# EFFECT OF STRUCTURE AND STEREOCHEMISTRY ON CYTOTOXICITY OF RUTHENIUM POLYPYRIDYL COMPLEXES

by

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ABSTRACT

EFFECT OF STRUCTURE AND STEREOCHEMISTRY ON CYTOTOXICITY OF RUTHENIUM POLYPYRIDYL COMPLEXES

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Efforts to develop novel non-platinum, metal-based antitumor drugs have been pursued by many groups and ruthenium complexes have drawn a lot of attention as prospective transition metal-based antineoplastic agents. Recently, it was discovered that complex $[(phen)_2Ru(tatpp)Ru(phen)_2]^{+4}$ shows biological activity both *in vitro* and *in vivo*. Expanding on this area of research, in depth cytotoxicity studies in human lung cancer cells and healthy cells were carried out in five different ruthenium(II) polypyridyl complexes as racemic mixtures and also their enantiopure forms. This study was carried out by performing MTT-

cytotoxicity assays on non small cell lung cancer cells (NSCLC), H358 – Human Bronchioalveolar Carcinoma and H226 – Squamous Lung Carcinoma as well as noncancerous/normal cells, HAVSMC – Human Aorta Vascular Smooth Muscle Cells and HUVEC – Human Umbilical Vein Endothelial Cells. The complexes screened in this cytotoxicity assay include $[Ru(phen)_3]^{2+}$, $[(phen)_2Ru(tpphz)]^{2+}$ **MZ$^{2+}$**, $[(phen)_2Ru(tpphz)Ru(phen)_2]^{4+}$ **Z$^{4+}$**, $[(phen)_2Ru(tatpp)]^{2+}$ **MP$^{2+}$** and $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ **P$^{4+}$,** where, phen – 1,10-phananthroline, tpphz - tetrapyrido[3,2-a:2'3'-c:3"2"-h:2"3"-j]phenazine and tatpp - 9,11,20,22-tetraazatetrapyrido[3,2-a:2'3'-c:3",2"-l:2"',3"'-n]pentacene.

The study showed some promising results which are listed below. The type of the bridging ligand attached to the metal center played a vital role in determining the potency of the drug, the longer tatpp ligand being more effective. The benefit of working with two metal centers as compared to one center was minimal; **MP$^{2+}$** and **P$^{4+}$** have similar $IC_{50}$ values. Stereochemistry had a measurable effect on cytotoxicity; the **Δ/ΔΔ** isomers were approximately twice more potent than the **Λ/ΛΛ** isomers. The stereochemistry around the dimeric complexes ought to be **ΔΔ** for it work best, meso complexes (**ΔΛ**) are equivalent in potency as the **ΛΛ** isomers. Complexes **MP$^{2+}$** and **P$^{4+}$** showed higher $IC_{50}$ values in healthy cells and so they have a larger therapeutic window which can be very useful in designing new cancer therapy drugs.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Role of Organometallics as Therapeutic Agents

Organometallics compounds present a wide array of possibilities in designing therapeutic agents for use in medicine and biology. Some applications include pharmaceuticals for cancer therapy, radiopharmaceuticals for diagnostics and therapy, probes for biosensors, to name a few. There has been particular interest in the three dimensional nature of octahedral transition-metal complexes, which makes them excellent candidates as spatial DNA probes to target cancer cells. The mode of action of these complexes is thought to be stereospecific/stereoselective binding to the chiral metal centers. A key factor in designing an anticancer drug is the optimization of the chemical reactivity of the drug to promote direct attack on the target site, e.g., DNA, and to reduce the chances of side-effects by minimizing the attack on other unrelated sites.[1-3]

## 1.2 Importance of Cytotoxicity Assay in Drug Development

Successful development of any drug requires both *in vitro* and *in vivo* screening. Unfortunately, many times, a drug does not make it through the entire development process due to its unacceptable toxicity levels. Cytotoxicity is a useful tool to assess the toxicity of the drug in the cellular level and thereby helps in identifying the optimal candidate in the drug designing process.

## 1.3 Anticancer Drugs Containing Platinum Centers

Nearly 30 years after its introduction, the remarkable effectiveness and success of the inorganic metal complex, cis-diamine-dichloroplatinum(II) (cisplatin), in the treatment of a number of solid tumor cancers continue to make it one of the most widely used chemotherapeutic agents in use. The ability of cisplatin to inhibit cell division was discovered in the 1960's by Rosenberg *et al.,*[4] and this activity is now attributed to its ability to form DNA adducts via the formation of inter- and intra- strand crosslinks in DNA, particularly those involving two adjacent guanine residues or two adjacent guanine-adenine bases leading to apoptosis.[5] Cisplatin, however, is not without drawbacks which include toxic side-effects like neurotoxicity and nephrotoxicity, a limited applicability to a relatively small range of tumors due to primary resistance to cisplatin, and the development of cisplatin-resistant tumors.[6] The second generation of platinum drugs, such as carboplatin and oxaliplatin, have wider clinical applications, mostly due to their lower overall toxicity.[7] One common feature in these mononuclear platinum compounds is the presence of two labile *cis* ligands on the platinum which aid in forming DNA adducts.

## 1.4 Ruthenium Complexes – An Emerging Class of Anticancer Drugs

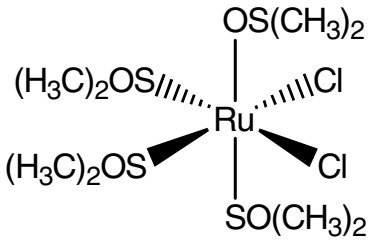
Over the past few decades, ruthenium complexes have gained importance in the search for new non-platinum based antineoplastic agents. Certain chemical properties, such as rate of ligand-exchange, range of accessible oxidation states, and ruthenium's ability to mimic iron in binding to certain

biological molecules are attractive for medical applications.[8] Another peculiar attribute of ruthenium, similar to that of Pt-drugs is that ruthenium is xenobiotic for all living systems and thus cells lack the proper machinery to neutralize its effects which can also be utilized in designing ruthenium-based anti-cancer agents.

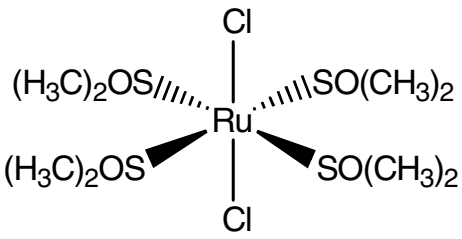
*1.4.1 Ruthenium Complexes with Displaceable Groups*

1.4.1.1 Ruthenium Complexes Containing Dimethylsulfoxide

Ruthenium(II) complexes containing dimethylsulfoxide, for example, *cis*- and *trans*- [Ru(II)(DMSO)$_4$Cl$_2$] exhibit promising antitumor activity and are comparatively less toxic with the LD$_{50}$ up to 1 g/kg, which corresponds to a larger dose required to obtain a therapeutic effect on animals. The structures of the two isomers, first characterized by Alessio *et al.* are given in Fig. 1.1.




*cis*-[Ru(II)(DMSO)$_4$Cl$_2$]




*trans*-[Ru(II)(DMSO)$_4$Cl$_2$]

Fig. 1.1 Structures of the two isomers of [Ru(II)(DMSO)$_4$Cl$_2$]

In aqueous solution, the *cis*-isomer rapidly loses the O-bonded DMSO whereas the *trans*-isomer undergoes the loss of two S-bonded DMSO almost immediately. Both the isomers then undergo slow reversible chloride dissociation to form cationic species. The *cis*-isomer has lesser reactive species

than the *trans*-isomer, in addition to the steric hindrance by the three DMSO ligands, making the *cis*-complex inert compared to the *trans*-complex. In an antitumor activity assay, the more reactive trans-isomer seemed to have a higher potency and is 20-fold more toxic than that of the other isomer, $LD_{0.05}$ – maximum tolerated dose, being 37 and 700 mg/kg for the *trans*- and the *cis*-complexes respectively.[9,10] DNA binding studies conducted by Novakova *et al.*[11] by using the technique of flameless atomic absorption spectrophotometry (FAAS) showed that the binding reached a maximum after 48 hours. CT-DNA was incubated with the ruthenium complexes. At specific time intervals, aliquots were withdrawn and the amount of free ruthenium (not bound to DNA) was determined by FAAS. The amount of ruthenium bound to DNA increased with time, which corresponded to approximately 40% and 75% of the molecules of *cis*- and *trans*-[Ru(II)$(DMSO)_4Cl_2$] respectively. This substantiates the result that the DNA binding of the *cis*-[Ru(II)$(DMSO)_4Cl_2$] is considerably less effective than that of *trans*- isomer.

1.4.1.2 Ruthenium Complexes with Heterocyclic Rings

NAMI-A (ImH[trans-$RuCl_4$(DMSO)Im]) has been extensively studied for its antitumor properties over the past few years, and has proved to be particularly more active and selective against tumor metastases with considerably less organ toxicity. The complexes, HInd[trans-$RuCl_4Ind_2$], (KP1019) and NAMI-A, are in Phase II clinical studies for cancer treatment where they have shown activity against cisplatin-resistant tumors and cisplatin-inactive tumors.[12]

Pluim *et al.* investigated the pharmacological effects of NAMI-A in comparison with cisplatin in two human ovarian tumor cell lines (Igrov-1 and 2008), and two human mammary tumor cell lines (MCF-7 and T47D), focusing mainly on the cytotoxicity, intracellular accumulation, and DNA adduct formation. Upon incubating the cells with increasing drug concentrations for 4 days in a growth inhibition assay, the $IC_{50}$ values for NAMI-A were 2000, 510, 800, and 900 μM and for cisplatin were 1.0, 0.37, 3.5, and 4.0 μM in Igrov-1, 2008, MCF-7 and T47D cell lines respectively. Cellular accumulation and the amount of drug bound to DNA in tumor cells was less in NAMI-A compared to cisplatin. Cytotoxicity of cisplatin correlated with DNA binding and cellular accumulation, but in the case of NAMI-A, correlation existed between cytotoxicity of NAMI-A and DNA binding and not with accumulation in cells. NAMI-A formed fewer intrastrand adducts with DNA than cisplatin which clarifies that lower cytotoxicity NAMI-A may be due reduced DNA reactivity. Thus, the antimetastatic mechanism of NAMI-A, unlike cisplatin, does not involve DNA binding as the most significant process, instead NAMI-A is thought to interfere with the type IV collagenolytic activity and reduce the metastatic potential of the tumors.[13,14]

*1.4.2 Ruthenium Complexes without Displaceable Groups*

1.4.2.1 Ruthenium Complexes Containing Polypyridyl Groups

Another promising class of antitumor ruthenium complexes is those possessing polypyridyl ligands. The DNA binding and cleavage properties of various ruthenium polypyridyl complexes have been intensively investigated.

Some of these complexes exist as chiral molecules capable of enantioselective recognition of DNA. Barton and co-workers showed that **Δ**-isomer of complex $[Ru(phen)_3]^{2+}$ shows preferential enantioselective binding to DNA compared to the **Λ**- isomer. There are two possible modes of action by which these complexes bind DNA non-covalently: (i) in a groove-bound fashion stabilized by a mixture of hydrophobic, electrostatic, and hydrogen-bonding interactions and (ii) through an intercalative association in which a planar, heteroaromatic moiety slides between the DNA base pairs. For the racemic complex, $[Ru(phen)_3]^{2+}$, the binding constant is around 6.2 x $10^3$ $M^{-1}$.[15-17] Hiort *et al.* investigated the interactions of the **Δ**- and **Λ**- $[Ru(phen)_2dppz]^{2+}$ with DNA. They observed some small changes in the binding affinities of **Δ**- and **Λ**- $[Ru(phen)_2dppz]^{2+}$ with intrinsic binding constant $K_b$ in the range of $10^7$-$10^8$ $M^{-1}$.[18] Lincoln and co-workers showed that there is preferential binding of the dimeric complex **ΔΔ**-$[(phen)_2Ru[dppz(11,11')dppz]Ru(phen)_2]^{4+}$ to the DNA as compared to the **ΛΛ**-isomer with $K_b$ ~ $10^{12}$ $M^{-1}$.[19] O'Reilly *et al.* showed that a bimetallic ruthenium complex, $[(bipy)_2Ru[Mebipy(CH_2)_nbipyMe]Ru(bipy)_2]^{4+}$ (n = 5,7) binds strongly to DNA and can photosensitise DNA strand break even at high ionic strengths, in contrast to its monomeric analogue $[Ru(bipy)_3]^{2+}$.[20]

1.4.2.2 Cytotoxicity of Ruthenium Polypyridyl Complexes

Ma *et al.* studied the DNA binding and cytotoxicity of ruthenium complexes containing 2-amino-4-phenylamino-6-(2-pyridyl)-1,3,5-triazine (appt) (Fig 1.2). The binding constant ($K_b$) of the complex $[Ru(^tBu_2bpy)_2(2\text{-appt})](PF_6)_2$

with CT-DNA, as determined by absorption titration, was (8.9 ± 0.5) x $10^4$ $dm^3$ $mol^{-1}$. The lower $K_b$ for this complex indicates that it interacts with DNA via groove binding. They also conducted modeling studies which suggested that the minor groove is the favored binding site for the complex.

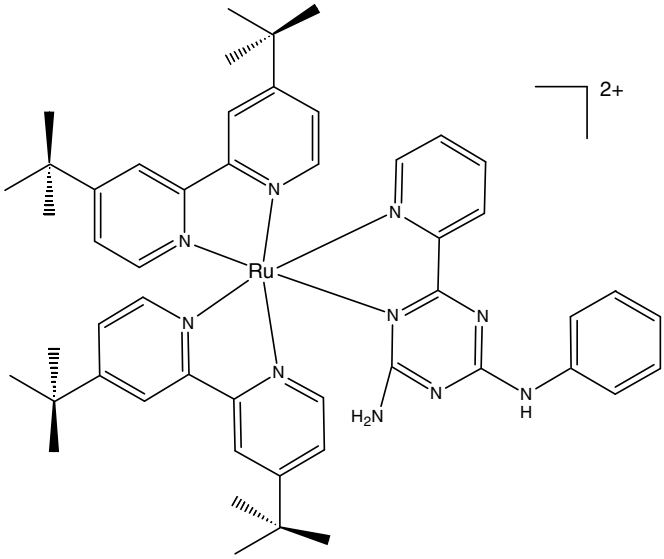



Fig 1.2 Structure of complex $[Ru(^tBu_2bpy)_2(2\text{-appt})]^{2+}$

MTT – Cytotoxicity assay was carried out in four human carcinoma cell lines (KB-3-1, KB-V-1, HepG2, HeLa) and noncancerous normal lung fibroblasts (CCD-19Lu) for the complex $[Ru(^tBu_2bpy)_2(2\text{-appt})](PF_6)_2$ against the control - cisplatin. The results are tabulated in Table 1.1. The cells were exposed with the drug for 48 hours and the $IC_{50}$ values were calculated using the MTT assay. The complex was moderately toxic compared to the control and there were not significant differences in the $IC_{50}$ values in cancer cells expect the multi-drug resistant cell line, KB-V-1. The drug was also less toxic to the noncancerous cell

line CCD-19Lu suggesting specific activity towards cancer cells. They also conducted the same experiment at pH 6.8 in HeLa cell line but there was no significant difference in the values at pH 6.8 compared to those determined at pH 7.2-7.4.[21]

Table 1.1 Cytotoxicity of $[Ru(^tBu_2bpy)_2(2\text{-appt})]^{2+}$ vs. Cisplatin in Human Cancer Cells and Noncancerous Cells

| Complex | $IC_{50}$ μM | | | | |
|---|---|---|---|---|---|
| | KB-3-1 | KB-V-1 | HepG2 | HeLa | CCD-19Lu |
| $[Ru(^tBu_2bpy)_2(2\text{-appt})]^{2+}$ | 52.3 | 199.0 | 30.2 | 59.7 | 151.0 |
| Cisplatin | 22.1 | 39.1 | 10.5 | 11.6 | 129.0 |

## 1.5 Scope of Thesis

Recent studies in our lab have shown that some large dimeric ruthenium complexes, such as $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$, are well-tolerated in mice toxicity studies and have promising antitumor properties. The relatively low animal toxicity and high DNA binding affinity for the dimer $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ suggest that it may be useful as an antitumor agent.[22] When prepared from racemic materials, this complex exists as a statistical mixture of three stereoisomers (**ΛΛ**, **ΔΔ** and **ΔΛ**). To date, all of the preliminary data on this complex (DNA binding, DNA cleavage, cytotoxicity and mouse tumor models) have been collected using the mixture, referred to as *mix*-$[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$. This work proposes to examine the effect of

stereochemistry on cytotoxicity (*in vitro*) of various monomeric and dimeric ruthenium complexes in normal and tumor cell lines, the details of which are given in Chapter 2. We expect one isomer of the complexes might have a better cytotoxicity than the other/s. Previous studies by Barton et al., Hiort et al. and Lincoln et al. suggest there is an enantiomeric preference in binding of the **Δ/ΔΔ** isomers to DNA.[15,18,19] Some small ruthenium polypyridyl complexes have been examined for toxicity to animals. Simple compounds such as $[Ru(phen)_3]^{2+}$ are highly toxic to animals leading to convulsions and death in minutes at relatively low doses (<6 mg/kg). Dwyer *et al.,*[23] have shown that $[Ru(phen)_3]^{2+}$ and $[Ru(bpy)_3]^{2+}$ are strong inhibitors of acetyl cholinesterase (AChE) which is their likely target *in vivo*, as AChE inhibition is known to lead to convulsions and rapid death due to a short circuiting of the nervous system. The authors also showed that the **Δ** enantiomer was more inhibitory than the **Λ** isomer, suggesting additional therapeutic benefit may be achieved by using a specific stereoisomer if structurally related complexes are to be used as drugs.

Chapter 3 elucidates the stereospecific synthesis and characterization of some monomeric tris phen ruthenium(II) compounds incorporating nitro and amino groups in one of its 1,10-phenanthroline ligands.

# CHAPTER 2

## STRUCTURE-ACTIVITY RELATIONSHIP OF RUTHENIUM POLYPYRIDYL COMPLEXES BASED ON CYTOTOXICITY

### 2.1 Introduction

Interest in metal-based antitumor drugs dates back to the early 1960's with tremendous success of *cis*-diammine-dichloroplatinum(II) (cisplatin) as a widely used and very effective chemotherapeutic agent, discovered by Rosenberg *et al.*[4] The presence of two labile *cis* chloride ligands on the platinum aid in forming adducts via the formation of inter- and intra- strand crosslinks in DNA, and thus inhibiting DNA replication, leading to cell death. Cisplatin, however, is not without drawbacks which include toxic side-effects like neurotoxicity and nephrotoxicity, a limited applicability to a relatively small range of tumors due to primary resistance to cisplatin, and the development of cisplatin-resistant tumors.[6]

Efforts to develop novel non-platinum, metal-based antitumor drugs have been pursued by many groups with the aim of improving clinical effectiveness, reducing general toxicity, broadening the spectrum of activity and treating cisplatin-resistant tumors. In the past decade, ruthenium complexes have drawn a lot of attention as prospective transition metal-based antineoplastic agents. It is conventionally believed that the pharmacological target of prospective

anticancer ruthenium drugs is the DNA, which is the same as platinum drugs. This is because the substitutional exchange rates for Ru-Cl bonds are similar to those of Pt-Cl bonds and their ability to bind DNA is almost always correlated with their cytotoxicity.[24,25] DNA binding is seen to result in any number of effects including inhibition of DNA replication, mutagenesis, inducing SOS repair, and reducing RNA synthesis.[8] Two promising ruthenium-based anticancer drugs are HInd[trans-$RuCl_4Ind_2$], (KP1019) and ImH[trans-$RuCl_4$(DMSO)Im], (NAMI-A) (where Ind = indole and Im = imidazole).[12] These complexes are less toxic on the host than platinum complexes and exhibit specific activity against metastasizing tumors. NAMI-A has advanced to Phase II clinical studies for cancer treatment where it has shown activity against MCa mammary carcinoma, Lewis lung carcinoma and TS/A adenocarcinoma.[26]

Another class of widely-studied DNA binding Ru(II) compounds are cationic ruthenium(II) polypyridyl complexes, such as $[Ru(phen)_3]^{2+}$ and $[Ru(phen)_2(dppz)]^{2+}$. These complexes lack labile chloride ligands but still show good to excellent DNA-binding properties with $K_b$'s of $10^4$ $M^{-1}$ to $10^7$ $M^{-1}$ for $[Ru(phen)_3]^{2+}$ and $[Ru(phen)_2(dppz)]^{2+}$, respectively. In these cases, however, binding is dominated by electrostatic and, for $[Ru(phen)_2(dppz)]^{2+}$, intercalative forces. Due to this DNA-binding affinity, this class of compounds has enjoyed considerable attention and a number of factors contributing to the DNA binding have been elucidated including: complex size, charge, hydrophobicity and stereochemistry. Even higher binding constants can be achieved by addition of a

second ruthenium(II) ion and further elaborations of the ligands or bridging ligand structure. For example, a $K_b$ of $10^{12}$ $M^{-1}$ is observed for the dimeric complex $[(phen)_2Ru[dppz(11,11')dppz]Ru(phen)_2]^{4+}$.[15,19,27-29] While such complexes exhibit interesting DNA binding behavior, few such complexes have demonstrated promising therapeutic action and photodynamic therapy.[30-32] One reason that few such complexes have been used therapeutically is that early studies showed these complexes to be quite toxic *in vivo*.[23]

Our lab has been investigating the photophysical and photochemical properties of monomeric and oligomeric Ru(II) polypyridyl complexes with a focus on synthesizing efficient light-harvesting assemblies which can catalyze a variety of redox reactions.[33,34] Recently, we examined the DNA-binding ability of the complex $[(phen)_2Ru(tatpp)Ru(phen)_2]^{+4}$ $\mathbf{P^{+4}}$ and its biological activity both *in vitro* and *in vivo*. Not surprisingly, we discovered that the complex $\mathbf{P^{4+}}$ binds DNA tightly ($K_b \sim 10^7$ $M^{-1}$), however, unlike its related analogues (e.g., $[Ru(phen)_3]^{2+}$ and $[Ru(phen)_2(dppz)]^2$), $\mathbf{P^{4+}}$ was shown to be an active DNA cleavage agent in the presence of mild reducing agents such as glutathione. Furthermore, the cleaving activity was potentiated under anaerobic conditions. This type of hypoxia-selective DNA cleavage is very unusual for any metal complexes and is intriguing in that such an agent potentially could be used to therapeutically target hypoxic cancer cells.[35]

In order to assess the toxicity of these complexes, we did a preliminary animal toxicity screen with mice. Both $\mathbf{P^{4+}}$ and $\mathbf{Q^{4+}}$ $[(phen)_2Ru(tatpq)Ru(phen)_2]^{+4}$

were surprisingly well tolerated with the maximum tolerable dose (MTD) being 67 mg/kg and 17 mg/kg, respectively. For comparison, $[Ru(phen)_3]^{2+}$ is far more toxic with a MTD of <6mg/kg. In another screening experiment, the *in vivo* activity of $\mathbf{P^{4+}}$ and $\mathbf{Q^{4+}}$ on melanoma (B16-F1) was examined using an orthotopic syngeneic mouse melanoma model. Nine mice were divided into three groups of three (Control, $\mathbf{P^{4+}}$ and $\mathbf{Q^{4+}}$) and injected with 1 million B16-F1 mouse melanoma cells. A total of 16 doses, (0.2 mg of $\mathbf{P^{4+}}$ / 0.1mg of $\mathbf{Q^{4+}}$**),** were administered intraperitoneally, every alternate day in the experimental groups while the control received only the buffer. Parameters of mouse weight and tumor volume were measured daily for a total of 45 days. While complex $\mathbf{Q^{4+}}$ showed little tumor inhibition, complex $\mathbf{P^{4+}}$ had a noticeable inhibitory effect on tumor growth.[22]

Given this promising set of preliminary data we decided to investigate a few ruthenium(II) polypyridyl complexes in depth for cytotoxicity in human lung cancer cells and healthy cells. Specifically, we wanted to examine the role of the bridging ligand in the cytotoxicity as well as the chirality at the metal centers. This study was carried out by performing MTT-cytotoxicity assays on non small cell lung cancer cells (NSCLC), H358 – Human Bronchioalveolar Carcinoma and H226 – Squamous Lung Carcinoma as well as noncancerous/normal cells, HAVSMC – Human Aorta Vascular Smooth Muscle Cells and HUVEC – Human Umbilical Vein Endothelial Cells. The complexes screened in this cytotoxicity assay include $[Ru(phen)_3]^{2+}$, $[(phen)_2Ru(tpphz)]^{2+}$ $\mathbf{MZ^{2+}}$, $[(phen)_2Ru(tpphz)Ru(phen)_2]^{4+}$ $\mathbf{Z^{4+}}$, $[(phen)_2Ru(tatpp)]^{2+}$ $\mathbf{MP^{2+}}$ and

$[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ **$P^{4+}$,** where, phen – 1,10-phananthroline, tpphz - tetrapyrido[3,2-a:2'3'-c:3"2"-h:2"3"-j]phenazine and tatpp - 9,11,20,22-tetraazatetrapyrido[3,2-a:2'3'-c:3",2"-l:2"',3"'-n]pentacene. (Figure 2.1) All these complexes were used as chloride salts for ease of solubility in water.

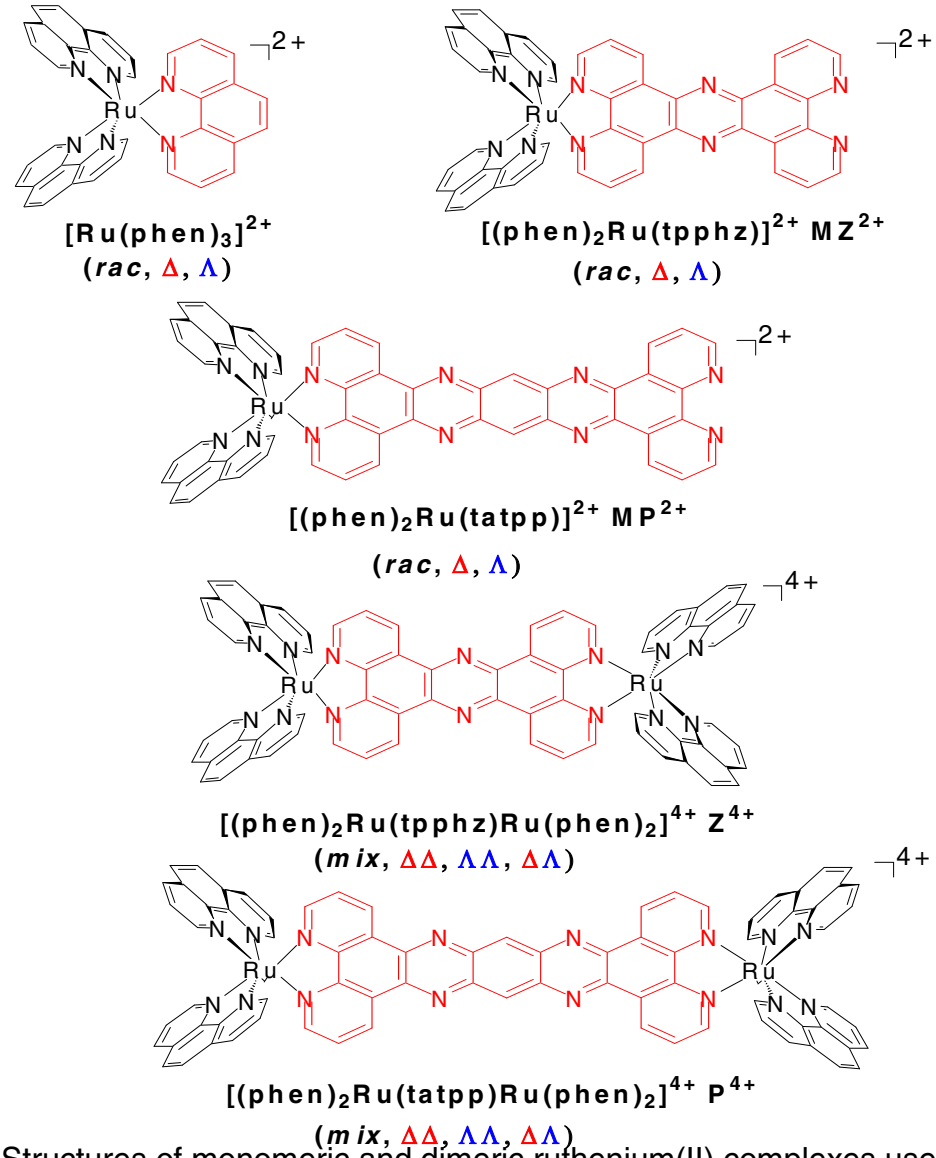



Fig 2.1 Structures of monomeric and dimeric ruthenium(II) complexes used in the cytotoxicity screening assay.

## 2.2 Experimental Section

### *2.2.1 Materials*

The cell lines H358, H226 and HAVSMC were kindly donated by Dr. Sanjay Awasthi. The cryopreserved primary culture of HUVEC was purchased from Lonza Walkersville, Inc. Maryland, USA. The NSCLC cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), 1% (v/v) Penicillin/Streptomycin solution (P/S), 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. HAVSMC cells were cultured in DMEM medium supplemented with 10% (v/v) FBS and 1% (v/v) P/S solution and the primary culture of HUVEC cells in EGM medium supplemented with BBE (Bovine Brain Extract), heparin, hEGF, Hydrocortisone, GA-1000 (Gentamicin, Amphotericin B) and FBS 10 ml. All the cells were cultured at 37°C in a humidified atmosphere of 5% $CO_2$.

The complexes $[Ru(phen)_3]^{2+}$ and $\mathbf{Z^{4+}}$ were synthesized as diastereomeric mixtures following known literature procedures.[36,37] The complexes, $\mathbf{MZ^{2+}}$, $\mathbf{MP^{2+}}$ and $\mathbf{P^{4+}}$, were synthesized by a colleague in our lab, Abhishek Yadav, as diastereomeric mixtures.[38-40] These complexes were also synthesized in their enantiopure form; the racemate (*rac*) monomeric complexes can be resolved as two enantiomers, namely **Δ** and **Λ** forms. The dimeric complexes were synthesized as a diastereotopic mixture of three isomeric forms (*mix*), namely **ΔΔ**, **ΛΛ**, and **ΔΛ.** The individual stereoisomers of the dimeric complexes were

also obtained by following a stereospecific synthetic approach. The syntheses and chiral resolution procedures of the building blocks of these complexes are already established in our lab. The enantiopurity of the stereoisomers were determined by HPLC experiment with cyclodextrin chiral stationary phase.[41]

*2.2.2 MTT-Cytotoxicity Assay*

MTT cytotoxicity assay of various ruthenium(II) complexes was carried out as described by Awasthi *et al.*[42] Cells in log phase growth were inoculated into each well of a 96-well plate, with each well containing a cell density of 20,000 tryphan-blue excluding cells in 160 μL of RPMI-1640 medium. Post 24-hour incubation, 40 μL aliquots of the drug in varying concentrations (500 nM – 150 μM) were added with 8 replicates at each concentration. Each experiment was conducted at least twice. The cells were exposed to the drug for a period of 96 hours after which 20 μL of 5mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS was added in each well. The cells were incubated for 2h at 37°C during which MTT is converted to formazan in the mitochondria of living cells. The medium was decanted and the remaining cell contents were solubilized in 100 μL of DMSO with gentle shaking over a period of 2h at room temperature. The absorbance was read at 570 nm using a microplate reader (BIO-TEK Instruments). $IC_{50}$ is defined as the concentration of the drug necessary to reduce the formazan (chromogen) formation by 50%.

The data acquired from each experiment was tabulated as a spreadsheet table (Table 2.1). Cell survival% at each concentration was calculated as the

percentage ratio of mean of $Abs_{570}$ in drug treated wells to that of control/untreated wells. The $IC_{50}$ value for each data set was obtained by fitting the data in a sigmoidal dose response curve using the Global Optimization by Stimulated Annealing (GOSA-fit) software by Bio-Log Scientific Software, France. The equation of the sigmoidal dose response curve is given as:

$$y = \min + \frac{(\max - \min)}{1 + 10^{(\log IC_{50} - \log x).slope}} \qquad \text{(Equation 1)}$$

where, y is the response (cell survival %), x is the dose of the drug in μM, $IC_{50}$ is the drug concentration at the halfway point between the minimum cell survival (min) and maximum cell survival max, and slope is the slope of the curve at its midpoint.

Each cytotoxicity assay was performed three times and the data fitted to equation 1 as described above. An analysis of variance (ANOVA) calculation was performed using Microsoft Excel and the 99% confidence intervals for the data sets were calculated.

## 2.3 Results and Discussion

The principal goal of this project was to evaluate the cytotoxicity of various monomeric and dimeric ruthenium(II) complexes in cancerous and normal human cells. Cell survival as a function of increasing drug concentration was measured using a standard colorimetric MTT assay - the underlying principle being the yellow MTT is reduced to purple formazan in the mitochondria of living cells (Fig 2.2).

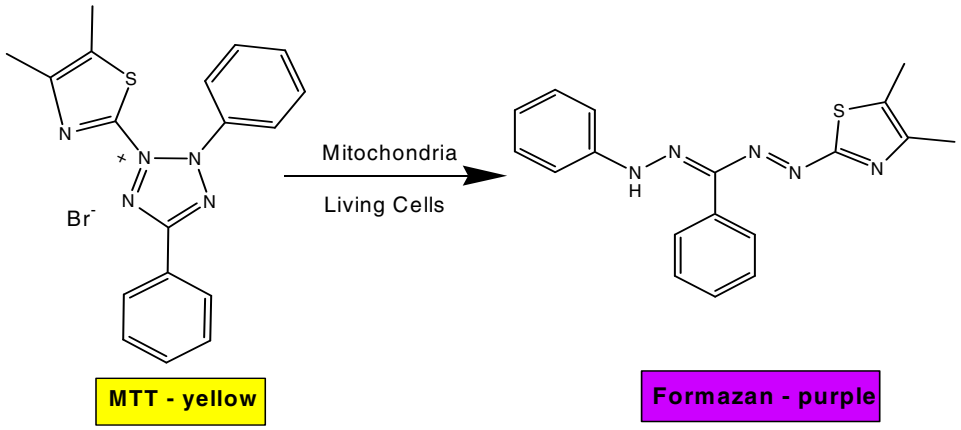



Fig 2.2 Reduction of MTT to formazan in the presence mitochondria of living cells

Table 2.1 MTT Assay Data Table for ***rac*- Ru(phen)$_3$Cl$_2$** in H226 Cells

12/12/2006 **Experiment 1** **Run 1** Cells used :**H226**

| | | | Concentrations of ***rac*- Ru(phen)$_3$Cl$_2$** μM | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | Blank | Cells only | **0.5** | **1** | **10** | **20** | **40** | **60** | **80** | **100** | **125** | **150** |
| A | 0.101 | 1.045 | 1.014 | 1.001 | 0.946 | 0.906 | 0.850 | 0.729 | 0.547 | 0.444 | 0.318 | 0.205 |
| B | 0.107 | 1.051 | 1.023 | 1.007 | 0.963 | 0.936 | 0.875 | 0.745 | 0.554 | 0.449 | 0.322 | 0.208 |
| C | 0.111 | 1.063 | 1.033 | 1.014 | 0.982 | 0.940 | 0.888 | 0.752 | 0.565 | 0.472 | 0.336 | 0.219 |
| D | 0.125 | 1.069 | 1.043 | 1.015 | 1.012 | 0.944 | 0.900 | 0.759 | 0.578 | 0.495 | 0.341 | 0.231 |
| Average | 0.111 | 1.057 | 1.028 | 1.009 | 0.976 | 0.932 | 0.878 | 0.746 | 0.561 | 0.465 | 0.329 | 0.216 |
| SD | 0.010 | 0.011 | 0.013 | 0.007 | 0.028 | 0.017 | 0.021 | 0.013 | 0.014 | 0.023 | 0.011 | 0.012 |
| Cell Survival % | | | 97.28 | 95.48 | 92.31 | 88.12 | 83.08 | 70.60 | 53.07 | 43.99 | 31.14 | 20.41 |

12/12/2006 **Experiment 1** **Run 2** Cells used : **H226**

| | | | Concentrations of ***rac*- Ru(phen)$_3$Cl$_2$** μM | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | Blank | Cells only | **0.5** | **1** | **10** | **20** | **40** | **60** | **80** | **100** | **125** | **150** |
| A | 0.103 | 1.049 | 1.020 | 1.002 | 0.946 | 0.911 | 0.860 | 0.736 | 0.550 | 0.445 | 0.318 | 0.205 |
| B | 0.110 | 1.060 | 1.025 | 1.012 | 0.979 | 0.940 | 0.884 | 0.751 | 0.560 | 0.470 | 0.331 | 0.211 |
| C | 0.117 | 1.066 | 1.043 | 1.015 | 0.986 | 0.941 | 0.898 | 0.758 | 0.567 | 0.487 | 0.340 | 0.229 |
| D | 0.130 | 1.070 | 1.048 | 1.023 | 1.015 | 0.996 | 0.906 | 0.785 | 0.592 | 0.500 | 0.350 | 0.268 |
| Average | 0.115 | 1.061 | 1.034 | 1.013 | 0.982 | 0.947 | 0.887 | 0.758 | 0.567 | 0.476 | 0.335 | 0.228 |
| SD | 0.012 | 0.009 | 0.014 | 0.009 | 0.028 | 0.036 | 0.020 | 0.021 | 0.018 | 0.024 | 0.014 | 0.028 |
| Cell Survival % | | | 97.43 | 95.45 | 92.48 | 89.23 | 83.58 | 71.37 | 53.45 | 44.80 | 31.54 | 21.50 |

The raw data from the MTT assay obtained upon treating H226 cells with racemic $[Ru(phen)_3]Cl_2$ are shown in Table 2.1. Each run contained 4 identically treaded samples (A-D) and the experiment was repeated at least twice (2 runs). The $IC_{50}$ was obtained from this data using the equation 1 which fit a sigmoidal dose response curve as shown in Fig 2.3.

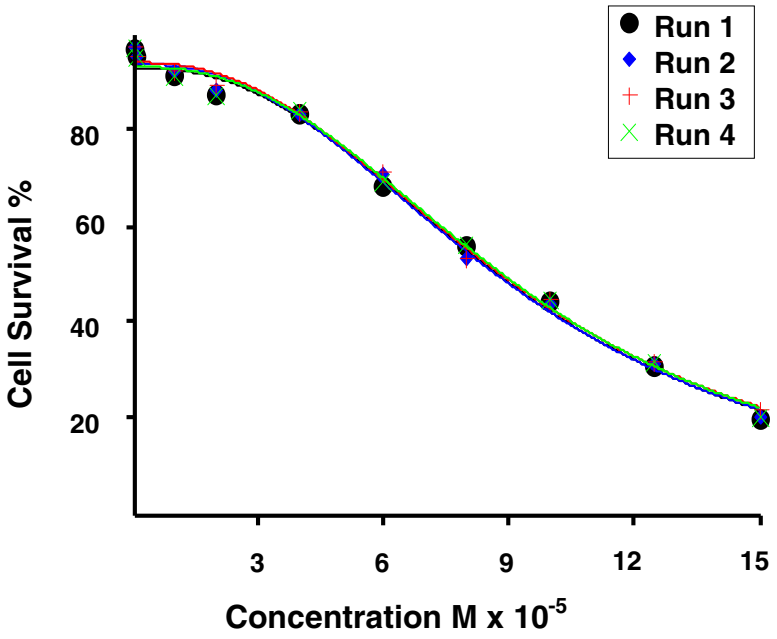



Fig 2.3 Sigmoidal dose response curve of [*rac*- $Ru(phen)_3]^{2+}$ in H226 cells

The $IC_{50}$ is the concentration of the drug at 50% cell survival. For complex *rac*- $[Ru(phen)_3]Cl_2$, $IC_{50}$ = 92.83 μM in H226 cells.

This assay was performed for the racemates of the monometallic complexes $[Ru(phen)_3]^{2+}$, **MZ**$^{2+}$, **MP**$^{2+}$ and the diastereotopic mixtures of **Z**$^{4+}$ and **P**$^{4+}$. In this case each diastereotopic mixture is a statistical distribution of 25 % **ΔΔ- Z**$^{4+}$, 25% **ΛΛ- Z**$^{4+}$ and 50% **ΔΛ- Z**$^{4+}$ (meso) and 25 % **ΔΔ- P**$^{4+}$, 25% **ΛΛ- P**$^{4+}$ and 50% **ΔΛ- P**$^{4+}$ (meso). For simplicity, we refer to the diasterotopic mixtures as *mix*-**Z**$^{4+}$ and *mix*- **P**$^{4+}$. The $IC_{50}$ values for all these complexes are plotted in the

bar graph shown in Figure 2.4 with data for H358 cells in light blue and data for H226 cells shown in dark blue. From these data, we can draw a number of conclusions. First, there is little difference in the activity of the complexes between the two cell lines with most of the data showing a slightly lower $IC_{50}$ for H358 cell line over the H226 line. Second, it is clear that the two complexes with the tatpp bridging ligand, **MP**$^{2+}$ and **P**$^{4+}$ are significantly more cytotoxic compared to compounds with tpphz ligand (**MZ**$^{2+}$, **Z**$^{4+}$) or the basic complex $[Ru(phen)_3]^{2+}$. Third, there is little difference in the cytotoxicity for structures containing two ruthenium centers over those with one.

It is also apparent that the non-phenanthroline ligand is a major determinant in the cytotoxicity. Replacement of one phenanthroline in $[Ru(phen)_3]^{2+}$ with tpphz leads to a two-fold improvement in cytotoxicity whereas replacement with tatpp leads to an approximate 6-fold increase in cytotoxicity. This shows that the types of ligands surrounding the metal center are a key factor in determining the toxicity of the complex. At this time we speculate that the complexes with the tatpp ligand are the most cytotoxic because of their DNA cleaving ability compared to tpphz complexes.[22,35] While these studies were conducted *in vitro*, it is reasonable to assume that these DNA binding complexes are acting on the cellular DNA and that tatpp complexes are better at damaging DNA than tpphz complexes.

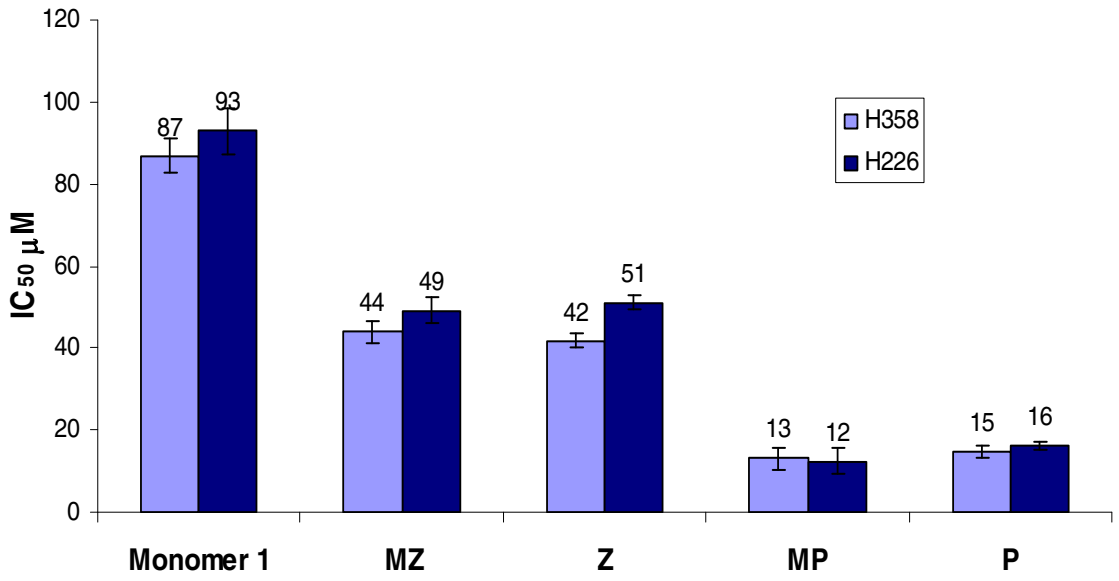
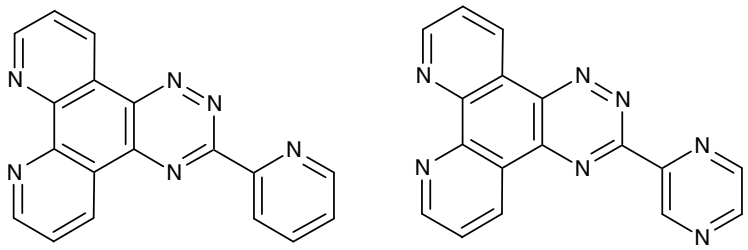



Fig 2.4 $IC_{50}$ of *rac/mix* Ru(II) complexes in H358 and H226 cells.

It is clear from these data that the cytotoxicity of cationic ruthenium polypyridyl complexes can be modulated to a significant degree by the nature of the attached ligands. With tatpp, we suspect it is the redox active nature of this ligand that is responsible, in part, for the observed cytotoxicity. We speculate that the complex intercalates into the cellular DNA and is readily reduced by common cellular reductants, such as glutathione (GSH), to form DNA cleaving species.

Liu et al also examined the cytotoxicity of some cationic ruthenium polypyridyl complexes in a number of cancer cell lines.[43] As seen in Table 2.2, these complexes have the general formula of $[Ru(bpy)_2(L\text{-}L)]^{2+}$, $[Ru(bpy)(L\text{-}L)_2]^{2+}$ or $[Ru(phen)_2(L\text{-}L)]^{2+}$, where L-L is 3-(pyrazin-2-yl)-1,2,4-triazino-[5,6-*f*]-1,10-phenanthroline (pztp) or 3-(pyridin-2-yl)-1,2,4-triazino[5,6-*f*]-1,10-phenanthroline) (pytp) both of which are shown in Figure 2.5.



**pytp** **pztp**

Fig 2.5 Structures of polypyridyl ligands in the cytotoxicity assay by Liu *et al.*[43]

Table 2.2 Cytotoxicity results of ruthenium polypyridyl complexes

| Complexes | $TC_{50}$ (μM) | | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|---|---|
| | MDCK | Vero | HL-60 | BEL-7402 | KB | HELA |
| $[Ru(bpy)_2(pztp)]^{+2}$ | 480 | 500 | 6.7 | 12.6 | 6.9 | 12.3 |
| $[Ru(pztp)_2(bpy)]^{+2}$ | 500 | 520 | 9.8 | 6.5 | 12.3 | 24.5 |
| $[Ru(bpy)_2(pytp)]^{+2}$ | 400 | 460 | 65.4 | 9.6 | 14.6 | 6.8 |
| $[Ru(phen)_2(pytp)]^{+2}$ | 410 | 480 | 98.6 | >100 | >100 | >100 |

The tumor cell lines used were HL-60 (human leucocytoma), BEL-7402 (human liver carcinoma), KB (human nasopharyngeal carcinoma), and HELA (human adenocarcinoma of the cervix). The control animal cells used were MCDK (Madin-Darby Canine Kidney cells) and Vero (kidney epithelial cell from African green monkey).

As in the case of **$MZ^{2+}$** and **$MP^{2+}$**, simple substitution of one bipyridine ligand with either pztp or pytp leads to complexes with $IC_{50}$ values on the order of 6 to 15 uM for most cell lines examined (Table 2.2). Substitution of two bipyridines seemed to have a negligible effect. Significantly, the complex with phenanthroline ligands $[Ru(phen)_2(pytp)]^{2+}$ was more than 10 times less cytotoxic than the bipyridine analogue $[Ru(bpy)_2(pytp)]^{2+}$. This suggests that the bpy

analogues of **MP**$^{2+}$ and **P**$^{4+}$ may be significantly more cytotoxic than the existing phenanthrolines and this needs to be tested.

Also shown in Table 2.2 are the $TC_{50}$ values for these complexes from which it is evident that these complexes are relatively non-toxic to normal animal cells. The complex $[Ru(bpy)_2(pztp)]^{+2}$ shows the best results compared to the other complexes in the study. On comparing these with the monomeric complexes in our study, we find that their $IC_{50}$ values are similar.

Having determined that the longer bridges on various ruthenium(II) polypyridyl complexes play an important part in the cytotoxicity, we wanted to further investigate if chirality could also contribute to the effect. All of the complexes under investigation contain either one or two chiral centers. So, we first tested the complex $[Ru(phen)_3]^{+2}$. It can be resolved in two enantiomers, **Δ-**form and **Λ-**form.

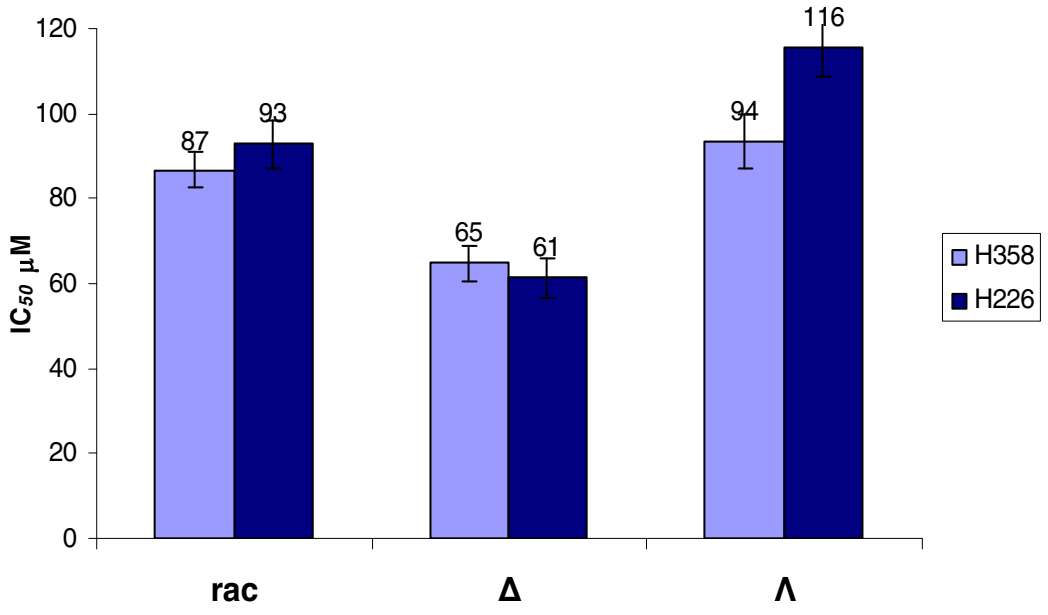



Fig 2.6 $IC_{50}$ of various isomers of $[Ru(phen)_3]^{2+}$ in H358 and H226 cells.

The MTT-assay involving the racemate and the individual enantiomers of the complex $[Ru(phen)_3]^{2+}$ clearly showed that the **Δ**-isomer is approximately twice as potent as **Λ**-isomer and shows favorable activity than the racemate (Fig 2.6). Barton *et al.* characterized the binding of **Λ**- and **Δ**- $[Ru(phen)_3]^{2+}$ to the DNA duplex. As shown in Fig. 2.7, one of the phenanthroline ligands of the **Δ**-isomer intercalates easily in-between the DNA base-pairs whereas the **Λ**-isomer does not fit into the right-handed DNA helical groove due to unfavorable steric interference.[15]

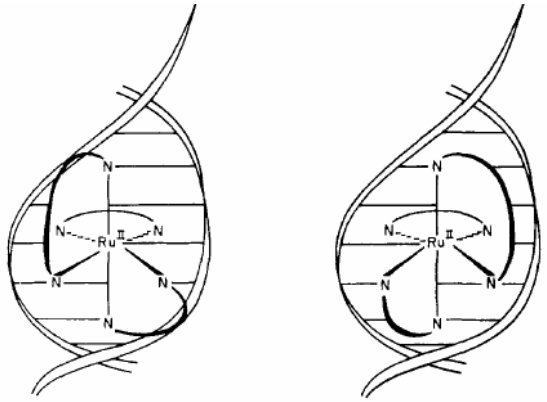



Fig 2.7 Pictorial Representations of (a) **Λ**-$[Ru(phen)_3]^{2+}$ and (b) **Δ**-$[Ru(phen)_3]^{2+}$ bound to B-DNA. Barton *et al. JACS* **1984**, *106*, 2176

Hiort *et al.* observed some changes in the binding affinities of Δ- and Λ-$[Ru(phen)_2dppz]^{2+}$ with intrinsic binding constant $K_b$ in the range of $10^7$-$10^8$ $M^{-1}$. Lincoln and co-workers showed that there is preferential binding of the dimeric complex **ΔΔ**-$[(phen)_2Ru[dppz(11,11')dppz]Ru(phen)_2]^{+4}$ to the DNA as compared to the **ΛΛ**-isomer with $K_b \sim 10^{12}$ $M^{-1}$. In an animal toxicity experiment by Dwyer *et al.,* **Δ**-$[Ru(phen)_3]^{+2}$ was twice as toxic as **Λ**-$[Ru(phen)_3]^{+2}$, the toxic doses being 18.4 mg/kg and 9.2 mg/kg. All these data suggest that stereochemistry

influences the efficacy of these complexes interacting with biological systems.[18,19,23] We also performed similar experiments with all other monomeric complexes and found that the activity of **Δ**-form almost always surpasses that of **Λ**- form (Fig 2.8).

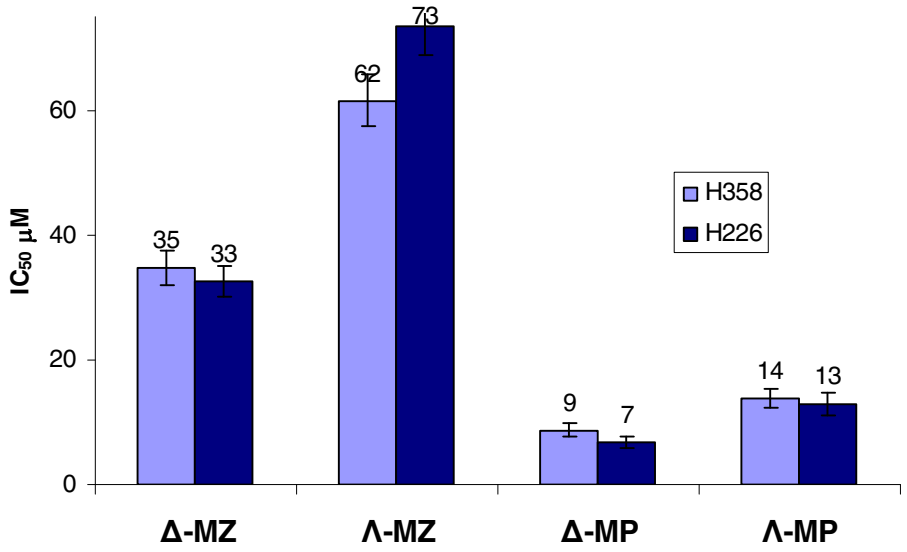
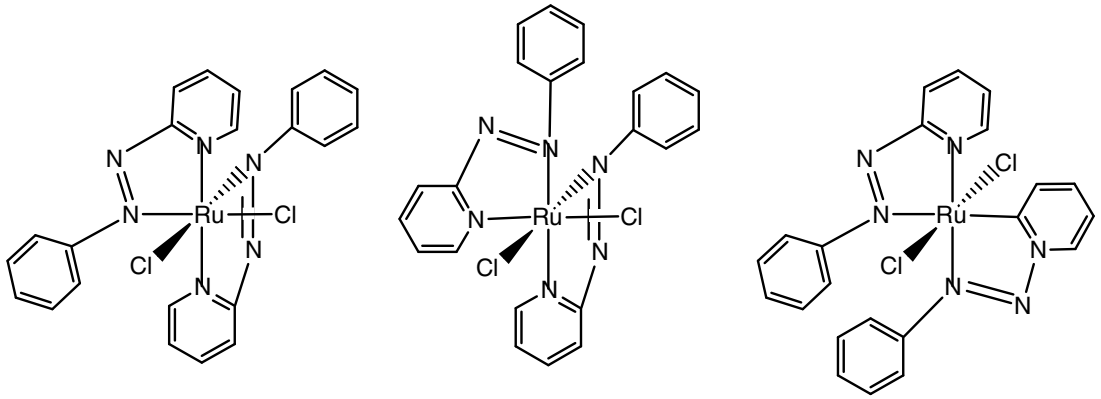



Fig 2.8 $IC_{50}$ of **Δ-** vs. **Λ-** enantiomers of complexes $\mathbf{MZ^{+2}}$ and $\mathbf{MP^{+2}}$

In support of our study, strong differences in the *in vitro* cytotoxicity of three isomeric forms of dichlobis(2-phenylazopyridine)ruthenium(II) complex were reported by Velders *et al.* The structures of the complexes α–$[Ru(azpy)_2Cl_2]$ (α–Cl), β–$[Ru(azpy)_2Cl_2]$ (β–Cl), γ–$[Ru(azpy)_2Cl_2]$ (γ–Cl) are given in Fig. 2.9.




**α–Cl** **β–Cl** **γ–Cl**

Fig. 2.9 Structures of three isomers of $[Ru(azpy)_2Cl_2]$

The cytotoxicity of the isomers, α–Cl, β–Cl, and γ–Cl was assayed in seven human tumor cell lines – MCF-7 (breast cancer), EVSA-T (breast cancer), WIDR (colon cancer), IGROV (ovarian cancer), M19 MEL (melanoma), A498 (renal cancer), and H226 (non small cell lung cancer) and compared with that of the well-known anticancer drugs, cisplatin and 5-fluorouracil. Of the three isomers, α–Cl was ten times more toxic than the other isomers in most of the cell lines under study and its $IC_{50}$ values were comparable with that of the commercial available drugs (Table. 2.3). It is clear from this data that there exists a stereochemical basis for the structure-activity relationship between the ruthenium complexes and the target molecules such as DNA in the physiological system. The co-ordination around the ruthenium metal center plays a crucial role in its biological activity. This may be the underlying reason for higher cytotoxicity of α–Cl than the γ–Cl complex, where the ligands are *cis* to each other. Steric hindrance of bidentate ligands towards the free co-ordination sites may be another imperative factor to be considered in lowering the potency of the γ–Cl

complex.[44] This result is closely related to our study, the only difference being the absence of labile ligands in our complexes. But it is already known in the literature that Ru(II) complexes with polycyclic planar heteroaromatic ligands intercalates between the DNA bases unlike those complexes with labile ligands which form adducts with the DNA.[5,28]

Table 2.3 $IC_{50}$ μM of the various isomers of $[Ru(azpy)_2Cl_2]$ in comparison with cisplatin (CPT) and 5-fluorouracil (5-FU).

| | MCF-7 | EVSA-T | WIDR | IGROV | M19 | A498 | H226 |
|---|---|---|---|---|---|---|---|
| α–Cl | 0.6 | 0.1 | 1.9 | 0.8 | 102 | 1.2 | 1.5 |
| β–Cl | 4.1 | 1.9 | 11.2 | 7.3 | 1647 | 8.8 | 10.0 |
| γ–Cl | 5.9 | 5.4 | 16.6 | 11.8 | 2437 | 15.3 | 14.8 |
| CPT | 5.8 | 3.7 | 1.7 | 2.3 | 442 | 1.1 | 2.6 |
| 5-FU | 2.3 | 1.4 | 3.2 | 0.6 | 558 | 7.5 | 10.9 |

The diastereotopic mixture of dimeric complexes (*mix*), such $\mathbf{Z^{4+}}$ and $\mathbf{P^{4+}}$**,** is a combination of three isomers, **ΔΔ**-, **ΛΛ**- which are chiral and an achiral isomer, **ΔΛ**-*meso*. The structural difference between $\mathbf{Z^{4+}}$ and $\mathbf{P^{4+}}$ is the bridging ligand; $\mathbf{P^{4+}}$ has tatpp ligand and $\mathbf{Z^{4+}}$ has tpphz ligand. The pictorial representation of the various isomers of $\mathbf{P^{4+}}$ is shown in Fig 2.11. We obtained the same result as the monomeric complexes; **ΔΔ**- was more effective than the other isomeric forms in both $\mathbf{Z^{4+}}$ and $\mathbf{P^{4+}}$. As mentioned earlier, complexes with tatpp bridging ligand, $\mathbf{MP^{2+}}$ and $\mathbf{P^{4+}}$**,** had the lowest $IC_{50}$ values compared to complexes with tpphz bridging ligand, $\mathbf{MZ^{2+}}$ and $\mathbf{Z^{4+}}$. The geometric configuration around both the Ru(II) chiral metal centers ought to be **Δ** in order to increase the efficiency of the complex to suppress cell growth. The *meso* complex which has

one metal centre in **Δ** configuration and the other in **Λ** configuration is not as effective as the **ΔΔ**-isomer in both dimers (Fig 2.10).

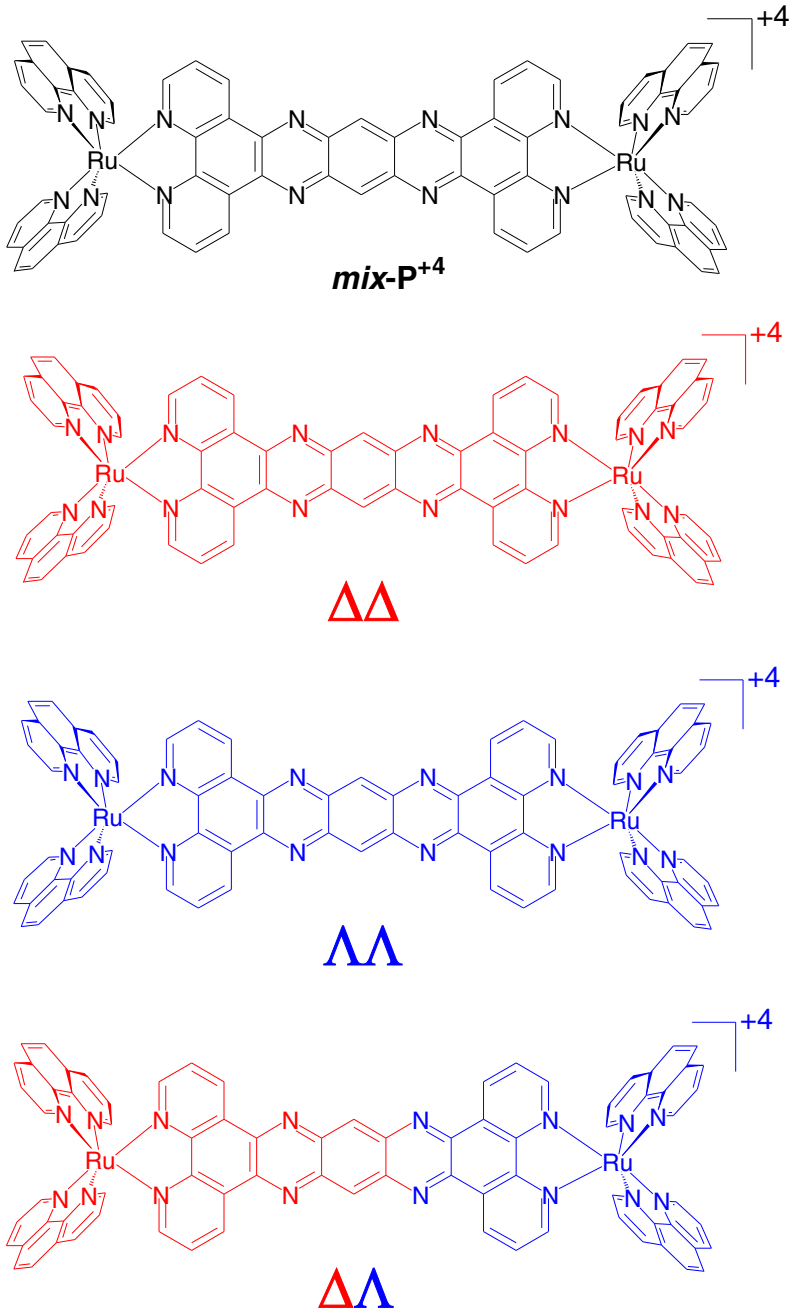
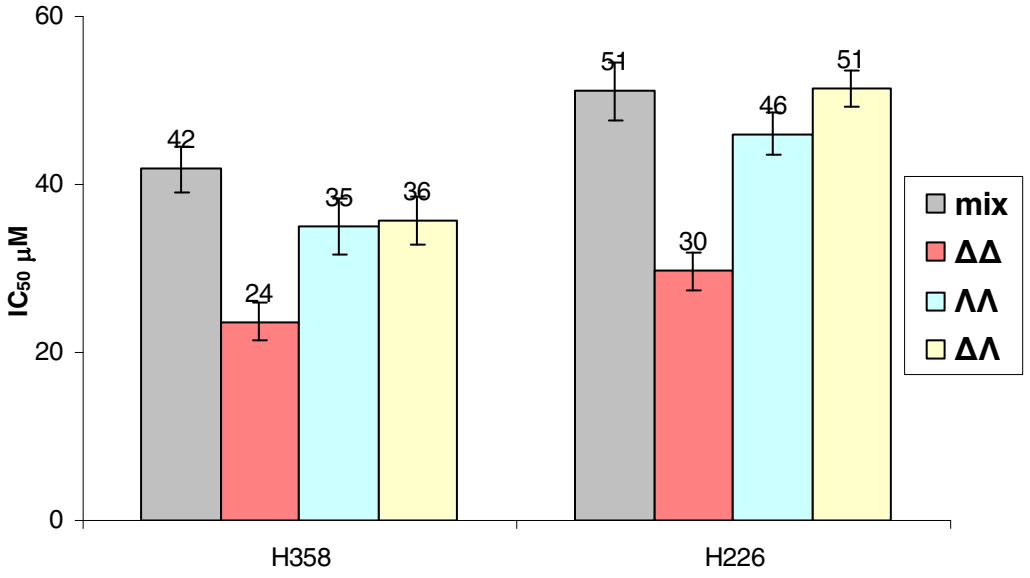
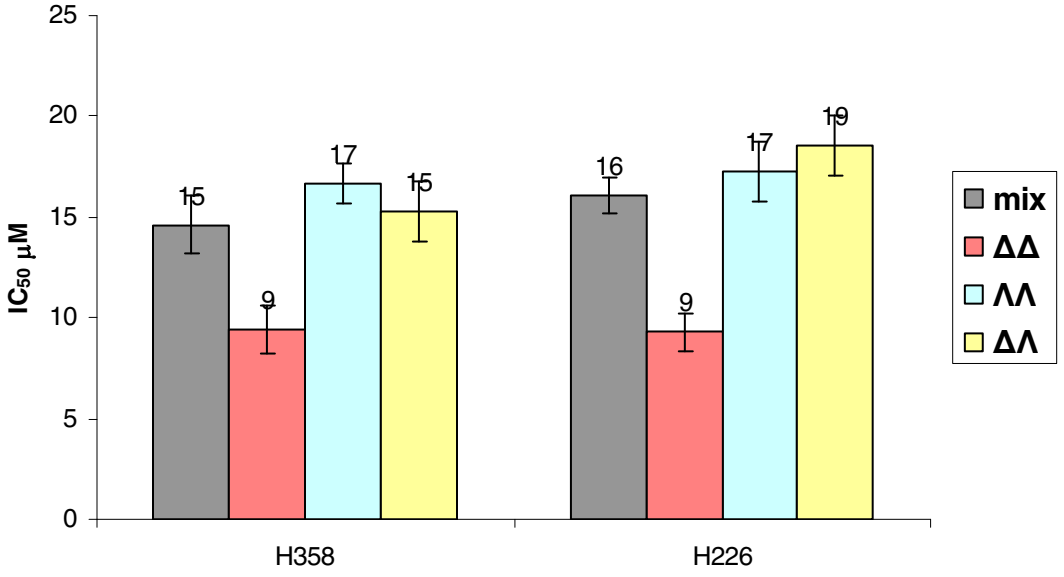



Fig 2.10 Structures of the diastereotropic mixture of complex **P$^{4+}$**




(a.)




(b.)

Fig 2.11 Effect of Stereochemistry in (a.) complex $\mathbf{Z}^{4+}$ and (b.) complex $\mathbf{P}^{4+}$

We took this investigation a step further and examined the effect of these Ru(II) complexes on healthy/noncancerous cells based on chirality and length of the bridging ligands. In designing any anticancer drug, the ideal scenario anticipated will be for the drug to be selectively toxic to cancer cells and relatively less toxic to normal cells. We performed the same MTT assay on two types of normal cells, namely Human Aorta Vascular Smooth Muscle Cells (HAVSMC) and Human Umbilical Vein Endothelial cells and the various isomers of complexes **$MP^{2+}$**, **$Z^{4+}$** and **$P^{4+}$** were tested. The results are tabulated in Table 2.4.

Table 2.4 $IC_{50}$ of some Ru(II) complexes in normal cell lines

| **Compound Name** | **Cell Line** | **$IC_{50}$ μM** | | | |
|---|---|---|---|---|---|
| | | **Mix** | **ΔΔ** | **ΛΛ** | ***Meso*** |
| **$Z^{4+}$** | HAVSMC<br>HUVEC | 78.7±4.6<br>86.8±5.0 | 93.6±5.7<br>80.2±5.2 | 98.1±6.3<br>88.5±5.8 | 89.4±5.3<br>85.4±4.8 |
| **$MP^{2+}$** | HAVSMC<br>HUVEC | 99.4±7.0<br>93.9±5.4 | 100.3±6.0<br>91.6±4.7 | 91.2±5.1<br>104.4±5.5 | N/A<br>N/A |
| **$P^{4+}$** | HAVSMC<br>HUVEC | 133.3±10.6<br>132.5±8.7 | 104.5±6.8<br>118.0±7.0 | 124.9±7.6<br>127.3±8.6 | 116.2±6.7<br>135.9±10.4 |

As shown in Table 2.4, there is no significant difference in the $IC_{50}$ values in the various isomers for a given complex in a particular cell line. It is explicit from the results that chirality has little role to play in toxicity of these complexes in normal cells. However, the important aspect of this assay in normal cells is the overall increase of $IC_{50}$ values compared to the ones in cancer cells. We can thereby deduce that some of these complexes are selectively toxic to cancer cells and presumably have a large therapeutic range. A therapeutic window is

defined as the range in drug concentration that can be administered without causing any toxic effect as a result of the drug treatment. For example, $IC_{50}$ of **ΔΔ-P$^{4+}$** in H358 cancer cells is 9.5 μM while the $IC_{50}$ of the same complex in HUVEC is 118.0 μM, an almost ten-fold increase, whereas the therapeutic window for complex **Z$^{4+}$** in the same cell line is only four-fold. Thus, as the therapeutic window increases, the potency of the drug also increases. The index for **Λ/ΛΛ-** also decreases as their $IC_{50}$ values are greater than that of their enantiomers. The therapeutic window for **Δ/ΔΔ-** isomers of complexes **Z$^{4+}$**, **MP$^{2+}$** and **P$^{4+}$** are given in Fig 2.12.

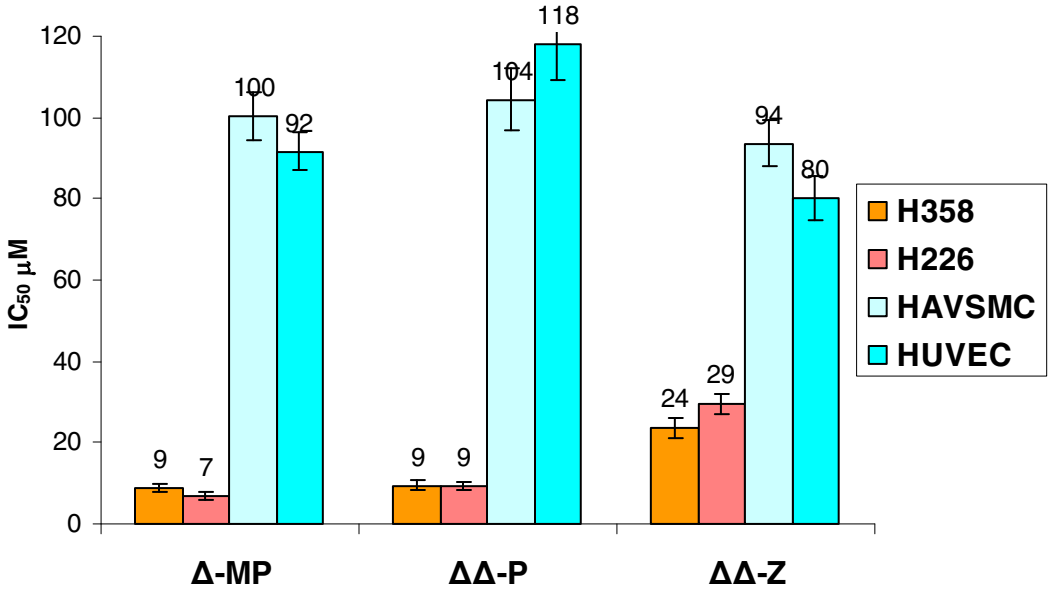



Fig 2.12 Therapeutic windows of **Δ-MP$^{2+}$**, **ΔΔ-P$^{4+}$** and **ΔΔ-Z$^{4+}$** in various cell lines.

## 2.4 Summary and Conclusion

Cytotoxicity screening of a series of monomeric and dimeric ruthenium(II) polypyridyl complexes as racemate/diastereotopic mixtures and their enantiopure forms was conducted with the objective of finding new antineoplastic agents for cancer therapy. Complex **MP**$^{4+}$ and its dimeric analog **P**$^{4+}$ showed encouraging results as potential antineoplastic agents with $IC_{50}$ values near those of cisplatin for H358 and H226 cells and significantly less cytotoxicity with normal healthy cells. The stereochemistry of these Ru(II) complexes has a measurable effect on the activity with **Δ or ΔΔ isomers being the most active.** The therapeutic index for complexes with tatpp ligand, **P**$^{4+}$ is by far the larger compared to the complex with tpphz ligand, **Z**$^{4+}$. Thus, in conclusion, a structure-toxicity relationship exists between the various stereoisomers of potential Ru(II) complexes which can be utilized in formulating a developing cancer remedy.

# CHAPTER 3

## RESOLUTION AND DETERMINATION OF THE ENANTIOPURITY OF RUTHENIUM POLYPYRIDYL COMPLEXES

### 3.1 Introduction

The helical chirality inherent in octahedral transition metal complexes bound by three bidentate ligands has fascinated chemists for over a century now. The right- and left-handed configurations of these metal complexes are referred to as **Δ-** and **Λ-** enantiomers, respectively (Figure 3.1).[45] Derivatives of $[Ru(bpy)_3]^{2+}$ and $[Ru(phen)_3]^{2+}$ have enjoyed a substantial amount of attention owing to the chemically robust nature of the complexes and the favorable electrochemical and photophysical properties. In addition, the skeletal rigidity and variable functionality of such Ru(II) complexes have made them potential candidates for chirooptical and chiral recognition applications.[46,47] They also have shown potential as DNA probes because of their stereoselective interactions with DNA.[18,43] They can be used as building blocks in the synthesis of a variety of supramolecular assemblies.[36,45,48]

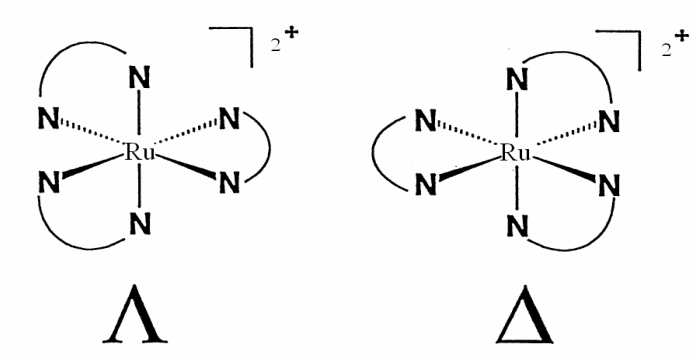
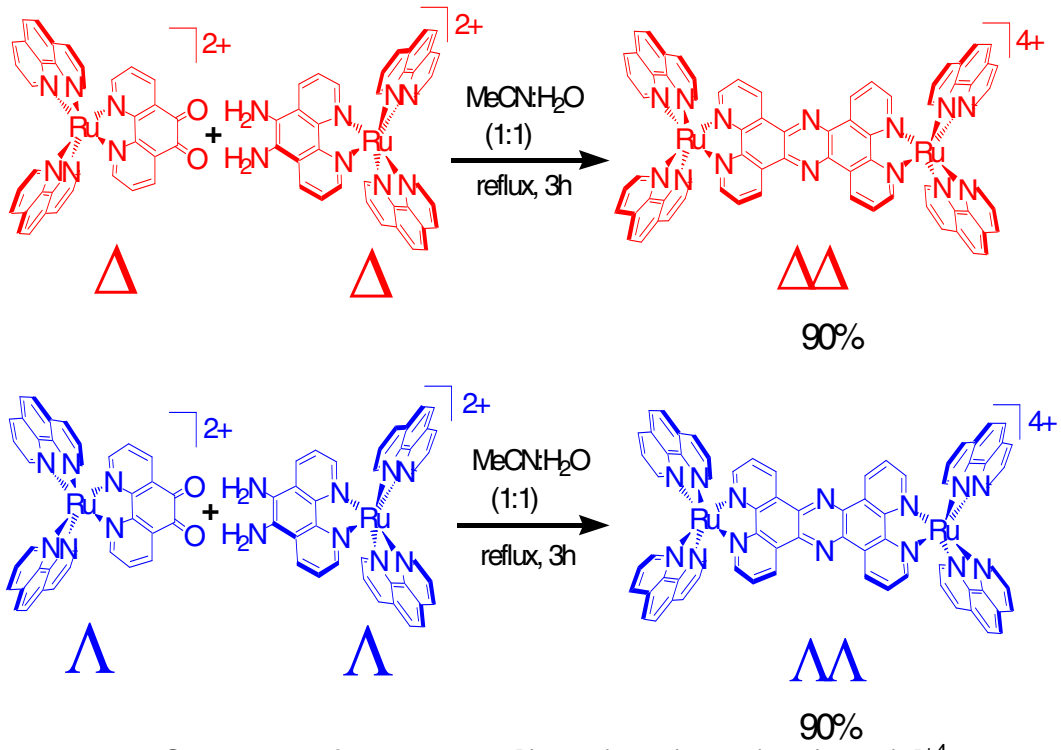



Figure 3.1 Mirror image relationship of monomeric Ru(II) trisdiimine enantiomers.

Many of these applications require stereochemically pure compounds or at least the knowledge of the stereochemical composition. The best approach to ensure stereochemical control in the synthesis of ruthenium tris-diimine complexes is to start with enantiomerically pure monomers, thereby forming only one isomer of the complex. Stereospecific synthesis of polynuclear complexes utilizing enantiopure, substitutionally inert ruthenium tris-diimine complexes as chiral synthons has already been reported in literature by our group.[37,45,49] To cite an example, the periphery of the coordinated ligands (eg. 1, 10-phenanthroline) can be modified to other ligands such as phendione and phendiamine, which can be coupled in an irreversible Schiff's base condensation to form the tpphz (tetrapyrido[3,2-a:2'3'-c:3"2"-h:2"'3"'-j]phenazine) bridging ligand between the two metal centers as shown in Scheme 3.1.




Scheme 3.1 Stereospecific synthesis $[(phen)_2Ru(tpphz)Ru(phen)_2]^{+4}$ complex

This approach guarantees retention of stereochemistry at the metal centers as it does not involve the making or breaking bonds at the metal stereocenter in contrast to the ligand displacement approach. Chiral dendritic assemblies can be obtained using branching chiral synthons, such as **Δ-**$[Ru(phendione)_3]^{2+}$, which can undergo the condensation reaction multiple times with excess **Δ-**$[Ru(phen)_2phendiamine]^{2+}$. The resulting first-generation metallodendrimer, $[(\mathbf{\Delta}(phen)_2Ru(tpphz))_3\text{-}\mathbf{\Delta}\text{-}Ru]^{4+}$, is a rigid and enantiopure assembly of 4 chiral metal centers with overall $D_3$ symmetry. Oxidation of the peripheral phenanthrolines on this tetramer to phendiones leads to a core which can be used to form enantiomerically and diastereomerically pure decametallic dendrimers of the type shown in Figure 3.2.[45,49] This high level of stereospecific

control can be used in the development of new chiral host complexes, chiral catalysts, chiral porous solids and other important aspects of nanotechnology.[50]

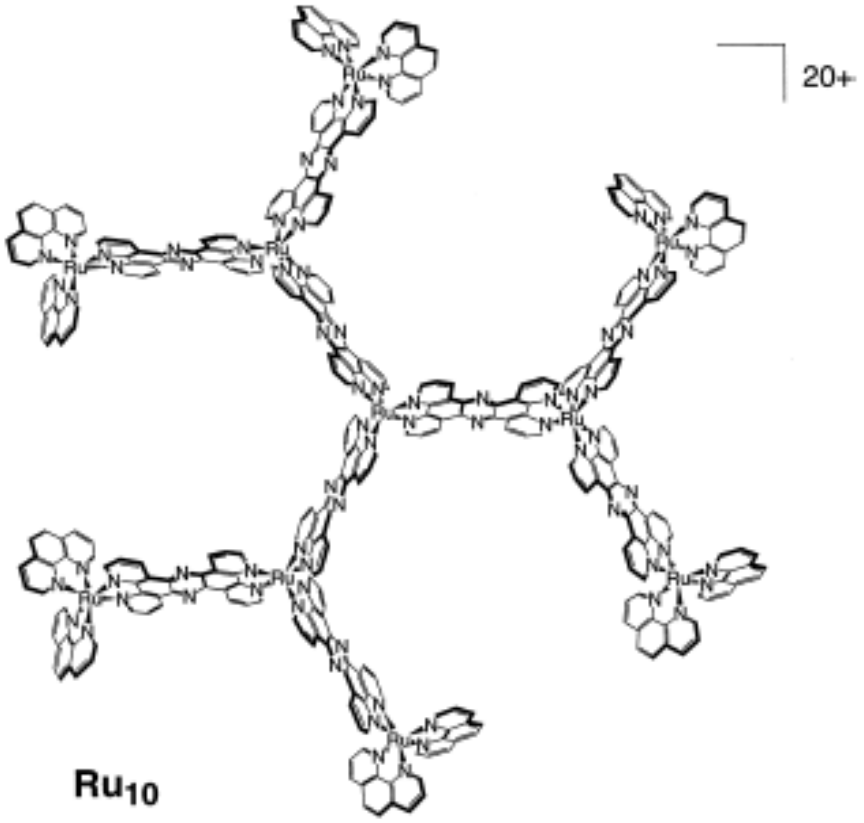



Fig 3.2 Structure of decametallic ruthenium dendrimer. MacDonnell *et al. Coord. Chem. Rev.* **1999**, *185-186, 535-549.*

In the synthesis of chiral multi-metallic species such as dimers, tetramers, dendrimers, and polymers, one approach, as discussed above, is to begin with enantiomerically pure monomers in order to establish stereochemistry. Another approach is to simply make the target without concern of the local stereochemistry and then to use chromatographic methods to separate the diastereomers and enantiomers. For example, the various stereoisomers of the tri-metallic complex $[(Ru(bpy)_2)_3(\mu\text{-HAT})]^{6+}$ (where HAT is the tritopic ligand

1,4,5,8,9,12-hexaazatriphenylene), and related mono- and di-metallic analogues have been separated by cation exchange chromatography by Keene and his coworkers. SP-Sephadex C25 is used as the support and the stereoisomers are eluted with aqueous sodium toluene-4-sulfonate, sodium (-)-O,O'-dibenzoy-L-tartrate, or sodium (+)-di-4-ditoluyl-D-tartrate solutions. They were able to separate the *meso* (**ΔΛ**) and *rac* (**ΔΔ/ΛΛ**) diastereomeric forms of the dimeric species $[(Ru(bpy)_2)_2(\mu\text{-HAT})]^{4+}$ and also the enantiomers of the *rac* form, **ΔΔ** and **ΛΛ**, separately. In addition to the dimers, the trinuclear ruthenium complex $[(Ru(bpy)_2)_3(\mu\text{-HAT})]^{6+}$ was successfully separated into their homochiral forms ($\mathbf{\Delta^3/\Lambda^3}$) and its heterochiral forms ($\mathbf{\Delta^2\Lambda/\Lambda^2\Delta}$) and enantiomers of both the forms were also isolated individually.[51] While this method works, the yields of each stereoisomer are determined statistically and the actual amounts of complexes that could be isolated in this manner are usually relatively small (e.g. 1-20 mg).

Resolution and synthesis of chiral synthons is a relatively simple process, the most common procedure being resolution by crystallization. When diastereomeric salts such as sodium arsenyl L(+) or D(-) tartrate salts are added to the racemate in solution, the **Δ-** or **Λ-** enriched crystals respectively precipitate out via the lock and key mechanism involving the stereocenters of the tartrate salt and metal complex. The enriched salts are isolated by filtration and arsenyl tartrate is knocked off with mild acid, leaving the pure enantiomer in solution. Enantiopurity of the enantiomer can be determined by various methods such as Circular Dichroism (CD), Nuclear Magnetic Resonance Spectroscopy (NMR) and crystallization techniques. But, CD spectra do not give the absolute purity of the

complexes. Torres *et al.* performed an NMR experiment with chiral lanthanide shift reagents to analyze the stereochemical outcome of a harsh oxidation reaction of Λ-$[Ru(phen)_3]^{2+}$ to Λ-$[Ru(phendione)_3]^{2+}$. As low as 5% of the minor enantiomer was detected by this method, this implies that the chirality of the complex is retained.[36] These are strictly analytical methods that can not be used to purify the individual enantiomers.

Over the past two decades, enantiomeric separations based on liquid chromatography (LC) using chiral stationary phases (CSPs) have gained importance in various fields such as pharmaceuticals, governmental bodies such as FDA, environmental sciences. In chemistry, high performance liquid chromatography (HPLC) with CSPs plays a key role in determining the enantiopurity of new synthesized chiral complexes. It is widely used both as an analytical method and as a preparative tool.

In this chapter, we describe the synthesis of two new ruthenium trisdiimine complexes, $[Ru(phen)_2nitrophen]^{+2}$ and $[Ru(phen)_2aminophen]^{+2}$, and a simplified procedure by which these and some related complexes can be resolved into enantiomers. We also describe the application of HPLC methods for the chiral separation of these complexes and apply this technique to determine the absolute enantiopurity to a high degree of accuracy, especially when compared to other more commonly used methods such as Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR). The monomeric chiral complex containing the amine functionality should be a useful chiral synthon for the stereospecific

syntheses and potentially as a chiral selector when immobilized on a chromatographic stationary phase.

## 3.2 Experimental Section

### *3.2.1 Materials*

The compounds 1,10-phenanthroline (phen), 20% fuming sulfuric acid (18-24% free $SO_3$), arsenic(III) oxide, L(+)- and D(-)-tartaric acid, tetra-n-butylammonium chloride hydrate and hydrazine monohydrate were purchased from Alfa Aesar and Ruthenium(III) Chloride trihydrate was purchased from Pressure Chemical Company and used without further purification. The column Cyclobond I 2000 RN (naphthylethyl carbamate, 250X46 mm) was obtained from Advanced Separation Technologies. Acetonitrile and methanol of HPLC grade were purchased from EMD and palladium on carbon (Pd/C, 10%) and ammonium nitrate from Sigma-Aldrich. $Ru(phen)_2Cl_2$ was prepared by following a reported literature procedure.[3,52]

### *3.2.2 Instrumentation*

$^1H$ NMR spectra were obtained on a JEOL Eclipse Plus 500 MHz spectrometer using solvent $CD_3CN$. Chemical shifts were given in ppm and referenced to TMS. UV-visible spectra were obtained on a Hewlett-Packard HP8453A spectrophotometer in $CH_3CN$. Circular Dichroism (CD) spectra were recorded on a Jasco 710 spectrophotometer in $CH_3CN$. The chromatographic system was a HP (Agilent Technologies, Palo Alto, CA, USA) 1050 system with a UV VWD detector, an autosampler, a quaternary pump and Chemstation software.

*3.2.3 Synthesis of Sodium Arsenyl (+) or (-) Tartrate, $Na_2(As(+)tart)_2$ and $Na_2(As(-)tart)_2$*

The sodium salts of arsenyl L(+) or D(-) tartrate were synthesized using the literature procedure first reported by G.G.Henderson[53] in 1895 and are reproduced here with a slight modification. Only the synthesis of $Na_2(As(+)tart)_2$ is described as the preparation of the enantiomeric (-) salt is identical except that D(-) tartaric acid is used instead of L(+) tartaric acid.

L(+) tartaric acid (20 g, 0.133 mol) and NaOH (5.33 g, 0.133mol) were dissolved in water (150 mL) and the solution was heated to reflux. $As_2O_3$ (13.1 g, 0.066 mol) was added and the resulting slurry refluxed for 15 min upon which the solution became clear. The solution was filtered while hot and 300 mL ethanol was added. The resulting solution was cooled to 4°C then filtered, washing with cold ethanol. The product was air dried. The salts were obtained in good yield of about 29g (90%).

*3.2.4 Synthesis of 5-nitro-1, 10-phenanthroline (nitrophen).*

The compound was prepared by following an established literature procedure[54,55], with slight modifications added to help remove sodium sulfate and 1,10-phenanthroline-5,6-dione (phendione) impurities. Furthermore the original literature did not report NMR data so this is included for completeness.

1,10 phenanthroline (15 g, 83 mmol) was added to 75 mL of 20% fuming sulfuric acid in small portions and stirred until completely dissolved. The reaction mixture was then heated to 120°C and 40 mL of concentrated $HNO_3$ was added dropwise. Temperature was maintained between 165-170°C using oil bath, and the mixture was refluxed for 1 h. 30 min. The resulting orange solution was

cooled to room temperature and poured over ice. Using aqueous NaOH (20 M), the stirred solution was slowly neutralized to a pH of approximately 6, during which time a yellow precipitate formed. The crude product was washed thoroughly with cold water and was recrystallized using absolute ethanol and filtered to rid the product of salts. The product was then recrystallized using hot water with phendione impurities remaining in the water. The pure product was filtered out. Yield – 11g (61%) Anal. Calcd for $C_{12}H_7N_3O_2.H_2O$: C, 59.26; H, 3.73; N, 17.28. Found: C, 58.74; H, 3.57; N, 17.31. $^1$H NMR (500 MHz, $CD_3CN$): 9.28 (1H, d, $^3J$ = 3 Hz), 9.23 (1H, d, $^3J$ = 4 Hz), 8.92 (1H, dd, $^3J$ = 9 Hz, $^4J$ = 2 Hz), 8.79 (s, 1H), 8.58 (1H, dd, $^3J$ = 8 Hz, $^4J$ = 2 Hz), 7.87 (1H, dd, $^3J$ = 8 Hz, $^4J$ = 3.5 Hz), 7.84 (1H, dd, $^3J$ = 8 Hz, $^4J$ = 3.5 Hz).

*3.2.5 Synthesis of [Ru(phen)$_2$nitrophen](PF$_6$)$_2$*

The compound $Ru(phen)_2Cl_2$ (1.05 g, 1.97 mmol) was dissolved in a 1:1 mixture of water and ethanol (50 mL) and heated under $N_2$ atmosphere. Once the reaction mixture began to reflux, 5-nitro-1,10-phenanthroline (0.525 g, 2.3 mmol) was added in 100mg portions. The mixture was refluxed 12 h and cooled to room temperature. The solution was filtered and the red product was precipitated as a $PF_6$ salt upon the addition of aqueous $NH_4PF_6$. The product was oven dried at 60°C in vacuo. Yield – 1.2g (70%). Anal. Calcd for $RuC_{36}H_{23}N_7O_2P_2F_{12}$: C, 44.27; H, 2.38; N, 10.04. Found: C, 43.65; H, 2.35; N, 10.11. $^1$H NMR (500 MHz, $CD_3CN$): 9.15 (1H, s), 9.06 (1H, d, $^3J$ = 9 Hz), 8.76 (1H, d, $^3J$ = 8 Hz), 8.63 (1H, br. S), 8.61 (2H, br. S), 8.59 (1H, d, $^3J$ = 3.5 Hz),

8.26 (4H, s), 8.20 (1H, d, $^3J$ = 5 Hz), 8.15 (1H, d, $^3J$ = 7 Hz), 8.04 (2H, m), 7.99 (2H, m), 7.73 (2H, m), 7.63 (4H, m).

*3.2.6 Resolution of* ***Λ*** *–[Ru(phen)$_2$(nitrophen)]Cl$_2$*

The chloride salt of the compound [Ru(phen)$_2$(nitrophen)](PF$_6$)$_2$ was obtained by separately dissolving the compound and tetra-n-butylammonium chloride hydrate in dry acetone. The two solutions were combined and the orange-brown precipitate was filtered out and oven dried at 60°C in vacuo. Racemic [Ru(phen)$_2$(nitrophen)]Cl$_2$ (1.0g) was dissolved in hot water (25 mL). A solution of sodium arsenyl L(-) tartrate (2.25 g in 30 ml hot water) was added into the racemic solution while stirring vigorously. The solution was chilled at 4°C overnight. The supernatant was captured by filtration and warmed to 80°C. A solution of sodium arsenyl D(+) tartrate (1.25g in 15 ml hot water) was added to the supernatant while stirring. The solution was again chilled overnight at 4°C and then filtered. The precipitate was suspended in hot $HNO_3$ (50 mL, 2M) until complete dissolution and the resulting solution was treated with aqueous $NH_4PF_6$. The greenish brown precipitate was filtered, washed with water, and oven dried at 60°C. Yield - 60%. UV/vis: [$\lambda_{max}$, nm ($\varepsilon$ $M^{-1}cm^{-1}$)]: 445 (18200). CD for Λ-[Ru(phen)$_2$(nitrophen)](PF$_6$)$_2$ [$CH_3CN$, $\lambda_{max}$, nm ($\Delta\varepsilon$, $M^{-1}cm^{-1}$)]: 414.8 (-15.6), 464.5 (15.3). The procedure for the resolution of **Δ-**[Ru(phen)$_2$(nitrophen)](PF$_6$)$_2$ was identical to the above mentioned procedure except for the order in which the sodium arsenyl tartrate salts were added. The other complexes such as [Ru(phen)$_2$(phendione)]$^{2+}$ and [Ru(phen)$_3$]$^{2+}$ were also chirally resolved into **Δ-** and **Λ-** using the same procedure mentioned above.

UV/vis for Λ-[Ru(phen)$_2$(phendione)](PF$_6$)$_2$ : [$\lambda_{max}$, nm (ε M$^{-1}$cm$^{-1}$)]: 434 (16800). CD for Λ-[Ru(phen)$_2$(phendione)](PF$_6$)$_2$ [CH$_3$CN, $\lambda_{max}$, nm (Δε, M$^{-1}$cm$^{-1}$)]: 413.9 (-11.3), 455.7 (18.3). UV/vis for Λ-[Ru(phen)$_3$](PF$_6$)$_2$ : [$\lambda_{max}$, nm (ε M$^{-1}$cm$^{-1}$)]: 452 (19200). CD for Λ-[Ru(phen)$_3$](PF$_6$)$_2$ [CH$_3$CN, $\lambda_{max}$, nm (Δε, M$^{-1}$cm$^{-1}$)]: 419.6 (-19.2), 463.3 (14.5).

The HPLC experiment was performed by Ping Sun in Dr. Armstrong's lab to check the purity of the enantiomers of the complex [Ru(phen)$_2$(nitrophen)]Cl$_2$ under the following experimental conditions. The mobile phase was composed of 80%CH$_3$OH/20%CH$_3$CN/0.2%NH$_4$NO$_3$ and was degassed by ultrasonication under vacuum for 5 min. The flow rate was 1.0 mL/min. and the injection volume was 5 μL. The detection wavelength was 254 nm. All experiments were carried out at room temperature. [Ru(phen)$_2$(nitrophen)]Cl$_2$ was dissolved in CH$_3$OH.

*3.2.7 Synthesis of [Ru(phen)$_2$(aminophen)](PF$_6$)$_2$.*

A solution containing [Ru(phen)$_2$(nitrophen)]Cl$_2$ (0.40 g, 0.53 mmol) and Pd/C (0.5 g) in ethanol (50 mL) was purged with N$_2$ gas for 15 min and then heated to a gentle reflux. To this mixture, a solution of 6 mL N$_2$H$_4$·H$_2$O in 20 mL ethanol was added dropwise over a period of 1 h. while refluxing the solution. The reflux was continued for another 6 h. The solution was cooled to 4°C and filtered through a pad of Celite. The pad was washed with 30 mL of additional ethanol and the combined filtrates were concentrated on a rotary evaporator to one-third of the original volume. Addition of 10 mL of an aqueous solution of NH$_4$PF$_6$ (10%) gave a reddish orange precipitate which was isolated by filtration, washed with 5 mL pure water and oven dried at 60°C in vacuo. Yield – 401 mg

(80%). Anal. Calcd for $RuC_{36}H_{25}N_7P_2F_{12}.H_2O$: C, 44.82; H, 2.82; N, 10.16. Found: C, 44.90; H, 3.33; N, 9.70. $^1$H NMR (500 MHz, $CD_3CN$): 8.59 (5H, m), 8.25 (4H, d, $^3J$ = 3 Hz), 8.21 (1H, d, $^3J$ = 8.5 Hz), 8.03 (5H, m), 7.63 (6H, m), 7.39 (1H, dd, $^3J$ = 8.5 Hz, $^4J$ = 5 Hz), 7.19 (1H, s), 5.57 (2H, s).

## 3.3 Results and Discussion

### *3.3.1 Syntheses*

The compound 1,10-phenanthroline can undergo electrophilic aromatic substitution in the presence of fuming sulfuric acid and concentrated nitric acid to give 5-nitrophen. Since the original synthesis[54] dates back to late 1940's, the pH of the solution was adjusted in a crude manner by first neutralizing the acidic reaction mixture with a base and then lowering the pH back with a mild acid. In our procedure, we directly adjusted the pH to 6. Only the melting point and CHN analysis were reported in the article, we now report the NMR spectra of this ligand. The compound was synthesized following literature procedure[54] for the most part but with slight changes. The synthesis of this compound is similar to the synthesis of 1,10-phenanthroline-5,6-dione (phendione),[56] as a result, phendione also forms during the process, and can be eliminated by recrystallization from ethanol. The neutralization of the acid mixture with NaOH resulted in the formation of sodium sulfate salt, which can be removed with repeated washings with cold water. In $^1$H NMR (Fig 3.3), the characteristic proton, $H_d$, appears as a singlet (8.8ppm) and is shifted downfield when compared to the same proton in the compound 1,10-phenanthroline which is attributed to the effect of the neighboring electron-withdrawing nitro group. The

proton $H_c$ is also shifted downfield due to the same effect mentioned above and the signal appears as a doublet of doublet (8.92ppm) while Hc' signal is at 8.58ppm. The signal for the protons closest to the nitrogen, Ha and Ha' fall at 9.2ppm.

Goss and Abruna[57] first reported the synthesis of $[Ru(bpy)_2phendione]^{2+}$ in 1985, a similar method was followed to synthesize $[Ru(phen)_2phendione]^{2+}$ and $[Ru(phen)_2nitrophen]^{+2}$. This is a one pot synthesis in which $Ru(phen)_2Cl_2$ and a slight excess of nitrophen or phendione are refluxed in water-ethanol (1:1) to form this tris diimine complex. The mixtures were refluxed overnight and the complexes can be precipitated as their $(PF_6)^-$ salts respectively. In $^1H$ NMR of nitrophen complex (Fig 3.4), it is very evident that the $H_d$ signal (9.15ppm) is shifted significantly downfield compared to all the other protons.

The compound $[Ru(phen)_2nitrophen]^{2+}$ was resolved into its enantiomers, Λ- and Δ- forms using sodium salts of L(+)- and D(-)- arsenyl tartrate. The syntheses of these arsenyl tartrate salts were published as early as the 1890's. In order to provide easy access to this simple and convenient synthesis, we reiterate the procedures once again in this report. Arsenious oxide was added to a boiling hot solution of sodium salts of L(+)- or D(-)- tartaric acid, refluxed for 30 min, filtered and recrystallized using cold ethanol. White crystalline salts were obtained in good yield.

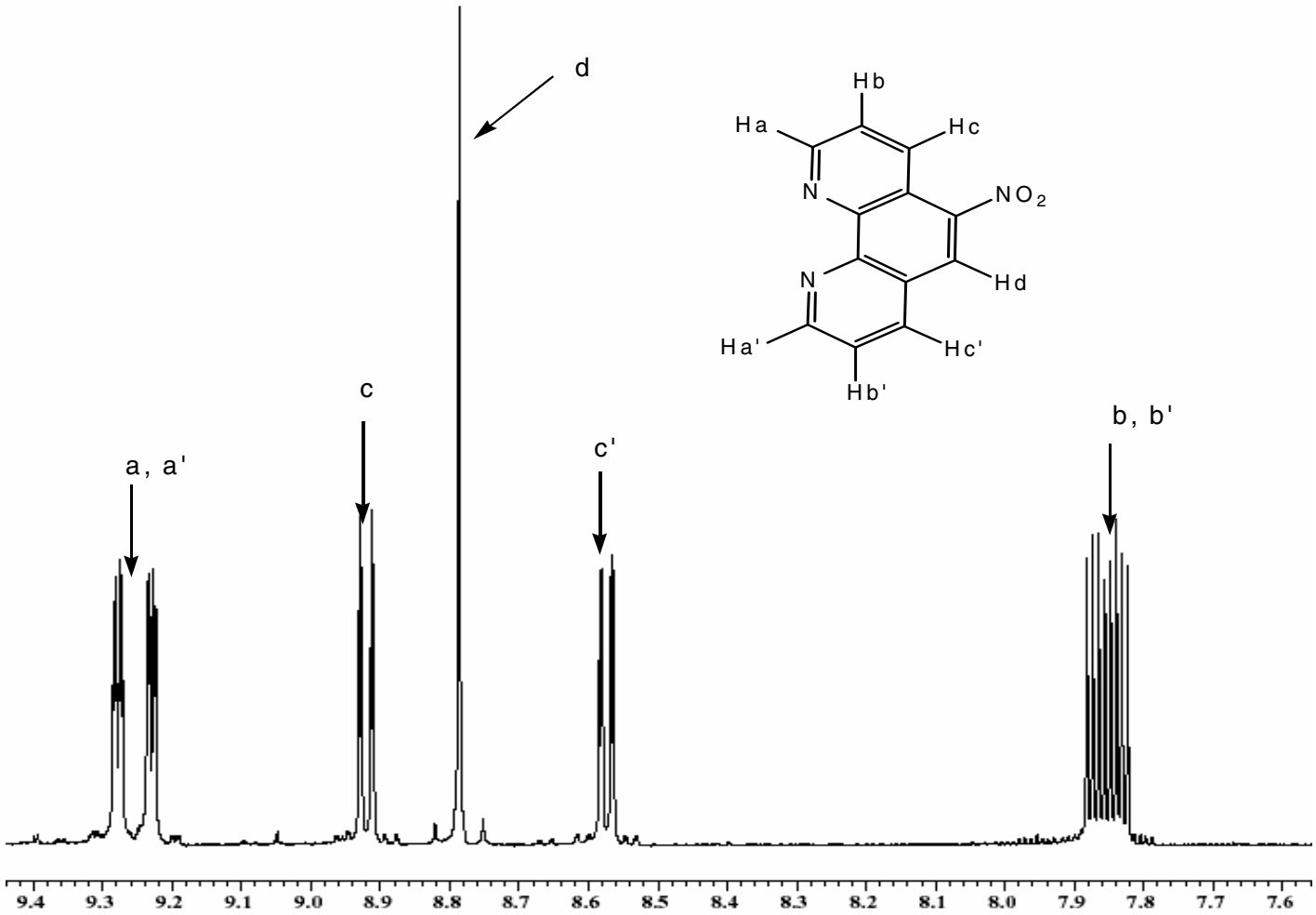



Fig 3.3 500 MHz $^{1}$H NMR spectrum of 5-nitro-1,10-phenanthroline

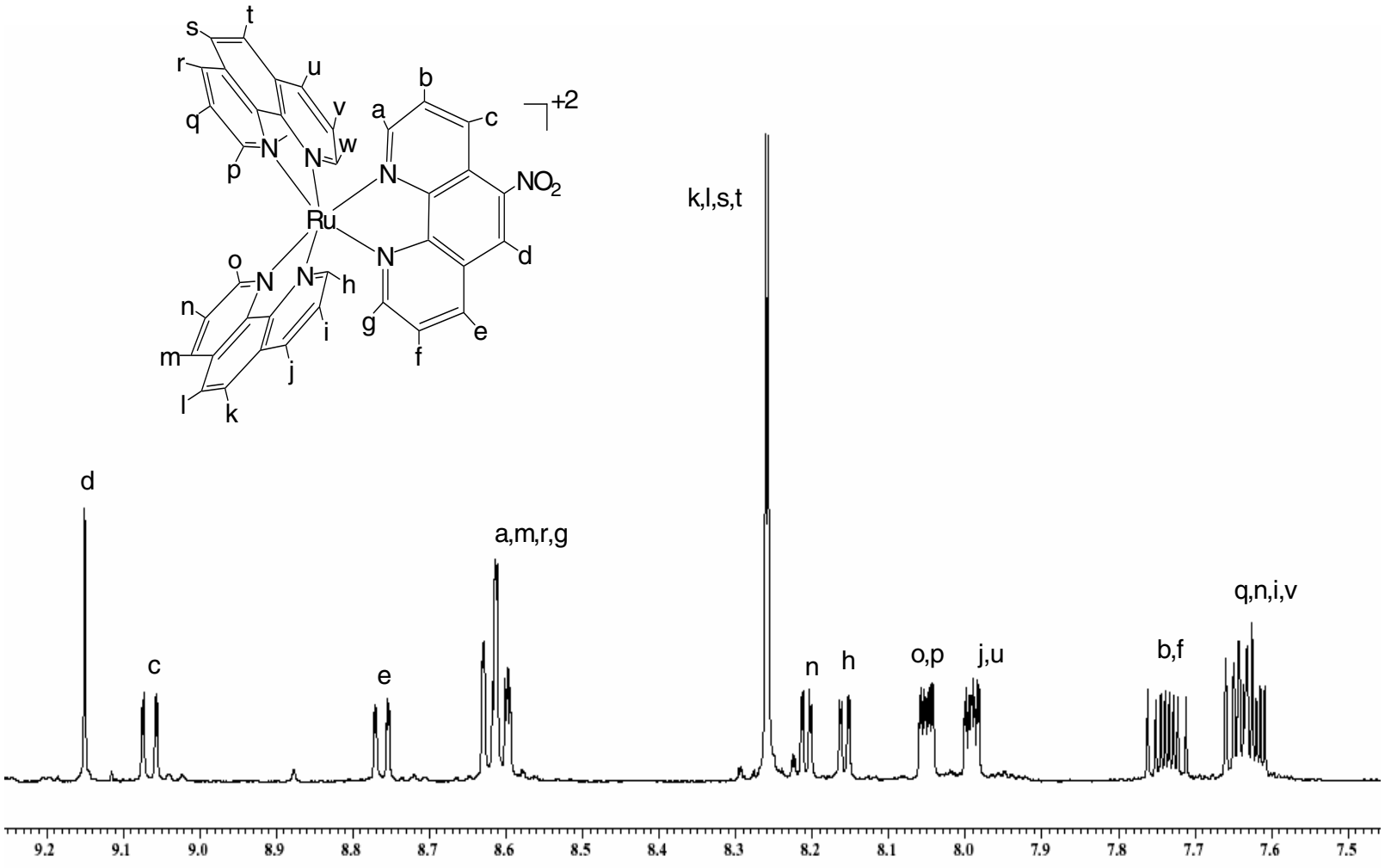



Fig 3.4 500 MHz $^1$H NMR spectrum of $[Ru(phen)_2nitrophen](PF_6)_2$

Our pursuit to innovate new chiral host complexes on silica support led to the synthesis of $[Ru(phen)_2(aminophen)](PF_6)_2$. The synthesis of this complex was accomplished by the reduction of nitro to amine group in the complex $[Ru(phen)_2(nitrophen)]Cl_2$ in the presence of hydrazine hydrate and with palladium on activated carbon as the catalyst. In order to synthesize the specific isomers of this complex, $[Ru(phen)_2(nitrophen)]Cl_2$ was first resolved chirally using sodium arsenyl tartrate salts and pure enantiomers were then reduced to give respective aminophen complexes. In $^1H$ NMR (Fig 3.5), the distinctive amine signal ($NH_2$ protons) appears at around 5.5ppm. The proton neighboring the amine group, $H_d$, is significantly shifted upfield and the singlet peak appears at 7.2ppm as compared to the same $H_d$ proton in the nitrophen complex.

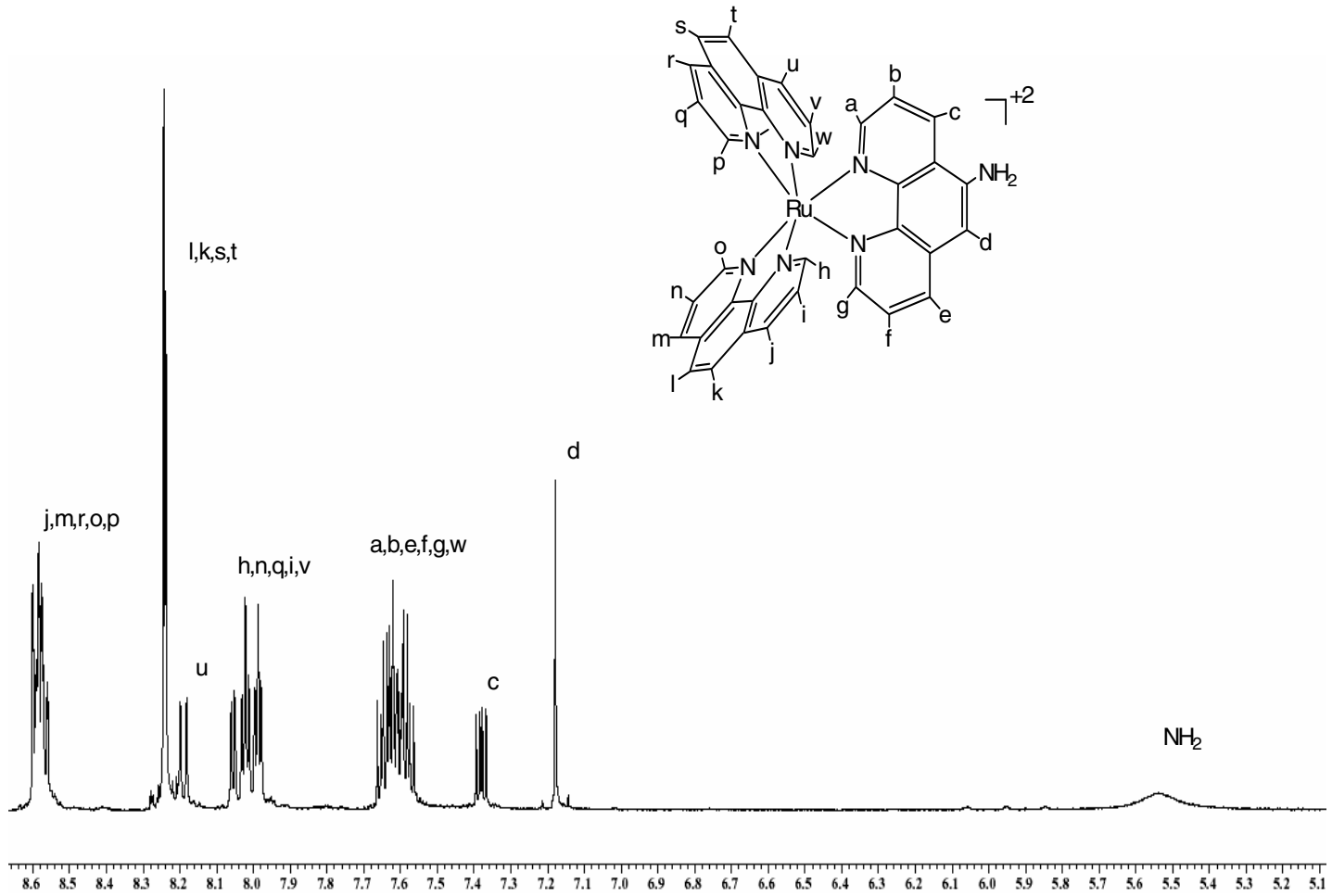



Fig 3.5 500 MHz $^{1}H$ NMR spectrum of $[Ru(phen)_2aminophen](PF_6)_2$

*3.3.2 Resolutions*

The chiral resolution of $[Ru(phen)_2phendione]Cl_2$ involving the principle of resolution by crystallization has been reported by Hiort *et al.*[18] The pure enantiomers were obtained in moderate yield, **Δ** enantiomer – 48% and **Λ** enantiomer – 64%. This is one shortcoming of chiral resolution of racemates as the composition of the enantiomers is 1:1 in the racemic mixture. The resolution procedure given by Hiort *et al.* is extensive and involves a recrystallization procedure. We have devised less complicated and more convenient procedure for separating the enantiomers of ruthenium(II) complexes like $[Ru(phen)_3]Cl_2$, $[Ru(phen)_2phendione]Cl_2$, $[Ru(phen)_2nitrophen]Cl_2$. For isolating **Λ** isomer, the chloride salt of the racemic ruthenium complex was treated first with L(+)-sodium arsenyl tartrate. The **Δ**-enriched L(+)-arsenyl tartrate diastereomer precipitated out, the supernatant was captured and treated with the D(-)-sodium arsenyl tartrate. The **Λ**-enriched D(-)-arsenyl tartrate diastereomer that precipitated was dissolved in hot dilute nitric acid to get rid of the arsenyl tartrate salt. The pure enantiomer was then isolated as hexafluorophosphate salt. An identical method was followed for separating **Δ**- isomer; only the order in which the arsenyl tartrate salts were added, was interchanged. The best result for the chiral separation was obtained when 1g of racemic ruthenium salt was used. The yield for these separations was around 70%. The precipitate obtained from the first treatment and the supernatant from the second treatment of the tartrate salts can be pooled and treated with hot dilute nitric acid. The starting material for this chiral

separation can thus be obtained and recycled. This procedure for separating the enantiomers of Ru(II) complexes is quick and easy to follow.

*3.3.3 Determination of Enantiopurity*

HPLC method on a teicoplanin stationary phase analyzed a series of chiral ruthenium(II) complexes, including $[Ru(L)_3]^{2+}$(L=bipyridine, phenanthroline, and 4,7-diphenylphenanthroline), two mixed-ligand complexes $[Ru(bpy)_2pztr]^+$ and $[Ru(bpy)_2pytr]^+$, and the dinuclear complex $[((Ru(bpy)_2)_2bptr]^{3+}$. However, the retention times were unsatisfiably long and the enantioselectivity is not sufficient in some cases.[58,59] Since the bonded cyclodextrin stationary phase was first commercialized in 1983, the cyclodextrin-based CSPs have proven to be successful for separating enantiomers.[60] Among them, the aromatic-derivatized cyclodextrin CSPs are multimodal and capable of working in three operational modes, which extends the range of enantiomers resolved. They have been widely applied to enantioseparate many different classes of compounds including transition metal polypyridyl complex enantiomers.

The HPLC chromatograms of **Λ** and **Δ** enantiomers of $[Ru(phen)_2(nitrophen)]Cl_2$ are shown in Figure 3.6. Analysis of the racemate demonstrates a separation of the **Δ** and **Λ** enantiomers with retention times of 4 and 5 minutes respectively. The peaks were identified by examining optically pure samples. The **Λ** sample contained a small amount of the **Δ** enantiomer, corresponding to an enantiomeric excess of 95.6%. Similarly, the chromatogram of the **Δ** enantiomer showed the presence of a small amount of **Λ**, resulting in an

enantiomeric excess of 94.5%. The enanatiomeric excess (e.e.) for a particular isomer is calculated with the formula,

**e.e.=[(Δ-Λ)/( Δ+Λ)]*100%**.

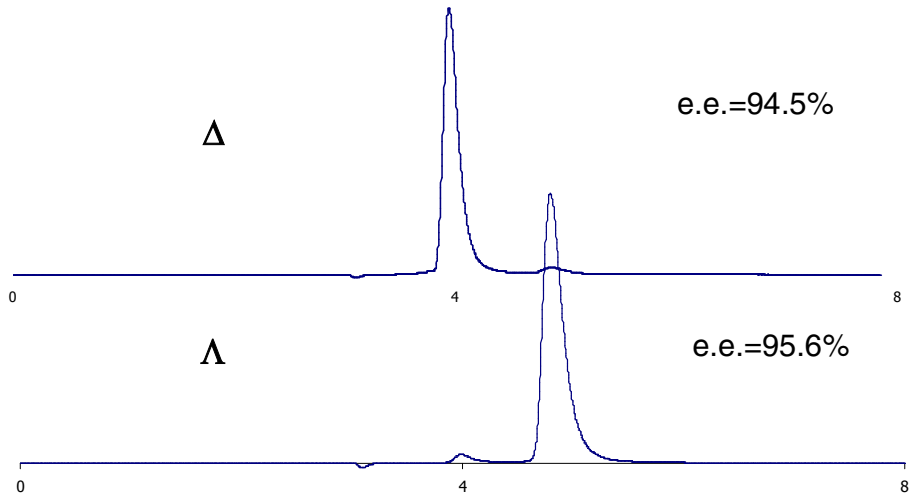



Fig 3.6 HPLC chromatograms of the two enantiomers of $[Ru(phen)_2nitrophen]Cl_2$

## 3.4 Summary and Conclusion

We successfully synthesized the ligand, nitrophen and incorporated it in some monomeric $[Ru(phen)_2X]$ complexes where X=nitrophen, aminophen. Though the syntheses of sodium arsenyl tartrate salts have been known for a long time, we provide a convenient and precise recipe of the salts in this report. Chiral separation by crystallization is revisited, and an easy scheme to separate the enantiomers is reported for monomeric ruthenium(II) complexes like $[Ru(phen)_2(nitrophen)]Cl_2$; the purity was determined by HPLC experiment (Table 3.1). It is difficult to isolate the stereoisomers from the racemate

$[Ru(phen)_2(aminophen)]Cl_2$ by ensuing resolution by crystallization. Our approach to this problem involved the chiral resolution of $[Ru(phen)_2(nitrophen)]Cl_2$ to the two isomers and followed by the reduction process.

Table 3.1 Resolution and Determination of Enantiopurity of Monomeric Ruthenium(II) Polypyridyl Complexes

| Compound | e.e % | Yield% |
|---|---|---|
| $[Ru(phen)_3]Cl_2$ | Δ: 99.9<br>Λ: 93.6 | Δ: 75<br>Λ: 77 |
| $[Ru(phen)_2nitrophen]Cl_2$ | Δ: 94.5<br>Λ: 95.6 | Δ: 70<br>Λ: 72 |
| $[Ru(phen)_2aminophen]Cl_2$ | Δ: 99.4<br>Λ: 95.2 | N/A<br>N/A |
| $[Ru(phen)_2phendione]Cl_2$ | Δ: 91.1<br>Λ: 97.9 | Δ: 60<br>Λ: 65 |

# APPENDIX A

## IC50 VALUES OF VARIOUS RUTHENIUM COMPOUNDS IN THE SCREENING

| Monomeric Compound | IC50 μM | | | | | |
|---|---|---|---|---|---|---|
| | H358 | | | H226 | | |
| | rac | Δ | Λ | rac | Δ | Λ |
| $[Ru(phen)_3]Cl_2$ | 86.7 ± 4.1 | 64.8 ± 4.2 | 93.6 ± 6.3 | 92.8 ± 5.7 | 61.4 ± 4.7 | 115.8 ± 6.9 |
| $[(phen)_2Ru(tpphz)]Cl_2$ | 43.7 ± 2.6 | 34.8 ± 2.6 | 61.6 ± 4.2 | 49.2 ± 3.3 | 32.4 ± 2.4 | 73.4 ± 4.5 |
| $[(phen)_2Ru(tatpp)]Cl_2$ | 13.2 ± 1.8 | 8.8 ± 1.0 | 13.8 ± 1.5 | 12.6 ± 1.9 | 6.7 ± 0.9 | 12.9 ± 1.9 |

| Dimeric Compound | IC50 μM | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | H358 | | | | H226 | | | |
| | mix | ΔΔ | ΛΛ | meso | mix | ΔΔ | ΛΛ | Meso |
| Z | 41.8 ± 2.7 | 23.6 ± 2.5 | 35.0 ± 3.3 | 35.5 ± 2.8 | 51.1 ± 3.4 | 29.7 ± 2.28 | 46.1 ± 2.5 | 51.4 ± 2.1 |
| P | 14.6 ± 1.4 | 9.5 ± 1.2 | 16.7 ± 1.0 | 15.3 ± 1.5 | 16.0 ± 0.9 | 9.3 ± 0.9 | 17.3 ± 1.5 | 18.6 ± 1.4 |

| Compound | IC50 μM | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HAVSMC | | | | HUVEC | | | |
| | mix/rac | ΔΔ | ΛΛ | meso | mix/rac | ΔΔ | ΛΛ | Meso |
| MP | 99.4 ± 6.9 | 100.3 ± 5.9 | 91.2 ± 5.1 | N/A | 93.9 ± 5.4 | 91.6 ± 4.7 | 104.4 ± 5.5 | N/A |
| Z | 78.8 ± 4.55 | 93.6 ± 5.7 | 98.1 ± 6.3 | 89.4 ± 5.3 | 86.8 ± 5.1 | 80.2 ± 5.2 | 88.5 ± 5.8 | 85.4 ± 4.8 |
| P | 133.3 ± 10.6 | 104.5 ± 6.8 | 124.9 ± 7.6 | 116.2 ± 6.7 | 132.5 ± 8.7 | 117.9 ± 6.9 | 127.3 ± 8.6 | 135.9 ± 10.4 |

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