administration of TP at the high dose level of 0.15 mg/kg was significantly increased compared to that in S-180-bearing control mice ($P < 0.01$), and the pathological spleen microphotographs suggested the immuno-suppressive status and extra-medullary haemopoiesis (data not shown). Therefore, the result that TP significantly increased the spleen index in tumour-bearing mice suggests the strong immuno-suppressive effect of TP on tumour-bearing mice. However, TP-PM and CTX had no obvious effect on the spleen index in S-180-bearing mice.

Both anti-tumour and immuno-suppressive effects of TP were observed in the same tumour-bearing mice in this study. The major objective of the study was to observe immuno-suppressive activity

at dose levels that exhibited obvious anti-tumour effects. In this study, the relatively high dose was designed to observe the maximum anti-tumour effects and the immuno-suppressive activity of TP. The results demonstrated that TP or TP-loaded polymeric micelles (TP-PM) showed nearly maximum anti-tumour activity in the designed dose ranges, and no dose–effect relationship was obvious. At these dose levels, TP showed obvious immuno-suppressive activity in the S-180-bearing mice while TP-PM did not. These results suggest that polymeric micelles alleviate the immuno-suppressive activity of TP and enhance the anti-tumour effect of TP. TP, as a hydrophobic drug, may be physically incorporated into the core of the polymeric micelles by hydrophobic interactions. Polymeric micelles might influence the disposition of TP in the body of the tumour-bearing mice as they could change the pharmacokinetic feature of other anti-tumour drugs in the body of the tumour-bearing animals [13,22,32,33]. Due to the characteristic structure of the polymeric micelles, in which the inner core is segregated by a dense PEG palisade, the properties or amount of the drugs wrapped within the polymeric micelles hardly affect their distribution after systemic administration; TP-PM may possess long-term circulation characteristics due to the efficient stealthy behaviour of the dense PEG shell. Further, it may avoid RES recognition due to a size smaller than 200 nm as well as its excellent biocompatibility [12]. At the same time, the long-term circulating polymeric micelles accumulate in the solid tumour due to the above-mentioned EPR effect; therefore, the distribution of TP-PM in immune organs (spleen, thymus, lymph nodes) may be decreased and the distribution of TP-PM in tumours may be enhanced. On the other hand, the target molecule and the target tissue for the anti-tumour effect may differ from those for the immuno-suppressive effect. Therefore, the intensity of anti-tumour activity may be different from the intensity of immuno-suppressive activity at equal dose levels.

4. Conclusion

TP-PM was conveniently prepared using the MePEG-PLA copolymer by a solvent evaporation method, with an average diameter of 78.9 nm, a core–shell structure and long-term stability. TP-PM exhibited anti-tumour effect on A2780 cells via a MePEG-PLA polymer micelles carrier. TP-PM also exhibited an anti-tumour effect and prevented the immuno-suppressive side effect of TP in S-180-bearing mice. This study demonstrated that TP encapsulated in polymeric micelles does not act as an immuno-suppressant without losing its anti-tumour effect. We have therefore demonstrated that polymeric micelles are a stable and effective drug delivery carrier of TP for the therapy of solid tumours. The pharmacokinetics and toxicity studies of TP-PM employing different animal models are under investigation.

Acknowledgements

This work was supported by the National Basic Research Program of China (Grant No. 2006CB933301). We thank the Analytical and Testing Center of Huazhong University of Science and Technology for the TEM analysis. We also thank Dr. Zhiqing Wang and Feng Xiao for their technical assistance.

References

[1] A.M. Brinker, J. Ma, P.E. Lipsky, I. Raskin, Medicinal chemistry and pharmacology of genus *Tripterygium* (Celastraceae), Phytochemistry 68 (2007) 732–766.
[2] T. Tengchaisri, R. Chawengkirttikul, N. Rachaphaew, V. Reutrakul, R. Sangsuwan, S. Sirisinha, Anti-tumor activity of triptolide against cholangiocarcinoma growth in vitro and in hamsters, Cancer Lett. 133 (1998) 169–175.
[3] E.W. Chan, S.C. Cheng, F.W. Sin, Y. Xie, Triptolide induced cytotoxic effects on human promyelocytic leukemia, T cell lymphoma and human hepatocellular carcinoma cell lines, Toxicol. Lett. 122 (2001) 81–87.
[4] T.M. Kiviharju, P.S. Lecane, R.G. Sellers, D.M. Peehl, Antiproliferative and proapoptotic activities of triptolide (PG490), a natural product entering clinical trials, on primary cultures of human prostatic epithelial cells, Clin. Cancer Res. 8 (2002) 2666–2674.
[5] S.M. Yang, J.G. Chen, Z. Guo, X.M. Xu, L.P. Wang, X.F. Pei, J. Yang, C.B. Underhill, L.R. Zhang, Triptolide inhibits the growth and metastasis of solid tumors, Mol. Cancer Ther. 2 (2003) 65–72.
[6] G.X. Zhou, X.L. Ding, J.F. Huang, H. Zhang, S.B. Wu, Suppression of 5-lipoxygenase gene is involved in triptolide-induced apoptosis in pancreatic tumor cell lines, Biochem. Biophys. Acta 1770 (2007) 1021–1027.
[7] H. Lu, M. Hachida, S. Enosawa, X.K. Li, S. Suzuki, H. Koyanagi, Immunosuppressive effect of triptolide in vitro, Transplant. Proc. 31 (1999) 2056–2057.
[8] S. Gross, P. Walden, Immunosuppressive mechanisms in human tumors: why we still cannot cure cancer, Immunol. Lett. 116 (2008) 7–14.
[9] A. Gaumann, H.J. Schlitt, E.K. Geissler, Immunosuppression and tumor development in organ transplant recipients: the emerging dualistic role of rapamycin, Transpl. Int. 21 (3) (2008) 207–217.
[10] F.L. Xiong, H.B. Chen, X.L. Chang, Y.J. Yang, H.B. Xu, X.L. Yang, Research progress of triptolide-loaded nanoparticles delivery systems, Conf. Proc. IEEE Eng. Med. Biol. Soc. 5 (2005) 4966–4969.
[11] Q. Zheng, T. Gong, X. Sun, Z.R. Zhang, Synthesis, characterization and in vitro evaluation of triptolide-lysozyme conjugate for renal targeting delivery of triptolide, Arch. Pharm. Res. 29 (2006) 1164–1170.
[12] N. Nishiyama, K. Kataoka, Current state, achievements and future prospects of polymeric micelles as nanocarriers for drug and gene delivery, Pharmacol. Ther. 112 (2006) 630–648.
[13] K. Kawano, M. Watanab, T. Yamamoto, M. Yokoyama, P. Opanasopit, T. Okano, Y. Maitani, Enhanced antitumor effect of camptothecin loaded in long-circulating polymeric micelles, J. Control. Release 112 (2006) 329–332.
[14] K. Yasugi, Y. Nagasaki, M. Kato, K. Kataoka, Preparation and characterization of polymer micelles from poly (ethylene glycol)–poly (D,L-lactide) block copolymers as potential drug carrier, J. Control. Release 62 (1999) 89–100.
[15] Y. Yamamoto, K. Yasugi, A. Harada, Y. Nagasaki, K. Kataoka, Temperature-related change in the properties relevant to drug delivery of poly(ethylene glycol)–poly(D,L-lactide) block copolymer micelles in aqueous milieu, J. Control. Release 82 (2002) 359–371.
[16] X.Y. Xu, L. Li, J.P. Zhou, S.Y. Lu, J. Yang, X.J. Yin, J.S. Ren, Preparation and characterization of N-succinyl-N′-octyl chitosan micelles as doxorubicin carriers for effective anti-tumor activity, Colloids Surf. B Biointerfaces 55 (2007) 222–228.
[17] H.M. Aliabadi, S. Elhasi, A. Mahmud, R. Gulamhusein, P. Mahdipoor, A. Lavasanifar, Encapsulation of hydrophobic drugs in polymeric micelles through co-solvent evaporation: the effect of solvent composition on micellar properties and drug loading, Int. J. Pharm. 329 (2007) 158–165.
[18] L.Y. Zhang, M. Yang, Q. Wang, Y. Li, R. Guo, X.Q. Jiang, C.Z. Yang, B.R. Liu, 10-Hydroxycamptothecin loaded nanoparticles: preparation and antitumor activity in mice, J. Control. Release 119 (2007) 153–162.
[19] H.M. Yuan, J.M. Song, X.G. Li, N. Li, J.C. Dai, Immunomodulation and antitumor activity of k-carrageenan oligosaccharides, Cancer Lett. 243 (2006) 228–234.
[20] R.W. Egan, M.A. Jones, A.L. Lehninger, Hydrophile–lipophile balance and critical micelle concentration as key factors influencing surfactant disruption of mitochondrial membranes, J. Biol. Chem. 251 (1976) 4442–4447.
[21] H. Schott, Solubility parameter and hydrophilic–lipophilic balance of nonionic surfactants, J. Pharm. Sci. 73 (1984) 790–792.
[22] S.C. Kim, D.W. Kim, Y.H. Shim, J.S. Bang, H.S. Oh, S.W. Kim, M.H. Seoa, In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy, J. Control. Release 72 (2001) 191–202.
[23] M.X. Liu, J. Dong, Y.J. Yang, X.L. Yang, H.B. Xu, Characterization and release of triptolide-loaded poly (D,L-lactic acid) nanoparticles, Eur. Polym. J. 41 (2005) 375–382.
[24] Y. Miyata, T. Sato, A. Ito, Triptolide, a diterpenoid triepoxide, induces anti-tumor proliferation via activation of c-Jun NH2-terminal kinase 1 by decreasing phosphatidylinositol 3-kinase activity in human tumor cells, Biochem. Biophys. Res. Commun. 336 (2005) 1081–1086.
[25] X.H. Jiang, B.C. Wong, M.C. Lin, H.F. Kung, G.H. Zhu, S.H. Jiang, Functional p53 is required for triptolide induced apoptosis and AP-1 and nuclear factor-kB activation in gastric cancer cells, Oncogene 20 (2001) 8009–8018.
[26] C. Fang, B. Shi, Y.Y. Pei, M.H. Hong, J. Wu, H.Z. Chen, In vivo tumor targeting of tumor necrosis factor-α-loaded stealth nanoparticles: effect of MePEG molecular weight and particle size, Eur. J. Pharm. Sci. 27 (2006) 27–36.
[27] K. Greish, Enhanced permeability and retention of macromolecular drugs in solid tumors: a royal gate for targeted anticancer nanomedicines, J. Drug Target. 15 (2007) 457–464.
[28] T. He, C. Tang, S. Xu, T. Moyana, J. Xiang, Interferon gamma stimulates cellular maturation of dendritic cell line DC2.4 leading to induction of efficient cytotoxic T cell responses and antitumor immunity, Cell. Mol. Immunol. 4 (2007) 105–111.
[29] D.M. Qiu, P.N. Kao, Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb *Tripterygium wilfordii* Hook f, Drugs R D 4 (2003) 1–18.

[30] J. Zhou, C. Xiao, L.H. Zhao, H.W. Jia, N. Zhao, C. Lu, D.J. Yang, J.C.-O. Tang, A.S.C. Chan, A.P. Lu, The effect of triptolide on CD4+ and CD8+ cells in Peyer's patch of SD rats with collagen induced arthritis, Int. Immunopharmacol. 6 (2006) 198–203.
[31] T.M. Oberyszyn, Non-melanoma skin cancer: importance of gender, immunosuppressive status and vitamin D, Cancer Lett. 261 (2008) 127–136.
[32] H. Miura, H. Onishi, M. Sasatsu, Y. Machida, Antitumor characteristics of methoxypolyethylene glycol–poly(D,L-lactic acid) nanoparticles containing camptothecin, J. Control. Release 97 (2004) 101–113.
[33] O. Molavi, Z. Ma, A. Mahmud, A. Alshamsan, J. Samuel, R. Lai, G.S. Kwon, A. Lavasanifar, Polymeric micelles for the solubilization and delivery of STAT3 inhibitor cucurbitacins in solid tumors, Int. J. Pharm. 347 (2008) 118–127.