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SYNTHESIS, IN VITRO, AND IN SILICO ANTI-CANCER STUDIES OF SUBSTITUTED BENZIMIDAMIDE AND ITS CU(II) COMPLEX

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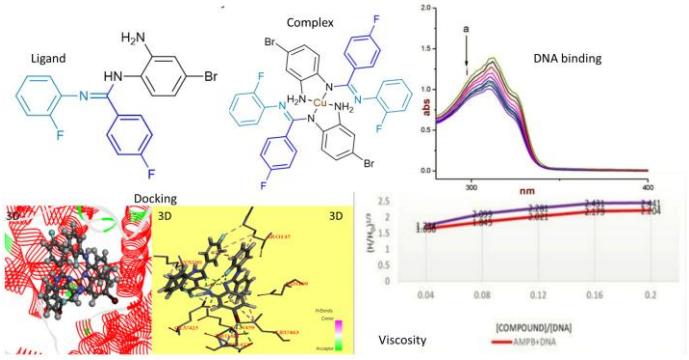
Copper complexes have a remarkable identity in the hope of curing cancer. In search of a non-toxic anticancer agent, a new ligand (*N*-(2-amino-4-bromophenyl)-4-fluoro-*N*'-(2-fluorophenyl)benzimidamide) having famous medicinal moiety, "benzimidamide" was synthesized for complexation with Copper. The synthesis of compounds was confirmed by FT-IR, multinuclear (^{1}H, ^{13}C) NMR spectroscopy. The carbon, hydrogen, and nitrogen composition were determined by CHN analysis. The compounds were also characterized by UV-vis, PXRD, and thermal (TGA/DTA) analysis. The absorption spectral titration experiments suggest that the ligand and its Cu(II) complex are bound to DNA through intercalation mode but Cu(II) complex is a good partial intercalator having a binding constant of less than 10^{6} M^{-1}, which was further verified by viscometry. The Cu(II) complex showed meagre nuclease activity as compared to ligand, in the absence of oxidant. However, the activity was improved in the presence of an oxidant expedited by the generation of hydroxyl free radicals. The reduced level of toxicity parameters in liver and kidney function tests is a clear indication of the nontoxic nature of the ligand and its Cu(II) complex. The cytotoxic effect on MDA-MB-231, MCF-7, WRL-68 and MCF-10A cell lines was observed and we found that Cu(II) complex is more active than the ligand against the cancer cell lines and remains inactive against human normal MCF-10 breast cells and WRL-68 hepatic cells. All the findings suggest that both the ligand and Cu(II) complex could bind with DNA, cleave DNA via intracellular reactive oxygen species, defunctionalize mitochondria, arrest the cell cycle and stimulate the programmed cell death. As a result of the docking process on human serum albumin (PDB code: 1AO6), it was found that the ligand and its Cu(II) complex have a higher affinity for human serum albumin. Cu(II) complex was found to have the least docking energy (-7.1 Kcal mol^{-1}).



INTRODUCTION

Some Cu(II) complexes have been considered good substitutes for the platinum antitumor drug due to their strong redox potential and comparatively good coordination with nucleobases due to the momentous role of copper in different cells, modulating several cytokines and growth

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factor mechanisms of action.[1] They have likewise been viewed as chemical nucleases and known to cleave DNA by different mechanisms viz. hydrolytic and oxidative.[2] Many studies have proven that the ligand attached to the metal centre greatly affects the cytotoxicity of the complex and planarity in the ligand enhances the DNA binding via grove binding or intercalation.[3] The chemical nature of coordinating ligand modifies the absorbency of the complex through the cell membrane and affects the redox-couple of the Cu(II) centre.[4] There are many examples in the literature that copper(II) complexes of benzimidamide have been used in the inhibition of tumour cell lines and can induce apoptosis.[5] The benzimidamide moieties act as drug carriers and as starting materials for the diversified heterocyclic molecule.[6] Due to hydrogen bond donor properties, benzimidamide moieties can approach biological targets, especially DNA to form new types of a bruise.[7] Presently, research is progressively concentrating on the proposal and preparation of targeted organic ligands having structure-specific edifices. The structure and bioactivities of Cu(II) complexes with their ligands, having high aromaticity and large planarity Phenyl-Phenyl-benzimidamide moieties might offer a valuable understanding of the biochemistry and chemistry of bioactive molecules. Thus herein, we are presenting the facile synthesis of a new ligand, "*N*-(2-amino-4-bromophenyl)-4-fluoro-*N*'-(2-fluoro-phenyl)benzimidamide and its Cu(II) complex, together with DNA binding affinity, DNA cleavage effect and anti-cancer activity.

RESULTS AND DISCUSSION

It is well known that microwave synthesis has an edge over conventional synthesis in that it takes a very short time for reaction completion and no or very less amount of solvent used. The ligand and its Cu(II) complex are non-hygroscopic, stable at room temperature, and insoluble in chloroform, n-hexane, ethyl acetate and water. The ligand shows very good solubility in ethanol while Cu(II) complex is only soluble in dimethyl sulfoxide (DMSO) and 1,4-dioxane. The FT-IR spectra and CHN analysis were mainly used to distinguish between the ligand and Cu(II) complex. Their experimental CHN values are in good agreement with the calculated ones.

1. Spectroscopic analysis

To confirm the deprotonation site and binding mode of the ligand FT-IR spectrum has been used. A strong IR band observed at 3445 cm^{-1} in the FT-IR spectrum of ligand has been assigned to NH_2 of the bromophenyl ring. The low νNH_2 value is probably due to intramolecular hydrogen bonding. A νNH band was observed at 3220 cm^{-1}. This strong band is absent in Cu(II) complex, suggesting the deprotonation of a proton from the nitrogen of imidamide[8] and the formation of covalent bonds between nitrogen and the Cu(II) ion. As we have not used any drastic conditions (n-butyl lithium or potassium t0butoxide). So, there is no chance of removal of a proton from the -NH_2 group. Therefore, it is concluded that upon coordination the proton is removed from the nitrogen of imidamide[8]. The band observed at 1638cm^{-1} in the IR spectrum of ligand, is assigned to >C=N- stretching vibration. This band is shifted to a lower frequency in Cu(II) complex informing the participants of azomethine nitrogen in chelation. The band observed at 444 cm^{-1} in the FT-IR spectrum of the Cu(II) complex is assigned to υCu –N stretching vibration. This band is absent in the IR spectrum of the ligand.

In the ^{1}H NMR spectrum of ligand, a singlet imidamide proton appeared at 11.04 ppm and a singlet of two protons of -NH_2 at 4.2 ppm confirms the synthesis of the ligand. All the aromatic protons appeared in the range of 8.23- 7.01 ppm. The ^{13}C spectrum of ligand justifies the structure of the ligand. The imidamidic carbon (-N=C) appears at 162.7 ppm. The two carbon atoms directly attached to fluorine appeared at 165.8 and 160.4 ppm. Carbon attached to the -NH_2 group appears at 153.4 ppm while carbon attached to -NH appeared at 147.8 ppm. The carbon attached to doubly bonded nitrogen (-N=) appeared at 151.6 ppm. All other carbons appeared in the range of 135.3-129.5 ppm while carbon attached to -NH appeared at 147.8 ppm. The carbon attached to doubly bonded nitrogen (-N=) appeared at 151.6 ppm. All other carbons appeared in the range of 135.3-129.5 ppm.

The finest characterization of the synthesized compounds can be seen from the CHN analysis. The theoretical calculations and their resemblance to experimental values confirm the absence of chlorine in the structure of the Cu(II) complex. From CHN analysis it is also confirmed that two ligands are coordinating with the copper centre and the Cu(II) complex could be in square planer geometry.

2. Thermal analysis

Thermal analysis (TGA and DTA) of the ligand and its Cu-complex was performed to calculate the weight loss happening during pyrolysis as a function of temperature (Fig. 1). TGA of ligand and its Cu-complex is comprised of two main thermal events: In the case of ligand, the first weight loss (20%) is due to incomplete decomposition and at 400°C, the second weight loss (80%) is due to the whole decay of ligand. While for Cu(II) complex, the organic moiety decomposed at 496°C in the form of major weight loss (54%). Above 496°C, there is no remarkable loss in weight, indicating the formation of CuO. In the DTA curve of ligand, two sharp peaks at 228°C and 388°C demonstrate the presence of wobbly intermediates through this filth process, while, these peaks shifted at high temperature, 235°C and 464°C respectively in the case of Cu(II) complex.

The stoichiometric calculations between the reactant and the end-product (in the case of Cu(II) complex) indicated the attachment of two ligands with one Cu metal ion. The final product was estimated as CuO which was later confirmed by the PXRD technique.

3. PXRD analysis

The amorphous nature is represented by ligand and its Cu(II) complex in the pattern of Powder XRD. as depicted in Fig. 2. While the TGA residue of the Cu(II) complex appeared as pure CuO in the PXRD pattern confirming the synthesis of the Cu(II) complex. The detected reflections represent the monoclinic structure having a space group (C2/c). The average crystallite size calculated through the Scherrer equation is 16nm.

$$D=0.9\lambda/\beta\cos\theta$$

4. DNA Binding Studies

4.1. UV-Visible Titrations

To inspect the interaction between the synthesized compound and CT-DNA electronic absorption spectra were taken. Broadly speaking the peak at, 310-330 nm appeared both in the case of ligand and its Cu(II) complex. A prominent shoulder at 300.10 nm was also aroused. Both compounds give hypochromism and hypsochromic shift (1 nm) observed by an increasing concentration of DNA. According to the literature, the peak shifting due to the interface of compounds with CT-DNA gives a strong clue about the mode of binding between compounds and DNA.[9] A hypsochromism may be noted due to the protonation of aromatic nitrogen resulting in the establishment of the cation which is the involvement of the lone pair of nitrogen that disturbs the conjugation in chromophore leading to a hypsochromic shift in the peaks. We can say that the addition of DNA changes the microenvironment of the compound in an aqueous solution.

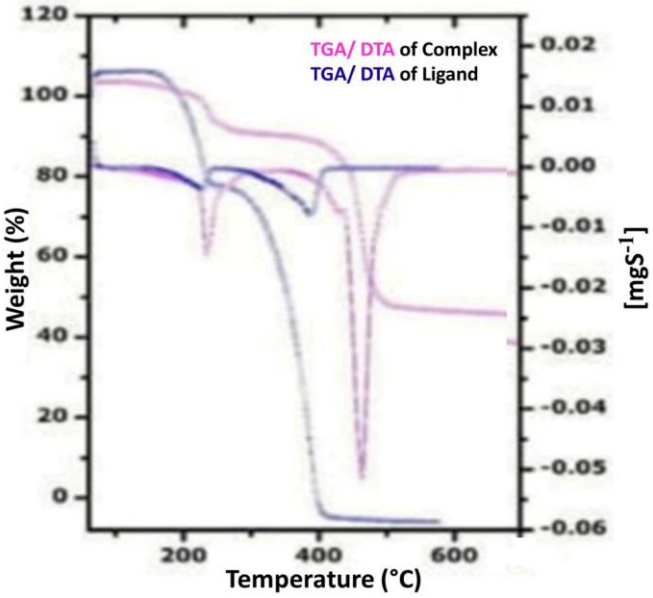


Fig. 1 – TGA/DTA curves of ligand and its Cu(II) complex.

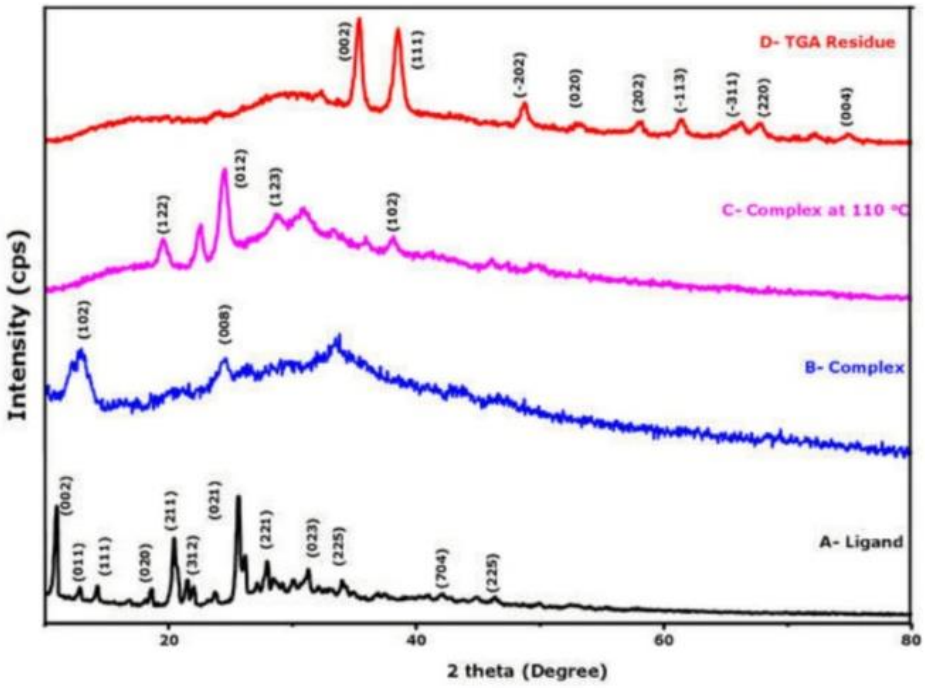


Fig. 2 – PXRD curves of ligand, Cu(II) complex and TGA residue.

A decrease in absorbance may be due to the proximity of the compound to the DNA bases as compared to the solvent. The hypsochromic shift may be due to the intercalation of the benzimidamide moiety of Cu(II) complex into the DNA helix, resulting in the shortening of the helical axis of the DNA and alteration of confirmation of DNA. The hyperchromic shift of absorbance results in the mutilation of the DNA double helix. We do not examine such a hyperchromism indicating that there is no mutilation to the DNA upon binding with ligand or Cu(II) complex (Fig. 3a-3b).

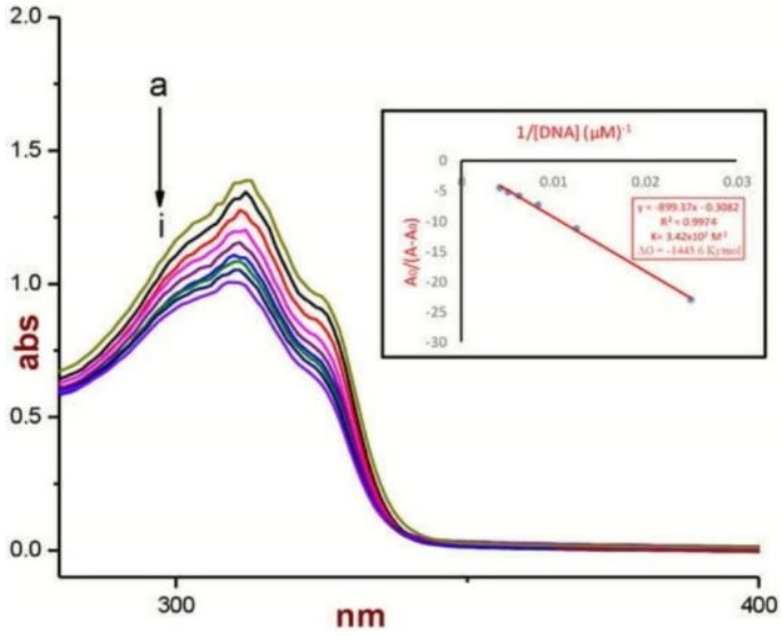


Fig. 3a – The absorption spectrum of the ligand in diverse [DNA] and calculations of Gibb's free energy (ΔG) and binding constant (K).

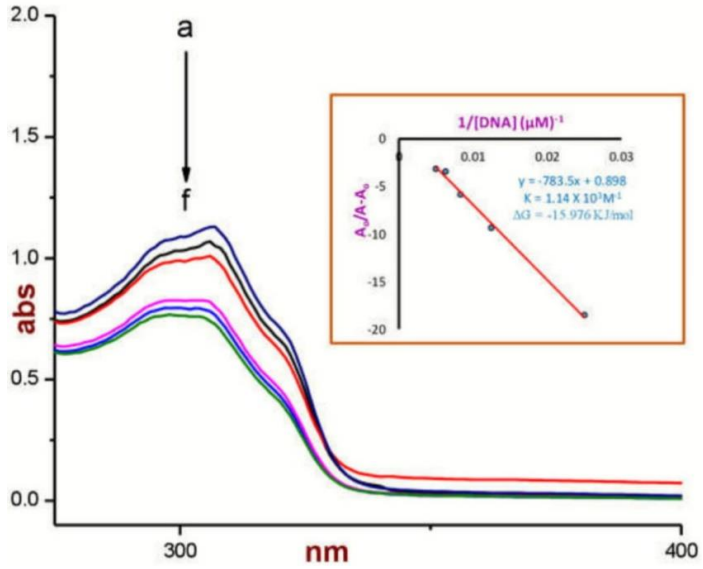


Fig. 3b – The absorption spectrum of the Cu(II) complex in diverse [DNA] and calculation of Gibb's free energy (ΔG) and binding constant (K).

It is expected that the hypochromic shift is due to a lessening of π-π* transition energy or lessening of transition possibilities due to the coupling of the π* orbital of the ligand or Cu(II) complex with a π orbital of base pairs of DNAs. Hence intercalative association/ binding constant, K emerges from the π-π* stacking contact and can be calculated by plotting $A_o/(A-A_o)$ vs 1/[DNA]. Where A_o is the absorbance of ligand plus DNA and A is the absorbance of Cu(II) complex plus DNA. The high value of 'K' may be accredited to the occurrence of electron-donating methyl group and nitrogen of NH_2 Group that can cause hydrogen bonding with DNA bases. DNA interaction studies give a strong clue about the anti-cancer potential of ligand and its Cu(II) complex.

4.2. Viscosity measurement

In the absence of crystallographic data, hydrodynamic methods sensitive to the length such as viscosity measurements are noticed as very critical tests of a binding mode in the solution phase.[10] In classical intercalation, the length of the DNA helix increases due to the separation of base pairs to accommodate the bounded compound. This results in the reduction of the effective length of the DNA helix and an increase in viscosity. A similar condition was aroused due to the presence of HS DNA in an aqueous solution of the compounds under test, indicating that the ligand/ Cu(II) complex may be inserted among the DNA base pairs and promote the separation of DNA base pairs at the site of intercalation result in the overall lengthening of DNA helix. This effect was more observed in the case of the Cu(II) complex which is exactly like the Cu(II) complex testified by Raman *et al*.[11] Conclusively the increase in DNA viscosity noticed in the Cu(II) complex, recommends a classical intercalative mode (Fig. 4).

5. DNA Cleavage Studies

Going into the depth of DNA interaction studies, the cleavage of pBR322 supercoiled plasmid DNA in the presence of ligand and Cu(II) complex was examined by agarose gel electrophoresis. In this regard conversion of a supercoiled form of DNA (DNA-SC, form I) into the nicked circular form of DNA (DNA-NC, form II) or linearized form of DNA (DNA-LN, form III) was inspected. It has been observed that the concentration of Cu(II) complex, 3μM (lane 7), 4μM (lane 8) and 6μM (lane 9) has the aptitude to moderately change DNA–SC to DNA–NC and DNA–LN. While the ligand (6μM) under the same conditions denoted no cleavage activity (lane 4, Fig. 5). It has also been examined that Cu(II) complex displayed no DNA scission in the absence of ascorbate activation, signifying the absence of a hydrolytic mechanism (lane 5, Fig. 5).

Fig. 4 – The plot of [ŋ/ŋ°]1/3 versus [COMPOUND]/[DNA] for ligand (red) and Cu(II) complex (purple).

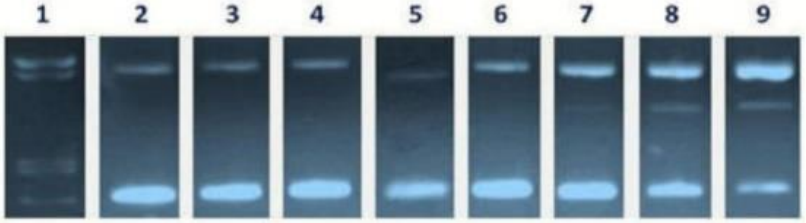


Fig. 5 – "1. λ DNA /EcoRI + Hind III Marker, 2. supercoiled DNA control; 3 supercoiled DNA control + ascorbate 6 mM; 4 ligand 6 μM; without ascorbate; 6. complex 1.8 μM; 7. complex 2 μM; 8. complex 4 μM; 9. complex 6 μM".

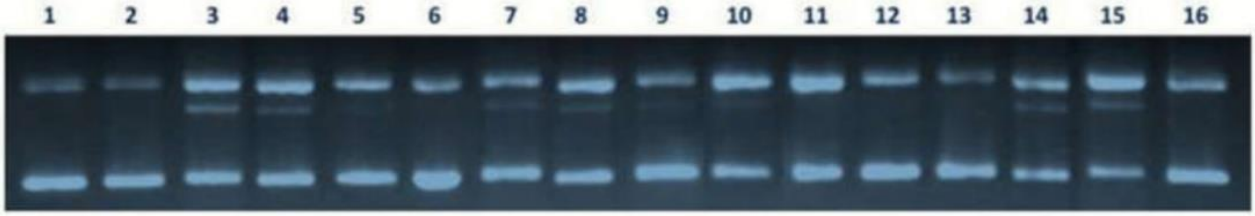


Fig. 6 – "1. λ DNA / EcoRI + Hind III Marker; 2. supercoiled DNA control; 3. complex 6 μM; 4. complex 6 μM + DMSO 0.4 M; 5. complex 6 μM + tert-butyl alcohol 0.4 M; 6. complex 6 μM-sodium formate 0.4 M; 7. complex 6 μM + urea 0.4 M; 8. complex 6 μM + tetramethyl piperidone 0.4 M; 9. complex 6 μM +DABCO 0.4 M; 10. complex 6 μM + TRION 100 mM; 11. complex 6 μM + neocuproine 12 μM; 12. complex 6 μM + neocuproine 120 μM; 13. complex 6 μM + neocuproine 240 μM; 14. complex 6 μM + distamicine 8 μM; 15. complex 6 μM + methyl green 1.25 μg/ mL; 16. complex 6 μM + catalase 650 U/ mL".

5.1. Mechanistic study

The mechanistic study of DNA cleavage facilitated by the Cu(II) complex was done with ROS scavengers. The addition of sodium formate and tertiary butyl alcohol inhibits the cleavage process indicating the involvement of hydroxyl radical (lanes 5 and 6, Fig. 6) while less inhibition in the presence of DMSO and urea does not entirely reject the connection of hydroxyl radical (lanes 8 and 9, Fig. 6) but indicating the involvement of singlet oxygen or a singlet-oxygen-like entity. It means that one of the active oxygen intermediates is accountable for DNA scission. The inhibition in the presence of TIRON divulges that superoxide anion (O^{2-}) is participating in the cleavage process (lane 10, Fig. 6).

Catalase also regulates the nucleolytic process (lane 16, Fig. 6) confirming the importance of hydrogen peroxide (H_2O_2) in the cleavage of DNA. Prior treatment of pUC18 DNA with distamycin and methyl green has also no effect on the cleavage process arbitrated by the compound (lanes 14 and 15, Fig. 6). The DNA cleavage inspection strongly states the no interaction of Cu(II) complex with two grooves of the double-stranded structure of DNA. Furthermore, the consistency of the cleavage process in the presence of a 12 μM concentration of neocuproine (lane 11, Fig. 6) and inhibition at 120 μM and 240 μM concentration of neocuproine affirms the participation of Cu(I) in the cleavage process.

The presence of neocuproine at a concentration does not interfere with the cleavage process Inhibition of the process is observed at higher concentrations of neocuproine (120 μM and 240 μM), indicating that Cu (I) is involved in the process. All this may be followed by the mechanism suggested by Bocarsly *et al.* fetching hydroxyl radicals, formed by various physical and chemical pathways linked to either Fenton or Haber-Weiss reactions mechanism.[12]
Fenton mechanism:

$$Cu^{2+}L + asH^{2-} \rightarrow Cu^{+}L + asH^{-}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+}L + OH^{-} + OH^{-}$$

Haber-Weiss reaction:

$$Cu^{+}L + O_2 \rightarrow Cu^{2+}L + O^{2-}$$

$$O^{2-} + H_2O_2 \rightarrow O_2 + OH^{-} + OH$$

6. Anti-cancer studies

The results from DNA interaction studies forced us to examine the potential of ligand and its Cu(II) complex against MDA-MB-231, MCF-7, WRL-68 and MCF-10A cell lines. Before the MTT assay, the in vivo toxicity of both the compounds was observed in liver and kidney function tests. This study has been approved by the Institutional Bioethics Committee (IBC) with the corresponding number, 4350/ ORIC.

6.1. Acute toxicity

Both the ligands and the Cu(II) complex have the potential to interact with the DNA. So it is necessary to study the effect of these compounds on the liver and kidney functioning because harmful effects may cause an alteration of many functionalities leading to a high risk of liver and renal cancer. The finding of this study indicates that the mice groups treated with the ligand and its Cu(II) complex exhibit normal levels in the biochemical tests of the liver and kidney. The reduced level of liver and kidney parameters relative to the control does not show any type of toxicity in the acute toxicity test which was conducted for ligand and its Cu(II) complex on liver function test at a dosage of 250 mg/kg (Fig. 7). However, in the case of the liver functioning test Cu(II) complex shows less toxicity as compared to the ligand. Overall there is no sign of renal or hepatic toxicity found.

6.2. MTT Cytotoxicity

MTT cytotoxicity assay was carried out to assess the anti-proliferation effect of the ligand and its Cu(II) complex on various cancer cell lines. The results show that both compounds prominently inhibited the proliferation of MCF-7 cells. However, they presented no suppressive activity against human normal MCF-10 breast cells and normal WRL-68 hepatic cells relative to the IC_{50} value of ligand and its Cu(II) complex toward MCF-7 cells. The IC_{50} values in the case of the Cu(II) complex are closer to the IC_{50} values in the presence of positive control doxorubicin. This may be due to the redox nature of copper. Fig. 8. It is well accepted that high toxicity towards cancer cells and low toxicity towards normal cells represent potent cancer inhibitor.[13]

6.3. Molecular Docking

The Autodock tool was used to further investigate the interaction pattern and check the potency of inhibitors against the target protein human serum albumin (PDB code: **1AO6**). The bonding interaction pattern (hydrogen/ hydrophobic) and minimum binding affinity values (kcal/mol) were used to evaluate the resultant docked complex. The docking results revealed that Cu(II) complex exhibited the lowest value of binding affinity (-7.1 kcal/mol), while ligand predicted the binding affinity of -6.4 kcal/mol.
Further analysis of the docked complex was performed based on hydrogen bonding, π-Alkyl, π-anion, and Van der Waals interactions. Docking analysis showed that both the docked compounds interacted with the active binding region of human serum albumin.[30] It has been observed that the interaction between the synthesized compounds and the target protein consists involved in hydrogen bonding and hydrophobic interactions as can be seen in the 3D and 2D pictures of the compounds (Fig. 9,10). Van-der-Waal interactions were also shown by compounds with the various amino acid residues in the binding pocket of human serum albumin. Overall, the docking study determines the good affinity of the reported compounds toward human serum albumin (PDB code: **1AO6**) and could be considered potent anti-cancer inhibitors in the future drug discovery process.

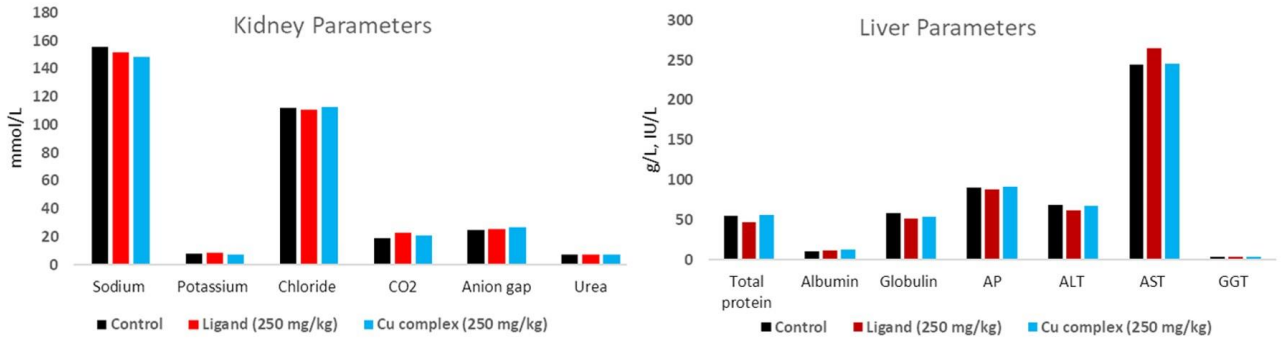


Fig. 7 – Effects of the ligand and its Cu(II) complex at dosage 250 mg/kg on liver and kidney function test.

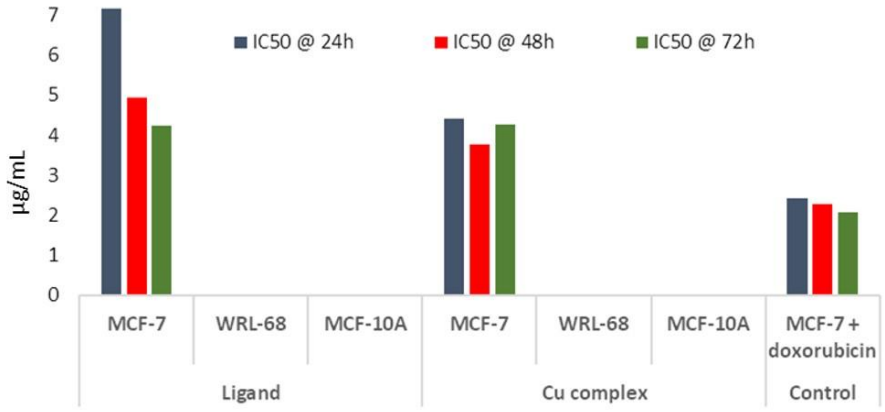


Fig. 8 – The IC_{50} concentration of the ligand and its Cu(II) complex against MCF-7, MCF-10A and WRL-68 cell lines after 24, 48 and 72h.

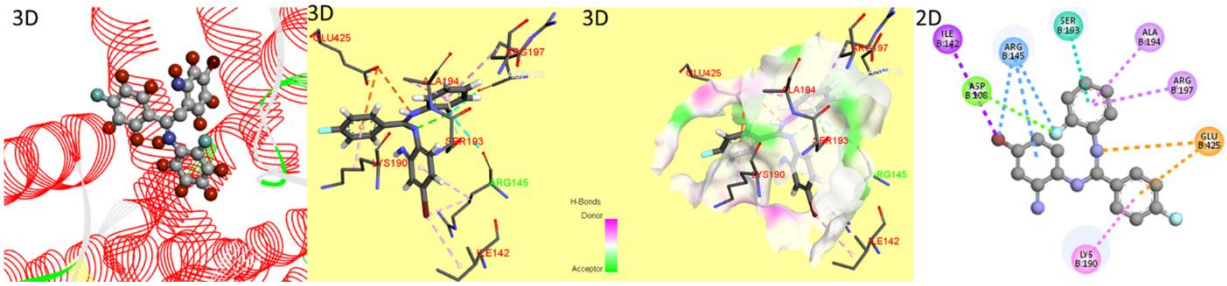


Fig. 9 – 3D and 2D depictions of docking of ligand in the active site of human serum albumin.

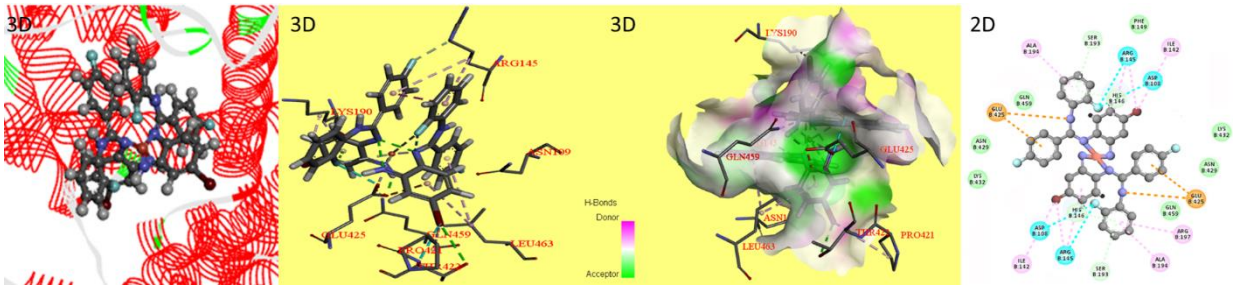


Fig. 10 – 3D and 2D depictions of docking of Cu(II) complex in the active site of human serum albumin.

MATERIALS AND METHODS

All chemicals were commercially accessible and were used without more refinement. All the chemicals and CT-DNA were procured from Sigma-Aldrich. FT-IR spectra were recorded in the range 4000-400 cm^{-1} with a. Bio-Rad Excaliber FT-IR FTS 3000 MX. Perkin Elmer 2400 CHNS

elemental analyzer was used to calculate the CHN analysis. The electronic spectra were recorded in DMSO with a Shimadzu UV-spectrophotometer. PXRD was recorded on a Burker D8 advance Cu K alpha (λ 1.54 Å) 2 theta range 3140 degrees. Thermal analyses were accomplished using a Perkin-Elmer Thermogravimetric Analyzer TG/DTA 6300 under an N_2 gas flow (20 $mLmin^{-1}$) at ambient pressure and a heating rate of 10 °$Cmin^{-1}$. Single-mode cavity discovers microwave (CEM Corporation, NC) generating non-continuous irradiation at 2450 MHz was used for irradiating reaction mixture.

1. Synthesis of Ligand

(i) Synthesis of N-(2-amino-4-bromophenyl)-4-fluorobenzamide

The synthesis was initiated by the dropwise addition of 4-fluorobenzoyl chloride at 4-bromobenzene-1,2-diamine in a 100 mL beaker under solvent-free reaction conditions (SFRC). To handle the vigorous nature of the reaction beaker was placed in an ice bath at 10°C. The reaction mixture has been continuously stirred with the rod until fumes have stopped or the order of 4-fluorobenzoyl chloride no longer is detected. After completion of the reaction, washed the mixture with cold water and dried the N-(2-amino-4-bromophenyl)-4-fluorobenzamide as a product.

(ii) Synthesis of N-(2-amino-4-bromophenyl)-4-fluoro-N'-(2-fluorophenyl)benzimidamide

The *N*-(2-amino-4-bromophenyl)-4-fluorobenzamide (4.0 mol) was ground thoroughly with 15 mol% CuO powder and made a paste with equimolar 2-fluoroaniline. The reaction mixture was placed in a 10 mL heavy walled-Pyrex tube sealed with aluminium crimped cap fitted with a silicon septum and then irradiated (80 W) for 15 minutes at 80°C in a microwave. After this reaction mixture was brought to room temperature. Aqueous workup results in separation of *N*-(2-amino-4-bromophenyl)-4-fluoro-*N*'-(2-fluorophenyl)benzimidamide as light yellow precipitates. Yield (84%); m.p., 198-200°C. IR spectrum (λmax, cm^{-1}): 1638 (C=N), 1687 (C=O), 3220 (NH), 3445 (NH_2). ^{1}H-NMR spectrum in DMSO-d_6 (δ, ppm): 11.04 (s, 1H, NH), 8.23-.8.20 (m, 4H, F-PhN), 7.76 (d, 2H, F-Ph), 7.59 (d, 2H, F-Ph), 7.43 (d, 2H, Br-Ph), 7.01 (s, 1H, Br-Ph), 5.05 (s, 2H, NH_2). ^{13}C-NMR spectrum in DMSO-d_6 (δ, ppm): 17.54, 19.75, 95.44 (C=C), 106.27 (C=C), 124.18, 129.94, 137.70, 150.08 (Ph), 162.76 (C=N), 163.64 (C=O), 163.83 (C=O). Found (%): C, 56.67; H, 3.76; N, 10.66; $C_{19}H_{14}BrF_2N_3$. Anal. calcd. (%): C, 56.73; H, 3.51; N, 10.45.

2. Synthesis of Cu(II) complex

(iii) (N-(2-amino-4-bromophenyl)-4-fluoro-N'-(2-fluorophenyl)benzimidamide)$_2$Cu

The synthesized ligand and copper(II) chloride were ground in a mortar in a pestle to make the mixture fully homogenized and then added 5 mL of ethanol in 25 mL round bottom flasks. Four drops of triethylamine were added. The reaction mixture was irradiated for 5 min at 70°C and 80 W to get the precipitations which were filtered, washed and dried in an oven at 100°C for 15 minutes to get the desired Cu(II) complex. Brown ppt. yield (69 %); IR spectrum (λmax, cm^{-1}: 444 (Cu-N), 542 (Cu-O), 1556 (C=C), 1596 (C=N), 1687 (C=O), 3335 (CH),3370 (NH). Found (%): C, 52.89; H, 3.79; N, 10.62. $C_{38}H_{26}Br_2CuF_4N_6$. Anal. calc. (%): C, 52.70; H, 3.03; N, 9.70.

3. DNA Interaction studies

3.1. UV–Visible titration assay

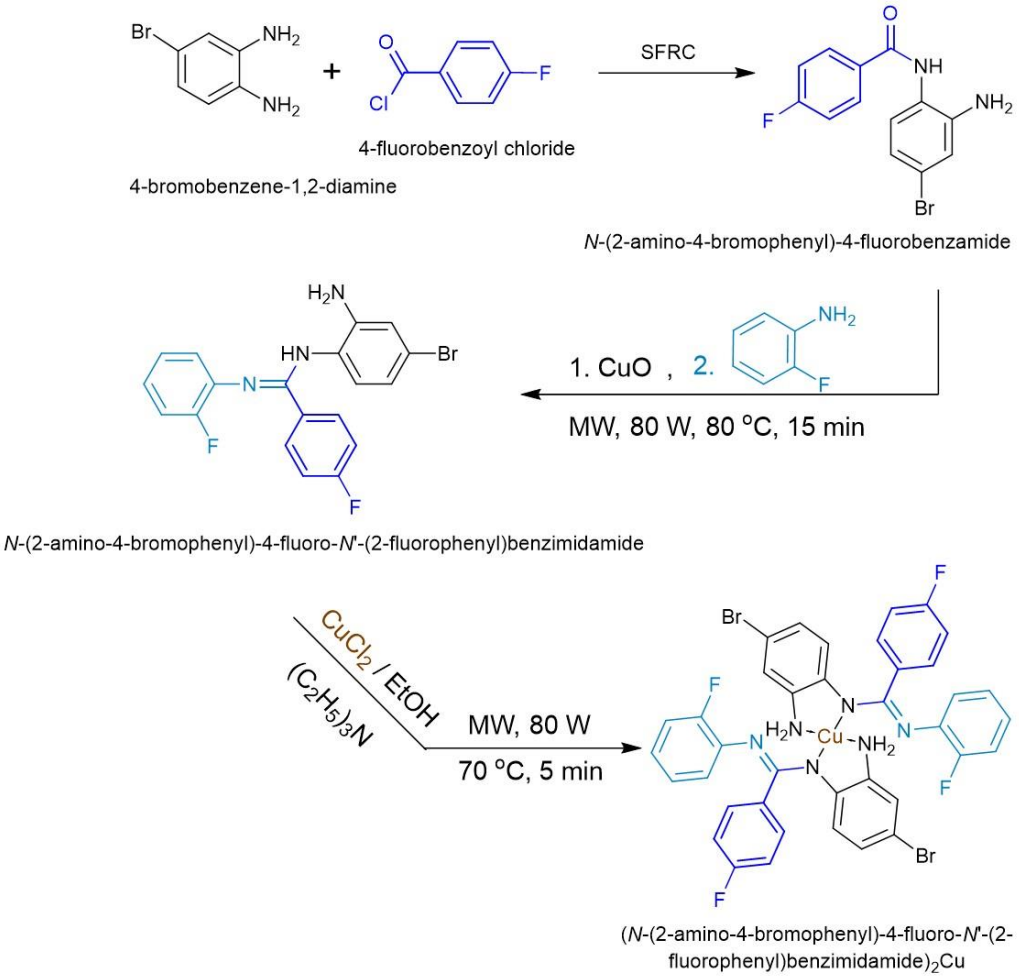
DNA-mediated hypochromic, as well as hypsochromic shifts, were observed in the ultraviolet-visible absorbance spectrum under the influence of synthesized compounds. At 25°C, an equal amount of DNA was kept in the reference cells and incubated for 10 minutes. Hypochromism was calculated by using a different concentration of CT-DNA in a fixed concentration of the test compound.[14]

The binding strength of the tested compound to DNA can be presented in terms of binding constant Kb. Kb was determined from the slope of the plot Ao/A-Ao vs. 1/[DNA] $(\mu M)^{-1}$ and was used for the calculation of ΔG using the equation ΔG=-RT ln Kb.

3.2. Viscometry assay

Viscosity measurements were taken by using a viscometer (SCHOT AVS 450) kept at 25.0 ± 0.5°C. About DNA samples (200 base pairs in length) were made by the sonication method to decrease the possible complexities resulting from DNA flexibility. A digital stopwatch was utilized to measure flow time. Flow time was measured thrice for each sample from which average flow was calculated. In the presence or absence of the compounds, the comparative viscosities for DNA were measured using the following equation.[15]

$$\eta \propto (t-t_o)$$



Scheme 1 – Synthesis of ligand and its Cu(II) complex.

where t represents the actual flow time of solution containing DNA while t_o is the flow time of phosphate buffer solution alone without using DNA samples. Data were represented as $(\eta/\eta_o)^{1/3}$ versus [complex]/[DNA], where ŋ and η_o refer to the viscosity of DNA in the presence of compound and viscosity of DNA alone respectively.

4. DNA Cleavage Assay

In this assay 6µL of a compound solution (50% DMF) at an increasing concentration between 1.8 to 6 µM, 7 µL of borate buffer (pH= 8.0), 1 µL of pUC18 (0.25 μgL^{-1}) and sodium ascorbate solution (6 µL) with 1000-fold molar excess relative to the concentration of the compounds prepared in borate buffer were incubated together at 37°C for 1 h. After that 3 µL of a quench of buffer solution (containing 0.25% bromophenol blue, 0.255 xylene cyanole) and 30 % glycerol were added to the incubated mixture. The resulting solution was subjected to Agarose Gel Electrophoresis (80 V for 2 h) using 0.8% agarose gel in 0.5x TBE buffer (0.045 mM TRIS, 0.045 mM boric acid and 1 Mm EDTA) which contained 2 µL per 100 mL of a solution of ethidium bromide (10 mg mL^{-1}). Gel printer plus TDI was used for photography.

"Mechanistic study was done by using different ROS (reactive oxygen species) scavengers; Standard hydroxyl radical scavengers including DMSO 0.4 M, sodium formate 0.4 M and urea 0.4 M, singlet oxygen scavengers including 2,2,6,6-tetramethyl-4-piperidone 0.4 M and DABCO 0.4 M, catalase 650U/ mL, tert-butyl alcohol 0.4 M, and a superoxide radical scavenger (TIRON 100 Mm). Major and minor groove binders were also used which are listed as follows. Ligand Neocuproine (strong chelator of Cu-I) with concentrations of 12, 120 and 240 µM was employed, which is used to check the reduction of Cu-II to Cu-I. Methyl green, (2.5 µL of a 0.1 $mgmL^{-1}$ solution) was employed as a major groove binder and distamycin with a concentration of 8 µM was used as a minor groove binder".[16]

5. Anti-cancer Studies

5.1. Acute cytotoxicity assay

To study the toxic effects of ligand and its Cu(II) complex thirty healthy female mice were used as model animals. The animals were subjected to animal standards set by the National Academy of Science's Guide regarding the Care and Use of Laboratory Animals. In our study, experiments were conducted on six groups of mice that were abstaining from all or some kinds of food or drink for 24 h[17]. 250 mg/kg dosage of synthesized compound was given to five groups of mice and the remaining group is used as normal control. Following feeding, the mice were evaluated for the upcoming 30 minutes and 2, 4, 24 and 48 h to decennary abnormal features. The above-mentioned dosage was continued, and the effects were observed until after 15 days, the mice were treated with an overdose of anaesthesia (ketamine and xylazine). The serological analysis was done on blood samples. Kidney and Liver histological analysis was carried out using eosin (H&E) and Haemotxylin.

5.2. MTT Cytotoxicity assay

Normal WRL-68 hepatic cells, human MCF-7 breast adenocarcinoma cells and normal MCF-10 Breast cells were purchased from ATTC (Manassas, VA, USA,). These cells were preserved in PRM-(1640) (Roswell Park Memorial Institute) medium which was accompanied by 10% FBS to perform a cytotoxicity test, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was employed [18]. In this assay, cells (5×10^4 cells/ mL) were implanted into sterile 96-well- plates (Nunc-, Germany). On the following day, the given cells were exposed to a varied concentration of each compound, whereas doxorubicin was used as +ve control. After 24, 48, and 72 h of incubation, incubated cells were stained with 20 µL of MTT (0.21×10^{-6} mol/L) for 3 hours. The resulting dark formazan crystals were dissolved in DMSO (100µL) and again incubated for 2 hours in dark. ELISA reader (Hidex, Turku, Finland) was used to calculate the absorbance (570 nm λ). After calculating IC_{50} values, the data were reported as the average of three replicates.

5.3. Molecular docking

Docking simulations were performed on Autodock vina by following the reported literature[19] with slight modifications. The 3D structure of human serum albumin (PDB code: 1AO6) was selected as target protein for the docking experiments with following parameters: size x = 20; size y = 20; size z = 20; center x = 25.2811; center y = 36.7115; center z = 23.1797.

CONCLUSIONS

A new ligand (N-(2-amino-4-bromophenyl)-4-fluoro-N'-(2-fluorophenyl)benzimidamide) and its Cu(II) complex was successfully synthesized through the simplest microwave approach with high efficiency. Both compounds have very good hypochromic and hypsochromic shifts on the attraction with DNA showing intercalative mode attraction which was further confirmed by viscometry. Which can damage DNA via the oxidative path. Mechanistic study about DNA cleavage unravels that Cu(II) complex, even at low concentration can cleave the DNA via an oxidative mechanism. The meagre nuclease activity of the Cu(II) complex in the absence of an oxidant is seemingly due to more stability of the Cu(II) complex. In determining cytotoxic parameters, the ligand, and the Cu(II) complex inhibits the proliferation of MCF-7 cells without harming the liver, kidney, and normal breast cells. Information acquired from the present *in silico* study gives new bits of knowledge for the identification and validation of a new inhibitor against a specific drug target. The good correlation between our model and the *in vitro/ silico* experimental data suggests that as compared to ligand, the Cu(II) complex could be a more promising anti-cancer agent.

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