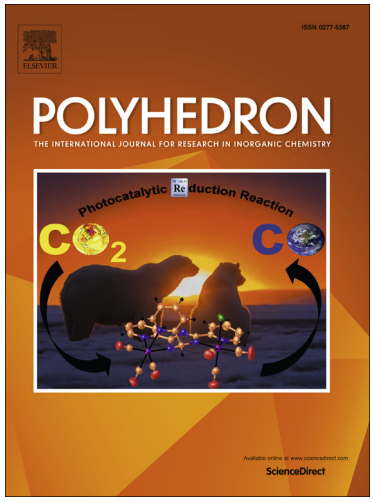

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Design of a mononuclear copper(II)-phenanthroline complex: Catechol oxidation, DNA cleavage and antitumor properties

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Abstract

A mononuclear copper(II) complex $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) [phen = 1,10-phenanthroline] has been synthesized and structurally characterized by different spectroscopic characterization methods including single crystal X-ray diffraction study. X-ray crystal structure analysis shows that **1** adopts square pyramidal geometry with CuN_2O_3 chromophore and the molecule crystallises in $P2_1/n$ space group. **1** has been evaluated as model system for the catechol oxidase enzyme by using 3,5-*di*-tert-butylcatechol (DTBC) as the substrate in methanol medium, revealing that **1** exhibits greater catalytic activity with K_{cat} value 3.91×10^3 $hour^{-1}$ compared to a few copper(II) complexes of the same class. Electrochemical analysis suggests that the mononuclar Cu(II) complex mimics the catechol oxidase enzyme in methanolic medium through radical pathway. The complex cleaves the double strand of pBR 322 plasmid DNA in a concentration-dependent manner so as for the DNA to change from super coiled form to both nicked circular and linear forms. The complex is cytotoxic to the human hepatocarcinoma cell HepG2, as revealed in MTT assay. AO/EB and Hoechst 33528 fluorescent staining techniques were used to find the mode of cell death. Interestingly, AO/EB and Hoechst stained cells observed at IC_{50} concentration shows that **1** brings about conformational change on DNA to induce apoptosis which would be the basis underlying its cytotoxic property.

Keywords: Copper(II); Crystal structure; Catecholase activity; DNA Cleavage; Antitumor activity

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1. Introduction

Synthesis of potential metal-based drugs, which may achieve wider spectrum of activity, enabling the administration of a lower dose, attack on different types of tumor cells, overcome drug resistance problems, and exhibit better selectivity and lower toxicity than *cis*-platin have received considerable curiosity [1-3]. So, there is substantial attention focused on the design of new metal-based anticancer drugs that exhibit enhanced selectivity and novel modes of DNA interaction like non-covalent interactions that mimic the mode of interaction of biomolecules [4-7]. Sigman and co-workers [8] developed the first chemical nuclease, bis(1,10-phenanthroline)copper(I) complex, that effectively cleaves DNA by binding the minor groove in the presence of hydrogen peroxide. Very recently, certain mixed ligand copper(II) complexes, which strongly bind and cleave DNA, have been shown to exhibit prominent anticancer activities and regulate apoptosis [9-11]. Therefore, designing suitable copper complexes for DNA binding and cleavage under physiological conditions is of remarkable importance in taking into account the advantages of processes that produce fragments similar to those formed by restriction enzymes [12-15].

On the other hand, investigation on the synthetic analogues of catechol oxidase has emerged as a field of modern research in bioinorganic chemistry to gain insight into functional mechanisms of catechol oxidase and elucidate different internal and external parameters like pH, nature of model substrates and solvents, the flexibility of the coordinating ligands, and the coordination environment of the copper centers which may influence the activity [16-21]. As the active site of catechol oxidase consists of a bridged-dicopper(II) moiety, several dinuclear copper(II) complexes derived from various chelating ligands have been widely studied for this purpose [22-26]. In addition to the dicopper(II) systems, a few mononuclear copper(II) complexes [27-29] and even a few copper(II) clusters and polymers have been found as active catalysts [30]. All these facts reflect that explorations of the possibility of exhibiting catecholase activity by new types of compounds are very much demanding, and our research group is engaged in this endeavor. In the present work, we report a mononuclear copper(II)-

phen complex of square pyramidal geometry with potential catecholase activity. Spectroscopic investigation and electrochemical analysis suggest rapid conversion of catechol to quinone via Cu(I)-superoxide intermediates with hydrogen peroxide as by product. The mononuclear copper(II) complex effectively cleaves DNA under physiological conditions. It is cytotoxic to a human hepatocarcinoma cell line (HepG2) and the mechanism of cell death is essentially apoptosis.

2. Materials and methods

2.1. Chemicals, solvents and starting materials

High purity 1,10-phenanthroline (E. Merck, India), copper(II) nitrate trihydrate (E. Merck, India), 3,5-di-*tert*-butylcatechol (Sigma-Aldrich, USA) and all reagents were purchased from the respective outlets and used as received.

2.2. Physical measurements

Infrared spectrum (KBr) was recorded with a FTIR-8400S SHIMADZU spectrophotometer in the range 400–3600 cm^{-1}. ^1H NMR spectrum in DMSO-d_6 was obtained on a Bruker Avance 300 MHz spectrometer at 25°C and was recorded at 299.948 MHz. Chemical shifts are reported with reference to $SiMe_4$. Ground state absorption was measured in a JASCO V-730 UV-vis spectrophotometer. Electrospray ionization (ESI) mass spectrum was recorded using a Q-tof-micro quadruple mass spectrometer. The pH value of the solutions was measured in Systronics pH meter at room temperature. Elemental analyses were performed on a Perkin Elmer 2400 CHN microanalyzer.

*2.3. Synthesis of [Cu(phen)(OH_2)$_2$(NO_3)](NO_3) (**1**)*

A methanolic solution (10 mL) of phen (0.180 g, 1 mM) was added drop-wise to an aqueous solution (15 mL) of $Cu(NO_3)_2.3H_2O$ (0.305 g, 1 mM). The blue solution was filtered and the supernatant liquid was kept in air for slow evaporation. Yield: 0.448 g (69% based on metal salt). Anal. Calc. for $C_{12}H_{12}N_4O_8Cu$ (**1**): C, 35.69; H, 3.00; N, 13.88. Found: C, 35.80; H, 2.95; N, 13.95%. IR (KBr, cm^{-1}): 3393 (ν_{OH}),1626, 1604 ($\nu_{C=N}$), 1384 (ν_{NO3}); UV-Vis (λ_{max}, nm): 278, 302, 695.

2.4. Crystal structure determination and refinement

Single crystal X-ray diffraction data were collected using a Rigaku XtaLABmini diffractometer equipped with Mercury375R (2x2 bin mode) CCD detector. The data were collected with graphite monochromated Mo-Kα radiation (λ=0.71073 Å) at 298(2) K using ω

scans. The data were reduced using Crystal Clear suite, and the space group determination was done using Olex2. The structure was resolved by direct method and refined by full-matrix least-squares procedures using the SHELXL-97 software package using OLEX2 suite [31, 32].

2.5. Catalytic oxidation of 3,5-DTBC

In order to examine the catecholase activity of the complex, a 10^{-4}M solution of **1** in methanol solvent was treated with 100 equiv. of *3,5-di*-tert-butylcatechol (3,5-DTBC) under aerobic conditions at room temperature. Absorbance vs. wavelength (wavelength scans) of these solutions were recorded at a regular time interval of 5 min in the wavelength range 300–500 nm.

The kinetics of oxidation of 3,5-DTBC were determined by the method of initial rates and involved monitoring the growth of the quinone band at 400 nm as a function of time [33]. Kinetic experiment was performed with **1** (at a constant concentration of 1×10^{-4} M) and 3,5-DTBC (varying the concentration from 1 × 10-3 M to 1 × 10-2 M) in methanol adopting UV-Vis spectrophotometer. The experimental procedure involved the preparation of stock solutions of the mononuclear Cu(II) complex and the substrate 3,5-DTBC at higher concentrations in methanol medium. 2 mL of 3,5-DTBC at appropriate concentration, obtained by accurate dilution from the stock solution, was taken into the UV-Vis quartz cell and kept for a while inside the cell holder which was attached with a thermostat to keep the temperature at 25 °C. Then, 0.04 mL of stock solution of the complex was added to it to achieve the ultimate concentration of the complex as 1×10^{-4} M. The formation of 3,5-DTBQ was monitored with time at a wavelength of 400 nm. The initial rate method was applied to determine the rate constant value for each concentration of the substrate and each experiment was repeated thrice.

2.6. Detection of presence of hydrogen peroxide in the catalytic oxidation of 3,5-DTBC

To detect the formation of hydrogen peroxide during the catalytic reaction, we followed a reported method [34]. Reaction mixtures were prepared as in the kinetic experiments. During the course of the oxidation reaction, the solution was acidified with H_2SO_4 to pH 2 to stop further oxidation after a certain time and an equal volume of water was added. The formed quinone was extracted three times with dichloromethane. To the aqueous layer were added 1 mL of a 10% solution of KI and three drops of a 3% solution of ammonium molybdate. The

formation of I^{3-} could be monitored spectrophotometrically because of the development of the characteristic I^{3-} band (λ_{max}= 353 nm) (Fig. S6).

2.7. Electrochemical analysis

The electrochemical analysis of **1** in 50% methanol in the presence of 0.1 M NaF as an supporting electrolyte was performed with Ag/AgCl, and saturated KCl, respectively, at the scan rate of 20 mVs^{-1}. The number of electrons involved in the reduction was confirmed by the chronocoulometry study. A plot of cathodic peak current (Ip_c) with the square root of the scan rate ($\sqrt{v}$) was studied using the following equation (1) to check whether the reduction is purely diffusion controlled or not and also to detect the presence of any adsorbed species on to the electrode surface.

$$I_{pc} = (2.69 \times 10^5)\, n^{3/2}\, D_O^{1/2}\, A\, C\, v^{1/2} \quad \ldots(1)$$

To find the catalytic role of copper complex, a mixture containing one part of copper complex and 100 parts of 3,5-DTBC was prepared and incubated for 1 minute which was then followed by cyclic voltammetry without purging of argon gas.

2.8. DNA cleavage studies

Cleavage of DNA was monitored by agarose gel electrophoresis technique, with the complex $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) at two different concentrations (25 and 50µg per mL). pBR 322 DNA was incubated with the complex for one hour at 37 °C. The reaction was also monitored in the presence of the radical activator (H_2O_2) and an inhibitor (DMSO). The inhibition reaction was carried out by the addition of the inhibitor reagent prior to the addition of the complex and the activator. After incubation 2 µL of bromophenol blue dye solution was mixed with the reaction mixture. The latter was then loaded carefully onto the electrophoresis chamber wells in the agarose gel when control DNA was loaded onto a separate well. TAE buffer was used as the running buffer and the electrophoresis was conducted at a constant current of 50 V per well for 30 min. The bands were observed in the gel documentation system and photographed to find the extent of DNA cleavage.

*2.9. Anticancer activity of the $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**)*

2.9.1. Cell culture

The HepG2 human hepatocarcinoma cell was obtained from the National Center for Cell Science (NCCS), Pune, India. The cells were cultured in DMEM medium (Sigma–Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Gibco) and 100

U/mL of penicillin and 100 µg/mL of streptomycin as antibiotics (Gibco), in 96 well culture plates, at 37 °C, in a humidified atmosphere of 5% CO_2, in a CO_2 incubator (Forma, Thermo Scientific, USA). All experiments were performed using cells from passage 15 or less.

2.9.2. Cytotoxicity assay (MTT assay)

The complex, in the concentration range from 50 to 500 µM in DMSO, was added to the wells 24 h after seeding of 5×10^3 cells per well in 200 µL of fresh culture medium. DMSO solution was used as the solvent control. A miniaturized viability assay using 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was carried out according to the method described by Mosmann [35]. After 24 and 48 h, 20 µL of MTT solution [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well and the plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. The purple formazan product was dissolved by addition of 100 µL of DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for three replicates each and used to calculate the mean. The percentage inhibition was calculated, from this data, using the formula:

$$= \frac{[\text{Mean OD of untreated cells (control)} - \text{Mean OD of treated cells (treated)}] \times 100}{\text{Mean absorbance of untreated cells (control)}}$$

From these data, the IC_{50} (the concentration at which the complex killed 50% of the cells at the respective durations of treatment) for 24 and 48 h treatment were arrived at.

2.9.3. Acridine orange (AO) and ethidium bromide (EB) staining

Acridine orange and ethidium bromide staining was performed as described by Spector et al. [36]. The cell suspension of each sample containing 5×10^5 cells, was treated with 25 µL of AO and EB solution (3.8 µM of AO and 2.5 µM of EB in PBS) and examined in a fluorescent microscope (Carl Zeiss, Jena, Germany) using an UV filter (450–490 nm). Three hundred cells per sample were counted in triplicate for each dose point. The cells were scored as viable, apoptotic or necrotic as judged by the staining, nuclear morphology and membrane integrity [36], and the percentages of apoptotic and necrotic cells were then calculated. Morphological changes were also observed and photographed.

2.9.4. Hoechst 33528 staining

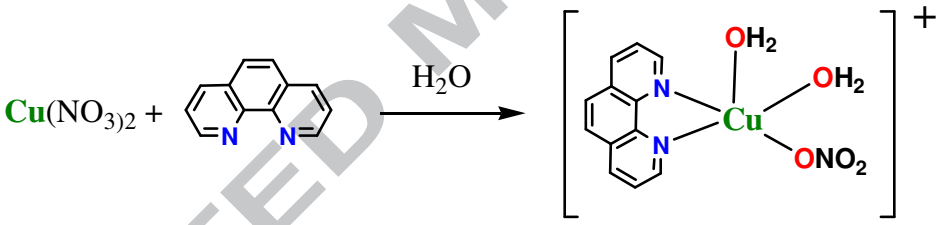
The HepG2 cells were cultured in 6-well plates and treated with the 24 h IC_{50} of the complex. After incubation, the treated and untreated cells were harvested and stained with

Hoechst 33258 (1 mg/mL, aqueous) for 5 min at room temperature. A drop of cell suspension was placed on a glass slide, and covered with a cover slip to reduce light diffraction. At random 300 cells, in duplicate, were observed at ×400 in a fluorescent microscope (Carl Zeiss, Jena, Germany) fitted with a 377–355 nm filter, and the percentage of cells reflecting pathological changes was calculated.

3. Results and discussion

3.1. Synthesis and formulation

The mononuclear Cu(II) complex was prepared out of reaction among copper(II) nitrate trihydrate and 1,10-phenanthroline in water medium. The coordination geometry of **1** was determined by mainly single crystal X-ray diffraction study along with different spectroscopic and analytical techniques. The blue crystals suitable for X-ray data collection were obtained by slow evaporation of resultant reaction mixture. The different formulations were confirmed by elemental analysis, IR, UV-vis, mass spectral analysis, thermogravimetric analysis and crystallographic structure analysis of the compound.



Scheme 1. Procedure for preparation of the copper(II) complex (1)

3.2. Crystal structure

The X-ray structural determination of compound **1** shows that the mononuclear cationic copper(II) complex adopts distorted square pyramidal geometry around Cu(II) in the space group $P2_1/n$. An ORTEP view of **1** with an atom labelling scheme is shown in Figure 1. The coordination geometry around copper(II) is described as distorted square pyramidal as evident from the value of the trigonal index, $\tau = 0.26$; [$\tau = |\beta - \alpha|/60$, where α=90.0° and β=106.132°; τ is **1** for a perfect trigonal bipyramidal geometry and is zero for a perfect square pyramidal geometry [37]. The coordination geometry around the Cu centre consists of equatorial plane which is formed by nitrato oxygen (O1), pyridine nitrogens (N1/N2) and the oxygen atom (O5) from water and the axial position is occupied by oxygen atom of water molecule (O4). The charge of cationic unit is counterbalanced by anionic charge of nitrate molecule. The

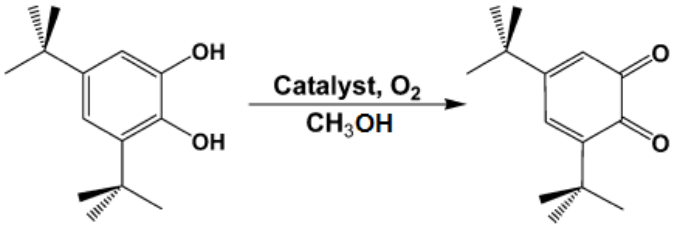
crystal packing shows an extended H-bonding scheme (Fig. S1). In fact, uncoordinated oxygen atoms of the nitrate molecule act as hosts to form H-bonds towards C-H/O-H bonds, and lead to one dimensional (1D) chain arrangement (Fig. S1). From supramolecular perception it is seen that oxygen atoms (O6,O7,O8) of counteranionic nitrate molecule hold diagonally two mononuclear copper(II) units through H-bonding interactions O···H-O (O8···H4A, 2.10 A°, O6···H5B, 2.66 A°, O7···H5B, 1.86 A°) between axial water (O^4H_2) and equatorial water (O^5H_2) while each of the mononuclear units in a layer extends via O3···H8-C8 (2.44 A°). The H-bonding interactions help to form self-assembled 1D chain along *b* axis. The crystallographic structural parameters of **1** are listed in Table 1. Selected bond lengths and angles are presented in Table S1, and the relevant hydrogen bonding parameters are summarized in Table S2.

*3.3. IR and electronic spectra of [Cu(phen)(OH_2)$_2$(NO_3)](NO_3) (**1**)*

A moderately strong, sharp peak due to a C=N stretching vibration, at 1626 cm^{-1} for the complex shows the presence of imine group in **1**. In complex **1**, there is a single band at 3393 cm^{-1}, indicating the presence of O-H stretching vibration of water. The IR spectrum shows close splitting of the band centered at 1384 cm^{-1} due to different coordination motifs of nitrate stretching [38,39]. The bands at 1273, 1421 and 1491 cm^{-1} and a single band at 1384 cm^{-1} for complex **1**, corroborate the presence of coordinated and ionic nitrate respectively (Figure S2). The electronic spectrum of the compound is recorded in methanolic solution at room temperature. In the higher-energy region of the optical spectrum, **1** shows characteristic bands at 272 and 302 nm which are assignable to π-π* transition of the C=N chromophore in phenanthroline while a low intensity broad band centered at 695 nm is observed in the visible region (Fig. S3), suggesting the square pyramidal geometry around copper centre [40]. The structural integrity in solution state for the copper(II) complex has been determined by ESI mass spectral analysis. The ESI mass spectral study of **1** in methanol medium exhibits the molecular ion peak, at m/z 277.9484 as $[Cu(phen)(H_2O)_2]^{2+}$-$H^+]^+$ (Fig. S4). The release of coordinated nitrate ion from the primary zone of coordination is also evident from the ESI-MS spectrum of **1** and further consolidates the solution instability.

3.4. Catechol oxidase activity

3,5-Di-*tert*-butylcatechol (3,5-DTBC) is the most widely used substrate for the study of potential catecholase activity of biomimicking coordination compounds, primarily for the following reasons: (i) its low reduction potential makes it easy to oxidize, (ii) the bulky tert-butyl substituents prevent further overoxidation reactions such as ring-opening, and (iii) the oxidation product, 3,5-di-*tert*-butylquinone (3,5-DTBQ), is highly stable with a characteristic absorption band maxima at 401 nm in pure methanol.



Scheme 2. Catalytic oxidation of 3,5-DTBC to 3,5-DTBQ in air-saturated methanol solvent

As a model of the catechol oxidase enzyme we have taken one mononuclear copper(II) complex and examined its efficiency in oxidation of 3,5-DTBC to 3,5-DTBQ. The catalytic activity of complex **1** was pursued by treating 1×10^{-4} M solution of the complex with 1×10^{-2} M of 3,5-DTBC. The reaction was monitored for 1 h after addition of 3,5-DTBC to the methanolic solution of the complex (Fig. 2). Upon addition of 3,5-DTBC, a new band is generated at 400 nm and with times to go the band exhibits significant increment of the absorbance at 400 nm. Since it is well established that 3,5-DTBQ shows band maxima at 400 nm in pure methanol,[41] the experiment unequivocally proves oxidation of 3,5- DTBC to 3,5-DTBQ catalyzed by **1**. 3,5-DTBQ was purified by column chromatography and isolated in high yield (73.7% for **1**).

Kinetic studies were performed to understand the extent of the catalytic efficiency of **1**. The kinetics of oxidation of 3,5-DTBC were determined by the method of initial rates and involved monitoring the growth of the quinone band at 400 nm as a function of time. The average rate constant values for **1** shows that the rate is first order at low concentrations of the 3,5-DTBC substrate but zero order at its higher concentrations. Figure 3 represents the dependency of initial rate on the concentration of catechol for complex **1** in methanol medium. The rates of reaction obtained for various 3,5-DTBC concentrations were fitted to the Michaelis–Menten equation (Fig. 3) and linearized by means of Lineweaver–Burk plot to

determine various enzymatic kinetic parameters (K_m, V_{max} and K_{cat}) as in Table 2 for the copper(II) compound. Comparing the turnover rate between our copper(II) complex and the reported ones by some renowned scientists it is found that ranging from 4.1 to 23.5 h^{-1} have been found which are comparable to those reported by Krebs et al. and Neves et al. [16,22] and to an order of hundred times more than the complexes (**2**) reported by Panja et al., Hundal et al. [47,48] but are significantly lower than those reported by Krebs et al., and Monzani et al. [24,25,41].

*3.5. Mechanistic insight of catecholase activity via electrochemical analysis of **1***

The cyclic voltammogram (Fig. 4a) of **1** in 50% methanol in the presence of 0.1 M NaF as the supporting electrolyte shows one-electron reduction peak (I_a) with the corresponding oxidation peak (I_b) at -150 mV and + 80 mV vs. Ag/AgCl, saturated KCl, respectively, at the scan rate of 20 mVs^{-1}. A plot of cathodic peak current (Ip_c) with the square root of the scan rate ($\sqrt{v}$) (Fig. 4b) shows a linear relationship passing through the origin which clearly indicates that this reduction is purely diffusion controlled (diffusion coefficient for reduction = 1.82×10^{-5} cm^2s^{-1}) and there is no adsorption on to the electrode surface. Comparing the experimental results of the present copper complex with that of earlier results [42] one can say that the reduction of the present complex is due to the formation of Cu(I)-semiquinone species in solution. In the complex the negative value of the reduction potential (-150 mV) suggests that the reduction is quite difficult which is due the fact that the ligands increase the electron density significantly on central Cu(II). This is the reason why the reduced species becomes unstable and is oxidised at a positive potential (i.e., + 80 mV). This means that the reduced species easily oxidises to the original complex (**1**). In addition there is a sharp reduction peak (II) in the cyclic voltammogram of the copper complex (Fig. 4a). A plot of cathodic peak current (Ip_c) of this peak with the scan rate (v) shows a linear relationship which clearly indicates that this reduction is solely due to the adsorption of the compound onto the electrode surface but not due to bulk electrolysis.

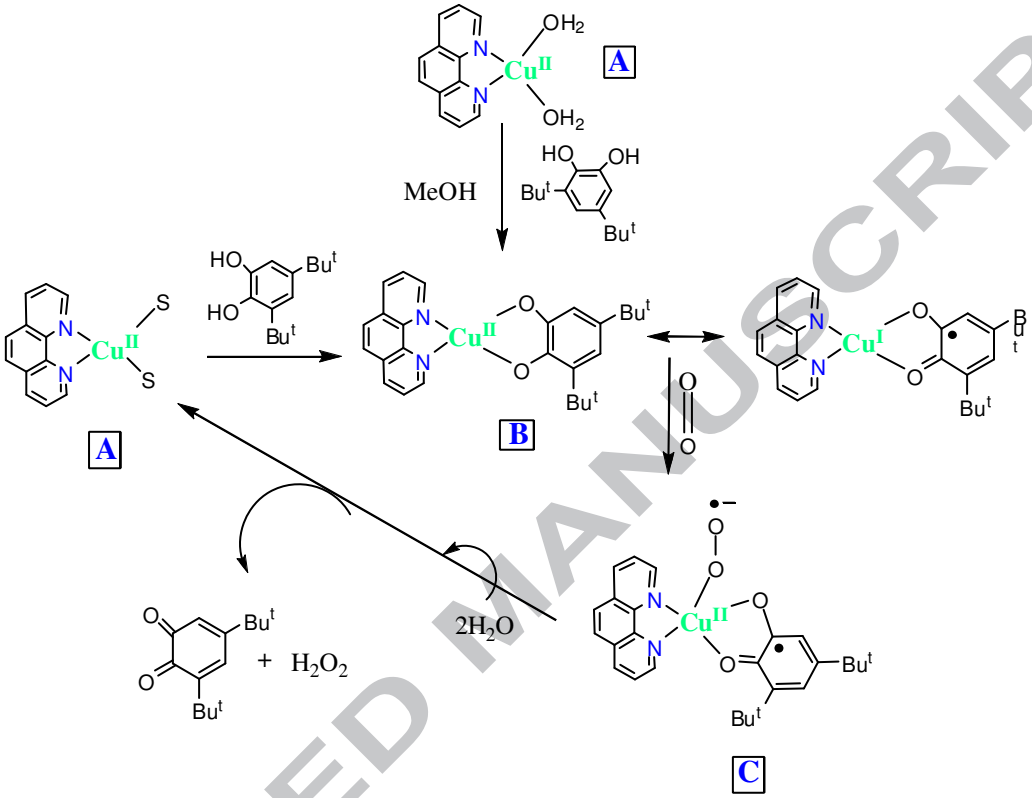
To find the catalytic role of copper complex, electrochemical investigation of a mixture containing one part of copper complex and 100 parts of 3,5-DTBC shows a reduction and an oxidation peak at -235 and +210 mV respectively (Fig. 5). In the cyclic voltammogram of the mixture 3,5-DTBC and copper complex an oxidation hump nearly at +50 mV was observed which is definitely due to the oxidation of Cu(I)-semiquinone generated in the catalytic pathways. The decrease in the intensity of the reduction peak of Cu(II) in the cyclic

voltammogram of 3,5-DTBC in the presence of copper complex is indicative of the reduction of Cu(II) to Cu(I) during the course of electro-catalytic effect of the complex.

3.6. Mechanistic inferences

Despite the clarification of the active sites of catechol oxidases being known from its crystal structure the catalytic mechanism is still a much debated issue. Krebs *et al.* [43] and Solomon *et al.* [44] have suggested largely accepted bio-mimetic mechanism of catechol oxidase enzyme involving different coordination nature of catechol to the copper centers. Previously, Chung-Yuan *etal.* [45] investigated a biomimetic study of a dicopper-phenanthroline complex encapsulated in silica towards catechol oxidase and showed that conversion of hydroxo-bridged dicopper(II) complexes into μ-peroxo dicopper(II) complexes in presence of molecular oxygen, and produced quinone with water as byproduct . To establish the pathway for the catalytic reaction, we initially detected the presence of hydrogen peroxide following a reported procedure [34] and found the band at 353 nm which is due to existence of I_3^- (see experimental section) using a 1×10^{-2} M concentration of catechol with a catalyst concentration of 1×10^{-4} M for **1** (Fig. S5). To obtain a mechanistic inference of the catecholase activity by **1** and to investigate possible complex–substrate intermediates, we recorded ESI-MS spectrum for a 1:100 mixture of the complex and 3,5-DTBC within 10 min of mixing in a methanol solvent (Fig. S6). The spectrum of **1** shows a base peak at m/z 243.9 (100%), which can be assigned to the quinone sodium aggregate $[3,5\text{-DTBQ-Na}^+]^+$. Other characteristic peaks at m/z 464.38 indicate the presence of the bidentate catecholate adduct B as $[Cu(phen)(3,5\text{-DTBC})]^+\text{-H}^+]^+$. Another important peak at m/z 501.0 possibly corroborates the formation of species C as $[Cu(phen)(O_2)(3,5\text{-DTBC})\text{-Na}^+]^+$. Alternatively, molecular oxygen exists in triplet state with two electrons occupying two degenerate molecular orbitals and as a diradical, it can directly react with the Cu(I)-radical species in a termination (radical-radical) reaction. This reaction pathway may be an alternative to the formation of species C. From electrochemical analysis and mass spectrometric investigation it is evident that in solution there is existence of Cu(I) species, semiquinone species, catecholate adduct and oxygen bound species in the catalytic cycle of the reaction. Now, we are at the stage to suggest a plausible pathway in which at the first stage active catalyst (B) is generated by the reaction of 3,5-DTBC with one equivalent of complex (Scheme 3). The catecholate-bound species exists in equilibrium with copper(I)-semiquinone adduct (B) which generates the superoxide intermediate (C) with aerial oxygen in which copper(I) center in B undergoes an

oxidation to a copper(II) center in C (Scheme 3). In the catalytic cycle, next step involves the elimination of quinone as major product with hydrogen peroxide as byproduct with concomitant origination of [Cu(phen)$(S)_2$] (A); where S = H_2O.



Scheme 3 Proposed catalytic cycle for the oxidation of 3,5-DTBC by the copper(II) complex

3.7. DNA cleavage studies

The irradiation of pBR322 plasmid DNA in the presence of the copper complex in a concentration (µg/mL) dependent manner was studied so as to determine the efficiency with which it sensitizes DNA cleavage. The conversion of circular supercoiled DNA to its nicked circular form was monitored. Though there was conversion of supercoiled form (Form I) to linear form (Form III) at both the concentrations, cleavage increased with the increasing concentration of the complex (50 µg) (Fig 6b,c). The cleavage activity was also observed using a cleavage activator (H_2O_2) and an inhibitor (DMSO). To find the involvement of oxygen species in the damage to the DNA, the hydroxyl radical scavenger DMSO was used along with the complex and the hydroxyl radical ($OH^{\bullet}$) (Fig 6d), which resulted in significant inhibition of DNA cleavage confirming the involvement of diffusible hydroxyl radical as the

reactive species causing strand scission. The nuclease activity of the complex increased in the presence of the activator along with the complex (Fig 6e). Sigman *et al*.,[46,47] proved that copper(I) complex of 1,10-phenanthroline (OP), $[(OP)_2\text{-}Cu^{I}]^{+}$, with H_2O_2 as a co-reactant, is a chemical nuclease that nicks DNA by either diffusing to the minor groove where it is oxidized by H_2O_2 or binds to minor groove where it gets reduced before reacting with H_2O_2 and the phenomenon is sensitive to the conformation of the nucleic acid and cleavage of DNA. For our molecule, the major reason behind this is that the hydroxyl radical's reactions with the deoxyribose sugar backbone are initiated by hydrogen abstraction from a deoxyribose carbon, and the predominant consequence is eventual strand breakage and base release. The DNA cleavage experiment clearly reveals that the addition of copper(II)-phen complex to the DNA led to the mild transition from Form I (Supercoiled) to Form III (Linear). But the addition of the activator led to the conversion of Form I to Form II (Nicked circular) which was absent with the addition of the hydroxyl radical scavenger.

*3.8. Anticancer activity of [Cu(phen)(OH$_2$)$_2$(NO$_3$)](NO$_3$) (**1**)*

3.8.1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

Since the present mononuclear copper(II) complex has the ability to cleave DNA in the presence and absence of a reductant, and since DNA cleavage is considered important for a drug to act as an anticancer agent [48,49], the cytotoxic effect of the complex, dissolved in DMSO, was investigated against a human hepatocarcinoma cell line (HepG2) adopting MTT assay. The IC_{50} values were obtained by plotting the cell viability against the concentration of the complex (Fig. 7). The results revealed that the IC_{50} at 48 h (60±0.2 μM) is lower than at 24 h (80±0.1 μM) clearly indicating that the complex exhibits cytotoxicity against HepG2 in a dose- and duration-dependent manner. Thus, the cytotoxicity exhibited by the complex is consistent with its strong binding with DNA, and its efficiency in cleaving DNA in the absence of an external agent is responsible for its potency to induce cell death.

3.8.2. Fluorescent staining for apoptosis

Apoptotic cell death is known as characterized by different cellular changes such as cell shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies. These apoptotic characteristics as produced in HepG2 human hepatocarcinoma cell by the [Cu(phen)(OH$_2$)$_2$(NO$_3$)](NO$_3$) (**1**) complex were analyzed adopting AO/EB staining. In this staining method, the fluorescence pattern depends on the

viability and membrane integrity of the cells. In general, dead cells only are permeable to ethidium bromide and fluoresce orange-red, whereas live cells are permeable to acridine orange only and thus fluoresce green. The cytological changes which were observed in the treated cells are classified into four types based on the fluorescence emission and morphological features of chromatin condensation in the AO/EB stained nuclei: i) viable cells, possessing highly organized nuclei, fluoresce green; ii) early apoptotic cells, which show nuclear condensation, emit orange-green fluorescence; iii) in late apoptotic cells, with highly condensed or fragmented chromatin, the nuclei fluoresce orange to red; and iv) cells undergoing necrosis fluoresce orange to red, with no indication of chromatin fragmentation. All these morphological changes were observed after the treatment of cancer cells with the complex. Figure 8 indicates the apoptotic morphologies induced by the complex at 24 h IC_{50}. Since the copper complex is a cationic molecule consisting of a planar hetero-polyaromatic phenanthroline ligand and two aqua molecules at Cu(II) centre, it has the natural ability to bind with DNA through heterogeneous base pair with ease. The replacement of labile aqua molecules by DNA base pair or intercalation of phenanthroline ring through base pair may very effectively cause apoptosis of the affected cells. Figure 9 shows the efficacy of the complex at its 24 h IC_{50} to induce apoptosis in close to 50% of cells as revealed in the AO/EB assay. The IC_{50} values and morphological changes consolidate the potential anticancer activity of the molecule. However, further studies are needed in this direction to confirm the mode of cell death induced by the complex.

3.8.3. Hoechst staining

Morphological changes in the nucleus and chromatin were revealed by Hoechst 33528 staining method. The cells treated with 24 h IC_{50} of the complex showed changes in the morphology of the nuclei. In the control cells the nuclei were round with intact chromatin while after treatment with the complex changes such as chromatin marginalization, condensation and fragmentation were observed (Figure 8), which are characteristic of apoptosis. It is interesting that close to 50% of treated cells exhibited nuclear morphologies indicating apoptosis (Fig. 10).

4. Conclusion

We have synthesized and characterized a mononuclear copper(II)-phenanthroline complex of the type $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ which can act as potential catalyst for the oxidation of

catechols to quinones, mimicking the catecholase activity of catechol oxidase with high turnover number, K_{cat} (h^{-1}) value as 3.91×10^3. The DNA cleavage experiment clearly reveals that the addition of **1** led to the partial transition from Form I (Supercoiled) to Form III (Linear). But the addition of an activator led to the conversion of Form I to Form II (Nicked circular) which was not the case following addition of the hydroxyl radical scavenger. Induction of apoptosis in the human hepatocarcinoma cell line (HepG2) was assessed in relation to the changes in cell viability, cellular morphology and nuclear cytology, which shows the efficacy of **1** to induce apoptosis in time and dose-dependent manner. Interestingly, the observed IC_{50} values reveal that **1** effects conformational change on DNA strongly. Moreover, detailed experimentation related to DNA interaction, cleavage and cytotoxicity on this complex will help to expound clinically relevant information for developing possible new anticancer drugs.

Supplementary data

Supplementary crystallographic data are available free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033; E-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk) upon request, quoting deposition number CCDC 1061531.

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Table 1. Crystallographic refinement parameters of $[Cu(phen)(H_2O)_2(NO_3)](NO_3)$ (**1**)

Crystal parameters	**1**
Empirical formula	$C_{20}H_{20}CoN_5Cl_2O_5$
Formula weight	540.21
Temperature	293 K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	P-1
Unit cell dimensions	a = 7.0995(3) Å α = 91.829(4)°
	b = 12.2760(6) Å ß= 91.508(4)°
	c = 13.1897(7) Å γ = 99.633(4)°
Volume	1132.16(10) $Å^3$
Z	2
Density (calculated)	1.573 Mg/m^3
Absorption coefficient	1.036 mm^{-1}
F(000)	544
Reflections collected	28913
Independent reflections	6566
R(int)	0.021
Completeness to theta	99.6 %
Goodness-of-fit on F^2	1.058
Final R indices [I>2sigma(I)]	R1 = 0.0786, wR2 = 0.2835
R indices (all data)	R1 = 0.0623, wR2 = 0.1488
Largest diff. peak and hole	0.610 and -0.426 e. $Å^{-3}$

Table 2. Kinetic parameters for the oxidation of 3,5-DTBC catalyzed by **1** in methanol

Complex	V_{max} ($M\ s^{-1}$)	K_m (M)	K_{cat} (h^{-1})	Ref
1*	1.08×10^{-3}	2.7×10^{-3}	3.91×10^{3}	Present
2	1.31×10^{-7}	2.27×10^{-3}	2.35×10^{1}	50
2	1.145×10^{-7}	2.12×10^{-3}	4.1	51

*Std. Error for V_{max} (MS^{-1}) = 6.40×10^{-6}; Std. Error for K_m (M)= 3.71×10^{-4}

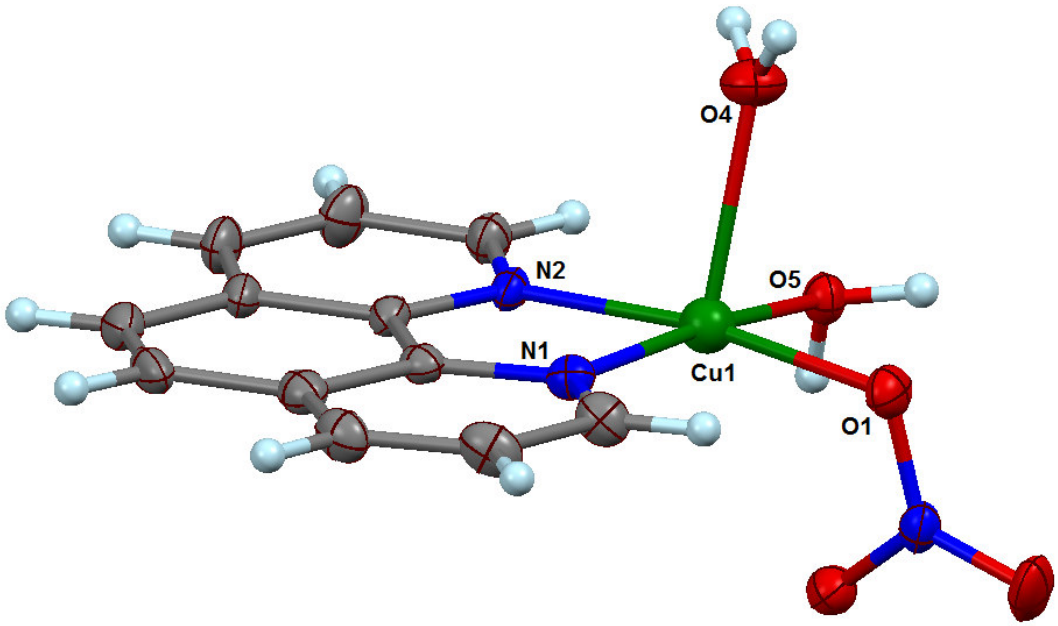


Fig 1. An ORTEP diagram of $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) with atom numbering scheme and 30% probability ellipsoids.

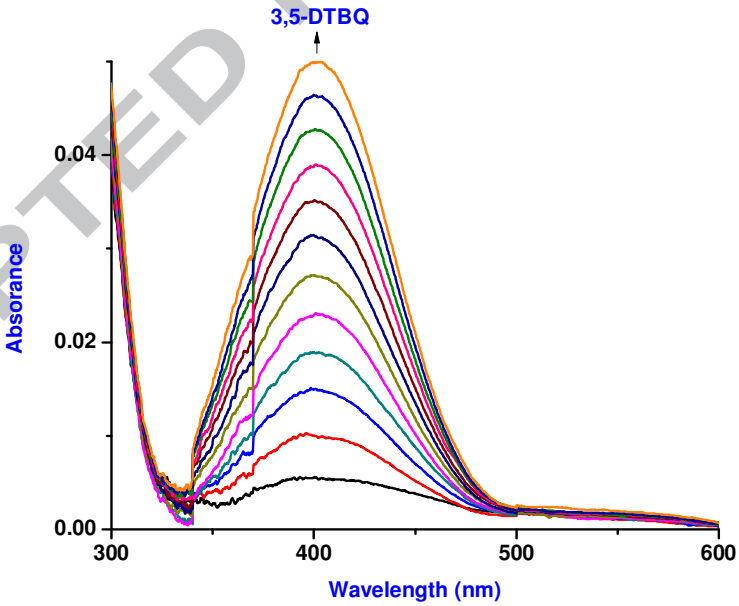


Fig 2. UV-vis spectral change of complex at a regular interval of 5 min with 3,5-DTBC.

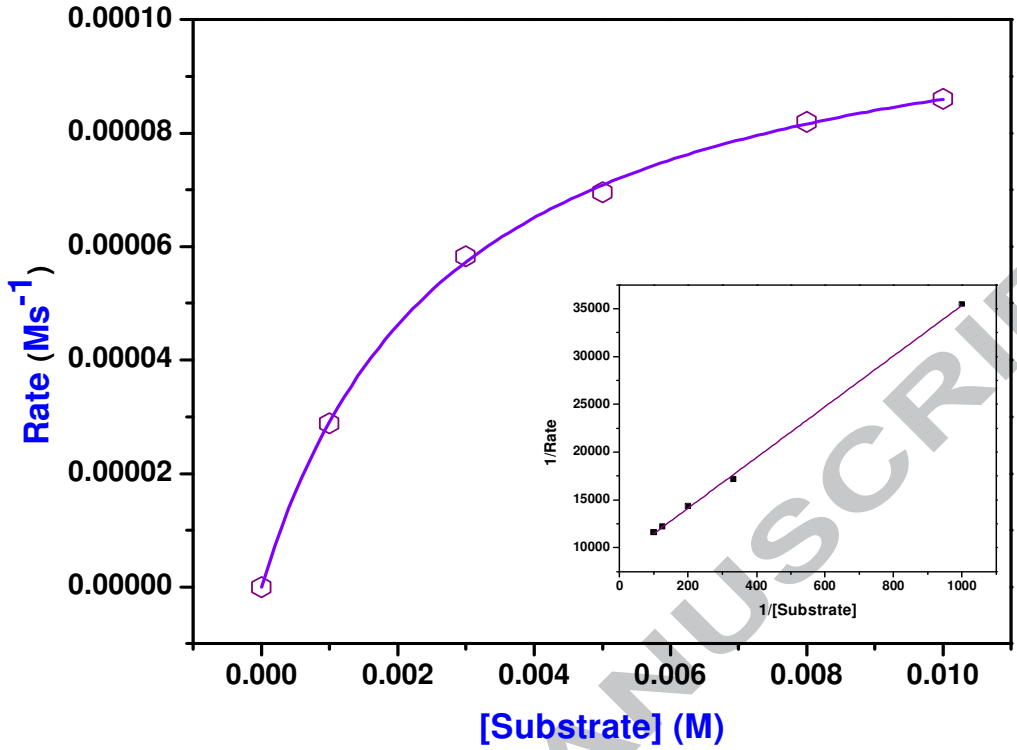


Fig. 3 Plot of rate vs. [substrate] in presence of **1** in MeOH; inset: Lineweaver-Burk plot

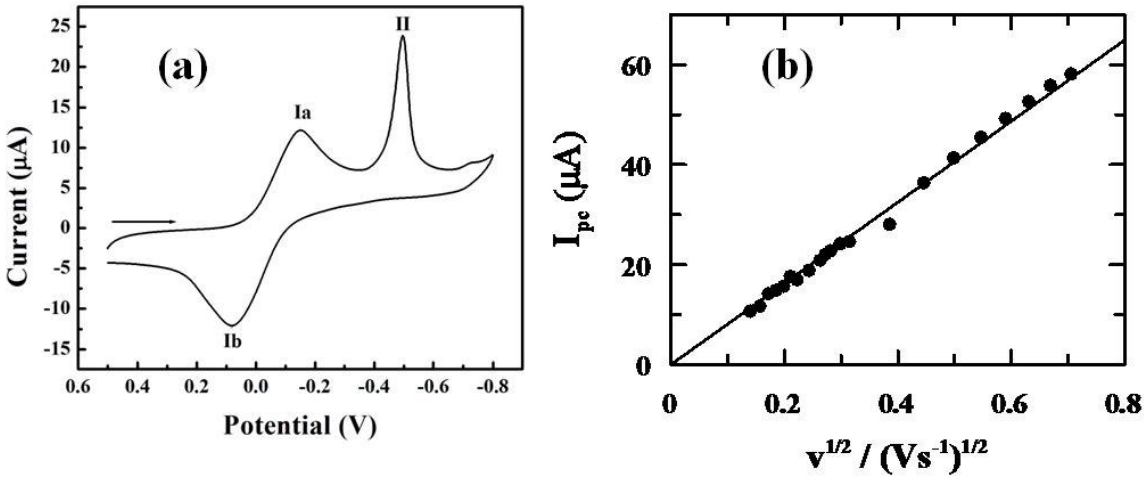


Fig. 4(a): Cyclic voltammogram of **1** in 50% methanol. Scan rate: 20 mVs^{-1}. [**1**] = 10^{-3} M, [NaF] = 0.1 M, T = 298.15 K; (b): Plot of cathodic peak current (I_{pc}) *vs.* the square root of the scan rate ($v^{1/2}$) for the reduction of **1**.

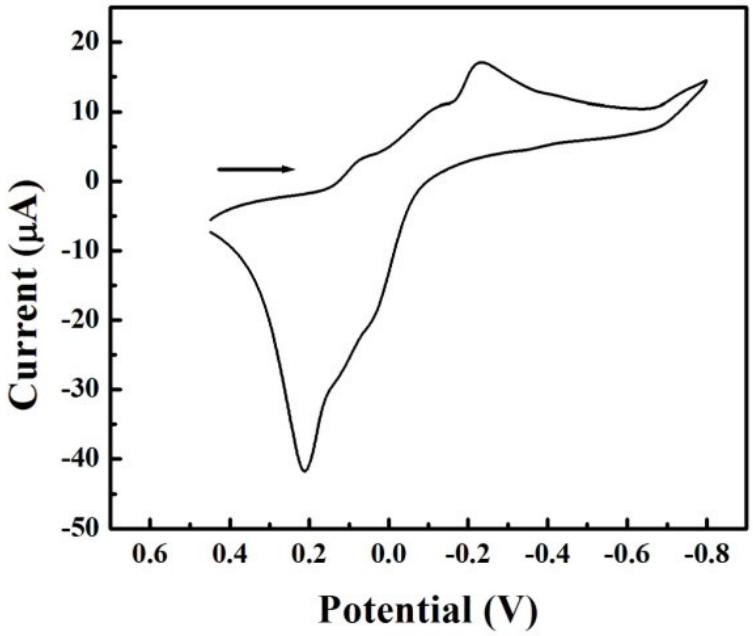


Fig 5. Cyclic voltammogram of **1** and 3,5-DTBC mixture in 50% methanol. Scan rate: 20 mVs^{-1}. [**1**] = 10^{-3} M, [3,5-DTBC], = 0.1 M, [NaF] = 0.1 M, T = 298.15 K.

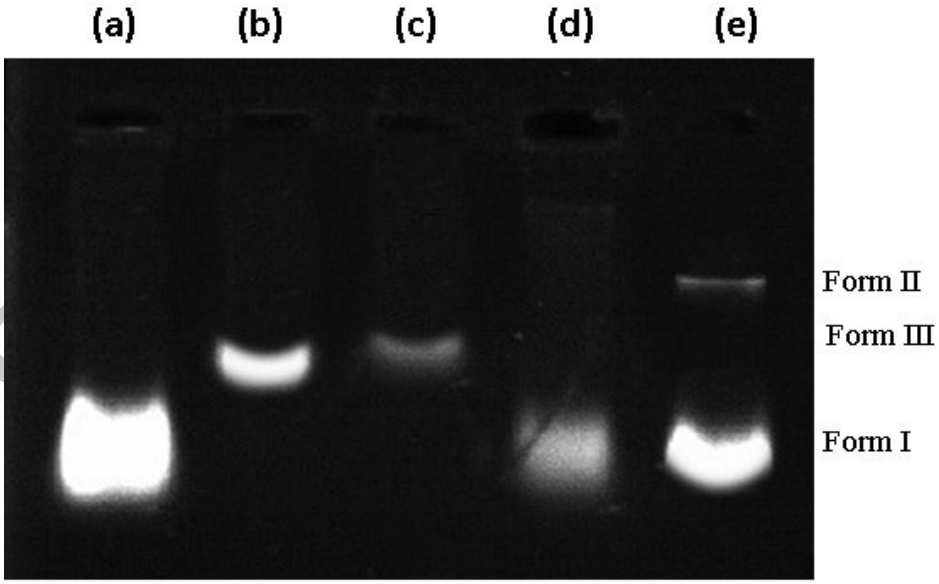


Fig 6. DNA cleavage activities of **1 [**Lane a: Control pBR 322 DNA, Lane b: pBR 322 DNA + 25 μg of **1**, Lane c: pBR 322 DNA + 50 μg of **1**, Lane d: pBR 322 DNA + 25 μg of **1+** 5 μl DMSO + 5 μl H_2O_2 , Lane 5: pBR 322 DNA + 25 μg of **1+** 5 μl H_2O_2]

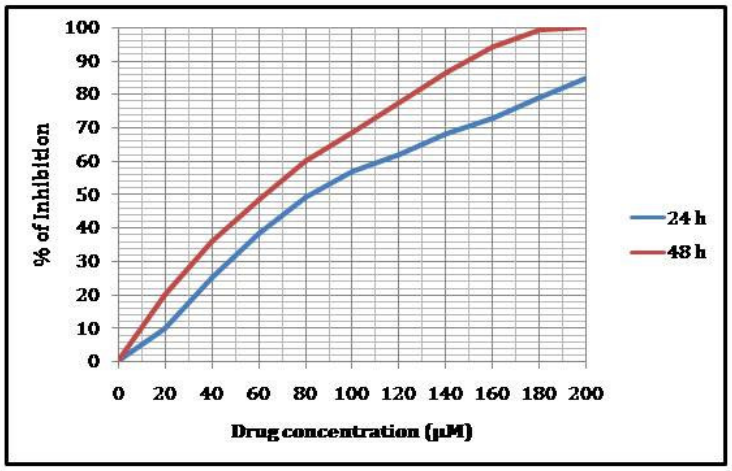


Fig 7. MTT assay of $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) in HepG2 cells treated for for 24 h and 48 h

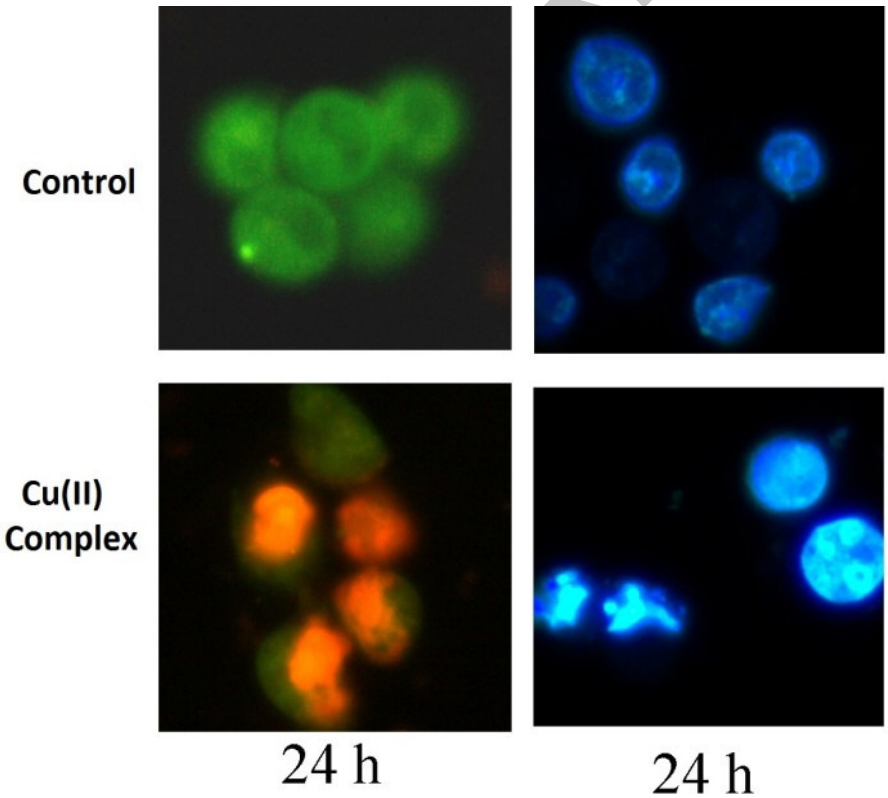


Fig 8. Representative morphological changes observed in HepG2 cells after 24 h incubation with $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) as revealed in AO/EB staining (left) and Hoechst staining (right)

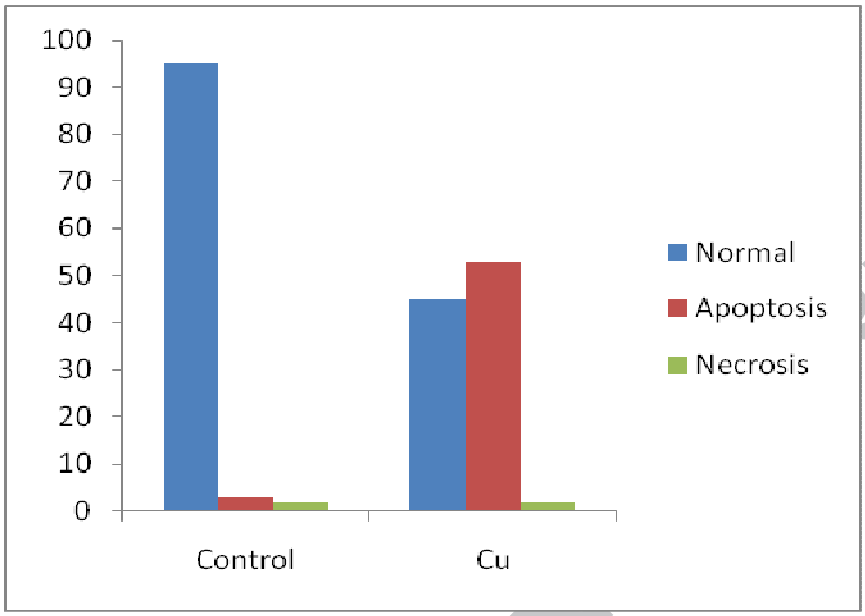


Fig 9. The relative percentage of morphological changes in HepG2 cells after 24 h incubation with $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) as revealed in AO/EB staining

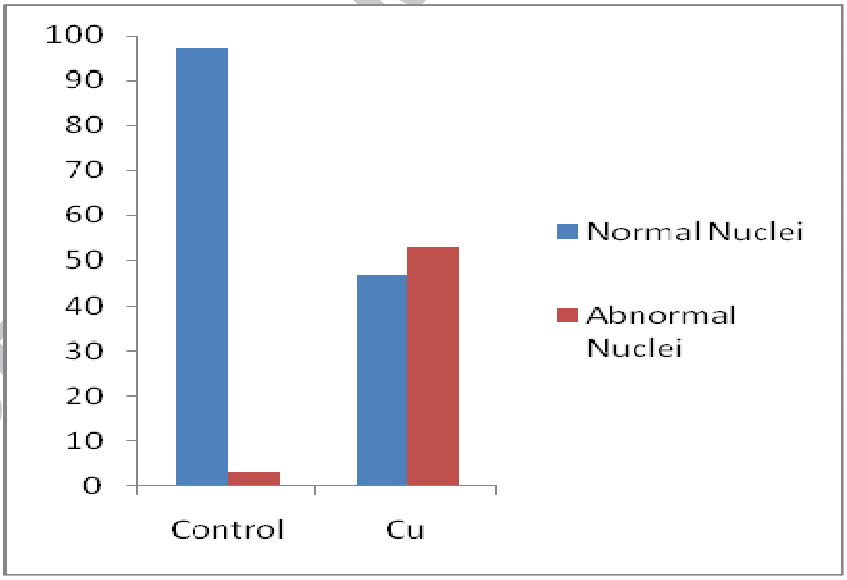
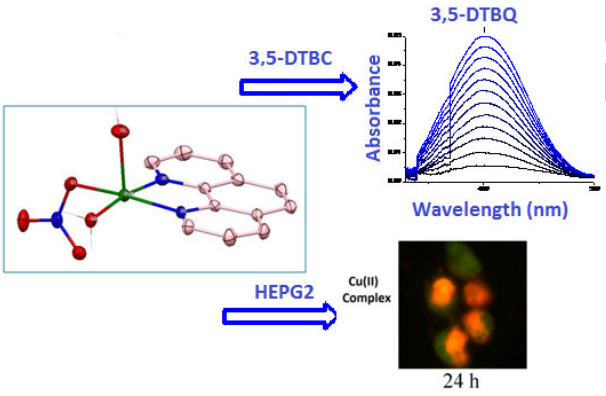


Fig 10. The relative percentage of morphological changes in HepG2 cells after 24 h incubation with $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) as revealed in Hoechst staining.

Design of a mononuclear copper(II)-phenanthroline complex: Catechol oxidation, DNA cleavage and antitumor properties

Dhananjay Dey,[a] Subrata Das,[a] Hare Ram Yadav,[b] Anandan Ranjani,[c] Loganathan Gyathri,[c] Sanjay Roy,[d] Partha Sarathi Guin,[d] Dharumadurai Dhanasekaran,[c] Angshuman Roy Choudhury,[b] Mohammad Abdulkadhar Akbarsha,[e] and Bhaskar Biswas,[a]*

G R A P H I C A L A B S T R A C T



A mononuclear copper(II) complex $[Cu(phen)(OH_2)_2(NO_3)](NO_3)$ (**1**) has been evaluated as model system for the catechol oxidase enzyme by using *3,5-di*-tert-butylcatechol (DTBC) as the substrate in methanol medium, revealing that **1** exhibits greater catalytic activity with K_{cat} value 3.91×10^3 $hour^{-1}$. The complex cleaves the double strand of pBR 322 plasmid DNA in a concentration-dependent manner and is cytotoxic to the human hepatocarcinoma cell HepG2.

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P I C T O G R A M