Electronic Supporting Information

An octopus-mimic PEGylated peptide as a specific integrin αvβ3 inhibitor for preventing tumor progression

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This PDF file includes:

1. Materials and Methods

Materials

All chemicals and solvents are of reagent grade unless otherwise indicated. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Peptide candidates were purchased from Karebay Biochem (ningbo, China). The deionized water was obtained by a Millipore NanoPure purification system (resistivity > 18.2 MΩ cm^{-1}). 8-arm PEG-MAL was purchased from ToYongBio Tech.Inc (Shanghai, China).

Cell lines and cell culture

MDA-MB-231 (ATCC HTB-26), PC-3 (ATCC CRL 1435), MCF-7 (ATCC HTB-22) and HUVEC (ATCC PCS-100-013) were obtained from ATCC (Manassas, VA). Human hepatic L02 cells (CAS GNHu 6) were obtained from the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were cultured in Leibovitz Medium (L-15, Gibco) supplemented with 10% fetal calf serum (FBS, Gibco) and antibiotics (penicillin: 80 U/ml; streptomycin: 80 μg/ml). PC-3 and L02 cells were cultured in RPM1640 medium (Gibco) supplemented with 10% fetal calf serum (FBS, Gibco) and antibiotics (penicillin: 80 U/ml; and streptomycin: 80 μg/ml). HUVEC cells were cultured in dublecco's modified eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FBS, Gibco) and antibiotics (penicillin: 80 U/ml; streptomycin: 80 μg/ml). All cells were incubated at 37 ℃ with 5% CO_2.

Animal experiments

Nude BALB/c mice were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, Jiangsu, China). All animal experiments were performed in accordance with the National Institute of Health Guidelines under the protocols, approved by the ethics committee at the Affiliated Drum Tower Hospital of Nanjing University Medical School.

Synthesis of octopus-R

octopus-R were synthesized according to the scheme shown in Fig. 2a. An aqueous solution of 8-arm PEG-MAL (1 mM) was added in MPA-ACP-RWrNR (10 mM) aqueous solution, followed by adding trimethylamine to adjust pH to about 9.0. The reaction mixture was kept at room temperature for 12 hours, and the product was purified with HPLC. HPLC column: VP-ODS C18 column, 150 × 4.6 mm, 5 μm. Flow rate: 1mL min-1. Solvent: solvent A: 0.1% trifluoroacetic acid in H2O, solvent B: 0.1% trifluoroacetic acid in acetonitrile. Gradient: 16%→32% solvent B from 0-20 min.

Characterization of octopus-R

UV absorption spectra were collected with a PerkinElmer Lambda 750 UV–vis-NIR spectrophotometer. High performance liquid chromatography (HPLC) chromatograms were conducted with SHIMADZU Prominence LC-20A. HPLC column: VP-ODS C18 column, 150 × 4.6 mm, 5 μm. Flow rate: 1mL min^{-1}. Solvent: solvent A: 0.1% trifluoroacetic acid in H2O, solvent B: 0.1% trifluoroacetic acid in acetonitrile. Gradient: 16%→32% solvent B from 0-20 min. The molecular weight of octopus-R was confirmed by MALDI TOF/TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics, USA).

The Serum Stability of octopus-R

Octopus-R was incubated in 50% FBS (diluted in PBS) at 37 °C for 24 hours, followed by measuring with Malvern Zetasizer Nano ZS. The size and zeta potential were measured at 0, 2, 4, 6, 8, 12 and 24 hours.

Octopus-R was incubated with 50% fetal bovine serum (Gibco, USA) at 37℃, and the samples (200 μL) were collected at different time points, followed by analyzing by HPLC.

MTT assay

MDA-MB-231, PC-3, HUVEC and L02 cells were seeded into 96-well plates at a density of 2×10^3 cells per well, and incubated for 24 hours, followed by culturing with different concentrations of c-RGDyK, RWrNR or octopus-R for 48 hours. Then 200 μl MTT solution (0.5 mg/ml) was added and incubated at 37 ℃ for additional 4 hours. The culture medium was removed, followed by adding 150 μl of DMSO. The absorbance was measured at 570 nm using Microplate Reader (TECAN, 200 pro, USA).

Chick chorioallantoic membrane model

Chick embryos were hatched in an incubator at 37 ℃ for 2 days, and filter papers containing 25 μM c-RGDyK, RWrNR or octopus-R were put on the chick embryo chorioallantoic membrane. The chick embryos were sealed with a plastic wrap, and incubated for 3 days. The chorioallantoic membranes were imaged by stereomicroscope and the newly born vessels were quantified by imageJ software.

Transwell assay

The cell migration was determined using costar 24-well plates containing cell culture inserts with an 8-μm pore size. The cells in fetal serum free medium (MDA-MB-231: 5×10^5 cells/ml and PC-3: 2×10^6 cells/ml) were mixed with 25 μM c-RGDyK, RWrNR or octopus-R, and cells in free medium were used as the control. Cells were then plated in the upper chamber, and cultured for a few of hours (MDA-MB-231: 8 hours and PC-3: 36 hours). The unattached cells were removed from the microporous membrane with a cotton swab. The membrane was fixed with polyformaldehyde for 15 min at 4℃, stained with 0.1% crystal violet and imaged by an inverted microscope (ZEISS). Inhibition rate was calculated as % migration = (cell numbers of the control group - cell numbers of the experiment group) / cell numbers of the control group × 100%.

Wound-healing assay. MDA-MB-231, PC-3 and MCF-7 cells (2×10^5) were seeded in 12-well plates and cultured for 12 hours. Scratch wounds were created using a micropipette tip. After the suspended cells were removed, cells were cultured in serum-free media containing octopus-R, RWrNR or c-RGDyK (25 μM) for 12 hours. Wound area recovery was imaged by an inverted microscope (ZEISS). The size of wound area was measured by ImageJ program.

Synthesis of octopus-R-Cy5

Octopus-R-Cy5 was synthesized by conjugating octopus-R with Cy5-NHS ester via the amidation reaction. Briefly, 100 μL aqueous solution of Cy5-NHS ester (1 mM) (Dalian Meilun Biotechnology CO., LTD) was added in 1 mL aqueous solution of octupus-R (100 μM), followed by adding trimethylamine to adjust pH to 9.0. The reaction mixture was kept at room temperature for 8 hours in dark, followed by purifying with dialysis (MwCO 3000).

Xenograft tumor bearing mice models. Male and female nude mice (6-8 weeks, 18-22 g) were purchased from the Experimental Animal Center of Nanjing Medical University. Female nude mice bearing MDA-MB-231 tumors were prepared by subcutaneously implanting MDA-MB-231 cells (10^7) in the right bake. Male nude mice bearing PC-3 tumors were prepared by subcutaneously implanting PC-3 cells (10^7) in the right bake. Tumor sizes were measured with the Vernier caliper and tumor volumes (mm^3) were calculated according to the following formula: volume = 0.5 × longest diameter × shortest diameter^2.

***In vivo* tumor targeting**

In vivo tumor imaging was performed using an IVIS Spectrum Imaging System (Perkin-Elmer, USA). The mice bearing MDA-MB-231 or PC-3 tumors were injected with octopus-R-cy5 (1 μM/kg) through the tail vein. Mice were then imaged at 2, 4, 8, 12, 24 hours after injection. After 24 hours, the mice were euthanized, and tumors and major organs were harvested and imaged with an IVIS Spectrum Imaging System. The imaging data were analyzed using the Living Imaging 4.5 Software.

***In vivo* blocking study**

Tumor bearing mice were intravenously co-injected with octopus-R-Cy5 (1 μM/kg) and unlabeled octopus-R (25 μM/kg), followed by imaging with IVIS Spectrum Imaging System at different time points. The imaging data were analyzed using the Living Imaging 4.5 Software.

***In vivo* antitumor effects**

The mice bearing MDA-MB-231 or PC-3 tumors were intravenously injected with 25 μM/kg of saline, c-RGDyK, RWrNR or octopus-R every other day for two weeks. The tumor sizes and body weights of mice were measured every day. After 14-day treatment, the mice were euthanized, and tumors and major organs were collected for the further assessment.

Histopathology and immunohistochemistry

Tumor tissues were sectioned and stained with H&E, caspase-3 and CD31 for evaluating anticancer effects. Major organs were sectioned and stained with H&E for evaluating biosafety.

Statistical analyses

All experiments were repeated at least 3 times with 6 replicates. Error bars represent standard error of the mean from independent samples assayed within the experiments. Statistical analysis was done with GraphPad Prism 6 software. Statistical significance was calculated using unpaired Student's *t*-test, and a p-value < 0.05 was considered to statistically significant.

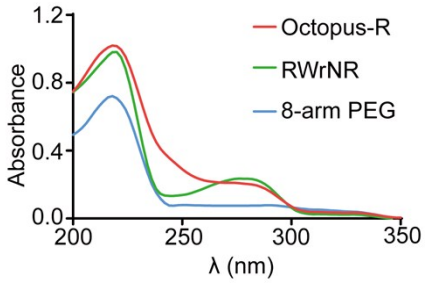

Fig. S1 UV absorption spectra of octopus-R, RWrNR and 8-arm PEG

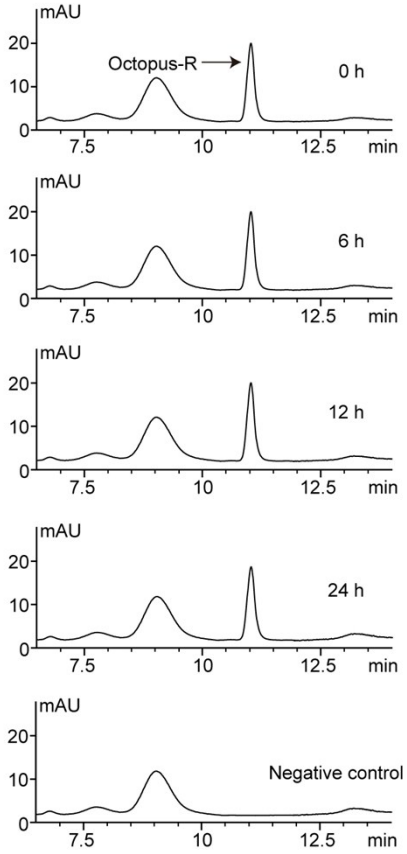

Fig. S2 The serum stability of octopus-R

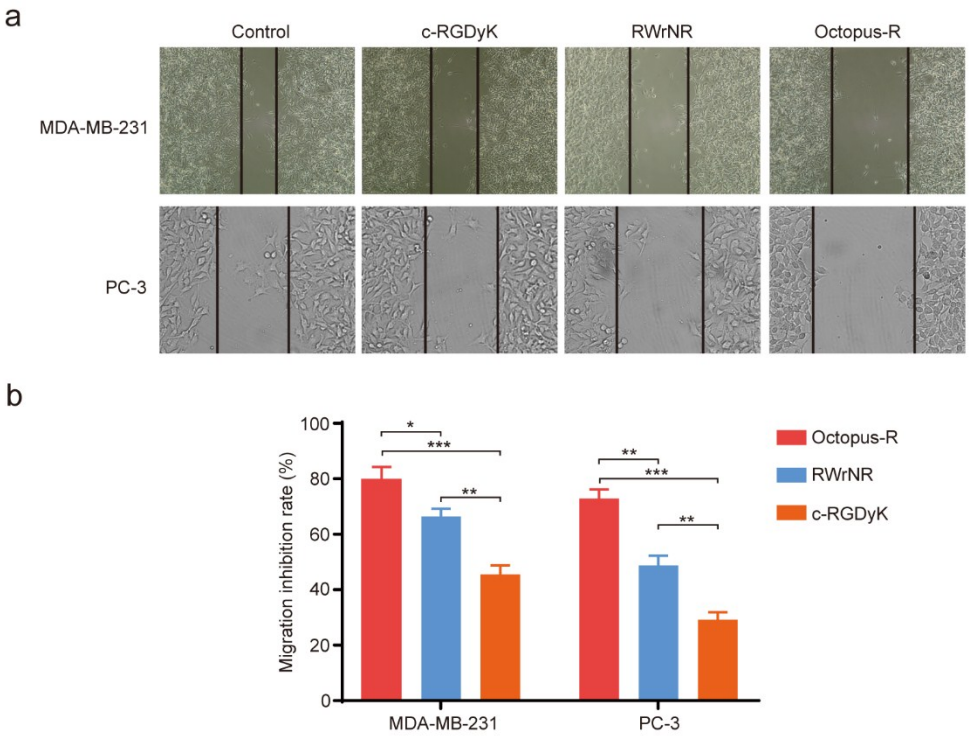

Fig. S3 a Microscopic images of the wound healing assay of MDA-MB-231, PC-3 and MCF-7 cells treated with octopus-R, RWrNR and c-RGDyK. Scale bar: 50 μm. **b** The qualification of migration inhibition of wound healing assay. Values are the mean ± SD, n = 6. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t* test, two tails)

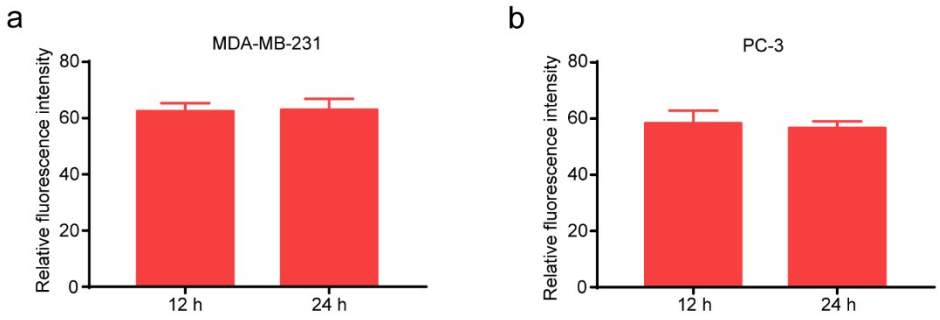

Fig. S4 a The fluorescence intensity of tumor in 12-h and 24-h after injection in MDA-MB-231 tumor bearing mice. **b** The fluorescence intensity of tumor in 12-h and 24-h after injection in PC-3 tumor bearing mice.

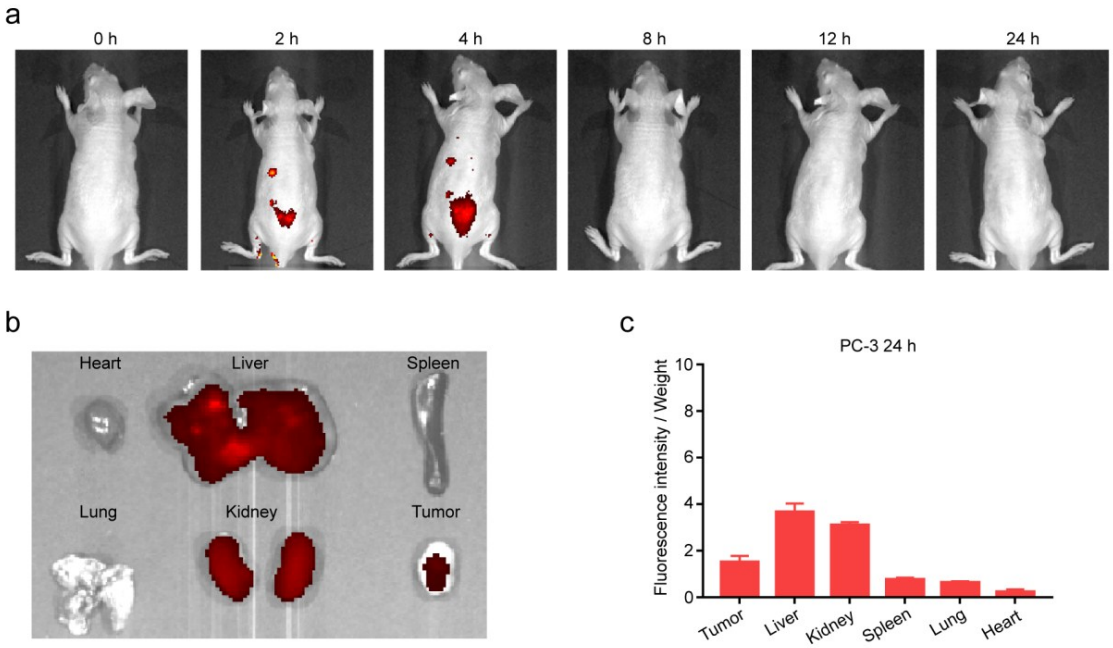

Fig. S5 a *In vivo* imaging of PC-3 tumor-bearing nude mice after intravenous administration of Cy5. Mice were systemically administrated with Cy5, and imaged with IVIS Spectrum Imaging System at 2, 4, 8, 12 and 24 hours. **b** *Ex vivo* fluorescent imaging of major organs after 24-h

intravenous injection of Cy5. **c** Quantification of the fluorescence intensity in major organs and tumors.

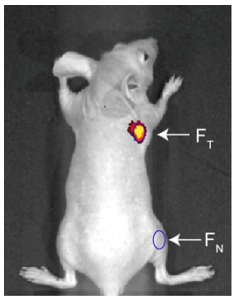

Fig. S6 The relative ratio of fluorescent signals between the tumor (F_T) and normal tissues (F_N)

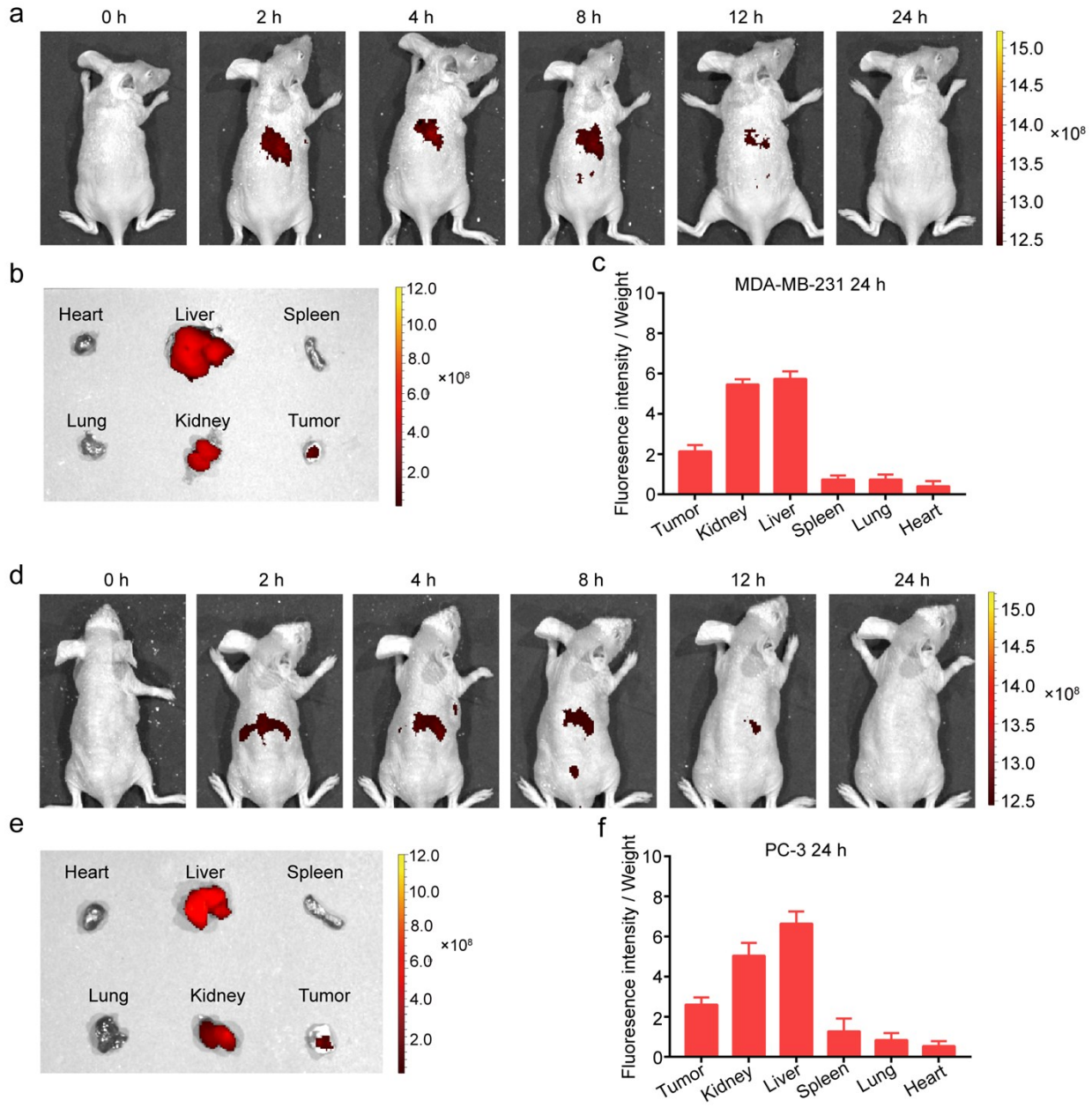

Fig. S7 a *In vivo* imaging of MDA-MB-231 tumor-bearing nude mice after intravenous co-injection with octopus-R-Cy5 (1 μM/kg) and unlabeled octopus-R (25 μM/kg), and imaged with IVIS Spectrum Imaging System at 0, 2, 4, 8, 12 and 24 hours. **b** *Ex vivo* fluorescent imaging of major organs after 24-h intravenous co-injection with octopus-R-Cy5 (1 μM/kg) and unlabeled octopus-R (25 μM/kg). **c** Quantification of the fluorescence intensity in major organs and tumors.

d *In vivo* imaging of PC-3 tumor-bearing nude mice after intravenous co-injection with octopus-R-Cy5 (1 μM/kg) and unlabeled octopus-R (25 μM/kg), and imaged with IVIS Spectrum Imaging System at 0, 2, 4, 8, 12 and 24 hours. **e** *Ex vivo* fluorescent imaging of major organs after 24-h intravenous co-injection with octopus-R-Cy5 (1 μM/kg) and unlabeled octopus-R (25 μM/kg). **f** Quantification of the fluorescence intensity in major organs and tumors.

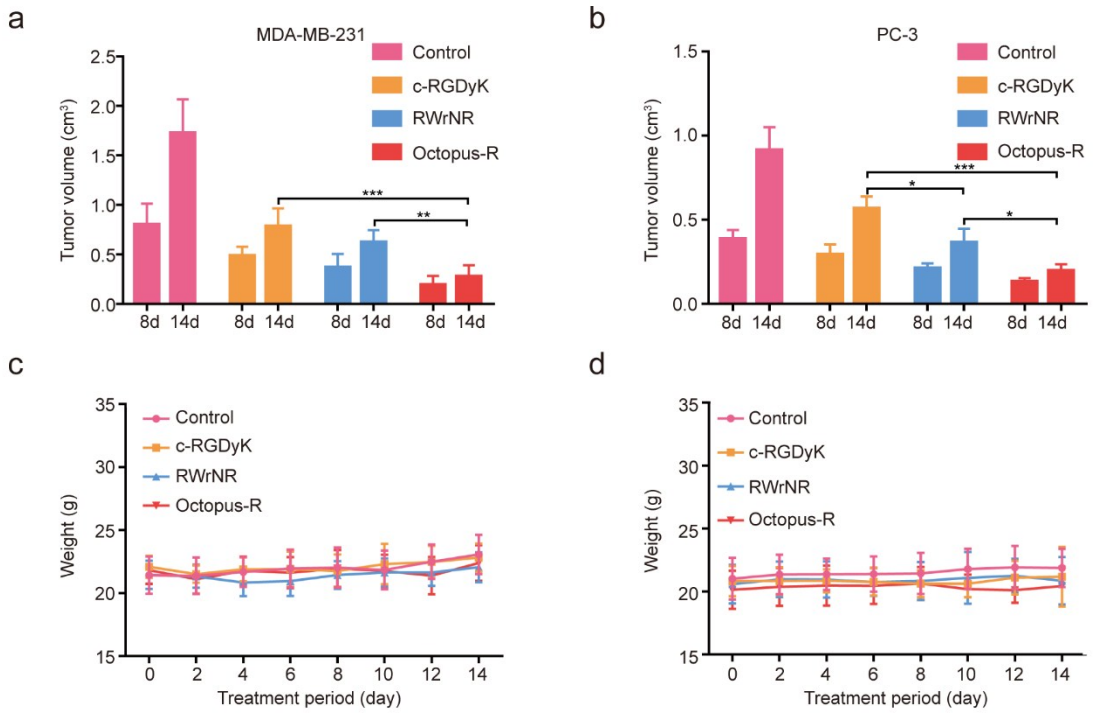

Fig. S8 a The comparison between 8-days treatment and 14-days treatment in MDA-MB-231 tumor bearing mice. **b** The comparison between 8-days treatment and 14-days treatment in PC-3 tumor bearing mice. **c** The weight of MDA-MB-231 tumor bearing mice during 14-days treatment. **d** The weight of PC-3 tumor bearing mice during 14-days treatment. Values are the mean ± SD, n = 4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t* test, two tails)

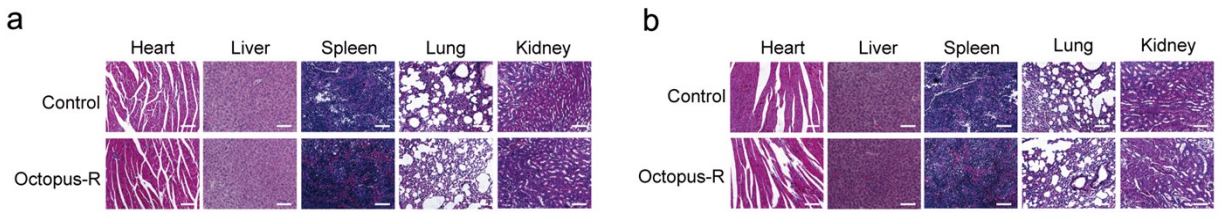

Fig. S9 a H&E staining of major organs in MDA-MB-231-tumor bearing mice, treated with octopus-R. **b** H&E staining of major organs in PC-3-tumor bearing mice, treated with octopus-R. Scale bars represent 200 μm.