Supporting Information

Near-infrared ^{10}B-BODIPY for the Precise Guidance of Tracer Imaging and Treatment in Boron Neutron Capture Therapy

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SUPPLEMENTAL MATERIALS AND METHODS

Experimental section

General Measurements. ^{1}H NMR and ^{11}B NMR was performed by 500 M digital nuclear magnetic resonance spectrometer (Avance NEO 500). Mass spectrum was performed by Matrix-assisted laser desorption / ionization time of flight mass spectrometry (MALDI-TOF). Ultraviolet absorption spectrum was performed by the ultraviolet spectrophotometer (PerkinElmer, Lambda 265). Fluorescence emission spectroscopy was performed by the fluorescence spectrophotometer (CARY-Eclipse). Cellular uptake was observed by Confocal laser scanning microscopy (CLSM, Olympus FluoView 3000). Differences and rates of cellular uptake was performed and analyzed by Flow cytometry (Amnis Flowsight) and Flowjo software. In vivo imaging observed and analyzed by the IVIS Lumina LT small animal live optical imaging system (PerkinElmer).

1. Materials and methods

1.1 Materials

2, 4-dimethylpyrrole and 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. Magnesium powder, 4-bromobenzaldehyde diethyl acetal and boron trifluoride diethyl etherate was purchased from Anhui Zesheng Technology Co., LTD. ^{10}B-Boric acid (>99%) was purchased from Dalian Boentan Sci&Tech Co., Ltd. RM-1 and B16F10 cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell counting kit-8 (CCK-8), Phosphate buffer saline (PBS) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from purchased from Beyotime Institute of Biotechnology (Shanghai, China). Kunming mice (4-5 weeks old, male, 18-20 g) and C57BL6/j (4-weeks old, female, 18-20 g) mice were provided by Jilin University, and animals were housed in SPF-grade rooms (25±2 °C, 50±5% relative humidity). All animal experiments were approved and carried out according to the guidelines of the Ethical Committee of Northeast Normal University (Approval numbers: 202402042).

1.2 Methods

Synthesis of major compounds

Synthesis of ^{10}B-trimethyl borate: ^{10}B-boric acid and methanol are heated at 55°C for 5 h, and then distilled to obtain a mixture of ^{10}B-boric acid and methanol. Adding anhydrous calcium chloride for shock, stratification, the supernatant is the target product.

Synthesis of BDP: Benzaldehyde (1 eq.) was dissolved in dichloromethane, followed by 2, 4-dimethylpyrrole (2.2 eq.) and a small amount of trifluoroacetic acid (0.05 eq.), and stirred at room temperature under nitrogen protection. After 12 h reaction, 2, 3-dichloro-5, 6-dicyano-p-benzoquinone was added. Continue stirring for 2 h and slowly add triethylamine (7 eq.) and boron trifluoride ether (9 eq.). The mixed solution was stirred for 2 h, washed with saturated salt water, collected the organic layer, dried with anhydrous magnesium sulfate, and then evaporated by rotation to obtain the crude product concentrate. Finally, the yellowish orange powder was obtained by silica gel column purification Yield:23%. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.49-7.47 (m, 3H), 7.29-7.27 (m, 2H), 5.98 (s, 2H), 2.56 (s, 6H), 1.38 (s, 6H).

Synthesis of ^{10}B-(4-formylphenyl) boronic acid: Under argon, THF (2M) and magnesium powder (2 eq.) are added to the flask. The THF solution (1 eq.) of 4-bromobenzaldehyde diethyl acetal is then dropped through a drip funnel for more than 15 minutes. After stirring under reflux for 1 h, the mixture cooled to room temperature. At -50°C, in trimethyl borate (2.2 eq.) dried tetrahydrofuran, a previously prepared solution of 4-(diethoxymethyl) phenyl magnesium bromide is dropped, the mixture is kept at this temperature for 1 h, and then it is allowed to heat to 0°C. Ether is added, then an aqueous solution of sulfuric acid (3 eq.) is added, the mixture is extracted with ether, and the organic phase is dried on anhydrous magnesium sulfate. The solvent was removed under reduced pressure and recrystallized to obtain the ^{10}B-(4-formylphenyl) boronic acid. Yield:35%. ^{1}H NMR (500 MHz, DMSO-d6) δ 10.02(s, 1H), 8.34 (brs, 2H), 7.96 (d, 2H, *J*=8.0 Hz), 7.85 (d, 2H, *J*=8.0 Hz).

Synthesis of PBA-BDP**:** ^{10}B-(4-formylphenyl) boronic acid: (2.5 eq.), BDP (1 eq.), acetic acid (20 eq.) and tetrahydropyrrole (20 eq.) were mixed, and the PBA-BDP was separated by column chromatography after reflux at 95°C under acetonitrile for 5 h. Yield:12%. **^{1}H NMR** (500 MHz, DMSO-d6) δ 7.57-7.56 (m, 9H), 7.46-7.44 (m, 6H), 7.40-7.39 (m, 4H), 6.91-6.85 (m, 4H), 6.17 (s, 2H), 1.39 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 157.54, 141.90, 137.61, 137.49, 135.02, 134.44, 133.73, 133.50, 133.33, 132.19, 131.08, 129.02, 128.96, 128.77, 120.52, 15.03.**^{11}B NMR** (160 MHz, DMSO-d6) δ 20.76, 0.93. MS (m/z) (MALDI): calcd for: $C_{33}H_{30}B^{10}B_2F_2N_2O_4^+$ $[M+H]^+$:587.3, found 587.2.

Analytical method of molecular docking

Among these receptors, the selectin family (e.g., E-selectin, L-selectin, and P-selectin) is a notable example. In this study, molecular docking with E-selectin—a key member of the selectin family—was employed to validate the rational design of the compound. To analyze the binding affinities and modes of interaction between the drug candidate and their targets, AutodockVina 4.0, a silico protein–ligand docking software was employed. The molecular structures of ENMD-2076 was retrieved from PubChem Compound (https://pubchem.ncbi.nlm.nih.gov/). The 3D coordinates of E-selectin (Uniprot ID P16581) were downloaded from the PDB (http://www.rcsb.org/pdb/home/home.do). For docking analysis, all protein and molecular files were converted into PDBQT format with all water

molecules excluded and polar hydrogen atoms were added. The grid box was centered to cover the domain of each protein and to accommodate free molecular movement. Molecular docking studies were performed by Autodock Vina 1.2.2 (http://autodock.scripps.edu/).

Cellular uptake of B16F10 or RM-1 cells and L929 cells

In 6 well plates containing cover glass, B16F10 or RM-1 cells were planted at a concentration of 5×10^4 /mL. After 12 h, solution of PBA-BDP were added at 0,1,2 and 4 h respectively to achieve a final concentration of 5 μg/mL. The cells were then washed 3 times with PBS, stained with DAPI, and washed again with PBS for 3 times. The cover glass was mounted upside down on a glycerol-containing tablet and sealed with nail polish. The results were observed using laser confocal microscopy or fluorescence microscopy.

Additionally, B16F10, RM-1 and L929 cells were cultured in 12 well plates at a suspension concentration of 1×10^5/mL. After 12 h, solution of PBA-BDP were added at 1, 2 and 4 h respectively to achieve a final concentration of 5 μg/mL. After incubation, the cells were washed once with PBS, digested by pancreatic enzymes, centrifuged to remove the supernatant, and then washed twice with 90% ethanol. Then 100-120 μL PBS was added and dispersed, and analyzed by flow cytometry.

Cellular uptake of B16F10 or RM-1 cells after treatment of PBA

In 6 well plates containing cover glass, B16F10 or RM-1 cells were planted at a concentration of 5×10^4 /mL. After 12 h, solution of PBA was added to achieve a final concentration of 100 μg/mL. After 24 h, solution of PBA-BDP were added at 0, 1, 2 and 4 h respectively to achieve a final concentration of 5 μg/mL. The cells were then washed 3 times with PBS, stained with DAPI, and washed again with PBS for 3 times. The cover glass was mounted upside down on a glycerol-containing tablet and sealed with nail polish. The results were observed using laser confocal microscopy or fluorescence microscopy.

Additionally, B16F10 or RM-1 cells were cultured in 12 well plates at a suspension concentration of 1×10^5/mL. After 12 h, solution of PBA was added to achieve a final concentration of 100 μg/mL. After 24 h, solution of PBA-BDP were added at 1, 2 and 4 h respectively to achieve a final concentration of 5 μg/mL. After incubation, the cells were washed once with PBS, digested by pancreatic enzymes, centrifuged to remove the supernatant,

and then washed twice with 90% ethanol. Then 100-120 μL PBS was added and dispersed, and analyzed by flow cytometry.

Killing efficacy of BNCT in vitro.

B16F10 and RM-1 cells were seeded in 96 well plates at a suspension concentration of 5000. After 24 h, the culture medium was removed, a new 0.2 mL culture medium was added, and different concentrations of PBA-BDP solution were added, so that the final concentrations were 50, 40, 30, 20, 10, 0 μg/mL, respectively. Two control groups were established: a group containing both medium and cells, and a group containing medium but no cells. The experiment was divided into two groups, one group was cultured for 48 h, the other group was cultured for 12 h, then received thermal neutron irradiation for 25 minutes, and then cultured for another 24 h. After 10% CCK-8 was added to each well, the medium was discarded after a further 4 h of incubation at 37°C. The absorbance at 450 nm was measured by enzyme-labeled instrument.

In vivo imaging

0.1 mL RM-1 cell suspension was adjusted to 5×10^6/mL, and then subcutaneously injected into the right leg of Male Kunming mice to establish a subcutaneous tumor model of RM-1. After tumor formation, 1 mg/kg PBA-BDP (PBS solution of 5% ethanol) was injected into the tail vein. At 0, 1, 2, 4, 6, 8 and 10 h after injection, the fluorescence distribution of PBA-BDP in mice and the fluorescence intensity of tumors and organs were observed by VIS Lumina LT small animal live optical imaging system (PerkinElmer).

In vivo release of PBA-BDP

The subcutaneous tumor model of prostate cancer was established by subcutaneous injection of RM-1 cells with a concentration of 5×10^6/mL into the right leg of male Kunming mice. After tumor formation, 100 mg/kg PBA-BDP (PBS solution of 5% ethanol) was injected into the tail vein. The mice were respectively euthanized at 2, 4, 6, and 8 h. Subsequently, the tumors were excised, rinsed with normal saline solution, weighed, digested using concentrated nitric acid and hydrogen peroxide, and finally analyzed for boron concentration using inductively coupled plasma emission spectrometry (ICP-OES). Determine the concentration of ^{10}B at each time.

Determination of ^{10}B in blood

The subcutaneous tumor model of prostate cancer was established by subcutaneous injection

of RM-1 cells with a concentration of 5×10^6/mL into the right leg of male Kunming mice. After tumor formation, 100 mg/kg PBA-BDP (PBS solution of 5% ethanol) was injected into the tail vein. After 2, 4, 6, and 8 h, blood samples were collected and weighed, followed by heating with concentrated nitric acid and hydrogen peroxide for subsequent determination of ^{10}B concentration by ICP-OES after clarification.

Tumor suppression assay.

The subcutaneous tumor model of prostate cancer was established by subcutaneous injection of RM-1 cells with a concentration of 5×10^6/mL into the right leg of male Kunming mice. Tumor-bearing mice were randomly divided into the control group, the PBA-BDP group, the neutron group and the BNCT group, with 7 mice in each group. The control group was not subjected to any form of intervention. The PBA-BDP exclusively received intratumoral injection of PBA-BDP solution. the neutron group was exclusively irradiated with thermal neutrons for 1 h. Mouse in the BNCT group were treated with thermal neutron radiation for 1 h after intratumoral injection of 100 mg/kg PBA-BDP (PBS solution of 5% ethanol).

2. Supporting Figures

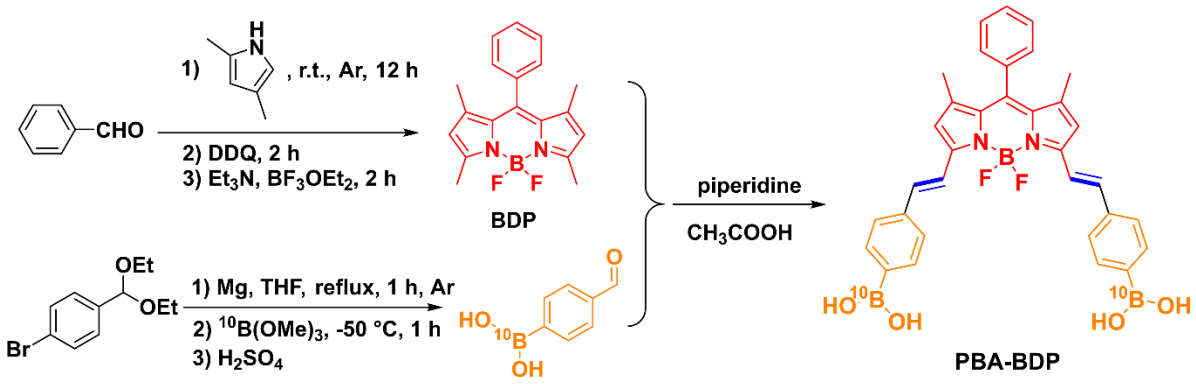

Figure S1 Synthetic route of PBA-BDP.

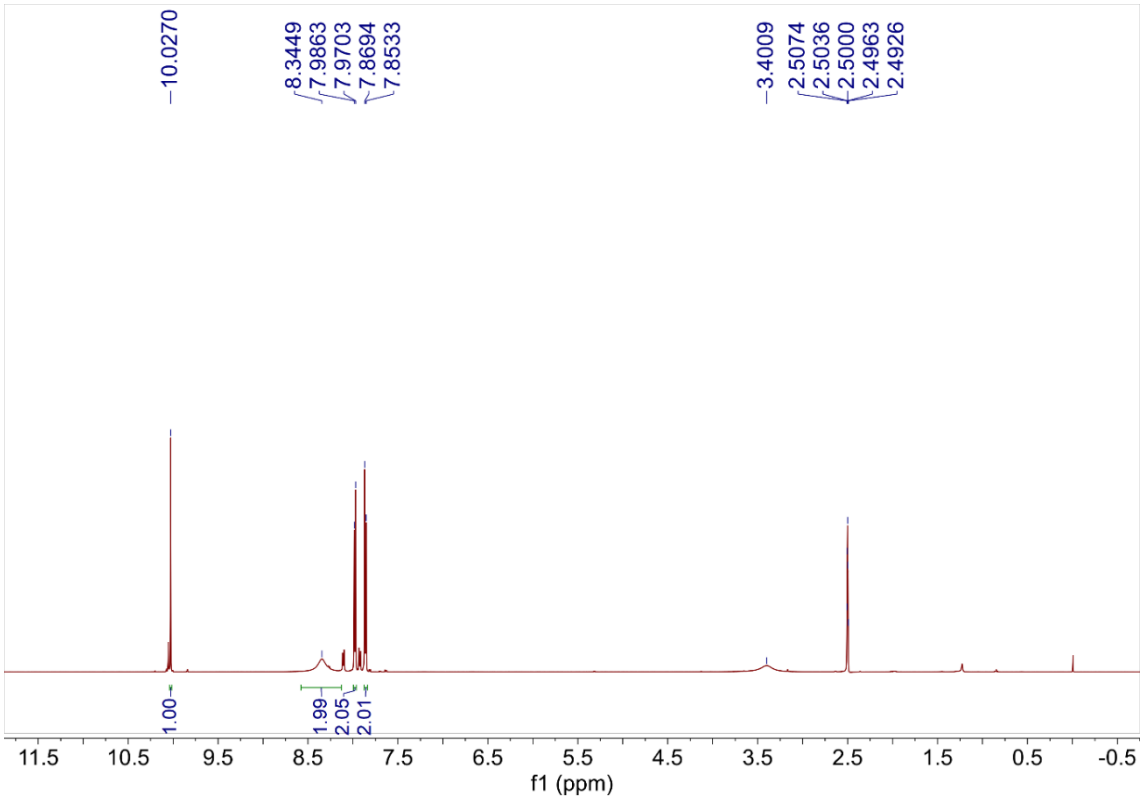

Figure S2 ^{1}H NMR of ^{10}B-(4-formylphenyl) boronic acid**.**

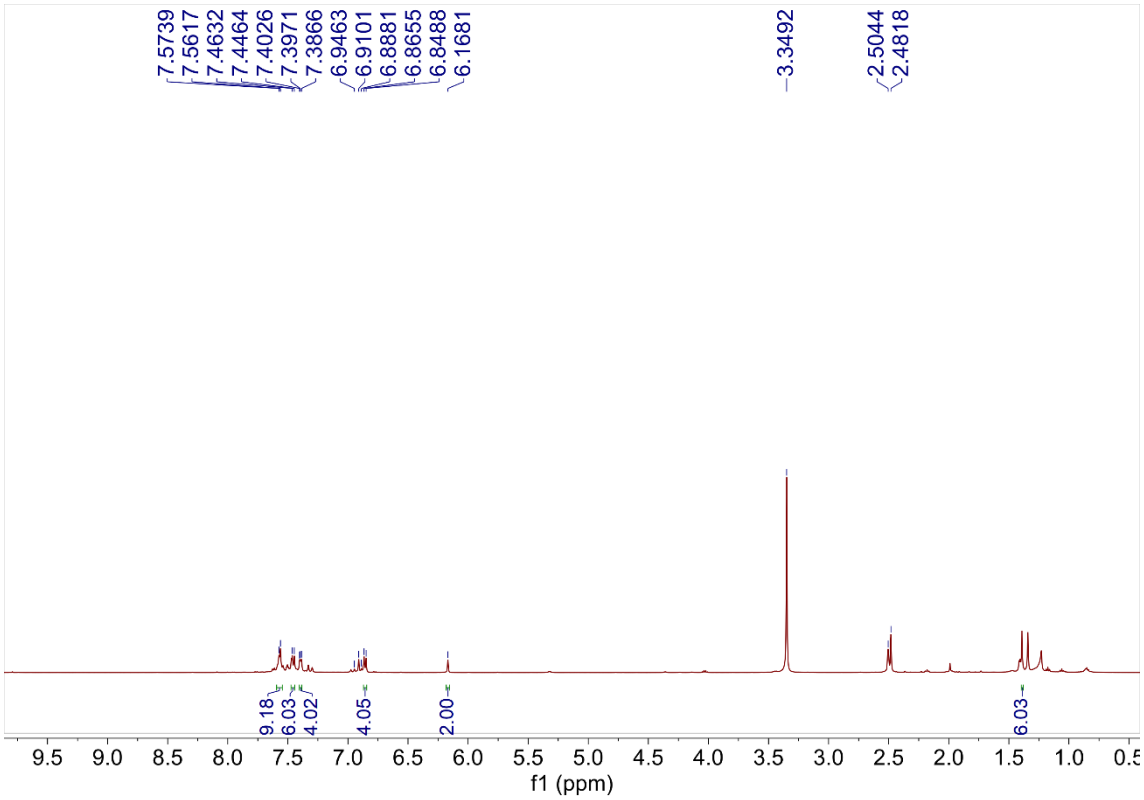

Figure S3 ^{1}H NMR of PBA-BDP.

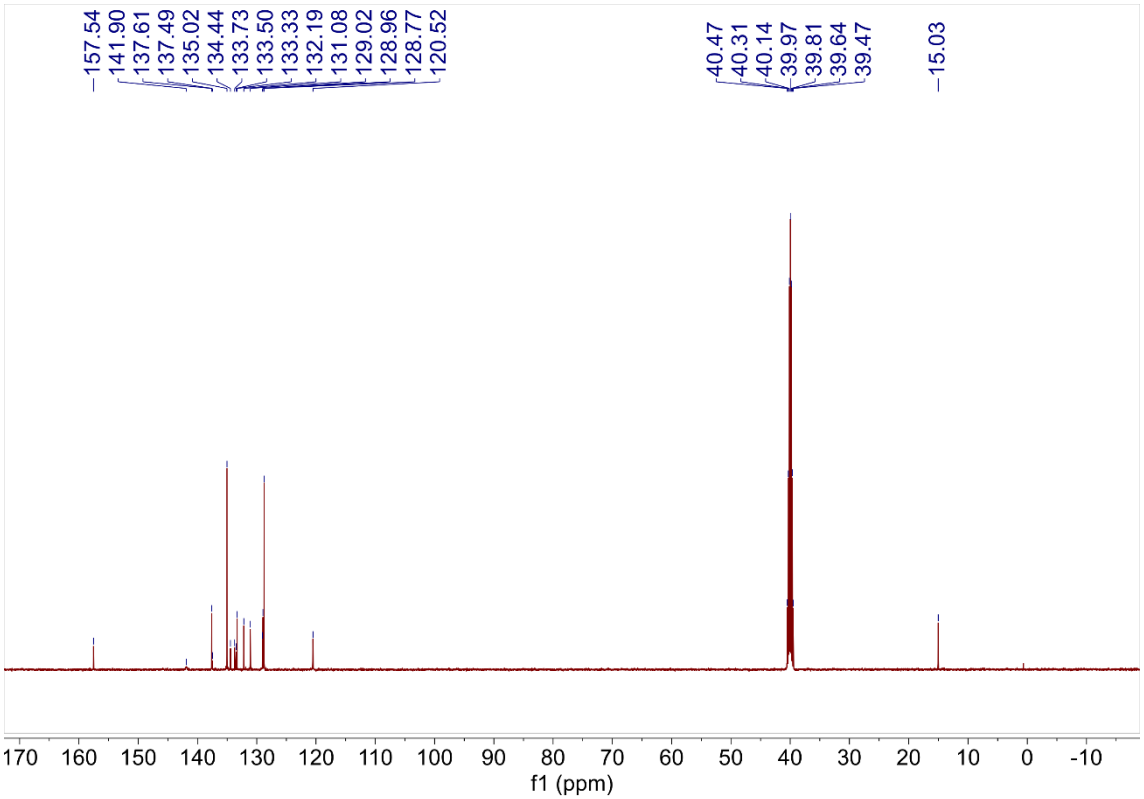

Figure S4 ^{13}C NMR of PBA-BDP.

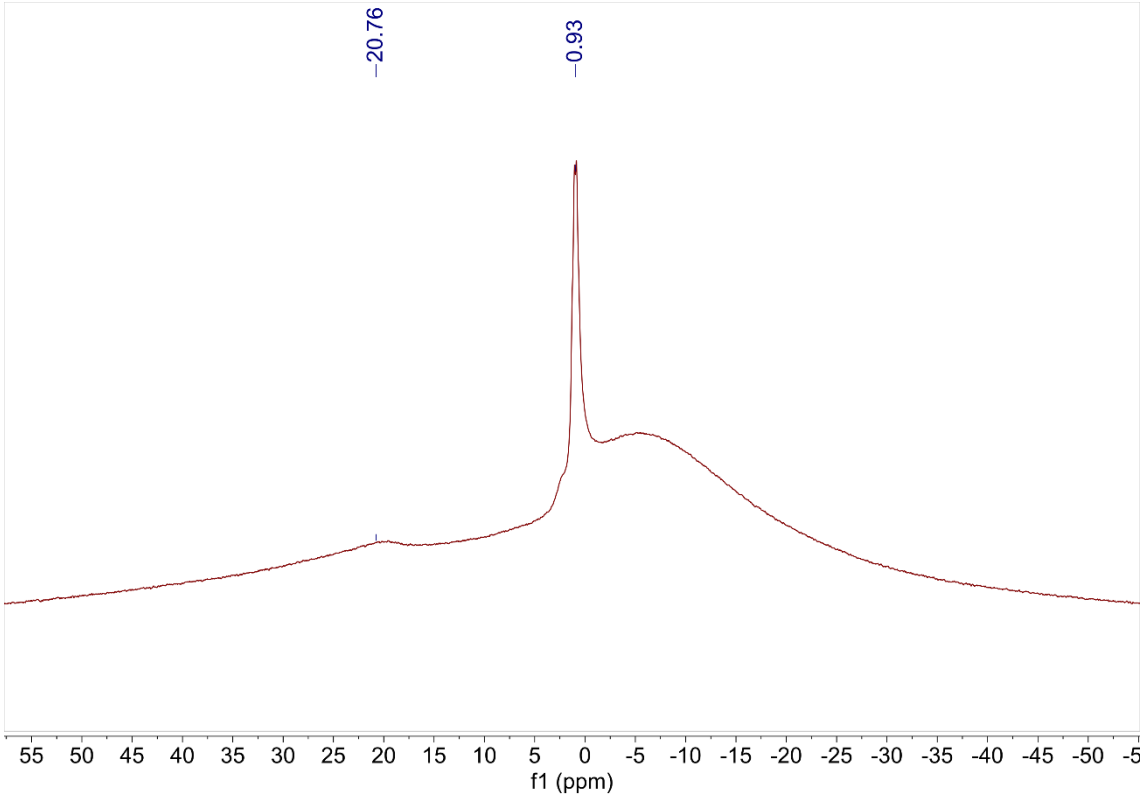

Figure S5 ^{11}B NMR of PBA-BDP.

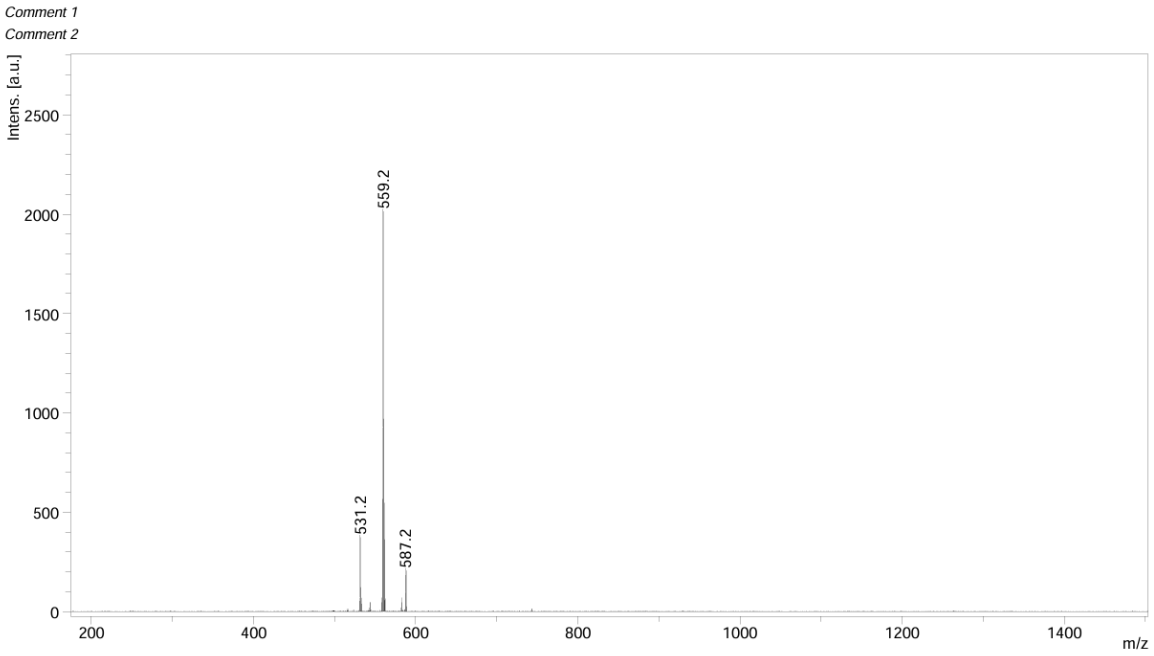

Figure S6 MS of PBA-BDP.

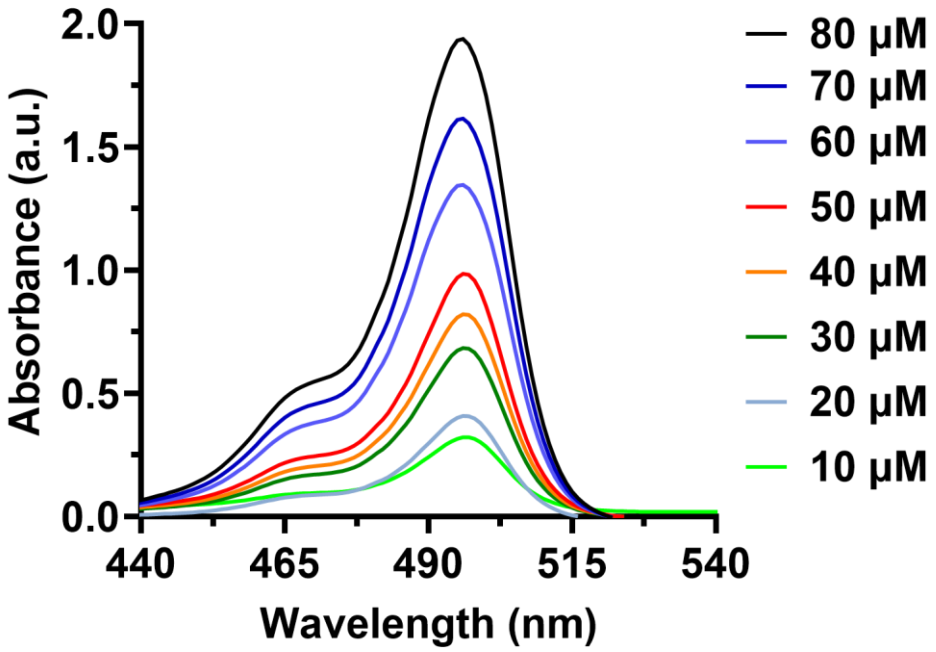

Figure S7 UV-VIS absorption spectra of ethanol with different concentrations of BDP.

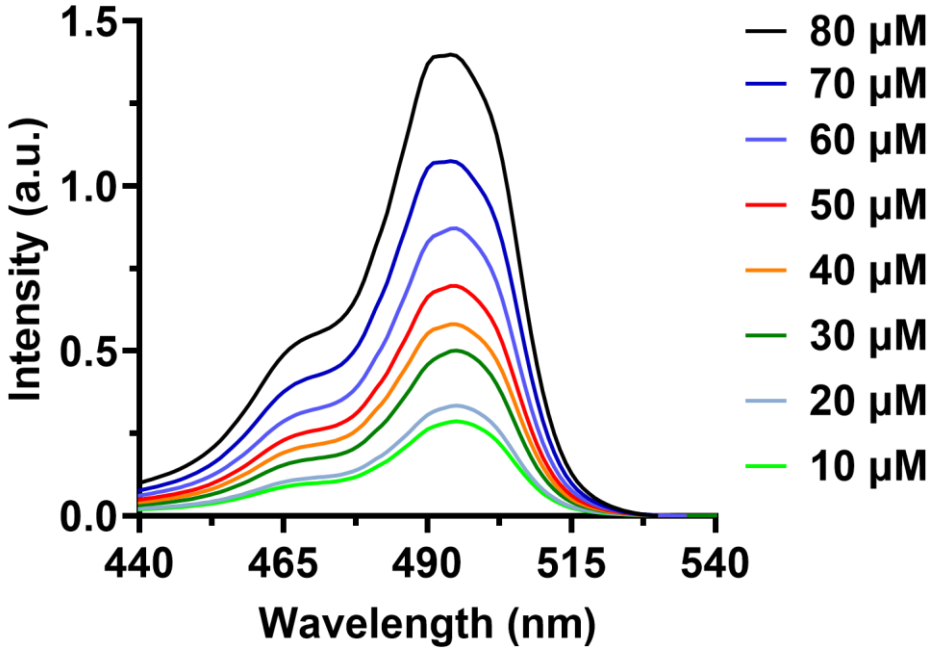

Figure S8 UV-VIS absorption spectra of PBS with different concentrations of BDP.

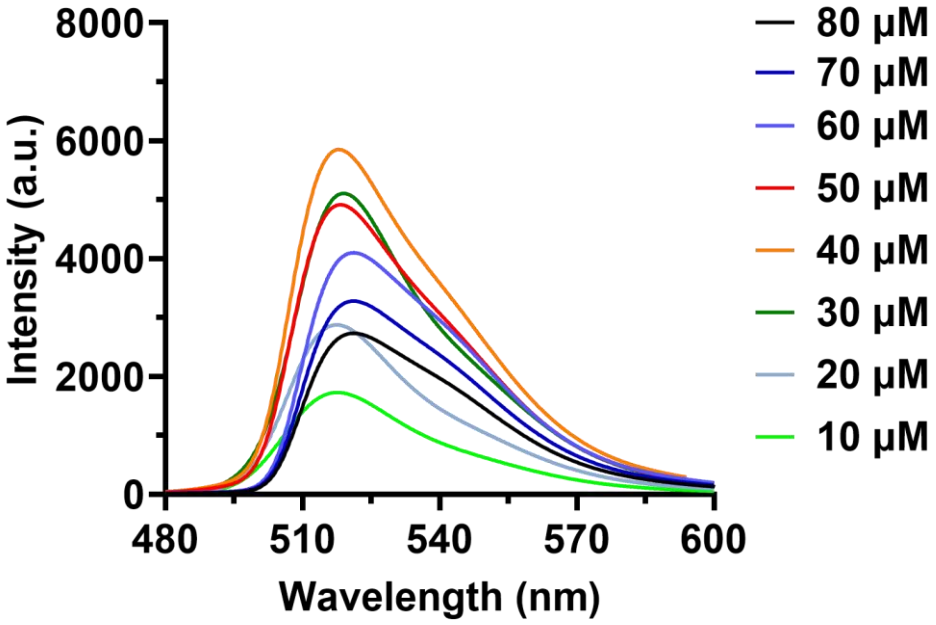

Figure S9 FL spectra of ethanol with different concentrations of BDP.

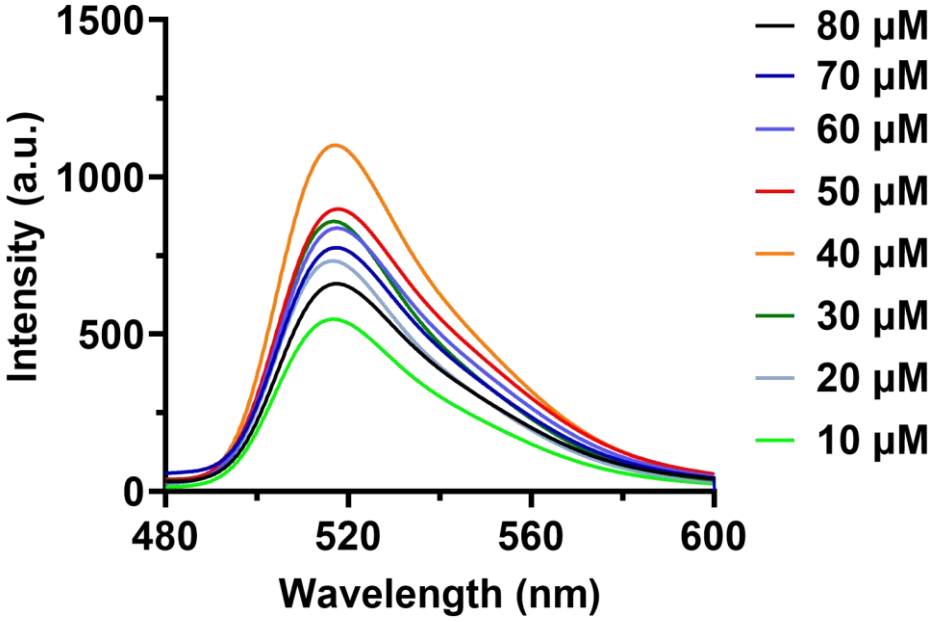

Figure S10 FL spectra of PBS with different concentrations of BDP.

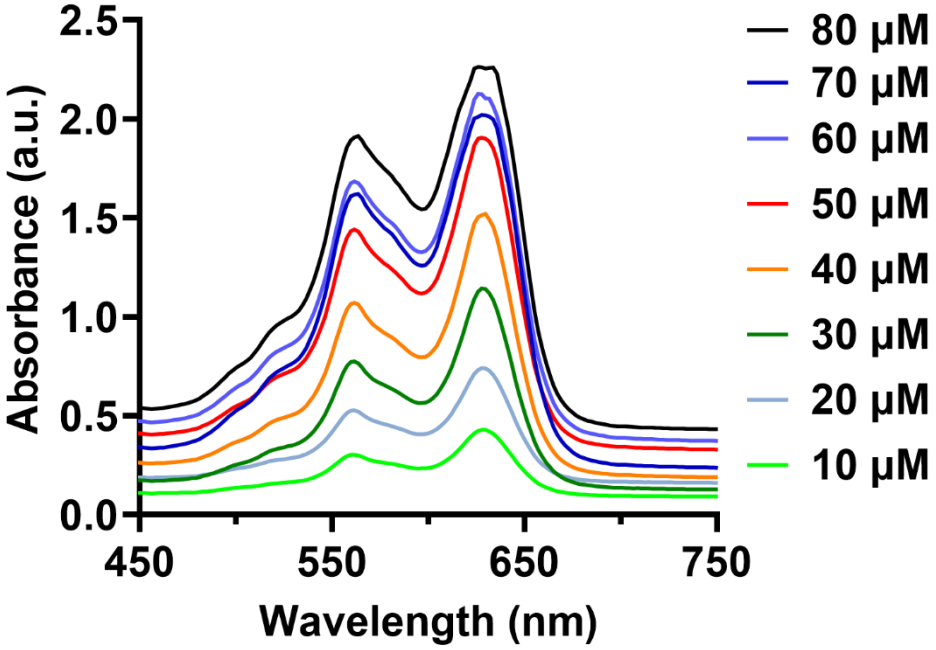

Figure S11 UV-VIS absorption spectra of ethanol with different concentrations of PBA-BDP.

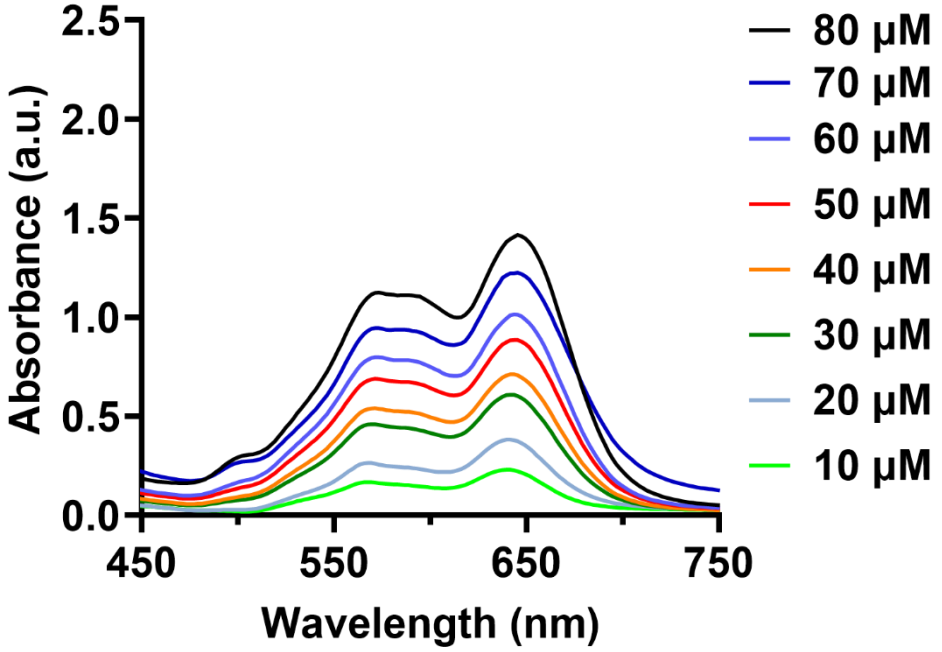

Figure S12 UV-VIS absorption spectra of PBS with different concentrations of PBA-BDP.

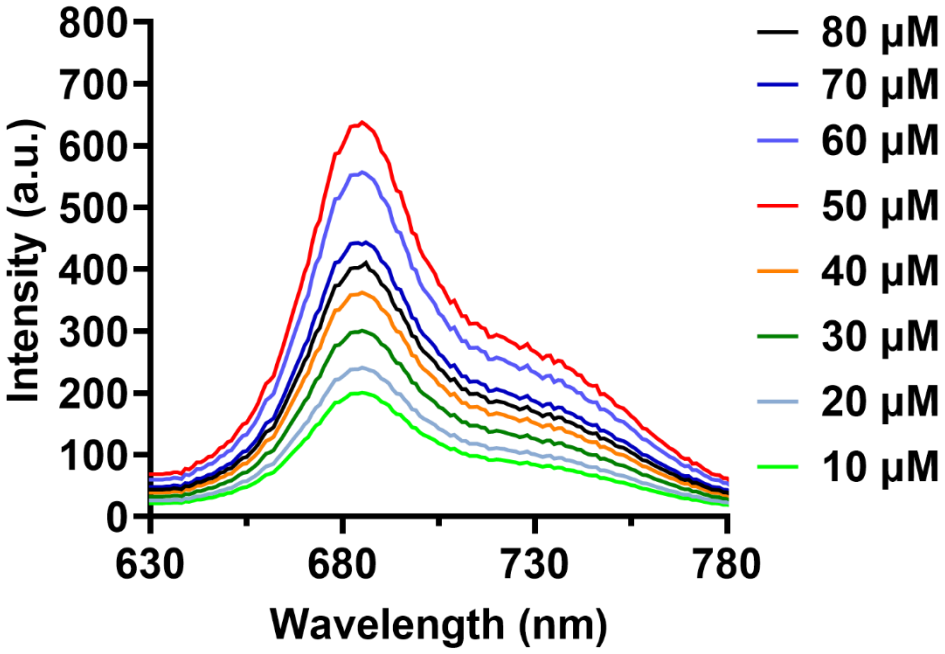

Figure S13 FL spectra of ethanol with different concentrations of PBA-BDP.

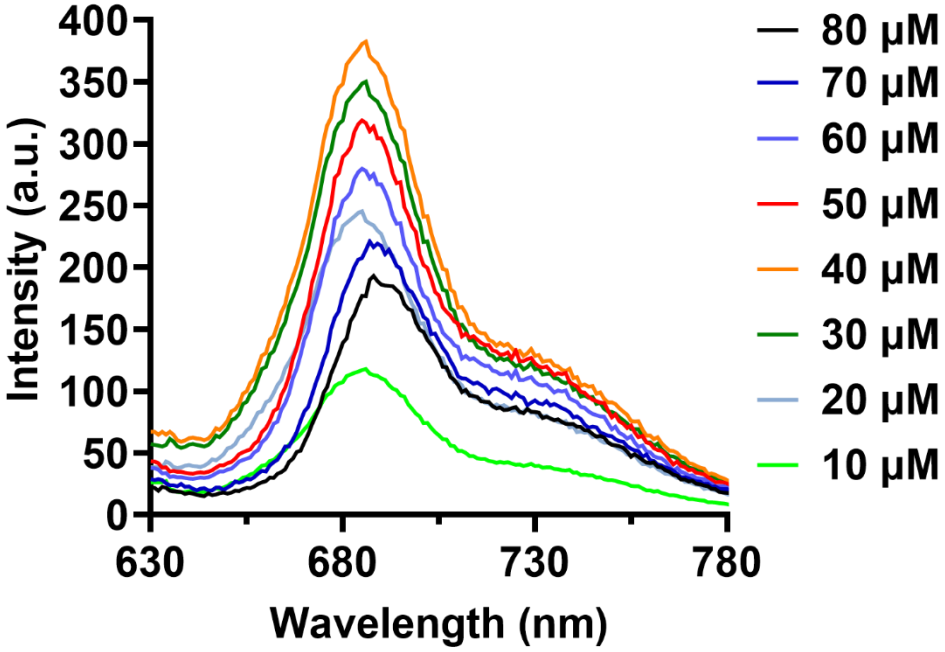

Figure S14 FL spectra of PBS with different concentrations of PBA-BDP.

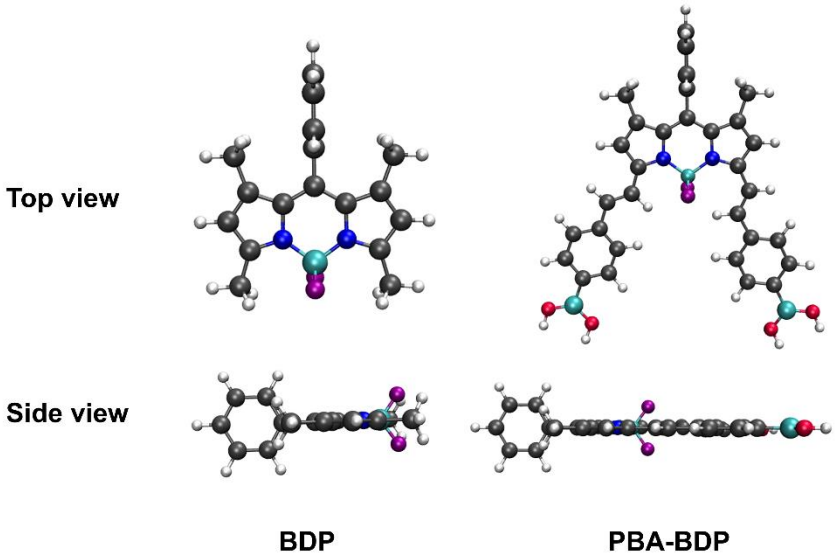

Figure S15 Top view and side view of optimized BDP and PBA-BDP. The atoms in the figure are represented by different colors. C: gray, H: white, N: blue, B: cyan, F: purple, O: red.

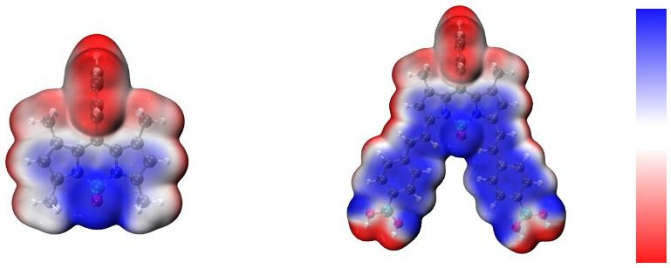

Figure S16 ESP analysis of BDP and PBA-BDP. Color scale: -0.03 a.u. ~ 0.03 a.u.

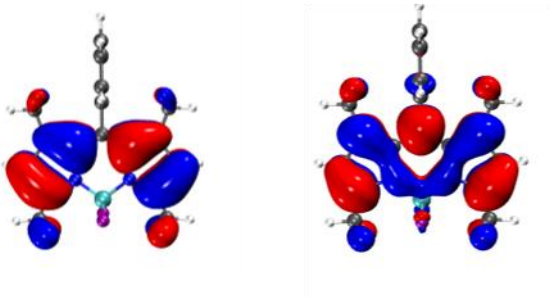

Figure S17 HOMO and LUMO electron cloud density distribution of BDP.

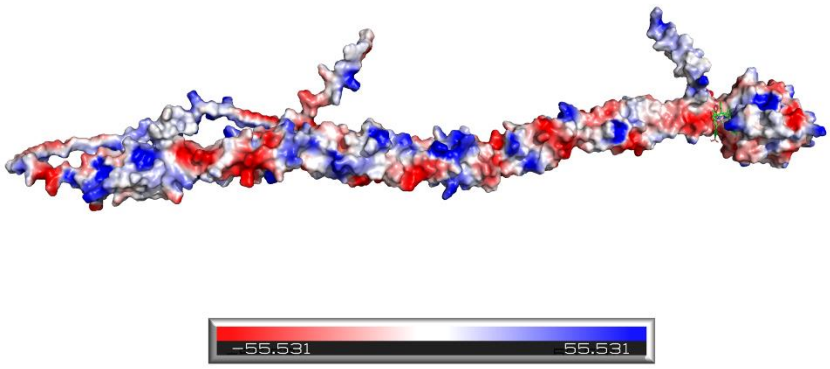

Figure S18 Interface structure of SELE and PBA-BDP.

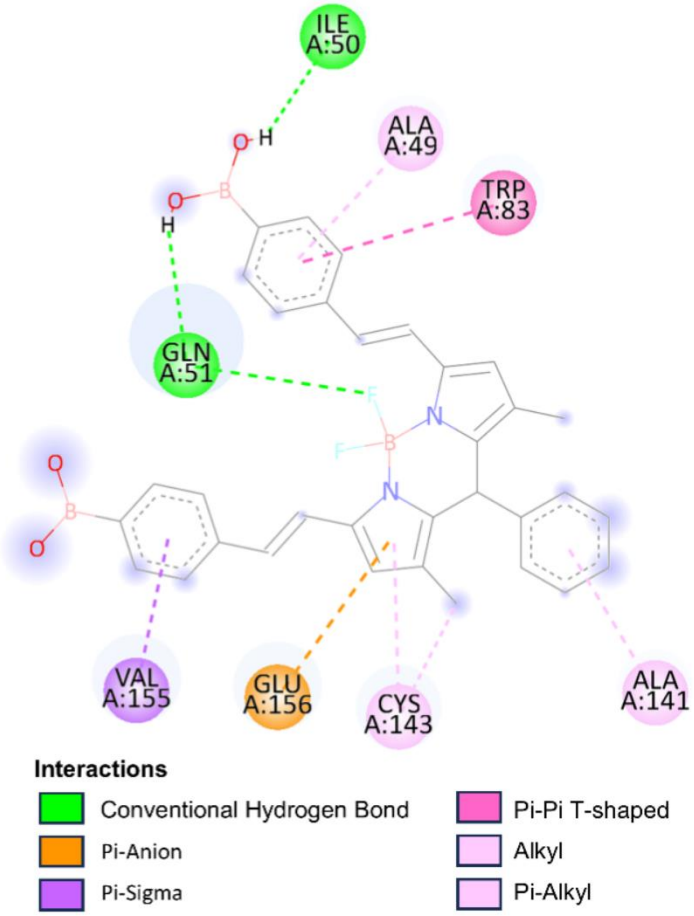

Figure S19 2D diagram of the interaction between SELE and PBA-BDP.

Table S1 Scoring terms for molecular docking in each binding mode.

mode	affinity(kcal/mol)	Cluster RMSD	Reference RMSD
1	-5.79	0	67.36
2	-5.72	0	65.86
3	-5.66	0	65.25
4	-5.33	0	70.73
5	-5.23	1.21	70.82
6	-5.2	0	70.19
7	-5.18	0.56	70.14
8	-5.18	0.72	69.81
9	-5.14	0	69.86

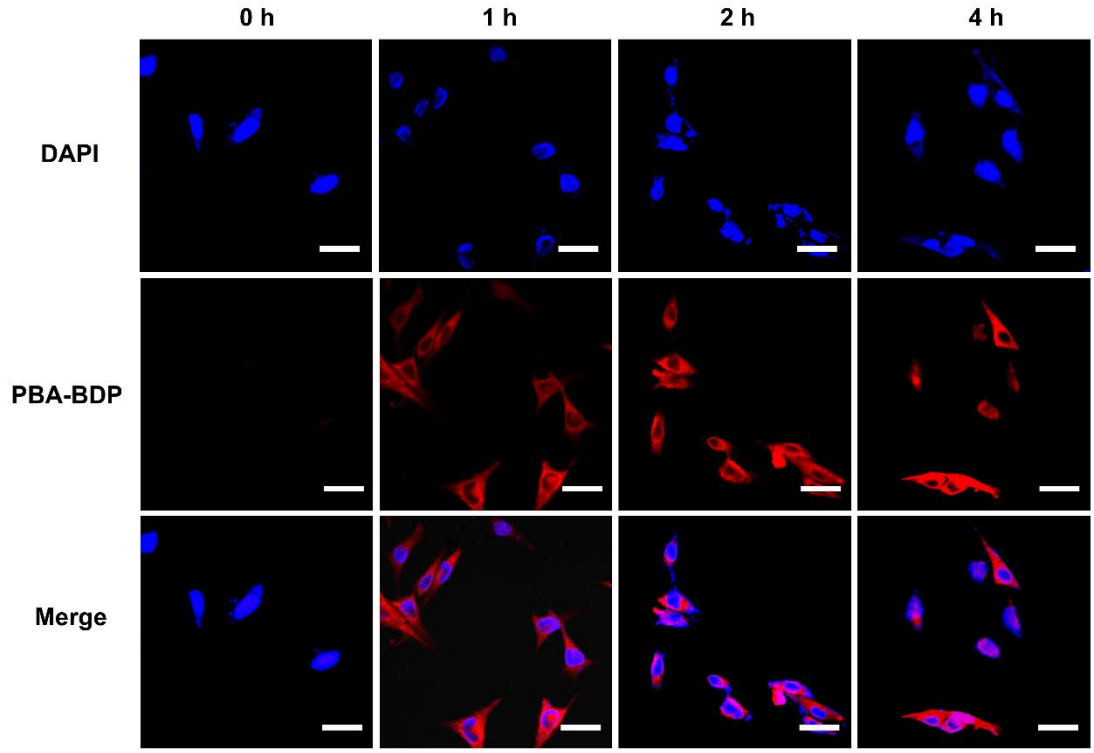

Figure S20 Representative fluorescence images of B16F10 cells treated with PBA-BDP at different incubation times (Scale bar: 20 μm).

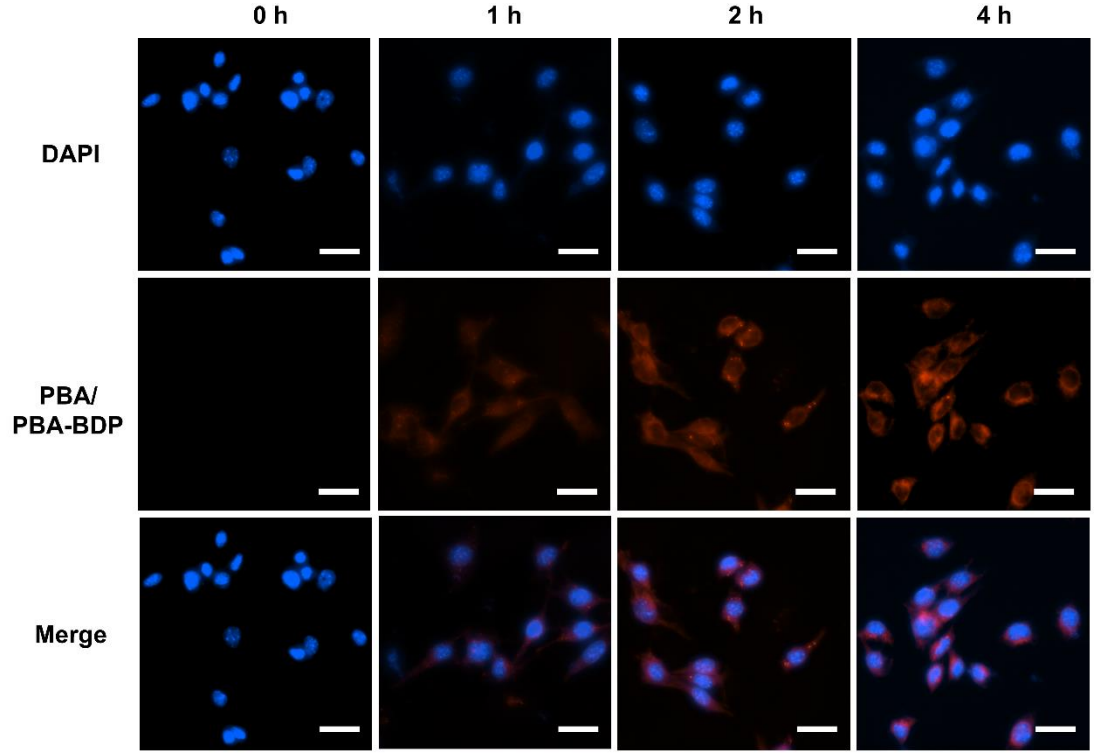

Figure S21 Representative fluorescence images of B16F10 cells treated with PBA-BDP at different incubation times after treatment of PBA (Scale bar: 20 μm).

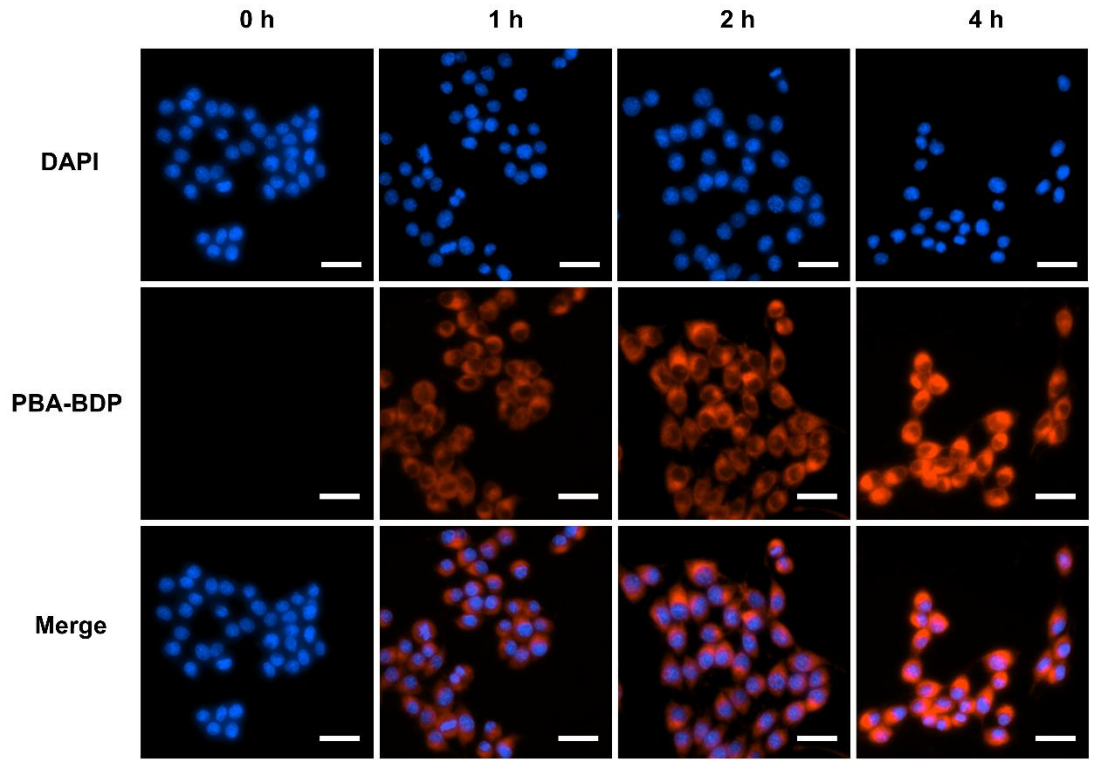

Figure S22 Representative fluorescence images of RM-1 cells treated with PBA-BDP at different incubation times (Scale bar: 20 μm).

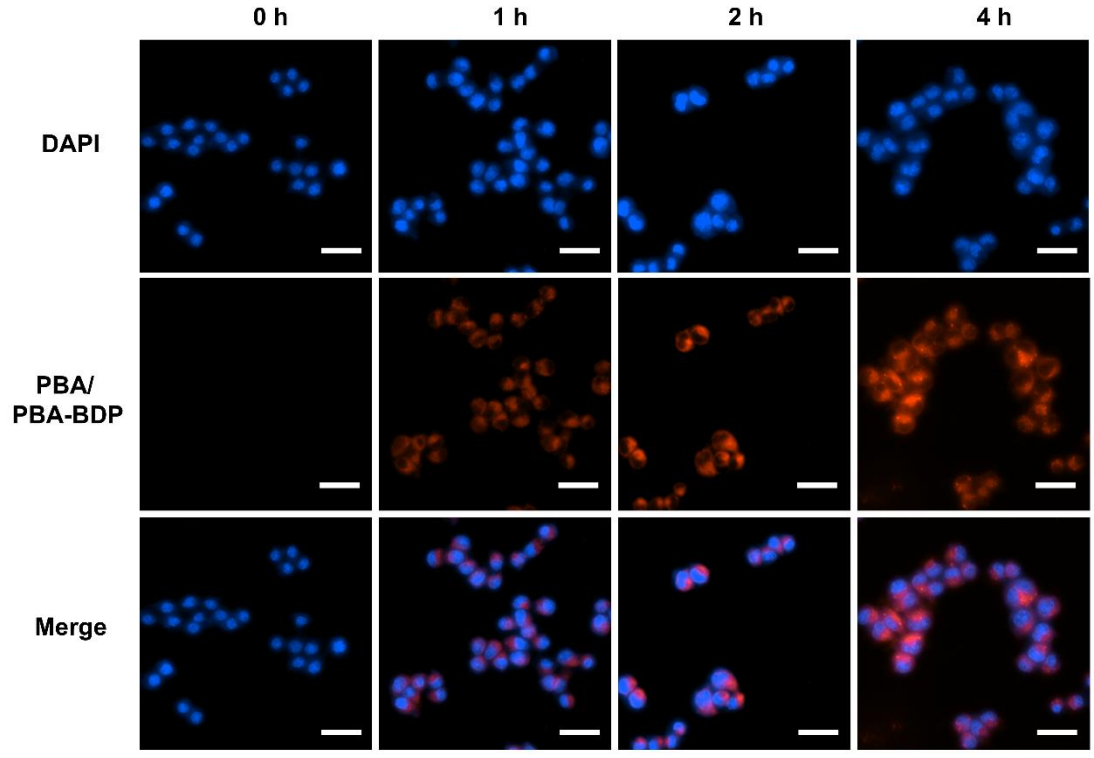

Figure S23 Representative fluorescence images of RM-1 cells treated with PBA-BDP at different incubation times after treatment of PBA (Scale bar: 20 μm).

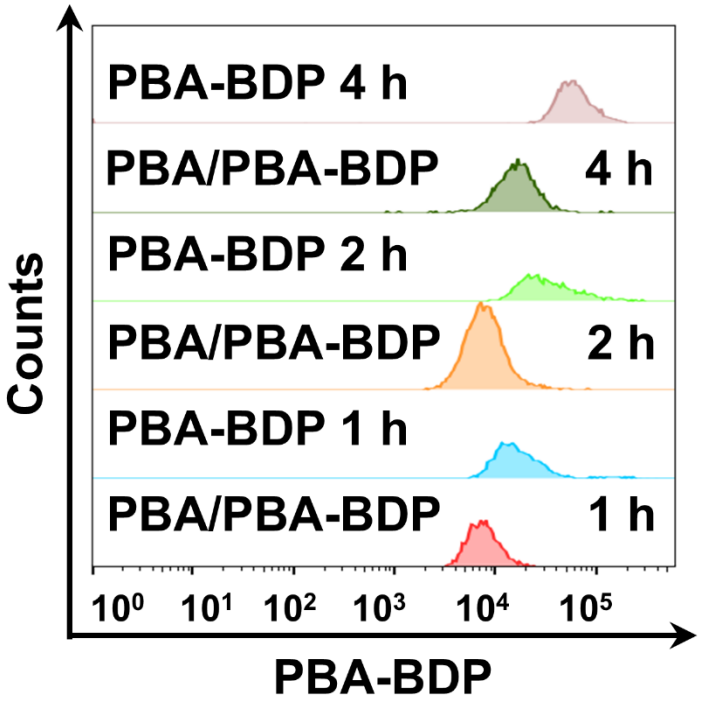

Figure S24 Histograms of B16F10 cells incubated with PBA/PBA-BDP and PBA-BDP.

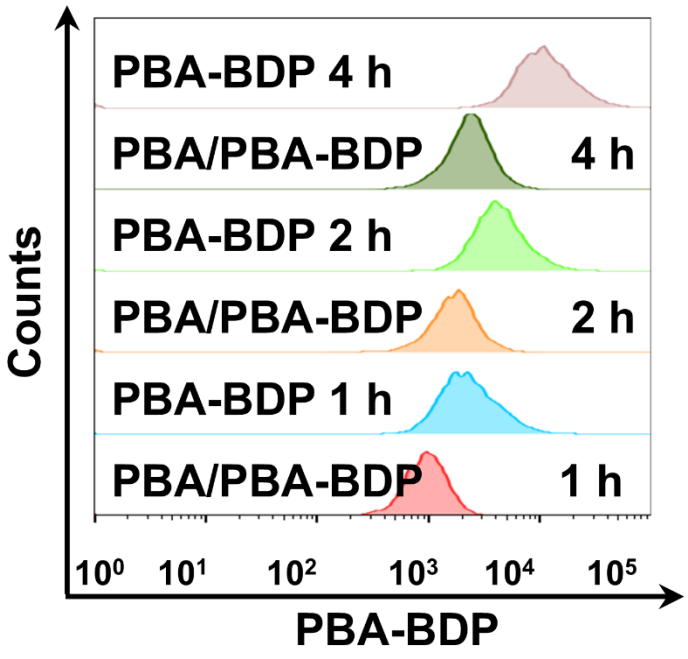

Figure S25 Histograms of RM-1 cells incubated with PBA/PBA-BDP and PBA-BDP.

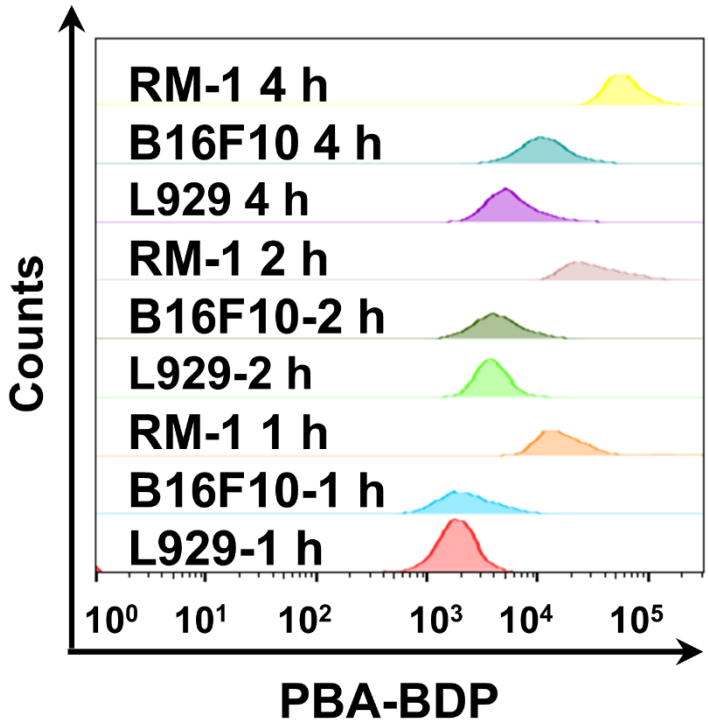

Figure S26 Histograms of RM-1, B16F10 and L929 cells incubated with PBA-BDP at different times.

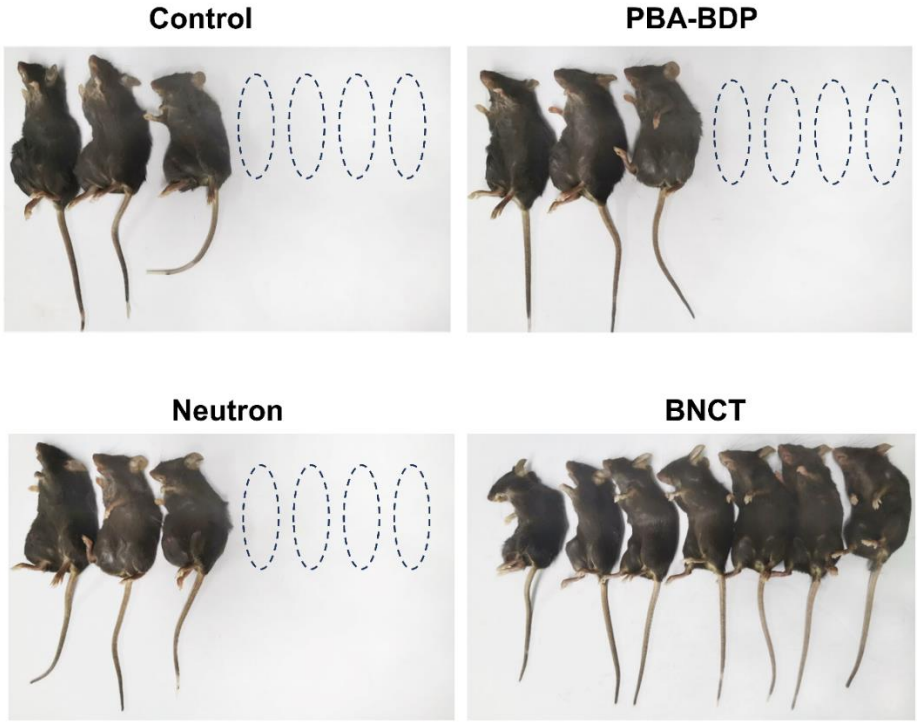

Figure S27 Photos of mice in each group.

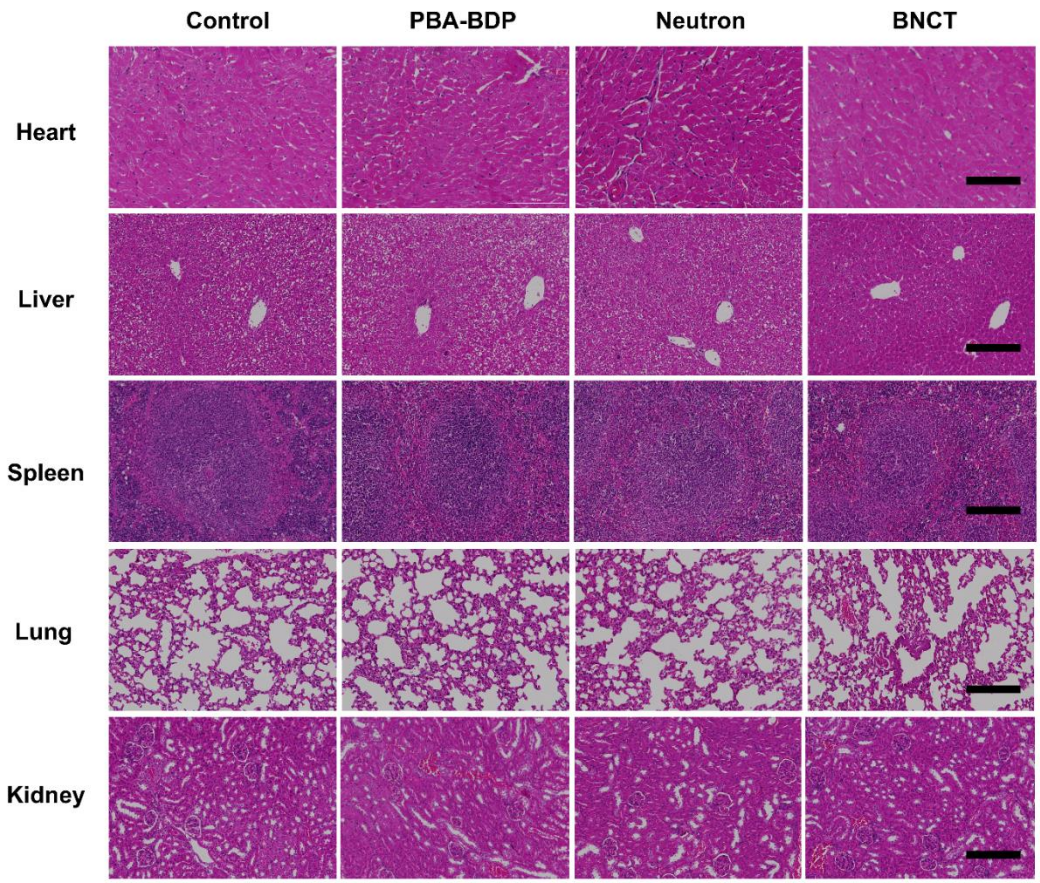

Figure S28 H&E staining analysis of major organs (n=7, scale bar: 200 μm).