CHAPTER VI

BIOLOGICAL STUDIES INVOLVING COBALT(III) SCHIFF BASE COMPLEXES CONTAINING LONG CHAIN ALIPHATIC AMINE LIGANDS

6.1 Introduction

Cancer is one of the major threats to human society. Among the various types of cancer, lung cancer is the most common cancer in men and women worldwide.\textsuperscript{162} There are many chemotherapeutic agents applied in the treatment of lung cancer. However due to side effects, resistance of cancer cells to the drugs and the extreme financial burden, there is still a crucial need to develop novel and more effective anti-cancer agents. Transition metal complexes containing Schiff bases are extensive interest because of their broad spectra of biological and pharmaceutical properties, such as anti-bacterial, anti-oxidant, anti-proliferative, anti-malarial and especially in anti-cancer activities. In recent years many metal complexes with Schiff bases like salen and salophen ligands have shown superior activity than \textit{cis}-platin. Interestingly, Schiff base containing metal complexes are very effective against \textit{cis}-platin resistance cancer cell lines.\textsuperscript{70} Over the years, metal complexes containing many tetradeinate Schiff base ligands have been reported as anti-cancer agents.\textsuperscript{37-41} Ronald Gust reported that fluorinated Fe(III) salophen complexes showed tumor cell selective effects dependent on the position and number of fluorine substituents.\textsuperscript{163} Mandal et al. have investigated the apoptotic activity of complexes of Mn(III) salophen with varying substituents (hydroxy and methoxy) at different positions and the results revealed that methoxy substituted derivatives induced more effective apoptosis than their corresponding hydroxy derivatives.\textsuperscript{164} These reports clearly indicate that the changes in the
substitution of suitable groups in the aromatic ring of the Schiff base ligand can influence the nature of anti-cancer activity of metal complexes with such type of ligands differ in their anti-cancer activity.

Previous reports have shown that metal complexes containing acacen Schiff base ligands show remarkable protein binding ability. Further the present study (chapter IV) shows that cobalt complexes containing acacen and Ph-acacen ligands display strong binding ability with DNA. Like acacen ligand, cobalt complexes containing napen and napophen ligands have also show strong binding affinity with DNA. As DNA is the fundamental molecule for many biological processes and DNA targeting is one of the important areas in anti-cancer drug designing it is essential to study their anti-proliferative activity of cobalt complexes containing those ligands with lung cancer cell lines to get more insight in the rational designing of cobalt based Schiff base complexes. In recent years the designing of metal complexes with anti-angiogenesis properties is an emerging area in cancer treatment. Recently metal complexes containing benzimidazole, naphthalimide, phenanthroline derivatives and arene ligands have been reported. Yet till date there is no report on anti-angiogenesis activity of cobalt complexes with above mentioned ligands.

Hence it was decided to determine the anti-cancer activity of cobalt complexes 1-8 with lung cancer cells, A549. Further their mode of cell death was studied by various staining assays. In addition to that their anti-angiogenesis activity was also evaluated by CEA assay.
6.2 Materials and methods

6.2.1 Cell culture

Human lung cancer cells, A549 were obtained from National Center for Cell Science Pune, India. A549 and HT-29 cells were acquired directly from Dr. M. Bally’s laboratory (BC Cancer Agency Research Center, Vancouver, BC). A549 cells were cultured at 37° C under a 5% CO₂ atmosphere in DMEM medium (Invitrogen) containing 10 % FBS (Sigma-Aldrich, St. Louis, Mo, USA), 100 µg/mL of penicillin and 100 µg/mL streptomycin as antibiotics (Himedia, Mumbai, India).

6.2.2 Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out as described previously. The cobalt(III) complexes 1-8 were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and prepared to final dilution of DMSO to 0.1% in buffer, was added to the well, 24 h after seeding 5000 cells (A549) per well of 96-well plates. The same solvent of 0.1% DMSO in buffer was used as the solvent control and cis-platin was used as the positive control. After 24 h incubation, 20 mL of MTT solution [5 mg mL⁻¹ in phosphate-buffered saline (PBS)] was added to each well, and the plates were wrapped with aluminium foil and incubated for 4 h at 37° C. The purple formazan product was dissolved by addition of 100 mL of 100% DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96-well plate reader (Bio-Rad, Hercules, California, USA). Data were collected for three replicates each and used to calculate the mean. The percentage inhibition was calculated from this data using the following formula:

\[
\text{Percentage inhibition} = \frac{\text{Mean OD of untreated cells (control)} - \text{mean OD of treated cells}}{\text{Mean OD of untreated cells (control)}} \times 100
\]
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The IC$_{50}$ value was find out as the concentration of the complexes that is required to reduce the absorbance to half that of the control.

6.2.3 Acridine orange (AO) and ethidium bromide (EB) staining

Acridine orange and ethidium bromide staining were performed as described by Spector et al.$^{170}$ A549 cells were seeded in 6-well plates and allowed to reach 70% confluency. The cells were then treated with the IC$_{50}$ concentration of the complexes for 24 h. The treated and untreated cells (25 mL of suspension containing 5000 cells) were incubated with acridine orange (AO) and ethidium bromide (EB) solutions (1 part of 100 μg/mL each of AO and EB in PBS) and examined in a fluorescent microscope (Carl Zeiss, Jena, Germany) using a UV filter (450–490 nm). Three hundred cells per sample were counted, in triplicate, for each time point and scored as viable or dead, and if dead, whether by apoptosis or necrosis as judged from the nuclear morphology and cytoplasmic organization. The percentages of apoptotic and necrotic cells were then calculated. Morphological features of interest were photographed.

6.2.4 Hoechst 33258 staining

The cell pathology was detected by staining the nuclear chromatin of trypsinized cells (5.0 × 10$^{4}$/mL) with 1 μL of Hoechst 33258 (1 mg/mL, aqueous) for 10 min at 37°C. Staining of suspension cells with Hoechst 33258 detected apoptosis. A drop of suspension was placed on a glass slide and a coverslip was laid over to reduce light diffraction. At random 300 cells were observed in a fluorescent microscope (Carl Zeiss, Germany) fitted with a 377–355 nm filter and observed at 400x magnification, and the percentage of cells reflecting pathological changes were calculated. Data were collected for triplicates and used to calculate the mean and the standard deviation.
6.2.5 Annexin V-Cy3 staining

Phosphatidylserine is present in the inner leaflet of plasma membrane. The translocation of phosphatidylserine from the inner to outer leaflet of the plasma membrane is one of the early apoptotic features. The presence of phosphatidylserine at cell surface was detected by phosphatidylserine binding protein annexin V conjugated with Cy3 using the commercially available Annexin V-Cy3 apoptosis detection kit (APOAC, Apoptosis Detection Kit, Sigma). Cells were cultured on cover slips and treated with IC$_{50}$ concentrations of cobalt complexes 1-8 and incubated for 24 h. The cell pellet was washed with PBS and then with binding buffer. The washed cell pellets were suspended in 50 mL of double label staining solution (Ann-Cy3 and 6-CFDA) and kept in incubation for 10 min in dark. Afterwards the excess label was removed by washing the cells with binding buffer. The annexin-Cy3 and 6-CFDA-labelled cells were observed in the fluorescent microscope. 300 cells at random were observed. This assay facilitated detection of live cells (green), necrotic cells (red), and apoptotic cells (red nuclei and green cytoplasm). The percentage of cells reflecting cell death (apoptotic and necrotic, separately) was manually calculated. Data were collected from three individual experiments, each in triplicate, and used to calculate the respective means and the standard deviations.

6.2.6 Cell cycle analysis

Cell phase distribution was determined by flow cytometry with DNA staining to reveal the total amount of DNA. Approximately 1 x 10$^6$ non-small cell lung cancer (A549) cells were treated with IC$_{50}$ concentrations of cobalt complexes 1-8. Then the treated cells were incubated for 24 h. After incubation, the cells were trypsinized, harvested, and fixed in 1 mL 80 % cold ethanol and incubated at 4 °C for 15 min. Then, cells were centrifuged at 1500 rpm for 5 min and the cell pellets
were resuspended in 500 μL propidium iodide (10μg/mL) containing 300μg/mL RNase (Sigma Chemical Co., St. Louis, MO, USA). The cells were then incubated on ice for 30 min and filtered in 53μm nylon mesh. Cell cycle distribution was analyzed using FACS (fluorescent activated cell sorter) (Becton-Dickinson, Sanjose, CA, USA) with 15 mW, 488 nm argon ion laser. PI (Propidium Iodide) signals were collected using a 585/42 band pass filter. The data were acquired and analyzed using Dean-Jett-Fox algorithm.

6.2.7 Chick embryo angiogenesis assay (CEA)

The anti-angiogenic activity was investigated by the chick embryo angiogenesis (CEA) assay using a reported protocol.\textsuperscript{85} Briefly, Specific pathogen free (SPF) fertile chicken eggs were obtained from a government poultry firm. The eggs were incubated at 37 °C under a humid atmosphere. After the fourth day of incubation, the shells of the eggs were cautiously broken using forceps and placed on a sterile petri-dish. Precautions were taken to prevent puncture of any of the blood vessels while transferring. The stock solutions were prepared by dissolving the cobalt complexes 1-8 in DMSO and then diluted suitably with the corresponding buffer to the required concentrations for all the experiments. The final DMSO concentration never exceeded 0.1 %. Sterile filter paper discs (6 mm dia) soaked in solution of complexes (10 μM) were placed in 3 different positions over the generating blood vessels. For the control experiments sterile filter paper disks (6 mm dia) soaked with 10 μM of 0.1% of DMSO in PBS were used. Images of blood vessels (i.e. 0 h) were taken using Canon IXUS 185 digital camera (Leica) according to earlier report.\textsuperscript{172} The petri-dishes were aseptically transferred in a humidified incubator at 37 °C and images were captured similarly after 4 h of incubation.
6.3. Results and discussion

6.3.1. Anti-cancer studies

The *in-vitro* cytotoxicity of the all the cobalt complexes 1-8 against A549 lung cancer cell lines at different concentrations was examined by MTT assay\(^{169}\) (Fig 6.1). Metal ion and ligands alone were tested separately with A549 cells and they show low cytotoxic towards lung cancer A549 cells (IC\(_{50}>100\mu M\)) compared to metal complexes. The IC\(_{50}\) values for the cobalt complexes 1-8 are given in Table 6.1. The order of the anti-cancer potential of these cobalt complexes is cobalt complexes 8>7>6>5>4>3>2>1. It is remarkable that this order is same as the order observed for these complexes in their DNA binding ability.

**Table 6.1** IC\(_{50}\) values of the complexes 1-4

<table>
<thead>
<tr>
<th>Complex</th>
<th>IC(_{50}) value(^{a}) ((\mu M)) at 24h A549 lung cancer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>complex 1</td>
<td>28</td>
</tr>
<tr>
<td>complex 2</td>
<td>19</td>
</tr>
<tr>
<td>complex 3</td>
<td>17</td>
</tr>
<tr>
<td>complex 4</td>
<td>15</td>
</tr>
<tr>
<td>complex 5</td>
<td>11</td>
</tr>
<tr>
<td>complex 6</td>
<td>10</td>
</tr>
<tr>
<td>complex 7</td>
<td>7</td>
</tr>
<tr>
<td>complex 8</td>
<td>5</td>
</tr>
<tr>
<td><em>Cis</em>-platin</td>
<td>80</td>
</tr>
</tbody>
</table>

\(^{a}\text{IC}_{50} = \text{Concentration of the drug required to inhibit growth of 50\% of the cancer cells (in \(\mu M\)).}\)
Fig. 6.1 Inhibitory effects of cis-platin and complexes 1-8 and on A549 lung cancer cells treated with different concentrations for 24. All the data were expressed in Mean ± SD of two experiments with two replicate wells.
Beside all the complexes are more effective than \textit{cis}-platin. However more biological studies are needed to clearly understand the mechanism of action of these complexes. The obtained results show that complex 8 is more active against lung cancer cells than previously reported cobalt(III) complexes of same nature.

\textbf{6.3.2 Cell death as revealed in fluorescent staining}

To determine the mode of cell death of cobalt(III) complexes 1-8, AO and EB staining methods were used and the cells were observed in a fluorescent microscope. The cytological changes observed have been classified into the following four types according to the fluorescence emission and features of the chromatin\textsuperscript{173} : (i) viable cells having uniformly green fluorescing nuclei with highly organized structure; (ii) early apoptotic cells (which still have intact membranes but have started undergoing DNA fragmentation) having green fluorescing nuclei, and peri-nuclear chromatin condensation visible as bright green patches or fragments; (iii) late apoptotic cells having orange to red fluorescing nuclei with condensed or fragmented chromatin; and (iv) necrotic cells, swollen to large sizes, having uniformly orange to red fluorescing nuclei with no indication of chromatin fragmentation. These morphological changes and manual counting data (Fig. 6.2) observed for complexes 1-8, show higher cytotoxicity, indicating that the cells are committed to very efficient apoptotic cell death and necrosis to a certain extent. The percentage of cells which have undergone apoptosis and necrosis is given in Fig. 6.3 as bar diagram.
Fig. 6.2. Photomicrographs of control and AO/EB stained A549 lung cancer cells treated with the complexes 1-8 for 24 h.
Fig. 6.3. The graph shows data on percentage of cells that are normal cells which afflicted with apoptosis and necrosis in the control and 24 h.

6.3.3 HOECHST 33248 staining

After the cells were treated with the respective IC₅₀ concentrations of the complexes for 24 h, the cells were observed for cytological changes adopting Hoechst 33258 staining. The observations revealed that the treatment with the complexes brought about marginalization and/or fragmentation of chromatin, bi-nucleation, cytoplasmic vacuolation, nuclear shrinkage, cytoplasmic blebbing and late apopt of dot-like chromatin (Fig. 6.4). These cytological changes strongly indicate that the cells were committed to cell death, more by apoptosis than necrosis.
Fig. 6.4. Hoechst 33258 stained A549 cells control and treated with complexes 1-8 for 24 h.

6.3.4. Annexin V-Cy3 staining

The translocation and accumulation of the phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface is used to determine the early indicator of apoptosis.\textsuperscript{175} The combination of annexin V-Cy3 (emits red) and 6-CFDA (emits green) allows the differentiation among early apoptotic cells (annexin V positive, 6-CFDA positive), necrotic cells (annexin V positive, 6-CFDA negative) and viable cells (annexin V negative, 6-CFDA positive).
Fig. 6.5. A549 lung cancer cells stained with Annexin V-Cy3 and 6-CFDA. Cells were treated with complexes 1-4 for 24 h.
Fig. 6.6. A549 lung cancer cells stained with Annexin V-Cy3 and 6-CFDA. Cells were treated with complexes 5-8 for 24 h.
The results obtained with annexin V binding assay of control and treated cells are represented in Fig. 6.5. and 6.6. When the cells were treated with cobalt complexes 1–8, there is a significant increase in number of cells positive for both annexin V and 6-CFDA indicating early stage of apoptosis. The results clearly indicate that the all the cobalt complexes have selectively triggered apoptotic mode of cell death. The percentage of live cell, apoptotic and necrotic cells was calculated which are given as bar diagram in Fig. 6.7.

**Annexin V-Cy3 staining**

![Annexin V-Cy3 staining](image)

**Fig. 6.7** The percentage of cells that are normal cells which afflicted with apoptosis and necrosis in the control and 24 h.

### 6.3.5. The cell cycle analysis

The possible cell cycle arrest was determined by flow cytometer which is used to distinguish and quantify the cells in different phases of the cell cycle. The lung cancer cells, A549 were treated with cobalt complexes 1-8 at respective IC$_{50}$ concentration. The treated and fixed cells were stained by propidium iodide and analysed for DNA/cell content. The DNA histogram (Fig. 6.8.) shows gradual
increase in the populations of sub-\(G_0/G_1\) cells in all the complexes. The increase in the cell accumulation is in sub \(G_0\) phase lead to the apoptosis. Besides complexes 7 and 8 show high amount of cell population than compare to control at \(G_2/M\) phase indicating that the cell cycle arrest occurs at this phase.

![Cell cycle distribution](image)

**Fig.6.8.** Effects of cobalt complex 1-8 on the cell cycle progression in lung cancer A549 cells. The cell cycle distribution was analyzed Dean-Jett-Fox software and depicted with the histogram.

### 6.3.6. Anti-angiogenesis activity

Chick embryo angiogenesis (CEA) assay is a well-known in vivo angiogenesis assay. To confirm the anti-angiogenic properties of the cobalt complexes 1-8, CEA assay has been performed. The results are given in Fig. 6.9 and the results show that the vascular sprouting slightly increases in vehicle control (0.1 % DMSO)
chick embryo after 4 h of treatment. But the blood vessels were significantly damaged in chick embryo incubated after the treatment with the cobalt complexes 1 - 8. Several angiogenic parameters such as vessel size, vessel length and number of vessel branches have been quantified by angioquant software according to our earlier literature\textsuperscript{178} and given as bar diagram (Fig 6.10) All these parameters are found to be decreased in the presence of cobalt complexes 1-8 compared to control, it clearly indicates that all the cobalt complexes have anti-angiogenic potential. Among cobalt complexes 1-8, complexes 8 show higher anti-angiogenic activity compared to other complexes. This trend is also in tune with DNA binding ability of the cobalt(III) complexes.
Fig. 6.9. CEA assay. The vascular sprouting has been damaged in the presence of complexes 8 (10 μM) compared to vehicle control (DMSO). C, 1-8 at 0h and C', 1'-8' at 4h

Fig. 6.10. The different angiogenic parameters, e.g., vessel size, vessel length and number of branches in the presence of cobalt complexes 1-8 compared to control
6.4. Conclusion

The anti-cancer activity of the cobalt complexes 1-8 with A549 human non-small cell lung cancer cell line has been determined by MTT assay. All the complexes exhibit cytotoxicity against A549 cell line with potency higher than the widely used anti-cancer drug, *cis*-platin. Among all the complexes, cobalt complex 8 shows remarkable cytotoxic potential with A549 cells than other cobalt complexes. The mode of cell death was assessed by adopting AO/EB, Hoescht 33258, Annexin V-Cy3 staining methods and the obtained results indicate that the mode of cell death is apoptosis. The cell cycle analysis confirms that the cobalt complexes 1-8 induce the apoptotic mode of A549 cell death by arresting of subG0/G1 phase of cell cycle progression and complexes 7 and 8 show G2/M phase arrest. Further anti-angiogenic activity of cobalt complexes 1-8 was determined by CEA assay. The results show that all the cobalt complexes have the ability to inhibit angiogenesis. Among all the cobalt complexes 1-8, complex 8 shows the highest anti-angiogenic activity.

The trend in anti-cancer and anti-angiogenesis properties of the cobalt complexes 1-8 of the present study is same has been observed for DNA binding ability of these cobalt complexes. That is higher is the binding ability of the cobalt complex with DNA higher is its anti-cancer activity. All the cobalt complexes show moderate binding ability with BSA which is a good indication that these complexes are good enough to be carried through the blood stream as well as can be released at the appropriate target site.