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Electronic Supplementary Information

Ultrasound-enhanced theranostics of orthotopic breast cancer through a multifunctional core-shell tecto dendrimer-based nanomedicine platform

Junli Gong,‡[a,b] Cong Song,‡[c] Gaoming Li,[b] Yunqi Guo,[b] Zhiqiang Wang,[b] Honghua Guo,[d] Jindong Xia,[d] Yuchen Tao,[e] Qiusheng Shi,[e] Xiangyang Shi,*[b] Mingwu Shen*[b]

[a] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, Shanghai Engineering Research Center of Nano-Biomaterials and Regenerative Medicine, College of Chemistry and Chemical Engineering, Donghua University, Shanghai 201620, People's Republic of China

[b] College of Biological Science and Medical Engineering, Donghua University, Shanghai 201620, People's Republic of China

[c] Medical Science and Technology Innovation Center, Shandong First Medical University, Jinan, Shandong 250117, People's Republic of China

[d] Department of Radiology, Shanghai Songjiang District Central Hospital, Shanghai 201600, People's Republic of China

[e] Department of Ultrasound, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, People's Republic of China

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* Corresponding author. E-mail addresses: mwshen@dhu.edu.cn (M. Shen) and xshi@dhu.edu.cn (X. Shi)

‡ Authors contributed equally to this work.

Additional experimental details

Materials

Generation 3 (G3) and generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers were purchased from Dendritech (Midland, MI). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), and doxorubicin hydrochloride (DOX.HCl) were from Sigma-Aldrich (St. Louis, MO). 1-Adamantaneacetic acid (Ada-COOH), β-cyclodextrin (β-CD), N,N-carbonyldiimidazole (CDI), and 1,3-propane sultone (1,3-PS) were from J&K Scientific Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) was from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). 2,2',2''-(10-(2-(2,5-Dioxopyrrolidin-1-yloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triy) triacetic acid (DOTA-NHS) was obtained from CheMatech (Dijon, France). Triethylamine and methanol were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). $Gd(NO_3)\cdot 6H_2O$ were from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). 2'-O-methyl microRNA 21 inhibitor (miR 21i) (sequence: 5'-UCA ACA UCA GUC UGA UAA GCU A-3') and FAM-labeled miR 21i (Carboxyfluorescein, Green fluorescence) were from Shanghai Gene Pharma (Shanghai, China). Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 1000, 3500, and 8000-14000 were from Secoma Biotechnology Co., Ltd. (Beijing, China). The Primary Amino Nitrogen (PANOPA) Assay Kit was from Megazyme (Wicklow, Ireland). Agarose was from Biowest (Nuaille, France). MDA-MB-231 cells (a triple-negative breast cancer (TNBC) cell line) were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and 0.25% trypsin-EDTA were from Gibco (Carlsbad, CA). Penicillin and streptomycin were from Gino Biomedical Technology Co., Ltd. (Hangzhou, China.). 4',6-Diamidino-2-phenylindole (DAPI) was from BestBio Biotechnology Co., Ltd. (Shanghai, China). Upstream and downstream primers of genes (GAPDH, PTEN, PDCD4, Bax, and Bcl-2) were designed and obtained from Generay Biotechnology Co., Ltd. (Shanghai, China). Cell counting

kit-8 (CCK-8), cell cycle, apoptosis analysis kit, RNAeasy™ Plus Animal RNA Isolation Kit with Spin Column, cDNA synthesis kit, SYBR Green qPCR (polymerase chain reaction) Mix (2X, Low ROX), bicinchoninic acid (BCA) protein assay kit, western blot kit, PTEN antibody, PDCD4 antibody, and HRP-labeled Goat Anti-Mouse IgG(H+L) were from Beyotime Biotechnology Co., Ltd. (Shanghai, China). GAPDH, Bax and Bcl-2 antibodies were from Servicebio Co., Ltd. (Wuhan, China). The MicroTissues®3D Petri Dish® micro-mold was from Microtissues (Providence, RI). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm.

Synthesis of G3-DOTA and Ad-G3-DOTA dendrimers

The G3-DOTA dendrimers were first prepared following procedures described in our earlier report.[1,2] In brief, G3 PAMAM dendrimers (18.98 mg) with surface amine groups were dissolved in 5 mL DMSO and were reacted with 5 molar equiv. of DOTA-NHS (10 mg) in 5 mL DMSO under intensive magnetic stirring at room temperature for 24 h. Subsequently, the mixture was dialyzed against water for 3 days (9 times, 2 L) using a dialysis membrane with an MWCO of 1000. After freeze drying, the G3-DOTA product was obtained.

In addition, G3.NHAc and G3.NHAc-DOTA were also synthesized according to the literature[3] in order to calculate the number of DOTA moiety modified onto each G3 dendrimer. In brief, excess triethylamine (10 mg, 80 μL) was dropped into a DMSO solution of G3 or G3-DOTA (10 mg for G3 and 13.31 mg for G3-DOTA, 5 mL) under continuous stirring for 30 min, and acetic anhydride (5.4 mg, 5 μL) was then dropped into the mixture solution while stirring at room temperature for 24 h to get the raw product of G3.NHAc or G3.NHAc-DOTA. Both products were dialyzed against phosphate buffered saline (PBS, 3 times, 2 L) for 1 day and water (6 times, 2 L) for 2 days using a dialysis membrane with an MWCO of 1000. After freeze drying, the G3.NHAc and G3.NHAc-DOTA products were obtained and stored at -20 °C for further use.

Next, Ad-G3-DOTA dendrimers were prepared *via* EDC coupling of the surface amine groups of G3-DOTA dendrimers with the carboxyl group of Ad. Briefly, EDC (7.9 mg) dissolved in DMSO (5 mL) was dropwise added to a DMSO solution of Ad-COOH (0.8 mg, 5 mL), and the mixture was

stirred at room temperature for 30 min, followed by addition of NHS (4.74 mg, 5 mL DMSO) while stirring for 3 h. The activated Ad-COOH was then dropwise added to a DMSO solution of G3-DOTA dendrimers (25.3 mg, 5 mL) under vigorous stirring at room temperature for 3 days. The molar feeding ratio of G3-DOTA, Ad, EDC, and NHS was set at 1: 1.5: 15: 15. The reaction mixture was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with an MWCO of 1000. After a further lyophilization process, the product of Ad-G3-DOTA dendrimers was obtained and stored at -20 °C for further use.

Synthesis of G5-CD dendrimers and G5-CD/Ad-G3-DOTA core-shell tecto dendrimers with DOTA modifcation (for short, CSTD-D)

To prepare the G5-CD dendrimers, β-CD (21.58 mg) dissolved in DMSO (5 mL) was mixed with a DMSO solution of CDI (31.15 mg, 5 mL) under stirring for 6 h. Then, the above mixture was dropwise added to a DMSO solution of G5.NH_2 dendrimers (22.01 mg, 5 mL) under stirring at 30 °C in a water bath for 60 h. The molar feeding ratio of G5, β-CD, and CDI was set at 1: 25: 250. The reaction mixture was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with an MWCO of 5000. A further lyophilization process gave rise to a white power of the product of G5-CD, which was stored at -20 °C for further use.

G5-CD dendrimers and Ad-G3-DOTA dendrimers at a molar ratio of 1: 12 were used to obtain CSTD-D. In brief, a water solution of G5-CD (5.12 mg, 5 mL) was mixed with a water solution of Ad-G3 (14.61 mg, 5 mL) under stirring at room temperature for 24 h. Subsequently, the raw product of CSTD-D was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with an MWCO of 8000-14000. A further lyophilization process gave rise to the product of CSTD-D, which was stored at -20 °C for further use.

Synthesis of 1,3-PS-G5-CD/Ad-G3-DOTA (PCSTD-D) and 1,3-PS-G5-CD/Ad-G3-DOTA(Gd) (PCSTD-Gd)

PCSTD-D were obtained by reacting CSTD-D (5.78 mg in 5 mL PBS) with 1,3-PS (449.6 mg, 323 μL) under stirring at room temperature for 24 h. Subsequently, the mixture was dialyzed against water (9 times, 2 L) for 3 days using a regenerated cellulose dialysis membrane with an MWCO of

1000 and freeze-dried to obtain the solid of PCSTD-D, which was stored at -20 °C for further use.

Next, $Gd(NO_3)_3 \cdot 6H_2O$ (2.74 mg) in 5 mL water was mixed with PCSTD-Gd (11.48 mg) in 5 mL water under strong stirring at room temperature for 24 h. Then, the raw product of PCSTD-Gd was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with an MWCO of 1000. After freeze drying, the PCSTD-Gd product was obtained. For comparison and characterization, the products of CSTD-Gd and G5-Gd were also synthesized under the same experimental conditions, respectively.

General characterization

^{1}H NMR spectra and 2D NOESY spectra were recorded on a Bruker AV 600 NMR spectrometer (Karlsruhe, Germany). All samples were dissolved in D_2O before measurements. Zeta potential and dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) coupled with a standard 633-nm laser. All samples were dispersed in water before measurements. Atomic force microscopy (AFM) was performed using a Molecular Force Probe 3D AFM (Asylum Research, Santa Barbara, CA). Each sample was prepared by dropping a diluted sample suspension onto a silicon wafer and nitrogen-dried before measurements. UV-vis spectra were collected using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Waltham, MA). All samples were dispersed in water before measurements. The final Gd contents in the PCSTD-Gd were determined *via* Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH). The colloidal stability of the PCSTD-Gd complexes was evaluated by exposing them to water or DMEM (2 mg/mL) for 7 days at room temperature to test their hydrodynamic sizes at different time points (1, 3, 5 and 7 days, respectively) or measuring the relative Gd contents (% of original) in the outer phase of the dialysis bag containing them after dialysis for 1, 2, 3, 4, 5, 6 and 7 days, respectively at 37 °C. The number of the primary amines on each dendrimer-related sample was determined using a Megazyme PANOPA Assay Kit (Wicklow, Ireland) according to the manufacturer's instruction.

The protein resistance property of PCSTD-Gd was evaluated by co-incubating BSA with PCSTD-Gd. In brief, the 500 μL of PCSTD-Gd (or CSTD-Gd) at different concentrations (0.25, 0.5,

1, 2 and 4 mg/mL, respectively, in water) were incubated with 500 μL of BSA (2 mg/mL, in water) at 37 °C for 4 h. Each mixture was centrifuged (8000 r/min, 15 min) and the supernatant was detected using UV-vis spectrophotometer according to the standard instruction of BCA quantitation kit. The difference in the concentration of BSA in the supernatant after CSTD incubation and that before CSTD incubation was used as an indicator to characterize the antifouling property of each sample.

Characterization of PCSTD-Gd/miR 21i polyplexes

The prepared PCSTD-Gd/miR 21i polyplexes were characterized by gel retardation assay, DLS, and zeta potential measurements according to protocols described in our previous work.[4] For gel retardation assay, the PCSTD-Gd/miR 21i polyplexes (1 μg miR 21i) with different N/P ratios (0.125, 0.25, 0.5, 1, 2, and 5, respectively) were prepared, mixed with 6 × loading buffer, and applied to 1% (w/v) agarose gel containing 4S Green plus for electrophoresis at 80 V for 40 min. Free miR 21i (1 μg) was also tested for comparison. Afterward, the retardation of miR 21i was imaged using a ChemiDocTM XRS + Molecular Imager (BIO-RAD, Hercules, CA). For DLS and zeta potential measurements, PCSTD-Gd/miR 21i polyplexes (5 μg miR 21i) under various N/P ratios (2, 5, 10, 15, 20 and 30, respectively) were formed and diluted to 1 mL with diethypyrocarbonate water before experiments.

***In vitro* drug release kinetics**

The formed PCSTD-Gd/DOX complexes were characterized with UV-vis spectroscopy, and *in vitro* DOX release kinetics of PCSTD-Gd/DOX complexes were also tested by UV-vis spectroscopy under two different pHs (pH = 7.4 and pH = 6.5) at 37 °C. The PCSTD-Gd/DOX complexes (2 mg) were dispersed in 1 mL phosphate buffer at pH 6.5 or pH 7.4, transferred to a dialysis bag (MWCO = 1000 Da), and submerged in the corresponding buffer medium (9 mL). The whole system was kept in a constant temperature vibration shaker at 37 °C for different time periods. At each scheduled time interval, 1 mL of the external buffer medium was collected and measured by UV-vis spectroscopy, and then the same volume of the corresponding buffer medium was replenished. The experiment was performed in triplicate for each sample.

Cell culture

MDA-MB-231 cells were regularly cultured and passaged in DMEM containing 10% FBS and 1% penicillin-streptomycin (named $DMEM^{++}$) in a Thermo Scientific cell incubator (Waltham, MA) at 5% CO_2 and 37 °C. *In vitro* studies were carried out to investigate the performances of PCSTD-Gd/miR 21i polyplexes, PCSTD-Gd/DOX complexes and PCSTD-Gd/DOX/miR 21i polyplexes, respectively.

Cytocompatibility assay

The cytocompatibility of PCSTD-Gd/miR 21i polyplexes was evaluated by CCK-8 assay kit. In brief, MDA-MB-231 cells were seeded in 96 well plates at a density of 5×10^3 cells per well with 100 μL $DMEM^{++}$ for each well and incubated overnight. The next day, the medium in each well was replaced with fresh $DMEM^{++}$ containing PCSTD-Gd or PCSTD-Gd/miR 21i polyplexes at various CSTD concentrations (100, 200, 500, 1000, 2000, and 3000 nM, respectively). After 24 h, each well was added with 10 μL CCK-8 solution and the cells were incubated for additional 2 h in the cell incubator. Finally, the absorbance of each well was recorded by a Thermo Scientific Multiskan MK3 ELISA reader at 450 nm. MDA-MB-231 cells treated with PBS were used as a control, and the amount of miR 21i was always kept at 1 μg/well. Each sample was tested in sextuplicate wells.

Transfection of PCSTD-Gd/miR 21i polyplexes

The cellular uptake of PCSTD-Gd/miR 21i polyplexes was carried out by FACS Calibur flow cytometer (Franklin Lakes, NJ). Firstly, MDA-MB-231 cells were seeded into a 12-well plate at a density of 1.0×10^5 cells per well and were cultured overnight at 37 °C and 5% CO_2. Secondly, the PCSTD-Gd/FAM-miR 21i polyplexes were prepared at different N/P ratios (N/P = 1, 5, 10, 15, or 20). Thirdly, the medium of each well was replaced with 500 μL $DMEM^{++}$ containing the PCSTD-Gd/FAM-miR 21i polyplexes, and the cells were incubated for 4 h. Finally, the cells were washed with PBS for three times, digested with trypsin, collected into tubes, resuspended in PBS after centrifugation, and finally measured in the FL1-H channel by flow cytometry (n = 3). Notably, MDA-MB-231 cells treated with PBS or free FAM-miR 21i were used as controls, and the amount of miR 21i was always kept at 1 μg/well.

Next, confocal laser scanning microscopy (CLSM) was performed to explore the intracellular localization of PCSTD-Gd/FAM-miR 21i polyplexes. Briefly, MDA-MB-231 cells were seeded in a confocal dish at a density of 1.0×10^5 cells per dish with 1 mL $DMEM^{++}$ and cultured overnight in a 37 °C incubator under 5% CO_2. After that, the medium of each dish was replaced with 500 μL $DMEM^{++}$ containing PBS, free FAM-miR 21i, or PCSTD-Gd/FAM-miR 21i polyplexes (N/P = 15, the optimal N/P ratio obtained from cellular uptake assay), respectively. The cells were transfected for 4 h, washed three times with PBS, fixed with 4% glutaraldehyde at 4 °C for 15 min, stained with DAPI for 15 min, washed with PBS for 3 times, and observed by CLSM to check their green and blue fluorescence signals.

In order to confirm the biological function of miR 21i after cancer cells were transfected with the PCSTD-Gd/miR 21i polyplexes, wound healing assay was carried out to examine the migration of cancer cells. According to the standard procedures reported in our previous work,[5] MDA-MB-231 cells were seeded into a 12-well plate at a density of 1.0×10^5 cells per well, and were cultured overnight at 37 ℃ and 5% CO_2. Then, a 10 μL pipette tip was used to make a scratch in each well and the cells were washed with PBS for three times. The cell medium was replaced with 1 mL of $DMEM^{++}$ containing PCSTD-Gd/miR 21i polyplexes (N/P = 15, 2 μg/well miR 21i) in each well. MDA-MB-231 cells treated with PBS or free miR 21i were used as controls, and the wound in each well was observed by Leica DM IL LED inverted phase contrast microscope at 0, 12, 24, and 48 h, respectively. Image J software (https://imagej.nih.gov/ij/download.html) was used to quantify of the migration area.

Combinational gene therapy and chemotherapy of TNBC cells *in vitro*

The anticancer effect *in vitro* was evaluated by CCK-8 assay. Firstly, MDA-MB-231 cells were seeded in 96-well plates at a density of 5×10^3 cells per well with 100 μL $DMEM^{++}$ and incubated overnight. The next day, the medium in each well was replaced with 100 μL fresh $DMEM^{++}$ containing PCSTD-Gd/DOX/miR 21i polyplexes, PCSTD-Gd/DOX complexes, or free DOX.HCl at various DOX concentrations (0.5, 1.25, 2.5, 5, 10, 25 and 50 μg/mL, respectively). After 24 h, each well was added with 10 μL CCK-8 solution and the cells were incubated for additional 2 h in the cell

incubator. Finally, the absorbance of each well was recorded by a Thermo Scientific Multiskan MK3 ELISA reader at 450 nm. The half-maximal inhibitory concentrations (IC_{50s}) of free DOX.HCl, PCSTD-Gd/DOX complexes, and PCSTD-Gd/DOX/miR 21i polyplexes were calculated using a GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Each sample was tested in sextuplicate wells.

The apoptosis of MDA-MB-231 cells induced by different miR 21i-related or DOX-related complexes was examined using an Annexin V-FITC/7-AAD apoptosis detection kit. In brief, MDA-MB-231 cells were cultured overnight in 6-well plates at a density of 2×10^5 cells per well in 2 mL of $DMEM^{++}$. The next day, the medium in each well was replaced with 2 mL fresh $DMEM^{++}$ containing free miR 21i, free DOX, PCSTD-Gd/miR 21i, PCSTD-Gd/DOX, or PCSTD-Gd/DOX/miR 21i ([DOX] = 3.12 μg/mL for all DOX-related groups, and the concentration of other groups corresponded to the DOX-incorporated groups). After 24 h, the cells in each well were washed, collected, resuspended in 195 μL of binding buffer, and added with 5 μL of Annexin V-FITC and 5 μL of 7-AAD according to the kit instruction. Then, each sample was incubated for 15 min in the dark before flow cytometry analysis (n = 3). Cells treated with PBS were used as control.

Next, cell cycle assay was also performed. Briefly, MDA-MB-231 cells were cultured in a 6-well plate at a density of 2.0×10^5 cells per well in 2 mL of $DMEM^{++}$ overnight, and exposed to 2 mL fresh medium containing free miR 21i, free DOX, PCSTD-Gd/miR 21i, PCSTD-Gd/DOX, or PCSTD-Gd/DOX/miR 21i ([DOX] = 3.12 μg/mL for all DOX-related groups, and the concentration of other groups corresponded to the DOX-incorporated groups). After 24 h incubation, cells were collected, washed twice with PBS, and fixed with 75% cold ethanol overnight at 4 °C. Then, samples were washed and stained with a mixed solution of 1% Triton X-100, 0.01 % RNase, and 0.05 % PI for 15 min at 37 °C in the dark for DNA quantification. The cells treated with PBS were used as control. The DNA content was measured by flow cytometry and the percentage of each cell cycle stages was calculated using the ModFit software (Verity Software House, Topsham, ME).

Real-time PCR (RT-PCR) was used to quantify the gene (PTEN, PDCD4, Bax, Bcl-2) levels of the cancer cells after different treatments. MDA-MB-231 cells were cultured in a 6-well plate at a

density of 2.0×10^5 cells per well in 2 mL of DMEM^{++} overnight, and exposed to 2 mL fresh medium containing free miR 21i, free DOX, PCSTD-Gd/miR 21i, PCSTD-Gd/DOX, or PCSTD-Gd/DOX/miR 21i ([DOX] = 3.12 μg/mL for all DOX-related groups, and the concentration of other groups corresponded to the DOX-incorporated groups). After 48 h, the total RNA was extracted from cells using RNAeasy™ Plus Animal RNA Isolation Kit and was reversely transcribed into cDNA by cDNA synthesis kit. The obtained cDNA, SYBR Green qPCR mix, and the upstream and downstream primers of the gene were mixed into a 20-μL reaction system according to standard protocols. Amplification reaction was carried out using 7500 fast RT-PCR system (Applied Biosystems, Foster City, CA) and the PCR was performed at 95 °C for 2 min, and 40 reaction cycles were applied for the process of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Relative quantification was conducted using amplification efficiencies derived from 2nd-strand cDNA. Data are gained as fold changes ($2^{-\Delta\Delta Ct}$) and were analyzed using Opticon Monitor Analysis Software V 2.02 (Thermal, Waltham, MA). The upstream and downstream primer sequences of each gene are shown in Table S3.

Western blot (WB) analysis was carried out to evaluate the expression of related target proteins (PTEN, PDCD4, Bax, and Bcl-2) of the cancer cells after different treatments. Briefly, MDA-MB-231 cells were seeded into a 6-well plate at a density of 2.0×10^5 cells per well and were cultured overnight. Then, the medium of each well was replaced with 2 mL DMEM^{++} containing free miR 21i, free DOX, PCSTD-Gd/miR 21i, PCSTD-Gd/DOX, or PCSTD-Gd/DOX/miR 21i ([DOX] = 3.12 μg/mL for all DOX-related groups, and the concentration of other groups corresponded to the DOX-incorporated groups). PBS was used as a negative control. After 48 h, the treated cells were washed, collected and analyzed *via* western blot assay to detect the protein expression according to the literature. In general, the miR 21 regulates the proliferation, migration and invasion of tumor cells. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is an oncogene suppressor, which is the target gene of miR 21. PTEN expression is suppressed by the expressed miR 21 through the activation of the P13K/PTEN/AKT signaling pathway, which further take effect on the downstream target genes (Bax and Bcl-2) to induce cancer cell apoptosis. Meanwhile, the

miR 21 targets programmed cell death protein 4 (PDCD4) and inhibits its expression, inducing a negative effect in cell cycle arrest, tumor cell proliferation inhibition and cell apoptosis promotion by SP1 transcription factor.

Preparation of sono vue microbubbles (MBs)

Sono Vue (Bracco diagnostics Inc., Geneva, Switzerland), an ultrasound contrast agent containing stabilized MBs filled with SF_6 gas, was used. It is available as a kit including one vial of SF_6 gas (59 mg) and powder (25 mg), and one pre-fixed syringe with a volume of 5 mL. According to the manufacture's instruction, 5 mL of normal saline (NS) was added to the purchased Sono Vue vial with a sterile needle. After that, the vial was shaken by hand for 20 s to form the MBs (SF_6 gas with the shell composed of lipid monolayer membrane) in a liquid suspension. The prepared Sono Vue solution was stored at 4 °C and used up within 6 h.

Ultrasound (US) apparatus

The UTMD experiments were conducted using Chattanooga Intelect Mobile Ultrasound Reference 2776 with 5 cm² applicator (DJO France SAS, Mouguerre, France). The Sono Vue solution was shaken to form homogeneous white emulsion before use. After the MDA-MB-231 cells reached 80-90% confluence, we replaced the cell medium with the fresh DMEM containing 20% microbubbles, treated the cells with coupling agent, and applied the ultrasound therapeutic instrument probe under a fixed main frequency of 1 MHz, power of 0.4 W/cm^2, and an irradiation time of 30 s for each well.

The effect of UTMD on the cell viability and cellular uptake after different treatments was investigated. For cell viability assay, 5×10^3 MDA-MB-231 cells were added into each well of a 96-well plate with 100 μL DMEM^{++} and incubated overnight. The next day, the medium in each well was replaced with 80 μL fresh DMEM^{++} containing PBS or PCSTD-Gd/miR 21i polyplexes at different N/P ratios (N/P =1, 2, 5, 10, 15, or 20) and 20 μL Sono Vue MBs. Then, the MDA-MB-231 cells in the 96-well plate were subjected to UTMD treatment (0.4 W/cm^2, 30 s per well). After 24 h of continuous cultivation, cells in each well were added with 10 μL CCK-8 solution and the cells were incubated for additional 2 h in the cell incubator. Finally, the absorbance of each well was recorded by a Thermo Scientific Multiskan MK3 ELISA reader at 450 nm. Notably, MDA-MB-231

cells treated with 100 μL fresh DMEM^{++} containing PBS or PCSTD-Gd/miR 21i polyplexes at different N/P ratios (N/P =1, 2, 5, 10, 15, or 20) without UTMD were also tested for comparison, and the amount of miR 21i was always kept at 1 μg/well. Each sample was tested in sextuplicate wells.

For *in vitro* cellular uptake experiment, 1 × 10^5 MDA-MB-231 cells were added into each well of a 12-well plate with 1 mL DMEM^{++} and incubated overnight. The next day, the medium in each well was replaced with 0.8 mL fresh DMEM^{++} containing PCSTD-Gd/FAM-miR 21i polyplexes at different N/P ratios (N/P = 1, 5, 10, 15, and 20, respectively) and 0.2 mL of the Sono Vue MBs. After that, the MDA-MB-231 cells in the 12-well plate were subjected to UTMD treatment (0.4 W/cm^2, 30 s per well). After the cells were incubated for 4 h, the cells were washed with PBS for three times, digested with trysin-EDTA, collected into the tubes, resuspended in PBS after centrifugation, and finally measured in the FL1-H channel by flow cytometry (n = 3). MDA-MB-231 cells treated with 0.8 mL fresh DMEM^{++} containing PBS or free FAM-miR 21i were used as controls, and the amount of FAM-miR 21i was always kept at 1 μg/well. For comparison, the same experiments were also carried out without UTMD.

Evaluation of UTMD-enhanced anticancer effect *in vitro*

For cytotoxicity assay under UTMD, 5 × 10^3 MDA-MB-231 cells were added into each well of a 96-well plate with 100 μL DMEM^{++} and incubated overnight. The next day, the medium in each well was replaced with 80 μL fresh DMEM^{++} containing PCSTD-Gd/DOX/miR 21i polyplexes at various DOX concentrations (0.625, 1.5625, 3.125, 6.25, 15.625, 31.25 and 62.5 μg/mL, respectively) and 20 μL Sono Vue MBs to get the final DOX concentrations of 0.5, 1.25, 2.5, 5, 10, 25 and 50 μg/mL, respectively. Then, the MDA-MB-231 cells in the 96-well plate were subjected to UTMD treatment (0.4 W/cm^2, 30 s per well). After 24 h of continuous cultivation, CCK-8 assay was carried out to detect the UTMD-enhanced cytotoxicity according to procedures described above.[6]

Penetration of multicellular tumor spheroids (MCTS)

MCTS composed of MDA-MB-231 cells were constructed according to the literature.[7] Briefly, 500 μL of agarose solution (2%, w/v, in sterile saline) was dropped into the micro-mold to form 3D Petri

Dish gels. Then, the solidified 3D Petri Dish gels were separated from the micro-mold, placed to each well of a 12-well plate, and equilibrated for more than 15 min with 2.5-mL $DMEM^{++}$. Then, the culture medium outside and inside the cell seeding chamber of the 3D Petri Dish was removed. After that, the cell suspension (190 μL) containing 5×10^5 MDA-MB-231 cells were carefully seeded into each 3D Petri Dish. Ten minutes later, additional fresh culture medium (2.5 mL) was slowly added to each well of the 12-well plate to allow the cells to aggregate and grow. After about 7 days of culture, the MCTS with a diameter of 200 μm were built up successfully.

To study the penetration of PCSTD-Gd/DOX/miR 21i polyplexes after UTMD treatment, the MCTS of MDA-MB-231 cells were incubated with PCSTD-Gd/DOX/miR 21i polyplexes at a DOX concentration of 3.9 μg/mL and 20 μL Sono Vue MBs to get the final DOX concentration of 3.12 μg/mL. Subsequently, the MCTS in the 12-well plate were subjected to UTMD treatment (0.4 W/cm^2, 30 s per well). After 6 h of continuous cultivation, the MCTS were washed carefully with PBS and observed by CLSM with Z-stack scanning from the top to bottom with 5 μm per section. The DOX fluorescence intensity in each MCTS sample was analyzed by Zeiss CLSM software. The MCTS treated with PCSTD-Gd/DOX/miR 21i polyplexes without UTMD were also tested for comparison.

Hemolysis assay

All animal experiments were performed in accordance with the guidelines of the Committee on Experimental Animal Care and Use of Donghua University and following the regulations of the National Ministry of Health. Mouse blood stabilized with heparin was first centrifugated for 5 min (3000 rpm), then washed with PBS for 5 times according to the procedure reported in the literature[8] in order to completely remove the serum and obtain red blood cells (RBCs). The collected RBCs were 5-times diluted with PBS. Then, 0.5 mL of RBC suspension was mixed with 0.5 mL PBS solution of PCSTD-Gd/DOX/miR 21i complexes with different DOX concentrations (0.5, 1.25, 2.5, 5, 10, 25 and 50 μg/mL, respectively) and incubated for 2 h at 37 °C. Subsequently, the mixture was centrifugated (10000 rpm, 5 min) and photographed. After that, the absorbance of the supernatants (hemoglobin) was determined at 540 nm *via* UV-vis spectrometry. The percentage of hemolysis was

calculated according to the following formula: hemolysis rate (%) = (sample absorbance − negative control absorbance) / (positive control absorbance − negative control absorbance) × 100%. PBS and water were used as the negative and positive controls respectively, and each measurement was repeated for 3 times.

T_1 MR relaxometry

The T_1 relaxation times of samples were measured by a 0.5 T NMI20-Analyst NMR analyzing and imaging system (Shanghai Niumag Corporation, Shanghai, China). The PCSTD-Gd and G5-Gd complexes were diluted in water with Gd concentrations in a range of 0.04-0.64 mM in 2-mL Eppendorf tubes, respectively. The longitudinal relaxation times (T_1) were measured (at 298 K) and the r_1 relaxivity was calculated by linearly fitting the inverse T_1 relaxation time ($1/T_1$) as a function of Gd concentration. The T_1-weighted MR images of samples were recorded using a clinical MR system (3.0 T, MAGNETOM VERIO, SIEMENS Medical Systems, Erlangen, Germany) with a spin-echo imaging sequence under the following parameters: point resolution = 156 mm × 156 mm, TR = 500 ms, TE = 20 ms, and matrix = 256 × 256.

***In vivo* MR imaging**

Female BALB/c nude mice (15-20 g, 3-4 weeks, Shanghai Slac Laboratory Animal Center, Shanghai, China) were used to establish the orthotopic breast cancer model for MR imaging. Each mouse was injected with 1×10^6 MDA-MB-231 cells (in 100 μL of PBS) under the right lower breast pad. When the tumor volume reached 100 mm^3 at about 4 weeks post-injection, the model was built up successfully for further use.

For MR imaging, the tumor-bearing mice were first anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and intravenously injected with PCSTD-Gd/DOX/miR 21i polyplexes ([Gd] = 5 mM, 100 μL for each mouse). Subsequently, MR imaging was executed before injection and at different time points post-injection using a clinical 3.0 T MR system (Siemens Medical Systems, Erlangen, Germany). The parameters were set as follows: TE = 15 ms, TR = 620 ms, FOV = 8 × 10 cm, and matrix = 256 × 171. MR images were acquired and the tumor MR signal-to-noise ratio (SNR) was quantified.

For UTMD-enhanced MR imaging, after intravenous injection of the PCSTD-Gd/DOX/miR 21i polyplexes ([Gd] = 5 mM, 100 μL) to each tumor-bearing mouse, the US transducer was positioned above the tumor, which was coated with an aqueous coupling agent, and Sono Vue solution (100 μL) was slowly injected *via* tail vein. Then, the tumor of each mouse was sonicated percutaneously for 2 min to achieve the longest active sonoporation time (1 MHz, 0.4 W/cm^2), followed by MR imaging at different time points.

***In vivo* Gd biodistribution**

At different time points (0, 1.5, 2, 3, 6, 12, and 48 h, respectively) post intravenous injection of the PCSTD-Gd/DOX/miR 21i polyplexes ([Gd] = 5 mM, 100 μL) or PCSTD-Gd/DOX/miR 21i polyplexes with UTMD treatment to each mouse, the MDA-MB-231 tumor-bearing mice were sacrificed. Major organs of the mice including heart, liver, spleen, lung, kidney, and tumor were extracted, weighed, cut into small pieces, and then lysed using an *aqua regia* solution (2 mL, hydrochloric acid/nitric acid, v/v = 3: 1) for 7 days. The Gd content was quantified by ICP-OES after the obtained lysate was diluted with water (n = 3 for each group).

***In vivo* antitumor activity and biosafety assays**

The orthotopic breast cancer model was first established. When the tumor volume reached about 100 mm^3, the mice were randomly divided into six groups (n = 5 in each group): PBS (I), PCSTD-Gd/miR 21i (II), DOX.HCl (III), PCSTD-Gd/DOX (IV), PCSTD-Gd/DOX/miR 21i (V), and PCSTD-Gd/DOX/miR 21i with UTMD treatment (VI). In all groups, each mouse was injected with materials dispersed in 100 μL PBS every 3 days for 5 consecutive times. Notably, the DOX dose was 5 mg/kg in each mouse for all DOX-related groups, and the concentration of other groups corresponded to the DOX-incorporated groups. For the group of PCSTD-Gd/DOX/miR 21i with UTMD treatment, the US transducer (1 MHz, 2 min, and 0.4 W/cm^2) was positioned above the tumor region, which was coated with an aqueous coupling agent, and then Sono Vue solution (100 μL) was slowly injected after injection of the PCSTD-Gd/DOX/miR 21i solution. During the treatment period, body weight and tumor volume of all groups were measured every 2 days. The tumor volume was calculated according to the formula of $V = W^2 \times L / 2$, where W and L represent

the length in minor and major axes, respectively. The tumor weight and tumor photographs of mice in each group were measured and collected on the last day after different treatments.

At the end of the treatments, US imaging was performed using the transducer in both contrast-enhanced ultrasound (CEUS) mode and conventional B-mode at the same time points. The ultrasound imaging was performed on a SIEMENS Healthineers Acuson sequoia (Siemens Shanghai Medical Equipment Ltd., Shanghai, China). Three mice were randomly selected from each group for US imaging after injection of Sono Vue (100 μL for each mouse) through orbital vein. CEUS images were used to record the perfusion process of tumors for 2 min. To investigate the perfusion process, the perfusion curve of a region of interest was generated after injection of Sono Vue, and optical imaging signal intensities were measured using the software supplied by the manufacturer.

In addition, the tumor-bearing mice in different treatment groups were sacrificed at 14 days post-injection, and the livers were extracted, photographed, and examined to count the number of nodules on the surface. Subsequently, the vital organs (heart, liver, spleen, lung, and kidney) and tumor tissues were excised, fixed in 4% paraformaldehyde overnight, and embedded in paraffin for hematoxylin & eosin (H&E) staining to observe the histological changes to assess biosafety of materials and to examine the tumor necrosis effect. The tumor sections were also TdT-mediated dUTP nick end labeling (TUNEL) and Ki67 stained according to standard protocols[1] to examine the tumor cell apoptosis and proliferation inhibition effects.

Statistical analysis

All experimental data ($n \geq 3$) are shown in the form of mean ± standard deviation (SD). One-way analysis of variance statistical method was adopted to evaluate the significant difference of data between groups using IBM SPSS Statistic 26 software (IBM, Armonk, NY). A p value of 0.05 was considered as the level of significance, and the associated data were marked as (*) for $p < 0.05$, (**) for $p < 0.01$, (***) for $p < 0.001$, respectively.

Table S1. Hydrodynamic sizes and zeta potentials of dendrimers and CSTDs (n =3).

Sample	Hydrodynamic size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
PCSTD-Gd	309.5 ± 39.4	0.53 ± 0.07	16.3 ± 3.8
PCSTD-D	279.5 ± 28.2	0.36 ± 0.04	15.3 ± 3.1
CSTD-D	248.0 ± 27.7	0.44 ± 0.02	29.3 ± 4.2
G5-CD	189.1 ± 37.8	0.41 ± 0.08	16.7 ± 4.9
Ad-G3-DOTA	153.3 ± 31.2	0.35 ± 0.03	24.8 ± 2.7
G3-DOTA	103.5 ± 18.6	0.33 ± 0.16	26.3 ± 2.1

Table S2. Mean number of primary amines per dendrimer/CSTD (n = 3).

Samples	Mw[a]	Mean number of primary amines
PCSTD-Gd	114763	83.8
PCSTD-D	111460	72.5
CSTD-D	105613	75.2
G5-CD	39626	60.2
Ad-G3-DOTA	9426	12.6
G3-DOTA	9193	12.6

[a] Mws were estimated by ^{1}H NMR spectra.

Table S3. The sequences of upstream and downstream primers.

Name	Sequences (5'-3')
GAPDH-F*	GGACCTGACCTGCCGTCTAG
GAPDH-R*	GTAGCCCAGGATGCCCTTGA
PDCD4-F	GGGAGTGACGCCCTTAGAAG
PDCD4-R	ACCTTTCTTTGGTAGTCCCCTT
PTEN-F	TTTGAAGACCATAACCCACCAC
PTEN-R	ATTACACCAGTTCGTCCCTTTC
Bax-F	TTTTGCTTCAGGGTTTCATCCA
Bax-R	TGCCACTCGGAAAAAGACCTC
Bcl-2-F	CGCCCTGTGGATGACTGAGTA
Bcl-2-R	GACAGCCAGGAGAAATCAAACAG

*F and R represent the upstream and downstream primer, respectively.

Table S4. IC_{50} values according to the DOX concentration of samples.

Sample	IC_{50} (μg/mL)
DOX	0.70
PCSTD-Gd/DOX	4.64
PCSTD-Gd/DOX/miR 21i	3.12
PCSTD-Gd/DOX/miR 21i + UTMD	1.50

Table S5. The tumor inhibition rate of samples.

Sample	Tumor inhibition rate (%)
DOX	36.89
PCSTD-Gd/ miR 21i	4.88
PCSTD-Gd/DOX	51.70
PCSTD-Gd/DOX/miR 21i	72.13
PCSTD-Gd/DOX/miR 21i + UTMD	78.89

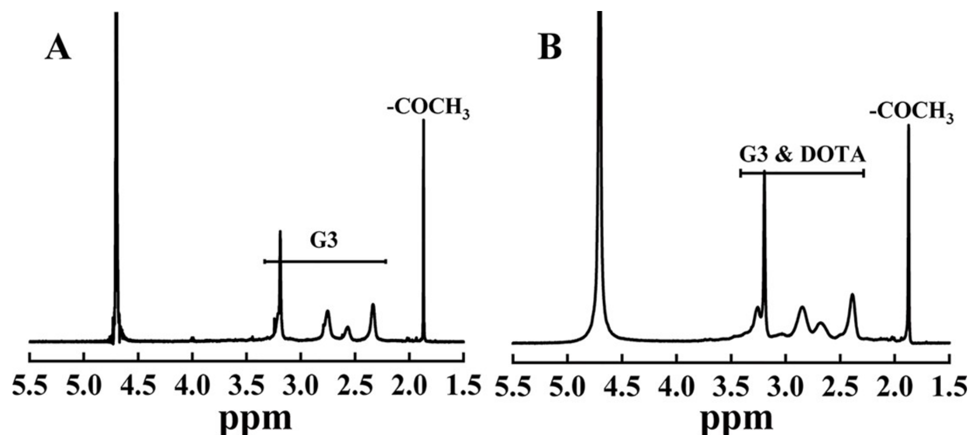

Figure S1. ^{1}H NMR spectra of G3.NHAc (A) and G3.NHAc-DOTA (B) dendrimers. Since the characteristic peaks of DOTA overlap with those of the G3 dendrimer, we calculated the amount of DOTA bonded to each G3 dendrimer by an indirect method. Firstly, we calculated the number of the acetyl groups in each fully acetylated G3 dendrimer (G3.NHAc) and in each fully acetylated G3-DOTA dendrimer (G3.NHAc-DOTA), respectively through NMR integration comparison. Through subtraction, the number of DOTA moiety per G3 dendrimer was calculated to be 3.4.

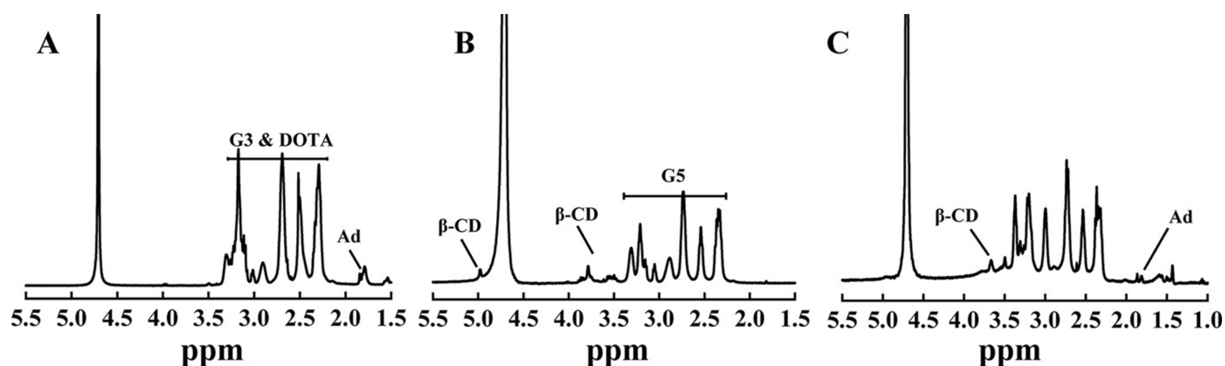

Figure S2. ^{1}H NMR spectra of Ad-G3-DOTA (A), G5-CD (B) and CSTD-D (C).

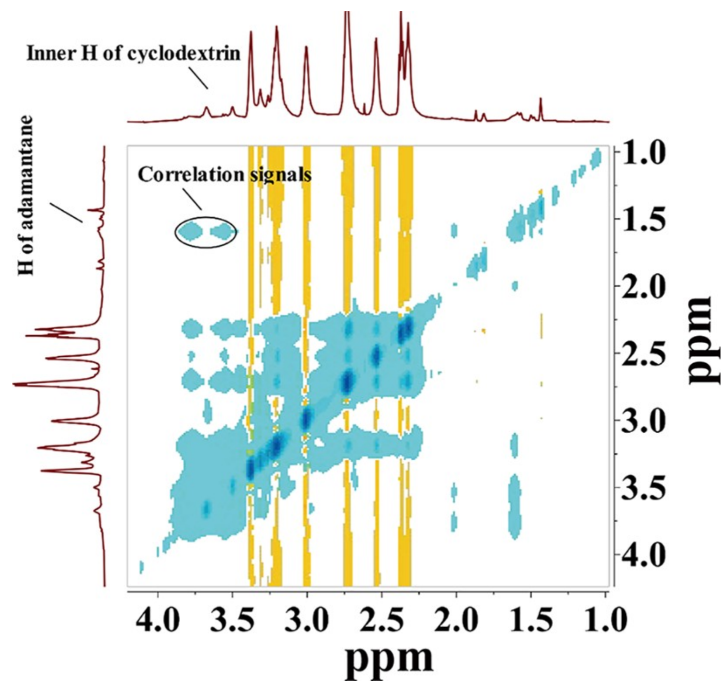

Figure S3. 2D NOESY spectrum of CSTD-D.

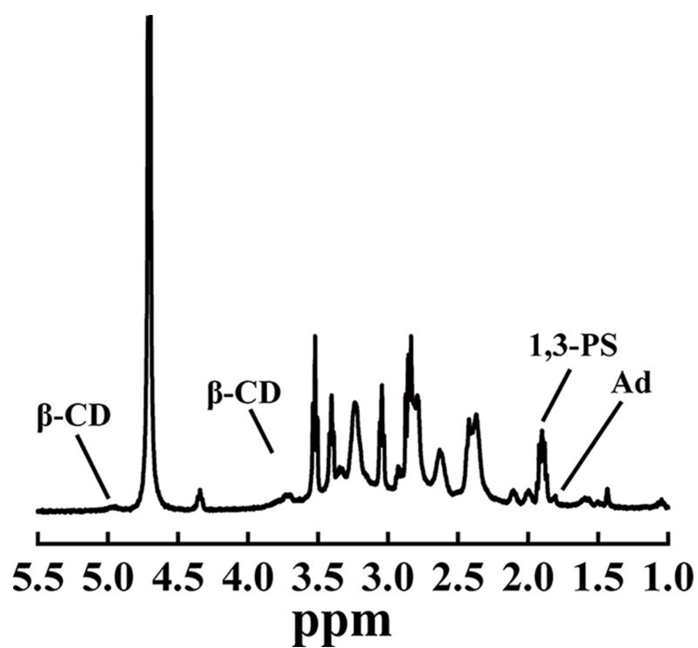

Figure S4. ^{1}H NMR spectrum of PCSTD-D.

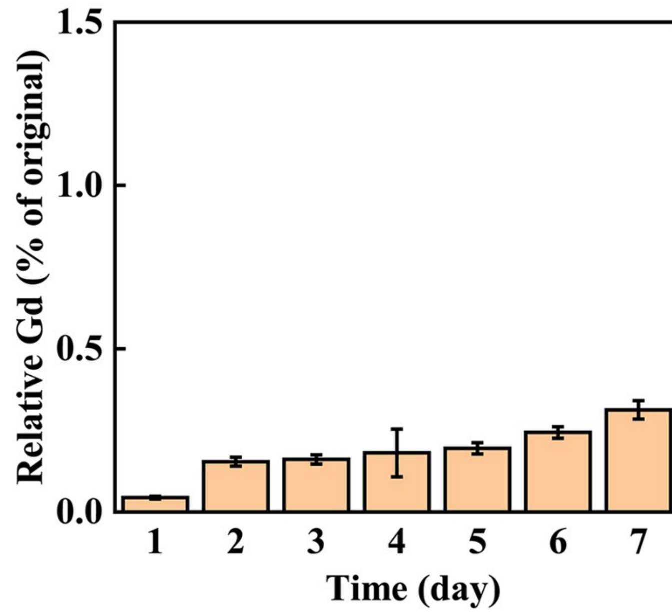

Figure S5. The relative Gd contents (% of original) in the outer phase of the dialysis bag containing the PCSTD-Gd complexes after dialysis within 7 days at 37 °C (n = 3).

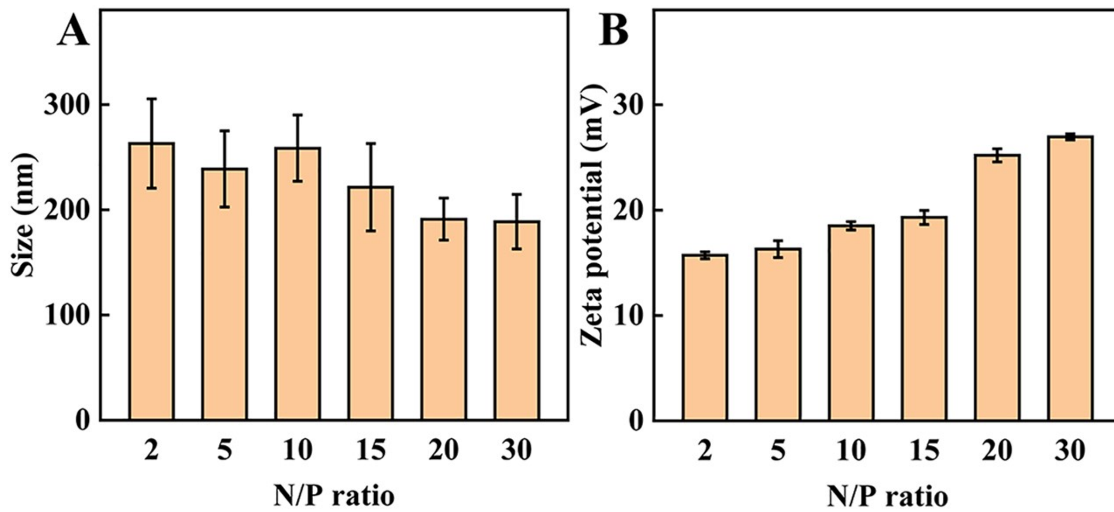

Figure S6. Hydrodynamic sizes (A) and zeta potentials (B) of the PCSTD-Gd/miR 21i polyplexes under different N/P ratios (n = 3).

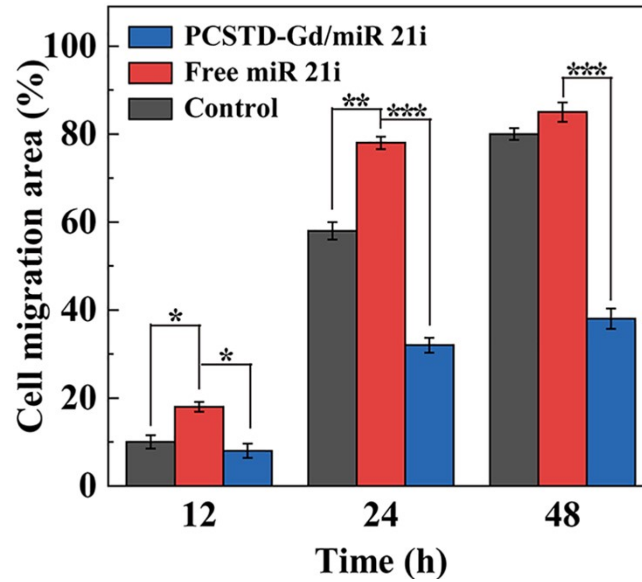

Figure S7. The corresponding cell migration of MDA-MB-231 cells after transfection with the PCSTD-Gd/miR 21i polyplexes for 0, 12, 24 and 48 h, respectively for the wound-healing assay (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001, respectively.

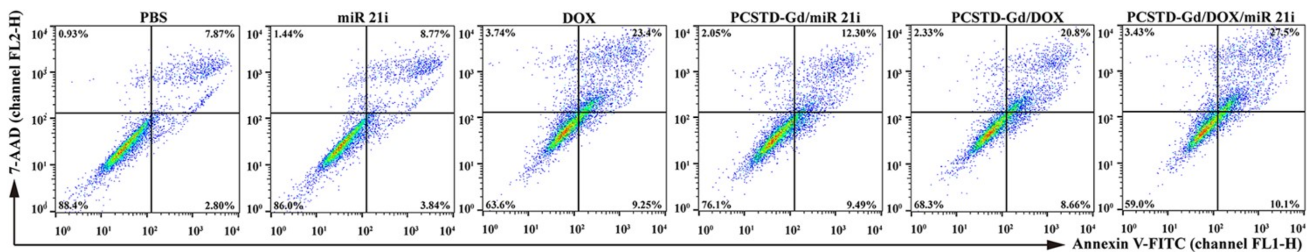

Figure S8. Apoptosis analysis of MDA-MB-231 cells after different treatments at a DOX concentration of 3.12 μg/mL for 24 h.

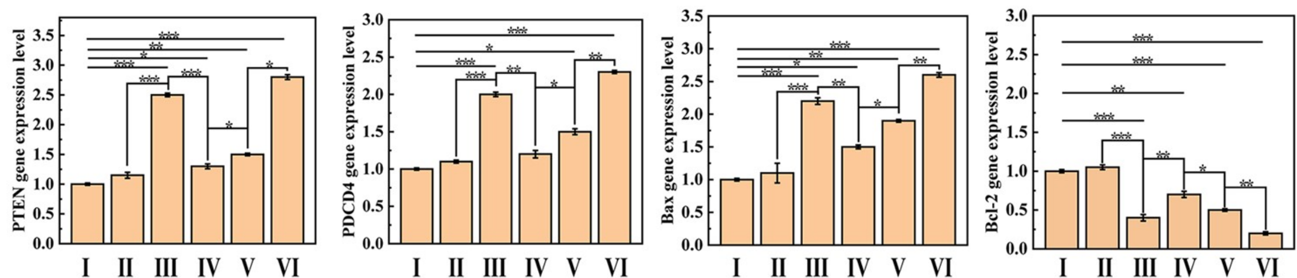

Figure S9. Quantitative analysis of gene expression based on RT-PCR assay. I: PBS, II: miR 21i, III: DOX, IV: PCSTD-Gd/miR 21i, V: PCSTD-Gd/DOX, and VI: PCSTD-Gd/DOX/miR 21i (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001, respectively.

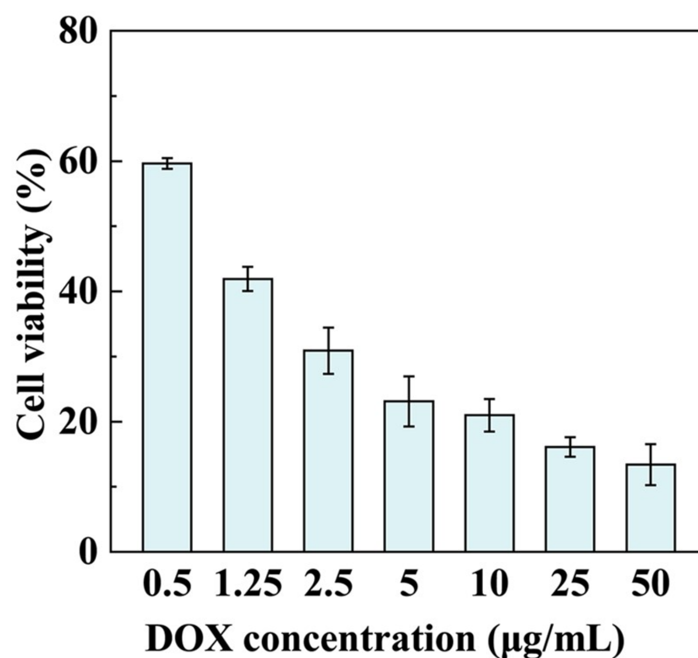

Figure S10. Viability assay of MDA-MB-231 cells after treated with the PCSTD-Gd/DOX/miR 21i under UTMD at different DOX concentrations for 24 h (n =6).

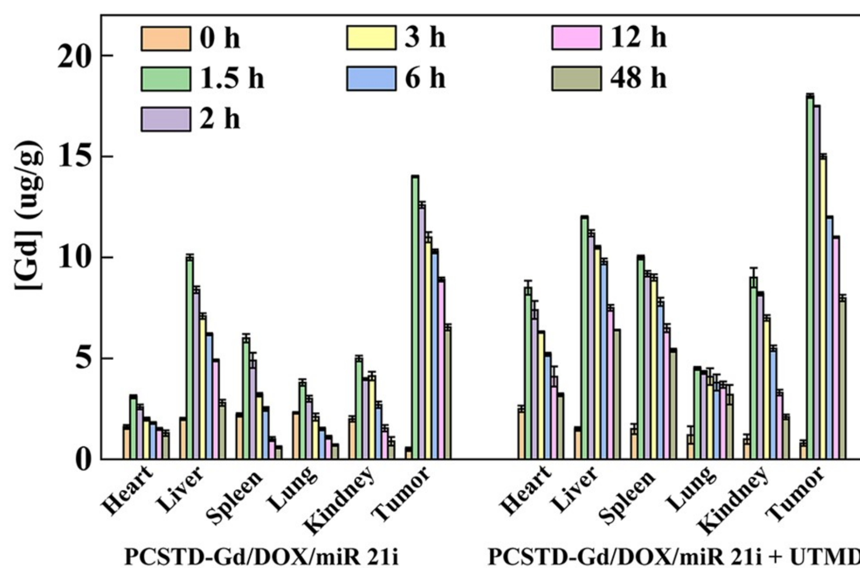

Figure S11. Biodistribution of Gd in the major organs and tumors of mice at different time points post intravenous injection of the PCSTD-Gd/DOX/miR 21i with or without UTMD ([Gd] = 5 mM, in 100 μL PBS, for each mouse, n =3).

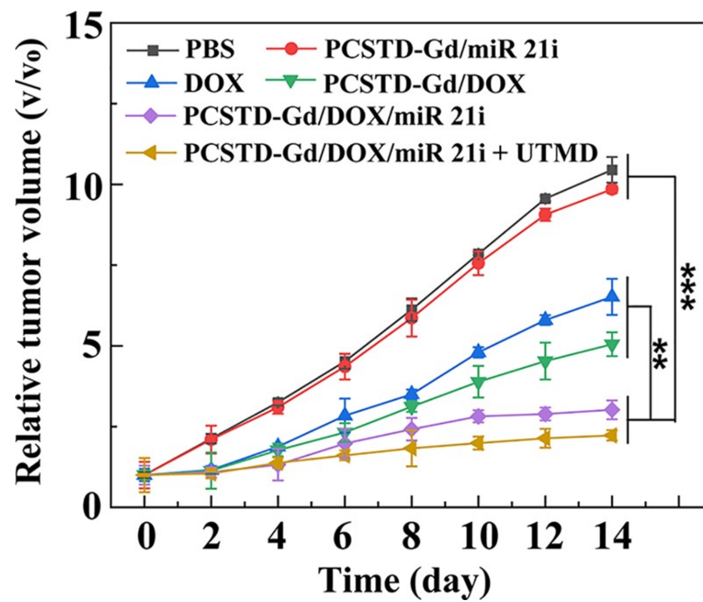

Figure S12. Relative tumor volume of tumor-bearing mice as a function of time in different treatment groups (n = 5). ***$p < 0.001$.

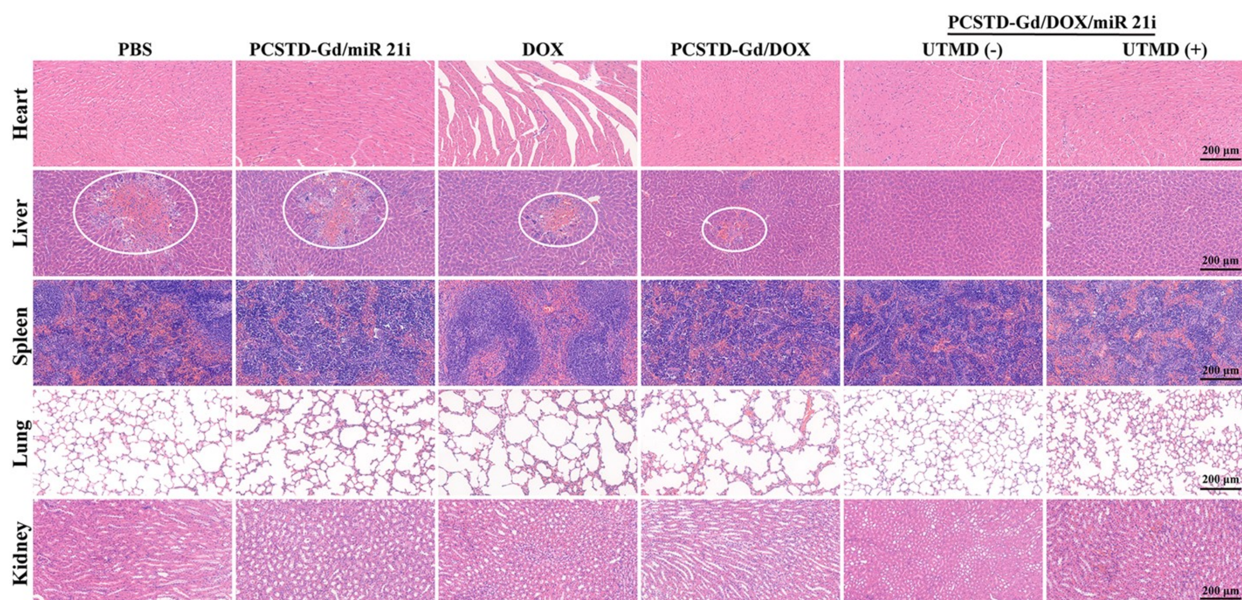

Figure S13. H&E staining of the sacrificed mouse organs on day 14 after different treatments (scale bar for each panel represents 200 μm).

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