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Inclusion complex of GA-13315 with cyclodextrins: Preparation, characterization, inclusion mode and properties

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ABSTRACT

GA-13315 (13-chlorine-3,15-dioxy-gibberllic acid methyl ester) was semi-synthesized by GA_3 (gibberellic acid) as a potential anticancer drug. To pursue its promising application, cyclodextrin was used for forming complexes to overcome its drawbacks such as poor water solubility and stability. So, GA-13315/CD complexes were prepared with native β-cyclodextrin and its derivatives (hydroxypropyl-β-cyclodextrin (HPβCD)) and their inclusion complexation behavior, characterization and binding ability in both solution and the solid state was studied by means of UV, XRD, DSC, SEM, ^{1}H and 2D NMR spectroscopy. Furthermore, preliminary in vitro cytotoxicity assay showed that the complexes still maintain antitumor activities, compared with GA-13315 or adriamycin (ADM, positive control) as the positive control. The results showed that the water solubility and stability of GA-13315 were obviously improved in the inclusion complex with cyclodextrins, suggesting the inclusion complexes as promising future therapeutic agents.

1. Introduction

Gibberellic acid (GA_3) is not only an important hormone (Nora, Silva, & Rombaldi, 2010), but also widely used as a starting material because it is available in abundant amounts from fermentation of the fungus *Gibberella fujikuroi* (Bomke & Tudzynski, 2009; Mander, 1992), which have been used in partial synthesis of a class of gibberellin derivatives. However, there is rare report on antitumor gibberellins (Banwell, Phillis, & Willis, 2006; Hanson, 2007; Liu, Mander, & Willis, 1998; Mander, 2003). Recently, a unique series of gibberellin derivatives were designed and synthesized, which bore two α, β-unsaturated ketone units, respectively, at the A-ring and D-ring, and displayed anticancer activities (Chen et al., 2009). One of these substances, 13-chlorine-3,15-dioxy-gibberllic acid methyl ester (Fig. 1) (GA-13315), indicated the inhibiting Topo I-catalyzed relaxation of supercoiled DNA (Chen et al., 2009). Further studies showed that GA-13315 possesses high antitumor and antiangiogenic activity in vitro and in vivo (Zhang et al., 2010). However, the poor aqueous solubility and stabilization restrains the usage of GA-13315 as an anticancer drug. It is necessary to search for an efficient and nontoxic carrier for GA-13315 to extend its therapeutic applications. The most frequently used method to improve physicochemical properties of drug molecules is the preparation of its inclusion complexes with cyclodextrins (CDs) (Davis & Brewster, 2004; Loftsson & Duchene, 2007; Loftsson, Hreinsdottir, & Masson, 2005).

Cyclodextrins (CDs) are a series of water-soluble cyclic oligomers consisting of six to eight D-glucose monomers linked by α-1,4-glucose bonds, which form hydrophobic central cavities, with hydrophilic external walls (Connors, 1997; Szejtli, 1998, 2004). They have found important applications in the pharmaceutical, cosmetic and food industries due to their feature – improving the solubility, bioavailability and stability of the guest molecule – to form inclusion complexes with a wide range of compounds (Liu & Chen, 2006; Misiuk & Zalewska, 2009; Roik & Belyakova, 2011; Wu, Liang, Yuan, Wang, & Yan, 2010). More recently, our group reported that the inclusion complexation of CDs with natural products such as taxifolin (Yang et al., 2011), crassicauline A (Chen et al., 2011), nimbin (Yang et al., 2010), artemether (Yang, Lin, Chen, & Liu, 2009), scutellarin (Yang, Yang, Lin, Chen, & Liu, 2009), and azadirachtin B (Yang, Chen, Lin, & Liu, 2008; Yang & Lin, 2009), significantly enhanced the water solubility and bioavailability of the products. So, taking into account of the ability of cyclodextrins,

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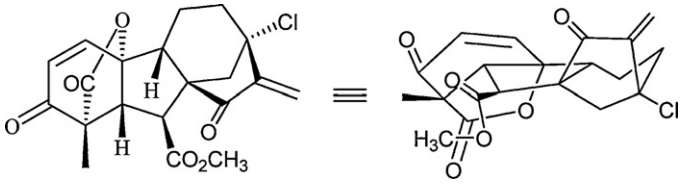


Fig. 1. The structure of GA-13315.

we are attempting to investigate the influence of the inclusion complexes of GA-13315 with cyclodextrins.

Among the natural cyclodextrins, in spite of the low solubility of β-CD in water, it appears particularly useful in the pharmaceutical industry because of its complexing ability, cavity dimensions, low cost and high productivity rate (Sancho, Gasull, Blanco, & Castro, 2011). In addition, its modified derivative hydroxypropyl-β-cyclodextrin (HPβCD) is widely studied in the field of drug encapsulation because of its inclusion ability as well as its high water solubility and nontoxicity by intravenous and oral administration (Eid et al., 2011). In this paper, we report the preparation and characterization of some water-soluble inclusion complexes formed by GA-13315 and β-cyclodextrin and its derivative 2-hydroxypropyl-β-cyclodextrin (HPβCD) (Fig. 1). We were particularly interested in exploring the solubilization effect of CDs on GA-13315 and the binding ability of the resulting inclusion complexes, which would provide a useful approach for obtaining novel GA-13315-based pharmaceutical drugs with high water solubility, high bioavailability and low toxicity.

2. Experimental

2.1. Materials

Pure GA-13315 (FW = 390), obtained from GA_3 by a semi-synthetic route (Chen et al., 2009), was provided by Prof. Zhang's group; β-CD (average substitution degree = 1135) and 2-hydroxypropyl-β-cyclodextrin (HPβCD, average substitution degree = 1380) were purchased from ABCR GmbH & Co. KG and used without further purification. Other reagents and chemicals were of analytical reagent grade. All experiments were carried out using ultrapure water.

2.2. Apparatus

NMR spectra were conducted on a Bruker Avance DRX spectrometer at 500 MHz and 25 °C in D_2O. The one-dimensional spectra of both solutions were run with FID resolution of 0.18 Hz/point. The residual HDO line had a line width at a half-height of 2.59 Hz. Two-dimensional (2D) ROESY spectra were acquired at 25 °C with presaturation of the residual water resonance and a mixing (spin-lock) time of 350 ms at a field of ~2 kHz, using the TPPI method, with a 1024 K time domain in F2 (FID resolution 5.87 Hz) and 460 experiments in F1. Processing was carried out with zero-filling to 2 K in both dimensions using sine (F2) and qsine (F1) window functions, respectively.

The UV–vis spectrum was recorded on a Shimadzu UV 2401 (Japan) equipped with a conventional 1 cm path (1 cm × 1 cm × 4 cm) quartz cell in a thermostated compartment, which was kept at 25 °C by a Shimadzu TB-85 Thermo Bath unit.

A powder X-ray diffraction spectrum was taken by a Rigaku TTRIII Rotating Target diffractometer with Cu Kα radiation (40 kV, 100 mA), at a scanning rate of 5°/min. Powder samples were mounted on a vitreous sample holder and scanned with a step size of $2\theta = 0.02°$ between $2\theta = 3°$ and 50°.

Differential scanning calorimetry (DSC) measurements were conducted on a 2960 SDT V3.0F instrument, and 3–3.5 mg of each sample was heated at a rate of 10 °C/min from room temperature to 400 °C under dynamic nitrogen atmosphere at a flow rate of 70 mL/min.

SEM photographs were determined on a FEI QUANTA 200. The powders were previously fixed on a brass stub using double-sided adhesive tape and then were made electrically conductive by coating with a thin layer of gold (approximately 300 Å) in a vacuum for 30 s and at 30 W. The pictures were taken at an excitation voltage of 15, 20 or 30 kV and a magnification of 1080, 1200, 1400 or 2000×.

2.3. Preparation of GA-13315/β-CD and GA-13315/HPβCD complexes

Herein, CD was used in excess to improve the yield of the complex. GA-13315 (0.02 mM, 7.8 mg) and CD (0.01 mM) were completely dissolved in a mixed solution of water and ethanol (ca. 7 mL, V:V = 4:1, given the poor water solubility of GA-13315, ethanol was used), and the mixture was stirred for 5 days at room temperature. After evaporating the ethanol from the reaction mixture, the uncomplexed GA-13315 was removed by filtration. The filtrate was evaporated under reduced pressure at 37 °C to remove the solvent and dried in vacuum to produce the GA-13315/CDs complexes. GA-13315/β-CD complex (yield 92%): ^{1}H NMR (500 MHz, D_2O, TMS): δ 6.14–6.32 (m, 2H, H-17′ of A ring protons for GA-13315), 5.67–5.76 (m, 2H, H-17′ of D ring protons for GA-13315), 5.02–5.03 (s, 7H, H-1 of β-CD), 3.49–3.89 (m, 47H, H-2–6 of β-CD and H-5′ of B ring protons for GA-13315 and methyl ester protons for GA-13315), 1.13–3.39 (m, 12H, H-6′, 9′, 11′, 12′, 14′ of B, C and D ring protons for GA-13315). GA-13315/HPβCD complex (yield 91%): ^{1}H NMR (500 MHz, D_2O, TMS): δ 6.01–6.18 (m, 1H, H-17′ of D ring protons for GA-13315), 5.63–5.70 (m, 1H, H-17′ of D ring protons for GA-13315), 5.00–5.15 (s, 7H, H-1 of HPβCD), 3.53–3.95 (m, 70H, H-2–6 and CH_2- and CH_3-2, 3, 6 of HPβCD and H-5′ of B ring and methyl ester protons for GA-13315), 1.09–3.25 (m, 12H, H-6′, 9′, 11′, 12′, 14′ of B, C and D ring protons for GA-13315).

2.4. Preparation of GA-13315/HPβCD physical mixture

The physical mixture, to test for possible inclusion, was performed by mixing the powders in a 1:1 molar ratio of GA-13315 and HPβCD in an agate mortar.

2.5. Spectral titration

Given the poor water solubility of GA-13315, a water/ethanol (V:V = 4:1) solution was used in the spectral measurements. The concentrations of GA-13315 were kept constant at 0.115 mM in β-CD and at 0.1535 mM in HPβCD. Then, an appropriate amount of CD was added, and the final concentrations varied from 0 to 3.25–3.95 mM (β-CD: 0, 0.681, 0.851, 1.064, 1.33, 1.66, 2.08, 2.6, 3.25 mM; HPβCD: 0, 1.036, 1.3, 1.62, 2.02, 3.16, 3.95 mM). The absorption spectra measurements were taken after 1 h. The measurements were done in the 200–400 nm spectral range. All experiments were carried out in triplicate.

2.6. Aqueous solubility of GA-13315/CDs

An adequate amount of the complex was added to 2 mL water (ca. pH 6.0) to ensure the solution reached saturation under nitrogen, sheltered from light, and the mixture was stirred for 1 h at 20 ± 2 °C. Then, the remaining solid in the solution was filtrated off using a 0.45 μm cellulose acetate membrane. The filtrate was

evaporated under reduced pressure to dryness and the residue was dosed by the weighing method.

2.6.1. Measurement of cytotoxicity (Chen et al., 2008)

The cytotoxicity of the samples on tumor cells was measured by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. K562 (human chronic myelogenous leukemia cell line) and MKN-28 (human gastric adenocarcinoma cell line) were seeded into 96-well microculture plates, for the adherent cells (MKN-28), the cells were allowed culture 24 h for adhesion before the samples addition, while suspended cells (K562) were seeded just before the samples addition. The cell densities were selected based on the results of preliminary tests, in order to maintain the control cells in an exponential phase of growth during the period of the experiment and to obtain a linear relationship between the optical density and the number of viable cells. The tumor cell was exposed to the samples at 1.0, 10 and 100 μM concentrations for different periods (adherent cells 72 h, suspended cells 48 h) and each concentration was tested in triplicate. At the end of exposure, 20 μl of 5 mg/mL MTT (Sigma chemical Co.) was added to each well and the plates were incubated for 4 h at 37 °C. Then triplex solution (10% SDS–5% isobutanol–0.012 M HCl) was added and the plates were incubated for 12–20 h at 37 °C. The optical density (OD) was read on a plate reader at 570 nm. DMSO was used as solvent for sample GA-13315, injection water was used as solvent for other samples (GA-13315/βCD and GA-13315/HPβCD) and adriamycin (ADM, positive control). DMSO and injection water control wells, in which sample was absent, were included in all the experiments, in order to eliminate the influence. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition}\,(\%) = \left(\frac{OD_{\text{control}} - OD_{\text{treated}}}{OD_{\text{control}}}\right) \times 100\%.$$

The cytotoxicity of the samples on tumor cells was expressed as IC_{50} values (the sample concentration reducing by 50% of the absorbance in treated cells, with respect to untreated cells), were calculated by LOGIT method.

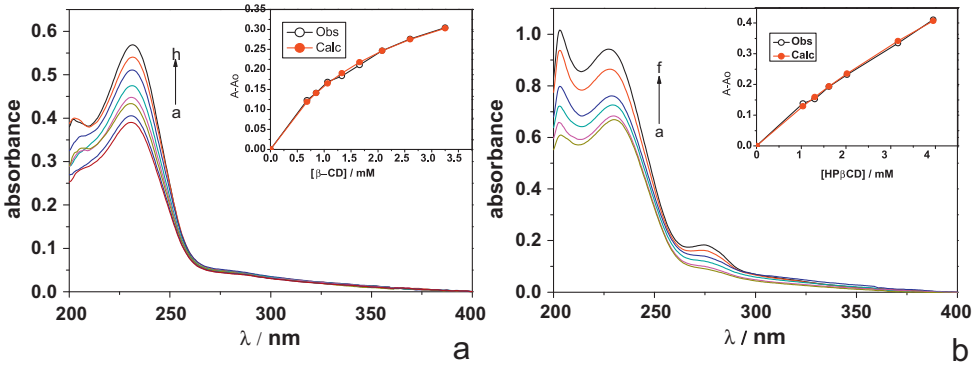
2.6.2. Stability test

Comparative tests involving the stability of aqueous solution of free and complexed GA-13315 with β-CD and HPβCD were tracked using the absorbance changes at 231 nm by means of UV spectra at room temperature (during 20 days, all experiments were carried out in triplicate at predetermined time intervals). The results were expressed as percentages of the remaining GA-13315, i.e., the $A/A_0 \times 100$ ratio, where A_0 is the initial concentration of GA-13315 alone or the GA-13315/CD complex and A is the concentration at time of the specified interval.

3. Results and discussion

3.1. Spectral titration

The spectrophotometric titration was measured to determine the inclusion complexation behavior of β-CD and HPβCD with GA-13315 in a water/ethanol (V:V = 4:1) solution. It has been observed that absorption intensities increase with increasing concentrations of CDs. As the size-fit, shape-fit, and charge-fit effects are the dominant controlling factors in the formation of inclusion complexes of CDs (Liu & Chen, 2006), these results indicate that the binding behavior is mainly dependent on the individual structural features of the host and guest. Assuming a 1:1 stoichiometry for the GA-13315/CD inclusion complex, the equilibrium of the inclusion complex formed between GA-13315 and CD could be written as Eq. (1), and the stability constant (K_s) could be calculated from Eq. (2), where [GA-13315/CD], [GA-13315] and [CD] are the equilibrium concentration of the GA-13315/CD inclusion complex, GA-13315 and CD, $[\text{GA-13315}]_0$ and $[\text{CD}]_0$ are the initial concentration of GA-13315 and CD, respectively, and $\Delta\varepsilon$ is the differential molar extinction coefficient of GA-13315 in the absence and the presence of CD. According to the Lambert–Beer Law, it was found that the concentration of the GA-13315/CD complex was equal to $\Delta A/\Delta\varepsilon$, Eq. (2). Then, Eq. (3) was derived from Eq. (2). Finally, the K_s was obtained from the analysis of the sequential changes of absorption

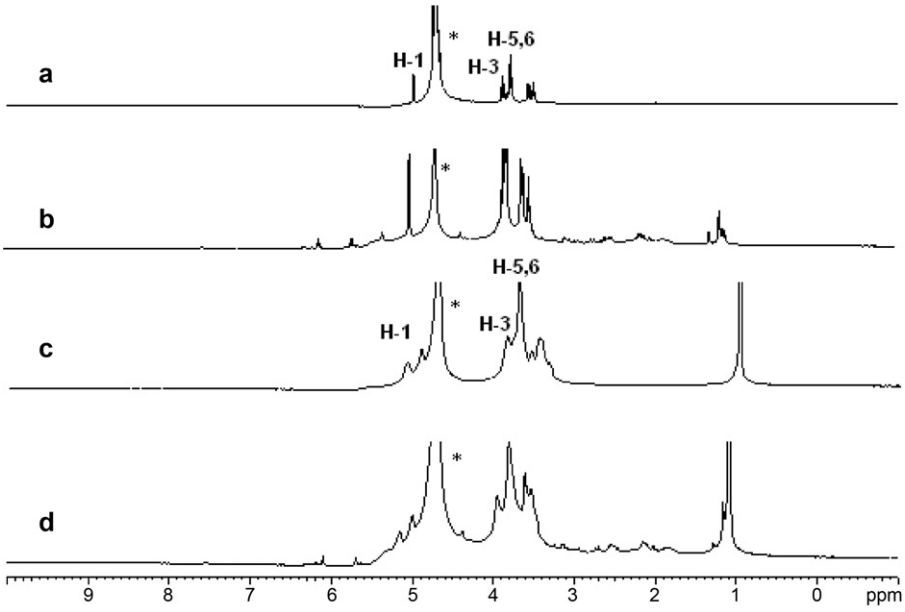


a b

c

Host	Ks / M^{-1}	log Ks	$-\Delta G^o$ / kJ·mol^{-1}
β-CD	472.3	2.6737	15.008
HPβCD	79.6	1.9006	10.668

Fig. 2. (a) UV–vis spectral of GA-13315 (0.115 mM) in different β-CD concentrations (0–3.25 mM, from a to h), (b) UV–vis spectral of GA-13315 (0.1535 mM) in different HPβCD concentrations (0–3.95 mM, from a to f) in a water/ethanol (V:V = 4:1, ca. pH 7.0) mixed solution, and the nonlinear least-squares analysis (inset) of the differential intensity (ΔA at 231 nm) to calculate the complex stability constant (K_s); and the stability constant (K_s and log K_s) and Gibbs free energy change ($-\Delta G^\circ$) for the inclusion complexation of CDs with GA-13315 guest in a water/alcohol (V:V = 4:1, ca. pH 7.0) mixed solution (c).



e

δ (ppm)

		β-CD	GA-13315/β-CD complex	HPβCD	GA-13315/HPβCD complex
H-1	d	4.99	5.03	4.92	5.00
H-2	dd	3.57	3.64	3.57	3.61
H-3	dd	3.88	3.88	3.87	3.95
H-4	dd	3.50	3.55	3.46	3.53
H-5	m	3.80	3.85	3.71	3.81
H-6	dd	3.77	3.82	3.71	3.81
H-Me	s	—	—	0.99	1.09

Fig. 3. ^{1}H NMR spectra of GA-13315 in the absence and the presence of β-CD and HPβCD in D_2O at 25 °C, respectively. (a) β-CD, (b) GA-13315/β-CD complex, (c) HPβCD, (d) GA-13315/HPβCD complex (asterisk highlights the water peak); and the chemical shifts (δ) of the β-CD, HPβCD, GA-13315/β-CD and GA-13315/HPβCD complexes (e).

(ΔA) at various CD concentrations, with a nonlinear least squares method according to the curve-fitting Eq. (3).

$$\text{GA-13315} + \text{CD} \overset{K_s}{\rightleftharpoons} \text{GA-13315} \cdot \text{CD} \quad (1)$$

$$K_s = \frac{[\text{GA-13315·CD}]}{[\text{GA-13315}][\text{CD}]} = \frac{\Delta A/\Delta\varepsilon}{([\text{GA-13315}]_0 - \Delta A/\Delta\varepsilon)([\text{CD}]_0 - \Delta A/\Delta\varepsilon)} \quad (2)$$

$$\Delta A = \frac{\Delta\varepsilon([\text{GA-13315}]_0 + [\text{CD}]_0 + 1/K_s) \pm \sqrt{\Delta\varepsilon^2([\text{GA-13315}]_0 + [\text{CD}]_0 + 1/K_s)^2 - 4\Delta\varepsilon^2[\text{GA-13315}]_0[\text{CD}]_0}}{2} \quad (3)$$

Using a nonlinear least squares curve-fitting method (Liu, Li, Wada, & Inoue, 1999), we obtained the complex stability constant for each host–guest combination. Fig. 2 (inset) shows a typical curve-fitting plot for the titration of GA-13315 with β-CD and HPβCD, which shows the excellent fit between the experimental and calculated data, confirming the formation of 1:1 GA-13315/CDs inclusion complexes. In the repeated measurements, the K_s values were reproducible within an error of ±5%. The stability constant (K_s) and Gibbs free energy change ($-\Delta G^\circ$) for the inclusion complexation of CDs with GA-13315 are listed in Fig. 2c.

3.2. Binding ability

Extensive studies have revealed that the size/shape-fit concept plays a crucial role in the formation of inclusion complexes of host CDs with guest molecules of various structures. Besides, several weak intermolecular forces such as ion–dipole, dipole–dipole, van der Waals, electrostatic, hydrogen bond, and hydrophobic interactions are known to cooperatively contribute to inclusion complexation. In this case, the host–guest size match may dominate the stability of the complexes formed between CDs and GA-13315. As can be seen from Fig. 2c, the binding constant for the complexation of GA-13315 with β-CD is larger than that with HPβCD. By comparing the enhancement effect of β-CD and its modified (HPβCD) for GA-13315, β-CD gave a stronger K_s value than HPβCD, which showed that β-CD can complex better with the guest GA-13315 than HPβCD. It was showed that the size-fit effect was the

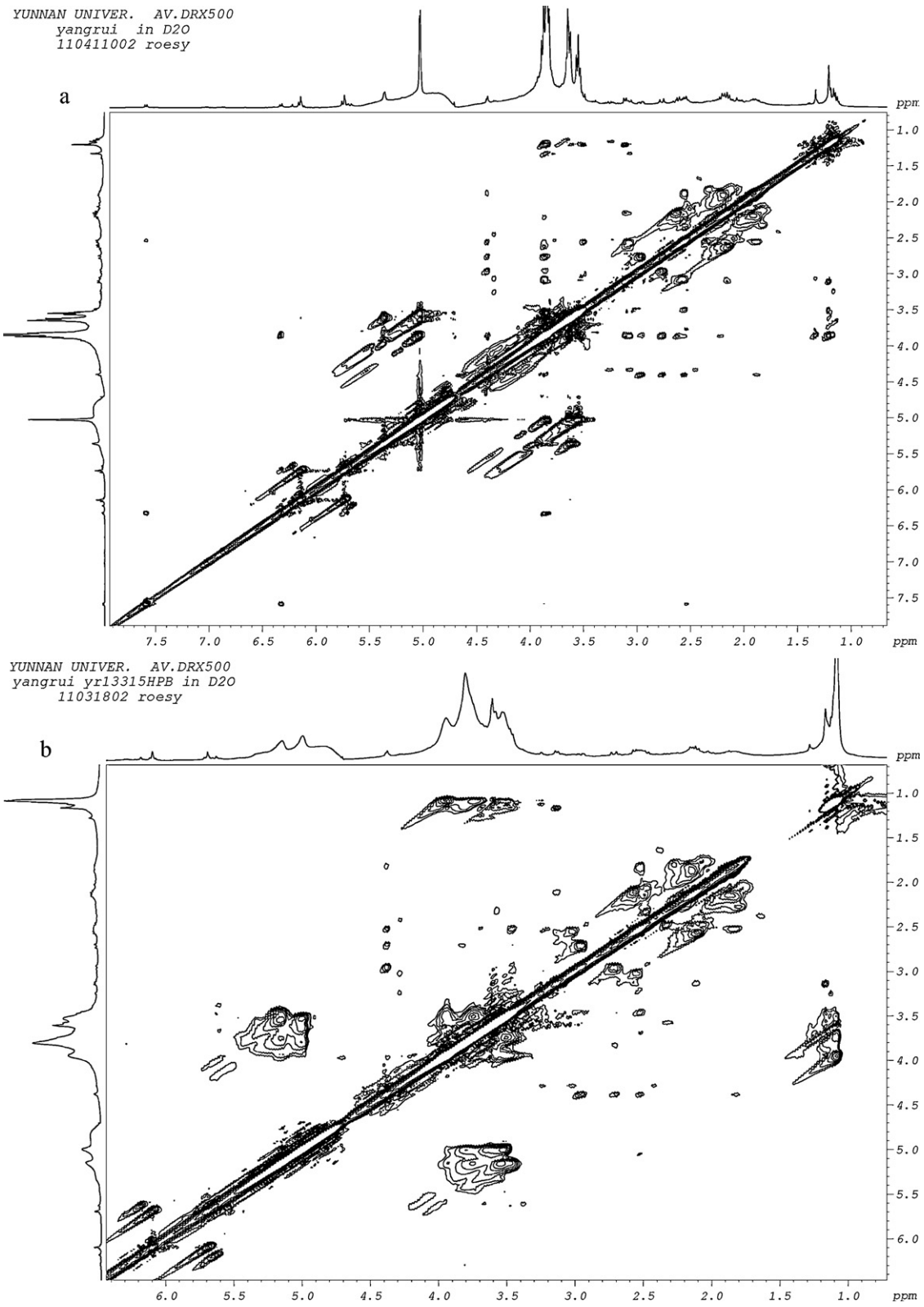


Fig. 4. ROESY spectrum of (a) GA-13315/β-CD complex and (b) GA-13315/HPβCD complex in D_2O.

dominant controlling factor in the formation of inclusion complexes of GA-13315/CDs.

3.3. ^1H and 2D NMR analysis

NMR spectroscopy provides some of the most powerful evidence for the study of host–guest chemistry in solution, and the information of the chemical shifts has been used to establish inclusion modes (Yang et al., 2010). Firstly, we compared the ^1H NMR spectra of GA-13315 in the presence of the host CDs (Fig. 3). GA-13315 is transparent to ^1H NMR under most conditions, even in D_2O, as a result of its poor water solubility. Assessment of the GA-13315 complex by ^1H NMR clearly demonstrated the presence of the framework protons of the GA-13315 molecule, consistent

with the significant solubilization. The ^{1}H NMR spectrum of GA-13315/CDs in Fig. 3 confirmed the existence of GA-13315 entrapped into CDs. By comparing the integration area of these protons with that of the CD's H-1 protons, we calculated the inclusion stoichiometry of the GA-13315/CDs, that is, 1:1 for the GA-13315/β-CD and GA-13315/HPβCD complexes.

Chemical shift variations of host and guest protons reflect the formation of a complex between them. It is well known that H-3 and H-5 protons of CD are positioned inside the cavity, with H-3 closer to the wider rim and H-5 on the opposite. Consequently, the chemical shift variation of H-3 and H-5 is a direct evidence of inclusion. Inclusion complexation with GA-13315 had a negligible effect on the δ values of the H-3 protons of β-CD (0 ppm). In contrast, the values of the H-1, H-2, H-4, H-5 and H-6 protons exhibited relatively weak but significant changes (0.04–0.07 ppm), which could have been caused by the hydrogen bond between the hydroxyl arms of β-CD and the oxygen atoms of GA-13315. It is worth noting that the H-5 protons shifted ca. 0.05 ppm, but the H-3 protons remain unchanged after inclusion complexation. This phenomenon may indicate that GA-13315 should have inserted into the β-CD cavity from the narrower rim. Similarly, all of the HPβCD protons showed appreciable shifts after inclusion complexation with GA-13315 (0.04–0.10 ppm). By comparing these chemical shifts, we found that the shifts of the H-5 and H-6 protons (0.10 ppm) were larger than those of the H-3 (0.08 ppm) proton, indicating that GA-13315 may enter the cavity of HPβCD from the narrower rim as well.

Afterwards, the mode of the inclusion complex was further confirmed by two-dimensional (2D) NMR spectroscopy, since cross-peaks in ROESY spectra are expected for protons that are closer than 0.4 nm in space (Correia et al., 2002). To gain more confirmation, we obtained 2D ROESY of the inclusion complexes of GA-13315 with CDs. The ROESY spectrum of the GA-13315/β-CD complex (Fig. 4a) showed appreciable correlation of the H-6′, H-9′ H-11′, H-14′ and H-18′ protons of GA-13315 with the H-3, H-5 and H-6 protons of β-CD. These results indicate that the A, B and C ring of GA-13315 was included in the β-CD cavity. The ROESY spectrum of the GA-13315/HPβCD complex (Fig. 4b) also showed significant correlations between the H-14′ and H-18′ proton of GA-13315 and the H-2, H-3, H-4 and H-5 protons of HPβCD. These results indicate that the A, B and C ring of GA-13315 was also included in the HPβCD cavity.

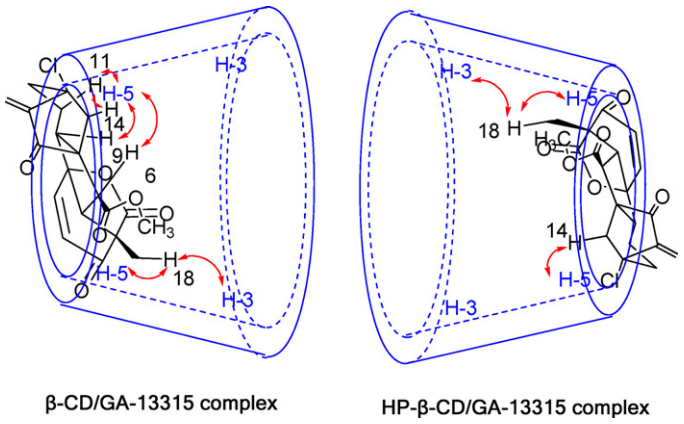


Fig. 5. Possible inclusion mode and significant NOESY (↔) correlations of the GA-13315/β-CD and GA-13315/HPβCD inclusion complexes.

Based on these observations, together with the 1:1 stoichiometry, we deduced the possible inclusion modes of GA-13315 with CDs as illustrated in Fig. 5.

3.4. XRD analysis

Powder X-ray diffractometry is a useful method for the detection of CD complexation in powder or microcrystalline states. Fig. 6A is the powder X-ray diffraction (XRD) spectra of GA-13315, β-CD, HPβCD and their inclusion complexes. As indicated in Fig. 6A, GA-13315 (Fig. 6A(a)) and β-CD (Fig. 6A(b)) showed intense and sharp peaks that prove both of them are in a crystalline form. On the other hand, HPβCD is amorphous (Fig. 6A(c)). In contrast, the XRD of the GA-13315/β-CD and GA-13315/HPβCD complexes (Fig. 6A(d) and A(e)) are amorphous and show halo patterns, which are evidently different from the superimposition of crystalline GA-13315 in β-CD

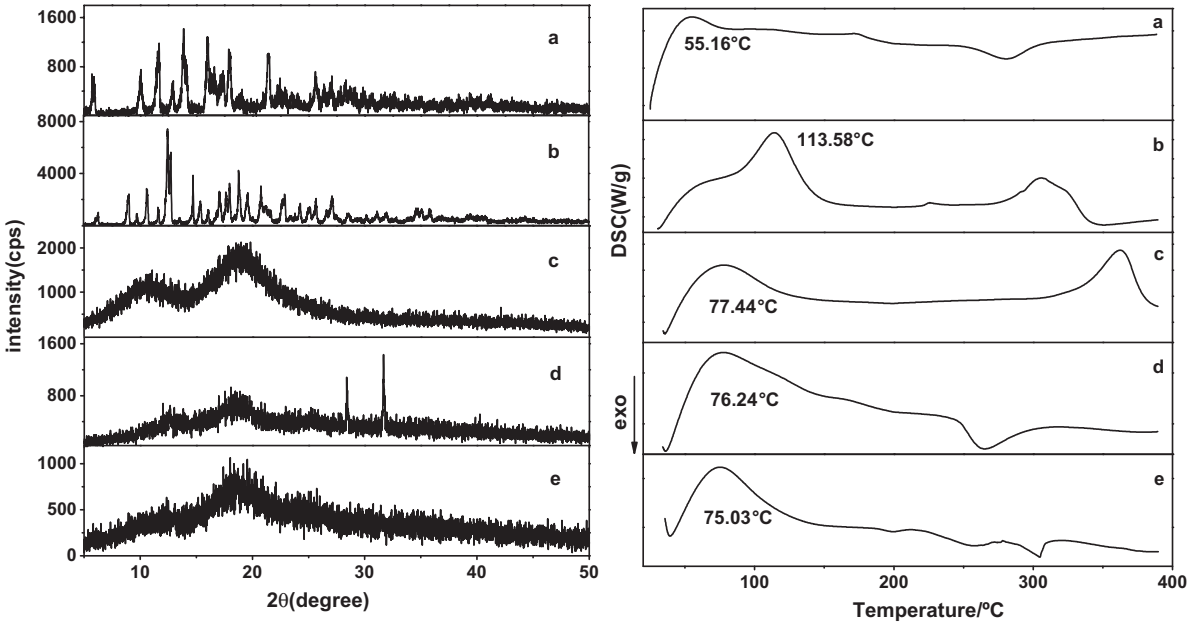


Fig. 6. (A) XRD patterns: (a) GA-13315, (b) β-CD, (c) HPβCD, (d) GA-13315/β-CD inclusion complex, (e) GA-13315/HPβCD inclusion complex; (B) DSC thermograms: (a) GA-13315, (b) β-CD, (c) HPβCD, (d) GA-13315/β-CD inclusion complex, (e) GA-13315/HPβCD inclusion complex.

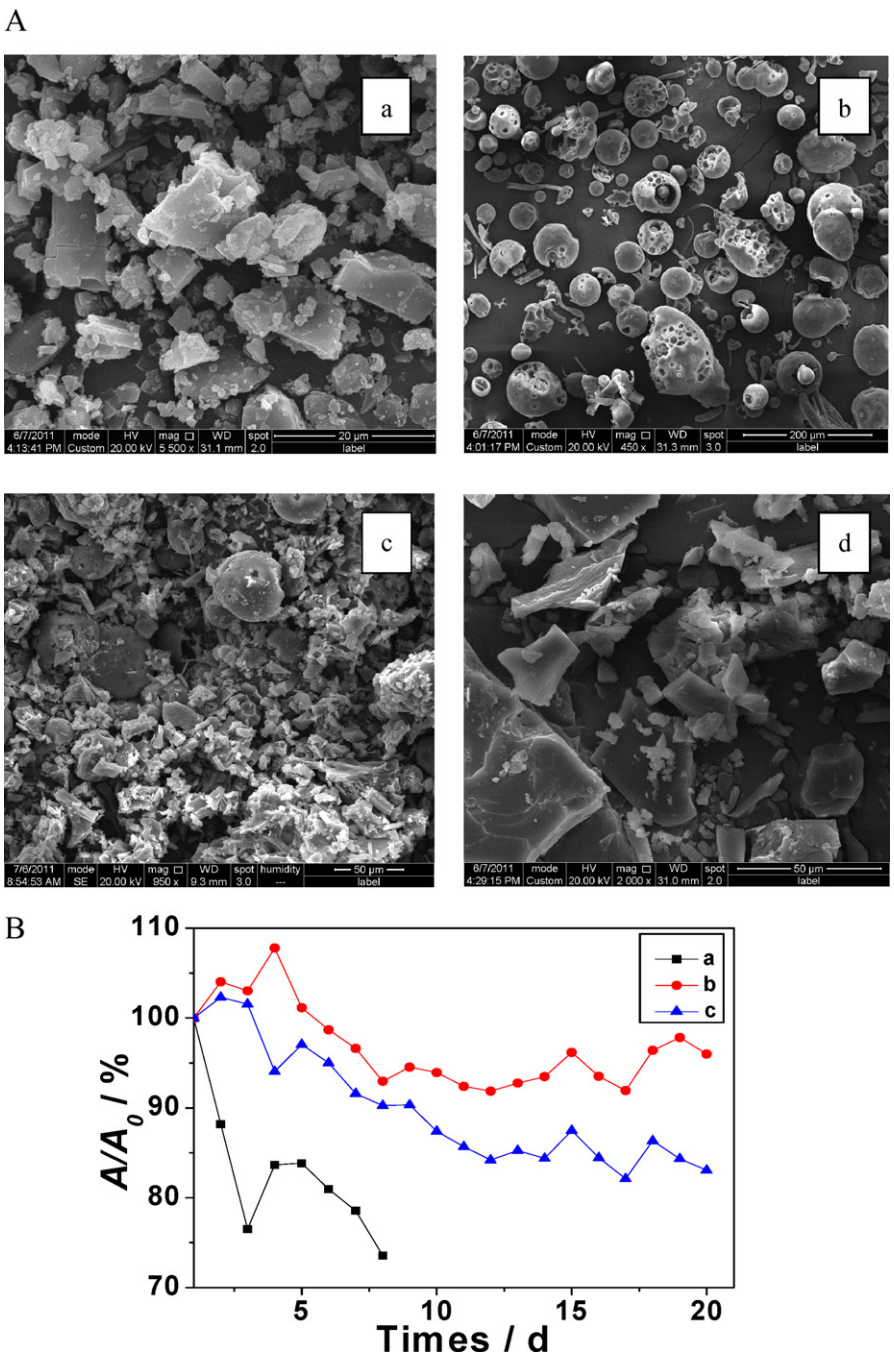


Fig. 7. (A) Scanning electron microphotographs: (a) GA-13315, (b) HPβCD, (c) GA-13315 and HPβCD physical mixture (1:1 molar ratio), (d) GA-13315/HPβCD inclusion complex; (B) degradation profiles of GA-13315 decomposition in an aqueous solution containing: (a) free GA-13315, (b) GA-13315/β-CD complex, (c) GA-13315/HPβCD inclusion complex, with an interval of 1 day.

and HPβCD, indicating the interaction between β-CD (or HPβCD) and GA-13315.

3.5. DSC analysis

The thermal properties of the GA-13315/β-CD and GA-13315/HPβCD complexes were investigated by differential scanning calorimetry (DSC); the thermogram reveals some information on the solid state interactions between the drug and cyclodextrin. As shown in Fig. 6B, the DSC curve of GA-13315 contained an endothermic peak at 55 °C (Fig. 6B(a)). In contrast, the DSC curves of pristine β-CD and HPβCD had an endothermic peak at 113 and 77 °C (Fig. 6B(b) and B(c)), respectively. However, in the DSC curves of the GA-13315/CD complexes, the endothermic peaks at about 55 °C corresponding to the free GA-13315 disappeared, coinciding with the appearance of a new endothermic peak at 76 and 75 °C in the case of the GA-13315/β-CD and GA-13315/HPβCD (Fig. 6B(d) and B(e)), respectively. These results further confirm the formation of GA-13315/CDs complexes.

3.6. Solubilization

The water solubility of the GA-13315/CD complex was assessed by the preparation of its saturated solution (Montassier, Duchêne, & Poelman, 1997). An excess amount of the complex was placed in 2 mL of water (ca. pH 6.0) and the mixture was stirred for

1 h. After removing the insoluble substance by filtration, the filtrate was evaporated under reduced pressure to dryness and the residue was dosed by the weighing method. The results show that the water solubility of this GA-13315, compared to that of free GA-13315 (ca. 40 μg/mL), was remarkably increased to approximately 1.5 and 1.4 mg/mL by the solubilizing effects of β-CD and HPβCD, respectively. In the control experiment, a clear solution was obtained after dissolving the GA-13315/β-CD (5.3 mg) and GA-13315/HPβCD (7 mg) complexes, which was equivalent to 1.5 and 1.4 mg of GA-13315, respectively, in 1 mL of water at room temperature. This confirmed the reliability of the obtained satisfactory water solubility of the GA-13315/CD complex, which will be beneficial for the medical utilization of this compound.

3.7. In vitro cytotoxicity

The cytotoxicity of free GA-13315 and complexed GA-13315 with β-CD and HPβCD on K562 and MKN-28 was individually evaluated through MTT assay (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987; Mossman, 1983) using adriamycin (ADM) as the positive control. The IC_{50} values are presented in Table 1 (IC_{50} value, defined as the concentration corresponding to 50% growth inhibition). Free GA-13315 displayed higher cytotoxicity compared to ADM against K562 cells. However, cytotoxicity against K562 and MKN-28 displayed that complexes still remain in anticancer activity. Typically, GA-13315/β-CD (IC_{50} 7.75 μM and 13.3 μM) was more active than GA-13315/HPβCD (IC_{50} 27.4 μM and 17.8 μM) against K562 and MKN-28.

3.8. SEM analysis

Scanning electron microscopy (SEM) is well suited to visualize the surface texture of the deposited films, i.e., CDs and drugs or the products obtained by different methods of preparation, such as physical mixing, solution complexation, coevaporation and others (de Araujo et al., 2008; Duchêne, 1987). The SEM photographs of HPβCD, GA-13315, their inclusion complexes and their physical mixtures are shown in Fig. 7A. Pure GA-13315 existed in irregularly shaped crystal (Fig. 7A(a)), and HPβCD was observed as a spherical crystal with cavity structures (Fig. 7A(b)). The physical mixture of GA-13315/HPβCD revealed some similarities with the crystals of the free molecules and showed both crystalline components (Fig. 7A(c)). In contrast, the GA-13315/HPβCD inclusion complex appeared in the form of compact and homogeneous plate-like structure with crystal particles in which the original morphology of both components disappeared (Fig. 7A(d)). The sizes and shapes of GA-13315 and HPβCD particles were different from those of the inclusion complex, which may indicate the formation of the GA-13315/HPβCD inclusion complex.

3.9. Stability

In order to evaluate the stability of GA-13315/CDs, we tracked the absorbance changes of GA-13315, GA-13315/β-CD and GA-13315/HPβCD (Yang et al., 2008). The solid GA-13315, GA-13315/β-CD or GA-13315/HPβCD was quickly dissolved thoroughly and kept at room temperature, and the absorbance was analyzed at 231 nm by UV/vis spectra each day. Fig. 7B illustrates the change trend of the relative absorbance A/A_0 of GA-13315, GA-13315/β-CD and GA-13315/HPβCD each day, respectively. The decomposition of free GA-13315 was found to be very marked upon dissolving in water. The relative absorbance of GA-13315 quickly diminished in the first 2 days, then slowed down, but accelerated again on the fifth day, whereas the absorbance at 231 nm disappeared on the ninth day. However, the degradation of GA-13315 was retarded when GA-13315 was included in CDs, as shown in Fig. 7B. Until the eighth day, the percentage of remaining GA-13315 in GA-13315/β-CD complex solution kept slowly decreasing, close to 93%, and then the percentage tended to stabilize. Nevertheless, the degradation of GA-13315 in GA-13315/HPβCD complex solution was close to 15% until the twelfth day, and tended to stabilize as well. This indicated that GA-13315/CDs are much more stable than free GA-13315.

Table 1
In vitro cytotoxic activities of GA-13315 and GA-13315/CDs.

	Anti-cancer activity IC_{50} (μM)	
	K-562	MKN-28
GA-13315	8.97×10^{-3}	11.2
GA-13315/βCD	7.75	13.3
GA-13315/HPβCD	27.4	17.8
ADM	4.07	2.4

4. Conclusions

The GA-13315 molecule forms inclusion complexes with β-CD and HPβCD. The results of UV–vis, NMR, DSC, XRD and SEM demonstrated that GA-13315/CDs have different physicochemical characteristics from GA-13315. The water solubility and stability of GA-13315 were significantly enhanced by inclusion. Given the limitation of applications for GA-13315 and the facile and environmentally friendly preparation of the GA-13315/CD complex, this inclusion complexation should be regarded as a promising strategy in the design of a novel formulation of GA-13315 for anticancer medicine.

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