Carbohydrate Research 400 (2014) 19–25

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Synthesis, characterization, and in vitro evaluation of artesunate-β-cyclodextrin conjugates as novel anti-cancer prodrugs

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ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form 6 August 2014
Accepted 28 August 2014
Available online 8 September 2014

Keywords:
Artesunate
β-Cyclodextrin
Conjugate
Prodrug
Carrier
Anti-cancer

ABSTRACT

A novel series of artesunate-β-cyclodextrin (ATS-β-CD) conjugates, in which artesunate (ATS) was coupled covalently to one of the primary hydroxyl groups of β-cyclodextrin (β-CD) through amino bond formation, were synthesized and characterized by ^{1}H NMR, HRMS, 2D NMR (ROESY), X-ray diffraction (XRD), and thermogravimetric analysis (TGA). The results showed that the aqueous solubility of ATS-β-CD conjugates was 26–45 times better than that of free ATS. The cytotoxicity of the ATS-β-CD conjugates was evaluated on human colon cancer cell lines HCT116, LOVO, SW480, and HT-29, and the results indicated that ATS-2NβCD exhibited a very high cytotoxicity against HCT116, LOVO, and HT-29 with IC_{50} values of 0.58, 1.62, and 5.18 μmol/L, respectively. In addition, the supposition of better cytotoxicity was further supported by the control experiment of fluorescent cyclodextrin.

1. Introduction

At present, cancer mortality has ranked second worldwide, only after that of cardiovascular and cerebrovascular diseases. Unfortunately, the conventional therapies for cancer including chemotherapy, radiotherapy, and surgery still are of limited efficacy in many cases.[1] Moreover, current anti-cancer regimens are frequently associated with significant levels of toxicity and the emergence of drug resistance.[2]

Exploring novel highly active drugs with cancer targeting but little side effects has become one of cancer research hotspots. Artemisinin (ART) is a sesquiterpene lactone with 1,2,4-tiroxane ring system isolated from the Chinese herb *qinghaosu* (*Artemisia annua* L.).[3,4] ART and its derivatives have been commonly used in the clinical treatment of malaria up to now. The possible mechanism is that the endoperoxide moiety of ART and its analogs is activated by reduced heme ($FPFe^{2+}$) or ferrous iron (Fe^{2+}), leading to cytotoxic carbon-centered radicals which are highly potent alkylating agents.[5–8] However, the exact mechanism of the antimalarial activity is under investigation. Interestingly, the potent anticancer action of ART and its derivatives, which can also be attributed to an intact endoperoxide moiety and shares the same antimalarial molecular mechanism, was proposed afterward.[9–14] Radicals which form the endoperoxide may play an important role in the cell alterations, such as apoptosis, arrest of growth, inhibition of angiogenesis, DNA damage, and so on. Moreover, the selectivity of ART and its derivatives may be boosted by preferential targeting of transferrin receptors which are responsible for iron uptake and overexpression in cancer cells.[11] But, several other studies have associated the cytotoxicity of ART and its derivatives with impaired cytokinesis, enhanced levels of oxidative stress, inhibition of tumor invasion, migration, and metastasis.[15] It was reported that human leukemia HL-60,[16] colorectal cancer HT-116,[17] fibrosarcoma HT-1080,[18] glioma cells C6,[19] melanoma A375, G361, LOX,[20] and colorectal HCT116[21] had responded to ART or its derivatives. The precise mechanism underlying the antiproliferative action of the ART and its derivatives, however, is still highly controversial. Artesunate (ATS, Scheme 1) is semisynthesized from dihydroartemisinin (DHA, the main active metabolite of ART) and has shown improved anticancer action. Despite its efficacy, ATS has pharmacokinetic limitations due to its special molecular structure. Naturally, ATS has low solubility in water, poor stability, and short half-life in vivo,[22,23] which cause difficulties in clinical usage.

Cyclodextrins (CDs, including α-CD, β-CD, and γ-CD) are truncated-cone cyclic polysaccharides that are mainly composed of

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http://dx.doi.org/10.1016/j.carres.2014.08.018
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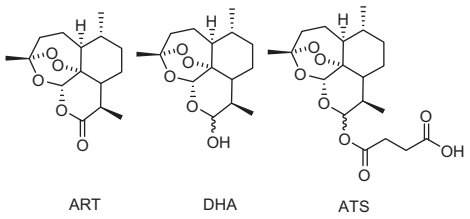


Scheme 1. The structures of artemisinin (ART), dihydroartemisinin (DHA), and artesunate (ATS).

six to eight D-glucose units, which possess numerous modifiable hydroxyl groups and a hydrophobic cavity. This unique structure enable CDs to possess good aqueous solubility and the ability to include various drug compounds in results that CDs are commonly used as drug delivery carriers.[24-29] Simultaneously, this hydrophobic cavity of CDs could adhere and accumulate onto the surface of tissues or cells by including accessible surface molecules.[30] It is well known that during tumor angiogenesis, the nascent capillaries supplying nutrients to the tumors possess large gaps between vascular endothelial cells compared to healthy tissues. The junction gap in the cancer cell membrane was loose, which allowed cyclodextrin conjugates to include cholesterols of the cancer cell membrane to release free drug into the cancer cells.[31,32] So ATS may act synergistically with β-CD, which can serve as drug carriers in cancer therapy.

The aim of this work was to develop a series of new active ATS-β-CD conjugates as prodrug of ATS with better aqueous solubility, stability, and anticancer activity. In this study, β-CD was first modified to a series of mono-substituted derivatives, then the ATS was bound covalently onto the mono-substituted group of β-cyclodextrins (β-CDs). ^{1}H NMR, 2D NMR, and HRMS were used to confirm the synthesis of these conjugates, which were further investigated by X-ray diffraction (XRD) and thermogravimetric analysis (TGA). Their solubilizations were evaluated as well as their cytotoxicities on human colon cancer cell lines HCT116, LOVO, SW480, and HT-29. In addition, the supposition of better cytotoxicity was further supported by the control experiment of fluorescent cyclodextrin.

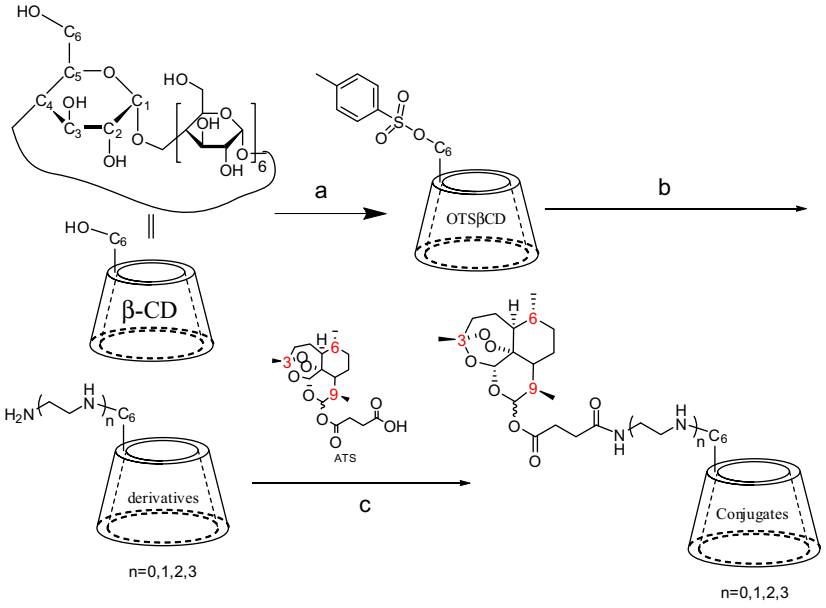
2. Results and discussion

2.1. Chemistry

1N-βCD was synthesized in three steps (Scheme 2). One of the primary alcohol groups of β-CD was tosylated using p-toluenesulfonyl chloride in anhydrous pyridine, followed by reacting with sodium azide in DMF to get N_3-βCD. 1N-βCD was finally obtained through reduction reaction from N_3-βCD with triphenylphosphine and water in DMF. 2N-βCD, 3N-βCD, and 4N-βCD were synthesized in two steps from β-CD. Firstly, β-CD was first modified to OTS-βCD in pyridine. Then, OTS-βCD was converted to 2N-βCD, 3N-βCD, and 4N-βCD on heat in excess ethylenediamino, diethylenetriamino, and triethylenetetraamino solution. It is important to note that the four derivatives are much more soluble in water than the parent β-CD and OTS-βCD. So, TLC (7:7:5:4 Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O) was carried out to monitor the synthesis process. The derivatives were further confirmed by ^{1}H NMR after purifying by Sephadex G-25. 1N-βCD, 2N-βCD, 3N-βCD, and 4N-βCD were coupled to the ATS carboxyl group which was first activated using EDCI in the presence of NHS (Scheme 2). The pure ATS-1NβCD, ATS-2NβCD, ATS-3NβCD, and ATS-4NβCD conjugates were finally obtained by purifying on a sephadex column LH-20. The formation of a 1:1 conjugation could be confirmed by the 7:3:6 ratio among the peak areas of H-1 (7H) of CD, 3-CH_3 (3H) of ATS and 6, 9-CH_3 (6H) of ATS (Fig. 1). In the high resolution mass spectrum, 1500.5632 $[M+H]^+$ (Fig. 2), 1543.6023 $[M+H]^+$, 1608.6407 $[M+Na]^+$, and 1651.6720 $[M+Na]^+$ were found, respectively. The results indicate that ATS is bound covalently to one of the primary hydroxyl groups of β-CD.

2.2. Powder X-ray diffraction (XRD) of ATS-β-CD conjugates

Powder XRD patterns allow examination of the medium and long range ordering of materials.[33] The powder X-ray diffraction patterns of ATS, ATS-1NβCD, ATS-2NβCD, ATS-3NβCD, and ATS-4NβCD are illustrated in Figure 4. We can see that free ATS (Fig. 3a) is in crystalline form, but the conjugates (Fig. 3b–e) are of amorphous structure.



Scheme 2. Synthesis of βCD derivatives and the ATS-β-CD conjugates. Reagents and conditions: (a) pyridine, TSCl; (b) NaN_3, PPh_3, H_2O, DMF, 75→25 °C (1N-βCD); or ethylenediamino, 75 °C (2N-βCD); or diethylenetriamino, 80 °C (3N-βCD); or triethylenetetraamino, 80 °C (4N-βCD); (c) EDCI, NHS, 0→25 °C.

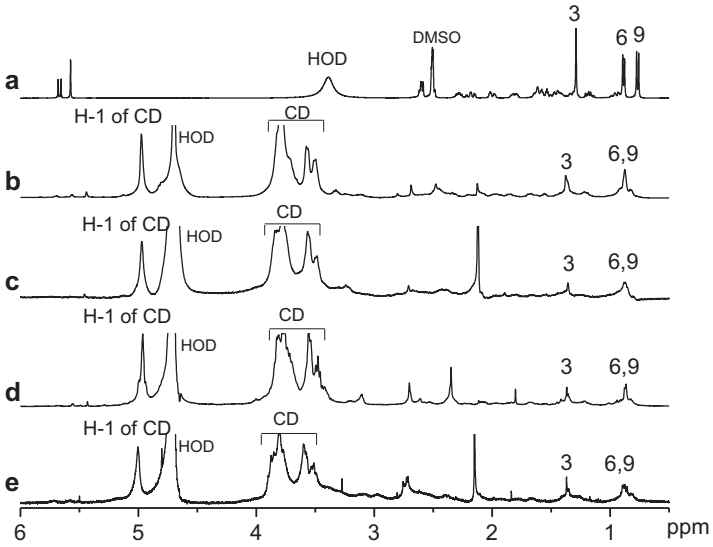


Figure 1. ^{1}H NMR spectra of ATS (a, DMSO-d_6); ATS-1NβCD (b, D_2O); ATS-2NβCD (c, D_2O); ATS-3NβCD (d, D_2O); and ATS-4NβCD (e, D_2O).

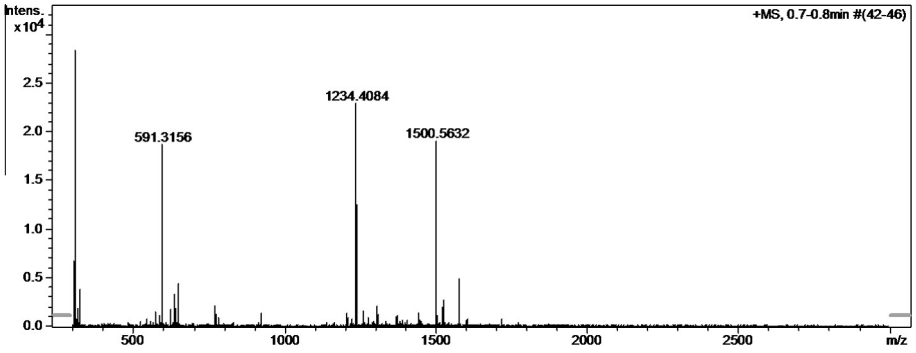


Figure 2. ESI-MS of ATS-1NβCD ($[M+H]^+$).

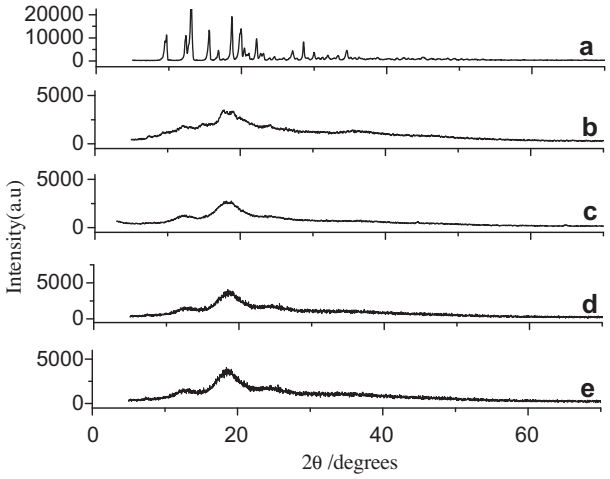


Figure 3. X-ray diffractograms (Cu-κα) for ATS (a); ATS-1NβCD (b); ATS-2NβCD (c); ATS-3NβCD (d); and ATS-4NβCD (e).

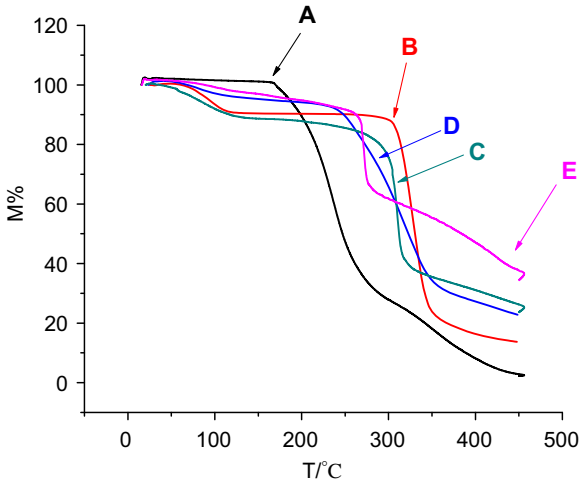


Figure 4. TG curves for: ATS (A); ATS-1NβCD (B); ATS-2NβCD (C); ATS-3NβCD (D); and ATS-4NβCD (E).

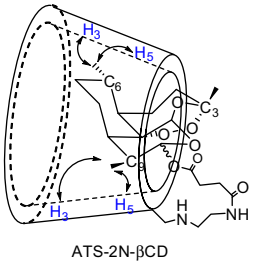
2.3. Thermogravimetric analysis (TGA) of ATS-β-CD conjugates

The thermal properties of the ATS-β-CD conjugates were investigated by thermogravimetric (TG) analysis. The analysis on TG curves showed that natural ATS decomposes at ca. 190 °C (Fig. 4A). However, the conjugates showed better thermal stability, that is, the decomposition temperatures of ATS-1NβCD, ATS-2NβCD, ATS-3NβCD, and ATS-4NβCD were ca. 290 °C, ca. 275 °C, ca. 250 °C, and ca. 255 °C (Fig. 4B–E, respectively).

Table 1
Aqueous solubility of ATS and four conjugates

Compound	MW	Aqueous solubility[a] (mmol/L)
ATS	384	0.52 ± 0.5
ATS-1NβCD	1499	13.6 ± 0.5
ATS-2NβCD	1542	18.4 ± 0.5
ATS-3NβCD	1585	22.9 ± 0.5
ATS-4NβCD	1628	23.7 ± 0.5

[a] In water at 298 K, pH 7.0.



Scheme 3. Possible self-inclusion mode of ATS-2NβCD.

2.4. Aqueous solubility of ATS-β-CD conjugates

The aqueous solubility of four conjugates is assessed by the preparation of its saturated solution. The results showed that the aqueous solubility of ATS was 0.52 ± 0.5 mmol/L at room temperature. As expected, the aqueous solubility of ATS-1NβCD conjugate increased to 13.6 ± 0.5 mmol/L, being >26 times, in molar concentration, than that of ATS alone. Moreover, the aqueous solubility achieved 23.7 ± 0.5 mmol/L, which was 45 times that of ATS, when 4N-βCD was bound to the ATS (Table 1).

The improvement in aqueous solubility is attributable to the phase change from a crystalline state of ATS with lower solubility to an amorphous state of the conjugates with higher solubility.[31] This suggestion was supported by the powder XRD patterns (Fig. 3). This amorphous state occurred probably because the conjugates were formed to self-inclusion complex.[34] This guess was proved by the experiments with 2D ROESY NMR spectroscopic study (Fig. 5), in which 6,9-CH_3 of ATS showed appreciable correlation with H-3,5 of CD, indicated that the ATS of ATS-2NβCD and ATS-4NβCD were partly included in the CD cavities, forming a self-inclusion complex (Scheme 3). But, with short amino linkage, ATS-1NβCD could not perform a self-inclusion pattern. This is the probable reason as to why ATS-1NβCD showed the worst aqueous solubility among four conjugates.

2.5. In vitro cytotoxicity studies

The cytotoxicity tests for ATS-β-CD conjugates were evaluated in vitro for antiproliferative activity against human HCT116, LOVO, SW480, and HT-29 cell lines by the MTT cytotoxicity assay in culture supernatants. Cells treated with the same amounts of ATS, dihydroartemisinin (DHA), 5-fluorouracil (5-Fu), and oxaliplatin (OXA) were used as positive controls. Cells treated with nothing were used as negative controls. The IC_{50} values were calculated and summarized in Table 2. These values can help us to quickly

Table 2
IC_{50} of some drugs and ATS-β-CD conjugates in various colon cancer cell lines

Samples	IC_{50}[a] (μmol/L)			
	HCT116[b]	LOVO[b]	SW480[b]	HT-29[b]
1N-βCD	ND[c]	ND	ND	ND
2N-βCD	ND	ND	ND	ND
3N-βCD	ND	ND	ND	ND
4N-βCD	ND	ND	ND	ND
OXA	0.76	1.51	30.10	9.70
5-Fu	5.40	23.0	30.80	38.50
DHA	1.76	2.11	14.10	21.10
ATS	1.04	2.60	1.82	9.11
ATS-1NβCD	2.67	3.0	20.0	28.0
ATS-2NβCD	0.58	1.62	3.89	5.18
ATS-3NβCD	4.10	3.15	17.67	43.50
ATS-4NβCD	20.90	24.60	44.80	60.20

[a] The IC_{50} values represent the concentration of a drug required for 50% reduction of cellular growth.
[b] Human colon cancer lines HCT116, LOVO, SW480, and HT-29 were cultured at 5×10^5/mL in RPMI 1640 supplemented with 10% FBS, 5% CO_2 at 37 °C.
[c] Not detected.

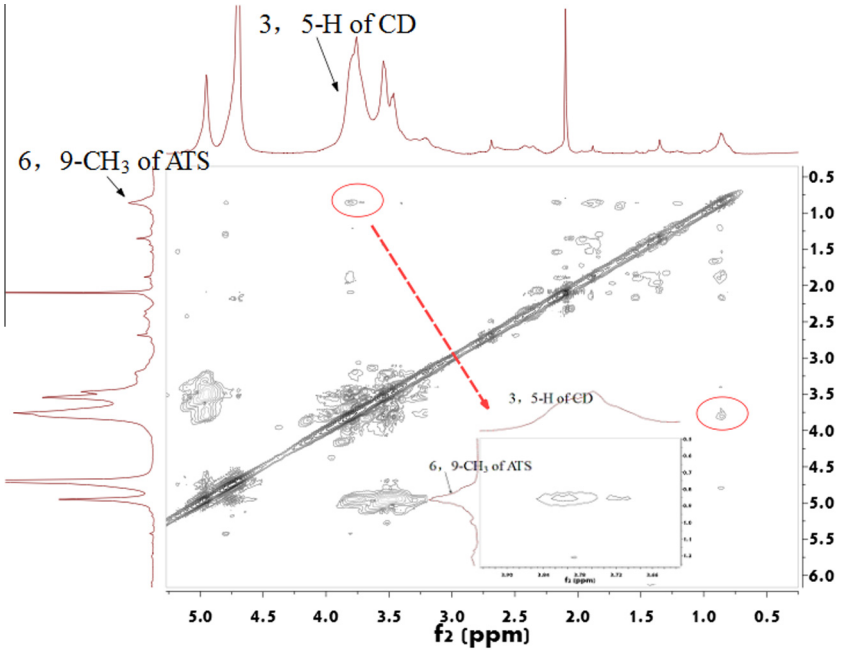
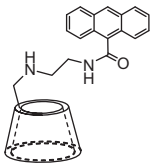


Figure 5. 2D ROESY spectrum of ATS-2NβCD.



Scheme 4. The chemical structure of anthracene-9-carboxyl-2N-βCD conjugate.

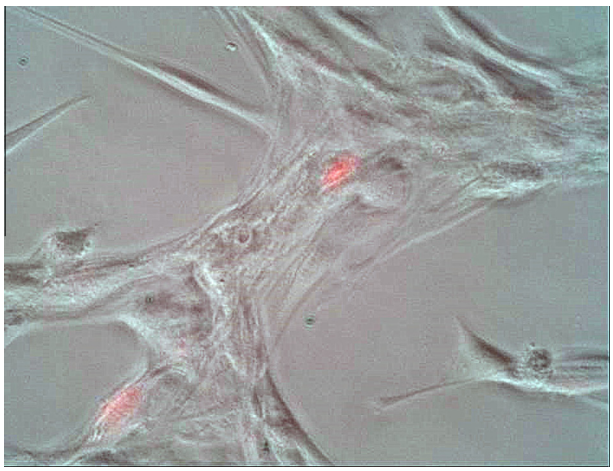


Figure 6. Confocal fluorescence image of the glioma cells C6 treated with anthracene-9-carboxyl-2N-βCD conjugate.

determine which conjugate would be useful for cancer therapy in vitro. As shown by the results in Table 2, the IC_{50} values of DHA and ATS were lower than those of 5-Fu in four colon cancer cell lines, and were as good or even superior in comparison with those of first-line drug OXA, which indicated that the ART derivatives have potentials of research.

These conjugates presented somewhat toxicity to the human colon cancer cells. The IC_{50} values of ATS-1NβCD and ATS-3NβCD were 4.10–2.67 μmol/L, 3.15–3.0 μmol/L, and 20.0–17.67 μmol/L, which were lower than those of 5-Fu (5.40 μmol/L, 23.0 μmol/L, and 30.80 μmol/L, respectively) against HCT116, LOVO, and SW480 cell lines. It is noteworthy that the IC_{50} values of ATS-2NβCD were 0.58 μmol/L, 1.62 μmol/L, and 5.18 μmol/L, which exhibited higher cytotoxicity than DHA (1.76 μmol/L, 2.11 μmol/L, and 21.10 μmol/L, respectively) and ATS (1.04 μmol/L, 2.60 μmol/L, and 9.11 μmol/L, respectively) in HCT116, LOVO, and HT-29 cell lines. Furthermore, the IC_{50} value of ATS-2NβCD against HCT116 was even lower than that of OXA (0.76 μmol/L). Unfortunately, the IC_{50} values of ATS-4NβCD, which were higher than all free drugs and conjugates tested, were not satisfactory. On the control tests, the IC_{50} values of β-CD derivative carriers were not detected. The mixtures of ATS and the β-CD derivatives showed almost similar IC_{50} values to the free ATS.

These data suggest that the 2N-βCD is the most appropriate carrier among these four β-CD derivatives for delivering ATS in the human colon cancer cell lines. However, the exact mechanisms remain unconfirmed. Nevertheless, this finding could be counted for in terms of the following reasons. The cholesterols of cancer cell membranes are inclined to be included by β-CD so that the ATS moiety was displaced competitively by the cholesterols out of the cavity and was hydrolyzed by peptidases from the conjugates to release into the cancer cells. This supposition was also supported by the control test, fluorescent anthracene-9-carboxyl-2N-βCD conjugate was synthesized (Scheme 4). Confocal fluorescence images showed that glioma cells C6 treated with this conjugate exhibited red fluorescence (Fig. 6), but, the cells treated with free anthracene-9-carboxyl acid did not exhibit any fluorescence. This phenomenon pointed that 2N-βCD could help the anthracene-9-carboxyl acid, which was bound covalently to the 2N-βCD, to release into C6 cells.

3. Conclusion

In summary, a series of novel bound covalently ATS-β-CD conjugates were synthesized and characterized for the first time to the best of our knowledge. The conjugates showed higher thermostability and aqueous solubility than free ATS. The cytotoxicity of ATS-β-CD conjugates was evaluated in vitro using human colon cancer cell lines HCT116, LOVO, SW480, and HT-29. Fortunately, ATS-2NβCD exhibited higher cytotoxicity than DHA, ATS in HCT116, LOVO, and HT-29 cell lines, and even more efficacy than OXA in HCT116 cell lines. The ability of cyclodextrin conjugates to release free drug into the cancer cells was supported by the fluorescence control experiment. These valuable properties of the conjugates could promote the exploration of novel highly active drugs for human colon cancer.

4. Experimental

4.1. Materials

β-CD (Mengzhou Huaxing Biochemistry Co.) was recrystallized twice by double distilled water. *N*,*N*-Dimethylformamide (DMF, Chengdu Kelong Chemical Co.) was dried over calcium hydride for 2 days and distilled under a reduced pressure prior to use. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDCI, Aladdin Industrial Co.) and *N*-hydroxysuccinimide (NHS, Aladdin Industrial Co.) were used without further purification. ATS was kindly supplied by Kunming Pharmaceutical Co. (Kunming, PR China). Other chemicals were of analytical-reagent grade and were used as received.

4.2. Chemistry

4.2.1. Synthesis of mono-6-*O*-(*p*-tolysulfonyl)β-cyclodextrin (OTS-βCD)

Mono-6-*O*-(*p*-tolysulfonyl)β-cyclodextrin (OTS-βCD) was synthesized by the method of Ueno et al.[35] (yield = 30.6%). White amorphous solid, R_f = 0.75 (Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O = 7:7:5:4); ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.78–7.73 (d, 2H, Ar-H of TSCl), 7.47–7.42 (d, 2H, Ar-H of TSCl), 5.89–5.57 (m, OH), 4.84 (s, 7H, H-1 of CD), 4.37–4.13 (m, OH), 3.68–3.20 (m, H-2, 3, 4, 5, 6 of CD), 2.39 (s, 3H, CH_3 of TSCl).

4.2.2. Synthesis of mono(6-amino-6-deoxy)β-cyclodextrin (1N-βCD)

Mono(6-azido-6-deoxy)β-cyclodextrin (N_3-βCD) was synthesized by the method of Nielsen et al.[36] (yield = 84.2%). White amorphous solid, R_f = 0.68 (Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O = 7:7:5:4); ^{1}H NMR (400 MHz, D_2O, δ): 4.90 (s, 7H, H-1 of CD), 3.80–3.38 (m, H-2, 3, 4, 5, 6 of CD).

Mono(6-amino-6-deoxy)β-cyclodextrin (1N-βCD) was synthesized from N_3-βCD, the details are described elsewhere (yield = 65.5%).[37] White amorphous solid, R_f = 0.45 (Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O = 7:7:5:4); ^{1}H NMR (400 MHz, D_2O, δ): 5.01 (s, 7H, H-1 of CD), 3.95–3.32 (m, H-2, 3, 4, 5, 6 of CD).

4.2.3. Synthesis of mono(6-ethylenediamino-6-deoxy)β-cyclodextrin (2N-βCD)

Mono(6-ethylenediamino-6-deoxy)β-cyclodextrin (2N-βCD) was synthesized by the method described below. 3.0 g OTS-βCD

(2.4 mmol) was dissolved in 25 mL anhydrous ethylenediamino solution, the mixture was stirred at 75 °C for 10 h under N_2 protection, the reaction was monitored by thin-layer chromatography. After the reaction was completed, the solution was concentrated on a rotary evaporator, and followed by the addition of acetone. The precipitate was collected by filtration and purified by Sephadex G-25 with aqueous solution (yield = 84.9%). Yellow amorphous solid, R_f = 0.35 (Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O = 7:7:5:4); ^{1}H NMR (400 MHz, D_2O, δ): 4.93 (s, 7H, H-1 of CD), 3.81–3.33 (m, H-2, 3, 4, 5, 6 of CD), 2.92–2.61 (m, 4H, CH_2CH_2 of ethylenediamine).

4.2.4. Synthesis of mono(6-diethylenetriamino-6-deoxy)β-cyclodextrin (3N-βCD)

Mono(6-diethylenetriamino-6-deoxy)β-cyclodextrin (3N-βCD) was synthesized by the method described below. 3.0 g OTS-βCD (2.4 mmol) was dissolved in 30 mL anhydrous diethylenetriamino solution, the mixture was stirred at 80 °C for 12 h under N_2 protection, the reaction was monitored by thin-layer chromatography. After the reaction was completed, the mixture was cooled to room temperature and precipitated in acetone. The precipitate was collected by filtration and purified by Sephadex G-25 with aqueous solution (yield = 64.5%). Yellow amorphous solid, R_f = 0.25 (Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O = 7:7:5:4); ^{1}H NMR (400 MHz, D_2O, δ): 5.03 (s, 7H, H-1 of CD), 3.90–3.39 (m, H-2, 3, 4, 5, 6 of CD), 2.85–2.65 (m, 8H, CH_2CH_2 of diethylenetriamine).

4.2.5. Synthesis of mono(6-triethylenetetraamino-6-deoxy)-β-cyclodextrin (4N-βCD)

The synthetic procedures for mono(6-triethylenetetraamino-6-deoxy)-β-cyclodextrin (4N-βCD) were similar to the 3N-βCD described above. (yield = 54.5%). Yellow amorphous solid, R_f = 0.19 (Ac_2O/*i*-PrOH/$NH_3{\cdot}H_2O$/H_2O = 7:7:5:4); ^{1}H NMR (400 MHz, D_2O, δ): 5.08 (s, 7H, H-1 of CD), 3.89–3.38 (m, H-2, 3, 4, 5, 6 of CD), 2.85–2.61 (m, 12H, CH_2CH_2 of triethylenetetraamine).

4.2.6. General procedure for synthesis of ATS-β-CD conjugates

Mono(6-artesunate-amino-6-deoxy)-β-cyclodextrin (ATS-1NβCD) was synthesized by the method described below. A solution of 0.5 g ATS (1.2 mmol), 0.4 g EDCI (2.0 mmol), and 0.3 g NHS (2.3 mmol) in DMF (20 mL) was stirred for 45 min in an ice bath, then 1.1 g 1N-βCD (1.0 mmol) was added to the reaction. The mixture was allowed to react for additional 2 h in an ice bath and subsequently for 15 h at room temperature. After the reaction was completed, the solution was removed by a rotary evaporator, and followed by the addition of double-distilled water. The clear solution was collected by filtration, and then was poured into acetone. The precipitate was collected and purified on a Sephadex LH-20 column with aqueous solution as eluent. The synthetic procedures for mono(6-artesunate-ethylenediamino-6-deoxy)-β-cyclodextrin (ATS-2NβCD), mono(6-artesunate-diethylenetriamino-6-deoxy)-β-cyclodextrin (ATS-3NβCD), and mono(6-artesunate-triethylenetetraamino-6-deoxy)-β-cyclodextrin (ATS-4NβCD) were similar to the ATS-1NβCD described above.

4.2.6.1. Mono(6-artesunate-amino-6-deoxy)-β-cyclodextrin (ATS-1NβCD). ATS-1NβCD was finally obtained from Sephadex LH-20 column chromatography and the yield was 57% (white amorphous solid). ^{1}H NMR (400 MHz, D_2O, δ): 4.97 (s, 7H, H-1 of 1N-βCD), 3.85–3.44 (m, H-2, 3, 4, 5, 6, 7 of 1N-βCD), 2.73–2.49 (m, 4H, CH_2CH_2 of ATS), 1.40–1.31 (s, 3H, 3-CH_3 of ATS), 0.95–0.75 (m, 6H, 6,9-CH_3 of ATS). HRMS (ESI, *m*/*z*): $[M+H]^+$ calcd for $C_{61}H_{98}NO_{41}$, 1500.5616; found, 1500.5632.

4.2.6.2. Mono(6-artesunate-ethylenedi-amino-6-deoxy)-β-cyclodextrin (ATS-2NβCD). ATS-2NβCD was finally obtained from Sephadex LH-20 column chromatography and the yield was 50% (yellow amorphous solid). ^{1}H NMR (400 MHz, D_2O, δ): 5.03 (s, 7H, H-1 of 2N-βCD), 3.95–3.24 (m, H-2, 3, 4, 5, 6, 7 of 2N-βCD), 2.70–2.39 (m, 8H, CH_2CH_2 of ATS, CH_2CH_2 of ethylenediamine), 1.50–1.40 (s, 3H, 3-CH_3 of ATS), 0.97–0.80 (m, 6H, 6, 9-CH_3 of ATS). HRMS (ESI, *m*/*z*): $[M+H]^+$ calcd for $C_{63}H_{103}N_2O_{41}$, 1543.6038; found, 1543.6023.

4.2.6.3. Mono(6-artesunate-diethylenetriamino-6-deoxy)-β-cyclodextrin (ATS-3NβCD). ATS-3NβCD was finally obtained from Sephadex LH-20 column chromatography and the yield was 43% (yellow amorphous solid). ^{1}H NMR (400 MHz, D_2O, δ): 5.00 (s, 7H, H-1 of 3N-βCD), 3.95–3.20 (m, H-2, 3, 4, 5, 6, 7 of 3N-βCD), 2.70–2.40 (m, 12H, CH_2CH_2 of ATS, CH_2CH_2 of diethylenetriamine), 1.45–1.35 (s, 3H, 3-CH_3 of ATS), 0.97–0.80 (m, 6H, 6,9-CH_3 of ATS). HRMS (ESI, *m*/*z*): $[M+Na]^+$ calcd for $C_{65}H_{107}N_3O_{41}Na$, 1608.6460; found, 1608.6407.

4.2.6.4. Mono(6-artesunate-triethylenetetraamino-6-deoxy)-β-cyclodextrin (ATS-4NβCD). ATS-4NβCD was finally obtained from Sephadex LH-20 column chromatography and the yield was 40% (yellow amorphous solid). ^{1}H NMR (400 MHz, D_2O, δ): 5.02 (s, 7H, H-1 of 4N-βCD), 3.95–3.34 (m, H-2, 3, 4, 5, 6, 7 of 4N-βCD), 2.70–2.40 (m, 16H, CH_2CH_2 of ATS, CH_2CH_2 of triethylenetetraamine), 1.40–1.30 (s, 3H, 3-CH_3 of ATS), 0.97–0.75 (m, 6H, 6,9-CH_3 of ATS). HRMS (ESI, *m*/*z*): $[M+Na]^+$ calcd for $C_{67}H_{112}N_4O_{41}Na$, 1651.6882; found, 1651.6720.

4.3. Characterization

^{1}H NMR and 2D ROESY NMR spectra were recorded on a Bruker Avance DRX 400 MHz spectrometer at 298 k. Chemical shifts (ppm) are given with respect to residual nondeuterated solvent protons (δ 2.50 ppm for DMSO and δ 4.80 ppm for HDO). Electrospray ionization mass spectra (ESI MS) were recorded on an Agilent LC/MSD TOF mass spectrometer in D_2O. Powder X-ray diffraction (XRD) was measured in a D/max-3B diffractometer using Cu-κα (κ = 15460 Å) with 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°. Thermogravimetric analysis (TGA) was performed under constant nitrogen flow (50 mL/min), with a heating rate of 10 °C/min using a NETZSCH STA449F3 instrument. The heating scans were performed on 8.0–12.0 mg of the samples in the temperature range of 25–500 °C.

4.4. Measurement of aqueous solubility

The aqueous solubility of the conjugates was assessed by preparation of their saturated aqueous solutions.[38] An excess amount of conjugate was put in 5 mL distilled water (pH ca. 7), and the mixture was stirred for 1 h. After removing the insoluble material by filtration, the filtrate was evaporated under reduced pressure to dryness and the residue was dosed by weighing method.

4.5. Cells and medium

Human colon cancer lines HCT116, LOVO, SW480, HT-29, and glioma cells C6 were cultured at 5×10^5/mL in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

4.6. Measurement of cytotoxicity

Cells were seeded at 5×10^4/mL and treated with the indicated amounts of ATS-β-CD conjugates. The effect of conjugates was evaluated as cell survival after treatment. Cells treated with the same amounts of ATS, dihydroartemisinin (DHA), 5-fluorouracil (5-Fu), and oxaliplatin (OXA) were used as positive controls. Cells treated with nothing were used as negative controls. Cell viability was evaluated by a microculture tetrazolium reduction assay using MTT (3-(4,5-dimethyltriazol-2-yl)2,5-diphenyltetrazo-lium bromide; Sigma). 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 was removed. Precipitated formazan was dissolved in 150 mL 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.

4.7. Fluorescence control experiment

Glioma cells C6 were incubated with the indicated amounts of anthracene-9-carboxyl-2N-βCD at 37 °C for various time intervals and then washed and analyzed by confocal microscopy.

Acknowledgment

This work was supported by National Natural Science Foundation of China (NNSFC) (Nos. 21362016, 21361014, 21302074 and 21162042), which is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2014.08.018.

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