ESIPT Based Promising Fluorescence Sensor for Cu\(^{2+}\) and CN\(^{-}\) Ions:
Investigation towards Logic Gate Behaviour, Anticancer Activities and Bioimaging Application

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Experimental section

**General methods.** All reagents practised for the synthesis work were purchase from sigma-Aldrich chemical. These reagents were stacked away under vacuum condition and were utilized without further refining. All the anions were taken in the form of sodium salts. Elemental analysis was performed by the help of CH 1760 E electrochemical workstation. IR spectra were taken with Perkin Elmer FT-IR 1000 spectrophotometer as films between KBr. The UV–Vis titration experiments were executed on UV-2450 (UV-vis spectrophotometer) double beam spectrophotometer with quartz cuvette featuring path length 1 cm. Fluorescence emission spectra were recorded using fluoromax-4 spectrofluorometer with quartz cuvette featuring path length 3 cm. \(^1\)H NMR and \(^{13}\)C spectra were taken on a Bruker DRX 400 MHz spectrophotometer by applying tetramethylsilane (TMS) as an internal standard.

**Cell culture.** HeLa (human cervical adenocarcinoma), MCF-7 (human breast adenocarcinoma), HepG2 (human hepatocellular carcinoma) and HEK-293 cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic mix (100 U/ml of penicillin and 100 µg/ml streptomycin) at 37 °C in CO\(_2\) incubator.

**Cell viability assay.** MTT assay was accomplished as portrayed previously (Mosmann, 1986). In brief, 5 X 10\(^3\) cells suspended in 200 ul of DMEM medium were plated in 96-well plates (Corning, NY, USA) and incubated under standard conditions. Dilutions of the test compounds were made in DMSO and added to the monolayer in triplicates. The final solvent concentration used for all dilutions (0.1%) was used as a vehicle control. Cultures were then assayed after 24 h by the addition of 20 µl of 5 mg/ml MTT and incubating it for another 4 h at 37 °C. The MTT containing medium was then aspirated, and 100 µl of DMSO was added to solubilize the formazan. The absorbances were determined on a FLUOstar optima.
microplate reader (BMG Labtech, Germany) at 570 nm. The IC\textsubscript{50} values were calculated by graph pad prism 6.0 software.

**Confocal microscopy.** 5 X10\textsuperscript{4} cells (MCF-7) were seeded on each cover slip placed in the well of 12 well plates and incubated for 24 hours. Thereafter cells were treated with probe 2, probe 2\textsuperscript{+}Cu\textsuperscript{2+} and 2\textsuperscript{+}Cu\textsuperscript{2+}+CN solution in 3 respective wells for 1 hour. After 1 hour incubation, the medium of each well was removed and the cells were further washed by PBS twice. Thereafter the cells were visualised under Confocal Laser Scanning Microscope (LSM 780, Carl Zeiss, Germany).

**Statistical analysis.** Data are specified as mean ± standard deviation (SD) of triplicate independent experiments and statistically evaluated using one way ANOVA and followed by Tukey post hoc test using Graph Pad Prism 6 (Graph Pad Software, San Diego, CA, USA). A p-value of less than 0.05 was considered to be statistically significant.

**Synthesis of probe 2 & 3**

Intermediate 1 was synthesized by the reported procedure. Intermediate 1 (5mM) in methanol solution was added to a R.B. flask and stirred at room temperature. After complete dissolution of 1, 3-formyl chromone (5mM) was added. Yellow coloured precipitate of 3-((E)-((2-((E)-((1H-pyrrol-2-yl)methylene)amino)phenyl)imino)methyl)-2-methoxy-2H chromen-4-ol (probe 2) was formed within 10 minutes and recrystallized using methanol.

Yield: 92%. Anal. Cal. (C\textsubscript{22}H\textsubscript{19}N\textsubscript{3}O\textsubscript{3}): C,70.76; H,5.13; N,11.25; O,12.85. Found: C,70.74; H,5.14; N,11.27; O,12.84. IR: 3467(-OH), 1647 (-C=N). \textsuperscript{1}H NMR (DMSO-\textsubscript{d}\textsubscript{6}, 400 MHz): 12.72 (d, 1H), 11.5 (s,1H), 8.55 (s, 1H), 8.23 (d, 1H), 7.91 (dd, 1H), 7.60 (d, 1H), 7.53 (t, 1H), 7.35 (dd, 1H), 7.29-7.23 (m, 2H), 7.16-7.09 (m, 2H), 6.91 (s, 1H), 6.32 (q,1H), 5.86 (s, 1H), 3.39 (s, 3H). \textsuperscript{13}C NMR (DMSO-\textsubscript{d}\textsubscript{6}, 400 MHz): 179.55, 155.34, 149.45, 143.68,138.72, 134.26,134.05, 131.07, 126.60, 125.78, 124.19,122.87, 121.93, 117.93, 117.59, 116.40, 113.49, 110.39, 103.74, 101.33, 101.31, 54.85.

To a solution of 1 (5mM) in ethanol 3-formyl chromone (5mM) was added and stirred for 1 H at room temperature. The solvent was reduced under pressure and yellow colored compound 3-((E)-((2-((E)-((1H-pyrrol-2-yl)methylene)amino)phenyl)imino)methyl)-2-ethoxy-2H chromen-4-ol (probe 3) formed and recrystallized by ethanol.

Yield: 87%. Anal. Cal. (C\textsubscript{23}H\textsubscript{21}N\textsubscript{3}O\textsubscript{3}): C,71.30; H,5.46; N,10.85; O,12.39. Found: C,71.29; H,5.46; N,11.10.86; O,12.40. IR: 3463(-OH), 1644 (-C=N). \textsuperscript{1}H NMR (DMSO-\textsubscript{d}\textsubscript{6}, 400 MHz): 11.06 (s,1H), 8.49 (s, 1H), 8.04 (d, 2H), 7.59 (d, 1H), 7.46 (t, 1H), 7.36 (d, 1H), 7.28 (d, 1H), 7.21 (s, 1H), 7.19 (t, 1H), 7.13 (t, 1H), 7.09 (t,1H), 7.02 (d, 1H), 6.73 (s, 1H), 6.34 (s, 1H),
5.85 (s, 1H), 3.91 (q, 1H), 3.73 (q, 1H), 3.49 (s, 1H), 1.21 (t, 3H). $^{13}$C NMR (DMSO-$d_6$, 400 MHz): 180.61, 161.88, 155.87, 146.50, 142.12, 138.82, 137.79, 135.16, 134.14, 133.34, 131.59, 131.34, 126.93, 126.40, 123.30, 120.07, 117.95, 117.07, 112.18, 110.61, 103.94, 100.87, 63.45, 15.20.

Scheme S1. Synthetic routes for intermediate 1 and molecular sensing probes 2 & 3.

Fig. S1 $^1$H-NMR of probe 2 in DMSO-$d_6$. 
Fig. S2  $\textsuperscript{13}$C-NMR of probe 2 in DMSO-$d_6$.

Fig. S3  $^1$H-NMR of probe 3 in CDCl$_3$. 
Fig. S4  
$^{13}$C-NMR of probe 3 in CDCl$_3$.

Fig. S5  
In situ selectivity studies of probe 2 towards various metal ions (Mn$^{3+}$, Pb$^{2+}$, Cr$^{3+}$, Zn$^{2+}$, Hg$^+$, Hg$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, Co$^{2+}$ & Na$^+$) (a) UV-vis spectral changes at 10 µM and (b) fluorescence changes at 1 µM (PBS buffer, pH 7.4, 1.0% DMSO).
Fig. S6  Binding stoichiometric study of probe 2 (10 µM) in aqueous medium (PBS buffer, pH 7.4, 1.0% DMSO) with Cu$^{2+}$ (a) Jobs plot & (b) Molar ratio plot.

Fig. S7 Fluorescence response of probe 2 (1 µM) in aqueous medium (PBS buffer, pH 7.4, 1.0% DMSO) in presence of various [Cu$^{2+}$] with an excitation at 410 nm and slit width is 1/1.
Fig. S8 Fluorescence response of in situ formed 2+Cu^{2+} complex (1 µM) in aqueous medium (PBS buffer, pH 7.4, 1.0% DMSO) in presence of various [CN⁻] with an excitation at 410 nm and slit width is 1/1 after 30 minutes.

Fig. S9 Cytotoxic assessment (a) morphological changes in HEK 293 cell lines after 24 h of exposure with (i) control (ii) probe 2 (iii) 2+ Cu^{2+} and (iv) probe 3 (v) 3 + Cu^{2+} complex. (images were taken by inverted phase contrast microscope at 200 X magnification. *p < 0.05) and MTT assay of HEK 293 cell lines (b) probe 2 & 2+ Cu^{2+} (c) probe 3 & 3 + Cu^{2+} complex.