University of Groningen

Binding behavior, water solubility and in vitro cytotoxicity of inclusion complexes between ursolic acid and amino-appended beta-cyclodextrins

Song, Shuang; Gao, Kai; Niu, Raomei; Yi, Wenlong; Zhang, Jihong; Gao, Chuanzhu; Yang, Bo; Liao, Xiali

Published in:
Journal of Molecular Liquids

DOI:
[10.1016/j.molliq.2019.111993](https://doi.org/10.1016/j.molliq.2019.111993)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Song, S., Gao, K., Niu, R., Yi, W., Zhang, J., Gao, C., Yang, B., & Liao, X. (2019). Binding behavior, water solubility and in vitro cytotoxicity of inclusion complexes between ursolic acid and amino-appended beta-cyclodextrins. *Journal of Molecular Liquids*, 296, [111993]. <https://doi.org/10.1016/j.molliq.2019.111993>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Binding behavior, water solubility and *in vitro* cytotoxicity of inclusion complexes between ursolic acid and amino-appended β -cyclodextrins

Shuang Song ^{a,1}, Kai Gao ^{a,1,2}, Raomei Niu ^{a,b}, Wenlong Yi ^a, Jihong Zhang ^{c,d},
Chuanzhu Gao ^a, Bo Yang ^a, Xiali Liao ^{a,*}

^a Faculty of Life Science and Technology, Kunming University of Science and Technology, 650500, Kunming, China

^b Jiangsu Xinchun Pharmaceutical Co., LTD, 222047, Lianyungang, China

^c Faculty of Medicine, Kunming University of Science and Technology, Kunming, 650500, China

^d Research Centre for Pharmaceutical Care and Quality Management, First People's Hospital of Yunnan Province, Kunming, 650500, China

ARTICLE INFO

Article history:

Received 24 June 2019

Received in revised form

11 October 2019

Accepted 20 October 2019

Available online 28 October 2019

Keywords:

Ursolic acid

Amino-appended β -cyclodextrin (ACD)

Inclusion complex

Binding behavior

Water solubility

In vitro cytotoxicity

ABSTRACT

Ursolic acid (UA) is a pentacyclic triterpenoid of naturally abundance with a broad spectrum of important biological activities and low toxicity. However, potential applications in pharmaceutical industry are severely hampered by its poor water solubility, which leads to low bioavailability. Herein, we harness the unique and superior inclusion capability of a series of amino-appended β -cyclodextrins (ACDs) to prepare solid inclusion complexes of UA/ACDs. These inclusion complexes were characterized in their solid state by scanning electron microscope (SEM), differential scanning calorimetry (DSC), thermogravimetric (TG) and powder X-ray diffraction (XRD) analyses. Furthermore, their supramolecular binding behavior in aqueous solution was investigated by one-dimensional (1D)- and two-dimensional (2D)-nuclear magnetic resonance (NMR) spectroscopic experiments and NMR-based phase solubility analysis. Binding stability constants (K_s) were determined (1799, 1410, 889 and 993 L mol⁻¹ for UA/a0, UA/a1, UA/a2 and UA/a3, respectively), and dynamic bimodal inclusion modes with a 1:1 inclusion stoichiometry for UA/ACDs systems were proposed. Water solubility of UA is dramatically promoted by more than 200-fold after formation of inclusion complexes. *In vitro* cytotoxicity of UA achieves significant elevation against human cancer cell lines HepG2, HT-29 and HCT116 by inclusion complexation from MTT assay, while these inclusion complexes show no cytotoxicity against human normal cell line LO2, which confirms their safety. These results would benefit to the further development of liquid formulation of UA for pharmaceutical uses.

© 2019 Elsevier B.V. All rights reserved.

1. Introduction

Ursolic acid (3 β -3-hydroxy-urs-12-en-28-oic-acid, UA) (Fig. 1) is a pentacyclic triterpenoid which occurs either as a free acid or an aglycone of terpenoid saponins in nature. UA exists in many plants, especially in sorts of fruits and herbs, such as apples, bilberries, cranberries, parsley, sage, rosemary and thyme, etc. [1,2]. A wide spectrum of important biological activities of UA such as antioxidant [3–5], antibacterial [6,7], anti-inflammatory [8,9], anticancer [10–13], anti-hyperlipidemic [14], hepatoprotective [15,16], anti-

diabetic [17,18], anti-Alzheimer's [19] and immunomodulatory [17] effects were reported during the last decade. UA is non-toxic and used in cosmetics and health products, either as an active component or as a natural scaffold for a wide range of novel and potent bioactive molecules [20]. In addition to its broad biological activities and low toxicity, the naturally abundance of UA also features significantly easier availability from nature in comparison to the scarce existence of most biologically active natural products. However, the highly hydrophobic pentacyclic backbone of UA results in very low water solubility, which leads to poor oral bioavailability. Therefore, strategies for increasing water solubility of UA are in urgent demand for the further development of its applications in pharmaceutical industry.

Cyclodextrins (CDs) are truncated-cone shaped cyclic oligosaccharides mainly composed of six to eight D-glucose units linked by α -1,4-glycosidic bonds. CDs have two hydrophilic outer faces, i.e., the primary and secondary faces decorated with primary and

* Corresponding author. Faculty of Life Science and Technology, Kunming University of Science and Technology, 650500, Kunming, China.

E-mail address: xialiliao@hotmail.com (X. Liao).

¹ These authors contributed equally to this work.

² Current address: Faculty of Science and Engineering, University of Groningen, Groningen, the Netherlands.

Fig. 1. The chemical structure of UA.

secondary hydroxyl groups, respectively, and a hydrophobic inner cavity which could accommodate varieties of inorganic, organic or bio-molecules by supramolecular inclusion complexation [21]. CDs are often used as drug carriers to improve water solubility, bioavailability, pharmacological and metabolic properties of drugs that are poorly soluble in water via covalent or non-covalent bindings [22,23]. Owing to its non-toxicity and the easiest accessibility, β -CD represents the most popular type of CDs, and is widely used in the fields of food, cosmetic, pharmaceutical and catalysis [24–26]. However, the use of β -CD is seriously restricted for its relatively poor water solubility when the liquid formulation is in demand. Strategies for chemical modification on native β -CD have thus emerged as an attractive option of addressing this notable problem, which would not only improve its water solubility, but also adjust the three-dimensional structure of the hydrophobic cavity in order to adapt to unprecedented substrates. Among them, the mono-substitution on β -CD has become the most popular and practical one because of its convenience of preparation and versatility [27]. Mono-substituted β -CDs with a variety of amino groups showed much greater water solubility and were frequently used for molecular recognition and supramolecular catalysis in recent years in our laboratory [28–31]. Herein, inclusion complexes of amino-appended β -CDs (ACDs) (Fig. 2) with UA were prepared and their binding behavior, water solubility and *in vitro* cytotoxicity towards human cell lines were studied.

2. Experimental

2.1. Materials

β -CD (MW = 1135.0, 98% purity) and UA (MW = 456.7, >98% purity) were purchased from YuanNuoTianCheng Tech (Chengdu, China) and Adamas Reagent (Shanghai, China), respectively, and were used without further purification. Other reagents were all of analytical grade. Ultrapure water was used throughout all experiments.

2.2. Preparation of solid inclusion complexes of UA with ACDs

The ACDs appended with different lengths of amino chains, i.e., mono-(6-deoxy-6-amino)- β -CD (**a0**), mono-(6-deoxy-6-ethylenediamine)- β -CD (**a1**), mono-(6-deoxy-6-diethylenetriamine)- β -CD (**a2**) and mono-(6-deoxy-6-triethylenetetraamine)- β -CD (**a3**) were synthesized according to our previous work [31]. Solid inclusion complexes of UA/ACDs were prepared by a suspension method. In brief, UA and ACD were added to water in a molar ratio of 3:1 and the resulting suspension was allowed to stir at 25 °C for 72 h in the dark. It was then filtered through a 0.45- μ m membrane to get rid of any undissolved solids. The filtrate was evaporated under reduced pressure followed by drying at 50 °C *in vacuo* to yield a solid inclusion complex of UA/ACD.

2.3. Preparation of physical mixtures of UA and ACDs

UA and ACDs were mixed thoroughly with a molar ratio of 1:1 in a small mortar at room temperature to give the 1:1 physical mixtures.

2.4. Scanning electron microscope (SEM) analysis

The SEM analysis of solid inclusion complexes was performed on a Jeol JSM-840 scanning electron microscope (Japan) to probe their microscopic morphology in solid state. Powders of solid inclusion complexes of UA/ACDs, along with UA, ACDs and their physical mixtures for comparison, were all evenly dispersed on a double-sided adhesive tape which was fixed on a brass stub before testing. Samples were gold sputter-coated make them electrically conductive. The micrographs were then obtained with an accelerating potential of 15 kV under reduced pressure.

2.5. Thermal analysis

Thermal analysis of solid inclusion complexes of UA/ACDs along with UA, ACDs and their physical mixtures including differential scanning calorimetry (DSC) and thermogravimetric (TG) measurements were performed on a NETZSCH STA449F3 instrument (Germany). Each sample was heated at a rate of 10 °C $\cdot$ min⁻¹ from room temperature to 450 °C under a dynamic nitrogen atmosphere at a flow rate of 100 mL $\cdot$ min⁻¹ to obtain its DSC and TG curves.

2.6. Powder X-ray diffraction (XRD)

For the powder XRD analysis, a D/Max-3B diffractometer (Japan, Cu-K α , λ = 1.5460 Å) was used for solid inclusion complexes of UA/ACDs along with UA, ACDs and their physical mixtures under

Fig. 2. The structure of ACDs.

conditions as follows to get their XRD patterns: voltage: 40 kV; scanning rate: $5^\circ \cdot \text{min}^{-1}$; scanning with a step size of $2\theta = 0.02^\circ$ between 5° and 70° (2θ).

2.7. Job plot

The inclusion stoichiometry between UA and ACDs was determined by Job plot [32] by fluorescence spectroscopic titration. Stock solutions of UA ($3 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$) and ACDs ($3 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$) were prepared using methanol and an aqueous buffer solution ($\text{NaHCO}_3\text{--Na}_2\text{CO}_3$, pH = 10.5), respectively. They were then mixed and diluted to a volume of 2 mL using the buffer above in colorimetric tubes with a constant total molar concentration ($3 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$), while the molar fraction of UA varied from 0.1 to 0.9. The relative fluorescence spectroscopic intensity (F) was measured on a RF-5301PC fluorescence spectrometer (Shimadzu Co., Japan) at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 346/454 \text{ nm}$.

2.8. Phase solubility experiments

Phase solubility experiments were carried out according to the method firstly reported by Higuchi and Connors [33] and a recent work by Isaacs and Zhang [34]. Excess amounts (about 2 equiv.) of UA were added to aqueous samples of ACDs with specific concentrations (3.0, 4.0, 5.0, 6.0 and $7.0 \text{ mmol} \cdot \text{L}^{-1}$) in D_2O (0.6 mL) in 5 mm NMR tubes. They were vibrated for 1 h at 25°C , followed by deposition by standing. Their proton nuclear magnetic resonance (^1H NMR) spectra were obtained on a Bruker Avance III HD spectrometer (600 MHz, Bruker BioSpin, Switzerland) at 25°C . The molar concentrations of UA were then derived from the comparison between integral areas of specific peaks of UA and ACDs.

2.9. Nuclear magnetic resonance (NMR) spectroscopy

All NMR analyses including ^1H and 2D ROESY NMR experiments were conducted on a Bruker Avance III HD spectrometer (600 MHz, Bruker BioSpin, Switzerland) at 25°C in D_2O (99.9% D).

2.10. Water solubility test

Water solubility of inclusion complexes of UA/ACDs was assessed by preparation of their saturated solution. Briefly, an excess amount of the solid inclusion complex was placed in water (pH 7.0, 2 mL), and the suspension was stirred in the dark for 1 h at $25 \pm 2^\circ\text{C}$, followed by filtration on a $0.45\text{-}\mu\text{m}$ membrane. The filtrate was evaporated under reduced pressure followed by lyophilization. The water solubility of UA in the form of inclusion complexes with ACDs was determined from the weight of freeze-dried inclusion complexes and the phase solubility diagrams.

2.11. In vitro cytotoxicity test (the MTT assay)

The *in vitro* cytotoxicity of the solid inclusion complexes of UA/ACDs were evaluated towards three human cancer cell lines HepG2, HT29 and HCT116 by the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay since these cancer cell lines were reported to be sensitive to UA. Meanwhile, cisplatin was used as the positive reference. To clarify the cytotoxic selectivity of these solid inclusion complexes against tumor and normal cells, the human normal liver cell line LO2 was used in this assay as well. Cells were cultured at $5 \times 10^5 \text{ mL}^{-1}$ in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h, and then were seeded at $5 \times 10^4 \text{ cm}^{-2}$, followed by being treated with indicated amounts of samples of UA, ACDs and their solid inclusion complexes at concentrations ranging from 0.001 to $10 \mu\text{M}$ in dimethyl sulfoxide

(DMSO). In brief, $50 \mu\text{L}$ of MTT stock solution (5 mg mL^{-1} in PBS) was added to $150 \mu\text{L}$ of cell cultures in 96-microwell flat-bottom plates for 4 h at 37°C . Plates were then centrifuged and the MTT-containing culture medium was removed. Precipitated formazan was dissolved in DMSO ($150 \mu\text{L}$). OD values were read within 15 min on a spectrometer at 490 nm.

3. Result and discussion

3.1. SEM analysis

The micro-morphologic variations of UA before and after the formation of inclusion complexes with ACDs are verified by SEM analysis. As shown in Fig. 3 (taking **a3** for example), the native UA aggregates as irregular blocky crystals (Fig. 3, A), while **a3** appears as irregular particles (Fig. 3, B). The 1:1 physical mixture of UA and **a3** displays a simple hybrid of both morphologic characteristics of UA and **a3** (Fig. 3, C). In contrast, after the formation of solid inclusion complex between UA and **a3**, the morphologic characteristics of UA fully disappear and it exists as unique and distinct broken brick-like crystals (Fig. 3, D), which is significantly different from the above-mentioned three. Thus it suggests the formation of the inclusion complex of UA/**a3**, which is unambiguously distinct from their physical mixture on the morphological characteristics. Similar results are obtained for other solid inclusion complexes of UA/ACDs (see Supplementary data).

3.2. Thermal analysis

Thermal properties of inclusion complexes of UA/ACDs are tested by DSC and TG analyses. DSC curves of OA, **a3** and their 1:1 physical mixture and solid inclusion complex are displayed in Fig. 4. UA displays a sharp endothermic peak at 285°C , which indicates the melting point of UA (Fig. 4, A), while the host molecule **a3** shows two broad and relatively shallow endothermic bands at 108 and 363°C , at which points **a3** begins to lose its water inside its cavity and decomposes itself, respectively (Fig. 4, B). From the DSC curve of their 1:1 physical mixture, a sharp endothermic peak at 282°C and also two broad peaks at 86 and 342°C could be seen, which represent both thermal characteristics of UA and **a3** (Fig. 4, C). In contrast, from the DSC curve of their solid inclusion complex, there are two broad peaks at 91 and 352°C which indicate features of **a3**, however, it is clear that the sharp characteristic peak of UA at 285°C disappears (Fig. 4, D), which confirms the formation of the inclusion complex. DSC curves concerning other solid inclusion complexes of UA/ACDs (**a0**, **a1** and **a2**) are similar to that of UA/**a3** discussed here (see Supplementary data).

Further information about thermal properties of the solid inclusion complexes between UA and ACDs are investigated by TG analyses. TG curves for UA, ACDs, and their 1:1 physical mixture and solid inclusion complex are obtained as shown in Fig. 5 taking **a3** for an example. UA begins to decompose at about 345°C (Fig. 5, a), which is much higher than its melting point at 285°C from its DSC curve (Fig. 4, A). The host molecule **a3** decomposes rapidly from 230 to 355°C and then slows down this process (Fig. 5, b). The 1:1 physical mixture of UA and **a3** begins to decompose at about 250°C (Fig. 5, c). However, the solid inclusion complex exhibits a thermal decomposition point at about 275°C (Fig. 5, d), which is quite different from the former three and suggests the formation of inclusion complex. TG curves concerning other ACDs (**a0**, **a1** and **a2**) are similar to that of **a2** discussed here (see Supplementary data).

3.3. Powder XRD analysis

Crystal patterns of the solid inclusion complexes of UA/ACDs are

Fig. 3. SEM microphotographs: (A) UA; (B) **a3**; (C) the physical mixture (1:1) and (D) the solid inclusion complex of UA/**a3**.

obtained from powder XRD analysis. As shown in Fig. 6 (taking **a2** for example), the XRD pattern of UA displays a high degree of crystallinity and exhibits characteristic peaks at $2\theta = 5.1, 10.3, 10.9, 12.9, 14.3, 21.1, 26.9$ and 42.2° (Fig. 6, a), while **a2** shows almost amorphous pattern with two broad peaks at $2\theta = 12.5$ and 18.3° (Fig. 6, b). The XRD pattern of their physical mixture (1:1) retains most characteristic crystal peaks of **a2**, with the intensity of peaks at $2\theta = 21.1, 26.9$ and 42.2° decreases significantly (Fig. 6, c). However, the pattern of the solid inclusion complex loses characteristic peaks of UA completely and exhibits a similar fashion to that of **a2**, strongly indicating the formation of the inclusion complex between UA and **a2** (Fig. 6, d). Similar results are obtained for **a0, a1** and **a3**, which also indicates the formation of inclusion complexes between UA and ACDs (see Supplementary data).

3.4. Inclusion stoichiometry

The inclusion stoichiometry of inclusion complexation between UA and ACDs is determined using Job plot. The Job plot curves are obtained from the fluorescence data as shown in Fig. 7 (taking UA/**a0** for example). From the fraction of **a0** at the peak value of relative fluorescence intensity (F), we can bring up an inclusion stoichiometry of 1:1 between UA and **a0**. The same results are obtained for

other UA/ACDs systems (see Supplementary data).

3.5. Phase solubility analysis

The phase solubility diagrams of UA/ACDs are constructed based on the molar concentrations of UA and ACDs ($\text{mmol} \cdot \text{L}^{-1}$). Since the latter are designed as 3.0, 4.0, 5.0, 6.0 and $7.0 \text{ mmol} \cdot \text{L}^{-1}$, the former are obtained from the comparison between integral areas of specific peaks of UA and ACDs, i.e., H12 of UA (the alkenyl proton) and H-1 of ACDs, from their ^1H NMR spectra (see Supplementary data). The resultant phase solubility diagrams show the water solubility of UA increases linearly with the increase of ACDs' concentration, which exhibits an A_L type of phase solubility profile for all four UA/ACDs systems (Fig. 8). This suggests the formation of 1:1 inclusion complexes between UA and ACDs, which agree with the results from the Job plot above. The stability constants (K_S) of the inclusion complexes are calculated from the slopes of the linear phase solubility plots using the following equation (Eq. (1))

$$K_S = \frac{\text{Slope}}{S_0(1 - \text{slope})} \quad (1)$$

where S_0 is the solubility of UA when the concentration of ACDs is

Fig. 4. DSC curves of (A) UA, (B) **a3**, (C) the physical mixture (1:1), and (D) the solid inclusion complex of UA/**a3**.

Fig. 5. TG curves of (a) UA, (b) **a3**, (c) the 1:1 physical mixture, and (d) the inclusion complex of UA/**a3**.

0 ($S_0 = 0.00001235 \text{ mol L}^{-1}$ [35]).

The binding stability constants (K_S) for the inclusion complexes of ACDs and UA are calculated from Eq. (1) as summarized in Table 1. It is obvious that K_S of inclusion complexation between UA and four ACDs increases in the following order: **a0** > **a1** > **a3** > **a2**. With the shortest amino side chain, **a0** exhibits the best binding ability. The K_S decreases with the extension of amino side chains, as indicated for **a1** and **a2**. However, K_S for **a3** is greater than **a2**, which suggests that a linear correlation between the stability constant and the length of amino side chains does not exist. The changes of standard Gibbs free energy (ΔG) are then calculated from the stability constant (K_S) as shown in Eq. (2):

$$\Delta G = -RT \ln K_S \quad (2)$$

where R is the molar gas constant ($8.31446 \text{ J} \cdot \text{mol}^{-1} \text{ K}^{-1}$) and T is

Fig. 6. XRD patterns: (a) UA; (b) **a2**; (c) the 1:1 physical mixtures of UA/**a2**; (d) the solid inclusion complex of UA/**a2**.

the absolute temperature (298 K).

As shown in Table 1, the inclusion complexation between UA and ACDs in water is accompanied with negative ΔG s, which indicates a spontaneous process.

3.6. Inclusion mode

Possible non-covalent interactions between UA and ACDs in their solution state are investigated using 1D and 2D-NMR analysis in D_2O . The ^1H NMR spectra for **a1** and the inclusion complex of UA/**a1** are chosen to be discussed here as displayed in Fig. 9. From the ^1H NMR spectrum of the inclusion complex of UA/**a1** (Fig. 9, B), we notice that signals belonging to protons of **a1** emerge, which indicates the inclusion complexation between UA and **a1** since UA is

Fig. 7. The Job plot curve for UA/a0 system at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 346/454$ nm ($[UA] + [a0] = 3 \times 10^{-3} \text{ mol} \cdot \text{L}^{-1}$).

Fig. 8. Phase solubility diagrams of UA with different ACDs in water.

scarcely dissolved in D_2O . Chemical shift changes for protons of **a1** in its ^1H NMR spectra before and after inclusion complexation with UA are determined. Downfield shifts for H-1 to H-6 protons of **a1** occur with variations ranging from 0.02 to 0.06 ppm, which are summarized in Table 2.

The 2D-NOESY or ROESY NMR experiments have recently become powerful tools for sophisticated elucidation of the intermolecular hydrogen bonding interactions between macromolecular hosts and their guest molecules. Generally, hydrogen-bonding interactions in spatial distance less than $0.5 \mu\text{m}$ between protons of host and guest molecules could be identified by the nuclear

Overhauser effect (NOE) correlations from a 2D-NOESY or ROESY spectrum. We record 2D-ROESY NMR spectra for inclusion complexes of UA/ACDs in D_2O to help elucidate their possible inclusion mode. As depicted in Fig. 10, we determine the affiliation of both **a1** and UA protons [36–38] and identify their NOE correlations individually. A noticeable cross peak linking to H12 alkenyl proton of UA and H-3 of **a1** could be found (Fig. 10, A). Besides, appreciable correlations between H23–27 and H29–30 methyl protons of UA and H-3 and H-5 protons of **a1** are observed, which indicates that these protons of UA are included in the CD cavity since H-3 and H-5 protons are located in the inner cavity of CD. Specifically, H24–27 and H29–30 protons correlate with both H-3 and H-5 protons of **a1**, while H23 only correlate with H-3 of **a1** (Fig. 10, B). As H-3 and H-5 protons are located near to the secondary and primary face of **a1**, respectively, both of ring A and E of UA should lie near to the primary face of **a1**. We also find cross peaks between ethylene protons on the amino side chain of **a1** and H-3 and H-5 of **a1** (Fig. 10, C). Also it could be identified that the proximal ethylene protons correlate with both H-3 and H-5 protons, while the remote ones only correlate with H-3 protons, which suggests that the amino side chain penetrates into the cavity of **a1** from the primary face to form self-inclusion. Therefore, we propose a possible inclusion mode of a dynamic equilibrium between UA and **a1** as depicted in Fig. 11, with the aid of the 1:1 inclusion complexation of UA/ACDs from the phase solubility diagrams and Job plot. It is better to derive such a bimodal binding mode, rather than a 1:2 one with each end of a UA molecule embedded in a CD cavity concurrently, or a single 1:1 one with the whole molecule of UA in a CD cavity. The driving force to urge this dynamic equilibrium could be hydrophobic interactions and molecular size matching between UA and inner cavity of ACDs. Such dynamic binding modes could also be found in the inclusion complexation between cinchona alkaloids and CDs reported by Liu et al. [39,40]. Similar results are obtained from the interpretation of 2D-ROESY spectra of inclusion complexes between UA and other ACDs (**a0**, **a2** and **a3**, see Supplementary data).

3.7. Water solubility

Water solubility of UA in the form of UA/ACDs inclusion complexes was assessed by preparing their saturated solutions in water. When the mass of the solute in the saturated solution was weighed, the water solubility of UA could be deduced from the phase solubility diagrams.

Results in Table 3 show that water solubility of UA is remarkably increased to 1.61, 1.42, 1.55 and 1.35 mg mL^{-1} , which are promoted by 284, 250, 273 and 238 folds, respectively, as compared with that of native UA ($0.00564 \text{ mg mL}^{-1}$) [35]. As one of commercial CD derivatives, (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD) is well-known for its excellent water solubility and safety and is used widely in pharmaceutical industry, which was used to increase the solubility of UA in water by Xu et al. [41]. To our delight, the fold increases of water solubility of UA by ACDs are all much higher than that of HP- β -CD as shown in Table 3, which suggests that ACDs are superior solubilizing agent than HP- β -CD for UA.

Table 1
Binding stability constants (K_s) of inclusion complexes of UA/ACDs at 25°C .

Complexes	Linear equation	R^2	$K_s/\text{L} \cdot \text{mol}^{-1}$	$\Delta G/\text{kJ} \cdot \text{mol}^{-1}$
UA/a0	$[UA] = 0.02147[a0] - 0.03291$	0.9941	1799	-18.58
UA/a1	$[UA] = 0.01712[a1] - 0.02213$	0.9933	1410	-17.92
UA/a2	$[UA] = 0.01086[a2] - 0.01499$	0.9976	889	-16.78
UA/a3	$[UA] = 0.01212[a3] - 0.01524$	0.9944	993	-17.05

Fig. 9. ^1H NMR spectra for (A) **a1**, and (B) the inclusion complex of UA/**a1** in D_2O .

Table 2

Chemical Shifts of protons of **a1** before and after inclusion complexation with UA at 25°C in D_2O .

Protons	Chemical shift (ppm)		
	δ_{a1}	δ_{complex}	$\Delta\delta$ ($\delta_{\text{complex}} - \delta_{a1}$)
H-1 of a1	4.91	4.95	0.04
H-2 of a1	3.51	3.54	0.03
H-3 of a1	3.81	3.87	0.06
H-4 of a1	3.43	3.46	0.02
H-5 of a1	3.69	3.73	0.04
H-6 of a1	3.73	3.78	0.05

3.8. In vitro cytotoxicity studies

The cytotoxicity of solid inclusion complexes of UA/ACDs is evaluated against three human cancer cell lines HepG2, HT-29 and HCT116, and the human liver cell line LO2 by the MTT assay using cisplatin as the positive drug. The IC_{50} values are calculated as shown in Table 4. The *in vitro* cytotoxicity of UA in the form of inclusion complexes with ACDs are all greater than that of native UA and ACDs, which have weak to negligible cytotoxicity against the selected cancer cell lines. Moreover, the cytotoxicity of UA in the

Fig. 10. The 2D-ROESY spectrum for the inclusion complex of UA and **a1** in D_2O .

Fig. 11. Possible dynamic inclusion mode between UA and a1.

Table 3

Water solubility of UA in the form of inclusion complexes with ACDs.

Inclusion complex	Mass of the inclusion complexes (mg)	Water Solubility of UA ($\text{mg} \cdot \text{mL}^{-1}$)	Fold increase than free UA
UA/a0	187.7	1.61	284
UA/a1	215.1	1.42	250
UA/a2	382.5	1.55	273
UA/a3	309.3	1.35	238
UA/HP- β -CD	/	0.176	30

Table 4

IC₅₀ (μM) of inclusion complexes of UA/ACDs by MTT assay.

Entry	Samples	IC ₅₀ (μM)			
		HepG2	HT-29	HCT116	LO2
1	cisplatin	4.32	8.04	5.18	12.35
2	UA	26.06	31.63	33.12	45.87
3	a0	>100	93.77	72.57	>100
4	a1	89.17	>100	>100	>100
5	a2	86.91	>100	>100	>100
6	a3	54.07	>100	>100	>100
7	UA/a0	6.59	5.11	5.23	>100
8	UA/a1	3.17	1.02	1.22	>100
9	UA/a2	1.61	2.12	2.44	>100
10	UA/a3	2.49	2.38	2.70	>100

form of inclusion complexes is even comparable to that of cisplatin. This might be attributed to the enhanced permeability and retention (EPR) effect of macromolecular assemblies towards solid tumors [42,43]. Particularly, the selectivity of cytotoxic effects of these inclusion complexes on between tumor and normal cells is also uncovered from the IC₅₀ values of >100 against LO2, compared to that of cisplatin and UA, which confirms their safety. Although the further *in vivo* antitumor activity and detailed mechanism of action for these inclusion complexes of UA/ACDs are still to be disclosed, these preliminary results could provide useful clues to a new formulation of UA for its development as an antitumor drug in the future.

4. Conclusion

In the present work, solid inclusion complexes between UA and ACDs were prepared and were characterized by SEM, DSC, TG, XRD and NMR analysis. Their binding behavior was studied. The stability constants (K_S) were measured using NMR-based phase solubility analysis, and dynamic bimodal inclusion modes were proposed through Job plot and 2D-ROESY experiments. After the formation of inclusion complexes, water solubility of UA was promoted dramatically by more than 200 fold than native UA. We also found that the cytotoxicity of UA against three human cancer cell lines, i.e., HepG2, HT-29 and HCT116, was increased significantly by

inclusion complexation from MTT assay, while the safety of these inclusion complexes is confirmed on the human normal liver cell line LO2. These solid inclusion complexes of UA/ACDs showed promising potential as a new liquid formulation of UA for its uses in pharmaceutical industry.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was financial supported by Yunnan Applied Basic Research Projects (Nos. 2018FB018 & 2018FA047) and the National Natural Science Foundation of China (NNFSC) (Nos. 21961017 & 81560601).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2019.111993>.

References

- [1] L. López-Hortas, P. Pérez-Larrán, M.J. González-Muñoz, E. Falqué, H. Domínguez, Recent developments on the extraction and application of ursolic acid. A review, *Food Res. Int.* 103 (2018) 130–149.
- [2] S.T. Cargnin, S.B. Gnoatto, Ursolic acid from apple pomace and traditional plants: a valuable triterpenoid with functional properties, *Food Chem.* 220 (2017) 477–489.
- [3] S.-J. Tsai, M.-C. Yin, Antioxidative and anti-inflammatory protection of oleoanolic acid and ursolic acid in PC12 cells, *J. Food Sci.* 73 (2008) H174–H178.
- [4] A.A. Ramos, C. Pereira-Wilson, A.R. Collins, Protective effects of ursolic acid and luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells, *Mutat. Res. Fund. Mol.* 692 (2010) 6–11.
- [5] P.G.G. Do Nascimento, T.L.G. Lemos, A.M.C. Bizerra, A.M.C. Arriaga, D.A. Ferreira, G.M.P. Santiago, R. Braz-Filho, J.G.M. Costa, *Molecules* 19 (2014) 1317–1327.
- [6] M. Gilibert, K. Marcinkevicius, S. Andújar, M. Schiavone, M.E. Arena, A. Bardón, Sesqui- and triterpenoids from the liverwort *Lepidozia chordulifera* inhibitors of bacterial biofilm and elastase activity of human pathogenic bacteria, *Phytomedicine* 22 (2015) 77–85.
- [7] K.I. Wolska, A.M. Grudniak, B. Fiecek, A. Kraczkiewicz-Dowjat, A. Kurek, Antibacterial activity of oleoanolic acid and ursolic acids and their derivatives, *Cent. Eur. J. Biol.* 5 (2010) 543–553.

- [8] K. Takada, T. Nakane, K. Masuda, H. Ishii, Ursolic acid and oleanolic acid, members of pentacyclic triterpenoid acids, suppress TNF- α -induced E-selectin expression by cultured umbilical vein endothelial cells, *Phytomedicine* 17 (2010) 1114–1119.
- [9] Y. Ikeda, A. Murakami, H. Ohigashi, Ursolic acid: an anti- and pro-inflammatory triterpenoid, *Mol. Nutr. Food Res.* 52 (2008) 26–42.
- [10] H. Chen, Y. Gao, A. Wang, X. Zhou, Y. Zheng, J. Zhou, Evolution in medicinal chemistry of ursolic acid derivatives as anticancer agents, *Eur. J. Med. Chem.* 92 (2015) 648–655.
- [11] J. Iqbal, B.A. Abbasi, R. Ahmad, T. Mahmood, S. Kanwal, B. Ali, A.T. Khalil, S.A. Shah, M.M. Alam, H. Badshah, Ursolic acid a promising candidate in the therapeutics of breast cancer: current status and future implications, *Biomed. Pharmacother.* 108 (2018) 752–756.
- [12] R. Yin, T. Li, J. Tian, P. Xi, R.H. Liu, Ursolic acid, a potential anticancer compound for breast cancer therapy, *Crit. Rev. Food Sci.* 58 (2018) 568–574.
- [13] E.W.C. Chan, C.Y. Soon, J.B.L. Tan, S.K. Wong, Y.W. Hui, Ursolic acid: an overview on its cytotoxic activities against breast and colorectal cancer cells, *Chin. J. Integr. Med.* 17 (2019) 155–160.
- [14] I. Kazmi, M. Afzal, S. Rahman, M. Iqbal, F. Imam, F. Anwar, Antiobesity potential of ursolic acid stearyl glucoside by inhibiting pancreatic lipase, *Eur. J. Pharmacol.* 709 (2013) 28–36.
- [15] M.H. Shyu, T.C. Kao, G.C. Yen, Oleanolic acid and ursolic acid induce apoptosis in HuH7 human hepatocellular carcinoma cells through a mitochondrial-dependent pathway and downregulation of XIAP, *J. Agric. Food Chem.* 58 (2010) 6110–6118.
- [16] Q. Huang, H. Chen, Y. Ren, Z. Wang, P. Zeng, X. Li, J. Wang, X. Zheng, Anti-hepatocellular carcinoma activity and mechanism of chemopreventive compounds: ursolic acid derivatives, *Pharm. Biol.* 54 (2016) 3189–3196.
- [17] S.-M. Jang, S.-T. Yee, J. Choi, M.-S. Choi, G.-M. Do, S.-M. Jeon, J. Yeo, M.-J. Kim, K.-I. Seo, M.-K. Lee, Ursolic acid enhances the cellular immune system and pancreatic beta-cell function in streptozotocin-induced diabetic mice fed a high-fat diet, *Int. Immunopharmacol.* 9 (2009) 113–119.
- [18] F.S.G. Silva, P.J. Oliveira, M.F. Duarte, Oleanolic, ursolic, and betulinic acids as food supplements or pharmaceutical agents for type 2 diabetes: promise or illusion? *J. Agric. Food Chem.* 64 (2016) 2991–3008.
- [19] K.-Y. Yoo, S.-Y. Park, Terpenoids as potential anti-Alzheimer's disease therapeutics, *Molecules* 17 (2012) 3524–3538.
- [20] L. López-Hortas, P. Pérez-Larrán, M.J. González-Muñoz, E. Falqué, H. Domínguez, Recent developments on the extraction and application of ursolic acid, a review, *Food Res. Int.* 103 (2018) 130–149.
- [21] G. Crini, Review: a history of cyclodextrin, *Chem. Rev.* 114 (2014) 10940–10975.
- [22] S.S. Jambhekar, P. Breen, Cyclodextrins in pharmaceutical formulations I: structure and physicochemical properties, formation of complexes, and types of complex, *Drug Discov. Today* 21 (2016) 356–362.
- [23] S.S. Jambhekar, P. Breen, Cyclodextrins in pharmaceutical formulations II: solubilization, binding constant, and complexation efficiency, *Drug Discov. Today* 21 (2016) 363–369.
- [24] P. Jansook, N. Ogawa, T. Loftsson, Cyclodextrins: structure, physiochemical properties and pharmaceutical applications, *Int. J. Pharm.* 535 (2018) 272–284.
- [25] G. Crini, S. Fourmentin, É. Fenyvesi, G. Torri, M. Fourmentin, N. Morin-Crini, *Environ. Chem. Lett.* 16 (2018) 1361–1375.
- [26] D.S. Dalal, D.R. Patil, Y.A. Tayade, β -Cyclodextrin: a green and efficient supramolecular catalyst for organic transformations, *Chem. Rec.* 18 (2018) 1560–1582.
- [27] M. Rezanka, Monosubstituted cyclodextrins as precursors for further use, *Eur. J. Org. Chem.* (2016) 5322–5334, 2016.
- [28] Y. Ren, Y. Liu, Z. Yang, R. Niu, K. Gao, B. Yang, X. Liao, J. Zhang, Solid inclusion complexes of oleanolic acid with amino-appended β -cyclodextrins (ACDs): preparation, characterization, water solubility and anticancer activity, *Mat. Sci. Eng.* 69 (2016) 68–76.
- [29] Y.-H. Deng, L.-N. Su, Y.-H. Pang, Y.-F. Guo, F. Wang, X.-L. Liao, B. Yang, Preparation, characterization and water solubility of inclusion complexes of daidzein with amino-modified β -cyclodextrins, *Chin. J. Anal. Chem.* 45 (2017) 648–653.
- [30] Y. Ren, B. Yang, X. Liao, The amino side chains do matter: chemoselectivity in the one-pot three-component synthesis of 2-amino-4H-chromenes by supramolecular catalysis with amino-appended β -cyclodextrins (ACDs) in water, *Catal. Sci. Technol.* 6 (2016) 4283–4293.
- [31] Y. Ren, B. Yang, X. Liao, Merging supramolecular catalysis and aminocatalysis: amino-appended β -cyclodextrins (ACDs) as efficient and recyclable supramolecular catalysts for the synthesis of tetraketones, *RSC Adv.* 6 (2016) 22034–22042.
- [32] P. Job, Formation and stability of inorganic complexes in solution, *Ann. Chim.* 9 (1928) 113–203.
- [33] T. Higuchi, K.A. Connors, Phase solubility techniques, *Adv. Anal. Chem. Instrum.* 4 (1965) 117–212.
- [34] B. Zhang, L. Isaacs, Acyclic cucurbit[n]uril-type molecular containers: influence of aromatic walls on their function as solubilizing excipients for insoluble drugs, *J. Med. Chem.* 57 (2014) 9554–9563.
- [35] H. Lin, F. Qin, J. Gao, Determination of equilibrium solubility and apparent oil/water partition coefficient of ursolic acid, *Chin. J. Mod. Appl. Pharm.* 29 (2012) 635–637.
- [36] W. Seebacher, N. Simic, R. Weis, R. Saf, O. Kunert, Complete assignments of ^1H and ^{13}C NMR resonances of oleanolic acid, 18α -oleanolic acid, ursolic acid and their 11-oxo derivatives, *Magn. Reson. Chem.* 41 (2003) 636–638.
- [37] M.G.V. Silva, Í.G.P. Vieira, F.N.P. Mendes, I.L. Albuquerque, R.N. dos Santos, F.O. Silva, S.M. Morais, Variation of ursolic acid content in eight *Ocimum* species from northeastern Brazil, *Molecules* 13 (2018) 2482–2487.
- [38] H.-C. Liu, Y.-S. Jin, H.-S. Chen, Study on triterpenoid and flavonoid contents of *Rabdosia japonica*, *Acad. J. Second Mil. Med. Univ.* 34 (2013) 1121–1124.
- [39] Y. Liu, L. Li, H.-Y. Zhang, Z. Fan, X.-D. Guan, Selective binding of chiral molecules of cinchona alkaloid by β - and γ -cyclodextrins and organoselenium-bridged bis(β -cyclodextrin)s, *Bioorg. Chem.* 31 (2003) 11–23.
- [40] Y. Liu, G.-S. Chen, Y. Chen, F. Ding, J. Chen, Cyclodextrin as carriers for cinchona alkaloids: a pH-responsive selective binding system, *Org. Biomol. Chem.* 3 (2005) 2519–2523.
- [41] R. Li, P. Quan, D.-F. Liu, F.-D. Wei, Q. Zhang, Q.-W. Xu, The influence of cosolvent on the complexation of HP- β -cyclodextrins with oleanolic acid and ursolic acid, *AAPS PharmSciTech* 10 (2009) 1137–1144.
- [42] H. Maeda, Macromolecular therapeutics in cancer treatment: the EPR effect and beyond, *J. Control. Release* 164 (2012) 138–144.
- [43] N. Qiu, X. Li, J. Liu, Application of cyclodextrins in cancer treatment, *J. Incl. Phenom. Macrocycl. Chem.* 89 (2017) 229–246.