Supporting Information

Evaluation of cyclooxygenase-2 fluctuation via a near-infrared fluorescent probe in idiopathic pulmonary fibrosis cell and mice models

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Contents:

S1. General Experimental Section

Instruments. Mice imaging was performed on Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System. Fluorescence spectra were determined using a HORIBA Scientific Fluoromax-4 spectro fluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on Thermo Scientific NanoDrop 2000/2000C spectrophotometer. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker spectrometer. CCK-8 assay was carried out by a microplate reader (Tecan, Austria). The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×60). Intracellular fluorescence detection was carried out on flow cytometry (Aria, BD) with excitation at 650 nm and emission at 750-800 nm.

Materials. All reactions were performed under argon protection and dark, monitored by TLC (Hailang, Yantai). Flash chromatography was carried out using silica gel (200-300 mesh). The purity of Cy-COX was separated on a Shimadzu LC-20AT HPLC system equipped with fluorescence and UV-vis absorption detectors. When it was used for imaging, the purity of Cy-COX was greater than 95%. All chemicals used in synthesis were analytical reagent grade, and were used as received. Ultrapure water was used throughout. N-Boc-1,6-hexanediamine, trifluoroacetic acid (TFA), COX-2, Tris-HCl were obtained from Sigma-Aldrich. CCK-8 kit was purchased from Dojindo. The rabbit antibodies for Western blot analysis were obtained from Abcam (COX-2: 1:1000; Collagen Ⅰ: 1:1000; α-SMA: 1:1000;). MRC-5 and RLE-6TN cells were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

Spectrophotometric experiments. Absorption spectra were obtained with 1.0-cm cuvette cells.

The probes Cy-COX was added to a 10.0-mL color comparison tube. After dilution to 0.5 μM with 5 mM Tris-HCl buffer, different concentrations of COX-2 were added. The mixture was incubated at 37 ^{o}C for 60 min before measurement. Fluorescence spectra were obtained with a 1.0-cm quartz cells by Xenon lamp. The probe Cy-COX was added to a 10.0-mL color comparison tube, respectively. After diluted to 10 μM with 5 mM Tris-HCl buffer, different concentrations of COX-2 were added. The mixture was incubated at 37 ^{o}C for 60 min before measurement.

Preparation of analytes. Cy-COX (1 mM) was prepared in DMSO and stored at 4°C in darkness. The solution of Cy-COX (1 mM) was prepared in dimethyl sulfoxide (DMSO) and then the probe was diluted to working concentration (10 μM) with Tris-HCl buffer (100 mM, pH 8.0). All other reagents and chemicals were all from commercial sources and used without further purification. Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

Cell Cultures. MRC-5 cells (human embryonic lung fibroblast cell line) and RLE-6TN cells (rat type two alveolar epithelial cell line) were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences. MRC-5 cells and RLE-6TN cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C.

Cell transfection: MRC-5 and RLE-6TN cells were chosen as the cell model of overexpression of COX-2 by Ribo FECTTM CP Transfection Kit (Ribo Bio Company, Guangzhou, China). The COX-2 genes were transiently overexpressed by a pIRES2-EGFP plasmid (OBiO Technology (shanghai) Corp.,Ltd.). Identical empty vectors lacking a cDNA insert were employed as control.

Flow cytometry. FCM assay was applied to detect the intramolecular generation of COX-2 with probe Cy-COX. The MRC-5 and RLE-6TN cells were cultivated at 2.0×10^5 cells/well in 6-well plates, and treated with 1 μM Cy-COX at 37 °C for 60 min, and then the MRC-5 and RLE-6TN cells with different treated. After harvest, MRC-5 and RLE-6TN cells were washed, and resuspended with PBS and then analyzed by flow cytometry.

Confocal Imaging. The fluorescence images of MRC-5 and RLE-6TN cells were treated with 1 μM Cy-COX for 60 min and then performed by a confocal laser scanning microscope (Japan

Olympus Co., Ltd) with an objective lens (×60). Excitation wavelength was 650 nm, and the emission was collected from 750 nm to 800 nm.

Molecular docking experiment. Molecular docking study was performed to investigate the binding mode between the compounds and the human COX-2 using Autodock vina 1.1.2.[1] The three-dimensional (3D) structure of the COX-2 (PDB ID: 5F19) was downloaded from RCSB Protein Data Bank (www.rcsb.org). The 2D structure of the compounds were drawn by ChemBioDraw Ultra 14.0 and converted to 3D structure by ChemBio3D Ultra 14.0 software. The AutoDockTools 1.5.6 package [2,3] was employed to generate the docking input files. The ligands were prepared for docking by merging non-polar hydrogen atoms and defining rotatable bonds. The search grid of the COX-2 site was identified as center_x: 6.26, center_y: 54.58, and center_z: 61.794 with dimensions size_x: 17.25, size_y: 21.75, and size_z: 15. In order to increase the docking accuracy, the value of exhaustiveness was set to 20. For Vina docking, the default parameters were used if it was not mentioned. The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMoL 1.7.6 software (www.pymol.org)

In vivo imaging in living mice. Mice model was constructed as described in the section of "Animal model". Then All the mice were given an intratracheal instillation of Cy-COX(100 μM, 50 μL in 1:99 DMSO/saline v/v). Finally, all groups of mice were anesthetized by i.p. injections of 4% chloral hydrate (0.25 ml). Images were taken by a Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System, with an excitation filter of 650 nm and an emission of 750-800 nm. The results were the mean standard deviation of five separate measurements.

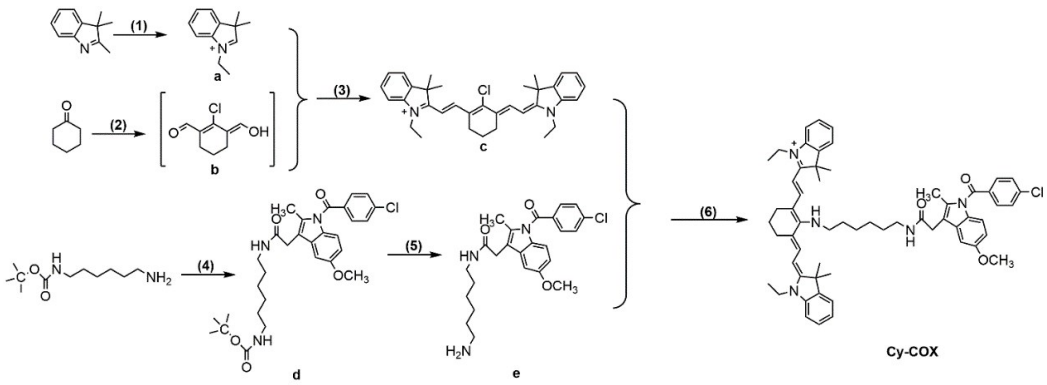
Western blot analysis. Lung cells were homogenized and protein was extracted with 200 μL RIPA lysis buffer including 2 μL PMSF (Solarbio, China). All the proteins were quantified with BCA protein assay kit (Biogot, China). Equal amount of protein (70 μg) from every sample was electrophoresed, and separated by 10% SDS-polyacrylamide gels (Bio-Rad, USA). After the electrophoresis, removed the glass plate, gently opend the upper plate, and cut the glue according to the Marker and the molecular weight of the target protein. Placed the transfer clips in the following order: black side down, line with sponge, three layers of filter paper. Put the cut strips on the filter paper and put them neatly, and kept them wet with the film transfer solution. The PVDF membrane (Millipore, Germany) was cut and marked according to the size of the cut

adhesive. The PVDF membrane was first placed in methanol for 2 min and then in ultrapure water for 5 min for activation. After the activated PVDF membrane was spread on the gel, three layers of filter paper were laid on the PVDF membrane. After removing the bubbles between the filter papers with glass rod, a sponge was placed on the filter paper to close the seal. The transfer clamp was placed in the groove of the transfer mold, the ice bag was placed in the groove to cool down, and an appropriate amount of transfer film liquid was poured into the constant current of 200 mA for transfer printing for 2h. Then the PVDF membranes were blocked with 7% milk for 3-6 h and treated with major antibodies overnight at 4 ^{o}C. Appropriate secondary antibody which was conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology, USA) was used to quantify protein and an enhance chemiluminescence (ECL) detection system was employed to monitor the signals. These results were accurately analyzed with Image J software.

H&E and Masson staining. Lung of mice models in every group were all excised and fixed in 10% formaldehyde and embedded in paraffin and stained with hematoxylin and eosin (H&E) to verify histology. Lung stained with Masson's trichrome.

S2. Synthetic routes for Cy-COX

Scheme S1. The synthetic approaches of Cy-COX



(1) Iodoethane, acetonitrile, refluxed, 12 h, 90%; (2) DMF, CH_2Cl_2, $POCl_3$, 45 °C, 3 h, 85%; (3) n-Butyl alcohol:benzene = 7:3 (v/v), refluxed, 3 h, 70%; (4) EDCl, N-Boc-1,6-hexanediamine, DMAP, indomethacin, room temperature, 24h, 55%; (5) Trifluoroacetic acid, CH_2Cl_2, room temperature, 3h; (6) ethylene glycol methyl ether, refluxed, 12h.

Synthesis of compound a

2,3,3-Trimethyl-3H-indolenine (12 g, 75 mmol) and iodoethane (11.5 g, 75 mmol) were mixed in 40 mL anhydrous acetonitrile in a 250 mL round flask. The mixture was refluxed for 12 h, then removed from heat and cooled. The precipitate was filtered through a Bu¨chner funnel and the solid product was washed with diethyl ether and dried in vacuum to afford a pink product in 90 % yield. ^{1}H NMR (DMSO-d_6, 500 MHz) δ(ppm): 8.04-8.02 (t, 1H), 7.91-7.89 (t, 1H), 7.66-7.62 (m, 2H), 4.57-4.53 (m, 2H), 2.92 (s, 3H), 1.58 (s, 6H), 1.49-1.46 (t, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 196.5, 142.4, 141.1, 129.4, 124.1, 115.8, 54.6, 43.8, 40.1, 22.4, 14.9, 13.3. LC-MS (ESI$^+$): m/z $C_{13}H_{18}N^+$ calcd. 188.1434, found [M^+] 188.1435.

Synthesis of compound b

A solution of 40 mL of anhydrous N,N-dimethylformamide (DMF) and 40 mL of anhydrous CH_2Cl_2 was placed in a 250 mL round-bottom flask. The solution was chilled to -10℃ and then stirred for 20 min. Phosphorus oxychloride (37 mL) in anhydrous CH_2Cl_2 (35 mL) was added dropwise into the above solution through a constant pressure drop of liquid funnel. 4-Cyclohexanone (10g, 101.9 mmol) was added into the mixture in batches; the solution immediately changed from colorless to yellow. Then the solution was slowly heated to 45℃ for 3 h, cooled, poured over ice, and allowed to stand overnight. The yellow solid was collected through a Bu¨chner funnel and dried in vacuum in 80 % yield. ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 8.12 (s, 1H), 8.08 (s, 1H), 7.14 (s, 1H), 2.38-2.71 (m, 2H), 2.53-2.45 (m, 2H), 2.28-2.26 (m, 2H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 191.8, 162.9, 148.5, 145.7, 142.4, 37.2, 31.7, 30.6. MS (ESI$^-$): m/z $C_8H_9ClO_2$ calcd. 172.0553, found [M-H]$^-$ 171.0481.

Synthesis of compound c

Compound a (0.84 g,2 mmol) and Compound b (0.17 g, 1 mmol) were dissolved in 100 mL mixed solution of n-butyl alcohol and benzene (7:3, v/v) in a 250 mL round flask, refluxed for 3 h, and dried in vacuum to obtain a green solid. The crude product was purified by silica gel chromatography using EtOAc/CH_3OH (8:1, v/v) as eluent to afford compound c as a green solid in 70 % yield. ^{1}H NMR ($CDCl_3$-d_1, 500 MHz) δ (ppm): 8.43-8.40 (d, 1H), 7.41-7.39 (m, 2H), 7.28-7.15 (m, 6H), 6.09-6.07 (d, 2H), 5.01 (s, 1H), 4.14-4.15 (m, 4H), 3.01-2.97 (m, 4H), 2.68-2.63 (m, 2H), 2.04 (m, 6H), 1.79 (m, 6H), 1.42-1.39 (m, 3H), 1.27-1.24 (m, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 172.2, 171.2, 150.3, 144.6, 141.6, 141.2, 128.9, 125.5, 122.3, 110.9, 100.6, 60.4, 49.5, 39.9, 38.0, 34.2, 28.1, 21.1, 14.2, 12.5. MS (ESI$^+$): m/z $C_{34}H_{40}ClN_2^+$ calcd. 511.3137, found [M^+] 511.3137.

Synthesis of compound d

N-Boc-1,6-hexanediamine (216 mg, 1 mmol), indomethacin (358 mg, 1 mmol), DMAP (122 mg, 1 mmol), EDCl (192mg, 1 mmol) was added to CH_2Cl_2 (20 ml). After argon was introduced into the reaction device, the mixture was stirred at room temperature for 24 hours.The mixed solution was evaporated in vacuum and the primary product was purified by silica gel column chromatography (20:1 CH_2Cl_2/CH_3OH) to give the light yellow solid compound d in 80 % yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 – 7.58 (m, 1H), 7.52 – 7.41 (m, 1H), 6.87 (dd, J = 21.6, 5.7 Hz,

1H), 6.68 (dd, J = 9.0, 2.5 Hz, 1H), 5.81 (s, 1H), 4.57 (s, 1H), 3.80 (s, 2H), 3.62 (s, 1H), 3.17 (dd, J = 13.3, 6.8 Hz, 1H), 3.02 (dd, J = 12.9, 6.5 Hz, 1H), 2.37 (s, 2H), 1.51 – 1.32 (m, 7H), 1.28 – 1.13 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.72, 168.25, 156.18, 155.96, 139.46, 136.21, 133.54, 131.11, 130.84, 130.31, 129.12, 115.01, 112.95, 112.17, 100.85, 78.93, 55.66, 40.10, 39.24, 32.18, 29.85, 29.29, 28.34, 26.10, 25.97, 13.19. MS (ESI): m/z $C_{30}H_{38}ClN_3O_5$ calcd. 555.2500. found 555.7483.

Synthesis of compound e

Compound d (555 mg, 1 mmol) was added to CH_2Cl_2 (2 mL), and the solution stirred at room temperature for 30 minutes to fully dissolve compound e. Trifluoroacetic acid (2 mL) was added to the solution and the mixed solution stirred at room temperature for 3h. After the solvent was evaporated in vacuo, compound e was got and directly put into the next reaction. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (t, *J* = 10.3 Hz, 1H), 7.44 – 7.34 (m, 1H), 6.93 – 6.78 (m, 1H), 6.65 (dd, *J* = 9.0, 2.5 Hz, 1H), 5.98 (t, *J* = 5.8 Hz, 1H), 3.89 – 3.71 (m, 2H), 3.59 (s, 1H), 3.15 (dd, *J* = 13.3, 6.8 Hz, 1H), 2.33 (s, 2H), 1.35 (dq, *J* = 31.5, 7.1 Hz, 2H), 1.18 (ddd, *J* = 21.9, 10.9, 4.3 Hz, 2H).

Synthesis of probe Cy-COX

Compound c (511.5 mg, 1 mmol), compound e (455 mg, 1 mmol), and triethylamine (101 mg, 1 mmol) were successively added to the ethylene glycol methyl ether solution (25 mL). The above solution was placed in a dark environment and heated to reflux at 90°C for 12h. After the mixture was concentrated, the crude product was purified by silica gel column chromatography (10:1 CH_2Cl_2/CH_3OH) to give the blue probe Cy-COX in 60 % yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.63 (s, 1H), 7.74 – 7.61 (m, 4H), 7.50 – 7.40 (m, 2H), 7.32 – 7.19 (m, 2H), 7.05 (dd, J = 9.3, 5.0 Hz, 3H), 6.86 (dd, J = 27.2, 8.5 Hz, 4H), 6.64 (dd, J = 9.0, 2.5 Hz, 1H), 5.56 (d, J = 12.2 Hz, 2H), 4.13 (q, J = 7.1 Hz, 2H), 3.82 (d, J = 9.2 Hz, 6H), 3.77 (t, J = 6.8 Hz, 2H), 3.69 (s, 2H), 3.26 (dd, J = 13.0, 6.7 Hz, 2H), 2.50 (t, J = 6.2 Hz, 4H), 2.39 (s, 3H), 1.95 – 1.86 (m, 2H), 1.81 (dd, J = 12.7, 6.4 Hz, 2H), 1.73 (s, 4H), 1.59 – 1.48 (m, 3H), 1.43 – 1.27 (m, 10H), 1.26 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.99, 168.35, 161.95, 161.67, 156.07, 139.42, 136.44, 133.46, 131.08, 130.95, 130.81, 130.54, 129.09, 128.70, 117.61, 115.29, 114.91, 112.92, 111.68, 101.26, 55.63, 53.40, 52.26, 39.61, 39.32, 31.68, 28.83, 26.95, 25.73, 25.41, 13.05. MS (ESI+): m/z $C_{59}H_{69}ClN_5O_3$+ calcd. 930.5083, found [M+] 930.5267.

S3. The Docking experiment results

The Cy-COX was docked into the binding site of the COX-2 and the results were shown in Figure S1 and Figure S2. The estimated binding energy was -8.4 kcal•mol−1 for Cy-COX. The probe Cy-COX adopted a compact conformation to bind at the site of the COX-2 (Figure S1). The IMC part of the probe Cy-COX was located at the hydrophobic pocket, surrounded by the residues Val-89, Leu-93, Ile-112, Val-116, Leu-359 and Leu-531, forming a strong hydrophobic binding (Figure S2). Detailed analysis showed that the phenyl group of the Cy-COX formed cation-π interaction with the residue

Arg-120, and formed anion-π interaction with the residue Glu-524. Importantly, one key hydrogen bond interaction was observed between the Cy-COX and the residue Arg-120 (bond length: 2.6 Å), which was the main interaction between the Cy-COX and the COX-2 (Figure S2). All these interactions helped Cy-COX to anchor in the binding site of COX-2. In summary, the above molecular simulations give us rational explanation of the interactions between the fluorescent probe Cy-COX and the COX-2.

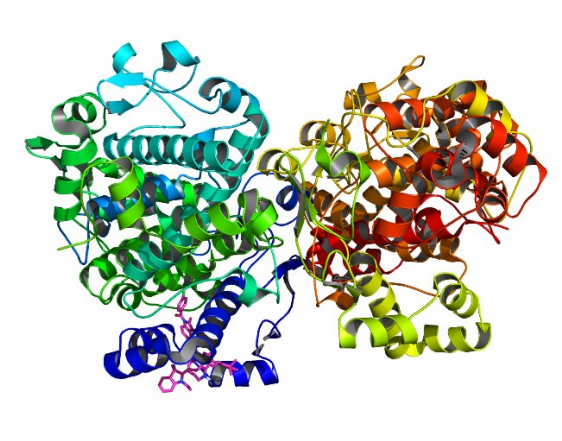


Figure S1. The Cy-COX was docked into the binding site of the COX-2 (total view).

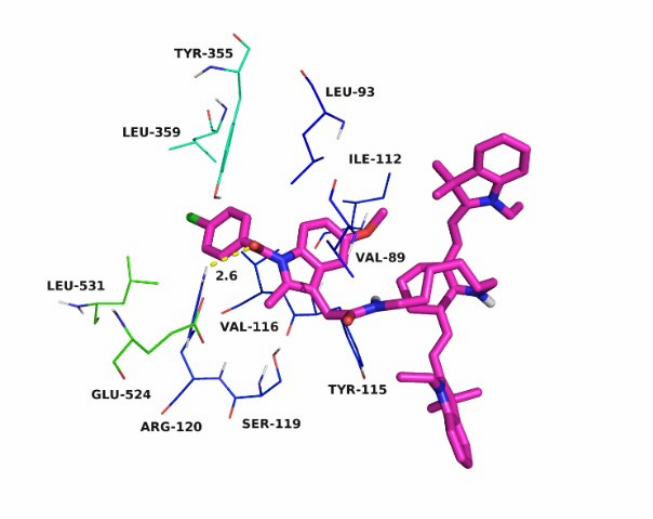


Figure S2. Detailed view of the binding mode between the Cy-COX and the COX-2. The representative binding residues of COX-2 were shown in lines; The Cy-COX was represented with rose red sticks; The hydrogen bonds were shown in yellow dotted lines.

S4. Absorption spectra of Cy-COX

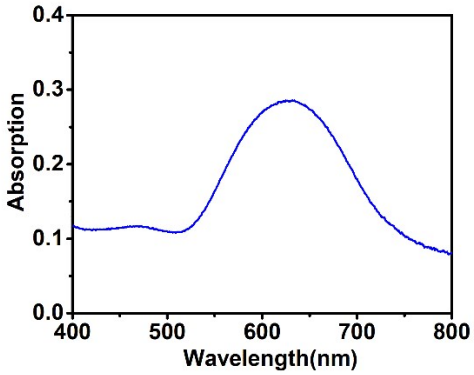


Figure S3. (A) UV-vis absorption 10 μM solution of Cy-COX in Tris-HCl (100 mM, pH 8).

S5. CCK-8 assay for Cy-COX

To evaluate the cytotoxicity of the probes, CCK-8 assays were carried out. MRC-5 cells (8000/cell), RLE-6TN (8000/cell) were planted into 96-well plates in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2 and 95% air and the cells allowed to adhere for 24h. Then the cells were incubated with 0, 10, 20, 30, 40, 50, 60, 70, 80, 100 μM (final concerntration) of Cy-COX for 24 h. Then, the previous media was threw away and 100 μL medium contain 10% CCK-8 solution (Dojindo, Japan) was added to each well, and about 30 min later, the absorbance was measured at 450 nm using a microplate reader (TECAN infinite M200pro). The effect of DMSO was removed by control groups. As shown in Figure S4, cells showed high vitality indicated that our probe was safety for cell detection.

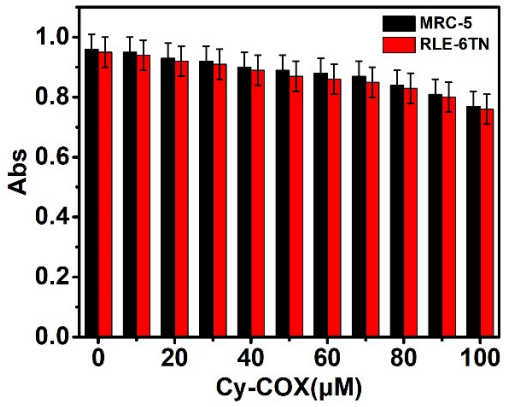


Figure S4. CCK-8 assay for Cy-COX. MRC-5 and RLE-6TN cells were treated with different concentrations of Cy-COX. The experiments were repeated three times and the data were shown as mean (±S.D.).

S6. The selectivity of Cy-GGT to various biospecies

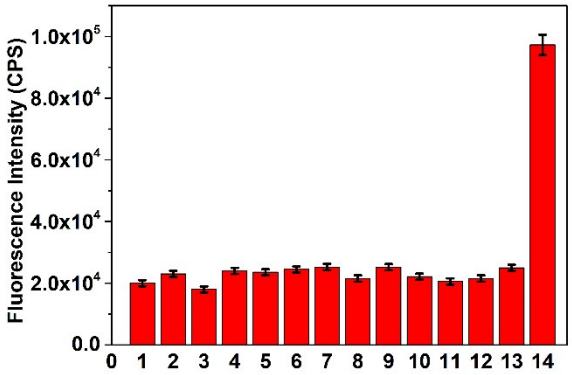


Figure S5. Fluorescence responses of Cy-COX (10 μM) toward various species : 1, control; 2, Cys; 3, Hcy; 4, GSH; 5, H_2O_2; 6, Na^+; 7, Mg^{2+}; 8, NaHS; 9, Na_2S_4; 10, S-nitrosoglutathione (GSNO); 11, L-arginine (L-arg); 12, GGT; 13, COX-1 and 14, COX-2.

S7. Bright-field cell images of those shown in Figure 2a (in manuscript)

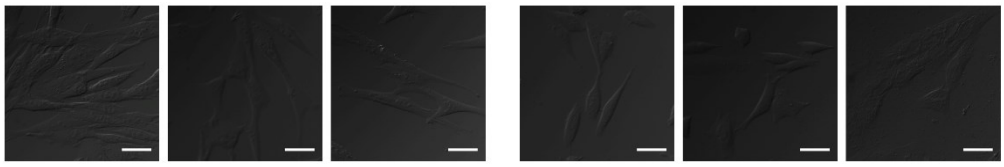


Figure S6. Bright-field images of Figure 2a (in manuscript). Scale bars: 20 μm.

S8. Bright-field cell images of those shown in Figure 3a (in manuscript)

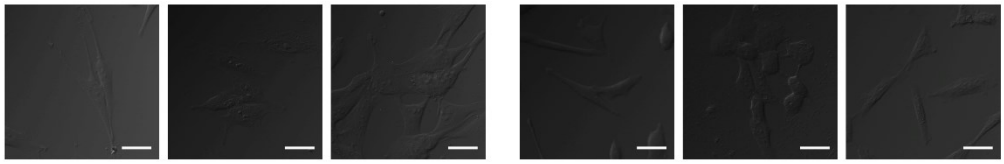


Figure S7. Bright-field images of Figure 3a (in manuscript). Scale bars: 20 μm.

S9. Bright-field cell images of those shown in Figure 4a (in manuscript)

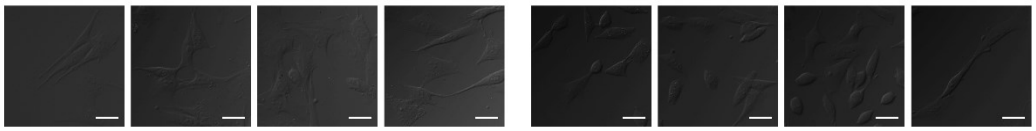
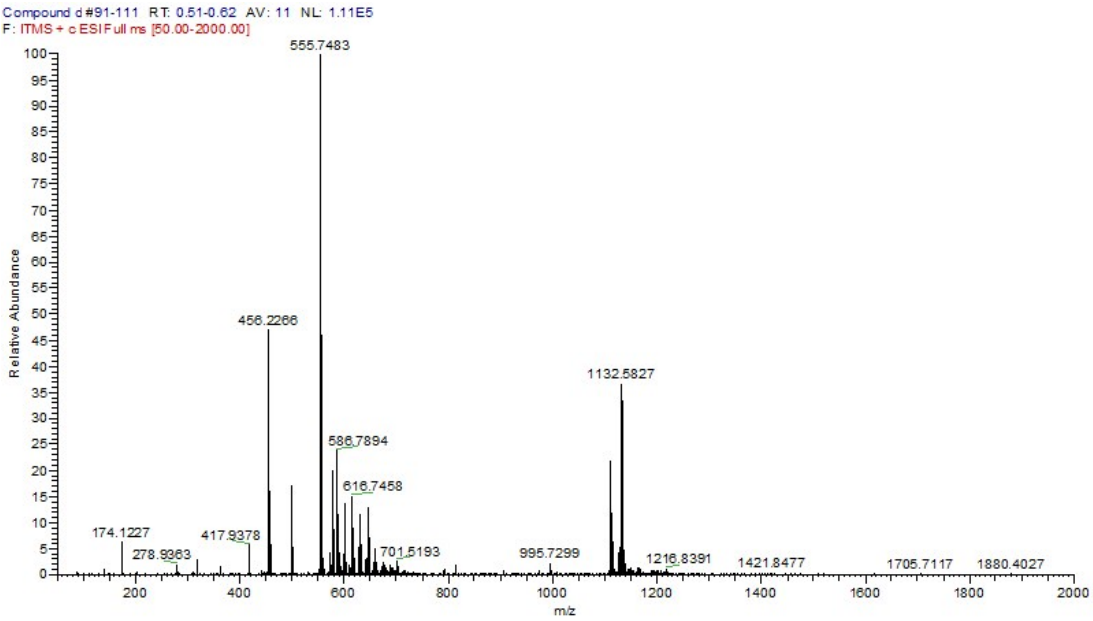


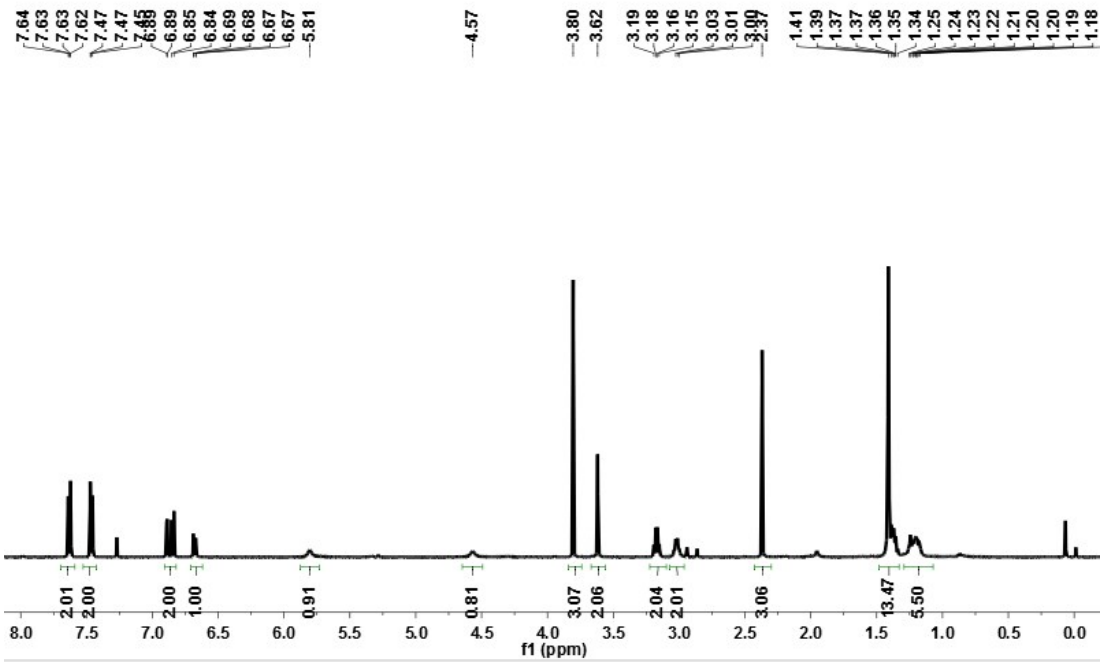
Figure S8. Bright-field images of Figure 4a (in manuscript). Scale bars: 20 μm.

S10. Characterization (^{1}H NMR, ^{13}C NMR and LC-MS) of compound d and Cy-COX

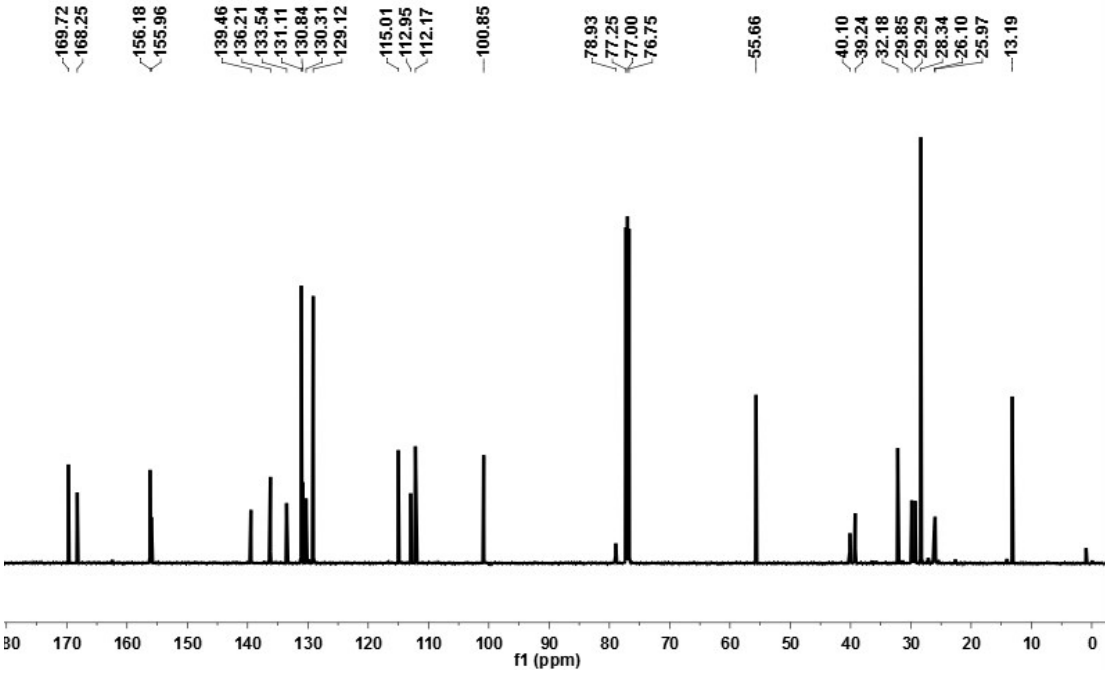
LC-MS of compound d



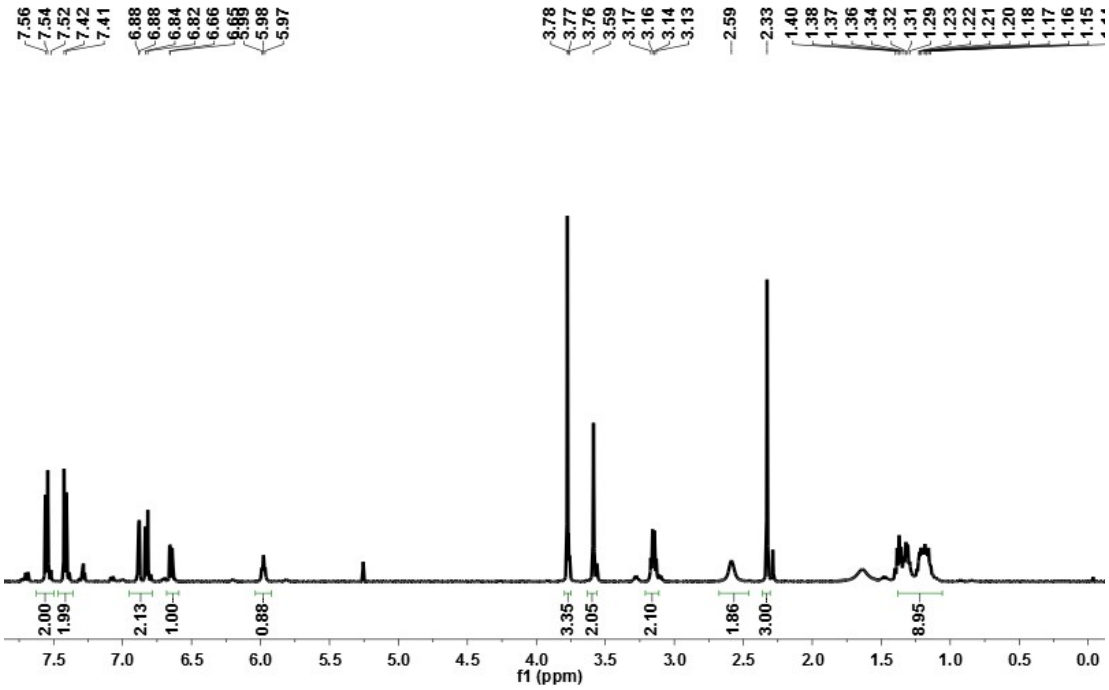
^{1}H NMR of compound d



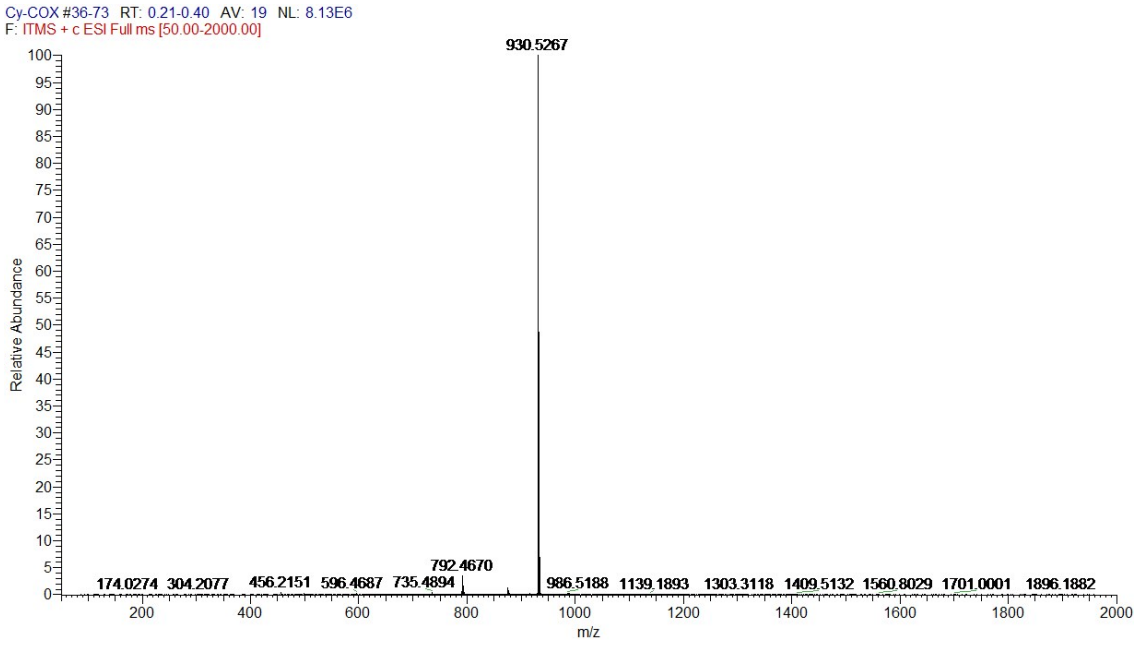
^{13}C NMR of compound d



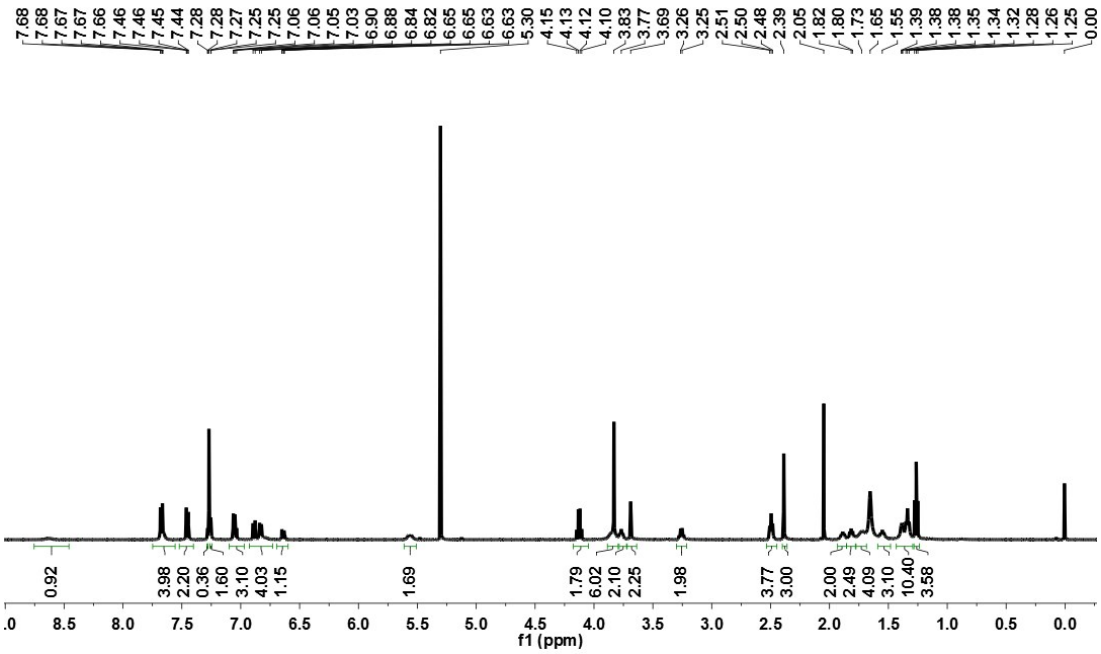
^{1}H NMR of compound e



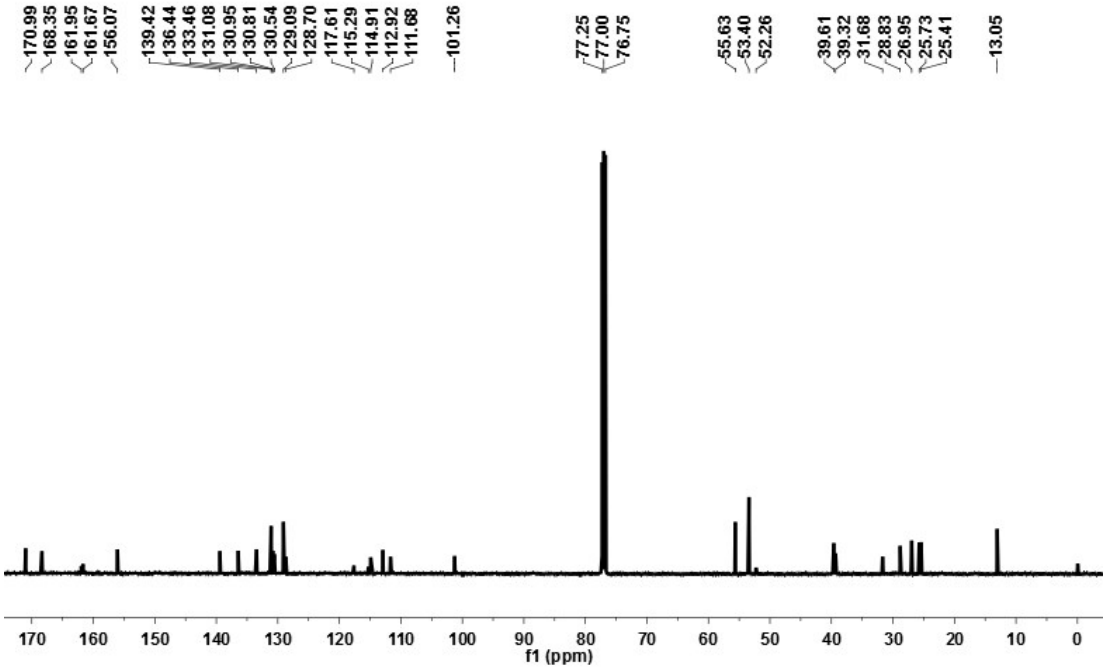
LC-MS of Cy-COX



^{1}H NMR of Cy-COX



^{13}C NMR of Cy-COX



S11. Comparison of the probe Cy-COX with reported COX-2 probes

Fluorescent Probe	Detection Limit	References
BTDAN-COX-2	1.0 nM	Ref.31
TPI-IMC	0.08 μg/mL	Ref.32
ANQ-IMC-6	0.11 μg/mL	Ref.33
NP-C6-CXB	0.05 μg/mL	Ref.34
NANQ-IMC6	0.01 μg/mL	Ref.35
Cy-COX	11 ng/mL	This work

S12. References

1 G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell and A. J. Olson, *Journal of computational chemistry*, 2009, **30**, 2785-2791.
2 M. F. Sanner, *Journal of molecular graphics & modelling*, 1999, **17**, 57-61.
3 G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell and A. J. Olson, *Journal of computational chemistry*, 2009, **30**, 2785-2791.