Synergistic Cascade Strategy Based on Modifying Tumor Microenvironment for Enhanced Breast Cancer Therapy

Huan Zhang

Sichuan University West China Hospital

Jinshun Xu

Sichuan University West China Hospital

Binyang Gao

Sichuan University West China Hospital

Hong Wang

Sichuan University West China Hospital

Jianbo Huang

Sichuan University West China Hospital

Jie Zhou

Sichuan University West China Hospital

Rui Yang

Sichuan University West China Hospital

Feng Yan

Sichuan University West China Hospital

Yulan Peng (✉ yulanpeng520@126.com)

West China School of Medicine: Sichuan University West China Hospital <https://orcid.org/0000-0003-3077-0746>

Research Article

Keywords: Breast cancer, Tumor microenvironment, Drug delivery, Hyperthermia, Nanoparticles

Posted Date: July 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-700346/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Frontiers in Pharmacology on November 15th, 2021. See the published version at <https://doi.org/10.3389/fphar.2021.750847>.

Abstract

Background: Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer with very few treatment options. Although tumor-targeted nanomedicines hold great promise for the treatment of TNBC, the tumor microenvironment (TME) continues to be a major cause of failure in nanotherapy and immunotherapy. To overcome this barrier, we designed a new synergistic cascade strategy (SCS) that uses mild hyperthermia and smart drug delivery system (SDDS) to alter TME resistance in order to improve drug delivery and therapeutic efficacy of TNBC.

Methods: Mild hyperthermia was produced by microwave (MW) irradiation. SDDS were formulated with thermosensitive polymer-lipid nanoparticles (HA-BNPs@Ptx), composed of polymer PLGA, phospholipid DPPC, hyaluronic acid (HA, a differentiation-44 targeted molecule, also known as CD44), 1-butyl-3-methylimidazolium-L-lactate (BML, a MW sensitizer) and paclitaxel (Ptx, chemotherapy drug). 4T1 breast tumor-bearing mice were treated with two-step MW combined with HA-BNPs@Ptx. Tumors in mice were pretreated with 1st MW irradiation prior to nanoparticle injection to modify TME and promote TME and promoting nanoparticle uptake and retention. The 2nd MW irradiation was performed on the tumor 24 h after the injection HA-BNPs@Ptx to produce a synergistic cascade effect through activating BML, thus enhancing hyperthermia effect, and instantly releasing Ptx at the tumor site.

Results: Multifunctional CD44-targeted nanoparticles HA-BNPs@Ptx were successfully prepared and validated *in-vitro*. After the 1st MW irradiation of tumors in mice, the intratumoral perfusion increased by 2 times and the nanoparticle uptake augmented by 7 times. With the 2nd MW irradiation, remarkable anti-tumor effects were obtained with the inhibition rate up to 88%. In addition, immunohistochemical analysis showed that SCS therapy could not only promote the tumor cells apoptosis, trigger the immune response of cytotoxic T lymphocytes, but also significantly reduce the lung metastasis.

Conclusions: The SCS using mild hyperthermia combined with smart drug delivery system, can significantly improve the efficacy of TNBC treatment in mice by modifying TME and hyperthermia-mediated EPR effects.

Background

Breast cancer is the most common cancer and one of the leading causes of cancer-related death in women worldwide [1]. Despite of the early detection and intervention, metastatic breast cancers remain largely incurable, especially triple-negative breast cancer (TNBC) [2, 3]. TNBC is aggressive subtype of breast cancers and accounts up to 10–20% of all breast cancer cases [4]. Due to the lack of specific targets and high probability of metastasis, currently available treatment options are very limited [5]. Metastatic breast cancer is characterized by a unique tumor microenvironment (TME), which differs from other subtypes. The components of TME including transformed extracellular matrix (ECM), soluble factors, immune suppressive cells, epigenetic modifications and re-programmed fibroblasts together hamper antitumor response and helps progression and metastasis of TNBC [6, 7]. Another barrier to

breast cancer is its high heterogeneity, which complicates treatment [8, 9]. For example, a small piece of tumor tissue obtained by biopsy does not necessarily represent all the tumor components [10]. In addition, high interstitial fluid pressure (IFP) generated by TME also severely limits drug delivery to tumor cells, especially in immunotherapy and nanotherapy [11]. High IFP in TME may compress blood vessels, resulting in reduced intratumoral blood flow and nanodrug delivery [12, 13]. Since the TME is involved in the proliferation, angiogenesis, apoptosis inhibition, immune system suppression and drug resistance of metastatic breast cancer, it becomes recently an important target of TNBC therapy [14–16].

Hyperthermia combined with nanoparticles loaded with chemotherapeutic agents holds great promise for the treatment of cancers [17]. However, the efficacy in solid cancer treatment has not been clinically proven. For example, numerous clinical trials have been conducted since 2006 using ThermoDox, doxorubicin-loaded low temperature sensitive liposomes (LTSLs) to treat hepatic, colorectal, prostate and breast cancer [18]. Hyperthermia can be achieved using different heating techniques, such as radiofrequency (RF), focused ultrasound (FUS) and microwave (MW) [19]. A phase I study (TARDOX) recently showed that the combined treatment of LTSLs and non-invasive FUS hyperthermia seems to be clinically feasible, safe, and able to enhance intratumoral drug delivery [20]. Although an increased intratumoral drug delivery has been demonstrated in preclinical study, most of phase II and phase III Trials of ThermoDox were failed to demonstrate the benefit of the combined treatment over chemo- or thermal-therapy alone [21, 22]. Therefore, the preclinical research and clinical translation of thermosensitive nanomedicines are still facing huge demands and challenges [23, 24].

Hyperthermia induced by MW is a promising adjuvant therapy, which can be used to induce the apoptosis of tumor cells and destroy ECM in TME [25]. MW can penetrate deeply into most of solid tumors in patients, does not produce drug resistance of TME and has no systemic side effects [26]. Mild MW hyperthermia can also induce apoptosis of tumor cells at 39–45 °C [17, 27]. However, there are very few reports using MW hyperthermia combined with Ptx-loaded nanoparticles to treat metastatic breast cancers. Therefore, the need for combination therapy to overcome the resistance of breast tumors and improve the efficacy of current treatment is very clear.

In the present study, we proposed a new approach to enhance the bioavailability of nanodrugs and the efficacy of hyperthermia to treat triple negative breast cancer called “Synergistic Cascade Strategy” (SCS). The strategy consists of using local MW hyperthermia combined with smart drug delivery system (SDDS) to treat TNBC in a 4T1 breast cancer-bearing mice model. SDDS was formulated with polymer-lipid nanoparticles (HA-BNPs@Ptx), composed of polymer PLGA, phospholipid DPPC, hyaluronic acid (HA, targeting CD44), 1-butyl-3-methylimidazolium-L-lactate (BML, a MW sensitizer) and paclitaxel (Ptx, chemotherapy drug). Our previous work [28] showed that MW responsive nanoplatform made of lipid nanoparticles, containing DPPC and BML could efficiently deliver doxorubicin and inhibit hepatocellular carcinoma (HCC) progression with distant lung metastasis. The rational of SCS is the following: (1) performing the 1st MW on tumor to induce mild hyperthermia (≈43°C), in order to reduce IFP in TME and increase intratumoral blood flow. The mild hyperthermia also allows “permeabilizing” tumoral vessels and alter the tumor surrounding matrix, thus promoting the extravagation and penetration of

nanoparticles to reach tumor cells; (2) injecting long circulating target PLGA-DPPC nanoparticles immediately following the 1st MW exposure to allow the maximal accumulation of nanoparticles at tumor sites; and (3) after nanoparticle accumulation, performing 2nd MW exposure to activate the sensitizer BML thus increasing rapidly the temperature inside the tumor and instantly release Ptx from nanoparticles to induce the tumor cell apoptosis and destroy ECM in TME through synergistic cascade effects of hyperthermia and chemotherapy agent. Scheme 1 illustrates the scenario of the above approach.

Materials And Methods

Materials

NH₂-PLGA-NH₂ (lactide:glycolide, 50/50, MW: 8 kDa) was obtained from Xi'an Rui-Xi biological technology Co. Ltd. (Xi'an, Shaan Xi, China). 1-butyl-3-methylimidazolium-L-lactate (BML, MW: 228.29 Da) was provided by Cheng Jie Chemical Co. Ltd. (Shanghai, China). Hyaluronic acid (HA, MW: 3.8 kDa) was purchased from Dalian Meilun Biotechnology Co. Ltd. (Dalian, Liao Ning, China). Paclitaxel (Ptx, MW: 853.93 Da) was purchased from Shanghai Aladdin Biotechnology Co. Ltd. (Shanghai, China). 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC, MW: 734.1 Da), polyvinyl alcohol (PVA, MW: 30-70 kDa), 2-(N-morpholino) ethane sulfonic acid (MES), N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). All reagents used in this work were of analytical grade without further purification.

Preparation of HA-BNPs@Ptx

The double emulsification method was used to synthesize Ptx & BML loaded DPPC-PLGA hybrid nanoparticles (BNPs@Ptx) following the previous report [29]. Briefly, 10 mg DPPC, 20 mg NH₂-PLGA-NH₂ and 2 mg Ptx were thoroughly dissolved in 2 mL dichloromethane (DCM). 400 µL BML (0.5 mg/mL, in deionized water) was mixed in the solution. The Ptx and BML solution was emulsified on ice bath (2 min, with a 5 s on-off duty cycle) using an ultrasonic oscillation instrument (SCIENTZ-IIID, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, Zhejiang, China) at 12% power (24 kHz, 600 W). 8 mL PVA (2% w/w, in water) solution was further added to the emulsion and emulsified as before. DCM was then evaporated in a fume hood for 3 h under magnetic stirring. Finally, BNPs@Ptx was collected by centrifugation at 10000 g for 10 min and washed twice with double distilled water. Nanoparticles targeting CD44 were prepared by covalently coupling hyaluronic acid to BNPs@Ptx via amino groups on PLGA [30]. Briefly, 2 mg HA was dissolved in 195 mg MES buffer (pH = 5.5). 27 mg EDC and 8.6 mg NHS were then added to HA solution. Subsequently, the mixture was incubated with 1 mL BNPs@Ptx suspension under continuous stirring for 24 h on ice bath. HA-BNPs@Ptx was collected and purified by centrifugation and washing with double distilled water 2 times.

Characterization of HA-BNPs@Ptx

Morphology and physicochemical properties of HA-BNPs@Ptx

Transmission electron microscopy (TEM, Tecnai G2 F30, FEI Co., Ltd., Hillsboro, Oregon State, USA) and scanning electron microscope (SEM, SU8020, Hitachi, Ltd., Tokyo, Japan) were used to observe the morphology of nanoparticles. The size distribution, polydispersity index (PDI) and zeta potential were obtained using dynamic light scattering (DLS, Brookhaven Omni, Brookhaven Instruments Inc., Holtsville, NY, USA). The Fourier transform infrared spectrometer (FTIR, Nicolet iS10, Thermo Fisher Scientific Co., Ltd, Massachusetts, USA) was used to record the spectra of HA, BNPs@Ptx and HA-BNPs@Ptx in the wavelength range of 500–4000 cm^{-1} . The drug loading (DL) and encapsulation efficiency (EE) of Ptx were quantified using a standard calibration curve measured at 229 nm on a visible ultraviolet spectrophotometer (L5S, INESA Analytical Instrument Co., Ltd., Shanghai, China, see Additional file 1, Methods 1.1 for details).

MW induced thermal effect and Ptx release of HA-BNPs@Ptx

To evaluate thermal effect of MW irradiation with sensitizer BML, 1 mL HA-BNPs@Ptx or HA-NPs@Ptx suspensions were added to 24-well plate and exposed to MW energy instrument (WZY-1, Beijing Muheyu Electronics co., Ltd, Beijing, China). The suspensions were irradiated for 4 min at different MW power (0.4, 0.8, 1.2 and 1.6 W cm^{-2} , 450 MHz). The temperature change of HA-BNPs@Ptx suspension was recorded every minute using an infrared thermal mapping instrument (FLUKE 572-2, Hawk-IR International, Inc., Everett, WA, USA). Ptx release kinetics upon MW heating was evaluated by using visible ultraviolet spectrophotometer.

In-vitro biocompatibility and targeting ability of HA-BNPs@Ptx

Cytotoxicity assay

Mouse breast cancer cell line 4T1, mouse macrophage cell line J774 and human umbilical vein endothelial cells (HUVEC) were purchased from Cellcook (Guangzhou Cellcook Cell Biotechnology, LTD., Guangzhou, Guangdong, China) and cultured according to the supplier's instructions. 4T1 cells and HUVEC were seeded into a 96-well plate (1×10^4 cells per well) and incubated overnight. 5 duplicate holes were set in each group. Subsequently, 100 μL HA-BNPs@Ptx at Ptx concentrations (2, 5, 10, 20 and 50 $\mu\text{g/mL}$) was introduced to each group. The cytotoxicity of HA-BNPs@Ptx was examined by CCK-8 viability assay (Boster Biological Technology Co., Ltd., California, USA). The optical density (OD) was measured at 450 nm by Varioskan Flash microplate reader (Synergy Mx, BioTek Instruments, Inc., Winooski, VT, USA). The cytotoxicity treated with HA-NPs (without drug loaded NPs) was evaluated using the same method.

Hemolytic activity

2% suspension of chicken red blood cells were used to determine hemolytic activity of HA-BNPs@Ptx. 100 μL of phosphate buffer saline (PBS), double distilled water or HA-BNPs@Ptx suspensions at Ptx concentrations of 2, 5, 10, 20 and 50 $\mu\text{g/mL}$ were added to erythrocyte suspensions (100 μL). PBS was used as negative control group, and double distilled water was used as positive control group. All

suspensions were incubated at 37 °C for 1 h and centrifuged at 800 g for 10 min. The OD was measured at 550 nm using the Varioskan Flash microplate reader.

CD44 receptor mediated nanoparticles targeting

4T1 cells were used to verify CD44 targeting ability of nanoparticles. The CD44 expression level of 4T1 cells was evaluated by an inverted fluorescence microscope (see Additional file 1, Methods 1.2 for details). The targeting test was performed using BNPs@Ptx, HA-BNPs@Ptx and HA-BNPs@Ptx with the cells in which CD44 receptor was presaturated by an excess amount of free HA [31]. To obtain stained nanoparticles, 1 mL BNPs@Ptx or HA-BNPs@Ptx suspensions were stained with 10 µL DiO (1:100). After overnight culture, 4T1 cells were co-incubated with 100 µL BNPs@Ptx or HA-BNPs@Ptx for 6 h. The targeting ability was visualized by the inverted fluorescence microscope (AX10 imager A2/AX10 cam HRC, Carl Zeiss, Co., Ltd, Jena, Germany). Flow cytometry (FCM, Cyto Flex, Beckman Coulter, Inc., CA, USA) was used to further evaluate the ability of HA-BNPs@Ptx to target 4T1 cells at different time intervals (30 min, 1, 3, 6, and 24 h).

***In-vitro* cellular uptake and intracellular tracking**

To examine cellular uptake and intracellular trafficking, BNPs@Ptx and HA-BNPs@Ptx were labelled with a red fluorescence probe Dil. Endocytosis/phagocytosis experiment were performed in 4T1 cells and J774 cells (murine macrophages), respectively. 1×10^4 4T1 cells per well were seeded in confocal dishes. After 24 h, 100 µL Dil-BNPs@Ptx and Dil-HA-BNPs@Ptx were added to co-incubate with 4T1 cells for 1, 3 and 6 h, respectively. At each time point, 20 µL LysoTracker Green was added to each confocal dish to stain endo/lysosomes. 1.5 h after incubated with LysoTracker Green, all cells were fixed with 4% paraformaldehyde for 10 min. 10 µL 4,6-diamidino-2-phenylindole (DAPI, blue) was finally added in fixed cells to stain the nuclei. Confocal fluorescence images of the fixed cells were obtained by a laser scanning confocal microscope (LSCM, A1R+MP, Nikon Co., Tokyo, Japan).

J774 cells were treated in the same way as 4T1 cells, except that 5×10^4 cells and 10 µL of PMA (100 ng/ml) were added to each dish and incubated for 48 h.

***In-vitro* synergistic anti-tumor ability**

Synergistic anti-cancer activity of thermal-chemotherapy was evaluated with 4T1 cells *in-vitro*. The cells were seeded into 6-well plate (7.5×10^5 cells per well) for 24 h and then incubated with (i) PBS, (ii) HA-BNPs@Ptx, (iii) MW, (iv) BNPs@Ptx + MW, (v) HA-BNPs@Ptx + MW for 6 h. In order to optimize the thermal effects of nanoparticles, HA-BNPs@Ptx/4T1 cells were further exposed to MW irradiation at different power (0.4, 0.8, 1.2 and 1.6 W cm⁻²) for 1, 2, 3 and 4 min, respectively (see Additional file 1, Methods 1.3 for details). After incubation, the cells of the last 3 groups were exposed to MW irradiation (0.8 W cm⁻², 4 min). The anti-cancer activity of each group was qualitatively examined by using Calcein-AM/propidium iodide (PI) double stain kit (Beyotime Biotechnology® Inc., Suzhou, Jiangsu, China). After the staining of Live/Dead cells, all cell samples were imaged under the inverted fluorescence microscope.

To quantitatively assess the synergistic chemo-thermal therapy, 4T1 cells were stained by Annexin V-(FITC)/PI apoptosis detection kit (4A Biotech Co., Ltd, Peking, China) and analyzed by flow cytometer.

***In vivo* TNBC models, biodistribution and targeting ability**

BALB/c mice (female, 18-20 g) were provided by Dashuo Biological Technology (Chengdu, Sichuan, China). All processes were in accordance with the Chinese Society of Laboratory Animals on animal welfare and approved by the Animal Use and Care Management Advisory Committee of West China Hospital of Sichuan University (Approval No. 2017014A). 4T1 cells (1×10^6 /wells) were implanted into the second axillary mammary fat pad on the right side. When the volume of tumor reached about 100 mm³, 4T1 breast tumor-bearing mice were randomly assigned into two groups (6 mice per group) to compare CD44-targeted (Dil-HA-BNPs@Ptx) and non-targeted (Dil-BNPs@Ptx) nanoparticles. Nanoparticles (0.5 µL/g) were injected into 4T1 breast tumor-bearing mice via tail vein. *In vivo* fluorescence images were collected before and after injection of nanoparticles at 1, 3, 6 and 24 h using IVIS Spectrum system (Lumina XR, Caliper Life Sciences, Boston, Massachusetts, USA). To examine the biodistribution of nanoparticles, the main organs (heart, liver, spleen, lungs, and kidneys) and tumors were isolated from mice to perform *ex-vivo* imaging using the same IVIS Spectrum.

Blood chemistry analysis of BALB/c mice after 7 days intravenously injected with HA-BNPs@Ptx (see Additional file 1, Methods 1.4 for details).

***In vivo* mild hyperthermia - the 1st MW irradiation**

The 1st MW irradiation aimed to generate mild hyperthermia to alter the TME to increase the uptake of nanoparticles. 6 mice in each group received mild hyperthermia at the tumor site with a MW power of 0.8 W cm⁻². The temperature of tumor was monitored in real time using the infrared thermal mapping instrument. After mild hyperthermia, Dil-HA-BNPs@Ptx (0.5 µL/g) were immediately injected into 4T1 breast tumor-bearing mice via tail vein. The mice were euthanized and the tumor tissue was removed after 24 h. The retention of nanoparticles (red fluorescence) in tumor tissues were revealed using a pathological section scanner Panoramic DESK (P-MIDI-P250, 3D HISTECH, Budapest, Hungary).

To further explore changes in TME, intratumoral perfusion and micro-vessel density of tumor were evaluated using contrast-enhanced ultrasound imaging (CEUS) and CD31 immunohistochemical analysis. Microbubbles (0.2 mL/kg, SonoVueTM, Bracco, Italy) were injected via tail vein 24 h after the 1st MW irradiation. CEUS was performed using an ultrasound scanner (iU22, Koninklijke Philips N.V., Eindhoven, Netherlands) with a 12-5 MHz transducer. Subsequently, mice were euthanized and tumors were sectioned and stained with CD31 (dilution 1:50, Wuhan Service bio Co., Ltd, Wuhan, Hubei, China) to evaluate the micro-vessel density. The pathological sections were imaged using a fluorescence microscope.

***In vivo* anti-tumor efficiency – the 2nd MW irradiation**

The 2nd MW irradiation was dedicated to activate the sensitizer BML and to release the chemotherapy agent Ptx in the nanoparticles. Thirty 4T1 breast tumor-bearing mice were divided into 5 groups: (1) PBS (control group, G1), (2) 1st MW + HA-BNPs@Ptx (G2), (3) 1st MW + HA-BNPs + 2nd MW (G3), (4) HA-BNPs@Ptx + 2nd MW (conventional SDDS, G4), and (5) 1st MW + HA-BNPs@Ptx + 2nd MW (G5). After treated with mild hyperthermia (4 min MW exposure at 0.8 W cm⁻², G2 G3 and G5), PBS, HA-BNPs or HA-BNPs@Ptx (0.5 mL/kg) were immediately injected into 4T1 breast tumor-bearing mice intravenously. 24 h after injection, the mice received a 2nd MW irradiation (0.8 W cm⁻²) for 4 min. The tumor temperature was monitored in real time by infrared thermal mapping instrument. The body weight and tumor volumes were recorded every three days. The tumor suppression rate (TSR) was calculated using the following formula:

$$\text{TSR (\%)} = (V_c - V_x) / V_c \times 100\%$$

V_c: volume of PBS group; V_x: volume of treatment group.

On day 18 after treatment, tumors and main organs were excised and fixed overnight in 10% buffered formalin. The tumors were sectioned and stained with H&E, Ki-67 antibody and TdT-mediated dUTP nick-end labeling (TUNEL) staining. And the main organs were sectioned and stained with H&E. The expression level of CD3+, CD4+, CD8+ of immune cells in tumor tissues were evaluated by immunofluorescence. The slides of tumor tissue were incubated with Anti-CD3 Rabbit pAb (P22646, 1:700), Anti-CD4 Rabbit pAb (P06332 1:800), and Anti-CD8 Rabbit mAb (P10966, 1:500), following the standard procedure of Wuhan Service bio Co., Ltd, (Wuhan, Hubei, China). Nucleus was labeled with DAPI. The images were obtained by using the pathological section scanner Panoramic DESK.

In addition, the peripheral blood was collected from treated mice before euthanasia to analyze the percentages of tumor antigen-specific CD3+, CD4+ and CD8+ T cells. Serum cells were incubated with 3 µL FITC anti-mouse CD3 antibody, 5 µL PE anti-mouse CD4 and 10 µL APC anti-mouse CD8 (4A Biotech Co., Ltd, Peking, China) for 1 h at room temperature, respectively. Finally, the cells were resuspended in PBS and analyzed by flow cytometry and Flow Jo software.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). The statistical analysis was carried out with GraphPad Prism Version 8.0 software (GraphPad, USA). Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA). And two-group comparisons were performed by Student's t-test. Statistical significance was indicated by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Results

The characterization of HA-BNPs@Ptx

Figure 1A illustrates the preparation procedure of nanoparticles HA-BNPs@Ptx. TEM and SEM images showed a core-shell structure of nanoparticles with a mean diameter of about 150 nm, spherical shape and smooth surface (Fig. 1B). DLS revealed that HA-BNPs@Ptx was 203.30 ± 7.51 nm and relatively homogenous (PDI: 0.172, Fig. 1C). HA-BNPs@Ptx was negatively charged with zeta potential of -22 mV (Fig. 1D) and stable at neutral pH (Additional file 1, Fig. S1 A, B). The vibration absorption peaks of amide bonds at 1656 cm^{-1} , 1546 cm^{-1} and 1334 cm^{-1} in FTIR spectrums confirmed the successful conjugation of HA to BNPs@Ptx nanoparticles (Fig. 1E). Ptx loading capacity of HA-BNPs@Ptx determined by spectrophotometer was 13.17 wt.%, corresponding to encapsulation efficiency of 85.62 wt.% (Fig. 1F).

In-vitro MW-induced BML thermal effect and drug release

In order to optimize the MW thermal effect and Ptx release, nanoparticles suspensions (HA-NPs@Ptx & HA-BNPs@Ptx) were exposed at different MW power and time. The infrared thermal imaging pictures showed that under the same irradiation, the heating effect of HA-NPs@Ptx without BML (Fig. 2A, B) was significantly lower than that of HA-BNPs@Ptx with the sensitizer (Fig. 2C, D). For HA-BNPs@Ptx, the temperature of the suspension increased rapidly from room temperature to $35.2\text{ }^{\circ}\text{C}$ (0.4 W cm^{-2}) and reached nearly $60\text{ }^{\circ}\text{C}$ (1.6 W cm^{-2}) under 4 min MW exposure. Due to the lack of BML, the temperature of HA-NPs@Ptx increased only to $30.4\text{ }^{\circ}\text{C}$ at 0.4 W cm^{-2} and $41.8\text{ }^{\circ}\text{C}$ at 1.6 W cm^{-2} . These results suggest that HA-BNPs@Ptx has a greater potential to trigger Ptx release in combination of thermal-chemotherapy.

Fig. 2 E, F show the release of Ptx from HA-BNPs@Ptx upon MW irradiation. The Ptx solution standard curve was established according to previous reports to quantify Ptx (Additional file 1, Fig. S2) [32]. A maximum release of 70% was obtained at 1.6 W cm^{-2} for 4 min. At this condition, the temperature was very high ($58\text{ }^{\circ}\text{C}$, Fig. 2 D), this would directly induce tumor necrosis [33, 34]. In order to demonstrate our synergistic cascade strategy, a lower MW power of 0.8 W cm^{-2} was chosen for subsequent *in-vitro* and *in-vivo* studies.

In-vitro targeting ability, cellular uptake and anti-tumor effect

Hyaluronic acid, as a CD44 specific ligand, is widely used in various targeted drug delivery systems for triple negative breast cancer [31, 35]. The expression of CD44 on 4T1 cells was verified (Additional file 1, Fig. S3) and the targeting ability of nanoparticles to 4T1 cells was compared between HA-BNPs@Ptx and BNPs@Ptx (Additional file 1, Fig. S4). Fig. S4 reveals that the nanoparticles contain hyaluronic acid bundled fairly to 4T1 cells and the binding was specific, because the binding could be inhibited by adding the free hyaluronic acid to 4T1 cells before the incubation with the nanoparticles [36]. Non-specific binding could be observed with BNPs@Ptx, but the number of fluorescent nanoparticles was significantly less than that of HA-BNPs@Ptx, suggesting that the specific binding of HA and CD44 could be used to improve the cellular internalization efficiency of nanoparticles.

The cellular uptake of HA-BNPs@Ptx was evaluated with 4T1 cells and J774 cells. The cell nuclei were stained by DAPI (blue), the endo/lysosomes were stained by LysoTracker Green (green), and the

nanoparticles were labelled with DiI (red). Figure 3A shows that HA-BNPs@Ptx were taken up both by 4T1 and J774 cells, and stayed in endo/lysosomes. The uptake of HA-BNPs@Ptx by 4T1 cells was quantified using flow cytometry. A maximum uptake was observed after 6 h incubation (Additional file 1, Fig. S5). A lower fluorescent intensity obtained at 24 h suggests probably a partial degradation of nanoparticles in endo/lysosomes.

The synergistic anti-tumor effect of MW thermal-chemotherapy was studied qualitatively by confocal microscope and quantitatively by flow cytometry. *In-vitro* hyperthermia experiments indicate that MW irradiation alone was sufficient to induce apoptosis of 4T1 tumor cells (Additional file 1, Fig. S6), and the effect increased with increasing exposure power and time. Figure 3B shows that the combination of MW and HA-BNPs@Ptx resulted in the most significant cell damage compared to the other tested conditions (PBS, BNPs@Ptx, MW, MW + BNPs@Ptx). The 4T1 cell apoptosis analyzed by flow cytometry presented similar results (Fig. 3C, D). The apoptosis was less than 8% for the control group (PBS), 32% for HA-BNPs@Ptx alone, 35% for MW irradiation only, 55.6% MW + BNPs@Ptx and 87% for MW+HA-BNPs@Ptx. These results suggest that there was a synergistic effect by combining hyperthermia and Ptx-loaded nanoparticles, and also confirm the superiority of CD44-targeted nanoparticles compared to non-targeting nanoparticles.

The biocompatibility of HA-BNPs@Ptx and HA-NPs was investigated by cell counting kit8 (CCK8) assay with HUVEC and 4T1 breast cancer cells. A high cell viability (> 92%) was obtained after incubation with HA-BNPs@Ptx and HA-NPs (without Ptx and BML) for 24 h for the two cell lines (Additional file 1, Fig. S7). Figure S8 (Additional file 1) shows that the hemolysis rate induced by HA-BNPs@Ptx is less than 5%. This confirmed that the nanoparticles had good biocompatibility and also showed good stability, with no leakage of Ptx during 24 h incubation [37].

***In-vivo* targeting ability and biodistribution of HA-BNPs@Ptx (without 1st MW)**

To verify the targeting ability and biodistribution of HA-BNPs@Ptx, 4T1 breast tumor-bearing mice model was established in the second axillary mammary fat pad on the right side. HA-BNPs@Ptx and BNPs@Ptx were injected intravenously into 4T1 breast tumor-bearing mice, respectively. The targeting ability and biodistribution of nanoparticles were studied in real-time using a whole animal fluorescence imaging. The intensity of HA-BNPs@Ptx and BNPs@Ptx at tumor sites reached the maximum level at 24 h (Fig. 4A). Compared with the non-targeted BNPs@Ptx group, HA-BNPs@Ptx group exhibited significantly higher uptake in the tumor, almost two times higher (Fig. 4B). Tumor tissues and major organs (heart, lung, kidney, liver, and spleen) were harvested for *ex-vivo* imaging (Fig. 4C). The fluorescence intensity of tumor tissues was also two times higher for the targeted nanoparticles than for non-targeted nanoparticles (Fig. 4D). For other organs, comparable results were observed in liver and spleen, a stronger fluorescence was observed in lung for HA-BNPs@Ptx, probably suggesting a higher CD44 expression in the lung of breast cancer model.

***In-vivo* mild hyperthermia-mediated EPR effect of HA-BNPs@Ptx**

The purpose of the 1st MW irradiation was to pretreat animals to modify TME, so as to improve the delivery of nanoparticles to tumor [11, 38]. The whole animal was continuously monitored by infrared thermography to control the temperature change of the tumor. Figure 5A and B show that the temperature of tumor was 36.7 ± 0.2 °C before MW irradiation and increased to 43.8 ± 0.4 °C after 4 min MW irradiation (0.8 W cm^{-2}). After irradiation, the nanoparticles Dil-HA-BNPs@Ptx were immediately injected into mice through tail vein ($\sim 0.5 \mu\text{L /g}$). After 24 h of treatment, the hyperthermia effect was measured by one of the following three methods. First, red fluorescence-labeled nanoparticles accumulated at tumor site was detected by tissue sections (Fig. 5C, D). Second, contrast-enhanced ultrasound (CEUS) imaging was performed to measure the change of intratumoral blood flow (Fig. 5E, F). Third, the morphology of tumor vasculature was examined using CD31 immunofluorescent staining (Fig. 5G, H).

The results showed that the retention rate of nanoparticles in the tumors irradiated by MW increased by seven times compared with the tumors not irradiated (Fig. 5C, D). The echo intensity of CEUS showed that pretreatment of tumor and its surrounding tissue by MW irradiation could significantly improve intratumoral perfusion (Fig. 5E, F). CD31 immunofluorescence images also showed increased tumor vascular density and/or intratumoral vasodilatation in the tumor compared with tumors not irradiated (Fig. 5F). Figure 5G showed that 24 h after MW exposure, the percentage of effective tumor vessels increased by a factor of about 4-fold, from $3.8 \pm 1.5\%$ to $15.3 \pm 4.3\%$. These results suggest that mild hyperthermia as a tumor pretreatment can improve the accessibility of nanoparticles to tumors, possibly by reducing interstitial fluid pressure, dilating and increasing tumor blood vessels, and thus improving intratumoral perfusion [39].

***In-vivo* evaluation of synergistic cascade antitumor effect**

The synergistic cascade antitumor effect was achieved by combining two step MW irradiations with target nanoparticles HA-BNPs@Ptx (Fig. 6A). The tested conditions included group 1 (G1), PBS; group 2 (G2), 1st MW + HA-BNPs-Ptx; group 3 (G3), 1st MW + HA-BNPs + 2nd MW; group 4 (G4), HA-BNPs-Ptx + 2nd MW) and group 5 (G5) with 1st MW + HA-BNPs-Ptx + 2nd MW. Notice the difference between G2 and G5, G2 did not receive 2nd MW irradiation (nanoparticles); the difference between G3 and G5, the former contained no Ptx (thermal therapy); the difference between G4 and G5 was that G4 did not receive the 1st MW irradiation (tumor pre-treatment). The group (G3, G4 and G5) received 2nd MW was irradiated under the same conditions as the 1st one (0.8 W cm^{-2} for 4 min), and the tumor temperature was monitored in real time by the infrared thermal mapping instrument. The expose temperature of G3 and G5 under 2nd MW irradiation were all up to 51 °C (Additional file 1, Fig. S9A and B). G4 reached to approximately 45 °C. This indicates that BML encapsulated in HA-BNPs-Ptx provided a supplement heating.

Fig. 6B shows that throughout the treatment period, there was no significant change of animal body weight in all groups, indicating that all treatment groups were well tolerated. Compared with the other groups, the tumor volume of G1 group increased rapidly, up to about 2000 mm³ (Figure 6 C, D, E). The antitumor effect of G5 group was the best, with the inhibition rate of tumor growth up to 88%. G2 and G3

inhibited tumor growth by 58.2% and 67.5%, respectively. G4 also has a good anti-tumor effect, the inhibition rate of tumor growth was about 72.5%.

The degree of tumor tissue damage was evaluated by immunostaining using H&E, proliferation marker Ki-67 and TUNEL essays. The results showed that the most severe morphological change and necrosis from tumor slices were observed in G5 (Fig. 6F top). G5 significantly increased the apoptosis of cancer cells (Fig. 6F middle) and had the lowest stained cells by Ki-67 (Fig. 6F bottom). The pathological results were consistent with the tumor size and volume, indicating that G5 had the best anti-tumor effect.

Metastasis is a major challenge for TNBC treatment [40]. 4T1 breast tumor-bearing mice treated with the combination of two-steps MW irradiation with HA-BNPs@Ptx exhibited a reduction in spontaneous lung metastasis (Fig. 7A, B). Intratumoral immune profiling was performed in 5 groups through immunofluorescence staining (Fig. 7C). The proportions of T cells (CD3+) were increased in the treated group compared to G1. Among T cells, the percentages of CD4+ T cells and CD8+ T cells were significantly increased in the G5. Analysis of peripheral blood by flow cytometry was consistent with above results (Fig. 7F). These results suggest that sequential treatment of SCS can stimulate and recruit CD4+ CD8+ T cells in breast cancer tissues, and thus effectively inhibit spontaneous lung metastasis.

Subsequently, cytotoxicity of HA-BNPs-Ptx was investigated *in-vivo*. There was no difference in liver and kidney function (analysis of serum levels for ALT, AST, BUN and CRE) between the PBS group and the HA-BNPs-Ptx group (Additional file 1, Fig. S10). This indicates that the nanoparticles have good biocompatibility. The histological analysis staining of main organs tissue was done by hematoxylin and eosin (H&E) (Additional file 1, Fig. S11). After the treatment to study the damage of mice. No tissue necrosis was observed in the main organs (heart, liver, spleen and kidney) for all groups, demonstrating that the SCS was safe *in-vivo*.

Discussion

In the present study, we have combined different therapeutic approaches to treat the most challenging solid cancer, TNBC. Our hypothesis is that to manage high metastasis and high recurrence of TNBC, it is necessary to have a very strong and aggressive treatment. For this, we have designed a “synergistic cascade strategy” based on modifying TME, which consists of a pretreatment of tumor and its surrounding tissue with mild hyperthermia and followed by applying SDDS. The SDDS is composed of two components: (a) intravenous injection of tumor CD44-targeted thermosensitive liposomes (TSL) preloaded with BML and Ptx, and (b) MW-induced hyperthermia to simultaneously achieve drug release and tumor ablation.

To overcome the resistance of TNBC and to improve the uptake and biodistribution of nanoparticles, the tumor was first irradiated by MW to induce a mild hyperthermia (~ 43 °C) to alter the TNBC microenvironment. This “hyperthermia-mediated EPR effect” allows to reduce the interstitial pressure of TME and increase intratumoral blood flow and thereby enhance the uptake of nanoparticles at the tumor site. Following the 1st MW exposure, the nanoparticles HA-BNPs@Ptx was injected into 4T1 tumor-

bearing mice via tail vein. The nanoparticles were made of thermosensitive phospholipid DPPC ($T_m \sim 41^\circ\text{C}$) and PLGA, that have been extensively used in nanomedicine formulations both for clinical and laboratory for breast cancer treatment. The 2nd MW irradiation allowed producing a hyper heating effect ($T > 50^\circ\text{C}$) due to the activation of BML and a rapid release of the Ptx from nanoparticles. The increased temperature at the tumor site could directly damage tumor cells and surrounding tissue, inducing apoptosis both *in-vitro* (Fig. 3) and *in-vivo* (Fig. 6).

Hyaluronic acid is an important component of extracellular matrix. It plays an important role in tumor microenvironment and is involved in tumor cell proliferation, invasion, immune escape, stem cell change and drug resistance [41]. HA is also ligand for lymphatic vessel endothelial hyaluronan receptor-1 and hyaluronan-mediated motility receptor. HA is a biocompatible, biodegradable, and nonimmunogenic biopolymer capable of actively targeting cluster of differentiation-44 (CD44) on cell surface receptors overexpressed in many cancer cells, including TNBC [42–45]. For all these reasons, we have selected HA as ligand to target 4T1 tumor cells that overexpress CD44. Compared with non-targeted nanoparticles, HA-BNPs@Ptx can specifically target TNBC tumor cells both *in-vitro* (Fig. S4) and *in-vivo* (Fig. 5). HA-BNPs@Ptx nanoparticles exhibited improved cellular uptake, probably via HA receptor-mediated endocytosis and phagocytosis (Fig. 3).

In our previous work [28], we have demonstrated that encapsulated BML in P-selectin targeted nanoparticles could be used to boost thermal effect of MW-generated hyperthermia and efficiently prevent tumor progression and lung metastasis in HCC tumor-bearing mice. In the present study, we further confirmed that the incorporation of BML in the liposomes can significantly enhance thermal effect induced by MW irradiation with an increase of temperature more than 15°C . It is worth noting that a mild MW irradiation (0.8 W cm^{-2} and 4 min) was sufficient to increase significantly the intratumoral blood flow as shown by CEUS imaging and tumor microvessel density. As a result, the retention of nanoparticles HA-BNPs@Ptx was increased by sevenfold (Fig. 4).

Currently, chemotherapy remains the main option for the treatment of TNBC [46, 47]. However, conventional chemotherapies employed in treatment of TNBC suffer from issues of poor bioavailability, poor cellular uptake, resistance, and undesirable off-site toxicities [48]. Abraxane, a nanoparticle formulation of "Taxol" (paclitaxel), was approved for the treatment of metastatic breast cancer, but the efficacy is limited due to the high heterogeneity and drug resistance of breast tumors [49]. In the past few years, extensive research has been conducted to overcome drug resistance and improve the prognosis of TNBC [48]. The combination of chemotherapeutics with SDDS, particularly with stimuli-responsive systems that are able to control drug biodistribution and release in response to specific stimuli, either exogenous (temperature, magnetic field, ultrasound, light or electric pulses) or endogenous (changes in pH, enzyme concentration or redox gradients) has been explored for metastatic breast cancer [50–52]. The main advantages of combination SDDS with chemotherapy are (i) the decrease of toxicity of chemotherapeutic agents [5, 53]; (ii) the local delivery of anticancer agents with higher payloads and optimal distribution [54]; (iii) the use of nanocarriers to delivery immunomodulatory agents that can activate immune cells and modulate TME [11, 14, 39]; (iv) the incorporation of diagnostic agents for

imaging the tumor or TME [9, 55], and (v) the incorporation of active targeting molecules and specific stimuli to have synergistic cascade effects to enhance anti-tumor efficacy. The results obtained in this study fully demonstrates the virtue of SDDS. Finally, the use of thermal effect to pretreat the tumor and its microenvironment, which allows significantly increasing nanodrug uptake, so called “hyperthermia-mediated EPR effect”, is a efficient and clinically translatable approach. This approach should be further investigated and explored.

Conclusion

In this study, mild MW hyperthermia was confirmed that can be used to pretreat tumors to promote vasodilation of tumor vessels, improve intratumoral perfusion, and thus increase retention of nanodrugs. The smart drug delivery system HA-BNPs@Ptx demonstrated its high efficiency in active targeting, MW stimulation and tumor cell apoptosis. In addition, the synergistic cascade effects by combining two-step MW irradiation and HA-BNPs@Ptx could effectively inhibit progression of triple negative breast tumor in mice and trigger a powerful immune memory response. Two-step hyperthermia combined with nanomedicine showed a good synergistic cascade anti-tumor effect both on solid breast tumor in situ and lung metastasis, thus providing a new approach for the treatment of breast cancer.

Abbreviations

TNBC: Triple negative breast cancer; TME: Tumor microenvironment; ECM: Extracellular matrix; EPR: enhanced permeability and retention; IFP: Interstitial fluid pressure; SCS: Synergistic cascade strategy; SDDSs: Smart drug delivery systems; MW: Microwave; HA: Hyaluronic acid; BML: 1-butyl-3-methylimidazolium-L-lactate; Ptx: Paclitaxel; CD44: Differentiation-44; HCC: Hepatocellular carcinoma; CTLs: cytotoxic T lymphocytes; LTSLs: Low temperature sensitive liposomes; RF: Radiofrequency; FUS: Focused ultrasound; DPPC: 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine; PVA: Polyvinyl alcohol; MES: 2-(N-morpholino) ethane sulfonic acid; NHS: N-hydroxysuccinimide; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DCM: dichloromethane; TEM: Transmission electron microscopy; SEM: Scanning electron microscope; PDI: Polydispersity index; DLS : Dynamic light scattering; FTIR: Fourier transform infrared spectrometer; DL: Drug loading; EE: Encapsulation efficiency; HUVEC: Human umbilical vein endothelial cells; OD: Optical density; PBS: Phosphate buffer saline; FCM: Flow cytometry; DAPI: 4,6-diamidino-2-phenylindole; LSCM: Laser scanning confocal microscope; PI: Propidium iodide; CEUS: Contrast-enhanced ultrasound imaging; TSR: tumor suppression rate; TUNEL: TdT-mediated dUTP nick-end labeling; SD: standard deviation; ANOVA: one-way analysis of variance.

Declarations

Acknowledgements

The authors are grateful to Yan Wang (Research Core Facility of West China Hospital Sichuan university) for her collaboration in Flow cytometer analysis.

Authors' contributions

HZ, JSX and YLP designed the study. HZ, GBY, HW, JBH, JZ and RY, performed experiments. HZ, GBY and FY wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research was funded by Science and Technology Project of Chengdu, (2017-CY02-00027-GX, 2019-YF05-00376-SN), Post-Doctor Research Project, West China Hospital, Sichuan University (2020HXBH003), Science and Technology Innovation talent of Sichuan (20CXRC0065) and Science and technology project of the health planning committee of Sichuan (20PJ011).

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript and its Supplementary Information.

Ethics approval and consent to participate

All processes were in accordance with the Chinese Society of Laboratory Animals on animal welfare and approved by the Animal Use and Care Management Advisory Committee of West China Hospital of Sichuan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Department of Ultrasound, West China Hospital, Sichuan University, Chengdu 610041, China. ² Laboratory of Ultrasound Imaging Drug, West China Hospital, Sichuan University, Chengdu 610041, China.

References

1. Tajbakhsh A, Hasanzadeh M, Rezaee M, Khedri M, Khazaei M, ShahidSales S, et al. Therapeutic potential of novel formulated forms of curcumin in the treatment of breast cancer by the targeting of cellular and physiological dysregulated pathways. *J Cell Physiol*. 2018;233:2183-2192.
2. Thakur V, Kutty R V. Recent advances in nanotheranostics for triple negative breast cancer treatment. *J Exp Clin Cancer Res*. 2019;38:430.

3. Mu Q, Wang H, Zhang M. Nanoparticles for imaging and treatment of metastatic breast cancer. *Expert Opin Drug Deliv.* 2017;14:123-136.
4. Ding Y, Yang R, Yu W, Hu C, Zhang Z, Liu D, et al. Chitosan oligosaccharide decorated liposomes combined with TH302 for photodynamic therapy in triple negative breast cancer. *J Nanobiotechnology.* 2021;19:147.
5. Hu Q, Shang L, Wang M, Tu K, Hu M, Yu Y, et al. Co-Delivery of Paclitaxel and Interleukin-12 Regulating Tumor Microenvironment for Cancer Immunochemotherapy. *Adv Healthc Mater.* 2020;9:e1901858.
6. Deepak KGK, Vempati R, Nagaraju GP, Dasari VR, S N, Rao DN, et al. Tumor microenvironment: Challenges and opportunities in targeting metastasis of triple negative breast cancer. *Pharmacol Res.* 2020;153:104683.
7. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature.* 2013;501:346-354.
8. Pashayan N, Antoniou A, Ivanus U, Esserman L, Easton D, French D, et al. Personalized early detection and prevention of breast cancer: ENVISION consensus statement. *Nat Rev Clin Oncol.* 2020;17:687-705.
9. Wang Z, Li Z, Sun Z, Wang S, Ali Z, Zhu S, et al. Visualization nanozyme based on tumor microenvironment "unlocking" for intensive combination therapy of breast cancer. *Sci Adv.* 2020;6:eabc8733.
10. Januškevičienė I, Petrikaitė V. Heterogeneity of breast cancer: The importance of interaction between different tumor cell populations. *Life Sci.* 2019;239:117009.
11. Yang S, Gao H. Nanoparticles for modulating tumor microenvironment to improve drug delivery and tumor therapy. *Pharmacol Res.* 2017;126:97-108.
12. Griffon-Etienne G, Boucher Y, Brekken C, Suit HD, Jain RK. Taxane-induced apoptosis decompresses blood vessels and lowers interstitial fluid pressure in solid tumors: clinical implications. *Cancer Res.* 1999;59:3776-3782.
13. Nia HT, Munn LL, Jain RK. Physical traits of cancer. *Science.* 2020;370: eaaz0868.
14. Feng C, Xiong Z, Wang C, Xiao W, Xiao H, Xie K, et al. Folic acid-modified Exosome-PH20 enhances the efficiency of therapy via modulation of the tumor microenvironment and directly inhibits tumor cell metastasis. *Bioact Mater.* 2020;6:963-974.
15. Lang T, Liu Y, Zheng Z, Ran W, Zhai Y, Yin Q, et al. Cocktail Strategy Based on Spatio-Temporally Controlled Nano Device Improves Therapy of Breast Cancer. *Adv Mater.* 2019;31:e1806202.
16. Nienhuis HH, Gaykema SB, Timmer-Bosscha H, Jalving M, Brouwers AH, Lub-de Hooge MN, et al. Targeting breast cancer through its microenvironment: Current status of preclinical and clinical research in finding relevant targets. *Pharmacol Ther.* 2015;147:63-79.
17. Dunne M, Regenold M, Allen C. Hyperthermia can alter tumor physiology and improve chemo- and radio-therapy efficacy. *Adv Drug Deliv Rev.* 2020;163-164: 98-124.

18. May JP, Li SD. Hyperthermia-induced drug targeting. *Expert Opin Drug Deliv.* 2013;10:511-527.
19. Markezana A, Ahmed M, Kumar G, Zorde-Khvaleyevsky E, Rozenblum N, Galun E, et al. Moderate hyperthermic heating encountered during thermal ablation increases tumor cell activity. *Int J Hyperthermia.* 2020;37:119-129.
20. Lyon P, Gray M, Mannaris C, Folkes L, Stratford M, Campo L, et al. Safety and feasibility of ultrasound-triggered targeted drug delivery of doxorubicin from thermosensitive liposomes in liver tumours (TARDOX): a single-centre, open-label, phase 1 trial. *Lancet Oncol.* 2018;19:1027-1039.
21. De Maar J, Suelmann B, Braat M, Van Diest P, Vaessen H, Witkamp A, et al. de novoPhase I feasibility study of Magnetic Resonance guided High Intensity Focused Ultrasound-induced hyperthermia, Lyso-Thermosensitive Liposomal Doxorubicin and cyclophosphamide in stage IV breast cancer patients: study protocol of the i-GO study. *BMJ open.* 2020;10:e040162.
22. Tak W, Lin S, Wang Y, Zheng J, Vecchione A, Park S, et al. Phase III HEAT Study Adding Lyso-Thermosensitive Liposomal Doxorubicin to Radiofrequency Ablation in Patients with Unresectable Hepatocellular Carcinoma Lesions. *Clin Cancer Res.* 2018;24:73-83.
23. Nardecchia S, Sánchez-Moreno P, De Vicente J, Marchal J A, Boulaiz H. Clinical Trials of Thermosensitive Nanomaterials: An Overview. *Nanomaterials (Basel).* 2019;9:191.
24. Dou Y, Hynynen K, Allen C. To heat or not to heat: Challenges with clinical translation of thermosensitive liposomes. *J Control Release.* 2017;249:63-73.
25. Paulides MM, Dobsicek Trefna H, Curto S, Rodrigues DB. Recent technological advancements in radiofrequency- and microwave-mediated hyperthermia for enhancing drug delivery. *Adv Drug Deliv Rev.* 2020;163-164:3-18.
26. Wu Q, Xia N, Long D, Tan L, Rao W, Yu J, et al. Dual-Functional Supernanoparticles with Microwave Dynamic Therapy and Microwave Thermal Therapy. *Nano Lett.* 2019;19:5277-5286.
27. Qi J, Li W, Lu K, Jin F, Liu D, Xu X, et al. pH and Thermal Dual-Sensitive Nanoparticle-Mediated Synergistic Antitumor Effect of Immunotherapy and Microwave Thermotherapy. *Nano Lett.* 2019;19:4949-4959.
28. Xu J, Cheng X, Tan L, Fu C, Ahmed M, Tian J, et al. Microwave Responsive Nanoplatfrom via P-Selectin Mediated Drug Delivery for Treatment of Hepatocellular Carcinoma with Distant Metastasis. *Nano Lett.* 2019;19:2914-2927.
29. Xu J, Liu Y, Li Y, Wang H, Stewart S, Van der Jeught K, et al. Precise targeting of POLR2A as a therapeutic strategy for human triple negative breast cancer. *Nat Nanotechnol.* 2019;14:388-397.
30. Alam N, Koul M, Mintoo M J, Khare V, Gupta R, Rawat N, et al. Development and characterization of hyaluronic acid modified PLGA based nanoparticles for improved efficacy of cisplatin in solid tumor. *Biomed Pharmacother.* 2017;95:856-864.
31. Zhang Y, Hu H, Tang W, Zhang Q, Li M, Jin H, et al. A multifunctional magnetic nanosystem based on "two strikes" effect for synergistic anticancer therapy in triple-negative breast cancer. *J Control Release.* 2020;322:401-415.

32. Furman C, Carpentier R, Barczyk A, Chavatte P, Betbeder D, Lipka E. Development and validation of a reversed-phase HPLC method for the quantification of paclitaxel in different PLGA nanocarriers. *Electrophoresis*. 2017;38:2536-2541.
33. Seynhaeve A, Amin M, Haemmerich D, Van Rhoon G, Ten Hagen T. Hyperthermia and smart drug delivery systems for solid tumor therapy. *Adv Drug Deliv Rev*. 2020;163-164:125-144.
34. Pan J, Xu Y, Wu Q, Hu P, Shi J. Mild Magnetic Hyperthermia-Activated Innate Immunity for Liver Cancer Therapy. *J Am Chem Soc*. 2021;143:8116-8128.
35. Sun W, Du Y, Liang X, Yu C, Fang J, Lu W, et al. Synergistic triple-combination therapy with hyaluronic acid-shelled PPY/CPT nanoparticles results in tumor regression and prevents tumor recurrence and metastasis in 4T1 breast cancer. *Biomaterials*. 2019;217:119264.
36. Cerqueira BBS, Lasham A, Shelling AN, Al-Kassas R. Development of biodegradable PLGA nanoparticles surface engineered with hyaluronic acid for targeted delivery of paclitaxel to triple negative breast cancer cells. *Mater Sci Eng C Mater Biol Appl*. 2017;76:593-600.
37. Nave M, Castro RE, Rodrigues CM, Casini A, Soveral G, Gaspar MM. Nanoformulations of a potent copper-based aquaporin inhibitor with cytotoxic effect against cancer cells. *Nanomedicine (Lond)*. 2016;11:1817-1830.
38. Hynynen K. Hyperthermia-induced drug delivery in humans. *Nat Biomed Eng*. 2018;2:637-639.
39. Chen Q, Hu Q, Dukhovlinova E, Chen G, Ahn S, Wang C, et al. Photothermal Therapy Promotes Tumor Infiltration and Antitumor Activity of CAR T Cells. *Adv Mater*. 2019;31:e1900192.
40. Li Q, Yang J, Chen C, Lin X, Zhou M, Zhou Z, et al. A novel mitochondrial targeted hybrid peptide modified HPMa copolymers for breast cancer metastasis suppression. *J Control Release*. 2020;325:38-51.
41. Vasvani S, Kulkarni P, Rawtani D. Hyaluronic acid: A review on its biology, aspects of drug delivery, route of administrations and a special emphasis on its approved marketed products and recent clinical studies. *Int J Biol Macromol*. 2020;151:1012-1029.
42. Wang Z, Sau S, Alsaab HO, Iyer AK. CD44 directed nanomicellar payload delivery platform for selective anticancer effect and tumor specific imaging of triple negative breast cancer. *Nanomedicine*. 2018;14:1441-1454.
43. Mattheolabakis G, Milane L, Singh A, Amiji MM. Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine. *J Drug Target*. 2015;23:605-618.
44. Safdar MH, Hussain Z, Abourehab MAS, Hasan H, Afzal S, Thu HE. New developments and clinical transition of hyaluronic acid-based nanotherapeutics for treatment of cancer: reversing multidrug resistance, tumour-specific targetability and improved anticancer efficacy. *Artif Cells Nanomed Biotechnol*. 2018;46:1967-1980.
45. Kari OK, Tavakoli S, Parkkila P, Baan S, Savolainen R, Ruoslahti T, et al. Light-Activated Liposomes Coated with Hyaluronic Acid as a Potential Drug Delivery System. *Pharmaceutics*. 2020;12:763.
46. Núñez Abad M, Calabuig-Fariñas S, Lobo de Mena M, José Godes Sanz de Bremond M, García González C, Torres Martínez S, et al. Update on systemic treatment in early triple negative breast

- cancer. *Ther Adv Med Oncol*. 2021;13:1758835920986749.
47. Oualla K, El-Zawahry HM, Arun B, Reuben JM, Woodward WA, Gamal El-Din H, et al. Novel therapeutic strategies in the treatment of triple-negative breast cancer. *Ther Adv Med Oncol*. 2017;9:493-511.
48. Bai X, Ni J, Beretov J, Graham P, Li Y. Triple-negative breast cancer therapeutic resistance: Where is the Achilles' heel?. *Cancer Lett*. 2021;497:100-111.
49. Gradishar WJ. Albumin-bound paclitaxel: a next-generation taxane. *Expert Opin Pharmacother*. 2006;7:1041-1053.
50. Yang Y, Qiao X, Huang R, Chen H, Shi X, Wang J, et al. E-jet 3D printed drug delivery implants to inhibit growth and metastasis of orthotopic breast cancer. *Biomaterials*. 2020;230:119618.
51. Rivera-Rodriguez A, Chiu-Lam A, Morozov VM, Ishov AM, Rinaldi C. Magnetic nanoparticle hyperthermia potentiates paclitaxel activity in sensitive and resistant breast cancer cells. *Int J Nanomedicine*. 2018;13:4771-4779.
52. Li X, Jeon YH, Kwon N, Park JG, Guo T, Kim HR, et al. In Vivo-assembled phthalocyanine/albumin supramolecular complexes combined with a hypoxia-activated prodrug for enhanced photodynamic immunotherapy of cancer. *Biomaterials*. 2021;266:120430.
53. Thakkar S, Sharma D, Kalia K, Tekade RK. Tumor microenvironment targeted nanotherapeutics for cancer therapy and diagnosis: A review. *Acta Biomater*. 2020;101:43-68.
54. Wang Y, Zhang Z, Zheng C, Zhao X, Zheng Y, Liu Q, et al. Multistage Adaptive Nanoparticle Overcomes Biological Barriers for Effective Chemotherapy. *Small*. 2021:e2100578.
55. Wang Z, Ju Y, Ali Z, Yin H, Sheng F, Lin J, et al. Near-infrared light and tumor microenvironment dual responsive size-switchable nanocapsules for multimodal tumor theranostics. *Nat Commun*. 2019;10:4418

Figures

Figure 1

Synthesis and characterization of nanoparticles used in synergistic cascade strategy study. A BNPs@Ptx preparation and HA conjugation. B TEM and SEM image of HA-BNPs@Ptx. C Size distribution and D Zeta potentials of BNPs@Ptx and HA-BNPs@Ptx determined by DLS (n=3). E FTIR spectrums of HA, BNPs@Ptx, and HA-BNPs@Ptx (Amide bond: 1656 cm⁻¹, 1546 cm⁻¹ and 1334 cm⁻¹). F Encapsulation efficiency (EE) and loading capacity (LC) of Ptx in HA-BNPs@Ptx and BNPs@Ptx determined by spectrophotometer (n=3).

Figure 2

In-vitro evaluation of MW thermal effect and Ptx release. A and B IR thermal imaging and temperature histogram of HA-NPs@Ptx (without thermosensitive agent BML) at different MW power and exposure time. C and D IR thermal imaging and temperature histogram of HA-BNPs@Ptx (with thermosensitive agent BML). E Schematic illustration of HA-BNPs@Ptx BML activation and Ptx release under MW

irradiation. F In-vitro release of Ptx from HA-BNPs@Ptx exposed at different MW power and irradiation time. The data were presented as Mean $\pm$ SD (n=3).

Figure 3

In-vitro endocytosis and apoptosis studies. A Representative confocal imaging of 4T1 breast cancer cells and J774 macrophages incubated with HA-BNPs@Ptx for 1, 3 and 6 h at 37 °C. The cell nuclei were stained by DAPI (blue), the endo/lysosomes by Lyso Tracker Green (green), and HA-BNPs@Ptx were labelled with Dil (red). Scale bars represent 20 μ m. B Fluorescence images of 4T1 cells treated with PBS,

HA-BNPs@Ptx, MW, MW + BNPs@Ptx or MW + HA-BNPs@Ptx. Live cells were stained by Calcein-AM (green) and dead cells by PI (red). Scale bars represent 50 μm . C After treatment, early and late apoptotic cells were determined by flow cytometry using the Annexin V-Alexa Fluor 488/propidium iodide apoptosis detection kit. D Total cell apoptosis rate detected by flow cytometry. The data were presented as mean $\pm$ SD ($n=3$, $**p < 0.01$).

Figure 4

In-vivo targeting ability and biodistribution of nanoparticles. A Representative in-vivo fluorescence images of 4T1 breast tumor-bearing mice obtained at pre-injection, 1, 3, 6 and 24 h after intravenous injection of Dil-BNPs@Ptx and Dil-HA-BNPs@Ptx (0.5 mL/kg). B Average radiant intensity determined from in-vivo fluorescence imaging for tumor-targeted (Dil-HA-BNPs@Ptx) and non-targeted (Dil-BNPs@Ptx) nanoparticles. C Ex-vivo fluorescence images of main organs and tumor collected from tumor-bearing mice 24 h after injection of nanoparticles. D Average radiant intensity of main organs and tumor

determined from ex-vivo fluorescence imaging for BNPs@Ptx and Dil-HA-BNPs@Ptx at 24 h after injection of nanoparticles. The data were presented as mean $\pm$ SD ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 5

In-vivo mild hyperthermia effects on nanoparticle uptake and retention, intratumoral flow and tumor vasculature on 4T1 breast tumor-bearing mice. A Representative IR thermal imaging of mice with or without MW irradiation (0.8 W cm⁻², 4 min). B Average tumor temperature before and after MW irradiation ($n=3$). C Representative fluorescence images of uptake/retention of Dil-HA-BNPs@Ptx in tumor bearing mice untreated and treated with MW irradiation. Images were obtained at 24 h after the treatment. D Quantitative analysis of excised tumors collected from mice 24 h after treatment ($n = 3$). D

In-vivo contrast-enhanced ultrasound images obtained at different time-points after injection of microbubble contrast agent SonoVue™. E Increased intratumoral perfusion was confirmed by quantifying contrast-enhanced echo intensity of MW-treated and untreated 4T1 tumors (n = 3). F Immunofluorescence expression of CD31 showed vascular dilation or opening within tumors, which was consistent with CEUS imaging finding. G Compared with untreated 4T1 tumor, the area of tumor blood vessels increased 4 times after MW irradiation. Scale bars represent 50 μm. The data were presented as mean ± SD (n = 5, **p < 0.01, ***p < 0.001).

Figure 6

In-vivo study of the anti-tumor effect of synergistic cascade strategy (double hyperthermia + nanomedicine). A Schematic illustration of the treatment process of 4T1 breast tumor-bearing mice by synergistic cascade strategy. B and C Body weight Tumor growth of mice after SCS treatment, evaluated every 3 days. D and E Changes in tumor volume and representative photograph of excised tumor of the 4T1 breast tumor-bearing mice 18 days after the treatment (G1-G5). F H&E staining, Ki-67 staining and TUNEL staining images of tumor tissue after different treatments (G1-G5) for 18 days. Scale bars represent 50 μ m. The data were presented as mean $\pm$ SD ($n = 6$, ** $p < 0.01$, *** $p < 0.001$).

Figure 7

In-vivo study of the anti-metastasis effect of synergistic cascade strategy. A H&E staining of lung metastatic sites (yellow borders) of 4T1 tumors. Scale bars represent 2000 μm for 0.6 $\times$ and 50 μm for 20 $\times$, respectively. B The number of pulmonary metastatic nodules after various treatments (G1-G5) was calculated according to H&E staining results. C Representative immunofluorescence images showing tumor-infiltrating CD3⁺ CD4⁺ and CD3⁺ CD8⁺T cells within 4T1 tumors in the different treatment groups (G1-G5). (D) Representative flow cytometric images of CTLs (CD4⁺ CD8⁺) gated on CD3⁺ CTLs in peripheral blood. The data were presented as mean $\pm$ SD (n = 6, **p < 0.01, ***p < 0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.tif](#)
- [SI.doc](#)
- [Scheme1.tif](#)