Chapter IV

Development of Ruthenium derivatives as topoisomerase II poisons.
Topoisomerase II is being reverd as one of potential target for cancer chemotherapy:

Introduction:

Topoisomerases II is one of the nuclear enzyme responsible for the topological maintenance of the cellular DNA inter conversions in the DNA are the major target for many anticancer drugs belonging to different classes of compounds like epipodophillotoxins (etoposide, tenoposide), anthracyclins (doxorubicin, daunorubicin), amino-acridines (amsacrine), ellipticines (ellipcinium), anthracene-diones (mitoxantrone) etc. These compounds interfere with one of the steps in the catalytic cycle of Topoisomerase II and stabilize the cleavable complex; the transient intermediate have double stranded nicked DNA covalently bound to the enzyme. The cleavable complex is a state of intense fragility of the DNA molecule.

Metal complexes gained importance as anticancer drugs with the discovery of cisplatin. This particular discovery provoked the discovery of the anticancer activity of complexes of other metals. Early transition and late transition series of periodic table have been vigorously tested for their anti cancer activity. Although very few of them matched the efficacy of cisplatin, some of the non-platinum compounds were active against tumors that were unresponsive to cisplatin and other anticancer drugs. Some of the non platinum metal complexes which showed promising anti cancer activity are Spiro germanium a germanium complex, gallium nitrate, titanium metal complexes like titanocene dichloride and budotitane, ruthenium complexes such as Trans-indazolium [bisindazole] tetrachloro Ruthenate and Imidazolium trans- imidazole dimethylsulfoxide tetrachlororuthenate (NAMI-A) complex. These complexes have shown to possess distinct interaction with
DNA, RNA, and protein, which define their anticancer activities. For example, Gallium salts interfere with the ribonuclotide reductase activity and inhibit the DNA nucleotide synthesis (Waller 1996). Titanocene complexes and Ruthenium complexes are shown to interact with type IV collagenolytic activity which corresponds to an increase in the extracellular matrix components in tumor parenchyma (Maragoudakis et al., 1994). This hinders metastasis formation and blood flow to the tumors (Sava et al., 1996, Morgunova et al., 1999).

Among the metals used in the anticancer metal complexes, ruthenium shows unique properties. It has strong complex forming activity with numerous ligands. Ample studies indicate that most ruthenium complexes bind covalently to DNA via the N-7 atom of purines and cause cytotoxicity by inhibiting cellular DNA synthesis (Kopf et al., 1994, Haiduc et al). The DNA binding property of the Ruthenium complexes has been associated with their anticancer activity. Also, ruthenium complexes make use of various biological mechanisms for transport and macromolecular binding and they coordinate with various biological macromolecules. This feature helps in the development of ruthenium complexes that interact with specific biological molecules to bring about targeted anticancer activity. Many of the ruthenium complexes appear to be transported in blood through transferrin (80%) and to a lesser extent through albumin (Srivastavastava et al., 1989, Kratz et al., 1994). Tumor localization of Ruthenium, Gallium, and Titanium complexes attributes to this transport mechanism as tumor cells express a large number of transferrin receptors on their membranes (Nejmeddine et al., 1998). Redox molecules like glutathione interact with heavy metals and the reduced form of this peptide is known to interact with complex, which would activate the metal
complexes to bind to biopolymers in the hypoxic environment of tumors. Ruthenium (III) compounds can also serve as diagnostic tumor imaging agents, using the nuclides $^{97}\text{Ru}$ or $^{103}\text{Ru}$ (Srinivasa et al., 1989).

Numerous ruthenium compounds have earlier been reported to possess anticancer activity, some of them are potent than cisplatin (Giraldi et al., 1977, Clarke 1989, Seva et al., 1984, 1989, Mestroni et al., 1989, Pacor et al., 1991, Keppler et al., 1990). Two of these compounds are trans-[indazolium bis 9indazole] tetrachlororuthenate (III), cis-[Ru II Cl$_2$ (dimethysulphoxide) and trans-[Ru III Cl (dimethysulphoxide) Imidazole] Na$^+$.

In vitro analysis of these ruthenium complexes show that these complexes interact with DNA and the topoisomerase II in a bi-directional manner resulting in the formation of a TopoII mediated DNA cleavage complex. The formation of such a cleavage complex is the main route for the anti cancer action of the TopoII poisons. Our laboratory synthesized and studied mechanism of organometallic ruthenium derivatives like RuBen (dmso), RuBen Pyr, RuBen Apy, RuBen Agu, RuBen Aba and analyzed for their anti cancer activity. Among these Ruben Apy showed significant topo II poisoning and anti cancer activities. Further to understand the structural component required for topo II poisoning by Ruben Apy, we used these molecules as lead and designed various structural analogues to identify the structural orientation required for Ruben Apy for topo II poisoning. Four compounds in which the amino pyridine group is replaced with pyridine derivatives like pyridine 3-carboxaldehyde, Pyridine 3-sulphonic, 3- hydroxy pyridine and nicotinamide derivatives. These complexes are analyzed for their inhibitory action on topo II catalytic activity and the antiproliferative activity.
Methodologies: the following methods are used in the present study. Ruthenium Organometallic Complexes:

Synthesis of the dimeric starting compound:

This complex was synthesized as previously described by (Zelonka et. al, 1972). Briefly, freshly synthesized 1, 3-cyclohexadiene (6 ml) was added to RuCl<sub>3</sub>. 3H<sub>2</sub>O (1.7 gm) in 100 ml of aqueous ethanol. The solution was maintained at 45 °C for 3 hrs to form a red precipitate which was washed in ethanol and dried in vacuum to give the dimeric complex of [RuCl<sub>2</sub>(C<sub>6</sub>H<sub>6</sub>)<sub>2</sub>]. This dimer was the starting compound for the synthesis of all the complexes of the 'RuBen' type.

**RuBen Pyridine 3-carboxaldehyde**

To the 25 mg RuBen dimer, equimolar concentrations of pyridine 3-carboxaldehyde was added and incubated to get a brick red colored precipitate, which was washed in ethanol and dried in a vacuum drier.

The structure of the complex was confirmed by infrared spectroscopy (spectra 1).

**RuBen Pyridine 3-sulphonic acid**

To 25 Mg of Ruben dimer equimolar concentrations of pyridine 3-sulphonic acid derivative was added and incubated to get a dark green colored precipitate, which was washed with ethanol and vacuum dried.

The structure of the complex was confirmed by infrared spectroscopy (spectra 2).
RuBen 3- hydroxy pyridine

To 25 mg of Ruben dimer equimolar concentrations of 3- hydroxy pyridine derivative was added to get a brown precipitate, which was washed with ethanol and vacuum dried. The structure of the complex was confirmed by infrared spectroscopy (spectra 3).

RuBen nicotinamide

To 25 mg of Ruben dimer equimolar concentrations of nicotinamide derivatives was added to get a brown colored precipitate. The structure of the complex was confirmed by infrared spectroscopy (spectra 4).

Molecular modeling of the Ruthenium derivatives

Structural conformations of the Ruthenium derivatives synthesized were determined by molecular modeling analysis. The models were generated using the PC SPARTAN molecular modeling software (wave function)

SPARTAN graphic interphase was used to generate the 3- dimensional models of the molecules, which were subjected to energy minimization. Optimized structures were used to understand the structural variation between amino pyridine and other derivatives.
Ruthenium Derivatives

Ruben Pyridine-3-Carboxaldehyde

Ruben Pyridine-3-Sulphonic Acid

Ruben-3-Hydroxy Pyridine

Ruben Nicotinamide
Methods:

- Protein estimation was done according to the colorimetric method described by Bradford (1976).
- SDS-PAGE electrophoresis was carried out according to the procedure of Laemmli (1970).
- Silver Staining of the SDS-PAGE protein gels was carried out according to the method of Blum et. al, (1987).
- Western Blotting was done following the procedure of Towbin et. al., (1979).

Purification of pRYG Negatively Supercoiled Plasmid DNA:

The negatively supercoiled pRYG plasmid DNA was purified from the E.coli HB101 strain containing the plasmid, using the alkaline lysis procedure of Wang and Rossman (1994). The procedure described is for a 1 litre culture, which can be scaled up to 4 liters. An overnight culture of the plasmid containing bacteria (grown in the presence of 70 \( \mu \)g/ml ampicillin) was used for purification of the plasmid.

Butters used in the purification:

* Lysis buffer: 50 mM glucose, 25 mM tris-HCl, pH 8.0, 10 mM EDTA and 5 mg/ml lysozyme.
* Alkaline solution: 0.2 N NaOH and 1% SDS.
* 3M Sodium Acetate solutions
* Tris buffer saturated Phenol
* Sodium acetate buffer: 50 mM Tris, 100 mM Sodium acetate
* Tris-EDTA buffer: 10 mM tris-HCl, pH 7.5 and 1mM EDTA.
Procedure:

**Bacterial cell growth and harvesting:**

25 ml of LB broth was inoculated with a single bacterial colony containing the plasmid. The culture was grown in a shaking incubator for 8 h at 36 °C. This culture was used for inoculating 1 liter of LB broth. The 1-liter culture was grown overnight (12 -14 h) at 37 °C in a shaker incubator. The purification procedures were carried out 4 °C.

Cells were harvested by centrifugation at 5000 rpm for 10 min. The cells were lysed with 40 ml of lysis buffer by constant stirring over a period of 15 min.

**Alkaline lysis:**

80 ml of freshly prepared alkaline solution was added and the constituents were mixed by swirling in a bottle. The mixture was placed on ice for 10 min. 50 ml of freshly prepared 3M-sodium acetate solution was added gently against the walls of the bottle. The bottle was placed on ice for 10 min. The precipitated proteins were removed by centrifugation at 12,000 rpm. The supernatant was clarified by filtering it through glass wool. Ice-cold isopropanol (0.7 volume) was added to the supernatant and placed on ice for 1 hour.

**Acidic phenol- chloroform extraction of RNA:**

The precipitated DNA was pelleted at 12,000 rpm. The supernatant was removed and the pellet allowed for drying. This pellet was dissolved in 40ml of sodium acetate buffer solution. After 5 min on ice, equal volumes of Tris saturated phenol and chloroform are added and vortex mixed for 2 min in 50 ml tubes. The tubes were centrifuged at 12,000
rpm for 10 min. The aqueous phase was taken in an autoclaved conical flask and the phenol phase was removed.

**Precipitation and dissolution of DNA:**

The aqueous phase containing the DNA was treated with 0.7 volume of ice-cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 4.2) and placed on ice for 20 min. The DNA was pelleted and washed twice with ice-cold ethanol (70%). The pellet was dissolved in a proper volume of Tris-EDTA buffer.
Agarose gel electrophoresis of pRYG plasmid DNA

**Lane 1** marker DNA
**Lane 2** supercoiled pRYG DNA
**Lane 3** BamH1 digest of pRYG
**Lane 4** Topo II $\alpha$ relaxed DNA
**Lane 5** Topo II $\beta$ relaxed DNA
Purification of Topoisomerase II from rat liver:

Topoisomerase II was purified from rat liver tissue following the procedure of (Galande and Muniyappa 1996). In principle, the procedure involves the isolation of enriched nuclei for minimization of protease action. From the nuclei, topoisomerase II is isolated by polymin P precipitation of chromatin, followed by salt extraction of proteins, ammonium Sulfate precipitation and finally two rounds of gradient elution in a hydroxyapatite column. All the steps were carried out in a cold room at 4 °C.

Buffers used in the purification:

Buffer A: 10 mM Tris-HCl (pH 7.5), 5 mM MgCl\(_2\), 25 mM KCl, 0.34 M sucrose and 0.1 mM PMSF.

Lysis Buffer: 5 mM potassium phosphate (pH 7.5), 100 mM NaCl, 10 mM 2-mercaptoethanol and 0.5 mM PMSF.

PR buffer: 20 mM potassium phosphate (pH 7.5), 10 mM NaHSO\(_3\), 10% glycerol, 10 mM 2-mercaptoethanol and 0.5 mM PMSF.

Storage buffer: 30 mM potassium phosphate (pH 7.5), 50% glycerol, 0.1 mM EDTA and 0.5 mM DTT.

Procedure:

Isolation of enriched nuclei:

400 gm liver from 2 month old rats (wistar strain) was washed twice in ice cold saline, minced thoroughly and homogenized in 2.5 liters of buffer A. The cell-free homogenate was centrifuged at 5000 rpm for 10 min. The pellet suspended in 700 ml of buffer A
containing 2.2 M sucrose and the supernatant was discarded. Enriched nuclei were obtained by ultracentrifugation of the reconstituted pellet at 28,000 \textbf{rpm} for 1 h in a Beckman Ti-70 rotor. The nuclear pellet was washed once at 15,000 rpm with 200 ml of buffer A containing 1 M sucrose followed by 200 ml of buffer A with 0.1\% triton X-100.

\textit{Lysis of Nuclei:}

The nuclear pellet was resuspended in lysis buffer and subjected to lysis in an MSE sonicator with a macroprobe for 4 times, 30 sec. each, with two min intervals.

\textit{Polymin P precipitation:}

10\% Polymin P (pH 7.8) was added slowly to the lysate, while stirring to a final concentration of 0.35\% during a period of 15 min. The precipitate was pelleted at 6000 rpm for 10 min. The Pellet was resuspended in 200 ml of PR buffer. Proteins were extracted from the chromatin-polymin P complex with 0.55 M NaCl, while stirring for 30 min. Nucleic acids were reprecipitated by adding extra polymin P up to concentration of 0.7\% while stirring for 15 min. The nucleic acid precipitate was removed by centrifugation and the supernatant was filtered through glass wool.

\textit{Ammonium Sulfate precipitation and dialysis:}

The clarified supernatant was subjected to ammonium Sulfate (60\%) precipitation with continuous stirring for 1 h. The precipitate was collected by centrifugation at 12,000 rpm for 20 min. The pellet was resuspended in 100 ml of PR buffer and dialyzed against 3 X 1
liters of the same buffer over a period of 15 h. A precipitate formed during the dialysis, which was removed by centrifugation at 26,000 rpm for 20 min.

*Separation of low molecular proteins by Amicon filters.*

The clarified supernatant obtained after ultra centrifugation was taken in Amicon filters with cut off range 100 and centrifuged at 1500 g for 30 minutes at 4°C. The retentate obtained contained the high molecular weight fractions with higher amounts of topoisomerase II.

**Characterization of topoisomerase II:**

The purification profile of topo II is shown in a silver stained SDS-PAGE gel (10%) in The protein was confirmed by western blotting, with a monoclonal antibody against topo II a. Protein concentration was determined by the Bradford method (1976).

Enzyme activity was determined using the relaxation assay described in the ‘Topoisomerase II Activity Assays’ sectiob

**Definition of enzyme activity:** One unit of topo II activity is defined as the minimal amount of the enzyme required to completely relaxing 0.3 \( \mu \)g (0.5 nM base pairs) of negatively supercoiled pBR322 plasmid DNA in the presence of Mg\(^{2+}\) ions, in a specified period of time at 30 °C.
Purification of Topoisomerase II from R=tl\£r

SDS-PAGE analysis
1 2 3 4

Western blot
1 2 3 4

\[ \text{-170 KDa} \]

lane 1 - Nuclear lysate
lane 2 - 60% ammonium sulfate precipitate
lane 3 - Topo II supernatant
lane 4 - Retentate of anion filter
DNA Binding studies:

Thermal denaturation studies of calf thymus DNA was carried out to determine binding affinity of the metal complexes to DNA:

Calf thymus DNA (sodium salt) was dissolved in 1 mM sodium phosphate buffer containing 1 mM sodium chloride. DNA concentration was adjusted to give an absorbance of 1.0 in 1 ml at 260 nm. The metal complexes were added to DNA at concentrations, which gave drug to nucleotide ratios of 1:40, 1:20, 1:10, 1:5 and 1:1 respectively. The samples were incubated in 1 ml quartz cuvettes for 2 minutes to allow drug-DNA interaction. A Hitachi 150-20 spectrophotometer was set to give a 1 °C rise in temperature per minute with a KPC-6 thermo-programmer and SPR-7 temperature controller. Increase in absorbance at 260 nm was recorded from 40 to 90 °C. $T_m$ was determined from the denaturation curves and the data was plotted.

Topoisomerase 11 activity assays to determine molecular mechanism of action of the metal complexes:

DNA relaxation assay:

Relaxation of the supercoiled DNA occurs in a stepwise manner. Each DNA relaxation step of topo II involves a change in the linking number of DNA by 2. Thus, in an incomplete reaction, the supercoiled DNA band (form S of DNA) disappears and a ladder of bands (each band differing in linking number by 2 from its successive band) in different stages of relaxation appear. Complete relaxation of the plasmid DNA can be visualized as a single band (form R) of DNA. This is because; all the bands in the ladder
are partially relaxed into form II DNA. In the present study, an incomplete relaxation reaction has been employed for the drug assays.

This assay was performed following the procedure of Osheroff et al. (1983). The reaction mixture (50 μL) contained relaxation buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM MgCl$_2$, 30 μg/mL BSA, 1 mM ATP), 0.6 μg of negatively supercoiled pRYG plasmid DNA and increasing concentrations of the RuBen drugs. The reaction was initiated by adding 2 units (~8 nmole) of topo II and incubated at 30 °C for 15 min. The reaction was stopped by adding 2 μL of 10% SDS. To this, 3 μL of loading dye (0.5% bromo-phenol blue, 0.5% xylene cyanol, 60% sucrose, 10 mM tris-HCl, pH 8.0) was added, and the products were separated on a 1% agarose gel in 0.5x TAE buffer (20 mM tris-acetate, 0.5 mM EDTA) at 50 V for 8 h. The gel was stained with ethidium bromide, visualized using a Photodyne UV transilluminator and photographed.

**DNA Cleavage Assay:**

In presence of a topo II poison, a ternary 'cleavage complex' consisting of 'cleaved DNA-drug-topo IF is formed. The formation of this complex is confirmed by treating the reaction products of the cleavage assay with SDS. This detergent treatment denatures the enzyme, thus liberating the cleaved DNA. Proteinase K treatment completely disrupts the associated enzyme with DNA. This cleaved DNA is visualized as form L (linear plasmid DNA) in an agarose gel.

The formation of cleavage complex was assayed following the procedure of Zechiedrich et al (1989). The 50 μL reaction mixture contained relaxation buffer (minus ATP), 0.6 μg
of pRYG supercoiled DNA and increasing concentrations of drugs. The reaction was initiated by adding 10 units (40 nmol) of topo II and incubated at 30 °C for 15 min. The reaction was stopped with 2 μL of 0.5 M EDTA and 2 μL of 10% SDS. The DNA bound protein was degraded by incubating the reaction mixture with 2 μL of 1 mg/ml Proteinase K at 45 °C for 1 h. The products were separated on 1% agarose gel for 8 h at 50 V in 1x TAE buffer (40 mM tris-acetate, 1 mM EDTA), stained and photographed. The linear DNA band was quantified in terms of percentage of total DNA in a UVP gel documentation system.

Anticancer Activity assay:

[3H]-Thymidine incorporation by proliferating cells was the assay used for determining the in vitro anticancer activity of the RuBen drugs. Colo-205 (colon adeno-carcinoma), cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (A-431 cells were grown in DMEM with 10% serum. 0.2 x 10^6 cells/200 μL were distributed in triplicates in 96 well microtiter tissue culture plates. The cultures were incubated for 16 hours at 37 °C in a CO2 incubator (Forma Scientific) maintaining 5% CO2 atmosphere. Increasing concentrations of the drugs were added to the cells in culture. The DNA intercalating topo II poison, m-AMSA, was used as a positive drug control (positive and negative controls always contained an equal amount of DMSO present in the drug treated samples). The drug treatment was stopped after 6 h by centrifugation and change of media. After this, the cultures were further incubated for 48 h. The cultures were then pulsed with 0.5 μCi of ^3H-thymidine and incubation was continued for 4 h to allow thymidine incorporation by the proliferating cells. In the adhering cell cultures Colo-205
the medium was removed and the adhering cells were treated with 10 μL of trypsin-EDTA (0.25% trypsin, 1mM EDTA) for 5 min at 37 °C to release the cells from the adhering surface. Trypsinization was stopped by adding 10 μL of serum to the cells. The original cultures were then added back to the wells and the cells were harvested as described above. Radioactivity was measured in a Wallac liquid scintillation counter. The mean result of three independent experiments conducted in triplicates was plotted as drug concentration versus percentage of proliferation.
Results:

The observations of the experiments performed in the presence of the ruthenium derivatives will be described as below. Various experiments revealing the topoisomerase II inhibition, DNA binding nature and antiproliferative nature of these derivatives was conducted as follows.

Topoisomerase II inhibition by ruthenium derivatives.

Relaxation assay:

The inhibition of the topoisomerase II mediated relaxation activity by the ruthenium derivatives is monitored in the relaxation assay. Increasing concentration of Ruthenium derivatives are used in this assay. The known Topoisomerase II inhibitor mAMSA used in this assay showed complete inhibition of the topoisomerase II activity at a concentration of 100μM. Various Ruthenium derivatives were tested for their topo II inhibitory activity, the results show that the pyridine 3-carboxaldehyde derivative showed complete inhibition of the relaxation activity at a concentration of 400 μM (figure 28 panel A), which is 4 times more than the standard inhibitor that is mAMSA. Pyridine 3-sulphonic acid showed partial inhibition at a concentration of 500μM (figure 28 panel B) with no further inhibition at a concentration of 600 μM. The nicotinamide derivative showed complete inhibition of relaxation activity at 400 μM concentration. (Figure 28 panel B) Where as the 3-hydroxy pyridine derivative showed partial inhibition only at 500μM concentration (figure 26 panel B).
Cleavage assay:
Topoisomerase II mediated cleavage of DNA double strand and the formation of the formation of enzyme -drug- DNA ternary complex is observed by the cleavage assay. In the presence of topoisomerase II inhibitors the enzyme interacts with the DNA and cleaves it, but fails to religate the double stranded break. The enzyme-inhibiting drug freezes the enzyme and the DNA in the ternary cleavage complex. The cleaved DNA in the cleavage complex is released by SDS and proteinase K treatment. The circular supercoiled plasmid DNA used in this assay is linearized and appears as the third nicked DNA band. This linear DNA is a measure of the cleavage complex formed by the topoisomerase II Inhibitors. The cleavage assay was carried out at increasing concentration of ruthenium derivatives. The results of the assay carried out in the presence of pyridine 3-carboxaldehyde showed that this derivative formed cleavage complex formation at a concentration of 300μM with a slight increase in the intensity of the nicked DNA band at a concentration of 400 and 500μM (Figure 29 panel A), the pyridine 3- sulphonic acid derivative showed cleavage complex formation at a concentration of 500μM (Figure 29 panel B), the 3- hydroxy pyridine derivative showed the nicked DNA in the cleavage assay with a drug concentration of 300μM, with an increase in the nicked DNA with increase in the drug concentration. The nicotinamide derivative showed the cleaved or nicked DNA at a concentration of 400 and 500μM, these results correlated with the drug concentrations at which the drugs showed inhibition towards the relaxation activity.
DNA binding of Ruthenium derivatives:

The DNA binding nature of the ruthenium derivatives was studied by the monitoring the melting of calf thymus DNA in the presence of ruthenium drugs. The melting temperature in the presence of drugs (at 1:1 concentration) showed an increase. The calf thymus DNA in the absence of the drug showed a Tm value of 67 °C whereas in the presence of mAMSA, which is a DNA intercalating agent showed an increase of 8°C with a Tm value of 74 °C. The ruthenium derivatives also showed an increase in the Tm value indicating the DNA binding nature of these drugs (figure 30). In the presence of Ruben pyridine carboxaldehyde the Tm value was found to be 71, RuBen pyridine 3-sulphonic acid showed a Tm of 73, Ruben 3-hydroxy pyridine showed a Tm of 75 and in the presence of Ruben nicotinamide the Tm was found to be 78. The change in the Tm values indicated that these compounds have DNA binding ability.

Anti proliferative MTT assay:

MTT assay was carried out to check if the ruthenium derivatives show anti proliferative or cytotoxic activity. Colo-205 cells which are stable in terms of proliferation and reproducibility of drug assays were used. The results of this assay suggest that the RuBen pyridine 3-carboxaldehyde, RuBen Pyridine 3-sulphonic acid, RuBen 3-hydroxy pyridine and Ruben nicotinamide show inhibition almost 90-100% inhibition of the proliferation of the Colo cancer cells at concentrations ranging from 300-350μM (figure 31).
Molecular modeling analysis:

The modeling was done using SPARTAN software. The molecular models show that the coordinated groups are in different structural orientation. The different orientation of these groups may contribute to their interaction with the enzyme. The group pyridine 3-carboxaldehyde show an orientation which is coplanar with the organomettalic bond and these complexes have shown inhibition of topo II activity at lower concentration when compared to pyridine 3- sulphonic acid, nicotinamide and 3- hydroxy pyridine in which the groups are away from the organomettalic bond of Ruthenium atom (figure 32).
Discussion:

Topoisomerase II is a major nuclear enzyme that maintains the DNA topology in the complex chromosomal environment. The enzyme resolves numerous torsional problems in the DNA, which arise during replication and transcription. It also helps in catenation / decatenation, condensation/ decondensation of DNA and segregation of chromosomes during cell division. The enzyme performs these functions by nicking a segment of DNA passing second strand through a gate formed by the nicked DNA and finally rejoining the nicked segment. This DNA breaking activity of topoisomerase II results in loss of genetic integrity and therefore is the target for anticancer drugs. These Topoisomerase II drugs inhibit the catalytic activity of the enzyme by enhancing the cleavage complex formation and block the religation activity. Cancer cells, which over express topoisomerase II when treated with the inhibitors will harbour numerous Topoisomerase II, induced DNA breaks formation. The damaged DNA Stimulates repair and recombination pathways leading to sister chromatid exchange, large insertions/ deletions translocations and chromosomal aberrations. The accumulation of these aberrations triggers a series of events, which ultimately result in cell death by apoptosis and necrosis.

The present study show that ruthenium compounds can be attributed the anticancer activity because of their topoisomerase II inhibition. Earlier studies on the ruthenium compounds like the RuBen (dms0) and derivatives have implicated this molecule as a potent anticancer drug, whose molecular target is topoisomerase II. This particular molecule served as a parent molecule for the development of potent derivatives in which the topoisomerase II interacting DMSO group will be replaced by the other ligands. Further studies in this area lead to the development of derivatives of ruthenium in which
the dmso group is substituted with pyridine, 3-aminopyridine, aminobenzoioc acid or aminogaunidine. These four molecules showed DNA binding capacity as well as Topoisomerase II inhibition at concentrations less than the RuBen (dmso) derivative. These derivatives showed potential anticancer activity also. Among these derivatives the amino group containing amino pyridine, amino guanidine and amino benzoic acid, showing that the amino group may be responsible for interaction with Topoisomerase II. [Vashisht Gopal et al 2002]. These studies showed that the spatial orientation shown by the single amino group containing RuBenApy made it a potent inhibitor for Topoisomerase II. The conformation and the special orientation of the interacting amino group is responsible for the cleavage complex formation. The Ruthenium atom in the complex binds to the DNA and the ligand binds to the topoisomerase II.

It was previously suggested that in aqueous solution the chlorides and the dmso groups on the RuBen dmso hydrolyze and the complex exists in an equilibrium between the aqua and the chloro ions as the Ru \((C_6H_6)(H2O)\). Hydrolysis of the chloride leaving groups enables the interaction of the Ruthenium atom with nucleotide phosphate of the DNA double strand. Long-lived DNA/ protein interaction and eventual hydrolysis of the Ruthenium complex, which leads to the formation of the covalent cross-links. Hydrolysis of the coordinated groups is an important determinant of the macromolecular interaction, by theses complexes.

In the present study we have made derivatives of ruthenium benzene in which the amino pyridine group is replaced with substituted pyridine with the carboxaldehyde, hydroxy, suphonic acid and nicotinamide groups. The DNA binding studies reveal that the derivatives have strong DNA binding affinity. All the ruthenium derivatives showed
similar DNA binding affinity suggesting that the ruthenium atom interacts with the DNA. Their interaction with DNA may be ionic bonding with the phosphate backbone or covalent binding of nucleotide without disturbing the helix. The ruthenium derivatives do not show an intercalative mode of DNA binding.

The results of the Topoisomerase II inhibition experiments help in proposing a probable mechanism for the inhibitory activity of the ruthenium derivatives. The metal atom interacts with covalently or non-covalently with DNA nucleotides and the ligands form cross links with the enzyme and prevent DNA religation leading to the formation of stable drug induced cleavage, which is a characteristic of the topoisomerase II inhibitors. Such topoisomerase II inhibitors forming the cleavage complex formation will harbor the DNA double strand breaks. The accumulation of such double strand breaks in the cells ultimately leads to the cell death by apoptosis/necrosis.

The molecular modeling analysis shows that the complexes with groups, which are oriented in plain with the organomettalic bond of the ruthenium atom, show higher topo II inhibition when compared to the complexes with groups going away from the organomettalic bond. The interaction of the metal complexes with topo II enzyme could be determined by the orientation of the groups in the complexes.
Conclusions

• The Topoisomerase II isoforms shows significant changes in rat brain tissue. The Topoisomerase II a is found to be negligible in rat brain, where as the β isoform is predominantly present. Topoisomerase II β is expressed at different levels in various regions of brain showing the highest level of expression and activity in the cerebellar region. This observation is confirmed by immunohistochemical analysis. An age dependent change is observed in the levels of Topoisomerase II P with maximum protein seen in the young rats. Among the two cell types studied The cerebellar neurons showed the highest activity when compared to the astrocytes. Topo II β activity in cerebellar neurons shown to be decreased with increasing age.

• The activities of the topoisomerase II a and β have shown variation in different tissues with the a isoform showing negligible activity in the brain tissues of young adult and old rats. In Liver and testes tissues the activity is found to be high indicating higher level of the enzyme in these tissues. In liver and testes the enzyme showed age dependent variation with highest activity seen in the young and adult testes tissues. Where as in the liver there is increase in the activity with age. The topoisomerase II β activity is found to be high in all the three tissues with variation seen in the brain tissue with age. As reported earlier the young extracts of brain have shown highest activity of topoisomerase II p. There was a slight decrease in the activity of topoisomerase II β in the adult rats when compared to young and old rats. No much change is seen in the testes extracts of
the three age groups for the topoisomerase II β activity. Topo II α shows high sensitivity towards Topo II poisons than the topoII β.

- RuBen Pyridine 3-carboxaldehyde indicate that this particular derivative inhibit the DNA relaxation activity of topoisomerase II at a concentration of 400 and 500 μM. The Ruben pyridine 3- sulphonic acid showed inhibition of the relaxation activity at a concentration of 500 μM with no increase in the inhibition at higher concentrations. The Ruben 3- hydroxy pyridine showed inhibition of the relaxation activity at a concentration of 400 μM with no further increase of inhibition seen at higher concentrations. The RuBen nicotinamide derivative showed inhibition of the relaxation activity at a concentration of 500 μM. Cleavage activity was also shown by the complexes at concentrations similar to that of the concentrations at which they show inhibition of the relaxation activity. These derivatives have shown DNA binding property by changing the melting temperature from 67 to 70, 71,75,78 by the Ruben pyridine3- carboxaldehyde, RuBen pyridine 3- sulphonic acid, RuBen 3- hydroxy pyridine, Ruben nicotinamide. The derivatives have shown antiproliferative activity at concentrations of 300- 350 μM with Ruben pyridine3- carboxaldehyde, Ruben pyridine 3- sulphonic acid, Ruben 3- hydroxy pyridine, RuBen nicotinamide respectively. Analysis of Topo II poisoning by RuBenPy derivatives, show that the interaction group of Pyridine should be in plane with ruthenium atom so as to allow its interaction with the enzyme.
Figure 28

Relaxation assay in presence of ruthenium derivatives;

~0.6 mg of supercoiled DNA (lane 1) was incubated with Topo II in absence (lane 2) or presence of 100μM mAMSA (lane 3) of 100, 200, 300, 400 and 500 μM RuBenCHO (panel A lane 4-8), RuBen SA (panel A lane 9-13), RuBen OH (panel B lane 4-8), RuBen NA (panel B lane 9-13). The products were resolved on 1% agarose gel, ethidium bromide stained and photographed.
Figure 28

A

B
Figure 29

Cleavage assay in presence of ruthenium derivatives:
Cleavage assay was performed by incubation supercoiled DNA with topo II in absence (lane 2) or presence of 100μM mAMSA (lane 3) of 100,200,300, 400 and 500 μM RuBenCHO (panel A lane 4-8), RuBen SA (panel A lane 9-13), RuBen OH (panel B lane 4-8), RuBen NA (panel B lane 9-13). The products were resolved on 1% agarose gel, ethidium bromide stained and photographed.
Figure 30

Tm analysis was carried out with the ruthenium derivatives and the values obtained are graphically represented in the figure. The changes in the Tm values are clearly seen with the shift in the peak.
Figure 30

Melting temperature studies
Figure 31
The anticancer activity of Ruben CHO, Ruben SA, Ruben oh and Ruben NA was examined using MTT assay on colon cancer cells. The results of the study show the antiproliferative potency of the ruthenium derivatives. The highest potency is shown by mAMSA.
Figure 31

MTT assay

% inhibition of proliferation vs Drug concentration (μM)

- ● mAMSA
- ○ RuBen pyridine 3-carboxaldehyde
- ▼ RuBen pyridine 3- sulphonic acid
- ▲ RuBen 3- hydroxy pyridine
- ■ RuBen nicotinamide
Figure 32

Ruthenium (benzene)Cl 2-Coordinated with

Nicotinamide 50uM

Pyridine 40uM

Pyridine 40uM

Pyridine DMSO \(^{(\ast)}\) 50uM

Pyridine 30uM

Pyridine \(\text{H}^{(\ast\ast)}\) 25uM
Figure 32
The molecular models of Ruben NA, Ruben DMSO, Ruben Apy, Ruben CHO, Ruben OH and Ruben SA show the 3-dimensional orientation of the groups around the organometallic bond of ruthenium. The angular orientation may be responsible for the complexes to intact with the enzyme and align them in to the active site.