AWARD NUMBER: W81XWH-15-1-0114

TITLE: Targeting the ECM to Enhance Drug Delivery in Nf1-Associated Nerve Sheath Tumors

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The focus of the proposal is to determine what factors limit delivery of drugs to tumors of the peripheral nerves, namely neurofibromas (NFs) and their derivative, the malignant peripheral nerve sheath tumor (MPNST). The drugs in question include small molecule inhibitors of mTOR and MEK kinases, as well as a traditional chemotherapeutic agent, doxorubicin (also called by its trade name Adriamycin). The major factor under study for limiting drug delivery is the extracellular matrix component hyaluronic acid (HA). During the last research period we expanded two colonies of genetically engineered mice that develop NFs and MPNSTs for study. We also tested 2 drugs (RAD001 and PD-0325901) in these models for efficacy and found both had moderate activity. We tested a drug called PEGPH20 for its ability to degrade HA in NFs and MPNST–like tumors in these mice when injected. PEGPH20 did reliably remove the HA from the NF and MPNST microenvironments and dramatically improve tumor blood vessel patency, and doxorubicin delivery to tumor cells in situ with no clear affect on tumor cell apoptosis or mitotic index. In the next year we will test PEGPH20 enhancement of doxorubicin, RAD001 and PD-0325901 delivery, therapeutic effect.
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1. **INTRODUCTION:**
The focus of the proposal is to determine what factors limit delivery of drugs to tumors of the peripheral nerves, namely neurofibromas (NFs) and their derivative, the malignant peripheral nerve sheath tumor (MPNST). The drugs in question include small molecule inhibitors of mTOR and MEK kinases, as well as a traditional chemotherapeutic agent, doxorubicin (also called by its trade name Adriamycin). The major factor under study for limiting drug delivery is the extracellular matrix component hyaluronic acid (HA). It is our hypothesis that HA limits drug delivery to NFs and MPNSTs by creating high tumor interstitial pressure, vessel collapse and hypoxia. We are exploring the hypothesis that using a new, long half life pegylated hyaluronidase called PEGPH20, it’ll be possible to degrade HA in the extracellular environment in peripheral nerve sheath tumors and enhance drug delivery and therapeutic response dramatically.

2. **KEYWORDS:**
Hyaluronic acid, PEGPH20, doxorubicin, neurofibroma, malignant peripheral nerve sheath tumor

3. **ACCOMPLISHMENTS:**

**What were the major goals of the project?**

*Task 1: To investigate the ability of stroma targeting therapy to disrupt the extracellular matrix and enhance delivery and distribution of small molecule therapeutics to plexiform neurofibromas and MPNSTs.*

Subtask 1a. Complete application and approval for all mouse studies (months 1-2). **100% complete**

Subtask 1b. Generate mice with plexiform-like neurofibromas (and MPNSTs) on the following genetic backgrounds; Dhh-Cre; Nf1Flox/Flox;PtenFlox/Flox and Dhh-Cre; Nf1Flox/Flox;PtenFlox/+ . Sixty mice of each genotype will be generated. (months 3-8) **75% complete**

Subtask 1c. Develop an effective protocol for use of volumetric magnetic resonance imaging (MRI) imaging to determine the number and size of plexiform neurofibromas and MPNSTs in two of the mouse models described in subtasks 1b and 1c (i.e. Dhh-Cre; Nf1Flox/Flox;PtenFlox/+ and NPCis mice). This procedure can be carried out at the University of Minnesota’s Center for Magnetic Resonance Research utilizing our 9.4 Tesla/31 cm bore machine. **0% complete**

Subtask 1d. Develop and implement protocol for treatment of tumor-bearing mice with PEGPH20 at several doses via i.v. injections, along with doxorubicin to measure diffusion of this small molecule into tumors. We will treat tumor bearing mice with doses of PEGPH20 in a range similar to that used in 3 other papers describing its use on mouse tumor models (0, 5, 10, and 15 mg/kg i.v.). Treatment of Dhh-Cre; Nf1Flox/Flox;PtenFlox/Flox mice, which become moribund at ~15-20 days of age, may require i.p. injection of PEGPH20 if i.v. infusion proves impossible. **100% complete**

Subtask 1e. Measurement of tumor parameters before (i.e. day 0) and at 24 hours after treatment with PEGPH20. As described before, PEGPH20 affects are maximal by 8 hours and persist for at least 72 hours (Jacobetz et al., Gut, 2013). Our goal is to obtain data from three or four mice for each concentration for these analyses (0, 5, 10, or 15 mg/kg PEGPH20). **75% complete.**

Subtask 1f. We will measure HABP staining, collagen levels, Ki67 positivity, and CD31+ vessel density in our tissue microarray of human dermal neurofibromas, plexiform neurofibromas, and MPNSTs. We will attempt to correlate these parameters among samples. **50% complete**

*Task 2: To test the efficacy of cytotoxic and molecularly targeted therapies in combination with PEGPH20 to improve therapeutic response in faithful murine models of plexiform neurofibromas and MPNSTs.*
Subtask 2a. Complete application and approval for all mouse studies (months 1-2) 100% complete

Subtask 2b. Generate mice with plexiform neurofibromas and MPNSTs on the following genetic backgrounds; Dhh-Cre; Nf1Flox/Flox; PtenFlox/Flox and Dhh-Cre; Nf1Flox/Flox; PtenFlox/+ . Ninety mice of each genotype will be generated. (months 18-24). 10% complete

Subtask 2c. Enroll mice in the treatment protocols as they become visibly affected by tumors or they can be detected by MRI (approximate ages are day 15-20 for Dhh-Cre; Nf1Flox/Flox; PtenFlox/Flox; day 175 for Dhh-Cre; Nf1Flox/Flox; PtenFlox/+ ; and day 175 for NPCis mice). Mice will be enrolled in either short-term or long-term treatment studies as described below. (months 18-30) 0% complete

Subtask 2d. Develop and implement protocol for short-term treatment of tumor-bearing mice with PEGPH20, PEGPH20 + doxorubicin, or only doxorubicin (5 mice per treatment arm). Doxorubicin will be administered at 5 mg/kg via i.p. injection on day 2 and 6 as described before in a mouse xenograft model of osteosarcoma (Huang et al, Cancer Res, 2012). We will choose a dose of PEGPH20, based on studies in Task 1, which reliably induces a change in doxorubicin tumor penetrance. PEGPH20 will be injected i.v. on days 1 and 5. Mice will be sacrificed on day 7. We will measure tumor size by volumetric MRI, Ki67 positivity, and cleaved caspase 3 levels by immunohistochemistry. We will also measure stromal modification, vascular architecture, and patency. Waterfall plots will be generated to measure changes in tumor volume after enrollment. (months 18-30) 10% complete

Subtask 2e. Develop and implement protocol for short-term treatment of tumor-bearing mice with PEGPH20, PEGPH20 + MEK inhibitor (PD0325901), or only MEK inhibitor (5 mice per treatment arm). PD0325901 will be administered on days 2, 3, 4, 5, 6, and 7, by oral gavage at 5 mg/kg as described before for Dhh-Cre, Nf1Flox/Flox mice (Jessen et al., J. Clin. Invest., 2013). We will administer PEGPH20 i.v. one day prior to the first PD0325901 dose on day 1, and then again on day 5. We will measure tumor size by volumetric MRI, Ki67 positivity, cleaved caspase 3 levels, and phospho-ERK levels. We will also measure stromal modification, vascular architecture, and patency. Waterfall plots will be generated to measure changes in tumor volume after enrollment. (months 18-30) 10% complete

Subtask 2f. Develop and implement protocol for long-term treatment of tumor-bearing mice with PEGPH20, PEGPH20 + doxorubicin, or only doxorubicin (15 mice per treatment arm). Doxorubicin will be administered at 5 mg/kg via i.p. injection twice weekly as described before in a mouse xenograft model of osteosarcoma (Huang et al, Cancer Res, 2012). We will choose a dose of PEGPH20, based on studies in Task 1, which reliably induces a change in doxorubicin tumor penetrance. We will administer doxorubicin twice per week and PEGPH20 every 7th day i.v. for up to 70 days. We will measure overall survival time, as well as tumor size, Ki67 positivity, and cleaved caspase 3 levels. Endpoint criteria will be defined as 20% body weight loss in addition to general morbidity, lethargy, lack of social interaction or development of large tumors that impair mobility significantly. Evidence of toxicity will also be sought via weekly complete blood cell counts and histological examination of the heart and other organs at sacrifice. (months 30-36). 10% complete

Subtask 2g. Develop and implement protocol for long-term treatment of tumor-bearing mice with PEGPH20, PEGPH20 + MEK inhibitor (PD0325901), or only MEK inhibitor (15 mice per treatment arm). PD0325901 will be administered once per day, 5 days per week, by oral gavage at 5 mg/kg as described before for Dhh-Cre, Nf1Flox/Flox mice (Jessen et al., J. Clin. Invest., 2013). We will administer PD0325901 five days per week and PEGPH20 every 7th day i.v. for up to 70 days. We will measure overall survival time, as well as tumor size, Ki67 positivity, cleaved caspase 3 levels, and phospho-ERK levels. Endpoint criteria will be defined as 20% body weight loss in addition to general morbidity, lethargy, lack of social interaction or development of large tumors that impair mobility significantly. Evidence of toxicity will also be sought via weekly complete blood cell counts and histological examination of the heart and other organs at sacrifice. (months 30-36). 10% complete
Subtask 2h. Perform Kaplan-Meier analysis to determine affect of treatments on latency to sacrifice

What was accomplished under these goals?

- Several major objectives were met during the last research period. This includes that during the last research period we obtained institutional approval for all planned mouse experiments. We developed extensive data showing that chronic PEGPH20 treatment was well tolerated in pre-weanling mice given 1.5 mg/kg weekly. We obtained solid data showing that PEGPH20 treatment consistently improves doxorubicin delivery to tumor cells in situ in living mice and measured other parameters of response to PEGPH20 in NFs and MPNST-like tumors. We developed doses and schedules for RAD001 and PD0325901 treatment that are well tolerated in mice with NFs and MPNST-like tumors.

- There were several specific objectives during the last research period. One objective was to obtain and maintain institutional animal care and use approval for all animal work and this was obtained (U of MN IACUC # 1509-33035A). A second objective was to generate breeding colonies and offspring sufficient for the proposed experiments. We now have 12 breeding colonies generating Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} and Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} mice. Another objective was to develop reliable dosing strategies for treatment of these mice with PEGPH20 for depletion of HA and to determine how this changes a variety of peripheral nerve sheath tumor parameters. We also found that treatment of Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} mice with once daily 5 mg/kg RAD001 by oral gavage starting at day 7 of life and given 5 days on with 2 rest days is well tolerated and extends the life-span of these mice while reducing the rate of tumor growth. We have similar data showing that treatment of Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} mice with 1.5 mg/kg PD0325901 daily by oral gavage at 7 days of age with 5 days on and 2 rest days is tolerated. This also extends life span.

- Several significant results were obtained during the last research period. Treatment of Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} mice with 1.5 mg/kg PEGPH20 depletes HA from peripheral nerve sheath tumors, increase tumor blood vessel diameter and patency, and improves doxorubicin delivery to peripheral nerve sheath tumor cells. These findings are very important because they establish that it is unlikely that PEGPH20 treatment will make peripheral nerve sheath tumors worse but dies clearly improve small molecule delivery to tumor cells.

A second major result is clear evidence that PEGPH20 treatment at 1.5 mg/kg weekly to normal mice starting at birth does not cause any long term abnormalities, weigh loss or failure to develop abnormally. These results are important because they suggest that PEGPH20 treatment may be safe to use in children and adolescent patients with peripheral nerve sheath tumors.

The third major result is that treatment with the mTOR kinase inhibitor RAD001 can be tolerated at clinically relevant doses that inhibit target activity in peripheral nerve sheath tumor cells, and extend the life span of mice harboring peripheral nerve sheath tumors. This work was done in Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} mice. These data are shown in Figure 1.

Figure 1. Dhh-Cre; Nf1^{Flx/Flx};Pten^{Flx/Flx} mice were treated daily with 5mg/kg RAD001(Everolimus) IP after being allowed to age for one week. Six vehicle control animals and 4 treated animals were included. The untreated animals lived 18-19 days while the treated animals should significant increase in longevity with one mouse even quadrupling control age.
We have developed a dose and schedule for treatment with the MEK inhibitor PD0325901 that is tolerated in Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice. This is 1.5 mg/kg daily by oral gavage starting at day 7 of life, given 5 days with 2 day rest periods. We found that this dose does inhibit phospho-Erk levels in peripheral nerve sheath tumors in Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice. These data are shown in Figure 2.

**Figure 2.** Acute IP injections of PD0325901 were performed at the endpoint of Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice at varying dosages from 0.25 mg/kg to 5 mg/kg. The Sciatic, Brachial Plexus, Dorsal Root Ganglion, and Trigeminal Nerves were extracted and analyzed for Phospho-ERK levels as a readout of the penetrance and efficacy of the MEK inhibitor. 0.25 mg/kg showed partial depletion of phospho-ERK while 5 mg/kg showed total ablation (box) in all peripheral nerves examined. 1 mg/kg and 2.5 mg/kg also showed definitive reduction in signaling. 0.5 mg/kg IP is tolerable in the mice and has preliminarily showed an increase in longevity as well.

- **What opportunities for training and professional development has the project provided?**
  - Although not intended to, this project has provided training opportunities for a graduate student and two postdoctoral fellows. Mr. Bryant Keller, B.S. has done the bulk of this work and learned to do mouse work, necropsies, biochemistry and gene expression analyses. Mr. Keller presented the project in a poster and oral presentation at the 2016 NF Conference (sponsored by the Children’s Tumor Foundation) in Austin, Texas. Dr. Kyle Williams Ph.D., and Dr. Stephen Scully, Ph.D., assisted in experimental design, immunohistochemistry and drug delivery studies.

- **How were the results disseminated to communities of interest?**
  - Results were presented in a poster and 5-minute oral presentation by Mr. Bryant Keller at the 2016 Neurofibromatosis Conference (sponsored by the Children’s Tumor Foundation) in Austin, Texas on June 21, 2016.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - During the next budget period we plan to do several things. 1. Import and establish colony of NPCis mice (with CIS configuration of Trp53 and Nf1 mutations). 2. Complete treatment studies in Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice using single agent PD0325901, dual agent RAD001 and PD0325901, and both single agents with weekly PEGPH20 treatment. 3. Validate that our treatment regimen using doxorubicin (administered at 5 mg/kg via i.p. injection twice weekly) is tolerated in growing Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice when treatment starts at day 7 of life. 4. Complete treatment studies in Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice using single agent doxorubicin and doxorubicin with weekly PEGPH20 treatment. 5. Complete studies on the acute and chronic effects of PEGPH20 on peripheral nerve sheath tumors in Dhh-Cre; Nf1<sup>Flox/Flox</sup>;Pten<sup>Flox/Flox</sup> mice (HABP staining, collagen levels, Ki67 positivity, and CD31+ vessel density).

4. **IMPACT:**
What was the impact on the development of the principal discipline(s) of the project?
- We have learned that the drug PEGPH20, which degrades a component of connective tissue called hyaluronic acid (HA), can safely be administered to young growing mice. This means that it may be safe to give in human children and adolescents with NF1 syndrome who have troublesome peripheral nerve tumors called neurofibromas. Using a mouse model we have also learned that PEGPH20 isn’t likely to make these tumors worse, and does remove tumor associated HA, allowing more chemotherapy drug to reach tumor cells. The experience we have gained in treating very young mice with NF1 syndrome associated peripheral nerve sheath tumors is unique and we hope to use PEGPH20 in the near future to improve treatment in NF1 patients.

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.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
- Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
- We encountered problems using the intended dose of PD0325901 in Dhh-Cre; Nfl^{FloxFloX}; Pten^{FloxFloX} mice. We had to resort to testing several lower and lower doses. We found that if we delayed treatment until day 7 of life and used 1.5 mg/kg PD0325901 given daily by i.p. injection the treatment was tolerated. 5 mg/kg given by oral gavage was not tolerated in young mice, although this dose and route of administration is tolerated by adult mice.

Changes that had a significant impact on expenditures
- Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of human subjects
- Nothing to report.

Significant changes in use or care of vertebrate animals.
- Nothing to report.

Significant changes in use of biohazards and/or select agents
- Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations
- Results were presented in a poster and 5-minute oral presentation by Mr. Bryant Keller at the 2016 Neurofibromatosis Conference (sponsored by the Children’s Tumor Foundation) in Austin, Texas on June 21, 2016.

Journal publications.
- Nothing to report.

Books or other non-periodical, one-time publications.
• Nothing to report.

- **Other publications, conference papers, and presentations.**
  - Nothing to report.

- **Website(s) or other Internet site(s)**
  - Nothing to report.

- **Technologies or techniques**
  - Nothing to report.

- **Inventions, patent applications, and/or licenses**
  - Nothing to report.

- **Other Products**
  - Nothing to report

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**
  - One new postdoctoral fellow worked on this project:

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<thead>
<tr>
<th>Name</th>
<th>Dr. Steve Scully, Ph.D.</th>
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<tr>
<td>Project Role</td>
<td>Postdoctoral Fellow</td>
</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
<td>NA</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>4</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Scully has performed work on immunohistochemical characterization of peripheral nerve sheath tumors in mice treated with PEGPH20.</td>
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<td>Funding Support:</td>
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- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Dr. Largaespada (1.2 cal. months) and Dr. Provenzano (2.4 cal. months) both have effort on a newly awarded Physical Sciences in Oncology U54 Center Grant from the National Cancer Institute [CENTER FOR MODELING TUMOR CELL MIGRATION MECHANICS]. 3U54CA210190-01S1. 17-Aug-2016 to 31-Jul-2021.

- **What other organizations were involved as partners?**
  - Nothing to Report

7. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** Not applicable

- **QUAD CHARTS:** Not applicable.

8. **APPENDICES:** None