ORIGINAL RESEARCH

BCESA: a nano-scaled intercalator capable of targeting tumor tissue and releasing anti-tumoral β-carboline-3-carboxylic acid

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Background: In the discovery of DNA intercalators, β-carbolines compose one member of the most interesting alkaloid family and are of clinical importance. In the efforts, N-(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl-Ser-Ala-OBzl (BCESA) was designed as a nano-scaled DNA intercalator without Dox-like toxicity.
Methods: Based on the structural analysis and CDOCKER energy comparison, BCESA was rationally designed as such a nano-scaled intercalator. The anti-tumor activity, the toxicity and the tumor targeting action of BCESA were evaluated on mouse models.
Results: The in vitro proliferation of cancer cells, but not non-cancer cells, was effectively inhibited by BCESA. On S180 mouse model BCESA dose-dependently slowed the tumor growth, and 0.01 μmol/kg/day was found as a minimal effective dose. Both BCESA and its moiety were found in the tumor tissue, but not in the organs and the blood, of S180 mice.
Conclusion: BCESA should be a nano-scaled intercalator capable of targeting tumor tissue to release anti-tumoral β-carboline-3-carboxylic acid and its 1-methyl derivative, while Ser-Ala-OBzl is a simple and desirable carrier.
Keywords: nano-medicine, β-carboline, intercalator, d(AGACGTCT)$_2$, anti-tumor

Introduction

As DNA intercalator β-carbolines are one member of the most interesting alkaloid family and of clinical importance.[1–5] This leads to the discovery of the diverse β-carbolines, such as tetrahydro-β-carbolines[6] and their hydrantoin hybrids,[7] β-carboline-benzimidazole conjugates,[8] N-heterocyclic carbenes from β-carboline,[9] various harmines and related β-carboline,[10–14] ruthenium (II) complexes of β-carbolines,[15] β-carbolines derived from harmine,[16,17] β-carbolines with phenylheterocycles,[18] as well as 1-aryl or 1-indolyl substituted β-carbolines.[19,20]

In our previous papers, the diverse intercalators of DNA were reported.[21–25] On the other hand, however, their effective dose reached 8.9 μmol/kg/day. Besides, the previous evaluation of N-[(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl]-AA-OBzl showed that N-[(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-OBzl and N-[(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ala-OBzl had the best efficacy. To decrease the effective dose, Ser-OBzl and Ala-OBzl were combined, used to replace the AA-OBzl of N-[(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl]-AA-OBzl and N-(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl-Ser-Ala-OBzl (BCESA) was designed. The rationality of the design was firstly examined by docking

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investigation. The CDOCKER energies indicate that among 6 intercalators BCESA has the lowest energy (Figure 1). Besides, the docking feature of BCESA in the active site of d(AGACGTCT)$_2$ shows 8 π-π stacking interactions and 6 hydrogen bond interactions, suggesting BCESA well fits the active site of d(AGACGTCT)$_2$. These encouraged us to hypothesize that BCESA could be able to target tumor tissue and release the anti-tumoral pharmacophores wherein. In this context, the synthesis, structural analysis, nano-scaled assembly, anti-tumoral evaluation, tumor targeting action and release profile of BCESA were performed here.

Materials and methods
Reagents and instruments
L-Amino acids, reagents and solvents for the synthesis were obtained commercially and used without further purification unless otherwise specified. Qingdao silica gel (GF254) was used for TLC. Silica gel (H60, Qingdao Haiyang Chemical Co. Ltd, Qingdao China) was used for chromatography. Melting points were measured on an XT5 hot stage microscope (Beijing key electro-optic factory). The spectra of ^{1}H NMR (300 MHz and 500 MHz) and ^{13}C NMR (75 MHz and 125 MHz) were recorded on Bruker AMX-300 and AMX-500 spectrometers. DMSO-d6 and TMS were used as the solvent and the internal standard, respectively. Perkin-Elmer 983 instrument was used to record IR spectra. ZQ 2000

(Waters, USA) mass spectrometer and 9.4 T SolariX Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker, USA) were used to record the electrospray ionization mass spectra (ESI-MS), both of which had ESI/ matrix-assisted laser desorption/ionization (MALDI) dual ion source. The high-performance liquid chromatography (HPLC) purity of BCESA was conducted on an Agilent Technologies 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA), and Eclipse XDB C$_{18}$ column (5 μm, 4.6×150 mm) was used. The column temperature was 40°C. BCESA was eluted with methanol/H$_2$O. The gradient consisted of 60% methanol (0–5 min), 70% methanol (5–10 min), 80% methanol (10–20 min) and 90% methanol (20–30 min). The flow rate was 0.8 mL/min. Human colorectal carcinoma (HCT-8), murine sarcoma (S180), human neuroblastoma (SH-sy5y), human leukemia (HL60), human non-small-cell lung cancer (A549) and human breast cancer (MCF-7) cell line as well as non-carcinoma cell human keratinocytes cell line (HaCaT) were purchased from KeyGen Biotech Co. Ltd. (Nanjing,China).

Animals and ethics
The in vivo assay was performed under the supervisory control of Ethics Committee of Capital Medical University. The committee approved that this assay can use ICR mice only. Male ICR mice (20–22 g) were commercially obtained from the Laboratory Animal Center of
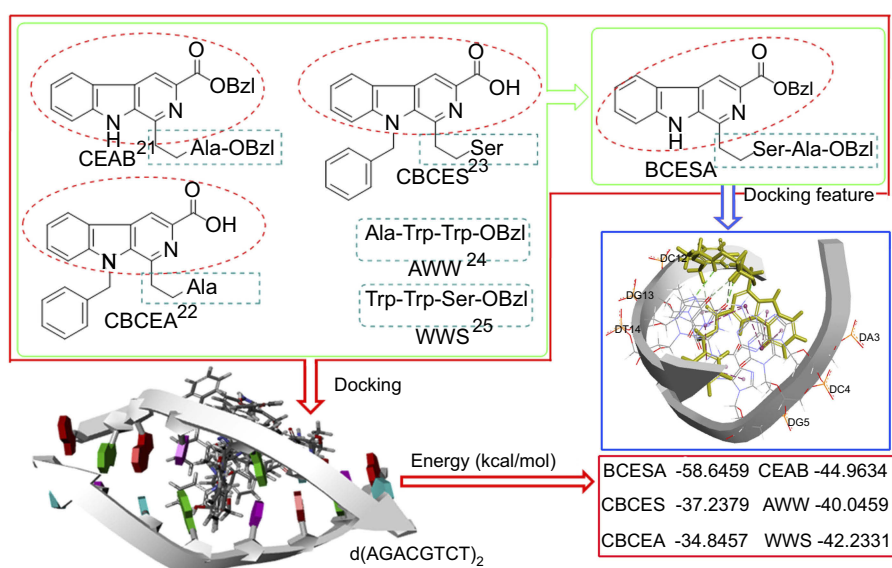

Figure 1 Pharmacophore analysis, CDOCKER energy comparison and docking feature resulted in a national design of BCESA.
Abbreviations: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl; CEAB, N-(3-Benzyloxycarbonylcarboline-1-yl)ethylalanine benzylesters; CBCES, N-(9-Benzyl-3-carboxyl-β-carboline-1-yl)-ethyl-serine; CBCEA, N-(9-Benzyl-3-carboxyl-β-carboline-1-yl) ethyl-alanine; AWW, Tripeptide sequence AWW benzyl ester; WWS, Tripeptide sequence WWS benzyl ester.

the University. The evaluation protocols were reviewed and approved by the committee. The committee assured that the requirements of Animal Welfare Act and NIH Guide for Care and Use of Laboratory Animals were maintained. With SPSS 19.0 program and one-way ANOVA all data resulted from the in vivo assay were statistically analyzed, and P-value <0.05 was considered statistically significant.

Docking investigation

By following the literature, the conformation of BCESA was generated to perform the docking investigation.[26,27] For this purpose, 10 energy optimized conformations of BCESA were docked into the active pocket of d(AGACGTCT)$_2$ (PDB:1N37).[28,29]

Synthesis of BCESA

Benzyl 1-(2,2-dimethoxyethyl)-β-4H-carboline -3-carboxylate (1)

At room temperature, a suspension of 20.0 mL (78.6 mmol) of 1,1,3,3-tetramethoxypropane, 20 mL of CF$_3$CO$_2$H and 200 mL of CH$_2$Cl$_2$ was stirred for 45 mins, into which 18 g (0.37 mol) of L-Trp-OBzl were added and stirred for additional 48 hrs. The reaction mixture was washed successively with saturated aqueous NaHCO$_3$ (200 mL ×3) and saturated aqueous NaCl (200 mL ×3), dried over anhydrous Na$_2$SO$_4$ for 12 hrs and then filtrated. The filtrate was evaporated under vacuum, the residue was chromatographically purified on silica gel column (petroleum ether/ethyl acetate, 2/1) to obtain **1** as pale yellow oil. Yield: 60%. ESI-MS (m/e): 395 [M + H][+.1]H NMR (300 MHz, CDCl$_3$) δ 8.50 (s, 1H), 5.22 (d, J =3.0 Hz, 2H), 4.69 (m, 1H), 4.42 (m, 1H), 4.02 (dd, J =7.6, 5.2 Hz, 1H), 3.44 (s, 3H), 3.38 (s, 3H), 3.20–3.13 (m, 1H), 3.08–2.97 (m, 1H), 2.13–1.99 (m, 2H).

Benzyl 1-(2,2-dimethoxyethyl)-β-carboline -3-carboxylate (2)

At 0ºC, the suspension of 10.8 g (20 mmol) of **1** in 100 mL of THF was stirred to form a clear solution. Into this solution 7.8 g (40 mmol) of KMnO$_4$ were added, stirred at 0ºC for 1 hr, at room temperature for 3 hrs, TLC (CHCl$_3$/MeOH, 15/1) indicated the complete disappearance of **1** and the formed precipitates were removed by filtration. The filtrate was evaporated under vacuum, and the residue was dissolved in 100 mL of ethyl acetate. The ethyl acetate solution was washed with saturated aqueous NaCl, dried over anhydrous Na$_2$SO$_4$ for 12 hrs and

evaporated under vacuum. The residue was chromatographically purified on silica gel column (petroleum ether/ethyl acetate, 3/1) to obtain **2** as yellow powders. Yield: 68%. ESI-MS (m/z): 391 [M+H][+.1]H NMR (300 MHz, DMSO-$d6$) δ 9.68 (s, 1H), 8.81 (s, 1H), 8.16 (d, J =8.0 Hz, 1H), 7.55 (m, 4H), 7.35 (m, 4H), 5.53 (s, 2H), 4.82 (s, 1H), 3.58 (d, J =4.0 Hz, 2H), 3.41 (s, 6H).

Benzyl 1-carbonylmethyl-β-carboline-3-carboxylate (3)

At room temperature, the mixture of 10 g (2.0 mmol) of **2**, 7.2 mL of acetic acid, 0.9 mL of hydrochloric acid and 0.9 mL of water was stirred at room temperature for 4 hrs. Into this mixture, 50 g of ice were added, and the formed precipitates were collected by filtration to provide **3** for the next reaction without purification. Yield: 67%. ESI-MS (m/e) 345 [M+H][+.1]H NMR (300 MHz, DMSO-$d6$) δ 10.97 (s, 2H), 9.79 (s, H), 8.79 (s, 1H), 8.27 (d, J =7.9 Hz, 1H), 7.40 (t, J =8.4 Hz, 4H), 7.10 (t, J =6.9 Hz, 2H), 6.99 (t, J =6.9 Hz, 2H), 6.23 (m, 2H), 5.50 (s, 2H), 4.67 (m, 2H).

Ser-Ala-OBzl

A solution of 2.7 g (13.30 mmol) of Boc-Ser, 2.16 g (16.02 mmol) of HOBt, 3.30 g (16.02 mmol) of DCC and 50 mL of anhydrous THF was stirred for 30 mins. Into this solution, a solution of 4.41 g (12.02 mmol) of Ala-OBzl in 10 mL of anhydrous THF was added. By using N-methylmorpholine (NMM) the reaction mixture was adjusted to pH 9, then at 0ºC stirred for 3 hrs and at room temperature for 12 hrs. By filtration the precipitates of DCU were removed, the filtrate was evaporated under vacuum and the residue was diluted with 30 mL of ethyl acetate. This solution was successively washed with saturated aqueous NaHCO$_3$ (20 mL ×3), 5% aqueous KHSO$_4$ (20 mL ×3) and saturated aqueous NaCl (20 mL ×3) and finally dried over anhydrous Na$_2$SO$_4$ for 12 hrs. After filtration, the filtrate was evaporated under vacuum and the residue was chromatographically purified on silica gel column (CH$_2$Cl$_2$/MeOH, 30/1) to give 3 g of Boc-Ser-Ala -OBzl in 63% yield.

At 0ºC, 1 g (2.73 mmol) of Boc-Ser-Ala-OBzl was stirred in a solution of hydrogen chloride in anhydrous ethyl acetate (10 mL, 4 M) for 2 hrs and evaporated under vacuum. The residue was dissolved in 10 mL of anhydrous ethyl acetate, and the solution was evaporated under vacuum. This procedure was repeated for 3 times to thoroughly remove the excess hydrogen chloride. The residue was triturated with ether to provide 0.6 g of Ser-Ala-OBzl as colorless powders. The yield was 83% and ESI(-)-FT-ICR-MS was 267.1275.

N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl (BCESA)

A mixture of 733 mg (2.42 mmol) of Ser-Ala-OBzl, 40.4 mg (1.01 mmol) of NaOH, 50 mL of MeOH and 695 mg (2.02 mmol) of **3** was stirred at room temperature for 30 mins, and then 127 mg (2.02 mmol) of $NaBH_3CN$ were added. The reaction mixture was stirred at room temperature for 20 hrs, adjusted to pH 2 with hydrochloric acid (3 M) and evaporated under vacuum. The residue was dissolved in 20 mL of water. This solution was washed with ether (50 mL ×3), adjusted to pH 8 with aqueous NaOH (1 M) and extracted with ethyl acetate (50 mL ×3). The ethyl acetate phase was washed with saturated aqueous NaCl and dried over anhydrous Na_2SO_4 for 12 hrs. After filtration, the filtrate was evaporated under vacuum, and the residue was chromatographically purified on silica gel column (petroleum ether/ethyl acetate, 5/1) to give 575 mg of BCESA as pale yellow powers. Yield: 48%. Mp 167–168ºC. $[\alpha]_D^{20}$=−2.43 (c=0.37, CH_3OH). ESI-FT-ICR-MS (m/z) of $[C_{34}H_{34}N_4O_6+ H]^+$: calcd 595.2551, found 595.2568. IR (KBr): 3230, 3088, 3062, 3032, 2935, 2868, 1712, 1651, 1568, 1500, 1456, 1379, 1348, 1307, 1247, 1213, 1153, 1139, 1103, 1053, 900, 786, 744, 696 cm^{-1}.^{1}H NMR (300 MHz, $CDCl_3$) δ 10.85 (s, 1H), 8.65 (s, 1H), 8.05 (d, J =7.8 Hz, 1H), 8.00 (d, 7.8 Hz, 1H), 7.48 (d, J =9.0 Hz, 4H), 7.39–7.32 (m, 3H), 7.27 (m, 6H), 5.42 (s, 2H), 5.09 (dd, J =30.4, 12.2 Hz, 2H), 4.61 (m, 1H), 3.97 (mz, 1H), 3.77 (m, 1H), 3.46–3.31 (m, 2H), 3.13 (m, 3H), 1.34 (d, J =7.2 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 172.8, 166.1, 144.2, 140.9, 136.4, 136.1, 136.0, 135.1, 128.5, 128.4, 128.3, 128.1, 127.9, 121.8, 121.6, 120.5, 116.7, 112.3, 67.2, 67.1, 66.3, 64.5, 63.0, 58.9, 47.9, 46.7, 34.2, 18.7, 17.8. HPLC Purity: 95.9%.

Characterization of BCESA

NOESY 2D^{1}H NMR spectrum of BCESA

To reveal the pattern of the molecular assembly of BCESA, the NOESY 2D^{1}H NMR spectrum was on Bruker 800 MHz spectrometer recorded by following the literature.[30,31]

FT-ICR-MS and qCID spectra of BCESA

To reveal the molecular assembly, the FT-ICR-MS and qCID spectra of BCESA were recorded on Bruker 9.4 T solariX FT-ICR mass spectrometer equipped with an ESI/MALDI dual ion source.[26,27]

SEM, TEM and AFM feature of BCESA

The nano-features of BCESA in solid state, in water and in mouse plasma were imaged by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) experiments, respectively.[32–34]

Mesoscale simulation of BCESA

By following the method of the literature the size of the nano-particles of BCESA a mesoscale simulation was performed.[35]

Faraday-Tyndall effect, particle size and zeta potential tests of BCESA

By following the method of the literature, the nano-property of BCESA in water was identified by Faraday-Tyndall effect, particle size and zeta potential.[36] Aimed at this, the solutions (10 μM, 1 μM, 0.1 μM, 0.01 μM) of BCESA in the ultrapure water of pH 6.8 and 1.2, as well as in PBS of pH 7.4 were prepared. The 650 nm laser was used for inducing Faraday-Tyndall effect.

Bioassays of BCESA

In vitro anti-proliferation assay of BCESA

In T-25 flasks, all the stock cultures were grown. The suspensions of the freshly trypsinized cells were seeded in 96-well microtiter plates, and the density was 5000 cells per well. At 37ºC, the cells were in the high glucose-DMEM or the RPMI-1640 medium cultured for 4 hrs. The medium contained 10% (v/v) fetal calf serum, 60 μg/mL of penicillin and 100 μg/mL of streptomycin. After the addition of 25 μL of the solution of Dox or BCESA, the cells were cultured for 48 hrs, then 25 μL of MTT solution (5 μg/mL) were added and the cells were incubated for additional 4 hrs. The medium was removed, and MTT-formazan was dissolved in 100 μL of DMSO. On a microplate reader, the optical density was measured at 570 nm. The IC_{50} was calculated based on the survival curve and linear regression analysis. Each test was repeated in quadruplicate, and the results were expressed as mean±SD.

In vivo anti-tumor assay of BCESA on S180 mouse model

In this evaluation ICR mice (male, 22±2 g) were used. For initiation of subcutaneous tumors S180 ascites tumor cells were obtained from the tumor-bearing mice that were serially transplanted once per week. By injecting 0.2 mL saline containing 1×10^7 viable tumor cells under the skin on the right armpit subcutaneous tumors were implanted, 24 hrs later the implanted mice were randomly divided into experimental groups (12 per group). To clarify the validity of the mouse model and the intercalation mechanism of BCESA,

Dox was used as the positive control, while the mice in this group were given a daily i.p injection of 2 μmol/kg/day of Dox for 9 consecutive days. To clarify the validity of the mouse model, 0.5% CMC-Na was used as the negative control, while the mice in this group were orally given 0.5% CMC-Na (10 mL/kg/day) for 9 consecutive days. The mice in BCESA groups were orally given a suspension of BCESA in 0.5% CMC-Na (0.01 μmol/kg/day or 0.1 μmol/kg/day or 1.0 μmol/kg/day) for 9 consecutive days. During the experimental period, the body weights of all mice were recorded every day, while 24 hrs after the last administration all mice were weighed, sacrificed by diethyl ether anesthesia and collected 1 mL of fresh blood. Then, the tumors and organs were dissected and weighed immediately.

In vivo liver and kidney injury of BCESA to S180 mice

The collected blood from the mice receiving in vivo antitumor assay was centrifuged at 1000 rpm for 15 mins to prepare the serum. By following the guidance of the kits (AST/GOT testing kit, ALT/GPT testing kit; JCBIO Co., Nanjing, People's Republic of China) the serum ALT and AST were measured. By following the standard protocol (creatinine assay kit, JCBIO Co., Nanjing, PR China), the serum Cr was measured.

UV spectrum and the interaction of BCESA towards CT DNA

To visualize the intercalation of BCESA towards calf thymus DNA (CT DNA) the ultraviolet (UV) spectrum assay was performed. In this assay, the UV spectra of CT DNA (10 μM, in PBS of pH 7.4) in the presence of BCESA (0 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 30 μM, 35 μM and 40 μM in PBS of pH 7.4) were monitored (Shimadzu 2550 spectrophotometer, 200–320 nm wavelength).

Fluorescent spectrum and the interaction of BCESA towards CT DNA

To visualize the intercalation of BCESA towards CT DNA, the fluorescent spectrum assay was performed. In this assay, the fluorescent spectra of BCESA (10 μM, in pH 7.4 PBS) in the presence of CT DNA (pH 7.4, 0 μM, 9 μM, 18 μM, 27 μM, 36 μM, 45 μM, 54 μM, 63 μM and 72 μM, in PBS of pH 7.4) were monitored (Shimadzu RF-5310PC spectrofluorometer, 258 nm of fluorescence excitation wavelength).

Relative viscosity and the interaction of BCESA towards CT DNA

To visualize the intercalation of BCESA towards CT DNA, the relative viscosity of CT DNA was measured on Ubbeholde viscometer immersed in a thermostated water bath of 25°C. In this assay into the solution of CT DNA in PBS (13 mL, 80 μM) the solutions of BCESA in PBS were added to get a series of ratios of [BCESA]/[CT DNA] that fell in a range of 0.13–0.82. After a thermal equilibrium time of 5 mins, the flow times of the ratios were measured. For this purpose, the equation, $\eta = (t - t_0)/t_0$, was used, wherein t_0 and t are the measured flow times in the absence and the presence of BCESA, respectively. The relative viscosity of CT DNA in the presence and the absence of BCESA are calculated and presented as $(\eta/\eta_0)^{1/3}$ versus binding ratio, wherein η and η_0 are the viscosities of CT DNA in the presence of BCESA and CT DNA alone, respectively.

CD spectrum and the interaction of BCESA towards CT DNA

To visualize the intercalation of BCESA towards CT DNA, the circular dichroism (CD) assay was performed. In this assay, the solution systems consisting of CT DNA in PBS (pH 7.4, 100 μM) and BCESA in PBS (pH 7.4, 50 μM, 100 μM, 150 μM and 200 μM) were incubated at 37ºC for 3 hrs to measure their CD spectra by following a standard procedure.
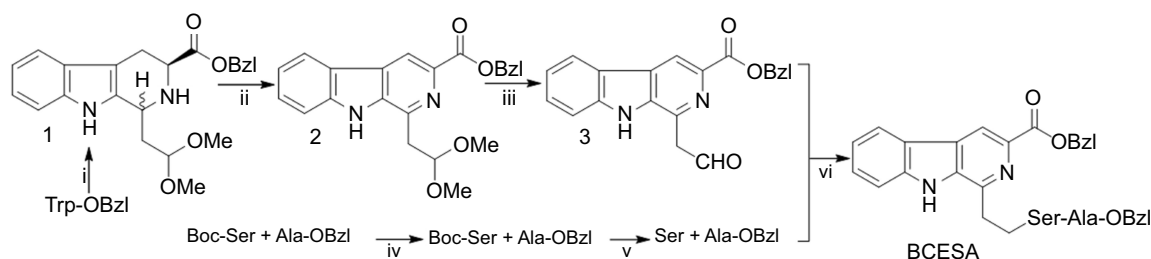
Results

BCESA was satisfactorily synthesized

To satisfactorily synthesize BCESA, a route of Scheme 1 was used. First *L*-Trp-OBzl and 1,1,3,3-tetramethoxypropane received Pictet–Spengler condensation to give benzyl 1-(2,2-dimethoxyethyl)-β-tetrahydro-carboline-3-carboxylate (**1**, 60% yield). Secondary the aromatization of **1** gave benzyl 1-(2,2-dimethoxyethyl)-β-carboline-3-carboxylate (**2**, 68% yield). Thirdly the hydrolysis of **2** gave benzyl 1-carbonyl-methyl-β-carboline-3-carboxylate (**3**, 67% yield). Besides, the solution-phase method provided Ser-Ala-OBzl in 52% yield. Finally, the coupling of **3** and Ser-Ala-OBzl generated BCESA in 48% yield.

Self-assembly and the tetramer of BCESA

The self-assembly of small molecule has been well documented.[29,37] Here the self-assembly of BCESA in ultrapure water (1 nM) was explored with its FT-MS spectrum. Figure 2A shows an ion peak at 2378.00696, the mass of a tetramer plus H. Besides, an ion peak at 1211.50060, the mass of a dimer plus Na and an ion peak at 617.24120, the mass of a monomer plus Na, are also indicated. To clarify the relationship between the tetramer, the dimer and the monomer, a qCID spectrum was recorded. Figure 2B indicates that

Scheme 1 Synthetic route of BCESA. (i) TFA, 1,1,3,3-tetramethoxypropane, CH$_2$Cl$_2$; (ii) KMnO$_4$, THF; (iii) HCl, HOAc, H$_2$O; (iv) DCC, HOBt, NMM; (v) hydrogen chloride in EtOAc (4 M); (vi) NaBH$_3$CN, MeOH, NaOH.

Abbreviations: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl; DCC, dicyclohexylcarbodiimide; HOBt, N-hydroxybenzotriazole; NMM, N-methylmorpholine.
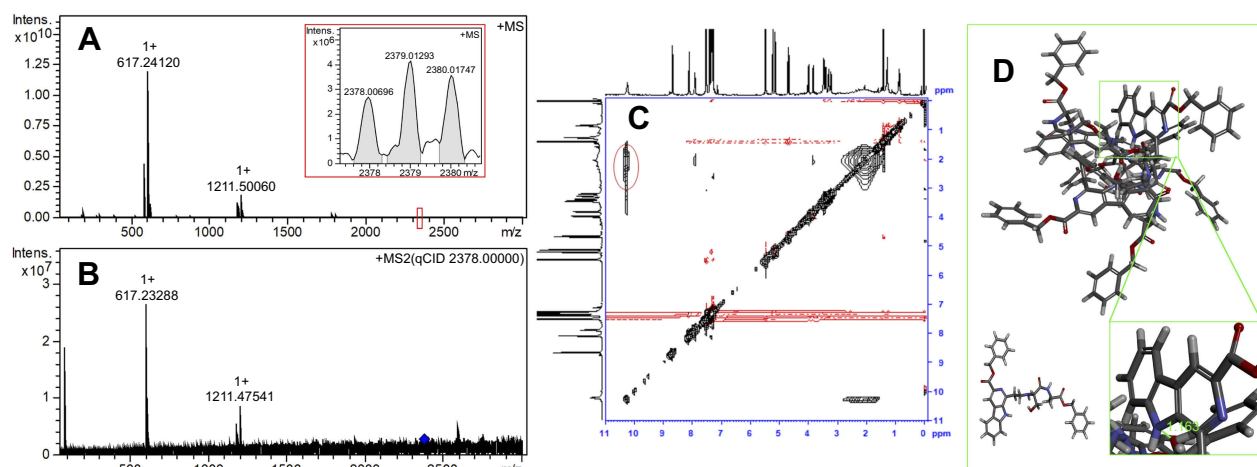

Figure 2 FT-MS and NOESY 2D^{1}H NMR spectra of BCESA. (**A**) FT-MS spectrum of BCESA in ultrapure water (1 nM), the spectrum of the tetramer (2378.00696) is locally amplified and inserted; (**B**) qCID spectrum of BCESA in ultrapure water (1 nM); (**C**) NOESY 2D^{1}HNMR spectrum of BCESA, in which an interesting cross-peak is marked with red ring; (**D**) Energy-minimized conformations of the monomer and the tetramer; in the impeller-like conformation of the tetramer the distance is locally amplified and inserted.

Abbreviation: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl.

in FT-MS condition the tetramer can be split to the dimer and the monomer.

To know BCESA how to form the tetramer its NOESY 2D^{1}HNMR spectrum was recorded. Figure 2C shows an interesting cross-peak labeled red circle. According to NOESY, this cross-peak is resulted from the interaction of the pyrrole H in the carboline moiety of one molecule and the hydroxyl H of Ser residue in another molecule. This means that the distance between these H is less than 4 . Based on this, the monomer of BCESA was first energy minimized, and then the assembly of four energy minimized monomers was manually accessed to form a tetramer. This operation leads the tetramer of BCESA to an impeller-like conformation (Figure 2D).

Nano-property of aqueous BCESA

To show the nano-property of aqueous BCESA the Faraday-Tyndall effect was tested. Figure 3A indicates that the radiation of 650 nm laser does not induce the ultrapure water occurring Faraday-Tyndall effect. Figure 3B-3E, Figure 3F-3I and Figure 3J-3M indicate that 650 nm laser-radiation induces the solution of BCESA in pH6.8 ultrapure water, in pH7.4 PBS and in pH 1.2 ultrapure water (10, 1, 0.1 and 0.01 μM) consistently occurring Faraday-Tyndall effect. This suggests that in tissue juice, in biological phase and in gastric juice BCESA has nano-property.

The nano-property of the solution of BCESA in ultrapure water and PBS was also examined on nano-laser particle size analyzer. Figure 3N indicates that during 7 days the sizes of BCESA in ultrapure water (0.01, 0.1 and 1 μM, pH 6.8 and 1.2) and PBS (0.01, 0.1 and 1 μM, pH 7.4) fall in the range of 150 nm to 275 nm, mirroring a typical nano-property.

The nano-property of the solutions of BCESA in ultrapure water and PBS was further examined on a ZetaPlus Potential Analyzer. Figure 3O-3Q indicates that the zeta potentials of the solutions of BCESA in ultrapure water of pH 6.8 (0.01
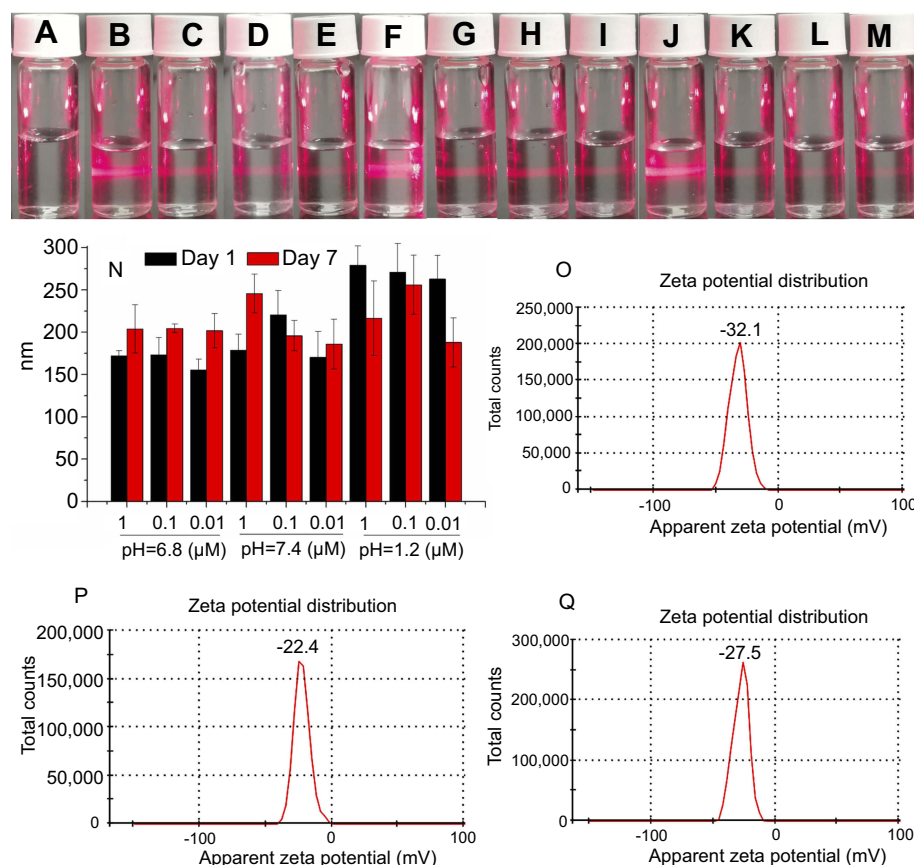
Figure 3 Faraday-Tyndall effect, zeta potential and nano-size of BCESA in water or PBS. (**A**) Ultrapure water radiated by 650 nm laser; (**B**) BCESA dissolved in ultrapure water (pH 6.8, 10 μM) and radiated by 650 nm laser; (**C**) BCESA dissolved in ultrapure water (pH 6.8, 1 μM) and radiated by 650 nm laser; (**D**) BCESA dissolved in ultrapure water (pH 6.8, 0.1 μM) and radiated by 650 nm laser; (**E**) BCESA dissolved in ultrapure water (pH 6.8, 0.01 μM) and radiated by 650 nm laser; (**F**) BCESA dissolved in PBS (pH 7.4, 10 μM) and radiated by 650 nm laser; (**G**) BCESA dissolved in PBS (pH 7.4, 1 μM) and radiated by 650 nm laser; (H) BCESA dissolved in PBS (pH 7.4, 0.1 μM) and radiated by 650 nm laser; (**I**) BCESA dissolved in PBS (pH 7.4, 0.01 μM) and radiated by 650 nm laser; (**J**) BCESA dissolved in ultrapure water (pH 1.2, 10 μM) and radiated by 650 nm laser; (**K**) BCESA dissolved in ultrapure water (pH 1.2, 1 μM) and radiated by 650 nm laser; (**L**) BCESA dissolved in ultrapure water (pH 1.2, 0.1 μM) and radiated by 650 nm laser; (**M**) BCESA dissolved in ultrapure water (pH 1.2, 0.01 μM) and radiated by 650 nm laser; (**N**) Nanosize of BCESA in pH7.0 and pH1.2 ultrapure water as well as pH 7.4 PBS (0.01 μM, 0.1 μM and 1 μM) during 7 days; (**O**) Zeta potential of BCESA in pH6.8 ultrapure water (0.01 μM); (**P**) Zeta potential of BCESA in pH7.4 PBS (0.01 μM); (**Q**) Zeta potential of BCESA in pH1.2 ultrapure water (0.01 μM).
Abbreviation: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl.

μM), in PBS of pH 7.4 (0.01 μM) and in ultrapure water of pH 1.2 (0.01 μM) are −32.1 mV, −22.4 mV and −27.5 mV, respectively, again mirroring a typical nano-property.

Nano-feature of BCESA

To visualize the nano-species of BCESA in water and in solids, the TEM and SEM features were imaged. Figure 4A and B indicates that in water (0.01 μM) BCESA forms the particles of 20–120 nm in diameter, and most diameters of the particles are less than 120 nm. Figure 4C and D indicates that the powders lyophilized from aqueous BCESA (0.01 μM) are the particles of 20–63 nm in diameter, and most diameters of the particles are less than 70 nm. Therefore, in solution and solid states, BCESA consistently exists as nanoparticles having similar size.

To visualize the nano-species of BCESA in ultrapure water of pH 6.8 and in mouse plasma, the AFM features were imaged. Figure 4E indicates that in ultrapure water of pH 6.8 (0.01 μM), BCESA forms nanoparticles of 17.06 nm in height. Figure 4F indicates that in mouse plasma (0.01 μM), BCESA forms nanoparticles of 53.58 nm in height. Figure 4G indicates that mouse plasma alone gives no any comparable nanoparticle.

Mesoscale simulation for nanoparticle of BCESA

To simulate the formation and predict the size of the nanoparticle of BCESA a calculation was performed by using mesoscale simulation software. Figure 5 shows that 324 tetramers, ie, 1296 molecules of BCESA can
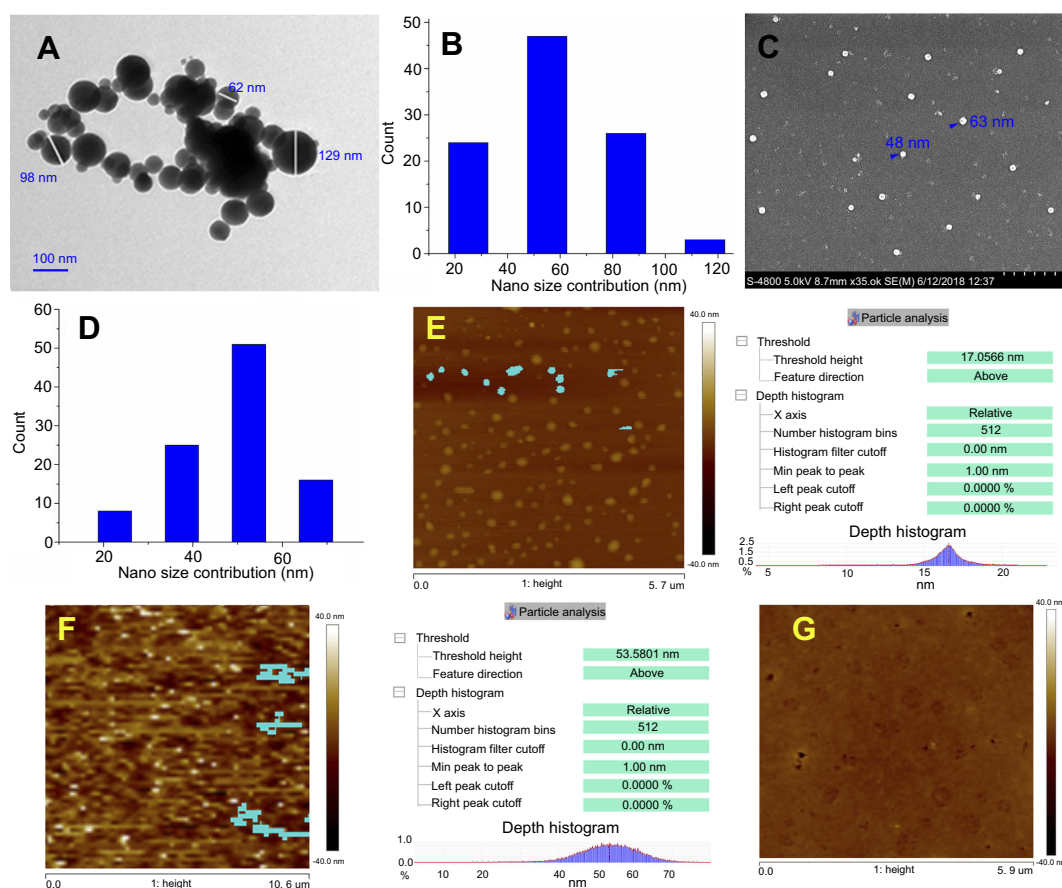
Figure 4 Nano-feature of BCESA in ultrapure water and mouse plasma imaged with TEM, SEM and AFM. (**A**) Nano-feature of BCESA in ultrapure water (pH 6.8, 0.01 μM) imaged with TEM; (**B**) Size distribution of the nano-particles of BCESA in ultrapure water (pH 6.8, 0.01 μM) imaged with TEM; (**C**) Nano-feature of the powders lyophilized from a solution of BCESA in ultrapure water (pH 6.8, 0.01 μM) imaged with SEM; (**D**) Size distribution of the powders lyophilized from a solution of BCESA in ultrapure water (pH 6.8, 0.01 μM) imaged with SEM; (**E**) AFM imaged particles of 17.06 nm in height of BCESA in ultrapure water (pH 6.8, 0.01 μM); (**F**) AFM imaged particles of 53.58 nm in height of BCESA in mouse serum (0.01 μM); (**G**) AFM image of mouse plasma alone.
Abbreviations: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl; TEM, transmission electron microscopy; SEM, scanning electron microscopy; AFM, atomic force microscopy.

form a nanoparticle of 61.59 nm in diameter. This simulation may help us to estimate how much molecules of BCESA are involved in a nanoparticle of exact size.

Intercalation of BCESA towards CT DNA

To examine the intercalation of BCESA towards CT DNA the UV, fluorescence, CD, viscosity and melting temperature experiments were performed.
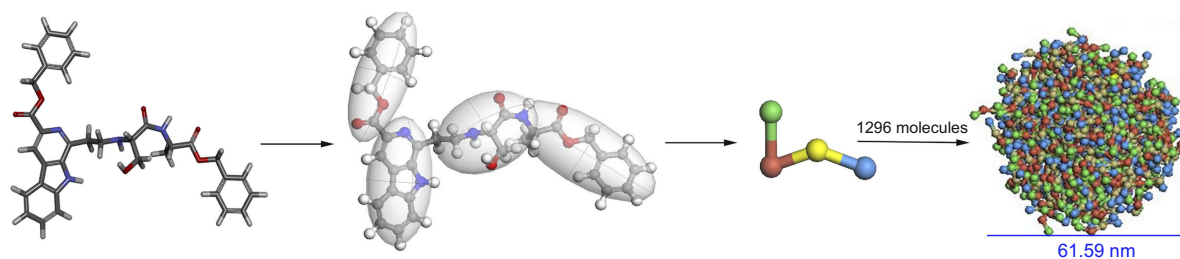

Figure 5 The course of mesoscale simulation to predict the formation and the size of a nanoparticle of BCESA.
Abbreviation: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl.

The UV spectra of CT DNA in PBS of pH 7.4 (10 μM) and CT DNA plus BCESA in PBS of pH 7.4 (final concentration: 0, 5, 10, 15, 20, 25, 30, 35 and 40 μM) were recorded in the region of 200–320 nm. Figure 6A shows that BCESA induces the bands at 218 nm and 258 nm of CT DNA to have hypochromic effect. The two bands also have small hypsochromic shift. The changes of CT DNA mean that BCESA intercalates the base pairs of CT DNA.

The fluorescence spectra of BCESA in PBS of pH 7.4 (10 μM) and BCESA plus CT DNA in PBS of pH 7.4 (final concentration: 0, 9, 18, 27, 36, 45, 54, 63 and 72 μM) were recorded. Figure 6B shows that with the concentration of CT DNA been increased from 0 μM to 72 μM the fluorescence intensity of BCESA is lowered from 360 to 170 (intensity is decreased by 52%), giving a sharp quenching phenomenon and suggesting the intercalation of BCESA towards the base pairs of CT DNA.

The CD spectra of CT DNA in PBS of pH 7.4 (200 μM) and CT DNA plus BCESA in PBS of pH 7.4 (final concentration: 50, 100, 150 and 200 μM) were recorded. Figure 6C shows that around 245 nm and 276 nm, the CD spectrum of CT DNA alone gives a negative peak and a positive peak, respectively. In the presence of 50, 100,

150 and 200 μM of BCESA, the ellipticities of the positive and negative bands of CT DNA been decreased by 46.5% and 60.6%, respectively. These mean that BCESA intercalates the base pairs of CT DNA.

The relative viscosities of [BCESA]/[CT DNA] in the ratios of 0.13–0.82 were recorded. Figure 6D indicates that with the increase of the concentration of BCESA the relative viscosity of CT DNA is increased steadily. These mean that BCESA intercalates the base pairs of CT DNA.

In thermal denaturation experiments, the curves of CT DNA alone (100 μM) and CT DNA (100 μM) plus BCESA (100 μM) were drawn to show the change of the Tm, and consequently to give the change of melting temperature (ΔTm) of 2.5°C (Figure 6E).

BCESA inhibiting the proliferation of cancer cells, but not non-carcinoma cells

The in vitro anti-proliferation activity of BCESA was evaluated on HCT-8, S180, SH-sy5y, HL-60, A549, MCF-7 and HaCaT by using MTT assay, and Dox was the positive control. Figure 7A shows that the IC_{50} of BECSA against HCT-8, S180, SH-sy5y, HL60, A549,
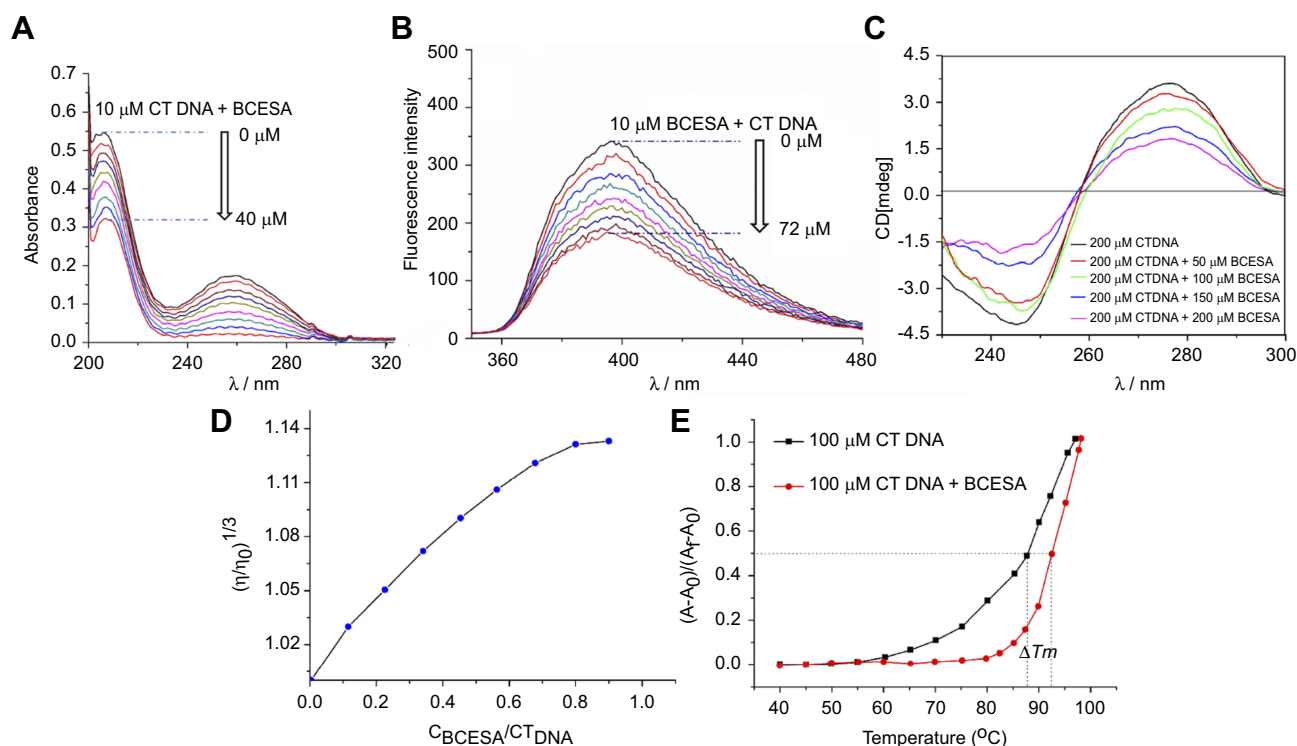

Figure 6 Intercalation of BCESA towards CT DNA. (A) BCESA changes the UV spectrum of CT DNA; (B) BCESA changes the fluorescence spectrum of CT DNA; (C) BCESA changes the CD spectrum of CT DNA; (D) BCESA changes the viscosity of CT DNA; (E) BCESA changes the Tm of CT DNA.
Abbreviations: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl; CT DNA, calf thymus DNA; UV, ultraviolet; CD, circular dichroism.
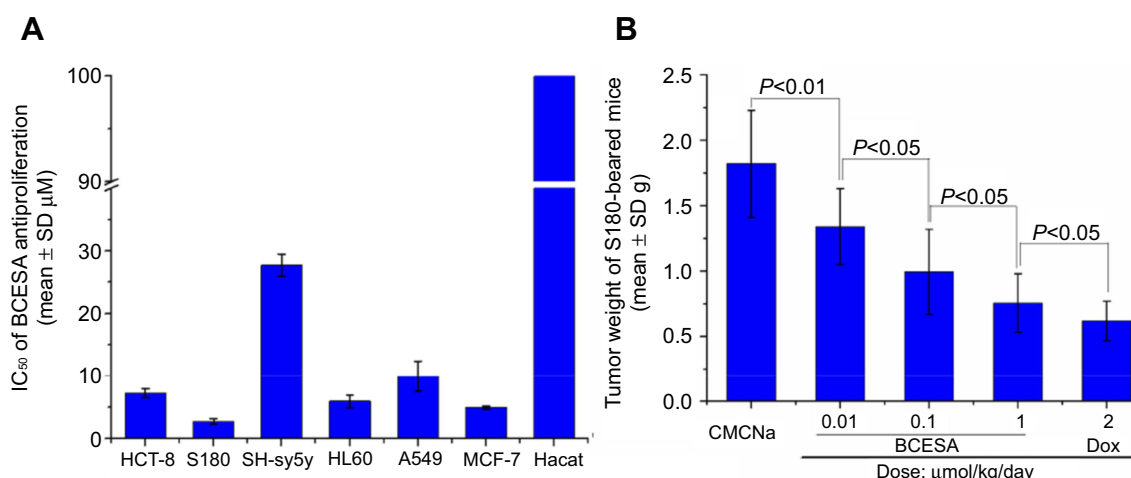
A

B

Figure 7 The anti-tumor activities of BCESA. (**A**) In vitro anti-proliferation activity of BCESA; (**B**) BCESA dose-dependently slows the tumor growth of S180 mice; n=12.
Abbreviations: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl; CMCNa, carboxymethylcellulose sodium; Dox, doxorubicin.

and HaCaT cells are 7.2 μM, 2.7 μM, 27.7 μM, 5.9 μM, 10.0 μM, 4.9 μM and >100 μM, respectively.

BCESA dose-dependently inhibits tumor growth

The dose-dependent assay of BCESA was performed on S180 mouse model and three doses (1.0 μmol/kg/day, 0.1 μmol/kg/day and 0.01 μmol/kg/day) were used, in which Dox was the positive control. Figure 7B indicates that when the dose of BCESA is from 0.01 μmol/kg/day via 0.1 μmol/kg/day increased to 1.0 μmol/kg/day, the tumor weight is gradually decreased, suggesting a dose-dependent inhibition.

BCESA has no systematic toxicity to S180 mice

The systematically chemotherapeutic toxicity consists of organ and body atrophy. To explain this toxicity the weights of the body, heart, liver, spleen, kidney and brain of the S180 mice treated with BCESA are shown in Figure 8A and B. As seen, the weights of the body, the liver and the spleen of S180 mice treated with 2.0 μmol/kg/day Dox, but not 1.0 μmol/kg/day BCESA, are significantly lower than those of S180 mice treated with CMCNa, suggesting Dox, but not BCESA, may induce body, liver and spleen atrophy. This comparison demonstrates that BCESA has no Dox-like side effects.

BCESA does not change the level of serum ALT, AST and Cr of S180 mice

To ensure the safety of BCESA therapy, the blood of S180 mice treated by CMCNa and BCESA (0.01 μmol/kg/day,

0.1 μmol/kg/day and 1.0 μmol/kg/day) was collected to prepare serum and to measure alanine transaminase (ALT), aspartate transaminase (AST) and creatinine (Cr). In the measurements, the guidance of AST/GOT, ALT/GPT and Cr testing kits was followed. Figure 8C, 8D and 8E indicates that the levels of ALT, AST and Cr in the serum of S180 mice treated with BCESA are equal to those of S180 mice treated with CMCNa.

BCESA targeting tumor tissue

By analyzing the FT-MS spectra of the extracts of the homogenates prepared with the tumor tissue, the brain, the heart, the lung, the liver, the spleen, the kidney and the blood of the treated S180 mice, a tumor-targeting action of BCESA was identified. Figure 9A is the FT(+)-MS spectrum of the extract of blood homogenate and only an ion peak of BCESA plus H occurs at 595.25368 (see the local amplified inset). This means that in blood circulation BCESA is delivered without degradation. Figure 9B is the FT(+)-MS spectrum of the extract of tumor tissue homogenate. The local amplified insets show that in the tumor tissue there are of BCESA and its metabolites, including β-carboline-3-carboxylic acid, 1-methyl-β-carboline-3-carboxylic acid, 1-carbonyl-methyl-β-carboline-3-carboxylic acid, 1-methyl-β-carboline-3-carboxylic benzyl ester, 1-carbonylmethyl-β-carboline-3-carboxylic benzyl ester, N-[(3-carboxyl- β-carboline-1-yl)ethyl]-Ser-Ala, N-[2-hydroxyl-(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl, N-[(3-benzyloxycar-bonyl-β-carboline-1-yl)ethenyl]-Ser-Ala- OBzl and Ser-Ala. Figure 9C-9H is the FT-MS(+)-MS spectra of the extracts of the homogenates prepared from the heart, the liver, the
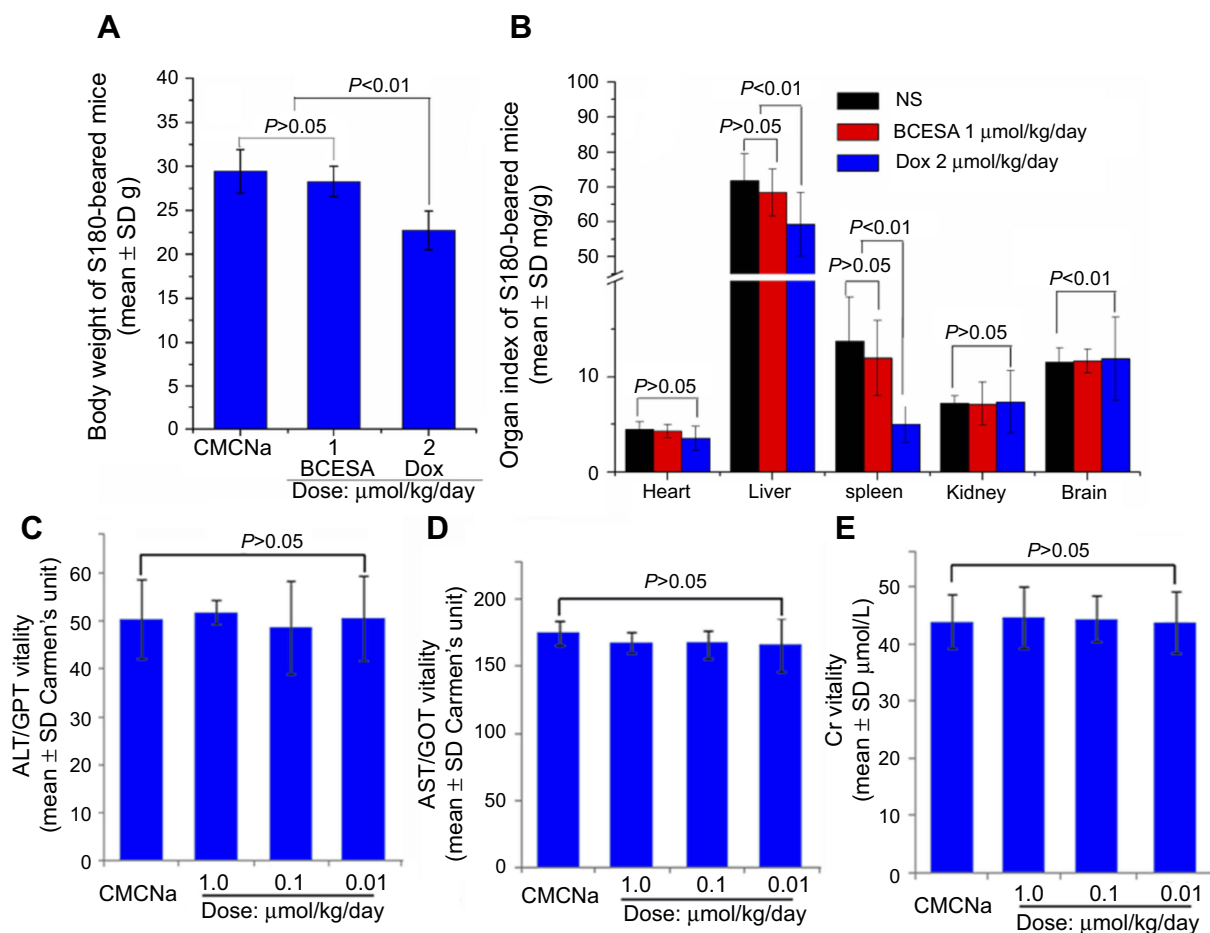
Figure 8 Effect of BCESA on the health of S180 mice. (**A**) Effect of BCESA on body weight of S180 mice; (**B**) Effect of BCESA on organ index of S180 mice; (**C**) Effect of BCESA on serum ALT of S180 mice; (**D**) Effect of BCESA on serum AST of S180 mice; (**E**) Effect of BCESA on serum Cr of S180 mice; n=12.
Abbreviations: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl; CMCNa, carboxymethylcellulose sodium; Dox, doxorubicin.

spleen, the lung, the kidney and brain. The local amplified insets show that these extracts give no any ion peak related to BCESA.

Discussion

The synthetic route could satisfactorily provide BCESA with desired yield and good purity. The experiments of docking, UV, fluorescence, CD, viscosity and melting temperature ensure that BCESA can intercalate the base pairs of CT DNA.

FT-MS spectrum of BCESA reveals that in water BCESA carries out self-assembly, thereby forms the tetramer as the sole and final form. NOESY 2D ^{1}H NMR spectra show that the self-assembly processes by the pyrrole H of the carboline moiety in one molecule to approach the hydroxyl H of Ser-residue in another molecule. Energy minimization gives the tetramer an impeller-like conformation. Mesoscale simulation software-assisted calculation predicts that 324 tetramers (1296

molecules) of BCESA can form a nanoparticle of 61.59 nm in diameter.

Faraday-Tyndall effect, nano-size analysis, SEM image, TEM image and AFM image show that in solution, solids and gastric juice BCESA consistently exists as similar nanoparticles. Besides, in blood circulation BCESA exists as the nanoparticles of small size, so that can effectively avoid macrophage phagocytosis and could be safely delivered.[38] This kind of nanoparticles should benefit BCESA to exhibit a targeting action.

In vitro MTT assay reveals that the IC_{50} values of BCESA against the proliferation of HCT-8, S180, SH-sy 5y, HL60 and A549 cells range from 2.7 μM to 27.7 μM. Besides, the IC_{50} value of BCESA against non-carcinoma cell, HaCaT, is more than 100 μM. This means that BCESA inhibits the proliferation of the cancer cells, but not the non-carcinoma cells. Furthermore, in this assay,
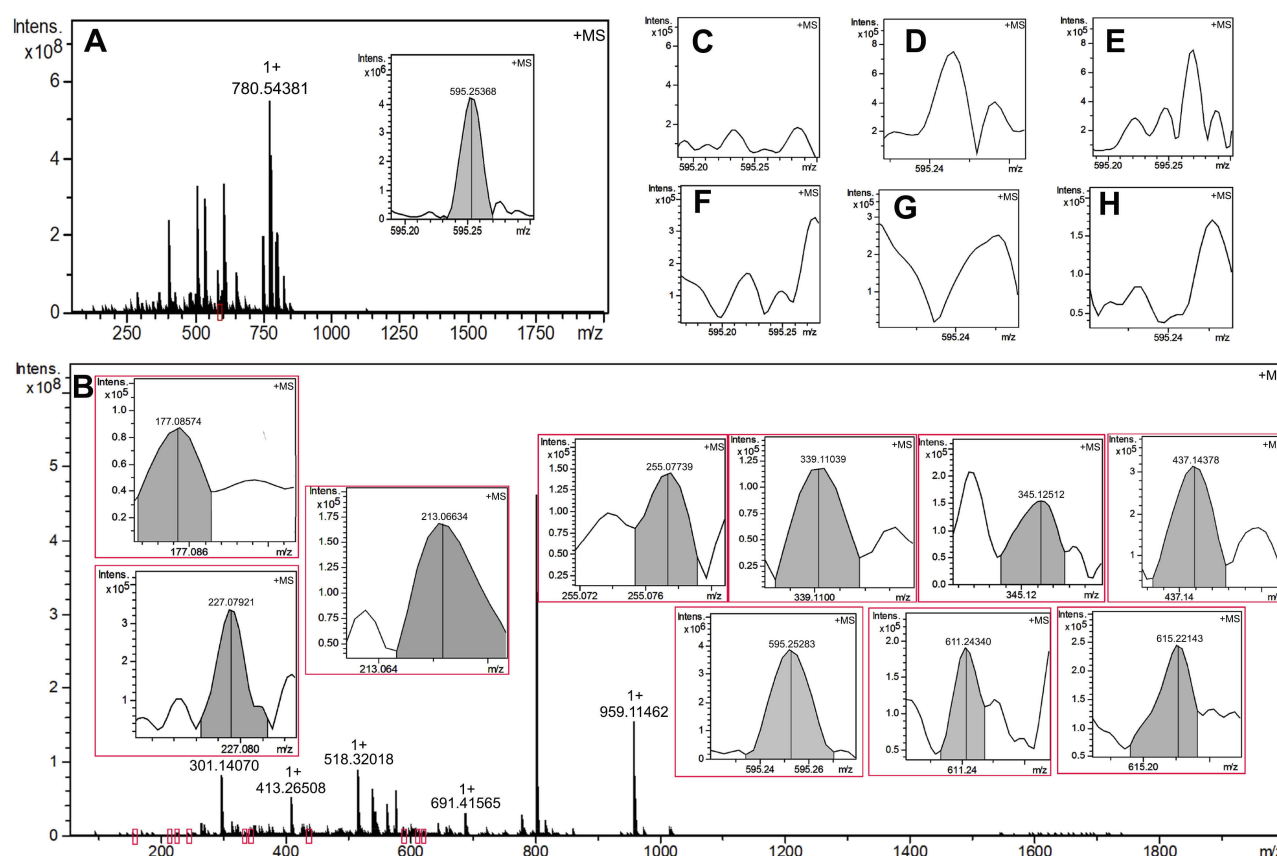
Figure 9 FT-MS(+)-MS spectra of the homogenate extracts of the tumor, the blood and the organs of S180 mice treated with 1.0 μmol/kg/day BCESA for nine consecutive days. (**A**) FT-MS(+)-MS spectra of the homogenate extract of the blood, in the local amplified inset only an ion peak of BCESA plus H is found at 595.25368; (**B**) FT-MS (+)-MS spectrum of the homogenate extract of the tumor tissue, in the local amplified insets the ion peaks of BCESA plus H, Ser-Ala plus H, β-carboline-3-carboxylic acid plus H, 1-methyl-β-carboline-3-carboxylic acid plus H, 1-carbonylmethyl-β-carboline-3-carboxylic acid plus H, 1-methyl-β-carboline-3-car- boxylic benzyl ester plus Na, 1-carbo- nylmethyl-β-carboline-3-carboxylic benzyl ester plus H, N-[(3-carboxyl-β-carboline-1-yl)ethyl]-Ser-Ala plus Na, N-[2-hydroxyl-(3-benzyloxycarbonyl-β-carboline-1-yl)-ethyl]- Ser-Ala-OBzl plus H and N-[(3-benzyloxycarbonyl-β-carboline-1-yl)-ethenyl]-Ser-Ala-OBzl plus Na occur at 595.25283, 177.08574, 213.06634, 227.07921, 255.07739, 339.11039, 345.12512, 437.14378, 611.24340 and 615.22143, respectively; (**C-H**) FT-MS(+)-MS spectra of the homogenate extracts of the heart, the liver, the spleen, the lung, the kidney and the brain, in which occur no any ion peak related to BCESA.
Abbreviation: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl.

S180 is the most sensitive cell to BCESA. This specific behavior led to the selection of S180 mouse model for the in vivo assay.

On S180 mouse model, the tumor weights of the mice treated with BCESA (0.01, 0.1 and 1.0 μmol/kg/day) for 9 consecutive days were lower than that of the mice treated with CMCNa, suggesting BCESA was an active antitumor agent and slowed the growth of the tumor in a dose-dependent manner. The activity of 1.0 μmol/kg/day of BCESA was equal to that of 2.0 μmol/kg/day of Dox, suggesting its activity was 2 folds of that of Dox.

The serum levels of ALT, AST and Cr of S180 mice treated with 1.0 μmol/kg/day of BCESA were equal to those of S180 mice treated with CMCNa. Therefore, at the dose of 1.0 μmol/kg/day, BCESA did not injure the liver and kidney of the mice.

FT-MS spectra of the extracts of the homogenates of the organs, the blood and the tumor tissue of the treated S180 mice showed that from the blood circulation BCESA entered the tumor tissue, but not the organs, and released the anti-tumoral β-carbolines wherein.

As a tumor target intercalator, the release profile of BCESA in the tumor tissue was addressed based on the FT-MS peaks of the extract of the homogenate of tumor tissue, and two release approaches were proposed (Figure 10).

In approach A, BCESA was first converted to N-[(3-benzyloxycarbonyl-β-carboline-1-yl)ethenyl]-Ser-Ala-OBzl and N-[2-hydroxyl-(3-benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl that was converted to Ser-Ala and 1-carbonyl-methyl-β-carboline-3-carboxylic benzyl ester. Next, 1-carbonylmethyl-β-carboline-3-carboxylic benzyl ester was converted to 1-methyl-β-carboline-3-carboxylic benzyl ester and

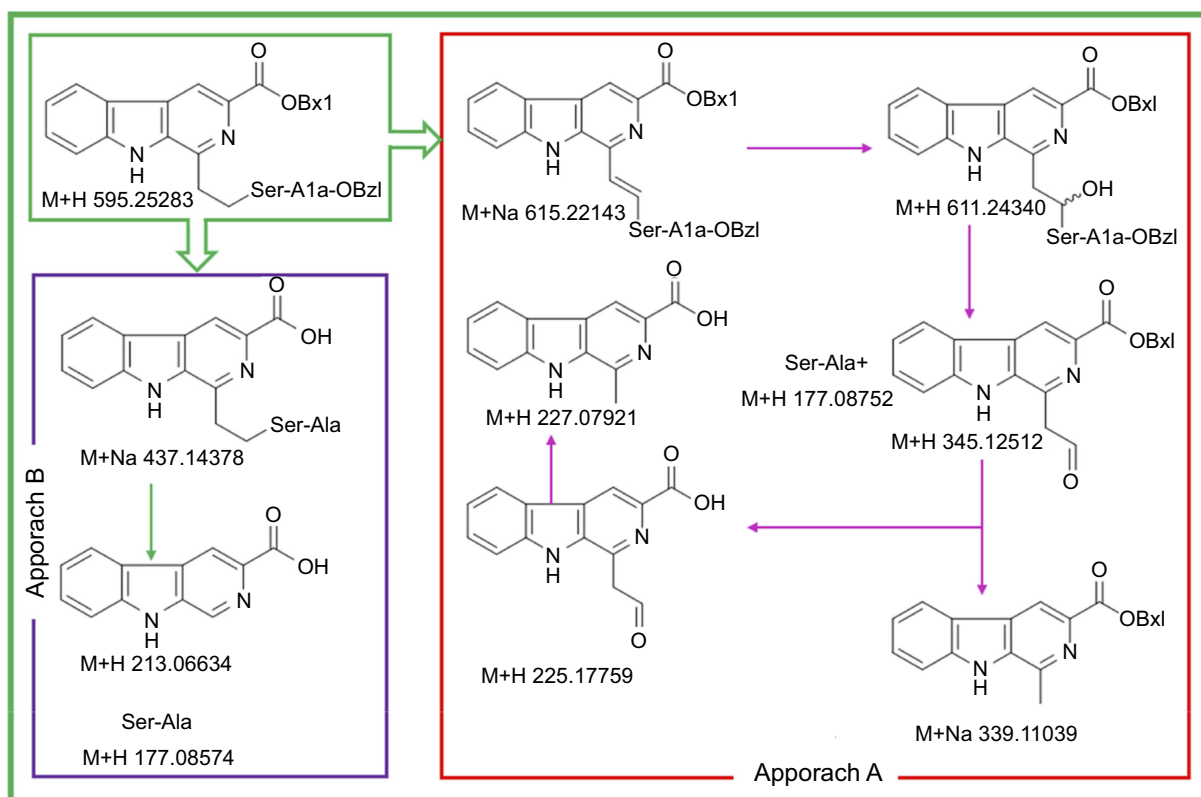**Figure 10** FT-MS(+)-MS spectra based degradation course of BCESA in tumor tissue.
Abbreviation: BCESA, N-[(3-Benzyloxycarbonyl-β-carboline-1-yl)ethyl]-Ser-Ala-OBzl.

1-carbonylmethyl-β-carboline-3-carboxylic acid. Finally, 1-carbonylmethyl-β-carboline-3-carboxylic acid was converted to 1-methyl-β-carboline-3-carboxylic acid. In approach B, BCESA was successively converted to N-(3-carboxyl-β-carboline-1-yl)-ethyl-Ser-Ala, β-carbo-line-3-carboxylic acid and Ser-Ala. According to the release profile of BCESA, Ser-Ala-OBzl should be the carrier of 1-methyl-β-carboline-3-carboxylic acid and β-carboline-3-carboxylic acid. In both approaches, Ser-Ala was from Ser-Ala-OBzl, while 1-methyl-β-carboline -3-carboxylic acid and β-carboline-3-carboxylic acid were known as the anti-tumoral agents.[39,40]

Conclusion
Anti-tumoral pharmacophore analysis and CDOCKER energy comparison resulted in the rational design of BCESA as an intercalator, which had no Dox-like toxicity. BCESA was a nano-scaled small molecule capable of targeting tumor tissue, releasing anti-tumoral 1-methyl-β-carboline-3-carboxylic acid and β-carboline-3-carboxylic

acid wherein. In our opinion, Ser-Ala-OBzl was a simple and desirable carrier of β-carbolines.

Disclosure
The authors report no conflicts of interest in this work

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