Supporting Information for

ORIGINAL ARTICLE

Selectively enhancing radiosensitivity of cancer cells *via in situ* enzyme-instructed peptide self-assembly

Yang Gao[a,†], Jie Gao[a,†], Ganen Mu[a], Yumin Zhang[a], Fan Huang[a], Wenxue Zhang[b], Chunhua Ren[a,*], Cuihong Yang[a,*], Jianfeng Liu[a,*]

[a]*Tianjin Key Laboratory of Radiation Medicine and Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300192, China*

[b]*Radiation Oncology Department, Tianjin Medical University General Hospital, Tianjin 300052, China*

[*]Corresponding authors.

E-mail addresses: renchunhua0815@126.com (Chunhua Ren), yangcuihong@irm-cams.ac.cn (Cuihong Yang), liujianfeng@irm-cams.ac.cn (Jianfeng Liu).

[†]These authors made equal contributions to this work.

1. Materials and methods

1.1. TEM sample preparation

The preparation of TEM samples of **1P**, **2P**, **1P**+ALP, and **2P**+ALP were consistent. At first, each sample (15 μL) was loaded on the carbon-coated copper grid. After being retained on the grid for 2 min, the excess sample was removed by filter paper carefully. Then 2% uranyl acetate solution (15 μL) was added to stain the grid, and removed by filter paper after 2 min. Afterwards, the grid was placed in a desiccator to dry.

1.2. Cell culture

The human cervical cancer cell line (HeLa cells) and human lung cancer line (A549 cells) were used to evaluate the effect of drugs and their mechanism of radiosensitization. HeLa cells and A549 cells were cultured in DMEM culture medium (for HeLa cells) or RPMI Medium 1640 basic (for A549 cells) supplemented with 10 % FBS and 1 % penicillin/streptomycin (pen/strep) at 37 ℃ in 5% CO_2.

1.3. Cell cycle distribution

The pretreated cells were incubated for another 24 h after irradiation. Then cells were collected, fixed with pre-cooled 70% ethanol solution and stored at 4 ℃ overnight. After washing with cold PBS, the cells were incubated with 500 μL PI staining solution for 10 min at 37 ℃ in the dark. The fluorescence intensities of PI were measured by flow cytometry (Attune NxT, Beijing, China). The quantitative analysis was performed using FlowJo 7.6.1.

1.4. Cell apoptosis detection

After being irradiated with γ-rays under 6 Gy, the pretreated cells continued to culture for 24 h at 37 ℃ 5% CO_2. The supernatant and cells were unified collected, and centrifuged. After washed with PBS, the cell sedimentation was dispersed with 1 mL PBS with 2% FBS, followed by bathing in 37 ℃ water for 10 min. Positive control cells bathed at 60 ℃ for 10 min. After washed with 1× Binding Buffer once, the cells were counted and adjusted at a density of 1×10^6/mL. FITC Annexin V (5 μL) was added for staining at room temperature for 20 min in the dark. Then PI (5 μL) was added and cells were stained for 10 min in the dark. Afterwards, 400 μL 1× Binding Buffer was added and mixed. The samples were examined with flow cytometry (Attune NxT).

1.5. Western blotting analysis

The expression of PARP, cleaved PARP, caspase 3, acetylated-H3 (Ac-H3) and acetylated-H4 (Ac-H4) were assessed by Western blotting. Among them, Ac-H3 and Ac-H4 were both extracted from the pretreated cells as previously described[1]. Then lysates of each cell type were run on 12% SDS-PAGE gels and transferred onto PVDF membranes, which were blocked with 5% non-fat milk in TBST for 1 h at room temperature, and then incubated with the corresponding primary antibody overnight at 4 ℃. Immunoreacted bands were detected using secondary antibodies and developed using a Gel Imaging System (Bio-rad, Gel Doc XR, Beijing, China). The internal control was β-Actin.

1.6. Confocal laser scanning microscope (CLSM) imaging

For cellular uptake and intracellular localization assays, HeLa and A549 cells were inoculated into the confocal dishes with a cell density of 2×10^5 cells per well and incubated for 24 h to allow the attachment of cells. The medium of the confocal dish was replaced by fresh medium containing L-phenylalanine (L-Phe, 100 μmol/L), and further incubated at 37 ℃ for 1 h to inhibit ALP. Following incubated with medium containing L-Phe and **2P** (50 μmol/L, or **2P**+ALP) for 4 h at 37 ℃. Upon completion, the cells were cultured with Lyso-Tracker-containing medium for another 30 min at 37 ℃, and then dyed with DAPI for 20 min at room temperature. Finally, the cells were imaged by CLSM (Nikon, Shanghai, China.

1.7. Flow cytometer analysis

HeLa and A549 cells were seeded into six-well plates in 2×10^5 cells per well and incubated for 24 h to allow the attachment of cells. The medium was removed, and the new medium containing endocytosis inhibitor (dynasore, 80 μmol/L) was added to incubate for 1 h at 37 ℃. Then 50 μmol/L of **2P** (or **2P**+ALP) was added to the above well. After being co-incubated for 4 h at 37 ℃, the cells were washed with PBS three times and then collected for quantitative detection by flow cytometry (Attune NxT).

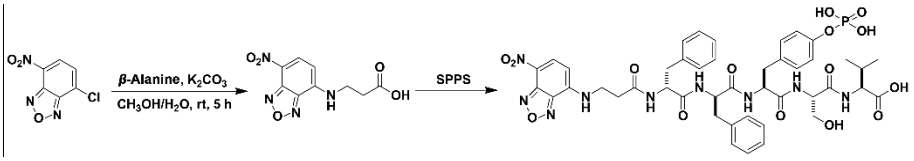

Figure S1 The synthesis route of NBD-^{D}F^{D}FpYSV (**2P**).

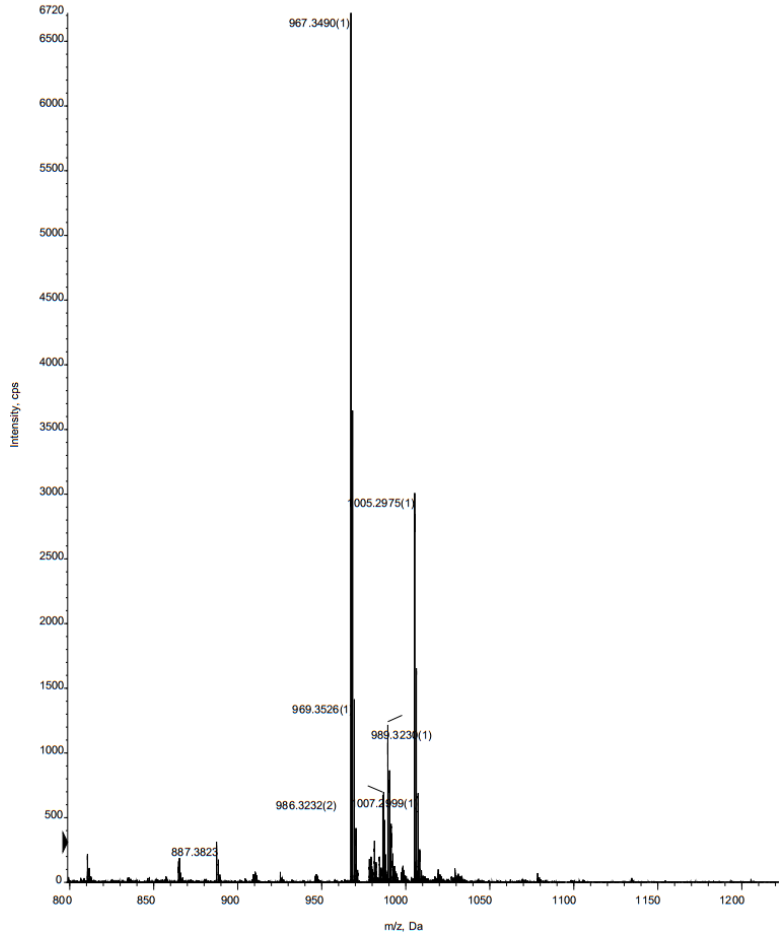

Figure S2 TOF-MS of Nap-G^{D}F^{D}FpYSV (**1P**).

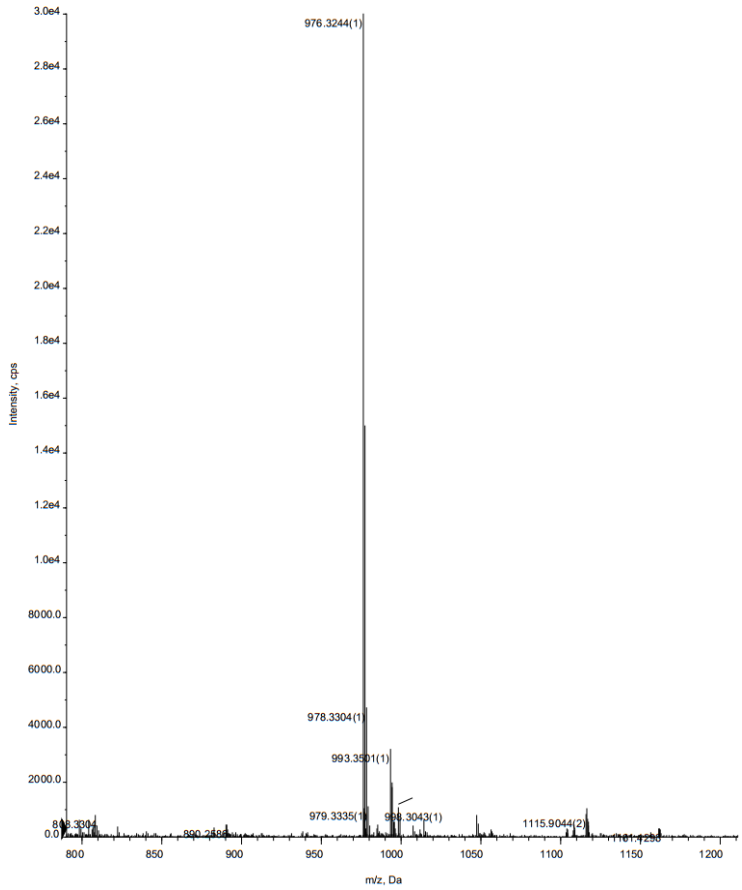

Figure S3 TOF-MS of NBD-^{D}F^{D}FpYSV (**2P**).

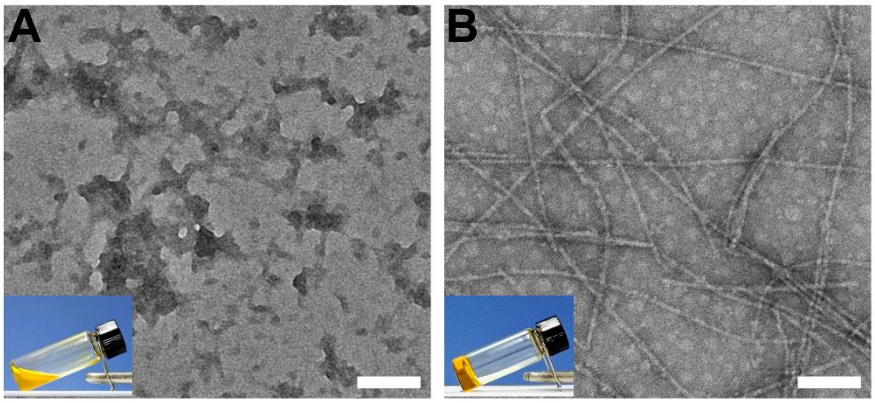

Figure S4 Characterization of enzymatic dephosphorylation of **2P**. Optical images and TEM images of (A) **2P** and (B) **2P**+ALP. Scale bar=100 nm, [**2P**]=4 mmol/L, [ALP]=10 U/mL.

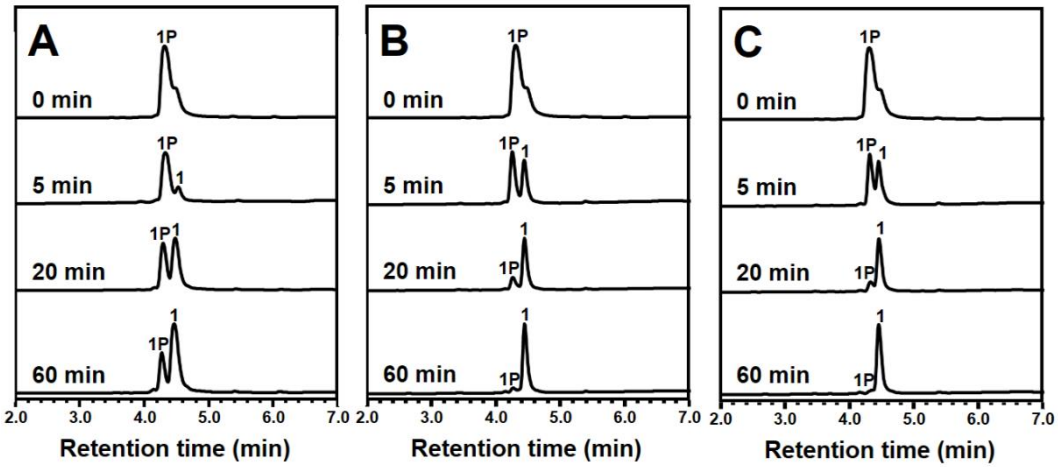

Figure S5 Representative HPLC spectra of the time-dependent dephosphorylation process of **1P** (4 mmol/L) in solution after 1 U/mL (A), 3 U/mL (B) and 10 U/mL (C) of ALP treatment.

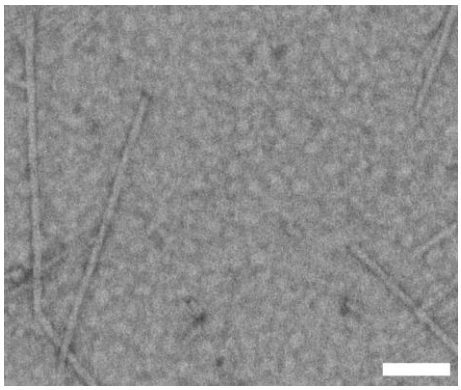

Figure S6 TEM image of 10 μmol/L of **1P**+ALP obtained by diluting the high concentration of **1P** hydrogel. Scale bar=100 nm.

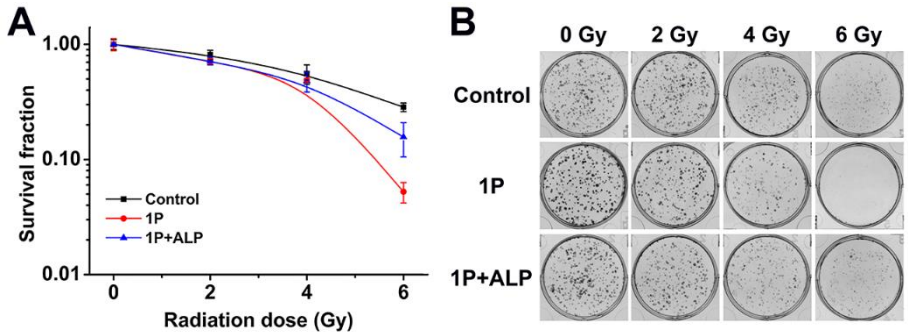

Figure S7 *In vitro* radiosensitization efficiency study in HeLa cells. Colony formation curves (A) and colony formation photographs (B) of HeLa cells after treatments with **1P** (10 μmol/L) and **1P**+ALP for 12 h. A: data are expressed as mean±SD, *n*=3.

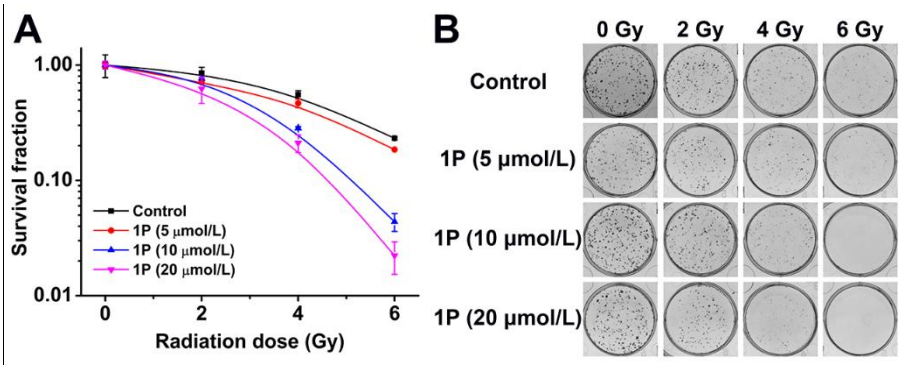

Figure S8 *In vitro* radiosensitization efficiency study in HeLa cells. Colony formation curves (A) and colony formation photographs (B) of HeLa cells after treatments with different concentrations of **1P** for 12 h. Data are expressed as mean±SD, *n*=3.

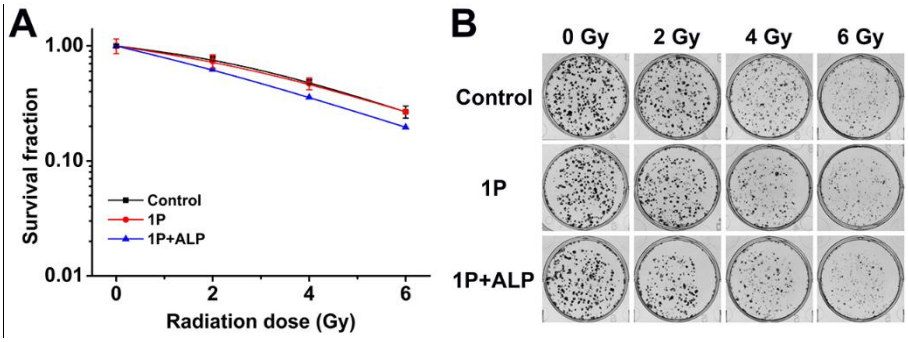

Figure S9 *In vitro* radiosensitization efficiency study in A549 cells. Colony formation curves (A) and colony formation photographs (B) of A549 cells after treatments with **1P** (10 μmol/L) and **1P**+ALP for 12 h. Data are expressed as mean±SD, *n*=3.

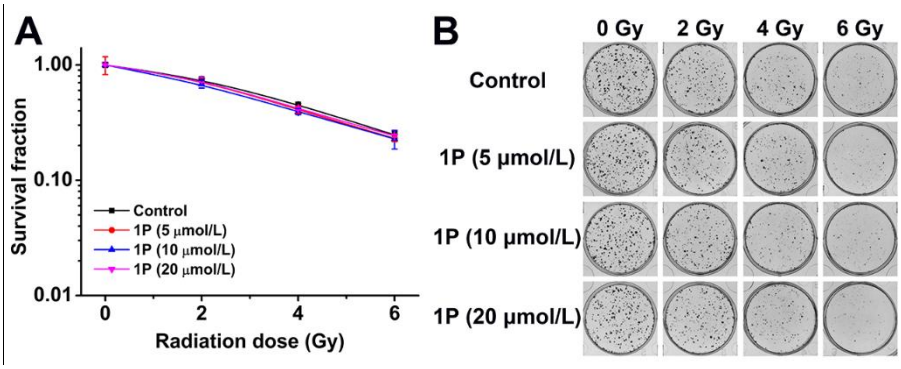

Figure S10 *In vitro* radiosensitization efficiency study in A549 cells. Colony formation curves (A) and colony formation photographs (B) of A549 cells after treatments with different concentrations of **1P** for 12 h. μM, μmol/L. Data are expressed as mean±SD, *n*=3.

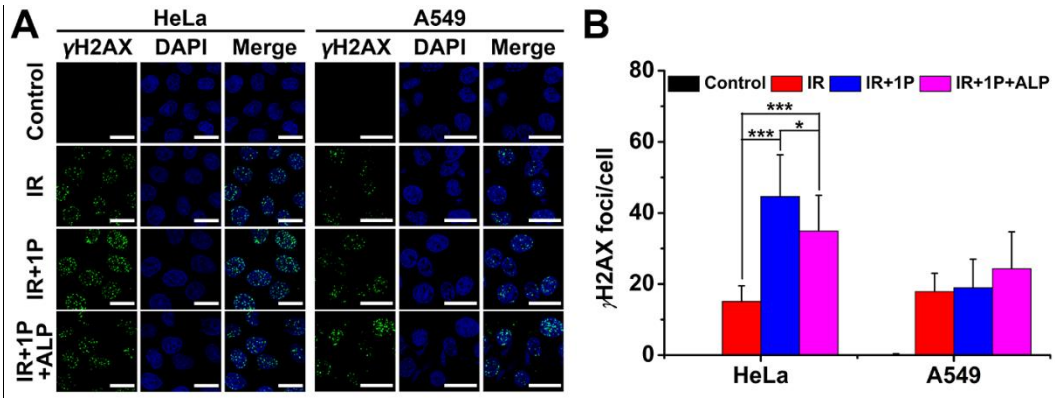

Figure S11 DNA damage detection by γH2AX immunofluorescence analysis. (A) Immunofluorescence images of γH2AX in HeLa cells and A549 cells treated with **1P** (10 μmol/L) and **1P**+ALP at 3 h after 6 Gy irradiation. Scale bar=20 μm. Nuclei were stained with DAPI (blue fluorescence). γH2AX was stained with Alexa Fluor 488 (green fluorescence). (B) Quantization of γH2AX expression in HeLa cells and A549 cells in (A, Data are expressed as mean±SD, n=20; $^{*}P$<0.05, $^{***}P$<0.001).

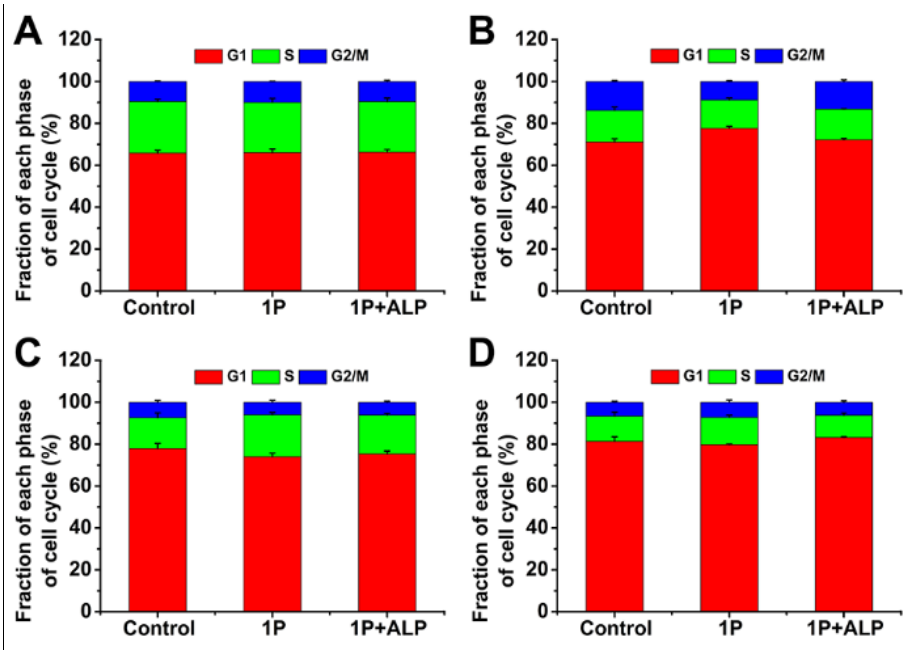

Figure S12 Cell cycle distribution measured by flow cytometry. Cell cycle distribution histograms of HeLa cells treated with various compounds for 12 h without (A) or with (B) 4 Gy irradiation. Cell cycle distribution histograms of A549 cells treated with various compounds for 12 h without (C) or with (D) 4 Gy irradiation. The cell cycle distribution was detected by flow cytometry 24 h after treatment. Data are expressed as mean±SD, n=3.

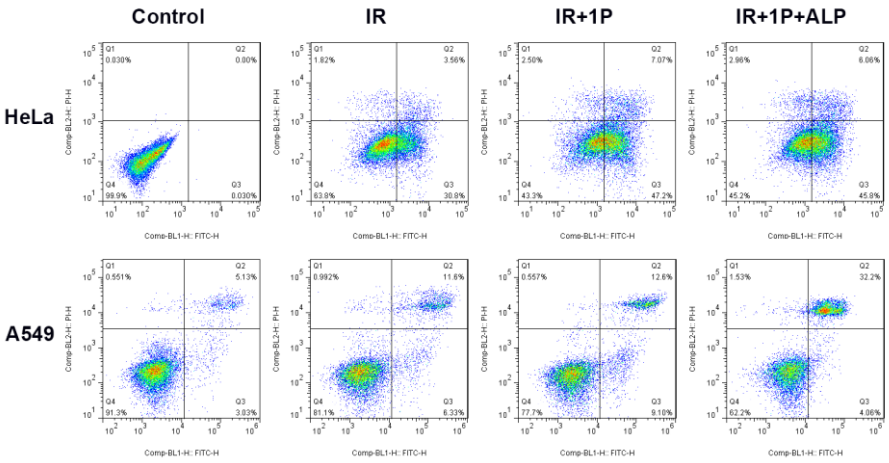

Figure S13 Apoptosis analysis of HeLa cells and A549 cells treated with **1P** (10 μmol/L) and **1P**+ALP for 12 h under 6 Gy irradiation. The apoptosis data were received by flow cytometry 24 h after treatment.

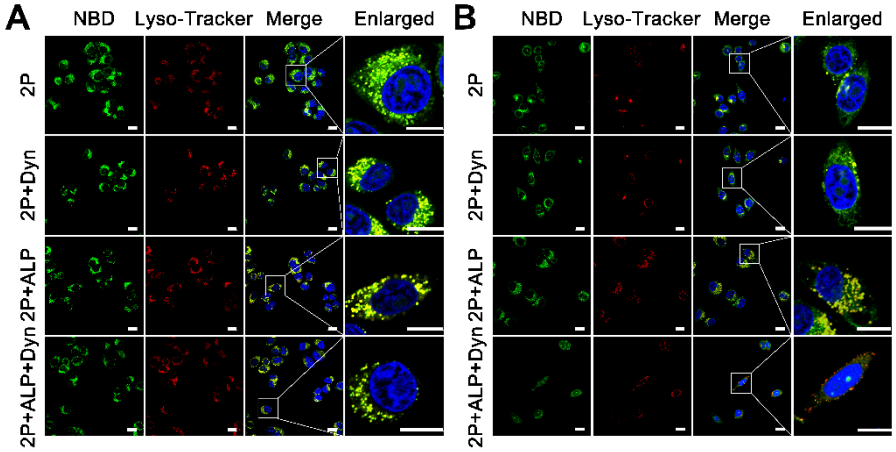

Figure S14 Endocytosis mechanism study by endocytosis inhibitor. Confocal laser scanning microscopy images of HeLa cells (A) and A549 cells (B) treated with **2P** (50 μmol/L) or **2P**+ALP for 4 h with or without dynasore (80 μmol/L). NBD: green, lyso-tracker: red, DAPI: blue, scale bar=20 μm.

References

1. Gao Y, Zhang C, Chang J, Yang C, Liu J, Fan S, et al. Enzyme-instructed self-assembly of a novel histone deacetylase inhibitor with enhanced selectivity and anticancer efficiency. *Biomater Sci* 2019;**7**:1477-85.