TOWARDS ESTABLISHING THE MECHANISM OF DNA CLEAVAGE BY REDOX-ACTIVE RUTHENIUM(II) POLYPYRIDYL COMPLEXES

by

CYNTHIA ANN GRIFFITH

Presented to the Faculty of the Graduate School of The University of Texas at Arlington in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN CHEMISTRY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2015
Acknowledgements

I would like to acknowledge my deepest gratitude to my advisor Dr. Frederick MacDonnell. My desire to achieve a Master's degree and the opportunity to discover my love for chemistry and research would never have been possible without the opportunity given to me by my greatest influence, Dr. Frederick MacDonnell. He took a chance on me and supported me against all odds so that I could achieve my goals in higher education. In addition to being an advisor he served as mentor, provided guidance not only in research but in life as well. He provided a working environment that allowed me to grow exponentially. Through his guidance I learned how to see past my own circumstances and maintain the necessary focus to complete my research and maintain a work life balance. In addition to Dr. MacDonnell, I would like to thank my committee members, Dr. Brad Pierce and Dr. Daniel Armstrong. They too contributed to my success at growth as a researcher and student. Furthermore all of committee members including my academic advisor, Dr. Kroll have shown me great patience and inspiration in the field of chemistry. There are no words to describe my gratitude and appreciation for their continued support.

I would like to also thank all of my lab mates over the years for their endless support and encouragement. A special thanks goes to Dr. Shreeyukta Singh and Steven Poteet. I am certain that without their faith in me, the rigor of completing my research would have been unbearable. Not only did they support me but they helped me greatly in the lab. My work could not have progressed as rapidly as it did without their help. In addition I would like to thank Kenneth Abayan, Nagham Alatrash, Joseph Aslan, Mahir Alrashdan, Pooja Ahuja, David Boston, Angie Dickinson, Kai-ling Huang, Eugenia Nahr, Matthew West and Yanling Chen for their help and support in the lab.

In addition, I would like to thank the dedication and support of the faculty and staff of the Chemistry and Biochemistry Department including, Debbie Cooke, Natalie
Croy, James Garner, and Jill Howard, for their friendship and assistance during these past years. They are all dedicated to the highest standards and success of all the students in the department. Due to their deepest desire to construct and maintain a department dedicated to the success of the students in academia as well as in industry, I am able to change not only my life but the lives of my family. Furthermore, I represent the opportunity this great university offers to all. I am a Hispanic single mother that has acquired a master’s degree at the age of 42. I have been given a second chance at life because of the dedication and support I received from family and this university.

Finally I would like to acknowledge the support and sacrifice my family has given and endured throughout this rigorous process. I would like to start with my children Elizabeth, Tanner, Anthony, Jessica, Michael, and Samantha Hennegan. I would also like to thank my father and stepmother, John and Kathy Griffith and my mother and stepfather, Guadalupe and Rocky Burnett. Last but not least I owe my greatest acknowledgment to my best friend, Matthew Noble. Had it not been for his continued support and faith in me I would not have been able to complete my research or this degree.

April 20, 2015
Abstract

TOWARDS ESTABLISHING THE MECHANISM OF DNA CLEAVAGE BY REDOX-ACTIVE RUTHENIUM(II) POLYPYRIDYL COMPLEXES

Cynthia Ann Griffith, M.S.

The University of Texas at Arlington, 2015

Supervising Professor: Frederick M. MacDonnell

In earlier reports, it was established that ruthenium (II) polypyridyl complexes, [Ru(tatpp)]^{2+} (3^{2+}) and [Ru(tatpp)Ru]^{4+} (4^{4+}) show potent antitumor properties in vivo in nude mouse lung cancer models. These RPC’s undergo in vitro reduction by glutathione (GSH) to form a species that induces DNA cleavage. It was also demonstrated that the mechanism of DNA cleavage follows an unusual dependence on dioxygen (O_2) concentration: the increase in cleavage activity of these RPC complexes are inversely proportional to O_2 concentration. In fact, cleavage is quenched in the complete absence of O_2.

In this work we currently postulate when 3^{2+} and 4^{4+} are singly reduced to species 3^{+} and 4^{3+}, which contains a radical anion localized on the tatpp ligand, abstracts a H atom from the deoxyribose moiety in DNA, leading to DNA cleavage. We describe our results from HPLC analysis of the scission products formed by the degradation of ctDNA and pUC 18 DNA, by carbon radical generated in vitro with 3^{2+} and 4^{4+}. We also show preliminary evidence suggesting that hydrolytic cleavage of the DNA backbone does not occur with 4^{4+} but does seem to occur with 3^{2+}. From this data we postulate the dual mode of action for DNA cleavage by 3^{2+} could possible explain the reason 3^{2+} has shown enhanced cleavage as compared to 4^{4+}. 
Table of Contents

Acknowledgements ........................................................................................................ iii

Abstract .......................................................................................................................... v

List of Illustrations ......................................................................................................... viii

List of Tables .................................................................................................................. ix

Chapter 1 Introduction to cancer and chemotherapy ...................................................... 1

1.1 Cancer ....................................................................................................................... 1

1.2 Chemotherapy .......................................................................................................... 1

1.3 cis-Diamminedichloroplatinum (II) (cisplatin) and its analogues ......................... 2

1.4 Ruthenium complexes ............................................................................................... 4

1.4.1 Ruthenium polypyridal complexes RPCs .............................................................. 4

1.4.2 Ruthenium chemotherapeutics with labile ligands .............................................. 5

1.4.3 Biological activity of RPC’s .................................................................................. 6

1.5 DNA cleavage activity of RPC’s .............................................................................. 8

1.5.1 Introduction to the DNA cleavage assay ............................................................... 8

1.6 Scope of the thesis .................................................................................................... 10

Chapter 2 Previous work done by the MacDonnell group exploring RPC chemistry ....... 11

2.1 Introduction ............................................................................................................... 11

2.2 Redox activity of RPCs ............................................................................................ 14

2.3 RPCs in the presence of radical scavengers ............................................................ 16

2.4 Sensitivity to dioxygen concentration ..................................................................... 20

2.5 Summary and conclusions ...................................................................................... 22

Chapter 3 Further elucidation of mechanistic pathway of $3^{2+}$ and $4^{4+}$ .................. 23

3.1 Introduction to known mechanisms of DNA cleavage ............................................ 23
List of Illustrations

Figure 1-1 Platinum based anticancer drugs .................................................. 3
Figure 1-2 Ruthenium complexes NAMI-A, KP1019 and RDC11 ...................... 6
Ruthenium cationic complexes ........................................................................ 7
Figure 1-4 Ruthenium complexes $3^{2+}$ and $4^{4+}$ ........................................... 8
Figure 1-5 Topoisomers of plasmid DNA: Form I, II and III .............................. 9
Figure 2.1 IC$_{50}$ of rac/mix- Ru(II) complexes- monomer vs. dimer .............. 12
Figure 2-2 IC$_{50}$ of Ru(II) complex in syngeneic mouse melanoma model: B16 ... 12
Figure 2-3 Tumor regression of NSCLC-H358 tumor cells ............................... 13
Figure 2-4 Redox protonation isomers of $3^{2+}$ .............................................. 14
Figure 2-5 Absorption spectra of $4^{4+}$ protonation states ............................... 15
Figure 2-6 DNA cleavage activity of $4^{4+}$ ..................................................... 16
Figure 2-7 DNA cleavage activity of $4^{4+}$ in presence of TEMPO .................. 18
Figure 2-8 DNA cleavage activity of $4^{4+}$ protonation states .......................... 19
Figure 2-9 Postulated mechanism of DNA cleavage by $[4]^{3+}$ species ............... 20
Figure 2-10 DNA cleavage activity of $4^{4+}$ under varying [O$_2$] ..................... 21
Figure 3-1 HPLC analysis of DNA scission products .................................... 30
Figure 3-2 T4 ligase assay with $3^{2+}$ and $4^{4+}$ ........................................... 32
Figure 3-3 Reduction potentials for GSH and RPCs of Interest ....................... 34
List of Tables

Table 2-1 Unique DNA scission product markers ................................................................. 24
Chapter 1

Introduction to Cancer and Chemotherapy

1.1 Cancer

Cancer is a group of approximately 100 diseases. Uncontrolled growth of abnormal (cancerous) cells and the ability of these cells to migrate from the original site of origin to a different organ system site (metastasis) are the two main characteristics of cancer. If metastasis occurs cancer may result in death. According to the National Vital Statistics (NVSS) by the Centers for Disease Control (CDC) on December 20, 2013, the top ten leading causes of death were reported for 2010 with 75% of the top ten causes accounting for all deaths in the United States. The report showed a total number of deaths at 2,596,993 at a rate of 821.5 deaths per 100,000 population with a life expectancy of 78.8 years. The leading cause of death was heart disease with 611,105 deaths. The second with 584,881 deaths was caused by malignant neoplasms (cancer or malignant tumor). All other causes were at or less than 149,205 deaths per remaining cause. Given the mortality rate of cancer much effort has been invested for drug development to treat cancer with minimal side effects.

1.2 Chemotherapy

Chemotherapy is the systemic treatment of cancer with the use of medicinal drugs. A chemotherapeutic agent is a general term used to identify drugs or molecular compounds used to treat cancer. The majority of these agents disrupt rapidly dividing cells that leads to cellular death or apoptosis. Chemotherapeutic agents can be classified into the following groups, alkylating agents, anti-tumor antibiotics, antimetabolites, topoisomerase inhibitors, mitotic inhibitors, corticosteroids and miscellaneous chemotherapy drugs. These classifications are based on factors such as
chemical structure, function and their relationship to other drugs. Furthermore, chemotherapeutic agents are not limited to any one group or classification. Transition metal based chemotherapeutic drugs have been used to treat cancer since the late 1970’s. More specific, cisplatin was approved for clinical use in 1978. However, platinum-based chemotherapeutics have limitations in low solubility, toxic side effects, and tumor resistance. Therefore, researchers have investigated other metal based chemotherapeutics that are able to overcome the side effects and potential resistance as seen with cisplatin and its derivatives. Considerations for alternative heavy metal complexes are the inherent toxicity of the metal complex(s) and the body’s ability to remove or prevent accumulation of metal ions.

Ruthenium polypyridal complexes (RPCs) have become up and coming drugs of interest due to their similar substitution kinetics of platinum based drugs and from extensive work by the Dwyer group showing these complexes do not accumulate in the body. In addition, RPC’s show different biological activity than cisplatin and its derivatives. This is significant as possible alternative treatments for tumors that develop resistance to cisplatin and its analogues.

1.3 cis-Diamminedichloroplatinum(II) (cisplatin) and its analogues
Cisplatin (cis-Pt(NH₃)₂Cl₂) (Figure 1-1), was discovered in 1965 by Barnett Rosenberg, Ph.D. at Michigan State University. It is best known for curing testicular cancer. Treatment of testicular cancer has decreased the death rate by two-thirds since 1975 relating to testicular cancer. In 2012 approximately 8,590 Americans were diagnosed with testicular cancer, of which 360 deaths were attributed to the diagnosis of testicular cancer. However, it is has also been known to be used but not limited to treating lung, bladder, cervical and ovarian cancers. Due to the highly toxic side effects of
cisplatin, analogues of cisplatin have been researched.\textsuperscript{9} Thousands of analogues have been researched for targeted cancer treatment and to combat drug resistance, but have not been FDA approved.\textsuperscript{16} The first successful FDA approved analogue, Carboplatin (cis-diammine(1,1-cyclobutanebicarboxylato)platinum (II)) (Figure 1-1) was approved in 1989 and is widely used because its effects are less toxic on patients. Oxaliplatin (trans-1,2-diamminocyclohexaneoxalaplatinum (II)) (Figure 1-1) is also FDA approved. It is used mostly for colon cancer.\textsuperscript{16, 17} Most recently JM-216 satraplatin (bis-(acetate)-ammine dichloro-(cyclohexylamine) platinum (IV)) (Figure 1-1) has been used in clinical trials and shows success in treatment of prostate cancer.\textsuperscript{17} It is unique to other platinum analogues this is due to its hydrophobic nature, allowing for greater solubility than other platinum drugs and can be taken orally by patients. It is not FDA approved but, is used in France and appears to show efficacy towards some cisplatin-resistant cell lines.\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{platinum_drugs.png}
\caption{Figure 1-1 Platinum based anticancer drugs.}
\end{figure}
Cisplatin and its derivatives, including satraplatin, are believed to be transported into the cell by the human copper transporter 1 (hCtr1) and copper efflux transporters ATP7A and ATP7B.\textsuperscript{18} Once inside the cell they are known to crosslink with DNA, form DNA adducts involving inter- and intra-strand crosslinks.\textsuperscript{17} These adducts ultimately disrupt DNA replication and transcription leading to cell cycle arrest and apoptosis.\textsuperscript{19} Although cisplatin and its analogs have shown tremendous success in treating cancer, it is well documented that many patients relapse and develop a resistance to treatment with platinum chemotherapeutics.\textsuperscript{18, 7, 20, 9} Recent studies have shown the copper transporter 2 (Ctr2) regulates Ctr1,\textsuperscript{20} the main transporter of platinum chemotherapeutics. This is significant in understanding and possibly reversing resistance to platinum based treatment. However, it could be many years before this technology is available to us. Therefore the need for alternative drugs with similar efficacy towards cisplatin resistant cell lines and less toxic side effects is being aggressively pursued.

1.4 Ruthenium complexes

1.4.1 Ruthenium polypyridal complexes RPCs

While research in metallopharmaceuticals continues to focus on platinum complexes, ruthenium polypyridyl or Ru(II) complexes (RPCs) have been explored as well, due to their interaction and or cleavage of DNA. In addition, RPC’s are studied for their stability, ease of synthesis, chiral properties and under physiological conditions has a range of oxidation states from Ru(II), Ru(III) and Ru(IV).\textsuperscript{21, 9, 22} The ability to undergo multiple oxidation states in physiological conditions was thought to be unique to platinum metals.\textsuperscript{22} Functionality of most metal based anticancer drugs is dependent upon their oxidation state thus making this feature quite significant. Moreover, they are of particular interest as their substitution kinetics are similar to that of platinum, while their side-effects
in vivo are often quite different. Meaning ruthenium therapeutics have been shown to be less toxic in animals with greater cellular cytotoxicity than cisplatin. Furthermore, it has been shown cellular uptake of some ruthenium based chemotherapeutics are dependent on the transferrin cycle and activation by reduction. It has been suggested the alternative pathway of cellular uptake of ruthenium anticancer drugs versus that of cisplatin is responsible for successful treatment against platinum resistant cell lines and the lower toxic effects. The promise of such compounds being their biological activity on tumors resistant to platinum-based drugs and potential applicability to a wider range of tumors with less severe toxicity relative to cisplatin. Most of the success to date with ruthenium derived compounds has been with complexes bearing one or more labile ligands which, like in cisplatin, allows the metal to directly bind with biological targets.

1.4.2 Ruthenium chemotherapeutics with labile ligands

The anti-tumor agents NAMI-A (Imidazolium [trans-imidazoliumdimethylsulfoxide-tetrachlororuthenate(III)], KP1019 (Indazolium [trans-tetrachlorobis(1H-indazole)ruthenate(III)]), RDC11 ([cis-bis(acetonitrile)-1,10-phenanthroline-2-phenylpyridineruthenium(II)] hexafluorophosphate), (Figure 1-2) and ruthenium-aryl-X complexes all contain labile ligands. The loss of these ligands and subsequent binding of the ruthenium to biological substrates is implicated in their biological activity. Interestingly NAMI-A failed primary cancer screening with an IC\textsubscript{60} panel, but seems to target secondary or metastatic cancerous cells while its counterpart shows promise towards primary cancer cell lines. It seems their activity towards cancerous cells is not dependent on DNA binding. Studies conducted to investigate their non-traditional behavior have allowed for a separation from classical to non-classical ruthenium-based
anticancer drugs. At this time NAMI-A and KP1019 are reported to have advanced to phase II clinical trials.

![Chemical structures of NAMI-A, KP1019, and RDC11](image)

**Figure 1-2** NAMI-A (Imidazolium [trans-imidazolidimethylsulfoxide-tetrachlororuthenate(III)], KP1019 (Indazolium [trans-tetrachlorobis(1H-indazole) rutherenate(III)]), RDC11 ([cis-bis(acetonitrile)-1,10-phenanthroline-2-phenylpyridineruthenium(II)])

### 1.4.3 Biological activity of RPCs

The biological activity of coordinatively saturated ruthenium (II) polypyridyl complexes RPCs, such as the trisphenanthroline complex (12+) and the trisbipyridine complex (22+) shown in Figure 1-3, was extensively studied by the groups of Dwyer and Schulman in the 1950’s and 60’s and even prior to that by Beccari in the late 1930’s. Since the discovery of the molecular-light switch behavior of [Ru(phen)₂dppz]²⁺ (Figure 1-3), there has been somewhat of a resurgence in this area recently with numerous studies exploring the DNA-binding activity of RPCs and to a lesser extent the cytotoxicity of RPCs,
These complexes differ from cisplatin and NAMI-A type complexes in that the metal ion is coordinatively saturated and unavailable to directly form bonds with biological targets. Instead, early studies with radiolabeled $^{106}$Ru(phen)$_3^{2+}$ showed that the intact complex cation was the bioactive unit, that this complex was not metabolized in vivo, did not accumulate in any organ, and nearly all the complex was recovered in the urine.\(^{31}\) Despite this lack of reactivity in vivo, these RPC’s were shown to possess biological activity both in vitro and in vivo.\(^{32,31,12,24,33}\)

The parent complexes $1^{2+}$ and $2^{2+}$ are modestly cytotoxic (IC$_{50}$'s between $10^{-3}$ and $10^{-4}$ μM) with enhanced cytotoxicity generally observed by increasing the lipophilicity of the complex.\(^{34}\) The 3,4,7,8-tetramethyl-1,10-phenanthroline derivative of $1^{2+}$ was shown to inhibit the growth of dispersed tumor cells (Landshultz ascites) in mice.\(^{32,35}\) As these RPCs have been shown to bind DNA, it is generally assumed that this is the biological target,\(^{35}\) although the mitochondria\(^{36,37,13}\) and the cell-cell interface\(^{38,25}\) have also been proposed as targets. It is not known how these complexes act on the molecular targets but given their general inertness, however it is postulated that these complexes bind at specific sites and thereby disrupt important cellular processes. It has been possible to track the intracellular distribution of many of these RPC’s due to their inherent
luminescence with both the nucleus and mitochondria often showing significant accumulation.\textsuperscript{36, 39} However, complexes (3\textsuperscript{2+}) and (4\textsuperscript{4+}) have not been found to luminesce regardless of the conditions tested. Therefore, other studies are being investigated to determine their intercellular and intracellular activities.

1.5 DNA cleavage activity of RPC's

While most RPCs will bind with DNA, few cause observable damage unless they are activated by an external factor, such as light irradiation.\textsuperscript{40} Recently, we have shown that the two ruthenium(II)-tatpp complexes, [(phen)$_2$Ru(tatpp)]$^{2+}$ (3\textsuperscript{2+}) and [(phen)$_2$Ru(tatpp)Ru(phen)$_2$]$^{3+}$ (4\textsuperscript{3+}), (shown in Figure 1-4) are effective DNA cleaving agents upon \textit{in situ} reduction by common biological reducing agents, such as glutathione (GSH).\textsuperscript{41} Both complexes bind to DNA through electrostatic interaction, dock in the minor groove of the DNA, then intercalate through the DNA resulting in DNA cleavage.\textsuperscript{41}

![Figure 1-4: (3\textsuperscript{2+}) and (4\textsuperscript{3+}) complexes.](image)

1.5.1 \textit{Introduction to DNA cleavage assay}

To determine the DNA cleaving potential of the RPC's investigated by the MacDonnell group, a DNA cleavage assay was used with pUC18 and gel agarose electrophoresis. The pUC18 plasmid DNA exhibits a supercoiled (SC or form I)
topological configuration prior to exposure to RPC’s in the assay. Upon exposure to the 
RPC of interest and GSH the $\text{sc}$DNA takes on an open circular (OC or form II) topological 
configuration when the DNA is nicked once by the RPC. Upon sequential nicks of the 
$\text{cc}$plasmid (form II) by the drug the topological configuration converts to linear DNA (form 
III). A general schematic of the changes in DNA topology is shown in Figure 1-5.

Figure 1-5 Topoisomers of plasmid DNA and the how the forms are tracked with 
agarose gel electrophoresis.

Significantly, the DNA cleavage activity is enhanced under low oxygen or hypoxic 
conditions which is mirrored by an increase in their potency as cytotoxic agents for 
treating H358 (Human bronchioalveolar carcinoma; non-small cell lung cancer (NSCLC)) 
and H226 (Squamous cell carcinoma; mesothelioma) tumor cells under hypoxic 
conditions. They also are active anti-tumor agents in vivo, causing a marked 
regression of tumor growth in nude mice bearing xenograph H358 tumors and more than 
doubling survival time. While direct proof is still lacking, we postulate the in vivo activity is 
also due to DNA damage and that the hypoxic conditions present in many tumors may 
lead to enhanced potency for these agents against those cell subpopulations.
1.6 Scope of this thesis

As mentioned earlier, two of the compounds studied in the MacDonnell group, \(3^{2+}\) and \(4^{3+}\), have shown unique behavior towards DNA and cancer cell lines under hypoxic conditions. Therefore, the need to understand their mechanism of activity towards DNA is of great importance. Chapter 2 discusses an introduction to the known chemistry of \(3^{2+}\) and \(4^{3+}\) and previous work done by the MacDonnell group towards understanding the activity of \(3^{2+}\) and \(4^{3+}\) under varying conditions. Results from previous discoveries have led us to postulate that complexes \(3^{2+}\) and \(4^{3+}\) induce DNA damage via a H-atom abstraction pathway. Chapter 3 will introduce known mechanisms for cleaving nucleic acids and the experiments used along with the results to support our hypothesis.
Chapter 2
Previous work done in the MacDonnell group exploring RPC chemistry

2.1 Introduction

The MacDonnell group has synthesized several RPC’s to study their potential DNA cleavage and biological activity.\textsuperscript{14,41} Two of the RPC’s synthesized, the dinuclear ruthenium (II) cationic polypyridyl complex \[(\text{phen})_2\text{Ru(tatpp)}\text{Ru(phen)}_2\]\textsuperscript{4+} (4\textsuperscript{4+}) (where tatpp = 9,11,20,22-tetraazatetrapyrido[3,2-a:2',3'-c:3'':2''-l:2'''-n]) and the corresponding mononuclear ruthenium(II) cationic complex \[(\text{phen})_2\text{Ru(tatpp)}\]\textsuperscript{2+} (3\textsuperscript{2+}), have shown consistent efficacy towards tumor cells \textit{in vivo} and \textit{in vitro}.\textsuperscript{41} Initial studies by Dr. Janaratne and Dr. Yadav using \textit{in vitro} cleavage assays with 3\textsuperscript{2+} and 4\textsuperscript{3+} found they are potent DNA cleaving agents under reducing conditions. The \textit{in vitro} cleavage assays were performed under a variety of conditions including in the presence and absence of O\textsubscript{2}. It was found the cleaving activity of 4\textsuperscript{4+} was strongly attenuated upon exposure to O\textsubscript{2}.\textsuperscript{14,41} To our knowledge, there are no metal-based complexes that show potentiated DNA cleavage under hypoxic conditions. Moreover, to our knowledge 3\textsuperscript{2+} and 4\textsuperscript{3+} are the first known ruthenium based complexes that cleave DNA by reduction activation versus irradiation. This discovery was important because few RPC’s show appreciable DNA cleaving ability unless excited by light irradiation.

Dr. Yadav also conducted cytotoxicity, animal toxicity and a tumor regression studies. The cytotoxicity was conducted with H538 and H226 tumor cell lines and the animal cytotoxicity with mouse melanoma B-16 tumor cells, resulting in promising cytotoxicity activity by complexes 3\textsuperscript{2+} and 4\textsuperscript{3+} in both studies. The tumor regression xenograft model H358 was conducted with H358 lung cancer tumor cells resulting in appreciable tumor regression for both complexes 3\textsuperscript{2+} and 4\textsuperscript{3+}. This data is shown in Figure 2-1, 2-2 and 2-3 respectively. Figure 2-1 clearly shows 3\textsuperscript{2+} and 4\textsuperscript{4+} has much
greater cytotoxicity than the control. The same trend is true and clearly shown in Figure 2-2 with the animal cytotoxicity study.

**Figure 2-1** IC$_{50}$ of rac/mix- Ru(II) complexes- monomer vs. dimer

**Figure 2-2** Animal toxicity data of RPC's with melanoma B-16 cells
Figure 2-3 Change in a tumor volume after injecting NSCLC-H358 lung cancer cells

Figure 2-3 also shows an appreciable regression in the tumors treated with $3^{2+}$ and $4^{4+}$ as compared to the control, where the tumors were not treated. The tumors in the rats that were treated with PBS buffer only served as the control group. The data for the control group clearly shows exponential tumor growth resulting in death of the rats 30 days into the experiment. The rats that were treated with complexes $3^{2+}$ and $4^{4+}$ did not grow to an appreciable size over the course of the experiment. The treated mice alive at the end of the experiment were summarily sacrificed. The results from the studies by Dr. Janaratne and Dr. Yadav led us to explore the possible mechanism of $3^{2+}$ and $4^{4+}$. In determining a suitable mechanism as to how these complexes inhibit tumor growth,
certain assumptions had to be made. The first assumption was that the Ru(II) complexes interact directly with the DNA.

2.2 Redox Activity of RPCs

RPCs $3^{2+}$ and $4^{4+}$ have been shown to have a number of stable redox and protonation isomers in aqueous solution, some of which are implicated in the observed DNA cleavage chemistry. As an understanding of these is needed to elucidate the observed DNA cleavage activity, a ladder scheme is shown for complex $3^{2+}$ in Figure 2-4 which gives the structures and relationships of the various reduced and protonated species, as well as the associated notation used to identify these species.

![Figure 2-4 Redox and protonation isomers for $3^{2+}$](image)

A nearly identical ladder scheme exists for $4^{4+}$ though the starting complex has greater charge (4+) and therefore has a different notation. Almost every species in these ladder schemes has been identified spectroscopically in previous studies, apart from the singly reduced-singly protonated, radical complexes ($H3)^{2+}$ and ($H4)^{4+}$ which are unstable with respect to disproportionation. Redox reactions are listed vertically and
protonation-deprotonation reactions horizontally. For clarity, some parasitic side-reactions, such as radical dimerization, which are not thought to be important in the DNA chemistry, are omitted.

In acetonitrile (MeCN, $4^{4+}$) undergoes two one-electron reductions at -0.18 and -0.54 V vs Ag/AgCl. It was also found these complexes are proton-coupled such that in aqueous solution at pH 7, the two one-electron processes in MeCN have merged to form a single bi-electronic process at -0.14 V vs Ag/AgCl. Furthermore, these potentials are clearly within the reducing potential of agents such as GSH, meaning, there may be a number of chemical species that are active in cleaving DNA. Moreover, changes in redox and/or protonation states of $3^{2+}$ and $4^{4+}$ were easily discernable in the visible absorption spectrum, allowing us to identify a particular species from a simple visible absorption spectrum as seen in Figure 2-5.

![Absorption spectra](image)

Figure 2-5 Absorption spectra of $4^{3+}$ and $(H_24)^{4+}$ in degassed water.
2.3 RPCs in the presence of radical scavengers

The biological activity of many RPCs have been studied and reported in literature. Therefore, it is generally accepted RPCs reversibly bind to DNA. In 2006 Rajput reported a binding constant for complex $4^{4+}$ as $-1.1 \times 10^8 \text{ M}^{-1}$. 43

Further studies were done to determine what species were involved in the cleaving of DNA. When $3^{2+}$ and $4^{4+}$ were activated with GSH in the presence of DNA it was found that the doubly protonated species, $[(\text{phen})_2R\text{u}(\text{H}_2\text{tatpp})\text{Ru}(\text{phen})_2]^{4+}$ ($[\text{H}_24^{4+}]$) showed the most cleavage activity. The initial studies for cleavage activity conducted by Dr. Janaratne showed that $4^{4+}$ required addition of a reducing agent, e.g. glutathione (GSH) to cut DNA, and was enhanced under anaerobic conditions compared to aerobic conditions, as shown in Figure 2-6. 41

Iron bleomycin (Fe-BLM) can cause single strand (ss) nicks under anaerobic conditions, but $O_2$ is required for double strand (ds) cleavage activity. Therefore, Fe-BLM was used as a positive control to show that the glove box was oxygen-free.

Figure 2-6 DNA cleavage assay of complex $4^{4+}$ under aerobic and anaerobic conditions

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 $[4]^{4+}$ or Fe-BLM complexes (12.8 μM) in a buffer of 0.5 mM GSH and 4 mM Na$_3$PO$_4$ (pH 7.0) under aerobic or anaerobic conditions (as indicated). Lanes M (marker lane of form I, II and III of pUC18 DNA), 1 (DNA without GSH), 2 (DNA plus GSH) and 3 (DNA plus $4^{4+}$) served as controls. Lane 4 and 5 are (DNA plus $4^{4+}$ with GSH) under aerobic and anaerobic conditions respectively. Lane 6 and 7 are (DNA plus Fe-BLM) and served as positive controls.
The results showed that complex $4^{4+}$ cleaved DNA under aerobic conditions and anaerobic conditions, but was enhanced under anaerobic conditions. To determine the species responsible for DNA cleavage Dr. Janaratne’s initial studies showed the most active species involved in DNA cleavage was $(H_24)^{4+}$ followed by the radical $4^{3+}$ and $4^{4+}$ respectively. As mentioned earlier the radical species $(H4)^{3+}$ is highly unstable due to disproportionation to produce $4^{4+}$ and $(H_24)^{4+}$. Therefore, it was not possible to measure the cleavage activity of $(H4)^{3+}$ with this assay. The nature of these results led us to believe further elucidation of the activity $(H4)^{3+}$ radical was needed and spurred the following questions:

I. Does $(H4)^{3+}$ contribute to DNA cleavage?

II. Are reactive oxygen species (ROS) involved in cleavage activity?

III. Can we derive a plausible mechanism supported by studies examining the effects of varied $[O_2]$?

Dr. Yadav continued the study by using radical scavengers to further elucidate the activity of the $H4^{3+}$ radical. He examined the cleavage activity in the presence of 2,2,6,6-tetramethylpipiridine-1-oxyl (TEMPO) which is a nitroxide radical species that effectively traps carbon and metal-centered radicals Figure 2-7 shows the results of the study in the presence of TEMPO.$^{14}$
Agarose gel (1%) stained with ethidium bromide of supercoiled pUC18 DNA (0.154 mM) cleavage products after incubation at 25°C for 2 h with [4]⁺⁺, GSH and TEMPO in 7 mM Na₃PO₄ buffer (pH 7.0) under aerobic and anaerobic conditions. Lane 1: DNA control; Lane 2: DNA plus GSH (0.513 mM); Lane 3: DNA plus [4]⁺⁺ (0.0128 mM); Lane 4: DNA, GSH (0.513 mM) plus [4]⁺⁺(0.0128 mM); Lane 5: DNA, GSH (0.513 mM) plus [4]⁺⁺(0.0128 mM) under anaerobic conditions; Lane 6: DNA, GSH (0.513 mM), [4]⁺⁺(0.0128 mM) plus TEMPO (2.04 mM); Lane 7: DNA, [4]⁺⁺(0.0128 mM) plus TEMPO (2.04 mM) under anaerobic conditions.

Figure 2-7 shows the DNA cleavage activity in vitro of [4]⁺⁺ and GSH in the presence TEMPO (lanes 6 (aerobic) and 7(anaerobic)) and in its absence (lanes 4 (aerobic) and 5 (anaerobic)). As seen in lanes 6 and 7, TEMPO quenches the cleavage activity in the presence or absence of [O₂], suggesting that a carbon-centered radical could be involved in the chemistry of cleavage activity here. In addition, an assay was done that involved the different redox species of [4]⁺⁺ to further elucidate the species involved in DNA cleavage. The results of this assay by Dr. Yadav are shown in Figure 2-8.
Figure 2-8 DNA Cleavage by $[4]^{4+}$, $[4]^{3+}$ and $[H_24]^{4+}$

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 $[4]^{4+}$, $[4]^{3+}$ and $[H_24]^{4+}$ in a buffer of 4 mM Na$_3$PO$_4$ (pH 7.0) 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 $4^{4+}$, 0.083 complex/DNA bp ratio), lane 3 (30.7 µM $4^{4+}$, 0.20 complex/DNA bp ratio), lane 4 (12.8 µM $4^{3+}$, 0.083 complex/DNA bp ratio), lane 5 (30.7 µM $4^{3+}$, 0.083 complex/DNA bp ratio), lane 6 (12.8 µM $H_24^{4+}$, 0.083 complex/DNA bp ratio), lane 7 (30.7 µM $H_24^{4+}$, 0.20 complex/DNA bp ratio).

As seen in lanes 2 and 3, $4^{4+}$ shows no significant damage to the DNA. The monoreduced complex $4^{3+}$ shows slightly more cleavage (lanes 4 and 5) than $4^{4+}$ but only marginally so. Interestingly, the doubly-reduced, doubly-protonated complex $(H_24)^{4+}$ causes extensive ss cleavage (lanes 6 and 7) with almost full conversion to circular DNA observed with 0.0307 mM $(H_24)^{4+}$ (lane 7). From these results were surmised the three main species involved in cleavage of DNA were $4^{4+}$, $4^{3+}$ and $(H_24)^{4+}$. The same studies were conducted for $3^{2+}$. They yielded very similar results suggesting the same mode of action as $4^{4+}$. Figure 2-9 shows the postulated scheme and relationship between these species.
2.3 Sensitivity to dioxygen concentration

Both RPCs $3^{2+}$ and $4^{4+}$ cleave DNA in the presence or GSH as shown by the conversion of supercoiled plasmid DNA (Form I) to circular DNA (Form II). They also showed enhanced cleavage activity when the experiment was conducted under anaerobic conditions (in a glove box). It was also found the doubly-reduced, doubly-protonated complex $(\text{H}_24)^{4+}$ cleaves DNA under hypoxic conditions without the requirement of an external reducing agent, such as GSH. This suggested DNA cleavage was not be due to reactions of the oxidized GSH or other reducing agents but must be due to the ruthenium complexes.

It was unclear how these complexes cleaved DNA with or without the presence of GSH. It was suspected the intermediate radical species, $3^+$ and $4^{3+}$, were more likely candidates for the cleavage reaction, which would mean that $(\text{H}_23)^{2+}$ and $(\text{H}_24)^{4+}$ would need to be oxidized by one-electron to be activated towards DNA cleavage. This led us to explore the role of dioxygen in the overall reaction scheme. Dr. Abayan
conducted a study using an oxygen sensitive electrode, results from studies revealed ~ 4 µM of O₂ was present in the glovebox where it was previously assumed to contain an anaerobic environment. Figure 2-10 shows the results of this study.

![Figure 2-10 Cleavage assay under varying [O₂]](image)

As seen in Lanes 2, 3, and 4, there was an appreciable increase of circular DNA as the [O₂] is lowered from 220 µM to 4 µM. However, in the complete absence of O₂ or within undetectable limits (0 µM) the cleavage was attenuated (Lane 5) such that it is even less than seen at 200 µM.

In order to explain this data, we proposed that there are three relevant forms of 3²⁺ all of which are intercalated with DNA and are reversibly interconverted by redox and protonation reactions. Equation (1) shows the interconversion of the three species associated with 3²⁺ bound to DNA. As seen, GSH drives the complexes towards the fully reduced species whereas O₂ reoxidizes the reduced complexes such that the relative amount of the three under steady-state conditions are dictated by the GSH/O₂ ratio. In the absence of GSH, all of the complex is converted to 3²⁺. Whereas, in the absence of O₂ but in the presence of GSH, all of the complex is reduced to (H₂3)²⁺, neither of which
is active for DNA cleavage. The reactive complex is the radical species $3^+$. While this species is unstable with respect to protonation and disproportionation at pH 7.2, intercalation gives the association complex ($3^+\cdot$-DNA) which alters the local environment at the protonation site and thereby decreases the pKa of (H3)$^{2+}$ such that $3^+$ is stable while bound to DNA. This unusual set of circumstances sensitizes the DNA bound complex to the cellular redox potential and potentiates the DNA cleavage activity when the [O$_2$] is low.

\[
\begin{align*}
\{3^{2+}\cdot\text{DNA}\} & \underset{+\text{GSH}}{\overset{+\text{O}_2}{\rightleftharpoons}} \{3^+\cdot\text{DNA}\} & \underset{+\text{GSH}}{\overset{+\text{O}_2}{\rightleftharpoons}} \{\text{H}_23^{2+}\cdot\text{DNA}\}
\end{align*}
\]

(1)

2.5 Summary and conclusions

The previous investigations of $3^{2+}$ and $4^{4+}$ by Dr. Janaratne, Dr. Yadav and Dr. Abayan have shown that both complex $4^{4+}$ and $3^{2+}$ cleave DNA, under reducing conditions in the presence of GSH. DNA cleavage activity was examined under both aerobic and anaerobic conditions. It was found that maximum cleavage activity was achieved under conditions of minimal oxygen. But when the system was “scrubbed” of any remaining oxygen, cleavage activity was almost completely stopped. This evidence further supports a mechanism in which a limiting amount of oxygen is needed and that the reaction is catalytic.

Cleavage assays in the presence of TEMPO suggested the presence a carbon based radical, when (H$_2$4)$^{4+}$ or (H$_3$3)$^{2+}$ was bound to DNA. From these results we postulate the radicals present in these systems are responsible for DNA cleave in vitro. In the following chapter (chapter 3) further elucidation of the how these radicals are cleaving DNA in vitro will be discussed.
Chapter 3

Further elucidation of mechanistic pathway of $3^{2+}$ and $4^{1+}$

3.1 Introduction to known mechanisms of DNA cleavage.

It is well known that DNA cleavage can occur through two pathways. Oxidants with free radical character can form an oxidative process leading to hydrogen atom (H-atom) abstraction from the carbohydrate backbone of DNA. $^{44,45}$ The other pathway is by hydrolysis of DNA backbone. However, lesions caused by H-atom abstraction, notably abstraction from the C1’ position of deoxyribose can give rise to premutagenic α-deoxyribonucleotides. $^{46}$ DNA damage resulting in a mutation is not known to have a repair mechanism for this type of damage in biological systems. If abstraction from a hydrogen atom occurs on the deoxyribose of DNA, a carbon based sugar radical is produced that may undergo rearrangement resulting in DNA strand scission. $^{45}$ If these lesions are not repaired they may lead to mutagenesis, carcinogenesis inherited disease, aging and cell death. $^{45}$

Hydrolysis does not affect the sugar moiety. It affects the phosphodiester bond in DNA. Damage by hydrolysis is easily repaired by ligase enzymes in biological systems.

If H-atom abstraction occurs on the sugar moiety each Cn’ position produces a unique scission product. The use of these scission products as a working model to show evidence of H-atom abstraction from the sugar moiety has been used with a wide variety of varying chemical species and is well accepted in the scientific community. $^{45,47,44}$ A table of these products is shown in Table 3-1.
Table 3-1 Unique scission product markers

<table>
<thead>
<tr>
<th>Marker Product</th>
<th>Scission Product Name</th>
<th>Position of Hydrogen Abstraction</th>
<th>Method of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="5-Methylene-2-furanone" /></td>
<td>5-Methylene-2-furanone</td>
<td>C-1’</td>
<td>HPLC</td>
</tr>
<tr>
<td><img src="image" alt="Base propenoate" /></td>
<td>Base propenoate</td>
<td>C-3’</td>
<td>HPLC</td>
</tr>
<tr>
<td><img src="image" alt="Oligonucleotide 3’-phosphoglycolaldehyde" /></td>
<td>Oligonucleotide 3’-phosphoglycolaldehyde</td>
<td>C-3’</td>
<td>PAGE</td>
</tr>
<tr>
<td><img src="image" alt="Oligonucleotide 3’-phosphoglycolate" /></td>
<td>Oligonucleotide 3’-phosphoglycolate</td>
<td>C-4’</td>
<td>PAGE</td>
</tr>
<tr>
<td><img src="image" alt="Base propenal" /></td>
<td>Base propenal</td>
<td>C-4’</td>
<td>reaction with thiobarbituric acid</td>
</tr>
<tr>
<td><img src="image" alt="Nucleotide 5’-aldehyde" /></td>
<td>Nucleotide 5’-aldehyde</td>
<td>C-5’</td>
<td>PAGE</td>
</tr>
<tr>
<td><img src="image" alt="Furfural (FUR)" /></td>
<td>Furfural (FUR)</td>
<td>C-5’</td>
<td>HPLC</td>
</tr>
</tbody>
</table>
In this work, we explore the mechanism by which these two complexes cleave DNA using a combination of DNA cleavage experiments under varying \([O_2]\) and analysis of the reaction solution for small molecule by-products of DNA cleavage. We had previously reported on the aqueous electrochemistry of \(3^{2+}\) and \(4^{4+}\) and have shown that the first two reductions, which occur at modest potentials, are localized on the tatpp ligand. Herein we propose that the observed DNA cleavage activity is due to the singly-reduced tatpp-radical complexes, \([\text{phen}]_2\text{Ru(tatpp)}]^+ (3^+)\) and \([\text{phen}]_2\text{Ru(tatpp)Ru(phen)}]^3+ (4.3^+), and that the sensitivity of DNA cleavage to the local \([O_2]\) is due changes in the steady-state concentrations of these radicals with respect to the overall cellular redox potential. This has therapeutic implications as many tumor cells are often under hypoxic stress.

3.2 Experimental

3.2.1 Chemicals

All chemicals were purchased commercially and used without further purification unless otherwise noted. Millipore (18Ω) water was used for all buffers and reactions that required water. All plasmid DNA (pUC18 and 19) and DNA ladders were purchased from Bayou Biolabs. Chemicals needed for the DNA electrophoresis assay, ethidium bromide, glutathione (GSH), trizma base, mono and dibasic phosphates, EDTA and agarose were purchased from Sigma Aldrich.

Chemicals for the T4ligase assay, T4 DNA ligase (HC), T4 DNA ligase 10X buffer, Acetylated Bovine Serum Albumin, EcoRI, Buffer H 10X Buffer were purchased from Promega.

Chemicals for the DNA scission products assay, furfural standard was purchased from Sigma Aldrich and the 5-methylene furanone (5-MF) standard was synthesized.
according to literature as described below. All reagents for the 5-MF synthesis were purchased from Sigma Aldrich.

The complexes \([\text{phen}]_2\text{Ru(tatpp)}\text{Ru(phen)}_2]^{4+}, [4^{4+}]\) and \([\text{phen}]_2\text{Ru(tatpp)}]^{2+}, [3^{2+}]\) were synthesized as described in literature by Nagham Alatrash.

3.2.2 Instrumentation

\(^1\)H NMR spectra were obtained on JEOL Eclipse Plus 300 or 500 MHz Spectrometers. UV-visible spectra were obtained on a Hewlett-Packard HP84535A spectrophotometer. Plasmid cleavage products were analyzed using an AlphalImage™ 2200 gel analysis system.

HPLC analysis of DNA scission products was carried out on a Agilent HPLC Infinity 1260, 1260 Pump G1311 1260 Quat VL, 1260 Auto sampler G1329B 1260 ALS and 1290 Detector G4212A 1290 DAD.

3.2.3 Buffer Preparations

6x Bromophenol blue loading buffer for DNA agarose gel assay, 10 mL To a glass vial was added 25 mg of bromophenol blue, 6.7 mL of ddH\(_2\)O (18Ω), and 3.3 mL of glycerol. Solution was stirred and stored at 4°C.

0.5M EDTA (pH 8.0), 1L

To a 1 L container was added 148 g of Ethylenediaminetetraacetic acid (EDTA), ~700 ddH\(_2\)O (18Ω) and solid sodium hydroxide (NaOH, ~30-40g) to adjust the pH to 8.0. Once the pH was adjusted ddH\(_2\)O (18Ω) was added to adjust volume to 1L.

Note: EDTA will not dissolve in water until the pH approaches 8.
50X TAE (tris-acetate-EDTA) Stock Solution 1L

To a 1 L container was added 242 g Tris Base ([tris(hydroxymethyl)aminomethane]) (MW=121.1), ~800 mL of ddH$_2$O (18Ω). The solution was stirred until all the tris base was dissolved. Next 57.1 mL Glacial Acetic Acid and 100 mL of 0.5 M EDTA was added respectively to the tris base solution. After solution was stirred thoroughly, ddH$_2$O (18Ω) was added to adjust the final volume to 1 L and was stored at room temperature.

Note: Final (1x) working concentration: 0.04 M Tris - Acetate 0.001 M EDTA

500 mM phosphate and 10 mM Nacl buffer @ pH 7.4

To a 1 L container was added 6 g of sodium phosphate monobasic anhydrous (F.W. 119.98 g/mol) and 584 mg of sodium chloride (NaCl). Next ~ 800 mL of ddH$_2$O (18Ω) was added. The pH was adjusted to 7.4 with 3 M NaOH. Once pH was adjusted ddH$_2$O (18Ω) was added to adjust the final volume to 1L.

3.2.4 DNA Cleavage Assay

All digestion reactions were carried out in a 1 mL Eppendorf tube with a total volume of 40 µL. The base pair (bp) concentration for pUC 18 or 19 DNA was 0.154 mM or 154 µM for all reactions. The concentration of complex used and condition of the experiment are given in the Figure legends. The concentration of buffer solution used to bring the volume to 40 µL was 50 mM phosphate and 10 mM NaCl at pH 7.4. After the digestion was complete the reactions were quenched with a dry ice and acetone ice bath. Loading buffer (30% glycerol in water with 0.1% w/v bromophenol blue) at 1/6 the total volume of the reaction was added to each reaction tube. Each tube was then vortexed 10 seconds each. Aliquots of each tube was added to the corresponding well into a previously made 1% DNA agarose gel immersed in TAE buffer (40 mM Tris-acetate, 1
mM EDTA, pH 8). The gel was electrophoresed at 70 V for 80-90 minutes. The DNA products were visualized by irradiation with ultra-violet light and the image recorded using a UVPGDS 8000 gel analysis system.

3.2.5 T4 Ligase Assay

Digested reactions with pUC19 DNA, GSH, $4^{4+}$ or $3^{2+}$ and phosphate buffer were treated with T4 ligase enzyme and buffer. All reactions had a total volume of 20 µL. To serve as a control pUC 19 plasmid DNA was digested with EcoRI for two hours. The EcoRI mixture containing 5 µL water, 8 µL of RE 10X buffer, 0.8 µL acetylated BSA, 4 µL of pUC19 plasmid DNA and 2 µL of EcoRI was heat inactivated at 65°C for ~20 minutes. Two reaction vials were made. Following heat inactivation 1.0 µL of T4 ligase 10X buffer and 0.5 µL of T4 DNA ligase was added to one of the two reaction vials to re-ligate the DNA for ~1 hour.

Two reaction vials for both $3^{2+}$ and $4^{4+}$ were prepared as well. These reaction vials were prepared with samples prepped for the DNA agarose assay. However, to one of the vials containing $3^{2+}$ and one containing $4^{4+}$ was added 1.0 µL of T4 ligase 10X buffer and 0.5 µL of T4 DNA ligase. The reactions were digested for ~ 1 hour. All samples were then analyzed with DNA gel electrophoresis and 6X loading buffer.

3.2.6 Synthesis of 5-MF standard

The synthesis of 5-Methylene furanone (5-MF) was done according to Crey et., al. Part I of the synthesis was the a four (4) day reaction to make the lactone 3,5-Di-O-p-toluoyl-2-deoxy-D-ribo-1,4-lactone needed for later conversion to 5-MF (part II). Several attempts were made to make the lactone successfully according to literature. However, it was found that an air sensitive environment was needed. Therefore
all work at the point of adding BF$_3$-Et$_2$O on day two and beyond was done under Argon or Nitrogen using schlenk line techniques for all synthesis including the evaporation.

The final product was confirmed with GCMS. The splitting pattern of 5-MF is well known and documented thoroughly in literature. The spectra confirming the product is shown in appendix A.

3.2.7 DNA Scission products assay

Preliminary reactions were conducted under the following conditions; (26μL) 200 μM pUC19 dsPlasmid DNA, (50 μL) 500 μM (4$^{+}$), (35.6 μL) 5000 μM GSH, (132 μL) 5 mM Tris, 50 mM NaCl Buffer @pH 7.4, digested aerobic and under argon (low Oxygen) for 2 hours, then heated @ 90°C for 1hr. The reaction was quenched with ice bath (dry ice/acetone), then extracted with 1mL DCM (dichloromethane) 3x, dried with MgSO$_4$ (magnesium sulfate) and concentrated. The conditions for HPLC analysis were as follows. The mobile phase of for HPLC was H$_2$O/MeCN 90/10, Flow Rate: 0.3mL/min, Injection Vol: 10 μL, Stationary Phase: Ascentis C18 (25cmx2.1mm) Supelco, Detection: UV 254nm.

Further experiments conducted to optimize results were conducted under the following conditions, (45.5 mL) 700 μM ctDNA, (4.1 mL) 58.3 μM (4$^{+}$), (19.9 mL) 5.8 mM GSH, (30.5 mL) 50 mM phosphate, 10 mM NaCl Buffer @pH 7.4. Digested at room temperature in air overnight, then heated @90°C for 1hr in a GC oven. The reaction was quenched reaction with ice bath (dry ice/aceton) extracted with 20mL DCM 3x, dried with MgSO$_4$ and concentrated. Samples were resuspended in pure MeCN for HPLC analysis. The mobile phase of for HPLC was 0.1 TFA/MeCN 90/10, Flow Rate: 0.1mL/min, Injection Vol: 10 μL, Stationary Phase: Zorbax Eclipse XDB-C18 4.6x150 column. The same method was conducted for 3$^{2+}$.
3.3 Results and discussion

3.3.1 Results for DNA scission products

In an effort to further establish the mechanism of action, we scaled-up the cleavage reaction and extracted the aqueous phase with CH$_2$Cl$_2$ to look for neutral, small-molecule DNA cleavage products characteristic of H-atom abstraction from either C1' or C5' of the deoxyribose units. While it is possible that the radical could attack any of the C-H bonds in the deoxyribose units, only abstraction from C1' or C5' leads to neutral dichloromethane (CH$_2$Cl$_2$) extractable by-products, which are characteristic of the cleavage reactions, 5-methylfuranone (5-MF) and furfural, respectively. The presence of furfural and 5-MF in the extracts were confirmed by HPLC and is shown in Figure 3-1.
The hydrogen at the C5' position is accessible for H-atom abstraction by a radical intercalator from inspection of simple DNA-intercalator models. The reaction is shown schematically in equation 2 and 3 which reveals an additional interesting point. The reaction of the radical with DNA ultimately generates the doubly-reduced form of the complex \((H_23)^{2+}\) which is free to bind and cleave DNA again upon re-oxidation to the radical. While we have yet to prove that the DNA cleavage reaction is catalytic in the tatpp complex, it would have obvious benefits in keeping the drug dosage low.

\[
\begin{align*}
\{3^{++}\} & \rightarrow \{H^{2+}\} \rightarrow \text{O} + H_2^{2+} \\
\{3^{++}\} & \rightarrow \{H^{2+}\} \rightarrow \text{CO} + H_2^{2+}
\end{align*}
\]

Initial estimates of the driving force of the H atom abstraction reaction by either radical species suggest an endergonic process, however there a number of mitigating factors which we believe will need to be considered for a truly accurate estimate of the thermodynamics of this reaction, including an accurate determination of the tatpp radical anion’s pKa and redox potential when bound to DNA. While these studies are in progress, it is interesting to note that the high stability and limited reactivity of the radical species is actually beneficial towards the desired application. Because the complex is properly positioned adjacent to the deoxyribose units by pre-binding and intercalation into the DNA, the formation of a ‘stable’ radical limits its reactivity to those substrates it encounters frequently or, more generally, to unimolecular reactions. This leads to a
relatively new paradigm in drug design, in which the reactivity of the drug is muted as much as possible and the reaction is gated by the DNA binding.

### 3.3.2 Results for DNA cleavage and T4 ligase assay

It seemed clear from the previous work done in the MacDonnell group that hydrolytic cleavage was not applicable towards a viable explanation for a possible mechanism. However, we felt it necessary to confirm this by using a known hydrolytic T4 ligase cleavage assay. As seen, in Figure 3-2 open circular DNA cleaved by $4^{4+}$ was not re-ligated in the presence of T4 ligase (lane 6). However, lane 8' indicated a small amount of relegation from DNA cleavage by $3^{2+}$. This further supports our hypothesis that cleavage primarily occurs via a H-atom abstraction from the sugar base moiety. The additional mode of cleavage by $3^{2+}$ may explain why it has shown to cleave more DNA and have greater cytotoxicity than $4^{4+}$.

![Figure 3-2](image)

In the first gel on the left is the control experiment for the hydrolytic cleavage assay. EcoRI a restriction enzyme was digested in the with pUC19 plasmid DNA. We chose pUC19 because it only has one site for EcoRI. Thus making a good model to show open circular DNA cleavage and also preventing multiple cuts that may inhibit the ability of T4 ligase to re-ligate the cut sites on the DNA by EcoRI. Lane 1 is a 1 kb DNA ladder.
Lane 2 is pUC19 DNA and buffer only. Lane 3 is pUC19 in the presence of EcoRI. Lane 4 is DNA cut by EcoRI after a digestion with HC T4 ligase. The gel on the right shows controls in lane 2'-4'. Lane 2' is DNA and buffer. Lane 3' is DNA. Lane 3 and 4 contain 4 and 3^{2+} with DNA and no GSH respectively. Lane 5' is 4^{4+}, GSH and DNA (24 hour incubation). Lane 6' is the same as lane 5 with the addition of HC T4 ligase. Lane 7' is 3^{2+} DNA and GSH (24 hour incubation). Lane 8' is the same as lane 7' with the addition of T4 ligase.

In summation we have shown complexes 3^{2+} and 4^{4+} cleave DNA more significantly under hypoxic conditions than under aerobic conditions. Furthermore, our studies show appreciable DNA cleavage is not primarily hydrolytic. Moreover, cleavage is attenuated in the presence of TEMPO indicating a carbon centered radical being responsible for DNA cleavage. The fact that cleavage is most active under hypoxic conditions is significant because it is assumed normal cells in oxygenated conditions will be less affected than tumor cells. We believe this because the core of tumor cells is less accessible to oxygen than normal cells. Since tumorous cells require more iron and oxygen receptors to supply nutrients to the cell, it is reasonable to deduce our purposed mechanism does not conflict with the current hypothesis that ruthenium complexes use the ferrin pathway for cellular uptake. It also important to remember complexes 3^{2+} and 4^{4+} are within the reduction potential of GSH versus previous RPCs that have been studied previously. This is summarized in Figure 3-3.
It is understood that experiments conducted *in vitro* are not a definitive explanation for complexes in biological systems. It is true the kinetics of $3^{2+}$ and $4^{4+}$ are very slow. It is also true if a repair pathway existed in a biological system for the damage the complexes cause to DNA, the repair would negate the activity of these drugs. The combined data we have shown suggests $3^{2+}$ and $4^{4+}$ are catalytic, responsible for tumor regression, increased DNA cleavage as $PO_2$ decreases and have low cytotoxicity. It is plausible to assume that if these drugs are able to interact with DNA in biological systems the mode of action would be H-atom abstraction of sugar moiety. Since these complexes are known to cleave at the C1’ position, which is known to give rise to premutagenic α-deoxyribo nucleotides, this would explain why DNA damage was not being repaired in the animal studies.
Appendix A

GCMS spectra of 5-MF
GCMS spectra of 5-MF
References


20. Öhrvik, H.; Nose, Y.; Wood, L. K.; Kim, B.-E.; Gleber, S.-C.; Ralle, M.; Thiele, D. J., Ctr2 regulates biogenesis of a cleaved form of mammalian Ctr1 metal transporter


Biographical Information

Cynthia Griffith was born in the United States in the State of Texas. She graduated from Nimitz High School in 1991. She returned to school in 2005. While completing her associates she enrolled in the University of Texas at Arlington (UTA) to pursue a Bachelors degree of Science in Biology and Chemistry. She began research for Dr. Frederick MacDonnell and held a teaching position as an undergraduate teaching assistant for general chemistry labs. While there she received the following awards, Undergraduate Teaching Award and Demonstration of Excellence in the presentation of Original Research in Chemistry, from the Chemistry and Biochemistry Society of UTA. In addition she represented the UTA and the MacDonnell group by presenting at two National conventions of the American Chemical Society in 2011 and 2012. Furthermore her collaborative work with Steven Poteet, as an undergraduate, in the MacDonnell group was published in the Journal of the American Chemical Society in 2013. She graduated in May of 2012 with a Bachelor’s of Science in Biology and Chemistry. In August of 2012 she began her graduate studies towards a Master’s of Science in Chemistry at UTA where she continued teaching and her research with Dr. MacDonnell. She was also a part of the Science Institute for Research Technologies (SIRT) at UTA, where she worked as a Research Scientist Assistant (RSA-1) in the Shimadzu Center for Advanced Analytical Chemistry (SCAAC). She received her Masters of Science in May 2015. She plans to continue with MacDonnell Group to pursue a PhD in Chemistry. After completing her PhD. she plans to continue her work in Industry and possibly explore teaching Chemistry.