Electronic Supplementary Information

Monoamine Oxidase-A Targeting Probe for Prostate Cancer Imaging and Inhibition of Metastasis

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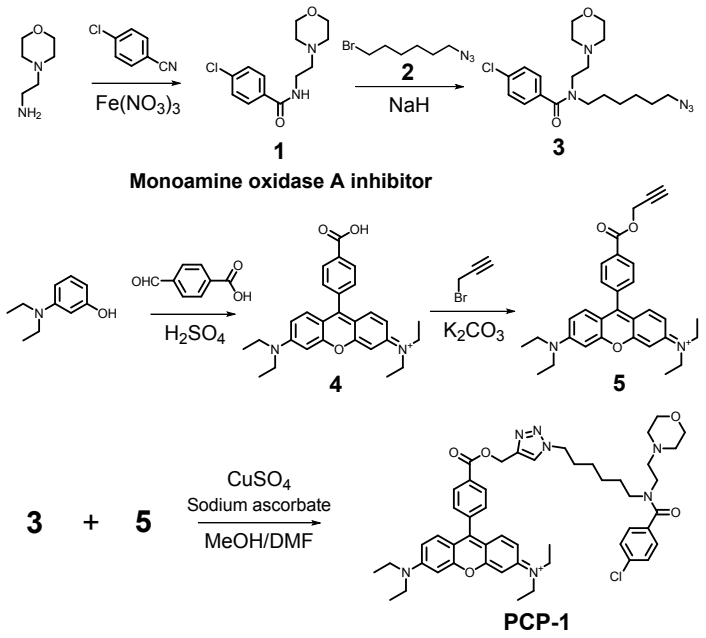
1. Experimental Section

1.1 Materials and Methods

Unless otherwise noted, all the materials used for the synthesis were purchased from the commercial suppliers (Sigma-Aldrich, Alfa, Samchun) and were used as received without further purification. All the reactions were carried out under the nitrogen atmosphere. All procedures for work-up and purification were carried out with reagent-grade solvents under ambient atmosphere. Column chromatography was performed with silica gel 60 (Merck, 0.063~0.2 mm) as a stationary phase. Analytical thin-layer chromatography (TLC) was performed using Merk 60 F254 silica gel (pre-coated sheets, 0.25 mm thick). ^{1}H and ^{13}C NMR spectra were collected in NMR solvents ($CDCl_3$) on a Bruker 500 MHz spectrometer. All chemical shifts are reported in ppm values using the peak of TMS as an internal reference. NMR data are reported as follows: chemical shifts, multiplicity (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, q: quartet, m: multiplet, br: broad signal), and coupling constants (Hz). The ESI mass spectra were recorded using a Shimadzu LC/MS-2020 Series instrument. UV-Vis spectra were recorded on a Scinco S-3100 spectrometer, and fluorescence spectra were obtained using a Shimadzu RF-5301PC instrument. Stock solutions of probe **PCP-1** were prepared in DMSO. All excitation and emission slit widths were set at 5 nm. The concentration of each of the samples was fixed at 10 μM in a total volume of 3 mL.

1.2 Synthesis



Scheme S1. Synthetic route for probe **PCP-1**.

Compound 1: Iron(III) nitrate nonahydrate (10 mol%) was added to the mixture of 4-chlorobenzonitrile (1.0 equiv, 1.10 g, 8.0 mmol) and 4-(2-aminoethyl)morpholine (8.0 equiv, 8.40 mL, 64.0 mmol). The reactor was sealed, and reaction mixture was allowed to stir at room temperature for 10 min before being heated (125 °C) for 24 h. The resulting reaction mixture was directly passed through silica column chromatography using (DCM:MeOH=98:2) as eluent to afford product (**1**) as a dark brown solid (1.6 g, 73%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72 (d, *J* = 8.65 Hz, 2H), 7.42 (d, *J* = 8.4 Hz, 2H), 3.73 (t, *J* = 4.6 Hz, 4H), 3.55 (dd, *J* = 11.0, 5.4 Hz, 2H), 2.61 (t, *J* = 6.0 Hz, 2H), 2.51 (t, *J* = 3.8 Hz, 4H) ppm.

Compound 2: To a solution of 1,6-dibromohexane (1.5 equiv, 6.92 mL, 45.0 mmol) in DMF at 55 °C, was added sodium azide (1.0 equiv, 1.95 g, 30.0 mmol) in portions during 25 min. The mixture was stirred overnight at 60 °C. The solvent was then removed under *vacuo* before diethyl ether (45 mL) and NaOH 1M (50 mL) were added. The aqueous layer was extracted with diethyl ether (2 x 45 mL); then, the organic layers were combined, dried over Na_2SO_4 and concentrated. The crude was purified by column chromatography on a silica gel column (Petroleum ether:Et_2O=100:0 then 97:3) to give the product (3.6 g, 58%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.41 (t, J = 6.8 Hz, 2H), 3.28 (t, J= 6.9 Hz, 2H), 1.87 (q, J = 6.9 Hz, 2H), 1.62 (q, J = 7.5 Hz, 2H), 1.49 (m, 2H), 1.41 (m, 2H) ppm.

Compound 3: Compound **1** (1.0 equiv, 580 mg, 2.15 mmol) was dissolved in DMF (20 mL), and the mixture was cooled to 0 °C with an ice bath. NaH (1.1 eqiv, 57.0 mg, 2.4 mmol) was added in small portions. The reaction mixture was warmed to RT and stirred for an additional 20 min. Thereafter, compound **2** (1.5 equiv, 667 mg, 3.2 mmol) was added dropwise. The reaction mixture was stirred at RT for 2 hours. Finally, reaction was quenched by the addition of water, extracted with ethyl acetate, washed with brine and dried over Na_2SO_4. The organic layer was filtered, concentrated and purified by column chromatography on silica gel (DCM:MeOH=98:2) to give the product (275 mg, 32%). ^{1}H NMR (500 MHz, MeOD): δ 7.47 (d, J = 8.35 Hz, 2H), 7.39 (m, 2H), 3.69 (m, 4H), 3.56 (m, 2H), 3.40 (m, 2H), 3.21 (m, 2H), 2.57 (m, 4H), 2.27 (m, 2H), 1.70 (m, 2H), 1.60 (m, 2H), 1.48 (m, 2H), 1.20 (m, 2H) ppm.

Compound 4: 4-Carboxybenzaldehyde (1.0 equiv, 1.0 g, 6.7 mmol) was added to 20 mL concentrated H_2SO_4.To this mixture, 3-diethylaminophenol (2.0 equiv, 2.2 g, 13.0 mmol) was added slowly. The reaction mixture was refluxed at 90 °C for 24 h. After which the reaction

was cooled to RT and quenched with crushed ice. A pH of the aqueous phase was neutralized with 1 N NaOH solution followed by extraction using ethyl acetate (50 mL x 3). The organic phase was washed with brine and dried over anhydrous Na_2SO_4. The organic solvent was evaporated and purified using silica gel column chromatography (DCM:EtOH=80:20) to yield dark purple solid (756 mg, 26%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.42 (d, J = 7.9 Hz, 2H), 7.38 (q, J = 0.25 Hz, 2H), 7.32 (d, J = 14.25 Hz, 2H), 6.86 (d, J = 10.0 Hz, 2H), 6.83 (m, 2H), 3.62 (q, J = 5.0 Hz, 8H), 1.33 (t, J = 5.0 Hz, 12H) ppm.

Compound 5: To a solution of compound **4** (1.0 equiv, 340 mg, 0.8 mmol) in anhydrous ACN (10 mL) was added potassium carbonate (2.0 equiv, 212 mg, 1.5 mmol) and propargyl bromide (1.5 equiv, 129 uL, 1.2 mmol). The mixture was stirred overnight at 80 °C. A saturated solution of ammonium chloride was added, and the mixture was extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column choromatography (DCM:EtOH=80:20) to yield product (310 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.32 (d, J = 8.45 Hz, 2H), 7.51 (d, J = 8.45 Hz, 2H), 7.23 (d, J = 9.55 Hz, 2H), 6.96 (d, J = 2.50 Hz, 2H), 6.91 (dd, J = 9.50, 2.45 Hz, 2H), 5.02 (d, J = 2.45 Hz, 2H), 3.67 (q, J = 7.25 Hz, 8H), 2.58 (t, J = 2.55 Hz, 1H), 1.34 (t, J = 7.15 Hz, 12H) ppm.

Probe **PCP-1**. A mixture of compound **3** (1.0 equiv, 445 mg, 1.1 mmol), **5** (1.0 equiv, 527 mg, 1.1 mmol), and sodium ascorbate (10 mol%) in DMF:MeOH (2:1 mL, degassed) was stirred for 15 min at RT. Then 5 mol% of $CuSO_4$ in 0.5 mL water was added to the reaction mixture, which was stirred for additional 6 h. After removal of the solvents under reduced pressure, the crude mixture was purified by column chromatography (DCM:EtOH=80:20) to yield product (786 mg, 79%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.31 (d, J = 8.2 Hz, 2H), 7.99 (s, 1H), 7.48 (d, J = 8.15 Hz, 2H), 7.37 (m, 2H), 7.35 (m, 2H), 7.26 (d, J = 9.55 Hz, 2H), 6.95 (dd, J = 9.7, 2.3 Hz, 2H), 6.84 (d, J = 2.1 Hz, 2H), 5.55 (s, 2H), 3.65 (q, J = 7.15 Hz, 8H), 3.47 (m, 2H), 3.34 (m, 2H), 3.24 (m, 2H), 2.59 (m, 4H), 2.42 (m, 2H), 2.27 (m, 4H), 2.03 (m, 2H), 1.88 (m, 2H), 1.66 (m, 2H), 1.50 (m, 2H), 1.33 (t, J = 7.05 Hz, 12H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.87, 165.44, 157.90, 155.83, 155.62, 155.12, 142.41, 136.44, 135.29, 131.74, 130.36, 129.58, 128.70, 128.17, 124.26, 114.56, 113.02, 96.58, 66.56, 65.85, 58.88, 53.45, 50.29, 46.25, 44.36, 30.11, 29.69, 28.43, 26.11, 22.69, 15.28, 12.69 ppm. ESI-MS: m/z calcd for $C_{50}H_{61}ClN_7O_5^+$ [M^+], 874.44; found 874.40.

1.3 Docking and Molecular Dynamics Simulations

The hMAO-A structure was obtained from Protein Data Bank (PDB ID: 2Z5X[1]). The spacing of 0.2 Å was set in grid box. Docking parameters for each cycle were set as 50 runs and 2,500,000 energy evaluations in Autodock 4.2 and AutodockTools 1.5.6[2] using the Lamarckian genetic algorithm. Finally, the 20 structures with lowest binding energy were obtained for further molecular dynamics (MD) simulations.

The force field of probe was obtained by DFT calculations using M06 exchange function with 6-31+G(d) basis sets. In MD simulations, the ff14SB and Tip3p water model were employed to perform 20 independent 30 ns MD simulations in Amber14 package.[3] SHAKE algorithm was applied to constrain bonds including hydrogen. Particle-mesh Ewald summation method was used to describe long-range electrostatics. The value of nonbonded cutoff was set as 12 Å. The distance between system and box edge was more than 12 Å. Temperature was controlled by Langevin thermostat with coupling constant of 1.0 ps. The pressure was controlled by Berendsen barostat with coupling constant of 2.0 ps. The time step was 1.0 ps. After equilibration evaluation, only last 10 ns equilibrated trajectories were used for binding free energy calculation via the molecular mechanics generalized-Born surface area (MM/GBSA) method.

1.4 Two-Photon Absorption Calculation

During calculation, all the molecular structures were optimized by Gaussian09[4] program in B3LYP level with 6-31+G(d) basis set and TPA calculations were performed by GAMESS[5] program in B3LYP level with 6-31G(d) basis set. In the present study, coumarin 307 and Fluorescein molecule were selected as reference dyes.[6]

1.5 Cell Culture

Human cancer cell lines (LNCap; prostate adenocarcinoma, MDA-MB-231; breast adenocarcinoma, HepG2; hepatocellular carcinoma and A549; lung carcinoma) were purchased from Korea Cell Line Bank (Seoul, South Korea). The cells were maintained in Minimum Essential Media or RPMI 1640 medium supplemented with 10% FBS (Hyclone, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

1.6 Cell Viability Assay

Approximately 1.5×10^4 cells were seeded on a 96 well microplate (SPL Life Science, South Korea) and incubated for overnight. After stabilization of cells, the cells were treated with either DMSO as a control or probe **PCP-1** for 24 h. To analyze the cell viability of cells in the presence and absence of **PCP-1**, the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega. USA) was used following the manufacturer's instructions. A SPECTRA MAX GEMINI EM microplate reader (Molecular Devices, USA) was used to measure the absorbance level. The wavelength was set at 490 nm. Cell viability assays were performed in triplicate, and the viability (%) was expressed as a percentage of measured absorbance relative to the control cells.

1.7 Two-photon Imaging

Cells (2×10^5 cells) were seeded on 35-mm glass-bottom confocal dishes (SPL Life Science) and allowed to stabilize for 24 h. When the cells reached confluency of 70 %, the cells were treated with probe **PCP-1** (10 μM in DMSO) at 37 °C for 1 h. Then, the cells were washed with PBS 2 times. Fluorescence microscopy images of probe-treated cells were obtained with an intravital multiphoton laser scanning microscope (IMP-LSM; SLM 780 NLO, Carl-Zeiss, Germany). The excitation wavelength of probe **PCP-1** was 830 nm while emission was collected at 500 - 700 nm. To obtain two-photon fluorescence images, internal PMTs were used to obtain the signals in 8 bit unsigned 512 × 512 pixels at a 400 Hz scan speed.

1.8 One-photon Imaging

Cells (2×10^5 cells) were seeded on 35-mm glass bottom confocal dishes (SPL Life Science) and allowed to stabilize for 24 h. When the cells reached confluency of 70 %, the cells were treated with probe **PCP-1** (10 μM in DMSO) at 37 °C for 1 h. The sub-organelles of cells were stained using 200 nM MitoTracker™ Green FM, 500 nM LysoTracker™ Green DND-26 and 500 nM ER-Tracker™ Green (BODIPY™ FL GLibenclamide) (Invitrogen, USA) for 30 min at 37°C. Then, the cells were washed with PBS 3 times. Fluorescent images were obtained using a confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc., Germany).

1.9 Probe Retention Assay

Cells were seeded in 96 well microplates. After incubated for overnight, the cells were treated with absence or presence of probe **PCP-1** (10 μM in DMSO) in each well for 48 h. After time-dependent manner incubation, fluorescence intensities were obtained using a Multi-Detection Microplate Reader system (HIDEX). The fluorescence channel was excited at 560 nm and the emission was collected by 600 nm band-pass filter.

1.10 Mitochondrial Membrane Potential (MMP) Assay

To assess the mitochondrial membrane potential, cells (5×10^5 cells) were seeded in 60 mm dishes and treated with 10 μM **PCP-1** for 24 h. After incubation, the cells were stained with 1 ml of a PBS solution containing 10 μg/ml JC-1 (Invitrogen) for 30 min at 37 °C with 5% CO_2. Supernatants were then collected and analyzed by FACS Carliber™ (BD, USA) using JC-1 according to the manufacturer's instructions.

1.11 Immunoblot Analysis

Immunoblot analysis was performed using antibodies specific for MAO-A (sc-271123), BAX (sc-7480), Caspase 3 (#9662), GAPDH (sc-47724) and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (sc-2357). They were purchased from Santa Cruz Biotechnology (Texas, USA) and Cell Signaling Technology (Massachusetts, USA). Briefly, cells were treated with probe **PCP-1** for 24 h. After incubations, cells were washed twice in ice-cold PBS and lysed in a radioimmunoprecipitation assay buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 % sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, and protease inhibitor cocktail (GeneDePot, South Korea). After lysed, the lysate was centrifuged, and the supernatant was recovered and loaded onto a 10% or 15% SDS-polyacrylamide gel for electrophoresis. To detect immunoreactive protein bands, enhanced chemiluminescence reagents (Merk Millipore, USA) were used according to the manufacturer's instructions.

1.12 Cell Migration Assay

Cell migration assay was performed using an IBIDI culture insert (ibidi GmbH, Munich, Germany) consists of two reservoirs separated by a 500 μm thick wall created by a culture insert in a 35 mm confocal dish. Cells were starved to inactivate cell proliferation and then an equal number of cells (3×10^4 cells/100 μl) were added into the two reservoirs of the same insert

and incubated at 37°C/5% CO_2. After 12 h, the insert was gently removed creating a gap of ~500 μm. The cells were treated with 10 μM **PCP-1** for 24 h. After incubation, the wounds were observed using JuLI^{TM} Stage microscopy and multiple images were taken at areas flanking the intersections of the wound.

2. NMR and ESI-MS spectra of PCP-1

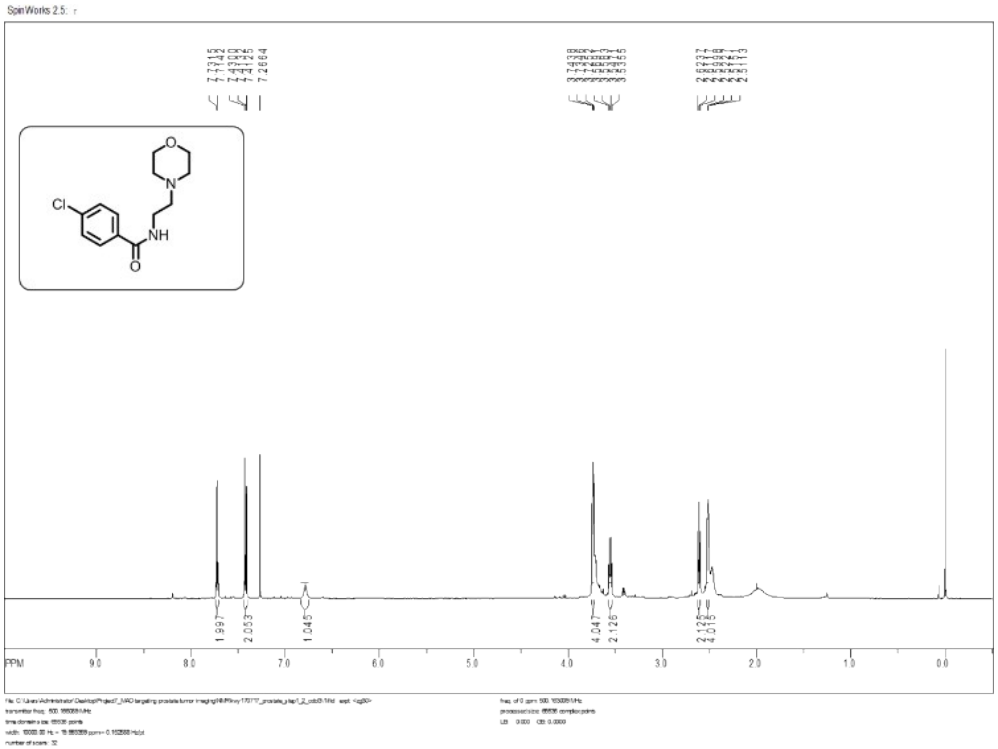


Fig. S1. ^{1}H NMR spectra (500 MHz) of **1** in $CDCl_3$.

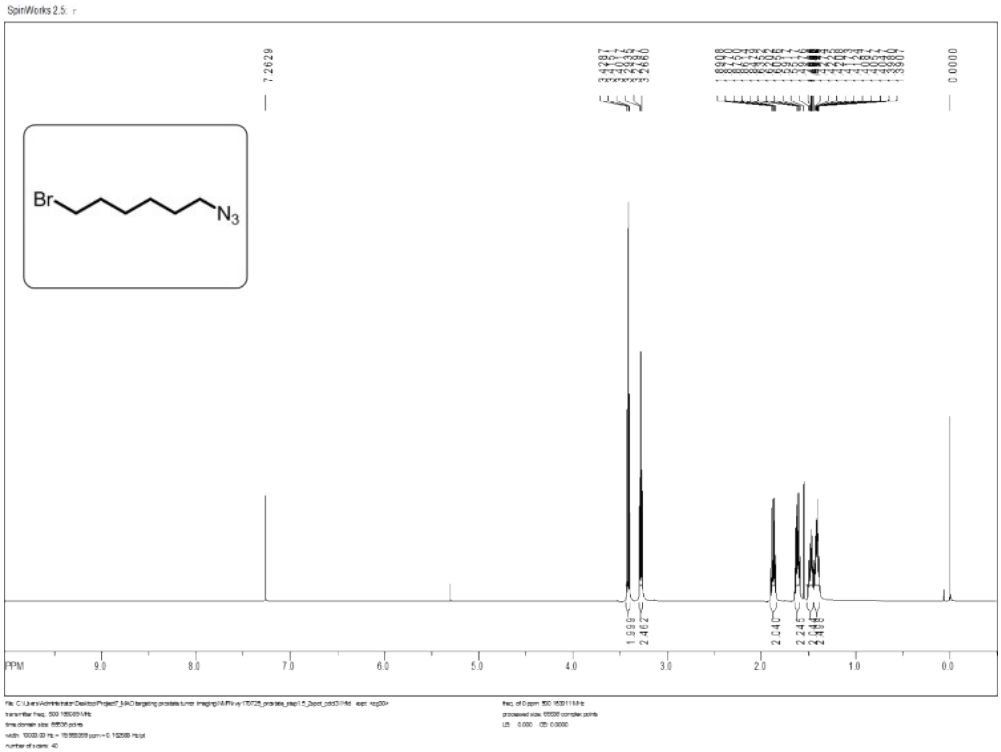


Fig. S2. ^{1}H NMR spectra (500 MHz) of **2** in $CDCl_3$.

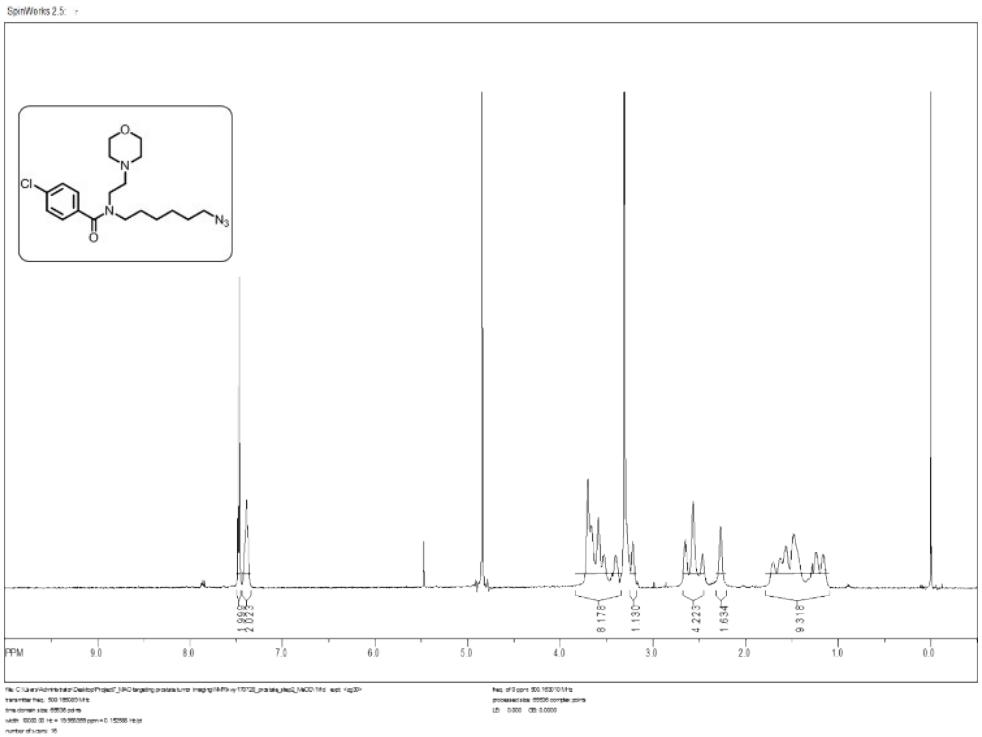


Fig. S3. ^{1}H NMR spectra (500 MHz) of **3** in MeOH-d_4.

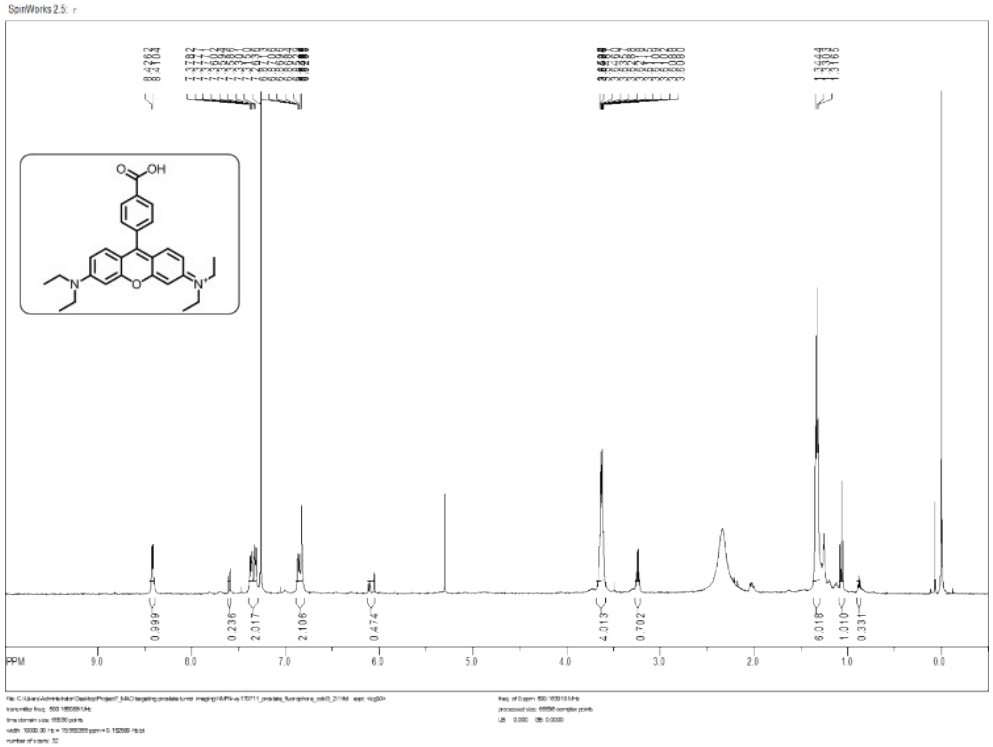


Fig. S4. ^{1}H NMR spectra (500 MHz) of **4** in $CDCl_3$.

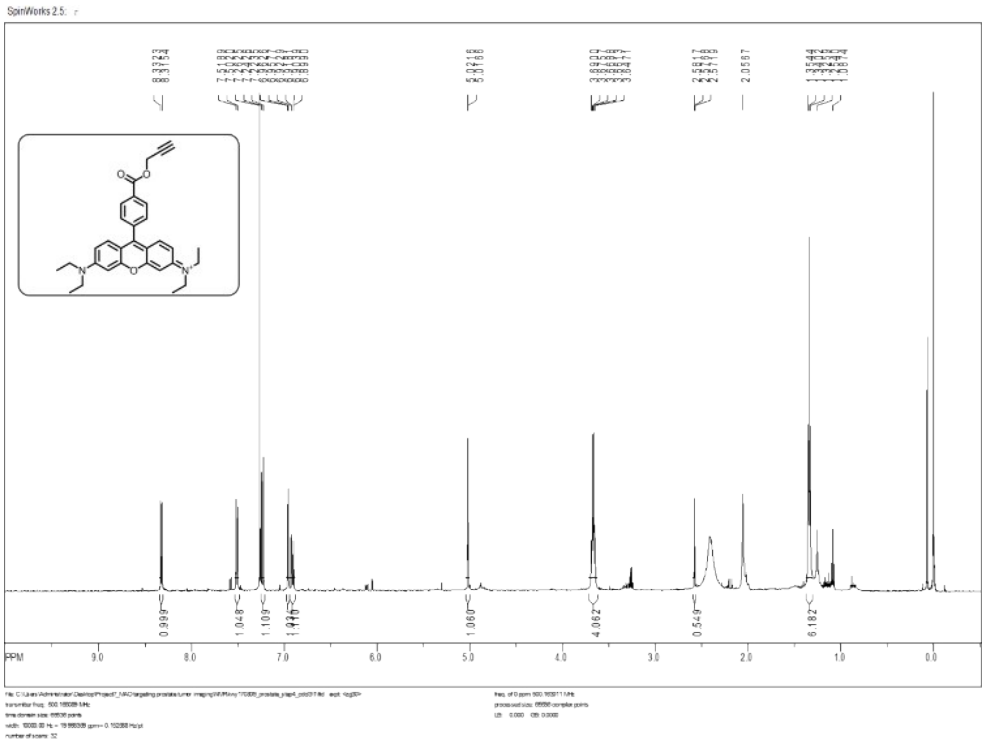


Fig. S5. ^{1}H NMR spectra (500 MHz) of **5** in $CDCl_3$.

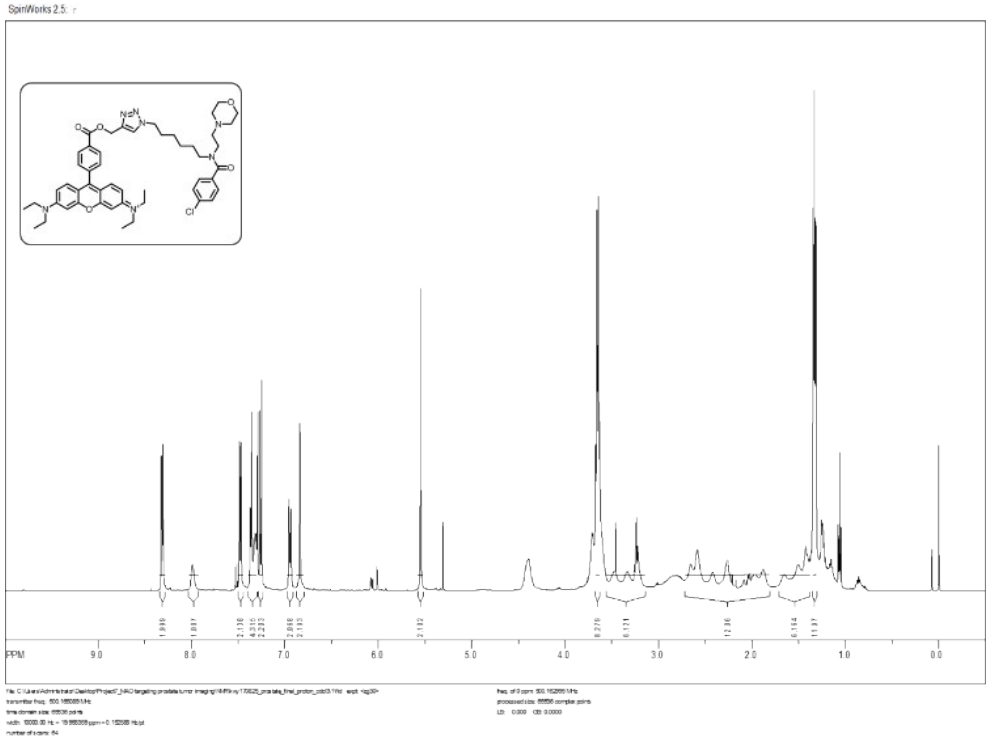


Fig. S6. ^{1}H NMR spectra (500 MHz) of **PCP-1** in $CDCl_3$.

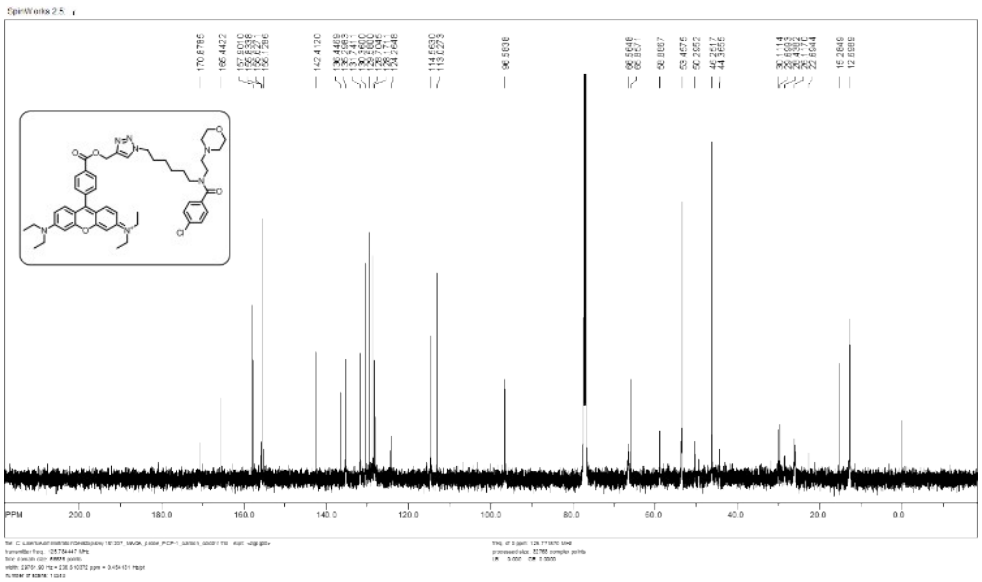


Fig. S7. ^{13}C NMR spectra (125 MHz) of **PCP-1** in $CDCl_3$.

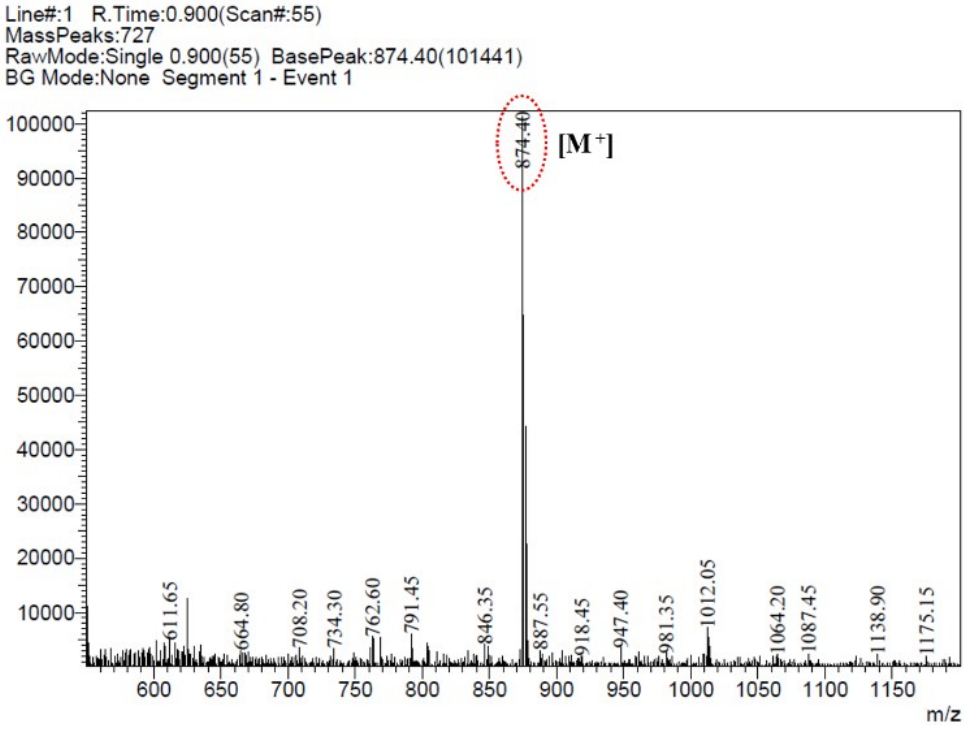


Fig. S8. ESI-MS spectrum of **PCP-1**.

3. Docking Study and Molecular Dynamics (MD) Simulations of PCP-1

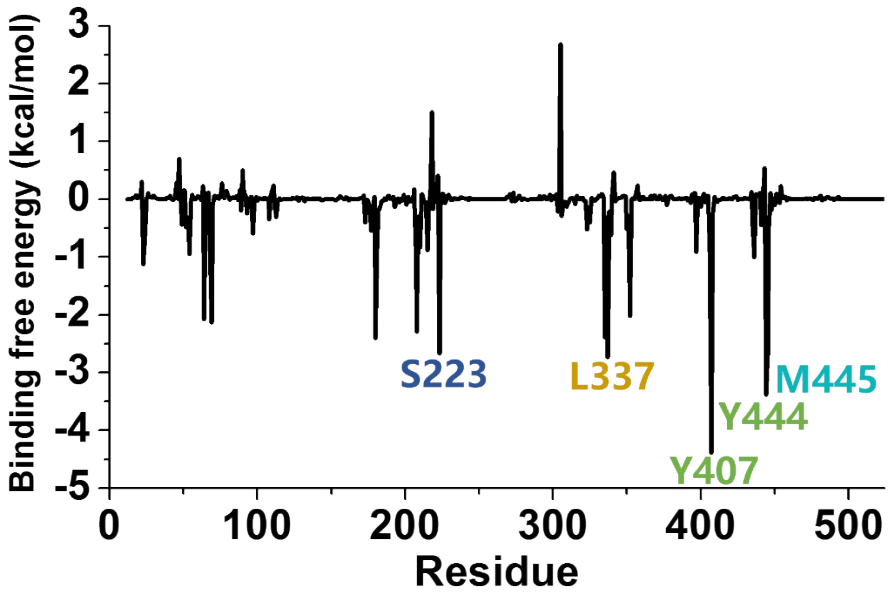


Fig. S9. The binding free energy acquired from molecular dynamics (MD) simulation.

Molecule	excited states	λ (nm)	δ_{TPA} (GM)
Rhodamine TP fluorophore	1	920	460
	2	808	404
	3	792	396
	4	768	384
Coumarin 307	1	686	685
	2	618	106
	3	583	5.0
	4	507	11.0
Fluorescein	1	945	0.0
	2	832	50.8
	3	779	2.7
	4	675	120

Fig. S10. The calculated two-photon absorption cross section (δ_{TPA}) of rhodamine TP fluorophore, coumarin 307, and Fluorescein molecules from first to fourth (excited states) in gas phases.

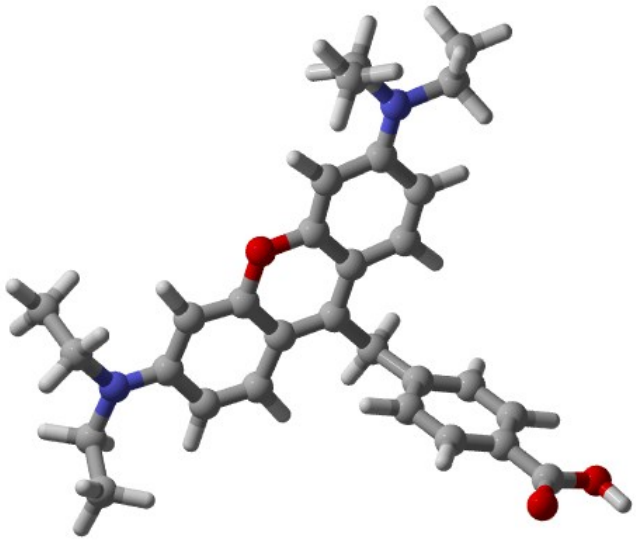


Fig. S11. The calculated structure of rhodamine TP fluorophore.

4. Optical Properties of PCP-1

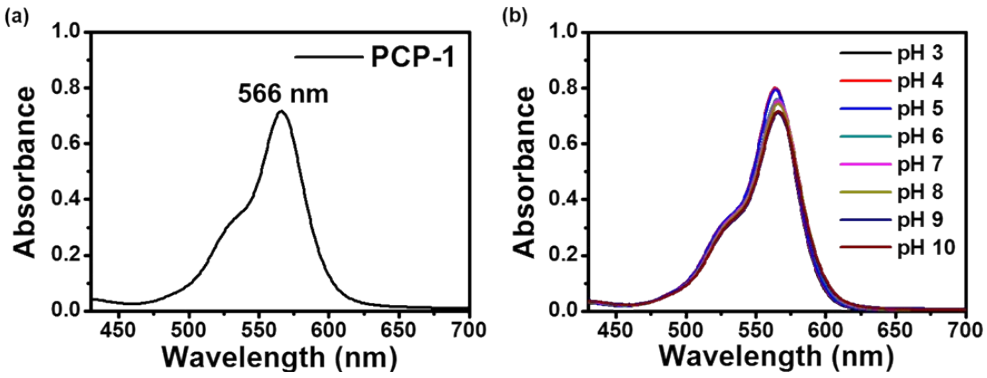


Fig. S12. (a) UV/Vis spectra of probe **PCP-1** (10 μM) in 10 mM PBS buffer (pH 7.4, 0.2% DMSO) (b) Absorbance change with varying pH range of the solutions (DMSO−water, 0.2% *v*/*v*).

5. PCP-1 Targets MAO-A in PCa Cells

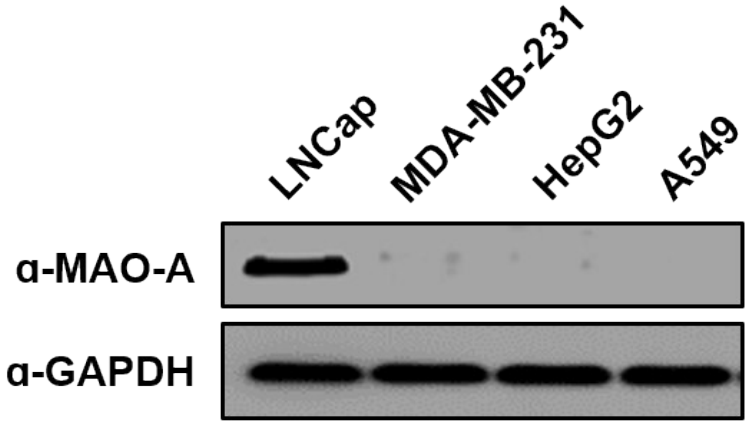


Fig. S13. Protein expression levels of hMAO-A in various cancer cell lines.

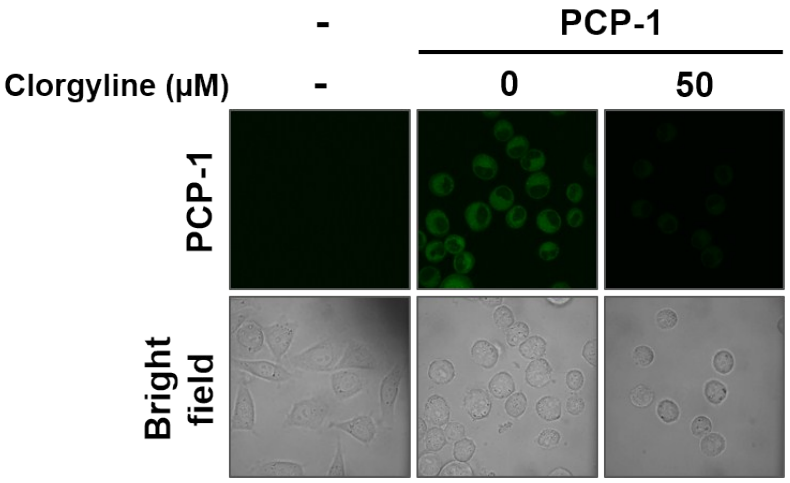


Fig. S14. TP cell images of **PCP-1** in the absence or presence of clorgyline in LNCap cells.

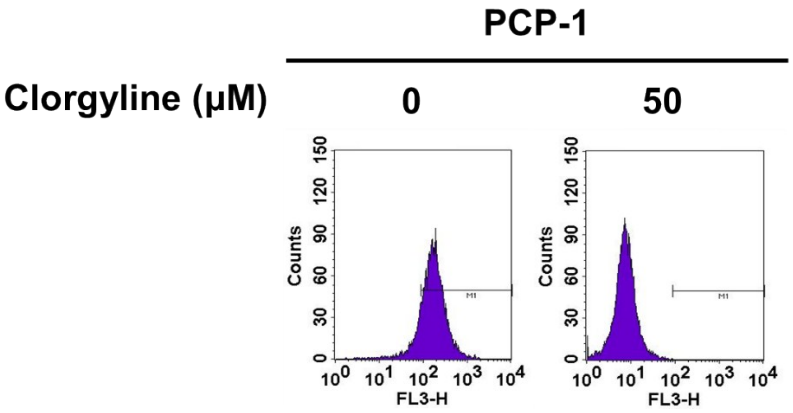


Fig. S15. Flow cytometry analysis on **PCP-1** levels in the absence or presence of clorgyline in LNCap cells.

6. PCP-1 Prevents PCa Cell Growth, Proliferation, and Metastasis.

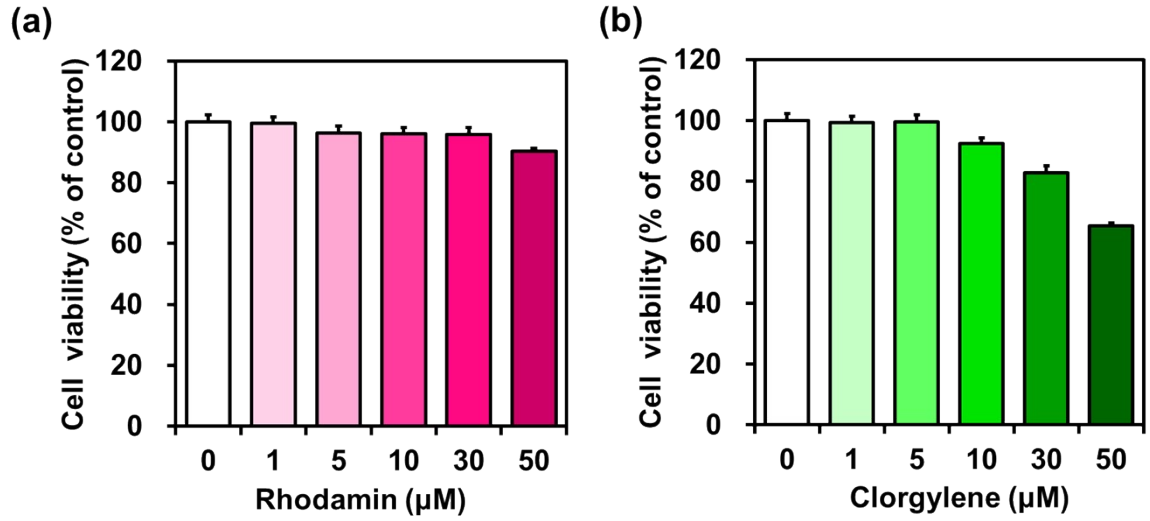


Fig. S16. **PCP-1** induced cell death. Cell viability of Rhodamine, and clorgyline. Cells were incubated with 0, 1, 5, 10, 30 and 50 μM of rhodamine (a), and clorgyline (b) for 24 h in LNCap cells. Values represent mean ± SE of three independent experiments performed in triplicate; *$p<0.05$.

7. Supplementary references

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