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Inclusion complex of barbigerone with hydroxypropyl-β-cyclodextrin: Preparation and *in vitro* evaluation

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ABSTRACT

The aim of this study was to improve the water solubility of barbigerone by complexing it with hydroxypropyl-β-cyclodextrin (HP-β-CD). The inclusion complexation behavior, characterization and interactions of barbigerone with HP-β-CD were investigated in both solution and the solid state by means of UV/VIS, ^{1}H NMR, FT-IR, PXRD, SEM. All the characterization information demonstrated the formation of barbigerone-HP-β-CD (bar-HP-β-CD) inclusion complex, and the bar-HP-β-CD inclusion compounds exhibited different spectroscopic features and properties from barbigerone. The results demonstrated that the water solubility of barbigerone was notably increased in the presence of HP-β-CD. Furthermore, preliminary *in vitro* cytotoxicity assay showed that bar-HP-β-CD still maintain the anticancer activity of barbigerone. These results suggest that HP-β-CD will be potentially useful in the delivery of water-insoluble anticancer agents such as barbigerone.

1. Introduction

Barbigerone (2′,4′,5′-trimethoxy-6″,6″-dimethylpyrano(2″,-3″:7,8) (Fig. 1a), a naturally occurring isoflavone, was first isolated from the seeds of *Tephrosia barbigerain* 1979 (Villain, 1980) and could be found in other plants (Wangensteen, Alamgir, Rajia, Samuelsen, & Malterud, 2005; Yenesew, Midiwo, & Waterma, 1998). It has been reported to have diverse pharmaceutical properties, including antioxidant, 15-lipoxygenase inhibitory activity (Wangensteen et al., 2006), and anti-plasmodial activity against the malarial parasite *Plasmodium falciparum* (Yenesew et al., 2003). Our previous studies have shown that barbigerone can inhibit murine lung cancer cell proliferation by inducing apoptosis via mitochondrial apoptotic pathway and by decreasing phosphorylated p42/44 MAPK and Akt (Li et al., 2009). In the present study, barbigerone was found to effectively suppress tumor angiogenesis and tumor growth in vitro and in vivo. Further studies indicated that barbigeronge inhibited tumor angiogenesis and tumor growth through VEGFR2 signaling pathways (Li, Wang, Ye, Peng, & Chen, 2012). Moreover, it exhibited less toxicity to non-cancer cells than tumor cells (Li et al., 2009). However, the aqueous solubility of barbigerone is almost nil, making it difficult for deep research. Therefore, it is important to introduce effective method to enhance the solubility of barbigerone.

Cyclodextrins, a family of cyclic amylose-derived oligomers with a hydrophilic outer surface and a lipophilic central cavity, are well known for its abilities to form inclusion complex with various guest molecules (Davis & Brewster, 2004). During the past decades, cyclodextrins has been successfully used as complexing agents to enhance the solubility, stability and bioavailability of drug molecules (Uekama, Hirayama, & Irie, 1998; Loftsson & Duchêne, 2007). The α-, β-, and γ-cyclodextrin are the most common cyclodextrins used as formulation vehicles consisting of six, seven, and eight D–(+)–glucopyranose units attached by α-1,4 linkage (Loftsson & Brewster, 1996). β-Cyclodextrin is the most useful and the lowest price, however, its solubility in water is relatively low (approximately 2%) and the toxicity of β-cyclodextrin limit its further application in pharmaceutical formulations (Davis & Brewster, 2004; Irie & Uekama, 1997). 2-Hydroxylpropyl-β-cyclodextrin (HP-β-CD, Fig. 1b) is an alternative to β-cyclodextrin having a higher aqueous solubility (above 60%) and may be slightly more toxicologically benign (Gould & Scott, 2005). Recently, HP-β-CD has been widely used to improve the solubility of poorly water-soluble drugs (Liu, Qiu, Gao, & Jin, 2006; Ma et al., 2012).

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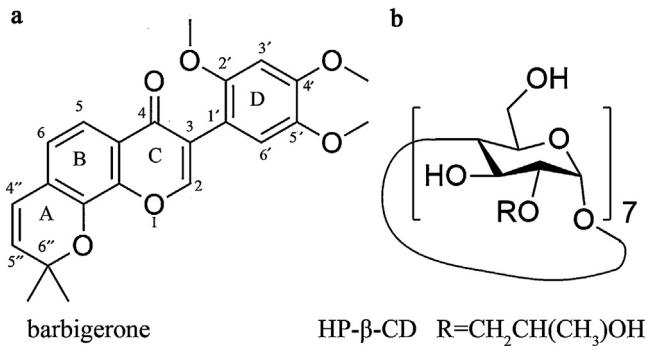

Fig. 1. The chemical structures of barbigerone and HP-β-CD.

However, to the best of our knowledge, no scientific study about the effect of cyclodextrins on enhancing solubility and dissolution rate of barbigerone has been reported.

In the present study, with the aim to increase the aqueous solubility of barbigerone, the complex of barbigerone with HP-β-CD was prepared by a simple coevaporation method. UV/Vis spectroscopy was measured to evaluate the formation of bar-HP-β-CD complex in aqueous solution. Drug/HP-β-CD interactions in solution were investigated by phase solubility analysis and the interactions in the solid state were characterized by ^{1}H NMR, FT-IR, XRD and SEM, aiming to verify the formation of inclusion complex. Finally, the inhibitory effects on cell growth of bar-HP-β-CD evaluated to prove the retaining bioactivity of free barbigerone.

2. Materials and methods

2.1. Materials

Barbigerone (≥98% in purity) was obtained from Millettia pachycarpa Benth, separated and purified in our laboratory, as reported previously (Ye et al., 2010). HP-β-CD was obtained as a gift from Shijiazhuang Pharmaceutical Group Co., Ltd. (Shijiazhuang, China). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) was purchased from Sigma (Sigma–Aldrich Inc.). Methanol [high-performance liquid chromatography (HPLC) grade] was purchased from Fisher Chemical (Loughborough, UK). All other reagents and solvents, which are analytic grade, were purchased from Ke-Long Chemical Reagent Factory (Chengdu, China) and used without further purification.

2.2. High-performance liquid chromatography analysis

Reverse-phase high performance liquid chromatography (HPLC, Waters 2695 separation module with a Waters 2998 photodiode array detector) was used to determine the concentration of barbigerone. Chromatographic analysis was performed on a C_{18} analytical column (Sunfire, 4.6 mm × 150 mm) with methanol/water (80/20, v/v%) as the mobile phase and UV detection at 264 nm. The injection volume was 10 μl and the flow rate was 1.0 ml/min.

2.3. Phase solubility study

The effects of HP-β-CD on the solubility of barbigerone were investigated according to the method established by Higuchi and Connors (1965). Excess amounts of barbigerone were added into 2 ml of aqueous HP-β-CD solutions with different concentrations (ranging from 0 to 20 mM). The obtained suspensions were shaken at 37 °C in a thermostatically controlled shaking air bath (100 rpm). After 48 h, aliquots were withdrawn, filtered through a 0.45 μm Millipore filtration to remove the excess barbigerone. The amount of barbigerone was then measured by the above-mentioned HPLC method. All experiments were carried out in triplicate.

2.4. Preparation of bar-HP-β-CD complex

A simple coevaporation method was used to prepare bar-HP-β-CD complex. Briefly, 150 mg HP-β-CD was completely dissolved in dehydrated alcohol. The solution was kept at 50 °C with moderate stirring for 30 min to let it freely swell. Then barbigerone (5 mg) was added into the system and stirred for 1 h. Then the organic solvent was evaporated under reduced pressure in a rotary evaporator at 50 °C and dried in vacuum to produce the bar-HP-β-CD complexes. The inclusion complex can be redissolved in distilled water or 5% glucose solution to obtain a clear solution for further use.

Physical mixtures: A physical mixture of barbigerone and HP-β-CD (1:1, molar ratio) were prepared by thoroughly mixing the two components in an agate mortar for 10 min.

2.5. Solubility study

Solubility studies were carried out according to the method reported previously (Hu, Zhang, Song, Gu, & Hu, 2012). In brief, excess amounts of barbigerone were added into 2 ml of water, then the suspensions were kept stirring at 25 °C for 48 h. After equilibrium attainment, samples were withdrawn and filtered through a 0.22 μm Millipore filtration to remove the excess barbigerone. The filtrate was centrifuged and injected into HPLC apparatus for analysis. The concentration of barbigerone was then calculated against a concentration-peak area calibration curve. The solubility of bar-HP-β-CD complex in water was tested in the similar procedure (Ma et al., 2012). Briefly, an excess amount of bar-HP-β-CD complexes were added to 2 ml water and the mixture were kept stirring for 1 h at 25 °C. After removing the insoluble substances, the filtrate was then injected into HPLC and the concentration of barbigerone was then determined by the standard curve of barbigerone.

2.6. Characterization

2.6.1. UV spectroscopy measurement

Complex formation between barbigerone and HP-β-CD was studied using the spectral shift method. Given the poor water solubility of barbigerone, a water/ethanol (V:V = 3:1) solution was used in the spectral measurements. The concentration of barbigerone was kept constant at 5 μg/ml while the HP-β-CD concentration was increased from 1 to 15 mmol/L. The mixtures were shaken for 1 h before the UV–vis spectrum was recorded on a Lambda 35 UV spectrophotometer (Perkin–Elmer, United States) equipped with a conventional 1 cm path (1 cm × 1 cm × 4 cm) quartz cell at 25 °C. The measurements were done in the wavelength range from 200 to 500 nm.

2.6.2. ^{1}H NMR spectroscopy

^{1}H NMR spectra were obtained on an Ascend 400 MHz NMR Spectrometer (Bruker, Switzerland). HP-β-CD, bar-HP-β-CD complexes and barbigerone were dissolved in D_2O, D_2O (or DMSO-d_6) and DMSO-d_6 respectively. Chemical shifts were reported in ppm with tetramethylsilane (TMS) as the internal standard.

2.6.3. Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectra were recorded on the Fourier transform infrared spectrometer (Nicolet 6700, Thermo Fisher Scientific, Waltham, MA) according to the KBr disk technique. Samples were prepared as KBr disks with 1 mg of complex in 100 mg of KBr. The

FTIR measurements were performed in the scanning range of 4000–400 cm^{-1} at ambient temperature.

2.6.4. Powder X-ray diffractometry (XRD)

X-Ray powder diffraction patterns were performed on a Philips X'Pert Pro diffractometer using Ni-filtered and Cu Kα radiation (45 kV, 35 mA). The scanning rate employed was 0.15°/min in a diffraction angle (2θ) range of 3°–40°.

2.6.5. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to evaluate the morphology of barbigerone, HP-β-CD, bar-HP-β-CD inclusion complex and the physical mixture. All samples were made electrically conductive by coating with a thin layer of gold before being examined using a JEOL JSM-7500F scanning electron microscope operated at 5.0 kV.

2.7. Cell culture

The human colon cancer cell line HCT116 and human liver cancer cell line HepG2 were purchased from the American Type Culture Collection (Manassas, Virginia). HCT116 and HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin, and maintained at 37 °C, 95% relative humidity, under 5% CO_2.

2.8. In vitro bioactivity study

The *in vitro* cytotoxicity of bar-HP-β-CD complex on HCT116 and HepG2 cell lines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test, and free barbigerone was used as control. Bar-HP-β-CD was dissolved in sterilized water and free barbigerone were dissolved in DMSO. Exponential growing cells were seeded in a 96-well plate at a seeding density of 4.0–5.0 × 10^{-3} per well, and cultured for 24 h. After that, harvested cells were exposed to bar-HP-β-CD or free barbigerone (diluted with DMEM, the final concentration of DMSO was less than 0.1%) at the indicated dosages. For each condition, three replicates were performed, six wells were left as blank controls. Cells were then incubated at 37 °C in a humidity atmosphere for 48 h and 20 μl MTT (5 mg/ml, dissolved in saline) was added to each well. After further incubation for 3 h at 37 °C, the incubated medium was removed and 150 μl of DMSO were added into each well to dissolve the formazan crystals. The absorbance was measured at 570 nm by 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 blank controls.

3. Results and discussion

3.1. Phase solubility studies

The phase solubility behavior of barbigerone in HP-β-CD solution was investigated at 37 °C and presented in Fig. 2A. The phase solubility study is a useful method for studying drug/cyclodextrin interactions because it provides not only the solubilizing ability of CDs but also the stability constant (K_s) of the inclusion complexes by analyzing the solubility curves (Higuchi & Connors, 1965). From the diagram, it could be observed that aqueous solubility of barbigerone increased linearly as the concentration of HP-β-CD increased, over the entire concentration range studied. The solubility curve has a slope of 0.0247 with a correlation coefficient squared values of 0.9951 ($r^2 > 0.990$) was regarded as a straight line and can be classified as A_L type. Since the slope is less than one, it was assumed that the solubility increase is due to the formation of 1:1 complex (Higuchi & Connors, 1965). The apparent 1:1 stability constants (K_c) of bar-HP-β-CD complexes was calculated from the slope of the linear plot of the diagram according to the equation K_c = slope/S_0 (1-slope), where S_0 is the solubility of pure barbigerone at the same temperature in water. The value of K_c was calculated to be 20,659 M^{-1}. The high K_c value indicates that the inclusion complex formed between barbigerone and HP-β-CD are quite stable.

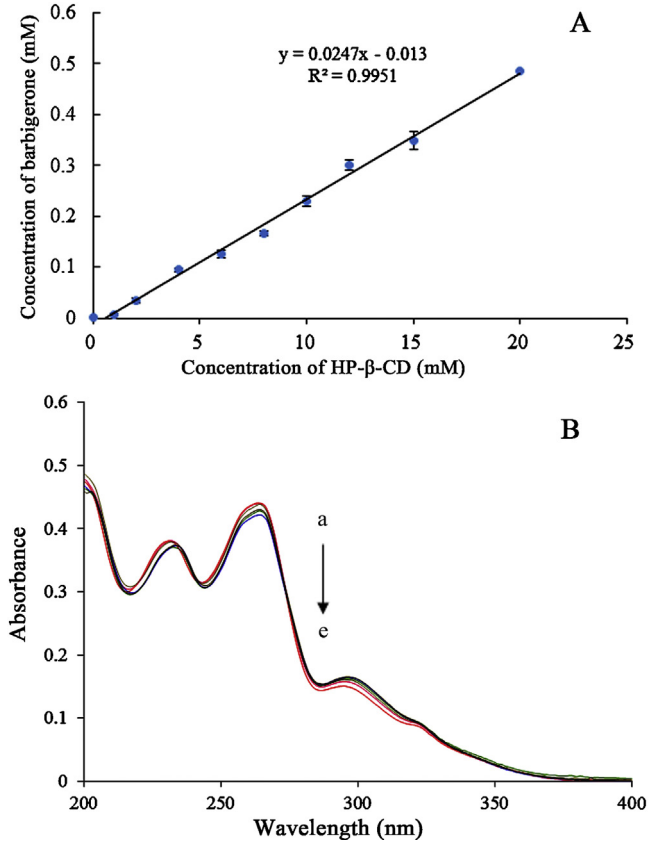

Fig. 2. (A) Phase solubility diagram of bar-HP-β-CD system. The HP-β-CD concentration was in the range of 0–20 mM. Each point is the mean of three determinations. (B) The effect of HP-β-CD concentration on the UV–vis spectra of barbigerone (5 μg/ml) in aqueous solution; the HP-β-CD concentration was (a) 0 mM; (b) 1 mM; (c) 2 mM; (d) 5 mM; (e) 15 mM, respectively.

3.2. Preparation of bar-HP-β-CD complex

In this study, a simple coevaporation method was used to prepare bar-HP-β-CD inclusion complex. Given the poor water solubility of barbigerone, absolute ethanol was used in the preparation. Barbigerone and swollen HP-β-CD were together dissolved in dehydrated ethanol instead of water, the bar-HP-β-CD inclusion complex was totally formed by self-association after stirring for only 1 h at 50 °C, while the complexation time in previous method needed at least 24 h and most of the drug still not embedded into HP-β-CD. During the experiment, the maximum mass ratio of barbigerone/HP-β-CD could reach up to 1:30 (molar ratio:1:7.9), when the amount of HP-β-CD increased, the amount of complexed barbigerone might also increase. The inclusion complex had a very good water solubility and can be directly dissolved in distilled water or 5% glucose solution. Moreover, the aqueous solution of bar-HP-β-CD complex was quite stable and can be kept at 4 °C for at least 12 weeks.

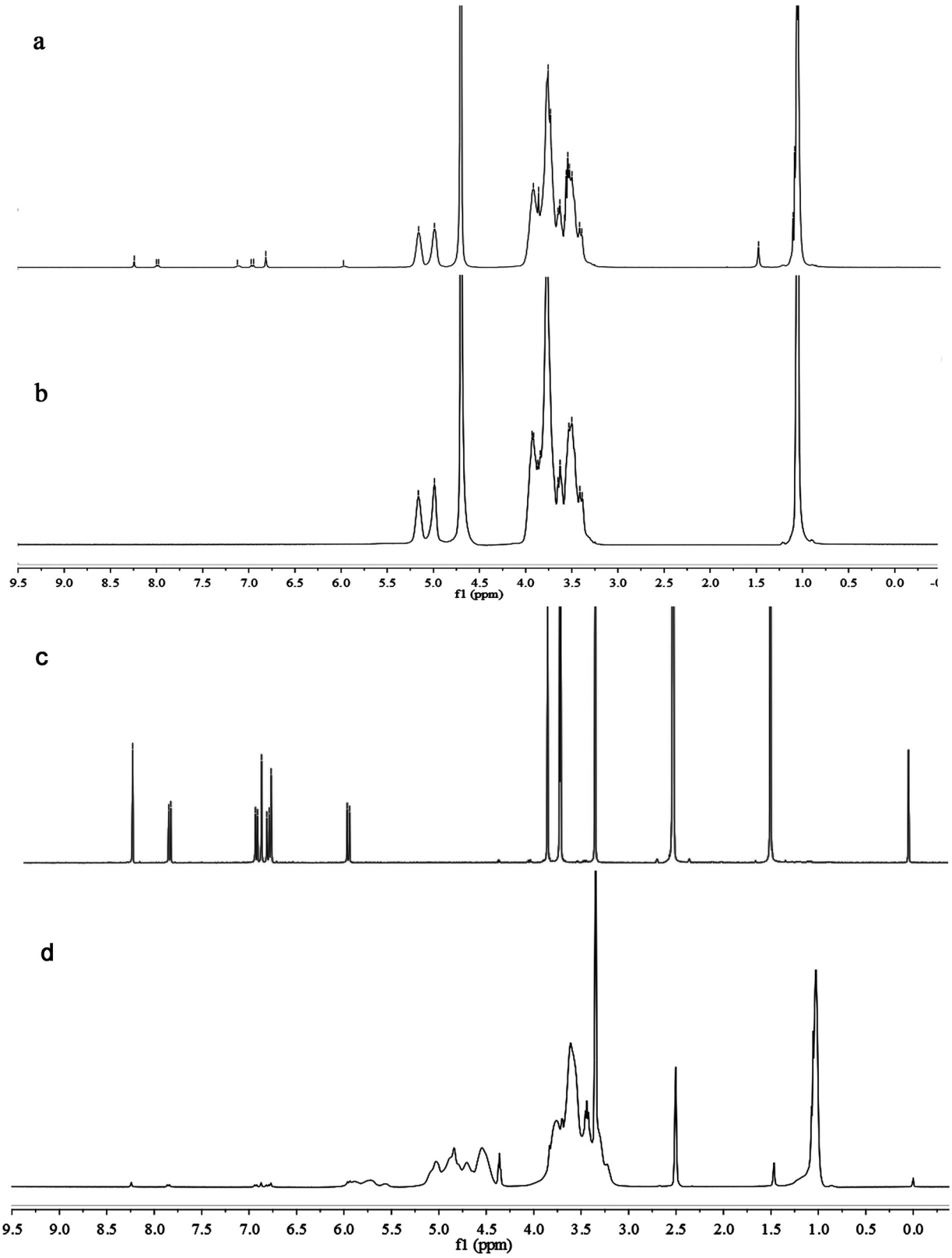

Fig. 3. ^{1}H NMR spectra.(a) Bar-HP-β-CD inclusion complex and (b) HP-β-CD in D_2O; ^{1}H NMR spectra of barbigerone (c) and (d) bar-HP-β-CD inclusion complex in DMSO-d6.

3.3. Solubility of barbigerone

The water solubility of barbigerone and complex were evaluated by preparation its saturation solution. An excess amount of barbigerone (or bar-HP-β-CD complex) was added into 2 ml distilled water and kept moderate stirring at 25 °C for 48 h. The insoluble substance was removed by filtration before the concentration of the filtrates were determined using HPLC. Although barbigerone has excellent solubility in organic solvents such as chloroform, dichloromethane and ethanol, the solubility is only about 0.5 μg/ml

Table 1
Chemical shift (δ, ppm) change values relating to the signals of HP-β-CD and barbigerone in the free and the complexed states.

Protons	HP-β-CD	Bar-HP-β-CD complex	$\Delta\delta$
HP-β-CD			
H-1	4.988	4.985	−0.003
H-2	3.531	3.541	−0.010
H-3	3.929	3.912	−0.017
H-4	3.499	3.495	−0.003
H-5	3.770	3.728	−0.042
H-6	3.770	3.754	−0.016
Me	1.045	1.041	−0.004
Barbigerone			
H-2	8.245	8.241	−0.004
H-3′	6.773	6.770	−0.003
H-6′	6.875	6.872	−0.003
H-5	7.839	7.838	−0.001
H-6	6.918	6.919	0.001
H-4″	6.793	6.793	0.000
—CH_3	1.467	1.466	−0.001

in water. In contrast, the water solubility of bar-HP-β-CD inclusion complex was remarkably increased to approximately 6.83 mg/ml. A clear solution can be obtained after dissolving the bar-HP-β-CD complex in water with a final concentration of barbigerone at 6 mg/ml. These results revealed that HP-β-CD notably improved the water solubility of barbigerone by the formation of bar-HP-β-CD inclusion complex and the water soluble bar-HP-β-CD complex will be beneficial to the medical utilization of this compound.

3.4. Characterization of bar-HP-β-CD complex

3.4.1. UV spectroscopy

UV–visible spectroscopy is an important tool to investigate the influence of the complexation on the absorption of barbigerone. The measurements were carried out in water/ethanol (V:V = 3:1) mixed solution owing to the rather low water solubility of barbigerone. As illustrated in Fig. 2B, two typical absorption peaks of barbigerone in the absence of HP-β-CD were observed at 264 nm and 231 nm, the spectrum of bar-HP-β-CD was very similar to those of barbigerone. It was noted that the absorbance intensity of barbigerone at 264 nm gradually decreased with increasing concentrations of HP-β-CD from 1 to 15 mmol/L. As the HP-β-CD concentration increased, a slightly red shift of absorption peak was observed in the curve of bar-HP-β-CD complex. These changes might be partly attributed to the shielding of chromophore groups in barbigerone molecule due to the inclusion into the HP-β-CD cavity (Chow & Karara, 1986; Liu et al., 2006). These results might suggest the possibility of the complex formation between barbigerone and HP-β-CD.

3.4.2. ^1H NMR spectroscopy

The ^1H NMR of HP-β-CD inclusion complex and bar-HP-β-CD (in D_2O) were shown in Fig. 3a and b. In the spectra of HP-β-CD, the signals for H-1, H-2, H-3, H-4, H-5 and H-6 located at 4.988, 3.531, 3.929, 3.499, 3.770 and 3.770, respectively (Table 1), were corresponding to the previous literature (Ma et al., 2012). In the spectrum for bar-HP-β-CD inclusion complex, appreciable chemical shift changes were observed with respect to the spectra for the free HP-β-CD. The chemical shifts of HP-β-CD protons in the absence and presence of barbigerone were listed in Table 1 ($\Delta\delta = \delta_{\text{bar-HP-β-CD complex}} - \delta_{\text{HP-β-CD}}$). From Table 1 we could see the signals of H-1, H-2 and H-4 protons on the outer surface of HP-β-CD changed only slightly with $\Delta\delta$ values of −0.003, −0.010 and −0.003 respectively. While significant chemical shift changes were exhibited by H-5 and H-3 protons in the inner surface of HP-β-CD with an up-field shift of 0.042 and 0.017 ppm, respectively. It is noteworthy that the chemical shift variation for H-3 was smaller than H-5 after resulting inclusion complex. Since both H-3 and H-5 protons from each sugar unit are located in the internal cavity of HP-β-CD, and H-3 protons are near the wide side of the cavity while H-5 protons near the narrow side (Bernini et al., 2004), we can propose from the ^1H NMR data that barbigerone was included in the HP-β-CD cavity, and should penetrate inside the cavity from the narrow side.

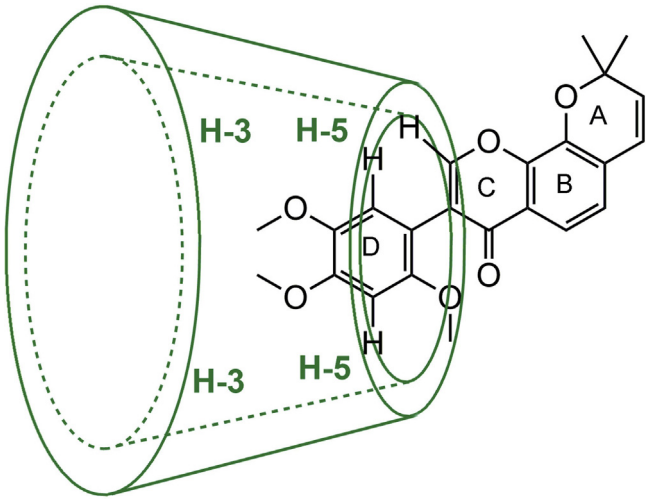

Fig. 4. Possible inclusion mode of bar-HP-β-CD complex.

To further explore the possible inclusion mode of the bar-HP-β-CD, we compared the ^1H NMR spectrum of barbigerone in the absence and presence of HP-β-CD (Fig. 3c and d). Owing to its poor water solubility, DMSO-d_6 was used as a solvent. As illustrated in Fig. 3c and d, the majority signals of barbigerone appeared at 5.9–8.3 ppm, which were distinct from the HP-β-CD protons (3.2–5.2 ppm). Most of the signals of barbigerone protons were emerged in the spectra of bar-HP-β-CD complex. However, the signals of barbigerone were much weaker than that of HP-β-CD, this phenomenon may be partly attributed to the low percentage of barbigerone in the inclusion complex. As expected, HP-β-CD-induced shift changes were also observed for barbigerone protons' signals between the free and complexed state (Table 1). From Table 1, we can see the most significant HP-β-CD-induced variations were observed in H-2, H-3′ and H-6′ protons, which were attributed to the aromatic protons (Fronza, Mele, Redenti, & Ventura, 1996) (Fig. 1a) from D ring of barbigerone. However, inclusion complexation with HP-β-CD had a negligible effect on the δ values of H-5, H-6, H-4″ and —CH_3 protons, which were assigned to the protons from A and B ring in barbigerone.

Based on these ^1H NMR results, we deduced the phenyl group (D ring) of barbigerone deeply inserted into the cavity of HP-β-CD and the possible inclusion modes for the bar-HP-β-CD complex was illustrated in Fig. 4.

3.4.3. Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra of barbigerone, HP-β-CD, bar-HP-β-CD inclusion complex and the physical mixture were shown in Fig. 5A. The FT-IR spectrum of barbigerone showed strong adsorption bands in the range of 1640–1510 cm^{-1}, 1460–1110 cm^{-1}, and 980–660 cm^{-1} (Fig. 5A(a)). The sharp absorption band appeared at 1641 cm^{-1} corresponding to the carbonyl (C=O) stretching vibration. The intense absorption was found at 1210 cm^{-1} attributed to the C—O stretching vibration. The vibration of —CH_3 could be found in the region 2980–2830 cm^{-1}. The FT-IR spectra of pure HP-β-CD (Fig. 5A(b)) illustrated intense broad absorption bands at 3600–3000 cm^{-1}, which corresponded to the free stretching vibration. The vibration of —CH_3 and —CH could be found at 2970 cm^{-1}. The large band appeared in the region 1150–1030 cm^{-1} could be ascribed to the C—O stretching vibration.

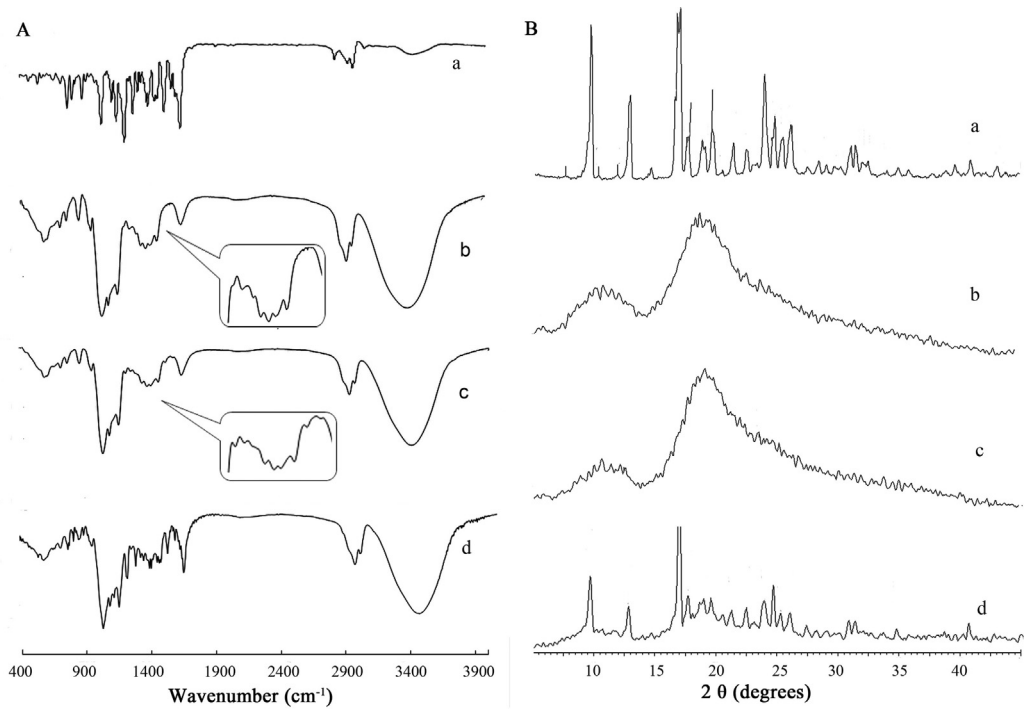

Fig. 5. (A) FT-IR spectra of (a) barbigerone, (b) HP-β-CD, (c) bar-HP-β-CD inclusion complex, (d) barbigerone and HP-β-CD physical mixture (1:1, molar ratio); (B) XRD patterns of (a) barbigerone, (b) HP-β-CD, (c) bar-HP-β-CD inclusion complex, (d) barbigerone and HP-β-CD physical mixture. (1:1, molar ratio).

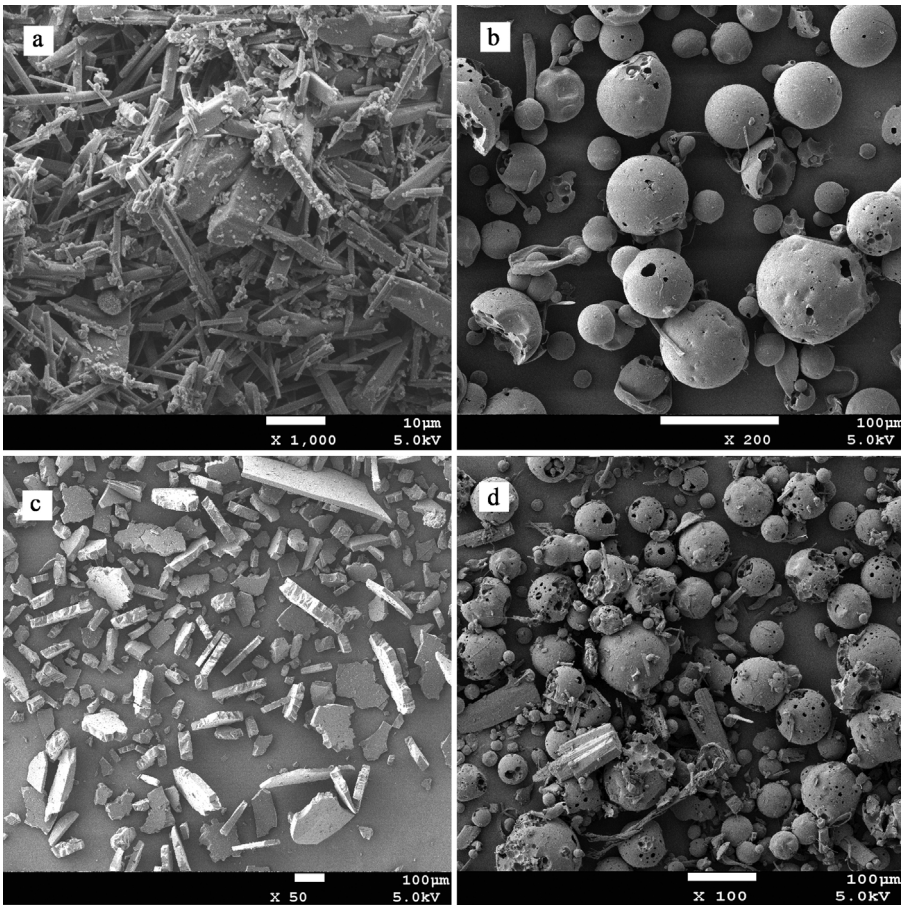

Fig. 6. Scanning electron microphotographs: (a) barbigerone, (b) HP-β-CD, (c) bar-HP-β-CD inclusion complex, (d) barbigerone and HP-β-CD physical mixture (1:1, molar ratio).

For the inclusion complex, the spectra was very similar to that of HP-β-CD (Fig. 5A(c)). The characteristic C=O stretching band of barbigerone at 1641 cm^{-1} and the $-CH_3$ stretching band in the region 2980–2830 cm^{-1} were masked by the intense band corresponding to the —OH bending vibration of HP-β-CD. This effect was expected due to the low barbigerone to HP-β-CD molar ratio (about 1:7.8), which resulted in the inclusion complexes containing less than 4% by weight. However, we can find the low intensity band with a leftward shift at 1569 cm^{-1}, 1515 cm^{-1} and 1207 cm^{-1}, which indicates the presence of barbigerone in the inclusion complex. In addition, no additional peaks were found in the spectrum of inclusion complex, suggesting the absence of any chemical reactions between HP-β-CD and barbigerone. However, the spectrum of physical mixture corresponds to the simple superposition of the spectra of the individual component (Fig. 5A(d)). Some characteristic absorption peaks of barbigerone were easy to be observed, indicating the natural structure of barbigerone still existed without any interactions with HP-β-CD.

3.4.4. XRD analysis

X-ray powder diffractometry is a useful method for the detection of CD complexation in powder or microcrystalline states. Fig. 5B showed the X-ray diffraction patterns of barbigerone, HP-β-CD, bar-HP-β-CD complex and physical mixture. The powder diffraction pattern of barbigerone displayed several sharp peaks at diffraction angels (2θ) of 9.8, 16.9, 17.1, 21.5, 24.0 (Fig. 5B(a)) suggesting that the compound existed as crystalline form. On the other hand, HP-β-CD was amorphous lacking crystalline peaks (Fig. 5B(b)). These observations are consistent with the previous reports (Hu et al., 2012; Yang et al., 2012). In the case of the physical mixture, the diffraction pattern (Fig. 5B(d)) was simply the superposition of the two patterns of the crystalline barbigerone and the amorphous HP-β-CD. In contrast, the XRD of their inclusion complex (Fig. 5B(c)) exhibited the amorphous halo pattern, which was quite different from a superposition of the XRD of HP-β-CD and the barbigerone. The spectrogram of bar-HP-β-CD complex was similar to that of the amorphous HP-β-CD and the characteristic crystalline peaks of barbigerone was disappeared, indicating some new complex compounds were formed and existed in amorphous state.

3.4.5. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is a qualitative method used to visualize the surface structure of raw materials or the prepared products. The SEM images of barbigerone, HP-β-CD, bar-HP-β-CD inclusion complex and their physical mixtures were illustrated in Fig. 6. Pure barbigerone existed in needle-like crystal with many different size (Fig. 6a), whereas HP-β-CD was observed as spherical particles with cavity structures (Fig. 6b). In the physical mixture, the characteristic HP-β-CD microspheres, which were mixed with barbigerone crystals or adhered to their surface, were clearly observed (Fig. 6d). However, the bar-HP-β-CD inclusion complex appeared in the form of irregular blocky particles (Fig. 6c) in which the original morphology of both components disappeared, which confirmed the formation of the inclusion complex of barbigerone and HP-β-CD.

3.5. In vitro bioactivity of bar-HP-β-CD complex

In this study, MTT assays were used to evaluate the bioactivity of bar-HP-β-CD complex. The MTT assay relies on the mitochondrial activity of cells and reflects their metabolic activity. The effects of bar-HP-β-CD complex and free barbigerone on HCT116 and HepG2 cells' viability were illustrated in Fig. 7. After incubation for 48 h, both bar-HP-β-CD complex and free barbigerone decreased the viability of HCT116 cells in a dose-dependent manner

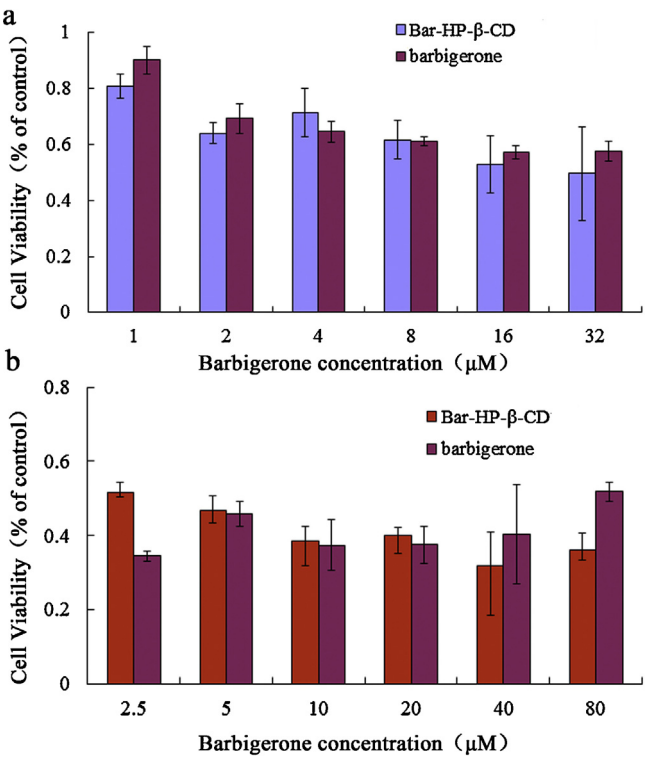

Fig. 7. Cell viability of bar-HP-β-CD inclusion complexes and free barbigerone on cancer cells. (a) Human colon cancer cells (HCT116) and (b) human liver cancer cell (HepG2; data were given as mean ± SD, $n = 3$).

(Fig. 7a), and bar-HP-β-CD complex showed slightly higher cytotoxicity than free barbigerone. Both the inclusion complex and free barbigerone exhibited significant cytotoxicity in HepG2 cells at various concentrations (Fig. 7b). These results indicated that the cell cytotoxicity of bar-HP-β-CD was close to free barbigerone and inclusion complexation with HP-β-CD retained the bioactivity.

4. Conclusion

In this study, barbigerone was successfully complexed with HP-β-CD to form an inclusion complex by a simple coevaporation method. The results of UV–vis, ^1H NMR, FT-IR, XRD and SEM all proved the formation of bar-HP-β-CD inclusion complex. The stability constant of the inclusion complexes was found to be 20,659 M^{-1}. The water solubility of barbigerone was remarkably increased by complexation with HP-β-CD. Further *in vitro* studies showed that bar-HP-β-CD inclusion complex retained the anticancer activity of barbigeorne. This inclusion should be regarded as a promising strategy in the delivery of water-insoluble anticancer agents such as barbigerone.

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