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ORIGINAL ARTICLE

Host–guest systems based on pH-sensitive acyclic cucurbit[n]urils for controlled release of camptothecin

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Abstract

Stimuli-responsive drug delivery systems may provide an effective way to treat cancer as they can release cargoes regularly according to changes in the human microenvironment. In this work, we design and prepare acid-controlled release complexes of camptothecin with three pH-sensitive acyclic cucurbit[n]urils. The inclusion complexes have been characterized by ^{1}H and 2D nuclear magnetic resonance, X-ray powder diffraction, and phase solubility diagram. Cells incubated with complexes have been analyzed by high-content analysis, and cytotoxicity tests have been completed by MTT assay. The results showed that complexes with different binding constants can release the drug substance in the physiological pH environment of cancer cells, maintain good anticancer activity, and have low cytotoxicity. This provides a strategy about targeted and responsive systems of CPT for clinical application.

Keywords Acyclic cucurbit[n]uril · Camptothecin · Inclusion complexes · Antitumor activity

Introduction

Acyclic cucurbit[n]urils (ACBs) are a new class of supramolecular host molecules that have developed rapidly in recent years, which have broad application prospects in the fields of drug delivery, gene delivery and supramolecular catalysis. These pioneering works have been done by Isaacs research group in the early research [1]. The typical ACBs comprise a central glycoluril tetramer unit and two of symmetric or asymmetric aromatic molecules, which present a C-shaped structure in three-dimensional space [2–4]. More researches showed that ACBs enhanced the solubility and bioactivity of many drugs that were poorly soluble in aqueous solutions through host–guest interactions [2, 5–10]. Due to the derivatization of aromatic molecules in result to adjust the cavity, ACBs can encapsulate guest molecules with different structures to have many potential applications [11, 12]. Ma's research group recently developed a type of "acid-degradable" ACBs, which could degrade and release encapsulated guests at mildly acidic pH [13]. It is well known that pH on the cell surface of tumor cells is slightly lower than that of normal tissue cells due to rapid proliferation of tumor cells [14, 15], So this provides a strategy about targeted and responsive systems of anticancer drug molecules.

CPT (Fig. 1) is a natural compound with high antitumor activity, and it is isolated from the bark of *camptotheca acuminata* in mainland of China [16]. Because of its excellent anti-tumor activity, CPT is widely used in the treatment of many cancers including colorectal cancer, liver cancer, lung cancer and bladder cancer [17–21]. In fact, natural CPT is a pentacyclic quinoline alkaloid and it has a lactone ring (active site) in spatial structure. However, CPT is very unstable and has poor solubility. The balance of the lactone and the carboxylate form of camptothecin is reversible, which is primarily affected by the pH in the environment [17, 22, 23]. Although the water solubility of CPT is enhanced when its structure changes from lactone to carboxylate, the antitumor activity is lowered and the side effects are also enhanced drastically [24–26].

More studies showed that the anticancer mechanism of CPT was that it can inhibit proliferation of cancer cells by

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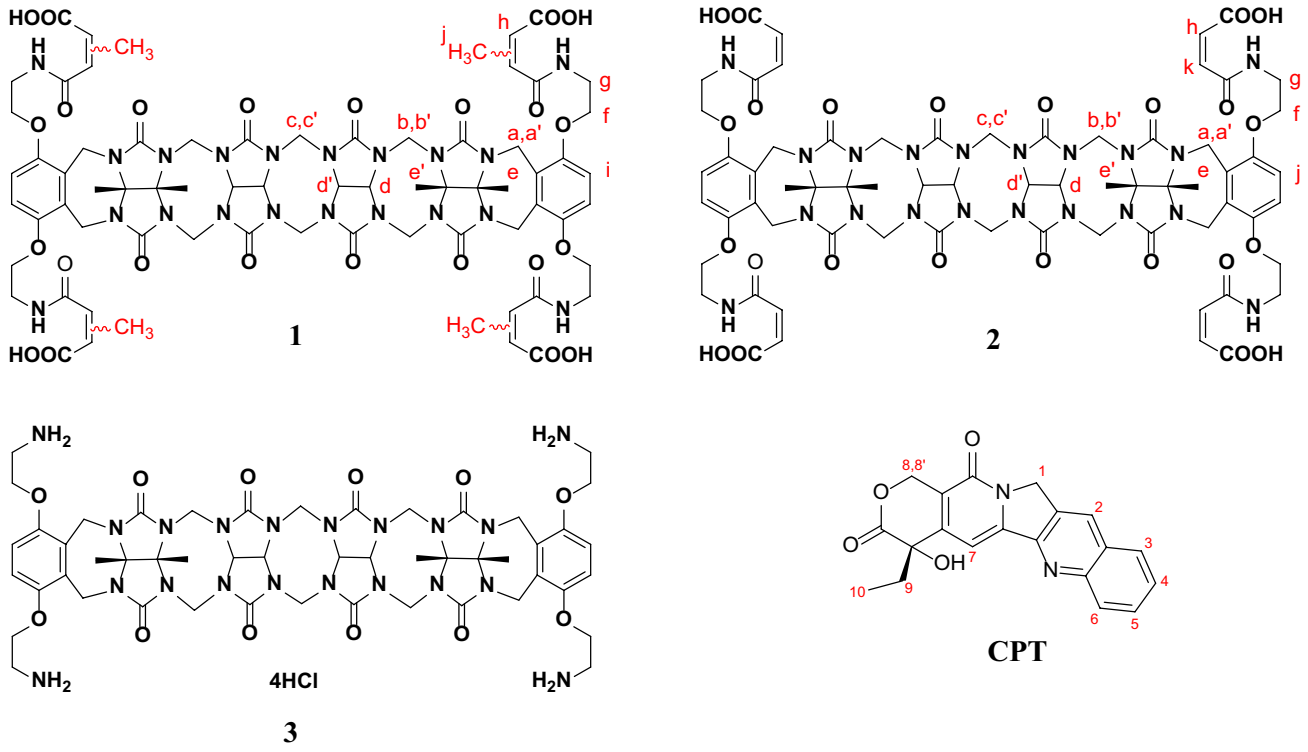

Fig. 1 Structure of acyclic CB [n] molecular hosts and Camptothecin (CPT)

binding to DNA, which was different from many other anticancer drugs. More specifically, CPT can bind to DNA replicase topoisomerase I, subsequently break down DNA strands during the S and G2/M phases of cell cycle (means during DNA replication), and this entire process was irreversible [27–30]. In the early 1970s, water-soluble sodium salt of CPT was used in clinical trials, but it was suspended for its low efficacy and unpredictable severe toxicity including hemorrhagic cystitis and bone marrow toxicity [26]. Therefore, it is meaningful to construct efficient and non-toxic carriers for the delivery of CPT. In order to overcome these disadvantages, many scholars studied and developed the derivatives of CPT. The two most widely used derivatives of CPT are Topotecan (Hycamtin®, GlaxoSmithKline, Brentford, UK) and Irinotecan (Campto®, Pfizer) Ltd., New York, New York, USA), which were approved for clinical application by the US Food and Drug Administration (FDA) [19, 31, 32]. Topotecan was approved for the treatment of ovarian cancer, cervical cancer and small cell lung cancer and Irinotecan was currently used for metastatic colorectal cancer [24]. Despite many studies and efforts have been devoted to improving the poor water solubility of CPT (2.94 ± 0.18 μg ml^{-1}) [33] and decreasing its cytotoxicity to normal cells [2], such as liposomes, nanoparticles, microspheres, and some molecular container like column aromatic hydrocarbons, cucurbituril, etc. [19, 21, 23, 25, 33, 34], further researches were still needed for clinical application of CPT.

Therefore, we prepared inclusion complexes of three ACBs named as host **1**, host **2**, host **3** (Fig. 1, The following are expressed as **1**, **2**, **3**) with CPT. To verify the water solubility, bioavailability and tumor cell targeting of inclusion complexes of ACBs with CPT, we investigated the interactions between **1**, **2**, **3** and CPT, as well as their binding behaviors and toxicity to cells. The results indicated that these two inclusion complexes could selectively deliver CPT to tumor cells. These acid-labile ACBs not only improved the water solubility of CPT, but also provided a new method for constructing new CPT formulations with high bioavailability.

Experimental section

Reagents and materials

All the reagents were purchased from commercial sources and used without further purification. Camptothecin (CPT, MW = 348.358, PC > 98%) used in this work was purchased from Chengdu Yuannuo Tiancheng Technology Co. Ltd. Other reagents were of analytical grade. All experiments were carried out by using ultrapure water.

Synthesis of acid-labile acyclic cucurbit[n]uril

Starting materials were purchased from commercial suppliers and were used without further purification or were prepared by literature procedures (see supplementary). The **3** was prepared according to literature procedures [13]. The **3** (370 mg, 0.31 mmol) was dissolved in water (3 ml), triethylamine (1001 mg, 9.91 mmol) was added in and stirred uniformly, then a solution of citraconic anhydride (550 mg, 4.91 mmol) in acetonitrile was added to the reaction solution, and the mixture was reacted at room temperature for 8 h. After the reaction is done, the solution was adjusted to pH 6 with a 0.5 mol hydrochloric acid solution. The precipitate was washed with acetone (15 ml × 3), and then adjusted to pH about 7 with 1 M NaOH solution. The precipitate was washed with acetone, distilled under reduced pressure to get a light brown product **1**, the yield can reach 71%. The preparation of **2** was similar to that of **1**, except that the anhydride used was maleic anhydride (552 mg, 4.92 mmol). The yield can reach 62%.

Preparation of inclusion complexes and physical mixture

The **1** (0.01 mmol) and CPT (0.03 mmol, 10.5 mg) were added to ultrapure water (10 ml), and the mixture was stirred for 3 days at room temperature in the dark. Then, the precipitate was removed by filtered with a 0.45 μm Millipore membrane, and the filtrate was evaporated under pressure to remove the solvent and dried in vacuum to obtain the CPT/**1** complexes. The inclusion complexes of **2** and CPT is also prepared with the same method. The physical mixtures of CPT and two acid labile acyclic CB [n] molecular containers were prepared by full mixing the powders of CPT and **1** (or **2**) with a 1:1 molar ratio in an agate mortar.

^{1}H NMR analysis

The ^{1}H NMR experiments were acquired on Bruke Avance DRX spectrometer at 600 MHz. Except from the experiment of only CPT, which was dissolved in 99.98% $CDCl_3$ other samples were in 99.98% D_2O or 99.9% DMSO. All samples were filtered before used. The reference used was tetramethylsilane (TMS).

Phase-solubility diagram

The phase-solubility diagram of the inclusion complex was studied by using the Higuchi and Connors methods [35]. A superfluous CPT was suspended in ultrapure water containing different increasing amounts of **1** and **2** (from 0.0015 to 0.012 M), respectively. After the mixture was sonicated in an ultrasonic wave at 25 °C for 2 h, then all the suspensions were centrifuged and the supernatant was collected and filtered with a 0.45 μm Millipore membrane. These experiments were repeated three times. The phase solubility curves were plotted by the solubility of CPT versus the concentrations of **1** and **2**, respectively. The apparent stability constant Ks for CPT/**1** and CPT/**2** complexes could be calculated from the slope and intercept of linear fit of the curve according to the following Equation (1):

$$K_S = \frac{Slope}{S_0\,(1 - Slope)} \tag{1}$$

The verification of acid-labile

The degradation of **1** has been demonstrated in the literature [13]. To validate the degradation of **2** under the acidic conditions, we dissolved **2** in D_2O for incubation and monitored the decomposition of **2** at pH 5.0 (at 3 h, 6 h, and 3 days respectively). The incubation temperature is 37 °C.

X-ray powder diffraction

The X-ray powder diffraction patterns were operated with a D/Max-3B diffractometer using Cu-Kα radiation (k = 1.5460 Å, 40 kV, 100 mA), and the scanning rate was 5°/min. Powder samples were placed on a vitreous sample holder and scanned with a step size of 2θ = 0.02° between 2θ = 5° and 70°.

Solubility test

A series of ethanol solution of camptothecin with different concentrations ranging 1.5–6.5 × 10^{-5} mol/L were prepared. The UV–Vis absorbance (A) at 360 nm was measured on Shimadzu UV-2550 spectrophotometer at 25 °C. A standard curve was made by using concentration (C, mol/L) as the horizontal axis and absorbance as the vertical axis. The fitted standard curve is: A = 13597C – .0098 (R^2 = 0.9967). Excess solid inclusion complexes were placed in dark water and the mixture was stirred at 25 ± 2 °C for 3 h. Then the solution was filtered on a 0.45 μm cellulose acetate membrane. The filtrate was diluted tenfold and its absorbance was recorded at 360 nm at 25° C. Finally, the water solubility is calculated from the standard curve.

In vitro cytotoxicity studies

The cytotoxicity tests for CPT and its inclusion complexes with **1** and **2** were evaluated in vitro for toxicity to normal cells (human HEK-293 cell line) and three cancer cells (human HePG2, HCT116, SH-SY5Y cell lines) by the MTT cytotoxicity assay. The IC_{50} values that represented the concentration of drug required for 50% reduction of

cellular growth have been calculated. Cells were cultured at 1×10^4 cells 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. Cells were seeded at 1×10^4 cells $ml^{-1}$and treated with the indicated amounts of the CPT, **1**, **2** and their inclusion complexes. The cytotoxic activities of the CPT and its inclusion complexes were evaluated as cell survival after treatment. Cell viability was evaluated by a microculture tetrazolium reduction assay using 3-(4,5-dimethyltriazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT).

Methods for cell assays

Dye Rhodamine B (RhB, 20 μg ml^{-1}) alone and in complexation with hosts (20 equiv) were incubated with HePG2 cells at 37 °C for 2 h respectively in three different pH media. The media were composed of DMEM containing 10% fetal bovine serum and 1% penicillin G-streptomycin. Dilute hydrochloric acid was used to adjust the pH to 6.4 or 5.5. Subsequently, cells were washed five times with PBS solution to remove surface-bound dye molecules. Cells were analyzed by high content analysis (HCA).

Results and discussion

^{1}H NMR analysis

^{1}H NMR spectroscopy studies were used to give a deep insight into the host–guest interactions. We compared the ^{1}H NMR of **1** or **2** in the presence and absence of CPT to explore the inclusion behaviors of CPT/**1** (Fig. 2a) and CPT/**2** complexes (Fig. 2b). Due to poor solubility of CPT, we used $CDCl_3$ as a solvent instead of D_2O in NMR test.

As shown in the ^{1}H NMR spectrum of CPT/**1** and CPT/**2** complexes, partial proton peaks of CPT were clear, which is significantly different from the proton peaks of individual **1** and **2**. In Fig. 2a, we can see that there are slight displacements of the protons of **1** in the presence of CPT compared with the absence of CPT. These results indicated the formation of inclusion complexes of CPT with **1** and **2**.

Phase-solubility diagram

In order to study the binding ability between hosts and CPT, we measured the phase solubility diagram showed in Fig. 3. The phase-solubility diagram of the CPT/ACBs systems (Fig. 3.) showed drug solubility increased linearly with increasing CPT concentration. This diagram can be classified as A_L type according to the model proposed by Higuchi and Connors' theory [35]. It proved that one CPT molecule forms a water-soluble complex with one ACBs molecule (1:1 complex). The regression equations of **1** was [CPT] = 0.0212 [Host 1] + 0.0741, $R^2 = 0.9927$. The regression equations of **2** was [CPT] = 0.0346 [Host 2] + 0.0080, $R^2 = 0.9966$.

The apparent stability constant (K_s) was calculated from the linear fit of the curve. according to the Eq. (1) and S_0 of CPT ($S_0 = 2.94$ μg ml^{-1}). The K_s values of the complexes of CPT with 1, 2 were 2566, and 4247 M^{-1}, respectively, to show that the ACBs have stronger binding ability with CPT. The K_s values of complex of CPT/3 can be considered as zero because of no change of CPT with the increasing of three.

Extensive studies have revealed that the size/shape–fit concept and dipolar interaction play the crucial role in the inclusion complexation of ACBs with guest molecules of various structures. On basis of the size/shape–fit concept, weak intermolecular forces such as ion–dipole, dipole–dipole, van der Waals, electrostatic, hydrogen bonding and hydrophobic interactions are known to co-operatively contribute to the inclusion complex.

By comparing the enhancement effect of all kinds of ACBs for CPT, **1**and **2** gave the higher Ks enhancement for CPT than that of 3. As 1 and 2 are anionic ACB and 3 is cationic ACB, it was demonstrated that anionic ACBs can enhance binding ability to CPT, which was proved by NMR analysis.

The verification of acid-labile

In order to verify that the hosts can be cleaved under mildly acidic conditions, the **2** was dissolved in D_2O under acidic condition (pH 5.0), and then the degradation of **2** was monitored by ^{1}H NMR at three different periods. In Fig. 4, a large number of **2** started decomposing what we could observe in (b) after incubation at pH 5.0 for 3 h. After incubation for 3 days, the **2** was nearly converted into **3** (in Fig. 4). The observation indicated the degradation of **2** under acidic condition. This degradation was induced by the "charge conversion" from anionic to cationic [36, 37]. To further explain that hosts could degrade under mildly acidic condition and release CPT, we took CPT/**1** inclusion complex as an example and demonstrated by ^{1}H NMR that the CPT/**1** inclusion complex degrades under mildly acidic conditions and releases CPT wrapped by **1**. It can be seen from Fig. 5 that under the same mildly acidic condition, as the incubation time of the CPT/**1** inclusion complex gradually increases, the CPT's peaks gradually disappeared, indicating that drug in the cavity was gradually released. After 6 h, when **1** was almost completely cleaved, the hydrogen proton signal of CPT in the ^{1}H NMR spectrum also disappeared. The **1** cracked into **3**, which cannot continue to wrap CPT, causing CPT to be released under mildly acidic condition.

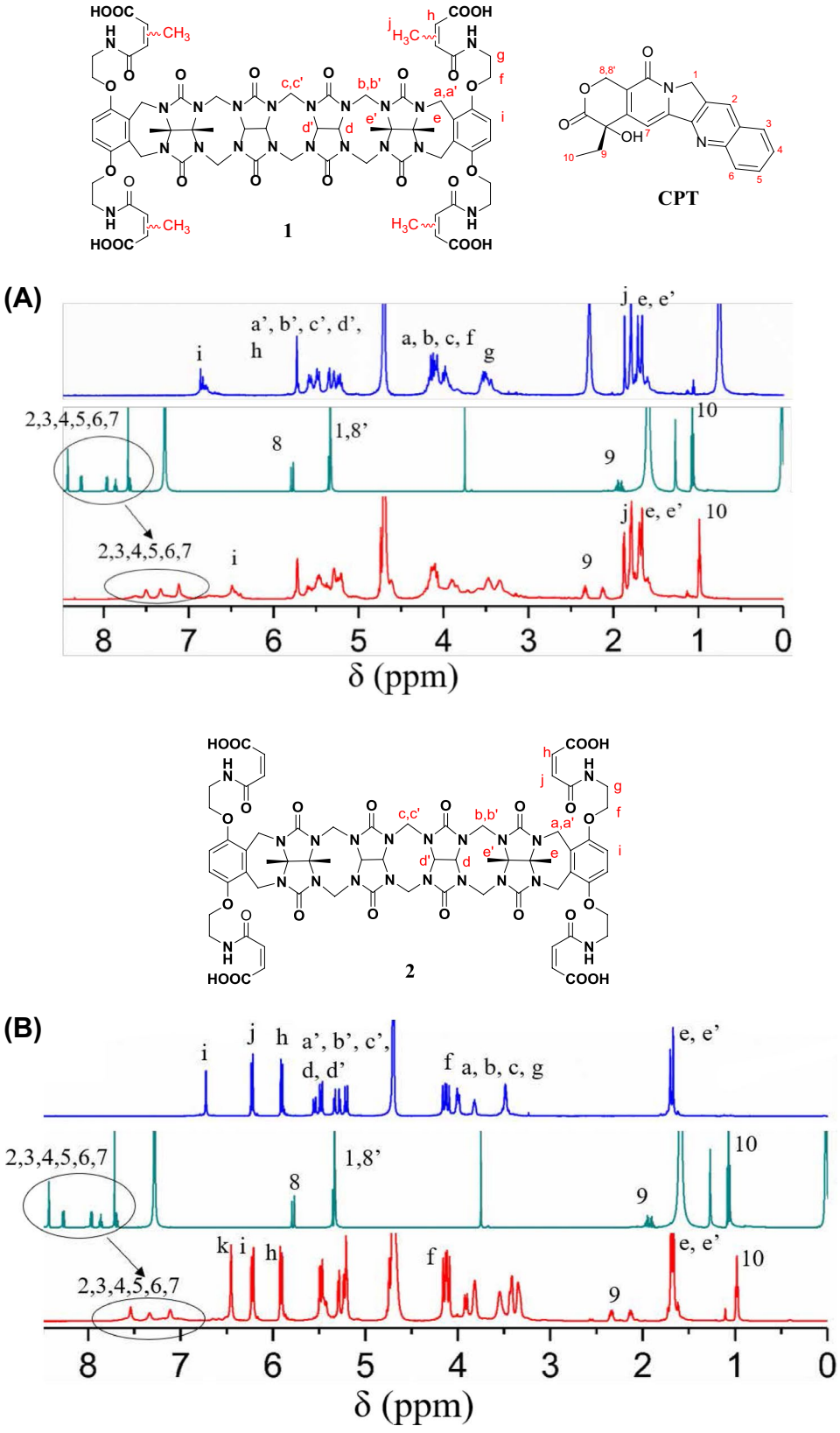

Fig. 2 **a** ^{1}H NMR spectra from top to bottom: **1** (in D_2O), CPT (in $CDCl_3$) and CPT/**1** complex (in D_2O). **b** ^{1}H NMR spectra from top to bottom: **2** (in D_2O), CPT (in $CDCl_3$) and CPT/**2** complex (in D_2O)

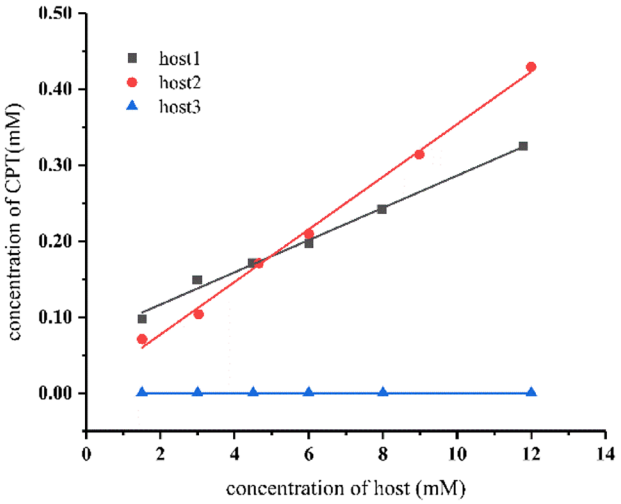

Fig. 3 Phase-solubility diagram of CPT/**1**, CPT/**2** and CPT/**3** complexes in aqueous solution (unbuffered, pH = 7.0) at 25 °C

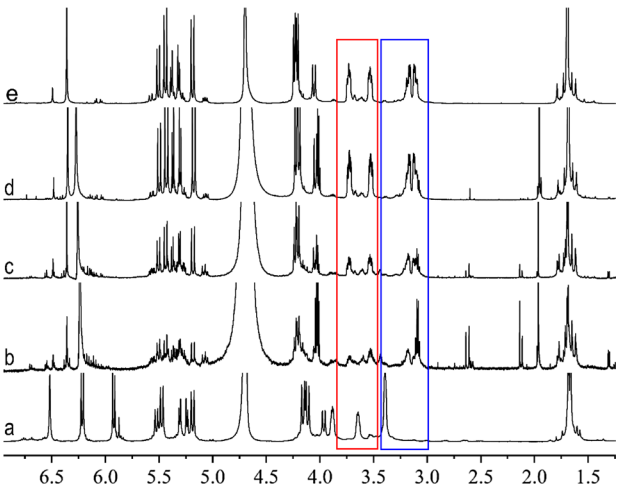

Fig. 4 ^{1}H NMR spectra (a) **2**, (b) 3 h, (c) 6 h, (d) 3d and (e) **3** (all of them are in D_2O of pH 5.0)

According to the above Ks value and the NMR signal, the CPT can be encapsulated in the cavity of hosts. In combination with the acid instability of the hosts, we can suspect that the complex will slowly shift to **3** under mildly acidic conditions, just as the Fig. 6 illustrated release process: the complex Cracks into **3**, while releasing the drug contained in the cavity under mildly acidic conditions. Consequently, it provides condition for designing anticarcinogen targeted release system for tumor cells. Subsequent analysis will further validate this conjecture.

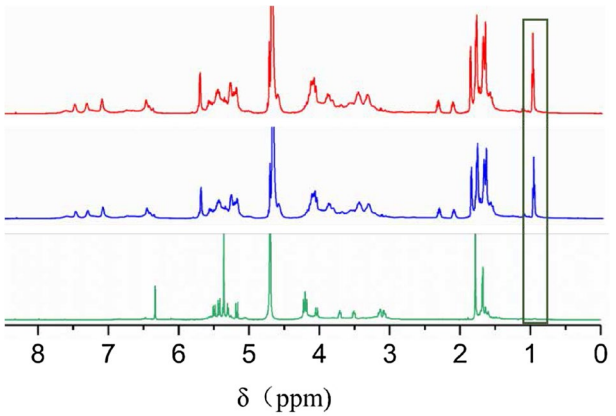

Fig. 5 ^{1}H NMR spectra from top to bottom: CPT/**1** inclusion complex, after incubation in pD 5.0 buffer for 1 h, after incubation in pD 5.0 buffer for 6 h

X-ray powder diffraction

The crystalline state of CPT, **1**, **2** and their inclusion complexes and physical mixtures were examined by X-ray powder diffraction respectively. As demonstrated in Fig. 7, CPT (Fig. 7a and Fig. S5a), free **1** and free **2** showed their characteristic peaks respectively, indicating that it is in crystalline form. In contrast, the inclusion complex of CPT/**1** (Fig. 7d) displayed a very different amorphous halo patterns in the diffractogram whose characteristic diffraction peaks of CPT were completely disappeared and the X-ray powder diffraction of CPT/**2** (Fig. S5d) also exhibited analogous amorphous structure. This indicates that there is substantially no free CPT in the inclusion complexes. However, two physical mixtures not only showed the CPT peaks, but also showed the hosts peaks (Fig. 7c and Fig. S5c), because they were just simply mixed. These results further demonstrated the formation of CPT/**1** and CPT/**2** inclusion complexes.

Water solubility

Water solubility of CPT/**1** and CPT/**2** inclusion complexes were evaluated by preparing their saturated solutions. The concentration of CPT in the aqueous solution of inclusion complex was calculated using a standard curve of ultraviolet spectrophotometer. The results showed that compared with the free CPT (about 2.94 μg ml^{-1}) [33], the water solubility of CPT was significantly increased to 5.748 mg ml^{-1} (16.5 mM) by inclusion complexation with **1**; By inclusion complexation with **2**, water solubility of CPT significantly increased to 5.156 mg ml^{-1} (14.8 mM). The two were about 1955 and 1751 times that of free CPT. This confirms the water solubility of CPT/**1** and CPT/**2** complexes were more satisfactory and reliable, which would facilitate the further development and utilization of camptothecin.

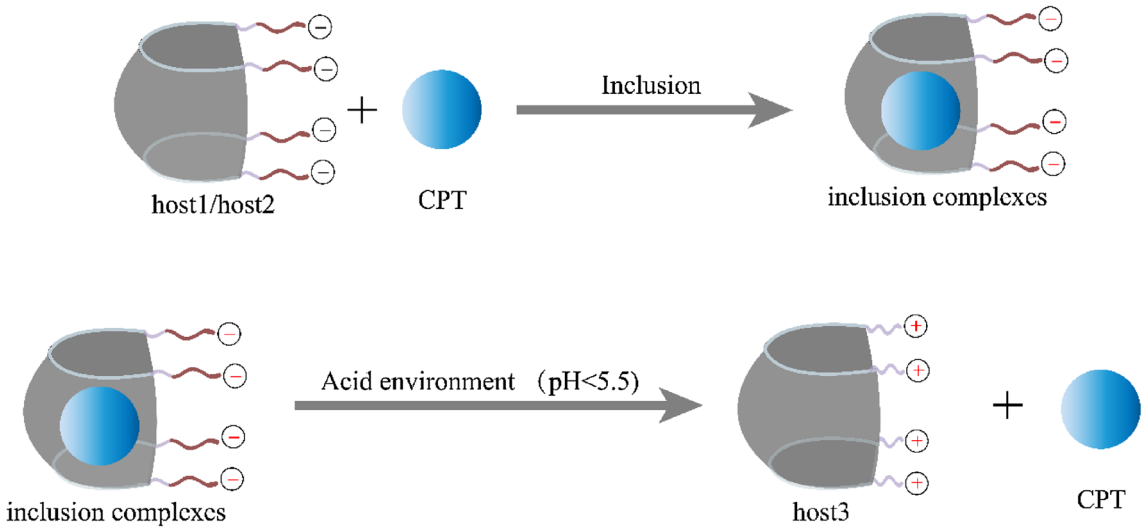

Fig. 6 The inclusion complexes of host/drug release concept in vivo

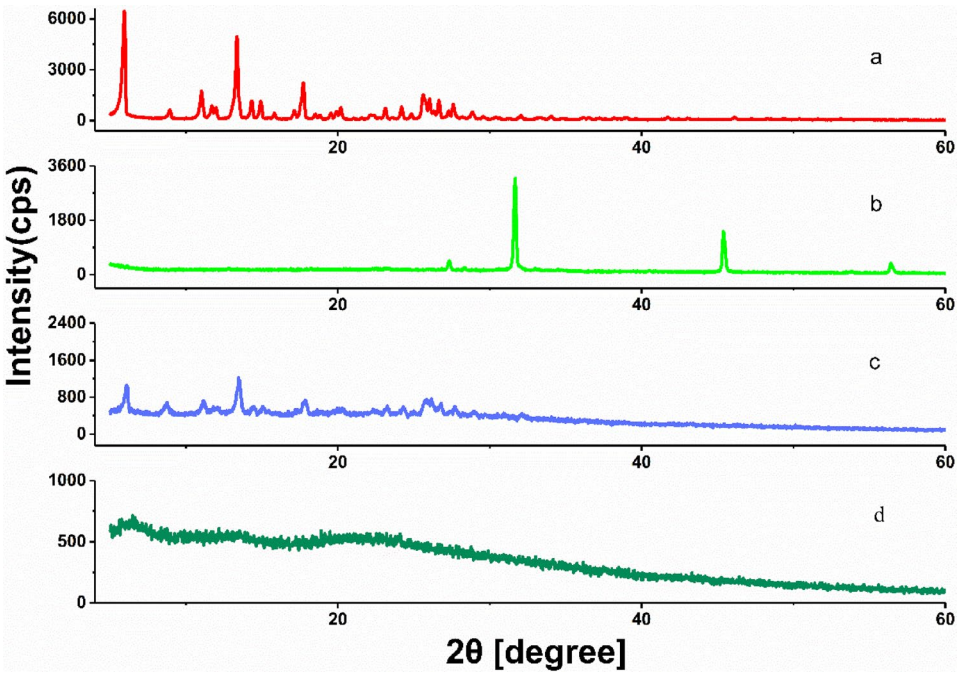

Fig. 7 XRD patterns: **a** CPT, **b 1**, **c** CPT/**1** physical mixture (1:1) and **d** CPT/**1** inclusion complex

In vitro cytotoxicity studies

To verify the anticancer toxicity and cytotoxicity of the inclusion complex, the cytotoxicity of CPT and its solid inclusion complexes with **1** and **2** were evaluated in vitro against human cancer cell lines HepG2, HCT-116 and SH-SY5Y by MTT assay with cisplatin and adriamycin as positive control. Among them, 293T cells are human normal cells. The IC_{50} values were calculated in Table 1. It could be seen from the table that CPT had lower toxicity to normal cells and maintained good cytotoxicity against cancer cells after being encapsulated by **1** and **2**. The result suggested that CPT/**1** and CPT/**2** inclusion complexes provided a novel approach to pharmaceutical formulations of CPT as a potential anticancer drug candidate. Although our works is a close system which is quite contrived and different from what goes on in vivo, we finished some works at cell level and provided a strategy about low cytotoxicity systems of CPT for next application.

As camptothecin is alkaloid with aromatic ring structure, anionic ACBs can bind camptothecin more strongly than most endogenous substances in vivo. In the next

Table 1 IC_{50} (μM) of CPT/**1** and CPT/**2** inclusion complexes to four kind of cells in vitro

Number	Sample	IC_{50}(μM)			
		293T	HePG2	HCT-116	SY5Y
1	CPT	12.84	0.074	0.11	0.75
2	**1**	> 100	> 100	> 100	> 100
3	**2**	> 100	> 100	> 100	> 100
4	CPT/**1**	> 100	15.21	10.83	10.11
5	CPT/**2**	> 100	13.34	10.52	8.59
6	Adriamycin	1.81	6.35	0.26	5.45
7	Cisplatin	2.17	27.2	8.13	13.38

work, we will design and prepare the nanometer systems based on pH-sensitive acyclic cucurbit[n]urils to be survive intact in the body long enough to reach the tumor.

3.7 pH dependent dye internalization assay

We designed a cellular uptake assay to mimic the process of controlled release of antitumor drugs in vitro. The dye Rhodamine (RhB) was used as a model drug. HePG2 cells were incubated with RhB for 1 h at pH 7.4, 6.4 and 5.5 in the absence or presence of hosts to mimic a neutral physiological environment or an acidic tumor extracellular environment. The cells were subsequently washed with PBS solution and the fluorescence values were observed by high content analysis to determine cell uptake efficiency. As shown in Fig. 8, at pH 6.4 and pH 5.5, RhB was efficiently internalized by HePG2 cells due to rapid degradation of citraconic amide and maleic amide compared to pH 7.4.

Moreover, cell uptake of RhB alone was more effective because hosts can encapsulate the drug and reduce its internalization efficiency. We measured the fluorescence values by using a plate reader to quantify cellular uptake, which

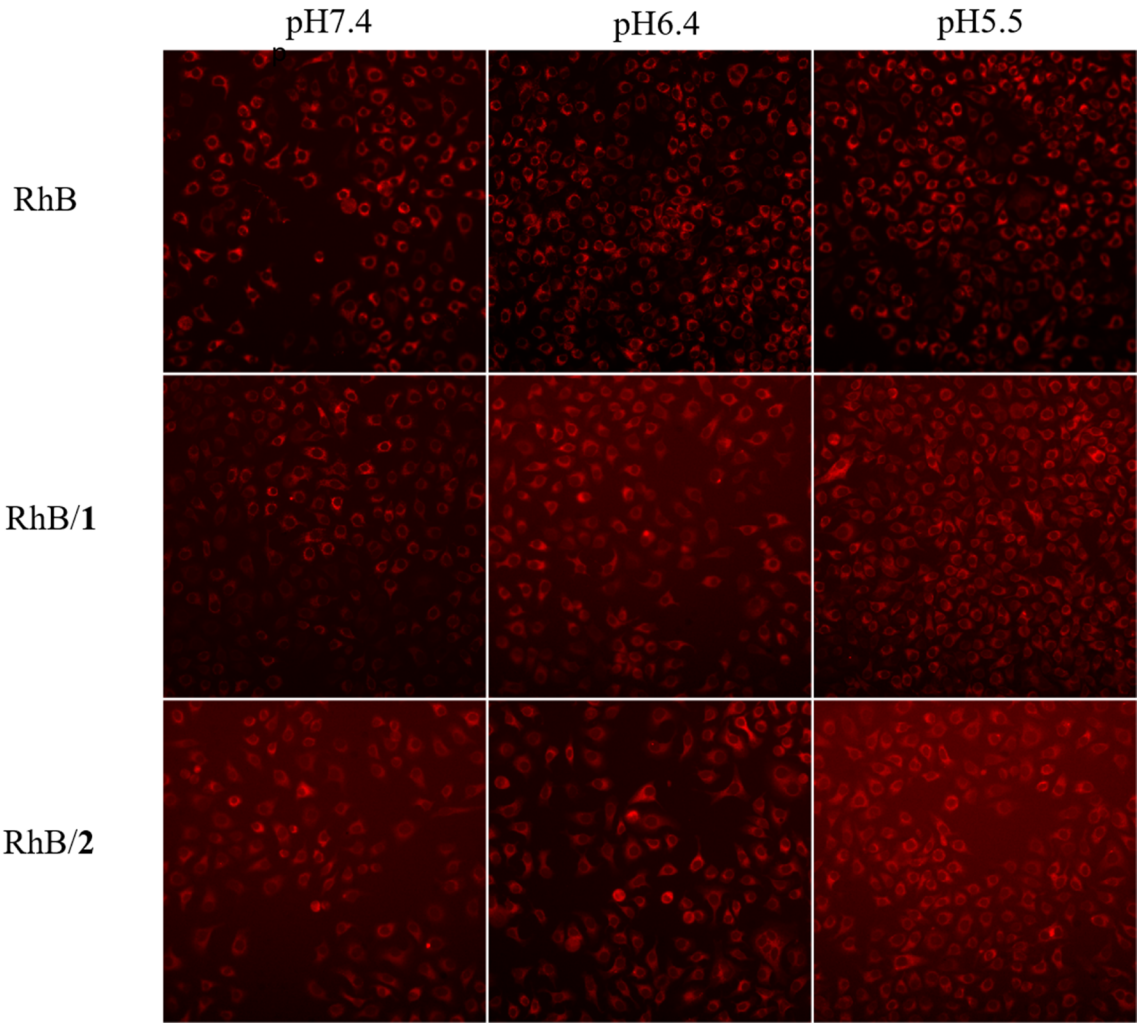

Fig. 8 pH-dependent cellular delivery of RhB by acid labile hosts. Fluorescence imaging for cellular uptake of RhB at different pH in HePG2 cells. From left to right: pH 7.4, pH 6.4, pH 5.5; From top to bottom: RhB, RhB/**1**, RhB/**2**

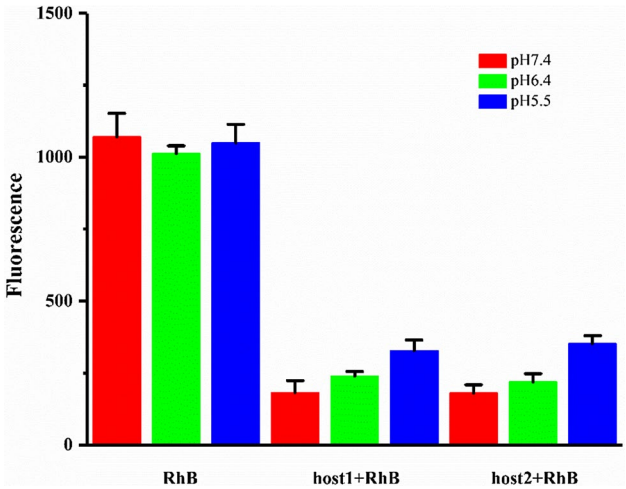

Fig. 9 Fluorescence values for cellular uptake of RhB at different pH. Fluorescence values shown as mean ± SD (n = 4)

was shown in Fig. 9. The inclusion complexes showed a rapid release of RhB at pH 5.5. In contrast, RhB was stably encapsulated to have inefficient cellular internalization when incubated with **1**, **2** or pH 7.4, So the inclusion complexes release encapsulated drug at mildly acidic pH with a regulable rate, which enables targeted delivery of HePG2 cells incubated under mildly acidic conditions. In accordance with the above findings, it will be confirmed that acid labile hosts could encapsulate the cargo and targeted delivery to cells with mildly acidic environment.

Conclusions

In this work, the inclusion complexes of CPT with two kinds of acid-labile acyclic cucurbit[n]uril were successfully prepared. Their characterization, inclusion complexes behaviors, binding ability and their in vitro cytotoxicity were studied. The results showed that the two acid-labile acyclic Cucurbit[n]uril significantly enhanced the water solubility of CPT. It indicated that CPT/hosts were less toxic than camptothecin monomer and their antitumor activity was almost equivalent to that of cisplatin in cytotoxicity assay. Cellular experiments indicated that two inclusion complexes can release CPT in tumor cells. With studies of the model drug-rhodamine dye, it was confirmed that the model molecule carried by hosts could be released under mildly acidic conditions, which contributed to target the release of anticancer drugs and provides an opportunity to expand clinical application of CPT.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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