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TITLE: Targeting Discoidin Domain Receptors in Prostate Cancer

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14. ABSTRACT We report our major findings on our studies focusing on the Discoidin Domain Receptors (DDR), a set of kinase receptors that signal in response to collagen. The project's goal is to define the expression and therapeutic potential of DDRs in prostate cancer. During the first funding period, we conducted immunohistochemical studies by staining a 200 case Grade/Stage tissue microarray (TMA) that was examined for DDR1 expression using a highly specific antibody. This TMA includes 1600 cores that are being evaluated for antigen expression/localization and association with Gleason score. These studies are ongoing. We examined the anti-tumor effect of a highly specific DDR1 blocking antibody in a mouse model of intraosseous tumor growth using the PC3 human prostate cancer cell line. We obtained a humanized anti-DDR1 antibody, which blocks receptor activation by collagen I. Mice were inoculated with PC3 cells and anti-DDR1 or control antibody treatment. The study showed an apparent reduced tumor burden in the treated mice using bioluminescence. However, several mice suffered bone fractures, which compromised analyses of intraosseous tumor growth by bone histomorphometry, and thus these studies were inconclusive. We are currently fine-tuning the experimental conditions to prevent excessive intraosseous tumor growth by testing different cell inoculums.					
15. SUBJECT TERMS Prostate cancer, bone metastases, discoidin domain receptors, kinases, targeted therapies, immunohistochemistry					
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1. INTRODUCTION

Subject: Treatment of prostate cancer (PCa) patients with bone metastases remains a challenge due to the limited arsenal of effective therapeutic drugs that reduce disease progression. Therefore, a major goal in PCa research is to identify specific targetable molecules to prevent and/or diminish the ability of PCa cells to survive within the intraosseous environment. The subject of our project is a set of receptor tyrosine kinases (RTKs), known as Discoidin Domain Receptors (DDR), which signal in response to collagen, the major organic component of the bone extracellular matrix.

Purpose: To investigate the expression, therapeutic potential, and regulation of DDRs in PCa bone metastases.

Scope: Studies are proposed to define the expression of DDRs in PCa tissue specimens, the ability of DDRs to contribute to intraosseous tumor growth and define the regulation of DDRs in PCa cells.

2. KEYWORDS

Discoidin Domain Receptors, prostate cancer, bone metastases, collagen, tyrosine kinase, targeted therapy, extracellular matrix, signaling, antibodies,

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim 1. To investigate the expression of DDRs in our cohort of human PCa specimens and its association with clinical, pathological, and outcome data.

Task 1: To select and purchase tissue microarrays (TMA) from the Prostate Cancer Biorepository Network (PCBN).

Task 2: Conduct immunohistochemical (IHC) studies

Specific Aim 2. To evaluate the anti-cancer effects of DDR1 inhibitors in preclinical human-mouse xenograft models of primary and intraosseous PCa.

Task 3: Evaluate function-blocking antibodies in the orthotopic model of PCa

Task 4: Evaluate function-blocking antibodies in the intraosseous model of PCa

Specific Aim 3. To define the molecular and cellular bases of DDR regulation and signaling in PCa cell lines in cell based-assays.

Task 5: Analyses of DDR regulation, function, and signaling

- **What was accomplished under these goals?**

1) Major activities:

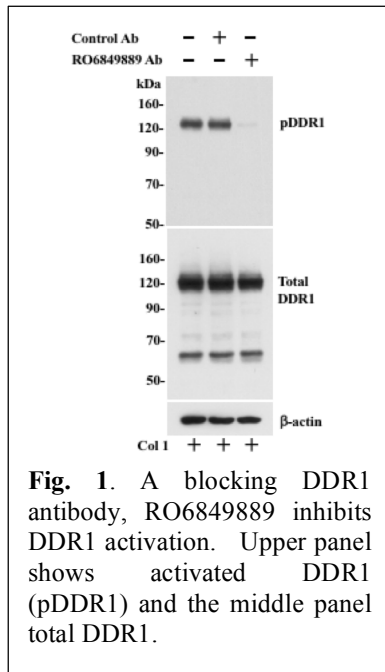
Since the starting of the funding period, we worked in the following Tasks (described above):

Task 1: We obtained the Tissue Microarray (TMA) from the Prostate Cancer Biorepository Network (PCBN) at the Johns Hopkins University Site. The PCBN is a public bioresource that provides tissue and other biospecimens to all prostate cancer investigators. It is supported in part by the Prostate Cancer Research Program of the CDRMP. We applied more than a year and half ago to request the TMA, which required an extensive explanation of the science, demonstration of the antibody specificity, and IRB approval. Finally, after complying with all the requirements, last year we received the 200 case Grade/Stage TMA set in 5 slides containing 1600 core tissues. The TMA provided information on tumor stage and grade and was blinded in relation to patient identification, as required. An IRB (exempt) was approved by Wayne State University for the use of this TMA as requested by the PCBN and provided to the CDMPR.

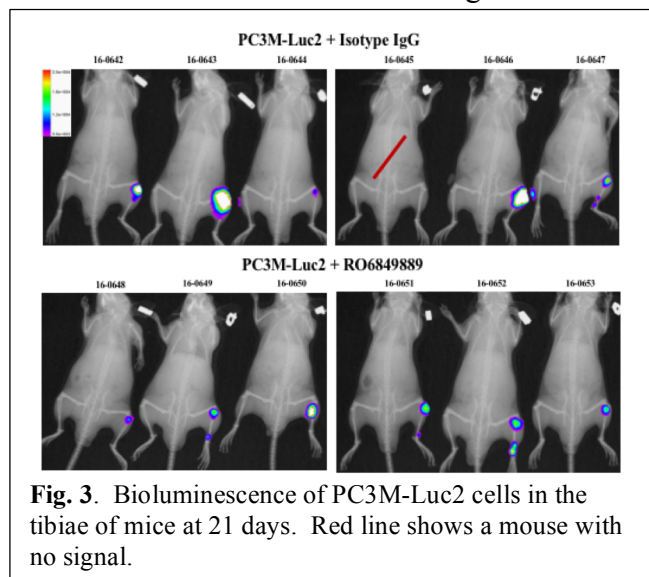
Task 2: We conducted preliminary IHC studies with a DDR1 antibody that was obtained from Dr. Marco Prunotto (Roche). The antibody was evaluated for specificity and optimal concentration using normal and tumor tissues of various sources. We also tested for specificity using cell lines with or without DDR1 expression.

Next, we stained the TMA using a protocol developed in our labs. Briefly, after deparafinization, hydration, and washing, the slides were subjected to antigen retrieval by microwaving. The staining of the tissues was performed using the ImmPRESS™ kit following the manufacturer instructions. After blocking with normal horse serum, the tissues were then incubated overnight with the rabbit DDR1 antibody (1:50) followed by the procedures and reagents of the kit. The 1600 cores of the TMAs are currently being evaluated by Dr. Dongping Shi, a pathologist with expertise in prostate cancer and co-investigator in this application. The data will be then analyzed by the Biostatistical Core of the Karmanos Cancer Institute. Preliminary analyses showed intense staining, which is mostly membranous. Any associations with stage/grade awaits a thorough analysis by the biostatistician.

Task 4: Through an MTA with Roche, we obtained a neutralizing antibody against DDR1 referred to as RO6849889 antibody. In the application, we reported the ability of the antibody to block DDR1 activation in response to collagen I in cancer cells. With award of the application and the animal protocol, Roche produced large quantities of RO6849889 for the mouse studies. This is a humanized rabbit antibody that only binds to human DDR1 and does not cross-react with mouse DDR1. Therefore, we set to test the new batch with MiaPaCa cells, which are pancreatic cancer cells that were transfected to overexpress DDR1b. We use these cells to easily visualize DDR1 activation by collagen I, and inhibition by the antibody. The cells were incubated in serum-free media with 2 µg/ml of either RO6849889 antibody or isotype control antibody for 30 min at 37°C before adding 20 µg/ml collagen I. After a 2-h incubation, the cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. The lysates were subjected to reducing 7.5% SDS-

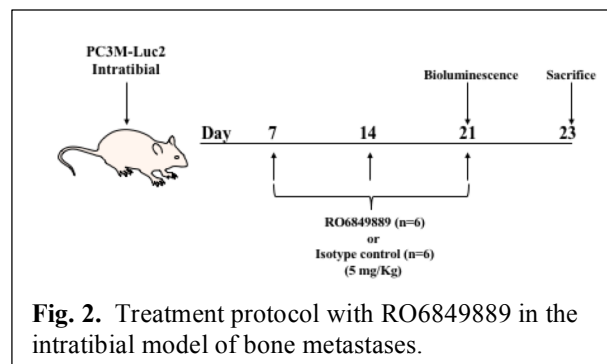


based on the results of the pharmacokinetic data obtained by Roche. Based on these results we decided to administer the compound with the schedule indicated in **Figure 2**. At day 21, the mice were examined for intraosseous tumor burden by bioluminescence after inoculation of luciferin and X-ray. This was performed using the In-Vivo Xtreme imaging system. Both types of images obtained were merged in order to detect and assess tumor burden through the bioluminescent image registered with the X-ray for anatomical context (**Figure 3**). Although the results of the bioluminescence images are not conclusive (without histomorphometry), we noticed



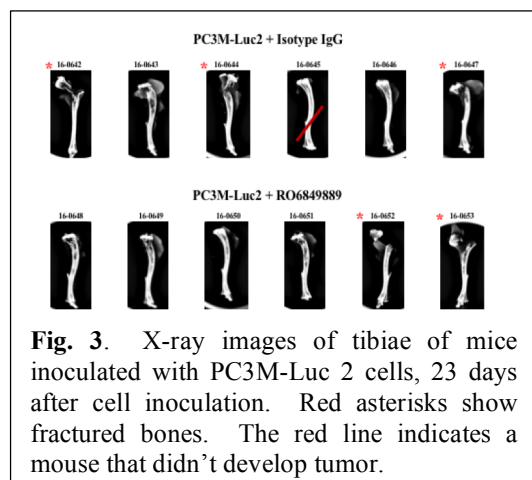
PAGE followed by immunoblotting phospho-DDR1 Y513 Ab from Cell Signaling Technology (CST) and the membrane was re-probed with DDR1 Ab, D1G6, from CST for total DDR1. As shown in **Figure 1**, RO6849889 Ab but not the isotype control antibody blocked DDR1-induced collagen I activation. These results gave us the justification to move into the mouse studies.

To evaluate the efficacy of the antibody, we used luciferase-transfected PC3 cells, referred to as PC3M-Luc2. These cells, originally derived from a patient's PCa bone metastasis, express endogenous DDR1 expression, which is activated in response to collagen I (data not shown), and luciferase. With the antibody and the cells in hand, we used the intratibial model, one of the most common techniques used to study tumor interaction with the host bone microenvironment, as stated in Task 4 of the SOW. Briefly, 7-weeks old male SCID mice were inoculated in the tibiae with 5×10^5 PC3M-Luc2 cells in a volume of 10 μ l and the mice were divided in two groups for administration of either RO6849889 or control antibody (5 mg/Kg). This antibody dose was selected



an apparent reduced intensity of luciferase in mice treated with RO6849889 (**Figure 3**). We also noticed that some mice had fractures of the proximal tibiae. Therefore, all the animals were sacrificed on day 23. After sacrifice, their tibiae were harvested and subjected to *ex vivo* X-ray imaging using the Trident Digital Specimen Radiography system. The X-ray images were used to determine bone response (osteolytic, osteosclerotic or mixed) in untreated or treated mice.

As shown in **Figure 4**, the X-ray images showed osteolytic response in all the tibiae that, in several cases were fractured, as shown



earlier with whole body imaging. The tibiae injected with the tumor cells and the contralateral tibiae (controls) were fixed, decalcified, and paraffin-embedded for ulterior longitudinal sectioning for H&E staining and iIHC for pan-cytokeratin, using our established protocols. Because of the presence of multiple tibial fractures that disrupted the continuity of the tumor tissue, we could not evaluate tumor burden by histomorphometry. Therefore, the results of this study were inconclusive.

2) Specific objectives:

The objectives during the period cover by this report were:

- a. Identify TMA resource and optimal antibodies and conduct immunohistochemical studies to define the expression of DDRs in prostate cancer and its association with disease progression.
- b. Initiate the mice studies with the blocking antibody.

3) Significant results or key outcomes:

Tasks 1 and 2: We have stained a TMA containing 200 cases (in 1600 cores) of prostate cancer samples obtained from the PCBN. We observed positive and strong staining and the complete analyses of the results are being conducted.

Task 4. We obtained and characterized a DDR1 neutralizing antibody RO6849889. The antibody blocks DDR1 phosphorylation in response to collagen I. We conducted studies to evaluate the efficacy of targeting DDR1 in a model of intraosseous tumor growth with PC3M-Luc2 cells. Positive outcomes: the cells formed intraosseous tumors that emitted detectable bioluminescence. Inoculation of the antibodies had no evident toxic effects on the mice. Negative outcomes: The results were inconclusive due to: 1. The aggressiveness of the cells, which caused bone fractures, and thus made it difficult to conduct histomorphometry analyses to measure tumor burden within the bone. 2. We need to improve the use of bioluminescence to better evaluate tumor burden. 3. Antibody schedule. Although the antibody is quite stable in mouse, we thought that it will be better to re-evaluate schedule. Because these cells are highly aggressive, we are considering to administer the first dose at earlier times to elicit a better anti-tumor effect before the cells establish a significant tumor burden.

Task 3: Not performed yet.

Task 5: Ongoing but nothing to report at this time.

4) Other achievements.

Nothing to Report.

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

Nothing to Report

- **How were the results disseminated to communities of interest?**

Nothing to Report

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

For the next reporting period we are planning the following studies based on the SOW:

Task 1 and Task 2: We will complete the analyses of the TMA staining with the 200 cases. Based on these results we will evaluate the next step, which may include correlations with development of bone metastases, and survival. We will discuss these results with the scientists at the PCBN, which can provide additional information in regards to the cases. We also plan to evaluate antibodies for DDR2.

Task 3: We will initiate the studies to evaluate the ability of the neutralizing antibody RO6849889 to elicit anti-tumor effect in orthotopic tumor growth of PC3M-Luc2 cells. We are planning first to inoculate a few mice to determine the optimal cell number and time of progression. Then we will design the treatment experiment.

Task 4: We will continue with the intratibial model. Because of the aggressive nature of the PC3M-Luc2 cells, we are currently evaluating the tumor cell inoculum in few mice to avoid causing bone fractures and keep an intraosseous tumor without breaching the cortex, which will make feasible the histomorphometrical analyses.

Task 5: We plan to conduct the studies proposed. Specifically, we will evaluate the roles of DDR1 in regulation of proliferation, migration and invasion. We will examine the cellular localization of DDR1 in PCa cell lines. Based on the results in mice, we will examine the ability of DDR1 activation to regulate the expression of osteolytic factors.

4. IMPACT

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

Nothing to Report.

- **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

- **Changes in approach and reasons for change**

There are no changes in the approach.

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

As stated, we encounter problems with the unexpected aggressiveness of the PC3M-Luc2 cells upon intratibial inoculation. This caused tumors to grow too fast and to produce bone fractures. Solution: we will inoculate a few mice with different amount of cells to determine optimal conditions.

- **Changes that had a significant impact on expenditures**

The hiring of a Research Scientist (5.40 calendar months) was delayed. Starting August 2016, this position will be filled by Dr. Anjum Sohail (5.40 calendar months), who will be conducting cell studies, as described in Task 5 of the SOW.

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

There were no changes in use or care of human subjects, vertebrate animals, and/or select agents. We do not anticipate future changes in these categories for the upcoming funding period.

6. PRODUCTS:

- **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?**

Name	Project Role	Nearest Person Months Worked	Contribution to Project	Funding Support
Rafael Fridman	PI	0.48	Design of experiments data analyses	This grant
Daniel Bonfil	Co-PI	0.48	Design of experiments data analyses	This grant
Dongping Shi	Co-PI	0.12	Analyses of TMA	This grant
Wei Chen	Biostatistician	0.12	Statistical analyses	This grant
Allen Saliganan	Research Scientist	5.40	Animal studies, immunostaining	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?**

New grant funded to Key Personnel in reporting period:

Rafael Fridman, PI in this grant:

Agency: DOD-BCRP

Grant #: Breakthrough Award L1 BC150621P1

Title: "Discoidin Domain Receptors: Novel Targets in Breast Cancer Bone Metastasis"

Period: 02/01/16-01/31/19
Effort: 0.72 Calendar Months
Role: Partnering PI

Daniel Bonfil, co-PI in this grant:

Agency: DOD-BCRP
Grant #: Breakthrough Award L1 BC150621P
Title: "Discoidin Domain Receptors: Novel Targets in Breast Cancer Bone Metastasis"
Period: 02/01/16-01/31/19
Effort: 1.2 Calendar Months
Role: Initiating PI

Agency: National Cancer Institute (NCI), National Institutes of Health (NIH),
Grant #: RO1 CA123362-05
Title: "PDGF D and Prostate Cancer Progression"
Period: 05/2016-04/2021
Effort: 0.6 Calendar Months
Role: co-I

Dongping Shi, co-PI in this grant:

Nothing to Report

- **What other organizations were involved as partners?**
 - **Organization Name:** Hoffmann-La Roche
 - **Location of Organization:** Basel, Switzerland
 - **Partner's contribution to the project**
 - **Other:** Supplied the neutralizing antibody to DDR1, referred to as RO6849889.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

Nothing to Report