Supporting Information
For

Dinuclear Zinc(II) Complexes Containing (Benzimidazo1-2-yl)benzene That Overcome Drug Resistance in Hepatocellular Carcinoma Cells through Induction of Mitochondria Fragmentation

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1. Materials and Methods

1.1 Reagent and Physical Measurement

$ZnCl_2$, *o*-phenylenediamine, *o*-phthalic acid and 1,2,4,5-benzenetetracarboxylic acid were purchased from Alfa Aesar. Elemental analyses were performed on an Elementar vario EL elemental analyzer. Infrared spectra were recorded with samples as KBr pellets using a Nicolet NEXUS 670 FTIR spectrophotometer. ^{1}H-NMR spectra were measured using a Mercury-Plus 300MHz nuclear magnetic resonance spectrometer with d^6-DMSO as solvent and TMS as internal reference. UV/Vis diffuse reflectance spectra were recorded on a Perkin Elmer Lambda 35 UV/Vis spectrophotometer. Emission spectrum was measured with Edinburgh FLSP920 Combined Fluorescence Lifetime and Steady State Spectrometer.

1.2. Synthesis of ligand

The ligand 1, 2-bis(2-benzimidazolyl)benzene was synthesized by the condensation of o-phenylenediamine with o-phthalic acid in polyphosphoric acid according to the literature [1].

1.3 Synthesis of the complex Zn(bbb)Cl_2·DMF·H_2O (1)

$ZnCl_2$ (273 mg, 2.0 mmol) dissolved in DMF solution (10 ml) was added to a DMF solution (10 ml) of 1, 2-bis(2-benzimidazolyl)benzene (620 mg, 2.0 mmol). The reaction mixture was stirred for 20 minutes at room temperature, then expose it in air, after a week, the block colorless crystal was obtained from the DMF solution. Yield: 650 mg, 60.52%. Anal. Calcd. for $C_{23}H_{23}N_5Cl_2O_2Zn$ ([Zn(bbb)Cl_2·DMF·H_2O]): C,

51.37; H, 4.31; N, 13.02%. Found: C, 51.41; H, 4.33; N, 12.98%. Selected IR data (KBr, cm^{-1}): 3557, 3465, 3000, 1649, 1433, 1326, 1279, 987, 740. ^{1}H NMR (300 MHz, d^{6}-DMSO) δ 13.95 (s, 2H), 8.50 (s, 2H), 7.99 (dd, J = 5.6, 3.4 Hz, 2H), 7.95 (s, 2H), 7.89 (d, J = 3.0 Hz, 2H), 7.64 (s, 2H), 7.38 (dd, J = 5.9, 2.9 Hz, 4H).^{13}C NMR (126 MHz, d^{6}-DMSO) δ151.07 (s), 139.66 (s), 133.11 (s), 132.31 (s), 131.00 (s), 127.54 (s), 124.39 (s), 123.42 (s), 118.12 (s), 112.35 (s).

1.4. Synthesis of 1,2,4,5-tetrakis(1H-benzo[d]imidazol-2-yl)benzene (tbb)

1, 2, 4, 5-benzenetetracarboxylic acid (1.27g, 0.005 mol) and o-phenylenediamine (2.16 g, 0.020 mmol) were added to polyphosphoric acid (20 mL), and the mixture was stirred at 190 ℃ for 4 h. The resulting dark brown solution was allowed to cool to room temperature and poured into water with vigorous stirring. A light brown solid formed, then was filtered off and the solid was stirred in 50 mL 10% ammonia water for 1 h. It was then filtered off, washed with hot water, dried under vacuum. Crude yield: 1.56 g, 57.0%. 1,2,4,5-tetrakis(1H-benzo[d]imidazol-2-yl)benzene is insoluble in most organic solvents and was used in the next step without further purification.

1.5. Synthesis of dinuclear Zn complex, $Zn_2(tbb)Cl_4$·4DMF (2)

Excess of $ZnCl_2$ (0.670 g) and crude 1,2,4,5-tetrakis(1H-benzo[d]imidazol-2-yl)benzene (0.400 g) were dissolved in DMF (40 mL). Then mixture refluxed on a oil bath for 1h under stirring, during which time most of the ligand dissolved, forming a red solution. The solution was discolored by charcoal, then filtered and the filtrate

was exposed to air. After 3 days, colorless crystals suitable for X-ray analysis. ESI-MS (in MeOH): *m/z* 543.7 ($[M]^+$). Anal. Calcd. for $C_{46}H_{50}N_{12}Cl_4O_4Zn_2$ ($[Zn_2(tbb)Cl_4 \cdot 4DMF]$): C, 49.88; H, 4.55; N, 15.18%. Found: C, 49.41; H, 4.43; N, 14.98%. Selected IR data (KBr, cm^{-1}): 3492, 3073, 2920, 1649, 1484, 1452, 1379, 1326, 1279, 1255, 1161, 1109, 1064, 1015, 990, 914, 859, 828, 807, 744, 662. ^1H NMR (300 MHz, d^6-DMSO) δ 14.24 (s, 4H), 8.72 (s, 4H), 8.54 (s, 4H), 7.95 (s, 4H), 7.67 (s, 4H), 7.43 (s, 8H).

1.6. X-ray crystallography

Single crystal structure determination for the complex was performed on a Siemens Smart-CCD diffractometer. Empirical absorption corrections were applied by using the SADABS program for the Siemens area detector. The structures were solved by direct methods by using program SHELXTL. Fourier difference techniques, and refined by full-matrix least-squares. All non-hydrogen atoms were refined anisotropic displacement parameters. All hydrogen atoms were theoretically added. Absorption correction adopt Semi-empirical from equivalents method. Refinement method was based on Full-matrix least-squares on F^2. Crystallographic data for the structural analysis have been deposited with the Cambridge Crystallographic Data Centre, with CCDC No of 939161 for complex **1** and 939162 for complex **2**.The crystal data are summarized in **Table S1**.

1.7. Stability of Zn complexes

The stability of the complexes in aqueous solution with or without the presence of

different concentrations of metallothioneins (MTs) at 25 ℃ for 72 h was examined by UV-Vis spectrometry.

1.8. Cell culture

Human hepatoma cell line (HepG2) was purchased from American Type Culture Collection (ATCC, Rockville, MD). The cell line was cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and 1% (v/v) penicillin‐streptomycin (PS, 10,000 U/ml, Gibco, USA) in a humidified atmosphere of 5% CO_2 in air at 37˚C. To development drug-resistant human hepatoma cell line (R-HepG2), HepG2 cells were cultured in the presence of doxorubicine (Dox). The survival cells were treated stepwise with a higher concentration of Dox from 0.1 to 100 μM during cell passages. After more than 10 rounds of selection, a clone of R-HepG2 cells with Dox drug resistance was then obtained. To maintain the drug resistance, R-HepG2 cells were cultured and maintained in medium containing 1.2 μM Dox. From time to time, the sensitivity of cells to Dox and other anti-cancer agents was analyzed to confirm their resistance to Dox [1].

1.9. Cell viability assay and determination of cellular uptake

Cell viability was determined by measuring the ability of the cells to metabolize 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan dye [1]. Briefly, cells were placed into 96-well plates at a density of 2.0×10^3 cells/well. After 24 h, different concentrations of PEG-SeNPs were added and incubated for 72 h. Then 20 μl/well of MTT solution (5 mg/ml in PBS buffer) was added and incubated for another 5 h. The medium was removed and replaced

with 150 μl/well of DMSO to dissolve the formazan crystals. Absorbance at 570 nm was taken on a 96-well microplate reader. After treatments, the intracellular Zn concentration was determined by ICP-MS analysis [2].

2.0. Flow cytometric analysis

DNA flow cytometric analysis was carried out according to our previous method [2]. Briefly, cells exposed to the complex for 72 h were harvested by centrifugation and washed with PBS. Cells were stained with propidium iodide (PI) (1.21 mg/mlTris, 700 U/mlRNase, 50.1 mg/l PI, pH8.0) after fixed with 70% ethanol at -20°C overnight. DNA content was analyzed on a Beckman Coulter Epics XL MCL flow cytometer (Miami, FL). Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). The proportion of cells in G0/G1, S and G2/M phases was represented as DNA histogram. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in cell cycle pattern. For each experiment, 10000 events per sample were recorded.

2.1. TUNEL assay and DAPI staining

After treatment with complex **2** for 72 h, the cells cultured in chamber slides were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS. After that, the cells were incubated with 100 μL/well TUNEL reaction mixture containing nucleotide mixture and terminal deoxynucleotidyl transferase (TdT) for 1 h and 1 g/ml of DAPI for 15 min at 37 ℃, respectively. The cells were then washed with PBS and examined under a fluorescence microscope (Nikon Eclipse 80i).

2.2. Determination of caspases activities

After treatment with complex **2** for 72 h, the activities of caspase-3, -8 and -9 were determined by fluorometric method as previously described [3]. Cells after treatment were collected, lysed and 100 μg protein specific caspase substrates (Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8 and Ac-LEHD-AMC for caspase-9). Plates were incubated at 37℃ for 1 h and caspase activity was determined by fluorescence intensity with the excitation and emission wavelengths set at 380 and 440 nm, respectively.

2.3. Evaluation of mitochondrial membrane potential (ΔΨm)

After treatment with complex **2** for 72 h, the cells cultured in 6-well plates were trypsinized and resuspended in 0.5 mL of PBS buffer containing 10 μg/ml JC-1. After incubation for 10 min at 37 °C in the incubator, the cells were immediately centrifuged to remove the supernatant and cell pellets were suspended in PBS and then analyzed by flow cytometry. The percentage of the green fluorescence from JC-1 monomers was used to represent the cells that lost ΔΨm [4].

2.4. Determination of intracellular ATP content

The intracellular ATP content was measured using a bioluminescence somatic cell assay kit (Sigma) according to the manufacturer's instructions. After treatment with complex **2** for 72 h, the cells were incubated with somatic cell ATP releasing reagent. After that, the supernatants were collected and mixed with the luciferase reagent. ATP measurements were determined using an Lmax luminometer (Molecular Devices).

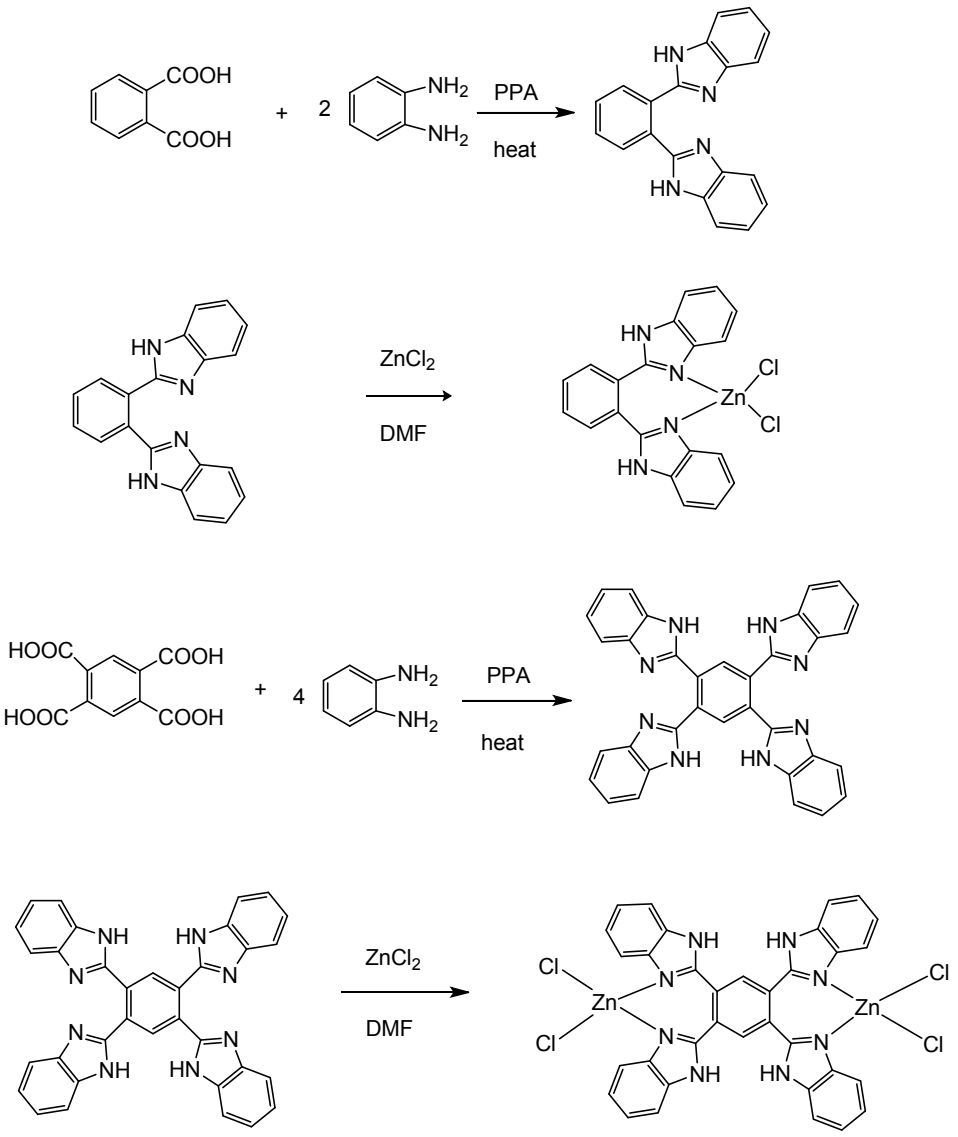
2.5. Living cell staining and microscopy

The cells were grown to 60% confluence on a glass coverslip. Mitochondria and nuclei were stained with 50 nM MitoTracker Red CMXRos and 1 μg/ml DAPA, respectively for 20 min. After washing with PBS twice, cells were cultured in fresh medium within a thermo-cell culture FCS2 chamber of Carl Zeiss Cell Observer (Jena, Germany), and then the complex at different concentrations were added into the medium. Cell images were captured with a monochromatic CoolSNAP FX camera (Roper Scientific, USA) and analysed by using AxioVision 4.2 software (Carl Zeiss).

2.6. Statistical analysis

Experiments were carried out at least in triplicate and results were expressed as mean ± SD. Statistical analysis was performed using SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc. Chicago, IL). Difference between two groups was analyzed by two-tailed Student's t test, and that between three or more groups was analyzed by one-way ANOVA multiple comparisons. Difference with $P<0.05$ (*) or $P<0.01$ (**) was considered statistically significant.

2. Results



Scheme S1. Schematic routes for synthesis of ligands and zinc complexes.

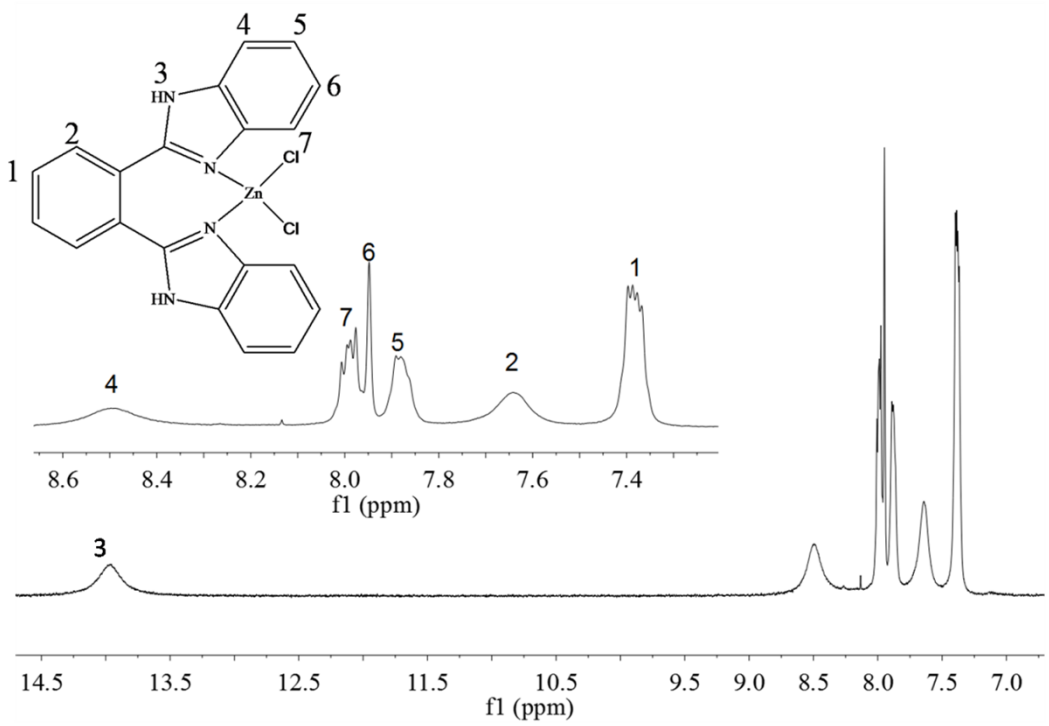


Fig. S1. The ^{1}H NMR spectra of complex **1.**

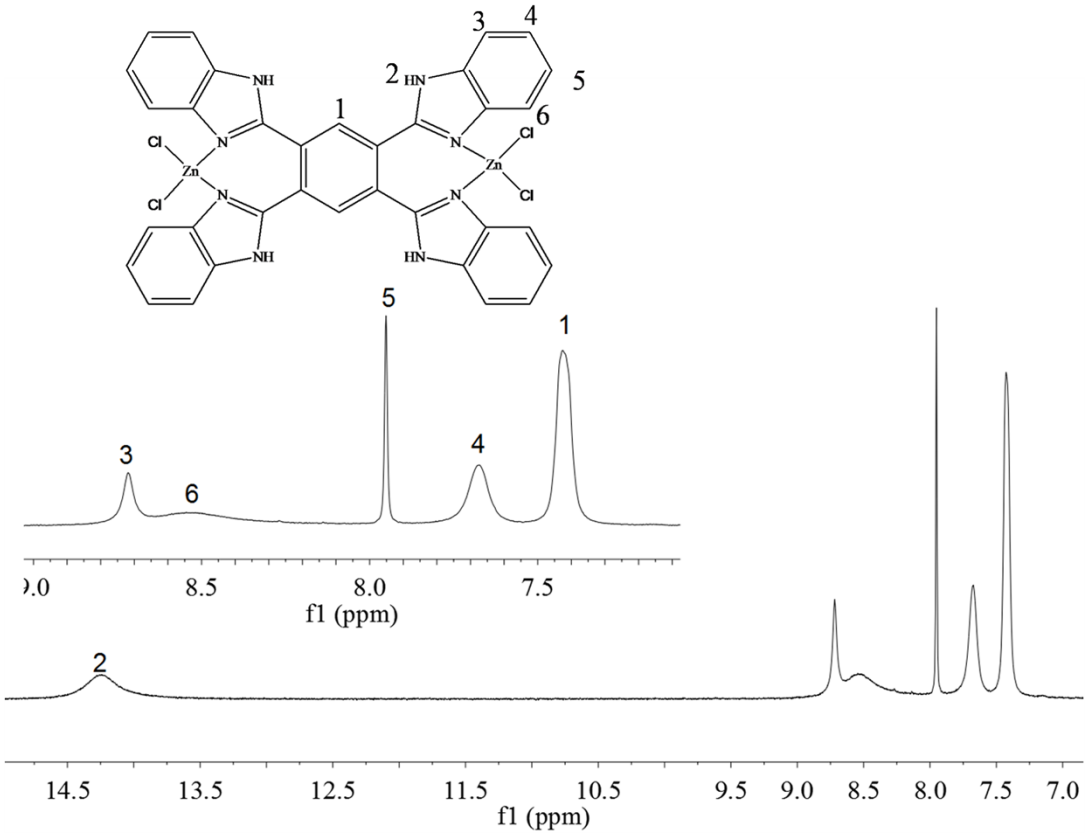


Fig. S2. The ^{1}H NMR spectra of complex **2.**

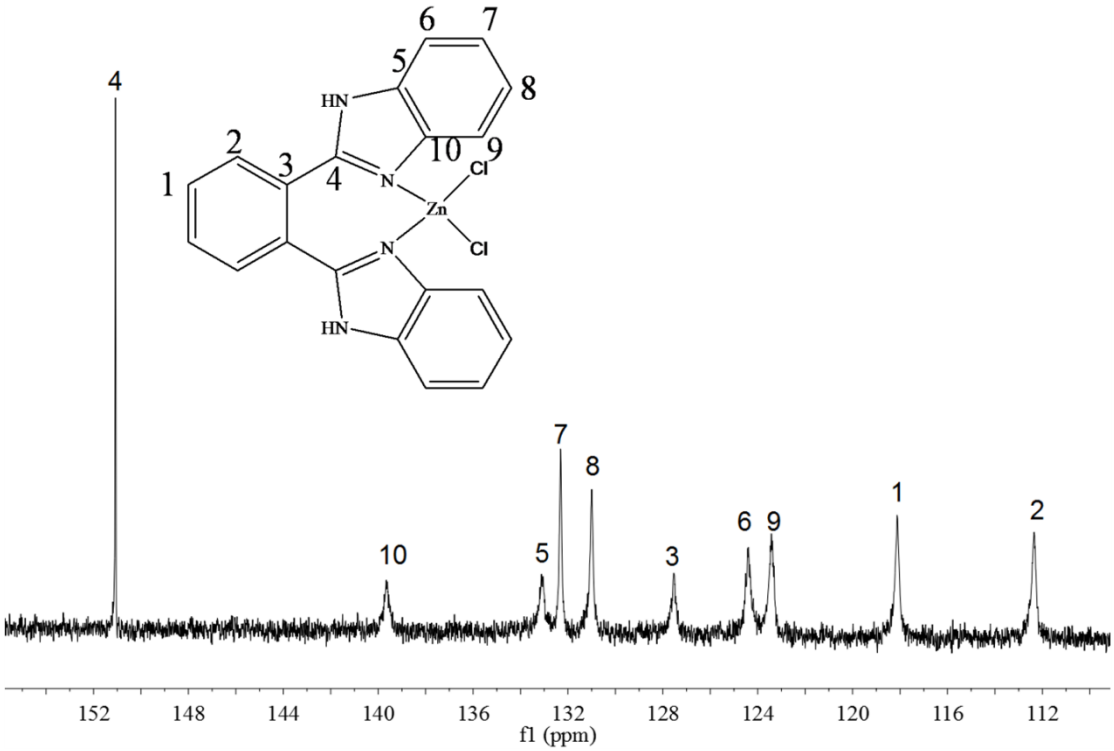


Fig. S3. The ^{13}C NMR spectra of complex **1.**

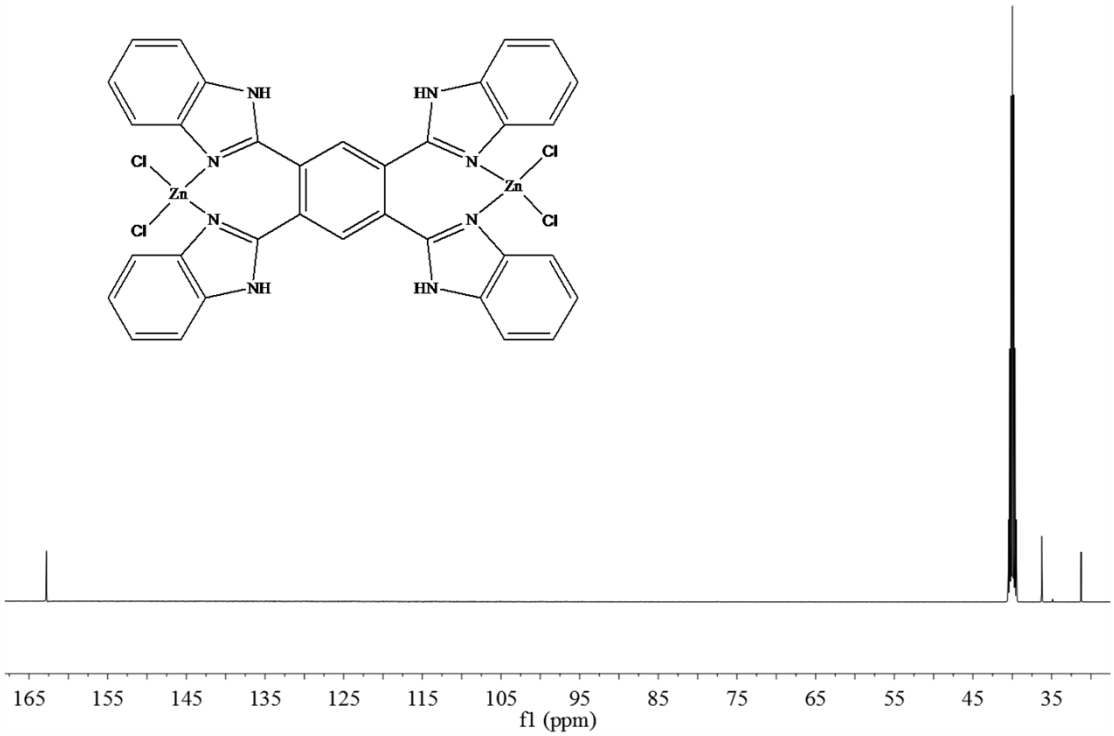


Fig. S4. The ^{13}C NMR spectra of complex **2.**

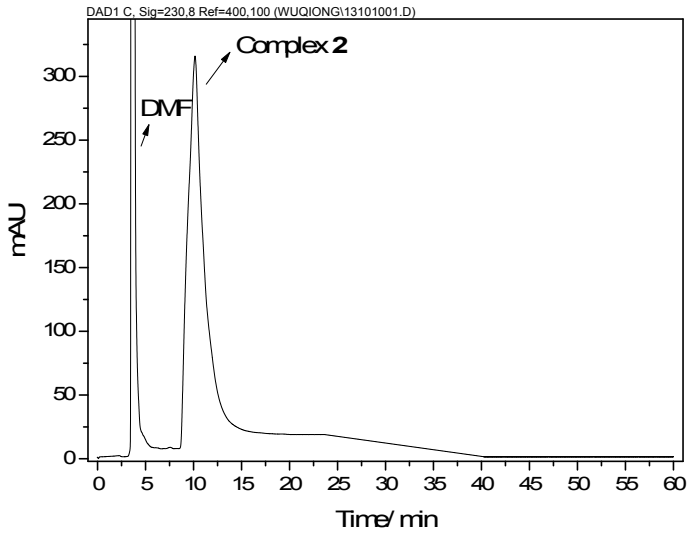


Fig. S5**.** HPLC analysis of complex **2**. The solvent of HPLC was methanol, and the signal was collected at 230 nm.

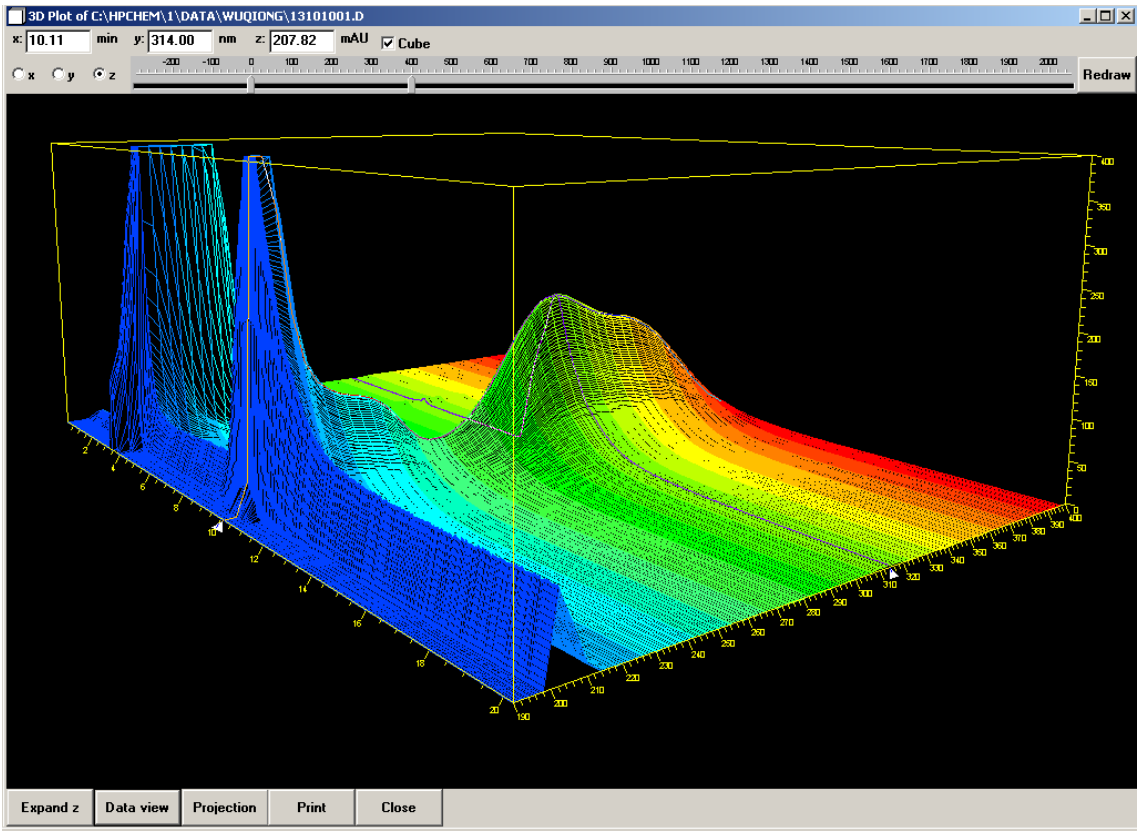


Fig. S6**.** 3D plot of HPLC analysis on complex **2.**

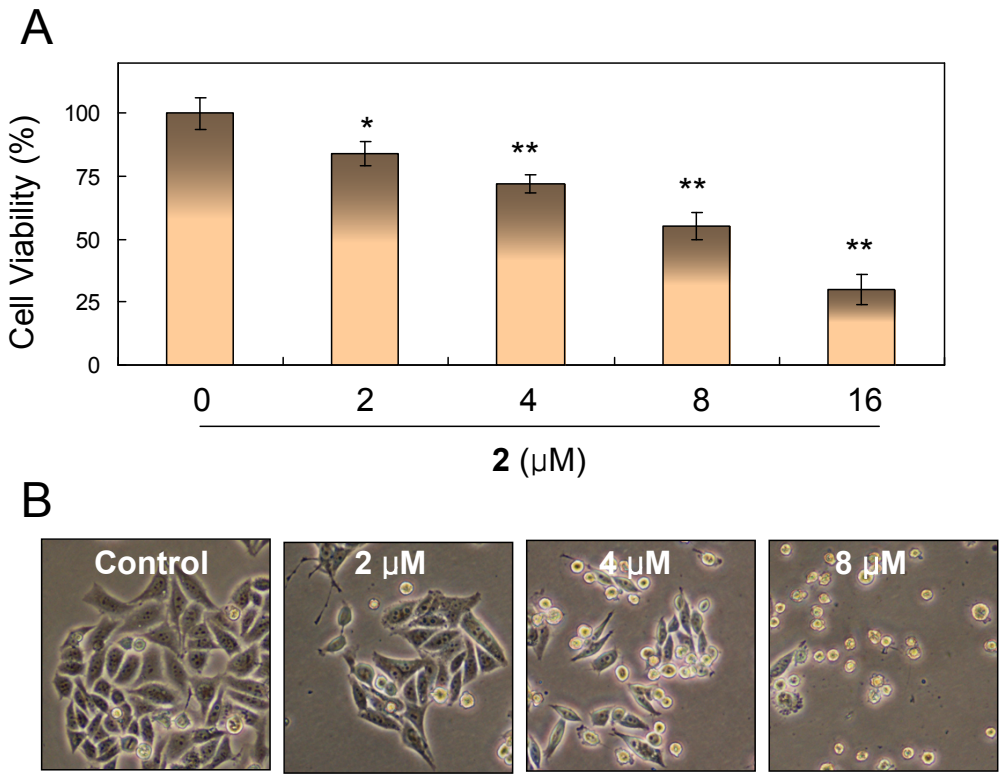


Fig. S7. *In vitro* anticancer activity of dinuclear Zn(II) complex against HCC cells. (A) R-HepG2 cells were treated with complex **2** for 72 h and cell viability was determined by MTT assay. (B) Morphological change induced by complex **2** for 72 h (magnification, 200×). Significant difference between treatment and control groups is indicated at $P<0.05$ (*) and $P<0.01$ (**) level.

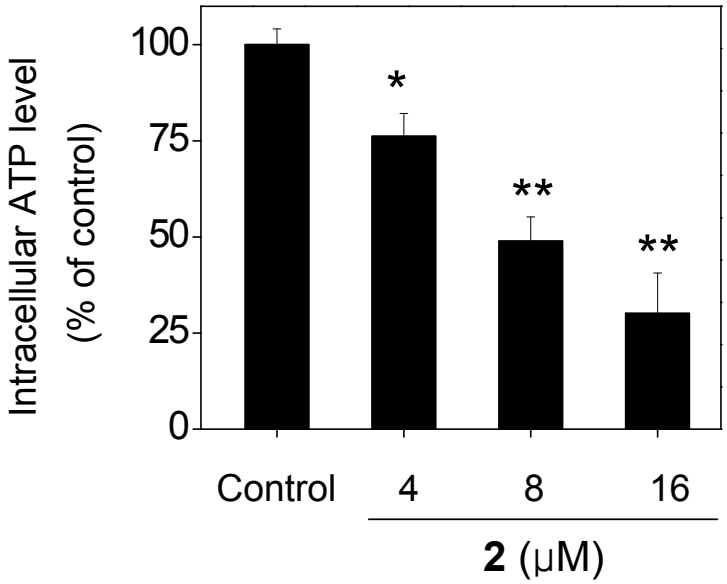


Fig. S8**.** Depletion of intracellular ATP in R-HepG2 cells by dinuclear Zn(II) complex (72 h). Significant difference between treatment and control groups is indicated at $P<0.05$ (*) and $P<0.01$ (**) level.

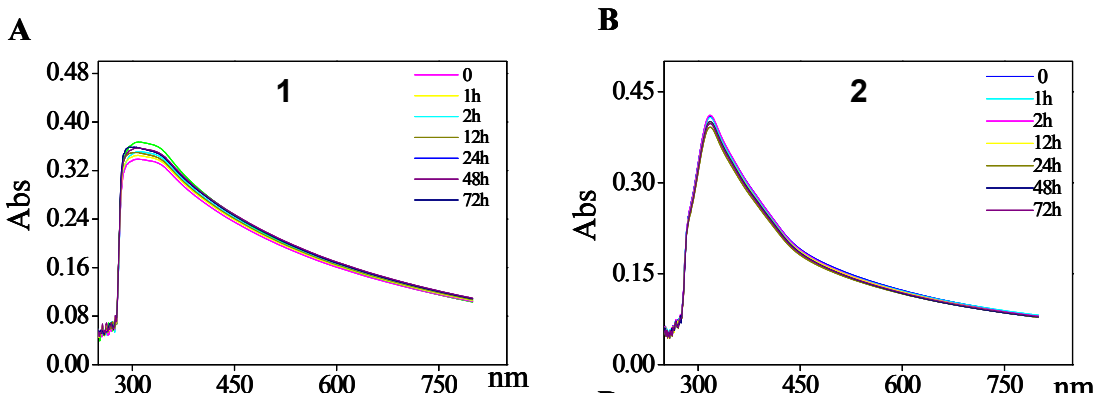


Fig. S9. UV-Vis spectra of complex **1** and **2** (20 μM) in aqueous solution during incubation at 25 ℃ for 72 h.

Table S1. Crystallographic data for complexes **1** and **2**.

	Complex **1**	Complex **2**
Formula	$C_{23}\ H_{23}\ Cl_2\ N_5\ O_2\ Zn$	$C_{46}\ H_{50}\ Cl_4\ N_{12}\ O_4\ Zn_2$
Formula weight	537.73	1107.56
Temperature	173(2) K	173(2) K
Wavelength	0.71073	0.71073
Crystal system	Monoclinic	Monoclinic
space group	P2(1)/c	P2(1)/n
a / Å	12.374	9.8612(10)
b / Å	10.3710	25.265(3)
c / Å	18.186	11.1161(11)
β/ deg	96.074	111.146
V / $Å^3$	2320.7	2583.0(5)
Z	4	4
Density calc.$Mg.m^{-3}$	1.539	1.424
Absorption coefficient (mm^{-1})	1.320	1.189
F(000)	1104	1140
Crystal size	0.46 x 0.22 x 0.18	0.46 x 0.43 x 0.39mm
Theta range for data collection	1.65 to 25.°	1.61 to 25.01°
Limiting indices	-14<=h<=12, -12<=k<=	-11≤h≤11, -30≤k≤27, -
Reflections collected / unique	9873 / 4055 [R(int) =	11185/4534[R(int) =
Data / restraints / parameters	4055 / 0 / 302	4534 / 0 / 341
GOF on F^2	1.056	1.052
Max. and min. transmission	0.7971 and 0.5819	0.6542 and 0.6108
Final R indices [I>2sigma(I)]	R1 = 0.0322, wR2 =	R1 = 0.0295, wR2 =
R indices (all, data) R_1, wR_2	R1 = 0.0476, wR2 =	R1 = 0.0382, wR2 =
Largest diff. peak and hole $e.Å^{-3}$	0.487 -0.306	0.450 and -0.253

Table S2. Selected bond lengths (Å) and angles (º) for the complex **1**.

Zn1-N4	2.003(2)	Zn1-Cl2	2.2278(8)
Zn1-N1	2.013(2)	Zn1-Cl1	2.2479(8)
N4-Zn1-N1	97.28(9)	N4-Zn1-Cl2	112.37(7)
N1-Zn1-Cl2	106.05(7)	N4-Zn1-Cl1	112.64(7)
N1-Zn1-Cl1	114.73(6)	Cl2-Zn1-Cl1	112.71(3)

Table S3. Selected bond lengths (Å) and angles (º) for the complex 2.

Zn1-N1	2.0263(18)	Zn1-N4	2.0360(17)
Zn1-Cl2	2.2131(6)	Zn1-Cl1	2.2173(7)
N1-Zn1-N4	90.87(7)	N1-Zn1-Cl2	114.02(5)
N4-Zn1-Cl2	113.33(5)	N1-Zn1-Cl1	109.36(5)
N4-Zn1-Cl1	109.36(5)	Cl2-Zn1-Cl1	116.45(3)

References

(1) Liu, S.; Cao, W.; Yu, L.; Zheng, W.; Li, L.; Fan, C.; Chen, T. *Dalton Trans* 2013, **42**, 5932-40.

(2) Chen, T.; Wong, Y. S. *Cell Mol Life Sci* 2008, **65**, 2763-75.

(3) Liu, W.; Li, X.; Wong, Y. S.; Zheng, W.; Zhang, Y.; Cao, W.; Chen, T. *ACS Nano* 2012, **6**, 6578-91.

(4) Huang, Y.; He, L.; Liu, W.; Fan, C.; Zheng, W.; Wong, Y. S.; Chen, T. *Biomaterials* 2013, **34**, 7106-16.