Electronic Supplementary Information

A nuclear-targeted titanium dioxide radiosensitizer for cell cycle regulation and enhanced radiotherapy†

Wei Pan,‡[a] Shaohua Gong,‡[a] Jianbo Wang,‡[b] Longhai Yu,[a] Yuanyuan Chen,[a] Na Li*[a] and Bo Tang*[a]

[a]*College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Institute of Molecular and Nano Science, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P. R. China.*

[b]*Radiation Department, Qilu Hospital of Shandong University, Jinan 250100, P. R. China.*

*E-mail: lina@sdnu.edu.cn; tangb@sdnu.edu.cn

Experimental details:

Reagents and Materials. Tetrabutyl titanate (TBOT), dimethyl sulfoxide (DMSO) and anhydrous alcohol were purchased from China National Pharmaceutical Group Corporation, China. (3-aminopropyl) triethoxysilane (APTES), N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethly-aminopropyl) carbodiimide (EDC) were acquired from Alfa Aesar (Tianjin, China). 7-ethyl-10-hydroxycamptothecin (SN-38) and polyethylene-polypropylene glycol (F127) were obtained from Macklin Reagent Co., Ltd., Shanghai, China. TAT (TAT: YGRKKRRQRRR) and RGD peptides were purchased from Chinese Peptide Company. 4T1-Luc cells were purchased from Shanghai AOLU Biological Technology Co., Ltd, China. Laser confocal petri dishes were purchased from Hangzhou Xinyou Biotechnology Co., Ltd., China. All chemicals were directly used without further purification. All of the aqueous solutions used in experiments were prepared using deionized water (18.2

MΩ·cm).

Instruments. Transmission electron microscopy (TEM) was carried out on HT7700 electron microscope (Hitachi, Japan). Changes of zeta potential were measured on Malvern Zeta Sizer Nano (Malvern Instruments). Fluorescence spectra were performed on a fluorescence spectrometer (FLS-980, Edinburgh, UK). UV-vis absorption spectra were measured on pharmaspec UV-1700 UV-visible spectrophotometer (Shimadzu, Japan). X-ray radiotherapy at a power of 6 MV for 4 Gy or 6 Gy, the dose rate of irradiation was 300 cGy/min and the source skin distance = 100 cm (Siemens Primus HI, Germany).

Preparation of $MTiO_2$ NPs. Firstly, 8 g F127 and 2 mL water were added to 200 ml anhydrous ethanol and the mixture was stirred at room temperature for 1 h. Then 2.9 mL tetrabutyl titanate was added to the above mixture dropwise. The mixture was stirred at room temperature for 8 h and white product was obtained. The white product was centrifuged (14000 rmp, 15 min) and washed several times with ethanol and water to remove F127. The white powder $MTiO_2$ NPs were obtained.

Verification of the generation of ROS. Two groups $MTiO_2$ solution (0.1 mg/mL, 2 mL) were prepared and added with 2-chloro-1,3-dibenzothiazoline cyclohexane, respectively. One group was performed with 4 Gy X-ray treatment while the other group with no X-ray treatment. Then the two groups solution were centrifugated (12000 rpm, 10 min). The fluorescence spectrum of the obtained supernatants was measured at 488 nm excitation.

Preparation of $MTiO_2$-NH_2 NPs. Aminating reagent APTES was used to modify the amino functional groups on the surface of $MTiO_2$ NPs. 1 mg $MTiO_2$ NPs were added to the solution containing 5 mL anhydrous ethanol and 50 μL secondary water. After stirring for 15 min at room temperature, 5 μL of APTES was added to the above mixed solution and stirred at room temperature for 12 h. Then the $MTiO_2$-NH_2 NPs could be acquired. The product was washed with ethanol several times to remove the dissociative aminating reagent. Finally, $MTiO_2$-NH_2 NPs were dispersed in 1 mL DMSO for further use.

Preparation of $MTiO_2$(SN-38) NPs. 10 mg SN-38 was dissolved in DMSO. Then 1

mL as-prepared $MTiO_2$-NH_2 NPs (1 mg/mL) was added and stirred at room temperature for 24 h. The surplus SN-38 was removed by centrifugation (10000 rpm, 10 min). Then repeat to wash several times with secondary water containing 5% DMSO. And continue to wash several times with secondary water to remove DMSO. The product was dispersed in 1 mL PBS buffer (10 mM, pH 7.4).

Quantification of SN-38 in $MTiO_2$ (SN-38) NPs. Finally, the capacity of SN-38 in $MTiO_2$ (SN-38) NPs was calculated using UV-vis absorption spectroscopy according to the standard linear calibration curve of SN-38. SN-38 has an ultraviolet absorption peak at 384 nm. The standard linear calibration curve of SN-38 could be plotted by UV-vis absorption spectroscopy. A series of concentrations of SN-38 solution were prepared, the absorbance at 384 nm was measured to obtain a standard linear calibration curve of UV-vis absorption of SN-38.

Preparation of $MTiO_2$(SN-38)-TAT nanoparticles. The TAT peptides were anchored to the $MTiO_2$(SN-38) NPs via an amide bond. Firstly, 3.2 μmol of TAT peptides, 16 μmol of EDC and 16 μmol of NHS were added to 4 mL of PBS buffer (10 mM, pH 7.4) to activate the carboxy terminal group of the TAT peptides for 1 h at room temperature. Subsequently, 2 mg of $MTiO_2$(SN-38) NPs were added to the above solution, and the solution was stirred at room temperature for 24 h. Finally, the excess TAT peptides, EDC and NHS was removed by washing several times with PBS buffer, and $MTiO_2$(SN-38)-TAT NPs were dispersed in PBS buffer (10 mM, pH 7.4) and stored at 4 °C.

Optimization the concentration of TAT peptides. Firstly, six groups of 4T1-Luc cells were inoculated in a confocal dish and cultured in a 37 °C, 5% CO_2 cell culture incubator for 24 h. Then, 1 mL of culture medium containing $MTiO_2$ (SN-38)-TAT (FITC) NPs (0.1 mg/mL) with different TAT peptides concentrations was added to the confocal dish, respectively. After 12 h, the cells were washed twice with PBS buffer to remove residual nanoparticles. Then the nuclei were stained with Hoechst 33342 at 37 °C for 15 min, and the cells were washed twice with PBS buffer. Finally, cells were observed by confocal laser scanning microscopy using 488 nm excitation for FITC (collection range 500-550 nm) and 405 nm excitation for Hoechst 33342

(collection range 430-480 nm).

Addition of TAT peptides (2 mg $MTiO_2$(SN-38) NPs were added to each group)

TAT (μmol)	NHS (μmol)	EDC (μmol)
0.2	1	1
0.4	2	2
0.8	4	4
1.6	8	8
2.4	12	12
3.2	16	16

Preparation of $MTiO_2$(SN-38)-TAT-RGD NPs. Firstly, $MTiO_2$(SN-38)-TAT NPs and 1 mg NHS-PEG2000-MAL were added to 4 mL PBS buffer (10 mM, pH 7.4), and the mixture was stirred for 12 h at room temperature to obtain $MTiO_2$(SN-38)-TAT-PEG-MAL NPs. Then, $MTiO_2$(SN-38)-TAT-PEG-MAL was stirred with 1.6 μmol of RGD peptides at room temperature for 24 h to react with the thiol group of RGD peptides to obtain $MTiO_2$(SN-38)-TAT-RGD NPs. Finally, $MTiO_2$(SN-38)-TAT-RGD NPs were washed several times with PBS buffer to remove excess RGD peptides. The production was dispersed in PBS buffer (10 mM, pH 7.4) and stored at 4 °C.

Optimization the concentration of RGD peptides. Firstly, six groups of 4T1-Luc cells were inoculated in a confocal dish and cultured in a 37 °C, 5% CO_2 cell culture incubator for 24 h. Then, 1 mL of culture medium containing $MTiO_2$(SN-38)-TAT(FITC)-RGD NPs (0.1 mg/mL) with different RGD peptides concentrations was added to the confocal dish, respectively. After 4 h, the cells were washed twice with PBS buffer to remove residual nanoparticles. Finally, cells were observed by confocal laser scanning microscopy using 488 nm excitation for FITC (collection range 500-550 nm). The addition amounts of different RGD peptides were 0/0.2/0.4/0.8/1.6/2.4 μmol (the amount of $MTiO_2$(SN-38)-TAT NPs in each group was 2 mg).

SN-38 release experiment. 5 mg $MTiO_2$(SN-38)-TAT-RGD NPs was dispersed in 5 mL PBS buffer. Then the solution were put into a dialysis bag with cut-off molecular weight (MW) of 3500 Da and dialyzed against PBS buffer at 37 ℃. The PBS buffer outside of the dialysis bag at different time point were collected and measured via UV-visible spectrophotometer to obtain the UV-vis absorption spectra.

Cell culture. 4T1-Luc cells were cultured in the RPMI 1640 culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37 ℃ in a humidified environment containing 5% CO_2.

Detection of cell cycle. Firstly, three groups of 4T1-Luc cells were seeded in a 90 mm cell culture dish and cultured in a 37 °C, 5% CO_2 cell culture incubator for 24 h. Then the cells were treated with PBS, $MTiO_2$-TAT-RGD NPs (0.1 mg/mL) and $MTiO_2$(SN-38)-TAT-RGD NPs (0.1 mg/mL), respectively. After 36 h, cells were digested and centrifuged (1,000 rpm/min, 5 min), and the cells were washed twice with PBS buffer (10 mM, pH 7.4). Then the cells were fixed with 70% pre-cold ethanol and placed in 4 °C. After 12 h, the cells were centrifuged and washed twice with pre-cooled PBS buffer (1,000 rpm/min, 5 min). Then the RNase A (100 μg/mL) was added and the cells were incubated in an incubator for 30 min and then washed twice with PBS buffer. The above solution was added with PI (50 μg/mL) and incubated for 30 min. Excess PI in each group of cells was removed via washing several times with PBS buffer. 200 μL PBS buffer without calcium and magnesium was added into each group of cells (note: the amount of PBS buffer without calcium and magnesium added depends on the number of cells). The DNA content was measured and the percentage of cells in each phase of the cycle was evaluated with ModFit LT software.

***In vitro* DNA damage assay.** DNA damage was detected using H2A.X Phosphorylation Assay Kit. 4T1-Luc cells were cultured with PBS, $MTiO_2$(SN-38)-TAT-RGD, X-ray, $MTiO_2$-TAT-RGD+X-ray, $MTiO_2$(SN-38)-TAT+X-ray, $MTiO_2$(SN-38)-RGD+X-ray, $MTiO_2$(SN-38)-TAT-RGD+X-ray (0.1 mg/mL) for 12 h. Then the cells were washed several times with PBS buffer to remove the unabsorbed nanoparticles. To fix the cells, 1X fixation solution was added at a cell density of 2×10^6 per mL and then incubated in ice for 20 min. After that, the fixative was removed by washing several times with PBS buffer. The cells pellets were then slowly resuspended in 1X permeabilization solution at a density of 2×10^6 cells per mL (50 μL per 1×10^5 cells). Subsequently, both negative control FITC conjugated normal mouse IgG and FITC-conjugated antiphosphohistone H2A.X (Ser139) (3.5 μL/1×10^5

cells) were added, and the cells were placed on ice for 20 min. One milliliter of 1X wash solution was added to remove the excess FITC-labeled antibody. Cell images were observed using confocal laser scanning microscopy with a 488 nm excitation for FITC (emission = 500-560 nm).

Colony formation assay. The 4T1-Luc cells were divided into 7 groups and seeded into 60 mm dishes and incubated at 37 °C under 5% CO_2 in cell culture incubator for 24 h. Each group of cells was received different treatment: PBS, $MTiO_2$(SN-38)-TAT-RGD, X-ray, $MTiO_2$-TAT-RGD+X-ray, $MTiO_2$(SN-38)-TAT+X-ray, $MTiO_2$(SN-38)-RGD+X-ray, $MTiO_2$(SN-38)-TAT-RGD+X-ray (0.1 mg/mL). After 12 h, the cells were washed three times with PBS buffer to remove residual nanoparticles and complete medium was added. Subsequently, a dose of 4 Gy of X-rays was administered and the cells were cultured for 10 days in a 37 °C, 5% CO_2 cell culture incubator. The cells were fixed with 4% paraformaldehyde for 15 min, washed slowly 3 times with PBS buffer and stained with 0.2% crystal violet for 45 min. The surviving fraction = (surviving colonies) / (cells seeded × plating efficiency). The mean surviving fraction was obtained from three parallel tests.

Cell migration assay. Seven groups of 4T1-Luc cells were seeded in 60 mm cell culture dishes and cultured in a 37 °C, 5% CO_2 cell culture incubator for 24 h. The cells were then treated with PBS, $MTiO_2$(SN-38)-TAT-RGD, X-ray, $MTiO_2$-TAT-RGD+X-ray, $MTiO_2$(SN-38)-TAT+X-ray, $MTiO_2$(SN-38)-RGD+X-ray, $MTiO_2$(SN-38)-TAT-RGD+X-ray (0.1 mg/mL), respectively. After 12 h, the cells were wounded by dragging a 10 μL pipette tip through the monolayer. Followed by three times washing with PBS, cellular debris was removed and images were acquired at the time of 0, 12, 24 and 48 h post-wounding. The data was quantified by AJ-VERT software.

Cell invasion assay. Invasive chamber coated with an artificial basement membrane was used for cell invasion experiment. The 4T1-Luc cells were divided into the same 7 groups as the above cell migration assay. 2×10^4 trypsinized cells were added into the upper compartment and further incubated for 24 h. The noninvasive cells on the upper surface of the membrane were removed by a cotton-tipped swab. The invasive cells which adhered to the lower surface of the membrane were subsequently fixed

with 4% paraformaldehyde and stained with 0.2% crystal violet before counting the number of invaded cells under microscope.

Animal tumor models. All animal experiments were carried out according to the 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. Balb/c mice (4-6 weeks old, female, weighing approximately 20 g) were fed under normal conditions of 12 h light and dark cycles, and food and water were obtained ad libitum. Tumor model establishment: 4T1-Luc cells were trypsinized, washed 3 times with RPMI 1640 serum-free medium, finally redispersed in RPMI 1640 serum-free medium, and then injected subcutaneously into the right forelimb of Balb/c mice. The tumor volume (V) was calculated by measuring the length (L) and width (W) of the tumor ($V = L \times W^2/2$). The relative tumor volume was calculated for each sample as V/V_0 (V_0 is the tumor volume at the start of treatment). When the tumor volume reached approximately 100 mm^3, the mice were received treatment.

Tumor growth inhibition *in vivo*. When the tumor volume reached about 100 mm^3, Balb/c tumor-bearing mice were randomly divided into 9 groups: PBS group, $MTiO_2$(SN-38)-TAT-RGD group, SN-38 group, X-ray group, SN-38+X-ray group, $MTiO_2$-TAT-RGD+X-ray group, $MTiO_2$(SN-38)-TAT+X-ray group, $MTiO_2$(SN-38)-RGD+X-ray group, $MTiO_2$(SN-38)-TAT-RGD+X-ray group. The corresponding treatments were received after the tumor volume reached 100 mm^3. 150 μL PBS, 150 μL $MTiO_2$(SN-38)-TAT-RGD NPs, 150 μL SN-38, 150 μL $MTiO_2$-TAT-RGD NPs, 150 μL $MTiO_2$(SN-38)-TAT NPs, 150 μL $MTiO_2$(SN-38)-RGD NPs were intravenously injected into the corresponding groups. The intravenous dose was 60 mg/kg. After 8 h, the tumor area of the tumor-bearing mice was supplied with a 6 Gy dose of X-ray irradiation. Changes in body weight and tumor volume of the mice were monitored every other day during the period until day 21.

Mouse survival rate experiment: Observing the mice treated with living tumors, and recording the tumor volume of mice every other day. When the mice die naturally or the tumor volume reaches 1000 mm^3, the mice were defined to death. The dead time

of mice and the number of mice was recorded to 40 days.

Haematoxylin and eosin (H&E) staining. Balb/c tumor-bearing mice were randomly divided into 7 groups and were received the same treatment as tumor growth inhibition experiment. Tumor damage: After 12 hours of intravenous injection, 7 groups of mice were treated accordingly. After 12 hours, the mice were killed. The dissected tumors were immersed in paraformaldehyde and stored in the dark. Damage of five major organ tissues: after 12 hours of intravenous injection, 7 groups of mice were treated accordingly. After 7 days, the mice were sacrificed. The five main organs (heart, liver, spleen, lung, kidney) were dissected in paraformaldehyde and stored in the dark.

Supplementary Figures:

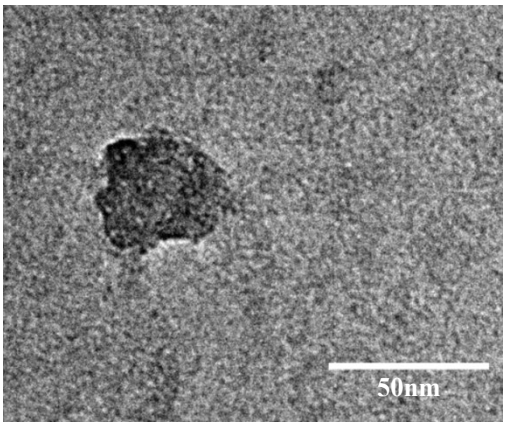

Fig. S1 The HR-TEM image of $MTiO_2$(SN-38)-TAT-RGD NPs.

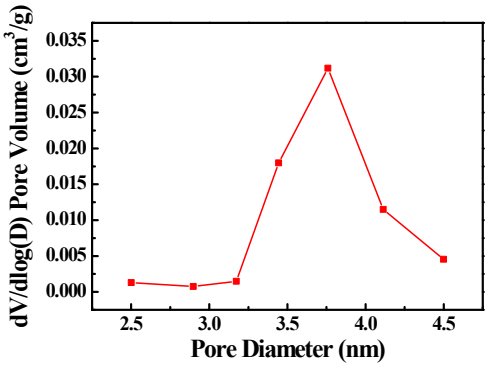

Fig. S2 The pore size distribution obtained by the Barrett-Joyner-Halenda (BJH) method.

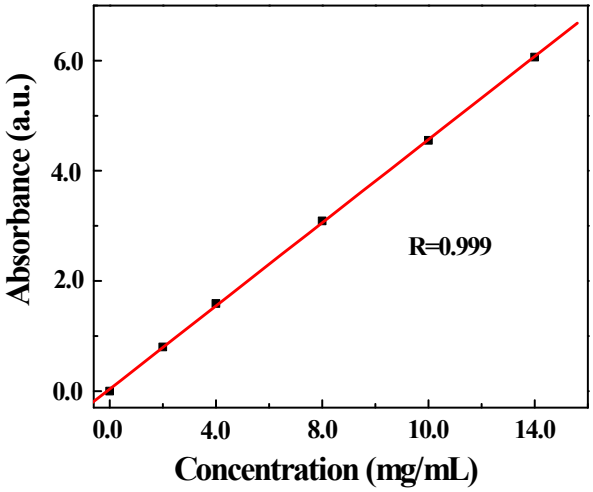

Fig. S3 The UV-vis absorption spectrum standard linear calibration curve of SN-38.

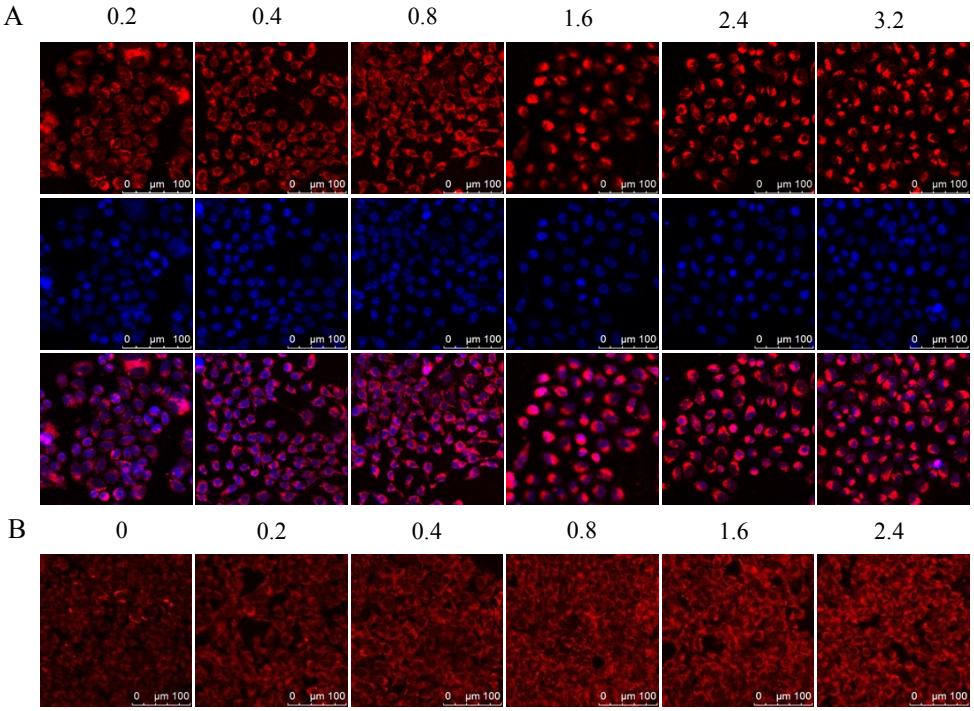

Fig. S4 (A) Confocal images of 4T1-Luc cells incubated with culture medium containing $MTiO_2$(SN-38)-TAT(FITC) attached with different concentration (0.2, 0.4, 0.8, 1.6, 2.4, 3.2 μmol) of TAT (FITC) peptides. (B) Confocal images of 4T1-Luc cells incubated with culture medium containing $MTiO_2$(SN-38)-TAT(FITC)-RGD attached with different concentration (0, 0.2, 0.4, 0.8, 1.6, 2.4 μmol) of RGD peptides.

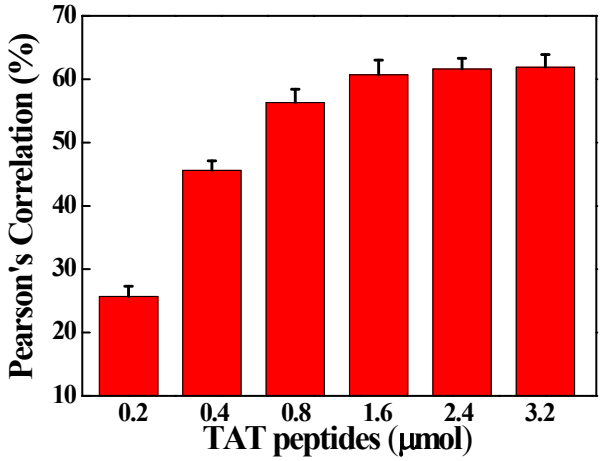

Fig. S5 The colocalization effect of $MTiO_2$(SN-38)-TAT NPs with different amount of TAT peptides.

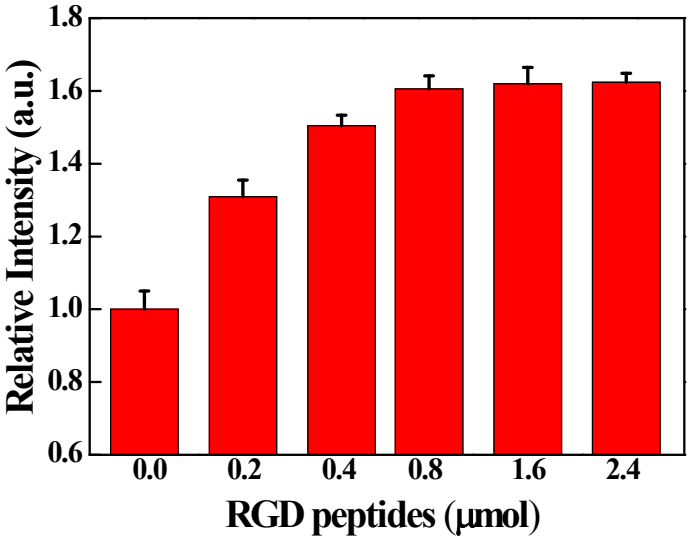

Fig. S6 Relative fluorescence intensity of 4T1-Luc cells treated with different concentration of RGD peptides for $MTiO_2$(SN-38)-TAT-RGD NPs.

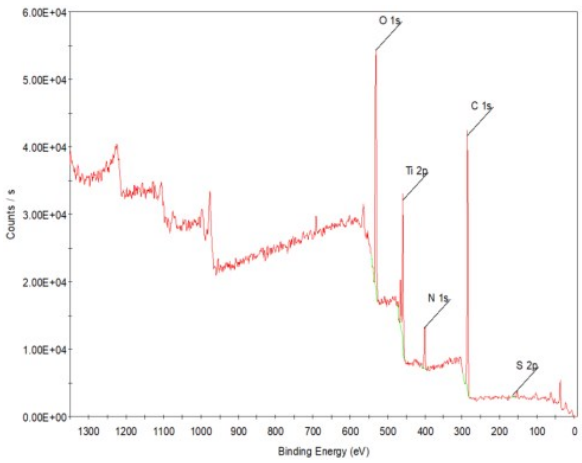

Fig. S7 The XPS spectrum of $MTiO_2$(SN-38)-TAT-RGD NPs.

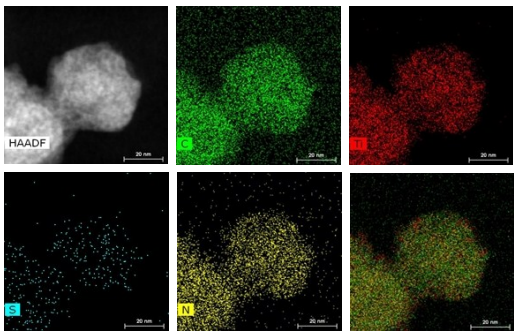

Fig. S8 The TEM mapping images of $MTiO_2$(SN-38)-TAT-RGD NPs.

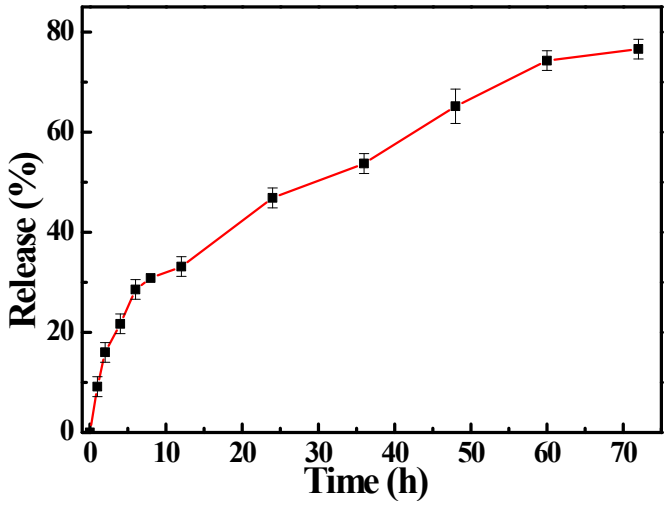

Fig. S9 The release profile of SN-38 from $MTiO_2$(SN-38)-TAT-RGD NPs.

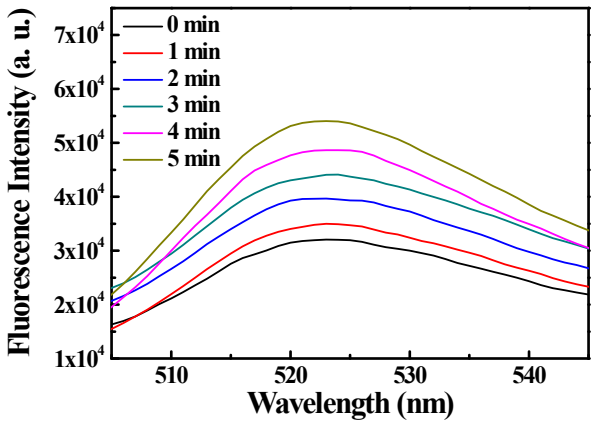

Fig. S10 The fluorescence spectra of DCFH-DA obtained from $MTiO_2$ NPs at different X-ray irradiation time.

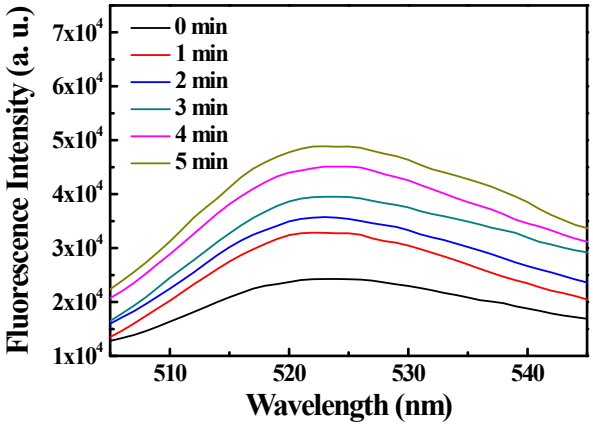

Fig. S11 The fluorescence spectra of DCFH-DA obtained from $MTiO_2$(SN-38)-TAT-RGD NPs at different X-ray irradiation time.

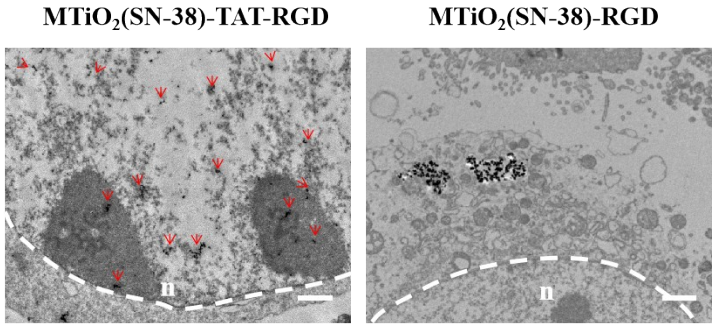

Fig. S12 Bio-TEM images of $MTiO_2$(SN-38)-TAT-RGD NPs and $MTiO_2$(SN-38)-RGD NPs. The red arrow indicated $MTiO_2$(SN-38)-TAT-RGD NPs in the cell nuclei. Scale bar=500 nm. n represents cell nucleus.

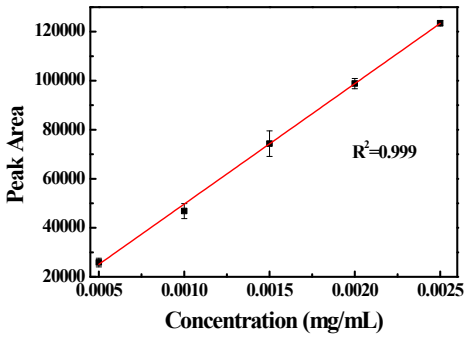

Fig. S13 The peak area standard linear calibration curve of SN-38 obtained by HPLC.

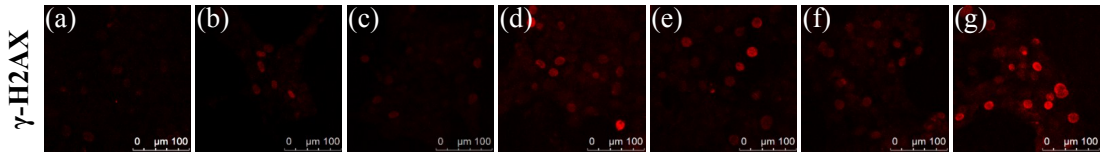

Fig. S14 DNA damage confocal images of 4T1-Luc cells with (a) PBS; (b) $MTiO_2$(SN-38)-TAT-RGD; (c) X-ray; (d) $MTiO_2$-TAT-RGD+X-ray; (e) $MTiO_2$(SN38)-TAT+X-ray; (f) $MTiO_2$(SN-38)-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT-RGD+X-ray.

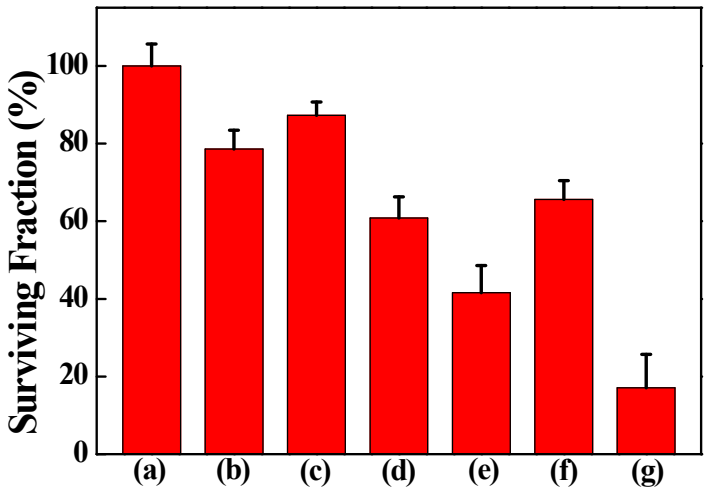

Fig. S15 Surviving fraction of 4T1-Luc cells after different treatments. (a) PBS; (b) $MTiO_2$(SN-38)-TAT-RGD; (c) X-ray; (d) $MTiO_2$-TAT-RGD+X-ray; (e) $MTiO_2$(SN-38)-TAT+X-ray; (f) $MTiO_2$(SN-38)-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT-RGD+X-ray.

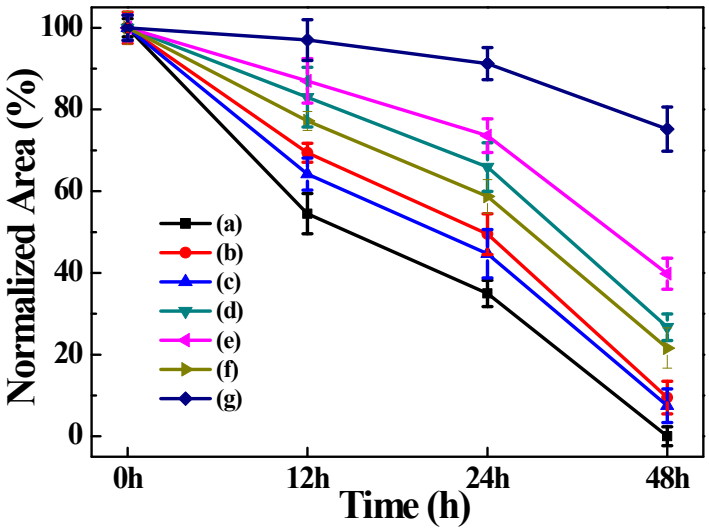

Fig. S16 Normalized area of 4T1-Luc cells acquired at different time point after different treatments. (a) PBS; (b) $MTiO_2$(SN-38)-TAT-RGD; (c) X-ray; (d) $MTiO_2$-TAT-RGD+X-ray; (e) $MTiO_2$(SN-38)-TAT+X-ray; (f) $MTiO_2$(SN-38)-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT-RGD+X-ray.

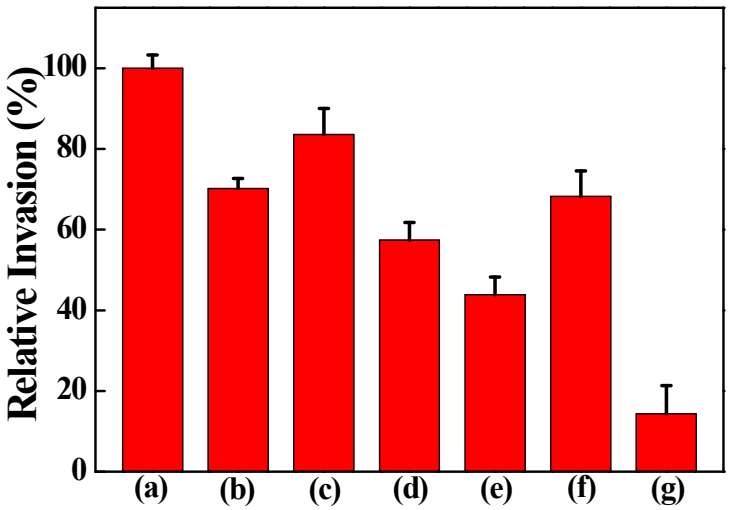

Fig. S17 Quantitative results of invading cells with different treatments. (a) PBS; (b) $MTiO_2$(SN-38)-TAT-RGD; (c) X-ray; (d) $MTiO_2$-TAT-RGD+X-ray; (e) $MTiO_2$(SN-38)-TAT+X-ray; (f) $MTiO_2$(SN-38)-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT-RGD+X-ray.

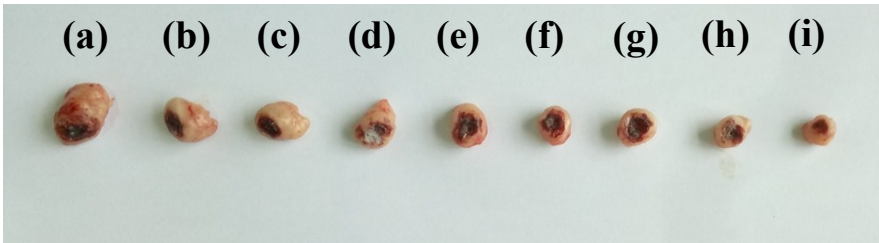

Fig. S18 The volume of tumors dissected from mice with different treatments. (a) PBS; (b) SN-38; (c) $MTiO_2$(SN-38)-TAT-RGD; (d) X-ray; (e) SN-38+X-ray; (f)$MTiO_2$-TAT-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT+X-ray; (h) TiO_2(SN-38)-RGD+X-ray; (i) $MTiO_2$(SN-38)-TAT-RGD+X-ray.

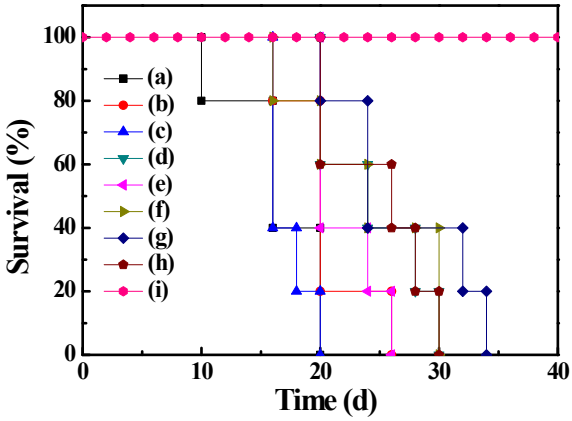

Fig. S19 Survival rates of mice after received corresponding treatments. (a) PBS; (b) SN-38; (c) $MTiO_2$(SN-38)-TAT-RGD; (d) X-ray; (e) SN-38+X-ray; (f)$MTiO_2$-TAT-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT+X-ray; (h) TiO_2(SN-38)-RGD+X-ray; (i) $MTiO_2$(SN-38)-TAT-RGD+X-ray.

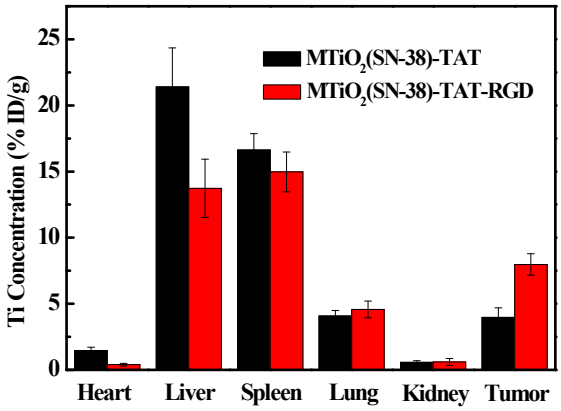

Fig. S20 Biodistribution of $MTiO_2$(SN-38)-RGD NPs and $MTiO_2$(SN-38)-TAT-RGD NPs.

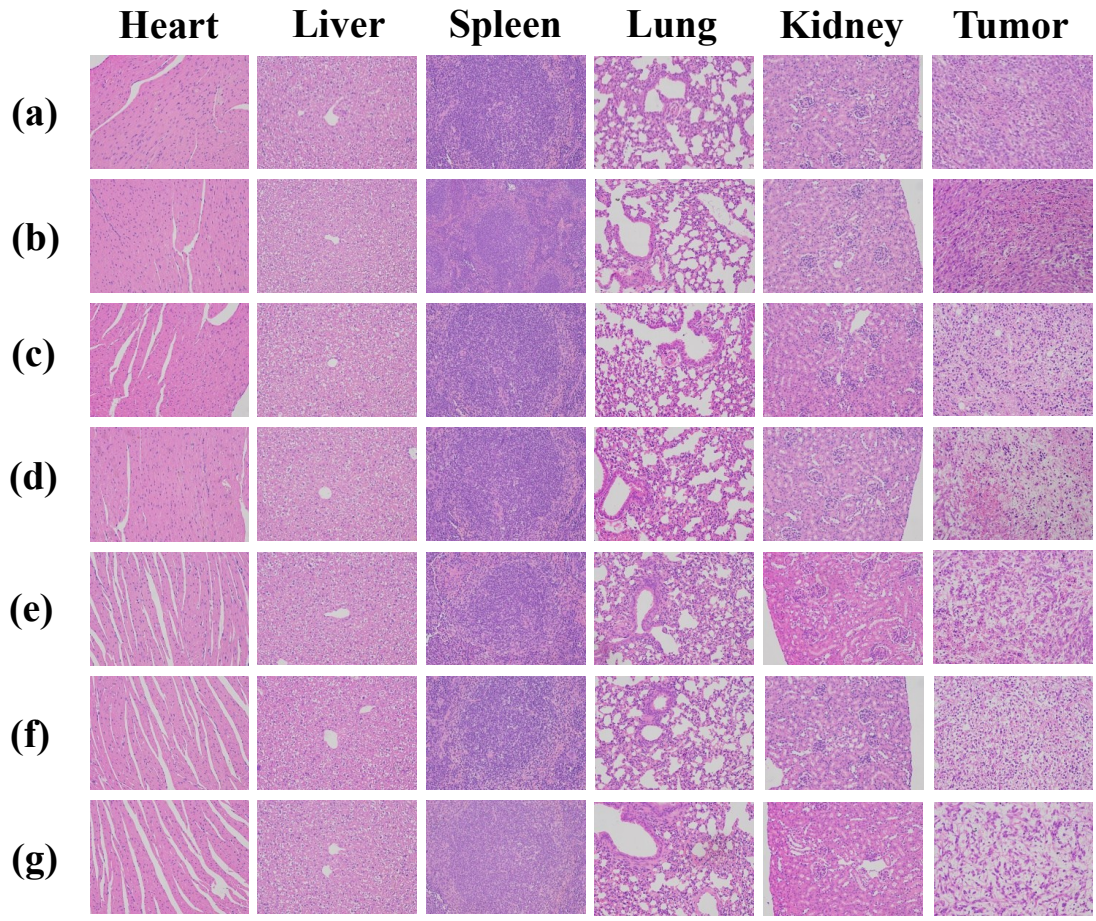

Fig. S21 H&E staining assay of major organs and tumor of mice after received with different treatments. (a) PBS; (b) $MTiO_2$(SN-38)-TAT-RGD; (c) X-ray; (d) $MTiO_2$-TAT-RGD+X-ray; (e) $MTiO_2$(SN-38)-TAT+X-ray; (f) $MTiO_2$(SN-38)-RGD+X-ray; (g) $MTiO_2$(SN-38)-TAT-RGD+X-ray.