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

by

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April 20, 2015

Abstract

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The University of Texas at Arlington, 2015

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In earlier reports, it was established that ruthenium (II) polypyridyl complexes RPCs, $[Ru(tatpp)]^{2+}$ (3^{2+}) and $[Ru(tatpp)Ru]^{4+}$ (4^{4+}) show potent antitumor properties *in vivo* in nude mouse lung cancer models.[2] 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+}.

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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 [3] 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. [4] The majority of these agents disrupt rapidly dividing cells that leads to cellular death or apoptosis.[5] 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.[6]

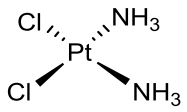
Transition metal based chemotherapeutic drugs have been used to treat cancer since the late 1970's. More specific, cisplatin was approved for clinical use in 1978. However, platinum-based chemotherapeutics have limitations in low solubility, toxic side effects, and tumor resistance.[7, 8] Therefore, 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. [9]

Ruthenium polypyridal complexes RPCs have become up and coming drugs of interested due to their similar substitution kinetics of platinum based drugs[10] and from extensive work by the Dwyer group showing these complexes do not accumulate in the body.[11, 12, 13] In addition, RPC's show different biological activity than cisplatin and its derivatives.[12] This is significant as possible alternative treatments for tumors that develop resistance to cisplatin and its analogues.[14]

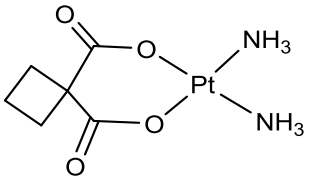
1.3 cis-Diamminedichloroplatinum(II) (cisplatin) and its analogues

Cisplatin (cis-$Pt(NH_3)_2Cl_2$) (Figure 1-1), was discovered in 1965 by Barnett Rosenburg, 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.[15] 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

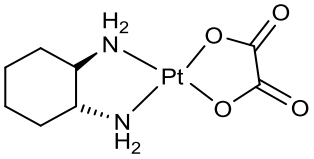
cisplatin, analogues of cisplatin have been researched.[9] Thousands of analogues have been researched for targeted cancer treatment and to combat drug resistance, but have not been FDA approved.[16] The first successful FDA approved analogue, Carboplatin (cis-diammine(1,1-cyclobutanebicarboxylato)platinum (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.[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.[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.[17]



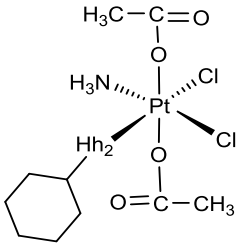
Cisplatin



Carboplain



Oxaliplatin



Satraplatin (JM216)

Figure 1-1 Platinum based anticancer drugs.

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.[18] Once inside the cell they are known to crosslink with DNA, form DNA adducts involving inter- and intra-strand crosslinks.[17] These adducts ultimately disrupt DNA replication and transcription leading to cell cycle arrest and apoptosis.[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.[18, 7, 20, 9] Recent studies have shown the copper transporter 2 (Ctr2) regulates Ctr1,[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). [21, 9, 22] The ability to undergo multiple oxidation states in physiological conditions was thought to be unique to platinum metals.[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.[21,23] Meaning ruthenium therapeutics have been shown to be less toxic in animals with greater cellular cytoxicity than cisplatin. [14, 21, 9] Furthermore, it has been shown cellular uptake of some ruthenium based chemotherapeutics are dependent on the transferrin cycle and activation by reduction. [24] 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.[22, 25, 24, 10] 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.[14]

1.4.2 Ruthenium chemotherapeutics with labile ligands

The anti-tumor agents NAMI-A (Imidazolium [trans-imidazoledimethylsulfoxide-tetrachlororuthenate(III), KP1019 (Indazolium [trans-tetrachlorobis(1H-indazole) ruthenate(III)]), RDC11 ([cis-bis(acetonitrile)-1,10-phenanthroline-2-phenylpyridineruthenium(II)] hexafluorophosphate),[26] (Figure 1-2) and ruthenium-aryl-X complexes[27] 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_{60} panel, but seems to target secondary or metastatic cancerous cells while its counterpart shows promise towards primary cancer cell lines.[28,29] 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.[22] At this time NAMI-A and KP1019 are reported to have advanced to phase II clinical trials.[28]

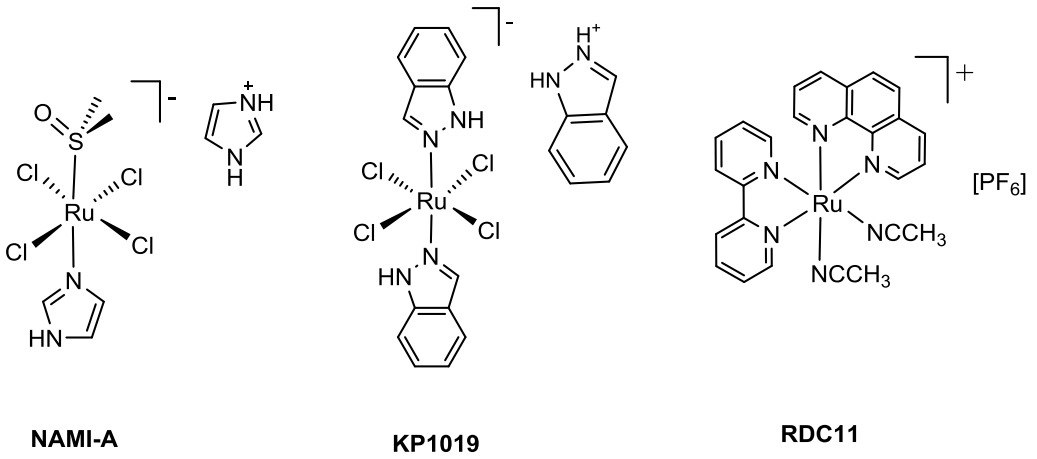
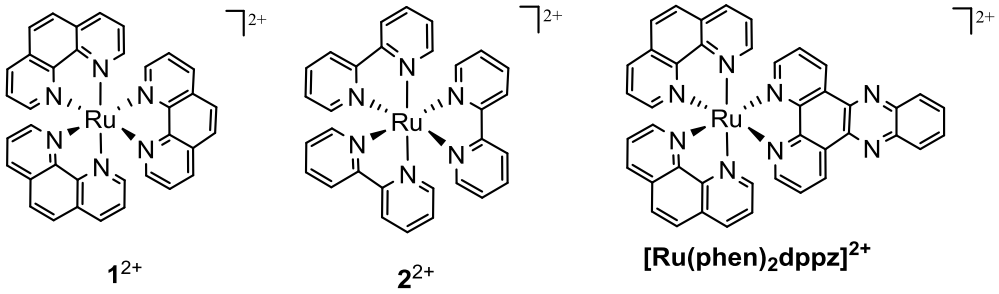


Figure 1-2 NAMI-A (Imidazolium [*trans*-imidazoledimethylsulfoxide-tetrachlororuthenate(III), KP1019 (Indazolium [*trans*-tetrachlorobis(1H-indazole) ruthenate(III)]), RDC11 ([*cis*-bis(acetonitrile)-1,10-phenanthroline-2-phenylpyridineruthenium(II)]

1.4.3 Biological activity of RPCs

The biological activity of coordinatively saturated ruthenium (II) polypyridyl complexes RPCs, such as the trisphenanthroline complex (1^{2+}) and the trisbipyridine complex (2^{2+}) shown in Figure 1-3, was extensively studied by the groups of Dwyer and Schulman[3] in the 1950's and 60's and even prior to that by Beccari in the late 1930's.[16, 30] 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,



$\mathbf{1}^{2+}$ $\mathbf{2}^{2+}$

Figure 1-3. Structures of cationic RPC's $[Ru(phen)_3]^{2+}$ (phen = 1,10-phenanthroline) (1^{2+}), $[Ru(bby)_3]^{2+}$ (bpy = bipyridine) (2^{2+}) and $[Ru^1 2dppz)]^{2+}$

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-pheanthroline 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.[36, 39] However, complexes (3^{2+}) and (4^{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 a light irradiation.[40] Recently, we have shown that the two ruthenium(II)-tatpp complexes, $[(phen)_2Ru(tatpp)]^{2+}$ (3^{2+}) and $[(phen)_2Ru(tatpp)Ru(phen)_2]^{3+}$ (4^{3+}), (shown in Figure 1-4) are effective DNA cleaving agents upon *in situ* reduction by common biological reducing agents, such as glutathione (GSH).[41] Both complexes bind to DNA through electrostatic interaction, dock in the minor grove of the DNA, then intercalate through the DNA resulting in DNA cleavage.[41]

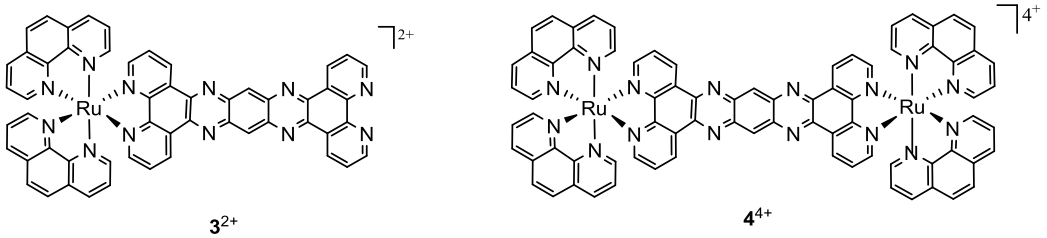


Figure 1-4 $[(phen)_2Ru(tatpp)]^{2+}$ (3^{2+}) and $[(phen)_2Ru(tatpp)Ru(phen)_2]^{3+}$ (4^{3+})

1.5.1 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 $_{SC}$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 $_{OC}$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.

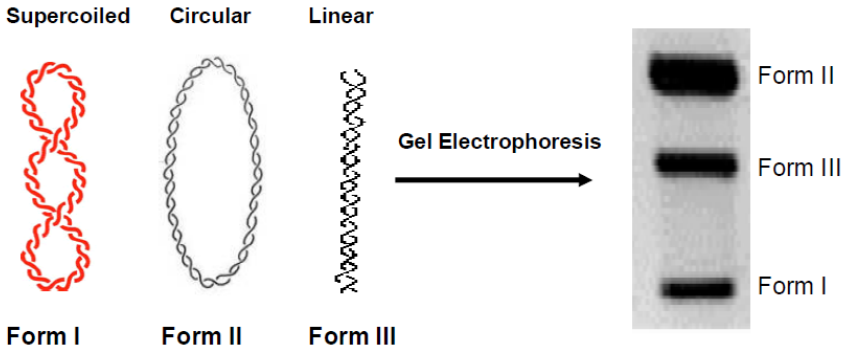


Figure 1-5 Topoisomers of plasmid DNA and the how the forms are tracked with agarose gel electrophoresis.

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.[14] 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.[14,41] Two of the RPC's synthesized, the dinuclear ruthenium (II) cationic polypyridyl complex $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ (4^{4+}) (where tatpp = 9,11,20,22-tetraazatetrapyrido[3,2-a:2',3'-c:3'',2''-l:2''',3'''-n) and the corresponding mononuclear ruthenium(II) cationic complex $[(phen)_2Ru(tatpp)]^{2+}$ (3^{2+}), have shown consistent efficacy towards tumor cells *in vivo* and *in vitro*.[41] Initial studies by Dr. Janaratne and Dr. Yadav using *in vitro* cleavage assays with 3^{2+} and 4^{3+} found they are potent DNA cleaving agents under reducing conditions. The *in vitro* cleavage assays were performed under a variety of conditions including in the presence and absence of O_2. It was found the cleaving activity of 4^{4+} was strongly attenuated upon exposure to O_2.[14, 41] To our knowledge, there are no metal-based complexes that show potentiated DNA cleavage under hypoxic conditions. Moreover, to our knowledge 3^{2+} and 4^{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^{2+} and 4^{3+} in both studies. The tumor regression xeno-graft model H358 was conducted with H358 lung cancer tumor cells resulting in appreciable tumor regression for both complexes 3^{2+} and 4^{3+}. This data is shown in Figure 2-1, 2-2 and 2-3 respectively. Figure 2-1 clearly shows 3^{2+} and 4^{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.

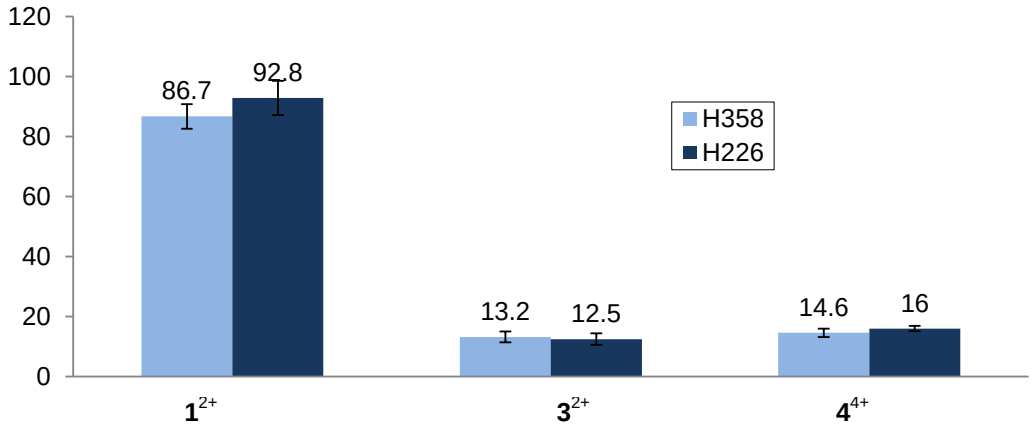


Figure 2-1 IC_{50} of rac/mix- Ru(II) complexes- monomer vs. dimer

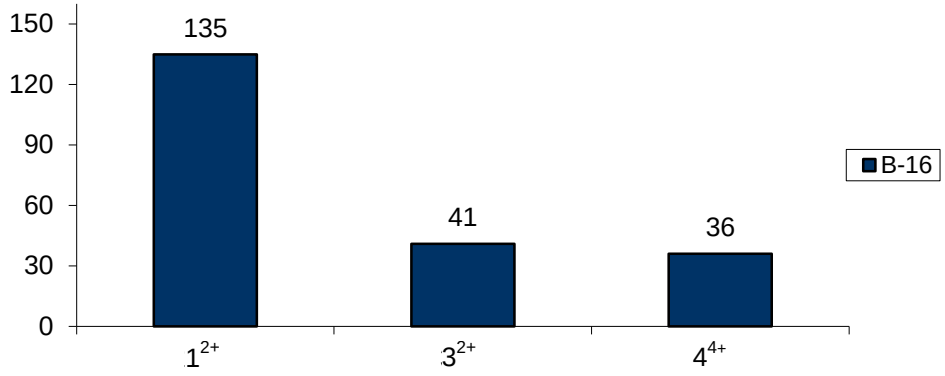


Figure 2-2 Animal toxicity data of RPC's with melanoma B-16 cells

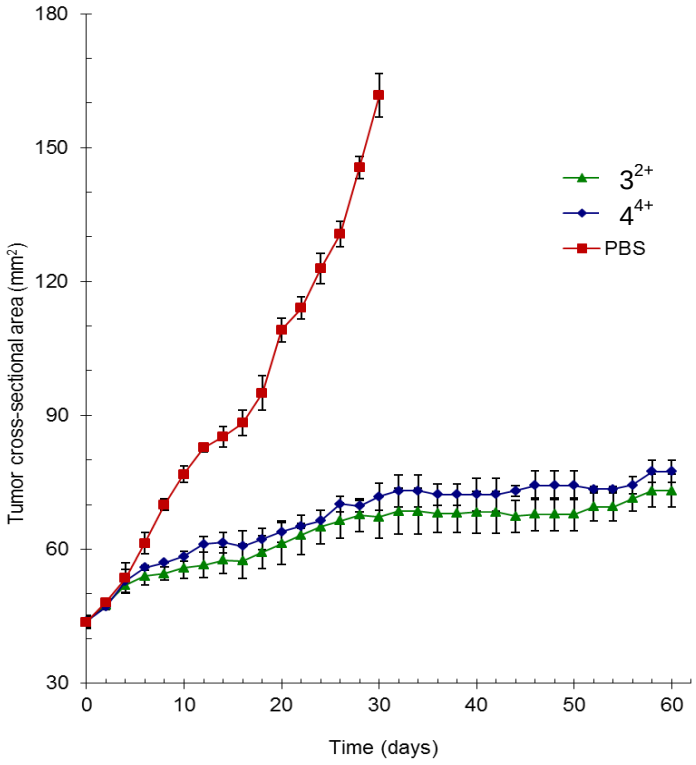


Figure 2-3 Change in a tumor volume after injecting NSCLC-H358 lung cancer cells

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.[14] 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.

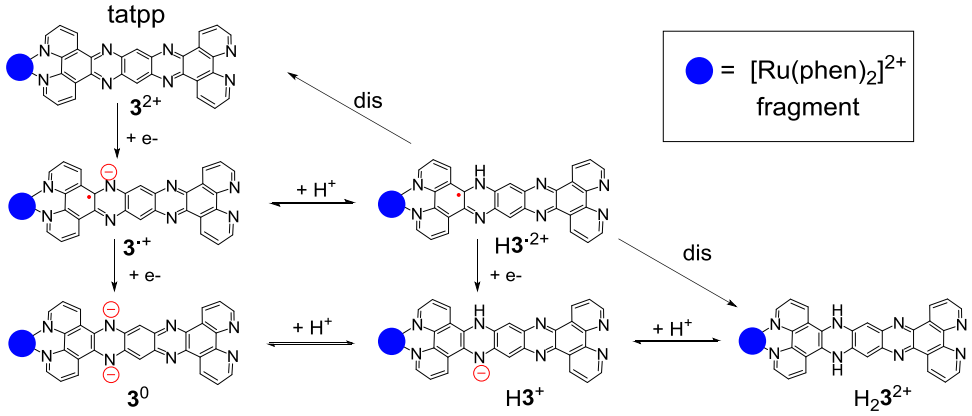


Figure 2-4 Redox and protonation isomers for 3^{2+}

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 $(H3)^{\cdot 2+}$ and $(H4)^{\cdot 4+}$ 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.[42] 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.[42] 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.

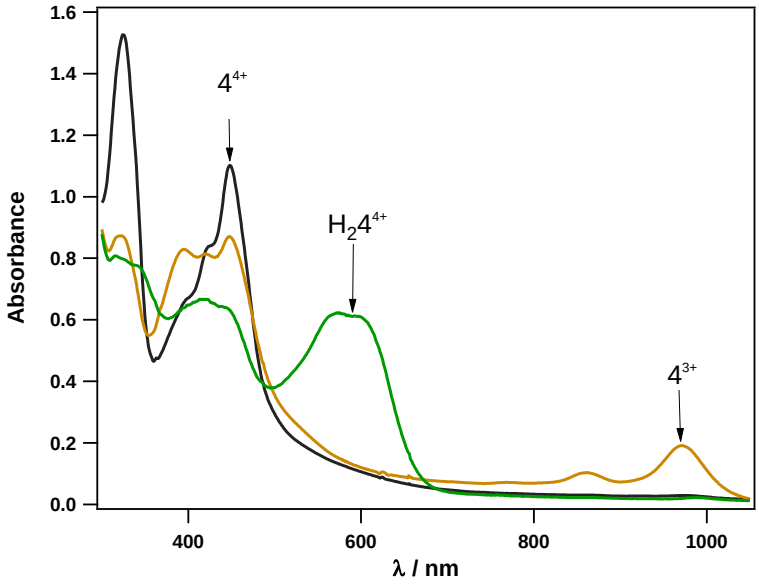


Figure 2-5 Absorption spectra of 4^{3+} and $(H_2 4)^{4+}$ in degassed water.

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 -1.1x10^{8} 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, $[(phen)_2Ru(H_2tatpp)Ru(phen)_2]^{4+}$ ($[H_24]^{4+}$) 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.

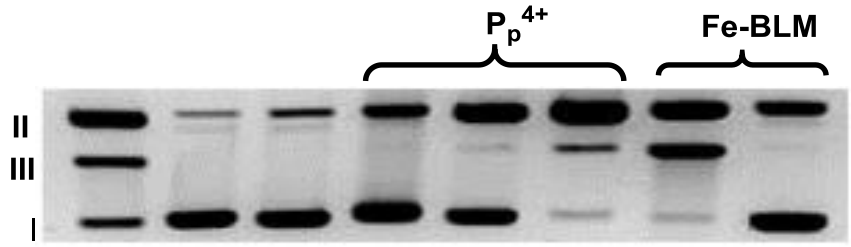


Figure 2-6 DNA cleavage assay of complex 4^{4+} under aerobic and anaerobic conditions

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_3PO_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.

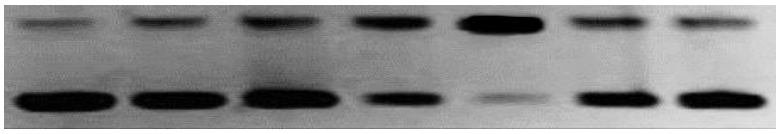
The results showed that complex 4^{4+} cleaved DNA under aerobic conditions and anaerobic conditions, but was enhanced under anaerobic conditions. To determine the species responsible for DNA cleavage Dr. Janaratne's initial studies showed the most active species involved in DNA cleavage was $(H_24)^{4+}$ 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]



1 2 3 4 5 6 7

Figure 2-7 DNA cleavage activity of $[P_p]^{4+}$ in presence of TEMPO

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 $[4]^{4+}$, GSH and TEMPO in 7 mM Na_3PO_4 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^{4+} (0.0128 mM); Lane 4: DNA, GSH (0.513 mM) plus 4^{4+}(0.0128 mM); Lane 5: DNA, GSH (0.513 mM) plus 4^{4+}(0.0128 mM) under anaerobic conditions; Lane 6: DNA, GSH (0.513 mM), 4^{4+}(0.0128 mM) plus TEMPO (2.04 mM); Lane 7: DNA, 4^{4+}(0.0128 mM) plus TEMPO (2.04 mM) under anaerobic conditions .

Figure 2-7 shows the DNA cleavage activity *in vitro* of 4^{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 $[O_2]$, 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^{4+} to further elucidate the species involved in DNA cleavage. The results of this assay by Dr. Yadav are shown in Figure 2-8.

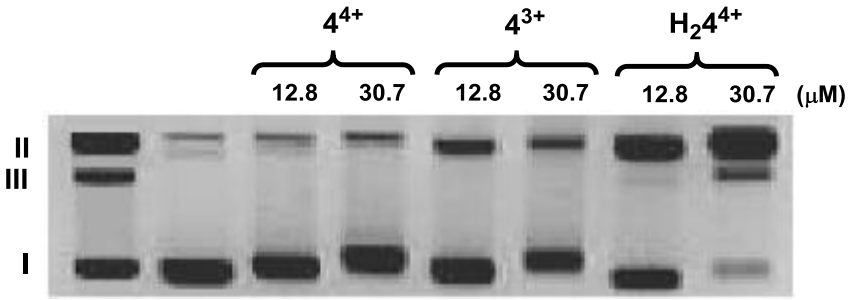


Figure 2-8 DNA Cleavage by $[4]^{4+}$, $[4]^{3+}$ and $[H_24]^{4+}$

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 $[H_24_p]^{4+}$ in a buffer of 4 mM Na_3PO_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.

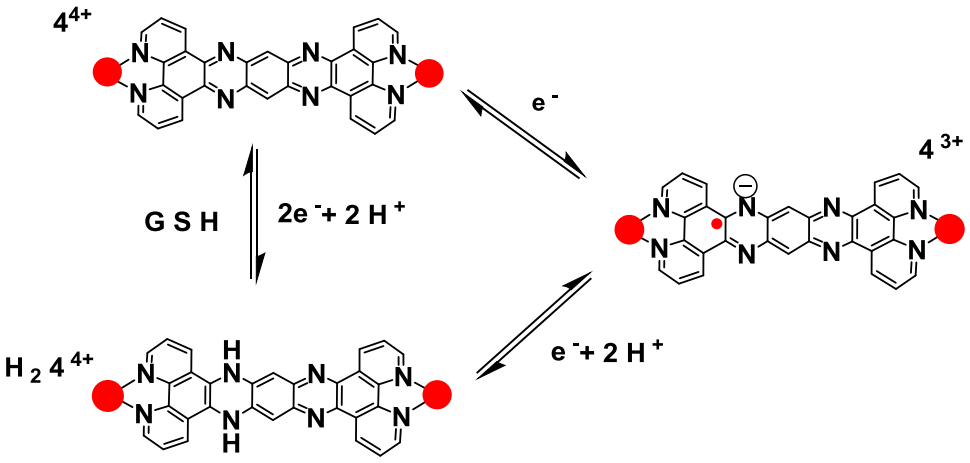


Figure 2-9: Postulated mechanism of DNA cleavage by $[4]^{3+}$ species

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 $(H_24)^{4+}$ 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, $3^{\cdot+}$ and $4^{\cdot3+}$, were more likely candidates for the cleavage reaction, which would mean that $(H_23)^{2+}$ and $(H_24)^{4+}$ 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 O_2 was present in the glovebox were it was previously assumed to contain an anaerobic environment. Figure 2-10 shows the results of this study.

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
DNA:	+	+	+	+	+
H_2MP^{2+}: 12.8 bp/M	-	+	+	+	+
DHB: 30.6 µM	-	-	-	+	+
Enyzme:	-	-	-	-	+
O_2 (conc µM):	4.0	200.0	4.0	4 .0	0.0

Incubation Time: 2hr
12.8 bp/M = 12.8 µM

Figure 2-10 Cleavage assay under varying [O_2]

As seen in Lanes 2, 3, and 4, there was an appreciable increase of circular DNA as the [O_2] is lowered from 220 µM to 4 µM. However, in the complete absence of O_2 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 3^{2+} 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 3^{2+} bound to DNA. As seen, GSH drives the complexes towards the fully reduced species whereas O_2 reoxidizes the reduced complexes such that the relative amount of the three under steady-state conditions are dictated by the GSH/O_2 ratio. In the absence of GSH, all of the complex is converted to 3^{2+}. Whereas, in the absence of O_2 but in the presence of GSH, all of the complex is reduced to $(H_23)^{2+}$, neither of which

is active for DNA cleavage. The reactive complex is the radical species $3^{\cdot+}$. 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 $(H3)^{\cdot 2+}$ such that $3^{\cdot+}$ 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.

$$\{\mathbf{3}^{2+}\ \text{DNA}\} \underset{+\ O_2}{\overset{+\ GSH}{\rightleftharpoons}} \{\mathbf{3}^{\cdot+}\ \text{DNA}\} \underset{+\ O_2}{\overset{+\ GSH}{\rightleftharpoons}} \{H_2\mathbf{3}^{2+}\ \text{DNA}\} \qquad (1)$$

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 $(H_24)^{4+}$ or $(H_23)^{2+}$ 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^{4+}

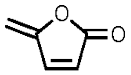
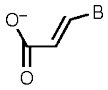
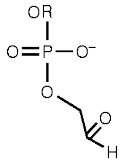
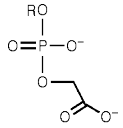
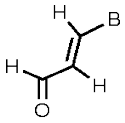
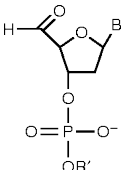
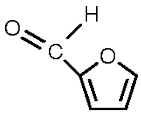
3.1 Introduction to known mechanisms of DNA cleavage.

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-deoxyribono nucleotides. [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 stand 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

Marker Product	Scission Product Name	Position of Hydrogen A bstraction	Method of Detection
	5-Methylene-2-furanone	C-1‘	HPLC
	Base propenoate	C-3‘	HPLC
	oligonucleotide 3‘-phosphoglyco-aldehyde	C-3‘	PAGE
	oligonucleotide 3‘-phosphoglycolate	C-4‘	PAGE
	base propenal	C-4‘	reaction with thiobarbituric acid
	nucleotide 5‘-aldehyde	C-5‘	PAGE
	Fufural (FUR)	C-5‘	HPLC

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, $[(phen)_2Ru(tatpp)]^{+}$ ($3^{\cdot+}$) and $[(phen)_2Ru(tatpp)Ru(phen)_2]^{3+}$ ($4^{\cdot3+}$), 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 $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$, $[4^{4+}]$ and $[(phen)_2Ru(tatpp)]^{2+}$, $[3^{2+}]$ 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 AlphaImage™ 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_2O (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_2O (18Ω) and solid sodium hydroxide (NaOH, ~30-40g) to adjust the pH to 8.0. Once the pH was adjusted ddH_2O (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(hydroxymehtyl)aminomethane]) (MW=121.1), ~800 mL of ddH_2O (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_2O (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_2O (18Ω) was added. The pH was adjusted to 7.4 with 3 M NaOH. Once pH was adjusted ddH_2O (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 uL 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-ribono-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_2O 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^{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_20/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^{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/acetone), 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_2Cl_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_2Cl_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.

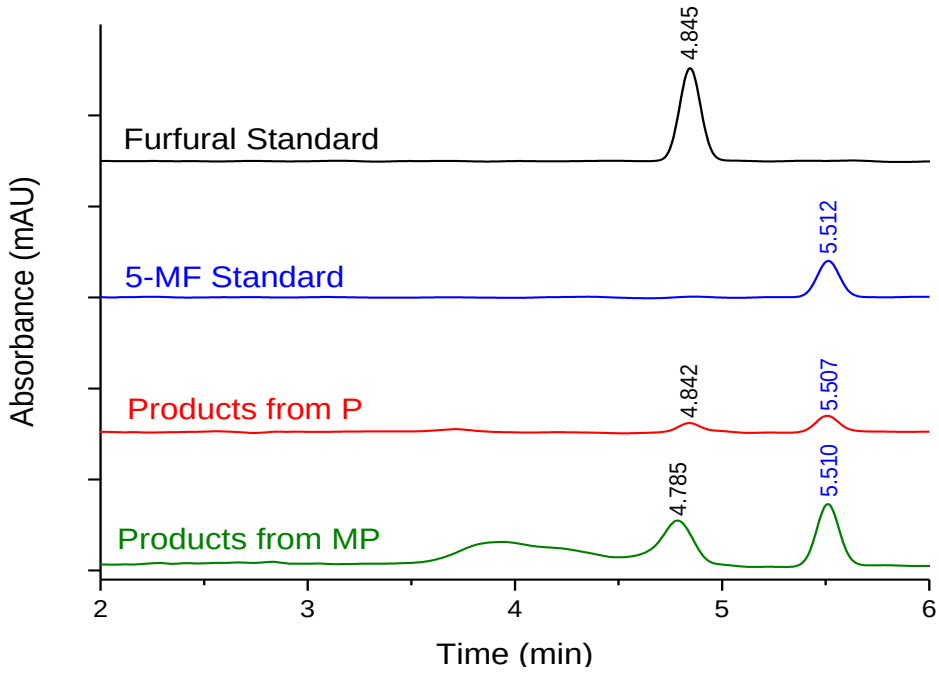
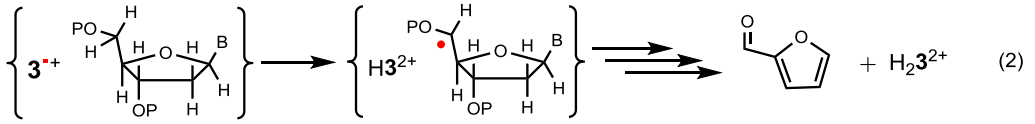
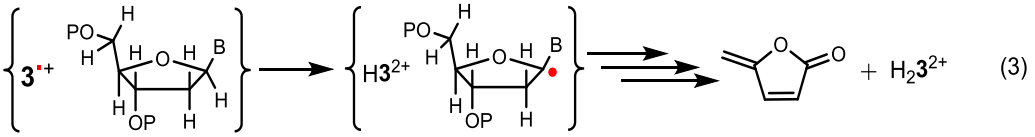


Figure 3-1 HPLC analysis of 5-MF and furfural at 254 nm

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 $(H_2\mathbf{3})^{2+}$ 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.



(2)



(3)

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+}.

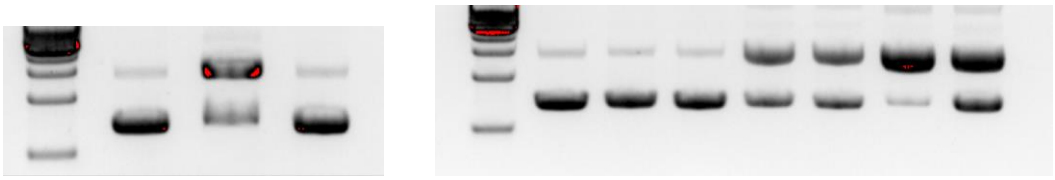


Figure 3-2 T4 ligase assay to show hydrolytic cleavage for 3^{2+} and 4^{4+}

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 contains 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.

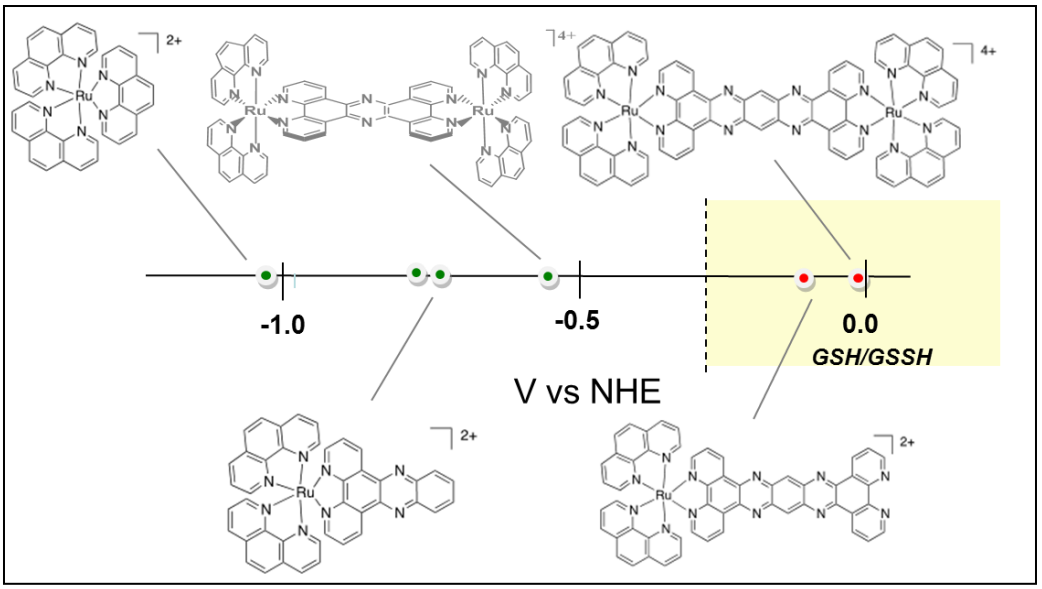
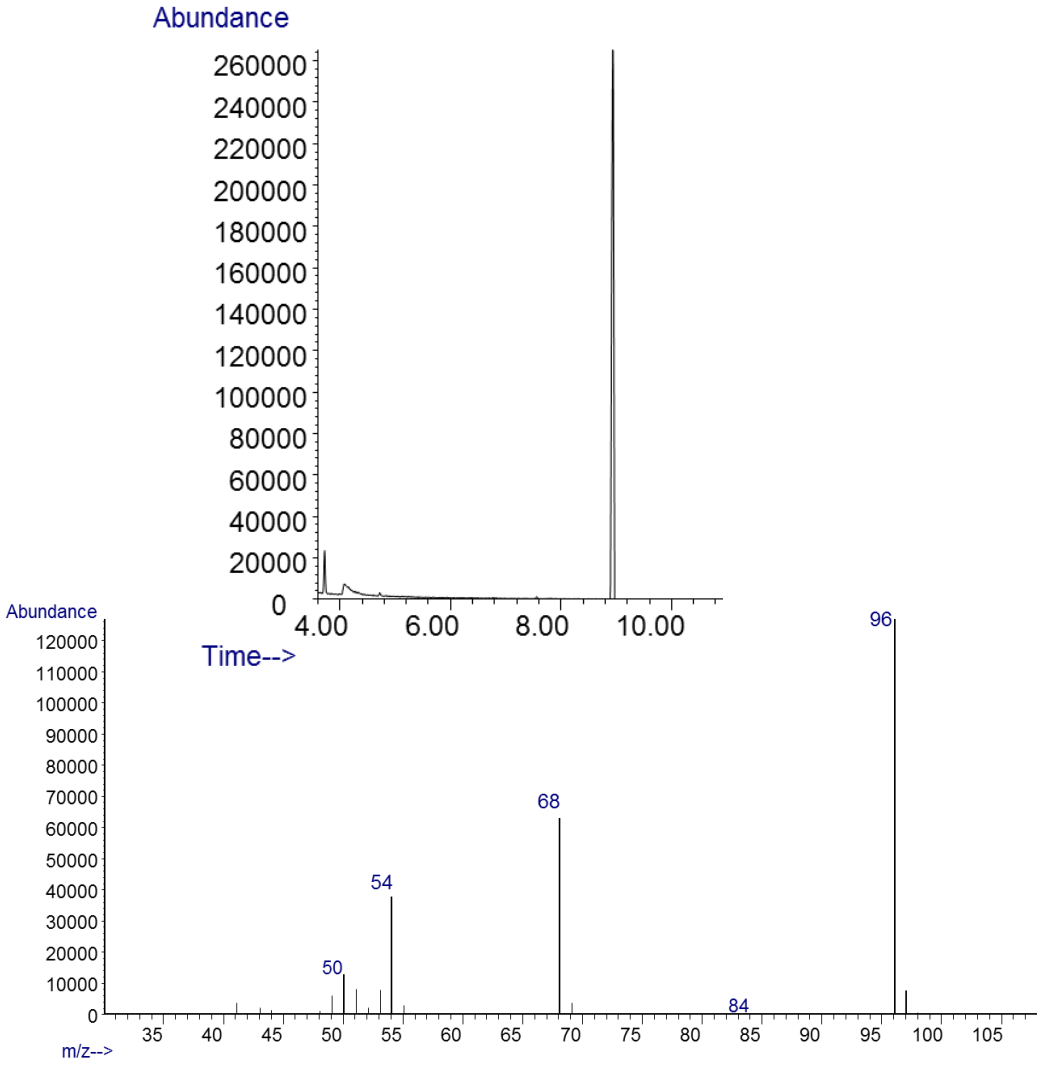


Figure 3-3 Reduction potentials of GSH and RPC's of interest

It 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 cytoxicity. 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-deoxyribono 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

1. Choi, S. Y. C.; Collins, C. C.; Gout, P. W.; Wang, Y., Cancer-generated lactic acid: a regulatory, immunosuppressive metabolite? *The Journal of Pathology* 2013, *230* (4), 350-355.

2. Yadav, A.; Janaratne, T.; Krishnan, A.; Singhal, S. S.; Yadav, S.; Dayoub, A. S.; Hawkins, D. L.; Awasthi, S.; MacDonnell, F. M., Regression of Lung Cancer by Hypoxia Sensitizing Ruthenium Polypyridyl Complexes. *Molecular Cancer Therapeutics* 2013.

3. Heron, M. P. D., Division of Vital Statistics, National Vital Statistics Reports. Deaths: Leading Causes for 2010 In *National Vital Statistics Reports*, 2013; Vol. 62.

4. Boulikas, T., *Anticancer Therapeutics*. Wiley-Blackwell A John Wiley & Sons, Ltd: 2008.

5. Elmore, S., Apoptosis: A Review of Programmed Cell Death. . *Toxicologic Pathology*
2007, *35* (4), 495-516.

6. Society, A. C. Chemotherapy Principles An In-depth Discussion of the Techniques and Its Role in Cancer Treatment http://www.cancer.org/acs/groups/cid/documents/webcontent/002995-pdf.pdf (accessed 3/18/15).

7. Zamble, D. B.; Lippard, S. J., Cisplatin and DNA repair in cancer chemotherapy. *Trends in Biochemical Sciences* 1995, *20* (10), 435-439.

8. Ishida, S.; Lee, J.; Thiele, D. J.; Herskowitz, I., Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proceedings of the National Academy of Sciences* 2002, *99* (22), 14298-14302.

9. Kristova, I., Platinum Complexes as Anticancer Agents. *Recent Patents on Anti-Cancer Drug Discovery* 2006, *1* (1), 1-22.

10. Clarke, M. J., Ruthenium metallopharmaceuticals. *Coordination Chemistry Reviews* 2003, *236* (1), 209-233.

11. Dwyer, F. P.; Goodwin, H. A.; Gyarfas, E. C., Mono- and Bis-(2,2'-bipyridine) and -(1,10-phenanthroline) Chelates of Ruthenium and Osmium. II. Bischelates of Bivalent and Tervalent Ruthenium. *Australian Journal of Chemistry* 1963, *16* (4), 544-548.

12. Dwyer, F. P.; Gyarfas, E. C.; Rogers, W. P.; Koch, J. H., Biological Activity of Complex Ions. *Nature* 1952, *170* (4318), 190-191.

13. Dwyer FP, M. E., Roe EMF, Shulman A., Inhibition of Landschuetz ascites tumor growth by metal chelates derived from 3,4,7,8 - tetramethyl - 1,10 - phenanthroline. *Br J Cancer* 1965, *19*, 195-9.

14. Yadav, A.; Janaratne, T.; Krishnan, A.; Singhal, S. S.; Yadav, S.; Dayoub, A. S.; Hawkins, D. L.; Awasthi, S.; MacDonnell, F. M., Regression of Lung Cancer by Hypoxia-Sensitizing Ruthenium Polypyridyl Complexes. *Molecular Cancer Therapeutics* 2013, *12* (5), 643-653.

15. Dwyer, F. P. G., E. C., The Chemistry of Ruthenium. Part VI. The Existence of the Tris-o-Phenanthroline Ruthenium (II) and Tris-o-Phenanthroline Ruthenium (III) Ions in Enantiomorphous Forms. *J. Proc. Roy. Soc. N. S. W.* 1949, *83*, 170-173

16. Institute, N. C. The "Accidental" Cure—Platinum-based Treatment for Cancer: The Discovery of Cisplatin. http://www.cancer.gov/aboutnci/servingpeople/cancer-research-progress/discovery/cisplatin (accessed 3/18/15).

17. Bhargava, A.; Vaishampayan, U. N., Satraplatin: leading the new generation of oral platinum agents. *Expert opinion on investigational drugs* 2009, *18* (11), 10.1517/13543780903362437.

18. Chen, H. H. W.; Yan, J.-J.; Chen, W.-C.; Kuo, M. T.; Lai, Y.-H.; Lai, W.-W.; Liu, H.-S.; Su, W.-C., Predictive and prognostic value of human copper transporter 1 (hCtr1) in patients with stage III non-small-cell lung cancer receiving first-line platinum-based doublet chemotherapy. *Lung Cancer* 2012, *75* (2), 228-234.

19. Lippert, B., *Cisplatin: chemistry and biochemistry of a leading anticancer drug*. Helvetica Chimica Acta ; Wiley-VCH: Zürich : Weinheim ; New York, 1999; p xii, 563 p.

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

lacking the copper- and cisplatin-binding ecto-domain. *Proceedings of the National Academy of Sciences* 2013, *110* (46), E4279-E4288.

21. Allardyce, C. S.; Dyson, P. J., Ruthenium in medicine: current clinical uses and future prospects. *Platinum Metals Review* 2001, *45* (2), 62-69.

22. Ang. W.E., D. P. J., Classical and Non-Classical Ruthenium-Based Anticancer Drugs: Towards Targeted Chemotherapy. *Eur. J. Inorg. Chem.* 2006, *2006* (20), 3993-4192.

23. Jakupec, M. A.; Galanski, M.; Arion, V. B.; Hartinger, C. G.; Keppler, B. K., Antitumour metal compounds: more than theme and variations. *Dalton Transactions* 2008, (2), 183-194.

24. Hartinger CG, J. M., Zorbas-Seifried S, Groessl M, Egger A, Berger W, et al., KP1019, a new redox-active anticancer agent – preclinical development and results of a clinical phase I study in tumor patients
Chem Biodivers 2008, *5*, 2140-55.

25. Zava, O.; Zakeeruddin, S. M.; Danelon, C.; Vogel, H.; GrÃ¤tzel, M.; Dyson, P. J., A cytotoxic ruthenium tris (bipyridyl) complex that accumulates at plasma membranes. *ChemBioChem* 2009, *10* (11), 1796-1800.

26. Liu, Y.-J.; Zeng, C.-H.; Liang, Z.-H.; Yao, J.-H.; Huang, H.-L.; Li, Z.-Z.; Wu, F.-H., Synthesis of ruthenium(II) complexes and characterization of their cytotoxicity in vitro, apoptosis, DNA-binding and antioxidant activity. *European Journal of Medicinal Chemistry* 2010, *45* (7), 3087-3095.

27. Dwyer, F. P. G., E. C., Persistance of Optical Activity in an Oxidation-Reduction Reaction
Nature 1949, *163*, 918.

28. Blunden, B. M.; Rawal, A.; Lu, H.; Stenzel, M. H., Superior Chemotherapeutic Benefits from the Ruthenium-Based Anti-Metastatic Drug NAMI-A through Conjugation to Polymeric Micelles. *Macromolecules* 2014, *47* (5), 1646-1655.

29. Antonarakis, E.; Emadi, A., Ruthenium-based chemotherapeutics: are they ready for prime time? *Cancer Chemotherapy and Pharmacology* 2010, *66* (1), 1-9.

30. Morris RE, A. R., Murdoch PdS, Chen H, Cummings J, Hughes ND, et al., Inhibition of cancer cell growth by ruthenium(II) arene complexes. *J Med Chem* 2001, *44*, 3616-21.

31. Meng X, L. M., Jenny M, Gross I, Benosman S, Fricker B, et al, Meng X, Leyva ML, Jenny M, Gross I, Benosman S, Fricker B, et al. *Cancer Res* 2009, *69*, 5458-66.

32. Lentz F, D. A., Lindauer A, Henke M, Hilger RA, Hartinger CG, et al, Pharmacokinetics of a novel anticancer ruthenium complex (KP1019, FFC14A) in a phase I dose-escalation study. *Anticancer Drugs* 2009, *20*, 97-103.

33. Shulman A, D. F., Metal chelates in biological systems. In: Dwyer FP, Mellor DP , editors. Chelating agents and metal chelates. *New York, NY: Academic Press* 1964, 383-439.

34. E., B., Pharmacological studies on the ferrous tri-2,2′-bipyridyl complex. IV. Relation between concentration in the blood and action on the central nervous system. *Boll Soc Ital Biol Sper* 1941, *16*, 216-18.

35. Augustyn KE, P. V., Barton JK, editors, Metallointercalators as probes of DNA recognition and reactions. Wiley encyclopedia of chemical biology London, England. John Wiley & Sons, Inc: 2009.

36. Koch JH, R. W., Dwyer FP, Gyarfas EC, The metabolic fate of tris-1,10-phenanthroline ruthenium-106 (II) perchlorate, a compound with anticholinesterase and curare-like activity. *Aust J Biol Sci* 1957, *10*, 342-50.

37. Koch, J. H.; Gallagher, C. H., Effect of some neuromuscular blocking agents on mitochondrial enzyme systems. *Nature* 1959, *184*, 1039.

38. Shulman, A. L., G.A., , Action of 1,10-phenanthroline transition metal chelates on P388 mouse lymphocyte leukaemic cells. *Chem Biol Interact* 1977, *16* (1), 89-99.

39. Koch JH, G. E., Dwyer FP., Biological activity of complexions. Mechanism of inhibition of acetylcholinesterase. *Aust J Biol Sci* 1956, *9*, 371-81.

40. White, D. O. H., A.W.; Cheyne, I.M.;Shew, M., , Actions of metal chelates of substituted 1,10-phenanthrolines on viruses and cells 3. Actions on cultured cells. *Aust J. Exp.Biol. Med. Sci.* 1969, *47(1)*, 81-89.

41. Janaratne, T. K.; Yadav, A.; Ongeri, F.; MacDonnell, F. M., Preferential DNA Cleavage under Anaerobic Conditions by a DNA-Binding Ruthenium Dimer. *Inorganic chemistry* 2007, *46* (9), 3420-3422.

42. de Tacconi, N. R.; Lezna, R. O.; Chitakunye, R.; MacDonnell, F. M., Electroreduction of the Ruthenium Complex [(bpy) 2Ru (tatpp)] Cl2 in Water: Insights on the Mechanism of Multielectron Reduction and Protonation of the Tatpp Acceptor Ligand as a Function of pH. *Inorganic chemistry* 2008, *47* (19), 8847-8858.

43. Rajput, C.; Rutkaite, R.; Swanson, L.; Haq, I.; Thomas, J. A., Dinuclear monointercalating RuII complexes that display high affinity binding to duplex and quadruplex DNA. *Chemistry-A European Journal* 2006, *12* (17), 4611-4619.

44. Tronche.C, G. K., Greenberg.M, DNA damage induced via independent generation of the radical resulting from formal hydrogen atom abstraction from the C1'-position of a nucleotide. *Chemistry and Biology* 1998, *5* (5), 263-271.

45. Pogozelski, W. K.; Tullius, T. D., Oxidative Strand Scission of Nucleic Acids: Routes Initiated by Hydrogen Abstraction from the Sugar Moiety. *Chemical Reviews* 1998, *98* (3), 1089-1108.

46. Jae-Tae.H, T. K., Greenberg.M, The reactivity of the 2-deoxibribonolactone lesion in single stranded DNA and its implication in reaction mechanisms of DNA Damage and repair. *Nucleic Acid Research* 1999, *27* (19), 3805-3810.

47. Zelenko, O.; Gallagher, J.; Xu, Y.; Sigman, D. S., Chemical Nuclease Activity of 1,10-Phenanthroline−Copper. Isotopic Probes of Mechanism. *Inorganic Chemistry* 1998, *37* (9), 2198-2204.

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Cynthia Griffith was born in the United States in the State of Texas. She graduated from Nimitz High School in 1991. She returned to 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.