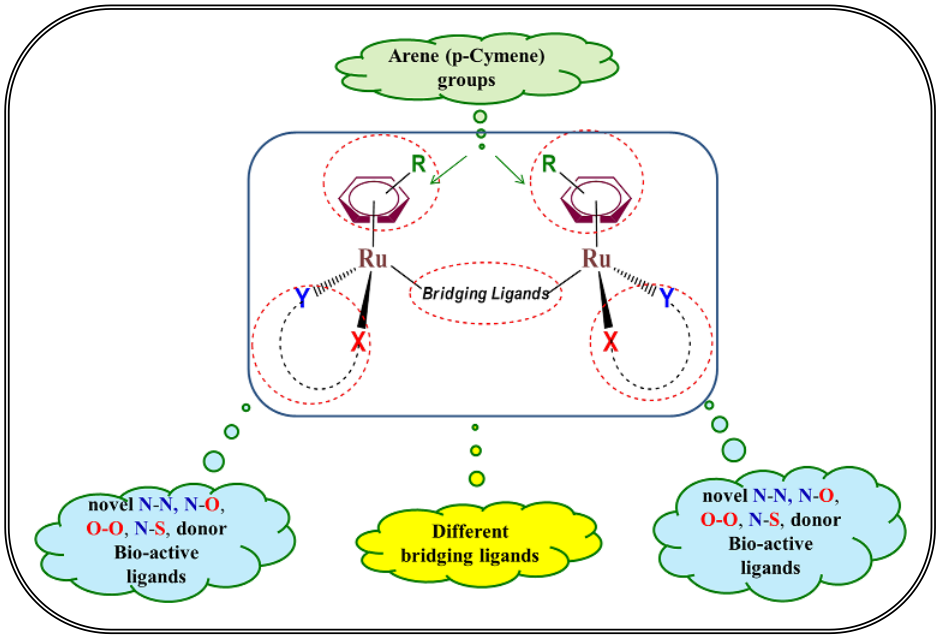
Synopsis of thesis entitled

"STUDIES ON ORGANOMETALLIC RUTHENIUM BASED BINUCLEAR COMPLEXES: SYNTHESIS AND EVALUATION OF THEIR BIOLOGICAL ACTIVITY"



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The Maharaja Sayajirao University of Baroda
For the Degree of

DOCTOR OF PHILOSOPHY
In
CHEMISTRY

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Content

Chapter-1 Introduction

Bio-organometallic chemistry is an emerging new discipline in chemical biology, and organometallic compounds are gaining continued importance in pharmaceuticals, as bio probe, tracers in immunoassay and biosensors. Design and evaluation of organometallic compounds as anticancer agents constitute an important area of research, particularly to target both primary and metastatic secondary tumors. Transition metal based organometallic compounds have excellent biological activities and such compounds are potent therapeutics for cancer, and as radiopharmaceuticals and antibiotics. The two major groups of organometallic complexes metallocenes and metal-arenes have been investigated for anticancer agents [1].

Ruthenium in Medicine:-

Medicinal inorganic chemistry is a field of increasing prominence as metal-based compounds offer possibilities for the design of therapeutic agents not readily available to organic compounds [2].

1. **Geometry**

   The possibility to occupy a high number of spatial positions with up to 30 stereoisomers due to their expanded set of octahedral coordination geometry. High number of potential accessory molecules that can be carried by the drug structure.

2. **Ligand Exchange**

   Ru (II) and Ru (III) complexes having similar ligand exchange kinetics to those compared with Pt (II) complexes are being investigated as potential anticancer drugs.

3. **Iron Mimic**

   Ruthenium has ability to mimic iron in binding to many biomolecules, including serum transferrin and albumin.

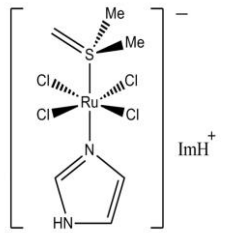
4. **Oxidation states**

   The oxidation states Ru (II), Ru (III) and Ru (IV) are all accessible under physiological conditions. Many Ru complexes with oxidation number (II) and (III) display anti-tumor activity, especially against metastatic stage.

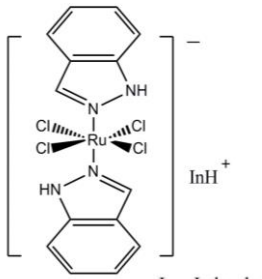
Ru complexes under clinical trials:-

In the last 35 years, basically three main classes of active Ru compounds (i.e. compounds that have demonstrated effectiveness *in vivo* against animal models or transplanted human tumors) have been discovered [3-5]:

Ru (III)-dmso compounds **Keppler-type Ru(III) complexes** **Organometallic Ru(II)-arene Compound**



NAMI - A



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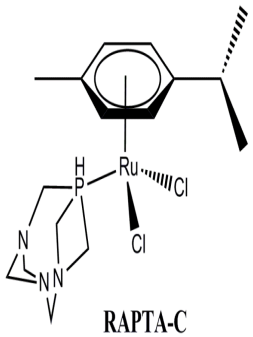
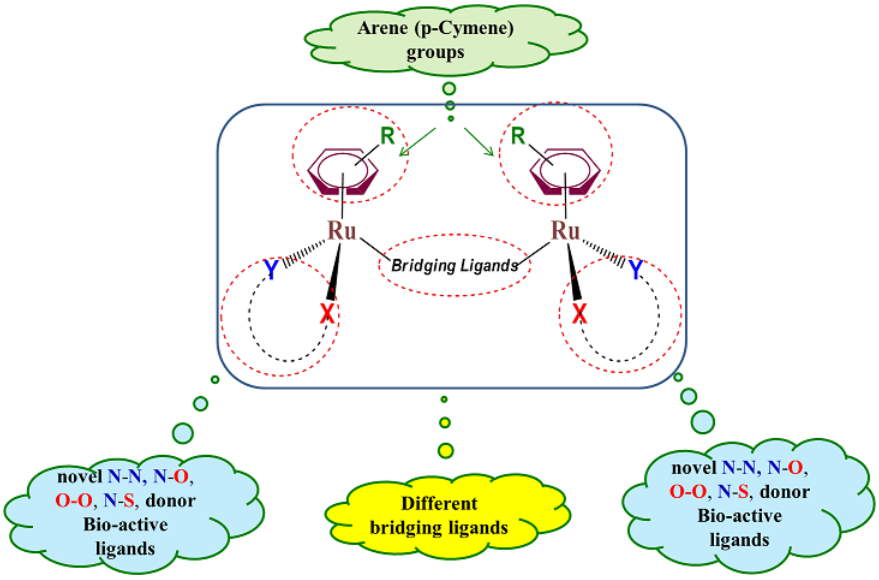


RAPTA-C

Importance of Binuclear compounds:-

Binuclear complexes offer considerable advantages compared with corresponding mononuclear complexes as probes for structural recognition of DNA. Mononuclear metal complexes are limited by their relatively small size, at best spanning only 4–6 base-pairs; typically they only have a footprint of two bases. To approaches the selectivity of nucleic acid-binding proteins, larger species such as di-, tri- and oligonuclear metal complexes are required. Binuclear complexes have a greater stereo chemical diversity than their mononuclear analogues, as the presence of two metal centres amplifies any possible chiral discrimination effects allowing them to more effectively probe the shape- and structure-recognition characteristics of nucleic acids than their mononuclear counterparts. Finally, binuclear complexes generally possess a larger cationic charge and a greater number of hydrophobic ligands than their corresponding mononuclear complex, hence should bind DNA with greater affinity and are more efficient at photosensitising DNA strand breaks than the mononuclear analogues [6].

➢ Synthetic & Investigative Objectives



➢ To synthesized and characterize bio-active ligands having different doner site N-N, N-O, O-O and N-S.

➢ To synthesized and characterize homo-binuclear Ru(II) arene complexes of *p*-cymene using bio active ligands and 4,4'- azopyridine / imidazole as bridging ligands.

➢ To study DNA and BSA binding efficacies of the synthesizes compounds employing UV-Vis and fluorescence spectroscopy

➢ In-cellulo cytotoxicity evaluation using MTT assay on HeLa i.e. human cervical cancer cell line and calculating the corresponding IC_{50} values of all the complexes

Chapter-2 Synthesis and characterization of bioactive ligands

1. Diphenyl pyrazol thiosemicarbazones:

Pyrazole has been the topic of interest for thousands of researchers across the world because of its wide spectrum pharmacological activities. Various structural modifications of pyrazole nucleus have been made to explore its characteristics and biological potential.Pyrazole analogs target various receptors such as protein kinase inhibitor, tyrosine kinase, tumor growth factor (TGF), cyclin dependent kinase (CDK) and fibroblast growth factor (FGF), which are significant for the management of cancer [7]. As shown in fig-1 these are the receptors targeted by pyrazole derivatives.

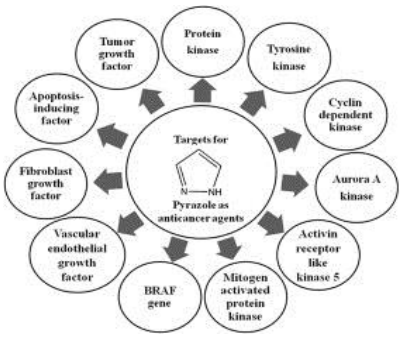
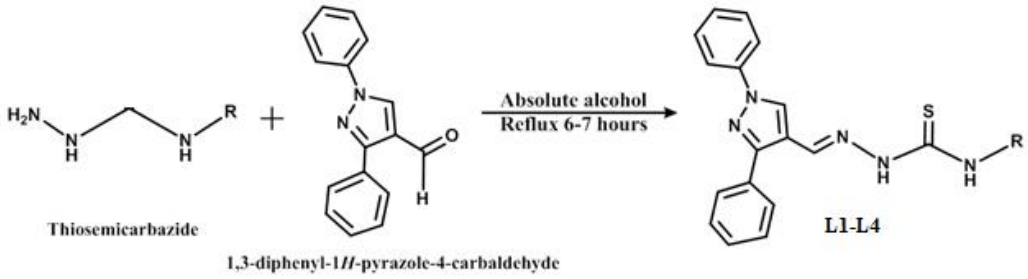


Figure: 1 List of Receptors targeted by Pyrazole derivatives

Synthetic Scheme

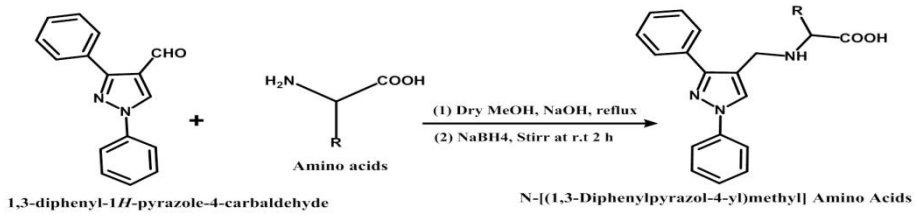


-R = -H **(L1)**, -CH_3 **(L2),** -C_6H_5 **(L3),** -$C_{10}H_7$ **(L4)**

2. Diphenylpyrazol α-Amino Acids derivatives:

Arylpyrazole derivatives play an important role in biologically active compounds and therefore represent an interesting template for medicinal chemistry. These compounds displayed diverse biological properties such as antiparasitic, antifungal, antibacterial and antidiabetic. In the research for antitumor agents, arylpyrazole derivatives exhibited promising antiproliferative properties against several kinds of human tumor cell lines [8].

Synthetic Scheme

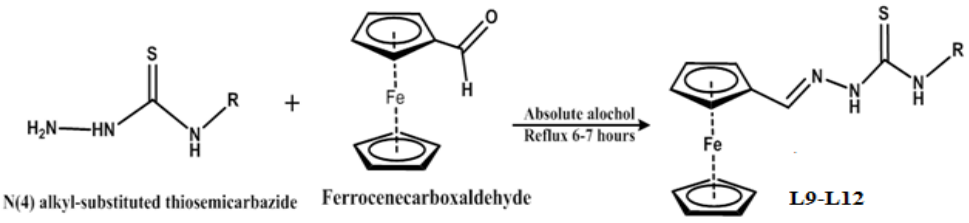


R = -CH_2 (4-hydroxyphenyl) **Fc-Tyr (L5)**;-CH_2Ph**Fc-Phe (L6)**; -$CH_2CH(CH_3)_2$**Fc-Leu (L7)**; -CH_2(3-indolyl)**Fc-Trp (L8)**

3. Ferrocenyl thiosemicarbazones:

Metal thiosemicarbazonate complexes are potential anticancer and chemotherapeutic agents which exhibit inhibitory activities against most of the cancers through inhibition of a crucial enzyme obligatory for DNA biosynthesis and cell division, viz. ribonucleotide diphosphate reductase (RDR). Thiosemicarbazones increase their antitumour activity by their ability to form chelates with specific metal ions. The anticancer activities of thiosemicarbazones are closely related to the parent aldehyde or ketone group, metal chelation ability and terminal amino substitution. Heterocyclic thiosemicarbazone show higher activity compared with aromatic thiosemicarbazones [9]**.**

Synthetic Scheme



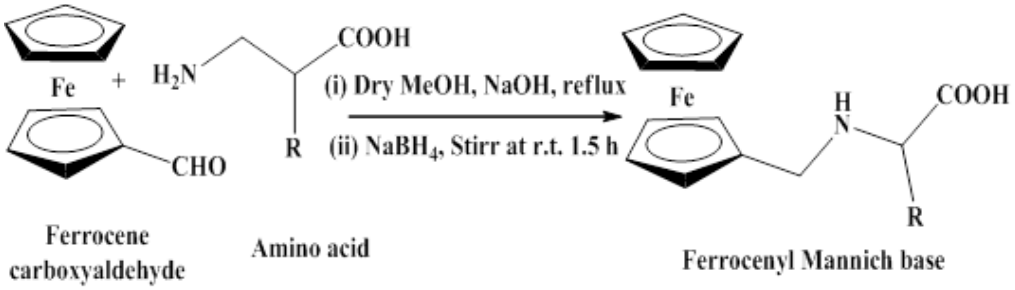
-R = -H **(L9)**, -CH_3 **(L10),** -C_6H_5 **(L11),** -$C_{10}H_7$ **(L12)**

4. Ferrocene mannich bases:

Many researchers have shown interest in design of unnatural ferrocenyl amino acids as shown below and peptides which further have been studied for their biomedical applications. Modification of proteins by incorporating such unnatural ferrocenyl amino acids helps the study of protein structure, activity and interaction with other biomolecules.These ferrocenyl amino acids have been targeted as it has been shown that tethering biologically active groups

to the ferrocenyl unit increases their potency, possibly due to the combined action of the organic molecule with the Fe-centre [10].

Synthetic Scheme:



R = -CH_2 (4-hydroxyphenyl) **Fc-Tyr (L13)**;-CH_2Ph**Fc-Phe (L14)**; -CH_2CH $(CH_3)_2$**Fc-Leu (L15)**; -CH_2(3-indolyl)**Fc-Trp (L16)**

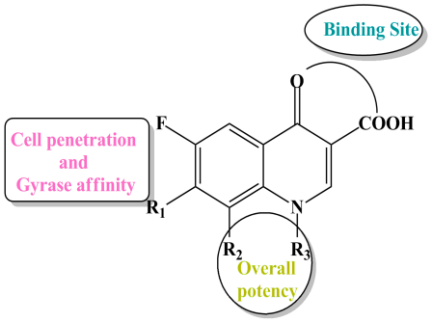
5. Fluoroquinolones:

Largely growing and most widely used group of antibacterials in the field of antibacterial chemotherapy, in the past few years. Inhibition of bacterial DNA Gyrase (Topoisomerase II) and topoisomerase IV which are important in DNA replication. Certain key advantages of fluoroquinolone therapy:

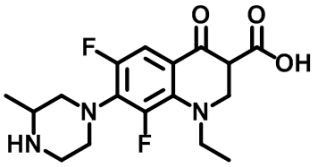
-facile penetration into cellular fluids

-attainment of higher concentration in the cell than serum levels

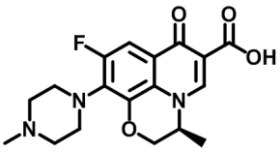
-concentration of fluoroquinolones in the lung reaches around 4-fold higher than serum levels Metal complexation plays an important role in the anticancer activities of quinolone compounds and the topic has been reviewed extensively by Turel. Some metal based fluoroquinolones have shown antitumor activity in cancer cell lines as well as in animal models [11,12].



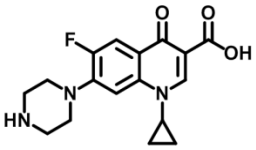
Looking at the anti-proliferative activity of the fluoroquinolones, we proposed to incorporate this series of ligand into our study. Here we took four different fluoroquinolones which were procured as generous gifts from local pharmaceutical companies with 99% HPLC purity**.**



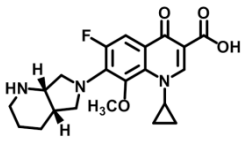
Lemofloxacin (L17)



Levofloxacin (L18)



Ciprofloxacin (L19)



Moxifloxacin (L20)

The characterisation of the ligands was done using **FT-IR, ESI-Mass, UV, ^{1}H NMR and ^{13}C NMR.**

Chapter 3: Synthesis and characterization of Binuclear Ru (II) arene complexes

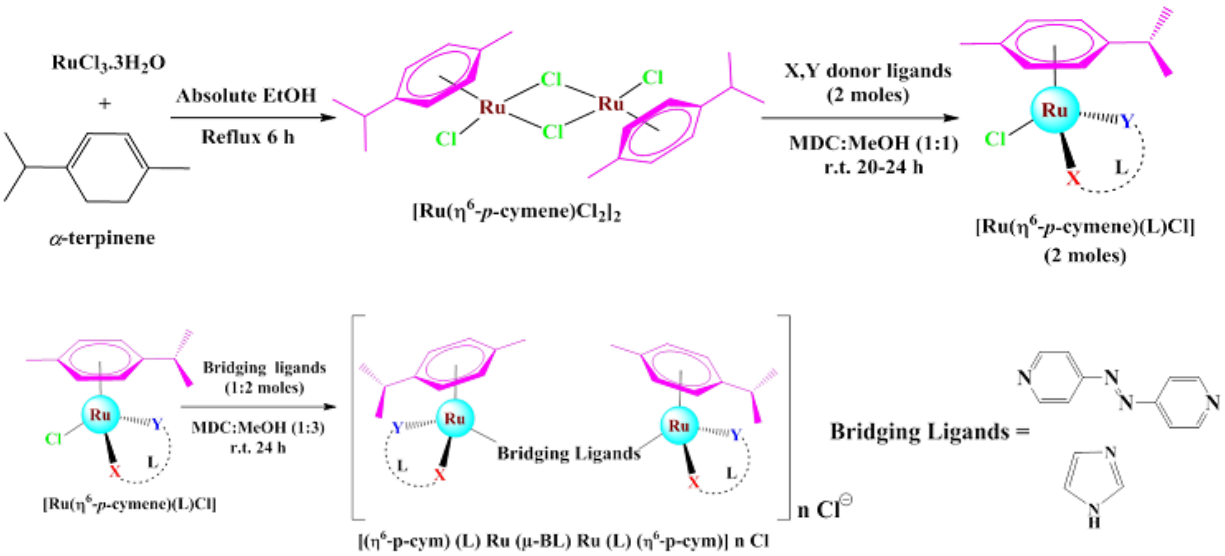
- **Cytotoxic Ru(II) arene complexes**

Ru^{II} arene complexes as potential anticancer agents has been explored they often possess good aqueous solubility and the arene ligand is relatively inert towards displacement under physiological conditions. Form adducts with nucleosides and nucleotides, forming Mononuclear or di-, tri-, and tetranuclear complexes.The discovery of the biological activity of organoruthenium complexes of the type [(η^6- arene)Ru(X)(Y)(Z)]*n*+ (where X and Y are either monodentate ligands or if they are linked, XY represents a chelating ligand; and Z is a leaving group) has opened up a new route towards the design and synthesis of novel anticancer agents. The aquation, particularly if chloride is the leaving group, is largely suppressed in extracellular fluids where high chloride concentrations are found (100 mM), whereas in the cell cytoplasm and nucleus where the chloride concentrations are lower (25 and 4 mM, respectively), the complex is predominantly in the reactive aqua form.Within the cell nucleus it is believed that these complexes bind to DNA forming monofunctional adducts with a high affinity for *N7* of guanine bases (G), as opposed to the bifunctional adducts formed by cisplatin.

- **<u>General synthesis of complexes: [(p-cym) (L) Ru (μ-Az) Ru (L) (p-cym)] n Cl^-</u>**

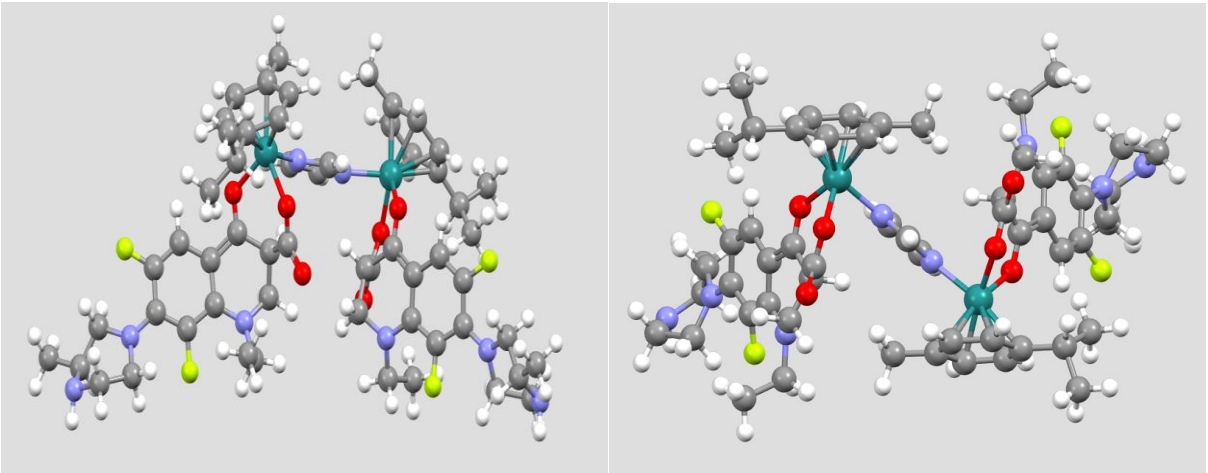
Complexes of general formula[(p-cym) (L) Ru (μ-L) Ru (p-cym)](Cl) were prepared by a typical μ-chlorido-bridge splitting reaction of [Ru(η^6-*p*-cymene)Cl_2]$_2$. To a solution of [Ru(η^6-*p*-cymene)Cl_2]$_2$ (in 2.5 ml CH_2Cl_2), the synthesized ligand **L** (in 2.5 ml methanol) was added on stirring in 1:2 mole ratio respectively. The reaction mixture was left on stirring for overnight

(20-24 h) at room temperature and then for slow evaporation. The resultant crystalline solid was then filtered, washed with pet ether and CH_2Cl_2 and dried in oven at 40°C for 1 h. The mononuclear complexes so obtained were recrystallized from dichloromethane and ether which resulted in reddish brown crystals. For the synthesis of binuclear complexes, the bridging ligand (L'= imidazole / 4,4'bis azopyridine) was added to the respective mononuclear complex in 2:1 mole ratio using MDC:MeOH (1:2) as a solvent. The reaction mixture was stirred for 22hr at r.t. The resultant reddish brown crystalline solid was then filtered, washed with pet ether and CH_2Cl_2 and dried in oven at 40°C for 1 h. The complexes so obtained were recrystallized from dichloromethane and ether which resulted in reddish brown crystals. The two step synthesis procedure is shown in **fig.** given below:



X =	Y =	Donor Ligand
N	S	Diphenylpyrazol thiosemicarbazide **(C1-C8)**
N	O	Diphenylpyrazol α-Amino Acids **(C9-C16)**
N	S	Ferrocenyl thiosemicarbazones **(C17-C24)**
N	O	Ferrocenyl amino acid mannich bases **(C25-C32)**
O	O	Fluoroquinolones **(C33-C40)**

The characterisation of the complexes was done using **FT-IR, ESI-Mass, UV, molar conductance and CHN analysis and DFT calculations to elucidate their structures.**

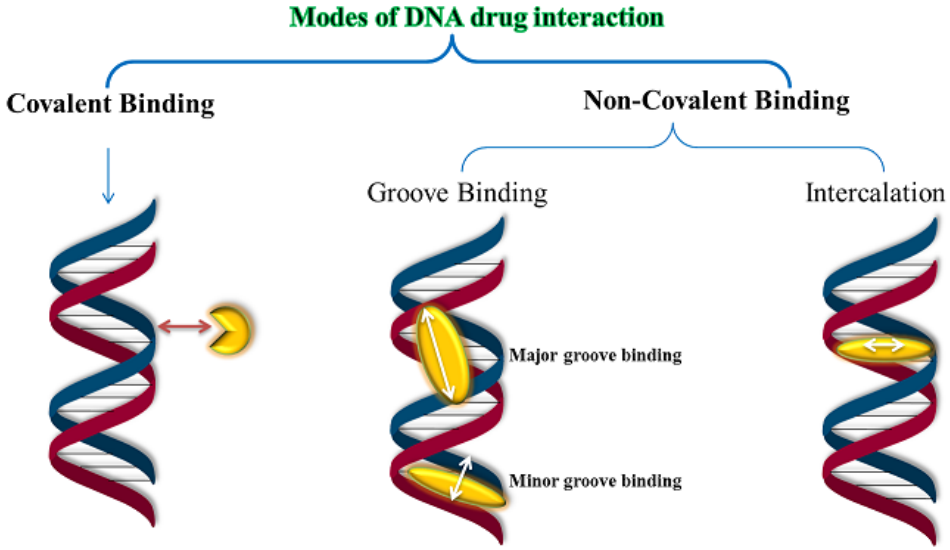


Chapter 4: *In-vitro* bioactivity of the Binuclear Ru (II) arene complexes

- In-vitro studies (outside cellular environment)
  - DNA binding studies
  - BSA binding studies

1. DNA binding studies

DNA as a cellular target Activation or inhibition of DNA function is required to cure or control a disease making it an important target in drug design [11].



Binding strength: covalent binding > Intercalation > groove binding > external binding

The Biological activities of both the series were carried out using UV-Vis and fluorescence spectroscopy.

- **DNA binding studies:** The DNA binding studies were carried employing viscosity measurements, UV-Vis and fluorescence spectroscopy.

The binding constant ($\mathbf{K_b}$) is calculated using Mehan's Equation [12] given below:

$$\mathbf{[DNA]/(\varepsilon_A-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f) + 1/K_b(\varepsilon_b-\varepsilon_f)}$$

K_b = binding constant

[DNA] = Concentration of DNA in base pair.

$\varepsilon_A = A_{obsb}/[compound]$

ε_f = Extinction co-efficient for the unbounded compounds

ε_b = Extinction co-efficient for the bounded compounds.

The Stern – Volmer quenching constant ($\mathbf{K_{sv}}$) [13] obtained from DNA-EB fluorescence quenching studies is calculated using the equation,

$$\mathbf{Io/I = 1 + K_{sv}[Q]}$$

K_{sv} = Stern – Volmer quenching constant

Io = fluorescence intensity in absence of quencher

I = fluorescence intensity in presence of quencher

[Q] = Conentration of quencher

❖ General conclusions:

- The nature of the plots (hypochromism) and viscosity measurements indicate that the compounds bind to DNA through non-covalent interactions (intercalation and hydrophobic interactions).
- Weak electrostatic interactions with the cationic complexes may also be present.
- The DNA binding constants (K_b) and the Stern-Volmer quenching constants (Ksv) for the ligands are in the range of 10^3 -10^4 M^{-1} whereas the K_b and Ksv values for the complexes are in the range 10^3 - 10^5 M^{-1}.
- In most of the cases, complexation of the ligands to the Ru(II) metal centres enhances their DNA binding efficacies.
- Nature of the bridging ligand does not seem to have any substantial impact on the binding of the complexes.

2. BSA binding studies

- **Serum albumin as a cellular target**

Serum albumins are proteins involved in binding and transport of drugs through the blood stream. Their binding with a drug may actually result in increase or decrease in the drug's efficacy making it important to investigate the interactions of prospective drugs with serum albumins. BSA is extensively studied, due to its structural homology with human serum albumin (HSA). HSA contains 585 amino acid residues with only one tryptophan located at position 214, while BSA has two tryptophans at positions 134 and 212 along the chain. BSA solutions exhibit a strong fluorescence emission with a peak at 343 nm, due to the tryptophan residues, when excited at 296 nm.

- **BSA binding studies:** The serum protein binding studies were carried employing fluorescence spectroscopy.The binding constant (Ka) and the number of binding sites on the protein (*n*) are calculated using the double logarithmic equation given below:

Log Io-I/I = log K_a + *n* log[Q]

K_a = Association binding constant.

n = number of binding sites on BSA

- The Stern –Volmer quenching constant (Ksv) is determined from the Stern-Volmer equation :

Io/I = 1 + K_{sv} [Q]

K_{sv} = Stern – Volmer quenching constant

Io = fluorescence intensity in absence of quencher

I = fluorescence intensity in presence of quencher

[Q] = Concentration of quencher

❖ General conclusions:

- Quenching of emission in the presence of the compounds (λ vs FI plot) may be owing to a variety of molecular interactions, collisional quenching, energy transfer, ground state complex formation and molecular rearrangements.
- These interactions cause a change in the micro environment around the protein molecule thereby causing quenching of the tryptophan residues of BSA.
- The linear nature of the Stern-Volmer plots reveals that only one quenching mechanism (either static or dynamic) is operative in the quenching process.
- The binding constant (K_b) values for the ligands and the complexes are in the range of 10^3 -10^7 M^{-1}.
- There is only one binding site (n ~ 1) on the macromolecule for binding of the compounds.
- Complexation of the ligands with the Ru(II) centres does not necessarily enhance their binding capabilities with the protein molecule.

➢ In-cellulo studies (within cellular environment)

- MTT assay against Human cervical cancer cell lines for all complexes.

❖ Need for novel anticancer agents:

- Development of multidrug resistance in patients.
- Long-term treatment with cancer drugs is also associated with severe side effects.
- Cytotoxic drugs have the potential to be very harmful to the body unless they are very specific to cancer cells.
- New drugs that will be more selective towards cancer cells

IC_{50} values of complexes

Complexes code	*IC_{50} (µM)*	*Complexes code*	*IC_{50} (µM)*
C1	24.4 ± 2.96	**C25**	75.5 ± 5.77
C2	17.3 ± 1.82	**C26**	31.1± 6.17
C3	29.6 ± 5.14	**C27**	115.5±4.65
C4	31.7 ± 3.24	**C28**	32.3±8.51
C5	32.6 ± 2.04	**C29**	39.8 ±9.91
C6	35.9 ± 5.71	**C30**	43.4 ± 2.95
C7	41.3 ±11.44	**C31**	56.5 ± 5.12
C8	23.7 ± 8.90	**C32**	31.2 ± 2.56
C9	**9.1 ± 7.60**	**C33**	61.0 ± 2.98
C10	54.12 ± 3.70	**C34**	50.8 ± 6.70
C11	23.5 ± 8.27	**C35**	120.9 ± 4.65
C12	32.6 ± 3.13	**C36**	30.1 ± 8.51
C13	10.9 ± 1.48	**C37**	98.5 ± 6.29
C14	**5.0 ± 3.7**	**C38**	31.7 ± 3.51
C15	**6.4 ± 5.35**	**C39**	69.0 ± 2.98
C16	15.7 ± 1.61	**C40**	73.1 ± 6.70
C17	15.5 ± 8.11		
C18	**4.2 ± 2.38**	**Cisplatin**	18.8 ± 3.4
C19	21.6 ± 22.26	**NAMI-A**	608.5 ± 55.4
C20	17.8 ± 3.62	**RAPTA-C**	>1600
C21	30.2 ± 15.51		
C22	22.3 ± 2.07		
C23	14.5 ± 15.68		
C24	11.5 ± 5.40		

❖ General conclusions:

- The cytotoxicity of the synthesized complexes were found to be in the order of 4-120 µM.
- The complexes are less toxic than cis-platin but more toxic than the Ru-complexes under clinical trials.
- An important feature of the mechanism underlying anti-tumour effects of DNA binding metal based compounds is repair of their DNA adducts.
- The level of DNA repair synthesis induced by the DNA adducts of Ru-arene complexes was found to be markedly higher than that induced by cis-platin.
- This implies that the adducts of Ru-arene compounds are removed from DNA more efficiently than cis-platin.
- This explains the lower cytotoxicity of these complexes compared to cis-platin.

Summary and general conclusions

In summary, homo-binuclear Ru(II) complexes of *p*-cymene and Bio active ligands with 4,4'-azopyridine/imidazole as a bridging ligand were synthesized and characterized using various spectral techniques. DNA and BSA binding studies showed that the binuclear complexes have good to strong binding affinities towards the macromolecules when compared to the ligands complexes due to more available binding sites as well as larger cationic charge and a greater hydrophobicity than their mononuclear counterparts. Such impressive binding efficacies widened their scope for in-vitro investigations as anticancer agents following which the cytotoxicity experiments were conducted employing MTT assay on Hela cancer cell line. Low IC_{50} values of the complexes can be explained by the presence of a hydrophobic arene moiety which facilitates cellular uptake of the complexes. Control experiment suggested that the there is no effect on cells in absence of the complexes.

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