Supporting information

From ancient medicine to targeted nanocarrier: A Sparganii Rhizoma-derived nanoparticle for diagnostic imaging and endocrine therapy in cancer

Yi-Zhou Wu ¹, Yi-Kai Shen ¹,², Yu-Jia Chen ¹,², Jie Sun ³

¹ Department of Cell Biology, School of Basic Medicine, Nanjing Medical University, Nanjing 211166, China
² First School of Clinical Medicine, Nanjing Medical University, Nanjing 211166, China
³ Safety Assessment and Research Center for Drug, Pesticide and Veterinary Drug of Jiangsu Province, School of Public Health, Nanjing Medical University, Nanjing 211166, China

* Corresponding Author:
Jie Sun, Ph.D.
E-mail: yssjmm@126.com

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1. Materials and Methods

1.1. Chemicals.

The following chemicals were purchased: 1-ethyl-3-(3-dimethylaminopropyl)-carbonized diamine (EDC), N-hydroxy succinimide (NHS), indocyanine green (ICG), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), IC182780, and chromatography reagents (Sigma, USA); G15, LY294002 and PD98059 (Cell Signaling Technologies, USA); ICI182780, and LY2157299 (Eli Lilly Company, USA); vinegar (Jiangsu Hengshun Vinegar-Industry, China).

1.2. Instruments.

A merlin field emission SEM system (Carl Zeiss, Germany), EDS (QUANTAX, Bruker, Germany), UV-visible spectrophotometer (UV3600, Shimadzu, Japan), photoluminescence spectrometer (FL-4600, Hitachi, Japan), NTA system (NanoSight NS300, Malvern Instruments, UK) and Zetasizer (Malvern Instruments, UK) were used.

1.3. Cell lines and cell culture.

The human breast cancer MCF-7 cell line and human embryonic kidney HEK293 cell line were obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 µg/mL streptomycin, and at 37°C in a humidified atmosphere containing 5% CO₂.

1.4. MTT assay.

The cells were seeded in 96-well tissue culture plates at a density of $2 \times 10^3$ cells per well. After 24 h, the cells were treated with different concentrations of SpaTA. Next, 20 µL MTT solution (5 mg/mL) was added to each well and the samples were incubated for 4 h, followed by removal of the supernatant and addition of 100 µL DMSO. The optical density was measured at a wavelength of 540 nm by using a multi-detection spectrophotometer (SpectraMax M2e, Molecular Devices, USA). All experiments were performed at least three times. The relative cell viability was expressed as compared to the control group.

1.5. Immunoblotting assay.

The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM sodium deoxycholate) with 1x phosphatase inhibitor cocktail (PhosSTOP, Roche),
sonicated, and centrifuged for 40 min at 12,000 ×g at 4°C, and the supernatants were collected. The protein concentration was measured using a BCA kit (Pierce, USA). Western blot analysis was performed using 10%-12% polyacrylamide gels for electrophoresis, transfer to PVDF membranes, blocking in 5% BSA for 1 h at room temperature and incubation with a primary antibody (1:1000 dilution) overnight at 4°C. Primary antibodies, including pAkt, Akt, pERK, ERK and pSMAD were from Abcam (UK); pFAK and FAK were from Becton Dickinson (USA); pEGFR, EGFR, Bax, cleaved caspase-3 and GAPDH were from Cell Signaling Technologies (USA). Then horseradish peroxidase (HRP)-labeled secondary antibody (Cell Signaling Technologies, USA) was added for 1 h at room temperature. The proteins were detected using enhanced chemiluminescence reagents (ECL, Pierce, USA) and imaged using a ChemDoc XRS system (Bio-Rad, USA).

1.6. Immunofluorescence assay.

The cells were fixed in 4% paraformaldehyde (Sigma, USA), permeabilized with 0.1% Triton X-100 in PBS, blocked with 10% serum and incubated with different primary antibodies (1:500 dilution) against ERα (Abcam,UK), pFAK or vinculin (Becton Dickinson, USA) for 1 h at room temperature, followed by incubation with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Jackson ImmunoResearch, USA) for 1 h. F-actin was directly stained with Alexa Fluor 647-conjugated phalloidin (Molecular Probes, USA). The nuclei were counter-stained with DAPI for 20 min. The intracellular localization of proteins was acquired and analyzed by confocal laser scanning microscopy (LSM710, Carl Zeiss, Germany).

1.7. TUNEL assay.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit was purchased from Roche (USA). Tumor tissue sections or cells on coverglass were permeabilized with 20 μg/mL proteinase K. Endogenous peroxidase was inactivated by 3% H2O2 in methanol. The apoptotic cells were detected by labeling the 3′-OH ends of the fragmented DNA with biotin-dNTP at 37°C for 1 h, followed by incubation with streptavidin-Alexa Fluor 488 for 30 min. The nuclei were counter-stained with DAPI for 20 min. Apoptotic cells were identified as bright green dots in the blue-stained nuclei observed under a fluorescence microscope (Observer A1, Carl Zeiss, Germany).

1.8. Wound-healing assay.
MCF-7 cells were grown to subconfluence in 6-well plates. A straight scratch was made across the monolayer in each well using a 200 µL micropipette tip and the cells were washed with fresh culture medium to remove the displaced cells. Thereafter, the cells were treated with different drug formulations. The wounds were imaged at t = 0 and then after 12 h and 24 h under a bright-field microscope (BX43, Olympus, Japan). According to 5 randomly selected images in each group, the scratch widths were measured and analyzed to determine statistically significant differences.

1.9. Transwell assay.

The ability of MCF-7 cells to pass through filters was measured using a 24-well insert Transwell chamber system (Corning Costar, USA). The inserts were pre-coated with 50 µL Matrigel (Becton Dickinson, USA) that was diluted in FBS-free DMEM medium. The MCF-7 cells were resuspended in serum-free medium and 200 µL cell suspension containing the different drugs was added to the upper chamber, while 800 µL medium containing 10% FBS served as a chemo attractant in the lower chamber. The cells were cultured for 24 h. Thereafter, the un-migrated cells were removed and the membranes were fixed with methanol for 5 min, followed by staining with 0.5% crystal violet for 1 h. The migrated cells were imaged under a bright-field microscope (BX43, Olympus, Japan). The number of migrated cells were counted and analyzed from 10 randomly selected images in each group.

1.10. Tumor xenograft model.

All animal experiments were approved by the Animal Research Ethics Board of Nanjing Medical University (Approval No. IACUC-1801015). Adult female BALB/c nude mice weighing 22 ± 2 g were used for establishing a tumor xenograft model. The MCF-7 cells (2 × 10⁶) in the logarithmic growth phase were subcutaneously inoculated into the right thoracic mammary fat pad of nude mice. The animals were maintained in a specific pathogen free (SPF) and controlled environment at 24 ± 2°C under a 12 h light/dark cycle and received food and water ad libitum. When the tumor volume reached approximately 50 mm³, the mice were treated with different drugs or PBS (control) by injection into the tail vein. After the treatment period, the mice were anesthetized and the tumors were harvested for further analysis. The tumor size was measured with a caliper and calculated using the equation \( V=\frac{\pi}{6} \times \frac{4}{3} \times (L+W)/2 \), where L represents the largest and W represents the smallest diameter of the tumor.

1.11. In vivo tumor imaging.
The MCF-7 cells were subcutaneously inoculated into two sides of the back of BALB/c nude mice (2 × 10^6 cells for each side) and allowed to grow for 28 d. The animals were then treated with 100 mg/kg SpaTAX or PBS by intravenous injection. Six hours later, the animals were anesthetized by sevoflurane inhalation and placed on a table with the detector positioned toward the tumor region. The NIR fluorescence was acquired and analyzed by a Xenogen IVIS Spectrum system (Caliper, USA) equipped with a 750-780 nm excitation filter and a 820-850 nm emission filter. This system was also used for NIR fluorescence imaging of SpaTAX solutions.

1.12. Immunohistochemistry assay.

Tumor tissues were fixed with formalin, embedded in paraffin and sectioned. The tumor sections were dewaxed by xylene and rehydrated through an ethanol gradient, treated with 3% H$_2$O$_2$ to neutralize any endogenous peroxidase and permeabilized with 0.1% Triton X-100 in PBS. The microwave-based antigen retrieval method using citrate buffer (pH 6.0) was applied. The sample background was blocked in 5% BSA for 1 h at room temperature. The tumor sections were incubated with different primary antibodies (1:500 dilution) overnight at 4°C respectively, followed by the HRP-labeled secondary antibody and visualized using a DAB staining kit (Cell Signaling Technologies, USA). The nuclei were counter-stained in blue with hematoxylin. For H&E staining, the cytoplasm was stained in red with eosin. After dehydration with an ethanol gradient, the tumor sections were sealed with resin. The tissue sections were imaged using a bright-field optical microscope (BX43, Olympus, Japan).


All data are presented as the mean ± standard deviation. The student’s t-test or one-way analysis of variance (ANOVA) were carried out according to the means of the experimental groups. The statistical significance of differences was determined by a value of $p < 0.05$. 
2. Supplemental Figures.

**Figure S1. Immunofluorescence assay of pFAK and F-actin.** The MCF-7 cells were treated with SpaTA (0.1 mg/mL) alone or combined with G1 (100 nM) and stained after 24 h for the intracellular proteins vinculin (green) and F-actin (red). The nuclei were stained with DAPI (blue). Scale bar: 10 μm.
Figure S2. **Wound-healing assay.** After a scratch across the monolayer, MCF-7 cells were treated with SpaTA (0.1 mg/mL) alone or combined with G1 (100 nM), and the migrated distance was measured after 12 h and 24 h respectively. Scale bar: 100 μm.

Figure S3. **Transwell assay.** MCF-7 cells were treated with SpaTA (0.1 mg/mL) alone or combined with G1 (100 nM) and assayed after 24 h. The migrated cells were stained by crystal violet. The small and white circles in the images were those pores in the membrane. Scale bar: 50 μm.
**Figure S4. HPLC assay of LY2157299.** The drug loading rate was analyzed by HPLC. The content of uncoupled LY2157299 (red) in the sample dialysis fluid was calculated according to a standard LY2157299 solution (blue), by the peak-area ratio method.

![HPLC Assay of LY2157299](image)

**Figure S5. NIR imaging of BALB/c nude mouse by IVIS system.** The mouse without tumor was intravenously injected with SpaTAX (100 mg/kg). The whole-body NIR fluorescence was imaged after 6 h later.

![NIR Imaging](image)

**Figure S6. H&E staining assay.** The MCF-7 tumor-bearing nude mice were treated with PBS (control), 1 mg/kg LY2157299, 100 mg/kg SpaTA or 100 mg/kg SpaTAX. The pathological changes in tumor tissues were analyzed by H&E staining. Scale bar: 20 μm.
Figure S7. **In vitro toxicity assay.** HEK293 cells were treated with the indicated concentrations of SpaTAX or PBS (control), and assayed at 72 h using the MTT assay. The relative cell viability is presented as compared to control group. The graph represents the results from three independent experiments. * p < 0.05.

Figure S8. **Body weight assay.** BALB/c mice (non-immunodeficient) were treated with PBS (control), 1 mg/kg LY2157299, 100 mg/kg SpaTA or 100 mg/kg SpaTAX by intravenous injection for a total of 14 days (treatment every 2 days). The body weight was measured at day 1, 3, 5, 7 and 14.
Figure S9. *In vivo toxicity assay.* Histological examination of heart, liver, spleen, lung and kidneys from (non-immunodeficient) BALB/c mice after PBS, 1 mg/kg LY2157299, 100 mg/kg SpaTA or 100 mg/kg SpaTAX treatments for a total of 14 days (treatment every 2 days). Scale bar: 20 μm.