Chapter-3
Interaction Of Mixed Ligand Copper(II) Complexes with DNA and its Anti-Proliferative Effects
3.1 BACKGROUND

The main concerns in the design of biologically important molecules are:

(i) need to bind with genetic materials
(ii) necessity to have nuclease activity
(iii) inhibition of translation process upon mutation in the genetic material

This is because (i) and (ii) are very important in enzymatic reactions, which are involved in biological processes and in biotechnological manipulation of gene materials. Drug design also calls for the study of in vitro and in vivo biological activities of the designed molecules. Nucleases are the enzymes, which hydrolyze the phosphodiester bond of genetic material such as DNA and RNA, much faster by a factor of $10^{12}$ than the non-enzymatic reactions [1]. A number of molecules have been identified to bring the hydrolytic cleavage in RNA [2-7], whereas very few reports are available on such reaction of DNA due to its relatively high thermodynamic stability against hydrolysis [8-10]. The presence of 2’ hydroxyl group in ribose ring makes the hydrolysis to proceed faster in the case of RNA, whereas the absence of hydroxyl group in the same position impedes the reaction in DNA, which enables the gene coding. Rational design of nuclease mimics is an intense area of research in the field of bioinorganic chemistry. Ligands
(primary and co-ligand) play a crucial role in the binding of metal complexes to DNA as well as tuning the redox property of the metal complex.

Hence, rationally designed mixed ligand complexes can bind to DNA strongly and can also act as a nuclease mimic. It was proposed that, the cytotoxic effects of the metal complexes depend on their binding ability towards DNA. Since, the importance of copper ions and its biological relevance have been discussed in Chapter 1, the current chapter focuses on the synthesis of mixed ligand copper(II) complexes, their interaction with DNA and anti-proliferative effects of the complexes on osteosarcoma, MG63 and normal fibroblast NIH3T3 cell lines.

1,10-Phenanthroline (phen) derivatives has been chosen as a bidentate co-ligands for the present investigation because phen and its derivatives possess following merits [11]

i. Planarity

ii. Rigidity

iii. Hydrophobicity

iv. Heteroaromaticity

v. Thermodynamically (entropically) favorable chelation

Moreover, ligands such as bipyridine and terpyridine possess relatively less metal binding aptitude due to their available free rotation about the bipyridyl C—C axis compared to phen. It is also interesting to note that,
the symmetric substitution in phen at the 2,9-positions enhances the fluorescence intensity [12]. The chemical structure of terpyridine (terpy), phenanthroline and bipyridine (bpy) has been presented in Figure 3.1.

![Chemical structures of terpyridine (terpy), phenanthroline (phen), and bipyridine (bpy)](image)

**Figure 3.1 Schematic representations of the ligand and co-ligands**

The second ligand chosen is terpy derivative. Terpyridine ligand was chosen due to its following merits [13]

i. good chelating agent

ii. ease of substitution at 4′ position

iii. π-stacking ability of the ligand

These salient features of phen and terpy can be collectively used to tune the steric/electronic properties and furthermore these ligands may favor five coordinate complexes with copper(II) center. Pentacoordinate square pyramidal or trigonal bipyramidal complexes are of interest because of the possible sixth vacant coordination site, and such metal complexes may be good candidates to bring about hydrolytic cleavage of DNA.
3.2 SYNTHESIS OF LIGANDS

3.2.1 Synthesis of 2-(4-(1H-imidazol-2-yl)-6-(pyridine-2-yl)pyridine (L1=itpy)

The ligand was synthesized by reported procedure using solventless aldol condensation and Michael addition method [14]. Sodium hydroxide (0.8 g, 20 mmol), 2-acetylpyridine (2.4 g, 20 mmol) and imidazole-2-carboxaldehyde (1.0 g, 10 mmol) were ground well using a mortar and pestle. The color of the mixture turned yellow and this yellow medium was aggregated until orange red powder was obtained. The powder was transferred to a suspension of ammonium acetate (5 g) in glacial acetic acid (10 mL) and the reaction mixture was allowed to reflux for 2h. The crude ligand was precipitated by the addition of H₂O (5-10 mL), which was collected, washed and recrystallized from ethanol/acetonitrile (2:5) mixture. The authenticity of the ligand was established by ESI-MS analysis where ligand itpy showed molecular ion peak as base peak at m/z 300 (M+H)⁺.

^1H NMR (500 MHz; DMSO-d₆; TMS) 7.16 (1H, s), 7.37 (1H, s), 7.50 (2H, t), 8.00 (2H, t), 8.63 (2H, d), 8.74 (2H, d), 8.97 (2H, s), 13.22 (1H, s).

3.2.2 Synthesis of 4-(2,6-di(pyridin-2-yl)pyridine-4-yl)pyridine (L2=ptpy)

The ligand ptpy was synthesized by adopting the same procedure as itpy. By employing pyridine-2-carboxaldehyde as the aromatic aldehyde the corresponding terpyridyl derivative was synthesized and characterized. The authenticity of the compound was confirmed through its ESI mass spectra, which showed the molecular ion peak at m/z 311 (M+H)⁺. ^1H NMR
(500 MHz; DMSO-d$_6$; TMS) 7.12 (2H, d), 7.16 (2H, s), 7.47 (2H, d), 7.50 (2H, t), 8.00 (2H, t), 8.63 (2H, d), 8.74 (2H, d)

3.2.3 Synthesis of 2-(4-(4-methoxyphenyl)-6-(pyridine-2-yl)pyridine-2-yl)pyridine (L3=meotpy)

By the same procedure as mentioned above meotpy was synthesized by having methoxybenzene as the aromatic aldehyde and the synthesized ligand was characterized by ESI-MS and $^1$H NMR techniques. ESI-MS of L3 showed molecular ion peak at $m/z$ 340 corresponding to (M+H)$^+$. $^1$H NMR (500 MHz; DMSO-d$_6$; TMS) 7.12 (2H, d), 7.37 (2H, d), 7.50 (2H, t), 8.00 (2H, t), 8.63 (2H, d), 8.74 (2H, d), 8.40 (2H, d), 3.73 (3H, s)

3.3 SYNTHESIS OF PENTA CO-ORDINTATE COPPER(II) COMPLEXES

Compared to conventional copper(II) complexes with four and six coordinate geometry the five coordinate complexes have been synthesized by reacting the equimolar ratio of tridentate ligand, bidentate ligand and copper(II) nitrate salt. Three copper(II) complexes were synthesized by the procedure described below.

3.3.1 Synthesis of [Cu(itpy)(dmp)](NO$_3$)$_2$·4.5H$_2$O (1)

Complex 1 was synthesized by stirring of Cu(NO$_3$)$_2$·3H$_2$O (0.12 g, 0.5 mmol) with itpy (0.15 g, 0.5 mmol) at room temperature for 10 minute followed by the addition of 2,9-dimethylphenanthroline (dmp) (0.11 g, 0.5 mmol) to the above solution and continued stirring for 15 minute. The
reaction mixture was filtered and set aside for slow evaporation. A green solid that separated out upon slow evaporation of the solvent was washed with diethyl ether and dried under vacuum. The complex [Cu(itpy)(dmp)](NO$_3$)$_2$ was recrystallized from acetonitrile/water (5:1). Yield: 79%. Found C, 50.03; H, 4.46; N, 16.41. Anal. Calcd. For C$_{32}$H$_{27}$CuN$_7$: C, 49.97; H, 4.59; N, 16.39%. FT-IR (KBr pellet) cm$^{-1}$: 3396, 2926, 1618, 1384, 1029, 794.

3.3.2 Synthesis of [Cu(ptpy)(dmp)](NO$_3$)$_2$·2H$_2$O (2)

Complex 2 was synthesized by employing the procedure as described above with ptpy (0.31 g, 1 mmol), dmp (0.21 g, 1 mmol) and Cu(NO$_3$)$_2$·3H$_2$O (0.24 g, 1 mmol). The complex was recrystallized from acetonitrile-water (5:1). Yield: 76%. Found: C, 55.17; H, 4.16; N, 15.21. Anal. Calcd. For C$_{34}$H$_{30}$CuN$_8$O$_8$: C, 55.06; H, 4.07; N, 15.12%. FT-IR (KBr pellet) cm$^{-1}$: 3395, 3024, 1595, 1384, 1337, 856.

3.3.3 Synthesis of [Cu(meotpy)(dmp)](NO$_3$)$_2$·H$_2$O (3)

Complex 3 was synthesized by using the procedure employed for complex 1 with meotpy (0.34 g, 1 mmol), dmp (0.21 g, 1 mmol) and Cu(NO$_3$)$_2$·3H$_2$O (0.24 g, 1 mmol). A green solid that separated out upon slow evaporation of the solvent was filtered, and washed with diethyl ether and dried under vacuum. It was recrystallized from acetonitrile:water (5:1). Yield: 79%. Found: C, 57.59; H, 4.04; N, 13.13. Anal Calcd for C$_{36}$H$_{31}$CuN$_7$O$_8$: C, 57.40; H, 4.15; N, 13.02. FT-IR (KBr pellet) cm$^{-1}$: 3399, 2918, 1599, 1384, 1017, 837.
3.4 CHARACTERIZATION OF THE COMPLEXES

To authenticate the synthesized complexes, characterization of the complexes by spectroscopic and crystallography techniques were adopted and discussed below.

3.4.1 Electronic Absorption Spectra

The electronic absorption spectra of the complexes 1-3 were recorded in acetonitrile and shown in Figure 3.2.

![Figure 3.2 Electronic absorption spectra of complexes 1-3 in acetonitrile](image)

The electronic spectrum of complex 1 showed strong charge transfer band at 343 nm and intraligand transitions at 218 and 275 nm. The corresponding transitions for complexes 2 and 3 have been observed at 350,
276, 212 nm and 343, 277, 218, respectively. The ligand field transitions have been observed as a broad band centered at 628, 623 and 660 nm for complexes 1, 2 and 3, respectively. Electron Paramagnetic Resonance spectra of complexes 1-3 recorded at 77 K in DMSO revealed typical axial spectrum. All the three complexes exhibited $g_{||} > g_{\perp}$ values indicating that the unpaired electron predominantly lies in the $d_{z^2}$ orbital (Figure 3.3).

**Figure 3.3** EPR spectra of complexes 1-3 in DMSO at 77 K (Microwave frequency = 9.23 GHz, power = 3 mW, modulation frequency = 100 kHz, modulation amplitude = 5 G)
The “g” values for the three complexes are presented in the Table 3.1. Using WINEPR SIMFONIA package, simulation of the spectra were obtained which was used to calculate $g_\parallel$, $g_\perp$ and $A_\parallel$ values. As a representative simulation spectra, complex 1 is presented with experimental and simulation spectra in Figure 3.3.

Table 3.1  Characterization of complexes 1, 2 and 3 by Electronic, EPR Spectroscopy and Mass Spectrometry

<table>
<thead>
<tr>
<th>Complexes</th>
<th>g value</th>
<th>$\lambda_{mm}$</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cu(itpy)(dmp)]NO$_3$ (1)</td>
<td>$g_\perp = 2.22$</td>
<td>628, 343, 275, 218</td>
<td>285.34</td>
</tr>
<tr>
<td></td>
<td>$g_\parallel = 2.05$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Cu(ptpy)(dmp)] NO$_3$ (2)</td>
<td>$g_\perp = 2.22$</td>
<td>623, 350, 276, 212</td>
<td>291.34</td>
</tr>
<tr>
<td></td>
<td>$g_\parallel = 2.06$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Cu(meotpy)(dmp)] NO$_3$ (3)</td>
<td>$g_\perp = 2.22$</td>
<td>660, 343, 277, 218</td>
<td>305.20</td>
</tr>
<tr>
<td></td>
<td>$g_\parallel = 2.04$</td>
<td></td>
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</tr>
</tbody>
</table>

3.4.2  Mass Spectrometry

The authenticities of the complexes were confirmed by mass spectrometry. Mass spectral analysis provides the evidence for the formation of five coordinate Cu(II) complexes. Complexes showed signals for their respective molecular ions, at (m/z) 285.34 for complex 1, at (m/z) 291.34 for complex 2 and for complex 3 at (m/z) 305.20. Five coordinate geometries of the complexes are evident since molecular ion peaks has been observed as a base peaks in all the three cases.
3.4.3 Crystal Structure Analysis

Single crystals were grown for complexes 1, 2 and 3 from acetonitrile/water mixture. The structures of all the three complexes were obtained by single crystal X-ray diffraction studies. Complexes 1, 2 and 3 crystallized with the molecular formula \([\text{Cu}(\text{itpy})(\text{dmp})](\text{NO}_3)_2 \cdot 4.5\text{H}_2\text{O}\), \([\text{Cu}(\text{ptpy})(\text{dmp})](\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}\) and \([\text{Cu}(\text{meotpy})(\text{dmp})](\text{NO}_3)_2 \cdot \text{H}_2\text{O}\) with space groups \(\text{P2}_1/\text{c}\), \(\text{C}2/\text{c}\) and \(\text{P}2_1\text{c}1\text{c}1\), respectively. Addison et al., introduced a very useful parameter known as trigonality index \((\tau)\) which provides a measure of the degree of distortion in square-based pyramidal versus trigonal bipyramidal geometry adopted by five-coordinate Cu(II) complexes. The parameter “\(\tau\)” is defined as \((\beta-\alpha)/60\), where \(\beta\) and \(\alpha\), are the largest coordination angles, and its value varies from 0 for square pyramidal (sp) to 1 for trigonal bipyramidal (tbp) \([15]\). The complete description of the crystal structures of the three complexes with their \(\tau\) values are discussed in the following sections.

The molecular structure of complex 1 shows mononuclear dicationic nature of the complex having chelating terpyridine and phenanthroline ligand derivatives. The ORTEP representation of the complex 1 is shown in the Figure 3.4a.
Figure 3.4a  ORTEP representation of [Cu(itpy)(dmp)](NO₃)₂·4.5H₂O (1)

Figure depicts the coordination of the ligands as distorted square pyramidal (4+1) and the \( \tau \) value was calculated to be 0.33. The basal plane of the square pyramid is constituted by N(1), N(12) and N(18) from terpy ligand and the fourth position is occupied by N(42) from dmp ligand. The apical Cu—N(31) bond is 2.219 Å, which is slightly longer than the other Cu—N bonds. To compare, in the case of [Cu(itpy)₂]²⁺ the axial Cu—N bond has been reported to be 2.0726 and 2.0909 Å [16]. The dmp ligand in complex 1 is roughly perpendicular to terpy ligand. The pyridyl rings of the dmp ligand lying on the apical position of complex 1 are \( \pi \)-stacked with one another and the packing diagram is represented in the Figure 3.4b. Most remarkably, in the octahedral complex [Cu(itpy)₂]²⁺ \( \pi \)-stacking was between layered terpyridyl moieties [16]. The crystal packing diagram shown that, the -NH- groups of imidazolyl moiety are involved in hydrogen bonding with one
another. It is interesting to note that, the imidazole group containing -N- and -NH- facing outward is freely available for H-bonding. This may behave as H-donor and acceptor with the base pairs of DNA.

Figure 3.4b Crystal packing structure of [Cu(itpy)(dmp)](NO₃)₂·4.5H₂O (1)

The important bond lengths/bond angles for the complexes are listed in Table 3.2.
Table 3.2 Bond lengths and bond angles of complexes 1-3

<table>
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<tr>
<th></th>
<th><a href="NO%5Ctextsubscript%7B3%7D">Cu(itpy)(dmp)</a>\textsubscript{2} •4.5H\textsubscript{2}O</th>
<th>Bond distance/Å</th>
<th>Bond angle (°)</th>
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<tr>
<td>Cu(1)-N(12)</td>
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<tr>
<td>Cu(1)-N(42)</td>
<td>1.980(2)</td>
<td>N(12)-Cu(1)-N(1)</td>
<td>79.58(9)</td>
</tr>
<tr>
<td>Cu(1)-N(1)</td>
<td>2.037(2)</td>
<td>N(42)-Cu(1)-N(1)</td>
<td>99.49(9)</td>
</tr>
<tr>
<td>Cu(1)-N(18)</td>
<td>2.053(2)</td>
<td>N(12)-Cu(1)-N(18)</td>
<td>79.95(9)</td>
</tr>
<tr>
<td>Cu(1)-N(31)</td>
<td>2.219(2)</td>
<td>N(42)-Cu(1)-N(18)</td>
<td>98.38(8)</td>
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<tr>
<td></td>
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<td>N(1)-Cu(1)-N(18)</td>
<td>156.11(8)</td>
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<td>N(12)-Cu(1)-N(31)</td>
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<td>N(42)-Cu(1)-N(31)</td>
<td>80.21(8)</td>
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<td>N(1)-Cu(1)-N(31)</td>
<td>102.44(8)</td>
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<td><a href="NO%5Ctextsubscript%7B3%7D">Cu(ptpy)(dmp)</a>\textsubscript{2} •2H\textsubscript{2}O</td>
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<tr>
<td>Cu(1)-N(12)</td>
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<td>N(12)-Cu(1)-N(18)</td>
<td>79.93(7)</td>
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<tr>
<td>Cu(1)-N(18)</td>
<td>2.0134(18)</td>
<td>N(12)-Cu(1)-N(1)</td>
<td>79.80(7)</td>
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<td>N(18)-Cu(1)-N(1)</td>
<td>159.73(8)</td>
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<td>Cu(1)-N(42)</td>
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<td>N(12)-Cu(1)-N(42)</td>
<td>146.53(9)</td>
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<td>Cu(1)-N(31)</td>
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<td>N(18)-Cu(1)-N(42)</td>
<td>95.89(8)</td>
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<td>101.00(8)</td>
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<td>N(12)-Cu(1)-N(31)</td>
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<td>N(18)-Cu(1)-N(31)</td>
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<td>N(42)-Cu(1)-N(31)</td>
<td>79.63(9)</td>
</tr>
<tr>
<td><a href="NO%5Ctextsubscript%7B3%7D">Cu(meotpy)(dmp)</a>\textsubscript{2} •H\textsubscript{2}O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu(1)-N(12)</td>
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<td>N(12)-Cu(1)-N(42)</td>
<td>169.55</td>
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<td>N(12)-Cu(1)-N(18)</td>
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<td>Cu(1)-N(31)</td>
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<td>N(12)-Cu(1)-N(18)</td>
<td>96.80</td>
</tr>
<tr>
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<td>157.11</td>
</tr>
<tr>
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<td>N(1)-Cu(1)-N(31)</td>
<td>98.37</td>
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<td></td>
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<td>N(1)-Cu(1)-N(42)</td>
<td>101.47</td>
</tr>
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</table>
For complex 2, the $\tau$ value was calculated to be 0.63 which suggests the geometry of the complex is in between trigonal bipyramidal and square pyramidal. The ORTEP representation of complex 2 is shown in Figure 3.5a. The basal plane is formed by the N(31) and N(42) from dmp ligand and N(12) of ptpy ligand. The N(1) and N(18) of terpy ligand occupies the apical position. The Cu-N (equatorial) and Cu-N (axial) distances are 2.03 Å and 2.02 Å, respectively. This indicates axial and equatorial positions have almost similar bond lengths.

In this case, the pyridyl head group has been twisted by about 0.45°. It may be relevant to compare the Cu—N bond length of complex 2 with that of [Cu(trien)(phen)]$^{2+}$ which is reported to possess distorted square pyramidal
structure. The axial Cu—N bond length has been reported to be 2.186 Å which is longer than that observed in complex 2 and the equatorial Cu—N bond length ranges from 2.00 to 2.04 Å [17]. Complex 2 is an independent molecule without any π-stacking and H-bonding ability which is evident from the crystal packing structure of complex 2 shown in Figure 3.5b. Since dmp occupies the equatorial position and both the apical positions are occupied by the pyridyl moieties from terpy, π-stacking may be unfavorable.

![Crystal Packing Structure of [Cu(ptpy)(dmp)](NO$_3$)$_2$.H$_2$O (2)](image)

**Figure 3.5b Crystal Packing Structure of [Cu(ptpy)(dmp)](NO$_3$)$_2$.H$_2$O (2)**

Complex 3, with methoxybenzyl head group, crystallized with distorted trigonal bipyramidal geometry. The ORTEP representation of complex 3 has been given in the Figure 3.6.
The $\tau$ value for this complex has been calculated to be 0.89. The two Cu—N bond distances, Cu—N(31) and Cu—N(42) in the basal plane, arising from the coordination of copper(II) ion to the bipyridyl ligand, were found to be 2.2216 and 1.9942 Å, respectively. The third Cu—N(12) bond distance in the basal plane, arising from the coordination of terpyridyl ligand to copper(II) ion was 1.9352 Å. The two apical position are occupied by N(1) and N(18) from terpy ligand whose bond length are found to be 2.0357 and 2.0483 Å.

3.5 DNA BINDING STUDIES

3.5.1 Electronic Absorption Spectral Studies

To determine the binding ability of the synthesized complexes with biomolecule such as DNA, electronic absorption spectral titration method has
been adopted to monitor the interaction of complexes 1, 2 and 3 to CT-DNA. The absorption spectrum of 20 μM of complexes in the absence and presence of CT-DNA has been shown in the Figure 3.7. It could be seen from the spectra that binding of the complexes to DNA lead to perturbation in their ligand centered band. The absorption bands of 1, 2 and 3 were affected with increasing concentrations of DNA. A strong hyperchromic effect in the intraligand transition was observed for complexes 1 and 2, whereas for complex 3 hypochromic effect was observed. It was reported that binding of a metal complex to DNA through intercalation usually results in hypochromism in the absorption spectral bands of the complex due to strong stacking interaction between aromatic chromophore and the base pairs of DNA [18-20]. On the other hand, groove binders have been reported to increase the absorbance (Hyperchromism). Observed spectral behaviors clearly rule out intercalative binding of the complexes 1 and 2 to DNA, since intercalation would have led to hypochromism in the spectral bands of these two complexes.

Literature reports indicate that the bidentate ligand phen with 2,9-disubstitution intercalates weakly between the base pairs of DNA [21]. DNA possesses several hydrogen bonding sites, both in the major and minor grooves [21]. Besides, compounds with free -N- and -NH- groups (from amide or indole) favor hydrogen bonding with AT base pairs. Interactions with the groove walls and phosphate groups can enhance overall complex stability [23]. The minor groove contains primarily H-bond acceptor groups,
the purine N(3) and pyrimidine O(2), at the floor of the groove walls [24] and it is possible that groove binders with H- bonding ability can interact with these H-bond acceptors. The spectral changes observed in the case of complex 3 showed hyperchromism with incremental amount of DNA, are clearly indicative of intercalative mode of binding of the complex. This could be due to planarity of the methoxybenzyl head group which led to π-stacking interaction towards DNA.

![Figure 3.7](image)

**Figure 3.7** Electronic spectral studies of (a) complex 1 (20 µM), (b) complex 2 (20 µM) and (c) complex 3 (20 µM) upon addition of CT-DNA (0-200 µM). Inset: Plots of [DNA]/(ε_a-ε_f) Vs [DNA]
To quantitatively calculate the binding affinity of complexes 1-3, the intrinsic binding constants $K_b$ of the complexes were determined by monitoring the changes in absorbance of the intraligand bands with increasing concentration of CT-DNA [25]. For complexes 1 and 2, the binding constants $K_b$ were determined to be $3.35 \pm 0.32 \times 10^5$ and $2.37 \pm 0.21 \times 10^5$ M$^{-1}$, respectively. These values suggest that complex 1 has marginally stronger binding affinity for calf thymus DNA than 2 among groove binders. Imidazolyl group in the tridentate ligand coordinated to complex 1 contains free -N- and -NH- groups which may involve in secondary interactions like H-bonding with DNA. The binding constant, $K_b$ was calculated as $(7.1 \pm 0.25) \times 10^4$ M$^{-1}$ for complex 3 which is comparable with other intercalators [26,27].

3.5.2 Viscosity Measurements

To further investigate the binding nature of the complexes to DNA, viscosity measurements on the solutions of DNA incubated with the complexes have been carried out. In the viscosity measurements, the rate of flow of the buffer (10 mM Tris), DNA (200 μM) and DNA with the copper complexes at various concentrations (0-200 μM) were measured and the relative specific viscosity was calculated. The plot of relative specific viscosity $(\eta/\eta_o)^{1/3}$ vs $1/R$ for the complexes 1-3 are shown in the Figure 3.8.
Figure 3.8  Effect of complexes 1, 2 and 3 (0–200 µM) on the viscosity of CT-DNA (200 µM)

It was known that a classical intercalator like ethidium bromide (EB) causes lengthening of the DNA duplex upon the insertion of EB between the stacked bases, and this increase the relative viscosity of DNA. On the other hand, bend or kink or random changes in the DNA helix results in decrease in the specific viscosity of DNA. Such behavior has been attributed to strong covalent binding of complexes with DNA bases [28]. Complex 1 brings about
negligible change in viscosity whereas complex 2 showed random changes in the viscosity of DNA with increase in concentration of the complex. The observed increase in the viscosity of DNA in the presence of complex 3 confirms intercalative binding of the complex to DNA. These results further substantiate the groove binding ability of complexes 1 and 2 to DNA and intercalative mode of binding of complex 3 to DNA.

3.6 GEL ELECTROPHORETIC ASSAY

3.6.1 DNA cleavage properties

The nuclease activities of synthesized complexes have been assessed by gel electrophoretic technique using plasmid DNA, pUC 18. Copper(II) complexes have been reported to bring about DNA cleavage even in the absence of any co-reagent which is otherwise called as hydrolytic cleavage [29]. The supercoiled (SC) DNA when subjected to electrophoresis will move faster (Form I). Single scission of the SC form will lead to nicked circular (NC) form (Form II) which moves slowly on electrophoresis. Further cleavage of DNA results in linear forms (Form III) which appear in between form I and form II in an electrophoresis gel. The DNA cleavage activity of complex 1 has been studied using SC pUC 18 DNA in a medium of Tris buffer. Complex 1 shows cleavage of SC DNA (form I) to form II and form III on 1h incubation at 37 °C. This result suggests that the nuclease activity of complex 1 does not involve any oxidative process. Copper(II) complexes [Cu(dmp)₂]⁺ and [Cu(itpy)₂]²⁺ on the other hand, have been reported to be efficient cleavers of DNA under photolytic condition [30] or in the presence
of reducing agents like ascorbic acid or peroxide [16]. **Figure 3.9(A)** shows the effect of concentration of the complex 1 on the hydrolytic cleavage of plasmid DNA. As can be seen from lanes 2, 3 and 4, 40, 60 and 80 μM of complex 1 was able to convert supercoiled DNA to nicked circular and linear form of DNA. Interestingly, any further increase in the concentration of complex 1 (100 and 120 μM) results in suppression of the DNA cleaving ability of the complex. It is possible that the lone pair of electrons on the imidazolyl moiety probably attack the phosphodiester, resulting in the DNA cleavage. At higher concentration of the complex, the imidazolyl -N- might be involved in H-bonding with the neighboring molecule and as a result, it is not capable of initiating phosphodiester cleavage.

![Figure 3.9](image.jpg)

**Figure 3.9** Concentration dependant cleavage of pUC 18 DNA upon addition of complexes 1 and 2. Gel electrophoresis diagram showing the cleavage of plasmid DNA (400 ng) incubated with complexes for 1 h in tris buffer (pH 7.2). (A) Lane 1, DNA control; Lane 2, DNA+1 (40 μM); L3, DNA+1 (60 μM); Lane 4, DNA+1 (80 μM); Lane 5, DNA+1 (100μM); Lane 6, DNA+1 (120 μM). (B) Lane i, DNA+2 (20 μM) alone; Lane ii, DNA+peroxide (1 μL) alone; Lane iii, DNA+2 (20 μM)+H₂O₂ (1 μL); Lane iv, DNA+2 (40 μM)+H₂O₂ (1 μL); Lane v, DNA+2 (60 μM)+H₂O₂ (1 μL). (C) Incubation of complexes 1 and 2 in the presence of minor groove binder distamycin. DNA was incubated with 50μM distamycin for 15 min in Tris buffer (pH 7.2). Lane a, DNA+Distamycin (10 μM) alone; Lane b, DNA+DMSO (2 μL) alone; Lane c, DNA+1 (20 μM)+distamycin (10 μM); Lane d, DNA+2 (20 μM) +distamycin (20 μM); Lane e, DNA+2 (20 μM)+H₂O₂ (2 μL)
Complex 2, on the other hand, does not bring about DNA cleavage in the absence of any co-reagent as can be seen from lane (i) in Figure 3.9(B). This complex however, brings about DNA cleavage in the presence of H_2O_2. The control experiments performed by using complex 2 and peroxide on separate lanes reveal no cleavage of DNA as seen in lanes i and ii, respectively. As can be seen from lanes iii, iv and v, 20, 40 and 60 μM of complex 2 was able to bring about DNA cleavage in the presence of H_2O_2. DNA cleaving experiments have also been performed in the presence of hydroxyl radical quencher, DMSO. The results show that DMSO inhibits the cleavage of DNA (lane e) in Figure 3.9(C). This clearly shows that hydroxyl radical is the reactive oxygen species for the observed cleavage in the presence of complex 2.

To probe the groove binding preferences of complexes 1 and 2, the minor-groove binder distamycin was used and the results of the experiments has been shown in the Figure 3.9 (C). Prior to the addition of complexes 1 and 2, plasmid DNA was incubated with distamycin. Control experiments performed with DNA in the presence of DMSO and distamycin show the absence of any significant cleavage (lanes a and b). The electrophoresis result on incubation with minor groove binder distamycin shows a complete inhibition in the presence of complex 1 (lane c) while complex 2 exhibits significant DNA cleavage (lane d). This suggests minor groove preference for the complex 1 and major groove binding for the complex 2. These results are
of significance as majority of the oxidative cleavage reagents generally bind in the minor groove rather than the major groove [31].

**Figure 3.10** exhibits the results of electrophoresis experiments carried out with pUC 18 DNA, incubated with various amounts of complex 3. It can be seen from the figure that 20, 40 and 60 μM of complex 3 (lanes 2, 3 and 4, respectively) was able to convert supercoiled form to nicked circular form of DNA without any additives (hydrolytic cleavage). Even in the presence of DMSO (lane 5) and sodium azide (lane 6), which are quenchers of hydroxyl radicals and singlet oxygen respectively, complex 3 was able to convert supercoiled form of DNA to the nicked circular form of DNA.

![Figure 3.10](image)

**Figure 3.10** Electrophoresis of DNA on incubation with complex 3. DNA (400ng) was incubated with the complex for 60 min in tris buffer (pH 7.2) Lane 1, DNA control; lane 2, DNA + 3 (20μM); lane 3, DNA + 3 (40μM); lane 4, DNA + 3 (60μM); lane 5, DNA + 3 (40μM); lane 6, DNA + 3 (40μM)

3.7 CYTOTOXICITY OF COMPLEXES 1-3 ON MG63 AND NIH3T3

3.7.1 MTT assay

The anti-proliferative effect of complexes 1, 2 and 3 to osteosarcoma and normal cell lines were measured by MTT reduction assay. The assay was based on the fact that only live cells reduce yellow MTT but not dead cells, to
blue formazan products. The metabolic activity of the cells were assessed by their ability to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue water-insoluble formazan crystal. In this case, we tested various concentrations of complexes 1-3 on cell viability and proliferation using MTT assay. The results obtained by the MTT shows that, the complexes possess inhibitory effect on the proliferation and growth on both cancerous MG63 and non-cancerous NIH3T3 cells. The IC₅₀ value of complex 1 for growth inhibition of NIH3T3 cells was 500 nM. In the case of osteosarcoma MG63 cells, at a much lower dosage of 125 nM of complex 1, 50% of the cell death was observed. In the case of complex 2 when varying concentrations of complexes were treated with MG63 and normal cell lines, the pattern of cell growth inhibition was observed to be similar in both NIH3T3 and MG63 cell lines. For complex 3, the IC₅₀ value was found to be 750 nM in the cancerous MG63 cells, whereas in the normal NIH3T3 cells the 50% inhibitory concentration was found to be 1500 nM. Any compound to be developed as a successful anticancer agent should have the ability to distinguish normal from malignant cells and selectively inhibit the growth of the malignant cells. In such case, complexes 1 and 3 which showed selectivity towards cancerous MG63 cell line observed in MTT assay. And this cell growth inhibition may be due to either apoptosis (programmed cell death) or necrosis (accidental cell death). Using Annexin-V and propidium iodide (PI) staining method, the mechanistic pathway of cell death (apoptosis or necrosis) was confirmed by
observing the morphological changes bring about by the complexes when treated with MG63 and NIH3T3 cell lines.

3.7.2 Annexin V-Propidium Iodide staining

FITC Annexin-V, propidium iodide (PI) staining was performed to analyze the morphological changes of cell death [32]. Viable cells do not get stained with PI since the membranes of intact cells are impermeable to PI, whereas the membranes of dead and damaged cells are permeable to PI. One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipids phosphatidylserine from the cell’s cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of annexin-V [33]. Complexes 1 and 3 with varying concentrations of 125 nM and 250 nM were treated with MG63 and NIH3T3 cell lines and stained with Annexin-V and PI. The stained cells were examined under a phase contrast and also with the fluorescence microscope. The results obtained at the concentration of 125 nM of each complex on MG63 cells and also the control cells in the absence of complexes were presented in Figure 3.11. The results show a gradient increase in the number of apoptotic cells with respect to increasing concentration of the complexes. It can be seen from the figure that complexes 1 and 3 treated cells upon staining with Annexin-V and PI exhibited prominent red orange stains and therefore penetration of PI inside the dead cells was evident.
Figure 3.11 Phase contrast image and Annexin-V:PI staining of MG63 cells incubated with complexes 1 (125 nM) and 3 (125 nM)

The control experiment was simultaneously carried out in the absence of complexes showed normal cell viability with no remarkable cell death as could be seen from Figure 3.12. When compared to NIH3T3 cells the complexes did not cause any significant apoptotic effects as indicated by negligible orange red stained cells observed under fluorescence microscope.
Whereas at the same concentration of the complexes we could see cell blebbing, cell shrinkage and cell debris indicating apoptotic events brought about by the complexes. These results indicate that complexes 1 and 3 at concentration of 125 nM are less toxic to NIH3T3 cells compared to MG63 cells.

Figure 3.12 Phase contrast image and Annexin-V:PI staining of NIH3T3 cells incubated with complexes 1 (125 nM) and 3 (125 nM)
3.7.3 Effect of Complexes 1 and 3 on Caspase 3 and 9

Cell death of complexes 1 and 3 on MG63 cell line were confirmed to be apoptosis by double staining analysis. In order to further understand the signaling events leading to apoptosis we analyzed the activity of caspase 3 and 9. Caspases are the cysteine-dependent aspartate-specific proteases that play key role in the apoptotic pathways. Two types of caspases (i) initiator caspases, caspase 8, 10, 9, 2 and (ii) effector caspases, caspase 3, 7, 6 are reported to play key role in mediating apoptosis [34,35]. The activation of initiator caspases are required to activate specific effector caspases that then proteolytically degrade a host of intracellular proteins to carry out the cell death program. We analyzed the effector caspase (caspase 3) and initiator caspase (caspase 9) activity in MG63 and NIH3T3 cell lines after treatment with complexes 1 and 3 and the results obtained are presented in the Figure 3.13. From the figure it is evident that complexes 1 and 3 (125 nM) treated with NIH3T3 cells have showed no effect on caspase 3 and 9. On the other hand, when treated with MG63 cells we observed a significant increase (two fold increase) in caspase 3 and 9 with complexes 1 and 3 at the similar concentration tested. These caspase activities indicate that the complexes 1 and 3 have little effect on normal NIH3T3 cells whereas these complexes showed caspase dependent apoptosis on MG63 cells.
Figure 3.13 Caspase 3 and 9 activities of complexes 1 (125 nM) and 3 (125 nM) on treatment with NIH3T3 and MG63 cell lines
REFERENCES


