

AWARD NUMBER: W81XWH-18-1-0561

TITLE: NOXA loss as a major mechanism of intrinsic resistance to targeted therapies in breast cancer

PRINCIPAL INVESTIGATOR: Anthony Faber PH.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University and Memorial Sloan
Kettering Cancer Center

REPORT DATE: OCTOBER 2020

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE OCTOBER 2020		2. REPORT TYPE Annual		3. DATES COVERED 30SEPT2019 - 29SEPT2020	
4. TITLE AND SUBTITLE NOXA loss as a major mechanism of intrinsic resistance to targeted therapies in breast cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-18-1-0561	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Anthony Faber, Maurizio Scaltriti Email: acfaber@vcu.edu; scaltrim@mskcc.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Site 1: Virginia Commonwealth University, 907 Floyd Avenue, Richmond, VA 23284 Site 2: Memorial Sloan Kettering Cancer Center 1275 York Avenue New York, NY				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We continue to make significant and timely progress for this latest period. Our grant hypothesis was that MCL-1 inhibition can sensitize HER2 inhibitors in HER2 amplified breast cancer and ER inhibitors in ER+ breast cancer. Highlights of the past 12 months include data supporting the use of the novel HER2 inhibitor, tucatinib, in combination with dinaciclib both in vitro and in mouse models of HER2 amplified breast cancer. We have uncovered a feedback activation of PI3K and MEK signaling from dinaciclib, which is abrogated by HER2 inhibitors in HER2 amplified breast cancer, adding a second layer of rationale to use these two classes of drugs together. In addition, we have found expression of other anti-apoptotic BCL-2 members other than MCL-1 do not impact the sensitivity of HER2 inhibitors and MCL-1 inhibitors, consistent with a dominant role of the NOXA-MCL-1 axis in sensitivity of HER2 inhibitors in HER2 amplified breast cancer. In ER+ breast cancer, we have characterized the BCL-2 protein complex changes that underline sensitivity of ER inhibitors plus MCL-1 inhibition through a series of immunoprecipitation experiments. For staining of breast cancer samples, we have rigorously validated a NOXA antibody that we will move forward with.					
15. SUBJECT TERMS MCL1, targeted therapy, apoptosis, resistance, NOXA					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 11	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	p.4
2. Keywords	p.4
3. Accomplishments	p.4
4. Impact	p.8
5. Changes/Problems	p.8
6. Products	p.8
7. Participants & Other Collaborating Organizations	p.8
8. Special Reporting Requirements	N/A

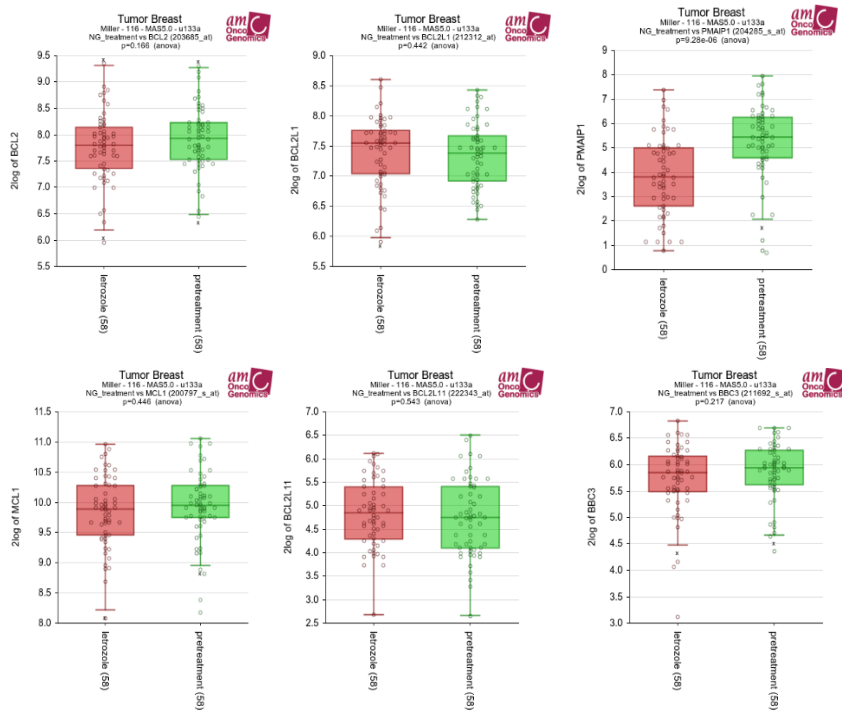


Figure 1. Changes in BCL-2 members over 2 weeks from Letrozole treatment. Of note, no significant changes except NOXA is markedly lower in the post-letrozole treatments.

MCL-1 BH3 mimetics or CDK inhibitors that block MCL-1 transcription-- can induce cell death.

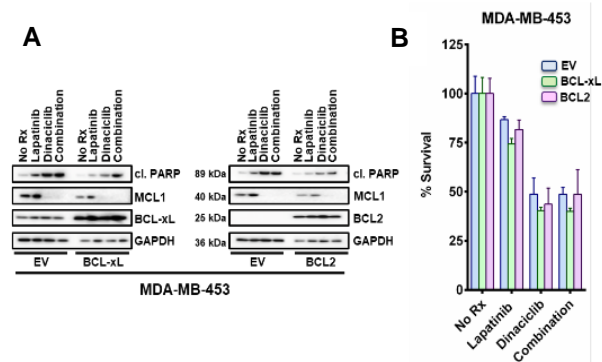


Figure 2. Dinaciclib functions mainly by inhibiting MCL-1. (A) MDA-MB-453 control or BCL2/BCL-XL expressing cells were treated with lapatinib, dinaciclib and their combination. Whole cell lysates were prepared, subjected to western blotting and probed for the indicated proteins. (B) MDA-MB-453 control or BCL2/BCL-XL - expressing cells were treated with lapatinib, dinaciclib and their combination and subjected to CellTiter-Glo.

1. Introduction

HER2-amplified breast cancers and Estrogen receptor (ER) positive breast cancers are susceptible to *HER2* inhibitors and ER inhibitors, respectively. However, treatment with these targeted agents elicit transient responses, and ways to sensitize these cancers further with the addition of rationally implemented targeted therapies continues to be the subject of intense research. In this grant, we have posited that low expression of the endogenous MCL-1 inhibitor, NOXA, in *HER2*-amplified breast cancers causes 1) resistance to *HER2* inhibitors through MCL-1 activity and 2) susceptible to combination therapy with MCL-1 inhibitors. The mechanism is through suppression of ER-mediated loss of NOXA transcription, which is mediated by miRNA4728, a coamplified gene with *HER2* in these cancers. In addition, by way of a overlapping mechanism, in ER+ breast cancers, treatment with ER inhibitors leads to loss of NOXA transcription. In both cases, addition of MCL-1 inhibitors –either

2. Keywords: *MCL1*, targeted therapy, apoptosis, resistance, NOXA

3. Accomplishments

Major Task 1

Characterize the miRNA4728/ER/NOXA axis in *HER2*-amplified breast cancers and its role in intrinsic resistance to *HER2i*

Subtask 1:

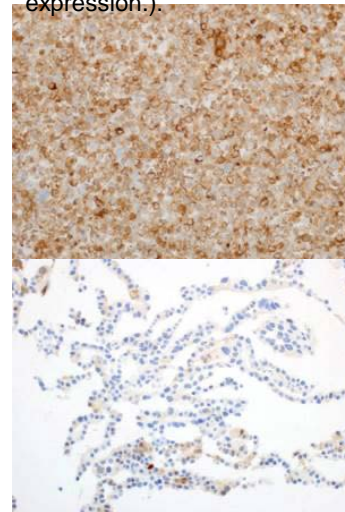
Here, we extended our data analyses to a dataset of ER+ breast cancer patients on the ER inhibitor, Letrozole. Pretreatment and Letrozole treatment tumors were assessed over 2 weeks in a publically available database. Interestingly, we found that the NOXA mRNA levels were

markedly suppressed following 2 weeks of letrozole treatment compared to the mRNA levels of the other BCL2 family members (BBC3: PUMA, BCL2L11: BIM, BCL2L1: BCL-XL) (Figure 1). This result of other BCL-2 members not changing supports our previous observation of low expression of NOXA in *HER2* amplified breast cancer as the important alteration following ER inhibition, and is consistent with that of our hypothesis.

Subtask 2: In collaboration with Dr. Edi Brogi, (Director of Breast Pathology, MSKCC), and Dr. Mikhail Dozmorov (Department of Biostatistics, VCU), we will evaluate 180 samples of clinically annotated *HER2*+ breast cancer specimens collected at MSKCC for *HER2* levels, NOXA levels, and MCL-1 levels by immunohistochemistry.

Immunohistochemical Detection of NOXA/PMAIP1

Image 1. Validation of NOXA AB 114C307. (Top) staining of cell line H1048 (high NOXA expression, 20x). (below) staining of cell line EFM-192A (low NOXA expression.).



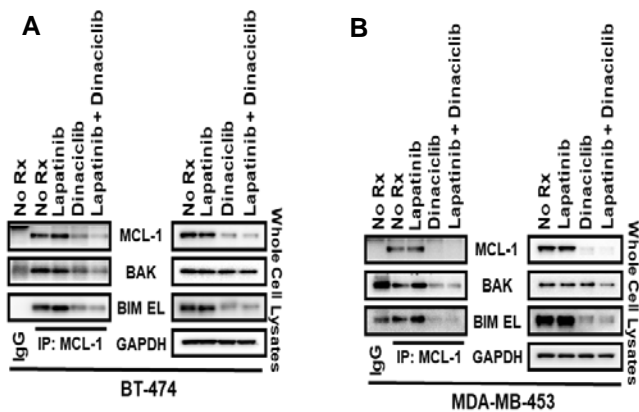


Figure 3. Dinaciclib synergizes with lapatinib to displace BIM and BAK from MCL-1. (A) and (B) MCL-1 complexes were immunoprecipitated from the indicated *HER2*-amplified breast cancer cell lines following overnight treatment with no drug, 1 μ M lapatinib, 100 nM dinaciclib, and their combination. An IgG-matched isotype antibody was served as an immunoprecipitation control. The interaction between MCL-1 and BIM EL/BAK proteins was investigated (“No Rx”: No drug).

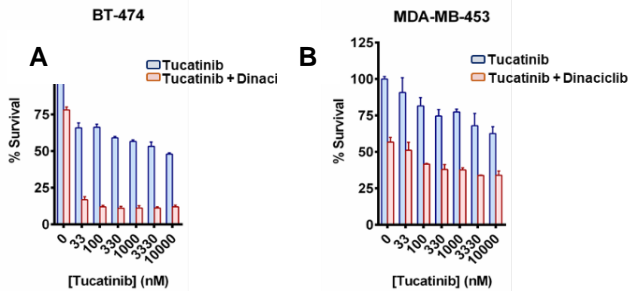


Figure 4. Dinaciclib sensitizes *HER2*-amplified breast cancer to tucatinib. BT-474 and MDA-MB-453 cells were treated with increasing concentrations of tucatinib and 100 nM dinaciclib for 24 and 72 h respectively, and the percentage of viable cells was determined.

In order to establish a rigorous assay for NOXA expression, Dr. Achim Jungbluth and Dr. Edi Brogi comprehensively evaluated NOXA/PMAIP1 commercial reagents. Due to the inherent inconsistency of polyclonal reagents, the search for suitable reagents was focused on monoclonal antibodies. All immunohistochemical stains were performed on a Leica Bond-3 (Leica, Buffalo Grove, IL) automated stainer platform and on formalin-fixed paraffin embedded tissues. Initial testing of clones was done on a panel of ten normal tissues. Optimization assays comprised of modifying titration steps as well as heat-based antigen retrieval steps using low pH citrate (ER1, Leica) and hipH TRIS buffer (ER2, Leica). A polymer based secondary kit (Refine, Leica) was used to detect the primary.

In the initial testing, clone D8L7U (#147665; Cell Signaling Technology, Danvers, MA) did not reveal any staining compatible with presence of NOXA/PMAIP1 (data not shown). Clone 114C307 (ab13654; Abcam, Cambridge, MA) showed staining in normal tissues compatible with expression of NOXA/PMAIP1. For example, strong staining was seen in tubules of the kidney cortex while testicular germ cells remained negative (data not shown). Specificity was further analyzed in formalin-fixed paraffin embedded pellets of various cell lines. NOXA/PMAIP1 mRNA levels were tested by rt-PCR or compared to publicly available databases of the Broad Institute (<https://portals.broadinstitute.org/ccl>). Sample staining are shown in Image 1. We validated these findings in human neoplasms as well as known controls and the data are consistent with a robust assay that can score for NOXA expression with specificity and potential for quantitation.

Human Breast Cancer Sample Testing

We have now obtained HPRO approval and MSK IRB

approval for our plans for testing breast cancers using this assay. We have further searched among an initial cohort of over 600 samples and unfortunately most of these were exhausted. We then searched for additional samples under our protocol and identified a cohort of over 400 samples and verified adequate remaining tumor in the majority of these. We have now begun cutting unstained slides of this and anticipate results in 4-6 months.

Subtask 3: In Dr. Scaltriti's and Dr. Faber's laboratory, we will determine the role of NOXA/MCL-1 in a panel of *HER2* amplified breast cancer intrinsic resistant models to diverse *HER2* inhibitors

As we observed in our previous *in vitro* results that dinaciclib sensitizes *HER2*-amplified breast cancer cells to *HER2* inhibitor lapatinib resulting in increased cell death and expression of the cell death marker, cleaved PARP, we performed further *in vitro* and *in vivo* experiments to characterize the role of the BCL2 proteins, how they provide intrinsic resistance to *HER2* inhibition, and the interaction with each other in sensitizing *HER2*+ breast cancer cells to the lapatinib and dinaciclib combination.

First, the selectivity of dinaciclib in inhibiting MCL-1 was tested once again, by treating BCL-xL or BCL2 overexpressing MDA-MB-453 cells with dinaciclib, lapatinib and their combination. In the BCL2 and BCL-xL overexpressing genetically engineered cells there was no protection from cell death markers (Fig. 2A) as well as in the cell viability assessed by cell titer glo (Fig. 2B), demonstrating that no other anti-apoptotic BCL2 proteins except for MCL-1 are involved in dinaciclib induced cell death, and in line with the hypothesis that dinaciclib is an effective and specific MCL-1 inhibitor that can be paired with *HER2* inhibitors rationally.

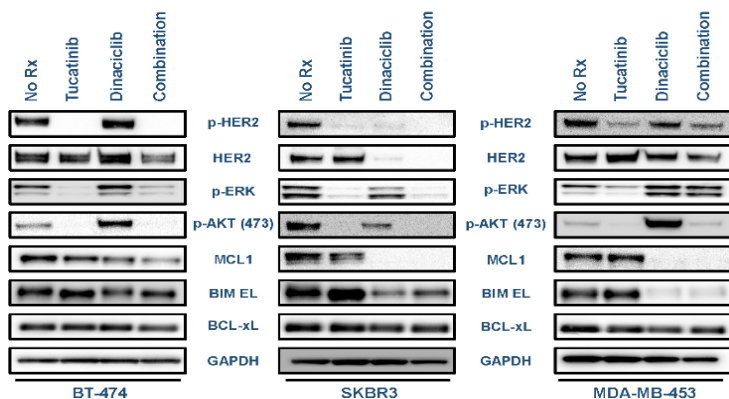


Figure 5. Tucatinib disrupts oncogenic signaling downstream of HER2. BT-474, SKBR3 and MDA-MB-453 cells were treated with no drug, lapatinib, dinaciclib and their combination. Whole cell lysates were prepared, subjected to western blotting, and probed for the indicated proteins.

To further understand the mechanism by which dinaciclib sensitizes HER2+ breast cancer cells to lapatinib, we immunoprecipitated MCL-1 in two HER2 amplified breast cancer cell lines, BT474 and MDA-MB-453. We observed that dinaciclib functions mainly by liberating the pro-apoptotic BCL2 effector molecule BAK from the pre-existing inhibitory complex with MCL-1 (Fig. 3). In addition, we assayed the efficacy of a selective HER2 inhibitor, tucatinib, in combination with CDK/MCL-1 inhibitor dinaciclib. Tucatinib is an FDA-approved, orally bioavailable small molecule that provides exceptional selectivity for the HER2 kinase compared to the related receptor tyrosine kinase EGFR. As it has quickly advanced with clinical activity in HER2+ advanced breast cancers (NCT02614794), we decided to focus on this HER2 inhibitor over the antibodies. To evaluate the sensitivity of the drug in combination

with dinaciclib, we performed cell viability assays in two *HER2*-amplified breast cancer cell lines, BT474 and MDA-MB-453. Both cell lines displayed remarkable toxicity after co-treatment with the two drugs, further validating our novel drug combination of HER2 inhibitors and MCL-1 inhibitors (Fig. 4). We further treated three HER2 positive breast cancer cell lines, BT-474, SKBR3 and MDA-MB-453 with tucatinib, dinaciclib and their combination to investigate whether the oncogenic signaling downstream of HER2 was blocked (Fig. 5). While dinaciclib enhanced the PI3K and MEK/ERK signaling, as evidenced by elevated p-AKT and p-ERK, tucatinib abrogated both feedback activations (Fig. 5). In order to gain further mechanistic insights, we immunoprecipitated MCL-1 in BT-474 and MDA-MB-453 cells and observed that dinaciclib functions mainly by liberating the pro-apoptotic BCL2 molecules BIM and BAK from the preexisting inhibitory complexes with MCL-1 (Fig. 6). These data provide evidence that tucatinib and dinaciclib is a viable combination therapy that is both clinically relevant and combines rationally by converging on MCL-1 and preventing feedback activation of PI3K and MEK pathways demonstrated by dinaciclib.

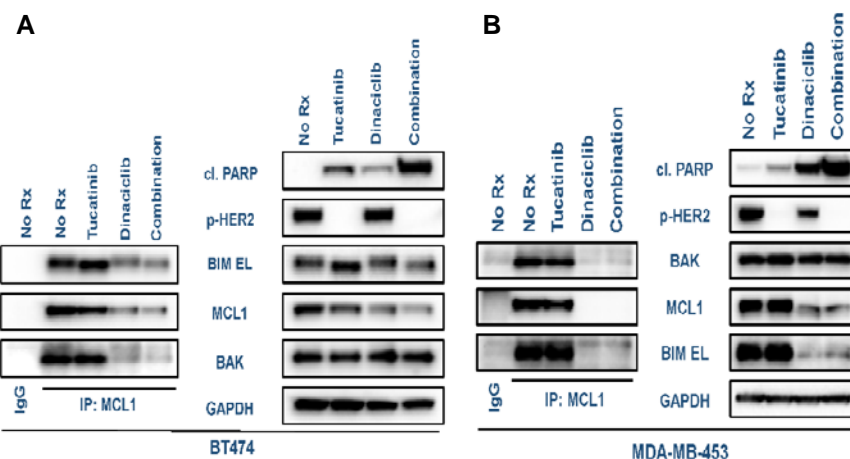


Figure 6. Dinaciclib synergizes with tucatinib to displace BIM and BAK from MCL-1. MCL-1 complexes were immunoprecipitated from the indicated *HER2*-amplified breast cancer cell lines following treatment with no drug, tucatinib, dinaciclib and their combination.

Major Task 2: Characterize the role of the ER-NOXA axis in response to anti-estrogens in ER+ positive breast cancer in vitro and in clinical specimens.

Subtask 1: In the Faber and Scaltriti laboratory, we will determine how the MCL-1 inhibitor S63845 is sensitizing to ER inhibitors

As we reported previously, the combination of ER inhibitor fulvestrant and MCL-1 inhibitor S63845 resulted in enhanced apoptotic cell death in various ER+ breast cancer cells (last year's report). In the ER+ breast cancer cell lines HCC1500 and T47D, we have looked further at these BCL-2 member complexes to determine the key changes that underline this sensitivity to ER inhibitors in combination with MCL-1 inhibitors. Indeed, we find consistent from the data from last report and the data from Fig. 1. above in the tumors, fulvestrant increases the amount of MCL-1 bound to BIM; S63845 abolishes this complex to induce cell death (Fig. 7).

Subtask 2: In collaboration with Drs. Edi Brogi, (Director of Breast Pathology, MSKCC), and Dr. Mikhail Dozmorov (Department of Biostatistics, VCU), we will analyze ~400 ER+ breast cancer samples and 180 HER2+ samples and their relationship to NOXA, MCL-1 and patient outcomes.

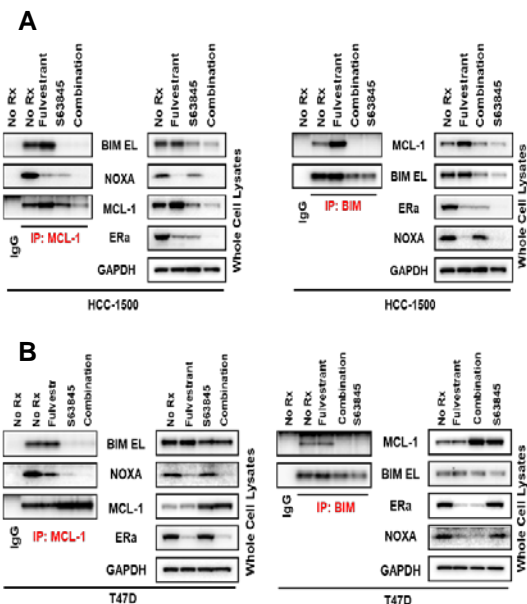


Figure 7. MCL1 inhibitor S63845 disrupts MCL1: BIM complexes. Immunoblots indicate MCL1 and BIM IP performed in (A) HCC-1500 and (B) T47D cells treated with no drug, Fulvestrant, S63845, and their combination.

As noted above, we have validated these samples and are in the process of cutting the slides for evaluation.

Subtask 3: In the Faber and Scaltriti laboratory, we will determine if microRNA 4728 is responsible for NOXA downregulation via estrogen receptor downregulation. These experiments will include miRNA quantification, miRNA silencing experiments, and miRNA overexpression experiments.

In the previous report, we have completed this subtask.

Major Task3: Assess the efficacy of dual HER2 and MCL-1 inhibition in diverse HER2 amplified breast PDX models and dual ER and MCL-1 inhibition in diverse ER+ breast PDX models.

We tested the efficacy of dinaciclib and lapatinib *in vivo*. Briefly, we injected HER2-amplified breast cancer cells BT474 in the fat mammary pads of NSG mice. We found that dinaciclib sensitized BT-474 xenografts to the HER2 inhibitor lapatinib when dosed twice a week (Fig. 8A). Mice remained healthy, based on their weight profiles (Fig. 8B). On-target inhibition of CDK9 was shown by suppression of the carboxyl-terminal domain (CTD) of RNA polymerase II and MCL-1 (Fig. 8C).

Additionally, in experimental data that was presented in part in last year's report, we have found stellar combination efficacy of the ER inhibitors fulvestrant and S63845 *in vivo*. These data are graphed per individual tumor in Fig. 9.

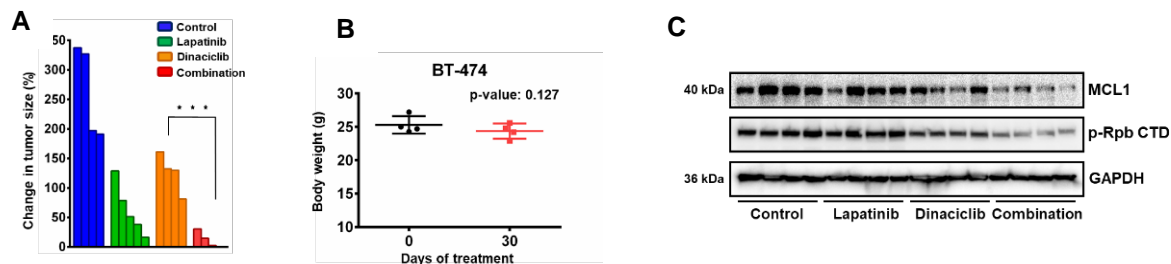
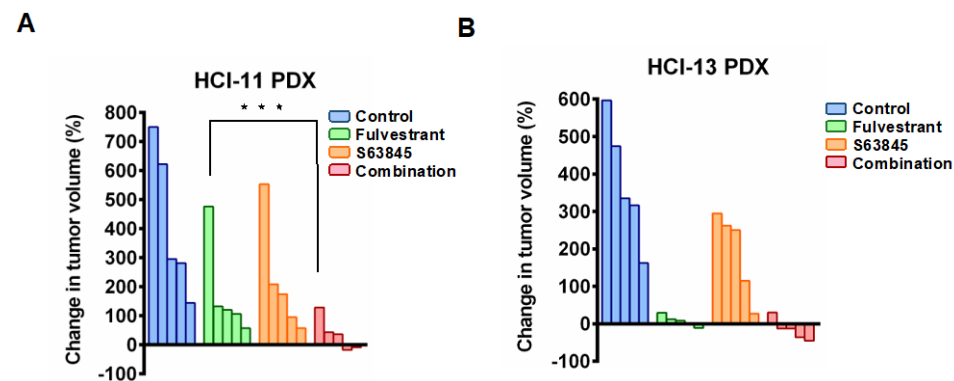


Figure 8. Combination treatment with lapatinib and dinaciclib leads to anti-tumor activity *in vivo*. (A) Approximately 15×10^6 BT-474 cells were injected orthotopically into each NSG mouse (both sides) and monitored for subsequent growth. When tumors were ~ 200 mm³, mice were randomized into treatment cohorts: control (no drug), 100 mg/kg lapatinib, 40 mg/kg dinaciclib and their combination. Dinaciclib was administered twice a week via IP injection. Lapatinib was given orally once a day for 5 consecutive days. Tumor measurements were performed daily, and the percentage (%) of changes in volume for each tumor is shown by a waterfall plot (control = 4 tumors, lapatinib = 5 tumors, dinaciclib = 4 tumors, combination = 4 tumors). (B) Weights of the combination cohort of the human xenograft-bearing mice of the combination cohort. P-value was calculated using the t test. (C) Tumors were harvested from BT-474 tumor-bearing mice approximately 2h after drugs administration and tumor lysates were subjected to western blot analyses and probed for the indicated proteins.

4. Impact

In all, our data demonstrate the *in vitro* and *in vivo* efficacy of both ER and HER2 inhibitors in combination with MCL-1 inhibitors in ER+ breast cancer and *HER2*-amplified breast cancer models respectively. We



demonstrated that MCL-1 inhibitor S63845 and CDK/MCL1 inhibitor dinaciclib sensitizes the cells to ER or HER2 inhibitors by disrupting the BAK:MCL-1 and/or BIM: MCL-1 complexes. Disruption of the MCL-1 complex releases BAK and/or BIM to then promote outer mitochondrial membrane permeabilization and cell death. In addition, we also demonstrated the *in vitro* efficacy of small molecule FDA-approved highly selective HER2 inhibitor tucatinib in combination with the CDK/MCL-1 inhibitor dinaciclib in *HER2*-amplified breast cancer cells.

Figure 9. Fulvestrant and S63845 combine to shrink ER+ breast cancer PDX tumors. Two models treated Change in tumor volume of ER breast cancer PDX+, divided into four group: treatment with vehicles, fulvestrant, S63845, and the combination. Fulvestrant was administered subcutaneously at a concentration of 5 mg/body/wk, and S63845 was administered intra venously biweekly at a concentration of 25mg/kg. Note tumor shrinkage with the combination of some of the tumors in both models.

Importantly, much of this work demonstrating MCL-1

blockade using the pan-CDK inhibitor dinaciclib with HER2 inhibitors like tucatinib or lapatinib to overcome therapy resistance in HER2-amplified breast cancer was submitted in a manuscript at Cell Death and Disease, was sent out for peer-review, and was well-reviewed. The manuscript is now in revisions and we anticipate re-submitting the revised manuscript within one month. The title of the manuscript is: Targeting transcription of MCL-1 overcomes NOXA-deficiency to sensitize HER2 amplified breast cancers to HER2 inhibitors.

Additionally, we have a second manuscript focusing on the ER inhibitor/MCL-1 inhibitor combination data that is written, and is awaiting finalization. The title of this manuscript is **Adaptive resistance to ER inhibition is overcome by targeting MCL-1.**

5. Changes/Problems

This year our work focused on understanding the mechanism involved in sensitizing *HER2*-amplified and ER+ breast cancers to HER2 and ER inhibitors when treated in combination with MCL-1 inhibitor S63845 or CDK/MCL-1 inhibitor dinaciclib. We have moved to tucatinib (Seattle Genetics) from some of the original other HER2 inhibitors (particularly the antibodies) since this drug has emerged as very clinically relevant, as in fact now FDA-approved for use in combination in HER2 amplified breast cancer. We will continue to develop these therapies to benefit patients resistant to HER2 and ER inhibitors. We are still working to obtain the specified number of ER+ breast cancer patient's specimens, which will be stained with NOXA and MCL-1 antibodies.

6. Products

N/A

7. Participants & Other Collaborating Organizations

The SOW has been faithfully followed for the contributions of VCU and MSKCC

The following individuals have worked on the grant at VCU:

- 1) Name: Anthony Faber
Project Role: Lead PI
Nearest person month worked: 1
Dr. Faber oversees the everyday experimentation in the laboratory related to the proposal

- 2) Name: Sheeba Jacobs
Project role: Postdoctoral Fellow
Nearest person month worked: 9
Dr. Jacobs participates in all aims at VCU as a scientist in the laboratory
- 3) Jennifer Ramachandran (Koblinski)
Project role: co-I
Nearest person month worked: 1
Dr. Ramachandran assists in all mouse-related work and pathology at VCU
- 4) Mikhail Dozmorov
Project role: co-I
Nearest person month worked: 1
Dr. Dozmorov assists in all statistical matters for this grant
- 5) Colin Coon
Project role: research technician
Nearest person month worked: 1
Dr. Coon assists Dr. Jacobs with the laboratory-related work from this grant

The following individuals have worked on the grant at MSKCC:

- 1) Maurizio Scaltriti
Project Role: Partnering PI
Nearest person month worked: 1
Dr. Scaltriti oversees all day-to-day experimentation in the laboratory related to this proposal and oversees the design and data analysis.
Funding Support: This award
- 2) Sarat Chandarlapaty
Project Role: Co-Investigator
Nearest person month worked: 1
Dr. Chandarlapaty directs the efforts in his laboratory on developing model systems, collects human samples from breast cancer patients treated at MSKCC and assesses the benefit of different therapeutic strategies.
Funding Support: This award
- 3) Edi Brogi
Project Role: Co-Investigator
Nearest person month worked: 1
Dr. Brogi analyzes tissue samples from breast cancer patients undergoing treatment and evaluates the purity of cancer tissues and performs immunohistochemistry assays.
Funding Support: This award
- 4) Yanyan Cai
Project Role: Research Scholar
Nearest person month worked: 4
Dr. Cai, a postdoctoral scholar in the Scaltriti Lab, leads all lab experiments, coordinates with genomics core and was responsible for animal work.
Funding Support: This award
- 5) Sophie Shifman
Project Role: Research Scholar
Nearest person month worked: 3
Ms. Shifman is a Research Technician in the Scaltriti Lab who assists with all aspects of this proposal, including tissue culture, cloning, biochemical assays, maintenance of patient-derived models needed for in vivo studies and sample preparation for sequencing.

Funding Support: This award

6) Name: Laura Baldino

Project Role: Research Technician

Nearest person month worked: 3

Ms. Baldino is a Research Technician in the Chandarlapaty Lab who conducts experiments in mouse models and tissue culture related to the efficacy of HER2 directed therapies in xenograft models.

Funding Support: This award

8. Special Reporting and Requirements

N/A