Coordinated regulation of BACH1 and mitochondrial metabolism through tumor-targeted self-assembled nanoparticles for effective triple negative breast cancer combination therapy

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Received 8 March 2022; received in revised form 30 May 2022; accepted 4 June 2022

Abstract  The poor prognosis of triple negative breast cancer (TNBC) results from a lack of approved targeted therapies coupled with aggressive proliferation and metastasis, which is associated with high recurrence and short overall survival. Here we developed a strategy by employing tumor-targeted self-assembled nanoparticles to coordinately regulate BACH1 (BTB domain and CNC homology 1) and mitochondrial metabolism. The BACH1 inhibitor hemin and mitochondria function inhibitor berberine derivative (BD) were used to prepare nanoparticles (BH NPs) followed by the modification of chondroitin sulfate (CS) on the surface of BH NPs to achieve tumor targeting (CS/BH NPs). CS/BH NPs were found to be able to inhibit tumor migration and invasion by significantly decreasing the amounts of tumor cell metabolites, glycolysis and metastasis-associated proteins, which were related to the inhibition of BACH1 function. Meanwhile, decreased mitochondrial membrane potential, activated caspase 3/9 and increased ROS production demonstrated coordinated regulation of BACH1 and mitochondrial metabolism. In a xenograft mice model of breast cancer, CS/BH NPs significantly inhibited tumor growth.

KEY WORDS
Coordinated regulation; BACH1; Mitochondrial metabolism; Hemin; Berberine derivative; Self-assembled nanoparticle; Triple negative breast cancer

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2022.06.009
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1. Introduction

Triple negative breast cancer (TNBC) is a breast cancer subgroup defined by a lack of expression estrogen receptor (ER) and progesterone receptor (PGR) as well as human epidermal growth factor receptor 2 (HER2)\(^1,2\). As an aggressive tumor, TNBC constitutes approximately 10%–20% of all breast cancers (BCs) with poor prognosis and high relapse rate after chemotherapy\(^3,4\). As of today, there are no targeted treatment options with a definite potential for TNBC13. Therefore, mitochondrial metabolism is potentially a target for the treatment of TNBC. Berberine (Ber) is a natural isoquinoline alkaloid compound isolated from many kinds of medicinal plants\(^16\). Ber not only induced mitochondrial membrane potential (\(\Delta \psi_{ mitochondrial} \)) depolarization, arrested G1 phase of cell cycle and cell apoptosis, but also inhibited the proliferation, migration and invasiveness of TNBC cells\(^7,18\). In addition, Ber, also a promising cancer immunotherapy candidate, could suppress the expression of indoleamine 2,3-dioxygenase 1 (IDO1)\(^19\). Our previous researches proved that 9-O-octadecyl substituted berberine derivative (BD) not only manifested as excellent antineoplastic agent but also as effective mitochondria-targeting ligand\(^20,21\).

Meanwhile, studies have shown that loss of BACH1 regulates mitochondrial metabolism, such as increased dependency on mitochondrial respiration and exhibiting increased sensitivity to agents that target these pathways. Therefore, the combination and synchronous delivery of hemin that degrades BACH1 and berberine derivative that suppresses the mitochondrial functions is an effective therapy for TNBC patients.

In the last decades, nano drug co-delivery system (NDCDS) has emerged as a promising carrier for combined therapy against cancer\(^22\). NDCDS possesses advantages in loading drugs with distinct properties into one single vehicle, thus simultaneously delivering them \textit{in vivo} and showing synergetic antitumor effect\(^23\). The enhanced permeation and retention (EPR) effect is the basis of anticancer-targeted therapy. Due to fundamental shortcomings in tumor passive transport, intelligent NDCDS based on active tumor targeting has been studied. Among them, self-assembled nanoparticles have attracted broad scientific interests due to its distinct characteristics, such as the ability to offer high encapsulation capacity of drugs and stability, and the simple preparation methods. Cluster determinant 44 (CD44) is a primary cell-surface receptor, highly overexpressed in breast cancer, which can be involved in the proliferation and metastasis of cancer cells. Therefore, CD44 is a promising target for TNBC treatment and specifically binds glycosaminoglycan-based biomaterials\(^24\). Chondroitin sulfate (CS), (sulfated glycosaminoglycans), is a natural anionic polysaccharide with strong affinity for the CD44 receptor\(^25\). CS has a good biocompatibility and biodegradability, which is often used for modifying drug carriers to improve tumor targeting as well as maintain high stability.

Based on this, our study aimed to develop self-assembled nanoparticles (CS/BH NPs) with tumor targeting to treat triple negative breast cancer by coordinating the regulation of BACH1 and mitochondrial metabolism. As illustrated in Scheme 1, BH NPs were fabricated with BACH1 inhibitor hemin and mitochondria function inhibitor BD by a nano-precipitation method.
To improve the stability and tumor targeting, the BH nanoparticles were further modified with chondroitin sulfate (CS) on the surface to afford CS/BH NPs. In in vitro experiments, the prepared CS/BH NPs resulted in decreased metabolism of tumor cells, glycolysis, metastasis-associated proteins expressions, as well as mitochondrial dysfunction in TNBC cells due to the suppressed BACH1 function mediated by hemin. Sensitized by hemin, BD more effectively induced depolarization, cell apoptosis and ROS production via mitochondria pathway. The efficacy and safety of CS/BH NPs were also tested in vivo using a xenograft mice model to demonstrate the synergetic effect of hemin and BD and their toxicities for major organs.

2. Materials and methods

2.1. Materials

9-0-Octadecyl substituted berberine derivative (BD) was synthesized and characterized in our laboratory. Hemin and 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). Calcein-AM/PI Assay kit was purchased from Shanghai Yeasen Biotechnology Co., Ltd. (Shanghai, China). Chondroitin sulfate (CS) was purchased from TCI Chemical Industry Co., Ltd. (Shanghai, China). 1,1’-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine iodide, Mitotracker™ Deep Red FM and Lyso-Tracker™ Red DND99 were purchased from Thermo Fisher Technology Co., Ltd. (Shanghai, China). Matrigel was purchased from Corning Incorporated (Corning, NY, USA). Seahorse XF Glycolysis Stress Test kit, Seahorse XF Cell Mito Stress Test kit, Seahorse XF DMEM medium (pH 7.4), Seahorse XF 1.0 mol/L glucose solution, Seahorse XF 100 mmol/L pyruvate solution, Seahorse XF 200 mmol/L glutamine solution and Seahorse XFe96 FluxPak were purchased from Agilent Technologies Co., Ltd. (Beijing, China). Glucose Uptake Colorimetric Assay kit, Lactate Colorimetric Assay kit, GSH-Glo™ Glutathione Assay kit and NADP/NADPH Assay kit were purchased from Promega Biotech Co., Ltd. (Beijing, China). ATP Assay kit, ROS Assay kit, MMP-1 ELISA Assay kit, VEGF ELISA Assay kit and JC-1 fluorescent dye were provided by Beyotime Institute of Biotechnology (Shanghai, China). Colorimetric Assay kits and cell cycle kit were purchased from KeyGEN BioTECH Corp., Ltd. (Nanjing, China). HK2 ELISA Assay kit was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). GAPDH ELISA Assay kit was purchased from CUSABIO Biological Engineering Co., Ltd. (Wuhan, China). All the materials used in this study were analytical grade and used without further purification.
2.2. Cell lines and animals

Human breast cancer cells (MDA-MB-231) were purchased from the Experimental Center of China Medical University (Shenyang, China). MDA-MB-231 cells were cultured with Dulbecco’s modified Eagle medium cell culture medium (DMEM) (Thermo Fisher Technology Co., Ltd., Shanghai, China) supplemented with 10% FBS (PAN-Biotech GmbH, Adenbach, Germany), penicillin (100 μg/mL) and streptomycin (100 μg/mL; Hyclone Corp., Logan, UT, USA) in 5% CO₂ at 37 °C in an incubator (Heal Force, Heal Force HF90, Shanghai, China).

Female BALB/c nude mice (8–10 g) were purchased from Laboratory Animal Center (Sun Yat-sen University). The animals were kept in a specific pathogen-free (SPF) environment in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and all procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University.

2.3. Preparation and characterizations of CS/BH NPs

Self-assembled nanoparticles were produced by modifying the nanoprecipitation method. Briefly, BD and hemin were separately dissolved with DMSO to obtain the original solution (10 mg/mL) and then mixed (10:7, volume ratio). Then 200 μL of the mixed solution was added dropwise into 1.8 mL of pure water by stirring at 800 rpm (IKA, Big squid, German) at room temperature and stirred for another 3 min to obtain BH NPs. Under these conditions, the self-assembly of berberine derivative-hemin nanoparticles occurred spontaneously. Then, CS/BH NPs were prepared by mixing 1 mg/mL chondroitin sulfate solution with BH NPs in equal volume.

The particle size distribution and zeta potential of the NPs were measured by dynamic light scattering technique (Malvern Instruments Ltd., Malvern Nano ZS 90, Malvern, UK). The morphology of the NPs was examined by transmission electron microscope (FEI Company, FEI Techai G2 Spirit, Hillsboro, OR, USA), operating at an accelerating voltage of 120 kV. The samples were negatively stained as follows: a drop (approximately 10 μL) of the sample solution was placed onto a carbon-coated copper grid and incubated for 5 min. The excess solution was taped with a filter paper and then air-dried, followed by an application of 0.01% phosphotungstic acid to mount nanoparticles on the grid.

The preparation of DiR-labeled BH/DiR NPs and CS/BH/DiR NPs was the same as described previously, except in the nanoprecipitation process, the fluorescent dye DiR was dissolved in DMSO with BD and Hemin. Hemin, BD and BH NPs solutions were incubated respectively with DMSO and 0.2% SDS for 2 h, then the absorption curve of hemin and BD was measured to explore possible mechanisms between hemin and BD.

Encapsulation efficiency, drug loading and in vitro drug release were detailed in Supporting Information.

2.4. Hemolysis test

Routine blood experiments were performed to further assess the biocompatibility of the CS/BH NPs. Fresh blood was collected from normal BALB/c mice and centrifuged at 1500 rpm (Eppendorf Corp., Centrifuge 5424, Hamburg, Germany) for 5 min. The plasma was discarded, and red blood cells (RBCs) were washed three times with 0.9% normal saline to get 2% RBCs suspensions. BH NPs or CS/BH NPs at different concentrations (10, 25, 50 and 100 μg/mL) in PBS was added to equal volume of 2% RBCs suspensions. Triton and water were employed as the positive controls with PBS as the negative control. After allowing the samples to stand for 0, 1, 3, and 6 h, the appearance of all samples was recorded. The morphology of the precipitated red blood cells resuspended with PBS was observed under a microscope.

2.5. Cellular uptake

MDA-MB-231 cells in logarithmic phase were digested and inoculated on conical dishes at a density of 2 × 10⁵ cells/well and incubated at 37 °C and 5% CO₂ for 24 h. Then, the cells were separately treated with free BD, free hemin, BD/hemin physical mixture, BH NPs or CS/BH NPs for 4 h. After incubation, they were washed with cold PBS twice for photo observation under confocal laser scanning microscopy (CLSM, Olympus Corp., FV3000, Tokyo, Japan). The experiment was repeated three times. Meanwhile, many research reported that hyaluronic acid (HA) and chondroitin sulfate (CS) could bind specifically with CD44 receptor²⁶,²⁷. To examine the uptake of CS/BH NPs was mediated by CD-44 receptors, cells were pretreated with excess free CS (5 mg/mL) or HA (5 mg/mL) for 1 h, respectively, then incubated with CS/BH NPs. The cellular uptake was determined by CLSM (Olympus Corp.) in which BD emitted green fluorescence itself. Afterward, the same procedures were adopted as described above for imaging and fluorescence intensity measurement.

2.6. Lysosome escape

CLSM was used for the lysosome escape assay. MDA-MB-231 cells were seeded in a special confocal microscopy dish (NEST) at a density of 2 × 10⁵ cells/well, respectively. After 24 h, the cells were separately treated with free BD, BH NPs or CS/BH NPs at an equivalent concentration of BD (5.0 μg/mL) and hemin (3.5 μg/mL). At predetermined time intervals (4 and 10 h), the cells were washed twice with cold PBS and then stained with 50 nmol/L LysoTracker™ Red DND99 for 50 min in dark at 37 °C in the dark. Subsequently, the cells were washed and observed by CLSM.

2.7. Co-localization in the mitochondria

CLSM was used to observe the colocalization between BD and mitochondria. MDA-MB-231 cells were seeded (2 × 10⁵ cells/well) and separately treated with free BD, BH NPs or CS/BH NPs for 10 h of co-incubation. After incubation, the cells were collected and washed with cold PBS twice and stained with 100 nmol/L MitoTracker™ Deep Red FM for 50 min in dark at 37 °C. Subsequently, the cells were washed with PBS twice and observed by CLSM.

2.8. In vitro tumor inhibition

2.8.1. In vitro cytotoxicity

The MTT and Calcein-AM/PI staining assay were used to investigate the in vitro anticancer activities of free BD, free hemin, physical mixture, BH NPs and CS/BH NPs against MDA-MB-231 cells. For MTT assay, MDA-MB-231 cells were seeded in the 96-well plates at a density of 5000 cells/well and cultured for 24 h.
The cells were treated with the above samples at different concentrations, respectively. After 24 h of incubation, 20 μL of MTT reagent (5 mg/mL) was given into each well and incubated at 37 °C for 4 h. The supernatant was then removed and formazan crystals in each well were dissolved in 150 μL of DMSO. The absorbance at 570 nm was detected using a microplate reader (BioTek Instruments, Inc., Synergy H1, Burlington, VT, USA). Untreated cells were used as control, and the inhibition rate was calculated as shown in Eq. (1):

\[
\text{Survival rate (\%) } = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100
\]

For Calcein-AM/PI staining assay, MDA-MB-231 cells were seeded in culture dishes (5 × 10^4 cells per dish) and cultured for 24 h. After the medium was removed, various samples [free BD (5.0 μg/mL), free hemin (3.5 μg/mL), physical mixture, BH NPs and CS/BH NPs containing equal concentration to the combo free drugs] dispersed in DMEM were added. After another 24-h incubation, the cells were washed and stained with Calcein-AM/PI according to the manufacturer’s protocol and the fluorescence was detected via confocal laser scanning microscopy.

For further quantitative analysis of cell viability after being treated with different drugs, the content of ATP in treated MDA-MB-231 cells was detected. Briefly, the MDA-MB-231 cells were seeded in the 96-well plates (5 × 10^3 cells per well) for 24 h, followed by treatments with different drugs [free BD (5.0 μg/mL), free hemin (3.5 μg/mL), physical mixture, BH NPs and CS/BH NPs containing equal concentration to the combo free drugs] for 24 h. Subsequently, at room temperature, the same volume of CellTiter-Lumi™ plus luminescent cell viability assay kit (Beyotime Biological Technology Co., Ltd., Shanghai, China) was added to every well and co-incubated with cells for 10 min under oscillation. The cell viability was calculated by comparing the luminescence intensity with that of the control group.

2.8.2. In vitro apoptosis-inducing effects

Apoptosis of MDA-MB-231 cells following incubation with different nanoparticles was evaluated using flow cytometry. The cells were seeded in 6-well plates (2 × 10^5 cells per well) and cultured for 24 h. Then, the cells were separately incubated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs at an equivalent concentration of BD (5.0 μg/mL) and hemin (3.5 μg/mL) for 24 h. After a thorough wash with PBS, the cells were collected, washed and stained with the Annexin V-FITC/PI apoptosis kit (ComWin Biotech Co., Ltd., Beijing, China) according to the manufacturer’s protocol. The prepared samples were analyzed by flow cytometer (Beckman Coulter, Inc., CytoFLEX S, Brea, CA, USA) within 1 h.

2.8.3. Cell cycle analysis with flow cytometry

The influence of drugs on the cell cycle was analyzed by cell cycle kit according to the manufacturer’s recommendations. The MDA-MB-231 cells were seeded in the 6-well plates at a density of 2 × 10^5 cells/well and cultured for 24 h. The cells were separately treated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs at an equivalent concentration of BD (5.0 μg/mL) and hemin (3.5 μg/mL). After 24 h, the cells were collected and washed with cold PBS three times. Then, the cells were resuspended with 200 μL of PBS and added dropwise to 70% ethanol. The samples were stored at −20 °C overnight. Next, the RNA in the samples was eliminated by RNase A at 37 °C for 30 min. Finally, the samples were stained with propidium iodide (PI) for 30–60 min at 4 °C. Changes in cell cycle were analyzed by flow cytometer (Beckman Coulter Inc.).

2.8.4. Inhibition of cell migration and invasion

The inhibitory effect of different formulations on the migration or invasion of MDA-MB-231 cells was measured by wound healing and Transwell invasion assays, respectively.

For migration assay, cells were seeded into 6-well plates at a density of 5 × 10^5 cells/well. When a confluent monolayer of cells was formed, the 200-μL sterile pipette tip was used to vertically scratch the cell layer and form a wound. The floating cells and debris were removed with PBS, and the medium was exchanged with culture medium separately containing free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL). The closure of the gap was imaged at 0 and 24 h with an inverted microscope (Olympus Corp., FV3000, Tokyo, Japan). The wound area was calculated using the ImageJ software, which reflected the cell migration ability.

The invasion assay was performed using the 24-well Transwell chambers (JET Biofil, Guangzhou, China). Firstly, 100 μL of matrigel (diluted with serum-free medium) was added into the Transwell chambers and incubated at 37 °C overnight. Then, 100 μL of MDA-MB-231 cells (1 × 10^4 cells) were seeded into the upper chambers with serum-free medium, and 600 μL of DMEM medium with 20% FBS were added into the bottom of the chambers. Meanwhile, 100 μL of serum-free medium with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 1 μg/mL, hemin: 0.7 μg/mL) was separately added into the upper chambers. After 24-h incubation, invasive cells that passed through the filter were fixed with 0.1% paraformaldehyde (20 min) and stained with 0.1% crystal violet solution (20 min). Next, the cells were washed with PBS and observed under an optical microscope. Finally, the invasive cells were subjected to 33% acetic acid, and absorbance was measured at 570 nm using a microplate reader.

2.9. In vitro antitumor mechanisms

2.9.1. Extracellular acidification and oxygen consumption rate assays

Cancer cells are known to reprogram their metabolism using different strategies to meet energetic and anabolic needs. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cancer cells are two key and measurable bioenergetics parameters that link metabolic reprogramming, metabolic phenotype, and substrate preference.

ECAR and OCR were examined using Seahorse XF Glycolysis Stress Test kit and Seahorse XF Cell Mito Stress Test kit, respectively, which were determined using the Extracellular Flux Analyzer (Agilent Technologies Co., Ltd., Seahorse XFe 96, Beijing, China). Experiments were performed according to the manufacturer’s protocols. Briefly, MDA-MB-231 cells were seeded into 96-well microplates at a density of 1 × 10^4 cells/well and separately treated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL) for 24 h. For ECAR, the culture media were replaced with 160 μL of cellular assay media supplemented with 2 mM/L glutamine and the plates were incubated for 1 h in a CO2-free incubator at 37 °C. After baseline measurements, glucose (10 mM/L), oligomycin (2 μM/L, the inhibitor of oxidative phosphorylation (OXPHOS)), and 2-deoxy-D-glucose (2-DG, 50 mM/L, the
inhibitor of glycolysis) were added sequentially into each well at indicated time points. For OCR, the culture media were replaced with cellular assay media supplemented with 1 mmol/L pyruvate, 2 mmol/L glutamine, and 10 mmol/L glucose, and the plates were incubated for 1 h in a CO₂-free incubator at 37 °C. Then, all samples were measured in response to consecutive addition of (i) oligomycin [2 μmol/L, the inhibitor of adenosine triphosphate (ATP) synthase], (ii) carbonyl cyanide p-trifluoromethoxyphenylhydrazone (1 μmol/L, FCCP, the mitochondrial uncoupler), and (iii) rotenone and antimycin A [1 μmol/L, the inhibitor of complex I (NADH dehydrogenase) and complex III (cytochrome c reductase)], respectively. Data were assessed by Seahorse XF-96 Wave software.

2.9.2. Measurement of glucose uptake, lactate, glutathione, NADPH and ATP production

MDA-MB-231 cells were seeded into 96-well plates (1 × 10⁴ cells/well) and 6-well plates (5 × 10⁵ cells/well) and cultured for 24 h, then separately treated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL) for 24 h. Glucose Uptake Colorimetric Assay kit, Lactate Colorimetric Assay kit, GSH-Glo™ Glutathione Assay kit, NADP/NADPH and ATP Assay kit were used to examine glucose uptake, lactate, glutathione, NADPH and ATP production according to the manufacturer’s protocol, respectively.

2.9.3. Mitochondrial membrane potential (ΔΨm)

JC-1, a cationic fluorescent dye when added to living cells, is known to be localized exclusively in mitochondria, and was applied to measure the ΔΨm. Briefly, MDA-MB-231 cells (1 × 10⁵ cells/well) were seeded into confocal dishes and cultured for 24 h, then separately treated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL) for 24 h. After being collected and washed with PBS, cells were stained by JC-1 according to the manufacturer’s instruction and determined using the flow cytometer.

2.9.4. ROS production

The intracellular ROS production was determined using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime Biotechnology Co., Ltd., Shanghai, China). Briefly, MDA-MB-231 cells were seeded into 6-well plates (1 × 10⁶ cells/well) and cultured for 24 h, then separately treated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL) for 24 h. After washing with PBS, the cells were incubated with DCFH-DA probe (1 mL/well) for 20 min. Images and fluorescence intensity were acquired using a fluorescence microscope (ex/em: 485/527 nm) and flow cytometry, respectively.

2.9.5. Caspase activities

The activities of caspase 9 and caspase 3 in MDA-MB-231 cells were detected using colorimetric assay kits (KeyGEN BioTECH Corp., Ltd., Nanjing, China). Briefly, MDA-MB-231 cells were seeded into 6-well plates (1 × 10⁵ cells/well) and cultured for 24 h, then separately treated with free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL) for 24 h. After treatment, the cells were collected, washed, and added with cold lysis buffer to react for 40 min on ice. The supernatants of cell lysates were collected after centrifugation at 10,000 rpm (Eppendorf Corp., Centrifuge 5810R, Hamburg, Germany) for 10 min at 4 °C to measure the protein concentration using a BCA protein assay kit. Meanwhile, the supernatants were added with caspase 9 or caspase 3 substrate to react for 4 h at 37 °C. The absorbance was measured at 405 nm via a microplate reader, and the activity ratio was calculated according to the kit instructions.

2.9.6. ELISA for HK2, GAPDH, MMP1 and VEGF

The supernatant of cell lysates was obtained using the same method as described in the caspase activities test, in which the expressions of tumor glycolysis and metastasis-associated proteins, HK2 (hexokinase 2), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), MMP1 (matrix metalloproteinase 1) and VEGF (vascular endothelial growth factor) in cancer cells were estimated using ELISA kits according to the manufacturer’s protocol by determining the absorbance of the samples at 450 nm.

2.10. In vivo tumor targeting and biodistribution

MDA-MB-231 murine breast cancer models were used to investigate tumor targeting and biodistribution of CS/BH NPs. Briefly, 1 × 10⁷ MDA-MB-231 tumor cells were resuspended in 200 μL of serum-free DMEM medium and injected into the armpit of the female BALB/c nude mice. Follow-up experiments were continued after tumor volume grew to an appropriate volume. A near-infrared reflection (NIR) fluorescence imaging system (Berthold Technologies, NightOwl II LB 983, Bad Wildbad, Germany) was used to evaluate the tumor targeting capability and biodistribution of DiR-loaded NPs in MDA-MB-231 tumor-bearing female BALB/c nude mice. The mice were randomly divided into four groups (n = 3) for the intravenous injection of 200 μL of saline, free DiR, BH/DiR NPs and CS/BH/DiR NPs at an equivalent DiR concentration (0.4 mg/kg), respectively. At the set time points (1, 4, 8, 12 and 24 h), the fluorescence images were captured using the NIR fluorescence imaging system. Finally, the mice were sacrificed at 24 h post-injection, the major organs (heart, liver, spleen, lungs, and kidneys) and tumors were excised and imaged using the in vivo imaging system.

2.11. In vivo antitumor efficiency

MDA-MB-231 tumor-bearing female BALB/c nude mice were randomly divided into six groups (n = 5), and separately treated with saline, free BD, free hemin, physical mixture, BH NPs or CS/BH NPs (BD: 1.0 mg/kg, hemin: 0.7 mg/kg), respectively, via the tail intravenous injection every two days. Then, the tumor volumes and body weights of mice were recorded every two days. At defined time points, mice were humanely sacrificed, and the tumors were excised, weighed, and sliced for hematoxylin and eosin (H&E) staining and TdT-mediated dUTP Nick-End Labeling (TUNEL, Wuhan Servicebio technology Co., Ltd., Wuhan, China) assay staining.

2.12. Biosafety

To evaluate the systemic toxicity of different drugs, blood was extracted from the mice’s eye socket and subjected to hematological analyses, including liver function tests, such as aspartate transaminase (AST) and alanine transaminase (ALT), and renal function indicators such as creatinine and blood urea nitrogen. Besides, at the experimental endpoint, the major organs (heart, liver, lung, spleen, and kidney) were also collected and fixed for H&E staining to assess the adverse effects.
2.13. Statistical analysis

All results are presented as the mean value with standard deviation, and each value was the mean of at least three replicate independent experiments performed in parallel. Statistical differences were analyzed by one-way analysis of variance (ANOVA) with Tukey post-hoc analysis, where \( P < 0.05 \) was considered a statistically significant difference.

3. Results and discussion

3.1. Preparation and characterizations of CS/BH NPs

Berberine derivative (BD), synthesized as described in our previous study, and hemin co-loaded nanoparticles were prepared by the nanoprecipitation method. Next, the chondroitin sulfate (CS) molecules were coated on the surface of the BH NPs (CS/BH NPs) by strong electrostatic interactions. In the prescription study, it was found that when the mass ratio of BD to hemin was 10:7 and CS concentration was 1.0 mg/mL, the prepared nanoparticles had the smallest particle size, the smallest PDI and good stability. The encapsulation efficiency of BD and hemin in CS/BH NPs is 98.3% and 98.9%, respectively, and the drug loading is 18.9% and 20.2%, respectively. Therefore, this parameter was fixed for the subsequent preparation of CS/BH NPs (Supporting information Figs. S1 and S2).

The CS/BH NPs had spherical shapes with homogeneous size distribution, as indicated by dynamic light scattering (DLS) results and transmission electron microscope image (Fig. 1A and B). The particle size of the CS/BH NPs was around 141.3 nm and yielded a \( \zeta \) potential of around \(-23.1\) mV. The particle size of NPs exhibited good stability in both water and FBS solution (Fig. 1C and D). No significant agglomerations were observed in particles, implying that the plasma protein had little influence on NPs and NPs could be kept intact in the blood circulation. As shown in UV–Vis absorption spectrum (Fig. 1G and H), the absorption of BH NPs at 400 nm significantly decreased and the absorptive peak at 500 nm was red-shifted compared to free hemin. The addition of sodium dodecyl sulfate (SDS) and DMSO into the BH NPs could enhance and eliminate this phenomenon, respectively. The changes of UV–Vis absorption spectrum preliminarily determined that BD and hemin self-assembled to nanoparticles through hydrophobic interaction and \( \pi-\pi \) stacking.

As shown in Fig. 1E and F, free BD and hemin were almost completely released within 2 h at pH 7.4. In contrast, the cumulative release rates of BD from BH NPs and CS/BH NPs were
33.2% and 24.7% at pH 7.4, respectively. The released percentages of BD from BH NPs and CS/BH NPs were 36.7% and 28.6% at pH 6.5, similar to that at pH 7.4. However, BD could be quickly released from BH NPs (99.6%) and CS/BH NPs (89.6%) at pH 48 h at pH 5.0. The release curves of hemin were similar to those of BD. The results showed that self-assembled NPs exhibited a remarkably prolonged release in neutral medium and rapid release in acidic medium.

3.2. Hemolysis test

A hemolysis assay was performed by RBCs incubated with the BH NPs or CS/BH NPs (Fig. 1I). The photographs of the blood supernatant dissolved in Triton and water were red (positive control), whereas transparent in PBS (negative control). No hemolysis was observed even when the concentrations increased to 100 μg/mL for both BH NPs and CS/BH NPs. Interestingly, the color of blood supernatant of CS/BH NPs was consistent with the pure NPs solution, but not for BH NPs. These results indicated that the positive charge of BH NPs adsorbed on the negatively charged cell surface, and the negative charge of CS/BH NPs reduced the combination with RBCs. All the results demonstrated that the modification of CS strongly reduced the interaction between CS/BH NPs and RBCs, and enhanced the biocompatibility of CS/BH NPs. Observation of the precipitated red blood cells showed that the red blood cells still maintained their original shape (Supporting information Fig. S4B).

3.3. Cellular uptake

Effective uptake of nanomedicine by tumor cells is an important prerequisite for its antitumor effect. Due to its fluorescence, BD showed an intrinsic green fluorescence after excitation, which can be used as a detection signal to represent the uptake of NPs. As shown in Fig. 2, the fluorescence intensity of CS/BH NPs group was much stronger than those of free BD and BH NPs groups. Moreover, CS or HA pre-treatment significantly reduced the uptake of CS/BH NPs, suggesting that endocytosis of CS/BH NPs might be similar to those of HA-coated nanoparticles, that is, the internalization of which were mediated by CD44 receptors.

3.4. Lysosomal escape

To target mitochondria, CS/BH NPs must escape from lysosomes after accumulation in the acidic lysosomes. Lysosome escape of different formulations was further investigated using MDA-MB-231 cells. Lysosome was stained as red fluorescence by LysoTracker, and BD emitted green fluorescence itself. The yellow fluorescence color demonstrated localizations of BD into the lysosomes. As shown in Fig. 3A, the cell treated with CS/BH NPs had the strongest green fluorescence, in which the majority of green fluorescence located in the lysosomes (yellow fluorescence) and endosomes at 4 h after incubation. In contrast, BH NPs and CS/BH NPs displayed separation from the lysosomes as the incubation time extended to 10 h, which was characterized by weak yellow fluorescence and strong red fluorescence. Moreover, the green fluorescence of CS/BH NPs was slightly stronger than that of BH NPs, which may be attributed to the higher cell uptake of CS/BH NPs than that of BH NPs at 4 and 10 h. Therefore, the results suggested that most BH NPs and CS/BH NPs could successfully escape lysosome at 10 h after incubation.

3.5. Co-localization in the mitochondria

After escaping from lysosomes, positively charged BD can achieve mitochondrial targeting. To analyze the co-localization of BD with mitochondria, we applied free BD, BH NPs and CS/BH NPs to MDA-MB-231 cells and monitored the co-localization using CLSM. According to Fig. 3B, free BD (green fluorescence) could partially accumulate into the mitochondria stained as red fluorescence by MitoTracker. Comparatively, higher accumulation of green fluorescence in mitochondria were observed after the cells incubated with BH NPs and CS/BH NPs. Among all the samples, CS/BH NPs group exhibited the highest accumulation in mitochondria, suggesting its targetability to cancer cell mitochondria.

Figure 2  In vitro cellular uptake of CS/BH NPs by CLSM on MDA-MB-231 cells at 4 h after incubation. BD emits green fluorescence itself. Scale bar = 20 μm.
Figure 3  Intracellular localization of NPs. (A) Lysosomes escape of free BD, BH NPs and CS/BH NPs on MDA-MB-231 cells observed by CLSM. The lysosomes were stained as red by LysoTracker Red. BD emits green fluorescence itself. Yellow fluorescence indicates the overlay BD and lysosomes. (B) Mitochondria targeting of free BD, BH NPs and CS/BH NPs in MDA-MB-231 cells. The mitochondria were stained as red by MitoTracker Deep Red. BD emits green fluorescence itself. Yellow fluorescence indicates the overlay of BD and mitochondria. Scale bar = 20 μm.
Figure 4  Cytotoxicity in tumor cells. (A) Effect of different treatments on viability of MDA-MB-231 cells by CLSM (scale bar = 20 μm). (B) Quantitative analysis of the cell viability of MDA-MB-231 cells after treatment with different drugs. (C) In vitro apoptosis-inducing effects on MDA-MB-231 cells of free BD, free hemin, the mixture of BD and hemin, BH NPs and CS/BH NPs by flow cytometry. (D) Quantitative analysis of apoptosis in MDA-MB-231 cells. (E) The changes in cell cycle distribution of MDA-MB-231 cells. (F) Quantitative analysis of cell cycle distribution in MDA-MB-231 cells. In all above tests, the concentrations of BD and hemin in every group were 5.0 and 3.5 μg/mL, respectively. Data are all presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; "P < 0.05, ""P < 0.01, """"P < 0.001 vs. BD; ΔΔP < 0.01, ΔΔΔP < 0.001 vs. hemin; •P < 0.05, ••P < 0.01, •••P < 0.001 vs. Mix; □□P < 0.01, □□□P < 0.001 vs. BH NPs.

Figure 5  In vitro CS/BH NPs inhibit the migration and invasion of breast cancer cells. (A) Wound healing assay results (scale bar = 1 mm). (B) Quantitative analysis of relative migration ratio on MDA-MB-231 cells. (C) Transwell invasion assay results. (D) Quantitative analysis of relative invasion ratio on MDA-MB-231 cells. (E–F) Expression of various proteins in MDA-MB-231 cells. MDA-MB-231 cells in all above tests were treated with free BD (5.0 μg/mL), free hemin (3.5 μg/mL), physical mixture, BH NPs and CS/BH NPs containing equal concentration to the combo free drugs. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; "P < 0.05, ""P < 0.01, """"P < 0.001 vs. BD; ΔΔP < 0.01, ΔΔΔP < 0.001 vs. hemin; •P < 0.05, ••P < 0.01, •••P < 0.001 vs. Mix; □□P < 0.01, □□□P < 0.001 vs. BH NPs.
Figure 6  The effect of free BD, free hemin, the mixture of BD and hemin, BH NPs and CS/BH NPs on ECAR level (A–C), OCR level (D–F), glucose uptake (G), lactate secretion (H), total GSH level (H), total NADPH level (H) and ATP production (I) of MDA-MB-231 cells at 24 h after treatment. The concentrations of BD and hemin are 5.0 and 3.5 μg/mL, respectively. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; ^P < 0.05, ^^P < 0.01, ^^P < 0.001 vs. BD; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. hemin; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. Mix; "P < 0.05, ""P < 0.01, """P < 0.001 vs. BH NPs.

Figure 7  Mitochondrial membrane potential of MDA-MB-231 cells after 24 h of incubation with free BD, free hemin, the mixture of BD and hemin, BH NPs and CS/BH NPs (BD: 5.0 μg/mL, hemin: 3.5 μg/mL), respectively, detected by CLSM using a JC-1 probe (red fluorescence and green fluorescence represent the high and low membrane potentials, respectively. Scale bar = 20 μm).
3.6. *In vitro* tumor inhibition

3.6.1. Cytotoxicity in tumor cells

The MTT was used to investigate the *in vitro* anticancer activities. The results (Supporting information Fig. S3) showed that free BD had a certain killing effect on MDA-MB-231 cells and the antitumor effect was enhanced when BD was combined with hemin. CS/BH NPs showed significantly increased dose-dependent anticancer activities. Furthermore, Calcein-AM/PI fluorescent staining and CellTiter-Lumi™ plus luminescent cell viability assays were applied to distinguish the live/dead cells and evaluate cell viability. Fig. 4A showed that all tested groups had obvious dead cells except free hemin group. Portion of MDA-MB-231 cells died after the cells were treated with free BD and the number of dead cells increased when treated by the physical mixture group. Comparatively, with the treatment of NPs, the dead cells were further increased, and all cells almost died in the CS/BH NPs group. As shown in Fig. 4B, quantitative analysis results showed that all tested groups had a significant decrease in cell viability at 24 h after incubation, whereas the CS/BH NPs exhibited a sharply decreased viability of 21.0%. These results indicated that CS/BH NPs showed stronger tumor cell killing which was attributed to the enhanced endocytosis mediated by CS and the synergistic effect of hemin and BD.

3.6.2. Apoptosis assay

The potential therapeutic efficacy of CS/BH NPs was investigated in MDA-MB-231 cells. The apoptosis-inducing effect was obtained by using an Annexin V-FITC/PI apoptosis detection kit. As shown in Fig. 4C and D, the total apoptosis rate induced by CS/BH NPs group was 51.7%, which was remarkably higher than those groups of BD 22.9%, hemin 6.7%, Mix 35.2% and BH NPs 43.0%.

3.6.3. Cell cycle analysis

Furthermore, cell cycle progression experiments were designed to examine changes in cell cycle phase distribution of different drugs on MDA-MB-231 cells *via* flow cytometric analysis. As shown in Fig. 4E and F, the percentages of G0/G1, S and G2/M phase of the control group represented 63.6%, 22.6% and 13.5%, respectively. G0/G1 phase cells decreased and G2/M phase cells increased for Mix group and the NPs groups, compared with the untreated control cells. Especially for CS/BH NPs group, the proportions change of G0/G1 and G2/M phase cells reached 50.8% and 23.7%, respectively. Taken together, it suggested that the G2/M-block is caused by drugs assigned to its capacity to antitumor activity.

3.6.4. Inhibition of migration and invasion

Metastasis was considered a critical factor for poor prognosis of TNBC\(^2\), and tumor cell migration and invasion were prerequisites. To investigate the inhibition effect of self-assembled NPs on MDA-MB-231 cell metastasis, *in vitro* wound healing and Transwell invasion assays were performed (Fig. 5A–D). The migration results of the control group showed that MDA-MB-231 cells had a strong migration ability. Compared to control group, all tested groups were...
able to inhibit the migration (free BD: 64.6%, free hemin: 52.3%, Mix: 40.5%, BH NPs: 24.6%, CS/BH NPs: 10.4%) and invasion (free BD: 80.9%, free hemin: 79.0%, Mix: 59.0%, BH NPs: 39.3%, CS/BH NPs: 25.3%) of tumor cells, and the inhibition efficiency of CS/BH NPs reached the maximum.

3.7. Antitumor mechanisms

3.7.1. Regulation of tumor metabolism

One of the pivotal metabolisms shifts of tumor cells is that, even in oxygen-rich condition, they preferred the lactic acid fermentation pathway to produce ATP, also known as “Warburg effect”\(^3\). In tumor cells, glucose uptake was increased and converted to pyruvate by hexokinase and glyceraldehyde-3-phosphate dehydrogenase, then oxidized to lactate. These metabolites and the associated H\(^+\) productions were secreted to the microenvironment and resulted in decreased pH of the extracellular environment\(^3\). Then, glucose uptake, lactate production and ECAR that link metabolic reprogramming, metabolic phenotype, and substrate preference in tumor cells are the main and easily measurable parameters of glycolysis.

Changes in the complexes of mitochondrial electron transport chain are also identified in cancer. Complex I plays an important role in maintaining the mitochondrial function and is involved in ATP and ROS production\(^3\). ROS in cancer cells plays a vital role in regulating cell death, metabolic reprogramming, and tumor microenvironment. GSH, as the ROS scavenger, inhibits ROS accumulation and reduces cell death. BD and hemin, all complex I inhibitors, may decrease \(\Delta G_m\) and ATP synthesis, trigger the production of ROS, and further promote proliferation arrest and tumor cell death. Meanwhile, the oxygen consumption rate (OCR) is also an important indicator for mitochondrial functions. Therefore, the effect of self-assembled NPs on cancer metabolism and its metabolites was investigated.

As shown in Fig. 6A–C, free BD and hemin could slightly decrease the levels of ECAR, basal glycolytic rate and maximal glycolytic rate of MDA-MB-231 cells. When tumor cells were treated with the mixture of BD and hemin, these values were further decreased and the CS/BH NPs group had the lowest level (7.5 mpH/min, 41.4% and 22.0%, ECAR, glycolysis and maximal glycolysis, respectively). Consistent with the results of ECAR, CS/BH NPs group also showed the strongest inhibitory effect on OCR levels. As shown in Fig. 6D–F, the values of OCR, basal OCR, and maximal OCR of CS/BH NPs group were 22 pmol/min, 21.8% and 26.6%, respectively. CS/BH NPs led to dramatic decreases in both OCR and ECAR indicating that mitochondrial and glycolytic metabolisms were reduced simultaneously to give a hypometabolic state\(^3\). Decreased ATP production further confirmed the inhibition effect of CS/BH NPs on mitochondrial respiration. CS/BH NPs induced an approximately 80.0% decrease in ATP production in MDA-MB-231 cells (Fig. 6I, 20.3% for the control). All these results also indicated that the

![Figure 9](image-url)
lipophilic BD had the ability to inhibit mitochondria metabolism in tumor cells. Meanwhile, tumor cell metabolites, including glucose uptake, lactate production, total GSH level and total NADPH were tested via corresponding assay kits. The trends of all results were consistent. Namely, the levels of these metabolites were slightly decreased in free BD and free hemin groups further decreased in the mixture of BD and hemin group, and the lowest in the CS/BH NPs group. The percentages of glucose uptake and extracellular lactate secretion of CS/BH NPs group (Fig. 6G, 35.0% and 33.4% of control, respectively) were decreased, which can effectively change the acidic environment of tumor site, and thus inhibit tumor proliferation and metastasis. The significant decrease of total GSH and NADPH levels of CS/BH NPs group (Fig. 6H, 42.4% and 41.4% of control, respectively) in tumor cells suggested that CS/BH NPs could induce tumor cell death via ROS accumulation. All the results demonstrated that CS/BH NPs possessed the potential to inhibit BACH1-related mitochondrial and glycolytic metabolisms of tumor cells, thereby blocking the brisk metabolic adaption of tumor cells, as a promising strategy for TNBC therapy.

3.7.2. Decrease mitochondrial membrane potential ($\Delta \psi _m$) In accordance with the above results, the self-assembled nanoparticles could inhibit the metabolism of tumor cells. Then the interference of nanoparticles on mitochondrial function was investigated. The assessment of $\Delta \psi _m$ in intact cells could reflect their physio-pathological conditions$^{34}$ and its Loss is a characteristic of apoptosis. JC-1, as a reliable fluorescent probe, can predominantly localize in mitochondria and qualitatively assess the $\Delta \psi _m$. Dissipation of $\Delta \psi _m$ leads to the depression of JC-1 mitochondrial accumulation, which leads to the decrease in JC-1 aggregate formation (red fluorescence) and the increase of JC-1 monomer formation (green fluorescence)$^{35}$. Accordingly, the fluorescent color of JC-1 changed from red to green. As shown in Fig. 7, the images of free BD group showed weak green

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**Figure 10** Antitumor activities of self-assembled NPs. (A) Tumor tissues excised from euthanized MDA-MB-231 tumor-bearing mice after applying different drugs for 14 days. (B) Tumor growth curves after the intravenous injection of different drugs. (C) The tumor inhibitory rate. The TIR is calculated using the following equation: TIR (%) $= (W_{control} - W_{treated})/W_{control} \times 100$. (D) Tumor weights of six tested groups at 14 days after treatment. (E) Histological observation of the tumor tissues after treatment. Scale bar $= 200 \mu m$. (F) TUNEL assay of tumor sections treated with free drugs, physical mixture and self-assembled NPs. Scale bar $= 100 \mu m$. Mice were treated with saline (control), free BD, free hemin, Mix, BH NPs and CS/BH NPs, respectively (BD: 1.0 mg/kg, hemin: 0.7 mg/kg). Data are presented as the mean $\pm$ SD ($n = 5$). **$P < 0.01$, ***$P < 0.001$ vs. Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. BD; $^{\Delta}P < 0.01$, $^{\Delta\Delta}P < 0.001$ vs. hemin; $^{\bullet}P < 0.05$, $^{\bullet\bullet\bullet}P < 0.001$ vs. Mix; $^{\circ}P < 0.05$ vs. BH NPs.
fluorescence. When tumor cells were treated with the mixture of free BD and free hemin, the green fluorescence increased, implying a further dissipation of $\Delta \psi_m$. By comparison, the dissipated $\Delta \psi_m$ of CS/BH NPs was the most significant and there was almost no red fluorescence. Meanwhile, the decreased $\Delta \psi_m$ in MDA-MB-231 cells indicated that CS/BH NPs-induced apoptosis was related with mitochondrial dysfunction.

### 3.7.3. ROS production

Excess ROS causes the damage of mitochondrial membrane, the release of cytochrome C, up-regulates caspase level and triggers apoptosis. The destruction of mitochondrial metabolism could influence intracellular redox homeostasis, so the ROS production of MDA-MB-231 cells after incubation with different drugs via DCFH-DA reagent was detected. As shown in Fig. 8A, the green fluorescence, representing ROS production, increased dramatically after applying CS/BH NPs. Meanwhile, the quantitative analysis showed the ROS level of free BD, free hemin, Mix, BH NPs and CS/BH NPs increased 3.3-, 2.5-, 3.2-, 6.5-, and 9.3-fold compared to the control group, respectively (Fig. 8B and C).

### 3.7.4. Caspase activities

The dissipated $\Delta \psi_m$ and increased ROS suggested mitochondrial dysfunction, which resulted in the release of cytochrome C into the cytoplasm, and then further activated caspase 9 and caspase 3.36. To further investigate whether the antitumor activity of CS/BH NPs attributed to this apoptotic pathway, the activities of intracellular caspase 3 and caspase 9 were detected by using the spectrophotometric method. Consistent with the results of ROS production, the activities of caspase 3 and caspase 9 of CS/BH NPs group were strongly increased (136.9% and 129.8% of control, respectively), while there was only slightly increase in free drug groups (Fig. 8D and E).

### 3.7.5. The expression of glycolysis and metastasis-associated proteins

Meanwhile, a similar trend was also observed in the expressions of proteins HK2, GAPDH, MMP1 and VEGF, which were associated with tumor metastasis (Fig. 5E and F). CS/BH NPs could dramatically down-regulate the expressions of four proteins, especially for the MMP1. HK2 and GAPDH are the pivotal proteins in the glycolysis of tumor cells. The high expressions of MMPs and VEGF in tumors are responsible for extracellular matrix (ECM) degradation and vascular permeability, respectively, then promoting tumor cell invasion and metastasis37. Therefore, CS/BH NPs could inhibit tumor proliferation and metastasis by down-regulating the expression of metabolism-related proteins and blocking the release of tumor cells from the ECM, indicating that the degraded BACH1 therapy method exerted significant antitumor activity in vitro.

In conclusion, after being endocytosed into tumor cells, hemin and BD exhibited their potency for antitumor. For example, CS/BH NPs could decrease oxygen consumption and oxidative phosphorylation, inhibit glycolysis, and reduce ATP production in TNBC cells. This energy deficiency, together with compromised $\Delta \psi_m$ and elevated oxidative stress, resulted in tumor cells apoptosis, finally inhibiting tumor proliferation and metastasis simultaneously.

### 3.8. In vivo imaging and biodistribution

Free DiR and DiR-loaded self-assembled NPs were administrated intravenously into MDA-MB-231 tumor-bearing BALB/c nude mice. A preliminary evaluation of safety of self-assembled NPs in vivo was performed. (A) Body weight changes of the tumor-bearing mice. (B) Hepatic and renal function indicators of mice bearing MDA-MB-231 tumor xenografts after treatment. (C) Hematoxylin and eosin (H&E) stained organ slices from the MDA-MB-231-bearing nude mice after treatment. Scale bar = 200 μm. These six groups of mice were treated with saline (control), free BD, free hemin, Mix, BH NPs and CS/BH NPs, respectively (BD: 1.0 mg/kg, hemin: 0.7 mg/kg). Data are presented as the mean ± SD (n = 5, independent animals were imaged and showed similar results). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.
mice for the evaluation of tumor targeting and biodistribution in vivo. The DiR was performed using the NIR fluorescence imaging system and quantified by region-of-interest (ROI) analysis. At all set time points, only weak fluorescence was observed in free DiR group at the tumor site (Fig. 9A). Although both BH/DiR NPs and CS/BH/DiR NPs groups showed strong fluorescence at 8 h compared with free DiR group (1.5- and 1.9-fold, respectively), only CS/BH/DiR NPs group remained in tumor region up to 24 h with the intense fluorescence (1.8-fold higher than that of free DiR, Fig. 9C).

Mice were sacrificed at 24 h and the major organs and the tumors were excised for ex vivo imaging. The results of ROI analysis showed that CS/BH/DiR NPs group had the strongest fluorescence signal in tumor, which was 2.4- and 1.5-fold higher than that of free DiR group and BH/DiR NPs group, respectively (Fig. 9B and D). Meanwhile, the fluorescence intensity of CS/BH/DiR NPs group was decreased in other major organs, especially in the liver and spleen. This suggested that the CS prevented the maximum amount of CS/BH NPs from being cleared by phagocytosis and the reticuloendothelial system, making CS/BH NPs effectively target the tumor site.

3.9. In vivo antitumor efficacy

The therapeutic effects of free drugs and our self-assembled nanoparticles were evaluated in a tumor xenograft nude mouse model established using MDA-MB-231 cells. For the treatment groups, nude mice respectively received i.v. injections of free BD, free hemin, Mix, BH NPs or CS/BH NPs every two days for a total of seven doses (BD: 1.0 mg/kg, hemin: 0.7 mg/kg). As shown in Fig. 10A–D, tumor growth in the saline group and the free hemin group was rapid, the tumor volume was 2245 and 1818 mm³, respectively. The free BD and Mix groups showed similar therapeutic efficacy (tumor volume, 1568 mm³ for free BD and 1454 mm³ for Mix). Comparatively, CS/BH NPs treatment significantly inhibited tumor progression: the tumor volume remained small (492 mm³, 4.6-fold smaller than that of control group) and TIR was up to 74.5% after 14-day treatment.

To further confirm the antitumor effect in vivo, the tumors were excised for H&E staining assay. The blue and pink respectively represented the nucleus and the cytoplasm. As shown in Fig. 10E, the control group showed typical features of the tumor tissues such as largely and deeply stained nucleus as well as less cytoplasm. In contrast, the tumor tissue of treated groups showed different levels of necrosis such as wrinkled nucleus and increased cytoplasm area. The CS/BH NPs group showed the largest area (80%–90%) of tumor necrosis. As shown in Fig. 10F (TUNEL assay results), CS/BH NPs showed the strongest apoptosis-inducing effects, indicating that the delivery of BD and hemin to tumor could effectively inhibit tumor growth. This result was consistent with the in vitro and in vivo studies, implying that CS/BH NPs had the strongest anti-tumor efficacy.

3.10. Preliminary evaluation of safety of NPs in vivo

Body weights were measured to assess the in vivo systematic toxicity of self-assembled NPs after i.v. administration (Fig. 11A). It was illustrated that none of the groups significantly altered the body weights of mice during intravenous injection progress. Besides, hematological parameters such as AST, ALT, creatinine and BUN were examined and had similar results with body weights. According to Fig. 11B, compared with the control group, the values of AST, creatinine and BUN in free BD group were significantly different, suggesting that free BD had slightly hepatorenal toxicity. A similar phenomenon was seen in the free hemin group, which obvious changes were observed in ALT and BUN values, also suggesting that free hemin had mild liver and kidney toxicity. For the BH NPs group, the levels of BUN and creatinine showed a slight increase, indicating mild kidney toxicity, which was possibly related to the positive charges BH NPs carried. However, almost no obvious changes were observed between CS/BH NPs group and the control group, except for the level of BUN, demonstrating that self-assembled nanoparticles were safer than free drugs in vivo. Additionally, mice treated with CS/BH NPs exhibited no noticeable major organ (heart, liver, spleen and kidneys) pathological abnormality and lung metastasis by H&E staining (Fig. 11C and Supporting information Fig. S5). These results indicated that tumor-targeted nanoparticles with potent antitumor activity elicited no significant toxicities to the major organs in mice.

4. Conclusions

Trapped by distinct cancer metabolism and metabolic heterogeneity, the combination therapy strategy of TNBC based on multiple mechanisms has attracted much more attention. In this work, we developed tumor-targeted self-assembled nanoparticles to coordinate regulate BACH1 and mitochondrial metabolism for the combination therapy of TNBC. BACH1 inhibitor hemin and mitochondria function inhibitor BD self-assembled to form BH NPs in a simple nano-precipitation method followed by coating chondroitin sulfate on the surface to enhance the stability and tumor targeting of the prepared CS/BH NPs. Besides the excellent tumor and mitochondria targeting of CS/BH NPs, hemin encapsulated in the nanoparticles effectively suppressed BACH1, resulting in decreased tumor cell metabolites, glycolysis, metastasis-associated proteins expressions and dissipating mitochondrial membrane potential. Moreover, BD, an effective inhibitor of mitochondrial metabolism, demonstrated to be more beneficial for inhibiting tumor cell migration and invasion with the combination of hemin. The results of uptake and biodistribution in vitro and in vivo revealed that CS/BH NPs could be effectively delivered to the tumor cells and target mitochondria. The tumor inhibition rate of CS/BH NPs in a tumor xenograft nude mouse model was up to 74.5% without observable toxicities to other organs, demonstrating the excellent efficacy and safety of CS/BH NPs. In conclusion, the design and application of CS/BH NPs via exerting influence on the regulation of BACH1 and mitochondrial metabolism could be a potential strategy for effective therapy of TNBC.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 81973264, 82104080 and 81773659); Guangdong Basic and Applied Basic Research Foundation, China (Nos. 2020A1515010593, 2019A1515011954 and 2021A1515012621); Guangdong Provincial Key Laboratory of Construction Foundation, Sun Yat-sen University (No. 2019B030301005, China); the Fundamental Research Funds for the Central Universities, Sun Yat-sen University (No. 22qntd4509, China).

Author contributions

Xuan Yang, Yalong Wang and Haiyan Hu designed the research. Xuan Yang carried out the experiments and performed data
analysis. Yalong Wang, Junke Zhao, Hehui Rong, Yujun Chen, Mengting Xiong and Xiaoxing Ye participated part of the experiments. Hayian Hu provided experimental drugs and quality control. Shihui Yu wrote the manuscript. Shihui Yu and Yalong Wang revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest
The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.06.009.

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