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Enhancing Effect of Hydroxypropyl-β-cyclodextrin on the Intestinal Absorption Process of Genipin

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Supporting Information

ABSTRACT: The purpose of this work is to investigate the effect of the genipin/hydroxypropyl-β-cyclodextrin (HP-β-CD) inclusion complex on the intestinal absorption of genipin and identify its mechanism of action. The phase solubility profile was classified as A_L type, indicating the formulation of a 1:1 stoichiometry inclusion complex. Fourier transform infrared spectroscopy, Differential scanning calorimetry, X-ray powder diffractometry, and ^{1}H nuclear magnetic resonance (NMR) and two-dimensional (2D) ^{1}H rotating-frame Overhauser enhancement (ROESY) NMR spectroscopies further confirmed the formulation of the inclusion complex with superior dissolution properties than the drug alone. The results of single-pass intestinal perfusion showed that the intestinal absorption of genipin was affected by P-glycoprotein (Pgp). The absorption rate and permeability value of the inclusion complex were significantly higher than the free drug, suggesting that its enhancing effect was involved in its solubilizing effect and Pgp inhibitory effect. The mechanisms of HP-β-CD on Pgp inhibition were demonstrated by restraining the Pgp ATPase activity rather than changing the fluidity of the cell membrane.

KEYWORDS: Genipin, hydroxypropyl-β-cyclodextrin, inclusion complex, P-glycoprotein (Pgp), intestinal absorption

INTRODUCTION

Genipin (Figure 1A) is the aglycone of geniposide extracted from the fruit of *Gardenia jasminoides* Ellis, named Zhi Zi, which has long been used for the treatment of diseases in China. In the food industry, genipin is used to produce natural colorants via a simple reaction with primary amines. Manipulation of the reaction conditions enables the production of blue, green, violet, and red colorants.[1,2] In medicine, genipin exhibits significant anti-inflammatory[3] and antithrombotic[4] effects, has the ability to protect the hippocampal neurons from the toxicity of Alzheimer's amyloid β proteins,[5] and suppresses *in vitro* fas-induced lethal liver apoptosis in primary cultured murine hepatocytes.[6] Recently, the antidepressant-like effects of genipin have gained increasing attention.[7] Pharmacokinetic studies suggest that geniposide, when it is orally administered, is hydrolyzed into genipin by enzymes produced by intestinal bacteria.[8] It is genipin and not geniposide that exhibits the pharmacological activities of gardenia.[9] However, poor intestinal absorption of genipin was a significant barrier to its clinical application.

Cyclodextrins (CDs) are truncated-cone polysaccharides mainly composed of six to eight D-glucose monomers linked by α-1,4-glucosidic bonds. They have a hydrophobic central cavity and hydrophilic outer surface and can encapsulate model substrates to form host−guest complexes or supermolecular species.[10] This usually enhances drug solubility in water and affects the physicochemical properties of the drug.[11−13] Hydroxypropyl-β-cyclodextrin (HP-β-CD; Figure 1B) has been extensively investigated for its relatively high water solubility, low toxicity, and satisfactory inclusion ability.[14−16] Several commercial formulations are composed of cyclodextrin inclusion complexes, illustrating the usefulness of this approach.[17−19] In the case of poorly water-soluble compounds in the developmental stage, possible interactions with P-glycoprotein (Pgp) closely involved in the absorption and exsorption of structurally and pharmacologically unrelated drugs in the intestine are taken into consideration. Many references have reported that the inhibitory mechanisms of Pgp by methylated β-CD were releasing transporters,[20] altering cholesterol levels,[21,22] increasing plasma membrane fluidity,[23] etc. While the Pgp inhibitory mechanisms of HP-β-CD remain unclear. Thus, the influence of HP-β-CD on membrane fluidity and Pgp ATPase were studied.

In a more general attempt to optimize the pharmaceutical properties and the absorption of genipin, this work aimed to investigate the effectiveness of HP-β-CD for improving the intestinal absorption of genipin. We used phase solubility techniques, molecular modeling by two-dimensional (2D) ^{1}H rotating-frame Overhauser enhancement (ROESY) nuclear magnetic resonance (NMR) spectroscopy and physicochemical characterizations by Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), powder X-ray diffractometry (XRD), and ^{1}H NMR spectroscopy. The main purpose of this study was to compare the intestinal absorption of the genipin/HP-β-CD inclusion complex to that of genipin and to identify HP-β-CD as

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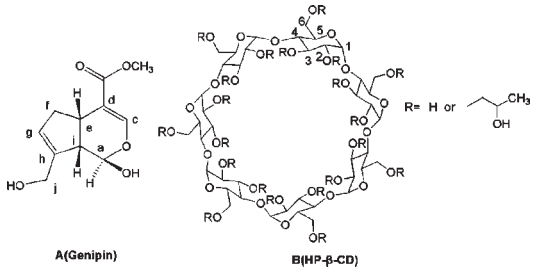

Figure 1. Structure and proton labeling of (A) genipin and (B) HP-β-CD.

a solubility enhancer and Pgp inhibitor. Moreover, the mechanisms by which HP-β-CD affects Pgp inhibition are investigated by a membrane anisotropy measurement and Pgp ATPase assay.

MATERIALS AND METHODS

Materials. Genipin was purchased from Linchuan Zhixin Bio-Technology Co. (Jiangxi, China). HP-β-CD (molecular mass, 1540; batch number, 74T014) was purchased from Wacker Chemie AG (Munich, Germany). The Pgp-Glo assay kit was from Promega Co. (Madison, WI). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; molecular mass, 734.04; batch number, 78K5203) and 1,6-diphenyl-1,3,5-hexatriene (DPH; molecular mass, 232.32; batch number, MKBD1354 V) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Acetonitrile and methanol for high-performance liquid chromatography (HPLC) analysis were obtained from Merck Co. (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water purification system (Molsheim, France). Other chemicals were commercially available and used as received.

Phase Solubility Studies. Phase solubility studies were carried out in water according to the method previously reported by Calabrò et al.[24] Briefly, excessive amounts of genipin (60 mg) were added to 3 mL of aqueous solution containing increasing amounts of HP-β-CD (0−40 mM) taken in a series of capped tubes. The suspensions were shaken at 25 °C for 7 days. After equilibrium was attained, the samples were filtered through 0.45 μm Millipore syringe filters and assayed by the HPLC method. The HPLC analysis was performed on a Waters 2695 system (Milford, MA) equipped with a 2487 dual λ absorbance detector. The samples of 10 μL were injected onto a HPLC reverse-phase column (250 × 4.6 mm, 5 μm) at 25 °C. All samples were detected with an ultraviolet (UV) detector at 238 nm (see the Supporting Information). The mobile phase consisted of a mixture of acetonitrile/water (15:85, v/v) containing 0.1% (v/v) formic acid. The flow rate was 1.0 mL/min. Each test group was performed in triplicate. Phase solubility profiles were obtained by plotting the solubility of genipin versus the concentration of HP-β-CD.

Preparation of the Inclusion Complex. The inclusion complex was prepared by freeze drying at a 1:1 molar ratio based on the results of phase solubility studies. In short, genipin was dissolved in small amounts of methanol at 45 °C. The solution was added to a HP-β-CD−water solution, and the resulting suspension was stirred continuously for 8 h before cooling slowly to room temperature. The prepared suspension was filtered quickly over 0.45 μm Millipore syringe filters to remove any free genipin. It was then frozen and lyophilized in a Savant ModulyoD freeze drier (Waltham, MA) at −45 °C and 100 mbar to obtain the inclusion complex. In parallel, physical mixtures, containing the same ratios of genipin and HP-β-CD, were prepared in a mortar.

Characterization of the Inclusion Complex. ^{1}H spectra for HP-β-CD, genipin, and the genipin/HP-β-CD inclusion complex were obtained using a Bruker Avance DRX500 spectrometer (Rheinstetten, Germany) at 298 K in D_2O. ROESY experiments were run on the same instrument. Samples were equilibrated for at least 24 h before measurement.

FTIR spectra were recorded on KBr pellets in the 4000−400 cm^{-1} wavenumber range using a Bio-Rad FTS 3000 spectrophotometer (Richmond, CA).

DSC was recorded on a Perkin-Elmer DSC 7 (Norwalk, CT). A total of 5−10 mg of samples was sealed in aluminum pans and heated at a heating rate of 10 °C/min from room temperature to 300 °C and under N_2 flow (100 mL/min).

XRD was measured in the range of 2θ = 4−50° by step scanning on the Rigaku D/MAX-2500 diffractometer (Rigaku, Japan), with Cu Kα radiation operated at 40 kV and 100 mA and a scanning rate of 2°/min.

Dissolution Studies. Dissolution studies were performed according to the stirring basket method, using a Sotax AT7 apparatus (Basel, Switzerland), by adding an excess of drug or an equivalent amount of inclusion complex to 500 mL of pH 7.2 phosphate buffer at 37 ± 0.5 °C while stirring constantly at 100 rpm. At pre-determined time intervals, suitable aliquots were withdrawn with 0.45 μm Millipore syringe filters and immediately replaced with the same volume of fresh dissolution medium that was at the same temperature. The drug amount in the samples was assayed by the above-described HPLC method. All experiments were repeated in triplicate.

Absorption Studies. *Animals.* Male Sprague−Dawley rats, weighing 200−220 g, were obtained from Tianjin Shanchuanhong Laboratory Animal Technology Co., Ltd. (SCXK 2009-0001, Tianjin, China). The rats were housed under standard conditions and fasted overnight with free access to water until the experiment. Animal experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and the protocol was approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine.

Perfusion Preparation. The Krebs−Ringer buffer, containing 20 μg/mL of phenol red, was used as the perfusion solution. Genipin (30 mg) in the absence or presence of Pgp inhibitor, verapamil (60 μg/mL), or an equivalent amount of the inclusion complex were dissolved in 1000 mL of perfusion solution. The concentrations of genipin used in the perfusion studies were determined by dividing the effective prescribed dose (50 mg/kg) by 250 mL, the standard volume for a glass of water advised to be taken with the dose and, hence, the accepted minimal gastric volume, to represent the maximal drug concentration present in the intestinal segment, and were within their intrinsic solubility in Krebs−Ringer buffer.[25] A total of 50 mg/kg of genipin, the effective prescribed dose to exert its pharmacologic effects, was decided according to the previous studies of our laboratory.[7]

Rat Experiment. Absorption tests using the rat single-pass intestinal perfusion model were performed according to the reported method.[26] After fasting overnight, male Sprague−Dawley rats were randomly assigned to different experimental groups (n = 4). Rats were anesthetized with urethane (1 g/kg, intraperitoneally) and affixed supine on a surface under suitable lighting to maintain body temperature. The jejunum was exposed by a midline abdominal incision, and two polyethylene cannulae (outer diameter, 5 mm; inner diameter, 3 mm) were inserted through small slits at the proximal and distal ends (about 10 cm). To clear the gut, saline solution at 37 °C was passed slowly through it until the effluent was clear. The perfusion solutions were incubated in a 37 °C water bath. The perfusion flow rate used was 0.2 mL/min, which was 10 times lower than that used in humans (2−3 mL/min). The outflow solution was collected every 15 min for 120 min, starting 15 min after the initiation of the perfusion when the steady state had been achieved (as assessed by the inlet/outlet concentration ratio of genipin, which approaches 1 at steady state). The perfusate samples were immediately assayed by HPLC. The length of the perfused intestinal segment was measured accurately at the termination of the experiment.

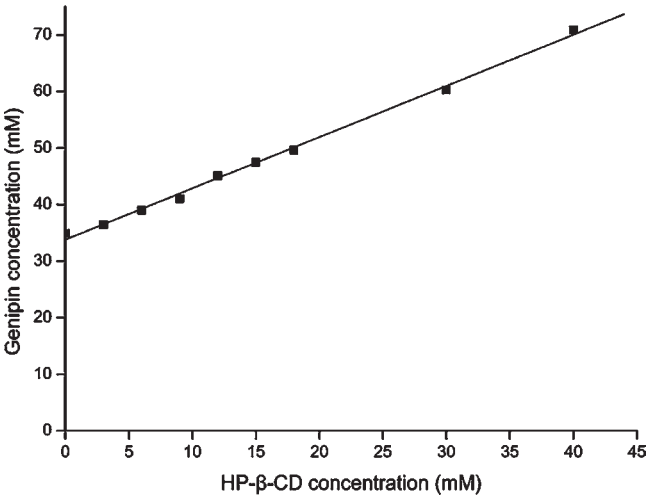

Figure 2. Phase solubility diagram for the genipin/HP-β-CD host–guest system at 25 °C.

HPLC Assay. The above-described HPLC method was used. Linearity was demonstrated over a concentration range of 3–36 μg/mL (R = 0.9998) for genipin, in Krebs–Ringer buffer, with a retention time of 12.8 min, and 2–24 μg/mL (R = 0.9997) for phenol red, with a retention time of 7.2 min. The method was precise and reproducible.

Data Analysis. The net water flux, resulting from both water absorption and efflux in the intestinal segment, was determined by the measurement of phenol red, a non-absorbed, non-metabolized marker. The measured C_{out}'/C_{in}' ratio was corrected for water transport according to the following equation:

$$C_{out}'/C_{in}' = C_{out}/C_{in} \times C_{in\ phenol\ red}/C_{out\ phenol\ red} \quad (1)$$

where $C_{in\ phenol\ red}$ is equal to the concentration of phenol red in the inlet sample and $C_{out\ phenol\ red}$ is equal to the concentration of phenol red in the outlet sample.

The drug absorption rate constant (K_a) and the effective permeability coefficient (P_{eff}) were calculated using the following equations:

$$K_a = (1 - C_{out}'/C_{in}')Q/V \quad (2)$$

$$P_{eff} = -Q \ln(C_{out}'/C_{in}')/2\pi rl \quad (3)$$

where Q is the perfusion buffer flow rate (0.2 mL/min) and V, r, and l are the volume, radius, and length of the intestine segment, respectively.

Mechanisms of Pgp Inhibition. *Measurement of Fluorescence Anisotropy.* The membrane fluidity was evaluated by anisotropy measurements with the fluorescent probe DPH.[27] Around 1 mg of DPPC was dissolved in 500 μL of chloroform and dried under a stream of nitrogen. After the lipids had been dried under a high vacuum for at least 2 h, they were hydrated by adding 100 μL of phosphate-buffered saline (PBS) of HP-β-CD (6.8 mmol/L) or an equivalent of the inclusion complex. Each sample was vortexed for 2 h at 60 °C to uniformly disperse the lipids. It was then subjected to 5 cycles of freeze and thaw in liquid nitrogen and a 40 °C water bath to ensure solute equilibration between trapped and bulk solutions. The resulting liposomes (0.15 mg of lipids/mL) were incubated for 30 min at 37 °C in PBS at pH 7.4 with 10 mM DPH, which was added as a 100-fold concentrated stock solution in tetrahydrofuran. The anisotropy values were determined with a Flexstation 3 microplate reader. The wavelengths of excitation and emission were 360 and 430 nm, respectively.

Determination of Pgp ATPase Activity. The ATPase activity of Pgp was determined using a luminescent ATP detection kit (Pgp-Glo assay kit, Promega, Madison, WI) according to the instructions of the manufacturer. Briefly, 0.5 mg/mL of Pgp membranes and 5 mM MgATP were incubated in the absence or presence of 100 μM sodium orthovanadate at 37 °C for 40 min, and the remaining ATP was detected as a luciferase-generated luminescent signal. Basal Pgp ATPase activity was determined as the difference between ATP hydrolysis in the presence or absence of vanadate. Verapamil-stimulated Pgp ATPase activity was measured in the presence of 200 μM verapamil.

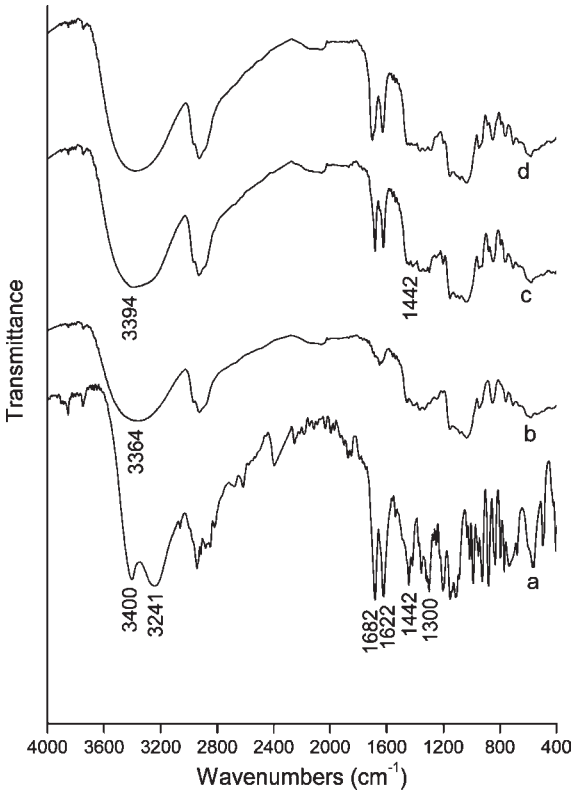

Figure 3. FTIR spectrograms of (a) genipin, (b) HP-β-CD, (c) the physical mixture, and (d) the genipin/HP-β-CD inclusion complex.

Statistical Analysis. Results are presented as the mean ± standard deviation (SD). Statistical differences were calculated by one-way analysis of variation (ANOVA) with Tukey post hoc test using Origin 7.5. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phase Solubility Studies. The phase solubility diagram of the genipin/HP-β-CD system (Figure 2) showed that drug solubility increased linearly with increasing HP-β-CD concentrations. The solubility of genipin increased significantly, by 72.55% at 30 mM HP-β-CD. The diagrams can be classified as A_L type according to the model proposed by Higuchi and Connors[28] and can be related to the formulation of inclusion complexes. The apparent 1:1 stability constant, K_c, was calculated from the phase solubility diagrams based on the following equation:

$$K_c = \text{slope}/S_0(1 - \text{slope}) \quad (4)$$

where slope is the value found in the linear regression and S_0 (8 mg/mL) is the water solubility of genipin in the absence of HP-β-CD (intercept). This gave a K_c of 283.81 M^{-1}, suggesting that a favorable interaction occurs because, in general, drug–CD association constants are reported in the range of 50–2000 M^{-1}.[11]

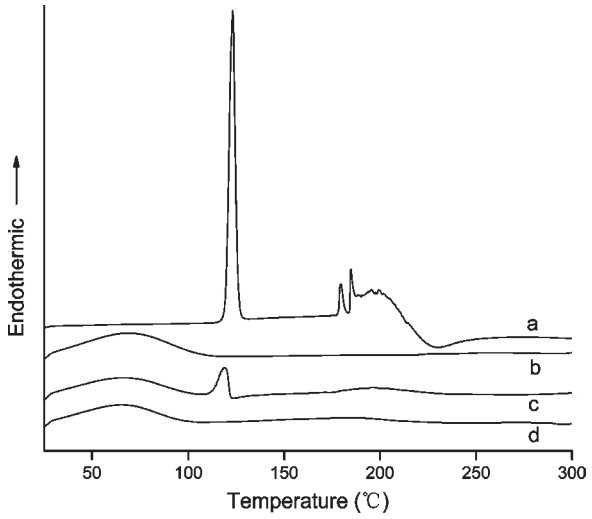

Figure 4. DSC thermograms for (a) genipin, (b) HP-β-CD, (c) the physical mixture, and (d) the genipin/HP-β-CD inclusion complex.

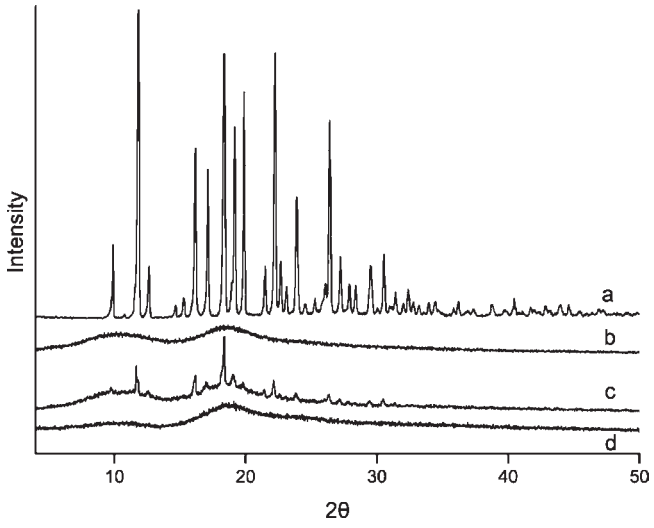

Figure 5. X-ray diffractograms for (a) genipin, (b) HP-β-CD, (c) the physical mixture, and (d) the genipin/HP-β-CD inclusion complex.

FTIR Spectroscopy. FTIR spectrograms are given in Figure 3. The free genipin had intense absorption peaks at 3400, 3241, 1682, 1622, 1442, and 1300 cm^{-1}, which were the characteristic peaks for identification. The spectrum of HP-β-CD presented characteristic peaks at 3364 cm^{-1}. The spectrum of the physical mixture was well-matched with that of the HP-β-CD spectrum in about 2930 cm^{-1} and the crystalline drug spectrum in about 3394 cm^{-1}, indicating that no interaction had occurred as a result of simply physically mixing the free drug with HP-β-CD. Less intense absorptions at around 1682, 1622, 1442, and 1300 cm^{-1} were shown in the physical mixture. This may be attributed to the noncovalent associations of HP-β-CD with genipin in water and the formation of large aggregates and the hydrogen-bonding interaction occurring between HP-β-CD and genipin. The spectrum of the genipin/HP-β-CD inclusion complex showed a broad peak at around 3364 cm^{-1} and less-intense absorptions at around 1682, 1622, 1442, and 1300 cm^{-1}. The sharp absorptions at 3400 and 3241 cm^{-1}, which were observed in the spectrum of genipin, had disappeared. These results indicated that some groups of genipin might be included in the cavity of the HP-β-CD molecules to form the inclusion complex.

DSC. DSC spectrograms are given in Figure 4. The thermogram of genipin was typical of that of a highly crystalline compound, showing a fusion endothermic peak at 123.30 °C (ΔH = 137.6 J/g), followed by exothermic effects because of decomposition phenomena at higher temperatures. The thermogram of HP-β-CD exhibited a very broad endothermic peak between 29 and 106 °C because of the loss of water molecules from the cyclodextrin cavity. In the thermal curve of the physical mixture, the fusion endothermic peak of genipin was much lower than that of the crystalline substance (ΔH = 21.5 J/g) and shifted to a lower temperature as a consequence of the interaction between genipin and HP-β-CD. However, this interaction could be induced by the thermal energy supplied to the sample in the DSC scan.[29] In contrast, there were no sharp peaks attributable to the crystalline form of genipin in the inclusion complexes, indicating that genipin was no longer present as a crystalline form but was converted into an amorphous state.

Powder XRD. XRD was another valid strategy used to confirm the cyclodextrin complexation in powder or microcrystalline states. The diffraction pattern of the drug/CD complex should be clearly distinct from that containing the individual components of the inclusion complex. As shown in Figure 5, XRD patterns clearly confirmed the crystalline nature of genipin and the amorphous state of HP-β-CD. The XRD pattern of the physical mixture confirmed the presence of both compounds as isolated solids, because the diffractograms showed both genipin peaks and the amorphous halo of HP-β-CD. The diffractogram of the inclusion complex indicated a loss of crystallinity of genipin, demonstrating the formulation of the genipin/HP-β-CD inclusion complex. Moreover, the intensity differences observed on diffractograms of HP-β-CD and the inclusion complex, in the 3−50° (2θ) range, strengthen the theory that an amorphous complex had been formed because an approximately 1.5-fold intensity decrease was observed after complexation.

Inclusion Mode. To explore the possible inclusion mode of the genipin/HP-β-CD inclusion complex, we compared the ^{1}H NMR spectra of genipin in the absence and presence of HP-β-CD (Figure 6). The ^{1}H NMR resonance of HP-β-CD was assigned according to the related literature.[10] The ^{1}H resonance of genipin was assigned as follows. ^{1}H NMR (500 MHz, D_2O) δ: 7.51 (s, H-c), 5.83 (brs, H-g), 4.90 (d, J = 7.0 Hz, H-a), 2.03 (m, H-f1), 2.55 (t, J = 6.5 Hz, H-i), 2.75 (dd, J = 7.5, 16.0 Hz, H-f2), 3.12 (dd, J = 7.0, 8.0 Hz, H-e), 3.68 (s, -OCH_3), 4.23 (d, J = 14.0 Hz, H-j2), 4.17 (d, J = 14.0 Hz, H-j1). The formulation of the genipin/HP-β-CD inclusion complex was confirmed by the shifts observed for all of the proton resonances of genipin.

Two-dimensional (2D) ^{1}H NMR is a powerful tool for investigating inter- and intramolecular interactions, because the presence of nuclear Overhauser effect (NOE) cross-peaks between the protons from two different species indicates spatial contact within 0.4 nm.[30] Furthermore, it is an effective method for studying spatial conformations of CD inclusion complexes.[31,32] To gain more conformational information, we obtained 2D ROESY of the inclusion complex of genipin with HP-β-CD (Figure 7). Cross-peaks were observed between the H-j proton of genipin and the H-3 proton of HP-β-CD (peak c) and between the H-c, H-g, H-e, and H-f1 protons of genipin and the H-3 and H-5 protons of HP-β-CD (peaks a, b, d, and e). These NOE cross-peaks indicate that genipin is deeply included in the hydrophobic

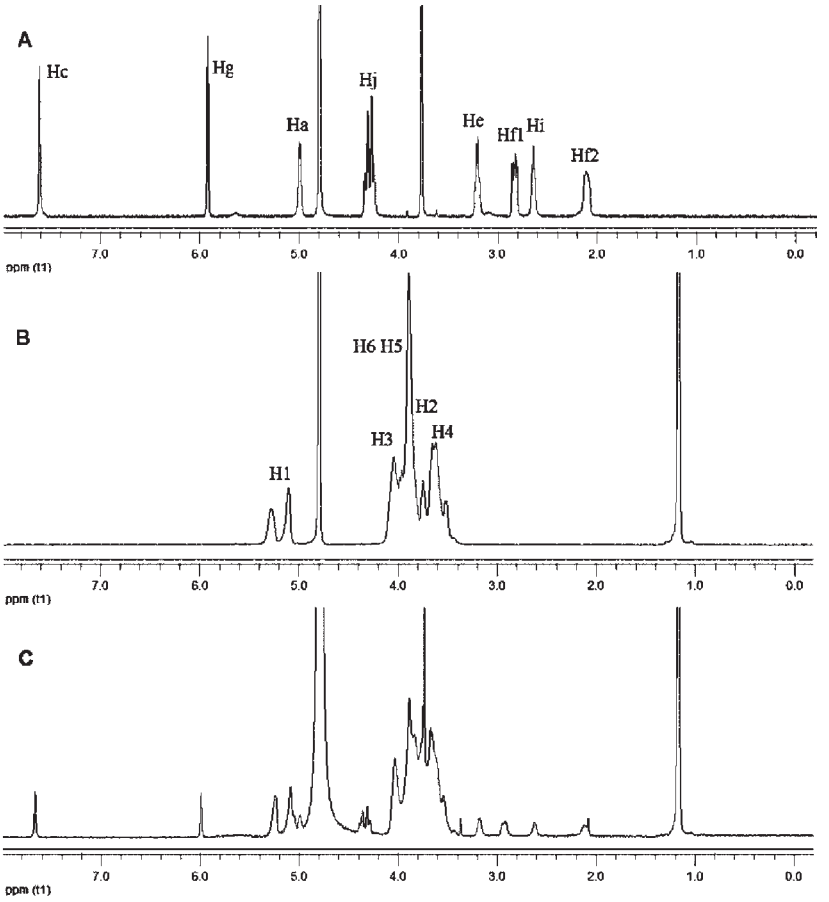

Figure 6. ^{1}H NMR spectra of (A) genipin, (B) HP-β-CD, and (C) the genipin/HP-β-CD inclusion complex in D_2O at 25 °C.

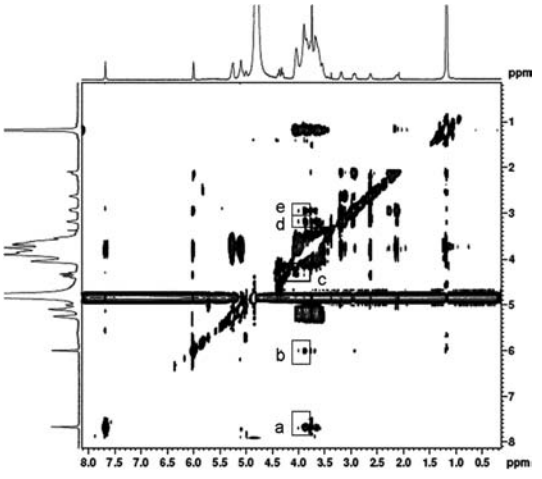

Figure 7. Two-dimensional (2D) ROESY NMR spectrum of the genipin/HP-β-CD inclusion complex in D_2O at 25 °C.

cavity of β-CD. Interestingly, the ROESY spectrum of the genipin/HP-β-CD sample presented several intermolecular NOE cross-peaks between the H-c, H-g, H-e, and H-f1 protons of genipin and the H-2 and H-4 protons of HP-β-CD. The simultaneous interaction with both internal and external HP-β-CD protons may be related to the polymeric structure of HP-β-CD.

Some insights into the inclusion mode could be gained by molecular dynamics (MD) simulations using the Amber force field and semi-empirical method PM3. The optimized structure of the genipin/HP-β-CD inclusion complex is shown in Figure 8.

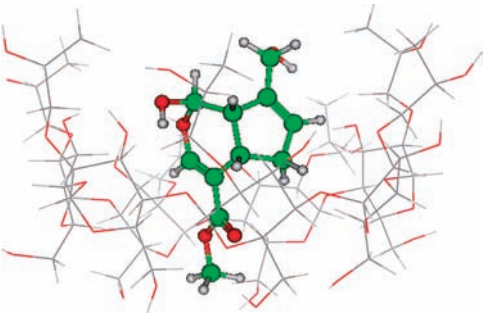

Figure 8. Possible inclusion model of the genipin/HP-β-CD inclusion complex.

Dissolution Studies. Dissolution profiles of genipin and the genipin/HP-β-CD inclusion complex are displayed in Figure 9. It can be seen that the genipin/HP-β-CD inclusion complex produced higher solubility values and a faster dissolution rate when compared to that of the genipin/β-CD inclusion complex[33] and the free drug. The solubility profile of the genipin/HP-β-CD inclusion complex showed that 94.25% of the drug had been released in 3 h, compared to only 51.96% of the free drug. This enhancement can be ascribed to the increased hydrophilicity of the system, which may reduce the interfacial tension between the drug and the dissolution media. Moreover, the inclusion complex

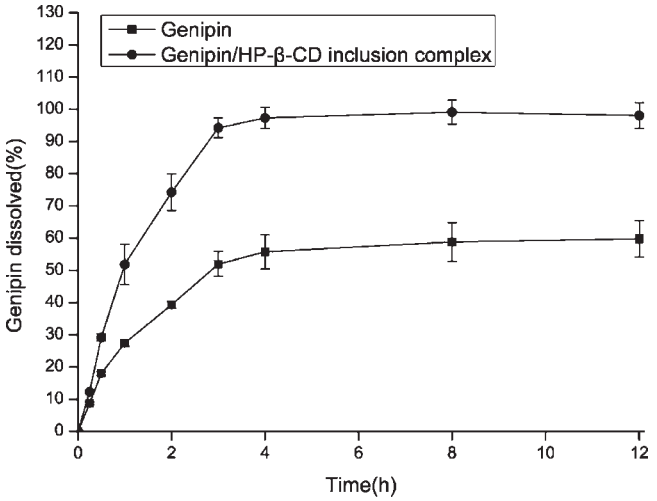

Figure 9. Dissolution profiles of genipin and an equivalent of the genipin/HP-β-CD inclusion complex.

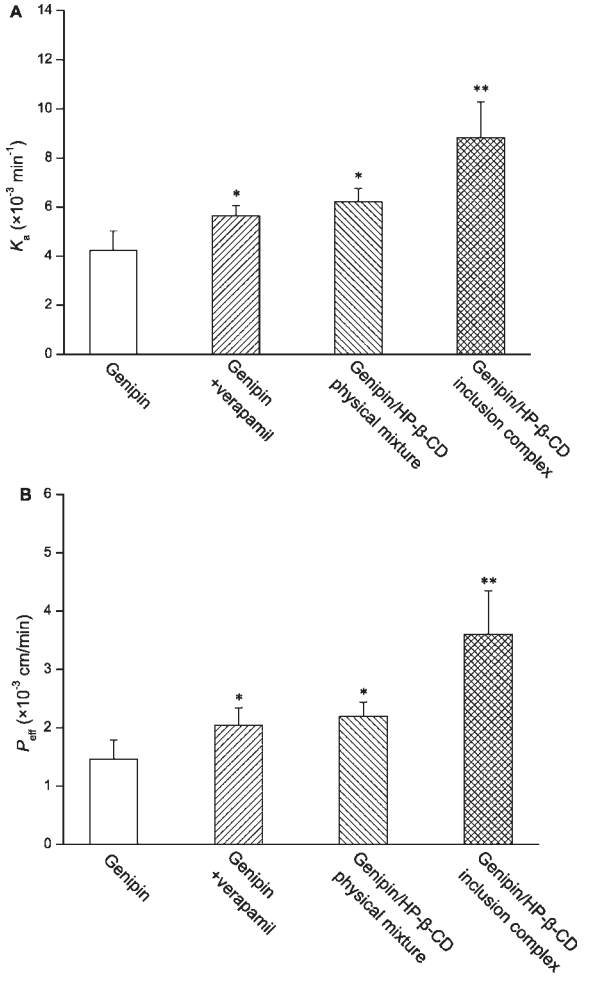

Figure 10. (A) Absorption rate and (B) permeability of genipin with and without verapamil, the physical mixture, and the genipin/HP-β-CD inclusion complex. (*) $p < 0.05$ and (**) $p < 0.01$, significantly different from genipin alone.

dissolved more rapidly than the free drug at the early stage of the dissolution process. The free genipin amount was 17.98% in 30 min and 27.42% in 1 h, while the genipin amount dissolved from the genipin/HP-β-CD inclusion complex was 29.18% in 30 min and 51.85% in 1 h. Hence, HP-β-CD can act on the hydrodynamic layer surrounding the drug particles, resulting in an *in situ* inclusion process that improves the solubility of the drug.[34]

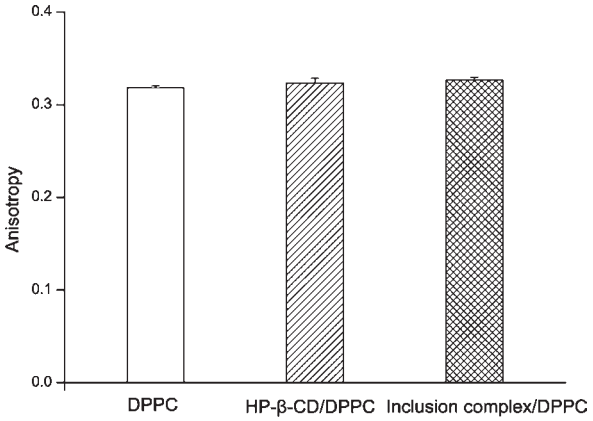

Figure 11. DPH anisotropy values of the DPPC bilayer membrane, containing either HP-β-CD or the genipin/HP-β-CD inclusion complex.

***In Situ* Absorption Studies.** We evaluated the effect of the inclusion complex on the absorption of genipin using an *in situ* perfusion technique because it provides a greater correlation with intestinal absorption in humans than Caco-2 and MDCK cell lines.[35] During the perfusion experiment, genipin was stable in the perfusion solution at 37 °C and no adsorption was observed to either the inlet and outlet tubes or the plastic vials. As shown in Figure 10, the presence of verapamil significantly increased the absorption rate and the permeability of genipin (K_a = 4.23 × 10^{-3} min^{-1} and P_{eff} = 1.46 × 10^{-3} cm/min). This improvement indicated that intestinal Pgp was involved in genipin intestinal absorption. The genipin/HP-β-CD physical mixture gave a 1.47- and 1.43-fold absorption rate and permeability, respectively, when compared to genipin alone. For the genipin/HP-β-CD inclusion complex, the absorption rate and permeability was further improved, 2.60- and 3.46-fold, respectively, higher than genipin alone. A more substantial increase in intestinal absorption was seen with the genipin/HP-β-CD inclusion complex. This enhancement may be attributed to both the solubility enhancement and the Pgp inhibitory effect of HP-β-CD.

Mechanisms of Pgp Inhibition. The mechanisms by which HP-β-CD inhibits Pgp activity may be different from that of other Pgp inhibitors, such as cyclosporine A, quinidine, and verapamil.[36] Pgp recognizes many compounds as substrates and tends to have high affinity with hydrophobic and positively charged compounds at physiological pH.[37] HP-β-CD appears not to be a substrate of Pgp because it is both a hydrophilic and electrically neutral cyclic oligosaccharide with a relatively high molecular weight. Furthermore, HP-β-CD should not compete with Pgp substrates because of its lack of cell permeability. Thus, HP-β-CD must have an alternative inhibitory effect on Pgp activity that differs from the other Pgp inhibitors described above.

Changing the membrane fluidity is considered to be one important mechanism of the Pgp inhibitory effect. To mimic the interactions between HP-β-CD and the cell membrane, a DPPC bilayer membrane was formed and fluorescence polarization measurements were used to detect any change in the dynamic properties of DPPC induced by HP-β-CD.[38] High anisotropy

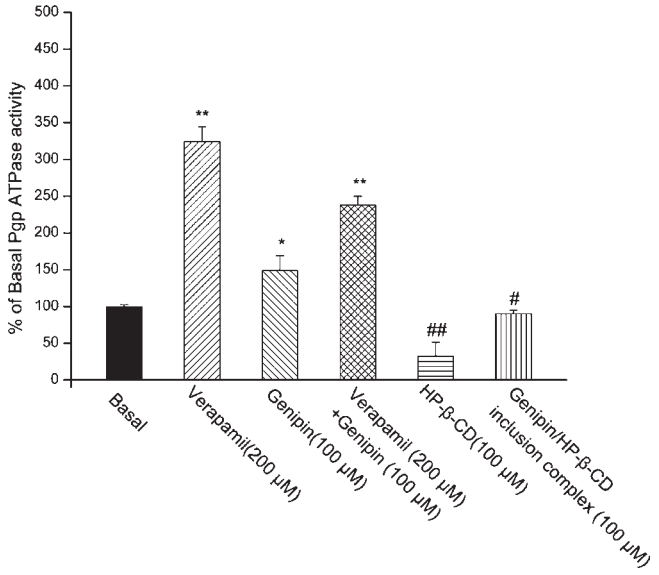

Figure 12. Effects of HP-β-CD on Pgp ATPase activity. (#) $p < 0.05$ and (##) $p < 0.01$, significantly stimulated the basal Pgp ATPase activity. (*) $p < 0.05$ and (**) $p < 0.01$, significantly inhibited the basal Pgp ATPase activity.

values indicate high membrane rigidity and low bilayer fluidity. As shown in Figure 11, HP-β-CD did not change the membrane fluidity, as compared to the DPPC bilayers.

Pgp is an ATP-dependent efflux pump; therefore, Pgp ATPase activity plays an important role in the mechanisms of Pgp inhibition.[39] The influence of HP-β-CD on the ATPase activity of Pgp was measured using human Pgp membranes (Figure 12). Verapamil, a known substrate of Pgp, stimulated Pgp ATPase activity. Genipin alone stimulated the basal Pgp ATPase activity and inhibited the verapamil-stimulated Pgp ATPase activity. These results suggest that genipin is a substrate of Pgp and competitively interacts at the drug-binding site of Pgp. HP-β-CD and the genipin/HP-β-CD inclusion complex significantly inhibited the Pgp ATPase activity and, hence, inhibits the Pgp efflux pump.

In conclusion, the genipin/HP-β-CD inclusion complex exhibits higher solubility and superior dissolution rates when compared to genipin alone. The intestinal transport of genipin was a Pgp-dependent way. The inclusion complex improved the absorption of genipin by both enhancing solubility and inhibiting the Pgp efflux pump. Unlike other Pgp inhibitors, the genipin/HP-β-CD inclusion complex does not alter membrane fluidity; rather, its inhibitory action is due to its effect on Pgp ATPase activity.

■ ASSOCIATED CONTENT

Supporting Information. UV spectra of genipin in the various concentrations of HP-β-CD (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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