Supporting Information

CuS Nanoparticles as a Photodynamic Nanoswitch for Abrogating Bypass Signaling to Overcome Gefitinib Resistance

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Experimental Section

Materials: $CuCl_2$, $N_2H_4 \cdot H_2O$ and Na_2S were obtained from J&K technology Co., Ltd. (China). Polyvinylpyrrolidone (PVP40, MW = 4000 Da) was obtained from Sigma-Aldrich (USA). Thiol-PEG-Carboxyl (COOH-mPEG-SH, MW = 5000 Da) was obtained from Shanghai Ponsure Biotechnology, Co., Ltd. Antibodies used for Western blotting (Erk, phospho-p42/44 Erk, Akt, phospho-Akt, IGF1R, phospho-IGF1R, phospho-IKK, IKKα, IKKβ, phospho-NF-κB, NF-κB, phospho-IκB, IκB and β-actin) were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody, Alexa Fluor 555-conjugated secondary antibody, DAPI were purchased from Invitrogen (Carlsbad, CA, USA). HCC827 cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. PC9 cells were provided by Dr. Qianggang Dong in Shanghai Cancer Institute, Shanghai Jiao Tong University School of Medicine. Gefitinib-resistant HCC827-GR and PC9-GR cells were established and provided by Dr. Lu Xu in the department of pharmacology and chemical biology, Shanghai Jiao Tong University School of Medicine.[1]

Preparation and characterization of CuS NPs: We synthesized CuS NPs following our previously described method with minor modifications.[2] Briefly, polyvinylpyrrolidone (PVP40, 0.48g) was dissolved into 50 μL deionized water in a round flask, followed by the addition of $CuCl_2$ solution (200 μL, 0.53 mol/L) under magnetic stirring at room temperature. After the mixture with NaOH solution (50 mL, pH 9.0), hydrazine hydrate (12.5 μL) was added to the suspension to form Cu_2O spheres. After 6 min of reaction, Na_2S solution (400 μL, 178 mg/mL) was added into the suspension. Then the flask was heated at 60 °C and stirred at 660 rpm for 3.5 h. CuS NPs were centrifuged at 12 000 rpm for 30 min at 10 °C and washed twice with deionized water. To formulate CuS@PEG NPs, CuS solution (0.5 mL, 2 mg/mL) was added into HS-PEG-COOH solution (1.2 mL, 10 mg/mL) for

reaction by gently rotating at room temperature. After 12 h, CuS@PEG NPs were centrifuged at 12 000 rpm for 20 min at 10 °C, and washed twice with deionized water. The TEM images of CuS NPs were obtained using a FEI Talos F200X operating at 200 kV. Dynamic light scattering (Nano ZS, Malvern) was utilized to record the hydrodynamic size distribution of CuS NPs. The UV-Visible absorption spectrum was measured using Nanodrop 2000c (Thermo, USA).

Cellular uptake of CuS NPs: Firstly, CuS NPs were labeled by Rhodamine B (RhB). Briefly, the aqueous solution of CuS NPs and RhB were mixed at a mass ratio of 1:10 (CuS NPs: RhB), and then the mixture was washed twice by distilled water after 4 h of rotation to remove the unbound RhB. HCC827-GR and PC9-GR cells (3×10^5) were seeded into glass bottom dishes and cultured overnight before incubation with CuS@RhB NPs medium solution (30 μg/mL) for 4 h. Subsequently, the cells were fixed by 4% paraformaldehyde for 15 min, and then the nucleus were stained by DAPI for 10 min. Finally, the cellular uptake was determined by confocal laser scanning microscopy (CLSM). The internalization of CuS@RhB NPs was also determined by flow cytometry (FCM). Briefly, after incubated with CuS@RhB NPs for 4 h, the cells were harvested and re-suspended in PBS to prepare single cell suspension, followed by FCM analysis.

ROS detection: Ce6 molecules or CuS NPs were dispersed into DCFH solution at a concentration of 30 μg/mL, and then the solution was respectively irradiated by 660 nm laser for 6 min or 808 nm laser for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval), followed by the detection of DCF fluorescence (488/525 nm). To detect the cellular ROS level, HCC827-GR (8×10^3) and PC9-GR cells (1×10^4) were seeded into 96-well plates and cultured overnight. The cells were then incubated with CuS NPs (30 μg/mL) medium for 4 h and irradiated by 808 nm NIR laser at a power density 2 W/cm^2 for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval). Subsequently, the cellular ROS was detected using ROS Assay Kit (Beyotime, Shanghai, China) according to the

manufacturer's instructions. In confocal Raman detection, the cells were incubated with Au/PATP (1.1 μM) and CuS NPs (30 μg/mL) for 4 h and irradiated by 808 nm NIR laser at a power density 2 W/cm^2 for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval). Afterwards, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and the excess paraformaldehyde was removed with distilled water, and then the samples were air-dried before SERS measurements. The SERS detection and imaging were performed on the Renishaw inVia Raman Microscope equipped with an 830 nm laser. The SERS spectra were collected through a 5×objective (Lecia). The laser power and acquisition time were 101 mW and 1s, respectively.

Analysis of protein expression: For *in vitro* detection, the HCC827-GR and PC9-GR cells were seeded into 6-well plates (3× 10^5 cells per well) and cultured overnight. After 4 h incubation with CuS NPs (30 μg/mL), the cells were collected and transferred to the 96-well plate, where cells were irradiated by an 808 nm NIR laser at a power density 2 W/cm^2 for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval), and the cells without CuS/Laser treatment served as a control. Subsequently, cells were re-seeded into 6-well plates. After 24 h, cells were collected and the total cell protein was extracted by lysing cell pellets in RIPA buffer (Beyotime, Shanghai, China) supplemented with protease inhibitor (Sangon Biotech, Shanghai, China) and phosphatase inhibitor (Beyotime, Shanghai, China). To test the effect of H_2O_2 treatment, HCC827-GR and PC9-GR cells were seeded into 6-well plates (3×10^5 cells per well) and cultured overnight. The growth medium was then replaced by a culture medium containing NAC (5 mM) and/or H_2O_2 (1 mM). After 24 h incubation, cells were collected and the total cell proteins were similarly extracted as described above. *In vivo* detection, when the tumor grown to about 100 mm^3, the nude mice were divided into 3 groups: control group, Ce6/Laser group and CuS/Laser group. Mice in Ce6/Laser and CuS/Laser groups were intravenously injected with Ce6 and CuS NPs (2 mg/mL, 200 μL) for 24 h circulation. After that, these tumors were

respectively irradiated by 660 nm laser for 6 min, or 808 nm laser for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval) , and then the tumor were collected for protein extraction after 24 h. In all treatment groups, BCA kit (Thermo, USA) was used to quantify the total proteins, and the expression of related proteins was detected by western blotting assay.

Immunofluorescent staining: HCC827-GR and PC9-GR cells were seeded into glass-bottom dishes (3×10^5 cells per dish) and cultured overnight. Then, the cells were incubated with CuS NPs (30 μg/mL) for 4 h and irradiated by 808 nm NIR laser at a power density 2 W/cm^2 for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval) and then cultured for 24 h, the cells untreated with CuS/Laser were as control. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2%Triton X-100 for 20 min, and then the cells were blocked with 1% BSA for 30 min. Thereafter, the cells were stained with IGF1R antibody overnight at 4 °C and then stained with Alexa Fluor 555-conjugated secondary antibodies for 1h at room temperature, and then the nucleus were stained by DAPI for 10 min. Finally, the cells were visualized by CLSM.

In vitro treatment: To evaluate the cytotoxicity of CuS NPs, HCC827-GR and PC9-GR cells were seeded into 96-well plates (1×10^4 cells per well) overnight. Cells were incubated with CuS NPs at a serial of concentrations (0, 12.5, 25, 50, 75, 100 and 200 μg/mL) for 4 h before replacing with fresh medium. After 12 h, the cell viability was measured by Cell Counting Kit-8 Assay (CCK8, Dojindo, Japan) according to the manufacturer's instructions. To explore the sensitization of CuS/Laser treatment in gefitinib resistant cells, HCC827-GR and PC9-GR cells were seeded into 96-well plates (8×10^4/ 1×10^5 cells per well). After 12 h of attachment, the cells were divided into 6 groups: control group, CuS NPs group (CuS), NIR laser group (Laser), CuS/Laser group, gefitinib group, and CuS/Laser/gefitinib group. The cells in CuS NPs group, CuS/Laser group and CuS/Laser/gefitinib group were incubated with CuS NPs (30 μg/mL) for 4 h, and then the cells in Laser group, CuS/Laser

group and CuS/Laser/gefitinib group were irradiated by 808 nm NIR laser at a density of 2 W/cm^2 for 6 min (pulsed mode with 30-ms stimulus and 20-ms interval), followed by the cells in gefitinib group and CuS/Laser/gefitinib were incubated with gefitinib (5 μM) for 48 h. Finally, the cell viability was detected by CCK8 assay. In the concentration gradient experiment, the HCC827-GR and PC9-GR cells in 96-well plates were divided into 2 groups: gefitinib group and CuS/Laser/gefitinib group. The cells in CuS/Laser/gefitinib group were incubated with CuS NPs (30 μg/mL) for 4 h and followed by the NIR laser irradiation. Next, the cells in two groups were treated with gefitinib as the indicated concentrations (0, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, 10^{-5} and 5×10^{-5} mol/L) for 48 h. The cell viability was measured by CCK8 assay.

Colony formation assay: HCC827-GR and PC9-GR cells were seeded into 6-well plates (3×10^5 cells per well). After the attachment of 12 h, the cells were incubated with CuS NPs (30 μg/mL) for 4 h, and then the cells were collected and transferred to a 96-well plate, followed by NIR laser irradiation. Subsequently, the cells treated with or without CuS/Laser were seeded into 6-well plates (2×10^3 cells per well) and cultured overnight before incubation with gefitinib-containing medium (5×10^{-6} mol/L). The medium was refreshed every other day. After treatment for 10 days, the grown colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for imaging.

In vivo treatment: BALB/c nude mice (4 weeks) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd (China). All experimental procedures were approved and reviewed in accordance with the guidelines for the care and use of laboratory animals at Shanghai Jiao Tong University. HCC827-GR (5×10^6) or PC9-GR (1×10^7) cells were subcutaneously inoculated in the flank of the nude mice. When the tumor size reached about 100 mm^3, defined as day 0, the mice were randomly divided into four groups (n = 5) and received the following treatment. Saline control group was intravenously injected with saline at day 0 and intragastrically injected with saline every other day

from day 1. Gefitinib group was intragastrically administrated with gefitinib (5 mg/kg) every other day from day 1; CuS/Laser group was intravenously injected with CuS NPs (2 mg/mL, 200 μL) at day 0 followed by laser irradiation (808 nm, 2 W/cm^2, 6 min with repeated 30-ms stimulus and 20-ms interval) at day 1; CuS/Laser/gefitinib group received the same treatment with CuS NPs and irradiation, followed by intragastrical administration of gefitinib (5 mg/kg) every other day from day 1. During the experimental period, the body weight and tumor size were monitored every other day. The tumor volume was calculated according to the following formula:

$$\text{Tumor volume (V)} = L \times W^2/2$$

L and W were the measured length and width of tumors, respectively. At the end of treatment, the mice were euthanized by injecting excessive anesthetic (4% chloral hydrate), the tumor tissues were isolated for weighting and photographing. The main organs (heart, liver, spleen, lung, and kidney) were isolated for hematoxylin-eosin (H&E) staining.

Statistical Analysis: Data are presented as mean ± SEM. Data analysis was carried out using GraphPad Prism 6.0 software (La Jolla, CA, USA). Differences between two groups were examined using unpaired Student's t-test, $p < 0.05$ was considered statistically significant.

References

1. Yue, J.; Lv, D.; Wang, C.; Li, L.; Zhao, Q.; Chen, H.; Xu, L. *Oncogene* 2018, *37*, 4300−4312.
2. Ramadan, S.; Guo, L.; Li, Y.; Yan, B.; Lu, W. *Small* 2012, *8*, 3143−3150.

Supporting Figures

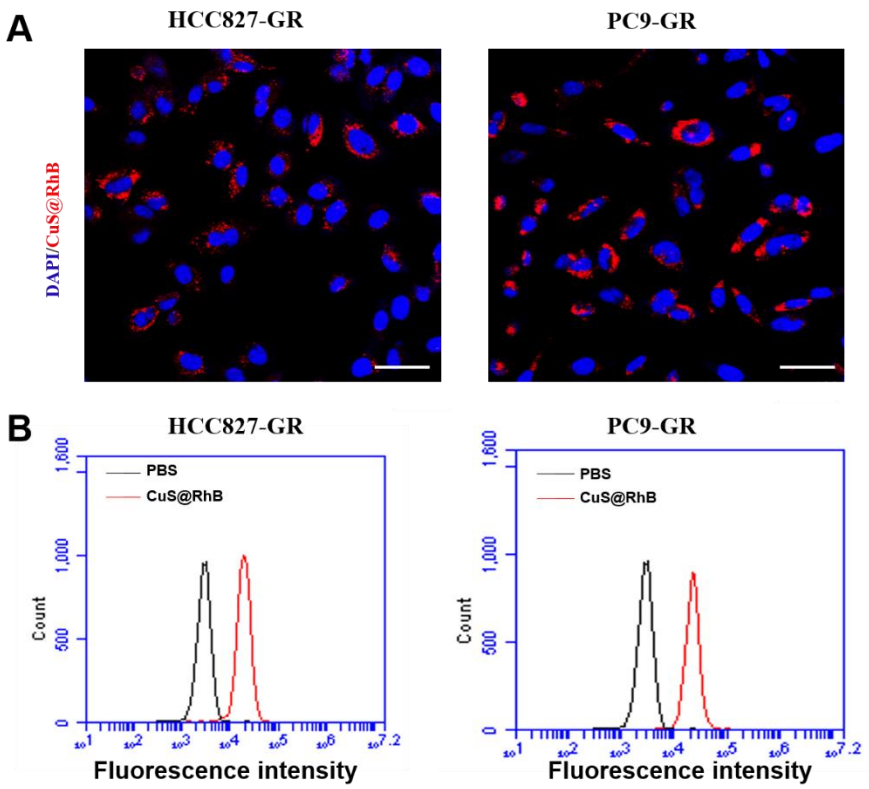

Figure S1. Cellular uptake of CuS NPs in HCC827-GR cells and PC9-GR cells measured by (A) Confocal imaging and (B) Flow cytometry. Blue: nucleus stained by DAPI; Red, RhB-labeled CuS NPs (scale bar: 50 μm).

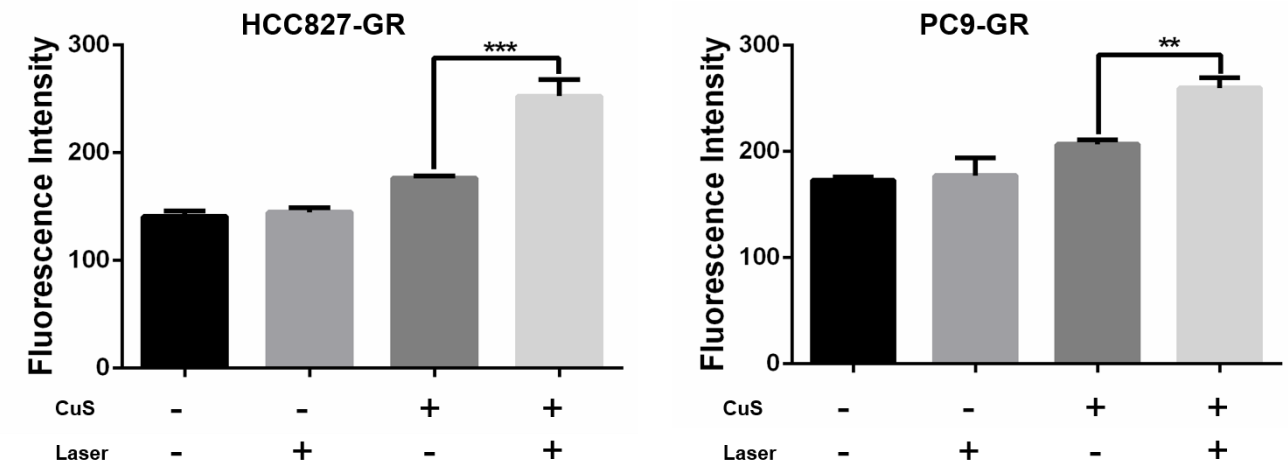

Figure S2. Intracellular ROS levels of HCC827-GR and PC9-GR cells with different treatments. Data are presented as mean ± SEM (n=3). ** $p<0.01$, *** $p<0.001$.

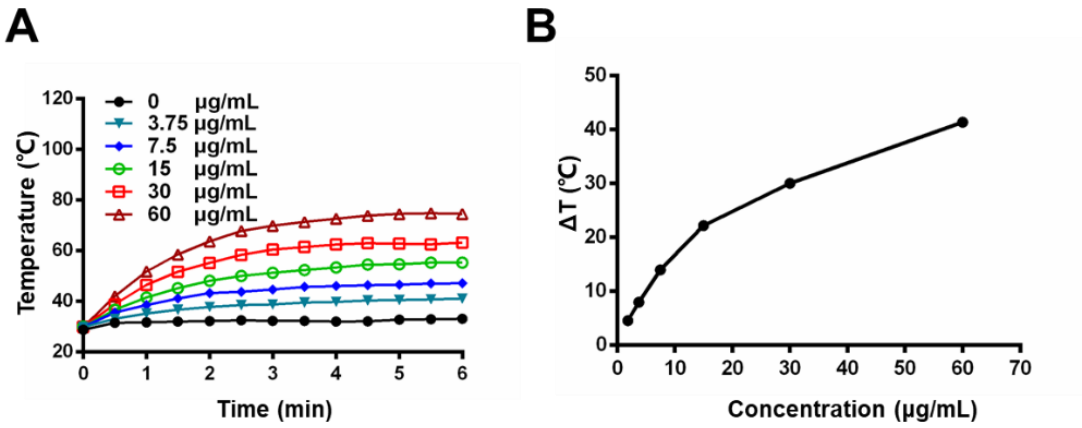

Figure S3. (A) Photothermal effect of CuS NPs at different concentration (0-60 μg/mL, 100 μL) under laser irradiation (808 nm, 2 W/cm^2). (B) Temperature rise (ΔT) of different concentration of CuS NPs after treated with 808 nm laser irradiation (2 W/cm^2) for 6 min.

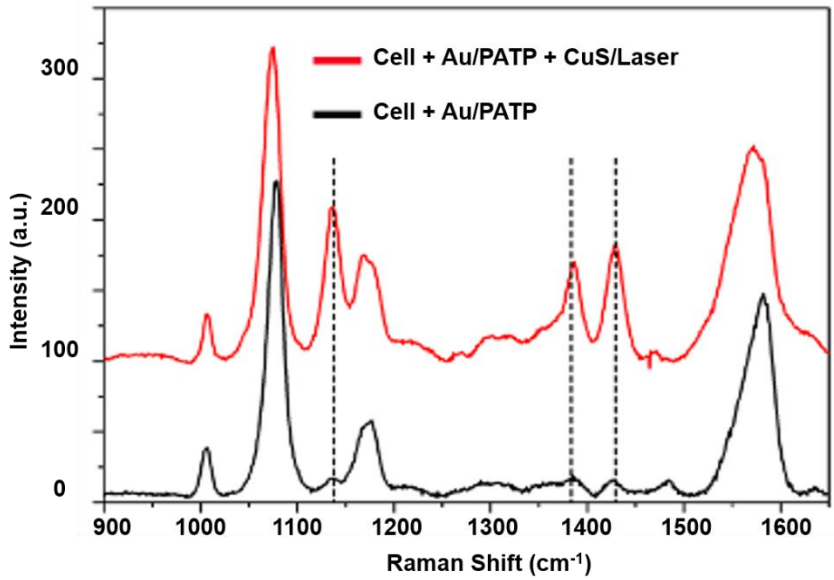

Figure S4. Representative Raman spectra of Au/PATP nanoprobe indicated intracellular ROS levels under different treatments.

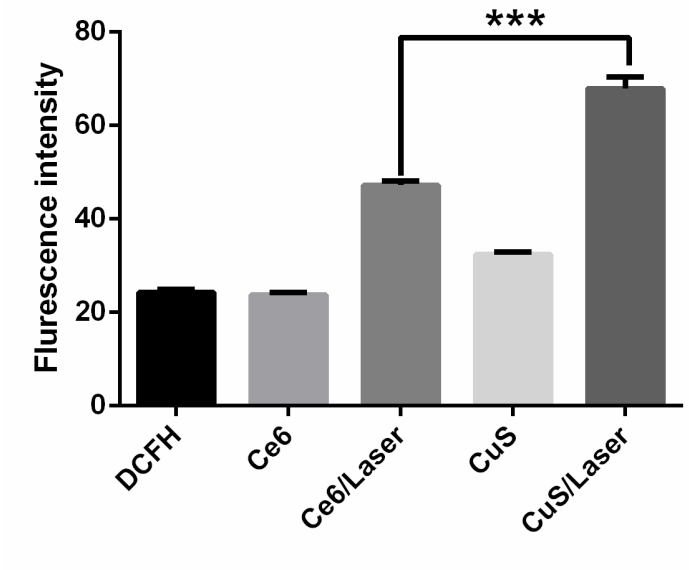

Figure S5. The ROS level in the laser-irradiated CuS NPs solution was significantly higher than that in the laser-irradiated Ce6 solution. Data are presented as mean ± SEM (n=3). *** $p<0.001$.

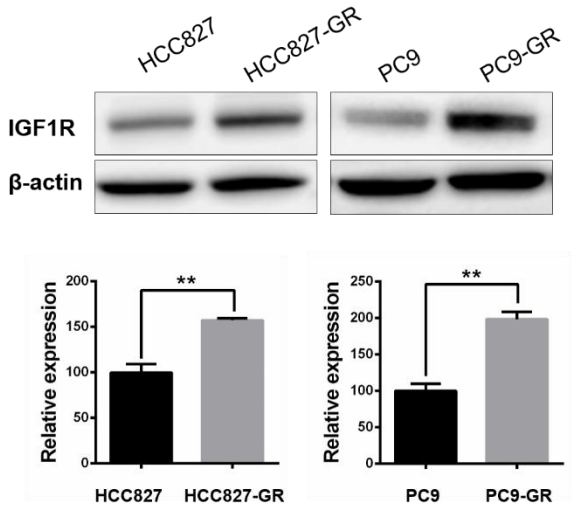

Figure S6. Western blotting showed the upregulation of IGF1R level in the two gefitinib-resistant NSCLC cell lines compared with their non-resistant counterpart cell lines. Quantitative data are relative to β-actin, normalized to non-resistant counterpart cells, presented as mean ± SEM (n=3), ** $p<0.01$.

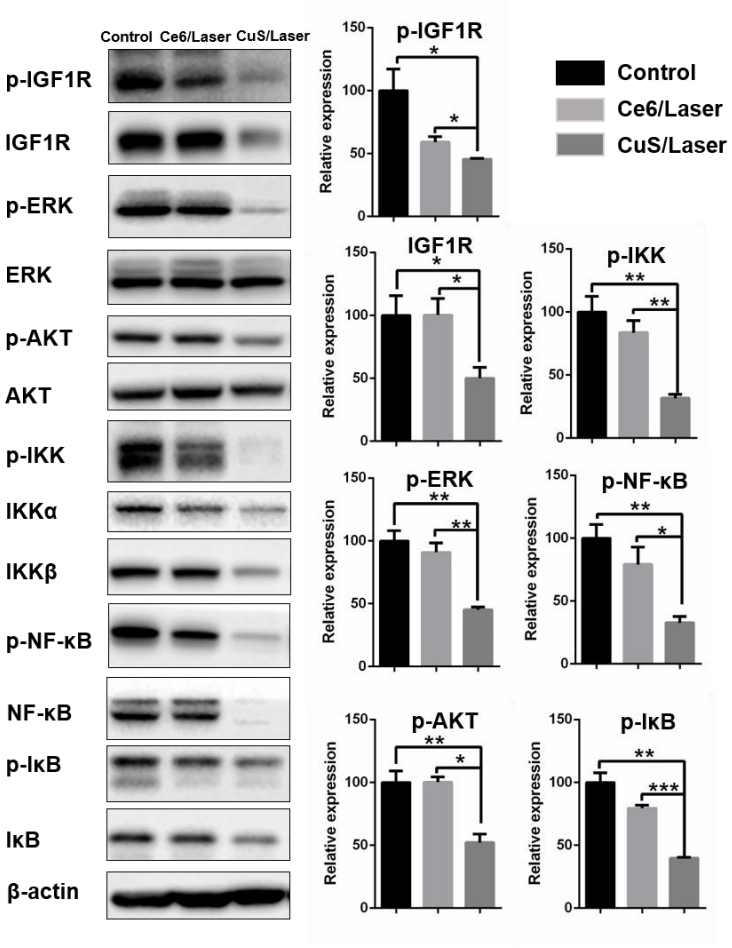

Figure S7. Western blotting showed the decreased p-IGF1R, IGF1R and the decreased phosphorylation levels of AKT, ERK, IKK, NF-κB and IκB at 24 h-post treatment with CuS/Laser in HCC827-GR-bearing tumors, while the expression levels of related proteins had no significant change in HCC827-GR-bearing tumors at 24 h-post treatment with Ce6/Laser. Control stands for untreated tumors. Quantitative data are relative to β-actin, normalized to control, presented as mean ± SEM (n=3), * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

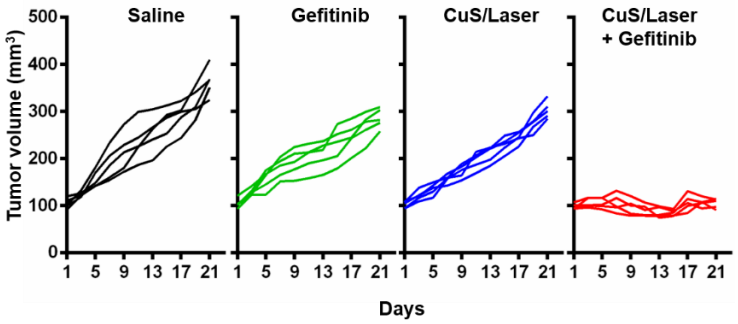

Figure S8. Tumor growth of every mice with various treatments in HCC827-GR-bearing tumor mice.

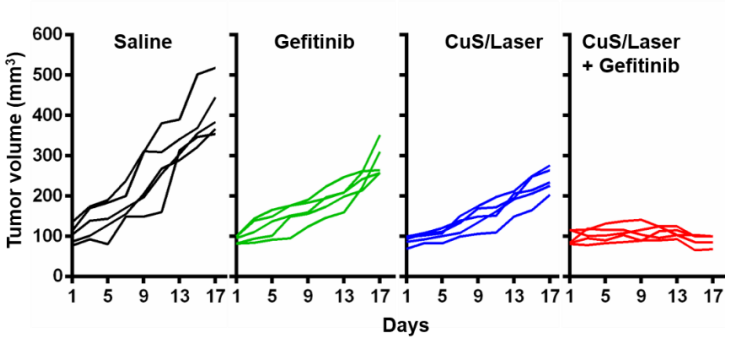

Figure S9. Tumor growth of every mice with various treatments in PC9-GR-bearing tumor mice.

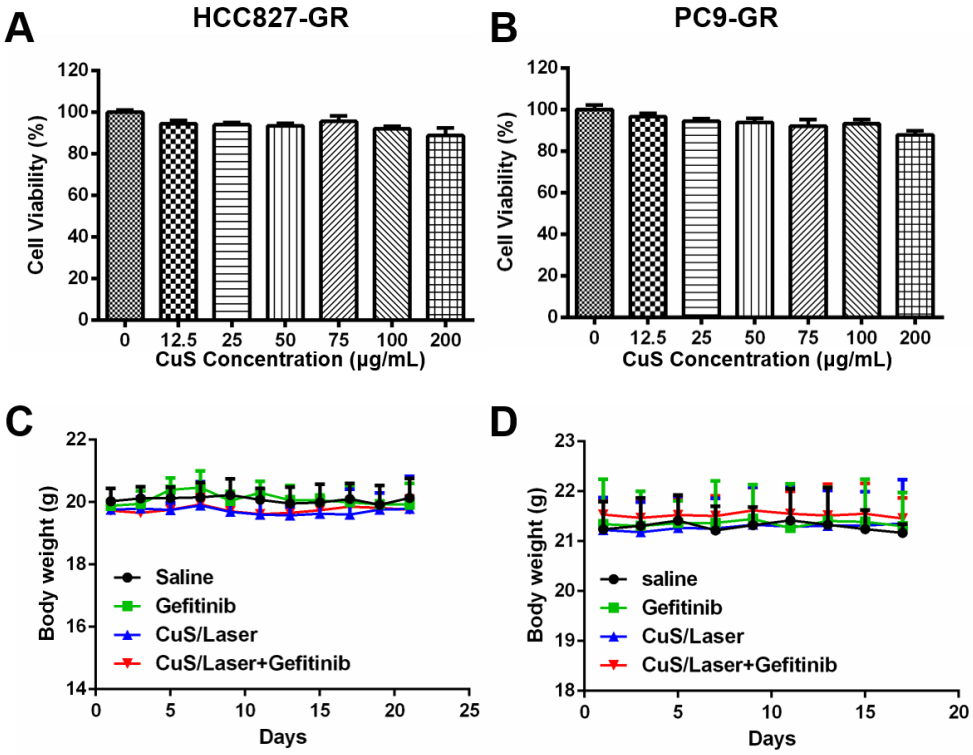

Figure S10. *In vitro* and *In vivo* safety of CuS NPs. The cytotoxicity of CuS NPs in (A) HCC827-GR and (B) PC9-GR cells. Body weight of (C) HCC827-GR-bearing and (D) PC9-GR-bearing tumor mice. Data are presented as mean ± SEM (n=5).

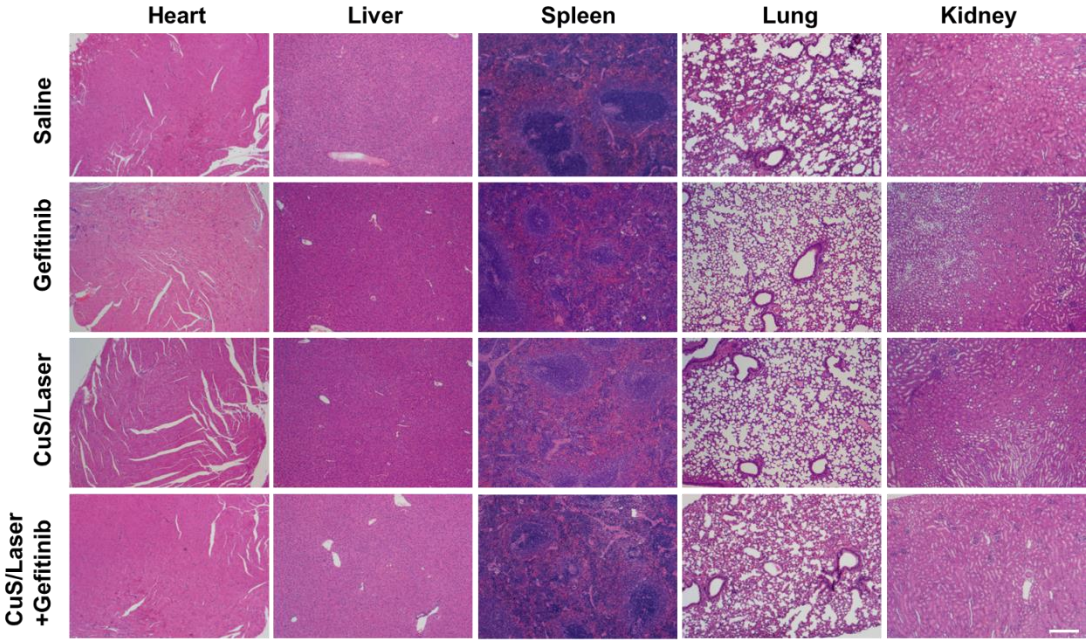

Figure S11. H&E staining of main organs collected from HCC827-GR-bearing tumor mice after different treatments (scale bar: 200 μm).

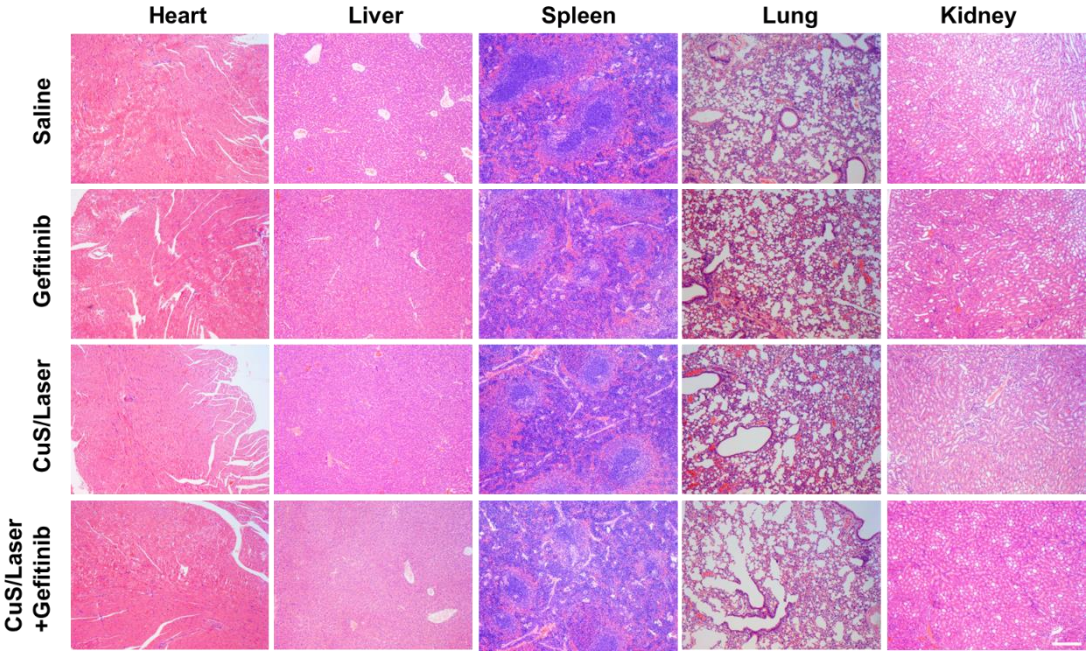

Figure S12. H&E staining of main organs collected from PC9-GR-bearing tumor mice after different treatments (scale bar: 200 μm).