Chapter-5

Investigation of interaction of copper(II) complexes of thiophenemethanamine derivatives with DNA and their cytotoxicity profile
5.1 BACKGROUND

The last couple of chapters contain discussions on the significance of terpyridyl derivatives and their corresponding copper(II) complexes wherein most of the findings shed light on the potential head groups of terpy in Cu(II) complexes. These interesting observations motivated to retain the head groups of terpy unit such as imidazole, benzimidazole and pyridine. This chapter aims at the design of Cu(II) complexes with these potential head group using thiophenemethanamine (thma) as a parent molecule in order to analyze the DNA targeting efficacy and its mechanistic pathway of cell death. Since, thiophene skeleton containing molecules have found a lot of interesting pharmaceutical applications such as antimicrobial, anticancer and anti-inflammatory activities [1-5] thma, a thiophene based molecule (Figure 5.1) has been designed, where thiophene, “S” and “N” from methylamine are expected to have metal chelation.

![Figure 5.1 Chemical structure of thiophenemethanamine](image)

There is scarce report about the DNA nuclease activity of thiophene carrying molecule or copper(II) complexes of ligands containing sulfur, along with nitrogen as the chelating atoms for their bioactivity [6,7]. Therefore, it is
interesting to observe the simultaneous effect of nitrogen along with sulfur when it has been coordinated to Cu(II) ion. This chapter deals with the synthesis of Cu(II) complexes possessing thma derivatives and aims to explain the following questions:

1. How does the copper(II) ion chelate with thma?

2. How does the transition from terpyridine to thma backbone influences the potential head groups of Cu(II) complexes?

3. What is the mode of binding of these complexes with DNA? Whether the binding ability has been dictated by the head groups of the parent molecules? Are $K_b$ values comparable with Cu(II) terpy derivatives?

4. Do these molecules promote DNA condensation/cleavage?

5. What could be the cytotoxic effects of the above synthesized complexes on MG63 and NIH3T3 cell lines?

### 5.2 SYNTHESIS OF LIGANDS

Thiophenemethylamine derivatives of imidazole, benzimidazole and pyridine ligands have been synthesized by the following procedures:

#### 5.2.1 Synthesis of 1-(1H-imidazol-2-yl)-N-(thiophen-2-ylmethyl) methanamine (L5=imthma)

2-(2-Aminomethyl)thiophene (0.57 mg, 5 mmol) was dissolved in 5mL of methanol and added dropwise to methanolic solution of
imidazole-2-carbaldehyde (0.48 g, 5 mmol). The mixture was stirred at room temperature for 3 h, followed by dropwise addition of sodium borohydride (10 mmol) in 10 mL of sodium hydroxide solution. The resulting solution was stirred for 3 h and concentrated under reduced pressure; the residue was then extracted with three 10 mL portions of chloroform. The combined extract was dried over anhydrous MgSO$_4$ and filtered, and the solvent was removed under reduced pressure to yield an oily product (L5). Yield: 0.466 g (62%); $^1$H NMR, δ ppm, (CDCl$_3$ 500 MHz, s, singlet; d, doublet; t, triplet): 12.1 (1H, s), 6.97 (4H, d), 6.79 (1H, t), 3.81 (2H, s), 3.91 (2H, s), 2.4 (1H, s). $^{13}$C NMR, δ ppm (DMSO-d$_6$): 146, 140, 128, 127, 125, 124, 121, 48.2, 46.2. ESI-MS of the ligand showed base peak at m/z 192 corresponding to the [M-1]$^+$ ion.

5.2.2 Synthesis of 1-(1H-benzimidazol-2-yl)-N-(thiophen-2-ylmethyl) methanamine (L6=btzma)

Following the above procedure ligand L6 was synthesized using benzimidazole-2-aldehyde (0.73 mg, 5 mmol) and 2-(2-Aminomethyl)thiophene (0.57 mg, 5 mmol) in methanol. Ligand 2 was obtained as half-white solid. Yield: 0.346 g (71%); $^1$H NMR, δ ppm(CDCl$_3$ 500 MHz, s, singlet; d, doublet; t, triplet): 7.60 (2H, d), 7.27 (2H, t), 6.90 (2H, d), 6.40 (1H, t), 5.10 (1H, s), 3.85 (2H, s), 3.78 (2H, s), 2.0 (1H, s). $^{13}$C NMR, δ ppm (CDCl$_3$): 143, 141, 140, 128, 126, 134, 123, 121, 115, 51.2, 47.3. In the case of L6 the base peak observed at m/z 244 accounts for [M+H]$^+$ ion.
5.2.3 Synthesis of 1-(pyridin-2-yl)-N-(thiophen-2-ylmethyl)methanamine (L7=pytma)

Ligand L7 was synthesized by the same above said procedure but in this case using pyridine-2-aldehyde (0.54 mg, 5 mmol) and 2-(2-Aminomethyl)thiophene (0.57 mg, 5 mmol) in methanol. Yield: 0.396 g (78%); \(^1\)H NMR, \(\delta\) ppm,(CDCl\(_3\) 500 MHz, s, singlet; d,doublet; t, triplet): 8.21 (1H, d), 7.84 (1H, t), 7.58 (1H,d), 7.38 (1H,t), 6.87 (1H, t), 6.72 (2H,d), 4.1, (2H,s), 3.71 (2H,s), 1.97 (1H, s). \(^{13}\)C NMR, \(\delta\) ppm (CDCl\(_3\)): 160, 150, 140, 138, 128, 127, 124, 123, 120, 50.8, 48.3. In the case of L7 the base peak observed at 205 which can be attributed to [M+H]\(^{+}\)ion. The schematic representation of ligand synthesis is shown in the Figure 5.2.

![Schematic representation of ligand synthesis](image.png)

**Figure 5.2 Representation of synthetic route of ligands L5, L6 and L7**
5.3 SYNTHESIS OF COPPER(II) COMPLEXES

Designed ligand environment which contains thiophene unit along with the head groups (imidazole, benzimidazole and pyridine) have been synthesized as mentioned above and their corresponding Cu(II) complexes were synthesized by the method described as follows:

5.3.1 Synthesis of the [Cu(imthma)_2](ClO_4)_2 (7)

A methanolic solution of L5 (0.39 mg, 2 mmol) was warmed for 5 min followed by the addition of Cu(ClO_4)_2.6H_2O (0.37 mg, 1 mmol). The solution was then refluxed for 15 minutes. The volume of the solution was reduced and the powder obtained was dried under vacuum to yield 62% product. The product was recrystallized from acetonitrile and water.

UV-visible (solvent: Acetonitrile): λ_max, intraligand transition at 208 nm; ligand field transition around 440-780 nm. Anal Calcd for C_{18}H_{24}Cl_2CuN_6O_9S_2: C, 32.41; H, 3.63; N, 12.60. Found: C, 32.52 %; H, 3.58 %; N, 12.55 %. FT-IR (KBr pellet) cm\(^{-1}\): 3326, 3319, 2922, 1384, 1029, 871.

5.3.2 Synthesis of [Cu(bzthma)_2](NO_3)_2 (8)

Complex 8 was obtained by refluxing methanolic solution of bzthma, L6 (0.49 mg, 2 mmol) with Cu(NO_3)_3H_2O (0.24 mg, 1 mmol) for 15 min. The product was recrystallized from acetonitrile and water. Yield: 67%.

UV-visible (solvent: Acetonitrile): λ_max, intraligand transition at 210, 270 and
a shoulder at 277 nm; ligand field transition around 520-800 nm. Anal Calcd, 
C_{26}H_{30}ClCuN_{6}O_{6}S_{2}: C, 45.54 %; H, 4.41 %; N, 12.24 %. Found: C, 45.42; 
H, 4.31; N, 12.37. FT-IR (KBr pellet) cm^{-1}: 3069, 1613, 1472, 1089, 790.

5.3.3 Synthesis of the [Cu(pythma)_{2}](NO_{3})_{2} (9)

Complex 9 was obtained by refluxing methanolic solution of pythma, 
L7 (0.49 mg, 4 mmol) with Cu(NO_{3}).3H_{2}O (2 mmol) for 15 minutes. The 
complex was obtained by evaporation of the solvent and recrystallized using 
acetonitrile and ether mixture. Yield: 72 %. UV-visible (solvent: Acetonitrile): 
\( \lambda_{\text{max}} \), intraligand transition at 208 and 260 nm; ligand field transition around 
550-800 nm. Anal Calcd, C_{22}H_{28}ClCuN_{4}O_{6}S_{2}: C, 43.49 %; H, 4.64 %; N, 9.22 
%. Found: C, 43.42; H, 4.54; N, 9.16. FT-IR (KBr pellet) cm^{-1}: 2926, 1618, 
1384, 1068, 764.

5.4 CHARACTERIZATION OF THE COMPLEXES

The copper(II) complexes were synthesized by refluxing a methanolic 
solution of the freshly prepared ligands L5-L7 with Cu(II) salt. The 
authenticities of the complexes were confirmed by various spectroscopic 
techniques which are discussed below.
5.4.1 Electrospray Ionization Mass Spectrometry and Electron Paramagnetic Resonance techniques

Mass spectral analysis of complex 7 showed base peak at \( m/z \) 547.93 assignable to the ion pair \([\text{Cu}(\text{imthma})_2\text{ClO}_4]^+\). As it is evident from the spectra given in Figure 5.3a, the ESI mass spectrum of this complex also showed peak at \( m/z \) 224 which corresponds to the molecular ion \([\text{Cu}(\text{imthma})_2]^2^+\).

![ESI-Mass spectra of complex 7 recorded in acetonitrile](image)

**Figure 5.3a** ESI-Mass spectra of complex 7 recorded in acetonitrile

**Figure 5.3b** shows the EPR spectrum of DMSO solution of the complex 7 recorded at 77 K. This complex shows a typical axial spectrum with \( g_\parallel \) of 2.069 and \( g_\perp \) value of 2.299. The \( A_\parallel \) value of 149 G indicates that...
the complex possesses tetrahedrally distorted square planar geometry, since perfect square planar complex possess $A_1$ value of 180-200 G.

Figure 5.3b  EPR spectra of complex 7 in DMSO at 77 K

The ESI-MS of complex 8 has been given in Figure 5.4a. In the case of complex 8, molecular ion peak appeared at $m/z$ 610.87. This peak is assignable to the ion pair [Cu(bzthma)$_2$.NO$_3$]$^+$. The base peak in the spectra of complex 8 however was observed at $m/z$ 548.07 and this peak can be assigned to [M-H]$^+$ species. The mass spectrum of this complex also exhibited a peak at m/z 274.60 which can be attributed to the molecular ion [Cu(bzthma)$_2$]$^{2+}$. 
EPR spectra of complex 8 shown in Figure 5.4b and the g values of this complex are found to be: $g_\perp$ is 2.082 and $g_\parallel$ value of 2.311 with almost similar $A_\parallel$ values as for the complex 7 of 149 G.

Figure 5.4a  ESI mass spectra of complex 8 in acetonitrile
Figure 5.4b EPR spectra of complex 8 in DMSO at 77 K

ESI-MS of complex 9 showed molecular ion peak at \( m/z \) 235.73 corresponding to the complex ion \([\text{Cu(pythma)}_2]^{2+}\). The base peak in the mass spectrum of this complex was observed at \( m/z \) 375.07 as could be seen from Figure 5.5a. This peak can be assigned to the species obtained due to the fragmentation of thma moiety from L7. EPR spectrum of complex 9 is shown in Figure 5.5b and in this case as well, the \( g \) values and \( A_i \) values are similar as for the other two complexes 7 and 8. The \( g_\perp \) for complex 9 is 2.083 and \( g_\parallel \) 2.306 with almost similar \( A_i \) values as for the complex 7 of 155 G. For the complex, more than four lines appear in the parallel region. This could be due to different site symmetries occurring during the freezing of the solution at 77 K.
Figure 5.5a  ESI mass spectra of complex 9 in acetonitrile

Figure 5.5b  EPR spectra of complex 9 in DMSO at 77 K
5.4.2 Crystal Structure

Bis-imthma copper(II) complex was obtained as dark green single crystals. The structure of this complex (complex 7) has been determined by single crystal X-ray diffraction at 293K. The crystal structure of the complex shows it to be a monomeric dicationic copper(II) complex. Even though the ligand possesses three coordinating sites, two nitrogens (N(1) from the imidazole group and N(3) from aliphatic amine group) and one sulfur atom, only the two nitrogens are coordinated to the Cu(II) ion and the sulfur is not coordinated to the metal ion. The ORTEP diagram shown in Figure 5.6 clearly indicates four coordinate geometry around the Cu(II) ion and presence of two perchlorate ions.
The crystal structure of 7 (ORTEP representation) shows the coordination of two bidentate ligands to Cu(II) center. The important bond lengths and bond angles of complex 7 are given in Table 5.1. It can be seen from the table that Cu-N(3) bond is longer than the Cu-N(1) bond by 0.112 Å. It is interesting to note that closely related ligands, (N-((1H-imidazole-2-yl)methyl)-2-(pyridine-2-yl)ethanamine) and (2-(pyridine-2-yl)-N-((pyridine-2-yl)methyl)ethanamine) act as tridentate ligands in their Cu(II) complexes [8] whereas in the present case the ligand behaves as bidentate.
Table 5.1. Bond length and bond angles of [Cu(imtma)_2]ClO_4 (7)

<table>
<thead>
<tr>
<th>Bond length (Å)</th>
<th>Bond angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-N(1)</td>
<td>1.9422(14)</td>
</tr>
<tr>
<td>Cu-N(3)</td>
<td>2.0542(13)</td>
</tr>
<tr>
<td>Cu-N(1)#</td>
<td>1.9422(14)</td>
</tr>
<tr>
<td>Cu-N(3)#</td>
<td>2.0541(13)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.5 DNA BINDING STUDIES

The understanding of how the synthesized complexes interacted with DNA was discussed using various spectroscopic techniques.

5.5.1 Fluorescence Spectral Studies

The emission spectra of complexes 7-9 have been measured in the absence and presence of CT-DNA. The emission of the complexes arises from the respective ligands. The ligands show strong emission at around 330 nm when excited at 280 nm. In the three complexes, the emission due to the ligand is greatly reduced due to the quenching by the paramagnetic ion. Nevertheless, the complexes show considerable emission intensity which can be exploited to monitor the binding of the complexes to DNA. The dependence of relative emission intensities as a function of DNA concentration is shown in terms of [DNA]/[Cu] (Figure 5.7). The fluorescence spectra of complex 7 and complex 9 showed an emission band around 330 nm when excited at 280 nm, for complex 8 emission was
observed around 295 nm when excited at 260 nm. With increase in the concentration of DNA decrease in the fluorescence intensity was observed in all the three complexes. Intercalation of the metal complex between the base pairs generally results in enhancement of the fluorescence of the intercalated molecule. This is because of the fact that upon intercalation, the metal complex finds itself in a relatively hydrophobic region and as a result energy transfer quenching of the photoexcited state of the metal complex by water molecules, is minimized [9-11]. The quenching of emission of the three complexes in the presence of DNA, observed in this study is indicative of non-intercalative binding towards DNA.

![Fluorescence spectra of complexes 7-9 (50 µM) with increasing concentration of CT-DNA (0-200 µM)](image)

**Figure 5.7** Fluorescence spectra of complexes 7-9 (50 µM) with increasing concentration of CT-DNA (0-200 µM)
To understand more clearly the interaction pattern of the complexes with DNA using fluorescence technique, a competitive binding experiment was performed. In this method Ethidium Bromide (EB) was used as a probe, which shows only a weak emission in aqueous buffer solution because of solvent quenching. However, EB emits intense fluorescent light in the presence of DNA due to its strong intercalation between the adjacent DNA base pairs. Both intercalators and groove binders are known to quench the emission of DNA bound EB due to its displacement or through energy transfer mechanism. In the present case the emission of EB was quenched upon addition of copper(II) complexes to DNA pre-incubated with EB which is evident from Figure 5.8.

**Figure 5.8** Fluorescence quenching studies of complexes 7-9 (0-200 µM) with DNA pretreated with EB
Quenching data were analyzed according to the following Stern–Volmer equation:

\[ \frac{I_0}{I} = K_q [Q] + 1 \]  \hspace{1cm} (5.1)

Here,

- $I_0$: emission intensity in the absence of complexes,
- $I$: emission intensity in the presence of complexes,
- $K_q$: quenching constant
- $[Q]$: metal complex concentration.

The $K_q$ value was obtained from the slope of the $I_0/I$ versus $[Q]$ plot and the obtained values for the complexes 7, 8, 9 were $1.03 \pm 0.18 \times 10^4$, $2.09 \pm 0.35 \times 10^4$, and $1.16 \pm 0.57 \times 10^4$ M$^{-1}$, respectively. In addition, the apparent binding constant ($K_{\text{app}}$) values were also obtained for the complexes using the following equation:

\[ K_{EB}[EB] = K_{\text{app}}[\text{complex}] \]  \hspace{1cm} (5.2)

where, the complex concentration is the value at which there is a 50% reduction in the fluorescence intensity of EB, $K_{EB} = 1.0 \times 10^7$ M$^{-1}$ and $[EB] = 2.0 \mu$M. The apparent binding constants were found to be $10.01 \pm 0.23 \times 10^5$, $6.30 \pm 0.31 \times 10^5$, $8.31 \pm 0.19 \times 10^5$ M$^{-1}$ for complexes 7, 8 and 9, respectively.
5.5.2 Steady-State Emission Quenching Using K₄[Fe(CN)₆]

Steady-state emission quenching experiments using [Fe(CN)₆]⁴⁻ as quencher can further support the interaction of these complexes with DNA [12]. Anionic quenchers such as [Fe(CN)₆]⁴⁻, very efficiently quench the emission of cationic copper(II) complexes which are free in solution. However, when these complexes are intercalated between the DNA base pairs, their positive charge is partially screened by the phosphate groups of DNA. As a result quenching of the photoexcited state of the copper(II) complex by [Fe(CN)₆]⁴⁻ ions will be less efficient. In the non-intercalative binding of the metal complexes to DNA, the screening of the positive charge on the complexes by the negatively charged phosphate of DNA is relatively less efficient. Hence the Stern-Volmer quenching constant in the case of non-intercalative binders will be almost similar to that of the free complex. Stern-Volmer quenching constants for complexes 7-9 in the absence and presence of DNA are given in Table 5.2. In the presence of DNA, the quenching pattern was the same as observed in the absence of DNA for all the three complexes. This study further provides evidence for the groove binding ability of the complexes with DNA. Intercalators show decrease in the Stern-Volmer quenching constant indicating strong binding of the complex to DNA [13-16].
Table 5.2  Emission spectral properties of the copper(II) complexes in the absence and presence of CT DNA

<table>
<thead>
<tr>
<th>Complex</th>
<th>Stern-Volmer quenching constant&lt;sup&gt;a&lt;/sup&gt; (M&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absence of DNA</td>
</tr>
<tr>
<td>7</td>
<td>4.61 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>2.33 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>3.90 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(a) Stern-Volmer constants for the quenching of the complexes by K₄[Fe(CN)₆] in the absence and presence of DNA.

5.5.3  Circular Dichroic Spectral Studies

Circular Dichroic spectra of CT-DNA were recorded in the wavelength range of 220 - 300 nm with increasing concentration of complexes 7-9 (5-120 μM). Figure 5.9 shows the CD spectrum of DNA (represented as “a”) with complexes 7-9. On incubation with the three Cu(II) complexes, the CD spectrum of DNA undergoes marginal changes both in its positive and negative bands. The CD spectrum of DNA in the presence of the three complexes has been represented as (b) complex 7, (c) complex 8 and (d) complex 9 in Figure 5.9. The negligible changes of the bands in CD spectra clearly rule out the possibility of intercalative mode of binding of these complexes to DNA. Groove binding and electrostatic interaction of the complexes with DNA has been shown to bring about only marginal changes in the intensity of negative band as well as the positive band of DNA. On the other hand intercalators are known to enhance the intensities of both these bands [17].
5.5.4 Viscosity Studies

In the hydrodynamic measurements, the rate of flow of the buffer, DNA (100 μM) and DNA incubated with copper complexes 7-9 at various concentrations (10-120 μM) were measured and the results are presented in Figure 5.10. Complex 7 brought about negligible change in the viscosity of DNA, indicating groove binding of the complex to DNA. Complex 8 brought about a marginal increase in viscosity of DNA followed by saturation with subsequent increase in the concentration of complex. Complex 9 brought about random changes in the viscosity of DNA with increase in the concentration of complex. The results of this study indicate that complexes 7,
8 and 9 are essentially DNA groove binders. However, in the case of complex 8, partial intercalation of the complex cannot be ruled out.

**Figure 5.10** Viscosity measurement of DNA (200 µM) in presence of complexes 7-9 (0-200 µM)

### 5.5.5 Molecular Docking with DNA

To further gain insight into mode of binding of complexes 7-9 to DNA, molecular docking was performed using the duplex sequence d(CGCGAATTCGCG)$_2$ dodecamer DNA (PDBID:355D). Molecular docking is a computational method used to assess non-covalent binding of small molecules (ligands) to macromolecule (receptor). This study helps to understand the preferable orientation of the small molecule towards
biomacromolecule such as DNA, protein. Atomic charges for each complex were obtained from quantum mechanical calculations using G09 package and these structures were further used for the docking studies. Minimum energy conformation obtained from docking of the complexes 7, 8 and 9 with d(CGCGAATTCGCG)$_2$ dodecamer. From the Figure 5.11a and 5.11c, it can be seen that the complexes 7 and 9 bind to grooves of DNA. In addition, complexes 7 and 9 were docked with DNA by alternating the distance of major groove nucleobases to check the extent of binding of the metal complexes through intercalative mode and the docked poses have been presented in Figure 5.11d and 5.11f. The molecular modeling study however showed that complexes 7 and 9 bind DNA preferably through major groove rather than intercalative mode. Figure 5.11b denotes the groove binding ability of complex 8 to DNA but it is also important to note from Figure 5.11e that the result of the docked position also confirms partial intercalation of this complex to DNA. This result is in agreement with the experimental results observed from viscosity measurements. Molecular modeling studies also show that all the complexes interact closely with adenine unit of the DNA. It is also clear from the docking studies that there is no interaction between thiophene moieties present in the ligands with the nucleobases of DNA.
Figure 5.11 View of the best docked poses of complexes 7, 8 and 9 with duplex sequence d(CGCGAATTCGCG)₂

5.6 DNA CLEAVAGE ABILITY

Complexes 7-9 incubated with DNA did not bring about any DNA cleavage, whereas in the presence of peroxide it showed significant DNA cleavage. The control experiments performed by DNA alone (lane 1 of Figure 5.12a), and DNA with H₂O₂ alone (lane 2 of Figure 5.12a) did not show any DNA cleavage. All the three complexes have been incubated with pUC 18 at 37 °C for 2h and the results obtained are discussed.
Figure 5.12 (a) Cleavage of plasmid DNA by complex 7 in the presence of coreagents. DNA (400 ng) was incubated with the complexes for 60 minutes in Tris buffer (pH 7.2) (a) (a) Lane 1, DNA control; lane 2, DNA+ H$_2$O$_2$ (1 μL); lane 3, DNA+ 1 (10 μM) + H$_2$O$_2$ (1 μL); lane 4, DNA+ 1 (20 μM) + H$_2$O$_2$ (1 μL); lane 5, DNA+ 1 (30 μM) + H$_2$O$_2$ (1 μL); lane 5, DNA+ 1 (30 μM) + H$_2$O$_2$ (1 μL); lane 5, DNA+ 1 (30 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 1 (40 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 1 (50 μM) + H$_2$O$_2$ (1 μL).

(b) Incubation of pUC 18 DNA upon addition of complex 8. Lane 1, DNA control; lane 2, DNA+ H$_2$O$_2$ (1 μL); lane 3, DNA+ 2 (10 μM) + H$_2$O$_2$ (1 μL); lane 4, DNA+ 2 (20 μM) + H$_2$O$_2$ (1 μL); lane 5, DNA+ 2 (30 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 2 (40 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 2 (50 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 2 (50 μM) + H$_2$O$_2$ (1 μL).

(c) Cleavage of SC DNA upon addition of complex 9. Lane 1, DNA control; lane 2, DNA+ H$_2$O$_2$ (1 μL); lane 3, DNA+ 3 (10 μM) + H$_2$O$_2$ (1 μL); lane 4, DNA+ 3 (20 μM) + H$_2$O$_2$ (1 μL); lane 5, DNA+ 3 (30 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 3 (40 μM) + H$_2$O$_2$ (1 μL); lane 6, DNA+ 3 (50 μM) + H$_2$O$_2$ (1 μL).

At a very low concentration of complex 7 (10-30 μM) as represented in the lanes 3, 4 and 5 of Figure 5.12a, a complete conversion of SC form to NC form and then to linear form were observed. In the case of complex 8 at the concentration of 10 and 20 μM, complete conversion of SC form to nicked circular form was observed. Mixture of nicked circular and linear form was predominant beyond 20 μM of complex 8 (Figure 5.12b). For complex 9, complete conversion of Form I to Form II was observed at 10, 20 and 30 μM of complex. Conversion to Form III was observed only beyond 30 μM of the complex (Figure 5.12c). 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 in all the three complexes.
This shows that hydroxyl radical is the reactive oxygen species for the observed cleavage of DNA by the synthesized copper(II) complexes in the presence of peroxide [18].

5.7 CYTOTOXICITY STUDIES

5.7.1 MTT Assay

The metabolic activities of the cells were assessed by MTT assay and the results show that the three complexes inhibited the growth of the cells. Complex 7 at the tested concentration of 5, 10, 20, 40, 60, and 100 μM showed cell growth inhibition to 50% in NIH3T3 as well as MG63 cells, suggesting a concentration independent effect of the complex on these cell lines. The complexes 8 and 9 on the other hand showed a concentration dependent effect on NIH3T3 and MG63 cell line. We observed that in the case of complex 8, the viability of normal cell line was not affected till the concentration of 20μM compared to MG-63 cells, where the cell proliferation was reduced to less than 50% at the metal complex concentration as low as 5μM. The pattern of growth inhibition in cells treated with complex 9 was similar to that observed for complex 2, except for the observation that the cell viability of normal fibroblast cell line was not inhibited at 5 μM of the complex, whereas less than 50% growth inhibition was observed for cancerous MG63 cell line at the same concentration. These results suggest that complexes 8 and 9 can be further studied for their biological activities owing to its specificity in inhibiting the cell growth of cancerous cells compared with the normal cells at concentration as low as 5μM. The growth
inhibitory effect of these complexes at low concentration was significantly high for MG63 cell line when compared to the normal fibroblast cell line; at low concentrations of these two complexes the viability and proliferation of the normal fibroblast cell line was not affected. The morphological changes and nuclear condensation observed in MG63 and NIH3T3 cells (Figure 5.13) were consistent with growth inhibition observed in MTT assay. Compared to the complex 7, complexes 8 and 9 showed greater specificity to induce cytotoxic effects on cancerous MG63 cells compared to the normal fibroblast cell line.

**Figure 5.13** Morphological changes observed with MG63 and NIH3T3 cells on treatment with complexes 7-9

5.7.2 **Annexin-V and Propidium Iodide Staining**

MG63 and NIH3T3 cells were treated with complexes 8 and 9 with different concentration and double-stained with Annexin-propidium iodide.
To visualize the mode of cell death, fluorescence microscopy was used. From the results, it has been observed that, the apoptotic body formation and the morphological changes in the cells, which are characteristic of apoptosis. The results of the Annexin-PI staining on cells treated with complex 8 (10 μM), complex 9 (10 μM) and also the cells treated in the absence of complexes are presented in Figure 5.14. Cells treated with complexes 8 and 9 showed prominent orange red stain corresponds to apoptosis. But it is also to be noted that the normal cells NIH3T3 was relatively affected at this concentration on complex 9. Therefore, according to the MTT assay, complex 7 which showed almost similar effects on MG63 and NIH3T3 cells was not subjected further for the mechanistic pathway of cell death. Complex 8, a benzimidazolyl derivative of thiophene methyl amine is effective amongst the three complexes. Since, the double staining assay reveals that complex 9 with the lower concentration of 10 μM also influences the normal cell line. The cytotoxic effect of complexes 7-9 follows,

Complex 8 > Complex 9 > Complex 7

To further assess the caspase dependent or independent pathways of apoptosis by the complexes 8 and 9 caspase activities assay using caspase 3 and 9 were performed.
Figure 5.14 MG63 and NIH3T3 cells treated with complex 8 (10 µM) and complex 9 (10 µM) and stained with Annexin-V and PI

5.7.3 Effect of Complexes 8 and 9 on Caspase 3 and 9

The caspase activities will further help to understand the cell signaling pathways of apoptosis. Complexes 8 and 9 with varying concentration were treated with MG63 and NIH3T3 cell lines and the results are shown in Figure 5.15. It was observed from the figure that caspase 3 and 9 activity in cells treated with MG63 cells were significantly enhanced in the presence of both the complexes. The activities of caspase 3 and caspase 9 increased remarkably in MG63 cells and they were statistically significant compared with caspase activity of normal NIH3T3 cells. This indicates that the apoptosis of MG63 cells was induced by complexes via the intrinsic
mitochondrial apoptotic pathway due to activation of caspase 9 and caspase 3. Thus it was concluded that complexes 8 and 9 brought about cell specific apoptosis in osteosarcoma cell line where complex 8 possesses much appreciable activities compared to complex 9.

Figure 5.15  Activities of complex 8 (10 µM) and 9 (10 µM) on caspase 3 and 9
REFERENCES


