8.1 INTRODUCTION

Several reviews have appeared discussing the roles of metal ions in biological systems [1-4]. The involvement of metal complex formation in normal life processes has led to reviews such as “The effects of chelating agents on organisms”[5], “Chelation in medicine” [6] “Metal binding in medicine” [7], “Metal chelates in biological systems”, [8] and “Structure and bonding in biochemistry” [9]. The aims of these reviews [10] are to draw the attention of coordination chemistry researches to focus upon metal complexes in biological systems. Many Schiff base complexes with transition metals have drawn wide attention because of their diverse biological and pharmaceutical activities [11,12]. The literature survey showed that the chelating Schiff base ligands derived from diamines and various carbonyl compounds encompass a highly remarkable class of compounds having a wide range of applications in clinical [13], biochemical [14,15] and physiological activities [16,17].
Deoxy ribonucleic acid (DNA) is the primary target molecule for most anticancer and antiviral therapies according to cell biologists. Investigations on the interaction of DNA with small molecules are important in the design of new types of pharmaceutical molecules. Since the chemical nuclease activity of transition metal complexes was discovered in the 1980s, there has been a great interest in studying the interaction model and the mechanism of transition metal complexes with DNA. There are metal complexes which interact with DNA and induce the breakage of DNA strands by appropriate methods [18-21]. DNA is an important genetic substance in organisms. Any errors in gene expression can often cause diseases and play a secondary role in the outcome and severity of human diseases. Thus, there is an increasing focus on the binding study of small molecules to DNA during the last decades. A more complete understanding of DNA binding is necessary to design a new drug. There are three DNA binding modes, they are intercalative binding, groove binding and external electrostatic binding. Among these interactions, intercalation and groove binding are the most important DNA binding modes as they invariably lead to cellular degradation. Intercalative binding results when small molecules or the drug intercalate into the nonpolar interior of the DNA helix. Groove binding interactions involve direct interactions of the bound molecule with edges of base pairs in either of the major (G-C) or (A-T) grooves of the nucleic acids. Electrostatic interaction happens in the case of positively charged molecules. They electrostatically interact with the negatively charged phosphate backbone of DNA chain. Geometry of the complexes is mainly responsible for the affinity of the metal complexes to DNA. The geometry of complexes depends on the metal ion type and different functional groups in the ligands. So the investigation on the interaction of the Schiff base transition metal complexes with DNA has a great significance for disease defense, new medicine design and clinical application of drugs.

Copper complexes are of particular interest with regard to DNA cleavage through oxidative pathways [19-22]. Biological activities such as antibacterial and anticancer properties of Cu(II) complexes have been also reported [23-24].
Transition metal complexes with tunable coordination environments and versatile spectral and electrochemical properties offer a great scope for the design of species that are suitable for DNA binding and cleavage activities. Hence, the synthesis of symmetrical and unsymmetrical binuclear Cu(II) complexes has gained more attention in recent years [25].

DNA binding activity of copper(II) complexes of Schiff base, \(N,N'-\text{bis}(3,5\text{-}
\text{tert-butylsalicylidene-2-hydroxy})-1,3\text{-propanediamine}\), has been reported [11]. These complexes bind to DNA by moderate intercalative binding modes. Furthermore, all these complexes can cleave plasmid DNA to nicked DNA in a sequential manner as the concentrations or reaction times are increased. Their cleavage activities are promoted in the presence of hydrogen peroxide. Liu et al. [26] reported the cytotoxic and DNA binding activity of Cu(II) Schiff base complexes, which was derived from 2-oxoquinoline-3-carbaldehyde.

Quinoxaline derivatives are present in several biologically active compounds and play an important role in the synthesis of the pharmaceuticals [27,28]. Based on these reports, the synthesized copper(II) Schiff base complexes were screened to know whether these complexes have any cytotoxic and DNA binding activities. The results of the cytotoxicity and DNA cleavage studies are presented in this chapter.

8.2 EXPERIMENTAL

8.2.1 Materials

The materials used for the preparation of Schiff base ligands and their copper(II) complexes are presented in Chapter 2. Dalton Lymphoma Ascites (DLA) cells (Amala Cancer Research Center, Thrissur, Kerala), phosphate buffer saline (PBS) [\(\text{NaCl 4 g, NaH}_2\text{PO}_4 0.72 \text{ g, KH}_2\text{PO}_4 0.1 \text{ g, KCl 0.1 g, distilled water 500 mL}\)], trypan blue, haemocytometer, agarose gel, ethydium bromide
(Sigma Aldrich), tris-acetate-EDTA buffer and pUC18 DNA (GeNei, Bangalore) were used in this study.

8.2.2 Methods

8.2.2.1 Synthesis of Schiff base ligands

The synthesis of Schiff base ligands, qch, qce, qcp, qcb, qcc and qco, are given in Chapter 2.

8.2.2.2 Synthesis of copper(II) nitrate complexes

The synthesis of copper(II) nitrate complexes are given in Chapter 5.

8.2.2.3 In vitro cytotoxicity studies of copper(II) complexes - Trypan blue exclusion method

The predictive value of in vitro cytotoxicity test is based on the idea that toxic chemicals affect basic functions of cells which are common to all cells, and that the toxicity can be measured by assessing cellular damage. The main focus of the research for the development of in vitro cytotoxicity assays is to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound. Evidence for the utility of in vitro cytotoxicity tests has led many pharmaceutical companies to screen compound libraries to remove potentially toxic compounds early in the drug discovery process. In the trypan blue exclusion method, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Trypan blue exclusion, as described in the above protocol, can be performed in 5 to 10 min [29,30].
In vitro cytotoxicity of the copper(II) nitrate complexes were studied on Dalton Lymphoma Ascites (DLA) cells by trypan blue exclusion method. The principle behind this method is that the drug at toxic concentration damages the cell and makes pores on the membrane through which trypan blue enters. The damaged cells are stained blue by trypan blue stain and can be distinguished from viable cells. Live cells are excluded from staining. The general procedure for this study is given below:

DLA cells were aspirated from the peritoneal cavity of tumour bearing mice. These cells were washed three times using phosphate buffered saline (PBS). The viability of the cells was checked by using trypan blue. Different dilution (10⁻¹, 10⁻² and 10⁻³ M) of cells was made. The number of cells in the 10⁻³ M dilution was counted by the use of haemocytometer and cell number was adjusted to 1x10⁷ cells/mL. The experiment was set up by incubating different concentration of the drug with 1x 10⁶ cells. The final volume of the assay mixture was made upto 1 mL using PBS and was incubated at 37 °C for about three hours. 1.0 mL of trypan blue was added after incubation and the number of dead cells was counted using a haemocytometer. The percentage of viable cells was calculated as follows:

\[
\text{Viable cells (\%) = } \frac{\text{Total number of viable cells per mL of aliquot}}{\text{Total number of cells per mL of aliquot}} \times 100
\]

8.2.2.4 DNA Cleavage Studies of Copper(II) Complexes - *Gel electrophoresis*

The cleavage of DNA by metal complexes was studied using agarose gel electrophoresis [31-34]. The ability of the copper(II) nitrate complexes for the DNA cleavage was also checked by agarose gel electrophoresis, which was performed by incubation at 37 °C for 1 h as follows: pUC18 plasmid DNA of 0.25 μg/μL concentration was used for the experiments. Stock solutions of the Cu(II) complexes (10⁻³ M) in demineralised water with DMSO were freshly
preparation before use. Aliquot parts of 3 μL of the Cu(II) complex solutions were added to aliquot parts of 5 μL of the pUC18 DNA in 20 μL of a Tris-acetate EDTA buffer solution. The reaction mixture was incubated at 37 °C for 1 h, and then 4 μL of charge marker were added to aliquots parts of 20 μL of the adduct complex/DNA. The mixtures were electrophoretised in agarose gel (1%) at 80 V for 1 h. After that the DNA was dyed with ethydium bromide solution (0.5 μg/μL in TBE) for 20 min. A sample of free DNA was used as a control. After electrophoresis, bands were visualised by UV light and photographed.

8.3 RESULTS AND DISCUSSION

8.3.1 In vitro cytotoxicity study

The complexes, [Cu(qch)NO$_3$(H$_2$O)]NO$_3$ 13, [Cu$_2$(qce)$_2$(NO$_3$)$_2$(OH)$_2$]·9H$_2$O 14, [Cu(qcp)NO$_3$(H$_2$O)]NO$_3$ 15, [Cu(qcb)NO$_3$H$_2$O]NO$_3$ 16, [Cu(qcc)(H$_2$O)$_2$](NO$_3$)$_2$ 17 and [Cu(qco)(NO$_3$)$_2$]·2H$_2$O 18, were studied for short term in vitro cytotoxicity using Dalton’s Lymphoma Ascites cells. The tumour cells were aspirated from the peritoneal cavity of tumour bearing mice, washed thrice with normal saline and checked for viability using trypan blue dye exclusion method. The cell suspension (1x10$^6$ cells in 0.1 mL) was added to tubes containing various concentrations of the test compounds and the volume was made upto 1mL using phosphate buffered saline (PBS). In the control tube only cell suspension was taken. These assay mixtures were incubated for 3 hour at 37 °C and percent of dead cells were evaluated by trypan blue exclusion method. Results of this study are given in Table 8.1.
Table 8.1: Effect of copper(II) complexes against DLA cell lines by trypan blue dye exclusion method

<table>
<thead>
<tr>
<th>Concentration of the complexes</th>
<th>Sample</th>
<th>Percent cell death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Cells were treated at concentrations ranging from 10-200 µg/mL of the complex for 48 h and then the percentage of cell viability was analysed. Viable cells which remained unstained by trypan blue were counted in a haemocytometer. The percentage cytotoxicity of the DLA cells at different concentrations ranging from 10-200 µg/mL was calculated. Results showed a drug (the copper(II) Schiff base complexes 13-18) dose dependent inhibition of the growth of DLA cells. The results are also represented in Figure 8.1. All the complexes except 14 produced 100% cytotoxicity at 200 µg/mL. The complexes 15, 16, 17 and 18 exhibit 100% cytotoxicity even at 100 µg/mL. Complex 15 [Cu(qcp)NO$_3$(H$_2$O)]NO$_3$ was found to have higher cytotoxicity effect than that for the other complexes. Complex 14 [Cu$_2$(qce)$_2$(NO$_3$)$_2$(OH)$_2$·9H$_2$O shows slightly lower activity, when compared to that of the other complexes. This study reveal the cytotoxicity nature of copper(II) Schiff base complexes against DLA cells.
8.3.2 DNA cleavage studies of the copper(II) complexes

The ability of the copper(II) complexes, [Cu(qch)NO$_3$(H$_2$O)]NO$_3$ 13, [Cu$_2$(qce)$_2$(NO$_3$)$_2$(OH)$_2$·9H$_2$O 14, [Cu(qcp)NO$_3$(H$_2$O)]NO$_3$ 15, [Cu(qcb)NO$_3$H$_2$O] NO$_3$ 16, [Cu(qcc)(H$_2$O)$_2$(NO$_3$)$_2$ 17 and [Cu(qco)(NO$_3$)$_2$]·2H$_2$O 18, to cleave DNA was tested by gel electrophoresis method. In our study pUC18 DNA was used as the sample. pUC18 is a plasmid DNA of 2686 base pairs. On agarose gel, pUC18 shows three distinct bands corresponding to the three different conformations of the plasmid, namely, open circular, linear and supercoiled forms. The three different conformations interchange, *ie*, supercoiled to open circular and open circular to linear, depending on different physical and chemicals factors. In the present study, the copper(II) complexes were tested for their DNA
binding property. The image of bands obtained after gel electrophoresis is shown in Figure 8.2.

**Figure 8.2: DNA Fragmentation by copper(II) complexes**

The image of DNA cleavage consists of several lanes and it is marked as following:

- Lane 1: 500 bp DNA marker
- Lane 2: pUC 18 DNA
- Lane 3: pUC 18 DNA + [Cu₂(qce)₂](NO₃)₂(OH)₂·9H₂O 14
- Lane 4: pUC 18 DNA + [Cu(qcp)NO₃(H₂O)]NO₃ 15
- Lane 5: pUC 18 DNA + [Cu(qch)NO₃(H₂O)]NO₃ 13
- Lane 6: pUC 18 DNA + [Cu(qcb)NO₃H₂O]NO₃ 16
- Lane 7: pUC 18 DNA + [Cu(qcc)(H₂O)₂](NO₃)₂ 17
- Lane 8: pUC 18 DNA + [Cu(qco)(NO₃)₂]·2H₂O 18
In the above gel photo, two bands are visible for pUC18 DNA (Lane 2) which corresponds to the open circular and supercoiled form of DNA. Only one band is seen in the third and sixth lane, which suggest that the binding of the copper(II) complexes (14 and 16) cause a change in the conformation of DNA from supercoiled to open circular form. Likewise, in lanes 4, 5, 7 and 8, only one band is seen which corresponds to supercoiled form of pUC18 DNA. Thus it could be concluded that the binding of the metal complex results in nicking of the DNA strand. Among these complexes, \([\text{Cu}_2(\text{qce})_2(\text{NO}_3)_2(\text{OH})_2\cdot9\text{H}_2\text{O}]\) 14 and \([\text{Cu(qcb)}\text{NO}_3\text{H}_2\text{O}]\)NO3 16, act as very good DNA cleavagers.

8.4 CONCLUSIONS

Results of the present study suggest that the copper(II) complexes could induce tumor cell death by physiological and pathological means. The potency of complexes, \([\text{Cu}_2(\text{qce})_2(\text{NO}_3)_2(\text{OH})_2\cdot9\text{H}_2\text{O}]\) 14 and \([\text{Cu(qcb)}\text{NO}_3\text{H}_2\text{O}]\)NO3 16 to bring about the cytotoxicity decreases with decrease of dose and they can also cleave the pUC18 plasmid DNA efficiently. Thus, the synthesized Cu(II) complexes exhibit a low DNA cleavage activity together with moderate cytotoxicity against DLA cell lines.

REFERENCES


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