Original Article
Preparation and antitumor effects of glaucocalyxin A-γ-cyclodextrin clathrate

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Abstract: Objective: To improve the water solubility of glaucocalyxin A (GLA) by the preparation of glaucocalyxin A γ-cyclodextrin clathrate (GLA-γ-CD) and to investigate the inhibitory effect of GLA-γ-CD on tumor growth in S180 cell xenografts. Materials and methods: GLA-γ-CD, γ-cyclodextrin (γ-CD) and GLA were combined at a mass ratio of 1:1, dissolved in 60 °C water by stirring. GLA completely entrapped by the γ-CD was verified by differential thermal analysis, the GLA content was determined. Phase solubility, solubility, and in vitro dissolution rate experiments were performed. The S180 xenograft mouse model was used to observe the tumor inhibitory effects of GLA-γ-CD and GLA, and the TUNEL assay was used to detect differences in their rates of tumor cell apoptosis induction. Results: After combination with γ-CD, the solubility of GLA-γ-CD was 21.78-fold greater than that of GLA. The in vitro dissolution rate of GLA-γ-CD was significantly greater than that of GLA, and reached more than 90% in 20 min. Furthermore, GLA-γ-CD was more effective than GLA as an inhibitor of S180 tumor cells; the inhibitory rate of the high-dose group reached 57.26%, which was 54.11% greater than the inhibitory rate of the GLA group at the same dose. In addition, GLA-γ-CD induced tumor cell apoptosis more effectively than did GLA. Conclusion: The water solubility of GLA significantly increased in combination with γ-CD resulting in the production of GLA-γ-CD. Furthermore, GLA-γ-CD was more effective than GLA as an inducer of S180 tumor cell apoptosis and an inhibitor of tumor growth.

Keywords: Glaucocalyxin A, γ-cyclodextrin, clathrate, tumor growth

Introduction

Glaucocalyxin A (GLA) is a diterpenoid compound isolated from plants of the genus Isodon from the Lamiaceae family, which has cardiovascular protective effects, as well as antibacterial and antioxidant activity [1-3]. Recently, the cytotoxic activity of GLA has become a focus of research. GLA inhibits the proliferation of HeLa cells, HL-60 cells, and other cell lines in vitro [4-6]. However, GLA has poor water solubility and low bioavailability, which interferes with its absorption and efficacy. Efforts to improve these limiting characteristics using organic solvents to enhance the solubility of GLA were unsuccessful, and increased its toxicity. To improve the water solubility of GLA, γ-cyclodextrin (γ-CD) was selected as the clathrate material in this study, in which we evaluated the clathrate phase solubility, solubility, and dissolution rate of GLA, and observed the impact of the GLA-γ-cyclodextrin compound (GLA-γ-CD) on mice that received S180 tumor cell transplants. The aims of this study were to find better clathrate materials and methods of GLA preparation, to explore the possible antitumor mechanisms of new GLA preparations, and to provide an experimental basis for the clinical application of GLA-clathrate combinations.

Materials and methods

Materials

Instruments: TU-1810PC spectrophotometer (Beijing Purkinje General Co.), ZRC-6FT intelligent drug dissolution apparatus (Tianjin Chong Hing Electronic Equipment Manufacturing Co., Ltd.), CRY-32P differential thermal analyzer (Shanghai Precision & Scientific Instrument Co., Ltd.).

Reagents: The GLA raw material (high-performance liquid chromatography (HPLC) with mass fraction > 96% as determined by peak area nor-
malization method) was provided by the Teaching and Research Section of the Department of Natural Medicine Chemistry of Xinxiang Medical University, the GLA reference material was provided by the Natural Medicinal Chemistry Department of Xinxiang Medical College (batch number: 20101006; mass fraction > 99%). γ-CD was obtained from Wuhan Xianghe Chemicals Co., Ltd. (batch number: 20091008), cyclophosphamide was obtained from Jiangsu Hengrui Medicine Co., Ltd. (batch number: 09123121), and the apoptosis assay kit was obtained from Wuhan Boster.

**Tumor lines and animals:** The sarcoma S180 tumor cell strain was purchased from Nanjing KGI Biological Co. Male and female kunming mice (specific pathogen-free) weighing 18-22 g were provided by the Experimental Animal Center of Henan Province (certificate of conformity of SCXK (Henan): 2005-0001).

**Preparation of GLA clathrate and determination of solubility**

**Clathrate preparation:** The inclusion material (γ-CD) and GLA raw material were combined at a mass ratio of 1:1. GLA was dissolved by adding a small amount of ethanol (with ultrasound if necessary) prior to use. The clathrate was dissolved with an appropriate amount of water using a magnetic stirrer in a 60°C water bath for 4 h, while the GLA-ethanol solution was added slowly. Ethanol was distilled using a rotary evaporator at 60°C under reduced pressure. The residual aqueous solution was dried by freezing to obtain GLA-γ-CD.

**Identification of GLA clathrate by thermal analysis:** Appropriate amounts of GLA and γ-CD were combined at a ratio of 1:1 (by volume) and analyzed by differential thermal analysis. The reference object for the measurements was an empty crucible. Temperature sampling was performed from 40°C with a heating rate of 10°C•min⁻¹ over a temperature range of 30-300°C, and the measured gas was static air.

**Measurement wavelength selection, standard curve plotting, and recovery determination:** GLA, γ-CD, and GLA-γ-CD were distilled with water to a suitable concentration and UV scanning of the solutions was performed at a wavelength range from 200 to 400 nm. The detection wavelength was selected according to the UV spectra figures. Ten milligrams of the GLA reference material was dried by phosphorus pentoxide and 100 mL of purified water was added to prepare 100 μg•mL⁻¹ GLA stock solution. Water was then added to the stock solution to prepare samples with GLA concentrations of 12.0, 15.0, 18.0, 21.0, 24.0, 27.0, and 30.0 μg•mL⁻¹. The absorbance (A) of each sample was detected at the appropriate wavelength. A linear regression equation was constructed using the drug concentration (C) and the sample absorbance. Nine samples of γ-CD were weighed according the various concentrations of GLA. GLA reference material was added to prepare 3 solutions with high, medium, and low concentrations. The measured absorbance of each solution was inserted into the regression equation to calculate the corresponding concentration and recovery rate.

**Preparation of clathrate phase solubility diagram:** γ-CD-water solutions were prepared at 0, 2, 4, 6, and 8 mmol•L⁻¹. GLA solution was added to the γ-CD-water solutions in a water bath oscillator at 25°C for 48 h, after which the resulting solution was filtered with a micro-pore membrane (pore size = 0.45 μm) (filtered after centrifugation if necessary). The resulting filtrate was diluted and GLA solubility was calculated. The solubility curve was plotted with the γ-CD concentration on the abscissa and the solubility of GLA on the ordinate.

**Determination of inclusion solubility:** Excess GLA, GLA-γ-CD, and the physical mixture were weighed, 5 mL distilled water was added to each sample, and the sample was shaken for 24 h at 25±0.5°C in a water bath oscillator. After shaking, a 0.45-μm filter membrane was used for filtering. The subsequent filtrate was diluted and the absorbance at 236 nm was measured and used to calculate GLA solubility in the solution.

**Determination of inclusion dissolution:** Inclusion dissolution was determined per the rotating basket method described in the 2010 edition of the “People’s Republic of China Pharmacopoeia”. Appropriate amounts of GLA, GLA-γ-CD, and the physical mixture (equivalent to 40 mg GLA) were weighed and placed in the rotation basket (the pad coarse filter paper was put at the bottom of the rotating basket). The release medium was distilled water, the volume
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was 900 mL, the water temperature was 37±0.5°C, and the rotary speed was 100 rpm. The GLA, GLA-γ-CD, and physical mixture were placed in the rotation basket. The basket was turned and the time required for the sample to contact the media was recorded. Samples (5 mL) were taken at 5, 10, 20, 30, 45, and 60 min, and filtered using a membrane filter (pore size = 0.45 μm), while the temperature was adjusted to dissolve the material. The resulting filtrate was diluted appropriately, the absorbance was measured, and the concentration of the drug was calculated. Next, the percentage of cumulative drug dissolution was calculated and the dissolution diagram was plotted.

**S180 xenograft model**

**Preparation of the S180 xenograft model mice, animal grouping, and administration:** Under sterile conditions, S180 mice of approximately 9 days of age were selected for drawing ascites intraperitoneally with well grown tumor cells, which appeared as a thick milky liquid. The cells were washed twice with sterile saline, counted, and adjusted to a concentration of 1×10^7 cells mL^1. These cells (0.15 mL per mouse) were subcutaneously inoculated in the left armpit to mimic the S180 xenograft model. The procedure was abandoned if the suspension flew out during the inoculation process. The procedure was repeated until the desired number of experimental animals was obtained. Twenty-four hours after inoculation, the animals were randomly divided into the control group, the cyclophosphamide group (10 mg·kg^-1), the GLA group (80 mg·kg^-1), and the high-, medium-, and low-dose GLA-γ-CD groups (80, 40, 20 mg·kg^-1) (n = 10 animals per group). Gavage was performed at 10:00 daily. The control group received saline for 12 d. During the experiment, the general conditions and body weight changes of mice in each group were observed.

**Gross tumor morphology and weight:** Twenty-four hours after the last administration, the mice were sacrificed, the tumor mass was collected, and the dispersion degree, necrosis, and tissue adhesion of the tumor mass were evaluated. The wet weight of the tumor was used to calculate the inhibitory rate of the administered compounds as follows:

Inhibitory rate (%) = (the average tumor weight of the control group - the mean tumor weight of the treatment group)/the average tumor weight of the control group × 100.

**Determination of the thymus index and spleen index:** The thymus and spleen were collected and weighed to allow us to calculate the thymus index and spleen index as follows:

Organ index = organ weight in mg/body weight in grams.

**TUNEL assay:** The tumor masses were paraffin-embedded and sliced, and subjected to the TUNEL assay. Apoptotic cell nuclei were identified by their small size and brownish yellow or brown coloration. One-thousand cells from

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**Figure 1.** Differential thermal analysis spectra. 1. γ-CD, 2. GLA, 3. GLA-γ-CD inclusion complex, 4. GLA and GLA-γ-CD mixture.

**Figure 2.** The UV absorption spectrogram. 1. GLA standard solution, 2. γ-CD solution, 3. GLA-γ-CD inclusion solution.
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Preparation and solubility determination of GLA-γ-CD

Identification of GLA-γ-CD by differential thermal analysis: GLA had an endothermic peak at 216°C, which was the peak melting point of the drug. γ-CD and the GLA-γ-CD clathrate did not have peaks at 216°C, while the physical mixture of GLA and γ-CD did have a peak at this temperature, showing that the added GLA was completely entrapped in the γ-CD (Figure 1).

Standard curve plotting, wavelength, and recovery determination: The UV spectra of GLA, γ-CD, and GLA-γ-CD are shown in Figure 2. GLA had a strong and stable absorption peak at 236 nm, and γ-CD showed no absorption at this wavelength; therefore, 236 nm was selected as the detection wavelength and used to plot the standard curve. The drug concentration (C) was used to plot a linear regression with the absorbance value (C = 40.644; A = 0.274 4; r = 0.9996, n = 7). Results indicated that GLA had a good linear relationship at 12.0-30.0 μg•mL⁻¹ concentration. The corresponding concentrations and recoveries were calculated by this regression equation. The average γ-CD recovery rate was 98.95±1.26% (RSD = 1.27%).

Results

Preparation and solubility determination of GLA-γ-CD

Figure 3. Phase solubility curves of GLA in different concentrations of γ-CD solutions.

Figure 4. Solubility diagram of GLA. **P < 0.01 compared to Free GLA; ΔΔP < 0.01 compared to GLA and γ-CD mixture.

Plotting the inclusion phase solubility diagram: The values of GLA solubility and γ-CD concentrations were used to perform linear regressions, and the apparent stability constants (Kc values) of the clathrate solutions were calculated according to the following formula:

Kc = slope/[-×S₀ (1 slope)]

The slope and S₀ from this calculation were used as the slope and intercept of the regression equation. The obtained equation was Y = 0.6351X + 0.0005 (r = 0.9992), Kc = 3480.95. The apparent stability constant Kc was an important parameter used to measure the stability of inclusion, which meant that there was a dynamic equilibrium between the free drug molecules in the solution and the clathrate of drug molecules. The Kc value reflected the strength of the binding force of the inclusion of γ-CD and drug molecules. The Kc value measured in this experiment was larger, which showed that the clathrate formed by GLA and γ-CD was stable. From the phase solubility

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Determination of the solubility of the clathrate complex: GLA-γ-CD solubility in water reached 5.01±0.52 mg·mL⁻¹ at 25°C, which was significantly greater than that of GLA solubility (0.23±0.07 mg·mL⁻¹) and the mixture of GLA and γ-CD (0.49±0.11 mg·mL⁻¹). The solubility of GLA-γ-CD was 21.78-fold greater than that of GLA (Figure 4).

Determination of clathrate dissolution: The dissolution rate of GLA-γ-CD reached more than 90% in 20 min (Figure 5).

Impact of GLA-γ-CD in the S180 xenograft model

Gross morphology of tumor mass: Tumor mass was palpable at the third day after inoculation. Obvious tumor masses appeared by day 7 after inoculation, and the size of these masses increased daily. Partial adhesions of the tumor mass and the chest wall muscle or upper limb muscles appeared on the 12th day in the control group, and no skin ulceration was observed. The GLA-γ-CD and GLA groups showed less tumor tissue invasion than was observed in the control group, and no skin ulceration was observed. Tumor growth was slow in the high-dose GLA-γ-CD group and the cyclophosphamide group, and none of the phenomenon mentioned above occurred.

Impact on S180 tumor growth: The mean tumor weights in each treatment group were significantly less than that of the control group. All tumor inhibition rates were greater than 30%, and the high-dose GLA-γ-CD group significantly inhibited tumor growth (P < 0.01) with an inhibitory rate of 57.26%, which was close to that of the CTX group. High-dose GLA-γ-CD also significantly reduced tumor weight in comparison with an identical dose of GLA. GLA-γ-CD significantly inhibited tumor growth with an inhibitory rate 54.11% greater than that of GLA (Figures 6, 7).

Effect on body weight of tumor-bearing mice: The body weight in each group increased gradually over time. The weight of the mice in the control group increased the fastest, followed by the GLA-γ-CD group and the GLA group, while mice in the cyclophosphamide group showed...
no obvious weight gain. The weight gain was significantly lower than that of the control group on the 6th, 9th, and 12th days of observation ($P < 0.05$) (Figure 8).

**Impact on thymus index and spleen index of tumor-bearing mice:** The thymus index and spleen index of the tumor-bearing mice in the GLA-γ-CD and GLA groups showed no significant changes in comparison with that of the control group ($P > 0.05$). Cyclophosphamide significantly reduced the thymus index and spleen index of the tumor-bearing mice ($P < 0.01$, Figure 9).

**Impact on S180 cell apoptosis:** The number of apoptotic cells and the apoptotic index in the high-dose GLA-γ-CD group, the GLA group, and the cyclophosphamide group were increased significantly in comparison with these measures in the control group. The apoptotic index of the high-dose GLA-γ-CD group was significantly improved in comparison with that of the GLA and cyclophosphamide groups (Figures 10, 11).

**Discussion**

Cyclodextrin (CD) products are formed from starch or straight-chain dextrin by cyclodextrin glycosyltransferase. CD is a cyclic polysaccharide consisting of 6, 7, or 8 D-glucopyranose units bonded by an α-1, 4-type glycosidic link. The hollow, tube-like cylindrical molecular structure of CD has a narrow width, an opening at both ends, and relative hydrophobicity in the internal cavity, while the hydroxyl groups are distributed outside the molecule. The internal lipophilicity and external hydrophilicity of CD allow it to entrap foreign compounds, which can greatly increase the water solubility of poorly water-soluble drugs, and thus improve their stability, bioavailability, and bioactivity, and their reduce side effects overall [7-10]. The main types of CD are designated α-CD, β-CD, and γ-CD according to the number of glucose units [11]. Compared with α-CD and β-CD, γ-CD had a larger cavity, good water-solubility, and few toxic side effects and irritant properties, which give it potential applications in many industries, and particularly in the food and pharmaceutical sectors [12].

GLA had poor water solubility and a relatively large 3-dimensional molecular structure. The structure of γ-CD allows it to entrap GLA. When the experimentally measured clathrate constant values were large, the clathrate had high material stability and solubilization and dissolution were significantly improved. Drugs must be
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dissolved in digestive solution in order to be
absorbed through the gastrointestinal epithelial membrane, so the solubility of drugs is a criti-
cal determinant of their absorption. GLA has very low solubility in water, but GLA-γ-CD showed solubility more than 21 greater than that of GLA. The dissolution rate of GLA-γ-CD was also significantly improved in comparison with that of GLA, which enhanced absorption of the drug in the body of the mice and lays a good foundation for in vivo drug studies. In addition, the present study used ethanol as a solvent to dissolve the GLA, and thus hazardous materials such as chloroform, metha-
ol, and other organic solvents were not required, and the safety of the drug was guaranteed.

Figure 10. Observation of apoptotic histopathology by TUNEL (×400) A: Control group B: 10 mg/kg CTX group, C: 80 mg/kg GLA group, D: 80 mg/kg GLA-γ-CD group.

Figure 11. Effect of GLA-γ-CD and GLA on the apoptotic index of S180-bearing mice. **P < 0.01 compared to control group; *P < 0.05 compared to GLA group.
GLA is a diterpenoid isolated from Varglaucocalyx that induces apoptosis and inhibits tumor growth in vitro [4-6]. In this study, GLA-γ-CD significantly inhibited S180 tumor growth in vivo in a dose-dependent manner. The inhibition rate observed in the high-dose group was similar to that of the cyclophosphamide group, and was significantly better than that of GLA at the same dose, which indicated that γ-CD improved the water-solubility and anti-tumor effects of GLA. Zhao et al. used γ-CD to package folic acid to form γ-CD-folic acid compounds, which were cytotoxic to HepG2 cells and had good biocompatibility [13].

Tumor occurrence and development are closely related to immune function decline. The thymus index and spleen index reflect the status of the immune system. We found that GLA treatment did not affect the thymus index or spleen index in mice, indicating that it had little effect on the immune system and that its anti-tumor effect may be unrelated to immune function regulation.

Apoptosis plays a key role in controlling cell proliferation, tumor incidence, and growth regulation. Tumor apoptosis is a critical mechanism that is induced by cancer prevention therapies [14]. The anti-tumor effects of compounds used in traditional Chinese medicine are also achieved by promoting apoptosis [15]. Using the TUNEL assay, we found that the number of apoptotic cells in tumor tissue was significantly increased after GLA treatment, suggesting that the antitumor mechanism of GLA may be related to the induction of apoptosis, which was a finding similar to the results of a recent study by Li et al. [4]. We also found that the number of apoptotic cells in the GLA-γ-CD group was greater than that of the GLA group, and the apoptotic index of the former group was improved significantly, suggesting that GLA-γ-CD induced apoptosis in tumor cells more effectively than did GLA, and it had minimal cytotoxicity in mice. The results of our studies with the GLA-γ-CD inclusion complex show that it has advantages over pure GLA, and have established a good foundation for the development of future applications.

Conclusion

The present work demonstrated the preparation of a glaucocalyxin A clathrate with γ-cyclodextrin (GLA-γ-CD). The solubility of GLA-γ-CD in water reached (5.01±0.52) mg·mL⁻¹, which was 21 times greater than that of pure GLA. In addition, the stability, solubility, and dissolution of GLA were significantly improved by entrapment in γ-CD. GLA-γ-CD induced S180 tumor cell apoptosis and inhibited tumor growth more effectively than did GLA, and it had minimal cytotoxicity in mice. The results of our studies with the GLA-γ-CD inclusion complex show that it has advantages over pure GLA, and have established a good foundation for the development of future applications.

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Disclosure of conflict of interest

None.

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