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14. ABSTRACT
The overarching goal of this project is to determine whether the unique metabolic phenotype of brain metastases arising from breast cancer presents new opportunities for therapeutic intervention. Specifically, we are addressing whether the nutrient environment of the brain increases the dependence of metastatic breast cancer cells on lysosomal function and autophagy, thereby sensitizing brain metastases to lysosomotropic agents that disrupt autophagy. We are also testing the hypothesis that drug combination strategies can be developed in which treatment with a metabolic inhibitor further increases the dependence of brain-metastatic breast cancer cells on lysosome function/autophagy, such that concomitant treatment with a lysosome inhibitor yields a synergistic response. During the present reporting period, we found that brain-metastatic triple-negative breast cancer (TNBC) cells are markedly sensitized to lysosome inhibitors in a physiological culture medium that models the nutrient composition of the brain interstitial fluid (ISF). Unexpectedly, the sensitivity of HER2+ brain-metastatic breast cancer models to lysosome inhibitors is unaffected by the nutrient environment, potentially reflecting a poor ability to activate autophagy when the HER2-mTORC1 axis is constitutively active. We have also found that lysosome inhibitors selectively synergize with OXPHOS inhibitors against brain-metastatic TNBC cells in physiological, but not standard, culture medium.

15. SUBJECT TERMS
Astrocytes, autophagy, brain metastasis, breast cancer, chloroquine, CRISPR screen, gap junctions, glutamine, lysosome, metabolism, metformin, OXPHOS, synergism

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The onset of brain metastases (BMs) is considered an end-stage event in breast cancer, with median survival after diagnosis of only 4-18 months and treatment options typically restricted to surgery and/or radiotherapy. The environment encountered by circulating breast cancer cells that cross the blood-brain barrier is hostile, in part due to the low abundance in the brain of many nutrients that fuel tumor growth at other sites in the body. Consequently, breast cancer BMs adopt a distinct metabolic phenotype from that of the primary tumor, with a marked shift towards more efficient energy metabolism and nutrient recycling *via* autophagy. In this project, we are testing the hypothesis that synergistic drug combination strategies can be developed in which a metabolic inhibitor forces dependence of brain-metastatic breast cancer cells on autophagy, and concomitant treatment with an autophagy inhibitor blocks their ability to engage in this adaptive response. We are focusing on drugs that are already approved for other indications in order to streamline possible future clinical application. Our overarching goal is to develop urgently needed new treatment options for patients with brain-metastatic breast cancer, which will decrease the mortality associated with this disease and limit the need for invasive treatments that adversely impact patient quality of life. The specific aims of the study are to **(1)** Develop drug synergy strategies that target autophagy dependence and metabolic reprogramming in breast cancer BMs. **(2)** Develop approaches to disrupt nutrient exchange between the metastatic niche and breast cancer BMs. **(3)** Conduct a CRISPR/Cas9 functional genomics screen to identify novel candidate metabolic drug targets in breast cancer BMs.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Astrocytes, autophagy, brain metastasis, breast cancer, chloroquine, CRISPR screen, gap junctions, glutamine, lysosome, metabolism, metformin, OXPHOS, synergism

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Specific Aim 1: Develop drug synergy strategies that target autophagy dependence and metabolic reprogramming in BCBMs.	**Timeline (months)**	**% complete**
Major Task 1: Determine the sensitivity of BCBM cells to autophagy inhibitors under physiological nutrient levels.	1-5	
Subtask 1: Measure dose response curves for MDA-MB-231 and MDA-MB231-BR, JIMT-1 and JIMT-1-BR, SUM-190PT and SUM-190PT-BR, and MDA-MB-361 cells to autophagy inhibitors chloroquine, hydroxychloroquine, quinacrine, and mefloquine in standard FBS-supplemented culture medium.	1-5	100
Subtask 2: Measure dose response curves for MDA-MB-231-BR, JIMT-1-BR, SUM-190PT-BR, and MDA-MB-361 cells to each autophagy inhibitor in brain physiological culture medium.	1-5	100

Subtask 3: Measure dose response curves for MDA-MB-231-BR, JIMT-1-BR, SUM-190PT-BR, and MDA-MB-361 cells to each autophagy inhibitor in brain physiological culture medium during 3D growth as tumor spheroids.	1-5	50
Milestone(s) Achieved: Comparison of the dose responses of parental and brain-metastatic breast cancer cell lines to autophagy inhibitors in standard cell culture conditions and in 2D/3D brain physiological nutrient culture.	5	80
Major Task 2: Evaluate drug synergy strategies that impose metabolic stress on brain-metastatic breast cancer cells and concomitantly block their ability to engage in autophagy.	5-12	
Subtask 1: Use the Chou-Talalay method to determine Combination Indices (a measure of drug synergy) for co-treatment of MDA-MB-231-BR, JIMT-1-BR, SUM-190PT-BR, and MDA-MB-361 cells. In this subtask, synergy between each autophagy inhibitor (chloroquine, hydroxychloroquine, quinacrine, and mefloquine) and the OXPHOS inhibitor metformin will be quantified in physiological medium, examining both 2D and 3D growth under normoxic conditions and in moderate hypoxia (1% O_2). The effect of inhibitor treatment on both cell proliferation and viability will be quantified.	5-12	75
Subtask 2: Determine Combination Indices for co-treatment of MDA-MB-231-BR, JIMT-1-BR, SUM-190PT-BR, and MDA-MB-361 cells. Synergy between each autophagy inhibitor and phenformin will be quantified.	5-12	25
Subtask 3: Determine Combination Indices for co-treatment of MDA-MB-231-BR, JIMT-1-BR, SUM-190PT-BR, and MDA-MB-361 cells. Synergy between each autophagy inhibitor and IACS-010759 will be quantified.	5-12	25
Milestone(s) Achieved: Quantification of synergies between metabolic (OXPHOS) inhibitors and autophagy inhibitors in physiological medium.	12	50
Major Task 3: Test the efficacy of synergistic drug combinations in animal models of brain-metastatic breast cancer.	12-18	
Subtask 1: Based on the data obtained from the *ex vivo* studies above, the most potent drug synergy will be evaluated in animals, initially using luciferase-expressing MDA-MB-231-BR cells. One group of mice will remain untreated, two groups will receive monotherapy of each inhibitor at the doses listed in the Project Narrative, and one group will receive combination therapy. BCBM growth will be read out every 4 days by bioluminescence imaging.	12-18	0
Subtask 2: Analysis of data obtained, and preparation of manuscript for publication.	10-18	25
Milestone(s) Achieved: A detailed understanding of the sensitivity of BCBMs to autophagy inhibitors, and evaluation of a synergy strategy – using drugs that are already either clinically approved or in clinical trials – for treating brain-metastatic breast cancer.	18	50

Specific Aim 2: Develop approaches to disrupt nutrient exchange between the metastatic niche and BCBMs.	Proposed timeline (months)	% complete to date
Major Task 1: Determine the influence of astrocytes and neurons on BCBM cell proliferation in physiological medium.	14-20	
Subtask 1: Measure the proliferation of luciferase-expressing brain-metastatic breast cancer cell lines (by bioluminescence read-out) growing in monoculture and in co-culture with astrocytes, using FBS-supplemented RPMI medium.	14-18	50
Subtask 2: Measure the proliferation of each luciferase-expressing brain-metastatic breast cancer cell line growing in monoculture and in co-culture with astrocytes, using brain physiological medium.	14-18	50
Subtask 3: Repeat the experiments in Subtask 2, but this time testing co-culture of BCBM cells with glutamatergic neurons and tri-culture with both astrocytes and glutamatergic neurons.	14-18	0
Milestone(s) Achieved: An expanded understanding of how astrocytes support the proliferation of brain-metastatic breast cancer cells in different nutrient conditions, and the effect that neurons have on this support.	18	25
Major Task 2: Test whether BCBMs hijack the astrocyte-neuron glutamine-glutamate cycle.	18-24	
Subtask 1: Measure brain-metastatic breast cancer cell proliferation (by bioluminescence read-out) in co-culture with astrocytes in which glutamine synthetase (GLUL) has been depleted by RNAi. Conduct control experiments using RNAi-resistant GLUL constructs.	18-22	0
Subtask 2: Express the FRET-based glutamine sensor FLIPQTV3.0-100m in GLUL-null brain-metastatic breast cancer cells, and read-out changes in BCBM cell glutamine levels during co-culture when astrocyte GLUL is blocked with small-molecule inhibitors.	18-22	0
Subtask 3: If the experiments above indicate that other metabolic supplies from astrocytes are critical for supporting BCBM cell growth, measure the metabolite secretome of astrocytes in physiological medium and use this as a guide for probing the roles of other biosynthetic pathways.	20-24	0
Milestone(s) Achieved: An understanding of whether astrocyte-mediated support of BCBM cell proliferation in low nutrient conditions is dependent on the supply of glutamine from astrocytes to BCBM cells. Or, if this is not the case, data to show which other metabolite exchanges are responsible.	24	0
Major Task 3: Evaluate strategies to disrupt nutrient exchange between the metastatic niche and BCBMs.	24-32	
Subtask 1: Using physiological medium, measure the dose response for inhibition of BCBM cell proliferation (by bioluminescence read-out) in co-culture with astrocytes, with 8 concentrations of drugs that inhibit the gap junction network. Express FLIPQTV3.0-100m in GLUL-null	24-30	0

BCBM cells, and read-out changes in BCBM cell glutamine levels during astrocyte co-culture when the gap junction network is disrupted with small-molecule inhibitors.		
Subtask 2: Use the Chou-Talalay method to test for drug synergies between inhibitors of the gap junction network and inhibitors of autophagy on growth inhibition of BCBM cells co-cultured with astrocytes in physiological medium under normoxic conditions and in moderate hypoxia (1% O_2). The effect of inhibitor treatment on both cell proliferation and viability will be quantified.	26-32	0
Subtask 3: If gap junction inhibitors are ineffective at blocking nutrient exchange between astrocytes and BCBM cells, develop and evaluate other strategies such as inhibiting BCBM cell-specific high affinity glutamine transporters. If Subtasks 1 and 2 yield positive results, proceed to test the most promising synergy strategy *in vivo*.	26-32	0
Milestone(s) Achieved: An understanding of whether pharmacological disruption of nutrient exchange between astrocytes and BCBM cells can inhibit BCBM cell proliferation in low nutrient environments.	32	0

Specific Aim 3: Conduct a CRISPR/Cas9 screen to identify novel candidate metabolic drug targets in BCBMs.	**Proposed timeline (months)**	**% complete to date**
Major Task 1: Conduct a CRISPR/Cas9 whole-genome screen in MDA-MB-231-BR cells growing in FBS-supplemented RPMI medium and in physiological medium.	28-36	
Subtask 1: Conduct screen as described in Project Narrative, using the Human CRISPR Knockout Pooled Library (Brunello) in MDA-MB-231-BR cells cultured for 14 population doublings in FBS-supplemented RPMI medium and in physiological culture medium.	28-33	25
Subtask 2: Analyze data from the screen to identify metabolism-related genes that are conditionally essential in BCBM cells in low-nutrient conditions encountered in the brain.	33-34	0

What was accomplished under these goals?

1. Major activities

Specific Aim 1, Major Task 1: Determine the sensitivity of BCBM cells to autophagy inhibitors under physiological nutrient levels.

- Dose-response curves for parental and brain-metastatic triple-negative breast cancer (TNBC) and HER2+ breast cancer cell lines treated with autophagy inhibitors (chloroquine, hydroxychloroquine, mefloquine, quinacrine) have been measured in standard cell culture conditions and in brain ISF-mimetic culture conditions. Based on a recent study of breast cancer cell lines with intrinsic brain metastasis tropism, we added two additional breast cancer cell lines to our panel, HCC1806 (TNBC) and HCC1954 (HER2+).

Specific Aim 1, Major Task 2: Evaluate drug synergy strategies that impose metabolic stress on brain-

metastatic breast cancer cells and concomitantly block their ability to engage in autophagy.

- Quantitative drug synergism analyses have been conducted for combinations of autophagy inhibitors with OXPHOS inhibitors applied to brain-metastatic breast cancer cells growing in standard culture conditions or brain ISF-mimetic conditions.

Specific Aim 2, Major Task 1: Determine the influence of astrocytes and neurons on BCBM cell proliferation in physiological medium.

- We have optimized a co-culture model for brain-metastatic cancer cells and astrocytes, and have collected preliminary data on the influence of astrocytes on brain-metastatic breast cancer cell proliferation in standard or brain-physiological culture conditions.

Specific Aim 3, Major Task 1: Conduct a CRISPR/Cas9 whole-genome screen in MDA-MB-231-BR cells growing in FBS-supplemented RPMI medium and in physiological medium.

- We have made an early start on the initial stages of the CRISPR/Cas9 whole-genome screen, preparing the pooled gRNA library, transfecting cells, and collecting genomic DNA at multiple time-points from cells growing in standard or brain-physiological culture medium.

2. Specific objectives

During this reporting period, we have evaluated differences in the autophagy pathway between brain-metastatic breast cancer (BMBC) cells and matched 'parental' breast cancer cells, and also between BMBC cells growing in standard *versus* brain-physiological nutrient conditions. We then determined the sensitivity of parental and BMBC cells to pharmacological lysosome/autophagy inhibitors. The underlying rationale for these experiments was based on preliminary data indicating that expression of autophagy-related genes is upregulated in breast cancer brain metastases relative to the primary tumor. This led to the hypothesis that lysosomal function and increased autophagic flux are important for the growth of metastatic breast cancer cells in the restricted nutrient environment of the brain ISF.

In previous studies, we found that the expression of oxidative phosphorylation (OXPHOS) genes is elevated in brain-metastatic, relative to parental, breast cancer cells. Moreover, BMBC cells are highly sensitized to inhibitors of OXPHOS when growing in physiological culture medium. Since activation of autophagy can enable cell survival upon OXPHOS blockade, we hypothesized that combination treatment with an OXPHOS inhibitor and an autophagy inhibitor would yield a synergistic response against BMBC cells in a physiological nutrient environment. A specific objective during this reporting period, therefore, was to conduct quantitative drug synergism analyses of combinations of pharmacological autophagy and OXPHOS inhibitors, focusing on those molecules that are already clinically approved for other indications.

We have also made an early start on some of the work proposed under Specific Aims 2 and 3. Aim 2 focuses on metabolic coupling between BMBC cells and astrocytes within the brain metastasis tumor microenvironment (TME). To address this, we proposed initially to use a co-culture system in which the cell populations are separated by a porous membrane that permits metabolite exchange without direct cell-cell contact. We then proposed to examine whether astrocytes influence the proliferation of BMBC

cells in standard (nutrient-rich) culture medium and/or in brain physiological culture medium. Other objectives for this Aim are for future reporting periods, and are not discussed here. Aim 3 involves conducting a CRISPR/Cas9 whole-genome screen to identify genes that are selectively essential for the growth of BMBC cells in culture conditions mimicking the nutrient environment of the brain ISF. The primary objective of this experiment is to obtain an unbiased 'global' understanding of genes that facilitate the metabolic adaptation of BMBC cells to the nutrient environment of the brain.

3. Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

Methodology summary

During this reporting period, all experimental work was performed *ex vivo*, in line with the Statement of Work. Cell proliferation assays were conducted using standard procedures, with end-point read-outs measured using CyQUANT (Thermo) assays or automated cell counting (TC20 automated cell counter), and EC_{50} values calculated used GraphPad software. Quantitative inhibitor synergism analysis was conducted using the Chou-Talalay method and CompuSyn software. For indirect co-culture experiments, Transwell (Corning) cell culture inserts with 0.4 µm pore polycarbonate membranes were used.

Parental and brain-metastatic breast cancer cells are equally sensitive to lysosome inhibitors in standard cell culture conditions

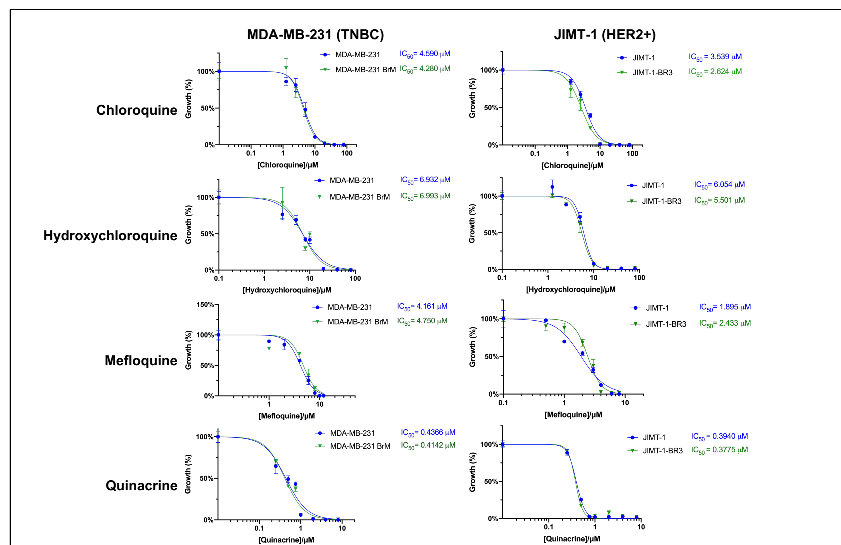

Figure 1. Sensitivity of parental and brain-metastatic breast cancer cells to lysosome inhibitors in standard cell culture media. Representative data for TNBC and HER2+ breast cancer cell lines are shown, with parental cell data in blue and brain-metastatic cell data in green.

We first assessed the sensitivity of parental breast cancer cell lines and their matched brain-metastatic derivatives to lysosome inhibitors in standard cell culture media. We hypothesized that under these conditions of supraphysiological nutrient availability, there would not be intrinsic differences in lysosome dependence between parental and brain-metastatic cells. Consistent with this hypothesis, across all TNBC and HER2+ breast cancer models, there were no substantial differences in sensitivity to lysosome inhibitors between the parental cells and their brain-metastatic derivatives (Figure 1). As a general trend, HER2+ breast cancer cells showed slightly greater sensitivity than TNBC cells to each inhibitor. Consistently, quinacrine

was the most potent drug at suppressing cellular proliferation.

TNBC but not HER2+ brain-metastatic breast cancer cells are sensitized to lysosome inhibitors in physiological culture medium

We next assessed the sensitivity of the BMBC models to lysosome inhibitors in a house-made culture medium that models the nutrient composition of the brain ISF. The underlying hypothesis for this experiment was that the restricted nutrient availability in the brain ISF would increase the dependence of metastatic breast cancer cells on lysosomal function and related processes including autophagy and/or macropinocytosis. Consistent with our hypothesis, we found that brain-metastatic TNBC cells are markedly sensitized to lysosomotropic agents when cultured in brain ISF-mimetic medium relative to conventional culture media (Figure 2). This sensitization is not merely reflecting general cell stress in physiological medium, as across a broad panel of inhibitors we have examined, sensitization is observed only with lysosome inhibitors and a small subset of metabolism-targeted inhibitors (not shown). **Unanticipated result:** In contrast to the TNBC models, HER2+ BMBC cells are not sensitized to lysosome inhibitors in physiological culture medium (Figure 3). Across the cell lines we have examined, all TNBC cells are sensitized to all lysosome inhibitors tested, whereas no HER2+ cells are sensitized to any of the inhibitors. In the case of quinacrine, HER2+ breast cancer cells even show increased resistance in physiological medium. This is considered neither a 'positive' nor a 'negative' result, but the apparent subtype-specific difference is of biological interest and warrants further investigation.

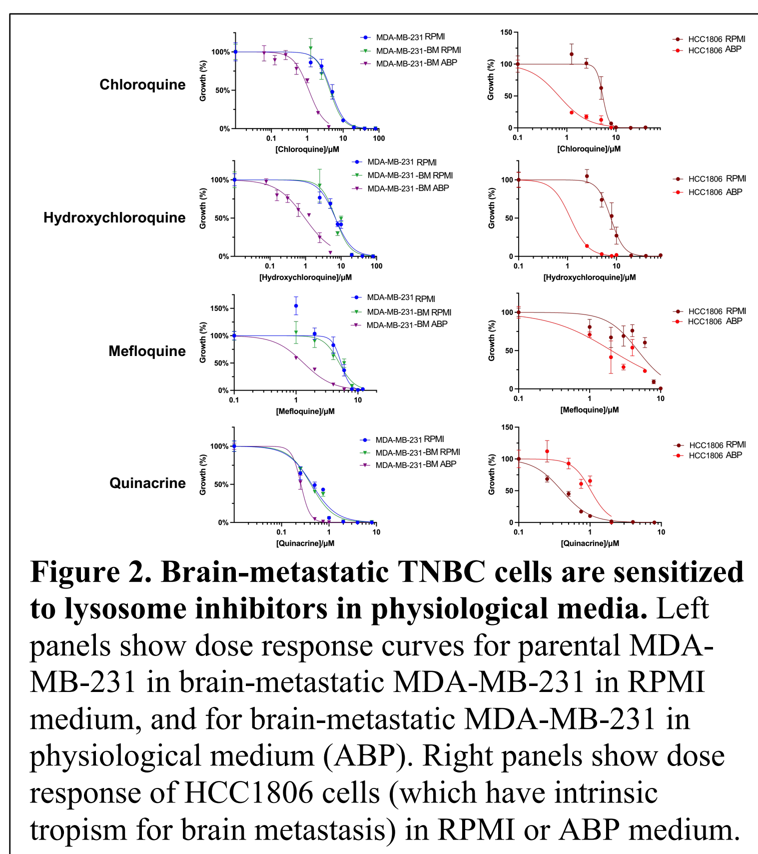

Figure 2. Brain-metastatic TNBC cells are sensitized to lysosome inhibitors in physiological media. Left panels show dose response curves for parental MDA-MB-231 in brain-metastatic MDA-MB-231 in RPMI medium, and for brain-metastatic MDA-MB-231 in physiological medium (ABP). Right panels show dose response of HCC1806 cells (which have intrinsic tropism for brain metastasis) in RPMI or ABP medium.

Signaling downstream of HER2 leads to sustained activation of mTORC1 in HER2+ breast cancers, which in turn signals to suppress autophagic flux. Thus, one possible explanation of our results is that HER2+ breast cancer cells are not able to activate autophagy effectively upon nutrient restriction, and rely on alternative pathways for cell survival and growth. A recent study demonstrated that baseline autophagy is critical for maintaining high levels of HER2 in the cytoplasmic membrane of HER2+ breast cancer cells (PMID: 33472043). Thus, another possible explanation is that HER2+ cells have an intrinsic dependence on baseline lysosome function (consistent with the lower IC_{50} values we measured for HER2+ cells treated with lysosome inhibitors), which is not exacerbated during metabolic stress.

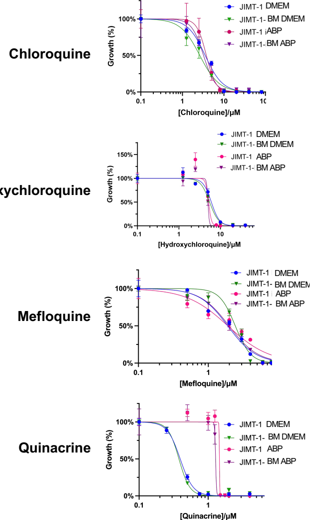

Figure 3. Brain-metastatic HER2+ cells are not sensitized to lysosome inhibitors in physiological media. Dose-response curves for parental and brain-metastatic JIMT-1 cells in standard medium (DMEM) or physiological medium (ABP).

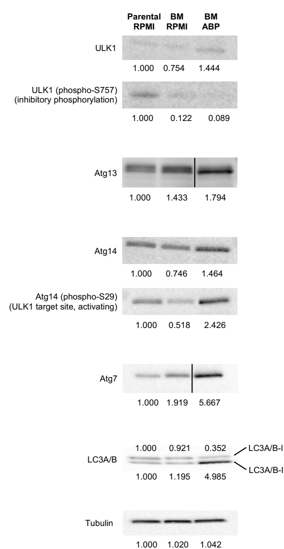

Figure 4. Upregulation of autophagy mediators in physiological media. Western blot analysis of parental (P) and brain-metastatic (BM) MDA-MB-231 cells.

Mediators of autophagosome formation are upregulated in brain-metastatic TNBC cells in physiological medium

In the above section, we found that brain-metastatic TNBC cells are sensitized to inhibitors of lysosome function during growth in physiological culture medium. We therefore considered whether increased sensitivity to lysosome inhibitors corresponds to an upregulation of key components of the autophagy pathway. Western blot analysis showed that in standard culture medium, parental and brain-metastatic TNBC cells generally had similar levels of autophagy-related proteins, with the exception of decreased inhibitory phosphorylation of ULK1 (an autophagy-initiating kinase) at residue S757 in brain-metastatic cells (Figure 4). However, during growth in physiological medium, brain-metastatic TNBC showed marked changes in autophagy-related proteins, including further decreased inhibitory phosphorylation of ULK1, corresponding increased phosphorylation of the ULK1 substrate ATG14 (involved in autophagosome formation) at residue S29, upregulation of ATG7 (involved in LC3 lipidation), and a corresponding shift from LC3-I to the phosphatidylethanolamine conjugate LC3-II, indicative of increased abundance of autophagosomes (Figure 4). Thus, during growth in physiological medium, brain-metastatic TNBC cells upregulate core components of the autophagy pathway and become sensitized to lysosome/autophagy inhibitors.

Lysosome inhibitors synergize with OXPHOS inhibitors to suppress the growth of brain-metastatic TNBC cells in physiological medium

In previous studies, we found that brain-metastatic TNBC cells have a signature of increased expression of OXPHOS genes relative to matched parental TNBC cells. Moreover, brain-metastatic TNBC cells are highly sensitized to OXPHOS inhibitors during growth in physiological culture medium. Since increased autophagy is a potential resistance mechanism to OXPHOS blockade, we hypothesized that the combination of OXPHOS and autophagy/lysosome inhibitors would synergize in suppressing BMBC cell growth in physiological nutrient environments. To test this hypothesis, we have used the Chou-Talalay method (and the associated software, CompuSyn) to conduct a quantitative drug synergism analysis. **Unanticipated result:** In standard culture medium, the combination of OXPHOS and lysosome inhibitors was

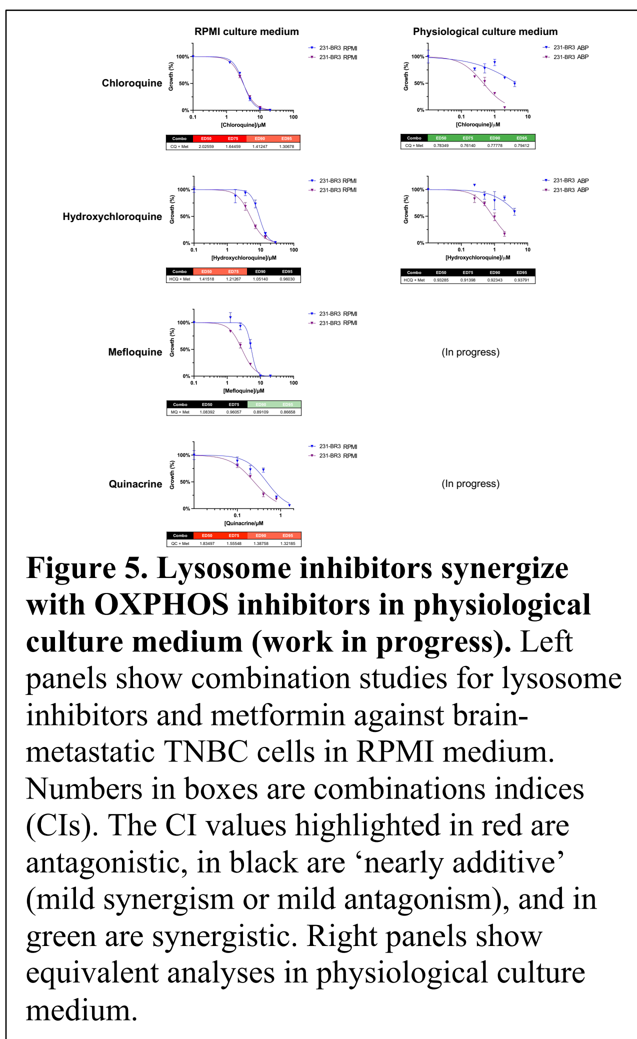

Figure 5. Lysosome inhibitors synergize with OXPHOS inhibitors in physiological culture medium (work in progress). Left panels show combination studies for lysosome inhibitors and metformin against brain-metastatic TNBC cells in RPMI medium. Numbers in boxes are combinations indices (CIs). The CI values highlighted in red are antagonistic, in black are 'nearly additive' (mild synergism or mild antagonism), and in green are synergistic. Right panels show equivalent analyses in physiological culture medium.

frequently antagonistic, implying that treatment with one class of inhibitor can protect cells from treatment with the other class. This antagonism was observed for combination of the OXPHOS inhibitor metformin with the lysosome inhibitors chloroquine, hydroxychloroquine, and quinacrine, but in the case of mefloquine there was slight or moderate synergism at doses above the EC_{50} (Figure 5, left panels). However, consistent with our hypothesis, in physiological culture medium the combination of lysosome and OXPHOS inhibitors was mildly to moderately synergistic (Figure 5, right panels), with synergy indicating that the presence of one class of inhibitor can enhance the efficacy of the other class.

Stated goals not met

Most of the goals for this reporting period have been met. The drug synergism experiments (Figure 5) are still in progress, and will be completed within the next month. Experiments to expand the *ex vivo* studies to include anchorage-independent and hypoxic conditions have turned out to require substantial optimization, as we have found that cell proliferation in physiological medium under these conditions is severely suppressed. Optimization efforts will continue during the next reporting period.

4. Other achievements

We have made some early progress on other components of the project, as indicated in the table above. This includes initial co-culture experiments with BMBC cells and astrocytes, and the early stages of the planned CRISPR/Cas9 screen. Our experiments so far support the hypothesis that astrocytes can provide metabolic support to brain-metastatic breast cancer cells, and this work will be pursued further in the next reporting period.

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

A postdoctoral fellow, graduate student, and research technician have all contributed to the project during the current reporting period. All have become members of the New York Academy of Sciences (NYAS), and attended a virtual symposium focused on cancer metabolism hosted by the NYAS. All have registered to attend a 12-week research education and training course in metabolomics, which will be hosted this year by Weill Cornell Medicine and Memorial Sloan Kettering Cancer Center. The graduate student has presented his research at a CSHL School of Biological Sciences mini-symposium.

How were the results disseminated to communities of interest?

Results from the early stages of this project have been presented internally at CSHL, including to the Cancer and Molecular Biology Program members and at the CSHL School of Biological Sciences symposium in summer 2021. We have been in touch with local high school students, and have arranged for a student to gain research experience by working on this project during summer 2022.

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

To date, the project is running largely in accordance with the approved Statement of Work, and no major changes to this are anticipated. The two major goals for the next reporting period are as follows.

1. Finalize the ongoing quantitative drug synergism analysis *ex vivo*, and then evaluate the efficacy of the most promising drug combination against BMBC models *in vivo*. Based on our unanticipated results indicating enhanced activity against TNBC over HER2+ breast cancer, we plan to focus on TNBC models for this work.
2. Examine the metabolic interactions that occur between BMBC cells and astrocytes and neurons, two other cell types that are present in the brain metastasis tumor microenvironment.

All of the planned experiments will follow the procedures outlined in the original research proposal. Since there has been a delay in receiving approval for the IACUC (and subsequent ACURO) approval for the animal work, the cell culture-based experiments for this reporting period will be started immediately, with the animal experiments beginning as soon as the necessary approvals are obtained.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

Although we are currently 1/3 of the way into the project and do not yet have a complete story to share with the research and patient communities, the key results obtained to date have the potential to be impactful as the project develops.

- Two medications that are widely prescribed for other indications synergize in their inhibitory effects on BMBC cell proliferation.
- This synergy is dependent on the nutrient environment, occurring selectively in nutrient-restricted but not nutrient-rich conditions.
- From our data so far, brain-metastatic TNBC cells, but not HER2+ cells, are sensitized to lysosome inhibitors in physiological medium.

The latter two points are potentially impactful to a broader research community, as they illustrate how the nutrient environment of a cell can profoundly affect its response to specific drugs. These data will contribute to a growing recognition that culture conditions, including the nutrient composition of the culture medium, must be carefully considered when designing and interpreting drug response experiments.

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.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

There are no major changes in objectives and scopes. As noted above, we have added two additional breast cancer cell lines with intrinsic brain metastasis tropism to our panel. To date, we have been unable to run experiments in hypoxic or anchorage-independent conditions, and efforts to optimize this are ongoing.

Actual or anticipated problems or delays and actions or plans to resolve them

As described above, the project is running broadly on schedule. Some experiments, such as completion of the drug synergism analysis, are 1-2 months behind schedule, but we have made earlier than planned progress on some other components. As noted above, experiments involving hypoxic and anchorage-independent growth have proved to be challenging, and efforts are ongoing to optimize this work.

The one significant delay in the new reporting period is caused by a delay in the approval of our IACUC/ACURO protocol for the project. However, since the co-culture experiments do not depend on the results from the animal experiments, we plan to focus initially on this *ex vivo* work. As long as we receive approval of the relevant animal protocols by September 2022, we do not anticipate this to cause a delay to the project as a whole.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

No significant change in planned experiments, but the work is on hold until the necessary IACUC and ACURO approval is obtained.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications.

Yang W-H, Qiu Y, Stamatatos O, Janowitz T, Lukey MJ. Enhancing the efficacy of glutamine metabolism inhibitors in cancer therapy.
Trends in Cancer 7, 790-804 (2021).
Status: Published.
Acknowledgement of federal support: Yes.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

- **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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Field	Details
Name:	Michael Lukey
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-3608-3883
Nearest person month worked:	2
Contribution to Project:	Michael Lukey has overseen all aspects of the project and contributed to experiment design and data interpretation.
Name:	Wen-Hsuan Yang
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0001-5780-6839
Nearest person month worked:	12
Contribution to Project:	Dr. Yang has designed and performed experiments and interpreted data.
Name:	Jed de Ruiter-Swain
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-5308-6835
Nearest person month worked:	1
Contribution to Project:	Jed de Ruiter-Swain has designed and performed experiments and interpreted data.
Funding:	CSHL School of Biological Sciences
Name:	Olivia Stamatatos
Project Role:	Research Technician 1
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	1
Contribution to Project:	Olivia Stamatatos has designed and performed experiments and interpreted data.
Funding:	Lukey lab start-up

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

CURRENT	
Title	2P30CA045508-34 CSHL Cancer Center Support Grant - Shared Resource Tech (PI: Tuveson)
Effort	0.12 Calendar
Supporting Agency	NIH
Grants Officer	Funmi Elesinmogun, MS Grants Management Specialist National Cancer Institute 9609 Medical Center Drive West Tower, 2W452 Rockville, MD 20850
Performance Period	08/01/2021 - 07/31/2026
Funding Amount	
Project Goals	The major goal of this project is research support.
Specific Aims	Research support.
Overlap	There is no scientific or budgetary overlap.
Title	(THIS AWARD) W81XWH-2110071Strategies to target metabolic addictions in brain-metastati breast cancer (PI: Lukey)
Effort	1.80 Calendar
Supporting Agency	Dept. of the Army - USAMRAA
Grants Officer	Jamie A. Shortall Grants Officer jamie.a.shortall.civ@mail.mil 301-619-2393
Performance Period	01/15/2021 - 1/14/2024
Funding Amount	
Project Goals	The overall objective of this proposal is to develop strategies that selectively att the aberrant metabolism of BCBMs, with the goal of providing new treatment options for patients with brain-metastatic breast cancer.
Specific Aims	Aim 1: Develop drug combination strategies to target autophagy dependence in BCBMs Aim 2: Develop approaches to block neuron/glia-BCBM nutrient exchange. Aim 3: Identify candidate metabolic drug targets in BCBMs using a Crispr/Cas9 functional genomics screen.

Title	2P30CA045508-34 CSHL Cancer Center Support Grant -Developmental Funds (PI: Lukey)
Effort	0.12 Calendar
Supporting Agency	NIH
Grants Officer	Funmi Elesinmogun, MS Grants Management Specialist National Cancer Institute 9609 Medical Center Drive West Tower, 2W452 Rockville, MD 20850
Performance Period	01/01/2022- 12/31/2022
Funding Amount	
Project Goals	The major goal of this project is research support.
Specific Aims	Research support
Overlap	There is no scientific or budgetary overlap.
Title	(PROJECT ENDED) METAvivor Early Career Research Award - Targeting the unique metabolic pr of brain metastases in breast cancer (PI: Lukey)
Effort	2.0 Calendar
Supporting Agency	METAvivor Research and Support, Inc.
Grants Officer	Sonya Negley Executive Director 1783 Forest Drive #184 Annapolis, MD 21401
Performance Period	04/01/2020 - 03/31/2020
Funding Amount	(recommended for funding)
Project Goals	Examine whether metformin--and/or more selective and potent inhibitors OXPHOS--might be valuable agents for treating stage IV breast cancer patients BMs.
Specific Aims	Aim 1: Determine the sensitivity of breast cancer BMs to OXPHOS inhibitors i different nutritional environments. Aim 2: Evaluate rational drug combinations for synergistic attack of BM metab Aim 3: Determine the molecular profile of BM cells and the metabolic response OXPHOS inhibition.
Overlap	There is no scientific or budgetary overlap. Drug synergy experiments in Aim 2 examining the combination of OXPHOS inhibitors with nucleotide metabolism inhibitors.
Title	(PROJECT ENDED) The Elsa U. Pardee Foundation
Effort	1.2 Calendar
Supporting Agency	The Elsa U. Pardee Foundation

Grants Officer	Julie Foye P.O. Box 2767 Midland, Michigan 48641-2767 foye@pardeefoundation.org
Performance Period	10/1/2020 - 9/30/2021
Funding Amount	Selected for funding
Project Goals	The goals of this project are to establish the mechanisms by which phosphatidic a modulates the activity and inhibitor sensitivity of glutaminase (GLS) in breast ca cells, and then to develop approaches that maximize the efficacy of GLS inhibito for breast cancer therapy.
Specific Aims	Aim 1: Define the role of phosphatidic acid (PA) and phospholipase D2 (PLD2) mediating resistance to glutaminase (GLS) inhibitors in triple-negative breast cancer (TNBC). Aim 2: Develop synergistic drug combination strategies targeting PLD2 and GLS in TNBC. Aim 3: Evaluate a dietary strategy to sensitize TNBC to inhibitors of glutamine Metabolism.
Overlap	There is no scientific or budgetary overlap.
PENDING	
Title	Defining the role of sleep in cancer immunity and metabolism (PI: Janowitz) *Recommended for funding*
Effort	0.36 Calendar
Supporting Agency	STARR Cancer Consortium
Grants Officer	Sylvie Le Blancq Executive Director of the STARR Cancer Consortium leblancs@mskcc.org 646-684-5862
Performance Period	01/01/2022 - 12/31/2023
Funding Amount	
Project Goals	
Specific Aims	Aim 1: Determine causality via optogenetic bi-directional control of sleep/wake states in mice with colorectal or pancreatic cancer. Aim 2: Demonstrate the translational potential of our findings in human subjects with pancreatic cancer. Aim 3: Apply novel machine learning/AI techniques to mouse and human data.
Overlap	There is no scientific or budgetary overlap.

What other organizations were involved as partners?

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

   COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

   QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.