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TITLE: Targeting Prolyl Peptidases in Triple-Negative Breast Cancer

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14. ABSTRACT
Triple negative breast cancer (TNBC) is an aggressive sub-type with limited treatment options and poor prognosis. The most life-threatening aspects of TNBC are therapy resistance and metastasis. To improve survival in TNBC patients it will be necessary block metastasis and decrease tumor cell survival. We identified a protein called PRCP (prolylcarboxypeptidase) that promotes metastasis and survival in breast cancer cells. We found high expression of PRCP in TNBC patients coincides with decreased recurrence-free survival (worse outcome). In a drug screen we identified a potent inhibitor of PRCP and its related family member prolyl endopeptidase (PREP) and showed that it has anti-tumor activity *in vivo*. The goals of this grant are 1) to determine the molecular mechanism by which PRCP and PREP promotes survival and metastasis and, 2) to test our drug candidate for its ability to reduce TNBC tumor growth and target metastatic TNBC tumors. These goals are pursued in two specific aims. Results obtained so far show that PRCP/PREP inhibition reduces IRS1 and IRS2 protein levels, blocks proliferation, and induces death in multiple TNBC cell lines of different sub-types. These effects appear to result, at least in part, through IRS1/2, AKT, and mTORC1 inhibition. Results also indicate the PRCP/PREP inhibitor we identified can inhibit growth of human TNBC tumors in mice, supporting PRCP/PREP as treatment targets and our inhibitor as a therapeutic agent.

15. SUBJECT TERMS
Triple negative breast cancer, Prolyl peptidases, Breast cancer treatment, Animal model

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1. Introduction:

Triple negative (TNBC) breast cancer patients have poor prognosis due to the aggressive nature of the tumors, the lack of therapeutic targets, and the high rate of metastasis [1,2]. Identifying factors that promote TNBC growth, survival, and metastasis is critical for development of new therapies [3-5]. Multiple signaling pathways can contribute to TNBC growth and metastasis, including the IGF-1R and EGFR/ErbB3 pathways. IRS-1 and IRS-2 are adaptor proteins that mediate signaling downstream of both IGF-1R and EGFR/ErbB3 [6-8]. Pathways activated downstream of IRS-1/2 include the AKT-mTORC1 pathway, the MEK-ERK pathway, and JAK-STAT pathway [6-8], all of which can promote survival, proliferation, and metastasis [9-13]. The AKT /mTORC1 pathway is of particular interest. Several AKT/mTORC1 inhibitors are in trials for various cancers including TNBC. However, complex negative feedback loops within the pathway limit their effectiveness. For example, AKT inhibitors cause increased expression of IGF1R/ErbB3 and, as a result, increase survival and proliferation signaling mediated by IRS-1 [14,15]. Further, mTORC1 inhibitors (e.g. rapamycin) cause feedback stabilization of IRS-1 and subsequent activation of AKT [16] and JAK2/STAT5 signaling [17]. *Drugs that target IRS-1/2 directly would have a distinct advantage over current AKT/mTORC1 inhibitors because they would not only inhibit the AKT/mTORC1 pathway but also block feedback activation of IRS-1/2 dependent survival and proliferation signaling.* PRCP and PREP are prolyl peptidases that can regulate GPCR signaling by cleaving various GPCR agonists [18]. In preliminary data for this proposal we found high expression of PRCP in TNBC patients coincides with decreased recurrence-free survival (worse outcome). In a drug screen we identified a potent inhibitor of PRCP/PREP called Y-ox, and showed that it has anti-tumor activity *in vivo*. Our hypothesis is that PRCP/PREP promotes TNBC proliferation, survival, and metastasis by stabilizing IRS1/2 and activating the AKT/mTORC1 pathway. The goals of this proposal are 1) to determine the molecular mechanism by which PRCP/PREP promotes TNBC cell survival and metastasis, and 2) to test our drug candidate Y-ox for its ability to reduce TNBC tumor growth and target metastatic TNBC tumors in mice.

2. Keywords:

PRCP:	prolylcarboxypeptidase
PREP:	prolylendopeptidase
IGF-1R:	Insulin like growth factor receptor 1
IRS1/2:	Insulin receptor substrates 1 and 2
AKT:	Survival kinase also known as Protein Kinase B
mTORC1:	mammalian target of rapamycin complex 1
GPCR:	G-protein coupled receptor
EGFR:	Epidermal growth factor receptor
ErbB3:	Human epidermal growth factor receptor 3
MTT:	Colorimetric assay for assessing cell metabolic activity. Commonly used to measure proliferatin and viability
S6K:	Ribosomal protein S6 kinase. This protein is phosphorylated by mTORC1

3. Accomplishments:

3a. What were the major goals of the project as stated in the approved Statement of Work?

There were four major goals/tasks in the approved Statement of Work.

Major Goal/Task number 1: Determine the effect of PRCP/PREP overexpression, knockdown, and inhibition on IRS1/2 levels and on signaling pathways downstream of IRS1/2 (AKT-mTOR, MEK-ERK, Jak-STAT).

Milestone to be achieved by completing task 1: Completion of task 1 will allow us to determine if PRCP and or PREP affects levels of IRS1/2 and signaling proteins downstream of IRS1/2 in TNBC cell lines.

Major Goal/Task number 2: Determine the effect of PRCP/PREP overexpression, knockdown, or inhibition on TNBC cell proliferation, survival, migration, and invasiveness.

Milestone to be achieved by completing task 2: Completion of task 2 will allow us to determine if PRCP and/or PREP affects TNBC cell proliferation, survival, and invasiveness.

Major Goal/Task number 3: Determine the ability of our drug candidate Y-ox, alone or in combination with rapamycin, to inhibit TNBC xenograft tumor growth in the mouse mammary fat pad and to block TNBC metastatic tumor growth in a mouse model of metastasis.

Milestone to be achieved by completing task 3: Completion of task 3 will allow us to determine in Compound Y-ox alone or in combination with rapamycin can inhibit TNBC primary and metastatic tumor growth in mice.

Major Goal/Task number 4: Perform statistical analysis of all data sets, finalize all results and figures.

Milestone to be achieved by completing task 4: Completion of task 4 will establish if Compound Y-ox alone or in combination with rapamycin inhibits primary and metastatic TNBC growth in mice in a statistically significant way.

3b. What was accomplished under these goals?

Major Activities for this funding period:

- Measured proliferation and viability/survival in multiple TNBC cell lines treated with Y-ox or Y-ox plus rapamycin.
- Determined IRS1/2 levels and levels of activated AKT, mTORC1, and ERK in multiple TNBC cell lines treated with Y-ox.
- Examined the effect of PRCP and PREP knockdown on viability/survival of TNBC cells.
- Tested the ability of Compound Y-ox to inhibit human TNBC xenograft tumor growth in mice.

Specific Objectives:

- to determine if Y-ox alone inhibits survival and reduces viability in TNBC cells.
- to determine if this associated with reduced IRS1/2 levels and reduced activity of pathways downstream of IRS1/2.
- to determine if knockdown of PRCP and PREP have similar effects to Y-ox.
- to determine if Y-ox in combination with rapamycin causes a greater inhibition of TNBC viability compared to Y-ox alone.
- to determine if Compound Y-ox can inhibit human TNBC xenograft tumor growth in mice.

Significant Results and Key Findings:

One of the Major Goals in the approved Statement of Work is to determine the effect of PRCP/PREP overexpression, knockdown, or inhibition on TNBC cell proliferation, survival, migration, and invasiveness. We started to address this goal with our drug candidate and PRCP/PREP inhibitor Y-ox. Multiple cell lines from different TNBC sub-types were treated with vehicle or increasing doses of Y-ox (1, 5, 10 μM). We then measured proliferation and viability/survival by measuring MTT absorbance 1 and 4 days after treatment. Increased MTT absorbance indicates an increased number of cells, while reduced MTT absorbance indicates loss of viability/increased cell death. We observed a pronounced loss of viability in all cell lines treated with drug Y-ox at the 10 μM dose (Fig 1), and visual examination confirmed Y-ox treated cells were dead (Fig 2). Our hypothesis was that Y-ox will reduce viability and kill TNBC cells by causing a reduction in IRS1/2 protein levels and subsequently inhibiting the AKT-mTORC1 pathway. Consistent with this we saw IRS1/2 protein levels were depleted in Y-ox treated cells (Fig 3). AKT is activated by phosphorylation at serine 473 (S473), and S6K phosphorylation at threonine 389 (T389) is an indicator of mTORC1 activity. The AKT-mTORC1 pathway was inhibited in Y-ox treated cells, evidenced by loss of pAKT(S473) and loss of pS6K(T389) (Fig 3). The MEK/ERK and Jak/STAT pathways are also activated downstream of IRS1/2. ERK is activated by phosphorylation at threonine 204 (T204) and tyrosine 202 (Y202). ERK (T204, Y202) were not affected by Y-ox (Fig 3). We were unable to detect phosphorylated (activated) levels of Jak2 and STAT5 in all TNBC cell

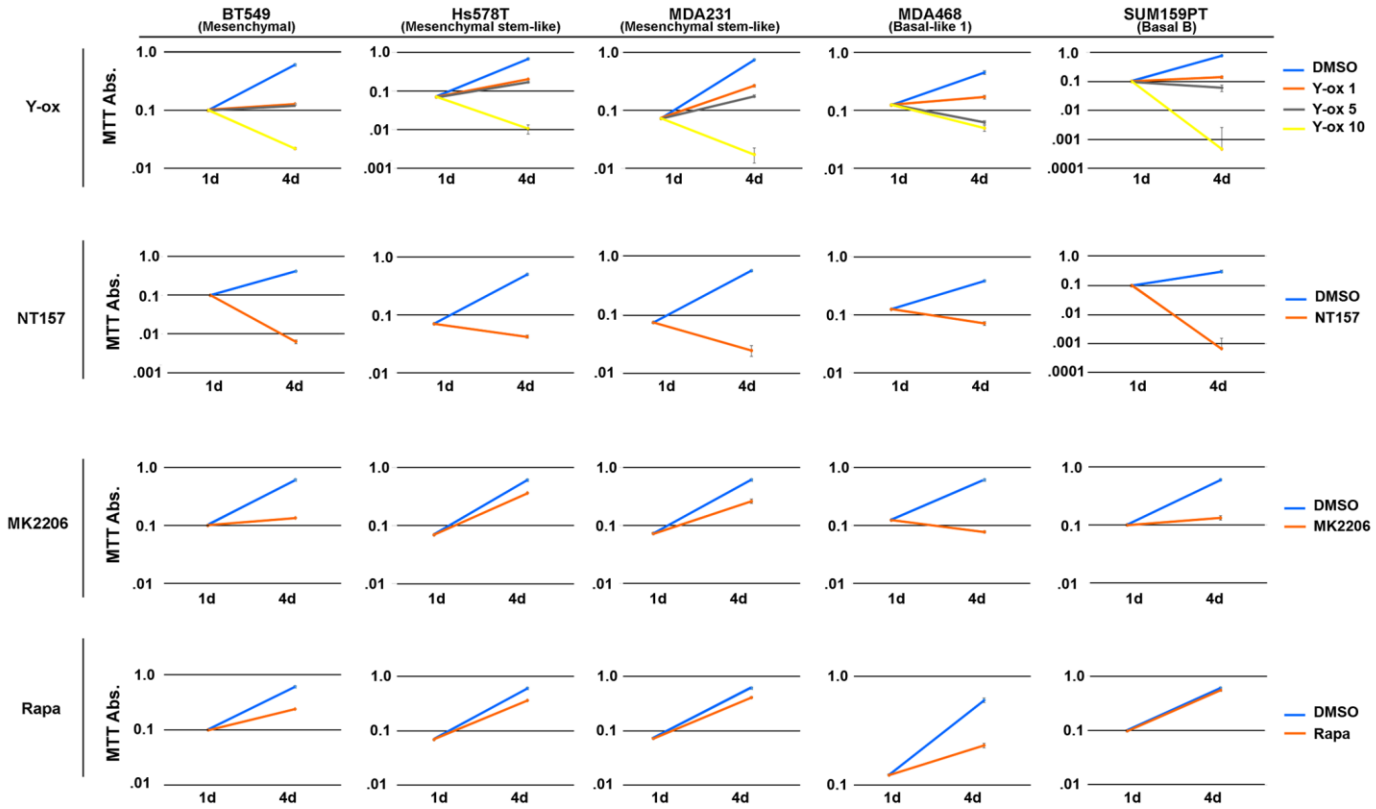

Fig 1. The effect of Y-ox and IRS1/2, AKT, mTORC1 inhibitors on TNBC cell viability. Five TNBC cell lines of different subtypes (indicated in parentheses) were treated with Y-ox (1, 5, or 10 μM), the IRS1/2 inhibitor NT157 (10μM), AKT inhibitor MK2206 (10μM), or mTORC1 inhibitor rapamycin (10 nM). Viability was assessed 1 and 4 days after treatment using the MTT assay kit from Thermo Fisher. An increase in MTT absorbance indicates increased cell proliferation, while decreased MTT absorbance reflects decreased number of viable cells (cell death).

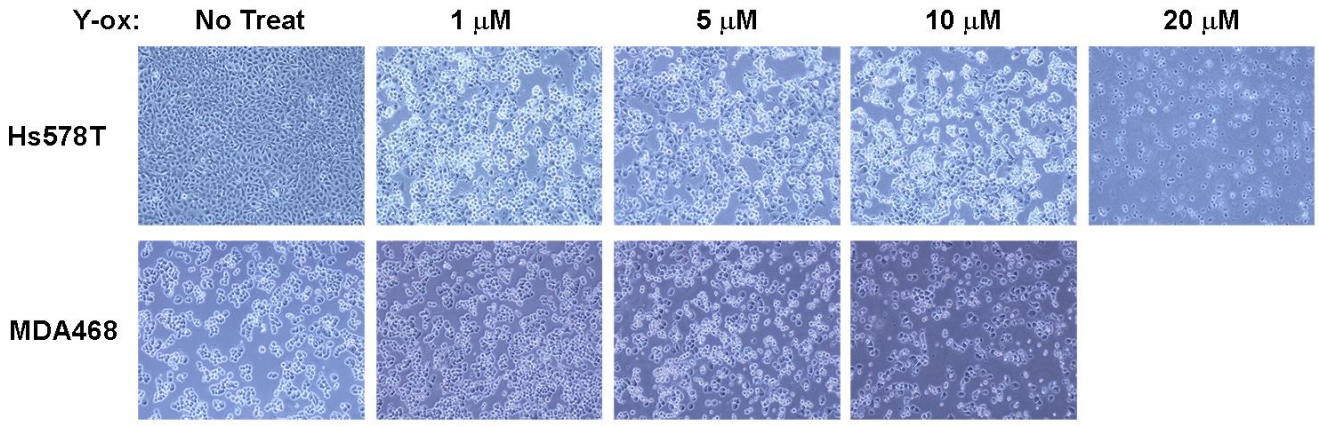

Fig. 2. Y-ox induces TNBC cell death. Hs578T and MDA468 cells were untreated or treated with the indicated dose of Y-ox for 24 hrs, and pictures of the cells taken. It can be seen that Y-ox kills the cells in a dose-dependent manner.

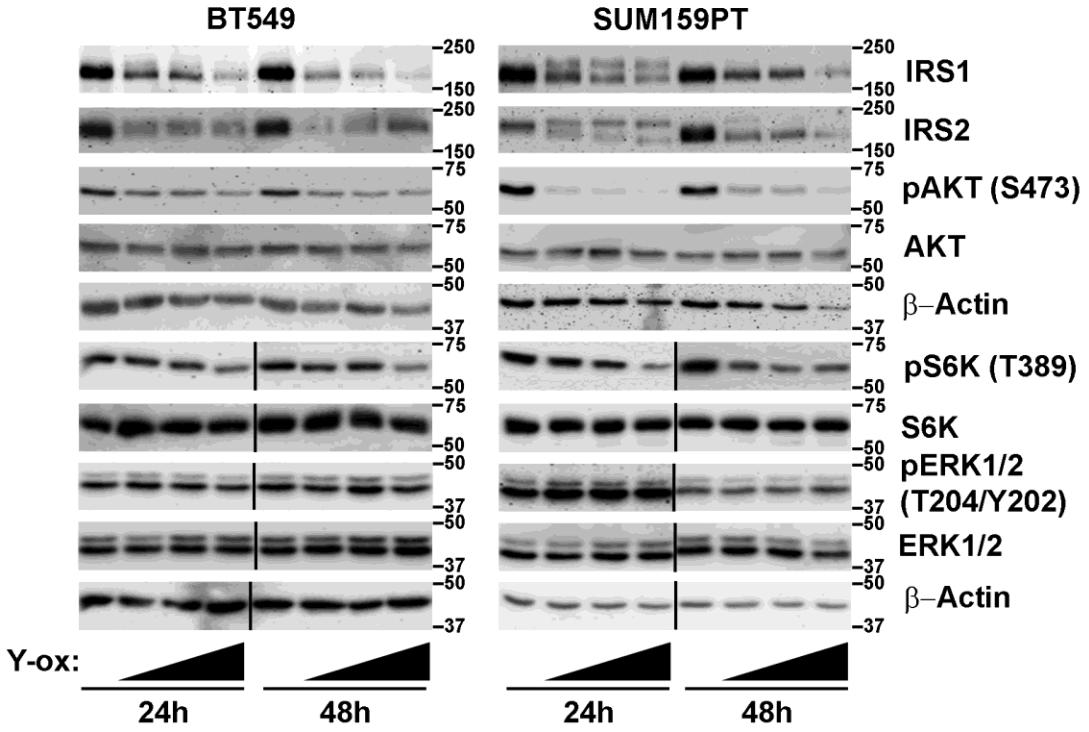

Fig 3. Y-ox causes IRS1 and IRS2 depletion and inhibits AKT and mTORC1 activity in TNBC cells. BT549 and SUM159PT were treated with increasing Y-ox doses (1, 5, 10μM) for 24 and 48 hrs, and levels of the indicated proteins determined by immunoblotting. IRS1/2, pAKT(S473), and total AKT were determined in one blot, and pS6K, total S6K, pERK1/2, and total ERK1/2 determined on another blot. β-actin levels were used in both blots as a loading control. Lines indicate where portions of the blots were pieced together.

lines tested. The results suggest loss of viability in TNBC cell lines treated with Y-ox could result from depletion of IRS1/2 and inhibition of the AKT-mTORC1 pathway. To test this, we treated TNBC cells with an IRS1/2 inhibitor (NT157), an AKT inhibitor (MK2206), and an mTORC1 inhibitor (rapamycin) and monitored proliferation and viability/survival by MTT absorbance. NT157 caused a pronounced loss of viability in all TNBC cell lines, similar to Y-ox (Fig 1). This is consistent with the idea that loss of viability in Y-ox treated cells results from IRS1/2 depletion. In contrast, MK2206 and rapamycin reduced proliferation partially in some but not all cells and did not cause the same pronounced loss of viability seen with Y-ox or NT157 treatment. The inability of MK2206 or rapamycin to cause loss of viability in these experiments could reflect feedback activation of alternative survival signaling pathways in the presence of these inhibitors. We conclude the PRCP/PREP inhibitor causes a pronounced loss of viability in TNBC cell lines due to depletion of IRS1/2. Inhibition of AKT-mTORC1 signaling may contribute partially to the effect.

We wished to know if knockdown of PRCP and/or PREP has the same effects as Y-ox. This is important because it will address whether the effect of Y-ox is through PRCP/PREP inhibition or some other mechanism. We proposed using lentiviruses to stably knockdown PRCP and PREP in TNBC cells, and then to measure the effect on IRS1/2, AKT, mTORC1, JAK2/STAT5, and ERK levels. However, given that PRCP/PREP inhibition by Y-ox caused such a pronounced loss of viability (Fig 1) it was unclear if stable knockdown cells would be viable and if they could be isolated. The shRNA lentivirus for PRCP knockdown encodes puromycin resistance

for selection, and the shRNA lentivirus for PREP knockdown encodes hygromycin resistance. SUM159PT cells (TNBC) were grown in culture dishes and either uninfected or infected with control shRNA lentiviruses or PRCP/PREP shRNA lentiviruses to knockdown PRCP and PREP. The cells were then placed in puromycin (Puro) or hygromycin (Hygro) to select and isolate the infected cells (Fig 4). The plates were stained with crystal violet to indicate cells that had survived. As expected, uninfected SUM159PT cells did not grow in puromycin or hygromycin (Fig 4). However, cells infected with shPRCP and its control virus grew in the presence of puromycin, and cells infected with shPREP and its control grew in hygromycin. As expcted cells doubly infected with both control viruses grew in puromycin and hygromycin. However, very few cells that were doubly infected with shPRCP and shPREP viruses remained on the plate in the presence of puromycin and hygromycin (Fig 4). This is consistent with Fig 1 and PRCP/PREP knockdown causing loss of viability. Consistent with loss of viability, we were unable to expand the remaining cells on the plate. We conclude PRCP/PREP knockdown causes loss of viability in these cells, as does Y-ox.

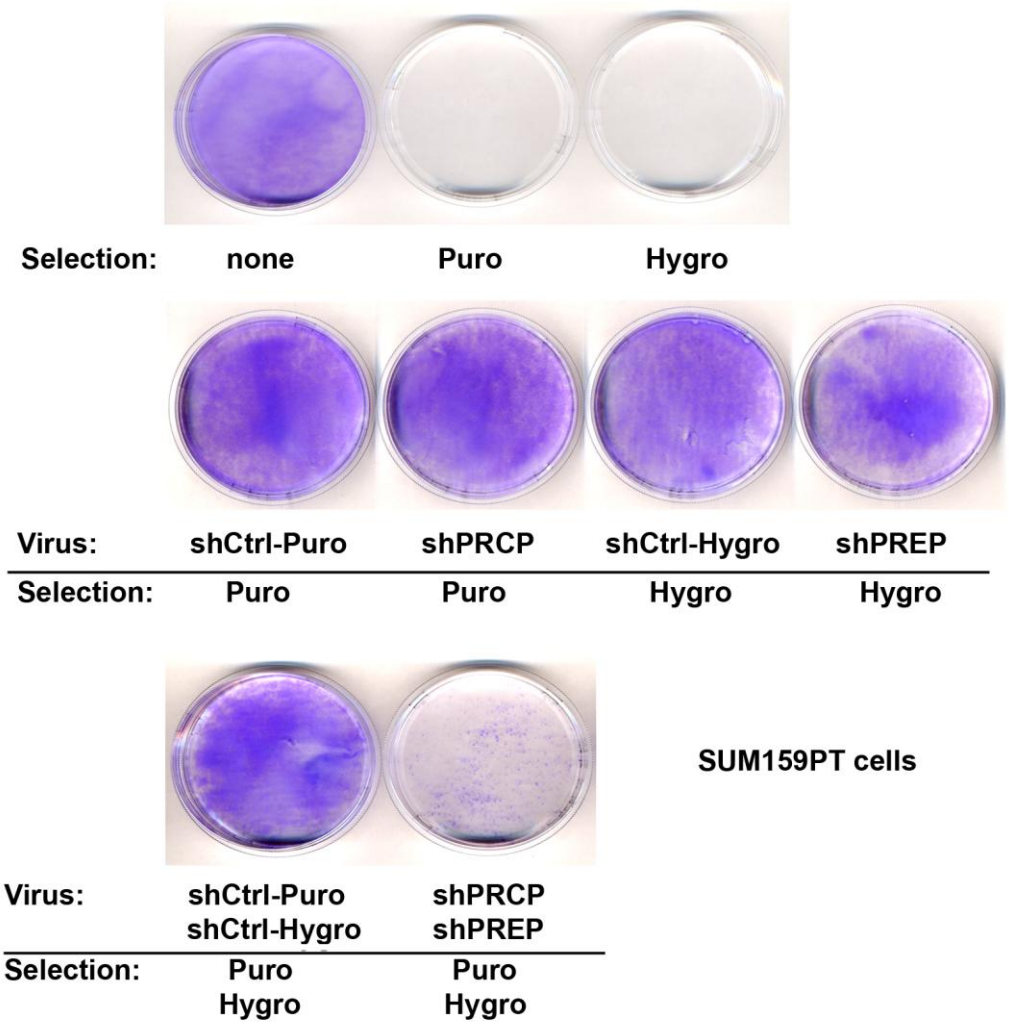

Fig 4. PRCP/PREP knockdown causes loss of TNBC cell viability. SUM159PT cells were uninfected, infected with shRNA control lentiviruses that also encode puromycin (Puro) or hygromycin (Hygro) resistance, or lentiviruses encoding shRNA against PRCP or PREP as indicated. The lentivirus encoding shRNA against PRCP also encodes Puro resistance, and lentivirus encoding shRNA against PREP encodes Hygro resistance. Infected cells underwent selection by growth in medium containing Puro and Hygro for 2 weeks as indicated. The cells were stained with crystal violet. Viable cells could not be isolated from SUM159PT doubly infected with shPRCP and shPREP lentiviruses (bottom row).

mTORC1 inhibitors have been tested in clinical trials for various cancers. However, these inhibitors have failed due, in part, to the fact that mTORC1 inhibition causes stabilization of IRS1 and activation of AKT, which promotes survival. Rapamycin is an mTORC1 inhibitor. In preliminary data for this grant we showed that Y-ox caused depletion of IRS1 and IRS2 in rapamycin treated TNBC cells and prevented activation of AKT. Based on those findings we proposed to ask if Y-ox and rapamcyin would cause an additive or synergistic killing of TNBC cells. To test this, we treated 6 different TNBC cell lines with Y-ox alone or in combination with two different doses of rapamycin. We measured survival and viability 4 days after treatment by MTT assay (Fig 5). Increasing doses of Y-ox reduced viability in all cell lines (the blue lines in Fig 5). Rapamycin alone at 1 and 10 nM caused a modest reduction in viability in most cell lines and a slightly greater reduction in viability, such as BT20 and MDA231. Rapamycin in combination with low doses of Y-ox (0.3 and 0.6 mM) caused an apparently additive reduction in viability in most cell lines, the exceptions being SUM159PT and Hs578T where combination of Y-ox with rapamycin had little effect to no effect. We conclude Y-ox in combination with rapamycin can cause a greater killing effect compared to either agent alone in some but not all TNBC cell lines, and the combined effect appears to be additive rather than synergistic.

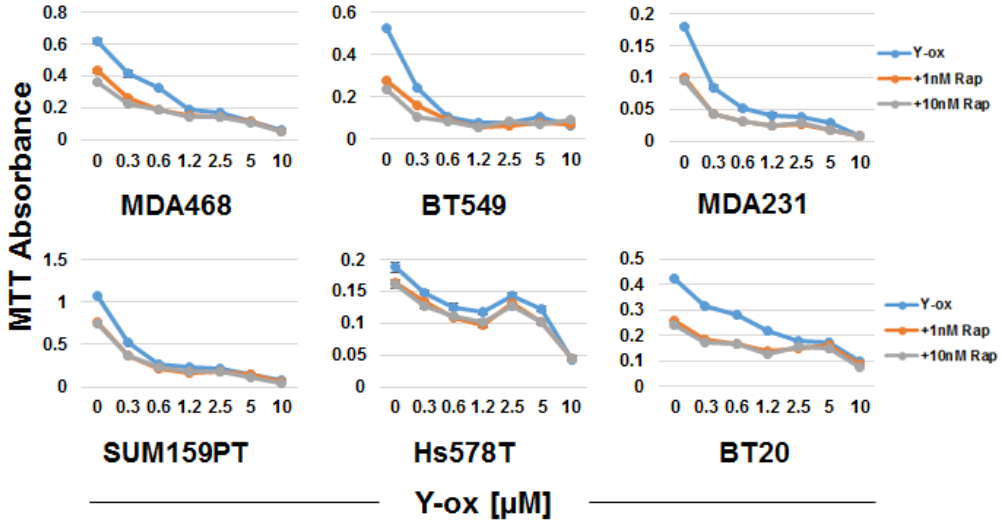

Fig 5. Effect of Y-ox plus rapamycin on TNBC viability. TNBC cell lines were treated with increasing doses of Y-ox alone or with 1 and 10 nM rapamycin, and viability determined at the 4 day time point by MTT assay.

A goal in this first year was to determine if our drug candidate Y-ox can inhibit TNBC tumor growth in mice. MDA231 human TNBC cells were grown in the mammary fat pad of immune compromised mice, and the mice were treated with vehicle or Y-ox when tumors reached 100-200 mm3. Treatment was daily (5days/wk) for 4 weeks and tumor measured every 4-5 days. As shown in Fig 6, Y-ox at both doses caused a pronounced inhibition of tumor growth. We conclude Y-ox has anti-tumor activity against MDA231 cell human TNBC tumor growth in mice.

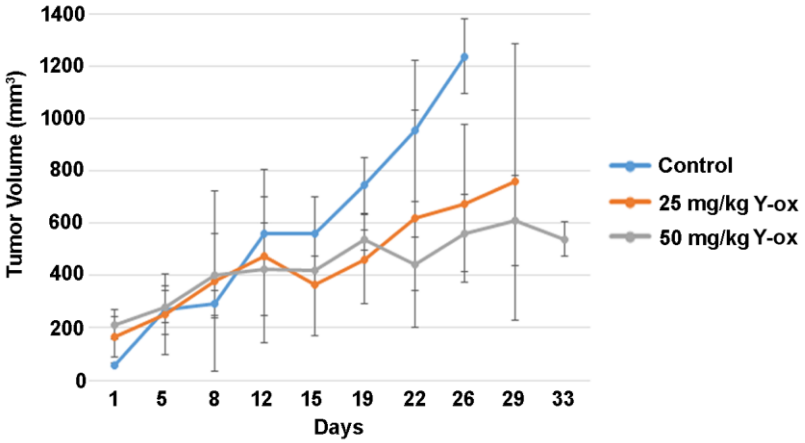

Fig 6. Y-ox inhibits TNBC tumor growth in mice. MDA231 cells were injected into the mouse mammary fat pad and allowed to form tumors. Treatment began when tumors were 100-200mm^3, as determined by caliper measure. Mice were treated daily with vehicle (control) or Y-ox at the indicated dose. Tumor growth was monitored for 4 weeks. The results show Y-ox at 25 and 50 mg/kg inhibited /reduced MDA231 tumor growth.

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Stated goals not met: We had proposed to test the ability of Y-ox to inhibit growth of two human TNBC cell lines xenograft tumors in mice during the first year. However, we only had enough drug (Y-ox) to test one (MDA231). The company from which we ordered synthesis of Y-ox had trouble producing it. However, they recently overcome the problem and sent us a 400 mg shipment that arrived on 2/24/2017. With the drug now in hand, we will move forward with testing the ability of Y-ox to inhibit growth of other human TNBC cell line xenograft tumors grown in the mouse mammary fat pad.

We proposed examining the effect of stable PRCP/PREP knockdown on IRS1/2 protein levels and on activation of signaling pathways downstream of IRS1/2. However, stable knockdown of PRCP and PREP caused a pronounced loss of viability in SUM159PT cells, similar to Y-ox (see Fig 4), and we were unable to isolate cells with stable knockdown of both proteins. Therefore, we were unable to examine the effect of stable PRCP/PREP knockdown on levels of the different proteins because we were unable to isolate cells with stable knockdown of both proteins. We expect this may be true in all cell lines. To overcome this problem we are planning on using siRNA to transiently knock down expression of PRCP and PREP in the various TNBC cell lines, and then testing if IRS1/2 is depleted and if downstream signaling pathways are inhibited.

We hypothesized PRCP/PREP over-expression would have the opposite effect than knockdown or inhibition. Namely, we predicted over-expression of PRCP/PREP would increase IRS1/2 levels and increase AKT-mTORC1 signaling and cell invasiveness. We recently isolated DNAs for generation of lentivirus that overexpress PRCP and PREP but have not yet generated these viruses. We plan to do so in the next funding period. However, we are also skeptical about obtaining overexpressing cells. This is based on our experience with MCF7, an estrogen receptor positive breast cancer cell line. We infected MCF7 with a PRCP overexpressing lentivirus and obtained a rare, single clone that overexpresses PRCP. The difficulty obtaining PRCP overexpressers suggests to us that cells cannot easily tolerate PRCP overexpression and that additional events must occur that allow cells to survive PRCP overexpression. If true, it may not be possible to obtain PRCP and PREP stable overexpressing cells.

3c. What opportunities for training and professional development has the project provided?
Nothing to report

3d. How were the results disseminated to communities of interest?
Nothing to report

3e. What do you plan to do during the next reporting period to accomplish the goals?
We have found that Y-ox reduces IRS1/2 levels and inhibits AKT activation in 4 TNBC cell lines (2 cell lines shown in Fig 3). During the next period we will determine if Y-ox inhibits IRS1/2 and inhibits AKT in at least one addition TNBC cell line, and also ask if MEK-ERK and Jak-STAT signaling are inhibited by Y-ox. We will test/confirm that Y-ox blocks IRS1 stabilization and AKT activation in TNBC cells treated with rapamycin. To test if PRCP and PREP depletion has the same effect as Y-ox we will use siRNA to deplete PRCP and PREP in TNBC cells and then ask if proliferation (cell counts) and viability (MTT assay) are inhibited, and if the IRS1/2-AKT-mTORC1 pathway is inhibited (by immunoblotting for IRS1/2, pAKT, and pS6K). Y-ox inhibited growth of MDA231 cells grown as xenograft tumors in mice (Fig 6). To ask if Y-ox has activity in mice against other TNBC tumors, we will grow 4 additional TNBC cell lines as xenograft tumors in the mouse mammary fat pad. We will then treat the mice with vehicle (control) or Y-ox and monitor tumor growth over time. We will isolate MDA231 and HS578T that overexpress firefly luciferase. These TNBC cells are metastatic and we will use them in year 3 to test if Y-ox can block metastatic TNBC tumor growth in mice. We will generate lentiviruses for overexpression of PRCP and PREP. We will then infect TNBC cells with these viruses and attempt to isolate overexpressing cells. If they can be isolated, we will determine by immunoblotting if they have increased IRS1/2 levels and increased AKT-mTORC1 pathway activation.

4. Impact

4a. What was the impact on the development of the principal discipline(s) of the project?
Nothing to report

4b. What was the impact on other disciplines?
Nothing to report

4c. What was the impact on technology transfer?
Nothing to report

4d. What was the impact on society beyond science and technology?
Nothing to report

5. Changes/Problems
We proposed examining the effect of stable PRCP/PREP knockdown on IRS1/2 protein levels and on activation of signaling pathways downstream of IRS1/2. However, stable knockdown of PRCP and PREP caused a pronounced loss of viability in SUM159PT cells, similar to Y-ox (see Fig 4), and we were unable to isolate cells with stable knockdown of both proteins. Therefore, we were unable to examine the effect of stable PRCP/PREP knockdown on levels of the different proteins because we were unable to isolate cells with stable knockdown of both proteins. We expect this may be true in all cell lines. To overcome this problem we are planning on using siRNA to transiently knock down expression of PRCP and PREP in the various TNBC cell lines, and then testing if IRS1/2 is depleted and if downstream signaling pathways are inhibited.

6. Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
7a. What individuals have worked on the project?

Name:	Ricardo Perez
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0001-5912-3505 (ORCID ID)
Nearest Person Months Worked:	12
Contribution to Project:	Mr Perez performed MTT assays, immunoblots, and conducted with Dr Duan the mouse studies examining Y-ox ability to inhibit MDA231 tumor growth.
Funding Support:	Rush University Graduate College funds

Name:	Lei Duan
Project Role:	Postdoc/Instructor, Key Personnel
Researcher Identifier (e.g. ORCID ID):	0000-0002-9531-0751 (ORCID ID)
Nearest Person Months Worked:	7.8
Contribution to Project:	Dr Duan was recently promoted to Assistant Professor and works with Dr Maki and in Dr Maki's lab. Dr Duan has directed the Graduate Student Ricardo Perez in his various experiments. Dr Duan worked with Ricardo Perez to carry out the mouse studies examining Y-ox ability to inhibit MDA231 tumor growth.
Funding Support:	

Name:	Carl Maki
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-1207-2250 (ORCID ID)
Nearest Person Months Worked:	3
Contribution to Project:	Dr Maki is directing all aspects of the project. He meets daily with Ricardo Perez and Dr Lei Duan to plan the experiments, troubleshoot problems, and discuss and interpret the results.
Funding Support:	

7b. 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

7c. What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. Appendices

none