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Novel biocompatible formate bridged 1D-Cu(II) coordination polymer induces apoptosis selectively on human lung adenocarcinoma (A549) cells

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Experimental Section:

Materials

Commercially obtained chemicals and solvents were used without further purification. $Cu(NO_3)_2.3H_2O$ (Merck), 3–formyl–6–methylchromone (Sigma–Aldrich), 8–Aminoquinoline (Sigma–Aldrich), 6X loading dye (Fermentas Life Science), and supercoiled pBR322 DNA (E. coli) (Sigma-Aldrich) were utilized as received. The disodium salt of CT-DNA was purchased from Sigma Chem. Co. and was stored at 4 ˚C.

The diagnostic kits, reagents, and other specified chemicals, for cytotoxic studies, were procured from Sigma Chemical Company Pvt. Ltd, St Louis, MO, USA. DMEM, antibiotics/antimycotics solution and FBS were purchased from Invitrogen, Life Technologies (USA). Plastic and Culture wares consumables used in this study were procured from Nunc, Denmark.

Physical measurements

Carbon, hydrogen, and nitrogen contents were carried out on CHN Elemental Analyzer (model: Elementar Vario EL III). Fourier−transform infrared (FT-IR) spectra were recorded on Spectrum Two PerkinElmer FT-IR spectrometers. Electronic spectra were recorded on PerkinElmer Lambda 35 UV−vis spectrometer in MeOH using 1 cm path length cuvette, and data were reported in $\lambda_{max/nm}$. Fluorescence measurements were determined on an RF–5301 PC spectrofluorophotometer (Shimadzu).

DNA binding

To quantify the binding affinity of complex with CT-DNA, the intrinsic binding constant, K_b was calculated from Wolfe–Shimer Eq. (S1), through a plot of [DNA]/ε_a–ε_f vs. [DNA], where [DNA] represents the concentration of DNA, and ε_a, ε_f and ε_b the apparent extinction coefficient (A_{obs}/[complex **1**]), the extinction coefficient for free metal complex, and the extinction coefficient for the free metal complex **1-3** in the fully bound form, respectively.

$$\frac{[DNA]}{\varepsilon_a - \varepsilon_f} = \frac{[DNA]}{\varepsilon_b - \varepsilon_f} + \frac{1}{K_b(\varepsilon_a - \varepsilon_f)} \qquad (S1)$$

The steady state fluorescence data was quantitatively assessed using Stern–Volmer equation [S2]:

$$F_o/F = 1 + K_{sv}[Q] \qquad (S2)$$

where, F_0 is fluorescence intensity of the native HSA; F is fluorescence intensity HSA in presence of complex; K_{SV} is Stern–Volmer constant; and [Q] is concentration of quencher.

Cell Culture

The human lung cancer A549 were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere of 95% of air and 5% CO_2. For evaluation of growth inhibition, cells were seeded in 12-well plates at a concentration of 5,000 cells/well and grown for 24 h in complete medium. Solutions of the compounds were prepared by diluting a freshly prepared stock solution DMSO of the corresponding compound in aqueous media DMEM. Stock solutions of the compounds were stable over several hours. The percentage of DMSO in the culture medium never exceeded 0.2 %: at this concentration, DMSO has no effect on the cell viability. Afterward, the intermediate dilutions of the compounds were added to the wells (200 µL) to obtain a final concentration ranging from 0 to 10 µM, and the cells were incubated for 24 h. After exposure of **PCU1** to the cells and were allowed to grow for the next 24 h, time after which the cells were harvested and analyses were performed to measure cell cycle and apoptosis.

MTT assay

Cell viability was determined by (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay by using a Cell Titre 96® non-radioactive cell proliferation assay kit (Promega, Madison, WI, USA). Briefly, the A549 cells (1×10^4 cells/ well) were allowed to adhere for 24 h in 96-well flat-bottom cell culture plates, and were then exposed to various concentrations. A negative control (DMSO) was also maintained for comparison. After 24 h of the incubation period, 15 µl of MTT reagent was added to each well and further incubated for 3 h at 37 °C. Finally, the medium with MTT solution was removed, and 200 µl of solubilization solution was added to each well and further incubated for 30 min by occasional vortexing. The optical density (OD) of each well was measured at 550 nm by using a Synergy microplate reader (BioTek, Winooski, VA, USA). Results were generated from three independent experiments, and each experiment was performed in triplicate. The percentage of cytotoxicity compared to the untreated cells was estimated in order to determine the IC_{50} value (the concentration at which 50% cell proliferation is inhibited).

Morphological changes analysis

A549 cells were dispensed in a 6-well plate at a density of 1×10^5 cells per well and grown for 24 h. Morphological changes were observed to determine the alterations induced by **PCU1** in A549 cells treated with two concentrations for 24 h. After the end of the incubation period, cells were washed with PBS (pH- 7.4) and observed under a phase-contrast inverted microscope (Olympus IX51, Tokyo, Japan) at 100× magnification, and photographs were captured.

Changes in mitochondrial membrane potential (Δψm)

Rhodamine 123 is a fluorescent probe that binds to metabolically active mitochondria, and a reduction in mitochondrial membrane potential (MMP) is an indicator of the early stages of apoptosis. A549 cells were seeded on a cover-slip-loaded 6-well plate at a density of 1×10^5 cells per well and allowed to adhere for 24 h then treated with the two concentrations of **PCU1** and further incubated for 24 h. The cells were washed with PBS (pH 7.4) and fixed with ice-cold 70% ethanol and incubated with 5μg/mL rhodamine 123 at 37 ℃ for 30 min. Finally, the cells were washed with PBS and were mounted onto a microscope slide in mounting medium, and images were collected using appropriate filter settings in a compound microscope (Olympus BX41, Japan) at 400 X magnification. For the quantitative estimation of MMP, A549 cells were grown and treated in 96-well black bottom microtiter plates. Then the cells were stained with rhodamine 123 as described above. The green fluorescence intensity was detected using a Synergy microplate reader (BioTek, Winooski, VA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The values were averaged from multiple wells and expressed as a percentage of fluorescence intensity relative to the control wells.

Changes in cell nuclear morphology

Cell nuclear morphology and chromatin condensation was evaluated by fluorescence microscopy following Hoechst 33342 staining. A549 cells were seeded on a cover-slip-loaded 6-well plate at a density of 1×10^5 cells per well and allowed to adhere for 24 h then treated with the two concentrations of **PCU1** and further incubated for 24 h. The cells were washed with PBS (pH7.4), fixed with ice-cold 70% ethanol, and 1 μM Hoechst 33342 was added to the cells and incubated for 15 min at 37°C. Finally, the cells were washed with PBS and were mounted onto a

microscope slide in mounting medium and images were captured using excitation and emission filter settings (345 nm - 460 nm) in a compound microscope (Olympus BX41, Japan) at 400 X magnification.

Apoptotic morphological changes by acridine orange – ethidium bromide dual staining method:

Acridine orange and Ethidium bromide dual staining method was adopted to differentiate between condensed apoptotic or necrotic nuclei from normal cells. A549 cells were seeded on a cover-slip-loaded 6-well plate at a density of 1×10^5 cells per well and allowed to grow overnight. Then the cells were exposed to the two sub-lethal concentrations of **PCU1** and further incubated for 24 h. After the end of the incubation period, cells were washed twice using PBS to remove the remaining media. Then, cells were stained by adding equal volumes of AO and PI (20 μg/mL in PBS). Finally, after washing with PBS, the stained cells were mounted onto a microscope slide in mounting medium, and images were collected using appropriate filter settings in a compound microscope (Olympus BX41, Japan) fitted with fluorescence attachment and CCD camera. Quantification of apoptotic and necrotic cells based on the uptake of acridine orange and propidium iodide in more than 300 cells was performed.

Detection of autophagy by acridine orange staining

As a marker of autophagy induction, acidic vesicular organelles (AVOs), which consist predominantly of autophagosomes and autolysosomes, were observed by fluorescence microscopy after staining of cells with acridine orange (AO). AO is a weak fluorescent base that accumulates in acidic vesicular spaces and fluoresces bright red. The intensity of the red fluorescence is proportional to the degree of AVO formation. A549 cells were seeded on a cover-slip-loaded 6-well plate at a density of 1×10^5 cells per well and allowed to grow overnight. Then the cells were exposed to the two sub-lethal concentrations of **PCU1** and further incubated for 24 h. Then, the cells were washed twice with PBS and then incubated with 5 µg/ ml working solution of AO in a serum-free medium at room temperature for 15 min in the dark. Finally, the AO stained cells were observed in a compound microscope (Olympus BX41, Japan) fitted with fluorescence attachment and a CCD camera.

Apoptosis, Cell cycle

The human lung cancer A549 were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere of 95% of air and 5% CO_2. For evaluation of growth inhibition, cells were seeded in 12-well plates at a concentration of 5,000 cells/well and grown for 24 h in complete medium. Solutions of the compounds were prepared by diluting a freshly prepared stock solution DMSO of the corresponding compound in aqueous media DMEM. Stock solutions of the compounds were stable over several hours. The percentage of DMSO in the culture medium never exceeded 0.2 %: at this concentration, DMSO has no effect on the cell viability. Afterward, the intermediate dilutions of the compounds were added to the wells (200 µL), and the cells were incubated for 24 h. After exposure of **PCU1** to the cells and were allowed to grow for the next 24 h, time after which the cells were harvested, and analyses were performed to measure cell cycle and apoptosis.

Antiproliferative activity:

Cell culture and treatment:

The human lung cancer cell line A549 grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) and 10 µg of streptomycin mL^{-1} at 37 °C in 5% CO_2. In the treatment group, A549 cells were treated for 48 h with tested complexes along with control alone (DMSO).

Cell proliferation Assay:

A549 cells were plated in quadruplicate at a density of ~60,000 cells per well in 2 mL of respective culture media in six-well plates. Cells were grown in the regular medium with 5% FBS in control and treated with nanoparticles. The growth curve was analyzed at a dose of 35 µM. Cells were trypsinized, and the number of viable cells was determined by 0.4% trypan blue and counted at different time points starting from day 0 to 48 h, using a Coulter Counter.

Quantitative real-time RT-PCR:

Total cellular RNA from control and nanoparticle treated A549 cells were extracted using Trizol according to the manufacturer's protocol. Two micrograms of total RNA were used for cDNA preparation. The cDNA was then used to quantify different genes by real-time PCR using

MMP2, TGF-β and GAPDH specific primers (Khan et al. 2014). The Ct (threshold cycle) value of each sample was calculated from the threshold cycles with the instrument's software (SDS 2.3), and the relative expression of MMP2, TGF-β mRNA was normalized to the GAPDH value. Data w analyzed using the comparative threshold cycle (2- ΔCT) method.

Statistical analysis

All experiments were carried out with three independent replicates, and values are presented as mean ± standard error of the mean (SEM). Data were statistically analyzed using the Student's *t*-test for comparison between the means of applying a significance level of $P < 0.05$.

Synthesis of ligand (L)

To a solution of 3-formyl-6-methylchromone (0.188 g, 1 mmol) in methanol (10 mL) was added 8-aminoquinoline slowly (0.144 g, 1 mmol) dissolved in methanol (7 mL). The ligand appears immediately as a yellow colored precipitate; the mixture was stirred for 1 h at 70 °C. The resulting yellow colored precipitate was filtered, washed with ether and hexane. Yield 76.5 %; M.P. 122 °C; Anal. Calc. for $C_{21}H_{17}N_2O_3$: C, 73.03; H, 4.96; N, 8.11%. Found: C, 72.98; H, 4.88; N, 8.06%. IR (KBr, cm^{-1}): 3434 (br, s), 2401 (br, m), 1652 (m), 1616 (s), 1560 (s), 1454 (s), 1396 (s), 1278 (s), 1159 (s), 1065 (s), 977 (s), 818 (m), 791 (s), 746 (s). ^{1}H NMR (400MHz, DMSO–d_6, δ, 297.7 K): 13.03 (br, s, 1H, Phenolic OH), 9.03 (dd, 1H, *J*= 4.4 Hz, H1,), 8.44 (dd, 1H, *J*= 8.4 Hz, H3), 8.37 (d, 1H, *J*= 12.8 Hz, H4), 7.88 (dd, 1H, *J*= 8.8 Hz, H6), 7.69 (m, 4H, H2, H5, H10, H15), 7.37 (dd, 1H, *J*= 8.4 Hz, H17), 7.03 (d, 1H, *J*= 8.0 Hz, H14), 3.35 (s, 3H, CH3). ^{13}C NMR (100MHz, DMSO–d_6; δ, 297.7 K): 180.29 (C19), 153.44 (C12), 149.59 (C10), 142.69 (C1), 137.52 (C8), 136.40 (C13), 136.01 (C9), 135.16 (C3), 130.91 (C16), 128.36 (C4), 126.99 (C15), 125.58 (C18), 122.55 (C17), 122.49(C6), 122.01 (C5), 117.81 (C7), 110.65(C14), 104.98(C2), 101.27 (C11), 20.21 (C20). UV– vis (1×10^3 M, MeOH, nm): 210, 269.

Table S1. Bond lengths (Å) of **PCU1**.

Atom	Atom	Length/Å	Atom	Atom	Length/Å
Cu1	O2	1.943(4)	C13	C19	1.400(8)
Cu1	O1	1.944(4)	C13	C14	1.392(8)
Cu1	O3[1]	2.362(5)	C12	C11	1.416(8)
Cu1	N1	1.946(5)	C1	C2	1.381(7)
Cu1	N2	2.007(6)	C1	C6	1.425(9)
O2	C22	1.257(7)	C11	C20	1.489(8)
O1	C12	1.280(7)	C19	C18	1.372(9)
O4	C19	1.371(7)	C2	C3	1.393(9)
O4	C20	1.437(7)	C14	C15	1.373(9)
O5	C20	1.405(7)	C6	C5	1.413(9)
O5	C21	1.433(7)	C9	C8	1.397(9)
O3	C22	1.229(7)	C18	C17	1.376(8)
N1	C10	1.307(7)	C5	C7	1.404(9)
N1	C1	1.389(8)	C5	C4	1.419(9)
N2	C6	1.372(7)	C17	C15	1.385(9)
N2	C9	1.315(7)	C7	C8	1.370(8)
C10	C11	1.380(8)	C15	C16	1.521(8)
C13	C12	1.469(9)	C4	C3	1.350(9)

Table S2. Bond angles of **PCU1**.

Atom	Atom	Atom	Angle/°	Atom	Atom	Atom	Angle/°
O2	Cu1	O1	90.18(18)	C2	C1	C6	117.4(7)
O2	Cu1	O3[1]	91.32(15)	C10	C11	C12	124.5(6)
O2	Cu1	N1	173.9(2)	C10	C11	C20	118.8(6)
O2	Cu1	N2	93.2(2)	C12	C11	C20	116.8(6)
O1	Cu1	O3[1]	92.67(18)	O4	C19	C13	121.3(7)
O1	Cu1	N1	93.5(2)	O4	C19	C18	118.0(6)
O1	Cu1	N2	171.5(2)	C18	C19	C13	120.7(6)
N1	Cu1	O3[1]	93.36(19)	C1	C2	C3	120.5(7)
N1	Cu1	N2	82.6(2)	C15	C14	C13	122.9(7)
N2	Cu1	O3[1]	95.04(19)	O3	C22	O2	127.7(7)
C22	O2	Cu1	116.5(5)	O4	C20	C11	115.0(5)
C12	O1	Cu1	125.1(4)	O5	C20	O4	109.1(6)
C19	O4	C20	115.3(5)	O5	C20	C11	108.1(6)
C20	O5	C21	113.5(5)	N2	C6	C1	116.3(6)
C22	O3	Cu1[2]	124.5(4)	N2	C6	C5	122.1(6)
C10	N1	Cu1	123.5(5)	C5	C6	C1	121.6(7)
C10	N1	C1	121.3(6)	N2	C9	C8	123.9(7)

C1	N1	Cu1	114.5(4)	C19	C18	C17	119.9(7)
C6	N2	Cu1	112.1(4)	C6	C5	C4	118.3(7)
C9	N2	Cu1	129.9(5)	C7	C5	C6	117.3(7)
C9	N2	C6	118.0(6)	C7	C5	C4	124.4(8)
N1	C10	C11	126.0(6)	C18	C17	C15	121.5(7)
C19	C13	C12	120.8(6)	C8	C7	C5	120.1(8)
C14	C13	C12	121.5(6)	C14	C15	C17	117.7(7)
C14	C13	C19	117.3(7)	C14	C15	C16	122.8(7)
O1	C12	C13	118.5(6)	C17	C15	C16	119.5(7)
O1	C12	C11	125.0(7)	C3	C4	C5	119.1(7)
C11	C12	C13	116.5(6)	C7	C8	C9	118.5(7)
N1	C1	C6	114.5(6)	C4	C3	C2	123.0(7)
C2	C1	N1	128.1(6)				

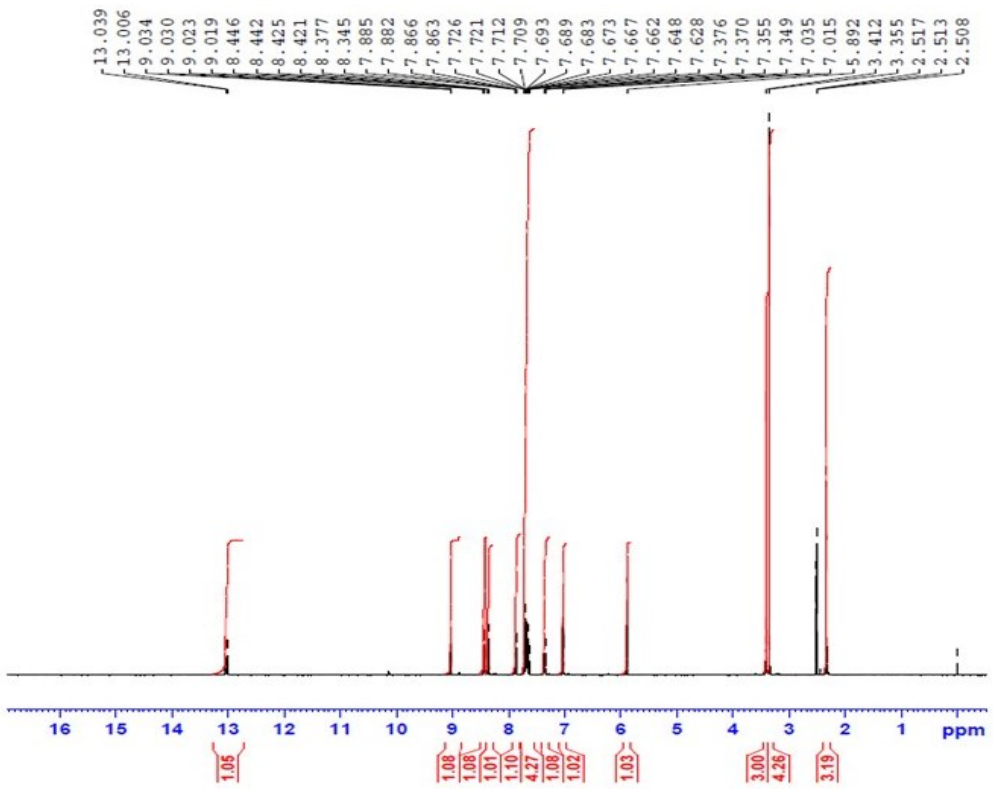


Fig. S1. ^{1}H NMR spectrum of ligand (L).

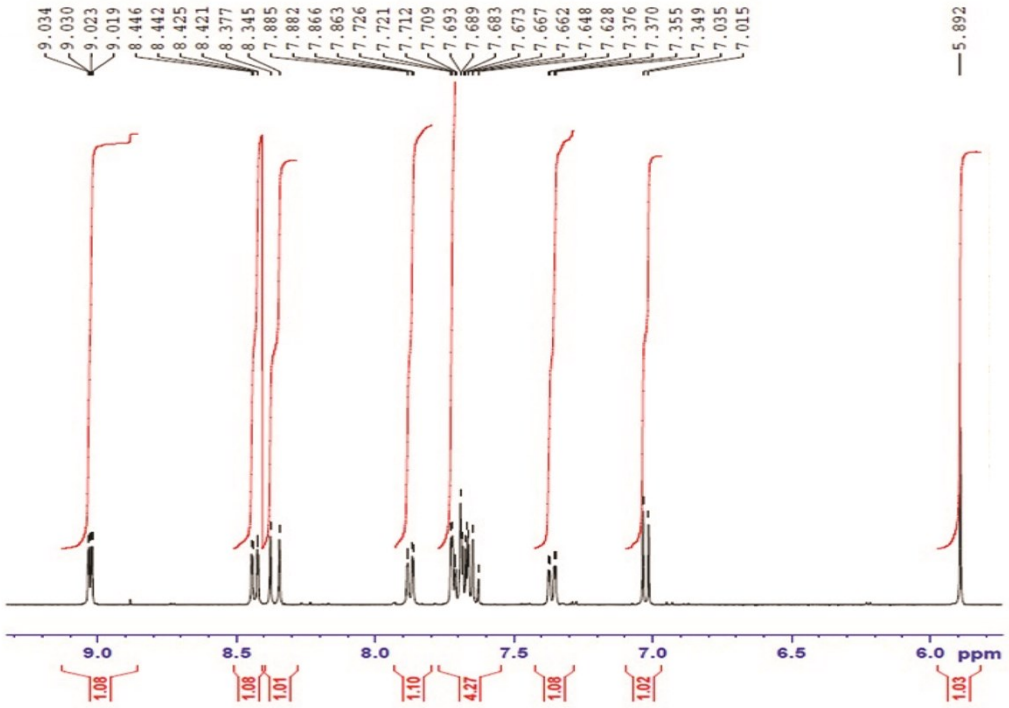


Fig. S2. ^{1}H NMR spectrum of ligand (L).

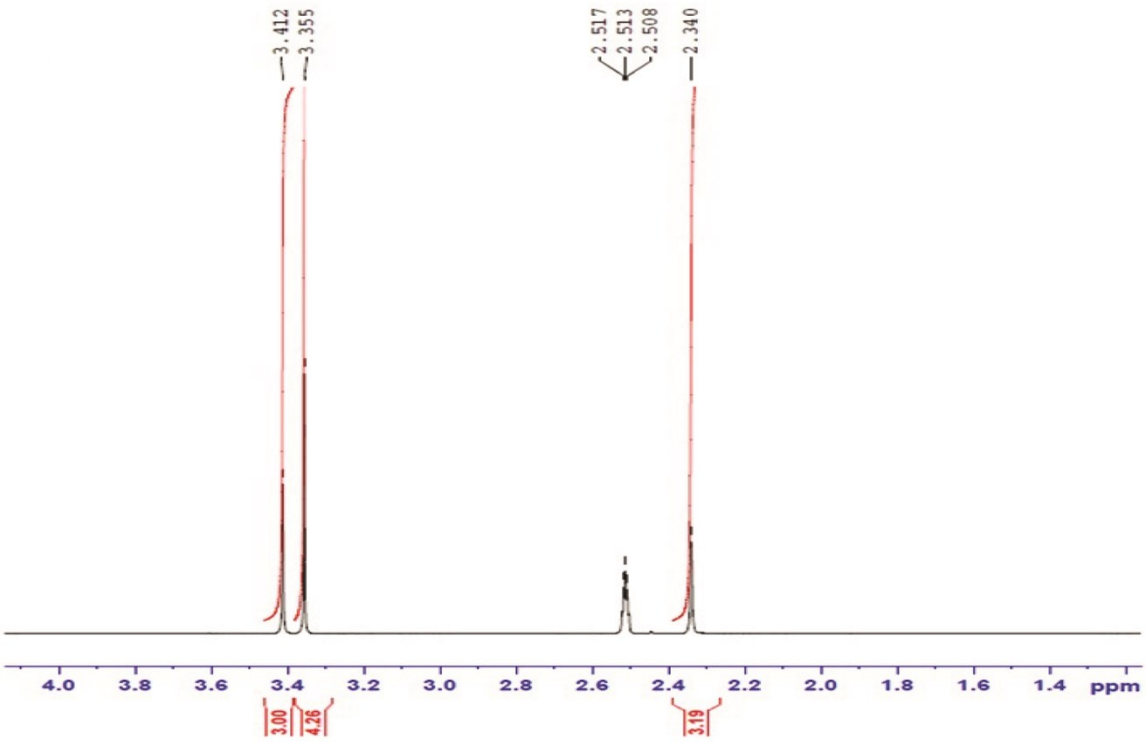


Fig. S3. ^{1}H NMR spectrum of ligand (L).

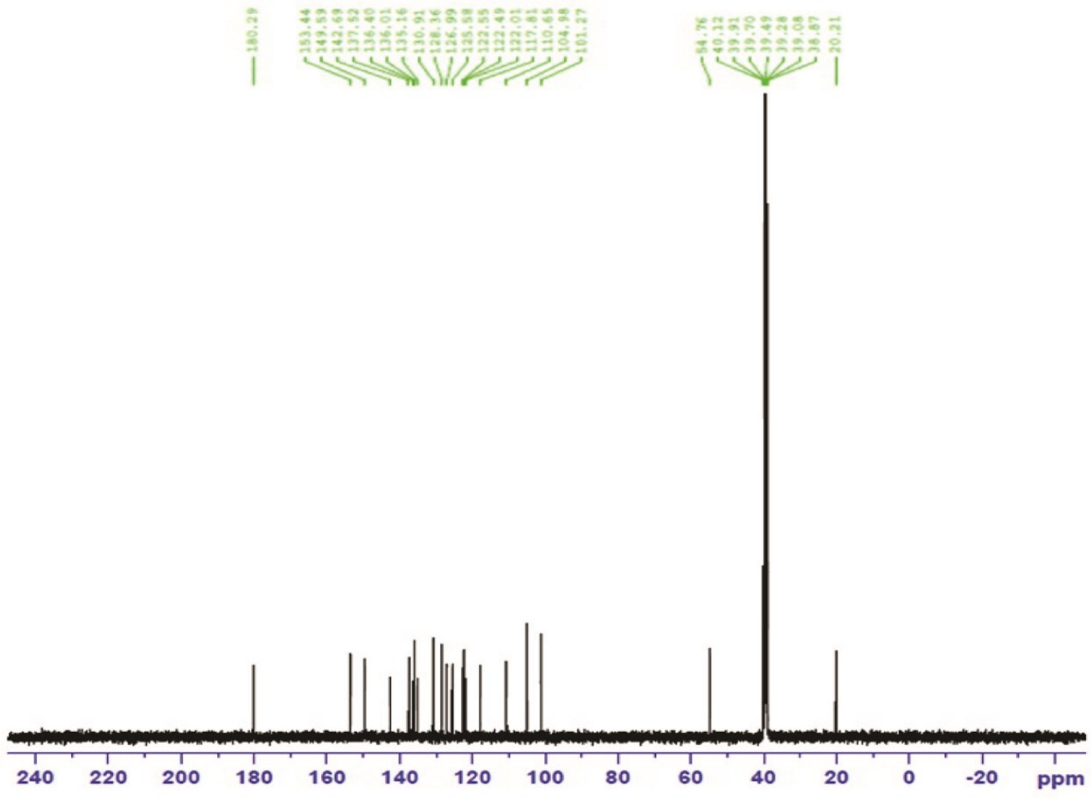
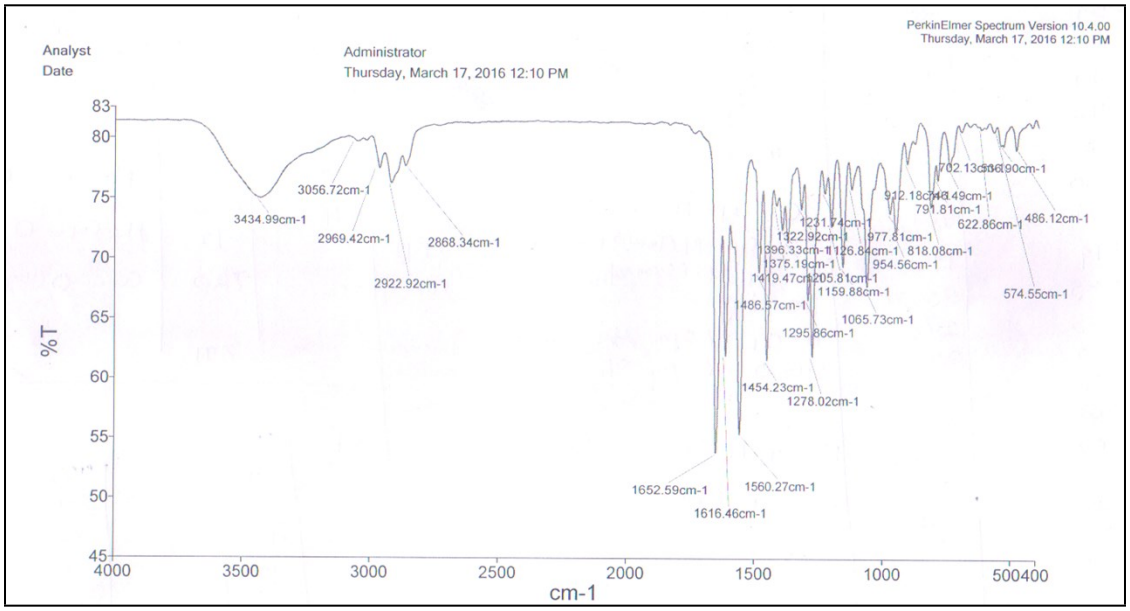


Fig. S4. ^{13}C NMR spectrum of ligand (L).

(a)



(b)

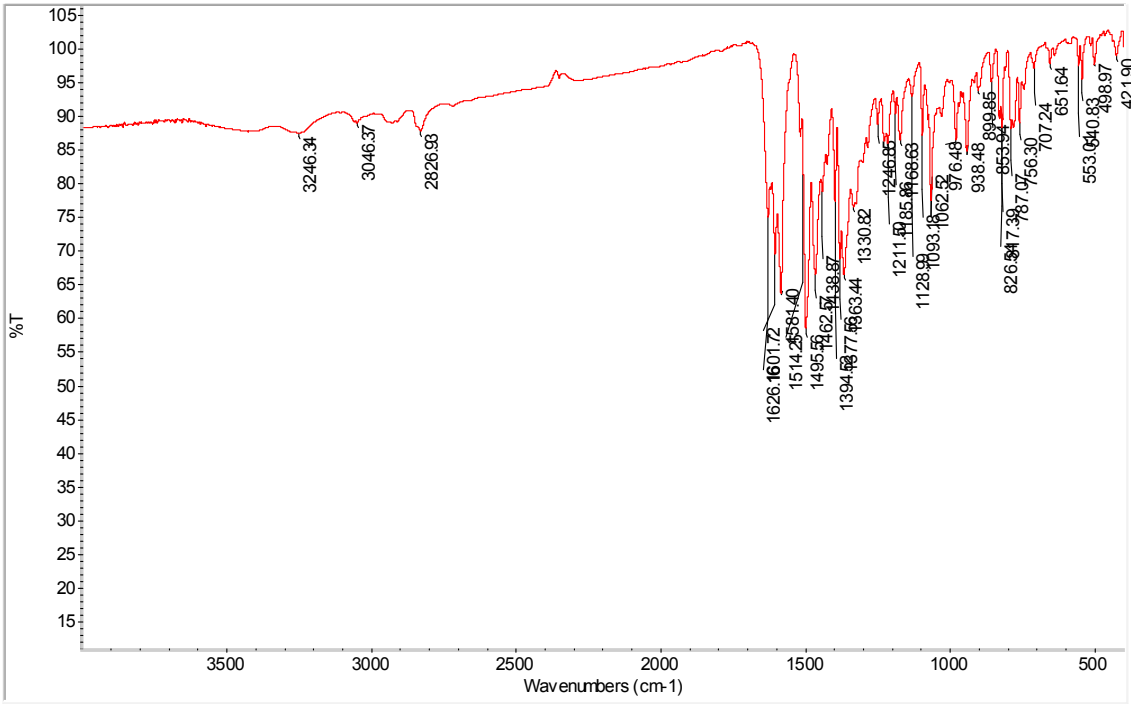


Fig. S5. FTIR spectra of (a) **Ligand** and (b) **PCU1**.

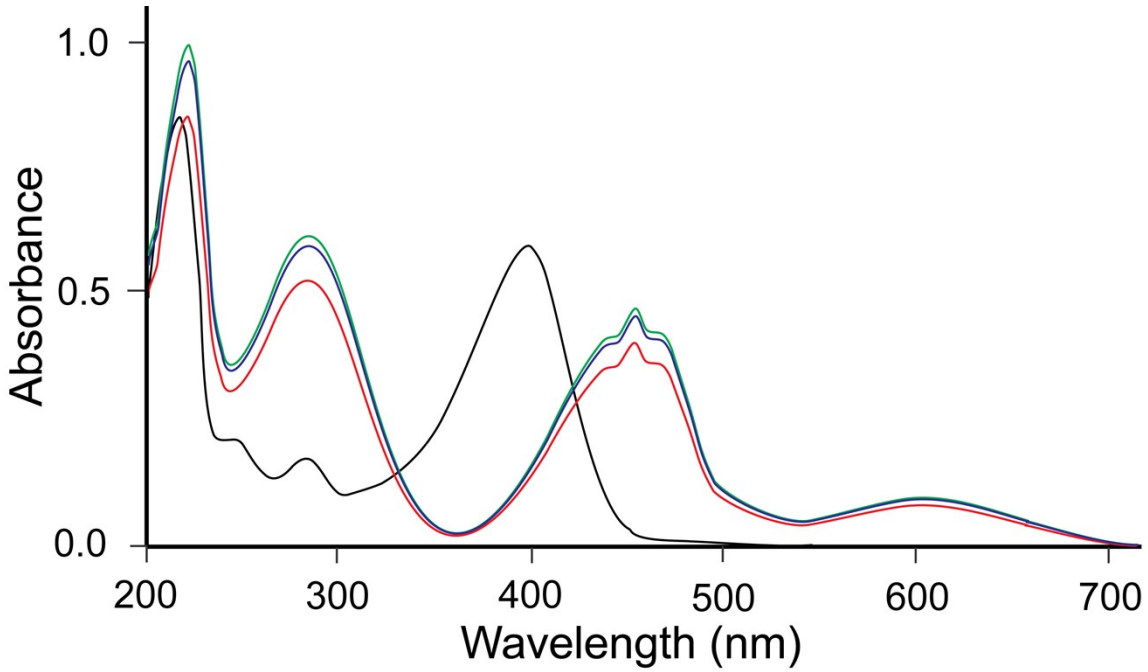


Fig. S6. UV-vis spectra of ligand (black), PCU1 in DMSO (green), and stability of **PCU1** in 10% DMSO+ 90% H_2O (blue) and 1% DMSO+Tris-HCl buffer(red) after 12h.

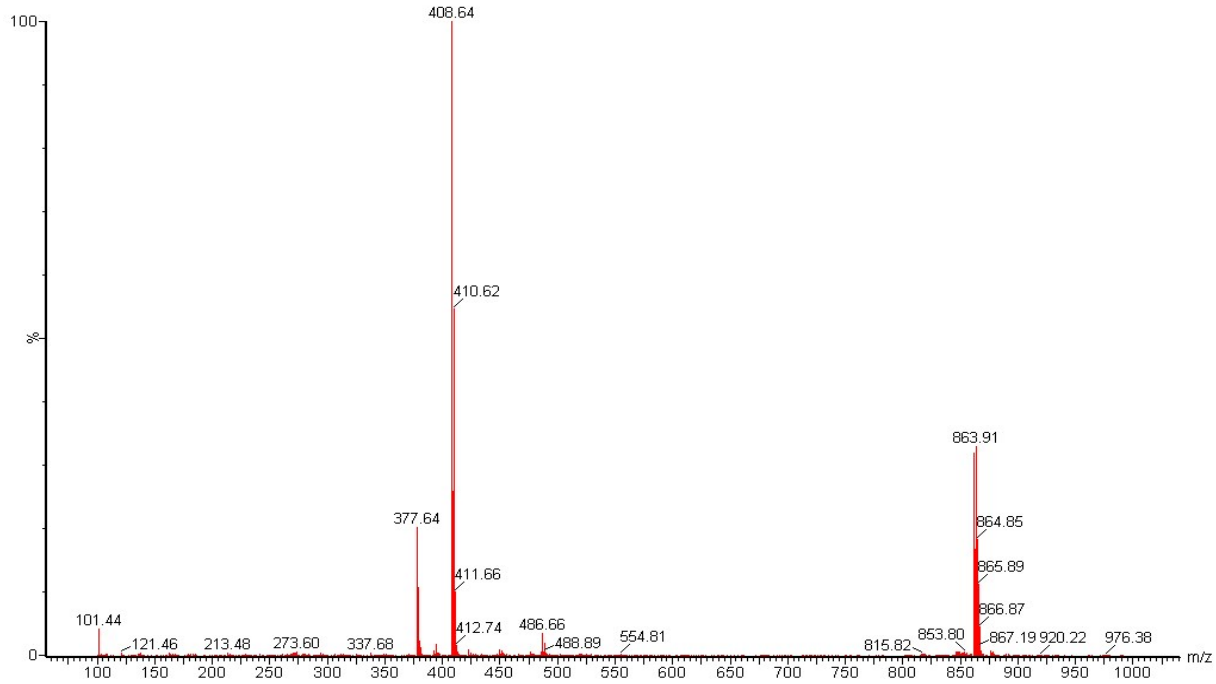


Fig. S7. ESI-Mass spectrum of complex **PCU1**.

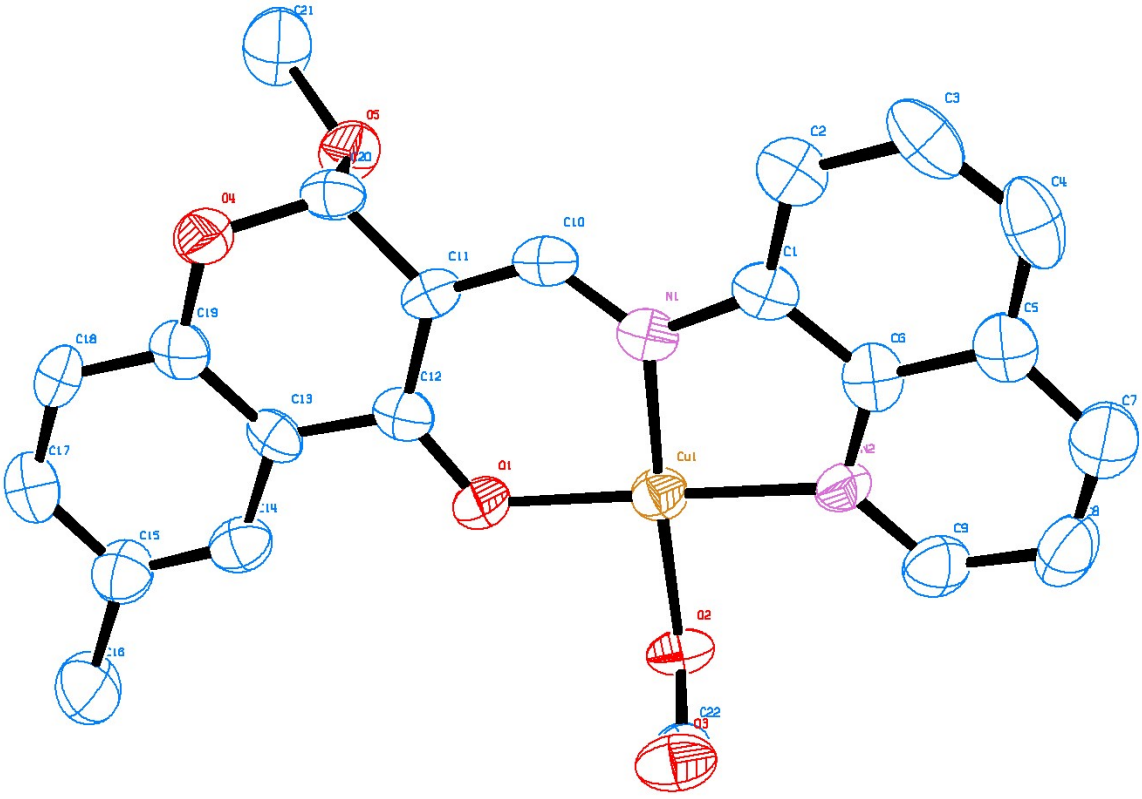


Fig. S 8. The ortep view of the **PCU1** with atomic numbering scheme with 50% ellipsoid and hydrogen atoms was omitted for the clarity of the structure.

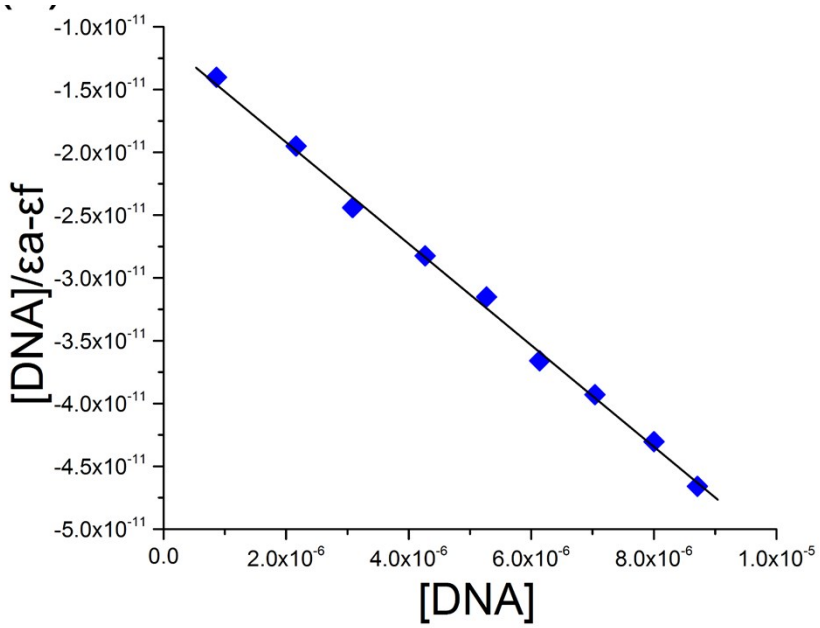


Fig. S9. Plots of [DNA]/ $\varepsilon_a - \varepsilon_f$ (M^2 cm) vs [DNA] for the titration of CT DNA with complex **PCU1** , experimental data points; full lines, linear fitting of the data. [Complex] = 3.33×10^{-5} M, [DNA] = (0–8.53) × 10^{-5} M. Arrow shows change in intensity with increasing concentration of DNA.

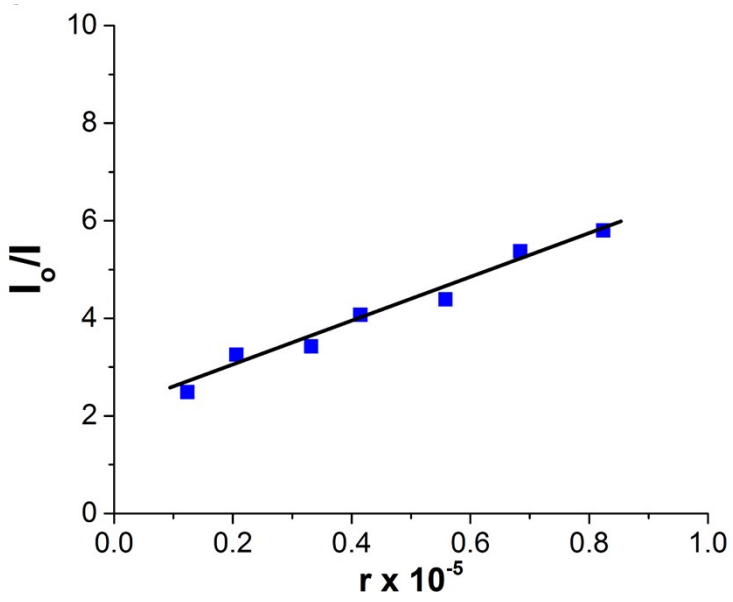


Fig. S10. Emission quenching plot of CT DNA bound ethidium bromide in the presence of complex **PCU1**, in buffer 5 mM Tris–HCl/50 mM NaCl, pH=7.2 at 25 ºC.

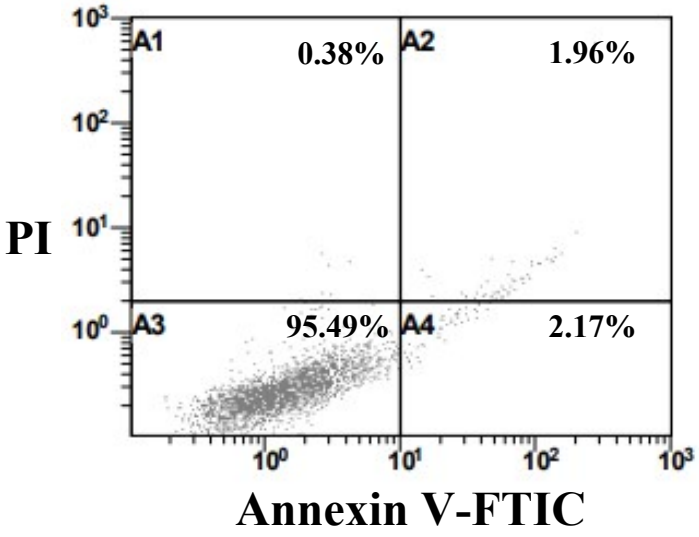


Fig. S11. The quantitative insight of apoptosis induced in A549 cells (control/untreated) by flow cytometry analysis.