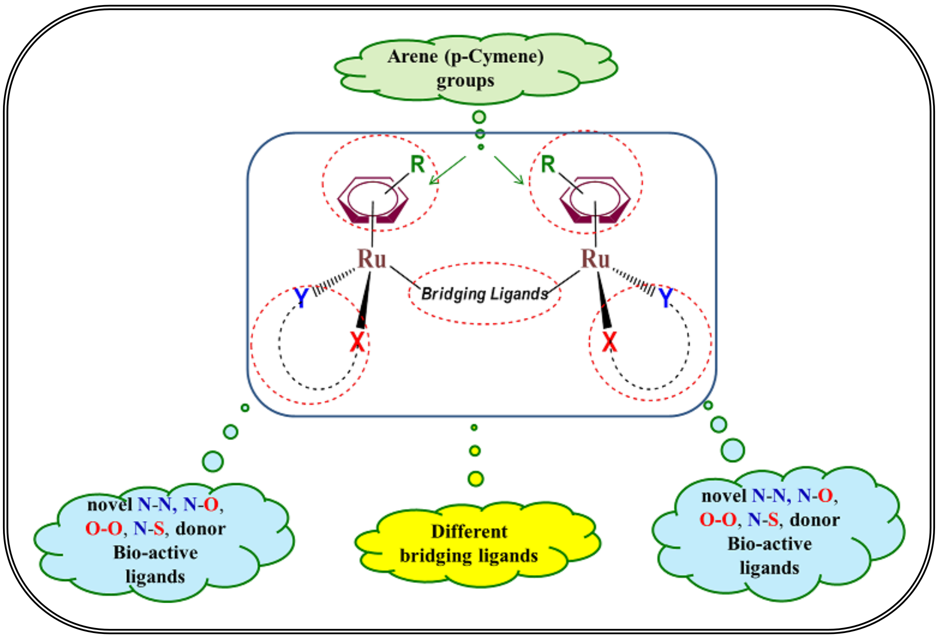
Summary of thesis entitled

"Studies on organometallic ruthenium based binuclear complexes: Synthesis and evaluation of their biological activity"



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For the Degree of

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In
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Content

Chapter-1

Introduction to Organometallic Binuclear Ruthenium complexes with their biological activities

Cancer: A worldwide epidemic: Need for novel metal based anticancer drugs:

Cancer is the uncontrolled growth of anomalous cells in the body [1]. Around 8.2 million people die of cancer within five years of diagnosis making it the leading cause of death worldwide. According to a WHO report, it is expected that annual cancer cases will rise to 26 million within the next two decades [2]. There have been tremendous efforts to combat cancer with current available drugs. Metal scaffolds play an important role in medicinal chemistry and drug design after the serendipitous discovery and development of platinum compounds [3]. Metallotherapeutics exert their action by inhibiting cancer cell division, trigger apoptosis by inducing DNA damage and disrupting DNA repair process [4]. The platinum-based drug cisplatin is one of the most common and effective drugs used by the oncologists in the treatment of numerous forms of human cancers. However, its effectiveness and therapeutic value is limited by serious side effects and observed drug resistance [5]. Many researchers are actively involved in the search for other alternative transition metal compounds, and new ruthenium compounds have been reported as promising antitumor metallotherapeutics [6].

1.1 Ruthenium complexes as anticancer agent:

Ruthenium-based anticancer metallotherapeutics are alluring alternatives over platinum-based therapeutics because they were found to have certain merits and different modes of action [7]. Several reviews on the anticancer ruthenium compounds have been published in the recent years [8-11]. Ruthenium compounds (i) have low side effects due to their higher selectivity for cancer cells compared with normal cells, (ii) are active against some cisplatin resistant cell lines, (iii) have higher selectivity for their targets which may be due to selective uptake by the malignant tissues and (iv) can mimic iron in binding to some biomolecules [12]. These favourable properties make these ruthenium scaffolds attractive alternatives for medicinal application. Ruthenium therapeutics has inherently low toxicity though ruthenium's ability to mimic iron is often erroneously linked with its low toxicity [13]. Ruthenium belongs to the same group as iron in the periodic table, which is reflected by its high affinity for transferrin and by its activation by reduction in cells [12, 14]. Some ruthenium compounds are excellent candidates for clinical development, due the low cytotoxicity and genotoxicity, different ligand exchange kinetics, facile transport, activation mechanisms, and high biological activity. Many medicinal chemists have designed new Ru (II) scaffolds that are being investigated in preclinical studies at various stages of development. In the past few decades several patents of antineoplastic ruthenium complexes with a range of different scaffolds have been reported [15].

Thus it can be concluded from the vast existing literature that ruthenium drugs have promising anticancer activity in the *in vitro* and *in vivo* models compared to platinum (II) compounds, due to their low systemic toxicity. In general, the activity of ruthenium compounds are mainly determined by the ligand combination and coordination geometry between ruthenium and its ligands, mostly with regard to their reactivity, hydrophobicity, binding, cellular uptake and intracellular distribution. In this regard, several Ru (II) compounds have high selectivity and targeting ability, which helps in improving their efficiency towards the cancer cells and further minimized their toxicity in normal cells. These complexes function in a different way to classical chemotherapies.

1.2 Aim and outline of this thesis:

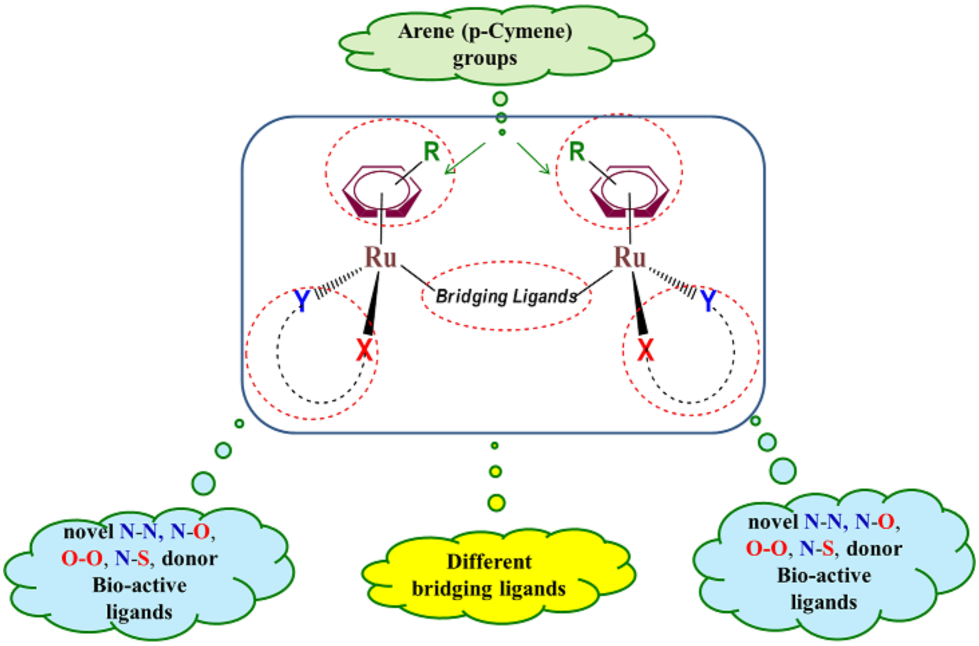


Fig. 1.1: *Aim and outline of this thesis*

- 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

2.1 Diphenyl pyrazol thiosemicarbazones:

Pyrazoles represent an important pharmacophore with various biological properties, and some pyrazole-containing derivatives have already been used as drugs for therapeutic purposes. Literature reveals that pyrazole derivatives are potent pharmacological compounds, and, therefore, their design and synthesis is an important area of research. The structural modifications of pyrazole, concerning the substituents at the 1-position, the carbon at the 3-position and the substituent at the 5-position have led to the preparation of new derivatives with a broad spectrum of biological activity. Structural modification on the different positions of the basic molecule allows for upgrading its pharmacological profile, providing it with antimicrobial, anticonvulsant, analgesic, anti-inflammatory, anti-viral, anti-malarial and anti-cancer properties. A comprehensive review by A. Ansari et al. focuses on the synthesis of diverse pyrazole derivatives and their biological activities [16].

2.1.1 Synthesis and characterization

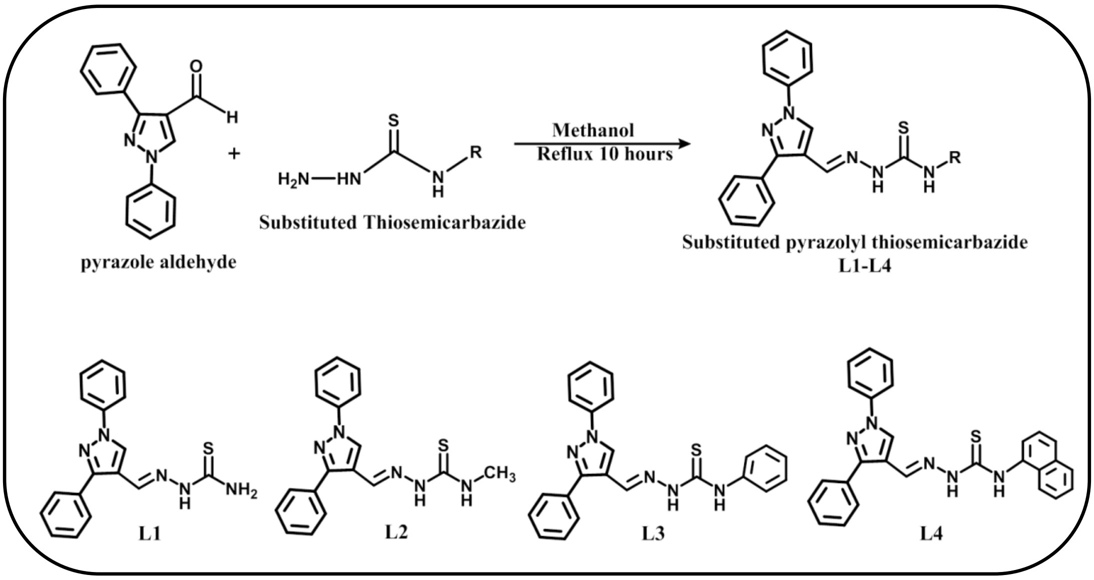


Fig 2.1: *General synthetic route to 1-substituted pyrazolyl thiosemicarbazone* ***L1-4***

The ligands were synthesized and characterised according to the literature [17].

2.2 Diphenylpyrazol α-Amino Acids derivatives:

Designing of new molecules as anticancer agents, require simulation of a suitable bioactive pharmacophore. The pharmacophore should not only be potent but must also be safe on normal cell. Amino acids are by far the most important low-molecular-weight ligands in biological systems. The therapeutic use of amino acids presents a viable and important option for natural medicine. Moreover, natural-occurring amino acids are interesting moieties in organic synthesis with several advantages, such as easy access, low cost, presence of one or more defined stereocenters and the possibility of functionalization in both the amine or carboxyl end. Furthermore, an increased amino acid uptake has been described for some types of cancer, making it a promising building block for enhancing the selectivity towards cancer cells. Finally, some anticancer drugs, such as Melphalan and Eflornithine, have a substituted amino acid scaffold whereas both of the well-known anticancer drugs, Paclitaxel and Docetaxel have amino acids side chain.

2.2.1 Synthesis and characterization:

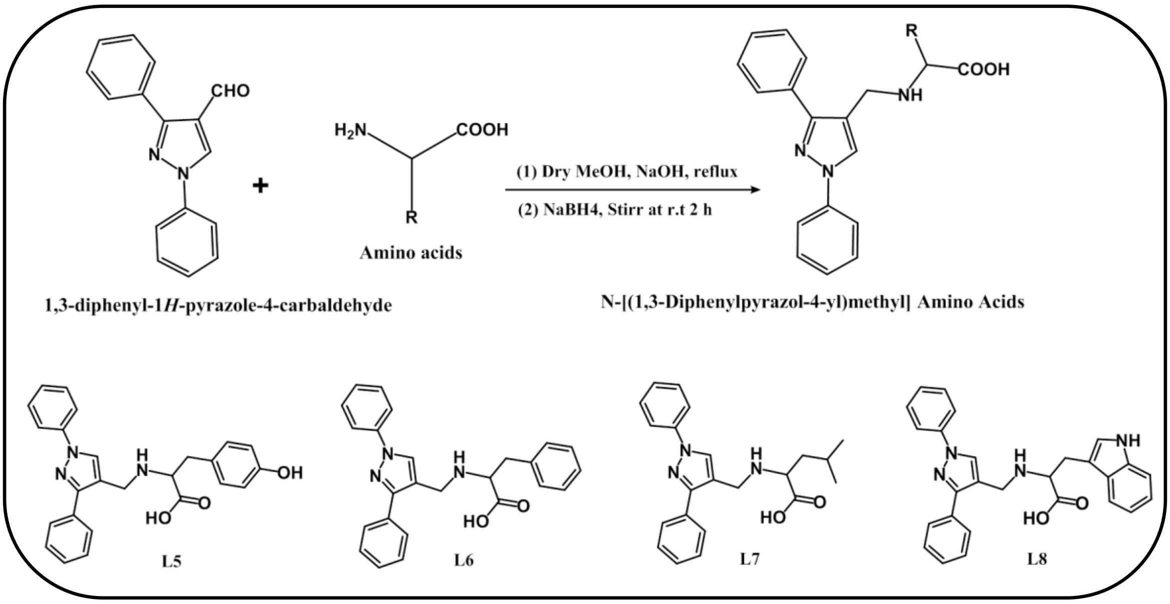


Fig 2.2 *General synthetic route to N-[(1,3-diphenylpyrazol-4-yl)methyl] α-amino acids* **L5-8**

The ligands were synthesized according to the literature [18].

2.3 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 [19]**.**

2.3.1 Synthesis and characterization

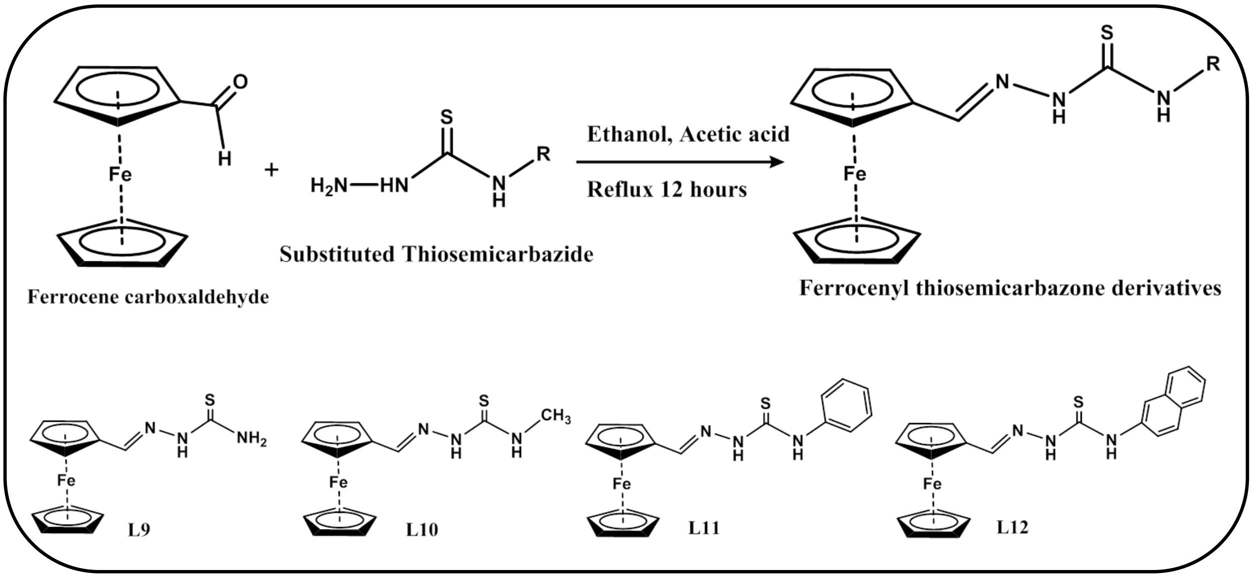


*Fig 2.3: General synthetic route to Ferrocenyl thiosemicarbazones **L9-12***

The ligands were synthesized and characterised according to the literature [20].

2.4 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 [21].

2.4.1 Synthesis and characterization

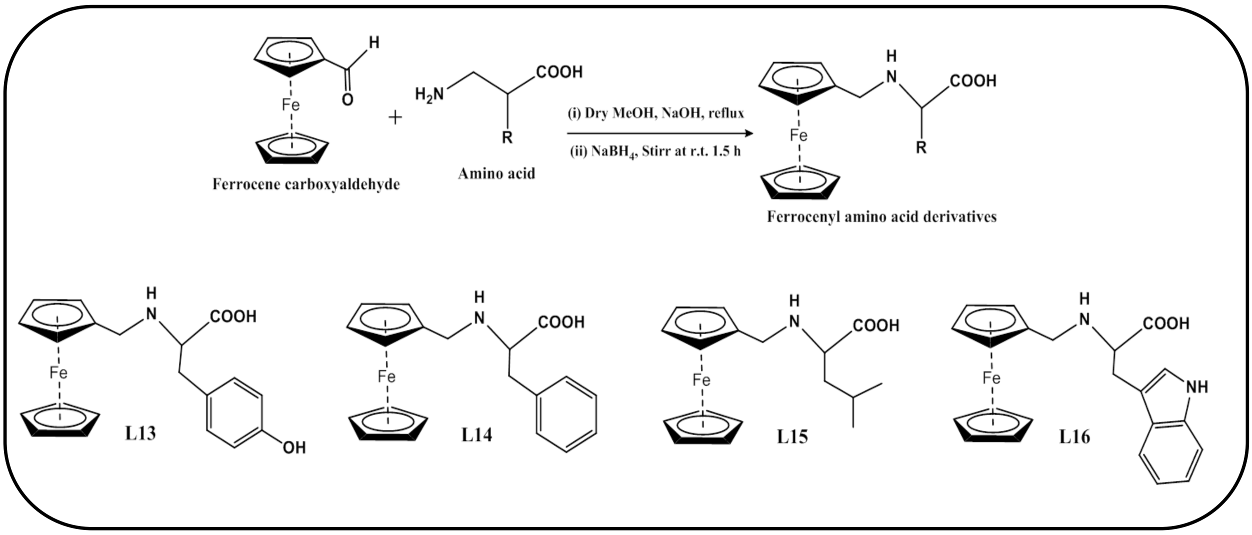


Fig 2.4: General synthetic route to Ferrocenyl Mannich bases ***L13-16***

All the Ferrocenyl amino acids ligands **L13-16** were synthesized by the above mention procedure and characterized by various spectral techniques to confirm their compositions as reported in literature [21].

2.5 Fluoroquinolones:

Despite being discovered serendipitously, the fluoroquinolones (FQs) are presently the largest class of antimicrobial agents used worldwide. Nalidixic acid the first quinolone recognized to exhibit antibacterial activity was isolated as a by-product during chloroquine synthesis in 1962. Over the next few years, extensive research led to discovery of the first generation of quinolones that overcame the limitations of parent molecule such as poor bioavailability and specificity only to gram-negative bacteria [22]. Later it was discovered that few structural modifications in the generic 4-quinolone backbone or its functional groups leads to enhanced bacterial cell penetration and prominent DNA gyrase inhibitory effect [23]. The quinolones are thus classified into four generations on the basis of their pharmacokinetic profile and antimicrobial activity (*Fig.2.5*). A fluorine atom at C-6 position and cyclic diamine piperazine molecule at C-7 position yielded the present-day FQs that possess significant potential against gramnegative, gram-positive and anaerobic bacteria. Fluoroquinolones (FQs) are well known to inhibit replication and transcription of bacterial

DNA either by targeting DNA gyrase or by targeting topoisomerase-II, both the enzymes being imperative in bacterial growth.

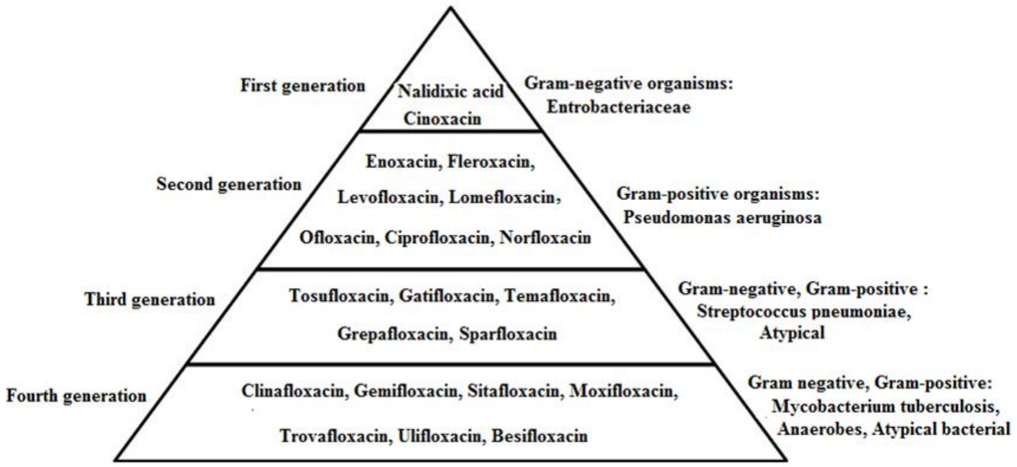
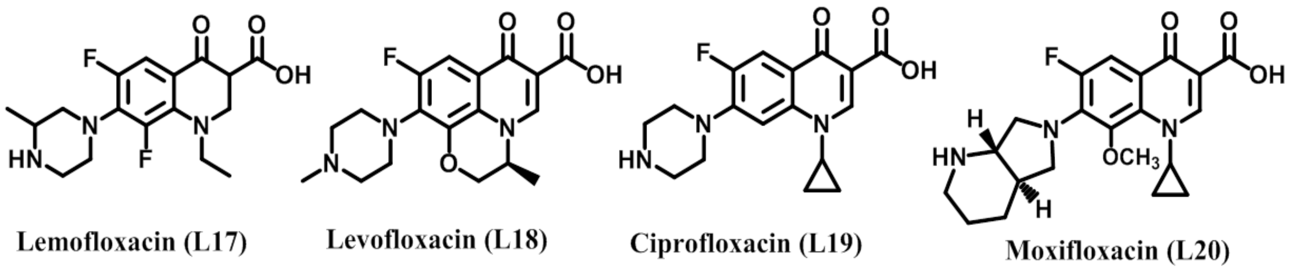


Fig. 2.5: *Classification of fluoroquinolones into four generations*

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



Lemofloxacin (L17) **Levofloxacin (L18)** **Ciprofloxacin (L19)** **Moxifloxacin (L20)**

Fig. 2.6: *Structures of the fluoroquinolones employed in the present study*

Chapter-3

Synthesis and characterization of Binuclear Ru (II) arene complexes

3.1 Homobinuclear Ruthenium (II)-arene complexes:

Ruthenium (II)-arene compounds are able to coordinate various types of ligands and therefore can modulate properties such as solubility, lipophilicity and pharmacokinetics. One of the first endeavour included coordination of metal arene moieties with bioactive ligands and a typical example of that approach is seen with [Ru(η^6-C_6H_6)Cl_2(metronidazole)] [24]. A viable approach for designing ruthenium complexes is the inclusion of biologically-active ligands that primarily aim at minimizing toxicity toward normal cells and provide ways to improve antiproliferative activity of metal-based drugs [25]. These ligands exhibit different coordination modes, facilitate compatibility of the complexes with the biological environment, and enhance cellular uptake. The diverse bio-relevant ligands incorporated as useful components of ruthenium complexes ranges from amino acids, peptides, proteins, carbohydrates, purine bases and oligonucleotides to steroids and other bioactive entities endowed with specific properties [26]. This methodology is confirmed by extensive studies on Ru complexes that provide unique rationale for design and production of potent ruthenium anticancer drugs with distinct transport pathways and mechanisms of action [27, 28].

3.2 General synthesis of [Ru_2 (μ-im / μ-azpy) (η^6-p-cym)$_2$ (L)$_2$]Cl_n complexes:

[Ru(η^6-*p*-cymene)Cl_2]$_2$ was prepared according to the procedure sited in literature [29, 30]. To a solution of [Ru(η^6-p-cymene)Cl_2]$_2$ (in 2.5 ml CH_2Cl_2), the synthesized ligand **L** (2.5 ml methanol) was added on stirring in 1:2 ratio. The reaction mixture was stirred overnight (20-24 h) and then left for slow evaporation at room temperature. The mononuclear complex [Ru(η^6-p-cym)(L)Cl] obtained was filtered, washed with pet ether and CH_2Cl_2 and dried in air. All the precursor mononuclear complexes synthesized were characterized using various spectral techniques and their composition confirmed. Synthesis of the binuclear complex was achieved by addition of a solution of the bridging ligand imidazole (im) / 4, 4'azopyridine (azpy) to a solution of the mononuclear complex [Ru(η^6-p-cym)(L)Cl], in 1:2 mole ratio using MDC:MeOH (1:2) as a solvent. In the synthesis of binuclear complexes with imidazole as bridging ligand, 1.0 M NaOH was added to raise the pH upto ~11 in order to deprotonate the –NH present in the imidazole ring [31]. The reaction mixture was stirred for 24 h under N_2 atmosphere. The reddish brown solid obtained was then filtered, washed with pet ether and CH_2Cl_2 and dried in oven at 40°C for 1 h. The complexes were recrystallized from dichloromethane and ether which resulted in reddish brown crystalline product. All the complexes are soluble in DMSO, MeOH, CH_2Cl_2 and partially soluble in water. *Fig. 3.1* shows the general synthetic route for preparation of the complexes.

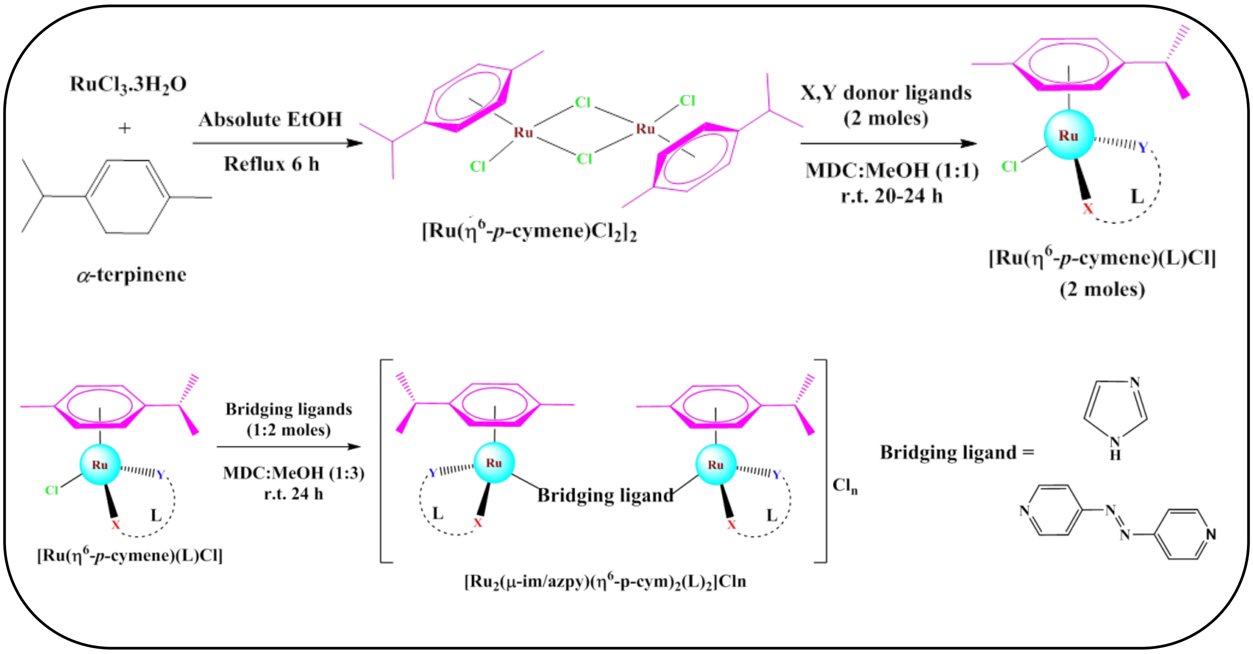


Fig. 3.1: General synthetic scheme of ***$[Ru_2(\mu\text{-}im/azpy)(\eta^6\text{-}p\text{-}cym)_2(L)_2]Cl_n$*** *complexes*

Table 3.1: List of the different ligand series used in the synthesis of $[(Ru_2(\mu\text{- }im/\mu\text{-}azpy)(\eta^6\text{-}p\text{-}cym)_2(L)_2]Cl_n$ complexes along with their coordinating sites to the Ru(II) centre.

Donor Ligand L	**Ligand code**	**Complex code**	**X =**	**Y =**
Diphenylpyrazol thiosemicarbazones	**L1-L4**	**C1-C8**	N	S
Diphenylpyrazol α-Amino Acids	**L5-L8**	**C9-C16**	N	O
Ferrocenyl thiosemicarbazones	**L9-L12**	**C17-C24**	N	S
Ferrocenyl amino acid mannich bases	**L13-L16**	**C25-C32**	N	O
Fluoroquinolones	**L17-L20**	**C33-C40**	O	O

The composition and structures of all the complexes have been confirmed by ESI Mass spectrometry, NMR, FTIR, UV-Vis spectroscopy and elemental analysis. Further geometry optimization of the complexes was done by performing DFT calculation. The analytical data are consistent with the proposed compositions and their molecular formulae.

Chapter-4

In-vitro bioactivity of the Binuclear Ru (II) arene complexes

4.1 In-vitro studies (outside cellular environment)

- DNA binding studies
- BSA binding studies

4.1.1 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 [32].

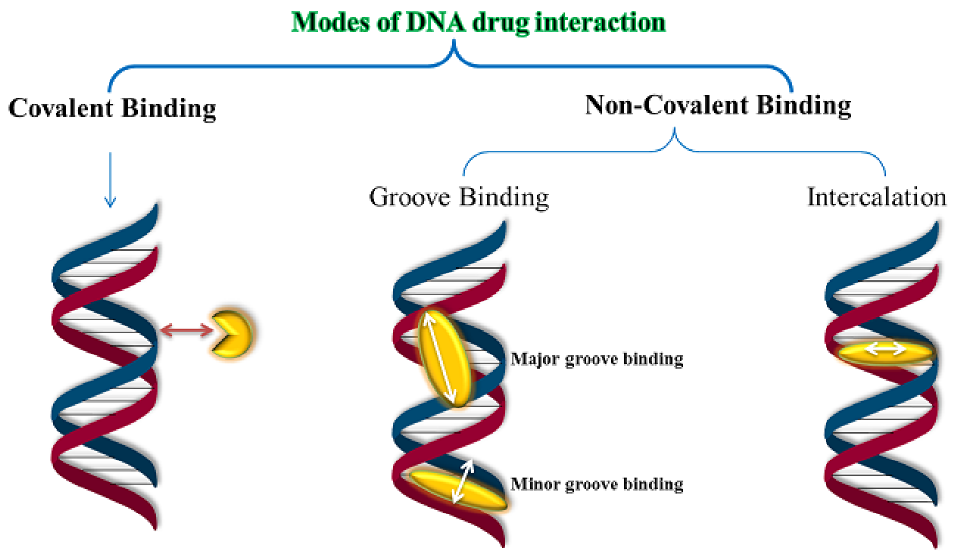


Fig. 4.1: *Different mode of DNA drug interaction*

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.

4.1.1.1 DNA binding studies:

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

The binding constant (**K_b**) is calculated using Mehan's Equation [33] given below:

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

Where [DNA] is the concentration of DNA in base pairs, $\varepsilon_A = A_{obsd}/$ [compound], ε_f is the extinction coefficient for the unbound compound and ε_b is the extinction coefficient for the compound in the fully bound form. K_b (binding constant) is given by the ratio of slope to the *y* intercept in plots $[\mathrm{DNA}]/(\varepsilon_A-\varepsilon_f)$ *versus* [DNA] according to equation

The standard Gibb's free energy for DNA binding was calculated from the relation below [34]

$$\Delta G^\circ_b = -RT \ln K_b$$

Where ΔG° Standard change in Gibb's free energy, R is universal gas constant (8.314 JK^{-1} mol^{-1}), T is temperature in Kelvin, ln K is natural log of K while K is equilibrium constant

The Stern – Volmer quenching constant (**K_{sv}**) [35] obtained from DNA-EB fluorescence quenching studies is calculated using the equation,

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

where Io and I are the emission intensities in the absence and the presence of the quencher (Q = compound) respectively, [Q] is the concentration of the quencher and K_{SV} is the Stern Volmer constant which can be obtained from the slope of the plot of Io/I *versus* [Q] and is often used to evaluate the quenching efficiency of each compound.

4.1.2 Result and Discussion:

- The nature of the plots (hypochromism) and viscosity measurements indicate that the complexes 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 (**K_{sv}**) for the ligands are in the range of $10^3 – 10^4$ M^{-1} whereas the **K_b** and **K_{sv}** 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.

4.1.3 BSA binding studies

Bovine serum albumin structure and biological functions:

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 shows very specific binding sites, of which most important one being referred is site-I and site-II, located in hydrophobic cavities of sub-domains IIA and IIIA respectively [36] as shown in *Fig. 4.1*. Site markers are small molecules, often used in studying the interaction of different ligands with the protein as they have specific binding locations in the albumin structure.

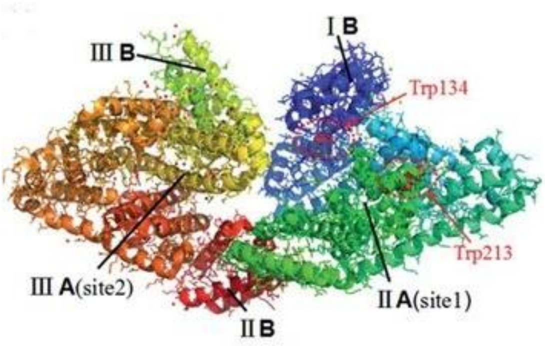


Fig. 4.1: *Discrete binding sites of BSA*

4.1.3.1 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:

$$\mathbf{Log\ Io\text{-}I/I = log\ K_a + \mathit{n}\ log\ [Q]}$$

where Io and I are the fluorescence intensities in the absence and the presence of quencher, and [Q] is the concentration of quencher (binuclear ruthenium complexes).The plot of log [(Io-I)/I] *versus* log [Q] for all the systems is linear and the values of $\mathbf{K_a}$ (M^{-1}) and ***n*** have been obtained from the intercept and slope, respectively.

The Stern –Volmer quenching constant ($\mathbf{K_{sv}}$) is determined from the Stern-Volmer equation:

$$\mathbf{Io/I = 1 + K_{SV}\ [Q] = 1 + k_q\, \tau_0 [Q]}$$

where Io is the initial tryptophan fluorescence intensity of BSA, I is the tryptophan fluorescence intensity of BSA after the addition of the quencher and $\boldsymbol{K_{SV}}$ the dynamic quenching constant, [Q] is the concentration of the quencher, k_q is the quenching rate constant of the biomolecule and $\boldsymbol{\tau_0}$ is the average lifetime of the molecule in the absence of the quencher. The dynamic quenching constant ($\boldsymbol{K_{SV}}$ M^{-1}) can be obtained from the slope of the plot Io/I *versus* [Q]. The $\boldsymbol{K_{SV}}$ plots for all the synthesized ligands and their corresponding binuclear Ru (II) complexes.

4.1.4 Result and Discussion:

- 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 ($\mathbf{K_b}$) 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.

4.2 In-cellulo studies (within cellular environment)

MTT assay against Human cervical cancer cell line (HeLa cell line) for all complexes.

4.2.1 Concept and principle of MTT assay:

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, coloured (dark purple) formazan product within the cell (*Fig. 4.2*). The cells are then solubilised with an organic solvent (e.g. DMSO) and the released, solubilised formazan reagent with an absorbance maximum near 570 nm is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. Viable cells with active metabolism convert MTT into formazan product but when cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood. Speculation in the early literature involving specific mitochondrial enzymes has led to the assumption that MTT is measuring mitochondrial activity, the reduction being caused by mitochondrial succinate dehydrogenase, but most likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT [37-39].

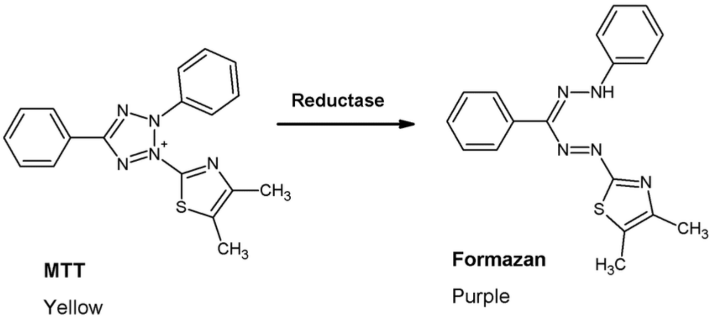


Fig. 4.19: *structure of MTT and colour formazan product*

Table 4.1: IC_{50} values of complexes obtained from MTT assay on HeLa cell lines. Values have been expressed in **µM** concentrations. Reported values of cisplatin and known ruthenium complexes NAMI-A and RAPTA have been provided for reference

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		

4.2.2 Result and Discussion:

- The cytotoxicity of the synthesized complexes was found to be in the order of 4.2 – 120 µM.
- The complexes are less toxic than cis-platin except few complexes 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 better cytotoxicity of these complexes compared to cis-platin.

Chapter-5

Summary, general conclusions and future perspectives

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 well 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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