*Supporting Information*

# Polarity-sensitive fluorescent probes based on imidazopyridazine derivatives for imaging lung cancer tissues

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# 1. Experimental section

## 1.1 Materials and apparatus

Unless otherwise stated, all reagents were purchased from Aladdin, Macklin or Qiangsheng (Suzhou, China) and used without further purification. Silica gel (200-300 mesh) was used to purify the product by flash chromatography. The synthesis of compound 2 was based on the reported literature.[1] Melting points were determined on an X-4 microscope electron thermal apparatus (Kerui, China). $^{1}$H NMR (400 MHz) and $^{13}$C NMR (151 MHz) spectra were respectively recorded with Varian and Bruker spectrometers at room temperature. HRMS data were achieved with a Finnigan MAT95 and Xevo G2-XS TOF mass spectrometer. UV–vis absorption spectra were obtained with a Shimadzu UV-1800 spectrometer and emission spectra were carried out on shimadzu RF-5301PC spectroscope in fused quartz cuvettes (10 mm × 10 mm) at room temperature. A Leica TCS SP5 II confocal laser scanning microscope was operated to record the fluorescence confocal images, and the images were processed with LAS-AF lite software. Cell samples were obtained from: Cell Bank of Chinese Academy of Sciences (TCHu150)-A549 and ATCC (CRL-1502)-WS1.

## 1.2 General synthetic procedure of 1a-1b.

Compound **2** (1.00 mmol), **3a-3b** (1.50 mmol), 1,1-bis(diphenylphosphino)ferrocene palladium dichloride (0.05 mmol), and sodium carbonate (2.5 mmol) were added to a toluene solution (5.0 mL). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 2 hours, and the reaction progress was monitored by TLC. Upon completion, the reaction was quenched, and the mixture was subjected to suction filtration to remove solid impurities, including the palladium catalyst and sodium carbonate. The filtrate was poured into 20 mL of water and extracted with dichloromethane ($CH_2Cl_2$, 3 × 25 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$, and the solvent was removed under reduced pressure to afford the crude products **1a-1b**. Purification by column chromatography using a $CH_2Cl_2$/MeOH mixture (20:1, v/v) as the eluent yielded the pure compounds **1a-1b**.

## 1.3 Characterization of compounds 1a-1b

**6-(4-(Diethylamino)phenyl)imidazo[1,2-b]pyridazine-3-carbonitrile(1a).** Green solid (150.5 mg), isolated yield: 50.4 %, mp: 169.3-171.1℃. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ (ppm) 8.15 (s, 1H, Ar-*H*), 7.96 (d, *J* = 9.6 Hz, 1H, Ar-*H*), 7.93 (d, *J* = 8.7 Hz, 2H, Ar-*H*) 7.68 (d, *J* = 9.6 Hz, 1H, Ar-*H*), 6.76 (d, *J* = 8.7 Hz, 2H, Ar-*H*), 3.45 (q, *J* = 7.1 Hz, 4H, 2×$CH_2$), 1.23 (t, *J* = 7.1 Hz, 6H, 2×$CH_3$). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ (ppm) 153.6, 149.7, 140.8, 140.2, 128.5, 125.5, 119.8, 119.1, 111.4, 110.4, 103.0, 44.5, 12.5. HRMS (TOF, ESI$^+$): m/z calcd $C_{17}H_{18}N_5^+$ for $[M+H]^+$ 292.1557, found: 292.1562.

**6-(4-(Diphenylamino)phenyl)imidazo[1,2-b]pyridazine-3-carbonitrile(1b).** Yellow solid (120.8 mg), isolated yield: 41.2 %, mp: 177.5-178.6℃. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ (ppm) 8.21 (s, 1H, Ar-*H*), 8.06 (d, *J* = 9.6 Hz, 1H, Ar-*H*), 7.89 (d, *J* = 8.9 Hz, 2H, Ar-*H*), 7.69 (d, *J* = 9.5 Hz, 1H, Ar-*H*), 7.36 – 7.29 (m, 4H, Ar-*H*), 7.20 – 7.10 (m, 8H, Ar-*H*). $^{13}$C NMR (101 MHz, $CDCl_3$) δ (ppm) 160.4, 152.3, 150.4, 146.7, 140.3, 129.5, 128.0, 126.9, 126.7, 125.5, 124.3, 121.7, 117.6. HRMS (TOF, ESI$^+$): m/z calcd $C_{25}H_{18}N_5^+$ for $[M+H]^+$ 388.1557, found: 388.1557.

**1.4 Photostability experiment of probes 1a-1b.**

The reference and test samples were dissolved in acetonitrile with a concentration of 1.0 × $10^{-5}$ M. A Philips iodine tungsten lamp (500 W) was used to irradiate them. The distance between the samples and the lamp was 25 cm. To reduce the interference of the heat and the short-wavelength light, a transparent pot with $NaNO_2$ (60 g/L, thickness: 8 cm) was placed between the samples and the lamp. Photostability was in terms of remaining absorption (%) calculated from the change of absorption intensity at the absorption maximum before and after irradiation.

**1.5 Preparation of the test solution.**

For the optical assay, the stock solution of probes **1a-1b** (1.0 mM) were prepared in 10 mL DMSO. UV-vis absorption and fluorescence emission spectra of **1a-1b** in different solvents including water ($H_2O$), methanol (MeOH), dimethyl sulfoxide (DMSO), ethyl acetate (EA), 1,4-dioxane, and toluene (TOL) with decreasing orientation polarizability ($\Delta f$) were recorded. The 100 μL stock solution of probes was transferred to 10 mL volumetric flasks, and diluted with corresponding solvent to the scale line, then mixed. The polarity-response test solutions were prepared from the 100 μL probes stock solution and the 1,4-dioxane-water mixed system. Furthermore, the volume percentages of 1,4-dioxane in the mixed system were 100%, 99%,98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 80% in turn. In all optical experiments, the

concentration of the test solutions was 10 μM. The excitation wavelength of **1a** was 395 nm, that of **1b** was 400 nm, and the slit widths were 3 nm/3 nm.

**1.6 Determination of the relative fluorescence quantum yield.**

The relative fluorescence quantum yields were measured using the following equation:

$$\Phi_x/\Phi_{st} = [A_{st}/A_x]\ [n_x^2/n_{st}^2]\ [D_x/D_{st}]$$

Where st was the standard; x is the sample; F was the quantum yield; A was the absorbance at the excitation wavelength; D was the area under the fluorescence spectra on an energy scale; and n was the refractive index of the solution. Coumarin-153 (Φ = 54.7% in ethanol) was used as the standard.

**1.7 Selectivity experiment.**

The stock solutions for selectivity were prepared in volumetric flasks (10 mL) with concentrations of 10 mM for $Ba^{2+}$, $Cu^{2+}$, $Pd^{2+}$, $Ni^{2+}$, $Na^{+}$, $K^{+}$, $Mn^{2+}$, $Cl^{-}$, $CO_3^{2-}$, $SO_4^{2-}$, $HSO_3^{-}$, $S_2O_5^{2-}$, Pro, Cys, GSH in double-distilled water (DDW). Each test solution was prepared in a volumetric flask with a 100 μL stock solution of probes and the corresponding volume stock solution of 1,4-dioxane, or interfering ions, or amino acid solutions, and then diluted with PBS buffer (pH = 7.4) to obtain a total volume of 10 mL.

As for the pH assay, test solutions of probes **1a-1b** with different pH values were prepared by mixing 1.0 mL stock solution of probes (100 μM) with 2.0 mL of 1,4-dioxane, and then adjusting the volume to 10 mL with PBS. The concentration of test solutions was 10 μM in all optical experiments.

**1.8 Cytotoxicity experiments.**

100 μL of A549 cells and WS1 cells suspension were respectively placed in a 96 well plates and then the plates were placed in a humidified incubator for pre-incubation for 24 h. After that, different concentrations of **1a-1b** (0 μM, 2.0 μM, 4.0 μM, 6.0 μM, 8.0 μM, 10 μM) were added respectively and co-incubated for 6 h. Finally, MTT solution (10 mL) was separately injected into each well and cultured for 1h. Cytotoxicity of **1a-1b** was evaluated as a percentage by the following formula:

$$\text{Survival rate (\%)} = (A_{sample} - A_b)/ (A_c - A_b).$$

$$\text{Inhibition rate (\%)} = (A_c - A_{sample})/ (A_c - A_b)$$

$A_c$: Negative control (including media and cells, no test substance), $A_b$: blank (including test substance and media, no cells).

**1.9 Cell culture and imaging methods.**

Prior to laser confocal experiments, A549 cells and WS1 cells were seeded onto glass bottom dishes at a density of no less than $2 \times 10^5$ cells and cultured in a $CO_2$ incubator (37°C, 5:95 = $CO_2$: air) for 48 h. The culture medium consisted of 10% calf serum, 100 U $mL^{-1}$ penicillin, 100 μg $mL^{-1}$ streptomycin, and $2.5 \times 10^{-4}$ M $L^{-1}$ glutamine. First, A549 cells were co-incubated with a commercial LDs red marker (Nile Red, 0.1 μM) and commercial mitochondrial markers (Mito-Tracker Red CMXRos, 3.0 μM) for 3 min. Then, **1a-1b** were added at a concentration of 0.5 μM and incubated for an additional 3 min. After incubation, the cell culture medium was removed and the cells were washed with PBS buffer (pH = 7.4). Confocal fluorescence images of stained cells were obtained using a laser scanning microscope with a 20 X objective. In cell experiments, fluorescence images of **1a-1b** were acquired under the same settings (green channel: excited at 405 nm, emission at 500-600 nm; red channel: excited at 561 nm, emission at 570-750 nm). The overlap tendency between **1a-1b** and the commercial marker was obtained by measuring the fluorescence intensity of the regions of interest (ROI).

In the investigation of LDs and mitochondrial alterations in the physiological activity, A549 cells were incubated with **1a** (0.5 μM) and **1b** (0.5 μM) for 3 h, with fluorescence imaging conducted every 30 min throughout the process. Meanwhile, in the experiment of changes in mitochondrial polarity and LDs polarity, **1a** (0.5 μM), **1b**(0.5 μM) and TLR4 activator (LPS) reagent were co-incubated with A549 cells for 1 h, and interval fluorescence imaging was performed every 15 min during this period. Meanwhile, probe **1a** (1.0 μM) was employed for treating A549 cells under starvation conditions (serum-free medium) for 150 min. Rapamycin (5.0 μM) and probe **1a** (1.0 μM) were adopted for incubating A549 cells for 150 min. Rapamycin (5.0 μM), 3-MA (5.0 μM) and probe **1a** (1.0 μM) were adopted for incubating A549 cells for 150 min. In the experiment for monitoring changes in mitochondria polarity, the interval fluorescence imaging was performed during this period.

**1.10 Fluorescence Imaging of Human Tissue.**

A total of three types of human carcinoma samples were collected at the Four Affiliated Hospital of Soochow University according to the principles of the Declaration of Helsinki. The

study was ap-proved by the Research Ethics Committee the Four Affiliated Hospital of Soochow University (241128). Informed consent was collected from all the patients.

Serial sections of paraffin-embedded blocks of 7 μm-thick hepatocellular carcinoma tissue were used for probes **1a-1b** staining. Sections were incubated three times in xylene for 10 min each; twice in anhydrous ethanol for 5 min each; once in 95% ethanol for 1 min each to completely remove paraffin; and then washed twice in water for 1 min each. Sections were incubated with probes **1a-1b** (10 μM in PBS) for 30 min and then fixed with neutral resin. The prepared probe sections were observed using a 20 X objective laser scanning microscope. Green channel ($\lambda_{ex}$ = 405 nm, $\lambda_{em}$ = 450-600 nm) prepared tissue sections were collected by LAF-Lite software, and data were processed using Image J software and expressed as mean deviation.

## 2. Table and Figures

**Table S1** Optical properties of probes **1a-1b** in different solvents.

| Probe | Solvents | $\lambda_{Abs,max}$/nm | $\lambda_{Em,max}$/nm | Stokes shift/nm | $\varepsilon\times10^4$ $M^{-1}$ $cm^{-1}$ | $\Phi^{[a]}$/% |
|---|---|---|---|---|---|---|
| **1a** | TOL | 386 | 487 | 101 | 1.72 | 77.2 |
| | 1,4-dioxane | 382 | 514 | 132 | 1.79 | 67.1 |
| | EA | 381 | 498 | 117 | 1.86 | 22.8 |
| | DMSO | 388 | 573 | 185 | 1.64 | 10.9 |
| | MeOH | 392 | ND | ND | 2.23 | ND |
| | $H_2O$ | 384 | ND | ND | 1.07 | ND |
| **1b** | TOL | 388 | 483 | 95 | 1.88 | 97.8 |
| | 1,4-dioxane | 381 | 518 | 137 | 1.85 | 95.3 |
| | EA | 382 | 491 | 109 | 1.92 | 42.3 |
| | DMSO | 384 | 583 | 199 | 1.71 | 12.5 |
| | MeOH | 384 | ND | ND | 1.78 | ND |
| | $H_2O$ | 399 | 518 | 119 | 1.33 | 8.30 |

[a] Coumarin-153 (Φ = 54.7% in ethanol) was used as the reference compound; ND indicates not detected.

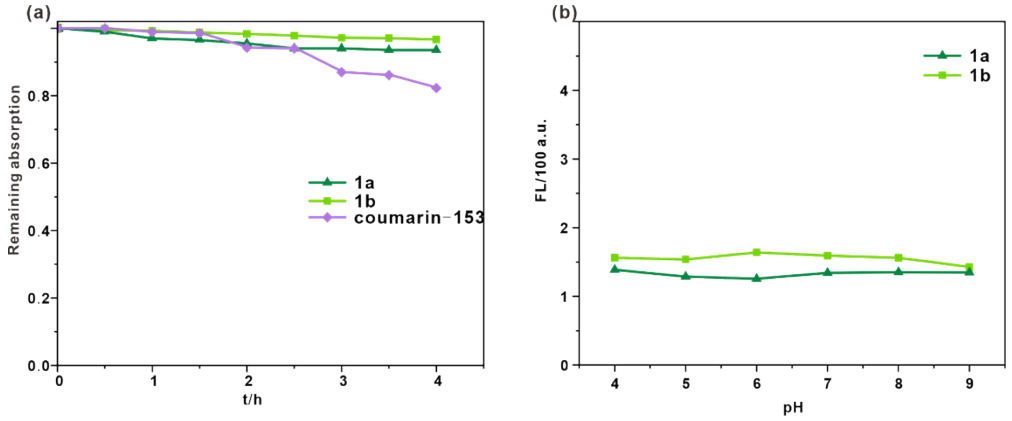


**Fig. S1 (a)** Photostability of probes **1a-1b** (10 μM) and coumarin-153 (10 μM) in acetonitrile. (b) Fluorescence intensity of probes **1a-1b** (10 μM) at physiological pH (4.0–9.0) in PBS buffer containing 1,4-dioxane (1,4-dioxane: PBS = 7: 3, v/v).

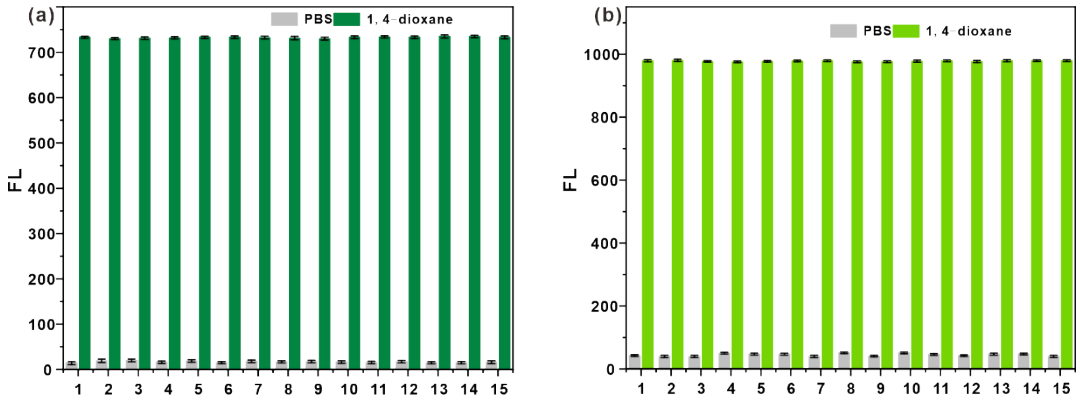


**Fig. S2** Selectivity experiments of **1a** (a) and **1b** (b) (10 μM) toward different analytes in PBS buffer containing DMSO (DMSO: PBS = 3: 7, v/v). 1.0 mM for 1. $Ba^{2+}$, 2. $Cu^{2+}$, 3. $CO_3^{2-}$, 4. Cys, 5. GSH, 6. $Na^+$, 7. $K^+$, 8. $Mn^{2+}$, 9. $Ni^{2+}$, 10. $Pd^{2+}$, 11. $S_2O_5^{2-}$, 12. $SO_4^{2-}$, 13. $HSO_3^-$, 14. $Cl^-$, 15. Pro. The data are shown as mean SD (n = 3).

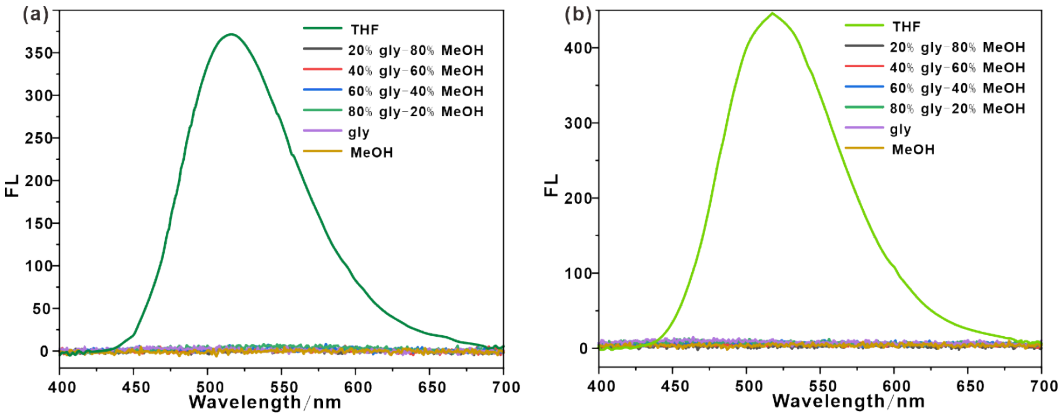


**Fig. S3** Probe **1a** (a) and **1b** (b) viscosity interference test.

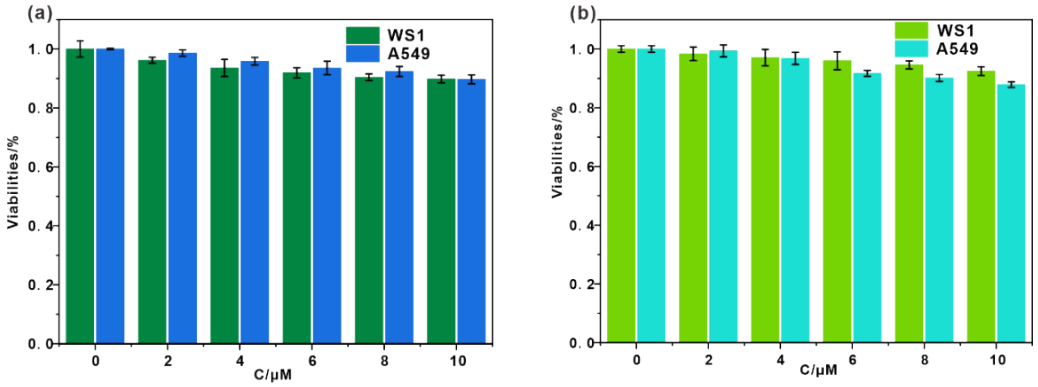


**Fig. S4** Cell viabilities of WS1 normal cells and A549 cancer cells treated with probes **1a** (a) and **1b** (b) of different concentrations (0, 2.0, 4.0, 6.0, 8.0, and 10 μM). The data are shown as mean SD (n = 3).

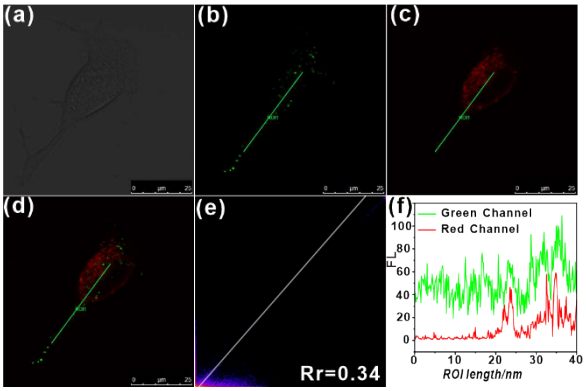


**Fig. S5** Fluorescence confocal images of A549 cells with probe **1b** (1 μM). (a) The brightfield images of A549 cells; (b) images (green channel) of A549 cells with **1b**; (c) images (Red channel) of A549 cells with Red Lyso-Tracker (1 μM); (d) the merged images of green channel and red channel; (e) the fluorescence intensity correlation plot; (f) intensity profiles of the regions of interest (ROIs) in the green channel and red channel.

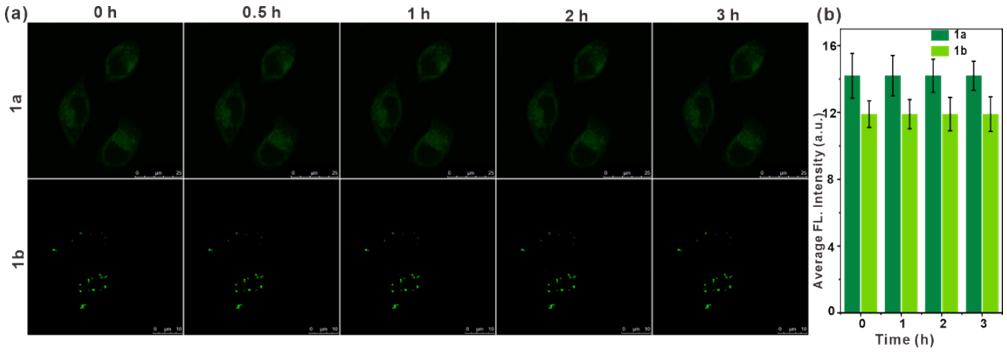


**Fig. S6** (a) Observation of physiological activity of probes **1a-1b** (0.5 μM) co-cultured with A549 cells for 3 h. (b) The average fluorescence intensity changes over time of **1a-1b**. The data are shown as mean SD (n = 3).

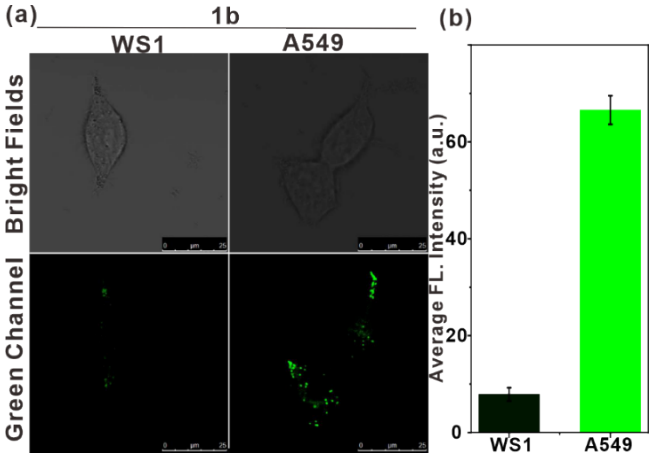


**Fig. S7** (a) Fluorescence images of cancer cells (A549), normal cells (WS1) and co-cultured for **1b** (0.5 μM). (b) The average fluorescence intensity of **1b** in cancer and normal cells. The data were shown as mean SD (n = 3).

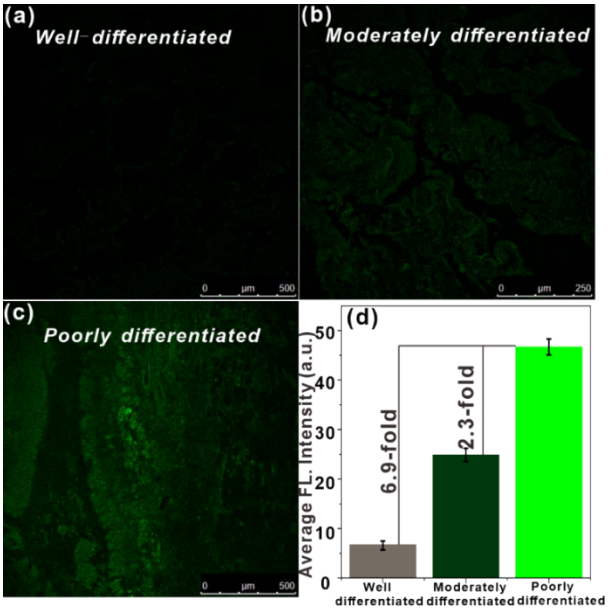


**Fig. S8** Confocal fluorescence images of paraffin sections of well-differentiated lung cancer tissues, moderately differentiated lung cancer tissues and poorly differentiated lung cancer tissues stained with probe **1b** (10 μM, 10 min). (b) Mean fluorescence intensities of probe **1b** stained living tissues in (a). The data are shown as mean SD (n = 3). (Excited at 405 nm, emission at 400-600 nm)

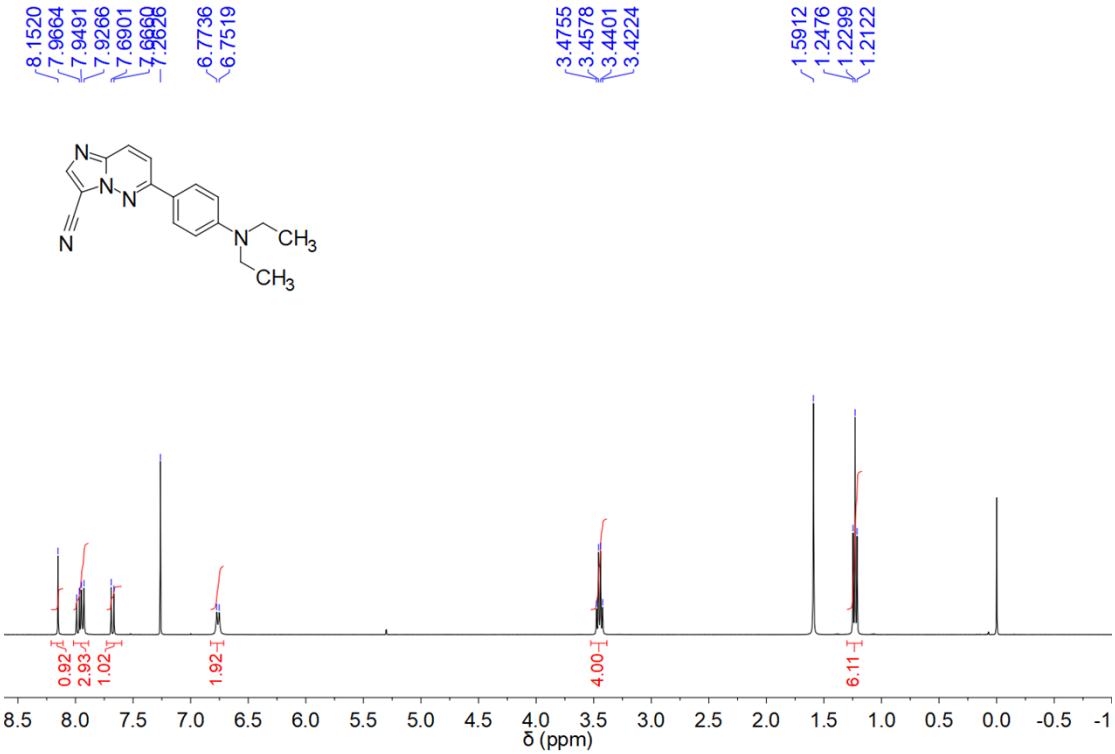


**Fig. S9** $^{1}$H NMR spectrum of probe **1a** (400 MHz, $CDCl_3$).

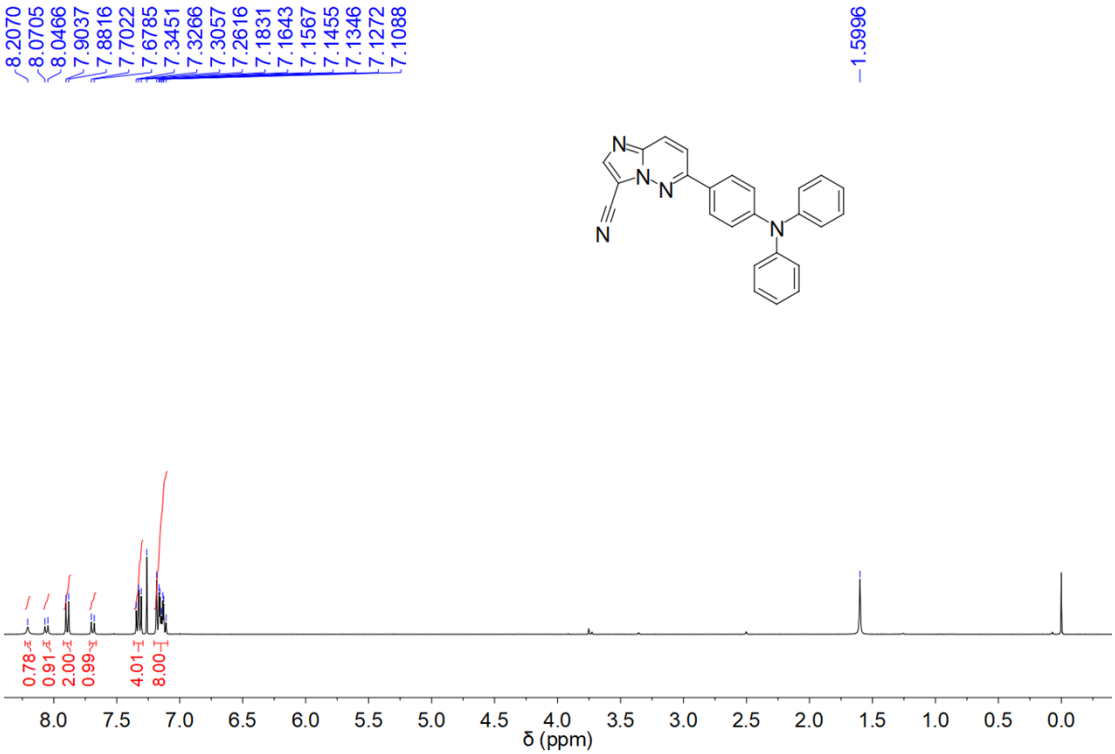


**Fig. S10** $^{1}$H NMR spectrum of probe **1b** (400 MHz, $CDCl_3$).

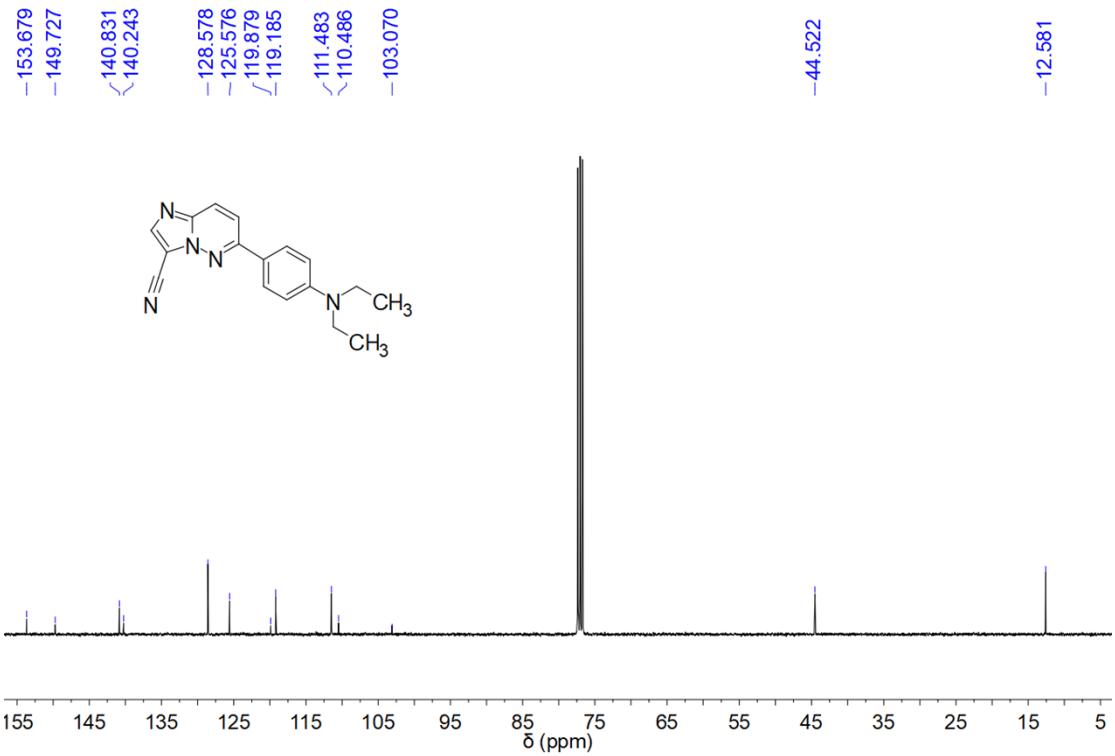


**Fig. S11** $^{13}$C NMR spectrum of probe **1a** (151 MHz, $CDCl_3$).

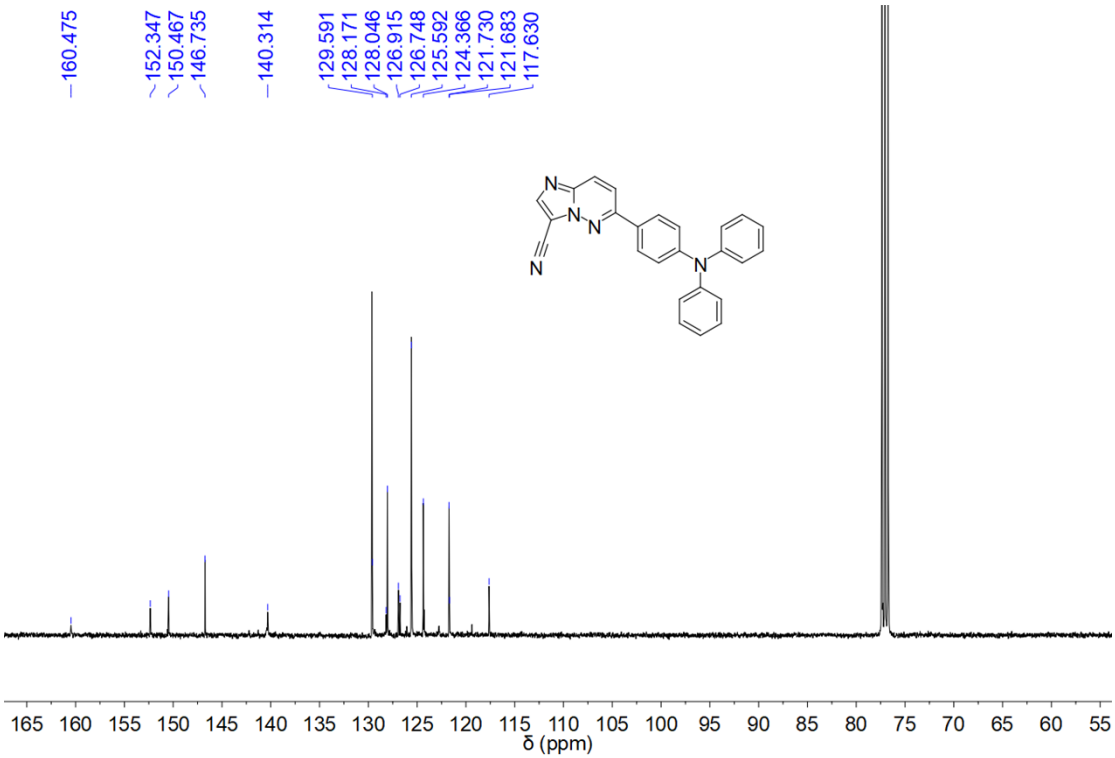


**Fig. S12** $^{13}C$ NMR spectrum of probe **1b** (151 MHz, $CDCl_3$).

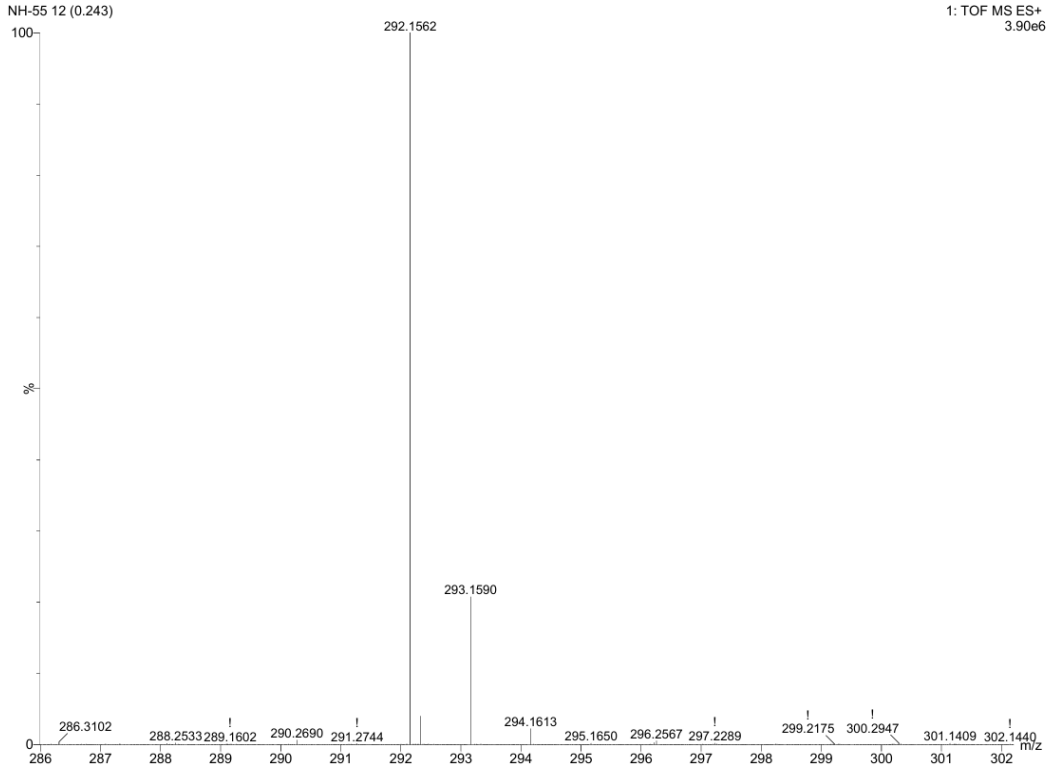


**Fig. S13** HRMS (TOF, $ESI^+$) spectrum of probe **1a**.

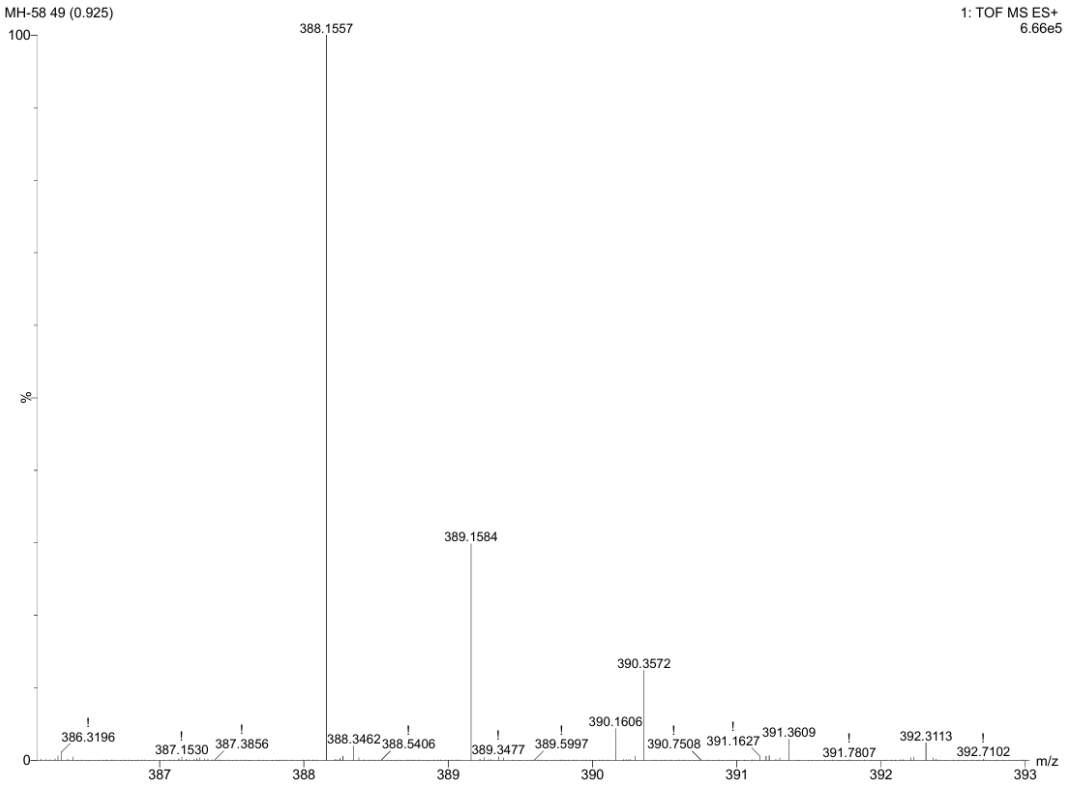


**Fig. S14** HRMS (TOF, ESI$^+$) spectrum of probe **1b**.

## 3. Reference

(1) Vetrichelvan, M.; Rakshit, S.; Chandrasekaran, S.; Chinnakalai, K.; Darne, C. P.; Doddalingappa, D.; Gopikumar, I.; Gupta, A.; Gupta, A. K.; Karmakar, A.; et al. Development of a Scalable Synthesis of the Small Molecule TGFβR1 Inhibitor BMS-986260. *Organic Process Research & Development* **2020**, *24* (7), 1310-1320. DOI: 10.1021/acs.oprd.0c00232.