A novel small-molecule fluorescent probe caused by minimal structural modifications for specifically staining of the cell nuclear membrane

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1. Materials and measurements

All materials were purchased from commercial sources. All chemical reagents used in the experiment were analytically pure and further re-evaporate to remove water. The solvents used for spectral test were chromatographic pure. The raw materials of synthetic drugs were purchased from BIDE PHARMATECH CO., LTD(China) and TCI(Japan). The water used in the experiments was ultrapure water. The DMEM and FBS used in biological experiments were purchased from HyClone and Gibico. NMR spectra were recorded on a Bruker Advance 400MHz (Germany) in $CDCl_3$ and DMSO-d_6. Shifts were referenced relative to the internal solvent signals. ESI-MS spectra were recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). The quoted m/z values represented the major peaks in the isotopic distribution. UV/Vis spectra were recorded on a U2910 spectrophotometer (Japan). One-photon fluorescence and excitation spectra of dilute solutions were obtained on a HITACH F-2700 spectrofluorimeter equipped with a 450-W Xe lamp (Japan). For imaging, confocal images was collected on the Olympus FV1200 Microscope with a 60u oil-immersion objective lens.

2. General procedure for spectra measurements.

Absorption and fluorescence spectra

The solvents used in all spectroscopic testing were commercial chromatography-pure solvents and further purified by an ultra-dry solvent system to <30ppm water content for testing. The probe stoke solution was tested at a concentration of 10mM. The stock solution was dispersed in the test solvent at a concentration of 10μM. All tests were conducted in dark and dark conditions.

Theoretical calculations

DOPC preparation: The water molecules in the initial structures were deleted and added hydrogen atoms. Ligand (probes) preparation: using the optimized structure, the partial atomic charges were obtained by restrained electrostatic potential (RESP) calculating with Gaussian 09 package at HF/6-31g* level. Following that, docking was carried out using AutoDock 4.2.6. The parameters were set as default except for the number of GA runs (150) and the maximum number of energy evaluations (2500000). The displayed images were processed with PyMOL.

3. Cell and animal experiments

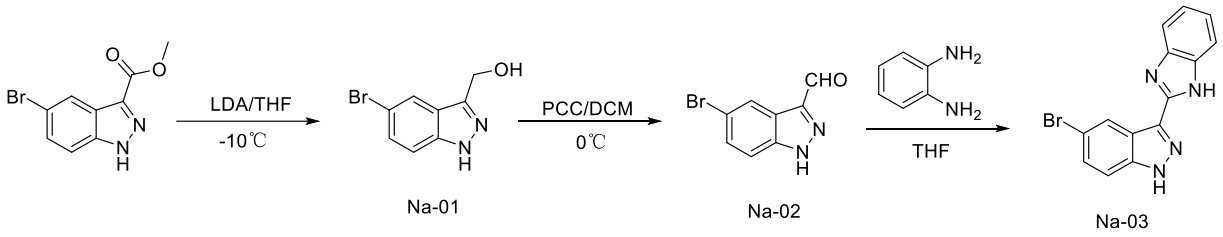
Cell culture and staining method

HeLa cells were cultured in DMEM containing 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin under 37ºC in a 5% CO_2 atmosphere. All samples were incubated with INDA-Nu(20μM), INDA-Numem(20μM) INDO-H(10μM) and INDO-Cl(10μM) in a glucose-free medium for 15min, and then images were collected with a confocal microscope.

MTT assay for the cell cytotoxicity

This involves the reduction of MTT tetrazolium to MTT formazan pigment by the metabolic activity of live-cells. Cells were seeded at a density of 1×10^5 cells/mL in a 96-well plate. After 24h of cell attachment, cells were treated with a probe for 24h. Six replicate wells were used for each control and tested concentrations. After incubation for 24h, the medium was removed and cells were washed with PBS twice. MTT tetrazolium solution (100mL of 0.5mg/mL in PBS) was added to each well, and the cells were further incubated at 37℃ for 4h in a 5% CO_2 humidified atmosphere. Excess MTT tetrazolium solution was then carefully removed and the colored formazan was dissolved in 100μL DMSO. The plate was shaken for 10min and the absorbance was measured at 590nm using a microplate reader.

4. Synthesis and characterization



Scheme S1 Synthesis routes of Na-03

Synthesis route of Na-01

Methyl-5-bromo-1H-indazole-3-carboxylate(2.5g,10mmol) was dissolved with 40mL of THF. Stirring the mixture under -10℃ at N_2 atmosphere for 10min, and then 10mL of $LiAlH_4$(1.6M in THF) was added. After stirring for 4h, the mixture was poured into ice water and extracted with ethyl acetate (3×10mL). The combined organic phase is distilled under vacuum pressure to remove the solvent, and the product Na-01 was obtained. The resulting product can be used directly for the next step without purification. The product does not need to be purified and can be used directly in the next step.

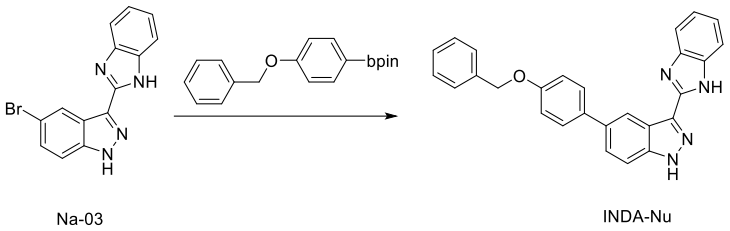
Synthesis route of Na-02

Na-01(2.27g,10mmol) was dissolved with 40mL of acetone. Stirring the mixture under 0℃ for 10min, and then PCC(4.5g, 20mmol) was added slowly. After stirring for 2h, the mixture was poured into ice water and extracted with ethyl acetate (3×10mL). The combined organic phase is distilled under vacuum pressure to remove the solvent. And then, the residue was purified by fast silica gel column chromatography through Flash Chromatography Instrument (CH_2Cl_2: Hexane= 1:20) and the product with the absorption spectra under both 365nm and 254nm was collected. State: Brown solid. Yield:1.96g (87.1%).

Synthesis route of Na-03

Na-02(225mg, 1mmol) and benzene-1,2-diamine(130mg, 1.2mmol) were dissolved in 10mL of THF and 7 drops of HCl(2N) were added. After stirring the mixture under 70℃ at N_2 atmosphere for 30min, 8mL of sodium

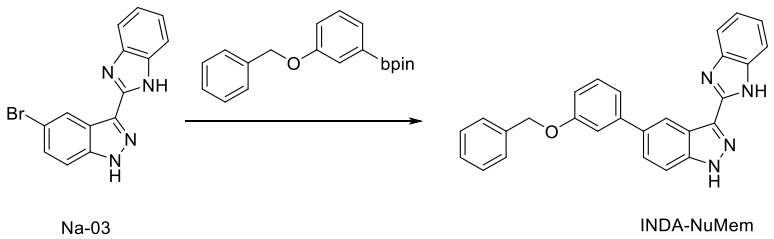
bisulfite(1N) was added. After refluxing the mixture for 16h, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. The product does not need to be purified and can be used directly in the next step. State: White solid. Yield: 246mg (78.6%). ^{1}HNMR (400MHz, DMSO-d_6) δ 14.35 (s, 1H), 10.18 (s, 1H), 8.26 (d, J = 2.1 Hz, 1H), 7.70 (d, J = 8.1 Hz, 1H), 7.62 (dd, J = 8.8, 1.9 Hz, 1H). ^{13}CNMR (101MHz, DMSO-d_6) δ 187.71, 143.21, 140.41, 130.64, 123.28, 122.35, 116.99, 113.91. MS: calced for $C_8H_5BrN_2O^+$[M+H]$^+$ 224.9658. Found for $C_8H_5BrN_2O^+$[M+H]$^+$ 224.9665.



Scheme S2 Synthesis routes of INDA-Nu

Synthesis route of INDA-Nu

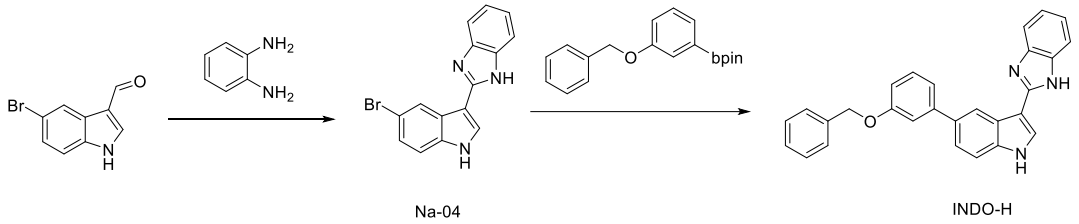
Na-03(156.6mg, 0.5mmol), K_3PO_4(212mg, 1mmol), (4-benzyloxy)phenylboronic(201.6mg, 0.65mmol), $PdCl_2(dppf)_2$(36mg. 0.05mmol) were added into 4.5mL of dioxane and heated under 100°C under N_2 atmosphere for 18h. And then, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. And then, the residue was purified by fast silica gel column chromatography through Flash Chromatography Instrument (CH_2Cl_2: Hexane= 1:20) and the product with the absorption spectra under both 365nm and 254nm was collected. State: White solid. Yield:132mg (63.4%). ^{1}HNMR (400MHz, DMSO-d_6) δ 13.84 (s, 1H), 13.06 (s, 1H), 8.74 (d, J = 32.4 Hz, 2H), 7.85 – 7.81 (m, 1H), 7.77 (d, J = 7.2 Hz, 1H), 7.67 (s, 2H), 7.65 (s, 1H), 7.64 (d, J = 1.9 Hz, 1H), 7.61 (t, J = 1.6 Hz, 1H), 7.52 (dd, J = 8.1, 1.4 Hz, 1H), 7.44 (d, J = 8.8 Hz, 1H), 7.38 (dd, J = 8.8, 1.8 Hz, 1H), 7.27 – 7.19 (m, 4H), 6.52 (s, 2H). ^{13}CNMR (101MHz, DMSO-d_6) δ 147.04, 146.33, 144.35, 143.38, 140.87, 140.43, 140.14, 139.85, 135.92, 134.65, 130.38, 130.09, 129.36, 124.51, 123.27, 122.72, 122.08, 119.44, 114.66, 113.32, 113.01, 111.93, 42.37. MS: calced for $C_{27}H_{20}N_4O^+$[M+H]$^+$ 416.1710. Found for $C_{27}H_{20}N_4O^+$ [M+H]$^+$ 416.1759.



Scheme S3 Synthesis routes of INDA-NuMem

Synthesis route of INDA-NuMem

Na-03(156.6mg, 0.5mmol), K_3PO_4(212mg, 1mmol), (3-benzyloxy)phenylboronic(201.6mg, 0.65mmol), $PdCl_2(dppf)_2$(36mg. 0.05mmol) were added into 4.5mL of dioxane and heated under 100℃ under N_2 atmosphere for 18h. And then, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. And then, the residue was purified by fast silica gel column chromatography through Flash Chromatography Instrument (CH_2Cl_2: Hexane= 1:20) and the product with the absorption spectra under both 365nm and 254nm was collected. State: Light yellow solid. Yield:166mg (79.7%).^{1}HNMR (400 MHz, DMSO-d_6) δ 13.71 (s, 1H), 13.02 (s, 1H), 8.72 (s, 1H), 7.76 (q, J = 8.7, 8.1 Hz, 3H), 7.53 (d, J = 7.4 Hz, 2H), 7.43 (q, J = 7.8 Hz, 4H), 7.34 (q, J = 7.4 Hz, 3H), 7.23 (d, J = 8.1 Hz, 2H), 7.09 – 7.03 (m, 1H), 5.23 (s, 2H). ^{13}CNMR (101 MHz, DMSO-d_6) δ 159.33, 147.56, 142.77, 141.28, 137.62, 136.85, 134.75, 130.61, 128.94, 128.31, 128.28, 127.15, 123.12, 121.99, 120.14, 119.45, 114.11, 113.86, 111.59, 69.77. MS: calced for $C_{27}H_{20}N_4O^+$[M+H]$^+$ 416.1710. Found for $C_{27}H_{20}N_4O^+$ [M+H]$^+$ 416.1762.



Scheme S4 Synthesis routes of INDO-H

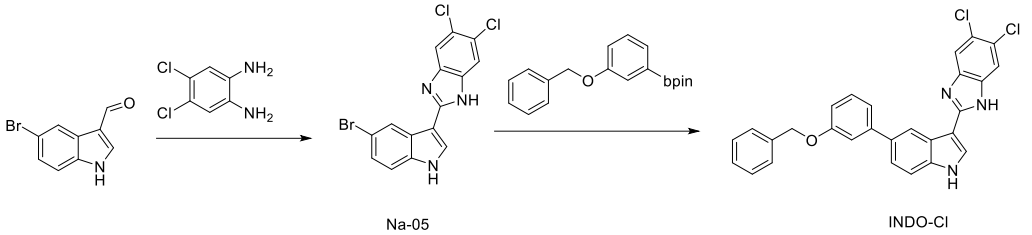
Synthesis route of Na-04

5-bromo-1H-indole-3-carbaldehyde(224mg, 1mmol) and benzene-1,2-diamine(130mg, 1.2mmol) were dissolved in 10mL of THF and 7 drops of HCl(2N) were added. After stirring the mixture under 70℃ at N_2 atmosphere for 30min, 8mL of sodium bisulfite(1N) was added. After refluxing the mixture for 16h, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. The product does not need to be purified and can be used directly in the next step. State: White solid. Yield:201mg (64.4%).

Synthesis route of INDO-H

Na-04(156.5mg, 0.5mmol), K_3PO_4(212mg, 1mmol), (3-benzyloxy)phenylboronic(201.6mg, 0.65mmol), $PdCl_2(dppf)_2$(36mg. 0.05mmol) were added into 4.5mL of dioxane and heated under 100℃ under N_2 atmosphere for 18h. And then, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. And then, the residue was purified by fast silica gel column chromatography through Flash Chromatography Instrument (CH_2Cl_2: Hexane= 1:20) and the product with the absorption spectra under both 365nm and 254nm was collected. State: Light yellow solid. Yield:152mg

(73.2%). ^{1}HNMR (400MHz, DMSO-d_6) δ 12.46 (s, 1H), 11.71 (s, 1H), 8.73 (s, 1H), 8.18 (d, J = 2.7 Hz, 1H), 7.66 (s, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.55 – 7.45 (m, 4H), 7.42 (t, J = 7.9 Hz, 3H), 7.37 – 7.27 (m, 3H), 7.14 (d, J = 5.3 Hz, 2H), 7.01 (dd, J = 8.2, 2.5 Hz, 1H), 5.22 (s, 2H). ^{13}CNMR (101 MHz, DMSO-d_6) δ 159.25, 149.87, 143.88, 137.69, 136.65, 133.29, 130.39, 128.93, 128.25, 127.43, 126.09, 122.21, 121.89, 121.38, 120.10, 119.91, 118.50, 114.05, 113.22, 112.79, 110.78, 107.55, 69.72. MS: calced for $C_{28}H_{21}N_3O^+$[M+H]$^+$ 416.1757. Found for $C_{27}H_{20}N_4O^+$ [M+H]$^+$ 416.1762.



Na-05 INDO-Cl

Scheme S5 Synthesis routes of INDO-Cl

Synthesis route of Na-05

5-bromo-1H-indole-3-carbaldehyde (224mg, 1mmol) and 2-(5-bromo-1H-indol-3-yl)-5,6-dichloro-1H-benzo[d]imidazole (224mg, 1.2mmol) were dissolved in 10mL of THF and 7 drops of HCl(2N) were added. After stirring the mixture under 70℃ at N_2 atmosphere for 30min, 8mL of sodium bisulfite(1N) was added. After refluxing the mixture for 16h, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. The product does not need to be purified and can be used directly in the next step. State: White solid. Yield:171mg (45.1%).

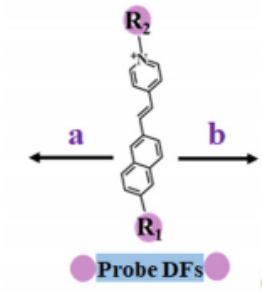
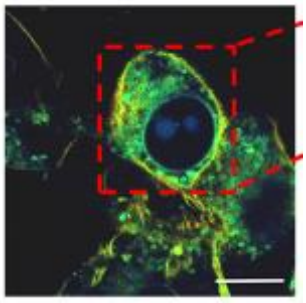
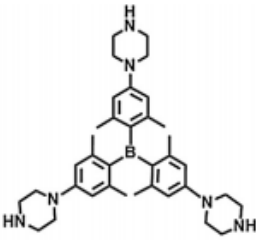
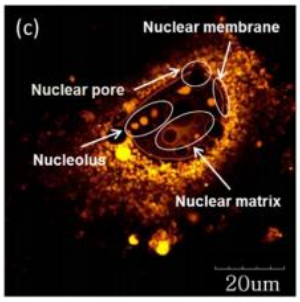
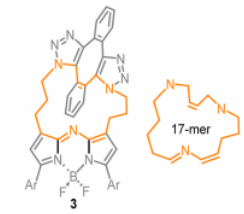
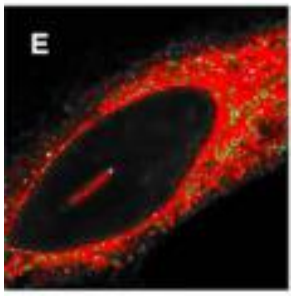
Synthesis route of INDO-Cl

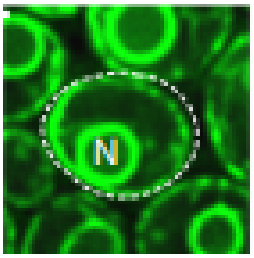
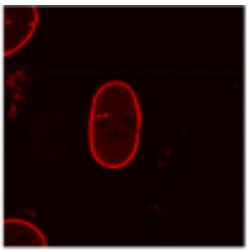
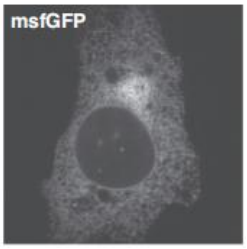
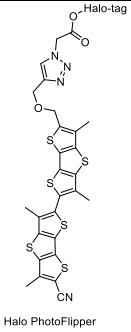
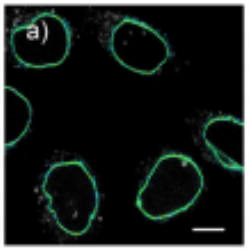
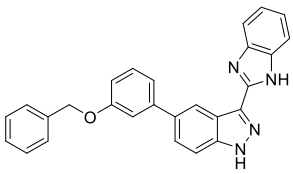
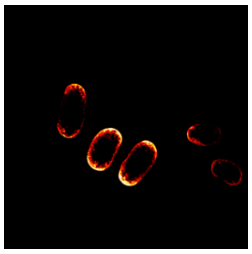
Na-05(191mg, 0.5mmol), K_3PO_4(212mg, 1mmol), (3-benzyloxy)phenylboronic(201.6mg, 0.65mmol), $PdCl_2(dppf)_2$(36mg. 0.05mmol) were added into 4.5mL of dioxane and heated under 100℃ under N_2 atmosphere for 18h. And then, the solution was cooled down to room temperature and diluted with ethyl acetate(25mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate. The combined organic phase is distilled under vacuum pressure to remove the solvent. And then, the residue was purified by fast silica gel column chromatography through Flash Chromatography Instrument (CH_2Cl_2: Hexane= 1:20) and the product with the absorption spectra under both 365nm and 254nm was collected. State: Light yellow solid. Yield:127mg (52.5%). ^{1}HNMR (400 MHz, DMSO-d_6) δ 12.79 (s, 1H), 11.82 (s, 1H), 8.66 (dd, J = 11.5, 1.9 Hz, 1H), 8.24 (s, 1H), 7.91 (s, 1H), 7.70 (s, 1H), 7.56 (dd, J = 26.5, 8.6 Hz, 4H), 7.41 (t, J = 7.3 Hz, 3H), 7.37 – 7.26 (m, 3H), 7.01 (dd, J = 8.2, 2.5 Hz, 1H), 5.22 (s, 2H). ^{13}CNMR (101 MHz, DMSO-d_6) δ 159.25, 152.52, 143.77, 137.68, 136.67, 133.63, 130.39, 128.92, 128.42, 128.28, 128.24, 125.99, 125.47, 123.84, 122.45, 120.12, 119.76, 119.44, 114.67, 114.06, 113.79, 113.29, 112.95, 106.67, 69.73. MS: calced for $C_{28}H_{19}Cl_2N_3O^+$[M+H]$^+$ 484.0978. Found for $C_{28}H_{19}Cl_2N_3O^+$ [M+H]$^+$ 484.0974.

5. Supplemental figures and Tables

Summary of reported nuclear membrane probes and INDA-NuMem

Scheme S6 Summary of reported nuclear membrane probes and INDA-NuMem

Probes	Species of target sites	Live cells or not	Stained objects
	Small Molecular Probe (10.1073/pnas.2316450121)	Yes	 Nuclear membrane, Cell membrane, and Endoplasmic Reticulum
	Small Molecular Probe (10.1016/j.snb.2018.01.161.)	Yes	 Nuclear membrane, Nucleolus, and Endoplasmic Reticulum
	Small Molecular Probe (10.1039/d4sc03489a)	Yes	 Nuclear membrane, Mitochondria, and Endoplasmic Reticulum

mGFP-mcrt1	Green Fluorescent Protein (10.1038/s41556-023-01207-8)	Yes	 Nuclear membrane
mApple-Lamin A	Red Fluorescent Protein (10.1021/acschembio.8b00219)	Yes	 Nuclear membrane
msfGFP	Green Fluorescent Protein (10.1111/j.1600-0854.2012.01336.x)	Yes	 Nuclear membrane, and Endoplasmic Reticulum
 Halo PhotoFlipper	Halo-tag protein (10.1002/anie.202113163)	No	 Nuclear membrane
 INDA-NuMem	Small Molecular Probe (This Paper)	Yes	 Nuclear Membrane

Absorption spectra and emission spectra

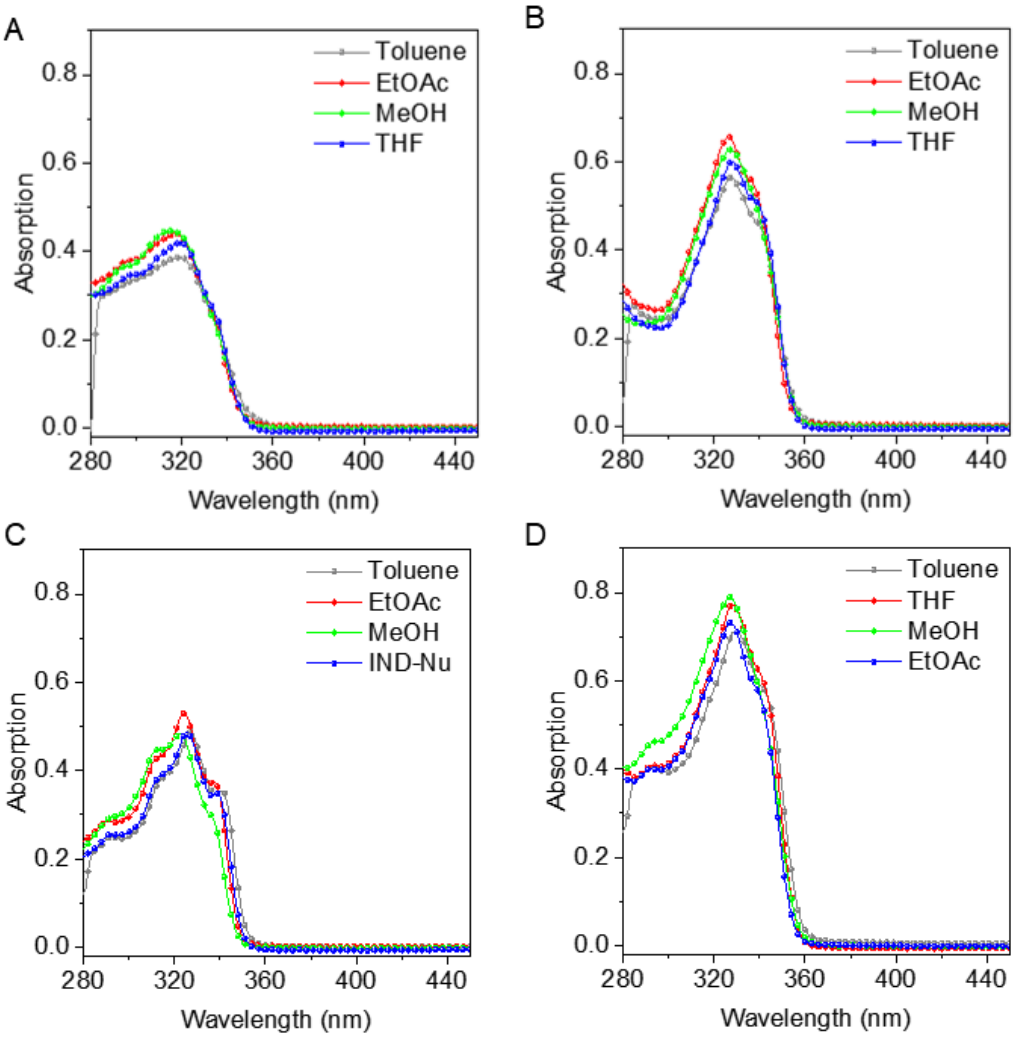


Figure S1 Absorption spectra of INDO-H(A), INDO-Cl(B), INDA-Nu(C), and INDA-NuMem(D) in different solvents. Testing concentration: 10μM.

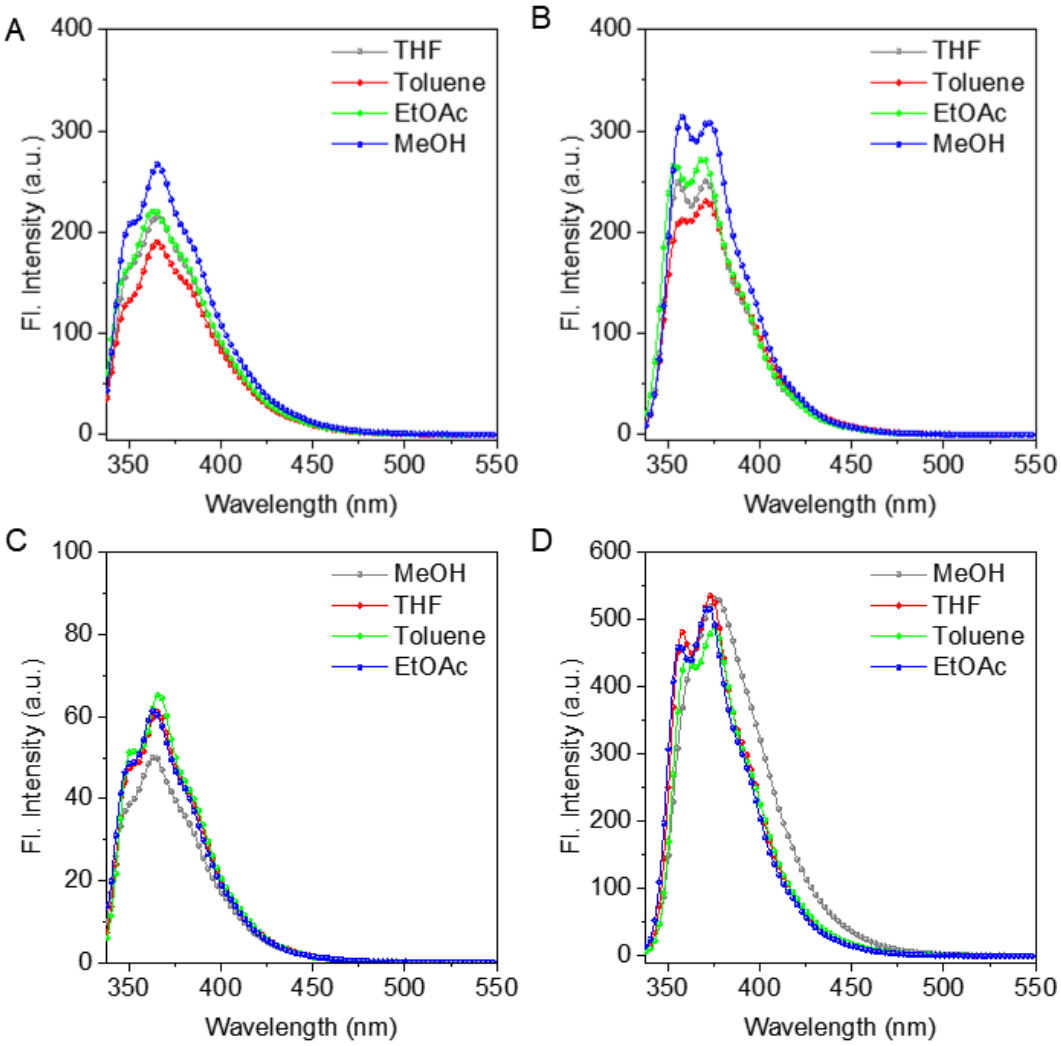


Figure S2 Fluorescent emission spectra of INDO-H(A), INDO-Cl(B), INDA-Nu(C), and INDA-NuMem(D) in different solvents. Testing concentration: 10μM.

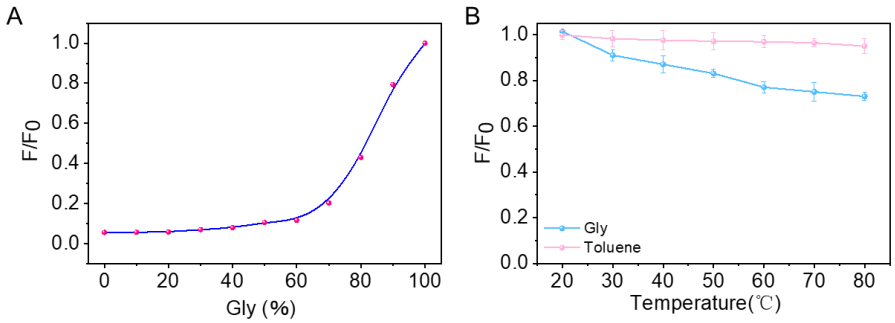


Figure S3 A.Relative fluorescence intensities of INDA-NuMem in different ratios of glycerol and water (F/F_{oil}) B. Fluorescence intensity ratio of INDA-NuMem at different temperatures ($F/F_{20°C}$) in different solvents(Blue line: Gly; Pink Line: toluene).

Photophysical data

Table S1 Photophysical Data for INDO-H, INDO-Cl, INDA-Nu and INDA-NuMem.

Comp.	Solvent	λ_{abs}/nm	$\varepsilon/10^4 M^{-1}cm^{-1}$	λ_{em}/nm	Φ_F	Stokes shift/cm^{-1}	Brightness ($\varepsilon*\Phi_F$)
INDO-H	Toluene	319	3.88	365.5	7.2%	3988	2774.2
	THF	320	4.04	365.5	6.3%	3890	2516.9
	EtOAc	317	4.41	363.0	7.1%	3998	3148.7
	MeOH	315	4.46	365.5	8.1%	4386	3599.2
INDO-Cl	Toluene	328	5.66	371.5	9.3%	3570	5241.2
	THF	328	6.00	355.5	10.1%	2358	6054.0
	EtOAc	327	6.55	353.5	11.2%	2292	7362.2
	MeOH	328	6.27	358.5	6.8%	2594	4269.9
INDA-Nu	Toluene	327	4.89	366.5	4.6%	3296	2234.7
	THF	324	4.78	365.0	3.9%	3467	1873.8
	EtOAc	324	5.30	363.0	4.8%	3316	2522.8
	MeOH	323	4.83	364.0	4.6%	3487	2226.6
INDA-NuMem	Toluene	329	7.13	361.0	16.7%	2694	11935.6
	THF	328	7.74	358.0	11.2%	2555	8676.5
	EtOAc	328	7.30	356.0	9.9%	2398	7270.8
	MeOH	327	7.89	378.0	10.2%	4126	8024.1

Cell Imaging

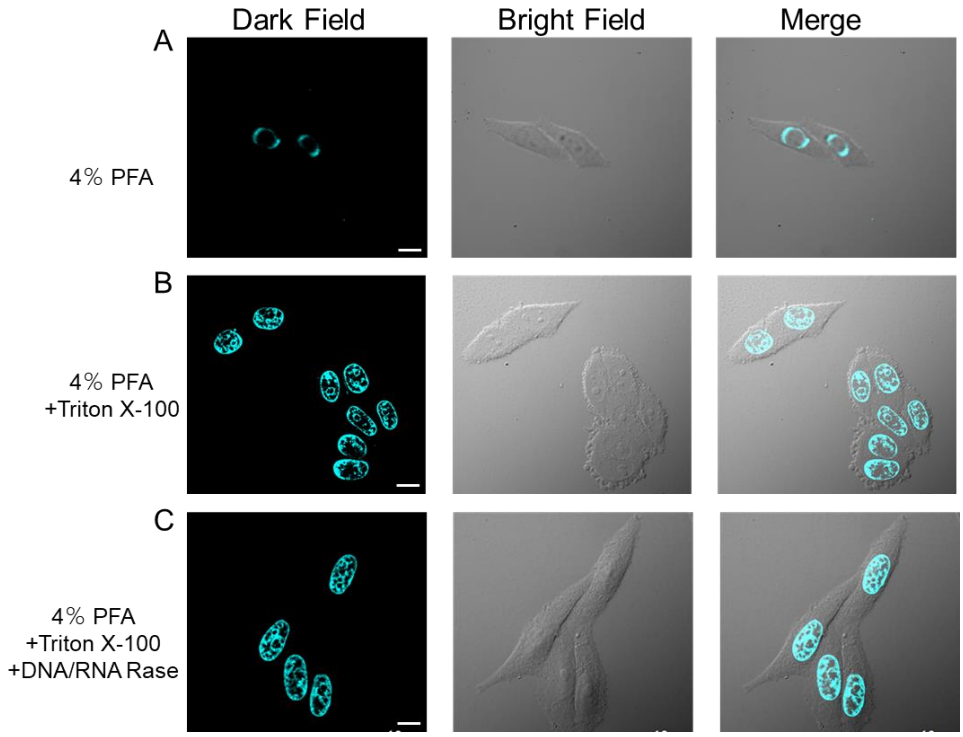


Figure S4 Confocal imaging of INDA-NuMem under different conditions. A. 4%PFA. B. 4%PFA and 0.5%Triton X-100. C. 4%PFA 0.5%Triton X-100 and DNA/RNA Rase.

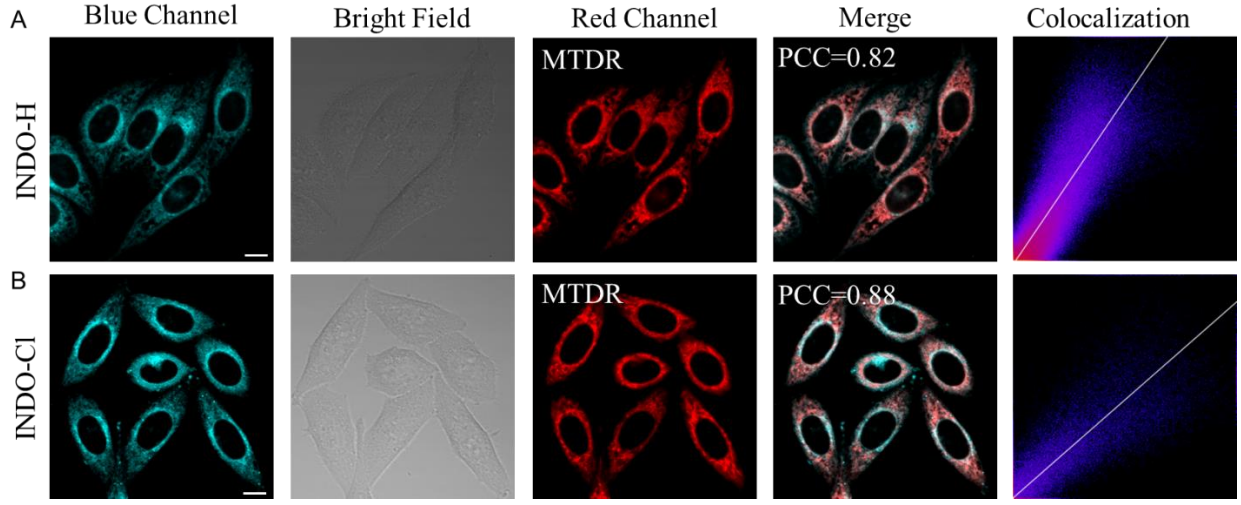


Figure S5 Live-cell co-localization confocal imaging of INDO-H(A) and INDO-Cl(B) with MTDR. Scale bar = 10μm, INDO-H: staining concentration = 20μM, staining time = 10min. λex=405nm, λem=415-465nm. INDO-Cl: staining concentration = 20μM, staining time = 10min. λex=405nm, λem=415-465nm. MTDR: staining concentration = 200nM, staining time = 30min. λex=633nm, λem=650-750nm.

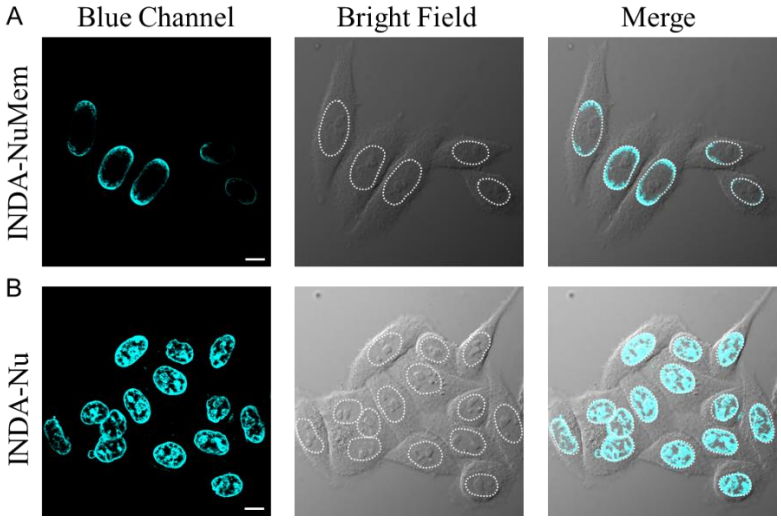


Figure S6 Colocalization analysis of Figure 4C and 4D. INDA-Nu(A) and INDA-NuMem(B) with bright field. Scale bar = 10μm, INDA-Nu: staining concentration = 20μM, staining time = 10min. λex=405nm, λem=415-465nm. INDA-NuMem: staining concentration = 20μM, staining time = 10min. λex=405nm, λem=415-465nm.

Note: We performed images in Figure 4 to illustrate the probe positioning position

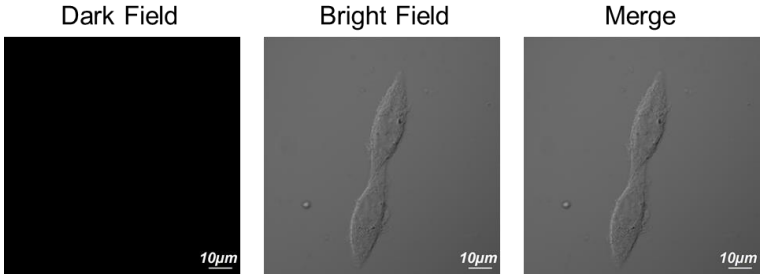


Figure S7 Live-cell confocal imaging unstained cells, using the same imaging conditions with INDA-NuMem. Scale bar = 10μm, λex=405nm, λem=415-465nm. Laser intensity: 20%.

Cell cytotoxicity

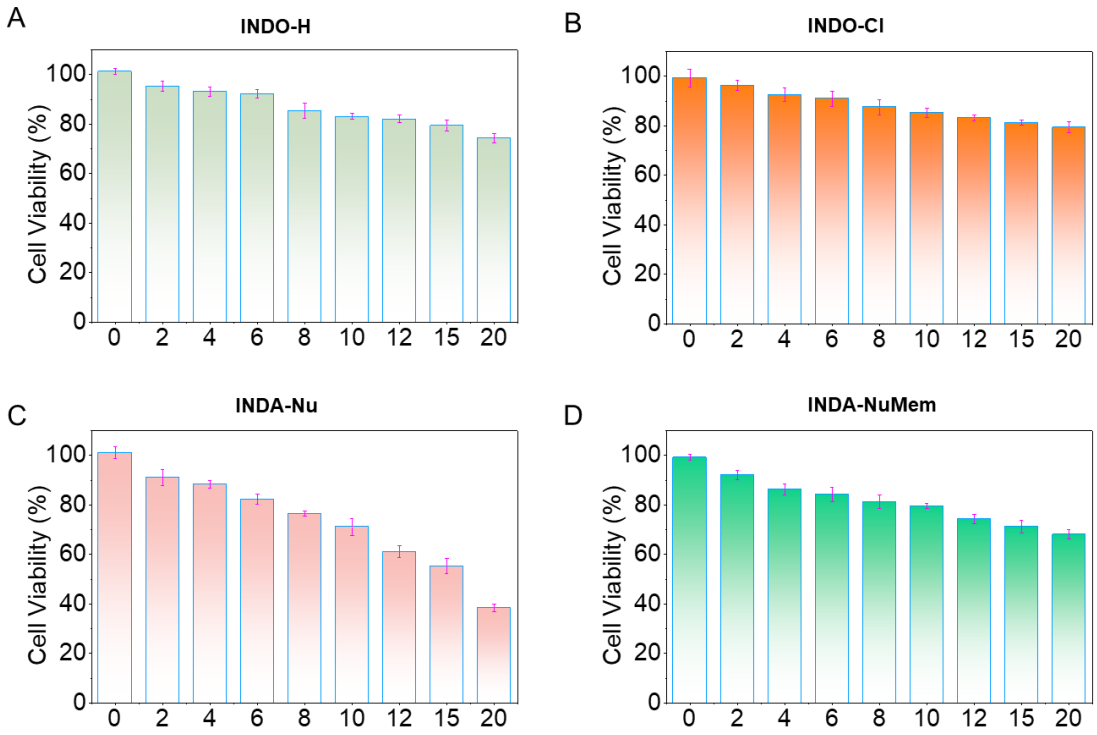


Figure S8 Cell cytotoxicity testing of INDO-H(A), INDO-Cl(B), INDA-Nu(C), and INDA-NuMem(D) under different concentration.

NMR spectra

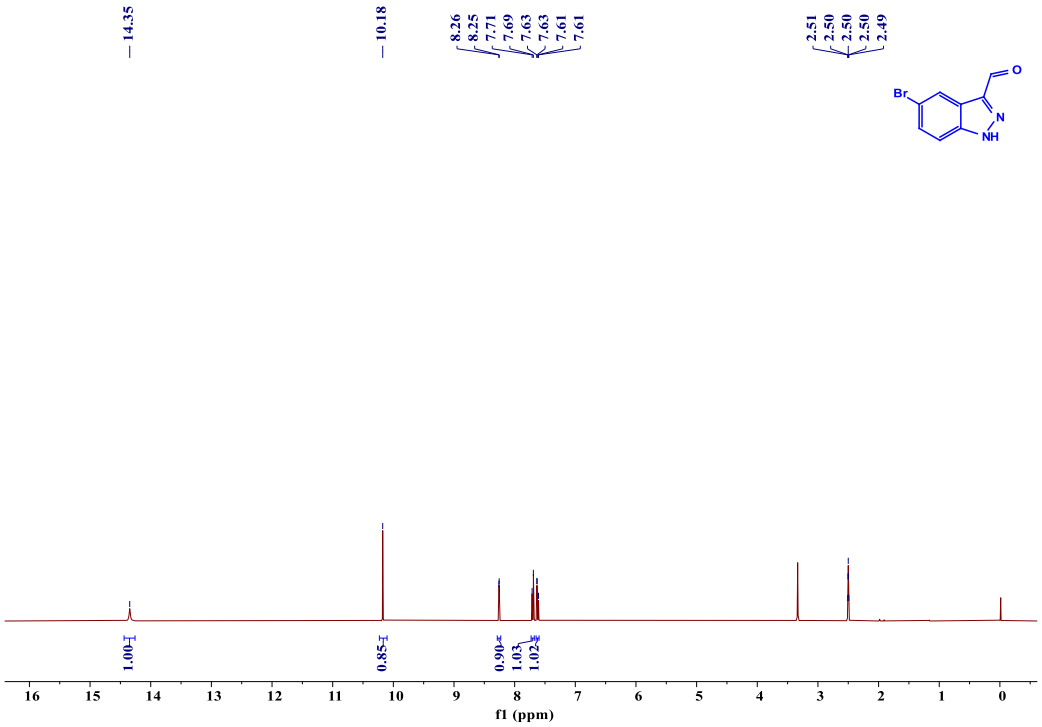


Figure S9 ^{1}HNMR of Na-02

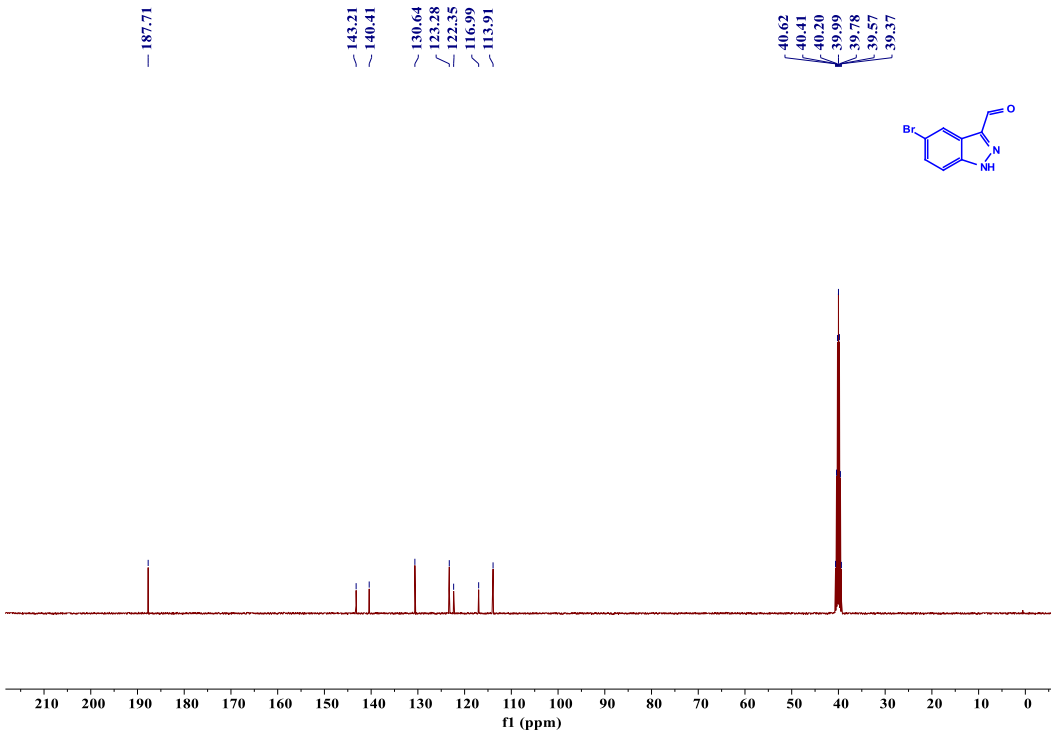


Figure S10 ^{13}CNMR of Na-02

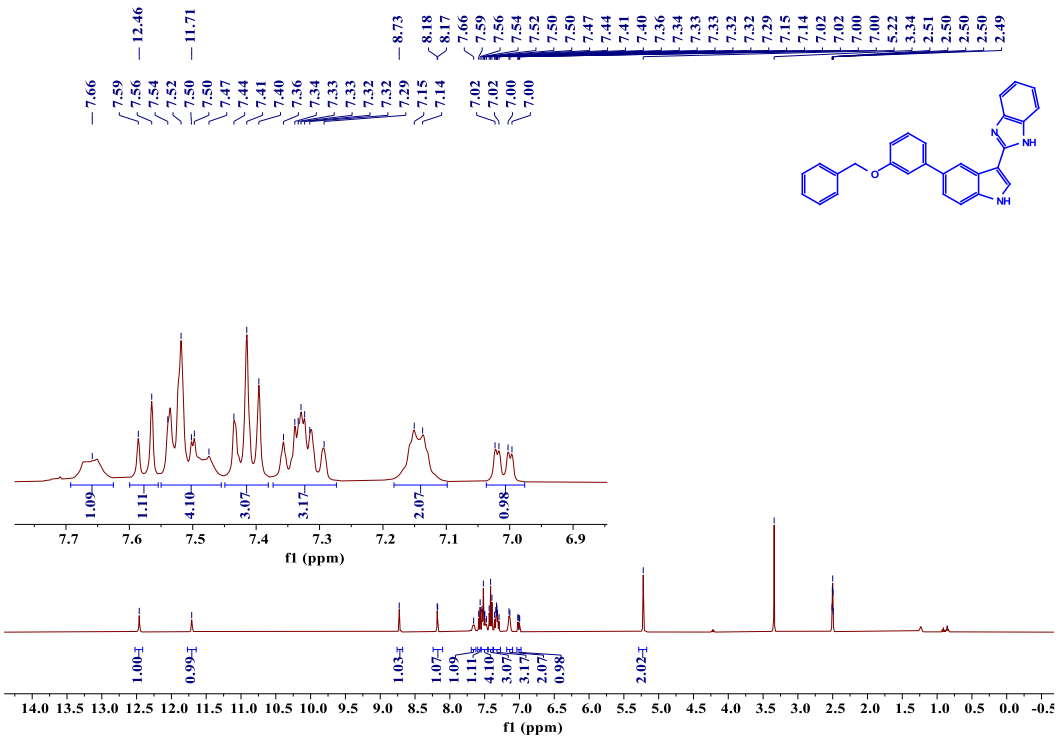


Figure S11 [1]HNMR of INDO-H

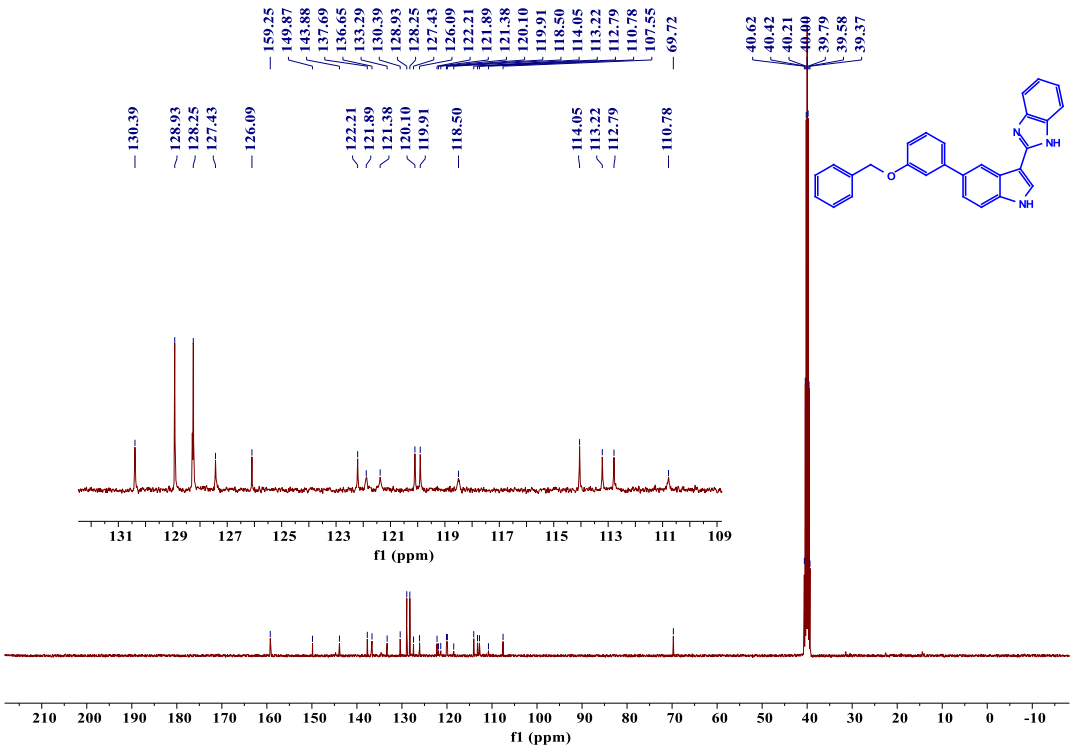


Figure S12 [13]CNMR of INDO-H

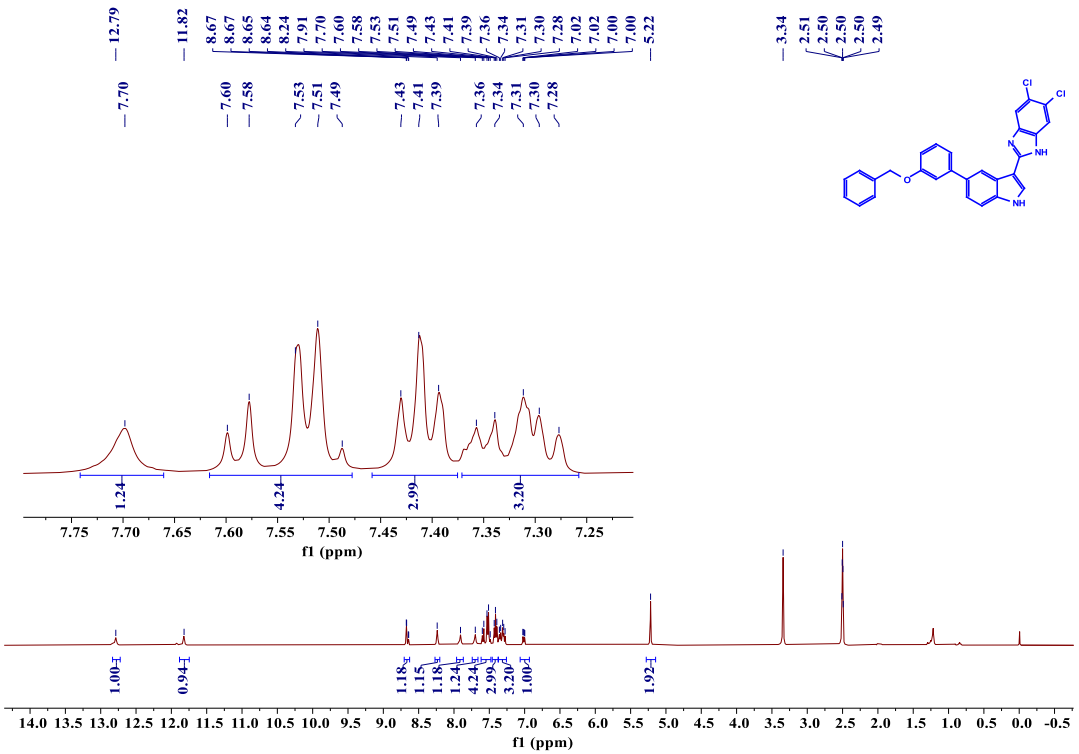


Figure S13 [1]HNMR of INDO-Cl

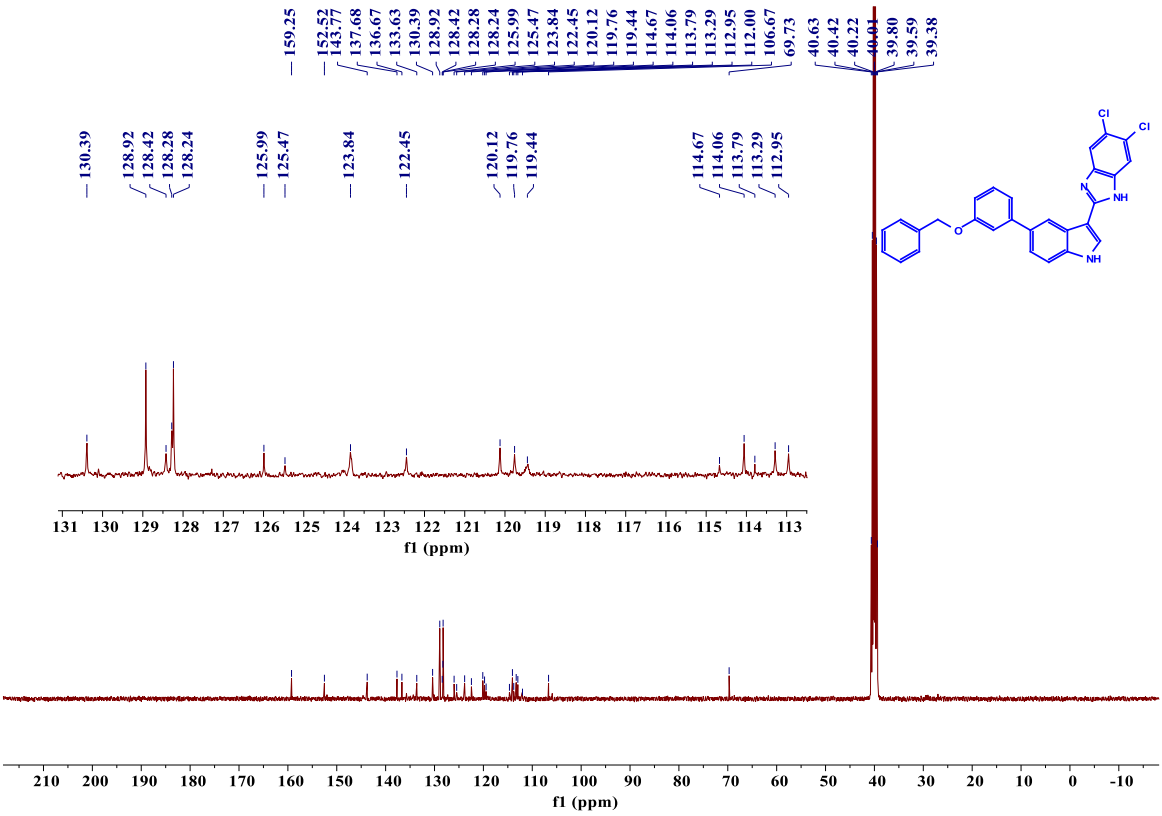


Figure S14 [13]CNMR of INDO-Cl

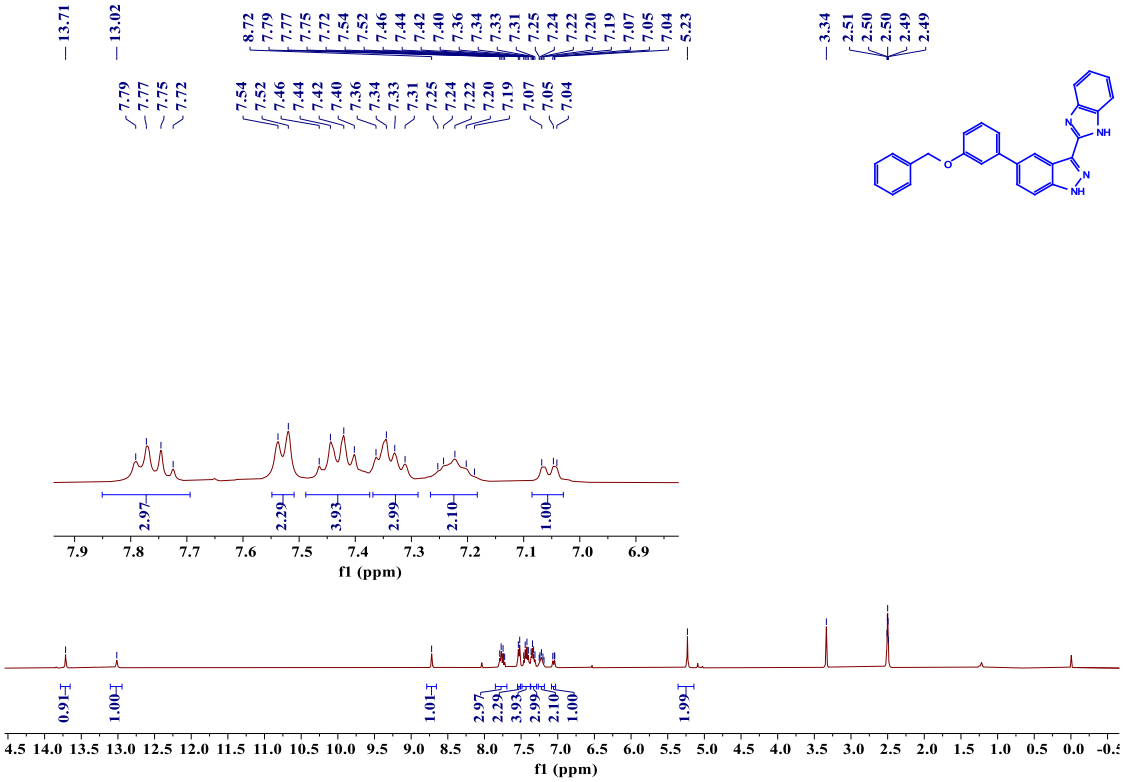


Figure S15 [1]HNMR of INDA-NuMem

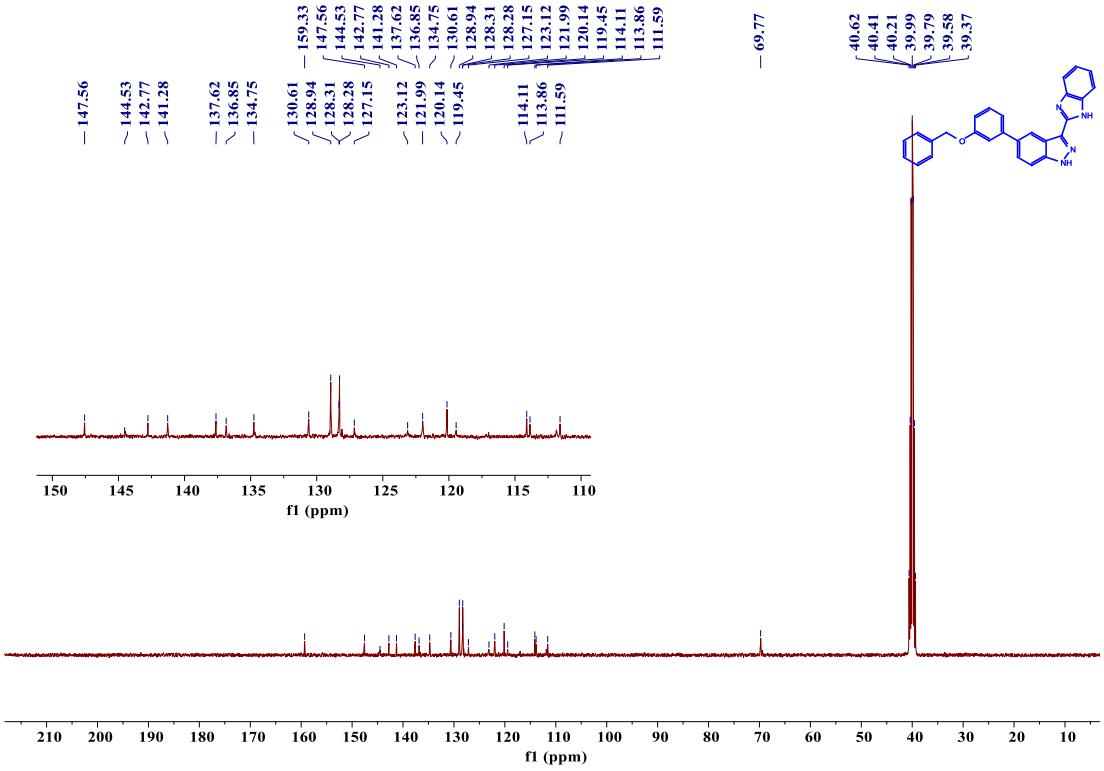


Figure S16 [13]CNMR of INDA-NuMem

High-resolution mass spectrometry

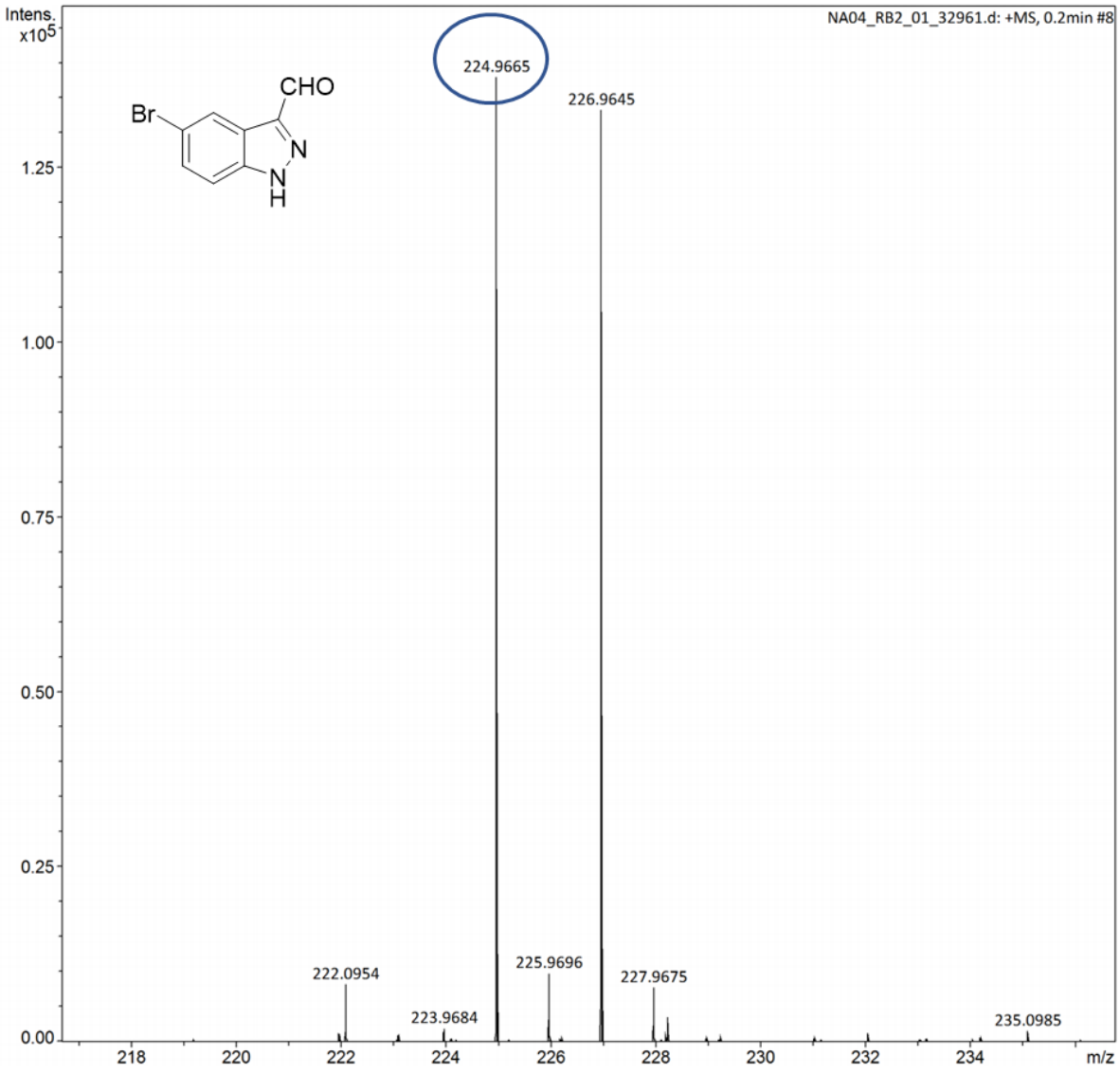


Figure S17 HRMS for Na-02

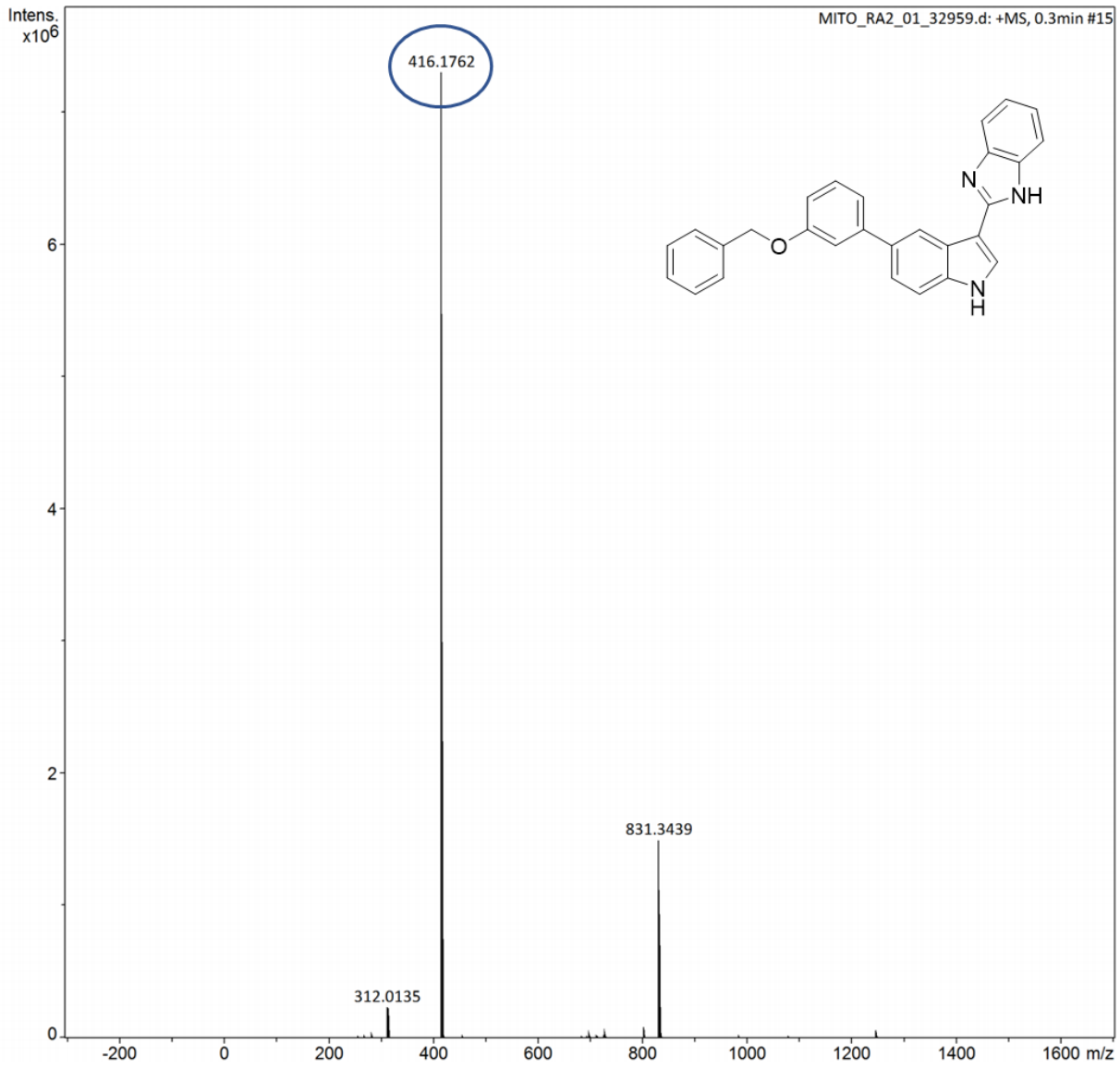


Figure S18 HRMS for INDO-H

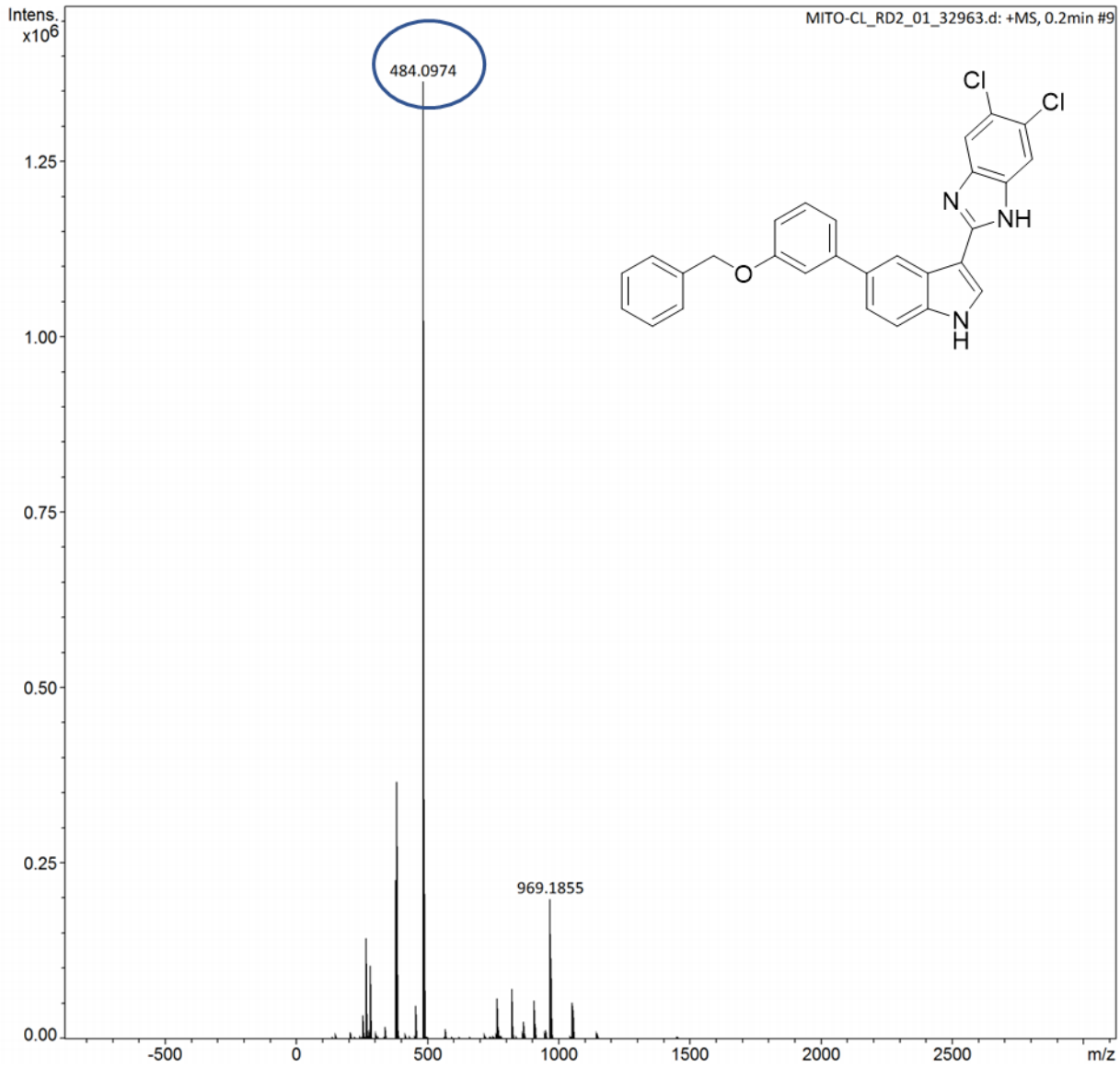


Figure S19 HRMS for INDO-Cl

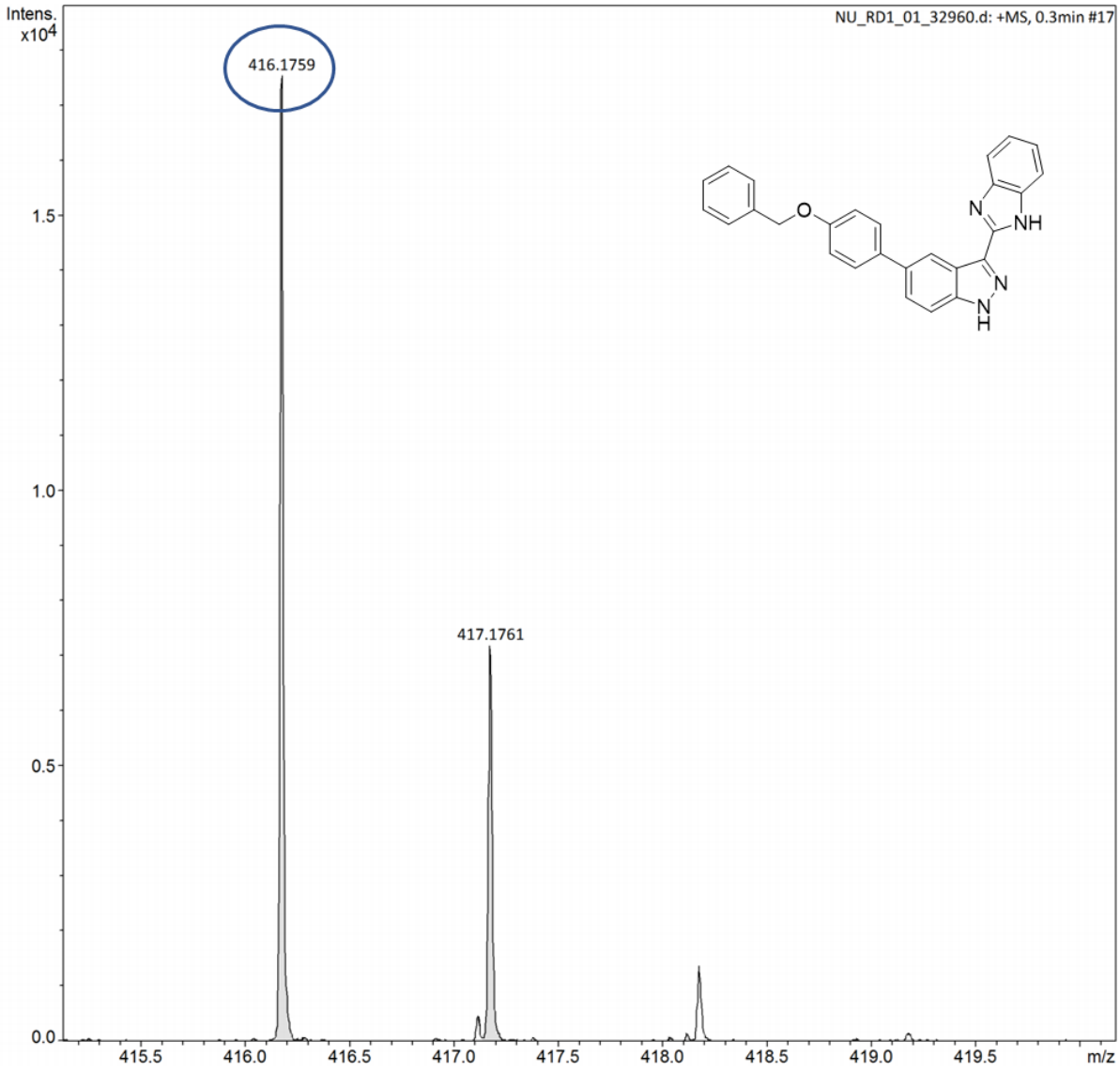


Figure S20 HRMS for INDA-Nu

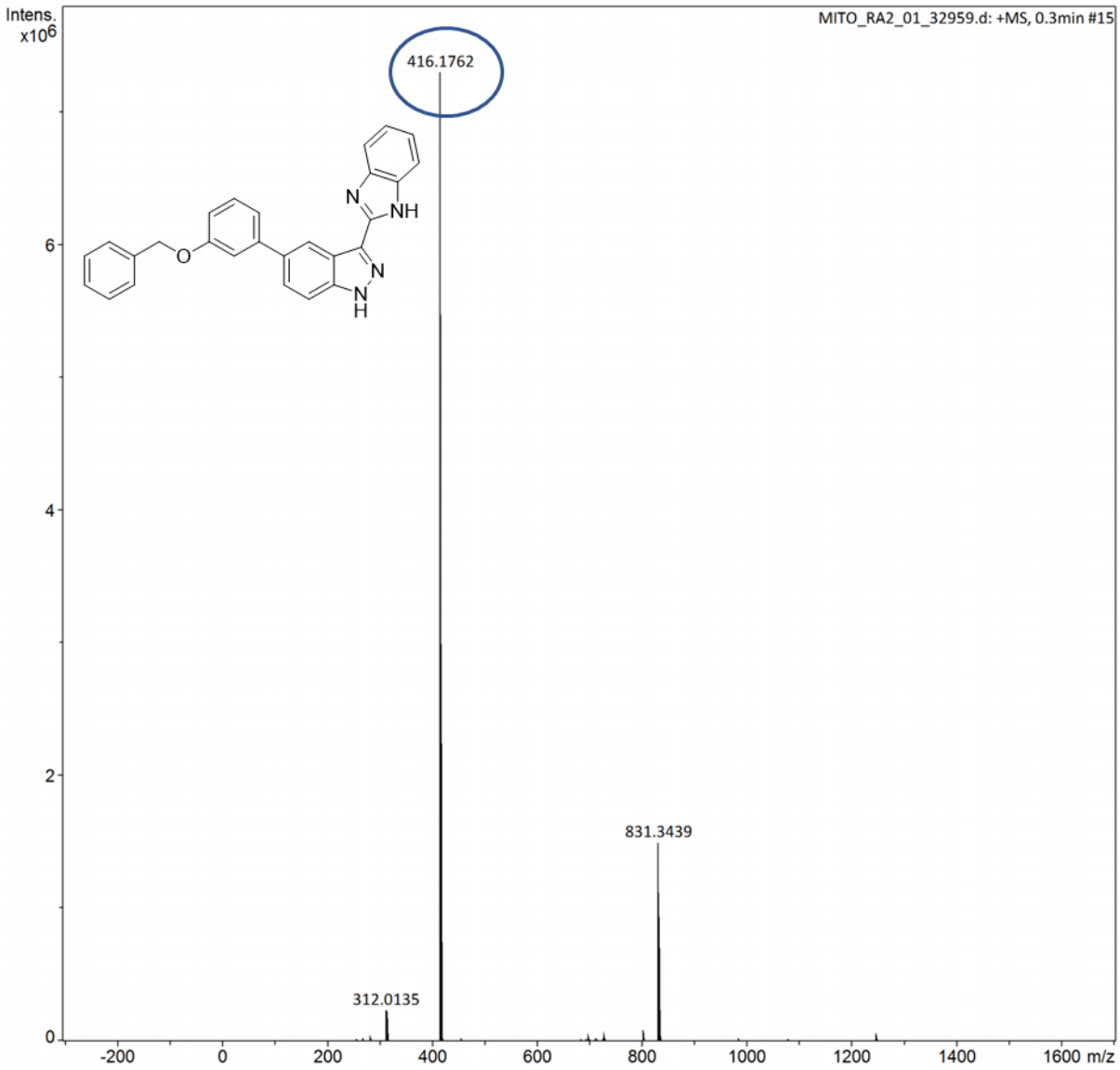


Figure S21 HRMS for INDA-NuMem