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14. ABSTRACT

This application proposes to inhibit the Discoidin Domain Receptors both with a single agent and in conjunction with drugs targeting RAS-MEK-ERK as a new possible treatment for Pancreatic Ductal Adenocarcinoma (PDAC). We hypothesize that DDRs mediate the crosstalk between mutant Kras "addicted" PDAC cells with collagen, and thereby activate signaling pathways that promote tumor cell survival and malignancy (MEK resistance). Thus, disrupting DDR function by pharmacological or genetic means may attenuate PDAC pro-survival/fibrotic pathways and enhance therapeutic efficacy drugs targeting Kras-driven (MEK) signaling networks. In the first funding period, we examined the sensitivity of human PDAC cell lines to Trametinib, a MEK inhibitor. We also tested the role of overexpression and downregulation of DDR1 on Trametinib sensitivity. We found that downregulation of DDR1 in CFPAC-1 cells significantly increased the sensitivity of the cells to Trametinib, as determined in cell proliferation assays. In contrast, overexpression of DDR1b in MiaPaCa cells, which are devoid of endogenous DDR1, had no impact on Trametinib sensitivity. We were also expanding the KPC component mouse colonies (KrasLSL-G12D, trp53LSL-R172H and Pdx1-Cre, for the conduct of the animal studies. We have also created over 50 organoids cultures, with ~15 being bankable and expandable for the studies proposed.

15. SUBJECT TERMS
Pancreatic cancer, discoidin domain receptors, collagen, drug resistance, receptor tyrosine kinases, chemotherapy, MEK inhibitors, kinome reprograming

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1. INTRODUCTION

Pancreatic Ductal Adenocarcinoma (PDAC) is the ninth most common cancer but its dismal survival rate elevates it to the third leading cause of cancer-related death in the United States, and is on track to become the second most common cause of cancer related death before 2020. Chemotherapy and radiation therapy have little impact on PDAC, leaving surgery as the most effective treatment. Unfortunately, only ~20% of patients that undergo surgery survive past five years.

One of the hallmarks of PDAC is the intense pro-fibrotic response (collagen deposition). The extensive fibrosis in PDAC occupies a large area of the tumor mass, and is characterized by a dense matrix rich in interstitial collagen, hyaluronic acid, high number of stromal cells, hypovascularity, and hypoxic conditions, all of which have been postulated to contribute to the aggressive nature of PDAC tumors and their resistance to cytotoxic therapies. Collagen, the major component of fibrotic stroma, has been shown to elicit some of the pro-malignant effects of the desmoplastic stroma on the PDAC cells, including migration, invasion, survival, and drug resistance via regulation of epithelial-to-mesenchymal transition (EMT), protease production, and activation of TGF-β signaling, just to mention a few. These data point to an important pro-malignant effect of collagens in PDAC that may facilitate disease progression and resistance to treatment. Consistent with this notion, recent studies have postulated that therapies targeting the PDAC stroma or the crosstalk between PDAC cells and the collagen matrix may represent promising approaches for the treatment of PDAC.

The Discoidin Domain Receptors (DDRs) are unique RTKs because they are the only kinase receptors that recognize collagens as their ligands. Upon collagen binding, DDRs activate signaling pathways that regulate cell proliferation, migration, survival, and differentiation. Importantly, DDRs are emerging as new players in cancer progression because they mediate the interactions of tumor cells with their immediate collagen environment. The DDR family comprises two distinct members, DDR1 and DDR2, which undergo receptor autophosphorylation in response to fibrillar collagens (DDR1 and DDR2) or non-fibrillar collagen IV (DDR1), with distinctive activation kinetics and downstream effectors. DDRs regulate tumor cell migration and invasion, cell survival, and drug resistance. DDRs have been implicated in drug resistance to targeted therapies, and thus targeting DDRs may aid to increase drug sensitivity. Thus, DDRs are good candidates for mediating the pro-survival effects of the PDAC fibrotic stroma.

New therapeutic approaches are urgently required to target the pro-oncogenic signaling networks activated in PDAC. In excess of 90% of PDAC harbor oncogenic Kras mutations that drive tumorigenesis and disease progression. Mutant Kras signals via the downstream components the RAS/RAF/MEK/ERK pathway, and major efforts have been invested to target Kras and its effectors. Direct inhibition of Kras has proven to be challenging. Thus, most approaches target downstream effectors such as MEK1/2 for the treatment of Kras-ERK driven PDAC. However, single agents targeting Ras and/or RTK downstream effectors have yielded disappointing results. In the case of MEK inhibitors, feedback reactivation of ERK or PI3K signaling was shown to be mediated in part by compensatory RTK activation pathways (kinome reprograming) leading to MEK inhibitor resistance.

Kinome reprograming is a process in which cancer cells can rewire their signaling networks to restore ERK activity and override the actions of MEK inhibition by reactivating MEK2, resulting in c-myc degradation, and transcriptional activation of several RTKs. Importantly, studies in breast cancer cells showed that DDR1 is one of the RTKs that appears to compensate for the inhibition of MEK1/2 in AZD6244-resistant triple negative breast cancer cells. Consistently, downregulation of DDR1 restored MEK inhibitor sensitivity. As we described in our application, we searched for resistance to AZD6244, a MEK1/2 inhibitor, in several human PDAC cell lines harboring mutant Kras using the COSMIC database (http://cancer.sanger.ac.uk/cosmic), and compared the extent of MEK inhibitor resistance in the cells in relation to the expression levels of DDR1. Interestingly, we observed a direct relationship between higher levels of DDR1 and resistance to AZD6244, suggesting that DDR1 expression is associated with MEK inhibitor resistance. MiaPaCa-2 cells, a mesenchymal PDAC cell line, which only express DDR2, is the most sensitive to AZD6244. Based on this preliminary, yet interesting association, and the potential role of DDR1 in kinome reprograming we hypothesized that DDR1, but not DDR2, expression may be part of the genomic make up of Kras-mutated PDAC tumors displaying greater MEK inhibitor resistance. Thus, a combination of DDR1 and MEK inhibition may produce synthetic lethality in MEK-dependent mutated Kras-driven PDAC tumors thriving within the collagen-rich environment.

2. KEYWORDS

Discoidin domain receptors, pancreatic cancer, receptor tyrosine kinases, collagen, chemotherapy, drug resistance, MEK inhibitors, kinome reprograming

3. ACCOMPLISHMENTS

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

Specific Aim 1: Evaluate the Role of DDRs in Resistance to MEK Inhibition and their Effectiveness as Potential Therapeutic Targets in the KPC Mouse Model.

> **Major Task 1:** Evaluate roles of DDRs in KPC cell lines in *in vitro* studies
>
> **Major Task 2:** Evaluate role of DDRs in KPC cell lines in the orthotopic syngeneic mouse model
>
> **Major Task 3:** Evaluate the therapeutic effect of a pan-DDR kinase inhibitor (Compound A) in the KPC model of pancreatic cancer

Specific Aim 2: Establish the Anti-Tumor Effect of Single or Combinatorial Lethality of DDR1 Inhibition on Human PDX and Matched Organoid Cultures.

> **Major Task 4:** Evaluate Compound A in Organoids from Primary Tumor Lines Derived from PDAC Patients (PDX, currently in hand)
>
> **Major Task 5:** Evaluate the Therapeutic Response of human PDX to DDR plus MEK

Inhibition

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

1) Major activities:

Specific Aim 1: Evaluate the Role of DDRs in Resistance to MEK Inhibition and their Effectiveness as Potential Therapeutic Targets in the KPC Mouse Model.

Task 1. As we proposed in the original application, we focused initially on investigating the sensitivity of Kras-mutated mouse KPC and human PDAC cell lines to pharmacological or genetic inhibition of DDRs and MEK inhibitor response.

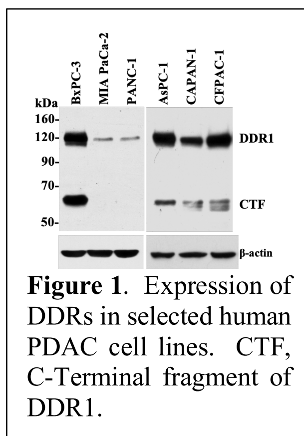

Figure 1. Expression of DDRs in selected human PDAC cell lines. CTF, C-Terminal fragment of DDR1.

To this end, we selected PDAC cell lines based on their profile of endogenous DDR1 expression (**Figure 1**). MiaPaCa and PANC1 cells, which are reported to be sensitive to the MEK inhibitor AZD6244, express low levels of DDR1, as we showed in the original application. In contrast, resistant cell lines usually express high levels of DDR1. To examine the role of DDR1 in MEK inhibitor response, we decided to utilize MiaPaCa and PANC-1 with modulated expression of recombinant DDR1. Thus, during the first funding period, we generated human PDAC MiaPaCa and PANC-1 cells overexpressing DDR1.

Figure 2 shows the expression and activation of two DDR1 isoforms, DDR1a and DDR1b, in the human MiaPaCa cell line. These stable transfectants were generated to express wild type (WT) DDR1a or DDR1b or DDR1 mutants. Specifically, the K618A (DDR1a) and K655A (DDR1b) mutants, which are unable to display kinase activity (referred to as kinase dead, KD), and the mutant R105A, which dampens binding to collagen, resulting in lack of ligand-stimulated activation.

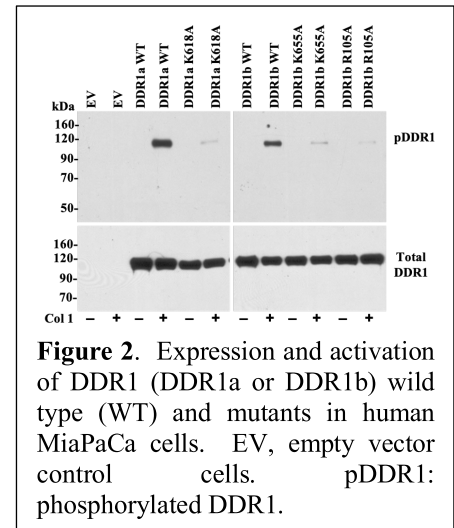

Figure 2. Expression and activation of DDR1 (DDR1a or DDR1b) wild type (WT) and mutants in human MiaPaCa cells. EV, empty vector control cells. pDDR1: phosphorylated DDR1.

Figure 3 shows the expression and activation of DDR1b, WT and K655A, and R105A mutants in human PANC-1 cells. As shown in **Figures 2 and 3**, WT DDR1 isoforms were activated in response to collagen I (COL1), a ligand for DDR1. In contrast, the K618A, K655A, and R105A mutants were not responsive, as expected. These figures also show that the level of total recombinant DDR1 expression in the transfectants is comparable.

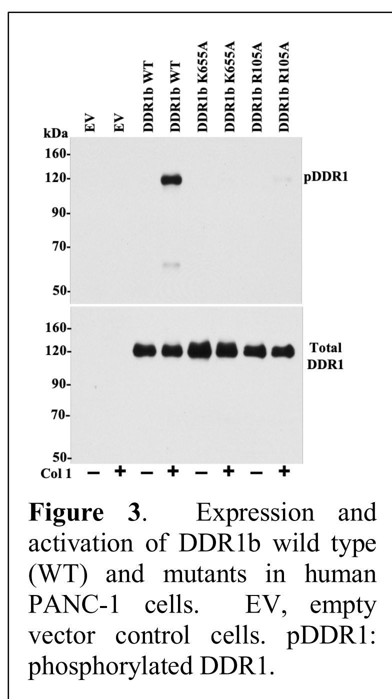

Figure 3. Expression and activation of DDR1b wild type (WT) and mutants in human PANC-1 cells. EV, empty vector control cells. pDDR1: phosphorylated DDR1.

Next, we tested the response of the human PDAC cell lines to MEK inhibition. To this end, we used Trametinib (Selleck Chemicals Cat. #: S2673), an FDA approved MEK inhibitor. First, we verified that the inhibitor inhibits constitutive ERK phosphorylation in MiaPaCa and ASPC-1 cells. These studies showed complete ERK1/2 phosphorylation by Trametinib, as expected (**Figure 4**).

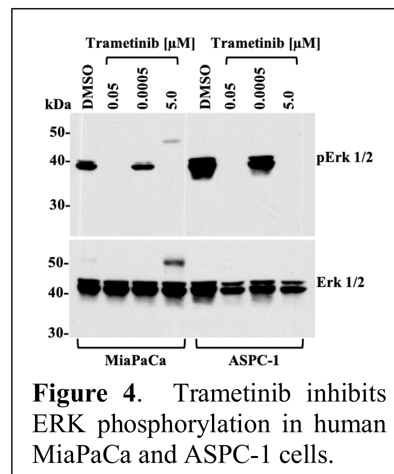

Figure 4. Trametinib inhibits ERK phosphorylation in human MiaPaCa and ASPC-1 cells.

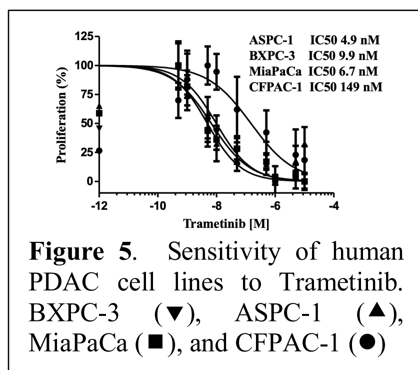

Figure 5. Sensitivity of human PDAC cell lines to Trametinib. BXPC-3 (▼), ASPC-1 (▲), MiaPaCa (■), and CFPAC-1 (●)

Next, we determined the sensitivity of ASPC-1, BXPC-3, CFPAC-1 and MiaPaCa cell lines to Trametinib. With the exception of MiaPaCa cells, the other cell lines express high levels of DDR1 (Fig. 1). The cells were incubated for 72 h with various doses of Trametinib in complete media and then analyzed with the MTT assay. No collagen was added in this experiment. As shown in **Figure 5**, CPFAC-1 cells were highly resistant to Trametinib with an IC50 of 149 nM. In contrast, ASPC-1, BXPC-3, and MiaPaCa cells displayed a comparable IC50: ~5-10 nM. From these preliminary results we conclude that sensitivity to Trametinib is not directly related to the levels of DDR1 expression in the absence of collagen stimulation. We are repeating this study with or without collagen.

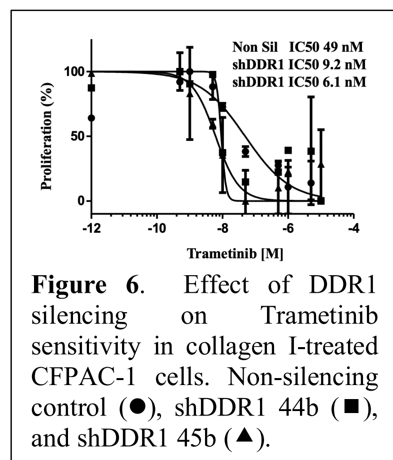

Figure 6. Effect of DDR1 silencing on Trametinib sensitivity in collagen I-treated CFPAC-1 cells. Non-silencing control (●), shDDR1 44b (■), and shDDR1 45b (▲).

Because CFPAC-1 cells express DDR1 and are highly resistant to Trametinib when compared to the other tested PDAC cell lines, we utilized two DDR1 silencing shRNAs (shDDR1 44b and 45b) to downregulate DDR1 in these cells. These stable cell lines with control non-silencing shRNA or shDDR1 were treated with various Trametinib concentrations in the presence of collagen I to activate the receptor. As shown in **Figure 6**, DDR1 downregulation with two different shRNA constructs reduced the IC50 of Trametinib by 5-7-fold compared to cells with the non-

silencing shRNA, suggesting that expression of DDR1 is associated with increased resistance to Trametinib, at least in CFPAC-1.

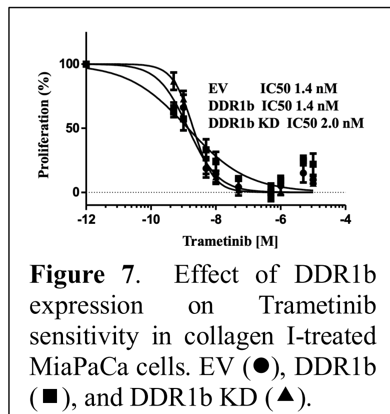

Figure 7. Effect of DDR1b expression on Trametinib sensitivity in collagen I-treated MiaPaCa cells. EV (●), DDR1b (■), and DDR1b KD (▲).

Having generated MiaPaCa cells overexpressing WT or KD DDR1b, we decided to conduct a preliminary experiment to examine whether ectopic expression of recombinant DDR1b alters sensitivity to Trametinib (**Figure 7**). To this end, the cells were treated with various Trametinib concentrations in the presence of collagen I to activate the receptor. These studies showed that in MiaPaCa cells, DDR1b overexpression and activation does not alter Trametinib sensitivity.

Task 2: Nothing to Report at this junction. However, we are initiating the studies on inhibitor response with the KPC cell lines. We are also modulating DDR expression in these cells.

Task 3: We are currently in the process of expanding the KPC component mouse colonies (Kras$^{LSL-G12D}$, trp53$^{LSL-R172H}$ and Pdx1-Cre. Previous plans to use a Ptf1a-Cre driver have been discarded due to a recent uptick in that KPC colony developing brain tumors leading to hind limb paralysis. This is a known, but underreported, phenotype of these mice that usually affects a negligible number of mice, but has afflicted over half our mice in the past year.

We have expanded these colonies sufficiently to maximize the generation of KPC mice for our preclinical studies, with the goal being to have 3 mice per litter available for enrollment. With this in mind, while enrollment will be "as available" this will maximize the number of mice of the same age, treated at the same time, minimizing some sources of variability.

Specific Aim 2: Establish the Anti-Tumor Effect of Single or Combinatorial Lethality of DDR1 Inhibition on Human PDX and Matched Organoid Cultures.

Task 4: We hired a technician, Mr. Daniel Paglia, to assist in the creation of organoid cultures derived from fine needle biopsies from pancreatic cancer patients. The advantage of this process is it is all-inclusive of pancreatic cancer patients, whereas organoid systems derived from resection surgery only includes ~20% of patients. With Mr. Paglia's assistance, we have created over 50 FNB organoids cultures, with ~15 being bankable and expandable for these studies. We are now ready to begin the treating the cultures as described in the proposal.

Task 5: Nothing to report

2) **Specific objectives:**

The objectives during the period covered by this report were:

a. To generate PDAC cell lines (human and mouse) with modulated DDR expression.

b. Conduct studies to examine the sensitivity of PDAC cell lines to Trametinib.

c. Examine the effects of DDR1 overexpression or downregulation on Trametinib sensitivity

d. Generate KPC mice for treatment with MEK and DDR1 inhibitor combinations.

e. Develop a small expandable library of organoid cultures for MEK and DDR1 inhibitor treatments.

3) Significant results or key outcomes:

Specific Aim 1, Task 1:

We generated several human PDAC cell lines with upregulated or downregulated expression of DDRs.

Conducted cell proliferation assays of these cell lines in response to Trametinib with cells displaying upregulated (MiaPaCa) or downregulated (CFPAC-1) DDR1 expression.

Specific Aim 1, Task 2:

Nothing to report

Specific Aim 1, Task 3:

Breeding colonies have been established and mice are currently being enrolled in the described preclinical trial.

Specific Aim 2, Task 4:

Sufficient numbers of expandable organoid cultures have been established for inhibitor studies.

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 period, we plan to perform the following studies, as per the SOW:

Specific Aim 1, Tasks 1-3:

a. We will regulate DDR expression in the KPC cell lines.

b. We will continue to examine the effects DDR1 expression and activation on Trametinib sensitivity in human and mouse PDAC cell lines.

c. We will examine effects of Compound A (DDR1 inhibition) on Trametinib sensitivity.

d. We will perform the animal studies.

Specific Aim 2.

a. KPC mice will be enrolled and treated with DDR1 and MEK inhibitor combinations.

b. Organoids will be treated with DDR1 and MEK inhibitor combinations and analyzed for effects on cell viability and proliferation.

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

As mentioned above, we have had to shift from the proposed KPC model driven by the Ptf1a-Cre allele to the Pdx1-Cre allele, due to prevalent brain tumor formation in the former model. This will change nothing about the proposed experiments. We have extensive experience with both models.

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

See above section.

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

Nothing to report.

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

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

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? See Note below Table**

Name	Project Role	Nearest Person Months Worked	Contribution to the Project	Funding Support
Rafael Fridman	PI	0.24	Design of experiments and data analyses	This grant
Anjum Sohail	Research Scientist	3		This grant
Howard Crawford (University of Michigan)	Co-I	0.24	Design of experiments and data analyses	Subcontract
Daniel Paglia (University of Michigan)	Research Technician	3	Establishment and maintenance of organoid cultures	Subcontract

- **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?**

Organization Name: Hoffmann-La Roche
Location of organization: Basel, Switzerland
Partner's contribution to the project: Supplied antibodies for DDR1 and a small molecule inhibitor for DDR1.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report.