Supporting Information

In-situ Imaging of Azoreductase Activity in the Acute and Chronic Ulcerative Colitis Mice by a Near-infrared Fluorescent Probe

Yang Tian,[†] Yongfei Li,[†,‡] Wen-Li Jiang,[†] Dong-Ye Zhou,[†] Junjie Fei,[†] and Chun-Yan Li*,[†]

[†]Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, College of Chemistry, Xiangtan University, Xiangtan, 411105, People's Republic of China

[‡]College of Chemical Engineering, Xiangtan University, Xiangtan, 411105, People's Republic of China

*Corresponding Author. E-mail: lichunyan79@sina.com.

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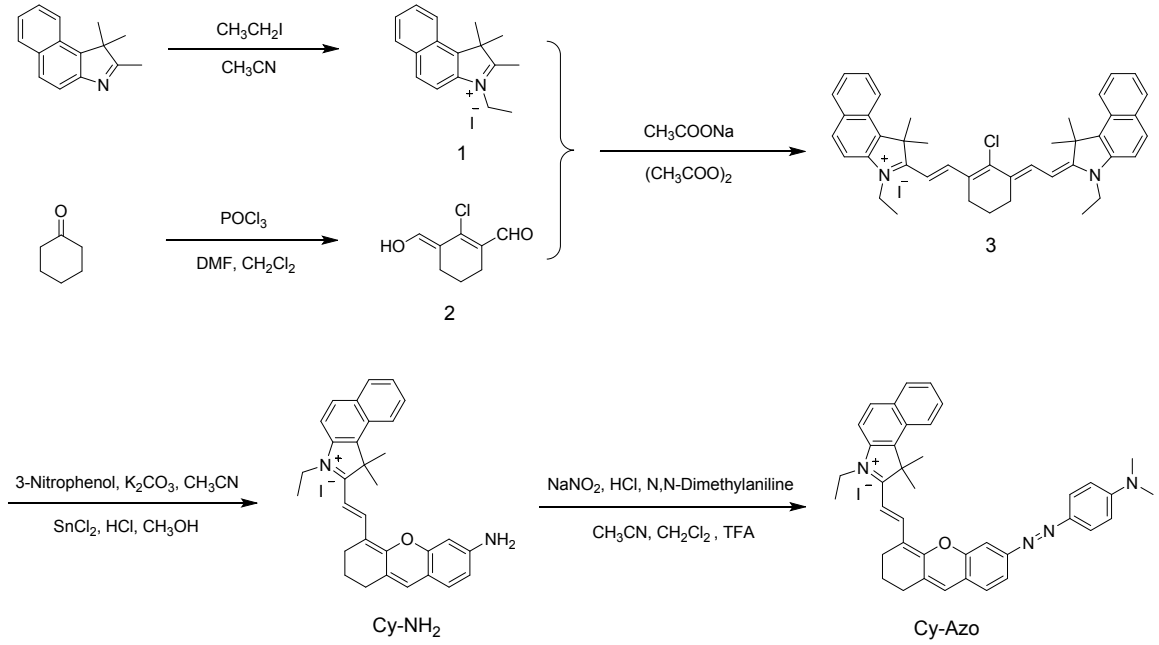
1. Synthesis of Cy-Azo

Scheme S1 Synthetic route for Cy-Azo



Compound 1. 1,1,2-Trimethylbenzoindolenine (1.05 g, 5.0 mmol) and iodoethane (1.17 g, 7.5 mmol) were dissolved in acetonitrile (20.0 mL). The reaction mixture was stirred and refluxed for 15 h. After cooling, the mixture was filtered and washed with ether to obtain a white solid product. Yield: 1.46 g (80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, J = 8.8 Hz, 1H), 8.08 (d, J = 8.8 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.81 (d, J = 8.8 Hz, 1H), 7.73 (m, 1H), 7.66 (m, 1H), 4.88 (q, J = 7.2 Hz, 14.8 Hz, 2H), 3.22 (s, 3H), 1.86 (s, 6H), 1.66 (t, J = 7.2 Hz, 3H). MS (TOF): 238.2.

Compound 2. A solution of 4.0 mL of dimethylformamide and 4.0 mL of methylene chloride was chilled in an ice bath. Phosphorus oxychloride (2.46 g, 16.0 mmol) dissolved in 3.0 mL of methylene chloride was then added dropwise with stirring, followed by the addition of cyclohexanone (0.39 g, 4.0 mmol). The reaction mixture was stirred and refluxed for 3 h. After cooling, the mixture was poured into 40.0 g ice and allowed to stand overnight. And then, the mixture was filtered to obtain

a yellow solid product. Yield: 0.45 g (65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.24 (s, 1H), 2.63 (s, 1H), 2.47 (t, J = 8.0 Hz, 4H), 1.71-1.68 (m, 2H). MS (TOF): 172.1.

Compound 3. Compound 1 (1.10 g, 3.0 mmol), freshly prepared compound 2 (0.26 g, 1.5 mmol), sodium acetate (0.25 g, 3.0 mmol) were dissolved in acetic anhydride (20.0 mL). The reaction mixture was heated to 70°C for 0.5 h. After the removal of solvent under reduced pressure, the crude product was washed with sodium bicarbonate saturated solution to obtain a brick red solid product. Yield: 0.83 g (75%). 1H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, J = 14.0 Hz, 2H), 8.13 (d, J = 8.4 Hz, 2H), 8.11-7.93 (m, 4H), 7.61 (m, 2H), 7.47-7.25 (m, 4H), 6.29 (d, J = 14.0 Hz, 2H), 4.39 (q, J = 7.0 Hz, 14.2 Hz, 4H), 2.79 (t, J = 6.0 Hz, 4H), 2.01 (s, 12H), 1.62 (s, 2H) 1.53 (t, J = 6.8 Hz, 6H). MS (TOF): 611.3.

Compound Cy-NH_2. 3-Nitrophenol (0.35 g, 2.5 mmol) and K_2CO_3 (0.35 g, 2.5 mmol) were dissolved in 15.0 mL CH_3CN in a flask, and the mixture was stirred at room temperature under nitrogen atmosphere for 10 min. Then, compound 3 (0.74 g, 1.0 mmol) in CH_3CN (2.0 mL) was introduced to the mixture via a syringe and the reaction mixture was stirred at room temperature for 4 h. The solvent was then evaporated under reduced pressure and the precipitate was dissolved in CH_2Cl_2, followed by washing with water for three times and drying over Na_2SO_4. The residue obtained by evaporation was dispersed in 30.0 mL CH_3OH for further use in the next step.

$SnCl_2$ (3.8 g, 20.0 mmol) dissolved in concentrated HCl (4.0 mL) was added the above solution under nitrogen atmosphere. The reaction solution was heated to 70°C and stirred overnight. Then, the solution was neutralized by saturated Na_2CO_3, and the precipitate was removed by filtration and washed with CH_2Cl_2. The collected filtrate and washings were treated thrice with water and dried over

Na_2SO_4. The solvent was removed by evaporation under reduced pressure, and the residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (50/3, v/v) as eluent to afford a dark blue solid product. Yield: 0.33 g (58%). ^{1}H NMR (400 MHz, DMSO): δ 8.54 (d, J = 14.4 Hz, 1H), 8.22 (d, J = 8.0 Hz, 1H), 8.12-8.06 (m, 2H), 7.80 (d, J = 8.8 Hz, 1H), 7.67 (t, J = 7.2 Hz, 1H), 7.61 (s, 1H), 7.53 (t, J = 15.2 Hz, 1H), 7.39 (d, J = 8.4 Hz, 1H), 6.72-6.69 (m, 2H), 6.30 (d, J = 14.8 Hz, 1H), 4.38 (q, J = 6.4 Hz, 7.2Hz, 2H), 2.71-2.66 (m, 4H), 1.95 (s, 6H), 1.83 (m, 2H), 1.36 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO): δ 175.1, 162.6, 156.1, 155.4, 141.6, 139.7, 138.4, 134.1, 132.1, 131.1, 130.5, 130.2, 128.4, 127.9, 125.6, 123.0, 122.5, 114.9, 114.3, 113.2, 112.2, 100.1, 97.9, 51.4, 46.3, 28.4, 27.8, 24.2, 20.7, 12.9. MS (TOF): 447.3.

Compound Cy-Azo. Cy-NH_2 (0.11 g, 0.2 mmol) was dissolved in CH_3CN/CH_2Cl_2 (1:1, 10.0 mL) containing 1% trifluoroacetic acid (TFA) and the mixture was stirred at 0°C under nitrogen atmosphere. Then, $NaNO_2$ (0.03 g, 0.4 mmol) was added, and stirring was continued at the same temperature for 30 min. Next, sulfamic acid (0.04 g, 0.4 mmol) was added and stirring for 10 min. Immediately, N,N-dimethylaniline (0.3 mL, 2.4 mmol) in CH_3CN (2.0 mL) was added and further stirred at 0°C for 1.5 h. The mixture was diluted with H_2O and extracted with CH_2Cl_2. The organic layer was collected, washed with brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (20/1, v/v) as eluent to afford a deep green solid product. Yield: 0.07 g (50%). ^{1}H NMR (400 MHz, DMSO): δ 8.73 (d, J = 15.2 Hz, 1H), 8.43 (d, J = 8.8 Hz, 1H), 8.21 (d, J = 8.8 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 7.98 (d, J = 9.2 Hz, 1H), 7.85 (d, J = 9.2 Hz, 2H), 7.77-7.70 (m, 3H), 7.65-7.63 (m, 2H), 7.44 (s, 1H). 6.86 (d, J = 9.2 Hz, 2H), 6.70 (d, J = 15.2 Hz, 1H), 4.60 (d, J = 7.2 Hz, 2H), 3.09 (s, 3H), 2.73 (t, J = 7.6 Hz, 3H), 1.85 (s,

2H), 1.43 (t, J = 6.8 Hz, 4H), 1.20 (s, 6H), 0.82 (t, J = 4.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO): δ 179.4, 159.2, 154.5, 153.6, 153.4, 144.8, 143.2, 139.0, 137.1, 133.0, 131.4, 131.3, 131.1, 130.5, 128.7, 128.6, 127.5, 126.9, 126.0, 123.3, 123.1, 119.5, 115.1, 113.0, 112.1, 108.9, 105.9, 52.9, 41.4, 29.5, 27.3, 24.1, 22.6, 20.3, 13.6. MS (TOF): 579.4. Elem. anal. (%) calcd. for $C_{39}H_{39}IN_4O$: C, 66.29, H, 5.56, N, 7.93. Found: C, 66.20, H, 5.57, N, 7.96.

2. Characterization of Cy-NH_2 and Cy-Azo

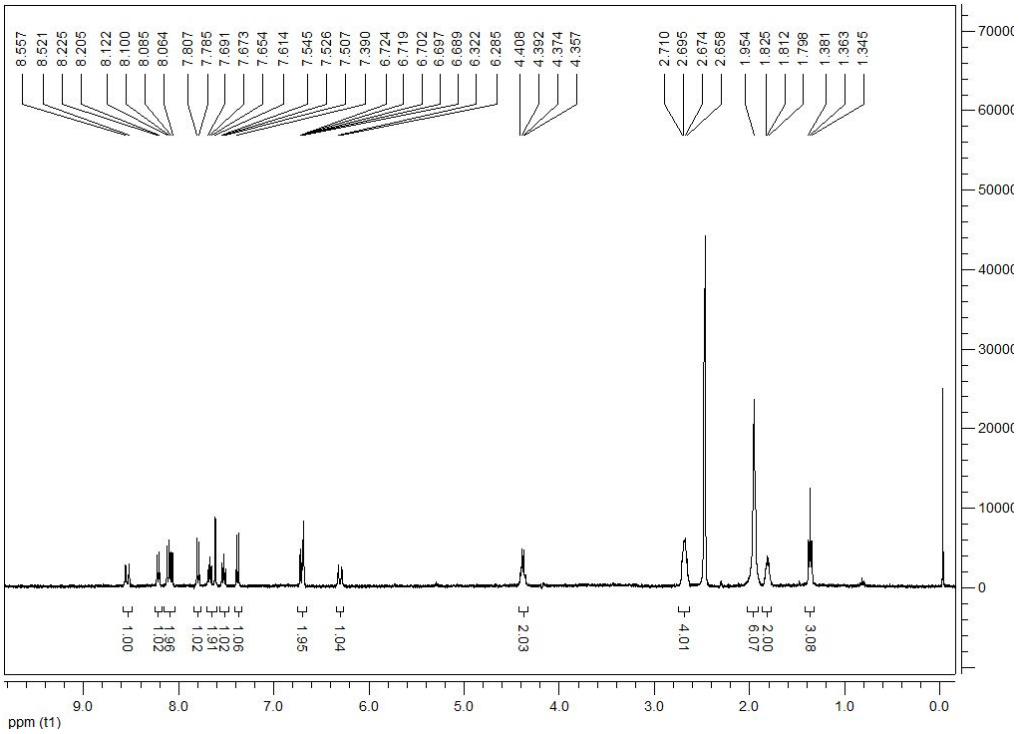


Figure S1. ^{1}H NMR spectra of Cy-NH_2 in DMSO.

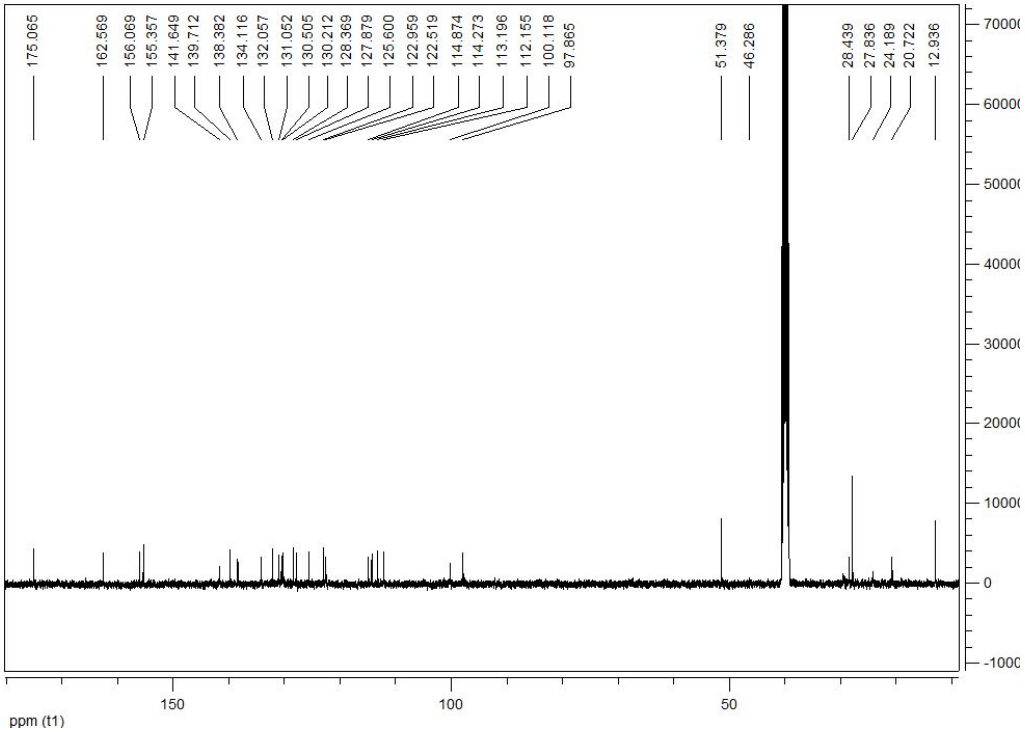


Figure S2. ^{13}C NMR spectra of Cy-NH_2 in DMSO.

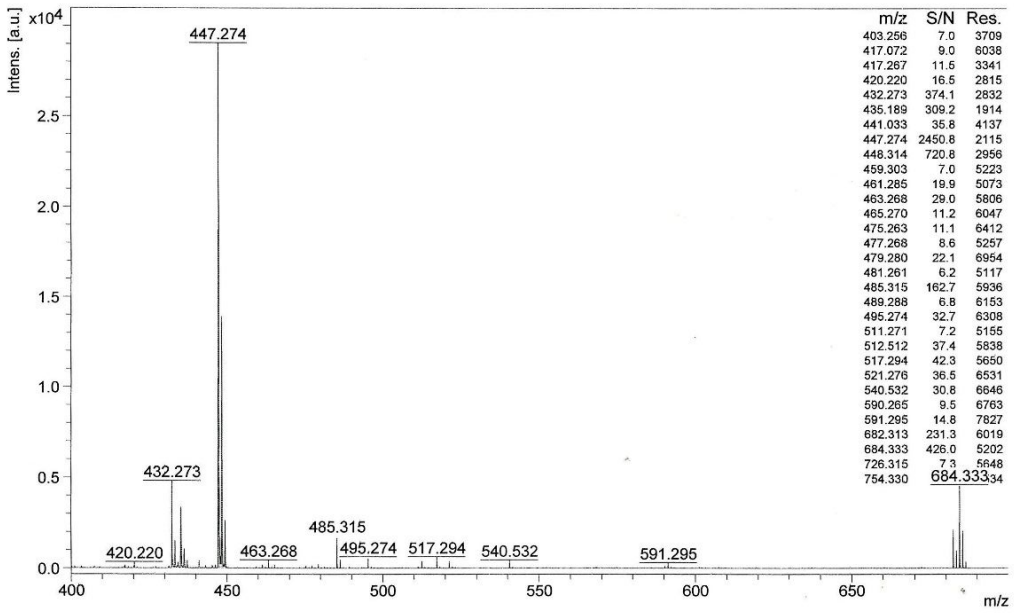


Figure S3. Mass spectra of Cy-NH_2.

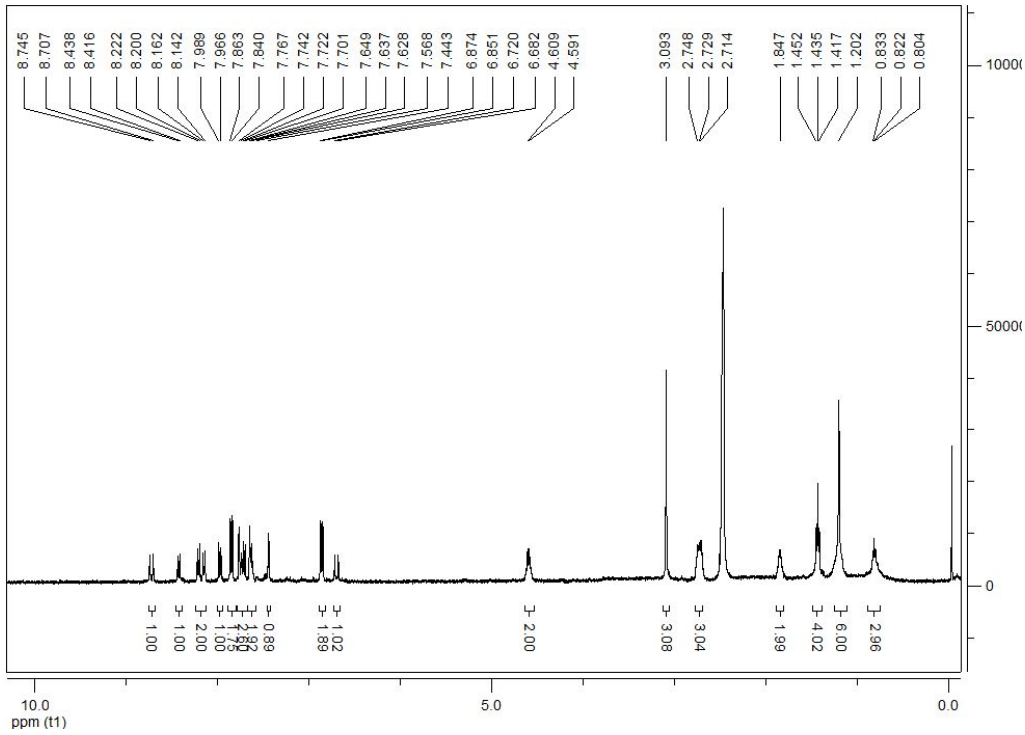


Figure S4. ^{1}H NMR spectra of Cy-Azo in DMSO.

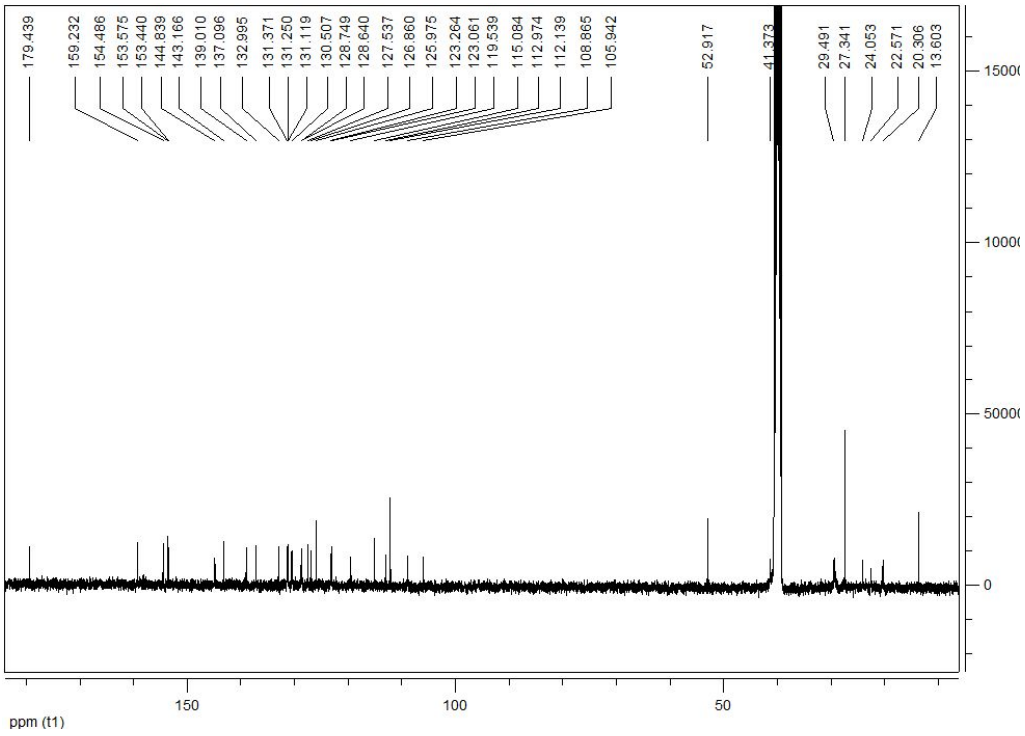


Figure S5. ^{13}C NMR spectra of Cy-Azo in DMSO.

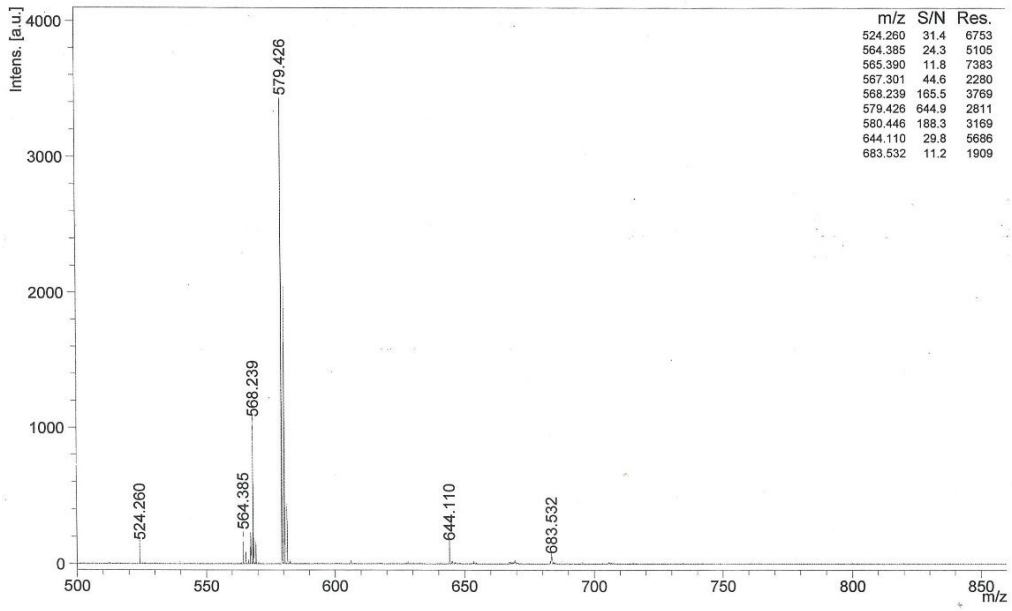


Figure S6. Mass spectra of Cy-Azo.

3. Spectroscopic response of Cy-Azo to AzoR

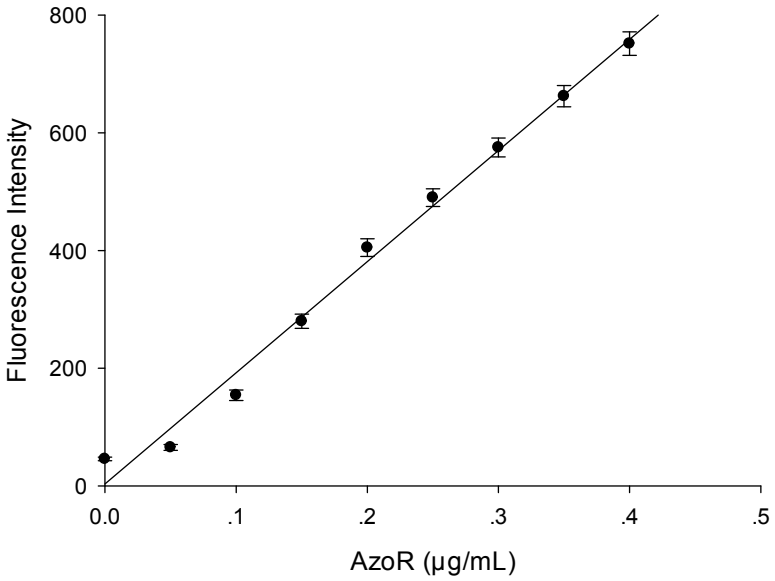


Figure S7. Linearity plots of the fluorescence intensity of Cy-Azo versus various enzyme activities.

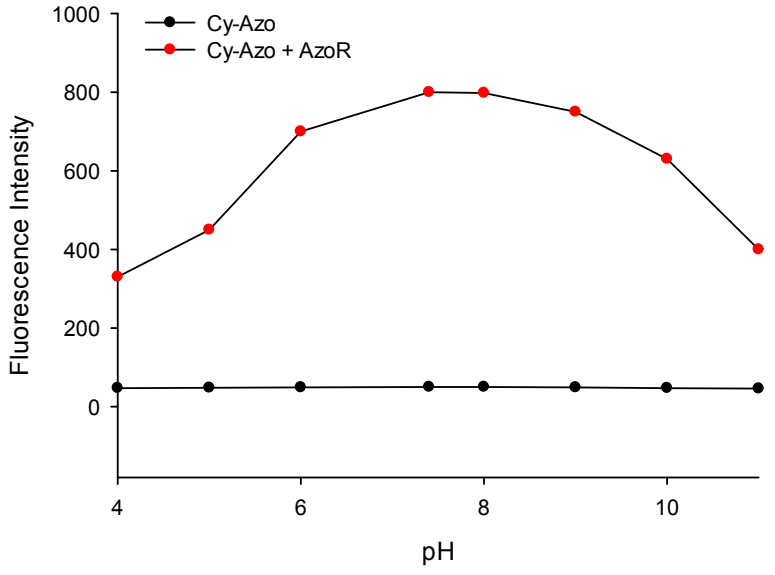


Figure S8. Effect of pH on the fluorescence intensity of Cy-Azo (10 μM) before and after the reaction with AzoR (0.4 μg/mL) with NADH (100 μM) in Tris-HCl buffer solution (pH 7.4, 50 mM, 1% DMSO).

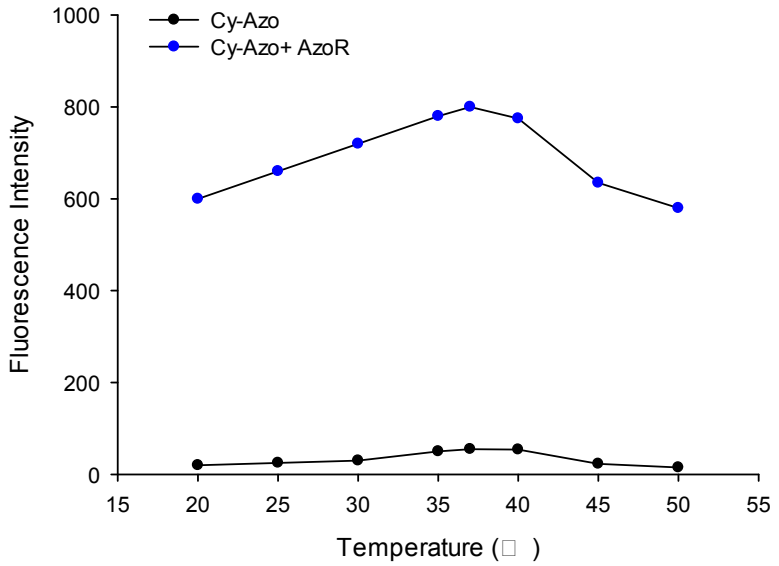


Figure S9. Effect of temperature on the fluorescence of Cy-Azo (10 μM) before and after the reaction with AzoR (0.4 μg/mL) with NADH (100 μM) in Tris-HCl buffer solution (pH 7.4, 50 mM, 1% DMSO).

4. Enzyme kinetics

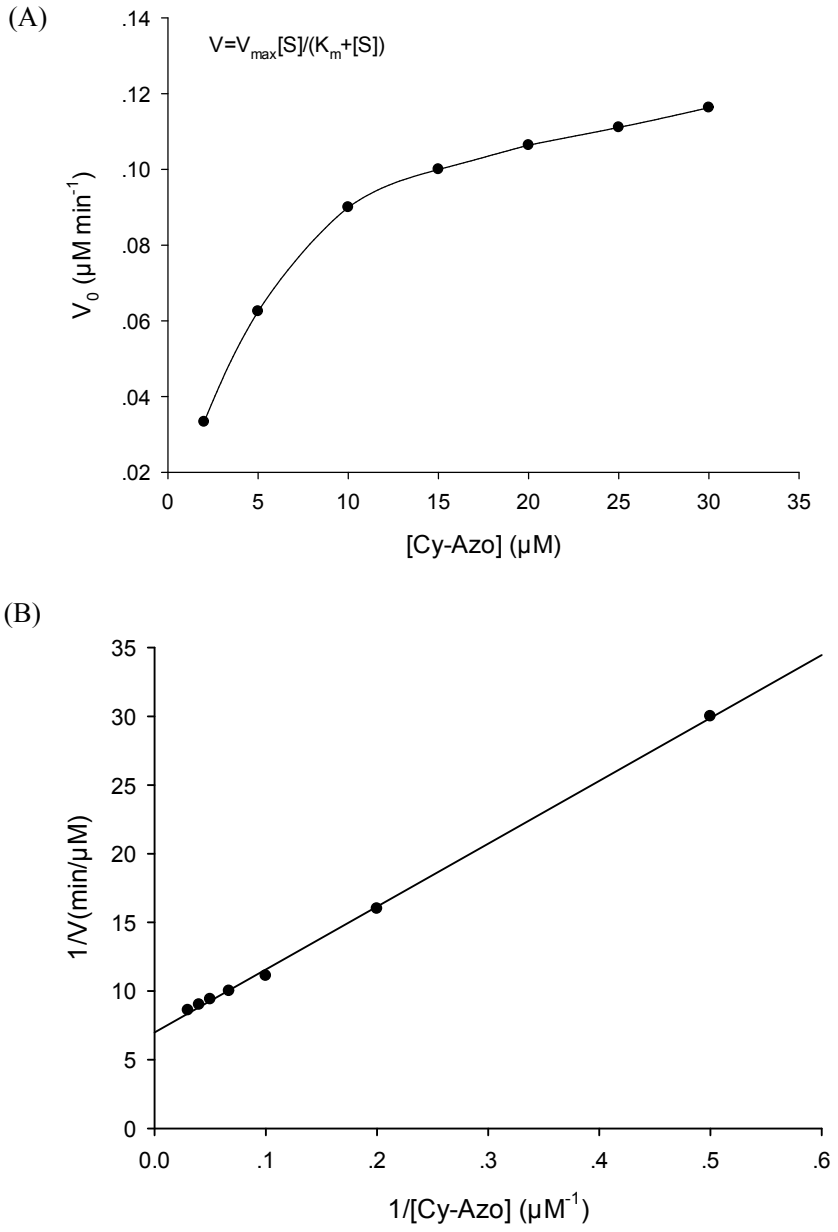


Figure S10. (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot for the reaction between Cy-Azo and AzoR (0.4 μg/mL) with NADH (100 μM).

5. Selectivity

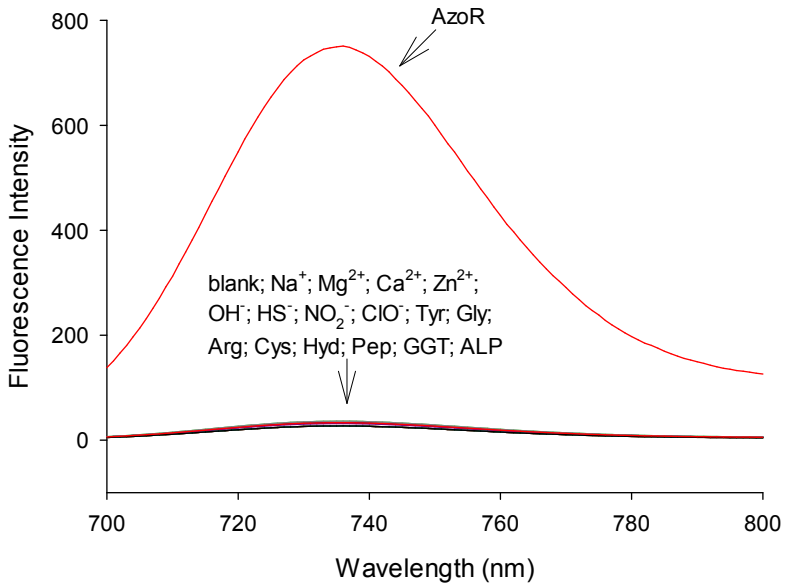


Figure S11. Fluorescence spectra of Cy-Azo (10 μM) to AzoR (0.4 μg/mL) with NADH (100 μM) and other various species in Tris-HCl buffer solution (pH 7.4, 50 mM, 1% DMSO).

6. AzoR inhibitor investigation

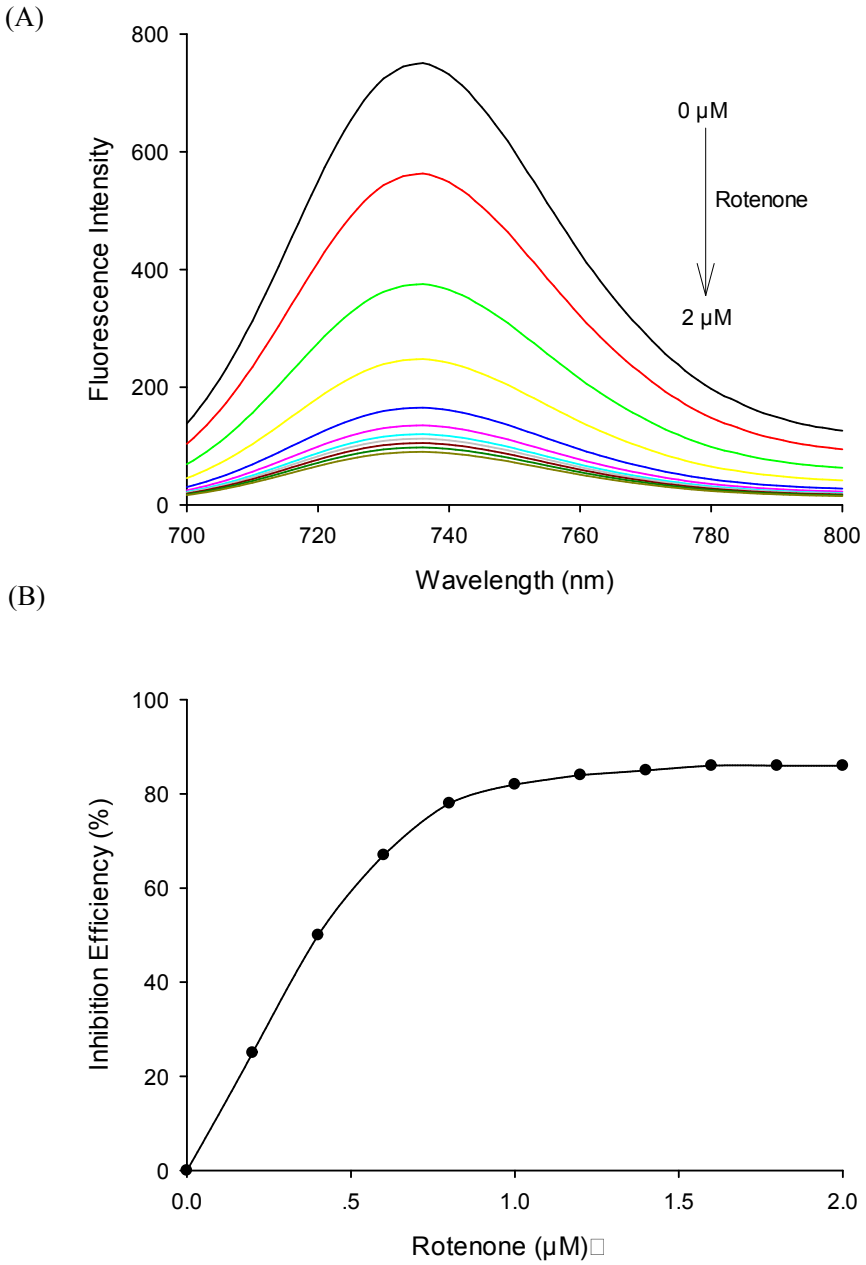


Figure S12. (A) Fluorescence spectra of Cy-Azo (10 μM) in the presence of AzoR (0.4 μg/mL) at different concentrations of rotenone (0-2 μM) in Tris-HCl buffer solution (pH 7.4, 50 mM, 1% DMSO). (B) The inhibition efficiency versus rotenone concentrations.

7. Effect of inhibitor on Cy-NH_2

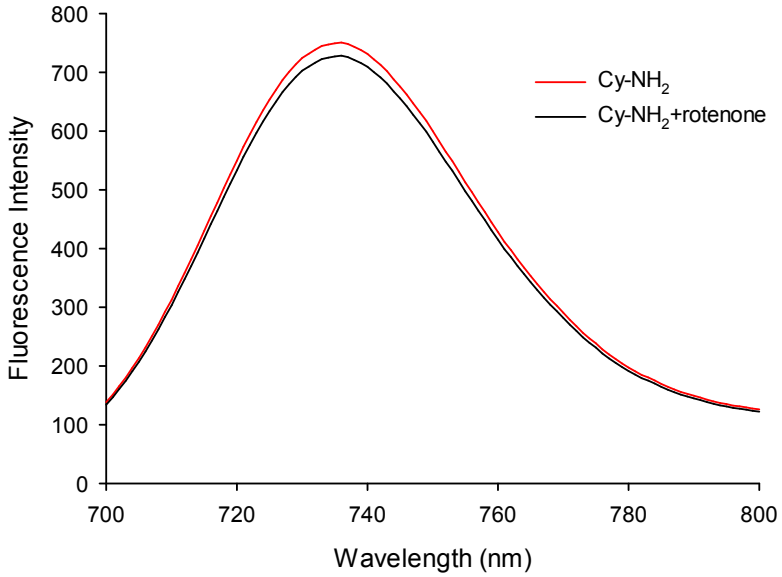
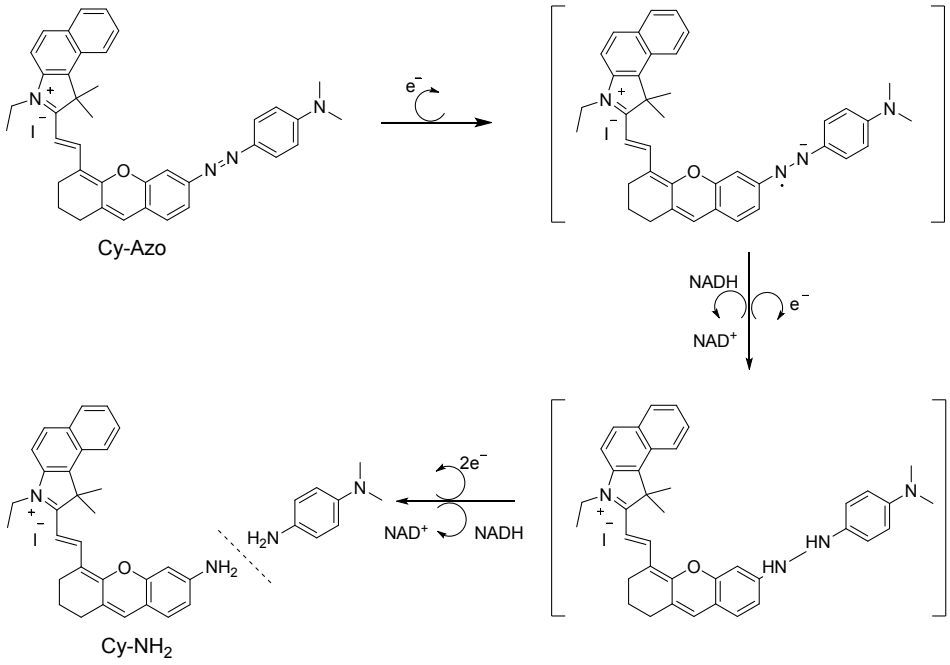


Figure S13. Fluorescence spectra of Cy-NH_2 (10 μM) before and after the addition of rotenone (2 μM) in Tris-HCl buffer solution (pH 7.4, 50 mM, 1% DMSO).

8. Mechanism investigation

Scheme S2 Proposed response mechanism of probe Cy-Azo with AzoR.



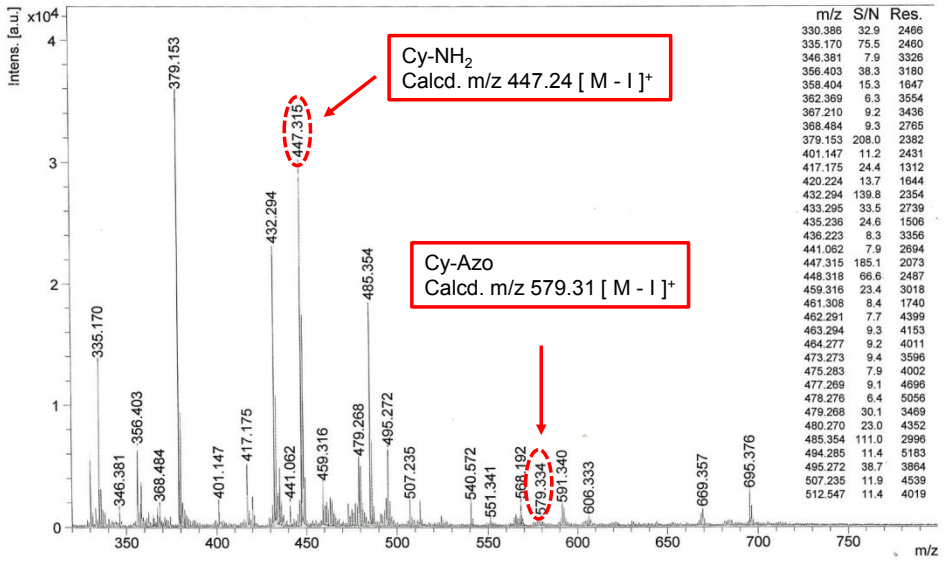
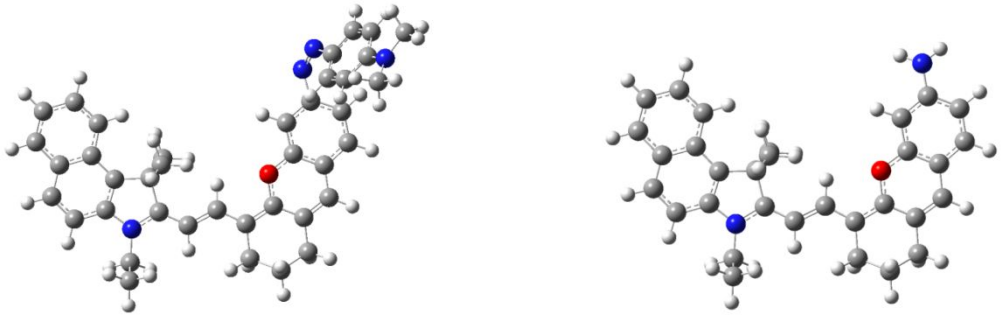


Figure S14. Mass spectra of Cy-Azo reacted with AzoR.



Cy-Azo Cy-NH_2

Figure S15. The optimized structures of Cy-Azo and Cy-NH_2.

9. Cell viability

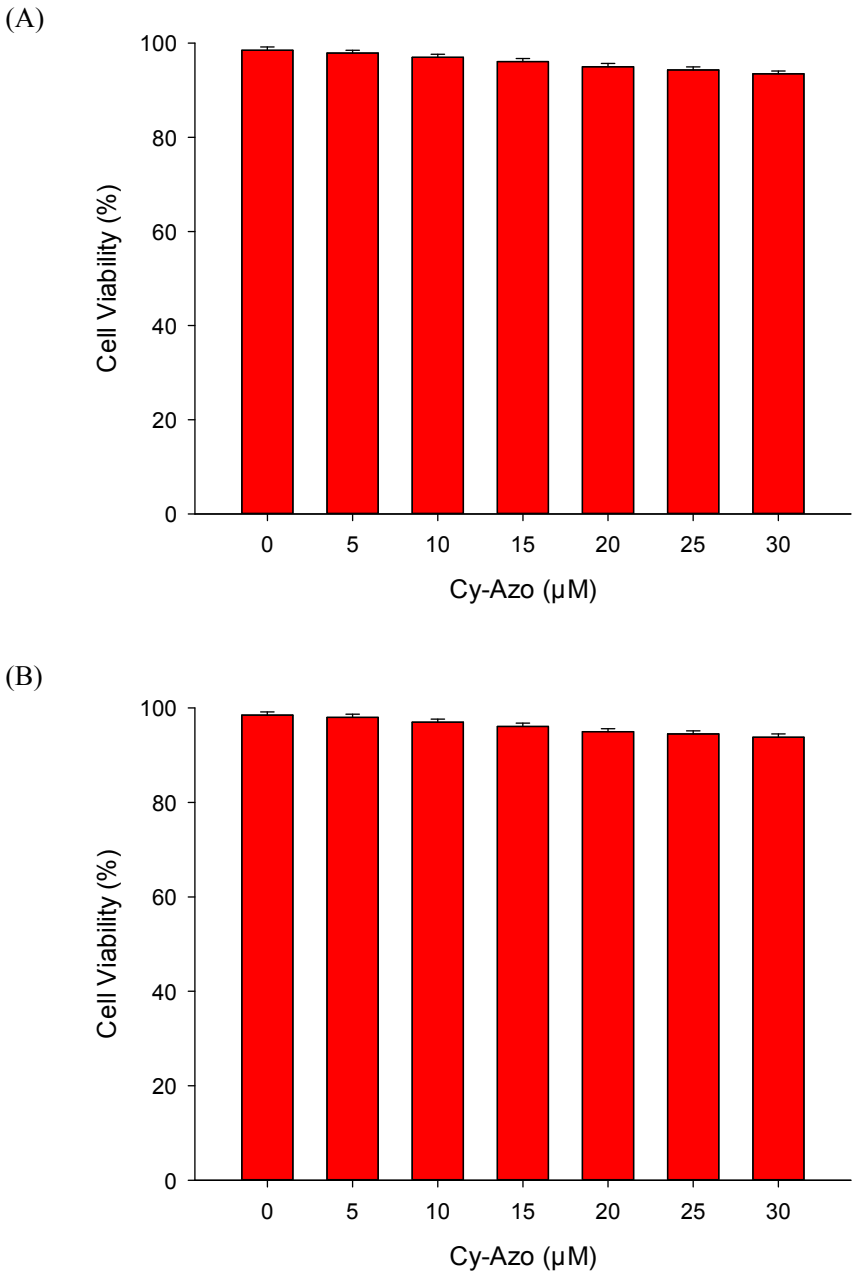


Figure S16. MTT assay for estimating cell viability (%) of (A) HCT116 cells and (B) HepG2 cells treated with various concentrations of Cy-Azo (0-30 μM) after 24 h incubation.

10. Fluorescence imaging in living cells

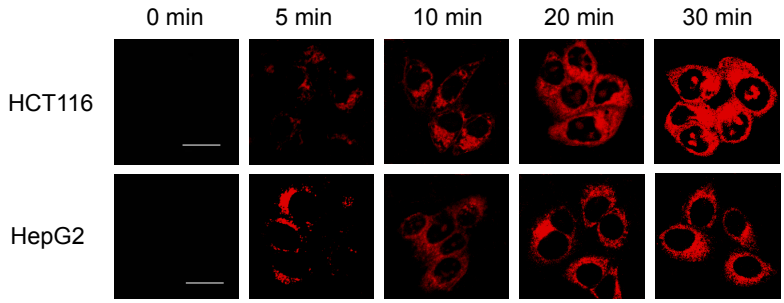


Figure S17. The time-dependent fluorescence response to AzoR activity in HCT116 cells and HepG2 cells. The cells were incubated with Cy-Azo (10 μM) at different time points: 0, 5, 10, 20 and 30 min. λ_{ex} = 640 nm, λ_{em} = 663-738 nm; Scale bar: 10 μm.

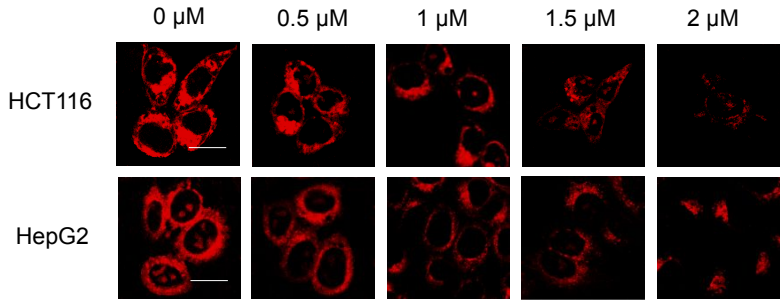


Figure S18. Fluorescence imaging of HCT116 cells and HepG2 cells incubated with different concentrations of AzoR inhibitor (rotenone, 0-2 μM) and Cy-Azo (10 μM). λ_{ex} = 640 nm, λ_{em} = 663-738 nm; Scale bar: 10 μm.

11. Fluorescence imaging in bacteria

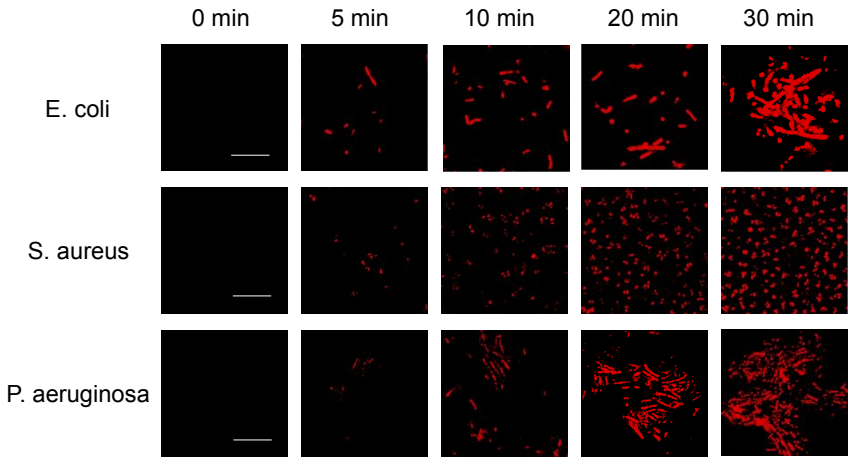


Figure S19. The time-dependent fluorescence response to AzoR activity in E. coli, S. aureus and P. aeruginosa. The bacteria were incubated with Cy-Azo (10 μM) at different time points: 0, 5, 10, 20 and 30 min. λ_{ex} = 640 nm, λ_{em} = 663-738 nm; Scale bar: 10 μm.

12. Fluorescence imaging in UC mice

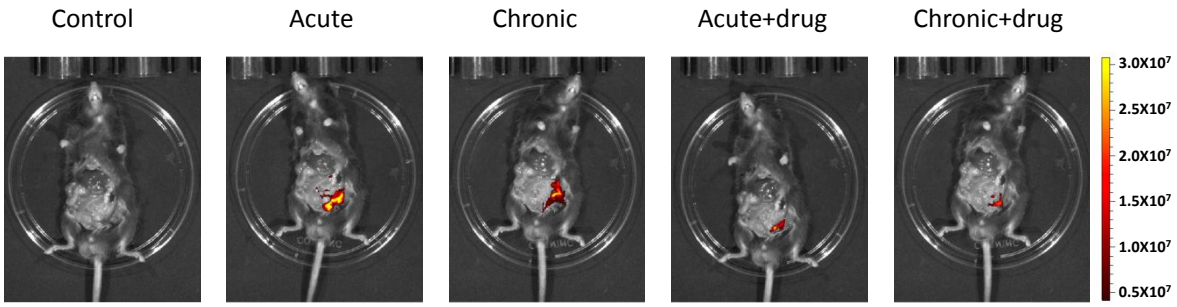


Figure S20. Fluorescence imaging of dissected mice from control group, acute UC group, chronic UC group, the therapy of acute UC group and the therapy of chronic UC group mice. λ_{ex} = 640 nm, λ_{em} = 695-770 nm.

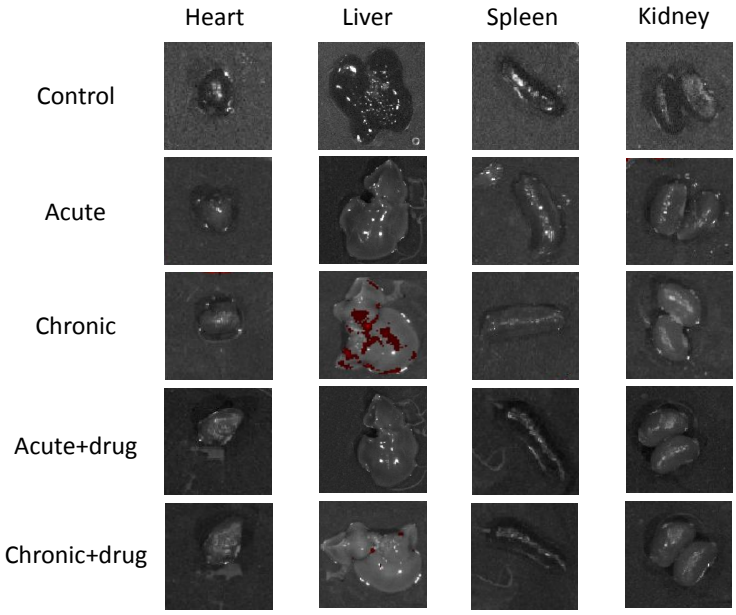


Figure S21. Fluorescence imaging of major organs (heart, liver, spleen, kidney) of mice from control group, acute UC group, chronic UC group, the therapy of acute UC group and the therapy of chronic UC group. λ_{ex} = 640 nm, λ_{em} = 695-770 nm.