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PRINCIPAL INVESTIGATOR: Malay Haldar, MD, PhD

CONTRACTING ORGANIZATION: University of Pennsylvania, Philadelphia, PA

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Malay Haldar

E-Mail: mhaldar@pennmedicine.upenn.edu

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University of Pennsylvania
3451 Walnut Street
Franklin Building, 5th Floor
Philadelphia, PA, 19104

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14. ABSTRACT

The objective of this proposal is to examine our hypothesis that *inhibition of RA signaling can elicit therapeutic immune responses in hepatocellular carcinoma*. We will test this hypothesis in murine models of HCC by (1) reducing RA production with novel RALDH1 inhibitors we developed, (2) inhibiting RA signaling with commercially available small molecule inhibitor of RAR/RXR, and (3) boosting T cell responses by anti-PD1. To our knowledge, this would be the first attempt to inhibit RALDH1 for immunotherapy of any cancer. We have made significant progress towards these goals in this reporting period (2021 – 2022). We demonstrate that our novel RALDH1 inhibitors can abrogate RA production in HCC cells. RA derived from HCC suppressed DC and promoted macrophage differentiation from monocytes; an effect that was reversed upon treatment of HCC cells with our RALDH1-inhibitors. *In vivo*, our RALDH1 inhibitors reduced immunosuppressive macrophages and suppressed tumor growth in HCC. Finally, we genetic deletion of RALDH1 in HCC and RALDH1 inhibitors showed similar tumor suppressive effects in HCC. Taken together, our findings thus far strongly supports RA inhibition as a therapeutic strategy in HCC.

15. SUBJECT TERMS
Retinoic Acid, Retinaldehyde Dehydrogenase, Hepatocellular Carcinoma, Immunotherapy

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1. Introduction

In the United States, nearly 33,000 people are diagnosed with liver cancer and 27,000 die from this disease each year. Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the third leading cause of cancer-related death worldwide. Once the disease metastasizes, treatment options are limited and the prognosis is very poor. Overall, the 5-year survival drops from about 32% in 'early stage' to less than 2% in metastatic HCC. Given this dismal prognosis, immune checkpoint blockade (ICB) therapy was recently approved for advanced metastatic HCC. However, ICB is not as effective here as in some other types of tumor and most patients do not respond. While the majority of current modalities in cancer immunotherapy act on T-cells, our laboratory is interested in targeting another key cell-type of the immune system – antigen-presenting cells. We have discovered that many solid tumors produce retinoic acid, which acts on antigen-presenting cells to create an immune-suppressive tumor microenvironment. Importantly, we showed that blocking retinoic acid in such tumors lead to anti-tumor immune responses. Hence, retinoic acid represents a new target for cancer immunotherapy. *The primary objective of this project is to determine whether targeting retinoic acid signaling is effective for HCC immunotherapy*. We have found that HCC expresses high levels of the RALDH1 enzyme to produce RA. Thus, we will examine whether RALDH1 inhibition with our novel compounds can reduce retinoic acid levels in HCC to promote anti-tumor immune responses and then test whether this approach alone or in combination with ICB can restrain HCC growth.

2. Keywords:

Retinoic acid, Retinaldehyde dehydrogenase, Dendritic cells, Macrophages, Hepatocellular Carcinoma.

3. Accomplishments

What were the major goals of the project?

Specific Aim 1: Impact of RALDH1 inhibition on HCC		
Major Task 1: To identify the impact of RALDH1 inhibition on RA production by HCC cells		
Subtask 1: Measure retinoic acid levels in human HCC cells with and without RALDH1 inhibitors using LC/MS. Cell lines used: SNU398 [Source: Simon Lab] Progress: Completed. RALDH1 inhibitors abrogated RA production in SNU398.	1-3	Haldar Lab
Subtask 2: Measure retinoic acid levels in mouse HCC cells with and without RALDH1 inhibitors using LC/MS. Cell lines used: Hepa 1-6 [Source: ATCC] Progress: Completed. The original proposed RALDH1 inhibitors C-86 and C-91 abrogated RA production in human HCC but, surprisingly, not in mouse HCC. As described further under the accomplishment section, we discovered the underlying reason being the differences between species in the drug binding residues. Importantly, in collaboration with scientists at NCATS, we were able to identify a compound within the same chemical genus of C-86 and C-91 that was active on mouse RALDH1. This compound is designated as C-99 and used for all experiments that require inhibition of murine RALDH1.	1-3	Haldar Lab
Major Task 2: To examine the impact of inhibiting HCC RA production on monocyte differentiation		
Subtask 1: Analyze human monocyte differentiation with reduced RA production by HCC. Cell lines used: SNU398 [Source: Simon Lab] and Primary human monocytes [Human immunology core, UPenn] Progress: Completed. As anticipated, HCC-derived RA had profound impact on monocyte differentiation, which was blocked in the presence of RALDH1-inhibitors.	3-6	Haldar Lab
Subtask 2: Analyze mouse monocyte differentiation with reduced RA production by HCC. Cell lines used: Hepa 1-6 [Source: ATCC] and Primary mouse monocytes [Haldar Lab] Progress: Completed. As anticipated, HCC-derived RA had profound impact on monocyte differentiation, which was blocked in the presence of RALDH1-inhibitors.	3-6	Haldar Lab
Major Task 3: To examine the impact of inhibiting HCC RA production on the tumor microenvironment		
Subtask 1: Analyze impact of RALDH1 inhibition on human HCC microenvironment. [3 treatment conditions X 3 endpoints X 8 mice per group X 5 overall experimental repeats	8-12	Haldar Lab

= 360 mice total] Cell lines and Model: SNU398 [Source: Simon Lab] and Immunodeficient NU/J mice [Jackson Laboratory] Progress: Completed. As described below under the accomplishments section, the SNU398 HCC cell lines did not grow well in immunodeficient mice. Therefore, we used another commonly used human HCC cell line – Huh7, for all xenotransplantation experiments. Of note, SNU398 and Huh7 both showed RALDH1-dependent RA production. Indeed, we have found that RALDH1-mediated high RA production is a common theme in all human and murine HCC cell lines that we tested.		
Subtask 2: Analyze impact of RALDH1 inhibition on murine HCC microenvironment. [3 treatment conditions X 3 endpoints X 8 mice per group X 5 overall experimental repeats = 360 mice total] Cell lines and Model: Hepa 1-6 [Source: ATCC] and Immunocompetent C57BL/6J mice [Jackson Laboratory] Progress: 50% completed. As described below in 'accomplishments', the Hepa 1-6 cell line showed significant T cell responses that often led to tumor rejection when transplanted into C57BL/6J mice. Therefore, we will use another commonly used murine HCC cell line – Hep55 for syngeneic transplantation experiments.	8-12	Haldar Lab
Major Task 4: To examine the general impact of RALDH1 inhibition on immune cells *in vivo*		
Subtask 1: Examine the impact of compound 86 on immune cells. [2 treatment conditions X 8 mice per group = 16 mice total] Cell lines and Model: Immunocompetent C57BL/6J mice [Jackson Laboratory] Progress: Not done. We anticipate starting these experiments soon.	12-14	Haldar Lab
Subtask 2: Examine the impact of compound 91 on immune cells. [2 treatment conditions X 8 mice per group = 16 mice total] Cell lines and Model: Immunocompetent C57BL/6J mice [Jackson Laboratory] Progress: Not done. We anticipate starting these experiments soon.	12-14	Haldar Lab
Milestone(s) Achieved: Upon completion of aim 1 we will uncovering the impact of RALDH1 inhibition by C-86 and C-91 on (1) RA production by human and mouse HCC, (2) intratumoral monocyte differentiation into macrophages vs. DCs, (3) anti-tumor immune responses in HCC, and (4) general immune cell distribution at steady state.	16	
Specific Aim 2: Targeting RALDH1 for immunotherapy of HCC		
Major Task 5: To test whether RA blockade can elicit therapeutic anti-tumor immune responses in HCC		
Subtask 1: To test single and combinatorial RA signaling blockade for HCC immunotherapy. [10 treatment conditions X 8 mice per group X 5 overall experimental repeats = 400 mice total] Cell lines and Model: Hepa 1-6 [Source: ATCC] and Immunocompetent C57BL/6J mice [Jackson Laboratory] Progress: 10% completed. As mentioned above, we will be using the Hep55 cells instead of Hepa 1-6. We have completed pilot experiments with Hep55 and anticipate performing experiments on a larger cohort shortly.	16-20	Haldar Lab
Subtask 2: To test single and combinatorial RA signaling blockade for HCC immunotherapy in a distinct HCC model. [10 treatment conditions X 8 mice per group X 5 overall experimental repeats = 400 mice total] Cell lines and Model: HCC-TM [Source: Simon Lab] and Immunocompetent C57BL/6J mice [Jackson Laboratory] Progress: Not done. We anticipate starting these experiments soon.	20-24	Haldar Lab
Subtask 3: To test single and combinatorial RA signaling blockade for HCC immunotherapy in an autochthonous murine model. [10 treatment conditions X 8 mice per group X 2 overall experimental repeats = 160 mice total] Cell lines and Model: Immunocompetent Sv129j mice [Jackson Laboratory] Progress: Not done. We anticipate starting these experiments soon.	24-32	Haldar Lab

Milestone(s) Achieved: Upon completion of Aim 2 we will have new insights into whether RA signaling blockade can be used for immunotherapy of HCC. We also anticipate publication of 1-2 peer reviewed papers and presentation of our work in 2-3 national or international meetings.	36	

What was accomplished under these goals?

Aim 1.1 -To identify the impact of RALDH1 inhibition on RA production by HCC cells: In our initial *in vivo* experiments, we found that the human SNU398 did not grow well in immunodeficient (NU/J) mice while the Hepa 1-6 cells showed immune rejection in subcutaneous syngeneic transplantation system. Thus, we explored other commonly used human and murine HCC cell lines suitable for our experimental strategies, eventually selecting Huh7 and Hep55 cells for human and murine HCC respectively. We had originally picked SNU398 and Hepa 1-6 based on availability and not due to any compelling scientific evidence for their superiority to other HCC lines. Indeed, all human and murine HCC lines we tested showed high RA driven by RALDH1 overexpression. Therefore, this change in cell lines does not affect our experimental strategy or conclusions. We found that C-86 (Figure 1a) and C-91 (not shown) abrogated RA production in *human* HCC cells but, surprisingly, not *murine* HCC. Homology modeling suggests that species-specific differences in amino acid residues at the drug-interacting regions may explain this species-specificity. Nonetheless, we identified another compound (C-99) within the same chemical series that inhibited murine RALDH1, albeit at higher concentrations (Figure 1B).

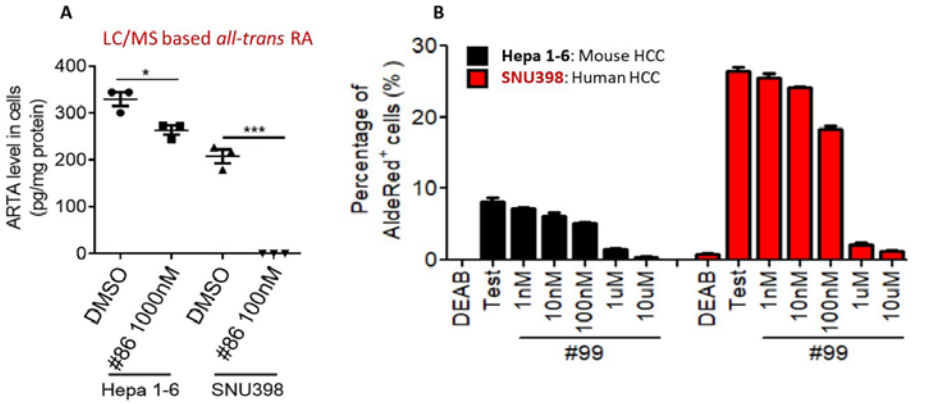

Figure 1: (**A**) All-trans retinoic acid (ATRA) measured by liquid chromatography/mass spectrometry (LC/MS) in lysates from murine (Hepa 1-6) or human (SNU398) HCC cells grown with or without C-86 inhibitor. C-86 is effective in human but not murine HCC. (**B**) Compound-99 (C-99) inhibits both human and murine RALDH1 at high (micromolar) concentration. Inhibition of RA production is measured by Aldefluor assay. DEAB: negative control.

Aim 1.2 – To examine the impact of inhibiting HCC RA production on monocyte differentiation. We found that RA derived from either human of murine HCC strongly suppressed monocyte differentiation into DCs (Figure 2a). Importantly, this effect on monocyte differention was abolished in the presence of RALDH1 inhibitors (Figure 2a). These findings were also confirmed at the level of gene expression, which showed corresponding changes in DC- and macrophage-associated transcription factors (Figure 2b).

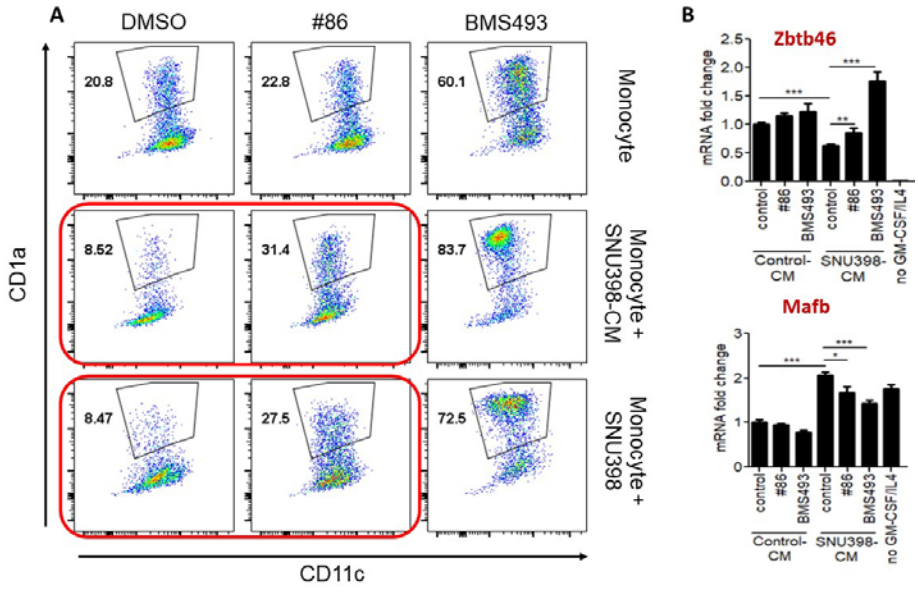

Figure 2: (**A**) Human monocytes were cultured alone (top row), or with SNU398 Cells (bottom row), or media taken from wells containing SNU398 Cells (conditioned media, CM, middle row). DMSO (control, left column), C-86 (RALDH1-inhibitor, middle column), or BMS493 (RAR-inhibitor, right column) were added to the cultures and cells analyzed after 4 days. Of note, for the co-culture experiments (cells or conditioned media), HCC cells were treated with C-86 prior to co-culture with monocytes. (**B**) RT-qPCR for DC-associated *Zbtb46* and macrophage associated *Mafb* transcription factors from monocytes differentiated under conditions outlined in (A). BMS493: RAR inhibitor.

Aim 1.3 – To examine the impact of inhibiting HCC RA production on the tumor microenvironment. Huh7 (human HCC) cells were transplanted into immunodeficient (NU/J) mice and treated with C-86. Remarkably, despite the absence of T cells, treatment with C-86 was associated with significantly slower tumor growth (Figure 3a) and altered tumor immune microenvironment (TIME). The most prominent effect on TIME was a reduction in tumor-associated macrophages (TAMs) with drug treatment (Figure 3).

Generating RALDH1- knockout HCC cells. Although not proposed in our original submission, we generated a RALDH1 knockout HCC cell line to validate whether the effects of RALDH1-inhibitors on tumors represent *bona fide* on target effects. Indeed, RALDH1-KO HCC cells show significantly reduced growth *in vivo* and, importantly, did not respond to RALDH1-inhibitors (Figure 4). Thus, the tumor inhibitory effects of C-86 represent on-target effects on RALDH1. The generation of this RALDH1-KO cell line will also serve as a control for future *in vivo* experiments.

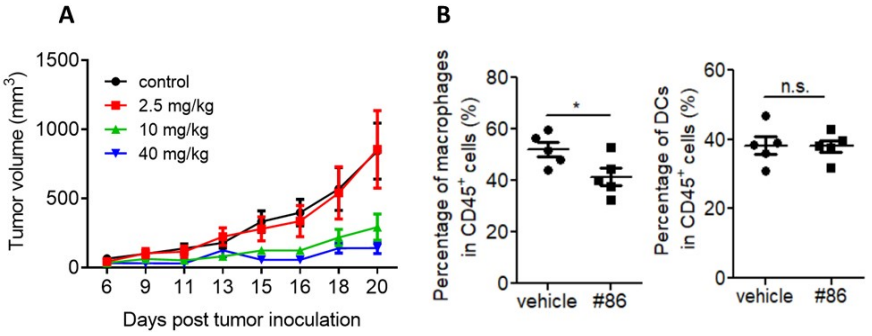

Figure 3: (**A**) Huh7 cells were transplanted subcutaneously into NU/J immunodeficient mice. After four days, mice were treated intrapertioneally with vehicle (control) or different doses of C-86. Shown are the tumor volume at different time points. (**B**) Quantification of macrophages and DCs in C-86 treated (10mg/kg) and control Huh7 tumors. Tumors were harvested at endpoint and analyzed by flow cytometry.

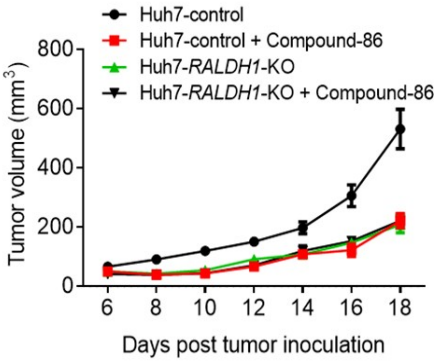

Figure 4: RADLH1 was deleted from parental Huh7 HCC cells by CRISPR/Cas9 (Huh7-RALDH1-KO) and transplanted into immunodeficient (NU/J) mice. Parental Huh7 (Huh7-control) served as control.RALDH1-KO tumors grew slower to the same extent as C-86 treated control tumors. Importantly, C-86 treatment did not affect growth of RALDH1-KO tumors.

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?

We plan to pursue the remaining experiments outlined in our original submission. Moving forward, we will incorporate new things that we have learnt in the past year, such as the appropriate choice of cell lines and species-specificity of the RALDH1- inhibitors, into our experimental strategies.

4. Impact

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

Results from this reporting period show that our RALDH1-inhibitors can abrogate RA production in HCC to prevent RA-mediated monocyte differentiation into immunosuppressive macrophages. Importantly, our inhibitors can suppress HCC growth *in vivo*. Thus, targeting RA production in cancer cells is a novel approach in immunotherapy of solid tumors.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Results from this research contributed towards the formation of a biotech startup company – 'Stage One Immunotherapeutics'. The primary goal of this newly formed company is to move RALDH1 inhibitors into the clinics.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change

There has been no change in experimental approaches. However, we made minor changes in some of the reagents as described above in detail. Briefly, we are now using Huh7 instead of SNU398 for human HCC cell lines, Hep55 instead of Hepa 1-6 for murine HCC cell lines, and C-99 instead of C-86 or C-91 to inhibit murine RALDH1.

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

Nothing to report.

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

Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Pengfei Yu, PhD
Project role:	Postdoctoral Research Associate

Researcher identifier:	33444744 (Upenn ID)
Nearest person month work:	12
Contribution to project:	Dr. Yu planned, performed, and analyzed experiments outlined in this progress report.
Funding support:	This grant (W81XWH-21-1-0592)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Not applicable as this is the first reporting period.

What other organizations were involved as partners?

Organization Name: National Center for Advancing Translational Sciences (NCATS)
Location of Organization: NIH, Bethesda, MD, USA
Partner's contribution to the project: Synthesize and provide RALDH1 inhibitors used in this project.

8. Special Reporting Requirements

Nothing to report

9. Appendices