Supplementary Material

Enhancement of mitochondrial ROS accumulation and radiotherapeutic efficacy using a Gd-doped titania nanosensitizer

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Materials and Reagents. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company; (4-carboxybutyl)triphenylphosphonium bromide (TPP), (3-aminopropyl)triethoxysilane (APTES), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimidehydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Alfa Aesar Chemical Ltd (Tianjin, China); tetrabutyl titanate (TBOT) was purchased from China National Pharmaceutical Group Corporation (Shanghai, China); Cyclosporine A (CsA) were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China); Caspase-3, Caspase-8 and caspase-9 were purchased from Boster Company; Mito-Tracker Green was purchased from Molecular Probes (Invitrogen, USA). The cell culture consumables were purchased from Shengyou Biotech Co., Ltd. (Hangzhou, China). All the chemicals were used without further purification. The human breast cancer cell line (MCF-7) was purchased from KeyGEN Biotechnology Company (Nanjing, China).

Characterization. The transmission electron microscopy (TEM) was carried out on a JEM-2100 electron microscope. Fluorescence spectra were obtained with FLS-980 Edinburgh. Fluorescence Spectrometer with a Xenon lamp and 1.0 cm quartz cell at the slits of 1.0/1.0 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi, China) with a combined glass-calomel electrode. Confocal fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscopy (Leica Germany) with an objective lens (×20). Energy dispersive spectra (EDX) were conducted out by using a Hitachi SU8010 SEM. X-ray photoelectron spectroscopy (XPS) were

carried out by using an Escalab250Xi instrument. Radiotherapy was carried out on a medical linear accelerator (Siemens Primus HI, Germany) at a power of 6 MV with a dose rate of 300 cGy/min and the source skin distance = 100 cm.

Cell Incubation and Mice Culture. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Israel) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 1.0% penicillin, and 1.0% streptomycin. The cells were seeded in confocal culture dishes, and then incubated for 24 h at 37 °C under a humidified atmosphere containing 5.0% CO_2.

All procedures of animal study were approved by Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. Female nude mice (approximately 4-6 weeks old, ~18 g) were housed under normal conditions with 12 h light and dark cycles and given access to food and water *ad libitum*.

Statistical analysis. All *in vitro* experiments were repeated at least three times (n=3 biologically independent experiments). Data is expressed as mean ± s. e. unless otherwise noted. Comparison between two groups were performed using an unpaired two-tailed *t*-test.

Experimental Procedures

Synthesis of TiO_2(Gd)-TPP and TiO_2(Gd)-TPP-IR806. First, amino-functionalized TiO_2(Gd) NPs (TiO_2(Gd)-NH_2) were prepared using (3-aminopropyl)triethoxysilane (APTES) as the amination reagent. Ethanol (40 mL) and

water (400 μL) were mixed, and TiO_2(Gd) NPs (8 mg) was dispersed into the solution. Then, 40 μL APTES (160 μmol) was added to the above solution, which was stirred for 15 min and then stored for 12 h. After centrifugation and washing with water three times, TiO_2(Gd)-NH_2 NPs were obtained and redispersed in MES buffer (20 mM, pH = 6.0). Subsequently, TiO_2(Gd)-TPP-IR806 was prepared through the EDC/NHS coupling method. Carboxylate groups of IR806 (8 μmol) and TPP (16, 24, 32, 40 μmol) were first activated by EDC (160, 240, 320, 400 μmol) and NHS (160, 240, 320, 400 μmol) at room temperature for 1 h. Then, the TiO_2(Gd)-NH_2 solution was added into the mixture and stirred for 24 h. Subsequently, the precipitates were collected *via* centrifugation and washed with methanol and PBS (10 mM, pH = 7.4) three times, and the final products were resuspended in PBS buffer. The content of TPP on the surface of TiO_2(Gd) NPs was quantified through UV-Vis absorption spectra according to the standard linear calibration curve.

Preparation of TiO_2(Gd)-TPP-HE. According to the method mentioned above, TiO_2(Gd)-NH_2 and TiO_2(Gd)-TPP were obtained. Carboxyl groups were then further modified on the surface of the TiO_2(Gd)-TPP NPs. Succinic anhydride (4 mg) and triethylamine (4 mg) were mixed in 16 mL DMSO, and then the TiO_2(Gd)-TPP NPs were added. Following stirring at 40 °C for 48 h, the precipitates were collected with centrifuging and washing with water three times and finally dispersed in MES buffer (20 mM, pH = 6.0). The HE molecule was modified on the surface of the TiO_2(Gd)-TPP NPs through amide bonds. Following activation of the carboxylate groups of the TiO_2(Gd)-TPP NPs by EDC (400 μmol) and NHS (400 μmol) for 1 h, HE (40 μmol) in

methanol was added and further stirred for another 24 h in the dark. Following centrifugation and washing with water and PBS buffer three times, TiO_2(Gd)-TPP-HE was successful synthesized and finally dispersed in PBS buffer.

Detection of mitochondrial membrane potential ($\Delta\Psi_m$). MCF-7 cells were first cultured in a confocal dish for 24 h at 37 °C in 5% CO_2 in DMEM. Then, PBS, TiO_2(Gd)-NH_2 (0.1 mg/mL) and TiO_2(Gd)-TPP (0.1 mg/mL) dispersed in DMEM were added to the confocal dishes. After incubation for 8 h, the cells were washed with PBS buffer twice to remove the residual NPs, and 2 mL of fresh DMEM was added. Following 4 Gy of X-ray irradiation, the cells were incubated for another 4 h. Next, Rhodamine 123 (5 μg/mL) was used to stain the cells for 15 min. Confocal images were acquired by CLSM with 488 nm excitation and collected with a bandpass filter with a range of 500-550 nm.

Cellular uptake and internalization pathways. MCF-7 cells were cultured at 37 °C in 5% CO_2 for 24 h and then incubated with different inhibitors including chlorpromazine (inhibitor of clathrin-mediated uptake, 10 μM) and ethylisopropylamiloride (EIPA, inhibitor of macropinocytosis, 50 μM) for 90 min prior to incubation with TiO_2(Gd)-TPP-IR806 NPs (0.1 mg/mL) for further 8 h. Subsequently, the medium was removed and the cells were washed three times with PBS. Confocal images were obtained by excitation of the samples at 633 nm.

Clonogenic survival assay. Cells were cultured in DMEM for 24 h in 5% CO_2 at 37 °C and were further subjected to the different treatment conditions (i.e., X-ray, TiO_2(Gd)+X-ray and TiO_2(Gd)-TPP+X-ray). Two hours after ionizing radiation, the

cells were washed, trypsinized and adjusted to specific densities. The cells were then seeded in a 6-well plate at different predetermined densities. After 10 days, colonies in each well were dyed by 0.5% crystal violet and counted when the colonies comprised more than 50 cells. Survival curve was plotted with Origin. The radio-enhancing effect was assessed by calculating Sensitizer Enhancement Ratio (Sensitization Enhanced Ratio; SER). A 10% death fraction was set to obtain the required dose of different agents. Thus, SER means the ratio when 10% of the total cells were alive.

Caspase 3 activation. MCF-7 cells were firstly cultured in confocal dishes for 24 h at 37 °C in 5% CO_2 in DMEM. Cells were further treated with different conditions (control, TiO_2(Gd)-TPP, X-ray, TiO_2(Gd)+X-ray, TiO_2(Gd)-TPP+X-ray), where the concentration of TiO_2(Gd)-TPP or TiO_2(Gd) were 0.1 mg/mL and the dose of X-ray was 4 Gy. After further incubated for 8 h, the cells were washed with PBS buffer twice to remove the residual NPs. The cells were incubated with primary antibody anti-caspase 3 after fixed with paraformaldehyde (4%) for 10 min. And then, enhanced secondary antibody were used to treat cells for 100 min at room temperature. Confocal images were acquired by CLSM with 633 nm excitation and collected with the range from 640 nm to 700 nm. Moreover, Cytochrome C, caspase 8 and caspase 9 were also verified using an immunofluorescence staining method. Cells were incubated with TiO_2(Gd)-TPP (0.1 mg/mL) for 8 h and then given 4 Gy X-ray radiation. After further incubation for 12 h, cells in different group were performed as the same procedure above.

Cell migration and invasion assay. (1) The cells were seeded into 60 mm dishes and incubated at 37 °C under 5% CO_2 in DMEM for 24 h. Cells were further treated with different conditions (control, TiO_2(Gd)-TPP, X-ray, TiO_2(Gd)+X-ray, TiO_2(Gd)-TPP+X-ray), where the concentration of TiO_2(Gd)-TPP or TiO_2(Gd) were 0.1 mg/mL and the dose of X-ray was 4 Gy. The monolayer was wounded using 10 μL sterile pipette tip and cell images were taken at time 0, 12, 24 and 36 h post-wounding. The area of wound healing was calculated using AJ-VERT software and each experiment was performed in triplicate. (2) Cell invasion assays were conducted using Matrigel coated invasion chambers with an 8-μm pore size in 24-well plates (BD Biosciences). MCF-7 cells were treated with the method mentioned above, 2×10^4 cells were added into the upper compartment and further incubated for 24 h. After removing the noninvasive cells on the upper surface of the membrane, the invasive cells were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet before counting the number of invaded cells under microscope.

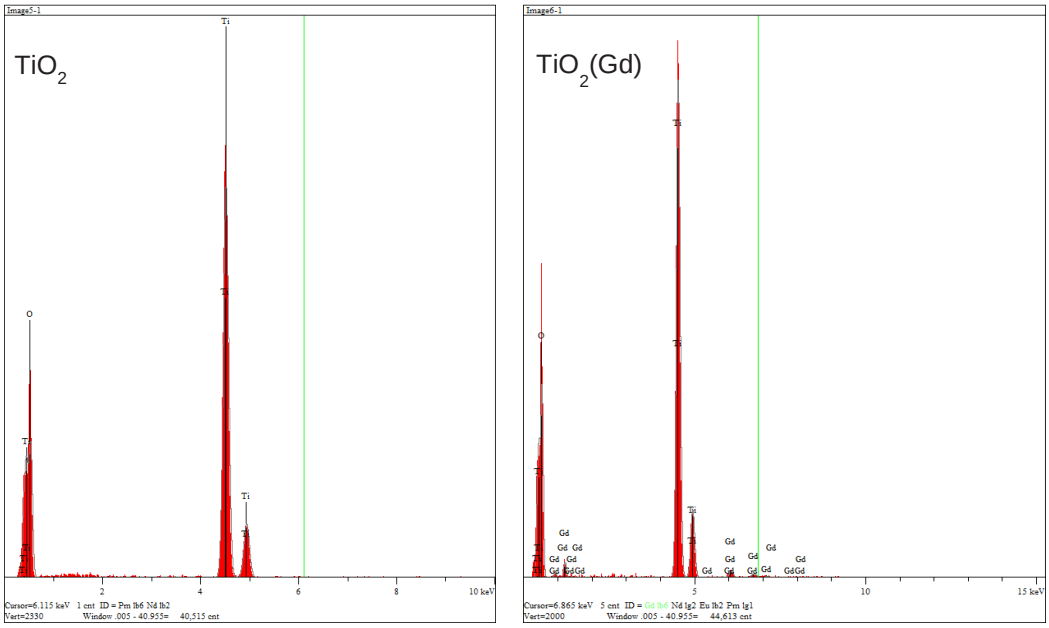

Figure S1. EDX analysis of TiO_2 and TiO_2(Gd) NPs.

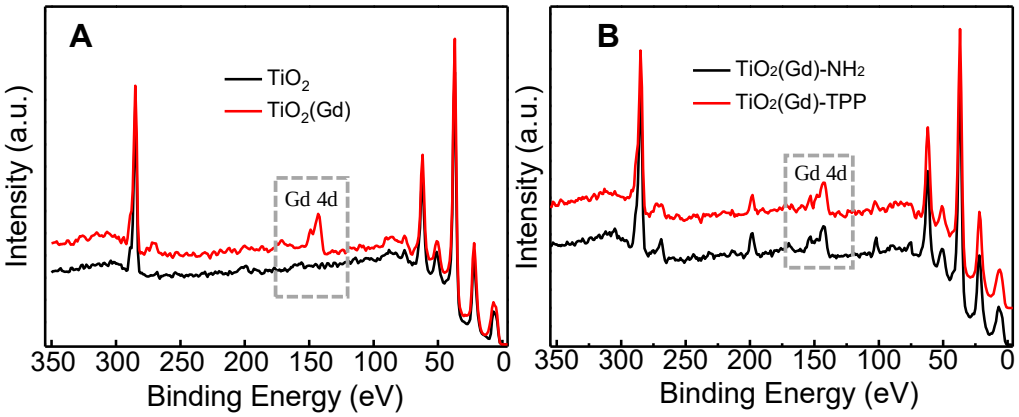

Figure S2. XPS spectra of the of (A) TiO_2 and TiO_2(Gd) NPs; (B) TiO_2(Gd)-NH_2 NPs and TiO_2(Gd)-TPP NPs.

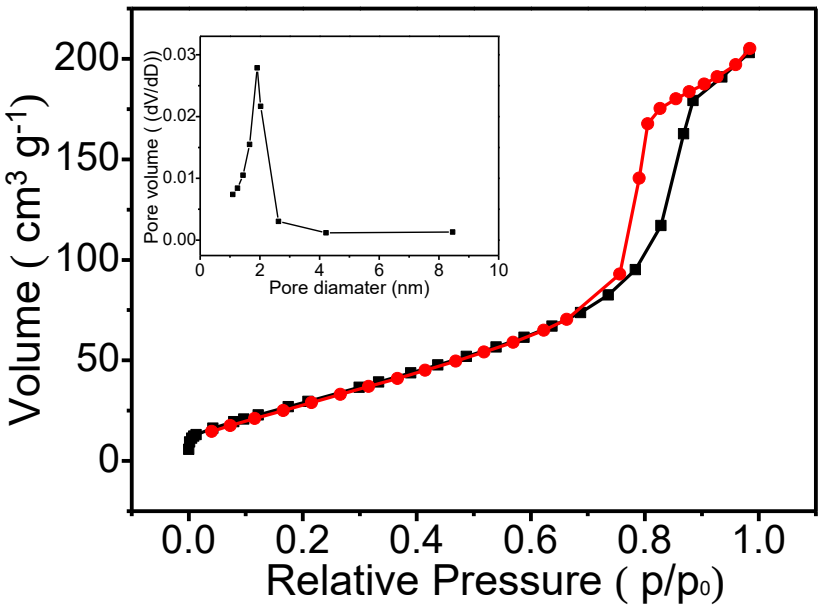

Figure S3. The N_2 adsorption-desorption isotherms. Inset: The pore size distribution of the TiO_2(Gd) NPs.

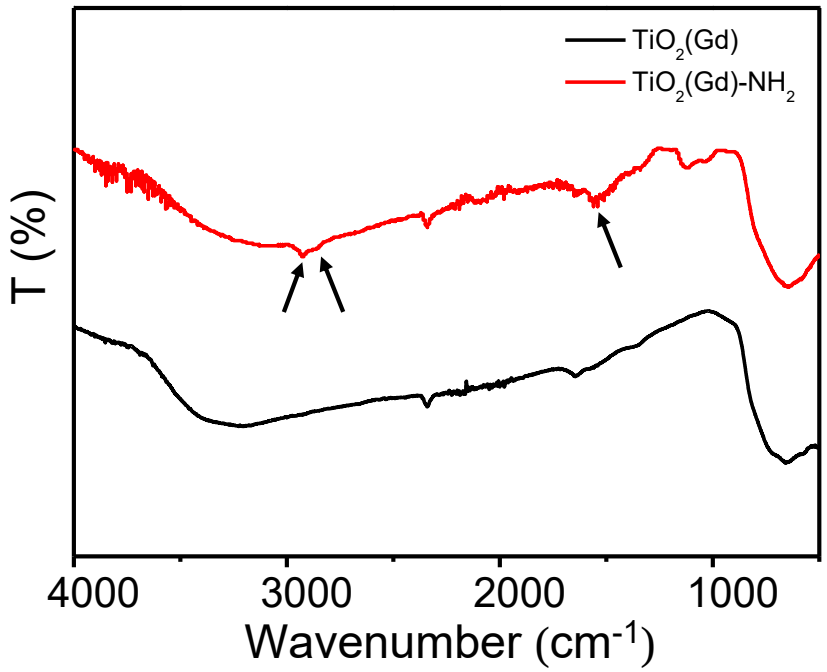

Figure S4. FTIR spectra of the TiO_2(Gd) NPs before and after surface modification with APTES. The new peaks at 2920 and 2852 cm^{-1} correspond to -CH_2- group of aminoethyl with APTES molecules and the peak at 1560 cm^{-1} is a characteristic absorption peak of R-NH_2.

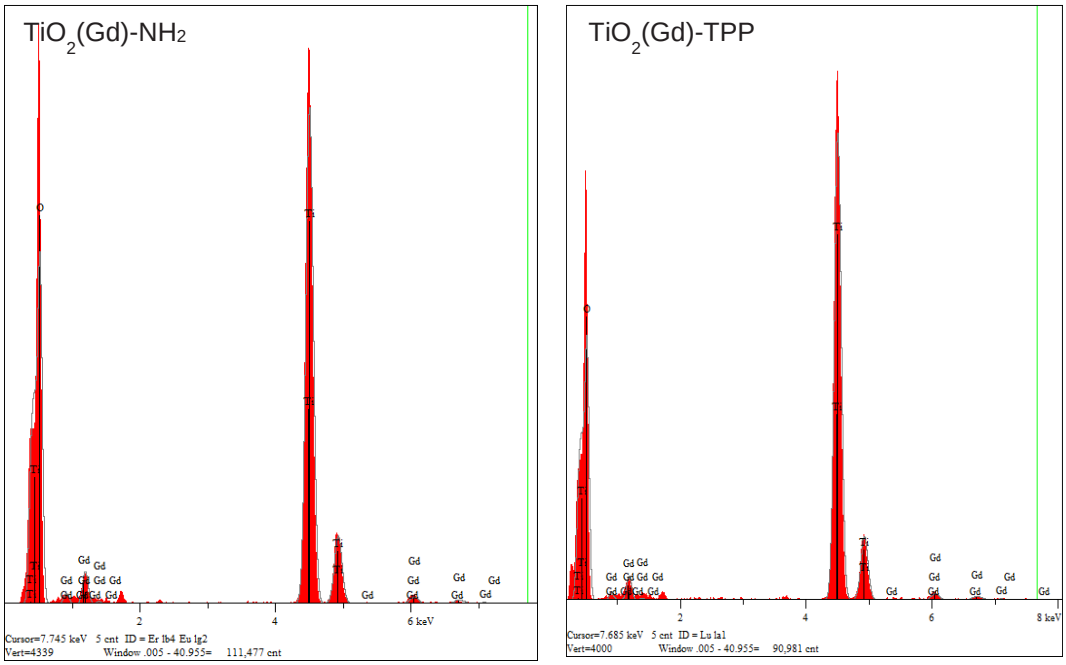

Figure S5. EDX analysis of TiO_2(Gd)-NH_2 and TiO_2(Gd)-TPP NPs.

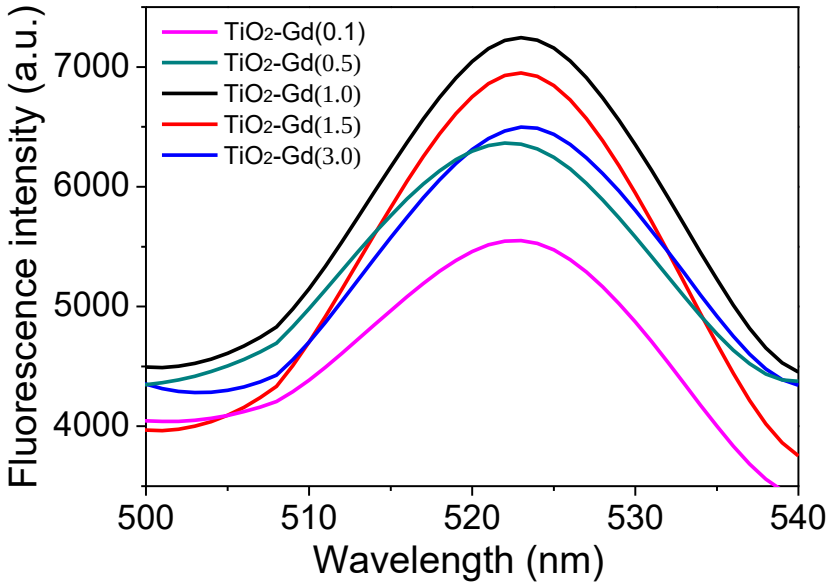

Figure S6. The generation of $O_2^{\bullet-}$ measured by the fluorescence intensity of DBZTC (λ_{ex} = 490 nm, λ_{em} = 500-540 nm) after X-ray irradiation.

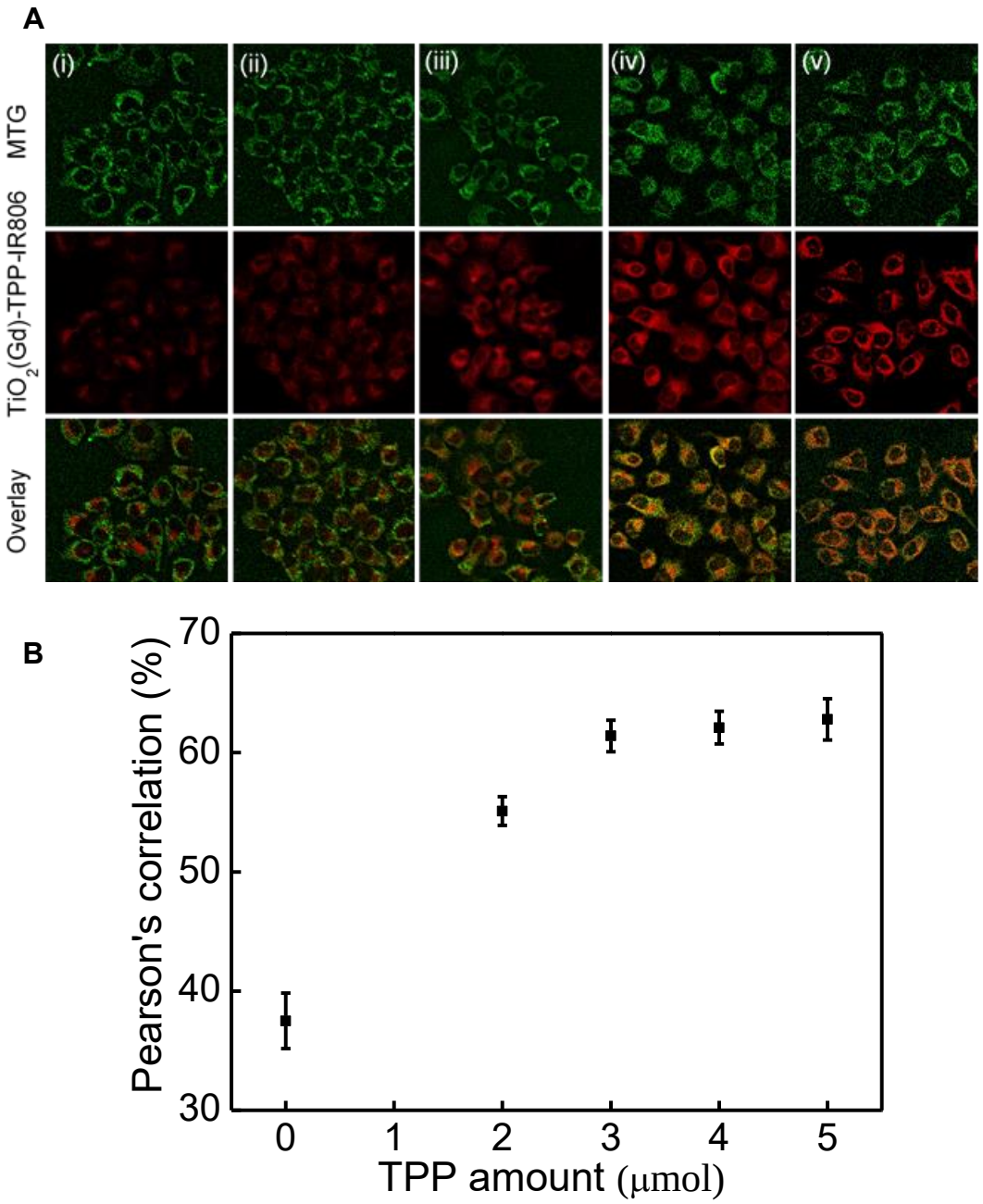

Figure S7. (A) Confocal images of TiO_2(Gd)-TPP-IR806 with different the amount of TPP (i-v: 0, 2, 3, 4, 5 μmol). IR806 (excitation = 633 nm, emission = 750-800 nm), Mito-Tracker Green (MTG) stained mitochondria (excitation = 488 nm, emission = 500-550 nm); (B) Pearson's correlation of TiO_2(Gd)-TPP-IR806 with different the amount of TPP was added.

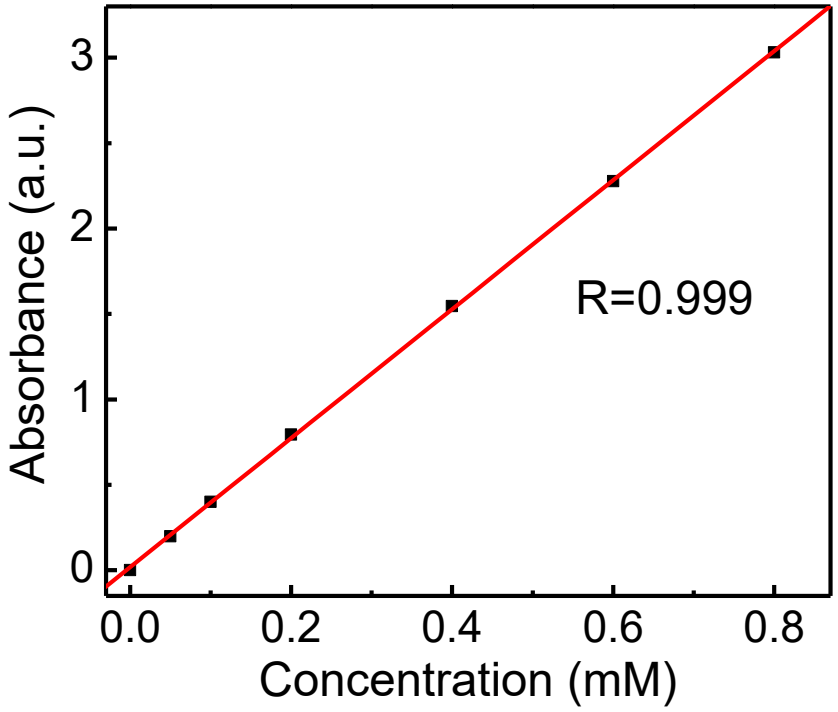

Figure S8. Standard linear calibration curve of TPP.

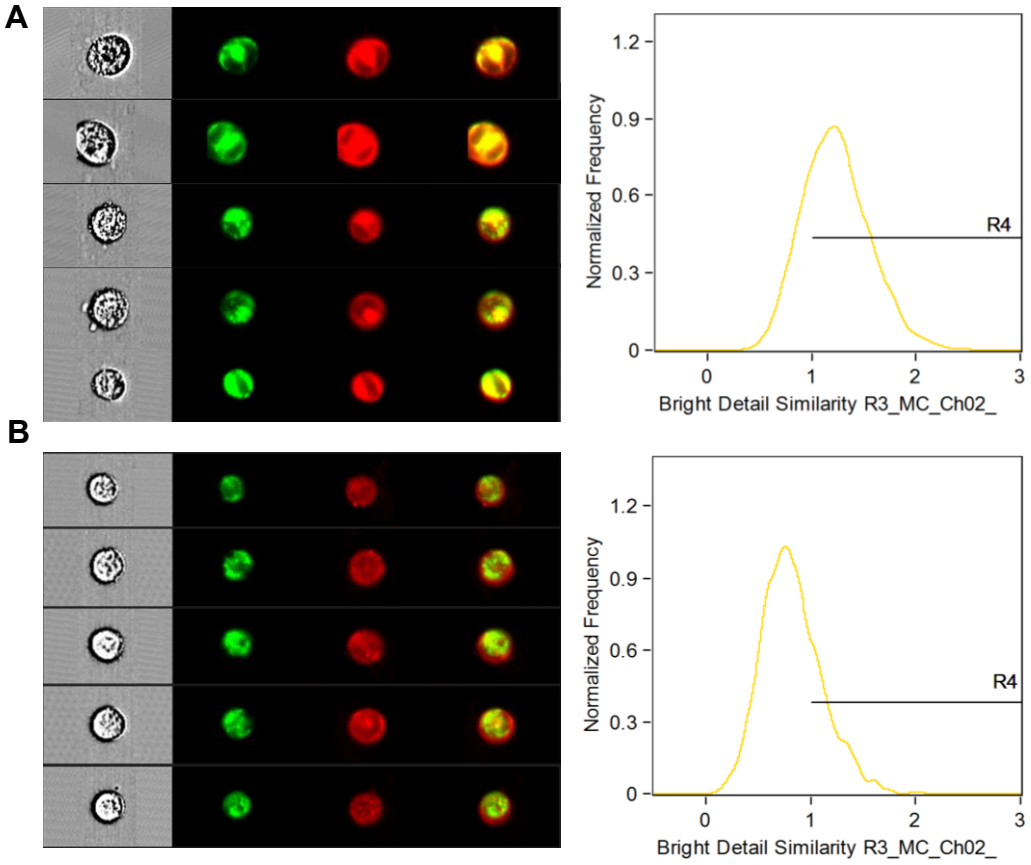

Figure S9. Co-localization images of the mitochondria targeted NPs using imaging flow cytometry. (A) TiO_2(Gd)-TPP-IR806 NPs and (B) TiO_2(Gd)-IR806 NPs. Right: Co-localization parameters of cells under different treatments. Flow cytometry images of nanosensitizer (red channel, excitation = 633 nm, emission = 750-800 nm), Mito-Tracker Green (MTG) stained mitochondria (green channel, excitation = 488 nm, emission = 500-550 nm), the overlay channel of nanosensitizer and mitochondria in last column.

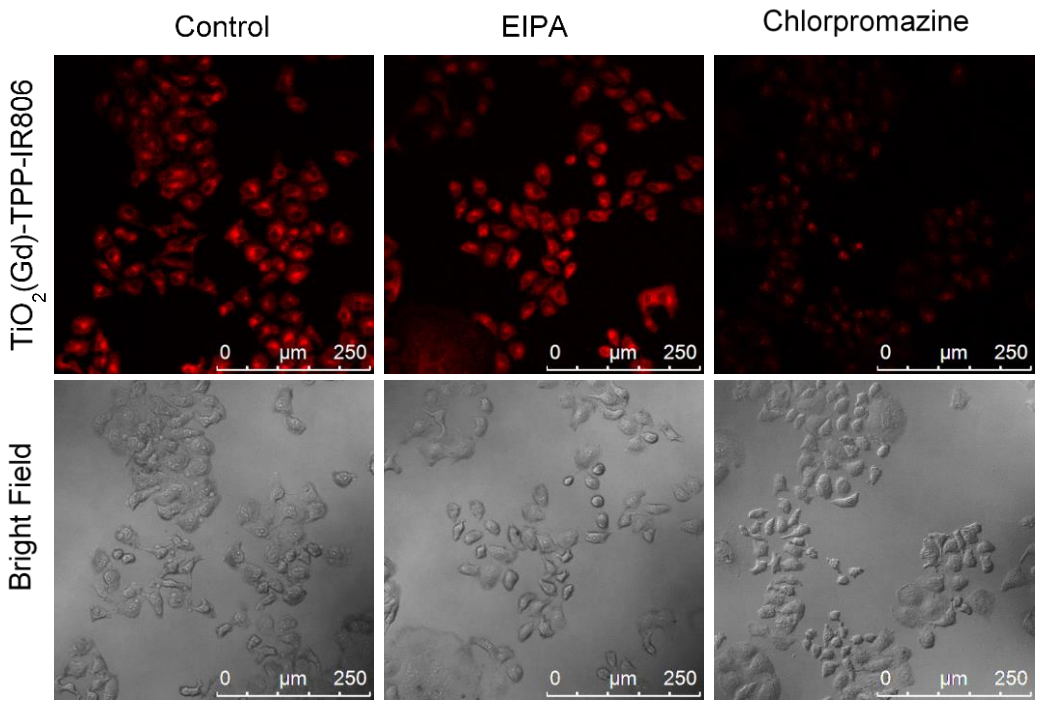

Figure S10. Confocal imaging of MCF-7 cells treated without (control) or with chlorpromazine (an inhibitor of clathrin-mediated uptake, 10 μM), ethylisopropylamiloride (EIPA, an inhibitor of macropinocytosis, 50 μM) before incubated with TiO_2(Gd)-TPP-IR806 (0.1 mg/mL).

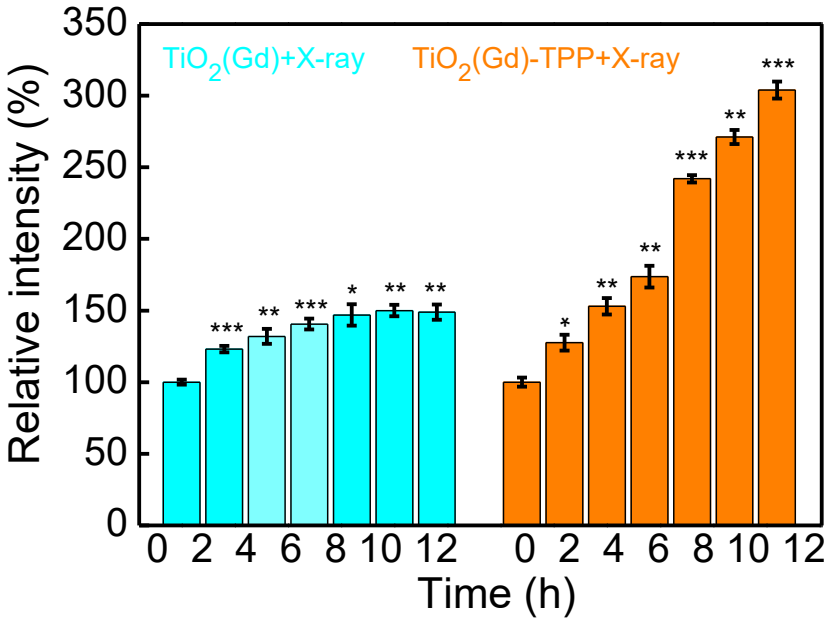

Figure S11. Relative fluorescence intensity of the cells for real-time detecting of intracellular $O_2^{\bullet -}$ burst. ***P < 0.001, **P < 0.01, *P < 0.05 versus 0 h in each treatment group.

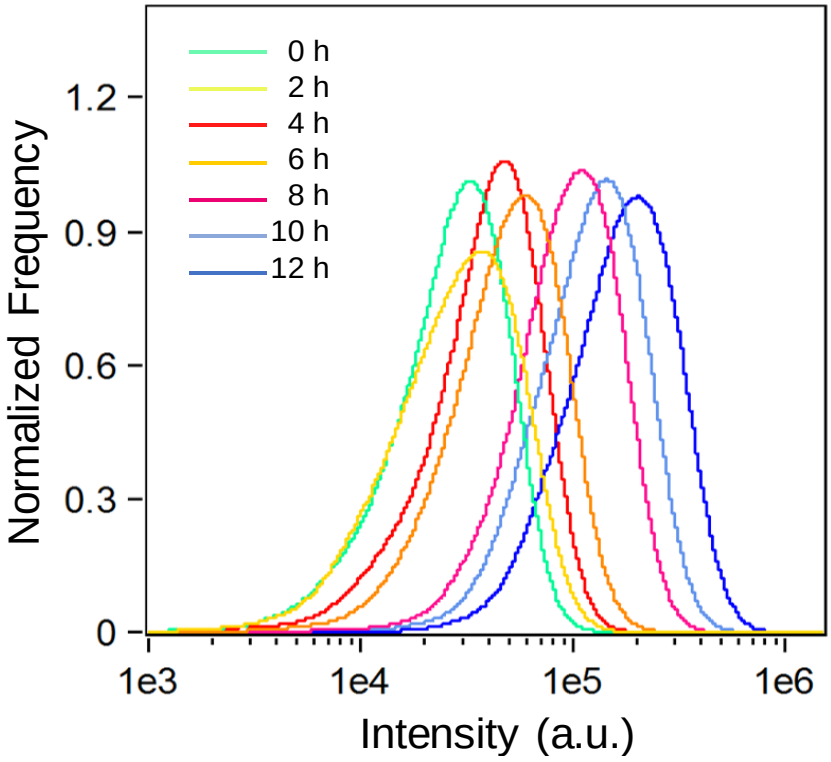

Figure S12. The *in vitro* intracellular $O_2^{\bullet-}$ generation was evaluated through flow cytometry analysis. The cells were incubated with TiO_2(Gd)-TPP NPs for 8 h followed by X-ray irradiation. The $O_2^{\bullet-}$ was detected by flow cytometry analysis for 12 h at 2 h intervals.

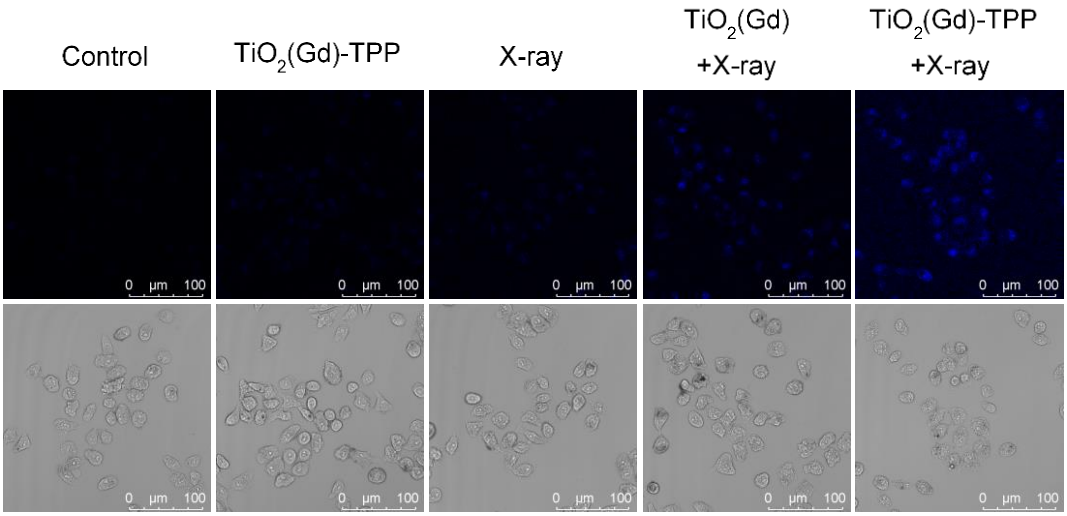

Figure S13. Intracellular hydroxyl radical detection by coumarin after X-ray irradiation 12 h.

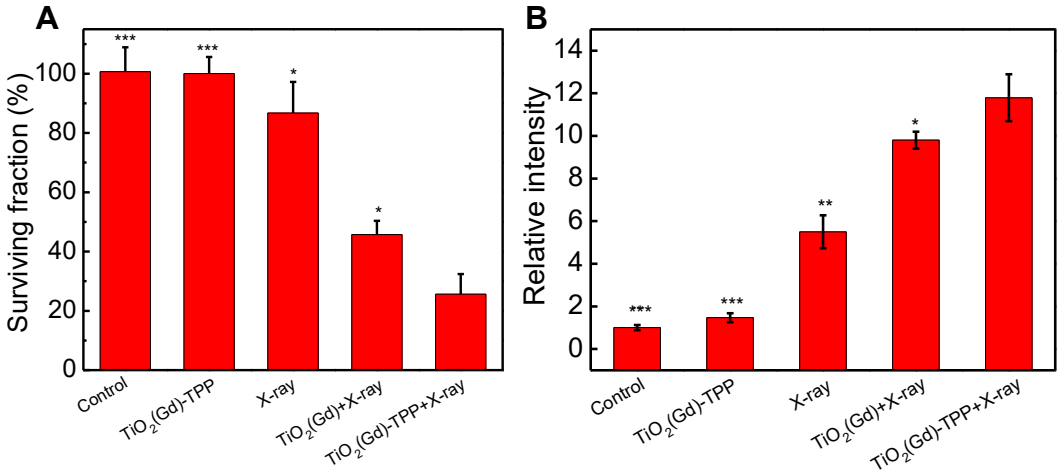

Figure S14. Cell proliferation by clonogenic survival assay and CFSE staining. (A) Survival fraction of MCF-7 cancer cells received various treatments. (B) The quantification of corresponding fluorescent intensity of cells. ***P < 0.001, **P < 0.01, *P < 0.05 compared with TiO_2(Gd)-TPP+X-ray group.

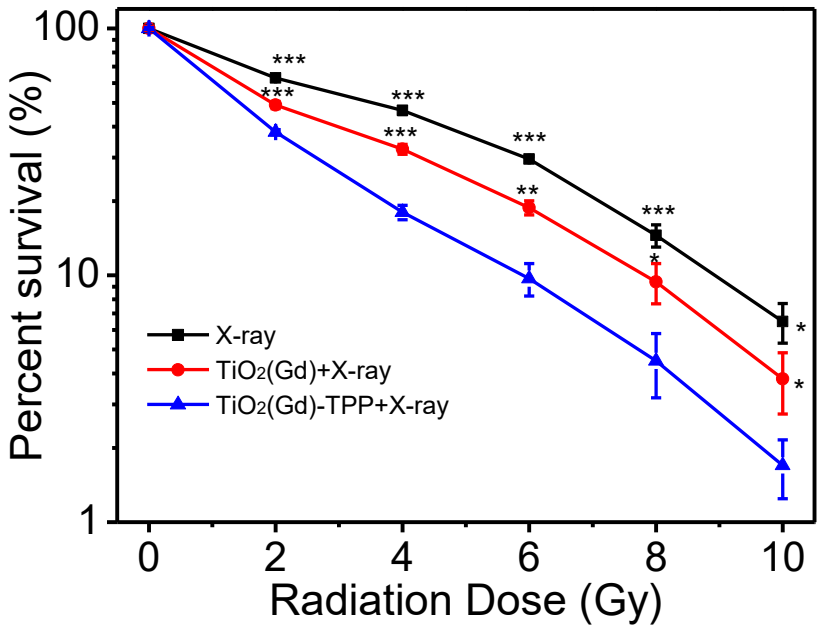

Figure S15. Survival curves of MCF-7 cancer cells treated with different condition. ***P < 0.001, **P < 0.01, *P < 0.05 compared with TiO_2(Gd)-TPP+X-ray group at each radiation dose.

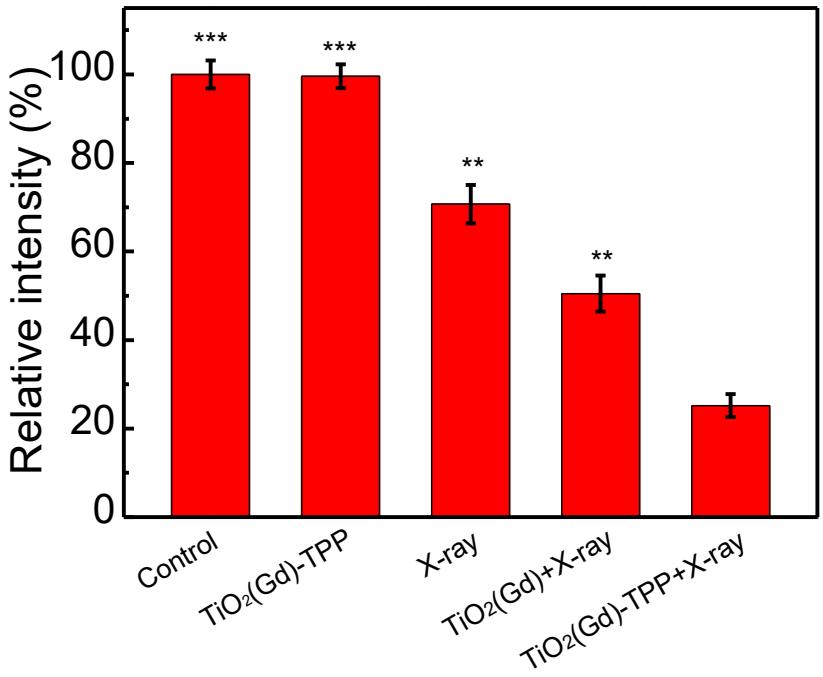

Figure S16. Relative fluorescence intensity of Rhodamine 123-stained cells with different groups for detecting mitochondrial membrane potential ($\Delta\psi_m$). ***P < 0.001, **P < 0.01, *P < 0.05 compared with TiO_2(Gd)-TPP+X-ray group.

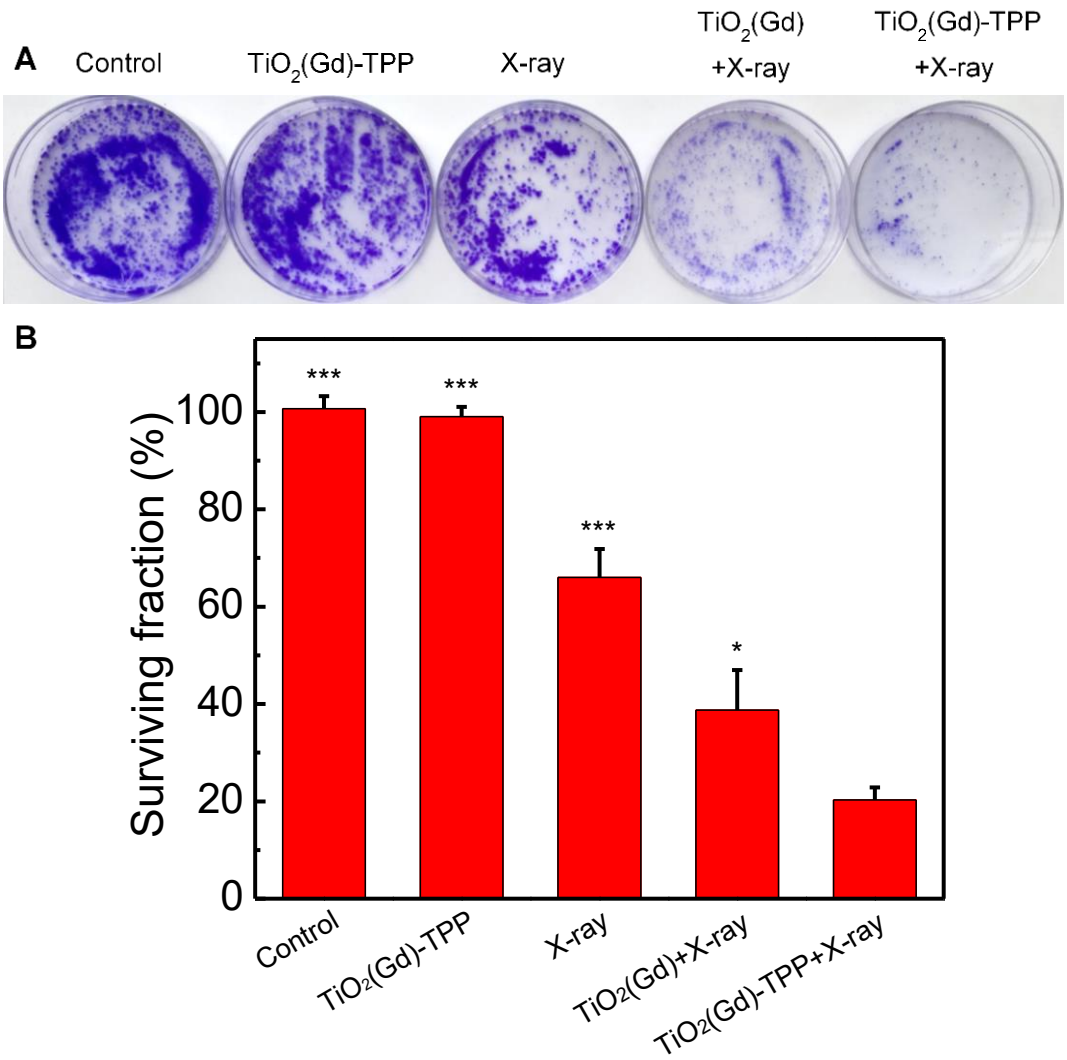

Figure S17. (A) Five groups of MCF-7 cells were treated with different conditions in the presence of CsA (1 μM). Representative photographs of colony formation. Cells were still incubated for 10 days and then fixed and stained; (B) Survival fraction of MCF-7 cancer cells received various treatments. ***P < 0.001, **P < 0.01, *P < 0.05 compared with TiO_2(Gd)-TPP+X-ray group.

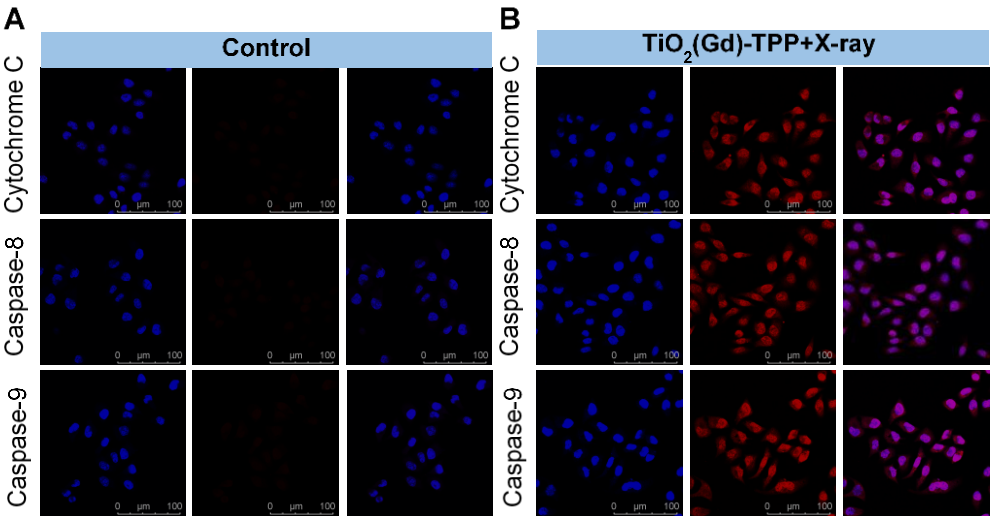

Figure S18. Immunofluorescent staining images of cytochrome C, caspase 8 and caspase 9. (A) MCF-7 cells without any treatment. (B) MCF-7 cells incubated with the nanosensitizer and irradiated with X-ray for 4 Gy. Confocal images were acquired at 12 h after irradiation. Cytochrome C, active caspase 8 and active caspase 9 are immunostained with Alexa-647-labelled secondary antibodies (red) and nuclei are stained with Hoechst 33342 (blue).

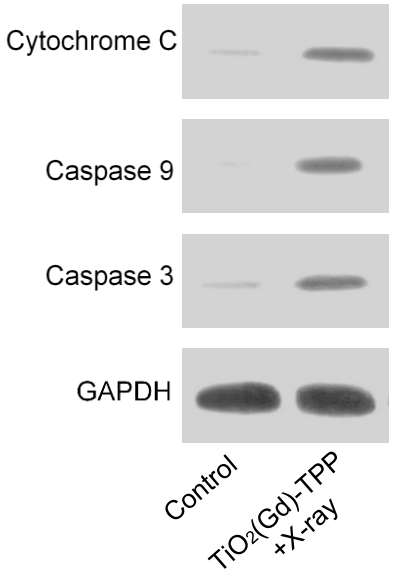

Figure S19. Western blotting analysis of cytochrome C, caspase 9 and caspase 3 levels. MCF-7 cells without any treatment as control; and MCF-7 cells incubated with the nanosensitizer (0.1 mg/mL) and irradiated with X-ray for 4 Gy.

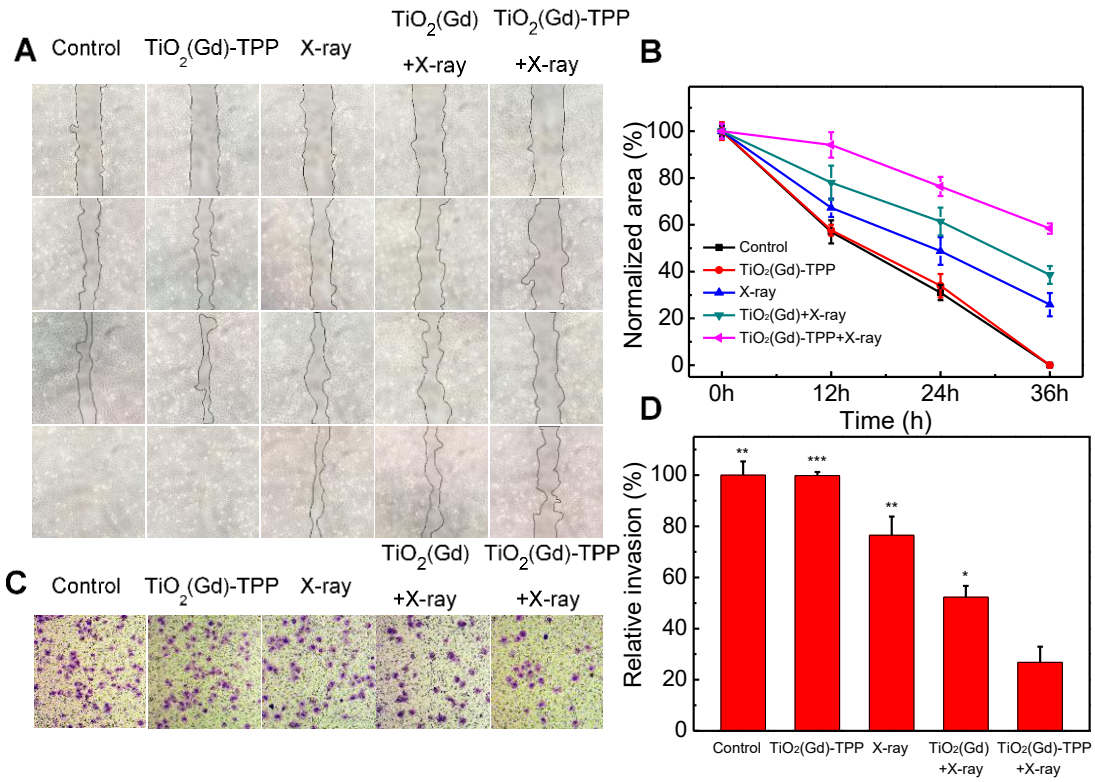

Figure S20. Representative photographs of cell migration and cell invasion. (A) Wound-healing assay of cells treated differently and then incubated for different time after wounding. The black curve indicated the wound edge; (B) Area ratio of wound healing at different time for cell migration; (C) The invasive MCF-7 cells were photographed following treating differently. (D) Quantitative results of invading cells. ***P < 0.001, **P < 0.01, *P < 0.05 compared with TiO_2(Gd)-TPP+X-ray group.