Supporting Information

Nuclease Activity via Self-Activation and Anticancer Activity of Mononuclear Copper(II) Complex: Novel Role of Tertiary Butyl Group in the Ligand Frame

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Reagents and materials

Phenylhydrazine, (S. D. Fine, Mumbai, India), 3, 5-Di-*tert*-butyl-2-hydroxybenzaldehyde (Sigma Aldrich, Steinheim, Germany), ethylenediamine tetraacetic acid and cupric chloride dihydrate (Merck Limited, Mumbai, India), sodium hydride, 2–chloropyridine (Acros organics, USA) were used as obtained. The supercoiled pBR322 DNA was purchased from Bangalore Genei (India) and stored at 4 °C. Agarose (molecular biology grade) and ethidium bromide were obtained from Sigma Aldrich. TBE (Tris–boric acid-EDTA) buffer (pH 8.2) and phosphate buffer (pH 7.2) were prepared in deionised water. 2-(1-phenylhydrazinyl)pyridine was prepared according to the reported method.[1]

Physical Measurements. Elemental analyses were carried microanalytically at Elementar Vario EL III. IR spectra were obtained as KBr pellets with Thermo Nicolet Nexus FT–IR spectrometer, using 16 scans and were reported in cm^{-1}. Electronic absorption spectra were recorded in acetonitrile solvent with an Evolution 600, Thermo Scientific UV–visible spectrophotometer. ^1H NMR spectrum was recorded on Bruker AVANCE, 500.13 MHz spectrometer in the deuterated dichloromethane. GC-MS data were obtained on a

quadrupole Perkin Elemer Clarus 500 MS coupled to a Perkin Elemer Clarus 500 GC with Elite-1 column and mass detector was operated at 70 eV. Molar conductivity was determined in dimethylformamide (DMF) at 10^{-3} M at 25 °C with a Systronics 304 conductometer. Cyclic voltammetric studies were performed on a CH–600 electroanalyser in DMF with 0.1 M tetrabutylammonium perchlorate (TBAP) as supporting electrolyte. The working electrode, reference electrode and auxiliary electrode were glassy carbon, Ag^+/AgCl and Pt wire electrode respectively. The concentration of the complex was in the order of 10^{-3} M. The ferrocene/ferrocenium couple occurs at $E_{1/2}$ = + 0.44 (80) V *vs* Ag^+/AgCl under the same experimental conditions.

Synthesis of (E)-2, 4-Di-*tert*-butyl-6-((phenyl(pyridin-2-yl)hydrazono)methyl)phenol (^{t}BuPhimpH): 3, 5-Di-*tert*-butyl-2-hydroxybenzaldehyde (0.234 g, 1.00 mmol) and 2-(1-phenylhydrazinyl)pyridine (0.185 g, 1.00 mmol) were dissolved in 10 mL of methanol. The reaction mixture was stirred at room temperature. Within 1 h, a white solid began to separate out and stirring was continued for another 3 h. The white precipitate was filtered, washed thoroughly with methanol, diethyl ether and then dried *in vacuo* and recrystallized in dichloromethane. Yield: (68%). GC-MS (methanol, *m/z*): 401 M^+ (4%), 169 (100%). Anal. Calcd for $C_{26}H_{31}N_3O$: C, 77.77; H, 7.78; N, 10.46, Found: C, 77.38; H, 7.65; N, 10.39. IR data [KBr, ν_{max}/cm^{-1}]: 3446 (ν_{OH}), 2949, 2918 ($\nu_{tert\text{-}butyl}$), 1612 ($\nu_{C=N}$), 1587, 1470, 1440, 1321, 1213, 766 cm^{-1}. UV–Vis [acetonitrile, λ_{max}/nm (ε/M^{-1}cm^{-1})]: 315 (19560), 343 (20440). ^{1}H NMR [$CDCl_3$, δ/ppm]: 1.25 (s, 9H), 1.50 (s, 9H), 6.78 (s, 1H), 6.84 (s, 1H), 7.21 (d, J = 7.55 Hz, 1H), 7.30 (d, J = 5.75 Hz, 3H), 7.44 (s, 1H), 7.53(t, J = 7.45 Hz, 1H), 7.64 (t, J = 7.70 Hz, 3H), 8.23 (d, J= 3.55 Hz, 1H), 11.75 (s, 1H).

Synthesis of [Cu(^{t}BuPhimp)(Cl)]·CH_3OH (1·CH_3OH): A batch (0.401 g, 1.0 mmol) of ^{t}BuPhimpH was dissolved in 8 mL of methanol and a solution of $CuCl_2·2H_2O$ (0.171 g, 1 mmol) with 3 mL of methanol was added dropwise to this solution. Immediately, we got a green solution and after 2 h of constant stirring, kept the solution for evaporation. Within three days we got square shaped green colored crystals which were suitable for single crystal X-ray diffraction. Yield: (67%). ESI-MS (acetonitrile, *m/z*): 503.6 [^{65}Cu(^{t}BuPhimp)^{37}Cl] (100%). Anal. Calcd for $C_{27}H_{34}N_3O_2ClCu$: C, 61.01; H, 6.45; N, 7.90, Found: C, 61.23; H, 6.52; N, 7.95. IR data [KBr, ν_{max}/cm^{-1}]: 3496 (ν_{OH}), 2958 and 2922 ($\nu_{tert\text{-}butyl}$), 1611 ($\nu_{C=N}$), 1565, 1479, 1447, 1340, 1171, 770 cm^{-1}. UV–Vis [acetonitrile, λ_{max}/nm (ε/M^{-1}cm^{-1})]: 282 (10330), 322(11278), 422 (8075), 650 (370). Conductivity [DMF, Λ_M/Ω^{-1}cm^{2}mol^{-1}]: 23.

Generation of the Phenoxyl Radical Complex. The phenoxyl radical species of the copper complex [Cu(^{t}BuPhimp)(Cl)] (**1**) was generated in situ by adding $(NH_4)_2[Ce^{IV}(NO_3)_6]$ (CAN, 50-200 μM) into a acetonitrile solution of this phenolate complex (100 μM) at room temperature. The decomposition rate of phenoxyl radical complex [**1**]$^{\bullet+}$ was monitored by a time dependent study at λ value of 565 nm.

DNA Cleavage Experiments. DNA cleavage was measured by the conversion of supercoiled (SC) pBR322 plasmid DNA to nicked circular (NC) and linear DNA forms (LC) in TBE (Tris–boric acid-EDTA) buffer (pH 8.2). Supercoiled pBR322 DNA (40 ng) was incubated at 37 °C with complex **1** (10 % acetonitrile) in the absence of any oxidizing or reducing agent. For mechanistic investigation, the complex **1** was also incubated with

radical scavengers like DMSO (20 mM), urea (20 mM), ethanol (20 mM), NaN_3 (20 mM), L-Histidine (20 mM), D_2O (20 mM) and catalase (1 U). The nuclease activity was also investigated in presence of varying concentrations of neocuproine (50-400 µM), berberine chloride (10-100 µM), and NaCl (50-400 µM). After incubation we added loading buffer (25% bromophenol blue and 30% glycerol). The agarose gel (0.8%) containing 0.4 µg/mL of ethidium bromide (EB) was prepared and the electrophoresis of the DNA cleavage products was performed on it. The gel was run at 60 V for 2 h in TBE buffer and the bands were identified by placing the stained gel under an illuminated UV lamp. The fragments were visualized by a UV illuminator (BIO RAD).

Procedure for maintaining anaerobic conditions:

(1) All the vials were filled with the requisite amount of acetonitrile, buffer and DNA and each vial was purged separately with nitrogen prior to the addition of the compound and sealed with parafilm.

(2) The stock solution of compound was prepared and purged with nitrogen thoroughly and sealed with parafilm.

(3) Now, the solution of the compound was placed on the wall inside the vial. The vial was again purged with nitrogen, the solution was mixed, again purged and parafilm was again applied for the incubation period.

(4) The procedure was repeated with each vial.

X-ray Crystallography. The X-ray data collection and processing for **1**·CH_3OH was performed on Bruker Kappa Apex-II CCD diffractometer by using graphite monochromated Mo-*Kα* radiation (λ = 0.71070 Å) at 273 K. Crystal structure was solved by direct methods.

Structure solution, refinement and data output were carried out with the SHELXTL program.[2,3] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in geometrically calculated positions and refined using a riding model. Images were created with the DIAMOND program.[4] Crystal data collection and refinement details of the structure of complex **1**·CH_3OH were summarized in Table S1.

During structure solution we encountered two different aspects which we would like to mention here. First, the molecule was found to be dinuclear (output file of check cif) and the Cu-Cl distances were 2.2479(3) Å and 3.057 Å, so the distance between the copper centre of one molecule and the Cl atom of the other molecule is beyond standard Cu-Cl bond distance.[5] Hence we describe this molecule as mononuclear. Second, in the assignment of solvent molecule, we encountered hard shapes. However, we described these Q peaks as two methanol molecules.

It has been found out that in the crystal lattice two molecules of **1**·CH_3OH constituted a roughly planar Cu_2Cl_2 unit (Figure S2). The distances between the Cu(1) of one molecule and Cl(1) from another molecule were 3.057 Å. Hence each copper atom was bonded to two chloride ions having distances 2.2479(3) Å and 3.057 Å giving rise to a planar Cu_2Cl_2 moiety. The Cu---Cu distance was 3.684 Å and this distance was close to the distances obtained from real chloro bridged dimeric copper complexes.[5,6] Considering complex **1**·CH_3OH with another molecule in the packing diagram (Figure S2), each Cu atom was penta-coordinated with distorted square-pyramidal geometry. The apical position at both the Cu(1) centre was occupied by the bridging Cl(1) anion. Both copper ions were in an N_2OCl_2 environment and the structural index parameter (τ) value[7] for both copper centers was found to be 0.18 which indicated a distorted square pyramidal geometry. Variable temperature

magnetic susceptibility data indicated weak antiferromagnetic coupling[6] and was described in Figure S4.

π-Stacking interaction with aryl hydrogen and hydrogen bonding network is important in supramolecular chemistry and crystal engineering.[8] In complex **1**·CH_3OH, the CH_3OH molecule showed hydrogen bonding interaction (2.511 Å) with coordinated chloride ion and a very strong interaction with the phenyl ring at a distance of 1.797 Å. The CH_3OH molecule showed the interaction (2.215 -2.368 Å) with the adjacent hydrogen atoms of *tert*-butyl group. The chloride ions were also involved in hydrogen bonding (2.864 Å) with the pyridine ring hydrogen. Each perpendicular phenyl ring afforded a non-covalent C-H----π interaction (2.896 Å) with the phenolato ring (Figure S3).

Magnetic properties. Magnetic measurements were carried out on crystalline sample of **1** with a Quantum Design MPMS XL SQUID magnetometer at temperature ranging from 5 to 300 K under an applied magnetic field of 1000 G. A plot of $\chi_m T$ vs. T is shown in Figure S4. A slight increase in $\chi_m T$ value was observed in the temperature range 300-10 K. We observed sudden increase in $\chi_m T$ value in going from 10-5 K. These observations are probably due to ferromagnetic coupling between two metal centres.[9]

Cell culture and Cytotoxicity assay[10,11]

Human breast cancer cells (MCF-7) were grown and maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen) and 100 IU/mL penicillin and 100 µg/ml streptomycin (Sigma). For the cytotoxicity analysis, ~10, 000 cells were seeded in each well of a 96 well micro titer plate containing 180 µL culture media,

grown overnight and then treated with 20 µL culture media containing the appropriate concentration of each compound (0.2 to 50 µM final concentrations). Equivalent volume of DMSO (vehicle) was added to control wells. After incubation for 96 h, 25 µL of MTT solution (stock 5 mg/mL in phosphate buffer saline) was added into each well and incubated for 4 h. The supernatant was removed and 100 µL of DMSO added to each well and incubated at room temperature for three hours. The absorption of formazan solution was measured at 560 nm using a micro plate reader (BMG Labtech). The percent viable cells (relative to control) were plotted against concentration for each compound to obtain the IC_{50} values. This experiment was repeated twice with 5 replicates each time.

For microscopic images, cells are grown on cover slip (to 60 % confluency) and treated with 10 µM of each compound (complex **1** or cisplatin) and incubated for 16 h and then visualized the cells under differential interference contrast (DIC) settings of a bright field microscope (NIKON TE2000).

Table S1 Summary of crystal data and data collection parameters for **1**·CH_3OH[Ψ]

Empirical formula	$C_{54}H_{60}N_6O_5Cl_2Cu_2$
Formula weight ($gmol^{-1}$)	1071.08
Temperature /K	273(2)
λ (Å) (Mo-Kα)	0.71073
Crystal system	Triclinic
Space group	P-1
a (Å)	8.4587(4)
b (Å)	12.3461(6)
c (Å)	13.1831(6)
α (°)	78.059(2)
β (°)	81.330(2)
γ (°)	74.426(2)
V ($Å^3$)	1290.86(11)
Z	1

Crystal size (mm)	0.23 x 0.19 x 0.16
F(000)	558
Theta range for data	2.83−28.35
Index ranges	$-11< h< 11, -16< k <16, -17< l<17$
Refinement method	Full matrix least–squares on F^2
Data/restraints/parameters	30646/0/306
GOF[a] on F^2	0.957
*R*1[b] [$I > 2\sigma(I)$]	0.0448
*R*1[all data]	0.0550
*wR*2[c] [$I > 2\sigma(I)$]	0.1363
*wR*2 [all data]	0.1477

[Ψ] In the crystal structure methanol is present on the crystallographic symmetric axis along with the isolated oxygen atoms. The complex appears to be monomer if it is seen in "DIAMOND" or "MERCURY" software however "Check cif" shows two Cu atoms bridged by two chlorine atoms possibly due to the close vicinity of the Cu-Cl centres.

Table S2 The IC_{50} values for MCF-7 cells of [Cu(^{t}BuPhimp)(Cl)] (**1**) and cisplatin

Compounds	**IC_{50} (μM)**
[Cu(^{t}BuPhimp)(Cl)] (**1**)	4.76 ± 0.14
Cisplatin	17.98 ± 1.18

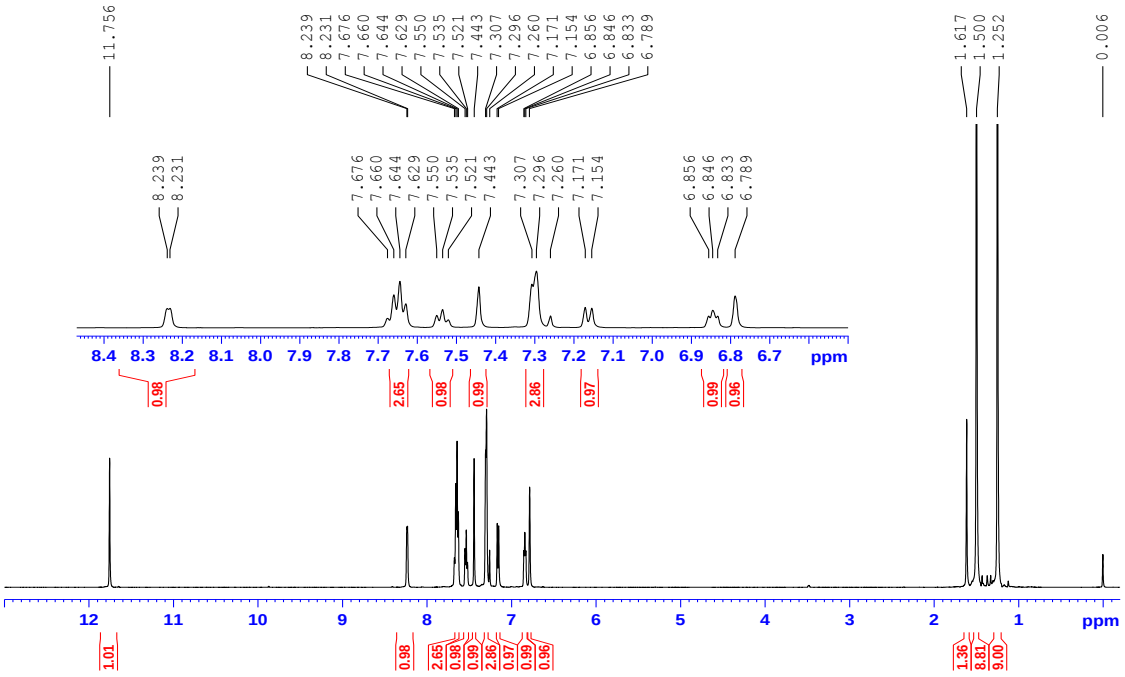


Figure S1 ^{1}H NMR spectrum of ligand ^{t}BuPhimpH in $CDCl_3$.

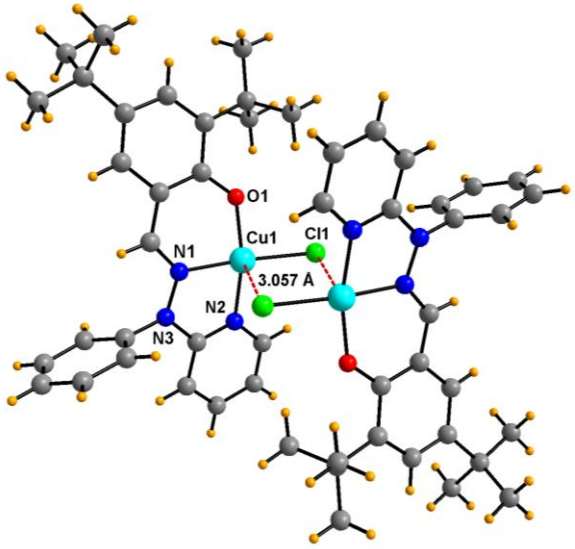


Figure S2 Ball-and-stick representation of the dinuclear formation of [Cu(^{t}BuPhimp)(Cl)]·CH_3OH (**1**·CH_3OH). Atoms are shown as spheres of arbitrary diameter.

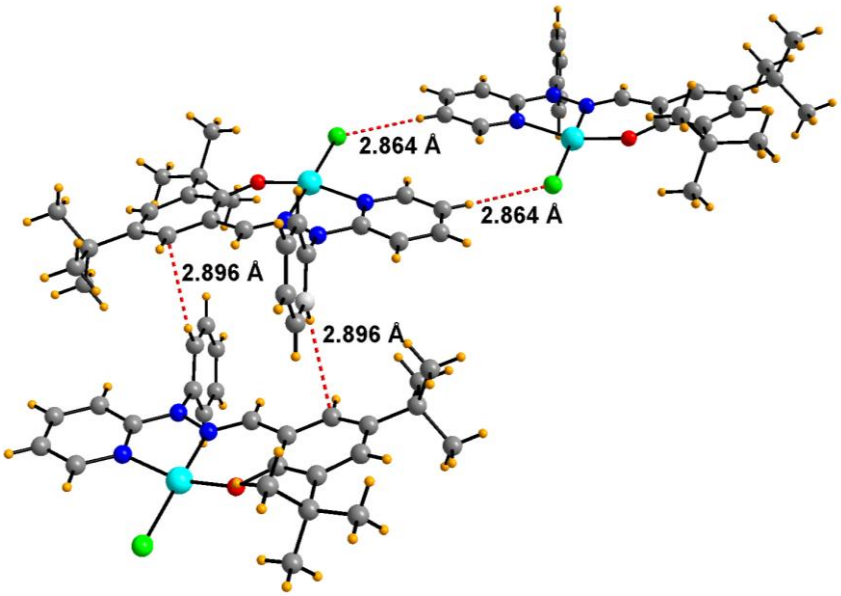


Figure S3 Hydrogen bonding and non-covalent interactions along with their distances in the packing diagram of [Cu(^{t}BuPhimp)(Cl)]·CH_3OH (**1**·CH_3OH)

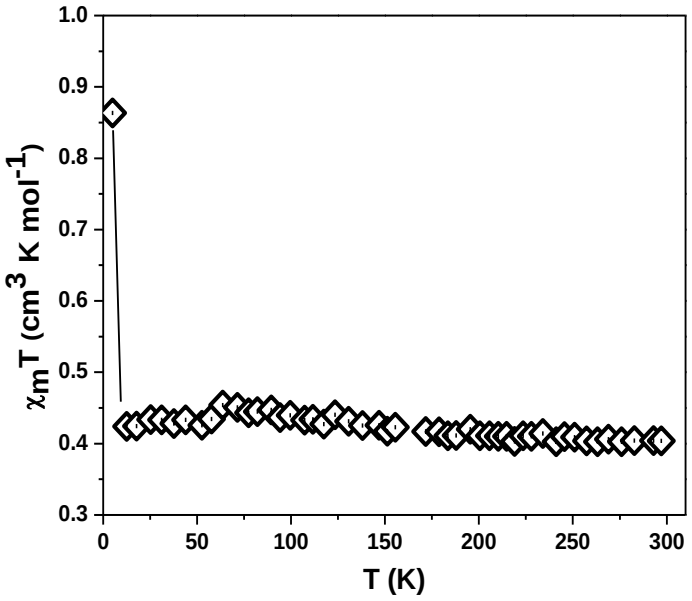
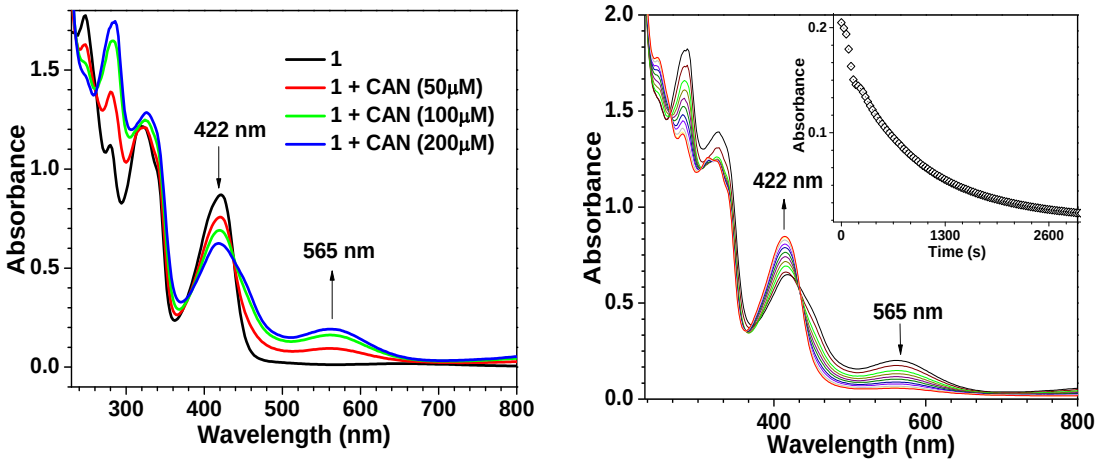


Figure S4 The plot of temperature dependance of χ_mT of **1** at 1000 G.



(a) **(b)**

Figure S5 (a) UV-vis spectra of generation of phenoxyl radical of **1** (100 μM) in the presence of CAN (50-200 μM) in acetonitrile. (b) UV-vis spectra of decomposition of $[1]^{\bullet+}$ within ~40 min. Inset: Time course of decomposition of $[1]^{\bullet+}$ ([1] = 100 μM, [CAN] = 200 μM) in acetonitrile at room temperature (λ_{max} = 565 nm)).

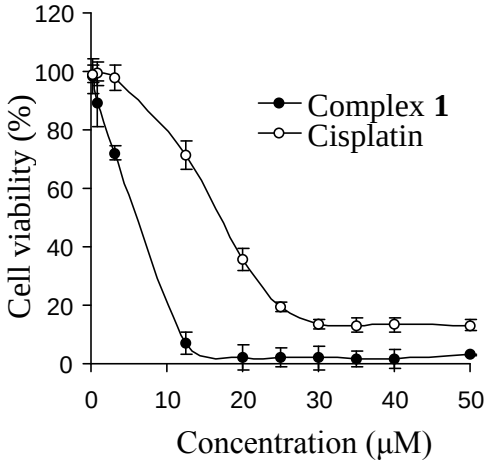


Figure S6 Cytotoxicity measurements:[10,11] MCF-7 cells were treated with varying concentration of complex **1** or cisplatin (positive control) for 96 h and then subjected to

MTT assay. The percent viable cells (relative to control) were plotted against concentration of each compound to determine the IC_{50}. Each experiment was repeated twice with 5 replicates each time. Bars indicate standard errors.

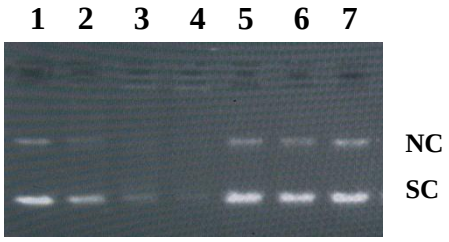


Figure S7 Gel electrophoresis separations showing the cleavage of supercoiled pBR322 DNA (40 ng) by **1** in buffer containing 10 % acetonitrile. Samples were incubated at 37 °C for 2 h. Lane 1, DNA; lane 2-4, DNA + [Cu(^{t}BuPhimp)(Cl)] (25, 35 and 50 μM respectively) in presence of oxygen; lane 5-7, DNA + [Cu(^{t}BuPhimp)(Cl)] (25, 35 and 50 μM respectively) in presence of nitrogen.

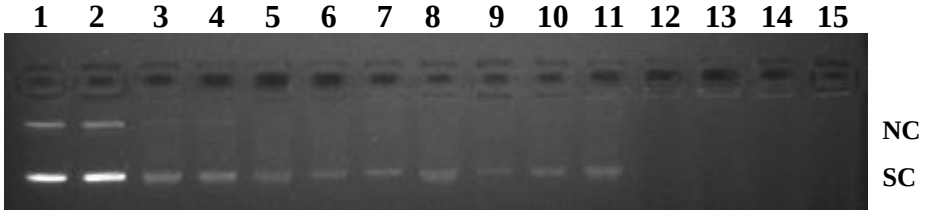


Figure S8 Gel electrophoresis separations showing the cleavage of supercoiled pBR322 DNA (40 ng) by **1** in buffer containing 10 % acetonitrile . Samples were incubated at 37 °C for 2 h. Keys: lane 1, DNA; lane 2, DNA + acetonitrile (10 %); lane 3, DNA + complex (50 μM); lane 4-7, DNA + complex (50 μM) + berberine chloride (10, 25, 50 and 100 μM respectively); lane 8-11, DNA + complex (50 μM) + NaCl (50, 100, 200 and 400 μM respectively); lane 12-15, DNA + complex (50 μM) + neocuproine (50, 100, 200 and 400 μM respectively).

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