Supporting Information

Enhanced antitumor effect for hepatocellular carcinoma in advanced stage using cyclodextrin-sorafenib chaperoned inclusion complex

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Methods

Materials

Sorafenib (purity > 98%) was a kind gift from Eastchina Pharm. Co., Ltd, China. β-cyclodextrin (purity >99%), γ-cyclodextrin (purity >99%) were purchased from Adamas Reagent Co.,Ltd. These compounds were initially characterized by X-ray powder diffraction (PXRD). All other materials and solvents were of analytical grade.

Preparation of Sor-β-CD

SCDs was prepared according to our previous procedure by co-crystallization method.

In brief, Sorafenib (0.5 g, 1.0 mmol), γ-CD (1.3g, 1.0 mmol) and 70% THF solvent (25 mL) were added into a 100 mL round bottom flask. The reaction mixture was stirred at 70°C until the solids dissolving completely. The solution was kept at 70°C for 12 h with stirring. After reaction, the solution was cooled slowly to room temperature, leaving overnight for crystallization, and the crystals of Sor-γ-CD were obtained.

Sorafenib (0.5 g, 1.0 mmol), β-CD (1.3g, 1.0 mmol) and 60% THF solvent (16 mL) were added into a 100 mL round bottom flask. The reaction mixture was stirred at 70°C until the solids dissolving completely. The solution was kept at 70°C for 24 h with stirring. After reaction, the solution was cooled slowly to room temperature, leaving overnight for crystallization, and the crystals of Sor-γ-CD were obtained.

Characterization, host-guest stoichiometry ratio and simulation of Sor-β-CD and Sor-γ-CD

Phase-solubility studies of Sor with β-CD and γ-CD were carried out according to the method reported by Higuchi and Connors at 37°C in 1 : 2 (volume ratio) mixture of ethanol-water and β-CD, γ-CD were various concentrations ranging from 2 to 20 mM. The stability constant (K_C) of the complexes was calculated by the following equation:

$$K_C = \frac{Slope}{S_o(1 - Slope)}$$

Where Slope is gradient of plot of Sor versus β-CD, γ-CD concentration, S_o is the solubility of Sor at 37◦C in absence of β-CD or γ-CD. The concentration of Sor was measured by UV spectrophotometric method at 265 nm by Thermo Scientific Evolution 300 UV–Vis spectrometer (Thermo Scientific, Waltham, MA). Mass spectra (MS)

were obtained using a Triple TOF5600 – AB SCIEX equipped with the electrospray ion source (ESI) and operated in the positive ion mode. UV–Vis spectrometer was recorded by Thermo Scientific Evolution 300 (Thermo Scientific, Waltham, MA). Nuclear magnetic resonance analysis (NMR) in DMSO-d6 spectra were recorded on a Bruker ADVANCE2B 500 MHz spectrometer and processed using MestReNova software. Fourier transform-infrared spectroscopy (FT-IR) spectra were recorded on the VECTOR-22 Fourier Infrared Spectrometer in the KBr diffuse reflectance mode. The diffraction patterns were measured on Rigaku D/Max-2550PC diffractometer using a rotating-anode Cu-target X-ray (λ= 1.5406 Å) generator operated at 40 kV and 250mA. The incidence Cu Kα X-ray beam was monochromatized by a vertical graded multiplayer mirror to remove the K_β radiation and to obtain a paralleled beam with a width of 0.8 mm. The scans were run from 3.0 to 40.0° 2θ, with an increasing step size of 0.02° and count time of 0.5~2 s. The morphology of channel-type structure of Sor-γ-CD was examined by transmission electron microscopy (TEM, HT-7700, Hitachi, Japan).

Single crystal X-ray diffraction (SC-XRD) data of Sor-β-CD was carried out using Rigaku RAXIS-RAPID-with Cu Kα radiation (λ = 1.5418 Å) operating at 50kV, 200mA and temperature at -120^{o}C. The structure was solved by direct method, refined and graphed molecular using SHELXL-97 (Sheldrick, 2008).

The crystal structures of β-CD (PDB code: 3CGT) and γ-CD (PDB code: 2ZYK) were extracted from protein data bank (PDB). The PDB file of sorafenib was geometrically optimized using the minimize energy method via Chemical Bio3D Ultra (Perkin Elmer, 14.0.0.117).

AutoDock Vina was one of widely used docking programs, which can predict free energy of ligand-receptor interaction. In our studies, the AutoDock Tools package (version 1.5.6) and AutoDock Vina (version 1.1.2) were adopted to conduct the docking studies. In docking simulation, sorafenib was used as a ligand, while cyclodextrins were used as receptors. Gasteiger charges were computed for each of the molecules. The grid maps were prepared using a 100 × 100×100 grid box with the distance between two grid points set at 0.375 Å centering on each cyclodextrins. All rotational bonds of sorafenib were set free, while those of cyclodextrin were held rigid. The docking studies were carried out using the empirical free energy function and applying the

standard protocol of the Lamarckian Genetic Algorithm (LGA). A total of 100 independent docking runs were carried out for each docking processes with a maximum number of 27,000 generations, a mutation rate of 0.02, a crossover rate of 0.8 and an elitism value of 1. All the other parameters were the default values by AutoDock Vina.

Solubility and dissolution measurement

The solubility and dissolution rate studies of Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD in water and gastric juice pH 1.2 (in the presence of 0.2% sodium lauryl sulfate-SLS) at 37^{o}C were reported by Thermo Scientific Evolution 300 UV–Vis spectrometer (Thermo Scientific, Waltham, MA). For solubility, excess quantities of Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD inclusion complex were dispersed in 5 mL of aqueous solvent in screw-capped vials and stirred at 100rpm in 24 h, 37^{o}C to obtain supersaturated solutions. Supersaturated solutions were filtered through Whatman's 0.45 μm syringe filter. For intrinsic dissolution rate measurements, 5 g sample was compressed to a 0.5 cm^2 disk using a hydraulic press at a pressure of 10 MPa for 5 min. The disk were sealed with wax and placed in a beaker with only one surface exposed to 250 mL of solvent which was preheated to 37^{o}C and rotated at 150 rpm. In all experiments, 3 mL of dissolution sample was withdrawn at specific time intervals for 240 min and replaced with an equal volume of the fresh medium to maintain a constant total volume.

All solution was measured the absorbance at their λ_{max}.

In Vitro Drug Release

Sor release profile from SCDs was studied in different aqueous media (added diatase solution, pH 3 solution and added 50% DMSO solution), using the dialysis bag technique. At specific times, 2 mL of release medium was withdrawn, replaced with an equal volume of fresh solution, and analyzed by HPLC. The amount of released Sor was expressed as percentage ratio between the weight of released drug at prefixed times and the total amount of entrapped Sor (Figure S6).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and HepG2 cells were purchased from American Type Culture Collection (ATCC, Rockville MD). HUVEC and HepG2 cells were cultured in RPMI-1640 medium, containing 10% foetal bovine srum (FBS), 100 units per ml penicillin, and 100 units per ml streptomycin, supplied by GE Life Sciences Co. Ltd. The two cell lines have passed the conventional tests of cell line quality control methods (e.g., morphology, isoenzymes, and mycoplasma). The cells grew as a monolayer and were detached upon confluence using trypsin (0.5% w/v in PBS buffer). The cells were cultured at 37 °C and 5% CO_2.

Evaluation of cytotoxicity

The cytotoxicity of Sor and SCDs against HepG2 cells were determined by MTT assays in a 96-well plate. All solutions and mediums were sterilized by filtration (0.22 μm) before use. The cells were seeded at a density of 8×10^3-10^4 cells per well in a 96-well cell culture plate, and incubated for 24 h for attachment. Then the cells were cultured with Sor and SCDs at various concentrations. At different intervals, the cells were washed with PBS buffer, and then 100 μL MTT solution (0.5 mg/mL) was added to each well. The MTT solution was removed after 4 h of incubation and the cells were washed with PBS for three times. A volume of 100 μL DMSO was added to each well to solubilize formazan crystals, and the absorbance of the formazan product was measured at 570 nm using a spectrophotometer (Bio-Rad Model 680). The untreated cells served as the 100% cell viability control, and the completely died cells served as the blank. All experiments were carried out with five replicates. The relative cell viability (%) related to control cells was calculated by the formula below:

$$V\% = \frac{[A]_{experiment} - [A]_{blank}}{[A]_{control} - [A]_{blank}} x100\%$$

Where V% is the percent of cell viability, $[A]_{experiment}$ is the absorbance of the wells culturing the treated cells, $[A]_{blank}$ is the absorbance of the blank, and $[A]_{control}$ is the absorbance of the wells culturing untreated cells

Western Blot Analysis

The cells were seeded into a six-well plate at 3×10^5 cells per well. After 12 hours, cells were treated with Sor and SCDs for 12 hours. Afterwards, cells were washed three times with PBS and lysed in ice-cold radioimmuno precipitation assay buffer containing protease inhibitors. The supernatant was collected by centrifugation at 12,000*g* for 5 min at 4°C. The concentration of total protein was quantified by the bicinchoninic acid (BCA)

method. The protein samples were loaded into SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred to poly-vinylidene fluoride membranes, followed by blocking in tris-buffered saline–Tween 20 (TBST) containing 5% nonfat dry milk at room temperature for 2 hours. Subsequently, each membrane was incubated at 4°C overnight with respective primary antibodies. Membranes were washed three times with TBST, followed by incubation with appropriate horseradish peroxidase–labeled antibodies at room temperature for 1 hour. Last, membranes were washed and then detected using chemiluminescence reagents.

Animals

The in vivo experiments were performed in accordance with the CAPN (China Animal Protection Law), which were approved by the Zhejiang University Institutional Animal Care and Use Committee in China (approval ID: 15232). Six week old (16–18 g) male BALB/c athymic nude mice that were provided from the Zhejiang Chinese Medical University were used for in vivo experiments. A pathogen-free environment under controlled humidity and temperature was used for maintaining the mice.

HPLC assay

HPLC method was used for determining the concentration of Sor in vivo experiment (Figure S1). The analytical column was a Agilent ZORBAX-SB-C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase was ACN: H_2O (7:3 v/v), the flow rate of mobile phase was 0.8 mL min^{-1} and the UV detector was at 265 nm.

In vivo pharmacokinetic and distribution study

In vivo pharmacokinetic and distribution study of Sor (from free Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD) in heart, liver, spleen, lung, kidneys, tumor and blood were carried out via oral administration with dose 20 mg kg^{-1} Sor. Sor concentration was determined by HPLC systems.

In vivo biosafety

Mice were randomly divided into four groups (4 for each group) for Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD. The mice were oral administration daily with dose of 20 mg kg^{-1} for one week. For each mouse, 0.3 mL blood was collected and analyzed using an automatic analyzer (Hitachi 7020, Japan).

Antitumor effect of SCDs via intratumoral injection and oral administration

As our definition, first, HCC tumor model in advanced stage was created by HepG2 cells-induced in BALB/c nude mice. BALB/c nude mice were injected 0.1 mL injection volume containing 4×10^6 HepG2 and transplanted subcutaneously at abdomen. After incubation, the tumor grew to 400 mm^3, the mice were randomly divided into 2 big groups (one group was investigated the in vivo antitumor efficacy of Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD via intratumoral injection and one group was studied these drug formulations anticancer effectiveness through oral administration). Each big group, we divided into 5 groups (4 mice per group): Control, Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD.

The first group was intratumoral injected with various drug formulations (three times per week, in three weeks, at dose of 16 mg Sor/1 kg, 100 μL/mouse).

The second group was orally adminstrated daily with Sor, Sor-Tos, SCDs for three weeks. The dose of 16mg kg^{-1} equivalent Sor, 200 μL/mouse (n = 4).

Tumor volume was measured every three days using digital caliper, and the tumor volume (V) was calculated as length (mm) × width $(mm)^2/2$. The relative volume (RTV) = $V_{day}/V_{first\ day}$. Body weight of the mice was recorded while measuring the tumor volume.

Tumor growth inhibition ratio (T/C %: treatment/control) was calculated using the formula:

$$T/C(\%) = \frac{\overline{V}_{treatment}}{\overline{V}_{control}}.100\%$$

Activity was defined as a T/C% ratio <40% [1, 2]

Mouse in vivo image

Magnetic resonance imaging (MRI) of the bearing tumor mice were scanned using a sequence (TR/TE = 400/20 ms, slice width of 3 mm, slice gap of 0.5 mm and 6 slices) on the MesoMR60 (Shanghai Niumag Corporation, Shanghai, China). Multispectral optoacoustic tomography (MSOT) was imaged using iTheraMedical GmbH,

Munich, Germany. Positron Emission Tomography (PET) Imaging: 18F-fluorodeoxyglucose (18F-FDG) micro PET imaging was performed on a micro PET R4 scanner (Concorde Microsystems, Knoxville, TN, USA).

In vivo immunohistochemistry assay

Mice were sacrificed after 3 weeks from the first oral administration; tumors were dissected and then fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin. Then the samples were cut into 3 μm sections and processed for hematoxylin and eosin (H&E), TUNEL staining and anti-mouse CD31 antibody. The sections were imaged on a Leica (DMLB & DMIL) microscope.

Statistical analysis

Data were expressed as mean ± standard deviation. Differences between groups were evaluated using the paired, two-sided Student t –test $p < 0.05$ was considered significant, and $p < 0.001$ was considered highly significant.

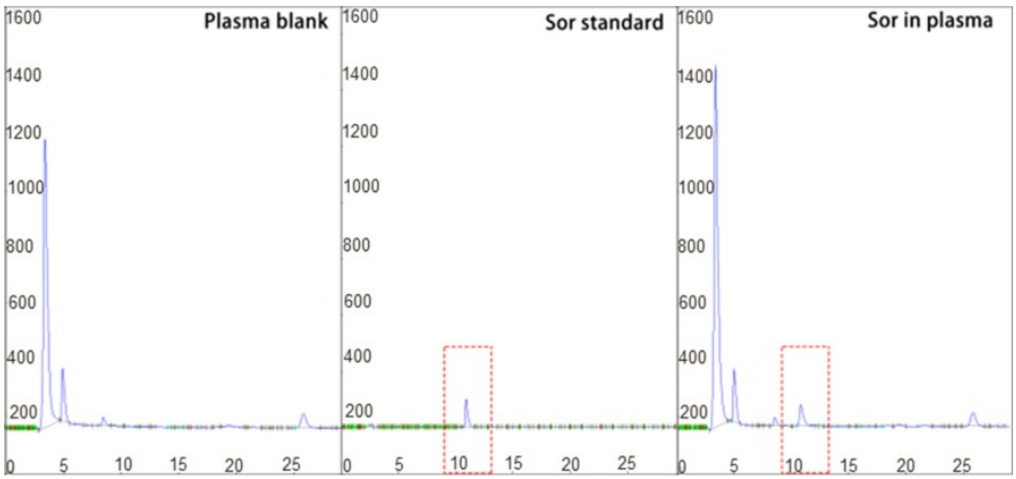

Figure S1. HPLC curves of free Sor, plasma with and without Sor.

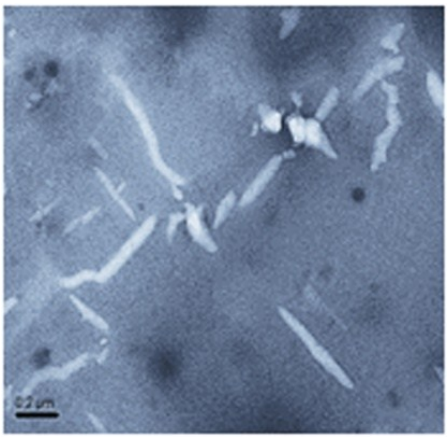

Figure S2. TEM image of Sor-γ-CD

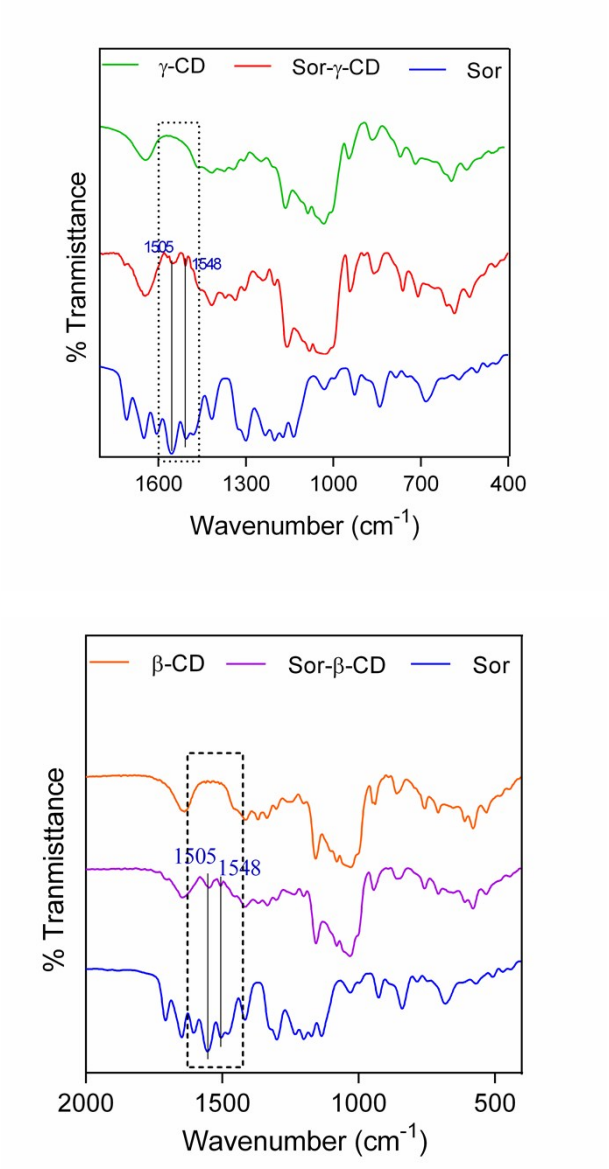

Figure S3. FT-IR Spectra of Sor-γ-CD (above), Sor-β-CD (under).

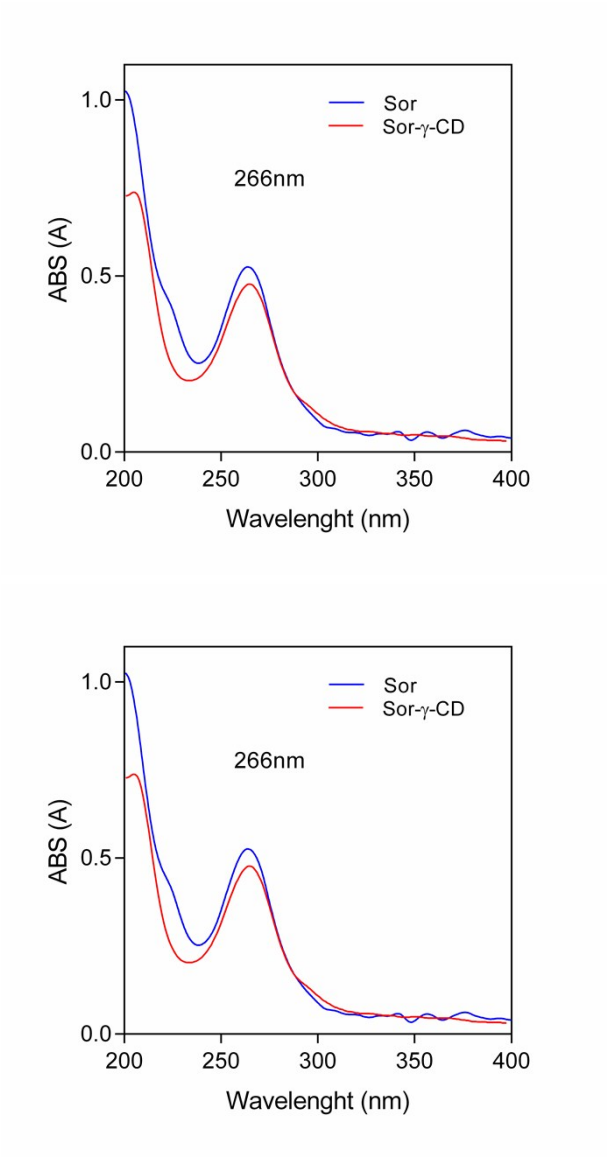

Figure S4. UV-Vis spectra of Sor and Sor-γ-CD (above) or Sor-β-CD (under) in water at 25°C, and the concentration of Sor is equivalent.

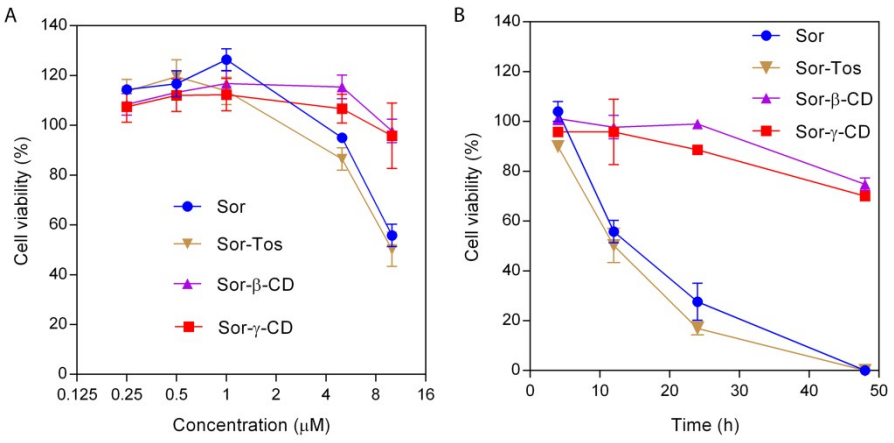

Figure S5. Cell viability assay of Sor, Sor-Tos, Sor-β-CD and Sor-γ-CD in HepG2 cell line A) with different concentration of formulations, B) at different time.

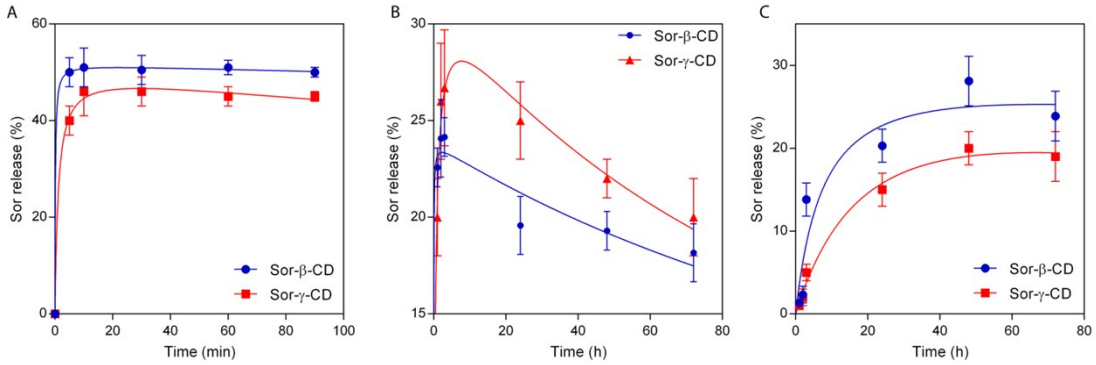

Figure S6. The release of Sor in aqueous solution A) added diastase solution, B) pH 3 solution, C) added 50% DMSO solution. The initial burst release and different in trend was observed in three solution. In the presence of metabolic enzymes (diatase) SCDs were released faster than pH 3 solution or in the presence of DMSO. Moreover, the released amount of Sor from SCDs in solution with the presence of diatase was highest, upto 50%.

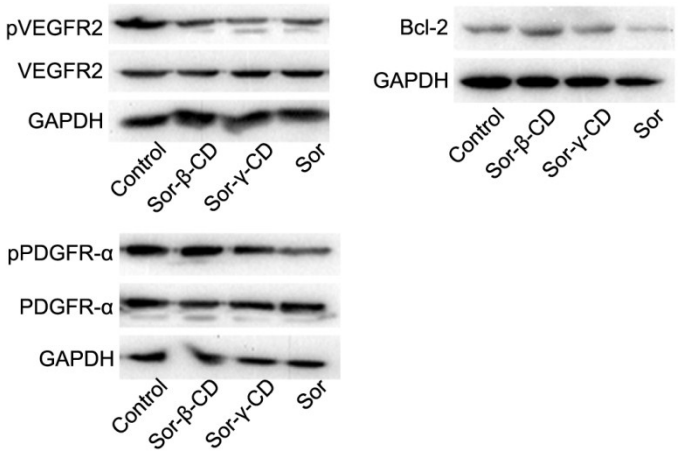

Figure S7. Western blotting analysis of related protein expression. The VEGFR and PDGFR evaluation was performed upon human umbilical vein endothelial cells (HUVECs) and the Bcl-2 expression was measured upon HepG2 cells.

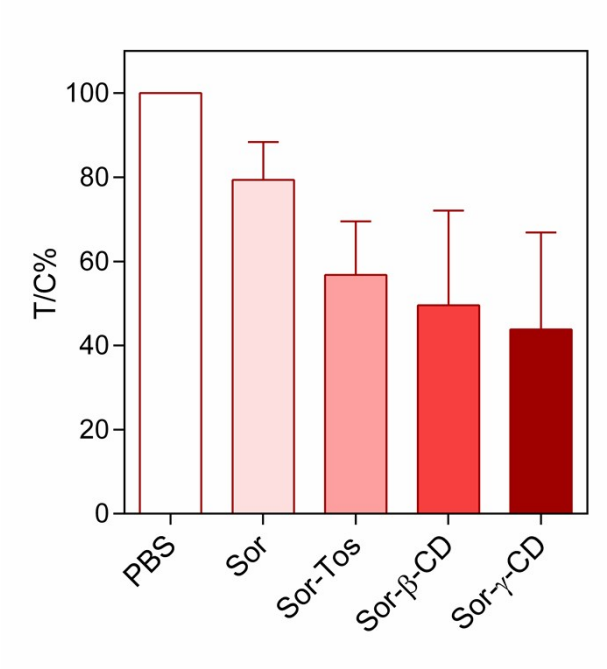

Figure S8. Tumor growth inhibition ratios (Treatment/Control T/C%) after 21 days of oral administration. The data were expressed as means ± s.d.

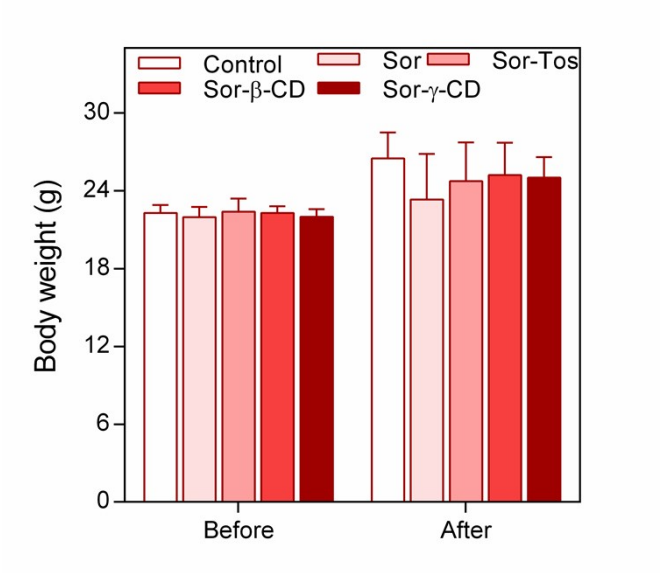

Figure S9. Body weight of mice before and after treatment through oral administration. The data were expressed as means ± s.d.

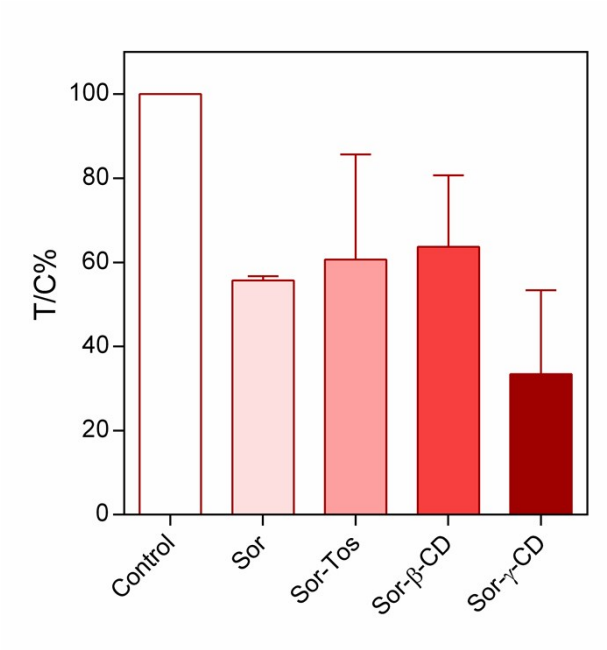

Figure S10. Tumor growth inhibition ratios (Treatment/Control T/C%) after three weeks of intratumoral injection. The data were expressed as means ± s.d.

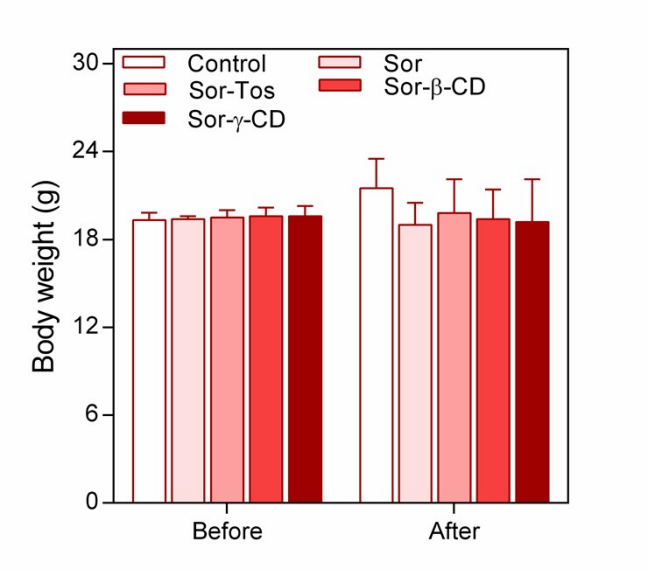

Figure S11. Body weight changed of the nude mice after three weeks of different treatments (n = 4). The data were expressed as means ± s.d.

Reference

1. N. P. Arango, E. Yuca, M. Zhao, K. W. Evans, S. Scott, C. Kim, A. M. Gonzalez-Angulo, F. Janku, N. T. Ueno, D. Tripathy, A. Akcakanat, A. Naing and F. Meric-Bernstam, *Breast Cancer Res.*, 2017, **19**.

2. F. H. Rad, H. Le Buanec, S. Paturance, P. Larcier, P. Genne, B. Ryffel, A. Bensussan, B. Bizzini, R. C. Gallo, D. Zagury and G. Uzan, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 2837-2842.