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Supporting Information

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Mitochondria-Targeted Cancer Therapy Using a Light-Up Probe with Aggregation-Induced-Emission Characteristics**

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Supporting information

Materials and Methods

1. Materials and characterization.

2,4-Dihydroxybenzaldehyde, 1,6-dibromohexane, hydrazine monohydrate, tripheylphosphine, Cs_2CO_3, ethanol, anhydrous DMF, dichloromethane, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were purchased from Sigma-Aldrich and used as received without further purification.

Dulbecco's Modified Essential Medium (DMEM) is a commercial product of National University Medical Institutes (Singapore). Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Bedford, United States). Fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Gibco (Life Technologies, AG, Switzerland). LysoTracker Red (LysoTracker), MitoTracker Red DND99 (MitoTracker) and propidium iodide were purchased from Life Technologies. DeadEnd™ colorimetric TUNEL system and cellTiter-Glo® luminescent cell viability assay were obtained from Promega.

The UV-vis absorption spectra were obtained using UV-vis spectrometer (Shimadzu, UV-1700, Japan). PL measurements were carried out on a Perkin-Elmer LS-55 equipped with a xenon lamp excitation source and a Hamamatsu (Japan) 928 PMT, using 90° angle detection for solution samples. ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm, $(CD_3)_2SO$ = 2.50 ppm or tetramethylsilane $Si(CH_3)_4$ = 0 ppm).

2. Synthesis of compound 2.

2,4-Dihydroxybenzaldehyde (276 mg, 2.0 mmol) and 1,6-dibromobutane (482 mg, 2.0 mmol) were first dissolved in DMF (10 mL), followed by addition of Cs_2CO_3 (652 mg, 2.0 mmol). The mixture was stirred at 60℃ under nitrogen for 12 h. After cooling to room temperature, the reaction mixture was extracted with dichloromethane (40 mL × 3). The

combined dichloromethane fractions were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was further separated by column chromatography (silica, petroleum ether:ethyl acetate = 20:1) to give **2** as colorless oil (402 mg, 67% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ11.48 (s, 1H), 9.71 (s, 1H), 7.42 (d, J = 8.4 Hz, 1H), 6.52 (d, J = 8.4 Hz, 2H), 6.40 (s, 1H), 4.01 (t, J = 6.0 Hz, 2H), 3.43 (t, J = 6.8 Hz, 2H), 1.91–1.80 (m, 4H), 1.52–1.50 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): 194.3, 166.3, 164.5, 135.2, 115.0, 108.7, 101.0, 68.3, 33.7, 32.6, 28.7, 27.8, 25.2; HRMS (ESI): m/z [M + H]+calcd for $C_{13}H_{18}BrO_3$: 302.1842; found: 302.2030.

3. Synthesis of AIE-Br.

4-[(6-Bromohexyl)oxy]-2-hydroxybenzaldehyde (300 mg, 1 mmol) was dissolved in absolute ethanol (10 mL), followed by addition of hydrazine monohydrate (25 mg, 0.5 mmol). The mixture was refluxed for 4 h. Precipitates were filtrated under vacuum and washed with absolute ethanol three times to yield **AIE-Br** as yellow powders after drying (253 mg, 85% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ11.74 (br s, 2H), 8.57 (s, 2H), 7.21 (d, J = 8.4 Hz, 2H), 6.50 (d, J = 8.4 Hz, 2H), 4.00 (t, J = 6.4 Hz, 4H), 3.43 (t, J = 6.4 Hz, 4H), 1.92–1.51 (m, 16H); ^{13}C NMR ($CDCl_3$, 100 MHz): 163.4, 162.7, 161.7, 133.5, 111.0, 107.9, 101.7, 68.0, 33.6, 32.7, 28.9, 27.9, 25.2. HRMS (ESI): m/z [M]+calcd for $C_{26}H_{34}Br_2N_2O_4$: 596.0885; found: 596.3950.

4. Synthesis of AIE-mito-TPP.

AIE-Br (298 mg, 0.5 mmol) and tripheylphosphine (262 mg, 1.0 mmol) were dissolved in acetonitrile (5 mL), and the mixture was stirred under reflux for 12 h. After completion of the reaction, the mixture was cooled to room temperature. The solvent was then removed by evaporation under pressure, and the residue was recrystallized from a mixture of hexane/ethyl acetate to yield **AIE-mito-TPP** as a yellow solid (510 mg, 91% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ11.66 (br s, 2H), 8.51 (s, 2H), 7.86–7.69 (m, 30H), 7.16 (d, *J* = 8.4 Hz, 2H),

6.46–6.43 (m, 4H), 3.93 (t, *J* = 6.0 Hz, 4H), 3.84–3.78 (m, 4H), 1.73–1.47 (m, 16H); ^{13}C NMR ($CDCl_3$, 100 MHz): 163.3, 162.6, 161.6, 135.0, 133.7, 133.6, 133.4, 130.5, 130.4, 118.8, 117.9, 111.0, 107.6, 101.9, 67.9, 29.8, 28.6, 25.5, 23.0, 22.6; HRMS (ESI): m/z [M–Br]+calcd for $C_{62}H_{64}BrN_2O_4P_2$: 1041.3519; found: 1041.3514.

5. Cell culture.

HeLa human cervix carcinoma cells, MDA-MB-231 human breast cancer cells, NIH-3T3 mouse embryonic fibroblast cells, U-87-MG human glioblastoma-astrocytoma, epithelial-like cells, HepG2 human liver hepatocellular carcinoma cells, MCF-7 human breast cancer cells, NHDF primary normal human dermal fibroblast cells, MRC5 human fetal lung fibroblast cells and HK2 human proximal tubular epithelial cells were provided by American Type Culture Collection (ATCC). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Invitrogen), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Thermo Scientific) and were maintained in a humidified incubator at 37 °C with 5% CO_2. Before experiments, the cells were pre-cultured until confluence was reached.

6. Intracellular localization of AIE-mito-TPP.

HeLa cells were cultured in the chambers (LAB-TEK, Chambered Coverglass System) at a density of 5×10^5 per mL for 18 h. The cells were incubated in 5% CO_2 with 1 μM **AIE-mito-TPP** at 37 °C for 2 h . For the co-localization experiments, the cells were washed with PBS, 200 nM MitoTracker and 100 nM LysoTracker were added and incubated at 37 °C for 45 min. The medium was removed and the cells were rinsed with PBS. The cells were imaged immediately by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany). For **AIE-mito-TPP**, the excitation was 405 nm, and the emission filter was 510-560 nm; for LysoTracker and mitoTracker imaging, the excitation was 543 nm, and the emission filter was 575-625 nm.

7. Cytotoxicity and anti-proliferation assay.

The cytotoxicity of the complexes was evaluated using the MTT assay. The HeLa, MDA-MB-231 and NIH-3T3 cells were seeded in 96-well plates at a density of 5000 cells/well. After incubation for 24 h, the medium was replaced with **AIE-mito-TPP** and **AIE-Br** at different concentrations and further incubated at 37 °C for 24 h. After washing the cells with PBS buffer, 0.5 mg mL^{-1} MTT solution was added and the cells were kept at 37 °C for 3 h. After MTT medium removal, the formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm using a microplate reader (Genios Tecan). The untreated cells served as the control and their viability was set as 100%.

Concentration-dependent anti-proliferation of AIE-mito-TPP in a panel of normal and cancer cell lines were assessed by MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). Cells were seeded in 96-well plates at 5000 cells/well, after 24 h, different concentrations of AIE-mito-TPP were added and incubated for 24 h. After this, supernatants were removed and each well was washed with PBS. The cells were then cultured in medium for additional 24 h. The MTS solution was added to the culture medium for 2-3 h according to the manufacturer's protocol. The absorbance was monitored at 492 nm using a microplate reader (Genios Tecan) for the cells immediately before drug addition (Az), after 48 h without any treatment (control growth, Ac) and with AIE-mito-TPP treatment (test growth, At). The percentage of growth inhibition was calculated as: [(At-Az)/(Ac-Az)] × 100%. The concentrations led to 50% growth inhibition (GI_{50}) were calculated by nonlinear regression analysis using Origin 8.0® software (OriginLab Corporation).

8. Cellular uptake assay.

HeLa and NIH-3T3 cells at a density of 2×10^5 cells per well were seeded onto 6-well plates and grown for 18 h to achieve 80% confluence. The cells without treatment were used as control. To study the concentration dependent cellular uptake, the cells were incubated

with 0.5, 1 and 2 μM **AIE-mito-TPP** in fresh DMEM medium at 37 °C for 2 h. For the time-dependent cellular uptake, 1 μM **AIE-mito-TPP** in fresh DMEM medium as incubated with cells for 0.5, 1 and 2 h. Then the cells were washed with PBS, trypsinized, and re-suspended in PBS. Flow cytometry measurements were conducted using Cyan-LX (Dako Cytomation) with excitation at 405 nm. The mean fluorescence was determined by counting 10,000 events.

For confocal imaging, the tested cells were cultured in the chambers (LAB-TEK, Chambered Coverglass System) at a density of 5×10^5 per mL for 18 h. The cells were incubated with 1 μM **AIE-mito-TPP** at 37 °C for 2 h, followed by staining with 200 nM MitoTracker red for 45 min. Then the cells were washed with PBS and imaged immediately by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany). The confocal setting was the same as before.

9. Mitochondrial membrane potential and ROS level detection.

Mitochondrial membrane potential was measured by tetramethylrhodamine, ethyl ester (TMRE) dye, which can accumulate in active mitochondria. After the HeLa and NIH-3T3 cells were treated with 1 μM **AIE-Mito-TPP** for 2 h at 37 °C, cells were washed three times with PBS and incubated with 50 nM TMRE for 30 min. Stained cells were washed, incubated with fresh medium and visualized under a laser confocal scanning microscope immediately. **AIE-mito-TPP** was excited at 405 nm and detected at 510-560 nm with green fluorescence; TMRE was excited at 543 nm and detected at 575-625 nm with red fluorescence. For quantitative analysis, cells incubated with different concentrations of **AIE-mito-TPP** were digested, collected and the fluorescence intensity of cells were analyzed by flow cytometry (Dako Cytomation). The histogram of each sample was obtained by counting 10,000 events using the PE-Texas Red filter sets (λ_{ex}= 488 nm, 630/20 nm band pass filter).

The intracellular ROS generation was detected by dehydroethidium (DHE). For confocal

imaging experiments, HeLa and NIH-3T3 cells were seeded in 8-well chamber overnight and 1 μM **AIE-mito-TPP** was incubated with the cells at 37 °C for 2 h. Then the cells were rinsed with PBS for 2-3 times and stained with 10 μM DHE for 30 min. Stained cells were washed, incubated with fresh medium and imaged immediately by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany). For quantitative analysis, different concentrations of **AIE-mito-TPP** treated cells were digested, collected and the fluorescence intensities of cells were analyzed by flow cytometry (Dako Cytomation) using the PE-Texas Red filter sets (λ_{ex}= 488 nm, 630/20 nm band pass filter). Mean intensity of red fluorescence for 10,000 cells was used to compare the ROS generation.

10. Cellular ATP activity assay.

Cellular ATP levels were measured using a firefly luciferase-based ATP assay kit (promega) according to the manufacturer's instructions. Briefly, HeLa cells were seeded in an opaque-walled 96-well for 18 h to achieve 80% confluence. After the cells were incubated with different concentrations of **AIE-mito-TPP** for 24 h, the cells were washed with PBS buffer and 100 μL of PBS was further added to the well. Another 100 μL of the diluted kit reagent was mixed with the cells for 2 min. The Luminance (relative luminescence units, RLU) was measured by a mirco-plate reader (GeniosTecan).

11. Cell cycle analysis.

After the cells were treated with 0.5 and 1 μM **AIE-mito-TPP** for 24 h, the cells were trypsinized, washed, and fixed by 70% ethanol at 4 °C overnight. To determine DNA content, samples were spun down and re-suspended in 500 μL of PBS staining solution containing 100 μg mL^{-1} RNaseA and 50 μg mL^{-1} propidium iodide and incubated at 37 °C for 30 min. The stained cells were analyzed by flow cytometry (Dako Cytomation).

12. Cell apoptosis assay.

The TUNEL assay was used to measure the cell apoptosis. **AIE-mito-TPP** treated cells

were washed and fixed with 4% paraformaldehyde for 20 min. The cells were washed with PBS, permeabilized in 0.2% Triton X-100 and labeled with terminal deoxynucleotidyl transferase (TdT) reaction mixture before further incubation at 37 °C for 60 min. Next, the cells were rinsed with cold PBS and re-suspended in 100 μL of streptavidin HRP for 30 min. The cells were then rinsed with PBS, stained with 100 μL of 3,3'-diaminobenzidine (DAB) solution until brown background appeared and the images were taken under microscopy with a 20× objection (Nikon).

13. Cell migration assay.

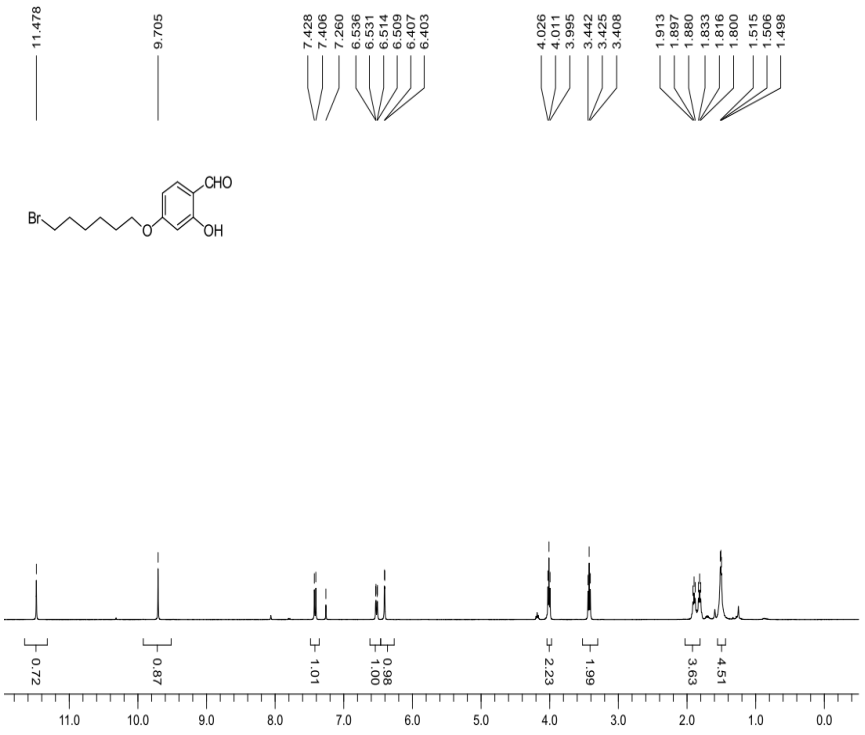
HeLa cells were seeded into 6-well plates and grown for 24 h. Cell scratch spatula was applied to make a scratch in the cell monolayer. After rinsing the HeLa cells with PBS, 0, 0.5 or 1 μM **AIE-mito-TPP** was added to the cells. The pictures of the scratches were taken using a digital camera coupled to a microscope with a 10× objection (Nikon). The cells were further incubated for 48 h, after which the pictures were taken again.

Table S1. Antiproliferative activity of **AIE-mito-TPP** in various normal and cancer cell lines (GI_{50} μM)[a]

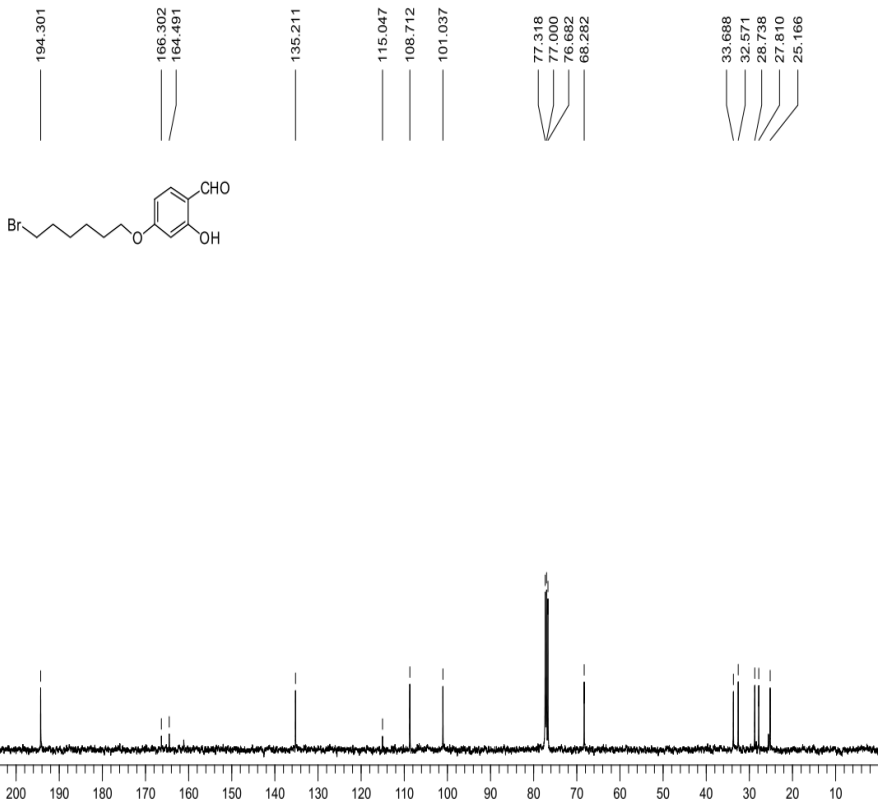
Cell lines	HeLa	U87-MG	HepG2	MDA-MB-231	MCF-7	NHDF	HK2	MRC5	NIH-3T3
GI_{50} (μM)	0.17± 0.01	0.23± 0.03	0.33± 0.05	0.19± 0.03	0.45± 0.06	2.35± 0.09	3.26± 0.12	1.31± 0.15	1.83± 0.10

[a]GI_{50} is the concentration that causes 50% cell growth inhibition. Values are the mean of the three experiments and calculated by nonlinear regression analysis using Origin 8.0® software (OriginLab Corporation).

HeLa: human cervix adenocarcinoma cells; U-87 MG: human glioblastoma-astrocytoma, epithelial-like cells; HepG2: human liver hepatocellular carcinoma cells; MDA-MB-231: human breast adenocarcinoma cells; MCF-7: human breast cancer cells; NHDF: normal-human primary dermal fibroblast cells; HK2: human proximal tubular epithelial cells; MRC5: human lung fibroblast cells; NIH-3T3: mouse embryo fibroblast cells.

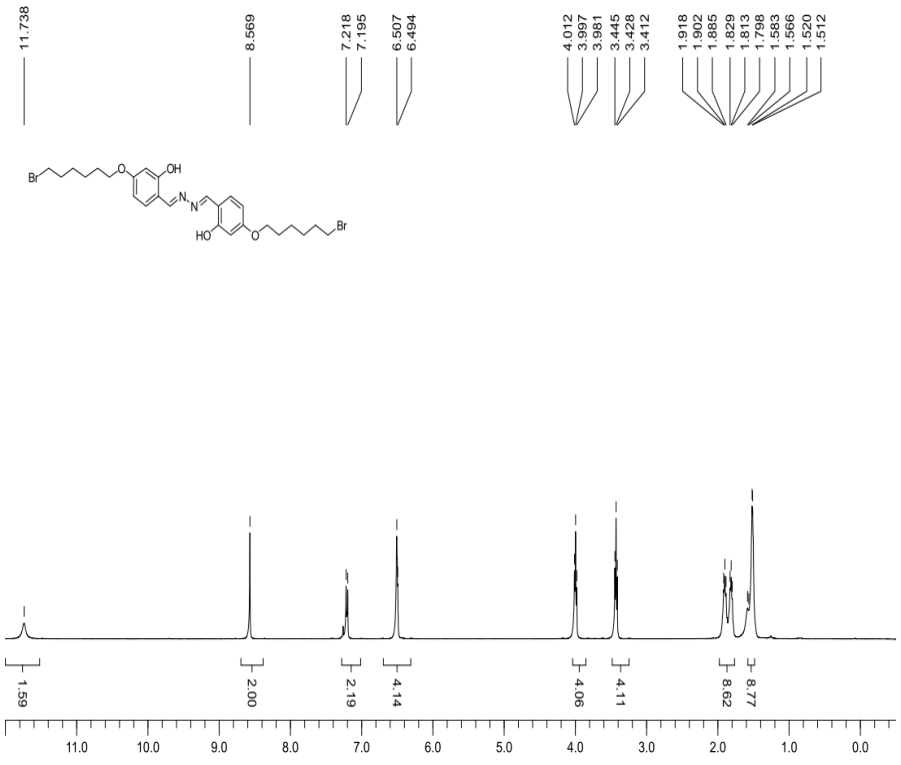


Chemical shift (ppm)

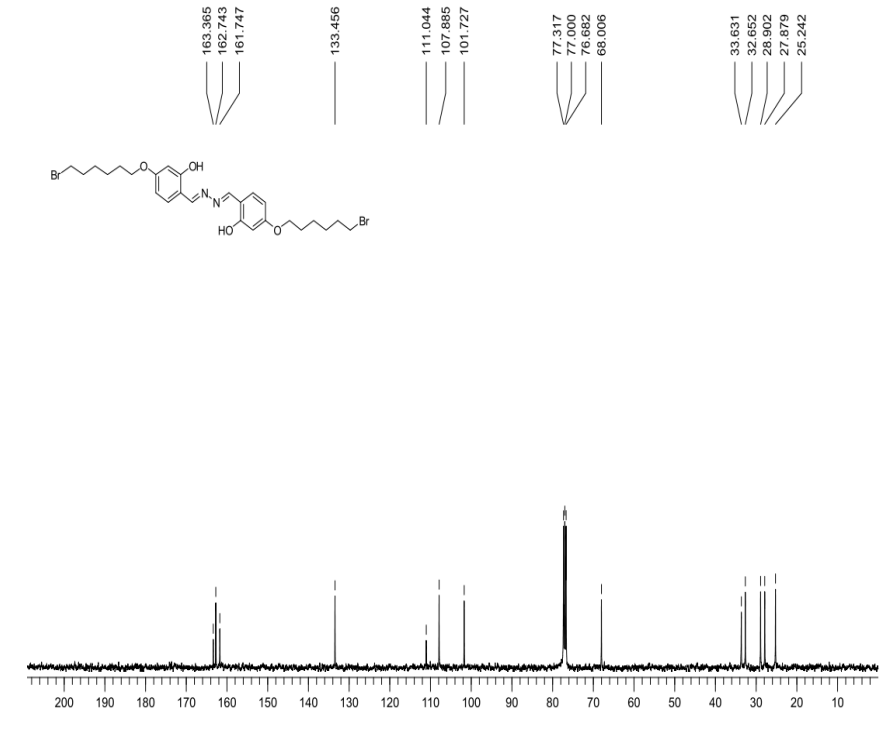


Chemical shift (ppm)

Figure S1. ^{1}H NMR and ^{13}C NMR spectra of **2** in $CDCl_3$.

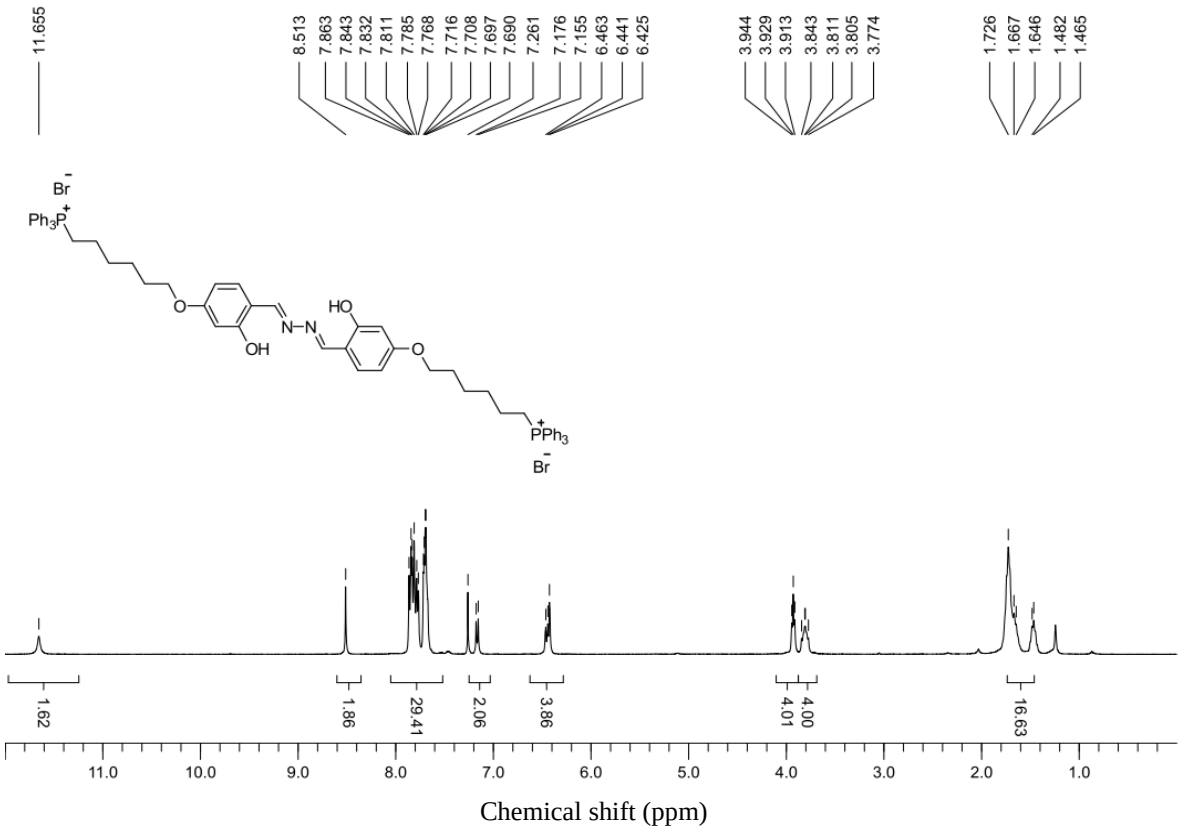


Chemical shift (ppm)

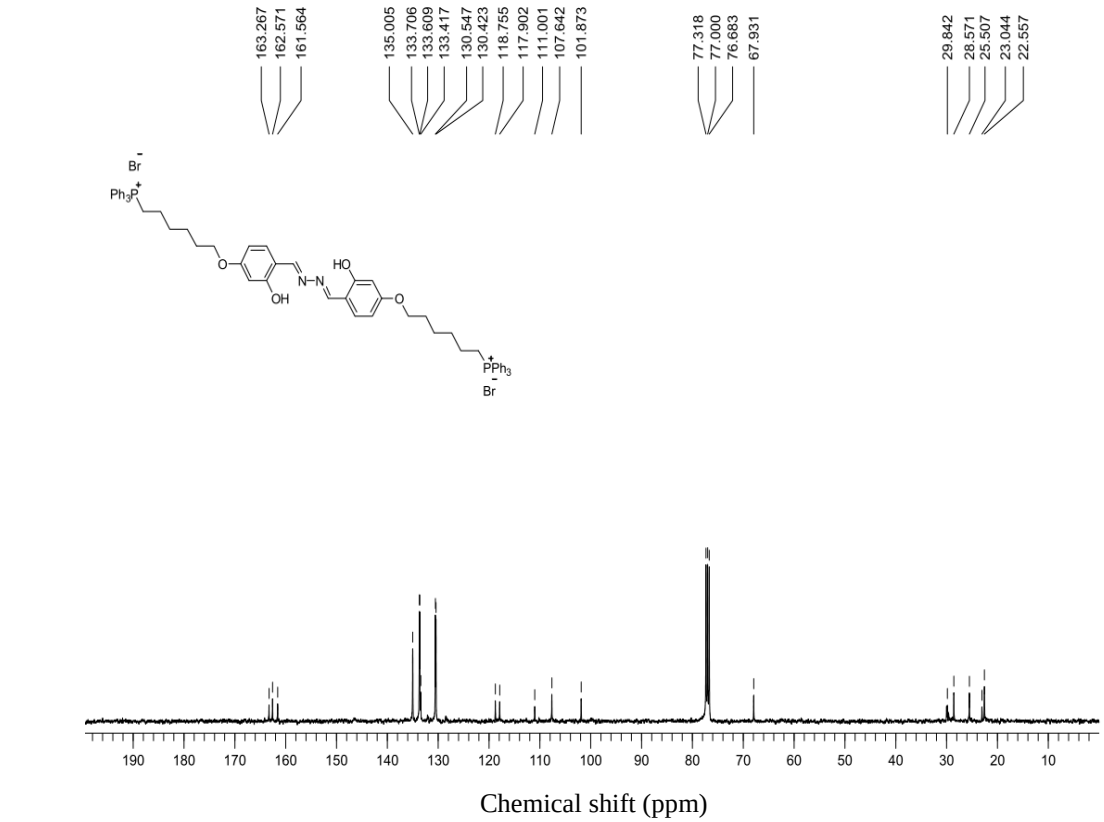


Chemical shift (ppm)

Figure S2. ^{1}H NMR and ^{13}C NMR spectra of **AIE-Br** in $CDCl_3$.



Chemical shift (ppm)



Chemical shift (ppm)

Figure S3. ^{1}H NMR and ^{13}CNMR spectra of **AIE-mito-TPP** in $CDCl_3$.

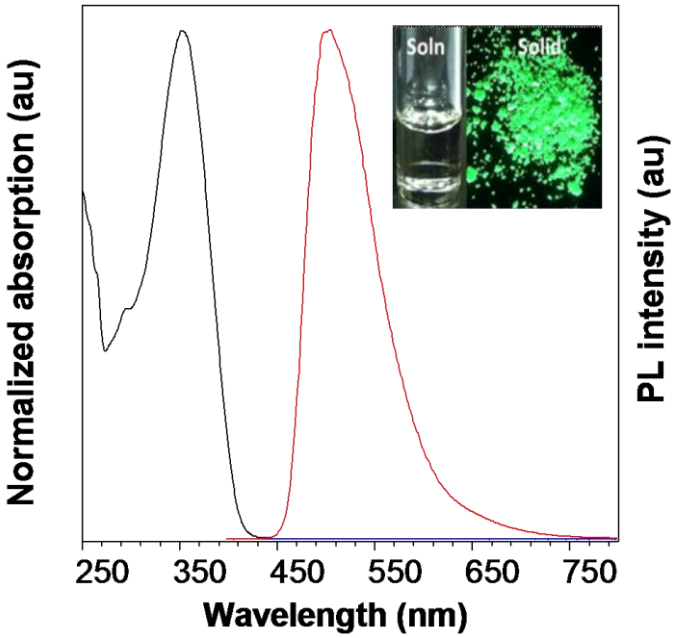


Figure S4. Normalized UV (black) and PL (blue) spectra of 10 μM **AIE-mito-TPP** in DMSO; the PL spectrum of **AIE-mito-TPP** in solid state is shown in red. Excitation wavelength: 356 nm.

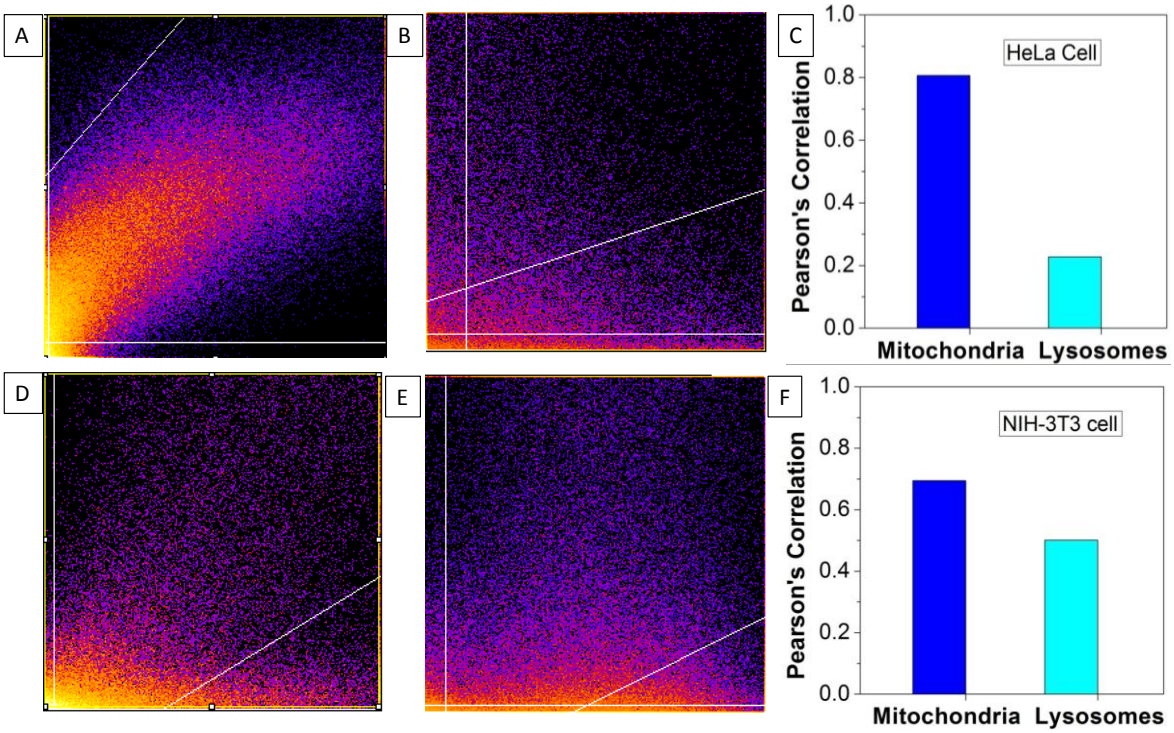


Figure S5. Colocalization scatter plot for **AIE-mito-TPP** in mitochondria and lysosomes of HeLa cells (A, B) and NIH-3T3 cells (D, E); comparison of calculated Pearson's correlation coefficients for **AIE-mito-TPP** in mitochondria and lysosomes of HeLa (C) and NIH-3T3 cells (F).

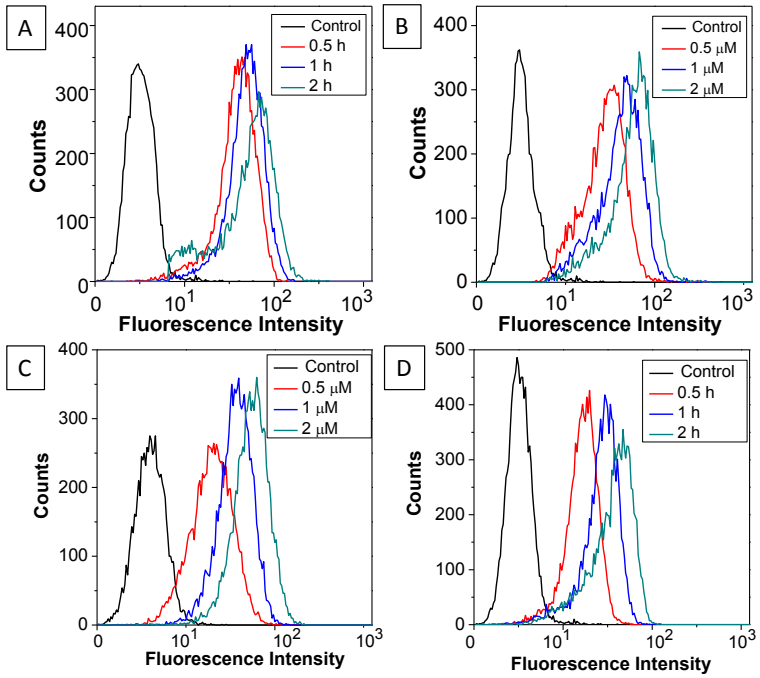


Figure S6. Dynamic fluorescence intensity of **AIE-mito-TPP** in HeLa and NIH-3T3 cells at different time points studied by flow cytometry (A, C); fluorescence intensities of HeLa and NIH-3T3 cells upon incubation with different concentrations of **AIE-mito-TPP** for 2 h (B, D).

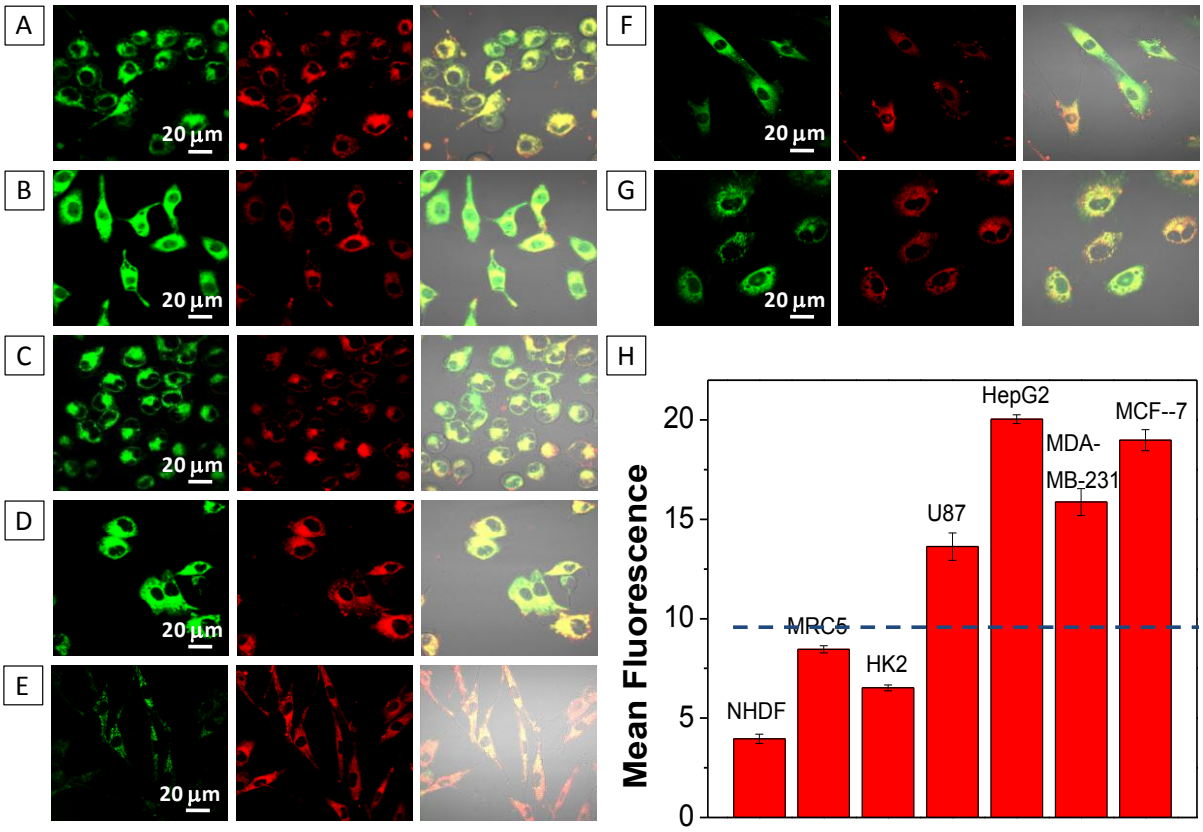


Figure S7. The confocal images of U87-MG (A), HepG2 (B), MDA-MB-231(C) and MCF-7 (D), NHDF cells (E), MRC5 (F) and HK2 (G) upon incubation with 1 μM **AIE-mito-TPP** at 37 °C for 2 h, followed by co-staining with 200 nM MitoTracker; (H) The mean fluorescence intensity of **AIE-mito-TPP** in a panel of cancer and normal cell lines quantified by Image J software. All images share the same scale bar of 20 μm.

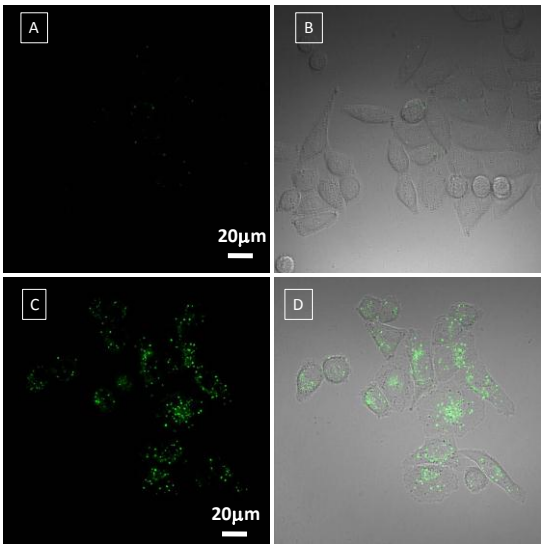


Figure S8. The confocal images of HeLa cells upon incubation with (A, B) 1 μM **AIE-Br** and (C, D) 10 μM **AIE-Br** at 37 °C for 2 h. All images share the same scale bar of 20 μm.

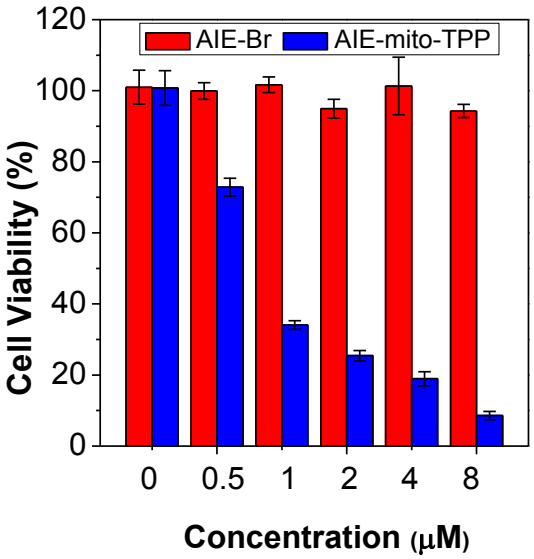


Figure S9. Cell viability of MDA-MB-231 cells after incubation with different concentrations of **AIE-Br** and **AIE-mito-TPP** for 24 h.

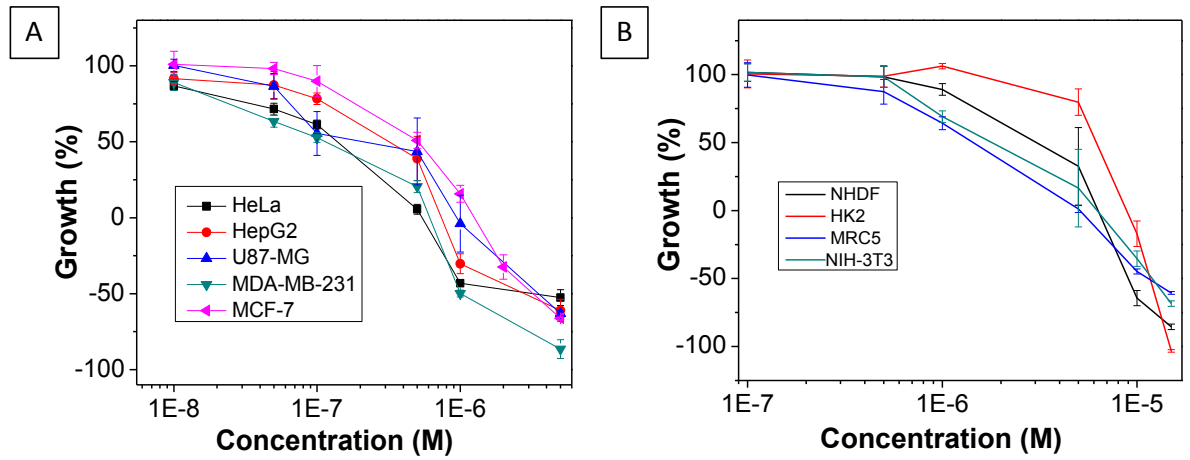


Figure S10. Concentration-dependent-response curves of **AIE-mito-TPP** in different normal cell lines (A) and cancer cell lines (B) analyzed by MTS assays after 24 h exposure to **AIE-mito-TPP** and further 24 h incubation.

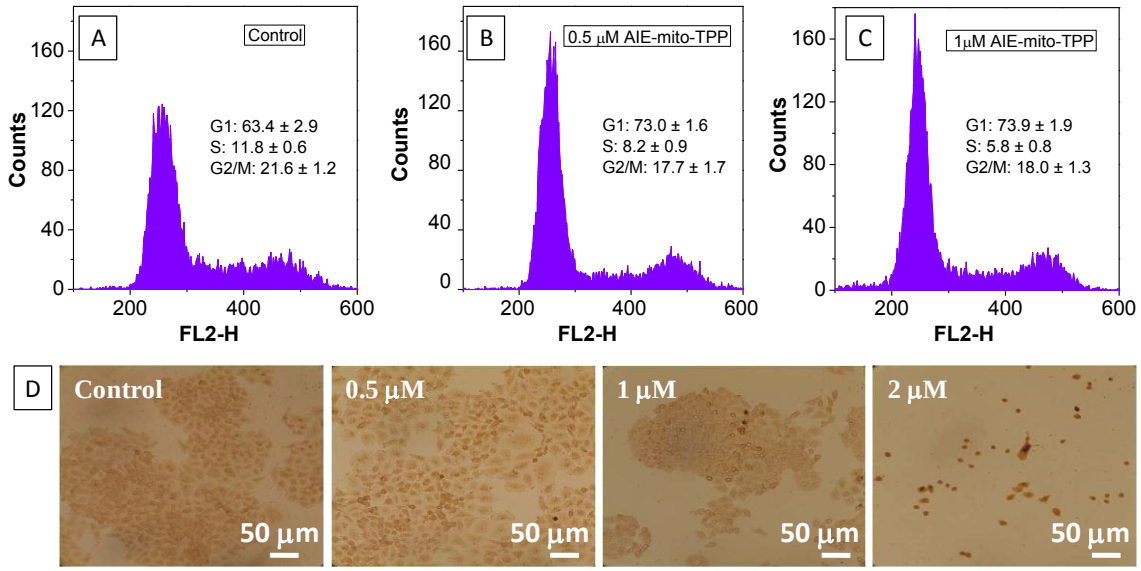


Figure S11. Flow cytometric analysis for cell cycle distribution of HeLa cells by staining with propidium iodide after treatment with 0 μM (A), 0.5 μM (B) and 1 μM (C) **AIE-mito-TPP** for 24 h; (D) TUNEL stained images of HeLa cells upon incubation with different concentrations of **AIE-mito-TPP** for 24 h.

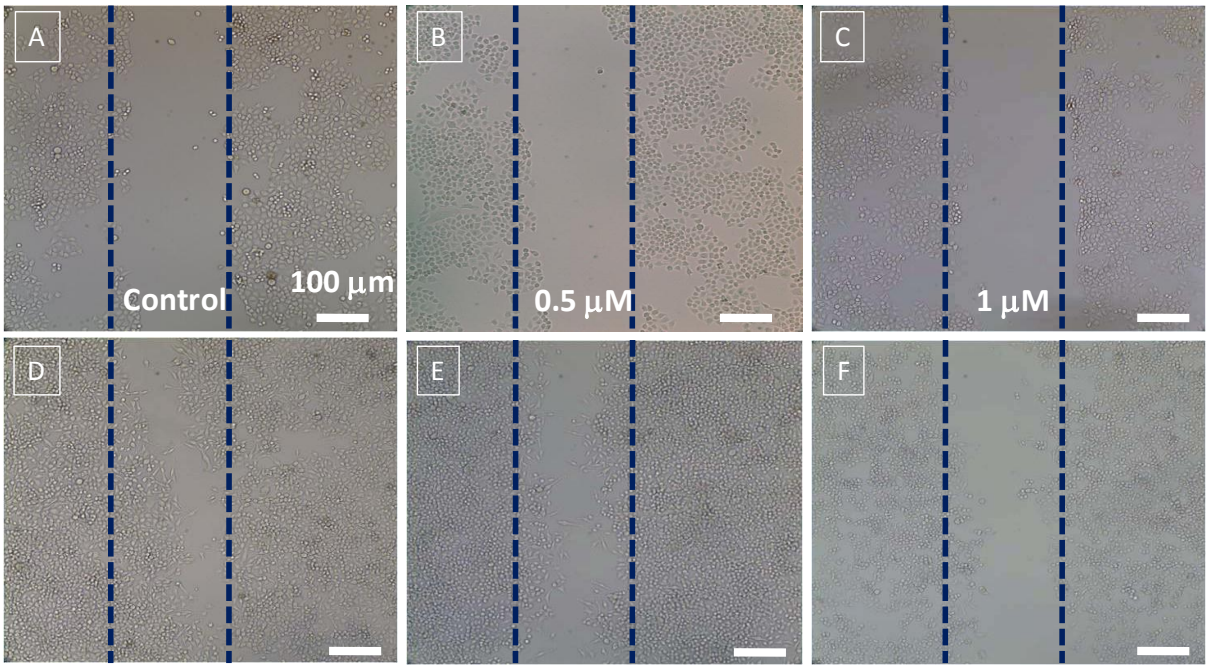


Figure S12. Phase contrast images of HeLa cells before (A, B and C) and after 48 h (D, E and F) incubation with 0.5 or 1 μM **AIE-mito-TPP.** All images share the same scale bar of 100 μm.