Design and development of ICCA as a dual inhibitor of GPIIb/IIIa and P-selectin receptors

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Background: The impact of upregulation of platelet membrane glycoprotein (GP)IIb/IIIa and P-selectin on the onset of arterial thrombosis, venous thrombosis, and cancer encourages to hypothesize that dual inhibitor of GPIIb/IIIa and P-selectin receptors should simultaneously inhibit arterial thrombosis, block venous thrombosis, and slow tumor growth.

Methods: For this reason, the structural characteristics and the CDOCKER interaction energies of 12 carbolines were analyzed. This led to the design of 1-(4-isopropyl-phenyl)-β-carboline-3-carboxylic acid (ICCA) as a promising inhibitor of GPIIb/IIIa and P-selectin receptors.

Results: The synthetic route provided ICCA in 48% total yield and 99.6% high-performance liquid chromatography purity. In vivo 5 μmol/kg oral ICCA downregulated GPIIb/IIIa and P-selectin expression thereby inhibited arterial thrombosis, blocked venous thrombosis, and slowed down tumor growth, but did not damage the kidney and the liver.

Conclusion: Therefore, ICCA could be a promising candidate capable of downregulating GPIIb/IIIa and P-selectin receptors, inhibiting arterial thrombosis, blocking venous thrombosis, and slowing down tumor growth.

Keywords: thrombosis, cancer, GPIIb/IIIa, P-selectin

Introduction

Deep venous embolism, arterial embolism, cerebral vascular embolism, and tumor are responsible for elevated mortality worldwide.¹-³ Discovery of lead compounds capable of simultaneously inhibiting arterial thrombosis, preventing venous thrombosis, and slowing down tumor growth is of clinical importance. This pushes the inhibitors of P-selectin and glycoprotein (GP)IIb/IIIa receptors into our view.

P-selectin in the α-granules of the resting platelets is expressed on the surface of the activated platelet and thereby is converted to a soluble form. Findings indicate that the soluble P-selectin plays an important role in thrombosis and tumor growth; eg, the increase of serum P-selectin reflects the risk of thrombotic disease attack, while relevant rise of P-selectin mirrors the enhancement of tumor growth.⁶-¹⁰ Several lines of evidence suggest that P-selectin antagonist can inhibit the attack of the thrombotic disease and slow down tumor growth.¹¹-¹⁴ As the most abundant complex on the surface of platelets, GPIIb-IIIa is Ca²⁺-dependent receptor and is one member of integrin family. On the surface of activated platelets, but not on the resting platelets, GPIIb/IIIa effectively binds plasma fibrinogen (FIB) and initiates platelet aggregation. Similarly, an antagonist of GPIIb/IIIa can inhibit the attack of thrombotic disease and slow down tumor growth.¹⁵-¹⁷ These findings imply that dual inhibitor of P-selectin and GPIIb/IIIa receptors should simultaneously inhibit arterial thrombosis, venous thrombosis, and tumor growth.

In this context, the structural characteristics of the previously reported carbolines were...
analyzed. These carbolines inhibited arterial thrombosis; targeted GPIIb/IIIa receptor thereby inhibited arterial thrombosis; targeted P-selectin receptor thereby inhibited arterial thrombosis and venous thrombosis, slowed down tumor growth, or simultaneously inhibited thrombosis and slowed down tumor growth, or targeted P-selectin receptor thereby simultaneously inhibited arterial thrombosis and slowed down tumor growth. The findings led to 10 known carbolines been docked into the active pockets of P-selectin and GPIIb/IIIa receptors. The CDOCKER interaction energy led to the design and the development of 1-(4-isopropylphenyl)-β-carboline-3-carboxylic acid (ICCA) as a candidate capable of simultaneously downregulating P-selectin and GPIIb/IIIa receptors.

Materials and methods

General

The reactions were performed under nitrogen (1 bar). On Bruker Avance II-300 spectrometers, 1H (300 MHz) and 13C (75 MHz) nuclear magnetic resonance (NMR) spectra were measured by using dimethyl sulfoxide (DMSO-d6) as the solvent and tetramethylsilane as the internal standard. A PerkinElmer® 983 instrument was used to record infrared spectra. A 9.4-T SolariX Fourier transform (FT) ion cyclotron resonance mass spectrometer (Bruker Corp., Billerica, MA, USA) was used to record electrospray ionization (ESI) (+/−)-ion cyclotron resonance–FT–mass spectrometry (MS) spectra. On a XT5 hot stage microscope (Beijing Keyi Electro-optic Instrument Factory, China), melting points were tested. Chromatography was done with sephadex-LH20 or silica gel GF254 or silica gel H60 (Qingdao Marine Chemical Plant, China). Before use, all solvents were distilled and dried by following literature procedures. High-performance liquid chromatography (HPLC) purities (C18 column, 4.6×150 mm; Waters Corporation, Milford, MA, USA) of all compounds were 95.34%−99.63%. Alliance separation module e2695 HPLC system (Waters Corporation) was used to separate the compounds by using a Waters XTerra module e2695 HPLC system (Waters Corporation) was used to perform the analysis, and a P-value <0.05 was considered statistically significant.

Synthesis

Preparation of (1R, 3S)-1-(4-isopropyl-phenyl)-2,3,4,9-tetrahydro-β-carboline-3-carboxylic acid benzyl esters (1)

At room temperature, a solution of 4.47 g (15.2 mmol) of L-Trp-OBzl, 3.0 mL (18.2 mmol) of cuminic aldehyde, 50 mL of CH2Cl2, and 5 mL of trifluoroacetic acid was stirred for 24 hours, adjusted to pH of 8 by using aqueous NaHCO3 (5%), and successively washed with aqueous NaHCO3 (5%) and saturated aqueous NaCl. The separated CH2Cl2 phase was dried over anhydrous Na2SO4, filtered, and evaporated under vacuum. The residue was separated on silica gel column (petroleum ether/aceton, 5:1) to give 3.85 g (60%) of (1) as colorless powders. 1H NMR (300 MHz, DMSO-d6): δ/ppm =10.60 (s, 1H), 7.54 (d, J =7.5 Hz, 1H), 7.22 (m, 12H), 5.31 (s, 1H), 5.13 (s, 2H), 3.88 (m, 1H), 3.10 (dd, J =4.8 Hz, J =15.3 Hz, 1H), 2.97 (dd, J =6.9 Hz, J =14.7 Hz, 1H), 2.89 (s, 1H), 2.86 (m, 1H), 1.18 (d, J =6.6 Hz, 6H); ESI-MS (m/e): 425 [M + H]+.

Preparation of benzyl 1-(4-isopropylphenyl)-β-carboline-3-carboxylate (2)

A mixture of 6.0 g (14.15 mmol) of (1), 50 mL of 1,4-dioxane, and 2.37 g (21.3 mmol) of SeO2 was stirred at 70°C for
6 hours. The formed precipitates were removed from the reaction mixture by filtration, the filtrate was evaporated under vacuum, and the residue was purified on silica gel column (petroleum ether/acetone, 8:1) to give 5.3 g (89%) of (2) as colorless powders. 1H NMR (300 MHz, DMSO-d6): δ/ppm = 11.93 (s, 1H), 8.94 (s, 1H), 8.43 (d, J = 6.0 Hz, 1H), 7.94 (d, J = 9.0 Hz, 2H), 7.70 (d, J = 9.0 Hz, 1H), 7.53 (m, 5H), 7.36 (m, 4H), 5.46 (s, 2H), 3.03 (m, 1H), 1.30 (d, J = 6.0 Hz, 1H); 13C NMR (75 MHz, DMSO-d6): δ/ppm = 165.89, 149.84, 142.81, 141.95, 137.13, 137.05, 135.68, 135.07, 129.49, 129.06, 128.97, 128.45, 127.20, 122.46, 121.62, 120.83, 117.06, 113.27, 66.48, 33.85, 24.29; ESI-MS (m/z): 421 [M + H]+.

Preparation of ICCA
A 0°C solution of 3.5 g (8.30 mmol) of (2) in 30 mL 1,4-dioxane was adjusted to pH of 12 by 15 mL aqueous NaOH (2M). This reaction mixture was stirred at 0°C for 24 hours, and the thin-layer chromatography (petroleum ether/acetone, 4:1) indicated the complete disappearance of (2). This reaction mixture was adjusted to pH of 2 by 5% aqueous KHSO4 to give 2.48 g (90%) of ICCA as light-yellow powders. Melt form light yellow precipitates. The precipitates were filtered from mixture was adjusted to pH of 2 by 5% aqueous KHSO4 and then washed with 10 mL of water and collected. The precipitates were then treated with 20 mL of water and the solution was evaporated to dryness under vacuum, at 25°C (1,000 g) for 10 minutes to prepare serum. To all wells, 20 μL matrix solution was added. To each standard well, 5 μL standard solution was added. To each testing well, 5 μL serum from healthy ICR mice, from S180 mice treated with CMCNa, or from S180 mice treated with ICCA (5 μmol/kg), or from healthy ICR mice. At 4°C, the blood was centrifuged (1,000 g) for 10 minutes to prepare serum. To all wells, 20 μL of sodium hydroxide solution (0.4 M) was added, and the plate was gently tapped for 15 minutes to ensure thorough mixing. The plate was read at 510 nm with microtiter plate reader to record OD value. By using the standard curve, serum concentration of ALT was calculated.

Measuring serum aspartate aminotransferase (AST)
Serum glutamic-oxaloacetic transaminase (GOT) was measured by using the procedure of the literature and AST/GPT assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, People’s Republic of China). To prepare serum, 0.5 mL blood was obtained from S180 mice treated with CMCNa, from S180 mice treated with ICCA (5 μmol/kg), or from healthy ICR mice. At 4°C, the blood was centrifuged (1,000 g) for 10 minutes to prepare serum. To all wells, 20 μL of sodium hydroxide solution (0.4 M) was added, and the plate was gently tapped for 15 minutes to ensure thorough mixing. The plate was read at 510 nm with microtiter plate reader to record OD value. By using the standard curve, serum concentration of ALT was calculated.

Antiproliferation in vitro assay
The in vitro viability assays of human myeloid leukemia cells (K562), ascites tumor cells (S180), human nonsmall cell lung cancer cells (A549), and nonsmall cell lung cancer cells (95D) were performed by using 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) procedure of the literature.22

Antitumor in vivo assay on S180 mice
The injection of doxorubicin (a known intercalator, 2 μmol/kg/day, positive control) in normal saline for 9 consecutive days, the oral carboxymethyl cellulose sodium (CMCNa; 0.5%, negative control) for 9 consecutive days, and the oral suspension of ICCA (5 μmol/kg/day) in CMCNa (0.5%) for 9 consecutive days were carried out on S180 mouse model (each 12) by using the procedure of the literature to perform the antitumor in vivo assay.25

Measuring serum alanine transaminase (ALT)
Serum glutamic-pyruvic transaminase (GPT) was measured by using the procedure of the literature and ALT/GPT assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, People’s Republic of China). To prepare serum, 0.5 mL blood was obtained from S180 mice treated with CMCNa, from S180 mice treated with ICCA (5 μmol/kg), or from healthy ICR mice. At 4°C, the blood was centrifuged (1,000 g) for 10 minutes to prepare serum. To all wells, 20 μL matrix solution was added. To each standard well, 5 μL standard solution was added. To each testing well, 5 μL serum from healthy ICR mice, from S180 mice treated with CMCNa, or from S180 mice treated with ICCA (5 μmol/kg) was added. The well was incubated at 37°C for 30 minutes. To all wells, 20 μL of dinitrophenylhydrazine was added. To each control well, 5 μL of distilled water was added, and the well was incubated at 37°C for 20 minutes. To each well, 200 μL of sodium hydroxide solution (0.4 M) was added, and the plate was gently tapped for 15 minutes to ensure thorough mixing. The plate was read at 510 nm with microtiter plate reader to record OD value. By using the standard curve, serum concentration of ALT was calculated.
through mixing. The plate was read at 510 nm with microtiter plate reader to record OD value. By using the standard curve, serum concentration of AST was calculated.

**Measuring serum creatinine (Cr)**

Serum Cr was measured by following the procedure of the literature and the kit (Nanjing Jiancheng Bioengineering Institute). To prepare serum, 0.5 mL blood was obtained from S180 mice treated with CMCNa, or from S180 mice treated with ICCA (5 μmol/kg), or from healthy ICR mice. At 4°C, the blood was centrifuged (1,000 g) for 10 minutes. To each standard well, 6 μL of the standard solution was added. To each control well, 6 μL of distilled water was added. To each testing well, 6 μL of serum from healthy ICR mice, from S180 mice treated with CMCNa, or from S180 mice treated with ICCA (5 μmol/kg) was added. To the well, 60 μL enzyme solution A was added. The well was incubated at 37°C for 5 minutes and was read at 546 nm by using a microtiter plate reader to record OD value (A₁). The well was incubated at 37°C for 30 minutes. To the well, 60 μL enzyme solution B was added. The well was incubated at 37°C for 5 minutes and read at 546 nm by using a microtiter plate reader to record OD value (A₂). The contents of Cr in samples were calculated by following the equation of the kit, which was shown in the Supplementary materials.

**Measuring serum blood urea nitrogen (BUN)**

The measurement of BUN was performed by following the procedure of the kit (Shanghai Lianshuo Biological Technology Co. Ltd., Shanghai, People’s Republic of China). To prepare serum, 0.5 mL blood was obtained from S180 mice treated with CMCNa, from S180 mice treated with ICCA (5 μmol/kg), or from healthy ICR mice. At 4°C, the blood was centrifuged (3,000 g) for 10 minutes. Then, 50 μL standard solution and 50 μL streptavidin–horseradish peroxidase (streptavidin–HRP) were added into each standard well, and 10 μL anti-BUN antibody and 50 μL streptavidin–HRP were successively added to each testing well having 40 μL serum from healthy ICR mice, from S180 mice treated with CMCNa, or from S180 mice treated with ICCA (5 μmol/kg). At 37°C, the well was incubated for 60 minutes and then washed five times. For coloration 50 μL chromogen solution A and 50 μL chromogen solution B (from the kit) were added to the well, and the well was incubated at 37°C in dark for 15 minutes. To stop the reaction, the well received 50 μL stop solution (from the kit). The OD value was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of BUN was calculated.

**Mouse and rat arterial thrombus weight assays**

Arterial thrombus weight assay was performed on male ICR mouse (22–25 g, each 12) model or on male Sprague Dawley rat (200–220 g, each 12) model by following the procedure of the literature. In mouse arterial thrombus weight assay, 0.5% CMCNa (10 mL/kg, negative control), a suspension of aspirin in 0.5% CMCNa (240 μmol/kg, positive control), or a suspension of ICCA in 0.5% CMCNa (5 μmol/kg) was given orally. In rat arterial thrombus weight assay, 0.5% CMCNa (3 mL/kg), a suspension of aspirin in 0.5% CMCNa (167 μmol/kg), or a suspension of ICCA in 0.5% CMCNa (5 μmol/kg) was given orally.

**P-selectin and GPIIb/IIIa expression assays**

P-selectin expression assay was based on the experiment of an enzyme-linked immunosorbent assay (ELISA), by using the kit (rat P-selectin ELISA kit; Wuhan Huamei Biotech Co., Ltd., Wuhan, Hubei Province, People’s Republic of China) and by following the procedure of the literature. GPIIb/IIIa expression assay was based on the experiment of the ELISA by using the kit (rat GPIIb/IIIa ELISA kit; Wuhan Huamei Biotech Co., Ltd.) and by following the procedure of the literature. In both assays, the serum was similarly obtained from male rats treated with 5 μmol/kg of ICCA, 167 μmol/kg of aspirin (positive control), or 0.5% CMCNa (negative control).

**Rat tail bleeding time assay**

This assay was performed by following the procedure of the literature. In this assay, the male Sprague Dawley rats (200–220 g) were orally treated with 0.5% CMCNa (3 mL/kg, negative control, 12 rats), suspension of aspirin in 0.5% CMCNa (167 μmol/kg, positive control, 12 rats), or suspension of ICCA in 0.5% CMCNa (5 μmol/kg, 12 rats).

**Rat venous thrombus weight assay**

This assay was performed by following the procedure of the literature. In this assay, male Sprague Dawley rats (250–300 g) were orally treated with 0.5% CMCNa (3 mL/kg, negative control, 12 rats), a suspension of warfarin in 0.5% CMCNa (4.87 μmol/kg, positive control, 12 rats), or a suspension of ICCA in 0.5% CMCNa (5 μmol/kg, 12 rats).

**Measuring serum prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), and FIB**

To measure serum PT, TT, APTT, and FIB, the blood of male Sprague Dawley rats (250–300 g) orally treated with 0.5% CMCNa (3 mL/kg, negative control, 12 rats) or a
Coagulation factor VII (FVII) expression assay

FVII expression assay was based on the experiment of ELISA by using kit (rat FVII ELISA kit; Shanghai Lianshuo Biological Technology Co. Ltd.). In this assay, the blood of male Sprague Dawley rats (250–300 g) orally treated with ICCA (5 μmol/kg, 12 rats) or 0.5% CMCNa (negative control, 12 rats) was collected by using standard procedure (ie, the blood been collected into a tube containing 3.8% sodium citrate at a ratio of 9:1 and centrifuged at 4°C and 3,000 rpm/min for 10 minutes) to prepare serum. The serum was received at 10-fold dilution with the diluents of the kit and incubated at 37°C for 3 minutes to prepare blank and test serum. To control well and test well, 100 μL of blank serum and 100 μL of test serum were added, respectively. At 37°C, the well was incubated for 120 minutes. After removing solvent to the well, 100 μL of biotin labeling antibody of the kit was added, and the well was incubated at 37°C for 60 minutes. The solution in the well was discarded, and the washing solution of the kit was added to wash the well (200 μL × 3). Then, 100 μL of HRP labeling avidin of the kit was added, and the well was incubated at 37°C for 60 minutes and then washed five times. For coloring, 90 μL of the substrate of the kit was added to the well, and the well was incubated at 37°C in dark for 30 minutes. To stop coloring, 50 μL stop solution of the kit was added. The OD value of the well was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of FVII was calculated.

Coagulation factor IX (FIX) expression assay

FIX expression assay was based on the experiment of ELISA by following the procedure of the kit (rat FIX ELISA kit; Shanghai Lianshuo Biological Technology Co. Ltd.). In this assay, the blood of male Sprague Dawley rats (250–300 g) orally treated with ICCA (5 μmol/kg, 12 rats) or 0.5% CMCNa (negative control, 12 rats) was collected by using standard procedure (ie, the blood been collected into a tube containing 3.8% sodium citrate at a ratio of 9:1 and centrifuged at 4°C and 3,000 rpm/min for 10 minutes) to prepare serum. Then, 50 μL standard solutions and 50 μL streptavidin–HRP of the kit were added into standard well; 10 μL anti-FIX-antibody and 50 μL streptavidin–HRP were successively added into the testing well having 40 μL serum from CMCNa- or ICCA (5 μmol/kg)-treated rats. The well was incubated at 37°C for 60 minutes and washed five times. For coloring, 50 μL chromogen solution A and 50 μL chromogen solution B of the kit were added into the well, and the well was incubated at 37°C in dark for 15 minutes. To stop coloring, 50 μL stop solutions of the kit were added. The OD value was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of FIX was calculated.

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Coagulation factor II (FII) expression assay

FII expression assay was based on the experiment of ELISA by using kit (rat FII ELISA kit; Shanghai Lianshuo Biological Technology Co. Ltd.). In this assay, the blood from male Sprague Dawley rats (250–300 g) orally treated with ICCA (5 μmol/kg, 12 rats) or 0.5% CMCNa (negative control, 12 rats) was collected by using standard procedure (ie, the blood been collected into a tube containing 3.8% sodium citrate at a ratio of 9:1 and centrifuged at 4°C and 3,000 rpm/min for 10 minutes) for preparing serum; 50 μL standard solutions and 50 μL streptavidin–HRP were added into the standard well, and 10 μL anti-FII-antibody and 50 μL streptavidin–HRP were successively added into the testing well having 40 μL serum from CMCNa- or ICCA (5 μmol/kg)-treated rats. The well was incubated at 37°C for 60 minutes and washed five times. For coloring, 50 μL chromogen solution A and 50 μL chromogen solution B of the kit were added into the well, and the well was incubated at 37°C in dark for 15 minutes. To stop coloring, 50 μL stop solutions of the kit were added. The OD value was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of FII was calculated.

Vitamin K1 (VK1) expression assay

VK1 expression assay was based on the experiment of the ELISA by using the kit (rat VK1 ELISA kit; Shanghai Lianshuo Biological Technology Co. Ltd.). In this assay, the blood of male Sprague Dawley rats (250–300 g) orally treated with ICCA (5 μmol/kg, 12 rats) or 0.5% CMCNa (negative control, 12 rats) was used to prepare serum by following the standard procedure. To standard well, 50 μL standard solutions and 50 μL streptavidin–HRP were added. To testing well having 40 μL of the serum, 10 μL of anti-VK1-antibody and 50 μL of streptavidin–HRP were successively added. At 37°C, the well was incubated for 60 minutes and washed five times. For coloring, 50 μL chromogen solution A and 50 μL chromogen solution B (from the kit) were added to the well, and then, it was incubated at 37°C in dark for 15 minutes. To stop the reaction, the well received 50 μL stop solution (from the kit). The OD value was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of VK1 was calculated.

 suspension of ICCA in 0.5% CMCNa (5 μmol/kg, 12 rats) was used. Serum PT, TT, APTT, and FIB were measured by semi-automatic blood coagulation instrument (Virtues Pacific Biological Polytron Technologies Inc., Tianjin, People's Republic of China).

Coagulation factor II (FII) expression assay

FII expression assay was based on the experiment of ELISA by using kit (rat FII ELISA kit; Shanghai Lianshuo Biological Technology Co. Ltd.). In this assay, the blood from male Sprague Dawley rats (250–300 g) orally treated with ICCA (5 μmol/kg, 12 rats) or 0.5% CMCNa (negative control, 12 rats) was used to prepare serum by following the standard procedure. To standard well, 50 μL standard solutions and 50 μL streptavidin–HRP were added. To testing well having 40 μL of the serum, 10 μL of anti-FII-antibody and 50 μL of streptavidin–HRP were successively added into the testing well having 40 μL serum from CMCNa- or ICCA (5 μmol/kg)-treated rats. The well was incubated at 37°C for 60 minutes and washed five times. For coloring, 50 μL chromogen solution A and 50 μL chromogen solution B of the kit were added into the well, and the well was incubated at 37°C in dark for 15 minutes. To stop coloring, 50 μL stop solutions of the kit were added. The OD value was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of FII was calculated.
Kit was added into the well. The OD value of the well was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of FIX was calculated.

**Coagulation factor X (FX) expression assay**

FX expression assay was based on the experiment of ELISA and by using the procedure of the kit (rat FX ELISA kit; Shanghai Lianshuo Biological Technology Co. Ltd.). In this assay, the blood of male Sprague Dawley rats (250–300 g) orally treated with ICCA (5 μmol/kg, 12 rats) or 0.5% CMCNa (negative control, 12 rats) was collected by using standard procedure (ie, the blood been collected into a tube containing 3.8% sodium citrate at a ratio of 9:1 and centrifuged at 4°C and 3,000 rpm/min for 10 minutes) to prepare serum. Then, 50 μL standard solutions and 50 μL streptavidin–HRP of the kit were added into standard well, and 10 μL anti-FX-antibody and 50 μL streptavidin–HRP were successively added into testing well having 40 μL serum from CMCNa- or ICCA (5 μmol/kg)-treated rats. The well was incubated at 37°C for 60 minutes and then washed five times. For coloration, 50 μL chromogen solution A and 50 μL chromogen solution B of the kit were added into the well, and the well was incubated 37°C in dark for 15 minutes. To stop coloration, 50 μL stop solution (from the kit) was added into the well. The OD value of the well was measured at 450 nm within 15 minutes of the addition of the stop solution. By using standard curve, serum concentration of FX was calculated.

**Docking toward active sites of P-selectin and GPIIb/IIIa**

To perform docking investigation, the conformation of β-carboxylic acids and their benzyl esters was generated by following the literature. According to the literature, 10 energy-optimized conformations of each β-carboxylic acid and benzyl ester were docked into the active sites of P-selectin and GPIIb/IIIa with CDOCKER protocol and using the procedure of the literature.

**Results and discussion**

ICCA was assigned as a dual inhibitor of P-selectin and GPIIb/IIIa receptors

To design a candidate capable of simultaneously downregulating P-selectin and GPIIb/IIIa receptors, the pharmacophores of 10 carbolines in the red box of Figure 1, of which the activities related to either P-selectin receptor or GPIIb/IIIa receptor were previously reported, were analyzed. The analysis of the pharmacophores marked with “oval a” led to a common pharmacophore, ie, β-carboline-3-carboxylic acid and its benzyl ester marked with “circle A.” The analysis of the 1-substituents marked with “square b” led to a common 1-substituent, ie, 4-isopropylphenyl marked with “square B.” Thus, the pharmacophore analysis and the 1-substituent analysis together led to the design of ICCA and its benzyl ester in the green box C of Figure 1 as the promising candidate capable of simultaneously downregulating P-selectin and GPIIb/IIIa receptors.

To rationally design ICCA and its benzyl ester, 10 β-carboxylic acids and their benzyl esters of the literature were docked into the active sites of both P-selectin and GPIIb/IIIa receptors by using CDOCKER protocol. Figure 1 shows that for P-selectin receptor the CDOCKER interaction energies fall within a range between −11.2 and −33.3 kcal/mol, and ICCA has the lowest CDOCKER interaction energy. Figure 1 also shows that for GPIIb/IIIa receptor the CDOCKER interaction energies fall within a range of −11.5 and −20.2 kcal/mol, and again ICCA has the lowest CDOCKER interaction energy.

To explain the possible reason of ICCA having the lowest energy, the docking features of ICCA in the active sites of P-selectin and GPIIb/IIIa are shown. Figure 1 emphasizes that ICCA, but not the others, can act on four important amino acid residues (Asn44, Glu80, Asn105, and Tyr48) of the active site of P-selectin and on five important amino acid residues (Ser101, Phe100, Asn99, Lys98, and Ala61) of the active site of GPIIb/IIIa.

To make the present CDOCKER interaction energies of 12 β-carboxylic acids and their benzyl esters been easy-to-read, Table 1 was added. The data emphasize that for P-selectin receptor the energies fall within a range of −11.2 and −33.3 kcal/mol, and ICCA has the lowest CDOCKER interaction energy. The data also emphasize that for GPIIb/IIIa receptor the CDOCKER interaction energies fall within a range of −11.5 and −20.2 kcal/mol, and again ICCA has the lowest CDOCKER interaction energy.

**Route suitable for the preparation of ICCA**

To process the investigations, ICCA was synthesized by following the route depicted in Scheme 1. Briefly, the Pictet–Spengler condensation of L-Trp-OBzl and cuminic aldehyde provided (1R, 3S)-1-(4-isopropyl-phenyl)-2,3,4,9-tetrahydro-β-carboline-3-carboxylic acid benzyl esters (1) in 60% yield. In the presence of SeO₂, it was converted to ICCA benzyl ester (2) in 89% yield. At 0°C and in aqueous NaOH (2M), the hydrolysis of (2) was performed, and ICCA was provided in 90% yield and 99.6% HPLC purity.
The proper reaction condition, the desirable yields, and the high purity emphasize that the synthetic route is suitable for the preparation of ICCA.

**ICCA effectively inhibits arterial thrombosis in vivo**

To evidence the rationality of the design, the arterial thrombotic assays of ICCA were performed on both mouse and rat models.\textsuperscript{19,21} Figure 2A shows that the arterial thrombus weights of the mice orally treated with 5 μmol/kg of ICCA are significantly lower than those of the mice orally treated with CMCNa and 24 μmol/kg of aspirin. This means that 5 μmol/kg oral ICCA effectively inhibits the mice to form arterial thrombus. The dose of aspirin is 4.8 folds of ICCA; this also means that the antithrombosis activity of ICCA is 4.8 folds higher than that of aspirin.

**Figure 1** Structural analysis and docking investigation led to ICCA been assigned as an inhibitor of both P-selectin and GPIIb/IIa receptors.

Abbreviations: GPIIb/IIa, platelet membrane glycoprotein IIb/IIa; ICCA, 1-(4-isopropylphenyl)-β-carboline-3-carboxylic acid.
Figure 2B shows that the arterial thrombus weights of the rats orally treated with 5 $\mu$mol/kg of ICCA are significantly lower than those of the rats orally treated with CMCNa and 16.7 $\mu$mol/kg of aspirin. This means that 5 $\mu$mol/kg oral ICCA effectively inhibits the rats to form arterial thrombus. The dose of aspirin is 3.3 folds of ICCA; this also means that the antithrombosis activity of ICCA is 3.3 folds higher than that of aspirin.

Table 1  CDOCKER interaction energies and activities of $\beta$-carboxylic acids and benzyl esters

| Carbone | Energy (kcal/mol) for | Carbone | Energy (kcal/mol) for |
|---------|----------------------|---------|----------------------|
|         | GPIIb/IIIa            |         | GPIIb/IIIa            |
|         | P-selectin            |         | P-selectin            |
|         | -12.9                 | -13.9   | -13.1                 |
|         | -18.3                 | -18.5   | -18.1                 |
|         | -11.5                 | -12.1   | -16.6                 |
|         | -14.1                 | -11.2   | -20.1                 |
|         | -12.8                 | -12.3   | -13.1                 |
|         | -15.9                 | -17.2   | -20.2                 |

Abbreviation: GP, glycoprotein.

Scheme 1 Synthetic route of ICCA: (i) TFA, CH$_2$Cl$_2$; (ii) SeO$_2$, 1,4-dioxane and 70°C; (iii) aqueous NaOH (2M), 1,4-dioxane and 0°C.

Abbreviations: CH$_2$Cl$_2$, dichloromethane; ICCA, 1-(4-isopropylphenyl)-$\beta$-carboline-3-carboxylic acid; SeO$_2$, selenium dioxide; TFA, trifluoroacetic acid; NaOH, sodium hydroxide.
ICCA as a dual inhibitor of GPIIb/IIIa and P-selectin receptors

**ICCA downregulates GPIIb/IIIa and P-selectin expression in vivo**

To evidence the rationality of the design, the in vivo expression of GPIIb/IIIa and P-selectin was examined by following the reported methods. Serum GPIIb/IIIa and P-selectin of the rats treated with 5 μmol/kg ICCA are significantly lower than those of the rats treated with CMCNa and are equal to those of the rats treated with 167 μmol/kg aspirin, respectively (Figure 2C and D). Thus, by downregulating GPIIb/IIIa and P-selectin expression, ICCA effectively inhibits arterial thrombus of rats. Besides, the efficacy of ICCA downregulating the expression of GPIIb/IIIa and P-selectin is 33 folds higher than that of aspirin.

**ICCA has no bleeding risk**

To estimate whether ICCA has a bleeding risk, the tail bleeding time assay was performed by following the reported method. It was found that 5 μmol/kg oral ICCA, but not 167 μmol/kg oral aspirin, did not prolong tail bleeding time of the treated rats (Figure 2E). Therefore, ICCA, but not aspirin, has no bleeding risk.

**ICCA effectively inhibits venous thrombosis in vivo**

To evidence the rationality of the design, the venous thrombotic assay of ICCA was performed on rat model. It was found that the venous thrombus weight of the rats orally treated with 5 μmol/kg ICCA was significantly lower than that of the rats orally treated with CMCNa and equal to that of the rats orally treated with 4.87 μmol/kg warfarin (Figure 3A). This means that 5 μmol/kg of oral ICCA effectively inhibits venous thrombosis in vivo. Besides, the efficacy of 5 μmol/kg of ICCA is equal to that of 4.87 μmol/kg of warfarin.

**ICCA is not warfarin- and heparin-like anticoagulant**

To ensure ICCA been downregulator of GPIIb/IIIa and P-selectin receptors, but not a warfarin- and heparin-like anticoagulant, the PT, TT, APTT, FIB, VK1, FII, FVII, FIX, and FX of venous thrombosis rats treated with 5 μmol/kg ICCA were measured by using the reported methods. Figure 3B and C indicates that 5 μmol/kg of ICCA does not alter serum levels of PT, TT, APTT, FIB, VK1, FII, FVII, FIX, and FX.

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**Figure 2** Efficacy of ICCA in the arterial thrombotic assays, n=12: (A) At 5 μmol/kg of oral dose, ICCA effectively inhibits the mice to form arterial thrombus; (B) at 5 μmol/kg of oral dose, ICCA effectively inhibits the rats to form arterial thrombus; (C) 5 μmol/kg ICCA decreases GPIIb/IIIa in arterial serum of the treated thrombosis rats; (D) 5 μmol/kg ICCA decreases P-selectin in arterial serum of the treated thrombosis rats; (E) 5 μmol/kg ICCA does not prolong the tail bleeding time of the treated thrombosis rats.

**Abbreviations:** CMCNa, carboxymethyl cellulose sodium; GPIIb/IIIa, platelet membrane glycoprotein IIb/IIIa; ICCA, 1-(4-isopropylphenyl)-β-carboline-3-carboxylic acid.
Figure 3 Efficacy of ICCA in venous thrombotic assay: (A) venous thrombus weight of rats orally treated with 5 μmol/kg ICCA, n = 12; (B) PT, TT, APTT, and FIB of the venous thrombosis rats treated with 5 μmol/kg ICCA, n = 12; (C) serum VKI, FII, FVII, FIX, and FX of venous thrombosis rats treated with 5 μmol/kg ICCA; n = 12.

Abbreviations: APTT, activated partial thromboplastin time; CMCNa, carboxymethyl cellulose sodium; FII, coagulation factor II; FVII, coagulation factor VII; FIX, coagulation factor IX; FX, coagulation factor X; FIB, plasma fibrinogen; ICCA, 1-(4-isopropylphenyl)-β-carboline-3-carboxylic acid; PT, prothrombin time; TT, thrombin time; VK1, vitamin K1.

of the treated rats, ie, the levels fall within the normal range. ICCA is a downregulator of GPIIb/IIIa and P-selectin receptors, but not warfarin- and heparin-like anticoagulants.

ICCA has no in vitro cytotoxicity
To examine the cytotoxicity of ICCA, the proliferation assays were performed on A549, 95D, K562, and S180 cell models by using MTT method. Figure 4A shows that the viabilities of 100 μM of ICCA-treated A549, 95D, K562, and S180 cells are <50%. The IC₅₀ values of ICCA against the proliferation of the cancer cells are >100 μM, ie, ICCA has no cytotoxicity for cancer cells. Therefore, ICCA should not be considered a cytotoxic agent.

ICCA effectively slows tumor growth in vivo
To evidence the rationality of the design, the tumor growth assay of ICCA was performed on S180 mouse model. Figure 4B shows that the tumor weight of S180 mice orally treated with 5 μmol/kg/day of ICCA for 9 consecutive days is significantly lower than that of S180 mice orally treated with CMCNa. Considering that the IC₅₀ of ICCA inhibiting S180 cell proliferation is >100 μM and ICCA is not a cytotoxic agent (Figure 4A), the in vivo efficacy of ICCA slowing tumor growth could be correlated with the in vivo downregulating GPIIb/IIIa and P-selectin expression.

To further evidence the correlation of ICCA slowing down tumor growth with the downregulation of GPIIb/IIIa and P-selectin expression, but not the inhibition of cancer cell proliferation, the existence of ICCA in the tumor tissue of S180 mice orally treated with CMCNa and 5 μmol/kg/day ICCA for 9 consecutive days was examined by ESI(−)-FT-MS qualitatively. However, ESI(−)-FT-MS spectra gave no peak related to ICCA. This ensures that the in vivo antitumor action of ICCA is independent on the direct cytotoxic activity and supports the impact of downregulating GPIIb/IIIa and P-selectin expression on tumor growth.

ICCA has no liver and renal toxicity
The serum ALT, AST, Cr, and BUN are commonly used to estimate the toxicity of the drug to kidney and liver. In this regard, the serum ALT, AST, Cr, and BUN of the S180 mice treated with 5 μmol/kg/day of ICCA for 9 consecutive days were measured by using the corresponding kits. Figure 4C–F shows that after receiving ICCA, the serum ALT, AST, Cr, and BUN of the S180 mice are equal to those of the healthy mice. Therefore, the therapy of 5 μmol/kg of ICCA does not injure the kidney and the liver.

Comparing the activities of 9 carbolines
To get insight into the dependence of bioactivity on the structure of β-carboline, the antiproliferation, antitumor, and antithrombosis activities of ICCA and carbolines were compared.
Figure 4 Antiproliferation activity in vitro, antitumor activity in vivo, and toxicity of ICCA: (A) cell viabilities of ICCA-treated A549, 95D, K562, and S180 cells, n=6; (B) tumor weight of S180 mice orally treated with 5 μmol/kg/day ICCA for 9 consecutive days, n=12; (C) Cr of S180 mice orally receiving 5 μmol/kg/day ICCA for 9 consecutive days, n=6; (D) ALT of S180 mice orally receiving 5 μmol/kg/day ICCA for 9 consecutive days, n=6; (E) AST of S180 mice orally receiving 5 μmol/kg/day ICCA for 9 consecutive days, n=6; (F) BUN of S180 mice orally receiving 5 μmol/kg/day ICCA for 9 consecutive days, n=6.

Abbreviations: 95D, nonsmall cell lung cancer cell line; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; CMCNa, carboxymethyl cellulose sodium; Cr, creatinine; DOX, doxorubicin; ICCA, 1-(4-isopropylphenyl)-β-carboline-3-carboxylic acid; K562, human myeloid leukemia; S180, ascites tumor cells; Kar, Karmen.

Table 2 shows that the IC_{50} values of ICCA inhibiting the proliferation of HeLa, MCF-7, HepG2, and K562 cells are >100 μM. This is in consistent with the finding that ICCA is a downregulator of P-selectin and GPIIb/IIIa receptors, but not a cytotoxic agent. Similar IC_{50} values are also given by 1-methyl-β-carboline-3-carboxylic acid, but it was reported as an intercalator.

Table 3 indicates that the tumor weight of 5 μmol/kg/day of ICCA-treated S180 mice is significantly higher than that of 0.1 μmol/kg/day of

| Carbolinc | IC_{50} inhibiting the proliferation of the following cells | References |
|----------|----------------------------------------------------------|------------|
| HeLa     | MCF-7 | HepG2 | K562 |
| ICCA     | >100 μM | >100 μM | >100 μM | >100 μM | – |
| Ala-OBzl | 19 μM | 30 μM | 49 μM | Not available | 22 |

(Continued)
Table 2 (Continued)

| Carboline | IC_{50} inhibiting the proliferation of the following cells | References |
|-----------|----------------------------------------------------------|------------|
|           | HeLa | MCF-7 | HepG2 | K562 |
| CMCNa     | 5  | 66 μM | 99 μM | 52 μM | Not available |
| ICCA      | 0.1 | 26 μM | Not available | 29 μM | 21 μM |
| NS        | 8.9 | 1.62±0.28 | 1.07±0.59 | 25 |
| NS        | 89  | 1.65±0.49 | 0.76±0.28 | 26 |

**Table 3** Tumor weights of ICCA and three carbolines-treated S180 mice

| Carboline | Dose (μmol/kg) | Tumor weight (mean ± SD g) | References |
|-----------|----------------|-----------------------------|------------|
| CMCNa     | –              | 2.17±0.31                   | –          |
| ICCA      | 5              | 1.54±0.49                   | –          |
| NS        | 0.1            | 0.68±0.21                   | 24         |
| NS        | 8.9            | 1.07±0.59                   | 25         |
| NS        | 89             | 0.76±0.28                   | 26         |

**Table 4** shows that only 5 μmol/kg of ICCA can simultaneously inhibit the formation of arterial and venous thrombus. Due to deep venous embolism and arterial embolism being responsible for elevated mortality worldwide, the therapy efficacies support the clinical importance of the design and the development of ICCA as the downregulator of P-selectin and GPIIb/IIIa receptors.

**Conclusion**

The analysis of structural characteristic and CDOCKER interaction energy of antiarterial thrombotic carboline, antivenous thrombotic carboline, and antitumor carboline leads to the successful design of ICCA. The synthetic route is able to provide ICCA in high yield and high purity. The in vivo bioassay ensures that oral ICCA effectively inhibits arterial thrombosis, venous thrombosis, and tumor growth, but does not injure the kidney and the liver. All data emphasized that ICCA is a promising candidate of the downregulator of GPIIb/IIIa and P-selectin receptors and thereby is worthy of considering for development.
Table 4 Thrombus weights ICCA and four carbolines-treated thrombosis rats

| Compound            | Dose (μmol/kg) | Thrombus weight (mean ± SD mg) | References |
|---------------------|----------------|-------------------------------|------------|
|                     |                | Arterial                      | Venous     |
| CMCNα               | –              | 41.64±8.00                    | 22.4±4.4   | –          |
|                     | 5              | 33.49±6.25                    | 11.2±3.5   | –          |
| NS                  | –              | 38.67±3.26                    | Not available | 19        |
|                     | 5              | 29.69±3.22                    | Not available | 19        |
| NS                  | –              | 37.86±2.94                    | Not available | 20        |
|                     | 1              | 27.69±3.55                    | Not available | 20        |
| NS                  | –              | 31.37±3.79                    | Not available | 20        |
|                     | 10             | 26.66±1.19                    | Not available | 23        |

Abbreviations: CMCNα, carboxymethyl cellulose sodium; ICCA, 1-(4-isopropyl-phenyl)-β-carboline-3-carboxylic acid; NS, normal saline.

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The authors report no conflicts of interest in this work.

References
1. Moretti A, Ferrari F, Villa RF. Pharmacological therapy of acute ischaemic stroke: achievements and problems. *Pharmacol Ther*. 2015;153:79–89.
2. Granger DN, Kvietys PR. Reperfusion therapy – what’s with the obstructed, leaky and broken capillaries. *Pathophysiology*. 2017;24:213–228.
3. Bonechi C, Lamponi S, Donati A, et al. Effect of resveratrol on platelet aggregation by fibrinogen protection. *Biophys Chem*. 2017;222:41–48.
4. van der Spuy WJ, Augustine TN. Ultrastructural investigation of the time-dependent relationship between breast cancer cells and thrombosis induction. *Micron*. 2016;90:59–63.
5. Castaman G. Risk of thrombosis in cancer and the role of supportive care (trans-fusion, catheters, and growth factors). *Thromb Res*. 2016;140: S89–S92.
6. Nasti TH, Bullard DC, Yusuf N. P-selectin enhances growth and metastasis of mouse mammary tumors by promoting regulatory T cell infiltration into the tumors. *Life Sci*. 2015;131:11–18.
7. Amano H, Ito Y, Ogawa F, et al. Angiotensin II type 1A receptor signaling facilitates tumor metastasis formation through P-selectin-mediated interaction of tumor cells with platelets and endothelial cells. *Am J Pathol*. 2013;182:553–564.
8. Pusch G, Debrabant B, Molnar T, et al. Early dynamics of P-selectin and interleukin 6 predicts outcomes in ischemic stroke. *J Stroke Cerebrovasc Dis*. 2015;24:1938–1947.
9. Wassel CL, Berardi C, Pankow JS, et al. Soluble P-selectin predicts lower extremity peripheral artery disease incidence and change in the ankle brachial index: the Multi-Ethnic Study of Atherosclerosis (MESA). *Atherosclerosis*. 2015;239:405–411.
10. Alfonso F, Angiolillo DJ. Targeting P-selectin during coronary interventions: the elusive link between inflammation and platelets to prevent myocardial damage. *J Am Coll Cardiol*. 2013;61:2056–2059.
11. Juenet M, Aid-Launais R, Li B, et al. Thrombolytic therapy based on fucoidan-functionalized polymer nanoparticles targeting P-selectin. *Biomaterials*. 2018;156:204–216.

12. Khan R, Spagnoli V, Tardif JC, L’Allier PL. Novel anti-inflammatory therapies for the treatment of atherosclerosis. *Atherosclerosis*. 2015;240:497–509.

13. Xu W, Zhao M, Wang Y, et al. Design, synthesis, and in vivo evaluation of benzyl N²-nitro-N²-(9H-pyrindo[3,4-b]indole-3-carbonyl)-L-arginine as an apoptosis inducer capable of decreasing the serum concentration of P-selectin. *Med Chem Commun*. 2016;7:1730–1737.

14. Chen H, Wang W, Zhang X, et al. Discovery of DEBIC to correlate P-selectin inhibition and DNA intercalation in cancer therapy and complicated thrombosis. *Oncotarget*. 2017.

15. Kanic V, Vollrath M, Penko M, Markota G, Kanic Z. GPIIb-IIIa receptor inhibitors in acute coronary syndrome patients presenting with cardiogenic shock and/or after cardiopulmonary resuscitation. *Heart Lung Circ*. 2018;27:73–78.

16. Emani S, Kaza AK, Almodovar M, Thiagarajan R, Emani SM. Intravenous GPIIb/IIIa inhibitor for secondary prevention of shunt thrombosis in a pediatric patient. *Ann Thorac Surg*. 2015;99:e151–e153.

17. Yan M, Jurasz P. The role of platelets in the tumor microenvironment: from solid tumors to leukemia. *Biochim Biophys Acta*. 2016;1863:392–400.

18. Goubran HA, Burnouf T, Radojevic M, El-Ekiaby M. The platelet-cancer loop. *Eur J Inter Med*. 2013;24:393–400.

19. Zhao M, Li B, Li W, et al. Synthesis of new class dipeptide analogues with improved permeability and anti thrombotic activity. *Bioorg Med Chem*. 2006;14:4761–4774.

20. Wang X, Wang Y, Wu J, et al. Docking based design of diastereoisomeric MTCA as GPIIb/IIIa receptor inhibitor. *Bioorg Med Chem Lett*. 2017;27:5114–5118.

21. Zhu H, Wang Y, Song C, et al. Docking of THPDTPI: to explore P-selectin as a common target of anti-tumor, anti-thrombotic and anti-inflammatory agent. *Oncotarget*. 2017;9:268–281.

22. Zhao M, Bi L, Wang W, et al. Synthesis and cytotoxic activities of β-carboline amino acid ester conjugates. *Bioorg Med Chem*. 2006;14:6998–7010.

23. Li C, Zhang X, Zhao M, et al. A class of novel N-(1-methyl-β-carboline-3-carbonyl)-N′-(aminoacid-acetyl)-hydrazines: aromatization leded design, synthesis, in vitro anti-platelet aggregation/in vivo anti-thrombotic evaluation and 3D QSAR analysis. *Eur J Med Chem*. 2011;46:5598–5608.

24. Li S, Wang Y, Zhao M, Wu J, Peng S. BPIC: a novel anti-tumor lead capable of inhibiting inflammation and scavenging free radicals. *Bioorg Med Chem Lett*. 2015;25:1146–1150.

25. Wu J, Li C, Zhao M, Wang W, Wang Y, Peng S. A class of novel carboline intercalators: their synthesis, in vitro anti-proliferation, in vivo anti-tumor action, and 3D QSAR analysis. *Bioorg Med Chem*. 2010;18(17):6220–6229.

26. Wu J, Zhao M, Qian K, Lee KH, Morris-Natschke S, Peng S. Novel N-(3-carboxyl-9-benzyl-beta-carboline-1-yl)ethylamino acids: synthesis, anti-tumor evaluation, intercalating determination, 3D QSAR analysis and docking investigation. *Eur J Med Chem*. 2009;44:4153–4161.

27. Jin S, Wang Y, Zhu H, et al. Nanosized aspirin-Arg-Gly-Asp-Val: delivery of aspirin to thrombus by the target carrier Arg-Gly-Asp-Val tetrapeptide. *ACS Nano*. 2013;7:7664–7673.

28. Gadi D, Brouham M, Aziz M, et al. Parsley extract inhibits in vitro and ex vivo platelet aggregation and prolongs bleeding time in rats. *J Ethnopharmacol*. 2009;125:170–174.

29. Kluft C, van Leuven CJ. Consequences for the APTT due to direct action of factor XIa on factor X, resulting in bypassing factors VIII–IX. *Thromb Res*. 2015;135:198–204.

30. Hu X, Zhao M, Wang Y, et al. Tetrahydro-β-carboline-3-carboxyl-thymopentin: a nano-conjugate for releasing pharmacophores to treat tumor and complications. *J Mater Chem B*. 2016;4:1384–1397.