SPANDIDOS PUBLICATIONS

Antitumor activity of monomethoxy poly(ethylene glycol)-poly (ε-caprolactone) micelle-encapsulated doxorubicin against mouse melanoma

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Abstract. Doxorubicin (Dox) is one of the most commonly used and highly effective antineoplastic agents, but the clinical application of this broad spectrum drug is largely hampered by its poor stability and serious toxicity to normal tissues. Hence, it is essential to improve the therapeutic effect and decrease the systematic toxicity for the administration of doxorubicin. In our study, doxorubicin was incorporated into monomethoxy poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) micelle by a self-assembly method. The cytotoxicity and cellular uptake efficiency of Dox-loaded MPEG-PCL (Dox/MPEG-PCL) micelle against B16-F10 murine melanoma cells was examined by the methylthiazol-tetrazolium (MTT) test and flow cytometry. The antitumor activity of Dox/MPEG-PCL was evaluated in C57BL/6 mice injected subcutaneously with B16-F10 cells. Toxicity was evaluated in tumor-free mice. Meanwhile, tumor proliferation, intratumoal angiogenesis and apoptotic cells were evaluated by PCNA, CD31 staining and TUNEL assay, respectively. Encapsulation of doxorubicin in MPEG-PCL micelle improved the cytotoxicity of doxorubicin and enhanced its cellular uptake on B16-F10 cell *in vitro*. Administration of Dox/MPEG-PCL micelle resulted in significant inhibition (75% maximum inhibition relative to controls) in the growth of B16-F10 tumor xenografts and prolonged the survival of the treated mice (P<0.05). These anti-tumor responses were associated with marked increase of tumor apoptosis and notable reduction of cell proliferation and intratumoral microvessel density (P<0.05). The system toxicity also decreased in the Dox/MPEG-PCL group compared with free doxorubicin group. Our data indicate that the encapsulation of doxorubicin in MPEG-PCL micelle improved the anti-tumor activity *in vivo* without conspicuous systemic toxic effects.

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Introduction

Doxorubicin is an anthracycline isolated from the pigment-producing *Streptomyces peucetius* early in the 1960s. It is one of the most commonly used and highly effective antineoplastic agents that is usually administered intravenously for the treatment of malignancies, including tumors of the breast, ovary, bladder, pancreas and thyroid (1). Nevertheless, the clinical use of this broad spectrum drug is also limited because of its poor stability and serious non-specific toxicity to normal tissues, which induces severe side effects such as acute dose limiting bone marrow toxicity and chronic cumulative cardiac toxicity (2-4). Also, the rapid clearance rate from the circulation by the reticuloendothelial system (RES) renders the extravasation of doxorubincin into tumor site extremely low, which accordingly weakens the drug efficacy. Strategies for improving the efficacy and safety of doxorubicin have moved along two medicinal chemistry lines: i) development of tumor-targeted formulations; and ii) development of new analogs. The pharmaceutical development of the first strategy is best exemplified by the widely used liposomal formulations of doxorubicin, which appear to improve the therapeutic effects (5-7). However, the usefulness of this formulation is limited by the increased uptake by RES (6,7). Alterations in the liposomal surface charge and hydrophilicity produce 'stealth liposome' with prolonged circulation half-time, slow drug release and changes in tissue distribution with increased deposition in tumors and reduced cardiotoxicity (8-12). However, the liposome doxorubicin is not devoid of adverse effects. Its toxicity profile is characterized by dominant and dose-limiting mucocutaneous reactions. Moreover, it has been reported that these liposomes can cause a poorly understood

immediate hypersensitivity reactions (HSRs) usually developed at the start of infusion in a relatively large number (up to 7%) of patients (13-15). Therefore, finding a novel drug delivery system is imperative to overcome these internal defects and to increase the anticancer efficacy of doxorubicin.

Recent development in nanotechnology provides researchers with new tools for cancer therapy. Biodegradable polymeric nanoparticles are highlighted as anticancer drug delivery system to improve anticancer effect and safety of the cargo. The nano-sized drug carriers exhibit prolonged circulation time by avoiding rapid renal clearance and unwanted uptake by the RES, resulting in enhanced permeability and retention (EPR) of tumor site (16-18). Polymeric micelles are spherical, nano-sized, and supramolecular assemblies of amphiphilic copolymers that possess a core-shell-type architecture. The advantages of block copolymer micelles include their nanosize, biodegradability, biocompatibility, long-circulation times, controllable drug-release profile, and tissue penetrating ability. Poly (ethyleneglycol)-poly(ε-caprolactone) (PEG-PCL) copolymers are biodegradable, biocompatible and amphiphilic. These properties make PEG-PCL nano-particles good candidates for advanced drug delivery systems. In addition, the PEG-PCL possesses excellent drug loading capability and is superior pharmaceutical excipients for drug delivery, which might serve as an available drug carrier to encapsulate doxorubicin and facilitate the uptake and retention of doxorubicin in cancers.

We successfully prepared doxorubicin-loaded MPEG-PCL micelle (~20 nm) by a self-assembly method, and proved that incorporation of doxorubicin in MPEG-PCL micelle improved anticancer effect and reduced systemic toxicity of doxorubicin, which suggested the potential clinical application of Dox/MPEG-PCL micelle in cancer therapy. The pre-clinical protocol of the present study aimed to evaluate the potential application of Dox/MPEG-PCL in treatment of B16-F10 cancer cell line *in vitro* and *in vivo*.

Materials and methods

Cell lines and culture. B16-F10 melanoma cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Auckland, NZ), 100 units/ml penicillin, and 100 units/ml streptomycin. The culture was maintained in 95% air-humidified atmosphere with 5% CO_2 at 37˚C.

Preparation and characterization of Dox/MPEG-PCL. Doxorubicin loaded MPEG-PCL micelle was prepared by a self-assembly method. Briefly, blank MPEG-PCL micelle was first prepared by heat-induced self-assembly method. Due to the amphiphilic properties, the MPEG-PCL copolymer self-assembled into micelles in water that triggered by temperature increasing to 50˚C. Then, doxorubicin-loaded MPEG-PCL micelle was prepared by a pH-induced self-assembly method. Doxorubicin aqueous solution was dropped into MPEG-PCL micelle in PBS (pH 7.4) under moderate stirring. Because of the low solubility of doxorubicin in PBS at pH 7.4, doxorubicin self-assembled into the hydrophobic core of MPEG-PCL micelle. Then, the Dox/MPEG-PCL was lyophilized for future application.

To determine the release kinetics of doxorubicin from micelle, the prepared 0.5 ml of doxorubicin loaded MPEG-PCL micelle was placed in a dialysis bag (molecular mass cut-off 8-14 kDa). The dialysis bags were incubated in 30 ml of phosphate buffer (pH 7.0 or 5.5) containing Tween-80 (0.5%) at 37˚C with gentle shaking, and incubation medium were replaced by fresh incubation medium at predetermined time points. The released drug was quantified by determining the absorbance at 485 nm using a spectrophotometer (M5, Molecular Corporation, US), and the cumulative release profile with time was demonstrated. This study was repeated 3 times, and result was expressed as mean value ± SD.

Assessment of cell uptake of Dox/MPEG-PCL. B16 cells were grown in 6-well plates and incubated for 24 h to 70% confluence. Subsequently, free doxorubicin, Dox/MPEG-PCL and control MPEG-PCL, were added to each well, respectively. After 24 h of co-incubation, cells were collected for measurement of doxorubicin fluorescence. The fluorescence from individual cells was detected with a flow cytometer (FACSAria BD, USA). For detection of doxorubicin-derived fluorescence, excitation was with the 488-nm line of an argon laser, and emission fluorescence was detected at 564 nm. For all experiments in which the intracellular doxorubicin was quantified using flow cytomentry, at least 10,000 cells were measured from each sample (FACS-DiVa BD).

Analysis of cytotoxicity. The cytotoxicity of Dox/MPEG-PCL micelle on B16-F10 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, B16-F10 cells were plated at a density of $2.5x10^3$ cells per well in 100 μl RPMI-1640 medium in 96-well plates and grown for 24 h. Then 0.1 ml fresh RPMI-1640 growth medium containing a series of concentrations of free doxorubicin, Dox/MPEG-PCL, MPEG-PCL and medium only was added to each well. Untreated cells in growth media were used as a control. After incubation for 24, 48 or 72 h, additional 20 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well. The plates were incubated for 4 h at 37˚C. After the incubation, the MTT solution was carefully removed from each well, and 150 μl DMSO was added to dissolve the MTT formazan crystals. Absorbance was measured at 570 nm using an ELISA microplate reader (Bio-Rad). The cell viability (%) was related to the control wells containing untreated cells with fresh cell culture medium and was calculated according to the following: cell viability (%) = $absorption_{test}/absorption_{control}$ x 100%. All dates are presented as the mean of three measurements (± SD).

In vivo tumor studies. The research protocol was reviewed and approved by the Institutional Animal Care and Treatment Committee of Sichuan University. C57BL/6 female mice (6-8 weeks old) were from Sichuan University Animal Center (Sichuan, Chengdu, China). The mice were injected subcutaneously in the right flank with 0.1 ml of cell suspension containing $2x10^5$ cells and maintained in RPMI-1640 medium. Tumor-bearing mice were randomly assigned to one of the four groups (n=10 mice/group) when tumors were palpable: i) mice treated with Dox/MPEG-PCL (5 mg/kg);

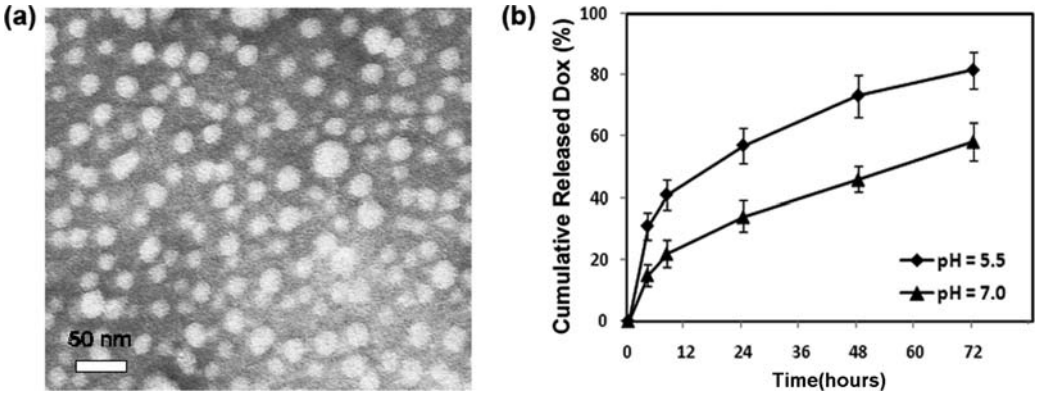

Figure 1. Characterization of Dox/MPEG-PCL micelle. (a) TEM image of Dox/MPEG-PCL micelle and (b) release profile of Dox/MPEG-PCL micelle at pH 7.0 or pH 5.5.

ii) mice treated with free doxorubicin (5 mg/kg); ii) mice treated with empty MPEG-PCL (100 mg/kg); iv) mice treated with 100 μl of normal saline (NS). Tumor growth was monitored every three days by measuring the two perpendicular diameters using a caliper and calculated using the formula vol = (a x b^2)/2, where vol is volume, a is the length of the major axis, and b is the length of the minor axis. The mice were dissected on the 17th day post-inoculation and the tumors were excised. For the life span study, the experiment was terminated on day 45.

Immunohistochemistry. The anti-intratumoral angiogenesis and cell proliferation effects were determined by immunohistochemistry with an antibody reactive to CD31 and PCNA. Briefly, tumor sections were dewaxed, rehydrated through graded ethanol washes and incubated with 3% hydrogen peroxide for 10 min. After antigen retrieval, non-specific binding of reagents was obturated by incubation of sections in 5% normal rabbit serum for 15 min. The sections were continuously incubated with goat anti-mouse immunoglobulin at 4˚C overnight, biotinylated rabbit anti-goat secondary antibody at 37˚C for 40 min and then streptavidin-biotin reagents at 37˚C for 30 min, resulting in the brown staining of microvessel endothelia cells. To quantify microvessel density (MVD) and PCNA-positive cell were selected by scanning tumor sections under a light microscope and three equal-sized fields were randomly chosen and analyzed. The number of microvessels per field was counted separately by two investigators.

The terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining was applied to detect apoptosis in tumor cells. With an *in situ* apoptotic cell detection kit according to the manufacturer's directions (Promega, Madison, WI, USA) as described previously. Images of the representative sections were taken by using a fluorescence microscope (Olympus, Tokyo, Japan). In tissue sections, cell nuclei were stained dark green, indicating apoptosis, and were recorded as TUNEL-positive nuclei. Three equal-sized fields were randomly chosen and analyzed. Density was evaluated in each field, yielding the density of apoptotic cells (apoptosis index).

Toxicological study. To further study the potential toxic effects of Dox/MPEG-PCL treatment, 40 mice without tumors were randomly assigned to one of four treatment groups (n=10 mice per group) and received the following treatment: NS, MPEG-PCL micelles alone (200 mg/kg each), free doxorubicin (10 mg/kg each), or Dox/MPEG-PCL (10 mg/kg each). After tail vein injection, weight was recorded daily. They were observed continuously for relevant indexes such as weight loss, ruffled fur, appetite, diarrhea, cachexia, skin ulceration or toxic deaths. The experiment was terminated on day 25 and the tissues of heart, liver, spleen, lung, kidney, and brain were fixed in 4% neutral buffered formalin solution and embedded in paraffin. Sections of 3-5 mm were stained with hematoxylin and eosin (H&E) and observed by two pathologists in a blinded manner.

Statistical analysis. Comparison of cellular proliferation inhibition was carried out by using Student's t-test. Comparisons of tumor volume among different groups were carried out by using one-way analysis of variance (ANOVA). Survival was assessed with the Kaplan-Meier method and statistical significance was determined by the log-rank test; P-values <0.05 were considered to be statistically significant. All statistical tests were two-sided.

Results

Preparation and characterization of Dox/MPEG-PCL. Doxorubicin was incorporated in monomethoxy poly (ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) micelle by a self-assembly method. This procedure of preparing Dox/MPEG-PCL micelle was very simple, and easy to scale up. Meanwhile, any surfactants, organic solvents and vigorous stirring were not applied in this procedure. These Dox/MPEG-PCL micelles had the drug entrapment efficiency of 92.8% and drug loading of 4.23%. These micelles were monodispersed (polydispersity index = 0.131), and had the mean particle size of 34.1 nm. According to TEM image (Fig. 1a), these doxorubicin loaded MPEG-PCL micelles were monodisperse and spherical with a diameter of ~20 nm. The doxorubicin release from Dox/MPEG-PCL was faster at pH 5.5 than at pH 7.0 (Fig. 1b).

Increased cellular uptake in vitro. To measure the internalization of doxorubicin quantitatively, cells cultured on 6-well plates were exposed to free doxorubicin or Dox/

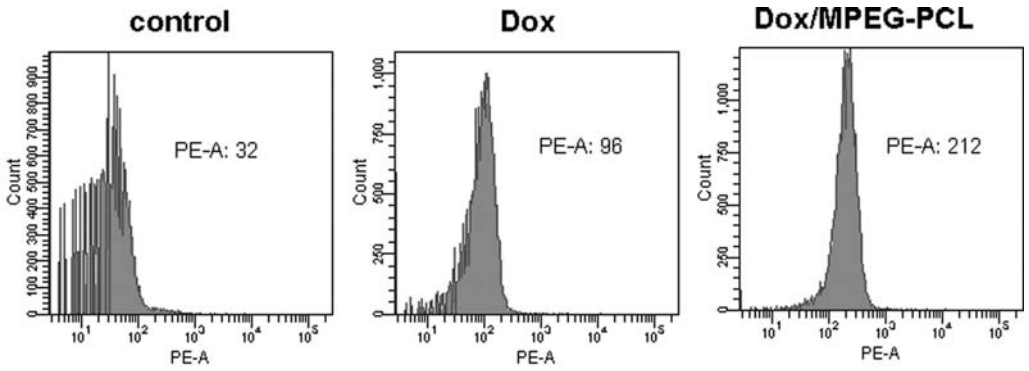

Figure 2. Penetration of the doxorubicin and Dox/MPEG-PCL in B16-F10 tumors analyzed by flow cytometry. B16 cells were grown in 6-well plates and incubated for 24 h to 70% confluence. Then, free doxorubicin (0.3 μg/ml), Dox/MPEG-PCL (0.3 μg/ml) and control MPEG-PCL were added to each well separately. After 24 h of exposure, the cells were collected for measurement of doxorubicin fluorescence.

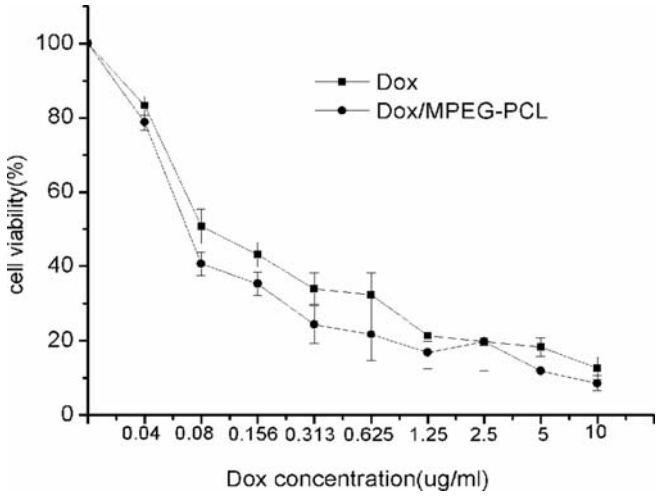

Figure 3. Viability inhibitory effect of Dox/MPEG-PCL on B16-F10 cell determined by the MTT assay. Cells were seeded in 96-well plates at an initial density of $2x10^3$ cells/well in 0.2 ml growth media and treated with the indicated concentrations of Dox/MPEG-PCL, Dox respectively for 48 h. The cell viability is significantly lower in Dox/MPEG-PCL group than Dox group ($^*P<0.05$). The data are expressed as percentages of viable cells relative to media-only treated control cells at each drug concentration. Means ± SD (n=3).

MPEG-PCL with the concentration of 0.3 μg/ml, and after incubation for 24 h, the cells were collected for analysis of doxorubicin-derived fluorescence by flow cytometry. Results indicated that B16-F10 cell more efficiently internalized Dox/MPEG-PCL micelle than free doxorubicin (Fig. 2).

Improved tumor cell proliferation inhibition in vitro. Inhibition of B16-F10 cell proliferation by doxorubicin encapsulated in MPEG-PCL was shown in Fig. 3. MTT assay shows that Dox/MPEG-PCL treatment resulted in a dose- and time-dependent inhibition of cell proliferation. Empty MPEG-PCL micelle at the concentration of 1 mg/ml did not suppress the proliferation of B16-F10 cells at all (data not shown). The concentration of doxorubicin encapsulated in MPEG-PCL that caused 50% mortality was much lower than that of free doxorubicin. These results indicate that the encapsulation of doxorubicin in MPEG-PCL had enhanced its cytotoxic activity.

Improved inhibition of B16-F10 melanoma carcinogenesis and prolong survival. To further study the *in vivo* anti-tumor efficiency of Dox/MPEG-PCL, subcutaneous melanoma model on mice was established and further investigated by treatment with Dox/MPEG-PCL and NS, MPEG-PCL or free doxorubicin respectively. One week after the last treatment, mice from each group were sacrificed and subcutaneous tumors were excised. As shown in Fig. 4A, in comparison with treatments with NS, MPEG-PCL or free doxorubicin, treatment with Dox/MPEG-PCL significantly suppressed tumor growth (P=0.000, 0.000, 0.001, respectively) as evaluated by measuring tumor volume at regular intervals. However, no significant difference of tumor weight was observed between NS and MPEG-PCL group. Therefore, administration of Dox/MPEG-PCL could reduce tumor weight by about 75% in comparison with that of controls.

The beneficial effects of incorporation of doxorubicin in MPEG-PCL to a subcutaneous carcinoma model *in vivo* were also reflected by the survival time. The mice in the Dox/MPEG-PCL-treated group lived dramatically longer than those in the NS, MPEG-PCL or free doxorubicin groups ($P<0.05$), as shown in the Kaplan-Meier survival curve displayed in Fig. 4B. There was no significant statistically difference in survival time between NS-treated mice and MPEG-PCL-treated mice in melanoma tumor model ($P>0.05$). Therefore, our study proved that the Dox/MPEG-PCL formulation could prolong the survival time as compared with that of DOX and control.

Decreased systematic toxicity in the Dox/MPEG-PCL-treated mice. Since earlier research confirmed the severe irreversible side effects of doxorubicin treatment, we compared the body weight and other side effects on mice treated with free doxorubicin or Dox/MPEG-PCL. The body weight was monitored every three days. Two mice that were administered free doxorubicin died before the planned endpoint (22 days) after a continuous decrease in body weight. In contrast, mice treated with the same dose of Dox/MPEG-PCL began to gain weight within 3-5 days of the start of treatment and attained body weight equal to that of control on day 11. Also, no difference of body weight was observed among the groups at the end of the study (Fig. 5A). After mice were sacrificed on day 25, their liver, lung, kidney, spleen, brain and heart were

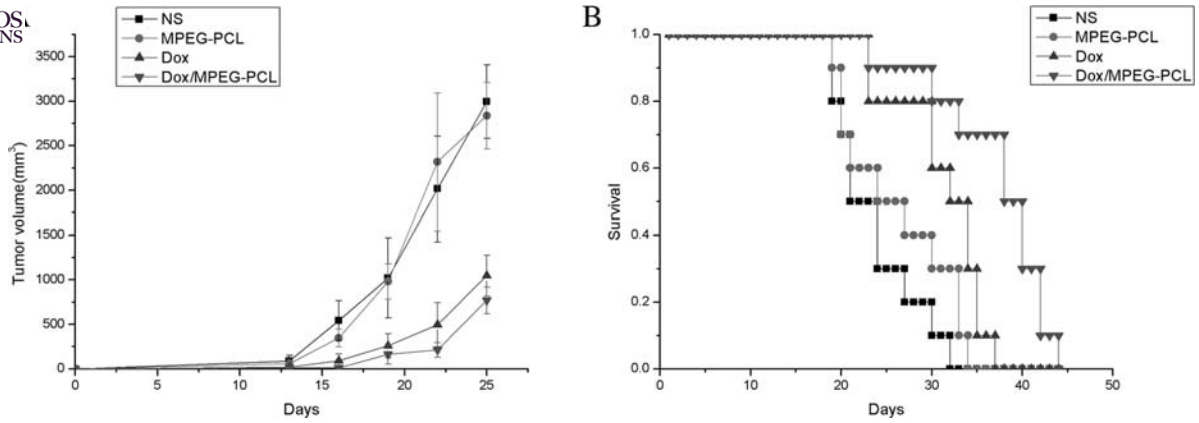

Figure 4. Tumor suppression and survival advantage in mice. (A) Dox/MPEG-PCL significantly inhibited B16-F10 tumor growth. After tumor inoculation, mice were randomly separated and administered with NS, MPEG-PCL, doxorubicin (5 mg/kg), and Dox/MPEG-PCL (5 mg/kg) respectively. Seven days after the last treatment, mice were sacrificed. Values are means ± SD; (*P<0.05) (B) Kaplan-Meier survival curve for tumor-bearing mice treated with NS, MPEG-PCL, doxorubicin (5 mg/kg), and Dox/MPEG-PCL (5 mg/kg). Survival was significantly longer in Dox/MPEG-PCL-treated mice compared with control groups (log-rank test, *P<0.05) and there was no statistical difference among control groups (P>0.05).

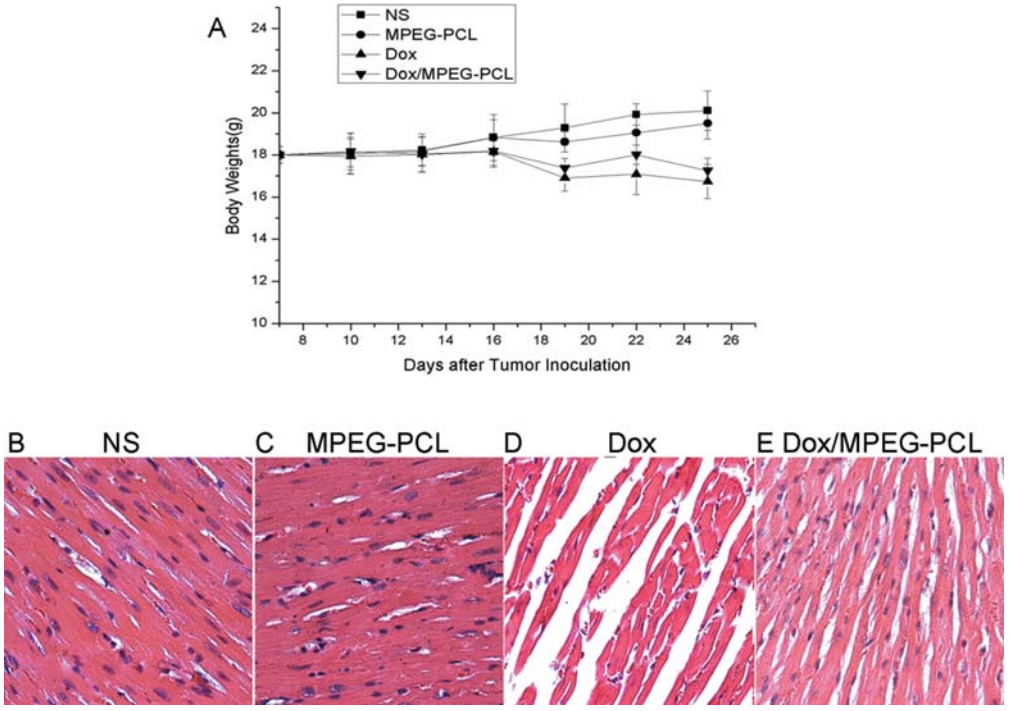

Figure 5. Systemic toxicity of MPEG-PCL encapsulated doxorubicin (Dox/MPEG-PCL) in C57BL/6 mice. (A) Mean body weights on days 7, 10, 13, 16, 19, 22 and 25 of mice treated with three doses of free doxorubicin (Free Dox, 10 mg/kg), Dox/MPEG-PCL (10 mg/ kg), empty MPEG-PCL micelles as control (10 mg/kg) and NS; error bars correspond to 95% confidence intervals. Values are means ± SD (n=10 mice per group). (B-E) Section of cardiac tissue obtained from C57BL/6 mice without tumors which received a single intravenous dose of Dox/MPEG-PCL (10 mg/kg) (E); free doxorubicin (5 mg/kg) (D), empty MPEG-PCL micelles (5 mg/kg) (C) or NS. Hearts were harvested at day 25 after intravenous injection.

harvested and H&E histological staining was performed. As observed by two pathologists in a blinded manner, we found that free doxorubicin caused severe histopathologic lesions that manifested as myocarditis. The muscle fibers showed varying degrees of damage ranging from loss of striation to complete fragmentation (Fig. 5D). However, mice treated with Dox/MPEG-PCL showed only slight changes of toxicity to muscle fibers in cardiac tissues (Fig. 5E), compared with the normal muscle fibers from mice receiving empty micelles (Fig. 5C). In addition, no conspicuous adverse effects in gross measures were observed, such as loss of appetite, feeding, ruffling of fur, or behavior change.

Improved inhibition of intratumoral angiogenesis in the Dox/MPEG-PCL-treated mice. One determinant of tumor growth is the ability of a nascent tumor to stimulate angiogenesis. Due to a growing body of evidence indicating an association between targeted doxorubicin and tumor angiogenesis (19), MVD was evaluated in the tumors harvested. We examined the tumor tissues with anti-CD31 and the most

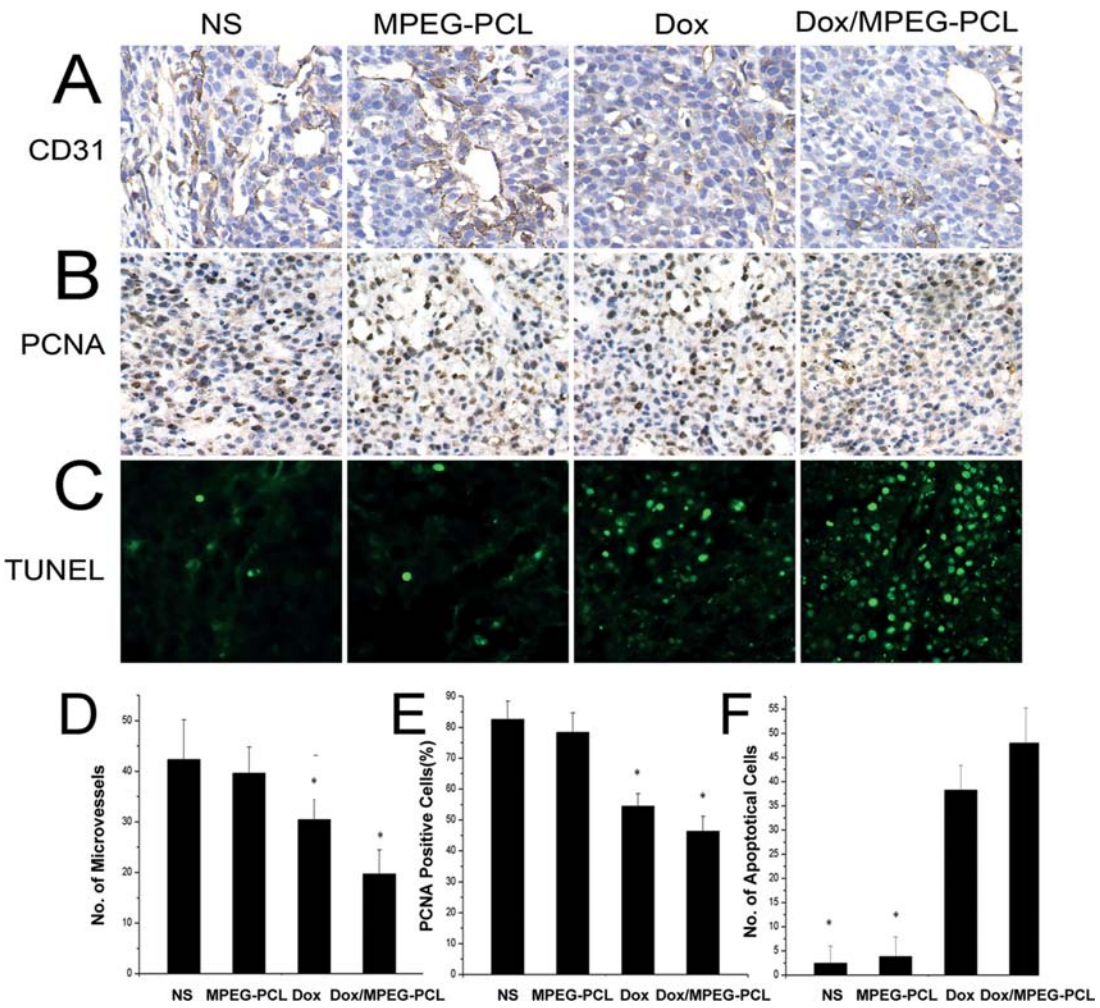

Figure 6. Effect of Dox/MPEG-PCL on cell proliferation, angiogenesis and apoptosis *in vivo*. (A) Angiogenesis within tumors was detected by CD31 staining of microvessels. (B) PCNA-positive cells were rich in NS and MPEG-PCL groups. (Magnification, x400); (C) Induction of apoptosis was indicated by TUNEL assay. (D) Quantification of vessels. The average number of microvessels per vascular hot spot was significantly decreased in Dox/MPEG-PCL-treated tissues compared with those in the three control groups (*P<0.05). (E) Quantification of PCNA staining. Whereas, Dox and Dox/MPEG-PCL groups tremendously decreased the positive expression. Percentages of PCNA-positive muclei in Dox/MPEG-PCL group was significantly lower than that in NS or MPEG-PCL group (*P<0.05). (F) Quantification of TUNEL staining. TUNEL-positive nuclei were significantly increased in Dox/MPEG-PCL-treated tissues compared with those in the control groups treated with NS, MPEG-PCL or doxorubicin (*P<0.05).

highly vascularized area of each tumor was identified and three high-powered fields were counted in this area for MVD. As shown in Fig. 6A, the most significant reduction of MVD was observed in the group treated with Dox/MPEG-PCL (19.7±4.8) compared with doxorubicin (30.4±4.1), MPEG-PCL (39.6.8±5.2) and NS treatments (42.3±7.9; P<0.05). No significant difference was found between MPEG-PCL group and NS group (P>0.05) (Fig. 6D). Thus, it may be hypothesized that the antitumoral effects of Dox/MPEG-PCL could be contributed to its ability of inhibit intratumoral angiogenesis.

Improved inhibition of cell proliferation in the Dox/MPEG-PCL-treated mice. To investigate potential mechanisms underlying the efficacy of Dox/MPEG-PCL-based therapy *in vivo*, we examined its effects on tumor cell proliferation using PCNA staining. The number of cancer cell nuclei that were strongly PCNA positive was counted as a ratio of immunoreactive positive cells to the total number of cells counted. Dramatical reduction of PCNA expression was noted in Dox/MPEG-PCL-treated group compared with free Dox, MPEG-PCL and NS groups (P<0.05, Fig. 6B). Our data showed the percentage of PCNA-positive cells reached 82.5±5.9, 78.3±6.3 and 54.4±4.1% in NS, MPEG-PCL and doxorubicin groups, respectively; whereas corresponding values in Dox/MPEG-PCL-treated group reached only 46.3±4.8% (Fig. 6E). Thus, Dox/MPEG-PCL led to decreased proliferation compared with controls *in vivo*.

Increased promotion of tumor apoptosis in the Dox/MPEG-PCL-treated mice. As previously reported, tumor growth is often considered as a balance between apoptosis and proliferation. Thereby, we applied the TUNEL assay to detect apoptosis of tumor cells within tumor tissue. Cell nuclei stained with dark green, as viewed by fluorescence microcopy (x400), indicated apoptosis, and were recorded as TUNEL-positive nuclei. The number of cells were counted in 3 random fields, avoiding areas of necrosis. As shown in Fig. 6C, significant increases of TUNEL-positive nuclei were found in Dox/MPEG-PCL-treated group compared with the two

 P<0.05) (Fig. 6F). These results suggested that the ation of Dox/MPEG-PCL could effectively trigger tumor cell apoptosis *in vivo*.

Discussion

Nanoparticles, are defined as microscopic particles with at least one dimension less than 100 nm. Nanotechnology is currently becoming the focus of biomedical field serving as a bridge between bulk materials and atomic or molecular structures. Numerous nano-sized drug carriers, such as polymer-drug conjugates, stealth liposome and microspheres have been investigated in order to enhance anticancer activity and minimize side effects of anticancer therapy (20-24). Polymeric carriers bearing physically entrapped or chemically conjugated drugs are an attractive strategy for improving the efficiency of tumor targeting. A portion of studies focused on the positive targeting of chemotherapy drugs into the tumor site and thus many researchers sought to develop conjugates bearing tumor-specific antibodies or peptides (25,26). More recent studies have reported that polymer-conjugated drugs and nanoparticulates showed prolonged circulation in the blood and can accumulate passively in tumor even in the absence of targeting ligands, suggesting the existence of a passive retention mechanism (25). In recent years, many polymer-conjugated drugs and polymer nano-particles have been explored with some success in maintaining or improving the anti-tumor activity and decreasing the systematic toxicity. But the large-scale synthesis of such conjugates is rather complicated and expensive. This is why we are attempting to develop a simple carrier system enabling tumour-specific drug delivery.

Doxorubicin is a promising antitumor drug but its clinical use was largely limited by its poor penetration and the systematic toxicity especially cardiac toxicity. In this research, we selected MPEG-PCL, an amphiphilic micelle which is biodegradable and biocompatible, as the nano-vector for doxorubicin. The Dox/MPEG-PCL micelle was readily prepared by a self-assembly method without using vigorous stirring, any unwanted organic solvents or surfactants. The monodisperse micelle had a small size and superior re-solubility after freeze drying. This feature can largely improve the selectivity of the drug in tumor tissue. Generally, the blood vessels in tumor tissues have large gaps (100-800 nm) between adjacent endothelial cells, and the capillary permeability of the endothelium in newly vascularized tumors are signi-ficantly wider than those of normal organs (27-29). Due to the defective vascular architecture and poor lymphatic drainage, nanoparticles can extravasate through these gaps into extravascular spaces and accumulate inside tumor tissues (26,27). This phenomenon is known as the enhanced permeability and retention (EPR) effect (28,30), which has been generally observed in many types of solid tumors. Since the prepared MPEG-PCL is small enough (~20 nm), it can increase the selectivity of the available cytotoxic agents by delivering them specifically to tumor tissue, which allows maximal accumulation and the deepest penetration into tumors. It is also interesting that the release of doxorubicin from MPEG-PCL was pH-dependent (fast release in pH 5.5, and slow release in pH 7.0). As known, in the blood circulation, the pH is neutral (pH 7.4) while the environment inside the tumor is much lower. Hence, the release of Dox/MPEG-PCL is triggered faster into the tumor site than in normal tissue which passively accumulated and consequently resulted in a local high dose of drug in the tested solid tumor. This characteristic was also observed by others (16,22). Therefore, MPEG-PCL is an excellent drug carrier which can provide controlled and targeted delivery of doxorubicin with better efficacy and fewer side-effects.

In the present study, Dox/MPEG-PCL micelles were administered in C57BL/6 mice bearing subcutaneous melanoma. MTT and flow cytometry indicated that Dox/MPEG-PCL showed superior cytotoxicity and cellular uptake. On the basis of *in vitro* studies, we applied the Dox/MPEG-PCL on a mouse melanoma model. As expected, administration of doxorubicin could only partially retard the tumor growth to some extent whereas the Dox/MPEG-PCL nanoparticles showed efficient inhibition of advanced tumor growth and prolonged the survival of the treated mice, without bringing about any gross toxicity of animals. These results may be induced by the triggered release of Dox by tumor extracellular pH (pH <7.0) after accumulation of Dox/MPEG-PCL micelle in the tumor sites via the EPR effect, and doxorubicin would be released faster into the tumor site compared with normal tissue. The property may present a more effective modality in tumor chemotherapy, providing higher local concentrations of the drug at tumor sites (targeted high-dose cancer therapy). Thus, the enhanced antitumor effect of Dox/MPEG-PCL might be due to the combination effects of the enhanced cytotoxicity, passive targeting doxorubicin to tumor tissues induced by EPR effect, and pH-dependent release profile.

The profound anti-tumor activity of Dox/MPEG-PCL *in vivo* guided us to perform immunohistochemistry staining with PCNA, CD31 and TUNEL staining of Dox/MPEG-PCL-treated tumor tissue to elucidate the potential anti-tumor mechanism *in vivo*. Our result showed that the MVD and PCNA-positive cells were significantly decreased in Dox/MPEG-PCL group, in comparison with those control with NS, MPEG-PCL or free doxorubicin (P<0.05), suggesting that the encapsulated micelles contribute partially to the inhibition of tumor angiogenesis and cell proliferation. TUNEL assay was also conducted in tumor tissue *in vivo* and proved increased apoptosis induction in the Dox/MPEG-PCL group when compared with other groups. Our findings were in line with the apoptosis promotion ability of doxorubicin reported previously (31). These results strongly indicate that the formulation of Dox/MPEG-PCL nanoparticles may be a valid and safe approach in cancer treatment.

In addition, we investigated the side effects of the new formulation. An optimized biodistribution of nanoparticles may lead to improved drug efficacy and, at the same time, reduced side effects. To investigate the systematic toxicity, we performed a toxicity test in tumor-free mice. As demonstrated by the study, the relative body weight among these groups fluctuated slightly but no significant difference was observed with the exception of the two mice treated with free doxorubicin that died before the planned endpoint. Besides, the pattern of the morphological changes of the internal organs exhibited significant difference. The heart tissue in mice

treated with free doxorubicin exhibited typical damage of focal destruction of myofibrils; however, the mice treated with Dox/MPEG-PCL suffered from moderate myocardial edema. Consistent with our current study, recent studies have shown encouraging results using other animal models, e.g. Pereverzeva *et al* evaluating the intravenous tolerance of nanoparticle-based formulation of doxorubicin in healthy rats and pointed out that the employment of the nanoparticles exhibits a more favorable safety profile (32). Furthermore, Tang *et al* used the HPLC to evaluate the tissue distribution of the free doxorubincin and micelle-encapsulated doxorubicin (M-Dox) and concluded that the mean doxorubicin concentrations 12 h after injection of doxorubicin in heart, liver, lung, spleen, and kidney of mice treated with M-Dox (10 mg/kg) are much lower than free doxorubicin (10 mg/kg) (33).

The administration of Dox/MPEG-PCL nanoparticles leads to significant inhibition of malignant melanoma cell growth *in vitro* and *in vivo* with low systemic toxicity. Our data suggest that administration of Dox/MPEG-PCL micelles strongly affects the proliferation, apoptosis and angiogenesis of malignant melanoma. Given its promising anti-tumor effect and minimal toxicity, Dox/MPEG-PCL micelles could serve as a potentially novel therapeutic agent in the treatment of tumors.

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