SUMMARY

The cis-dichlorotetrakis (dimethylsulphoxide) ruthenium (II) ([Ru(DMSO)_4Cl_2]) is a well known compound among the ruthenium complexes. Here, we report a unique binding of this compound with Calf Thymus DNA (CT-DNA) by spectroscopic and electrophoresis studies. The biological studies of this compound carried out in vivo and in vitro experiments on mice bearing with Dalton’s lymphoma were performed. The six coordinated [Ru(DMSO)_4Cl_2] complex was prepared and the structure has been characterized by FT-IR, ^1H NMR and X-ray diffraction studies. The evidences of DNA binding by this complex have been indicated from Fluorescence, UV-Visible and electrophoresis studies. Also, the anticancer property of this complex against Dalton’s Lymphoma is found interesting.
9.1 INTRODUCTION:

Platinum based complexes have been clinically used in cancer therapy. The parent compound of this class of anticancer agents, cisplatin (cis-PtCl₂(NH₃)₂) is highly effective towards testicular, and ovarian cancers, cervical, head and neck, esophageal, and small cell lung cancers [1-2]. Ruthenium complexes are also important as antitumor agents over platinum (II) complexes, and some are currently under clinical trials [3]. It follows a novel mechanism of action with the prospect of non-cross-resistance, reduced toxicity and acquires different activity [4-5]. The Ru complexes are suitable towards cisplatin resistance cancer cells, and efficiently exert antitumor action, which may be in part due to the ability of ruthenium complexes to mimic the binding of iron to certain biological molecules that in fact exploits a mechanism for non-toxic transport of iron inside the body [6-9]. This property is especially attractive for ruthenium complexes. The ability of ruthenium to mimic iron in binding to certain biological molecules make these complexes well suitable for medicinal use, and as an alternative for the platinum anticancer drugs in the treatment of cancer cells, resistant to cisplatin and its analogues [11-13]. The water soluble Ru complexes are usually suitable for medicinal use, and several Ru based drugs have shown good anticancer or antibacterial properties [15-16]. The Ru complexes have certain advantages than the platinum complexes due to its solubility in water and low toxicity [14-17]. Although there are several reports on the synthesis and medicinal property of ruthenium complexes, the design of DNA targeted ruthenium complexes may be useful [10, 17]. Many of these complexes acquire anticancer activity, and therefore used in chemotherapy. The ruthenium complexes like cis-[RuCl₂(NH₃)₄]Cl and trans-H1Im [RuCl₄Im₃] can bind with DNA, and the cytotoxicity and DNA binding of these complexes usually correlates in most cases [14, 25]. The complex [Ru(H₂O)(NH₃)₃] binds selectively with guanine nucleobase of both single and double strand DNAs, but there are other complexes that bind within adenine and cytosine residues [26-28].

There are abundant ruthenium complexes that can bind with DNA, and many of these compounds acquire anticancer activity [20-25]. Although several investigations on the anticancer properties of Ru complexes containing mixed ligands with DMSO are
available, the biological studies on these complexes are not reported. It is essential to analyze the chemical and biological properties of such complexes to understand the effect of other ligands in the overall assessment of therapeutic values. Hence, the spectroscopic studies on the binding of calf thymus DNA (CT-DNA) with this compound may be carried out, and the in vitro and in vivo anticancer activity may be studied.

Here, we intend to synthesize cis-[Ru(DMSO)₄Cl₂] complex, subsequently the crystal so formed have been taken for analysis. The structure of cis-[Ru(DMSO)₄Cl₂] is octahedral, with two chlorine and four DMSO molecules, where three DMSO molecules are S-bonded in a facial configuration and one DMSO is O-bonded. It is also possible to prepare trans-[Ru(II)(DMSO)₄Cl₂], with S-bonded DMSO molecules. The cis-[Ru(DMSO)₄Cl₂] complex when dissolved in water undergoes lost of the O-bonded DMSO ligand, whereas the trans compound rapidly loses the two S-bonded DMSO ligands to yield cis-diaqua species. Both these isomers can undergo hydrolysis slowly with the dissociation of chloride ion to form cationic compound [23, 29, 33]. The presence of the three remaining DMSO ligands in the cis isomer produces considerable steric hindrance, which makes the cis aqua species inert relative to the trans isomer. So, the study has been taken up to assess the biological property of this cis-[Ru(DMSO)₄Cl₂] complex.

9.2 EXPERIMENTAL SECTION:

9.2.1 MATERIALS:

Analytical grade RuCl₃·3H₂O Calf thymus DNA (CT-DNA) and tris-buffer were purchased from Sigma-Aldrich chemical company. CT-DNA was dissolved in tris-buffered saline (pH 7.6, TBS), and dialyzed overnight against the same buffer so that A₂₆₀/A₂₈₀ of the dialyzed solution can remain >1.8. Dimethyl sulfoxide (DMSO) and ethanol were also used as received.
A) Synthesis of cis-dichlorotetrakis (dimethylsulphoxide) ruthenium (II):

The RuCl$_3$.3H$_2$O was dissolved in excess of DMSO at 60°C for 20 minutes, and then heated at 80°C for 3 hours until the solution becomes wine red. The solution was cooled and added to large volume of acetone. The solution was kept for crystallization. After a few days yellow crystalline product was formed, and 78% yields was found.

B) Characterization Procedure:

The IR spectra of the compound were recorded as KBr pellets on a Shimadzu IR Affinity-1 spectrometer (model). The UV spectra were taken in aqueous solution on a shimadzu UV-2401 PC Spectrophotometer and the $^1$H NMR spectra was recorded on a Bruker Ultrashield 300 MHz NMR spectrometer using TMS as the internal standard.

C) Crystallography of [Ru(DMSO)$_4$Cl$_2$] Complex:

Single crystal X-Ray diffraction data were obtained at 100 K with Brunker smart AXS diffractometer with graphite-monochromatised Mo-K$_\alpha$ radiation by $\phi$-$\omega$ scans. We have used full matrix least square on F$^2$. The molecular graphic structure was analyzed by ORTEP plot program. The structure (Figure 9.1) was refined by using SHELXL-97, other materials were prepared by wingx publication routine [30].

D) Infrared Spectra:

Selected IR peaks with tentative assignments ($\nu_{\text{max}}$/cm$^{-1}$) at 1646 (C=N aromatic), 1103 (S=O for S boned DMSO), 933 (S=O for O boned DMSO), 3012, 2920, 2854 (C-H methyl) 460 and 424 (Ru-O and Ru-S) were recorded.

E) UV-Visible:

The UV-visible absorbance spectra of the complex (Figure 9.2) in water show two distinct peaks at $\lambda_{\text{max}}$ 357 nm and other at 308 nm.
F) $^1$H NMR:

The $[\text{Ru(DMSO)}_4\text{Cl}_2]$ complex is found diamagnetic. It corresponds to bivalent state of ruthenium (low spin $d^6$, $S=0$). $^1$H NMR spectra of the complex were recorded in D$_2$O solution. We observed signals at around 3.5 ppm for H (CH$_3$) and at 2.60 ppm, which are due to S-bonded and O-bonded DMSO.

9.2.2 SPECTROSCOPIC STUDIES ON DNA BINDING:

The spectroscopic and electrophoresis studies on the binding of cis-dichlorotetrakis (dimethylsulphoxide) ruthenium (II) complex with Calf Thymus DNA (CT-DNA) were carried out. The distinct spectral shift of CT-DNA mixed Ru complex from the spectra of free complex shows evidence of CT-DNA binding.

A) UV-VISIBLE ABSORPTION TITRATION:

The UV-visible absorption spectra of the cis-[Ru(DMSO)$_4$Cl$_2$] with various concentrations of CT-DNA. Tris-buffer at pH 7.6 was used for preparing the solution. The spectra were shown in Figure 9.2 and Table 9.1. The concentration of the complex remains unchanged and while the concentrations of CT-DNA was taken from $0.59 \times 10^{-5}$ M to $4.10 \times 10^{-5}$ M. The two distinct intense transitions at 357 nm and 308 nm was found in the spectra, which may be due to d-d and intra-ligand p–p* transitions. There observed a peak at 432 nm other than at 357 nm within the concentrations of CT-DNA from $0.59 \times 10^{-5}$ M to $4.10 \times 10^{-5}$ M. The binding strength of $[\text{Ru(DMSO)}_4\text{Cl}_2]$ was found quantitatively, the intrinsic binding constants $K_b$ was calculated from the following equation [31-32].

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

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 the $K_b$ was obtained from the slope of the plot shown in Figure 9.2b. It is found to be $3.09 \times 10^4 \text{ M}^{-1}$. 
B) **FLUORESCENCE EMISSION TITRATION:**

The interaction between the complex and CT-DNA was examined by fluorescence titration experiment. The emission spectra of the \([\text{Ru(DMSO)}_4\text{Cl}_2]\) complex and in presence of varying amounts of CT-DNA at 270 nm are shown in **Figure 9.3.** The concentrations of CT-DNA was increased from 0.59 \(10^{-5}\) M to 2.96 \(10^{-5}\) M, the intensity of original apexes observed at 430 nm and 543 nm of the complex diminished gradually, which become more pronounced at 430 nm, as shown in **Table 9.2.**

C) **ELECTROPHORESIS EXPERIMENT:**

The binding of \([\text{Ru(DMSO)}_4\text{Cl}_2]\) complex with CT-DNA was further monitored by electrophoresis experiment as shown in **Figure 9.4.** The solution of the complex and the CT-DNA was prepared in tris-HCl buffer at pH 7.6. The binding of the complex with CT-DNA was monitored by preparing three solutions of complex mixed with different concentrations of CT-DNA, which was again incubated for 24 hours at 37º C before running Gel Electrophoresis. The three solutions were placed at different lanes 1, 2 and 3 in the gel having concentrations 2 mM, 3 mM and 6 mM respectively. The CT-DNA of 5 mM concentration was placed in lane C. The samples were run from –ve to +ve potentials 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 80V) on a 1% agarose gel in tris-borate EDTA. The gel was then photographed under UV light.

9.2.3 **BIOLOGICAL STUDIES OF CIS-DICHLOROTETRAKIS (DIMETHYLSULPHOXIDE) RUTHENIUM (II) COMPLEX:**

A) **EFFECT OF DIFFERENT CONCENTRATIONS OF CIS-DICHLOROTETRAKIS (DIMETHYLSULPHOXIDE) RUTHENIUM (II) 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 \(3\times10^6\) cells/mice. After 4 days of post-tumor transplantation,
experimental mice were treated with single i.p. injection of different concentrations of ruthenium 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 day survivors and tumor free survivors were calculated. The % increase in the life span was calculated as

\[
\%\text{ILS} = \left( \frac{T - C}{C} \right) \times 100
\]

where \( T \) is the mean survival time of experimental mice, and \( C \) is the mean survival time of control mice [38]. Each set was repeated several times.

All the concentrations of injected ruthenium complex can increase the mean survival time of tumor bearing mice. As usual the control animals could survive for 9 days whereas tumor bearing mice treated with 20-50 mg/kg ruthenium complex were able survive up to 45 days (Figure 9.5). Increase in the life span of tumor bearing animals was found with the increase of concentrations of ruthenium complex up to 50 mg/kg injected to the tumor bearing mice. The life span of control animals were standardized for this experiment [34-35].

B) EFFECT OF CIS-DICHLOROTETRAKIS(DIMETHYLSULPHOXIDE) RUTHENIUM (II) 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 ruthenium 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, collected in sterile petridishes and incubated at 37\(^\circ\) 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 [36]. It was found that viability is directly
proportional to the concentrations of ruthenium complex injected. The LC$_{50}$ was found to be 40 mg/kg (Figure 9.6).

C) **Effect of cis-dichlorotetrakis(dimethylsulphoxide) ruthenium (II) complex on the survival of Dalton’s lymphoma cells in vitro:**

For cytotoxicity assay in vitro, Dalton’s lymphoma cells were plated 8x10$^7$ cells/dish at time zero in DMEM containing fetal calf serum, 10 mM NaHCO$_3$, 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 ruthenium complex [36-37]. 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$^0$C. The cells were trypsinized after incubation and viable cells were counted by trypan blue exclusion test shown in Figure 9.7.

### 9.3 Results and Discussion:

The cis-dichlorotetrakis (dimethylsulphoxide) ruthenium (II) complex was prepared from RuCl$_3$.3H$_2$O dissolved in excess of DMSO at 60$^0$C for 20 minutes, and then heated at 80$^0$C for 3 hours. Excess amount of acetone was added on cooling, and it was kept for crystallization. The crystalline compound so obtained was analyzed by XRD studies, and ORTEP structure is shown in Figure 9.1. The absorption spectra of the complex were characterized by two distinct λ$_{max}$ observed at 357 nm and 308 nm. The $^1$H NMR studies show bands at around 3.5 ppm and 2.60 ppm, which is due to S-bonded and O-bonded DMSO (CH$_3$) to Ru metal. Further studies are carried out to understand the binding of this compound with CT-DNA. Interestingly, distinct spectral shift from the original visible peak of complex at 357 nm was observed, and another peak appears at 432 nm on increasing the concentrations of CT-DNA from 1.7X10$^{-5}$ M to 4.7 X10$^{-5}$ M (Figure 9.2 and Table 9.1). Such changes indicate the evidences of CT-DNA binding by this complex and the intrinsic binding constant, K$_b$ is found to be 3.09 X10$^4$ M$^{-1}$. The fluorescence spectra of free and CT-DNA mixed complexes were recorded at 270 nm (excitation) as shown in Figure 9.3. On increasing the
concentrations of CT-DNA, the intensity of original apexes indicated at 430 nm and 543 nm for the complex diminishes gradually, and the effect becomes more pronounced at 430 nm (Table 9.2). It is the indication of CT-DNA binding by Ru complex.

Moreover, we have performed electrophoresis experiment to understand the binding of CT-DNA with the complex from the mobility and change of brightness. Lane C moves slightly faster from –ve to +ve potentials than that of lanes 1, 2 and 3 as shown in Figure 9.4. The electrophoretic mobilities and intensity of the bands have been decreased from lane 1 to 3 with the increase of concentration of the complex form 2 mM to 6 mM. It might be due to the binding of the [Ru(DMSO)₄Cl₂] complex with CT-DNA. We have also carried out biological studies (in vitro and in vivo) of this complex. The effect of this complex on Dalton’s lymphoma isolated from the peritoneal cavity of tumor bearing mice are found promising. The survival of Dalton’s lymphoma (in vivo) with different concentrations of complex i.e 10, 20, 30, 40 and 50 mg/kg of body weight are shown in Figure 9.6. It has been found that the viability of cells is directly proportional to the concentrations of Ru complex, and the LC₅₀ was found to be 40 mg/kg (Figure 9.6). Similarly, the survival of cells (in vitro) with increasing concentrations of [Ru(DMSO)₄Cl₂] were carried (Figure 9.7). Highest dose of [Ru(DMSO)₄Cl₂] complex was found 50 mg/kg, and 20% tumor free survivor was observed with this dose (Figure 9.5).

9.4 CONCLUSION:

From the spectroscopic studies (UV-Vis), the binding of the complex with CT-DNA is prominent with the intrinsic binding constant, Kₜ of 3.09X10⁴ M⁻¹. The results of electrophoresis studies support the binding of this complex with CT-DNA. The biological activity is found promising with the LC₅₀ of 40 mg/kg. 20% tumor free survivor is also detected when administered with this complex.

9.5 REFERENCES:


**Table 9.1.** The wavelengths and corresponding absorbances of the complex with and without mixing CT-DNA at different concentrations.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Wavelengths (nm)</th>
<th>Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ max (1)</td>
<td>λ max (2)</td>
</tr>
<tr>
<td>a DNA</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>b Complex</td>
<td>357</td>
<td></td>
</tr>
<tr>
<td>c Complex +0.59 ×10^-5 M DNA</td>
<td>336</td>
<td>432</td>
</tr>
<tr>
<td>d Complex +1.10 ×10^-5 M DNA</td>
<td>334</td>
<td>432</td>
</tr>
<tr>
<td>e Complex +1.78 ×10^-5 M DNA</td>
<td>335</td>
<td>432</td>
</tr>
<tr>
<td>f Complex +2.37 ×10^-5 M DNA</td>
<td>337</td>
<td>432</td>
</tr>
<tr>
<td>g Complex +2.96 ×10^-5 M DNA</td>
<td>339</td>
<td>432</td>
</tr>
<tr>
<td>h Complex +3.50 ×10^-5 M DNA</td>
<td>339</td>
<td>432</td>
</tr>
<tr>
<td>i Complex +4.10 ×10^-5 M DNA</td>
<td>339</td>
<td>432</td>
</tr>
</tbody>
</table>

**Table 9.2.** The emission peaks and fluorescence intensities of the complex with and without mixing CT-DNA at different concentrations.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Apex (nm)</th>
<th>Intensities</th>
</tr>
</thead>
<tbody>
<tr>
<td>a DNA</td>
<td>563</td>
<td>33.8</td>
</tr>
<tr>
<td>b Complex</td>
<td>430</td>
<td>25.11</td>
</tr>
<tr>
<td></td>
<td>543</td>
<td>24.98</td>
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<tr>
<td>c Complex +0.59 ×10^-5 M DNA</td>
<td>430</td>
<td>5.339</td>
</tr>
<tr>
<td></td>
<td>543</td>
<td>4.980</td>
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<tr>
<td>d Complex +1.10 ×10^-5 M DNA</td>
<td>430</td>
<td>1.912</td>
</tr>
<tr>
<td></td>
<td>543</td>
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<tr>
<td>e Complex +1.78 ×10^-5 M DNA</td>
<td>430</td>
<td>1.544</td>
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<tr>
<td></td>
<td>543</td>
<td>2.656</td>
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<tr>
<td>f Complex +2.37 ×10^-5 M DNA</td>
<td>430</td>
<td>0.920</td>
</tr>
<tr>
<td></td>
<td>543</td>
<td>2.480</td>
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<tr>
<td>g Complex +2.96 ×10^-5 M DNA</td>
<td>430</td>
<td>0.439</td>
</tr>
<tr>
<td></td>
<td>543</td>
<td>1.729</td>
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</table>
**Figure 9.1.** Structure of cis-dichlorotetrakis(dimethylsulphoxide) ruthenium(II) complex.

**Figure 9.2.** (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 $\varepsilon_b - \varepsilon_f$ vs. $\varepsilon_a - \varepsilon_f$ against reciprocal of concentration of CT-DNA ($1/[\text{DNA}]$).
Figure 9.3. Fluorescence intensity of the complex with and without mixing CT-DNA at different concentrations along with simple CT-DNA.

Figure 9.4. Gel electrophoresis of the complex with CT-DNA in tris-HCl buffer at pH 7.6 (concentration increases from lane 1-3).
**Figure 9.5.** Effect of [Ru(DMSO)$_4$Cl$_2$] complex on mice bearing Dalton’s lymphoma. 20% tumor bearing mice became tumor free after treatment 50 mg/kg Ru complex. Rest of the animals also (p< 0.05) increase in their life span compared to control.

**Figure 9.6.** Effect of this 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 9.7. The results are an average of five (n=5) independent experiments in triplicate and represented as mean ± SE. p < 0.05 vs control.