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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 originally 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 now developed several enhanced activity derivatives of the drugs (F3-8-60 and C4-180) which we will use 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 2:

i. Specific Aim 1,

Sub-task1.

MPNST Cell line xenografts/metastasis assays:

i. We have tested our anti-RAS compound F3-8-60 and anti-RALGDS compound C4-180 against S46.2TY cells in xenograft experiments (Figure 1- left panel). We observed no significant effect. This was a surprise as the compounds are excellent in soft agar assays. Indeed, F3-8-60 almost wipes out RAS signaling in these cells (Figure1 right panel). However, we are cautious interpreting the experiment as the room the animals were housed in suffered an outbreak of *c.bovis* and most of the tumors showed ulceration, likely due to the bacteria. This promoted a premature termination of the experiment for animal welfare reasons. The infection has now been resolved and we intend to repeat the experiment. In addition, we have now determined that we can treat animals with the F3-8-60 compound via gavage to obtain higher doses, which we will incorporate in the repeat. We observed no lung metastasis in these experiments.

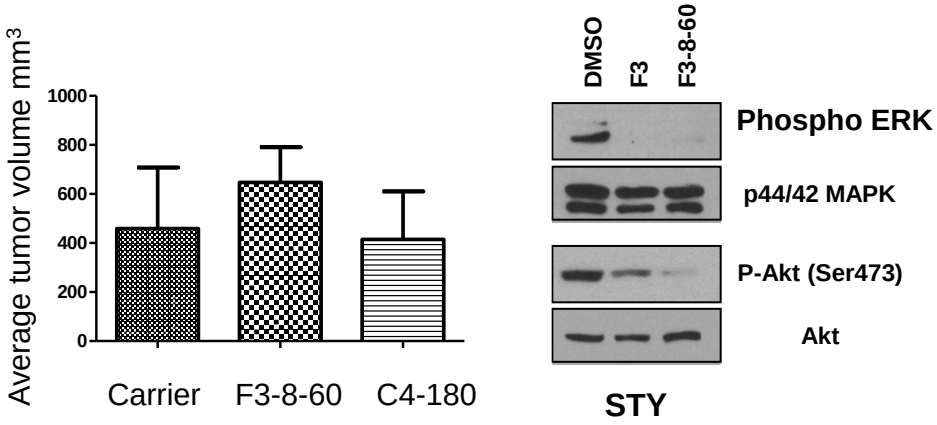

Figure 1. Drug treatment of S46.2TYcells.
Left panel: Xenografts of S46.2TYcells treated with anti-RAS and anti-RAL. No significant differences were observed.
Right panel- Western blots showing drug shut down of RAS/MAPK and RAS/PI3K/AKT signaling in S46.2TYcells.

ii. We have tested the enhanced activity derivatives of our compounds (F3-8-60 and C4-180) against xenografts of STS-26T cells. The cells did not make primary tumors, but were highly metastatic. This was particularly obvious in the spleen. Drug was treatment was via gavage. The anti-RAS F3-8-60 almost completely blocked the metastasis. The C4-180 anti-RALGDS did not. Representative spleens are shown in Figure 2.

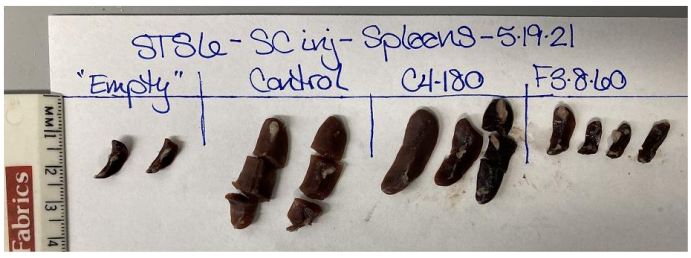

Figure 2. Suppression of metastasis of STS-26T cells. 1 x 10^6 cells were injected into NRG mice. After 2 weeks animals were treated with 40mg/kg of C4-180 by ip injection or 40mg/kg F3-8-60 by gavage.

Sub-task 3- pdx xenograft studies:

Pandemic associated issues have delayed this part of the project, but it is partially done.

Our original plan was to test three separate tumor fragments. We have completed the testing on the first two pdx (see Figure 3 below). Each track represents the changes in each tumor size over the treatment regime. The experiment appears to have worked and the reduction in average tumor growth is statistically significant. By the time of the second pdx study we had determined that F3-8-60 exhibited about 10% oral availability. So this study was performed with Gavage for the F3-8-60 arm. The third pdx is almost ready to enter testing. We have another two pdx that we may be able to add to the studies, resources permitting, to enhance overall robustness.

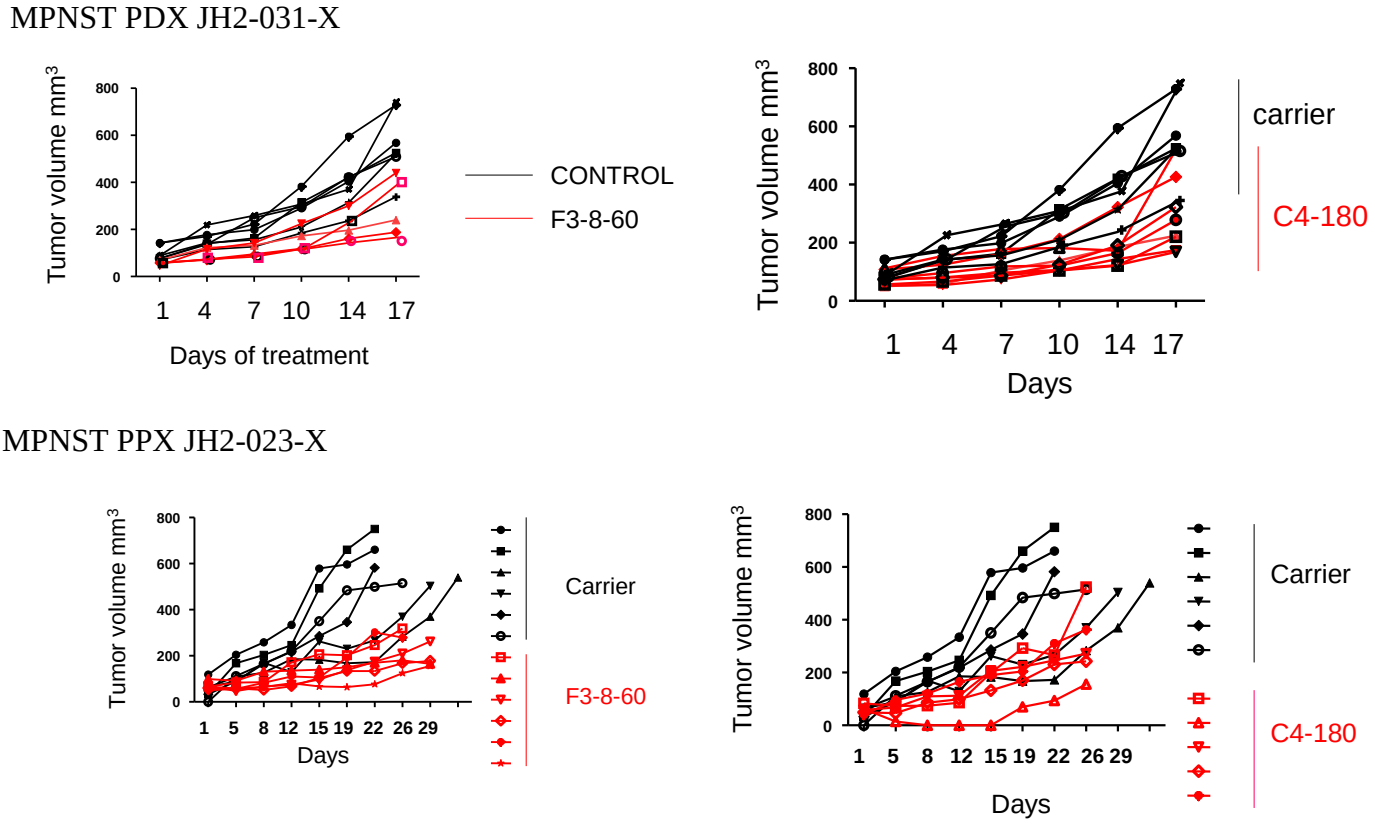

Figure 3. Inhibition of pdx by F3-8-60 anti-RAS and C4-180 anti-RALGDS.
Two MPNST pdx were challenged with F3-8-60 (40mg/kg gavage) or C4-180 (40mg/kg ip). When an implanted tumor fragment reached between 4-100mm3 in volume it was randomly assigned to a study group and entered into treatment.

MEK co-operation studies: RAS inhibitors may provoke feedback loops or select for the development of downstream mutations that promore resistance. RALGDS inhibitors do not affect the MAPK pathway. Therefore, it is possible that combination therapy including clinically approved MEK inhibitors could enhance anti-tumor activity of the agents. We treated S46.2TYcells with F3-8-60 and C4-180 (500nM) in the presence of absence of the MEK inhibitor Trametinib (1nM). We do appear to observe a synergistic effect and we may incorporate these results into the xenograft assays.

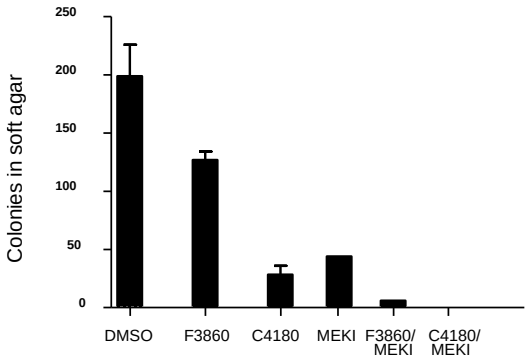

Figure 4. Co-operation between anti-RAS, anti-RALGDS and anti-MEK compounds.- Soft agar assays were performed on S46.2TYcells using agents alone and in combination.

Specific Aim 2- testing the inhibitors against a transgenic model of MPNST.

Introduction:

This part of the proposal involved obtaining transgenic mice with in-Cis mutations of p53 and NF1 from collaborator Parada. These animals spontaneously develop MPNST. Unfortunately, pandemic issues made it impossible for Dr. Parada to supply the animals in a timely manner. Consequently, we had Jackson Labs thaw out the strain and obtained it from them. We have now established the animals in the laboratory. Genotyping has allowed us to identify hz individuals (there are no hm individuals as these are embryonic lethal) (Figure 5). We are now setting up randomized cohorts for drug testing which will be carried out in rolling batches. The first drug treatments are about to start. Therefore, we have recovered this part of the project and appear to be back on target.

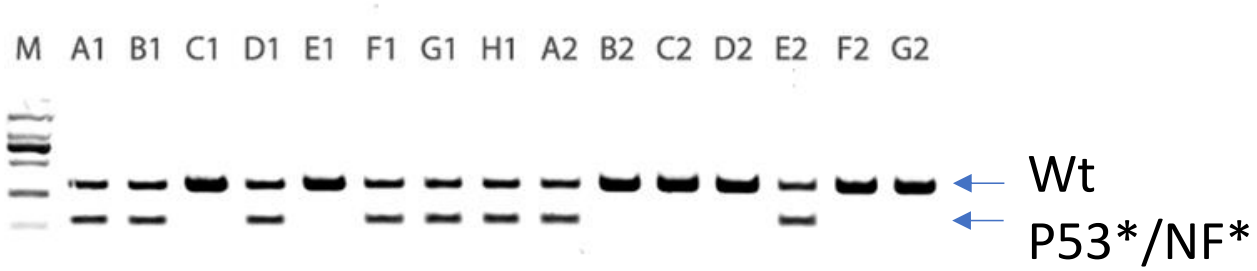

Figure 5. Example of genotyping of P53/NF1 Cis mice.

DMPK studies:

We also proposed performing PK studies to facilitate the oral administration of the agents. We have now determined that the characteristics are sufficient to allow in vivo evaluation:

F3-8-60

<u>p.o. (10 mg/kg)</u>
%F (absolute oral bioavailability) = 10.7 %
Cmax = 31.5 ng/mL = ~49 nM)
AUC = 212 ng/mL

These levels suggest that a treatment of 100mg/kg should give us a Cmax of around 500nM, which is a levels at which we observe inhibition of RAL signaling and inhibition of cell growth in soft agar. This fits well with the pdx inhibition observed in Figure X using 100mg/kg.

C4-180:

Using ip injections of 10mg/kg we observed a cmax of around 500nM- an effective dose in vitro. The shorter T1/2 than F3-8-60 justifies the use of 30mg/kg injections. Examination of oral availability is ongoing.

PK parameters	Unit	Mouse 1	Mouse 2	Mean
T1/2	h	0.856	0.834	0.845
Tmax	h	0.5	0.5	0.5
Cmax	ng/mL	304	237	271
AUClast	h*ng/mL	456	351	404
AUCInf	h*ng/mL	477	366	421

Training:
None

Dissemination of Results:

i. Invited Poster Presentation at the 7th International RASopathies Symposium (Virtual), July 23-25th.

"*Novel RAS inhibitors for NF1 disease*".
AACR
NCI cancer meeting Frederick MD

Plans for the next reporting period
We intend to pursue the remaining pdx studies and complete the transgenic studies. We have two additional pdx available and we may incorporate these into the experimental process to enhance robustness of the results. We intend to repeat cell line xenograft/metastasis studies with clean systems and higher doses.

4. IMPACT

Nothing to report yet

5. Changes/Problems

There have been no significant changes in objectives or approach. However, we are still somewhat behind schedule due the Covid induced shut-down. This project was particularly sensitive to the pandemic as it is heavily animal dependent, and we had to down-size our colony dramatically to comply with the institutional response. We then have had to wait while we expanded the population back up for experimentation. While other investigators were doing the same, someone managed to introduce c.bovis into the animal facility that added a further (now resolved) complication. In addition, we had to reacquire pdx samples from the biobank as we lost the originals in a freezer crash that went unnoticed because all the staff had been sent home for the pandemic. Actually, this produced only a modest additional delay as we did not have animals ready anyway. Even the transgenic arm of the project was affected by the pandemic as the laboratory of Dr. Parada suffered a shut down and could not supply us with the transgenic line we needed. To circumvent this obstacle, we had to request a thaw-back of the frozen line from Jackson laboratories. This has been successfully accomplished, genotyping established, and the experiment is underway.

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: Rachel Ferrill
Role Technician
Effort 12 months effort
Contribution Tissue culture assays, animal colony expansion, in vivo experiments and pdx animal experiments.
Funding CDMRP.

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:

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

10. Special Reporting Requirements

None

11. Appendices

None