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ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 527–534
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

The antitumor effect of novel docetaxel-loaded thermosensitive micelles

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Received 11 February 2007; accepted in revised form 4 January 2008
Available online 26 January 2008

Abstract

To further evaluate the novel docetaxel-loaded micelle based on the biodegradable thermosensitive copolymer poly(*N*-isopropylacrylamide-co-acrylamide)-*b*-poly(DL-lactide) that we had synthesized before, in this paper, we studied its in vitro cytotoxicity in three different tumor cell lines by standard MTT assays using different tumor cell lines, followed by studies of acute toxicity and the tumor distribution studies which were conducted in Kunming mice. Meanwhile, the in vivo antitumor efficacy as well as toxicity of the micelle was evaluated in C57BL/6 mice. According to our findings, the in vitro cytotoxicity of docetaxel-loaded micelles was lower than that of the conventional docetaxel formulation at 37 °C, while hyperthermia greatly enhanced the efficacy of drug-loaded micelles. The acute toxicity study showed reduced toxicity of docetaxel-loaded micelle compared to that of conventional docetaxel formulation. Moreover, docetaxel-loaded micelle enabled a prominent higher docetaxel concentration in tumor than conventional docetaxel formulation. Furthermore, a significantly higher antitumor efficacy was observed in mice treated with docetaxel-loaded micelles accompanied by hyperthermia; docetaxel-loaded micelles also caused less body weight loss of mice. This study demonstrates an increased antitumor efficacy and reduced toxicity of the novel docetaxel-loaded micelle and indicates its prospect of clinical applications.

Keywords: Docetaxel; Targeted drug delivery; Hyperthermia; Thermosensitive micelle; In vivo

1. Introduction

Chemotherapy is a standard of care in clinical oncological practice, but its efficacy is usually limited due to the inadequate concentration of drug at the tumor tissue and/or the toxicity effects on normal tissues. Poor solubility in water is also a handicap in bringing many anticancer drugs into optimal clinical use [1]. Docetaxel is a case in point. Docetaxel and its analog paclitaxel, which were developed in the past two decades, represent a new catalog of chemotherapeutic agents with high potency against many solid tumors including breast cancer, non-small cell lung cancer and gastric cancer, etc. [2–5]. However, side effects caused by docetaxel have considerably overshadowed its clinical use. Firstly, like most other classic chemotherapeutic agents, docetaxel distributes throughout the body in a nonspecific manner; secondly, as docetaxel is poorly soluble in water, non-ionic surfactant Tween 80 (polysorbate 80) and ethanol (50:50, v/v) were used to dissolve the agent in its current commercial formulation. The use of docetaxel in the present formulation leads to the well-known adverse drug reactions due to either the agent itself (e.g., fluid retention, neurotoxicity, musculoskeletal toxicity and neutropenia) or to the solvent system (e.g., hypersensitivity, fluid retention) [6]. Development of new

Abbreviation: LCST, low critical solution temperature; LD_{50}, median lethal dose; LLC, Lewis lung carcinoma; poly(IPAAm-co-AAm)-*b*-PDLLA, poly(*N*-isopropylacrylamide-co-acrylamide)-*b*-poly(DL-lactide); RES, reticuloendothelial system.

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doi:10.1016/j.ejpb.2008.01.015

approaches are required to make the chemotherapeutics achieve adequate local concentration at the tumor site and, at the same time, reduce side effects caused by nonspecific delivery.

In recent years, targeted drug delivery systems that deliver drugs specifically to a desired site have been developed [7–10]. Targeted drug delivery systems increase the efficacy of the therapeutic agents and in turn may slow down the development of cellular drug resistance which is induced partially by exposure to sub-lethal doses of chemotherapeutics [11]. One of these systems, the thermosensitive drug delivery system has drawn more and more attention [12–18]. The thermosensitive drug carrier undergoes a structural transition as a response of temperature increase, resulting in the deposition of the drug and easier drug absorption by cells. With regard to the thermal targeting strategy, the low critical solution temperature (LCST) is the most important parameter. Below the LCST, the thermosensitive polymer is well soluble in water but at a temperature above the LCST, it will become hydrophobic and then aggregate and precipitate [19]. However, the LCST of most reported thermosensitive micelles was below physiological body temperature (37 °C). As a result, the temperature of clinical hyperthermia (above 40 °C) cannot be used as targeting trigger because these micelles will become hydrophobic and deposit as soon as they are injected into human body. In order to solve this problem, the novel copolymer, poly(*N*-isopropylacrylamide-co-acrylamide)-*b*-poly(DL-lactide), [poly(IPAAm-co-AAm)-*b*-PDLLA], with the LCST of 41 °C has been synthesized and used as the carrier for delivery of docetaxel in a series of our studies [21]. The drug-loaded micelle and its synthesis process have been authorized with the national invention patents of P.R.C. (Patent NO: 200510040570.4; 200510040571.9).

In this paper, we compared the cytotoxicity of the docetaxel-loaded micelle with conventional docetaxel formulation as a control formulation in 3 widely used cancer cell lines and the antitumor efficacy in Lewis lung carcinoma (LLC)-bearing C57BL/6 mice. The tumor distribution in the S180-bearing mice and the acute toxicity in tumor-free mice were also evaluated in this study.

2. Materials and methods

2.1. Materials

Conventional docetaxel formulation (Docetaxel Injection, molecular weight: 807.88) was kindly provided by *Hengrui* Pharmaceutical Co. LTD., Jiangsu, China. Poly(*N*-isopropylacrylamide-co-acrylamide)-*b*-poly(DL-lactide) was prepared by our laboratory. RPMI 1640 (Gibco, NY, USA), Endothelial Cell Medium (EGM) (Clonetics, CA, USA), fetal bovine serum (Amersco, SF, USA), calf blood serum (Amersco), dimethylthiazoly-2,5-diphenyltetrazolium bromide (MTT) (Amersco) were used as received. Acetonitrile (Merck, Germany) was of chromatogram grade. All the other chemicals were of analytical grade and were used without further purification.

Human hepatocellular carcinoma cell line SMMC-7721, human gastric carcinoma cell line BGC823 and murine Lewis lung carcinoma (LLC) cell line were obtained from Shanghai Institute of Cell Biology (Shanghai, China).

Kunming (KM) mice were purchased from Central Animal Laboratory of China Pharmaceutical University (Jiangsu, China). SPF C57BL/6 mice were provided by Model Animal Research Center of Nanjing University (Jiangsu, China).

2.2. Preparation and properties of docetaxel-loaded micelles

Docetaxel-loaded micelles were prepared according to the method we have reported [21]. Briefly, 20 mg of docetaxel and 50 mg of poly(IPAAm-co-AAm)-*b*-PDLLA prepared by ring-opening polymerization were dissolved in acetonitrile. After stirring for 2 h, the mixture was immediately heated to 40 °C by soaking into a water bath and then slowly cooled down to room temperature. After removing the non-entrapped docetaxel by filtration, the obtained micellar solutions were frozen and lyophilized and then stored at 4 °C.

The Mw of the copolymer was 22,600 and Mn was 9600. LCST of the copolymer was 41 °C. The sizes of the micelles were around 80 nm and temperature-dependent. The micelle size decreased a little first in the range of 40–41 °C, and then dramatically increased after temperature reached 42 °C. The docetaxel-loaded micelles showed similar sizes which also changed with temperature. The maximum loading content of docetaxel was 27.1% (w/w) and the entrapment efficiency was 57.8%.

2.3. In vitro cytotoxicity studies

For in vitro study, three different tumor cell lines BGC823, SMMC-7721 and LLC were used.

The in vitro cytotoxicity of docetaxel-loaded micelles and conventional docetaxel formulation were determined by standard MTT assays. Cells were seeded in a 96-well plate at a density of 5000 cells per well and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was 1640 medium supplemented with 10% calf blood serum and changed every other day until 80% confluence was reached. The medium was then replaced with 200 μl medium with docetaxel-loaded micelles and conventional docetaxel formulation of different concentrations. One row of 96-well plates was used as control with 200 μl of culture medium only. The cells were incubated at 43 °C for 30 min to simulate hyperthermia and then were incubated at 37 °C for 20 h. After incubation, 20 μl of 10 mg/ml MTT solution was added to each well and the plate was incubated for 4 h, allowing the viable cells to reduce the yellow MTT into dark-blue formazan crystals, which were dissolved in 200 μl of dimethyl sulphoxide (DMSO). The absorbance of individual wells was measured at 570 nm by an ELISA

reader (Huadong, DG-5031, Nanjing). Cell viability was determined by the following formula:

$$\text{cell viability } (\%) = \frac{\text{Abs test cells}}{\text{Abs reference cells}} \times 100\%$$

In vitro cytotoxicity of cells without hyperthermia was also evaluated in the same way except for incubating cells at 43 °C for 30 min after replacing the medium.

All the results obtained from both cytotoxicity assays were confirmed by repeating the experiment on three independent occasions and testing in triplicate each time.

2.4. Acute toxicity studies

The LD_{50} (the median lethal dose) values of docetaxel-loaded micelles were investigated in Kunming mice (male/female, 4–6 weeks, 18–22 g). Ten mice were used per group and single i.v. doses of 570.0, 369.0, 238.8, 154.5 and 100.0 mg/kg docetaxel-loaded micelles were used for each group with and without hyperthermia at 43 °C for 30 min. The mice were observed for 2 weeks in all groups, and the number of mice survived was recorded. The median lethal dose (LD_{50}) was determined by the Weil method using Labcat Module [22].

2.5. Tumor distribution studies

Ascites of Kunming (KM) mice implanted sarcoma S180 was diluted with saline in the ration 1:4 in volume. Then 0.2 ml dilution was subcutaneously injected into the right flank of each KM mice (female, 8 weeks, 20 ± 3 g). Tumor dimensions were measured with vernier calipers and volumes were calculated as follows:

$$\text{Tumor volume } (\text{mm}^3) = \frac{\pi}{2} \times \text{width}^2 \times \text{length}$$

When tumors were 100–400 mm^3 in size, the mice were injected intravenously through the tail vein with 20 mg/kg of conventional docetaxel formulation and 74 mg/kg docetaxel-load micelles (which is an amount equivalent to 20 mg/kg of conventional docetaxel formulation, calculated assuming a maximum loading of docetaxel into the micelles) in a volume of 20 ml/kg. Hyperthermia was performed after injection: right hind limbs of mice including tumors were immerged in water bath at 43 °C for 30 min. Three mice were sacrificed at 3, 30 min, 1, 2, 3, 4, 8, 12 and 24 h after drug administration. The animals were dissected and tumors were collected. Tumors were stored at −80 °C until analyzed for docetaxel concentration. Tumor distribution without hyperthermia at 43 °C was also studied in the same way.

2.6. Analysis of in vivo docetaxel concentration

Concentrations of docetaxel in tumors were determined by a tandem mass spectrometer equipped with a binary high-performance liquid chromatography (LC/MS) system. Briefly, tumors were extracted with four volumes of acetonitrile. Then the organic phase was separated and evaporated. The residue was dissolved in 250 μl of methanol and the solution was centrifuged at 15,000 rpm for 10 min. Clear supernatant was collected and injected into LC/MS system.

Docetaxel was identified and quantified using negative-ion electrospray mass spectrometry in multiple-reaction monitoring mode with the transition ion (806.5 > 654.4 [mass/charge], or parent > major product). Chromatographic conditions included a rapid methanol gradient (10 mM ammonium acetate; pH 8.5) starting at 20% methanol and ending at 95% methanol within 5 min. The flow rate was 250 μl/min, and the column temperature was 30 °C. The chromatographic separation of docetaxel from background noise was accomplished using an analytical column (3 μl; 2 × 100 mm; Xterra C18; Waters, Milford, Mass). The lower limit of quantitation for docetaxel in the tandem mass spectrometer was 10 ng.

2.7. In vivo antitumor efficacy

The antitumor efficacy was investigated in LLC (Lewis lung carcinoma) cells bearing C57BL/6 mice.

For in vivo implantation, LLC cells were washed in Hanks' balanced salt solution (HBSS) and injected subcutaneously at 2×10^6 cells in 0.1 ml HBSS in the right hind limb of SPF C57BL/6 mice (male and female in half, 8 weeks, 20 ± 3 g). Tumor dimensions were measured as the method described in *tumor distribution studies*. When tumors were 200–300 mm^3 in volume (7–12 days after tumor cell implantation), treatments were started and this day was designated as day 0. On day 0, mice were randomly divided into groups of 6 mice. They were injected intravenously through the tail vein with 20 mg/kg/day of conventional docetaxel formulation, 74 mg/kg/day docetaxel-load micelles on days 0, 4, 8 and then treated with or without hyperthermia. Mice injected with saline with and without hyperthermia were set up as control groups to evaluate the respective role of hyperthermia and the micelles. As to the groups with hyperthermia, after injection, the right hind limbs of mice including tumors were totally immerged in water bath at 43 °C for 30 min.

Tumor size and the body weight of the mice were measured daily. The antitumor efficacy of docetaxel-loaded micelles was compared with that of conventional docetaxel formulation with and without hyperthermia by observing tumor volume daily and measuring tumor weight after execution. The tumor inhibition rate was also used to evaluate the antitumor efficacy, which was calculated by the following formula:

$$\text{Tumor inhibition rate } (\%) = \frac{\text{tumor weight of treated group} - \text{tumor weight of saline group}}{\text{tumor weight of saline group}} \times 100(\%)$$

The in vivo toxicity of both groups was evaluated by analyzing the body weight loss (BWL) of the mice [23].

All the in vivo studies of this research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85–23, revised in 1985).

2.8. Statistical analysis

Statistical analyses of data were done using Student's t test. Differences of $P < 0.05$ were considered statistically significant.

3. Results

3.1. The cytotoxicity of docetaxel-loaded micelles in tumor cells

The concentrations required to inhibit tumor cell growth by 50% (IC_{50} values) were evaluated from the dose–response curves (Fig. 1) and are shown in Table 1.

The viabilities of three cell lines with hyperthermia (43 °C) were all lower than those without hyperthermia ($P < 0.05$). The viabilities of all cell lines in conventional docetaxel formulation group were lower at 43 °C than those at 37 °C. Docetaxel-loaded micelles showed far less cytotoxicity than conventional docetaxel formulation ($P < 0.01$) at 37 °C, but demonstrated cytotoxicity similar to that of conventional docetaxel formulation at 43 °C ($P > 0.05$).

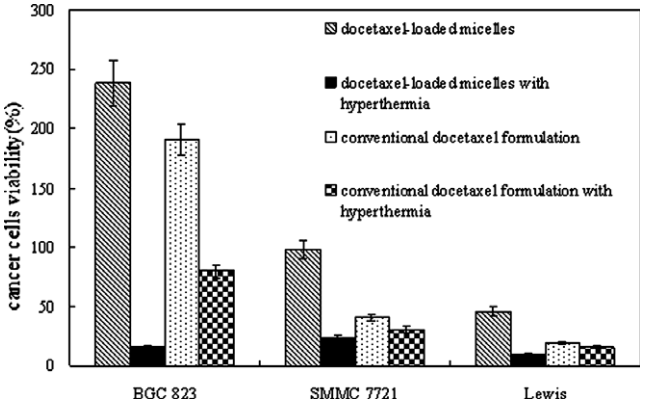

Fig. 1. Viability of different cancer cell lines treated by docetaxel-loaded micelles and conventional docetaxel formulation with and without hyperthermia. The in vitro cytotoxicity of docetaxel-loaded micelles and conventional docetaxel formulation was determined by standard MTT assays. Hyperthermia was done by incubating cells at 43 °C for 30 min after replacing the medium.

3.2. The acute toxicity of docetaxel-loaded micelles in mice

In Kunming mice, LD_{50} of docetaxel-loaded micelles was 278.07 mg/kg in room temperature (25 °C). With hyperthermia at 43 °C for 30 min, however, LD_{50} of docetaxel-loaded micelles increased to 330.94 mg/kg. They were both higher than the LD_{50} of conventional docetaxel formulation which was reported as 138 mg/kg.

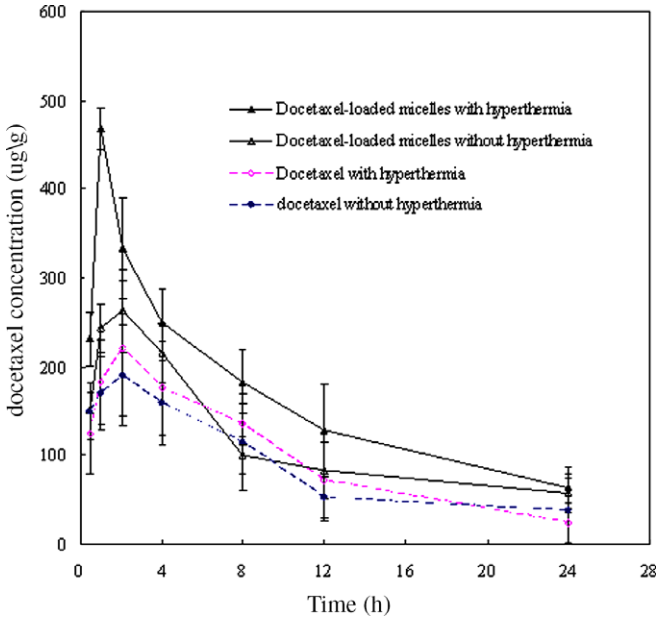

Fig. 2. Docetaxel concentration curve in tumors of S180-bearing mice treated with docetaxel-loaded micelles and conventional docetaxel formulation with and without hyperthermia. Xenograft experiments were done as described in Section 2. The start day of treatment was marked as day 0. The mice were injected intravenously through the tail vein with 20 mg/kg of conventional docetaxel formulation and 74 mg/kg docetaxel-load micelles (containing docetaxel 20 mg/kg) in a volume of 20 ml/kg. Hyperthermia was performed after injection, right hind limbs of mice including tumors were immerged in water bath at 43 °C for 30 min. Three mice were sacrificed at 3, 30 min, 1, 2, 3, 4, 8, 12 and 24 h after drug administration. The animals were dissected and tumors were collected. Tumors were stored at −80 °C until analyzed for docetaxel concentration. Tumor distribution without hyperthermia at 43 °C was also studied in the same way.

Table 1
IC_{50} (μg/ml) of different cell lines treated with docetaxel-loaded micelles and conventional docetaxel formulation with and without hyperthermia

	BGC823	SMMC-7721	LLC
Docetaxel-loaded micelles	238.2	97.9	45.8
Docetaxel-loaded micelles + hyperthermia[a]	15.3	23.7	10.1
Conventional docetaxel formulation	190.9	40.6	19.5
Conventional docetaxel formulation + hyperthermia[a]	79.9	30.7	15.3

[a] Incubating cells at 43 °C for 30 min after replacing the medium.

3.3. The docetaxel concentration in tumor after treatment of docetaxel-loaded micelles

The highest docetaxel concentration was found at 1 h in the group that received i.v. administrated docetaxel-loaded micelles with hyperthermia at 43 °C for 30 min. The other three groups (docetaxel-loaded micelles without hyperthermia group, conventional docetaxel formulation group and conventional docetaxel formulation with hyperthermia group) all presented the highest docetaxel concentration at 2 h after treatment. The maximum concentration was 470 mg/g for the group that received i.v. administrated docetaxel-loaded micelles with hyperthermia, higher than the other three groups.

3.4. The in vivo antitumor efficacy of docetaxel-loaded micelles and the toxicity

As shown in Figs. 3, 4 and Table 2, conventional docetaxel formulation inhibited tumor growth both with and without hyperthermia and the difference was not significant (53.0% vs. 66.7%, $P > 0.05$). The antitumor efficacy that was observed in all mice that received docetaxel-loaded micelles with hyperthermia was greater than that of conventional docetaxel formulation (82.1% vs. 53.0%, $P < 0.05$), and also than that of docetaxel-loaded micelles without hyperthermia (82.1% vs. 41.2%, $P < 0.05$). On the other hand, the weights of mice given docetaxel-loaded micelles did not decrease prominently while that of mice given conventional docetaxel formulation showed obvious loss of body weight (Fig. 4).

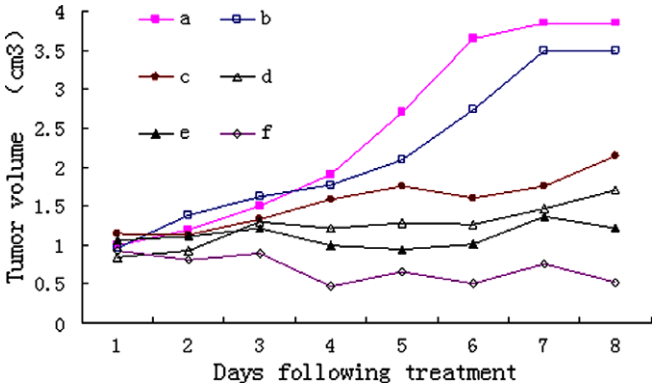

Fig. 3. In vivo antitumor efficacy of conventional docetaxel formulation and docetaxel-loaded micelles with and without hyperthermia in LLC-bearing C57BL/6 mice. Xenograft experiments were done as described in Section 2. The start day of treatment was marked as day 0. The mice were treated with 20 mg/kg/day of conventional docetaxel formulation, 74 mg/kg/day docetaxel-load micelles on days 0, 4, 8 with or without hyperthermia, respectively. Mice injected with saline with and without hyperthermia were set up as control groups. As to the groups with hyperthermia, after inject, right hind limbs of mice including tumors were totally immerged in water bath at 43 °C for 30 min. We measured the tumor volume of each mouse daily after the treatment. (a) Saline, (b) hyperthermia, (c) docetaxel-loaded micelles, (d) conventional docetaxel formulation with hyperthermia, (e) conventional docetaxel formulation, (f) docetaxel-loaded micelles with hyperthermia.

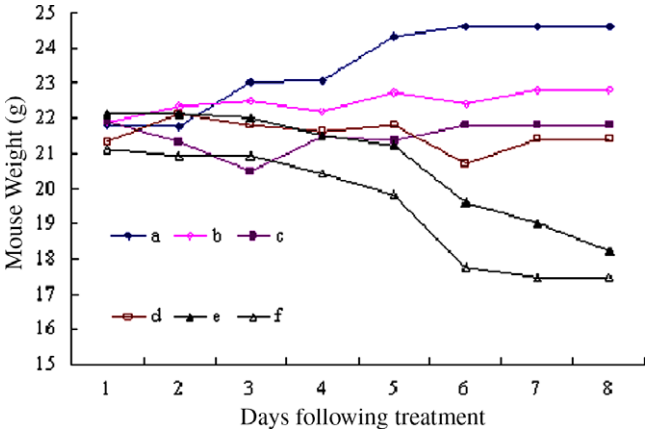

Fig. 4. Weight changes observed in LLC-bearing C57BL/6 mice treated with different modalities. Xenograft experiments were done as described in Section 2. The start day of treatment was marked as day 0. The mice were treated with 20 mg/kg/day of conventional docetaxel formulation, 74 mg/kg/day docetaxel-load micelles on days 0, 4, 8 with or without hyperthermia, respectively. Mice injected with saline with and without hyperthermia were set up as control groups. As to the groups with hyperthermia, after inject, right hind limbs of mice including tumors were totally immerged in water bath at 43 °C for 30 min. We measured the body weight of each mouse daily after the treatment. (a) Saline, (b) hyperthermia, (c) docetaxel-loaded micelles, (d) docetaxel-loaded micelles with hyperthermia, (e) conventional docetaxel formulation, (f) conventional docetaxel formulation with hyperthermia.

Table 2
Tumor weight and tumor inhibition rate observed in LLC-bearing C57BL/6 mice treated with saline, conventional docetaxel formulation, docetaxel-loaded micelles with and without hyperthermia

	Tumor weight (g)	Tumor inhibition rate (%)
Saline	3.9 ± 0.8	–
Saline with hyperthermia	3.7 ± 0.9	9.0 ± 10.0
Conventional docetaxel formulation	1.3 ± 0.3	66.7 ± 0.4
Conventional docetaxel formulation with hyperthermia	1.9 ± 0.7	53.0 ± 12.5
Docetaxel-loaded micelles	2.3 ± 0.9	41.2 ± 12.4
Docetaxel-loaded micelles with hyperthermia	0.7 ± 0.2	82.1 ± 0.4

The data are presented as means ± SD.
As to the groups with hyperthermia, after inject, right hind limbs of mice including tumors were totally immerged in water bath at 43 °C for 30 min.

4. Discussion

In recent years, targeted delivery strategies such as antibody-[24], receptor-[25], magnetic-[26], enzyme-[27] PH-[28] and thermo-targeting have gained more and more popularity. With the development of hyperthermia instruments and increasing clinical study of hyperthermia in the treatment of solid malignant tumors [29–32], the thermo-targeting has become a research interest [12–18].

In the present study, we evaluated the antitumor effect of the novel thermosensitive micelle with conventional docetaxel formulation. The prominent result was obtained

due to the targeted delivery of docetaxel and synergistic effects of chemotherapy and hyperthermia. At human physiological temperature (37 °C) which is below the LCST, the drug carrier will self-assemble to compose a hydrated outer shell formed by thermosensitive copolymer and a hydrophobic inner core formed by PDLLA. It is also capable of evading from nonselective reticuloendothelial system (RES) scavenging and acquires longer circulatory time due to its core-shell structure and its small size approximately at 80 nm in diameter. Moreover, the micelle presented a sustained release pattern. It is also a very important characteristic, which together with longer circulatory time leads to better effect and less toxicity to normal tissues. Once circulating to malignant tissue where local temperature is above the LCST after local hyperthermia, the outer shells of these micelles will become hydrophobic and the drugs inside can be absorbed into cells via hydrophobic interaction. The anticancer drugs will accumulate in cancer cells and reach a level high enough to kill them [21]. According to the comprehensive reviewing by Hildebrandt et al. [29], hyperthermia also enhances drug accumulation at the tumor tissue by increasing tumor blood flow and tumor microvascular permeability preferentially compared to normal tissue [18,19]. Besides, hyperthermia itself exerts certain antitumor effects on tumors by different mechanisms including affecting fluidity and stability of cellular membranes, inhibiting DNA-repair enzymes, etc. Furthermore, hyperthermia can increase the cytotoxicity of various antineoplastic agents including docetaxel by thermal chemosensitization [20].

According to the in vitro study, viabilities of the three tumor cell lines treated with hyperthermia (43 °C) were all lower than those without hyperthermia, which was observed both in the micelle group and the conventional docetaxel formulation group. The result suggests that hyperthermia itself has certain cytotoxicity to tumors and presented synergistic effect with docetaxel [21]. However, this synergistic effect was more prominent in the group of docetaxel-loaded micelles. After heating at 43 °C for 30 min, the cytotoxicity of docetaxel-loaded micelles increased more prominently than that of the conventional docetaxel formulation. When compared the cytotoxicity increase against SMMC-7721 cell line (measured by the decrease of IC_{50}) in the conventional docetaxel formulation group and docetaxel-loaded micelle group, the cytotoxicity increased by 32.2% in the conventional docetaxel formulation group but by 3.13-folds in the docetaxel-loaded micelle group. Similarly, as to the LLC and BGC823 cell lines, the cytotoxicity increased by 27.5% and 138.9% in the conventional docetaxel formulation group but by 3.53-folds and 14.57-folds in the docetaxel-loaded micelle group, respectively. It is suggested that docetaxel-loaded micelles could present targeted cytotoxicity after being heated at 43 °C. At 37 °C, the toxicity of docetaxel-loaded micelles to all cell lines was lower than that of the conventional docetaxel formulation. This result is consistent with our previous finding that poly(IPAAm-co-AAm)-*b*-PDLLA showed very low toxicity to HUVEC [21]. We can prospect less impairment of the micelles to tissues at human physiological temperature.

To be most effective, anticancer drugs should reach the tumor tissue in a concentration sufficient to exert the therapeutic effect. A lot of anticancer drugs have limited distribution from blood vessels to solid tumors, which limits their effectiveness [33]. Therefore, a tumor distribution study was conducted to better evaluate the effectiveness of the micelle. Fig. 2 shows the docetaxel concentration in tumors at various time intervals after administrations of conventional docetaxel formulation and docetaxel-loaded micelles. After hyperthermia, the concentration of docetaxel in tumors increased in both of the micelle groups and the conventional formulation groups because hyperthermia itself can increase local drug concentration [29]. However, this phenomenon was more evident in the micelle group than in the conventional formulation group. Probably, in the docetaxel-loaded micelle group, hyperthermia had also enhanced drug distribution via other mechanisms, e.g., the micelles will aggregate because of the absence of hydrophobic shell in micelles after hyperthermia and according to our previous study, docetaxel entrapped by micelles would be internalized to tumor cells easier after hyperthermia [21]. The group of docetaxel-loaded micelles with hyperthermia exhibited a prominent higher docetaxel concentration in tumor than the other three groups at all the tested time points, which may be responsible for the preferable antitumor effect of this novel micelle in vivo. Furthermore, docetaxel entrapped in the micelle would distribute to tumor more specifically, so that the drug concentration in other organs would be lowered, alleviating the side effects of docetaxel.

Study conducted in vivo to evaluate the antitumor efficacy has indicated that the conventional docetaxel formulation delayed tumor growth both with and without hyperthermia and the difference was not significant. Docetaxel-loaded micelles without hyperthermia had lower in vivo antitumor efficacy compared to conventional docetaxel formulation due to the entrapment of the docetaxel into the hydrophilic micelles when the temperature is below LCST. However, as the temperature increased above LCST, a striking antitumor response was observed in all mice that were treated with docetaxel-loaded micelles accompanied by hyperthermia, significantly higher than those treated with conventional docetaxel formulation (no matter with or without hyperthermia). This result implied the targeted drug delivery triggered by temperature transition from below to above the LCST. These findings are consistent with the results obtained in the in vitro toxicity study, the tumor distribution study as well as our previous findings [21].

The acute toxicity study and BWL observation were conducted to evaluate the toxicity of the micelle. The acute toxicity study showed the LD_{50} of docetaxel-loaded micelles with hyperthermia was higher than that of those without hyperthermia and both of them were higher than

the LD_{50} of conventional docetaxel formulation. The docetaxel-loaded micelle was hence supposed to be less toxic than the conventional formulation. In the study of the BWL, compared to the control group, all mice in the other groups showed reduced body weight. The BWL presented a trend as: conventional docetaxel formulation + hyperthermia > conventional docetaxel formulation > docetaxel-loaded micelles + hyperthermia > docetaxel-loaded micelles > hyperthermia > control (Fig. 4). At 37 °C, the reduced toxicity of the docetaxel-loaded micelles was due to the sustained release pattern of the micelles observed in our previous study [25]. After hyperthermia, the toxicity observed in the docetaxel-loaded micelle group was significantly lower than that in the conventional docetaxel formulation group because of the local rather than systemic increased uptake of the drug. The results above also suggested that hyperthermia itself exerted toxicity on mice because hyperthermia limits weight gain by the mice independently [34]. Hence it should be noticed that the BWL observed in the docetaxel-loaded micelle group with hyperthermia was caused by synergistic toxicity of hyperthermia and the heated docetaxel-loaded micelles. Nevertheless, it was still lower than the BWL in conventional docetaxel formulation group without hyperthermia. The results clearly indicated limited toxicity and preferable tolerability of reported drug delivery system here.

As to the delivery of taxanes, most of the studies concentrate on the delivery of paclitaxel. Among strategies for delivering docetaxel, fibrinogen-coated droplets of Olive Oil [35,36] cannot be delivered intravenously because of the μm scale size, which limits their applications. Recently, lipid nanocapsules [37] and liposomes [38] have been reported to deliver docetaxel but the present work focused on the synthesis of drug carrier, the drug loading and biodistribution of docetaxel. No data on the in vitro or in vivo antitumor effect are revealed in previously published works. For the targeting strategies, Chilkoti et al. [14] reported a thermosensitive copolymer with a LCST above 37 °C, however, the copolymer presented a linear structure in water, and a copolymer–drug conjugation was needed for drug delivery. Apparently, the formation of chemical conjugation requires a certain chemical group on the drug for reaction. As a result, only a limited number of drugs can be delivered in that way. Although prodrug protocol is another research focus to deliver taxanes targetedly [39], these prodrug strategies need formation of special chemical bonds. Therefore, they cannot be applied into other kinds of chemotherapeutic agents. In contrast, our micelle delivering drugs without any changes of their original structure allows a wider spectrum of drugs to be delivered.

Docetaxel has presented high potency against many solid tumors. However, in our study, only limited types of cell lines were used for in vitro evaluation and their hyperthermia response varies among cell lines. As to in vivo study, only the mice bearing LLC were used for in vivo evaluation. Therefore, more studies on other cell lines and animal models are needed to evaluate the efficacy of this novel docetaxel formulation. In addition, to comprehensively evaluate the micelle, a series of succeeding studies such as long-term toxicity, systemic distribution, pharmacokinetics, etc. will be part of our future work.

On the basis of the results obtained in the study, we propose here that the novel docetaxel-loaded micelles improved antitumor efficacy in combination with hyperthermia, more significantly than the conventional docetaxel formulation both in vitro and in vivo. This drug delivery system also attenuated the side effects when used in vivo. As the local heating process can be achieved using the commercialized hyperthermia instrument in clinical use, the results from these studies suggest that this micelle delivery system can be used clinically.

Acknowledgements

This work was supported by National Nature Science Foundation of China (No. 30670958) and Medical Technology Development Foundation of Nanjing.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2008.01.015.

References

[1] A.N. Lukyanov, V.P. Torchilin, Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs, Adv. Drug Deliv. Rev. 56 (2004) 1273–1289.
[2] A.T. van Oosterom, D. Schrijvers, D. Schrijvers, Docetaxel (Taxotere): a review of preclinical and clinical experience, Part II: clinical experience, Anticancer Drugs 6 (1995) 356–368.
[3] M.C. Bissery, G. Nohynek, G.J. Sanderink, F. Lavelle, Docetaxel (Taxotere): a review of preclinical and clinical experience. Part I: preclinical experience, Anticancer Drugs 6 (1995), 339–355, 363–368.
[4] J.A. Ajani, Chemotherapy for advanced gastric or gastroesophageal cancer: defining the contributions of docetaxel, Expert Opin. Pharmacother. 7 (2006) 1627–1631.
[5] E. Bouvier, S. Thirot, F. Schmidt, C. Monneret, First enzymatically activated Taxotere prodrugs designed for ADEPT and PMT, Bioorg. Med. Chem. 12 (2004) 969–977.
[6] L. van Zuylen, J. Verweij, A. Sparreboom, Role of formulation vehicles in taxane pharmacology, Invest. New Drugs 19 (2001) 125–141.
[7] K. Shao, Q. Hou, W. Duan, M.L. Go, K.P. Wong, Q.T. Li, Intracellular drug delivery by sulfatide-mediated liposomes to gliomas, J. Control. Release 115 (2006) 150–157.
[8] D.A. Groneberg, M. Giersig, T. Welte, U. Pison, Nanoparticle-based diagnosis and therapy, Curr. Drug Targets 7 (2006) 643–648.
[9] V.P. Torchilin, Lipid-core micelles for targeted drug delivery, Curr. Drug Deliv. 2 (2005) 319–327.
[10] J.K. Vasir, V. Labhasetwar, Targeted drug delivery in cancer therapy, Technol. Cancer Res. Treat. 4 (2005) 363–374.
[11] R.Y. Cheung, A.M. Rauth, P.T. Ronaldson, R. Bendayan, X.Y. Wu, In vitro toxicity to breast cancer cells of microsphere-delivered mitomycin C and its combination with doxorubicin, Eur. J. Pharm. Biopharm. 62 (2006) 321–331.

[12] J.V. Reisen, G.T. Polley, P.J. Verheijen, Structural targeting for heat integration retrofit, Appl. Therm. Eng. 18 (1998) 283–294.
[13] G. Kong, M.W. Dewhirst, Hyperthermia and liposomes, Int. J. Hyperther. 15 (1999) 345–370.
[14] A. Chilkoti, M.R. Dreher, D.E. Meyer, D. Raucher, Targeted drug delivery by thermally responsive polymers, Adv. Drug Deliv. Rev. 54 (2002) 613–630.
[15] O. Soga, C.F. van Nostrum, M. Fens, C.J. Rijcken, R.M. Schiffelers, G. Storm, Thermosensitive and biodegradable polymeric micelles for paclitaxel delivery, J. Control. Release 103 (2005) 341–353.
[16] F. Kohori, K. Sakai, T. Aoyagi, M. Yokoyama, Y. Sakurai, T. Okano, Preparation and characterization of thermally responsive block copolymer micelles comprising poly(*N*-isopropylacrylamide-*b*-DL-lactide), J. Control. Release 55 (1998) 87–98.
[17] J.E. Chung, M. Yokoyama, M. Yamato, T. Aoyagi, Y. Sakurai, T. Okano, Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(*N*-isopropylacrylamide) and poly(butylmethacrylate), , J. Control. Release 62 (1999) 115–127.
[18] M.H. Gaber, N.Z. Wu, K. Hong, S.K. Huang, M.W. Dewhirst, D. Papahadjopoulos, Thermosensitive liposomes: extravasation and release of contents in tumor microvascular networks, Int. J. Radiat. Oncol. Biol. Phys. 36 (1996) 1177–1187.
[19] H.G. Schild, Poly(*N*-isopropylacrylamide): Experiment, theory and application, Prog. Polym. Sci. 17 (1992) 163–249.
[20] F. Mohamed, O.A. Stuart, O. Glehen, M. Urano, P.H. Sugarbaker, Docetaxel and hyperthermia: factors that modify thermal enhancement, J. Surg. Oncol. 88 (2004) 14–20.
[21] M. Yang, Y.T. Ding, L.Y. Zhang, X.P. Qian, X.Q. Jiang, B.R. Liu, Novel thermosensitive polymeric micelles for docetaxel delivery, J. Biomed. Mater. Res A 81 (2007) 847–857.
[22] F.J. Paumgartten, O.A. Presgrave, M.A. Menezes, F.F. Fingola, J.C. Freitas, R.R. Carvalho, F.Q. Cunha, Comparison of five methods for the determination of lethal dose in acute toxicity studies, Braz. J. Med. Biol. Res. 22 (1989) 987–991.
[23] O.C. Farokhzad, J. Cheng, B.A. Teply, I. Sherifi, S. Jon, P.W. Kantoff, J.P. Richie, R. Langer, Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo, Proc. Natl. Acad. Sci. USA 103 (2006) 6315–6320.
[24] M. Kovár, T. Mrkvan, J. Strohalm, T. Etrych, K. Ulbrich, M. Stastný, B. Ríhová, HPMA copolymer-bound doxorubicin targeted to tumor-specific antigen of BCL1 mouse B cell leukemia, J. Control. Release 92 (2003) 315–330.
[25] T.W. Moody, L.C. Sun, S.A. Mantey, T. Pradhan, L.V. Mackey, N. Gonzales, J.A. Fuselier, D.H. Coy, R.T. Jensen, In vitro and in vivo antitumor effects of cytotoxic camptothecin-bombesin conjugates are mediated by specific interaction with cellular bombesin receptors, J. Pharmacol. Exp. Ther. 318 (2006) 1265–1272.
[26] H. Nobuto, T. Sugita, T. Kubo, S. Shimose, Y. Yasunaga, T. Murakami, M. Ochi, Evaluation of systemic chemotherapy with magnetic liposomal doxorubicin and a dipole external electromagnet, Int. J. Cancer 109 (2004) 627–635.
[27] T.L. Andresen, S.S. Jensen, K. Jorgensen, Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release, Prog. Lipid Res. 44 (2005) 68–97.
[28] K. Sakai, E.G. Smith, G.B. Webber, C. Schatz, E.J. Wanless, V. Bütün, S.P. Armes, S. Biggs, PH-responsive diblock copolymer micelles at the silica/aqueous solution interface: adsorption kinetics and equilibrium studies, J. Phys. Chem. B 110 (2006) 14744–14753.
[29] B. Hildebrandt, P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix, H. Riess, The cellular and molecular basis of hyperthermia, Crit. Rev. Oncol. Hematol. 43 (2002) 33–56.
[30] I. Takahashi, Y. Emi, S. Hasuda, Y. Kakeji, Y. Maehara, K. Sugimachi, Clinical application of hyperthermia combined with anticancer drugs for the treatment of solid tumors, Surgery 131 (2002) S78–S84.
[31] A.G. van der Heijden, G. Verhaegh, C.F. Jansen, J.A. Schalken, J.A. Witjes, Effect of hyperthermia on the cytotoxicity of 4 chemotherapeutic agents currently used for the treatment of transitional cell carcinoma of the bladder: an in vitro study, J. Urol. 173 (2005) 1375–1380.
[32] M. Takemoto, M. Kuroda, U.M. Rano, Y. Nishimura, K. Sawasaki, H. Kato, The effect of various chemotherapeutic agents given with mild hyperthermia on different types of tumours, Int. J. Hyperthermia 19 (2003) 193–203.
[33] A.I. Minchinton, I.F. Tannock, Drug penetration in solid tumours, Nat. Rev. Cancer 6 (2006) 583–592.
[34] E.D. Werts, K.M. Smith, Temporal response of murine bone marrow to local hyperthermia, Int. J. Radiat. Oncol. Biol. Phys. 10 (1984) 2315–2321.
[35] A.S. Jakate, C.M. Einhaus, A.P. DeAnglis, G.S. Retzinger, P.B. Desai, Preparation, characterization, and preliminary application of fibrinogen-coated olive oil droplets for the targeted delivery of docetaxel to solid malignancies, Cancer Res. 63 (2003) 7314–7320.
[36] C.M. Einhaus, A.C. Retzinger, A.O. Perrotta, M.D. Dentler, A.S. Jakate, P.B. Desai, G.S. Retzinger, Fibrinogen-coated droplets of olive oil for delivery of docetaxel to a fibrin(ogen)-rich ascites form of a murine mammary tumor, Clin. Cancer Res. 10 (2004) 7001–7010.
[37] M.N. Khalid, P. Simard, D. Hoarau, A. Dragomir, J.C. Leroux, Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors, Pharm. Res. 23 (2006) 752–758.
[38] G. Liang, Z. Jia-Bi, X. Fei, N. Bin, Preparation, characterization and pharmacokinetics of *N*-palmitoyl chitosan anchored docetaxel liposomes, J. Pharm. Pharmacol. 59 (2007) 661–667.
[39] T. Ganesh, Improved biochemical strategies for targeted delivery of toxoids, Bioorg. Med. Chem. 15 (2007) 3597–3623.