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Harnessing Se=N to develop novel fluorescent probes for visualizing the variation of endogenous hypobromous acid (HOBr) during the administration of immunotherapeutic agent

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1. Materials and instrumentation

All chemicals were purchased from Adamas Reagent, Ltd. (China), and analytical grade solvents were used without further purification. All aqueous solutions were prepared using ultrapure water (ultrapure water, 18 MΩ cm^{-1}). MTT was purchased from Sigma Corporation, column chromatography silica gel (200-300 mesh) was purchased from Qingdao Haiyang Reagent Co., Ltd. The DMEM medium, penicillin/streptomycin and fetal calf serum was purchased from Gibco Corporation. Eosinophil Peroxidase (EPO) Human Eosinophils was purchased from BioVision Corporation. LysoTracker Red DND-99 was purchased from Xiangsheng Biotechnology Co., Ltd, Shanghai.

HeLa cells were purchased from Procell Life Science & Technology Co., Ltd.

Fluorescence data was measured by a F-4700 fluorescence spectrophotometer (Hitachi) at room temperature (slit for ABT-Se: 2.5 nm, 2.5 nm; slit for NDI-Se: 5 nm, 5 nm). The absorption spectra were measured on a UV-1700 spectrophotometer (Shimadzu, Japan). The mass spectra were obtained by Maxis MHR-TOF ultra-high resolution quadrupole time of flight mass spectrometer (Bruker Germany). The ^{1}H NMR and ^{13}C NMR spectra were acquired on a nuclear magnetic resonance spectrometer (400 MHz, Bruker Co., Ltd., Germany). The δ value represents the shift of the spectrum relative to TMS ($(CH_3)_4Si$ = 0.00 ppm), expressed in ppm. The LC-Mass were performed on a high-performance liquid chromatography-mass spectrometer (LC-16, Shimadzu, Japan). Two-photon images were acquired with the Zeiss LSM 880 NLO (Zeiss, Germany) with a 20× water objective. A Ti: sapphire laser was used to excite the specimen at 800 nm with a laser power of 80 mW. MTT assay was measured with a microplate reader (TRITURUS).

Preparation of various interference substances. All reagents were used right after they were ready. Cys, Hcy, GSH and vitamin C (Vc) were all used as received. HS^-, S^{2-} and HSO_3^- were all used as their sodium salt and prepared as the stock solutions.

K^+, Na^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} and Ag^+ were all used as their chloride salt and prepared as the stock solutions. All these compounds were

commercial available with analytical purity and used directly.

We prepared reactive oxygen species (ROS) as follows:

Peroxynitrite ($ONOO^-$): hydrochloric acid (0.6 M) was added to the mixture of $NaNO_2$ (0.6 M) and H_2O_2 (0.7 M), then NaOH (1.5 M) was added. The resulted faint yellow solution was split into small aliquots and stored at lower than -20 °C. The concentration of the prepared peroxynitrite was determined by testing the absorption of the solution at 302 nm. The extinction coefficient of $ONOO^-$ solution is 1670 M^{-1} cm^{-1} at 302 nm. $C_{ONOO^-} = Abs_{302nm}/1.67$ (mM).

Hydroxyl radical (•OH) was prepared by the reaction of Fe^{2+} with H_2O_2 (1:6), and the concentration of •OH is equal to the concentration of Fe^{2+}.

Singlet oxygen ($^1O_2$) was prepared in situ by addition of the H_2O_2 stock solution into a solution containing 10 eq of NaClO.

Superoxide solution ($O_2^{\bullet-}$) was prepared by adding KO_2 to dry dimethylsulfoxide and stirring vigorously for 10 min.

Hypochlorous acid (HClO) was obtained by diluting commercial aqueous solutions. The concentration was determined by measuring the absorbance at 292 nm with a molar extinction coefficient of 391 M^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) was diluted appropriately in water. The concentration of H_2O_2 was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Nitric oxide (NO) was used from a stock solution prepared by sodium nitroprusside.

Hypobromous acid (HOBr): To ultrapure water (7.5 mL) was added liquid bromine (50 μL), the mixture was titrated with $AgNO_3$ solution at 0 °C until the solution was colorless. After filtering, the filtrate was reserved for use. UV-Vis spectra was performed to measure the absorbance at 260 nm and [HOBr] determined by Lambert−Beer's law (ε_{260} = 160 L M^{-1} cm^{-1}).

2. Fluorescence analysis

Fluorescence titration profiles of the two probes were constructed by mixing ABT-Se (10 μM)/NDI-Se (10 μM) with different level of HOBr (0-100 μM) in PBS buffer solution (1 % DMSO, 100 mM, pH=7.4, 37 °C). The measurement was carried out at $\lambda_{ex}/\lambda_{em}$ = 380/443 nm for ABT-Se and 440/560 nm for NDI-Se respectively. The specificity experiments of the two probes towards HOBr were carried out by incubation of the probes with HOBr and other biorelevant species, including reactive oxygen and nitrogen species (•OH, $^1O_2$, $O_2^{\cdot-}$, HOCl, H_2O_2, NO, $ONOO^-$ and HOBr), reactive sulfur species (GSH, Cys, Hcy, S^{2-} and SH^-), reductive species ($HSO_3^-$and Vc), and metal ions (K^+, Na^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} and Ag^+) in PBS buffer solution (1 % DMSO, 100 mM, pH=7.4, 37 °C). The kinetic studies of fluorescence responses were performed by incubating the probes (10 μM) with HOBr (100 μM) at $\lambda_{ex}/\lambda_{em}$ = 380/443 nm for ABT-Se and 440/560 nm for NDI-Se respectively.

Fluorescence titration profile of NDI-Se towards EPO was constructed by mixing NDI-Se (5 μM), Br^- (10.0 mM) and H_2O_2 (10.0 mM) with different [EPO] (0-100 ng/mL) in acetic acid buffer solution (0.05 M, pH = 5.0, 37 °C). The measurement was carried out at $\lambda_{ex}/\lambda_{em}$ = 440/560 nm after incubation for 1 h.

Determination of Fluorescence Quantum Yield. Using Rhodamine B (the fluorescence standard, Φ_F = 0.97 in EtOH) as a reference, the absorbance of ABT-Se, NID-Se and the corresponding products was adjusted to ca. 0.05. Taking the determination of fluorescence quantum yield of as an example. The absorption spectra of NDI-Se and Rhodamine B were measured by UV spectrophotometer. Then their maximum absorption spectra are used as excitation wavelengths to obtain the corresponding fluorescence spectra. Finally, the respective fluorescence spectra are integrated to obtain F_X. The parameters of the substances used are as follows.

ABT-Se (2.0 μM), NDI-Se (2.0 μM), HOBr (20.0 μM), Rhodamine B (5.0 μg/mL).

The fluorescence quantum yield (Φ_F) was calculated by the standard equation:

$$\Phi_{F(X)} = \Phi_{F(S)} \times \left(\frac{F_X}{A_X}\right) \times \left(\frac{A_S}{F_S}\right) \times \left(\frac{n_X}{n_S}\right)^2$$

where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the emission curve, and n is the refractive index of the solvents used. Subscripts S and X refer to the standard and the samples to be tested, respectively.

Measurement of Two-Photon Absorption Cross-Section. The TPA cross-sections (δ) of ABT-Se, NID-Se and the corresponding products were measured by using the TPA imaging method. Using fluorescein as a reference, the fluorescence intensity of ABT-Se and the corresponding product were measured by two-photon excitation at 760 nm. The fluorescence intensity of NDI-Se and the corresponding product were measured by two-photon excitation at 800 nm. The parameters of the substances used are as follows.

ABT-Se (10.0 μM), NDI-Se (10.0 μM), HOBr (100.0 μM), fluorescein (5×10^{-7} M).

The two-photon absorption cross sections (δ) was calculated by two-photon excitation fluorescence spectra, according to the equation:

$$\frac{\delta_x}{\delta_s} = \frac{F_x C_s n_s \varphi_s}{F_s C_x n_x \varphi_x}$$

where δ stands for the two-photon absorption cross-section, F stands for the two-photon fluorescence intensity, φ stands for the fluorescence quantum yield, C stands for molar concentration, n stands for the solvent refractive index, and the subscripts X and S denote the evaluated sample (ABT-Se, NID-Se and the corresponding products) and the standard reference (fluorescein, in NaOH of 0.1 M), respectively.

3. Synthesis and characterization

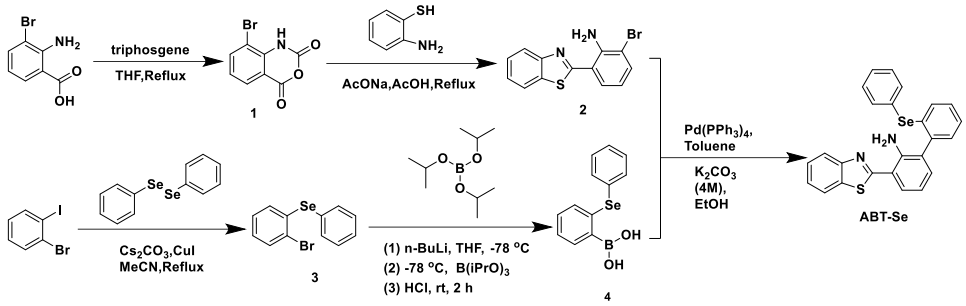


Fig. S1 Synthesis procedure of ABT-Se.

Synthesis of compound 1 [1]

To 2-amino-3-bromobenzoic acid (10 mmol, 2.16 g) dissolved in dry THF (20 mL) was added triphosgene (3.3 mmol, 980 mg), the mixture was refluxed for 4 h. After cooling to room temperature, water (30 mL) was added to the mixture to quench the reaction. Then the precipitated solid was filtered and washed with methanol. After drying the resulted yellow solid, compound 1 (2.01 g) was obtained (yield: 83.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08-8.10 (m, 1H), 7.89-7.91 (m, 1H), 7.18-7.22 (t, J = 16 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 157.79, 145.77, 139.93, 137.96, 129.83, 125.25, 111.82, 108.69. HRMS (ESI) (m/z): calculated for $C_8H_4BrNO_3$ $[M+Na]^+$ 263.9266; found 263.9275.

Synthesis of compound 2 [2]

To compound 1 (8.3 mmol, 2.01 g) and anhydrous sodium acetate (7.9 mmol, 0.65 g) dissolved in glacial acetic acid (60 mL) was added 2-aminobenzenethiol (12.7 mmol, 1.4 mL), the mixture was refluxed for 1.5 h. After cooling to room temperature, ethyl acetate (100 mL) was added to the mixture. Then sodium bicarbonate was used to quench the reaction. The organic phase was collected and washed with water (2 × 50 mL), saturated sodium chloride (50 mL) successively. After removing the solvent under reduced pressure, the residue was purified by silica gel column (petroleum ether: dichloromethane = 4:1 v/v), compound 2 (1.86 g) was obtained (yield: 73.4%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99-8.01 (m, 1H), 7.87-7.90 (m, 1H), 7.69-7.71 (m, 1H), 7.52-7.54 (m, 1H), 7.46-7.50 (m, 1H), 7.36-7.40 (m, 1H), 6.61-6.65 (t, J = 16 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.30, 153.41, 144.07, 134.62, 133.40, 129.61,

126.24, 125.25, 122.67, 121.22, 116.96, 116.17, 110.97. HRMS (ESI) (m/z): calculated for $C_{13}H_9BrN_2S$ $[M+H]^+$ 304.9742; found 304.9745.

Synthesis of compound 3 [3]

To 1-bromo-2-iodobenzene (5 mmol, 1.415 g) and diphenyl diselenide (3.5 mmol, 1.092 g) dissolved in dry acetonitrile (20 mL) was added cuprous iodide (0.25 mmol, 48 mg) and cesium carbonate (15 mmol, 4.889 g), the mixture was refluxed for 24 h. After removing the solvent under reduced pressure, the residue was purified by silica gel column (petroleum ether), compound 3 (1.52 g) was obtained (yield: 97.4%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64-7.66 (m, 2H), 7.50-7.52 (m, 1H), 7.37-7.43 (m, 3H), 7.00-7.09 (m, 2H), 6.84-6.87 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 136.47, 136.27, 133.04, 132.78, 130.53, 129.92, 129.40, 129.04, 128.56, 127.93, 127.39, 123.51. HRMS (ESI) (m/z): calculated for $C_{12}H_9BrSe$ $[M+Na]^+$ 334.8942; found 334.8941.

Synthesis of compound 4 [4]

To compound 3 (1 mmol, 312 mg) dissolved in dry THF (10 mL) was added n-butyllithium (0.46 mL, 2.5 M, 1.15 mmol) over 15 min at -78 °C. The mixture was stirred for 20 min at -78 °C, then triisopropyl borate (0.27 mL, 1.15 mmol) was added dropwise over 10 min. After the resulted mixture was warmed to room temperature gradually and stirred overnight at room temperature, hydrochloric acid (5 mL, 6 M) was added and stirred for another 2 h. After removing THF, ethyl acetate (5 × 25 mL) was used to extracted the mixture. The combined organic phase was dried and the solvent was removed under reduced pressure. The residue was purified by silica gel column (dichloromethane), compound 4 (130 mg) was obtained (yield: 46.9%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08-8.11 (m, 1H), 7.68-7.70 (m, 1H), 7.37-7.46 (m, 3H), 7.29-7.31 (m, 2H), 7.22-7.23 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.85, 137.19, 136.82, 136.56, 134.19, 131.90, 130.88, 129.71, 129.49, 128.78, 127.17. HRMS (ESI) (m/z): calculated for $C_{12}H_{11}BO_2Se$ $[M-H]^-$ 276.9936; found 276.9973.

Synthesis of ABT-Se [5]

To compound 2 (0.47 mmol, 143.1 mg) and compound 4 (0.47 mmol, 130 mg)

dissolved in toluene (8 mL), ethanol (1 mL) and potassium carbonate (3 mL) was added tetrakis(triphenylphosphine) palladium (0.047 mmol, 54.3 mg). The mixture was stirred at 80 °C for 24 h. After removing the solvent under reduced pressure, the residue was purified by silica gel column (petroleum ether: ethyl acetate = 4:1 v/v), ABT-Se (46 mg) was obtained (yield: 21.3%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94-7.96 (d, J = 8 Hz, 1H), 7.88-7.90 (d, J = 8 Hz, 1H), 7.79-7.81 (m, 1H), 7.52-7.55 (m, 2H), 7.43-7.47 (m, 1H), 7.34-7.38 (m, 2H), 7.29-7.32 (m, 4H), 7.22-7.23 (m, 2H), 7.14-7.17 (m, 1H), 6.81-6.85 (t, J = 16 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.35, 153.65, 144.17, 138.71, 135.61, 135.12, 133.37, 132.75, 131.21, 130.76, 130.40, 129.45, 129.41, 128.90, 128.22, 128.12, 127.01, 126.04, 124.92, 122.46, 121.17, 116.42, 115.53. HRMS (ESI) (m/z): calculated for $C_{25}H_{18}N_2SSe$ $[M+H]^+$ 459.0429; found 459.0336.

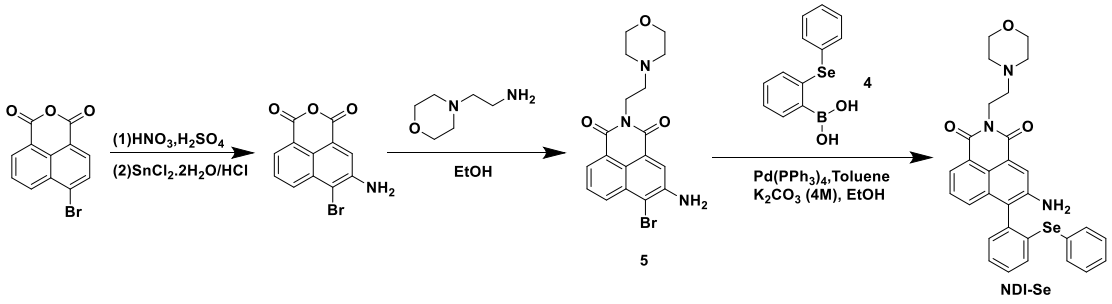


Fig. S2 Synthesis procedure of NDI-Se.

Synthesis of compound 5[6]

5-amino-6-bromo-1H,3H-benzo[de]isochromene-1,3-dione was synthesized according to literature method.

To 5-amino-6-bromo-1H,3H-benzo[de]isochromene-1,3-dione (120 mg, 0.41 mmol) dissolved in absolute ethyl alcohol (10 mL) was added 4-(2-aminoethyl) morpholine (106 mg, 0.82 mmol), the mixture was refluxed for 9 h. After cooling to room temperature, precipitated solid was filtered and wash with ethyl alcohol. Then the solid was dried and compound 5 (130 mg) was obtained (yield: 78.4%). ^{1}H NMR (400 MHz, d_6-DMSO): δ 8.21-8.24 (d, J = 12 Hz, 1H), 8.15-8.17 (d, J = 8 Hz, 1H), 8.12 (s, 1H), 7.76-7.80 (m, 1H), 6.32 (s, 2H), 4.12-4.16 (t, J = 16 Hz, 2H), 3.51-3.53 (t, J = 8 Hz, 4H), 2.53-2.56 (t, J = 12 Hz, 2H), 2.45 (s, 4H); ^{13}C NMR (100 MHz,

d_6-DMSO): δ 163.80, 163.52, 145.88, 132.11, 130.39, 129.00, 126.39, 122.76, 122.44, 122.34, 121.71, 107.13, 66.70, 55.99, 53.88, 37.32. HRMS (ESI) (m/z): calculated for $C_{18}H_{18}N_3O_3Br$ $[M+H]^+$ 404.0604; found 404.0632.

Synthesis of NDI-Se

To compound 5 (0.47 mmol, 190 mg) and compound 4 (0.47 mmol, 130 mg) dissolved in toluene (8 mL), ethanol (1 mL) and potassium carbonate (3 mL) was added tetrakis(triphenylphosphine) palladium (0.047 mmol, 54.3 mg). The mixture was stirred at 80 °C for 24 h. After removing the solvent under reduced pressure, the residue was purified by silica gel column (dichloromethane: methanol = 4:1 v/v), NDI-Se (64.6 mg) was obtained (yield: 24.7%). ^{1}H NMR (400 MHz, CD_3OD): δ 8.17-8.19 (m, 1H), 8.15 (s, 1H), 7.43-7.47 (m, 2H), 7.34-7.39 (m, 5H), 7.19-7.27 (m, 4H), 4.31-4.34 (t, J = 12 Hz, 2H), 3.66-3.68 (t, J = 8 Hz, 4H), 2.70-2.74 (t, J = 16 Hz, 2H), 2.61 (s, 4H); ^{13}C NMR (100 MHz, CD_3OD): δ 164.77, 164.33, 144.13, 136.20, 134.99, 132.32, 132.11, 131.09, 129.89, 129.45, 129.16, 129.07, 128.88, 127.94, 127.59, 126.59, 126.02, 123.80, 122.66, 122.15, 122.08, 121.86, 66.36, 55.92, 53.58, 36.49. HRMS (ESI) (m/z): calculated for $C_{30}H_{27}N_3O_3Se$ $[M+H]^+$ 558.1292; found 558.1320.

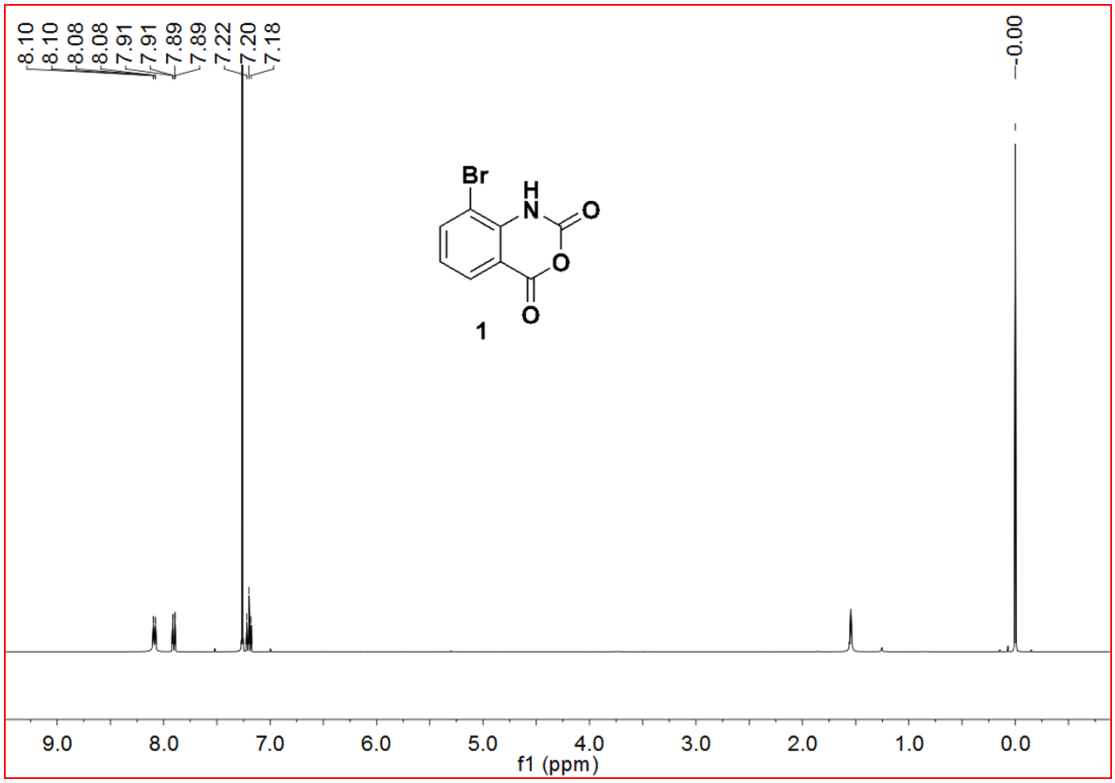


Fig. S3 ^{1}H NMR spectrum of compound 1 in $CDCl_3$.

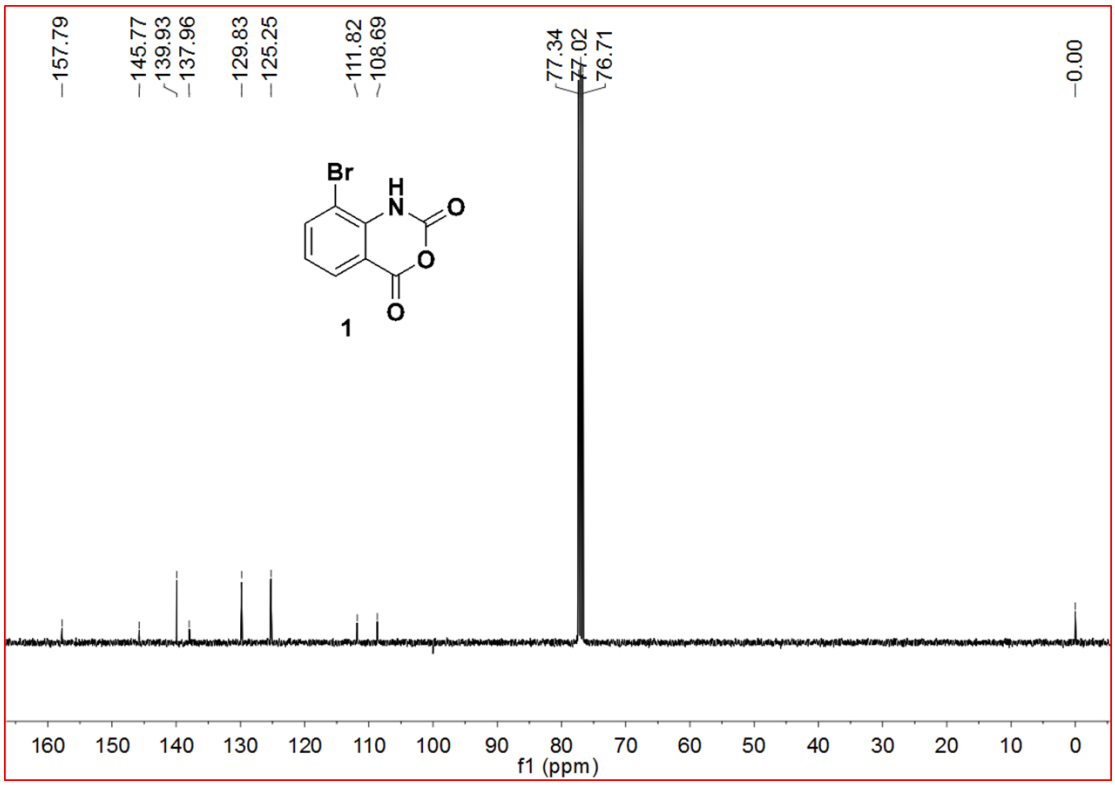


Fig. S4 ^{13}C NMR spectrum of compound 1 in $CDCl_3$.

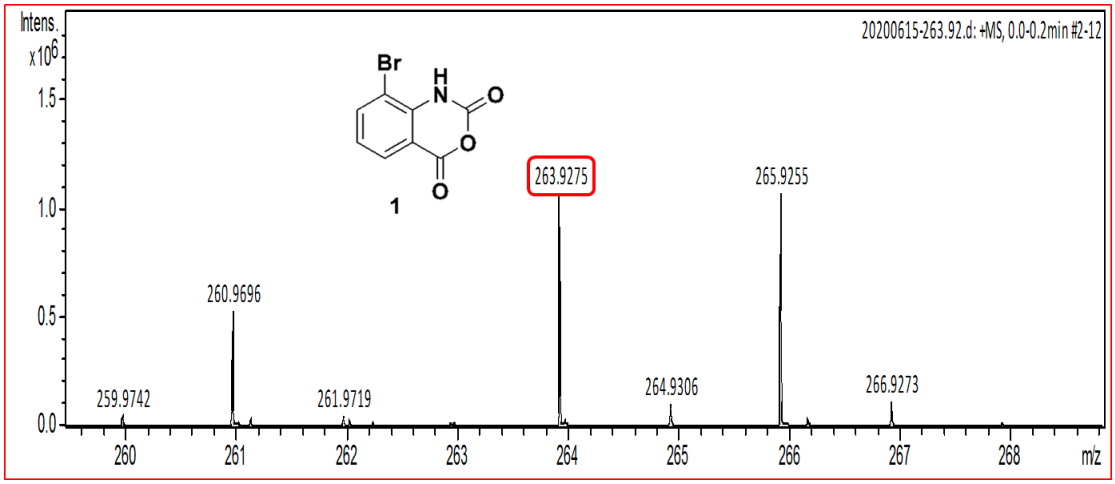


Fig. S5 HRMS of compound 1.

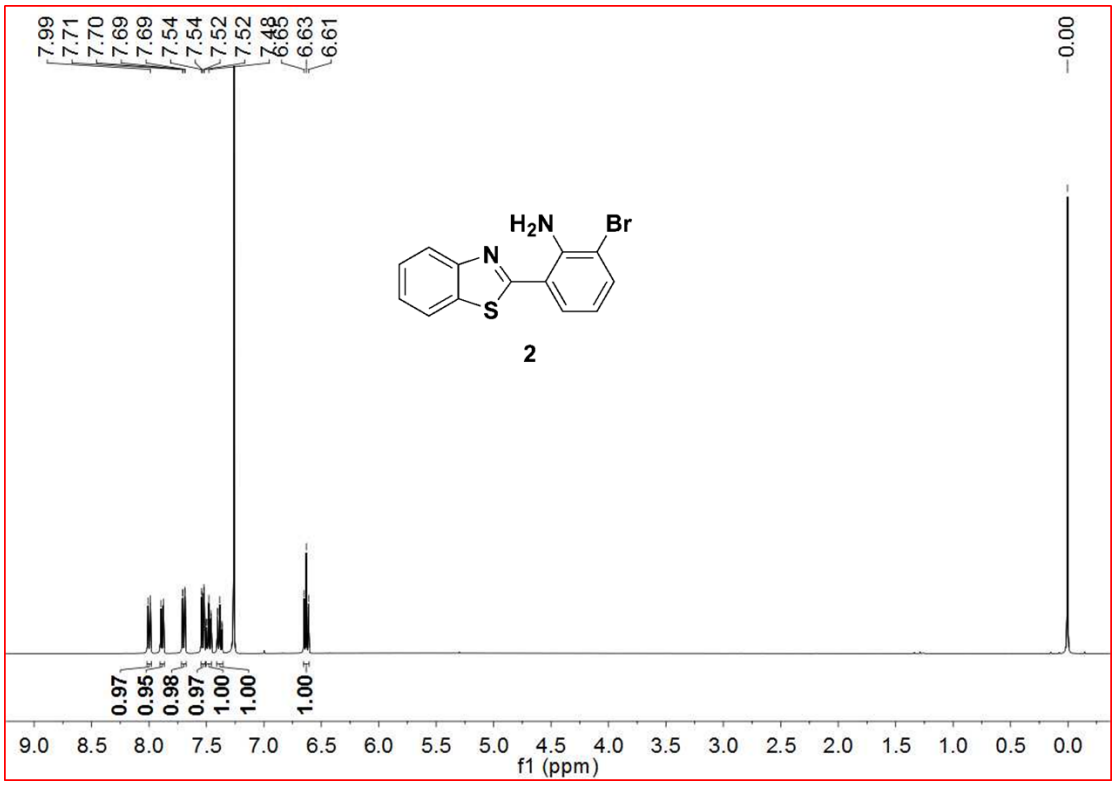


Fig. S6 ^{1}H NMR spectrum of compound 2 in $CDCl_3$.

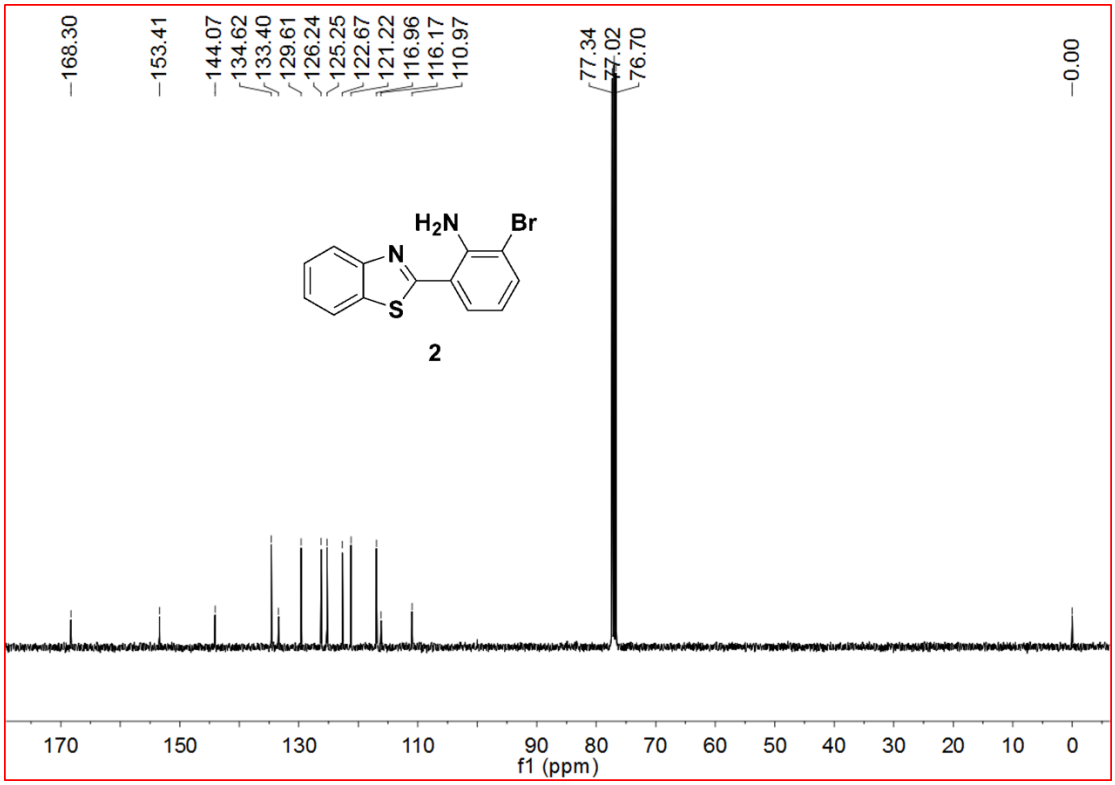


Fig. S7 ^{13}C NMR spectrum of compound 2 in $CDCl_3$.

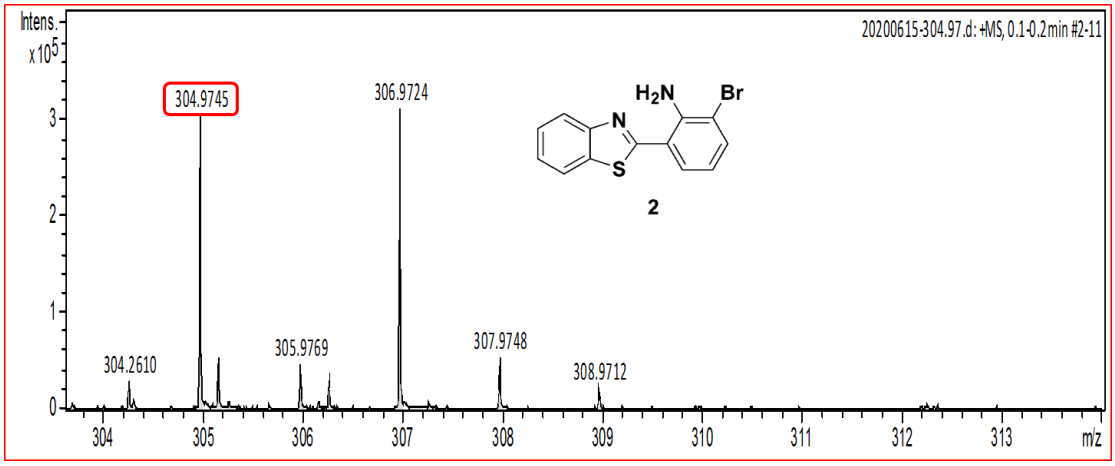


Fig. S8 HRMS of compound 2.

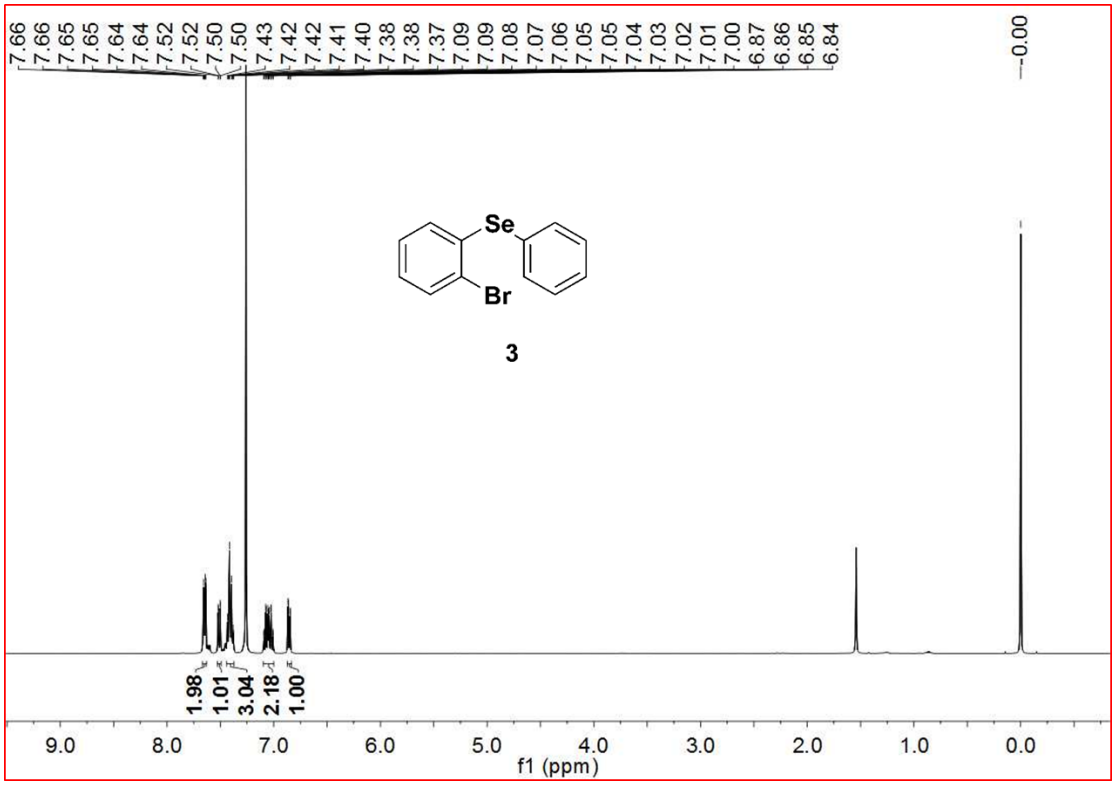


Fig. S9 ^{1}H NMR spectrum of compound 3 in $CDCl_3$.

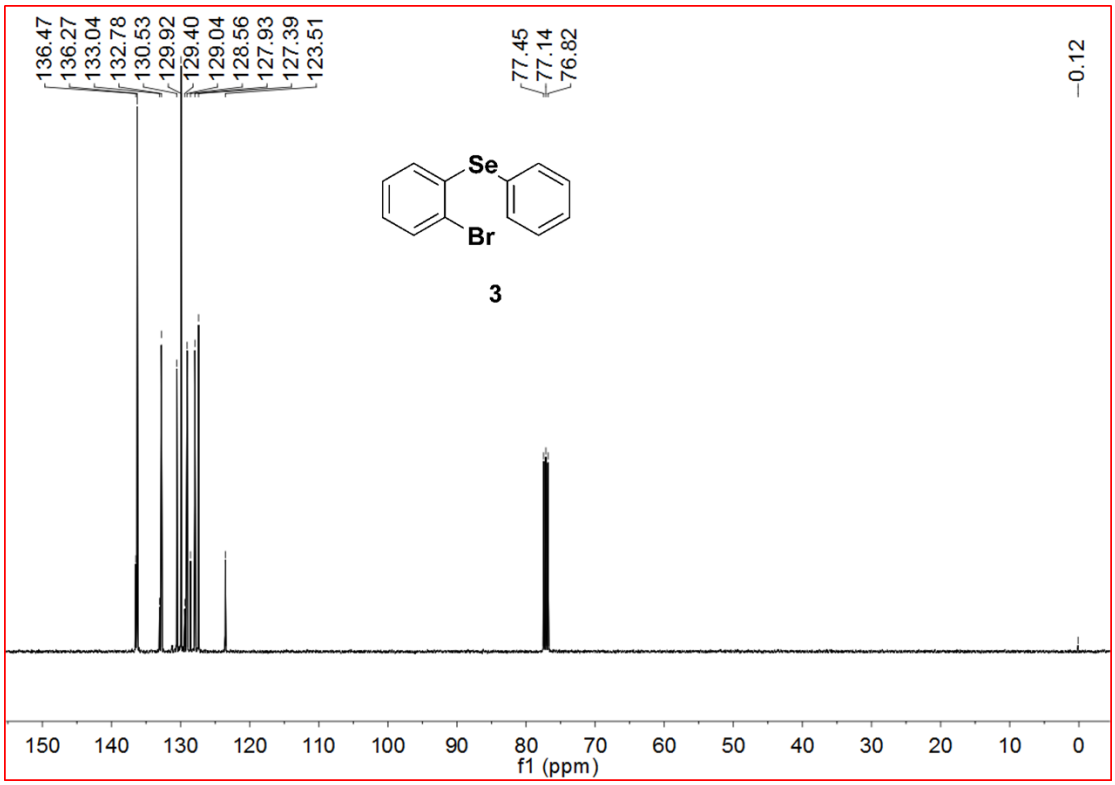


Fig. S10 ^{13}C NMR spectrum of compound 3 in $CDCl_3$.

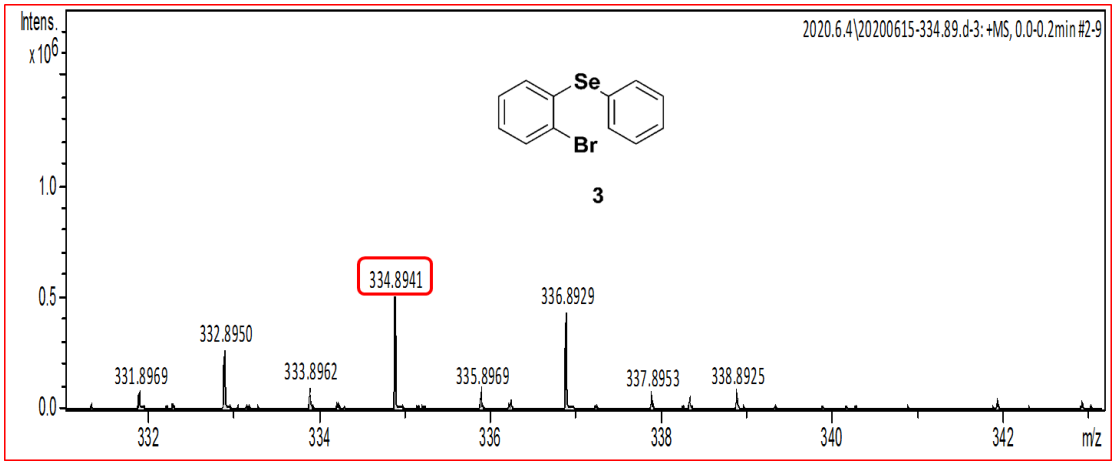


Fig. S11 HRMS of compound 3.

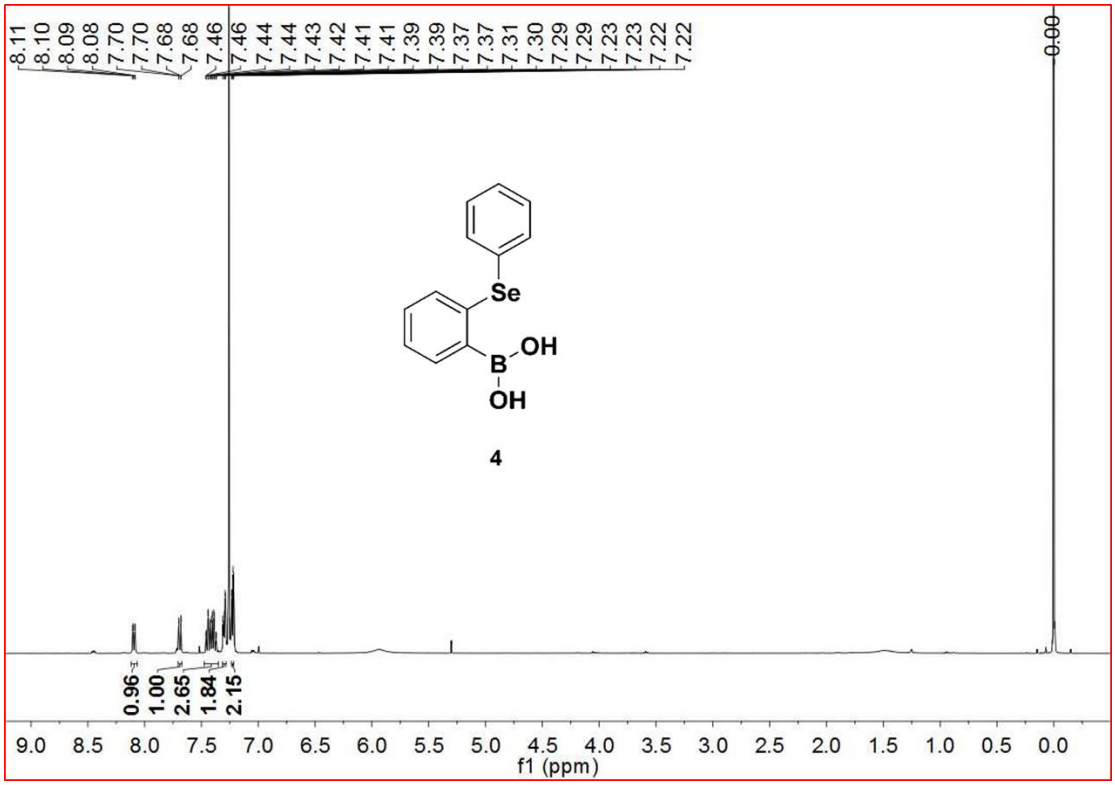


Fig. S12 ^{1}H NMR spectrum of compound 4 in $CDCl_3$.

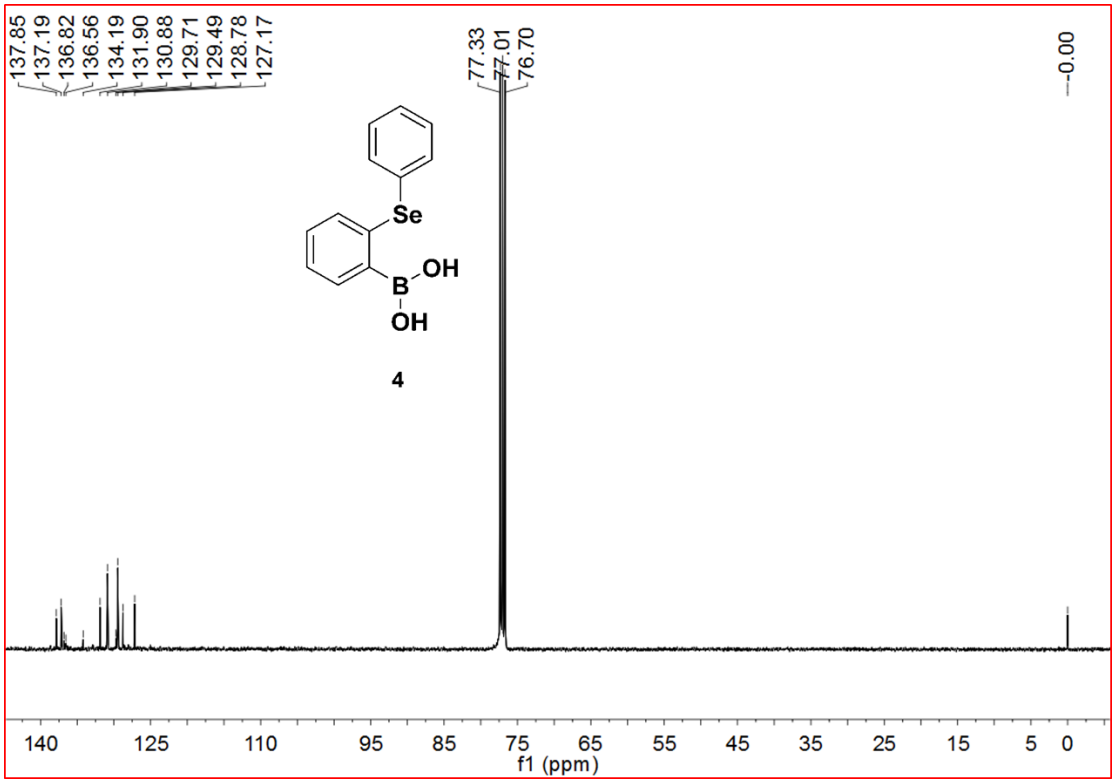


Fig. S13 ^{13}C NMR spectrum of compound 4 in $CDCl_3$.

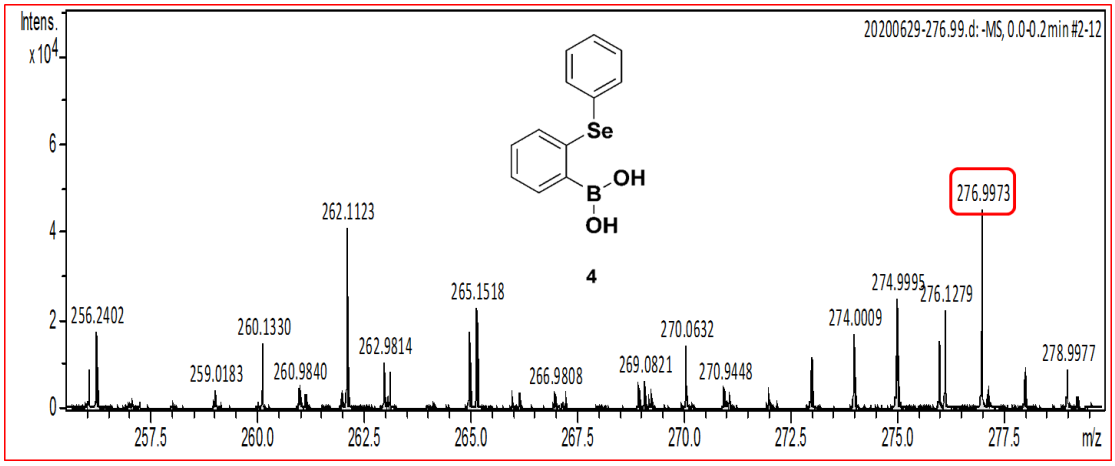


Fig. S14 HRMS of compound 4.

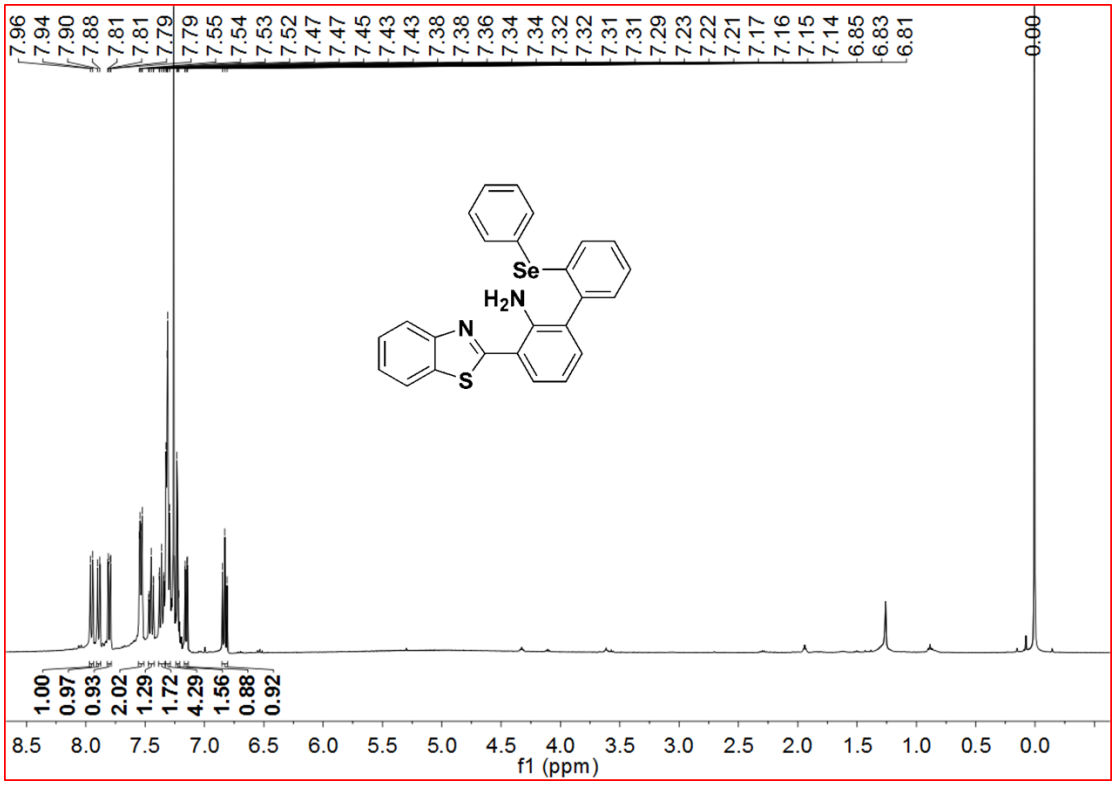


Fig. S15 ^{1}H NMR spectrum of ABT-Se in $CDCl_3$.

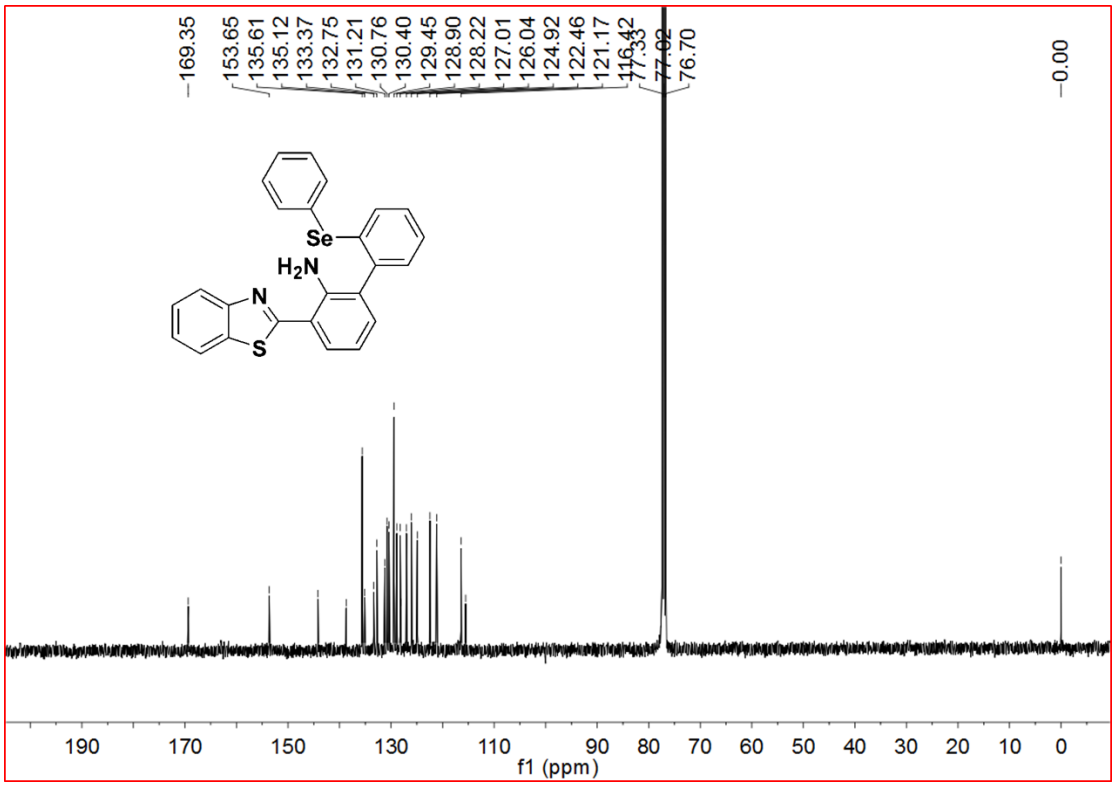


Fig. S16 ^{13}C NMR spectrum of ABT-Se in $CDCl_3$.

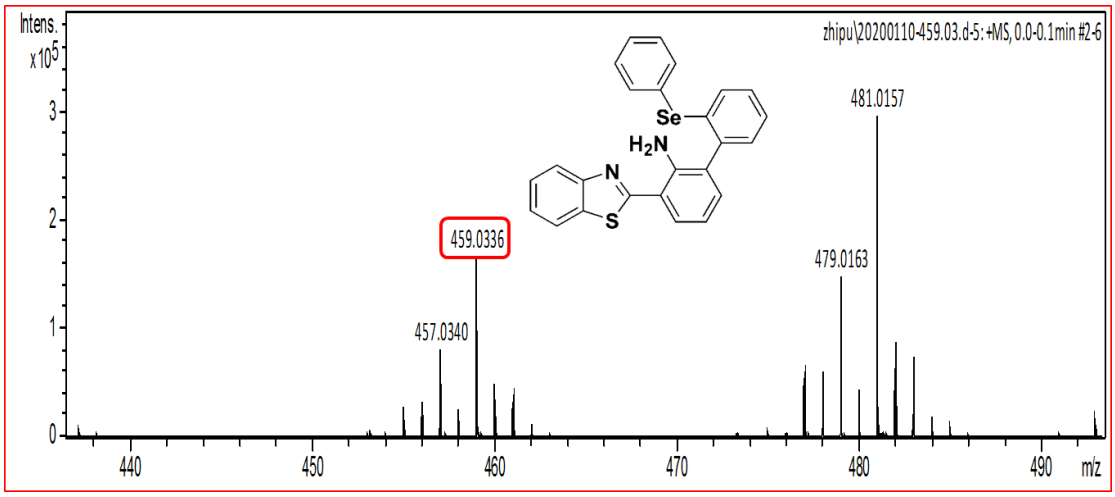


Fig. S17 HRMS of ABT-Se.

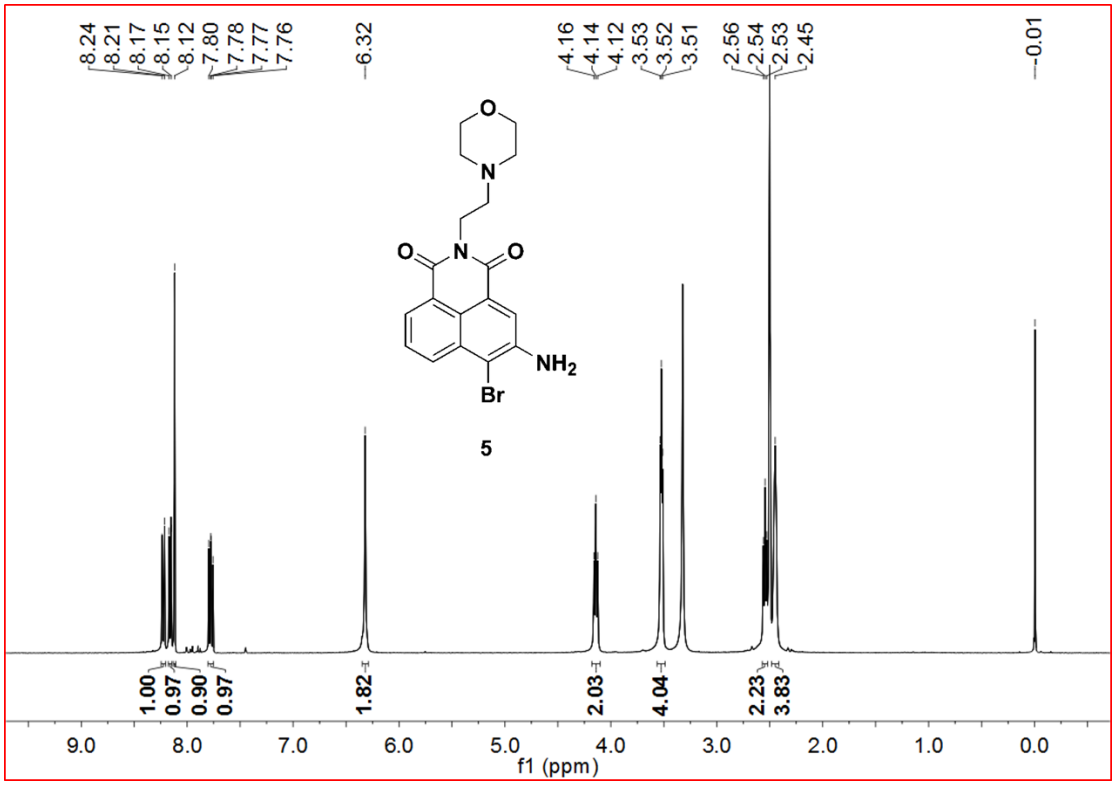


Fig. S18 ^{1}H NMR spectrum of compound 5 in d_6-DMSO.

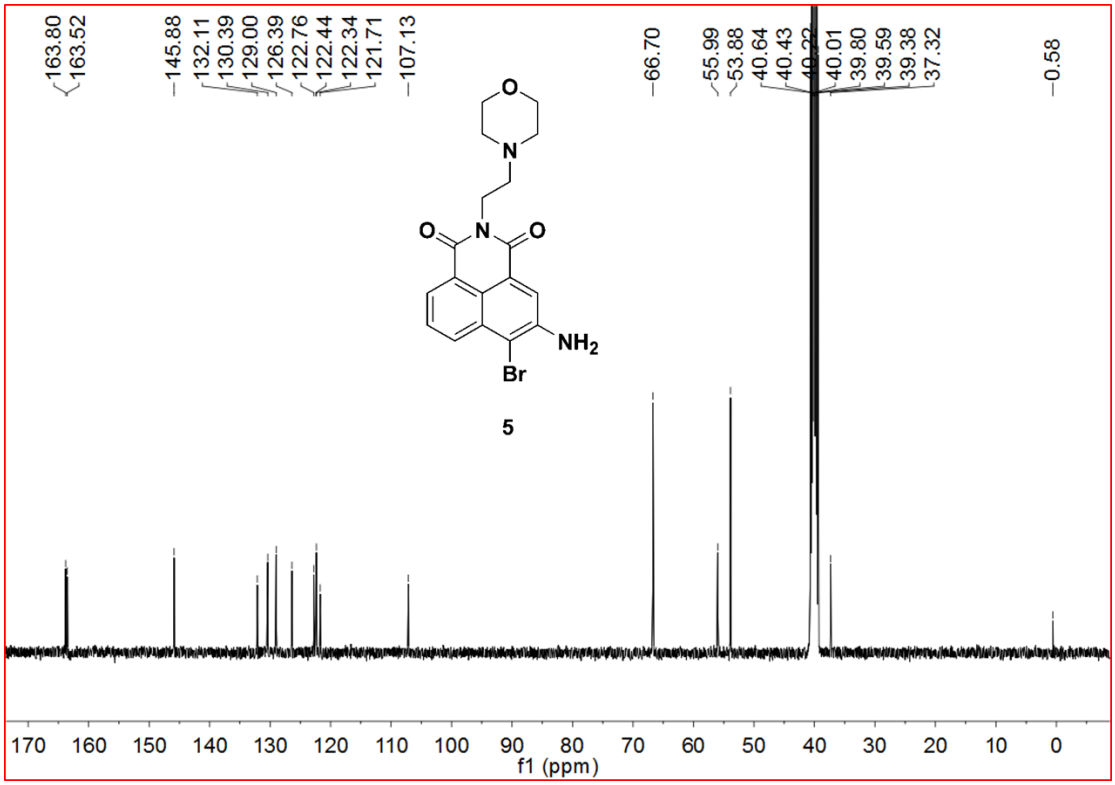


Fig. S19 ^{13}C NMR spectrum of compound 5 in d_6-DMSO.

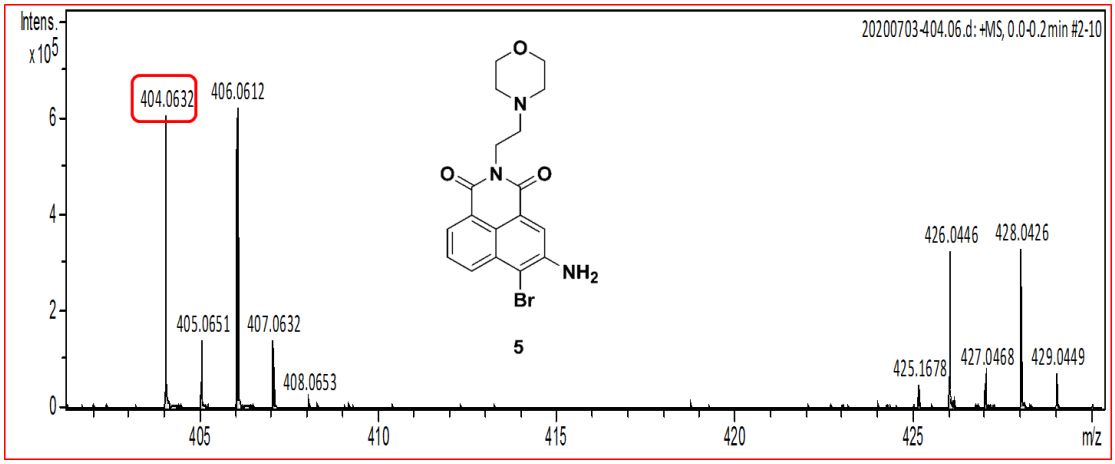


Fig. S20 HRMS of compound 5.

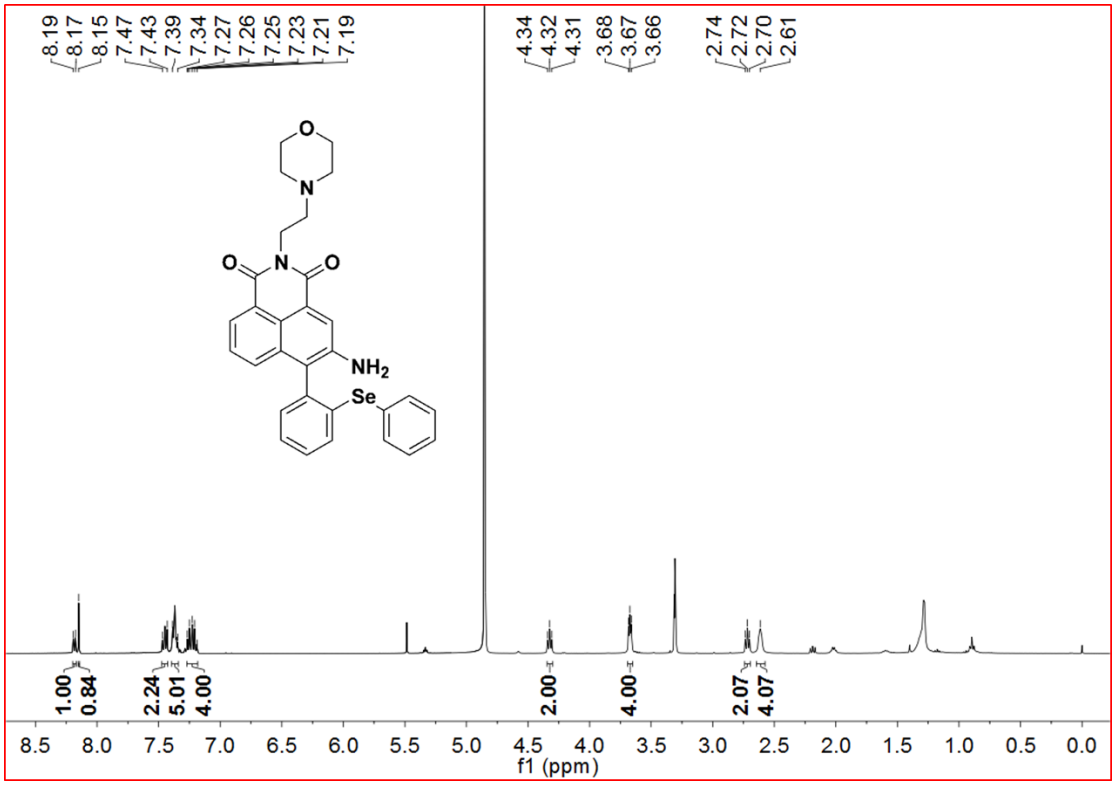


Fig. S21 ^{1}H NMR spectrum of NDI-Se in CD_3OD.

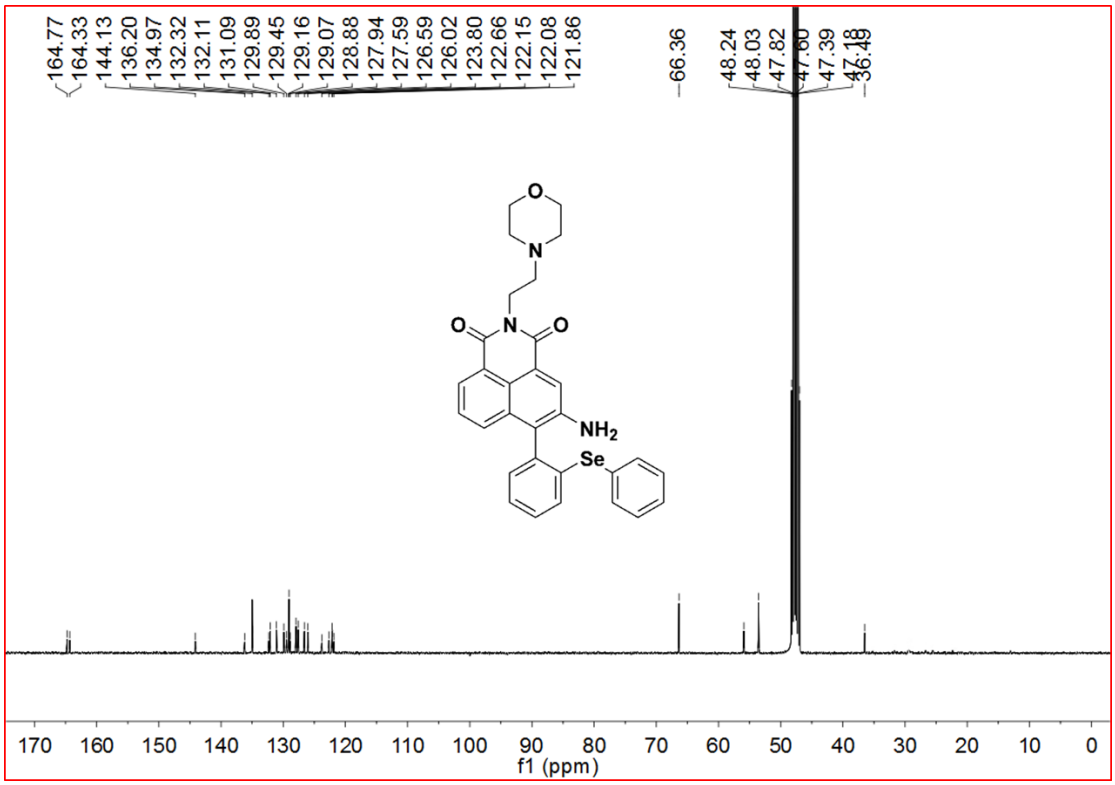


Fig. S22 ^{13}C NMR spectrum of NDI-Se in CD_3OD.

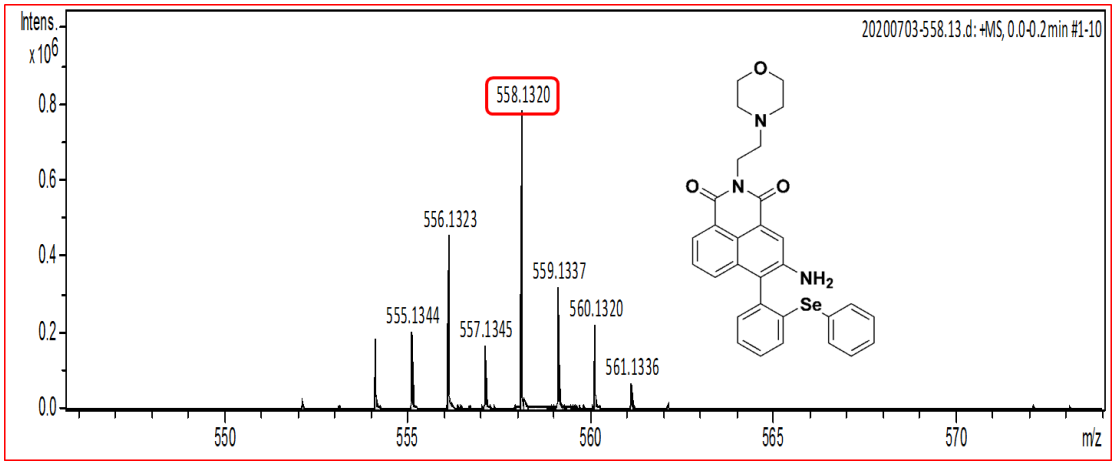


Fig. S23 HRMS of NDI-Se.

4. Cell culture

The Hela cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 1 % 100 U mL^{-1} antibiotics penicillin/streptomycin and 10 % fetal bovine serum (FBS) at 37 °C under a humidified atmosphere containing 5 % CO_2.

5. MTT assay

The cytotoxicity of the two probes was tested in HeLa cells using a standard MTT assay. The IC_{50} value was calculated according to the method of Huber and Koella[7]. It was displayed that the value of IC_{50} was 106.1 μM and 154.8 μM for ABT-Se and NDI-Se respectively (Fig. S38 and S39), which indicated the good biocompatibility for cell imaging.

6. Cell imaging

HeLa Cells were employed to perform the imaging.

Colocalization assay: HeLa Cells were washed with PBS three times and then co-incubated with Lyso Tracker Red DND-99 (50.0 nM) and NDI-Se (5.0 μM) for 15 min at 37 °C. After washing with PBS again, the cells were imaged by confocal laser scanning microscope. LysoTracker Red DND-99: $\lambda_{ex}/\lambda_{em}$=543 nm/580-640 nm; NDI-Se: $\lambda_{ex}/\lambda_{em}$=458 nm/510-570 nm.

Two-photon confocal fluorescent imaging of endogenous HOBr in HeLa cells with ABT-Se:

The cells were washed with PBS for three times firstly. Group a: HeLa cells were incubated with ABT-Se (10.0 μM) alone for 20 min, after removing ABT-Se solution, PBS was added again to image as control group. Group b: HeLa cells were pretreated with KBr (100 μM) for 20 min and washed with PBS for three times; After culturing with ABT-Se (10.0 μM) for 20 min, the ABT-Se solution was removed and the imaging was carried out. Group c: HeLa cells were pretreated with KBr (100 μM) and H_2O_2 (100 μM) for 20 min and washed with PBS for three times; After culturing with ABT-Se (10.0 μM) for 20 min, the ABT-Se solution was removed and the imaging was carried out. Group d: HeLa cells were pretreated with KBr (100 μM) and NAC (100 μM) for 20 min and washed with PBS for three times; After culturing with

ABT-Se (10.0 μM) for 20 min, the ABT-Se solution was removed and the imaging was carried out. The fluorescence images were obtained with 760 nm excitation and 420-520 nm collection.

Two-photon confocal fluorescent imaging of endogenous HOBr in HeLa cells with NDI-Se:

The cells were washed with PBS for three times firstly. Group a: HeLa cells were incubated with NDI-Se (5.0 μM) alone for 15 min, after the residual NDI-Se solution was washed off, PBS was added again to image as control group. Group b: HeLa cells were pretreated with KBr (100 μM) for 20 min and washed with PBS for three times; after culturing with NDI-Se (5.0 μM) for 15 min, the ABT-Se solution was washed off and the imaging was carried out. Group c: HeLa cells were pretreated with KBr (100 μM) and H_2O_2 (100 μM) for 20 min and washed with PBS for three times; after culturing with NDI-Se (5.0 μM) for 15 min, the NDI-Se solution was removed and the imaging was carried out. Group d: HeLa cells were pretreated with KBr (100 μM) and NAC (100 μM) for 20 min and washed with PBS for three times; After culturing with NDI-Se (5.0 μM) for 15 min, the NDI-Se solution was removed and the imaging was carried out.

7. Establishment of tumor xenograft 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. Female nude mice (5-week-old) were purchased from Shengwei Biotechnology Co., Ltd, Jinan. and housed under normal conditions with free access to food and water randomly. HeLa cells (1×10^6 cells per mouse, 100 μL PBS) were injected into the right flank of nude mice subcutaneously to establish HeLa tumor model. After one week of tumor growth, the mice were performed to two-photon fluorescence imaging.

8. Two-photon imaging on tumor site

The two-photon fluorescence imaging on tumor site during the course of cancer

immunotherapy was performed as follows: PBS/BEC (20 mg/kg for one treatment) was injected into living mice bearing HeLa tumor model for different times via the tail vein. After 12 h at the end of treatment, NDI-Se (50.0 μM) was injected intra-peritonelly, and the fluorescence imaging on tumor site was monitored after the mice were sacrificed. Group a: PBS (75 μL); Group b: BEC was administered for one treatment at day 0; Group c: BEC was administered for two treatments at day 0 and 2; Group d: BEC was administered for three treatments at day 0, 2 and 4. The fluorescence images were obtained with 800 nm excitation and 510-610 nm collection.

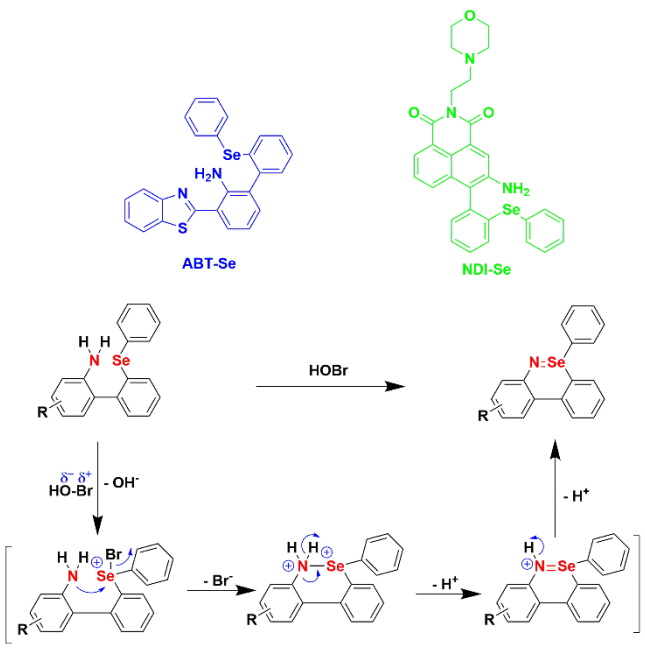
9. Immunohistochemistry Staining and Analysis

The tumor of mice in each imaging group were extracted after imaging. 4 % paraformaldehyde and 30 % sucrose were used to fixed and dehydrated the tumor tissues respectively. After embedding in frozen optimal cutting temperature (O.C.T.) medium, tumor sample was cut into sections at a thickness of 10 μm.

The immunohistochemistry staining and analysis on tumor site during the course of BEC-administration was performed as follows: (1) the tumor sections were dewaxed and dehydrated firstly. (2) inactivation of endogenous peroxidase: Endogenous peroxidase blocker was added to the tumor slices, which were then incubated at room temperature for 10 min, and washed with deionized water. (3) antigen repair: the slice was put into EDTA antigen repair solution, and heated by microwave at high heat for 8 min, then cooled naturally for 8 min, then heated at high heat for 8 min again, and cooled to room temperature. (4) blocking: 5 % BSA blocking solution was added to the slice, which was incubated at 37 ℃ for 30 min. Shake off excess liquid without washing. (5) primary antibody incubation: diluted primary antibody was added to the slice respectively and incubated at 4 ℃ overnight, then 37 ℃ for 30 min and washed with PBS (pH 7.2-7.6) for 5 min and 3 times (5 min×3). (6) secondary antibody incubation: biotin-labeled sheep anti-rabbit IgG was added and incubated at 37 ℃ for 30 min, then washed with PBS (pH 7.2-7.6) for 5 min and 3 times (5 min×3). (7) incubated with SABC: the slice was treated with

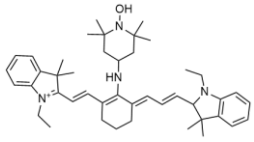
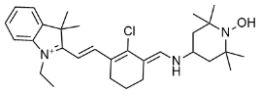
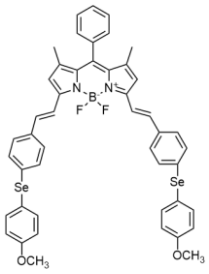
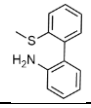
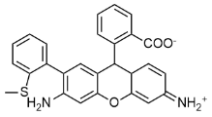
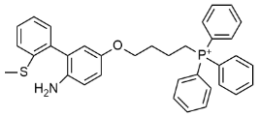
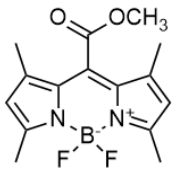
SABC-POD (rabbit IgG) kit and incubated at 37 ℃ for 30 min, then washed with PBS (pH 7.2-7.6) for 5 min and 4 times (5 min×4). (8) coloration: the slice was treated with DAB assay and observed with microscope. (9) counterstaining: the slice was treated with Mayor' hematoxylin and incubated at room temperature for 1 min, then washed with PBS (pH 7.2-7.6) and reblued with an alkaline solution. (10) sealing: the slice was sealed with neutral gum. (11) observing: microscope (Leica DM2500 and CCD camera) was used to observed the staining.

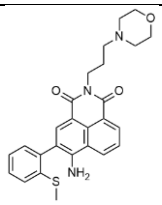
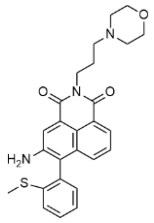
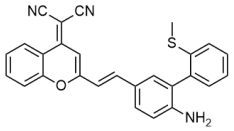
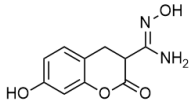
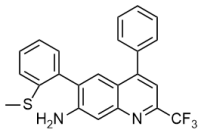
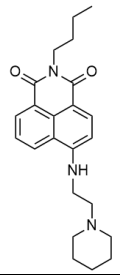
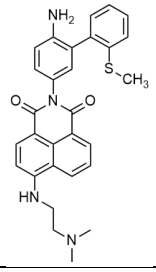
Analysis method: Three different 400-fold fields from each section were selected and analyzed with ImageScope software. All the dark brown color on the tissue sections were set as strong positive, brown yellow as moderate positive, light yellow as weak positive, and blue nuclei as negative. The area (unit: pixel) of strong positive, moderate positive, weak positive and negative tissue points were identified and analyzed, as well as the percentage of positive tissue points. Finally, histochemistry score (H-score) [8]was performed. Histochemistry score (H-SCORE) is a histological scoring method to deal with the results of immunohistochemistry. The number of positive cells in each section and their staining intensity were converted into corresponding values to achieve the semi-quantitative of tissue staining. H-SCORE = percentage of strong positive × 3 + medium positive × 2 + percentage of weak positive × 3.

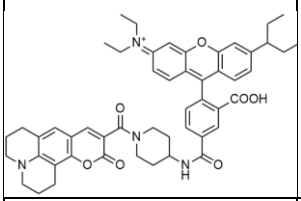
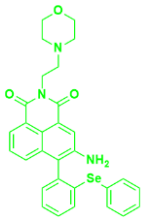
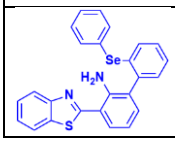


Scheme S1 The molecular structure of ABT-Se/NDI-Se and the diagram of proposed mechanism based on the formation of Se=N mediated by HOBr.

Table S1. Summary of the fluorescent probes for HOBr

Probe structure (including references)	Response mechanism	Type of signal transmitted	λ_{ex}/ λ_{em} (nm)	Limit of detection	Biological applications	
					In vitro (Cell line)	In vivo model
1[9] 	Oxidation of hydroxylamine	Ratiometric	445/ 550 610/ 632	——	RAW264.7	——
 2[9]	Oxidation of hydroxylamine	Turn-off	702/ 755	——	RAW264.7	——
 3[10]	Oxidation of selenium	Ratiometric	610/ 635 610/ 711	0.97 μM	RAW264.7	——
4[11] 	Formation of S=N	Turn-on	480/ 525	17 nM	HepG2 HL-7702	Zebrafish
 5[12]	Formation of S=N	Turn-on	624/ 663	20 pM	HepG2 HL-7702	Zebrafish
6[13] 	Formation of S=N	Ratiometric	435/ 437 502/ 528	1.8 nM.	RAW264.7	——
7[14] 	bromination	Ratiometric	480/ 581 480/ 616	3.8 nM	RAW 264.7 HCT116 A549 CCD-18Co MRC-5	The Salmonella-infected mice

8[15] 	Formation of S=N	Turn-off	430/ 540	33.5 nM cells	Hela	Kunming Mice
9[16] 	Formation of S=N	Ratiometric	365/ 555 365/ 610	99 nM	Hela	——
10[17] 	Formation of S=N	Turn-off	480/ 655 480/ 700	660 nM	MCF-7	——
11[18] 	Oxidation of amidoxime	Turn-on	395/ 460	30.6 nM	____	Arthritis model mice
12[19] 	Formation of S=N	Ratiometric	460/ 505 460/ 540	92 nM	RAW 264.7	Zebrafish
13[20] 	Cyclization of 1-(2-aminoethyl) piperidine	Turn-on	438/ 530 424/ 505	200 nM	RAW 264.7	Zebrafish
14[21] 	Formation of S=N	Turn-on	420/ 530	240 nM	HeLa	——

15[22] 	Bromination	Ratiometric	440/ 580 440/ 491	11.9 nM	HeLa	Zebrafish
Our work 	Formation of Se=N	Turn-on	440/ 560	296 nM	HeLa Two photon imaging of tumor site Immuno-histochemi stry analysis	HeLa xenografts
Our work 	Formation of Se=N	Tutn-off	380/ 443	711 nM	HeLa	——

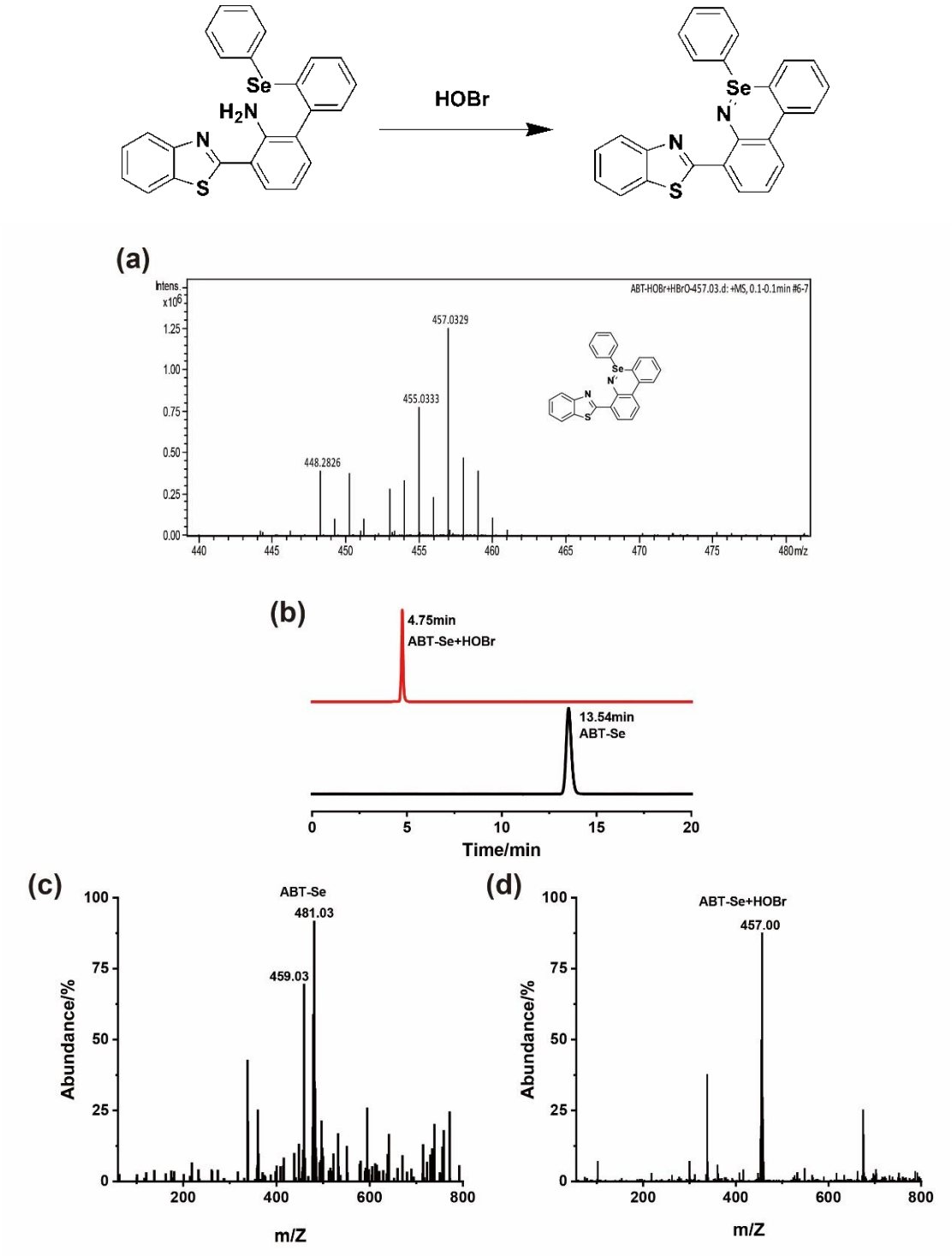


Fig. S24 Validation of the proposed mechanism for ABT-Se. (a) The HRMS spectra of the solution containing ABT-Se (10 μM) and HOBr (50 μM); (b, c, d) The HPLC-MS analysis of ABT-Se (60 μM) with/without the treatment of HOBr (300 μM). The mobile phase was methanol and the flow rate was 0.3 mL/min.

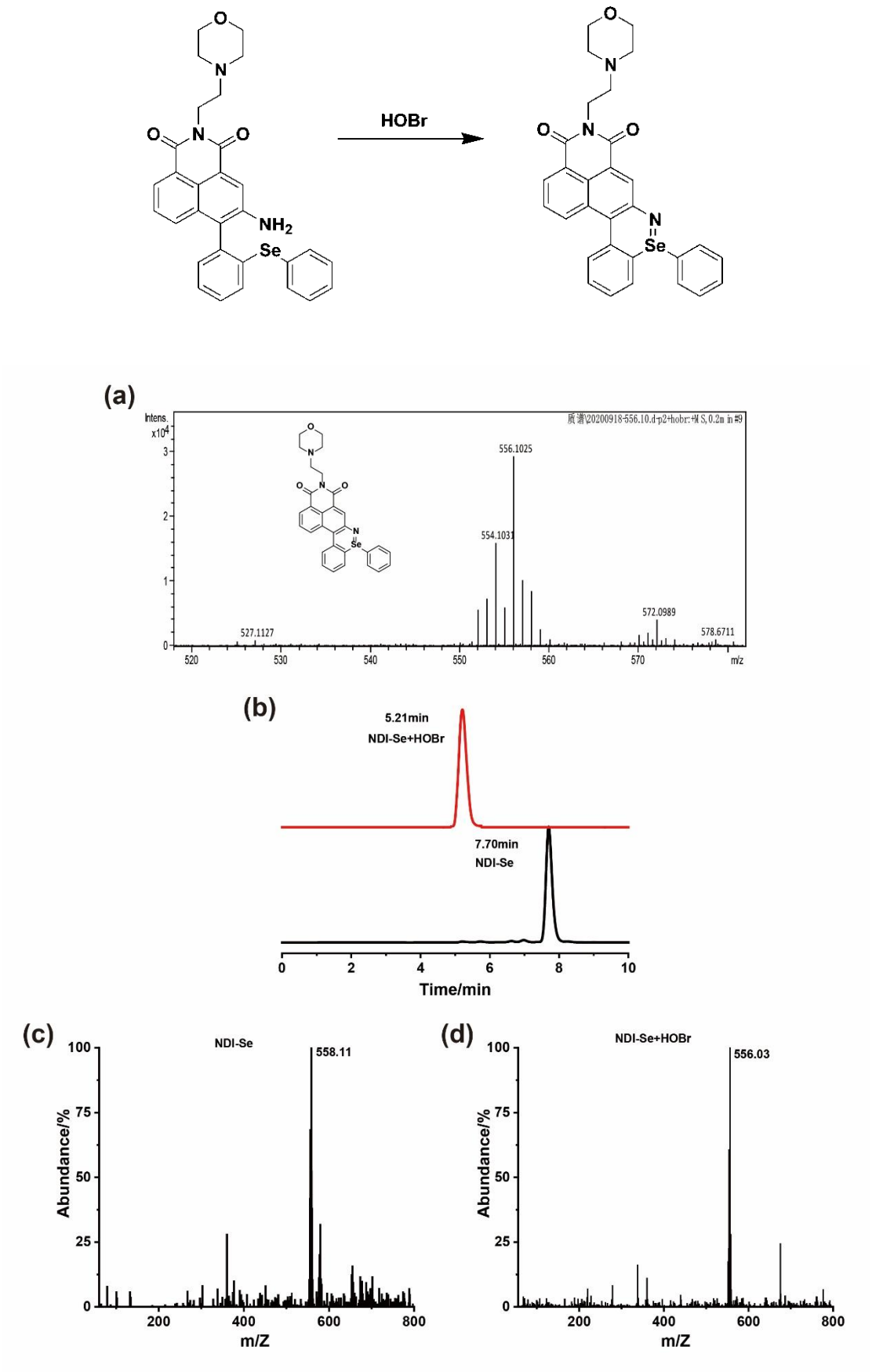


Fig. S25 Validation of the proposed mechanism for NDI-Se. (a) The HRMS spectra of the solution

containing NDI-Se (10 μM) and HOBr (50 μM); (b, c, d) The HPLC-MS analysis of NDI-Se (40 μM) with/without the treatment of HOBr (200 μM). The mobile phase was methanol and the flow rate was 0.3 mL/min.

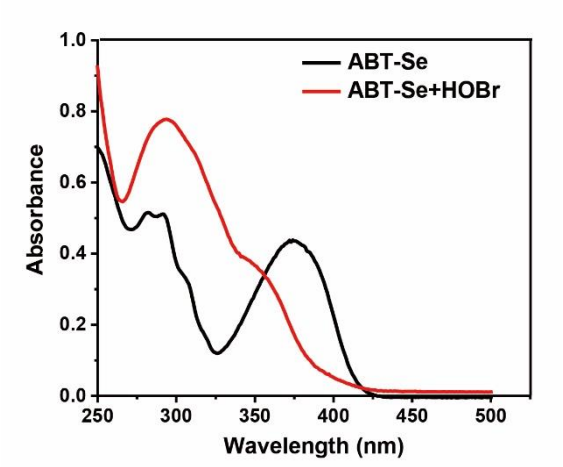


Fig. S26 Absorption spectra of ABT-Se (10 μM) in PBS buffer (0.5 % DMSO 100 mM, pH 7.4) before (black) and after (red) the addition of HOBr (50.0 μM).

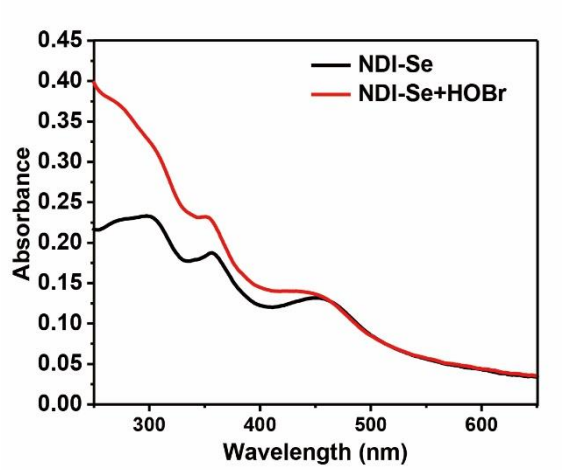


Fig. S27 Absorption spectra of NDI-Se (10 μM) in PBS buffer (1 % DMSO 100 mM, pH 7.4) before (black) and after (red) the addition of HOBr (50.0 μM).

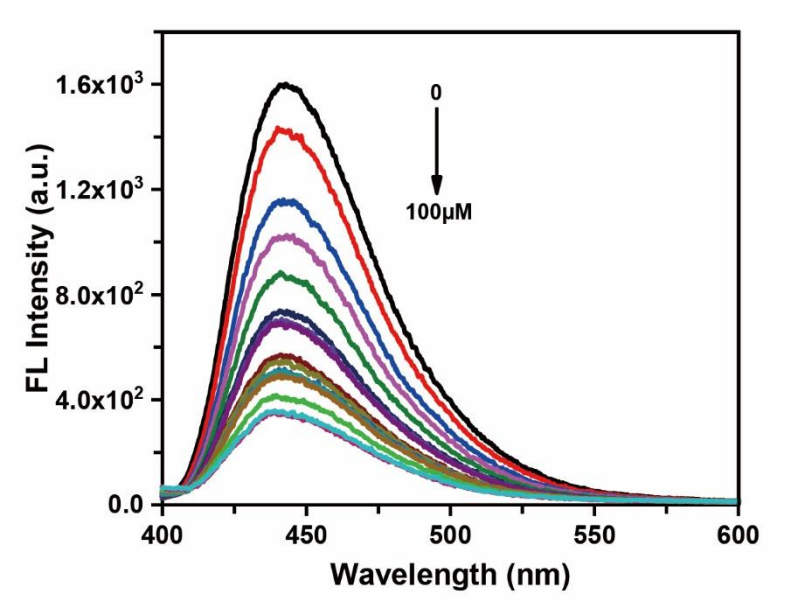


Fig. S28 Fluorescence titration profile of ABT-Se (10.0 μM, λex/λem=380 nm/443 nm) with HOBr (0-100.0 μM).

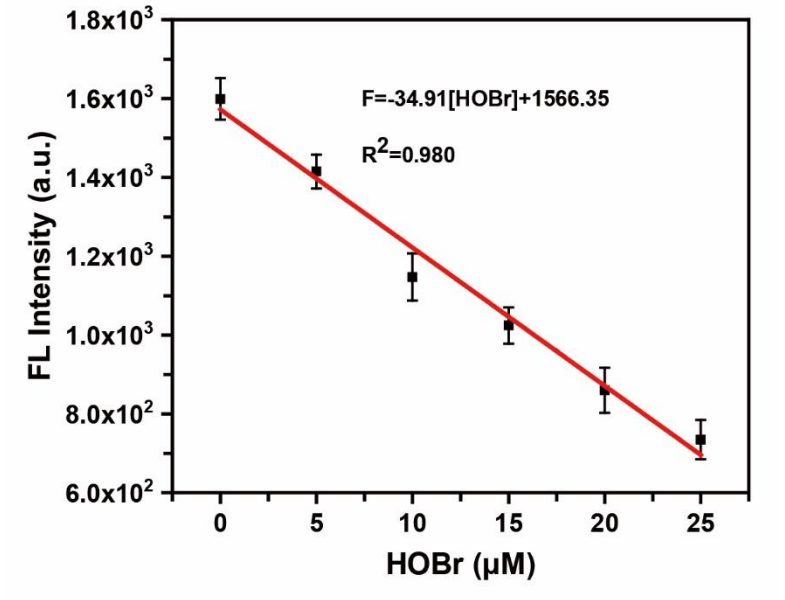


Fig. S29 The calibrated curve derived from Fig. S28.

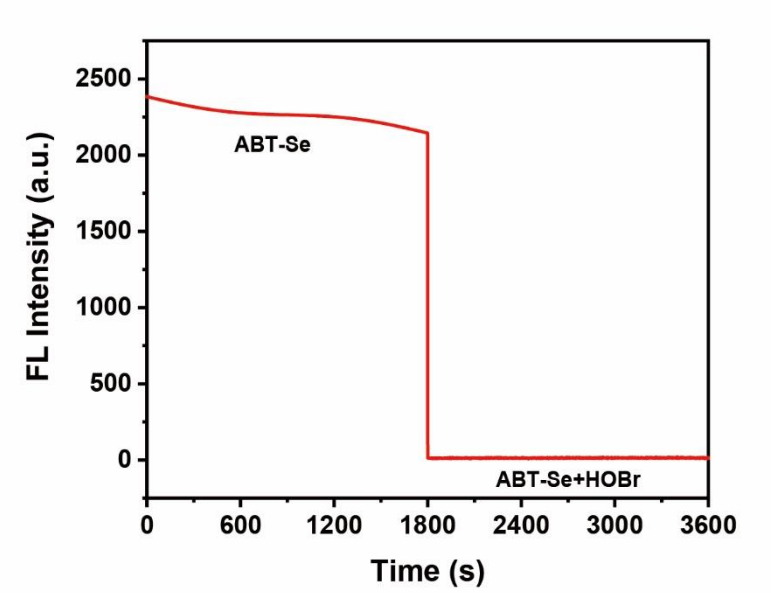


Fig. S30 Kinetic profile of ABT-Se (10.0 μM) with the addition of HOBr (100.0 μM). $\lambda_{ex}/\lambda_{em}$=380 nm/443 nm.

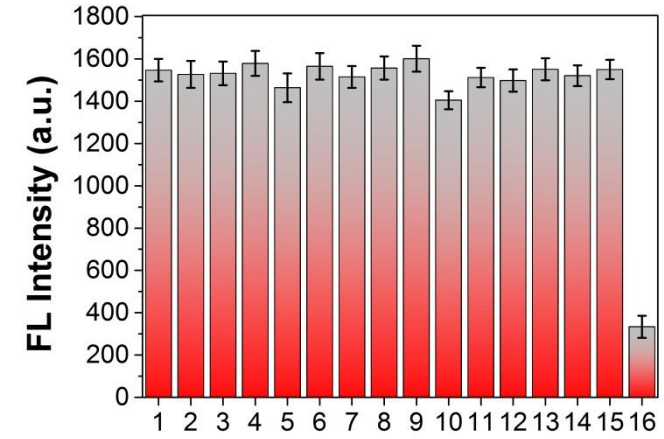


Fig. S31 Fluorescence of ABT-Se (10.0 μM) in the presence of various reactive species: 1. ABT-Se, 2. •OH (200.0 μM), 3. $^1O_2$ (200.0 μM), 4. $O_2^{\bullet-}$ (200.0 μM), 5. HOCl (200.0 μM), 6. H_2O_2 (200.0 μM), 7. NO (200.0 μM), 8. $ONOO^-$ (200.0 μM), 9. Na_2S (200.0 μM), 10. NaHS (200.0 μM), 11. $NaHSO_3$ (200.0 μM), 12. Vc (1.0 mM), 13. Cys (1.0 mM), 14. Hcy (1.0 mM), 15. GSH (1.0 mM), 16. HOBr (100.0 μM).

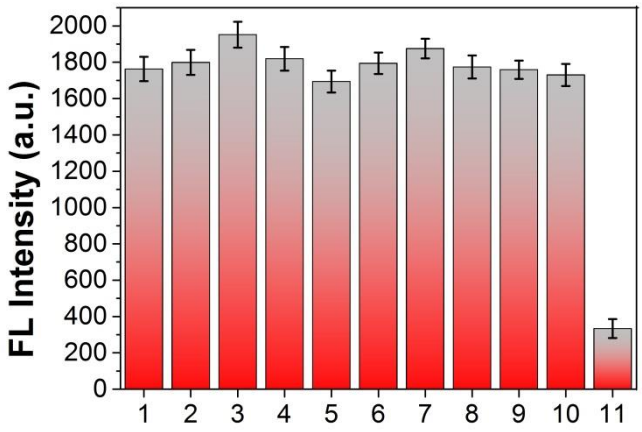


Fig. S32 Fluorescence of ABT-Se (10.0 μM) in the presence of various metal ions: 1. ABT-Se, 2. K^+ (1.0 mM), 3. Na^+ (1.0 mM), 4. Mg^{2+} (1.0 mM), 5. Ca^{2+} (1.0 mM), 6. Cu^{2+} (1.0 mM), 7. Zn^{2+} (200.0 μM), 8. Fe^{2+} (200.0 μM), 9. Fe^{3+} (200.0 μM), 10. Ag^+ (200.0 μM), 11. HOBr (100.0 μM).

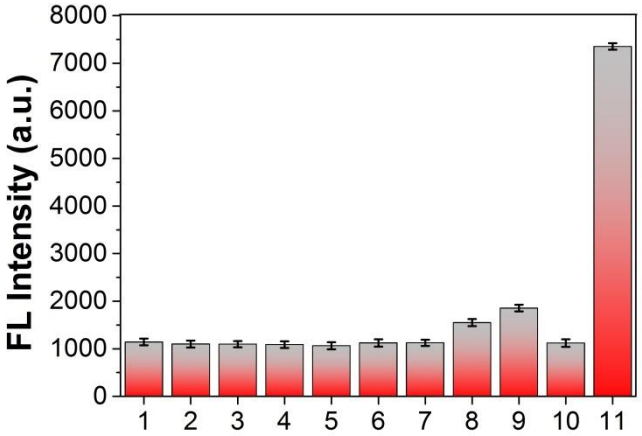


Fig. S33 Fluorescence of NDI-Se (10.0 μM) in the presence of various metal ions: 1. ABT-Se, 2. K^+ (1.0 mM), 3. Na^+ (1.0 mM), 4. Mg^{2+} (1.0 mM), 5. Ca^{2+} (1.0 mM), 6. Cu^{2+} (1.0 mM), 7. Zn^{2+} (200.0 μM), 8. Fe^{2+} (200.0 μM), 9. Fe^{3+} (200.0 μM), 10. Ag^+ (200.0 μM), 11. HOBr (100.0 μM).

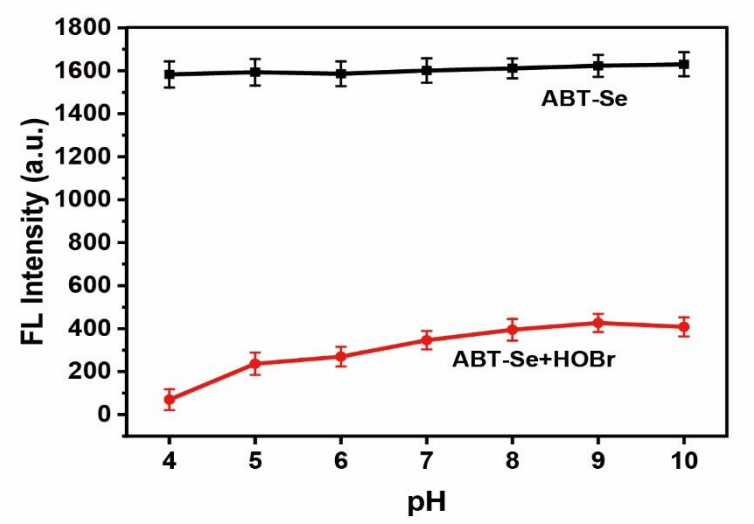


Fig. S34 Effects of pH on the fluorescence intensity of ABT-Se (10.0 μM) in the absence (black) and presence (red) of HOBr (100.0 μM) at room temperature. $\lambda_{ex}/\lambda_{em}$=380 nm/443 nm.

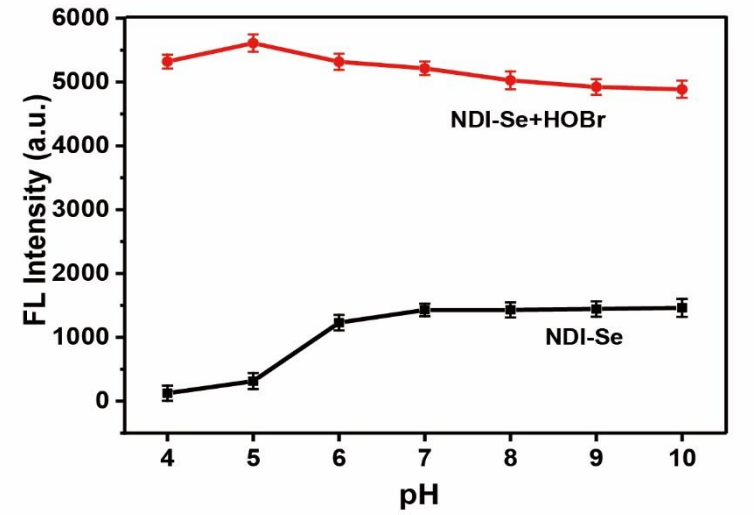


Fig. S35 Effects of pH on the fluorescence intensity of NDI-Se (10.0 μM) in the absence (black) and presence (red) of HOBr (100.0 μM) at room temperature. $\lambda_{ex}/\lambda_{em}$=440 nm/560 nm.

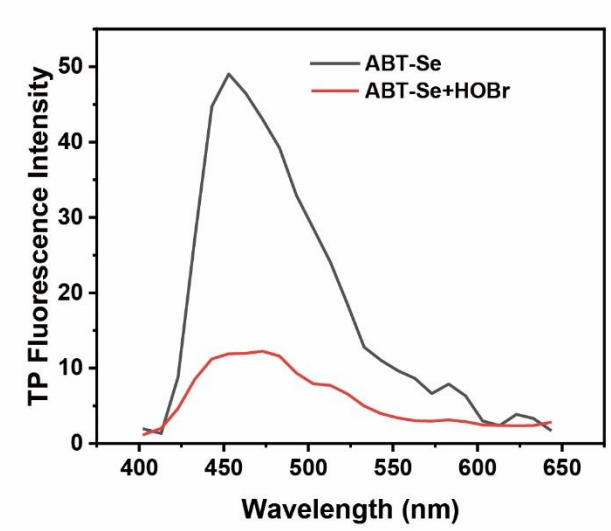


Fig. S36 The two-photon fluorescence spectrum of ABT-Se (black) and ABT-Se+HOBr (red). The two-photon absorption cross section was determined as 53.1 GM for ABT-Se (10 μM) and 17.1 GM for ABT-Se+HOBr (10 μM). ABT-Se: λ_{ex}=760 nm, λ_{em}=452 nm.

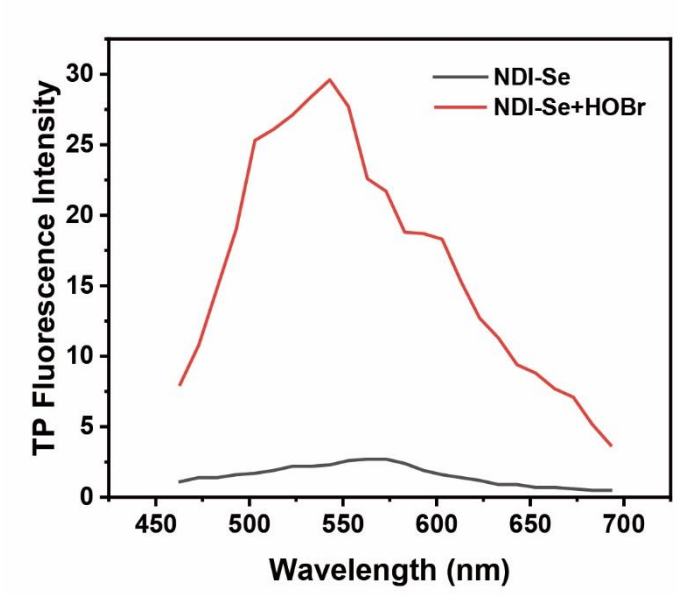


Fig. S37 The two-photon fluorescence spectrum of NDI-Se (black) and NDI-Se+HOBr (red). The two-photon absorption cross section was determined as 2.0 GM for NDI-Se (10 μM) and 21.8 GM for NDI-Se+HOBr (10 μM). NDI-Se: λ_{ex}=800 nm, λ_{em}=550 nm.

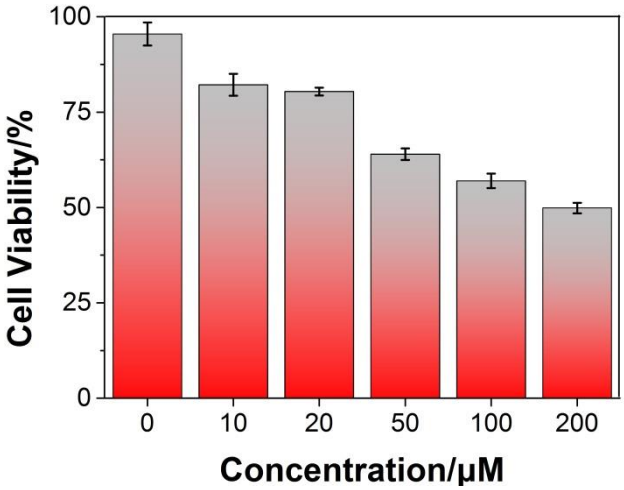


Fig. S38 MTT assay of HeLa cells with different concentrations of ABT-Se (0 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM).

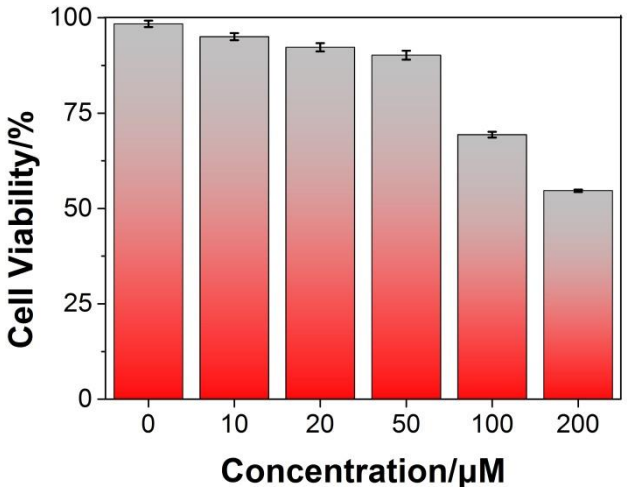


Fig. S39 MTT assay of HeLa cells with different concentrations of NDI-Se (0 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM).

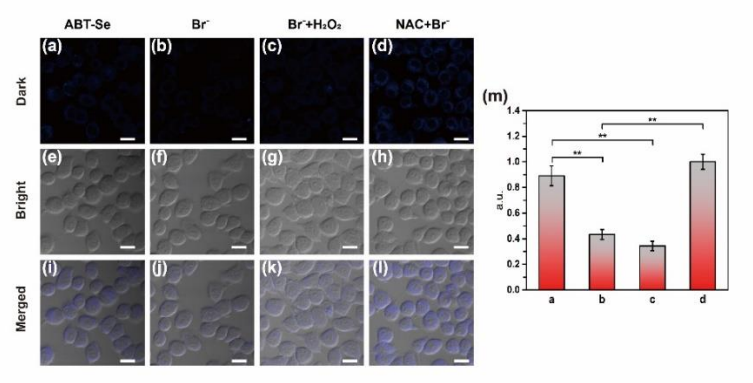


Fig. S40 Confocal fluorescent imaging of endogenous HOBr in HeLa cells. (a, e, i) HeLa cells were incubated with ABT-Se (10.0 μM) alone for 20 min; (b, f, j) HeLa cells were pretreated with KBr (100 μM) for 20 min, and then incubated with ABT-Se (10.0 μM) for 20 min; (c, g, k) HeLa cells were pretreated with KBr (100 μM) and H_2O_2 (100 μM) for 20 min, and then incubated with ABT-Se (10.0 μM) for 20 min; (d, h, l) HeLa cells were pretreated with KBr (100 μM) and NAC (100 μM) for 20 min, and then incubated with ABT-Se (10.0 μM) for 20 min; (m) The relative fluorescence intensity of (a−d). The fluorescence images were obtained with 760 nm excitation and 420-520 nm collection. The values are the mean ± s.d. for n =3, *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 20 μm.

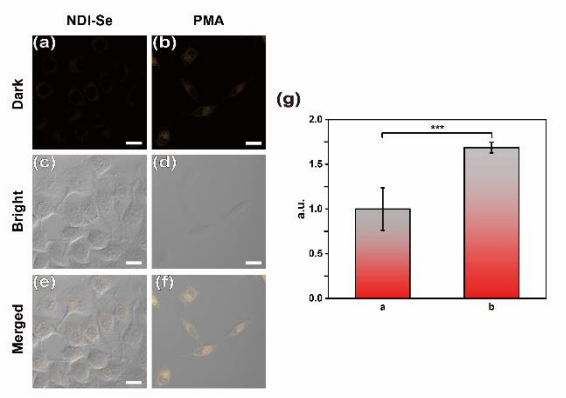


Fig. S41 Confocal fluorescent imaging of endogenous HOBr in HeLa cells. (a, c, e) HeLa cells were incubated with NDI-Se (5.0 μM) alone; (b, d, f) HeLa cells were pretreated with PMA (100 μM) for 20 min, and then incubated with NDI-Se (5.0 μM) for 15 min; (g) The relative fluorescence intensity of (a−b). The fluorescence images were obtained with 800 nm excitation and 510-610 nm collection. The values are the mean ± s.d. for n =3, ***p < 0.001. Scale bar: 20 μm.

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