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TITLE: Diagnostic and Therapeutic Strategy to Prevent Trauma-Induced Upper-Extremity Muscle Fibrosis and Heterotopic Ossification

PRINCIPAL INVESTIGATOR: Benjamin Levi, MD

CONTRACTING ORGANIZATION: Regents of the University of Michigan

Ann Arbor MI 48109-1340

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Benjamin Levi

E-Mail: blevi@umich.edu

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Regents of the University of Michigan
Kathryn Dewitt
503 Thompson St
Ann Arbor MI 48109-1340

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14. ABSTRACT

Heterotopic ossification (HO) is a devastating condition in which ectopic bone forms inappropriately in the soft tissue following large surface area burns, musculoskeletal trauma, and many orthopedic surgeries. The military population has been shown to be particularly at risk of HO following battlefield wounds.

Clinical detection of HO in patients is currently limited by poor visualization at early time points. At present, HO is often only visualized after it has formed, as diagnosis relies on the use of CT and MRI to identify ectopic lesions (6,7). Early diagnosis would allow for pharmacological treatment in order to arrest bone development, instead of surgical excision, often the only treatment strategy currently available, which often leads to additional ectopic bone formation (8). Here, we aim to further develop a novel high frequency spectral ultrasound imaging system as a method to detect HO formation at early time points and then appropriately deliver timed, guided treatments to prevent bone development. Additionally, we will investigate targeted delivery of FDA approved inhibitors of hypoxic signaling (rapamycin and amphotericin B) following HO detection using SUSI imaging to direct treatment.

15. SUBJECT TERMS Heterotopic ossification, rapamycin, amphotericin, fibrosis, ultrasound, SUSI, mTOR/HIF1α,					
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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The central goal of this grant is to demonstrate the efficacy of timed, image guided mTOR/ HIF-1α inhibitors (Rapamycin and Amphotericin) to attenuate muscle fibrosis, HO and joint contracture development without negatively affecting wound healing processes. We aim to change the current treatment paradigm of fibrosis and HO management in military, veteran and civilian populations at risk from one of delayed diagnosis and excision to one of early detection and timed, precise prevention. We hypothesize that early mTOR/HIF-1α signaling is critical for fibrosis and HO formation and that this destructive process can be mitigated through imaged guided delivery of mTOR/HIF-1α inhibitors. Specifically, we will deploy Rapamycin and Amphotericin B for timed, image guided drug delivery to block HO and fibrosis.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Heterotopic ossification, rapamycin, amphotericin, fibrosis, ultrasound, SUSI, mTOR/HIF1α,

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Regulatory Tasks:
Subtask 1: IACUC/ACURO Approval
Status: Status: USU IACUC protocol number: SUR-18-065 approved 2018-12-26, UM IACUC protocol number: PRO00007930 approved 2017-09-20. ACURO protocol number: OR170174 approved at UM and USU.

Specific Aim 1: *To validate timed, image guided prevention of heterotopic ossification with FDA approved inhibitors of hypoxic signaling.*
Status: In progress Y1Q4

Subtask 1: Validate imaging and histology and demonstrate that image guided HIF1a/mTOR inhibition with our therapeutics prevents HO.
Status: In progress Y1Q4

Subtask 2: Define the time point at which treatment is best administered.
Status: In progress Y1Q4

Subtask 3: Validate timed treatment starting when early HO is detected by imaging and histology.

Status: In progress Y1Q4

Milestone: Completion of validation of timed, image guided prevention studies and data analysis. Preparation and submission of peer-reviewed manuscripts.
Status: In progress Y1Q4

Specific Aim 2: *To mitigate post-traumatic muscle fibrosis through timed inhibition of mTOR/HIF-1α signaling with Amphotericin and Rapamycin separately or as combined therapy.*
Status: In progress Y1Q4

Subtask 1: Demonstrate that mTOR/HIF-1α inhibitors decrease post traumatic extremity muscle fibrosis.
Status: In progress Y1Q4

Subtask 2: Validate mTOR/HIF-1α inhibitors as separate and combined treatment modalities in extremity muscle fibrosis.
Status: In progress Y1Q4

Subtask 3: Define the time point at which treatment is best administered.
Status: In progress Y1Q4

Milestone: Completion of muscle fibrosis timed inhibition studies and data analysis. Preparation and submission of peer-reviewed manuscripts.
Status: In progress Y1Q4

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 1: *To validate timed, image guided prevention of heterotopic ossification with FDA approved inhibitors of hypoxic signaling.*
Status: In progress Y1Q4

Subtask 1: Validate imaging and histology and demonstrate that image guided HIF1a/mTOR inhibition with our therapeutics prevents HO.
Status: In progress Y1Q4

Methods and results for Specific Aim 1 for the reporting period:

1A. Heterotopic Ossification and Range of Motion in mouse model after burn tenotomy with Amphotericin B and Rapamycin Treatment

Heterotopic ossification (HO) is a devastating condition in which ectopic bone forms inappropriately in the soft tissue following large surface area burns, musculoskeletal trauma, and many orthopedic surgeries. The military population has been shown to be particularly at risk of HO following battlefield wounds, with occurrences in approximately 63-65% of blast-related extremity amputations and approximately 62% of limb sparing procedures (1-3). Previously, rapamycin has shown efficacy in inhibiting HO in rat model of blast trauma and in a mouse burn-tenotomy model by inhibition of Hif1a (4,5).
Despite potential pharmacological treatments, clinical detection of HO in patients is currently limited by poor visualization at early time points. At present, HO is often only visualized after it has formed, as diagnosis relies on the use of CT and MRI to identify ectopic lesions (6,7). Early diagnosis would allow for pharmacological treatment in order to arrest bone development, instead of surgical excision, often the only treatment strategy currently available, which often leads to additional ectopic bone formation (8). We have previously shown that high frequency spectral ultrasound imaging (SUSI) can effectively visualize traumatic HO formation in our burn-tenotomy model of HO at early time points (9).
Here, we aim to further develop this SUSI imaging system as a method to detect HO formation at early time points and then appropriately deliver timed, guided treatments to prevent bone development. We will expand upon previous research to investigate targeted delivery of FDA approved inhibitors of hypoxic signaling (rapamycin and amphotericin B) following HO detection using SUSI imaging to direct appropriate treatment.

First, in order to investigate HO formation following administration of HIF1a inhibitors, male C57BL/6 mice were given rapamycin at 2.5 mg/kg; liposomal amphotericin B (Ambisome®) at 10 mg/kg; or half doses of both rapamycin (1.25 mg/kg) and Ambisome (5 mg/kg) in combination. Two different dosing periods were investigated. In the first dosing scheme, all drugs were administered daily for one week. Total HO formation, range of motion about the injured ankle, and bone development analysis with high-frequency spectral ultrasound imaging (SUSI) was performed.

A burn-tenotomy surgical procedure was performed on all animals in order to incite HO formation at the ankle joint. Briefly, a scald burn was created on approximately 30% of the total body surface area and the Achilles tendon of the left leg was severed. This procedure has been shown to consistently cause HO formation. After the burn tenotomy procedure, mice were treated daily for two weeks (10 mg/kg Ambisome for 10 days), range of motion was investigated weekly for 9 weeks, and HO formation was quantified after nine weeks.

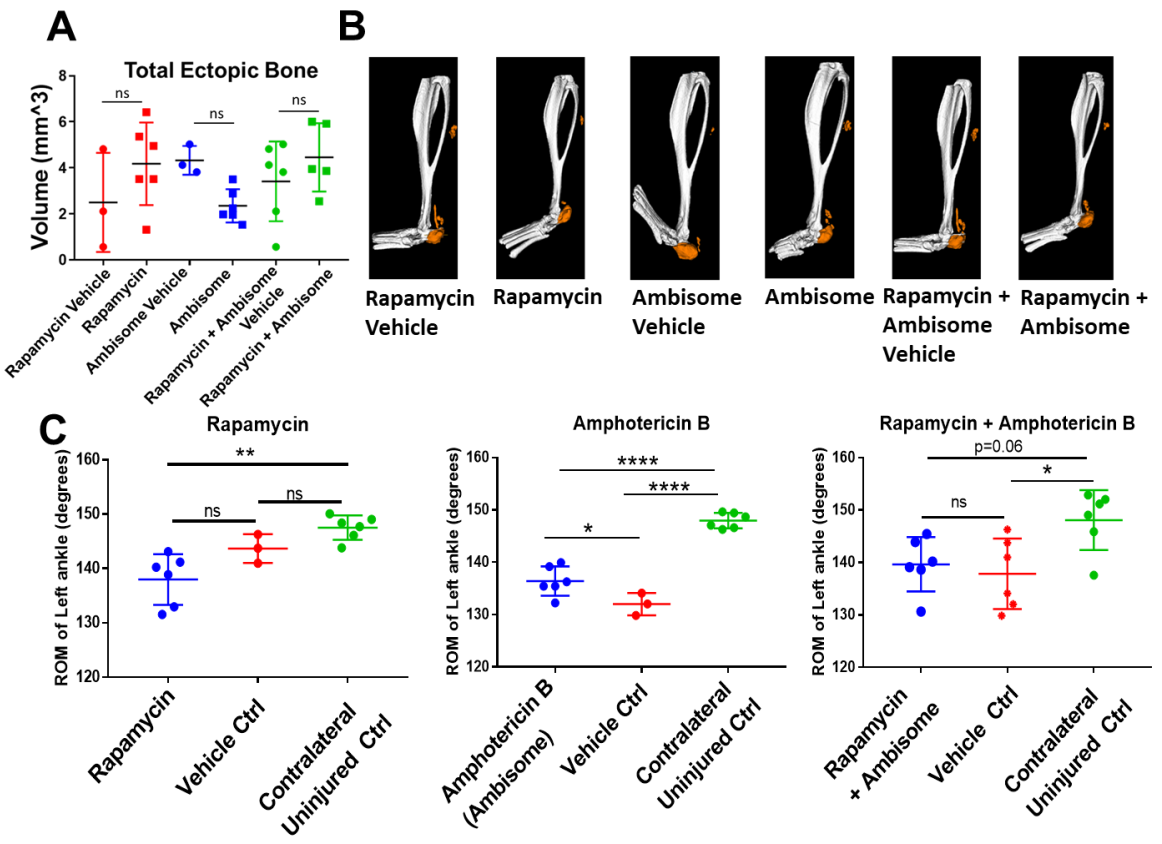

Figure 1: **Daily administration for 7 days of rapamycin (2.5 mg/kg daily), amphotericin B, (Ambisome®, 10 mg/kg daily), or combination of rapamycin and Ambisome® (1.25 mg/kg and 5 mg/kg respectively) does not decrease HO formation or increase range of motion at 9 weeks post injury.** A) Total ectopic bone formation after 9 weeks in mice treated with indicated treatments for one week. n≥3. When compared to vehicle control, no treatment showed significant difference in the volume of ectopic bone formed. Treatments and respective controls analyzed using independent samples t-test or Mann-Whitney U-test for nonparametric samples. B) Representative images of HO formation 9 weeks post burn tenotomy. Mice treated with indicated treatment for 7 days. Orange areas indicate ectopic bone formation. Representative of n≥3 per group. C) : Range of motion about the ankle 9 weeks post burn tenotomy in mice treated for one week with Rapamycin, Amphotericin B, or Rapamycin plus Amphotericin B or the respective vehicle control treatment. Mice treated with Amphotericin B presented with an increased range of motion at 9 weeks compared to vehicle control treated mice. Ordinary one way

In the second cohort of mice, all drugs were administered daily for two weeks, except Ambisome (10 mg/kg), which was administered daily for 10 days due to observed toxicity. Each group had ≥ 3 mice. Mice were also given the vehicle control in order for proper comparison. Vehicle control for the rapamycin was 3.125% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS). Vehicle control for the Ambisome was equal parts PBS and 5% dextrose, in order to most closely match the manufacturer's preparation instructions of the Ambisome products. All doses were injected intraperitoneal (IP).

A burn-tenotomy surgical procedure was performed on all animals in order to incite HO formation at the ankle joint. At nine weeks, mice were sacrificed and legs were scanned with a microCT scanner in order to quantify HO formation. The total ectopic bone volume was calculated. Test article treated groups were compared to their respective vehicle control group. A significant decrease in bone formation in groups treated with rapamycin or amphotericin B was not found. Interestingly, mice treated with the combination of rapamycin + amphotericin B were

found to have increased bone formation than mice treated with the vehicle control. Range of motion about the injured ankle was also investigated in treated and untreated mice. Briefly, 40g weight was attached with a string to a clip, and the clip was gently placed on the mouse's paw. The weight extends the ankle to its full range of motion and the ankle is digitally photographed. Image processing software is used to quantify the angle formed by the ankle. When comparing all treatments over the 9 week period, a significant difference is not seen between any groups at any time point. **Figure 2** shows total HO formation and range of motion about the injured ankle in mice post burn-tenotomy treated with rapamycin, Amphotericin B (Ambisome®), combination, or vehicle control.

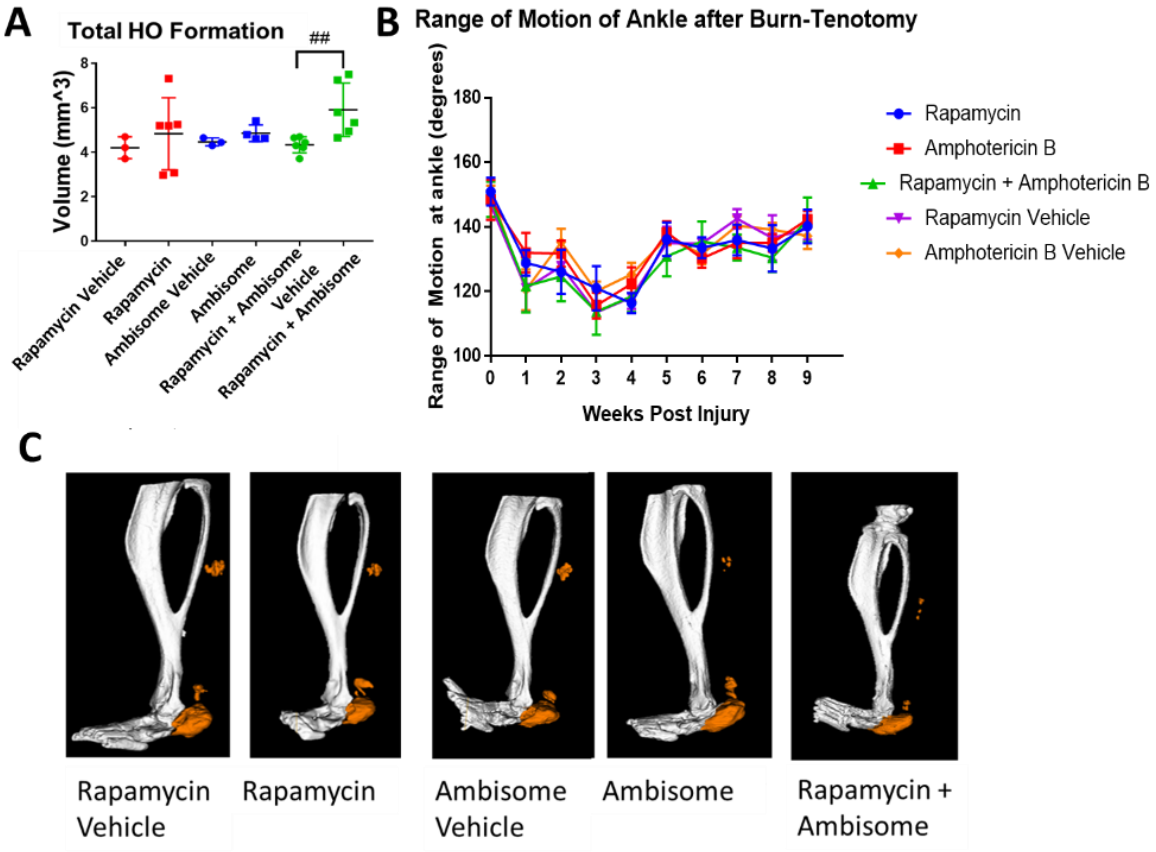

Figure 2: **Administration of rapamycin (2.5 mg/kg daily for 14 days), amphotericin B, (Ambisome®, 10 mg/kg daily for 10 days), or combination of rapamycin and Ambisome® (1.25 mg/kg and 5 mg/kg respectively) does not decrease HO formation at 9 weeks or increase range of motion.** A) Total ectopic bone formation after 9 weeks in mice treated with indicated treatments for two weeks (Amphotericin B for 10 days). ## indicates $p<0.01$, $n\geq3$. Treatments and respective controls analyzed using independent samples t-test or Mann-Whitney U-test for nonparametric samples. B) Range of motion about the injured ankle joint in mice that underwent a burn-tenotomy procedure to incite HO and were treated with the indicated treatment groups. A significant difference is not seen between any groups at any time point when all groups are compared over the 9 week time period using a repeated measures one-way ANOVA with Greenhouse-Geisser correction and Sidak's multiple comparisons test. C) Representative images of HO formation 9 weeks post burn tenotomy. Mice treated with indicated treatment for 2 weeks (Amphotericin B for 10 days). Orange areas indicate ectopic bone formation. Representative of $n\geq3$ per group.

Discussion and Conclusions

The rapamycin, Ambisome, and combination rapamycin-Ambisome treatments did not significantly reduce HO formation at 9 weeks post burn-tenotomy when given daily for 7 or 14 days. When Ambisome was given daily for 7 days post burn-tenotomy, mice presented with an

increased range of motion about the injured ankle at 9 weeks post injury compared to vehicle treated control mice. However, a difference in range of motion was not seen between any other treatment groups in the dosing schemes investigated. Going forward, mice may need to be dosed for a longer period of time than was done in these studies. While Qureshi, et al. showed that daily administration of rapamycin for 14 days was sufficient to attenuate total new bone and soft tissue ectopic bone at postoperative day 84 in a rat model of blast induced HO, this dosing scheme may not translate to the murine model (4). Agarwal, et al, have previously shown that inhibition of HIF1a with rapamycin in the murine burn tenotomy model is sufficient to reduce HO formation at 9 weeks post injury, however rapamycin was given every other day throughout the 9 week period (5). In the future, dosing of the rapamycin in the murine model may need to be increased in order to attenuate the HO formation. Secondly, a liposomal form of Amphotericin B (Ambisome) was used in the present studies. Because Amphotericin B can lead to lethal nephroxicity in the mouse model, Ambisome was chosen because of its improved safety profile (10). However, this formulation may change the mechanisms of HIF1a interaction with amphotericin B, leading to a decreased inhibition efficiency. Therefore, in the future we will dose non-encapsulated amphotericin B at a lower dose to ensure efficacy and safety.

1B. Effect of Rapamycin and Amphotericin B Treatment on Heterotopic Ossification in a combat trauma rat model of HO

Adult male Sprague Dawley rats (450-500 g) were housed in clean plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow) and fresh water ad libitum. The study protocol (**SUR-18-065**) was reviewed and approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, in compliance with all applicable Federal regulations governing the protection of animals in research. We used our established rat model of blast-related HO which incorporates the critical injury patterns associated with combat-related extremity injury including 120 ±7 kPa systemic blast overpressure exposure (120kPa), followed by femur fracture, quadriceps crush injury, tourniquet application for 3 hours prior to trans femoral amputation through the zone of injury. Animals were single housed post-surgery.

Treatments with either rapamycin (R, 2.5 mg/kg; Cayman Chemicals), Amphotericin B (A, 2 mg/kg; water-soluble formulation from X-Gen) or both (R+A, 1.25 mg/kg and 1 mg/kg, respectively) were started on post-operative day 1 (POD1) and administered i.p daily. Rapamycin was administered for 14 days and amphotericin B for 10 days. Vehicle control group was included only for rapamycin (3% DMSO in saline), since amphotericin B was dissolved in water. **Table 1** details the cohorts for this study.

Treatment groups	Animal number
Rapamycin alone **(R, 2.5 mg/kg)**	8
Amphotericin alone **(A, 2 mg/kg)**	8
Rapa+ Ampho **(R@ 1.25 mg/kg + A@ 1 mg/kg)**	8
Carrier **(C, 3% DMSO in saline)**	8
Naïve **(N, no injury)**	8
Total animal number	**40**

Weight loss was observed for all injury groups in the first 2 weeks post injury. The R+A drug combination group demonstrated the highest and persistent weight loss among all groups. However, all cohorts, except the combination group regained their post-operative weights by week 4 post injury **(Fig. 3A)**, thereby establishing the safety of the drugs and corresponding dosage used. The combination group also demonstrated recurrent wound dehiscence, which needed multiple revisions **(Fig. 3B)**, indicating that the combination of rapamycin and amphotericin B potentially interferes with wound healing.

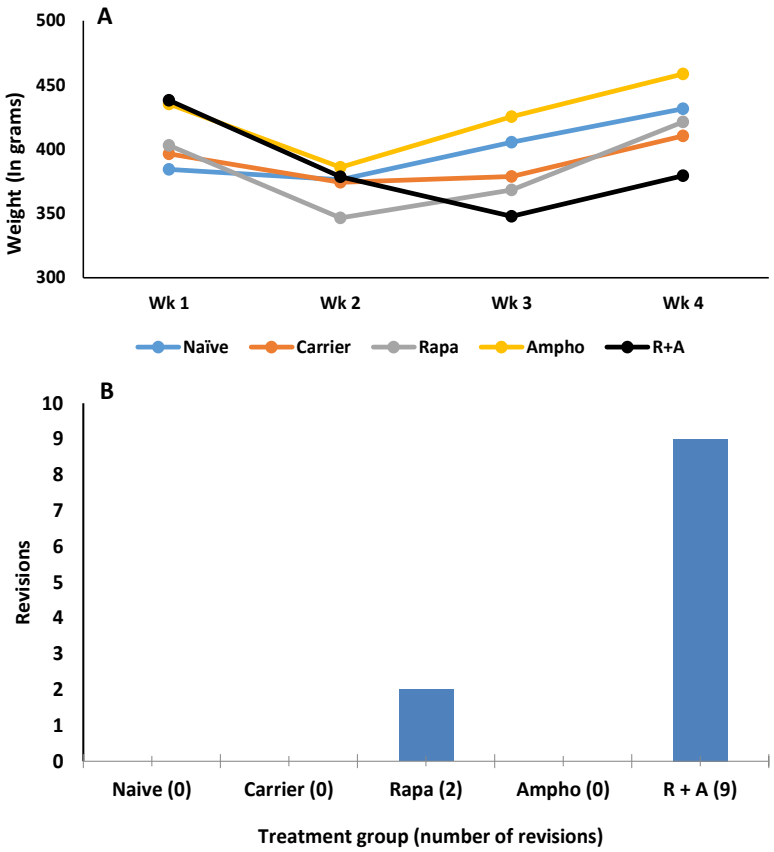

Figure 3. **A.** Longitudinal post-operative weight monitoring for the four cohorts; **a**. Naïve **b**. Carrier, **c**. Rapamycin **d**. Amphotericin **e**. Rapa + Ampho from post-operative week 1 through post-operative week 4. **B.** Frequency of wound dehiscence which needed revisions at the zone of amputation for each cohort.

This is an observational study, with the aim of longitudinal monitoring of ectopic bone formation and the efficacy of the treatment arms. MicroCT imaging was carried out for each cohort every 2 weeks for 8 weeks. Representative microCT images for the first three cohorts at POD14 (2 weeks) is shown in **Fig. 4**. The volumetric analysis of these images is currently underway

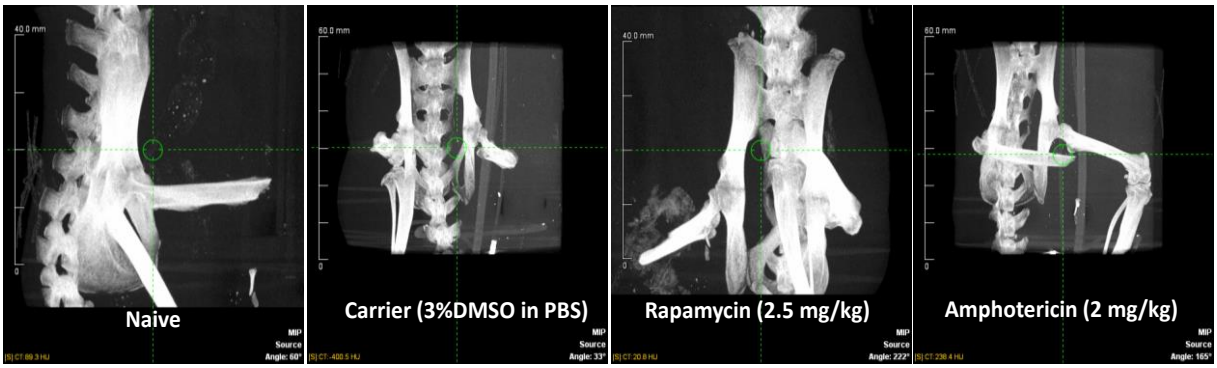

Figure 4. Representative microCT images of the injured and the contralateral limbs taken on the Inveon small animal scanner (Siemens) at 2 week
These images were acquired at 435 milliseconds exposure at Bin2 and represent the maximum intensity projection (MIP) view.

2. SUSI Analysis

2.1 SUSI Analysis in the mouse burn-tenotomy model of HO

Male C57BL/6 mice underwent a BT procedure, and were treated daily for one week with rapamycin (2.5 mg/kg), Ambisome (10 mg/kg), combination of rapamycin (1.25 mg/kg) and Ambisome (5 mg/kg), or the respective vehicle control. Both the injured and uninjured legs and ankles were imaged with SUSI weekly. At nine weeks, the volume of ectopic bone was quantified and compared between SUSI and CT methods.

In order to investigate the capabilities of SUSI, the injured leg at nine weeks post burn tenotomy was compared to the contralateral non-injured leg. First, the lower limb was imaged from just proximal to the insertion of the gastrocnemius to the distal end of the lower limb of both the injured leg and the uninjured contralateral control leg. Next, the grayscale images were analyzed and areas of potential HO were identified. The same anatomical region was identified on the scans of the contralateral uninjured leg of each animal. These regions were analyzed in order to determine the average acoustic concentration (AAC), average scatter diameter (ASD), and grayscale values. Injured legs were compared to non-injured legs. The average acoustic concentration, average scatter diameter, grayscale values were significantly different between the injured and non-injured legs, confirming that SUSI successfully distinguishes ectopic bone (HO) from uninjured tissue, as shown in **Figure 5.**

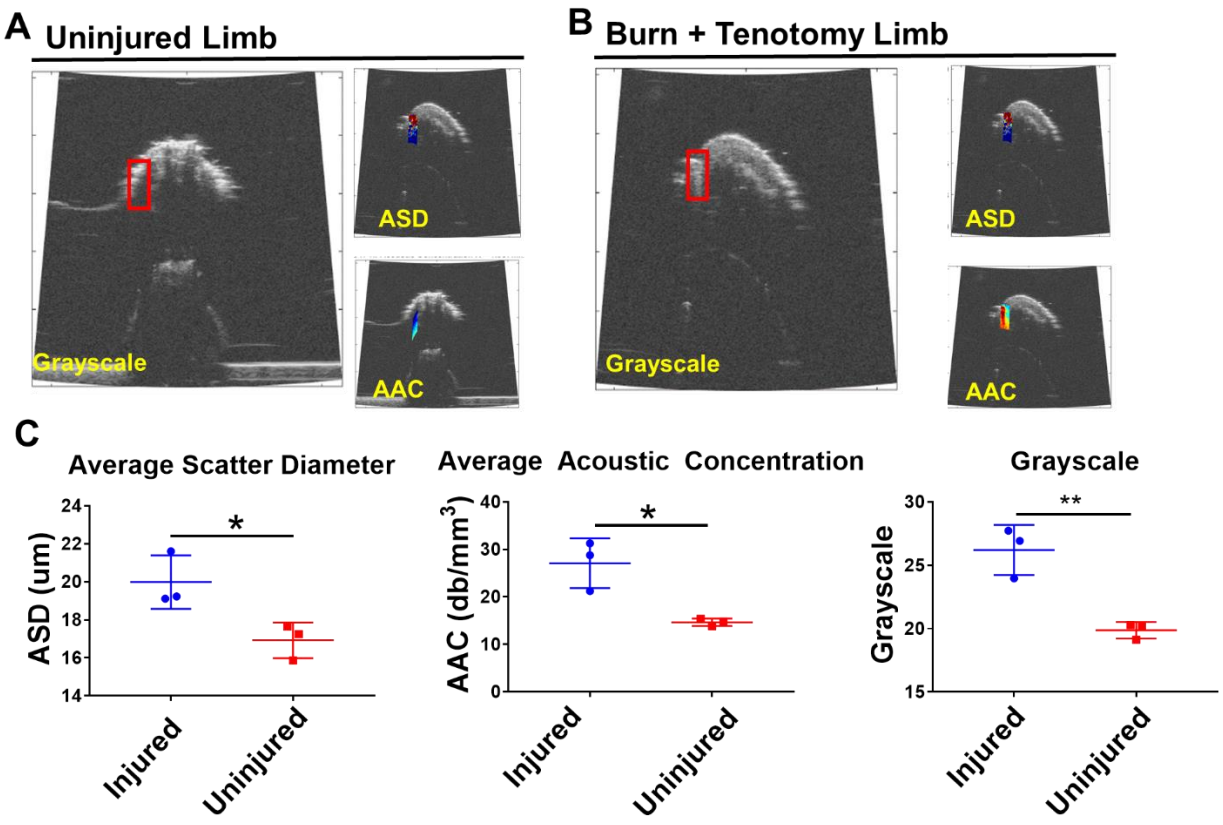

Figure 5: **AAC, ASD, and slope are significantly different between injured and contralateral non-injured legs at 9 weeks post burn tenotomy.** A) Representative grayscale, ASD, and AAC images of uninjured limb. Red outline indicates analyzed region of tissue that corresponds to location of HO formation in the injured limb. B) Representative grayscale, ASD, and AAC images of limb at 9 weeks post burn tenotomy. Red outline indicates HO formation. C) Quantitative analysis of ASD, AAC, and grayscale values show significant difference between ectopic bone and uninjured tissue at 9 weeks post burn-tenotomy. Significance determined using student's t-test to compare injured legs to uninjured legs. n=3 per group. * indicates $p<0.05$; ** indicates $p<0.01$.

Now that we have shown that SUSI successfully identified ectopic bone formation, weeks 1-8 after injury will be analyzed in order to identify earlier time points at which HO can be identified.

Analysis of ectopic bone formation between treatment groups is also currently in process. SUSI results between treatment groups will be compared to data obtained using microCT to measure volume of ectopic bone.

2.2 SUSI Analysis in the rat blast model of HO

Combat-related complex injury was modeled in rats as described in **Section 1B**. Injured and contralateral uninjured limbs were harvested at post-operative days (POD) 3, 6, 9, and 12, followed by fixation of the limbs in 10% formalin for 96 hours. The fixed limbs from each time point were imaged using SUSI in order to determine at which time point ectopic bone could be recognized.

New analysis methods to identify ectopic bone formation in the rat model were developed. First, the amputation site was scanned and the femur was identified. During the analysis, ectopic bone formation was identified near the distal end of the femur, matching past formation sites in this model (4). Next, the grayscale, average scatter diameter (ASD), and average acoustic concentration (AAC) were determined. **Figure 6** shows representative images and HO parameters at 12 days post injury. We are currently in the process of performing the same analysis on samples from earlier time points. All samples will be analyzed using the same methods in order to determine the earliest time point at which HO formation can be detected.

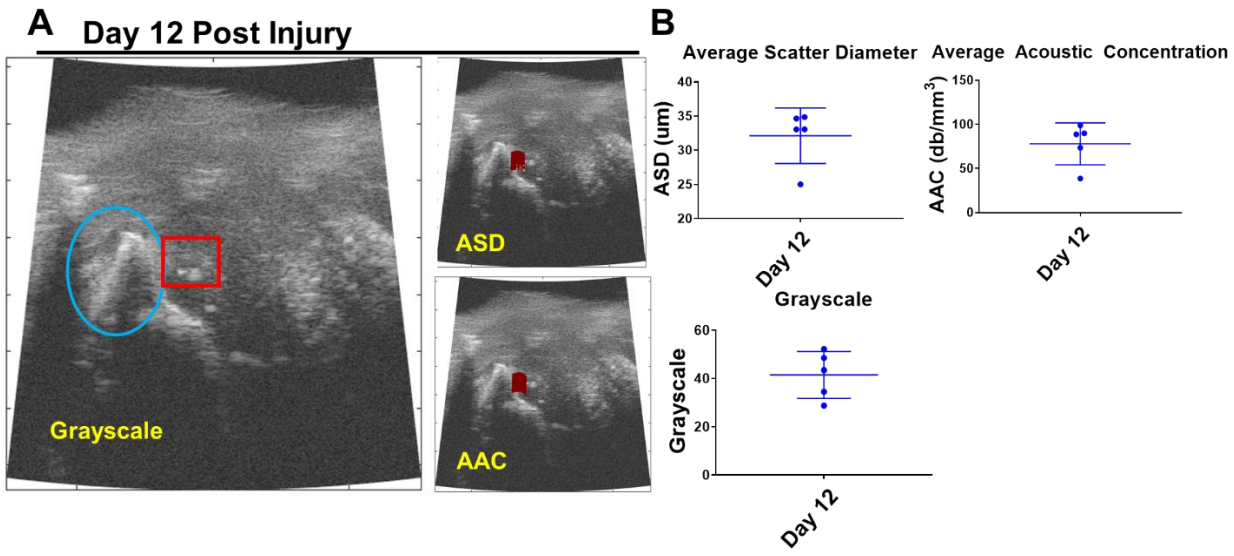

Figure 6: SUSI identifies HO formation in the rat blast model. A) Grayscale, average scatter diameter (ASD), and average acoustic concentration (AAC) image of the injured limb at 12 days post injury. Blue oval indicated the distal end of the femur. Red outline indicates area of HO formation that was analyzed. B) ASD, AAC, and Grayscale values for ectopic bone at twelve days post injury. Analysis of earlier time points is currently underway.

Discussion and Conclusions

We have validated that SUSI technology distinguishes HO formation in the burn-tenotomy model from healthy tissue at 9 weeks post injury. The average acoustic concentration, average scatter diameter, and grayscale values are significantly different in the HO site of the injured leg than the corresponding anatomical region of the uninjured leg. Earlier time points in the HO formation progression are currently under analysis in order to determine the earliest time point after injury that SUSI technology detects HO formation.

We have also extended this SUSI technology to analyze ectopic bone in a rat model of blast-related HO formation. In the rat model of blast-related HO, SUSI is able to identify HO at post injury day 12 in an injured limb. We have determined the AAC, ASD, and grayscale values of ectopic bone in this model, and are currently in the process of analyzing fixed tissues from earlier time points in order to determine the capability of the SUSI technology to identify HO earlier in its formation.

Specific Aim 2: *To mitigate post-traumatic muscle fibrosis through timed inhibition of mTOR/HIF-1α signaling with Amphotericin and Rapamycin separately or as combined therapy.* Status: To start Y2Q1

Subtask 1: Demonstrate that mTOR/HIF-1α inhibitors decrease post traumatic extremity muscle fibrosis.

Subtask 2: Validate mTOR/HIF-1α inhibitors as separate and combined treatment modalities in extremity muscle fibrosis.

Subtask 3: Define the time point at which treatment is best administered.

Milestone: Completion of muscle fibrosis timed inhibition studies and data analysis. Preparation and submission of peer-reviewed manuscripts.

Experimental design for the muscle fibrosis studies in the rat and murine models have been completed, and initial experiments with the muscle fibrosis models will begin at the beginning of Y2Q1.

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What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The post-doctoral fellow who is working on this project has been trained to perform the burn-tenotomy surgery. She has also been trained to perform the analyses for SUSI imaging.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Data from this project was shared with colleagues in orthopaedic surgery and trauma surgery.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During the next reporting period, all SUSI data that has been collected will be analyzed for the burn-tenotomy and blast-induced models of heterotopic ossification. The injured and

contralateral blast injured rat legs at postoperative day 56 will be harvested, imaged and analyzed with SUSI. Muscle fibrosis studies will begin. Rapamycin, amphotericin B, and combination of rapamycin and amphotericin B will be investigated to mitigate muscle fibrosis. Methods for detection of muscle fibrosis using SUSI technology will be developed.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have shown that the SUSI technology can be used to detect heterotopic ossification in the burn-tenotomy model. We have also made progress in the use of SUSI technology to detect HO formation on a blast induced model of HO.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

These findings will also have impacts on development of medical imaging technology and the use of rapamycin and amphotericin B as HIF1a inhibitors for other indications.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

A minor amendment for OR170174 was submitted September 19 2019 to decrease the Amphotericin B dose to 2 mg/kg when dosing alone and 1 mg/kg when dosing in combination with Rapamycin. We had previously dosed at 10mg/kg when alone and 5 mg/kg when with rapamycin. We believe that these changes will address the concerns of non-encapsulated Amphotericin B at higher doses over long dosing periods.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

None.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee

(or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Human subjects are not included in this project.

Significant changes in use or care of vertebrate animals.

None.

Significant changes in use of biohazards and/or select agents

None.

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**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the*

publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*

- *new business creation; and*
- *other*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	Benjamin Levi
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Planning of experimentation.
Name:	Nicole Edwards
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1-No effort on project as she is being covered by a T32 training grant.
Contribution to Project:	Dr. Edwards has been the project lead planning of experimentation.
Name:	Kaetlin Vasquez
Project Role:	Animal and lab manager
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Assist in coordination of project and regulatory documentation.
Name:	Shuli Li
Project Role:	Animal and lab manager
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	3
Contribution to Project:	Assist in planning of experimentation and coordination of project.
Name:	Thomas Davis
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1

Contribution to Project:	Planning of experimentation.
Name:	Devaveena Dey
Project Role:	Staff scientist
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Planning of experimentation.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *financial support;*
- *in-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *facilities (e.g., project staff use the partner's facilities for project activities);*
- *collaboration (e.g., partner's staff work with project staff on the project);*
- *personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *other*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.

QUAD CHARTS: In addition to embedding an updated Quad Chart within this annual / final technical report, also submit a standalone copy as an attachment in PowerPoint file only (.ppt or .pptx) to CDMRP Reporting at usarmy.detrick.medcom-cdmrp.mbx.cdmrp-reporting@mail.mil and copy the assigned CDMRP Science Officer.

Diagnostic and therapeutic strategy to prevent trauma induced upper extremity muscle fibrosis and heterotopic ossification

PI: Benjamin Levi **Org: University of Michigan, Ann Arbor, MI**
Contract Investigator: Thomas Davis PhD **Org:** Uniformed Services University of the Health Sciences **Award Amount:** $750,000

Study/Product Aim(s)

Primary Goal: The goals of this grant are to establish the efficacy of hypoxic signaling inhibition for HO and muscle fibrosis prevention, and to optimize these therapies using a non-invasive, clinically translatable diagnostic method to precisely identify the optimal timing and duration of treatment (Fig 1).
Research Idea: In this grant, we will demonstrate the ability of two therapeutics that target mTOR/HIF-1α signaling to prevent muscle fibrosis, HO and subsequent joint contractures. We will identify the optimal timing and deploy these image guided therapies after high risk injuries for to maximize prophylaxis and limit delayed wound healing.

Approach

- **Specific aim 1:** To validate timed, image guided prevention of heterotopic ossification with FDA approved inhibitors of hypoxic signaling.
- **Specific aim 2:** To mitigate post-traumatic muscle fibrosis through timed inhibition of mTOR/HIF-1α signaling with Amphotericin and Rapamycin separately or as combined therapy.

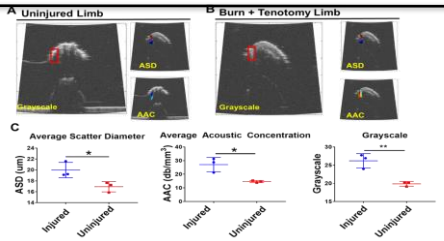

Fig. 1: SUSI Identifies HO. Representative grayscale, ASD, and AAC images of A) uninjured limb and B) burn-tenotomy injured limb at 9 weeks post injury. Red outline indicates analyzed region of tissue that corresponds to location of HO formation in the injured limb. C) Quantitative analysis of ASD, AAC, and grayscale values show significant difference between ectopic bone and uninjured tissue at 9 weeks post burn-tenotomy. Significance determined using student's t-test to compare injured legs to uninjured legs. n=3 per group. * indicates p<0.05; ** indicates p<0.01.

Timeline and Cost

Activities CY	18-19	19-20
Validate timed, ultrasound guided delivery of mTOR/HIF-1α inhibitors to mitigate post-traumatic HO.		
Demonstrate inhibitory efficacy of Amphotericin b and Rapamycin on muscle fibrosis.		
Verify efficacy and minimal toxicity of treatment strategies.		
Requested Budget ($750,000)	**$371,000**	**$379,000**

Goals/Milestones

CY18-19 Goals
- ❑We will use our Burn/tenotomy and Blast/Amputation/MRSA-infection rodent models to validate the effect of image guided mTOR/HIF-1α inhibitors (Rapamycin and Amphotericin) on HO *in vivo*. (USUHS, UM)
- ❑Demonstrate that Rapamycin or Amphotericin B delivered during abbreviated periods of time, or guided by spectral ultrasound (SUSI) minimizes treatment duration and off target effects. (USUHS,UM)

CY19-20 Goals
- ❑Demonstrate that our treatment strategy does not alter wound healing or cause off target toxicity
- ❑We will demonstrate that inhibitors of mTOR/HIF-1α (Rapamycin and Amphotericin) mitigate post-injury muscle fibrosis using proven animal models. (USUHS,UM)

Comments/Challenges/Issues/Concerns: N/A
Budget Expenditure to Date
Projected Expenditure: $750,000
Actual Expenditure: UM $123,626

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.