INVESTIGATION OF RUTHENIUM (II) POLYPYRIDYL DIMERS AS POTENTIAL CHEMOTHERAPEUTIC AGENTS

by

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ABSTRACT

INVESTIGATION OF RUTHENIUM (II) POLYPYRIDYL DIMERS AS POTENTIAL CHEMOTHERAPEUTIC AGENTS

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The exploration of transition metal complexes as chemotherapeutic agents is still a relatively unexplored but promising area of research. Chapter 1 highlights the most successful anticancer drugs and potential drugs based on metal complexes, with an emphasis of platinum and ruthenium complexes.

In Chapter 2, the biological activity of a series of novel ruthenium dimers is investigated with special attention given to evaluating their potential as anticancer chemotherapeutic agents. A partial list of the compound prepared includes: [Ru(bpy)(CO)$_2$]tpphz]$^{4+}$ ($7^{4+}$), [Ru(bpy)(CH$_3$CN)$_2$]tpphz]$^{4+}$ ($8^{4+}$),...
[Ru(bpy)(C$_2$O$_4$)$_2$tpphz]$_2$$^{2+}$ (9), [Ru(bpy)(CO)(Cl)$_2$tpphz]$_2$$^{2+}$ (10$^{2+}$),
[(bpy)$_2$Ru(tpphz)Ru(bpy)$_2$]$^{4+}$ (11$^{4+}$), [(phen)$_2$Ru(tpphz)Ru(phen)$_2$]$^{4+}$ (12$^{4+}$),
[(phen)$_2$Ru(tatpp)Ru(phen)$_2$]$^{4+}$ (P$_p$$^{4+}$), [(phen)$_2$Ru(tatpq)Ru(phen)$_2$]$^{4+}$ (Q$_p$$^{4+}$),
[(bpy)$_2$Ru(tatpp)Ru(bpy)$_2$]$^{4+}$ (P$_b$$^{4+}$) and [(bpy)$_2$Ru(tatpq)Ru(bpy)$_2$]$^{4+}$ (Q$_b$$^{4+}$). Systematic changes in functions, such as overall charge (+4, +2, 0), the presence or absence of labile ligands, long and short bridging ligands between metal centers, and redox inactive (tpphz) and active (tatpp and tatpq) ligands allowed us to complete a structure-activity evaluation of this class of potential anticancer drugs.

Most promising were the cationic dimers containing long, redox active bridging ligands, P$_p$$^{4+}$, P$_b$$^{4+}$, Q$_p$$^{4+}$ and Q$_b$$^{4+}$, which showed very high DNA binding constants ($K_b=10^7$ to $10^9$ M$^{-1}$) and good cytotoxicity against cancer cell lines (NSCLC). Animal toxicity studies (mice) showed most cationic complexes to be acutely toxic at relatively lower doses. However, the cationic dimers containing the long tatpp or tatpq bridging ligands were well tolerated in mice with maximum tolerable doses in the range of 67-167 mg/kg for P$_p$$^{4+}$ and 6.7-17 mg/kg for Q$_p$$^{4+}$ as the chloride salts. These promising results led to a study of the antitumor activity of P$_p$$^{4+}$ and Q$_p$$^{4+}$ in vivo using a mouse melanoma model. Excitingly, complex P$_p$$^{4+}$ seems to inhibit tumor growth in vivo although a little difference in survival times was observed. Nonetheless, the results are promising in that this was an initial screen in which numerous parameters including dosage, frequency of treatment, tumor type, etc., remains unoptimized.

The ability of these complexes to damage DNA was evaluated in Chapter 3 by using a plasmid DNA assay that shows if a complex can induce single or double-strand
cuts in the DNA molecule. None of the complexes causes any cleavage reactions unless an external reductant is added. However, upon addition of a common biological reductant (glutathione, dithiothritol or ascorbic acid), complex \( P_{p}^{4+} \) and \( Q_{p}^{4+} \) could be shown to induce single-strand cuts. Importantly, the DNA cleaving ability of \( P_{p}^{4+} \) is potentiated under anaerobic conditions, showing that the cleavage is not via \( O_{2} \) activation processes. Further studies established that complex \( P_{p}^{4+} \) is doubly reduced under the assay conditions and the doubly reduced product, denoted \( H_{2}P_{p}^{4+} \), is the cleavage agent. As this species is oxygen sensitive and readily reoxidized to \( P_{p}^{4+} \) upon exposure to air, the \([O_{2}]\) 'regulates' the nuclease activity by controlling \([H_{2}P_{p}^{4+}]\).

The ability of the ruthenium complexes to inhibit or poison topoisomerase I and II was evaluated and is reported in Chapter 4. The intercalating complexes showed the most significant topoisomerase I and II inhibition with complex \( P_{p}^{4+} \) standing out again for its potent biological activity.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................ iii
ABSTRACT ..................................................................................................................................... v
LIST OF ILLUSTRATIONS........................................................................................................ xiv
LIST OF TABLES.................................................................................................................... xx
LIST OF SCHEMES .................................................................................................................. xxii
LIST OF ABBREVIATIONS....................................................................................................... xxiii

Chapter

1. INTRODUCTION....................................................................................................................... 1
   1.1 Background of Cancer ..................................................................................................... 1
   1.2 Metal Complexes as Anticancer Drugs ......................................................................... 1
   1.3 Platinum-Based Anticancer Drugs ............................................................................... 2
       1.3.1 Mononuclear Platinum Complexes ...................................................................... 2
       1.3.2 Multinuclear Cationic Platinum Complexes ..................................................... 4
   1.4 Ruthenium-Based Anticancer Drugs ......................................................................... 6
       1.4.1 Ruthenium-DMSO Complexes ......................................................................... 6
       1.4.2 Neutral Ruthenium Polypyridyl Complexes with Labile Chlorides ................. 12
       1.4.3 Cationic Ruthenium Polypyridyl Complexes ............................................... 13
1.5 Scope of Thesis Dissertation ................................................................. 19

2. SYNTHESIS AND SCREENING OF RUTHENIUM POLYPYRIDYL COMPLEXES AS POTENTIAL ANTICANCER AGENTS ................................................................. 22

2.1 Introduction ..................................................................................... 22

2.2 Results and Discussion ................................................................. 26

2.2.1 Synthesis of Neutral Tris-heteroleptic Ru(II) Monomers .......... 26

2.2.2 Synthesis of Neutral Tris-heteroleptic Ru(II) Dimers ............. 33

2.2.3 Synthesis of Partially Neutralized Dimer, [{Ru(bpy)(CO)(Cl)₂tpphz}²⁺] ................. 41

2.2.4 Absorption Spectra of Tris-heteroleptic Ru(II) Monomers and Dimers ................................................................. 43

2.2.5 DNA Binding Studies with Ruthenium Dimers ................. 45

2.2.6 Cytotoxicity of Ru(II) Dimers Towards Non-Small Cell Lung Cancer Cell Lines (NSCLC) ................................................................. 50

2.2.7 Animal Toxicity ........................................................................ 52

2.2.8 In vivo Antitumor Activity ............................................................. 54

2.3 Summary and Conclusions .......................................................... 60

2.4 Materials and Methods ............................................................... 61

2.4.1 General ..................................................................................... 61

2.4.2 Physical Measurements ............................................................ 62

2.4.3 Synthesis .............................................................................. 63

2.4.4 Cytotoxicity in NSCLC cells .................................................... 69

2.4.5 Animal Toxicity and MTD of Ru(II) Dimers ......................... 70
2.4.6 *In vivo* Antitumor Activity of \( P_{p}^{4+} \) and \( Q_{p}^{4+} \) ........................................ 70

2.4.7 Equilibrium Binding Constants \( (K_b) \) ......................................................... 71

3. RUTHENIUM (II) DIMERS AS HYPOXIA SELECTIVE DNA DAMAGING AGENTS. ............................................................ 73

3.1 Introduction ........................................................................................................... 73

3.2 Results and Discussion ......................................................................................... 76

3.2.1 DNA Cleavage Assay ....................................................................................... 76

3.2.2 DNA Cleavage by Different Ruthenium Polypyridyl Complexes ...................................................................................... 78

3.2.3 Effect of Oxygen on DNA Cleavage Efficiency by \( P_{p}^{4+} \) ................. 82

3.2.4 Effect of Thiyl Radicals on DNA Cleavage Efficiency by \( P_{p}^{4+} \) .......... 84

3.2.5 Concentration Dependence of \( P_{p}^{4+} \) on DNA Cleavage ............... 86

3.2.6 Identification of the Active Reduced Form of \( P_{p}^{4+} \) ....................... 92

3.2.7 Identification of Reduced Species Responsible for DNA Cleavage ................................................................. 98

3.2.8 pH Independent DNA Cleavage by \( P_{p}^{4+} \) ......................................... 104

3.2.9 Establishing a Potential Mechanism for DNA Cleavage by \( H_2P_{p}^{4+} \) ................................................................. 104

3.2.10 DNA Cleavage by Different Analogues Consisting of tatpp Bridging Ligand ........................................ 109

3.3 Summary and Conclusions .............................................................................. 113

3.4 Materials and Methods ....................................................................................... 115

3.4.1 Chemicals ................................................................. 115

3.4.2 Instrumentation ........................................ 115
3.4.3 DNA cleavage by $P_{p}^{4+}$ Under Reduced Environment .......... 116
3.4.4 Anaerobic Reactions ................................................................. 116
3.4.5 Product Analysis and Quantitation ........................................... 117
3.4.6 Synthesis of $[P_{p}]Cl_{3}$ ................................................................. 117
3.4.7 Synthesis of $[H_{2}P_{p}]Cl_{4}$ .............................................................. 118
3.4.8 Preparation of a Solution of Zn(BF$_{4}$)$_{2}$ and tatpp ($Z^{4+}$) for DNA Cleavage Studies ................................................................. 119
3.4.9 Determination of Paramagnetic Susceptibility by the Evans Method ................................................................. 119

4. RUTHENIUM DIMERS AS DUAL TARGETS OF DNA TOPOISOMERASES ................................................................. 121

4.1 Introduction .......................................................................................... 121
   4.1.1 Identification of Catalytic Activity of Topoisomerases by Agarose Gel Electrophoresis .............................. 131
   4.1.2 CPT is a Known Topo I Targeting Anticancer Drug ............... 131
   4.1.3 m-AMSA is a Known Topo II Targeting Anticancer Drug ......... 133
4.2 Results and Discussion .......................................................................... 135
   4.2.1 Evaluation of the Inhibitory Properties of Cationic Ruthenium Polypyridyl Complexes on Topo I ..................... 135
   4.2.2 Evaluation of the Inhibitory Properties of Cationic Ruthenium Polypyridyl Complexes on Topo II ................. 139
   4.2.3 Concentration Dependence Study of Topo I Inhibition by $P_{p}^{4+}$ and $Q_{p}^{4+}$ .................................................... 141
   4.2.4 Concentration Dependence Study of Topo II Inhibition by $P_{p}^{4+}$ and $Q_{p}^{4+}$ .................................................... 142
   4.2.5 Concentration Dependent Inhibition of Catalytic Activity of Topo II by $P_{p}^{4+}$ .................................................... 143
4.2.6 Inhibition of the Catalytic Activity of Topo II by $P_{p}^{4+}$ Initiates Through Intercalating with the DNA .................. 144

4.2.7 Inhibition of Topo II by $P_{1}^{2+}$ .................................................... 146

4.2.8 Inhibition of Catalytic Activity of Topo II by $[\text{Ru(phen)}_{2}\text{dppz}]^{2+}$ .................................................. 147

4.3 Summary and Conclusions ...................................................................... 148

4.4 Materials and Methods ............................................................................ 151

4.4.1 General ......................................................................................... 151

4.4.2 Synthesis ....................................................................................... 151

4.4.3 Topo I Inhibition Assay ............................................................... 151

4.4.4 Topo II Inhibition Assay ............................................................... 152

APPENDIX .......................................................................................... 154

A $^1$H NMR, $^{13}$C NMR, COSY AND UV-VIS SPECTRA OF $[\text{Ru(bpy)}(\text{dppz})(\text{CO})_{2}]^{2+}$ ................................................................. 154

B $^1$H NMR, $^{13}$C NMR, COSY AND UV-VIS SPECTRA OF $[\text{Ru(bpy)}(\text{dppz})(\text{CH}_3\text{CN})_{2}]^{2+}$ ............................................................... 159

C $^1$H NMR, UV-VIS, COSY AND MASS SPECTRA OF $[\text{Ru(bpy)}(\text{dppz})(\text{C}_2\text{O}_4)]^{2+}$ ................................................................. 164

D $^1$H NMR, $^{13}$C NMR, MASS AND UV-VIS SPECTRA OF $[\{\text{Ru(bpy)}(\text{CO})_{2}\}_{2}\text{tpphz}]^{4+}$ ................................................................. 170

E $^1$H NMR, $^{13}$C NMR, MASS AND UV-VIS SPECTRA OF $[\{\text{Ru(bpy)}(\text{CH}_3\text{CN})_{2}\}_{2}\text{tpphz}]^{4+}$ ................................................................. 175

F MASS AND VIS SPECTRA OF $[\{\text{Ru(bpy)}(\text{C}_2\text{O}_4)\}_{2}\text{tpphz}]$ ................................................................. 180

G $^1$H NMR, $^{13}$C NMR, COSY AND UV-VIS SPECTRA OF $[\{\text{Ru(bpy)}(\text{CO})(\text{Cl})\}_{2}\text{tpphz}]^{2-}$ ................................................................. 183

H Crystal Structure of $[(\text{bpy})_{2}\text{Ru(tatpq)}_2\text{Ru(bpy)}_{2}]\text{Cl}_4$ ........................................................................ 188
## LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structures of widely used platinum containing anticancer drugs</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Structures of Di- and Trinuclear complexes</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>The Structures of some of the widely studied ruthenium anticancer complexes; DMSO = dimethylsulfoxide</td>
<td>7</td>
</tr>
<tr>
<td>1.4</td>
<td>Chemical structures of (ImH)[\textit{trans}-[Ru(Cl)₄(Im)]₂] (Im = Imidazole) and (IndH)[\textit{trans}-[Ru(Cl)₄(Ind)]₂] (Ind = Indazole)</td>
<td>8</td>
</tr>
<tr>
<td>1.5</td>
<td>Structures of ruthenium complexes use to treat tumor metastases</td>
<td>10</td>
</tr>
<tr>
<td>1.6</td>
<td>Structures of neutral ruthenium complexes, [Ru(phen)₂(Cl)]₂ (phen = 1,10-phenanthroline) and [Ru(DIP)₂(Cl)]₂ (DIP = 4,7-diphenyl-1,10-phenanthroline)</td>
<td>12</td>
</tr>
<tr>
<td>1.7</td>
<td>Structure of [Ru(phen)₃]²⁺ and its stereoisomerisms, Λ and Δ</td>
<td>14</td>
</tr>
<tr>
<td>1.8</td>
<td>Absolute configurations of the Δ and Λ enantiomers of [Ru(phen)₂dppz]²⁺</td>
<td>15</td>
</tr>
<tr>
<td>1.9</td>
<td>Structures of Ruthenium dimers; [µ-c₄(cpdpbz)₄(phen)₄Ru₂]⁴⁺ (1⁴⁺) and [µ- (11,11’-bidppz)(phen)₄Ru₂]⁴⁺ (2⁴⁺)</td>
<td>17</td>
</tr>
<tr>
<td>1.10</td>
<td>Structures of the ruthenium dimers, I, II, III and IV</td>
<td>18</td>
</tr>
<tr>
<td>2.1</td>
<td>DNA bound by the ruthenium metallointercalator, [Ru(bpy)₂dppz]²⁺</td>
<td>23</td>
</tr>
<tr>
<td>2.2</td>
<td>Structures of 1⁴⁺ and 2⁴⁺</td>
<td>24</td>
</tr>
<tr>
<td>2.3</td>
<td>Structures of dppz, tpphz, tatpp and tatpq</td>
<td>25</td>
</tr>
<tr>
<td>2.4</td>
<td>H NMR spectrum of <a href="PF%E2%82%86">4</a>₂ in d₃ MeCN</td>
<td>29</td>
</tr>
<tr>
<td>2.5</td>
<td>H NMR spectrum of <a href="PF%E2%82%86">4</a>₂ in d₃ MeCN</td>
<td>30</td>
</tr>
</tbody>
</table>
2.6 $^1$H NMR spectrum of [5](PF$_6$)$_2$ in $d_3$MeCN ................................................... 31

2.7 $^{13}$C NMR spectrum of [5](PF$_6$)$_2$ in $d_3$MeCN ................................................... 31

2.8 $^1$H NMR spectrum of 6 in DMSO-$d_6$ ........................................................................ 33

2.9 $^1$H NMR spectrum of cis and trans [7](PF$_6$)$_4$ mixture in $d_3$MeCN. ............... 35

2.10 $^{13}$C NMR spectrum of cis and trans mixture of [7](PF$_6$)$_4$ in $d_3$MeCN........... 36

2.11 $^1$H NMR spectrum of cis and trans mixture of [8](PF$_6$)$_4$ in $d_3$MeCN............ 38

2.12 Comparison of $^{13}$C NMR spectra of cis and trans mixture of A) [7](PF$_6$)$_4$ and B) [8](PF$_6$)$_4$ in $d_3$MeCN. ...................................................... 39

2.13 $^1$H NMR spectra of a) [Ru(bpy)$_2$tpphz](PF$_6$)$_2$ and b) [10](PF$_6$)$_2$ in $d_3$MeCN ........................................................................................................ 42

2.14 $^{13}$C NMR spectrum of [10](PF$_6$)$_2$ in CD$_3$CN. ................................................. 42

2.15 UV-Vis spectra of [4]Cl$_2$ (black line), [5]Cl$_2$ (red line), and 6 (purple line) in DMSO........................................................................................................ 43

2.16 UV-Vis spectra of [7]Cl$_4$ (black line), [8]Cl$_4$ (red line) and 9 (purple line) in DMSO. ........................................................................................................ 44

2.17 The structures of $P_b^{4+}$, $P_p^{4+}$ and $Q_p^{4+}$ .................................................................. 45

2.18 Displacement titration based on quenching of DNA-bound [Ru(phen)$_2$dppz]$^{2+}$ by $P_p^{4+}$, $Q_p^{4+}$, 7$^{4+}$ and 8$^{4+}$. .................................................. 47

2.19 Displacement titration based on quenching of DNA-bound [Ru(phen)$_2$dppz]$^{2+}$ by $P_p^{4+}$, $P_p^{4+}$ and $Q_p^{4+}$ .......................................................... 48

2.20 Cytotoxicity data of Ruthenium (II) dimers, $Q_p^{4+}$, $P_p^{4+}$, 7$^{4+}$, 8$^{4+}$, 10$^{2+}$ and 9 against NSCLC cells. ................................................................. 51

2.21 Number of mice survived vs. days after injection of mouse melanoma cells in C57B mice................................. 56

2.22 Tumor volume vs. days after injection of mouse melanoma cells in C57B mice. ........................................................................... 57

2.23 Average body weight vs. days after injection of mouse melanoma
3.1 Structures of hypoxia selective antitumor drugs, TPZ and AQ4N .......... 74
3.2 Structure of $P_p^{4+}$ pointing to its planar extended tatpp bridging ligand...... 76
3.3 Topoisomers of plasmid DNA: Form I, II and III................................. 77
3.4 Photograph (negative image) of an ethidium bromide-stained agarose gel showing the separation of the supercoiled, circular and linear pUC18 DNA (Lane 1) and of purified supercoiled pUC18 DNA (Lane 2) ........................................................................................................... 78
3.5 Chemical structures of the Ruthenium polypyridyl complexes, $P_p^{4+}$, $Q_p^{4+}$, $3^{2+}$, $12^{4+}$ and $13^{2+}$, under study.......................... 80
3.6 1 % agarose gel stained with ethidium bromide (negative image) of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with various ruthenium complexes (12.8 µM) in 4 mM Na$_3$PO$_4$ buffer (pH 7) buffer in the presence or absence of 0.64 mM GSH under aerobic conditions......................................................... 81
3.7 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with $P_p^{4+}$ or Fe-BLM complexes (12.8 µM or 0.083 of $P_p^{4+}$ to DNA bp ratio) in 4 mM Na$_3$PO$_4$ (pH 7) buffer and in the presence of 0.5 mM GSH under aerobic or anaerobic conditions (as indicated)................................................................. 83
3.8 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with $P_p^{4+}$ in 4 mM Na$_3$PO$_4$ (pH 7) buffer in the presence of different reducing agents............................................ 85
3.9 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with different concentrations of $P_p^{4+}$ in 4 mM Na$_3$PO$_4$ buffer containing 0.5 mM GSH or DTT (pH 7) under anaerobic conditions.............................................................. 87
3.10 Conversion of supercoiled (Form I) DNA to circular (Form II) and
3.11 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24°C for 2 h with different concentrations of $\text{P}_{p}^{4+}$ in a buffer of 4 mM Na$_3$PO$_4$ (pH 7) under anaerobic conditions .......................................................... 89

3.12 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24°C for 2 h with different concentrations of $\text{P}_{p}^{4+}$ in 4 mM Na$_3$PO$_4$ (pH 7) buffer under aerobic and anaerobic conditions ......................... 90

3.13 Conversion of supercoiled (Form I) DNA to circular (Form II) and linear (Form III) as a function of increasing $[\text{P}_{p}^{4+}]$ under anaerobic conditions .............................................................................................. 92

3.14 Ladder Scheme of the Redox and Protonations Processes for $\text{P}_{p}^{4+}$ .................. 93

3.15 Absorption spectra of $\text{P}_{p}^{4+}$ and its singly reduced $\text{P}_{p}^{3+}$ and doubly reduced $\text{P}_{p}^{2+}$ in degassed CH$_3$CN ................................................................. 94

3.16 Absorption spectra of doubly reduced $\text{P}_{p}^{2+}$ and its protonated versions doubly reduced singly protonated $\text{H}\text{P}_{p}^{3+}$ and doubly reduced doubly protonated $\text{H}_2\text{P}_{p}^{4+}$ in degassed CH$_3$CN ................................................................. 95

3.17 Absorption spectra of $\text{P}_{p}^{4+}$ and $\text{H}_2\text{P}_{p}^{4+}$ made with excess (10 equivalent) glutathione (GSH), dithithretol (DTT) and ascorbic acid (AA) .................................................... 97

3.18 Absorption spectra of $\text{P}_{p}^{4+}$ (12.8 µM) with GSH (0.256 mM) at different pH in degassed deionized water .................................................................................. 98

3.19 $^1$H NMR spectra of $[\text{P}_{p}(\text{PF}_6)_4]$, $[\text{P}_{p}(\text{PF}_6)_3]$ and $[\text{H}_2\text{P}_{p}(\text{PF}_6)_4]$ in degassed CD$_3$CN .................................................................................. 99

3.20 Absorption spectra of chloride salt of $\text{P}_{p}^{4+}$, $\text{P}_{p}^{3+}$ and $\text{H}_2\text{P}_{p}^{4+}$ in degassed water .............................................................................................................................. 100

3.21 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24°C for 3 h with different concentrations of $\text{P}_{p}^{4+}$, $\text{P}_{p}^{3+}$ and $\text{H}_2\text{P}_{p}^{4+}$ in a buffer of 4 mM Na$_3$PO$_4$ (pH 7) under anaerobic conditions ................................................................. 102
3.22 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 3 h with \( P_p^{4+} \) at different pH's in 4 mM Na\(_3\)PO\(_4\) buffer (pH 7) under anaerobic conditions ....................................................... 103

3.23 The postulated structures of singlet and triplet states of \( H_2P_p^{4+} \) ..................... 105

3.24 The postulated structures of \( H_3P_p^{4+} \) and \( H_4P_p^{4+} \) .............................................. 106

3.25 Change of absorption spectra of \( H_2P_p^{4+} \) itself and \( H_2P_p^{4+} \) intercalated into DNA with time in 4 mM Na\(_3\)PO\(_4\) buffer at pH 7 ........................................ 107

3.26 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 3 h and 20 h with \( H_2P_p^{4+} \) (12.8 µM, 12 DNA bp per \( H_2P_p^{4+} \)) in 4 mM Na\(_3\)PO\(_4\) (pH 7) buffer under anaerobic conditions........ 108

3.27 Structures of \( P_1^{2+} \) and \( Z^{4+} \) ............................................................................... 110

3.28 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after anaerobic incubation at 24 °C for 3 h with different concentrations of \( P_p^{4+} \), \( P_1^{2+} \) and \( Z^{4+} \) in a 4 mM Na\(_3\)PO\(_4\) buffer (pH 7) containing 0.5 mM GSH ................................................................. 111

3.29 Absorption spectra of \( H_2P_p^{4+} \), \( H_2P_1^{2+} \), \( Z^{4+} \) and \( H_2Z^{4+} \) in degassed water........................................................................................................ 113

4.1 Electron micrographs of the topoisomeric forms of plasmid DNA showing the transition from circular (relaxed) DNA to supercoiled DNA ........................................................................................................ 122

4.2 Separation of relaxed and supercoiled DNA by gel electrophoresis.............. 123

4.3 A proposed reaction mechanism for mammalian DNA Topo I ...................... 124

4.4 Chemical structures of some topo I poisons, camptothecin, topotecan and CPT-11 ................................................................. 126

4.5 Chemical structures of some topo II poisons, m-AMSA, adriamycin, etoposide, ellipticine and mitosantrone.............................. 127

4.6 Chemical structures of some clinically developed topoisomerase inhibitors, Intoplicine, TAS-103, F 11782 and XR 5000........ 129
4.7 Chemical structures of some metal containing topo poisons and inhibitors ................................................................................................... 130

4.8 An agarose gel picture screening, Lane 1, the original supercoiled DNA (pUC18); Lane 2, Relaxation of pUC18 supercoiled DNA by topo I; Lane M is the marker of pUC18 consisting three isomers, supercoiled DNA, circular DNA and linear DNA .................................................. 131

4.9 Concentration dependent inhibition of topo I catalytic activity by 2-250 µM (Lane 2-5) of CPT ............................................................................ 133

4.10 Concentration-dependent inhibition of topo II catalytic activity by 6.4-250 µM (Lane 2-6) m-AMSA .................................................................... 134

4.11 Represent the structures of [(phen)_2Ru(tatpp)Ru(phen)_2]^{4+} (P_p^{4+}), [(phen)_2Ru(tatpq)Ru(phen)_2]^{3+} (Q_p^{4+}), [(phen)_2Ru(bpm)Ru(phen)_2]^{4+} (14^{2+}), [Ru(phen)_2tatpp]^{2+} (P_1^{2+}), [Ru_2(phen)_2dpdz]^{2+} (3^{2+}) and [Ru(phen)_3]^{2+} (12^{2+}) .......................................................................................................................... 136

4.12 The DNA topo I relaxation assay was carried out in the presence of different ruthenium complexes ........................................................................ 138

4.13 The DNA topo II relaxation assay was carried out in the presence of different ruthenium complexes ........................................................................ 140

4.14 Inhibition of catalytic activity of topo I by two ruthenium dimers, P_p^{4+} and Q_p^{4+} ........................................................................................................ 141

4.15 The DNA topo II relaxation assay was carried out in the presence of two ruthenium dimers, P_p^{4+} and Q_p^{4+} ........................................................................ 143

4.16 Inhibitory effect of P_p^{4+} on topo II ............................................................................... 144

4.17 Effect of enzyme concentration on inhibition of topo II mediated catalytic activity by P_p^{4+} ................................................................................ 145

4.18 Inhibitory effect of P_1^{2+} on topo II ........................................................................ 146

4.19 Inhibitory effect of [Ru(phen)_2dpdz]^{2+} (7^{2+}) on topo II ........................................ 148
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Effects of treatment with NAMI-A in comparison with ICR and with cisplatin on survival time of surgered animals</td>
</tr>
<tr>
<td>1.2</td>
<td>Cytotoxicity results of neutral Ruthenium (II) complexes</td>
</tr>
<tr>
<td>1.3</td>
<td>IC$_{50}$ of the ΔΔ- enantiomers of complexes (I), (II), (III), (IV) and their known mononuclear complex Δ[Ru(phen)$_2$(dppz)]Cl$_2$ (3$^{2+}$)</td>
</tr>
<tr>
<td>2.1</td>
<td>Summary of Equilibrium DNA Binding Constants (K$_b$) and binding site size (s), for Ruthenium (II) dimers</td>
</tr>
<tr>
<td>2.2</td>
<td>IC$_{50}$ of the following Ruthenium (II) dimers and cisplatin</td>
</tr>
<tr>
<td>2.3</td>
<td>Toxicity of 1 mg/mL dose of Ruthenium (II) complexes in Vivo</td>
</tr>
<tr>
<td>2.4</td>
<td>MTD data of P$<em>{p4+}$ and Q$</em>{p4+}$</td>
</tr>
<tr>
<td>2.5</td>
<td>IC$_{50}$ and MTD data of Ruthenium (II) dimers</td>
</tr>
<tr>
<td>3.1</td>
<td>Relative yields of DNA cleavage products for Figure 3.6</td>
</tr>
<tr>
<td>3.2</td>
<td>Relative yields of DNA cleavage products under aerobic and anaerobic conditions as shown in Figure 3.7</td>
</tr>
<tr>
<td>3.3</td>
<td>Relative yields of DNA cleavage products upon incubation of complex P$_{p4+}$ with GSH, AA and DTT reducing agents</td>
</tr>
<tr>
<td>3.4</td>
<td>Relative yields of DNA cleavage products upon titration with complex P$_{p4+}$ under anaerobic conditions as shown in Figure 3.9</td>
</tr>
<tr>
<td>3.5</td>
<td>Product yields from the cleavage assay from Figure 3.12 as determined from densitometry</td>
</tr>
<tr>
<td>3.6</td>
<td>DNA cleavage products distribution after treatment of</td>
</tr>
</tbody>
</table>
supercoiled pUC18 DNA with $P_p^{4+}$, $P_p^{3+}$ and $H_2P_p^{4+}$ .................................................... 102

3.7 Cleavage of supercoiled pUC18 DNA by $P_p^{4+}$ with GSH at different pH ................................................................. 104

3.8 DNA product distribution from cleavage assay shown in Figure 3.31 ............................................................................ 108

3.9 Comparison of cleavage of supercoiled pUC18 DNA by $P_p^{4+}$, $P_1^{2+}$ and $Z^{4+}$ ................................................................. 112
LIST OF SCHEMES

Scheme

2.1 Synthetic strategy for tris(heteroleptic) neutral complexes, for instance, [Ru(bpy)(dppz)(C_2O_4)] (6). ........................................................ 27

2.2 Synthesis and structural formula of [Ru(bpy)(CO)_2(CF_3SO_3)_2] and 4^{2+}....... 28

2.3 Synthesis and structural formula of [Ru(bpy)(CO)_2(CF_3SO_3)_2] and 4^{2+}....... 30

2.4 Synthesis and structural formulas of 5^{2+} and 6. .............................................. 32

2.5 Synthesis route and structural formula of cis and trans [{Ru(bpy)(CO)_2}tpphz]^1+ (7^1+). .......................................................... 34

2.6 Synthesis route and structural formula of cis and trans mixture of 8^{4+}......... 37

2.7 Synthesis and structural formulas of cis and trans mixtures of 8^{4+} and 9 ...... 40

2.8 Synthesis and structural formula of [Ru(bpy)_2tpphz]^{2+} and 10^{2+}................. 41
LIST OF ABBREVIATIONS

cisplatin \(cis\)-diamminedichloroplatinum (II)

ACR \((\text{ImH})[\text{trans-Ru(Cl)}_4(\text{Im})_2]\)

NAMI \(\text{Na}[\text{trans-Ru(Cl)}_4(\text{DMSO})\text{Im}]-2\text{DMSO}\)

NAMI-A \(\text{ImH}[\text{trans-Ru(Cl)}_4(\text{DMSO})\text{Im}]\)

AM-A \(\text{NH}_4[\text{trans-Ru(Cl)}_4(\text{DMSO})(\text{NH}_3)]\)

Im Imidazole

Ind Indazole

\(\text{IC}_{50}\) the concentration of the drug, which leads to 50% inhibition of the cell growth

NSCLC non-small cell lung cancer cell lines

Phen 1,10-phenanthroline

bpy 2,2’-bipyridyl

bpm 2,2’-bipyrimidine

DIP 4,7-diphenyl-1,10-phenanthroline

dppz dipyrido[3,2-a:2’,3’-c]phenazine

tpphz tetrapyrido[3,2-a:2’3’-c:3’’,2’’-h:2’’’',3’’’’-j]phenazine

tatpp 9,11,20,22-tetraazatetrapyrido[3,2-\(a\):2’,3’-c:3’’,2’’-I:2’’’',3’’’’-n]-pentacene
tatpq  9,11,20,22-tetraazatetrapyrido[3,2-a: 2’,3’-c: 3″,2″-I:
2””,3””-n]-pentacene-10,21-quinone

MeOH  methanol

CO  carbonyl ligand

MeCN/CH$_3$CN  acetonitrile

TMNO  trimethylamine N-oxide

C$_2$O$_4$  oxalato ligand

DMSO  dimethyl sulfoxide

EtOH  ethanol

MTT  (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

TMS  tetramethylsilane

NMR  Nuclear Magnetic Resonance

IR  Infra red

MALDI  Matrix Assisted Laser Desorption Ionization

TOF  Time-of-flight

ESI  Electrospray Ionization

$1^4^+$  [μ-c4(cpdppz)$_2$(phen)$_4$Ru$_2$]$^{4+}$

$2^4^+$  [μ-(11,11’-bidppz)(phen)$_4$Ru$_2$]$^{4+}$

$3^2^+$  [Ru(phen)$_2$dppz]$^{2+}$

$4^2^+$  [Ru(bpy)dppz(CO)$_2$]$^{2+}$

$5^2^+$  [Ru(bpy)dppz(CH$_3$CN)$_2$]$^{2+}$
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>[Ru(bpy)dppz(C$_2$O$_4$)]</td>
</tr>
<tr>
<td>7$^{4+}$</td>
<td>[{Ru(bpy)(CO)$_2$}$_2$tpphz]$^{4+}$</td>
</tr>
<tr>
<td>8$^{4+}$</td>
<td>[{Ru(bpy)(CH$_3$CN)$_2$}$_2$tpphz]$^{4+}$</td>
</tr>
<tr>
<td>9</td>
<td>[{Ru(bpy)(C$_2$O$_4$)}$_2$tpphz]</td>
</tr>
<tr>
<td>10$^{2+}$</td>
<td>[{Ru(bpy)(CO)(Cl)}$_2$tpphz]$^{2+}$</td>
</tr>
<tr>
<td>11$^{4+}$</td>
<td>[(bpy)$_2$Ru(tpphz)Ru(bpy)$_2$]$^{4+}$</td>
</tr>
<tr>
<td>12$^{4+}$</td>
<td>[(phen)$_2$Ru(tpphz)Ru(phen)$_2$]$^{4+}$</td>
</tr>
<tr>
<td>13$^{4+}$</td>
<td>[Ru(phen)$_3$]$^{2+}$</td>
</tr>
<tr>
<td>14$^{2+}$</td>
<td>[(phen)$_2$Ru(bpm)Ru(phen)$_2$]$^{4+}$</td>
</tr>
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<td>P$_p$$^{4+}$</td>
<td>[(phen)$_2$Ru(tatpp)Ru(phen)$_2$]$^{4+}$</td>
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<tr>
<td>P$_b$$^{4+}$</td>
<td>[(bpy)$_2$Ru(tatpp)Ru(bpy)$_2$]$^{4+}$</td>
</tr>
<tr>
<td>Q$_p$$^{4+}$</td>
<td>[ (phen)$_2$Ru(tatpq)Ru(phen)$_2$]$^{4+}$</td>
</tr>
<tr>
<td>Q$_b$$^{4+}$</td>
<td>[(bpy)$_2$Ru(tatpq)Ru(bpy)$_2$]$^{4+}$</td>
</tr>
<tr>
<td>P$_{1}$$^{2+}$</td>
<td>[Ru(phen)$_2$tatpp]$^{2+}$</td>
</tr>
<tr>
<td>K$_b$</td>
<td>Equilibrium Binding Constant</td>
</tr>
</tbody>
</table>

s binding site size

GSH Glutathione

DTT Dithiothritol

AA Ascorbic acid

DNA bp DNA base pairs

ss single-strand

ds double-strand
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLM</td>
<td>Bleomycin</td>
</tr>
<tr>
<td>Iron-Bleomycin</td>
<td>Fe-BLM</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum Tolerance Dose</td>
</tr>
<tr>
<td>topo I</td>
<td>Topoisomerase I</td>
</tr>
<tr>
<td>topo II</td>
<td>Topoisomerase II</td>
</tr>
<tr>
<td>topo poison</td>
<td>Topoisomerase poison</td>
</tr>
<tr>
<td>topo suppressors</td>
<td>Topoisomerase suppressors</td>
</tr>
</tbody>
</table>
1.1 Background of Cancer

According to the National Cancer and Health statistics in 2001, approximately 22.9% of total deaths in the US were caused by cancer. It is the second most deadly disease next to heart disease in USA. Nearly half of all men and over one third of all women will develop cancer during their lifetime.

Once the cancer started, it often grows hidden until the later stages at which the cancer is not limited to the primary site, but it extended to other parts of the body (metastasized). Under these circumstances, the likelihood of a cure is minimum even after surgery. While there is a considerable arsenal of anticancer drugs, our ability to routinely cure cancer via chemotherapy is highly dependent on the type and stage of the disease, with many cancers remaining untreatable. Therefore, drug discovery remains an important research endeavor.

1.2 Metal Complexes as Anticancer Drugs

The vast majority of anticancer drugs are based on natural products or their derivatives. Drugs based on transition metal complexes are frequently viewed with skepticism, because they perceived as being highly toxic, unstable and very difficult to
get through the regulatory system and into clinical use. As a result, most pharmaceutical
companies and government screening agencies view anticancer drugs containing
transition metal ions as unlikely drug candidates.\textsuperscript{1,2} In this sense, there is a pervasive
bias towards organic compounds in anticancer drug research. This is unfortunate in that
one of the most widely used and successful anticancer drug in clinical use is the heavy
metal complex, cisplatin (\textit{cis}-diamminedichloroplatinum(II)). Fortunately, this
discovery and advances in our understanding of the roles of metal ions in
metalloproteins,\textsuperscript{3} metal ion transport and in many diseases\textsuperscript{4} have prompted numerous
scientists to further explore metal complexes as potential anticancer drugs, thus far with
a strong emphasis on platinum complexes.

1.3 Platinum-Based Anticancer Drugs

1.3.1. Mononuclear Platinum Complexes

In the 1960’s, Barnett Rosenberg made the serendipitous discovery that cisplatin
(Figure 1.1) could be used to treat cancer.\textsuperscript{5} Forty years later, it remains one of the three
most widely used anticancer drugs in the world with the high cure rates against
testicular carcinomas, ovarian tumors, head and neck tumors, bladder tumors and
osteosarcomas.\textsuperscript{6-8} With the testicular cancer, the cisplatin cure rates are more than 90%.
Unfortunately, cisplatin is not universally active and is not useful for the treatment of
many common malignancies including non-small cell lung carcinomas, lung
adenocarcinomas and adenocarcinomas of the colon and rectum.\textsuperscript{9} Furthermore, its use is
limited by its severe toxic side effects, which include nephrotoxicity, vomiting and nausea, ototoxicity, myelosuppression and neurotoxicity.

Despite its limitations, the success of cisplatin has inspired extensive research towards the development of new platinum-based drugs with less toxicity and improved pharmacological properties. It has also prompted intense research as to its mode of action. It is postulated that the anticancer activity of cisplatin is due to the formation of interstrand and intrastrand adducts with DNA. The two chlorides in cisplatin are labile but remain bound to the Pt(II) ion in the bloodstream due to the high chloride ion concentration in the blood. Once cisplatin passes into the cell, however, the chloride ligands are replaced by aqua ligands, as the overall chloride ion concentration is lower than in the blood. The aqua complex is cationic and preferentially, forms adducts with DNA by binding to N7 atoms of two adjacent guanine residues. The resulting adducts inhibit DNA replication, ultimately leading to cell death.\textsuperscript{8,10-12}

As a result of extensive research into structurally related complexes, two analogues of cisplatin, carboplatin (structure in Figure 1.1) \textit{(cis-diammine(1,1-cyclobutanedicarboxylato)platinum (II))}\textsuperscript{1,13} and oxaliplatin (structure in Figure 1.1) \textit{((D,L) trans-1,2-diamminocyclohexaneoxalatoplatinum(II))}\textsuperscript{14} have emerged from clinical trials as second generation platinum drugs with improved toxicity profiles. Today there are few clinical regimens of combination of chemotherapy that do not include one platinum compound.
1.3.2. Multinuclear Cationic Platinum Complexes

In an effort to expand the spectrum of anticancer activity of platinum-based drugs and to develop new complexes that can treat 'drug-resistant' tumors, multinuclear platinum complexes were explored and shown to have some promise. In this new class of compounds, two or more Pt(II) ions are linked by flexible H₂N-(CH₂)_n-NH₂ ligands. For example, [{trans-PtCl(NH₃)₂}₂{µ-trans-Pt(NH₃)₂(H₂N(CH₂)₆NH₂)₂}]⁴⁺ or BBR3464 (shown in Figure 1.2) is a highly charged (+4) trinuclear platinum complex, which is not only biologically active, but shows a significantly different activity profile than cisplatin. BBR3464 is forty- to eighty-fold more potent than cisplatin on molar dose basis and is active against cisplatin sensitive
and resistant tumors. Unlike cisplatin, BBR3464 shows prolonged tumor growth inhibition after discontinuation of the drug treatment.\(^{18}\)

It is likely that the anticancer activity of these multinuclear platinum complexes is also a result of interstrand and intrastand cross links with DNA. It is suggested that the charge and hydrogen bonding capacity within the central linker of these multinuclear complexes enhance the kinetics of DNA binding, as well as the relative ratios of the intrastrand and interstrand cross links.\(^{17,19}\) One of the biggest surprises in the success of this complex was the ease with which the cationic trimetallic complex crosses the cell membrane. This result was counter to the whole school of thought that said that metal complexes must be neutral to transverse the cell membrane and opened the door for the study of other charged metal complexes as potential therapeutics. The story on the usefulness of BBR3464 is still unfinished, but the complex has shown enough promise to merit clinical trials for the treatment of small cell lung, non-small cell lung, ovarian, and gastric cancer.\(^{20,21}\)

![Figure 1.2 Structures of Di- and Trinuclear complexes.](image-url)
1.4 Ruthenium-Based Anticancer Drugs

Among the remaining transition metals investigated thus far as anticancer agents, octahedral ruthenium complexes are among the most promising candidates for drug development. Ruthenium (II) and platinum (II) complexes have similar lability profiles, but differ in their preferred coordination numbers and stereochemistry. It is hoped that the similarities between Ru(II) and Pt(II) would be sufficient enough for biological activity and the differences enough such that a different spectrum of activity, toxicity and biodistribution are accessed.

1.4.1. Ruthenium-DMSO Complexes

The chloro-ammino ruthenium(III) complexes, $[\text{fac-Ru(Cl]}_3(\text{NH}_3)_3]$ and $[\text{cis-Ru(Cl)}_2(\text{NH}_3)_4]\text{Cl}$,\(^{22}\) and two chloro-dimethylsulfoxide-Ru(II) complexes, $\text{cis}$ and $\text{trans}$-$[\text{Ru(Cl)}_2(\text{DMSO})_4]$ (DMSO = dimethylsulfoxide) do show promising antitumor activity.\(^{23,24}\) Interestingly, the two amine complexes, $[\text{cis-Ru(Cl)}_2(\text{NH}_3)_4]\text{Cl}$ and $[\text{fac-Ru(Cl)}_2(\text{NH}_3)_4]\text{Cl}$ showed significant activity against the platinum resistant P388 leukemia (P388/DDP) and the $[\text{Ru(Cl)}_2(\text{DMSO})_4]$ isomers are highly active in tumor systems including the metastasizing Lewis lung tumors. For these tumors, the ruthenium complexes are considerably less toxic and more active than cisplatin.\(^{25}\)
Figure 1.3 The structures of some of the widely studied ruthenium anticancer complexes; DMSO = dimethylsulfoxide.

The poor solubility of several of these neutral complexes led to the development of negatively charged complexes of the type shown in Figure 1.4. Keppler and coworkers reported that the anionic bis-imidazole-tetrachloro ruthenate(III) complexes; (HL)[trans-Ru(Cl)$_4$(L)$_2$] (where L is a heterocyclic nitrogen ligand), (ImH)[trans-Ru(Cl)$_4$(Im)$_2$] (ACR) and (IndH)[trans-Ru(Cl)$_4$(Ind)$_2$], were active against platinum-resistant colorectal tumors in rats. However, their further clinical development has been delayed, because of toxic side effects and solubility remains an issue.
Sava and coworkers made several small structural modifications of the Keppler type of Ru(III) complexes including the substitution of one heterocyclic ring (Im or Ind) with DMSO to produce the most promising yet anionic Ru(III) antitumor complexes. These complexes show considerably less host toxicity, high solubility and specific activity against solid metastasizing tumors. Initial results were obtained with Na[trans-Ru(Cl)₄(DMSO)Im]-2DMSO (NAMI: see Figure 1.5), which was active against Lewis lung carcinoma, B16 melanoma and Mca mammary carcinoma. Unlike cisplatin, NAMI appears to be highly active and selective towards tumor metastases.
Also unlike cisplatin, it was almost free of organ toxicity towards liver, kidney and lungs.\textsuperscript{5}

NAMI is hydroscopic and decomposes rapidly upon exposure to moisture. Replacement of the counter ion, Na\textsuperscript{+}, with imidazolium ion (ImH\textsuperscript{+}) yields ImH[\textit{trans}-Ru(Cl)\textsubscript{4}(DMSO)Im] (NAMI-A: see Figure 1.5) and with ammonium ion (NH\textsubscript{4}\textsuperscript{+}) yields NH\textsubscript{4}[\textit{trans}-Ru(Cl)\textsubscript{4}(DMSO)NH\textsubscript{3}] (AM-A: see Figure 1.5). These new salts are no longer hydroscopic and are very stable in the solid state. Studies of NAMI-A show that it is, in some cases, more active than NAMI against solid tumor metastases.

As mentioned earlier, most of deaths from cancer come not from the primary sites, but also from the metastases. Even if surgery and/or radiotherapy successfully cure the primary site, many tumors develop distance metastases that bring sudden death after treatment. While metastatic cancers are difficult to cure, the establishment of ruthenium complexes, NAMI and NAMI-A, to target metastases, represent an important discovery in anticancer drug development.
Figure 1.5 Structures of ruthenium complexes used to treat tumor metastases.

The following table (see Table 1.1) evaluates the percent survived mice with Lewis lung carcinoma in which the primary tumor was removed by surgery on day 14 and the treatments were continued with NAMI-A, ICR, and cisplatin. Of the three treatments, NAMI-A had the best survival rates at the end of the study with 29% of the animals surviving compared to 14% for cisplatin (this was used as the control drug).
Table 1.1 Effects of treatment with NAMI-A in comparison with ICR and with cisplatin on survival time of surgereed animals.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dosage ** mg/Kg/die</th>
<th>% of survived animals in days following the surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>Day 14: 67% Day 22: 33% Day 32: 11%</td>
</tr>
<tr>
<td>NAMI-A</td>
<td>100</td>
<td>Day 14: 100% Day 22: 57% Day 32: 29%</td>
</tr>
<tr>
<td>ICR</td>
<td>40</td>
<td>Day 14: 100% Day 22: 29% Day 32: 0%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>4</td>
<td>Day 14: 100% Day 22: 43% Day 32: 14%</td>
</tr>
</tbody>
</table>

**: Equitoxic dosages for the treatment schedule used.

NAMI-A was the first ruthenium complex to progress to clinical trials. Phase I clinical trials were completed in the fall of 2002 at the Netherlands Cancer Institute (Amsterdam) and phase II clinical trials to examine its activity towards metastatic tumors have begun.

The biological target of NAMI type complexes is not firmly established. It is known that they are able to alter the ratio between of messenger RNAs for certain metalloproteinases (e.g., MMP-2) and specific inhibitor of these metalloproteinases, such as TIMP-2. The net effect of this is to increase to extracellular matrix components in the tumor parenchyma and surrounding tumor blood vessels, which prevents the tumor cells from invading the surrounding tissue.
**1.4.2. Neutral Ruthenium Polypyridyl Complexes with Labile Chlorides**

Neutral ruthenium(II) complexes containing two labile chloride ligands in a *cis* configuration, such as \([\text{Ru}(\text{phen})_2(\text{Cl})_2]\) have substitution and geometric similarities with cisplatin. *In vitro*, cytotoxicity studies revealed that both \([\text{Ru}(\text{phen})_2(\text{Cl})_2]\) and \([\text{Ru}($\text{DIP}$)_2(\text{Cl})_2]\) (see Figure 1.6) are extremely toxic towards mouse leukemia cell lines, L1210 and P815 (data contain in Table 1.2)\(^{43}\) with the cytotoxicity of \([\text{Ru}($\text{DIP}$)_2(\text{Cl})_2]\) complex approximately 11 times more potent than the \([\text{Ru}(\text{phen})_2(\text{Cl})_2]\) complex.

![Figure 1.6 Structures of neutral ruthenium complexes, $[\text{Ru}(\text{phen})_2(\text{Cl})_2]$ (phen = 1,10-phenanthroline) and $[\text{Ru}($\text{DIP}$)_2(\text{Cl})_2]$ (DIP = 4,7-diphenyl-1,10-phenanthroline).](image-url)
Table 1.2 Cytotoxicity results of neutral Ruthenium (II) complexes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell Line</th>
<th>IC$_{50}$ (µg/mL)</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ru(phen)$_2$(Cl)$_2$]</td>
<td>L1210</td>
<td>4.7</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>P815</td>
<td>7.0</td>
<td>8.4</td>
</tr>
<tr>
<td>[Ru(DIP)$_2$(Cl)$_2$]</td>
<td>P815</td>
<td>3.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>L1210</td>
<td>0.44</td>
<td>0.53</td>
</tr>
</tbody>
</table>

L1210 and P815 are mouse leukemia cell lines.

It is established that [Ru(phen)$_2$(Cl)$_2$] binds covalently to duplex DNA with the striking enantiomeric selectivity of ligand substituted Λ-(phen)$_2$Ru$^{2+}$ moiety to the B-DNA helix. Binding process is somewhat slow and takes 3.5 hours to achieve a maximum binding ratio of 0.045 (complex per 11 DNA base pairs). This slower DNA binding pattern, considers [Ru(phen)$_2$(Cl)$_2$] as an octahedral analogue for cisplatin.$^{44}$

1.4.3. Cationic Ruthenium Polypyridyl Complexes

The simple cationic Ru(II) polypyridyl complexes, such as [Ru(bpy)$_3$]$^{2+}$, [Ru(phen)$_3$]$^{2+}$, etc., have been discovered as antibacterial and antileukemic agents, in vitro.$^{45-48}$ However, they were also found to be acutely toxic at relatively low doses in mice.$^{49}$ Enzymatic assays revealed that these complexes are potent inhibitors of acetylcholine esterase (AChE), which is possibly be the reason for the observed acute toxicity.$^{49}$ [Ru(phen)$_3$]$^{2+}$ as do all tris-chelate complexes, exists as a pair of enantiomers identified as the Δ and Λ stereoisomers (see Figure 1.7). Especially, the chirality of the
complex plays a major roll in inhibiting AchE with $\Delta$-[Ru(phen)$_3$]$^{2+}$ giving 90% AchE inhibition compared to 20% inhibition for the same amount of the $\Lambda$ enantiomer.

The interactions of these cationic complexes with DNA have been the object of numerous investigations, yet its binding mode is still a matter of controversy. Barton and coworkers have proposed that it binds to DNA through three non-covalent modes: 1) electrostatic binding between cationic metal center and anionic phosphate backbone of DNA polymer, 2) hydrophobic binding with minor groove of DNA and 3) partial intercalation of one of the phenanthroline ligands into the DNA base stack from the major groove side.$^{50-52}$ DNA binding constants of $\sim 6.2 \times 10^3$ M$^{-1}$ have been reported for the racemic complex. Satyanarayana et al. measured the change in solution viscosity for the mixture of both enantiomers of [Ru(phen)$_3$]$^{2+}$ with DNA. The results showed no difference between enantiomers and further confirmed that classical intercalation could not be the binding mode.$^{53,54}$

Figure 1.7 Structure of [Ru(phen)$_3$]$^{2+}$ and its stereoisomers, $\Lambda$ and $\Delta$. 
The complexes [Ru(phen)$_2$dppz]$^{2+}$ and [Ru(bpy)$_2$dppz]$^{2+}$ are known to bind DNA much more avidly than their parent homoleptic complexes with $K_b$'s of $\sim 10^8$ M$^{-1}$. The enhanced binding is due to the large planar aromatic dppz ligand, which now favors intercalative binding to the DNA. These compounds are also known as “molecular light switches” because they do not luminesce in aqueous solution, but become luminescent when bound to DNA, thus 'lighting up' when DNA is present. Interestingly, this property provides a potential tool to monitor the DNA binding process.

Hiort et al. synthesized the $\Delta$ and $\Lambda$ isomers of [Ru(phen)$_2$dppz]$^{2+}$ (see Figure 1.8) in order to study the differences of their interactions with DNA. Surprising, only small differences were observed in their DNA binding, with both enantiomers having equilibrium binding constants of $\sim 10^8$ M$^{-1}$ in solutions containing 10 mM NaCl.$^{55}$

![Figure 1.8 Absolute configurations of the $\Delta$ and $\Lambda$ enantiomers of [Ru(phen)$_2$dppz]$^{2+}$](image-url)
Inspired by the tight DNA binding of monomeric Ru(II) complexes with DNA, dimeric metallointercalators were investigated as probes of nucleic acid structure. In contrast to monomeric ruthenium complexes, dinuclear ruthenium complexes have increased charge, and increased variations in shape and size, which show more specificity in binding with DNA.\textsuperscript{64-67} Lincoln and coworkers showed that by simply linking two [Ru(phen)\textsubscript{2}dppz]\textsuperscript{2+} complexes via a tether, as shown in Figure 1.9, gives complexes with extremely high DNA binding constants, in the range of \(10^{10}\) to \(10^{14}\) M\textsuperscript{-1}, which exhibit very slow dissociation kinetics.\textsuperscript{64,65} The stereochemistry does affect the binding with the \(\Delta\Delta\) enantiomers bind better that the \(\Lambda\Lambda\) enantiomers.\textsuperscript{65}
Figure 1.9 Structures of Ruthenium dimers: [μ-c4(cpdppz)₂(phen)₄Ru₂]⁴⁺ (1⁴⁺) and [μ-(11,11'-bidppz)(phen)₄Ru₂]⁴⁺ (2⁴⁺).

Because of the extreme DNA binding affinity, a number of enantiomerically pure analogues of 1⁴⁺ (ΔΔ stereoisomers), shown in Figure 1.10 were prepared and examined for tumor cell cytotoxicity against a number of cell lines. For the comparison, they also examined in parallel, Δ-[Ru(phen)₂dppz]²⁺ (3²⁺) and their results are given in Table 1.3. The concentration of the complex, which leads to 50% inhibition of the cell
growth, is reported as the IC$_{50}$, with lower values indicating more effective tumor killing ability.

Figure 1.10 Structures of the ruthenium dimers, I, II, III and IV.
Table 1.3 IC_{50} of the ΔΔ- enantiomers of complexes (I), (II), (III), (IV) and their known mononuclear complex Δ[Ru(phen)₂(dppz)]Cl₂ (3^{2+}).

<table>
<thead>
<tr>
<th>Tumor Cell Line</th>
<th>(I)</th>
<th>(II)</th>
<th>(III)</th>
<th>(IV)</th>
<th>(3^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung carcinoma HBT182</td>
<td>9</td>
<td>8</td>
<td>19</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>Melanoma</td>
<td>19</td>
<td>30</td>
<td>19</td>
<td>45</td>
<td>&gt;50*</td>
</tr>
<tr>
<td>SUDHL 1</td>
<td>14</td>
<td>12</td>
<td>14</td>
<td>22</td>
<td>39</td>
</tr>
<tr>
<td>LAMA</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>16</td>
<td>43</td>
</tr>
</tbody>
</table>

*17% growth inhibition at 50 mM.

For the most part, it is seen that the dimers have lower IC_{50} values than the mononuclear Δ-[Ru(phen)₂(dppz)]Cl₂ complex and the most of the complexes show IC_{50} values below 10 µM for several tumor lines. This was considered a promising start for such complexes, but problems, such as animal toxicity\(^{49}\) and mutagenic properties\(^{68}\) seem likely to limit their application as drugs. It appears that cationic ruthenium polypyridyl complexes have promising biological activity, which could warrant their use as drugs if the issue of toxicity could be overcome.

1.5 Scope of Thesis Dissertation

This thesis describes our investigation of a new class of cationic, ruthenium (II) polypyridyl monomers and dimers as prospective anticancer agents. These complexes differ from the prior work reported in that the metal centers are bridged by rigid, tetradentate ligands of the type shown in Figure 2.3 (Figure of tpphz, tatpp, tatpq). Not
only, do these planar aromatic ligands lead to intercalative DNA binding, but also two of the three, tatpp and tatpq, are redox active at biologically accessible potentials leading to new modes of biological activities.

In this work, a series of complexes with variable overall charge (0, +2, +4) containing labile (CH$_3$CN, oxalato, chloro) and non-labile (bpy, phen, tpphz, tatpp, CO) ligands, short tpphz or long (tatpp or tatpq) bridging ligands, and redox inactive (tpphz) and active (tatpp and tatpq) bridging ligands were prepared and studied for their DNA binding properties and tumor cell cytotoxicity. Promising candidates were further examined for animal (mouse) toxicity and antitumor activity, in vivo. Subsequent studies into the biological activity of the most promising antitumor agents examined their DNA cleaving activity, under both normal and hypoxic conditions, and their ability to inhibit topoisomerase I and II. (topo I and II) A partial list of the compounds prepared includes: [Ru(bpy)(CO)$_2$]$_2$tpphz]$^{4+}$ (7$^{4+}$), [Ru(bpy)(CH$_3$CN)$_2$]$_2$tpphz]$^{4+}$ (8$^{4+}$), [Ru(bpy)(C$_2$O$_4$)$_2$]$_2$tpphz] (9), [Ru(bpy)(CO)(Cl)$_2$]$_2$tpphz]$^{2+}$ (10$^{2+}$), [(bpy)$_2$Ru(tpphz)Ru(bpy)$_2$]$^{4+}$ (11$^{4+}$), [(phen)$_2$Ru(tpphz)Ru(phen)$_2$]$^{4+}$ (12$^{4+}$), [(phen)$_2$Ru(tatpp)Ru(phen)$_2$]$^{4+}$ (P$_p^{4+}$), [(phen)$_2$Ru(tatpq)Ru(phen)$_2$]$^{4+}$ (Q$_p^{4+}$), [(bpy)$_2$Ru(tatpp)Ru(bpy)$_2$]$^{4+}$ (P$_b^{4+}$) and [(bpy)$_2$Ru(tatpq)Ru(bpy)$_2$]$^{4+}$ (Q$_b^{4+}$).

Chapter 2 provides a detailed description of the synthesis and characterization of a number of novel tris-heteroleptic ruthenium (II) monomers and dimers. The cytotoxicity of the most of these complexes was screened by determining the IC$_{50}$ values for these agents against non-small cell lung cancer cells (NSCLC). Two of the most promising complexes, P$_p^{4+}$ and Q$_p^{4+}$ were further screened for animal toxicity and
antitumor activity *in vivo*. Complex $P_p^{4+}$ showed sufficient activity against mouse melanoma cancer *in vivo* that the molecular studies to determine its mode of action were begun. These studies are the basis of chapters 3 and 4, which examine its ability to cleave DNA and to inhibit topoisomerases (I and II), respectively.

Chapter 3 examines the ability of $P_p^{4+}$ to cause single and double-strand breaks to DNA under variety of conditions. DNA was assumed as a likely biological target given the high DNA binding affinity of the complex, as established in Chapter 2. A number of the ruthenium complexes were examined and only $P_p^{4+}$ and $Q_p^{4+}$ showed appreciable DNA cleavage ability and then only when external reductants, such as glutathione (GSH), were present. Studies to establish the mechanism of cleavage surprisingly, revealed that the DNA cleaving ability of $P_p^{4+}$ is enhanced under hypoxic conditions, which is not only unusual, but has exciting therapeutic potential for cancer treatment. Ultimately, we established that the active chemical nuclease is the doubly reduced complex, $P_p^{2+}$, which is also doubly protonated at pH 7.

Chapter 4 examine the inhibition of DNA topoisomerase I and II by $P_p^{4+}$, as many intercalators are known to inhibit or poison this important class of enzymes. Studies of the function of topoisomerase I and II in the presence of $P_p^{4+}$ reveal that it is a potent inhibitor of both topo I and II at relatively low concentrations. A comparison of the topoisomerase activity of $P_p^{4+}$ and a number of structural variants reveals the importance of intercalating planar extending bridging ligand (tatpp) and the complex charge of 4+ for the existing activity.
CHAPTER 2
SYNTHESIS AND SCREENING OF RUTHENIUM POLYPYRIDYL COMPLEXES
AS POTENTIAL ANTICANCER AGENTS

2.1 Introduction

Many anticancer drugs act at the DNA level. They preferentially bind DNA and inhibit the DNA replication and transcription, which is the basis of their anticancer activity.\(^\text{69-74}\) Ruthenium (II) polypyridyl complexes with the planar extended aromatic ligands have received significant attention in part, due to their extremely tight and intercalative binding to DNA.\(^\text{55,58,64,67,75-78}\) The dipositive charge of these complexes leads to bind DNA via electrostatic attraction. For example, DNA binding constants for \([\text{Ru(bpy)}_3]^{2+}\) and \([\text{Ru(phen)}_3]^{2+}\) are \(7.0 \times 10^2 \text{ M}^{-1}\) and \(3 \times 10^4 \text{ M}^{-1}\),\(^\text{53}\) respectively. Substitution of one of the bpy or phen ligands with large extended planar ligands, like dppz, for example, \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) and \([\text{Ru(bpy)}_2\text{dppz}]^{2+}\) lead to intercalation and much tighter binding, on the order of \(10^6 - 10^8 \text{ M}^{-1}\).\(^\text{55,61}\) A picture of a DNA fragment bound by several mononuclear \([\text{Ru(bpy)}_3\text{(dppz)}]^{2+}\) intercalators is shown in Figure 2.1.\(^\text{50,80}\) Intercalation was first coined by Lerman in 1961 and means that the molecule can reside in the spaces between two adjacent DNA base pairs where the intercalator is sandwiched tightly between the aromatic, heterocyclic base pairs and stabilized electronically in the helix by \(\pi-\pi\) stacking and dipole-dipole interactions.\(^\text{81}\)
Complexes containing two Ruthenium (II) ions connected to each other by two planar dppz ligands and thus possessing an overall +4 charge, show even tighter binding. For example, the dimers \([\text{(L-L)}_{2}\text{Ru(μ-c4(cpdpmpz)}_{2}\text{Ru(L-L)}_{2}]^{4+}\) and \([\text{(L-L)}_{2}\text{Ru(μ-(11-11′-bidppz)}\text{Ru(L-L)}_{2}]^{4+}\) have binding constants of \(\sim10^{12}\) M\(^{-1}\) and \(\sim10^{10}\) M\(^{-1}\) (25 °C and 50 mM NaCl), respectively (L-L can either be phen or bpy; see Figure 2.2 for their structures).\(^{64,66,82}\) This tight DNA binding may lead to useful biological activity as complex, \(1^{4+}\) shows some antitumor activity against platinum resistant tumor types in vitro.\(^{83}\)

The development of cationic complexes containing polypyridyl ligands as drugs has been tempered by the observation that most such complexes are acutely toxic when administered intraperitoneally (typical LD\(_{50}\) in mice was 6 mg/kg) and ineffective when administered orally. Dwyer and coworkers\(^{45,46,49}\) have shown that \([\text{Ru(phen)}_{3}]^{2+}\) and \([\text{Ru(bpy)}_{3}]^{2+}\) are potent inhibitors of acetylcholine esterase (AchE),\(^{49}\) which is their likely target in vivo. AchE function is essential for the nervous system and inhibition is known to lead to convulsions and rapid death. Notably, the chirality of the complexes strongly affects their inhibitory properties with \(Δ-[\text{Ru(phen)}_{3}]^{2+}\) giving 90% AchE inhibition compared to 20% inhibition for the same amount of the \(Λ\) isomer.\(^{84,85}\) The
preliminary toxicity studies, of the ruthenium polypyridyl dimer, 
[(bpy)_2Ru(tpphz)Ru(bpy)_2]^{4+} (11^{4+}), also confirmed that such dimers are acutely toxic in mice.\textsuperscript{86}

We hypothesized that the high cationic charge was largely responsible for the toxicity and set out to reduce the overall charge by replacing the ancillary ligands (phen or bpy) with anionic ligands. Ultimately, this required us to develop synthetic
procedures to construct tris-heteroleptic Ru(II) complexes. Keene and coworkers were first to report practical procedures for tris-heteroleptic Ru(II) of the type we pursued here.\textsuperscript{87-90}

This chapter describes our efforts to expand on this synthetic chemistry and synthesize neutral monomeric and dimeric Ru(II) complexes containing intercalating ligands such as dppz, tpphz, tatpp (tatpp = 9,11,20,22-tetraazatetrapyrido[3,2-a: 2’,3’-c: 3”,2”-I: 2””,3”’-n]-pentacene) and tatpq (tatpq = 9,11,20,22-tetraazatetrapyrido[3,2-a: 2’,3’-c: 3”,2”-I: 2””,3”’-n]-pentacene-10,21-quinone; see Figure 2.3).

Figure 2.3 Structures of dppz, tpphz, tatpp and tatpq.
These complexes were then screened for DNA binding and tumor cell cytotoxicity. Promising candidates were further evaluated for animal toxicity and antitumor properties. Ultimately, we discovered that the neutral complexes, we developed were not particularly promising as drug agents, however, some of the related cationic complexes were not only promising for antitumor properties, but also were surprisingly well tolerated by mice in toxicity studies, suggesting therapeutic potential.

2.2 Results and Discussion

2.2.1. Synthesis of Neutral Tris-heteroleptic Ru(II) Monomers

Our initial target complex was the neutral intercalator, [Ru(bpy)(dppz)(C₂O₄)] and the synthetic route we pursued is given in scheme 2.1. The key intermediate being the carbonyl complex, [Ru(bpy)(dppz)(CO)₂]²⁺ (4²⁺), which contains both an intercalating diimine (dppz) and a bpy ligand. It is known that coordinated CO ligands can be removed by oxidation with trimethylamine N-oxide (TMNO), leading to a reactive solvato intermediate that have the same *cis* stereochemistry.
Scheme 2.1 Synthetic strategy for tris-heteroleptic neutral complexes, for instance, [Ru(bpy)(dppz)(C\textsubscript{2}O\textsubscript{4})] (6).
As seen in scheme 2.1, [Ru(bpy)(CO)₂(Cl)₂] is synthesized from RuCl₃ formaldehyde and bpy according to a published procedure. Test reactions established that the direct replacement of chlorides in [Ru(bpy)(CO)₂(Cl)₂] by a second chelate ligand (L-L) is not possible, presumably due to the trans arrangement of the two chloride groups. The stereochemistry can be altered to the desired cis arrangement by conversion to the triflate intermediate, [Ru(bpy)(CO)₂(CF₃SO₃)₂], which has the cis configuration of leaving groups. The triflato complex readily undergoes substitution with a second chelate ligand to give [Ru(bpy)(L-L)(CO)₂]²⁺, as shown in scheme 2.2. The triflato intermediate is, however, sensitive to decomposition upon exposure to air, and is not isolated but instead used immediately. Addition of dppz gives the tris-heteroleptic carbonyl complex, 4²⁺, which was isolated as the PF₆⁻ salt.

Scheme 2.2 Synthesis and structural formula of [Ru(bpy)(CO)₂(CF₃SO₃)₂] and 4²⁺.
Complex [4]PF₆₂ was characterized by ¹H NMR, ¹³C NMR, IR and elemental analysis. The observation of 18 peaks in ¹H NMR is expected as the complex lacks any symmetry elements and all the protons are unique (see Figure 2.4). The spectrum was assigned as shown on the basis of a COSY spectrum to establish coupled protons and by comparison of the chemical shifts with related complexes. The normal downfield disposition of the dppz H_c and H_c' peaks is observed with the two resonance coming at very different chemical shifts, because one is trans to a bpy nitrogen and the other is trans to a CO group. Both the IR and ¹³C NMR data support a cis configuration of CO ligands with two carbonyl peaks observed at 2101 and 2045 cm⁻¹ in IR and two ¹³C chemical shifts at 191.2 and 191.3 ppm (see Figure 2.5).
Oxidative decarbonylation of \([\text{4}]\)(\(\text{PF}_6\))\(_2\) was performed as reported by Keene et al. using TMNO as an oxidant to give the solvato intermediate\(^{87,88,93,94}\) \([\text{Ru}(\text{bpy})(\text{dppz})(\text{CH}_3\text{CN})_2]^{2+}\) (\(5^{2+}\)) in high yield. The \(^1\text{H}\) NMR and \(^{13}\text{C}\) NMR data for \([\text{5}]\)(\(\text{PF}_6\))\(_2\) are shown in Figures 2.6 and 2.7, respectively, and confirm the retention of the \textit{cis} configuration by the acetonitrile groups. In particular, two methyl proton peaks at 2.37 and 2.23 ppm are observed in the \(^1\text{H}\) NMR spectrum as expected for the \textit{cis}
configuration and the disappearance of carbonyl peaks in the $^{13}$C NMR confirmed the substitution of carbonyls with acetonitrile ligands.

![Diagram of [5](PF$_6$)$_2$ in $d_3$ MeCN](image)

Figure 2.6 $^1$H NMR spectrum of [5](PF$_6$)$_2$ in $d_3$ MeCN.

![Diagram of [5](PF$_6$)$_2$ in $d_3$ MeCN](image)

Figure 2.7 $^{13}$C NMR spectrum of [5](PF$_6$)$_2$ in $d_3$ MeCN.
The neutral oxalato complex was obtained by the reaction of $5\text{[PF}_6\text{]}_2$ with excess sodium oxalate (10 equivalents) in ethanol/water mixture. The initial orange-red solution turned into dark purple as the reaction proceeded and ultimately a purple solid was isolated. Compared with the cationic complexes, the neutral complex, $6$ is only sparingly soluble, however, a saturated DMSO-$d_6$ solution was concentrated enough to obtain a $^1$H NMR spectrum, which is shown in Figure 2.8. The $^1$H NMR is consistent with the proposed structure with 18 different proton signals as expected. Unfortunately, $^{13}$C NMR data were not obtained due to the poor solubility. The compound was further characterized by elemental analysis and mass spectrometry, which both support the proposed structure.
2.2.2. Synthesis of Neutral Tris-heteroleptic Ru(II) Dimers

The synthesis of the neutral tpphz containing dimer, \([\{\text{Ru(bpy)}(\text{C}_2\text{O}_4)\}_2\text{tpphz}\}\) (9) was similar to that of the neutral monomer, \([\text{Ru(bpy)}\text{dppz}(\text{C}_2\text{O}_4)]^{2+}\) (6). In this case, \([\text{Ru(bpy)}(\text{CO})_2(\text{CF}_3\text{SO}_3)]\) was refluxed with the tetradeinate tpphz ligand in ethanol for 5h under an inert atmosphere (see Scheme 2.5) to give the dimer, \([\{\text{Ru(bpy)}(\text{CO})_2\}_2\text{tpphz}\]^{2+}\) (7\(^{4+}\)) in 40% yield. Compared to the monomer, longer reaction time and larger solvent volume were required to obtained decent yields because of the poor solubility of the tpphz ligand.

The dimer was characterized by \(^1\text{H NMR}, \ ^{13}\text{C NMR},\ \text{IR and elemental analysis}. As shown in Figure 2.9, a total of 28 peaks are observed in the \(^1\text{H NMR} spectrum. Four of these peaks are observed in the downfield region characteristic of the H-atoms lying nearest to the pyrazine nitrogen on tpphz. The presence of \textit{cis} and \textit{trans} isomers for 7\(^{4+}\) is expected as shown in Scheme 2.5 and thus 4 'H\(_c\)' resonances (labeled H\(_c\), H\(_c\)' , H\(_c\)'', and H\(_c\)'') are not unexpected. The areas of the four peaks are approximately equal indicating
a 1:1 mixture, which is expected on a purely statistical basis. Attempts to separate two isomers by chromatography have not been successful and thus the mixture is used in subsequent syntheses and studies.

Scheme 2.5 Synthesis route and structural formula of cis and trans [{Ru(bpy)(CO)$_2$}$_2$tpphz]$^{4+}$ ($7^{4+}$).
Figure 2.9 $^1$H NMR spectrum of cis and trans [7](PF$_6$)$_4$ mixture in $d_3$ MeCN.
Figure 2.10 $^{13}$C NMR spectrum of cis and trans mixture of [7](PF$_6$)$_4$ in $d_3$ MeCN.

The neutral dimer, [{Ru(bpy)(C$_2$O$_4$)$_2$}$_2$tpphz] (9), was prepared in a manner similar to that for the dppz monomer. Decarbonylation of 7$^{4+}$ with TMNO in MeCN gave the solvato complex [{Ru(bpy)(CH$_3$CN)$_2$}$_2$tpphz][PF$_6$]$_4$ (8[PF$_6$]$_4$), which was characterized by $^1$H NMR and $^{13}$C NMR, as shown in Figures 2.11 and 2.12, respectively. Again, the presence of the cis and trans geometrical isomers is observed in a 1:1 ratio. In this case, not only are four 'H' resonances seen in the downfield region, four methyl resonances around 2.2 – 2.4 ppm are observed in the $^1$H NMR. In the $^{13}$C NMR, the carbonyl resonances at 191.2 and 190.9 ppm are gone and the methyl carbon peaks seen between 2.9 and 5.0 ppm (see Figure 2.12) verifying the substitution of carbonyls with acetonitrile ligands. Separation of these two geometrical isomers was not attempted at this stage owing to the lability of the CH$_3$CN ligands.
Scheme 2.6 Synthesis route and structural formula of *cis* and *trans* mixture of $8^{4+}$. 

(CH$_3$)$_3$NO in CH$_3$CN Argon
Figure 2.11 $^1$H NMR spectrum of cis and trans mixture of [8](PF$_6$)$_4$ in $d_2$ MeCN.
Figure 2.12 Comparison of $^{13}$C NMR spectra of cis and trans mixture of A) [7](PF$_6$)$_4$ and B) [8](PF$_6$)$_4$ in $d_3$ MeCN.
The neutral oxalato dimer was obtained by reaction of [8](PF₆)₄ with excess Na₂C₂O₄ in 1:1 EtOH/H₂O under N₂. During the reaction, the color changed from orange to dark purple and a purple solid precipitate formed. This solid was not soluble enough to obtain good NMR data and characterization was limited to mass spectrometric data and absorption spectroscopy. The MALDI-TOF spectrograph showed a parent ion peak consistent with the proposed structure for 9. The absorption spectra are discussed in detail in section 2.2.4, however, the features of the spectrum of 9 were very similar to those for the mononuclear dppz complex, [Ru(bpy)(dppz)(C₂O₄)].

Scheme 2.7 Synthesis and structural formulas of cis and trans mixtures of 8⁺⁺ and 9.
2.2.3. Synthesis of Partially Neutralized Dimer, \([\{\text{Ru(bpy)(CO)(Cl)}\}_2\text{tpphz}\}]^{2+}\)

A dimer with an overall charge of +2, was prepared by reacting \([\text{Ru(bpy)}_2\text{tpphz}]^{2+}\) and \([\text{Ru(CO)}_2(\text{Cl})_2]_x\) in a 1:1 ratio to yield \([\{\text{Ru(bpy)(CO)(Cl)}\}_2\text{tpphz}\}]^{2+}\ (10^{2+})\). As shown in Figures 2.13 and 2.14, the $^1$H NMR, $^{13}$C NMR spectra support this structural assignment. As with the other complexes, the proton assignments were made by comparison to known complexes of similar structure and with the aid of COSY NMR.

Scheme 2.8 Synthesis and structural formulas of \([\text{Ru(bpy)}_2\text{tpphz}]^{2+}\) and \(10^{2+}\).
Figure 2.13 $^1$H NMR spectra of a) [Ru(bpy)$_2$tpphz](PF$_6$)$_2$ and b) [10](PF$_6$)$_2$ in $d_3$ MeCN.

Figure 2.14 $^{13}$C NMR spectrum of [10](PF$_6$)$_2$ in $d_3$ MeCN.
2.2.4. Absorption Spectra of Tris-heteroleptic Ru(II) Monomers and Dimers

Absorption spectra of the Ru(II) monomers and dimers as recorded in DMSO are shown in Figures 2.15 and 2.16, respectively. The corresponding carbonyl, acetonitrile and oxalate monomers and dimers gave nearly identical spectra except that the molar extinctions for the dimers were more than twice that of the monomers. All of the complexes, except of carbonyl species, exhibit broad bands in the 400-700 nm region, which can be attributed to the Ru(II) dπ to diimine (π*) MLCT transitions. The diimines vary but include, bpy, dppz and tpphz.

![Absorption Spectra](image)

Figure 2.15 UV-Vis spectra of [4]Cl₂ (black line), [5]Cl₂ (red line), and 6 (purple line) in DMSO.
All the spectra display sharp bands between 325 to 400 nm corresponding to \(n\rightarrow\pi^*\) and \(\pi\rightarrow\pi^*\) ligand centered transitions (LC). These transitions are seen in the free dppz or tpphz ligands and are only slightly perturbed upon coordination. The intense bands below 350 nm are attributed to a number of \(\pi\rightarrow\pi^*\) transitions for the aromatic ligands and were not examined in detail.

Figure 2.16 UV-Vis spectra of \([7]\text{Cl}_4\) (black line), \([8]\text{Cl}_4\) (red line) and \(9\) (purple line) in DMSO.
2.2.5. DNA Binding Studies with Ruthenium Dimers

All of the dimeric complexes prepared thus far: [{Ru(bpy)(CO)₂tpphz]⁴⁺ (7⁺), [{Ru(bpy)(CH₃CN)₂tpphz]⁴⁺ (8⁺), [{Ru(bpy)(C₂O₄)₂tpphz] ⁴⁺ (9)

[(phen)₂Ru(tatpp)Ru(phen)₂]⁴⁺ (Pₚ⁴⁺), [(bpy)₂Ru(tatpp)Ru(bpy)₂]¹⁺ (Pₚ⁴⁺), and

[(phen)₂Ru(tatpq)Ru(phen)₂]⁴⁺ (Qₚ⁴⁺) (See Figure 2.17 for Pₚ⁴⁺, Pₚ⁴⁺ and Qₚ⁴⁺)

Figure 2.17 Structures of Pₚ⁴⁺, Pₚ⁴⁺ and Qₚ⁴⁺.
structures) have chiral centers at the metal sites and therefore exist as either racemates (when just one chiral center is present) or as diastereotopic mixtures (when two chiral centers are present). All of these complexes were prepared without regard to the stereochemistry at the metal centers and therefore exist as either a racemate or a statistical mixture of diastereomers. The local metal stereochemistry is also likely to have some effect on any observed biological activity as well as the DNA binding affinity. While it is possible to prepare many of these complexes in diastereomerically and enantiomerically pure form, we decided to first investigate the DNA binding and cytotoxicity of the complexes 'as prepared' with the assumption that any promising drug candidates discovered could be 'reinvestigated' with respect to stereochemical purity. Thus for the remainder of this thesis, a complex, regardless of its stereochemical makeup, was viewed as a single compound and we evaluated its aggregate properties.

DNA binding studies were carried out using a dye displacement assay in which a luminescent intercalating dye with a known binding constant is displaced by binding of the ruthenium complex. Upon displacement, the dye is no longer fluorescent and thus the DNA binding can be followed by fluorescence spectroscopy. None of the ruthenium complexes under investigation here showed fluorescence in solution, regardless of the presence or absence of DNA. In these studies, the fluorescent dye used was a ruthenium complex, $\Delta$-[Ru(phen)$_2$dpdz]$^{2+}$, which has a $K_b$ of $5 \times 10^7 \text{ M}^{-1}$ and shows intense fluorescence when bound to DNA.$^{58,95}$
Figures 2.18 and 2.19 show the decrease in luminescence of a solution of calf-thymus DNA presaturated with $\Delta$-[Ru(phen)$_2$dppz]$^{2+}$ upon titration with the various ruthenium complexes indicated.

Because, we know the $K_b$ and the binding site size (s) for $\Delta$-[Ru(phen)$_2$dppz]$^{2+}$ are 5 x10$^7$ M$^{-1}$ and 2 respectively, we can establish the $K_b$ for each complex in this competitive binding experiment. As this fitting procedure is complicated and requires simulation, our raw data was analyzed by a noted expert in this field, Dr. Per Lincoln.
(Calamus University, Sweden),\textsuperscript{55,58,82,96} and fit according to the procedure describe in the literature (McGhee-von Hipple equation).\textsuperscript{97} All the studies were performed with calf thymus DNA in buffer (5 mM Tris, 10 mM NaCl, pH 7.5) at room temperature. The $K_b$ and the s that were determined by fitting this data are presented in Table 2.1.

Figure 2.19 Displacement titration based on quenching of DNA-bound \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) by $P_p^{4+}$, $P_b^{4+}$ and $Q_p^{4+}$.

![](image)
Table 2.1 Summary of Equilibrium DNA Binding Constants ($K_b$) and binding site size (s), for Ruthenium (II) dimers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_b$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7$^{4+}$</td>
<td>$2.6 \times 10^7$ (1.3)</td>
</tr>
<tr>
<td>8$^{4+}$</td>
<td>$9.4 \times 10^9$ (2.5)</td>
</tr>
<tr>
<td>P$p^{4+}$</td>
<td>$2.4 \times 10^9$ (2.2)</td>
</tr>
<tr>
<td>P$b^{4+}$</td>
<td>$2.0 \times 10^9$ (2.1)</td>
</tr>
<tr>
<td>Q$p^{4+}$</td>
<td>$3.4 \times 10^8$ (1.7)</td>
</tr>
</tbody>
</table>

All of the ruthenium dimers efficiently displace $\Delta$-[Ru(phen)$_2$dppz]$_{2^{2+}}$ and binding constants in the range of $10^7$ to $10^9$ M$^{-1}$ were obtained. The carbonyl dimer, 7$^{4+}$ showed the weakest binding. Surprisingly, simply changing the carbonyl ligands to acetonitriles resulted in a 361-fold increase in binding affinity (7$^{4+}$ to 8$^{4+}$). We speculate that the MeCN complex may be able to bind in more than one fashion, e.g., via intercalation and/or via direct coordination of the Ru(II) ion to the DNA by displacement of one of the labile MeCN ligands. The dimers bridged by the longer tatpp or tatpq ligand also showed extremely tight binding with the quinone complex being about 7 fold less tightly bound. Interestingly, replacement of the peripheral phen ligands in P$p^{4+}$ with bpy ligand in P$b^{4+}$ has a little effect on the observed binding affinity (Figure 2.19). Unlike cationic dimers, the neutral dimer, 9 did not show any evidence of binding to DNA.
2.2.6. Cytotoxicity of Ru(II) Dimers Towards Non-Small Cell Lung Cancer Cell Lines (NSCLC)

The strong interaction of these complexes with DNA suggested that they might have activity as antitumor agents by inhibiting the DNA replication. In order to evaluate the cytotoxicity of these complexes towards cancer cells, we treated H358 bronchioalveolar non-small cell lung carcinoma cells (NSCLC) with these complexes and followed the cytotoxicity using a standard MTT drug sensitivity assay.\textsuperscript{98-100} The data from these assays are plotted in Figure 2.20, which shows the fraction of cells surviving (alive) after incubation with different concentrations of the drug complex for a specified time period. The IC\textsubscript{50} is defined as the drug concentration required to kill 50\% of the tumor cell population relative to a control\textsuperscript{101,102} and is obtained directly from the plot in Figure 2.20. The IC\textsubscript{50} values of these dimers, and the clinically used anticancer drug, cisplatin\textsuperscript{100} are presented in Table 2.2.

All of the complexes tested, except the neutral oxalate complex, 9, showed IC\textsubscript{50} values less than 100 µM. The two most cytotoxic complexes are the dimers, P\textsubscript{p}4+ and Q\textsubscript{p}4+, which are bridged by the long tatpp and tatpq ligands. Cationic dimers bridged by the shorter tpphz ligand also showed good cytotoxicity. Neutral complexes showed the least cytotoxicity. As can be seen from Table 2.2, almost all of the cationic ruthenium complexes had IC\textsubscript{50} values comparable with that for cisplatin.
Figure 2.20 Cytotoxicity data of Ruthenium (II) dimers, $Q_p^{4+}$, $P_p^{4+}$, $7^{4+}$, $8^{4+}$, $10^{2+}$ and 9 against NSCLC cells.

Table 2.2 IC$_{50}$ of the following Ruthenium (II) dimers and cisplatin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>&gt;100 ± 10</td>
</tr>
<tr>
<td>$7^{4+}$</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>$8^{4+}$</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>$10^{2+}$</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>$P_p^{4+}$</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>$Q_p^{4+}$</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10 ± 2$^{100}$</td>
</tr>
</tbody>
</table>
2.2.7. Animal Toxicity

A number of cationic ruthenium polypyridyl complexes including \([\text{Ru(phen)}_3]^{2+}\) and \([\text{Ru(bpy)}_3]^{2+}\) are known to be acutely toxic in animals at relatively low doses.\(^{45,46,49}\) As this structural component is common to many of the complexes investigated here, we screened a number of complexes for toxicity in mice. The initial animal toxicity screen was performed by intraperitoneal injection to a single mouse (C57BL/6NTac mice, ~ 30 g each) with 0.2 mg of the ruthenium complex (as the chloride salt for the cationic complexes) in 200 \(\mu\)L Tris buffer, pH 7.5. This dosage corresponds to approximately 6.7 mg complex per kilogram of mouse body weight (6.7 mg/kg).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Toxicity of 1 mg/mL dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Survived</td>
</tr>
<tr>
<td>7(^{4+})</td>
<td>Survived</td>
</tr>
<tr>
<td>8(^{4+})</td>
<td>Died</td>
</tr>
<tr>
<td>10(^{2+})</td>
<td>Died</td>
</tr>
<tr>
<td>11(^{4+})</td>
<td>Died*</td>
</tr>
<tr>
<td>(\text{P}_p)(^{4+})</td>
<td>Survived</td>
</tr>
<tr>
<td>(\text{Q}_p)(^{4+})</td>
<td>Survived</td>
</tr>
</tbody>
</table>

\(^*\text{Tpphz dimer, [(bpy)_2Ru(tpphz)Ru(bpy)_2]}^{4+}\), which we used for our \textit{in vivo} studies

The results from this study are given in Table 2.3. All of the tpphz bridged complexes, except the cationic carbonyl dimer (7\(^{4+}\)) and neutral oxalate complex (9), were acutely toxic at this dosage and the animals were observed to have seizures and die within minutes (see Table 2.3). Interestingly, the two complexes with longer bridging ligands, \(\text{P}_p\)\(^{4+}\) and \(\text{Q}_p\)\(^{4+}\), showed no toxicity in this initial screen. The neutral
dimer, 9 was poorly soluble and could only be administered up to a maximum dose of 15 mg/kg, at which no toxicity was observed.

The maximum tolerable doses (MTD) of the most promising complexes, $P_p^{4+}$ and $Q_p^{4+}$ were determined by injecting mice ($n = 3$) with 200 µL of a freshly prepared solution of the complexes (chloride salt) in Tris buffer, pH 7.5. Drug concentrations examined were 50 mg/mL, 25 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1mg/mL. At first, a single mouse was treated with a single dose of the drug complex and observed. If the mouse died in short order, this dosage was considered lethal and the next smaller dosage was examined. If the mouse survived, then two additional mice were injected and the group was monitored over the next 30 days. For $P_p^{4+}$ all three mice survived a dosage of 10 mg/mL or 67 mg/kg mouse. For $Q_p^{4+}$ all three mice survived a dosage of 1 mg/mL or 6.7 mg/kg mouse, whereas none survive the next highest dose of 2.5 mg/mL.

<table>
<thead>
<tr>
<th>Drug Dose (mg/mL)</th>
<th>$P_p^{4+}$</th>
<th>$Q_p^{4+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>25</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>10</td>
<td>Survived</td>
<td>Died</td>
</tr>
<tr>
<td>5</td>
<td>Survived</td>
<td>Died</td>
</tr>
<tr>
<td>2.5</td>
<td>Survived</td>
<td>Died</td>
</tr>
<tr>
<td>1</td>
<td>Survived</td>
<td>Survived</td>
</tr>
</tbody>
</table>
These initial studies suggest a structure-activity relationship with respect to animal toxicity. The neutral complex, 9 is not noticeably toxic, but neither is it active against tumor cells. The dimeric complexes bridged by the short tpphz bridging ligand are acutely toxic to animals, whereas the dimers bridged by the longer tappa and tatpq bridging ligands, e.g., $\text{P}^4_\text{p}$ and $\text{Q}^4_\text{p}$, are considerably less toxic (see Table 2.5). These latter complexes are also extremely tight DNA binders and show good cytotoxicity against NSCLC cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\text{IC}_{50}$ (µM)</th>
<th>MTD (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>$&gt;100 \pm 10$</td>
<td>$&gt;15^a$</td>
</tr>
<tr>
<td>$7^4+$</td>
<td>$12 \pm 1$</td>
<td>$&gt;6^b$</td>
</tr>
<tr>
<td>$8^4+$</td>
<td>$21\pm 2$</td>
<td>$&lt;6^b$</td>
</tr>
<tr>
<td>$10^2+$</td>
<td>$8 \pm 1$</td>
<td>$&lt;6^b$</td>
</tr>
<tr>
<td>$\text{P}^4_\text{p}$</td>
<td>$8 \pm 1$</td>
<td>67</td>
</tr>
<tr>
<td>$\text{Q}^4_\text{p}$</td>
<td>$1 \pm 0.1$</td>
<td>17</td>
</tr>
</tbody>
</table>

(a) maximum dose that could be given due to poor solubility, (b) acutely toxic with single animal tested with 0.2 mg complex in 200 µL buffer (ca. 6mg/kg).

2.2.8. In vivo Antitumor Activity

The promising combination of low animal toxicity and high tumor cytotoxicity of $\text{P}^4_\text{p}$ and $\text{Q}^4_\text{p}$ warranted a further investigation of their antitumor properties in vivo. The complexes were investigated for antitumor activity in vivo using an orthotopic syngeneic mouse melanoma model. A total of 9 male C57BL/6NTac mice (Taconic
Animal Labs) were injected with 1 million B16 mouse melanoma cancer cells subcutaneously in the left rear thigh (Day 0) and then divided into three groups of three mice each: Control group (Group C); Group P ([P_p]Cl_4 treated) and Group Q ([Q_p]Cl_4 treated). Twenty four hours (Day 1) after the injection of the cancer cells, the each mouse was injected intraperitoneally with 0.2 mg P_p^{4+} (Group P) or 0.1 mg Q_p^{4+} (Group Q) dissolved in 200 µL Tris buffer (5mM Tris,10 mM NaCl, pH 7.5). The Control group was injected with 200 µL Tris buffer alone. Thereafter, Group P and Group Q were injected with an identical dose of the appropriate ruthenium complex every other day until a total of 16 doses had been administered. Group C was treated with an identical number of doses of Tris buffer. Every other day, each mouse was weighed and examined for tumor growth. When tumors were visible, the tumor volume was recorded by measuring the tumor long (b) and short (a) axes with calipers and calculating the volume according to the formula $V = \frac{\pi}{6}a^2b$. The experiment was conducted over a period of 45 days after which all of the mice had died.

The results of this study are summarized in the following three figures. Figure 2.21 shows the lifetime data for the three groups under study. As can be seen, the mice in Group Q were the quickest on average to die, with all three mice dead in 37 days. The difference between the Control group and Group P was less significant with the Control group losing its first mouse on day 24 and Group P on day 29. In both of these groups, the last surviving mouse died on day 45. From this data, we see that the complex Q_p^{4+} shows chronic toxicity at this dosage and regimen. The roughly comparable lifetime performance of Group P and the Control group show that complex P_p^{4+}, at a minimum,
is not overly toxic at this dosage and regimen. We also conclude, however, that neither $P_{p}^{4+}$ nor $Q_{p}^{4+}$ is effective at extending mouse survival time at this dosage and regimen.

Figure 2.21 Number of mice survived vs. days after injection of mouse melanoma cells in C57B mice. (3 mice in each group).

If we examine the mouse tumor volume as a function of time and treatment (see Figure 2.22), we observe that the tumor growth for Group P is retarded relative to the Control group and accelerated for Group Q. This data is complicated by the fact that individual mice died during the course of the experiment (as shown in Figure 2.21) and thus the number of mice were not constant. Colored arrows numbered 1, 2 and 3 denote the day mouse 1, mouse 2 and mouse 3 in each group died. Two important trends in the tumor volume measurements are seen. First, mice treated with $P_{p}^{4+}$ (Group P) show
smaller tumor volumes than the Control group suggesting that $P_p^{4+}$ is inhibiting melanoma growth in vivo. Second, the group treated with $Q_p^{4+}$ (Group Q) shows no significant difference in tumor volume relative to the Control group, suggesting no therapeutic effect (at this dosage and regimen).

Figure 2.22 Tumor volume vs. days after injection of mouse melanoma cells in C57B mice. (3 mice in each group). For each group, a death of a mouse is indicated using the same colored arrows.

Finally, we followed the average mouse body weight during the course of this experiment and the data are shown in Figure 2.23. None of the treated mice lost weight during the treatments, which were considered a positive sign as this meant that the mice continued to eat and drink despite repeated injections of these complexes. With time, all three groups of mice showed weight gain, which approximately mirrored the growth in
tumor volume, suggesting that the weight gain was largely due to the tumor growth. Group P showed the least gain in body mass and by far the smallest percent change in body mass. For example, if we measure the percent change in body mass for the Control group and Group P on day 30 (where n=2 for both groups) we find a 7% increase in mass for Group P compared to a 23% increase in mass for the Control group. By this measure, complex $P_{p}^{4+}$ is clearly inhibiting tumor growth. We also note that the type of tumor under treatment here, B16 mouse melanoma, is known to be a resilient and a cancer type difficult to treat. We chose this tumor model as promising results of this cancer type bode well for the treatment of less aggressive and drug-resistant cancers.

On the other hand, the mice treated with $Q_{p}^{4+}$ fared much less well compared to the Control group. Group Q showed the largest weight gain, which may be due in part to toxic side effects of this complex. If this complex weakens the mouse immune system, then it is possible that the tumor growth in these animals would be accelerated relative to the Control group (which has a healthy, though insufficient, immune response to the cancer).
Figure 2.23 Average body weight vs. days after injection of mouse melanoma cells in C57B mice. (3 mice in each group). For each group, a death of a mouse is indicated using the same colored arrows.

Another study of mouse melanoma tumor inhibition, which involved higher doses of $P_{p}^{4+}$ (2.5 to 10 mg/mL of $P_{p}^{4+}$) was begun, but was terminated before any meaningful data could be obtained regarding antitumor activity. At these higher doses, the animals grew skin blisters (lumps) at the site of the injection, which then led to necrosis and loss of the blister. We postulate that this blistering may be due to precipitation of the drug in the intraperitoneal cavity and thus would need to administer less concentrated solutions.
2.3 Summary and Conclusions

A series of monomeric and dimeric ruthenium polypyridyl complexes were prepared in which factors known to affect the DNA binding affinity and postulated to affect the complex toxicity were systematically varied. Some of the specific properties that were varied include: overall charge (0, +2, +4), number of metals (monomers vs. dimmers), size (short (tpphz) vs long (tatpp, tatpq) bridging ligands) and lability and the ruthenium centers (non-labile ligands such as bpy, phen, tpphz, tatpp, CO versus labile ligands such as MeCN, oxalato, chloro). The syntheses of several of these complexes required the development or application of new synthetic routes to the products.

The complexes were subjected to a number of screens to evaluate their potential as chemotherapeutic agents for cancer treatment. The first screen was an evaluation of the complexes cytotoxicity towards NSCLC cells \emph{in vitro}. The IC\textsubscript{50} values for two of complexes, specifically \( P_p^{4+} \) and \( Q_p^{4+} \), were impressive (<10 µM) and suggested the complexes warranted further study. DNA binding studies using a variety of spectroscopic techniques showed the tetracationic intercalating complexes, \( P_p^{4+} \) and \( Q_p^{4+} \), bind extremely tightly to DNA with \( K_b \)'s on the order of \( 10^9 \) M\textsuperscript{-1}.

As the toxicity of this class of compounds is a frequent cause for concern, promising complexes were screened for animal (mouse) toxicity. While most complexes proved to be acutely toxic at low doses, the two long bridged dimers \( P_p^{4+} \) and \( Q_p^{4+} \) were surprisingly well tolerated by mice. MTDs of 6.7-167 mg/kg and 6.7-17 mg/kg, respectively, for \( P_p^{4+} \) and \( Q_p^{4+} \), were measured in mice. Importantly, the two most cytotoxic complexes against NSCLC \emph{in vitro}, \( P_p^{4+} \) and \( Q_p^{4+} \), turned out to be two
of the least toxic in vivo. These promising results lead to a study of the antitumor properties of $P_{p}^{4+}$ and $Q_{p}^{4+}$ in vivo using a mouse melanoma model. While the results from this preliminary study were diverse, the complex, $P_{p}^{4+}$ appears to inhibit the growth of the melanoma and shows no chronic toxicity during the time course of the experiment. Complex $Q_{p}^{4+}$, on the other hand, was less effective at controlling tumor growth and did show the signs of chronic toxicity over the course of the experiment at this dosage and therapeutic regimen.

In summary, complex $P_{p}^{4+}$ has emerged as a promising anticancer drug from these studies. Given the extremely tight DNA binding, we assume that the cellular target for bioactivity is the DNA. The next chapter explores the ability of this complex and many of the related Ru complexes reported in this chapter to cleave DNA under some notable environmental conditions.

2.4 Materials and Methods

2.4.1. General

Hydrated RuCl$_3$. 3H$_2$O (Pressure Chemicals), carbon monoxide (Matheson gas; 98% purity), trifluorometanesulfonic acid (Alfa Aesar; 99% assay), MTT (3-4,5-cyanotetrazolium-3-yl)-2,5-diphenyltetrazolium bromide) (Aldrich), neutral alumina (Aldrich), Na$_2$C$_2$O$_4$ (J. T. Baker Chemicals), K$_2$C$_3$O$_4$ (EM Science), 2,2′-Bipyridyl (Alfa Aesar), TMNO (Aldrich), 1,10-phenanthroline (Alfa Aesar), 1,2-dichloroobenzene (Aldrich) were used without further purification. The ligands dppz (dipyrido[3,2-
a:2′,3′-c]phenazine\(^1\) and tpphz (tetrapyrido[3,2-a:2′,3′-c:3′′,2′′-h:2′′′,3′′′-j]phenazine\(^2\) and the ruthenium complexes \([\text{Ru(CO)}_2\text{(Cl)}_2]_x\), \([\text{Ru(bpy)}(\text{CO})_2\text{(Cl)}_2]_y\), \([\text{Ru(bpy)}(\text{CO})_2\text{(CF}_3\text{SO}_3)_2]_z\), \([\text{(bpy)}_2\text{Ru(tpphz)}\text{Ru(bpy)}_2]^{4+}\) \(^{11}\), \(P_p^{4+}, P_b^{4+}, Q_p^{4+}\) and \(Q_b^{4+}\) were synthesized according to the literature procedures. All organic solvents were of reagent grade and used as received. Non-small cell lung cancer (NSCLC) lines H358 (bronchioalveolar) and B16 mouse skin melanoma were purchased from American Type Culture Collection (Manassas, VA). Cancer cells were incubated at 37 °C in a humidified atmosphere of 5% CO\(_2\) in the appropriate medium: RPMI 1640 (NSCLC) or DMEM (B16 mouse melanoma) medium supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) P/S solution, 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. RPMI 1640 and DMEM mediums, penicillin/streptomycin solution (P/S), fetal bovine serum (FBS), phosphate buffered saline 7.4 (PBS), trypsin-EDTA and trypan blue were purchased from Life Technologies, Inc., Grand Island, NY. Unless otherwise noted all the \textit{in vitro} and \textit{in vivo} studies were performed using the chloride salt of the ruthenium complex.

\begin{align*}
\text{2.4.2. Physical Measurements}
\end{align*}

\(^1\)H and \(^13\)C NMR spectra were recorded on a JEOL Eclipse plus 500 MHz or 300 MHz spectrophotometers. Spectra were referenced to tetramethylsilane (TMS) or residual solvent peaks from the deuterated solvent. UV-visible spectra were obtained on a Hewlett-Packard HP8453A spectrometer. Infrared spectra were acquired by using a
JASCO FT-IR 410 spectrometer. Elemental analyses were performed using a perkin-Elmer model 2400 CHN analyzer. Mass spectra were obtained using Matrix Assisted Laser Desorption/ Ionization- Time Of Flight (MALDI-TOF) and Electrospray Ionization Time Of Flight (ESI-TOF) mass methods at Scripps Research Institute at La Jolla, CA.

2.4.3. Synthesis

\[[\text{Ru(bpy)(dppz)(CO)\textsubscript{2}}]\textsuperscript{2+} (4\textsuperscript{2+}): \text{dppz} (1.17 \text{ g}, 4.2 \text{ mmol}), [\text{Ru(bpy)(CO)}\textsubscript{2}(\text{CF}_3\text{SO}_3)\textsubscript{2}]\textsuperscript{2+88,91} (1.27 \text{ g}, 2.1 \text{ mmol}) in \text{400 mL} \text{ of absolute ethanol were refluxed for 3 \text{ hours under nitrogen. After the reaction, the ethanol was removed (300 mL) under reduced pressure and the excess dppz was filtered. Aqueous solution of NH}_4\text{PF}_6 \text{ was added to the filtrate to precipitate the product as a yellow-green solid. The product was further purified by dissolving it in a minimum volume of acetone and precipitating with ethanol. Typical isolated yield was 0.92 \text{ g} (50\%).}\] 1H NMR (CD\textsubscript{3}CN, δ): 10.09 (dd, \textit{J} = 8.0 Hz and \textit{J} = 1.5 Hz, 1H), 9.83 (dd, \textit{J} = 8.0 Hz and \textit{J} = 1.5 Hz, 1H), 9.53 (dd, \textit{J} = 5.5 Hz and \textit{J} = 1.5 Hz, 1H), 9.20 (d, \textit{J} = 5.5 Hz, 1H), 8.65 (d, \textit{J} = 8.0 Hz, 1H), 8.50 (m, 4H), 8.41 (dd, \textit{J} = 9.0 Hz and \textit{J} = 5.0 Hz, 1H), 8.16 (m, 3H), 7.98 (m, 2H), 7.84 (dd, \textit{J} = 5.5 Hz and \textit{J} = 1.5 Hz, 1H), 7.44 (dd, \textit{J} = 6.0 Hz and \textit{J} = 1.0 Hz, 1H) and 7.34 (t, \textit{J} = 7.0 Hz, 1H). 13C NMR (δ, 125 MHz, CD\textsubscript{3}CN): 191.3, 191.2, 159.8, 158.4, 157.1, 156.1, 153.1, 151.4, 150.0, 149.4, 144.2, 143.5, 143.3, 140.3, 140.2, 139.1, 138.9, 134.3, 134.3, 133.2, 132.5, 131.0, 130.9, 130.9, 130.4, 129.9, 129.6, 127.2 and 126.4. IR (Nujol): ν\textsubscript{co} = 2101 and 2046 cm\textsuperscript{-1}. Vis: [\textit{λ}_{\text{max}}, \text{nm (ε} \times 63
$10^3 \text{M}^{-1}\text{cm}^{-1}$): 383 (3.43) and 366 (3.51). Anal. Calcd for C$_{30}$H$_{18}$N$_6$O$_2$F$_{12}$P$_2$: C 41.07, H 2.40, N 9.40. Found: C 40.69, H 2.05, N 9.49.

$[\text{Ru}(\text{bpy})(\text{dppz})(\text{CH}_3\text{CN})_2]^{2+}$ (5$^{2+}$): $[\text{Ru}(\text{bpy})(\text{dppz})(\text{CO})_2](\text{PF}_6)_2$ (0.2 g, 0.23 mmol) was dissolved in 100 mL of acetonitrile and the resulting solution was deaerated by bubbling argon through it for 30 minutes. The reaction mixture was heated to reflux and trimethylamine N-oxide (0.051 g, 0.68 mmol) was added in one portion after which the reflux was continued for another 90 minutes. After cooling to room temperature, the solvent was removed under reduced pressure to leave a dark solid. The solid was redissolved in a minimum of acetonitrile and loaded onto a 15 cm neutral alumina column (packed using the solvent, MeCN). A single orange colored band was eluted from the column with acetonitrile. This band was collected and the 90% of the solvent was removed under vacuum and the final product precipitated with the aqueous solution of NH$_4$PF$_6$. The solid was filtered and dried under vacuum at 40 °C. Typical isolated yield was 0.14 g (70%). $^1$H NMR (CD$_3$CN, δ): 9.74 (dd, $J_1 = 5.5$ Hz and $J_2 = 1.0$ Hz, 1H), 9.69 (dd, $J_1 = 8.0$ Hz and $J_2 = 1.0$ Hz, 1H), 9.45 (d, $J = 5.0$ Hz, 1H), 9.35 (d, $J_1 = 8.5$ Hz and $J_2 = 1.5$ 1H), 8.58 (d, $J = 8.0$ Hz, 1H), 8.41 (d, $J = 8.0$ Hz, 1H), 8.35 (m, 2H), 8.30 (m, 2H), 8.05 (m, 2H), 8.00 (dd, $J_1 = 5.5$ Hz and $J_2 = 1.5$ Hz, 1H), 7.92 (m, 2H), 7.67 (m, 2H), 7.16 (t, $J = 6.0$, 1H), 2.37 (s, 3H) and 2.23 (s, 3H). $^{13}$C NMR (δ, 125 MHz, CD$_3$CN): 158.1, 157.5, 155.4, 154.3, 153.8, 152.7, 151.5, 150.7, 142.7, 142.7, 139.8, 139.7, 138.7, 138.2, 134.0, 133.6, 132.6, 130.9, 130.2, 129.6, 129.6, 127.7, 127.6, 126.7, 126.7, 126.4, 126.2, 124.1, 123.7, 3.7 and 3.6. IR (Nujol): $\nu_{\text{CN}} =$
2000 and 2061 cm\(^{-1}\). Vis: \([\lambda_{\text{max}}, \text{nm} (\varepsilon \times 10^3 \text{ M}^{-1}\text{cm}^{-1})]\): 363 (3.71), 370 (3.62) and 431 (2.18).

\[\text{[Ru(bpy)(dppz)(C_2O_4)] \ (6): [Ru(bpy)(dppz)(CH_3CN)_2](PF_6)_2 \ (0.2, 0.22 \text{ mmol})}\]

and Na\(_2\)C\(_2\)O\(_4\) (0.12 g, 0.88 mmol) in 50 mL of EtOH and 50 mL of water, refluxed for 12 hours under N\(_2\) atmosphere. After the reaction, the ethanol was removed under reduced pressure to get dark colored precipitate suspended in water. The solid was filtered and washed with excess water (200 mL) and acetonitrile (100 mL) and dried in a vacuum oven at 40 \(^\circ\)C for 12 hours. Yield, 0.11 g (80%). \(^1\)H NMR (DMSO-\(d_6\), \(\delta\)): 9.59 (d, \(J = 8.0\) Hz, 1H), 9.31 (d, \(J = 5.0\) Hz, 1H), 9.24(d, \(J = 8.0\) Hz, 1H), 8.99 (d, \(J = 5.5\) Hz, 1H), 8.82 (d, \(J = 8.5\) Hz, 1H), 8.66 (d, \(J = 8.0\) Hz, 1H), 8.52 (d, \(J = 8.5\) Hz, 1H), 8.46 (d, \(J = 9.5\) Hz, 1H), 8.35 (dd, \(J_1 = 8.0\) Hz and \(J_2 = 5.5\) Hz, 1H), 8.21 (t, \(J = 8.0\) Hz, 1H), 8.14 (m, 3H), 7.91 (t, \(J = 6.5\) Hz, 1H), 7.79 (t, \(J = 8.0\) Hz, 1H), 7.69 (m, 2H) and 7.11 (t, \(J = 7.5\) Hz, 1H). Vis: \([\lambda_{\text{max}}, \text{nm} (\varepsilon \times 10^3 \text{ M}^{-1}\text{cm}^{-1})]\): 357 (3.76), 366 (3.96), 375 (4.25) and 542 (2.71). Anal. Calcd for \([\text{Ru(bpy)(dppz)(C}_2\text{O}_4)]\).H\(_2\)O or C\(_{30}\)H\(_{20}\)N\(_6\)O\(_5\): C 55.81, H 3.12, N 13.02. Found: C 55.69, H 3.08, N 12.85. ESI-TOF (m/z): 628 (M), 629 (M+), 651 (MNa\(^+\)), 538 (M-C\(_2\)O\(_4\)), 1168 (M-M).

\[\text{[\{Ru(bpy)(CO)_2(tphpz)}^{4+} (7^{4+}): [Ru(bpy)(CO)_2(Cl)_2] \ (0.25 \text{ g, 0.65 mmol}) was}\]

suspended in 40 mL of 1,2-Dichlorobenzene and the whole solution was deaerated with N\(_2\) for 30 minutes. Slow drop wise addition of CF\(_3\)SO\(_3\)H eventually led to complete dissolution of the solid upon which the addition was ceased. The solution was heated to 110 \(^\circ\)C for 90 minutes and then cooled to 0 \(^\circ\)C on ice. With gently stirring, 200 mL of anhydrous ether was added and the stirring then continued for another hour during
which time a precipitate forms. The precipitate was filtered and washed with diethyl ether (3x) and dried under N₂ (room temperature). This triflato complex was used immediately by adding 350 mL of EtOH and then tpphz (0.12 g, 0.36 mmol) to give a suspension. The suspension was refluxed for 5 hours under N₂ until it became clear. After cooling the reaction to room temperature the ethanol was removed under reduced pressure. When only ~ 20 mL of ethanol remained, ~ 20 mL of water was added. The solution was filtered and the product precipitated with aqueous solution of NH₄PF₆. The resulting precipitate was filtered and washed with water (100 mL) and dried in a vacuum oven. The isolated product was further purified by dissolving in a minimum volume of acetone and reprecipitating it with ethanol. The solid was filtered, washed with EtOH and dried under vacuum at 50 °C for 3 hours. Yield 0.05 g (50 %).

H NMR (δ, 500 MHz, CD₃CN): 10.44 (dd, J₁ = 8.0 Hz and J₂ = 1.5 Hz, 1H), 10.34 (dd, J₁ = 8.5 Hz and J₂ = 1.5 Hz, 1H), 10.18 (td, J₁ = 8.0 Hz and J₂ = 1.5 Hz, 1H), 10.09 (td, J₁ = 8.5 Hz and J₂ = 1.5 Hz, 1H), 9.67 (m, 2H), 9.23 (t, J = 4.0 Hz, 2H), 8.67 (t, J = 6.5 Hz, 2H), 8.52(m, 6H), 8.13 (m, 4H), 7.99 (m, 4H), 7.48 (m, 2H) and 7.34 (m, 2H). 

C NMR (δ, 125 MHz, CD₃CN): 191.2, 190.9, 160.7, 158.5, 157.1, 156.1, 154.1, 151.6, 151.5, 151.9, 151.4, 151.4, 150.2, 149.7, 143.5, 143.2, 141.4, 139.8, 139.8, 139.7, 139.6, 139.5, 132.3, 132.2, 131.6, 131.6, 131.7, 131.5, 130.9, 130.6, 130.5, 130.5, 130.4, 129.9, 129.9, 129.7, 129.7, 127.3 and 126.4. IR(Nujol): νCO = 2050 and 2104 cm⁻¹. Vis: [λmax, nm (ε× 10³ M⁻¹cm⁻¹)]: 376 (7.38) and 394 (8.05). Anal. Calcd for [{Ru(bpy)(CO)₂}₂tpphz] or C₄₆H₂₈N₁₀F₂₄O₄P₄Ru₂: C 36.22, H 1.77, N 8.81. Found:

$$\{\text{Ru(bpy)}(\text{CH}_3\text{CN})_2\text{tpphz}\}^4^+ (0.40 \text{ g}, 0.25 \text{ mmol})$$ was dissolved in 150 mL of CH$_3$CN and the solution was deaerated with argon for more than 30 minutes. After heating to reflux, Me$_3$NO (0.11 g, 1.5 mmol) was added in one portion and the solution refluxed for 3h. The solution was removed under reduced pressure and the crude product precipitated upon addition of aqueous NH$_4$PF$_6$. The solid was filtered and redissolved in a minimum of acetonitrile and loaded onto a 15 cm neutral alumina column. Elution with acetonitrile gave a single orange colored band, which was collected and the solvent was removed under reduced pressure to a volume of $\sim$ 30 mL and the product precipitated upon addition of aqueous solution of NH$_4$PF$_6$. The solid was isolated by filtration, washed with water (200 mL) and EtOH (100 mL) and dried under vacuum at 40 °C for 4 hours. Yield 0.3 g (73%). $^1$H NMR ($\delta$, 500 MHz, CD$_3$CN): 10.22 (dd, $J_1$ = 8.0 Hz and $J_2$ = 1.5 Hz, 1H), 10.12 (dd, $J_1$ = 8.0 Hz and $J_2$ = 1.5 Hz, 1H), 9.91 (dd, $J_1$ = 8.0 Hz and $J_2$ = 1.5 Hz, 1H), 9.84 (dd, $J_1$ = 5.5 Hz and $J_2$ = 1.5 Hz, 1H), 9.81 (m, 2H), 9.44 (t, $J$ = 5.0 Hz, 2H), 8.58 (m, 2H), 8.47 (dd, $J_1$ = 8.0 Hz and $J_2$ = 5.5 Hz, 1H), 8.42 (m, 3H), 8.34 (m, 2H), 8.12 (m, 2H), 7.92 (m, 5H), 7.83 (dd, $J_1$ = 8.0 Hz and $J_2$ = 5.5 Hz, 1H), 7.66 (m, 2H), 7.14 (m, 2H), 2.37 (s, 3H), 2.36 (s, 3H), 2.17 (s, 3H) and 2.17 (s, 3H). $^{13}$C NMR ($\delta$, 125 MHz, CD$_3$CN): 159.4, 158.8, 157.4, 156.4, 155.0, 154.1, 152.9, 152.1, 141.8, 141.7, 141.6, 141.6, 139.9, 139.5, 136.3, 135.9, 135.8, 135.5, 135.4, 131.2, 131.1, 130.9, 130.8, 129.0, 128.1, 128.0, 127.9, 127.8, 127.4, 125.8, 124.9, 4.9 and 4.8. Vis: [$\lambda_{max}$, nm ($\varepsilon \times 10^3$ M$^{-1}$ cm$^{-1}$)]:

67

[{Ru(bpy)(C₂O₄)}₂ tpphz] (9): [{Ru(bpy)(CH₃CN)}₂(tpphz)]⁴⁺ (0.18 g, 0.11 mmol) and Na₂C₂O₄ (0.1 g, 0.75 mmol) were mixed with 30 mL EtOH and 30 mL water. The resulting suspension refluxed for 12 hours under an argon atmosphere. The solution cooled down to room temperature and the EtOH was removed under reduced pressure and the product was filtered and washed with excess water (200 mL) and CH₃CN (200 mL) and dried in the vacuum oven at 40 °C overnight. Yield 0.083 g (70%). Vis: [λ_max, nm (ε×10³ M⁻¹ cm⁻¹)]: 362 (7.53), 381 (10.64) and 547 (6.63). MALDI-TOF m/z: 1077 (MH⁺), 989 ([M-C₂O₄]⁺), 809 ([M-3C₂O₄]⁺).

[{Ru(bpy)(CO)(Cl)}₂tpphz]²⁺ (10²⁺): [Ru(bpy)₂(tpphz)]Cl₂ (0.13 g, 0.15 mmol) was dissolved in 30 mL MeOH and the solution was deaerated for 30 minutes under N₂. Then it was heated to reflux and [Ru(CO)₂(Cl)₂]ₙ (0.068 g, 0.30 mmol) was added in one portion and the solution was refluxed for another 2 h. The resulting solution was filtered and the product precipitated by adding aqueous solution of NH₄PF₆. The solid was isolated by filtration, washed with water (200 mL) and EtOH (200 mL) and dried under vacuum at 40 °C for 12 h. Yield 0.16 g (80%). ¹H NMR (δ, 500 MHz, CD₃CN): 10.11 (d, J = 8.3 Hz, 1H), 9.92 (d, J = 8.3 Hz, 1H), 9.71 (d, J = 8.3 Hz, 1H), 8.55 (dd, J₁ = 7.8 Hz, J₂ = 10 Hz, 2H), 8.29 (d, J₁ = 5.0 Hz, 1H), 8.24 (t, J = 12.3 Hz, 1H), 8.13 (t, J = 8.0 Hz, 1H), 8.00 (m, 2H), 7.87 (d, J = 5.0 Hz, 1H), 7.75 (d, J = 5.5 Hz, 1H), 7.48 (t, J = 6.6 Hz, 1H), and 7.26 (t, J = 6.5, 1H). ¹³C NMR (δ, 125 MHz, CD₃CN): 197.3, 158.3,
158.0, 156.3, 155.7, 153.3, 153.1, 151.5, 149.2, 141.4, 141.1, 139.1, 139.1, 137.6, 134.9, 130.9, 130.7, 128.8, 128.7, 128.6, 125.4, and 125.4. IR(Nujol): ν CO = 2007 and 2069 cm⁻¹. Vis: [λ max, nm (ε × 10³ M⁻¹cm⁻¹)]: 362 (7.93), 380 (10.72) and 449 (6.53). Anal. Caled for [{Ru(bpy)(CO)(Cl)}₂tpphz] or C₄₆H₂₈N₁₀Cl₂F₁₂O₂P₂Ru₂: C 41.97, H 2.15, N 10.65. Found: C 43.15, H 2.69, N 11.20.

2.4.4. Cytotoxicity in NSCLC cells

All reagents were handled in a sterile vacuum hood. Approximately 20,000 cells (200 µL) were plated into each well of a 96-well flat-bottomed microtiter plate and the plate was incubated at 37 °C for 24 h. Then the cells were diluted with the medium (100 µL of RPMI-1640) containing the test ruthenium complex of 5 different concentrations (0.01, 0.1, 1.0, 10 and 100 µM) as the chloride salts. Analysis was performed at the end of the 96 h incubation time. Briefly, 20 µL of MTT (2 mg/mL) was added to all wells followed by incubation at 37 °C for 1 h. At the end of incubation the cells were centrifuged in Eppendorf tubes at 28,000 g for 10 minutes. The supernatant was discarded and the pellet thoroughly solubilized in 50 µL of DMSO, followed by addition of 1 mL of isopropanol. The optical density of each extract was measured at 560 nm on a Gilford Response spectrophotometer. Eight replicate wells were used in each concentration of ruthenium complex tested. The IC₅₀ (the ruthenium complex concentration that reduced formazan formation by 50%) for these ruthenium complexes were determined from a plot of percentage surviving cells (compared with control cells) versus ruthenium complex concentration.
2.4.5. Animal Toxicity and MTD of Ru(II)Dimers

An initial animal toxicity screen was performed by intraperitoneal injection in C57BL/6Ntac mice (approximate weight 30 g each) with 0.2 mg of the ruthenium complex \( \left( \text{7}^{4+}, 8^{4+}, 9, 10^{2+}, P_p^{4+}, Q_p^{4+} \right) \) and \( \left[ \text{bpy} \right] \left[ \text{Ru(tpphz)} \text{Ru(bpy)} \right]_2 (11^{4+}) \) as chloride salts) in 200 \( \mu \)L Tris buffer, pH 7.5. Two complexes, \([P_p]\text{Cl}_4\) and \([Q_p]\text{Cl}_4\) were subjected to additional toxicity screens.

The maximum tolerable dose (MTD) of a particular complex was determined by injecting mice (n = 3) with 200 \( \mu \)L of a freshly prepared solution of the complexes (chloride salt) in Tris buffer, pH 7.5. Drug concentrations examined were 50 mg/mL, 25 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1mg/mL. At first, a single mouse was treated with a single dose of the drug complex and observed. If the mouse died in short order, this dosage was considered lethal and the next smaller dosage was examined. If the mouse survived, then two additional mice were injected and the group observed over the next 30 days. Every other day their physical behavior was monitored. Based on the number of mice survived after giving these doses for three months, MTD was calculated.

2.4.6. In vivo Antitumor Activity of \( P_p^{4+} \) and \( Q_p^{4+} \)

The in vivo antitumor activity of \( P_p^{4+} \) and \( Q_p^{4+} \) (as chloride salts) was examined using an orthotopic syngeneic mouse melanoma model. Nine male C57BL/6NTac mice (Taconic Animal Labs) were injected with 1 million B16 mouse melanoma cells subcutaneously in the left rear thigh (Day 0) and divided in to three groups of three
mice (Control, P and Q). Twenty four hours (Day 1) after the injection, the mice were injected intraperitoneally with 200 µL Tris buffer (5mM Tris, 10 mM NaCl, pH 7.5) containing 0.2 mg $\text{P}_4^{4+}$ (Group P) or 0.1 mg $\text{Q}_4^{4+}$ (Group Q) and or just buffer (Group C). All three groups were injected with a repeat dose every other day until a total of 16 doses had been administered. Throughout the experiment, tumor volumes and body weights were monitored of all groups every other day. Tumor volume was calculated by measuring the tumor with calipers on the long (b) and short (a) axes using the formula $V = (\pi/6)a^2b$.\textsuperscript{103}

2.4.7. Equilibrium Binding Constants ($K_b$)

Fluorescence studies were performed with calf-thymus DNA in the buffer (5 mM Tris, 10 mM NaCl, pH 7.5) using an intercalator displacement titration where the intercalator was the ruthenium complex, $\Delta\text{-[Ru(phen)$_2$(dppz)$_2$]}^{2+}$. This complex has $K_b = 5 \times 10^7 \text{M}^{-1}$, binding site size of 2 and only fluoresces when intercalated into the DNA.\textsuperscript{109,110}

Calf thymus DNA (4 µM) and excess $\Delta\text{-[Ru(phen)$_2$(dppz)$_2$]}^{2+}$ (10 µM) were mixed in 3.00 mL buffer (5 mM Tris Cl, 10 mM NaCl, pH 7.5) in a 1 cm quartz cuvette. After mixing the solution, the original fluorescence intensity was measured at 610 nm. Then the ruthenium complex was titrated in very small aliquots (2 µL) and the fluorescence quenching was measured at each addition. Equilibrium binding constant ($K_b$) and binding site size ($s$) were obtained in collaboration with Prof. Per Lincoln (Chalmers University, Sweden) by fitting the fluorescence quenching titration data to the McGhee-
von Hippel type of noncooperatorative competitive binding isotherm\textsuperscript{97,111} using MATLAB 6.5.1 mathematical computing software.\textsuperscript{55,58,111}
CHAPTER 3
RUTHENIUM (II) DIMERS AS HYPOXIA SELECTIVE DNA DAMAGING AGENTS

3.1 Introduction

Due to the rapid growth of cancer cells, they can become relatively isolated from the blood supply and this becomes increasingly difficult for nutrients and specially oxygen to diffuse to them resulting in existence of some areas of tumor under low oxygen environment recognized as hypoxic conditions. The low oxygen tension (hypoxic) existing in the tumor is a major problem in controlling many different cancer types since they are resistant to radiotherapy\textsuperscript{112-115} and are less sensitive to most chemotherapeutic agents\textsuperscript{116-118} and also stimulate metastases.\textsuperscript{119,120} While the hypoxic microenvironment present in solid tumors is the major draw back in treating cancer, it offers an attractive target in designing hypoxia selective anticancer drugs, which may be truly specific only for tumors.

A few drugs have been established to target cancer cells under low oxygen environment (hypoxic conditions). Bioreductively activated drugs for instance, TPZ (tirapazamine, 3-Amino-1,2,4-benzotriazine 1,4-dioxide), AQ4N (1,4-bis-\{[2-(dimethylamino-N-oxide)ethyl]amino\}5,8-dihydroxyanthracene-9,10-dione)\textsuperscript{121-123} etc., (see Figure 3.1) have gained attention towards targeting hypoxic cancer cells.
Figure 3.1 Structures of hypoxia selective antitumor drugs, TPZ and AQ4N.

Tirapazamine (SR4233, TPZ; 3-amino-1,2,4-benzotriazine 1,4-dioxide; structure in Figure 3.1)\textsuperscript{124,125} is the most successful hypoxia selective N-oxide analogue recently investigated. It has the highest hypoxic to poxic ratio observed of any bioreductively activated drugs. TPZ is currently in phase II and III trials in combination with radiotherapy and with cisplatin based chemotherapy for the treatment of head and neck cancers, glioblastoma, and non-small cell lung cancer\textsuperscript{126-129}. It has been proved that TPZ mediates its biological activity through DNA strand breaks generated from the action of an oxidizing radical\textsuperscript{130,131}.

The use of transitions metal complexes for therapeutics has enjoyed extensive attention given the tremendous success of cisplatin\textsuperscript{132} as a chemotherapeutic agent and the ability of many metal complexes to interact with and damage cellular structures, particularly DNA. Although not the mechanism of action of cisplatin, the majority of metal complexes cleave DNA via the activation of dioxygen to generate reactive oxygen species (ROS), such as hydroxyl radical and superoxide radical.\textsuperscript{133} These ROS are ultimately responsible for the DNA cleavage and thus complexes acting in this
manner are less active under hypoxic conditions. There are also a number of metal complexes that damage DNA via an oxygen independent mechanisms, such as metal-based oxidants,\textsuperscript{134,135} photoactivated complexes,\textsuperscript{136-138} complexes that form DNA adducts\textsuperscript{5,10} and complexes which hydrolyze DNA.\textsuperscript{133} Such complexes may act on hypoxic subpopulations of cancerous cells, but they do not specifically target hypoxic tumor cells over normal cells.

To our knowledge, there are no metal-based complexes that show potentiated DNA cleavage under hypoxic conditions. This chapter describes our evaluation of the DNA cleaving properties of a number of structurally related ruthenium polypyridyl complexes using a plasmid DNA cleaving assay. These \textit{in vitro} cleavage assays were performed under a variety of conditions including in the presence and absence of O\textsubscript{2}. During the course of this study, we discovered that one complex in particular, P\textsubscript{p}\textsuperscript{4+} (see Figure 3.2), is a potent DNA cleaving agent under reducing conditions. Importantly, the cleaving activity of P\textsubscript{p}\textsuperscript{4+} is strongly attenuated upon exposure to O\textsubscript{2}. As mentioned above, this type of hypoxia selective cleaving activity is unprecedented in metal complexes and has the potential to be therapeutically useful in targeting hypoxic cancer cells. This chapter describes these results and our efforts to establish exact chemical species responsible for the cleavage and its mechanism of action.
3.2 Results and Discussion

3.2.1. DNA Cleavage Assay

The use of supercoiled plasmid DNA, such as pUC18, pUC19, pBR322, etc., as a substrate for DNA cleavage experiments is well established. Plasmid DNA can exist in three different topological conformations: supercoiled DNA (Form I), circular DNA (Form II) and linear DNA (Form III) (see Figure 3.3). These three topoisomers are easily separated by agarose gel electrophoresis and visualized under UV light after staining the gel with ethidium bromide as shown in Figure 3.4.
If supercoiled DNA (Form I) is used as a substrate, agents that cause single-strand (ss) nicks will convert the Form I to the relaxed, circular form (Form II). If the nuclease agent induces double-strand (ds) cuts, the supercoiled DNA is converted to the linear form (Form III) thus both ss and ds DNA cutting agents can be identified. If a ss cutting agent induces multiple nicks in both DNA strands and these nicks occur in close proximity (within 16 bp of each other), then it is possible for a nicking agent to lead to ds cuts. Typically, the relative yields of the circular and linear forms can be compared in a statistical manner to see if an agent is causing 'true' ds cuts or multiple closely spaced nicks.140-142
3.2.2. DNA Cleavage by Different Ruthenium Polypyridyl Complexes

The ability of ruthenium polypyridyl complexes to bind with DNA is well established, nonetheless, a few such complexes have shown appreciable DNA cleaving ability unless excited by light irradiation.\(^{143-145}\) In order to draw comparisons, we concurrently screened the non-intercalating complex, [Ru(phen)$_3$]$^{2+}$ and four intercalating ruthenium polypyridyl complexes for DNA cleaving ability. The four intercalating complexes, $P_p^{4+}$, $Q_p^{4+}$, [(phen)$_2$Ru(tpphz)Ru(phen)$_2$]$^{4+}$ (12$^{4+}$) and [(phen)$_2$Ru(dppz)]$^{2+}$ (3$^{2+}$) (see Figure 3.5 for their structures) all differ by the nature of the intercalating ligand and in the case of 2, the number of ruthenium centers. Except [Ru(phen)$_3$]$^{2+}$ (13$^{2+}$), all are tight binders with the binding constants in range of $10^6$ to $10^9$ M$^{-1}$. For 13$^{2+}$ binding is thought to be primarily via electrostatic forces.

A typical assay involved incubation of the complex with purified supercoiled pUC18 DNA at a ratio of 1:12 complex to DNA base pair (DNA bp) for a period of 1-2 h. Care was taken to work under low light conditions, as many of the Ru complexes are...
photochemically active and we wished to examine the non-photochemical reactivity of the complexes. Reactions were quenched by precipitation of the DNA by addition of ethanol and the products were determined by agarose gel electrophoresis.

No cleavage activity was observed in experiments in which $\text{P}_{p}^{4+}$, $\text{Q}_{p}^{4+}$, $3^{2+}$, $12^{4+}$ or $13^{2+}$ were incubated with supercoiled DNA and buffer in air (results not shown). However, upon addition of glutathione (GSH, 0.64 mM), several of the complexes show some nuclease activity as shown in Figure 3.6. These results were quantitated by densitometry and are tabulated in Table 3.1. By comparing lanes 1 and 2, we observe that GSH alone causes a small but noticeable amount (~7%) of ss nicking. This is most likely due to reduction of trace metal ions (e.g. Cu$^{2+}$, Fe$^{3+}$) and subsequent O$_2$ activation to generate reactive oxygen species. Addition of the monomeric complexes, $3^{2+}$ and $13^{2+}$ to the GSH mixture does not result in additional DNA cleavage activity as seen by comparing lanes 6 and 7 to lane 2. On the other hand, addition of $12^{4+}$, $\text{Q}_{p}^{4+}$ or $\text{P}_{p}^{4+}$ does lead to a measurable increase in circular DNA in the presence of GSH. Relative yields of circular DNA increase from 21% for $12^{4+}$ (Lane 5) to 29% for $\text{Q}_{p}^{4+}$ (Lane 4) to 67% $\text{P}_{p}^{4+}$ (Lane 3) revealing $\text{P}_{p}^{4+}$ to be, by far, the most active nuclease agent. Lane 3 even shows a trace amount of linear DNA, suggesting that $\text{P}_{p}^{4+}$ may induce some ds breaks.
Figure 3.5 Chemical structures of the Ruthenium polypyridyl complexes, \( \text{P}_p^{4+} \), \( \text{Q}_p^{4+} \), \( 3^{2+} \), \( 12^{4+} \) and \( 13^{2+} \), under study.
Figure 3.6 1% agarose gel stained with ethidium bromide (negative image) of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with various ruthenium complexes (12.8 µM) in 4 mM Na₃PO₄ buffer (pH 7) in the presence of 0.64 mM GSH under aerobic conditions. Lanes M (marker lane of form I, II and III of pUC18 DNA), Lane 1 (DNA without GSH); and Lane 2 (DNA plus GSH) served as controls. Lanes 3-7 are same as Lane 2 except with the 12.8 µM Ru complex as given in the legend. At these concentrations the Ru complex to DNA bp ratio was 0.083.

<table>
<thead>
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<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
<tbody>
<tr>
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<td>DNA control</td>
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</tr>
<tr>
<td>2</td>
<td>GSH control</td>
<td>90 10 0</td>
</tr>
<tr>
<td>3</td>
<td>Pₚ⁴⁺/GSH</td>
<td>32 67 1</td>
</tr>
<tr>
<td>4</td>
<td>Qₚ⁴⁺/GSH</td>
<td>71 29 0</td>
</tr>
<tr>
<td>5</td>
<td>12⁴⁺/GSH</td>
<td>79 21 0</td>
</tr>
<tr>
<td>6</td>
<td>3²⁺/GSH</td>
<td>89 11 0</td>
</tr>
<tr>
<td>7</td>
<td>13²⁺/GSH</td>
<td>88 12 0</td>
</tr>
</tbody>
</table>

All experiments were conducted at a metal complex concentration of 12.8 µM and 0.64 mM GSH in 4 mM Na₃PO₄ (pH 7) unless otherwise stated.
3.2.3. Effect of Oxygen on DNA Cleavage Efficiency by \( P_p^{4+} \)

Of the five complexes investigated above, \( P_p^{4+} \) is both the most easily reduced complex\(^{108,146}\) as well as the most active nuclease. Cleavage of DNA by ROS is a common mechanism of action, when reducing agents and metal complexes are present and was our initial postulate on how \( P_p^{4+} \) was causing the observed DNA damage. To test this hypothesis, the DNA cleavage assay was performed under both aerobic and anaerobic conditions. The latter would presumably prevent any DNA damaging reactions if ROS are involved. Anaerobic conditions were achieved by performing the incubations in a nitrogen atmosphere glovebox using thoroughly degassed solutions. As a positive control, we employed the known oxygen dependant DNA cleaving agent, iron(II)-bleomycin (Fe-BLM). Under anaerobic conditions, Fe-BLM does not induce ds cuts and its nicking ability is strongly attenuated, when exposed to \( O_2 \), however, Fe-BLM is an effective ds nuclease.\(^{147-149}\) For the purposes of comparison, identical concentrations of \( P_p^{4+} \) and Fe-BLM (12.8 \( \mu \)M or one complex per 12 DNA bp) were employed. The Fe-BLM stock solution was prepared by mixing one equivalent of BLM with two equivalent of \([\text{Fe(II)(NH}_4)_2(\text{SO}_4)_2]\) strictly under anaerobic conditions. A sample of this stock solution was exposed to oxygen for 60 seconds prior to incubation with the pUC18 DNA in order for the experiments conducted under aerobic conditions.\(^{150}\)
Figure 3.7 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with P₄⁺ or Fe-BLM complexes (12.8 µM or 0.083 of P₄⁺ to DNA bp ratio) in 4 mM Na₃PO₄ (pH 7) buffer and in the presence of 0.5 mM GSH under aerobic or anaerobic conditions (as indicated). Lanes M (marker lane of form I, II and III of pUC18 DNA), 1 (DNA without GSH), and 2 (DNA plus GSH) served as controls.

The results from this experiment were striking! Figure 3.7 shows agarose gel from this experiment and the relative yields of supercoiled, circular and linear DNA as determined from densitometry are reported in Table 3.2. The mixture of GSH and P₄⁺ under anaerobic conditions (Lane 5) showed enhanced DNA cleavage over the same mixture in the presence of O₂ (Lane 4), which was exactly opposite to our initial prediction. The positive control, Fe-BLM (Lanes 6 and 7), behaved exactly as expected with some ss nicking observed in the absence of O₂ and near complete conversion of supercoiled DNA to linear and circular DNA in its presence (Lane 6). As the anaerobic incubations of Fe-BLM and P₄⁺ were performed side-by-side, we are assured that the cleavage seen in lane 5 is oxygen independent. Under aerobic conditions a mixture of P₄⁺ and GSH yields 48% circular DNA and a trace formation of linear DNA (2%).
Under anaerobic conditions, the same mixture yields 73% circular DNA and a 14% linear DNA.

Table 3.2 Relative yields of DNA cleavage products under aerobic and anaerobic conditions as shown in Figure 3.7

<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>DNA control</td>
<td>90</td>
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<tr>
<td>2</td>
<td>GSH control</td>
<td>85</td>
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<tr>
<td>3</td>
<td>$P_p^{4+}$</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td>$P_p^{4+}$/GSH/O$_2$</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>$P_p^{4+}$/GSH</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>Fe-BLM/O$_2$</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>Fe-BLM</td>
<td>65</td>
</tr>
</tbody>
</table>

All experiments were conducted under anaerobic conditions unless otherwise noted.

3.2.4. Effect of Thiyl Radicals on DNA Cleavage Efficiency by $P_p^{4+}$

This potentiated DNA cleaving ability under anaerobic conditions (see Figure 3.7) is rare for metal complexes and the nuclease activity could be attributed to thiyl radicals. Sulfur-centered radicals are highly reactive and are capable of hydrogen atom abstraction reactions. For example, Jain et al.$^{151}$ have shown that mixtures of Cu(II) and GSH lead to DNA cleavage under anaerobic conditions and postulate that the thiyl radical (GS$^\cdot$), generated in situ, is responsible for the observed DNA damage. Given the similarity of the results, we next examined the effect of the reducing agent on the observed nuclease activity.
The DNA cleavage assay was performed using three common biological reducing agents: GSH, ascorbic acid (AA) and dithiothritol (DTT). Both GSH and DTT contain sulfhydryl groups and can potentially form reactive thyl radicals, however, AA does not have this possibility. Any radical species generated via the oxidation of AA will rapidly decompose and should not attack DNA. The results from the cleavage experiments are shown in Figure 3.8 and the relative yields of cleavage products are reported in Table 3.3. As can be seen, solutions of \( P_p^{4+} \) with each of the three reducing agents show similar distributions of DNA cleavage products under aerobic (Lanes 2, 5 and 8) or anaerobic (Lanes 3, 6 and 9) conditions. Yields of circular DNA (Form II) for experiments conducted under aerobic conditions were 31, 27 and 49 % for GSH, AA and DTT, respectively. Under anaerobic conditions, yields of Form II DNA
increased to 57, 54 and 71 %, respectively. As before, the reactions conducted under anaerobic conditions showed greater yields of the cleavage products supporting an oxygen independent cleavage mechanism. Importantly, these results suggest that it is some reduced form of $P_p^{4+}$ is the active nuclease in these experiments.

Table 3.3 Relative yields of DNA cleavage products upon incubation of complex $P_p^{4+}$ with GSH, AA and DTT reducing agents.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
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<tr>
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<td>I</td>
</tr>
<tr>
<td>1</td>
<td>GSH control</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>GSH/ $P_p^{4+}$/O$_2$</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>GSH/$P_p^{4+}$</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>AA control</td>
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<tr>
<td>5</td>
<td>AA/ $P_p^{4+}$/O$_2$</td>
<td>63</td>
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<tr>
<td>6</td>
<td>AA/$P_p^{4+}$</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>DTT control</td>
<td>77</td>
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<tr>
<td>8</td>
<td>DTT/ $P_p^{4+}$/O$_2$</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>DTT/$P_p^{4+}$</td>
<td>16</td>
</tr>
</tbody>
</table>

All experiments were conducted under anaerobic conditions unless otherwise noted. [Reducing agent] was 0.13 mM and [P$_p^{4+}$] was fixed at 12.8 µM.

3.2.5. Concentration Dependence of $P_p^{4+}$ on DNA Cleavage

A concentration dependent study of the cleaving activity of complex $P_p^{4+}$ was conducted under anaerobic conditions using GSH or DTT as reducing agents. As seen in Figure 3.9, the DNA cleaving ability scales directly with the concentration of the complex added. The relative amount of each form of DNA (I, II or III) for each reaction
is given in Table 3.4 and a plot of the conversion of supercoiled (Form I) to Forms II and III as a function of \([\text{P}_p^{4+}]\) is given in Figure 3.10. This dose dependent cleavage is indicative of the active nuclease being some reduced form of complex \(\text{P}_p^{4+}\). The fact that either GSH or DTT can function as the reducing agents further supports this supposition.

![Figure 3.9 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with different concentrations of \(\text{P}_p^{4+}\) in 4 mM Na₃PO₄ buffer containing 0.5 mM GSH or DTT (pH 7) under anaerobic conditions. Lanes M (marker lane of form I, II and III of pUC18 DNA), 1 (DNA alone), 2 (DNA plus GSH), and 6 (DNA plus DTT) served as controls. Lanes 3, 4 and 5 contain 12.8, 25.6, and 51.2 µM of \(\text{P}_p^{4+}\), respectively and 0.5 mM each of GSH. Lanes 7, 8 and 9 contain 12.8, 25.6, and 51.2 µM of \(\text{P}_p^{4+}\), respectively and 0.5 mM each of DTT. For both titrations, the ratio of complex \(\text{P}_p^{4+}\) to DNA bp increases from 0.083 to 0.17 to 0.33.](image-url)
Table 3.4 Relative yields of DNA cleavage products upon titration with complex $P_{p}^{4+}$ under anaerobic conditions as shown in Figure 3.9.

<table>
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</thead>
<tbody>
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<tr>
<td>1</td>
<td>DNA control</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>GSH control</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>$P_{p}^{4+}$ (12.8 µM)/GSH</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>$P_{p}^{4+}$ (25.6 µM)/GSH</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>$P_{p}^{4+}$ (51.2 µM)/GSH</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>DTT control</td>
<td>73</td>
</tr>
<tr>
<td>7</td>
<td>$P_{p}^{4+}$ (12.8 µM)/DTT</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>$P_{p}^{4+}$ (25.6 µM)/DTT</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>$P_{p}^{4+}$ (51.2 µM)/DTT</td>
<td>0</td>
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Figure 3.10 Conversion of supercoiled (Form I) DNA to circular (Form II) and linear (Form III) as a function of increasing $[P_{p}^{4+}]$ in the presence of 0.5 mM GSH under anaerobic conditions.
The cleaving ability at lower concentrations of \([\text{P}_{p}^{4+}]\) was also examined under anaerobic conditions as shown in Figure 3.11. Similar to the experiments conducted at higher concentrations, we see conversion of Form I to II with a trace amount of III. As before, the amount of cleavage observed is proportional to the concentration of complex \(\text{P}_{p}^{4+}\) added. The fact that the nuclease activity is observed even at very low loadings (0.02 complexes per DNA bp) show the complex is a very efficient nuclease. In this experiment, the GSH/\(\text{P}_{p}^{4+}\) ratio was fixed at 20 (as in Figure 3.11). Given the observed cleavage activity, it seems only modest excess is required and the most important thing is the amount of \(\text{P}_{p}^{4+}\) intercalated into DNA under reduced environment.

![Figure 3.11](image)

Figure 3.11 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with different concentrations of \(\text{P}_{p}^{4+}\) in a buffer of 4 mM Na\(_3\)PO\(_4\) (pH 7) under anaerobic conditions. Lanes M (marker lane of form I, II and III pUC18 DNA), 1 (DNA alone) and 2 (DNA plus 0.3 mM GSH) served as controls. Conditions for remaining lanes: 3 (12.8 \(\mu\)M \(\text{P}_{p}^{4+}\)/0.26 mM GSH); 4 (10.3 \(\mu\)M \(\text{P}_{p}^{4+}\)/0.21 mM GSH); 5 (7.68 \(\mu\)M \(\text{P}_{p}^{4+}\)/0.15 mM GSH; 6 (5.13 \(\mu\)M \(\text{P}_{p}^{4+}\)/0.10 mM GSH); 7 (3.07 \(\mu\)M \(\text{P}_{p}^{4+}\)/0.062 mM GSH). The ratio of complex to DNA bp decreased as follows: 0.083, 0.067, 0.05, 0.33 and 0.02.
The above experiment was repeated with higher ratio of $P_p^{4+}$ to DNA bp as shown in Figure 3.12. The yield data is given in Table 3.5. As can be seen, dose dependent cleavage was observed under both aerobic and anaerobic conditions, but experiments conducted under anaerobic conditions showed appreciably more cleavage products. Figure 3.13 shows a plot of the relative yield of Form I, II and III DNA both as a function of complex concentration and of the environmental conditions (aerobic and anaerobic).

![Figure 3.12 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 2 h with different concentrations of $P_p^{4+}$ in 4 mM Na$_3$PO$_4$ (pH 7) buffer under aerobic and anaerobic conditions. Lanes M (marker lane of form I, II and III pUC18 DNA), 1 (DNA + aerobic), 2 (DNA + 1 mM GSH + aerobic) and 3 (DNA + 1 mM GSH + anaerobic) served as controls. Remaining lanes were incubated under conditions as indicated in the figure with the GSH to complex ratio maintained at 20.](image-url)
Table 3.5 Product yields from the cleavage assay from Figure 3.12 as determined from densitometry.

<table>
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<td></td>
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<td>I</td>
</tr>
<tr>
<td>1</td>
<td>DNA/O₂</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>GSH/O₂</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>GSH</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>Pₚ⁴⁺ (12.8 µM)/GSH (0.256 mM)/O₂</td>
<td>53</td>
</tr>
<tr>
<td>5</td>
<td>Pₚ⁴⁺ (12.8 µM)/GSH (0.256 mM)</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>Pₚ⁴⁺ (25.6 µM)/GSH (0.256 mM)/O₂</td>
<td>51</td>
</tr>
<tr>
<td>7</td>
<td>Pₚ⁴⁺ (25.6 µM)/GSH (0.512 mM)</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>Pₚ⁴⁺ (51.2 µM)/GSH (1.02 mM)/O₂</td>
<td>28</td>
</tr>
<tr>
<td>9</td>
<td>Pₚ⁴⁺ (51.2 µM)/GSH (1.02 mM)</td>
<td>23</td>
</tr>
</tbody>
</table>

Anaerobic conditions unless stated otherwise.

In all cases, the cleavage products for the anaerobic experiments exceed those for the aerobic ones, although the differences become less pronounced at high [Pₚ⁴⁺]. Higher Pₚ⁴⁺ concentrations were not examined because the complex tends to precipitate the plasmid DNA, which then becomes very difficult to redissolve in buffer.
Figure 3.13 Conversion of supercoiled (Form I) DNA to circular (Form II) and linear (Form III) as a function of increasing \([\text{P}^{4+}]\) under anaerobic aerobic conditions.

3.2.6. Identification of the Active Reduced Form of \(\text{P}^{4+}\)

The redox chemistry of complex \(\text{P}^{4+}\) has been extensively studied and it is known that \(\text{P}^{4+}\) can undergo multiple reductions centered on the planar tatpp bridging ligand.\(^{108,146}\) The ladder scheme given in Figure 3.14 shows a number of redox and protonated isomers of \(\text{P}^{4+}\), which are obtainable in aqueous solution at modest potentials as a function of pH. Of the six complexes shown in Figure 3.14, only complex \(\text{HP}^{4+}\) is unstable in solution and rapidly disproportionates as shown in reaction 1. In this scheme, complexes are stacked vertically to indicate redox changes and horizontally to indicate changes in the protonation state.
In acetonitrile, \( \text{P}_{\text{p}}^{4+} \) undergoes two one-electron reductions at -0.18 and -0.54 V vs Ag/AgCl. These redox processes are strongly proton-coupled such that in aqueous solution at pH 7, the two one-electron processes in MeCN have merged to form a single bielectronic process at -0.14 V vs Ag/AgCl.

These potentials are clearly within the reducing potential of agents such as GSH, DTT and AA and as such, we have a number of chemical species that may be the active DNA cleaving agent. Fortunately as will be shown, changes in redox and/or protonation state of \( \text{P}_{\text{p}}^{4+} \) lead to easily discernable changes in the visible absorption spectrum,
which allows us to readily identify a particular species from a simple visible absorption spectrum. Stoichiometric chemical reduction of $P_p^{4+}$ with cobaltocene in acetonitrile was used to generate $P_p^{3+}$ and $P_p^{2+}$ in solution and their absorption spectra are shown in Figure 3.15. In order to separate redox processes from protonation processes, these titrations were performed in dry acetonitrile. Subsequently, the doubly reduced product $P_p^{2+}$ was protonated by addition of one and then two equivalents of trifluoroacetic acid to generate $H P_p^{3+}$ and $H_2P_p^{4+}$, respectively, whose spectra are shown in Figure 3.16.

![Figure 3.15 Absorption spectra of $P_p^{4+}$ and its singly reduced $P_p^{3+}$ and doubly reduced $P_p^{2+}$ in degassed CH$_3$CN.](image-url)
These characteristic absorption spectra are not strongly perturbed when the solvent is changed to water; however, the pH of the solution is critical as to what species is dominant in solution. The pKₐ's for the singly reduced complex, Pₚ³⁺, and the doubly reduced complex, Pₚ²⁺, have been estimated from spectroelectrochemical data and are shown in reactions 2-4. When these data are considered in light of reaction 1, at pH 7 the dominant reduced species in aqueous solution is H₂Pₚ⁴⁺.

\[
\begin{align*}
\text{HP}^{4+} &\rightarrow \ Pₚ^{3+} + H^+ & \text{pK}_a &\approx 8 \\
\text{H₂P}^{4+} &\rightarrow \ HPₚ^{3+} + H^+ & \text{pK}_{a1} &\approx 9 \\
\text{HP}^{3+} &\rightarrow \ Pₚ^{2+} + H^+ & \text{pK}_{a2} &\approx 10
\end{align*}
\]
Importantly, all of the reduced species are rapidly reoxidized to \( \text{P}_p^{4+} \) upon exposure to air whether they are in acetonitrile or aqueous solution. From handling numerous solutions of these complexes, we have noted that the doubly reduced complexes, \( \text{P}_p^{2+}, \text{HP}_p^{3+} \) and \( \text{H}_2\text{P}_p^{4+} \), are the most oxygen sensitive with \( \text{P}_p^{3+} \) being noticeably slower to reoxidize.

When a solution of \( \text{P}_p^{4+} \) in 4 mM phosphate buffer (pH 7) was treated with 10 equivalents of GSH, DTT or AA, the yellow solution rapidly turned green, which is indicative of the doubly reduced species. The absorption spectra from these reductions are shown in Figure 3.17. All three reducing agents produce a common product, \( \text{H}_2\text{P}_p^{4+} \), as identified by the strong broad absorption at 590 nm. The intensity of this band for GSH reduced species is slightly less than that observed for DTT and AA. The reasons for this discrepancy are unclear. Nonetheless, it is clear that under the conditions most frequently employed in the DNA cleaving assays, \( \text{H}_2\text{P}_p^{4+} \), is the dominant complex in solution.
Figure 3.17 Absorption spectra of $P_p^{4+}$ and $H_2P_p^{4+}$ made with excess (10 equivalent) glutathione (GSH), dithiothritol (DTT) and ascorbic acid (AA).

If the pH of the buffer is varied, then a different product distribution is evident in the absorption spectra of the doubly reduced complex. As seen in Figure 3.18, raising the pH from 6 to 10 results in the appearance of a new band at 710 nm. This band is indicative of the presence of the monoprotonated complex, $HP_p^{3+}$ and is not unexpected given that the pK$_{a2}$ of $H_2P_p^{4+}$ is 9.
Figure 3.18 Absorption spectra of $P_p^{4+}$ (12.8 µM) with GSH (0.256 mM) at different pH in degassed deionized water.

3.2.7. Identification of Reduced Species Responsible for DNA Cleavage

Two of the reduced species, $P_p^{3+}$ and $H_2P_p^{4+}$, were prepared as described in the materials and methods section and isolated as $[P_p]Cl_3$ and $[H_2P_p]Cl_4$. Both complexes showed paramagnetically broadened NMR spectra, as shown in Figure 3.19. While this would be expected for the monoreduced species $P_p^{3+}$, it was unexpected for $H_2P_p^{4+}$ and ultimately supports the role of radical species in the chemistry of $H_2P_p^{4+}$. The
complexes identity was confirmed by taking their absorption spectra in degassed water, as shown in Figure 3.20.

Figure 3.19 $^1$H NMR spectra of $[\text{P}_p](\text{PF}_6)_4$, $[\text{P}_p](\text{PF}_6)_3$ and $[\text{H}_2\text{P}_p](\text{PF}_6)_4$ in degassed $d_3$ MeCN.
With these reduced complexes in hand, DNA cleavage experiments were conducted, which directly compared the cleavage activity of $\text{P}_p^{4+}$, $\text{P}_p^{3+}$ and $\text{H}_2\text{P}_p^{4+}$. The incubations were performed under anaerobic conditions in the absence of any external reducing agents, such as GSH or DTT. The resulting gel is shown in Figure 3.21 and the product distribution as determined by densitometry is given in Table 3.6. As seen in lanes 2 to 5, neither $\text{P}_p^{4+}$ (Lanes 2 and 3) nor $\text{P}_p^{3+}$ (Lanes 4 and 5) show appreciable ss or ds cleavage activity, although $\text{P}_p^{3+}$ does seem to be a slightly more active nicking agent. In contrast, the doubly reduced, doubly protonated complex, $\text{H}_2\text{P}_p^{4+}$, shows...
considerable nuclease activity with both ss and ds cuts observed (Lanes 6 and 7). At the loadings typically employed in previous cleavage assays (0.083 complex/DNA bp or one complex every 12 base pairs DNA), \( H_2P_p^{2+} \) yields 48% form II and 2% form III cleavage products. Increasing the loading to one complex per five DNA bp (0.20 complex/DNA bp) increases the yields to 68% and 17% for form II and III, respectively. This dose dependent nuclease activity reveals \( H_2P_p^{4+} \) as the agent responsible for the DNA damage. Given that, it is readily generated in the presence of GSH, AA or DTT at pH 7 shows it to be the active nuclease in the prior studies and that the role of the reducing agents is to generate \( H_2P_p^{4+} \) \textit{in situ}. Presumably, aerobic conditions result in reoxidation of this complex. Given that, the excess GSH (or other reducing agents are present), it seems likely that the overall process is catalytic and \( H_2P_p^{4+} \) is constantly being oxidized and regenerated under aerobic conditions. The relative decrease in DNA nuclease activity under aerobic vs anaerobic conditions is therefore just a function of the lower equilibrium concentration of \( H_2P_p^{4+} \).
Figure 3.21 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 3 h with different concentrations of \( P_p^{4+} \), \( P_p^{3+} \) and \( H_2P_p^{4+} \) in a buffer of 4 mM \( Na_3PO_4 \) (pH 7) under anaerobic conditions. Lanes M (marker lane of form I, II and III pUC18 DNA), and 1 (supercoiled DNA) served as controls. Lane 2 (12.8 µM \( P_p^{4+} \), 0.083 complex/DNA bp ratio), Lane 3 (30.7 µM \( P_p^{4+} \), 0.20 complex/DNA bp ratio), Lane 4 (12.8 µM \( P_p^{3+} \), 0.083 complex/DNA bp ratio), Lane 5 (30.7 µM \( P_p^{3+} \), 0.083 complex/DNA bp ratio), Lane 6 (12.8 µM \( H_2P_p^{4+} \), 0.083 complex/DNA bp ratio), Lane 7 (30.7 µM \( H_2P_p^{4+} \), 0.20 complex/DNA bp ratio).

Table 3.6 DNA cleavage products distribution after treatment of supercoiled pUC18 DNA with \( P_p^{4+} \), \( P_p^{3+} \) and \( H_2P_p^{4+} \).

<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>DNA control</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>( P_p^{4+} ) (12.8 µM)</td>
<td>90</td>
</tr>
<tr>
<td>2'</td>
<td>( P_p^{4+} ) (30.7 µM)</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>( P_p^{3+} ) (12.8 µM)</td>
<td>79</td>
</tr>
<tr>
<td>3'</td>
<td>( P_p^{3+} ) (30.7 µM)</td>
<td>81</td>
</tr>
<tr>
<td>4</td>
<td>( H_2P_p^{4+} ) (12.8 µM)</td>
<td>50</td>
</tr>
<tr>
<td>4'</td>
<td>( H_2P_p^{4+} ) (30.7 µM)</td>
<td>15</td>
</tr>
</tbody>
</table>

All experiments were conducted under anaerobic conditions.
3.2.8. pH Independent DNA Cleavage by $P_{p}^{4+}$

Given that, the protonation of the doubly reduced complex can be altered with pH as was shown in Figure 3.18, the ability of the doubly reduced complex to cleave DNA as a function of pH in the range of 6 to 10 was examined. The doubly reduced complex ($HP_{p}^{3+}$ or $H_{2}P_{p}^{2+}$) was generated \textit{in situ} by addition of an excess of GSH and anaerobic conditions were maintained. The results are shown in Figure 3.22 and the product distributions are tabulated in Table 3.7.

![Figure 3.22](image)

The most significant result from this study is that relatively constant amount of cleavage observed regardless of the pH. The product distribution in lane 3 (pH 6) and lane 10 (pH 10) is virtually identical with each showing yields of ~ 65-75 % circular (Form II) and ~ 3-8 % linear (Form III) DNA. From the data obtained in Figure 3.18,
we know that the dominant species at pH 6 is $\text{H}_2\text{P}_p^{4+}$ and $\text{HP}_p^{3+}$ at pH 10. Clearly, the difference in protonation has little effect towards the cleavage ability of the doubly reduced complexes.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA alone</td>
<td>92 8 0</td>
</tr>
<tr>
<td>2</td>
<td>GSH/pH 6</td>
<td>82 18 0</td>
</tr>
<tr>
<td>3</td>
<td>GSH/$\text{P}_p^{4+}$/pH 6</td>
<td>19 75 6</td>
</tr>
<tr>
<td>4</td>
<td>GSH/pH 7</td>
<td>76 24 0</td>
</tr>
<tr>
<td>5</td>
<td>GSH/$\text{P}_p^{4+}$/pH 7</td>
<td>29 69 3</td>
</tr>
<tr>
<td>6</td>
<td>GSH/$\text{P}_p^{4+}$/pH 8</td>
<td>32 64 4</td>
</tr>
<tr>
<td>7</td>
<td>GSH/$\text{P}_p^{4+}$/pH 9</td>
<td>22 75 3</td>
</tr>
<tr>
<td>8</td>
<td>DNA alone/pH 10</td>
<td>86 14 0</td>
</tr>
<tr>
<td>9</td>
<td>GSH/pH 10</td>
<td>77 23 0</td>
</tr>
<tr>
<td>10</td>
<td>GSH/$\text{P}_p^{4+}$/pH 10</td>
<td>17 75 8</td>
</tr>
</tbody>
</table>

In all cases the concentration of $[\text{P}_p^{4+}]$ is 12.8 µM and [GSH] is 0.25 mM.

3.2.9. Establishing a Potential Mechanism for DNA Cleavage by $\text{H}_2\text{P}_p^{4+}$

The mechanism by which doubly reduced $\text{P}_p^{4+}$ cleaves DNA is unknown and a few literature precedents exist for DNA cleavage by reduced metal complexes. The paramagnetically broadened NMR spectrum for $\text{H}_2\text{P}_p^{4+}$ suggests the role of radical species (Figure 3.19). The proton signals on the tatpp ligand are the most strongly broadened and, as this is the site of reduction, we believe that the radical species are most likely localized here. Typically, one would anticipate a diamagnetic, singlet state
electronic structure for \( \text{H}_2\text{P}_p^{4+} \) as shown in Figure 3.23 (left), however, another possibility, consistent with the observed paramagnetism and cleavage activity, is the presence of a diradical (triplet) ground or low-lying excited state, as shown in Figure 3.23 (right).

![Figure 3.23 The postulated structures of singlet and triplet states of \( \text{H}_2\text{P}_p^{4+} \).](image)

Magnetic susceptibility measurements of solid \([\text{H}_2\text{P}_p][\text{PF}_6]_4\) gave confusing results, in that the effective magnetic moment per complex was too high for even a triplet state. Given the likelihood for \(\pi-\pi\) stacking in the solid state, we suspect that intermolecular electronic coupling is responsible for this unusual data. In order to eliminate intermolecular interactions, the magnetic susceptibility of \([\text{H}_2\text{P}_p][\text{PF}_6]_4\) was measured in solution (CD\(_3\)CN) using the Evans NMR method.\(^{153-157}\) The details and raw data of this experiment are reported in appendix J. From this experiment, \(\mu_{\text{eff}}\) of 1.13 BM for \([\text{H}_2\text{P}_p][\text{PF}_6]_4\) is obtained, which corresponds to less than one unpaired electron per complex. Theoretical spin only values of \(\mu_{\text{eff}}\) for \(S = 1/2\) and \(S = 1\) spin systems are 1.73 and 1.83 BM, respectively.\(^{158}\) The interpretation of this result is still uncertain, however, one model to explain the data postulates a singlet ground state in thermal equilibrium with a low-lying triplet state. If this is true, then measurements of the
temperature dependence of $\mu_{\text{eff}}$ will allow us to determine the singlet-triplet energy gap. Currently, our best explanation for the observed DNA cleavage activity is that thermal population of this diradical state gives a species capable of H-atom abstraction from the DNA deoxyribose rings, which subsequently is known to lead to DNA strand scission.$^{5,10}$ Diradicals have been implicated in DNA cleavage for the enediyne family of chemical nucleases$^{159}$ as well as for some photoexcited metal complexes bound to DNA.$^{160}$ It is interesting to note that because $\text{H}_2\text{P}_{p}^{4+}$ intercalates upon binding DNA, these tattp-centered radicals would be closely positioned to the proposed site of attack, the deoxyribose rings.

The fate of $\text{H}_2\text{P}_{p}^{4+}$ after cleaving DNA is not clear. If we assume that the diradical is involved and the DNA is attacked via H-atom abstraction, then possible products include $\text{H}_3\text{P}_{p}^{4+}$ and $\text{H}_4\text{P}_{p}^{4+}$ (as shown below), which correspond to single and double H-atom abstraction processes, respectively. We decided to monitor the absorption spectrum of the complex during the cleavage reaction to see if the fate of the complex could be determined. A large-scale plasmid cleavage assay was carried out in a quartz cuvette such that aliquots of DNA could be examined by agarose gel
electrophoresis to determine the extent on the reaction while we simultaneously monitored the absorption spectrum of the complex. The cuvette contained a solution of supercoiled pUC18 DNA (0.154 mM), $\text{H}_2\text{P}_\text{p}^{4+}$ (12.8 µM) in 4 mM Na$_3$PO$_4$ buffer (pH 7.0) under anaerobic conditions. A control cuvette containing $\text{H}_2\text{P}_\text{p}^{4+}$ in the same buffer and anaerobic conditions but lacking DNA was also prepared and monitored alongside the cleavage experiment. The evolution of the absorption spectra for time zero, after 3 h and after 20 h is shown in Figure 3.25. The agarose gel showing the extent of the DNA cleavage in aliquots removed at the same times is shown in Figure 3.26 and the yields of the cleavage products as determined by densitometry are given in Table 3.8.

![Figure 3.25 Change of absorption spectra of $\text{H}_2\text{P}_\text{p}^{4+}$ itself and $\text{H}_2\text{P}_\text{p}^{4+}$ intercalated into DNA with time in 4 mM Na$_3$PO$_4$ buffer at pH 7.](image)

Figure 3.25 Change of absorption spectra of $\text{H}_2\text{P}_\text{p}^{4+}$ itself and $\text{H}_2\text{P}_\text{p}^{4+}$ intercalated into DNA with time in 4 mM Na$_3$PO$_4$ buffer at pH 7.
Figure 3.26 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after incubation at 24 °C for 3 h and 20 h with $H_2P_{Pp}^{4+}$ (12.8 µM, 12 DNA bp per $H_2P_{Pp}^{4+}$) in 4 mM Na$_3$PO$_4$ (pH 7) buffer under anaerobic conditions. Lane M, marker lane consisting three different isomers of pUC18, Form I, Form II and Form III; Lane 1, pUC18 DNA alone after 3 hours; Lane 2, DNA + $H_2P_{Pp}^{4+}$ (12 DNA bp per $H_2P_{Pp}^{4+}$) after 3 hours; Lane 3, DNA alone after 20 hours; Lane 4, DNA + $H_2P_{Pp}^{4+}$ after 20 hours.

Table 3.8 DNA product distribution from cleavage assay shown in Figure 3.26.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>DNA control/3 h</td>
<td>84.9</td>
</tr>
<tr>
<td>2</td>
<td>$H_2P_{Pp}^{4+}$/3 h</td>
<td>67.4</td>
</tr>
<tr>
<td>3</td>
<td>DNA control/20 h</td>
<td>82.9</td>
</tr>
<tr>
<td>4</td>
<td>$H_2P_{Pp}^{4+}$/20 h</td>
<td>86.0</td>
</tr>
</tbody>
</table>

All experiments were conducted under anaerobic conditions with only 12.8 µM $H_2P_{Pp}^{4+}$ present.

Surprisingly, the absorption spectrum of $H_2P_{Pp}^{4+}$ is unchanged over the course of the experiment even though the cleavage assay indicates that the DNA is being cleaved. At the same time, $H_2P_{Pp}^{4+}$ in the absence of DNA is slowly disappeared, presumably due to oxidation from trace oxygen leaking into the cuvette. Taken together, these data indicate that $H_2P_{Pp}^{4+}$ is stabilized or regenerated in the presence of DNA. Given that, the
cleavage reaction is observed, one must assume that the DNA is either cleaved in reaction that does not involve a redox change on the complex, which seems very unlikely, or that $H_2P_p^{4+}$ is reformed in the process. One speculative reaction sequence that could explain this data would be the evolution of $H_2$ by $H_4P_p^{4+}$ or $H_3P_p^{4+}$ as indicated in reactions 5 and 6. At this time, we lack the data to clearly establish a cleavage mechanism. It seems likely that whatever mechanism we find, it will be very unusual and possibly unprecedented.

$$2 \ H_2P_p^{4+} \ \rightarrow \ 2 \ H_2P_p^{4+} \ + \ H_2 \ \ \ (5)$$

$$H_4P_p^{4+} \ \rightarrow \ H_2P_p^{4+} \ + \ H_2 \ \ \ (6)$$

3.2.10. DNA Cleavage by Different Analogues Consisting of tatpp Bridging Ligand

As indicated in the previous sections, the tatpp bridging ligand is the site of the relevant redox and protonation chemistry. This ligand, by itself, is insoluble in most common solvents including water and coordination of the $[\text{Ru(phen)}_2]^{2+}$ units acts to solubilize it and the cationic charge of the complex is no doubt an important factor in the observed DNA binding affinity. As mentioned, the role of the Ru(II) ions in the cleavage mechanism is not clear and may only be consequential as per the solubility and binding issues. To address this issue, we examined the DNA cleaving ability of two new tatpp complexes: mono-metallated complex, $[\text{Ru(phen)}_2\text{tatpp}]^{2+}$ ($P_1^{2+}$) and a Zn(II)
adduct formulated as \([\text{ZntatppZn}](\text{BF}_4)_4\) \((\text{Z}^{4+})\), which are shown in Figure 3.27. The Zn(II) adduct is prepared \textit{in situ} by mixing \textit{tatpp} with excess hydrated \textit{Zn(BF}_4)_2 in MeCN and the open coordination sites on the Zn(II) ions are assumed to be solvent molecules. The concentrated solution of \(\text{Z}^{4+}\) in MeCN was diluted with the water and buffer to perform DNA cleavage studies in which the final solution contained less than 6% of MeCN.

![Figure 3.27 Structures of \(\text{P}_1^{2+}\) and \(\text{Z}^{4+}\).](image)

DNA cleavage was studied with the constant concentration of GSH and increasing concentration of \(\text{P}_1^{2+}\) (Lane 4-6) and \(\text{Z}^{4+}\) (Lane 7-9) and compared with the \(\text{P}_p^{4+}\) data (Lane 3). Similar to \(\text{P}_p^{4+}\), \(\text{P}_1^{2+}\) and \(\text{Z}^{4+}\) showed DNA cleavage, however, their activity gets diminished (see Figure 3.28).
Figure 3.28 1% agarose gel (negative image) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM bp) cleavage products after anaerobic incubation at 24 °C for 3 h with different concentrations of $P_p^{4+}$, $P_1^{2+}$ and $Z^{4+}$ in a 4 mM Na$_3$PO$_4$ buffer (pH 7) containing 0.5 mM GSH. All experiments except lanes M and 1 were performed in the presence of 0.5mM GSH. Lanes M (marker lane of form I, II and III pUC18 DNA), Lane 1 (supercoiled DNA without added GSH), Lane 2 (DNA with GSH), Lane 3 (25.6 µM $P_p^{4+}$; 0.17 $P_p^{4+}$ per DNA bp), Lane 4 (12.8 µM $P_1^{2+}$; 0.083 $P_1^{2+}$ per DNA bp), Lane 5 (25.6 µM $P_1^{2+}$; 0.17 $P_1^{2+}$ per DNA bp), Lane 6 (51.2 µM $P_1^{2+}$ 0.33 $P_1^{2+}$ per DNA bp), Lane 7 (12.8 µM $Z^{4+}$ $0.083 Z^{4+}$ per DNA bp), Lane 8 (25.6 µM $Z^{4+}$ $0.17 Z^{4+}$ per DNA bp), Lane 9 (51.2 µM $Z^{4+}$; 0.33 $Z^{4+}$ per DNA bp).

The densitometric scanning results (Table 3.9) of Figure 3.28 clearly show the percent conversion of plasmid DNA by each complex. It is interesting to know at the concentration of 25.6 µM, $P_p^{4+}$ is able to form 71% ss and 19% ds nicks whereas to obtain similar activity, twice of the concentration of $P_1^{2+}$ required. DNA cleavage by $Z^{4+}$ under reduced conditions even lower than both cationic complexes and also does not enhance with the increasing loading of $Z^{4+}$ (~40-50% ss and ~10% ds nicking).
Table 3.9 Comparison of cleavage of supercoiled pUC18 DNA by $P^4+$, $P^{2+}$ and $Z^{4+}$

<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
<th>% Form of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA control</td>
<td>85 15 0</td>
</tr>
<tr>
<td>2</td>
<td>GSH (0.5 mM) control</td>
<td>76 24 0</td>
</tr>
<tr>
<td>3</td>
<td>GSH (0.5 mM) + $P^4+$ (25.6 µM)</td>
<td>10 71 19</td>
</tr>
<tr>
<td>4</td>
<td>GSH (0.5 mM) + $P^{2+}$ (12.8 µM)</td>
<td>32 57 11</td>
</tr>
<tr>
<td>5</td>
<td>GSH (0.5 mM) + $P^{2+}$ (25.6 µM)</td>
<td>21 64 15</td>
</tr>
<tr>
<td>6</td>
<td>GSH (0.5 mM) + $P^{2+}$ (51.2 µM)</td>
<td>14 68 18</td>
</tr>
<tr>
<td>7</td>
<td>GSH (0.5 mM) + $Z^{4+}$ (12.8 µM)</td>
<td>45 47 8</td>
</tr>
<tr>
<td>8</td>
<td>GSH (0.5 mM) + $Z^{4+}$ (25.6 µM)</td>
<td>49 42 9</td>
</tr>
<tr>
<td>9</td>
<td>GSH (0.5 mM) + $Z^{4+}$ (51.2 µM)</td>
<td>37 53 10</td>
</tr>
</tbody>
</table>

Since $P^{2+}$ and $Z^{4+}$ showed DNA cleavage under reduced environment, we have carried out the chemical reductions with GSH similar to $P^4+$ to further confirm that the same active species involved in DNA cleavage. The procedure for chemical reduction of $P^{2+}$ and $Z^{4+}$ are given under the materials and methods. It is evident that the ligand centered (LC) absorption spectrum moves to lower energy (450 to 550 nm) to give characteristic spectrum of doubly reduced doubly protonation form with all these complexes (see Figure 3.29).
Figure 3.29 Absorption spectra of $H_2P_p^{4+}$, $H_2P_1^{2+}$, $Z^{4+}$ and $H_2Z^{4+}$ in degassed water.

### 3.3 Summary and Conclusions

$P_p^{4+}$ is a ruthenium polypyridyl dimer with the redox active tatpp bridging ligand, which binds tightly to DNA through intercalation.

We have successfully carried out DNA cleavage studies with $P_p^{4+}$ using supercoiled pUC18 DNA under different conditions. $P_p^{4+}$ itself has no activity in cleaving DNA. But under reducing conditions, $P_p^{4+}$ readily cleaves plasmid DNA both under aerobic and anaerobic conditions to give circular (Form II) and linear (Form III) products. Interestingly, the cleavage ability gets potentiated under anaerobic conditions.
Parallel experiments, which we have carried out with oxygen dependent DNA cleaving agent, Fe-BLM further supports this argument.

$P_{p}^{4+}$ cleaves plasmid DNA in the presence of thiol containing reducing agents as well as nonthiol containing reducing agents, eliminating the thiyl radical ($GS^+$) mediated DNA cleavage, which generates after reduction of $P_{p}^{4+}$. The parallel experiments done with different reduced species of $P_{p}^{4+}$ show that the actual species responsible for DNA cleavage is doubly reduced doubly protonated $H_2P_{p}^{4+}$. Singly reduced $P_{p}^{3+}$ has no activity in cleaving DNA.

The DNA cleavage by $H_2P_{p}^{4+}$ is independent of pH. From pH6 to 10, $H_2P_{p}^{4+}$ has the potential in cleaving DNA.

Other than $P_{p}^{4+}$ its monomer $P_{1}^{2+}$ as well as the tatpp bridging ligand that dissolved using Zn(BF$_4$)$_2$ ($Z^{4+}$) have the potential of cleaving DNA. However, their efficiencies diminish due to the less probability of intercalation of reduced form of planar tatpp ligand with decreasing charge ($P_{p}^{4+}$ to $P_{1}^{2+}$).

Comparison of chemical reduction data of $P_{p}^{4+}$, $P_{1}^{2+}$ and $Z^{4+}$ proves that the same doubly reduced doubly protonated tatpp bridging ligand is involved in initiating lethal single and double-strand nicks to DNA.
3.4 Materials and Methods

3.4.1. Chemicals

All chemicals were reagent grade and were used as received. Supercoiled DNA, pUC18 were purchased from Bayou Biolab (New England), glutathione (GSH), Blenoxane, agarose (molecular biology grade), ethidium bromide, dithiothritol (DTT), ascorbic acid (AA) and trisma base were purchased from Sigma-Aldrich. Zinc tetrafluoroborate and cobaltocene were purchased from Alfa-Aesar.

The complexes, \([\text{Ru(phen)}_3]^{2+}\), \([\text{Ru(phen)}_2\text{dppz}]^{2+}\), \([\text{Ru(phen)}_2\text{tpphz}]^{4+}\), \([\text{Ru(tatpp)}_2\text{Ru(phen)}_2]^{4+}\) \((\text{P}_4^{4+})\) \([\text{Ru(tatpq)}_2\text{Ru(phen)}_2]^{4+}\) \((\text{Q}_4^{4+})\) and tatpp were synthesized as described in the literature methods.

3.4.2. Instrumentation

\(^1\)H NMR spectra were obtained on JEOL Eclipse Plus 300 or 500 MHz Spectrometers. Spectra were referenced to tetramethysilane (TMS) or residual solvent peaks from the deuterated solvent. UV-visible spectra were obtained on a Hewlett-Packard HP84535A spectrophotometer. Plasmid cleavage products were analyzed and quantitated using an AlphaImager™ 2200 complete gel documentation and analysis system.
3.4.3. DNA cleavage by $P_p^{4+}$ Under Reduced Environment

DNA Cleavage reactions were carried out in a total volume of 20 µL in 0.5 mL Eppendorf tubes in a 7 mM Na$_3$PO$_4$ buffer medium (pH 7) containing 2 µL of supercoiled pUC18 DNA (1 µg/1 µL, 0.154 mM DNA base pairs). Conditions and equilibrating time were varied as indicated in the legend. After equilibration, the DNA precipitated by adding 2 µL sodium acetate (pH 5.2) and 80 µL ethanol followed by cooling overnight at –20 °C. The precipitated DNA was pelleted out by centrifuging the Eppendorfs at 13000 rpm (round per minutes) for 15 minutes. Ethanol solution was decanted out and the samples air-dried for about 30 minutes and were resuspended in 40 µL of a storage buffer (e.g., 40 mM Tris-Cl, 1 mM EDTA at pH 8.0), 65 µL deionized water and 12 µL of a loading buffer (e.g., 30% glycerol in water with 0.1% w/v bromophenol blue). After then 20 µL of each sample was loaded on 1% agarose gel containing ethidium bromide (0.2 µL/1 mL) and electrophoresed at 80 V for 90 minutes using TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). Bands were visualized by ultra violet light and photographed followed by estimation of the percent intensity of the DNA bands by densitometric scanning.

3.4.4. Anaerobic Reactions

Anaerobic conditions were generated by degassing of all the reagents including DNA, before equilibration, for five freeze-pump-thaw cycles under N$_2$. The degassed reagents were taken into a glove box and all the solutions were prepared inside it to minimize further contamination with oxygen. Quenching of the activity of $P_p^{4+}$ and Fe-
BLM was done anaerobically by precipitating the DNA using 2 µL of degassed sodium acetate at pH 5.2 and 80 µL degassed ethanol under N₂ inside the glove box.

3.4.5. Product Analysis and Quantitation

The extent of supercoiled pUC18 DNA cleavage was determined via densitometric analysis of ethidium bromide stained agarose gels using the volume quantitation method in AlphaImager™ 2200 complete gel documentation and analysis system. The sum of intensity of the existing bands in each lane was standardized to 100%. The correction coefficient of 1.47±0.3 was applied for decreased stainability of Form I DNA vs Form II and III.¹⁶³

3.4.6. Synthesis of [Pₚ]Cl₃

All experimental manipulations were performed in a nitrogen-filled glove box using degasses solvents. Complex [Pₚ](PF₆)₄ (200 mg; 1.00 ×10⁻⁴ mol) in 100 mL degassed CH₃CN was mixed with the cobaltocene (19 mg, 1.00 ×10⁻⁴ mol) in 100 µL of CH₃CN. The solution was stirred for 30 minutes and the product precipitated by adding a minimum volume of degassed ether (~ 200 mL). The dark solid was filtered and dried under vacuum. The formation of [Pₚ][PF₆]₃ was confirmed by obtaining an absorption spectrum of the solid in degassed acetonitrile. The solid was then dissolved in a minimum volume of acetone and the chloride salt precipitated by drop wise addition of a saturated solution of tetrabutylammonium chloride in acetone. The dark solid was isolated by filtration, washed with acetone (~ 50 mL) and dried inside the glove box.
The $^1$H NMR spectra of $[\text{Pp}]\text{(PF}_6\text{)}_3$ in CD$_3$CN showed considerable paramagnetic broadening and is shown in appendix I. Importantly, the NMR was free of any peaks indicative of the cobatocenium co-product, $[\text{Co(Cp)}_2]\text{Cl}$. The absorption spectrum of this product as dissolved in deionized water is shown in Figure 3.20 and shows the characteristic peaks at 970 and 860 nm for the $\text{P}^{3+}$ complex.

3.4.7. Synthesis of $[\text{H}_2\text{Pp}]\text{Cl}_4$

All experimental manipulations were performed in a nitrogen-filled glove box using degasses solvents. Complex $[\text{Pp}]\text{Cl}_4$ (0.2 g; $1.29 \times 10^{-4}$ mol) was dissolved in 100 mL of degassed water and a solution of glutathione (0.2 g, $6.51 \times 10^{-4}$ mol) in 50 mL of water was added. After stirring for 2 h, the complex precipitated by addition of an aqueous solution of NH$_4$PF$_6$. The solid was isolated by filtration, washed with water (∼200 mL) and ether (∼100 mL), and dried inside the glove box. The formation of $[\text{H}_2\text{Pp}]\text{[PF}_6\text{]}_4$ was confirmed by obtaining an absorption spectrum of the solid in degassed acetonitrile. The solid was then dissolved in a minimum volume of acetone and the chloride salt precipitated by drop wise addition of a saturated solution of tetrabutylammonium chloride in acetone. The dark solid was isolated by filtration, washed with some acetone (∼50 mL) and dried inside the glove box. The $^1$H NMR spectrum of $[\text{H}_2\text{Pp}]\text{(PF}_6\text{)}_4$ in CD$_3$CN showed considerable paramagnetic broadening and is shown in appendix I. Importantly, the $^1$H NMR spectrum of $[\text{H}_2\text{Pp}]\text{Cl}_4$ in D$_2$O was free of any peaks indicative of GSH or related by-products. Due to the difficulty in thoroughly drying the solid in the glove box, some solvent peaks for ether, acetone and
acetonitrile are seen in the $^1$H NMR spectrum. The absorption spectrum of the chloride salt, $[\text{H}_2\text{P}_p]\text{Cl}_4$, in deionized water is shown in Figure 3.20 and shows the characteristic peaks at 580 nm for $\text{H}_2\text{P}_p^{4+}$.

3.4.8. Preparation of a Solution of Zn(BF$_4$)$_2$ and tatpp ($Z^{4+}$) for DNA Cleavage Studies

The bridging ligand, tatpp (8 mg) was suspended in excess hydrous Zn(BF$_4$)$_2$ (50 mg) in acetonitrile (5 mL) in a 10.00 mL volumetric flask. The solution was warmed until the solid completely dissolved and was diluted up to 10.00 mL volume with acetonitrile. After the solution ($Z^{4+}$) was prepared, it was filtered through 0.2 microns filter disk to remove excess Zn(BF$_4$)$_2$ xH2O. Then the clear solution was degassed by five freeze-pump-thaw cycles under N$_2$ and was immediately taken into a glove box to minimize further contamination with oxygen. Finally the solutions for DNA cleavage were prepared by diluting the stock solution to required concentrations with degassed water inside the glove box.

3.4.9. Determination of Paramagnetic Susceptibility by the Evans Method

In this $^1$H NMR method, two CD$_3$CN solutions were prepared containing 2% (CH$_3$)$_4$Si (TMS) inside the glove box. A known amount of the substance ($P_p^{4+}/P_p^{3+}/H_2P_p^{4+}$) was dissolved in one CD$_3$CN sample in which the density of the solution was already determined. The CD$_3$CN containing the TMS (reference sample) was incorporated in a capillary tube while the CD$_3$CN solution of the paramagnetic species ($P_p^{4+}/P_p^{3+}/H_2P_p^{4+}$) whose susceptibility was to be determined was placed in the
NMR sample tube. The paramagnetic solution also contained TMS, which existed chemical shift difference compared with the reference TMS, was used to calculate the molar susceptibility ($\chi^m_{\text{para}}$; see Appendix J).\textsuperscript{153,154}
CHAPTER 4
RUTHENIUM DIMERS AS DUAL TARGETS OF DNA TOPOISOMERASES

4.1 Introduction

Topoisomerases are important class of enzymes, which are responsible for catalyzing a number of conformational and topological changes in DNA.\textsuperscript{164-169} As shown in Figure 4.1, a loop of DNA can exist in a number of topoisomeric forms with the simplest form being circular DNA, which is considered to be 'relaxed' with little to no torsional stress. On the other hand, the DNA can be twisted or wound such that to relieve torsional stress the DNA loop coils up to form so called 'supercoiled' DNA. Supercoiling is found in natural plasmid DNA and is a common way for cells to store the DNA in a compact form.

From the mathematical treatment of this topological problem, where DNA is viewed as a circular ribbon, three parameters are used to describe supercoiling: the linking number ($L_k$), the twist ($T_w$) and the wrythe ($W_r$) and are related by equation 1.\textsuperscript{170}

\begin{equation}
L_k = T_w + W_r
\end{equation}

$L_k$ and $T_w$ are functions of the edge of the ribbon, and $W_r$ is a function of the ribbon (or helix) axis. Importantly, the extent of supercoiling can be expressed in terms of $L_k$, \textsuperscript{121}
which described the number of times the DNA crosses itself upon coiling. Furthermore, it is possible to separate topoisomers of DNA with different linking numbers (Lk) using agarose gel electrophoresis as shown in Figure 4.2, thus providing a convenient way for monitoring the function of topo I or II in screening assays.

![Figure 4.1](image)

Figure 4.1 Electron micrographs of the topoisomeric forms of plasmid DNA showing the transition from circular (relaxed) DNA to supercoiled DNA.\cite{171,172}

The winding or twisting of the DNA about the helical axis can either be with the natural twist of the helix, thus over winding the helix and is known as positive supercoiling or against the natural twist of the helix, thus under winding the helix and is known as negative supercoiling. Most supercoiled plasmid DNA is negatively supercoiled and incubation with either topoisomerase I or II typically leads to the observation of the relaxed or partially relaxed forms. It can be seen that topoisomerases help managing torsional stress, catenation, and knotting of the DNA within the cell. Drugs, which interfere with their action, are often lethal to the cell and thus they are attractive targets for cancer chemotherapy.
Based on the fundamental differences in their reaction mechanisms, DNA topoisomerases are broadly categorized into two groups, topoisomerase I (topo I) and topoisomerase II (topo II). A typical pathway by which both topo I and II function includes the following steps: 1) binding of the enzyme to double-stranded DNA, 2) the enzyme transiently breaks one (topo I) or both (topo II) phosphodiester strands and forms a transient adduct known as the “cleavable complex”, 3) the transient breaks allows either the passage of one (topo I) or both DNA strands (topo II) through the break(s), 4) religation of the phosphodiester bond(s) of the DNA and 5) dissociation of the enzyme from the DNA. As mentioned, topo I and II differ in their breakage step (step 2 of the mechanism) with topo I (see Figure 4.3) inducing transient ss breaks in the DNA backbone whereas topo II induces transient ds breaks. Topo I does not require ATP for the function. Topo II involves a transient complex, which has a four base pair stagger between the cuts and then requires ATP for the relegation of the phosphodiester linkages.
Figure 4.3 A Proposed reaction mechanism for mammalian DNA topo I.\textsuperscript{173}
Not surprisingly, DNA topoisomerases have been identified as the primary molecular target for a number of clinically prescribed antitumor drugs. The drugs predominantly function by either acting as inhibitors of the enzyme function (topo suppressors) or by freezing the activity of the topoisomerases at a particular step of the reaction. Agents that freeze the enzymes such that the DNA is not religated (by stabilizing the 'cleavable complex') often lead to high levels of DNA strand breaks. These agents are referred to as topoisomerase poisons (topo poisons), as opposed to the suppressors (inhibitors), which simply slow the rate of enzyme activity.

Most topo poisons are intercalators. The chemical structures of some of the most widely used topo I poisons; camptothecin, topotecan [(s)-9-dimethylaminomethyl-10-hydroxycamptothecin hydrochloride], irinotecan / CPT-11 (7-ethyl-10[4-(piperidino)-1-piperidino] carboxyloxy-camptothecin), are shown in Figure 4.4, while Figure 4.5 shows the structures of some well known topo II poisons, including m-AMSA, adriamycin, ellipticine, and etoposide, which are used clinically to treat many different types of cancers.
Figure 4.4 Chemical structures of some topo I poisons, camptothecin, topotecan and CPT-11.
Figure 4.5 Chemical structures of some topo II poisons, **m-AMSA**, adriamycin, etoposide, ellipticine and mitosantrone.
A number of known and widely-used topo suppressors, TAS 103,^{196,197} XR5000,^{198,199} intoplicine,^{200} F11782 (Tafluposide),^{201,202} ICRF193^{203} are shown in Figure 4.6. They are also commonly intercalating agents, but they are thought to prevent the binding of topoisomerase to DNA, instead of stabilizing the 'cleavable complex'. Most of the known topo suppressors work on both topo I and topo II and a number of these are particularly promising in overcoming tumors showing multi-drug resistance (MDR).^{204-207}
Figure 4.6 Chemical structures of some clinically developed topo inhibitors, Intoplicine, TAS-103, F 11782 and XR 5000.
Even though a large number of organic compounds have been established as topoisomerase targeting anticancer drugs, the application of transition metal complexes for this aspect is almost non-existent. Gopal and coworkers reported the inhibition of topo II by the ruthenium complexes: RuIm, RuInd and RuSAL (shown in Figure 4.7). These complexes proved effective antitumor agents in animal models, however, their clinical development has been slow due to toxic side effects in human body. Studies suggest that RuIm and RuInd are topo II poisons, whereas RuSAL acts as a topo II inhibitor.

Figure 4.7 Chemical structures of some metal containing topo poisons and inhibitors.
4.1.1. Identification of Catalytic Activity of Topoisomerases by Agarose Gel Electrophoresis

The activity of topo I or II is readily followed by incubation with supercoiled plasmid DNA and following its conversion to more relaxed forms by gel electrophoresis. As shown in Figure 4.8, topo I induces relaxation of supercoiled pUC18 DNA. The action of potential topo suppressors and poisons can thus be examined by performing this assay in the presence and absence of the potential drug.

![Diagram of agarose gel electrophoresis](image)

Figure 4.8 An agarose gel picture screening, Lane 1, the original supercoiled DNA (pUC18); Lane 2, Relaxation of pUC18 supercoiled DNA by topo I; Lane M is the marker of pUC18 consisting three isomers, supercoiled DNA, circular DNA and linear DNA.

4.1.2. CPT is a Known Topo I Targeting Anticancer Drug

CPT is a water soluble derivative of camptothecin and is approved for the treatment of refractory colorectal cancer. It has been shown that its antitumor activity is correlated with its ability to stimulate topo I but not topo II mediated DNA
cleavage \textit{in vitro}. Other than stimulating topo I mediated cleavages (topo poisoning), \textbf{CPT} is also able to inhibit the catalytic activity of topo I (topo I suppressor).\textsuperscript{216}

Wang \textit{et al.} has tested the ability of \textbf{CPT} to inhibit the catalytic activity of purified human topo I \textit{in vitro} by measuring the relaxation of supercoiled (SC) plasmid DNA (pRYG) substrate in a topo I specific reaction buffer (Figure 4.9).\textsuperscript{217} Lane 1 represents the fully relaxation of pRYG plasmid DNA by 1 unit of topo I, which is identified by the complete conversion of SC band to relaxed forms (R). Lane 2-5, in which the similar relaxation assays carried out in the presence of increasing concentration of \textbf{CPT} confirm that the inhibitory effect of topo I by \textbf{CPT} is concentration dependent. \textbf{CPT} is ineffective in inhibiting the catalytic activity of topo I at 2 \textmu M (Lane 2), but increasing concentration from 10-250 \textmu M \textbf{CPT} (Lane 3-5) retrieves inhibition of relaxation of topo I that are associated with the decrease of amount of the relaxed forms (R) and concomitant increase of the intensity of original supercoiled band (SC). However, even the concentration as high as 250 \textmu M of \textbf{CPT}, the inhibition is not completed and would be able to inhibit \textasciitilde 50\% of catalytic activity (Lane 5). In this study Lane 6 was included to demonstrate that \textbf{CPT} itself has no effect towards pRYG plasmid DNA.
Figure 4.9 Concentration dependent inhibition of topo I catalytic activity by 2-250 µM (Lane 2-5) of CPT. R: relaxed pRYG DNA marker; SC: supercoiled pRYG DNA substrate.

4.1.3. m-AMSA is a Known Topo II Targeting Anticancer Drug

m-AMSA is a DNA intercalator and specifically target topo II for anticancer activity. The Figure 4.10 shows the topo II relaxation assays, which Wang et al. have carried out with the m-AMSA. At low concentrations, m-AMSA acts as a topo II poison through stabilizing the topo II mediated “cleavable complex” formation (topo II poison), but at high concentrations, it starts to inhibit the catalytic activity of topo II (topo II suppressor). Similar to CPT, inhibition of catalytic activity of topo II by m-AMSA is also concentration dependent. Raising the concentration of m-AMSA, 6.4-250 µM (Lane 2-6), produces decrease in intensity of relaxed bands and increase in intensity of original supercoiled band confirming the inhibition catalytic activity of topo II.
Table 4.10 Concentration dependent inhibition of topo II catalytic activity by 6.4-250 μM (Lane 2-6) m-AMSA. L: linear DNA marker; R: relaxes pYRG DNA marker; SC: supercoiled pYRG plasmid DNA substrate. 

As described in Chapter 2, there are a number of cationic ruthenium polypyridyl complexes, which tightly bind to DNA via intercalation. One of these complexes ($P_p^{4+}$) even shows promise as an antitumor agent in a mouse tumor model study. Chapter 3 explored the DNA cleaving ability of these ruthenium complexes from which it was discovered that one complex, $P_p^{4+}$, does induce DNA single and double strand breaks in the presence of GSH or other suitable reductants. It is possible that this DNA cleaving activity is responsible for the observed antitumor activity in mice; however, it is also possible that the antitumor activity is related to inhibition of topoisomerase. We have used DNA relaxation assays as described in materials and methods to investigate the ability of the cationic ruthenium polypyridyl complexes shown in Figure 4.11, to inhibit topo I and II. As will be seen, complex $P_p^{4+}$ again stands out for its unusual activity compared to the rest of the structurally related complexes and more detailed studies of its activity of inhibition of topo I and II were performed. For comparison, many of the
studies included the known topo I inhibitor, CPT\textsuperscript{216,218} and the topo II inhibitor, m-AMSA as positive controls.

4.2 Results and Discussion

4.2.1. Evaluation of the Inhibitory Properties of Cationic Ruthenium Polypyridyl Complexes on Topo I

All of the complexes shown in Figure 4.11 were studied as inhibitors of topo I or/and II using the supercoiled plasmid DNA relaxation assay previously described.\textsuperscript{217,211,219} We screened a range of complexes exhibiting dramatically different DNA binding constants and modes of interactions. \([\text{Ru(phen)}_3]^{2+} (13^{2+})\) and \([(\text{phen})_2\text{Ru(bpm)}\text{Ru(phen)}_2]^{4+} (14^{4+})\) bind via electrostatic forces with the tetracation binding more avidly, because of the increased charge.\textsuperscript{51} The remaining three complexes all bind by intercalation, however, electrostatics also plays an important role. The monometallic complexes, \([\text{Ru(phen)}_2\text{dppz}]^{2+} (3^{2+})\textsuperscript{55}\) and \(\text{P}_1^{2+}\) have binding constants on the order of \(10^5\) to \(10^7\) M\(^{-1}\) whereas the dimers, \(\text{P}_p^{4+}\) and \(\text{Q}_p^{4+}\), bind considerably more tightly at \(2.4 \times 10^9\) and \(3.4 \times 10^8\) M\(^{-1}\), respectively.
The results of the inhibition of topo I by these complexes, are measured by the pUC18 DNA relaxation assay, as shown in Figure 4.12. Out of all, $P_p^{4+}$ and $Q_p^{4+}$
decrease the intensity of topo I mediated relaxed forms and increase the intensity of original supercoiled DNA (Form I in Figure 4.12) more efficiently than all the other ruthenium complexes as well than the known topo I inhibitor, CPT demonstrating potent inhibition of catalytic activity of topo I by $P_{p}^{4+}$ and $Q_{p}^{4+}$. Under the similar experimental conditions the short bridging dimer, $14^{4+}$ (Lane 6), and the monomer, $13^{2+}$ (Lane 9) as well as $P_{1}^{2+}$ (Lane 7) and the known monomeric intercalator, $3^{2+}$ (Lane 8) did not inhibit the relaxation activity of topo I. Taken together, the results show that $P_{p}^{4+}$ and $Q_{p}^{4+}$ are potent inhibitors of topo I and their activity is presumably a function of the tight binding and intercalation via the extended tatpp or tatpq bridging ligand and the high charge (4+).
Figure 4.12 The DNA topo I relaxation assay was carried out in the presence of different ruthenium complexes. Supercoiled plasmid DNA, pUC18 (Lane 1, 31 µM DNA bp) was incubated with topo I (Lane 2, 5 units) in the presence of 100 µM CPT, (Lane 3, 1.6 DNA bp/CPT), 1.5 µM P_p^{4+} (Lane 4, 20 DNA bp/P_p^{4+}), 1.5 µM Q_p^{4+} (Lane 5, 20 DNA bp/Q_p^{4+}), 1.5 µM 14^{2+}, (Lane 6, 20 DNA bp/14^{2+}), 1.5 µM P_1^{2+} (Lane 7, 20 DNA bp/P_1^{2+}), 1.5 µM 3^{2+} (Lane 8, 20 DNA bp/3^{2+}) and 1.5 µM 13^{2+} (Lane 9, 20 DNA bp/13^{2+}). Lane M in this figure is the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA.
4.2.2. Evaluation of the Inhibitory Properties of Cationic Ruthenium Polypyridyl Complexes on Topo II

We have also screened different types of ruthenium complexes as shown in Figure 4.11 against topo II relaxation assay to identify their ability to inhibit the catalytic activity of topo II. In this study, the complete relaxation of pUC18 DNA was achieved using 5 units of calf thymus topo II (Lane 2). Inhibition of catalytic activity of topo II was monitored using 1.2 μM of different ruthenium complexes. In this assay, the electrostatic binders, \(13^{2+}\) and \(14^{4+}\) as well as monomeric intercalators, \(P_1^{2+}\) and \(3^{2+}\) are inactive towards inhibiting the catalytic activity of topo II. Again the two most promising complexes are the dimers \(P_p^{4+}\) and \(Q_p^{4+}\) (Lanes 4 and 5), which are able to completely inhibit the catalytic activity of topo II. Lack of inhibition of topo II relaxation by \(P_1^{2+}\) and \(14^{4+}\) confirm the involvement of the planar extended bridging ligand and the charge for the inhibition of the catalytic activity of topo II. The DNA relaxation assays performed with identical concentration of standard topo II inhibitor, \(m\text{-AMSA}\) (Lane 3) was not able to inhibit the catalytic activity, demonstrating that \(P_p^{4+}\) and \(Q_p^{4+}\) are able to inhibit the catalytic activity of the enzyme even at low concentrations, in which \(m\text{-AMSA}\) is inactive.
Figure 4.13 The DNA topo II relaxation assay was carried out in the presence of different ruthenium complexes. Supercoiled plasmid DNA, pUC18 (Lane 1, 23 μM DNA bp) was incubated with topo II (Lane 2, 5 units) in the presence of 1.2 μM m-AMSA, (Lane 3, 20 DNA bp/m-AMSA), 1.2 μM P\textsuperscript{4+} (Lane 4, 20 DNA bp/P\textsuperscript{4+}), 1.2 μM Q\textsuperscript{4+} (Lane 5, 20 DNA bp/Q\textsuperscript{4+}), 1.2 μM 14\textsuperscript{4+} (Lane 6, 20 DNA bp/14\textsuperscript{4+}), 1.2 μM P\textsubscript{1}\textsuperscript{2+} (Lane 7, 20 DNA bp/P\textsubscript{1}\textsuperscript{2+}), 1.2 μM 3\textsuperscript{2+} (Lane 8, 20 DNA bp/3\textsuperscript{2+}) and 1.2 μM 13\textsuperscript{2+} (Lane 9, 20 DNA bp/13\textsuperscript{2+}). Lane M in this figure is the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA.
4.2.3. Concentration Dependence Study of Topo I Inhibition by $P_{p}^{4+}$ and $Q_{p}^{4+}$

A concentration dependent study of the inhibition of catalytic activity of topo I by $P_{p}^{4+}$ and $Q_{p}^{4+}$ was conducted as shown in Figure 4.14. The standard topo I inhibitor CPT (100 µM) was used in parallel as a positive control (Lane 3). Under these experimental conditions, 5 units of topo I was required to relax supercoiled pUC18 DNA. As seen, both $P_{p}^{4+}$ and $Q_{p}^{4+}$ show complete inhibition of topo I mediated relaxation (Lanes 4 and 7, respectively) at 3 µM, wherein it is far below that of CPT (100 µM). Even at

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<tr>
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<th>CPT (100 µM)</th>
<th>Topo I (5 units)</th>
<th>$P_{p}^{4+}$ (µM)</th>
<th>$Q_{p}^{4+}$ (µM)</th>
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Figure 4.14 Inhibition of catalytic activity of topo I by two ruthenium dimers, $P_{p}^{4+}$ and $Q_{p}^{4+}$. Supercoiled plasmid DNA, pUC18 (Lane 1, 31 µM DNA bp) was incubated with topo I (Lane 2, 5 units) in the presence of 100 µM standard topo I inhibitor CPT (Lane 3, 1.6 DNA bp/CPT) and 3.1 (Lane 4, 10 DNA bp/$P_{p}^{4+}$), 1.5 (Lane 5, 20 DNA bp/$P_{p}^{4+}$) and 0.6 µM (Lane 6, 50 DNA bp/$P_{p}^{4+}$) of $P_{p}^{4+}$ and the same concentration of $Q_{p}^{4+}$ (Lane 7-9). Lane M in this figure is the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA.
concentrations as low as 1.5 µM, the activity of topo I is completely inhibited by both of these complexes. Titration down to 0.5 µM of the complexes was required to see formation of some relaxed plasmid DNA and was comparable to the activity seen with 100 µM CPT.

4.2.4. Concentration Dependence Study of Topo II Inhibition by $P_p^{4+}$ and $Q_p^{4+}$

A concentration dependent study of the inhibitory action of $P_p^{4+}$ and $Q_p^{4+}$ on topo II was conducted as shown in Figure 4.15. In this experiment, m-AMSA (50 µM) was used in parallel as a positive control (Lane 3). As before, inhibition of catalytic activity of topo II (5 units) was evaluated by comparing the decrease intensity of relaxed forms and increase intensity of original supercoiled DNA. As with the topo I results, both $P_p^{4+}$ and $Q_p^{4+}$ inhibit the catalytic activity of topo II in a concentration dependent manner. Again the ruthenium complexes are far more effective even with the concentration as low as 0.6 µM of $P_p^{4+}$ (Lane 6) or $Q_p^{4+}$ (Lane 9) in which the catalytic activity of topo II is still partially inhibited, indicating the potent inhibition of topo II enzyme.
Figure 4.15 The DNA topo II relaxation assay was carried out in the presence of two ruthenium dimers, \( \text{P}_p^{4+} \) and \( \text{Q}_p^{4+} \). Supercoiled plasmid DNA, pUC18 (Lane 1, 23 µM DNA bp) was incubated with topo II (Lane 2, 5 units) in the presence of 50 µM standard topo II inhibitor \( \text{m-AMSA} \) (Lane 3, 0.46 DNA bp/\text{m-AMSA}) and 2.3 (10 DNA bp/\( \text{P}_p^{4+} \)), 1.15 (20 DNA bp/\( \text{P}_p^{4+} \)) and 0.46 µM (50 DNA bp/\( \text{P}_p^{4+} \)) of \( \text{P}_p^{4+} \) and the same concentration of \( \text{Q}_p^{4+} \) (Lane 7-9). Lane M in this figure is the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA.

### 4.2.5. Concentration Dependent Inhibition of Catalytic Activity of Topo II by \( \text{P}_p^{4+} \)

Out of all, only \( \text{P}_p^{4+} \) expressed the promising results against treating melanoma cancer in mice. Hence inhibition of topo II activity by \( \text{P}_p^{4+} \) was tested for wide range of concentration (2.3 – 0.11 µM). According to the data it is clear that inhibitory effect by \( \text{P}_p^{4+} \) primarily depends on the concentration of \( \text{P}_p^{4+} \) (see Figure 4.16). \( \text{P}_p^{4+} \) is ineffective at 0.11 µM (Lane 8), but increasing the concentrations of \( \text{P}_p^{4+} \), 0.23-2.3 µM (Lane 3-7)
result in gradual decrease in intensity of relaxed forms, followed by concomitant increase of intensity of original supercoiled bands demonstrating the inhibition of catalytic activity of topo II is concentration dependent.

**Topo II (5 units)**

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<tr>
<th>Pp$^{4+}$ (µM)</th>
<th>1</th>
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\[ Pp^{4+} (\mu M) \]

Figure 4.16 Inhibitory effect of Pp$^{4+}$ on topo II. Lane M the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA; Lane 1, 23 µM pUC18 DNA substrate; Lane 2, same as Lane 1 except that the 5 units of enzyme was used; Lane 3-7, same as Lane 2 except that the concentration of Pp$^{4+}$ was decreased to 2.3 (10 DNA bp/Pp$^{4+}$), 1.15 (20 DNA bp/Pp$^{4+}$), 0.46 (50 DNA bp/Pp$^{4+}$), 0.23 (100 DNA bp/Pp$^{4+}$) and 0.11 (200 DNA bp/Pp$^{4+}$) µM respectively.

4.2.6. Inhibition of the Catalytic Activity of Topo II by Pp$^{4+}$ Initiates Through Intercalating with the DNA

An alternative, experiment was carried out to confirm whether the inhibition of topo II activity by Pp$^{4+}$ is due to tighter binding of Pp$^{4+}$ with the DNA or with the topo II enzyme. In this relaxation assay, a constant amount of Pp$^{4+}$ and the DNA substrate were incubated with the increasing concentration of topo II and the changes in inhibition pattern were observed by agarose gel electrophoresis.$^{220}$ As seen in the Figure 4.17, inhibition of the topo II mediated relaxation of the pUC18 DNA substrate by 3
µM of $P_p^{4+}$ was not released with the increasing amount of the enzyme (Lane 3-7). Enzyme activity was raised by 8 times from the required activity and still did not regain the catalytic activity. This experiment clearly shows that the inhibition of catalytic activity of topo II is mainly mediated through the intercalation of $P_p^{4+}$ with DNA, but not with the enzyme.

![Image](image.png)

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<tr>
<td>Topo II (units)</td>
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<td>5</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>$P_p^{4+}$ (3 µM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
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Figure 4.17 Effect of enzyme concentration on inhibition of topo II mediated catalytic activity by $P_p^{4+}$. The DNA topo II relaxation assay was carried out in the presence of increasing concentration of the topo II enzyme and a constant concentration of $P_p^{4+}$, 3 µM (10 DNA bp/$P_p^{4+}$). Lane M is the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA; Lane 1, no drug, 23 µM base pairs pUC18 supercoiled DNA; Lane 2, no drug, pUC18 DNA and 5 units of topo II enzyme; Lane 3, same as Lane 2 but 2.3 µM of $P_p^{4+}$ was added; Lane 4-7, same as Lane 3 except that the amount of enzyme was increased to 10, 20, 30 and 40 units respectively.

Time-resolved electrophoretic analysis done for chiral forms of $P_p^{4+}$ ($\Delta\Delta$ & $\Lambda\Lambda$ forms) clearly revealed that both the enantiomeric forms of $P_p^{4+}$ bind to DNA by intercalation. This is further supported by spectroscopic binding studies of $P_p^{4+}$ carried out in chapter 2 ($K_b = \sim 10^9$ M$^{-1}$). Therefore, similar to ethidium bromide, intercalation of tight binding $P_p^{4+}$ to DNA may cause unwinding of the DNA helix.
resulting in freezing the ability of recognizing the activity site of DNA by topo II for catalytic activity. 195,222-225

4.2.7. Inhibition of Topo II by $P_1^{2+}$

Other than $P_p^{4+}$ its monomer $P_1^{2+}$ was also subjected to concentration dependent topo II assay to monitor its potential of inhibition of topo II enzyme (see Figure 4.18). Unlike $P_p^{4+}$, $P_1^{2+}$ did not show potent inhibitory effect. At 2.3 µM of $P_1^{2+}$ we find the partial inhibition of topo II activity where it with $P_p^{4+}$ would be complete inhibition (see Figure 4.16). Therefore the data clearly illustrate that removal of one ruthenium center from $P^{4+}$ dimer clearly diminishes the topo II inhibition.

![Figure 4.18 Inhibitory effect of $P_1^{2+}$ on topo II. Lane M the marker, which includes supercoiled (Form I), circular (Form II) and linear (Form III) DNA; Lane 1, 23 µM pUC18 DNA substrate; Lane 2, same as Lane 1 except with the 5 units of topo II enzyme; Lane 3-7, same as Lane 2 except that the concentration of $P_1^{2+}$ was introduced into the medium as 2.3 (Lane3, 10 DNA bp/$P_1^{2+}$), 1.2 (Lane 4, 20 DNA bp/$P_1^{2+}$), 0.46 (Lane 5, 50 DNA bp/$P_1^{2+}$), 0.23 (Lane 6, 100 DNA bp/$P_1^{2+}$) and 0.11 µM (Lane 7, 200 DNA bp/$P_1^{2+}$), respectively.]
4.2.8. Inhibition of Catalytic Activity of Topo II by $[\text{Ru(phen)}_2\text{dppz}]^{2+}$

$[\text{Ru(phen)}_2\text{dppz}]^{2+}$ ($3^{2+}$) is an extensively studied DNA intercalator, which binds tightly to DNA (Equilibrium binding constant, $K_b \sim 10^7$ M$^{-1}$). Based on viscosity measurements,$^61$ linear dicroism,$^{55,75}$ fluorescence energy transfer experiment,$^61$ unwinding assays$^58$ etc., intercalative binding mode is suggested for $3^{2+}$. Inhibitory effect of topo II relaxation by $3^{2+}$ was monitored with the increasing concentration of this complex. Similar to $\text{P}_p^{4+}$, $3^{2+}$ also inhibit the catalytic activity of topo II in a concentration dependant manner. At 2.3 $\mu$M of $3^{2+}$, the catalytic activity of topo II is completely inhibited comparable to $\text{P}_p^{4+}$. However, reducing the concentration of $3^{2+}$ by half (1.15 $\mu$M) results in reappearing the catalytic activity of topo II where as with $\text{P}_p^{4+}$ it is still inhibited, showing the enhanced activity in inhibiting the topo II by $\text{P}_p^{4+}$ than $3^{2+}$. In contrast, comparing the data of topo II inhibition by two intercalators, $3^{2+}$ (Figure 4.18) and $\text{P}_l^{2+}$ at 2.3 $\mu$M (Figure 4.17), show that $3^{2+}$ is a better inhibitor than $\text{P}_l^{2+}$. Taken together, the results confirm that extending the planar bridging ligand from dppz to tatpp (see Figure 4.10 for structures) reduces the potential of inhibiting catalytic activity of topo II.
| 72+ (µM) | - | - | 2.3 | 1.2 | 0.46 | 0.23 | 0.12 |
| Topo II (5 units) | - | + | + | + | + | + | + |

Figure 4.19 Inhibitory effect of [Ru(phen)$_2$dppz]$^{2+}$ (32+) on topo II. Lane M is the marker, which includes supercoiled (form I), circular (form II) and linear (form III) DNA; Lane 1, 23 µM pUC18 DNA substrate; Lane 2, same as Lane 1 with the 5 units of topo II enzyme; Lane 3-7, same as Lane 2 except that the concentration of 32+ was introduced as 2.3 (10 DNA bp/32+), 1.2 (20 DNA bp/32+), 0.46 (50 DNA bp/32+), 0.23 (100 DNA bp/32+) and 0.11 µM (200 DNA bp/32+) respectively.

### 4.3 Summary and Conclusions

The expression of topo I does not vary significantly during the cell cycle, whereas topo II expression increases in response to DNA replication and reaches a peak at G$_2$/M phase. Since the mechanisms and expression of topo I and II differ within the cell cycle, inhibitors of both enzymes (dual inhibitors) have been shown to have significant therapeutic advantages over agents targeting one type of topoisomerase enzyme to overcoming MDR.

As mentioned earlier in this chapter, several dual inhibitors of topo I and II have been identified; XR5000 (DACA), TAS103, intoplicin, XR11576, etc. (see Figure 4.5), which are in clinical evaluation. The advantage of these type of dual inhibitors is that they are able not only to target both enzymes simultaneously, which
controls the topology of DNA, but are also able to overcome MDR that associated with
the topo I or II targeting drugs.

Understanding the possible mechanisms for the observed cytotoxicity of $P_p^{4+}$
and $Q_p^{4+}$ against NSCLC cell lines *in vitro* as well as the activity of $P_p^{4+}$ against mice
melanoma *in vivo* were the main motivations of this investigation.

Because of $P_p^{4+}$ and $Q_p^{4+}$ bind tightly to DNA ($K_b = \sim 10^8-10^9 \text{ M}^{-1}$) through
intercalation,$^{221}$ a series of topo I and II relaxation assays were carried out to explore the
tight DNA binding towards topoisomerases activity.

Concentration dependent topo inhibition assays carried out in this chapter
clearly show that both $P_p^{4+}$ and $Q_p^{4+}$ are potent inhibitors of catalytic activity of topo I
as well as topo II enzymes in a concentration dependent manner. Further, the parallel
studies done with standard topo I inhibitor, CPT and topo II inhibitor, m-AMSA clearly
show that $P_p^{4+}$ and $Q_p^{4+}$ are more efficient dual inhibitors of topo I and II than either of
them.

$P_p^{4+}$ and $Q_p^{4+}$ have high positive charge (4+), which involves in strong
electrostatic binding to anionic backbone of DNA. To confirm whether this high charge
primarily involved in inhibiting the enzyme, parallel assays were carried out with a
similar charge dimer, $[(\text{phen})_2\text{Ru(bpm)Ru(phen)}_2]^{4+}$ (144+) with a short bridging ligand
(bpm). Lack of inhibition of topo I or topo II by 144+, specifies the importance of planar
extended aromatic bridging ligand (tatpp or tatpq) for the inhibition of the catalytic
activity of the enzyme.
Other than the dimer version, $P_{p}^{4+}$, we have accomplished in synthesizing the monomer version, $P_{t}^{2+}$, which has the extended bridging ligand, tatpp, but with one ruthenium center. Assays done with both topo II, clearly demonstrate that $P_{t}^{2+}$ is not as efficient as $P_{p}^{4+}$. Additional experiments done with the known DNA intercalators, $3^{2+}$, $P_{t}^{2+}$ and $P_{p}^{4+}$ demonstrate the effect of extending the planar bridging ligand from dppz to tatpp without raising the charge reduces the inhibiting activity of the enzyme. These results also confirm that the raising the charge along ($2^{+}$ to $4^{+}$) with the extending the planar ligand (dppz to tatpp), for instance $P_{t}^{2+}$ to $P_{p}^{4+}$ potentiate the enzyme inhibiting activity much more efficiently than changing one parameter per instance.

Even though $P_{p}^{4+}$ is a known intercalator, an extra experiment was conducted with a topo enzyme (topo II) to determine whether, the inhibition of the enzyme by $P_{p}^{4+}$ would be initiated through intercalation of $P_{p}^{4+}$ into DNA or interaction of $P_{p}^{4+}$ with the enzyme. Under these conditions, the amount of enzyme was raised while keeping the DNA substrate and the drug ($P_{p}^{4+}$) constant. Inability to regain the catalytic activity of the enzyme even after 8-fold excess, confirms that strong binding of $P_{p}^{4+}$ to DNA, prevents interaction of the enzyme with the DNA for the catalytic activity.

Shortage of many specific topo I or topo II targeting drugs are mainly due to their inability to overcome the MDR. However, recently established dual inhibitors of catalytic activity of topo I and II such as XR 5000,$^{198}$ XR11576,$^{230}$ etc., (see Figure 4.5 for their structures), which are in clinical evaluation, are able to circumvent MDR. Hence the dual inhibition of topo I and II by $P_{p}^{4+}$ is a very promising path in the discovery of drugs to avoid MDR.
4.4 Materials and Methods

4.4.1. General

Human Topo I and calf thymus Topo II were purchased from USB Corporation, Topo I inhibitor, CPT and topo II inhibitor, m-AMSA were purchased from TopoGEN Inc., Supercoiled plasmid DNA, pUC18 was purchased from Byou Biolab, ATP, Sodium dodecyl sulfate, Tris base, EDTA, spermidine, boric acid, agarose, glycerol, were purchased from Sigma-Aldrich, Bromophenol blue, ethidium bromide, Bovine serum albumin (BSA), bpm were purchased from Lancaster.

4.4.2. Synthesis

The dimers, [(phen)$_2$Ru(tatpp)Ru(phen)$_2$]$^{4+}$ ($P_p^{4+}$)$_{108}$ and [(phen)$_2$Ru(tatpq)Ru(phen)$_2$]$^{4+}$ ($Q_p^{4+}$)$_{108}$ and [Ru(phen)$_2$tatpp]$^{2+}$ ($P_1^{2+}$)$_{108}$ were synthesized according to the literature procedures developed in this lab. [Ru(phen)$_3$]$^{2+}$, [Ru(phen)$_2$dppz]$^{2+55}$ and [(phen)$_2$Ru(bpm)Ru(phen)$_2$]$^{4+231}$ were prepared as described elsewhere.

4.4.3. Topo I Inhibition Assay

A typical assay for examining the inhibition of topo I by drug molecules involved examination of the relaxation of supercoiled plasmid DNA by the enzyme in the presence and absence of the drug. The DNA products were separated and visualized using standard gel electrophoresis techniques. A typical assay was conducted as follows. A solution with of total volume of 25 uL was made up to contain 0.5 µg
supercoiled pUC18 DNA (31 µM in base pairs) and 1.5-15 µM $P_p^{4+}$ in topo I assay buffer (10 mM Tri-HCl (pH 7.9), 150 mM NaCl, 1 mM EDTA, 0.1% BSA, 0.1 mM Spemidine, and 5% glycerol). CPT (100 µM) was used as a positive control. Reactions were started by the addition of topo I (5 units; 5 units/µL) and incubated at 37 °C for 30 minutes. The reaction was quenched by addition of 5 µL of stop buffer (5% SDS, 0.05% bromophenol blue and 30% glycerol). The final DNA samples were loaded on 1% agarose gel (made up with the running buffer, 89 mM Tris-borate (pH 8), 2 mM EDTA) and subjected to electrophoresis at 5 V/cm for 4 hours in the running buffer. After electrophoresis, the gel was stained in an aqueous solution of ethidium bromide (10 µg/ mL) for 30 minutes and destained in water and photographed under ultraviolet illumination.

4.4.4. Topo II Inhibition Assay

A typical assay for examining the inhibition of topo II by drug molecules involved examination of the relaxation of supercoiled plasmid DNA by the enzyme in the presence and absence of the drug. The DNA products were separated and visualized using standard gel electrophoresis techniques. A typical assay was conducted as follows. A solution with a total volume of 20 uL was made up to contain 0.3 µg supercoiled pUC18 DNA (23 µM in base pairs) and 1.5-15 µM $P_p^{4+}$ in topo II assay buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl$_2$, 0.1 mM EDTA, 0.15 mg/ mL BSA, 1 mM ATP). 100 µM Amsacrine (m-AMSA) was used as a positive control. Reactions were started by the addition of topo II (5 units) in 5 units/uL.
After incubation at 37 °C for 30 minutes, the reactions were quenched by addition of 5 µL stop buffer (5% (W/V) SDS, 0.05% bromophenol blue, 30% glycerol). The final samples were loaded on a 1% horizontal agarose gel and it was subjected to electrophoresis at 5 V/ cm for four hours in the running buffer (100 mM Tris-borate, pH 8.3, 2 mM EDTA). After electrophoresis, the gel was stained in an aqueous solution of ethidium bromide (10 µg/ mL) and destained in water and photographed under ultraviolet illumination.
1H NMR, 13C NMR, COSY AND UV-VIS SPECRA OF [Ru(bpy)(dppz)(CO)2]2+
$^1$H NMR of [Ru(bpy)(dppz)(CO)$_2$]$^{2+}$ in $d_3$ MeCN
$500 \text{ MHz } ^1\text{H COSY NMR of } [\text{Ru}($\text{bpy}$)($\text{dppz}$)($\text{CO}$)$_2$]$^{2+}$ in $d_3$ MeCN
$^{13}$C NMR of $[\text{Ru(bpy)(dppz)}(\text{CO})_2]^{2+}$ in $d_3$ MeCN.
UV-Vis Spectrum of [Ru(bpy)(dppz)(CO)₂](PF₆)₂ in DMSO
APPENDIX B

$^1$H NMR, $^{13}$C NMR, COSY AND UV-VIS SPECRA OF [Ru(bpy)(dppz)(CH$_3$CN)$_2$]$^{2+}$
$^1$H NMR of [Ru(bpy)(dppz)(CH$_3$CN)$_2$]$^{2+}$ in $d_3$ MeCN
$^{13}$C NMR of [Ru(bpy)(dppz)(CH$_3$CN)$_2$]$^{2+}$ in d$_2$ MeCN
UV-Vis Spectrum of [Ru(bpy)(dppz)(CH$_3$CN)$_2$](PF$_6$)$_2$ in DMSO
APPENDIX C

$^1$H NMR, COSY, MASS AND UV-VIS SPECTRA OF [Ru(bpy)(dppz)(C$_2$O$_4$)]
$^1$H NMR of [Ru(bpy)(dppz)(C$_2$O$_4$)] in $d_6$ DMSO
500 MHz $^1$H COSY Spectrum of [Ru(bpy)(dppz)(C$_2$O$_4$)] in DMSO-$d_6$
UV-Vis Spectrum of [Ru(bpy)(dppz)(C$_2$O$_4$)] in DMSO
MS of [Ru(bpy)dppz(C₂O₄)] in MeOH
APPENDIX D

$^1$H NMR, $^{13}$C NMR, MASS AND UV-VIS SPECTRA OF [$\{\text{Ru(bpy)(CO)}_2\}_2\text{tpphz}\}^4^+$
$^1$H NMR of [(Ru(bpy)(CO)$_2$]tphz$]^{4+}$ in $d_3$ MeCN
$^{13}$C NMR of [(Ru(bpy)(CO)$_2$tpphz)]$^{4+}$ in $d_3$ MeCN
MALDI-TOF REFLECTRON

MS of \( \{\text{Ru(bpy)(CO)}_2\text{tpphz}\}^{4+} \) in MeCN
UV-Vis Spectrum of [{Ru(bpy)(CO)}₂tpphz](PF₆)₂ in DMSO.
APPENDIX E

$^1$H NMR, $^{13}$C NMR, MASS AND UV-VIS SPECTRA of 
$\left\{\text{Ru(bpy)(CH}_3\text{CN)}_2\text{tpphz}\right\}^{4+}$
$^1$H NMR of $\{(Ru(bpy)(CH_3CN)_2)tpphz\}^{4+}$ in $d_2$ MeCN
$^{13}$C NMR of $[\{\text{Ru(bpy)}(\text{CH}_3\text{CN})_2\}^2_{\text{tpplhz}}]^{4+}$ in $\text{d}_3$ MeCN
MALDI-TOF REFLECTRON

Method: R20394H
Mode: Reflector
Accelerating Voltage: 20000
Grid Voltage: 70.000 %
Guide Wire Voltage: 0.000 %
Delay: 150.00
Sample: 30

Counts

15000-

10000-

5000-

0-

500 1000 1500 2000 2500 3000 3500 4000
Mass (m/z)

MS of [{(Ru(bpy)(CH3CN)2tpphz}]4+ in MeCN
UV-Vis Spectrum of [{Ru(bpy)(CH$_3$CN)$_2$}$_2$tpphz](PF$_6$)$_2$ in DMSO.
APPENDIX F

MASS AND UV-VIS SPECTRA OF [{Ru(bpy)(C₂O₄)}₂tpphz]
UV-Vis Spectrum of [{Ru(bpy)(C$_2$O$_4$)$_2$tpphz}] in DMSO.
APPENDIX G

\[ ^1\text{H NMR, } ^{13}\text{C NMR, COSY AND UV-VIS SPECTRA OF} \]
\[ [\{\text{Ru(bpy)(CO)(Cl)}\}_2\text{tpphz}\}]^{2+} \]
$^1$H NMR of $\{\text{Ru(bpy)}(\text{CO})(\text{Cl})_2\text{pphZ}\}^{2+}$ in $d_3$ MeCN
$^{13}$C NMR of $\{\text{Ru(bpy)}(\text{CO})(\text{Cl})\}_2\text{tpphz}}^{2+}$ in $d_3$ MeCN
500 MHz $^1$H COSY Spectrum of [(Ru(bpy)(CO)(Cl)$_2$tpphz)]$^{2+}$ in CD$_3$CN
UV-Vis Spectrum of [{Ru(bpy)(CO)(Cl)}₂tpphz](PF₆)₂ in CH₃CN.
APPENDIX H

Crystal Structure of \([(\text{bpy})_2\text{Ru(tatpq)Ru(bpy)_2}]\text{Cl}_4\) \([Q_h]\text{Cl}_4\)
RU1-N1C = 2.04 Å
RU1-N2C = 2.05 Å
N1C-C1C = 1.33 Å
C1C-C2C = 1.38 Å
C2C-C3C = 1.50 Å
C3C-C4C = 1.41 Å
C4C-C5C = 1.35 Å
C5C-C6C = 1.42 Å
C6C-C7C = 1.45 Å
C7C-C8C = 1.45 Å
C8C-C9C = 1.53 Å
C9C-C10C = 1.42 Å
N2C-C6C = 1.32 Å
C7C-C11C = 1.39 Å
C4C-C17C = 1.46 Å
C17C-N4C = 1.35 Å
C11C-N3C = 1.41 Å
N3C-C13C = 1.37 Å
C13C-C14C = 1.51 Å
C15C-C14C = 1.40 Å
C14C-O1C = 1.20
APPENDIX I

$^{1}H$ NMR of $P_{p}^{4+}$, $P_{p}^{3+}$ and $H_{2}P_{p}^{4+}$
$^1$H NMR of \([P_F(P_F_6)_3]\) in $d_3$ MeCN
^1^H NMR of [H_2P]_4(Cl)_4 in d_2H_2O
$^1$H NMR of $[\text{H}_2\text{P}_4]\text{Cl}_4$ in DMSO-$d_6$
APPENDIX J

EVANS METHOD DATA OF $P_p^{4+}$, $P_p^{3+}$ and $H_2P_p^{4+}$
The shift of proton resonance line of an inert substance, such as (CH$_3$)$_4$Si (TMS) due to the presence of paramagnetic ions (unpaired electrons) is given by the following theoretical expression.

\[
\chi_{\text{para}}^{m} = \frac{3}{4\pi} \frac{\Delta f(T)MW \cdot g(\text{solv})}{f \cdot m \cdot d(T)} - \frac{\chi_v MW}{d(T)} - \chi_{\text{dia}} \text{ emu/mol}
\]

\(\chi_{\text{para}}^{m}\) = molar susceptibility of solute (emu/mol)

\(\Delta f(T)\) = difference in Hz between diamagnetic TMS (Trimethylsilane) and paramagneti TMS

\(f\) = frequency of spectrometer

\(MW\) = molecular weight of solute (paramagnetic) g/mol

\(m\) = mass of solute in 1 mL solution at room temperature (R.T.) (g)

\(g(\text{solv})\) = mass of 1.0 mL solvent at R.T.

\(d(T)\) = density of solvent at the temperature under study

\(\chi_v\) = volume susceptibility emu/mol

\(\chi_{\text{dia}}\) = diamagnetic susceptibility (Pascal constant, emu/mol)
The 500 MHz $^1$H NMR spectra of $P_p^{4+}$, $P_p^{3+}$ and $H_2P_p^{4+}$ in CD$_3$CN at 298 K with the spinner on. Only the proton peaks of (CH$_3$)$_4$Si (TMS) are shown.
The 500 MHz $^1$H NMR spectra of $\text{P}_p^{4+}$, $\text{P}_p^{3+}$ and $\text{H}_2\text{P}_p^{4+}$ in CD$_3$CN at 298K with the spinner off. Only the proton peaks of (CH$_3$)$_4$Si (TMS) are shown.
APPENDIX K

DNA CLEAVAGE BY $P_p^{4+}$ UNDER DIFFERENT ENVIRONMENTAL CONDITIONS
DNA cleavage by $P_{pp}^{4+}$ (0.128 mM), in the presence of varying amounts of GSH and different environmental conditions (aerobic and anaerobic). Reactions were incubated at 24 °C for 2 h in 4 mM Na$_3$PO$_4$, pH 7.0 containing 0.154 mM of supercoiled pUC18 DNA. Lane M, Marker consisting form I, II and III; Lane 1, pUC18 DNA alone, under aerobic condition; Lane 2, 0.128 mM $P_{pp}^{4+}$ alone, under anaerobic conditions; Lane 3, 0.641 mM GSH alone, under anaerobic condition; Lane 4, 0.128 mM $P_{pp}^{4+}$ + 0.256 mM GSH, under aerobic conditions; Lane 4’, same as Lane 4, under anaerobic conditions; Lane 5, 0.128 mM $P_{pp}^{4+}$ + 0.385 mM GSH, under aerobic conditions; Lane 5’, same as Lane 5, under anaerobic conditions; Lane 6, 0.128 mM $P_{pp}^{4+}$ + 0.513 mM GSH, under aerobic conditions; Lane 6’, same as Lane 6, under anaerobic conditions.
DNA cleavage by $P_{4+}$ (0.0128 mM) and $Q_{4+}$ (0.0128 mM) in the presence of GSH (0.256 mM) at different concentrations of salt ($Na_3PO_4$), under anaerobic conditions. Reactions were incubated at 24 $^\circ$C for 2 h at different buffer conditions, pH 7 containing 0.154 mM of supercoiled pUC18 DNA. Lane M, marker lane consisting three different isomers of pUC18, Form I, Form II and Form III; Lane 1, pUC18 + GSH (0.256 mM) in 7 mM $Na_3PO_4$ buffer, pH 7; Lane 2, $P_{4+}$ + GSH in deionized water; Lane 3, $P_{4+}$ + GSH in 4 mM $Na_3PO_4$ buffer, pH 7; Lane 4, $P_{4+}$ + GSH in 7 mM $Na_3PO_4$ buffer, pH 7; Lane 5, $Q_{4+}$ + GSH in deionized water; Lane 6, $Q_{4+}$ + GSH in 4 mM $Na_3PO_4$ buffer, pH 7; Lane 7, $Q_{4+}$ + GSH in 7 mM $Na_3PO_4$ buffer, pH 7.
Absorption spectra of $Z^{4+}$ and $HZ^{4+}$ ($Z^{4+} + \text{Co(Cp)}_2$) resulted with one equivalent Co(Cp)$_2$ and $H_2Z^{4+}$ ($Z^{4+} + \text{Co(Cp)}_2 + \text{EtOH}$) resulted with two equivalent Co(Cp)$_2$ in degassed water/acetonitrile.
Absorption spectra of $\text{HZ}^{4+}$ resulted with two equivalent $\text{Co(Cp)}_2$ in degassed acetonitrile and $\text{H}_2\text{Z}^{4+}$ resulted after the protonation of $\text{HZ}^{4+}$ in degassed EtOH/acetonitrile.
Absorption spectra of $Z^{4+}$, and $H_2Z^{4+}$ prepared with excess GSH (5 equivalent) in degassed water/acetonitrile.
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218


223


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