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14. ABSTRACT

Mutations in NF1 lead to the aberrant activation of the Ras oncoproteins. Upregulated Ras activity promotes the development of potentially lethal MPNST in NF1 patients. Genetic and pharmaceutical anti-Ras approaches can inhibit MPNST in experimental systems of NF1 dysfunction. However, currently there are no anti-Ras therapeutics that are clinically effective. The development of such agents could revolutionize treatment options for NF1 disease. We have developed two small molecules, one a direct and one an indirect inhibitor of Ras function. We have confirmed that these two molecules are active against MPNST tumor cells in vitro. Moreover, we have shown that they have low toxicity and are active against Ras driven tumor cell systems *in vivo*. Here, we seek to test the molecules against MPNST model systems in vivo.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION

Neurofibromatosis type 1 is a syndrome caused by mutations in the NF1 gene (1). These mutations can be hereditary, but also occur spontaneously at a significant rate. It is one of the most common genetic disorders of the nervous system and results in the propensity for the growth of benign tumors called Neurofibromas (2, 3). Some of the benign tumors can develop into malignant peripheral nerve sheath tumors (MPNST). These tumors are typically resistant to conventional therapy (4) and are the leading cause of mortality in Neurofibromatosis patients (5). In addition, approaching 70% of NF1 patients exhibit a spectrum of cognitive/learning disorders of variable penetrance (6).

NF1 is a large multifunctional protein but is it appears that its most important function is to act as a negative regulator, or GAP (GTPase activating protein), for the RAS oncoprotein (7, 8). Defects in NF1 function, result in an up-regulation of RAS activity, which appears to be a driving event (9, 10) in NF1 defective tumors, as it is in many other cancers (11). Indeed, in experimental systems, inhibition of RAS can revert MPNST (12). Moreover, both genetic and pharmacological studies in NF1 deficient mice have shown that excessive RAS signaling also appears to be responsible for many of the cognitive defects due to NF1 deficiency (13, 14). Thus, RAS directed therapy is the most logical approach to defeat the cancer and to treat learning/cognitive defects. However, to date, no clinically effective anti-RAS treatments have been successfully developed (15).

We have developed and patented two novel small molecules that are designed to specifically inhibit hyper-active RAS function. One binds directly to RAS and prevents it communicating with downstream effectors. This is designated F3. The other binds directly to a key RAS effector called RALGDS and prevents its activation by RAS. This is designated C4. We have already demonstrated efficacy against other RAS driven tumor systems in vitro and in vivo for these agents. We have confirmed low toxicity. We have also developed several enhanced activity derivatives of the drugs which we are including in the experiments. This proposal seeks to evaluate the potential for these compounds in suppressing the development of MPNST in animal models.

2. KEYWORDS:

NF1, MPNST, RAS, small molecule inhibitors, Neurofibromatosis

3. ACCOMPLISHMENTS

A. Major goals for year 1:

i. Specific Aim 1,

Sub-task1.

Local IRB/AICUC approval obtained. Scheduled to be approved by month 3, actually approved by month 1.

HRPO/ACURO approval obtained. Scheduled to be approved by month 6, actually approved by month 2.

Sub-task 2. Mechanistic studies on Cell lines: Scheduled to be completed by month 6. Delayed by pandemic. Partial completion, estimate 60% (see below).

i. **F3 and enhanced activity derivatives suppress the activation of the RAS/MAPK pathway in S462.TY MPNST cells**.

We have confirmed that the F3 RAS inhibitor suppress the activation of the MAPK pathway in MPNST cells (S462.TY) and identified several F3 derivatives that appear to be more effective.

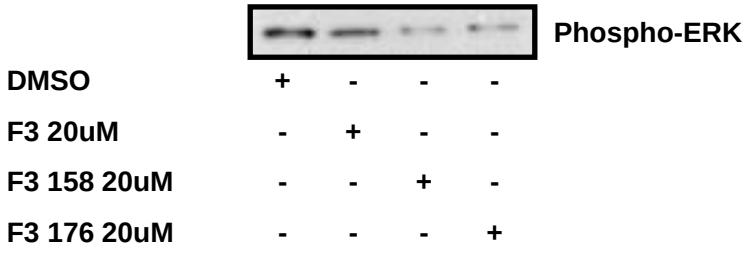


Figure 1. F3 and its derivatives suppress the MAPK pathway. *The MPNST cell line S462.TY was treated with F3 and 2 F3 derivatives for one hour before the cells were lysed and equal quantities of protein examined by Western blot to measure the degree of MAPK pathway activation using a phospho ERK specific antibody.*

ii. F3 but not C4 blocks matrigel induced differentiation of MPNST progenitor cells

MPNST arise from non-malignant Plexiform Neurofibroma cells. The molecular mechanism responsible for the conversion of PFN to MPNST remains unclear. However, a tumor suppressor called RASSF1A is almost always inactivated during this process. We knocked down RASSF1A in a PFN cell line resulting in a cell line with many of the characteristics of MPNST, including the ability to grow in soft agar. This MPSNT precursor, deficient for RASSF1A, also reacquires the ability to differentiate in matrigel. The anti-RAS F3 compound suppresses the matrigel-induced differentiation at doses that have no effect on normal 2D growth (Figure 2). The C4 anti-RALGEF compound had no such effect.

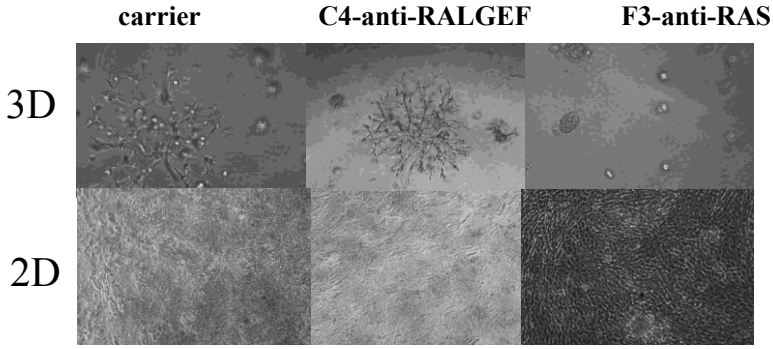


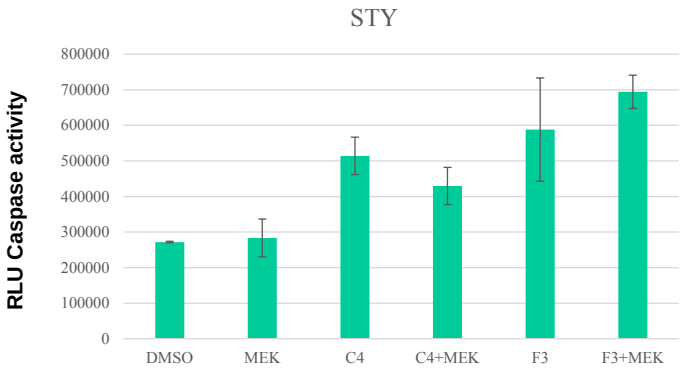
Figure 2. F3 suppresses the differentiation of MPNST-precursor cells. *PFN 96.1 cells were stably transfected with shRNA against RASSF1A. Cells were*

plated in matrigel and found to differentiate. F3 but not C4 suppressed the differentiation (top panels) whereas cells grown in 2D in the same drug concentration showed no adverse effects.

Thus, we have identified a key role for RASSF1A loss in the generation of MPNST and shown that the F3 compounds can be active against early stage MPNST.

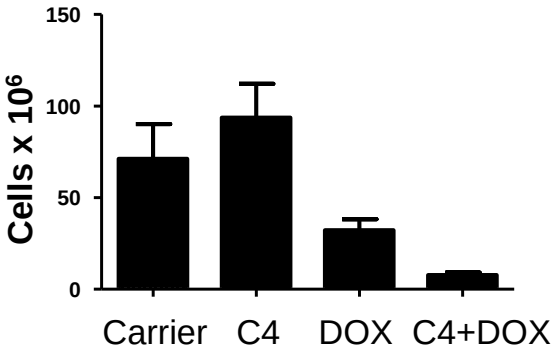
iii. C4 and F3 induce anoikis in S462.TY MPNST cells.
Most normal non-hematopoietic cells depend upon signals from surface attachment to survive. Loss of this signaling results in a form of apoptosis called anoikis. Hyper-activation of RAS signaling protects tumor cells from anoikis, allowing them survive during the metastatic process. We suspended MPNST cells in adhesion-proof plates and treated with drugs overnight. We then assayed for the induction of caspase activation as a measure of anoikis using a commercial luciferase based kit. In addition, we included an FDA approved MEK1 inhibitor in order to examine the effects of suppressing another major RAS pathway. Both F3 and C4 stimulated anoikis. However, the addition of MEK1 exerted no additional effect, suggesting that the anoikic effect is already maximally activated by the C4 or F3 treatment.

Figure 3. Anoikis induction.
MPNST cells (*S462.TY)* were treated with drugs alone or in combination in suspension culture. Caspase activation was measured using a commercial luciferase based kit.



iv. **C4 and Doxorubicin exhibit cooperative action:**
The RAL pathway has been implicated in chemotherapeutic drug resistance. Therefore, we performed experiments to determine if the RALGDS inhibitor C4 could co-operate with Doxorubicin. Cells were grown in 2D and treated with a dose of C4 that has little effect on cell growth or survival. However, when combined with Doxorubicin there was a striking decrease in cell survival.

Figure 4. C4 and Doxorubicin show a synergistic effect on cell death. *S462.TY MPNST cells were treated with C4, Doxorubicin or a combination of the drugs. C4 was used at a dose which does not promote cell death in 2D culture (10 uM). The two drugs had an enhanced effect on tumor cell death.*



Sub-task 3. Initiate studies on MPNST pdx: We have obtained three frozen pdx samples of MPNST tumors from the Johns Hopkins repository. The first has been implanted for growth and expansion.

Goals not met:

Some experiments still need to be repeated for an additional cell line to ensure data robustness.

Training:

MD/PhD Student Desmond Harrell-Stewart (Historically under-represented minority) gained training on pdx manipulation and drug action studies. He has now successfully defended his thesis.

Dissemination of Results:

i. Invited Poster Presentation at the 6th International RASopathies Symposium, Baltimore Maryland, August 2-4,2019.

"*Novel RAS inhibitors for NF1 disease*".
ii. Invited Seminar, Cincinnati Children's Hospital Rasopathies group, December 10th 2019.
"*Novel RAS inhibitors for NF1 disease*"

Plans for the next reporting period
I intend to bring in one, possibly two technicians to speed up the project to make up for the COVID-19 induced delays. We will finish up the mechanistic studies and proceed with the in vivo drug testing in xenograft models. We will 'warm up" the frozen pdx and establish experimental groups. We will establish the MPNST transgenic system from collaborator Dr. Parada.

4. IMPACT

Nothing to report yet

5. Changes/Problems

There have been no significant changes in objectives or approach. However, the Covid-19 pandemic has had a significant (temporary) negative impact on the project. The laboratory went to shift work, then full remote working and is now back at shift work pending resumption of normal service. In addition, there has been a delay in hiring personnel. This has resulted in slower progress and lower than anticipated expenditures in this first year.

1. Some of the mechanistic studies have been delayed, and remain ongoing.
,
2. Collaborator Dr. Parada has been unable to provide the transgenic line as his laboratory in New York was put into stasis. He has promised to send the animals as soon as he can get the breeding back up. In the event that this is not just a temporary setback, we will pursue the alternative approach of obtaining the individual NF1 and p53 mice from Jackson Laboratories and attempting to breed our own Cis bi-transgenic mouse system.

3. Much our mouse work, including the breeding up of experimental populations for xenograft drug experiments, had to be shut down and we have only recently been able to restart. I am optimistic we can still complete all the work, but it seems likely there will be delays.

6. Products

Nothing to report

7. Participants and Collaborating Organizations

Name: Geoff Clark
Role: PI
Effort: 2 Months effort

Contribution: Supervised project and performed cell culture experiments.
Funding: CDMRP, NIH, Qualigen LLC.

Name: Desmond Harrel-Stewart
Role MD/PhD student
Effort 10 months effort
Contribution Tissue culture assays, animal colony expansion, and pdx animal experiments.
Funding NIH,

Name Howard Donninger
Role Instructor
Effort 2 months%
Contribution Signaling analysis and tissue culture assays
Funding NIH, Jewish Fund For Excellence.

Changes in active support:
Nothing to report

Other Organizations involved:

Memorial Sloan Kettering Hospital, New York NY- Dr. Parada – Collaboration.

Johns Hopkins University- Baltimore MD- Dr. Pratilias- Collaboration.

10. Special Reporting Requirements

None

11. Appendices

None