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TITLE: Sequential versus Combined Medical Therapies as a Novel Heterotopic Ossification Prevention Strategy

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14. ABSTRACT The long-term treatment of military warfighters who sustain severe battlefield blast-related extremity injuries and/or multiple limb amputations is one of the major challenges for military healthcare providers. A common complication facing modern combat casualties is the extra-skeletal development of bone within damaged/healing tissue resulting in soft tissue heterotopic ossification (HO). HO is more prevalent in military trauma, occurring in approximately 65-67% of amputations and nearly 62% of limb sparing procedures. Importantly, clinicians describe HO as the single most important barrier to meaningful functional mobility, independence, and return to military service. In the proposed research, we will use our physiologic model of blast and extremity trauma-induced heterotopic ossification (HO) to test and investigate two drugs (Palovarotene and Rapamycin) which are FDA approved and are currently used clinically for various indications, so that our goal of reaching clinical trials in human patients within five years can become realistic. Our physiologic model of blast-related HO extremity injury incorporates many of the same critical injury patterns detected in combat service members casualties with acute extremity injuries/amputation, including blast overpressure exposure, a comminuted femur fracture, and crush injury to the surrounding musculoskeletal tissue.					
15. SUBJECT TERMS Combat related amputations, heterotopic ossification, fracture healing					
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1. INTRODUCTION:

With the combined effects of improved body armor, tourniquets, aggressive resuscitation techniques, and improvements in the training of medical personnel, an increased number of service members survive to reach tertiary combat care units. The physiological insults produced by blast overpressure trauma results in severe extremity injury trauma accounting for approximately 70% of all combat-related war injuries. Compared to previous conflicts, there is a relative decrease in lower extremity and torso wounds, with a concomitant increase in the head, neck and upper extremity wounds. Heterotopic ossification (HO), the development of ectopic bone within non-skeletal tissues, affects two thirds of patients with high-energy blast-related injuries and more specifically HO develops in 30% of injured service members with complex upper extremity wounds wherein the residual limb is amputated. In the civilian trauma setting, HO is most commonly encountered following musculoskeletal trauma, severe burns, spinal cord injury or following acetabular or elbow surgery. In contrast, combat-related blast injuries are heterogeneous and complex, in regard to severity, distribution, mechanism of injury and level of contamination. These devastating multisystem wounds involving lacerations, crush injury, burns, fractures, systemic overpressure exposure on tissues, traumatic brain injury, extensive soft tissue, neural, vascular and osseous destruction, amputations, ischemia reperfusion injury and infection provide significant clinical management and tissue reconstruction challenges.

The clinical complications of HO formation include lifestyle-limiting pain, skin ulceration and poor tolerance of prosthetic wear, where conservative interventions such as multi-modal pain regimens, physiotherapy and prosthetic modification fail to alleviate symptoms and prevent surgical excision in approximately 41% of patients. Surgical excision of mature HO, although largely definitive, can be wrought with complications and when successful, can result in further delays of rehabilitation

The clinical impact of upper extremity HO differs from that of lower extremity HO based on the differing functions of the upper and lower extremities. The lower extremity functions in weight-bearing and locomotion, and bone-spikes of HO can greatly interfere with comfortable socket wear. The upper extremity exists to move the hand in space, and an injured upper extremity with 2-opposable fingers and decreased range of motion at the wrist and elbow is still often more functional than a prosthetic. Preservation of length and distal function guide the treatment of upper extremity injuries, while creation of a functional limb with robust soft tissue envelope for weight bearing is the goal of treating lower extremity injuries. As such, HO in the upper extremity effects the function differently, not just through limiting prosthetic tolerance (which is usually greater), but also by inhibiting range of motion (ROM) for positioning the terminal device in space for fine motor activities and activities of daily living. Upper extremity HO can also limit function and signal capture from muscle groups necessary for myoelectric device control.

2. **KEYWORDS:**

Combat related amputations, research, heterotopic ossification, fracture healing, effect palovarotene, effect of rapamycin, combined effect of palovarotene and rapamycin, sequential effect of palovarotene and rapamycin,

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Regulatory Tasks (months 0-3)

Subtask 1: Submission of IACUC Applications.

Subtask 2: Submission of IACUC protocol for ACURO and DoD approval of animal studies.

Milestone 1 - ACURO approval. (Completed 12OCT2016)

PROTOCOL:

Protocol [ACURO Assigned Number]: OR150080

Title: "Evaluation of Target Therapies for Inhibiting Combat-Related Heterotopic Ossification in a Rat (*Rattus norvegicus*) Model of Extremity Injuries,

SUBMITTED TO AND APPROVED BY:

- IACUC protocol **15-OUMD-19S** was approved by the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD on 18-MAR-2015 and then subsequently received SAMRMC Animal Care and Use Review Office (ACURO) approval (OR150080).
- Protocol OR150080 entitled, " Evaluating targeted therapeutics, diagnostics and molecular mechanisms underlying combat-related heterotopic ossification in a rat (*Rattus norvegicus*) model of polytraumatic injury.," IACUC protocol number **18-OUMD-10S**, Protocol Principal Investigator Matthew Bradley, approved as of 18-MAR-2018.
- Protocol OR150080 entitled, " Evaluating targeted therapeutics, diagnostics and molecular mechanisms underlying combat-related heterotopic ossification in a rat (*Rattus norvegicus*) model of polytraumatic injury.," IACUC protocol number **21-OUMD-21S**, Protocol Principal Investigator Stephen Kaba, approved as of 02-JUNE-2021.

STATUS: ACTIVE

Protocol 21-OUMD-21S replaced Protocol number 18-OUMD-10S.

Milestone 2 – Completion of *in vitro* studies and data analysis by end of the 9th month. Determination of the best-of-breed angiogenic/osteogenic inhibitor and/or combination.

Specific Aim 2 - To investigate the effects of multiple drug combination strategies in preventing HO formation in blast-related extremity injury model of HO (9-15 months). 80% completed.

- A. Determine the efficacy of different combinations of Rapamycin and RAR- γ agonist in attenuating HO formation.
- Determine the optimal doses of Rapamycin and RAR- γ agonist that radiographically demonstrate the most significant attenuation of HO at 12 weeks. (Completed 30OCT2017).
 - Histological and molecular analysis of wound healing/repair and tissue chondrogenesis, angiogenesis, and osteogenesis.

Milestone 3 – Determination of best-of-breed treatment strategy to prevent HO formation. Submission of manuscript entailing the combined *in vitro* and *in vivo* optimal doses and time dependent efficacy of Rapamycin and Palovarotene in attenuating osteogenic, chondrogenic and endotheliogenic differentiation.

Specific Aim 3 - Assess the effect of Rapamycin and Palovarotene independently and/or concomitantly on fracture healing (months 15-22). 80% completed.

- A. Test the effects of RAR- γ agonist and Rapamycin alone and in combination on fracture healing.
- Monitor fracture healing radiographically using micro-CT (2-, 3-, 6-, and 8-weeks post injury). (100% complete)
 - Assess the progression and quantitate fracture callus volume following prophylactic treatment of the rats, using microCT and biomechanical testing.
 - Histological and molecular assessment of early fracture healing/repair. (100% complete)

Milestone 4 – Completion of *in vivo* studies assessing the effects of therapeutic strategies on fracture healing. Submission of a peer-reviewed manuscript covering these findings (months 22-24). (100% complete)

What was accomplished under these goals?

Milestone 1: Submitted an IACUC protocol, "Evaluation of target therapies for inhibiting combat-related heterotopic ossification in a rat (*Rattus norvegicus*) model" and acquired ACURO and DoD approval of animal studies (completed 12OCT2016)

- Protocol OR150080 entitled, "Evaluation of Target Therapies for Inhibiting Combat-Related Heterotopic Ossification in a Rat (*Rattus norvegicus*) Model of Extremity Injuries," IACUC

protocol number 15-OUMD-19S, Protocol Principal Investigator Jonathan Forsberg, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 12-OCT-2016.

Specific Aim 1: Demonstrate the *in vitro* effect of Palovarotene and Rapamycin on the growth and osteogenic and angiogenic differentiation of muscle-derived rat MSCs (months 4-8). 100% completed.

- A. Isolated and characterized rMSCs from skeletal muscle of naïve Sprague Dawley rats.
 - *Immune-phenotype and functionally characterized the isolated rMSCs. (Completed 21DEC2016)*
 - *Established a working inventory of stock rMSCs to conduct Specific Aim-1. (Completed 21DEC2016)*
- B. Determine the dose response for Rapamycin and Palovarotene *in vitro*.
 - *Determine optimal dose and combination of Rapamycin, Palovarotene to significantly attenuate osteogenic and endotheliogenic differentiation. (Completed 30NOV2017)*

In vitro drug combinations experiments using mesenchymal stem cells (MSCs). We used frozen bone marrow derived Sprague Dawley rats MSCs (rMSCs) obtained from section 'A' of SA1 above to assess the efficacy of different drug combinations of Palovarotene and Rapamycin on the growth and osteogenic differentiation of rMSCs:

1. Effect of sequential treatment of Palovarotene, followed by rapamycin (**P/R**) on connective tissue progenitor (CTP) frequency and osteogenic differentiation *in vitro*
2. Effect of sequential treatment of rapamycin, followed by Palovarotene (**R/P**) on connective tissue progenitor (CTP) frequency and osteogenic differentiation *in vitro*
3. Effect of combined treatment of Palovarotene and rapamycin (**PR**) (simultaneous) on connective tissue progenitor (CTP) frequency and osteogenic differentiation *in vitro*

***In vitro* effect of sequential and combined treatment of Palovarotene and Rapamycin on the growth and osteogenic differentiation of bone marrow-derived rat mesenchymal stem cells (rMSCs)**

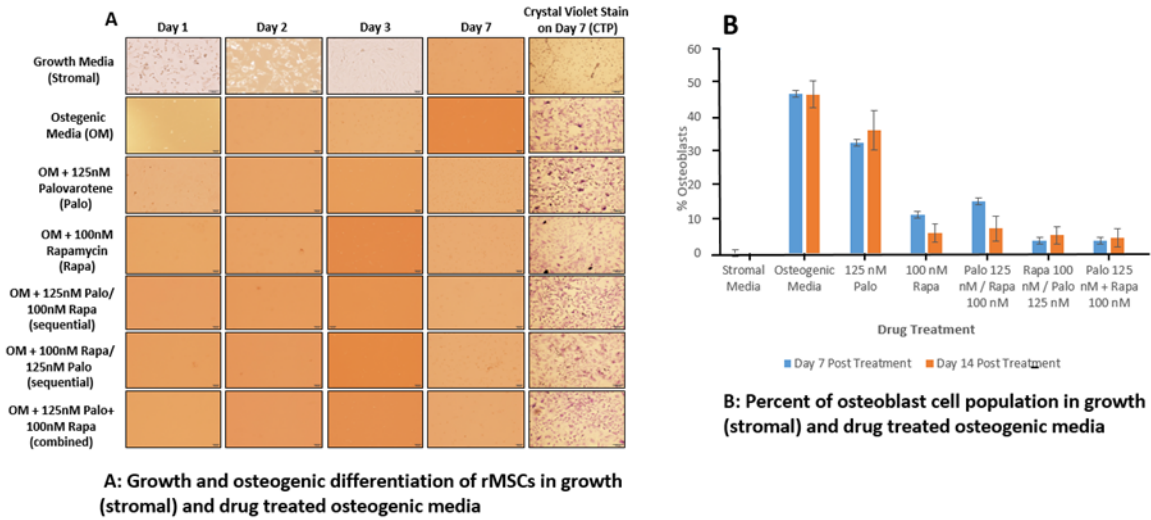


Figure 1: *In vitro* effect of sequential and combined treatment of Palovarotene and Rapamycin on the growth and osteogenic differentiation of bone marrow-derived rat mesenchymal stem cells (rMSCs)

Previously isolated bone-derived rat mesenchymal stem cells (rMSCs) from the naïve donor rats (2nd passage) were seeded in triplicate at a density of 1×10^3 cells/well in 6-well plates in normal growth media (stromal) supplemented with 10% FBS, 100 U/ml penicillin, and for 24 h at 37°C in fully humidified 5% CO₂ in air atmosphere. For the differentiation study group, normal growth media was changed to osteogenic media and supplemented with different combinations of Palovarotene (125 nM) and/or Rapamycin (100 nM) with media changes every 3 days. After

7 or 14 days, adherent cell colonies were rinsed twice with PBS, fixed with 100% methanol for 5 min at room temperature, air-dried, stained with Crystal violet solution for 5 min, and then rinsed with distilled water to remove residual dye. Connective tissue progenitor osteoblast (CTP-O) colonies were counted using light microscopy by a reader (TAD) that was blinded to the treatment groups. **A:** Shows the growth and osteogenic differentiation of rMSCs in stromal and osteogenic media with/without Palovarotene and/or Rapamycin treatments. **B:** Alteration of CTP frequency and osteogenic differentiation after sequential and/or combined Palovarotene and Rapamycin treatment. Palovarotene alone decreased osteogenic connective tissue progenitor (CTP-O) the least, while non-treated osteogenic media did not show any observable decrease *in-vitro*. Treating rMSCs first with Rapamycin followed Palovarotene 30 min later (Rapa/Palo) as well as simultaneous treatment of both drugs showed the least percentage in osteoblast population *in-vitro*.

In vitro drug combinations experiments using mesenchymal stem cells (MSCs): We used frozen bone marrow derived Sprague Dawley rats MSCs (rMSCs) obtained from SA1, above to assess the efficacy of different drug combinations of Palovarotene and Rapamycin on the growth and osteogenic differentiation of rMSCs

- Effect of sequential treatment of Palovarotene, followed by rapamycin (**P/R**) on alkaline phosphatase (ALP) level and activity associated with on osteogenic differentiation *in vitro*
- Effect of sequential treatment of rapamycin, followed by Palovarotene (**R/P**) on ALP level and activity associated with on osteogenic differentiation *in vitro*
- Effect of combined treatment of Palovarotene and rapamycin (**PR**) (simultaneous) on ALP level and activity associated with on osteogenic differentiation *in vitro*

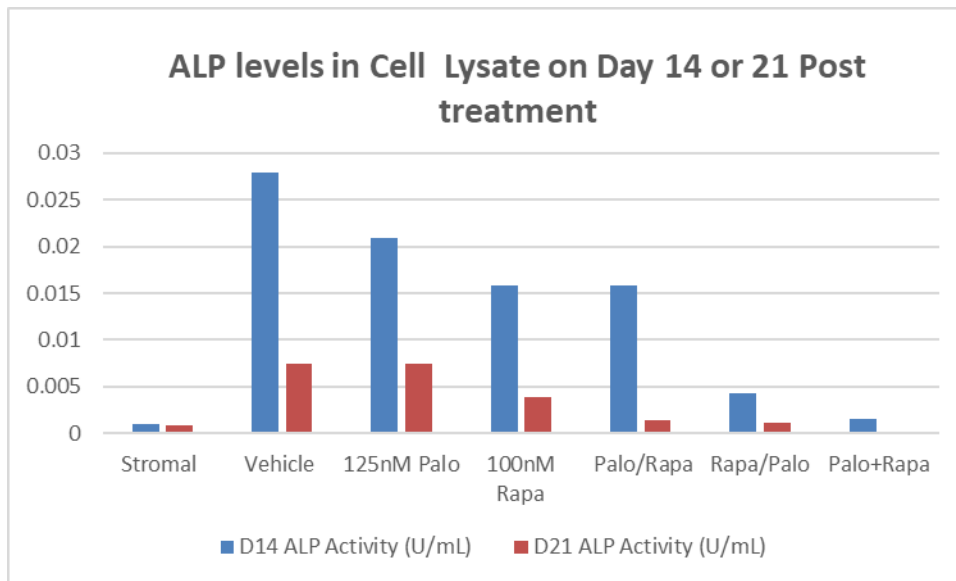


Figure 2: ALP measured in marrow-derived rat mesenchymal stem cells (rMSCs) cell lysate supernatants showing activity (U) per litre (L) of tested sample. Samples were diluted 3-fold. Previously isolated bone-derived rat mesenchymal stem cells (rMSCs) from the naïve donor rats (2nd passage) were seeded in triplicate at a density of 1×10^3 cells/well in 6-well plates in normal growth media (stromal) supplemented with 10% FBS, 100 U/ml penicillin, and for 24 h at 37°C in fully humidified 5% CO₂ in air atmosphere.

For the differentiation study group, normal growth media was changed to osteogenic media and supplemented with different combinations of Palovarotene (125 nM) and/or Rapamycin (100 nM) with media changes every 3 days for 14 or 21 days. For the quantification of cellular alkaline phosphatase (ALP) activity (ALP assay; abcam, colometric), media was discarded, cell washed once with cold PBS, re-suspended 50uL in assay buffer and homogenized using the “Bullet Blender Gold” (Next Advance). The cell lysate was then centrifuged (15000 rpm, 4°C, 15min). The Supernatant was collected and used for the ALP assay.

The ALP levels measured after 21 days was lower 14 days in all treatment groups. Levels of ALP measured following the sequential treatments of P/R or R/P was the lowest but no observable difference in ALP activity between the two treatments on day 21.

Effect of *in vitro* RAR γ agonist treatment on muscle derived CTP colony formation

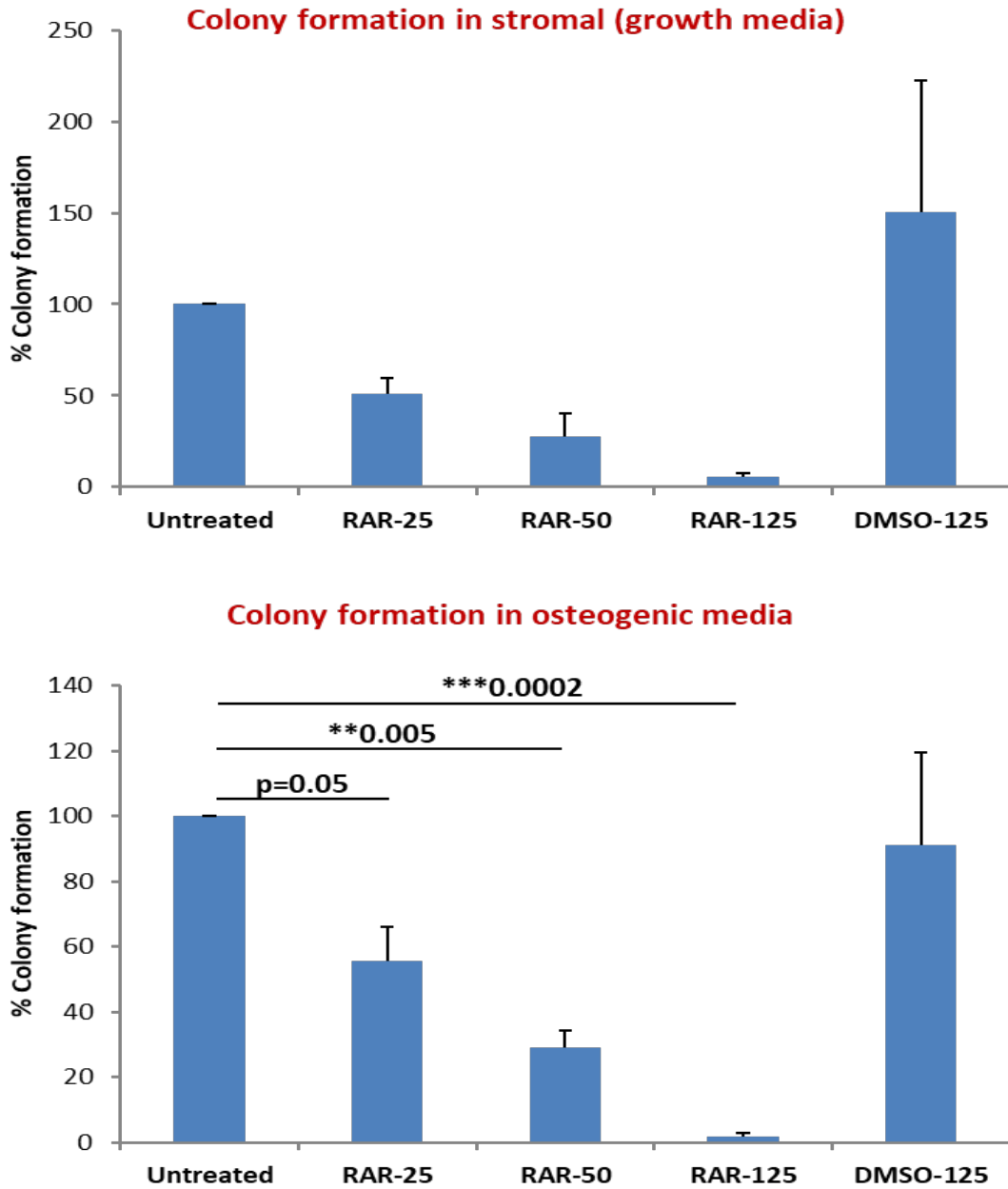


Figure 3: Palovarotene treatment has a significant dose dependent effect (0-125nM) on rat muscle tissue-derived CTP-O colony formation *in vitro* under MSC stromal growth (not shown) and osteogenic culture conditions

- Assay gene expression of osteogenic, chondrogenic and angiogenic markers for the optimal dose of the drug (100% Completed)

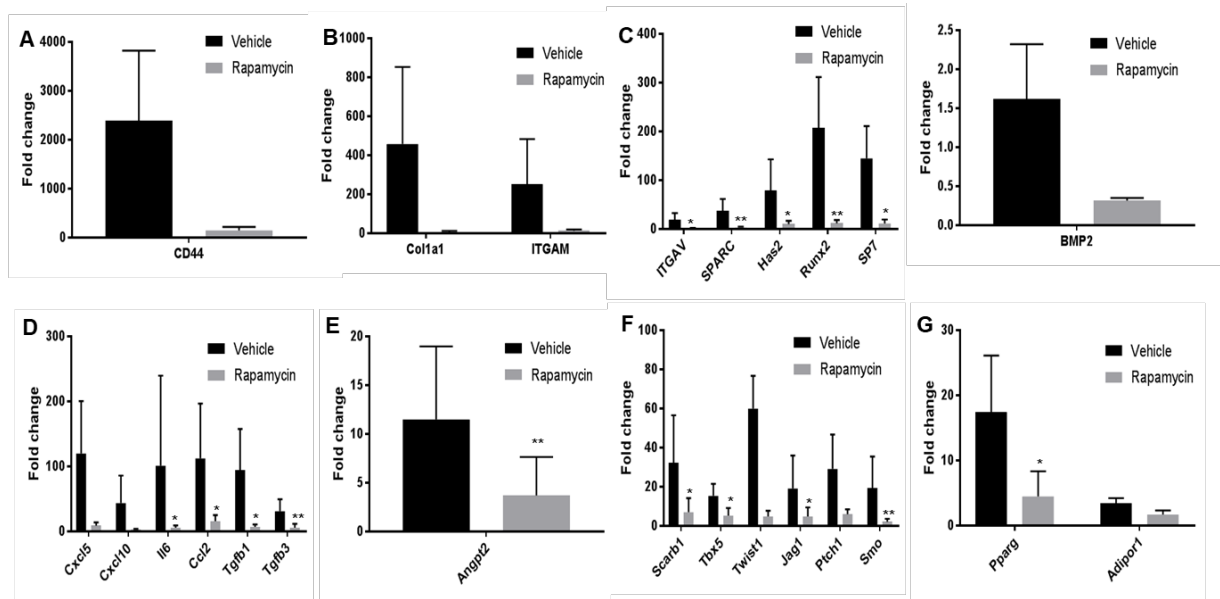


Figure 4: Early rapamycin treatment downregulates key genes involved in extracellular matrix synthesis (A-C), osteogenesis (C-D), inflammation (E), and angiogenesis (F-G)

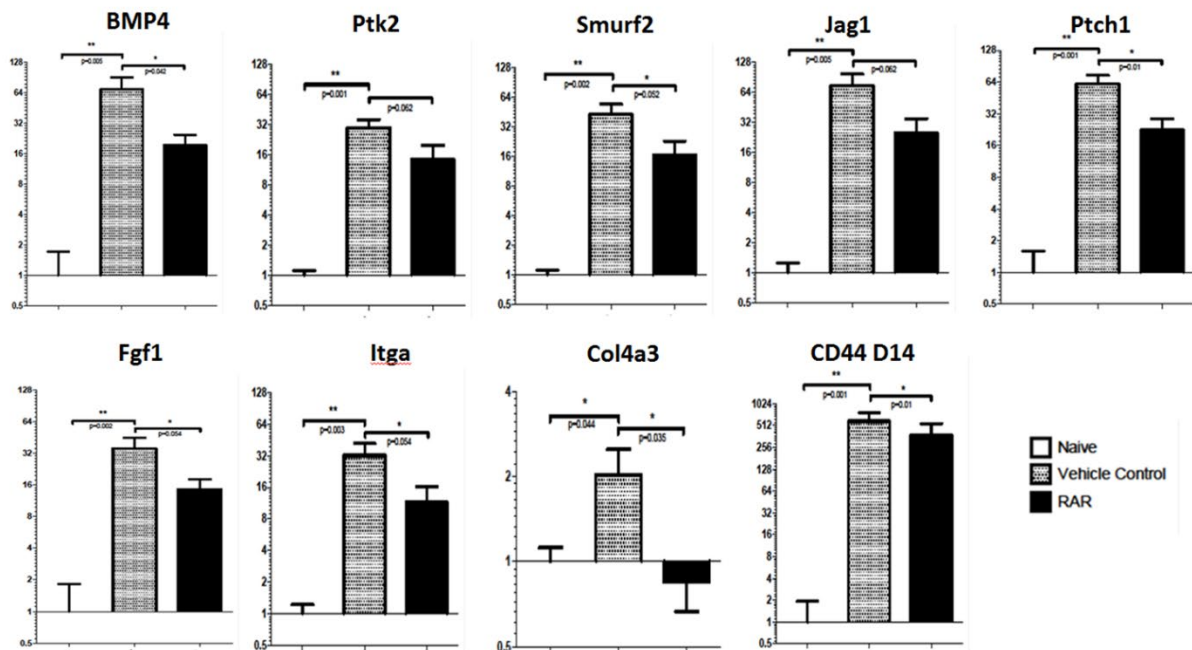


Figure 5: Palovarotene treatment suppresses early osteogenic and extracellular matrix gene expression in injured muscle

- *Quantitate the effects of drugs alone and/or in combination on cellular mineralization, endothelial cell vessel formation and cartilage at optimal and cytotoxic doses. (Completed 100%).*

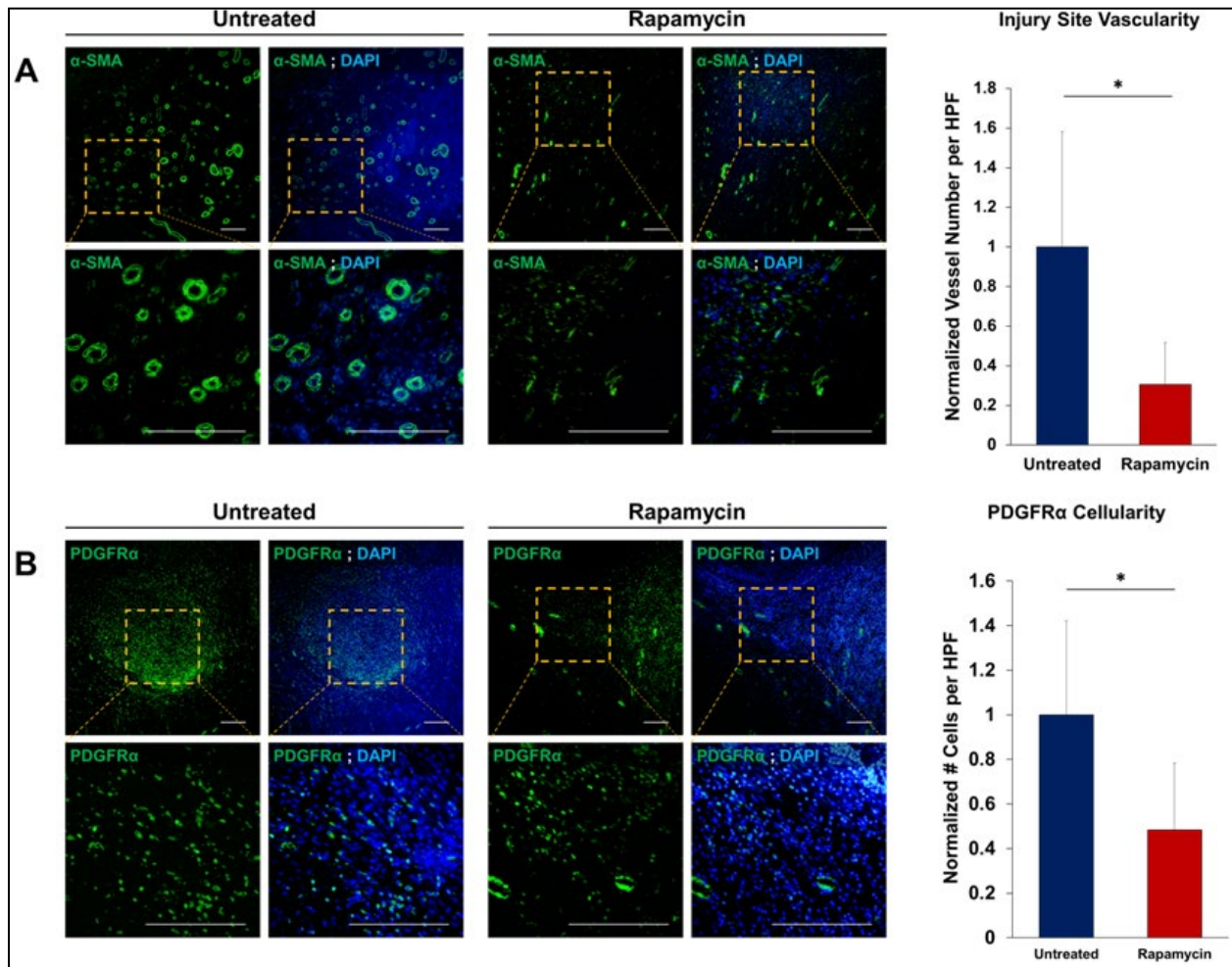


Figure 6: Rapamycin treatment resulted in significant reduction in vascular density (α -SMA blood vessels) and the frequency of PDGFR α progenitor cells

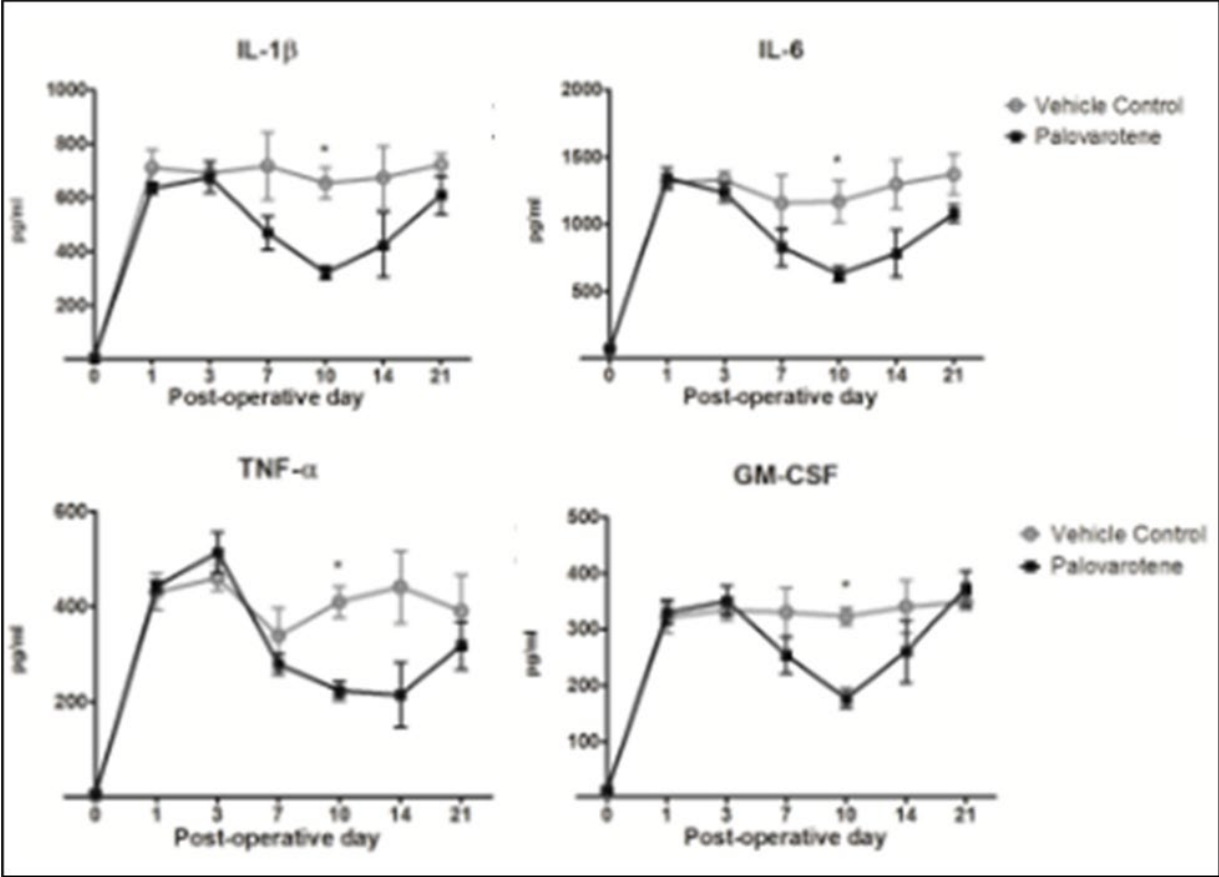


Figure 7: Palovarotene treatment decreases the systemic inflammatory response following a blast injury

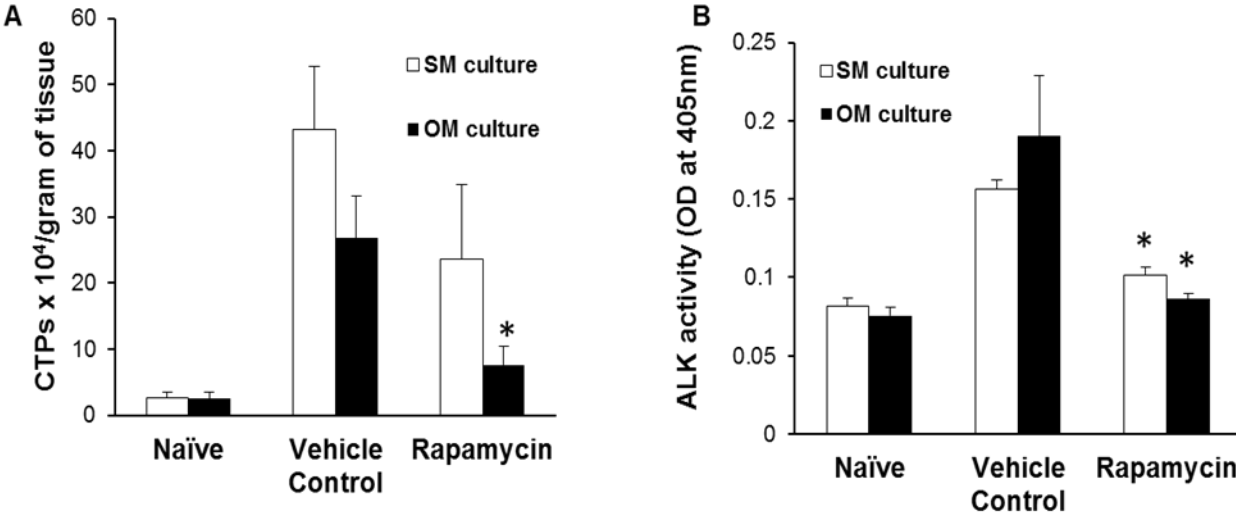


Figure 8: Rapamycin treatment was found to decrease the frequency (left panel) as well as osteogenic differentiation (right panel) of CTPs under both stromal media (SM) and osteogenic media (OM)**Milestone 2** – Completion of *in vitro* studies and data analysis by end of the 9th month. Determination of the best-of-breed angiogenic/osteogenic inhibitor and/or combination.

Some of the results obtained in SA1 and shown here have been published in peer-reviewed journals

- Qureshi AT, Dey D, Sanders EM, Seavey JG, Tomasino AM, Moss K, Wheatley B, Cholok D, Loder S, Li J, Levi B, Davis TA. Inhibition of Mammalian Target of Rapamycin Signaling with Rapamycin Prevents Trauma-Induced Heterotopic Ossification. *Am J Pathol.* 2017 Nov;187(11):2536-2545. doi: 10.1016/j.ajpath.2017.07.010. Epub 2017 Oct 10. PMID: 29029772; PMCID: PMC5809339.
- Pavey GJ, Qureshi AT, Tomasino AM, Honnold CL, Bishop DK, Agarwal S, Loder S, Levi B, Pacifici M, Iwamoto M, Potter BK, Davis TA, Forsberg JA. Targeted stimulation of retinoic acid receptor- γ mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. *Bone.* 2016 Sep;90:159-67. doi: 10.1016/j.bone.2016.06.014. Epub 2016 Jun 28. PMID: 27368930; PMCID: PMC5546218.

Specific Aim 2: Investigate the effects of multiple drug combination strategies in preventing HO formation in blast-related extremity injury model of HO (9-15 months). 50% completed.

- A. Determine the efficacy of different combinations of Rapamycin and RAR- γ agonist in attenuating HO formation
 - *Determine the optimal doses of Rapamycin and RAR- γ agonist that radiographically demonstrate the most significant attenuation of HO at 12 weeks. (completed 30OCT2017)*

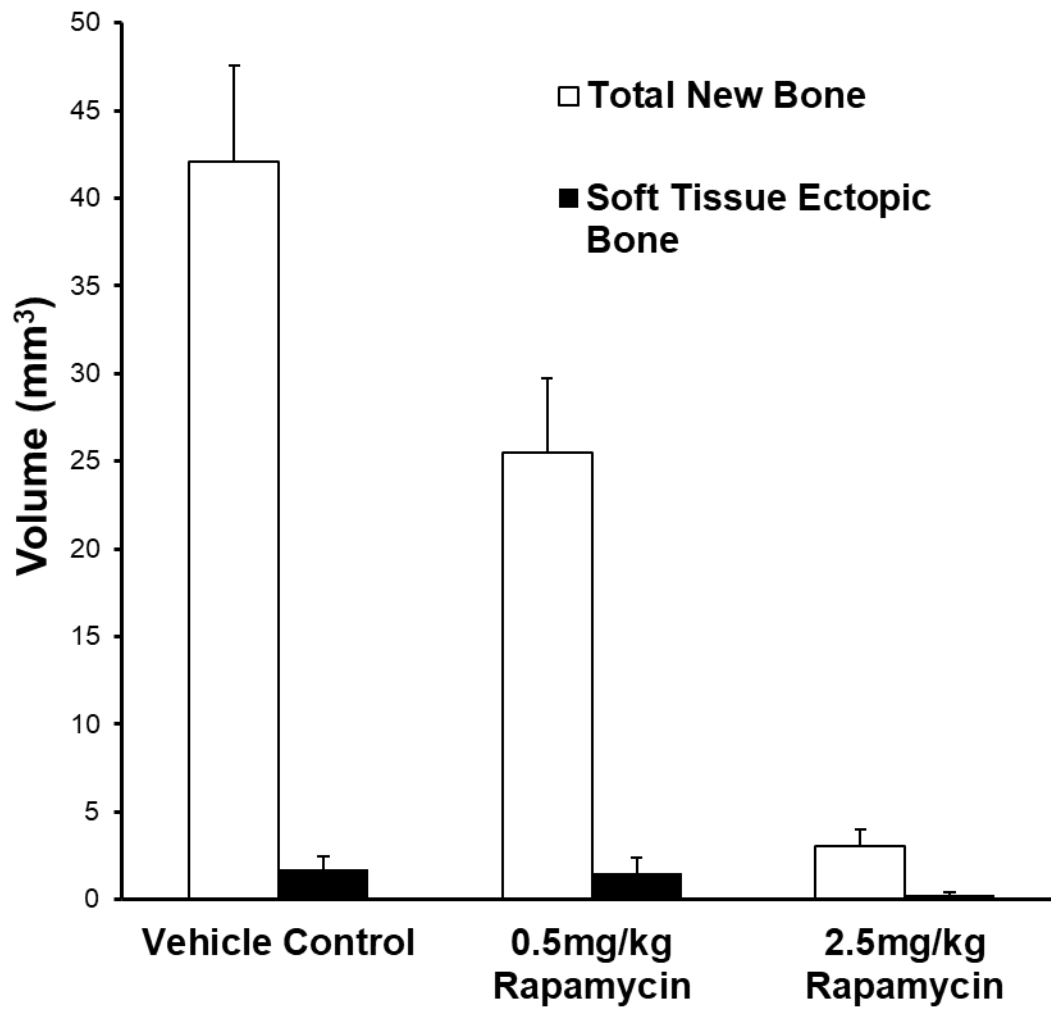


Figure 9: Rapamycin treatment significant decreased the volume of both soft ectopic tissue and total new bone.

In vivo combination drug experiments of Rapamycin and Palovarotene

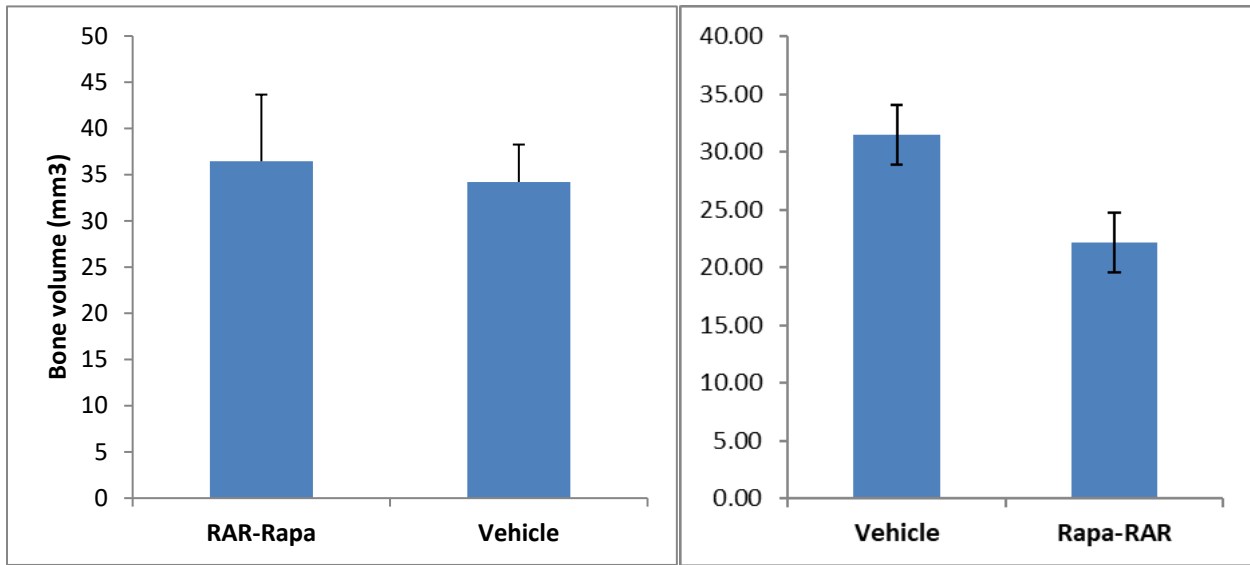


Figure 10: Volumetric Analysis for Different Drug Combinations: A) *Palovarotene-Rapamycin Combination*: All animals (n=8) received Palovarotene (RAR; 1mg/Kg in 100uL) by oral gavage every other day in the first week and Rapamycin (Rapa; 2.5mg/Kg in 100uL) by intraperitoneal (IP) injection every day in the second week. **B) *Rapamycin- Palovarotene Combination*:** Each animal (n=8) received the same dose of the drugs as described in “A” but in the reverse order. Control animals received 100uL of the solvent (Vehicle) for the respective drug. The results shown here represents the average volume of bone formed for three rats 12 weeks after surgery.

No statistically significant difference was observed in the volume of bone formed between the two treatments despite the relatively lower bone volume seen between the Vehicle control and the Rapa-RAR combination compared to the RAR-Rapa combination.

- *Histological and molecular analysis of wound healing/repair and tissue chondrogenesis, angiogenesis, and osteogenesis.*

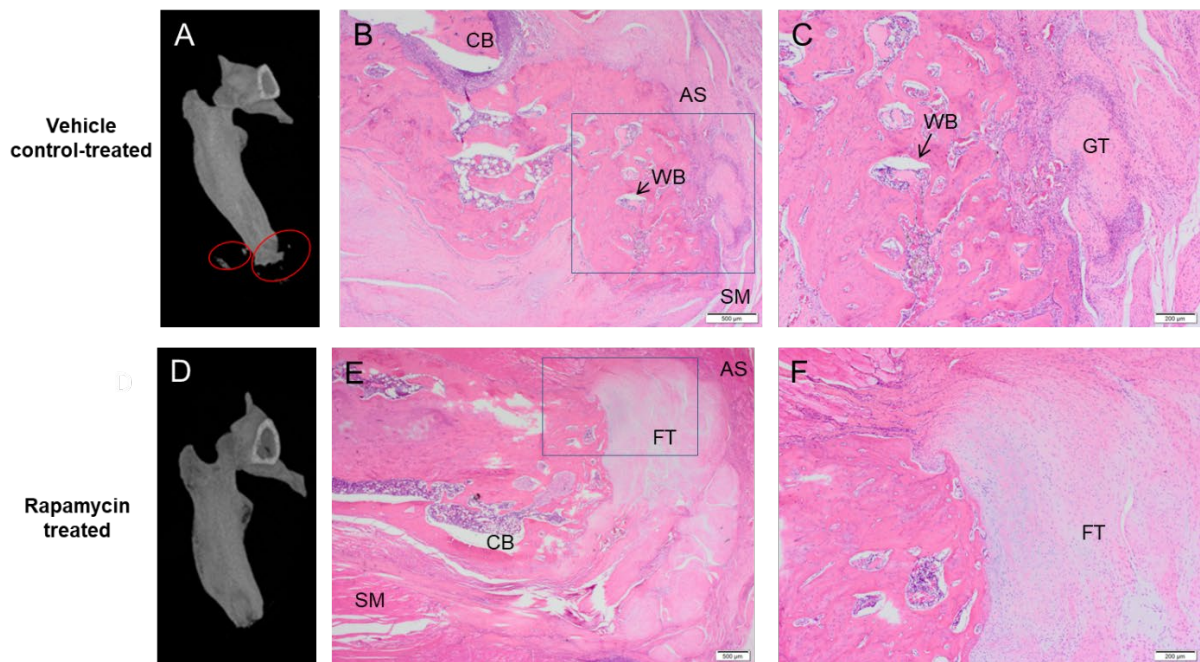


Figure 11: Rapamycin treatment attenuates the formation of ectopic bone in the blast-related extremity injury/methicillin-resistant *Staphylococcus aureus* infection model of heterotopic ossification (HO). A and E: Representative three-dimensional rendered micro-computed tomography images of rat femurs at postoperative day 84 treated with vehicle control (3% dimethyl sulfoxide/saline; A) or rapamycin (2.5 mg/kg per day for 14 days, i.p; E). Areas of HO formation (red circles). BeD and FeH: Histologic assessment of hematoxylin and eosin-stained tissue sections from animals treated with vehicle control (BeD) and rapamycin (FeH). Areas of interest in near the distal end of the residual femur (boxed areas) in B, C, F, and G were examined at higher magnification and are shown in C, D, G, and H, respectively. Scale bars: 500 mm (B, C, F, and G); 200 mm (D and H). AS, amputation site; CB, cortical bone; FH, femoral head; FT, fibroblastic tissue; GT, granulation tissue; SM, skeletal muscle; WB, woven bone.

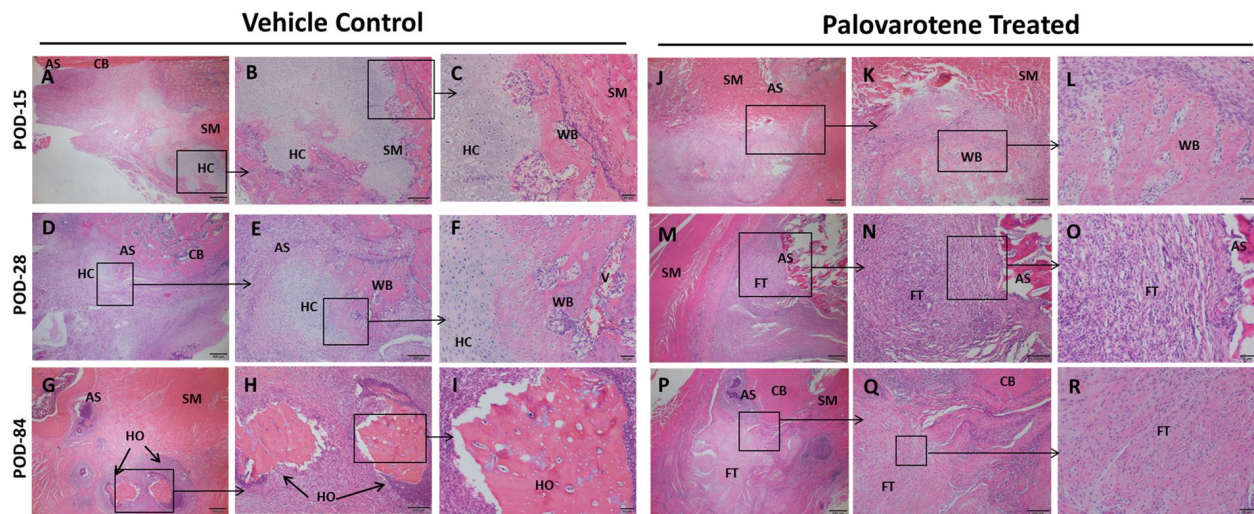


Figure 12: Palovarotene treatment inhibits the initial phases of HO. Histologic and microscopic assessment of HO formation in vehicle- control rats at 15 days (A–C), 28 days (D–F) and 84 days (G–I), and in Palovarotene treated rats at 15 days (J–L), 28 days (M–O) and 84 days (P–R) post injury. CB = cortical bone, AS = amputation site; FT = fibroblastic tissue; HC = immature and hypertrophying cartilage; SM = skeletal muscle; WB = woven bone; V = vascularization and HO =heterotopic ossification. The scale bars in the images (A), (D), (G) (M) and (P) correspond to 500 μ m. Scale bars in images (B), (E), (H), (K), (N) and (Q) corresponds to 200 μ m. Scale bars in images (C), (F), (I), (L), (O) and (R) correspond to 50 μ m

Milestone 13 – Determination of best-of-breed treatment strategy to prevent HO formation. Data entailing the combined *in vitro* and *in vivo* optimal doses and time dependent efficacy of Rapamycin and Palovarotene in attenuating osteogenic, chondrogenic and endotheliogenic differentiation was published as follows:

- Wheatley, BM et al., 2018. Palovarotene inhibits connective tissue progenitor cell proliferation in a rat model of combat-related heterotopic ossification. *J Orthop Res* 36:1135–1144.

Specific Aim 3 - Assess the effect of Rapamycin and Palovarotene independently and/or concomitantly on fracture healing (months 15-22). 80% completed.

- Test the effects of RAR- γ agonist and Rapamycin alone and in combination on fracture healing.

- Monitor fracture healing radiographically using micro-CT (2-,3-, 6- and 8-weeks post injury). (100% complete).

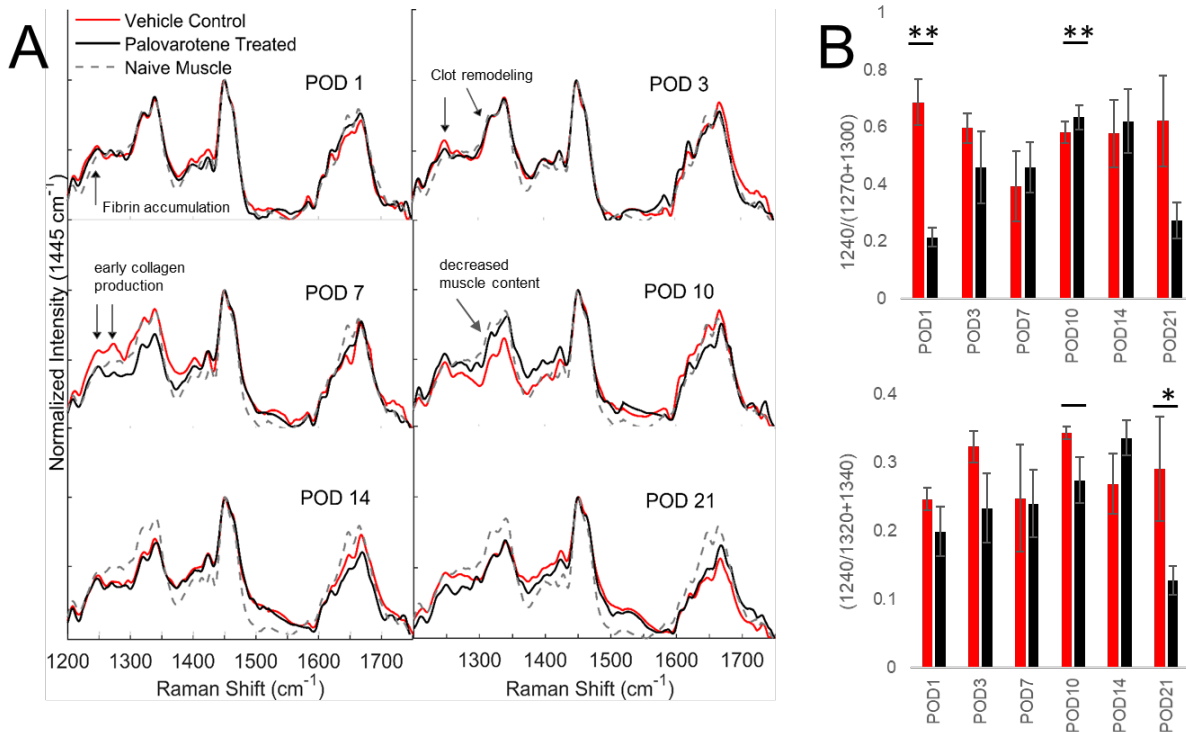


Figure 14: Raman Spectroscopy Detects Compositional Tissue Changes Associated with Early Heterotopic Ossification: (A and B) Comparison of mean adipose, bone subtracted Raman spectra (A) and calculated band area ratio (BAR) metrics (B) of vehicle control and Palovarotene treated animals at PODs 1, 3, 7, 10, 14, and 21 (n=5 rats/time point). Spectra are normalized to 1445cm⁻¹, a marker of total protein content. Amide III protein disorder/order (1240/[1270+1300]cm⁻¹) BAR, is measure of β -sheet and disordered protein over helical, ordered protein and protein disorder/normal muscle (1240/[1320+1340] cm⁻¹) is a measure of β -sheet and disordered protein over CH₂CH₃ twist and wag bands characteristic to muscle tissue. Vehicle control animals exhibited significantly increased spectral signatures suggestive of fibrin accumulation at POD-1 and replacement of injured muscle with collagen at POD-7, 10, and 21 when compared to Palovarotene treated animals. Asterisks (*) indicate p<0.05, (**) indicate p<0.001. Error bars represent standard error of the mean.

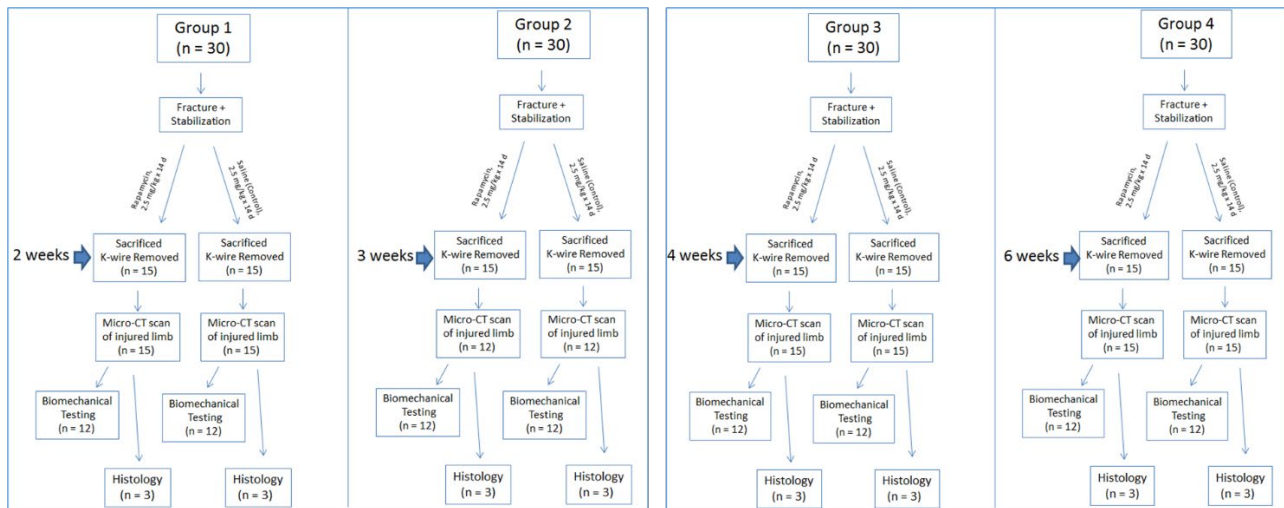
- Access the progression and quantitate fracture callus volume following prophylactic treatment of the rats, using microCT and biomechanical testing.

Briefly, the methodology is as described:

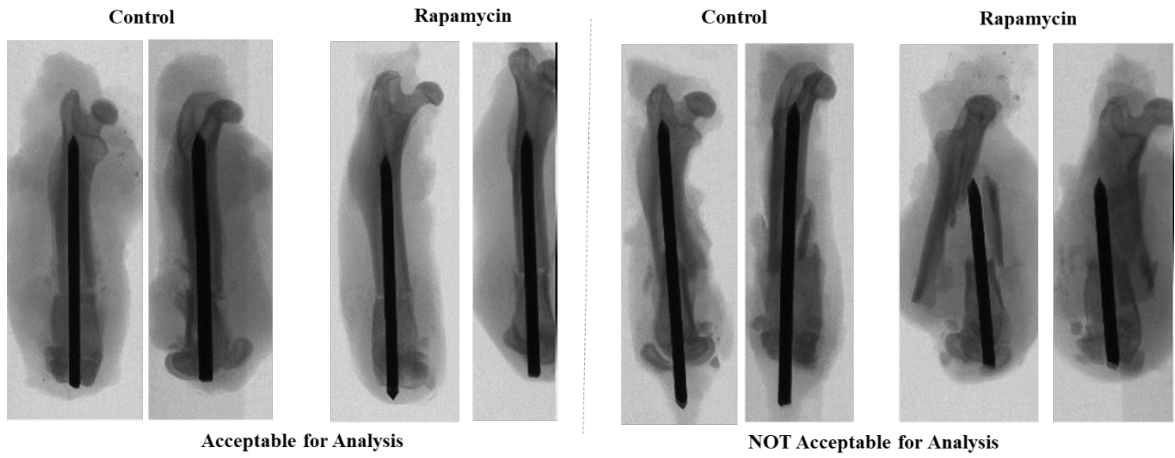
An open femoral osteotomy is performed and stabilized with a retrograde k-wire. Subjects are then randomized to treatment (rapamycin 2.5 mg/kg IP daily for 14 days) or control (3% DMSO 2.5 mg/kg IP daily for 14 days). Remaining subjects from cohorts of 30 were then euthanized as 2, 3, 6, or 8 weeks. Radiographic and biomechanical testing of healing fractures was then performed.

An open femoral osteotomy model was developed in order to produce consistent transverse fractures. As compared to a closed model of fracture, this has resulted in a more consistent fracture pattern that allows for comparison between treatment and control groups. The model is not without complication, however. Of the 116 rats randomized to control or treatment, 8 (6.9%) were euthanized due to wound complications and 20 (17.2%) were euthanized due to loss of fixation. An additional 10 rats (8.6%) were not included in the analysis due to inadequate fracture patterns discovered at the time of necropsy. Ultimately, 76 of 116 rats (65.5%) were included for final analysis. This limits the strength of our conclusions, however it should be noted that there was no significant difference in complications between treatment and control and therefore this represents a reflection of the model rather than the treatment effect.

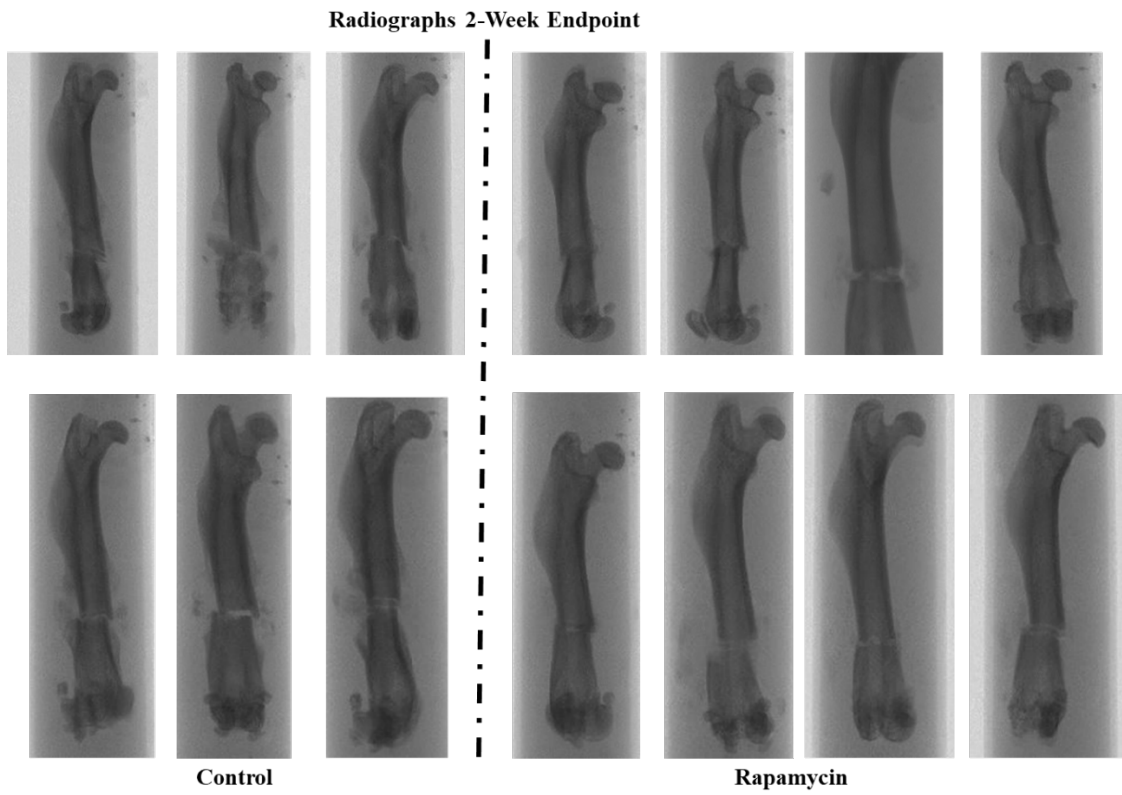
Study Design:



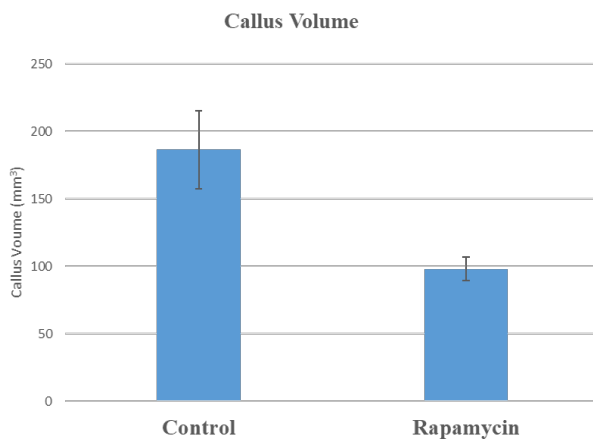
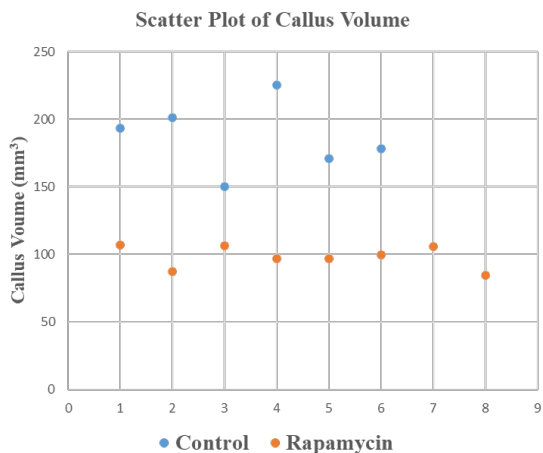
Fracture Patterns:



Results:



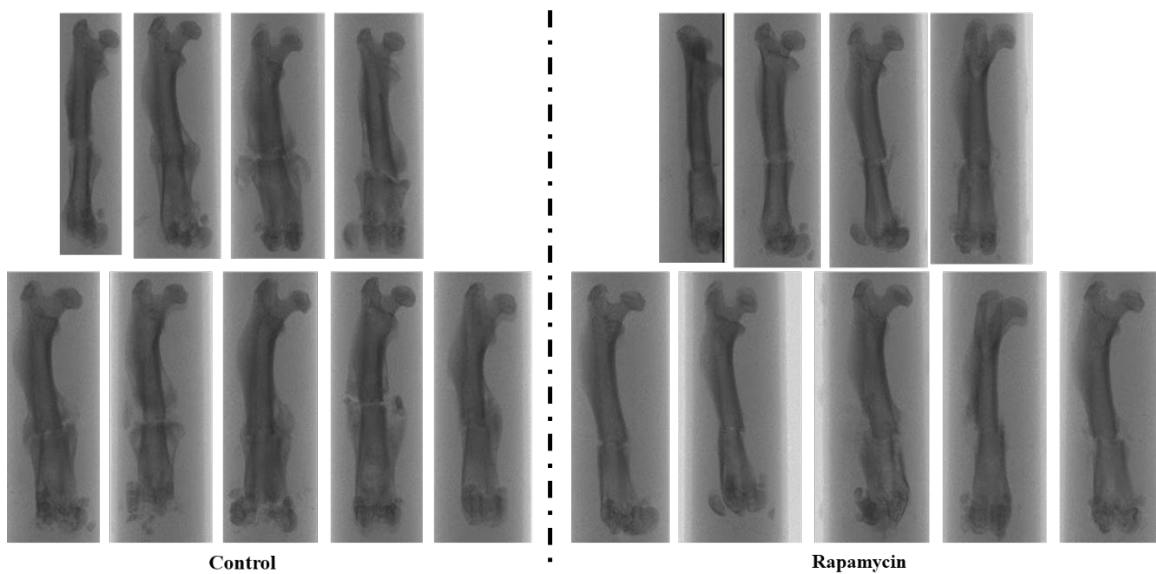
Callus Volume 2-Week Endpoint



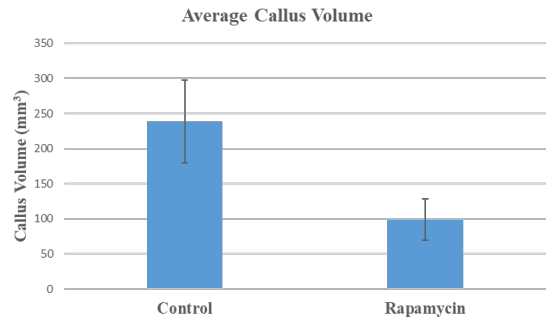
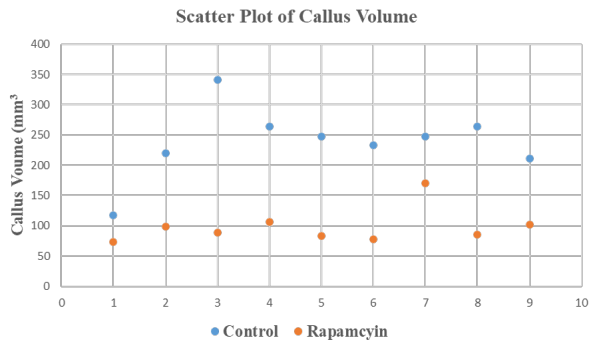
Due to complications (wound, loss of fixation, inadequate fracture pattern), only 6 from treatment and 8 from control were included for analysis

47.4% reduction, $p = 0.0002$

Radiographs 3-Week Endpoint



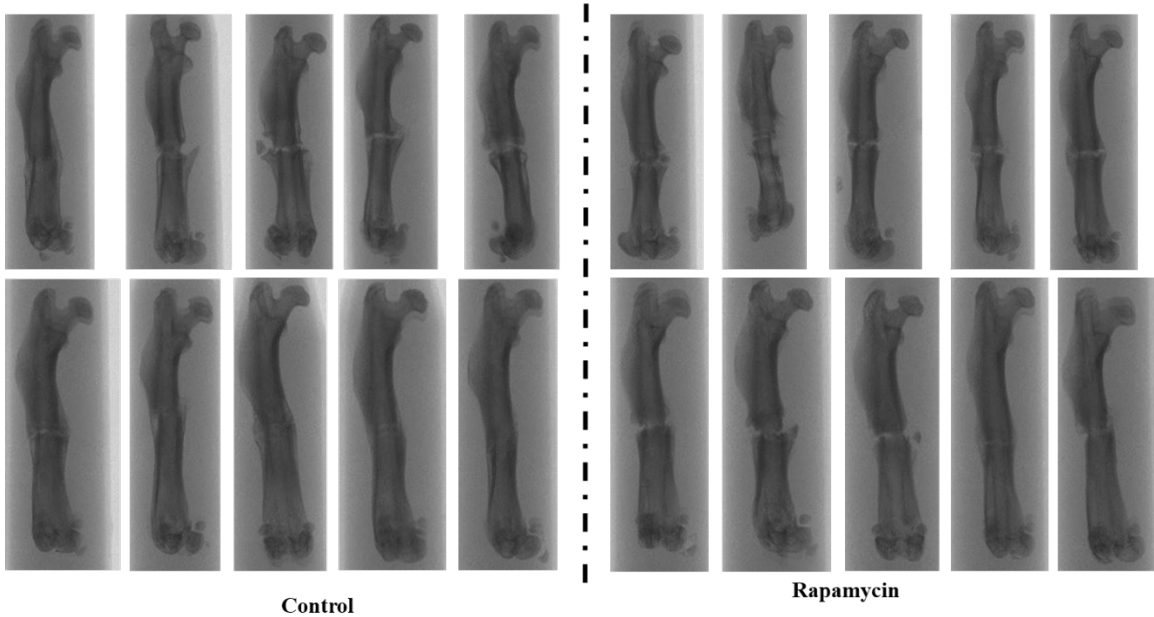
Callus Volume 3-Week Endpoint

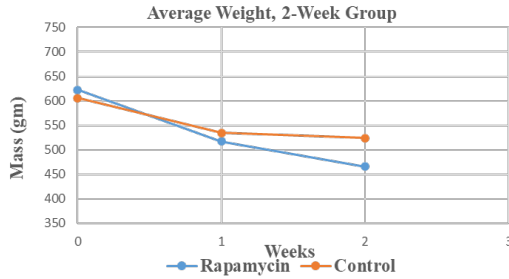


Due to complications (wound, loss of fixation, inadequate fracture pattern), only 9 from treatment and 9 from control were included for analysis

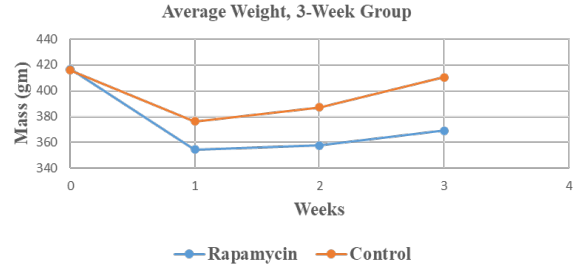
58.6% reduction, $p = 0.00004$

Radiographs 6-Week Endpoint

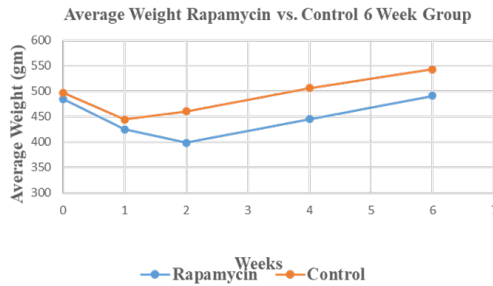




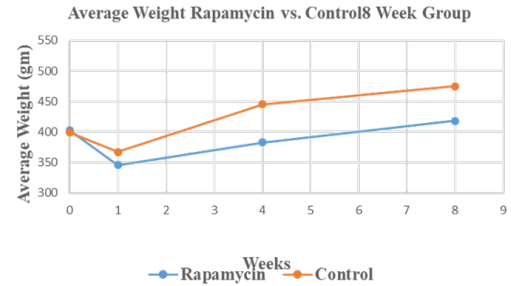
Av. Rapa 2-week weight loss = 25.1%; Av Control 2-week weight loss = 13.5%



Peak Weight Loss Rapa = 14.9%; Peak Weight Loss Control = 9.5%



6-Week group: Control group loses less weight than rapamycin treated group



8-Week group: Control group loses less weight than rapamycin treated group

Summary/Conclusions on fracture healing project.

- End points defined as week 2, 3, 6, & 8 to align with existing fracture healing literature.
- All surgeries for the rapamycin study completed.
- The method of analysis of callus volume has been optimized by utilizing segmentation to distinguish callus from higher density cortical bone and lower density muscle, air or soft tissue.
- Analysis of callus volume at the 2-week timepoint revealed a 47.4% reduction in callus volume (Average +/- SD, control = 186.4 +/- 28.9 mm³, rapamycin = 98.1 +/- 8.6 mm³, p=0.0002).
- Analysis of callus volume at the 3-week time revealed a 58.6% reduction in callus volume (Average +/- SD, control = 238.45 +/- 58.9 mm³, rapamycin = 98.66 +/- 29.23 mm³, p=0.00004).
- Analysis of callus volume at the 6-week time revealed no significant difference between groups. (Average +/- SD, control = 165.66 +/- 47.39 mm³, rapamycin = 154.97 +/- 29.07 mm³, p=0.55).
- At the 8-week time point, the previously described method of segmentation (point iii) could not distinguish between new callus and original cortical bone and thus preventing volumetric analysis to complete project aims.
- Specimens from the 2- and 3-week time point had only formed soft callus (as determined during intramedullary k-wire removal at the time of necropsy) and thus biomechanical testing cannot be performed as proposed in the project aims. Subsequent biomechanical testing for the 6- and 8-week groups was not completed.

Final reporting

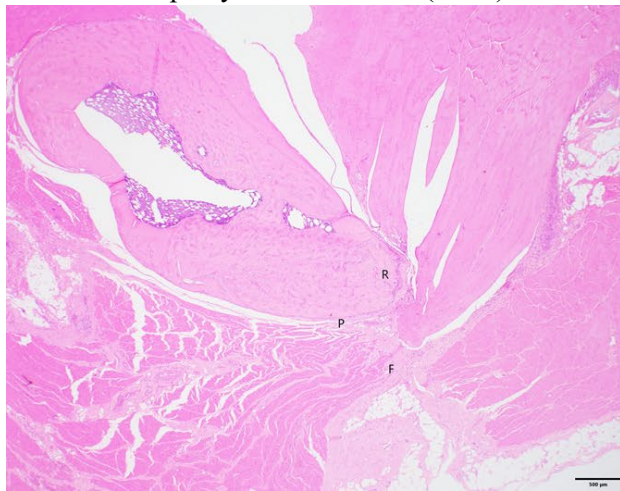
- a. Complication rates resulted in only 40-60% of subjects available for analysis
- b. Rapamycin caused differential weight loss which confounded results
- c. Unknown serum concentration of rapamycin
- d. Primarily soft callus only at 2- and 3-week time points, precluding use of 3 point bending test
- e. There remains unclear clinical significance of this data

- Histological and molecular assessment of early fracture healing/repair. (100% complete).

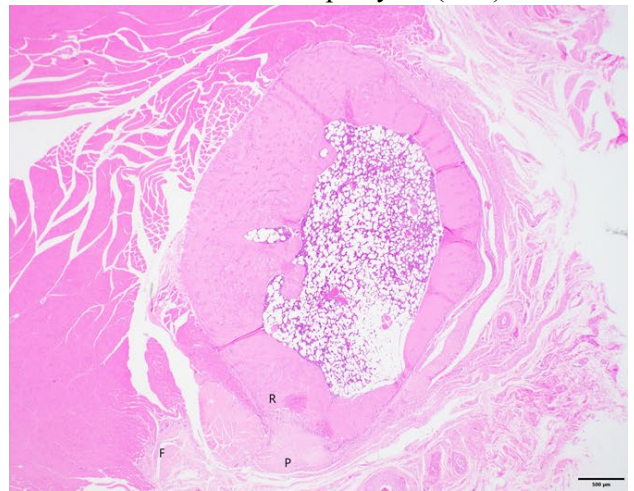
Histological assessment

Representative histological H&E-stained sections

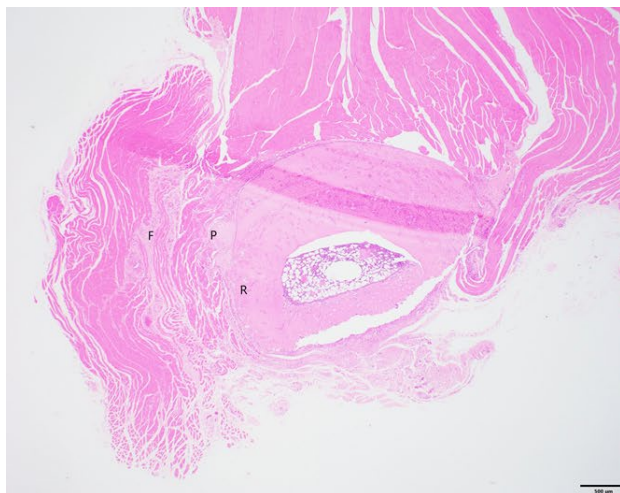
Rapamycin Palvarotene (RaPa)



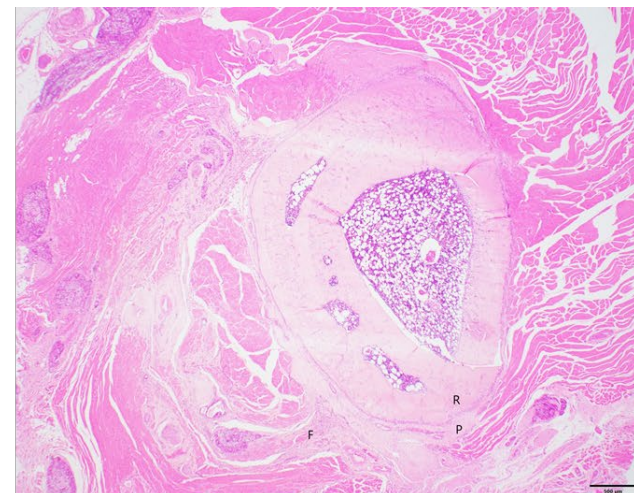
Palovarotene Rapamycin (PaR)



Palovarotene + Rapamycin (PR)



Injured control



Representative images of H&E stained histology slides. Transverse images are of the amputation sites of the injured limb. Three outcome measures were evaluated using a pathologist scoring system. R = bony remodeling, P = periosteal reaction, F = fibrosis in muscle. There was no significant difference between any of groups.

Table 1. Descriptions of the histological assessment site and type of H&E-stained cross sections of injured and contralateral limbs.

Bone, Injured Limb	Skeletal muscle/soft tissue, Injured Limb	Skeletal muscle, contralateral
Bone degeneration/necrosis	Skeletal muscle degeneration/necrosis	Infiltrate, mononuclear
Bone resorption/remodeling	Skeletal muscle inflammation mononuclear	Inflammation, mixed
Bone sequestra	Skeletal muscle inflammation PMN	Edema
Bone bacteria	Skeletal muscle bacteria	Hemorrhage
Bone marrow inflammation mononuclear	Skeletal muscle fibroplasia	Fibroplasia
Bone marrow inflammation PMN	Foreign body presence	Perivascular inflammation
Bone marrow bacteria	Periosseous necrosis	Myocyte degeneration/necrosis
Myelofibrosis	Soft tissue/muscle mineralization	Bacteria
Periosteum fibroplasia	Distal soft tissue necrosis	Mineral
Periosseous inflammation mononuclear		
Periosseous inflammation PMN		
Periosseous bacteria		
Periosteum osteoid		
Periosteum hyperchondrosis		
Periosteum hyperostosis		
Heterotopic bone		

Cross-sectional images were stained for H&E and evaluated by a veterinary pathologist at three different sites, injured bone, skeletal muscle/soft tissue, and contralateral skeletal muscles for multiple injury associated pathophysiological process, using a numerical grading system. A One-way ANOVA showed no statistically significant difference between the treatment groups and the injured control.

Conclusions:

Taken together, the histologic analysis demonstrates that there is no histologically significant effect of sequential or combined treatment of rapamycin and palovarotene on attenuating heterotopic bone formation, inflammation, or other osteo-chondro-associated pathophysiology.

Molecular assessment

Gene/ function	Rapamycin- Palovarotene	Palovarotene- Rapamycin	Palovarotene + Rapamycin
<i>Extracellular matrix synthesis</i>			
Acan	↓***	↓***	↓***
Col4a1	↓***	↓***	↓***
Comp	↓****	↓****	↓****
Ibsp	↓***	↓***	↓***
Sparc	NS	↓*	↓*
<i>Osteo chondro adiogenesis</i>			
Adipoq	↓*	↓**	↓**
AdipoR1	↓*	↓**	↓**
Alpl	↓****	↓***	↓****
Fabp4	↓****	↓****	↓****
Pdgfa	↓****	↓****	↓****
Pou5f	↓***	↓***	↓***
Smo	NS	NS	↓*
Tbx5	↓***	↓***	↓***
Bmp2	↓****	↓****	↓****
Bmp4	NS	↓*	↓*
Bmp6	↓**	↓**	↓**
Hdac1	↓****	↓****	↓****
Hif1a	↓****	↓****	↓****
Omd	↓*	↓**	↓**
Phex	↓**	↓**	↓**
Pparg	↓***	↓**	↓***
Ptch1	↓****	↓****	↓****
Smurf1	↓****	↓***	↓****
Smurf2	↓****	↓****	↓****
Sox2	↓***	↓***	↓***
Sp7	↓****	↓****	↓****
Sp1	NS	↓*	↓*
Tert	↓*	NS	↓*
Twist1	↓**	↓**	↓***
<i>Angiogenesis</i>			
Angpt2	NS	↓*	↓*
Fgf1	↓*	NS	↓**
Fgf2	↓****	↓****	↓****
Gli2	↓*	NS	↓*
Hif1a	↓****	↓****	↓****
Kdr	↓****	↓****	↓****

Sox2	↓***	↓***	↓***
Vegfa	↓****	↓****	↓****
<i>Inflammation</i>			
C-001	↓***	↓****	↓****
Cd44	NS	NS	↓*
CSf3	↓**	↓***	↓***
Cxcl12	↓***	↓****	↓****
Cxcl5	↓****	↓****	↓****
Eng	↓***	↓**	↓***
Has1	↓**	↓**	↓**
Has2	↓**	↓*	↓**
Hat1	↓***	↓***	↓***
Hdac1	↓****	↓****	↓****
Hnfla	↓**	↓**	↓**
Igf2	↓**	↓*	↓**
IL10	↓*	↓*	↓**
IL1b	↓****	↓****	↓****
IL6	↓**	↓**	↓**
Itga1	↓**	↓**	↓***
Itga2	NS	NS	↓*
Itgax	NS	NS	↓*
Itgev	NS	↓*	↓*
Jag1	↓****	↓****	↓****
MMP9	NS	↓*	↓*
Notch1	↓**	↓**	↓**
Ptk2	↓**	↓**	↓**
Rhoa	↓****	↓****	↓****
Runx2	↓***	↓****	↓****
Scarb1	↓*	↓*	↓*
Sox2	↓***	↓***	↓***
Sp1	NS	↓*	↓*
Sp7	↓****	↓****	↓****
Sparc	NS	↓*	↓*
Tgfb1	↓***	↓***	↓***
Tgfb3	↓***	↓***	↓***
Tnf	↓*	↓*	↓**

Table 1.

RT-PCR gene array analysis. Animals were treated sequentially with rapamycin (0.1mL IP)-palovarotene (0.1mL oral gavage), palovarotene-Rapamycin, or a combination of rapamycin and palovarotene. Control animals did not receive any treatment. 20ug of RNA extracted from injured skeletal muscle was assayed using a Bio-Rad 384 gene panel. A one-way ANOVA

followed by Dunnett's multiple comparisons test was conducted to compare the three treatment groups to untreated injured controls. NS= non-significant, * p<.05, **p<.01, ***p<.001, ****p<.0001. ↓= downregulated.

Molecular assessment of injured skeletal muscle tissue proximal to the amputation site from the treated and untreated control rats was assessed for expression levels of mRNA transcripts for 83 genes. The genes included extracellular matrix (ECM) synthesis, adipogenesis, angiogenesis, osteogenesis, injury and inflammation.

Extracellular matrix synthesis

Gene expression for ECM synthesis transcripts was significantly downregulated in all groups for Can, Col10a, Col2a, Col4a1, Comp, Ibsp. Interestingly, compared to the sequential Rapamycin/Palovarotene (RaPa) group, Sparc was decreased only in the sequential Palovarotene/Rapamycin (PaR) and combinatorial Palovarotene and Rapamycin (PR) treatment groups.

Osteo-chondro-adipogenesis

This trend was also observed in the osteo-chondro-adipogenesis gene set with significantly decreased expression of BMP4 and SP1 in the PaR and PR groups but not the RaPa. The expression level of Smo was significantly lower in the RaPa or PaR groups but was in the PR. Tert was significantly decreased in the RaPa and PR but not the PaR group. Osteo-chondro-adipogenesis associated genes, Adipoq, AdipoR1, Alpl, Bmp2, Bmp6 Fabp4, Hdac1, Hif1a, Omd, Pdgfa, Phex, Pou5f, Pparg, Ptch1, Smurf1, Smurf2, Sox2, Sox2, Sp7, Tbx5 and Twist1 were significantly downregulated in all groups.

Angiogenesis

Angiogenic mRNA transcripts, Fgf2, Hif1a, Kdr, Sox2, and Vegfa, were downregulated in all groups. Angpt2 was downregulated in the PaR and PR group but not the Rapa, while Fgf1 and Gli2 were downregulated in the RaPa and PR groups.

Inflammation

The assayed inflammation-associated genes were downregulated in all treatment groups compared to untreated controls, except Cd44, Itga2, Itgax, Itgev, MMP9, Sp1, and Sparc. In particular, Cd44, Itga2, and Itgax were downregulated in the PR group, and Itgev, MMP9, Sp1 and Sparc were downregulated in the RaPa group.

Conclusions

Collectively, the data demonstrate a trend for suppression of expression of genes associated with extracellular matrix remodeling, osteogenesis, chondrogenesis, angiogenesis and inflammation following sequential treatment with rapamycin and Palovarotene or a combination of the two drugs. In the absence of a radiographic assessment of heterotopic ossification at the amputation site, we are unable to draw conclusions regarding the relationship between the downregulated genes and the development of HO.

Milestone 4 – Completion of *in vivo* studies assessing the effects of therapeutic strategies on fracture healing. Data covering these findings published in peer-reviewed journals as follows:

- Wheatley BM, Cilwa KE, Dey D, Qureshi AT, Seavey JG, Tomasino AM, Sanders EM, Bova W, Boehm CA, Iwamoto M, Potter BK, Forsberg JA, Muschler GF, Davis TA. Palovarotene inhibits connective tissue progenitor cell proliferation in a rat model of combat-related heterotopic ossification. *J Orthop Res.* 2018 Apr;36(4):1135-1144. doi: 10.1002/jor.23747. Epub 2017 Nov 16. PMID: 28960501.
- Pavey GJ, Qureshi AT, Tomasino AM, Honnold CL, Bishop DK, Agarwal S, Loder S, Levi B, Pacifici M, Iwamoto M, Potter BK, Davis TA, Forsberg JA. Targeted stimulation of retinoic acid receptor- γ mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. *Bone.* 2016 Sep;90:159-67. doi: 10.1016/j.bone.2016.06.014. Epub 2016 Jun 28. PMID: 27368930; PMCID: PMC5546218.

What opportunities for training and professional development has the project provided?

Andrew Yurko: Research Associate, designed and executed some phases of the cell culture study. Developed cell culture skills and troubleshooting maintenance of cell lines.

Sohaib Alvi: Research Assistant, helped in the design and monitored cell culture during some phases of the study. Study provided opportunity to learn cell culture and assist in project planning.

Keegan Loveless: Research assistant mentored during the course of the project. Learned surgical techniques and animal monitoring.

Babita Parajuli: Research assistant mentored during the course of the project. Developed critical skills for study planning, execution of surgical procedures, and performed analysis of harvested specimens.

Peter Leung: Research assistant mentored during the course of the project. Developed critical skills for study planning, execution of surgical procedures, and performed analysis of harvested specimens.

Crystal Card: Research assistant mentored during the course of the project. Developed critical skills for study planning, execution of surgical procedures, and performed analysis of harvested specimens.

Patrick Grimm, MD: Orthopaedic surgery resident who designed and executed fracture healing study

Aaron Brown: Research assistant mentored during the course of the project. Developed critical skills for study planning, execution of surgical procedures, and performed analysis of harvested specimens.

James Carr: Research assistant mentored during the course of the project. Developed critical skills for study planning, execution of surgical procedures, and performed analysis of harvested specimens.

Ryan Haskins, PhD: Technical scientific oversight of the project and laboratory studies

Michael Shaughness, PhD: Executed the gene expression and histological analysis for specific aim 3.

How were the results disseminated to communities of interest?

Results were disseminated to communities of interest by publication of results in multiple peer-review journals and presentations at multiple conferences and meetings as described in the body of the reporting above.

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

Nothing to report.

4. IMPACT:

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

Early results suggest that rapamycin may inhibit early fracture healing which should be considered if further efforts are made to utilize rapamycin as a prophylactic agent for heterotopic ossification, particularly in the setting of the multiply injured combat patient. Furthermore, given the significant complication rate, mostly due to loss of fixation, future fracture models should consider alternate methods such as the use of threaded or partially threaded k-wire, or a radiolucent external fixation system for fracture stabilization.

What was the impact on other disciplines?

The potential increased risk of delayed fracture healing should be considered for transplant patients using rapamycin in their immunosuppressant regimen.

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

During the final year of this project, the MicroCT, an essential piece of equipment for analysis was deemed no longer functional so completion of the in-vivo aims was not possible. The remaining work performed was to conclude the histologic and genetic analysis outlined in the project aims to close out the project.

Actual or anticipated problems or delays and actions or plans to resolve them

- Fall 2017: Work-site construction delayed scheduling surgeries which require the use of a blast-tube to induce the injuries in our full trauma-model.
- Summer 2018: Critical parts for biomechanical testing were backordered and arrived August 2018. Additionally, worksite construction causing intermittent access to the blast tube necessary for performing surgeries for in vivo studies.
- We found significantly less attenuation than anticipated for both of our combinational strategies with Rapamycin and Palovarotene. We believe this effect is due to inferior quality of drug from a new distributor and not a synergistic effect of treatment. Testing empirically with Rapamycin purchased from original distributor utilized in single drug treatment experiments resulted in significant delays.
- Performing surgeries for the fracture healing studies: progress was delayed due to method development. We are now using an open fracture model and have completed three of five designated time points.
- Osteogenic Media shortage: Supplier of our osteogenic media ran out and to endure long-term backorder. Lonza is the only distributor of our media. Due to the shortage, they recommended researchers to find different media to use, required initial testing/experiments assessment and formulation of an in house option for media.
- March 2020: During the COVID-19 Pandemic Animal work on Non-COVID-19 related studies were halted or drastically restricted until November 2020 when it was reinstated in limited quantities with significant restrictions on manpower regulations on base/in the lab. The work progress was substantially slowed and was not able to be fully resumed until April 2021.

- October 2020: The micro CT scanner which is an essential equipment for this project is non-functional and was ultimately deemed unable to be repaired in March 2021.

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:

Publications, conference papers, and presentations

Journal publications.

Peer-reviewed journal publications:

- Pavey, GJ. et al, 2016. Targeted stimulation of retinoic acid receptor- γ mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. *Bone* 90: 159-167
- Qureshi et al., 2017. Inhibition of mammalian target of rapamycin signaling with rapamycin prevents trauma-induced heterotopic ossification. *Am J Pathol.* 187: 2536-45.
- Wheatley, BM et al., 2018. Palovarotene inhibits connective tissue progenitor cell proliferation in a rat model of combat-related heterotopic ossification. *J Orthop Res* 36:1135–1144.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Presentations:

- Qureshi, AT et al, Palovarotene treatment suppresses osteogenic cell activity and ectopic bone formation in a trauma-induced rodent model of heterotopic ossification. Poster. Military Health Service Research Symposium (MHSRS) Kissimmee, FL, 2 August 2017.
- Wheatley et al., 2017 Palovarotene Inhibits Connective Tissue Progenitor Cell Proliferation in a Rat Model of Combat-Related Heterotopic Ossification. Oral Presentation. Society of Military Orthopaedic Surgeons (SOMOS) 59th Annual Meeting, Scottsdale, AZ, 12 December 20170

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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: LCDR Carolyn Gosztyla, MD
Project Role: PI
Nearest Person month worked: 7
Contribution to the Project: New PI. Assumed responsibility for closeout of this project and all final reporting.

Name: Stephen Kaba
Project Role: Senior Scientist
Nearest Person month worked: 8
Contribution to the Project: No change

Name: Andrew Yurko
Project Role: Surgical and Laboratory Research Assistant
Nearest Person month worked: 8
Contribution to the Project: Assist with animal manipulation, monitoring and sample collection & analysis

Name: Crystal Leonhardt
Project Role: Surgical and Laboratory Research Coordinator
Nearest Person month worked: 8
Contribution to the Project: No change

Name: Michael Shaughness
Project Role: Post-doctoral research fellow
Nearest Person month worked: 2
Contribution to the Project: Completed gene analysis and histology analysis for Aim 3

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Sequential versus Combined Medical Therapies as a Novel Heterotopic Ossification Prevention Strategy Following Blast-Related Extremity Injury

Log Number: OR150080

Award Number: W81XWH-15-PRORP-ARA

PI: LCDR Carolyn Gosztyla

Org: Naval Medical Research Center

Award Amount: \$500,000

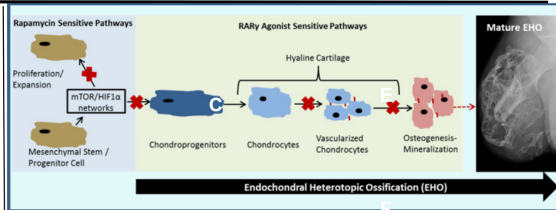


Study Aims

- Specific Aim 1:** Characterize the *in vitro* effect of Palovarotene and Rapamycin on osteogenic and angiogenic differentiation of mesenchymal cells. (1-8 months)
- Specific Aim 2:** Investigate the effects of multiple drugs combinations in preventing endochondral heterotopic ossification formation in blast-related extremity injury model of endochondral heterotopic ossification (EHO). (6 -24 months).

Approach

Our central hypothesis is that an early multi-treatment modality that targets multiple complex and intrinsic molecular signaling pathways deemed important for osteogenic and angiogenic progenitor cell recruitment, proliferation, growth and differentiation within a permissive hypoxic microenvironment would be a more effective strategy in preventing EHO formation. We will assimilate the findings of two defined and proven EHO intervention strategies developed in our lab with progress towards complete inhibition of EHO by targeting both Palovarotene and Rapamycin sensitive pathways involved in the recruitment of progenitor cells and endochondral ossification. More specifically, we will evaluate timing as well as concurrent and sequential treatment strategies that target the regulation of key micro-environmental molecular signaling components/inductive factors that are critical in the development of the extra skeletal bone tissue formation in damaged/traumatized ischemic tissue.



Accomplishments: IACUC and ACURO approval. Development and characterization of rMSC cell lines for *in vitro* cytotoxicity assay testing.

Goals/Milestones

- Q4 FY16** – IACUC protocol submission and *in vitro* testing of Palovarotene and Rapamycin
- IACUC protocol renewed (June 2021)
- Run cytotoxicity assays for drugs-single agents and combinations (100%)
- Run *in vitro* differentiation assays to potency of test agents on inhibition of MSC-derived chondrogenesis, osteogenesis and angiogenesis (dose & timing) (100%)
- FY17** - Assessment of therapeutic strategies in a blast-related model of EHO
- Characterize sequential versus combined treatments of chondrogenic/osteogenic inhibitors (dose and timing) (100%)
- Correlate radiographic data with histopathology (quantitative and qualitative assessment of ectopic bone inhibition) (100%)
- Assess drug therapy effects on normal fracture healing (80%)
- FY18 - Q3 FY19** – In depth cellular and molecular assessment of EHO inhibition
- Early chondrogenic, angiogenic and osteogenic gene transcript analysis (100%)
- Quantitate the *in vivo* effects of inhibitor treatment on tissue-derived osteogenic progenitor cells (80%)
- Final data analysis and manuscript submission (50%)

Comments/Challenges/Issues/Concerns:

Equipment (microCT scanner) problem has been diagnosed and will not be able to be utilized. Xray will be used for analysis. Last phase of project, animal models, will start soon.

Budget Expenditure to Date
Projected Expenditure: \$500,000

Timeline and Cost

Activities	FY 16	17	18	19/20/21/22
IACUC approval & in vitro cytotoxicity of inhibitors		█		
Effects of inhibitors MSC differentiation		█		
Assess inhibitors in Rat blast model		█	█	█
Data analysis, manuscript submission		█	█	█
Estimated Budget		\$239,312	\$260,688	

Updated: SEP 29, 2022

9. APPENDICES: