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Traceable In-cell Synthesis and Cytoplasm-to-Nucleus Translocation of Schiff base Zinc Complexes as A Simple and Economical Anticancer Strategy

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MATERIALS AND METHODS

$ZnCl_2$, NaOH, cystamine dihydrochloride, salicylaldehyde, 2-cyanophenol were purchased from J&K Scientific Ltd, cisplatin, 2-aminoethanethiol hydrochloride, DMSO(dimethylsulfoxide), MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylt etrazo liumbromide)(Sigma Aldrich), Triethylamine anhydrous were purchased from J&K Scientific Ltd.; LTG(Lyso Tracker Green), MTDR, SYTO-59 were purchased from Life Technologies(USA), DAPI (Sigma Aldrich, USA), PUC19 DNA, (Life Technologies, USA). All the tested compounds were dissolved in DMSO and diluted by PBS just before the cellular experiments, and the concentration of DMSO was less than 1% (v/v). NMR spectra were recorded on a Bruker Avance 400 spectrometer (Germany). ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). Microanalysis (C, H, and N) was carried out using an Elemental Vario EL CHNS analyzer (Germany). UV/Vis spectra were recorded on a Varian Cary 300 spectrophotometer (USA). Emission measurements were conducted on an FLS 920 (Japan). Cell images were captured by a Zeiss LSM 710 Confocal.

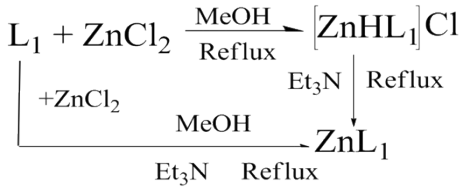
Synthesis and characterization

Synthetic procedure of ligand $\mathbf{L_1}$: NaOH (2 mmol) was dissolved in ethanol was added into a suspension of cystamine dihydrochloride (1 mmol) stirred for 4h. The solvent was evaporated in vacuo and the white solid extracted with 20 ml CH_2Cl_2, and then mixed with salicylaldehyde (2 mmol) which was dissolved in 20 ml CH_2Cl_2, and refluxed for another 6 h, the mixture was concentrated in vacuo and recrystallized in diethyl ether, Yield:72%. ESI-MS (CH_3OH): m/z calcd for $[M+H]^+$, 361.10; found: 361.00. Elemental analysis calcd (%) for $C_{18}H_{20}N_2O_2S_2$: C, 59.91; H, 5.59; N,7.77; C/N,7.71; found: C, 59.89; H, 5.57; N,7.76, C/N,7.72. ^{1}H NMR (400 MHz, DMSO) δ 13.30 (s, 2H), 8.59 (s, 2H), 7.38 (ddd, J = 15.5, 8.0, 3.9 Hz, 4H), 6.99 – 6.64 (m, 4H), 3.89 (t, J = 6.3 Hz, 4H), 3.09 (t, J = 6.4 Hz, 4H).

Synthetic procedure of $\mathbf{ZnHL_1}$: Zin complex was easily obtained by reacting $ZnCl_2$ with $\mathbf{L_1}$ in methanol, the mixture was refluxed for 3 h, the precipitated complexes were filtered off after cooling, washed with water and methanol. These ligands and metal complexes were characterized by ESI-MS and ^{1}H NMR. ESI-MS (CH_3OH): m/z calcd for $[M+H_2O+H]^+$, 442.85; found: 442.60. Elemental analysis calcd (%) for $[\mathbf{ZnHL^1}]Cl{\cdot}2H_2O$ ($C_{18}H_{23}ClN_2O_4S_2Zn$): C, 43.56; H, 4.67; N,5.64; C/N,7.72; found: C, 43.51; H,4.63; N,5.64, C/N,7.71. ^{1}H NMR (400 MHz, DMSO) δ 13.25 (s, 1H), 8.56 (s, 2H), 7.42 (d, J = 7.5 Hz, 2H), 7.31 (t, J = 7.7 Hz, 2H), 6.94 – 6.79 (m, 4H), 3.87 (t, J = 6.3 Hz, 4H), 3.06 (t, J = 6.3 Hz, 4H).

Synthetic procedure of $\mathbf{ZnL_1}$: $\mathbf{ZnL_1}$ was obtained in a similar way as $\mathbf{ZnHL_1}$ except $\mathbf{L_1}$ was deprotonated in CH_3OH by Et_3N in advance. Elemental analysis calcd (%) for $\mathbf{ZnL_1}{\cdot}H_2O$: C,

48.93; H, 4.56; N,6.34; C/N,7.71; found: C, 48.92; H, 4.55; N,6.35, C/N, C/N,7.70.^{1}H NMR (400 MHz, DMSO) , δ 8.51 (s, 2H), 7.39 – 7.21 (m, 4H), 6.75 – 6.51 (m, 4H), 3.96 (d, J = 49.3 Hz, 4H), 3.02 (d, J = 59.2 Hz, 4H). It is noted that ESI-MS spectrum is not obtained due to the electric neutrality and high quality NMR signals can not be obtained due to the extremely poor solubility of **ZnL_1** in DMSO as well as other organic solvents.



Scheme S1 Synthetic routes of **ZnL_1** and **$ZnHL_1$**.

Cell lines and culture conditions

A549, MCF-7, Hela and HLF cells were obtained from Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Cells was maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco BRL) or RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium at 37 °C under 5% CO_2. Cells treated with (1% DMSO) vehicle control were used as the reference group in each experiment.

Cellular uptake and distribution

Confocal microscopy was used for tracing cytoplasm-to-nucleus translocation of in-cell generation of **$ZnHL_1$**. A549 cells were incubated with ligand **L_1** (40 μM) for 6 h, and then 100 μM of $ZnCl_2$ was added for another up to 12 h incubation. The excitation wavelength is 405 nm, the emission is collected at 450 ± 20 nm (blue channel), 520 ± 20 nm (green channel), respectively.

For colocalization investigation, A549 cells were treated with **L_1** (40 μM, 6 h) and $ZnCl_2$ (100 μM, another 2 h or 12 h), followed by the co-incubation with mitochondria-specific dye MTDR (100 nM), lysosome specific dye LTG (150 nM), or nucleus-specific dye STYO-59 for another 30 min at 37 °C. Cells were washed three times with PBS before confocal microscopy. For MTDR, $\lambda_{ex}/\lambda_{em}$ = 644/665± 20 nm, for LTG, $\lambda_{ex}/\lambda_{em}$ = 504/511 ± 20 nm; for STYO 59, $\lambda_{ex}/\lambda_{em}$ = 622/645 ± 20 nm.

ICP-MS measurements

A549 cells were seeded in 10 cm culture dishes with a density of 1 × 10^5 cells/ml in 5 ml cell medium. Cells were incubated with concentrations of **L_1** (0, 20 and 40 μM) at 37 °C for 12 h, then the same amount of Zn^{2+} (100 μM) was added into the culture medium for another 12

h incubation. After digestion in trypsin−EDTA solution, cells were counted and digested in 60% HNO_3 at room temperature overnight, then diluted with Milli-Q H2O to obtain 2% HNO_3 solutions for ICP-MS (ThermoElemental, USA) measurement of the whole cell zinc contents. Nuclear fractions were separated by a cell nucleus and cytoplasm extraction kit (Sangon, Shanghai, China). The standards for Zinc calibration were freshly prepared by diluting a $Zn(NO_3)_2$ stock solution with 2% HNO_3 in Milli-Q H_2O.

Induction of cancer cell apoptosis

The Annexin V/PI assay was carried out according to the manufacturer's protocol. A549 cells were seeded in 6-well plates and indicated concentrations of compounds were added and incubated for a total period of 36 h. The cells were harvested and stained with Annexin V and PI as described above at room temperature for 15 min in the dark, and analyzed immediately by flow cytometry (λ_{ex} = 488 nm). Data were analyzed by FlowJo Software (TreeStar, USA).

Immunofluorescence staining

A549 cells were seed into 6-well plates with poly-L-lysine-treated coverslips (Nest) for 24 h, and then treated with compounds as indicated concentrations for a total incubation time of 36 h. The cells were then fixed with 4% paraformaldehyde, and permeablized in 0.5% Triton X-100 (in 1×PBS). After that, cells were incubated with primary antibodies against γH2AX at 4 °C overnight. Next, cells were washed three times with TBST, and incubated with secondary antibodies, DyLight 549-conjugated anti-mouse (Multi Sciences, China), for 2 h at room temperature. Cells were then marked by DAPI. Fluorescence was detected by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany).

DNA cleavage

DNA gel electrophoresis assay: pUC19 DNA (200 ng/mL) was incubated with different compounds ($ZnCl_2$, $\mathbf{L_1}$, and $\mathbf{ZnHL_1}$) in Tris-HCl buffer (20 mM Tris-HCl, 20 mM Na_2HPO_4, pH 7.4) at 37 °C for 4 h. The DNA samples were analyzed by electrophoresis (100 V, 1 h) on a 0.8% agarose gel in 1×TBE buffer (18 mM Tris-borate acid, 0.4 mM EDTA, pH 8.3). The gel was stained with 2 μL Gel-Red and the images were captured on FluorChem M (ProteinSimple, Santa Clara, CA).

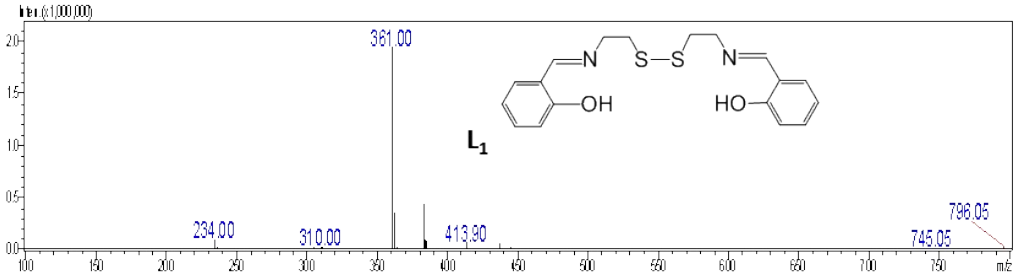
***In vivo* anti-tumor investigations**

The Female BALB/c nude mice aged 4−5 weeks were randomly divided into 5 groups (four

mice per group). MCF-7 cells (2×10^6) were suspended in 150 μL PBS, and the xenografts were established by subcutaneous injection. When the tumor grew to 130 mm^3, mice were intratumorally injected with 50 μL of PBS, $\mathbf{L_1}$, $ZnCl_2$, $\mathbf{ZnHL_1}$ and $ZnCl_2$ + $\mathbf{L_1}$ as indicated dose within the first day, and same dose of compounds were injected in the fourth day and eighth day. Tumor sizes were measured by a vernier caliper and mice weight were measured simultaneously every two day. Tumor volume was defined as $V=A^2B/2$, A and B are the length and width diameter of tumors.

Supporting Figures and Tables

(A)



(B)

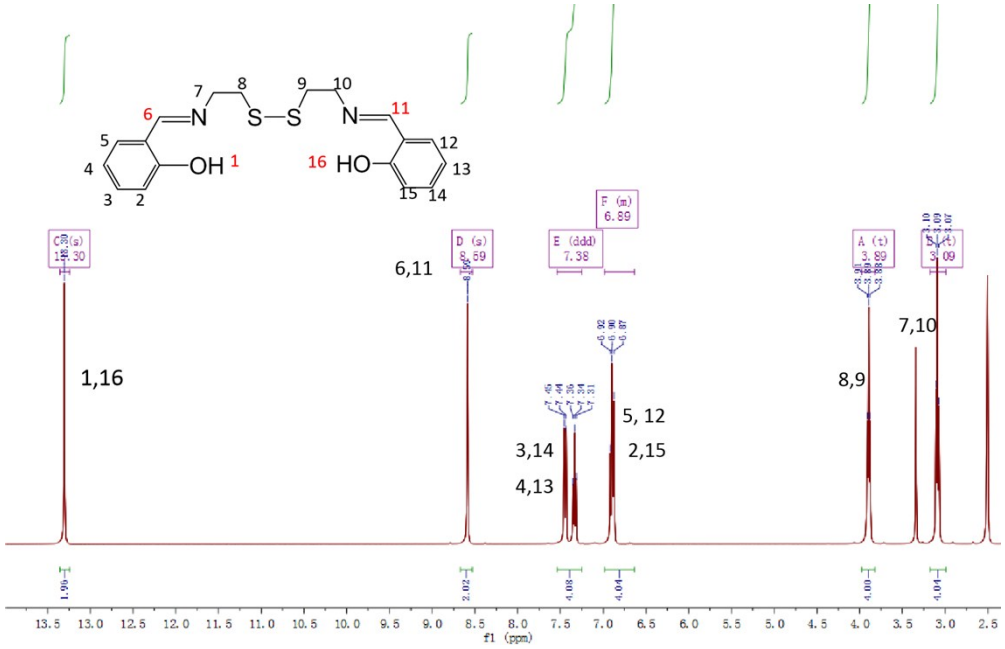
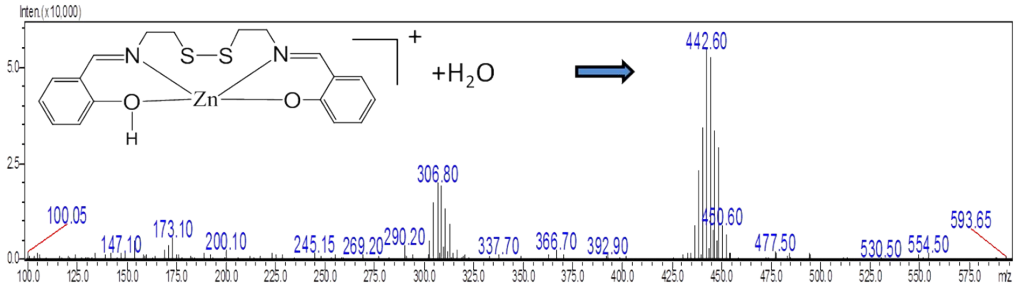


Figure S1 (A) ESI-MS of $\mathbf{L_1}$. m/z = 361.00 $[\mathbf{L_1}+H]^+$; (B) ^1H NMR spectrum of $\mathbf{L_1}$ recorded in DMSO-d_6.

(A)



(B)

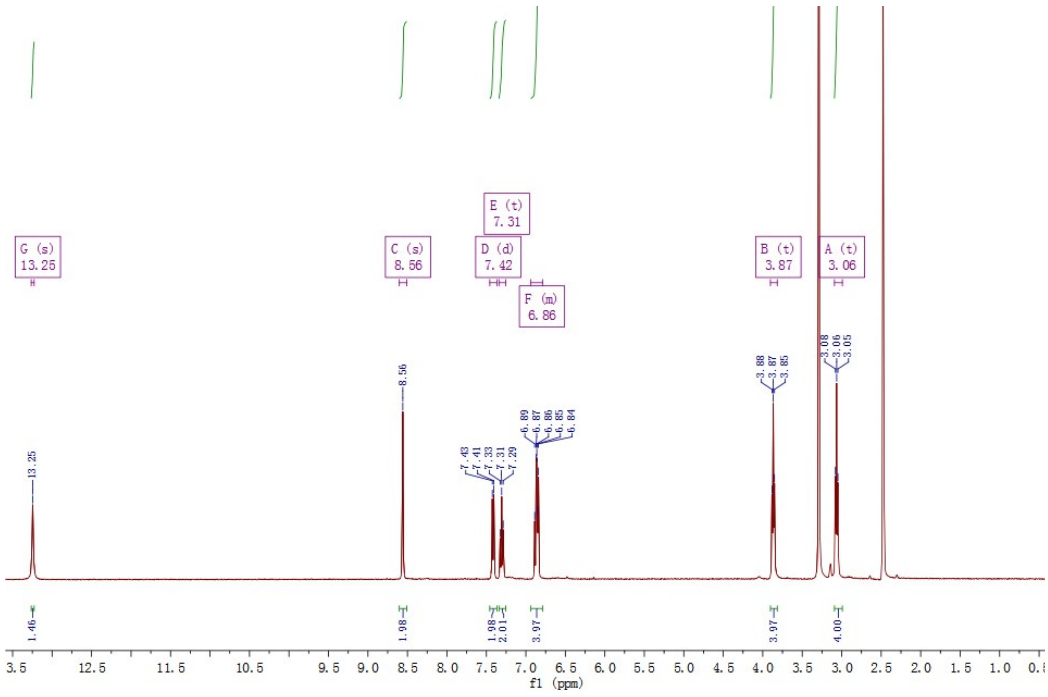


Figure S2 (A) ESI-MS spectrum of **ZnHL$_1$** complex, m/z = 442.60 [**ZnHL$_1$**+H_2O]$^+$; (B) ^{1}H NMR spectrum of **ZnHL$_1$** recorded in DMSO-d_6.

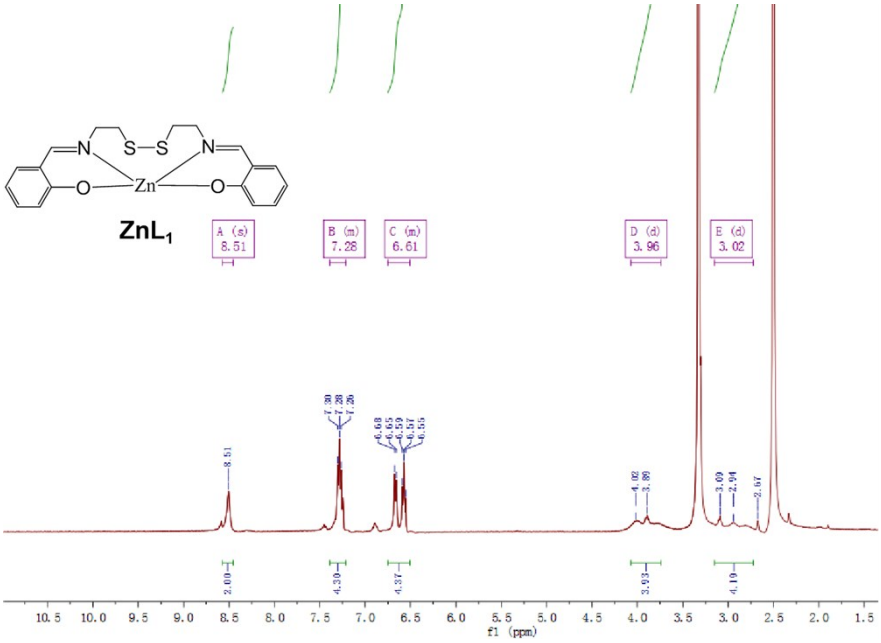


Figure S3 ^{1}H NMR spectrum of **ZnL$_1$** recorded in DMSO-d$_6$ (high quality signals can not be obtained due to the poor solubility of **ZnL1** in DMSO).

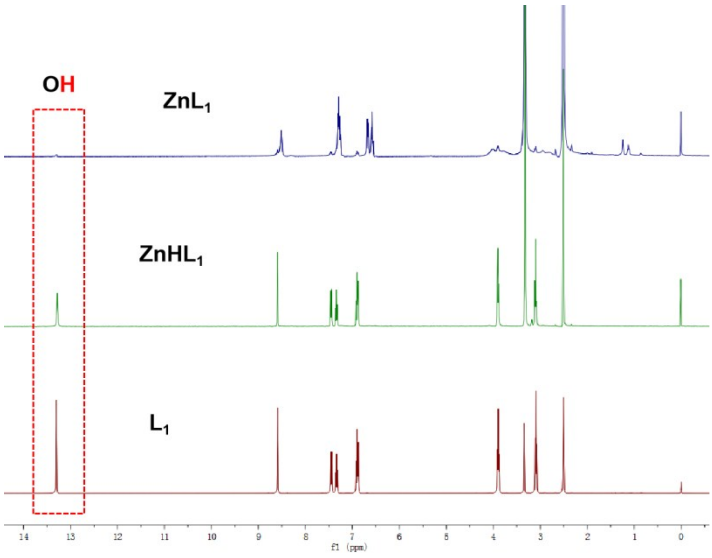


Figure S4 ^{1}H NMR stacking spectra of **ZnL$_1$**, **ZnHL$_1$** and **L$_1$** comparing the O-H signal at 13.30 ppm.

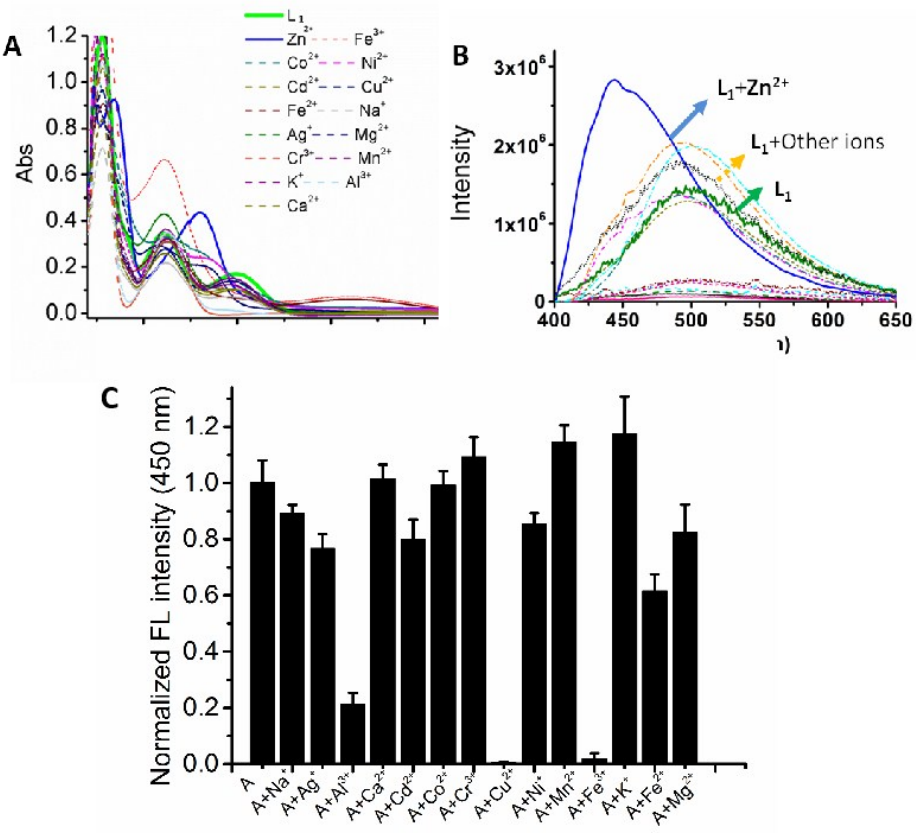


Figure S5 (A) UV-Vis absorption and (B) fluorescence spectra of **L_1** (30 μM) in DMSO-H_2O (1:1, v/v) solvent in the presence of different metal ions (90 μM); (C) Fluorescence response of **L_1** (30 μM) containing 30 μM Zn^{2+} to other indicated metal ions (90 μM) in DMSO-H_2O (1:1, v/v) solvent (A = L_1 + Zn^{2+}; in mixed solution L_1 : Zn^{2+} : other metal ions = 1:1:3)

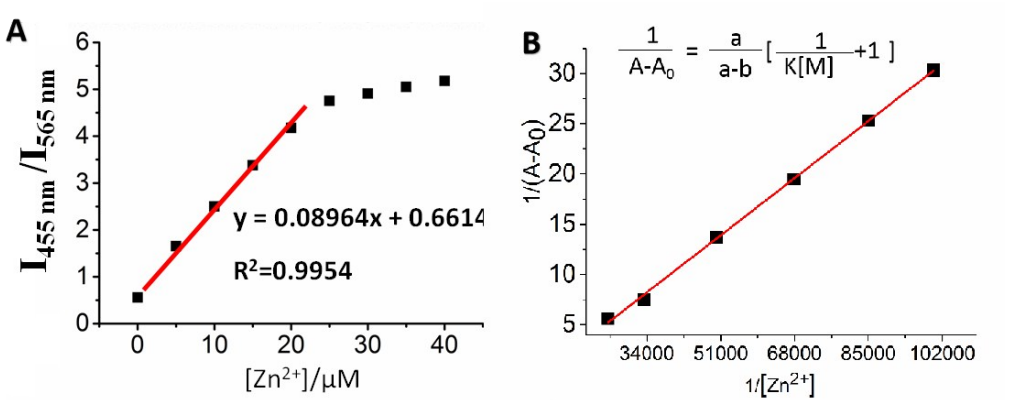


Figure S6. (A) Fluorescence response ($I_{455\ nm}$ / $I_{565\ nm}$) of $\mathbf{L_1}$ (30 μM) as a function of zinc ion concentrations (λ_{ex} = 365 nm); (B) Benesi-Hildebrand plot (B-H plot) obtained from absorption changes of $\mathbf{L_1}$ at 362 nm as a function of zinc ion concentrations. (R^2 = 0.998). Binding constant is calculated to be $0.93\times10^4\ M^{-1}$.

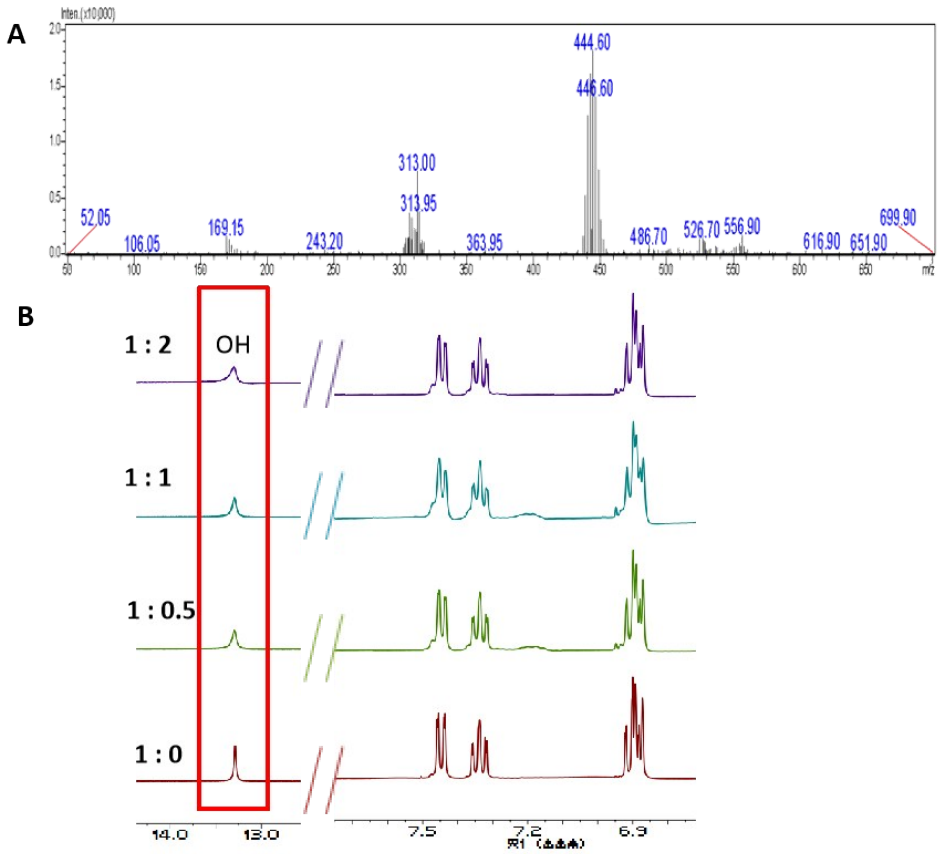


Figure S7 (A) ESI-MS spectrum of $\mathbf{L_1}$ after $ZnCl_2$ addition. Cluster peaks centered at m/z = 446.60 can be assigned to $[\mathbf{ZnHL_1} + Na]^+$ (Calculated m/z = 446.02); (B) ^1H NMR titration of $\mathbf{L^1}$ (1 mM) with increasing concentrations of $ZnCl_2$ in DMSO-d_6.

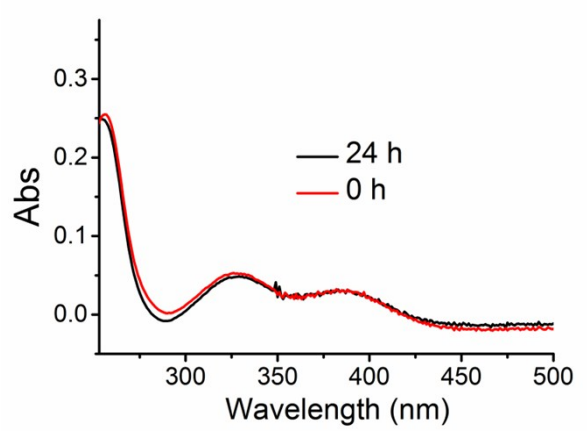


Figure S8 UV-Vis spectra of $\mathbf{L_1}$ in Na_2HPO_4/Crictic acid buffer (pH = 7.0) recorded at different time..

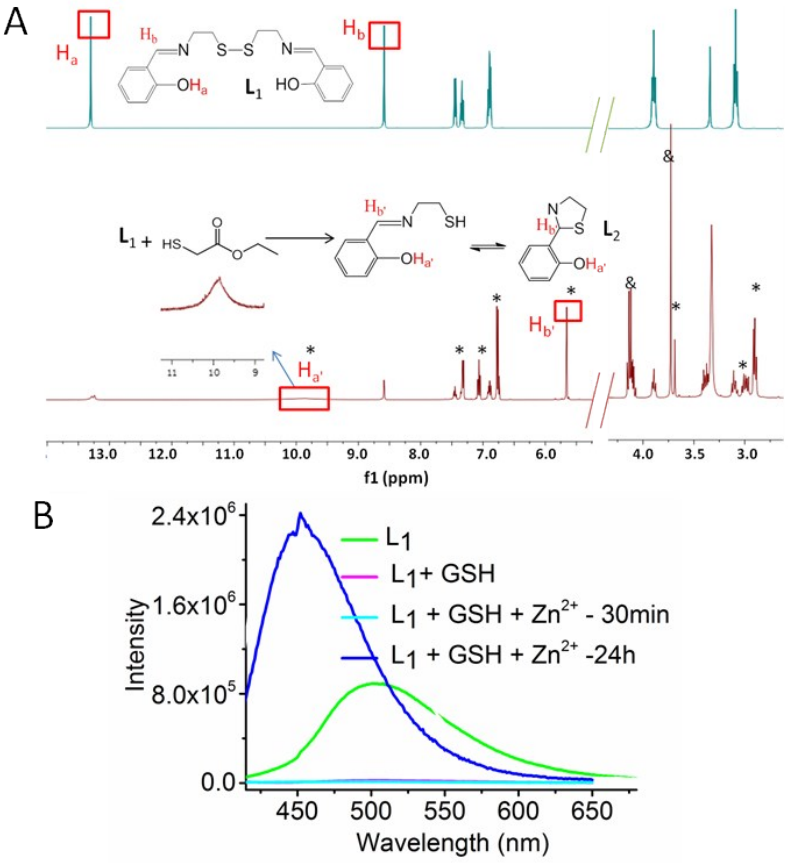


Figure S9 (A) ^{1}H NMR spectra of $\mathbf{L_1}$ in the absence and presence of ethyl thioglycolate measured in DMSO-d_6 solvent, in which * represents *in situ* generated $\mathbf{L_2}$ and & represents ethyl thioglycolate; (B) Changes in the fluorescence spectra of $\mathbf{L_1}$ (30 μM) in H_2O/DMSO (1:1) solvent before and after adding GSH (5 mM) and $ZnCl_2$ (40 μM) in turn.

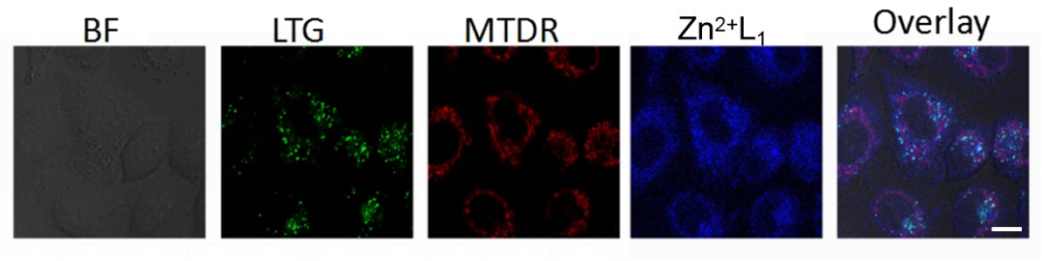


Figure S10 Colocalization investigation of intracellular synthesized **ZnHL$_1$** with LTG (Lyso Tracker Green) and MTDR (MitoTracker Deep Red). A549 cells were incubated with **L$_1$** (40 μM, 6 h) followed with $ZnCl_2$ treatment (100 μM, 2 h), then co-incubated with LTG (100 nM) and MTDR (150 nM) for 30 min at 37 °C. (MTDR**:** λ_{ex}= 644 nm, λ_{em} = 665± 20 nm ; LTG: λ_{ex}= 504 nm, λ_{em} = 511± 20 nm; **ZnHL$_1$**: λ_{ex}= 405 nm, λ_{em}= 450 ± 20 nm). Scale bar: 5 μm.

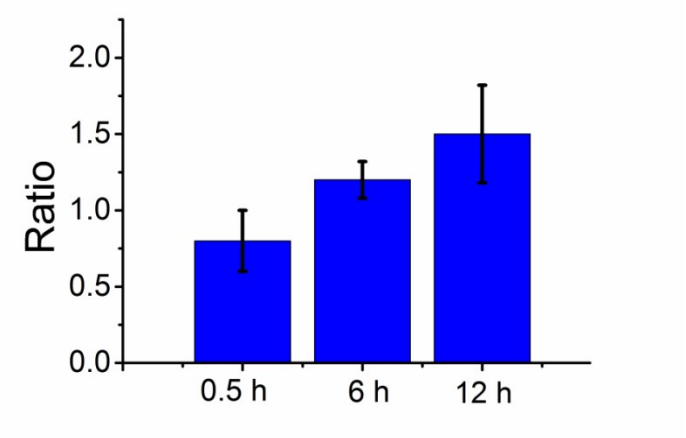


Figure S11 Average F_{blue}/F_{green} ratios were measured from five cells in Fig.2A, λ_{ex}=405 nm, F_{blue} :λ_{em}= 450 ± 20 nm; $F_{green:}$:λ_{em}= 520 ± 20 nm.

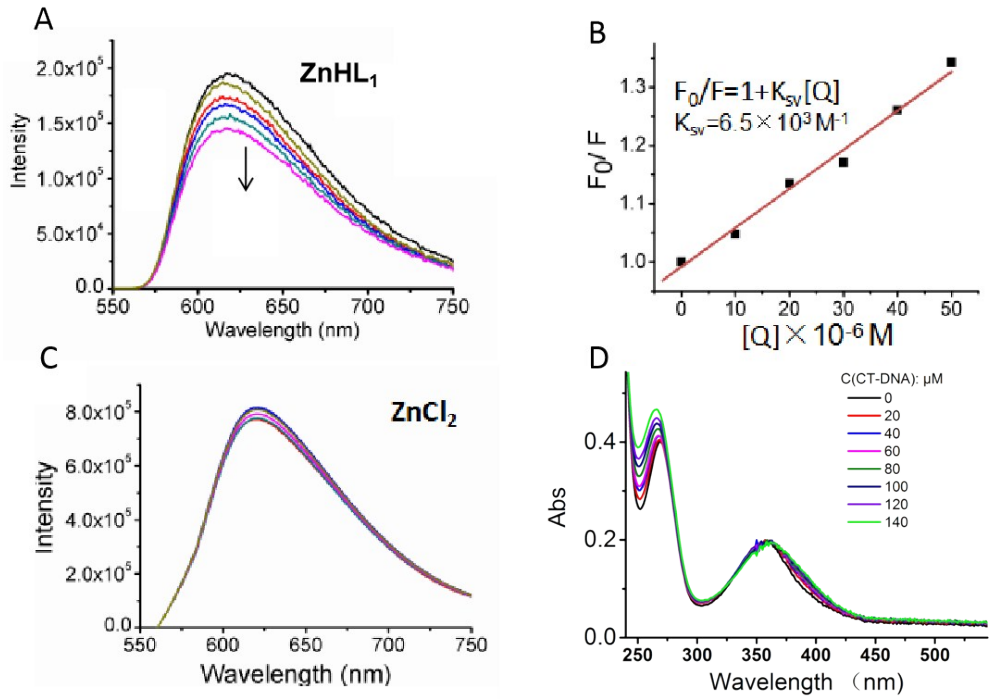


Figure S12 (A and C) Emission spectra of Ethidium bromide (EB, 3 μM) bound to ctDNA (30 μM) in the presence of increasing concentrations of **$ZnHL_1$** (0~50 μM) and $ZnCl_2$ (0-250 μM), respectively. λ_{ex} = 525nm. (B) Plot of F_0\F *vs* [**$ZnHL_1$**] with experimental data points from Fig. 12A and for liner fitting; (D) UV-Vis spectra of **$ZnHL_1$** (20 μM) in the presence of increasing amounts of ctDNA.

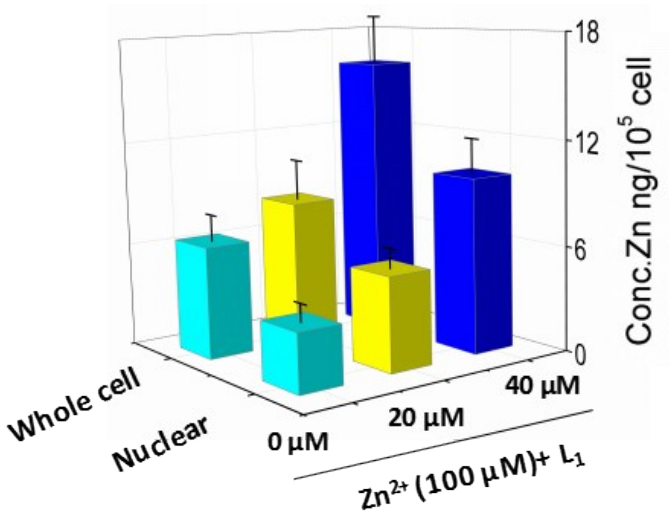


Figure S13 Quantification of the intracellular synthesized **$ZnHL_1$** in nucleus and the whole A549 cell measured by ICP-MS.

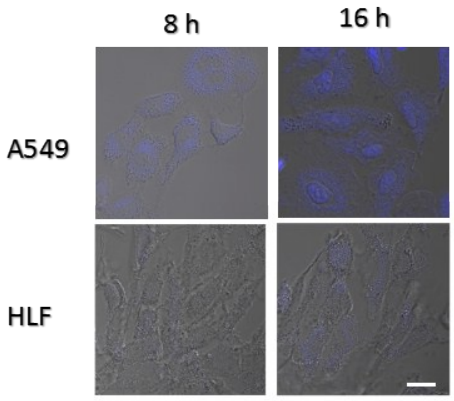


Figure S14 Cellular uptake of intracellular synthesized **ZnHL$_1$** (40 μM) for 8 and 16 h by cancer cells A549 and normal cells HLF measured by confocal microscopy. Both A549 and HLF cells were incubated with **L$_1$** (40 μM, 6 h) followed with $ZnCl_2$ treatment (100 μM, 8 and 16 h), respectively. Scale bar: 5 μm.

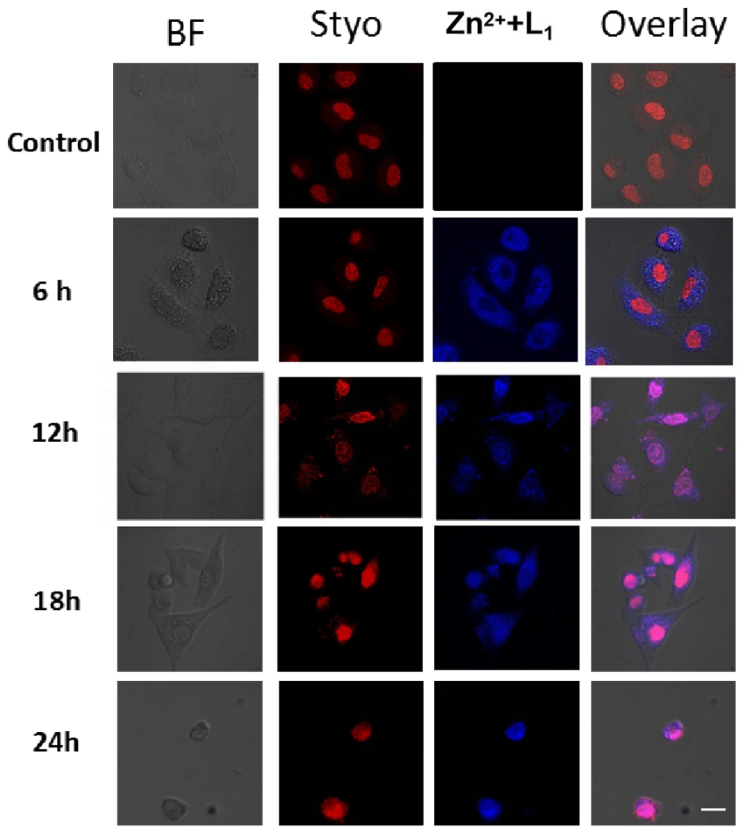


Figure S15 Tracing the changes in the nuclear morphology of A549 cells after treatment with intracellular synthesized **ZnHL$_1$** (40 μM, up to 24 h); STYO 59 dye is used for co-staining ($\lambda_{ex}/\lambda_{em}$ = 622/645 ± 20nm); Scale bar: 5 μm.

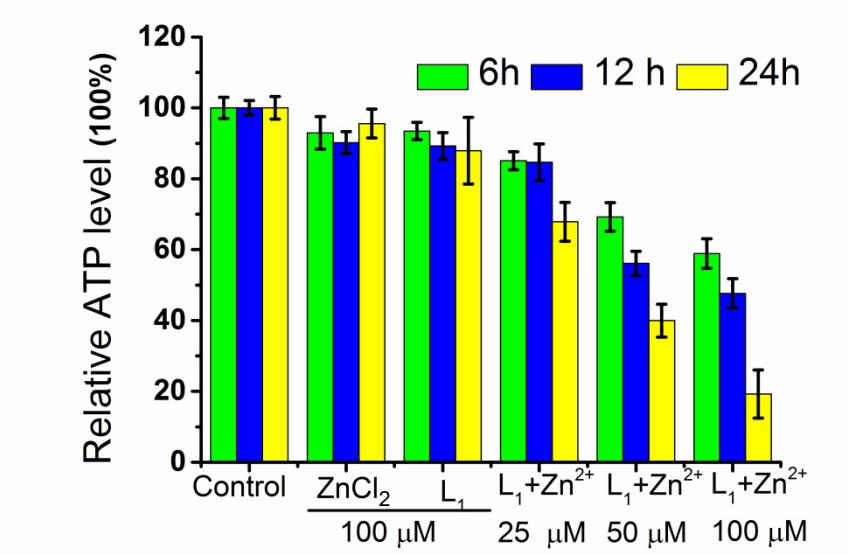


Figure S16 (A) Intracellular ATP levels in A549 cells at different treatment stage (6 h, 12 h and 24 h).

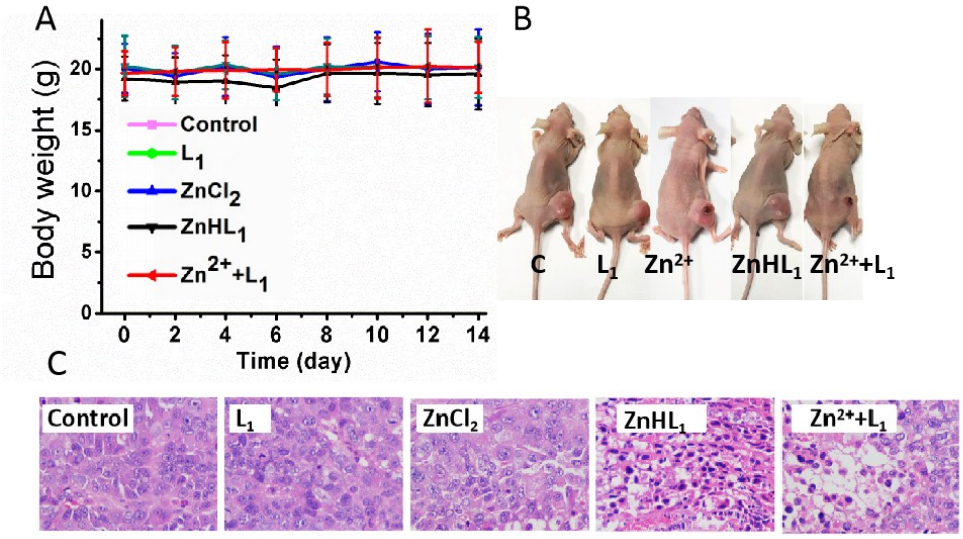


Figure S17 *In vivo* anti-tumor efficacy of PBS (control), $\mathbf{L_1}$, $ZnCl_2$, as-prepared $\mathbf{ZnHL_1}$ and in-cell synthesized $\mathbf{ZnHL_1}$ (Zn^{2+}+$\mathbf{L_1}$). (A) Body weight changes of mice after various treatment; (B) Photos of resected tumors and MCF-7 tumor-bearing mice before sacrificed; (C) Haematoxylin and eosin staining of tumor tissues harvested from mice on day 14.