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TITLE: Targeting BRAF V600E and Autophagy in Pediatric Brain Tumors

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Introduction:

Despite advancement in treatment for childhood central nervous system (CNS) tumors, they remain the leading cause of death in pediatric oncology. One potential therapeutic intervention is targeting the autophagic pathway, a complex catabolic process that contributes to tumor cell survival. Recent data has shown $BRAF^{V600E}$ mutations in a range of these tumors and my own research finds that these tumors show a level of autophagy-dependence not seen in $BRAF^{WT}$ tumors. There is also evidence that autophagy inhibition is a potential mechanism in preventing or reversing resistance to direct inhibition against activated $BRAF^{V600E}$. The current study examined the interaction of the $BRAF^{V600E}$ mutation and autophagy in brain tumors. I hypothesized that $BRAF^{V600E}$ identifies tumors that will respond to combination therapy with autophagy inhibition with enhanced tumor cell death, establishing a basis for future rational clinical trial design for pediatric brain tumor patients harboring the mutation.

Keywords:

Autophagy

BRAF

Brain tumor

Pediatric

Resistance

Accomplishments:

The major aims of this project were: **(1)** Determine how the $BRAF^{V600E}$ mutation results in sensitization of CNS tumor cells to autophagy inhibition by (a) evaluating ERK activity in $BRAF^{V600E}$ mutant cells compared to WT controls and (b) assessing MEK-inhibition as a potential secondary target in this patient population. **(2)** Evaluate the effectiveness of autophagy inhibition to improve small molecule targeted therapy in $BRAF^{V600E}$ CNS tumors in vivo by using combination therapy with chloroquine (autophagy inhibition) and vemurafenib (Raf-inhibitor).

The major goals of the project as outlined in the SOW and accomplishments are as follows:

Major Task 1: Training and educational development in nuero-oncology and autophagy research *(only applicable to training award mechanisms)*
I have continuously attended a weekly lab meeting with neuro-oncology as well as a separate meeting with an autophagy focus. I attend the Neuro-oncology meeting weekly on Fridays and present my research approx. every 6 weeks. I also attend the autophagy focused meeting on Fridays and present my research in progress report once a quarter as well as well as a journal club. I also present research at the montly campus neuro-oncology research in progress meeting. I had goals to attend a national scientific meeting in relevant scientific field. I submitted an abstract of my most recent findings to the Society of Neuro-Oncology Pediatric Neuro-Oncology Basic and Translational Research Conference. It was presented in poster format 05/7/15-05/08/15. I also submitted an abstract to the AACR Advances in Brain Cancer Research focused

meeting. The abstract was presented in poster format 05/28/15-05/30/15.

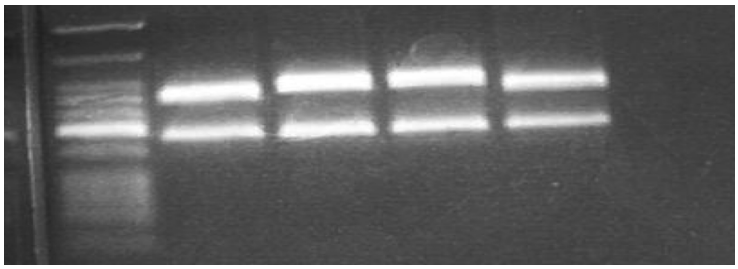
Research-Specific Tasks:

Specific Aim 1: *Determine how the BRAF^{V600E} mutation results in sensitization of CNS tumor cells to autophagy inhibition.*

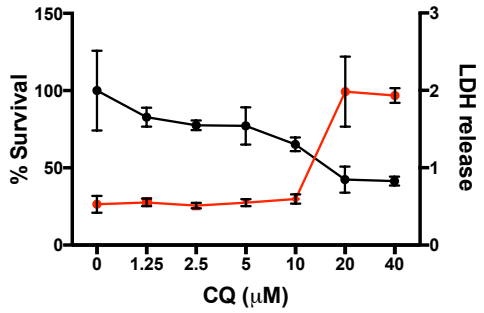
Major Task 1: Evaluate genetic alterations following activation of BRAF^{V600E}

Cell lines used: BRaf$^{CA/WT}$Ink4alox/lox mouse neurospheres (Cell line provided by Dr. Claudia Petritsch, UCSF). We have been able to successfully grow the neurospheres and attemped the Ad:cre activation. Initial evaluation of the activated cells did not show positive activation. We re-peated this and shown successful activation by PCR.

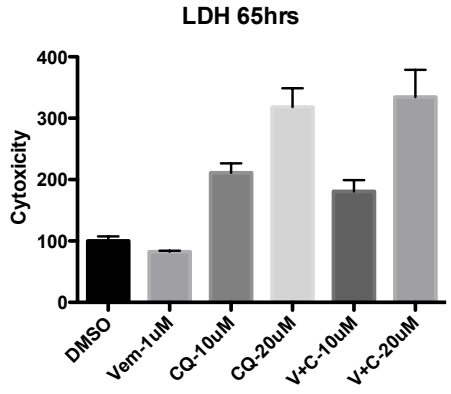
CA/WT p3	V600E/WT p3	V600E/WT p6	CA/WT p6



Activated cells also show a significant growth difference with activated cells growing much faster than un-activated cells. Further analysis of these cells has shown a dose dependent response to vemurafenib as well as autophagy inhibition response in a dose dependent manner to chloroquine as shown by MTS and LDH.



Cytotoxic response to vemurafenib and autophagy inhibition is shown below as measured by LDH release in 6390 BRAF^{V600E} activated cells.



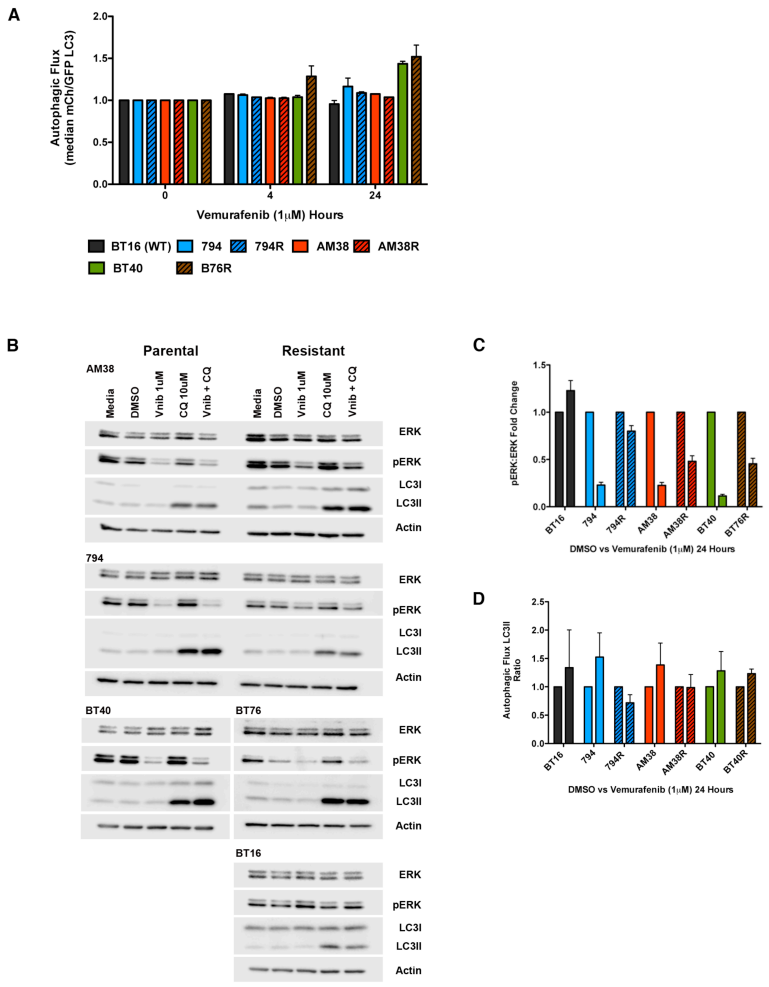
I have also completed an evaluation of human brain tumor cells lines to show that the MAP kinase pathway is targeted by treatment of these cells with the BRAF inhibitor vemurafenib. Note the difference in pERK suppression in resistant lines. An assessment of autophagy response is also shown. An example comparing the parental (sensitive) and resistant lines. (see western blot shown in Major Task 2).

Remaining work following completion of this award will focus on completing Affymetrix U133-plus chip analysis for a more complete evaluation of genetic changes within these cells.

Major Task 2: Evaluation of autophagy in isogenic lines for direct

An evaluation of CNS tumor lines both sensitive and resistant to BRAF inhibition has been completed. Evaluation of MAP kinase pathways in relation to changes in level of autophagy in $BRAF^{V600E}$ cells. As can be seen in the figure below, inhibition of autophagy through pharmacologic methods with chloroquine (CQ) does not appear to alter pERK/ERK. Additional studies of pMEK/MEK will be undertaken, as well as inhibition of autophagy genetically through shRNA to specifically inhibit the process and avoid off-target drug effects.

Also note the evaluation of autophagy levels in paired cells. Note the induction of resistance in these cells does not cause an overall increase in autophagy levels when compared to parental/sensitive controls.



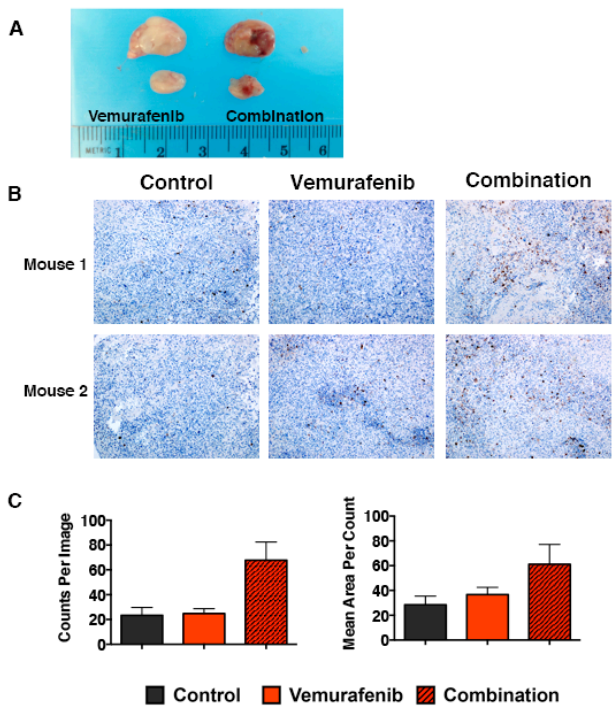
Major Task 3: Evaluation of MAP kinase pathways in relation to changes in levels of autophagy in BRAF^{V600E} cells

The award was completed prior to work starting on this Task. Follow up plan after completion fo this award will continue to evaluate these tasks.

Specific Aim 2: *Evaluate the effectiveness of autophagy inhibition to improve small molecule targeted therapy in BRAF^{V600E} CNS tumors in vivo.*

Major Task 1: Mouse studies with intracranial xenografts

Due to the early closure of this grant, only early progress was made on this Task. I did complete a caspase activation analysis on a subset of preliminary in vivo experiments that were completed just prior to activation of this award (see below). I have also established two cells lines in the intracranial model (AM38 and 794). Additional in vivo work will continue following closure of this award. Initial results are promising.



Combination RAF and autophagy inhibition results in greater cell death *in vivo*. Mice with IC tumors were treated with saline, vemurafenib, or a combination of vemurafenib and CQ. (A) Gross inspection of tumors, evidence of hemorrhage indicating cell death in combination treatment. (B) IHC staining for caspase is more prominent in combination treated samples than control or vemurafenib only. (C) Quantification of the number of caspase positive area and the area of caspase positive areas. Both are higher in combination treated mice compared to control or vemurafenib only mice.

Opportunities for training and professional development:

Please see Major Task 1 under training and educational development. In addition to the information above, I continued to have weekly on-on-one meetings with both co-mentors Dr. Andrew Thorburn and Dr. Nicholas Foreman. I was able to attend the conferences as noted above as well.

How were the results disseminated to communities of interest:

I had poster presentations at two major conferences as noted above. In addition, I have completed writing a manuscript that is currently under review at Cancer Research. This manuscript focuses on the ability of autophagy inhibition to improve response to vemurafenib, specifically when used in the context of cells resistant to BRAF inhibition.

Plan for the next reporting period:

Nothing to report (final report).

Impact:

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

I published in *Cancer Discovery* the first evidence that the $BRAF^{V600E}$ mutation causes brain tumor cells to become dependent on autophagy and that targeting of autophagy in these cells was an effective therapeutic intervention. Additionally, we demonstrated that autophagy inhibition is synergistic with vemurafenib, a kinase inhibitor directed at BRAF. The work completed over the DOD award period is the natural progression of these findings and in addition to studying the mechanism related to the benefit of autophagy inhibition, it addresses the biggest problem in using kinase inhibitors for cancer therapy, acquired resistance. Specifically, I found that inhibition of a completely different process (autophagy) that is essential for brain tumor cells driven by the targeted kinase can improve targeted BRAF inhibition and additionally, circumvent clinically acquired kinase inhibitor resistance.

This data provides several important conceptual advances from my previous work. I now have direct evidence from tumor tissue from people who acquired resistance to the drug during clinical treatment, and we can show direct reversal of this resistance by the addition of autophagy inhibition. Modeling of resistance in tumor cell lines directly supports this. The biggest conceptual advance is more general, providing a fundamentally different way to overcome acquired resistance (current approaches target the same or parallel kinase pathway a different way) that may apply to different resistance mechanisms and be longer lasting.

What was the impact on other disciplines? Nothing to report.

What was the impact on technology transfer? Nothing to report.

What was the impact on society beyond science and technology? Nothing to report.

Change/Problems? Nothing to report.

Products: Nothing to report

Publications, conference papers, and presentations

Mulcahy Levy, JM; Zahedi, S; Griesinger, AM; Davies, KD; Aisner, DL; DeMasters, BK; Fitzwalter, BE; Goodall, ML; Amani, V; Donson, AM; Birks, DK; Mirsky, DM; Hankinson, TC; Handler, MH' Foreman, NK; Thorburn, A. *Autophagy Inhibition Overcomes Clinically Acquired Resistance to BRAF Inhibition in Brain Tumors*. Cancer Research, under review. Acknowledgement of federal support: yes. When accepted for publication a copy of the manuscript will be forwarded.

Conference poster presentations reported above.

Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Jean M. Mulcahy Levy
Project Role:	PI
Nearest person month worked:	5

Contribution to Project:	Dr. Mulcahy Levy oversaw all aspects of this project in addition to performing experiments.
Funding Support:	No change
Name:	Andrea Griesinger
Project Role:	Professional Research Assistant
Nearest person month worked:	6
Contribution to Project:	Ms. Griesinger was primarily in charge of performing experiments.
Funding Support:	No change

Since the last reporting period the PI of this project was awarded an NIH/NCI K08 Mentored Research Scientist award. The DOD award was relinquished prior to the start of the K08 award per granting requirements.

What other organizations were involved as partners? Nothing to report.

Special reporting requirements: None
Appendices: None