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TITLE: Targeting CaSR/GABAB R1 Heterodimers to Treat Bone Metastases in Breast Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project are to test whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can antagonize the growth and/or survival of breast cancer cells exposed to high extracellular calcium *in vitro* or grown in animal models of bone metastases *in vivo*. In the first year, we have made considerable progress in achieving goals in Aims 1 and 2. We found that knocking down CaSR expression or inhibiting CaSR function with the calcilytic compound, NPS-2146, in breast cancer cells resulted in a reduction in the cAMP and PTHrP responses to high extracellular calcium levels. It also blunted proliferation and increased calcium-induced apoptosis. These changes in cell turnover were accompanied by an increase in p27 levels and an increase in nuclear AIF levels. We have generated stable GABABR1-knockdown MDA-MB231.1866 cells and are beginning to characterize their responses in the same manner as the CaSR-knockdown cells. We have also begun to examine how inhibition of the CaSR and GABABR1 may sensitize cancer cells to DNA damaging agents.

15. SUBJECT TERMS

Calcium-sensing receptor, Gaba B receptors, breast cancer, osteolytic bone metastases, parathyroid hormone-related protein, G-protein-coupled receptors

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Progress Report

1. Introduction.

This project focuses on a signaling pathway by which the formation of CaSR/GABABR1 heterodimers allows breast cancer cells to upregulate PTHrP production in response to high extracellular calcium concentrations. In turn, PTHrP acts in the nucleus to stimulate the proliferation of tumor cells and to protect them from the toxic effects of high extracellular calcium. Our hypothesis is that this pathway is critical for tumor cells to thrive in the bone microenvironment and that inhibiting CaSR/GABABR1 heterodimers will kill breast cancer cells in osteolytic bone metastases. The purpose of the project is to test this hypothesis by examining whether genetic or pharmacologic inhibition of the CaSR and/or GABABR1 will sensitize breast cancer cells to DNA-damaging agents in vitro and in bone metastases in vivo.

2. Keywords

Calcium-sensing receptor, Gaba B receptors, breast cancer, osteolytic bone metastases, parathyroid hormone-related protein, G-protein-coupled receptors

3. Accomplishments

Major Goals of the Project

The goal of this project remains the same: to test whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can antagonize the growth and/or survival of breast cancer cells exposed to high extracellular calcium *in vitro* or grown in animal models of bone metastases *in vivo*. We continue to work towards the original 3 specific aims:

Aim 1 - to determine whether genetic knockdown of the CaSR or the GABAB R1 inhibits PTHrP production, reduces proliferation and increases apoptosis of breast cancer cell lines exposed to high extracellular calcium.

Aim 2 – to determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can synergize with radiation or PARP activation to kill breast cancer cells at high extracellular calcium.

Aim 3 – to determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models.

Progress towards accomplishing Goals

We will report our progress and accomplishments as organized in the Statement of Work.

Aim 1, Task 1: Create stable GABAB R1 knockdown cell lines in BT474, 4T1 and MDA-MB231.1833 breast cancer cells.

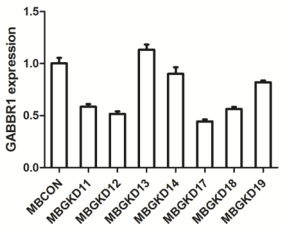

Fig. 1. Separate clones of MDA-MB231 cells with stable knockdown of GABBR1 expression. We chose to use clone 17, which had the greatest knockdown.

We had difficulty generating stable BT474 and 4T1 cell lines with significant knockdown of GABABR1, suggesting that loss of this protein was somehow toxic to these cells and/or put them at a competitive growth disadvantage. However, we did succeed with creating a stable knockdown cell line for MDA-MB231.1833 cells. We selected a cell line with about 55-60% reductions in GABABR1 expression to grow up and use in subsequent experiments (clone 17, see Fig. 1). Once we characterize this cell line, we will need to confirm key experiments in the other planned cell lines using transient knockdown strategies.

Milestone of Creating GABAB R1-knockdown cells is mostly achieved

Aim 1, Task 2: Examine cAMP levels, PTHrP production, cell proliferation and cell death in 4T1 and MDA-MB231.1833 CaSR-knockdown and control breast cancer cells.

We had been working on these experiments while the grant was under review and they were finished and published in *Cancer Research* just before the start of the current grant. We found that knocking down CaSR expression or inhibiting CaSR function with the calcilytic compound, NPS-2146, in breast cancer cells resulted in a reduction in the cAMP and PTHrP responses to high extracellular calcium levels. It also blunted proliferation and increased calcium-induced apoptosis. These changes in cell turnover were accompanied by an increase in p27 levels and an increase in nuclear AIF levels (see Fig. 2 and Kim et al Cancer Res 76:5348, 2016).

Milestone of Measuring effects of CaSR knockdown on cell growth in breast cancer cells has been achieved.

Aim 1, Task 3: Examine cAMP levels, PTHrP production, cell proliferation and cell death in BT474, 4T1 and MDA-MB231.1833 GABAB R1-knockdown and control breast cancer cells.

We are in the process of performing these experiments in GABABR1-knockdown MDA-MB231.1866 cells. Thus far, we have observed that knocking down GABBR1 in these cells appears to increase the baseline levels of cAMP and PTHLH mRNA expression but the increase in PTHrP in response to 5mM calcium is preserved. These data are not what we had expected and suggest that co-expression of the CaSR and GABBR1 tends to dampen the responsiveness of CaSR signaling without specifically altering its G-protein usage. These data will need to be repeated and the experiments will need to be performed in different cell lines before we can be sure whether this is the case. However, our results do show that the GABABR1 can modulate the CaSR-PTHrP axis in response to high extracellular calcium concentrations. We will also examine how GabaBR1 knockdown affects cell proliferation and apoptosis in the knockdown cells.

Milestone of Measuring the effects of GABAB R1 knockdown on cell growth in BT474, 4T1 and MDA-MB231.1833 cells is only partly achieved.

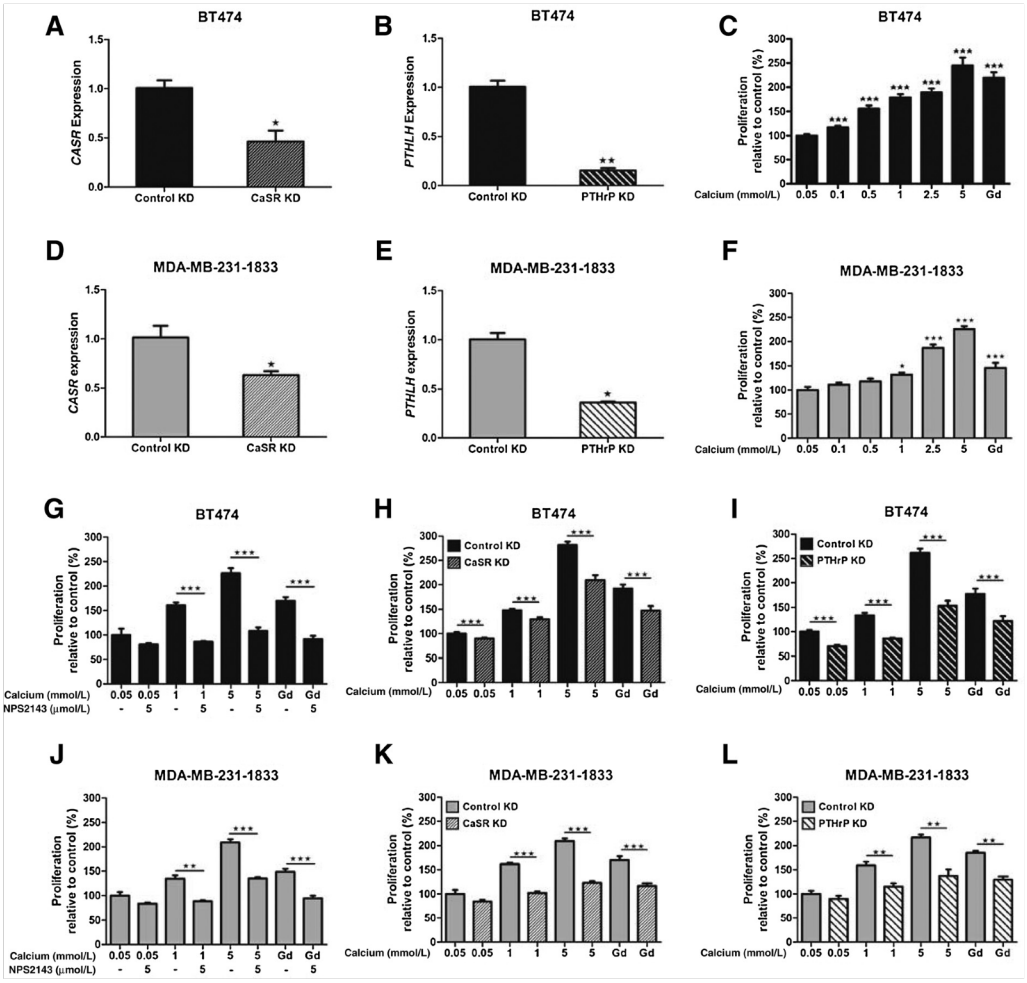

Figure 2

CASR and *PTHLH* mRNA levels in control and CaSRKD or PTHrPKD BT474 (**A** and **B**) or MDA-MB-231.1833 (**D** and **E**) cells. **C** and **F,** BrdUrd incorporation in response to increasing calcium or gadolinium (100 μmol/L) in control BT474 (**C**) and MDA-MB-231.1833 (**F**) cells. **G** and **J,** BrdUrd incorporation in control BT474 (**G**) or MDA-MB-231.1833 (**J**) cells ± treatment with NPS2143. **H** and **K,** BrdUrd incorporation in control vs. CaSRKD BT474 (**H**) or MDA-MB-231.1833 (**K**) cells. **I** and **L,** BrdUrd incorporation in control versus PTHrPKD BT474 (**I**) or MDA-MB-231.1833 (**L**) cells. Bars represent mean ± SEM, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Aim 2, Task 1: Examine whether CaSR-knockdown or GABAB R1-knockdown cells are more susceptible to cell death after treatment with MNNG or radiation
Aim 2, Task 2: Examine whether treatment with NPS2143 sensitizes breast cancer cells to MNNG or radiation

We have begun these experiments using MNNG treatment with CaSR-knockdown BT474 and MDA-MB-231.1877 cells. Our initial experiments demonstrate that loss of the CaSR or treatment with calcilytics appears to synergize with MNNG to promote cell death in these cells.

These experiments need to be repeated but our results thus far are consistent with the notion that loss of the CaSR-PTHrP may sensitize cells to the effects of PARP activation. If repeated experiments confirm our initial results, we will examine whether targeting the CaSR increases the accumulation of AIF into the nucleus of these cells.

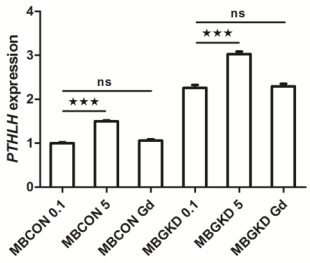

Fig. 3 PTHLH expression in control and GABBR1 KD cells in response to activation of the CaSR

Milestones to determine if genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers sensitizes breast cancer cells to MNNG or radiation have been partially achieved.

Specific Aim 3 - To determine whether inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models.

These experiments, which were planned to be performed between 18-36 mos into the project, have not been initiated yet.

Additional Experiments. While not part of the original proposal, we have recently observed that knockdown of the *CASR* or *PTHLH* genes is associated with decreased levels of Bcl-2 and a greatly increased Bax/Bcl-2 ratio. This is potentially significant since Bcl-2 and its family members have been described to regulate the exit of cleaved AIF out of the mitochondria and into the nucleus. Furthermore, previous papers have suggested that PTHrP regulates Bcl-2 mRNA expression in chondrocytes. Therefore, we hypothesize that the CaSR-PTHrP axis may regulate nuclear AIF levels indirectly, by controlling Bcl-2 expression.

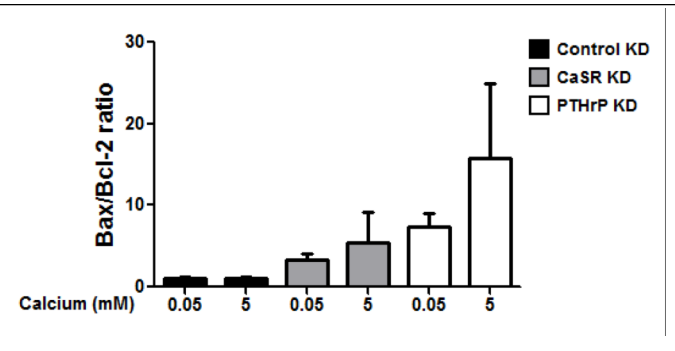

Fig. 4. Bax/Bcl-2 mRNA ratios in control, CaSR KD and PTHrP KD BT474 cells at low and high extracellular calcium concentrations

Opportunities for training and professional development

Nothing to report.

Dissemination of Results to Communities of Interest

Nothing to report.

Plans to Accomplish Goals During Next Reporting Period.

We plan to continue our work as organized in the SOW.

Aim 1, Task 1: Create stable GABAB R1 knockdown cell lines in BT474, 4T1 and MDA-MB231.1833 breast cancer cells

We will continue attempting to generate stable GABABR1-knockdown BT474 and 4T1 cells as outlined in the original proposal. If we continue to be unsuccessful in these cell lines, we will use the GABABR1-knockdown MDA-MB-231.1833 cells already established and will also use the same lentiviral shRNA constructs to attempt to create transient GABABR1-knockdown cells to use in some critical experiments.

Aim 1, Task 3: Examine cAMP levels, PTHrP production, cell proliferation and cell death in BT474, 4T1 and MDA-MB231.1833 GABAB R1-knockdown and control breast cancer cells

We will finish characterizing cAMP, PTHrP production as well as cell proliferation and cell death in the GABABR1-knockdown MDA-MB-231.1833 cells. As we are successful in creating GABBR1-knockdowns in the other cell lines, we will also perform the same experiments in those cells.

Aim 2, Task 1: Examine whether CaSR-knockdown or GABABR1-knockdown cells are more susceptible to cell death after treatment with MNNG or radiation

In the coming year, we plan to treat CaSR-knockdown cells and GABABR1-knockdown cells with MNNG in the presence of 0.5mM or 5mM calcium and assess cell viability and apoptosis. We will also examine total and nuclear AIF levels by immunoblot.

We will also examine the effects of knocking down the CaSR and GABABR1 on cell viability and apoptosis after radiation-induced DNA damage.

Aim 2, Task 2: Examine whether treatment with NPS2143 sensitizes breast cancer cells to MNNG or radiation

In the coming year, we plan to treat a series of breast cancer cell lines as well as dissociated cells from several different PDX breast cancer tumors with the calcialytic agent, NPS2143 combined with either MNNG or radiation. We will evaluate whether pharmacologic inhibition of the CaSR can sensitize cells to DNA damage in the presence of high extracellular calcium.

Aim 3, Task 1: Determine whether genetic knockdown of either CaSR or GABAB R1 expression inhibits the growth of osteolytic lesions

In the coming year, we plan to initiate these experiments by introducing CaSR-knockdown, MDA-MB-231.1833 cells into nude mice and examine whether this reduces the number and/or size of osteolytic bone metastases.

4. Impact

Impact on the principal discipline.

It is still early on in this project. However, this grant did support a review article written by Dr. Wonnam Kim and myself published in Frontiers in Physiology as part of a special series on the Calcium-Sensing Receptor. This article and a subsequent presentation at the 3rd International Symposium on the Calcium-Sensing Receptor in Florence Italy in the Spring of 2017 have placed the hypothesis that the CaSR is a potential target for breast cancer bone metastases out into the scientific community most interested in the physiology and pathophysiology of the CaSR. As we generate more publications, this project will also impact the wider breast cancer research community.

Impact on other disciplines.

Nothing to report

Impact on Society.

Nothing to report

5. Changes/Problems

Nothing to report.

6. Products

Kim, W and **Wysolmerski, JJ**. Calcium-sensing receptor in breast physiology and cancer. *Frontiers in Physiology* 7:440, 2016, PMCID: PMC5043011, doi: 10.3389/fphys.2016.00440.

7. Participants and Other Collaborating Organizations.

Individuals working on this project

Name: John Wysolmerski
Project Role: PI
Research Identifier:
Nearest person month worked: 1
Contribution to Project: Oversaw the entire project. Supervised Dr. Kim and Ms Dann.

Name: Wonnam Kim
Project Role: Post-doctoral Associate
Research Identifier:
Nearest person month worked: 12
Contribution to Project: Performed all experiments described in this report.

Name: Pamela Dann
Project Role: Senior Research Associate
Research Identifier:
Nearest person month worked: 2
Contribution to Project: Helped Dr. Kim with cell culture and routine proliferation and cell death assays.

Changes in Other Support for the PI

There has been a change in the PI's Other Support, although no changes that alter his effort on the current project.

The updated Other Support for Dr. Wysolmerski is contained in the following pages:

OTHER SUPPORT - WYSOLMERSKI, JOHN J

ACTIVE SUPPORT

1 R01 HD076248-01 Wysolmerski (PI) 04/01/2014 – 03/31/2019 2.4 calendar months
NIH/NICHD $219,540 (direct costs/yr)
PMCA2 Regulates Mammary Gland Involution
The major goals of this project are: 1) To determine whether continued transgenic expression of PMCA2 will prevent or delay the onset of cell death during mammary gland involution; 2) to examine whether interactions between NHERF1, NHERF2 and PMCA2 regulate PMCA2 localization and/or function during lactation and early involution; and 3) To examine whether PMCA2 and NHERF1 interact to regulate ErbB2 activity during lactation and early involution.
Role: PI
Funding Agency Contact: Daniel J. Raiten, raitend@mail.nih.gov; Eunice Kennedy Shriver National Institute of Child Health and Human Development
Overlap: None

BC151665 Wysolmerski (PI) 09/01/16 – 9/30/19 0.6 calendar months
DOD/BRCP $125,000 (direct costs/yr)
Targeting CaSR/GABAB R1 Heterodimers to Treat Bone Metastases in Breast Cancer
The major goals of this project will be 1) To determine whether genetic knockdown of the CaSR or the GABAB R1 inhibits PTHrP production, reduces proliferation and increases apoptosis in breast cancer cell lines exposed to high extracellular calcium; 2) To determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can synergize with radiation or PARP activation to kill breast cancer cells at high extracellular calcium; 3) To determine whether genetic or pharmacologic inhibition of CaSR/GABAB R1 heterodimers can inhibit the growth of osteolytic bone metastases in mouse models
Role: PI
Funding Agency Contact: Ms. Danielle Rickey (Danielle.l.reckley.civ@mail.mil) or Ms. Wendy Baker (wendy.a.baker.civ@mail.mil), DOD BCRP Grant Specialists
Overlap: None

1R21 AR070717-01 Wysolmerski (PI) 03/01/2017 – 02/28/2019 0.6 calendar months
NIH/NIAMS $150,000 (direct costs/yr)
FGF23 Contributes to the Pathophysiology of Humoral Hypercalcemia of Malignancy
The major goals of this project will be 1) To explore whether overexpression of PTHrP in breast tumors Induces osteocyte FGF23 production; 2) To explore whether inhibiting FGF23 function increases 1,25 $(OH)_2$ vitamin D levels and bone formation rates in mice with HHM; 3) To assess whether elevations in FGF23 contribute to tumor progression in HHM.
Role: PI
Funding Agency Contact: Ms. Stephanie Kreider (skreider@mail.nih.gov), NIAMS, NIH
Overlap: None

Yale Diabetes Research Center Pilot Wysolmerski (PI) 2/1/2017 – 1/31/2018 0.12 months
NIH/NIDDK $22,000 (direct costs)
PTHrP Causes Cancer Cachexia by Inducing White Adipocyte Browning
The major goals of this project are 1) to determine whether tumor-derived PTHrP disturbs systemic energy metabolism to induce cancer cachexia; and 2) to determine whether hypercalcemia contributes to the cachexia phenotype.
Role: PI
Funding agency Contact: Kathleen Marcucio (kathleen.marcucio@yale.edu), Yale School of Medicine
Overlap: None.

PENDING SUPPORT

1 R01 DK118739-01 Wysolmerski (PI) 04/01/2014 – 03/31/2019 3.6 calendar months
NIH/NIDDK $486,154 (direct costs/yr)
Heterodimerization of the Calcium-Sensing Receptor with the GabaB Receptors in the Breast
The major goals of this project are: 1) To examine whether heterodimerization with the GabaB receptors alters CaSR expression, signaling and PTHrP production in breast epithelial cells *in vitro*; 2) To use genetically modified mice to examine whether heterodimerization with GabaB receptors alters CaSR expression and function in the lactating breast *in vivo*; and 3) To use genetically modified mice to examine whether heterodimerization with GabaB receptors alters CaSR expression and function in breast cancers.
Role: PI
Overlap with current proposal: None

1 R21 AR073146-01 Wysolmerski (PI) 04/01/2018 – 03/31/2020 0.6 calendar months
NIH/NIAMS $150,000 (direct costs/yr)
PTHrP and Cancer Cachexia
The major goals of this project are: 1) To assess energy, fat and muscle metabolism in transgenic mice that overexpress PTHrP in breast cancers; 2) to explore whether hypercalcemia contributes to the metabolic effects of PTHrP to cause cancer cachexia; and 3) to examine energy, fat and muscle metabolism in a genetically modified mouse model of primary hyperparathyroidism.
Role: PI
Overlap with current proposal: None

BC171804 Wysolmerski and Bothwell (partnering PIs) 09/01/18 – 8/31/21 0.9 calendar months
DOD/BRCP $200,000 (direct costs/yr with partnering-PI, Alfred Bothwell)

B7-H4 in HER2-Positive Breast Cancer

The major goals of this project will be 1) To determine whether trastuzumab or lapatinib treatment increases B7-H4 levels in tumor and stromal cells, and alters TIL accumulation or composition within HER2-positive PDX tumors grown in mice with a "humanized" immune system and reconstituted with the patient's own PBMCs; and 2) To determine whether treatment of the above-mentioned PDX model with anti-B7-H4 blocking antibodies increases stromal TILs, alters TIL composition, and/or augments the therapeutic efficacy of trastuzumab.

Role: Submitting PI
Overlap: None