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Targeting Pediatric Glioma with Apoptosis and Autophagy Manipulation

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Gliomas are the most common and most deadly solid tumors that affect children. Treatment options are limited and cure rates are dismal. My laboratory has established that Mer and Axl receptor tyrosine kinase are aberrantly overexpressed in gliomas, and that inhibition of these RTKs leads to increased glioma cell apoptosis, decreased tumor cell survival and profoundly improved chemosensitivity. However, I have also recognized that Mer and Axl inhibition is associated with increased autophagy. Based on this new discovery, I hypothesize that Mer and Axl RTK signaling regulates autophagy pathway activation in glioma cells, and this regulation determines the efficiency of glioma cell killing.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Body</td>
<td>1</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>9</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>10</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>Appendices</td>
<td>10</td>
</tr>
</tbody>
</table>
INTRODUCTION:
Gliomas are the most common and most deadly solid tumors that affect children. Treatment options are limited and cure rates are dismal. My laboratory has established that Mer and Axl receptor tyrosine kinase are aberrantly overexpressed in gliomas, and that inhibition of these RTKs leads to increased glioma cell apoptosis, decreased tumor cell survival and profoundly improved chemosensitivity. However, I have also recognized that Mer and Axl inhibition is associated with increased autophagy. Based on this new discovery, I hypothesize that Mer and Axl RTK signaling regulates autophagy pathway activation in glioma cells, and this regulation determines the efficiency of glioma cell killing. Better understanding of the role autophagy plays in pediatric glioma and its interaction with RTK inhibition and apoptotic pathway activation will enable us to develop efficacious clinical trials for pediatric glioma that include the appropriate manipulation of autophagy.

BODY:
Copied below is the statement of work (from 4/22/11) with comments (in italics) regarding details of progress and results.

**Statement of Work (v. 4/22/11)**

<table>
<thead>
<tr>
<th>Specific Aim 1</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
<tr>
<td>1</td>
<td>1a</td>
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<tr>
<th>Specific Aim 2</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
<tr>
<td>4</td>
<td>4a</td>
<td>4a</td>
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<td>5</td>
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<td>5a</td>
<td>5a</td>
</tr>
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</table>

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<th>Specific Aim 3</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
<tr>
<td>6</td>
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<tr>
<th>Publication #1</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
<tr>
<td>9</td>
<td>9a</td>
<td>9b</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Publication #2</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Task</td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
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<td>10b</td>
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**SA1:** To test the hypothesis that Mer/Axl RTK inhibition results in increased levels or activity of known effector molecules of autophagy.

**Task 1:** Inhibit Mer or Axl RTK expression/signaling in G12, A172, U251, U118, and SF188 glioma cell lines.

1a. Transduction of constitutive and doxycycline-inducible shMer or shAxl into U251, U118 and SF188 cells. This has already been completed for G12 and A172 cells, needs to be done for U251, U118, SF188. [Months 1-3]

**Final Report Sept 2014---**U251 and SF188 cell lines, a pediatric glioma line, were transduced with Mer and Axl constitutive shRNA, single cell sorted, and clonal lines developed (Fig. 1ai). We have also more recently developed short term siRNA inhibited lines which removes some of the long term clonal changes that occur off target in shRNA inhibited lines as both Mer and Axl inhibition provide such a strong signal to find alternate pathways (Fig. 1aii).
1b. Apply ligand sequestration, antibody treatment or small molecule inhibitors to all cell lines as alternate modes of inhibition. [Months 10-18]

Final Report Sept 2014---We have successfully inhibited Mer and Axl RTK signaling with several different small molecule inhibitors (SMI) which competitively bind in the ATP binding pocket and block activation of the RTK. Using a commercially available SMI, Foretinib (GlaxoSmithKline), in cooperation with an MTA we have found that we can very effectively inhibit the activation of MerTK and Axl (Fig. 1bi). Most recently we have begun to use a proprietary molecule developed by an academic collaborator at the University of North Carolina that is more specific for MerTK and has less off targeted effects (Fig. 1bii).

Final Report Sept 2014---We have been using conversion of LC3I to LC3II as a marker of autophagic flux. We have found that we can successfully measure autophagic flux in G12 (1), A172, SF188 and U251 cell lines with this immunoblot method, and have again reiterated the increase in autophagic flux following Mer or Axl RTK inhibition

Task 2: Evaluate autophagic flux following RTK inhibition.

2a. Measure p62 and BHMT levels. [Months 1-3 and 16-18]
in several different glioma cell lines. We have evaluated p62 by immunoblot and found no major differences in autophagy by this method, likely due to low antibody sensitivity on immunoblot.

**Figure 2a.** Evaluation for autophagic flux following RTK inhibition. Immunoblot for levels of LC3II and LC3I (red boxes) following RTK inhibition with shRNA show increased conversion to LC3II compared to control shRNA or parental line indicating increased flux.

2b. Time-lapse microscopy of fluorescently tagged proteins. [Months 4-6 and 19-21]

**Final Report Sept 2014—**We have now developed GFP tagged and GFP-mCherry tagged u251 and SF188 cells in addition to the U87 cells. We have optimized our flow protocols to assess autophagy flux under many conditions with a high throughput analysis (Fig 2bi). We are also able to image cells with confocal microscopy and evaluate the fluorescently tagged proteins changes in real time and follow the movement of autophagy proteins through the cell and docking of autophagosomes with the lysosome (Fig 2bii).

**Figure 2bi.** Increased autophagy following MerTK and Axl inhibition can be recognized with a novel flow based analysis. Control U251 (shNTV, top) and U251 shRNA inhibited Mer (shMer, middle) or Axl (shAxl, lower) were transduced with LC3II-GFP-mCherry tag. Cells that are undergoing high rates of autophagic flux, shAxl and shMer, shift to the right in this flow cytometric evaluation.

**Figure 2bii.** Autophagy can be tracked at a cellular level with fluorescent microscopy. U251 cells were transfected with an LC3II tagged tandem GFP (green)-mCherry (red) construct. When autophagy is induced, the LC3II is localized to the autophagosomes, and appears as punctate green. As autophagy progresses through the cycle, the autophagosome fuses with the lysosome, lowering the pH, and quenching the GFP (green) signal leaving the punctate red fluorescent.

**Final Report Sept 2014—**Using this new fluorescent evaluation of autophagy activity, we have been able to reiterate the effects of Mer inhibition leading to upregulated autophagy using clinical methods of Mer inhibition (Fig. 2bii a). Furthermore, one important and novel finding is that the most commonly used cytotoxic therapies in glioma, temozolomide and radiation, also result in increases in autophagy (Fig. 2biii b,c), again reiterating the pro-survival effects of autophagy.
Task 3: Evaluate levels of autophagy effector molecules, Vps34, beclin1, ATG3, ATG5, ATG7, ATG12, Lamp2 and the Rab7 GTPase.

3a. qRT-PCR evaluation of transcript levels and immunoblot for protein levels. [Months 4-9 and 25-27]

Final Report Sept 2014--- Our data support the hypothesis that late stage autophagosome fusion with the lysosome and degradation of the components and recycling of the macronutrients is critical to the pro-survival mechanism of autophagy upregulation. Therefore we have now focused our efforts on downregulating the critical ‘late stage’ effectors of autophagy, Rab7 and Lamp 2. We are now introducing siRNA against Rab7 and Lamp2 to reiterate the effects of Chloroquine inhibition of autophagy.

SA2: To test the hypothesis that Mer/Axl RTK inhibition in combination with manipulation of autophagy will lead to greater glioma tumor cell killing efficiency in vitro.

Task 4: Our working hypothesis is that Mer or Axl RTK inhibition in combination with autophagy inhibition will result in the largest tumor cell kill. Therefore we will begin with constitutive shMer and Axl knockdown G12 and A172 (already available) glioma cells and treat with (and without treatment as control) chloroquine to inhibit autophagy.

4a. Evaluate cell survival following above with MTT, develop IC50 with 95% CI. Evaluate long term non-adherent colony numbers and size with soft agar. Statistically compare constitutive shMer/Axl knockdown cells treated versus untreated by chloroquine. [Months 1-6]

Final Report Sept 2014--- We have plated a variety of cell lines, including U251, A172 and G12, with constitutive Mer and Axl inhibition in soft agar and treated with and without chloroquine. We have found that A172 Mer KD (shMer) cells with autophagy inhibition with chloroquine treatment profoundly inhibited growth compared to those not this same autophagy inhibition effect has not been noted in the parental or shGFP control transduced lines. We have expanded our testing to utilize SF188, a pediatric glioma line, and using these and previously mentioned lines we have been doing MTT, soft agar, and short term clonogenic assessments. We have also added in radiation treatment, as this induces autophagy and is a standard therapeutic agent, to evaluate the response to autophagy inhibition and Mer/Axl regulation. In all cases we have found that the combination of autophagy blockade with apoptosis inhibition profoundly impacts cellular survival.

Figure 2b.ii. Autophagy increases with translational methods of Mer/Axl inhibition and standard cytotoxic therapies. U251 cells were transfected with an LC3II tagged tandem GFP (green)-mCherry (red) construct for autophagy measurements in real time. a.) When Mer and Axl are inhibited by the tyrosine kinase small molecule inhibitors UNC2025 or Foretinib or Mer expression is reduced by monoclonal antibody vMer590, there is a huge increase in autophagy. b.) Temozolomide and c.) radiation lead to large increase in pro-survival autophagy activation.

Figure 4a. Combination of MerTK and autophagy inhibition profoundly reduces survival. U251 (blue) were treated with 10 uM chloroquine (CQ; blue stripe) or shRNA against Mer (shMer; green), or the combination of CQ and shMer (green stripe) and cellular survival was assessed by xCELLigence analysis over 72 hours.
4b. Apply additional pharmacological methods of autophagy inhibition, including 3-MA, and Bafilomycin A1 and repeat assessments as described in 4a. [Months 7-9]

**Final Report Sept 2014**—We have applied both bafilomycin and 3-MA to our analyses, and have found that chloroquine treatments give the most reliable inhibition of autophagy without being directly cytotoxic. Bafilomycin can continue to be used for less robust inhibition of autophagy (at higher doses it has direct cellular toxicity) (Fig. 4bi and 4bii). 3-MA does not appear to work in concert with Mer/Axl inhibition likely indicating that the inhibition of late autophagy, as occurs with CQ, and not the earlier stages, as happens with 3-MA results in the highest effects on cell survival. See explanation of late stage autophagy inhibition in section 3a.

4c. Apply genetic methods of autophagy inhibition using siRNA directly targeting those effectors of autophagy found to be upregulated in SA1 and repeat assessments as described in subtask 4a. [Months 13-15]

**Final Report Sept 2014**—Most recently we have moved our genetic autophagy inhibition approach to introduction of shRNA against RAB7. We chose this because RAB7 similar to chloroquine, effects late stage autophagy with lysosomal fusion to the autophagosome. Thus far inhibition of Rab7 has not had a profound effect on cell survival when combined with Mer and Axl inhibition. We believe that Rab7 performs more survival functions, such as potentially mediating FADD death complexes on the surface of the autophagosome, beyond that of just autophagy activity regulation.

4d. Test or reiterate important findings in additional cell lines once subtask 1a completed. [Months 16-18]

**Final Report Sept 2014**—We have only successfully been able to model xenografts of U251 cells in our athymic nude mouse population. Therefore we have not expanded our in vitro work into other lines yet. However, we did instead expand our methods of inhibition to include more clinically relevant Mer and Axl inhibition approaches, such as a novel proprietary molecule UNC 2025 (Fig. 4d).
Figure 4d. A novel and proprietary method to inhibit Mer and Axl also works well in combination with late stage autophagy inhibition. U251 were treated with vehicle (DMSO), Chloroquine 10 μM (CQ), or 1 nM Bafilomycin A (Baf-A) and/or UNC2025 and small molecule tyrosine kinase inhibitor of Mer and Axl and cellular survival was assessed by cell counting analysis over 72 hours.

**Task 5:** Our working alternate hypothesis is that Mer or Axl RTK inhibition in combination with autophagy upregulation will result in the largest tumor cell kill. Therefore we will begin with constitutive shMer and Axl knockdown G12 glioma cells and treat with (and without treatment as control) rapamycin to increase autophagy.

5a. Evaluate cell survival following above with MTT, develop IC50 with 95% CI. Evaluate long term non-adherent colony numbers and size with soft agar. Statistically compare constitutive shMer/Axl knockdown cells treated versus untreated by rapamycin. [Months 19-24]

5b. Apply additional pharmacological methods of autophagy upregulation, including trehalose and repeat assessments as described in 5a. [Months 25-27]

5c. Apply genetic methods of autophagy upregulation using induced expression of Atg1 or activated DAP kinase and repeat assessments as described in subtask 5a. [Months 28-30]

5d. Test or reiterate important findings in additional cell lines once subtask 1a completed. [Months 31-33]

**Final Report Sept 2014---We have not yet begun investigations into upregulation of autophagy. Instead we have chosen to explore the mechanism of decreased cell number noted with combination Mer/Axl inhibition and late stage autophagy manipulation. We have investigated effects on cell cycle, necroptosis, apoptosis, and proliferation (Fig. 5).**

Figure 5. Mer and Axl inhibition in combination with autophagy manipulation result in decreased cellular proliferation and increased apoptosis. SF188 cells were treated with vehicle (DMSO), Chloroquine 10 μM (CQ), and/or UNC2025 and small molecule tyrosine kinase inhibitor of Mer and Axl and cellular proliferation was assessed by BrdU incorporation (left) while apoptosis was measured with Yo-Yo staining (above).
SA3: To test the hypothesis that Mer/Axl RTK inhibition in combination with autophagy manipulation will lead to less in vivo glioma tumor growth.

Task 6: Write and submit animal protocol for the USAMRMC Office of Research Protection.


Task 7: Initial in vivo investigations will be designed taking into consideration results from SA1 and SA2. Our current working hypothesis for these investigations is that Mer or Axl RTK inhibition with autophagy inhibition will prevent in vivo tumor growth most significantly; therefore we will begin with that combination.

7a. Develop SQ murine glioma models using parental (already completed) and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) chloroquine to inhibit autophagy. Measure tumor growth (mm3) in chloroquine treated and untreated mice by external caliper measurement and luciferin tagged imaging. Statistically compare volumes over time between groups. [Months 7-15]

Final Report Sept 2014—We have successfully developed the luciferase tagged U87 and U251 cell lines and used them to develop human glioma tumors in the subcutaneous tissues of mice. We have recently completed Mer and Axl inhibition strategies at both genetic and small molecule inhibitor levels and published these single agent effects (Fig 7a). We are now comparing our different strategies for Mer/Axl inhibition to determine best agent going forward. Additionally we are combining our top two strategies (Foretinib and UNC2025) with standard cytotoxic therapies (lomustine/CCNU) and Chloroquine.

Figure 7a. Mer and Axl inhibition with Foretinib is an effective treatment for glioma tumors and increases overall survival. U251 cells were subcutaneously implanted and tumors were allowed to establish over 8 weeks. Mice were treated every other day (QOD) by oro-gastric (OG) administration with: a. Vehicle only, Foretinib 30 mg/kg QOD OG initiated at 8 weeks post injection (Foretinib MRD), or Foretinib (with same dosing regimen), initiated when the tumor reached 125 mm3 (Foretinib ET). Tumors were measured twice weekly by caliper. a. Vehicle (n=10) and Foretinib MRD (n=7) treated mice, which started therapy at 8 weeks post injection, had tumor volumes compared with a paired two-tailed t-test (p-values are provided) and b. a Kaplan-Meier analysis of survival (survival endpoint defined as sacrifice due to tumor size in excess of 2500 mm3 or morbidity due to tumor) with a log rank Mantel-Cox test statistical comparison (p-values are provided). c. A subset of the Foretinib MRD treated mouse (n=4) had therapy discontinued at 8 weeks (arrow) and tumor volumes were compared to vehicle and continuous Foretinib therapy (n=3). An ANOVA with Tukey’s correction was used for statistical comparison to vehicle control. d. Two tumors (vehicle lanes 1-3 and foretinib treated lanes 4-6) were resected and immediately homogenized in lysis buffer with protease inhibitor with freshly prepared pepsin (lanes 2 and 5) and without (lanes 3 and 6). Lanes 1 and 4 are samples that were not homogenized. Samples were resolved with SDS PAGE and then blotted for the activated phospho-form (p-Mer) and stripped and re-probed for the total form (t-Mer) to test for target inhibition. e. Vehicle (n=10) and Foretinib ET (n=5) treated mice, which started therapy when the tumor exceeded 125 mm3, had tumor volumes compared with a paired two-tailed t-test (p-values are provided) and f. a Kaplan-Meier analysis of survival with a log rank Mantel-Cox test comparison (p-values are provided)

7b. Develop orthotopic frontal lobe murine glioma models using parental and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) chloroquine to inhibit autophagy. Measure tumor growth (mm3) in chloroquine treated and untreated mice by luciferin tagged imaging and MRI. Statistically compare volumes between groups. [Months 22-30]

Final Report Sept 2014—Similar to the update on 7a, we have successfully developed an intracranial orthotopic xenograft of human glioma using both U87 and U251 cell lines and have recently published the effects of inhibition
of MerTK, which profoundly inhibited tumor growth in both SQ and IC tumors (Fig. 7b). We are now beginning autophagy manipulation in combination to evaluate the in vivo effects.

7c. Consider use of other available cell lines, additional inhibitors of autophagy, and alternate methods of RTK inhibition based on results from SA1 and SA2. [Months 30-36]

Final Report Sept 2014—We have only successfully been able to introduce one other glioma line into intracranial tumor modeling, the U87 line. However, those tumors in our lab and in other published reports, grow very differently than the typical glioma, with very circumscribed and non-invasive border cells. Therefore we have not explored models with this line in more depth. We have however begun to use alternate methods of RTK inhibition and the addition of autophagy manipulation with both Chloroquine and Rapamycin.

Task 8: Our current alternate working hypothesis for these investigations is that Mer or Axl RTK inhibition with autophagy upregulation will prevent in vivo tumor growth most significantly; therefore we will also evaluate this combination.

8a. Develop subcutaneous murine glioma models using parental (already completed) and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) rapamycin to upregulate autophagy. Measure tumor growth (mm3) in rapamycin treated and untreated mice by external caliper measurement and luciferin tagged imaging. Statistically compare volumes over time between groups. [Months 7-15]

Final Report Sept 2014—See update for task 7c.

8b. Develop orthotopic frontal lobe murine glioma models using parental and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as
control) rapamycin to upregulate autophagy. Measure tumor growth (mm$^3$) in rapamycin treated and untreated mice by luciferin tagged imaging and MRI. Statistically compare volumes between groups. [Months 22-30]

Final Report Sept 2014—See update for task 7c.

8c. Consider use of other available cell lines, additional activators of autophagy, and alternate methods of RTK inhibition based on results from SA1 and SA2. [Months 30-36]

Final Report Sept 2014—See update for task 7c.

Task 9: Peer-reviewed manuscript describing the effects of Mer/Axl RTK inhibition on autophagy activation, molecular signaling, and cellular survival.

9a. Preparation and submission [Months 16-18]
9b. Revision and publication [Months 19-21]

Task 10: Peer-reviewed manuscript describing the effects of Mer/Axl RTK inhibition in combination with autophagy manipulation and proof of principle for clinical trial development and translational applications.

10a. Preparation and submission [Months 30-33]
10b. Revision and publication [Months 33-36]

Final Report Sept 2014—We have prepared several competitive abstracts and publications related to this grant.


KEY RESEARCH ACCOMPLISHMENTS:
-Transduction of U251 and SF188 glioma cell lines with GFP and tandem GFP-mCherry, allowing for real time cellular level microscopy as well as high throughput flow analysis, which can be used in all further tasks.
-Successful inhibition of MerTK and Axl RTK with several translational small molecule inhibitors, one of which is already in human clinical trial for non-CNS malignancies and may be able to rapidly be applied to glioma.
- Development of both a subcutaneous and intracranial xenograft model of human glioma for preclinical testing of our hypothesis.
- The addition of radiation therapy and chemotherapies, standard therapeutics used in GBM, to our analysis to assess the level of autophagy and evaluate our outcomes regarding tyrosine kinase inhibition and the combination with standard therapy.

**REPORTABLE OUTCOMES:**
See publications above.

**CONCLUSION:**
Mer and Axl RTKs regulate autophagic flux in human glioma cell lines, likely through inhibition of the PI3K/Akt to mTOR pathways. We hypothesize that this results in cell survival by increased recycling of needed macronutrients to support cell survival, repair, and proliferation after stresses such as cytotoxic therapy. Through inhibition of this upregulation at late stages of autophagy we can impair the recycling of these important macronutrients and improve glioma cell killing in response to standard cytotoxic therapies. Understanding the effects of autophagy manipulation in the setting of tyrosine kinase inhibition will help us design the most appropriate approach to targeted therapeutics for glioma.

**REFERENCES:**

1. A. K. Keating *et al.*, Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. *Mol Cancer Ther* 9, 1298 (May, 2010).

**APPENDICES:** None