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TITLE: Mixed-Lineage Kinases as Novel Targets for the Treatment of
Endocrine-Resistant, ER-Positive Breast Cancer

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14. ABSTRACT Previous research in our laboratory has demonstrated that a small molecule inhibitor of Mixed Lineage Kinases (MLKs) induces a cell cycle arrest and apoptosis in estrogen receptor (ER) - positive breast cancer cells in culture, while having minimal effects on non-tumorigenic mammary epithelial cells. The overall goal of the current research is to test the efficacy of this inhibitor in pre-clinical mouse xenograft models of human ER-positive breast cancer. During the current reporting period, we developed an accurate and sensitive mass spectrometry based method for detection of the compound in mouse serum and tissue, and optimized conditions for administration of the drug to animals. We also established methods for monitoring tumor growth using both cell lines and patient derived xenografts in immune compromised mice. Experiments are in progress to test the ability of the MLK inhibitor to block the growth of endocrine sensitive and endocrine resistant human breast cancer cell lines and patient derived xenografts in these pre-clinical mouse models.					
15. SUBJECT TERMS ER-positive breast cancer, mixed lineage kinases, patient derived xenografts, kinase inhibitor, pre-clinical models					
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1. Introduction: A majority of breast tumors express estrogen receptor alpha (ER) and require estrogen to grow. Although endocrine therapies such as antiestrogens (AEs) and aromatase inhibitors (AIs) have been very successful, resistance to these treatments is a serious clinical problem. We previously demonstrated in cell culture that a small molecule inhibitor of a family of kinases known as Mixed Lineage Kinases (MLKs) induces cell cycle arrest and apoptosis in ER⁺ breast cancer cell lines, including one with acquired endocrine resistance. The purpose of the research supported by this grant is to evaluate the potential efficacy of this inhibitor, CEP-1347, as a therapeutic for ER⁺ breast cancer using preclinical animal models. In these models, human breast cancer cell lines (Aim 1) and patient derived xenografts (Aim 2) will be introduced into the mammary fat pad of immune compromised mice, and the effects of CEP-1347, alone or in combination with an antiestrogen, on tumor growth and metastasis will be evaluated. Aim 3 will identify mechanisms and pathways affected by CEP-1347 treatment in these animal models. CEP-1347 progressed through Phase II clinical trials as a potential therapeutic for Parkinson's disease. While it did not slow disease progression, no major toxicity was noted during more than 18 months of daily treatment. If CEP-1347 is effective in blocking ER⁺ tumor growth or metastasis in our pre-clinical models, it could rapidly progress to clinical trials for breast cancer treatment.

2. Keywords: ER-positive breast cancer, mixed lineage kinases, patient derived xenografts, kinase inhibitor, pre-clinical models.

3. Accomplishments:

- **Major Goals**

The major goals for the initial 12 month period of this grant were as follows:

Major Task 1:

Obtain ACURO Approval (Months 1-3), Accomplished.

Establish conditions for CEP-1347 treatment (Months 1-6), accomplished (see below).

Major Task 2: Evaluate the effects of CEP-1347 on MCF-7 and LCC9 Xenografts (Months 6-14). In progress (see below).

Major Task 3: Establish PDX derivatives expressing RFP and Luc2 (months 4-12). In progress (see below).

- **What was accomplished under these goals?**

Major Task 1: Establish Conditions for CEP-1347 treatment. Our first goals after obtaining ACURO approval for animal studies were to establish conditions for detecting CEP-1347 in the plasma and tissues of treated mice, and to establish the optimal method of administering CEP-1347 to mice in order to obtain therapeutic levels in the plasma. Progress on these goals is reported below.

1) Establishing conditions for detecting CEP-1347. We have established accurate and sensitive methods to detect CEP-1347 in mouse plasma and tissues. Various amounts of the compound were spiked into normal mouse plasma, then extracted with 4 parts Acetonitrile (ACN) to one part plasma. The resulting samples were analyzed by High Performance Liquid Chromatography (HPLC) and both targeted and untargeted Mass Spectrometry (MS). As shown in Figure 1, untargeted MS analysis detected plasma concentrations as low as 2 nM, while the lowest concentration detected by HPLC was 25 nM. Detection by targeted MS was intermediate between the two (not shown). Untargeted ESI-MS was therefore used as the detection method in subsequent experiments.

2) Establishing method for CEP-1347 administration. A pilot experiment was carried out in which MCF-7 cells containing both luciferase (Luc) and RFP genes were injected into the mammary fat pad of athymic nu/nu mice. Tumors were allowed to grow until they reached a size of approximately 100-200 mm³, at which point mice were treated daily with CEP-1347 or vehicle. The method of administration was oral gavage, 10 mg/kg CEP-1347 in Softigen/MYRJ, and was based on previous reports. Treatment of the remaining mice was continued for several weeks, after which they were sacrificed, and plasma and tumor tissue were collected. Surprisingly, MS analysis indicated that CEP-1347 levels were virtually undetectable in both short (2 day) and long term treated animals. The CEP-1347 used in these experiments was a gift of Teva Pharmaceuticals. It had been stored for several years, so we considered the possibility that it had degraded. CEP-1347 is now available commercially, so we purchased fresh compound from Tocris and compared it to the TEVA compound for chemical purity by MS and for efficacy *in vitro* by testing its ability to inhibit the growth of MCF-7 cells. The results of these experiments indicated that the TEVA compound was superior to the TOCRIS, demonstrating both greater purity by MS (not shown) and greater efficacy in cell based studies (Figure 2).

Having demonstrated that the compound we obtained from TEVA was chemically pure and effective *in vitro*, we proceeded to conduct studies to determine the optimum formulation, dose and method of administration. The methods of administration tested included oral gavage, subcutaneous injection and intravenous injection. Based on the results of these studies, we have established the following dosing schedule: 60 mg/kg CEP-1347 daily by oral gavage in Gelucire:PG (3:1). Using this method, we obtain therapeutic levels (>100 nM) in plasma. We are currently in the process of conducting a second pilot tumor growth experiment using these conditions.

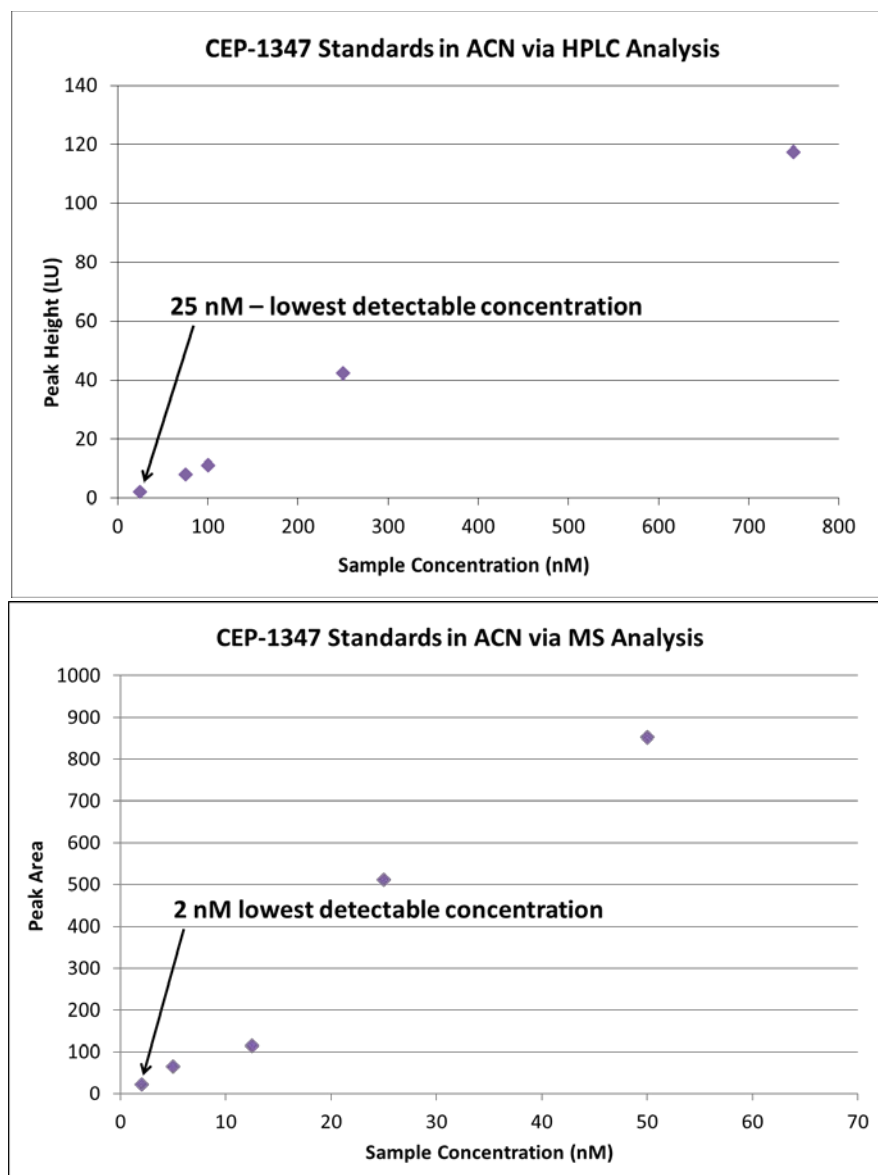


Figure 1: Analysis of CEP-1347 levels in plasma by HPLC and ESI-MS Analysis. Various amounts of CEP-1347 were added to normal mouse plasma. 20 μ l of plasma was added to 80 μ l of acetonitrile (ACN). Samples were vortexed, then spun at 14,000 rpm for 30 minutes in a microfuge. The ACN phase was collected and analyzed as follows. Top curve: HPLC using a XDB-C18 column with fluorescence detection. Bottom curve: Mass spectrometry on the Xevo G2-XS Qtof mass spectrometer using an untargeted method with ES ionization.

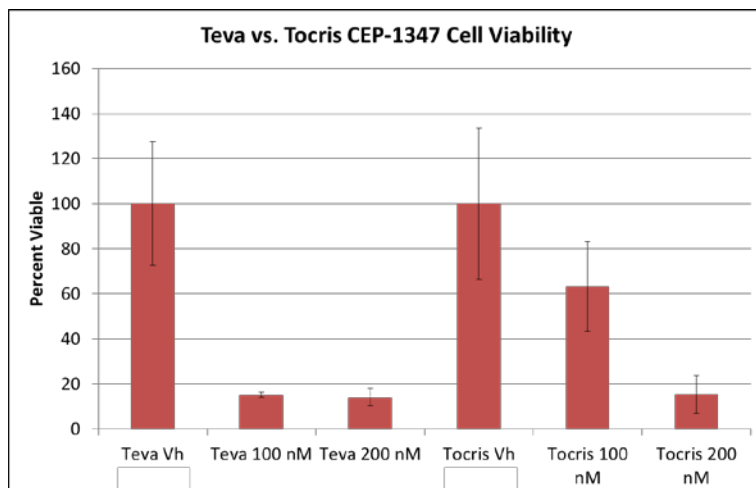
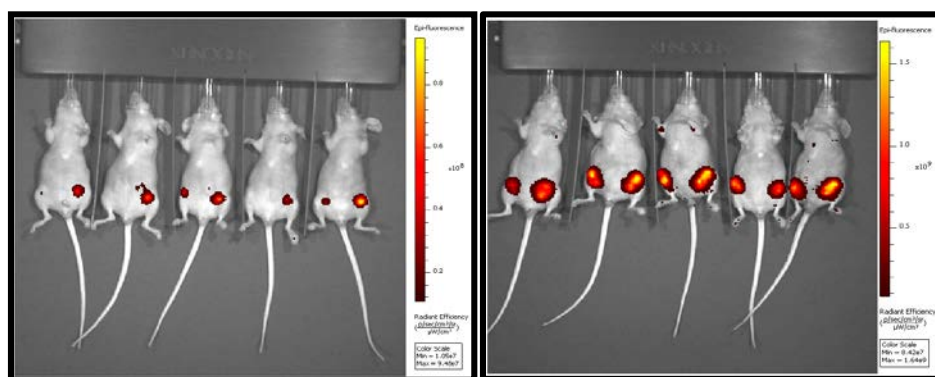


Figure 2: Comparison of Teva vs. Tocris CEP-1347 on cell viability. MCF-7 cells (1000 cells per well) were plated in triplicate in a 96 well dish. Treatments were added after 24 hr, and replenished after an additional 72 hours. On day 6 of treatment, CCK (Cell Counting Kit 8, Dojindo) reagent was added, and absorbance at 450 nm was read. For each compound, treatments were normalized to vehicle controls.

Task 2: Evaluate the effects of CEP-1347 on MCF-7 and LCC9 Xenografts. As described under Major Task 1 above, we did not obtain therapeutic plasma levels of CEP-1347 in our initial pilot experiment and, not surprisingly, did not see effects of CEP-1347 treatment on tumor growth (not shown). However, this experiment did provide us an opportunity to demonstrate and monitor MCF-7 cell tumor growth using caliper measurements, and RFP fluorescence (Figure 3) and luminescence (not shown) using the IVIS system. In addition, tumors removed from these animals at the time of sacrifice were fixed, sectioned, and analyzed for proliferation by Ki-67 staining (Figure 4), allowing us to establish these procedures in our laboratory.



DAY 4

DAY 47

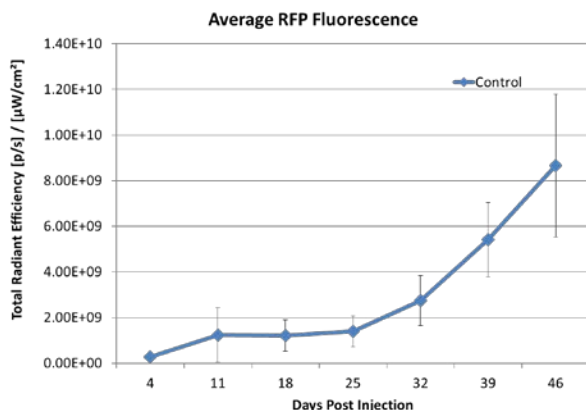


Figure 3: Growth of MCF-7 tumors in athymic nu/nu mice measured by RFP fluorescence. One million RFP-Luc MCF-7 cells were injected bilaterally into the 4th mammary fat pad of athymic nu/nu mice containing implanted estrogen pellets. Tumor growth was monitored by red fluorescence in an IVIS Spectrum (Caliper Life Sciences). Top: Representative images on Day 4 and Day 47 after cell injection. Bottom: Tumor growth curve obtained by weekly imaging. Similar growth curves were obtained by caliper measurement and luminescence (not shown).

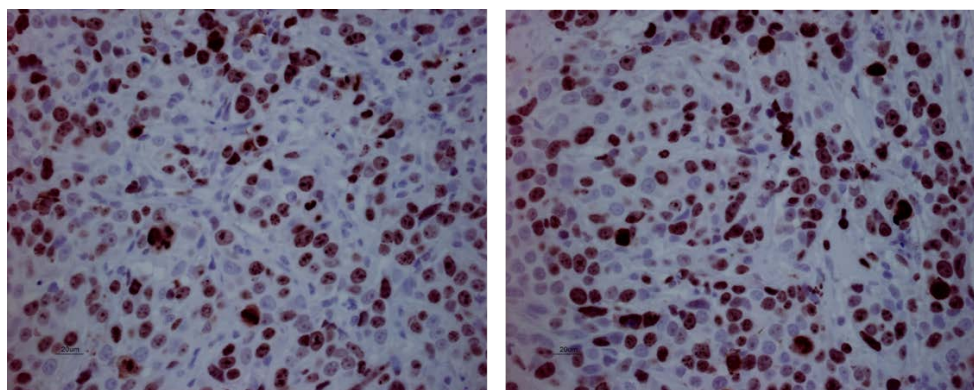


Figure 4: Assaying tumor cell proliferation by Ki67 staining. Tumors from estrogen treated mice were fixed in 10% formaldehyde, sectioned, and stained with an antibody to Ki67 as a marker of proliferation. Two representative sections are shown, demonstrating a high percentage of proliferating (brown) cells.

Major Task 3: Establish PDX derivatives expressing RFP and Luc2. As indicated in our original proposal, the final choice of PDX lines to investigate was made at the time of funding. After execution of a Material Transfer Agreement between Michigan State University and the University of Utah, we have obtained the following ER-positive PDX lines from Dr. Alana Welm at the Huntsman Cancer Institute at the University of Utah. The characteristics of each line are indicated below. All are ER⁺, PR⁺, HER2⁻. They represent both primary tumors and metastases, ductal and lobular carcinoma, and estrogen dependent and estrogen-independent tumors. All of these PDX lines form metastases in mouse xenografts.

HCl-003: Derived from primary Invasive Ductal Carcinoma (IDC). This line is estrogen dependent, and metastasizes to lung and lymph node.

HCl-011: Derived from a pleural effusion (metastatic) of an IDC. This line was derived from a patient who was treated with fulvestrant, so the metastatic tumor had developed endocrine resistance. The PDX line is estrogen independent but responsive, and metastasizes to lung and lymph node.

HCl-013: Derived from a pleural effusion of an Invasive Lobular Carcinoma. The patient from whom this line was derived was previously treated with letrozole, exemestane and tamoxifen, so the tumor had developed endocrine resistance. The xenograft is estrogen responsive.

HCl-013 EI: An estrogen independent derivative of HCl-013.

We have established procedures for implantation of tumor fragments into the cleared mammary fat pad of 4-5 week old NOD/SCID mice in our laboratory, and are currently in the process of amplifying the PDX lines described above for future transduction with RFP/Luc2 expressing lentivirus and/or tumor studies.

- **Opportunities for training and professional development.**

The MD/PhD student working on this project, Ms. Sonia Kumar, has been trained in animal handling and surgery by our collaborator Dr. Sandra O'Reilly. In addition, Ms. Kumar attended and presented her research at the Gordon Research Conference on Hormonal Cancers (see below).

- **Dissemination:** Nothing to Report

- **Plans for next reporting period.** We will carry out extensive *in vivo* experiments during the next funding period. To help facilitate this, a ½ time technician has been hired and begun to work on the project along with the graduate student Ms. Kumar. The specific experiments that will be conducted are outlined below.

- 1) Using our newly established treatment method, we will determine the effects of CEP-1347 treatment on MCF-7 and LCC9 tumor growth in athymic nu/nu mice.
- 2) MCF-7 and LCC9 tumors will be examined by immunostaining to determine the effects of treatment on tumor cell proliferation, invasion and signaling.
- 3) Organoids will be prepared from amplified PDX lines and infected with RFP/Luc expressing lentiviruses. RFP/Luc expressing sublines will be selected for treatment trials.
- 4) The RFP/Luc expressing PDX lines will be used to initiate experiments to examine the effects of CEP-1347 treatment on PDX tumor growth and metastasis. In the event that we are not successful at obtaining RFP/Luc expressing lines (since this has rarely been done), tumor studies will be conducted with the unlabeled PDX lines.

4. Impact: Nothing to report.

5. Changes/Problems:

- **Changes in approach.** The basic approach to these experiments has not been changed.
- **Problems or delays and plans to resolve them.** Large scale tumor studies with MCF-7 and LCC9 cells were delayed due to the need to establish optimal conditions for CEP-1347 detection, formulation and treatment. A second pilot experiment to test the effects of CEP-1347 on MCF-7 tumor growth is currently ongoing using our new formulation and dosing strategy. We anticipate carrying out the large scale experiments with MCF-7 and LCC9 cells during the next year.
- **Changes that had significant impact on expenditures.**
 - 1) One of the graduate students who initially intended to work on this project moved to another laboratory, and there was not another student available to take his place. We eventually hired a ½ time technician, Ms. Eva Miller, to assist with these studies, but

there was a gap before this occurred, which decreased expenditures during this funding period.

2) Large scale animal studies to test the effect of CEP-1347 on tumor growth were delayed due to the fact that therapeutic levels of CEP-1347 in plasma were not achieved using our initial formulation and dosing strategy. This resulted in decreased expenditures during this funding period, but the experiments will be conducted during the next period as described under “plans for next reporting period”.

- **Significant changes in vertebrate animal use.**

The overall design of our animal experiments to investigate the effects of CEP-1347 on tumor growth remains unchanged. However, upon review of the literature, we found that many groups remove an implanted estrogen pellet after tumors enter exponential growth, but prior to drug treatment. We therefore amended our AUF to allow us to remove the estrogen pellet from animals prior to drug treatments. This change was approved by the MSU institutional IACUC (approval date May 19, 2016) and has been reported to the agency.

6. Products:

- **Journal publications:** Nothing to Report
- **Books or other one time publications:** Nothing to report
- **Conference presentations:** Targeting Cellular Signaling Pathways in ER Positive Breast Cancer, Sonia Kumar, Kathleen Gallo and Susan E. Conrad. Poster Presentation, Gordon Research Conference on Hormone Dependent Cancers, August 15-21, 2015.
- **Websites, technologies, inventions, patent applications, other products:** Nothing to report.

7. Participants and other Collaborating Organizations.

Name:	<i>Susan Conrad</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>3</i>
Contribution to Project:	<i>Dr. Conrad provides overall direction for the project. She plans and oversees experiments, and reviews data.</i>
Funding Support:	
Name:	<i>Kathleen Gallo</i>

Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	1.5
Contribution to Project:	Dr. Gallo reviews data and participates in the planning of experiments.
Funding Support:	
Name:	Sonia Kumar
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	11
Contribution to Project:	Ms. Kumar has carried out most of the experiments on this project.
Funding Support:	n/a
Name:	Eva Miller
Project Role:	Lab Technician
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	1
Contribution to Project:	Ms. Miller assists Ms. Kumar with experiments.
Funding Support:	

- **Changes in Active/Other Support of PD/PI:**

S. Conrad (PI): Nothing to report.

K. Gallo (co-PI): R21 CA185163 (Gallo, co-I), Ended 3/31/16

- **Other Organizations:** Nothing to report.