

Development of 13-Cys-BBR as an Agent Having Dual Action of Anti-Thrombosis and Anti-Inflammation

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Background: There is a correlation between tumor and inflammation. The activity of 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine (13-Cys-BBR) slowing tumor growth is higher than that of BBR. Whether the anti-inflammation activity of 13-Cys-BBR is higher than that of BBR remains unknown. There is a correlation between thrombosis and inflammation. Whether 13-Cys-BBR is an inhibitor of thrombosis remains unknown.

Purpose: The object of this investigation is to compare the activities of 13-Cys-BBR inhibiting thrombosis and inflammation to those of BBR.

Methods: In vivo anti-thrombosis assay was performed on rat model of arterial and venous thrombosis. In vivo anti-inflammation assay was performed on mouse model of xylene induced ear edema.

Results: At oral dose of 66.7 nmol/kg, 13-Cys-BBR, but not BBR, inhibited the rats to form both venous thrombus and arterial thrombus. At oral dose of 2 μmol/kg, 13-Cys-BBR, but not BBR, inhibited the ears of the mice to occur edema.

Conclusion: The anti-venous thrombosis activity, anti-arterial thrombosis activity and anti-inflammation activity of 13-Cys-BBR were significantly higher than those of BBR. 13-Cys-BBR is a promising preclinical candidate.

Keywords: berberine, 13-Cys-BBR, thrombosis, inflammation

Introduction

Berberine (BBR) is a typical representative of the active components of traditional herbs.¹ BBR exhibited diverse pharmacological actions. In vitro BBR decreased the accumulation of sodium palmitate-induced lipid accumulation,² attenuated the proliferation of tumor cells,³ lowered interleukin-2 secretion,⁴ broke the regioselective hydroxylation of estradiol catalyzed by CYP1,⁵ triggered neuronal differentiation,⁶ ended neuro-degeneration induced by glutamate,⁷ fragmented DNA double-strand,⁸ regulated the secretion of glucagon like peptide 1,⁹ blocked HaCaT cell apoptosis,¹⁰ inhibited the replication of human cytomegalovirus,¹¹ arrested cell cycle,¹² and inhibited inflammation induced by lipopolysaccharide.¹³ In vivo BBR inhibited the growth and metastasis of endometrial cancer,¹⁴ inhibited the growth and lung metastasis of hepatocellular carcinoma,¹⁵ remitted chronic ulcerative colitis,¹⁶ treated type 2 diabetes,¹⁷ and managed musculoskeletal disorders (osteoporosis, osteoarthritis and rheumatoid).¹⁸

However, the poor oral bioavailability seriously limits the clinical utility of BBR. To overcome this shortcoming some preparations, such as the complex of BBR and γ-cyclodextrin,¹⁹ the conjugate of BBR and baicalein,²⁰ the composition of BBR and

5-aminosalicylic acid,²¹ as well as the supramolecule dendrimer-BBR,²² were well documented. To enhance the anti-tumor activity the 13-position of BBR was modified by CH₂CO-Cys(Bzl)-OBzl, CH₂CO-Glu(OBzl)-OBzl, CH₂CO-Ile-OBzl and CH₂CO-Asp(OBzl)-OBzl. It was found that CH₂CO-Cys(Bzl)-OBzl modification enhanced the anti-tumor activity of BBR, and the anti-tumor activity of the formed 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine (13-Cys-BBR) was significantly higher than that of BBR.²³

As mentioned above, BBR is an inhibitor of inflammation,¹³ but whether the anti-inflammation activity of 13-Cys-BBR is higher than that of BBR remains unknown. It is known that thrombosis and inflammation share common mechanism, but whether 13-Cys-BBR has dual action of anti-thrombosis and anti-inflammation remains unknown. In the context, this investigation is to know whether 13-Cys-BBR has dual action of anti-thrombosis and anti-inflammation.

Materials and Methods

General

L-Amino acids were commercially purchased from Sigma. All reactions were carried out in nitrogen (1 bar). ¹H (300 and 800 MHz) and ¹³C (75 and 200 MHz) NMR spectra were recorded on Bruker AMX-300 and AMX-800 spectrometers, dimethyl-sulfoxide (DMSO-d₆) was the solvent and tetramethylsilane was the internal standard. ESI(±)-FT-ICR-MS spectra were recorded on 9.4 T solarix Fourier transform ion cyclotron resonance mass spectrometer (Bruker Corp, Billerica, MA, USA). Chromatographic packing was Sephadex-LH₂₀ or Qingdao silica gel GF₂₅₄ or Qingdao silica gel H₆₀. Solvents were distilled and dried for use. HPLC was done with Agilent Technologies 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) on Eclipse XDB C18 column (5 μm, 4.6 mm × 150 mm); column temperature: 40°C; mobile phase: water and methanol; gradient: 60% methanol (0–5 min), 70% methanol (5–10 min), 80% methanol (10–20 min) and 90% methanol (20–30 min); flow rate: 0.8 mL/min; ultra-violet (UV) absorption spectrum was recorded online; UV detector was set to a scanning range of 200–400 nm; the chromatogram was monitored at a wavelength of 280 nm.

Animals

Male ICR mice and male Sprague Dawley rats (AEEI-2019-179) were purchased from the Laboratory Animal Center of Capital Medical University. The protocol of the

evaluations was reviewed and approved by ethics committee of Capital Medical University. Animal welfare fulfilled the requirements of Animal Welfare Act and NIH Guide for Care and Use of Laboratory Animals.

Statistical analysis and multiple group comparison were performed with ANOVA and LSD, respectively. SPSS 19.0-program was used to address *P*-value, and the value less than 0.05 was considered statistically significant.

Chemistry

Synthetic Route to 13-Cys-BBR

To practice the development of 13-Cys-BBR a procedure consisted of 4-step reactions was used (Figure 1). In contrast to 3 directly coupling Cys(Bzl)-OBzl,²³ here the activated ester of N-hydroxysuccinimide of 3 coupled Cys(Bzl)-OBzl. This led to a substantial increase of the yield of 13-Cys-BBR, and will benefit preparing sample of preclinical investigation. Figure 1 indicates that the activated ester of N-hydroxysuccinimide in the square brackets is not isolated, and the corresponding UV/Vis, FTIR, HRMS, ¹H NMR and ¹³C NMR spectra were not measured.

Preparation of 7,8-Dihydro-8-CH₂COCH₃-Berberine (1)

In to a solution of 5 g (13.6 mmol) of BBR in 26 mL of aqueous NaOH (5 M) 5 mL of acetone was added dropwise (within 20 min) and stirred at room temperature for 34 h. Then, this mixture was stirred at 0°C and adjusted to pH 7 by adding hydrochloric acid (10 M). The mixture was filtered to collect the precipitates. After washing with water 4.88 g (85%) of 1 was obtained.

Preparation of 13-CH₂CO₂C₂H₅-Berberine (2)

A solution of 4 g (9.3 mmol) of 1, 1.4 mL of ethyl bromoacetate, 120 mg of NaI and 100 mL of acetonitrile was stirred at 85°C for 64 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated in vacuum, the residue was purified on a column of silica gel (CH₂Cl₂/MeOH, 15/1) to give 3.56 g (83%) of 2 as yellow powders. ESI(+)/MS(*m/e*): 422 [M-Br]⁺. ¹H NMR (DMSO-d₆, 300 MHz) δ/ppm = 10.021 (s, 1H), 8.254 (d, *J* = 9.6 Hz, 1H), 8.015 (d, *J* = 9.6 Hz, 1H), 7.187 (s, 1H), 7.097 (s, 1H), 6.696 (s, 2H), 4.436 (s, 2H), 4.238 (dd, *J*₁ = 7.2 Hz, *J*₂ = 13.8 Hz, 2H), 4.116 (s, 3H), 4.093 (s, 3H), 3.120 (t, *J* = 7.2 Hz, 2H), 1.266 (t, *J* = 13.8 Hz, 3H).²³

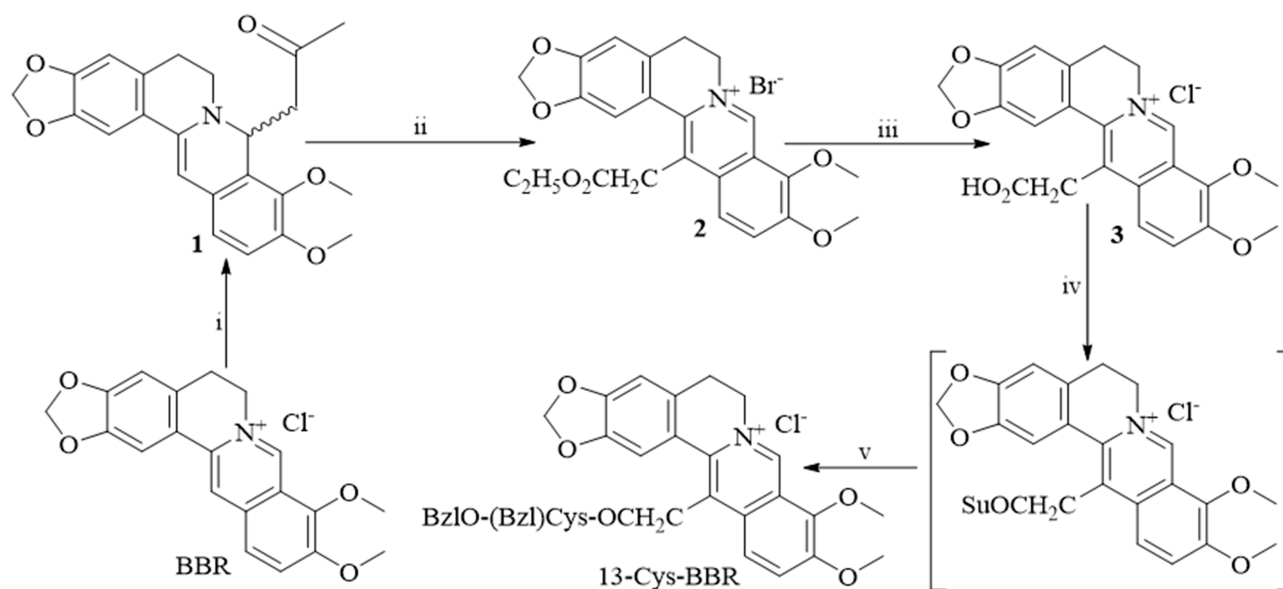


Figure 1 Improved synthetic route to 13-Cys-BBR: (i) aqueous NaOH (5M), acetone, 3 hours and room temperature; (ii) ethyl bromoacetate, NaI, CH₃CN, 6 hours and 80 °C; (iii) methanol NaOH (2 M), HCl, 10 hours, rt; (iv) N-hydroxysuccinimide (HOSu), dicyclohexylcarbodiimide (DCC) and HBTu; (v) Cys(Bzl)-OBzl, N-methylmorpholine, 48 hours and room temperature.

Abbreviations: BBR, berberine; 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine.

Preparation of 13-CH₂CO₂H-Berberine (3)

At room temperature and with stirring into a solution of 3.56 g (7.7 mmol) of 2, 5 mL of methanol and 2 mL of aqueous NaOH (2 M) was added dropwise, the pH of the reaction mixture was kept 13, and TLC (CH₂Cl₂/MeOH, 10/1) indicated the complete disappearance of 2. The reaction mixture was evaporated in vacuum, and with dilute hydrochloric acid the pH of the residue was adjusted to 3 to form precipitates. The precipitates were collected by filtration to give 3.02 g (91%) of 3 as yellow powders. ESI (+)/MS(m/e): 394 [M-Cl]⁺. ¹H NMR(DMSO-d₆, 300 MHz) δ/ppm = 10.040 (d, *J* = 9 Hz, 1 H), 8.254 (dd, *J*₁ = 9 Hz, *J*₂ = 9.6 Hz, 1 H), 8.014 (dd, *J*₁ = 9 Hz, *J*₂ = 15 Hz, 1 H), 7.195 (d, *J* = 15 Hz, 2 H), 6.192 (s, 2 H), 4.817 (s, 2 H), 4.483 (s, 1 H), 4.357 (s, 1 H), 4.114 (s, 3 H), 4.088 (s, 3 H), 3.112 (s, 2 H).²³

Preparation of 13-[CH₂CO-Cys(Bzl)-OBzl]-Berberine (13-Cys-BBR)

At room temperature a solution of 860 mg (2 mmol) of 3, 250 mg (2.2 mmol) of HOSu, 440 mg (2.1 mmol) of DCC, and 2 mL of N-methylmethane-sulfonamide in 4 mL of tetrahydrofuran was stirred, the pH was adjusted to 7 by N-methylmorpholine, and stirred for 8 h. Into the reaction mixture 676 mg (2 mmol) of Cys(Bzl)-OBzl was added and stirred for another 16 h. The reaction mixture was evaporated in vacuum and the residue was purified on

silica gel column (CH₂Cl₂/MeOH, 100/1) to give 978 mg (69%) of 13-Cys-BBR as yellow powders. FT-ICR-MS (m/e): 677.23418 [M-Cl]⁺ (calculated value: 677.2316). ¹H NMR (DMSO-d₆, 800 MHz): δ/ppm = 9.971 (s, 1 H), 9.312 (d, *J* = 7.2 Hz, 1 H), 7.974 (s, 2 H), 7.599 (s, 1 H), 7.408 (s, 5 H), 7.247 (s, 5 H), 7.172 (s, 1 H), 6.152 (s, 2 H), 5.189 (s, 2 H), 4.849 (m, 1 H), 4.723 (m, 1 H), 4.303 (s, 2 H), 4.103 (s, 3 H), 4.018 (s, 3 H), 3.805 (s, 2 H), 3.106 (m, 2 H), 2.927 (m, 3 H). ¹³C NMR (DMSO-d₆, 200 MHz), δ/ppm = 170.7, 170.4, 150.8, 149.9, 147.3, 145.9, 144.7, 138.4, 137.9, 136.2, 134.5, 133.4, 129.4, 128.9, 128.4, 128.5, 128.2, 127.8, 127.4, 126.3, 121.5, 121.4, 120.5, 109.7, 108.9, 102.5, 66.9, 65.4, 62.5, 57.5, 57.4, 52.3, 37.7, 35.6, 32.6, 27.7. HPLC purity: 98.3%.²³

Bioassays

In vivo Anti-Inflammatory Assay

Male ICR mice (25 ± 2 g) were housed in a 12/12 light/dark cycle (21 ± 2°C) for 2 days before use. Food and water were supplied ad libitum. The mice were randomly divided into 0.5% CMC-Na group (blank control), BBR group (parent compound control, oral dose: 2 μmol/kg), 13-Cys-BBR group (oral dose: 2 μmol/kg) and aspirin group (positive control, oral dose: 1100 μmol/kg), each 9 mice. Thirty min later 0.03 mL of xylene was evenly applied to the anterior and posterior surfaces of the right ear of the mice, while the left ear was reserved as

a control. 2 h later the mice were weighed, sacrificed by ether anesthesia to collect blood and remove ears. A rubber plug punch of 7 mm aperture was used to take the circular sections of the ears for weigh. Xylene induced ear edema was represented by the difference in weight of xylene treated right ear section and xylene untreated left ear section.

Measuring Serum TNF- α of Ear Edema Mice

Into 50 μ L of aqueous sodium citrate (3.8%) 450 μ L of blood was added, which were collected from inflammatory mice treated by CMC-Na (blank control) or BBR (parent compound control, oral dose: 2 μ mol/kg) or 13-Cys-BBR (oral dose: 2 μ mol/kg) or aspirin group (positive control, oral dose: 1100 μ mol/kg). The anti-coagulated blood was centrifuged at 200 g for 20 min to get the serum, and the TNF- α in the serum was measured according to the specification of mouse TNF- α ELISA kit (Xitang Biotechnology Co., Shanghai, People's Republic of China).

Arterial Thrombus Weight Assay

Arterial thrombus weight was used to represent the anti-arterial thrombotic activity and measured on Sprague Dawley rat model (male, 240–270 g, each 10) by following the procedure of the literature.²⁵ In this assay 0.5% CMC-Na (blank control, oral dose: 10 mL/kg) or a suspension of aspirin in 0.5% CMC-Na (positive control, oral dose: 167 μ mol/kg) or a suspension of 13-Cys-BBR in 0.5% CMC-Na (oral dose: 66.7 nmol/kg) or a suspension of BBR in 0.5% CMC-Na (parent compound control, oral dose: 66.7 nmol/kg) were given to the rats. 30 min after oral administration the rat was anaesthetized with sodium pentobarbital (80.0 mg/kg, ip), then the right carotid artery and left jugular vein of the rat were separated. A polyethylene tube having an exactly weighted thread (6 cm in length) was filled by the solution of heparin sodium in NS (50 IU/mL). One end of this polyethylene tube was inserted into the left jugular vein and another end of this polyethylene tube was inserted into the right carotid artery. Prior to this procedure the anticoagulant (heparin sodium) was injected, and then the blood was allowed through the polyethylene tube to flow for 15 min. Finally, the thread inside the polyethylene tube was taken out to weigh, and the increased weight of the thread was calculated.

Venous Thrombus Weight Assay

Venous thrombus weight was used to represent the anti-venous thrombotic activity and measured on Sprague Dawley rat

model (male, 240–270 g, each 12) by following the procedure of the literature.²⁵ Inferior vena cava (IVC) ligation model was used to evaluate the activity. In brief, rats were orally given 0.5% CMC-Na (blank control, oral dose: 10 mL/kg) or a suspension of warfarin in 0.5% CMC-Na (positive control, oral dose: 4870 nmol/kg) or a suspension of 13-Cys-BBR in 0.5% CMC-Na (oral dose: 66.7 nmol/kg) or a suspension of BBR in 0.5% CMC-Na (parent compound control, oral dose: 66.7 nmol/kg). 30 min after the administration the rats were anaesthetized with sodium pentobarbital (80.0 mg/kg, ip), and then a midline laparotomy was performed. From the body cavity, the small bowel was exteriorized and slightly moved to the left of the animal surrounding moistened gauze to separate the infrarenal IVC. With braided black silk suture of 4/0, the junction of the IVC and left renal vein on the IVC were ligated. The warm saline was sprayed, into the abdominal cavity the tissues were back set, with braided black silk suture of 4/0 the muscle layer and skin were gradually sutured. 4 h later the abdominal cavity was cut again. The ligatures were made near the bifurcation of the IVC and around all side branches of the ligated IVC segment. Consequently, 2 cm of venous segment bellow the ligation site was excised, the thrombus was removed, the blood was blotted with filter paper, and the thrombus was weighed to represent the activity.

Measuring Serum P-Selectin of Venous Thrombosis Rats

The blood of the rats treated by 0.5% CMC-Na (blank control, oral dose: 10 mL/kg) or a suspension of warfarin in 0.5% CMC-Na (positive control, oral dose: 4870 nmol/kg) or a suspension of 13-Cys-BBR in 0.5% CMC-Na (oral dose: 66.7 nmol/kg) or a suspension of BBR in 0.5% CMC-Na (parent compound control, oral dose: 66.7 nmol/kg) was collected into a tube containing 3.8% aqueous sodium citrate at a ratio of 9 parts aqueous sodium citrate to 1 part blood. The blood was immediately centrifuged at 1000 rpm for 15 min. The top layer was collected as the platelet-rich plasma (PRP), and received 10-fold-dilution with diluents solution from the kit of rat P-selectin ELISA kit (Cusabio, Biotech, USA) to get PRP sample. Serum P-selectin of the blood was measured by use of the kit. The wells containing 980 μ L of PRP from the blood were at 37°C incubated for 120 min. After removing solvent, into each well 100 μ L of biotin labeling antibody (from the kit) was added and at 37°C incubated for 60 min. The solution in the well was discarded, by adding 200 μ L of washing solution (from the kit) the well was washed for three times, into each well 100 μ L of horseradish peroxidase labeling avidin (from the kit) was added, at 37°C incubated for 60 min,

and then washed with washing solution (from the kit) for five times. For coloration, into each well 90 μL of the substrate solution (from the kit) were added and then in dark at 37°C incubated for 20 min. To stop the reaction into each well 50 μL of the stop solution (from the kit) was added. The OD value of the well was measured at 450 nm within 15 min. P-Selectin level was calculated by using the standard curve (from the kit standard samples).

Investigation of Docking Towards P-Selectin

To compare the feature of 13-Cys-BBR and BBR in the active site of P-selectin the docking investigation was performed by using the procedure of the literature.²⁵ In brief, the conformations of 13-Cys-BBR and BBR (parent compound control) were generated and energy-optimized. Ten energy-optimized conformations of BBR and 13-Cys-BBR were selected and individually docked into the active site of P-selectin by using CDOCKER protocol to get docking scores.

ESI(+)-FT-ICR-MS Analysis of Homogenate Extracts

To examine thrombus targeting action of 13-Cys-BBR the homogenate extracts of the thrombus, blood, brain, heart, kidney, liver and spleen of the arterial thrombosis rats treated by CMC-Na (blank control) or 13-Cys-BBR, as well as the venous thrombosis rats treated by CMC-Na or 13-Cys-BBR were homogenized. The homogenates were extracted with methanol and the extracts received ESI (+)-FT-ICR-MS analysis.²⁴

Results and Discussion

The Improvement of Synthetic Route Increased the Yield of 13-Cys-BBR

By coupling Cys(Bzl)-OBzl with active ester of 3, i.e. 13- $\text{CH}_2\text{CO}_2\text{Su}$ -berberine, but not 3 itself, the synthetic route of

the literature was improved. The improvement of the synthesis increased the yield of 13-Cys-BBR from previous 32% to present 69%. Therefore, this synthetic route is better than that of the literature,²³ and is good enough for providing 13-Cys-BBR to receive preclinical investigations.

The Anti-Inflammation Activity of 13-Cys-BBR Is Higher Than That of BBR

The anti-inflammatory activities of 13-Cys-BBR and BBR (parent compound control) were assayed on ear edema mice. In the assay CMC-Na was used as the blank control and aspirin was used as the positive control. Figure 2A shows that 13-Cys-BBR, but not BBR, orally inhibits the ear edema of the mice. The activity of 2 $\mu\text{mol/kg}$ of 13-Cys-BBR is equal to that of 1100 $\mu\text{mol/kg}$ of aspirin. Thus, the anti-inflammatory activity of 13-Cys-BBR is not only higher than that of BBR but also is 550 times of aspirin.

13-Cys-BBR Effectively Decreases Serum TNF- α of Ear Edema Mice

The serum TNF- α of ear edema mice treated by 13-Cys-BBR and BBR (parent compound control) was tested with ELISA kit. In this assay CMC-Na was used as the blank control and aspirin was used as the positive control. Figure 2B shows that the serum TNF- α of the mice orally treated by 2 $\mu\text{mol/kg}$ of 13-Cys-BBR, but not BBR, is significantly lower than that of the mice treated by CMC-Na, and is equal to that of the mice treated by 1100 $\mu\text{mol/kg}$ aspirin. Therefore, the efficacy of 13-Cys-BBR decreasing serum TNF- α is significantly higher than that of BBR and is 550 times of aspirin.

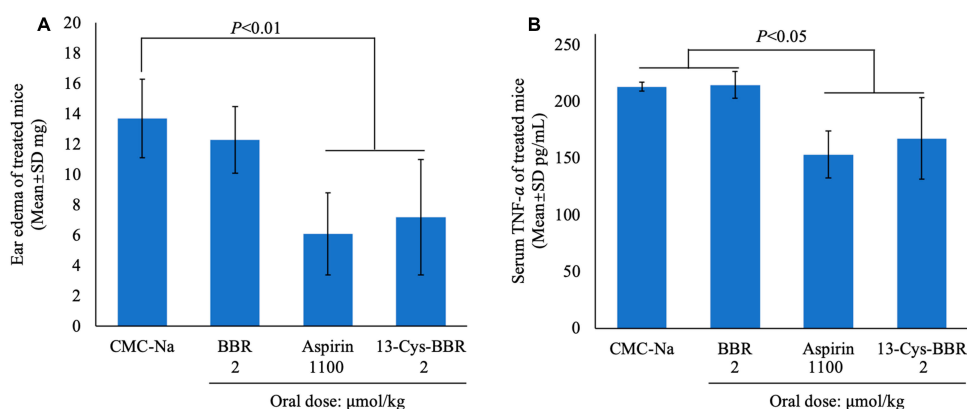


Figure 2 Effect of 13-Cys-BBR on ear edema and serum TNF- α : (A) effect of 13-Cys-BBR on ear edema; (B) effect of 13-Cys-BBR on serum TNF- α of ear edema mice; $n=9$. **Abbreviations:** BBR, berberine; 13-Cys-BBR, 13-[$\text{CH}_2\text{CO}_2\text{Cys(Bzl)-OBzl}$]-berberine.

The Anti-Venous Thrombosis Activity of 13-Cys-BBR Is Higher Than That of BBR

The anti-venous thrombosis activities of 13-Cys-BBR and BBR (parent compound control) were assayed on rat model. Figure 3A shows that the weight of the venous thrombus of the rats orally treated by 66.7 nmol/kg of 13-Cys-BBR, but not BBR, is significantly lower than that of the rats orally treated by CMC-Na (blank control) and is equal to that of the rats orally treated by 4.87 μ mol/kg of warfarin (positive control). Therefore, the anti-venous thrombosis activity of 13-Cys-BBR is significantly higher than that of BBR, and is 73 times of warfarin.

The Anti-Arterial Thrombosis Activity of 13-Cys-BBR Is Higher Than That of BBR

The anti-arterial thrombosis activities of 13-Cys-BBR and BBR (parent compound control) were assayed on rat model. Figure 3B shows that the weight of the arterial thrombus of the rats orally treated by 66.7 nmol/kg of 13-Cys-BBR, but not BBR, is significantly lower than those of the rats orally treated by CMC-Na (blank control) and aspirin (positive control, oral dose: 16.7 μ mol/kg). Therefore, the anti-arterial thrombosis activity of 13-Cys-BBR is significantly higher than that of BBR, and is more than 250 times of aspirin.

13-Cys-BBR Effectively Decreases Serum P-Selectin of Venous Thrombosis Rats

The serum P-selectin of venous thrombosis rats treated by 13-Cys-BBR and BBR (parent compound control) was tested with ELISA kit. Figure 3C shows that the serum

P-selectin of the rats orally treated by 66.7 nmol/kg of 13-Cys-BBR, but not BBR, is significantly lower than that of the rats orally treated by CMC-Na (blank control), and is equal to that of the rats orally treated by 4.87 μ mol/kg of warfarin (positive control). Therefore, the efficacy of 13-Cys-BBR decreasing serum P-selectin is significantly higher than that of BBR and is 73 times of warfarin.

The Active Site of P-Selectin Is Suitable for 13-Cys-BBR, but Not BBR

The docking investigation of 13-Cys-BBR and BBR (parent compound control) disclosed that the score of the former (docking score: 160.26) was higher than that (docking score: 103.16) of the latter (Figure 4). In addition, the interactions of 13-Cys-BBR and the amino acid residues of the active site are more than those of BBR and the amino acid residues of the active site (see the locally amplified illustrations of Figure 4). These distinct interactions suggest that the active site of P-selectin is suitable for 13-Cys-BBR, but not BBR, and all biological advantages of the former to the latter could be attributed to the interactions of P-selectin with them. It was well documented that the anti-thrombotic agent down-regulating the expression of P-selectin, thereby decreasing serum P-selectin and inhibiting both venous and arterial thrombosis.²⁵ Thus, the docking investigation theoretically explains why the venous thrombus weight and the serum P-selectin of the rats treated by 13-Cys-BBR are lower than those of the rats treated by BBR. The docking investigation also explains why at 66.7 nmol/kg of oral dose 13-Cys-BBR, but not BBR, is able to inhibit the rats to form arterial thrombus and venous thrombus.

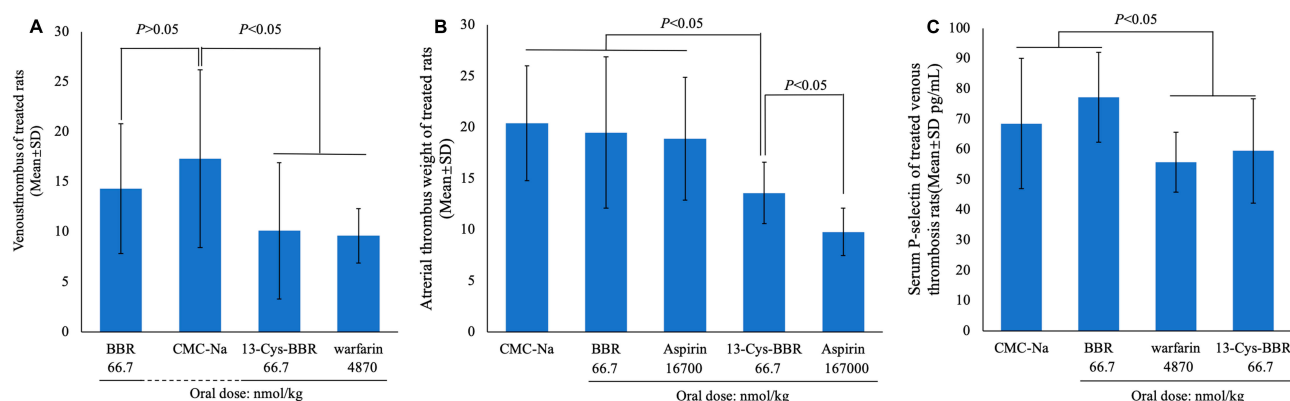


Figure 3 Effect of 13-Cys-BBR and BBR on thrombosis and P-selectin in vivo: (A) anti-venous thrombosis activity of 66.7 nmol/kg of oral 13-Cys-BBR and BBR, n=10; (B) anti-arterial thrombosis activity of 66.7 nmol/kg of oral 13-Cys-BBR and BBR, n=10; (C) inhibition of 66.7 nmol/kg of oral 13-Cys-BBR and BBR to P-selectin in the serum of treated rats in venous thrombosis assay, n=10.

Abbreviations: BBR, berberine; 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine.

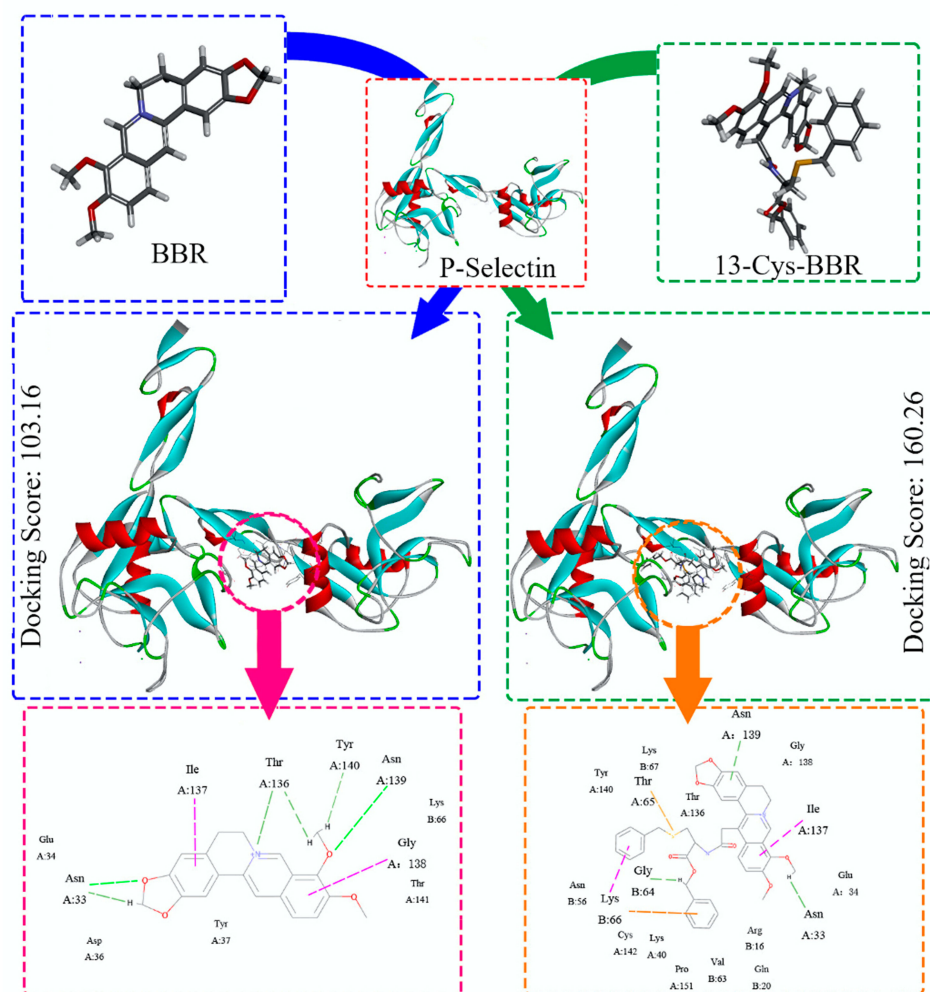


Figure 4 Docking feature of 13-Cys-BBR and BBR in the active site of P-selectin, as well as the docking scores of them.

Abbreviations: BBR, berberine; 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine.

13-Cys-BBR Targets Arterial Thrombus

To show whether 13-Cys-BBR targets thrombus of arterial thrombosis rats the ESI(+)-FT-ICR-MS spectra of the homogenate extracts of the thrombus, blood, brain, heart, kidney, liver and spleen of arterial thrombosis rats orally treated by CMC-Na (blank control) and 66.7 nmol/kg of 13-Cys-BBR were recorded. It was found that the ESI(+)-FT-ICR-MS spectra of the homogenate extracts of the arterial thrombus, blood, brain, heart, kidney, liver and spleen of the rats treated by CMC-Na gave no ion peaks related to 13-Cys-BBR. Similarly, the ESI(+)-FT-ICR-MS spectra of the homogenate extracts of the blood, brain, heart, kidney, liver and spleen of the rats treated by 13-Cys-BBR gave no any ion peaks related to 13-Cys-BBR.

Figure 5 is the ESI(+)-FT-ICR-MS spectrum of the homogenate extract of the arterial thrombus of the rats

treated by 13-Cys-BBR, and 8 ion peaks including the ion peaks of 8-demethylation 13-Cys-BBR at 663.21058, 8,9-demethyl-13-Cys-BBR at 649.20030, 8,9-demethyl-13-debenzyl-13-Cys-BBR at 573.16900, 8-demethylation 13-carbonylmethyl-BBR at 378.13360, 2,3-demethylene-8,9-demethyl-13-Cys-BBR at 637.20030, BBR at 336.12302, 8-demethylation-BBR at 322.10738 and CH₃CO-Cys(Bzl)-OBzl at 343.12421 are observed. These ion peaks are locally amplified and inserted into Figure 5.

These ion peaks evidence that 13-Cys-BBR only enters into the arterial thrombus, releases 8-demethyl-13-Cys-BBR, 8,9-demethyl-13-Cys-BBR, 8,9-demethyl-13-debenzyl-13-Cys-BBR, 8-demethyl-13-carbonylmethyl-BBR, 2,3-demethylene-8,9-demethyl-13-Cys-BBR, BBR, 8-demethyl-BBR and CH₃CO-Cys(Bzl)-OBzl, thereby inhibits the formation of arterial thrombus.

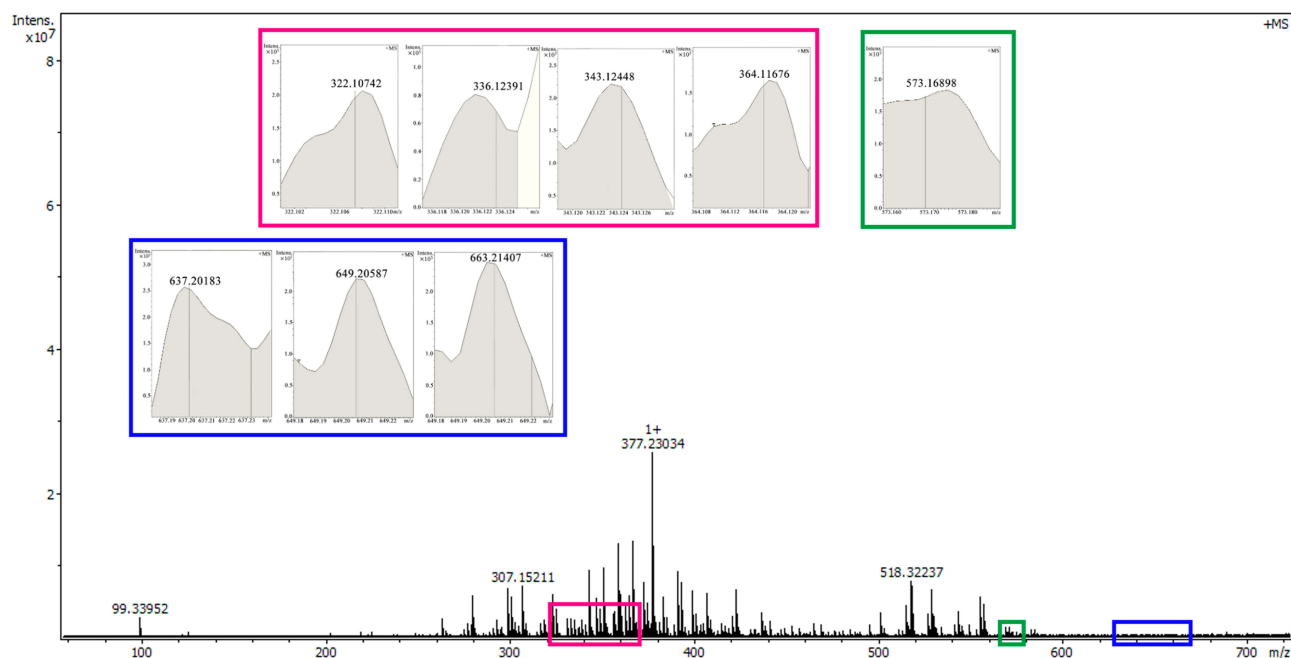


Figure 5 ESI(+)-FT-ICR-MS spectrum of the homogenate extract of the arterial thrombus of the rats treated by 13-Cys-BBR gave 8 ion peaks, the homogenate of the arterial thrombus was prepared and extracted by using the procedure of the literature.²⁶

Abbreviation: 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine.

13-Cys-BBR Targets Venous Thrombus

To show whether 13-Cys-BBR targets thrombus of venous thrombosis rats the ESI(+)-FT-ICR-MS spectra of the homogenate extracts of the thrombus, blood, brain, heart, kidney, liver and spleen of the venous thrombosis rats treated by CMC-Na (blank control) and 13-Cys-BBR were recorded. It was found that the ESI(+)-FT-ICR-MS spectra of the homogenate extracts of the thrombus, blood, brain, heart, kidney, liver and spleen of the venous thrombosis rats treated by CMC-Na gave no ion peaks related to 13-Cys-BBR. Similarly, the ESI(+)-FT-ICR-MS spectra of the homogenate extracts of the blood, brain, heart, kidney, liver and spleen of the venous thrombosis rats treated by 13-Cys-BBR gave no ion peaks related to 13-Cys-BBR.

Figure 6 is the ESI(+)-FT-ICR-MS spectrum of the homogenate extract of venous thrombus of the rats treated by 13-Cys-BBR, and 4 ion peaks including the ion peaks of 8-demethylation 13-Cys-BBR at 663.21058, 8-demethylation 13-carbonyl-methyl-BBR at 378.13108, 8-demethylation BBR at 322.10923 and CH₃CO-Cys(Bzl)-OBzl at 343.12502 are observed. These ion peaks are locally amplified and inserted into Figure 6.

These ion peaks evidence that 13-Cys-BBR only enters into the venous thrombus, released 8-demethylation 13-Cys-BBR, 8-demethylation 13-carbonyl

l-methyl-BBR, 8-demethylation BBR and CH₃CO-Cys(Bzl)-OBzl, thereby inhibits the formation of venous thrombus.

13-Cys-BBR Has No Liver and Kidney Toxicity

To show the safety of 13-Cys-BBR health rats (240–270 g) were orally gave 10 mL/kg/day of CMC-Na (blank control), 66.7 nmol/kg/day of 13-Cys-BBR and 66.7 nmol/kg/day of BBR (parent compound control) for 7 consecutive days. On the next day of the last dose the rats received ether anesthesia to sample arterial blood for testing serum uric acid (UA), serum alanine transaminase (ALT) and serum creatine kinase (CK). Figure 7A–C shows that the serum UA, ALT and CK of the rats treated by CMC-Na are equal to those of the rats treated by 13-Cys-BBR, while the serum UA, ALT and CK of the rats treated by CMC-Na are significantly lower than those of the rats treated by BBR. These data evidence that at 66.7 nmol/kg of oral dose 13-Cys-BBR, but not BBR, does not injure the liver and the kidney of the rats.

Conclusion

Based on the facts that there is a correlation between tumor and inflammation, the activity of 13-Cys-BBR slowing tumor growth is higher than that of BBR, and

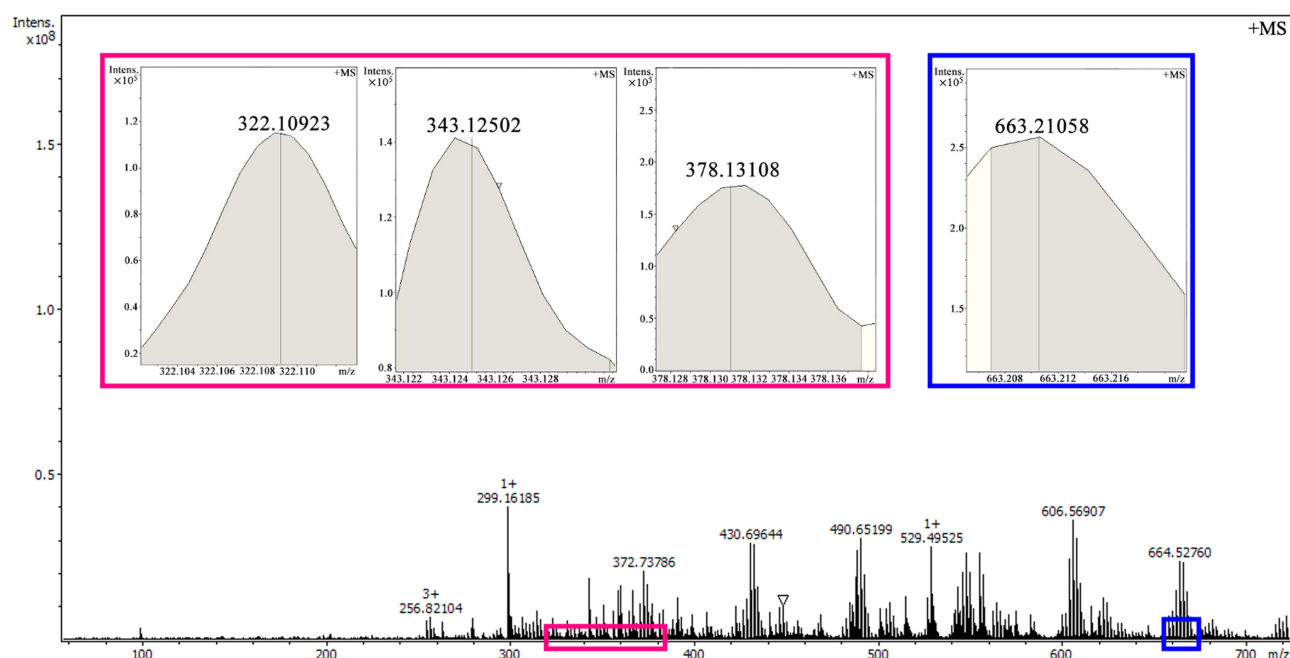


Figure 6 ESI(+)-FT-MS spectrum of the homogenate extract of the venous thrombus of the rats treated by 13-Cys-BBR gave 4 ion peaks, the homogenate of the venous thrombus was prepared and extracted by using the procedure of the literature.²⁶

Abbreviation: 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine.

there is a correlation between thrombosis and inflammation. The activities of 13-Cys-BBR inhibiting thrombosis and inflammation were hypothesized been higher than those of BBR. The essential assays consistently evidence that 13-Cys-BBR, but not BBR, is the excellent inhibitor of inflammation, venous thrombosis, arterial thrombosis, serum TNF- α and serum P-selectin. 13-Cys-BBR enters venous thrombus to release 8-demethyl-13-Cys-BBR, 8-demethyl-13-carbonylmethyl-BBR, 8-demethyl-BBR and CH₃CO-Cys(Bzl)-OBzl; enters arterial thrombus to release

8-demethyl-13-Cys-BBR, 8.9-demethyl-13-Cys-BBR, 8.9-demethyl-13-debenzyl-13-Cys-BBR, 8-demethyl-13-carbonylmethyl-BBR, 2.3-demethylene-8,9-demethyl-13-Cys-BBR, BBR, 8-demethyl-BBR and CH₃CO-Cys(Bzl)-OBzl. The release manner of 13-Cys-BBR targeting venous thrombus and arterial thrombus is summarized in Figure 8. The thrombus targeting leads 13-Cys-BBR having no liver and kidney toxicity as well.

The impact of arterial thrombosis and venous thrombosis on the outcome of the cancer patients is well

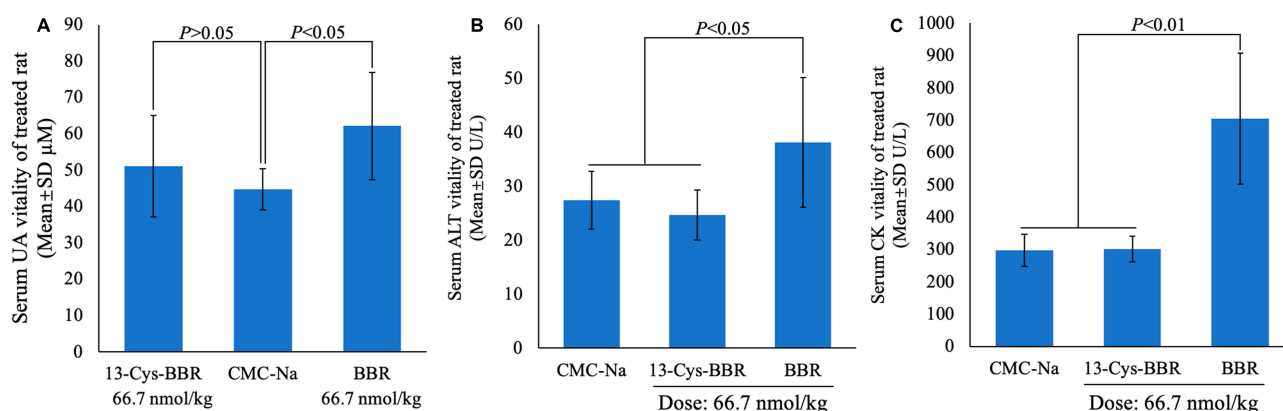


Figure 7 Liver and kidney toxicity of 13-Cys-BBR and BBR in vivo: (A) effect of 13-Cys-BBR and BBR on serum UA of health rats, n=3; (B) effect of 13-Cys-BBR and BBR on serum ALT of health rats, n=3; (C) effect of 13-Cys-BBR and BBR on serum CK of health rats, n=3.

Abbreviations: BBR, berberine; 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine; UA, uric acid; ALT, alanine transaminase; CK, creatine kinase.

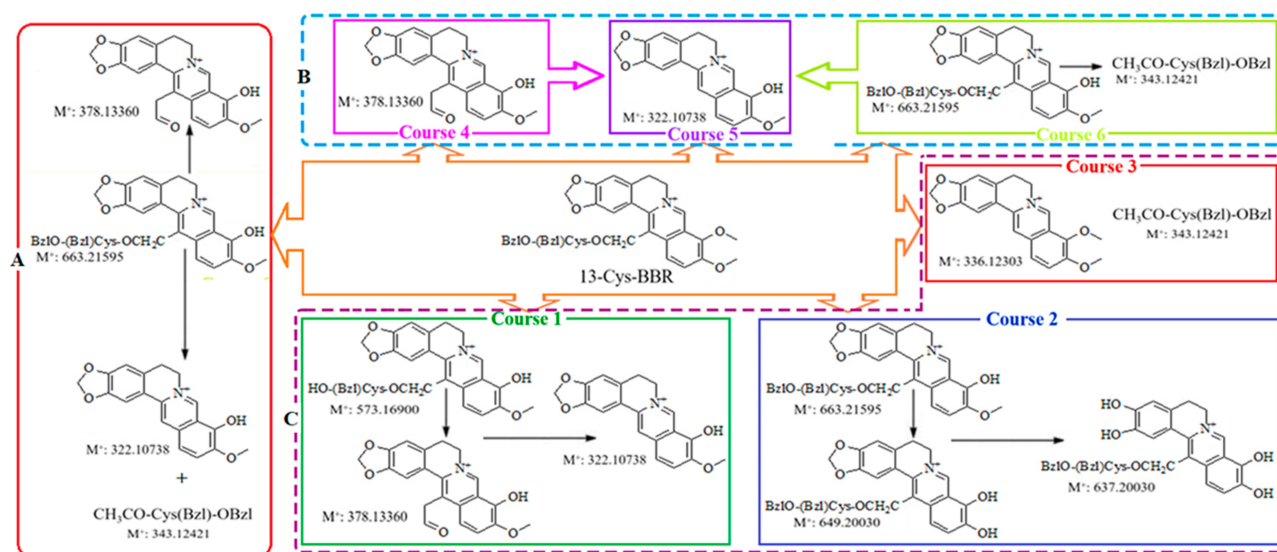


Figure 8 Thrombus targeting action and release of 13-Cys-BBR: (A) venous thrombus targeting action and release of 13-Cys-BBR; (B & C) arterial thrombus targeting action and release of 13-Cys-BBR.

Abbreviations: BBR, berberine; 13-Cys-BBR, 13-[CH₂CO-Cys(Bzl)-OBzl]-berberine.

established.^{24,26-29} And the thrombotic research is often linked to inflammatory response.^{30,31} The biological findings of 13-Cys-BBR in the present paper with the effect of arterial and venous thrombosis on the outcome of the cancer patients together suggest that 13-Cys-BBR should be a promising preclinical candidate.

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Disclosure

The authors report no conflicts of interest in this work.

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