Antitumor activity and toxicological properties of doxorubicin conjugated to α,β-poly[(2-hydroxyethyl)-L-aspartamide] administered intraperitoneally in mice

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A polymer–drug conjugate was developed by conjugating doxorubicin (DOX) to α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA) with a succinic spacer. The suitability of PHEA–DOX in intraperitoneal chemotherapy was investigated both *in vitro* and *in vivo*. The results showed that the release rate of DOX from PHEA–DOX in S180 ascites was faster than that in mouse serum or in buffer solutions. An in-vivo antitumor study revealed that PHEA–DOX was more effective than DOX against solid S180 tumor after intraperitoneal injection at the same dose of 10 or 15 mg (DOX eq.)/kg, respectively. At a high dose of 28 mg (DOX eq.)/kg, which was lethal for free DOX to mice, PHEA–DOX could inhibit 61.5% of solid S180 tumor growth and markedly prolonged the survival time of ascetic S180-bearing mice. The toxicological effects of PHEA–DOX injected intraperitoneally in normal mice were assessed by using LD_{50}, body weight increment, electrocardiography, blood biochemical indices, and myocardium histology, giving evidence that PHEA–DOX displayed considerably reduced systemic and cardiotoxicity compared with free DOX. All results suggest that PHEA–DOX has great potential for intraperitoneal chemotherapy because of its high therapeutic effects and few adverse side effects. *Anti-Cancer Drugs* 21:362–371 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Intraperitoneal (i.p.) chemotherapy is encouraged as a surgical adjuvant in patients with peritoneal mesothelioma or carcinomatosis by gastrointestinal and gynecologic cancers [1,2]. Doxorubicin (DOX) is an effective antineoplastic drug and is one of the earliest chemotherapy agents used in clinical trials through the i.p. route [3]. However, large doses of i.p. DOX have multiple adverse side effects such as severe abdominal pain with instillation resulting in intestinal sclerosis and obstruction. The clinical application of DOX is also restricted because of its cumulative cardiotoxicity and suppression of bone marrow function [4,5]. At present, DOX is given only in combination with cisplatin in i.p. chemotherapy. One of the options to improve i.p. therapeutic effect of DOX is to develop new formulations of the drug.

In the past decades, various drug delivery systems including liposome [6], nanoparticle [7], and macromolecular conjugate [8,9] have been explored to improve antitumor activity and minimize the side effects of DOX. A polymer–drug conjugate is a good strategy to optimize the physicochemical and biological properties of DOX. Compared with free drug, polymer–drug conjugates show considerable benefits of enhanced tumor distribution, sustained drug delivery, and increasing drug solubility and stability. Particularly, polymer–drug conjugates have a prolonged retention in the peritoneal carcinomatosis after i.p. administration, suggesting that i.p. chemotherapy of polymer–drug conjugates can be theoretically carried out because of increasing local drug exposure and less systemic toxicity [10,11].

Polymers provide an ideal platform for the design of polymer–drug conjugates [12]. The synthetic polymer α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA), because it is water soluble, biocompatible, easily obtained and modified [13] has been studied intensively and proposed as the drug carrier for paclitaxel [14,15], zidovudine [16,17], ganciclovir [18], for example. Much effort has been made in the investigation of in-vitro drug release properties, cytotoxicity, and in-vivo tissue distribution of these PHEA–drug conjugates. However, there has been little work concerning the pharmacodynamics and safety of these conjugates.

In this study, PHEA was succinoylated and linked to DOX to form a PHEA–DOX conjugate. The antitumor

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effects of PHEA–DOX were investigated on Hela and HepG2 cells *in vitro*, and on solid and ascetic sarcoma 180-bearing mice after i.p. administration *in vivo*. The safety of PHEA–DOX as an i.p. chemotherapy agent was evaluated by measuring its acute systemic toxicity and cardiotoxicity in normal mice. All results show that PHEA–DOX has more therapeutic effects and fewer adverse side effects than free DOX, suggesting that PHEA–DOX has the potential to be used for i.p. chemotherapy.

Materials and methods

Materials

L-aspartic acid, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St Louis, Missouri, USA). DOX hydrochloride (purity > 98%) was purchased from Standard Pharma Ltd (Italy). Enzymatic reagent kits for the determination of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) were purchased from Jiancheng Biochem Ltd (Nanjing, China). Methanol and acetonitrile were of chromatographic grade. Succinic anhydride, *n*-butanol, ethanolamine, *N,N*-dimethylformamide (DMF), 4-dimethylaminopyridine, sulfo-*N*-hydroxysuccinimide sodium salt, triethylamine, $AgNO_3$, and all other chemicals were of analytical grade and were used without further purification.

Animal models

Mouse sarcoma S180 cells, obtained from America Type Culture Collection, were propagated by i.p. implantation to a female ICR mouse. ICR mice (18–22 g) were obtained from the Experiment Animal Center of Nanjing Medical University (Nanjing China), and maintained in plastic cages at $21 \pm 2^{\circ}C$ with free access to pellet food and water. All mice received humane care in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). To generate the heterotopic tumor models, 2.4×10^6 cells were inoculated subcutaneously at the axillary region of animals to prepare solid S180-bearing mice, or injected i.p. to obtain ascitic S180-bearing mice.

Preparation and characterization of PHEA–DOX conjugate

PHEA was synthesized according to the method reported [13]. Briefly, L-aspartic acid (10 g) was mixed with 5 g of 85% phosphoric acid at 180°C for 8 h. The vitreous mass obtained was dissolved in 50 ml DMF and the solution was poured into a beaker containing 200 ml of water and centrifuged to get a precipitation. The precipitate was washed and centrifuged several times until the pH was neutral, then resuspended in distilled water and lyophilized to produce poly-L-succinimide (PSI). A total of 6 ml of ethanolamine was added drop-by-drop to a solution of PSI (2.1 g) in 11 ml of DMF and stirred at room temperature for 24 h. The solution was then acidified with glacial acetic acid to pH 4, dialyzed against distilled water for 4 days and lyophilized.

PHEA–suc was prepared as follows. PHEA (0.5 g) was dissolved in 10 ml of DMF. Succinic anhydride (0.15 g) was added, followed by 4-dimethylaminopyridine (30 mg) as a catalyst. The reaction was conducted for 6 h at room temperature. Then the solution was dialyzed against distilled water for 4 days and lyophilized. The succinyl content of PHEA–suc was quantified by titration against a standard of NaOH (4×10^{-3} mol/l) using bromothymol blue as an indicator [19].

DOX was conjugated to PHEA–suc using the method reported earlier [19]. PHEA–suc (0.5 g) was dissolved in 10 ml DMF. EDC (0.5 g) and sulfo-*N*-hydroxysuccinimide sodium salt (0.27 g) were dissolved in 20 ml of DMF and mixed with the PHEA–suc solution. This was allowed to react at room temperature for 30 min. DOX (125 mg) was added to the mixture and then triethylamine (60 µl) was added. The solution was gently stirred at room temperature for 24 h. The conjugate was purified by gel filtration on a Sephadex G25 column with distilled water as the eluent, and then lyophilized. This process was kept from light as much as possible.

The DOX content in the PHEA–DOX conjugate was determined after cleavage from the polymer [20] using a Shimadzu high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) consisting of two pumps (LC-10Avp and LC-10AS) and an SPD-10Avp ultraviolet detector. Free DOX encapsulated in the conjugate was measured by HPLC after the extraction of DOX from the aqueous PHEA–DOX solution into isopropanol–chloroform (25: 75, v/v). HPLC analysis was performed using an Extend-C18 column (4.6×250 mm I.D., 5 µm) with ultraviolet detection at 227 nm. The mobile phase was the methanol–acetonitrile–phosphate buffer (pH 5.0; 0.2 mol/l) (50 : 20 : 30, v/v/v) and the flow rate was 0.5 ml/min.

The molecular weight of PHEA was determined by aqueous size exclusion chromatography based on polyethylene oxide/polyethylene glycol standards. PHEA, PHEA–suc, and PHEA–DOX were characterized by Fourier transform infrared spectrometer (Nicolet 170SX, Japan) and proton nuclear magnetic resonance (^{1}H-NMR) spectrometry (Bruker DRX-500, Switzerland).

In-vitro release study

DOX release from PHEA–DOX was studied respectively in the buffering solutions at pH 5.5 (HAc, NaAc) and pH 7.4 (Na_2HPO_4, NaH_2PO_4), 50% mouse serum, and 50% ascites of S180-bearing mice at 37°C. Serum was obtained from normal mice, diluted by saline. S180

ascites were drawn 7 days after the mouse was injected i.p. with 2.4×10^6 cells and diluted by saline. A 0.5 ml release medium was added into several tubes. In each tube, the initial PHEA–DOX concentration was 0.5 mmol/l (DOX eq.). The sample was then treated as reported [20]. Briefly, the incubation medium in a tube was removed from the incubator at 37°C in the predetermined time interval and then extracted with 1.5 ml isopropanol–chloroform (25 : 75, v/v). After the upper aqueous phase in a tube had been removed, the lower organic phase containing DOX was evaporated in a vacuum. The DOX content of the residue was tested using HPLC after the residue had been dissolved in the mobile phase.

In-vitro cytotoxicity assay

HeLa and HepG2 cells obtained from America Type Culture Collection were grown and maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum. Cells were seeded at a density of 1.0×10^4 cell/well in 96-well plates and allowed to grow for 24 h before treatment. Cells were treated with various concentrations of DOX, PHEA–DOX, and PHEA–suc for 24 h at 37°C. Cell number was measured by the MTT cell viability assay. IC_{50} curves were fit to an exponential equation using Microsoft Excel (Microsoft Corporation, Washington, USA). The reported IC_{50} represents an average of at least three independent experiments, and significance was determined using Student's *t*-test.

In-vivo antitumor study

Antitumor activity of PHEA–DOX was examined in both solid and ascitic S180-bearing mice. Mice bearing solid tumors were randomly divided into seven groups with six mice in each group. DOX, PHEA–DOX, and PHEA–suc dissolved in saline were injected i.p. on day 4 and day 8 after S180 cells had been implanted. Saline was injected i.p. as the control. The mice were killed on day 11 and the tumors were excised and weighed. The tumor weight of the treated group (T) was compared with that of the control group (C). Tumor inhibition rate was calculated as $(1 - T/C) \times 100\%$.

Ascitic S180-bearing mice were randomly divided into four groups and six mice were used in each group. DOX, PHEA–DOX, and PHEA–suc were i.p. administeed on day 4 after S180 cells were implanted while saline was used as the control. The survival time of mice and the number of long-term survivors were recorded from the timepoint when the tumor cells were inoculated.

Toxicological study in healthy mice

The toxicity of PHEA–DOX, DOX, and PHEA–suc was assessed in normal female ICR mice for 2 weeks. Fourteen groups of mice were used with 10 mice in each group. PHEA–DOX and DOX were dissolved in saline at the concentration of 2 mg (DOX eq.)/ml and injected i.p. at doses of 6.0, 10.0, 16.7, 27.7, 46.3, and 77.3 mg (DOX eq.)/kg, respectively, which increased in geometric progression. Accordingly, the mice groups were designated as PHEA–DOX1 to PHEA–DOX6, and DOX1 to DOX6. The high dose of 46.3 or 77.3 mg (DOX eq.)/kg was divided in half and injected twice after a 4-h interval. PHEA–suc dissolved in saline was injected i.p. at a single dose of 250 mg/kg, which is equivalent to that contained in PHEA–DOX4 [27.7 mg (DOX eq.)/kg].

Mice were carefully observed for any behavioral changes during the first 5 h. Body weight, toxic symptoms, and mortality were recorded thereafter for 14 days. The median lethal dose (LD_{50}) was determined by the Bliss method.

At the end of 14 days, the mice that survived were anesthetized with pentobarbital and electrocardiograms were recorded with standard 12-lead synchronization high-frequency electrocardiogram system. Blood was collected from the heart and serum was tested for ALT, AST, CK, LDH, and BUN with enzymatic reagent kits. A thorough autopsy was carried out on all the mice to examine internal abnormalities. The heart was fixed in a 10% buffered neutral formalin solution. Paraffin sections of the heart were prepared and stained with hematoxylin and eosin for microscopic observation.

Statistical analysis

Statistical analysis was performed by Student's *t*-test for pairs of groups and one-way analysis of variance for multiple groups. All results were expressed as the mean ± standard deviation (SD) unless otherwise noted. A probability (*P*) of less than 0.05 was considered statistically significant.

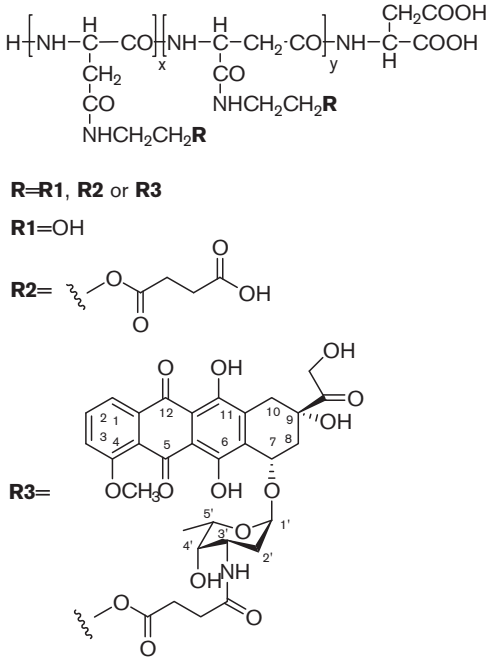
Results

Preparation and characterization of PHEA–DOX

PHEA was synthesized by the complete aminolysis of PSI in a DMF solution at room temperature. Then it was succinoylated and linked to the amino group of DOX, forming the PHEA–DOX conjugate. The chemical structure of PHEA–DOX is outlined in Fig. 1.

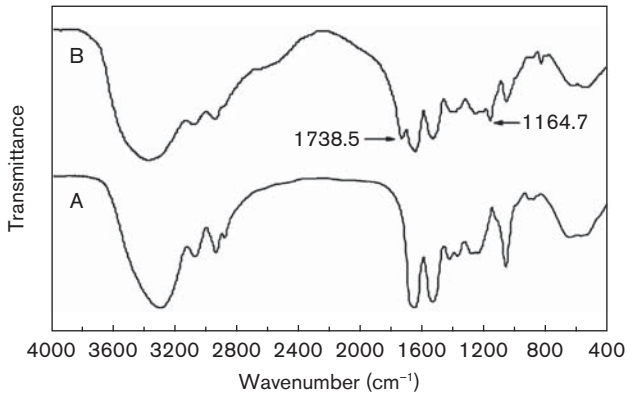
Figure 2 shows the Fourier-transform infrared spectra of PHEA and PHEA–suc. Compared with PHEA, there are typical ester bonds corresponding to succinyl branches of PHEA–suc ($\upsilon_{c=o}$: 1738.5/cm, $\upsilon_{as\ c-o-c}$: 1164.7/cm). The structure of PHEA–suc is further confirmed by ^{1}H-NMR (D_2O) analysis. It can be seen in Fig. 3(c) that the typical signals from PHEA and succinyl groups can be observed in the spectrum of PHEA–suc at 2.55 ppm (suc: $COCH_2CH_2CO$), 2.68 ppm (PHEA: $CHCH_2CONH$), 3.25 ppm (PHEA: $NHCH_2CH_2OH$), 3.55 ppm (PHEA: CH_2CH_2OH), and 4.61 ppm (PHEA: $NHCH(CO)CH_2$). New signals appear at 3.38 ppm (PHEA: CH_2CH_2OCO) and 4.01 ppm (PHEA: CH_2CH_2OCO) because of the graft structure.

Fig. 1



Chemical structures of α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA) (**R**=**R1**), PHEA–suc (**R**=**R1** and **R2**), and PHEA–doxorubicin (**R**=**R1**, **R2**, and **R3**).

Fig. 2

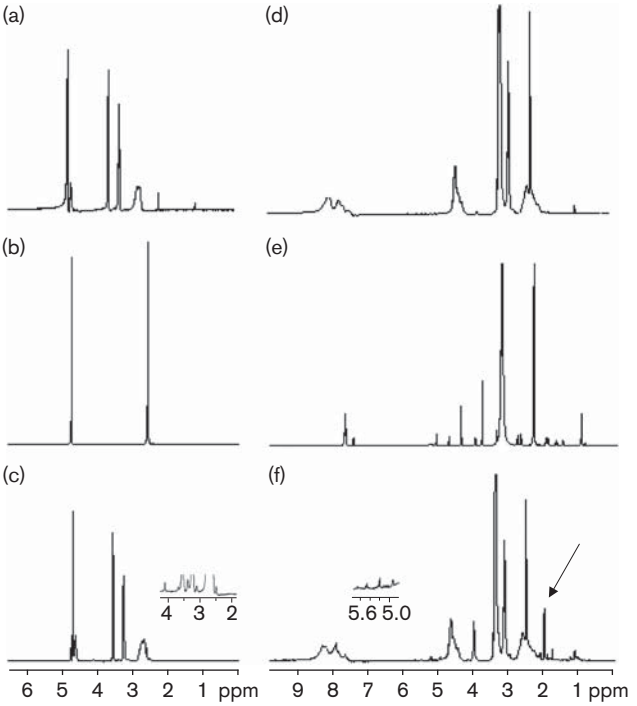


Fourier-transform infrared spectra of α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA) (A) and PHEA–suc (B).

Formation of PHEA–DOX is confirmed by ^{1}H-NMR analysis in DMSO-d_6 as shown in Fig. 3(f). Significantly, a new signal at 2.005 ppm appears because DOX was covalently bound to the polymer. In addition, the typical signals of DOX at 3.99 ppm (H_3COC-4) and 5.23 ppm (HC-1′) are detected in spectrum of PHEA–DOX.

The weight-average molecular weight of PHEA was determined as 23 kDa ($M_w/M_n = 1.76$) through gel permeation chromatography measurement. The degree

Fig. 3



^{1}H-NMR spectra of α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA) (a), succinic anhydride (b), and PHEA–suc (c) dissolved in D_2O; ^{1}H-NMR spectra of PHEA–suc (d), doxorubicin (DOX) (e) and PHEA–DOX (f) dissolved in DMSO-d_6.

of succinoylation of PHEA–suc was determined by titration as 11.8 mol%. The amount of DOX linked to PHEA was detected as 11.1 wt% of the conjugate. No remarkable amount of free DOX (less than 0.1 wt% of the conjugate) was found in the PHEA–DOX conjugate by HPLC after purification.

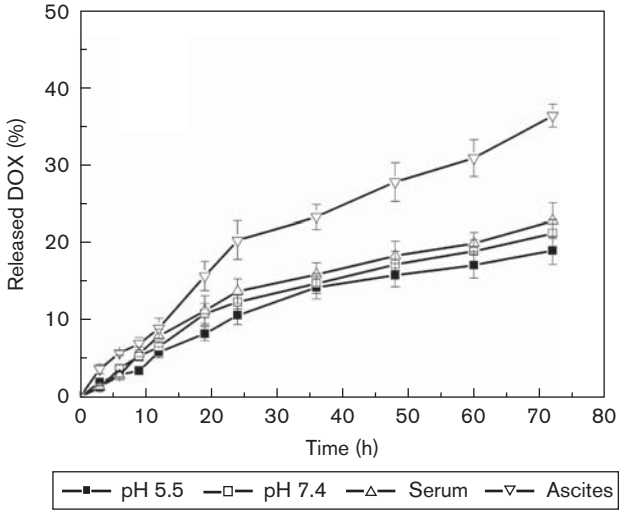
In-vitro release study

DOX release behavior from the conjugate was studied in different solutions including buffering solutions, mouse serum, and mouse ascites. As shown in Fig. 4, DOX was released in a sustained manner from PHEA–DOX in each solution. Although drug release at pH 5.5 was a little slower than that in the pH 7.4 buffer, the release rate was not quite different. PHEA–DOX also exhibited a similar drug release property in the mouse serum to that in buffer solutions. However, the DOX release rate was obviously accelerated in S180 ascites. Compared with that in mouse serum, about 1.5 times more DOX was released from PHEA–DOX in S180 ascites.

In-vitro cytotoxicity assay

Human cervix adenocarcinoma cells, HeLa, and human hepatocellular carcinoma cells, HepG2, were used to investigate the cytotoxicity of PHEA–DOX in comparison with free DOX. The results of cell viability treated by

Fig. 4



Release curve of doxorubicin from α,β-poly[(2-hydroxyethyl)-L-aspartamide]–doxorubicin (DOX) *in vitro* at pH 5.5 (■) and pH 7.4 (□) buffer solutions, in mice serum (△) and in S180 ascites (▽).

PHEA–DOX and DOX are shown in Fig. 5. The IC_{50} values of the free DOX at 24 h are 2.57 μg/ml on HeLa cells and 4.36 μg/ml on HepG2 cells, respectively, which are consistent with earlier reports [21–23]. PHEA–DOX, with IC_{50} values of 29.80 μg (DOX eq.)/ml on HeLa cells and 45.93 (DOX eq.) μg/ml on HepG2 cells, was less cytotoxic than DOX.

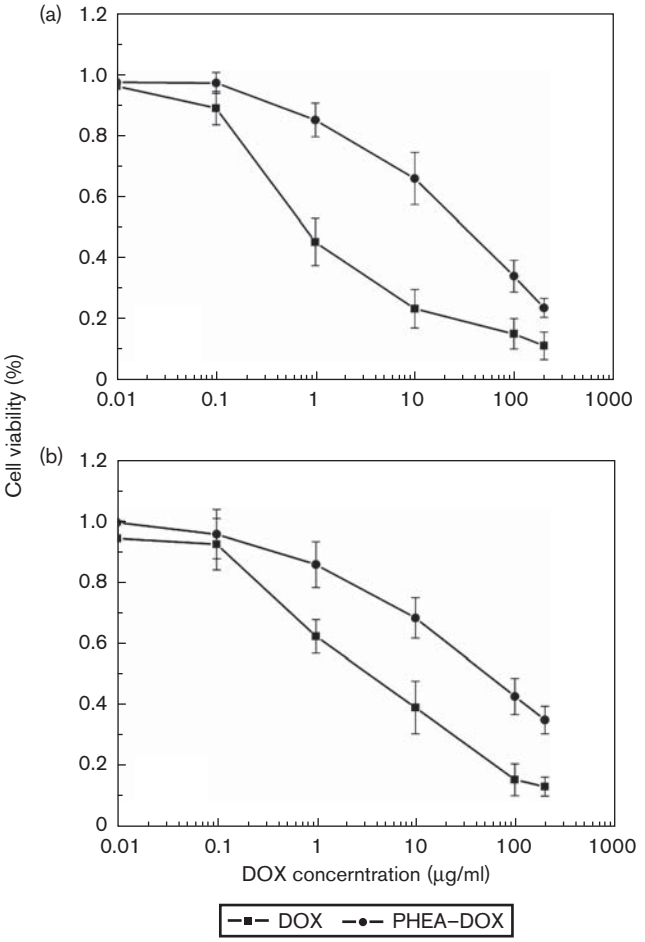
In-vivo antitumor study

The therapeutic effects of PHEA–DOX and DOX on both solid and ascitic sarcoma 180-bearing mouse models are summarized in Table 1. PHEA–suc does not have any antitumor effect on both tumor models. For the solid S180 tumor model, although the antitumor activity of both PHEA–DOX and DOX increases with the enhancement of dosage, PHEA–DOX is much more effective than DOX at each dose. At a high dose of 28 mg (DOX eq.)/kg, which is lethal for free DOX to mice, PHEA–DOX has 61.5% of the tumor inhibition rate. For the ascetic S180 tumor model, all mice injected with DOX at 15 mg (DOX eq.)/kg died within 1 week, with survival time being much shorter than that of the control group, indicating that DOX has high toxicity to ascetic S180-bearing mice. However, mice treated with PHEA–DOX at 28 mg (DOX eq.)/kg exhibited a significant prolongation of the survival time. At the end of the monitored time of 70 days, half of the mice in the PHEA–DOX group survived with complete ascites eradication.

Toxicology of PHEA–DOX in healthy mice

Both DOX and PHEA–DOX caused transient and reversible changes in the behavioral reactions of mice. Several minutes after i.p. injection, all mice behaved

Fig. 5



In-vitro cytotoxicity evaluations of doxorubicin (DOX) and α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA)–DOX against Hela cells (a) and HepG2 cells (b) after incubation for 24 h.

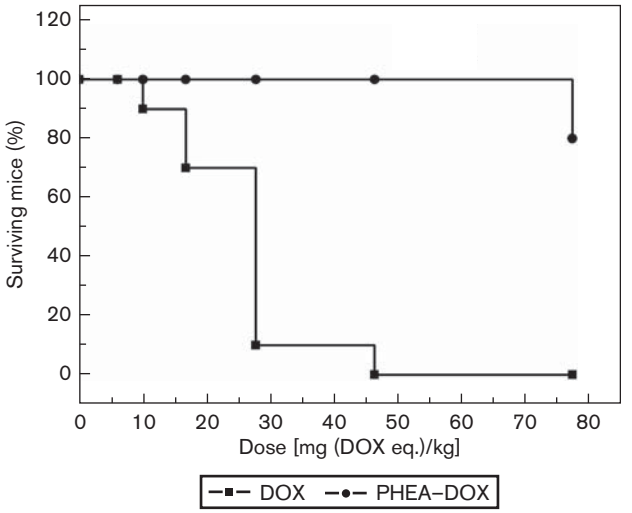
stereotypically with an increase of ventilation rates and then fell asleep. These changes became intense at high dosages, but all mice resumed motor activity within 2 h. The mice were then observed for 14 days. DOX-treated groups were associated with more profound piloerection, adynamia and hypodynamia than PHEA–DOX-treated groups for several days. The number of surviving mice was recorded for 2 weeks after i.p. administration. It can be seen in Fig. 6 that DOX exhibited serious toxicity, which resulted in a much higher mortality rate of mice on day 14 in a dose-dependant manner. LD_{50} of DOX was calculated to be 19.63 mg/kg with the 95% confidence interval ranging from 16.12 to 24.55 mg/kg. In contrast, PHEA–DOX showed remarkably reduced toxicity, which caused fewer deaths than free DOX. Only 20% mice treated by PHEA–DOX died at 77.3 mg (DOX eq.)/kg whereas no death occurred at other doses, indicating that the LD_{50} of PHEA–DOX was greater than 77.3 mg (DOX eq.)/kg. Administration of PHEA–suc was not associated with mortality.

Table 1 Antitumor effect of DOX and PHEA–DOX on solid and ascitic S180-bearing mice after intraperitoneal administration

Sarcoma 180	Drug	Dose [mg (DOX eq.)/kg]	Tumor weight (g)	Tumor inhibition rate (%)	Mean survival time (days)	Ratio of long-term survivors (%)
Solid	Saline control	–	0.732 ± 0.231	–	–	–
	DOX	10	0.566 ± 0.219	22.7	–	–
	DOX	15	0.532 ± 0.117	27.3	–	–
	PHEA–DOX	10	0.509 ± 0.204	30.5	–	–
	PHEA–DOX	15	0.455 ± 0.231	37.9	–	–
	PHEA–DOX	28	0.282 ± 0.137*	61.5	–	–
	PHEA–suc[a]	250	0.717 ± 0.278	–	–	–
Ascites	Saline control	–	–	–	16.5 ± 2.03	0
	DOX	15	–	–	8.6 ± 3.79	0
	PHEA–DOX	28	–	–	>46.5**	50%
	PHEA–suc[a]	250	–	–	17.1 ± 2.51	0

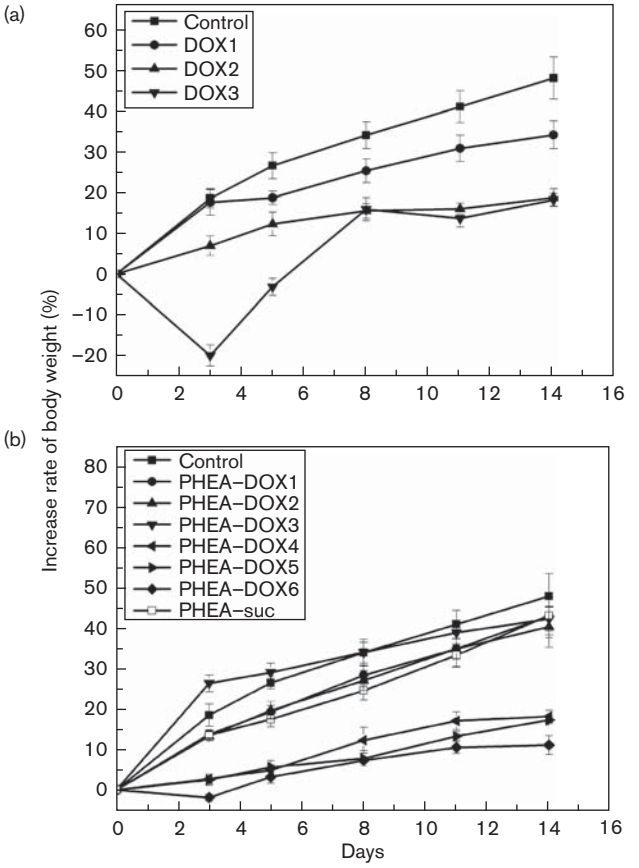
DOX, doxorubicin; PHEA–suc, succinoylated α,β-poly[(2-hydroxyethyl)-l-aspartamide].
[a]PHEA–suc was injected at 250 mg/kg.
*Significantly different from control ($P < 0.05$).
**Significantly different from control ($P < 0.001$).

Fig. 6



Mice surviving rate after administration of DOX and α,β-poly [(2-hydroxyethyl)-L-aspartamide] (PHEA)–doxorubicin (DOX).

Fig. 7

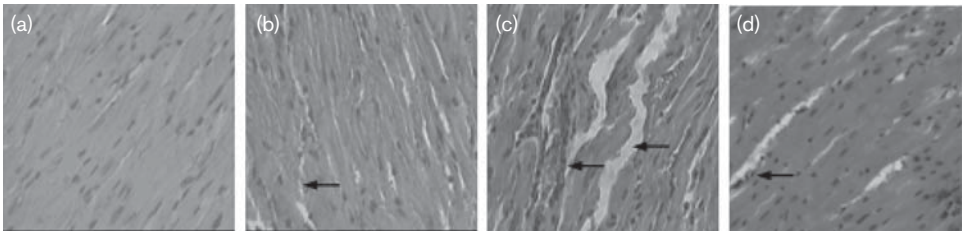


The change of mice body weight after administration of (a) doxorubicin 1 (DOX1) (● 6.0 mg/kg), DOX2 (▲ 10.0 mg/kg), DOX3 (▼ 16.7 mg/kg), (b) α,β-poly[(2-hydroxyethyl)-L-aspartamide] (PHEA)–DOX1 [● 6.0 mg (DOX eq.)/kg], PHEA–DOX2 [▲ 10.0 mg (DOX eq.)/kg], PHEA–DOX3 [▼ 16.7 mg (DOX eq.)/kg], PHEA–DOX4 [◀ 27.7 mg (DOX eq.)/kg], PHEA–DOX5 [▶ 46.3 mg (DOX eq.)/kg], PHEA–DOX6 [◆ 77.3 mg (DOX eq.)/kg] and PHEA–suc (□ 250 mg/kg).

Figure 7 shows body weight changes in mice that received i.p. injections of saline, free DOX, or PHEA–DOX at different doses. PHEA–DOX obviously exhibited low toxicity because it caused less weight loss than DOX at the same doses. Compared with the initial weight, mice treated with DOX3 [16.7 mg (DOX eq.)/kg] significantly lost weight because of anorexia and diarrhea during the first several days, whereas mice treated with PHEA–DOX6 [77.3 mg (DOX eq.)/kg] only exhibited a slight decrease in body weight.

Treatment with DOX caused massive myocardial degeneration (Fig. 8c and d), which always resulted in the increase of the plasma level of CK and LDH [27] (Table 3). This toxicity was extremely relieved when DOX was delivered from PHEA–DOX (Fig. 8d and Table 3). As shown in Fig. 8c and d, DOX groups displayed remarkable pathological changes, in which the myocardium was severely damaged with pronounced vacuolation and breakage of myofibers, atrophy, and widening of the gap of myofibers associated with the lymphoid infiltrations at the dose of 27.7 mg/kg. However,

Fig. 8



Mice myocardium stained by hematoxylin–eosin. (a) Control, ×250; (b) doxorubicin 2 (DOX2), 10 mg/kg, ×250; (c) DOX4, 27.7 mg/kg, ×250; (d) PHEA–DOX4, 27.7 mg (DOX eq.)/kg, ×250.

Table 2 Electrocardiographic parameters at 14 days after drug administration

Group	Dose [mg (DOX eq.)/kg]	Heart rate (beat/min)	PR-interval (ms)	QT-interval (ms)
Saline control	–	317.1 ± 44.97	52.8 ± 7.63	28.4 ± 0.72
DOX1	6	292.7 ± 55.98	59.6 ± 8.5	33 ± 2.76
DOX2	10	407.3 ± 128.95	69.7 ± 7.45*	38.1 ± 0.18*
DOX3	16.7	526.9 ± 127.89*	67.2 ± 2.26*	36.2 ± 1.28*
PHEA–DOX1	6	354.6 ± 144.94	58.4 ± 9.18	31.1 ± 5.69
PHEA–DOX2	10	347.1 ± 153.21	65.2 ± 5.66	28.8 ± 2.32
PHEA–DOX3	16.7	412.5 ± 81.39	62.3 ± 4.88	32.6 ± 2.83
PHEA–DOX4	27.7	499.1 ± 132.09*	63.2 ± 7.31	33.4 ± 1.65
PHEA–DOX5	46.3	498.3 ± 138.33*	73.9 ± 2.53*	26.3 ± 1.29
PHEA–DOX6	77.3	532.4 ± 45.27*	72.5 ± 1.64*	28.6 ± 0.57
PHEA–suc[a]	250	273.4 ± 51.36	52.4 ± 8.25	31.9 ± 4.58

DOX, doxorubicin; PHEA–suc, succinoylated α,β-poly[(2-hydroxyethyl)-l-aspartamide].
[a]PHEA–suc was injected at 250 mg/kg.
*Significantly different from control ($P < 0.05$).

mice treated with PHEA–DOX at 27.7 mg (DOX eq.)/kg displayed fewer minor lesions of myofibers compared with the DOX2 group at 10 mg/kg (Fig. 8c).

Cardiac damage can further be characterized by electrocardiography (Table 2) [24,25]. When drug dosage was up to 10 mg/kg, DOX turned to induce significant lengthening of P–R and Q–T interval and pronounced tachycardia. However, PHEA–DOX could reduce the cardiac toxicity of DOX. The increase in the Q–T interval with statistical difference was not observed in any group treated with PHEA–DOX and the prolongation of the P–R interval was observed only at a dosage higher than 46.3 mg (DOX eq.)/kg.

As summarized in Table 3, hepatotoxicity of PHEA–DOX was not observed as the ALT, AST, and AST/ALT ratio had no obvious changes, in which the AST/ALT ratio had been proposed as an indicator of liver damage [26], whereas the AST/ALT ratio of DOX was higher than control at a dosage of 16.7 mg/kg, suggesting hepatotoxicity of DOX. In addition, nephrotoxicity of PHEA–DOX was not found because the BUN level did not change significantly.

Discussion

Intraperitoneal chemotherapy is a therapeutic option in the management of peritoneal carcinomatosis by exposing the cancers to high drug concentrations. Many chemotherapeutic agents, involving DOX, have been tried in i.p. chemotherapy. However, DOX is not widely injected i.p. in clinical application because of serious toxicity effects. The therapeutic potency of DOX could be improved by forming polymer–DOX conjugates, some of which have entered clinical trials [28,29]; however, the chemotherapeutic effects of polymer–DOX conjugates after i.p. administration have scarcely been investigated. In this study, the PHEA–DOX conjugate was developed and used for biological evaluation for i.p. administration.

The PHEA–DOX conjugate was prepared successfully with a succinic spacer. During the reaction, the succinyl group was first linked to PHEA and then reacted with the amino group of DOX, in which DOX reacted for only one step in the synthetic route and could keep more drug activity than earlier reports [14–18], in which drugs were first succinoylated and then attached to the backbone.

An in-vitro drug release study showed that DOX was released in a sustained manner from PHEA–DOX, but a little more slowly at pH 5.5 than at the pH 7.4 buffering solution. The pH-dependent release property of PHEA–DOX is similar to those conjugates with succinic spacers, such as PHEA–zidovudine, PHEA–paclitaxel, and chitosan–MMC [15,16,30]. Such pH-dependent release behavior is not desirable as an ideal polymer–drug conjugate should be stable in neutral blood and be converted to active drug in acidic tumor sites. In fact, it is too simple to use different pH values to mimic physiological compartments. Abundant enzymes *in vivo* rather than the pH value alone would contribute to the degradation of PHEA–DOX. Therefore, fresh mouse serum and S180 ascites were used to examine the release property of the PHEA–DOX conjugate. It is notable that the drug was released from the conjugate much faster in S180 ascites than in mouse serum. The results suggest that the PHEA–DOX conjugate would have prolonged circulation and could be released in a sustained manner in tumor sites *in vivo*.

An in-vitro cytotoxicity assay indicated PHEA–DOX had less cell inhibition efficacy than DOX on both HeLa and

Table 3 Serum biochemical indices at 14 days after drug administration

Group	Dose [mg (DOX eq.)/kg]	ALT (U/l)	AST (U/l)	AST/ALT	CK (U/ml)	LDH (U/l)	BUN (mg/l)
Saline control	–	27.3 ± 2.3	31.5 ± 2.6	1.15	0.22 ± 0.18	474.4 ± 74.3	10.5 ± 3.7
DOX1	6	25.7 ± 2.6	26.8 ± 1.8*	1.06	0.20 ± 0.07	534.6 ± 51.0	6.8 ± 1.2
DOX2	10	29.4 ± 2.4*	29.6 ± 3.8	1.0	1.2 ± 0.17**	544.3 ± 46.8	8.4 ± 2.8
DOX3	16.7	21.7 ± 1.6*	32.2 ± 6.9*	1.48	1.05 ± 0.53**	554.4 ± 58.9*	6.3 ± 1.2
PHEA–DOX1	6	27.7 ± 3.1	26.3 ± 2.9	0.95	0.35 ± 0.17	480.5 ± 51.2	8.1 ± 2.7
PHEA–DOX2	10	28.1 ± 2.3	27.5 ± 3.2*	0.98	0.32 ± 0.09	484.3 ± 98.5	12.1 ± 6.3
PHEA–DOX3	16.7	27.8 ± 7.4	28.6 ± 6.1	1.03	0.48 ± 0.24*	494.1 ± 79.9	11.2 ± 5.3
PHEA–DOX4	27.7	28.0 ± 2.7	25.7 ± 3.2*	0.92	1.28 ± 0.12**	524.1 ± 30.4	8.1 ± 2.0
PHEA–suc[a]	250	27.6 ± 1.2	33.2 ± 2.2	1.20	0.26 ± 0.31	479.0 ± 63.4	9.6 ± 4.0

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; DOX, doxorubicin; LDH, lactate dehydrogenase; PHEA–suc, succinoylated α,β-poly[(2-hydroxyethyl)-l-aspartamide].
[a]PHEA–suc was injected at 250 mg/kg.
*Significantly different from control ($P<0.05$).
**Significantly different from control ($P<0.001$).

HepG2 cells. This is probably owing to the fact that the conjugate enters cells more slowly than free DOX and the DOX release from the conjugates is also a relatively slow process [27,31]. As reported, free DOX could be accumulated in the cell nucleus in a relatively high quantity through cell diffusion transportation after 90-min incubation with cancer cells, then induced fragmentation of nuclear DNA and triggered cell apoptosis [30]. However, polymer-bound drugs were usually internalized by endocytosis rather slowly. Even after entering cells, DOX released in a sustained manner from polymer–DOX conjugates might not be as sufficient to inhibit cell growth as DOX [30].

PHEA–DOX showed more effective antitumor results than DOX in both solid and ascetic tumor models after i.p. administration. Intraperitoneal chemotherapy is able to provide a greater concentration of cytotoxic agents and expose peritoneal tumors for a longer period than does intravenous administration. However, the accumulated DOX in the peritoneal region could damage major organs and limit its clinical indications. Polymer–drug conjugates crossed the peritoneal barrier more slowly than free drug, leading to higher elimination half-life than that of free drug [11]. A prolonged retention and local sustained release of PHEA–DOX in the peritoneal space will contribute to its high effectiveness against ascitic S180 tumor in spite of the fact that DOX exhibits high local toxicity. As for solid S180 tumor, PHEA–DOX had enhanced antitumor efficacy compared with DOX. This is presumably because of not only the slow elimination properties but also the 'enhanced permeability and retention (EPR) effect'. Polymer–drug conjugates are usually transported passively to solid tumors because of their unique pathophysiologic characteristics. The EPR effect is always enhanced by the increasing molecular weight and aggregate size of the polymer conjugate [32–34]. PHEA–DOX is an amphiphilic conjugate, which is likely to form aggregates in biological fluids [35] and contribute to the EPR effect. It may help to improve the antitumor activity of the PHEA–DOX conjugate on subcutaneously solid tumor despite relatively low molecular weight.

The toxicological properties of PHEA–DOX were evaluated at various doses in normal mice, and a tolerable therapeutic dose of PHEA–DOX was established. Both PHEA–DOX and DOX exhibited dose-dependent toxicity except for ALT, AST, and BUN data. It seems confusing but the nonlinearity relationship always appeared in clinical experience. This kind of nonlinearity relationship can also be seen in some reported papers [36,37]. Acute toxicity of DOX in normal mice was largely relieved when it formed the conjugate of PHEA–DOX, with LD_{50} improving more than four times. PHEA–DOX could remarkably increase body weight of mice compared with DOX, indicating its low gastrointestinal toxicity. It was also confirmed by necropsy of the dead mice, showing that i.p. DOX caused severe organ damage, whereas PHEA–DOX kept these organs from severe damage. From a clinical standpoint, it should be remarked that in patients that are prone to receive i.p. chemotherapy, survival is usually severely diminished, which makes cardiotoxicity a less important problem. Cardiotoxicity can be a problem in breast cancer, in which survival is much better than, for example, in ovarian cancer, in which i.p. administration of drugs is traditionally carried out. As PHEA–DOX exhibited much relieved systemic toxicity, we are still concerned about its cardiotoxicity after i.p. administration. PHEA–DOX exhibited significantly low cardiotoxicity compared with DOX. In a combination of the toxicity manifestations, it can be deduced that the dose of 27.7 mg/kg is a tolerable dosage for PHEA–DOX in i.p. chemotherapy. The lower adverse side effects of PHEA–DOX can be explained by the alteration of pharmacokinetics. Sustained released of DOX could produce lower peak plasma concentrations [38], which in turn were responsible for the lower toxicological properties of PHEA–DOX.

This study showed that PHEA–DOX had better therapeutic effects but significantly lower adverse side effects than free DOX after i.p. administration. In the future, pharmacokinetic profiles of PHEA–DOX will be evaluated to comprehensively investigate the mechanism of its antitumor ability and toxicological properties.

In summary, the PHEA–DOX conjugate was developed and found to enhance the therapeutic effects and reduce the side effects of the parent drug after i.p. administration. PHEA–DOX was more effective than DOX against subcutaneously transplanted solid S180 tumor. PHEA–DOX can enhance antitumor activity against solid S180 tumor and prominently prolong the survival time of ascitic S180-bearing mice at a tolerable high dose, which was lethal for DOX to mice. The acute toxicity study showed that PHEA–DOX led to relieved side effects compared with free DOX including higher LD_{50}, reduced cardiosystemic and systemic toxicity. These results indicate that PHEA–DOX is a promising polymeric drug and has potential for i.p. chemotherapy.

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