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14. ABSTRACT

Leptomeningeal metastases (LM) are growths of breast cancer in the linings of the brain and spinal cord and/or in the cerebrospinal fluid (CSF). LM represent 11-20% of central nervous system (CNS) metastases. They are prevalent in younger patients. LM occur in all subclasses of breast cancer and are currently treated with intrathecal (IT) methotrexate or liposomal cytarabine or radiation therapy. Although 68% of patients initially responded to chemotherapy treatment, median overall survival was 18 weeks, resulting from LM progression. Severe complications arise from LM. There is an urgent need for research into this devastating form of breast cancer progression to identify new potential preventives and treatments. We have now derived three models for colonization of the leptomeninges and spinal cord from an intrathecal injection. We have preclinical LM prevention experiments planned.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Leptomeningeal metastases (LM) are growths of breast cancer in linings of the brain (arachnoid mater and pia mater) and spinal cord, and/or in the cerebrospinal fluid (CSF)[1, 2]. LM represent 11-20% of central nervous system (CNS) metastases and have an extremely dire prognosis. They are most prevalent in breast cancer, particularly in the triple-negative and lobular subtypes. An analysis of retrospective studies totaling 446 LM cases from breast cancer found an average age at diagnosis of LM disease of 48.7 years [3]. Diagnosis was made by CSF cytology or MRI findings. Concurrent parenchymal brain metastases occurred in 42.8% of cases and 83.9% of patients had active systemic disease. Where reported, 48.1% of cases were hormone receptor positive (HR+), 27.2% were HER2+ and 27.6% were triple-negative. Included among the HR+ were a disproportionate number of cases of lobular breast cancer. Treatment for LM is typically intrathecal (IT) chemotherapy, either methotrexate or liposomal cytarabine. Although clinical response to treatment was 68.5%, median overall survival was 18.1 weeks [3]. Other potential treatments for LM include radiation therapy for palliation of symptoms and CSF blockages. There are no effective treatments for LM, nor are there adequate mouse models with which to generate supporting preclinical data. The purpose of this grant is to make a panel of mouse models of LM disease, and to use these models to credential potential therapies.

2. KEYWORDS:

Leptomeningeal, metastasis, breast cancer, CNS, CSF

3. ACCOMPLISHMENTS: What were the major goals of the project?

Work to date addresses Aim 1 (90% completion) and Aim 3 (ongoing).

Aim I. Establish multiple preclinical models of LM, either from extension of parenchymal brain metastases or direct colonization of the CSF.

a. Test existing brain metastasis models for extension into the CSF.

b. Develop new models of LM from direct injection into the CSF, prioritizing immune competent models.

c. For each model system, develop quantifiable endpoints. Determine the permeability of the blood-CSF barrier.

d. Molecularly profile the model systems using RNAseq to identify potential mechanistic and druggable pathways.

Since our previous report we have fully developed three models of LM colonization, from an intrathecal injection to the brain meninges and down the spinal cord. Our models have more quantifiable endpoints than any other reported, including bioluminescent imaging, MRIs that show both tumor and extensive edema, histopathological analysis of stained sections of brain and spinal cord, and isolation of tumor cells from CSF that are capable of *in vitro* growth. We are currently conducting RNA sequencing profiling on all the developed models to identify potential mechanistic and druggable pathways.

Aim 2. Profile human CSF specimens with from patients with LM using RNASeq. Analyze data to identify similarities to mouse models, potential driver pathways and potential druggable pathways. Profile infiltrating immune cells in CSF for comparison to models. Along with molecular analysis from Aim I, select at least two pathways/drugs to test *in vivo*.

We have not had the opportunity to collect these specimens.

Aim 3. Determine the contribution of the immune system to LM progression.
a. Profile LM model systems for infiltration of immune cell subtypes.
b. Determine the preclinical efficacy of systemic and/or IT immune checkpoint therapy.

We will start immune profiling the current stablished LM models (ongoing). For the HER2+ model, we elected to test standard of care therapeutic (trastuzumab) and a recently approved antibody-drug conjugage (T-DxD). These experiments are underway. For the lobular breast cancer model, we have launched a new drug screen for potential therapeutics. These therapeutics have been analyzed *in silico* for brain permeability and *in vitro* characterization of the top four compounds is starting.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

To date, we were able to fully develop three LM model systems. We have had to adapt the published methodology of Dr. Adrienne Boire, MSKCC. Her initial reports used intrathecal injections into the brain ventricle to produce LM metastases. These were harvested, cultured and reinjected three times. The resulting culture was injected intracardiacally to produce LM[4,5,6]. Dr. Boire sent us her MDA-MB-231LM cell line, and we visited the lab to learn the injection technique. We were unable to repeat this schema using the line that she sent us, or the lines described below. Rather, we developed a more limited colonization assay from intrathecal injections.

We have now developed intrathecal-injection based models for: (a) the HER2+ JIMT1-BR line (JIMT1-BR/LM4), (b) the ER+ lobular carcinoma cell line SUM44PE (SUM44PE-LM), and (c) the

triple-negative syngeneic 4T1-BR line (4T1-BR/LM). Figure 1 shows the schema for these models. Pertinent details for each are listed below.

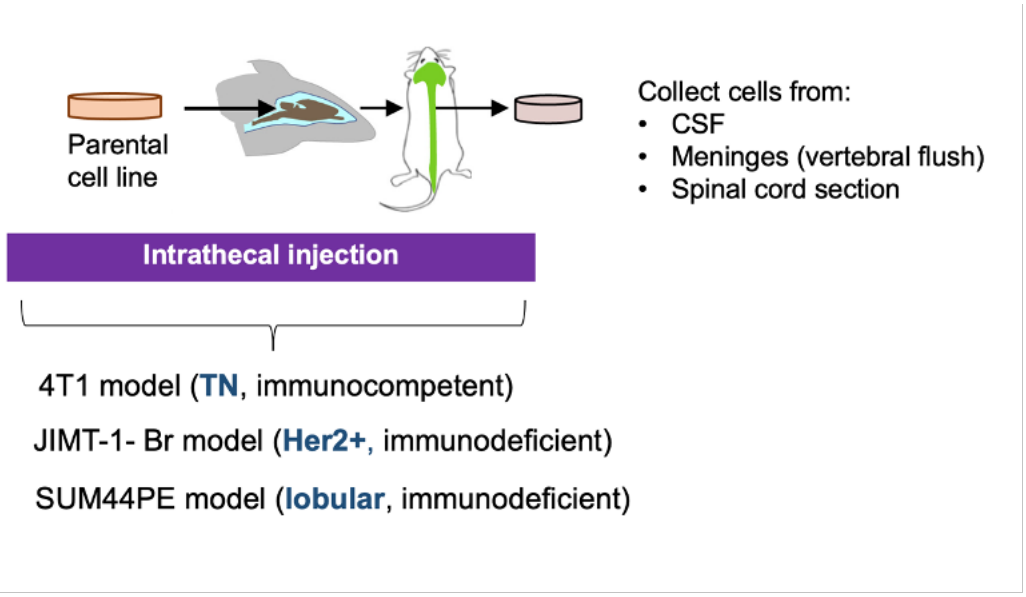

Figure 1. Approach to establish preclinical models of leptomeningeal metastases from breast cancer. Adapted from Boire et al., *Cell* 2017.

HER2+ breast cancer JIMT-1-BR, JIMT1-BR/LM4 (immunodeficient):

The JIMT1-BR/LM4 model system was derived through four rounds of intrathecal injection and harvesting of tumor cells. Besides bioluminescence of tumor cells, we have been able to validate several additional endpoints that may provide information on other aspects of the disease **(Figure 2**). These include MRI, which shows both LM deposits but also prominent edema. Using the LASP histology section we have obtained sections of the brain meninges, showing metastases but also the spinal cord, a difficult organ to section. These sections demonstrate LM deposits. Histopathology can therefore be used to document LM spread and the degree of invasion of the spinal cord. Finally, we were able to harvest minute amounts of cerebrospinal fluid (CSF) from the mice and can see tumor cells in it, which documents this nonadherent part of LM; we can also successfully culture these tumor cells, demonstrating their viability.

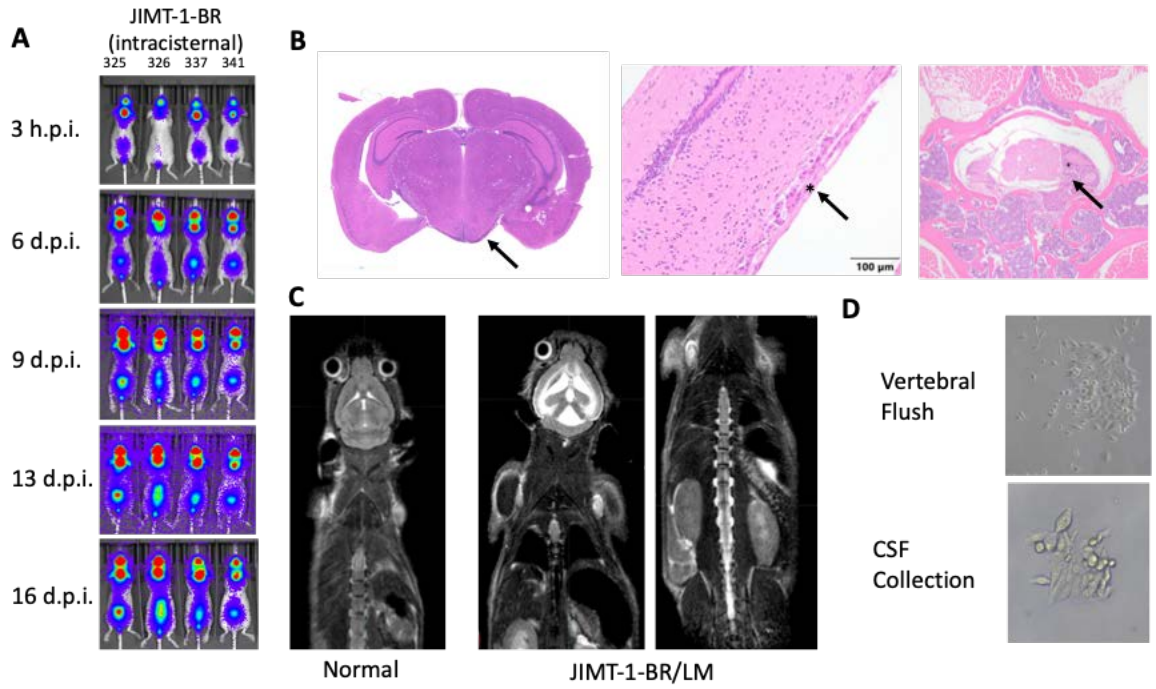

Figure 2. Endpoints for the HER2+ JIMT1-BR/LM4 model system. A. Bioluminescent imaging over 16 days post-intrathecal/intracisternal injection. B. H&E-stained histologic analysis showed tumor cell layers in the meninges of brain (arrow, left image), in the spinal cord (arrow, middle image) and tumor cells ensheathing a nerve in the spinal cord (arrows, right image). Scale bar 100 um. C. MRI analysis showing a normal mouse (left) and images of the head and spinal column of a JIMT1-BR/LM4 mouse (right) showing edema. D. *In vitro* culture of tumor cells from a vertebral flush and CSF demonstrating live cells. h.p.i= hours post-injection; d.p.i.= days post-injection.

Because this model system is immunocompromised (human tumor cells in a nude mouse), it is inappropriate for immune checkpoint therapy investigations. Our first planned experiment will test standard of care trastuzumab in a prevention setting, versus Trastuzumab deruxtecan (T-Dxd). We also added a trastuzumab plus nab-paclitaxel arm since our oncology colleagues note that this is an often used combination. We hypothesize that T-Dxd will show better efficacy than trastuzumab alone or in combination (**Figure 3**). A prevention endpoint is planned (ie, early and continuous drug treatment). Endpoints will be those developed for the model. In addition, we will attempt pHER2 staining of tissue sections to see activation status.
Depending on the results, several rounds of optimization are expected including iv versus intrathecal drug administration, prevention versus treatment endpoints, and rational combinations.

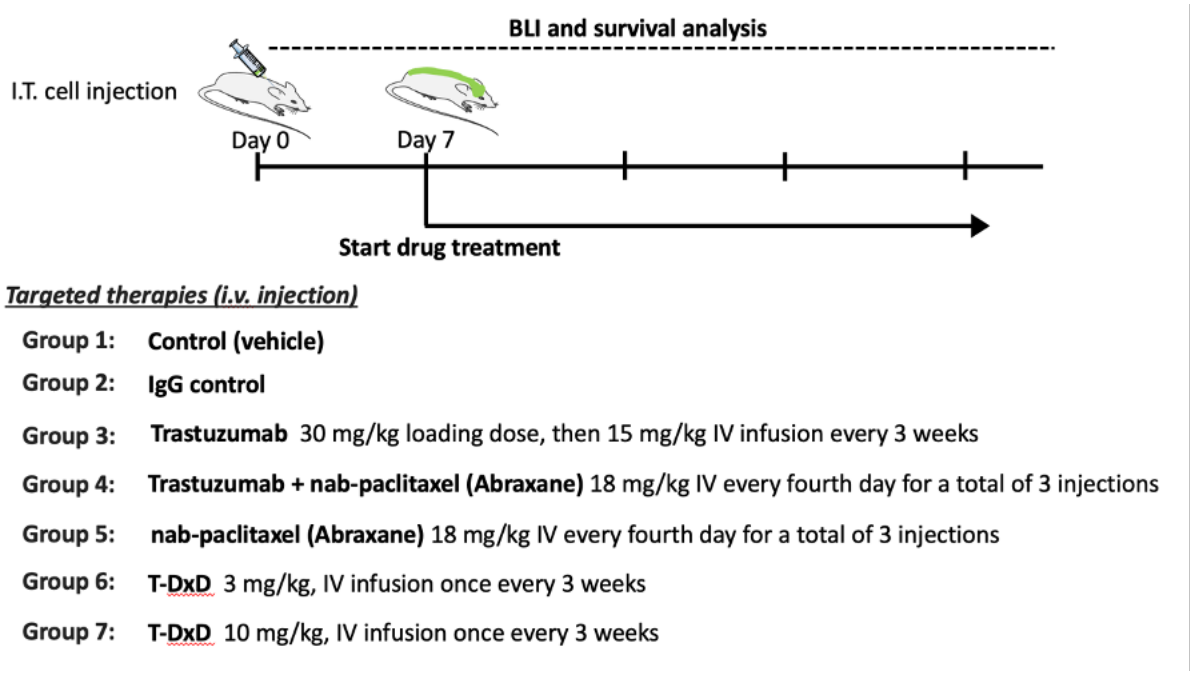

Figure 3. Schema of drug treatments experiments for the Her2+ LM model.

Lobular breast cancer LM model, SUM44PE-LM (immunodeficient):

Infiltrating lobular cancer (ILC) is typically ER+ and HER2-, in sections it forms single file linear patterns of epithelial cells dispersed in a fibrous stroma. It is characterized by a low proliferative rate and loss of E-cadherin expression (CDH1). ILC has a higher risk of recurrence than luminal a breast cancer and is often lumped into ER+ breast cancer trials where it is a minority of the cases. ILC metastasizes to the bones, gastrointestinal tract, ovaries, peritoneum, retroperitoneum, and leptomeninges [7]. Preclinical models for ILC are few, including GEMs of CDH1 loss and other deletions, injection of cell line into milk ducts and PDXs [8]. We used the SUM44-PE ILC cell line through 1 round of intrathecal injection and harvest to obtain the endpoints shown on **Figure 4**. Note that this line, extraordinarily slow growing *in vitro*, took almost a half year to produce a LM model. We attempted to speed the process by multiple injections, which failed (**Figure 5**). RNA sequencing is ongoing for further characterization.

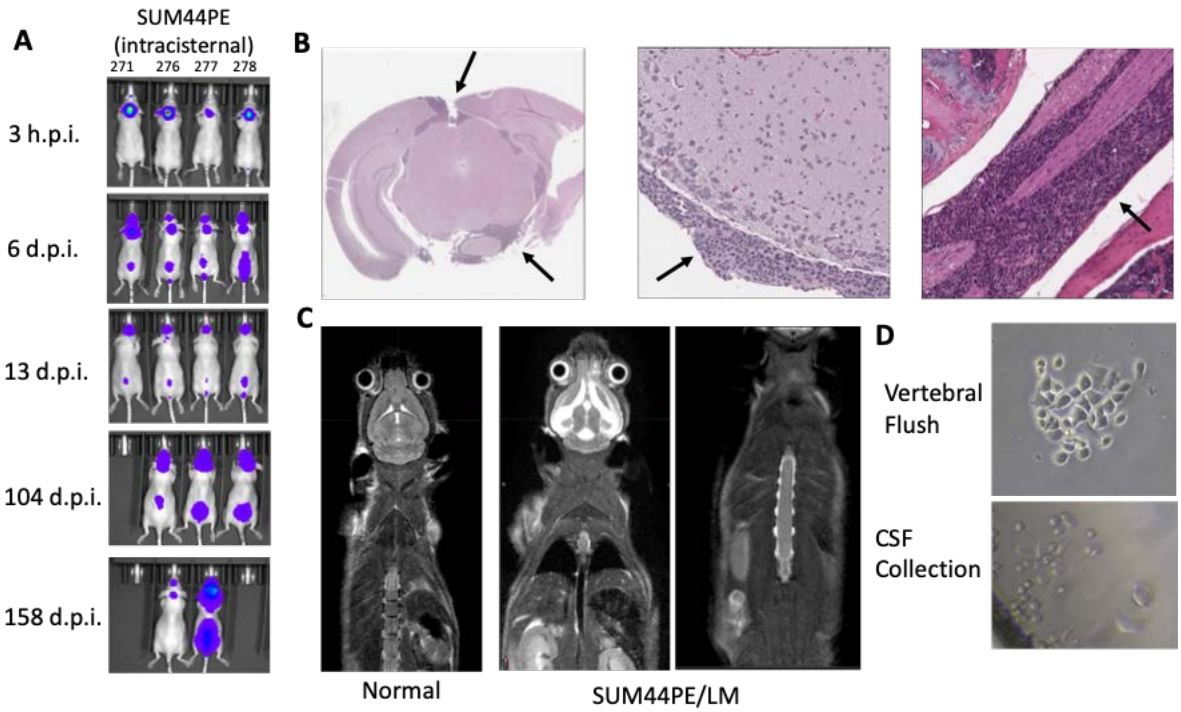

Figure 4. Endpoints for the SUM44-PE-LM model system. A. Bioluminescence imaging after intrathecal injection of tumor cells. B. H&E-stained histologic sections showing leptomeningeal cancer growth in the brain (arrow, left and middle images) and spinal cord (arrow, right image). C. MRI showing edema in brain and spinal cord. D. *In vitro* growth of cancer cells following vertebral wash (left) and in CSF (right).

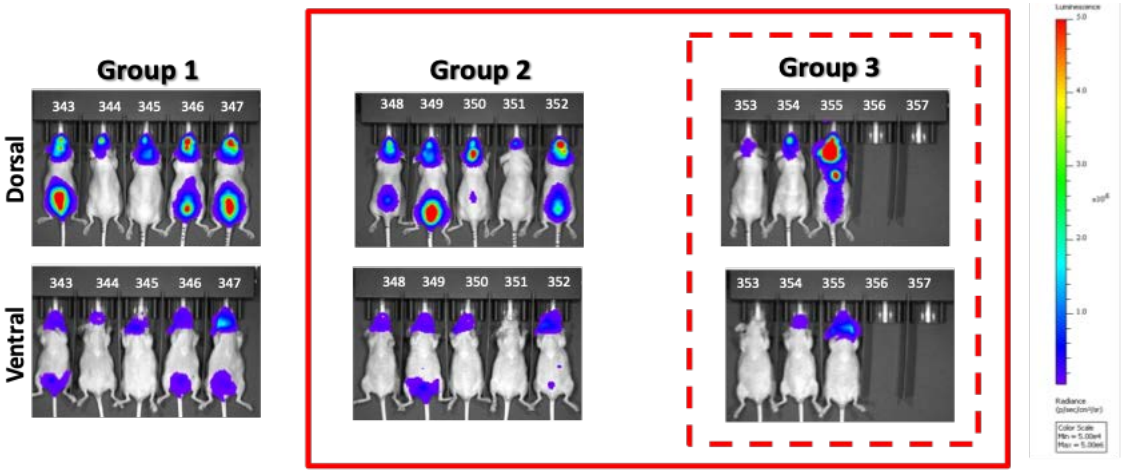

Figure 5. Bioluminescence images of SUM44-PE-LM model system where Group 1 received one round of intracisternal cancer cell injection as in Figure 4, Group 2 received two rounds and Group 3 received three rounds of injections spaced 1 week between injections. Images are at

approximately 4 months post-first injections. Note that missing mice in group 3 were euthanized before imaging. This technique failed to speed up or alter the metastatic spread of the line.

The SUM44PE-LM model is also immunodeficient, as it is human tumor cells in a nude mouse, so it is inappropriate for checkpoint immune therapy experiments. There are few therapeutics that are effective for lobular carcinoma. One reason is that, being ER+ and relatively rare, these patients are grouped together with other ER+ patients in trials. Thus, little of the data generated applies to lobular breast cancer. A second reason is that lobular cancer is aggressive, but this tends to happen relatively later.

Given the need for new therapeutics aimed specifically at ILC we turned to Dr. Craig Thomas, National Center for Advanced Translational Sciences (NCATS), NIH who has a high throughput drug screen for cell lines [9]. Dr. Thomas required $3x10^7$ cells for the screen which took months of culture for this slow-growing line. He delivered a list of 2480 drugs with varying degrees of *in vitro* activity against these cells. We then debated how to best use this list. Most drugs are brain impermeable due to the blood-brain barrier. The leptomeninges are protected by the blood-CSF barrier, which is poorly characterized. Reasoning that features that make a drug more permeable through the blood-brain barrier would also facilitate blood-CSF barrier passage, we asked Dr. Paul Lockman, W. Va. University, to use in silico analyses to predict those with *in vitro* activity that would be most brain permeable. He analyzed the top 50 compounds ranked by AUC values and predicted brain penetration. A portion of these data are listed on **Figure 6**.

After reviewing the literature on these drugs, we have elected to start with : (a) ARRY162 (binimetinib), a MEK 1-2 with activity in B-RAF mutant melanoma[10]. It also has activity in melanoma brain metastases[11,12]; (b) Torin 2, an M-TOR inhibitor that induces autophagy and has preclinical radiation sensitization activity[13,14]; (c) M2698, a dual P70S6K/AKT inhibitor is confirmed to cross the BBB and had activity in combination with tamoxifen or trastuzumab in breast cancer [15,16]; (d) Acilisib, on the intermediate list, a PI3 subunit δ inhibitor with clinical activity in blood cancers[17]; (e) Lurbinectedin, which binds DNA minor grooves and triggers DNA strand breaks[18,19,20]. Lurbinectedin has been tested in BRCA mutant breast cancer and other settings [21,22]; (f) Dinacyclib, a cdk 1,2,5,9 inhibitor approved for ER+ breast cancer. Each drug will be tested *in vitro* for inhibition of proliferation, migration and invasion; molecular pathways that should be impacted; responsiveness to drugs and radiation in colony formation assays. Based on these data we will select several for initial *in vivo* experiments, following the design above for the HER2+ model, ie, initially systemic dosing in a prevention setting.

Sample Name	MW	LogD Predicited	Equation (LogD)-sqrt(mw)	Log PS (Y)
Cdk4/6 Inhibitor IV	456.6	4.58	3.25	1.18
Tipifarnib	489.4	4.37	3.03	1.02
ML-390	406.4	3.96	2.66	0.75
Cyproterone acetate	416.9	3.62	2.31	0.50
ARRY-162	441.2	3.51	2.19	0.42
Torin-2	432.4	3.47	2.15	0.39
Verdinexor	442.3	3.09	1.77	0.12
M2698	449.9	2.96	1.63	0.02
GB-83	548.7	5.16	3.79	1.56
NMS-873	520.7	4.15	2.79	0.85
RO-4987655	565.3	3.85	2.47	0.62
Docetaxel	807.9	3.54	2.09	0.34
Telaprevir (VX-950)	679.8	3.2	1.78	0.13
YK-4-279	366.2	2.86	1.58	-0.02
Proscillaridin	530.6	2.85	1.49	-0.08
PDHK RIKEN	296.3	2.6	1.36	-0.17
NVP-AEW541	439.6	2.58	1.26	-0.25
KW-2478	574.7	2.61	1.23	-0.27
Acalisib	401.4	2.52	1.22	-0.28
Omacetaxine mepesuccinate	545.6	2.23	0.86	-0.53
Tazemetostat	572.7	2.24	0.86	-0.53
Biochanin A	284.3	1.97	0.74	-0.62
AZD-5438	371.5	1.97	0.69	-0.66
Delanzomib	413.3	1.91	0.60	-0.72
AMG-511	517.58	1.62	0.26	-0.96
MLN-7243	519.5	1.6	0.24	-0.98
Lurbinectedin	519.518	1.6	0.24	-0.98
Methylstat	505.6	1.37	0.02	-1.14
VS-5584	354.4	1.12	-0.15	-1.26
Dinaciclib (SCH727965)	396.5	0.93	-0.37	-1.41
Triptolide	360.4	0.67	-0.61	-1.58
Phlorizin	436.4	0.02	-1.30	-2.08
Echinomycin	1101.3	0.21	-1.31	-2.09
Tasquinimod	406.4	-0.02	-1.32	-2.10
Gedatolisib	615.7	-0.34	-1.73	-2.39
Actinomycin D	1255.4	-2.39	-3.94	-3.97

Figure 6. Dr. Lockman's analysis of the NCATS drug screen for the SUM44-PE cell line. Those hits with the highest predicted brain permeability are green, followed by moderate permeability in yellow and drugs to avoid in red. Log D is a distribution coefficient, Log PS is the octanol : water permeability, both measures of predicted brain permeability.

Triple-negative breast cancer LM model 4T1-BR/LM4, (immunocompetent):

Figure 7 shows endpoints for the 4T1 model system, derived through four rounds of intrathecal injection and harvesting of tumor cells. Disease location was confirmed as in the previous models in the meninges of the brain and spinal column. We have also acquired MRI scans that are currently being processed, and live, proliferative tumor cells cultured from the spinal column for RNA sequencing.

Once we have fully validated the model, it will be characterized for immune profile. We will stain for innate and acquired immunity to determine the number and localization of immune cells. If immune cells are found, we plan to determine the efficacy of immune checkpoint therapy on this model system. This would be a nab-paclitxel and anti-PD-1 therapy, as is FDA approved for triple-negative breast cancer. The experiment would test each drug as monotherapy and the combination, versus vehicle control, in a prevention setting.

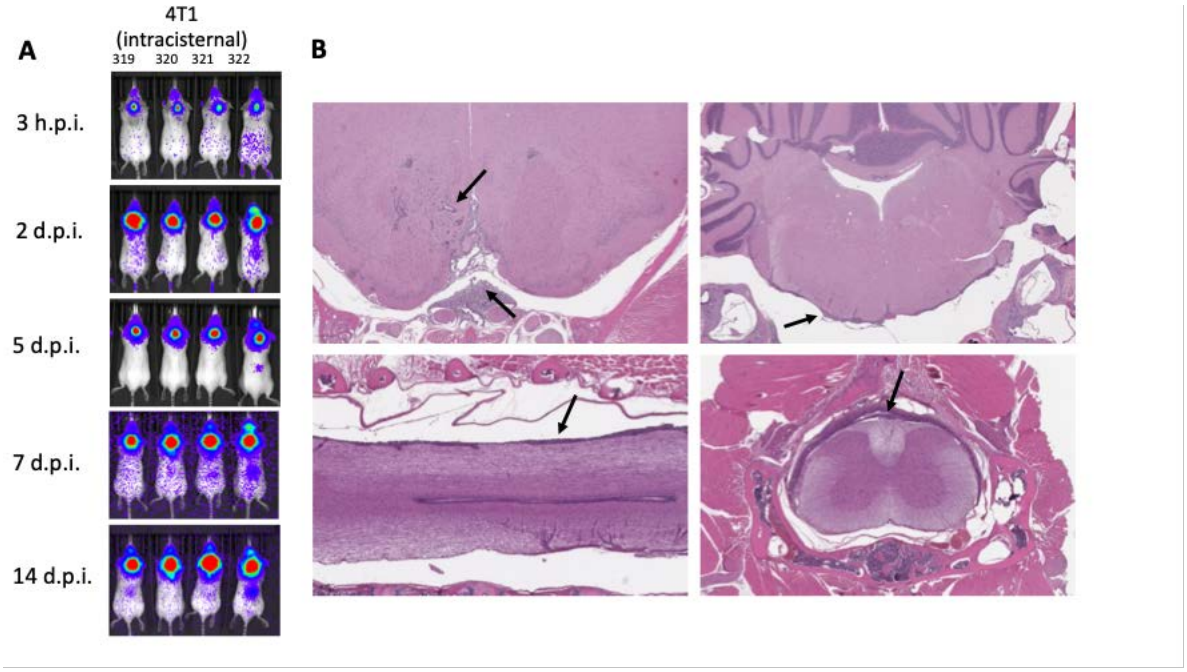

Figure 7. Endpoints for the 4T1-BR/LM4 model system. A. Bioluminescence imaging after intrathecal injection of tumor cells. B. H&E-stained histologic sections showing leptomeningeal cancer growth in the brain (arrows, top images) and spinal cord (arrows, bottom images).

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

Dr. Vanesa Silvestri has worked on this project since its inception, though she is now paid under an NIH postdoctoral fellowship to save funds for the critical animal work. This work has greatly expanded her professional capabililties.

How were results disseminated to communities of interest?

I intend to publish each model separately when we have preclinical drug data.

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

To date, we were able to fully develop three LM model systems. Given our models of LM colonization, we expect that systemic drug delivery may have some preventive activity, particularly T-Dxd. As mentioned, if no activity is seen by systemic delivery, then intrathecal delivery through an Omaya reservoir is planned. Combinations with radiation therapy will be attempted. The ILC drug development experiments are high risk, high impact, and we hope to improve our chances by testing as many drugs as possible.

4. **IMPACT:**

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

These data expand the number of preclinical LM models from the one published by Dr. Boire to four. The models cover relevant breast cancer subtypes, particularly the lobular breast cancer subtype. They offer the possibility of rationally credentialling preventatives and therapeutics.

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:

Nothing to report

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

Our only delays are caused by waiting periods for animal experiments to be conducted at LASP. They are still backed up from the pandemic and difficulty hiring.

Changes that had a significant impact on expenditures

We previously moved Dr. Silvestri to a NIH postdoctoral fellowship. The departure of Dr. Reed from Purdue University was fixed by adding funds for histopathologic analyses at LASP.

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

None

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

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

Journal publications.

Nothing to report

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

Nothing to report

Other publications, conference papers and presentations.

This work has been presented in the seminar series for the Women Malignancies Branch, CCR, NVI, NIH.

- **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?

Dr. Patricia Steeg, PI, “no change”

Dr. Vanesa L. Silvestri, postdoctoral fellow, “no change”

Animal Research Technical Support (ARTS) and Gnotobiotics Facility, Laboratory Animal Sciences Program (LASP) “no change”

Dr. Tiffany Lyle left Perdue University and no longer contributes to this project.

Dr. Craig Thomas, NCATS, NIH; collaborator; Dr. Thomas run a high throughput drug screen on the ILC cell line. Unpaid by this grant.

Dr. Paul Lockman, West Virginia University; collaborator; Dr. Lockman analyzed the drug screen to predict brain penetration based on molecular weight, lipophilicity etc. Unpaid by this grant.

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?

Other unpaid collaborators listed above.

8. SPECIAL REPORTING REQUIREMENTS
COLLABORATIVE AWARDS:

9. APPENDICES: