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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 discovered that this class of compounds is highly effective against cisplatin-resistant ovarian cancer cells, suggesting that these agents might be valuable for use in relapsed ovarian cancer. The mechanism of action of these compounds was also investigated, revealing that they induce via a non-apoptotic pathways. Furthermore, the intracellular localization, determined via confocal fluorescence microscopy, indicates that these compounds accumulate in the lysosome and endosomes. Lastly, in vivo tumor xenograft studies demonstrate that this class of compounds effectively inhibits tumor growth with minimal toxic side effects.

15. SUBJECT TERMS
cisplatin resistance, rhenium, folate receptor, technetium, theragnostic

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I. INTRODUCTION

Ovarian cancer is the most lethal type of gynecological disease. The platinum-based drugs, cisplatin and carboplatin, comprise the first-line treatment for this malady. Despite the clinical success of these drugs, they suffer from several key limitations. For example, they induce a number of long-term toxic side effects, such as nephrotoxicity and ototoxicity, which diminish patient quality of life. Additionally, ovarian cancer responds well to platinum chemotherapy during the first round of treatment, but becomes highly resistant or non-responsive in the relapsed form. Lastly, the platinum-based drugs are not amenable to direct in vitro or in vivo imaging. The inability to track these compounds in vivo prevents a real-time assessment of their efficacy in patients. In this project, we will overcome the limitations of the platinum-based drugs for the treatment of ovarian cancer by exploring new therapeutic agents based on the element rhenium. These compounds are designed to exhibit minimal toxic side effects, overcome cisplatin resistance mechanisms, and possess spectroscopic properties that are suitable 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 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. As part of this objective, we will also study these complexes' biological mechanisms of action and propensity to circumvent resistance in ovarian cancer by evading NER mechanisms.

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

i) Milestones: Identification of lead Re drug candidates that exceed the potency of existing platinum drugs, circumvent resistance, and operate effectively independent of cell NER status. Publish a manuscript on the structure-activity relationships of Re complexes.

ii) Status: 80% complete; our first manuscript studying the SAR and mechanism of action of this class of novel rhenium anticancer agents was published; see *J. Am. Chem. Soc.* **2017**, *139*, 14302. The compounds still need to be screened against NER-deficient cell lines.

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

ii) Status: 20% complete; the synthesis of these conjugates is still being troubleshot. As described below, we have found promising alternative synthetic routes to access these compounds.

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

i) Milestone: 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: 20% complete; this work depends on the synthesis of the Re-folate conjugates above. We have currently not isolated this compounds successfully; however, we have carried out in vivo antitumor studies with the 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 microscopy 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.*

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.

ii) Status: 20% complete; we have studied and prepared the ^{99m}Tc analogues of the unfunctionalized lead compound. We used this compound to investigate biodistribution in mice.

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, 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(dmphen)(OH$_2$)(CO)$_3$]$^+$ in these non-cancerous lung cells. *This result indicates that, even though [Re(dmphen)(OH$_2$)(CO)$_3$]$^+$ 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)$_3$]$^+$ 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.

	IC$_{50}$ (μM) or RF	
Cell Line	cisplatin	[Re(dmphen)(OH$_2$)(CO)$_3$]$^+$
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(dmphen)(OH$_2$)(CO)$_3$]$^+$ 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(dmphen)(OH$_2$)(CO)$_3$]$^+$ was probed in the presence of the necroptosis inhibitor necrostatin-1. Necrostatin-1 had no effect on the cytotoxic activity of [Re(dmphen)(OH$_2$)(CO)$_3$]$^+$, 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(dmphen)(OH$_2$)(CO)$_3$]$^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$, 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 $[Re(dmphen)(OH_2)(CO)_3]^+$. This compound did not lead to an increase in intracellular ROS nor did it depolarize the MMP. $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$

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

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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ was detectable well above the background autofluorescence within the cells (Figure 1). The yellow emission of the rhenium was distributed

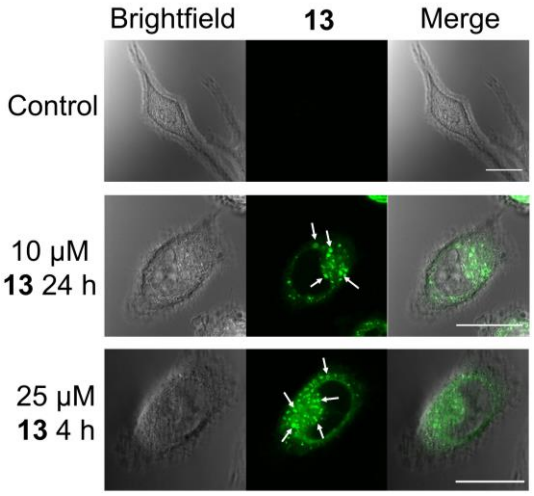

Figure 1. Confocal fluorescence microscope images of $[Re(dmphen)(OH_2)(CO)_3]^+$ (labeled as **13** in this figure), showing lysosomal uptake in HeLa cells.

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.

To further explore the localization of the rhenium complexes, HeLa cells were treated with $[Re(dmphen)(OH_2)(CO)_3]^+$ and different organelle-localizing dyes or transfected to express organelle-specific proteins fused with a fluorescent protein. These co-localization studies readily reveal that $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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]

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 $[Re(dmphen)(OH_2)(CO)_3]^+$, 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 $[Re(dmphen)(OH_2)(CO)_3]^+$. 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 2). 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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, $[Re(dmphen)(OH_2)(CO)_3]^+$ exhibits higher uptake in the kidneys and accelerated blood clearance properties than its ^{99m}Tc analogue.

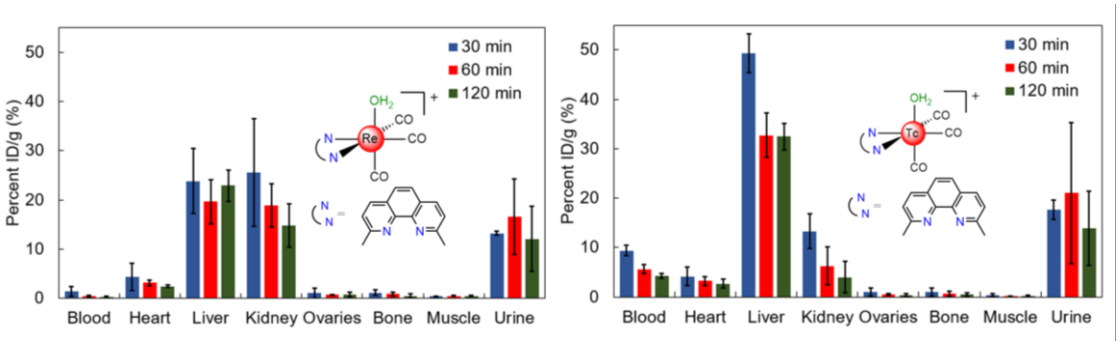

Figure 2. Biodistrubtion of $[Re(dmphen)(OH_2)(CO)_3]^+$ (left) and $[^{99m}Tc(dmphen)(OH_2)(CO)_3]^+$ (right) in naïve C57Bl6 mice as measured by ICP-MS and gamma counting, respectively.

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 $[Re(dmphen)(OH_2)(CO)_3]^+$ 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 Fig. 3a, 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 (Fig. 3b). 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.

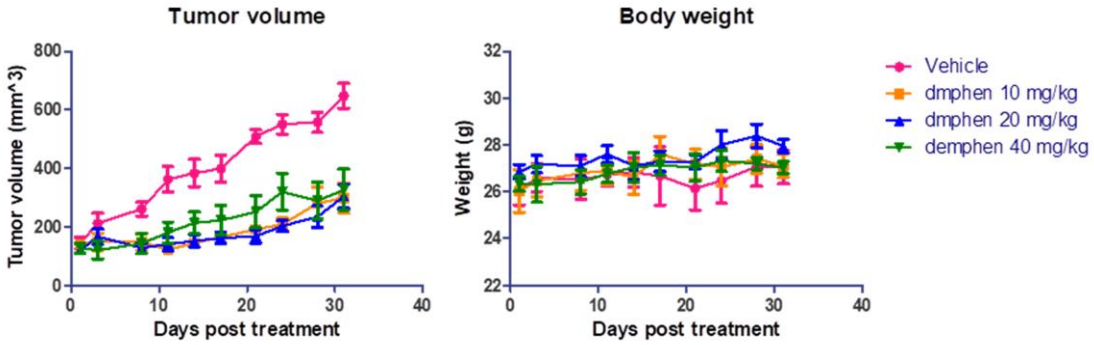

Figure 3. The novel rhenium anticancer agent $[Re(dmphen)(OH_2)(CO)_3]^+$ is effective in treating cancer in vivo with minimal toxic side effects. (a) Tumor volume of NSG mice bearing ovarian cancer patient-derived xenografts when treated with either the vehicle control, 10 mg/kg, 20 mg/kg, or 40 mg/kg of $[Re(dmphen)(OH_2)(CO)_3]^+$. At all administered doses, this compound significantly inhibits tumor growth. (b) Body weight of ovarian cancer PDX-bearing mice when treated with the vehicle control, 10 mg/kg, 20 mg/kg, or 40 mg/kg of $[Re(dmphen)(OH_2)(CO)_3]^+$. No significant body weight decreases are observed, indicating that there are few toxic side effects of this compound.

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 4.

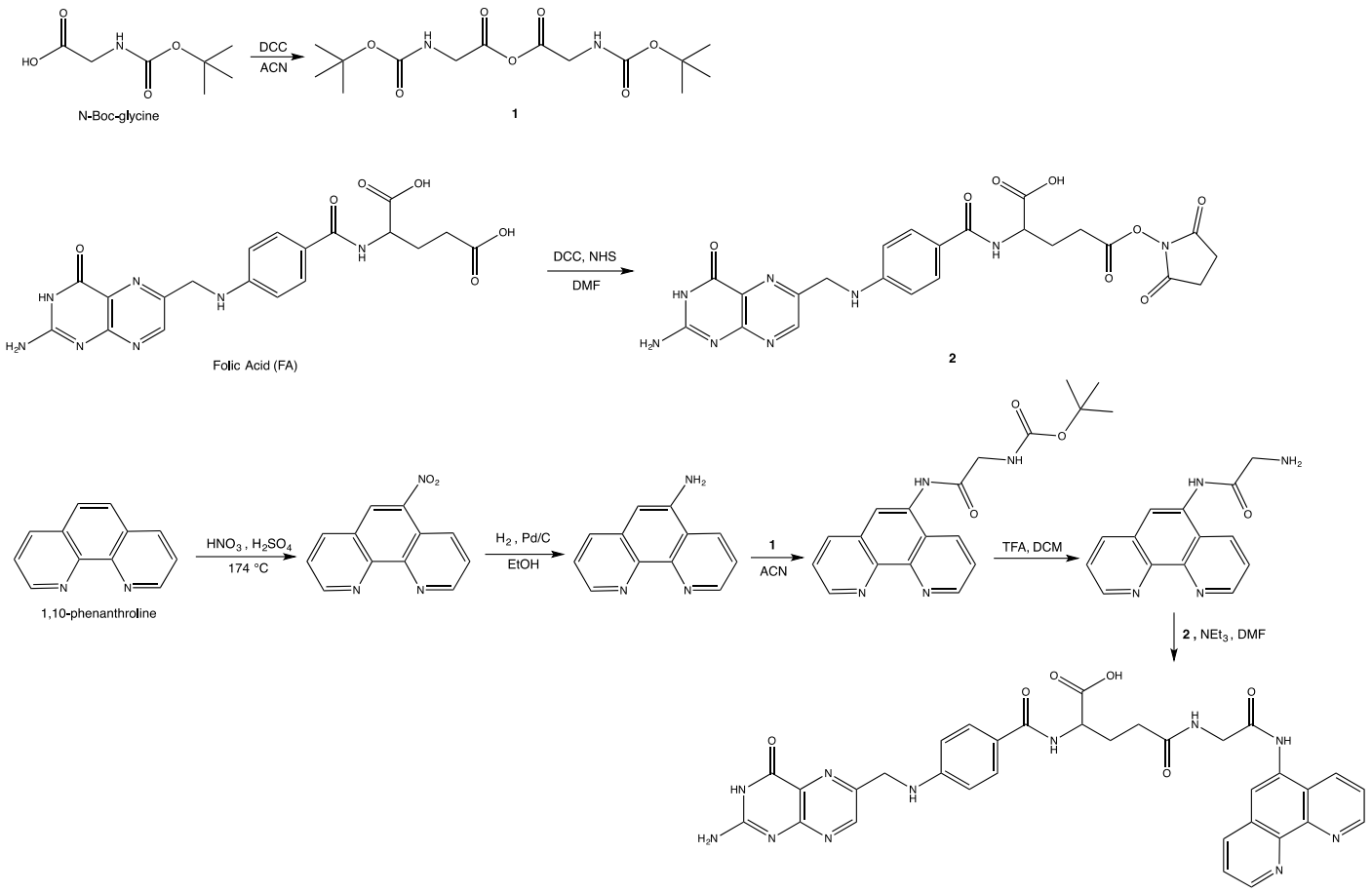

Figure 4. Synthesis of a phenanthroline-folate ligand. This ligand was found to be too insoluble for further investigation.

Although we were able to successfully synthesize the phenanthroline-folate ligand shown in Figure 4, 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 5).

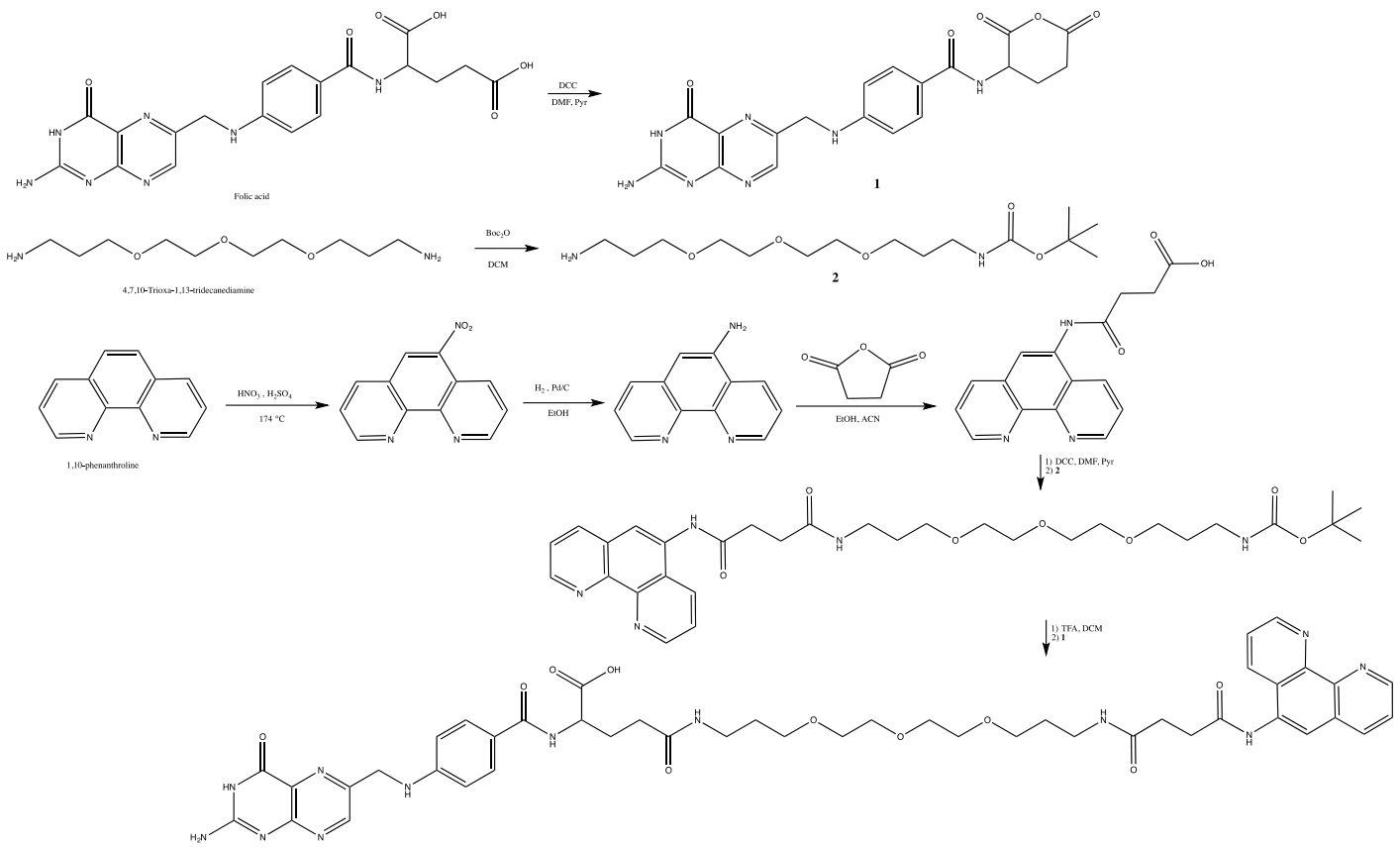

Figure 5. Attempted synthesis of PEG-linked phenanthroline-folate conjugate.

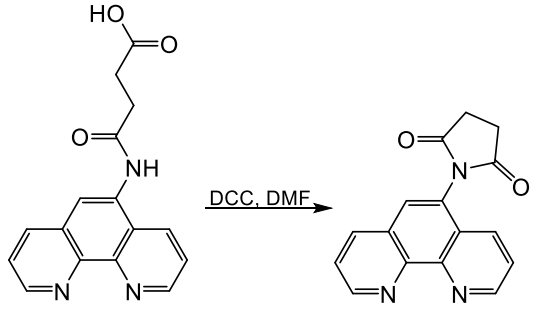

Figure 6. Unexpected formation of the cyclic succinimide product upon the use of an amide coupling reagent.

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 6. Based on these synthetic challenges, we will pursue alternative routes in the next funding period, as discussed in Section V.

C) TRAINING AND PROFESSIONAL DEVELOPMENT

1) Graduate Student Training. Three graduate students, Mr. Kevin Knopf, Ms. Charlene Konkankit, and Ms. Sierra Marker, have been working on various aspects of this project over the first year of its duration. As part of their work on this project, they have been receiving training in various aspects of molecular and cell biology. For example, Mr. Knopf learned how to carry out Western blots to check for protein expression levels. They have also been receiving training in synthetic chemistry. For example, Ms. Konkankit has significantly improved with respect to organic chemistry synthetic skills.

2) Graduate Student Conference Attendance. Ms. Marker attended the 6th Georgian Bay International Conference on Bioinorganic Chemistry (CanBIC) to present a poster on several of the results from this project. The conference allowed Ms. Marker to interact with internationally renowned scientists to expand her professional network and receive scientific feedback on her project.

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 "Radiation: It's not superpowers, it's better! Empowering women through science, not fantasy." 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) Research Experience for Teachers. The Cornell Center for Materials Research organizes a summer Research Experience for Teachers program where K–12 teachers carry out research alongside scientists at Cornell. The Wilson group has hosted a teacher to work on components of this project. Furthermore, Dr. Wilson has given a presentation of his research to the larger group of teachers in this program.

E) FUTURE PLANS

1) Investigate the role of NER in mediating the activity of these rhenium complexes. With respect to the Statement of Work objectives, we will probe the role of the NER in mediating the cytotoxicity of rhenium. In addition to using NER deficient cell lines, as described above, we will also use inhibitors of NER. The data will provide direct evidence for the role of genomic DNA as a target for this class of rhenium complexes.

2) Complete synthesis of rhenium-folate conjugates. We will continue efforts to design rhenium-folate conjugates.

3) Evaluate the in vitro and in vivo anticancer activity of the rhenium-folate conjugates. As described above, we will test the resulting rhenium-folate conjugates against ovarian cancer cells that express the folate receptor.

4) Evaluate ^{99m}Tc SPECT imaging of the folate conjugates. The ^{99m}Tc analogues of the rhenium-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 this class of compounds acts via a novel mechanism of action that is distinct from that of the platinum-based drugs. A consequence of this observation is that these compounds are not cross-resistant to cisplatin, rendering them useful for the treatment of platinum-resistant relapsed ovarian cancer. Furthermore, we have demonstrated that this class of compounds also exhibit anticancer activity in in vivo mouse models while giving rise to minimal toxic side effects. 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 Part III 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, a minor change in our approach is to develop rhenium-folate conjugates that do not have cleavable linkers. This change will facilitate our synthetic efforts. Because our most recent data indicates that these rhenium compounds enter cells through endocytosis and are localized in the lysosomes, the lack of cleavable linker should not be problematic because folate enters and localizes in the cells in a similar manner.

B) PROBLEMS OR DELAYS

The synthesis of rhenium-folate conjugates has provided unexpected challenges. As described above, amide coupling reactions with the succinate-functionalized phenanthroline ligand gave the cyclic succinimide product (see Part III B above) rather than the desired folate conjugates. Moving forward, we will follow the synthetic scheme proposed below (Figure 7), using azide-alkyne click chemistry to conjugate the two moieties. We anticipate that we will be able to make up for lost time here; the student working on this aspect of the project, Ms. Konkankit, has become significantly more skilled in synthetic chemistry over this year of additional training.

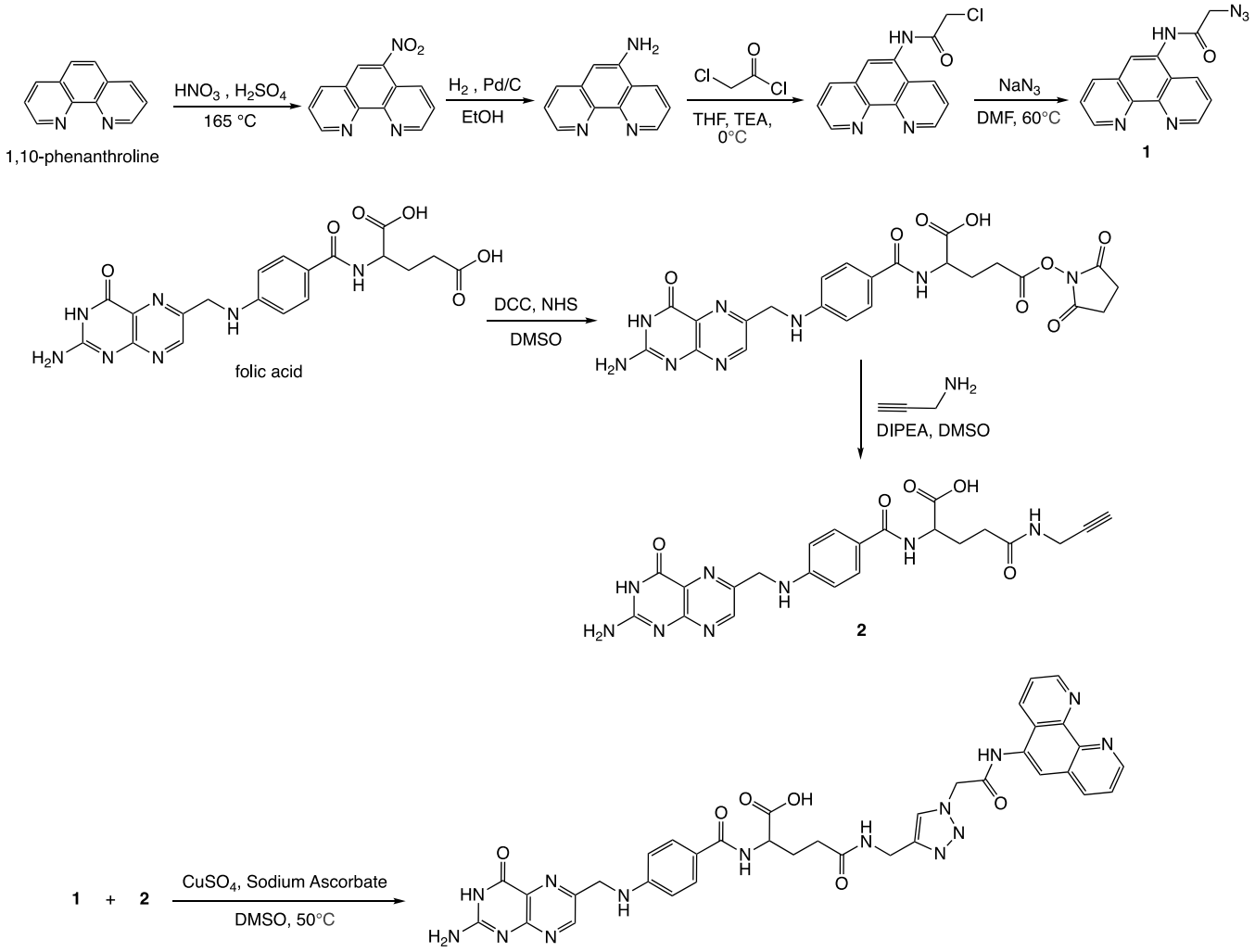

Figure 7. Synthetic pathways that we are currently pursuing to synthesize new rhenium-folate conjugates.

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) 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.

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).

C. PATENT APPLICATIONS

1) Justin J. Wilson, Kevin M. Knopf. "Composition and Methods Comprising Rhenium." *U.S. Provisional Patent Application No. 62/354,209*, June 24, 2016.

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:	Charlene Konkankit
Project Role:	Graduate Student
ORCID:	N/A
Nearest Person Month Worked:	6
Contribution to Project:	Ms. Konkankit worked to synthesize folate-targeted rhenium complexes.
Funding Support:	Teaching Assistantship

Name:	Sierra C. Marker
Project Role:	Graduate Student
ORCID:	N/A
Nearest Person Month Worked:	6
Contribution to Project:	Ms. Marker worked on the synthesis of new rhenium anticancer agents with the goals of designing improved analogues. She also worked with Mr. Knopf recently to study the mechanisms of action of these complexes.
Funding Support:	Teaching Assistantship

Name:	Jeremy Baskin
Project Role:	Collaborator
ORCID:	0000-0003-2939-3138
Nearest Person Month Worked:	0.5
Contribution to Project:	Prof. Baskin assisted students in the Wilson group in carrying out and analyzing confocal fluorescence microscopy images of the novel rhenium anticancer agents.
Funding Support:	Cornell University Startup, NIH K99 Award

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

Nothing to report.

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.

VIII. SPECIAL REPORTING REQUIREMENTS

Not applicable.

IX. APPENDICES

Not applicable.