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14. ABSTRACT

In this project, novel rhenium-based anticancer agents were explored as alternatives to the platinum drugs for the treatment of ovarian cancer. Over the course of this project, we explored the in vitro and in vivo anticancer activity of a range of different rhenium-based anticancer agents. We have shown that these compounds are highly effective in platinum-resistant ovarian cancer cells, suggesting that they may be valuable in this context. Furthermore, in vivo studies verified that these compounds are also effective in mice and appear to have minimal toxic side effects. Detailed mechanistic studies of these complexes show that they induce endoplasmic reticulum stress in ovarian cancer cells. This novel mechanism of action may be more broadly useful as a therapeutic strategy for the treatment of ovarian cancer. In summary, through this project we have identified a subset of rhenium complexes as promising anticancer agents for use in ovarian cancer.

15. SUBJECT TERMS

cisplatin resistance, rhenium, folate receptor, technetium, theragnostic

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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: 100% 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). We have since published several other manuscripts that describe the anticancer activities of a range of Re compounds and a thorough evaluation of their mechanisms of action (*Chem. Eur. J.* **2019**, *25*, 9206; *Chem. Commun.* **2020**, *56*, 6515; *J.*

Organomet. Chem. **2020**, *907*, 121064; *Angew. Chem. Int. Ed.* **2020**, doi: 10.1002/anie.202004883).

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: 60% 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. In collaboration with Prof Boros, we have also made a new ^{99m}Tc of a promising ER stress-inducing Re-based anticancer agent.

B. ACCOMPLISHMENTS

1) Mechanistic understanding of $Re(CO)_3$ complexes in cancer cells. We have tested the in vitro anticancer activity of our lead rhenium compound, $[Re(dmphen)(OH_2)(CO)_3]^+$, where dmphen is 2,9-dimethyl-1,10-phenanthroline (**Scheme 1**, Re-aqua), in an additional series of wild-type (A549, H460) and cisplatin-resistant (A549cisR, H460cisR) lung cancer cell lines. The results, shown in Table 1, reveal that this compound is equally effective in both wild-type and cisplatin-resistant cancer cell lines, signifying this compound to be generally effective for treating platinum-resistant cancers. Because toxic side effects are of significant concern in platinum-based chemotherapy, we evaluated the cytotoxicity of cisplatin and our lead rhenium compound in non-cancerous MRC-5 lung fibroblasts. Cisplatin is 10-fold more toxic than Re-aqua in these non-cancerous lung cells. *This result indicates that, even though Re-aqua has a higher IC_{50} value and therefore is less active than cisplatin against wild-type cancer cells, it has a greater therapeutic index, potentially allowing for safer patient administration.*

Table 1. IC_{50} values of cisplatin and $[Re(dmphen)(OH_2)(CO)_3]^+$ (Re-aqua) in wild-type and cisplatin-resistant cell lines. These results demonstrate that $[Re(dmphen)(OH_2)(CO)_3]^+$ is equally effective in wild-type and cisplatin-resistant cell lines.

Cell Line	IC_{50} (μM) or RF cisplatin	Re-aqua
KB-3-1	1.0 ± 0.3	0.92 ± 0.20
KBCP20	36 ± 7	1.6 ± 0.4
RF[a] (KB-3-1)	36	1.7
A2780	0.23 ± 0.07	2.2 ± 0.2
A2780CP70	8.2 ± 1.8	3.0 ± 0.7
RF[a] (A2780)	36	1.4
A549	3.0 ± 1.8	6.7 ± 4.9
A549 CisR	12.4 ± 8.5	5.4 ± 1.8
RF[a] (A549)	4.1	0.8
H460	0.75 ± 0.43	4.5 ± 0.7
H460 CisR	3.4 ± 1.6	5.3 ± 2.9
RF[a] (H460)	4.5	1.2
MRC-5	0.43 ± 0.14	4.1 ± 0.9

[a] RF is the resistance factor, which is the IC_{50} in the cisplatin-resistant cell line divided by the IC_{50} in the non-resistant matched cell line.

The mechanism of cell death was also investigated by evaluating the cytotoxicity of Re-aqua in the presence of various cell death inhibitors. The autophagy and paraptosis inhibitors 3-methyladenine and cycloheximide had no effect on the cytotoxicity of this compound, further ruling out these mechanisms of cell death. Because necroptosis, a regulated form of necrosis, was characterized as the cell death pathway induced by rhenium(V)-oxo compounds, the cytotoxicity of Re-aqua was probed in the presence of the necroptosis inhibitor necrostatin-1. Necrostatin-1 had no effect on the cytotoxic activity of Re-aqua, suggesting that necroptosis is not operative. Because the vacuoles induced by this compound are endolysosomal in origin (see below), the possibility of cell death induced by lysosomal proteases was investigated with the serine and cysteine protease inhibitor leupeptin. Again, no decrease in the cytotoxic effects of Re-aqua was observed in the presence of this protease inhibitor. Caspases are proteases that regulate programmed cell death. Their activation is implicated in apoptosis, and their downregulation in cancer cells has been linked to drug resistance. The use of the pan-caspase inhibitor Z-VAD-FMK revealed that this compound retains its cytotoxicity when caspases are inhibited and therefore induces cell death in a caspase-independent manner.

Western blots were performed to evaluate protein expression levels that might be altered by different cell death modes. A Western blot for PARP and cleaved PARP in HeLa cells treated with Re-aqua showed no significant alteration of the expression levels of these proteins, further ruling out apoptosis. Levels of LC3 were also unaffected by this compound, indicating that autophagy was not operative. Western blots for ERK and p-ERK, proteins activated from ER stress related to paraptosis, showed no change in expression level either. These studies validate the novel mode of cell death induced by Re-aqua, indicating that this class of compounds is mechanistically novel compared to the platinum-based drugs.

Further studies were carried out to investigate the potential role of ROS and depolarization of the MMP in mediating the cell death induced by Re-aqua. This compound did not lead to an increase in intracellular ROS nor did it depolarize the MMP. Re-aqua did give rise to flipping of phosphatidylserine to the outer membrane. Both paraptosis and necrosis give rise to an overproduction of ROS within the cell and apoptosis is known to depolarize the MMP. Thus, the cell death mechanism of this compound does not categorically fit within any of these descriptions. Although the flipping of phosphatidylserine is usually associated with apoptosis, alternative forms of cell death such as necrosis may also give rise to this phenomenon. Therefore, although Re-aqua produces a small population of annexin positive living cells, it is not caused by apoptosis based on the other assays showing non-apoptotic characteristics of cell death. Additionally, the annexin/PI histogram of this compound is much different than that of etoposide, a known apoptosis-inducer.

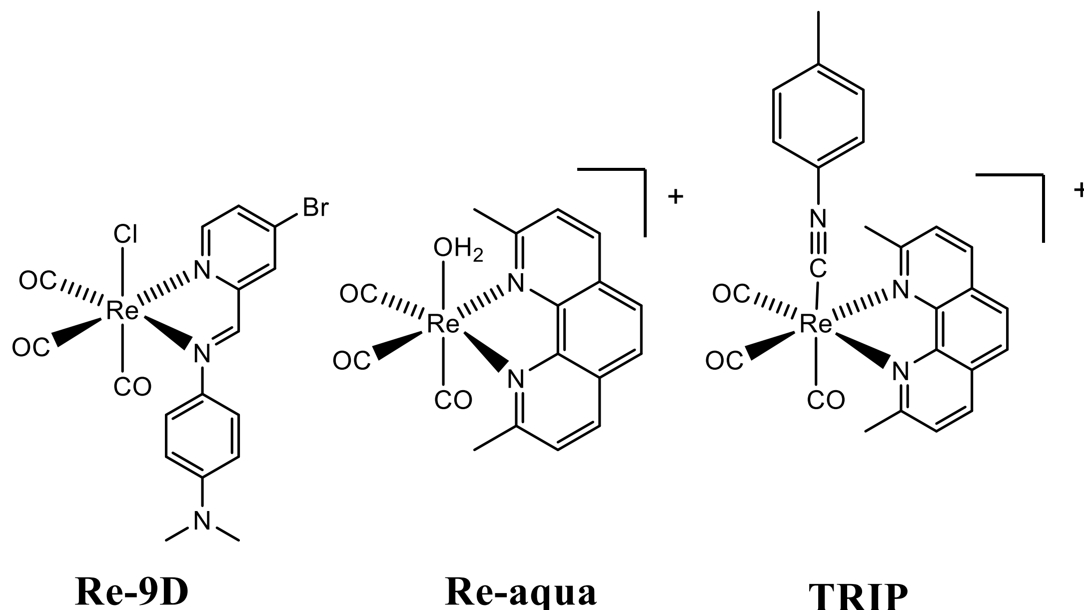
Cell cycle analysis indicates that Re-aqua arrests cells in the G2/M phases implying that it may have antimetastatic effects. Anticancer drugs like celastrol and taxol also inhibit cells in these phases. By contrast, cisplatin, a DNA-binding agent, inhibits cells predominantly in the S-phase. To further probe the mechanism of action of this compound, we have submitted it to the NCI60 tumor cell panel screening. In this service provided by the NCI, an anticancer drug candidate is screened in 60 distinct cancer cell lines, and the relative cytotoxicity of the compound against those cells is determined. The differences in activity of an experimental compound in the 60 cell lines can be compared to those of other compounds with established mechanisms of activity. This analysis is computed using the COMPARE algorithm created by the NCI. Compounds that correlate strongly with one another often act by similar mechanisms of action. The results of the COMPARE analysis for this $Re(CO)_3$ compound are shown in Table 2. The two top correlations are for the natural products macbecin II and rifamycin SV. Macbecin II is an established inhibitor of the protein Hsp90.[54] Rifamycin SV is an RNA polymerase inhibitor, but has known Hsp90-inhibitory activity. These results suggest that our rhenium complexes may likewise be inhibitors of Hsp90 or of the downstream processes related to this protein.

Table 2. NCI60 COMPARE Analysis Results for $[Re(dmphen)(OH_2)(CO)_3]^+$ (Re-aqua)

Pearson Correlation Coefficient (PCC)	Compound	NSC Number
0.649	macbecin II	S330500
0.625	rifamycin SV	S133100
0.605	L-cysteine analogue	S303861
0.585	pibenzimol hydrochloride	S322921
0.572	diglycoaldehyde	S118994
0.572	actinomycin D	S3053
0.557	CHIP (iproplatin)	S256927
0.557	anguidine	S141537
0.550	paclitaxel (Taxol)	S125973
0.541	5-azacytidine	S102816

Inspired by the success of our original lead complex, Re-aqua, 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 (TRIP). Based on the high potencies of both Re-9D and TRIP, 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.



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.

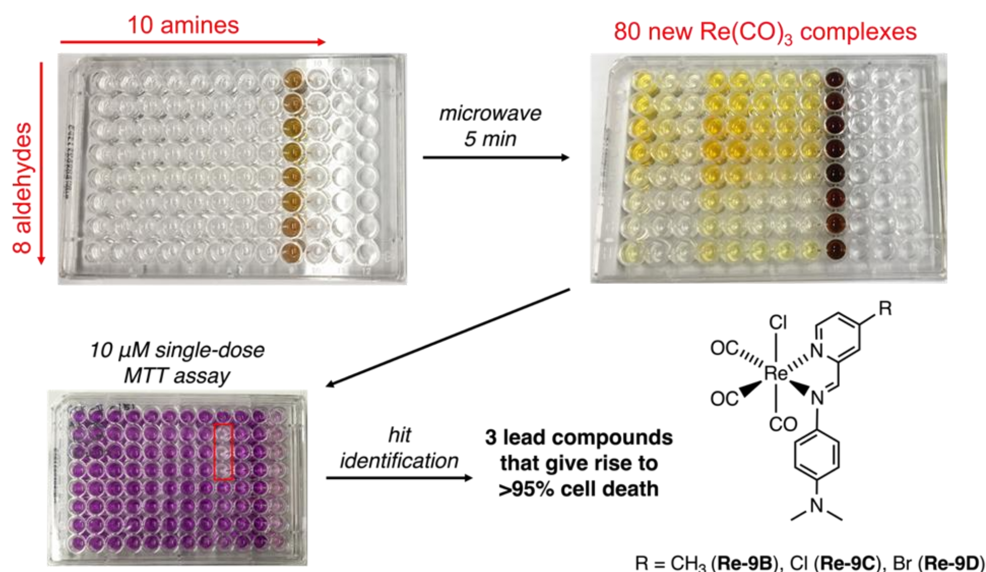
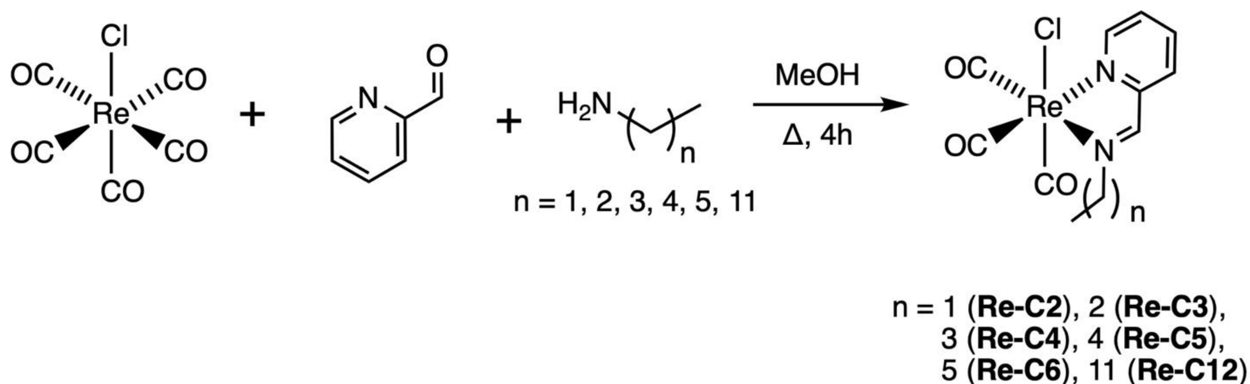


Figure 1. Scheme of combinatorial synthesis and cytotoxicity screen of rhenium Schiff base complexes that led to the identification of three lead complexes, including Re-9D.

Building upon this work, we have also synthesized and characterized a series of pyridylime complexes bearing different alkyl chain groups (**Re-Chains**, **Scheme 2**). The motivation for these complexes was to alter their lipophilicities. In measuring the cytotoxic activities of these complexes, it was observed that they are all equivalently active after a 48-h incubation period. However, at shorter time periods, the more lipophilic compounds exhibit greater potency (**Figure 2**). Thus, this result is significant because it demonstrates how the lipophilicity of these Re compounds may only affect cytotoxicity on a short time scale.



Scheme 2. General synthetic approach and structures of **Re-Chains** complexes.

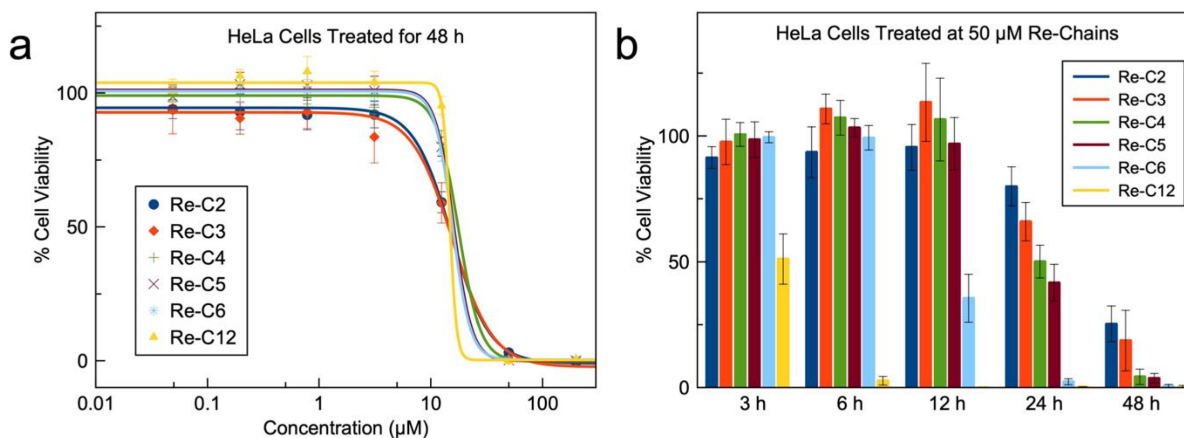


Figure 2 (a) Dose-response curves of HeLa cervical cancer cells and (b) time-dependent cell viability studies of HeLa cells treated with **Re-C2** (navy blue), **Re-C3** (red), **Re-C4** (green), **Re-C5** (maroon), **Re-C6** (light blue), **Re-C12** (yellow). The error bars represent the standard deviation from six replicates.

TRIP ($[Re(CO)_3$(dmphen)(*p*tolICN)$]^+$) 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 TRIP 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 TRIP exhibited greater potency. This result indicated that TRIP 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 TRIP 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 TRIP (**Figure 3**). Together, these results indicate that TRIP induces apoptosis through an UPR-mediated mechanism of action.

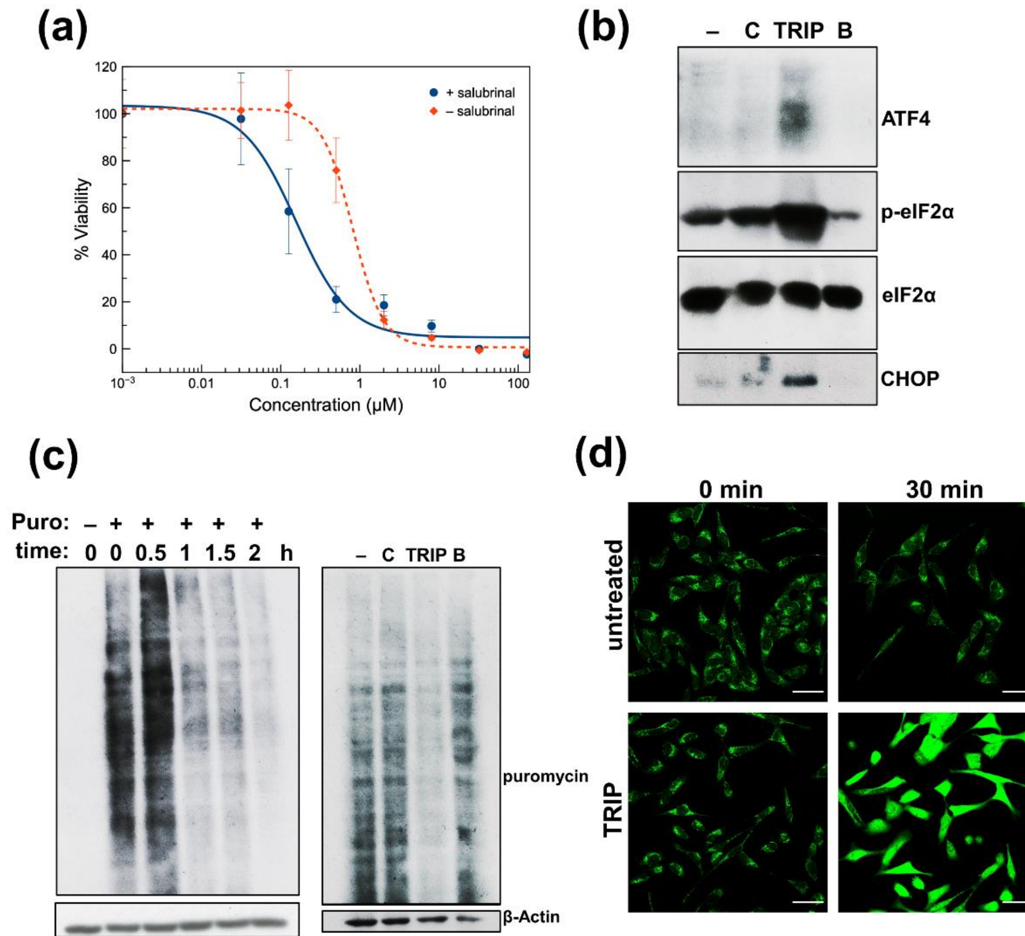


Figure 3. (a) Dose-response curve of A2780 cells treated with TRIP in the presence of 25 μM salubrinal (blue) or absence of salubrinal (red). (b) western blot of untreated (–), cisplatin (C, 10 μM), TRIP (5 μM), or bortezomib (B, 25 nM) for 24 h in A2780 cells. (c) Western blot of A2780 cells incubated with TRIP (5 μM) over 0, 0.5, 1, 1.5, and 2 h with puromycin (10 min, left blot) and A2780 cells untreated (–), cisplatin (C, 10 μM), TRIP (5 μM), or bortezomib (B, 25 nM) treated for 24 h with puromycin (10 min, right blot). (d) Confocal microscopy images of HeLa cells treated with ThT (5 μM) at 0 and 30 min in the absence (top panels) and the presence (bottom panels) of TRIP (5 μM) at 0 and 30 min. Scale bar = 50 μm.

Once we determined that TRIP 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 TRIP. 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 TRIP 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 TRIP 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 TRIP 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 TRIP 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 TRIP after just 30 min, a large fluorescence intensity increase from Thioflavin T was observed (**Figure 3**). Together, these results indicate that TRIP 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.

To further understand the mechanism of TRIP in ovarian cancer cell lines, we developed an ovarian cancer cell line with resistance to this compound. The A2780 ovarian cancer cell line was cultured in 2 μM increasing increments of TRIP over the duration of 12 months to generate resistance. The new resistant cell line, A2780TR, exhibited a 9-fold decrease in sensitivity to TRIP. We then evaluated the cytotoxicity of related anticancer drugs in this TRIP-resistant cell line. Notably, the platinum-based drugs cisplatin and oxaliplatin are not cross-resistant with TRIP in this cell line. However, we did observe a large cross-resistance with taxol (184-fold difference in activity). The large cross-resistance mechanisms of TRIP with taxol suggested that these two compounds are susceptible to similar resistance pathways. It is known that taxol is a substrate of the drug efflux transporter, Pgp. Therefore, we explored the Pgp mRNA expression levels with reverse transcriptase quantitative PCR (RT-qPCR) in wild-type A2780 and resistant A2780CP70 cell lines. These RT-qPCR results reveal that the resistant cell lines have 800-fold greater amounts of Pgp mRNA in comparison to the wild-type cell line (**Figure 4**). Therefore, this result suggests that Pgp overexpression is a mechanism of ovarian cancer cell resistance to TRIP. This result is important because it implicates a metal-based drug candidate in efflux from the Pgp transporter.

Furthermore, it highlights approaches, namely inhibition of Pgp, that may be able to increase the potency of this drug candidate.

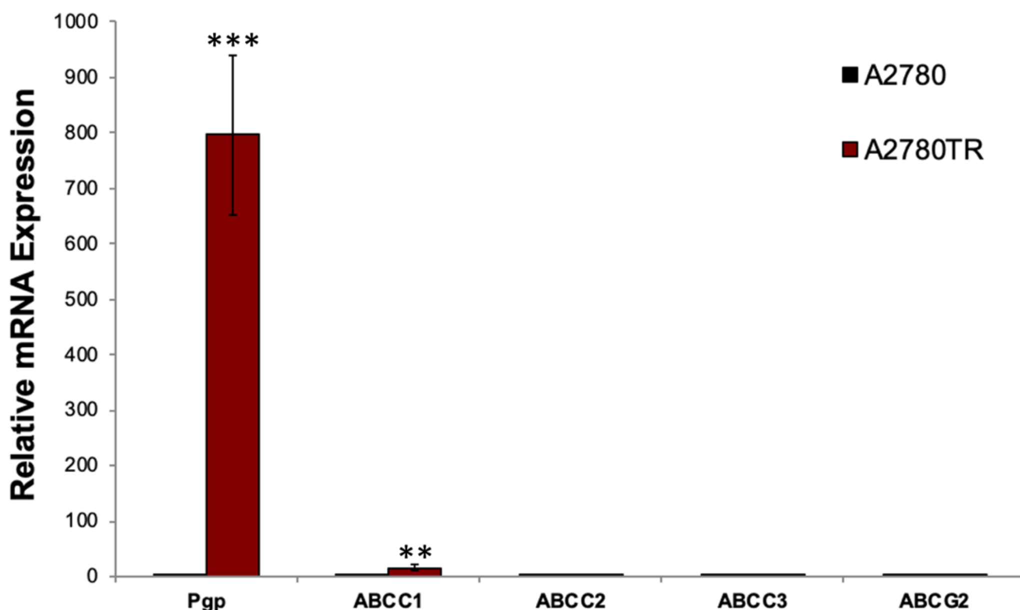
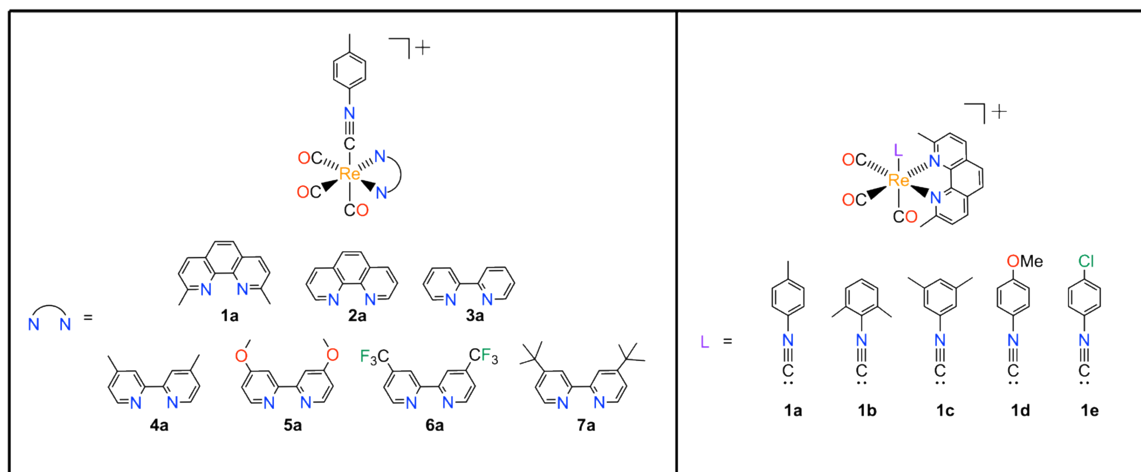


Fig. 4. mRNA expression levels of ABC transporters in A2780 and A2780TR cell lines. The mRNA levels of ABCC2, ABCC3, and ABCG2 in both A2780 and A2780TR cells were below detectable levels. (** = $p < 0.01$, *** = $p < 0.005$)

We have also synthesized a small library of related rhenium isonitrile complexes to assess the structure-activity relationships within this class of compounds. The analogues shown in **Scheme 3** were fully synthesized and characterized.



Scheme 3. New analogues of TRIP synthesized and characterized.

We then assessed the in vitro anticancer activities of these complexes. Correlation between the electron-richness of the compounds and their cytotoxicities was found. Thus, installing more electron-donating ligands is a means of increasing their cytotoxic potency. In addition, we carried out cell biology experiments on this series of compounds to verify that the act via the same mechanism of action as the parent compound TRIP. As shown in **Figure 5**, all of these compounds similarly cause mitochondrial fission, protein aggregate accumulation, and CHOP expression, signifying that they induce ER stress. Importantly, the extent to which these compounds induce these features is correlated to their cytotoxic activity, indicating that these features are important for mediating their cytotoxic activities.

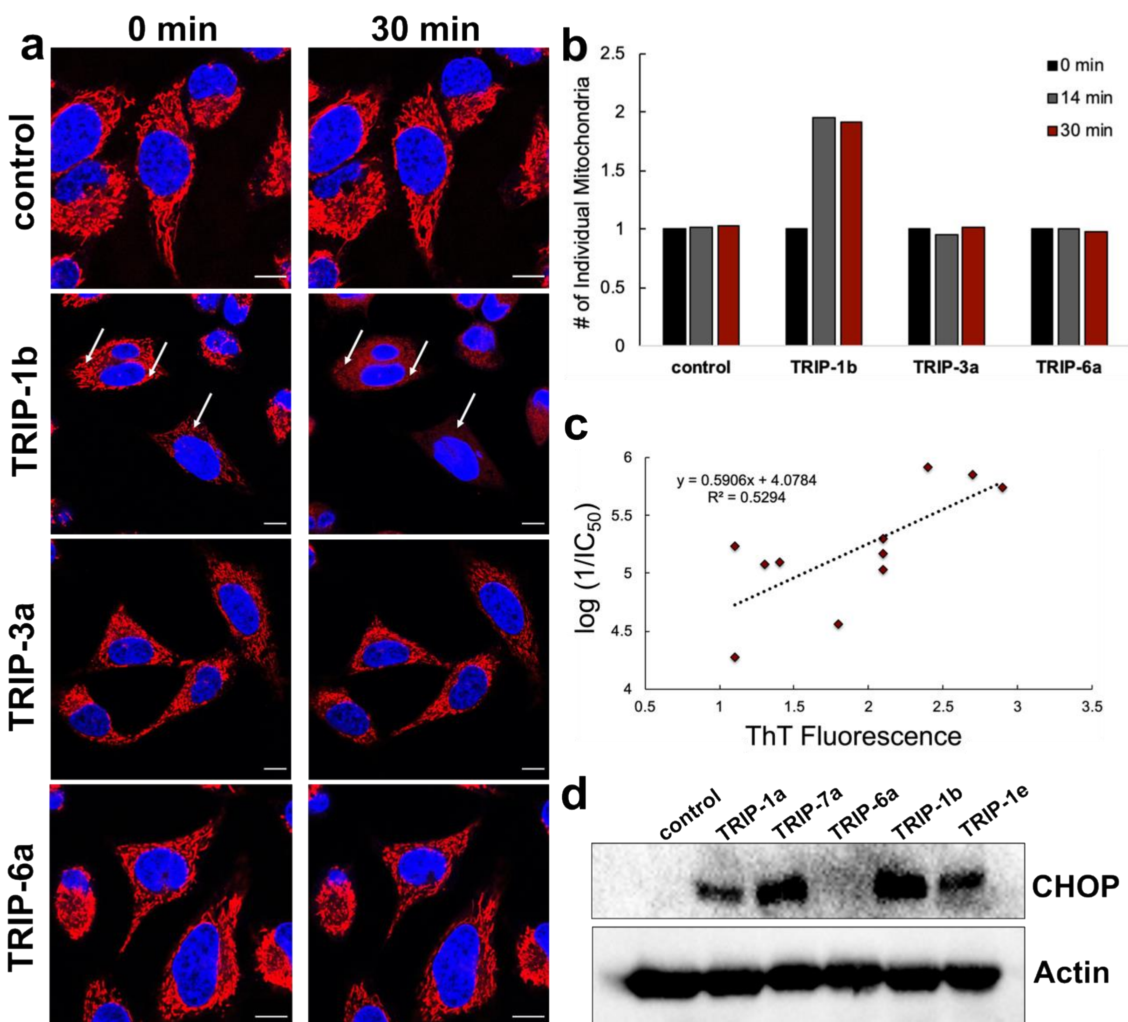


Fig. 5. (a) HeLa cells stained with MitoTracker Red (red channel) and Hoechst (blue channel) before (0 min) and after (30 min) exposure to 10 μM of a TRIP complex. For further characterization of this process, **Videos 1–4** are included in the **SI**. **(b)** Quantification of the number of individual mitochondria after treatment with 10 μM of a TRIP complex at 0, 14, and 30 min using ImageJ. **(c)** Correlation of ThT fluorescence in HeLa cells upon treatment with the TRIP derivatives vs the anticancer activity. **(d)** CHOP Western blot of selected TRIP derivatives after 24-h treatment in HeLa cells.

2) Intracellular localization and mechanism of cell uptake with confocal fluorescence microscopy. The ability to image the intracellular localization of the most potent rhenium complex Re-aqua by confocal fluorescence microscopy was investigated. HeLa cells were treated with this compound and incubated for 4 or 24 h prior to imaging. The emission of Re-aqua was detectable well above the background autofluorescence within the cells (**Figure 6**). The yellow emission of the rhenium was distributed throughout the cytosol. Notably, cytoplasmic vacuoles were observed, an apparent effect of the rhenium complex. The outer membranes of these vacuoles were brightly luminescent, indicating a large accumulation of the rhenium complexes.

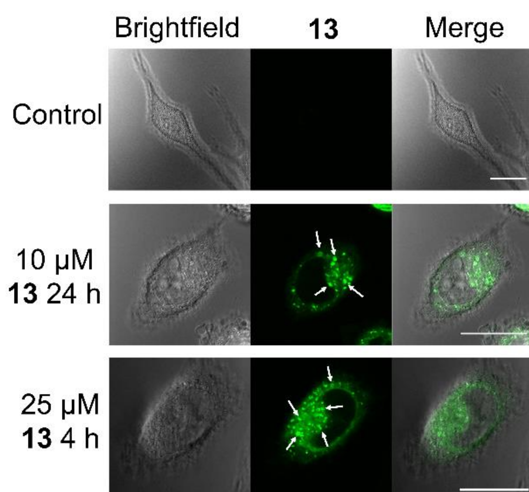


Fig. 6. Confocal fluorescence microscope images of Re-aqua (labeled as **13** in this figure), showing lysosomal uptake in HeLa cells.

To further explore the localization of the rhenium complexes, HeLa cells were treated with Re-aqua and different organelle-localizing dyes or transfected to express organelle-specific proteins fused with a fluorescent protein. These co-localization studies readily reveal that Re-aqua does not accumulate in the nucleus, mitochondria, or endoplasmic reticulum. In addition to its cytosolic distribution, the rhenium complex localizes to the large cytoplasmic vacuoles. The nature of these vacuoles was probed by transfecting the cells to express RFP-Rab5 and RFP-2×FYVE fusion proteins. Rab5 is a GTPase that localizes to the outer membrane of the early endosomes, and 2×FYVE is a tandem arrangement of a protein domain that binds to the lipid phosphatidylinositol 3-phosphate (PI3P), which is highly abundant in early endosomes and in the internal vesicles of multivesicular endosomes. The fluorescence microscopy images indicate that Re-aqua co-localizes with RFP-Rab5, and partially with the RFP-2×FYVE conjugate. This observation suggests that this compound accumulates in some populations of endosomes and further implies that the cytoplasmic vacuoles are endosomal in origin. The lysosomal marker LysoTracker Red DND-99 was also employed. The fluorescent images indicate that the intracellular localization of Re-aqua also correlates strongly with the lysosomes. This result suggests that the vacuoles also have lysosomal character and may be part of a compromised endosome-lysosome fusion process, or that this compound marks a broad population of endosomes and lysosomes. The cells were also transfected to express an RFP-LC3 fusion protein. LC3 is a

protein that accumulates on autophagosomes, digestive double-membrane vacuoles that occur during the process of autophagy. Fluorescence microscopy images indicate that the cytoplasmic vacuoles induced by this compound are not autophagosomes.[74]

Like the previous Re-aqua complex, the second-generation TRIP 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 7**). Although, there was some localization in the lysosomes and Golgi apparatus, the majority of the luminescence observed was cytosolic.

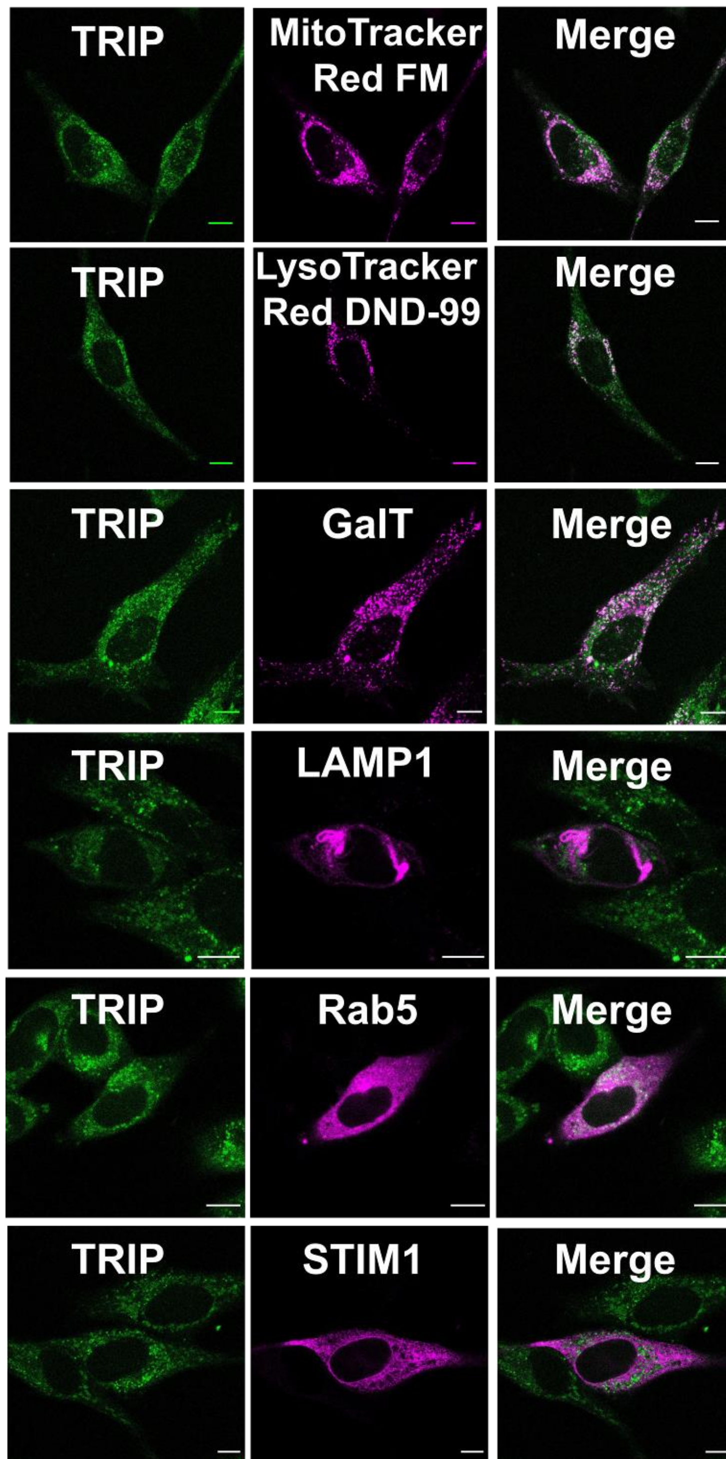


Fig. 7. Confocal fluorescent microscope images of HeLa cells treated with TRIP (5 μM, 4 h). Cells were additionally stained with the indicated transfection or dye. Transfected GalT-dsRed (galactosyltransferease 1) colocalizes with the Golgi apparatus, LAMP1-mRFP (Lysosomal Associated Membrane Protein 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 TRIP 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 TRIP 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 8**).

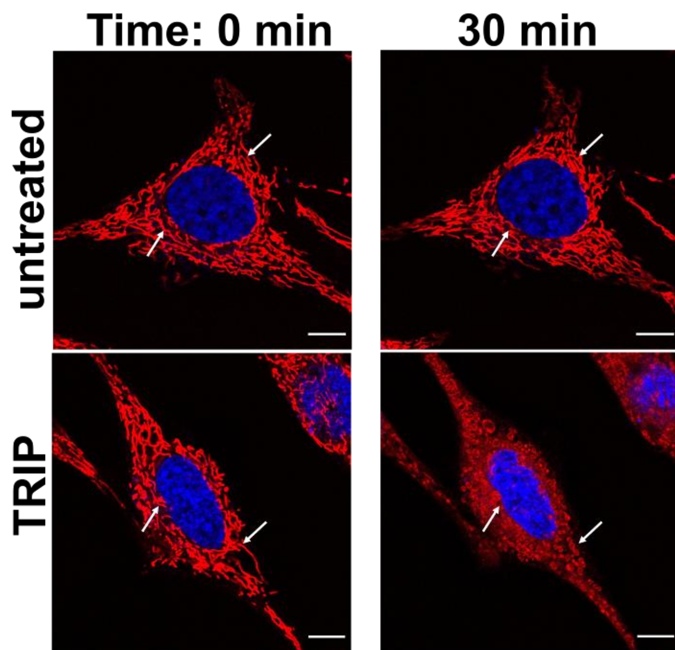
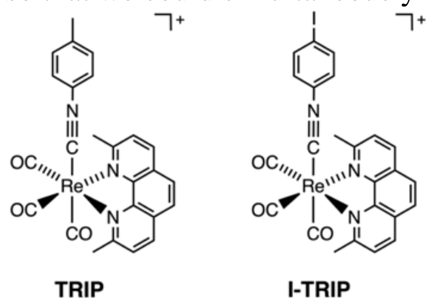


Fig. 8. Confocal fluorescence microscope images of HeLa cells untreated or treated with TRIP (labeled as TRIP in this figure, 5 μM). Cells were stained 15 and 30 min prior to compound treatment with Hoechst dye (blue) and MitoTracker Red (red), respectively.

In addition to using the intrinsic luminescent properties of these Re compounds, we have also used the presence of the exogenous element to carry out synchrotron-based X-ray fluorescence (XRF) imaging studies in collaboration with Professor Hugh Harris in the University of Adelaide. We have prepared an analogue of TRIP that contains an iodine atom on the axial isonitrile ligand so that we could simultaneously image this element as well (Scheme 4).



Scheme 4. Structures of TRIP and its iodo-analogue I-TRIP.

The XRF images are shown in **Figure 9**. As shown, the Re distributes through the cytosol. Importantly, the iodine matches the distribution pattern of the Re. This result indicates that the complex remains intact in cells.

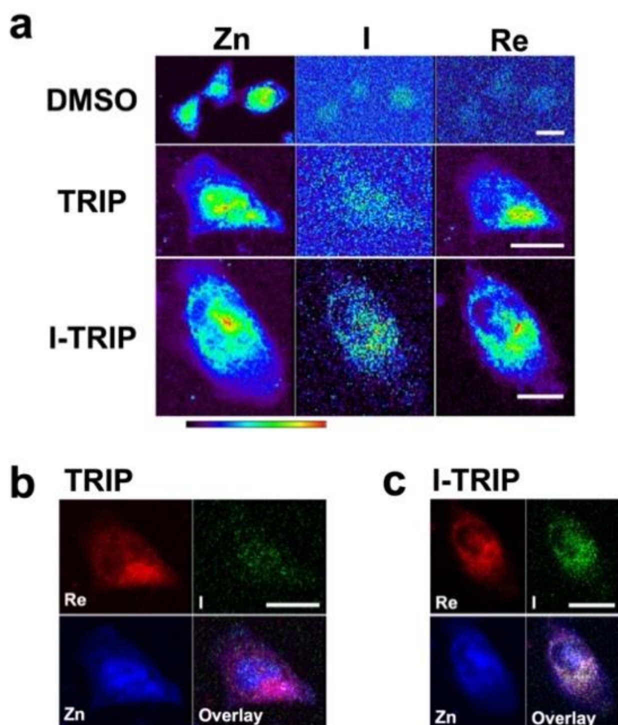


Figure 9. (a) XFM elemental distribution maps of HeLa cervical cancer cells treated with DMSO (0.06% v/v), 2 μM **TRIP**, or 3 μM **I-TRIP**. Correlation analysis on the alignment of Re, I, and Zn distribution maps for (b) **TRIP** and (c) **I-TRIP**. Scale bar = 20 μm.

We have also evaluated the mechanism of cell uptake of the pyridyl imine Re complexes shown in **Figure 10** by ICP-OES. The cell uptake was measured both at 37 °C and 4 °C, a temperature at which active transport is disfavored. The cell uptake increased with increasing complex lipophilicity. However, the uptake also decreased at low temperature. These results indicate that a combination of active and passive transport mediate the cellular uptake of the Re-based anticancer agents.

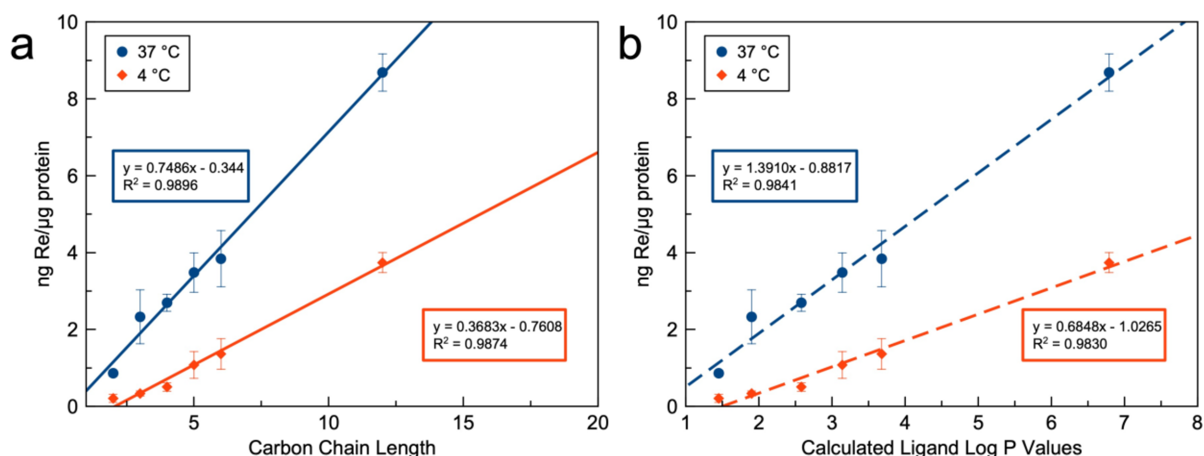


Fig. 10. Cellular uptake of **Re-Chains** after incubating for 3 h at 37 °C (blue) and 4 °C (red) in relation to (a) carbon chain length and (b) calculated log *P* values for the free ligands. The error bars represent the standard deviation from three replicates.

3) ^{99m}Tc analogues synthesis and comparative in vivo biodistribution studies. Tc is the lighter congener of Re, and exhibits similar chemistry. This similarity enables the use of ^{99m}Tc analogues of these rhenium anticancer agents as diagnostic partners for SPECT imaging or biodistribution studies. To assess in vivo behavior of Re-aqua, we synthesized its ^{99m}Tc analogue $[^{99m}Tc(dmphen)(OH_2)(CO)_3]^+$. The ^{99m}Tc analogue was prepared from the well-known precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$ and the dmphen ligand, and purified using preparative HPLC. After removal of the organic solvent, $[^{99m}Tc(dmphen)(OH_2)(CO)_3]^+$ was reconstituted and administered to naïve C57Bl6 mice via tail vein catheter simultaneously with a 0.10 µmol/kg dose of Re-aqua. Biodistribution was carried out at 30, 60 and 120 minutes post injection. Residual activity in select organs, tissues, and fluids (blood, heart, liver, kidney, ovaries, bone, muscle, urine) was quantified (**Figure 11**). We observed rapid renal and hepatic clearance of $[^{99m}Tc(dmphen)(OH_2)(CO)_3]^+$. No significant non-specific uptake was observed in any organs studied, paving the way for future studies of the distribution of $[^{99m}Tc(dmphen)(OH_2)(CO)_3]^+$ in models of disease.

We also assessed biodistribution and the metabolic profile of Re-aqua in naïve C57Bl6 mice. After allowing for decay of ^{99m}Tc, the rhenium concentration in select organs, tissues, and fluids (blood, heart, liver, kidney, ovaries, bone, muscle, urine) was quantified using inductively coupled plasma–mass spectrometry (ICP-MS). Biodistribution of Re-aqua revealed comparable behavior to its ^{99m}Tc analogue in most organs (Figure 2), suggesting the suitability of using the ^{99m}Tc analogue as a diagnostic partner. Notably, Re-aqua exhibits higher uptake in the kidneys and accelerated blood clearance properties than its ^{99m}Tc analogue.

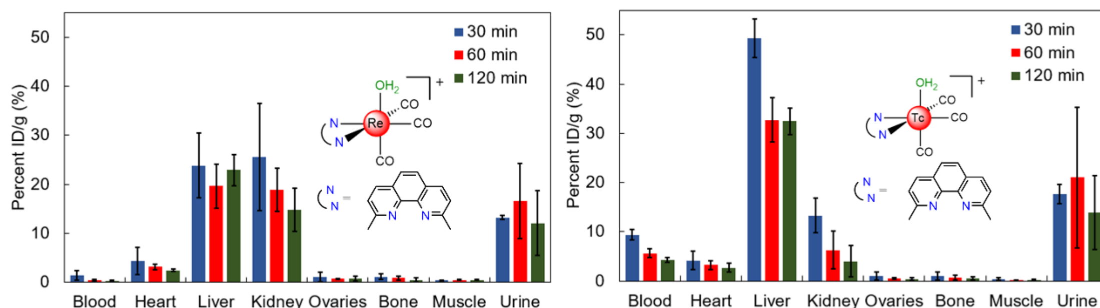


Figure 11. Biodistrubtion of Re-aqua (left) and $[^{99m}Tc(dmphen)(OH_2)(CO)_3]^+$ (right) in naïve C57Bl6 mice as measured by ICP-MS and gamma counting, respectively.

The ^{99m}Tc analogues of both the Re-9D and TRIP complexes were also 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 12a**). Similar to Re-aqua complex and its ^{99m}Tc analogue, this compound also exhibited hepatobiliary and renal clearance. The urine (**Figure 12b**) 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.

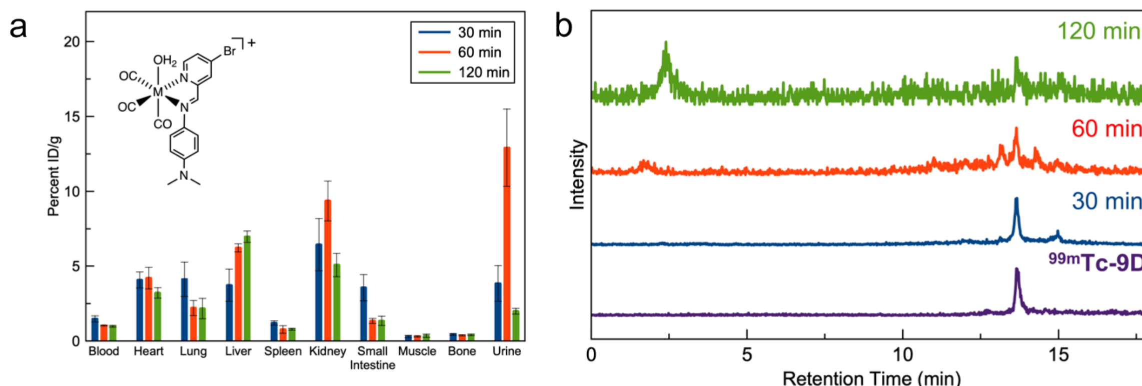


Fig. 12. (a) Biodistribution of ^{99m}Tc-9D(H_2O) in BALB/c mice detected using a gamma counter, in which M is ^{99m}Tc, after 30 (blue), 60 (red), and 120 (green) min. (b) Direct HPLC traces of urine samples collected from mice treated with ^{99m}Tc-9D(H_2O) after 30 (blue), 60 (red), and 120 (green) minutes. Reference chromatogram of ^{99m}Tc-9D(H_2O) is shown (purple).

We have also synthesized the ^{99m}Tc analogue of TRIP 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 13**). 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.

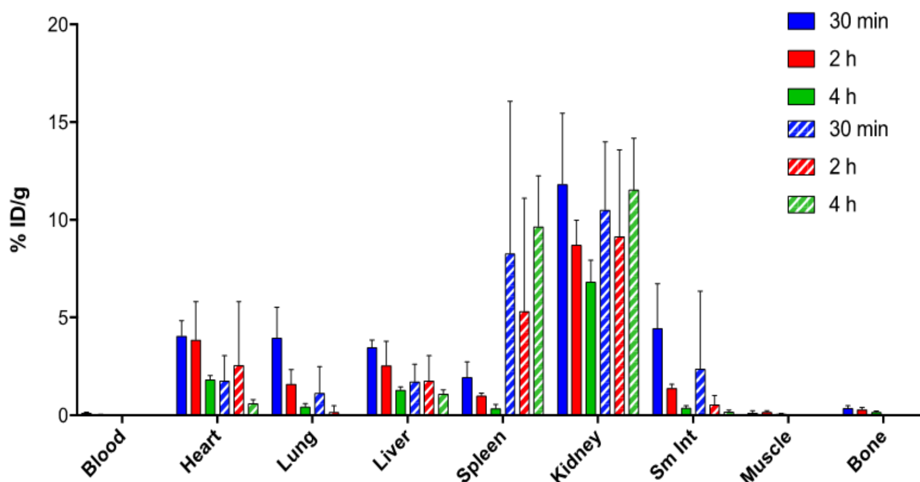


Fig. 13. Biodistribution of TRIP (solid bars) and ^{99m}Tc-ICN (striped bars) after injection in female BALB/c mice. Re content was quantified using ICP-OES and ^{99m}Tc content was determined using a gamma counter.

4) In vivo antitumor activity. These promising in vitro studies were further corroborated by in vivo studies in mice, which were carried out in the Center for Developmental Therapeutics at Northwestern University. We first evaluated the maximum tolerated dose of Re-aqua in NSG mice. *No adverse signs were observed up to an administered dose of 40 mg/kg.* By contrast, the MTD of cisplatin is around 20 mg/kg in this same mouse model, indicating that cisplatin is substantially more toxic than our lead rhenium compound. The in vivo antitumor activity was then measured using an ovarian cancer patient-derived xenograft model. The xenograft-bearing mice were treated with the compound twice weekly via tail vein injection. As shown in **Figure 14a**, this rhenium compound significantly inhibits tumor growth at all administered doses, *verifying that this compound possesses effective anticancer activity in vivo.* Furthermore, over the duration of this study, no decrease in mouse body weight was observed (**Figure 14b**). This result verifies that this rhenium compound gives rise to only minimal in vivo toxicity in contrast to cisplatin, which is known to substantially decrease mouse body weight when administered at therapeutically relevant concentrations.

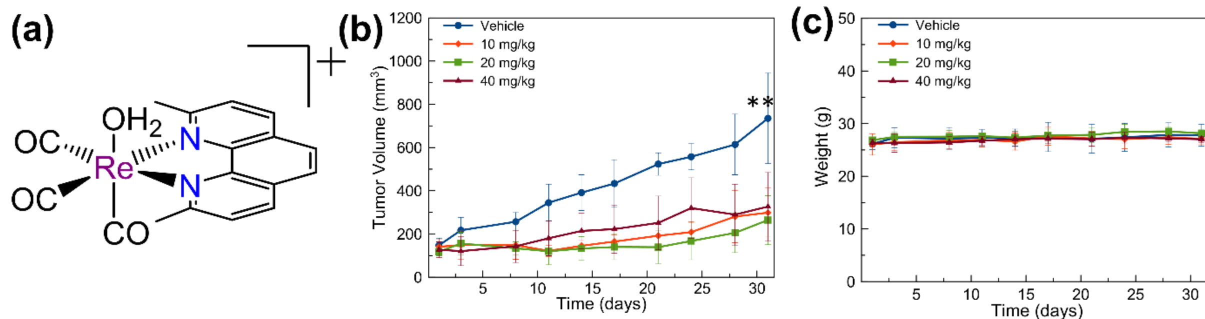


Figure 14. (a) Structure of our first-generation rhenium anticancer agent, Re-aqua. (b) Tumor volumes of mice during treatment periods with different doses of Re-aqua. (c) Mouse body weight during the treatment period. These data show that Re-aqua can inhibit tumor growth without negatively affecting the body weight of mice. Statistical significances of differences between values of the last time point (Panel B) were analyzed by the Student's t test: ** $0.001 < p < 0.01$.

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 3**). 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 15**). 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 3. 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

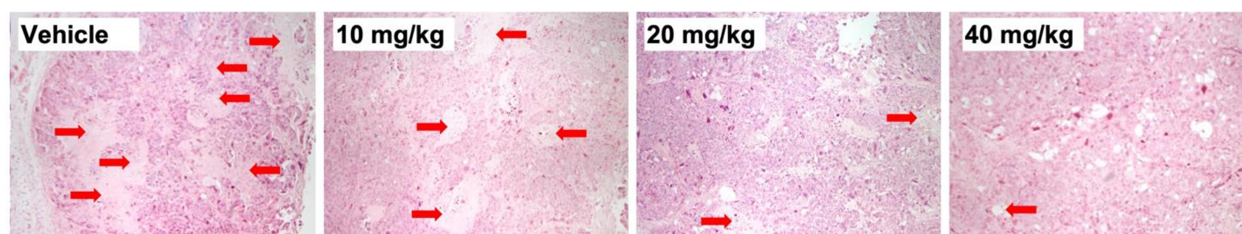


Fig. 15. H&E stained slides of tumors harvested from mice treated with vehicle or Re-aqua (10, 20, and 40 mg/kg). Red arrows indicate regions of necrosis.

Based on the promising in vitro activity of TRIP, we also evaluated it in vivo activity. Prior to testing the in vivo efficacy of TRIP, we performed a maximum tolerated dose (MTD) study, which showed no obvious sign of distress up to injections of 40 mg/kg. Based on the MTD, mice bearing the A2780 cancer xenografts were treated with 5, 10, or 20 mg/kg of this complex, administered in 20% DMSO/PBS solutions via tail vein injection, twice weekly. Although TRIP is soluble up to 2 mM in water or PBS, a DMSO cosolvent was needed to achieve the concentrations for in vivo dosing. Their tumor growth rates and overall health were monitored over 27 days (**Figure 16a**). The lower doses (5 and 10 mg/kg) of TRIP have no effect on tumor growth rate. At 20 mg/kg, however, TRIP significantly inhibits tumor growth as shown in **Figure 16a** and **16b**. From days 10 to 20 after beginning treatment, the 20 mg/kg cohort exhibited tumors with less than half the average volume of the vehicle-treated group. Thus, although TRIP was not able to entirely eradicate these tumors, it was able to markedly decrease the tumor growth rate, demonstrating that its anticancer activity is also retained in vivo. These in vivo data can also be analyzed by comparing the survival time of the treated mice relative to the untreated control. Such survival data may be more useful than tumor volumes in comparing the efficacy of drug candidates because different tumor types undergo a wide range of growth rates. The effects of TRIP on mouse survival were thus analyzed. In these experiments, mice were sacrificed and did not "survive" if their body weight decreased by 25% or if their tumor burden increased beyond 5500 mm^3. As shown in **Figure 16c**, 20 mg/kg of TRIP prolonged the survival of the tumor-bearing mice by 150% relative to the control group. These results demonstrate that this compound is able to significantly increase mice survival to levels that are comparable to several clinically investigated Ru anticancer agents. Additionally, the mice treated with the highest concentrations of TRIP exhibited minimal side effects, as evidenced by minimal body weight changes These data represents only the fifth example in the literature where the in vivo anticancer activity of a rhenium

tricarbonyl complex is demonstrated, and it supports the continued investigation of this interesting class of compounds as antitumor agents.

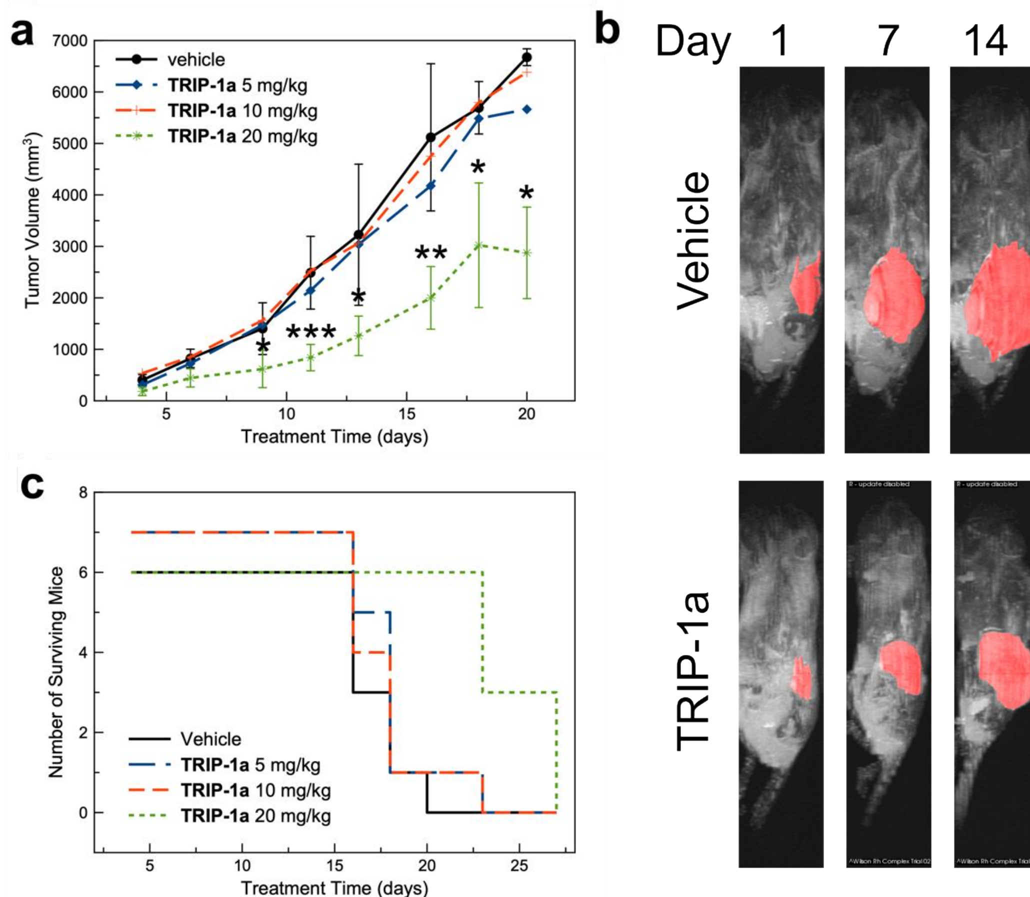


Fig. 16. (a) Tumor volume of mice bearing A2780 ovarian cancer xenografts (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$). **(b)** Magnetic resonance images of mice bearing A2780 ovarian cancer xenografts treated with the vehicle control (left) or TRIP at 20 mg/kg (right) over the course of 14 days. **(c)** Kaplan-Meier survival plot of mice treated with TRIP throughout the duration of the study.

5) Toward the synthesis of Re-folate conjugates. Because we demonstrated that these rhenium compounds localize to the lysosomes and endosomes, we considered that the use of a cleavable linker to attach the rhenium conjugate to folate would not be necessary for these compounds to mediate their activity (see Section V below for more information). As such, our initial synthetic efforts focused on preparing non-cleavable rhenium-folate conjugates. Our strategy was to conjugate folic acid to the phenanthroline ligand first, then coordinate the phenanthroline-folate ligand to a $Re(CO)_3$ core. The synthesis of the folate-phenanthroline conjugate followed the reaction scheme shown in **Figure 17**.

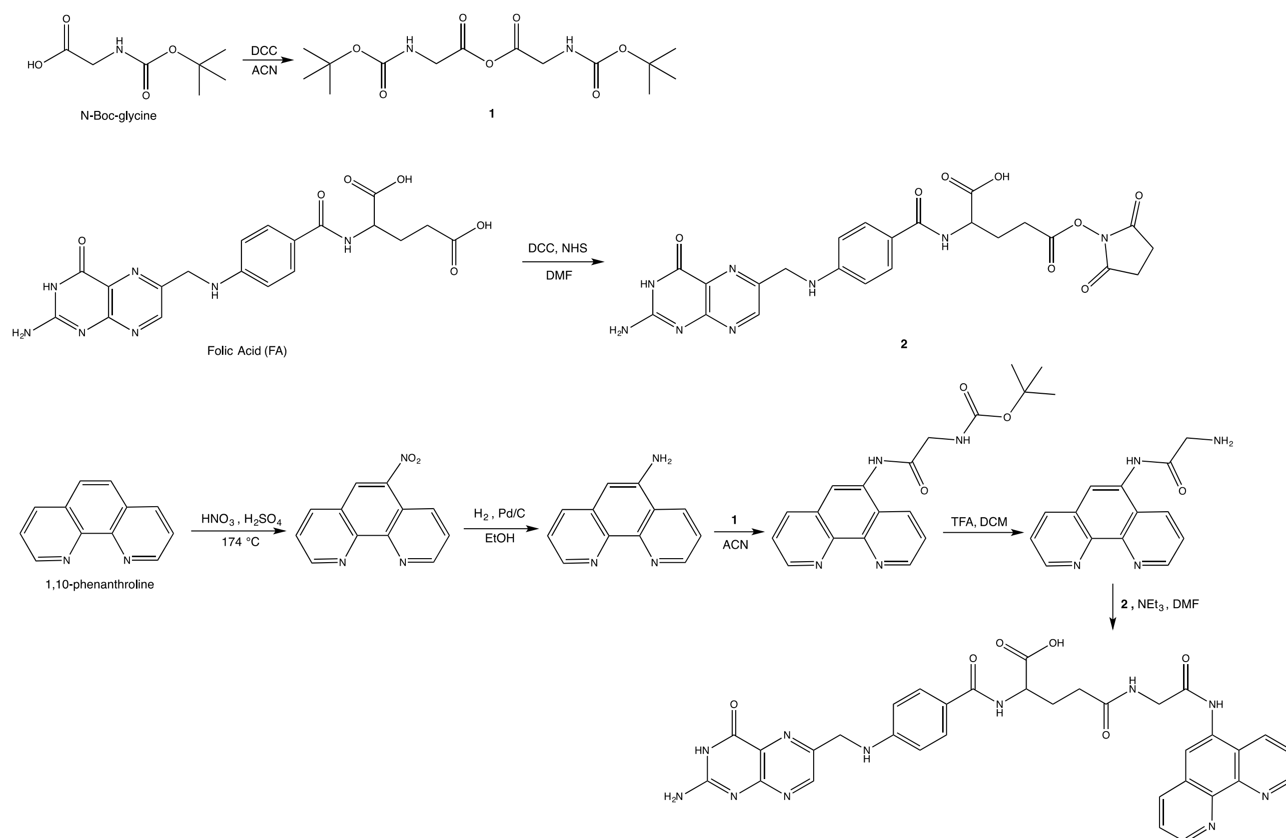


Fig. 17. Synthesis of a phenanthroline-folate ligand. This ligand was found to be too insoluble for further investigation.

Although we were able to successfully synthesize the phenanthroline-folate ligand shown in **Figure 17**, its solubility was poor in nearly all conventional organic solvents and water, thus hindering our efforts to attach it to rhenium. To improve the solubility of these ligands, we switched our synthetic efforts to incorporate a polyethylene glycol chain between the phenanthroline and folate (**Figure 18**).

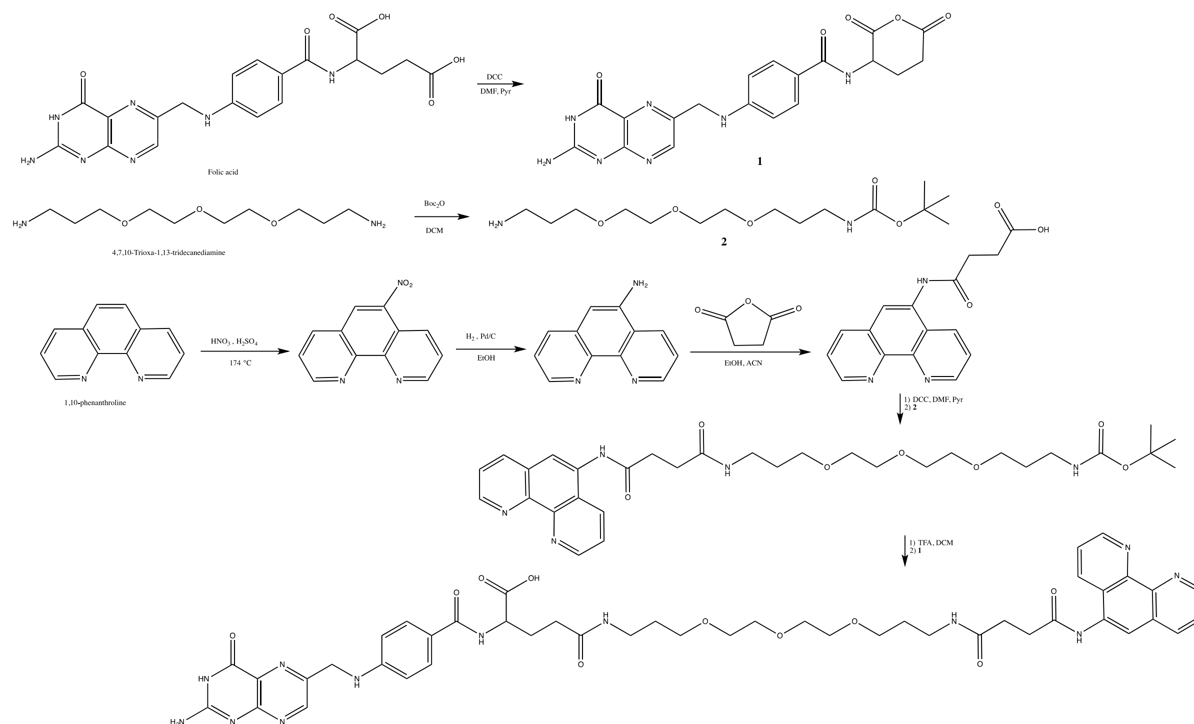


Fig. 18. Attempted synthesis of PEG-linked phenanthroline-folate conjugate.

The multi-step synthesis was successful up until the final amide coupling reaction between the phenanthroline ligand and the polyethylene glycol folate. The failure of the simple amide coupling reaction was determined to arise from cyclization of the succinate tail of the phenanthroline ligand to yield the undesired product shown in **Figure 19**.

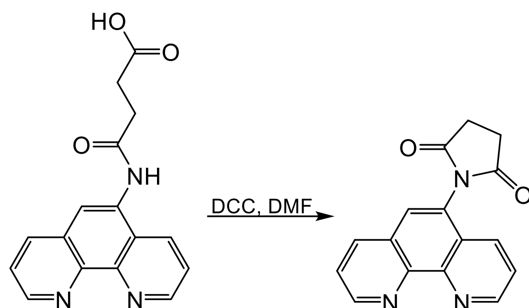


Fig. 19. Unexpected formation of the cyclic succinimide product upon the use of an amide coupling reagent.

We have pursued alternative routes shown in **Figure 20**.

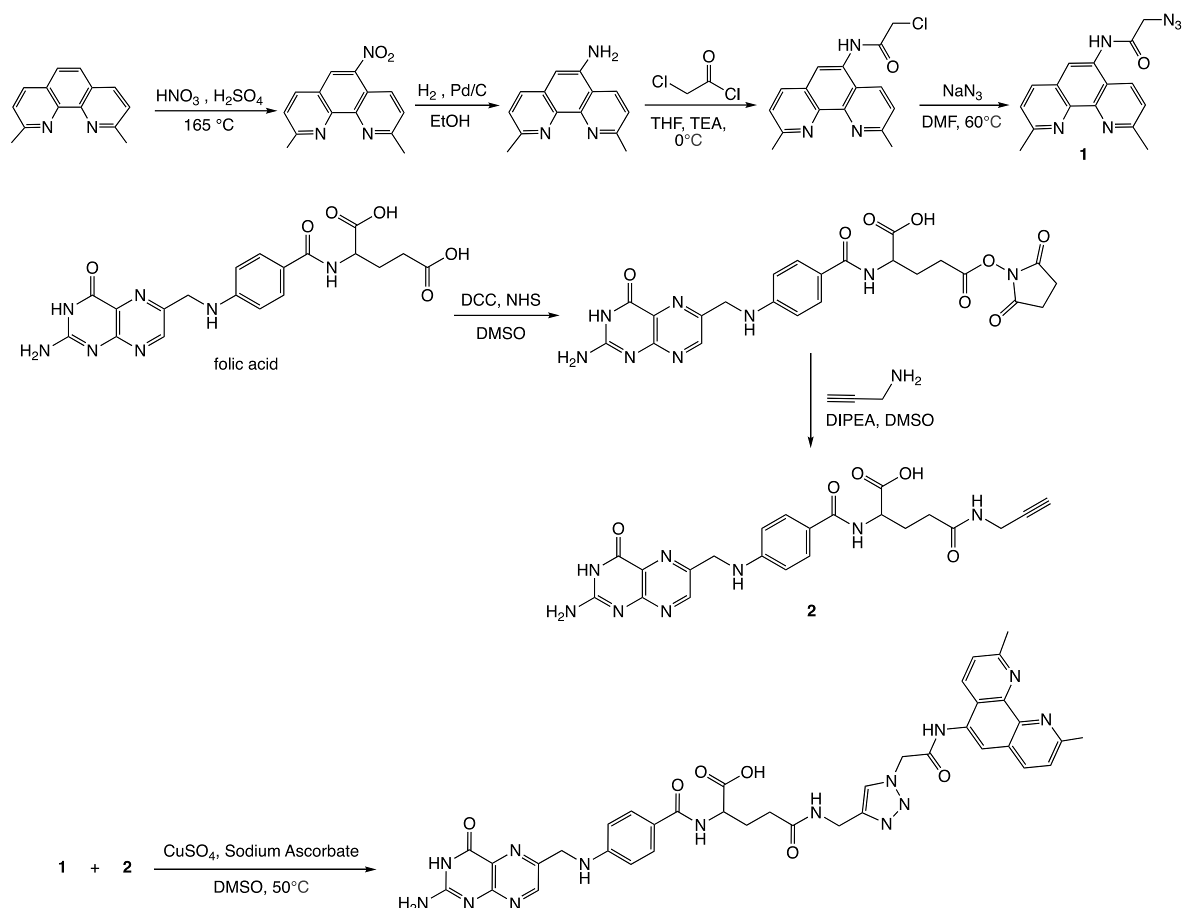


Fig. 20. Attempted synthesis of a dmphen-folate ligand.

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 tried pursuing alternative routes shown in **Figure 21**.

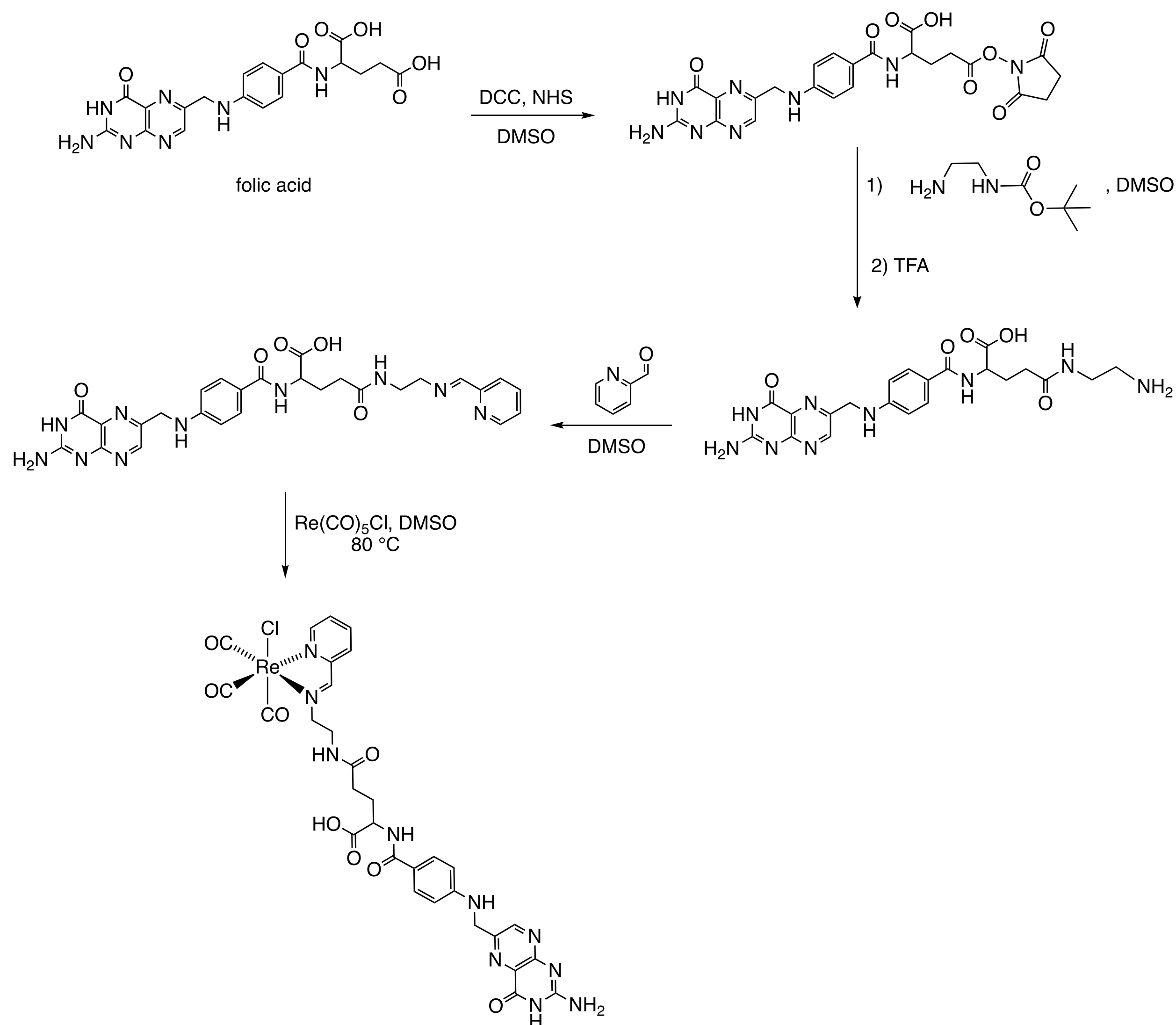


Fig. 21. Current synthetic scheme for Re-folate complex.

We have successfully synthesized the ethylene diamine-folate conjugate. However, we anticipate issues with solubility after isolation of the Re-folate conjugate.

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, the Gordon Research Conference for Metals in Medicine, and the National Meeting of the American Chemical Society. 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

Nothing to report.

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 led to the focus on the TRIP 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 detailed in previous annual reports. The major limitations have resulted from the poor solubility of such 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) Kevin M. Knopf, Brendan L. Murphy, Samantha N. MacMillan, Jeremy M. Baskin, Martin P. Barr, Eszter Boros, Justin J. Wilson. "In Vitro Anticancer Activity and in Vivo Biodistribution of Rhenium(I) Tricarbonyl Aqua Complexes." *J. Am. Chem. Soc.* **2017**, *139*, 14302-14314.

2) Sierra C. Marker, Samantha N. MacMillan, Warren R. Zipfel, Zhi Li, Peter C. Ford, Justin J. Wilson. "Photoactivated in Vitro Anticancer Activity of Rhenium(I) Tricarbonyl Complexes Bearing Water-Soluble Phosphines." *Inorg. Chem.* **2018**, *57*, 1311-1331.

3) 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.

4) 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.

5) 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." *Chem. Eur. J.* **2019**, *25*, 9206–9210.

6) Sierra C. Marker, Chilaluck C. Konkankit, Mark C. Walsh, Daniel R. Lorey II, Justin J. Wilson. "Radioactive World: An Outreach Activity for Nuclear Chemistry." *J. Chem. Ed.* **2019**, *96*, 2238–2246.

7) 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." *ACS Med. Chem. Lett.* **2019**, *10*, 822-827.

8) Brendan L. Murphy, Sierra C. Marker, Valencia J. Lambert, Joshua J. Woods, Samantha N. MacMillan, Justin J. Wilson. "Synthesis, characterization, and biological properties of rhenium(I) tricarbonyl complexes bearing nitrogen-donor ligands" *J. Organomet. Chem.* **2020**, *907*, 121064.

9) Chilaluck C. Konkankit, James Lovett, Hugh H. Harris and Justin J. Wilson. "X-Ray fluorescence microscopy reveals that rhenium(I) tricarbonyl isonitrile complexes remain intact *in vitro*" *Chem. Commun.* **2020**, 56, 6515–6518.

10) Chilaluck C. Konkankit, Brett A. Vaughn, Zhouyang Huang, Eszter Boros, Justin J. Wilson. "Systematically altering the lipophilicity of rhenium(I) tricarbonyl anticancer agents to tune the rate at which they induce cell death" *Dalton Trans.* **2020**, doi: 10.1039/d0dt01097a

11) Sierra C. Marker, A. Paden King, Robert V. Swanda, Brett Vaughn, Eszter Boros, Shu-Bing Qian, Justin J. Wilson. "Exploring Ovarian Cancer Cell Resistance to Rhenium Anticancer Complexes" *Angew. Chem. Int. Ed.* **2020**, doi: 10.1002/anie.202004883

12) A. Paden King, Justin J. Wilson. "Endoplasmic Reticulum Stress: An Arising Target for Metal-Based Anticancer Agents" *Chem. Soc. Rev.* **2020**, doi: 10.1039/d0cs00259c

13) Sierra C. Marker, A. Paden King, Samantha Granja, Brett Vaughn, Joshua J. Woods, Eszter Boros, Justin J. Wilson. "Exploring the In Vivo and In Vitro Anticancer Activity of Rhenium Isonitrile Complexes" *Inorg. Chem.* **2020**, accepted for publication, June 19, 2020.

B. CONFERENCE PRESENTATIONS

1) "Anticancer Potential of Rhenium(I) Complexes." Justin J. Wilson, Kevin M. Knopf, Sierra C. Marker. 8th Asian Biological Inorganic Chemistry Conference, Auckland, New Zealand, Dec. 4–Dec 9, 2016 (invited talk).

2) "Rhenium as an Alternative to Platinum? Value-Added Metalloanticancer Agents." Justin J. Wilson, Eszter Boros, Kevin M. Knopf, Sierra C. Marker, Chilaluck Charlene Konkankit. 6th Georgian Bay Conference on Bioinorganic Chemistry, Parry Sound, Ontario, Canada, May 23–May 27, 2017 (invited talk).

3) "Metals in Medicine: Coordination Chemistry to Control Biological Activity." Justin J. Wilson. SUNY Potsdam, Potsdam, NY, October 17, 2017 (invited seminar presentation).

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

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

6) “Rhenium as an Alternative to Platinum for the Treatment of Cancer” 256th American Chemical Society National Meeting, Boston, MA, August, 19, 2018 (contributed talk).

7) “The Heavy Metal Rhenium as an Alternative to Platinum-Based Chemotherapy” 2nd Annual Cornell Cancer Research Symposium, Ithaca, NY, October 2019, Invited Talk.

8) “Organometallic Rhenium Complexes as Anticancer Agents: Mechanisms of Action and In Vivo Potential” 9th International Symposium on Bioorganometallic Chemistry, York, United Kingdom, August 2019, Plenary Lecture.

C. PATENT APPLICATIONS

2) A. King, Sierra Marker, Shu-Bing Qian, Robert Swanda, Justin Wilson. “Rhenium-Isonitrile Complexes and Methods of Use for Treating Cancer.” International Patent Application No. PCT/US2019/046732, August 16, 2018.

1) Kevin M. Knopf, Sierra C. Marker, Justin J. Wilson. “Rhenium Complexes and Methods of Use for Treating Cancer.” U.S. Patent Application No. 16/311,961, December 20, 2018; International Patent Application No. PCT/US2017/038972, June 23, 2017.

VII. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

A) INDIVIDUALS

Name:	Kevin M. Knopf
Project Role:	Graduate Student
ORCID:	N/A
Nearest Person Month Worked:	12
Contribution to Project:	Mr. Knopf was lead author on the *JACS* paper that resulted from this work. He led studies on complex synthesis and characterization of biological activity.
Funding Support:	N/A

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 TRIP 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 TRIP 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 TRIP 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 TRIP 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:	Hugh Harris
Project Role:	Collaborator
ORCID:	0000-0002-3472-8628
Nearest Person Month Worked:	1
Contribution to Project:	Prof. Harris analyzed samples by X-ray fluorescence for us.
Funding Support:	N/A

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

CHE-1750295 (PI: Wilson) **07/01/2018–06/30/2023**
National Science Foundation $76,077 direct costs per year
CAREER: A Toolkit to Modulate the Mitochondrial Calcium Uptake Machinery

The major goal of this project is to develop ruthenium-based inhibitors for mitochondrial calcium uptake.

Cottrell Scholar Award (PI: Wilson) **07/01/2019 – 06/30/2022**
Research Corporation for Science Advancement $33,333 direct costs per year
Capturing the Heavy Alkaline Earth Elements: Ligand Design to Sequester Radioactive Strontium, Barium, and Radium

The major goal of this award is to design chelating agents for alkaline earth elements for environmental and industrial purposes. For example, the sequestration of naturally occurring radioactive materials and dissolution of barite scale.

R21 EB027282-01A1 (PI: Wilson) **09/16/2019–6/30/22**
National Institutes of Health $128,014 direct costs per year
Expanding the Therapeutic Potential of the Alpha Emitter Radium-223

The major goal of this project is to develop chelators for radium-223, design bifunctional analogues of these chelators, and then conjugate those bifunctional chelators to antibodies and evaluate their in vivo biodistribution.

R01 EB029259-01 (PI: Wilson) **03/05/20 – 11/30/23**
National Institutes of Health $263,926 direct costs per year
Chelation strategies for s-, p-, and f-block radionuclides for targeted alpha therapy

This project will design chelators for main group and f-block radionuclides to be used in targeted alpha therapy.

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.

6) **Organization Name:** University of Adelaide

Location of Organization: Adelaide, Australia
Partner's Contribution to Project: Professor Hugh Harris carried out and analyzed XRF of cells treated with these compounds.

VIII. SPECIAL REPORTING REQUIREMENTS

Not applicable.

IX. APPENDICES

Not applicable.