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TITLE: Investigating the Interactions of Glutamine Amidotransferases and MEK-ERK Signaling to Develop Novel Therapeutic Strategies for NF1-Associated Tumors

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14. ABSTRACT

People with the genetic condition neurofibromatosis type I (NF1) have a considerably increased risk for cancer compared to the general population. Many such tumors cannot be removed with surgery and do not respond well to traditional chemotherapy. The goal of the proposed research is to investigate whether a new type of medicine called a *glutamine amidotransferase inhibitor* can effectively treat tumors in NF1 patients. Glutamine amidotransferases, of which there are at least 8 in human cells, are metabolic proteins that use the amino acid glutamine to build nutrients that cancer cells need to grow and survive. Our prior research has shown that glutamine amidotransferase inhibitors, including the compound DRP-104, are effective treatments in tumor models, but this finding has not yet been explored thoroughly in NF1-associated tumor models including malignant peripheral nerve sheath tumor or glioma. The goal of the proposed research is to investigate the interactions of glutamine amidotransferases with signaling pathways that are active in NF1-associated tumors and to examine how effective DRP-104 is as a single agent or in rational combinations in NF1-associated tumor models – both in cells and in animal models. In this Year 1 progress report we describe efforts to date in these investigations. Both cell based studies and animal studies using DRP-104 have been initiated.

15. SUBJECT TERMS
Neurofibromatosis, glutamine, metabolism, MEK signaling, cancer

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1. INTRODUCTION

Improved understanding and therapies are needed to effectively treat tumors in patients with the cancer predisposition syndrome neurofibromatosis type I (NF1). NF1 is a negative regulator of the RAS-MEK-ERK pathway. MEK-ERK signaling regulates glutamine amidotransferases in RAS-driven tumors, but these interactions have not been explored in NF1-associated tumors. Glutamine amidotransferases are enzymes involved in multiple metabolic pathways in tumors, including glutamine-dependent nucleotide synthesis. Our prior work showed that a preclinical glutamine amidotransferase inhibitor inhibits growth of NF1-MPNST with prominent effects on tumor nucleotide synthesis. These findings warrant further mechanistic and translational investigations to identify the effect of MEK-ERK signaling on glutamine amidotransferases and evaluate the therapeutic utility of the newly discovered clinical-stage glutamine amidotransferase inhibitor DRP-104 in NF1-associated tumors. We hypothesize that NF1-associated tumor glutamine metabolism is modulated by interactions between MEK-ERK signaling and glutamine amidotransferases, and these interactions can be targeted for therapeutic benefit using the prodrug glutamine amidotransferase inhibitor DRP-104. The proposed research will test the above hypothesis by developing improved characterization of interactions between glutamine amidotransferases and MEK-ERK signaling in NF1-associated tumor models and by evaluating *in vitro* and *in vivo* efficacy of the clinical GA inhibitor DRP-104 both as a single agent and in rational therapeutic combinations including with MEKi. The proposed research will have both basic science and a translational impact in the NF1 community. If positive, the focus on clinically available agents will enable drug combinations to be rapidly translatable to a clinical trial in NF1 patients with tumors.

2. KEYWORDS:

Neurofibromatosis, glutamine, metabolism, MEK signaling, cancer

3. ACCOMPLISHMENTS:

a) What were the major goals of the project?

The Specific Aims of this project and associated milestones for Year One were:

Specific Aim 1: Characterize the relationship between glutamine amidotransferases and MEK-ERK signaling in NF1-associated tumor cells.

Milestone 1: *We will determine the effects of perturbing glutamine amidotransferases and MEK-ERK signaling on NF1-associated MPNST and glioma cell use of glutamine for biosynthesis.*

Milestone 2: *We will characterize the adaptive effects of glutamine amidotransferases at the translational and post-translational level to MEKi in NF1-associated tumor cells.*

Milestone 3: *We will characterize the response of MEK-ERK signaling to inhibition of glutamine amidotransferases.*

Specific Aim 2: Evaluate the *in vitro* efficacy of the glutamine amidotransferase inhibitor DRP-104 in genetically heterogeneous NF1-associated tumor cells both as a single agent and in rational combinations, including with MEK inhibition.

Milestone 1: *We will characterize the sensitivity and markers of growth arrest and cell death in response to DRP-104 across a panel of genetically heterogeneous NF1-associated tumor cell lines.*

Milestone 2: *We will determine the in vitro antitumor efficacy of combination DRP-104 plus MEKi and combination DRP-104 with additional rationally-selected agents in MPNST and glioma cells.*

Specific Aim 3: Evaluate the *in vivo* efficacy of the glutamine amidotransferase inhibitor DRP-104 as a single agent and in rational combinations, including with MEK inhibition, in NF1-associated syngeneic and PDX MPNST and glioma models.

Milestone 1: *We will determine the in vivo antitumor efficacy of single agent DRP-104 in MPNST and glioma mouse models.*

Milestone 2: *We will determine the in vivo antitumor efficacy of DRP-104 plus MEKi or other rational combinations in MPNST and glioma mouse models.*

b) What was accomplished under these goals?

Major tasks of the project completed year one
Aim 3, Major Task 1, Subtask 0: Obtain ACURO protocol approval – completed

Major tasks of the project ongoing year one:
Aim 1, Major Task 1, Subtask 1: Determine dose response of parental and resistant MPNST cell lines (ST-8814P, ST-8814R) to DRP-104 and MEKi (months 1-2) – completed dose response of ST-8814P to DRP-104 and MEKi. Ongoing evaluation in ST-8814R.

Aim 1, Major Task 1, Subtask 2: Prepare samples of $^{15}N_2$-glutamine and $^{13}C_5$-glutamine-labeled MPNST cells (ST-8814P, ST-8814R) treated with DRP-104, MEKi, the combination, or vehicle (months 3-10) – to be prepared after completion of dose response of ST-8814R in subtask 1 determined. Samples will be shipped to UTSW for glutamine flux analysis under Subtask 3.

Aim 1, Major Task 1, Subtask 4: Generate MEKi resistant JHH-NF1-PA1 glioma cell line; determine dose response of parental (P) and resistant cells (R) to DRP-104 and trametinib (month 3-9) – ongoing cell culture.

Aim 1, Major Task 2, Subtask 1: Measure effect of MEKi treatment on p-PFAS, p-CAD, and total PFAS, CAD, PPAT, CTPS, and GMPS in MPNST cell lines (sNF96.2, JH2-002, ST-8814, sNF90.8) by immunoblotting – ongoing.

Aim 1, Major Task 2, Subtask 2: Measure effect of MEKi treatment on p-PFAS, p-CAD, and total PFAS, CAD, PPAT, CTPS, and GMPS in glioma and melanoma/breast cancer cell lines (JHH-NF1-PA1, SF188, MeWo, MB-231) by immunoblotting – to be completed after Aim 1, Major Task 2, Subtask 1.

Aim 1, Major Task 2, Subtask 3: Examine effect of a second MEKi on glutamine amidotransferases affected in cell lines evaluated under Subtasks 1 & 2 by immunoblotting. – will initiate once Major Task 2, Subtasks 1&2 completed.

Aim 1, Major Task 2, Subtask 1: Measure effect of DRP-104 on p-ERK, total ERK, and other downstream MEK-ERK targets (e.g., p-RSK, cyclin D1) in MPNST cells (sNF96.2, JH2-002, ST-8814, sNF90.8) by immunoblotting – ongoing

Aim 2, Major Task 1, Subtask 1: Examine dose response of six MPNST cell lines to single-agent DRP-104; determine IC_{50} values – ongoing

Aim 2, Major Task 1, Subtask 2: Examine dose response of three glioma cell lines, one melanoma cell line, and one breast cancer cell line to single-agent DRP-104; determine IC_{50} values – ongoing

Aim 3, Major Task 1, Subtask 1: Examine tolerability of DRP-104 in B6 and NSG mice at three dose levels. Evaluate animal weights, body condition scores, and clinical lab values. – ongoing

Aim 3, Major Task 1, Subtask 3: Propagate MPNST PDX (JH2-002; JH2-031) to NSG mice for efficacy studies – Completed for JH2-031 PDX. Ongoing for JH2-002.

Aim 3, Major Task 1, Subtask 4: Examine antitumor efficacy of DRP-104 in MPNST PDX models – ongoing

C) Accomplishments from goals

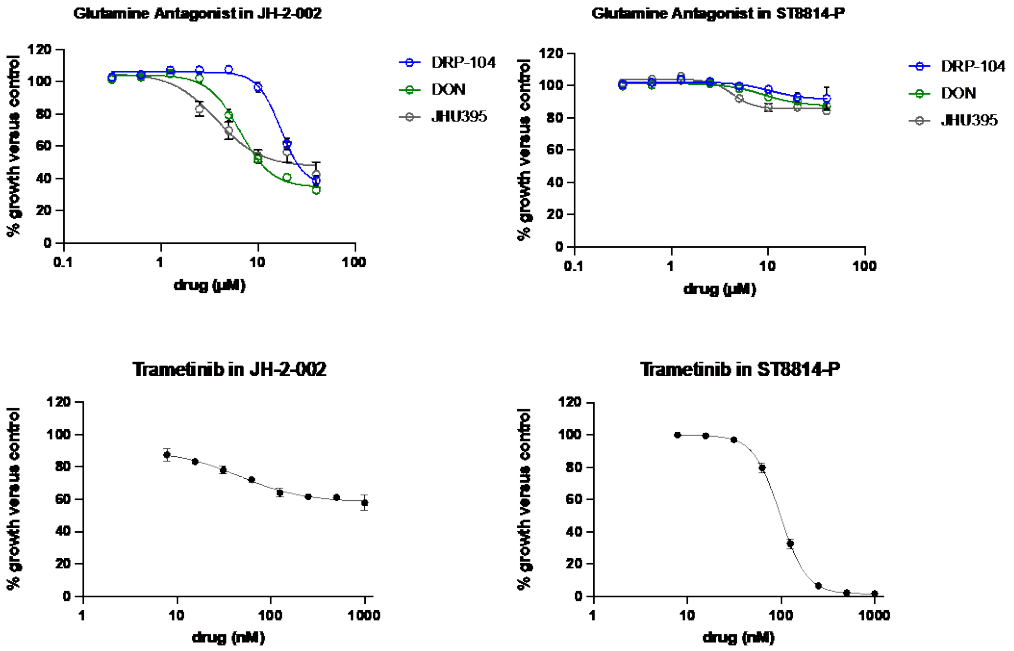
C1 & 2) Major activities and specific objectives: The major activities for year one were to characterize the relationships between glutamine metabolism and MEK-ERK signaling in cell culture models of NF1-associated tumors and to develop knowledge of the dose response of NF-1associated tumor cell lines to the novel

glutamine amidotransferase inhibitor DRP-104. Additionally, we aimed to begin to evaluate DRP-104 tolerability and anti-tumor efficacy in mouse models of NF1-associated tumors.

C3) The specific accomplishments in year one:

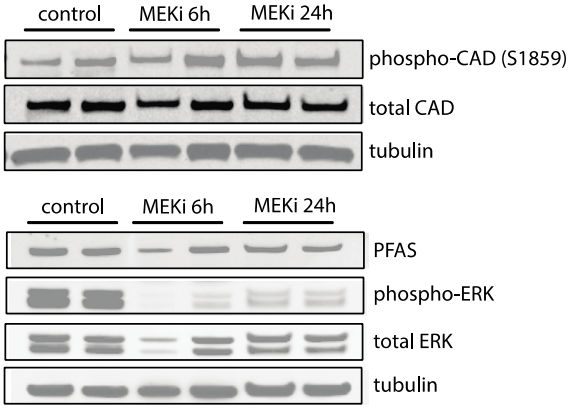
Evaluate the dose response of MPNST cell lines to DRP-104 and MEKi:

Dose response of JH-2-002 and ST-8814P human MPNST cell growth inhibition to the glutamine antagonist DRP-104 and trametinib (MEKi) were examined using Alamar blue fluorescence. GA dose response to DRP-104 was compared to the parent compound DON as well as to a nervous tissue penetrant GA used previously, JHU395. In these cell lines we observed a reciprocal relationship between cell growth inhibition to MEKi and GA. IC50 values for JH-2-002 cells were in the 3.7-17.2 micromolar range, with maximal percent efficacy ranging from 53-66% growth inhibition at 40 micromolar drug; however JH-2-002 cells were relatively insensitive to growth inhibition by trametinib at 1 micromolar, in line with prior published reports. By contrast ST-8814P cell growth showed limited inhibition by DRP-104 and other glutamine antagonists, while IC50 for trametinib was ~100 nM with nearly 100% efficacy at 10 micromolar drug. These findings lead us to hypothesize that NF1-associated tumor cell lines may have reciprocal sensitivity to MEKi and GA drugs, which may be exploited for future therapeutics studies. Going forward we will test this hypothesis in the remaining NF1-associated tumor cell lines undergoing dose response evaluation to DRP-104 and MEKi.



Measure effect of MEKi treatment on glutamine amidotransferases in MPNST cell lines:

We examined the effect of trametinib (20 micromolar, 6h and 24h) on MEK-ERK signaling and GA expression in JH-2-002 cells. Trametinib as expected partially inhibited signaling through MEK to phospho-ERK (bottom panel). Changes in total PFAS were not observed. Slightly more phospho-CAD was observed in trametinib treated cells than control (top panels). Going forward we will aim to quantify and replicate these results and examine this signaling pathway in other NF1-associated tumor cell lines.



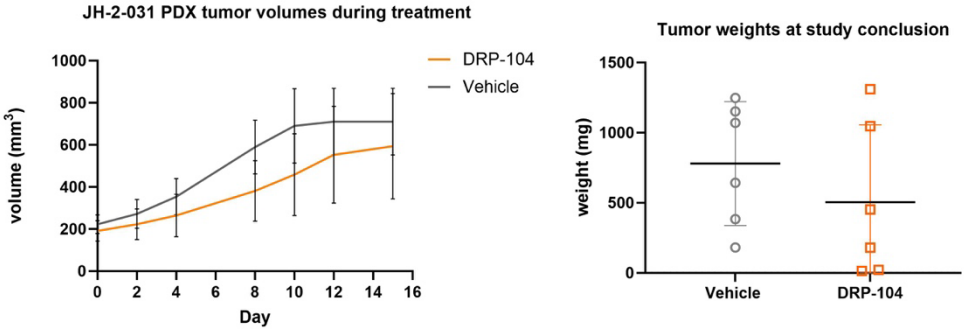
Examine tolerability of DRP-104 in mice at 3 dose levels

We examined the tolerability of DRP-104 in female athymic nude mice (~10 weeks of age). Mice were treated with vehicle or DRP-104 at 4 dose levels. The highest dose was 8 mg/kg s.c. = 3 mg/kg DON equivalent, shown in other strains to be above the MTD. Three additional dose levels at ½ log scale were also tested. We observed that the 8 mg/kg dose led to weight loss approaching ~15% baseline in less than two weeks. Mice receiving this dose also appeared pale, in line with significantly decreased hemoglobin at time of tissue harvest indicative of anemia. Thus in the nude mice strain 2.7 mg/kg s.c. (1 mg/kg DON equivalent) administration was determined to be the MTD and taken forward for further in vivo activity studies.

C4) Other accomplishments

Examine antitumor efficacy of DRP-104 in an MPNST PDX model

Nude mice (n = 6 per group, females) with established flank JH2-031 MPNST PDX were treated with DRP-104 2.7 mg/kg s.c. 5 days/week or vehicle. At day 15 from start of treatment, there was a trend towards DRP-104 treated animals having lower tumor volumes compared to vehicle (DRP014 mean volume 593 mm^3 versus vehicle mean volume 710 mm^3) though the difference was not statistically significant. There was also a trend towards smaller mean tumor weights in the DRP-104 treated tumors at tissue harvest.



D) Opportunities for training and professional development:

During year one of the grant I attended the Children's Tumor Foundation annual meeting in Scottsdale AZ. There I had the opportunity to take part in a metabolism working group with Dr. Miriam Bornhorst, an NF1 researcher from Children's National Medical Center, and Dr. Vincent Riccardi, an NF researcher with a longstanding interest in metabolism in NF. I also had the opportunity to present my work on targeting glutamine metabolism in cancer at the Strategic Advances in Sarcoma Science meeting at the National Cancer Institute in September 2023.

E) How were results disseminated to communities of interest?

Nothing to report

F) What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period we plan to continue ongoing experiments evaluating the interactions between glutamine metabolism and MEK-ERK signaling in a range of NF1-associated cell culture models. A priority will be to complete the sample preparation of $^{15}N_2$-glutamine and $^{13}C_5$-glutamine flux in MEKi treated MPNST and glioma cells and ship these samples to UTSW for bioanalysis. In addition we plan to complete the experiments evaluating the effect of MEKi on glutamine amidotransferase expression and the counter point experiments evaluating DRP-104 effect on MEK signaling. Finally, we will evaluate DRP-104 tolerability and activity in additional in vivo NF1-associated tumor models.

4. IMPACT:

a) Impact on development of the principal disciplines of the project

Nothing to report

b) Impact on other disciplines

Nothing to report

c) Impact on technology transfer

Nothing to report

d) Impact on society beyond science and technology

Nothing to report

5. CHANGES/PROBLEMS:

a) Changes in approach and reasons for change

Nothing to report

b) Actual or anticipated problems or delays and actions or plans to resolve them:
For the tasks under Aims 1 & 2 involving human cell lines the PI encountered delays during the reporting period. First, the PI received HRPO authorization to begin work with the cell lines proposed for this project on 3/25/2023. The initial submission to the local IRB for this research was completed prior to the start of Year 1, but there were delays in local IRB approval. Second, JHU and the company to which DRP-104 is licensed were not able to negotiate an MTA for the compound; therefore this was synthesized in house and received in April 2023. Third, in spring 2023 the PI began relocating to independent lab space elsewhere on the JHU campus, which affected initiation of some experiments. At this writing these delays have been resolved and the PI does not anticipate further problems in the research.

The phospho-PFAS antibody that was available at the time the proposal was written was removed from the catalogue by the vendor and is not available at this time. We continue to seek another source of this antibody and have been in contact with the prior vendor to express interest.

Due to laboratory staff turnover and a delay in hiring, a new research technician providing effort on this project started in Dr. Lemberg's lab in mid-August 2023.

c) Changes that had a significant impact on expenditures
Due to the timing of HRPO approval allowing initiation of Aim 1 experiments, samples for metabolomic analysis have not yet been shipped to the Metabolomics Core at UTSW. This will be carried out and the relevant funds spent in Year Two.

The PI had additional mice left after purchase from a prior award available for studies of DRP-104 in vivo in Year One. Thus less was spent from the current award on animals for the studies. However going forward as nude mice are available from Charles River we anticipate ongoing expenditures on this strain.
A new research technician was hired in August 2023 to focus on the proposed research for this project and her salary (50%) will be paid from this award going forward.

d) Significant changes in use or care of human subjects
Nothing to report

e) Significant changes in use or care of vertebrate animals
The PI has been able to propagate MPNST PDX in athymic nude mice in addition to NSG mice. Due to the fact that nude mice are less immunocompromised than NSG mice and therefore may better model the microenvironment of human cancers, nude mice have been used in PDX studies. Dr. Lemberg's approved IACUC protocol includes approval for tumor studies in nude mice.

F) Significant changes in use of biohazards and/or select agents
Nothing to report

6) PRODUCTS:
a) Publications, conference papers, and presentations
Nothing to report

b) Website(s) or other Internet site(s)
Nothing to report

c) Technologies or techniques
Nothing to report

d) Inventions, patent applications, and/or licenses
Nothing to report

e) Other Products
Nothing to report

7) PARTICIPANTS AND COLLABORATING ORGANIZATIONS:
a) Individuals working on the project

Name	Kathryn Lemberg
Project Role	Principal Investigator
Researcher Identifier	0000-0003-1511-9332
Nearest person month worked	4
Contribution to Project	Obtaining HRPO and ACURO approval. Cell culture. Dose response analysis. Western blotting. Mouse tumor propagation. Mouse tolerability study. In vivo efficacy study. Data analysis.

b) Change in active other support of the PD/PI or senior/key personnel since last reporting period
The PI previously had a Stetler Research Fund Award which ended in June of 2022 and a CureSearch Young Investigator Award (with a one year No Cost Extension) which closed in June of 2023.
The PI received an Hyundai Hope on Wheels Young Investigator Award in December of 2022 which is providing partial salary support (3 calendar months).
The PI also received laboratory start up support from her academic department upon promotion to Assistant Professor in July 2022.

c) Organizations involved as partners
Nothing to report

8) SPECIAL REPORTING REQUIREMENTS: None

9) APPENDICES: None