Supplementary Information

Nanodiamonds-based non-canonical autophagy inhibitor synergistically induces cell death in oxygen-deprived tumors

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Experimental Section

Reagents and cell lines

Nanodiamonds (NDs) were supplied by Gansu Gold Stone Nano. Material. Co. Ltd (China). HeLa, HEK293, Huh-7 and MCF7 cell lines were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai). dimethyloxalylglycine (DMOG), Minimum Essential Media (MEM), Fetal Bovine Serum (FBS), Thiazolyl blue tetrazolium bromide (MTT), Rapamycin, bafilomycin A, chloroquine and ATTO 550 were purchased from Sigma-Aldrich (USA). Sorafenib was purchased from Abmole. Primary antibody against LC3 was from Novus Biologicals (USA). Primary antibodies against P62, β -actin, HIF-1 α and Caspase-3 were purchased from Abcam (USA).

Preparation and characterization of NDs

The apparent hydrodynamic size and the zeta potential of nanoparticles were measured using a Zetasizer (nano ZS90, Malvern Instruments). To prepare fluorescence-labeled NDs, 50 μ g/mL NDs were mixed with 4.21 μ M Atto 550 in MEM medium for 2 h with shaking. Unbound fluorophore was removed by extensive washing. For transmission electron microscopy, solution containing NDs was dropped onto carbon coated copper grids to evaporate excess solvent, and examined with TEM (Jeol 2010, 200KV).

Cell culture and hypoxia treatment

HeLa, HEK293 and MCF7 cell lines were grown in MEM with 10 % heat-inactivated FBS and antibiotics (100 g/mL of streptomycin and 100 g/mL of penicillin) at 37 °C with humidified atmosphere (5 % CO₂). For hypoxia treatment, Cells were plated one day before incubation at 37

°C, 5% CO₂, 1% O₂ in a Hypoxia incubator (YCP-50S, Changsha Hua Xi Electronics Technetronic Co., Ltd) or incubated with 1 μ M DMOG for indicated time.

Determination of cell viability

Cell viability was determined using MTT assay and direct cell counting. Cells were seeded into 24-well plates at a concentration of 4×10^5 cells/mL one day before treatment. For MTT assay, 5 mg/mL MTT solution was added to each well, followed by 4 h incubation at 37 °C. Then cells were lysed with 10% acid SDS solution (pH 2~3). After centrifugation, the absorbance of supernatant was determined at 570 nm using a microplate reader (Bio-Rad 680, USA). For cell counting assay, cells were harvested and stained with Trypan blue. Numbers of viable cells were counted using an inverted microscope (CKX41SF, OLYMPUS).

Assay of Caspase-3 activity

The activity of Caspase-3 was determined using the Caspase-3 activity kit (Beyotime, China) according to the manufacturer's protocol. A reaction mixture of 10 μ L cell lysate, 80 μ L reaction buffer (20 mM Tris–HCl, pH 7.5, 1% NP-40) and 10 μ L Caspase-3 substrate (Ac-DEVD-pNA) (2 mM) were prepared and incubated at 37 °C for 4 hrs. Absorbance at 405 nm was measured using a microplate reader (Bio-Rad 680, U.S.A.). Average percentage and standard deviations of three biological replicates are shown.

Immunohistochemistry and Western Blotting

For detection of LC3 puncta, cells were cultured on poly-L-lysine-coated coverslips. Following treatment with 50 μ g/mL NDs, 2 μ M Baf A, 1 μ M DMOG or 2 μ M rapamycin, cells were fixed in 4% paraformaldehyde for 20 min. After washing by PBS for three times, cells were incubated

with blocking solution (1% BSA in PBS) for 30 min at RT and LC3 antibody (1:200) overnight at 4°C. After washing and incubation with Alexa 488-labeled secondary antibody and Hoechst 33258, Coverslips were mounted using DAKO fluorescence mount. To measure the protein levels of LC3, P62, HIF-1 α , Caspase-3 and β -actin, cells were harvested by SDS-loading sample buffer immediately after they were taken out of the incubator, followed by western blotting analysis with anti-LC3 (1:1000), anti-P62 (1:1000), anti-HIF-1 α (1:1000), anti-Caspase-3 (1:1000), anti- β -actin (1:1000).

TEM imaging of cell and tumor tissues

For TEM imaging of HeLa cells and tumors, samples were washed with PBS and collected, fixed sequentially with 2.5 % glutaraldehyde and 1 % osmium tetroxide, dehydrated and cut into ultrathin sections. The sections were stained with uranyl acetate and lead citrate, and imaged with a transmission electron microscope (JEOL-1230; JEOL, 80 KV).

Quantitative RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed into complementary DNA with the Superscript III First-Strand Synthesis kit (Invitrogen). Quantitative PCR was performed with SYBR Green Real-time PCR Master Mix (TOYOBO), and gene expression was analyzed on the StepOne Real-Time PCR System (Applied Biosystems). Expression levels of Actin were used as an internal control for normalization of gene expression.

Sequences of primers used in Quantitative PCR analysis.

| | Forward | Reverse |
|------|----------------------|---------------------|
| PHD3 | ATCGACAGGCTGGTCCTCTA | GATAGCAAGCCACCATTGC |

| BNIP3 | CTGAAACAGATACCCATAGCATT | CCGACTTGACCAATCCCA | |
|--------|-------------------------|------------------------|--|
| BNIP3L | CCAAGGAGTTCCACTTCAGACA | AGTAGGTGCTGGCAGAGGGTGT | |
| Glut1 | TGTGCTTCCAGTATGTGGAG | AAGAACAGAACCAGGAGCAC | |
| PDK | CCGCTCTCCATGAAGCAGTT | TTGCCGCAGAAACATAAATGAG | |
| Actin | CCAACCGCGAGAAGATGA | TCCATCACGATGCCAGTG | |

Fluorescence imaging of live cells

HeLa cells were transfected with EGFP-LC3 plasmid using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol one day before the treatments. Lysosomes were stained using 0.2 μ M LysoTracker Red (Life technology, USA) for 50 min before fluorescence imaging with a laser confocal microscope (Leica TCS SP8, Germany). The co-localization between LC3-GFP with NDs-Atto 550 and LysoTracker was quantified with the ImageJ software.

In vivo anti-tumor effects

Nude mice (female, 18–21 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., China. Huh-7 cells (1×10^6 per mouse) were subcutaneously injected in the forelimbs of nude mice. Mice were randomly assigned to one of the treatment groups (n=5 in each group). For Sorafenib-treated groups: 8 days after tumor inoculation, daily intragastric administration of sorafenib tosylate (30 mg/Kg) was given until day 23. For NDs-treated groups: 4 days after tumor inoculation, mice were injected with 80 µg NDs from tail veins every 4 days, from day 4 to day 20. The tumor volumes were measured every two days (from day 4 to day 24) in two dimensions with Vernier calipers. The tumor volumes were calculated using the following formula: length × width² × 0.5. At day 24, mice were sacrificed and the tumors were surgically excised. Tumor tissues were collected for western blot and immunohistochemical analysis. The animal use and experimental protocols were reviewed and approved by the Institutional Animal

Care and Use Committee (IACUC) of the Institute for Nutritional Sciences, SIBS, CAS.

Statistical Analysis

In all experiments, significance was determined using the t-distribution (two-tailed; two-sample equal variance) or one-way analysis of variance (ANOVA) using SPSS 11.5. *** p < 0.001; **p < 0.01; * p < 0.05.



Figure S1. Effects of three carbon nanomaterials on autophagy in HeLa cells. (a) Protein levels of LC3 isoforms and P62 were analyzed by western blotting for cells treated with indicated drugs or nanoparticles. Baf A, bafilomycin A1; Rapa, Rapamycin. (b) &(c) Quantitative analysis of protein levels in (a) with ImageJ software.



| | Water | | МЕМ | |
|---------|---------------|-------|---------------|-------|
| | Diameter (nm) | PDI | Diameter (nm) | PDI |
| 0-hour | 191.0 | 0.272 | 289.6 | 0.357 |
| 2-hour | 201.9 | 0.229 | 300.4 | 0.027 |
| 24-hour | 197.1 | 0.291 | 318.5 | 0.181 |



Figure S2. Characterization of NDs. (a) TEM images of NDs. (b) Stability of NDs in water or MEM medium was measured by dynamic light scattering (DLS) at indicated time points. (c) Size and zeta potential of NDs and NDs-550 particles were measured by dynamic light scattering (DLS). (d) Fourier transform infrared spectra of NDs.



Figure S3. NDs block autophagic flux in various cell types. HeLa (a) MCF-7 (b) and HEK293 (c) cells were incubated NDs (20 and 50 μ g/mL) for 24 hours respectively. Protein levels of LC3-II and P62 were analyzed by western blotting. Quantitative analysis of protein levels were performed with the ImageJ software.



Figure S4. Blockade of autophagy under hypoxia triggers cell death. (a) Microscopic images showing morphological changes in HeLa cells exposed to 20 μ g/mL or 50 μ g/mL NDs for 48 hours under normoxia (21% O₂) or in hypoxia (0.5% O₂). MCF7 cells were incubated with NDs (20 and 50 μ g/mL) or Baf A (15 nM) for 48 hours under normoxia or hypoxia. (b) Cell viability was analyzed with MTT assay. (c) Apoptosis was indicated by activities of Caspase-3. (d) Protein levels of cleaved Caspase-3 were analyzed by western blotting.



Figure S5. Hypoxia induces autophagy in HEK293 and MCF7 cells. (a) MCF7 and (b) HEK293 cells were treated with DMOG (1 μ M), hypoxia (0.5% O₂) or Rapamycin (2 μ M) for 24 hours. The protein levels of LC3-II were analyzed by western blotting.



Figure S6. HIF activity was not affected by NDs. (a) HeLa cells were treated with NDs (20 and 50 μ g/mL) under normoxia or hypoxia for 24 hours. Protein levels of HIF-1 α were analyzed by western blotting. (b) HeLa cells were treated with Baf A and hypoxia for 24 hours. Protein levels of HIF-1 α were analyzed by western blotting. (c) The mRNA levels of indicated genes were measured by qRT-PCR and normalized to Actin.



Figure S7. HIF levels were not affected by NDs in MCF7 cells. MCF7 cells were treated with NDs (20 and 50 μ g/mL) under normoxia or hypoxia for 24 hours. Protein levels of HIF-1 α were analyzed by western blotting (a) and quantified (b&c).



Figure S8. Baf A treatment triggers cell death both under normoxia and hypoxia. HeLa cells were incubated with NDs (50 μ g/mL) or Baf A (15 nM) for 48 hours under normoxia or hypoxia. Cell viability was analyzed with MTT assay.



Figure S9. HeLa cells expressing GFP-LC3 were incubated with NDs for 24 hours and imaged by confocal microscopy. Percentage of NDs-Atto550 colocalized with GFP-LC3 (left) or GFP-LC3 colocalized with LysoTracker red (right) were quantified with the ImageJ software (Wayne Rasband, NIH) ($n \ge 10$).



Figure S10. TEM images of Huh-7 xenografts prove that injected NDs (indicated by red arrows) entered the cytoplasm of tumor cells.



Figure S11. NDs treatment blocked autophagic flux *in vivo* and showed a mild inhibition on tumor growth. After tumor xenograft, mice were intravenously injected with 80 μ g NDs every 4 days, from day 4 to day 20. (a) Tumor volumes were measured and calculated every two days, from day 4 to day 24. Data are expressed as mean \pm SD (n=5). ***p < 0.001. (b) Images of isolated tumors. (c) Protein levels of LC3-II and P62 in the tumor tissues were analyzed by western blotting. (d) Tumor sections were immunostained with anti-P62 antibody.