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

PRINCIPAL INVESTIGATOR: John Wysolmerski

CONTRACTING ORGANIZATION: Yale University
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14. ABSTRACT The goal of this project is 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 <i>in vitro</i> or grown in animal models of bone metastases <i>in vivo</i> . Progress was slowed this past year due to change in personnel. Dr. Kim left to take a faculty position in South Korea and, after an interval of several months, we hired Dr. Julie Hens to take Dr. Kim's place. Dr. Hens is an accomplished researcher with experience in developmental and cancer biology. She is in the process of developing tetracycline-regulated GABAB R1-knockdown cell lines to circumvent the cell death caused by stable chronic knockdown cell lines to study the effects of loss of GABB R1 on cAMP production, PTHrP expression and cell proliferation and apoptosis. She has validated that the blocking the CaSR with NPS2143 synergizes to increase cell death caused by a DNA damaging agent, MNNG and is now examining whether this agent will sensitize to radiation damage or platinum-based chemotherapy. Finally, she is begging to study the effects of NPS2143 on the growth of breast cancer cells in bone <i>in vivo</i> .					
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 proposed to study a signaling pathway in 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 was that this pathway is critical for tumor cells to thrive in the bone microenvironment and that inhibiting CaSR/GABABR1 heterodimers would 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 would 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.

Overall it should be noted that progress this year was slowed by the departure of Dr. Wonnam Kim, who left Yale to assume a faculty position in South Korea. After a period of 3-4 months,

we were able to hire Dr. Julie Hens, who is an Associate Research Scientist and will be performing most of the experiments going forward. We anticipate that progress will again accelerate in year 3 of the award.

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

Given our initial difficulty in generating stable BT474 and 4T1 cell lines with significant knockdown of GABABR1 and the suboptimal knockdown of GABABR1 expression in the MDA-MB231.1833 cells, when Dr. Hens took over this project, we decided to attempt to create regulated knockdown cell lines using a tetracycline-regulated system hoping to be able to study the effects of acute loss of GABAB R1 on the behavior of breast cancer cells and CaSR signaling. We will use the T-RexTM System with the pcDNATM6TR plasmid from Thermo-Fischer Scientific to generate the tetracycline-regulated plasmids to express shRNA against GABA BR1. We have previously successfully used this system to drive regulated expression of wild-type or mutant PMCA2 calcium pump expression in T47D breast cancer cells. We will concentrate our studies on MDA-MB231.1833 and 4T1 cells in order to examine the potential effects on bone metastases. Once we develop and characterize inducible knockdown cell lines for GABBR1, we will proceed with the experiments in Aim 1, Task 3.

Milestone of Creating GABAB R1-knockdown cells is in progress.

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.

These experiments were completed and published in *Cancer Research* in year 1 (Cancer Res 76:5348, 2016).

Milestone of Measuring effects of CaSR knockdown on cell growth in breast cancer cells was 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.

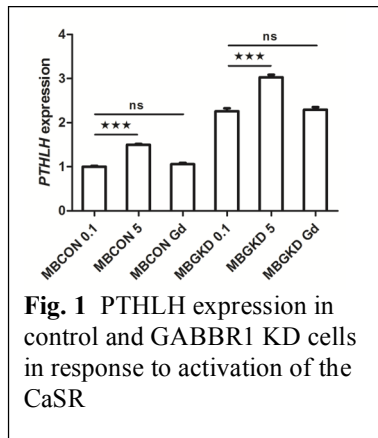


Fig. 1 PTHLH expression in control and GABBR1 KD cells in response to activation of the CaSR

Dr. Kim began performing these experiments in GABABR1-knockdown MDA-MB231.1866 cells before he left the laboratory. In cells in which we only achieved about 55% knockdown of GABBR1 mRNA, he noted that baseline levels of cAMP and PTHLH mRNA expression were increased and they were further increased by exposure to high extracellular calcium (Fig. 1). These data were contrary to our original hypothesis but they are consistent with the notion that GABBR1 alters CaSR signaling. However, we felt that it was difficult to fully interpret these experiments given the relatively modest reduction in GABBR1, which would presumably still allow for significant numbers of heterodimeric receptor complexes. Therefore, we plan to revisit these questions once we have better doxycycline-inducible knockdown cell lines. 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.

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

Initial experiments by Dr. Kim had suggested that combining MNNG treatment with CaSR-

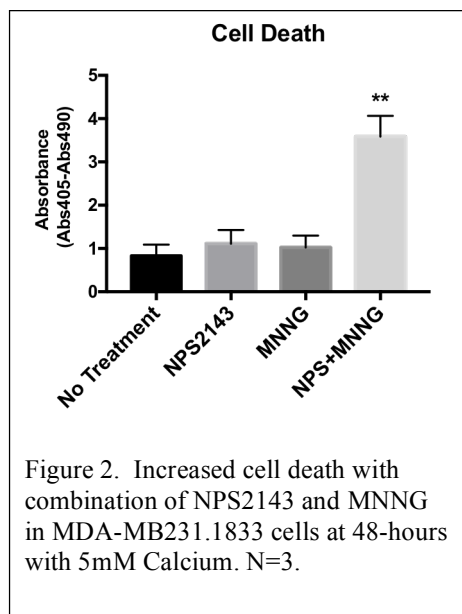


Figure 2. Increased cell death with combination of NPS2143 and MNNG in MDA-MB231.1833 cells at 48-hours with 5mM Calcium. N=3.

knockdown synergized to augment cell death. Dr. Hens has tried to extend these finding using the combination of NPS2143 and MNNG. As shown in Fig. 2, her initial experiments showed that the combination of NPS2143 with MNNG resulted in increased killing of MDA-MB-231.1833 cells at 5mM calcium. However, it has been very difficult to work with MNNG, which proves to be very toxic to these cells. In repeat experiments, the toxicity of MNNG alone has made it difficult to determine whether the combination with NPS 2143 is synergistic in a reproducible manner. She will next determine whether the same phenomenon occurs with radiation exposure or with other DNA damaging agents such as platinum-based chemotherapeutic agents.

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.

With the switch in personnel, we are behind schedule in starting these experiments. However, we have identified new collaborators who are performing intratibial injections of breast cancer cells routinely and we will pursue these experiments this year.

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 develop tetracycline-regulated GABBR1 knockdown cells as described above..

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

After developing tetracycline-regulated GABBR1 knockdown cells, we will proceed with the characterization of cAMP, PTHrP and proliferation assays.

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 or platinum 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

If we succeed with task 1, we plan to treat a series of dissociated cells from several different PDX breast cancer tumors with the CaSR-inhibiting agent, NPS2143 combined with either MNNG, platinum 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 and GABBR1-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.

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

Nothing to report.

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: Julie Hens, PhD

Project Role: Associate Research Scientist

Research Identifier:

Nearest person month worked: 12

Contribution to Project: Performed 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. Hens 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

PENDING SUPPORT

1 R21 AR073146-01A1 Wysolmerski (PI) 04/01/2019 – 03/31/2021 0.6 calendar months
NIH/NIAMS \$150,000 (direct costs/yr)

PTHrP and Cancer Cachexia

The major goals of this project are: 1) To determine the contributions of lipolysis, inflammatory cytokines and anorexia to the PTHrP-induced weight loss in a transgenic model of breast cancer; and 2) to explore whether hypercalcemia contributes to the metabolic effects of PTHrP to cause cancer cachexia.

Role: PI

Overlap with current proposal: None

Received 8th percentile score

1 R01 DK118739-01 Wysolmerski (PI) 04/01/2019 – 03/31/2013 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 how heterodimerization with GABBR1 or GABBR2 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

Received 25th percentile score