CYTOTOXICITY, CELLULAR LOCALIZATION AND FLUORESCENT MICROSCOPY OF MALIGNANT CELLS TREATED WITH RUTHENIUM (II) COMPLEXES OF THE TETRAAZATETRAPYRIDOPENTACENE LIGAND

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN CHEMISTRY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2015

Acknowledgments

I would like to express my deep appreciation to my supervising professor Dr. Frederick MacDonnell. His patience and mentoring through these last several years was paramount to my development, not only a graduate student but as a person. I express my gratitude for his patience and skill in my scientific research development. I would not have had this opportunity without his help and confidence in me. He has given me the tools to trust and guide myself in my research endeavors as and shown me that hard work that can accomplish many things.

I want to thank my committee members, Dr. Brad Pierce, Dr. Subhrangsu Mandal and Dr. Frank Foss for their support and the plethora of their ideas and contrasts, which helped the development of this thesis.

I would like to express my deep gratitude for Dr. Malgosia Wilk who has been a guide before my graduate career even started. The use of her laboratory, that she has graciously allowed me to use and perform these experiments, I will forever be grateful.

A special thank you to all my current and former lab mates Dr. David Boston, Dr. Nagham Alatrash, Dr. Shreeyukta Singh, Cynthia Hennegan, Pooja Ahuja, Eugenia Narh, Chen Yanling, Kai-ling Huang and Matthew West who have supported me personally and professionally. They kept me grounded when I needed to be and have been an amazing ear to speak to in all matters. Their encouragement and support guided and helped me immensely.

I would like to mention Dr. William Cleaver and Dr. Brian Edwards who have been an ear to my ideas and guides when I had strayed off the course from time to time. To Dr. Heidi Conrad, I am deeply thankful for your hard work with this writing of this thesis. Dr. Peter Kroll, thank you for your support and work with me the past couple of years. Thank you to my graduate school friends Emmanuel Varona, Joya Singh, Shawn Riddlen, John Paul Nimmo , Akop Yapremyan, Diego Lopez, Andy Seal and Curran Parpia for their friendship and support in so many ways that I can’t count on both hands.

I would like to give a special thank you to Dr. Dasgupta and his lab for the use and technical skill with my ICP-MS experiments. From his group, Dr. Charles Phillip Shelor and Brian

Stamos, I thank you for your patience and education with these experiments. Also, Dr. Mandal and your group, thank you so much for the use of your lab equipment.

I would like to thank all the Chemistry and Biochemistry and Biology department staff for all their hard work and continuous support through my career. They have not only aided me in day to day operations but are also my friends. A special thank you goes to Jim Garner and Linda Taylor for their tirelessly hard work.

I would like to give a deep loving thank you to my family Adib, Helen and Jennifer Dayoub who have been my unwavering support through the dark and hard times and an inspiration through the good. Also, Bryan and Annette Haddad, who spiritually guide me endlessly as well as my godmother Nawal Dockstader; she was taken from us due to colon cancer and is a driving force for my research. They remind me of where I come from and my mission in life.

I graciously conclude this acknowledgment and thank you to my fiancé Vilma Veronica Vasquez who for two years has supported me with endless love and understanding. I would not have come so far without her pushing me and knowing what I am capable of accomplishing. I thank her for all the patience, time and love through all this work. She makes me a better person and brings a smile to my face every day. Thank you so much for your love and support.

April 20, 2015

Abstract

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The ruthenium (II) polypyridyl complexes $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$, (P) and $[(phen)_2Ru(tatpp)]^{2+}$, (MP) are promising anticancer activity candidates due to their observed cytotoxic effect against multiple cancer cell lines. These complexes contain a redox-active (tatpp) bridge and a cytotoxic selectivity against cancer lines that are of interest in cellular biological systems. They also exhibit the ability to regress tumor growth in mouse models and have been shown to cleave DNA with an inverse dependence with (O_2). Significantly, these complexes may show a higher cytotoxic action in a hypoxic environment similar to that of tumors *in vivo.*

This thesis is a direct test of the following hypothesis. We postulate that the ruthenium(II) polypyridyl complexes P and MP exhibit enhanced cytotoxicity in a hypoxic environment. We believe that against multiple cancer cell lines, P and MP will show enhanced cytotoxicity in a reduced (O_2) environment. We also postulate that dsDNA, mitochondrial potential damage or a combination of both are responsible for the cytotoxic values exhibited by P and MP. The hypothesis of this thesis is that one or multiple compartments are being damaged as well as different methods of cell membrane entry attributing to the separate mechanistic ability of these complexes *in situ*. Chapters 2 and 3 of this thesis develop the hypothesis by an analysis of prior literature research and our biological screening approach to test the cytotoxic and intracellular mechanistic ability of these complexes.

In chapter 2, the details of the enhanced cytotoxicity in a hypoxic environment are discussed for complexes P and MP against cell lines H358, HCC-2998, HOP-62 and Hs766T. The study also contrasts and compares these findings in a normoxic environment as well as against complexes that show low cytotoxicity in the same environments.

Chapter 3 presents the method of cellular entry, compartment localization and possible location of cytotoxic action within the cancer cells utilizing ICP-MS and fluorescent microscopy to determine if their location corresponds to their cytotoxic effect. In 3rd chapter we will show stark contrasts between complexes P which is localized between several compartments vs. MP which was found to be highly in the cytoskeleton of cell lines H358 and HCC-2998. It was also found that these complexes exhibit active cellular membrane transport and separate endocytosis transport channels in Hs766T cells. Whereas P and MP, were both found to utilize the clathrin mediated transport channels, MP exhibited the use of lipid raft dependent endocytosis vs. P utilizing GTP couple protein transport. This study also shows the mitochondrial potential damage by complexes P and MP, utilizing fluorescent microscopy live and fixed cell imaging. Propidium iodide and JC-1 mitochondrial potential stains were used to discern the location of action between the nuclear dsDNA and mitochondrial membrane potential within H358 cells.

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Chapter 1

Ruthenium Polypyridyl Complexes and Possible Anti-Cancer Effects

1.1 Cancer Introduction

Cancer is a group of diseases characterized by uncontrolled cell growth and the spread of these abnormal cells. If the uncontrolled spreading of these cells is not constrained, death can occur.[1] External factors known as mutagens, such as tobacco, infection, chemicals and radiation, can cause cancer.[1] According to the American Cancer Society an expected 1.67 million people will be diagnosed with cancer in 2015. With the number of cancer patients increasing to record highs, new and evolving methods of combating cancer are being investigated. Chemotherapy, which is a cornerstone of cancer treatment, is also facing turmoil in the presence of new and emerging cancer types. The new cancer types, such as some ovarian and neck cancers are demonstrating resistance to standard treatments.[2] A standard for antitumor activity is *cis*-$[PtCL_2(NH_3)_2]$, cisplatin. Since its discovery as an inhibitor of cell replication in the 1960's by Rosenberg,[20] cisplatin [fig 1.1] has gone on to become one of the most widely used anti-cancer therapeutics.

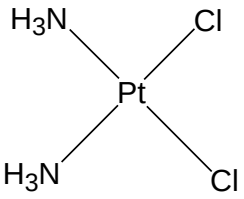


Fig 1.1 Cisplatin.

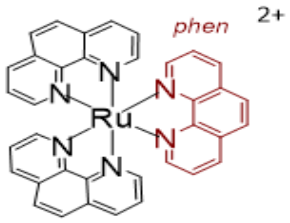
Cisplatin's putative mechanism is by the formation of DNA-platinum adducts.[3] After interaction, either DNA binding proteins signal DNA repair or apoptosis (cell death) occurs. The latter is observed more often leading to its chemotherapeutic effect.[3,4] Since this discovery, there have been numerous attempts to replicate the success of cisplatin with other metal complexes that interact with DNA, either through covalent or non-covalent interactions.[20] The success of cisplatin has ushered in many metal based drug research projects aimed at cancer treatment.

This is a driving force that is encouraging researchers to investigate new and other possible treatment methods that may provide relief for disease sufferers of cancer. Many treatment investigations include new classes of compounds, such as bleomycin, doxorubicin, and carboplatin which are considered part of the targeted therapy revolution.[2] Chemotherapy, being cytotoxic not only to cancer cells but all healthy cells in the body, has lead research down a new path of tumor targeting. Targeting tumor sites while relieving the stress on healthy cells would be highly advantageous to the patient.[3]

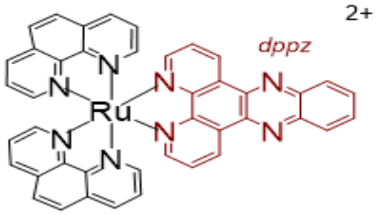
1.2 Biological Activity and Description of Certain Ruthenium Polypyridyl Complexes and current Laboratory Findings

Ruthenium polypyridyl complexes, RPCs, are a family of complexes being developed and used in anticancer drug discovery.[7] In this work we focused on coordinately saturated and kinetically inert ruthenium (II) complexes containing polypyridyl ligands. [fig 1.2]. RPCs tend to have reasonable cytotoxicity with the half maximal inhibition concentrations, IC_{50}'s, on the scale of 10^{-4} to 10^{-7} M.[7] Some RPCs do not metabolize *in vivo*, rather they are excreted and unchanged indicating that the complex is the bioactive unit.[8,10]

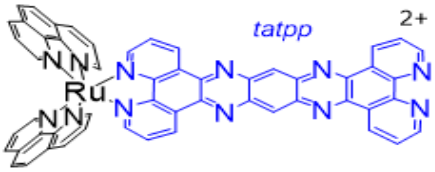
Chemotherapy is cytotoxic to many healthy cells as well as cancer cells, as such; targeting a complex to cancer cells versus healthy cells would be highly beneficial for cancer treatment. Many RPC's are highly toxic to all cell types such as $[Ru(phen)_3]^{2+}$, (phen=phenanthroline) and $[Ru(DIP)_3]^{2+}$, (DIP=diphenylphenanthroline) [fig 1.2], with IC_{50}'s around 1-2 µM.[16,18,19] We initially studied these RPC's and have synthesized planar bridging ligands that could possibly intercalate or target DNA similar to the DPPZ ligand (dipyridophenazine) in RPC, $[Ru(phen)_2dppz]^{2+}$ [fig 1.2].[9,18] In particular , RPCs containing the tatpp (tatraazatetrapyridopentacene) ligand in dinuclear complex $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$, (P) and mononuclear complex $[(phen)_2Ru(tatpp)]^{2+}$, (MP) [fig 1.2] are of interest as promising anticancer activity complexes in mouse tumor models.



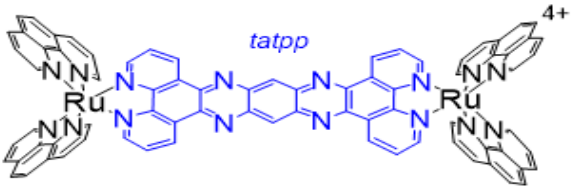
$[Ru(phen)_3]^{2+}$ **Ru**



$[(phen)_2Ru(dppz)]^{2+}$ **Dppz**

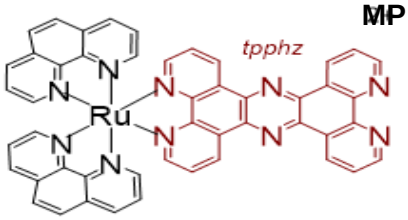


$[(phen)_3Ru(tatpp)]^{2+}$

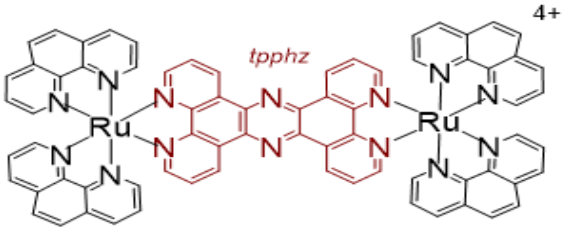


$[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ **P**

MP

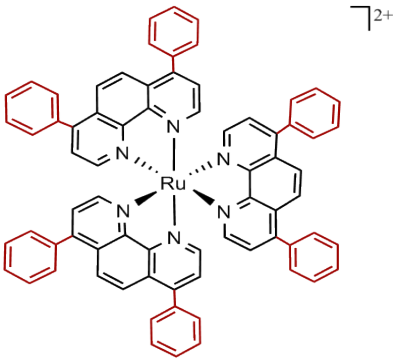


$[(phen)_3Ru(tpphz)]^{2+}$ **MZ**



$[(phen)_2Ru(tpphz)Ru(phen)_2]^{4+}$

Z



$[Ru(dip)_3]^{2+}$ **DIP**

Fig 1.2 Various ruthenium polypyridyl complexes (RPCs) and their nomenclature letters.

Redox activity of the tatpp bridge in P and MP both cut DNA *in vitro*, however it is not known if this is occurring *in situ* within the nucleus of a cell.[16,18] This information is salient because some RPCs are known to cause apoptosis through mitochondrial damage.[11] Differentiating the source of apoptosis is paramount to our understanding of the anticancer activity by these complexes.[11,12] Our work has also shown that P and MP are not acutely toxic in mice with a maximum tolerable dose (MTD) ranging from 65 – 68 (mg/Kg).[18] The antitumor activity was seen when H358 tumors were grown in nude mice and then treated by IP injection with P and MP. The tumor growth in the mice then halted and regressed. [fig 1.3].[16,18] IC_{50} studies have also revealed micro molar concentrations in the ranges of 8 – 31 μM of both P and MP to cancer lines H358 (NSCLC), H226 (Squamous CLC)[18], HCC-2998 (Human Colon Cancer Adenocarcinoma) and Hs7662 HTB-134 (Pancreatic Carcinoma Lymph Node. A detailed table is found in [tab 1.1]. P and MP may be able to separate themselves from other antitumor agents by showing selectivity towards carcinoma cells versus normal functioning healthy (non-carcinoma) cells.

Normal non-carcinoma cells: HUVEC (Human Umbilical Vein Endothelial Cells), and HAVSMC (Human Aorta Vascular Smooth Muscle Cells) showed a low response to complex treatment. A high response to complex treatment was observed in carcinoma cells, H358 (NSCLC) and H226 (Squamous CLC) [fig 1.4].[18] Preliminary studies have also shown their cytotoxic capabilities to be more efficacious in hypoxic environments.[16,18] This hypoxic environment reveals a possible anti-cancer pathway because most tumors *in vivo* are hypoxic in nature, due to low vascularization.[15] In an experiment conducted under normoxic and hypoxic conditions, H358 showed an appreciable greater sensitivity to MP under hypoxic conditions as compared to normoxic conditions.[16] [fig 2.2 see chapter 2]

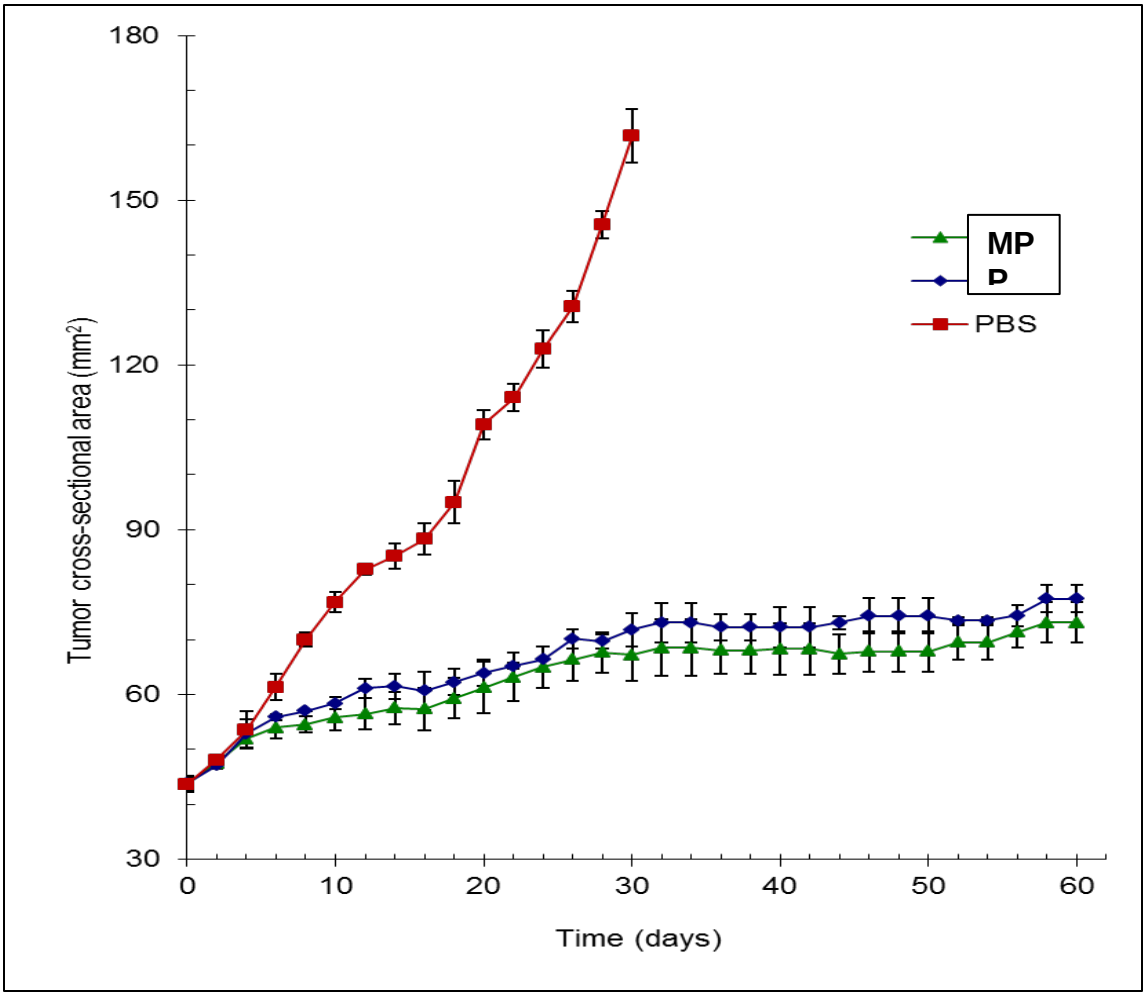


Fig 1.3 Mouse tumor regression model. Phosphate buffer saline (PBS) shows increased tumor growth while P and MP show tumor growth regression over a 60 day period.[18]

Tab 1.1 shows various IC_{50} values based on concentration of complex in μM. Carcinoma lines H358, Hcc-2998 and Hs766T are compared to normal non-carcinoma lines HUVEC and HAVSMC.

RPCs	H358 μM	HCC-2998 μM	Hs766T μM	HUVEC μM	HAVSMC μM
$[P]^{4+}$	9.5 ± 5.2	13 ± 5.1	10.0 ± 5.1	119.0 ± 5.0	104.0 ± 4.0
$[MP]^{2+}$	8.8 ± 5.0	20.0 ± 5.0	31.0 ± 5.9	92.0 ± 5.0	100.0 ± 5.0
DIP	1.7 ± 2.3	N/A	N/A	N/A	N/A

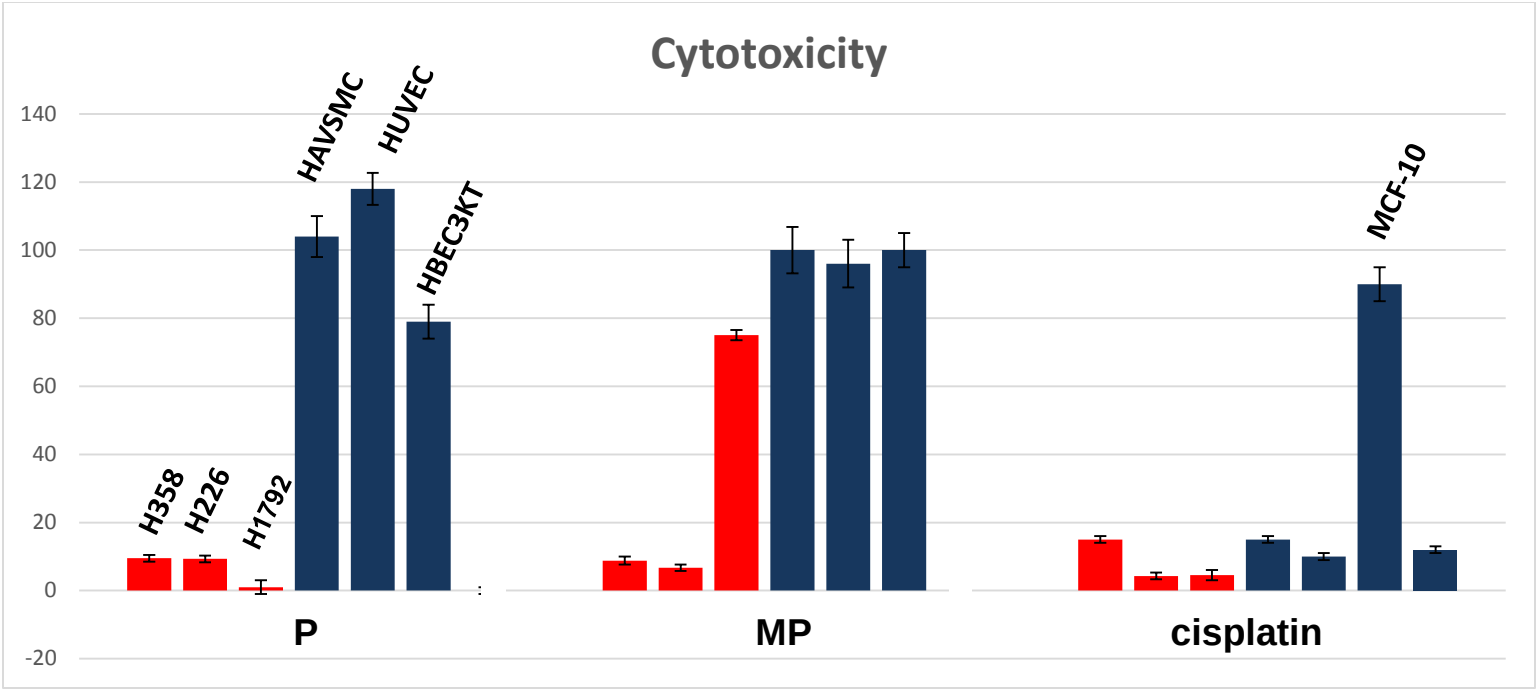


Fig 1.4 IC_{50} values of carcinoma lines H358, H226 and H1792 as compared to non-carcinoma lines HAVSMC, HUVEC and HBEC3KT.

1.3 Scope of Thesis

It is postulated that ruthenium polypyridyl complexes (RPC)s $[(phen)_2Ru(tatpp)Ru(phen)_2]^{2+}$, (P) and $[(phen)_2Ru(tatpp)]^{2+}$, MP exhibit enhanced cytotoxicity in a hypoxic environment, similar to carcinomic tumor environments *in vivo* [fig 2.2]. Against multiple cancer cell screens it is believed that these RPCs will show enhanced cytotoxicity when O_2 levels are reduced to hypoxic levels. It is also postulated that dsDNA, mitochondrial potential damage or a combination of both is responsible for the seen cytotoxic IC_{50} values of RPCs P and MP. One hypothesis of this thesis is that one of the two compartments are being damaged as well as both RPC complexes will show unique and different mechanism ability for their transport, localization and cytotoxic effect *in situ*.

Chapter 2

Examination of the Cytotoxicity of RPCs under Normoxia and Hypoxia

2.1 Introduction

Molecular oxygen (O_2) plays a substantial role in normal cell biological function. O_2 levels between 13-18% must be maintained for normal cell function.[21] Cancer cells form tumors in the body and typically become hypoxic due to low vascularization around the tumor site.[22] One of the hallmark of cancer cells is their fast proliferation rate.[23] The rapid mitosis of these cells allows for rapid tumor development. In some cases, cells can escape hypoxia mediated death by lowering expression of p53 with an increase in HIF-1 mechanisms.[24] The hypoxia can potentially provide a useful tumor targeting mechanism as normal human cells are not under hypoxic conditions. As stated in chapter 1, complexes P and MP both cut DNA *in vitro* and show potentiated cleavage in a hypoxic environment.[25] However, the mechanism of cytotoxicity for P and MP in cells is still unknown and, even if one assumes that DNA cleavage is responsible for the observed apoptosis, there is no assurance that the complexes will behave the same in cells as they do *in vitro*. In this chapter, we examine the cytotoxicity of P and MP in cells under normoxia and hypoxia by two separate incubation environments with DPPZ as a control. If an increase in cytotoxicity is observed in the hypoxic environment, it could provide support for DNA as the drug target and favors better destruction of cells under hypoxia, which are typically malignant cells. If observed, this could lead to effective therapies that show enhanced activity against one of the more chemo resistant sub-populations of malignant cells, and a population which is largely unaffected by oxygen dependent drugs such as bleomycin and doxorubicin.[26]

2.2 Experimental Section

2.2.1 Chemicals

All solvents were reagent and cell culture grade. All experimentation was performed with adherence to reagent manufacturer guidelines as according to ATCC guidelines. Cell culture

media DMEM (ATCC) and RPMI-1640 (Sigma) and supplements Fetal Bovine Serum (FBS), Penicillin/Streptomycin (PenStrep), BME Vitamins 100x (Sigma) were used as received. Cytotoxicity assay reagents 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl Sulfoxide (DMSO), 0.04% Trypan Blue (Sigma) and Phosphate Buffer Saline 10x (Biorad) were used as received and as described in literature unless otherwise noted. RPC complexes P, MP and DPPZ were used as received from the MacDonnell laboratory.[19]

2.2.2 Instrumentation

Normoxic incubation was maintained by a HeriCell CO_2 Incubator (Thermofisher). Hypoxic incubation was maintained by a Galaxy 14s Dual Channel CO_2/N_2 Incubator (New Brunswick). Cytotoxicity data was obtained with a FLUOstar Omega plate reader (BMG Labtech).

2.2.3 Cell Culture Lines/Maintenance

Carcinoma cell lines used were NCI-358m Bronchiolo-Aveolar Carcinoma, HCC-2998 Human Colon Adenocarcinoma, HOP-62 Human Lung Adenocarcinoma (NCI-Frederick Cancer DCTD Tumor/Cell Line Repository) and Hs-766T Pancreas Lymph Node (ATCC). NCI-H358m, HCC-2998 and HOP-62 were cultured in RPMI-1640 medium with 10% FBS, 1.1% PenStrep and 1x BME Vitamins at 37 ^{o}C in a 95% H_2O humidified atmosphere with 5% CO_2. Hs-766T were cultured in DMEM high glucose medium with 10% FBS, 1.1% PenStrep and 1x BME Vitamins at 37 ^{o}C in a 95% H_2O humidified with of 5% CO_2. All cell lines were seeded and passaged according to established protocols by ATCC.

2.3 Hypoxia and Normoxia Experimentation

2.3.1 Cell Seeding and Complex Dosing

To determine the cytotoxic effects of complexes P and MP on cell growth populations H358, HCC-2998, HOP-62 and Hs766T were seeded in microliter well plates consisting of 96 wells at a cell density of approximately $2x10^4$ cells/well. Lane A of the well plates is not seeded as

it is a negative control. Cell line passaging and seeding density were determined by hemocytometer with 0.04 % Trypan Blue staining. Cells were seeded and allowed to adhere for 24 hours before adding medium containing varying concentrations of ruthenium polypyridyl complexes [fig 2.1]: 0.1 µM, 1.0 µM, 10 µM, 25 µM and 50 µM. Once cell inoculation was completed, the cells were incubated for 72 hours.

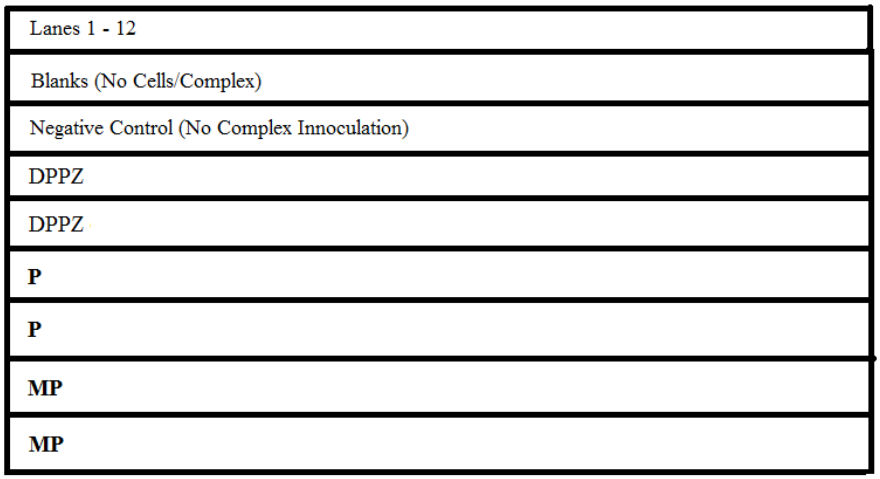


Fig 2.1 Concentrations of various ruthenium complexes: 0.1 µM, 1.0 µM, 10 µM, 25 µM and 50 µM were administered doubly in 2 wells/lane.

2.3.2 Hypoxia and Normoxia Incubation

To obtain the optimal atmosphere for normoxic and hypoxic experimentation, two separate incubators were used. The normoxic incubator was set to a humidified atmosphere of 95% H_2O with 16% O_2, 5% CO_2 and 79% N_2 at 37 ^oC. The hypoxic incubator was set to a humidified atmosphere of 95% H_2O with 1.1% O_2, 5% CO_2 and 94% N_2 at 37 ^oC. To ensure proper and equal timing of dosing, an additional 6 hours were used to reference the hypoxic incubator to appropriate O_2 levels of 1.1% before normoxic cell dosing experiments commenced.

2.3.3 MTT Assay and Cytotoxicity Determination

After 72h incubation time of the RPC inoculation in both normoxic and hypoxic atmospheres were reached, MTT assay was used to determine inhibition concentrations (IC_{50}) as follows: complex medium mixture was aspirated and cells were washed 2 times with PBS, cells were administered 25 µL of MTT/PBS reagent (5 mg/ml) for 3.5 hours, MTT reagent was then removed and 120 µL of DMSO was added to each well, plates were attached to plate shaker for 20 minutes and absorbance was read at 570 nm. IC_{50} for each cell line was then determined by inhibition of cell growth to 50% as compared to controls.

2.4 Results and Discussion

Two types of experiments were done to see if a hypoxic environment affected the complexes ability to kill cells. The first measured cell population after 48 h incubation as a function of drug concentration and environment (normixic or hypoxic). This data is shown in [fig 2.2]. Because of the short time frame, not all cell population were reduced by 50% even though they normally would do so at longer incubation periods. We purposely kept the timeframe short so that the hypoxic environment would, at least in theory, not contribute significantly to difference in cell growth. As seen in [fig 2.2], compounds cisplatin, MP and Dppz were examined in both normoxic and hypoxic environments. In this preliminary work we found that MP had a greater cytotoxic effect on H358 cells as compared to cisplatin and Dppz.

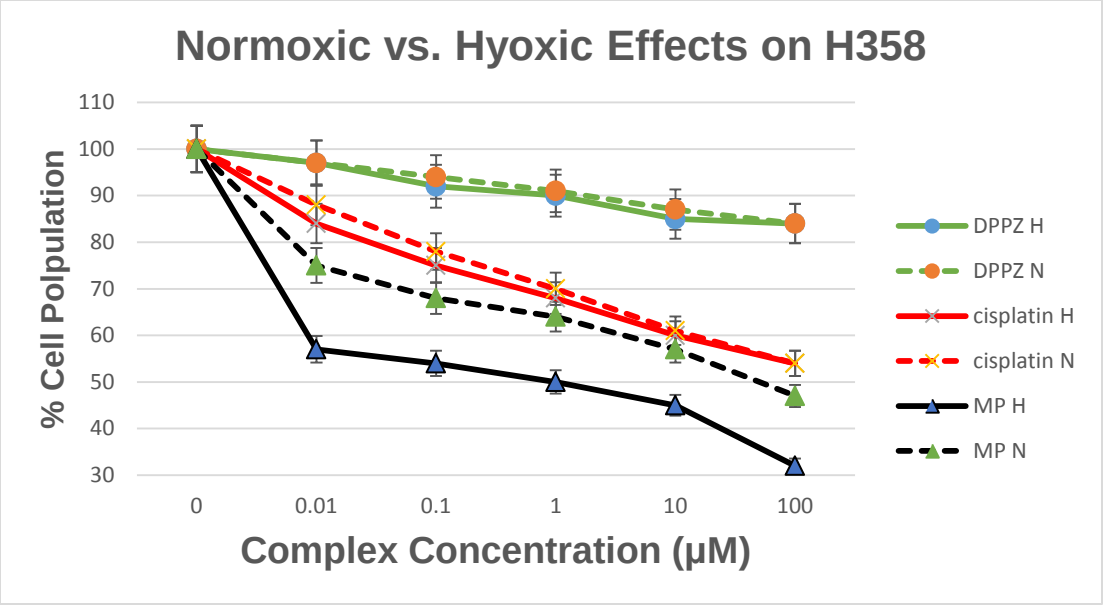


Fig 2.2 IC_{50} growth curve in hypoxic (1.1% O_2) environment with complexes Dppz, Cisplatin and MP ($[(phen)_2Ru(tatpp)]^{2+}$. MP shows an increase of cytotoxicity in a hypoxic environment as compared to the normoxic environment.

A second experiment was done at longer incubation periods and the IC_{50} data is collected in [tab 2.1]. The P and MP complexes showed better cytotoxicity as compared to the Dppz.[23] Cell lines H358 and HCC-2998 showed a slight hypoxic effect with MP and no significant difference with P. However, with cell lines Hs766T and HOP-62 a significant hypoxic effect, almost 2 fold, was observed with MP. P also exhibited a significant effect with HOP-62. While not all cell lines showed a significant increase in the inhibitory power of the complexes in a hypoxic environment, they were no less effective.

The second IC_{50} screening shows the difference in IC_{50} results while cell lines were inoculated with complex in a hypoxic (1.1% O_2) environment. In [fig 2.3], the HOP-62 cell line screening is shown with complex P, MP and Dppz with the difference in cytotoxicity curves based on the atmosphere differences from normoxia. As seen in [fig 2.3] HOP-62 cells treated with the control Dppz, showed little difference in the response to hypoxic stress. HOP-62 cells that were

treated with P and MP showed a significant decrease in cell growth over a 72 h period. For example MP does reach the IC_{50} for HOP-62 in both cases of atmosphere differences but the concentration of which they reach this inhibitory property is of interest. This result shows the possible heightened cytotoxic effect in a hypoxic environment, possibly as in a tumor. As shown in [fig 2.3] the cytotoxicity of P and MP were enhanced in a hypoxic environment. More importantly they show a 20 fold difference in the IC_{50} contrast between normal cell lines HUVEC and HUVSMC as shown in [fig 1.4].

For example, *in vivo*, bleomycin with Fe cofactors and O_2 forms activated bleomycin complexes that cleave single and double stranded DNA.[23,27] The lack of oxygen prevents this mechanism of action from taking place. Being that these are a common example of DNA cleaving agents via ROS; P and MP can be examined as an alternate cytotoxic mechanism in low O_2 settings. As previously mentioned, the unique ability of P and MP to cleave dsDNA with their redox ability can be increased with GSH as a reducing agent in the cellular cytoplasm. The low O_2 environment created a steady-state like mechanism that could be responsible for these observations.

Tab 2.1 Table of all IC_{50} values in normoxic and hypoxic environments. Values are expressed in concentration of complex with cell medium.

IC_{50} of RPCs	Conditions	H358	HCC-2998	Hs766T HTB-134	HOP-62
P^{4+}	normoxia	9.5 ± 3.2 μM	10.0 ± 3.1 μM	9.0 ± 3.1 μM	>80.0 μM
	hypoxia	11 ± 3.2 μM	9.0 ± 3.0 μM	9.0 ± 3.1 μM	55.0 ± 3.1 μM
MP^{2+}	normoxia	8.8 ± 3.0 μM	13.0 ± 3.0 μM	31.0 ± 3.9 μM	35.0 ± 3.1 μM
	hypoxia	9.0 ± 3.2 μM	10.0 ± 3.0 μM	15.0 ± 3.0 μM	25.0 ± 3.0 μM
DPPZ	normoxia	>50.0 μM	>50.0 μM	>50.0 μM	>50.0 μM
	hypoxia	>50.0 μM	>50.0 μM	>50.0 μM	>50.0 μM

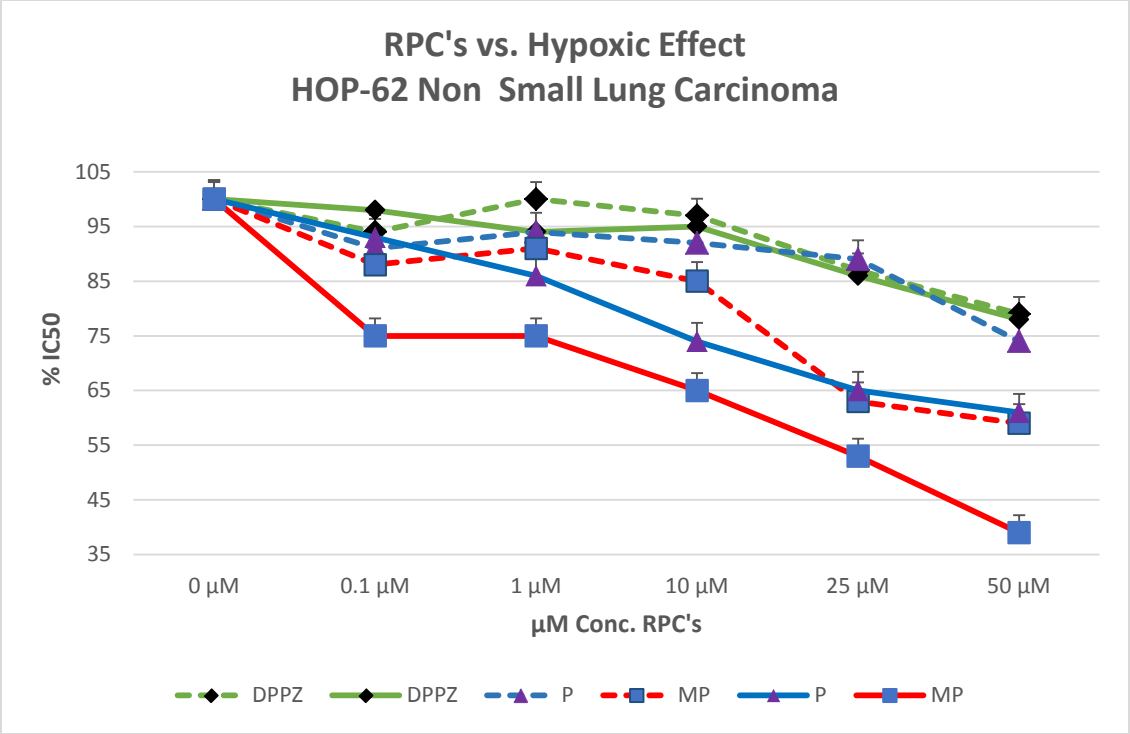


Fig 2.3 Cell line HOP-62 was treated with various concentrations of Dppz, P and MP for 72 h in normoxic and hypoxic environments. IC_{50} was determined by MTT assay and absorbance was read at 570 nm. The crossing of the 50% mark indicates the IC_{50} value has been reached.

In summary, these observations confirm that complexes P and MP are highly cytotoxic in cancer lines H358, HCC-2998, HOP-62 and Hs766T. In addition, P and MP show an increase in cytotoxicity, in a hypoxic environment, with cell lines HOP-62 and H766T. Tumor cells are hypoxic in nature and have a uniquely different metabolism and mechanism biochemistry, therefore these processes may be responsible for the IC_{50} differences noted in certain cell lines in hypoxia. The location of the complex action within the cancer cell lines are studied and discussed in the next chapter.

Chapter 3

Examination of RPCs Cellular Location, Mechanism of Membrane Entry and Cytotoxic Effect and Visualization

3.1 Introduction

While complexes P and MP appear to be promising anti-tumor drugs, little is known about how or even if these complexes enter cells, and if so where do they accumulate within a cell. In this chapter, we examined if and how these RPCs are entering cells and where they accumulate. Inductively coupled plasma- mass spectrometry (ICP-MS) was used to quantify Ru ion content in whole cells as well as cellular fractions as a function of temperature, to demonstrate active or passive diffusion, and as a function of selective inhibition of specific transport mechanisms. In particular, active transport mechanisms: clathrin mediated, lipid raft and GTP couple protein channels were probed to determine the method of active transport entry through the cell membrane. Lastly, we used confocal laser microscopy in conjunction with several well-known fluorescent dyes to examine the activity of these RPCs in the nuclei and mitochondria.

Propidium iodide, a nuclear stain, fluoresces strongly in the red (~595 nm) when intercalated in dsDNA.[36] When intercalation is not achieved due to dsDNA cleaving or damaging of the helix, a greatly diminished signal is observed. To determine the mitochondrial membrane potential function we used the JC-1 live imaging stain. JC-1 aggregates form with both functioning and failed mitochondria potential. Failed mitochondria potential forms a green aggregate (~529 nm) while functioning mitochondrial potential forms a red aggregate (~590 nm) with JC-1. Using these two fluorescent dyes in conjunction with RPC inoculation we were able to visualize the possible cellular location of their cytotoxic efficacy.

3.2 Experimental Section

3.2.1 Chemicals

All solvents used were of reagent and cell culture grade. All experimentation was performed with adherence to reagent manufacturer guidelines according to ATCC. Cell culture media DMEM (ATCC) and RPMI-1640 (Sigma) and supplements: Fetal Bovine Serum (FBS), Penincillin/Streptomycin (PenStrep), BME Vitamins 100x (Sigma) were used as received. Cytotoxicity assay reagents 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl Sulfoxide (DMSO), 0.04% Trypan Blue (Sigma) and Phosphate Buffer Saline 10x (Biorad) were used as received and as described in literature unless otherwise noted.[1] Ruthenium complexes P, MP and Dppz from the MacDonnell laboratory were used as received. The Qproteome Cell Compartment Kit (QIAGEN) was used as received according to all manufacturer guidelines. Cell membrane diffusion blockers Sodium Azide, 2-deoxy-D-Glucose, Sucrose, Dynasore hydrate and Nystatin (Sigma) where used as received. Cell culture grade confocal microscopy probes Propidium Iodide (Sigma) and JC-1 Mitochondrial Potential Kit (VWR) were used as received.

3.2.2 Instrumentation

Normoxic incubation was maintained by a HeriCell CO_2 Incubator (Thermofisher) Cytotoxicity data was obtained with a FLUOstar Omega plate reader (BMG Labtech). Ruthenium ion concentrations were determined using ICP-MS 1000 (Thermofisher). Confocal microscopy was performed using a Zeiss Axio-Plane 540 with mercury lamp and argon laser (488 nm, 534 nm and 560 nm) and 40x oil immersion objectives (Zeiss).

3.2.3 Cell Culture Lines/Maintenance

Carcinoma cell lines used were NCI-358m Bronchiolo-Aveolar Carcinoma, HCC-2998 Human Colon Adenocarcinoma (NCI-Frederick Cancer DCTD Tumor/Cell Line Repository) and Hs-766T Pancrease Lymph Node (ATCC). NCI-H358m and HCC-2998 were cultured in RPMI-

1640 medium with 10% FBS, 1.1% PenStrep and 1x BME Vitamins at 37 ^{o}C in a humidified atmosphere of 95% H_2O with 5% CO_2. Hs-766T was cultured in DMEM high glucose medium with 10% FBS, 1.1% PenStrep and 1x BME Vitamins at 37 ^{o}C in a humidified atmosphere of 95% H_2O with 5% CO_2. All cell lines were seeded and passaged according established protocols by ATCC.

3.2.4 Ruthenium Complex Loci Determination ICP-MS

H358 and HCC-2998 cell lines were seeded in 60x60 mm dishes and grown to an 80% confluency not to exceed $5x10^6$ cell density. Cell lines were then treated with 20 µM concentrations of Dppz, DIP, P and MP for 12 h. Cell lines were trypsined and centrifuged for 5 min at 1000 g to created cell pellets. Pellets were washed 3x with ice cold PBS. Each complex/cell treatment pellet was placed into separate 15 ml centrifuge tubes and treated with 4 buffers (in sequence per tube) to perform 4 distinct cell compartment isolations. Compartment protocol and buffers are as follows: Buffer 1 (cystolic proteins) was made to 1 ml with 10 µL of 100x protease inhibitor, Buffer 2 (Mitochondrial, ER and Lysosome proteins) was made to 1 ml with 10 µL of 100x protease inhibitor, Buffer 3 (Nuclear proteins) was made to 0.5 ml with 5 µL of 100x protease inhibitor and Buffer 4 (Cytoskeletal proteins) was made to 0.5 ml. Cell pellets, per cell line complex treatment, were administered and spun down gravity, as stated in table 3.1 to isolate cell compartments. This is further elucidated in [tab 3.1]. After compartment separation each cell protein compartment was diluted in 5 ml in Millipore DI H_2O with 1% HNO_3 to remove any cell membrane debris. Each compartment solution was then analyzed for Ru ion concentration using ICP-MS.

Tab 3.1 Cell pellets with various complex concentrations were treated and fractioned as described in table 3.1. After each centrifugation the supernatant containing that specific cell compartment protein were removed. Each of those supernatant fractions was diluted to 5 ml in 1% HNO_3 in H_2O which served as the ICP-MS working solution.

QIAGEN KIT	Buffer 1 (1ml)/pellet	Buffer 2 (1ml)/pellet	Buffer3 (0.5ml)/pellet	Buffer 4 (0.5ml)/pellet
DPPZ	10 min/ 4 ^{o}C shaking 1000 g/10 min/ 4 ^{o}C	30 min/ 4 ^{o}C shaking 6000 g/10 min/ 4 ^{o}C	15 min/ 25 ^{o}C still 10 min/4 ^{o}C shaking 6800 g/10 min/4 ^{o}C	Buffer 4 is added to remaining cell matter and this is the last fraction.
P	10 min/ 4 ^{o}C shaking 1000 g/10 min/ 4 ^{o}C	30 min/ 4 ^{o}C shaking 6000 g/10 min/ 4 ^{o}C	15 min/ 25 ^{o}C still 10 min/ 4 ^{o}C shaking 6800 g/10 min/4 ^{o}C	Buffer 4 is added to remaining cell matter and this is the last fraction.
MP	10 min/ 4 ^{o}C shaking 1000 g/10 min/ 4 ^{o}C	30min/ 4^{o}C Shaking 6000 g/10 min/ 4 ^{o}C	15 min/ 25 ^{o}C still 10 min/ 4 ^{o}C shaking 6800 g/10 min/4 ^{o}C	Buffer 4 is added to remaining cell matter and this is the last fraction.
DIP	10 min/ 4 ^{o}C shaking 1000 g/10 min/ 4 ^{o}C	30 min/ 4 ^{o}C shaking 6000 g/10 min/ 4 ^{o}C	15 min/ 25 ^{o}C still 10 min/ 4 ^{o}C shaking 6800 g/10 min/4 ^{o}C	Buffer 4 is added to remaining cell matter and this is the last fraction.

3.2.5 Mechanisms of Cellular Uptake of Ru complexes

A series of known cell membrane blockers were used to determine if complexes P and MP enter the carcinoma cell via active or passive transport.[28] Hs766T were seeded in 60mm x 80mm tissue culture dishes at a cell density of approximately $2x10^6$ cells in DMEM medium, for 6 h. The cells were then treated with endocytosis inhibitors for 1 h except for Nystatin, which was treated for 30 min, prior to innoculation. The concentrations were as follows: 10 mM Sodium Azide (NaN_3) and 2-deoxy-D-Glucose (DOG), 1 M Sucrose, 300 µM Dynasore and 50 µM Nystatin. Cells were treated with 40 µM of various RPCs for a 6 h period. ICP-MS was used to determine Ru ion concentration. For temperature active diffusion experiments Hs766T cells were seeded as previously mentioned and inoculated with Complexes P and MP for 6 h in 4 ^oC and 37 ^oC environments. ICP-MS was used to determine Ru ion concentration.

3.2.6 Ruthenium Complex Loci Confocal Microscopy

Confocal Microscopy was performed to further elucidate the location of various ruthenium polypyridyl complexes within the cellular compartments. H358 cells were grown to confluency, passaged and grown on microscope cover slips in RPMI-1640 media. Cells were treated with complex P and MP for 24 and 48 h. After each analysis period, cells were prepared for staining and fluorescent probing. Propidium Iodide (PI) was used as a nuclear stain as follows: Media was removed from cover slip and washed 2x with PBS, cells were fixed with ice cold methanol for 10 min, cell membranes were permeabilized with 1 ml of 0.25% Triton/PBS for 10 min in a 37 ^oC incubator, 300 µL of 1% PI/PBS solution was administered for 10 min in a 37 ^oC incubator and lastly 1 ml of 3% Bovine Serum Albumin was used as a blocking agent for 30 min in a 37 ^oC incubator. The cover slip was adhered to a microscope slide and scanned using a Zeiss axioplane fluorescent microscope at 488 nm excitation and an emission band path of 585-615 nm. The nuclear stains were then pseudo-colored blue. JC-1 mitochondrial potential tracker was used as a mitochondrial stain and was performed as follows: media was removed from cover slip and washed 2x with PBS, 300 µL of JC-1 stain buffer kit solution was administered for 15 min in a

37 ^{o}C incubator. The cover slip was adhered to a microscope slide and scanned using a Zeiss axioplane fluorescent microscope at 488/543 nm excitation and a dual tracker channel emission band path of 505-535 nm and 585-615 nm respectively, dependent upon mitochondrial potential function. JC-1 aggregates were pseudo-colored green (apoptotic cell via mitochondrial membrane potential absence) and red (JC-1 aggregates indicating functional mitochondrial membrane potential).

3.3 Results and Discussion

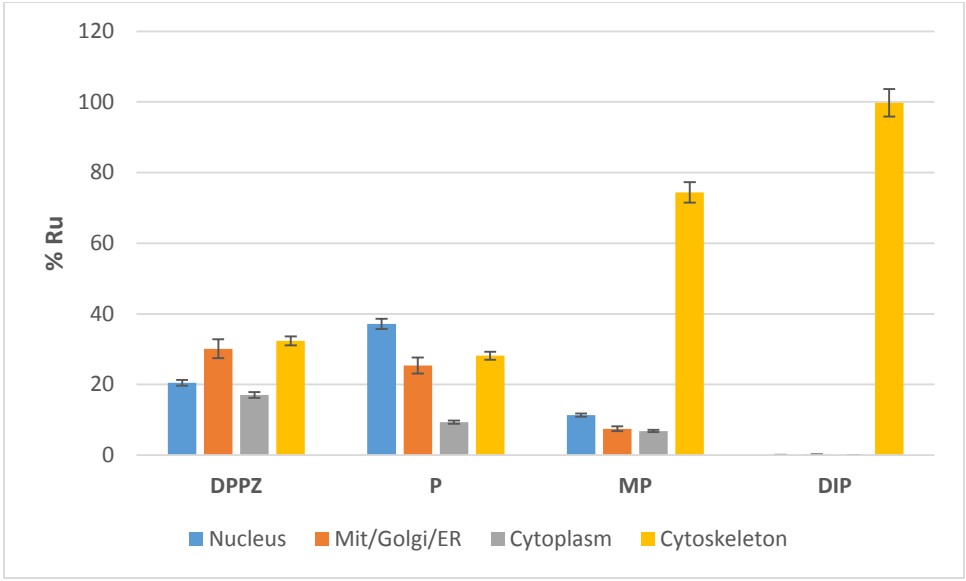
3.3.1 Ruthenium Complex Accumulation in Various Cellular Compartments

Cellular localization and accumulation is a critical first step to understanding biological activity of RPCs. The possible therapeutic value must exert itself by first reaching its target in the cell. Determining whether or not the RPCs are distributing evenly throughout a cell or localized in different concentrations in separate compartments is imperative to understanding their function. Also, the location can be a preview as to where the cyotoxic action of the RPCs occurs. Exact localization of RPCs P and MP was screened using ICP-MS measurements to quantify the Ru ion concentration in separate cellular compartments within cells lines H358 and HCC-2998. A QIAGEN compartmentalization kit was used to isolate 4 distinct cell compartments: cytoplasm, cell membranes (mitochondria, golgi and endoplasmic reticulum), nuclear membrane and cytoskeleton. Cell lines H358 and HCC-2998 were treated with 20 µM of complexes: Dppz, DIP, P and MP for 12 h. These compartments were separated with ultra-high centrifugation and dissolved in 5 mL of mmH_2O with 1% HNO_3. Results for compartment loci can be seen in [fig 3.1] with A as (H358) and B as (HCC-2998). Ru ion content, measured in ppb and then later translated to a percentage based on the total amount of Ru ion in the whole cell, P was nominalized for 1 ruthenium per MP so as to reflect whole complex localization per cell compartment. This data is shown in bar graph format in [fig 3.1]

Complexes Dppz and DIP were also examined as a control representing a closely-related but non-redox active cationic ruthenium polypyridyl complex. As seen in [fig 3.1 A/B],

approximately 20% localization was observed in the nucleus in H358 vs. 18% in the nucleus of HCC-2998. P showed an increase in nuclear compartment localization up to 38% in H358 and 30% in HCC-2998 showing a significant difference from Dppz. Barton et. al. demonstrated that Dppz intercalates but does not cleave DNA in the nucleus and the nuclear center of the cell is the target due to its hydrophobicity.[29] P shows a greater presence in the nucleus as compared to Dppz. P and Dppz also showed appreciable mitochondrial localization where cytotoxic ability might occur. A 30% localization of Dppz and a 25% localization of P in the mitochondrial regions of H358 and 19% equally of Dppz and P in the mitochondrial regions of HCC-2998 was observed. Markedly, a stark contrast was found with complexes DIP and MP being mostly localized in the cytoskeleton of both cells H358 and HCC-2998. DIP shows an almost 99% location within the cytoskeleton region of H358 and HCC-2998. MP was also pronounced in the cytoskeleton at approximately 75% in H358 and 90% in HCC-2998.

A



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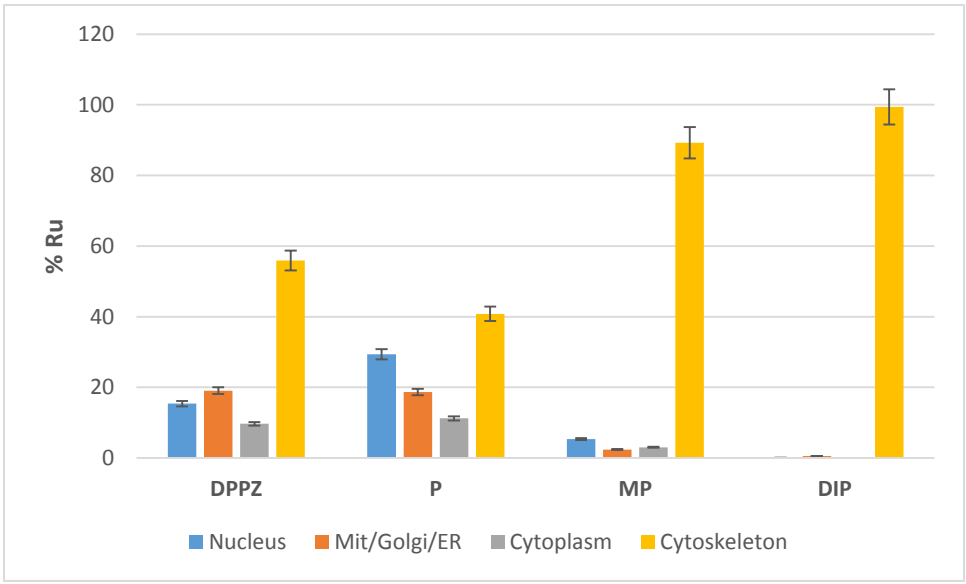


Fig 3.1 A/B Cell lines H358 (A) and HCC-2998 (B) were inoculated with 20 µM of Dppz, P, MP and DIP for 12 h. Cells were then compartmentalized by QIAGEN Compartment Kit and Ru ion was analyzed using ICP-MS. P was nominalized as to be compared to mononuclear complex MP and DIP.

The contrasts between cell locations for P and MP could serve as a good indicator of their activity location. It should be noted that the cytotoxic effects of P and MP could still be exerted through other means. Wang J. et. al. observed RPC bearing 2-phenylimidazol[4,5][1,10]phenanthroline (PIP) ligands induced mitochondrial damage leading to apoptosis, however, these complexes were located mostly in the cytoplasm.[31] One can argue that location could be a starting point for an apoptosis cascade to commence leading to cell death but not necessarily from that particular cell compartment.

3.3.2 Ruthenium Complex Cellular Membrane Transport

The location of P and MP in the cell lines H358 and HCC-2998 was examined in the previous section; now the route of cellular entry will be elucidated. Pluckett et. al. reported that endocytosis and active transport through the cellular membrane is energy dependent, whereas passive diffusion is not energy dependent.[29] Endocytosis, being an energy dependent pathway, is susceptible to modulation. Passive diffusion is an energy independent pathway less prone to modulation.[32] Barton et. al stated that processes requiring energy can be blocked by low temperatures.[29,33] To examine this possible mechanism and cell membrane transport entry pathways; a two-fold study was performed. First, the mechanism of cellular entry was examined. The focus was to determine if cellular entry follows an energy dependent endocytosis or non-energy dependent passive diffusion pathway and the type of active transport, if any, allowing entry through cell membranes. Cell line Hs-766T was inoculated with 40 µM of RPCs DIP, P and MP for 6 h in 4^{o} and 37 ^{o}C. As shown in [fig 3.2] the uptake of RPCs increased with temperature, indicating an energy dependent active transport through the cell membrane. Small amounts of RPC cell entry at 4 ^{o}C indicates some passive diffusion may also have occurred; however, active energy dependent transport is the most abundant.

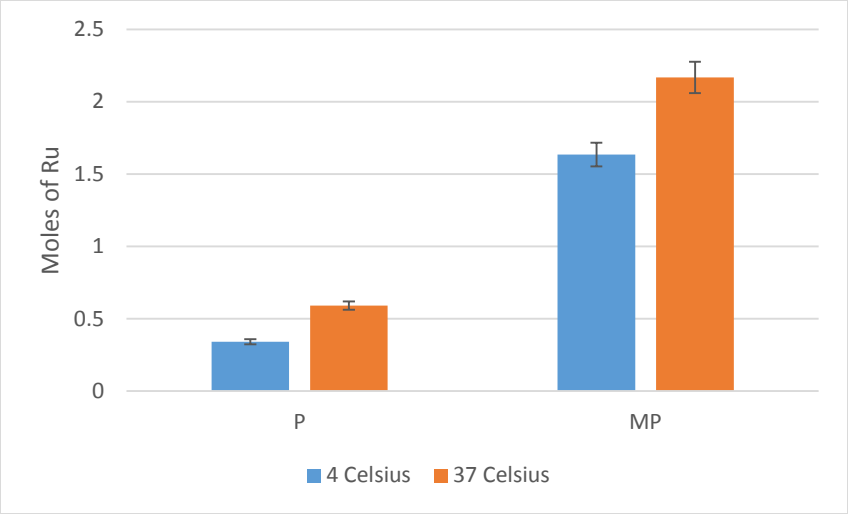


Fig 3.2 Diffusion of complexes P and MP through the cell membrane of Hs766T based on temperature difference of 4 ^{o}C and 37 ^{o}C. ICP-MS was used to quantify Ru ion content. P was nominalized as to be compared to mononuclear complex MP.

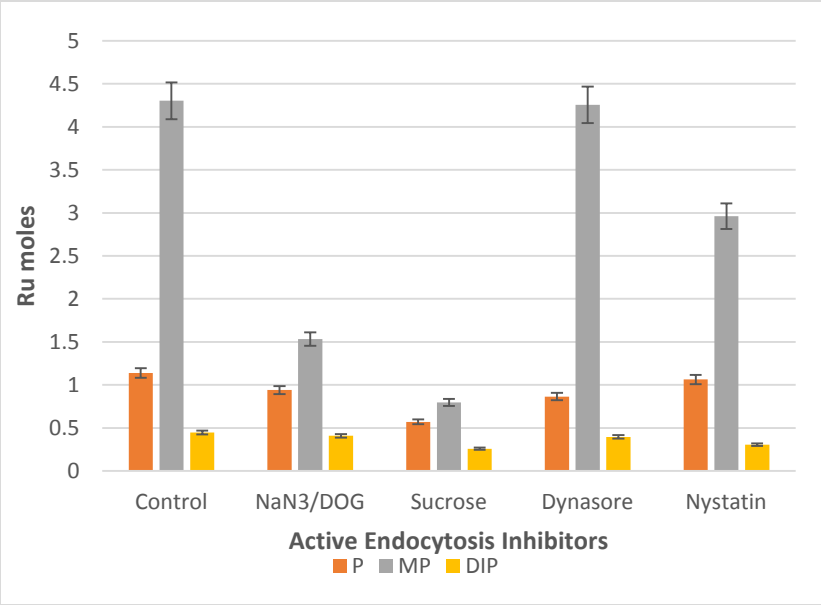


Fig 3.3 Separate active mediated endocytosis inhibitors were inoculated to Hs766T cell population separately and were inoculated with 40 µM of various complexes. Detection of Ru ion content was performed with ICP-MS. The control is 40 µM of various complexes without any inhibitor. P was nominalized as to be compared to mononuclear complex MP and DIP.

In the second study ICP-MS was used to detect Ru ion content in cells with the treatment of active mediated endocytosis inhibitors to examine the possible cell membrane transport receptors used by complexes P and MP. Treatments of sodium azide (NaN_3) and 2-deoxy-D-glucose (DOG) are known active transport energy dependent inhibitors.[30] A 33% inhibition of P was observed as compared to internalization of the control. An appreciable 65% inhibition of MP was found compared to the control as well. This finding further proves that these RPCs are entering the cell membrane by energy dependent pathways. Inhibition of lipid raft, clatherin and active receptor mediated endocytosis were studied as the three main routes of cell entry.[3] Phagocytosis inhibition was not studied since Hs766T cell lines and most cancer cell lines do not exhibit this function.[29,30] Sucrose, a clathrin mediated endocytosis inhibitor, did show a difference of 50% inhibition of P and 81% inhibition of MP entry as compared to the control. DIP showed a 50% inhibition. This suggests the clathrin transport mechanism is the main action of entry for these RPCs. In addition, nystatin, a lipid raft mediated endocytosis inhibitor, showed a 32% inhibition entry for MP and a 25% inhibition for DIP but no appreciable difference in entry inhibition with P. This shows some form of lipid raft mediated endocytosis is being utilized by MP. Dynasore, a GTP-binding protein inhibitor, which is a main component of receptor mediated endocytosis, also showed 33% inhibition of P entry into the cell membrane as compared to the control. No appreciable change can be seen with MP and DIP. Studies by Guo W. et. al. showed that RPCs can bind to extracellular serum proteins which can promote cellular uptake.[32] For this reason fetal bovine serum (FBS) was not removed from the media as in human blood a similar plasma is present.

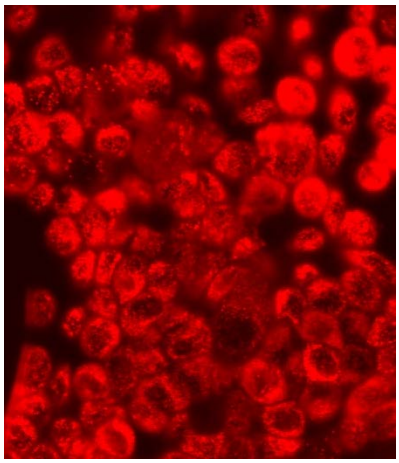
It can be concluded that P and MP are exhibiting highly active mediated endocytosis into the outer cell membrane and using different mediated cell membrane receptors to accomplish their entry. While P and MP are both using clathrin mediated endocytosis they exhibit differences in other forms of cell membrane mediated receptor entry. In addition to both P and MP using the clathrin mediated channel, P utilized a GTP binding endocytosis mechanism whereas MP used a

lipid raft dependent endocytosis. This further proves the diversity of both complexes and their method of entry into the cancer cell through active transport.

3.3.3 Ruthenium Complex Loci/Activity via Confocal Microscopy

Since the discovery of $[(bpy)_2Ru(dppz)]^{2+}$ and Dppz and their fluorescence capability when intercalated with dsDNA there has been an interest in using these fluorescent properties to observe RPC complexes in live cells.[34,35] Barton et. al. coined this term the “light switch effect”. Upon reversible binding of dsDNA the luminescence appears quenched in aqueous media but in the excited state fluoresces when intercalated with dsDNA.[34] Thomas et. al. did similar studies involving the $[(phen)_2Ru(tpphz)]^{2+}$ family of complexes using live imaging to determine their location of cytotoxic action within MCF-7 cancer cells.[34] The advantage is the ability of the RPC complexes to illuminate when intercalated with dsDNA, making them usable visible targets inside of cancer cells. The (tatpp) bridges on complexes P and MP do not show fluorescence, however the same concept can be applied from a “reverse engineering” standpoint. To further study the action of complexes P and MP specific compartment fluorescent dye labeling, which fluoresces when bound to functioning cell compartments or systems, was used. The actions of P and MP on the mitochondrial membrane potential and the nuclear dsDNA within H358 cancer cells were examined. JC-1 mitochondrial potential health dye was used to study the possible cytotoxic action of P and MP upon the mitochondria membrane potential. JC-1 is widely used in apoptotic studies to monitor mitochondrial health. JC-1 dye exhibits a fluorescent emission shift from green (~529 nm) to red (~590 nm) within live cell imaging. Green JC-1 aggregates are formed when mitochondrial potential has failed whereas red JC-1 aggregates are formed in the presence of functioning mitochondrial proton motive force.[3] Cell line H358 was inoculated with 20 μM of complex P and MP for a period of 24 and 48 h. A collection of these images in real live time is presented in [fig 3. 4-3.6].

A



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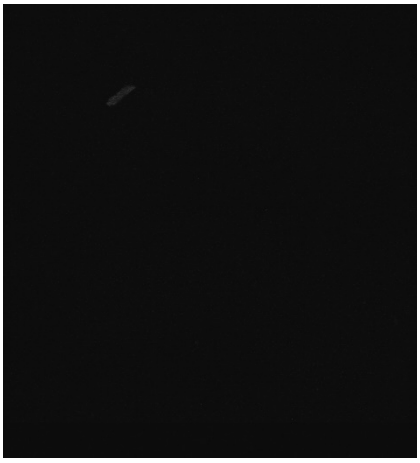

Fig 3.4 Real time JC-1 mitochondrial potential stains in cell line H358. Control images A (healthy mitochondrial potential) and B (ceased mitochondrial potential function) are shown without P and MP inoculation.

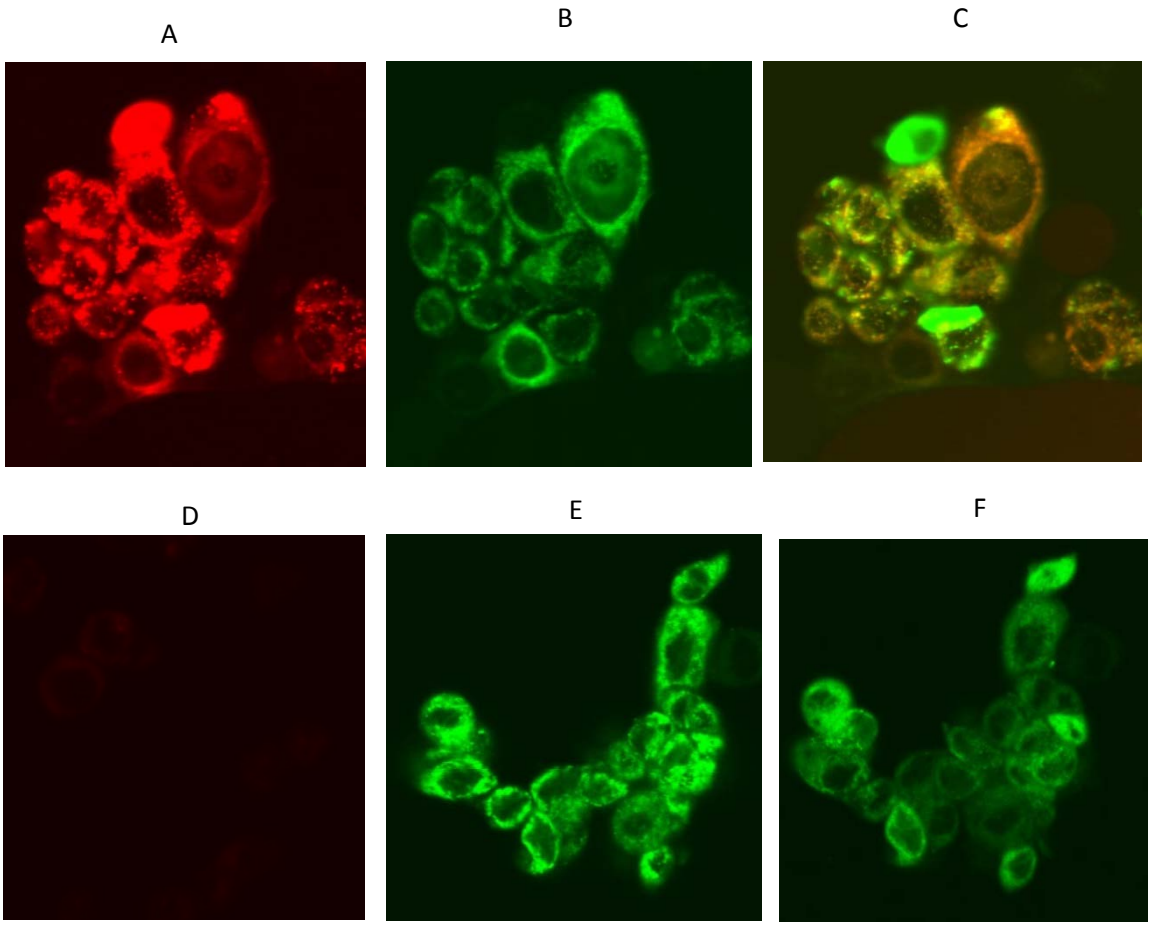

Fig 3.5 Real time JC-1 live imaging stain with complex P showing failed mitochondrial potential (green), functioning potential (red) and merged image (red/green) for 24 h (A-C) and 48 h (D-F).

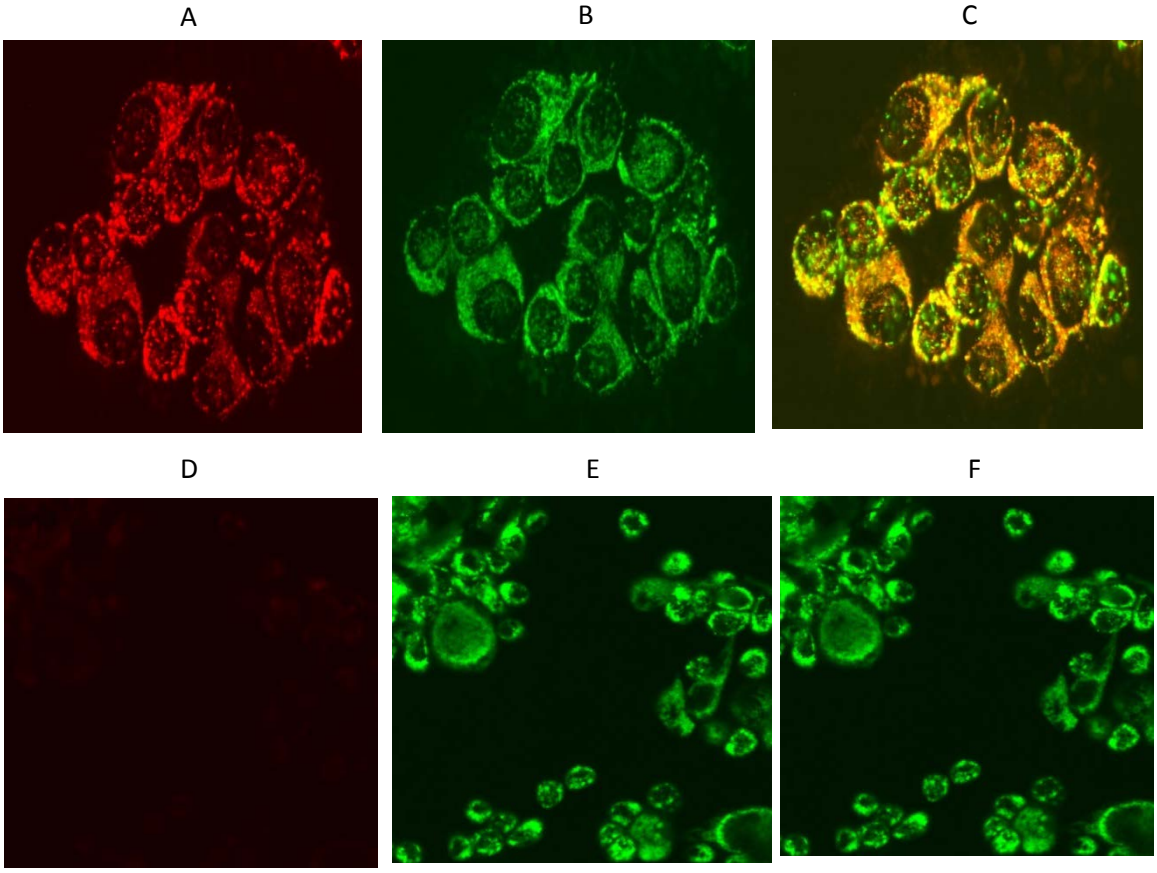

Fig 3.6 Real time JC-1 live imaging stain with complex MP showing failed mitochondrial potential (green), functioning potential (red) and merged image (red/green) for 24 h (A-C) and 48 h (D-F).

Image A [fig 3.4] shows the control of H358 cells without complex, indicating healthy and functional mitochondrial membrane potential. The JC-1 red aggregate is clearly observed confluent throughout the image. Image B shows no green JC-1 aggregate indicating all cells are operating normally with respect to the mitochondrial membrane potential function. Image tracks [fig 3.5 A-C] (P) and [fig 3.6 A-C] (MP) exhibit the first 24 h inoculation of complex separately. The JC-1 green aggregates are clearly forming after 24 h. The red aggregates are still visible in [fig 3.5 A] and [fig 3.6 A] as all mitochondrial potential has not ceased as of yet. Images [fig 3.5 B]

and [fig 3.6 B] shows the green JC-1 aggregate exhibiting the commencement of mitochondrial damage. In images [fig 3.5 C] and [fig 3.6 C] the images are merged to show the ratio between JC-1 aggregate dyes. Image tracks [fig 3.5 D-F] (P) and [fig 3.6 D-F] (MP) exhibit inoculation after 48 h. It can be seen that in [fig 3.5 D] and [fig 3.6 D] there is a miniscule JC-1 aggregate fluorescing. When these images are merged the JC-1 [fig 3.5 F and fig 3.6 F] green aggregate is the only signal that is showing. These results correlate with Wang and Qian et. al. that many RPCs are mitochondrial targeting toxins in cancer cells.[37]

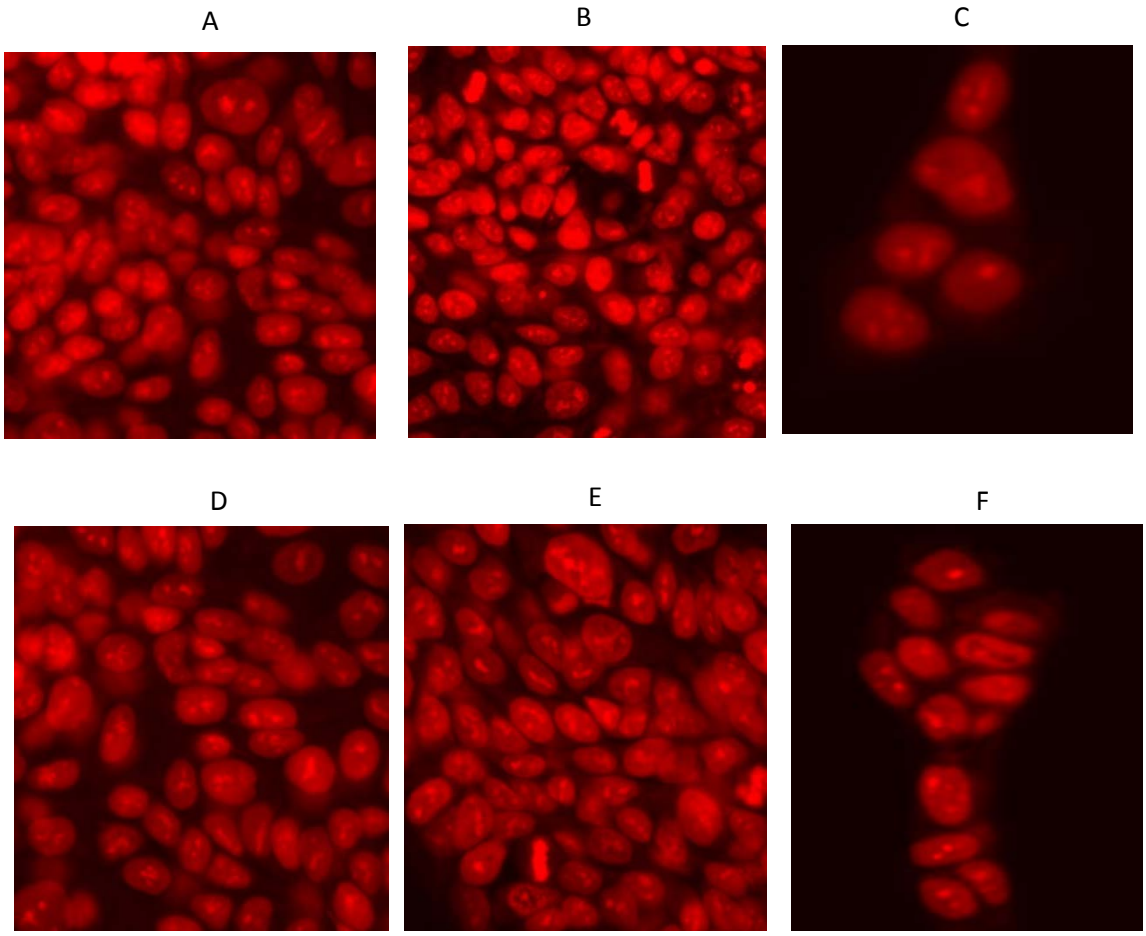

Fig 3.7 Propidium Iodide stains of dsDNA chromatin packaging. Tracks A and D are controls with no complex inoculation. P tracks B and C exhibit 24 and 48 h inoculation and MP tracks E and F exhibit 24 and 48 h inoculation. H358 cells were fixed, permiabilized and stained.

In addition to this work the effects of P and MP on the nuclear dsDNA within the H358 cell line have been examined. Propidium Iodide (P.I.) is a dsDNA intercalator and fluoresces brightly in the red visible region (~595 nm) when bound to intact dsDNA.[4] As stated in chapter 1 P and MP cleave dsDNA *in vitro*. To observe this effect *in situ* one can conclude that there will be a remission of signal from dsDNA breakage within the nuclear envelope. The control as before was prepared as H358 cells with no complex inoculation. In [fig 3.7] image tracks A and D are the

controls. The fluorescing chromatin dsDNA packaging is visibly intact and no breakage is noticed. After 24 h of inoculation with P in track B and MP in track E the chromatin dsDNA packaging is signaling brightly indicating no breakage of dsDNA. After 48 h inoculation with P in track C and MP in track F the chromatin packaging is still signaling bright and intact. The reduced cell count in the image C and F are now known to be due to the mitochondrial potential death rather than the nuclear damage that might have occurred. Apoptosis initiated by nuclear dysfunction is a cascade system that is not instantaneous but rather takes an allotted amount of time. If dsDNA broke where occurring reduced and fading signal from the chromatin packing would be seen which is not visible here. The nuclear dsDNA is still intact after 48 h inoculation with P and MP.

In summary, this study has shown that complexes P and MP are two separate and unique cancer cytotoxic effecting complexes. A contrast in cellular localization in non-small lung carcinoma H358 and human colon cancer HCC-2998 was observed. P localized in multiple cell compartments in various amounts whereas MP was highly localized in the cytoskeleton of the cell. This study has also elucidated the entry into pancreatic cancer cell Hs766T as being kinetically driven and multiple active mediated endocytosis receptors are in play. Again P and MP exhibit a contrast in their method of entry. P and MP both utilize clathrin receptor mediated entry. In addition, P is also utilizing the GTP coupled active transport receptor whereas MP is utilizing a lipid raft active transport mechanism. Lastly, this study has shown that complexes P and MP are highly toxic to the mitochondrial potential in non-small lung carcinoma H358 while no nuclear dsDNA damage is occurring indicating the mitochondria might be the target of cytotoxic action by these complexes. It is still unknown if this is the only cause of apoptotic death due to these RPCs but it is definitely a major player in the role of their anticancer activity.

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Biographical Information

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Adam is currently working on possible anti-cancer complexes with the Frederick MacDonnell lab at the University of Texas at Arlington.

He is an avid deep sea fishing enthusiast as well as an accomplished concert pianist.