iTEP nanoparticle-delivered salinomycin displays an enhanced toxicity to cancer stem cells in orthotopic breast tumors

Material and Method

Expression and purification of iTEP

The gene that codes iTEP containing 70 repeats of hexmers (GAGVPG) was previously generated in our lab (Chen, personal communication). The primers used to generate this sequence are: iTEP-sense,

5'- CGCGGGTGTGCCGGGCGGCGCCGGTGTTCCAGGGGGCGCGGGTGTGCCGGGAGGCGCAGG

TGTCCCTGGGGGCGCTGGTGTACCGGGAGG -3', and iTEP-antisense, 5'-TCCCGGTACACCAGCGCCCCCAGGGACACCTGCGCCTCCCGGCACACCCGCGCCCCCTGGAACACCGGCGCCGCCCGGCAC ACCCGCGCC-3'. The coding gene for the new iTEP with a multi-cysteines extension was generated using recursive directional ligation.[1] To generate a transformed *E. coli* clone that produces the new iTEP, 100 ng of the plasmids that contained the iTEP coding gene was transformed to BLR competent cells by heat shock 45 seconds at 37 °C water bath and 1 minute on ice. The cells were then spread onto agar plate containing 0.1 mg/mL ampicillin. After overnight growth at 37 °C, single colonies were picked up from the agar plate and used to inoculate starter culture medium, 5 mL LB media with 0.1 mg/mL ampicillin supplemented. The starter culture was incubated in a shaker incubator (MAXQ 4450, Thermo Fisher Scientific Inc., MA, USA) for 16 hours at 37 °C. The *E. coli* cells in the starter culture were then spun down at 3,500 rpms for 15 minutes and resuspended in 1mL fresh LB media for each sample. Then, the resuspended cells were used to inoculate 1L TB media in 4L culture flask. The incubation was performed in a shaker incubator at 230 rpms for 24 hours at 37 °C.

After 24 hours of culture, the cells were harvested by centrifugation at 4,600 rpms for 25 minutes at 4 °C. The cells pellet was collected. For cells from each 1 L culture, 20 mL PBS was used to resuspend the cells and the cells suspension was stirred and mixed until no obvious chunk of cells was present. Then, the cells in suspension were lysed by 30 rounds of sonication using a 505 Sonic Dismembrator (Thermo Fisher Scientific Inc. (MA, USA) with a

setting including 10-second on and 20-second off on ice. After the sonication, 4 mL polyethyleneimine (PEI) was added to cell lysate and vigorously vortexed with the lysate in order to precipitate nucleic acid. The cell lysate with PEI was centrifuged at 16,000 g for 15 minutes at 4 °C (cold spin) to precipitate any insoluble impurities. The supernatant containing iTEPs was transferred to a new tube and added sodium chloride to a final concentration of 2.5 M. The solution was then heated at 75 °C for 10 minutes to induce aggregation of iTEPs before centrifuged under 16,000 g for 15 minutes at 40 °C (hot spin). The pellet was collected and resuspended with appropriate volume of cold PBS with 20 mM TCEP (pH 7.0) to prevent undesired disulfide bond formation. The above process, inverse transition cycling (ITC) was usually repeated for 10 rounds.[2] The purity of iTEP was analyzed by SDS-PAGE. The purified iTEP solution was lyophilized and stored at -20 °C.

The SDS-PAGE characterization was performed under manufacture instructions. 12% gel was used and the loading amount is 100 μg/well. After developing the gel, the 0.5 M Copper Chloride was applied to stain the gel for 15 minutes and washed with water.

Synthesis of MPBH-Sali conjugates

Sali was reacted with MPBH under the catalysis by N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (>98%, Sigma–Aldrich, MO, USA) and 1-Hydroxybenzotriazole hydrate (HOBt) (wetted with not less than 20 wt. % water, 97%, Sigma–Aldrich, MO, USA) based on the manufacture's protocol. In detail, Sali sodium salt (1 e.q.), 4-(4-N-Maleimidophenyl)butyric acid hydrazide-HCl (MPBH-HCl) (Pierce, MA, USA) (0.8 e.q.), HOBt (1.5 e.q., with 20% water), EDC-HCl (2 e.q.), and triethylamine (TEA) (2 e.q.) were dissolved in acetonitrile. The reaction was stirred and kept in room temperature for overnight. Then, the acetonitrile was first removed by rotary evaporator (RE-300, Yamato Scientific Co., Ltd, CA, USA) and the precipitate was resuspened in 0.5 mL Dichloromethane (DCM). The reaction mixture was loaded into a silica column (60 Å, 200-400 mesh, Sigma–

Aldrich, MO, USA) and eluted by DCM-methanol mixture (20:1) to purify the conjugation product. The product was dried by rotary evaporator to generate light yellow crystals. The final yield was about 75% relative to Sali.

The presence of MPBH-Sali conjugates was confirmed by both the 1H NMR and Mass Spectrometry. The 1H NMR was performed in CD_2Cl_2 and measured on ARX-400 (proton at 400 MHz) (Bruker Corporation, MA, USA). The chemical shift was analyzed and integrated.

For ESI-MS, the purified reaction product was prepared in an 50% acetonitrile aqueous solution containing 0.1% TFA, loaded onto a sinapinic acid matrix, and examined using a QTOF 2 Mass Spectrometer (Waters, MA, USA) equipped with a nitrogen laser (337 nm).

Synthesis of iTEP-Sali

iTEPs were first dissolved in phosphate buffer (pH 7.0, 1 M NaPO4, 1 mM EDTA) to a concentration about 20 mg/mL and reduced by TCEP (20 mM, pH=7.0) for 1 hour at room temperature.[3] After reduction, purified MPBH-Sali conjugates were dissolved in acetonitrile (about 15 mg/mL) and added directly to the iTEP solution. The final solvent volume ratio is 1.3 between acetonitrile to water. The molar ratio between MPBH-Sali and thiol groups of the iTEPs is about 5:1. The reaction lasted for overnight at room temperature under stirring at 300 rpms. Then, the acetonitrile was removed; the reaction mixture was centrifuged for 15 minutes at 16, 000 g at 4 °C in order to precipitate and remove the unreacted MPBH-Sali. The supernatant was collected and further purified for 5 rounds by Amicon Ultra-15 centrifugal filter units (MW cutoff=3,000 Da) (EMD Millipore Corporation, MA, USA) in order to remove unreacted MPBH-Sali. The purity of iTEP-Sali conjugate was confirmed by HPLC. After

the purification, iTEP-Sali conjugate was lyophilized and stored at -20 °C. When needed for other experiments in this study, the dry iTEP-Sali conjugates were reconstituted in PBS.

Analysis of Sali or its conjugates using HPLC in combination with pre-column derivatization

Sali was detected as previously described by pre-column derivitization.[4] 100µL aqueous TFA solution (500 mg/mL) was mixed to 700 µL of samples containing Sali and incubated for 10 minutes at room temperature. Then, 100 µL of 1 mg/mL 2, 4-Dinitrophenol (DNPH) (moistened with water, ≥97.0%, Sigma–Aldrich, MO, USA) in methanol was added in the above mixture. The resulted mixture was mixed for 20 seconds by vortexing, then the reaction was allowed to progress for 20 minutes at 55 °C.

After the mixtures were cooled down, they were filtered and injected into a Symmetry C18 column (100Å, 3.5 µm, 4.6 mm X 150 mm, Waters, MA, USA) connected with an Agilent Infinity-1260 LC system (CA, USA). The analysis was perform using water (solvent A) and Acetonitrile (solvent B) (0.05% TFA) at a flow rate of 1.0 mL/min. The gradient was gradually increased from 80% B to 100% B from 0 to 20 minutes. The peak area corresponding to Sali and recorded at 392 nm was integrated. The quantities of Sali in each sample were calculated using the area values and a standard curve of area values of known concentrations of Sali processed by an identical procedure.

Analysis of thermally-induced transition behaviors of iTEPs

The thermally-induced transition was tracked by following changes of the absorbance of iTEP or iTEP-Sali conjugate solution at 350 nm with changes of temperature. Temperatures were increased or decreased at a rate of 1 °C/min. The absorbance was recorded using a UV–visible spectrophotometer (Cary 100 Bio, Varian

instruments) equipped with temperature-controlled multicellular holder. The absorbance values were plotted against temperature to give transition curves of samples. The T_t of a sample was defined as a given temperature when the derivative (dOD_{350}/dT) of the corresponding transition curve reached its maximum.

Measurement of critical micelle concentration (CMC)

The measurement method was adapted from McDaniel et, al 2013[5] with some adjustments. In detail, measurements were carried out in Costar™ 96-Well Black Clear-Bottom Plates purchased from Corning Inc. (NY, USA). iTEP-Sali conjugates were serially double-diluted in PBS starting from 100µM. The samples were prepared as triplicates. The final volume for these dilutions is 100µL. Then, 5µL ethanol containing 200µM pyrene was added to these dilutions. After mixed by pipetting with a multichannel pipette, fluorescence values of these sample were obtained immediately using an Infinite 200 PRO (Tecan Trading AG, Switzerland) plate reader with the setting: Ex: 334 nm with a slit of 10 nm, Em: ranging 360-390nm with a slit of 2.5 nm. Two emissions, $I_{370\text{-}373nm}$ and $I_{381\text{-}384nm}$ were our interest. To identify CMCs of samples, ratio of the emission peak number 1 ($I_{370\text{-}373nm}$) and the emission peak number 3 ($I_{381\text{-}384nm}$) of pyrene (I_1/I_3) was plotted as a function of conjugate concentrations at log 10 scale. The data points were fitted to a sigmoidal-dose response curve and the CMC was defined as the inflection point of the curve.

Generation of 4T1 mammospheres and mammosphere cells

The protocol for culturing mammospheres was adapted from a publication by Dontu et, al (2003)[6] and Gupta et, al (2009).[7] Monolayer 4T1 cells were removed from regular 4T1 cell culture after incubating the culture with 0.05% trypsin, 0.53 mM EDTA-4Na for 15 minutes (Sigma–Aldrich, MO, USA). The collected 4T1 cells were vigorously pipetted to generate single cell suspension. The cells were then plated in ultralow attachment plates

at various densities. A density of 20,000 viable cells/mL was used to generate 4T1 mammospheres. The culture media was supplemented with B27 (1X, Invitrogen, CA, USA), 20 ng/mL EGF, 20 ng/mL bFGF (BD Biosciences, NJ, USA), 4 µg/mL heparin (Sigma–Aldrich, MO, USA), and 0.5% methylcellulose (Stem Cell Technologies, NJ, USA).

In order to obtain single 4T1 mammosphere cells, the 5-day old mammospheres from 6-well plates were dissociated as described previously by Dontu et,al (2003).[6] The cells were spun down at 1,500 rpms for 5 minutes and washed once with a RPMI-1640 medium containing 10% serum to stop the digestion reaction. Then, mechanical pipetting was applied using a fire-polished Pasteur pipette to dissociate mammospheres into single cells. The cells finally obtained were confirmed microscopically for a single cell suspension. Cells obtained were counted and stored on ice before being plated into 96-well plate or used for flow cytometry.

Results

Design and generation of iTEPs

A parent, hydrophilic iTEP was modified into a new iTEP containing 32 cysteines (Figure S1). The new iTEP was generated by *E. coli* cells as a recombinant protein. The purified new iTEP displayed as a band between 46 kDa and 58 kDa markers on SDS-PAGE, which was consistent with its theoretical value, 50.1 KDa (Figure S2). The new iTEP had a transition temperature higher than 75°C (Figure S3), which indicates its highly hydrophilic nature. This new iTEP was paired and linked with hydrophobic Sali to form amphiphilic conjugates.

Synthesis of MPBH-Sali conjugates

A bi-functional linker, MPBH, was expected to react with Sali through the carboxyl of the latter under our reaction condition. The presence of expected MPBH-Sali conjugates in the purified reaction product was

suggested by NMR analysis results of the product (Figure S4). On the 1H NMR spectrum of the product, chemical shifts that represent carboxylic acid (δ around 11) of Sali disappeared. Meanwhile, a chemical shift representing amide bond (δ=8.2) emerged, together with chemical shifts of benzene ring (δ=7.2~7.4) as well as maleimide moiety (δ=6.9), two characteristic groups of MPBH. On the other hand, two shifts, δ=5.9 and δ=5.8 were assigned to the two hydrogen atoms at the both ends of the only carbon-carbon double bond of Sali.[8] These results collectively suggested that the purified product was likely the conjugates between Sali and MPBH through an amide bond as it NMR spectrum had signatures of MPBH, Sali, and amide bond. The possibility wass reinforced by the result of mass spectrometry analyses of the purified product, in which that the product yield a mass value of 1006.3 (m/z) (Figure S5). This number was consistent with the theoretical mass of MPBH-Sali conjugates. The presence of MPBH-Sali was also supported by the several mass peaks which matched various sodium and potassium salt forms of the conjugates. The absence of peak representing Sali (m/z=751) in the purified product indicated that the purification is complete.

Synthesis of iTEP-Sali conjugates

MPBH-Sali was further conjugated to our iTEP through a reaction between maleimide group of MPBH and multiple thiol groups of the iTEP. Purified products from the reaction retained inverse transition properties of the iTEP with a T_t of 52.30 °C at 25 μM in PBS (Figure S3). The T_t of the reaction product was much lower than the T_t of iTEP which is higher than 75°C. The decreased transition temperature suggested that the product is more hydrophobic than free iTEP, an indicator of addition of MPBH-Sali to iTEPs since MPBH-Sali a highly hydrophobic molecule.

Characterization of iTEP-Sali NPs

The critical micelle concentration of the iTEP-Sali NPs were measure using a pyrene assay. The CMC for the particle in PBS solution was 15.32 μM with a 95% CI=11.02-21.30 μM (Figure S6). The zeta potential for iTEP-Sali NP2 is -0.0204 mV±5.71 mV and for iTEP-Sali NP3 is 0.046 mV±3.17 mV. So both particles are neutral as we expected for the neutral iTEP used. (Figure S7).

Phenotypes of the cells isolated from 4T1 mammospheres and regular 4T1 monolayer culture

We utilized flow cytometry to study phenotypes of the cells collected from 4T1 monolayer culture or from 4T1 mammospheres. Practically, positive and negative expressions of both CD24 and CD44 by these cells were defined by referencing to fluorescence profiles of the same cells stained by isotype control antibodies (Figure S9). Because CSCs are normally cancer cells with a phenotype of $CD24^-/CD44^+$,[9] and 93.92±4.51% of 4T1mammosphere cells displayed a $CD24^-/CD44^+$ phenotype (Figure S7). Therefore, the 4T1 mammosphere cells were used as an operational model of CSCs for our *in vitro* studies. Further, staining profiles of the entire 4T1 mammosphere cells were used to gate CSCs among cells isolated from 4T1 orthotopic tumors (Figure S9 and S11).

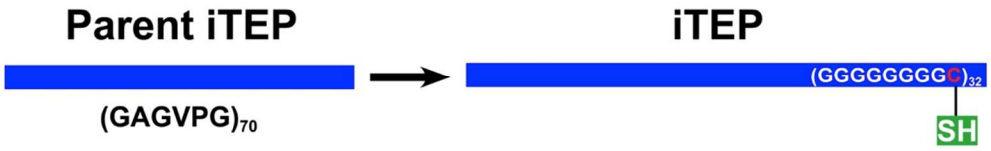

Figure S1. A scheme for iTEP design. The sequences for parent iTEP and the iTEP used in this study are shown in the scheme. The number "70" and "32" mean the number of hexmer repeats.

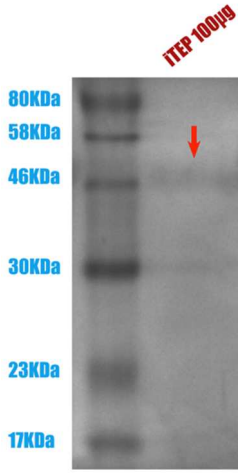

Figure S2. A copper-stained SDS-PAGE picture of purified iTEPs after 10 rounds of ITC purification. The loading amount is 100 μg/well. And the band was highlighted with red arrow.

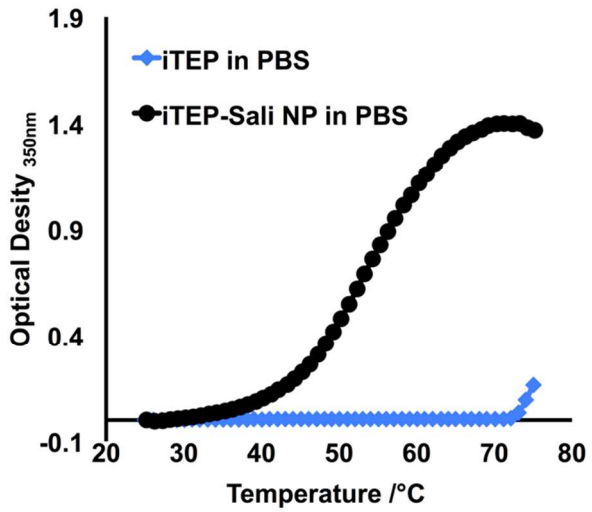

Figure S3. Thermally-induced phase transition curves of iTEPs and iTEP-Sali conjugates in PBS. Concentrations of both samples are 25 μM. The scans were performed in a temperature range from 25 °C to 75 °C.

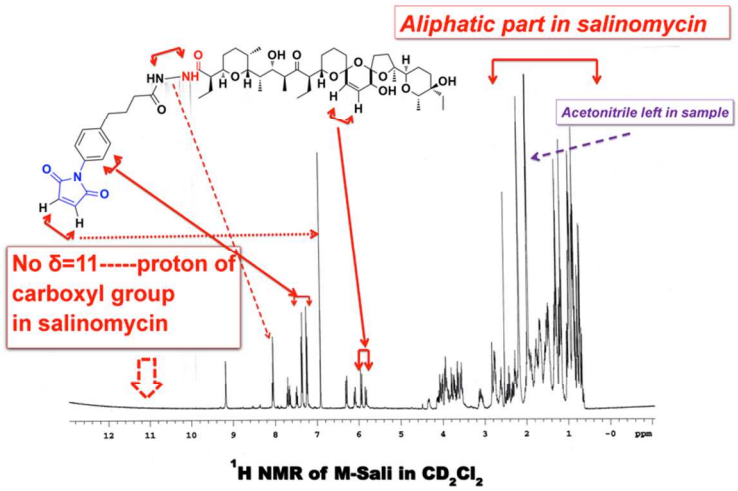

Figure S4. A 1H NMR spectrum of a purified reaction product between MPBH and Sali. The insert is the expected chemical structure of MPBH-Sali conjugates through an amide bond.

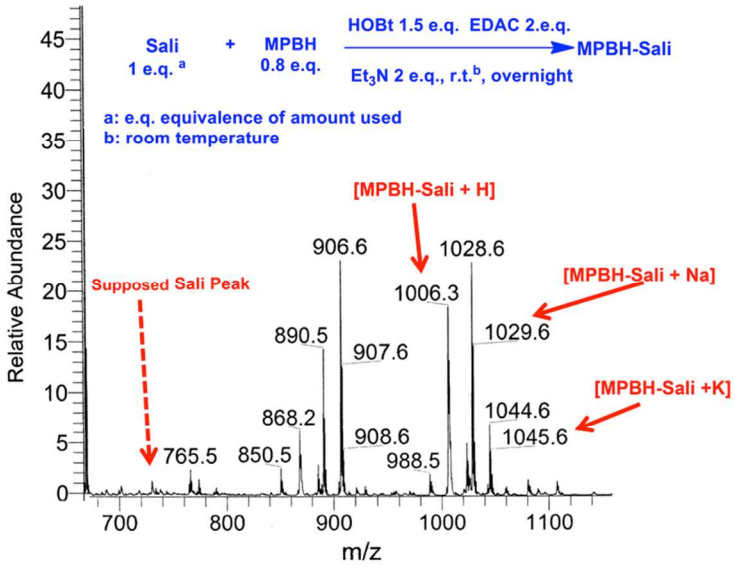

Figure S5. An ESI-MS spectrum of a purified reaction product between MPBH and Sali. The insert is a reaction equation describing the reaction condition used.

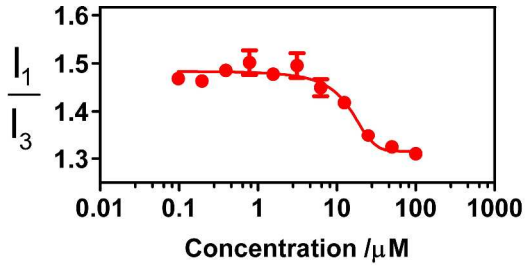

Figure S6. The ratio of the fluorescence emission intensities of the number 1 ($I_{370\text{-}373nm}$) and the number 3 ($I_{381\text{-}384nm}$) peaks of pyrene (I1/I3) in iTEP-Sali conjugate solutions was plotted as a function of iTEP-Sali conjugate concentrations at log 10 scale. The error bars indicate the standard deviation of each data point with n=3. The data was analyzed using a sigmoidal response model. The fitting curve was shown in the figure. [10]

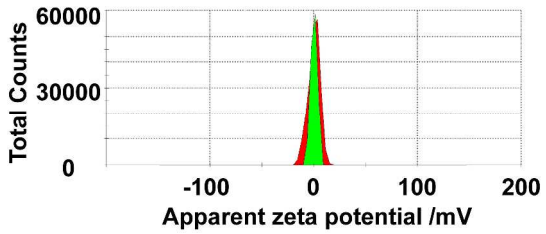

Figure S7. Zeta potential profiles of the iTEP-Sali NP2 (red filled curve) and the iTEP-Sali NP3 (green filled curve). The studies were performed in PBS at 37 °C using the Malvern Zetasizer Nano (Malvern, Chester County, PA, USA). The settings were water dispersant RI= 1.330, viscosity= 0.6864 cP, Dispersant Dielectric Constant: 74.4.

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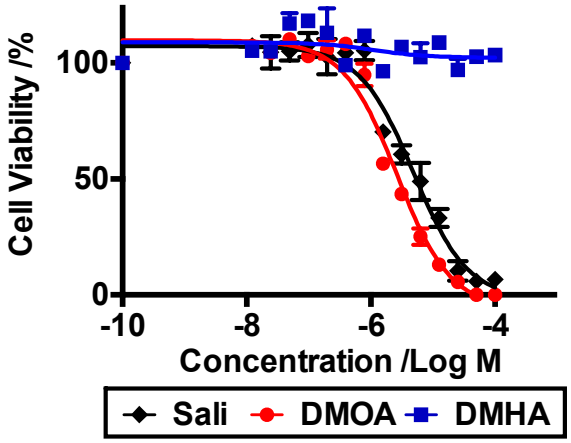

Figure S8. Viability profiles of 4T1 cells after they were exposed to different concentrations of Sali, DMOA, or DMHA for 48 hours. The IC_{50} of Sali is 4.85 µM with a 95% CI= 3.76~6.25 µM. The IC_{50} of the DMOA is 2.62 µM with a 95% CI = 2.01~3.30 µM. The DMHA did not show any toxicity up to 100 µM.

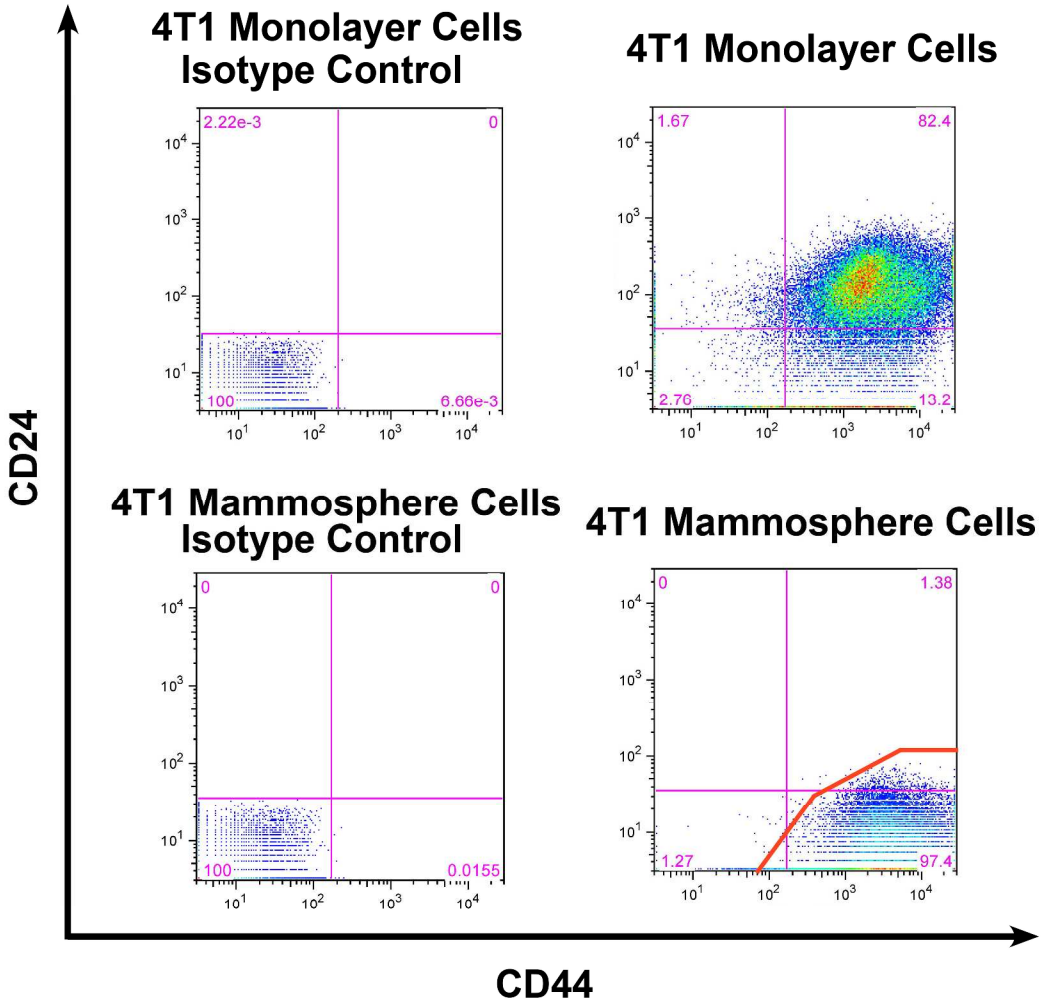

Figure S9. Representative flow cytometry graphs of cells isolated from 4T1 monolayer culture and mammospheres. These cells were stained with anti-CD44-APC and anti-CD24-PE antibodies, or isotype controls antibodies. In graph of the 4T1 mammosphere cells (right bottom), the entire cell population was circled together. The CD44 and CD24 expression profiles of the entire mammosphere cell population were used as a gating condition for CSCs in 4T1 orthotopic tumors.

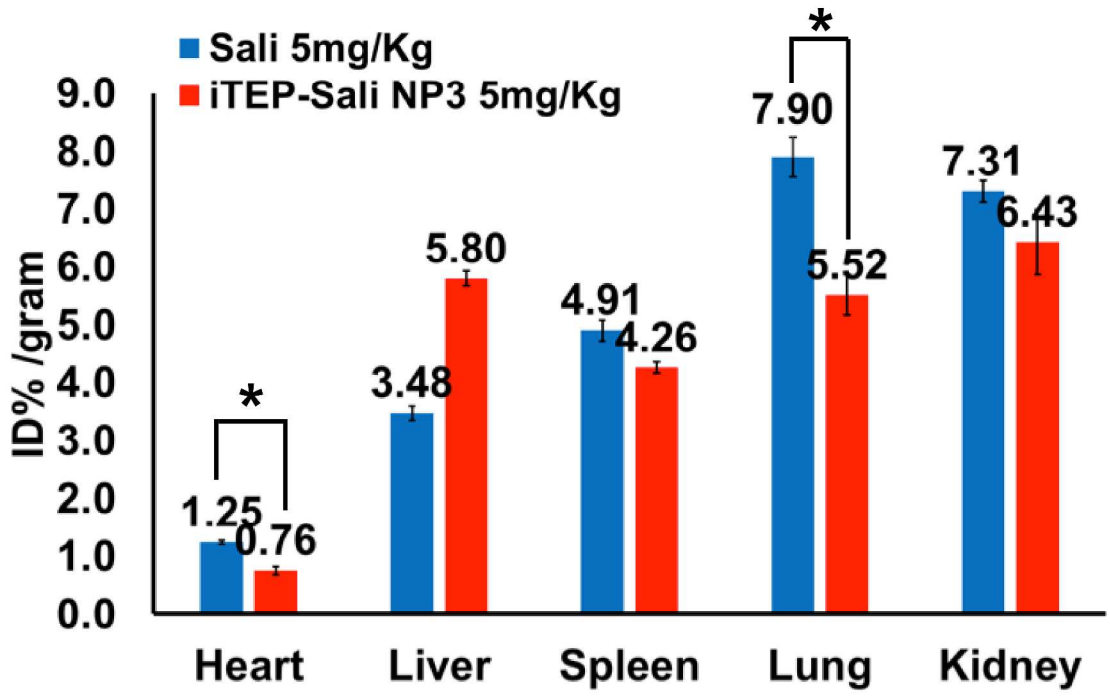

Figure S10. Sali concentrations in different organs of mice after Sali was administered to these mice as free form or encapsulated form in the NP3. Tissues were collected from the mice at 12 hour post intravenous injection. * indicate there is a statistic difference between two different formulations (for heart, p=0.0024, for lung, p=0.0011. p values determined by one-way ANOVA).

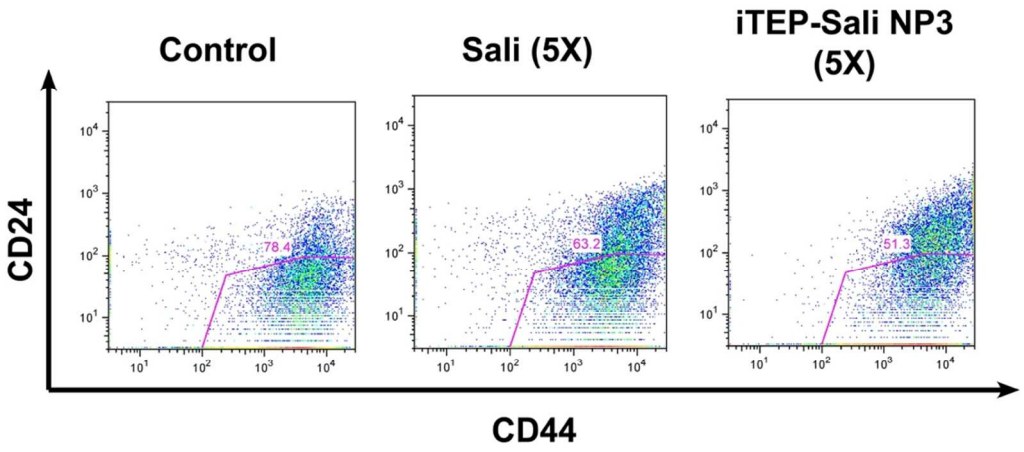

Figure S11. Representative flow cytometry graphs for cells isolated from harvested from 4T1 orthotopic tumors after corresponding treatment. The treatment applied for each group is indicated in the upper part of the figure. The percentages of cells that carry the CSC phenotype same as 4T1 mammosphere cells were labeled on top of the grated areas.

Reference

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