CHAPTER 8

SYNTHESIS, CHARACTERIZATION, DNA BINDING AND ANTICANCER PROPERTY OF TRICHLORODIMETHYLSULPHOXIDE-S-(1,10-PHENANTHROLINE) RUTHERNIUM (III)
SUMMARY

Synthesis of trichlorodimethylsulphoxide-S-(1,10-phenanthroline) ruthenium (III) ([Ru(phen)(DMSO)Cl₃]) was carried out to analyze the DNA binding and biological property. FT-IR, ¹H NMR and X-ray diffraction studies were used for the characterization. Octahedral geometry was found from the single crystal X-ray structure analysis. The bond lengths, bond angles and dihedral angles of this octahedral structure were also analyzed. The binding of this complex with Calf Thymus DNA (CT-DNA) was investigated from spectroscopic, electrochemical and electrophoresis studies. The evidences of CT-DNA binding by this complex have been found from these studies. The anticancer activity of this compound against Dalton’s lymphoma (in vitro and in vivo) was also found significant.
8.1 INTRODUCTION:

There are transition metal complexes which are modified from the existing potential anticancer agents. The effect of various ligands as anticancer or antitumor agents are well known, and it is essential to design superior agents in terms of effectiveness compared to the already available agent such as cisplatin [1]. Most aromatic organic molecules are used as ligands and subsequently, 1-10 phenanthroline (phen), acridine and m-AMSA are reported to acquire good potency [8-9]. These aromatic ligands are very useful in coordination chemistry because of their binding modes with transition metals. On the other hand, the coordination ability of ligand depends on the presence of chelating groups in the ligand [2-5]. However, there are certain advantages of using mixed ligands that may have better effect than the one type of ligand [5-7]. Moreover, there are extensive studies on the biological properties of few ligands used in potential anticancer agents, and synthesis of ruthenium complexes of these ligands are the interest of the present study.

Ruthenium complexes offer a potential role as antitumor agents over platinum (II) complexes, which are in currently clinical trials, with the properties of a novel mechanism of action, the prospect of non-cross-resistance, reduced toxicity and a different spectrum of activity [1,4]. There are number of reviews that illustrate the different properties of ruthenium complexes including their unique DNA binding modes and antitumor effects [5-7]. The compounds cis-[RuCl₂(NH₃)₄]Cl, fac-[RuCl₃(NH₃)₃] and trans-HIm[RuCl₄(Im)₂] acquire anticancer activity against primary tumour cells [15-17]. Ruthenium (II) arene complex, Ru(η₆-arene)Cl(en)[PF₆] (en = ethylene diamine and arene = benzene, p-cymene, tetrahydroanthracene etc.) show remarkable cytotoxic properties as demonstrated from the in vitro as well as in vivo experiments [18-20]. Recently reported Ru(II) complexes bearing isatin, thio semicarbazones, and chloro-fluoro-phenyl have in vivo and in vitro cytotoxic activity [21]. Many ruthenium complexes containing phenanthroline ligand have been reported and some of them acquire anticancer activity [22-23]. The complexes can be active anticancer compound, but sometimes low solubility in water is the disadvantage. In fact, the solubility may be enhanced by using DMSO molecules as ligand in ruthenium complexes, which again may produce some effect on cancer cells. Dimethyl sulfoxide (DMSO) complexes of both Ru(II) and Ru(III) are as good as cisplatin in terms of
anticancer activity [10-14]. The cisplatin can exist as neutral complex in blood serum that may be easier to cross the lipid bilayer, where the chloride loss and DNA binding could probably result from the lower chloride concentration in intracellular medium [24]. Similarly, NAMI emerges out as antimetastatic agent, but only small fraction of the compound can reach the tumour target. It is also a good agent to inhibit metastasis [25-26]. The [Ru(acac)₃] and [Ru(tfac)₃] (acac=acetylacetonate and tfac = trifluoroacetylacetonate) ruthenium complexes show different levels of antitumor activity (in vitro and in vivo), which may undergo different mechanisms of action as well as diverse perspectives in cancer treatment [27].

Initially, we have synthesized few Ru complexes using known ligands, which in fact acquire good anticancer activity. Hence, we have chosen various ligands that acquire good anticancer activity to synthesize new potential ruthenium complexes. Ruthenium is an important metal in inorganic and organometallic chemistry and also less toxic compared to platinum and its complexes [26-27]. Here, the synthesis, characterization and DNA binding of Ru(III) complex containing 1,10-phenanthroline and a DMSO molecule have been taken up, and the antitumor activity against Dalton’s Lymphoma was also examined.

8.2 EXPERIMENTAL SECTION:

8.2.1 MATERIALS:

Analytical grade RuCl₃·3H₂O was purchased from Sigma, and used without purification. Calf Thymus DNA (CT-DNA), tris-buffer and tetrabutylammonium perchlorate (TBAP) were obtained from Sigma-Aldrich chemical companies, USA. CT-DNA was dissolved in tris-buffered saline at pH 7.6 (TBS) and dialyzed overnight against the same buffer so that A₂₆₀/A₂₈₀ of the dialyzed solution should be > 1.8 [19]. The 1,10-phenanthroline (phen) and other solvents were used in the synthesis as received.
A) Synthesis of Trichlorodimethylsulphoxide-S-(1,10-Phenanthroline) Ruthenium (III) Complex:

Initially, the compound cis-Ru(phen)Cl₄.2H₂O was prepared by refluxing RuCl₃.3H₂O with phenanthroline in ethanol and water mixture for 1 hour as per usual procedure [28]. Then, DMSO was added and refluxed again for half an hour. The solvent was evaporated by heating in water bath and purified in column. The purity of the compound was checked by thin layer chromatography (TLC). Suitable crystals, for X-ray diffraction were obtained by slow evaporation of solvent from the solution. The compound was collected as a light red colored solid, and 80% yield was collected. The reaction steps are shown in Scheme 8.1.

![Scheme 8.1](image)

B) Characterization Procedure:

The IR Spectra of the compound was recorded as KBr pellets on a Perkin-Elmer FT-IR spectrophotometer. The UV-visible spectra were recorded in a Shimadzu UV-2401 PC Spectrophotometer, and the ¹H NMR spectra was recorded on a Bruker Ultrashield 300 MHz NMR spectrometer using TMS as the internal standard.

C) Crystallography of [Ru(phen)(dmsO)Cl₃] Complex:

Single crystal X-ray diffraction data was obtained at 100 K with Brunker smart AXS diffractometer with graphite-monochromatised Mo-Kα radiation by ϕ-ω scans. We used full matrix least square on F². The molecular graphic structure was analyzed by ORTEP plot program. The crystallographic data and refinement details are given in
Table (appendix). The structure was refined by using SHELXL-97, other materials were prepared by wingx publication routine (Figure 8.1) [29].

D) INFRARED SPECTRA:

Selected IR peaks with tentative assignments ($v_{\text{max}}$/cm$^{-1}$) at 1646 (C=N aromatic), 1090 (S=O), 3064 (C-H, SP$^2$ Carbon), 3012, 2926, 2852 (C-H methyl) 1591, 1538, 1413 (C=C aromatic), 450 and 429 (Ru-N and Ru-S) were observed.

E) UV-VISIBLE:

The UV-visible absorbance spectra of the [Ru(phen)(DMSO)Cl$_3$] complex (Figure 8.2) shows two $\lambda_{\text{max}}$ one at near visible region 400 nm and other at 273 nm.

F) $^1$H NMR:

The $^1$H NMR spectra of [Ru(phen)(DMSO)Cl$_3$] was recorded in DMSO solution. The complex is paramagnetic in nature, which corresponds to trivalent oxidation state of ruthenium (d$^5$ configuration), and the spectra of all the protons could not be detected clearly.

8.2.2 SPECTROSCOPIC STUDIES ON DNA BINDING:

The spectroscopic, electrochemical and electrophoresis studies of this Ru complex with Calf Thymus DNA (CT-DNA) were carried out. The distinct spectral shift in CT-DNA mixed Ru complex from the free Ru complex indicates the binding of complex with CT-DNA.

A) UV-VISIBLE ABSORPTION TITRATION:

The UV-visible absorption spectra of the [Ru(phen)(DMSO)Cl$_3$] complex in presence of CT-DNA at various concentrations is shown in Figure 8.2. This experiment was performed by maintaining a constant concentration of the complex while varying the concentrations of CT-DNA from 0.59X10$^{-5}$ M to 2.96X10$^{-5}$ M. The absorption spectra of the complex are characterized by two distinct intense transitions at 400 nm and 273 nm, which may be due to Ru (d-d) and intra-ligand (IL) p–p* transitions.
respectively. As the increase of CT-DNA concentrations from 0.59X10^{-5} M to 2.96X10^{-5} M, absorbance intensity of both the two peaks decreased without observing any shifting in wavelength, which are shown in Table 8.1 and Figure 8.2. The spectral characteristics suggest the interactions between the complex and CT-DNA. To estimate quantitatively the binding strength of [Ru(phen)(DMSO)Cl\_3], the intrinsic binding constants $K_b$ was calculated from the following equation [30-31].

$$\frac{\varepsilon_b-\varepsilon_f}{\varepsilon_a-\varepsilon_f} = \frac{1}{[\text{DNA}]} + \frac{1}{K_b}$$

where $\varepsilon_a$, $\varepsilon_f$ and $\varepsilon_b$ are the extinction coefficients of observed solution, free complex and the complex with maximum CT-DNA respectively. The value of $K_b$ was obtained from the slope of the plot shown in Figure 8.2b, which is found to be 2.77 X 10^4 M^{-1}.

**B) FLUORESCENCE EMISSION TITRATION:**

To further investigate the interaction of the complex with CT-DNA fluorescence titration experiment was performed. On excitation at 280 nm, the emission spectra of the complex, and in presence of varying amounts of CT-DNA are shown in Figure 8.3. On increasing the concentrations of CT-DNA from 0.59 X10^{-5} M to 4.1X10^{-5} M, a new peak appeared at 445 nm in addition to the original peak of complex (at 324 nm). The intensities of both the original and the new peaks have been increased with increasing concentrations of CT-DNA as shown in Table 8.2 and Figure 8.3.

**C) ELECTROCHEMISTRY:**

Cyclic voltammetric study for [Ru(phen)(DMSO)Cl\_3] complex was carried out in DMSO (0.005 M) solution containing 0.1 M TBAP as supporting electrolyte using Ag/Ag\^+ as reference electrode, and a glassy carbon was used as working electrode. The inert environment was maintained by passing N\_2 gas through the solution to remove oxygen. The voltamogram shows distinct oxidation and reduction peaks of a reversible electron transfer couple at scan rate 100 mVs^{-1}. The shift of redox potential ($E_{1/2}$) from 222 mV to 256 mV on addition of CT-DNA is observed (Figure 8.4).
D) ELECTROPHORESIS EXPERIMENT:

The binding of trichlorodimethylsulphoxide-S-(1,10-phenanthroline) ruthenium (III) complex to CT-DNA was further supported by electrophoresis experiment. The solution of CT-DNA was prepared in tris-HCl buffer at pH 7.6. The interaction between the complex and CT-DNA was monitored by preparing three solutions of the complex with CT-DNA at different concentrations, and incubated for 24 hours at 37º C before running Gel Electrophoresis. These three solutions were placed at three different lanes, 1, 2 and 3 in the gel having concentrations 6 mM, 3 mM and 2 mM respectively along with C, for CT-DNA (5 mM). The samples were run from -ve to +ve potential for 3 hours at different voltages (half an hour at 50 V, 1 hour at 60 V, half an hour at 70 V and 1 hour at 80 V) on a 1% agarose gel in tris-borate EDTA. After photographed the gel under UV light, it appears that lane C moves from –ve to +ve potential of the gel faster than that of lane 1, 2 and 3 and also brighter than others as shown in Figure 8.5.

8.2.3 BIOLOGICAL STUDIES OF TRICHLORODIMETHYLSULPHOXIDE-S-(1,10-PHENANTHROLINE) RUTHENIUM (III) COMPLEX:

A) EFFECT OF DIFFERENT CONCENTRATIONS OF TRICHLORODIMETHYLSULPHOXIDE-S-(1,10-PHENANTHROLINE) RUTHENIUM (III) COMPLEX ON MICE BEARING DALTON’S LYMPHOMA:

Control and experimental animals were selected randomly and divided into groups of 10 mice each according to randomized block design. Each animal was transplanted with 3x10⁶ cells/mice. After 4 days of post-tumor transplantation, the experimental mice were treated with single i.p. injection of different concentrations of Ru complex. Control animals were injected with equal amount of PBS. In each group mean survival time, % increase in the life span, % of more than 60 days survivors, and tumor free survivors were calculated. % increase in the life span was calculated as

\[
\%\text{ILS} = \frac{T - C}{C} \times 100
\]
where \( T \) is the mean survival time of experimental mice and \( C \) is the mean survival time of control mice [32-33]. Each set was repeated thrice and the results were pooled together.

For all the concentrations of this Ru complex, the increase of the mean survival time of tumor bearing mice was observed. Control animals could survive for 9 days whereas tumor bearing mice treated with 20-50 mg/kg Ru complex were able survive up to 45 days (Figure 8.6). The increase in the life span of tumor bearing animals was found when treated with different concentrations of trichlorodimethylsulphoxide-S-(1,10-phenanthroline) ruthenium (III) as indicated in Figure 8.6.

B) Effect of Trichlorodimethylsulphoxide-S-(1,10-Phenanthroline) Ruthenium (III) Complex on the Survival of Dalton’s Lymphoma Cells in Vivo Studies:

Dalton’s lymphoma cells were isolated from the peritoneal cavity of tumor bearing mice (control and treated with different concentrations of this Ru complex). 2-3 ml of sterile phosphate buffered saline (PBS) was injected into the peritoneal cavity and the fluid containing the tumor cells was withdrawn, and collected in sterile petridishes for incubation at 37\(^{0}\)C for 2 hours. The cells of macrophage lineage adhered to the bottom of petridishes to form a confluent monolayer. The non adherent population of lymphoma cells was gently aspirated out and washed repeatedly with PBS. The viability was tested by trypan blue exclusion test. It was found that viability is directly proportional to concentrations of injected [Ru(phen)(DMSO)Cl\(_3\)], and the LC\(_{50}\) was found to be 50 mg/kg (Figure 8.7) [34].
C) **Effect of Trichlorodimethylsulphoxide-S-(1,10-phenanthroline) Ruthenium (III) on the survival of Dalton’s Lymphoma Cells in Vitro Studies:**

For cytotoxicity assay in vitro, Dalton’s lymphoma cells were plated at high density (8x10⁷ cells/dish) at time 0 in DMEM containing fetal calf serum, 10 mM NaHCO₃, 0.3% glutamine and different concentrations of Ru complex for a fixed treatment duration of 1 hour. Control dishes were treated with equal amount of PBS used as a solvent for trichlorodimethylsulphoxide-S-(1,10-phenanthroline)ruthenium(III). At the end of drug treatment, cells were harvested and washed with PBS, suspended again in ADM with DFCS and incubated for 72 hours at 37°C. After incubation, cells were trypsinized and viable cells were counted by trypan blue exclusion test [35-36]. There exists a linear correlation between the concentration of, [Ru(phen)(DMSO)Cl₃] in the medium and % survival of lymphoma cells, the IC₅₀ was found to be 40 μg/ml (Figure 8.8).

**8.3 Results and Discussion:**

The [Ru(phen)(DMSO)Cl₃] is an octahedral complex that consist of one 1,10-phenanthroline (phen), one DMSO and three chlorine coordinated with the ruthenium metal. The compound was prepared from a mixture of RuCl₃.3H₂O and phen ligand. The solution was refluxed in ethanol, and DMSO was added after cooling. The mixture was again refluxed for half an hour, and the various steps are shown in Scheme 8.1. Initially, the two Cl atoms might be replaced by phen ligand and then DMSO molecule reacts with Ru(phen)Cl₄.2H₂O to replace the inner Cl atom to give [Ru(phen)(DMSO)Cl₃]. However, the crystalline compound so obtained was analyzed by XRD studies and ORTEP, which is shown in Figure 8.1. The absorption spectra of the complex were characterized by two distinct λₘₐₓ at 273 nm and 400 nm. The measured infrared (cm⁻¹) spectra of the complex are at 1086, 449, and 414. The potential bonds so observed correspond to γ (S=O), γ (Ru-N) and γ (Ru-S). The result of ¹H NMR study is not clear to detect the peaks of corresponding ligands. Analysis of the results show the compound as [Ru(phen)(DMSO)Cl₃].

Further studies were carried out to understand the binding of this complex with CT-DNA. Absorbance intensity of both the peaks at 400 nm and 273 nm decreased
with the increase of concentrations of CT-DNA from $0.59 \times 10^{-5}$ M to $2.96 \times 10^{-5}$ M as shown in Table 8.1 and Figure 8.2. The spectral characteristics suggest strong interactions between the $[\text{Ru(phen)(DMSO)Cl}_3]$ complex with CT-DNA. The intrinsic binding constant, $K_b$, is found to be $2.77 \times 10^4$ M$^{-1}$. On excitation at 280 nm, the fluorescence spectra of the free and CT DNA mixed complex was recorded (Figure 8.3). In addition to the original apex at 324 nm of the complex, a new peak at 445 nm is observed, which becomes more intense on increasing the concentrations of CT-DNA from $0.59 \times 10^{-5}$ M to $4.1 \times 10^{-5}$ M, as shown in Table 8.2 and Figure 8.3. Such observable spectral shift in intensity indicate the binding of the $[\text{Ru(phen)(DMSO)Cl}_3]$ complex with CT-DNA.

The potentials obtained from Osteryoung Square Wave Voltammetry (OSWV) of free complex and in presence of CT-DNA were found at 224 mV and 256 mV respectively (Figure 8.4). This +ve shift in redox potential (34 mV) after mixing with CT-DNA suggests to binding of the complex with CT-DNA. Moreover, we have performed electrophoresis experiments to understand clearly the binding of CT-DNA with $[\text{Ru(phen)(DMSO)Cl}_3]$ complex. Lane C moves faster from -ve to +ve side in the gel electrophoresis than that of lanes, 1, 2 and 3 and brighter those others. The electrophoretic mobilities and brightness of the bands are found decreased as the concentrations of the complex increases from lane 3 to 1. It might be due to the binding of complex with CT-DNA (Figure 8.5).

We have carried out biological studies (in vitro and in vivo) of this complex to understand the effects of this complex on the Dalton’s lymphoma isolated from the peritoneal cavity of tumor bearing mice. The survival of Dalton’s lymphoma (in vivo) with different concentrations of complex, i.e. 10, 20, 30, 40, 50 mg/kg of body weight are shown in Figure 8.7. It was found that the viability is directly proportional to concentrations of injected Ru complex. The LC$_{50}$ was found to be 50 mg/kg (Figure 8.7). Similar experiment was carried out for in vitro studies, and the survival of cells with increasing concentrations of Ru complex is shown in Figure 8.8. There exists a linear correlation between the concentrations of trichlorodimethylsulphoxide-S-(1,10-phenanthroline) ruthenium (III) and % survival of lymphoma cells. The IC$_{50}$ was found to be 40 μg/ml (Figure 8.8). However, no tumor free survivor was observed and the highest dose of this complex (50 mg/kg) were found to be most effective (Figure 8.6).
8.4 CONCLUSION:

The synthesis, characterization and biological evaluation of [Ru(phen)(DMSO)Cl₃] complex have been performed. The structure analyzed by single crystal XRD study shows six coordinated octahedral geometry. The results characterized by various techniques show evidences of CT-DNA binding. On the basis of spectroscopic shift, the intrinsic binding constant (Kₘ) is 2.77X10⁴ M⁻¹. Based on the observed distinct electrochemical E₁/₂ and electrophoresis band shifts, the complex is expected to bind with CT-DNA. The biological properties show that the highest dose of this complex is 50 mg/kg, but no tumor free mice were found after treatment with this complex.

8.5 REFERENCES:


Table 8.1. The wavelengths and absorbances of the complex with and without mixing CT-DNA at different concentrations.

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<thead>
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<th>Samples</th>
<th>Wavelengths (nm)</th>
<th>Absorbances</th>
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<td></td>
<td>λmax (1)</td>
<td>λmax (2)</td>
</tr>
<tr>
<td>a DNA</td>
<td>260</td>
<td>400</td>
</tr>
<tr>
<td>b Complex</td>
<td>273</td>
<td>400</td>
</tr>
<tr>
<td>c Complex +0.59 X10⁻⁵M DNA</td>
<td>273</td>
<td>400</td>
</tr>
<tr>
<td>d Complex +1.10 X10⁻⁵M DNA</td>
<td>273</td>
<td>400</td>
</tr>
<tr>
<td>e Complex +1.78 X10⁻⁵M DNA</td>
<td>273</td>
<td>400</td>
</tr>
<tr>
<td>f Complex +2.37 X10⁻⁵M DNA</td>
<td>273</td>
<td>400</td>
</tr>
<tr>
<td>g Complex +2.96 X10⁻⁵M DNA</td>
<td>273</td>
<td>400</td>
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</table>

Table 8.2. The emission peaks and fluorescence intensities of the complex with and without mixing CT-DNA at different concentrations.

<table>
<thead>
<tr>
<th>Samples</th>
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<th>Intensities</th>
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<td>b Complex</td>
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<td>76.47</td>
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<td></td>
<td>445</td>
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<td>d Complex +1.78X10⁻⁵M DNA</td>
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<td>124.9</td>
</tr>
<tr>
<td></td>
<td>445</td>
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<tr>
<td>e Complex +2.37X10⁻⁵M DNA</td>
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**Figure 8.1.** Structure of trichlorodimethylsulphoxide-S-(1,10-phenanthroline) ruthenium(III) complex.

**Figure 8.2.** (a) UV-visible spectra of the complex with and without mixing CT-DNA at different concentrations, along with simple CT-DNA and (b) plot of $\varepsilon_{b} - \varepsilon_{t} / \varepsilon_{a} - \varepsilon_{t}$ against reciprocal concentration of CT-DNA ($1/[DNA]$).
Figure 8.3. Fluorescence intensity of the complex with and without mixing CT-DNA at different concentrations, along with simple CT-DNA.
Figure 8.4. (a) Cyclic voltammogram of the complex at different scan rate, (b) Cyclic voltammogram of the complex with and without mixing CT-DNA, (c) and (d) are the square wave voltammogram for the complex and the complex in presence of CT-DNA, respectively.

Figure 8.5. Gel electrophoresis of trichlorodimethylsulphoxide-S-(1,10-phenanthroline) ruthenium (III) complex with CT-DNA studied in gel electrophoresis in tris-HCl buffer at pH 7.6 (concentration increases from lane 3-1).
Figure 8.6. Effect of this Ru complex on mice bearing Dalton’s lymphoma. No tumor bearing mice became tumor free after treatment 50 mg/kg of the complex. Rest of the animals also (p< 0.05) increase in their life span compared to control.

Figure 8.7. Effect of the complex on the survival of Dalton’s lymphoma cells in vivo. The results are an average of five (n=5) independent experiments in triplicate and represented as mean ± SE. p < 0.05 vs control.
Figure 8.8. The results are an average of five (n=5) independent experiments in triplicate and represented as mean ± SE. p < 0.05 vs control.