Supporting Information

Design, synthesis, *in vivo* evaluations of benzyl N^{ω}-nitro-N^{α}-(9H-pyrido[3,4-b]indole-3-carbonyl)-L-argininate as apoptosis inducer capable of decreasing serum concentration of P-selectin

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Experimental Section

1. General

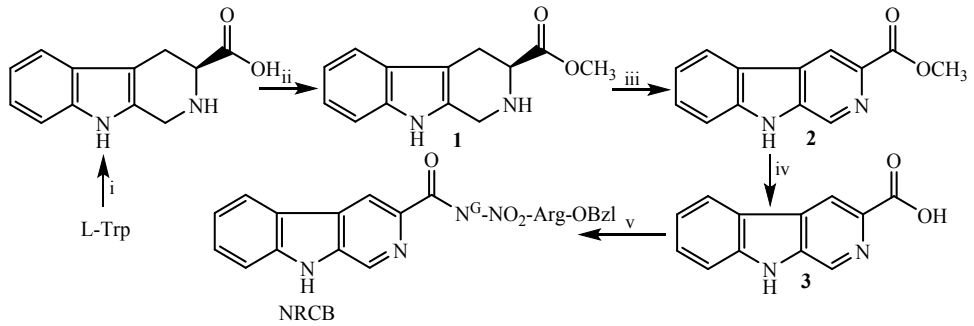
Sprague Dawley rats and ICR mice were purchased from the Animal Center of Peking University. Work performed was based on a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures that the welfare of the animals was maintained in accordance with the requirements of the Animal Welfare Act. Statistical analyses of all the biological data were carried out by use of analysis of variance. *P*-values<0.05 were considered statistically significant.

The protective amino acids (*L*-configuration) and sodium citrate (analytically pure) were available commercially (Sigma-Aldrich Corp, St Louis MO, USA). Column chromatography was performed on silica gel of 200-300 mesh. Anhydrous solvents were dried and purified by standard methods prior to use. The purity of intermediates and products was measured by using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC, C18 4.6×150 mm; Kromasi, AkzoNobel, Bohus, Sweden), and was higher than 95%. Reactions were monitored by TLC on glass plates coated with silica gel with a fluorescent indicator. Melting points (Mp) were determined on a XT5 hot stage apparatus (Beijing Keyi Electro-Optic Factory, Beijing, PR China) and uncorrected. Proton nuclear magnetic resonance (^{1}HNMR) spectra of 8 mg of NRCB were recorded on a Bruker 800 MHz spectrometer with in 0.5 mL of deuteron dimethyl sulfoxide (DMSO-d_6) mixed with tetramethylsilane (TMS). The probe temperature was 298 K, and the spectra were recorded by using a simple pulse-acquire sequence zg30. Digital zero filling to 64 K and a 0.3 Hz exponential function were applied to the FID before Fourier transformation. Typical acquisition parameters consisted of 64 K points covering a sweep width of 16447 Hz, a pulse width (pw90) of 8.63 μs, and a total repetition time of 24 s to ensure full relaxation of the ^{1}H resonances.

Electrospray ionization mass spectrometry (ESI-MS), was measured on a ZQ 2000 (Waters Corp, US) and a 9.4 T solariX Fourier transform (FT) ion cyclotronresonance (ICR), FT-ICR, mass spectrometer (Bruker Corp, Billerica, MA, USA), with an

ESI/matrix-assisted laser desorption/ionization (MALDI) dual ion source.

2. Synthesis of NRCB



Scheme 1 Synthetic route of NRCB.

i) CH_2O, H_2O and concentrated sulfuric acid; ii) CH_3OH and $SOCl_2$; iii) Acetone and KM nO_4; iv) Methanol (MeOH) aqueous NaOH (4 M); v) L-Arg(NO_2)-OBzl, N-methylmorpholi ne, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-hydroxybenzotriazol (HOBt) an dN,N-dimethylformamide (DMF).

Preparing 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid (1)

To a mixture of 5 g (24.5 mmol) of L-tryptophane and 10 mL formaldehyde in 400 mL of H_2O, 0.2 mL of concentrated sulfuric acid was added. The reaction mixture was stirred at room temperature (rt) for 6 h and adjusted to pH 6 with concentrated ammonia liquor. After filtration, 5.2 g (98.3%) of the title compound was obtained as yellow powders. Electrospray ionization (ESI)–mass spectrometry (MS) (m/e): 215 $[M - H]^-$.

Preparing methyl 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate (2)

At 0°C to 5.2 g (24.1 mmol) compound 1, 120 mL MeOH and 6.4 mL $SOCl_2$ was added dropwise, and the reaction mixture was stirred at rt for 48 h. The mixture was concentrated in vacuum to remove excess $SOCl_2$ and MeOH. The residue was dissolved in 100 mL of ethyl acetate and washed with saturated aqueous sodium chloride. The ethyl acetate phase was dried by anhydrous sodium sulfate. After filtration and evaporation under vacuum, the residue was purified on a silica gel column (CH_2Cl_2:MeOH=30:1) to provide 2.4 g (43%) of the title compound as yellow powders. ESI-MS (m/e): 231 $[M + H]^+$.

Preparing methyl 9H-pyrido[3,4-b]indole-3-carboxylate (3)

With stirring to the solution of 1 g (4.3 mmol) of compound 2 in 100 mL of acetone, 1 g (6.3 mmol) of potassium permanganate was added. The reaction mixture was stirred

at rt for 6 h. After filtration and evaporation under vacuum, the residue was purified on a silica gel column (Petroleum ether:Acetone=1:1) to provide 460 mg (47%) of the title compound as yellow powders. ESI-MS (m/e): 227 $[M + H]^+$.

Preparing 9H-pyrido[3,4-b]indole-3-carboxylic acid (4)

To a solution of 260 mg (1.2 mmol) compound 3 in 15 mL MeOH, aqueous NaOH (4 M) was added dropwise to adjust pH 12, and the reaction mixture was stirred at rt for 20 h. The reaction mixture was adjusted to pH 7 with saturated aqueous citric acid and evaporated under vacuum. To the residue 10 mL H_2O was added and the solution was adjusted to pH 2 with 5% aqueous citric acid. After filtration and evaporation 215 mg (88%) of the title compound was obtained as yellow powders. ESI-MS (m/e): 211 $[M - H]^-$.

Preparing benzyl N^{ω}-nitro-N^{α}-(9H-pyrido[3,4-b]indole-3-carbonyl)-*L*-argininate (NRCB)

At 0°Cto a solution of 826 mg (1.7 mmol) of L-Arg(NO_2)-OBzl, 0.5 mL (4.5 mmol) of N-methyl morpholine, 201 mg (1 mmol) of 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide (EDC), 123 mg (0.9 mmol) of N-hydroxybenzotriazol (HOBt) and 30 mL of N,N-dimethylformamide (DMF), 186 mg (0.9 mmol) of compound 4 was added. After stirring at 0°C for 1 h, the reaction mixture was stirred at rt for 5 h, and TLC (chloroform/MeOH, 30/1) indicated the complete disappearance of compound 4. The reaction mixture was evaporated under vacuum. The residue was dissolved in 100 mL of ethyl acetate and washed successively with 5% aqueous sodium bicarbonate, 5% aqueous citric acid, and saturated aqueous sodium chloride. The ethyl acetate phase was dried by anhydrous sodium sulfate. After filtration and evaporation under vacuum, the residue was purified on a silica gel column (CH_2Cl_2:MeOH=30:1) to provide 120 mg (27%) of the title compound as colorless powders. ESI-MS (m/e): 504 $[M + H]^+$. Mp: 115-116ºC. $[\alpha]_D^{25}$=+24.5 (c = 1.0, CH_3OH); ^{1}HNMR (800 MHz, DMSO-d_6): δ/ppm = 11.98 (s, 1H), 8.94 (s, 1H), 8.90 (d, *J*=7.2 Hz, 1H), 8.86 (s, 1H), 8.52 (d, *J*=4.0 Hz, 1H), 8.41 (d, *J*=8.0 Hz, 1H), 7.67 (d, *J*=8.0 Hz, 1H), 7.61 (t, *J*=7.2 Hz, 1H), 7.39-7.35 (m, 4H), 7.32 (t, *J*=7.2 Hz, 1H), 5.19 (s, 2H), 4.67 (dd, J_1=10.4 Hz, J_2=12.8 Hz, 1H), 3.19

(m, 2H), 1.99-1.95 (m, 2H), 1.61 (s, 2H), ^{13}C NMR (200 MHz, DMSO-d_6): δ/ppm =172.31; 165.50; 141.56; 139.44; 137.81; 136.41; 132.91; 129.17; 128.88; 128.66; 128.50; 128.24; 122.74; 121.43; 120.54; 114.77; 112.77; 66.57; 52.49 and 28.77.

^{1}HNMR spectra of NRCB

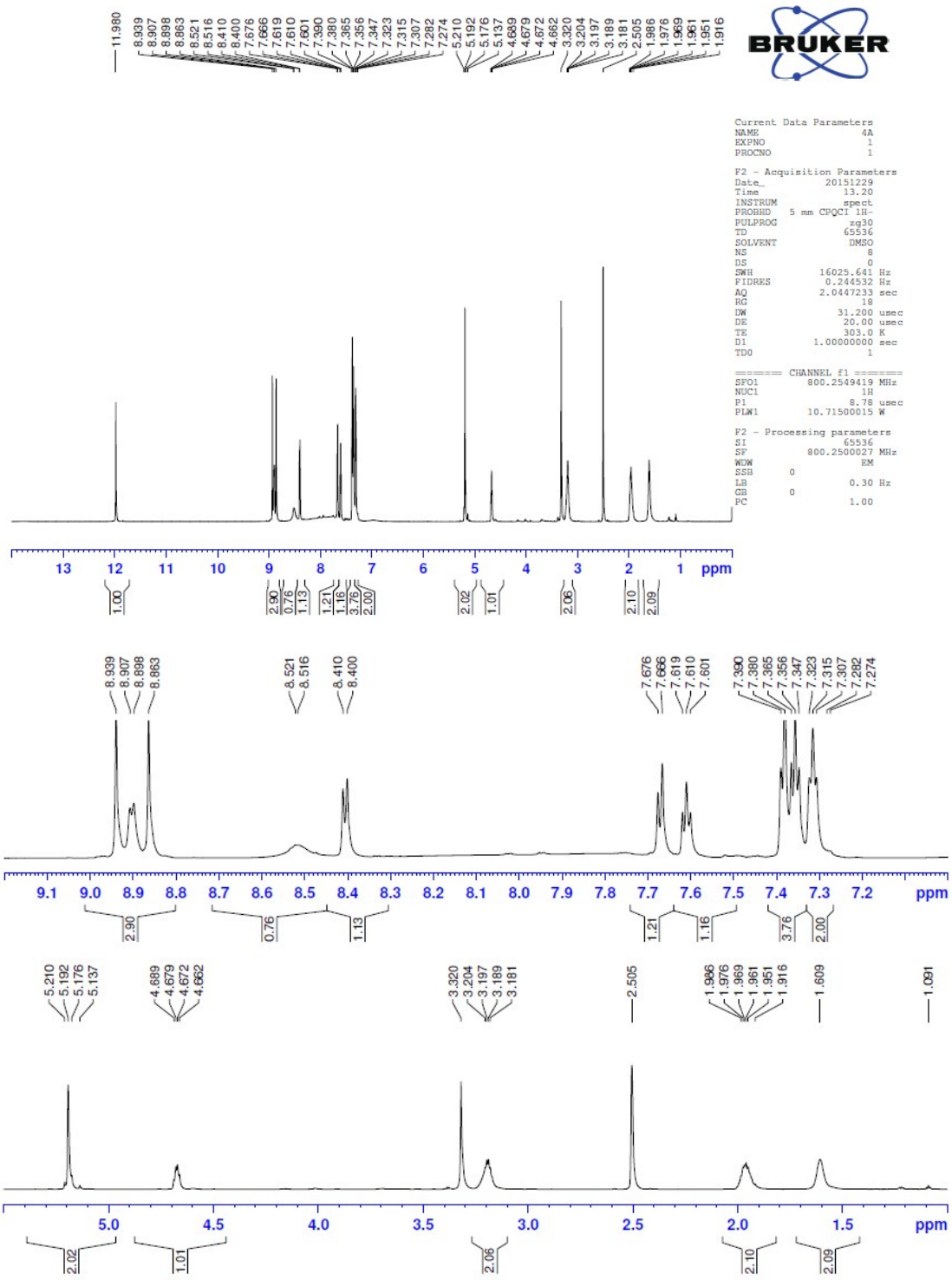


Fig. S1 ^{1}HNMR of NRCB (800 MHz, DMSO-*d6*).

^{13}CNMR spectrum of NRCB

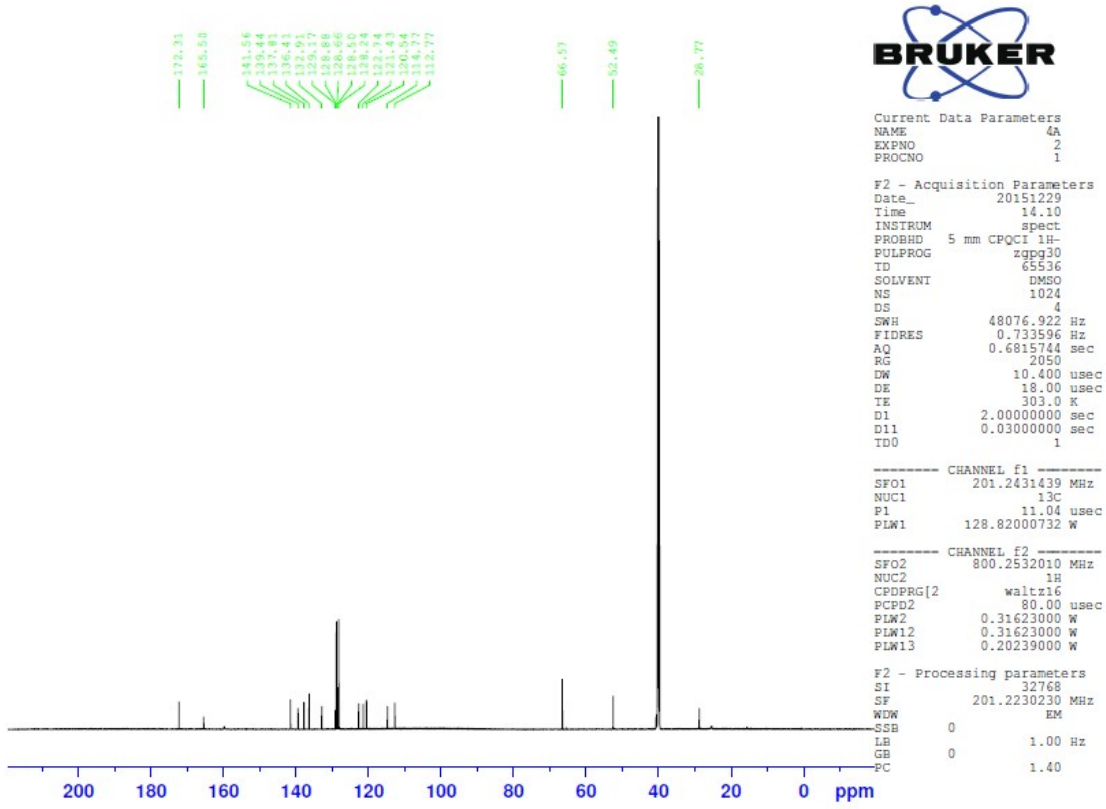


Fig. S2 ^{13}C NMR (200 MHz, DMSO-d_6) of NRCB.

NOESY 2D ^{1}HNMR of NRCB

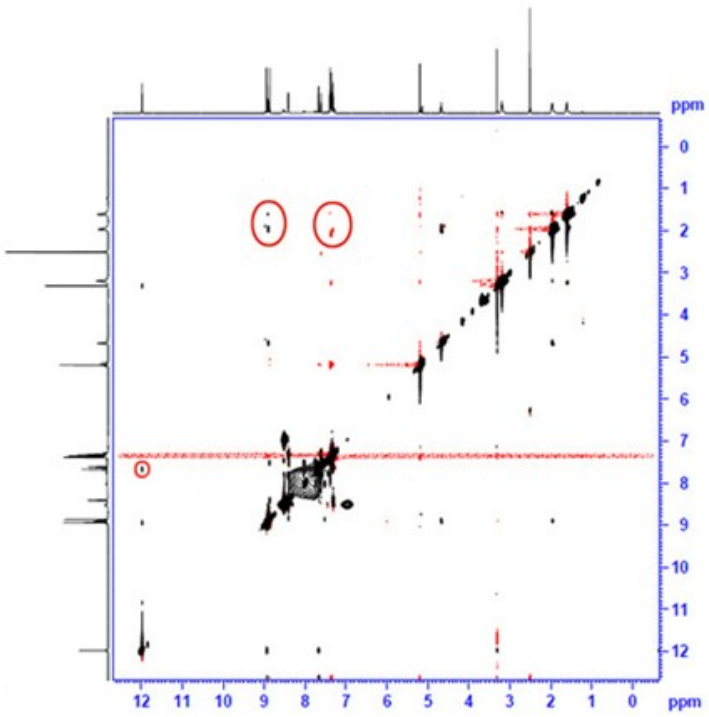


Fig. S3 NOESY 2D ^{1}HNMR spectrum (800 MHz, DMSO-*d6*): the cross-peaks labeled with red circles define intermolecular interaction of NRCB.

ESI(+)/FT-MS spectra of NRCB

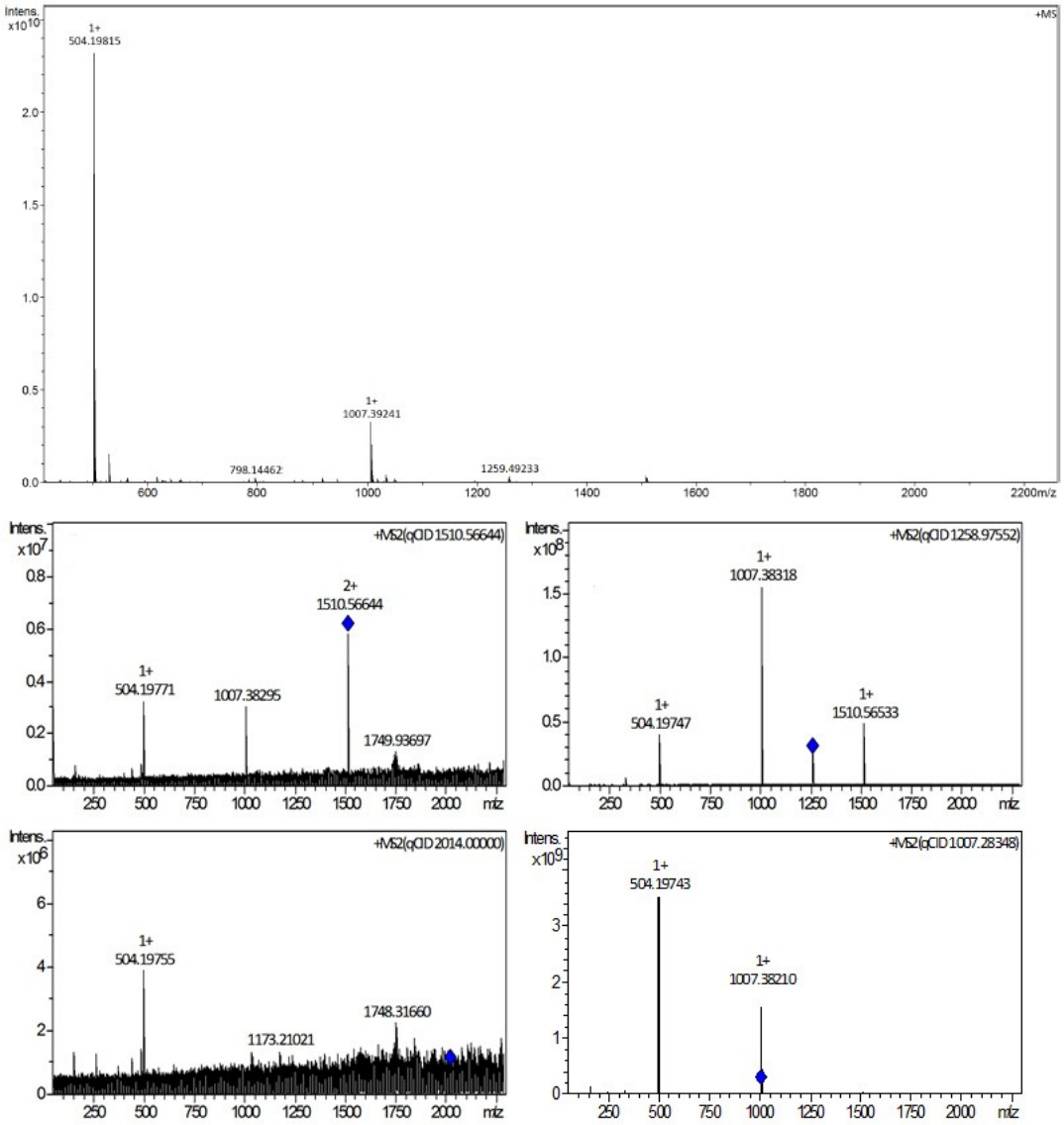


Fig. S4 ESI(+)/FT-MS full scan spectrum (up) and qCID ion scan spectrum (down).

HPLC purity of NRCB

An Agilent Technologies 1100 Series HLPC system (Agilent Technologies, Santa Clara, CA, USA) was used to measure the HPLC purity of NRCB. The sample was separated on a AkzoNobel Kromasil C18 reversed-phase column (4.6×150 mm, 5 μm; AkzoNobel, Bohus, Sweden)protected by a guard column of the same material (5×10 mm, 5 μm). The column thermostat was maintained at 25° C. To the column, 10 μL of a solution of NRCB in MeOH was injected for analysis. The flow rate was 0.5 mL/minute. The column was washed with water and MeOH (25:75), and equilibrated

to initial conditions for 12 minutes. Ultraviolet (UV) absorption spectra were recorded online. The UV detector was set to a scanning range of 200-400 nm, and a wavelength of 270 nm was used to monitor NRCB. The chromatogram was recorded and gave NRCB a retention time of 9.379 minutes and a single peak.

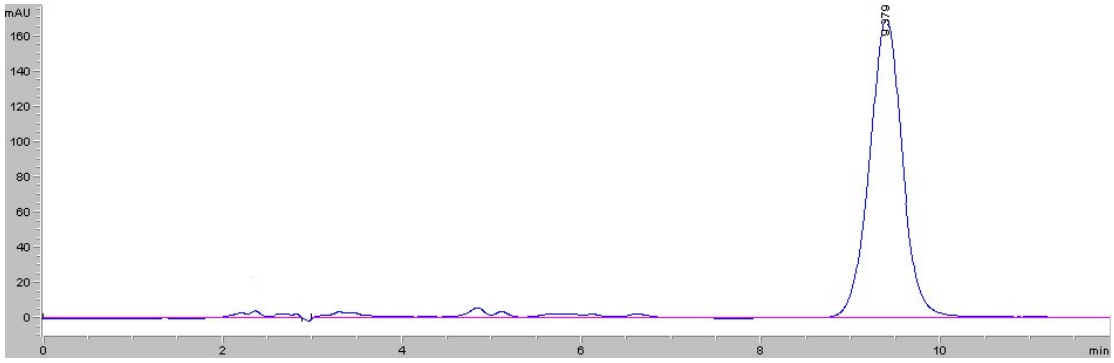


Fig. S5 HPLC program of NRCB.

3. Characterization

Flow cytometry assay

To explore the apoptosis activity of NRCB, flow cytometry assay was recorded. K562 cells (10^6 cells/mL) were incubated in 1640 Medium [containing 10% (v/v) fetal calf serum; 60 μg/mL of penicillin, and 100 μg/mL of streptomycin] at 37°C in a humidified atmosphere containing 5% CO_2 for 4 h. A solution of NRCB (final concentration: 10 μM, in complete 1640 medium as above; 2.5 mL/well) was added, and cells were incubated at 37°C in a humidified atmosphere (containing 5% CO_2) for 12 h and 24 h. After removing the medium, the cells were washed by fresh medium (1 mL×2) and stained with Annexin V-FITC (KeyGEN Biological Technology Co., Ltd., Nanjing, PR China) and Propidium Iodide (PI) for 10 minutes. PI and FITC fluorescence for cells was analyzed for ~10,000 events (counts) per sample aliquot.

TEM test

Shape and size examinations of the NRCB nanospecies were performed with transmission electron microscopy (TEM, JSM-6360 LV, JEOL, Tokyo, Japan). An aqueous solution of NRCB (pH 7.0) was dripped onto a formvar-coated copper grid, and then a drop of anhydrous ethanol was added to promote water removal. Then the grid was first allowed to dry thoroughly in air and it was then heated at 37°C for 24 hours. The samples were viewed under TEM. The shape and size distributions of the

nanospecies were determined by counting >100 species in randomly selected regions on the TEM copper grid. All of the determinations were carried out on triplicate grids. The TEM was operated at 80 kV (the electron beam accelerating voltage). Images were recorded on an imaging plate (Gatan Bioscan Camera Model 1792; Gatan, Inc., Pleasanton, CA, USA) with 20 eV energy windows at 6,000-400,000× and they were digitally enlarged.

SEM test

The shape and size of the nanospecies in lyophilized powders (from a solution of NRCB in ultrapure water) were measured by scanning electron microscopy (SEM, JEM-1230; JEOL) at 50 kV. The lyophilized powders were attached to a copper plate with double-sided tape (Euromedex, Souffelweyersheim, France). The specimens were coated with 20 nm gold-palladium using a JEOL JFC-1600 Auto Fine Coater. The coater was operated at 15 kV, 30 mA, and 200 mTorr (argon) for 60 seconds. The shape and size distributions of the nanoparticles were measured by examining >100 particles in randomly selected regions on the SEM alloy. All measurements were performed on triplicate grids. Images were recorded on an imaging plate (Gatan Bioscan Camera Model 1792; Gatan, Inc.) with 20 eV energy windows at 100–10,000×, and they were digitally enlarged.

AFM test

Atomic force microscopy (AFM) images were obtained using the contact mode on a Nanoscope 3D AFM (Veeco Instruments, Inc., Plainview, NY, USA) under ambient conditions. Samples of NRCB in rat plasma (10^{-6} M) were used for recording the images.

Faraday-Tyndall effect of NRCB

To explore the nano-property of the aqueous solution of NRCB, Faraday-Tyndall effect was tested, 1 μM solution of NRCB inultrapure water were irradiated with laser beam of 650 nm. In addition, the zeta potential and size were determined on a Malvern's Zetasizer (Nano-ZS90; Malvern Instruments) with the DTS Program.

Theoretically predicted nanoparticle size of NRCB

To theoretically predict the formation and the size of the nanoparticles the mesoscale

simulation software was used to perform the calculation. The molecule of NRCB was built and optimized simply in the visualizer window. “Beads” were constructed from atomistic simulations and placed at the center-of-mass of groups of the atoms corresponding to particular parts of a molecule.

Generation of 3D structure for NRCB docking

The 2D structure of NRCB was biult in ChemDraw Ultra 14.0, converted to 3D conformation in Chem3D 12.0, and then energy minimized in Discovery Studio4.0 with a Merck molecular force field (Merck & Co.) until the minimum RMS reached 0.001 in Chem3D Ultra 12.0. In the whole conformational space of NRCB the energy optimized conformations were sampled with systematic search and BEST method of Discovery Studio 4.0 with a SMART minimizer using CHARMM force field. The energy threshold was 20 kcal/mol at 300 K. The minimization root mean squared (RMS) gradient was 0.1 Å and the maximum minimization steps were 200. The maximum generated conformations were 255 with a RMS deviation (RMSD) cutoff of 0.2 Å. Top 10 energy optimized conformations of NRCB were selected for the docking to P-selectin.

Docking of NRCB toward active site of P-selectin

Software AutoDock 4 was used to perform the molecular docking of 10 energy optimized conformations of NRCB toward P-selectin. The P-selectin was prepared by AutoDock Tools 1.5 and treated as rigid: merging nonpolar hydrogens and assigning gasteiger charges and autodock elements. The 10 energy optimized conformations of NRCB were treated as rigid ligands and prepared by AutoDockTools 1.5: merging nonpolar hydrogens, assigning gasteiger charges, finding root and aromatic carbons, detecting rotatable bonds, and setting torsions. The grid box dimensions were set to 50 Å × 50 Å × 50 Å with a grid spacing of 0.375 Å. The Lamarckian genetic algorithm (LGA) was used to find the orientations, appropriate binding positions, and the conformations of NRCB in the active site pocket of P-selectin. The global optimization was performed with parameters of a population of 300 randomly positioned individuals. The Solis and Wets local search was performed with a maximum number of 3000. The maximum number of energy evaluations was increased to 2.5×10^7, and the maximum

number of generations in the LGA algorithm was increased to 2.7 $\times 10^5$. During the molecular docking experiments, 200 runs were carried out for each small molecular ligand. The resulted 200 conformations of each ligand were clustered with an rms tolerance of 2.0 Å and scored by the lowest binding energy.

***In vitro* anti-proliferation assay**

In vitro cell viability assays were carried out using 96-well microtiter culture plates and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining, according to the standard procedures. HeLa, SH-sy5y, HT-29 and HCT-8 cells (5×10^4 cells/mL) were grown in DMEM medium [containing 10% (v/v) fetal calf serum; 60 μg/mL of penicillin, and 100 μg/mL of streptomycin].

HL60, K562 and A549 cells (5×10^4 cells/mL) were grown in 1640 medium [containing 10% (v/v) fetal calf serum; 60 μg/mL of penicillin, and 100 μg/mL of streptomycin]. Stock solution of NRCB was prepared in DMSO and diluted with culture medium to desired concentrations. Cultures were propagated in a humidified atmosphere (with 5% CO_2) at 37°C for 4 h, and then NRCB solution was added. After 48 h of treatment, MTT solution was added (5 μg/mL, 25 μL per well), and cells were incubated for an additional 4 hours. The optical density was measured at 490 nm by a microplate reader, after adding 100 μL of DMSO to dissolve the MTT-formazan product (n =9).

UV spectrum assay reflecting the interaction of NRCB and CT DNA

UV spectrum assay can visualize the intercalation of NRCB toward DNA, and this assay was performed. In brief, after recording the UV spectrum (Shimadzu 2550 spectrophotometer, 200-320 nm wavelength; Shimadzu Corporation, Kyoto, Japan) of 10.0 μM of NRCB in phosphate buffered saline (PBS, 1 mL, pH 7.4), 10 μL of CT DNA in PBS (pH 7.4, 100.0 μM, final concentration: 0 μM; 1.0 μM; 2.0 μM; 3.0 μM; 4.0 μM; and 5.0 μM) was added and the spectra of NRCB plus CT DNA was recorded to monitor the effect of CT DNA on the UV spectrum of NRCB.

Fluorescent spectrum assay for NRCB intercalating toward CT DNA

Fluorescent spectrum assay can visualize the intercalation of NRCB toward DNA, and this assay was performed. In brief, after recording the fluorescent spectrum of NRCB in PBS (3 mL, pH 7.4, 10 μM; Shimadzu RF-5310PC spectrofluorometer, 280 nm of

fluorescence excitation wavelength; Shimdazu Corporation), a series of solutions of CT DNA in PBS (20 μL, pH 7.4, final concentration: 0 μM; 0.6 μM; 1.2 μM; 1.8 μM; 2.4 μM and 3.6 μM) was added, and the spectra of NRCB plus CT DNA were recorded to monitor the effect of CT DNA on the fluorescent spectrum of NRCB.

Circular dichroic spectrum assay for NRCB intercalating toward CT DNA

Recording the circular dichroic spectrum assay can visualize the intercalation of NRCB toward DNA, and this assay was performed. In brief, the solution of CT DNA in PBS (pH 7.4, 900 μL; 10^{-4}M) and 100 μL of solution of NRCB in PBS (pH 7.4, final concentration:2×10^{-6}M, 1×10^{-6} M, 5×10^{-7}M, 2.5×10^{-7}Mor 1.25×10^{-7}M) was mixed, incubated at 37°C for 3 h, and then the curve was recorded according to a standard procedure (on a J-810, JASCO, Halifax, Canada) (scan speed, 500 nm/minute; resolution step, 0.2 nm; sensitivity, 50 mdeg; k=200-350 nm; response, 0.5 seconds; bandwidth, 15 nm; accumulations, 5).

Cell uptake of NRCB

HeLa or SH-sy5y or HT-29 cells (5×10^4 cells/mL) were incubated in Dulbecco's Modified Eagle's Medium (DMEM) [containing 10% (v/v) fetal calf serum, 60 μg/mL of penicillin, and 100 μg/mL of streptomycin] at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. After removing the original medium, a solution of NRCB (final concentration, 40 μM in complete DMEM medium as above; 2.5 mL/well) was added, and cells were incubated at 37°C in a humidified atmosphere (containing 5% CO_2) for 12 hours and 24hours. After removing the medium, the cells were washed with fresh medium (1 mL×3) and resuspended in fresh medium (1 mL/well). NRCB on the cell surface and in the cytoplasm were analyzed by use of an Axio Observer A1 fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany) and TCS SP5 confocal microscopy (Leica Microsystems, Wetzlar, Germany), respectively. Fluorescence intensity was measured by Leica application suite advanced fluorescence lite 1.8.2 (Leica Microsystems, Wetzlar, Germany).

***In vivo* antitumor assay of NRCB**

Male ICR mice (purchased from Capital Medical University) were maintained at 21°C with a natural day/night cycle in a conventional animal colony. Mice were 10 weeks

old at the beginning of the experiment. S180 ascites tumor cells were subcutaneously injected to form solid tumors. To initiate subcutaneous tumors, cells obtained in ascitic form from tumor-bearing mice were serially transplanted once per week. Subcutaneous tumors were implanted under the skin at the right armpit by injecting 0.2 mL of normal saline (NS) containing 2×10^6 viable tumor cells. Twenty-four hours after implantation, mice were randomly divided into treatment groups (10 per group). Treatments were NRCB (0.01 μmol/kg, 0.1 μmol/kg or 1 μmol/kg), doxorubicin (2 μmol/kg, positive control) or vehicle only (negative control, CMCNa, 0.2 mL). Injections were given intraperitoneally every day for 10 days. Twenty-four hours after the last injection, mice were weighed, sacrificed by ether anesthesia, and dissected to immediately obtain and weigh the tumor.

***In vivo* anti-inflammatory assay of NRCB**

The anti-inflammatory assay of aspirin and NRCB was performed with xylene-induced ear edema, and the clinical dose of aspirin in the anti-inflammatory therapy was converted for the mice. In brief, male ICR mice (18 ± 2 g) were randomly divided into three groups, each 10 mice. The mice in vehicle control group were orally given 0.5 % CMCNa at a dose of 0.2 mL/mice, and the mice in positive group were orally given the suspension of aspirin in CMCNa at 167 and 16.7 μmol/kg of dose, and the mice in NRCB group were orally given the suspension of NRCB in 0.5 % CMCNa at a dose of 1 μmol/kg. Thirty minutes after the administration, 30 μL of xylene was applied to the anterior and posterior surfaces of the right ear, with the left ear as control. Two hours after xylene application, the mice received euthanasia to remove both ears. By using a cork borer of 7 mm in diameter the circular slices were collected and weighed. The increased weight caused by the irritant was calculated by subtracting the weight of the untreated left ear slice from that of the treated right ear slice.

Determination of serum P-selectin and TNF-α of S180 mice

To estimate the effect of NRCB on P-selectin and TNF-α, the blood of the treatedS180 mice was collected and centrifuged to obtain serum for measuring P-selectin by using mouse P-selectin elisa kit (Cusabio Biotech Co., Ltd, Wuhan, PR China, n=5) and mouse TNF-α elisa kit (Westang Biotech Co., Ltd, Shanghai, PR China, n=5) on a

microtiter plate reader within 15 minutes and recording OD value. According to the standard curve, the serum concentrations of P-selectin and TNF-α were calculated.

Determination of serum TNF-α and IL-2 of xylene treated mice

To estimate the effect of NRCB on TNF-α and IL-2, the blood of the xylene treated mice was collected and centrifuged to obtain serum for measuring TNF-α by using mouse TNF-α elisa kit (Westang Biotech Co., Ltd, Shanghai, PR China, n=3) and mouse IL-2 elisa kit (MyBioSource, Inc., San Diego, United States, n=3) on a microtiter plate reader within 15 minutes and recording OD value. According to the standard curve, the serum concentrations of TNF-α and IL-2 were calculated.