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DETERMINATION OF MITOCHONDRIAL TOXICITY AND POTENTIAL APOPTOTIC PATHWAY OF RUTHENIUM POLYPYRIDYL COMPLEXES [(phen)2Ru(tatpp)]Cl2 AND [(phen)2Ru(tatpp)Ru(phen)2]Cl4

Ali Mohamedi

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by

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ABSTRACT

DETERMINATION OF MITOCHONDRIAL TOXICITY AND POTENTIAL APOPTOTIC PATHWAYS OF RUTHENIUM POLYPYRIDYL COMPLEXES $[(phen)_2Ru(tatpp)]Cl_2$ AND $[(phen)_2Ru(tatpp)Ru(phen)_2]Cl_4$

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Faculty Mentor: Frederick Macdonnell

The solution to cancer has been elusive due to the toxicity inadvertently involved with healthy cells and the increasing level of resistance to current chemotherapeutics. This includes the gold standard cancer therapeutics such as the platinum based complex, cisplatin. As a result, other alternative metal pharmaceuticals, such as ruthenium-based complexes, have been sought after due to their low cytotoxicity profile towards healthy cells and increased cytotoxicity towards malignant cells. Previous *in cellulo* experiments showed the ruthenium polypyridyl complexes $[(phen)_2Ru(tatpp)]Cl_2$ (MP^{2+}) and $[(phen)_2Ru(tatpp)Ru(phen)_2]Cl_4$ (P^{4+}) accumulating in the mitochondria. Here the complexes were tested to see if both MP^{2+} and P^{4+} are mitochondrial toxins due to their accumulation in the mitochondria and whether the mitochondria is a main site of action of

one or both complexes. In addition the investigation included insight into interplay between the complexes and the Warburg effect in galactose based medium.. The non -small cell lung cancer cell line H358 was chosen due to its deficiency in the tumor suppressor gene p53, inevitably negating any effects of p53 mediated apoptosis by certain mitochondrial drugs. The Promega Mitochondrial ToxGlo multiplex assay was used to measure the degree of membrane permeability and adenosine triphosphate (ATP) synthesis decoupling from ATP synthase, resulting in either apoptosis or necrosis. Due to both P^{4+} and MP^{2+} currently being filed for a patent and current data unpublished, only the controls Oligomycin, digitonin, and ruthenium polypyridyl complex DIP were discussed. Oligomycin was replicated as a mitochondrial poison, digitonin as a necrotic agent, and DIP as a weak mitochondrial poison. All three matched their respective toxicity profiles seen in previous experiments validating the experiment as well as helping to elucidate a deeper outlook of the mechanism of action of P^{4+} and MP^{2+}.

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CHAPTER 1

INTRODUCTION

1.1 Ruthenium Polypyridyl Complexes

Since the work done by Francis Dwyer in the 1950's on metal based therapeutics, metal based complexes have been shown to have a variety of properties ranging from chemotherapy, imaging of cells, topical agents, and even antimicrobials (1). Success shown by platinum based complexes, such as clinical gold-standard chemotherapeutic cisplatin (cis-$Pt(NH_3)_2Cl_2$) (Figure 1.1) and its derivatives in treating malignancies, have led to the investigation of other possible chemotherapeutic metal based complexes such as those formed by ruthenium (1,2,3,4). Specifically, ruthenium polypyridal complexes (RPC's) have been shown as effective anticancer therapeutics due to the stable octahedral nature of the ruthenium cation and their ability to bind critical biopolymers such as DNA *in vivo* (1,5). These include complexes such as NAMI-A and KP1019, both in clinical trials Phase I and Phase II respectively. They have also been used in cellular based imaging and photodynamic therapy as shown by Dr. Jim Thomas et. al (5). These complexes are similar to platinum based complexes in reactivity, but have been shown to be less cytotoxic then their platinum counterparts avoiding unwanted side effects such as inhibition of acetylcholinterase that can lead to neurotoxicity and subsequent neurological damage (2,3,4). Due to their affinity for serum proteins such as albumin and transferrin, and similarity to iron, the compounds are more selective than platinum-based complexes (3,5).

Two specific RPC's have been created in Dr. Frederick Macdonnell's lab including $[(phen)_2Ru(tatpp)]^{2+}$ (MP^{2+}) and $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ (P^{4+}), using a bridging ligand, tetraazatetrapyridopentacene (tatpp) , that has been linked to antiproliferative activities such as regression of lung cancer under hypoxic conditions with the cell line H358 (Table 1.1) (Figure 1.1) (6). Due to the anticancer therapeutic potential the drugs were further studied to see their activities against DNA, mechanism of entry into a cell, as well as possible sites of action. The work done by Dr. Yadav in Dr. Macdonnell's lab showed that the complexes P^{4+} and MP^{2+} have been effective as agents that bind and cleave DNA and are redox active (7). Ruthenium polypyridyl complexes have also been investigated for the mechanism of transport and action previously mostly done by the work of Dr. Jacqueline Barton and Dr. Cindy Puckett by using the fluorescent complex $Ru(DIP)_2dppz^{2+}$, which has been used as a control in our own lab and was shown to be a mitochondrial poison (8,9). Modeled after this work, PhD candidate, Adam Dayoub, conducted a similar study with P^{4+} and MP^{2+} showing mechanisms of entry of active transport using clathrin coated vesicles, lipid rafts, and G-protein mediation inhibitors in addition to passive diffusion as major contributors (10). This work was further supplemented by the work of Dr. Nagham Alatrash showing the complexes P and MP to be very lipophilic increasing their potential as future therapeutics (11). It has been recently hypothesized that P^{4+} has an unknown apoptotic mechanism linked to binding nuclear DNA damage with the possibility of subsequent mitochondrial induced damage. The drug MP^{2+} has also shown promise as a mono-ruthenium complex in apoptotic cancer cells as shown by confocal laser scanning microscopy (CLSM), and possibly driven by mitochondrial induced apoptosis (5,10). From these previous studies and results we wanted

to further study the effects on the mitochondria by the two RPC's and determine whether there is a correlation between ATP uncoupling and membrane integrity of the cells when the drugs are imposed. This brings us to the Warburg Effect.

1.2 The Warburg Effect

Tumor causing and malignant immortalized cell lines tend to heavily rely on glycolysis for ATP production, maintaining metabolic growth, and homeostasis. Interestingly, although glycolysis is known to produce fewer amounts of ATP than oxidative phosphorylation, it is more heavily relied upon during tumorigenesis despite the presence of intact mitochondria in malignant cell lines (12). Cancer cell lines have also been shown to frequently switch between glycolysis and mitochondrial oxidative phosphorylation depending on the concentration of glucose available in the cell. This is commonly referred to the "Crabtree Effect" and goes hand in hand with the Warburg Effect (13). It also well known that during normal mitochondrial respiration ROS species are formed, which the malignant cells tend to avoid or neutralize (14). This particular phenomenon is known as the Warburg Effect and is seen in many different forms of cancer and multiple malignant cell lines. Since glycolysis is an anaerobic process, many cell lines have also been shown to survive or thrive under hypoxic conditions (15,16). As shown in the study done for the regression of lung cancer (Yadav et. al), many of the cell lines such as H358 and HCC-2998 in particular were unchanged in growth with no decrease in viability under hypoxic vs. normoxic conditions (7). Both drugs MP and P were shown to be effective against cancerous cells under hypoxic conditions giving further evidence that both drugs could be effective at controlling and killing aggressive "hard to reach" tumors (7). In a previous study shown to test for the Warburg Effect, several known mitochondrial

toxins were tested to see their effects on both the mitochondria and cell viability (17) (Figure 1.1). When grown under normal high glucose conditioned RPMI media, some of the drugs caused little drop in ATP production with varying levels of destruction of membrane integrity. This showed that cells are indeed under the Warburg Effect and are utilizing glycolysis as a main source of ATP production as evident by the steady or increased rate of ATP being produced despite the presence of the toxin (12). Another similar study was conducted to test for membrane integrity using fluorogenic substrates and it was determined that these two tests can in fact be combined to give more data (7). It is here where we were able to conduct a multiplex assay using Promega's Mitochondrial ToxGlo kit to assess both the level of ATP production and membrane structural integrity.

Here we will discuss the possible effects of both drugs alongside other known control mitochondrial poisons and the implications of apoptosis vs. necrosis on cancerous cells and surrounding tissue *in vitro.* This will further enlighten the possible mechanism of action of P^{4+} and MP^{2+} *in vitro* and how this might affect chemotherapeutics for future reference in metal therapy concerning transition metal chemistry. It is the lab's hope that by elucidating the mechanism of action of P^{4+} and MP^{2+} that a path to more *in vivo* studies and clinical trials may be paved in the future. Hopefully these drugs will be another safe and effective addition to the wide plethora of chemotherapeutics available today in the clinical sphere.

Table 1.1: Cytotoxicity of RPC's IC_{50} of H358 Non-Small Cell Lung Carcinoma

Cytotoxicity of Ruthenium Polypyridyl Complexes on H358 Cell Line	
RPC and Control	IC_{50} Values for H358 (μM)
mix-$[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ (P)	15.2 ± 1.8
rac-$[(phen)_2Ru(tatpp)]^{2+}$ (MP)	13.2 ± 1.8
rac-$[Ru(DIP)_2dppz]^{2+}$ (DIP)	≈1-2
rac-$[Ru(phen)_3]^{2+}$	86.7 ± 4.1
Oligomycin from *S. diastochromogenes*	≈1.0

The IC_{50} values shown here are specifically for the bronchiole alveolar malignant cell line H358 for the drugs P, MP, and DIP. The IC_{50} value is also shown for the drug $[Ru(phen)_3]^{2+}$, which was one of the first ruthenium polypyridyl complexes shown to have a cytotoxic effect towards malignant cell lines as well as infectious bacteria by Dr. Francis Dwyer in the 1950's (1). The abbreviation rac stands for racemic mixture, and although the ruthenium complexes have been shown to be separable into enantiomeric forms, only racemic mixtures of the compounds were tested (6,7). This data was found by Dr. Abishek Yadav, during his tenure in Dr. Macdonnell's lab.

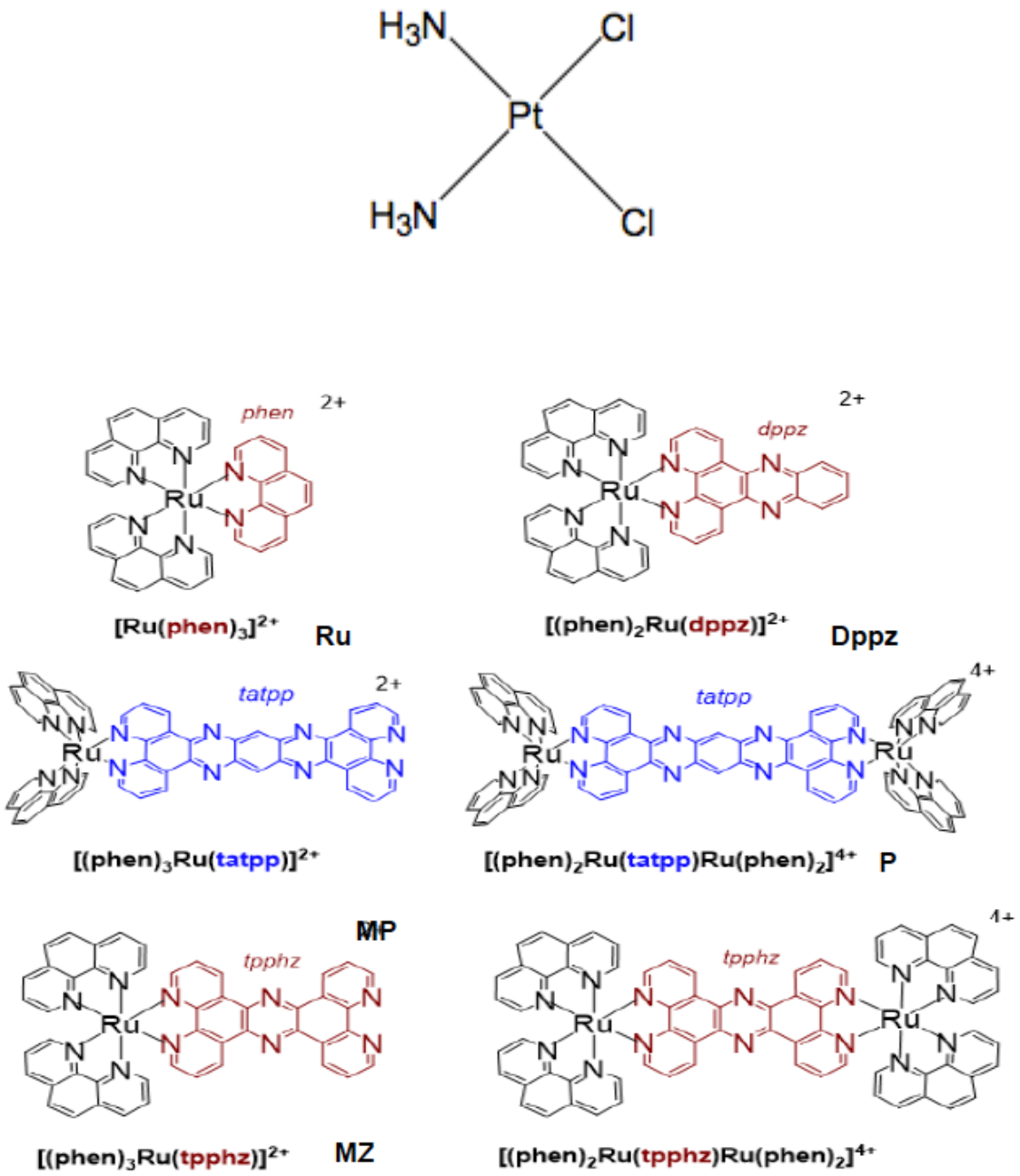

Figure 1.1: The Chemical Structure of Cisplatin and RPC's (Ruthenium Polypyridyl Complexes) Commonly Used in the Fred Macdonnell Lab Including P^{4+} and MP^{2+} (6,7,10)

CHAPTER 2

METHODOLOGY AND MATERIALS

2.1 Synthesis of RPC's

Synthesis of P^{4+} and MP^{2+} ruthenium polypyridyl complexes was carried out as mentioned before in previous experiments done by the Macdonnell lab (6).

2.2 Chemical Reagents

The ruthenium complexes were stored in stock solutions like all the other drugs. All control mitochondrial poisons were obtained from Sigma Aldrich including Oligomycin from *Streptomyces diastatochromogenes* (Sigma O4876), Carbonyl cyanide 3-cholorphenylhydrazone (CCCP)(Sigma C2759), Antimycin A from *Streptomyces sp.* (Sigma A8674), and sodium azide (NaN_3) (Sigma S2002). The carbohydrate D-(+)-galactose was also obtained from Sigma Aldrich (Sigma G0750).

2.3 Cell Culture Line Growth and Maintenance

The p53 deficient non-small cell lung cancer line NCI-358m H358 was grown from liquid nitrogen cryogenic storage in 100 mm^2 Falcon gamma sterilized tissue culture dishes (Fisher Scientific 08-772E). The cell line was maintained using Roswell Park Memorial Institute-1640 500 ml. growth medium supplemented with L-glutamine, glucose, and sodium bicarbonate buffer (Sigma R8758). The medium was further supplemented with 5.5 ml. Penicillin-Streptomycin solution (Sigma P4333), 0.5 ml of RPMI-1640 100X Vitamin solution (Sigma R7256), and 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma F4135). The cell line was kept in a Heracell incubator throughout growth at 5% CO_2 and

37°C simulating physiological conditions. The cell line was subcultured and passaged according to ATCC cell culture guidelines to maintain sufficient and constant growth and malignancy. All cell culture work was performed in a sterile environment in a Biosafety level 2 sterile hood using 70% ethanol as disinfectant to provide protection against outside infection.

2.4 Preparation of Stock Solutions

The ruthenium complexes were initially prepared into stock solutions consisting of 600 μM concentrations by dissolving the compounds in 100 μl of DMSO and then supplementing them with RPMI supplemented growth medium. The same was done for the control mitochondrial poisons making stock solutions consisting of 6 mM Oligomycin, 157 mM CCCP, 17 mM Antimycin A, and 12 mM NaN_3. These same stocks were then made into 300 μM stock solutions using Dulbeco's Modified Eagle's Medium (DMEM) without glucose and supplemented with 5 mM D-(+)-galactose (Sigma G0750) (17). It should be noted that the DMEM medium supplemented with D-(+)-galactose was not supplemented with FBS, unlike the RPMI-1640 growth medium, due to FBS containing a high concentration of glucose. All compounds were stored at a temperature of -20ºC and were thawed using a 37ºC water bath. Oligomycin was chosen as the primary mitochondrial control toxin to be used along side P, MP, and DIP due to it being the only control not having a mechanism of apoptosis driven by the tumor suppressing gene p53 (18).

2.5 Promega Mitochondrial ToxGlo Assay

The malignant cell line H358 was first grown in a 96 well Greiner flat-bottom sterile plate at 37ºC and 5% CO_2 over a period of 24 hours. A cell count of approximately

40,000 cells, with a total volume of 180 µl, was done for each well. The cell count was performed using the viability stain Trypan Blue (Sigma T1845) and a hemocytometer. After 24 hours of incubation, following the work of Marroquin et. al and Rodriguez et. al, the plate was dosed with DMEM medium supplemented with galactose with 100 µl in each well to induce the Warburg Effect (17,19). A second 96 well plate was used to make two fold serial dilutions of the compounds mentioned before starting at 40 µM by adding 100 µl volumes of culture medium to all wells, and then following the layout provided by the manufacturer's protocol (Promega G8000) (Table 1). A volume of 100 µl was transferred from each of the three columns for each drug (each drug was done in triplicate) to create a two fold a serial dilution with the last 100 µl being discarded. On row H, columns 4-12, the wells served as untreated medium controls for ATP chemistry (Table 1). The positive toxicity control provided by the kit, Digitonin, was subsequently diluted to 800 µg/ml in galactose supplemented DMEM medium and added in 10 µl volumes to row H columns 1-3. A second two fold serial dilution was carried out by finally adding the diluted compounds from the stock 96 well plate to the plate with H358 cells. The plate was incubated at 37ºC following the manufacturer's protocol accordingly. Then continuing to follow the manufacturer's protocol, the 5X Cytotoxicity reagent provided was used to measure the proportion of membrane integrity based on the protease activity of the fluorogenic substrate bis AAF-R110 (20). Fluorescence was measured at 485 nm. excitation and 520-530 nm. emission. The plate was then measured for ATP production using the kit's ATP Detection reagent following the manufacturer's protocol while shaking. Luminescence was measured to find the proportion of ATP produced by the cells. Both fluorescence and luminescence were measured using the FluoStar Omega spectrophotometer courtesy of Dr. Subranghsu

Mandal's lab. For both forms of measurement, the gain had to be adjusted for optimal measurement. Fluorescence and luminescence were measured in relative fluorescence units (RFU).

2.6 Data Analysis and Compilation

Data Analysis was performed using the program Microsoft Excel. Standard statistical methods were applied including the student's t-test, F-test, standard deviation, and ANOVA etc. The data compiled was compared to control data for the control mitochondrial toxins CCCP, imprimine, and digitonin (all arrest mitochondrial oxidative phosphorylation). This was used in comparison to see the damage done by the ruthenium complexes and if the damage was apoptotic or necrotic induced. Data was compiled according to the logarithmic method shown by Promega for comparison and proper analysis. It should be noted that the data during compilation was inverted due to the nature of the serial dilution being from high concentrations to subsequent low concentrations when performing the dilution.

Table 2.1: 96 Well Stock Plate Layout of Mitochondrial ToxGlo Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Oligo	Oligo	Oligo	P^{4+}	P^{4+}	P^{4+}	MP^{2+}	MP^{2+}	MP^{2+}	DIP	DIP	DIP
B	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2
C	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
D	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
E	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
F	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
G	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
H				UT	UT	UT	UT	UT	UT	UT	UT	UT

The stock plate that was used to create the initial drug dilution of the mitochondrial poison oligomycin (Oligo) and the ruthenium complexes P^{4+}, MP^{2+}, and DIP is shown with serial dilution ratios. A two-fold serial dilution was carried out using a multichannel pipette going down row A-G using the concentrations mentioned before of 40 μM, 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM, and 0.625 μM. These values would then be halved again in the experimental plate. The abbreviation UT stands for untreated control (i.e. only Galactose supplemented DMEM media, no complex) that would remain the same in the experimental plate. The wells H1-H3 were reserved for Digitonin (800 μg/ml) that was added in the experimental plate.

Table 2.2: 96 Well Experimental Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Oligo	Oligo	Oligo	P^{4+}	P^{4+}	P^{4+}	MP^{2+}	MP^{2+}	MP^{2+}	DIP	DIP	DIP
B	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2
C	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
D	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
E	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
F	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
G	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
H	D	D	D	UT	UT	UT	UT	UT	UT	UT	UT	UT

The experimental plate that was used to create the initial drug dilution of the mitochondrial poison, oligomycin (Oligo), and the ruthenium complexes P^{4+}, MP^{2+}, and DIP is shown with serial dilution ratios. Another two-fold serial dilution was carried out halving the previous concentrations used to 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, and 0.3125 μM.. The wells H1-H3 were reserved for Digitonin (D) (800 μg/ml) that was added to the experimental plate as a positive toxicity control comparison.

CHAPTER 3

EXPERIMENTAL RESULTS

The results of this experiment concerning the ruthenium polypyridyl complexes P^{4+} and MP^{2+} cannot be discussed due to lab's pursuance of a patent of both complexes as well as this data currently being unpublished. Subsequently, the results of the controls Oligomycin (Figure 3.1), Digitonin (Figure 3.2), and DIP (Figure 3.3) will be discussed with a comparison against toxcitiy profiles provided by Promega. ATP production and Membrane integrity were based off relative fluorescence units (RFU) based on the fluorescence of bis-AAF-R110 and the complexes themselves. Oligomycin showed significant cytotoxicity with the inhibitory concentration of 50% of the cell population (IC_{50}) previously reported at 1 μM with higher concentrations of the drug showing increase in relative fluorescence units (>200-250) (RFU) for membrane integrity, with a constant RFU (<100) value for ATP production (Figure 3.4). No sign of membrane integrity being compromised was seen initially, however as the concentration reached 20 μM the RFU values were shown to rise. Similarly, we also saw the ruthenium control DIP show significant mitochondrial toxicity at a concentration of 1μM (IC_{50} for H358 cell line) (Figure 3.5). The other toxicity profiles shown by Promega revealed the different possibilities that occur showing mitochondrial permeability vs. ATP production (22, Figure 3.6) (Figure 3.7). This data was used to compare the ruthenium complexes data. All three controls DIP, Oligomycin, and digitonin in the experiment matched the similar toxicity profiles given by Promega indicating a valid experimental analysis (Figure 3.6).

The digitonin profile was also similar to the profile given by Promega displaying a reduction in ATP production levels and simultaneous rise in membrane integrity (Figure 3.7). The ATP levels were shown to be slightly higher initially at the point of dosage (~300-350 RFU) for DIP, with the lowest concentration of drug, but showed a steady decrease in ATP production. The known mechanisms of mitochondrial toxicity were used to theorize about the possibilities of looking into further sites of action in future studies such as nuclear regions and other organelles in the cell (6).

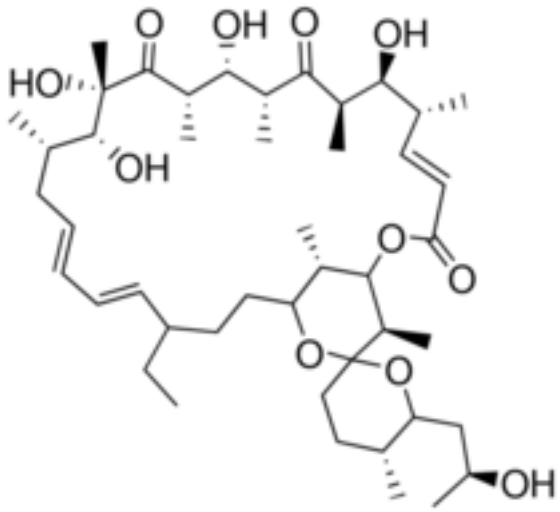

Figure 3.1: Chemical Structure of Oligomycin from *Streptomyces diastochromogenes* (Sigma-Aldrich O4786)

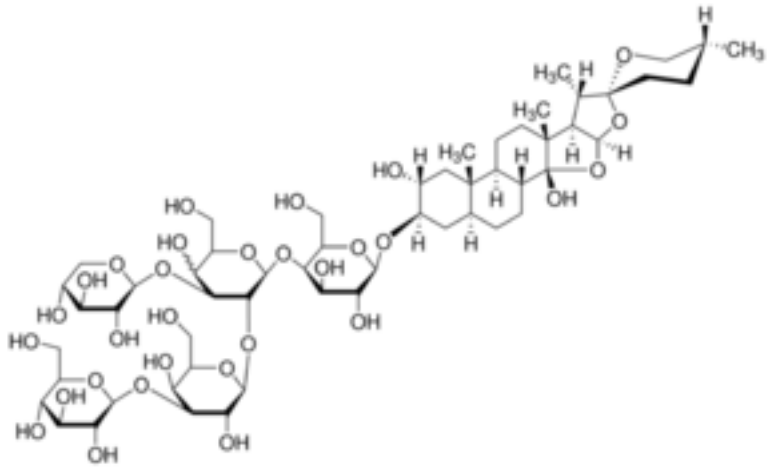

Figure 3.2: Chemical Structure of Digitonin
Digitonin was provided in Promega's Mitochondrial ToxGlo Kit (Sigma Aldrich D141, Promega Mitochondrial ToxGlo G8000)

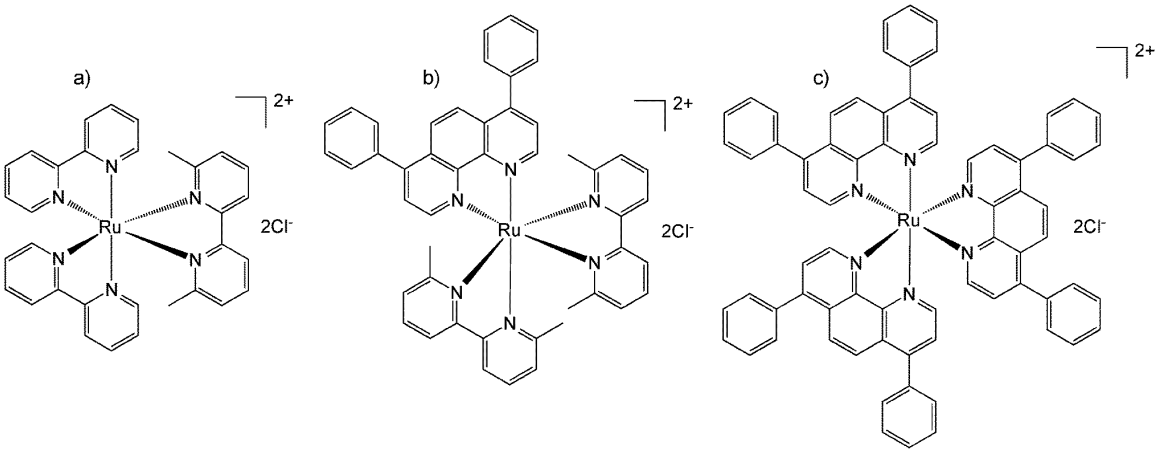

Figure 3.3: Chemical structure of $[Ru(DIP)_2dppz^{2+}$ (DIP) far right

DIP= 4,7-diphenyl-1,10-phenanthroline and dppz=dipyridophenazine. The far right structure represents the structure of DIP and was used by Jacqueline Barton and Cindy Puckett and hypothesized to be a mitochondrial poison (9). It is due to this evidence that

DIP was chosen as a mitochondrial poison RPC control. This complex was also shown to be light sensitive and autofluorescent with the ability to be used as an imaging agent (21).

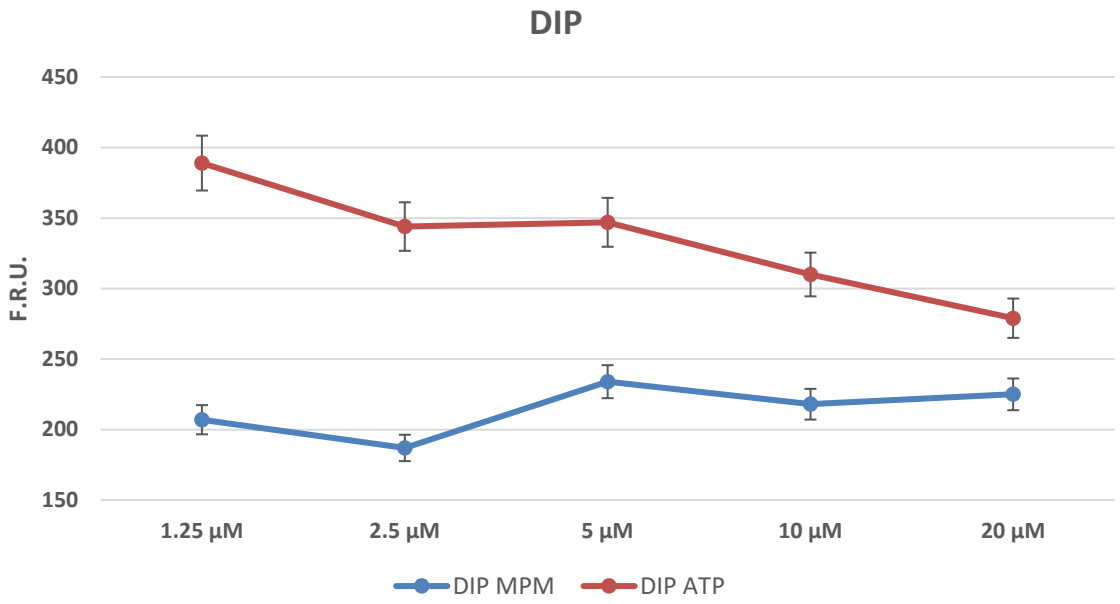

Figure 3.4: Mitochondrial Toxicity Data $[Ru(DIP)_2dppz]^{2+}$

DIP was used as an RPC control and tested against MP^{2+} and P^{4+}. Mitochondrial membrane integrity was measured using the bis AAF-139 substrate measured by fluorescence at 485 nm_{Ex}/520-530 nm_{Em} and can be seen in orange. The blue line represents ATP production and was measured using Promega's ultraglo luciferase enzyme measuring luminescence produced. Both fluoresecne and luminescence were measured using the FluoStarOmega spectrophotometer courtesy of Dr. Mandal's laboratory. Here DIP is shown to be a weak mitochondrial poison due to the steady concentration of ATP being produced at low levels, while mitochondrial membrane integrity being relatively stable from a start point of ~400 relative fluorescence units.

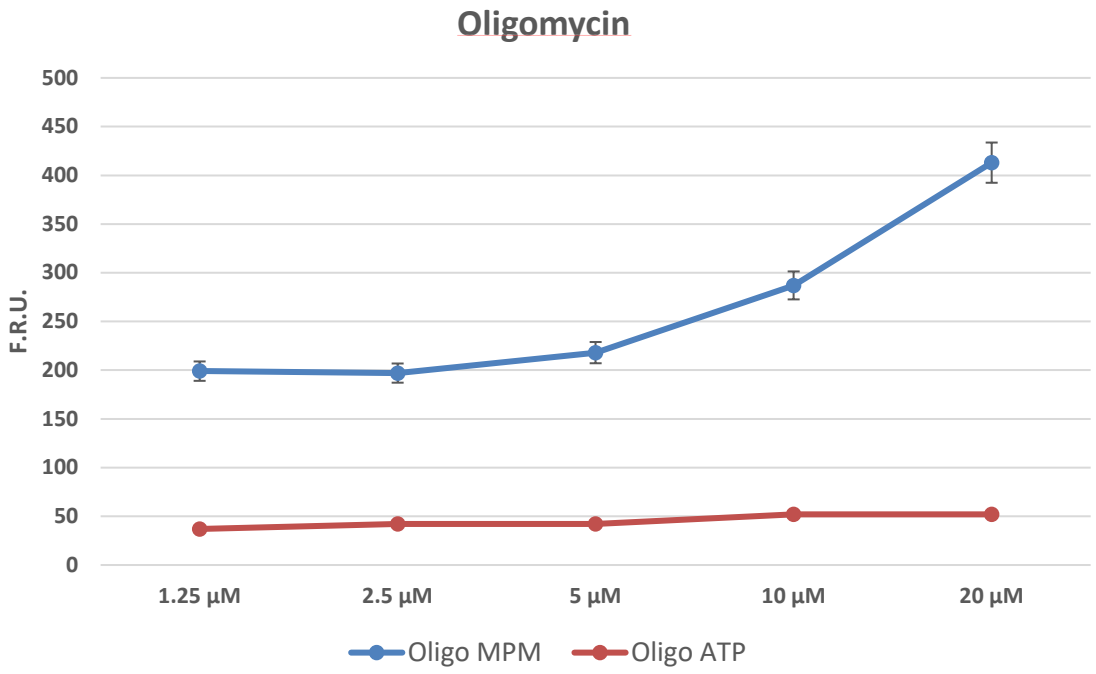

Figure 3.5: Mitochondrial Toxicity Data Oligomycin

Oligomycin was tested on H358 as a known mitochondrial poison against the RPC's tested in the ToxGlo multiplex assay. Similar to DIP and the other complexes, mitochondrial membrane integrity was measured by measuring fluorescence of the protease substrate bis AAF-139 at 485 nm_{Ex}/520-530 nm_{Em} shown in blue. ATP production was measured using the ultraglo luciferase enzyme. Again all complexes tested were measured using the FluoStarOmega multiplex spectrophotometer courtesy of Dr. Mandal's laboratory. Oligomycin was shown to be a mitochondrial poison with ATP production (orange) very low and mitochondrial membrane integrity (blue) slowly eroding with the increase in relative fluorescence units.

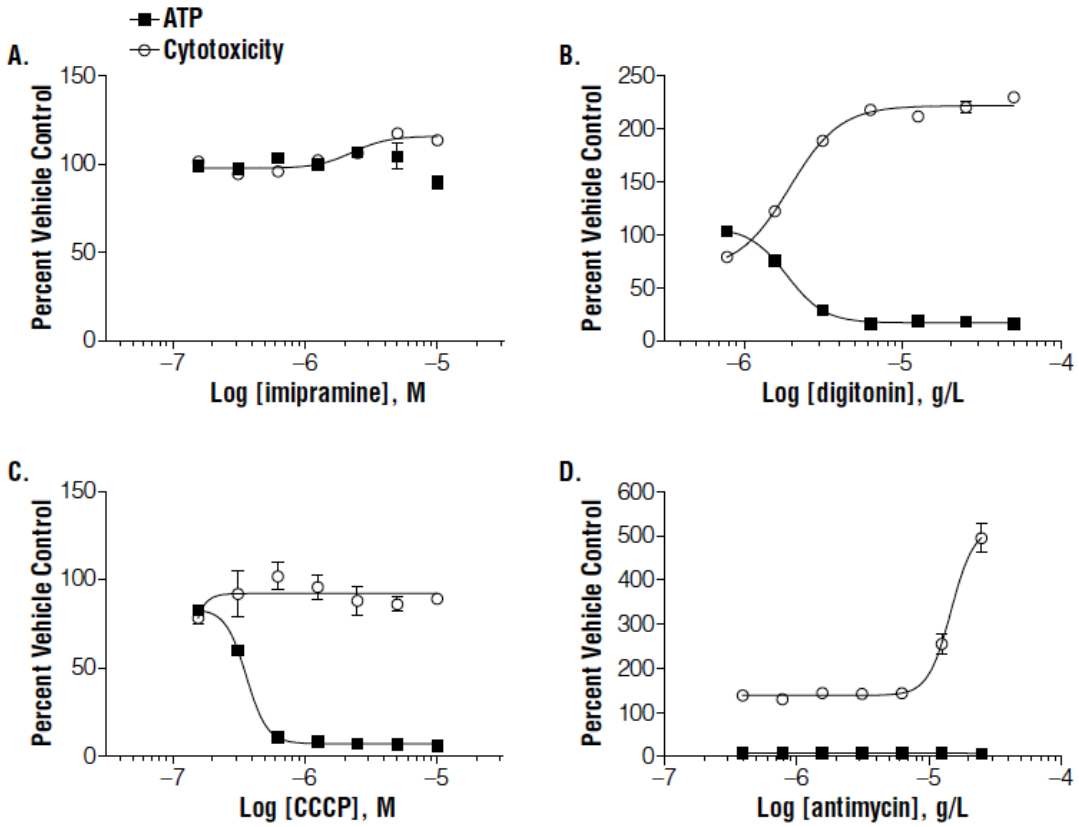

Figure 3.6: Mitochondrial ToxGlo Toxicity Profiles Promega

The graphs shown show the variety of control profiles given by Promega in their Mitochondria ToxGlo Assay (G8000) protocol that could be obtained and were used to compare against the RPC's P^{4+} and MP^{2+}. Panel A shows the drug imipramine revealing no change in ATP levels or cytotoxicity from the vehicle control indicating that it is not a mitochondrial poison and is not cytotoxic in general. Panel B showing an increase in cytotoxicity (increase in membrane permeability) and simultaneous reduction in ATP is indicative of primary necrosis as opposed to a mitochondrial poison. The mitochondrial poison CCCP is shown to have a reduction in ATP according to the percent vehicle control,

however the membrane of the mitochondria stays intact. Promega's last control, antimycin, also shows a mitochondrial poison with similar results.

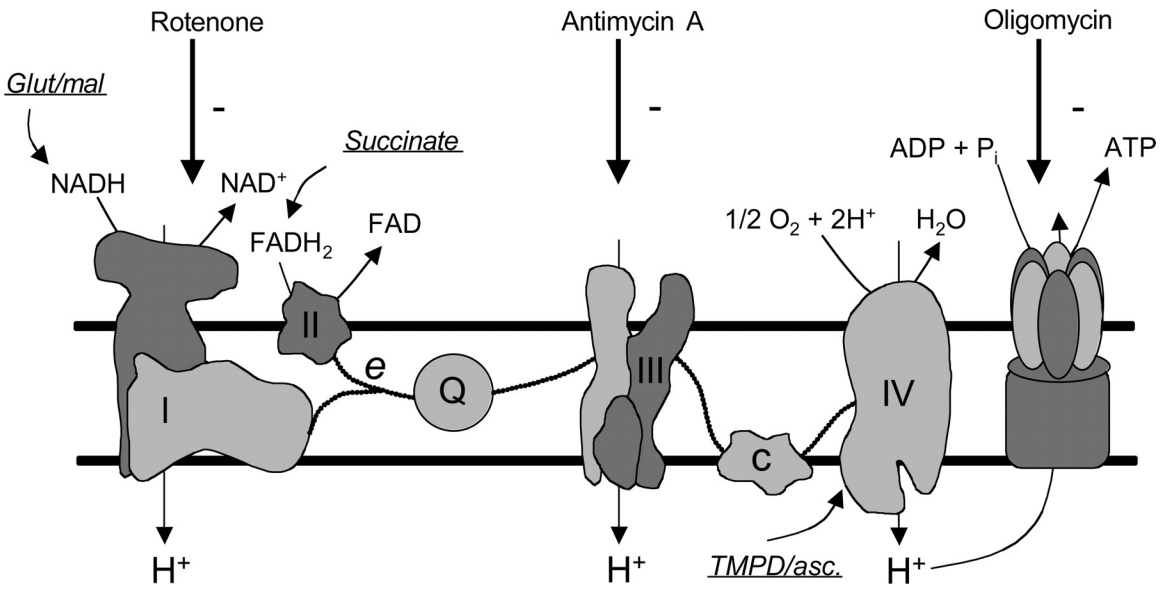

Figure 3.7: Mitochondrial Oxidative Phosphorylation Uncoupling

A physiological and molecular approach shown of the different sites of action of various mitochondrial poisons revealing different effects along the electron transport chain. This would subsequently affect mitochondrial oxidative phosphorylation and cause a variety of issues with the mitochondrial proton potential and Ca^{2+} permeability as shown by Marcil, et. al (22). In particular, Oligomycin is known to attack the F_0 subunit of ATPase (Complex V) inducing a p53-independent apoptosis mechanism that can tell us much about the mutations of the specific neoplasm and how it may affect the mitochondria.

CHAPTER 4

EXPERIMENTAL DISCUSSION

As mentioned before, the data pertaining to the ruthenium complexes P^{4+} and MP^{2+} cannot be discussed due to the drugs being filed currently for a patent and the data being unpublished. The control toxins were similar to those shown in Promega's Mitochondrial ToxGlo assay. Both oligomycin and digitonin matched their profiles and confirmed themselves as a mitochondrial poison and non-mitochondrial poison respectively. More importantly DIP being shown as mitochondrial poison confirmed the data shown by Dr. Jacqueline Barton and Adam Dayoub in his Master's thesis (9,10). Although the data for the RPC's cannot be discussed, it is important to realize that that simply recognizing the potential of the drugs acting on the mitochdondria does not directly correlate to their mechanism of action *in cellulo*. In relation with DIP, although it has previously been shown to accumulate in the mitochondria, it is very possible that similar ruthenium compounds might be acting on various other factors such as genetic regulation or modulation of glycolysis and the TCA cycle. In conjunction with the Warburg Effect, it can be postulated that if malignant cell lines are forced into a vulnerable metabolic state (i.e. where glucose was replaced with galactose as a substrate) then they will be more susceptible to certain therapeutics (13, 23). It was shown by Frey that in fact the glycolytic flux is slower when galactose is introduced as a substrate as opposed to glucose through the Leloir pathway (24). This would allow an increased dependence on mitochdonrial oxidative phosphorylation. It was even shown in the study by Birsov et.al that limiting the amount

of glucose from tumors indeed increases reliance on oxidative phosphorylation as well as sensitivity to inhibitors of the process, including the drug class of biguanidines (25). This could possibly lead to mitochondrial DNA mutations that can be further detrimental to the cell, usually inducing apoptosis.

Due to the high positive charge found in DIP and our ruthenium complexes, it can be safe to say that these complexes could be interacting with the membrane (as well as membrane proteins) and be a direct factor in disrupting the membrane integrity. Although the complexes were shown by Adam Dayoub in our lab as mainly utilizing clathrin-mediated, lipid raft, and G-protein coupled active transport, it is possible with a decrease in ATP production that prolonged periods of incubation entry into the cell would be decreased (9,10,15). DIP, according to the profile obtained, was also shown to be a mitochondrial poison by showing a decrease in ATP production indicating that the proton gradient across the mitochondrial membrane was more than likely disrupted, however not by a significant amount, possibly indicating this as a side effect (24). This would likely lead to caspase mediated apoptosis ending in caspase 3 cleavage. This is in contrast to the other toxicity control digitonin, which showed a classic profile as a necrotic agent and not a mitochondrial poison due to the disruption of the membrane integrity and a spillage of cellular contents.

Interestingly, it was shown by Weinberg et. al that the Warburg effect and tumoregenicity is in fact enhanced by the pentose phosphate pathway as opposed to glycolysis (26). It would be prudent to carry out further studies that would possibly be able to quantify the number of protons fluxing throughout the membrane to quantify a possible decrease and also to look further towards nuclear genetic regulation and if there are more

levels of regulation involved at the transcriptional, translational, or even post-translational level. It could also warrant further investigation into the Warburg effect's effect through the pentose phosphate pathway precursor ribulose and how that might play a role if affected by the complexes in different signaling cascades (26). Different biochemical processes will be explored as studies continue with the goal of creating a biochemical global pathway connecting different signaling cascades for a mechanism of action for both P^{4+} and MP^{2+}.

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BIOGRAPHICAL INFORMATION

Ali was born in Arlington, Texas on July 14, 1995 and has spent most of his life here with a small two-year stay in the state of Maine. Ali chose the University of Texas at Arlington for his undergraduate career due to its close proximity to home as well as a cost effective means to gain entry into a professional medical school. Pursuing interests in microbiology and cancer related studies, he was able to join Dr. Frederick Macdonnell's Inorganic Chemistry lab his third semester. Dr. Macdonnell's lab focuses on a variety of fields including bioinorganic cancer research and energy conversion of carbon dioxide to ethanol for fuel using various inorganic synthesis methods.

Ali's work alongside PhD candidate Adam Dayoub in the bioinorganic cancer research has resulted in work with DNA double-stranded break studies involving phosphorylation of histone H2AX, synergy, and Mitochondrial Toxicity Assays involving the ruthenium complexes created in Dr. Macdonnell's lab. Ali has also been involved working alongside Adam Dayoub at UT Southwestern Medical Center for various other projects. Currently, Ali is working on investigating the antimicrobial properties of the ruthenium polypyridyl complexes. In addition Ali has been working in UT Arlington's Microbiology labs for fours semesters, greatly enriching his scientific knowledge. Ali is grateful for all the opportunities he has been offered and will be attending UT Southwestern's School of Medicine this fall in pursuit of a career as a physician.