AWARD NUMBER: W81XWH-19-1-0417

TITLE: Novel Inhibitors of MPNST

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CONTRACTING ORGANIZATION: University of Louisville, Louisville, KY

REPORT DATE: August 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2023	Annual	15Jul2022-14Jul2023

4. TITLE AND SUBTITLE

Novel Inhibitors of MPNST

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0417

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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303 E. Market St. Suite 300
Louisville, KY 40202

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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.

16. SECURITY CLASSIFICATION OF:	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
			USAMRDC

a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	12	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

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

i. Specific Aim 1:

To determine if our novel Best-in-Class anti-RAS molecule or our First-in-Class anti-RALGDS molecule inhibitors can serve as effective targeted therapy for human MPNST xenografts.

Sub-task1:

Validation of enhanced activity derivatives of F3 and C4. By the time the application was funded, we had developed enhanced activity variants of the parental F3 anti-RAS and C4 anti-RALGDS compounds. These new agents are designated **F3-8-60** and **C4-180**. They will be used throughout the report. We repeated our initial signaling and soft agar assays to confirm the new agents worked better. Both new agents were superior in soft agar assays on S46.2TY cells (Figure 1).

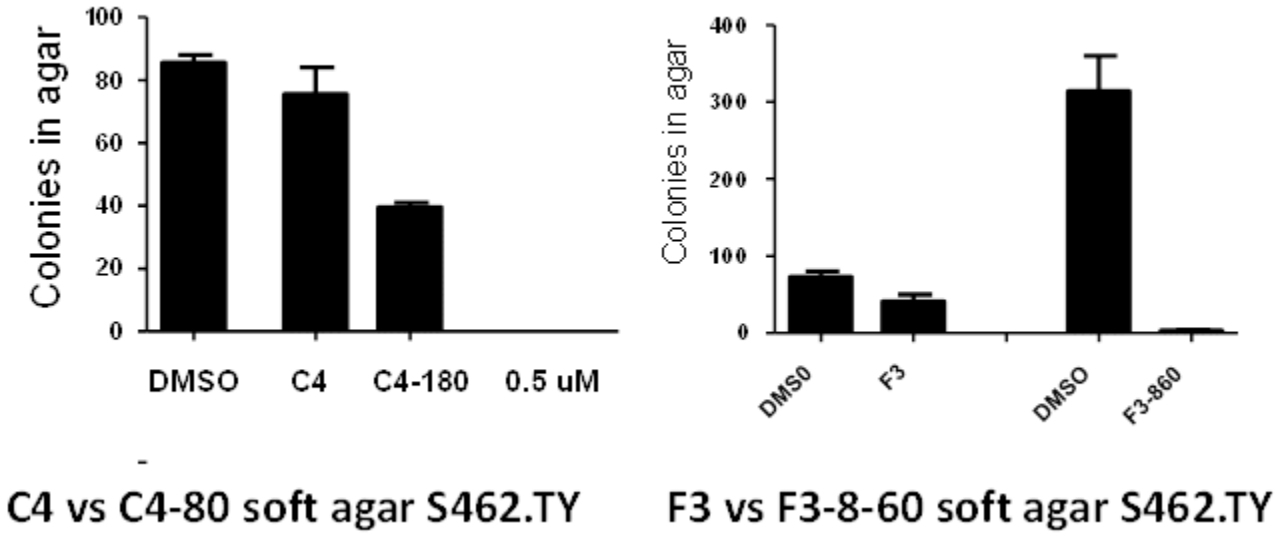


Figure 1. Soft agar assays of F3-8-60 and C4-180 in the MPNST cell line S462.TY. Cells *were plated in soft agar in the presence or absence of 500nM each drug. Colonies were scored after two weeks.*

There are three main RAS mitogenic effector pathways (Figure 2). These are the RAS/RAF/MAPK pathway which can be measured by determining the ration of phosphorylated ERK (active) to total ERK. The RAS/PI3-K/AKT pathway that can be measured by determining the levels of phosphorylated (active) AKT. As well as the RAS/RALGDS/RAL pathway that can be measured by determining the ratio of active (GTP bound) RAL to total RAL.

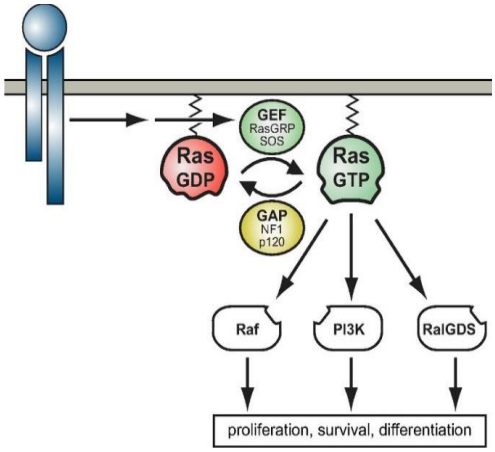


Figure 2. Scheme of classic RAS mitogenic signaling pathways. *Ras activates three main mitogenic pathways. ERK levels), the PI-3K/AKT pathway (measured by phospho AKT) and the RALGDS/RAL pathway (measured by levels of active RAL-GTP).*

We measured the activity of these pathways with the two drugs in S462.TY and STS-26T MPNST cell lines (Figure 3). Cells were treated for one hour before lysing and performing Western analysis.

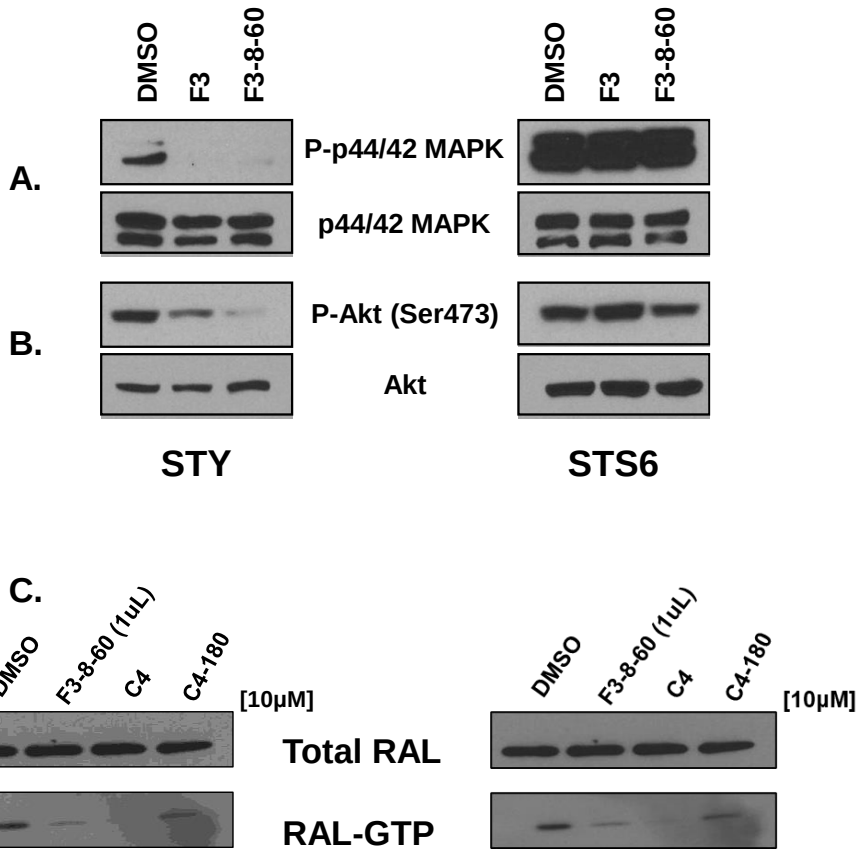


Figure 3. Inhibition of RAS pathway signaling in MPNST cell lines*. A. MAPK pathway activity after 1 hour treatment. Total ERK serves as the control. B. Phospho AKT measures the PI3K pathway activity, total AKT serves as the control. C. RAL-GTP levels were measured by pull down assay. Total RAL serves as a control. STY: S462.TY. STS6: STS-26T cells.*

S462.TY has a pathogenic NF1 mutation leading to loss of function and therefore elevation of RAS activity. F3-8-60 suppresses the MAPK pathway (A), the PI3K pathway (B) and the RAL pathway (C). STS-26T exhibits NF1 LOH, but also has a RAF mutation. This means that the RAF pathway is not suppressed by a RAS inhibitor (because it is downstream), but the RAL pathway is. C4-180 inhibits the RAL pathway in both cell lines as RAF is not upstream of RALGDS.

Sub-task 2: Do the compounds cause established MPNST xenografts to regress?

We have performed the S462.TY and STS-266 cell line xenograft assay and obtained statistically significant inhibition of tumor growth. The exception was with the F3-860 and S462.TY cells. Tumor variation One large tumor) invalidated the statistical significance (just), but a clear anti-tumor trend was visible. We used a

gavage approach to treatment with F3-8-60 with C4-180 being administered via ip injection.. At the doses tested we did not observe tumor regression, but a marked slowing of tumor growth. However, we have recently identified F3-8-60 related compounds with enhanced RAS binding and antitumor activity which can be tolerated by animals at much higher doses. We would propose to repeat these experiments with the new agents in to order to obtain tumor regression.

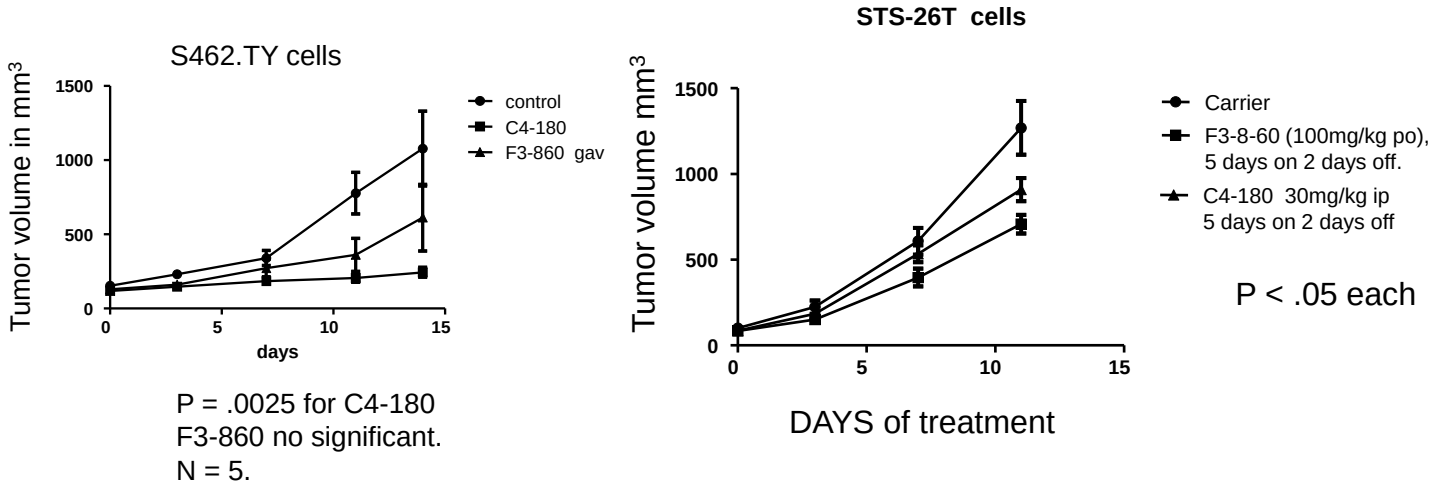


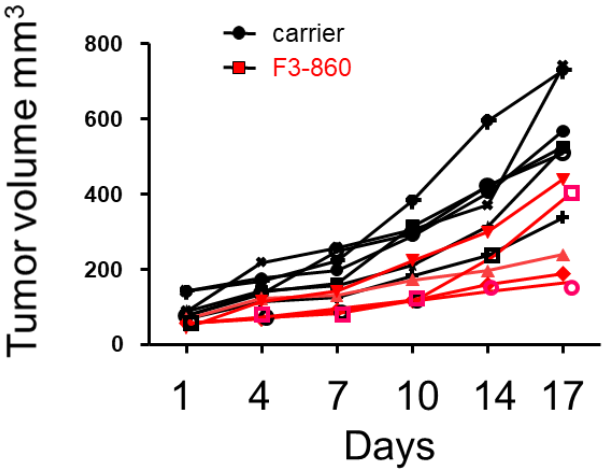
Figure 4. Inhibition of tumor growth of S462.TY and STS-26T cells in NSG mice by RAS and RALGDS inhibitors.

Mice were inoculated with tumor cells and when tumors reached > 50mm³ animals were randomly assigned to an experimental group (~ equal mix of male and female). Drug was administered 5 days on/2 days off for two weeks. N = 8. F3-8-60 was gavaged at 100mg/kg 5 days on two days off, C4-180 was administered by ip at 40mg/kg. n: >= 5.

Sub-task 3- pdx xenograft studies:

Cell lines have adapted to artificial tissue culture conditions and although easy to use tend to be a poor model for real human tumors. Primary tumor grafts (pdx) derived from human tumors that have only been propagated in mice and never placed in tissue culture maintain many of the actual properties of the original tumor growing in the patient. Therefore, they are the best model for testing therapeutics. We obtained 4 pdx systems from the NTAP (Neurofibromatosis Therapeutic Acceleration Program at Johns Hopkins). We have tested our agents against them.

The first two pdx (JH20023 and JH20031) both showed a statistically significant slowing of tumor growth. We observed one tumor show apparent regression at the doses tested, suggesting that higher does or a better agent might be able to provoke regression (Figure 5 top four panels). The third pdx (JH0051) failed to demonstrate any response to F3-8-60 and grew the most aggressively of the three (Figure 5 bottom panel). As it failed to respond to F3-8-60 we felt a response to C4-180 would be unlikely and did not perform these experiments for ethical reasons. The fourth pdx (JH20055) grew very slowly in the donor host and when transplanted into the experimental population failed to give tumors. Therefore, we could not analyze it.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F3-860
Tumor volume mm3
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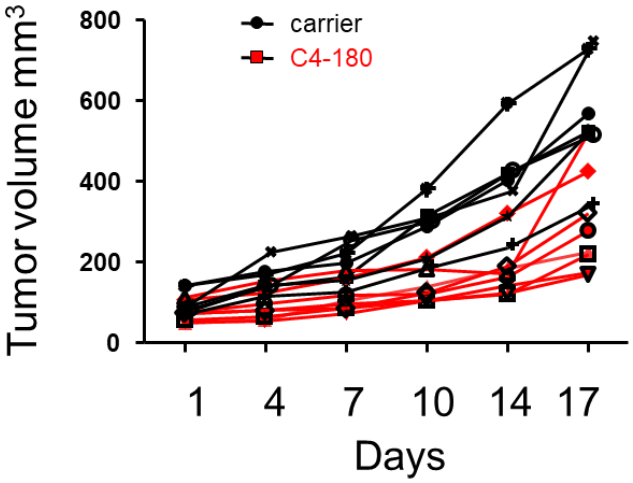
600
400
200
0
1 4 7 10 14 17
Days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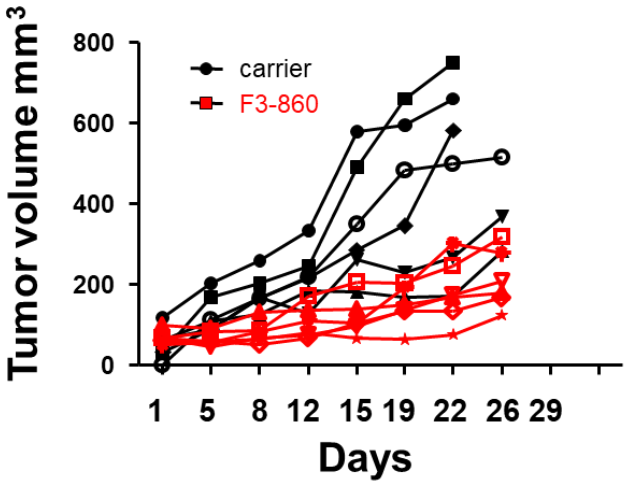
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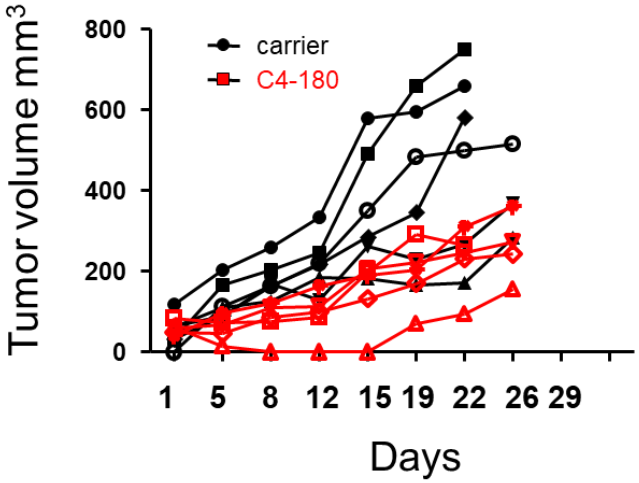
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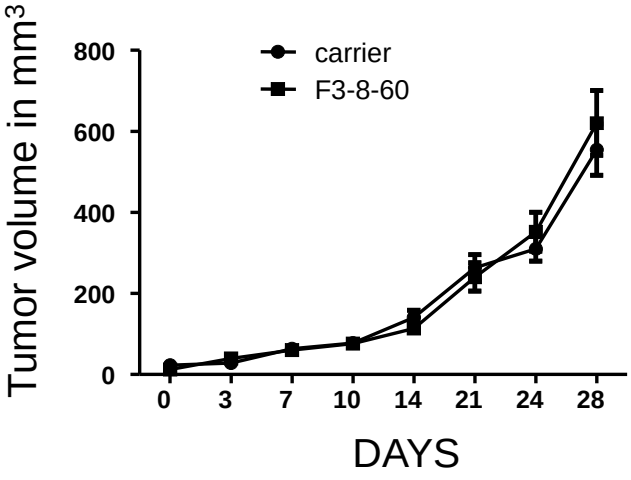
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F3-8-60
Tumor volume in mm3
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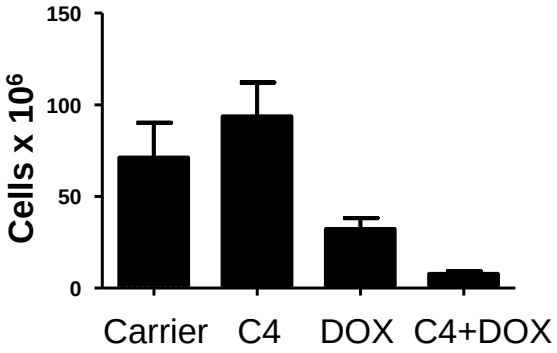
400
200
0
0 3 7 10 14 21 24 28
DAYS

Figure 5. Effects of F3-8-60 anti-RAS and C4-180 anti RALGDS on three different MPNST pdx. *Four pdx were obtained from the NTAP biobank. Pdx JH2-0023 (top two panels) JH2-0031 (Middle two panels) were treated with 80mg/kg F3-8-60 by gavage 5 days on 2 days off, C4-180 treatment: ip injection of 40mg/kg 5 days on 2 days off. Each track describes the behavior of one tumor implant. This makes it possible to see that there was considerable variation in response between individual implants, with some showing temporary regressions. Overall, both showed a significantly decreased overall growth of the tumors with both agents. Pdx JH2-051 showed no response to F3-8-60 and as there were no real differences in tumor growth we combined the results (n=6). As F3-8-60 failed we felt C4-180 was unlikely to succeed and so we did not test this agent for ethical reasons. We also used a fourth pdx: JH2-055 but this pdx refused to grow well enough to use.*

i. Do the RAS or RALGEF inhibitors sensitize tumor cells to chemotherapy?

The RAL protein uses RALBP1 as an effector. This is a multi-functional protein but one of its attributes is that it can serve as a non ABC drug transporter to shuttle chemotherapeutics out of a cell. Therefore, its inhibition could sensitize tumor cells to Doxorubicin. When we performed tissue culture experiments combining C4 with doxorubicin we found that the combination enhanced MPNST cell growth inhibition.

Figure 6. C4 RALGDS inhibitor enhances the sensitivity of S462.TY cells to Doxorubicin. *SF462.TY Cells were plated in 6 well plates and challenged with 10 nM Doxorubicin and 1.5 uM C4 alone or in combination. After 3 days cell numbers were counted in a hemocytometer.*



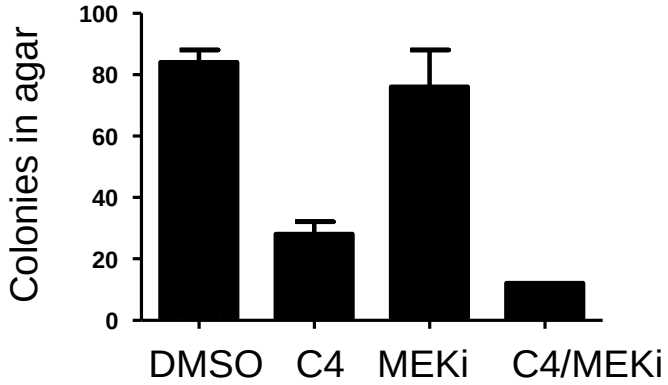
vii. Does the RALGDS inhibitor synergize with MAPK pathway inhibitors?

In Figure 2 we can see that RAS activates three classic mitogenic pathways. The C4 class of inhibitors suppresses only the RALGDS pathway. Therefore, we anticipate it will have an additive or synergistic effect when combined with an inhibitor specific to the RAF/MAPK pathway. Trametinib is an approved inhibitor of the kinase MEK that specifically suppresses the RAF/MAPK pathway.

We combined the MEK inhibitor with C4. We detected an enhanced suppressive activity of the combined compounds in soft agar assays against the MPNST cell line S462.TY (Figure 7)

Figure 7. C4 and MEK inhibitors co-operate to suppress MPNST cell growth in agar.

S462.TY cells were plated in soft agar with 5 uM C4 or 100nM MEKi (Trametinib) alone or in combination. Colonies were scored after 2 weeks. The combination showed a greater than additive effect.



Specific Aim 2: TASK 2.

Do the inhibitors suppress MPNST in a transgenic mouse model:

A transgenic mouse system of MPNST development has been established that uses a in cis mutation of p53 and NF1 to predispose the animals to MPNST. Groups of 12 animals were randomly assigned to experimental groups and treated with F3-8-80 by gavage and C4-180 by ip injection. Animals were euthanized at end point (any signs of distress of weight loss >10%). Neither group exhibited a statistically significant increase in survival ($p > .05$). The F3-8-60 group showed less survival, perhaps suggesting some toxicity from prolonged exposure to the drug.

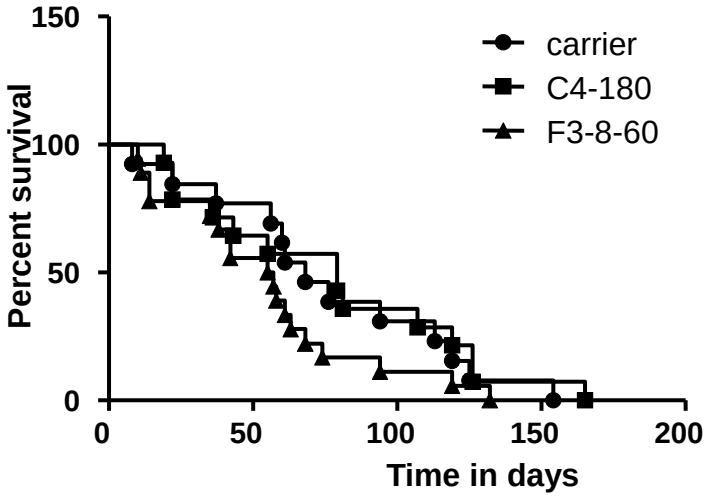


Figure 3. Response of P53/NF1 hz mice to anti-RAS drugs: Animals were bred, genotyped and positive animals fed into the system as they arose randomly. Drug was administered by gavage for F3860 (100mg/kg) and ip injections for C4-180 (40mg/kg). Animals were entered into the study at ages between 112-120 days. Time point 0 is the start of the drug treatment regime. N >= 12 for each group ~50/50 M/F.

Training:

Historically under-represented minority student Raphael Jigo underwent training on RAS signal analysis.

Dissemination of Results:

1. **Poster presented NCI RAS symposium conference October 2022. Frederick Maryland.**
2. **Poster presented at Rasopathies biannual meeting August 2019 Baltimore**

Plans for the next reporting period (if permitted):

1. Perform testing of a new anti-RAS molecule based on a different chemical scaffold to determine if we can turn growth suppression into tumor regression with the enhanced activity agent.

4. IMPACT

Manuscript under preparation.
NIH R01 application based on the data submitted.

5. Changes/Problems

There have been no significant changes in the original objectives or approach. However, the project fell a little 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. Even the transgenic arm of the project was affected by the pandemic as the laboratory of collaborator 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.

While we were pursuing this project, we continued to perform Medicinal Chemistry optimization of our anti-RAS agent as part of a separate project. We have now developed compounds that bind x 100 better to recombinant RAS protein, have better PK properties and that almost completely suppress pancreatic tumor growth in vivo. As we have some funds left over due to the delays, I would propose a second NCE which would allow us to repeat the in vivo experiments using the new improved agents.

6. Products

Established proof of principal for the use of pan RAS inhibitors against MPNST.

7. Participants and Collaborating Organizations

Name:	Geoff Clark
Role:	PI
Effort:	1.2 Months effort
Contribution:	Supervised project and performed cell culture experiments.
Funding:	CDMRP, NIH, Qualigen LLC.

Name:	Becca von Baby
Role	Technician
Effort	2.4 months effort
Contribution	Tissue culture assays, animal colony expansion, in vivo experiments and pdx animal experiments.
Funding	CDMRP. Qualigen LLC.

Name	Howard Donninger
Role	Instructor
Effort	2.4 months%
Contribution	Signaling analysis and tissue culture assays
Funding	NIH, Jewish Fund For Excellence.

Name	Raphael Jigo
Role	Graduate student
Effort	100%
Contribution	Signaling and in vitro growth assays.
Funding	CDMRP, IPIBS, American Lung.

Changes in active support:
American Lung Association Grant to examine the RALGDS inhibitors in lung cancer.

CDMRP Breast cancer award to examine the role of RAS inhibitors in Luminal B breast cancer

CDMRP Ovarian cancer award to examine the role of RAS inhibitors in High grade serous ovarian cancer.

KY pediatric cancer Foundation award to examine the role of RAS inhibitors in High grade pediatric glioma.

Qualigen LLC Sponsored Research Agreement.

Other Organizations involved:

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

8. Special Reporting Requirements

None

9. Appendices

None