Published in final edited form as:

Acta Biomater. 2019 January 15; 84: 378–390. doi:10.1016/j.actbio.2018.12.010.

Repurposing suramin for the treatment of breast cancer lung metastasis with glycol chitosan-based nanoparticles

Bei Cheng, Feng Gao, Erica Maissy, and Peisheng Xu

Department of Drug Discovery and Biomedical Sciences, College of Pharmacy, University of South Carolina, 715 Sumter St., Columbia, SC 29208, United States

Abstract

Suramin (SM), a drug for African sleeping sickness and river blindness therapy, has been investigated in various clinical trials for cancer therapy. However, SM was eventually withdrawn from the market because of its narrow therapeutic window and the side effects associated with multiple targets. In this work, we developed a simple but effective system based on a nontoxic dose of SM combined with a chemotherapeutic agent for the treatment of metastatic triple-negative breast cancer (TNBC). SM and glycol chitosan (GCS) formed nanogels because of the electrostatic effect, whereas doxorubicin (DOX) was incorporated into the system through the hydrophilic and hydrophobic interactions between DOX and GCS as well as the ionic interactions between DOX and SM to yield GCS-SM/DOX nanoparticles (NPs). GCSSM/DOX NPs have a size of approximately 186 nm and a spherical morphology. *In vitro* experiments showed that GCS-SM NPs could effectively inhibit cancer cell migration and invasion, as well as angiogenesis. Furthermore, in a TNBC lung metastasis animal model, GCSSM/DOX NPs significantly reduced tumor burden and extended the lifespan of animals, while not inducing cardio and renal toxicities associated with the DOX and SM, respectively. As all the components used in this system are biocompatible and easy for large-scale fabrication, the GCSSM/DOX system is highly translatable for the metastatic breast cancer treatment.

Graphical Abstract

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

suramin; doxorubicin; nanoparticle; antimetastasis; antiangiogenesis; triple-negative breast cancer

1. Introduction

Breast cancer is the most common cancer among women worldwide, and metastasis is the major cause of human cancer death [1]. Metastasis involves multiple steps, including cell invasion from the primary tumor, intravasation and extravasation in circulation system, and growth in the distant location [2]. Ideally, the most effective way of treating metastatic cancer is to kill cancer cells before their dissemination from the primary site. However, because of the lack of clinically effective tools in accurately detecting small tumor mass and killing 100% of cancer cells, many patients eventually die from cancer metastasis. When a patient with breast cancer is diagnosed with distant metastasis, the five-year survival rate drops sharply from 98.6% to 23.4% [3]. Currently, various treatments are available for patients with different stages of metastasis. However, at the early stage before the diagnosis of circulating tumor cells (CTC), there are barely any FDA-approved treatments other than prophylactic and vaccination. Once a patient is diagnosed with CTC, or even micro-metastasis, surgery or radiation together with systemic therapy will be applied. For high-risk patients, metronomic chemotherapy and antiangiogenesis might be necessary [2].

Angiogenesis, a hallmark of malignant disease, is a process to form new blood vessels based on the original ones, which is critical for tumor progression. CTCs upregulate several proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to adapt to the new tumor microenvironments [4]. The combination of bevacizumab, an inhibitor of VEGF, with paclitaxel/carboplatin significantly increased the median survival month for patients diagnosed with metastatic colorectal cancer or advanced nonsmall cell lung cancer in a phase III trial [5]. However, for patients with late-stage breast cancer, the best treatment thus far only provides survival advantage of a few months [6]. FGFs and their receptors (FGFRs) play crucial roles in many fundamental processes from embryogenesis to adult life, such as proliferation, differentiation, migration, angiogenesis, and wound healing [7–13]. Like many other mitogens, FGFs act like a double-edged sword and deregulate the signaling, thereby causing various types of human cancers including lung, breast, ovarian, and prostate cancers [7, 14–16]. Therefore, FGFs/FGFRs have been extensively studied as potential targets for cancer treatment. Many selective or nonselective pharmaceutical inhibitors for FGFs and FGFRs have been developed [17–21].

Suramin (SM), a polysulfonated naphthylurea that nonspecifically inhibits VEGF and bFGF, is reported as an antiangiogenic agent [22–27]. SM reversed the FGF-induced drug resistance at a concentration of 1–17 μ M in human prostate PC3 cells in the presence of doxorubicin (DOX) [25]. The combination of SM and various chemotherapeutic agents has been proven effective in many mice models and entered clinical trials. The combination of SM and paclitaxel inhibited the brain metastatic cancer at doses of 10 mg/kg for SM and 10 mg/kg for PTX [27]. The nontoxic doses of SM with DOX were suggested for the treatment of prostate cancer [25, 28]. The co-delivery of SM enhanced the activity of DOX in dogs

with spontaneous cancers [29]. In a xenograft prostate mouse model, the addition of SM to DOX inhibited tumor growth by 60% [25]. Although SM has a direct impact on cancer cells, a high dose of SM not only causes cytotoxic effects but also initiates tolerance response [30]. A recent study showed that a polymer-drug conjugate of low-molecular-weight heparin and SM can significantly inhibit the tumor progression in a xenograft squamous cell tumor model [31].

In this study, a simple and effective nanoparticle (NP) system is developed for the treatment of breast cancer lung metastasis. SM and glycol chitosan (GCS) forms a nanogel system due to the electrostatic effect, while DOX is encapsulated in the GCS/SM system due to the hydrophilic and hydrophobic interactions between DOX and GCS as well as the ionic interactions between DOX and SM. Both SM and GCS showed an inhibitory effect on the migration and invasion of MDA-MB-231 breast cancer cells. SM, at a nontoxic dose, was able to enhance the anticancer efficacy of DOX both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials and cell lines

GCS, suramin sodium salt (SM), doxorubicin (DOX), nuclei isolation kit (nuclei EZ prep), eosin Y, and BCA kit were purchased from Sigma-Aldrich (St. Louis, MO). Acidic fibroblast growth factor (aFGF) and bFGF were purchased from PeproTech (NJ, USA). Luciferin was obtained from Merck Millipore. Gill's Hematoxylin No. 2 was purchased from VWR. AntiCD31 antibody (ab28364) was purchased from Abcam. Dako envision kit was purchased from Dako (CA, USA).

The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin G sodium, and 100 µg/mL of streptomycin, and 10 ng/mL of a/b FGF. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (MD, USA) and incubated with complete EGM. Murine breast cancer cell line (E0771) was cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin G sodium, and 100 µg/mL of streptomycin. All these cells were maintained at 37°C in an atmosphere of 5% CO₂ and 95% air. MDA-MB-231/Luc-GFP cells, which stably express luciferase and green fluorescent protein, were a kind gift from Dr. Shirin Bonni (Faculty of Medicine, University of Calgary).

2.2. Preparation of DOX-loaded GCS-SM nanoparticles (GCS-SM/DOX NPs)

GCS-SM/DOX NPs were prepared by the ionic gelation technique through the electrostatic effect between the negatively charged sulfate groups of SM and positively charged amine groups of GCS. Typically, GCS was dissolved in phosphate-buffered saline (PBS, pH 7.0, 20 mM) to achieve the concentration of 2.8 mg/mL. SM was dissolved in the same buffer at the concentration of 0.5 mg/mL. DOX hydrochloride was dissolved in ddH₂O at the concentration of 1 mg/mL. The SM solution prepared above (1.5 mL) was mixed with the DOX solution first, and then, this solution was injected slowly to 2.5 mL of the GCS

solution at the speed of 0.2 mL/min with a microinjection pump (Harvard apparatus) with stirring (400 rpm) at room temperature (r.t.). The mixture was stirred for 30 min to allow the formation of GCS-SM/DOX NPs. After that, the mixture was centrifuged at 1,000 rcf for 5 min to remove large aggregates. The resulting NPs were lyophilized with 1% trehalose (w/w) and stored at 4 °C before use. Empty NPs (GCSSM) were prepared following the same protocol for GCS-SM/DOX NPs except that no DOX was added. To investigate the effect of SM concentration, pH of the buffer solution, volume of SM, and DOX feeding ratio, GCS-SM/DOX NPs were prepared using the same method as described above by tuning one parameter while keeping all the other parameters unchanged.

The size and surface charge (ξ -potential) of the NPs were measured using a Zetasizer Nano-ZS (Malvern, UK) at pH 7.4. The morphology of the GCS-SM/DOX NPs was observed under a transmission electron microscope (Hitachi H-800 TEM) using formvar/carbon-coated Copper Grids (Electron Microscopy Science). The original particle suspension was dropped on a grid, washed with ddH₂O (3 ×) to remove the remaining salt, and dried with a filter paper. DOX and SM concentrations were determined using a UV–Vis spectrometer (Beckman, DU-650) at 480 nm and 312 nm, respectively.

2.3. Encapsulation efficiency of GCS-SM/DOX NPs

To evaluate the encapsulation of DOX in the GCS-SM/DOX NPs, DOX was loaded into the NPs at different feeding ratios (1.3%, 3.6%, 9.0%, weight ratio of DOX:GCS). The unencapsulated DOX was separated from the NP suspension using a Millipore centrifugal 30K device at the speed of 3000 rcf for 30 min. The concentration of free DOX was determined using a fluorescent plate reader.

2.4. Release kinetics of GCS-SM/DOX NPs

Two milliliters of GCS-SM/DOX NPs was loaded in dialysis bags (MWCO: 6–8 KDa; Spectrum Laboratories), and the bags were immersed into 25 mL of PBS (pH 7.4, 100 mM) at 37°C under continuous stirring. At predesigned time points, 1 mL of media was withdrawn. Concentrations of SM or DOX were determined using a UV–Vis spectrometer (Beckman, DU650) at 312 nm and 480 nm, respectively. All experiments were carried out in triplicate independently.

2.5. Colloid stability of GCS-SM/DOX NPs

GCS-SM/DOX NPs were suspended in PBS of pH 7.4 supplemented with different amounts of FBS, ranging from 10% to 30%, at the final SM concentration of 100 μ M at 37°C. The size of the NPs was measured at predetermined time points with DLS.

2.6. Quantification of cellular uptake of GCS-SM/DOX NPs

Cellular uptake of GCS-SM/DOX NPs was quantified by flow cytometry and confocal microscopy. The intracellular distribution of DOX was measured by cell fraction assay. MDAMB-231 cells were seeded in 12-well plates at a density of 200,000 cells/well and treated with DOX or GCS-SM/DOX NPs at the DOX concentration of 1 μ M for 3 h. Afterwards, cells were washed 3 times with cold PBS to remove unbounded DOX or NPs.

Cells were collected, fixed in 4% formaldehyde, and analyzed using a FACS device (BD Accuri C6, BD Biosciences).

To further investigate the uptake behaviors of NPs, confocal microscopy was employed. MDA-MB-231 cells were seeded in petri dishes and treated with DOX or GCS-SM/DOX NPs at the DOX concentration of 1 µg/mL. After 3 h of co-incubation, cells were washed for 3 times and images were taken with a Zeiss 710 LM confocal microscope.

GCS-SM/DOX NPs were incubated with MDA-MB-231 cells at 37°C for 1 h. Cells were washed 3 times and then collected by scraping. Cells were subjected to subcellular fractionation after the treatment. In brief, the cells were resuspended in a harvest buffer (0.01 M HEPES, 50 mM NaCl pH 7.9, containing 0.5 M sucrose, 0.5% Triton X-100) and pelleted at 1,000 rpm for 10 min. The supernatant was collected as a cytoplasmic protein fraction. The pellets were washed once with buffer (0.01 M HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) and followed by further incubating with another buffer (0.01 M HEPES, 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40) for 15 min on ice. The nuclear fraction was further sonicated (3 × 10 s, with an interval of 1 min). The nuclear fraction was collected by pelleting the suspension through centrifugation at 400 rcf for 10 min. Cytosolic and nuclear fractions obtained from subcellular fractionation of MDA-MB-231 cells were further cleared by centrifugation at 10,000 rcf for 15 min at 4°C. DOX uptake amount was quantified by the fluorescence intensity at the excitation wavelength of 485 nm and the emission wavelength of 585 nm. The protein concentration in cytosol and nuclear fractions was quantified using the BCA assay kit.

2.7. Wound healing assay

The migration of breast cancer cells was evaluated by a wound healing assay. Living videos of cell migration in a 24-well plate were acquired with a Zeiss 710 LM confocal microscope. MDA-MB-231 cells were pretreated with a/b FGF for 24 h. After that, a wound was created by scraping the cell monolayer in a straight line with a p200 tip, and cell debris were removed by washing with PBS for three times. Cells were incubated with different treatments and allowed to grow in a 37 °C chamber with 5% CO₂ condition for 24 h. Videos were taken using a confocal microscope with a transmittance channel continuously at 10× magnification.

2.8. Cell invasion assay

BD cell culture inserts with a pore size of 8 µm (BD Biosciences) were precoated with 100 µL of Matrigel at the concentration of 0.2 mg/mL (dilution from BD Matrigel stock 10 mg/mL with coating buffer). MDA-MB-231 cells were seeded at the density of 70,000 cells per 50 µL in the inserts in FBS-free DMEM. SM or GCS-SM NPs was then added to the inserts together with 50 µL of serum-free medium. The lower chamber was added with 1.2 mL of 10% FBS-containing medium supplemented with different treatments, which is the same as the upper chamber. After 16 h of incubation, the inserts were washed with PBS and fixed with methanol. Subsequently, cells were stained with hematoxylin and washed twice with PBS. The membrane in the inserts was then cut and mounted onto a coverslip, and the cell number was determined with a light microscope under 10× magnification. Each well

was imaged at 5 different fields, and the invasion rate was expressed as the average number of cells per microscopic field.

2.9. Endothelial tube formation assay

HUVECs were cultured and maintained in a complete EGM. Cells were stained with CellTracker Deep Red (1 μ M) for 30 min before the experiment. Geltrex® (150 mL; Thermo Fisher Scientific) was added to each well in a 24-well plate and allowed to polymerize for 30 min at 37 °C. HUVECs were seeded in each well at a density of 2×10^5 cells/mL. SM, GCS, and GCS-SM NP at 10 and 20 μ M were mixed with cells before seeding in the coated gel layer. The tube formation was photographed at 6 h and 20 h with an inverted phase-contrast microscope (Canon PowerShot A640) in a randomly selected field under 10 $\times$ magnification; the number of tubes formed and length of the tubes were quantified manually using Image J. Fluorescence images of cells were photographed using Zeiss 710 LM confocal microscope under 10 $\times$ magnification.

2.10. Cell viability assay

The anticancer activities of the combination of DOX and SM were investigated by the MTT assay. Cells with or without a/b FGF treatment were seeded in 96-well plates at an initial density of 20,000 cells/well. After 24 h of incubation, the medium was replaced with 150 μ L of fresh medium containing different treatments, namely, DOX, SM, and combination of DOX and SM, and the cells were continued to be incubated for an additional 24 h or 48 h. Afterwards, the medium was replaced with 100 μ L of fresh medium containing 1 mg/mL MTT reagent, and the cells were incubated for another 4 h. The MTT crystal formed was dissolved with a stop solution, and finally, the optical density of the medium was measured using a microplate reader (ELx808, BioTek Instrument, Inc.) at $\lambda = 595$ nm. A combination index (CI) analysis based on the ChouTalalay method was performed using CalcuSyn software.

2.11. In vivo experiment

All animal experiments were conducted in accordance with NIH regulations and approved by the Institutional Animal Care and Use Committee of the University of South Carolina. Female *athymic* mice of age 6 to 8 weeks (nu/nu) were obtained from Jackson Laboratory. MDA-MB-231/Luc-GFP cells were implanted by tail vein injection (2×10^6 cells in 100 μ L of PBS). Mice were then randomly divided into 5 groups and received the following treatments: 1) saline only; 2) SM at the dose of 3.5 mg/kg once per week; 3) free SM and DOX at the dose of 3.5 mg/kg of SM and 0.5 mg/kg of DOX once per week; 4) GCS-SM NPs at the dose of 3.5 mg/kg of SM once per week; and 5) GCS-SM/DOX NPs at the dose of 3.5 mg/kg of SM and 0.5 mg/kg of DOX (molar ratio of SM to DOX is 17:1) once per week. The whole treatment procedure lasted for 2 months, and the progression of tumors was monitored every week through the luminescence signal generated after administering an i.v. injection of luciferin (30 mg/mL, 50 μ L) in the mice using an IVIS Lumina system. Body weights of the mice were recorded at the same time. At the endpoint of each mouse, organs were isolated and collected. The weights of the lungs were recorded. Fluorescence images of lungs were also captured. The collected organs were fixed in 10% neutralized formalin solution and embedded in paraffin.

2.12. Immunohistochemistry and histological analysis

Tissue sections of 5 μm thickness were obtained from the formalin-fixed, paraffin-embedded tissue blocks with a microtome. The standard hematoxylin and eosin staining was performed in the lung, liver, and kidney tissue sections of each group. To study the tumor neovasculture, CD31 IHC was applied to identify the intratumor blood vessel distribution. The section was deparaffinized and treated for 30 min with 0.5 M Tris buffer (pH 10) at 95 °C for antigen retrieval. Sections were blocked in 5% goat serum for 20 min at r.t. The sections were blocked with peroxidase block for 5 min and incubated with a primary antibody (rabbit polyclonal anti-CD31 antibody) diluted with 1% BSA (1:50) for 1 h at r.t, followed by staining with a peroxidase-labeled polymer for 30 min at r.t. The sections were then developed with the substrate DAB for 5 min at r.t. and counterstained with Gill's Hematoxylin for 10 s. The slides were mounted with ProLong® Gold Antifade Mountant and imaged at 40 $\times$ magnification with a microscope.

2.13. Statistical analysis

Statistical analysis was performed by the one-way ANOVA using SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA). The values of $*P < 0.05$ and $^{\#}P < 0.01$ were determined as statistically significant. Data were expressed as mean $\pm$ standard deviation (SD).

3. Results

3.1. DOX-encapsulated GCS-SM (GCS-SM/DOX) NP preparation

It has been reported that chitosan can form hydrogels with tripolyphosphate (TPP) [32, 33]. Many factors can affect the size of NPs, especially the TPP-to-chitosan ratio, pH of the buffer, and the ionic strength of the dissolution medium [33–36]. A few reports showed that chitosan was able to form a hydrogel with sodium lauryl sulfate [37, 38]. Inspired by this NP fabrication technology, we came up with the idea of creating GCS-SM NPs through the gelation process between the sulfate groups on SM and amine groups on GCS, where SM acted as an anionic compartment and GCS acted as a cationic counterpart (Figure 1). Moreover, as the anionic and cationic compounds account for the significant fraction of this delivery system, the loading capacity of a therapeutic agent could be greatly improved if one of them coincidentally was an active pharmaceutical ingredient.

To examine the effect of SM concentration on the NP size, we first fixed the GCS concentration at 2.8 mg/mL in PBS buffer (pH 7.4). With the SM concentration declining from 25% to 5% (w/w, SM/GCS), the hydrodynamic size of NP was slightly increased from 220 nm to 277 nm along with the increased polydispersity index (PDI). The relationships among particle size, SM content in the final formulation, pH of the fabrication buffer, and SM concentration are shown in Figure 2A–D. When the pH of the fabrication buffer varied from pH 6 to 7.4, the size of NPs changed from 210 nm to 298 nm accordingly. It was revealed that very low or very high pH inhibited the formation of NPs. We postulate that this is because very high or very low pH could prevent the ionization of one component of the system. Interestingly, within the range of 0.1 to 0.5 mg/mL, SM concentration did not have a significant effect on the size of the NP. However, when SM concentration reached 1 mg/mL,

the resulting NPs had a final size of more than 600 nm with a PDI of 0.25 (Figure 2C). In addition, it was found that the loading of DOX had little impact on the hydrodynamic size of the NP. Therefore, the payload amount could be tuned without significantly affecting the size of the NP.

3.2. Characterization of GCS-SM/DOX NP

On the basis of the above investigation, we fabricated NPs with a SM-to-chitosan ratio of 16% (w/w) in 20 mM PBS buffer (pH 7.0) at a SM concentration of 0.5 mg/mL. DLS results revealed that the resulting NPs have a hydrodynamic size of 186.1 nm coupled with a slightly positive surface charge (+1.2 mV) as shown in Figure 3A. Transmission electron microscopy results revealed that these NPs were in spherical shape with the average size of 49.0 nm (Figure 3B). We observed that the size difference between DLS and TEM results was due to the following reasons: (1) DLS measures the hydrodynamic diameter of the NPs suspended in PBS, whereas TEM measures the size of dried NPs. (2) Particularly in the case of GCS-based NPs, the high density of the more hydrophilic ethylene group grafted onto the backbone of chitosan further increases the interaction with the aqueous medium, which consequently extends the thickness of the water layer associated with the particle. The free DOX in the final NP suspension was less than 3% regardless of the feeding ratio of DOX to GCS in the tested range, thus suggesting that GCS-SM is a reliable carrier system for DOX (Figure S1).

Figure 3C shows the release behaviors of SM and DOX from the NPs. It was revealed that DOX and SM shared a similar release pattern, where after 8 h of incubation in PBS of pH 7.4, both the DOX and SM had reached a plateau of approximately 80%. The simultaneous release pattern for DOX and SM ascertained that the optimized ratio between the two drugs remained unchanged from the point of the preparation to their active site. As the complex was slightly positive, there is a concern about the colloid stability of the NPs. The stability of the NPs was evaluated through the long-term incubation of NPs in the PBS and short-term incubation of NPs supplemented with FBS. Figure 3D shows that these NPs were stable and did not aggregate in 10% serum-containing media. NPs were very stable in the PBS environment as evidenced by no size increase after 2 months of incubation at 37 °C (Figure 3E).

3.3. GCS-SM NP inhibitory effect on cell migration and invasion

To investigate the effect of SM or SM-loaded NPs on breast cancer cell migration and invasion, wound healing assay and Transwell invasion assay were carried out, respectively. Both MDA-MB-231 human breast cancer cell line and E0771 murine breast cancer cell line were tested in these assays. In the wound healing assay, effects of different treatments on cell migration were analyzed by live cell imaging (Figure 4 and Videos S1–6). The average speed for cell migration was calculated by the wound distance/the time consumed to heal the wound. Figure 4A and 4B shows that SM only slightly inhibited cell migration, while its inhibitory effect was significantly enhanced in the GCS-SM NP form. The control group had an average migration speed of 28.2 $\mu\text{m/h}$ (Figure 4C). Interestingly, GCS itself also showed a noticeable inhibition effect on cell migration with an average speed of 18.2 $\mu\text{m/h}$. A study showed that 100 $\mu\text{g/mL}$ glycosylated chitosan significantly inhibited the migration of both 4T1

and MDA-MB-231 cells, and the author reasoned that the cause of the inhibition was because glycosylated chitosan upregulates E-cadherin and downregulates slug and twist 1 expression [39]. On the basis of the structural similarity, we postulate that GCS has a similar (if not the same) function as that of glycosylated chitosan. To investigate whether the inhibitory effect of SM and SM NP on cell migration was due to its cytotoxic effect, cell viability assay was carried out. It was found that neither SM nor GCS-SM NP inhibited cell proliferation at concentrations up to 200 μ M within 24 h of treatment (Figure 4D), which indicates that the inhibition of cell migration could not be attributed to their cytotoxic effects.

To investigate the inhibitory effect of SM and GCS-SM NPs on cell invasion, a Transwell invasion assay was carried out with MDA-MB-231 and E0771 cells. Figure 4 E and F showed that the number of invaded MDA-MB-231 cells in each view area was significantly decreased from 952 in the control group to 718 in the group treated with 40 μ M SM. GCS-SM NP treatment further decreased the number to 50% of the control group. Figure S2 shows that after treating E0771 with SM or SM NPs at 40 μ M concentration, the invaded cells were significantly decreased compared with the nontreated group. GCS-SM NP did not inhibit cell proliferation up to 25 μ M after 24 h of incubation as evidenced by the MTT assay (Figure S3). However, GCS-SM NPs (10 μ M) exhibited an enhanced inhibitory effect on cell invasion as compared with free SM. Thus, we proved that SM or GCS-SM NPs inhibited both cell migration and invasion at a dose that does not induce cytotoxicity.

To investigate the antiangiogenic effect of SM, an *in vitro* tube formation assay was employed. When HUVECs were seeded on the top of Geltrex[®], elongated and robust tube-like structures were formed. The effects of GCS-SM NPs on the ability of HUVECs to form a tube-like structure were evaluated by measuring the length and the number of formed tubes. Figure 5A and 5B shows that the tube formation ability of HUVECs was significantly inhibited by treatment with SM or GCS-SM NPs. At the concentration of 20 μ M, GCS-SM NPs were able to inhibit the tube formation in aspects of both tube number and tube length for 50%. Free SM also showed an inhibitory effect on tube formation, where we speculated that it is partially due to the interaction of sulfate groups in SM and the Geltrex layer (Figure 5C and 5D).

3.4. Cellular uptake and subcellular distribution of GCS-SM/DOX NPs

To measure the uptake efficiency of GCS-SM/DOX NPs *in vitro*, MDA-MB-231 cells pretreated with a/b FGF were incubated with free DOX and GCS-SM/DOX NPs. After 1, 3, and 6 h of incubation, cells were analyzed by flow cytometry (FACS) and confocal microscopy. Figure 6A showed that free DOX entered cells much slower than GCS-SM/DOX NPs. After 1 h of treatment, NP-treated cells exhibited a much stronger red fluorescence signal inside the cell than those in the free DOX treatment group. It was revealed that almost all of the DOX that has been delivered by NPs was in the nuclei, while a lot of the DOX from the free DOX treatment remained in the cytosol after 3 h of treatment. We further compared the cellular uptake behaviors of free DOX or GCS-SM/DOX NPs at 3 h and 6 h with the presence of a/b FGF using FACS (Figure 6B). In the presence of a/b FGF, GCS-SM/DOX NP-treated cells showed a much better DOX uptake than free DOX. However, without a/b FGF treatment, no significant difference in uptake was observed

between free DOX and GCS-SM/DOX NP treatments at either 3 h or 6 h (Figure S4). It is an interesting phenomenon that a/b FGF causes the differences in DOX uptake between GCS-SM/DOX NPs and free DOX; we reason that FGF might induce chemoresistance, while SM could sensitize those drug-resistant cells [40].

Nuclear localization of DOX has always played an important role for its potency in killing cancer cells. A higher localization in the nuclei has been closely related to its DNA-binding capacity. After treating cells with a/b FGF, we found that the 1 h DOX uptake in the treatment group of GCS-SM/DOX NP was significantly enhanced in the nuclear subcellular fraction, thus suggesting a higher anticancer efficacy against MDA-MB-231 cells (Figure 6C).

3.5. SM and DOX have synergistic growth inhibitory effect on MDA-MB-231 cells

SM showed biphasic effects on the proliferation of cancer cells [41]. On the one hand, as a nonspecific growth factor inhibitor, it inhibits angiogenesis of the cancer cells. On the other hand, a high concentration of SM kills cancer cells directly. Although it has been reported that SM kills cancer cells in a dose- and time-dependent manner [23, 26, 42], the cell killing mechanism remains not fully understood.

As SM was reported to induce the cross-resistance of the drug against DOX and amsacrine and cause neurologic toxicity at a high concentration [43], it is crucial to determine a ratio that ensures the combination of DOX and SM achieves a synergistic effect while not inducing side effects. After 24 h of treatment with 200 μ M SM in the form of either free SM or SM NPs, no cytotoxicity was observed in MDA-MB-231 cells (data not shown). However, after 48 h of treatment, the IC_{50} of SM and SM NPs was 200 μ M and 63 μ M, respectively (Figure 6D). In this study, we decided to test SM at a nontoxic dose (10 μ M, 20 μ M and 100 μ M) when combined with DOX. We first investigated whether free drug combinations at a nontoxic dose of SM increase DOX cytotoxicity in the presence of a/b FGF. Figure 6E shows that the cytotoxicity of DOX was greatly enhanced by the addition of 10 μ M SM, but a further increase in SM concentration (20 μ M or 100 μ M) did not additionally boost its cytotoxicity.

SM was reported to enter human microvascular endothelial cells through an active process involving the caveolae system [44], whereas a zwitterionic NP may enter the cell membrane through membrane penetration [45]. We reasoned that the synergistic effect was caused by the efficiency of delivering SM. To optimize the synergistic effect, different ratios of GCS-SM NPs to DOX were tested in MDA-MB-231 cells. The results in Figure 6F show that synergistic growth inhibitory effects were observed at a relatively low ratio of SM to DOX (Additive effect with $CI = 1$, synergism with $CI < 1$, and antagonism with $CI > 1$) [46]. We further created a profile of synergistic growth inhibition of MDA-MB-231 cells under different concentrations of SM at three different GCS-SM/DOX ratios (from 3.5 to 20) (Figure 6G).

3.6. GCS-SM/DOX NP inhibits proliferation and metastasis of cancer cells in a tumor-bearing mouse model

On the basis of the *in vitro* results, we further tested the antimetastatic efficacy of GCSSM/DOX NP in a breast cancer lung metastasis mouse model. There are two frequently used methods to generate breast cancer lung metastasis animal models, namely, orthotopic implantation of cancer cells in the mammary gland and tail vein injection of cancer cells. We adopted the tail vein injection method not only because it is faster but also because it yields tumors mainly in the lung tissue with similar genetic profiles [47–49]. It was previously reported that if a treatment was started on the first day of the cell inoculation, the number of metastasis would be greatly inhibited by the combination of chemotherapy and SM [27]. To allow cancer cells to adapt to a new environment, we started the treatment on the second day post cell inoculation. The effect of combination treatment on the breast cancer lung metastasis was evaluated by measuring bioluminescence signals in the ventral images and overall survival time. Figure 7A shows the high sensitivity of luminescence imaging system and validates that the luminescence signal was highly correlated with cell number in the range of 125–8,000 cells/well.

The mice were given different treatments for 8 weeks according a predesigned schedule (Figure 7D). The whole-body photoemission rate was measured to indicate the cell proliferation and the progression of tumor metastasis for the next 6 weeks (Figure 7B). The results showed that the luminescence lung signal of MDA-MB-231 cells in the control group was remarkably increased after week 5. For the free SM or GCS-SM NP treatment groups, both the proliferation of cancer cells and metastasis were slowed down compared to the control group. For the free drug combination treatment, the proliferation of cancer cells was remarkably inhibited and no detectable lung metastasis was observed during the experimental period, whereas GCS-SM/DOX NPs completely eradicated the cancer cells in the treated mice. Furthermore, the injection of free SM, free DOX/SM combination, GCS-SM NPs, and GCS-SM/DOX NPs did not cause significant weight loss after 8 weeks of treatment (Figure 7C).

Kaplan–Meier survival analysis was carried out for 12 weeks (Figure 7E). The saline treated group showed 100% lethal by 68 days. SM and GCS-SM NP treatments had moderately increased survival rates. The median survival time for control mice was 64.5 days, while for SM treatment alone, it reached 76.5 days. For the free drug combination group and GCS-SM/DOX NP group, the median survival time was significantly improved ($P=0.0038$ and 0.021 , respectively). After week 12, the free drug combination group had the survival rate of 50%, whereas the GCS-SM/DOX NP group achieved 60%. Correspondingly, the median survival time was extended to 81.5 days for the free drug combination group, whereas for the GCS-SM/DOX NP treatment group, the median survival time was extended to higher than 85 days.

When a tail vein inoculation method was used, the metastatic sites established cell colonies instead of discrete tumors [50, 51]. Thus, we further evaluated the metastatic status of each group by measuring their lung weights. Neither SM nor GCS-SM NP groups showed a significant effect on lung weight compared to the control group, while the free drug

combination and the GCS-SM/DOX NP treatments reduced lung weight by 53% ($P=0.022$) and 56% ($P=0.0089$), respectively (Figure 7F).

At the end of the experiment, *ex vivo* organ images were obtained by injecting luciferin (i.p.) to mice before they were sacrificed (Figure S5). Images of the control group were taken separately because no mice had survived at the end of the whole experiment, but the same imaging protocol was carried out throughout all groups. Compared to the control group, surviving mice from other groups showed no significantly visible luminescence signal, thus indicating that the metastasis was highly inhibited in these mice (Figure S5A and B). As injected MDA-MB-231 cells were constitutively expressing both GFP and luciferase proteins, we were able to directly visualize the metastatic cancer cells in the lung in GFP channel. It was found that the GFP expression was positively correlated with the luminescent signal shown above, and GCS-SM/DOX NP-treated mice showed the least GFP signal in the lung (Figure S5C).

3.7. Evaluation of GCS-SM/DOX NP treatment by transmission electron microscopy, histology, and immunohistochemistry analysis

Histological analysis of the lung and liver from each treatment is shown in Figure 8A. On the basis of the boundary between the normal and tumor tissues, we found that the lung in the control group was almost fully covered by cancer cells, while both free SM and GCS-SM NP-treated groups have a mild reduction in the occupied area ratio between tumor and normal tissue sizes. In the free drug combination and GCS-SM/DOX NP groups, no visible tumor was found in their tissue sections. There was no significant difference in histology among the livers from all groups. Surprisingly, free drug combination of DOX and SM resulted in severe glomerulonephritis, where the glomeruli were surrounded by severe inflammatory infiltrate. As we expected, the GCS-SM/DOX NP-treated group showed a normal kidney histologic structure, similar to those in control, free SM, and GCS-SM NP-treated groups.

DOX is an effective anticancer drug with significant cardiotoxic effects [52]. It is suggested that the cardiotoxic effects primarily result from iron accumulation in mitochondria and the production of ROS. DOX-dependent cardiac damage often results in irregularly aligned mitochondria and reduced perivascular fibrosis, which can be detectable by transmission electron microscopy [52, 53]. Analysis of the cardiovascular pathology using a TEM revealed that the free drug combination-treated group exhibited misaligned mitochondria (Figure 8B), thus indicating the consequence of cardiotoxicity. As we expected, no abnormal mitochondria alignment was detected in the GCS-SM/DOX NP-treatment group.

It has been reported that SM inhibits tumor growth partially through its antiangiogenic effects. Thus, we evaluated the tumor neovasculature with IHC by examining the density of CD31-stained microvessels in tumor tissues from different treatment groups (Figure 11C). Different from other reports, SM alone did not decrease the formation of new blood vessels, which may be due to the relatively low SM concentration used in our *in vivo* study. It was revealed that the GCS-SM/DOX NP and the free drug combination of SM + DOX can significantly reduce the number of newly formed microvessels in tumor tissues, thus

suggesting that the increase in the survival rate of mice in those groups could partially attribute to the antiangiogenic effect of the drug combination.

4. Discussion

Cancer cell migration and metastasis significantly reduce the survival rate of patients with breast cancer. Therefore, an effective approach to control circulating tumor cells (CTC) and prevent them from forming tumors at remote sites such as lung, liver, brain, and bone is crucial in fighting against breast cancer. Research found that triple-negative breast cancer (TNBC) is more prone to spread to other organs after treatment. Thus, we adopted the TNBC lung metastasis model in this study.

With the help of TPP, chitosan has been formulated into various NPs through ionotropic gelation for the delivery of active pharmaceutical ingredients. TPP is a gelating agent and serves as a nonactive excipient in those dosages. SM, a century-old drug that has been approved for the treatment of African sleeping sickness and river blindness [54, 55], was recently proposed to sensitize chemotherapy drugs for the treatment of many types of cancers both in mouse models and in clinical trials [25, 28]. As the structure of SM contains six negatively charged sulfonic acid groups, we proposed to use it as a dual functional agent in formulating DOX-loaded NPs, thus serving as not only a gelating agent but also an active pharmaceutical ingredient.

When negatively charged SM was mixed with positively charged GCS, size controllable GCSSM NP (approximately 200 nm) formed spontaneously (Figure 3). The nanogel is slightly positively charged and stable in both PBS and serum-containing environments, thus indicating that it could be a reliable drug carrier for *in vivo* application. As shown in Figure 3C, both DOX and SM demonstrated sustained release from the NPs for 8 h, which was much slower than free drug (approximately 75% release of free DOX within 1 h) [56].

The NP form of SM (GCS-SM NPs) showed better antimigration and anti-invasion effects than free SM (Figure 4). Similar to the results reported by others, both free SM and GCSSM NP decreased the tube number and tube length in a cultured HUVEC assay (Figure 5), thus suggesting that GCS-SM has an antiangiogenic effect *in vitro*. MTT assay found that the combination of DOX and SM shows a synergetic effect in killing MDA-MB-231 cells at a wide range of concentrations (Figure 6). In the cellular uptake study, cells were treated with a/b FGF to induce drug resistance. Free DOX was transported into the cells through diffusion; however, the p-gp overexpressed on the membrane of the drug-resistant cells can pump out the free DOX quickly. In contrast, NPs have the potential to overcome drug resistance, as they can bypass p-gp through endocytosis. A similar result has also been reported by another study [57]. As we expected, GCS-SM/DOX NP enhanced the cytotoxicity of DOX by counteracting the effect of the a/b FGFs, which was due to the boosted cellular uptake of the DOX-loaded NP as compared with free DOX alone (Figure 6).

To evaluate the anticancer effect of the GCS-SM/DOX NP, a breast cancer lung metastatic mouse model was adopted. *In vivo* imaging revealed that both GCS-SM/DOX NP and the combination of free SM and DOX could effectively inhibit the growth of lung metastatic

tumor after 6 weeks of treatment (Figure 7). In addition, GCS-SM/DOX NP and the free drug combination extended the median survival time from 64.5 days to 85.0 days and 81.5 days, respectively. Correspondingly, the lung weights (tumor burdens) of the two treatment groups were significantly lighter than that of the control group (Figure 7F). The immunohistochemistry results of CD31 staining (Figure 8C) suggested that reduced tumor burden in the GCS-SM/DOX NP and the free drug combination groups was at least partially attributed to their potent antiangiogenic effect.

It has been reported in many studies that the use of DOX and SM causes cardiotoxicity [58, 59] and renal damage [60], respectively. To study the potential systemic toxicity of the GCS-SM/DOX NP treatment, both histology analysis and TEM were carried out at the end of the treatment. Although both the GCS-SM/DOX NP and its free drug counterpart exhibited a similar tumor growth inhibitory effect, no abnormal feature was identified in the tissues of the GCS-SM/DOX NP-treated group as revealed in Figure 8A and B, while the free drug combination induced significant toxicity in both the kidney and the heart as evidenced by the glomerulonephritis in the kidney and the damaged mitochondria in cardiomyocytes. Therefore, the GCS-SM/DOX system can eliminate the cardio and renal toxicities associated with the combination of free DOX and free SM (Figure 8B), respectively. In conclusion, we proved that the GCS-SM/DOX ternary NP system could be a safe and effective tool for preventing and treating TNBC lung metastasis.

5. Conclusion

In summary, we developed a GCS-based dual-payload nanocarrier system, which contains an antiangiogenic drug (SM) and a chemotherapeutic drug (DOX). The repurposed SM serves as a gelator for the formation of the NP and an active pharmaceutical ingredient to sensitize the cancer cell, thereby inhibiting their migration and invasion and blocking neovascularization *in vitro*. The *In vivo* experiment proved that GCS-SM/DOX NP could greatly inhibit breast cancer lung metastases and improve the survival time for mice while not inducing the cardiotoxicity and renal toxicity associated with its free drug counterpart. Because of the simplicity of the NP preparation procedure, the developed system could be easily scaled up and further tested in a clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

The authors thank National Institutes of Health (1R15CA188847-01A1 and 1R01AG054839-01A1) for the financial support of the research. They also thank the University of South Carolina Center for Targeted Therapeutics Microscopy and Flow Cytometry Core Facility (National Institutes of Health 5P20GM109091) for technical support.

References

- [1]. DeSantis C, Ma J, Bryan L, Jemal A, Breast cancer statistics, 2013, CA: a cancer journal for clinicians 64(1) (2014) 52–62. [PubMed: 24114568]

- [2]. Steeg PS, Theodorescu D, Metastasis: a therapeutic target for cancer, *Nature clinical practice. Oncology* 5(4) (2008) 206–19.
- [3]. Redig AJ, McAllister SS, Breast cancer as a systemic disease: a view of metastasis, *Journal of internal medicine* 274(2) (2013) 113–26. [PubMed: 23844915]
- [4]. Ledzewicz U, Maurer H, Schattler H, Optimal and suboptimal protocols for a mathematical model for tumor anti-angiogenesis in combination with chemotherapy, *Math Biosci Eng* 8(2) (2011) 307–23. [PubMed: 21631132]
- [5]. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH, Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer, *The New England journal of medicine* 355(24) (2006) 2542–50. [PubMed: 17167137]
- [6]. Wan L, Pantel K, Kang Y, Tumor metastasis: moving new biological insights into the clinic, *Nature medicine* 19(11) (2013) 1450–64.
- [7]. Turner N, Grose R, Fibroblast growth factor signalling: from development to cancer, *Nature reviews. Cancer* 10(2) (2010) 116–29. [PubMed: 20094046]
- [8]. Holland EC, Varmus HE, Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice, *Proceedings of the National Academy of Sciences of the United States of America* 95(3) (1998) 1218–23. [PubMed: 9448312]
- [9]. Rogelj S, Klagsbrun M, Atzmon R, Kurokawa M, Haimovitz A, Fuks Z, Vlodavsky I, Basic fibroblast growth factor is an extracellular matrix component required for supporting the proliferation of vascular endothelial cells and the differentiation of PC12 cells, *The Journal of cell biology* 109(2) (1989) 823–31. [PubMed: 2760114]
- [10]. Tomlinson DC, Lamont FR, Shnyder SD, Knowles MA, Fibroblast growth factor receptor 1 promotes proliferation and survival via activation of the mitogen-activated protein kinase pathway in bladder cancer, *Cancer research* 69(11) (2009) 4613–20. [PubMed: 19458078]
- [11]. Hanahan D, Folkman J, Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, *Cell* 86(3) (1996) 353–64. [PubMed: 8756718]
- [12]. Akita S, Akino K, Imaizumi T, Hirano A, Basic fibroblast growth factor accelerates and improves second-degree burn wound healing, *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 16(5) (2008) 635–41.
- [13]. Klein-Soyer C, Beretz A, Cazenave JP, Driot F, Maffrand JP, Behavior of confluent endothelial cells after irradiation. Modulation of wound repair by heparin and acidic fibroblast growth factor, *Biology of the cell / under the auspices of the European Cell Biology Organization* 68(3) (1990) 231–8.
- [14]. Beenken A, Mohammadi M, The FGF family: biology, pathophysiology and therapy, *Nature reviews. Drug discovery* 8(3) (2009) 235–53. [PubMed: 19247306]
- [15]. Haugsten EM, Wiedlocha A, Olsnes S, Wesche J, Roles of fibroblast growth factor receptors in carcinogenesis, *Molecular cancer research : MCR* 8(11) (2010) 1439–52. [PubMed: 21047773]
- [16]. Gao FJ, Hebbar S, Gao XA, Alexander M, Pandey JP, Walla MD, Cotham WE, King SJ, Smith DS, GSK-3beta Phosphorylation of Cytoplasmic Dynein Reduces Ndel1 Binding to Intermediate Chains and Alters Dynein Motility, *Traffic* 16(9) (2015) 941–61. [PubMed: 26010407]
- [17]. Daniele G, Corral J, Molife LR, de Bono JS, FGF receptor inhibitors: role in cancer therapy, *Current oncology reports* 14(2) (2012) 111–9. [PubMed: 22311684]
- [18]. Dieci MV, Arnedos M, Andre F, Soria JC, Fibroblast growth factor receptor inhibitors as a cancer treatment: from a biologic rationale to medical perspectives, *Cancer discovery* 3(3) (2013) 264–79. [PubMed: 23418312]
- [19]. Takano S, Gately S, Neville ME, Herblin WF, Gross JL, Engelhard H, Perricone M, Eidsvoog K, Brem S, Suramin, an anticancer and angiosuppressive agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator, *Cancer research* 54(10) (1994) 2654–60. [PubMed: 7513254]
- [20]. Hierro C, Rodon J, Tabernero J, Fibroblast Growth Factor (FGF) Receptor/FGF Inhibitors: Novel Targets and Strategies for Optimization of Response of Solid Tumors, *Seminars in oncology* 42(6) (2015) 801–19. [PubMed: 26615127]
- [21]. Kumar SB, Narasu L, Gundla R, Dayam R, A.R.P.S. J, Fibroblast growth factor receptor inhibitors, *Current pharmaceutical design* 19(4) (2013) 687–701. [PubMed: 23016864]

- [22]. Waltenberger J, Mayr U, Frank H, Hombach V, Suramin is a potent inhibitor of vascular endothelial growth factor. A contribution to the molecular basis of its antiangiogenic action, *Journal of molecular and cellular cardiology* 28(7) (1996) 1523–9. [PubMed: 8841939]
- [23]. Song S, Wientjes MG, Walsh C, Au JL, Nontoxic doses of suramin enhance activity of paclitaxel against lung metastases, *Cancer Res* 61(16) (2001) 6145–50. [PubMed: 11507065]
- [24]. Song S, Wientjes MG, Gan Y, Au JL, Fibroblast growth factors: an epigenetic mechanism of broad spectrum resistance to anticancer drugs, *Proc Natl Acad Sci U S A* 97(15) (2000) 8658–63. [PubMed: 10890892]
- [25]. Zhang Y, Song S, Yang F, Au JL, Wientjes MG, Nontoxic doses of suramin enhance activity of doxorubicin in prostate tumors, *The Journal of pharmacology and experimental therapeutics* 299(2) (2001) 426–33. [PubMed: 11602651]
- [26]. Song S, Yu B, Wei Y, Wientjes MG, Au JL, Low-dose suramin enhanced paclitaxel activity in chemotherapy-naïve and paclitaxel-pretreated human breast xenograft tumors, *Clin Cancer Res* 10(18 Pt 1) (2004) 6058–65. [PubMed: 15447990]
- [27]. Singla AK, Bondareva A, Jirik FR, Combined treatment with paclitaxel and suramin prevents the development of metastasis by inhibiting metastatic colonization of circulating tumor cells, *Clinical & experimental metastasis* 31(6) (2014) 705–14. [PubMed: 24965002]
- [28]. Tu SM, Pagliaro LC, Banks ME, Amato RJ, Millikan RE, Bugazia NA, Madden T, Newman RA, Logothetis CJ, Phase I study of suramin combined with doxorubicin in the treatment of androgen-independent prostate cancer, *Clin Cancer Res* 4(5) (1998) 1193–201. [PubMed: 9607577]
- [29]. Kosarek CE, Hu X, Couto CG, Kisseberth WC, Green EM, Au JL, Wientjes MG, Phase I evaluation of low-dose suramin as chemosensitizer of doxorubicin in dogs with naturally occurring cancers, *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine* 20(5) (2006) 1172–7.
- [30]. Bowden CJ, Figg WD, Dawson NA, Sartor O, Bitton RJ, Weinberger MS, Headlee D, Reed E, Myers CE, Cooper MR, A phase I/II study of continuous infusion suramin in patients with hormone-refractory prostate cancer: toxicity and response, *Cancer chemotherapy and pharmacology* 39(1–2) (1996) 1–8. [PubMed: 8995493]
- [31]. Park J, Kim JY, Hwang SR, Mahmud F, Byun Y, Chemical Conjugate of Low Molecular Weight Heparin and Suramin Fragment Inhibits Tumor Growth Possibly by Blocking VEGF165, *Molecular pharmaceutics* 12(11) (2015) 3935–42. [PubMed: 26448404]
- [32]. Gan Q, Wang T, Cochrane C, McCarron P, Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery, *Colloids Surf B Biointerfaces* 44(2–3) (2005) 65–73. [PubMed: 16024239]
- [33]. Jonassen H, Kjoniksen AL, Hiorth M, Stability of chitosan nanoparticles cross-linked with tripolyphosphate, *Biomacromolecules* 13(11) (2012) 3747–56. [PubMed: 23046433]
- [34]. Csaba N, Koping-Hoggard M, Fernandez-Megia E, Novoa-Carballal R, Riguera R, Alonso MJ, Ionically crosslinked chitosan nanoparticles as gene delivery systems: effect of PEGylation degree on in vitro and in vivo gene transfer, *J Biomed Nanotechnol* 5(2) (2009) 16271.
- [35]. Santander-Ortega MJ, Peula-Garcia JM, Goycoolea FM, Ortega-Vinuesa JL, Chitosan nanocapsules: Effect of chitosan molecular weight and acetylation degree on electrokinetic behaviour and colloidal stability, *Colloids Surf B Biointerfaces* 82(2) (2011) 571–80. [PubMed: 21071187]
- [36]. Huang Y, Lapitsky Y, Monovalent salt enhances colloidal stability during the formation of chitosan/tripolyphosphate microgels, *Langmuir* 27(17) (2011) 10392–9. [PubMed: 21749043]
- [37]. Elsayed A, Al-Remawi M, Qinna N, Farouk A, Al-Sou'od KA, Badwan AA, Chitosan–Sodium Lauryl Sulfate Nanoparticles as a Carrier System for the In Vivo Delivery of Oral Insulin, *Aaps Pharmscitech* 12(3) (2011) 958–964. [PubMed: 21761276]
- [38]. El-Gibaly I, Meki AM, Abdel-Ghaffar SK, Novel B melatonin-loaded chitosan microcapsules: in vitro characterization and antiapoptosis efficacy for aflatoxin B1-induced apoptosis in rat liver, *International journal of pharmaceutics* 260(1) (2003) 5–22. [PubMed: 12818806]
- [39]. Chen YL, Wang CY, Yang FY, Wang BS, Chen JY, Lin LT, Leu JD, Chiu SJ, Chen FD, Lee YJ, Chen WR, Synergistic effects of glycated chitosan with high-intensity focused ultrasound on

suppression of metastases in a syngeneic breast tumor model, *Cell death & disease* 5 (2014) e1178. [PubMed: 24743733]

- [40]. Li S, Payne S, Wang F, Claus P, Su Z, Groth J, Geradts J, de Ridder G, Alvarez R, Marcom PK, Pizzo SV, Bachelder RE, Nuclear basic fibroblast growth factor regulates triple-negative breast cancer chemo-resistance, *Breast Cancer Res* 17 (2015) 91. [PubMed: 26141457]
- [41]. Foekens JA, Sieuwerts AM, Stuurman-Smeets EM, Dorssers LC, Berns EM, Klijn JG, Pleiotropic actions of suramin on the proliferation of human breast-cancer cells in vitro, *Int J Cancer* 51(3) (1992) 439–44. [PubMed: 1592534]
- [42]. Li H, Li H, Qu H, Zhao M, Yuan B, Cao M, Cui J, Suramin inhibits cell proliferation in ovarian and cervical cancer by downregulating heparanase expression, *Cancer Cell Int* 15 (2015) 52. [PubMed: 26052253]
- [43]. Lelievre S, Benchokroun Y, Larsen AK, Altered topoisomerase I and II activities in suramin-resistant lung fibrosarcoma cells, *Molecular pharmacology* 47(5) (1995) 898–906. [PubMed: 7746278]
- [44]. Gagliardi AR, Taylor MF, Collins DC, Uptake of suramin by human microvascular endothelial cells, *Cancer Lett* 125(1–2) (1998) 97–102. [PubMed: 9566702]
- [45]. Wang T, Bai J, Jiang X, Nienhaus GU, Cellular uptake of nanoparticles by membrane penetration: a study combining confocal microscopy with FTIR spectroelectrochemistry, *ACS nano* 6(2) (2012) 1251–9. [PubMed: 22250809]
- [46]. Chou TC, Drug combination studies and their synergy quantification using the ChouTalalay method, *Cancer Res* 70(2) (2010) 440–6. [PubMed: 20068163]
- [47]. Rashid OM, Nagahashi M, Ramachandran S, Dumur CI, Schaum JC, Yamada A, Aoyagi T, Milstien S, Spiegel S, Takabe K, Is tail vein injection a relevant breast cancer lung metastasis model?, *J Thorac Dis* 5(4) (2013) 385–92. [PubMed: 23991292]
- [48]. Khanna C, Hunter K, Modeling metastasis in vivo, *Carcinogenesis* 26(3) (2005) 513–23. [PubMed: 15358632]
- [49]. Elkin M, Vlodavsky I, Tail vein assay of cancer metastasis, *Current protocols in cell biology / editorial board, Bonifacino Juan S. ... [et al.] Chapter 19* (2001)
- [50]. Plaksin D, Gelber C, Feldman M, Eisenbach L, Reversal of the metastatic phenotype in Lewis lung carcinoma cells after transfection with syngeneic H-2Kb gene, *Proc Natl Acad Sci U S A* 85(12) (1988) 4463–7. [PubMed: 3380801]
- [51]. Bugge TH, Kombrinck KW, Xiao Q, Holmback K, Daugherty CC, Witte DP, Degen JL, Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice, *Blood* 90(11) (1997) 4522–31. [PubMed: 9373263]
- [52]. Ichikawa Y, Ghanefar M, Bayeva M, Wu R, Khechaduri A, Naga Prasad SV, Mutharasan RK, Naik TJ, Ardehali H, Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation, *J Clin Invest* 124(2) (2014) 617–30. [PubMed: 24382354]
- [53]. Miranda CJ, Makui H, Soares RJ, Bilodeau M, Mui J, Vali H, Bertrand R, Andrews NC, Santos MM, Hfe deficiency increases susceptibility to cardiotoxicity and exacerbates changes in iron metabolism induced by doxorubicin, *Blood* 102(7) (2003) 2574–80. [PubMed: 12805055]
- [54]. Zaniboni A, Suramin: the discovery of an old anticancer drug, *Med Oncol Tumor Pharmacother* 7(4) (1990) 287–90. [PubMed: 2283894]
- [55]. McGeary RP, Bennett AJ, Tran QB, Cosgrove KL, Ross BP, Suramin: clinical uses and structure-activity relationships, *Mini reviews in medicinal chemistry* 8(13) (2008) 1384–94. [PubMed: 18991754]
- [56]. Lu J, Zhao W, Huang Y, Liu H, Marquez R, Gibbs RB, Li J, Venkataramanan R, Xu L, Li S, Li S, Targeted delivery of Doxorubicin by folic acid-decorated dual functional nanocarrier, *Mol Pharm* 11(11) (2014) 4164–78. [PubMed: 25265550]
- [57]. Ke XY, Coady DJ, Yang C, Engler AC, Hedrick JL, Yang YY, pH-sensitive polycarbonate micelles for enhanced intracellular release of anticancer drugs: a strategy to circumvent multidrug resistance, *Polymer Chemistry* 5(7) (2014) 2621–2628.
- [58]. Turdi S, Xu P, Li Q, Shen Y, Kerram P, Ren J, Amidization of doxorubicin alleviates doxorubicin-induced contractile dysfunction and reduced survival in murine cardiomyocytes, *Toxicol Lett* 178(3) (2008) 197–201. [PubMed: 18467042]

- [59]. He H, Markoutsas E, Zhan Y, Zhang J, Xu P, Mussel-inspired PLGA/polydopamine coreshell nanoparticle for light induced cancer thermochemotherapy, *Acta biomaterialia* 59 (2017) 181–191. [PubMed: 28688985]
- [60]. Smith A, Harbour D, Liebmann J, Acute renal failure in a patient receiving treatment with suramin, *American journal of clinical oncology* 20(4) (1997) 433–4. [PubMed: 9256906]

The doxorubicin-loaded glycol chitosan-suramin nanoparticle (GCS-SM/DOX) is novel in the following aspects:

- 1. SM acts as not only a gelator for the first time** in the preparation of the nanoparticle but **also an active pharmaceutical agent in the dosage form.**
- 2. GCS-SM/DOX NP significantly reduced tumor burden and extended the lifespan** of animals with **triple-negative breast cancer lung metastasis.**
- 3. GCS-SM/DOX NPs attenuate cardio and renal toxicities** associated with the DOX and SM.
- 4. The GCS-SM/DOX system is highly translatable** because of its **simple, one-pot, and easy-to-scale-up** preparation protocol.

Fig. 1. Scheme of the assembly of GCS-SM/DOX NP and its action in treating breast cancer lung metastasis.

Fig. 2.

The effects of (A) SM amount (GCS and SM were dissolved in PBS, pH 7.0; SM concentration of 0.5 mg/ml), (B) pH of the buffer (with SM concentration of 0.5 mg/ml), (C) SM concentration, and (D) the loading content of DOX (in PBS, pH 7.0) on the hydrodynamic size (black) and PDI (red) of the nanoparticle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Characterization of GCS-SM/DOX NP. (A) Hydrodynamic size distribution and surface charge (inserted). (B) Transmission electron microscopy images of GCS-SM/DOX NPs with 100 K magnification. Scale bar is 100 nm. (C) In vitro release of suramin and doxorubicin in PBS. (D) Hydrodynamic size of GCS-SM NPs when co-incubated with 10%–30% FBS medium. (E) Long-term stability GCS-SM NPs in PBS.

Fig. 4. The effect of GCS-SM NP on the migration and invasion of MDA-MB-231 cells. (A) Images of cell migration of cells after treated with SM, GCS or GCS-SM NP at different concentrations at 0, 4, 8, 12, 16, 20 h. Scale bars are 150 μ m. (B) Quantification of migration distance of each time point. (C) Average migration speed of each treatment groups. (D) Cytotoxicity of SM or GCS-SM NPs at different concentrations against MDA-MB-231 cells for 24 h. (E) Representative images of MDA-MB-231 cells in the invasion assay. Scale bars are 200 μ m. (F) Cell counting result after 16 h of invasion study. (n = 5 pictures per treatment, #P < 0.01).

Fig. 5.

The *in vitro* HUVEC tube formation assay. (A) Photographs of representative tubes formed in different treatments. Magnification: 10 $\times$. Scale bar: 200 μ m. (B) Confocal microscopy images of representative tubes formed in different treatments. Magnification: 100 $\times$. Scale bar: 50 μ m. (C) Quantitative data for endothelial cell tube formation expressed as length of tubes/field $\pm$ Std. (D) Quantitative data for endothelial cell tube formation expressed as number of tubes/field $\pm$ Std. ** $P < 0.001$ compared with control.

Fig. 6.

The uptake and cytotoxicity of GCS-SM/DOX nanoparticles and DOX for MDAMB-231 cells. (A) Confocal microscopy of the uptake of DOX or GCS-SM/DOX NP in MDAMB-231 cells for 3 h. Hoechst 33,342 (blue), DOX (red). Scale bars are 20 μm . (B) FACS spectra of MDA-MB-231 cells treated with free DOX or GCS-SM/DOX NP for 3 h or 6 h. (C) DOX distribution in the cytosol and nuclei after treating MDA-MB-231 with DOX or GCS-SM/DOX NP for 1 h. (D) The cytotoxicity of suramin and GCS-SM NP after 48 h of treatment. (E) Cell viability of DOX in combination with different concentrations of suramin in cells pretreated with a/b FGF. (F) Combination index (CI) of DOX and GCS-SM NPs mixed at different ratios. (G) Cell viability of combinational nanoparticles at different ratios. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

The inhibitory effect of GCS-SM/DOX NP on tumor growth *in vivo* using bioluminescence assay. (A) Cell luminescence intensity as a function of cell number. (B) Representative luminescence images of mice from different treatment groups from week 1 to week 6. (C) Mice body weight change curves over the experiment. GCS-SM/DOX NP increased survival rate and reduced side effects. (D) Timeline of the *in vivo* experiments. (E) Survival curves of different treatments. (F) Lung weight in different treatments. (* $P < 0.05$, ** $P < 0.01$).

Fig. 8.

Histology analysis and TEM analysis of organs from different treatment groups. (A) H&E stained images of lungs, livers, and kidneys. Black arrows indicate the tumor (T) area in the lung. Green arrows indicate the glomerulonephritis areas in the kidney. (B) Transmittance electron microscopy images of heart tissues from the control, free drug combination, and GCS-SM/DOX NP treatment groups. Scale bars are 2 μm. (C) IHC detection of CD31 in tumor tissues of different treatment groups. IHC staining (CD31) of tumor blood vessels (brown), microvessels (red arrow). Scale bars are 50 μm. The tumors were cut into 5 μm thick sections, stained with CD31, and imaged (10 fields per section) (* $P < 0.05$ compared with control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)