Spring 2019

Development of Ruthenium Drugs as Anticancer Agents

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2019

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CHEMISTRY THESIS

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Introduction:

Finding the cure for cancer is a major area of focus in today's medicinal world. Statistics stated in 2013 that 1 in 4 deaths in the United States can be attributed to cancer.[1] The matter of curing cancer is highly complex. Practically, it is difficult to create a compound that will kill diseased tissues without affecting healthy tissues.[2] In general cancerous cells are analogous to healthy tissues; they simply have upregulated metabolic function leading to uncontrolled growth. This does lead to differences that can be targeted but finding these differences and creating therapeutics for these purposes requires a great deal of research. When researching new drugs, it is important to create experiments that emulate biological conditions. Tumors are not easy to replicate in a lab setting.[3] This leads researchers to not only test viability of new drugs on diseased cells, but also to critically analyze the methods by which testing occurs. It is important to utilize *in vitro* method that bridges the gap between the lab and the clinical setting.

One drug that has been developed and used affectively for the treatment of cancer is Cisplatin. This medication is a platinum-based compound that readily accepts the unpaired electrons of sulfur and nitrogen containing compounds to form dative bonds. This allows the platinum to bind with amino acids cysteine and methionine as well as many of the nitrogen bases of nucleotides deforming nuclear DNA.[4] The damaging of the DNA initiates apoptosis.[5] Apoptosis is the desired method by which cancerous tissue is removed, because it is a controlled method of cell death that causes little inflammation. Alternately, necrosis, a method of cell death leading to inflammation and the damage of adjacent cells, is undesirable.[6,7] Cisplatin is injected systematically into a patient's circulatory system, and has been in use since FDA approval in 1978.[8] It has been considered an "essential medicine" by the World Health Organization.[8]

The mechanism by which Cisplatin affects DNA begins when the two chlorine ligands are hydrolyzed, replaced by water molecules that donate a lone pair from the oxygen to the metal center. Plasma has a high water-content so it is possible that hydrolysis will occur prior to reaching diseased tissue. While this is the case, the chloride ion concentration is high in the blood when compared to the inside of a cell (100μM versus between 4 and 12μM). This difference allows the Cisplatin to maintain its integrity well enough in the blood to reach and act within cells.[5] With this in mind, researchers often seek to create drugs with analogous mechanisms to Cisplatin's.

Although Cisplatin has been highly beneficial for treating a wide range of cancers, it has drawbacks that require discussion. A report by Hazlitt *et. al* indicates that this drug causes an incidence of hearing loss for 63% of patients. The extent of hearing loss varies between individuals. Cisplatin reacts within auditory cells causing apoptosis and the generation of reactive oxygen species (ROS), a decrease in antioxidant enzymes, and an increase in proinflammatory cytokines.[8] Resistance within center cancer types is another issue that diminishes the efficacy of Cisplatin's use. Lung carcinoma tumor A549/CDDP is an example of a known resistant cell type.[2] Currently, research is seeking the mechanism by which resistance occurs, but none of the accessed literature indicated a causative conclusion. To this point only hypotheses have been proposed. Researchers are currently unable to monitor all the reactions that Cisplatin undergoes within the cell due to miniscule concentrations. One theory is that tumors cells release excess amounts of glutathione (GSH). Cisplatin theoretically binds to the sulfur in GSH prior to the being able reacting with DNA.[4] The main reason for the side effects generally associated with chemotherapy is Cisplatin's inability to affect diseased tissue without damaging

healthy tissues. These include nausea and vomiting, loss of appetite, and hair loss. Since the regular cells grow back and tumor cells do not, the side effects are tolerated.[7]

Ruthenium Complexes: Targeting DNA

Current research is investigating ways to use ruthenium complexes as a substitute for Cisplatin in hopes of finding medicines that are more specific to cancerous cells and that reduce the side effects of chemotherapy. Due to the success of Cisplatin, many of these new drugs seek to target the DNA of diseased tissues. Cisplatin has a square planar geometry[5] while ruthenium complexes have an octahedral geometry. This helps to increase the variability in the ligands attached to the metal center allowing for higher specificity in targeting diseased tissues.[9] Many ruthenium complexes are used in medicine currently,[7] but the ruthenium complexes specified for anticancer therapy have yet to become staples in treatment. It is desirable that some of them will be applied to regular use in the near future. In this summary, the attractive aspects of ruthenium complexes and the methods by which their efficacy is tested are investigated.

Within the precious metal therapeutics, ruthenium is the only metal that forms stable complexes in oxidation states 2+ through 4+ under biological conditions. Ru(III) complexes are less biologically active when compared to Ru(II) and Ru(IV) compounds.[7] Ruthenium is also in the same d-block triad as iron and are able to bind to the protein transferrin that is found in a higher amount on the outside of tumor cells as opposed to healthy cells. This diminishes the chances of uptake of Ru complexes into healthy cells. Transferrin binds to the complex and actively transports it into the cell.[10] Once there, the molecules are in an environment with low oxygen levels. The hypoxic environment of diseased tissue leads to the reduction of Ru(III) to Ru(II), the more reactive species.[6,11] Even if some of the Ru(III) compound were to be moved into a healthy cell, the higher amount of oxygen would decrease the likelihood that reduction

would occur. As the reduced form, the complex theoretically will damage cancerous cell DNA by causing cross-links that lead to apoptosis. One theory of the incidence of cross-linking is that the octahedral geometry of ruthenium drugs leads to a "steric restriction." This proposed mechanism differs from the mechanism that Cisplatin employs to cause DNA deformation. Research believe this may be why the drugs with Ru overcome the resistance some tumors show towards the platinum drug.[7]

Another aspect of ruthenium complexes that makes them of interest is their similarity to platinum's ligand exchange.[7] Ligand exchange determines biological activity of a complex. Metal complexes are often activated when the ligands attached to the metal center react with compounds with which they come in contact. If the ligands do not remain bound to the metal center until they reach the desired location, the anticipated outcome will not result.[7,9] In the case of anti-cancer agents, this result is stopping metastasis and/or causing cell death of diseased tissue. Ruthenium complexes have shown to bind to DNA just as platinum based drugs do, but many lack solubility which makes it difficult for them to be transported through the circulatory system.[6,7] Using the dialkyl sulfoxide derivative dimethylsulfoxide (DMSO) researchers were able to create a soluble compound called NAMI-A. This compound binds to DNA much weaker than Cisplatin[12], and *in* vivo trials did not indicate DNA damage as the anticancer mechanism.[7] This was considered the most promising Ru(II) complex in 2001[7] due to the antimetastatic capabilities.[12] These antimetastatic capabilities are the reason it is called NAMI which means "new anticancer metastasis inhibitor."

Researchers have a litany of ligands to choose from that affect the characteristics of the complex. Since anticancer agents are injected through into the blood stream, researchers must create drugs that are able to dissolve in the blood without reacting. Patra, *et. al* describe a method

of incubation in human plasma that is utilized to test whether or not a substance degrade in an environment that simulates blood flow.[13] The substance in question is added along with an equivalent volume of diazepam solution to blood plasma obtained commercially. This solution is incubated with a gentle stirring of 300 rpm at 37°C for a period 24 hours or more. The researchers use this length of time because it far overshoots the time should remain in the blood prior to reaching its desired location. After incubation Patra, et. al used a 2:1 ratio by volume of methyl-tert-butyl ether/dichloromethane solution to stop any reaction that may be occurring. The solvent was allowed to evaporate, and the solution was centrifuged so the organic layer could be analyzed in HPLC-MS. A reverse phase column was used for the separation and ESI mode was used for the mass spectrometry analysis.[13] Huang *et. al* used the same overall method as Patra *et. al* only making slight modifications they saw necessary for the testing of their proposed ruthenium anti-cancer therapeutics.[2] Neither group explains explicitly how the decomposition of their respective target substances are compared. They only stated that each maintain integrity when incubated under the conditions previously described. It seems reasonable to assume that the substance and diazepam solution is simply tested through the same analytical methods, HPLC-MS, without incubation and compared to the results aforementioned. When viewing results, diazepam is an internal standard with a distinct peak that will show up in the mass spectrum. Since equivalent amounts of diazepam and the complex being tested are added, the proportion of the peaks that result should be the same between the incubated and the non-incubated analytes.

Once the complex travels through the blood, it must be taken up by the cells. It is indicated that cisplatin is taken up by cells mostly by passive diffusion.[5] Huang *et. al* sought the mechanism by which the complex is taken up by the cell by incubating it in the presence of cell

uptake inhibitors. Eight different inhibitors of energy dependent and energy independent entrance pathways were tested. By using Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) the group found that $[Ru(bpy)(phpy)(dppz)]^+$ is not hindered by any uptake inhibitors that block energy dependent pathways. Meaning it is most likely taken up by passive diffusion. $[Ru(bpy)(phpy)(dppz)]^+$ is only hindered by a high K^+ concentration around the cytoplasm. This high K^+ concentration increases the membrane potential from around -70 to 0mV. Researchers hypothesize that the positive charge on the complex makes it susceptible to changes in the cell membrane potential.[2]

The localization of the drug in cells aids in creating the comprehensive dataset necessary to begin formulating the mechanism of the therapeutic in question. Huang, *et. al* used ICP-MS (Inductively coupled plasma mass spectrometry) to test the amount of different ruthenium complexes that are able to reach the nucleus following incubation. ICP detects minute concentrations as small as one part per 10^{15}, the MS portion allows for quantification. The machine nebulizes the analyte and moves it through plasma at a temperature high enough to completely atomize the sample. This allows all the atoms present to be quantified individually. In the case of precious metal cancer drugs, the metal center is the focus of this method. These metals have a high mass relative to the other atoms present in the complex. Due to this element, the issue of resolution in the spectra is diminished.

To test the difference of uptake into the nucleus versus the cytoplasm, a “nuclear and cytoplasmic protein extraction kit.” This method requires that technicians start with a known concentration of cells and counted following incubation in trypsin—EDTA solution. Next, consecutive applications of centrifugation, collection of pellets, and resuspension in the various solutions indicated by the kit are needed to separate the cytoplasmic and nuclear fractions

respectively. Once separated each fraction is tested in the ICP-MS separately and recorded. Huang *et.* al indicated that 90% of the focal ruthenium complex was taken up to the nuclear proteins after two hours (Figure 1).[2]

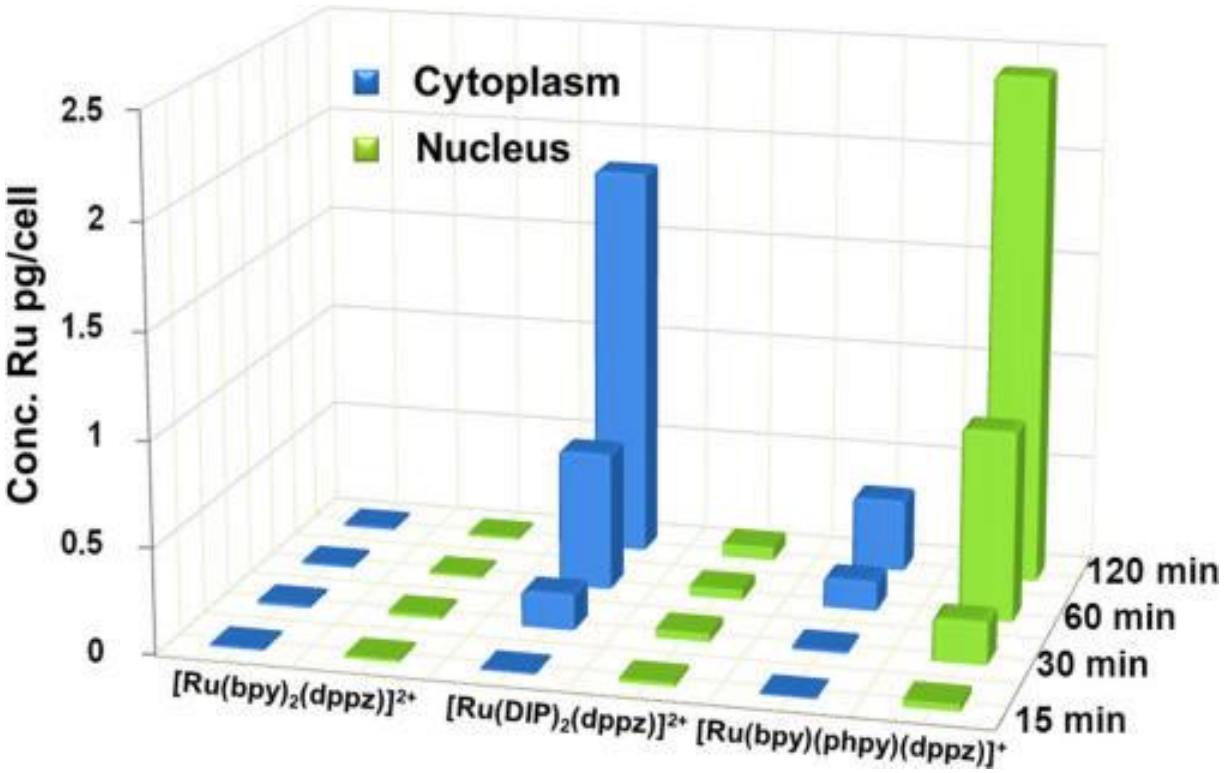


Figure 1. A representation of the change of concentration in the cytoplasm and the nucleus of the three ruthenium complexes tested by Huang *et. al* over time. [2]

Although accumulation in the nucleus is important for an anti-cancer drug, it does not necessarily mean the drug will be cytotoxic. Huang, *et. al* tested cytotoxicity by 2D cancer cell culture and 3D multicellular tumor spheroids (MCTSs). 2D analysis requires that a known number of cells are cultured on a 96-well flat-bottomed multiwell plate. Huang *et. al* used 8 tumor cell lines and one human cell line. Since Cisplatin is the current drug widely used, it was also used as an independent variable for the 2D analysis along with $[Ru(bpy)_2(dppz)]^+$ and $[Ru(bpy)(phpy)(dppz)]^+$.[2] 2D analysis is commonly used because many tests can be run can be run at a time and results are reached quickly with high reproducibility.[3] Incubation for this study was 48 hours. For all eight cases of tumor cell lines tested, $[Ru(bpy)(phpy)(dppz)]^+$ is more

cytotoxic than Cisplatin (Figure 2-2D). An interesting find by this group shows that $[Ru(bpy)(phpy)(dppz)]^+$ is even cytotoxic to a Cisplatin resistant line, A549/CDDP. Also, $[Ru(bpy)(phpy)(dppz)]^+$ is less cytotoxic to normal human cells. Cytotoxicity was represented as IC_{50} in µM, which is 50 percent of the concentration needed to inhibit cell growth. In the case of tumor cells, a lower number is desirable because this means the drug poignantly affects the growth mechanism. For healthy cells, the ultimate goal is for a drug that will show as high of a concentration as possible. Ultimately, a large disparity between the IC_{50} of diseased tissue versus normal tissue indicates that it is unlikely that the drug will affect both. IC_{50} for $[Ru(bpy)(phpy)(dppz)]^+$ on healthy tissue is shown as 11.5µM, while the number is less than 4.3µM. This means that $[Ru(bpy)(phpy)(dppz)]^+$ could be more selective than Cisplatin if used in cancer therapy.[2] While IC_{50} is the number used to represent the cytotoxicity of a therapeutic, it has nothing to do with the actual dosage that would be used when a drug is utilized in a clinical setting. The dosage is determined during the first stage of clinical trials. Cytotoxicity measurements simply are one of the steps taken *in vitro* to determine whether or not further testing of a drug should be pursued.

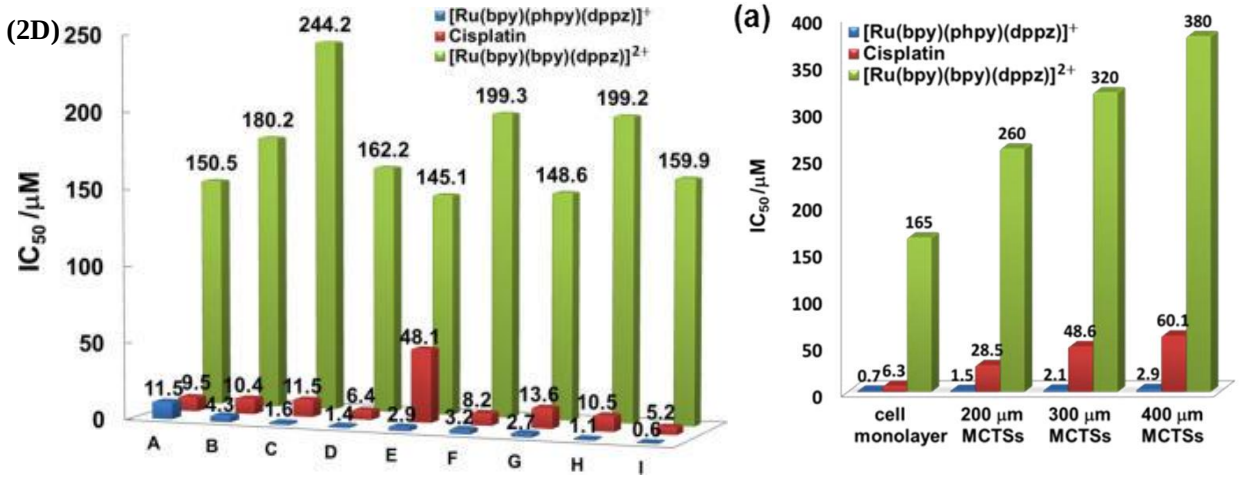


Figure 2. '2D' shows IC_{50} values for $[Ru(bpy)(phpy)(dppz)]^+$, Cisplatin, and $[Ru(bpy)_2(dppz)]^+$ when incubated using 2D cytotoxic analysis. The focus is the comparison of the blue and the red especially for culture A and E. A is normal cells while E is a Cisplatin resistant tumor cell type.

The rest from B-I are other cancerous tissue types. 'a' gives IC_{50} values for the 3 complexes aforementioned by comparing the effects on a monolayer versus three sizes of MCTSs.[2]

The viability of 2D analysis is questioned because there is often a notable deviance between *in vitro* and *in vivo* results for cytotoxicity.[3,14] De Witt Hamer *et. al* tested how the genomes of 2D cell lines and 3D MCTSs changed over a period of two weeks. In the majority of the cases, 2D cultures showed low correlation after a two-week period. The samples that did not show deviation after two weeks were tested at six and twelve weeks to find if changes may occur at those points. Two of the cases in which a high correlation was maintained after two weeks were further tested. Results showed that the genome of the primary cell cultures deviate further from the parent tumor than the spheroid cultures when tested at 6 and 12 weeks. MCTSs show a higher correlation to the parental tumor genome than primary cell cultures after twelve weeks. The genetic change is significant, but the study does not claim that this variance is the reason that differing results in a lab setting versus testing on small animals. There is simply evidence that the biology of 2D cultures are less representative of the tumors. Correlation data is tested by using a "genome-wide array comparative genomic hybridization (array CGH). [3]

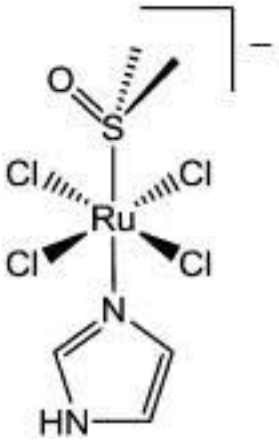
Huang, *et. al* included testing of MCTSs to give further information on the cytotoxicity of their complexes (Figure 2-a). As previously mentioned, the genomic character of these spheroids maintains a better correlation. Other aspects of these cultures that may also contribute to a higher reflection of a true tumor are cellular heterogeneity, nutrient and oxygen gradients, and proximity interactions of cell matrices. Creating MCTSs requires culturing a cell type followed by dissociation into a single cancer cell isolate with a trypsin/EDTA solution. Once isolated the cell is cultured to form the spheroid. Huang *et. al* grew their spheroids to 200, 300, and 400 µm and found the cytotoxicity of $[Ru(bpy)(phpy)(dppz)]^+$ as compared to Cisplatin. As the size of the MCTSs increased, the cytotoxity decreased. In each case $Ru(bpy)(phpy)(dppz)]^+$ is more

cytotoxic when compared to Cisplatin. The ruthenium complex shows IC_{50} values that are roughly twenty times lower at each spheroid size.[2] The development of 3D cytotoxic analysis indicates the importance of researchers continuing to explore better methods of testing. As more drugs fail to reach clinical use, researchers must take a step back and evaluate whether or not the protocol of getting them there is viable. From the research done on MCTSs, an image of how this is done is gained. The more realistic *in vitro* testing can be; the more confidence scientists can have that the therapeutics they create will work.

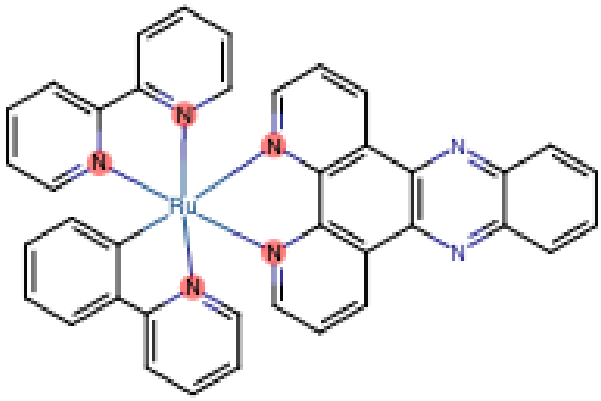
One of the ways anticancer drugs can lead to apoptosis is by blocking transcription. To do this, the drug must first be taken up the diseased tissue into the nucleus and bind to DNA. Once bound to DNA, the complex can block RNA polymerase and/or transcription factors from binding. Compound $[Ru(bpy)(phpy)(dppz)]^+$ showed the ability to block transcription and specifically a transcription factor called, NF-κB. The in vitro uptake of $[Ru(bpy)(phpy)(dppz)]^+$ into the nucleus is 90% after 2 hours of incubation making it an ideal compound to reduce the formation of RNA and ultimately proteins through translation once bound. This is the mechanism proposed by Huang *et. al* by which anticancer action occurs.[2] Based on these positive results, it is likely that researchers will continue to study the molecule discussed by Huang *et. al.*

Another way researchers begin to create an understanding of the mechanism is through computational chemistry. Vargiu *et.* al used the computation method of Density Functional Theory (DFT) to compute the energy levels of the hydrolysis products of NAMI-A to begin the process of understanding its mechanism of action.[12] DFT is a method that is commonly applied to syntheses. This group compared the energies of the complex when the metal in the plus three and plus two oxidation states. As previously noted, ruthenium compounds are stable biologically in both oxidation states and the difference in the oxidation can be beneficial for uptake into

cells.[7] Vargiu *et.* al found that reduction was favored under biological conditions and even more so in tumors due to the hypoxic environment of diseased tissue.[12,15] Also, the group found that hydrolysis of DMSO competes when ruthenium is in the plus three state. While no conclusions on how the hydrolysis affects the mechanism were made, the understanding of what hydrolysis products are formed helps to move research toward specified study.[12] Researchers could use this information Unfortunately, more recent literature explains the compound was not successful in phase 2 of clinical trials.[6] Therefore, the study to deduce the overall mechanism will not be continued unless NAMI-A makes an unforeseen resurgence.



NAMI-A



[Ru(bpy)(phpy)(dppz)]$^{+}$

Scheme 1. A display of the structures discussed in "Ruthenium Complexes: Targeting DNA" section.

Photodynamic Therapy: Targeting the Mitochondria

A completely new angle has been sought since finding a ruthenium drug with an analogous mechanism to Cisplatin has yet to be created. Photodynamic therapy (PDT) is a method that requires a photosensitizer (PS) to be energized by light. Without light radiation, a good photosensitizer will be inert in the body. The energy transfers to molecular oxygen species that yields reactive oxygen species (ROS).[11,14,16] The ROS cause oxidative stress leading to tissue

damage[8] to the immediate area in which the photosensitizers accumulate. Being that the mitochondria is the powerhouse of the cell, it is an ideal location for the photosensitizers to accumulate. Mitochondrion are the primary target because death that starts in these organelles tend to lead to apoptosis rather than necrosis of the cell.[14] Also, mitochondria targeting ruthenium complexes have shown promising cytotoxic effects on Cisplatin resistant tumor types[9], making PDT agents a desirable replacement for Cisplatin if drugs can be applied clinically.

Many PDT agents yield positive results when tested *in vitro*, but *in vivo* results are not as promising. The need for a more realistic *in vitro* test of cytotoxicity to bridge the results[2,3] leads researchers of PDT agents to focus conclusions of cytotoxicity with the results gathered when testing with multicellular spheroids (MCSs). Liu *et. al* explain that a MCS larger than 200µM has a structure able to mimic the environment of tumor better than traditional monolayer cultures.[14] This is justifiable because the larger the spheroid, the more likely it will be for its heterogeneity to be increased.

Another issue these compounds face is being irradiated once entering the tumor cells coupled with the often hypoxic environment of tumors.[14,15] The wavelengths of light used for radiation can only penetrate so deep when it comes into contact with tissues. Generally, PS are excited by light in the UV to the near infrared (NIR). NIR light is able to penetrate deeper than light in the UV-Visible range but is still limited. Using UV light can also be damaging to the cells by itself. Think about it. Skin cancer can occur from too much exposure to the UV rays of the sun. Therefore, it is best to stay away from this type of light since side effects are bound to arise.

There are two methods of irradiation within PDT that are used to excite the compounds. Both involve a method called 'one-photon activation' uses light from UV-Visible range to excite compounds most effectively. When the one-photon method uses light from the NIR, there is not enough energy to activate complexes and create ROS. Fortunately, a method that irradiates complexes with two photons simultaneously is effective in the NIR. The majority of original PS were made within one-photon activation in mind. [17] Because of the desirable penetration of the two-photon method, drugs that are receptive to this form of irradiation are highly sought after.[11,17]

A paper by Liu *et. al* investigates four ruthenium complexes (Scheme 2) with 4,7-diphenyl-1,10-phenanthroline (DIP) with the third ligand varying slightly for each. The third ligand for RuL1 and RuL2 are available commercially. The difference between L1 and L2 in the addition of an arene ring in place of the hydrogen. The L3 and L4 that were used could not be acquired commercially and the group synthesized them prior to forming the complexes. L3 required a four-step synthesis and L4 needs three reactions to be complete. The yield of L3 with the methods describe yielded 75% of the theoretical while L4 was 56%. Both are light yellow precipitates when formed and purified.[14] The ligands for L3 and L4 were created because RuL2 yields better PDT results than RuL1. The addition of triphenylphosphine (TPP) is added directly to the non-DIP ligand in L2 to form L3 and indirectly via a flexible alkyl oxide chain attached to L2 with an ether bond. The TPP addition is added to increase the solubility of the complexes and in an attempt to add higher selectivity for mitochondria.[14] In some cases, increasing the solubility negatively affects the cellular uptake of a therapeutic,[18] but this is not the case for the TPP ligands 3 and 4 used in the study by Liu *et. al*.[14] The researchers noted that ES-MS, IR, ^{1}HNMR, and ^{31}PNMR are the methods used to characterize the complexes. This paper describes the

experiments needed to test PDT agents *in vitro.* The group sought the two-photon absorption (TPA) cross-section, the localization of the substances in cells, and made a comparison of ROS production via the one-photon and two-photon methods

To find the wavelength at which excitation occurs, the TPA cross section (σ_2), spectroscopy is used. Equation 1 is dictated as the method for calculating the σ_2 at different wavelengths. 'C' is the concentration in M, 'I' is the integrated photoluminescent spectrum, and 'n' is the refractive index. The subscripts 'S' and 'R' represent whether the variable is sample or reference respectively. Rhodimine B is the reference that Liu *et. al* used for their calculations.[14] The method by which reference values for Rhodimine B are calculated is reported by Xu *et. al.*[19] Zeng *et. al* follow a similar methodology in their study. The calculations by Zeng *et. al* are performed at intervals of 10nm in the 2-photon range (760-900 nm[14,17]) and represented in graphical for in Figure 3 with σ_2 value on the y-axis and wavelength on the x-axis. A higher value is desirable because it indicates that less radiation is needed to excite the drug. The highest values for RuL1-L4 occur within the wavelengths 810-830.[14] It is reasonable to find such similarity for these four complexes due to the high degree of similarity in their ligands. Liu *et. al* report the σ_2 values in GM (1 GM = 1 x 10^{-50} cm s^4 $photon^{-1}$ $molecule^{-1}$). For the complexes tested, the GM values are 124-198, which is much higher than bioactive molecular probes, such as; H_2TPP. This higher number is inversely proportional to the intensity of the light needed to cause excitation of the material. Therefore, the higher the σ2 is the lower the intensity of the light used to irradiate the material needs to be. For RuL1-L4, L4 shows the highest GM value of 198 at 830nm.[14] Performing this tests allows researchers to know what wavelengths to use once testing of a PS on tumor cells commences. Also, it gives them an idea of which complexes should need less light radiation to be activated.

Equation 1: $\sigma_S = \sigma_R \frac{\phi_R C_R I_S n_S}{\phi_S C_S I_R n_R}$

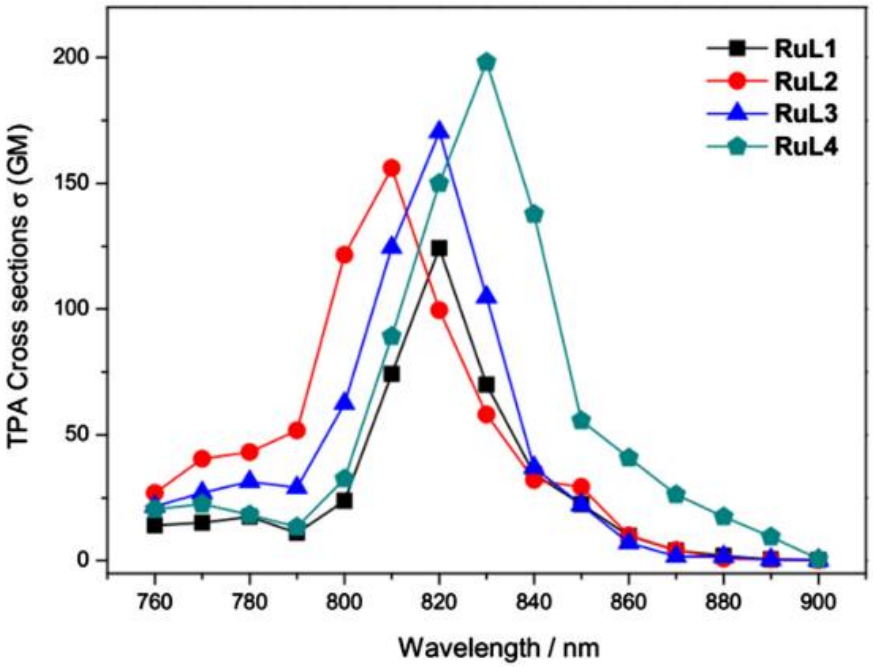


Figure 3. A graph showing the TPA cross-sections for the four ruthenium complexes tested by Liu *et. al* at 10 nm intervals.

Liu *et. al* used an analogous method to Huang *et. al* to characterize the uptake location of the ruthenium complexes into the cytoplasm and the nucleus. As previously stated, ICP-MS is the analytical machine that is able to quantify the small concentrations and their distribution. In this case the drug should accumulate in the cytoplasm, since that is the location of the mitochondrion. RuL1-RuL4 all accumulate at concentrations above 90% of the incubated amount into the cytoplasmic proteins after two hours.

The step that determines whether or not the drug is targeting the mitochondrion specifically distinguishes the experimentation from other types of tracking methods. The mitochondrion can be stained and viewed with one- and two-photon luminescent imaging with a Laser Scanning Confocal microscope when incubated with MitoTracker Green (MTG). As the name suggests, the mitochondrion show up as a bright green color on the image. The Ruthenium

complexes show up in a bright red color with the same imaging when excited by 458nm light. These two images are then compared, and a correlation can be made. RuL4 shows a correlation of 0.88 to the MTG image. This correlation factor then is multiplied by the percent concentration of the ICP-MS results to yield how much of the substance localizes in the target location. 'RuL3, RuL2, RuL1, RuL4' is the list of the results in increasing order for percent uptake into the mitochondria given by Liu *et. al* when preforming this method. It is interesting to note that the addition of TPP directly gave a less preferential accumulation to the mitochondria. It can be concluded that the addition of the TPP does target mitochondrion more preferential when the alkyl group spaces it from the non-DIP ligand.[14]

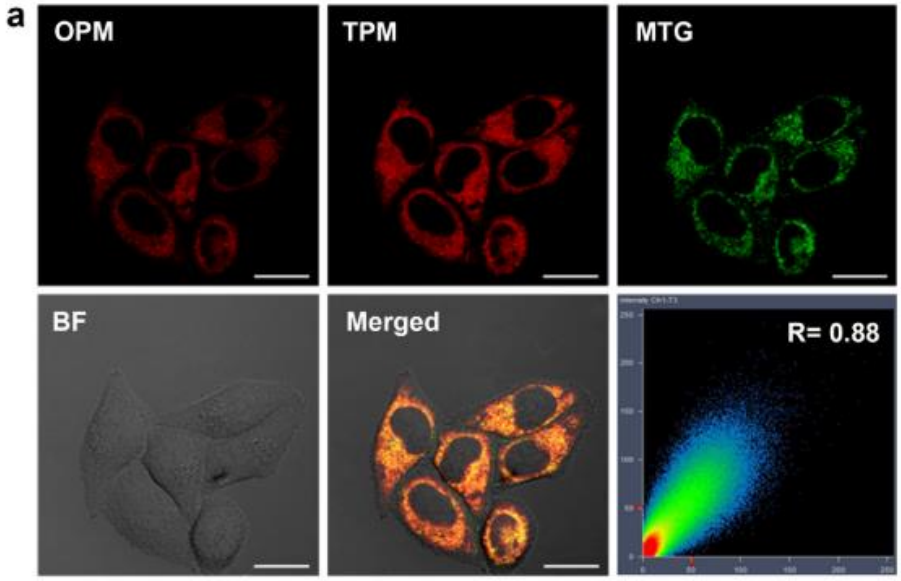


Figure 4. Images showing the Confocal Laser Microscope images of RuL4 and how the images are merged to create the correlation of the complex localization in the mitochondria.

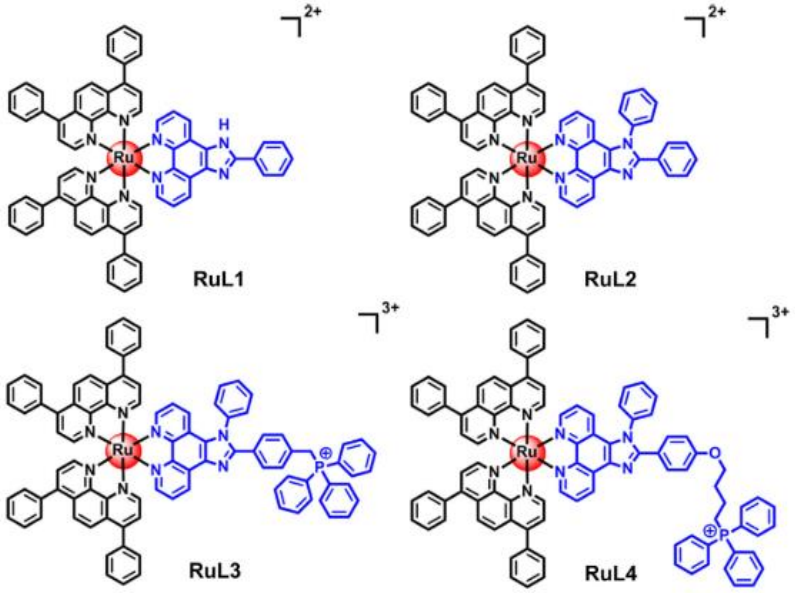
Since the creation of ROS is key to PDT agents inducing apoptosis, testing for the formation of ROS is an important piece of research. This involves incubating the therapeutic in question with a molecule like 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA) that is hydrolyzed by esterase enzymes to DCFH. DCFH fluoresces when it comes in contact with singlet oxygen (a ROS). This can be imaged with a confocal microscope with the intensity of

fluorescence directly correlating to the amount of singlet oxygen being formed. Each of the compounds show significant fluorescence when irradiated by two-photon light.[14] Therefore, ROS are being created when the complexes are irradiated.

While ROS formation gives confidence that cell death will occur, cytotoxicity tests remain an iteration in the research formula. Specifically, cytotoxicity in the dark must be measured to ensure the complexes maintain the desired inert capability until irradiated. This method is 2D and 3D analysis just as described by Huang *et. al.*[2] In 2D monolayer analysis, IC_{50} values for RuL1-L4 have no cytotoxic effects in the dark with concentrations upwards of 100 μM. Once irradiated with J/cm^2, RuL1-RuL3 show IC_{50} values near twelve, but RuL4 demonstrations highly positive results as it yields an IC_{50} of 3.5 μM. This result is expected since RuL4 has the highest accumulation in the mitochondrion and high ($\sigma 22$) value.[14] Since RuL4 gave such positive results in the area of cytotoxicity and has such a large TPA cross section, the group performed extra tests to see if the complex would remain cytotoxic when the amount of radiation was diminished. This testing was performed using 2D analysis. For the main testing of cytotoxicity, the light energy was 12 J/cm^2. When the light dosage is diminished to 2 J/cm^2 with a concentration of 10μM, RuL4 kills 60% of cells after 2 hours. 50% are killed under the same conditions when the light is reduced to 1.7 J/cm^2. These results point to the positive effects of a high TPA cross section coupled with high localization in the mitochondria.[14] While 3D analysis is considered the best indicator of cytotoxicity, 2D analysis remains an important step for PDT cancer research.[14,20]

In regard to 3D analysis, the MCTSs are important since few monolayers can be tested with two-photon emission. As previously mentioned, the larger the diameter of the MCTS, the better,[14] and the increase theoretically gives a closer representation of a tumor in a clinical

setting. Also, spheroids above 600 µm can produce secondary necroses.[20] This is important because it will give information as to which type of cell death a complex with induce. If necrosis is caused by the cytotoxicity of a therapeutic *in vitro*, then researchers can critically consider whether or not it is worth moving the drug on in testing. With this in mind, Liu *et. al* report using an 800 µm spheroid[14], doubling the size mentioned by Huang *et. al*.[2] Testing of uptake into the spheroid takes place in an analogous manner to the uptake into the cells of a monolayer. In the case of MCTSs, the proposed drug is incubated for 8 hours and then viewed with fluorescence microscopy. Each section of depth is recorded to show luminescence. Also, the creation of singlet oxygen in the MCTSs is more prevalent near the outside of the culture.[14] This is explained by the tendency of tumors and spheroids to have a hypoxic core.[14,15] RuL4 shows the best luminescence for the ROS creation experiment. Finally, the cytotoxicity of RuL4 in the 3D analysis is lowest for two-photon absorption at 1.9 µM compared to 9.6 µM for the one-photon method.[14] All in all, each of the four compounds[14] show positive results following these *in vitro* methods. The next step is to perform *in vivo* testing in hopes that the results continue and lead these types of drugs into clincal testing. This paper does not include information on *in vivo* methodology or data.

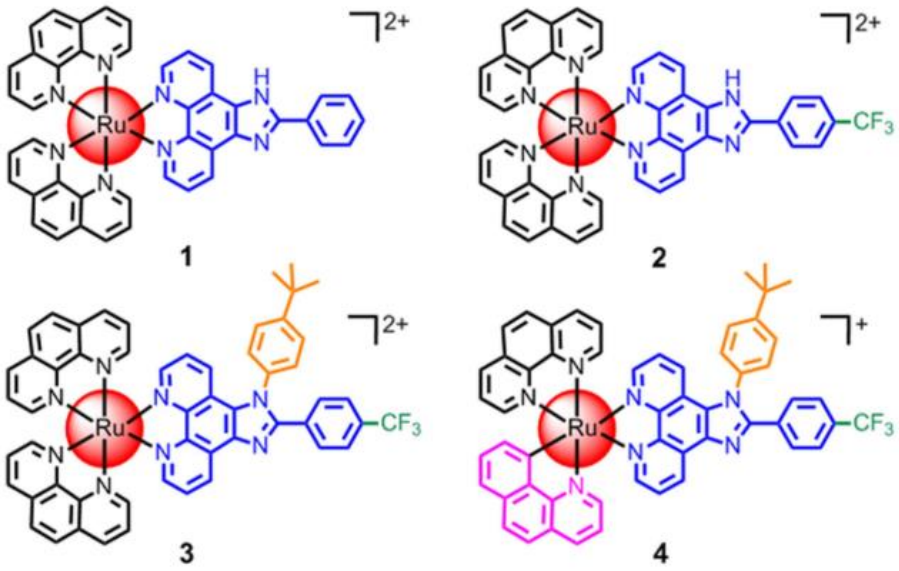


Scheme 2. Compounds reported by Liu *et. al*.[14]

Zeng *et. al* used four polypyridyl compounds (Scheme 3) with octupolar organization with high chemical and photo stability.[9] These compounds are quite similar to those used by Liu *et. al* in that they are polypyridyl compounds. Polypyridyls are able to stabilize electrons in the excited state which is the why they are often used in PDT. Zeng's compounds main difference is that they begin to include fluorine substituents that increase lipophilicity. This group focused on seeing the uptake of their complexes into different types of cancers cells rather than the normal HeLa cells used for this portion of testing by most other groups.[2,14] There is not a substantial difference for the uptake of the 4 ruthenium complexes into the 3 cacncerous tissues. The important things to note from this study about uptake is that Zeng 4 gave the most positive results. This compound is the most lipophilic, which indicates that lipophilicity and the uptake are directly correlated.[9] No indication of the reason for the correlation is given. Next, the group also tested uptake into normal cells. For all four ruthenium compounds, the uptake into normal cells is noticeably less. This shows some type of preference for tumor cells is can be concluded. Since these compounds are PDT agents, it does not seem that this should be as important. It

seems that this is included because in clinical settings it may be difficult to focus light only onto a tumor. There is usually healthy cell surrounding a tumor. If there is not compound in a healthy cell, then the possibility of any negative effect to healthy tissue is diminished. Finally, the uptake of cisplatin was compared. This was compared because a cisplatin resistance strain was used for the research. This is important because the results show that the ruthenium complexes are able to localize in a tumor type that cisplatin's uptake is seriously diminished for. This results shows that these complexes could be replacements for cisplatin resistant cancers.

Zeng *et. al* also focuses on the Thioredoxin system as a target for its compounds. This system is studied because it includes thioredoxin reductase (TrxR) which activates Trx. Trx seeks ROS helping to keep them from causing apoptosis. Also, Trx is involved in protein-protein interactions that regulate multiple cellular functions. Multiple cancers upregulate TrxR which makes it a possible drug target.[9,21] Also, its function in reducing ROS allows it to play a role in the resistance of Cisplatin. The tumor lines that are resistant commonly have a high TrxR amount or upregulate the production of the compound. When the upregulation of TrxR occurs, the resistance is considered "acquired" resistance.[21] Zeng *et. al* tested the effect of their most promising probe on TrxR expression. The results show that Zeng's fourth complex (Zeng 4) reduces the expression of TrxR and its activity by 45% which leads to the increase of ROS. Ultimately, this causes cell death. The ROS production is monitored with a confocal microscope, once again, after incubation with DCFH-DA.[9,14] This means the inhibition of TrxR plays a role in Zeng 4's anti-cancer mechansim.



Scheme 3. Ruthenium complexes discussed by Zeng *et. al*.[9]

Conclusion:

Overall, I found it difficult to find multiple further articles on the ruthenium complexes {[Ru(bpy)(phpy)(dppz)]$^{+}$}^{2} and PDT agent RuL4.[14] I believe these two complexes show the capability for further investigation of research. Furthering research will create clarity in the conclusions and will provide more information about mechanisms of action. Many of these articles indicate the importance of *in vivo* testing and the difficulties and variance that often arise when moving to that step. Therefore, seeing the iterations for the complexes is imperative to the process of instating new drugs into chemotherapy. Also, I would like to have seen *in vivo* based articles solely for the purpose of gathering a better understanding into how those types of experiments are formatted. It would be beneficial to compare how tumors are cultured and how the small animals receive/integrate the tumors. Also, I wonder if cytotoxicity is measured and if it is how this is performed. I assume that the process would be highly analogous.

I was encouraged on the one hand to see the multitude of ligands that are being studied and have literature that can be assessed. It is interesting to see how varying structures do give

very different functions. I wish there was more information that explained the functionality of the ligands rather than simply broad description. Most of the papers simply talk about the results the new ligands have and how solubility/lipophilicity are the main factor that change the action of the compounds.[22] This reasoning was shallow to me because was not developed more by the authors. I think this issue would diminish if the drugs showed more success and more research focused on a specific drug continued. All the different structures show the lack of success that is being had in this area of study. The *in vitro* results are easier to come by due to more reproducibility. The way that spheroids are cultured does lead to some variance, but those differences are exacerbated once testing moves on to live testing. Different DNA reacts to drugs differently. It is similar to allergies. One person is able to eat gluten without issue while others have extreme struggles with the protein. The further into the process of testing the drugs, the more factors must be taken into account. I suppose if one takes the Thomas Edison approach, then contentment can be maintained. All the failure in research brings scientists one step closer to finding more beneficial ways to address cancer.

In the end, PDT agents do a good job of taking a novel approach to the problem. But, the practicality of radiation to activate hinders the ability to use PDT for cancers in deep tissues. The movement toward two-photo excitation aids in this process, but I do not know that PDT alone with give a cure-all. It seems that cancer arises in too many forms to simply have one method that will negate this type of disease. Also, I thought the focus on the Trx system and its part in drug resistance is the kind of information that needs to be continually uncovered for better therapeutic designs. The more we understand about how the ligands play vital roles in the process of cancer treatment; the easier it will be to tune complexes with the correct ligands.

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