AWARD NUMBER: W81XWH-17-1-0097

TITLE: Multi-Modal Theragnostic Anticancer Complexes of Rhenium to Circumvent Plantinum Resistance in Relapsed Ovarian Cancer

PRINCIPAL INVESTIGATOR: Justin J. Wilson

CONTRACTING ORGANIZATION: Cornell University
Ithaca, NY 14850

REPORT DATE: April 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| April 2019 | Annual | 20 Mar 2018 – 19 Mar 2019 |

**4. TITLE AND SUBTITLE**

Multi-Modal Theragnostic Anticancer Complexes of Rhenium to Circumvent Plantinum Resistance in Relapsed Ovarian Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-17-1-0097

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Justin J. Wilson

E-Mail: jjw275@cornell.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Cornell University

373 Pine Tree Rd
Ithaca, NY 14850-2820

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Novel rhenium-based anticancer agents were explored as alternatives to the platinum drugs for the treatment of ovarian cancer. Over the course of this reporting period, we explored the in vivo anticancer activity of one of these promising candidates. These results show that this compound can inhibit ovarian tumor growth in mice. Histopathological analysis further showed that this activity was accompanied by minimal toxic side effects. Additionally, we have developed a new combinatorial synthetic strategy for screening a library of these compounds. These combinatorial efforts have led to the discovery of another promising lead compound that induces necrotic cell death in ovarian cancer cells. Lastly, we have discovered a highly potent rhenium anticancer agent that induces cell death via endoplasmic reticulum (ER) stress. Given the status of ER dysregulation in cancer cells, this compound represents a promising alternative to conventional platinum-based drugs.

**15. SUBJECT TERMS**

cisplatin resistance, rhenium, folate receptor, technetium, theragnostic

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 23 | 19b. TELEPHONE NUMBER *(include area code)* |

## Table of Contents

**Page**


**1. Introduction…….1**

**2. Keywords…….1**

**3. Accomplishments……..……...1–15**

**4. Impact……...……15–16**

**5. Changes/Problems...…..……16–17**

**6. Products…….……..…..…….17–18**

**7. Participants & Other Collaborating Organizations……18–20**

**8. Special Reporting Requirements……20**

**9. Appendices……20**

## I. INTRODUCTION

Ovarian cancer is the worldwide leading cause of death for women, and its five-year survival rate has only marginally improved in the past few decades. The first-line treatment plans for this disease use the platinum-based chemotherapeutic drugs, cisplatin and carboplatin. Despite their clinical success, many limitations arise from these drugs. For example, long-term side effects, such as nephrotoxicity and ototoxicity, are often associated with these drugs and result in poor patient quality of life. Despite the initial success of these platinum-based anticancer agents, ~70% of ovarian cancer patients relapse, and the disease becomes highly resistant or non-responsive to these drugs. Additionally, these compounds are not amenable to direct in vitro or in vivo imaging, and this limitation prevents our ability to assess their efficacy in real-time. In this project, we will illustrate the potential of rhenium-based chemotherapeutic agents and their abilities to overcome the limitations of current platinum drugs. Our rhenium compounds are designed to exhibit minimal toxic side effects, overcome cisplatin-resistant pathways, and have rich spectroscopic properties ideal for imaging.

## II. KEYWORDS

cisplatin, relapsed ovarian cancer, folate receptor, rhenium, technetium, SPECT imaging, theragnostic

## III. ACCOMPLISHMENTS

### A) MAJOR GOALS:

**1) Specific Aim 1: Determine the structure-activity relationships (SARs) of $[Re(NN)(OH_2)(CO)_3]^+$ complexes related to cytotoxicity and overcoming platinum resistance**

Because few studies have been carried out on the anticancer activity of $Re(CO)_3$ complexes, the goal of Aim 1 is to investigate the structural features of this class of compounds that impart cytotoxic activity. These studies will enable the rational design of improved analogues. We will also study the biological mechanisms of action and propensity of such complexes to circumvent resistance in ovarian cancer by evading nucleotide excisions repair (NER) mechanisms.

a) *Major Task 1: Compound evaluation in ovarian cancer cell lines.*

*i) Milestones:* Identification of lead Re drug candidates that exceed the potency of existing platinum drugs, circumvent cisplatin resistance, and operate effectively independent of cell NER status. Modification of the axial ligand increased compound potency in cancer cells and maintained theragnostic properties. Published a manuscript on the SARs of Re complexes. Published a manuscript of the combinatorial synthesis and screening of non-symmetric Re complexes. Submitted another manuscript on the modification of the axial ligand bearing the same $Re(CO)_3$ core as the original lead Re complex . Published a comprehensive review article on Re-based anticancer agents.

*ii) Status:* 95% complete; our first manuscript studying the SAR and mechanism of action of this class of Re anticancer agents was published (*J. Am. Chem. Soc.* **2017**, *139*, 14032). Additionally, we have published a manuscript on Re complexes bearing non-symmetric diimine ligands which were studied for SARs, ability to overcome cisplatin resistance pathways, and mode of cell death (*Inorg. Chem.* **2019**, *58*, 3895). Furthermore, we submitted a manuscript on a second-generation complex related to our lead compound identified in our first report. This manuscript

was submitted in March 2019. Investigation of the discrete molecular target of second-generation Re complexes is currently underway.

**2) Specific Aim 2: Target delivery of $Re(CO)_3$ cytotoxic payloads to ovarian cancer cells**

The toxic side effects of platinum drugs and other chemotherapeutic agents arise from collateral damage to non-cancerous cells. We will minimize broad cytotoxic damage of healthy cells by attaching functional groups to the $Re(CO)_3$ drug entities that will target ovarian cancer cells.

a) *Major Task 1: Synthesis and characterization of Re-folate cleavable conjugates.*

*i) Milestones:* Synthesis of a Re-folate conjugate that cleaves under the acidic conditions found in the endosome.

*ii) Status:* 30% complete; the synthesis of these conjugates is still being troubleshot. As described below, we have found promising alternative synthetic routes to access these compounds. In collaboration with the Boros Lab at Stony Brook University, we have made a Re-folate conjugate, but the compound exhibits poor water solubility and formulation for in vivo injection needs to be further addressed. Synthetic routes exploring more soluble compounds are currently underway.

b) *Major Task 2: In vitro and in vivo evaluation of Re-folate conjugates.*

*i) Milestones:* Demonstration of both in vitro and in vivo FRα-dependent anticancer activity of the Re-folate conjugates. Manuscript on in vivo efficacy of Re-folate conjugates.

*ii) Status:* 30% complete; this work depends on the synthesis of the Re-folate conjugates above. We have currently not screened Re-folate conjugates in cancer cells, but we have isolated a Re-folate compound which showed poor water solubility. We have carried out in vivo antitumor studies with several non-functionalized Re complexes.

**3) Specific Aim 3: Exploit the theragnostic utility of the $Re(CO)_3$ complexes.**

Unlike platinum-based drugs, the $Re(CO)_3$ core bears spectroscopic handles that we will harness for imaging. In particular, these complexes have long-lived triplet metal-to-ligand charge transfer ($^3$MLCT) luminescence that can be exploited for live-cell imaging. Additionally, in vivo imaging will be performed using $^{99m}$Tc analogues for single-photon emission computed tomography (SPECT) imaging. These imaging modalities will play an integral role in our development and selection of candidates for further preclinical studies.

*a) Major Task 1: In vitro confocal fluorescence microscopy of Re anticancer agents.*

*i) Milestone:* Determination of intracellular localization of Re complexes.

*ii) Status:* 100% complete; we have reported on these studies in our paper in *J. Am. Chem. Soc.* In continuation of this aim, we have evaluated the localization of second-generation Re complexes bearing axial isonitrile (ICN) ligands.

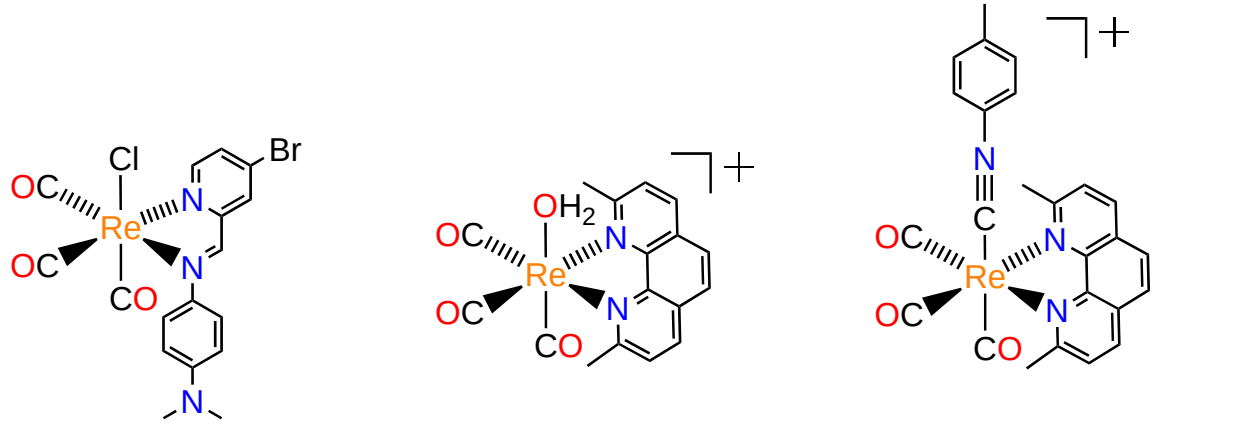
*b) Major Task 2: In vivo SPECT imaging of $^{99m}$Tc analogues.*

*i) Milestone:* Demonstration of in vivo FRα-dependent imaging of the Re-folate conjugates. Manuscript on imaging applications of Re anticancer agents. Synthesis of $^{99m}$Tc analogues of second-generation rhenium complexes.

*ii) Status:* 30% complete; we have studied and prepared the $^{99m}$Tc analogues of the unfunctionalized lead compound, as well as two other complexes bearing the $Re(CO)_3$ core. The biodistribution of these complexes was then investigated in vivo. We are currently studying the imaging capabilities of second-generation rhenium complexes that are highly luminescent and show promising anticancer properties.

## B) ACCOMPLISHMENTS:

**1) Mechanistic understanding of $Re(CO)_3$ complexes in cancer cells.** Inspired by the success of our original lead complex, Re-aqua ($[Re(CO)_3(dmphen)(OH_2)]^+$), we have synthesized an expanded library of complexes bearing the $Re(CO)_3$ core (**Scheme 1**). We have developed combinatorial synthetic methods, which allowed for facile variation of the equatorial diimine ligand (Re-9D). We have screened the resulting complexes for anticancer activity to develop an SAR. We have also varied the axial ligands of the rhenium complexes. After testing a variety of axial ligands, we identified complexes bearing the isonitrile (ICN) ligand, *p*tolICN = *p*-toluene isonitrile, as a potent anticancer agent (Re-ICN). Based on the high potencies of both Re-9D and Re-ICN, we evaluated their mechanisms of action. Like the original lead complex, the new rhenium analogues exhibit anticancer activity in both wild-type and cisplatin-resistant ovarian cancer cell lines. In addition, the second-generation complexes exhibit different mechanisms of action from cisplatin. Despite their similar structures, all three classes of $Re(CO)_3$ compounds induce different modes of cell death in ovarian cancer cells.




Re-9D
$[Re(CO)_3(Schiff\ base)Cl]$

Re-aqua
$[Re(CO)_3(dmphen)OH_2]^+$

Re-ICN
$[Re(CO)_3(dmphen)(ptolICN)]^+$

**Scheme 1.** Rhenium complexes investigated in these studies.

In our combinatorial synthesis and screening study, we identified three lead compounds, which were then synthesized on a preparative scale and fully characterized (**Figure 1**). Dose-escalation studies were carried out to determine the activity of the most potent of the three compounds, Re-9D ($[Re(CO)_3(Schiff\ base)Cl]$), and this complex was further analyzed for its ability to overcome cisplatin resistance and mechanism of action. We found that neither the presence nor the depletion of glutathione (GSH), an antioxidant and cytoprotective agent, had any effects on the activity of Re-9D. However, the toxicity of cisplatin decreased with higher

concentrations of GSH and increased with depleted GSH amounts. This result suggests that the insensitivity of this class of compounds to GSH may be a key feature that renders them non-cross-resistant to the platinum-based drugs. Flow cytometry and microscopy studies revealed that Re-9D induces cell death via a necrotic pathway, which is distinct from cisplatin and our previously studied Re-aqua complex.

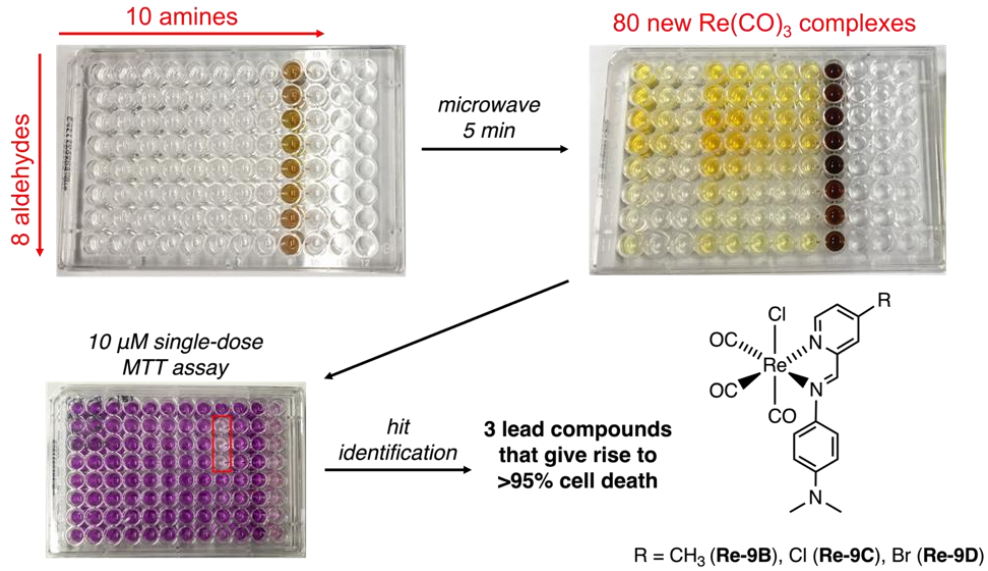



**Figure 1.** Scheme of combinatorial synthesis and cytotoxicity screen of rhenium Schiff base complexes that led to the identification of three lead complexes, including Re-9D.

The Re-ICN ($[Re(CO)_3(dmphen)(ptolICN)]^+$) complex exhibited potent anticancer activity (< 2 μM $IC_{50}$) in a panel of cancer cell lines and showed no cross resistance in cisplatin-resistant ovarian cells. To further investigate the high potency of the complex, we probed its mode of cell death induction using a variety of cell death pathway inhibitors, including inhibitors of necroptosis, paroptosis, and apoptosis. Treatment with Re-ICN and the pan-caspase inhibitor, Z-VAD-FMK, showed a significant decrease in toxicity. This result suggested that the complex was inducing cell death via a caspase-dependent mechanism. Further investigation through various assays including immunohistochemistry and flow cytometry confirmed that the complex was inducing caspase-dependent apoptosis. To explore how the complex induces apoptosis, we began by co-treating HeLa cells with inhibitors of certain cellular stresses, including endoplasmic reticulum (ER) stress, mitochondrial stress, and lysosomal stress. Upon treatment with the ER stress inhibitor, salubrinal, we found that Re-ICN exhibited greater potency. This result indicated that Re-ICN was acting through an ER stress pathway similar to that of salubrinal. Salubrinal is involved in preventing the dephosphorylation of the eukaryotic initiation factor 2α (eIF2α), which is responsible for activating the unfolded protein response (UPR). To determine if our complex was operating through this pathway, we collaborated with Prof. Shu-Bing Qian (Cornell University) and his graduate student, Mr. Robert Swanda. Prof. Qian is an expert in UPR-mediated nutrient starvation cell death pathways in eukaryotic cells. Western blot analysis of HeLa cell lysates treated with Re-ICN for 24 h showed a significant increase in phosphorylated eIF2α in comparison to both untreated and cisplatin-treated cells. To confirm that we were inducing the UPR, we also blotted for downstream

markers of ER stress, including the activation factor proteins ATF4 and CHOP. These proteins are activated downstream of eIF2α, and their expression correlates with both ER stress and apoptosis. Western blot analysis confirmed that both ATF4 and CHOP are activated after 24-h treatment with Re-ICN (**Figure 2**). Together, these results indicate that Re-ICN induces apoptosis through an UPR-mediated mechanism of action.

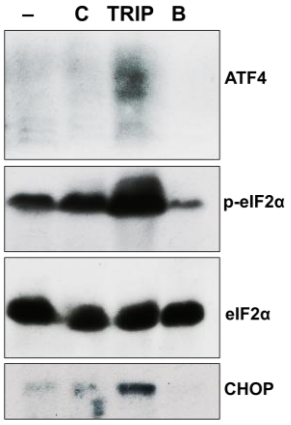



**Figure 2.** Western blot of HeLa cells untreated (–), treated with cisplatin (C), Re-ICN (labeled as TRIP in this figure), or bortezomib as positive control for ER stress (B). HeLa cells were treated for 24 h with 5 μM Re-ICN.

Once we determined that Re-ICN was inducing ER stress through a UPR-dependent mechanism, we began our investigation to determine a potential discrete molecular target of this complex. We used a combination of confocal fluorescence microscopy, western blot, and flow cytometry experiments to determine the direct cause of the ER stress. According to prior studies reported in the literature, the main triggers of UPR activation are either proteasome inhibition, heat shock protein inhibition, calcium dysfunction, or misfolded protein accumulation. We first investigated whether the proteasome was the main target for the observed ER stress induced from Re-ICN. To do so, we blotted for ubiquinated proteins, which will accumulate if the proteasome is being inhibited by the complex. The proteasome is responsible for removing misfolded proteins that have been marked for degradation with a ubiquitin tag. If our complex was inhibiting the proteasome, then we would expect to see an increase in the level of ubiquinated proteins in comparison to untreated cells. However, we saw no increase in ubiquinated proteins compared to untreated cells and the positive control, bortezomib. Next, we explored the possibility that Re-ICN was inducing ER stress through heat shock protein (HSP) inhibition. HSPs are chaperone proteins that are responsible for maintaining protein folding and function. The most abundant and commonly explored HSP is HSP90. To probe whether HSP90 inhibition was occurring, we looked for expression level changes in HSP70 and AKT using western blot analysis. HSP70 will be upregulated in response to HSP90 inhibition and AKT will be downregulated. We found that treatment with Re-ICN in HeLa cells showed no changes in either HSP70 or AKT levels, indicating that HSP90 is most likely not the molecular target. Calcium levels were next explored using the calcium-sensitive dye, Fluo-4AM. Upon increases in cytosolic calcium levels, Fluo-4AM will

exhibit an increase in fluorescence intensity. Using confocal fluorescence microscopy, we determined that treatment with Re-ICN resulted in no changes in Fluo-4AM fluorescence intensity in comparison to cells treated with the positive control, thapsagargin. Thapsagargin induces ER stress through inhibition of the SERCA pump, which is responsible for pumping $Ca^{2+}$ from the cytosol into the ER. Lastly, we investigated whether Re-ICN was causing an accumulation of misfolded proteins. The build-up of misfolded or aggregated proteins will eventually lead to downstream ER stress and activation of the UPR. We explored this possibility by using confocal fluorescence microscopy and the dye, Thioflavin T. Thioflavin T is a small molecule that is typically used to for the detection of amyloid β aggregation levels in Alzheimer's disease. When the dye comes in contact with protein aggregates, the dye will increase in fluorescence intensity. We were able to analyze the fluorescence intensity increase of Thioflavin T using confocal fluorescence microscopy. We found that upon treatment with Re-ICN after just 30 min, a large fluorescence intensity increase from Thioflavin T was observed (**Figure 3**). Together, these results indicate that Re-ICN induces the accumulation of misfolded proteins, leading to ER stress and apoptosis. This mode of cell death induction is distinct from currently FDA-approved metal anticancer drugs, including cisplatin, which generally operate through DNA damage.

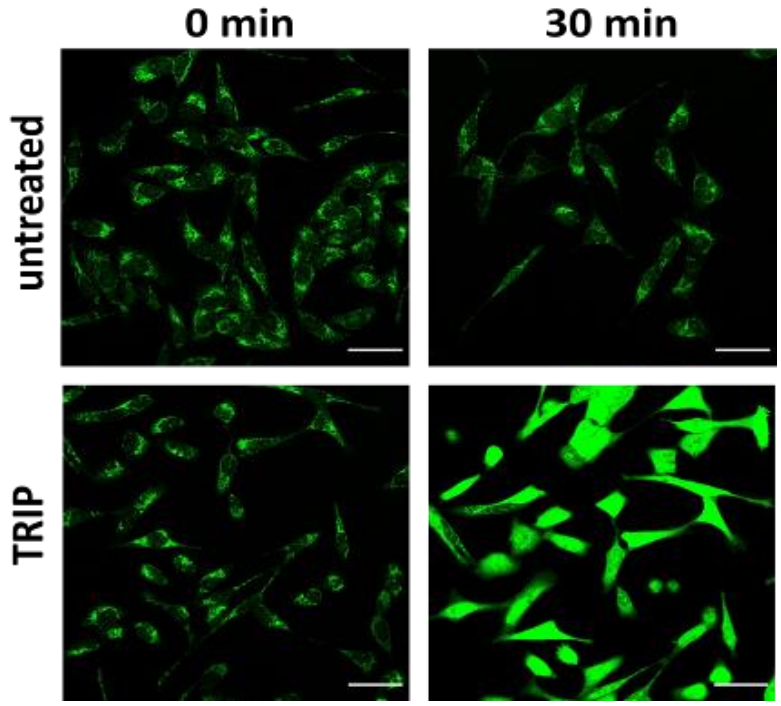



**Figure 3.** Confocal microscopy images of HeLa cells treated with Thioflavin T (5 μM) at 0 and 30 min in the absence (top panels) and the presence (bottom panels) of Re-ICN (labeled as TRIP in this figure, 5 μM) at 0 and 30 min. Scale bar = 50 μm.

**2) Intracellular localization and mechanism of cell uptake with confocal fluorescence microscopy.** Like the previous Re-aqua complex, the second-generation Re-ICN complex exhibits intrinsic luminescence, allowing for confocal fluorescence imaging. The complex was imaged in

HeLa cells cotreated with various organelle-localizing dyes or transfected to express organelle-specific proteins. After 4 h treatment, the complex exhibited minimal colocalization with the lysosomal dye, LysoTracker Red DND-99, as well as the fusion protein GalT-dsRed. GalT-dsRed is a galactosyltransferase 1 that localizes to the Golgi apparatus (**Figure 4**). Although, there was some localization in the lysosomes and Golgi apparatus, the majority of the luminescence observed was cytosolic.

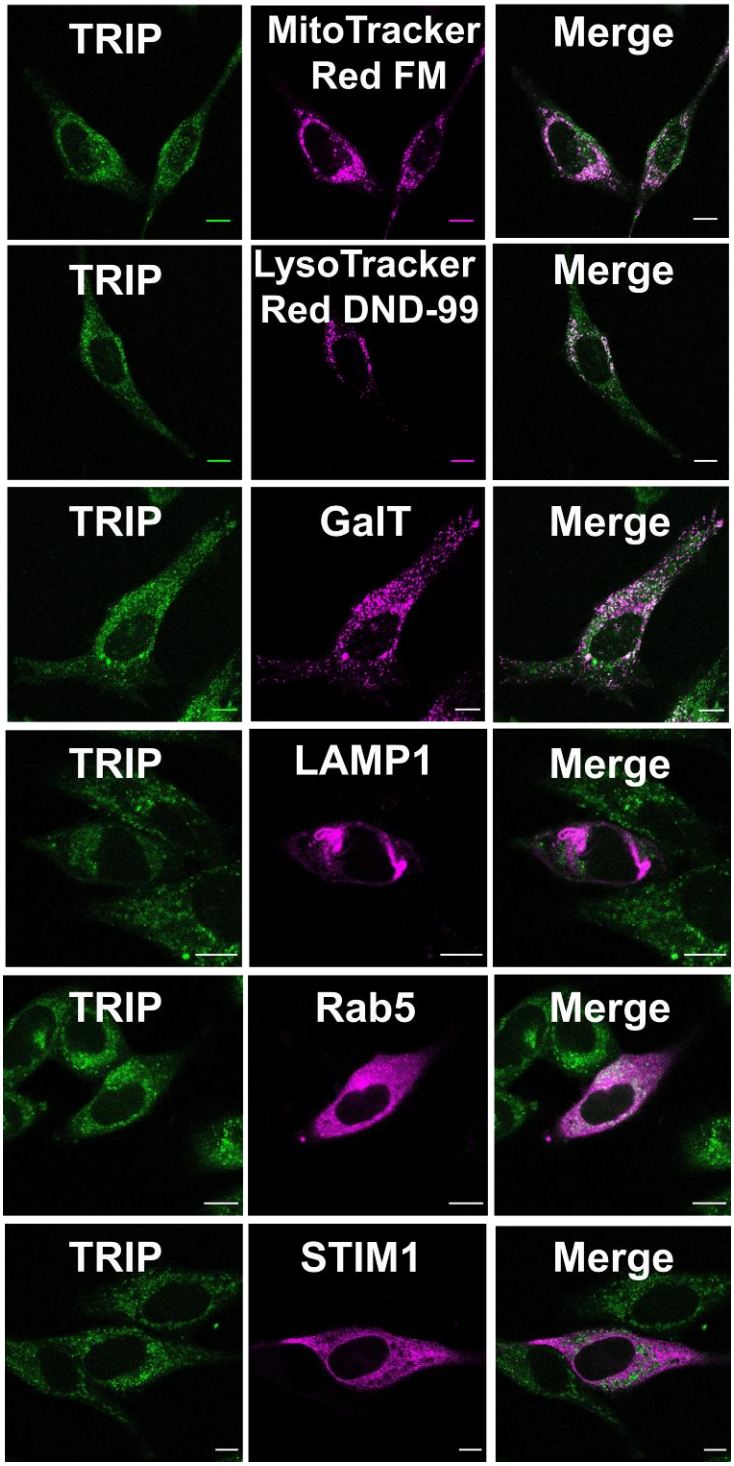


**Figure 4.** Confocal fluorescent microscope images of HeLa cells treated with Re-ICN (labeled as TRIP in this figure, 5 μM, 4 h). Cells were additionally stained with the indicated transfection or dye. Transfected GalT-dsRed (galactosyltransferease 1) colocalizes with the Golgi apparatus, LAMP1-mRFP (Lysosomal Associated Membrane Protein 1) colocalizes with lysosomes and late endosomes, Rab5-mRFP (Ras-associated binding 5) colocalizes with early endosomes, and STIM1-mRFP (Stromal Interaction Molecule 1) colocalizes with the ER. Scale bar = 10 μm.

In addition to the observed localization of the complex, we discovered that Re-ICN induces a morphological cellular change, specifically to the mitochondria. During confocal fluorescence imaging experiments, we noticed that HeLa cells treated with the complex and the mitochondria-localizing dye, MitoTracker Red, for 4 h had mitochondria that were rounded and punctate. These mitochondria were distinct from that of untreated HeLa cells, which exhibited elongated mitochondria. To understand further how Re-ICN was affecting the mitochondria, we imaged HeLa cells over the course of 30 min and found that within this time, the complex was inducing rapid mitochondrial fragmentation (**Figure 5**).

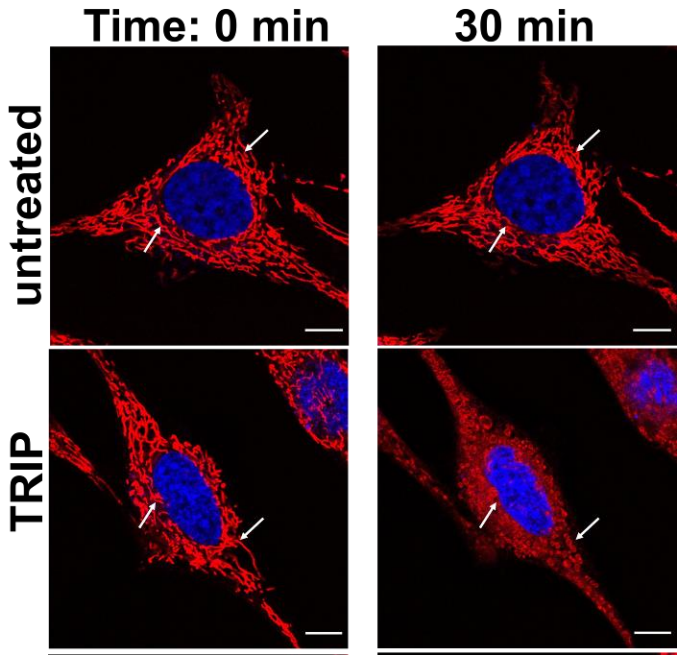



**Figure 5.** Confocal fluorescence microscope images of HeLa cells untreated or treated with Re-ICN (labeled as TRIP in this figure, 5 μM). Cells were stained 15 and 30 min prior to compound treatment with Hoechst dye (blue) and MitoTracker Red (red), respectively.

**3) $^{99m}$Tc analogues synthesis and in vivo biodistribution studies.** Tc is the lighter congener of Re, and it exhibits similar chemical properties. This similarity enables the use of $^{99m}$Tc analogues of these Re anticancer agents as diagnostic partners for SPECT imaging or biodistribution studies. The $^{99m}$Tc analogues of both the Re-9D and Re-ICN complexes were prepared from the well-known precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$. This trisaquo precursor was subsequently treated with the

Schiff base or dmphen ligand and analyzed by HPLC. The $^{99m}$Tc analogue of Re-9D was isolated with a water axial ligand ($^{99m}$Tc-9D($H_2O$)). Naïve BALB/c mice were injected with the $^{99m}$Tc-9D($H_2O$), and the percent injected dose per gram (%ID/g) biodistribution was determined at the 30, 60, and 120 min time points (**Figure 6a**). Similar to Re-aqua complex and its $^{99m}$Tc analogue, this compound also exhibited hepatobiliary and renal clearance. The urine (**Figure 6b**) and blood of mice injected with $^{99m}$Tc-9D($H_2O$) were analyzed by HPLC coupled to a radiation detector. The resulting chromatograms reveal that this compound is relatively stable in vivo. The intact complex ($t_r$ = 13.7 min) is observed in the urine at 30 and 60 min time points. Beyond 120 min, the majority of this compound has been cleared from the mice. Further analysis on the nature of these species would aid in understanding the in vivo behavior of $^{99m}$Tc-9D($H_2O$). Overall, the analysis of $^{99m}$Tc-9D($H_2O$) in urine samples show that an appreciable amount of this compound remains intact prior to and after renal and hepatic clearance.

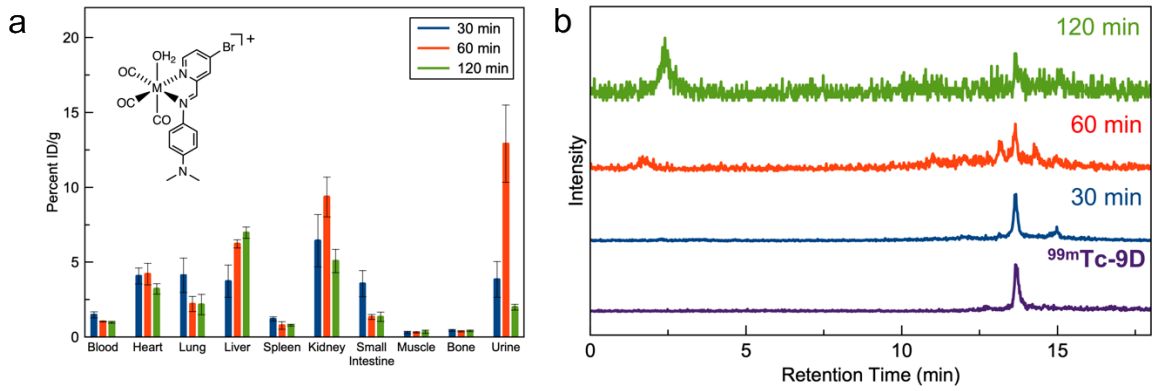



**Figure 6.** (a) Biodistribution of $^{99m}$Tc-9D($H_2O$) in BALB/c mice detected using a gamma counter, in which M is $^{99m}$Tc, after 30 (blue), 60 (red), and 120 (green) min. (b) Direct HPLC traces of urine samples collected from mice treated with $^{99m}$Tc-9D($H_2O$) after 30 (blue), 60 (red), and 120 (green) minutes. Reference chromatogram of $^{99m}$Tc-9D($H_2O$) is shown (purple).

We have also synthesized the $^{99m}$Tc analogue of Re-ICN and evaluated the biodistribution of both the Re and $^{99m}$Tc complexes in mice. The $^{99m}$Tc complex was prepared using established literature methods for the synthesis of similar "2+1" complexes. Metabolite analysis reveals that the $^{99m}$Tc complex may be detected in both urine and blood up to four hours after administration, indicating that the complex is suitably stable for imaging applications. The biodistribution of both the Re and $^{99m}$Tc complexes is similar, with the exception of high spleen uptake for the Re complex, which may be due to aggregation. The high levels of Re and $^{99m}$Tc in the kidneys indicate renal excretion, whereas the smaller uptake in the intestines and liver indicates minor hepatobiliary excretion (**Figure 7**). Together these results highlight the theragnostic potential of the isonitrile complexes, for the Re and $^{99m}$Tc complexes both exhibit high stability and similar distribution profiles.

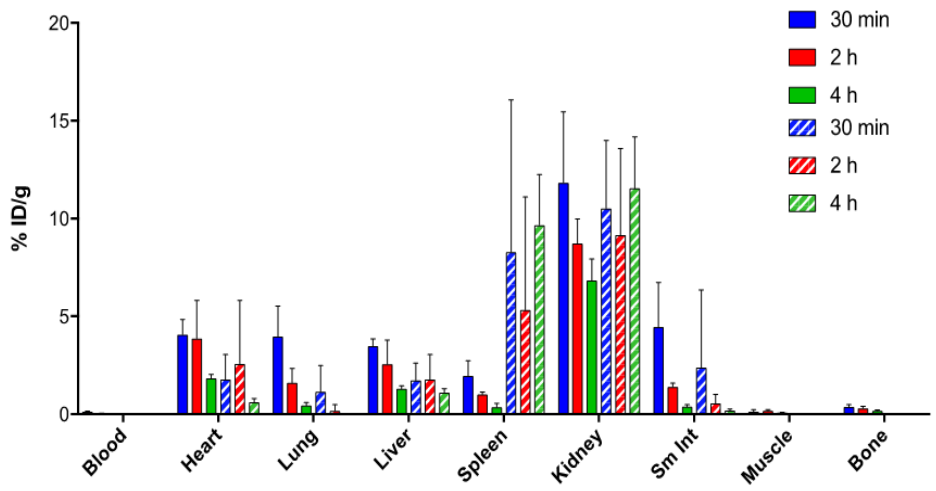



**Figure 7.** Biodistribution of Re-ICN (solid bars) and $^{99m}$Tc-ICN (striped bars) after injection in female BALB/c mice. Re content was quantified using ICP-OES and $^{99m}$Tc content was determined using a gamma counter.

**4) In vivo antitumor activity.** We have previously corroborated our in vitro studies with in vivo antitumor experiments, which were carried out in the Center for Developmental Therapeutics at Northwestern University. These studies included the evaluation of the maximum tolerated dose (MTD) of Re-aqua in NSG mice. No adverse side effects were observed up to an administered dose of 40 mg/kg. Notably, the MTD of cisplatin in the same mouse model is 20 mg/kg, indicating that the Re complex is better tolerated. We previously showed how the in vivo antitumor activity was measured in NSG mice bearing patient-derived ovarian cancer xenografts, which showed tumor growth inhibition at all administered doses without inducing changes in mice body weights throughout the duration of treatment. After these studies were conducted, we investigated the potential side effects of Re-aqua in NSG mice. The major organs of the mice treated with 10 mg/kg Re-aqua were harvested, fixed in 10% formalin, and digested in a heated solution of $HNO_3$ and $H_2O_2$, and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (**Table 1**). The organs with the highest concentrations of Re were the kidneys and liver, consistent with our previous biodistribution studies in naïve C57Bl6 mice. Moderate levels of rhenium were observed in the heart and lungs, and tissues with the lowest levels were the brain and tumor. We hypothesize that the low rhenium accumulation in the brain may reflect the poor permeability of Re-aqua through the blood-brain barrier. The lower levels of rhenium are somewhat surprising, given the tumor growth inhibitory activity of this compound. We hypothesize that improving the tumor-targeting capabilities of these compounds should drastically improve their observed biological activity. Furthermore, we analyzed the major organs for the extent of tissue damage by staining them with hematoxylin and eosin (H&E) (**Figure 8**). No obvious damage was observed in the liver, heart, or brain. Tumor sections, however, revealed lower percentages of necrotic tissue with increased concentrations of Re-aqua. This unexpected trend of decreasing necrosis with higher dose treatment may arise from the ability of Re-aqua to inhibit cell growth rather than kill cells in vivo. Rapidly growing tumors lack sufficient blood supply, depriving them of nutrients, and become hypoxic; these characteristics give rise to extensive necrotic cell death in the interior of the tumor. Thus, we hypothesize that the tumor growth inhibition induced by Re-aqua aided in

preventing the formation of necrotic tissue. Additionally, these necrotic regions exhibit less staining by H&E than healthy tissue, illustrating less densely packed cells. Taken together, these results confirm that Re-aqua is well tolerated, does not cause significant toxic side effects, and alters tumor morphology.

**Table 1.** Rhenium content in tissues for mice treated with 10 mg/kg Re-aqua after euthanasia. The error represents the standard error from three different mice.

| Tissue | pg Re/mg tissue |
|---|---|
| Kidneys | 6.0 ± 0.1 |
| Liver | 10.7 ± 0.6 |
| Heart | 2.4 ± 0.3 |
| Lungs | 2.3 ± 0.2 |
| Brain | 0.84 ± 0.04 |
| Tumor | 1.0 ± 0.3 |

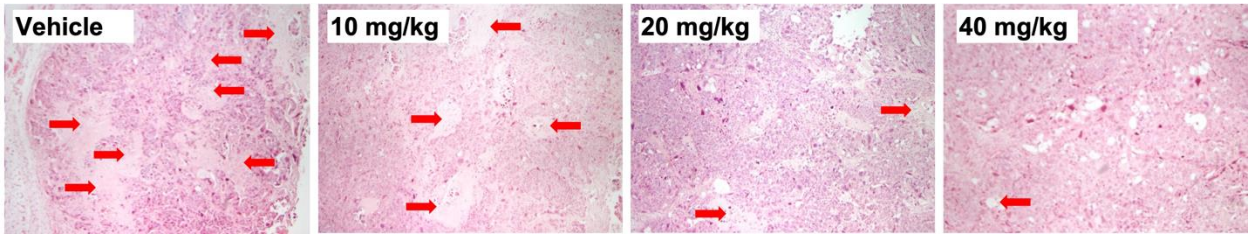
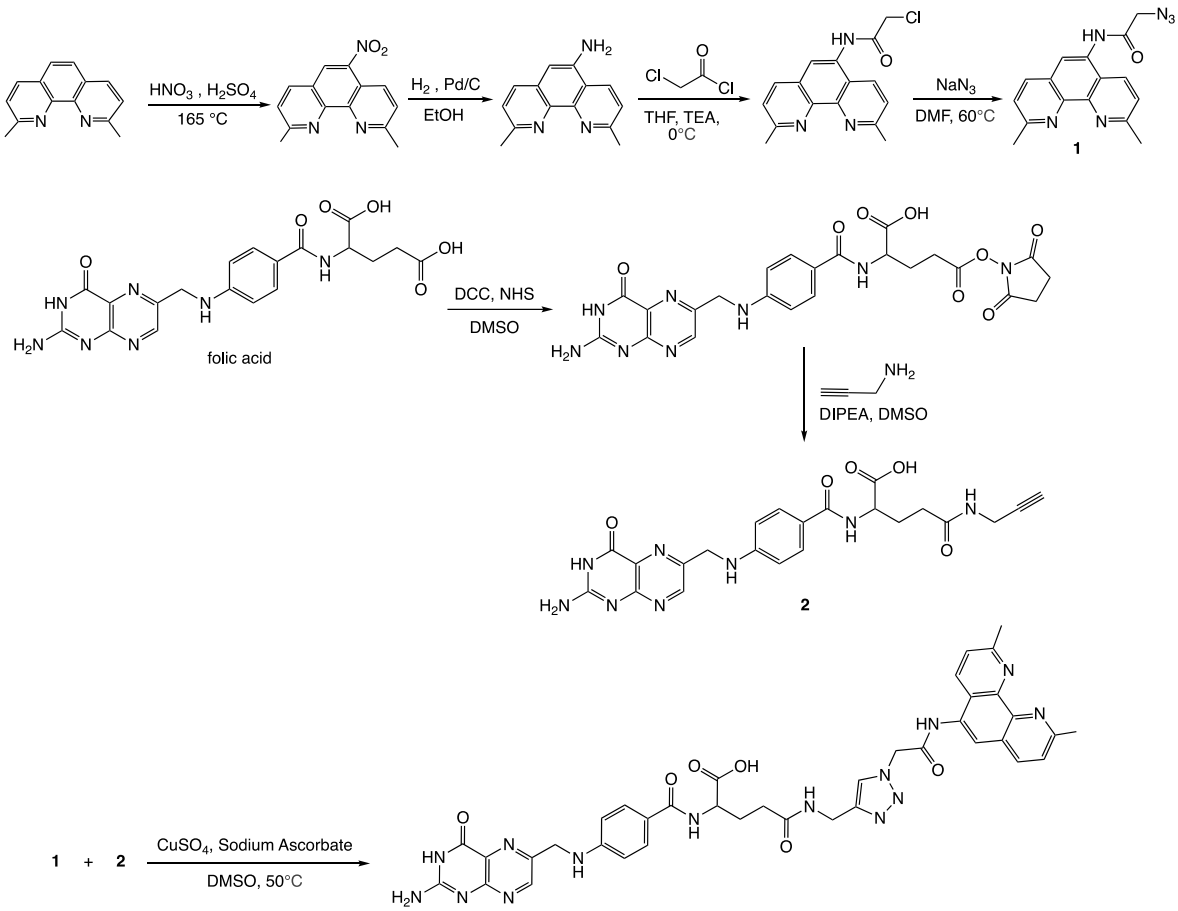


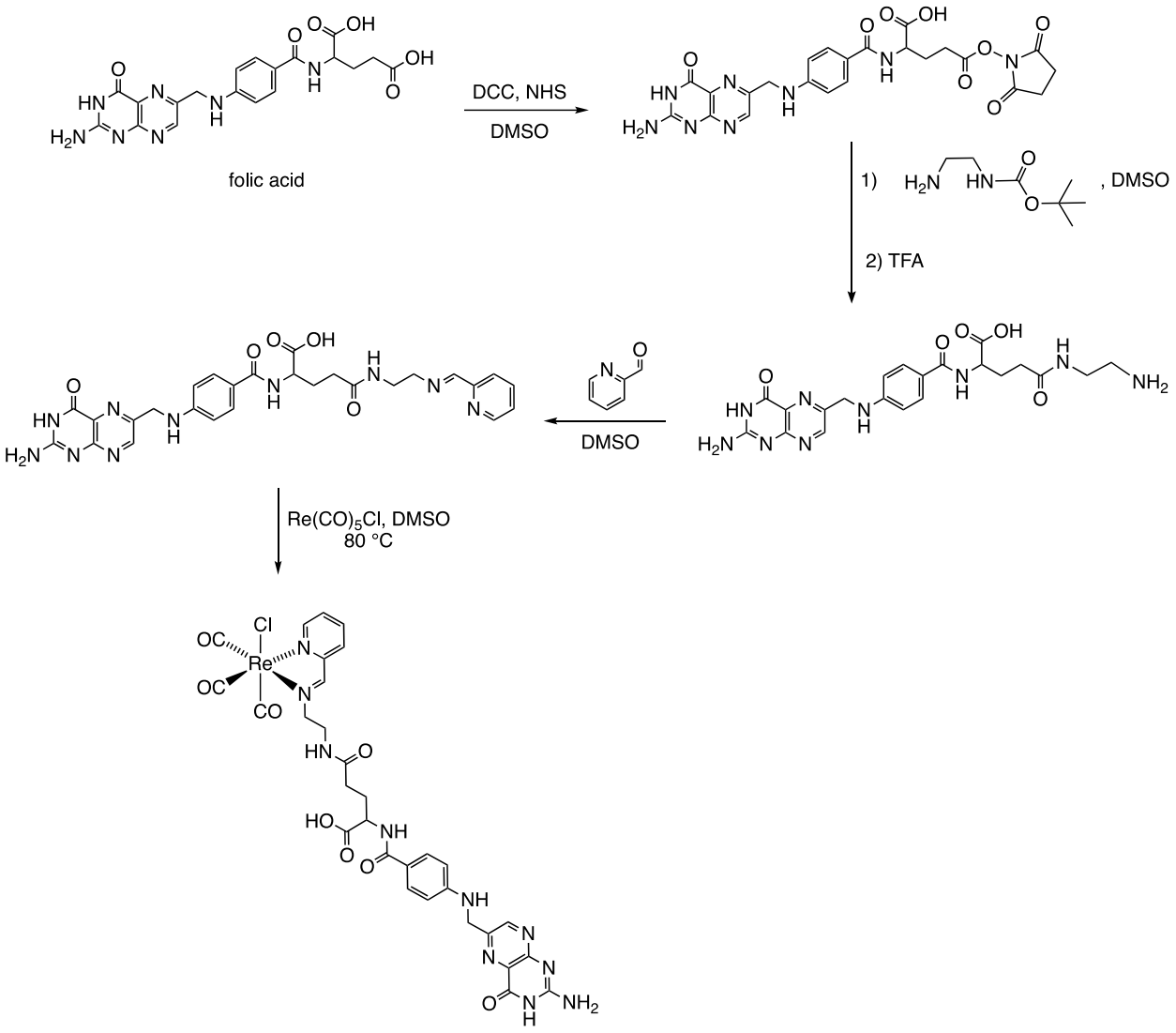

**Figure 8.** H&E stained slides of tumors harvested from mice treated with vehicle or Re-aqua (10, 20, and 40 mg/kg). Red arrows indicate regions of necrosis.

**5) Toward the synthesis of Re-folate conjugates.** Previously, we were able to successfully synthesize and isolate a phenanthroline-folate ligand. However, this complex had poor solubility in most organic solvents and water. This limitation led us to incorporate a polyethylene glycol chain. The multi-step synthesis was successful up until the final amide coupling reaction between the phenanthroline ligand and the polyethylene glycol folate, which unexpected formed a cyclic succinimide product. We have pursued alternative routes shown in **Scheme 2**.




**Scheme 2.** Attempted synthesis of a dmphen-folate ligand.

The synthesis of rhenium-folate conjugates has provided unexpected challenges. We attempted to incorporate an alkyne on folate and azide on dmphen, where dmphen is 2,9-dimethyl-1,10-phenanthroline, hoping to use azide-alkyne click chemistry to conjugate the two moieties. Dmphen was chosen instead of phenanthroline due to our previously published results illustrating higher cytotoxic effects induced by Re-aqua than $[Re(CO)_3(phenanthroline)(OH_2)]^+$. We have successfully isolated the alkyne-folate, but synthesis and isolation of the azide-dmphen moiety has been more difficult. Based on these synthetic challenges, we are pursuing alternative routes shown in **Scheme 3**.




**Scheme 3**. Current synthetic scheme for Re-folate complex.

We have successfully synthesized the ethylene diamine-folate conjugate. However, we anticipate issues with solubility after isolation of the Re-folate conjugate. For this purpose, we have illustrated a potential synthetic scheme, discussed in Section V, which incorporates a polyethylene glycol linker to improve solubility.

## C) TRAINING AND PROFESSIONAL DEVELOPMENT

**1) Graduate Student Training.** Three graduate students, Mr. Paden King, Ms. Charlene Konkankit, and Ms. Sierra Marker, have been working on various aspects of this project. As part of their work on this project, they have been receiving training in various aspects of molecular and cell biology. For example, Ms. Konkankit learned how to carry out flow cytometry experiments and ex vivo work, Ms. Marker has become skilled in confocal microscopy and photophysical experiments, and Mr. King has developed multinuclear and heteronuclear NMR expertise. They have also been receiving training in synthetic chemistry and continue to improve their skills with respect to analytic techniques and organic synthetic chemistry.

**2) Graduate Student Conference Attendance.** Mr. King, Ms. Marker, and Ms. Konkankit all attended and presented at the CBI symposium held at Cornell University, the Western NY Inorganic Chemistry Conference, and the Gordon Research Conference for Metals in Medicine. These conferences allowed the students to interact with internationally renowned scientists to expand their professional network and receive feedback on their projects.

## D) DISSEMINATION OF RESULTS TO THE COMMUNITY

**1) Expanding Your Horizons Outreach.** Our research group participates in the Expanding Your Horizons (EYH) program at Cornell. EYH is an annual event that brings more than 400 middle school girls to campus for various workshops developed by the students and faculty. The goal of the program is to stimulate the girls' interest in pursuing STEM degrees. The Wilson group developed a new workshop for EYH titled, "Radioactive World." The purpose of this workshop is to introduce the concept of radiation in daily life. Our activities allowed participants to measure radioactivity in everyday objects, such as smoke detectors and pitchblende. As part of this workshop, we also developed a game called Isotope Rummy, the goal of which is to add and subtract neutrons and protons to arrive at a stable isotope. Evaluations of the workshop were positive, indicating that the girls learned a great deal about radioactivity. We connect this activity to the use of SPECT imaging agents, like $^{99m}$Tc, used in this project.

**2) CHAMPS Program.** The Cornell-HHMI Accelerating Medical Progress through Scholarship (CHAMPS) program pairs undergraduate students of underrepresented minority groups with biomedical labs to carry out summer research. The Wilson group has hosted students from this program. These students are exposed to the research carried out in this project during weekly lab meetings. Additionally, Dr. Wilson has given formal research talks to all students in the CHAMPS program, discussing relevant aspects of this project.

**3) CBI Program.** The Chemistry Biology Interface (CBI) Training Program at Cornell is designed to "train graduate students with the core principles and techniques of chemistry so that they can address the most current and important problems in biology and medicine." Dr. Wilson regularly participates in meetings with graduate students in this program. Specifically, he has given two presentations to this group regarding research in this project.

**4) Cornell STEM Teacher Workshop.** In conjunction with the Cornell Center for Materials Research and the NY State Master Teacher Program of the Southern Tier Region, Dr. Wilson organized a workshop designed for teachers in NY to learn about current scientific research done at Cornell University. He was the keynote speaker and also delegated tasks to his students. For instance, Ms. Marker and Ms. Konkankit led one of the workshop events.

## E) FUTURE PLANS

**1) Investigate a library of rhenium isonitrile complexes bearing a variety of equatorial and axial ligands.** We are currently building a library of third-generation complexes bearing various diimine ligands including bipyridine and phenanthroline derivatives with the same axial ICN

ligand. We will investigate how the diimine ligand affects cytotoxicity as well as the photophysical properties of these complexes in order to develop an SAR.

**2) Determine the biomolecular target of Re-ICN complex.** We have submitted our Re-ICN complex for RNA sequencing to determine the discrete molecular target. These results will be confirmed using western blot analysis.

**3) Modify axial ligands on lead compound from combinatorial study.** We are currently investigating how the role of the axial ligand affects the cytotoxicity of the Re-9D complex.

**4) Complete synthesis of rhenium-folate conjugates.** We will continue efforts to design Re-folate conjugates.

**5) Evaluate the in vitro and in vivo anticancer activity of the rhenium complexes.** As described above, we will test the resulting Re-folate conjugates against ovarian cancer cells that express the folate receptor. We have also begun testing the in vivo anticancer activity of the lead Re-ICN complex.

**6) Evaluate $^{99m}$Tc SPECT imaging of the folate conjugates.** The $^{99m}$Tc analogues of the Re-folate conjugates will be evaluated in mice bearing ovarian cancer tumor xenografts.

## IV) IMPACT

### A) IMPACT ON BIOINORGANIC CHEMISTRY

Although the platinum-based drugs have long been used for the treatment of ovarian cancer, the successful implementation of alternative metal complexes as chemotherapeutic agents has progressed substantially slower. In the data obtained over this project period, we have demonstrated that $Re(CO)_3$ complexes act via mechanisms of action distinct from that of platinum-based drugs. These compounds do not exhibit cross-resistance with cisplatin, rendering them useful for the treatment of platinum-resistant relapsed ovarian cancer. We have also shown how this class of compounds has antitumor properties, illustrating their potential for use in the clinic. A significant impact that this research will have on the field of bioinorganic chemistry is that it will expand the search for new anticancer agents to metals other than platinum.

### B) IMPACT ON OTHER DISCIPLINES

The research carried out over the course of this last project period will have a significant impact broadly on the field of medicine. This research has demonstrated that inorganic complexes, other than those of platinum, can be valuable for use in medicine. The further clinical development of these rhenium complexes as anticancer agents, which is warranted based on their promising activities, will have a substantial impact for the treatment of ovarian cancer patients.

### C) IMPACT ON TECHNOLOGY TRANSFER

A patent describing the use of these rhenium compounds as anticancer agents for the treatment of ovarian cancer has been filed. Furthermore, discussions are underway with Andarix Pharmaceuticals, a startup company that explores radiotherapy applications of the radioactive $^{188}$Re isotope, to license the technology developed in this project.

## D) IMPACT ON SOCIETY

The extensive outreach efforts by the Wilson Group will have the positive impact on societal perceptions of the role of heavy metals in biology. These outreach efforts (see Section III, Part D above) help improve the public attitude on the use of metals in medicine.

# V) CHANGES AND/OR PROBLEMS

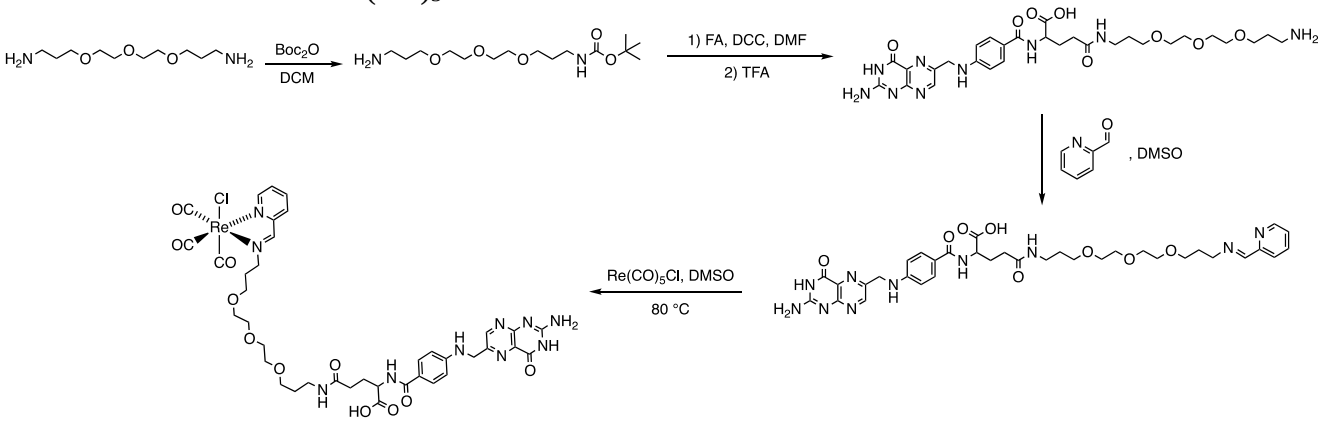
## A) CHANGES IN APPROACH

The chemistry to develop rhenium-folate conjugates was more challenging than anticipated. To further simplify the chemistry associated with multi-step synthesis, we attempted to develop synthetic schemes with fewer steps. This approach should also prevent loss in yield percentages and simplify purification processes.

We have currently been focusing efforts to develop more effective rhenium complexes than our first-generation, Re-aqua species, which has lead to the focus on the Re-ICN complex. We have found that this complex has significantly greater potency in a panel of cancer cell lines, operates through a distinct pathway than that of cisplatin, and has good photophysical properties for in vitro imaging. We have begun pursuing new complexes of this type as a consequence of the success of this complex. We believe that complexes of this type will have many benefits and success over the first-generation rhenium complexes.

## B) PROBLEMS OR DELAYS

We have faced a number of challenges in the synthesis of Re-folate. As described above, one major problem for these conjugates is poor solubility. In anticipation of this issue, we will attempt the synthetic scheme proposed below (**Scheme 4**) using Schiff-base chemistry to form a diimine ligand for coordination onto the $Re(CO)_3$ core.



**Scheme 4**. Synthetic pathways we will attempt in order to improve the solubility of Re-folate conjugates.

## C. CHANGES IN EXPENDITURES

No significant changes in expenditures.

## D. CHANGES IN HUMAN SUBJECTS, VERTERBRATE ANIMALS, BIOHAZARDS, SELECT AGENTS

No significant changes in these aspects.

# VI. PRODUCTS

## A. JOURNAL PUBLICATIONS

1) Chilaluck C. Konkankit, Sierra C. Marker, Kevin M. Knopf, Justin J. Wilson. "Anticancer Activity of Complexes of the Third Row Transition Metals, Rhenium, Osmium, and Iridium." *Dalton Trans.* **2018**, *47*, 9934–9974.

2) Chilaluck C. Konkankit, Brett A. Vaughn, Samantha N. MacMillan, Eszter Boros, Justin J. Wilson. "Combinatorial Synthesis to Identify a Potent, Necrosis-Inducing Rhenium Anticancer Agent." *Inorg. Chem.* **2019**, *58*, 3895–3909.

3) A. Paden King, Sierra C. Marker, Robert V. Swanda, Joshua J. Woods, Shu-Bing Qian, Justin J. Wilson. "A Rhenium Isonitrile Complex Induces Unfolded Protein Response-Mediated Apoptosis in Cancer Cells." *Submitted.*

4) Sierra C. Marker, Chilaluck C. Konkankit, Mark C. Walsh, Daniel R. Lorey II, Justin J. Wilson. "Radioactive World: An Outreach Activity for Nuclear Chemistry." *Submitted.*

5) Chilaluck C. Konkankit, A. Paden King, Kevin M. Knopf, Teresa L. Southard, Justin J. Wilson. "In Vivo Anticancer Activity of a Rhenium(I) Tricarbonyl Complex." *In revision.*

## B. CONFERENCE PRESENTATIONS

1) "Metals in Medicine:Coordination Chemistry to Control Biological Activity" National Institutes of Health, Molecular Imaging Program, Bethesda, MD, April 26, 2018 (invited seminar).

2) "Rhenium(I) Complexes as Anticancer Agents: Challenges and Opportunities" Metals in Medicine Gordon Research Conference, Andover, NH, June 28, 2018 (invited talk).

3) "Rhenium as an Alternative to Platinum for the Treatment of Cancer" 256$^{th}$ American Chemical Society National Meeting, Boston, MA, August, 19, 2018 (contributed talk).

## C. PATENT APPLICATIONS

1) A. Paden King, Sierra C. Marker, Justin J. Wilson. "Water Soluble and Luminescent Rhenium-Based Complexes as Anticancer Agents" *U.S. Provisional Patent Application No. 62/765,059*, Aug 16, 2018

## VII. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### A) INDIVIDUALS

| | |
|---|---|
| Name: | A. Paden King |
| Project Role: | Graduate Student |
| ORCID: | N/A |
| Nearest Person Month Worked: | 12 |
| Contribution to Project: | Mr. King worked to synthesize the lead Re-aqua compound. He synthesized and developed the Re-ICN complex and is currently working on the investigation of future rhenium isonitrile complexes. |
| Funding Support: | Teaching Assistantship |

| | |
|---|---|
| Name: | Charlene Konkankit |
| Project Role: | Graduate Student |
| ORCID: | N/A |
| Nearest Person Month Worked: | 18 |
| Contribution to Project: | Ms. Konkankit worked to synthesize a library of $Re(CO)_3(NN)Cl$ compounds and the folate-targeted rhenium complexes. She also worked on the in vivo antitumor studies and histopathology analysis. |
| Funding Support: | Teaching Assistantship |

| | |
|---|---|
| Name: | Sierra C. Marker |
| Project Role: | Graduate Student |
| ORCID: | N/A |
| Nearest Person Month Worked: | 18 |
| Contribution to Project: | Ms. Marker explored the biological and mechanistic studies of the Re-ICN complex. She is currently developing new rhenium isonitrile derivatives and investigating their photphysical and biological activity. |
| Funding Support: | Teaching Assistantship |

| | |
|---|---|
| Name: | Shu-Bing Qian |
| Project Role: | Collaborator |
| ORCID: | 0000-0002-4127-1136 |
| Nearest Person Month Worked: | |

| | |
|---|---|
| Contribution to Project: | Prof. Qian assisted in western blot analysis of Re-ICN complex and discussion and analysis of results. |
| Funding Support: | NIH grants R01GM1222814 and R21CA227917, Howard Hughes Medical Institute (award number 55108556) |

| | |
|---|---|
| Name: | Robert V. Swanda |
| Project Role: | Graduate Student |
| ORCID: | N/A |
| Nearest Person Month Worked: | |
| Contribution to Project: | Mr. Swanda performed all western blot experiments for the Re-ICN complex. |
| Funding Support: | CBI Fellowship |

| | |
|---|---|
| Name: | Eszter Boros |
| Project Role: | Collaborator |
| ORCID: | 0000-0002-4186-6586 |
| Nearest Person Month Worked: | 1 |
| Contribution to Project: | Prof. Boros carried out in vivo biodistribution and animal metabolite studies. |
| Funding Support: | Stony Brook University Startup, NIH K99 Award |

| | |
|---|---|
| Name: | Justin J. Wilson |
| Project Role: | Principal Investigator |
| ORCID: | 0000-0002-4086-7982 |
| Nearest Person Month Worked: | 2 |
| Contribution to Project: | Prof. Wilson supervised graduate students on this project. He assisted with data acquisition, data analysis, and manuscript writing. |
| Funding Support: | Cornell University Startup, 9-month teaching |

**B) CHANGE IN ACTIVE SUPPORT**

Cornell Technology Acceleration and Maturation (CTAM) Fund
01/01/19 – 12/31/19
Exploring the In Vivo Therapeutic Activity of a Potent ER Stress-Inducing Rhenium Anticancer Agent
Role: PI
$50,000 (direct)

Seed funding to carry out in vivo studies of the rhenium isonitrile compound.

National Science Foundation CAREER
07/01/18 – 06/30/22

A Toolkit to Modulate the Mitochondrial Calcium Uptake Machinery
Role: PI
$380,386 (direct)

No overlap.

## C) ORGANIZATIONS INVOLVED

1) **Organization Name:** Stony Brook University
**Location of Organization:** Stony Brook, NY
**Partner's Contribution to Project:** Collaboration and facilities; Prof. Boros from Stony Brook University collaborated with Prof. Wilson to carry out in vivo animal studies, as described above.

2) **Organization Name:** Center for Developmental Therapeutics, Northwestern University
**Location of Organization:** Evanston, IL
**Partner's Contribution to Project:** Facilities; tumor xenograft studies were carried out at this organization.

3) **Organization Name:** Section of Anatomic Pathology within the Animal Health Diagnostic Center at Cornell University
**Location of Organization:** Ithaca, NY
**Partner's Contribution to Project:** Facilities; histopathology analysis was carried out at this organization.

4) **Organization Name:** SUNY ESF
**Location of Organization:** Syracuse, NY
**Partner's Contribution to Project:** Facilities; ICP-MS studies were carried out at this organization.

5) **Organization Name:** Division of Nutritional Sciences at Cornell University
**Location of Organization:** Ithaca, NY
**Partner's Contribution to Project:** Collaboration and facilities; Prof. Qian collaborated with Prof. Wilson to carry out western blot analysis.

## VIII. SPECIAL REPORTING REQUIREMENTS

Not applicable.

## IX. APPENDICES

Not applicable.