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TITLE: Mechanisms of Action and Resistance to CDK4/6 Inhibitors in Breast Cancer

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14. ABSTRACT

CDK4/6 inhibitors combined with endocrine therapy (ET) are mainstay to treat metastatic estrogen receptor (ER) positive patients. Yet, almost 60% develop resistance to CDK4/6 inhibition within 2 years of initial treatment. An ongoing clinical challenge has thus been identifying biomarkers of response to predict patients that will either respond or not respond to palbociclib. Further, there is an unmet need to identify actionable targets for patients that have progressed on CDK4/6 blockade regimens. Currently, the only biomarker being used to identify patients for anti-CDK4/6 therapy is estrogen receptor (ER) by IHC. The goal of my study was thus to identify the therapeutic vulnerabilities of CDK4/6 inhibitor resistance cells and identify key markers that can longitudinally correlate with development of resistance. My data suggests that resistance to palbociclib results in a cascade of events initiating with induction of autophagy and senescence, leading to the promotion of senescence-associated secretory phenotype (SASP) that affects surrounding cells and promotes tumor growth.

15. SUBJECT TERMS

Breast cancer, estrogen receptor-positive, CDK4/6, cell cycle, drug resistance, autophagy, senescence, STAT3, IL-6, Beclin-1, p62, ULK1, patient-derived xenograft, disease progression, PARP inhibitor, TTI-101, SASP, chemokine, cytokine

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INTRODUCTION:

The proposed research aims to lead to a solution for the following overarching challenges: (1) Revolutionize treatment regimens by replacing them with ones that are more effective, less toxic, and impact survival. (2) Conquer the problems of over-diagnosis and overtreatment. (3) Eliminate the mortality associated with metastatic breast cancer. Various studies have tried to understand the mechanism of the CDK4/6 inhibitors, but no study to date examined the dose dependent effect of the inhibitor. This proposal will be elucidating the dose-dependent mechanisms of the CDK4/6 inhibitors and will identify key pathways to inform additional therapeutic strategies and improve personalization of therapy. Close examination of the mechanisms of palbociclib resistance suggests that resistance to palbociclib results in a cascade of events initiating with induction of autophagy and senescence, leading to the promotion of senescence-associated secretory phenotype (SASP) that affects surrounding cells and promotes tumor growth. Currently, the only biomarker being used to identify patients for anti-CDK4/6 therapy is estrogen receptor (ER) by IHC. The goal of my study was thus to identify the therapeutic vulnerabilities of CDK4/6 inhibitor resistance cells and identify key markers that can longitudinally correlate with development of resistance.

KEYWORDS: Breast cancer, estrogen receptor-positive, CDK4/6, cell cycle, drug resistance, autophagy, senescence, STAT3, IL-6, Beclin-1, p62, ULK1, patient-derived xenograft, disease progression, PARP inhibitor, TTI-101, SASP, chemokine, cytokine

ACCOMPLISHMENTS:

What were the major goals of the project & what was accomplished under these goals?

The following aims and tasks that have been addressed to reach the goals of the project:

Specific Aim 1: Identify the molecular mechanism by which CDK4/6 regulates autophagy pathway

✓ Major Task 1: Generate CDK4, CDK6, CDK4-KD, and CDK6-KD protein expression vectors

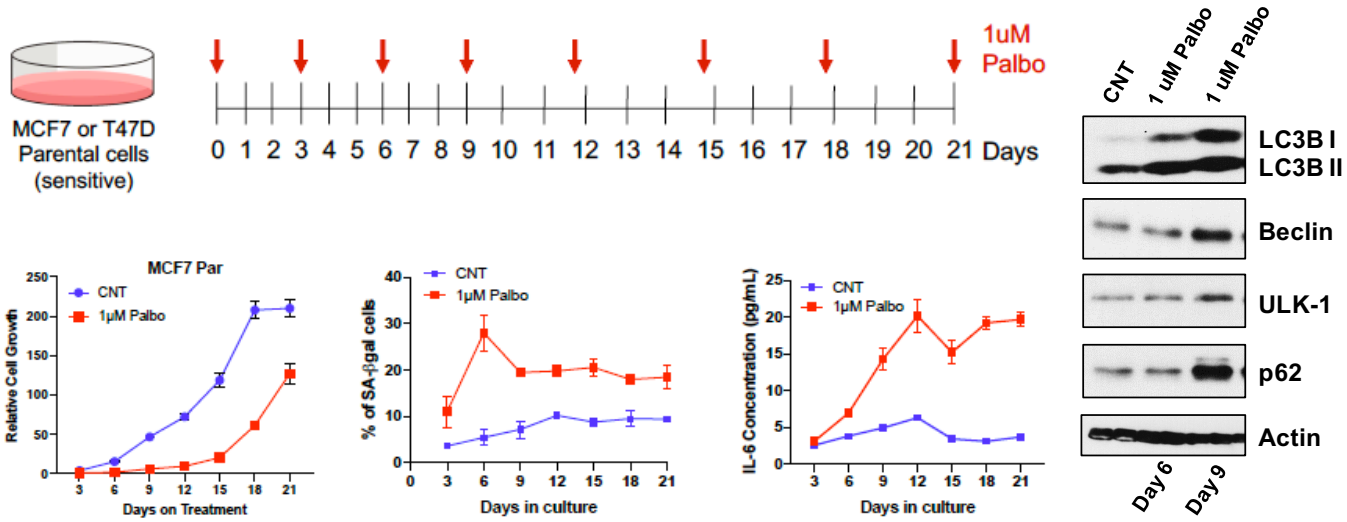
Complete as reported in year 1 and year 2 progress report.

✓ Major Task 2: Evaluate status of Beclin-1 activating and inhibitory complexes *in vitro*

The role of Beclin-1 in the mechanism of CDK4/6 inhibition is still under investigation. As reported in year 2 progress report there is a switch from autophagy and senescence when Beclin-1 is knocked down. Recent publications have shown that palbociclib (the first FDA-approved CDK4/6 inhibitor) has off-target effect of on ULK1 kinase activity. ULK induces the initiation of autophagy by activation of Beclin-1. The effect of palbociclib on ULK activity has not been investigated and I will elucidate the interaction of beclin-1, ulk1 and cdk4/6 in the next reporting period to complete this task.

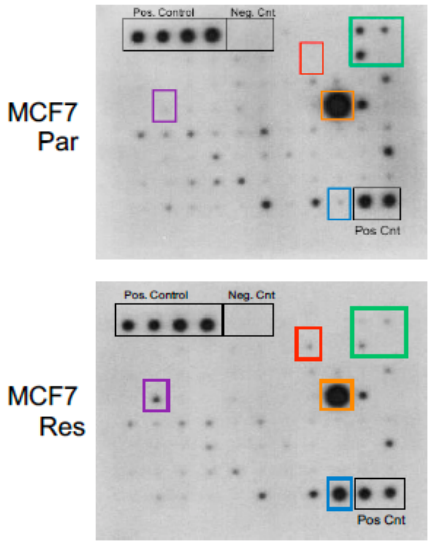
✓ Major Task 3: Identify phosphorylation events by CDK4/6 on autophagy proteins

Based on my findings reported in my last progress report, the effect of CDK4/6 on autophagy is most likely an indirect mechanism. Additionally publications have shown that there is a complex relationship between autophagy and senescence. These publications suggest that key protein invovled may be Beclin, ULK1, p62 and GATA4. I have proposed a time course (see figure below) to evaluate the changes in autophagy and senescence over time in repsonse to treatment with palbocilcib (palbo). Cells treated with palbo begin to overcome the treatment around day 18 (relative cell growth) and this is correlated with increased IL-6 levels suggesting increase SASP. Addditionally, there is a change in the levels of autophagy markers LC3B, Beclin, ULK1 and p62.



Due to the pandemic, I had to stop all in vitro experiments and it took multiple experiments to optimize the growing conditions of the cells for the experiment above. This time course experiment will be repeated during the next reporting period.

With the observation that IL-6 is significantly increased and is the most prominent SASP cytokine is interleukin-6 (IL-6), I investigated other interleukins, inflammatory cytokines, and growth factors, which comprise the senescence-associated secretory phenotype (SASP) that can affect surrounding cells and promote tumor growth. I performed a semi-quantitative cytokine array identifying 80 chemokines and cytomkines. Since chemokines and cytokines are sercreted proteins, I took the media from sensitive and resistant cells cultured for 6 days and incubated the media on a immunblot dotted with 80 cytokines (see figure below).



Semi-quantitative
Human 80 Cytokine Array

Human Cytokines	Fold Change in Signal Intensity (MCF7 Res vs Par)
TIMP-2	22.68
RANTES (CCL5)	7.84
IL-6	5.45
PDGF-BB	3.36
MDC (CCL22)	0.65
IL-4	0.52
M-CSF	0.25
OSM	0.18
VEGF-A	-0.19
TIMP-1	-0.25
MIP-1 beta (CCL4)	-0.25
IL-8 (CXCL8)	-0.26
IGFBP-2	-0.43
LIF	-0.49
OPN (SPP1)	-0.54
IGF-1	-0.55
BDNF	-0.58
TPO	-0.60
FGF-9	-0.60
TGF beta 2	-0.64
GRO alpha (CXCL1)	-0.74
IP-10 (CXCL10)	-0.86
IL-16	-0.87
GRO a/b/g	-0.92

I observed that there are global changes in cytokines (see figure above) that will be investigated further in the next reporting period. The identification of key cytokines as potential circulating markers indicating response will guide future analysis beyond this proposal as clinical biomarkers of response.

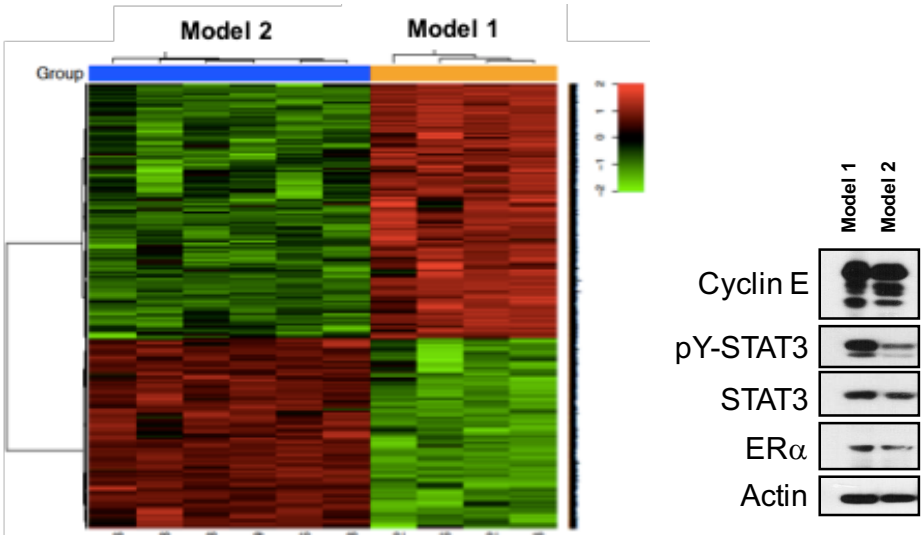
✓ Major Task 4: Evaluate the global changes in CDK4/6 phosphorylation upon palbociclib treatment in vitro

I plan to complete the analysis of the molecular mechanisms by which CDK4/6 regulates the autophagy and senescence pathway. This will allow additional biomarkers of sensitivity and resistance to CDK4/6 inhibition to be identified. There have been some technical issues due to low quality of immunoprecipitation (IP) experiments and troubleshooting of IP experiments is ongoing with new antibodies and will be reported in the final reporting period.

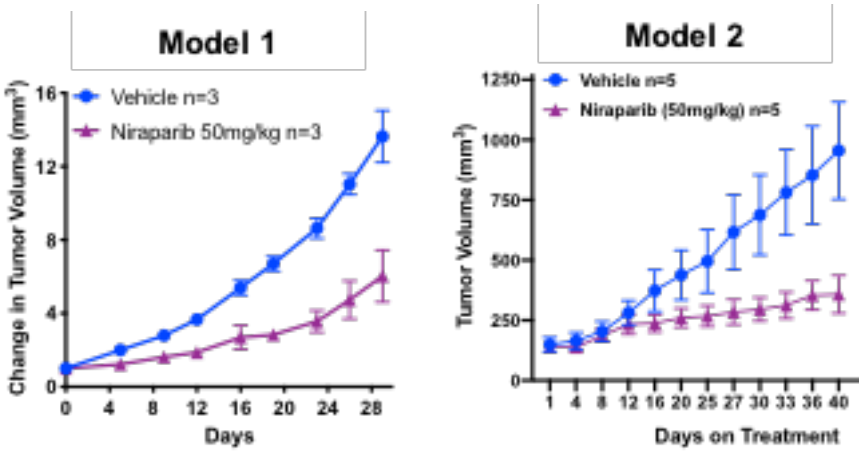
Specific Aim 2: Examine the mechanisms of resistance to palbociclib and identify treatment strategies to circumvent such resistance

✓ Major Task 6: Examine the effect of IL-6/STAT3 inhibitors in combination with drugs targeting DNA damage *in vitro* and *in vivo*

As mentioned in my last progress report, I have collaborated with Dr. Meric-Bernstam to obtain patient-derived xenografts (PDX) to test the treatment strategies as discussed as an alternative in the original proposal. I obtained 2 models: model 1 is a PDX from a patient who was on palbociclib + endocrine therapy for greater than 6 months prior to progression & model 2 is from a patient who was on therapy for less than 6 months prior to progression. I tested the effect of STAT3 inhibitor (TTI-101) in both models in the prior reporting period and model 1 showed a significant delay in tumor growth upon treatment with TTI-101, but not model 2. Due to the differences, I have performed RNA-seq just prior to the suspension of research in March 2020 and I was able to work on the bioinformatics during this reporting period to determine the difference in response between to the two models.



Additionally, I was able to maintain the mouse during the suspension of research. Once research resumed I was able to have the mouse models ready for testing PARP inhibition in model 1 and 2 as shown below.

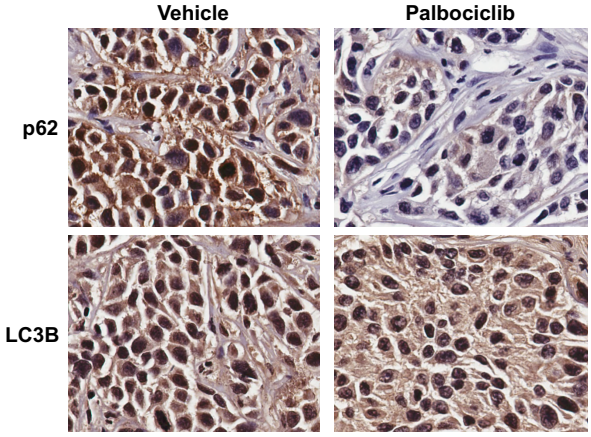


Both PDX models respond to PARP inhibition (niraparib). Using the RNA-seq results and additionally molecular analysis that will be carried the next reporting period, I will determine what biomarkers will determine how to select the appropriate therapy for individual patients. Additionally, I will be confirming the therapeutic benefit of STAT3 and PARP inhibition in 2 additional PDX models. Overall, I have identified two pathways which become altered in palbociclib resistant cells and can be potentially targeted clinically.

Specific Aim 3: Classify clinically applicable biomarkers to identify patients with intrinsic or acquired resistance to palbociclib.

✓ Major Task 7: Optimize antibody conditions of candidate biomarkers of response or resistance identified in Aim 1 & 2

I have begun testing potential biomarker candidates in autophagy, p62 and LC3B. I used PDX samples from previous reporting period that was treated with palbociclib to test the antibodies for p62 and LC3B. I have preliminary results (see figure below) but I need to further optimize the conditions.



Additionally, based on additional findings in aim 1 and 2 there could be better candidates that will be tested in the next reporting period.

✓ Major Task 8: Validate optimized antibody conditions in cohort of breast cancer patients

pY-STAT3 staining conditions are optimized, however ongoing efforts in collaboration with Dr. Sahin are being conducted to optimize the scoring of the stain. This will determine the percent of pY-STAT3 that will determine selection for treatment with STAT3 inhibitor. Once additional antibodies are validated, Dr. Sahin will assist with the pathological scoring.

✓ Major Task 9: Obtain breast cancer patients samples whose disease has progressed after palbociclib treatment

This task was delayed due to the suspension of collection of archival clinical samples for research due to the pandemic. At the end of this reporting period I have been allowed to request these slides and with the extension of my funding period, I will be able to validate the current identified biomarkers as well as identify/validate additional biomarkers as the experiments outlined above are completed. Overall, by the validation of these biomarkers we can make a case to bring the combination therapies we have identified to a clinical trial proposal.

Overall accomplishment summary

My main accomplishment for this reporting period is the results reported in Aim 2 indicating that PARP inhibition is safely tolerated and efficacious at the dose tested and PARP inhibition may be a suitable target for those tumors that are resistant to palbociclib that do not have overexpression of pSTAT3. Additionally, comparing the two tumor models in Aim 2 has identifying distinct therapeutic vulnerabilities of palbociclib resistance based on molecular analysis. The distinct therapeutic vulnerabilities will be further validated by biomarkers evaluated in Aim 3 in the next reporting period and these findings will allow for personalized medicine for patients.

What opportunities for training and professional development has the project provided?

Date of Training or Professional Development	Event	Description
04/23/2021	Committee meeting	Presentation updating research progress to my collaborators
04/09/2021	Frontiers Oncology Reviewer	Provided detailed feedback for manuscript under consideration for publication
02/10/2021	Houston Livestock Show & Rodeo Youth Agricultural Science Fair	Served as official judge for Senior division prelims
02/02/2021	Bench to bedside brainstorm meeting	Presentation to clinical collaborators updating preclinical research progress
01/12/2021	MD Anderson Cancer Canter Dept. of Experimental Radiation Oncology - SPDR Seminar Series	Oral presentation on "IL-6 promotes endocrine therapy and palbociclib resistance in estrogen receptor positive breast cancer cells"
08/13/2020	CPRIT CURE Summer Undergraduate Program - Virtual	Lead a small group of 10 undergraduate students in journal club discussion
06/15/2020 - 08/21/2020	CPRIT CURE Summer Undergraduate Program - Virtual	Virtually mentored and trained a CPRIT CURE Summer undergraduate student
06/08/2020	CPRIT CURE Summer Undergraduate Program - Virtual	Gave 20-minute lecture on circadian clocks in cancer biology to all 70 undergraduate students in the program

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Over the next reporting period, I will be the finalizing the research project. I will elucidate the interaction of beclin-1, ulk1 and cdk4/6 to complete the mechanisms of action of autophagy by CDK4/6 inhibition. I will validate the switch of autophagy to senescence by CDK4/6 inhibition. The ongoing IP experiments with new antibodies will be completed and determine if regulation of autophagy and senescence is a direct or indirect

mechanism of CDK4/6. I will be confirming the therapeutic benefit of STAT3 and PARP inhibition in 2 additional PDX models and complete the application of the biomarkers to the retrospective patient samples. The completion of the validation of identified biomarkers in addition to the further validating the therapeutic benefits of the proposed targeted therapies will provide guidance to my collaborators for use in clinical trials.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The precise biological mechanism(s) of the action or resistance to CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) are still unknown and there are no independent biomarkers to predict response and/or resistance to these inhibitors. Patients who experience resistance to this class of agents, are likely to have a less favorable biology (including resistance pathways), requiring novel combination strategies to delay progression and improve survival. Over this past year I confirmed the therapeutic benefit of PARP inhibition in preclinical mouse models of palbociclib resistance. This will provide rationale to propose novel clinical trials to my collaborators in Breast Medical Oncology and Investigational Cancer Therapeutics at MD Anderson Cancer Center leading to better outcomes for our patients. Additionally, I have identified some key mediators (or drivers) of palbociclib resistance that could be used as biomarkers to predict the development of resistance and allow earlier intervention of next-line therapy improving overall clinical outcomes.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS:

Due to the impact of COVID-19 pandemic, there was suspension of research from March 2020 - September 2020. I was allowed limited access during the 6-month period to only maintain some of my in vivo studies. All *in vivo* and *in vitro* experimental work were suspended and this caused a delay in completing the proposed aims of my study. I have outlined the delays and the remaining research tasks that need to be completed and above in the accomplishments section. With the approval of my NCE request, I will be able to finalize this research project no later than April 14th, 2022.

PRODUCTS:

Journal publications

Nothing to Report

Books or other non-periodical, one-time publications

Nothing to Report

Other publications, conference papers, and presentations

Please refer to table on page #10 which lists all presentations made during the last year.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	Nicole M. Kettner
Project Role:	PI
Researcher Identifier:	ORCID: 0000-0003-2043-4407
Nearest person month worked:	12
Contribution to Project:	Dr. Kettner has performed all the work that has been performed this reporting period
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: Nothing to Report

QUAD CHARTS**:** Nothing to Report

APPENDICES:

Nothing to Report