Electronic Supplementary Information for

A Lysosome-Targeting and Polarity-Specific Fluorescent Probe for Cancer Diagnosis

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Experimental section

Materials and apparatus
Phosphorus oxychloride, 3-(dimethylamino)phenol, ethyl acetoac, 4-phenylmorpholine and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich company. LysoTracker Green DND-26 was commercially available from Invitrogen (USA). All other chemicals and solvents with spectroscopical pure were bought from Tianjin chemical reagent company.

NMR spectra were recorded on Varian NMR System 300 MHz (Mecury, Varian, Inc., USA) using tetramethylsilane (TMS) as the internal standard in the solvent deuterated deuterated chloroform (CDCl₃) with 300 MHz for ¹H NMR and 100 MHz for ¹³C NMR, respectively. HR-MS was measured by an Agilent Accurate-Mass-Q-TOF MS 6520 system equipped with an electrospray ionization (ESI) source (Agilent, USA). Deionized water was obtained with a Milli-Q water purification system (Millipore). The pH value was performed on a Beckman pH-3c digital meter (Shanghai LeiCi Device Works, Shanghai, China). The UV-visible spectra were taken on a TU-1901 double-beam UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., LTD, Beijing, China). Fluorescence spectra were carried out on a FLS-920 Edinburgh Fluorescence Spectrophotometer (Edinburgh Co., Ltd., England) equipped with a xenon discharge lamp using 1 ml Fluor Micro Cell. Live cells and tissue fluorescence images were taken on a confocal laser scanning microscope (Zeiss, LSM880, Germany) with red channel (Ex = 458 nm, Em = 560 - 650 nm) for CPM. In vivo images were then taken by using a Bruker small animal in vivo Xtreme imaging system, with an excitation filter of 470 nm and an emission filter of 600 nm.
Synthesis and characterization

Synthesis routine of CPM

Scheme S1 The synthetic routine of CPM

Synthesis of Compound 1. 7 mL phosphorus oxychloride (75 mmol) was added dropwise to the solution of 3-(dimethylamino)phenol (5.00 g, 36.5 mmol) in 15 mL dry DMF at 0 °C over 5 min, then the reaction mixture was stirred for 30 min at room temperature, followed for 1 h at 65 °C. The solution was poured into 70 mL ice water, and the product was extracted with ethyl acetate three times and saturated sodium chloride aqueous solution one time. The organic layer was dried over anhydrous magnesium sulfate filtered and evaporated, affording compound 1 as a white powder 4.00 g (66 % yield). The crude product compound 1 was used for the subsequent synthesis without further purification and characterization.

Synthesis of Compound 2. A mixture of 1.00 g (6.05 mmol) compound 1, 0.99 g (8.53 mmol) ethyl acetoac and 0.50 ml piperidine was refluxed in 12 mL absolute ethanol for 3h. The reaction was cooled to room temperature. Then the precipitated solid was concentrated under vacuum and washed with absolute ethanol, affording compound 2 as a yellow powder 1.30 g (92 % yield). $^1$H NMR (CDCl$_3$, 300 MHz) δ (ppm): 8.44 (s, 1H, Ar-H), 7.42-7.24 (d, 1H, Ar-H), 6.65-6.61 (m, 1H, Ar-H), 6.46-6.45 (m, 1H, Ar-H), 3.11 (s, 6H, -CH$_3$), 2.60 (s, 3H, -CH$_3$).

Synthesis of Compound 3. 3.4 mL phosphorus oxychloride (36 mmol) was added
dropwise to the solution of 4-phenylmorpholine (3.00 g, 18.4 mmol) in 10 mL dry DMF at 0 °C over 5 min, then the reaction mixture was stirred for 30 min at room temperature, followed for 1 h at 65 °C. The solution was poured into 40 mL ice water, and the product was extracted with ethyl acetate three times and saturated sodium chloride aqueous solution one time. The organic layer was dried over anhydrous magnesium sulfate filtered and evaporated. The residue was purified by chromatography on a silica gel column using ethyl acetate/hexane (5/1, V/V) as the eluent, affording compound 3 as a white powder (2.80 g, 80 % yield). ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 9.80 (s, 1H, -CHO), 7.78-7.75 (d, 2H, Ar-H), 6.94-6.91 (d, 2H, Ar-H), 3.87-3.84 (m, 4H, -CH₂-), 3.36-3.33 (m, 4H, -CH₂-).

Synthesis of CPM. A mixture of 1.00 g (4.32 mmol) compound 2, 0.83 g (4.44 mmol) compound 3 and 0.53 ml piperidine are stirred in 15 ml absolute ethanol at room temperature for 10 min, followed at 65 °C over night. Then the reaction was cooled to room temperature, and the precipitated solid was collected by filtration. Then the red power (1.50 g, 3.71 mmol) was obtained after the precipitate washed with absolute ethanol and dried. Yield: 86 %. ¹H NMR (CDCl₃, 600 MHz) δ (ppm): 8.57 (s, 1H, Ar-H), 8.05-8.00 (d, 1H, Ar-H), 7.83-7.78 (d, 1H, Ar-H), 7.65-7.62 (m, 3H, Ar-H), 6.67-6.64 (m, 3H, Ar-H), 6.51-6.50 (m, 1H, Ar-H), 3.93-390 (m, 4H, -CH₂-), 3.31-3.29 (m, 4H, -CH₂-), 3.13 (s, 3H, -CH₃). ¹³C NMR (CDCl₃, 150 MHz) δ (ppm): 186.36, 160.87, 158.17, 154.71, 152.60, 148.56, 143.82, 131.38, 130.48, 126.46, 121.52, 114.57, 109.94, 108.99, 97.10, 66.70, 48.06, 40.30. HR-MS m/z: [M+H]⁺ calculated for C₂₄H₂₄N₂O₄⁺, 405.1809; measured, 405.1805.

UV-vis and fluorescence measurements

A stock solution of CPM (1.0 mM) was prepared in DMSO. 10 μM of CPM was used in spectroscopic determination by addition of 20 μL stock solution to 2.0 mL different polarity solvents. Excitation and emission bandwidths were both set at 2.0 nm, and the excitation wavelength was fixed at 465 nm. All spectroscopic experiments were measured at room temperature. The relative fluorescence quantum yield of CPM in different polarity solvents was determined with rhodamine B (Φᵣ =
0.89 in ethanol) as a fluorescence standard.

**Cell culture and cell cytotoxicity assay**

All the cells lines were kindly provided by Modern Research Center for Tradition Chinese (Shanxi University, Taiyuan, China). SMMC-7721, A549, HIC, HeLa, LO2, BEAS-2B and HIEC cells were cultured in RPMI1640 or DEME medium supplemented with 10 % fetal bovine serum and 1% antibiotics at 37 °C in a 5 % CO₂ atmosphere. The cell cytotoxicity of CPM to living SMMC-7721 cells was performed by a standard CCK-8 assay (cell counting kit-8). About $1 \times 10^4$ cells/well in 200 μL cell culture medium were seeded in 96-well microplate and then the medium was replaced with fresh medium that containing CPM with various concentrations of 0 μM, 5 μM, 10 μM, 15 μM and 20 μM for 24 h, respectively. After washing the cells with fresh medium three times, 20 μL CCK-8 in 180 μL PBS was loaded to each well for another 4 h. Then each well was analysed with an ELISA microplate reader and the absorbance was detected at 450 nm. The cell viability was expressed as relative to the control cells taken as 100 % metabolic activity.

**Cell imaging and colocalization experiments**

For live cell imaging, CPM was added to the cultured cells in a glass-bottomed dish for 10 min and washed with cold phosphate buffered saline (10 mM PBS, pH 7.4) three times. After replacement of the medium, cells were imaged using a confocal laser scanning microscope (Zeiss, LSM880) with red channel (Ex = 458 nm, Em = 560 - 650 nm), and a 63 × oil-immersion objective lens. For colocalization experiments, SMMC-7721 cells were plated on glass-bottomed dishes at a density of $1\times10^5$ for 24 h. CPM dissolved in DMSO (10 μL, 1 mM) were added to the cells medium (1 mL) at 5 μM or 3 μM final concentration for 10 min, then cells were washed with PBS (pH 7.4) three times. 1.0 μM LysoTracker Green DND-26, 0.2 μM MitoTracker Green or 1.0 μM ER-Tracker Green was then added and co-incubated for additional 30 min, respectively. After washed with PBS (pH 7.4) three times, fluorescence images of the cells were carried out on a confocal laser scanning microscope (Zeiss, LSM880) with a 63 × oil-immersion objective lens, and red
channel (Ex = 458 nm, Em = 560 - 650 nm) for CPM, Green channel (Ex = 488 nm, 500 – 550 nm) for Lyso Tracker DND-26, MitoTracker Green and ER-Tracker Green, respectively.

**Preparation of living organs, tumor-bearing mice and in vivo imaging**

KM mice (18-20 g) were purchased from Laboratory Animal Center of Shanxi Cancer Hospital (Taiyuan, China) for in vivo imaging. All animal experiments were performed in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document no. 55, 2001) and approved by the Animal Care and Use Committee of Shanxi University. We have taken great efforts to reduce the number of animal used in these studies and also taken effort to reduce animal suffering from pain and discomfort.

Tumor-bearing mice were prepared by subcutaneous injection of HeLa cells into the left axillae of mice over 10 days. For living organs imaging, the organs (heart, spleen, liver and kidney) and tumor were isolated from the mice. After washing by PBS (pH=7.4) for three times, these isolated organs and tumor were loaded with CPM (15 μM, 20 min), respectively, and finally subjected to in vivo imaging.

For living mice imaging, CPM (100 μL, 15 μM) was hypodermic injected into the tumour position of the tumor-bearing mice and the corresponding position of the normal mice, respectively. Before imaging, the mice were first anesthetized by an intraperitoneal injection of 4 % chloral hydrate (200 μL) for 5 min. Then, in vivo imaging were taken by using a Bruker small animal in vivo Xtreme imaging system, with an excitation filter of 470 nm and an emission filter of 600 nm.

**Preparation of human normal tissue, human cancer tissue cryosections and fluorescence imaging**

The harvested surgical specimens of patients, including human normal tissues (breast and thyroid) and their corresponding human cancer tissues (breast carcinoma in situ and thyroid microcarcinoma), were obtained from Taiyuan Central Hospital. Informed consent was obtained for any experimentation with human subjects. All the human tissues slices harvested from surgical specimens of patients and determined by doctors,
were cryosectioned as 5 μm thicknesses. The slices were incubated with CPM (5 μM) in PBS for 10 min, and then washed with PBS three times. The fluorescence images are carried out on a confocal laser scanning microscope (Zeiss, LSM880) with red channel (Ex = 458 nm, Em = 560 - 650 nm), and a 20 × water-immersion objective lens.

![Absorption spectra of CPM (10 μM) in different H₂O/THF solvent mixtures (water from 0% to 70%).](image)

**Fig. S1** Absorption spectra of CPM (10 μM) in different H₂O/THF solvent mixtures (water from 0% to 70%).

**Table S1** Spectral properties of CPM in various solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric Constant (ε)</th>
<th>Refractive Index (n)</th>
<th>Δf</th>
<th>λ_{abs} (nm)</th>
<th>λ_{em} (nm)</th>
<th>Δλ (nm)</th>
<th>Extinction Coefficient (10^4 M⁻¹ cm⁻¹)</th>
<th>Φ_f (%)</th>
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<tbody>
<tr>
<td>Toluene</td>
<td>2.4</td>
<td>1.496</td>
<td>0.0153</td>
<td>453</td>
<td>550</td>
<td>97</td>
<td>4.046</td>
<td>25.09</td>
</tr>
<tr>
<td>Dioxane</td>
<td>2.21</td>
<td>1.4224</td>
<td>0.0205</td>
<td>450</td>
<td>565</td>
<td>115</td>
<td>3.597</td>
<td>10.53</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>6.1</td>
<td>1.372</td>
<td>0.2012</td>
<td>459</td>
<td>602</td>
<td>143</td>
<td>2.506</td>
<td>9.85</td>
</tr>
<tr>
<td>THF</td>
<td>7.5</td>
<td>1.407</td>
<td>0.2087</td>
<td>454</td>
<td>605</td>
<td>151</td>
<td>4.381</td>
<td>8.46</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>25.9</td>
<td>1.5289</td>
<td>0.2356</td>
<td>450</td>
<td>605</td>
<td>155</td>
<td>3.578</td>
<td>6.25</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>37.5</td>
<td>1.346</td>
<td>0.3055</td>
<td>473</td>
<td>608</td>
<td>135</td>
<td>3.123</td>
<td>2.25</td>
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<tr>
<td>Methanol</td>
<td>33.6</td>
<td>1.326</td>
<td>0.3101</td>
<td>458</td>
<td>550</td>
<td>152</td>
<td>3.428</td>
<td>1.28</td>
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<tr>
<td>Water</td>
<td>80.1</td>
<td>1.3333</td>
<td>0.3212</td>
<td>417</td>
<td>565</td>
<td>195</td>
<td>0.995</td>
<td>0.73</td>
</tr>
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</table>
The dielectric constant data of the solvents were measured at 25 °C. Solvent-dependent spectra are often interpreted in terms of the Lippert-Mataga equation.

\[
\begin{align*}
    f (\varepsilon) &= (\varepsilon - 1) / (2\varepsilon + 1) \\
    f (n^2) &= (n^2 - 1) / (2n^2 + 1) \\
    \Delta f &= f (\varepsilon) - f (n^2)
\end{align*}
\]

Where \( \varepsilon \) and \( n \) are the dielectric constant and the refractive indices of the solvent, respectively.

**Fig. S2** (a) The 2D scatter fitting curve of fluorescence intensity of CPM versus dielectric constant in different solvents in Table S1, with a low relationship coefficient of \( R^2 = 0.58153 \). (b) The 2D scatter fitting curve of fluorescence intensity of CPM versus dielectric constant in different solvents in Table S1 (except for toluene), with a high relationship coefficient of \( R^2 = 0.90283 \).

As shown in **Table S1**, although the dielectric constant of toluene (2.4) is larger than that of dioxane (2.21), according to the Lippert-Mataga equation, the polarity parameter (\( \Delta f \)) of toluene (0.0153) is smaller than that of dioxane (0.0205), due to its higher refractive index (\( n = 1.496 \)) than that of dioxane (\( n = 1.4224 \)). In a lower polarity media, CPM would exhibit less charge separation and weaker interaction with solvents, leading to a stronger and shorter fluorescence emission.\(^1\) In contrast, when the environment polarity increased, CPM would perform larger charge separation and the excited state energy can be dissipated due to the dipole-dipole interaction between the probe and solvent. As a result, the energy was rapidly consumed through nonradiative relaxation, leading to a weaker fluorescence and
longer emission wavelength. In our experiment, when the solvent polarity decreases from water (0.3212) to toluene (0.0153), the fluorescence quantum yield of CPM increases from 0.73 to 25.09 (Table S1 and Fig. S4 in revised SI). So the fluorescence quantum yield of CPM in toluene (25.09) is much higher than that of dioxane (10.53), making toluene is the outlier points.

**Fig. S3** Absorption spectra of CPM (10 μM) in different solvents.

**Fig. S4** Fluorescence emission spectra of CPM (10 μM) in different solvents ($\lambda_{ex} = 465$ nm).
Fig. S5 HOMO and LUMO of CPM by DFT calculations at the base level of B3LYP/6-31G via Gaussian 09 program. (A) Ground State; (B) Excited State. The highest occupied molecular orbital (HOMO) of CPM is localized from the donor (D) morpholine part onto the phenylethylene linker (C=C), whereas the lowest unoccupied molecular orbital (LUMO) is localized on the acceptor coumarin group (A). CPM might undergo a substantial ICT process from morpholine to the coumarin group displaying a typical D-π-A structural feature, which is in accord with our design strategy.

Fig. S6 Linearity of quantum yield of CPM (10 μM) versus the solvent parameter Δf in different solvents.
Fig. S7 The fluorescence intensity of CPM (10 μM) to various relevant analytes in phosphate buffer (pH 7.4). 1. Blank; 2, Ca^{2+}; 3, Na^{+}; 4, K^{+}; 5, Cu^{2+}; 6, Fe^{2+}; 7, Fe^{3+}; 8, Pb^{2+}; 9, Ba^{2+}; 10, Ag^{+}; 11, Cl^{-}; 12, NO_{3}^{-}; 13, SO_{3}^{2-}; 14, ClO^{-}; 15, ClO_{4}^{-}; 16, ONOO^{-}; 17, H_{2}O_{2}; 18, Arg; 19, Asn; 20, Asp; 21, Cys; 22, Thr; 23, Ser; 24, Gln; 25, Glu; 26, His; 27, Tyr; 28, GSH.

Fig. S8 The fluorescence spectra of CPM (10 μM) in methanol-glycerol system under different viscosity. THF and methanol have almost the same viscosity (0.53 cP vs 0.60 cP) but different polarity (0.21 vs 0.31). The fluorescence intensity of CPM displayed huge difference in them. The fluorescence intensity changed little with increasing viscosity from 0.60 cP to about 100 cP.
Fig. S9 The fluorescence spectra of CPM (10 μM) under different water contents (10% and 100%) in H₂O/THF solvent mixtures with different pH values (5.0 and 7.4). \( \lambda_{\text{ex}} = 465 \, \text{nm} \).

Fig. S10 a) The fluorescence spectra of CPM (10 μM) under different water contents (50% and 100%) in H₂O/THF solvent mixtures with different pH values (from pH 2.0 to pH 10.0). b) Changes in the fluorescence intensity of CPM under different water contents (50% and 100%) in H₂O/THF solvent mixtures with different pH values (from pH 2.0 to pH 10.0). \( \lambda_{\text{ex}} = 465 \, \text{nm} \).
**Fig. S11** Cell viability of CPM on SMMC-7721 cells by a standard CCK-8 assay. 1, control; 2, 5 μM; 3, 10 μM; 4, 15 μM; 5, 20 μM. Data are expressed as mean values ± standard error of the mean of three independent experiments, each performed in three triplicate.

**Fig. S12** Fluorescence images of 5 μM CPM (a, e), images of co-labeled with 0.2 μM MitoTracker Green (b), 1.0 μM ER-Tracker Green (f), merged images (c, g) in SMMC-7721 cells. The Pearson's co-localization correlations of CPM with MitoTracker Green (d, -0.16) and ER-Tracker Green (h, 0.05), respectively. The red channel image was collected at 560 - 650 nm (λ_ex = 458 nm) for CPM. The green channel image was collected at 496 - 536 nm (λ_ex = 488 nm) for MitoTracker Green, and ER-Tracker Green. Scale bar: 5 μm.
**Fig. S13** Time-dependent fluorescence images of CPM in SMMC-7721 cells mediated by DMSO. Control cells with only CPM (3 μM) treated for 10 min (a), followed by treating with DMSO (10 μL) (b-e). The dark-field transmission images (f-j). The red channel images were collected at 560 - 650 nm ($\lambda_{ex} = 458$ nm). Scale bar: 20 μm.

**Fig. S14** Fluorescence images of CPM induced by LPS in live SMMC-7721 cells. (a) Control cells with only CPM (3 μM) incubated for 10 min. SMMC-7721 cells were mediated by LPS (50 μg/mL) for 30 min (b), 60 min (c), or 120 min (d), subsequently incubated with CPM (3 μM) for 10 min. (e-h) The bright-field cells images. The red channel images were collected at 560 - 650 nm ($\lambda_{ex} = 458$ nm). Scale bar: 20 μm.
Fig. S15 (a) Fluorescence images of CPM in five types of cancer cells (SMMC-7721, A549, HIC, HeLa and B16F10 cells) and three types of normal cells (LO2, BEAS-2B and HIEC cells) during 20 min. The cells were incubated with 5 μM CPM for 10 min at 37 °C and then washed with RPMI1640 or DEME medium at pH 7.4. (b) Mean fluorescence intensities of CPM-stained live cells. Error bars represent mean deviation (± S. D.), n = 5. The red channel images were collected at 560 - 650 nm (λ<sub>ex</sub> = 458 nm). Scale bar: 10 μm.
References
$^1$H NMR and $^{13}$C NMR spectra and HR-MS analysis of compound 2-3 and CPM
<table>
<thead>
<tr>
<th>Sample</th>
<th>Formula (M)</th>
<th>Ion Formula</th>
<th>Measured m/z</th>
<th>Calc m/z</th>
<th>Diff (ppm)</th>
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<tbody>
<tr>
<td>CPM</td>
<td>C₂₄H₂₄N₂O₄</td>
<td>[M+H]^+</td>
<td>405.1805</td>
<td>405.1809</td>
<td>-0.99</td>
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