

# Barbigerone-in-hydroxypropyl- $\beta$ -cyclodextrin-liposomal nanoparticle: preparation, characterization and anti-cancer activities

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**Abstract** Lipophilic drugs have limited solubility in phospholipid systems, hence maximum entrapment levels in liposomes are known to be low. Barbigerone (Bar), an anti-cancer isoflavone, is water insoluble and an effective delivery route is through encapsulation in cyclodextrins (CDs) followed by a second encapsulation in liposomes. In this study, Bar or its inclusion complex [Bar/2-hydroxypropyl- $\beta$ -CD (HP- $\beta$ -CD)] were incorporated into liposomes prepared by the ethanol injection method. A 4.6-fold increase of encapsulation efficiency was achieved for the liposome-Bar/HP- $\beta$ -CD complex (Bar/HP- $\beta$ -CD/liposome) in comparison with the conventional Bar/liposome. The size of the Bar/HP- $\beta$ -CD/liposome was  $87.76 \pm 1.45$  nm and the zeta potential was  $-35.02 \pm 0.50$  mV. In addition, the liposomes remained stable in liquid form at 4 °C for at least 3 months. The Bar/HP- $\beta$ -CD/liposome was evaluated for anti-cancer activity in hepatocarcinoma HepG2 and colon cancer C26

cells and showed comparable toxicity to that of plain Bar. In vivo studies in hepatic cancer xenografted nude mice model showed that Bar/HP- $\beta$ -CD/liposome significantly inhibited tumor growth with a substantial increase in animal survival. In conclusion, the encapsulation of the Bar/HP- $\beta$ -CD complex into liposomes could provide an alternative means for its potential use in cancer therapy.

**Keywords** Barbigerone · Hydroxypropyl- $\beta$ -cyclodextrin · Liposome · HepG2 · Anti-cancer

## Introduction

Barbigerone [Bar, which is 2',4',5'-trimethoxy-6'',6''-dimethylpyrano(2'',3'':7,8) isoflavone] (Fig. 1a) is an active compound isolated and purified from *Tephrosia barbigerona* [1]. Previous reports have demonstrated that Bar exhibits a variety of biological activities, such as antioxidant activity, 15-lipoxygenase inhibitory activity [2] and anti-plasmodial activity against the malarial parasite *Plasmodium falciparum* [3]. Recent studies by our group have shown that Bar exhibits an anti-cancer potential as it causes apoptosis of murine lung-cancer cells [4]. Bar could inhibit tumor-angiogenesis, tumor growth and lung metastasis via down-regulation of the MEK3/6/p38 MAPK signaling pathway [5]. In tumor-bearing mice treated with Bar, tumor-growth and metastasis were significantly suppressed, the life span was prolonged while little adverse effects were observed [6]. These research works have shown its potential for acting as an anticancer agent. However, the efficacy and utility of Bar are compromised by its intrinsic poor water solubility. Hence, the development of an improved delivery system for Bar is of high importance.

Lulu Cai is the co-first author, who worked equally with Neng Qiu on this paper.

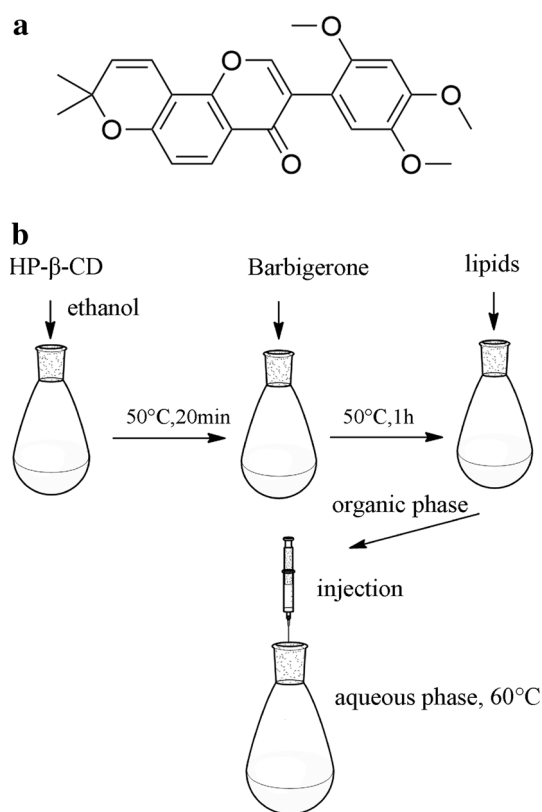
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**Fig. 1** **a** The chemical structure of barbigerone (Bar), and **b** the procedure and schematic diagram of preparation of Bar/HP- $\beta$ -CD/liposome

Over recent decades, many effective methods have been introduced to increase the aqueous solubility of water insoluble drug. Some traditional and novel approaches, including liposomes, micelles, emulsions, complexation, and pharmaceutical salts, have been widely used for water-insoluble drug delivery. Liposomes are spherical vesicles with an aqueous core surrounded by lipid bilayers. This unique structure allows liposomes to encapsulate both hydrophilic and lipophilic materials [7]. Currently, liposomes have been extensively investigated for their ability to improve the dissolution properties and release profile of chemotherapeutic agents, in efforts to increase therapeutic efficacy and decrease toxicity to normal cells [8, 9]. However, the amount of lipophilic drug incorporated into the conventional liposome bilayer is often limited in terms of drug-to-lipid mass ratios and the carrier functions of liposomes cannot be effectively exploited by drugs, such as Bar.

Cyclodextrins (CDs), a family of cyclic amylose-derived oligomers, have been successfully used as complexation agents to enhance the solubility, stability and bioavailability of drug molecules [10, 11]. The approach of combined liposomes and CD complexes of lipophilic drugs by

forming drug-in-CD-in-liposome can increase the entrapment efficiency and modulate in vivo dissociation of the drug, thereby contributing to improvements in the pharmacokinetic profile of the drugs [12, 13]. In this system, hydrophobic drugs can be entrapped in the aqueous phase of liposomes through water-soluble drug-CD inclusion complexes [14, 15]. This strategy merges the relative advantages of the two types of carrier into a single system and avoids the use of unnecessary organic solvents in the accommodation of water-insoluble drugs in the lipid bilayer of liposomes [16].

Therefore in this study, we tested this strategy and found that the enhanced solubility of Bar in aqueous 2-hydroxypropyl- $\beta$ -CD (HP- $\beta$ -CD) solution offers an improvement in Bar encapsulation within the liposomes. Bar/HP- $\beta$ -CD complexes were encapsulated into liposomes using the ethanol-injection method. The Bar/HP- $\beta$ -CD-liposome (Bar/HP- $\beta$ -CD/liposome) were characterized for morphological properties using transmission electron microscopy (TEM), while their particle size, zeta potential, and entrapment efficiency were determined using light scattering and gel-exclusion chromatography techniques, respectively. The in vitro release and stability of Bar/HP- $\beta$ -CD/liposome were also evaluated. The anti-cancer efficacy of Bar/HP- $\beta$ -CD/liposome was tested in vitro on C26 and HepG2 cell lines, and in vivo on HepG2 xenograft mice model.

## Materials and methods

### Materials and reagents

Bar ( $\geq 98$  % purity) was prepared in our laboratory. HP- $\beta$ -CD was purchased from Shijiazhuang Pharmaceutical Group Co., Ltd. (Shijiazhuang, China). Soya phosphatidyl choline (SPC) and monomethoxy polyethylene glycol 2000-distearoyl phosphatidyl-ethanolamine (MPEG<sub>2000</sub>-DSPE) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol was obtained from Shanghai Bio Science and Technology Co., Ltd. (Shanghai, China) and Vitamin E was purchased from Zhejiang Xinhecheng Co., Ltd. (Zhejiang, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol [high performance liquid chromatography (HPLC) grade] was purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). All the other solvents and chemicals were of analytical grade purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). Water was purified using a Millipore (Fairbanks, Alaska) laboratory ultra pure water system (0.2  $\mu$ m filter).

## Cell lines

The human liver cancer cell lines HepG2 and mouse colon cell lines C26 were purchased from the American Tissue Culture Collection (USA). HepG2 cells and C26 cells were incubated in Dulbecco's-modified Eagle's medium (Life Technologies, Bedford, Massachusetts) supplemented with heat-inactivated fetal bovine serum (Gibco, Auckland, New Zealand), penicillin (100 U/ml, Sigma-Aldrich) and streptomycin (100 µg/ml, Sigma-Aldrich), and maintained at 37 °C with 5 % CO<sub>2</sub> in a humidified incubator.

## Preparation of conventional barbigerone–liposome (Bar/liposome)

Conventional Bar liposomes (Bar/liposomes or CD-free liposomes) were prepared by the ethanol-injection method. In brief, SPC (250 mg), cholesterol (6.25 mg), MPEG<sub>2000</sub>–DSPE (2.5 mg) and Vitamin E (2.5 mg) were dissolved in absolute ethanol and the solution was kept stirring at 50 °C for 20 min in a round-bottomed flask. 10 mg Bar was added into the solution and kept stirring for 10 min. Meanwhile, 5 % glucose solution was heated to 60 °C in a water bath. The organic phase was injected vertically into the aqueous phase using a 10 ml syringe under vigorous stirring where the conventional liposomes were formed. The organic solvent was removed at 50 °C on a rotary evaporator (Buchi, Switzerland) to obtain the Bar liposome aqueous solution. Then the liposomal solution was filtered through polycarbonate filters (0.2 µm, Millipore) to remove the free Bar.

## Preparation of Bar/HP-β-CD/liposome

Preparation of the Bar/HP-β-CD/liposome was performed by a method [17] (Fig. 1b) similar to the conventional Bar liposome. Briefly, 300 mg HP-β-CD were dissolved in absolute ethanol and the solution was kept stirring at 50 °C for 20 min in a round-bottomed flask. Bar (10 mg) was added into the solution and kept stirring for 30 min to form the inclusion complex named Bar/HP-β-CD. Then SPC (70 mg), cholesterol (18 mg), MPEG<sub>2000</sub>–DSPE (12 mg) and Vitamin E (1 mg) were added into the Bar/HP-β-CD solution. The solvent mixture was kept at 50 °C under moderate stirring until a clear solution was obtained. Meanwhile, 5 % glucose solution was heated to 60 °C in a water bath. The organic phase was injected vertically into the aqueous phase using a 10 ml syringe under vigorous stirring where the Bar/HP-β-CD/liposome complex was formed. The organic solvent was removed at 50 °C on a rotary evaporator (Buchi, Switzerland) to obtain the Bar/HP-β-CD/liposome aqueous solution. A 5 % glucose solution was added to

achieve a final Bar concentration of Bar 1 mg/ml and the HP-β-CD–Bar–liposome aqueous solution was filtered through a polycarbonate filter (0.2 µm, Millipore). Finally, the prepared HP-β-CD–Bar–liposome solution was sealed in a cillin vial and stored at 4 °C for further experiments. A blank HP-β-CD liposome solution free of Bar was similarly prepared.

## Characterization of Bar/HP-β-CD/liposome

### High performance liquid chromatography analysis

The concentration of Bar and drug content of Bar/HP-β-CD/liposome were determined by reverse-phase HPLC (Waters e2695 using a Waters 2998 photodiode array detector). Chromatographic separations of Bar was performed on a reversed phase C<sub>18</sub> column (4.6 × 150 mm, 5 µm, Sunfire column) with methanol/water (80/20, v/v %) as the mobile phase. The flow rate was 1 ml/min and the injecting volume was 10 µl.

### Determination of encapsulation efficiency

The liposome encapsulation efficiency (EE) was determined by gel-exclusion chromatography. Non-entrapped Bar and/or the corresponding inclusion complex (Bar/HP-β-CD) were separated from drug loaded liposomes by a Sephadex G-50 (Sigma) column with distilled water as the eluent. The column was presaturated with blank HP-β-CD–liposome. The collected liposomes were lysed by methanol and subsequently analyzed for drug content by HPLC. The percent encapsulation efficiency (EE%) was then calculated according to the following equation:

$$EE\% = (\text{drug in the vesicles}/\text{total drug}) \times 100.$$

### Determination of particle size and zeta potential

The average particle size and zeta potential of Bar/HP-β-CD/liposome were calculated by laser diffraction particle sizer (Nano-ZS, Malvern Instruments, Malvern, UK) at a temperature of 25 ± 0.1 °C. The intensity of the laser light scattered by the samples was detected at an angle of 90° with a photomultiplier. For particle size measurement, the Bar/HP-β-CD/liposome solution was suitably diluted by distilled water to avoid multiple-scattering phenomena. All the results were the mean of at least three test runs.

For the zeta potential determination, Bar/HP-β-CD/liposome was properly diluted with distilled water and measured at 25 °C by laser Doppler spectroscopy (Nano-ZS, Malvern Instruments, Malvern, UK), where each sample was analyzed at least three times.

### Transmission electron microscopy (TEM)

The morphology of the prepared Bar/HP- $\beta$ -CD/liposome was observed under a transmission electron microscope (TEM, H-6009IV, Hitachi, Japan). The Bar/HP- $\beta$ -CD/liposome was diluted with distilled water and placed on a copper grid and covered with nitrocellulose. Then the sample was negatively stained with phosphotungstic acid and dried at room temperature before observation.

### In vitro release study

The in vitro release kinetics of Bar from Bar/HP- $\beta$ -CD/liposome was analyzed using the dialysis technique against phosphate buffer solution (pH 7.4) at 37 °C. Briefly, 0.3 ml of Bar/HP- $\beta$ -CD/liposome was placed in a dialysis bag (molecular mass cutoff 8000–14,400), and 0.3 ml of Bar solution in water (1 mg/ml, where HP- $\beta$ -CD was used to increase the solubility of Bar) was used as control. The dialysis bags were subsequently sealed and then incubated in 40 ml PBS (pH 7.4) containing Tween-80 (0.5 %, w/v). The system was placed on a thermostatic shaker (37 °C, 100 rpm). At predetermined time intervals, 0.5 ml of the release buffer was withdrawn for analysis and an equal volume of fresh PBS was added. The concentration of Bar released from Bar/HP- $\beta$ -CD/liposome was determined by HPLC.

### Stability of Bar/HP- $\beta$ -CD/liposome

The stability of Bar/HP- $\beta$ -CD/liposome was studied over 12 weeks by incubation of the liposomal suspensions at 4 °C. During the incubation period, the macroscopic appearance and particle size were monitored at specific time intervals. The liposome formulations were sized by laser diffraction particle sizer (Nano-ZS, Malvern Instruments, Malvern, UK) at a temperature of  $25 \pm 0.1$  °C. All the results were the mean of at least three test runs.

### Hemolytic test in vitro

Hemolysis measurements were performed on Bar/HP- $\beta$ -CD/liposome according to the ChP 2010 [18]. In brief, a 0.3 ml sample (1 mg/ml) was added into a test tube. After dilution with 2.5 ml normal saline, a 2.5 ml rabbit erythrocyte suspension (2 % in normal saline) was added at 37 °C. Distilled water and normal saline were used as positive and negative controls, respectively. After incubation at 37 °C for 3 h, the color of the supernatant was compared with those of the controls. If the supernatant solution was red, it suggested that hemolysis occurred. In contrast, the absence of hemolysis was indicated by a corresponding absence of color in the supernatant solution.

### In vitro cytotoxicity assay

HepG2 cells and C26 cells were chosen for this study. In vitro cytotoxicity of Bar/HP- $\beta$ -CD/liposome was evaluated by MTT test, and free Bar was used as control. Typically, 100  $\mu$ l samples of exponentially growing cells were seeded in a 96-well plate at a final density of  $4 \times 10^4$  cells/ml, and cultured for 24 h. After that, the cells were exposed to Bar/HP- $\beta$ -CD/liposome (in sterilized water) and free Bar (in DMSO, the final concentration of DMSO in medium is less than 0.3 % v/v) at the indicated dosages, respectively. After incubation at 37 °C in 5 % CO<sub>2</sub> for 48 h, 20  $\mu$ l MTT (5 mg/ml, dissolved in normal saline) were added into each well and incubated at 37 °C for another 3 h. After removing the incubated medium, the plate was treated with 150  $\mu$ l of DMSO and were gently shook for 10 min to dissolve the formazan crystals. The absorbance was measured at 570 nm using a Spectramax M5 microtiter plate luminometer (Molecular Devices, USA). Cell viability was given as the ratio of the absorbance of treated cells to that of a blank control.

### In vivo tumor growth inhibition study

#### Subcutaneous xenograft mice model

Female athymic BALB/C nude mice (6–7 weeks) were obtained from Beijing HFK Biotechnology Co., Ltd. (Beijing, China) and kept in an SPF facility with free access to standard chow and water. Our investigations were performed after approval by the Institutional Animal Care and Use Committee at Sichuan University and in accordance with principles of Laboratory Animal Care. To prepare tumor-bearing mice, HepG2 cells at a number of  $3\text{--}4 \times 10^6$  were suspended in a 0.1 ml of culture medium and subcutaneously inoculated at the right flank of mice using a 1.0 ml syringe.

#### In vivo antitumor activity

When the tumor grew up to 100 mm<sup>3</sup> in volume, the mice were randomly divided into three groups (n = 10). Bar/HP- $\beta$ -CD/liposome (10, 5 mg/kg) and normal saline were administered intravenously through the tail vein every day for 3 weeks. Group one was treated with Bar/HP- $\beta$ -CD/liposome (5 mg/kg of Bar) and group two was treated with Bar/HP- $\beta$ -CD/liposome (10 mg/kg of Bar). Normal saline was injected into the control group. Tumor volume was measured with a vernier caliper in two dimensions, and was estimated according to the formula  $V$  (mm<sup>3</sup>) =  $ab^2 \times 0.50$ , where a and b are the largest and smallest tumor diameters, respectively, and V is the tumor volume in mm<sup>3</sup>. Tumor volume and body weight were checked every other day,

while mice mortality was checked daily. At the end of the experiment, four mice of each group were killed by cervical dislocation and the entire tumor mass was quickly removed and weighed. The remaining six were kept for observation of life span. For survival studies, mice showing more than 20 % body loss or tumors larger than 2500 mm<sup>3</sup> were considered moribund and sacrificed.

## Statistics

Statistical analysis was performed using the SPSS 17.0 software. One-way ANOVA was used for analysis of significance of the difference between controls and treatment groups.  $P < 0.05$  was considered to be statistically significant.

## Results

### Bar/HP- $\beta$ -CD/liposome preparation and encapsulation efficiency

In our study, to incorporate the Bar/HP- $\beta$ -CD inclusion complex into liposomes, the simple and reproducible ethanol-injection method was used. The physical appearance of the liposomal complexes were found to be affected by the mass ratios of Bar/HP- $\beta$ -CD/lipid. As the mass ratio of Bar/HP- $\beta$ -CD/lipid was 1:30:10, a transparent solution with bluish opalescence can be obtained before filtration by 0.2  $\mu$ m filters and the prepared liposomal solution were stable for at least 48 h with no free drug precipitation (Table 1). The mass ratios of Bar/HP- $\beta$ -CD/lipid also play a significant role in the final vesicle EE. When an initial Bar/HP- $\beta$ -CD/lipid mass ratio of 1:30:10 was used for Bar/HP- $\beta$ -CD/liposome, the calculated EE was less than 15 %. As the lipid-to-Bar/HP- $\beta$ -CD complex mass ratio of Bar/

HP- $\beta$ -CD/liposome increased, the amounts of Bar loaded into the liposome obviously increased (Table 1). As the mass ratio was 1:30:35, the EE increased to 60.78 %, almost 4.6 times higher than that of conventional liposome (in the absence of HP- $\beta$ -CD, Bar/liposome, where the drug-to-lipid mass ratio was 1:35, EE% <13 %). These results suggest that the amount of Bar entrapped by the liposome increased in case Bar was encapsulated in the form of Bar/HP- $\beta$ -CD inclusion complex in comparison with Bar alone, and a relatively high EE could be achieved as the lipid-to-Bar mass ratio was increased from 10, 20, 30 up to 35 (Table 1).

### Liposome properties

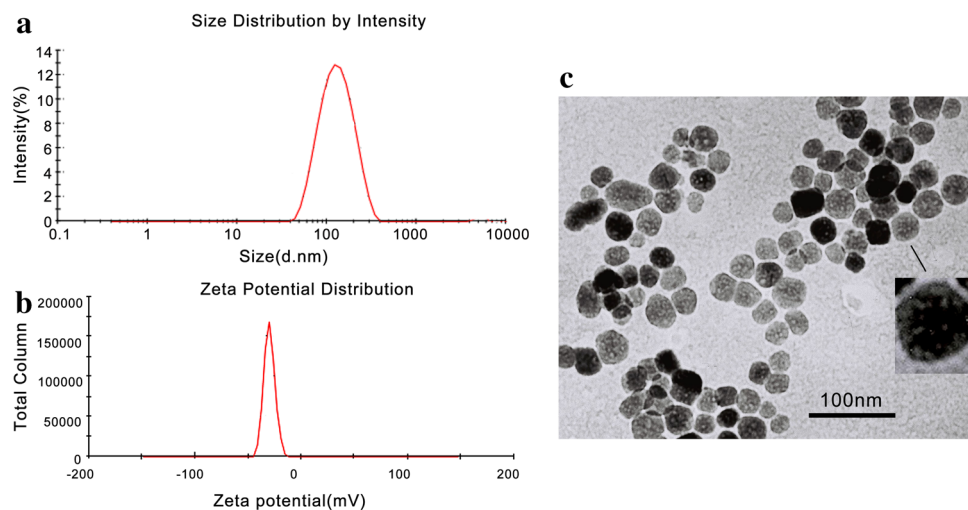
The mean particle size and zeta potential of Bar/HP- $\beta$ -CD/liposome are shown in Fig. 2a, b. The average size of the liposomes was  $108.2 \pm 3.37$  nm. When filtration was used in the liposome preparation, a better control over particle size was obtained. After final filtration through 0.2  $\mu$ m pore size, the average size was significantly reduced to  $87.76 \pm 1.45$  nm with a polydispersity index (PDI) of  $0.244 \pm 0.002$ . This relatively low PDI indicated that the liposome populations were homogeneous in size. As to the surface charge, significant differences were found between the  $\zeta$  potentials values of conventional Bar/liposomes ( $2.02 \pm 0.16$  mV) and liposomes encapsulating Bar/HP- $\beta$ -CD inclusion complexes ( $-35.02 \pm 0.50$  mV). The zeta potential indicates the degree of repulsion between same charged particles. A colloid dispersion with a high zeta potential, either negative or positive, will confer stability. Thus, the relatively high magnitude of zeta potentials (<30 mV) of Bar/HP- $\beta$ -CD/liposome indicated that the liposomal particles exhibited sufficient interparticle repulsion to prevent aggregation.

**Table 1** Effect of variation of the lipophilic phase composition on the stability and percent drug encapsulation efficiency (EE) of the Bar/HP- $\beta$ -CD/liposome

| Barbigerone | HP- $\beta$ -CD | Lipids <sup>a</sup> | Physical appearance                 | Time for precipitation | EE (%) |
|-------------|-----------------|---------------------|-------------------------------------|------------------------|--------|
| 1           | 0               | 35                  | Turbid                              | <5 min                 | 12.78  |
| 1           | 0               | 20                  | Turbid                              | <5 min                 | <10    |
| 1           | 10              | 10                  | Turbid                              | <5 min                 | –      |
| 1           | 20              | 10                  | Turbid                              | <5 min                 | –      |
| 1           | 20              | 20                  | Cloudy                              | 0.5–1 h                | –      |
| 1           | 30              | 10                  | Transparent with bluish opalescence | >48 h                  | 14.72  |
| 1           | 30              | 20                  | Transparent with bluish opalescence | >48 h                  | 29.86  |
| 1           | 30              | 30                  | Transparent with bluish opalescence | >48 h                  | 50.40  |
| 1           | 30              | 35                  | Transparent with bluish opalescence | >48 h                  | 60.78  |

<sup>a</sup> Lipids: SPC, MPEG<sub>2000</sub>-DSPE and cholesterol

**Fig. 2** Characterization of HP- $\beta$ -CD–Bar–liposome. **a** The size distribution spectrum, **b** the zeta potential, and **c** the transmission electron microscopic (TEM) image of liposomes encapsulating Bar/HP- $\beta$ -CD inclusion complex

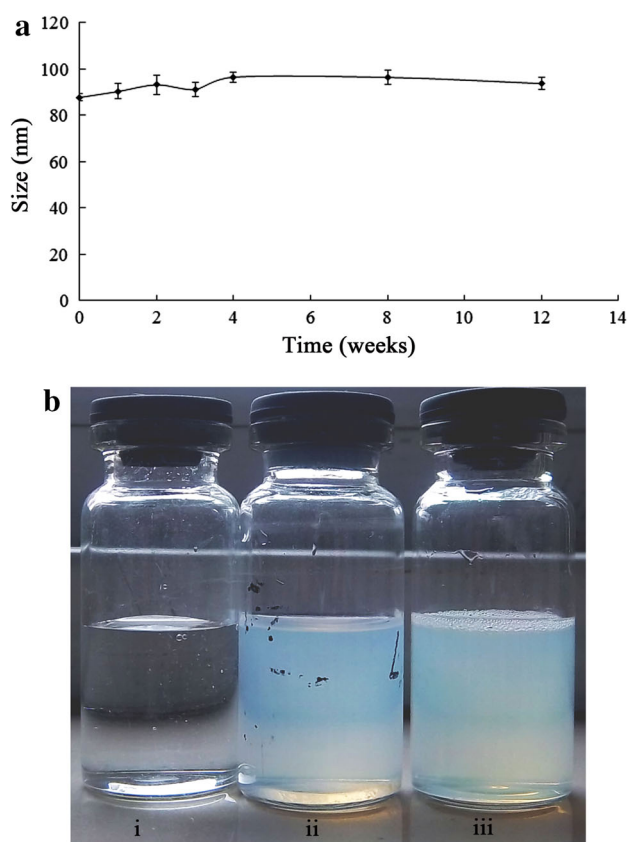


The morphologies adopted by Bar/HP- $\beta$ -CD/liposomes are depicted in Fig. 2c, where it is obvious from the TEM image that all liposomes are spherical and well-formed with a homogeneous sizes below 60 nm. The higher magnification inset in the TEM images illustrates finer details of Bar/HP- $\beta$ -CD/liposomes.

The authors studied the *in vitro* stability of Bar/HP- $\beta$ -CD/liposomes over 12 weeks by incubating them at 4 °C. The change in particle size was evaluated and the results of stability are presented in Fig. 3. As shown in Fig. 3, the Bar/HP- $\beta$ -CD/liposomes maintained its stability for at least 12 weeks. There was no significant changes in the liposome particle sizes and the liposomal suspensions maintained their bluish opalescence without precipitation throughout the incubation period.

### In vitro release studies

In order to maintain the sink condition during the release study, 0.5 % (v/v) of Tween-80 was added in the release medium (PBS, pH 7.4). Although the solubility of Bar increased up to 48.25  $\mu$ g/ml with the addition of 1 % (v/v) Tween-80 in the release medium, 0.5 % (v/v) Tween-80 already achieved a solubility of 17.54  $\mu$ g/ml (Bar) high enough to maintain the sink condition (Table 2). Figure 4 shows the release profiles of Bar/HP- $\beta$ -CD inclusion complex and Bar/HP- $\beta$ -CD/liposomes in PBS (pH 7.4, 37 °C). A sustained and slower Bar release from Bar/HP- $\beta$ -CD/liposomes than from the corresponding Bar/HP- $\beta$ -CD was observed. Bar was more quickly released from the Bar/HP- $\beta$ -CD inclusion complex. For example, approximately 80 % of Bar was released from the inclusion complex in the first hour, whereas only 35 % of Bar was released from the Bar/HP- $\beta$ -CD/liposomes within the same time period. The release of Bar from the liposomal formulation showed an initial burst release of about 25 % of Bar within the first



**Fig. 3** Stability of Bar/HP- $\beta$ -CD/liposome. **a** Mean particle size variation with time of storage, and **b** photographs of (i) Bar/HP- $\beta$ -CD inclusion complex, (ii) Bar/HP- $\beta$ -CD/liposome and (iii) blank HP- $\beta$ -CD/liposome after 12 weeks of storage

0.5 h, but the release became steady at a lower rate thereafter up to 12 h. These results indicate that it takes more time for Bar to be released once entrapped into liposomes as an inclusion complex, where a depot effect could be achieved when compared with the inclusion

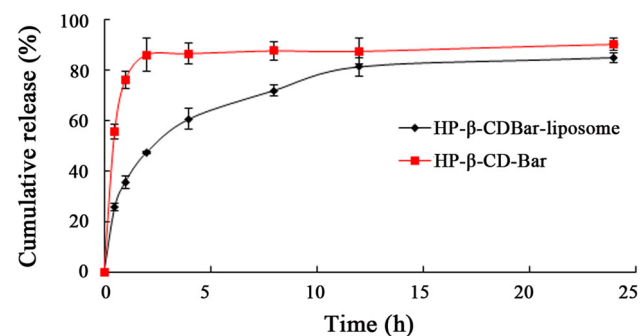
**Table 2** Effect of Tween-80 on the solubility of barbigerone (37 °C)

| Medium             | Ionized water | PBS   | 0.5 % Tween-80 in PBS | 1 % Tween-80 in water | 1 % Tween-80 in PBS |
|--------------------|---------------|-------|-----------------------|-----------------------|---------------------|
| Solubility (µg/ml) | 0.481         | 0.392 | 17.54                 | 42.428                | 48.246              |

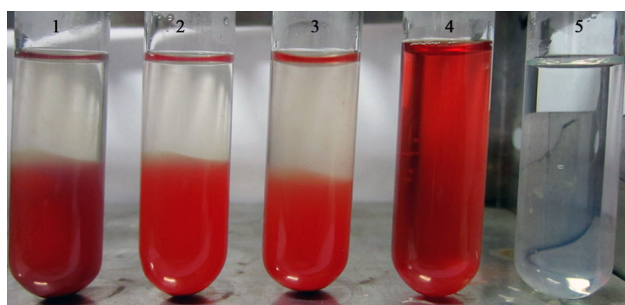
complex formulation. Therefore, the liposome entrapped Bar/HP-β-CD complex appears suitable for further sustained release formulation studies of Bar prior to a probable anticancer treatment.

### Hemolysis

Hemolysis is the destruction of red blood cells resulting in the release of hemoglobin into the surrounding fluid, which is highly toxic to the body. CDs have been reported to cause hemolysis [19, 20]. Thus, the hemolytic activity of Bar/HP-β-CD/liposomes was tested based on ChP. As shown in Fig. 5, Bar/HP-β-CD/liposomes proved non-hemolytic similar to the negative control (normal saline), thus indicating that the prepared liposomal inclusion



**Fig. 4** In vitro drug release profiles of the Bar/HP-β-CD inclusion complex and Bar/HP-β-CD/liposome in PBS solution at pH 7.4

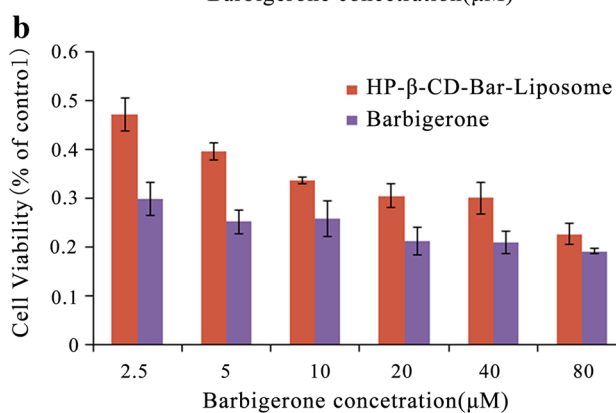
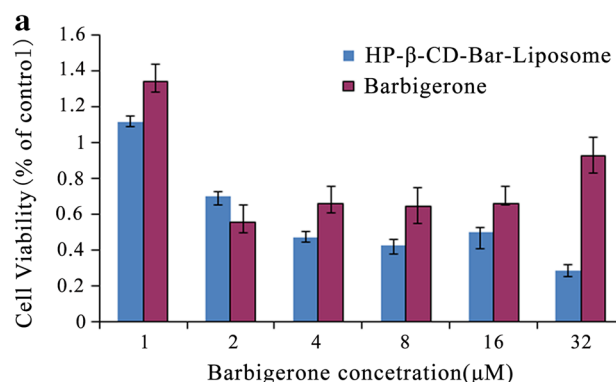


**Fig. 5** Hemolytic test of the Bar/HP-β-CD/liposome. This image was taken 3 h after reaction began. (1), (2) Bar/HP-β-CD/liposome, (3) normal saline used as negative control, (4) distilled water used as positive control, and (5) erythrocyte suspension

complex might serve as a safe vector for intravenous injection.

### In vitro cytotoxicity study

To investigate the cytotoxicities of Bar/HP-β-CD/liposome and free Bar, HepG2 cells and C26 cells were exposed to a series of equivalent concentrations of the Bar/HP-β-CD/liposome and of free Bar for 48 h, and the viability of tumor cells was tested using an MTT assay. As shown in Fig. 6a, the viability of C26 cells decreased as the Bar/HP-β-CD/liposome concentration increased, while Fig. 6b shows the viability of HepG2 cells using different concentrations of Bar/HP-β-CD/liposome and of free Bar. After 24 h incubation, both Bar/HP-β-CD/liposome and



**Fig. 6** In vitro anticancer activity of Bar/HP-β-CD/liposome in **a** murine colon cancer C26 cells and **b** human hepatocarcinoma HepG2 cells (**b**) after 48 h treatment with plain Bar or Bar/HP-β-CD/liposome (data were given as mean ± SD, n = 3)

free Bar could dramatically inhibit HepG2 cell growth in a dose-dependent manner. It is interesting to note that the Bar/HP- $\beta$ -CD/liposome formulation was almost as equipotent as free Bar in both cell lines. These results suggest that for both human and mouse tumor cells, Bar/HP- $\beta$ -CD/liposome did not lose its toxicity, thus comparing well with free Bar.

### Inhibition of tumor growth

The antitumor efficacy of Bar/HP- $\beta$ -CD/liposome was studied in HepG2 liver cancer bearing nude mice. The progress of tumor growth is shown in Fig. 7a. It was found that for groups injected with the Bar/HP- $\beta$ -CD/liposome, tumor growth was more significantly inhibited than in groups injected with normal saline (Fig. 7a–c). At the dose of 10 mg/kg, tumor weight on day 21 was 50.74 % of that of the saline-treated group. When analyzed by ANOVA, the difference between control and treatment groups (10 mg/kg) were significant ( $P < 0.05$ ), whereas the differences between the two dose groups were not significant ( $P > 0.1$ ).

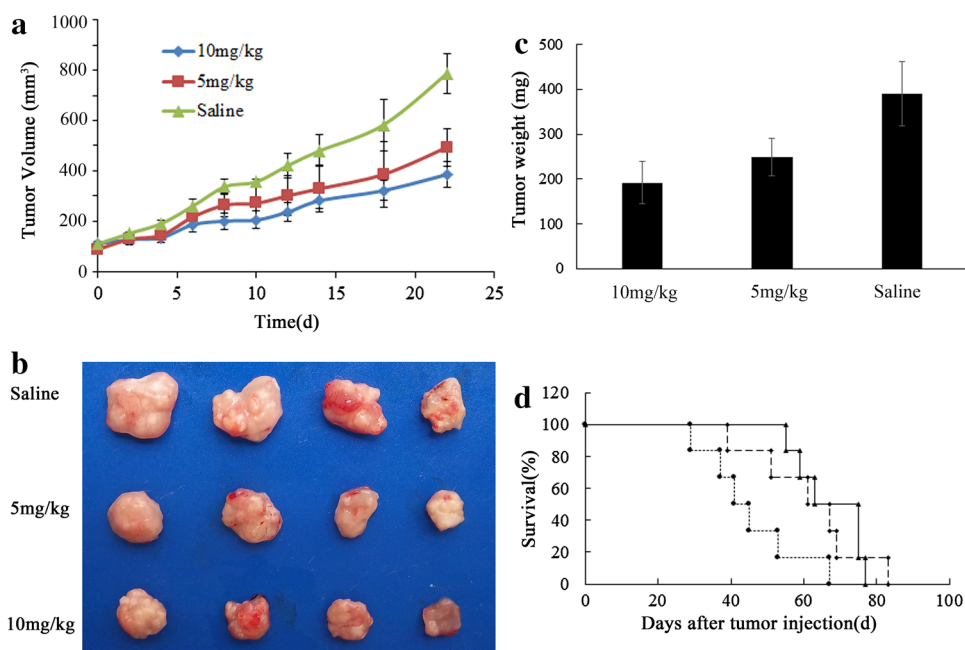
The survival time of tumor-bearing mice was evaluated following the treatment with Bar/HP- $\beta$ -CD/liposome. As shown in Fig. 7d, a significant extension of the mean survival time was observed, following the treatment of Bar/HP- $\beta$ -CD/liposome in HepG2-bearing mice in a dose-dependent manner. The mean survival time for mice treated with normal saline was  $45.33 \pm 5.43$  days. However, treatment with 5 mg/kg of Bar/HP- $\beta$ -CD/liposome increased the survival time to  $61.27 \pm 6.23$  days, while treatment with 10 mg/kg

of Bar/HP- $\beta$ -CD/liposome significantly increased the survival time to  $67.33 \pm 3.88$  days (Fig. 7d). The difference of life span (survival time) between treatment groups and control group were shown to be significant ( $P < 0.05$  for 5 mg/kg, and  $P < 0.01$  for 10 mg/kg). These results indicate that Bar/HP- $\beta$ -CD/liposome could prolong the survival time of tumor-bearing mice.

### Discussion

Liposomes can entrap hydrophobic drugs in the lipid bilayers and hydrophilic drugs in the aqueous phase. However, the hydrophobic nature of Bar limits the amount of drug that can be delivered by liposomes. Our initial attempts at preparing Bar/liposome using conventional techniques resulted in poor EE. For example, the EE was less than 13 % when the conventional thin film method or ethanol injection methods were used for liposome preparation. Apparently, the lipid bilayer of the liposomes did not effectively entrap Bar, while the water solubility of Bar was not high enough for its accommodation within the aqueous core. Recently, entrapment of water-insoluble drug into the aqueous phase of liposome in the form of water-soluble CD inclusion complexes has been investigated as a new potential strategy for merging the relative advantages of the two types of carriers into a single system [14]. The utilization of such a combined carrier system may prove useful in increasing drug solubility, stability and providing a better control over the release rate of poorly soluble drugs [21, 22]. To increase the EE and stability of

**Fig. 7** The effect of Bar/HP- $\beta$ -CD/liposome on the inhibition of tumor growth in nude mice inoculated with HepG2 cells. **a** Tumor volume, **b** photograph of tumors, **c** tumor weight, and **d** survival rate. The results were shown as mean  $\pm$  SE





liposome, HP- $\beta$ -CD, which is a highly water-soluble member of the CD family, was chosen as the water soluble carrier to load Bar. By forming a water-soluble complex with HP- $\beta$ -CD, the water solubility of Bar increases from 0.5  $\mu\text{g/ml}$  to 6.83  $\text{mg/ml}$  [23]. This  $\sim 10^4$ -fold increase in aqueous solubility allows the possibility of the Bar/HP- $\beta$ -CD complex entrapment into the aqueous phase of vesicles.

In this study, Bar/HP- $\beta$ -CD/liposome was successfully prepared using a simple ethanol injection method. liposome entrapment of the Bar/HP- $\beta$ -CD inclusion complex resulted in an appreciable EE enhancement over that of the conventional Bar/liposome. The EE of conventional Bar/liposome only reached up to 12.78 %, while that of the Bar/HP- $\beta$ -CD/liposome approached 60.78 % (Table 1), which was 4.6 times that of the conventional Bar/liposome. The drug-to-lipid mass ratio of liposome plays a significant role in the final liposome EE. As the amount of lipids increased, the amount of Bar might also increase. It is clear from the TEM image (Fig. 2c) that the prepared Bar/HP- $\beta$ -CD/liposome are spherical and well formed. However, the mean sizes of the liposomal complexes estimated through DLS typical measurements were relatively higher than those obtained from TEM micrographs, which is likely a consequence of the enhanced scattering from the minimal number of aggregated vesicles that are presented, even at the high dilutions used in DLS measurements. The conflicting mean particle size estimated by DLS and TEM have also been reported in the recent literature [24, 25].

The data and images described in Fig. 3 demonstrate that Bar/HP- $\beta$ -CD/liposome remained stable on storage in liquid form for at least 12 weeks. The increase in mean particle size estimates due to the aggregation or fusion of unstable liposomes during the preparation processing and/or upon storage is typical of the instability observed in liposome formulations. For example, as liposomes encounter one another in a suspension, they are prone to adhere and fuse to form larger aggregates [26]. An increase in liposome particle size commonly results in rapid uptake by the reticuloendothelial system with subsequent rapid clearance and, hence, a short half-life [27]. Thus, it is of critical importance to maintain and control liposomes at small and uniform sizes in developing a viable liposomal product. It is interesting to note that the particle sizes of Bar/HP- $\beta$ -CD/liposome remained almost constant for up to 12 weeks. Though the interaction between CDs and lipid molecules may lead to some kind of instability, the special composition of liposomal membrane (with PEG-DSPE in the liposome composition) and the relatively low zeta potential may have reduced liposome aggregation thus leading to the observed stability. Moreover, liposomes with large negative or positive zeta potentials ( $-35.02 \pm 0.50$  mV) do repel each other and remain monodisperse thus imparting more stability. It was

generally understood that liposomes with zeta potentials either in excess of +30 mV or below  $-30$  mV remain stable in the aqueous phase. In addition, it was demonstrated that PEG modification can prevent in vitro aggregation of negatively charged liposomes [28]. Therefore, the stability observed for Bar/HP- $\beta$ -CD/liposome suggests that the liposomal inclusion complex of Bar meets the requirement for a potentially viable drug delivery system.

The in vitro release study described in Fig. 4 showed that Bar/HP- $\beta$ -CD/liposome exhibited delayed release characteristics that might be related to a favorably combined CD inclusion complex action plus liposome action. The drug release from Bar/HP- $\beta$ -CD/liposome could be sustained-release and continuously supplied the dose in the treatment of cancer avoiding the associated adverse effects. The in vitro cytotoxicity study showed that Bar/HP- $\beta$ -CD/liposome retained potent anticancer activity close to that of free Bar. In aspects of in vivo efficacy study, the Bar/HP- $\beta$ -CD/liposomes seemed to provide benefits in terms of reducing tumor volume against hepatic cancer, which correlated with a substantial increase in animal survival.

## Conclusion

The present study demonstrated that Bar/HP- $\beta$ -CD/liposome formulations, which incorporated Bar into liposomes in the form of Bar/HP- $\beta$ -CD inclusion complex, giving rise to a significant enhancement of EE over that of the conventional Bar/liposome. This liposomal inclusion complex remained stable in liquid form at 4 °C for at least 3 months. In terms of in vitro efficacy studies, the Bar/HP- $\beta$ -CD/liposome exhibited comparable cytotoxicities against colon cancer and hepatic cancer cells. Further in vivo studies showed that the Bar/HP- $\beta$ -CD/liposome inhibited hepatocarcinoma cell growth in murine xenograft models with a substantial increase in animal survival. Our study clearly indicated that Bar/HP- $\beta$ -CD/liposome provides an alternative means of overcoming the low aqueous solubility of Bar, on the other hand, and an encapsulation of the inclusion complex would very likely serve as a potent Bar delivery vehicle for future cancer chemotherapy.

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