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Scutellarin-cyclodextrin conjugates: Synthesis, characterization and anticancer activity

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ABSTRACT

A series of scutellarin-cyclodextrin conjugates (SCU-CD conjugates), in which scutellarin was covalently bound to one of the primary hydroxyl groups of β-CD, were prepared, and their structures were determined using NMR and MS. These conjugates were further characterized by XRD and TG. The results showed that the aqueous solubility of the conjugates was much higher than that of scutellarin, and the conjugates could hardly be hydrolyzed to scutellarin in aqueous solutions. The cytotoxicity of SCU-CD conjugates on human colon cancer cell lines HT-29, SW480, Lovo and HTC116 indicated that the antitumor activities of the conjugates were better than that of scutellarin. This high antitumor activity, along with the satisfactory aqueous solubility and high stability of the conjugates, will be potentially useful for their application on human colon cancer chemotherapies.

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1. Introduction

In recent years, anticancer activities of flavonoid components from medicinal plant extract have been reported (Himeji et al., 2007; Li-Weber, 2009). Scutellarin (SCU, Scheme 1) is the main effective flavonoid component of breviscapine, which is an extract from the dried whole plant of *Erigeron breviscapus* (Vant.) Hand-Mazz. It was reported to attenuate H_2O_2-induced cytotoxicity, intracellular accumulation of reactive oxygen species (ROS) and Ca^{2+}, lipid peroxidation, and loss of mitochondrial membrane potential (MMP) and DNA, which may represent the cellular mechanisms for its neuroprotective action (Hong & Liu, 2004). The chemically standardized extract, including some scutellarin, from *Scutellaria barbata* can induce cell death in the human colon cancer lines (Goh, Lee, & Ong, 2005). Scutellarin can also inhibit tumor cell proliferation and migration, and regulate cell adhesion in oral squamous cell carcinoma (OSCC) (Li et al., 2010). However, it has poor water solubility and low antitumor activity, which inevitably hinder its application in vivo (Li-Weber, 2009; Yang, Yang, Lin, Chen, & Liu, 2009). Cyclodextrins (CDs) are truncated-cone polysaccharides mainly composed of six to eight D-glucose monomers linked by α-1,4-glycosidic bonds, which possess a hydrophobic cavity and numerous hydroxyl groups. Simultaneously, cyclodextrins also possess the ability to include various organic and biological substrates within its hydrophobic cavity (Szejtli, 1998). This property may enable it to adhere to the surface of tissues or cells by including accessible surface molecules in the cavity (Wenz, 1994). Therefore, the conjugates, which covalently linked drug molecular to cyclodextrin, are used for drug delivery (Kamada et al., 2002; Udo et al., 2010; Uekama, Minami, & Hirayama, 1997; Yano, Hirayama, Kamada, Arima, & Uekama, 2002).

In this work, scutellarin-cyclodextrin conjugates (amino-SCU-β-CD conjugate, enamino-SCU-β-CD conjugate and dienamino-SCU-β-CD conjugate, Scheme 1) were prepared, in which SCU is covalently bound to one of the primary hydroxyl groups of β-CD, and their structures were identified by ^{1}H NMR and MS. These conjugates were characterized by Powder X-ray diffraction and thermal analysis. Their solubilization and stabilization effects were evaluated as well as their anti-colon cancer activities on HT-29, SW480, Lovo and HTC116 cell lines. These may provide a useful approach to develop a highly effective drug candidate for human colon cancer chemotherapies.

2. Materials and methods

2.1. Materials

Scutellarin (>99%) was obtained from Kunming Pharmaceutical Corporation in Yunnan Province, PR China. β-Cyclodextrin was commercially available. *N,N*-Dimethyl formamide (DMF) was

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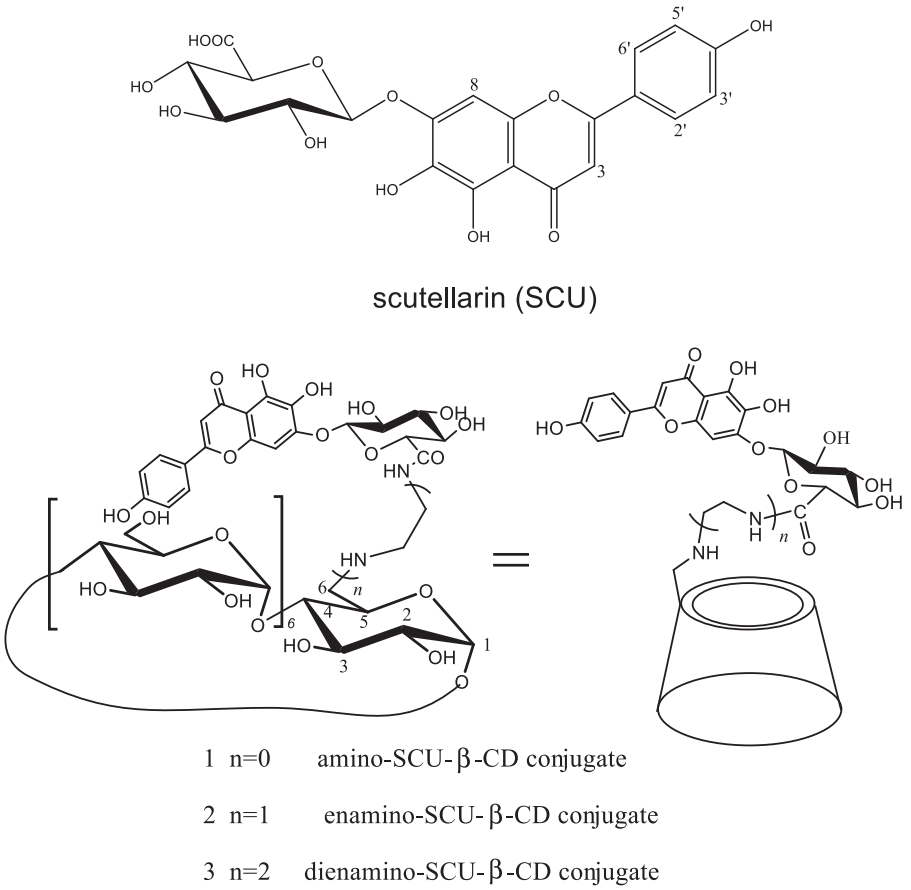

Scheme 1. Chemical structure of scutellarin and scutellarin-β-cyclodextrin conjugates.

predried over calcium hydride for 2 days and then distilled under a reduced pressure prior to use. Dicyclohexylcarbodiimide (DCC) was commercially available (Shanghai Reagent Factory) and used without further purification. Other chemicals and solvents were of analytical-reagent grade, and deionized double-distilled water was used throughout the study.

2.2. Preparation of mono[6-(scutellarin)amino-6-deoxy]-β-cyclodextrin (amino-SCU-β-CD conjugate)

Mono(6-deoxy-6-amino)-β-CD (amino-β-CD) was obtained in three steps from the parent β-CD and purified by Sephadex chromatography with aqueous solution (Belanger & Perly, 1992; Croft & Bartsch, 1983). A solution of amino-β-CD (2.2 g, 2 mmol), scutellarin (SCU, 1.1 g, 2.4 mmol), DCC (0.56 g, 3 mmol) and a small amount of 4 Å molecular sieves in DMF (50 mL) were stirred for 1 h in an ice bath and subsequently for 24 h at room temperature. The insoluble materials were removed by filtration. The filtrate was poured into 300 mL of acetone. The precipitate was collected and subsequently purified on a Sephadex G-25 column with water as eluent. The residue was dried in vacuo, and amino-β-CD conjugate was obtained in yield of 46%. UV/Vis λ_{max} (H_2O)/nm (log ε) 332.0 (4.10), 284.5 (3.95); ^{1}H NMR (500 MHz, D_2O): δ (ppm) 7.68–7.53 (m, H-2′, 6′ of SCU), 7.35–7.25 (m, H-3′, 5′ of SCU), 7.08 (s, H-3 of SCU), 6.37 (s, H-8 of SCU), 4.95 (s, H-1 of amino-β-CD), 3.88–3.62 (m, H-3, 5, 6 of amino-β-CD), 3.62–3.28 (m, H-2, 4 of amino-β-CD); ESI MS *m*/*z* 1577.4407 $[M+H]^+$ (Anal. Calcd for $C_{63}H_{88}NO_{45}$, 1577.4550).

2.3. Preparation of mono[6-(scutellarin)ethyleneamino-6-deoxy]-β-cyclodextrin (enamino-SCU-β-CD conjugate)

Mono(6-deoxy-6-ethyleneamino)-β-CD (en-β-CD) was obtained in two steps from the parent β-CD (May, Kean, Easton, & Lincoln, 1997; Petter, Salek, Sikorski, Kumaravel, & Lin, 1990). To a solution of DMF (50 mL) containing 2.4 g (2 mmol) of en-β-CD and 0.62 g (3 mmol) of DCC was added 0.92 g (2 mmol) of scutellarin in the presence of a small amount of 4 Å molecular sieves. The reaction mixture was stirred for 2 days in an ice bath and another 2 days at room temperature, and then allowed to stand for 1 h. The precipitate was removed by filtration and the filtrate was poured into 300 mL of acetone. The precipitate was collected and subsequently purified on a Sephadex G-25 column with water as eluent. After the residue was dried in vacuo, enamino-SCU-β-CD conjugate was obtained in 52% yield. UV/Vis λ_{max} (H_2O)/nm (log ε) 330.0 (4.04), 285.5 (4.07); ^{1}H NMR (500 MHz, D_2O): δ (ppm) 7.65–7.51 (dd, H-2′, 6′ of SCU), 7.25 (t, H-3′, 5′ of SCU), 6.81 (s, H-3 of SCU), 6.62 (s, H-8_a of SCU), 6.42 (s, H-8_b of SCU), 4.92 (s, H-1 of en-β-CD), 3.85–3.58 (m, H-3, 5, 6 of en-β-CD), 3.55–3.25 (m, H-2, 4 of en-β-CD), 3.15–2.85 (m, H of ethyl of en-β-CD); ESI MS *m*/*z* 1621.4992 $[M+H]^+$ (Anal. Calcd for $C_{65}H_{93}N_2O_{45}$, 1621.4972).

2.4. Preparation of mono[6-(scutellarin)diethyleneamino-6-deoxy]-β-cyclodextrin (dienamino-SCU-β-CD conjugate)

Mono(6-deoxy-6-diethyleneamino)-β-CD (dien-β-CD) was obtained in two steps from the parent β-CD (May et al., 1997;

Petter et al., 1990). A solution of dien-β-CD (2.4 g, 2 mmol), scutellarin (1.1 g, 2.4 mmol), DCC (0.62 g, 3 mmol) and a small amount of 4 Å molecular sieves in DMF (50 mL) were stirred for 1 h in an ice bath and subsequently for 24 h at room temperature. The insoluble materials were removed by filtration. The filtrate was poured into 300 mL of acetone. The precipitate was collected and subsequently purified on a Sephadex G-25 column with water as eluent. After the residue was dried in vacuo, dienamino-SCU-β-CD conjugate was obtained in a yield of 54%. UV/Vis λ_{max} (H_2O)/nm (log ε) 333.0 (4.25), 292.5 (4.17); ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 7.68–7.45 (dd, H-2′, 6′ of SCU), 7.20–7.12 (t, H-3′, 5′ of SCU), 6.95 (s, H-3 of SCU), 6.80 (s, H-8 of SCU), 4.83 (s, H-1 of dien-β-CD), 3.71–3.52 (m, H-3, 5, 6 of dien-β-CD), 3.48–3.25 (m, H-2, 4 of dien-β-CD), 2.85–2.55 (m, H of ethyl of dien-β-CD); ESI MS m/z 1662.5052 $[M-H]^-$ (Anal. Calcd for $C_{67}H_{92}N_3O_{45}$, 1662.5394).

2.5. Measurement of aqueous solubility

The aqueous solubility of the conjugates was assessed by preparation of its saturated aqueous solution (Montassier, Duchêne, & Poelman, 1997). An excess amount of conjugate was put in 5 mL of water (pH ca. 7), and the mixture was stirred for 1 h. After removing the insoluble substance by filtration, the filtrate was evaporated under reduced pressure to dryness and the residue was dosed by weighing method.

2.6. Characterization of the conjugates

UV/Vis spectra were performed on a Shimadzu UV 3600 spectrophotometer, and pH 7.2 buffer solution was used in the spectral measurements.

^{1}H NMR experiment was performed on a Bruker Avance DRX500 spectrometer at 298 K in a D_2O or DMSO-d_6. Samples were kept at least 24 h before measurement for equilibration.

Electrospray ionization mass spectra (ESI MS) were recorded on an Agilent 6210 TOF mass spectrometer in D_2O.

Powder X-ray diffraction (XRD) was measured in a D/max-3B diffractometer using Cu-κα (k = 15460 Å) with 30 mA, 40 kV, and a scanning rate of 5°/min. Powder samples were mounted on a sample holder and scanned with a step size of $2\theta = 0.02°$ between $2\theta = 3°$ and 70°.

Thermal analyses (TG) were recorded on a NETZSCH STA449F_3 instrument, with a 10 °C/min heating rate from room temperature to 500 °C under N_2 flow (100 mL/min).

2.7. Hydrolysis of SCU-β-CD conjugate

The conjugates in aqueous solution were added to phosphate buffer solutions of pH 1.5 or 7.2 at 37 °C. The final concentration of the conjugates was 4.0×10^{-4} M. At appropriate intervals, 200 μL of the reaction solutions were collected, and 60 μL of which was subjected to HPLC analysis under the following conditions: Lichrospher C_{18} HPLC column (Hanbon, 5 μm, 150 mm × 4.6 mm, CHA), a flow rate of 0.5 mL/min, a mobile phase of 1.67% acetic acid solution–acetonitrile (7:3, v/v), a detection at 335 nm.

2.8. Cell culture and treatments

Cells were cultured at 5×10^5 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. They were seeded at 5×10^4 mL^{-1} and treated with the indicated amounts of scutellarin and its conjugates.

Table 1
Some physicochemical properties of SCU and SCU-β-CD conjugates.

Compound	MW	Solubility (mmol/L)[a]
Scutellarin	462	0.35 ± 0.01
Amino-SCU-β-CD conjugate	1576	16 ± 0. 5
Enamino-SCU-β-CD conjugate	1620	20 ± 0.5
Dienamino-SCU-β-CD conjugate	1663	25 ± 0.5

[a] In water at 25 °C.

2.9. Measurement of cytotoxicity

The cytotoxic activities of scutellarin and its conjugates were evaluated as cell survival after treatment. Cell viability was evaluated by a microculture tetrazolium reduction assay using MTT (3-(4,5-dimethyltriazol-2-yl) 2,5-diphenyltetrazolium bromide) assay. Briefly, 50 mL of MTT stock solution (2 mg/mL in PBS) was added to 150 mL cell cultures in 96-microwell flat-bottom plates for 72 h incubation at 37 °C. Plates were then centrifuged and MTT containing culture medium were removed. Precipitated formazan was dissolved in 150 mL of DMSO. Results were read with 15 min in a spectrometer at 490 nm, and the means of triplicates were calculated. Cell inhibition rate is expressed as percentage of control samples.

3. Results and discussion

3.1. Chemistry

Mono(6-deoxy-6-amino)-β-CD (amino-β-CD) was prepared in three steps according to the method of previous study (Fig. 1) (Belanger & Perly, 1992; Croft & Bartsch, 1983). The mono [6-*O*-(*p*-toluentsulfonyl)]-β-CD (6-OTs-β-CD) was converted to mono(6-azido-6-deoxy)-β-CD by the reaction with sodium azide in water. The compound was reduced by treatments with triphenylphosphine in DMF and then with concentrated ammonium hydroxide to obtain amino-β-CD. Mono(6-deoxy-6-ethyleneamino)-β-CD (en-β-CD) and mono(6-deoxy-6-diethyleneamino)-β-CD (dien-β-CD) were prepared in two steps according to the method of previous study (Fig. 1) (May et al., 1997; Petter et al., 1990). Firstly, one of the primary alcohol group of β-CD was tosylated using *p*-toluenesulfonyl chloride in alkaline aqueous solution. Then, 6-OTs-β-CD was converted to mono en-β-CD or dien-β-CD on heating in excess ethylenediamine or diethylenetriamine. The amino-β-CD, en-β-CD and dien-β-CD were coupled to the scutellarin carboxyl group which was first activated using DCC in the presence of a small amount of 4 Å molecular sieves. The scutellarin-cyclodextrin conjugates were obtained by purifying on a Sephadex column with water as eluent. The chemical structure of the enamino-SCU-β-CD conjugate was determined by MS and NMR spectra as shown in Fig. 2.

In the high resolution mass spectrum, the enamino-SCU-β-CD conjugate gave a quasi-molecular ion of 1621.4992 $[M+H]^+$. The formation of a 1:1 conjugation could be confirmed by the 7:6 ratio between the peak areas of H1 proton of β-CD (7 protons) and aromatic proton (6 protons) of SCU in the ^{1}H NMR spectrum. The result indicates that SCU is introduced to one of the primary hydroxyl groups of β-CD.

From Table 1, it is of interest to note that the conjugates were highly soluble (16–25 mmol/L) in water, the solubility being >40 times, in molar concentration, than that of SCU. This increase in solubility is attributable to the phase change from a crystalline state of SCU with lower solubility to an amorphous state of the conjugates with higher solubility. The suggestion was supported by the powder XRD patterns (Fig. 3). This amorphous state occurred probably because the conjugate was formed to self-inclusion complex.

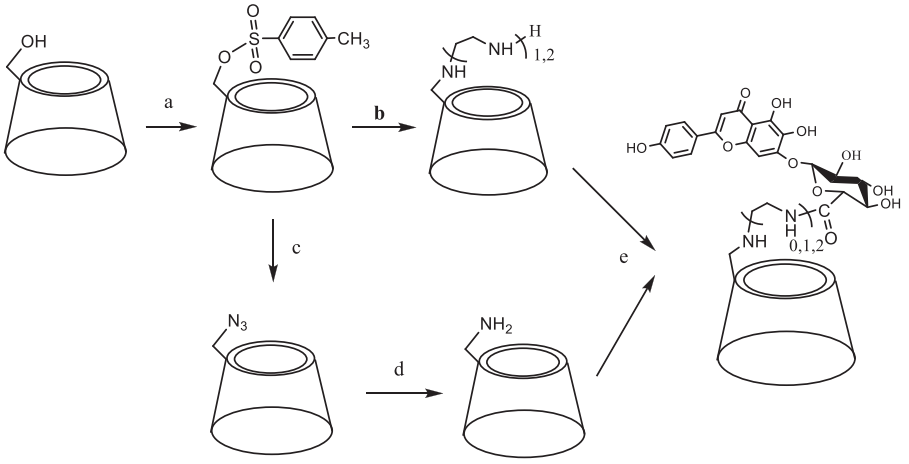

Fig. 1. Preparation of SCU-β-CD conjugate. Reagents: (a) *p*-toluenesulfonyl chloride; (b) ethylenediamine or diethylenediamine; (c) sodium azide; (d) triphenylphosphine, ammonium hydroxide; (e) scutellarin.

As reported previously, the conjugates are due to the formation of the self-inclusion complexes, which inhibits the intermolecular stacking association of the conjugates to form less soluble crystals (Yano, Hirayama, Arima, & Uekama, 2001). The supposition was proved by the experiments with two-dimensional (2D) NMR spectroscopic study, which part of the enamino-SCU-β-CD conjugate and dienamino-SCU-β-CD conjugate were intramolecularly included in the CD cavity, forming a self-inclusion complex. With short amine linkage, the amino-SCU-β-CD conjugate could not be showed the formation of the self-inclusion complex (see Supporting Information). This is the probably reason with the difference of aqueous solubility of three conjugates.

3.2. X-ray diffraction of the conjugate

Powder XRD patterns allow examination of the medium and long range ordering of materials (de Araújo et al., 2008). In contrast

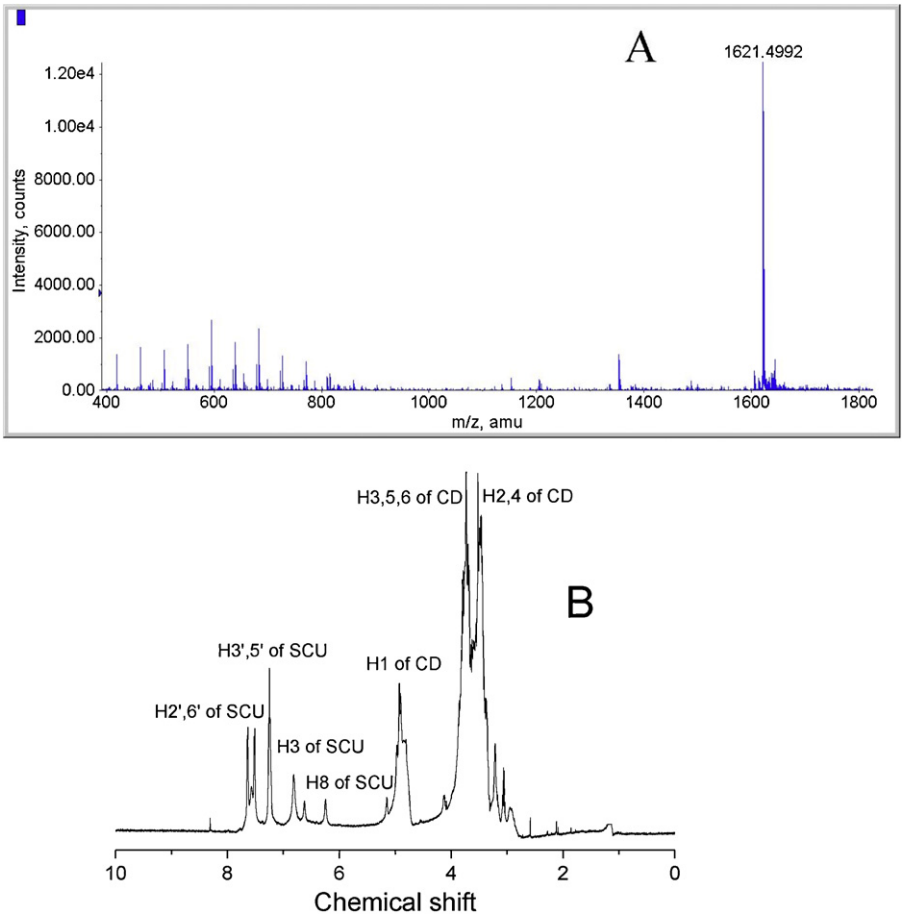

Fig. 2. ESIMS (A), and ^{1}H NMR (B) spectra of enamino-SCU-β-CD conjugate.

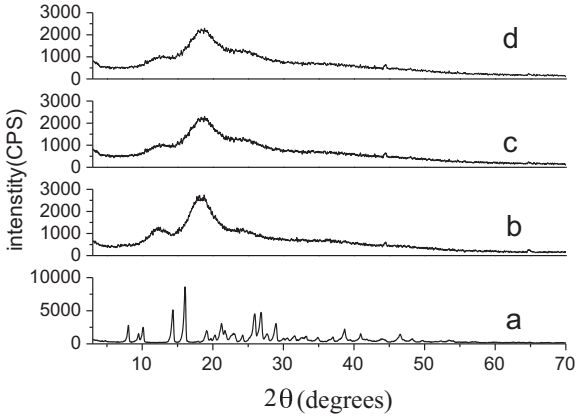

Fig. 3. Powder X-ray diffractograms (Cu-κα) for: (a) SCU, (b) amino-SCU-β-CD conjugate, (c) enamino-SCU-β-CD conjugate, (d) dienamino-SCU-β-CD conjugate.

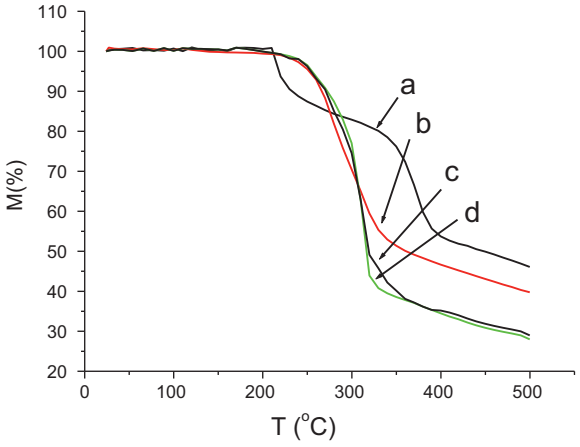

Fig. 4. TG curves for: (a) SCU, (b) amino-SCU-β-CD conjugate, (c) enamino-SCU-β-CD conjugate, (d) dienamino-SCU-β-CD conjugate.

to the crystalline character of SCU (Fig. 3a), All the conjugates have amorphous structures (Fig. 3b–d).

3.3. Thermal analysis of the conjugate

The thermal properties of the SCU-β-CD conjugates were investigated by thermogravimetric analysis (TG). The TG curves showed that the three conjugates decompose at ca. 305 °C (Fig. 4b–d). However, the SCU had different thermal stability with a melting point of ca. 220 °C and a decomposition temperature of ca. 360 °C (Fig. 4a).

3.4. Release behavior of conjugates in aqueous solutions

In order to reach the colon as an intact form, the colon-targeting drugs should survive the passage through stomach and small intestine. The physiological factors which affect on drug release from drugs are pH values, secretion of esterase and bile acids, intestinal contents and microflora. Herein, we investigated the effects of pH values on drug release behavior of the conjugate. Fig. 5 shows the hydrolysis rate-pH profile of the conjugate in aqueous solutions. The conjugates were markedly stable under physiological conditions of pH 1.5 or 7.2 at 37 °C. The results suggest that the conjugates may not be significantly subject to the chemical degradation until they reach to the colon.

3.5. In vitro cytotoxicity studies

The cytotoxicity tests for the conjugates were evaluated in vitro for antiproliferative activity against human cancer cell lines HT-29, SW480, Lovo and HTC116 by the MTT assay using camptothecin as reference compound. The IC_{50} values were calculated as showed in Table 2. As expected, these conjugates presents more satisfactory antiproliferative activity than free scutellarin; Excitingly, the IC_{50} values of enamino-SCU-β-CD conjugate and dienamino-SCU-β-CD conjugate were 7.7×10^{-5}–7.6×10^{-5} mol/dm^3 and 4.8×10^{-5}–4.6×10^{-5} mol/dm^3 respectively, which were much lower than that of free scutellarin (3×10^{-4} mol/dm^3 and 2.1×10^{-4} mol/dm^3) in HT-29 and SW480 colon cancer cell. On control tests, the IC_{50} values of amino-β-CD, enamino-β-CD and dienamino-β-CD exceed 5 mM in HT-29, SW480, Lovo and HTC116 colon cancer cell, indicating that these derivatives of β-CD were very nontoxic to all cell lines tested. The mixtures of scutellarin and amino-β-CD, enamino-β-CD and dienamino-β-CD showed almost similar IC_{50} values to free scutellarin (see Supporting Information). These results proved that the increase in cytotoxicity were attributable to the formation of conjugates (SCU-CD conjugates), in which scutellarin were covalently bound to one of the primary hydroxyl groups of β-CD.

However, the exact mechanisms remain unconfirmed. Nevertheless, the plausible explanation for these data could be that the conjugates were likely induce their passive accumulation on colon cancer cell lines tested in results of increase in cytotoxicity. In previous reported, the β-CD cavities of the conjugates, in which a molecule were covalently bound to one of the hydroxyl groups of β-CD, had the capability of including cholesterol or lecithin of the damaged cell membranes (Liu, Zhang, Chen, & Wang, 2007;

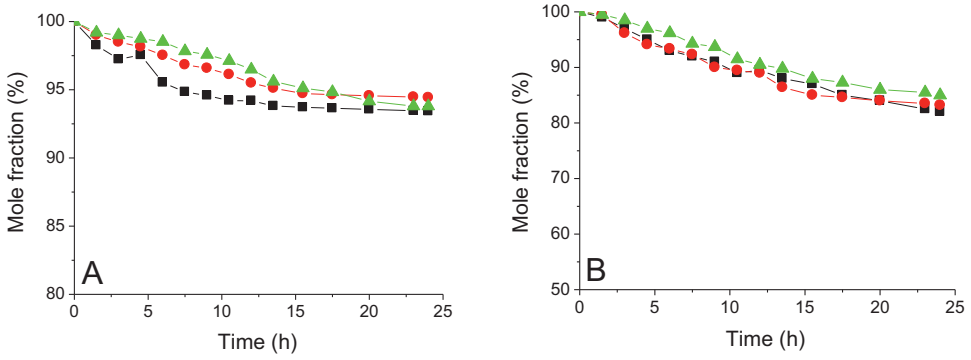

Fig. 5. Time courses for disappearance of SCU-β-CD conjugate and appearances of scutellarin in phosphate buffer at 37 °C. (A) at pH 1.5; (B) at pH 7.2. Key: (■) amino-SCU-β-CD conjugate; (●) enamino-SCU-β-CD conjugate; (▲) dienamino-SCU-β-CD conjugate. Each point represents the mean ± SE of 3 experiments.

Table 2
IC_{50} (mM) of SCU and SCU-β-CD conjugates in various colon cancer cell lines.

Cell line	Scutellarin (mM)	Amino-SCU-β-CD conjugate (mM)	Enamino-SCU-β-CD conjugate (mM)	Dienamino-SCU-β-CD conjugate (mM)
HT-29	0.30	0.16	0.076	0.077
SW480	0.21	0.15	0.048	0.046
Lovo	0.077	0.052	0.039	0.057
HTC116	0.078	0.045	0.034	0.048

Zhang, Chen, Yu, & Liu, 2009). It has been known that during tumor angiogenesis, the nascent capillaries supplying nutrients to the tumors possess large gaps in between vascular endothelial cells when compared to healthy tissues (Matsumura & Maeda, 1986). The junction gap in the cancer cell membrane was loose, which allowed conjugates to include cholesterols of the cell membrane. So the conjugates tend to collect and accumulate in the interstitial space of the tumor. With long amine linkage, the enamino-SCU-β-CD and dienamino-SCU-β-CD conjugate could be inclined to insert into the hydrophobic regions of the bilayers in result of easy anchor at the loose bilayer of the cancer cell membrane. In the case of the short amine linkage, amino-SCU-β-CD conjugate could not insert into the hydrophobic regions of the bilayers. So the amino-SCU-β-CD conjugate showed lower cytotoxicity than enamino-SCU-β-CD and dienamino-SCU-β-CD conjugate.

This supposition was also supported by the control experiments with stained yeast cells by benzenesulfonamidoquinolino-β-cyclodextrin (HQAS-1-CD and HQAS-2-CD, Fig. S8 a and b). As yeast cells have many features analogous to mammalian cells, the pre-cultured yeast cells were transferred to a solution of HQAS-1-CD and HQAS-2-CD, and fluorescence microscopic images were recorded after ca. 30 min with HQAS-2-CD, some damaged cells which had loose junction gap in the cell membrane (including splinter and dead cells) exhibited weak fluorescence at cell division domains (Fig. S9 a and b), but most of the yeast cells did not exhibit any appreciable fluorescence (see Supporting Information). In addition, HQAS-1-CD showed poorer stained yeast cell program than HQAS-2-CD (Liu et al., 2007; Zhang et al., 2009). This phenomenon pointed that healthy cells which possess a compact and integrated cell membrane had the ability to exclude conjugate molecule of cyclodextrin. The self-included conformation of HQAS-2-CD also restricted the its interaction with cell membranes. Therefore, HQAS-2-CD gave no response with healthy cells. However, with damaged cells, the junction gap in the cell membrane increased, which allowed the β-CD cavity of HQAS-2-CD to include competitively exposed molecules of the cell membrane and anchor in the loose bilayer of the cell. With long amine linkage, HQAS-2-CD could be easy to insert into the hydrophobic regions of the bilayers than HQAS-1-CD.

4. Conclusion

In conclusion, we successfully prepared a series of scutellarin-β-cyclodextrin conjugates (amino-SCU-β-CD conjugate, enamino-SCU-β-CD conjugate and dienamino-SCU-β-CD conjugate), which had much higher aqueous solubility than scutellarin, and could hardly be hydrolyzed to scutellarin in aqueous solutions. The conjugates showed higher antiproliferative activities than free scutellarin. These valuable properties of the conjugates will enable their potential application on new treatment for human colon cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.10.012.

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