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Targeted Stimulation of Retinoic Acid Receptor- γ Mitigates the Formation of Heterotopic Ossification in an Established Blast-Related Traumatic Injury Model

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Abstract

Heterotopic ossification (HO) involves formation of endochondral bone at non-skeletal sites, is prevalent in severely wounded service members, and causes significant complications and delayed rehabilitation. As common prophylactic treatments such as anti-inflammatory drugs and irradiation cannot be used after multi-system combat trauma, there is an urgent need for new remedies. Previously, we showed that the retinoic acid receptor γ agonist Palovarotene inhibited subcutaneous and intramuscular HO in mice, but those models do not mimic complex combat injury. Thus, we tested Palovarotene in our validated rat trauma-induced HO model that involves blast-related limb injury, femoral fracture, quadriceps crush injury, amputation and infection with methicillin-resistant *Staphylococcus aureus* from combat wound infections. Palovarotene was given orally for 14 days at 1 mg/kg/day starting on post-operative day (POD) 1 or POD-5, and HO amount, wound dehiscence and related processes were monitored for up to 84 days post injury. Compared to vehicle-control animals, Palovarotene significantly decreased HO by 50 to 60%

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Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research. This work performed at the Naval Medical Research Center, Silver Spring, MD, USA.

Conflict of interest

The other authors have no financial or personal conflicts of interest to disclose.

regardless of when the treatment started and if infection was present. Histological analyses showed that Palovarotene reduced ectopic chondrogenesis, osteogenesis and angiogenesis forming at the injury site over time, while fibrotic tissue was often present in place of ectopic bone. Custom gene array data verified that while expression of key chondrogenic and osteogenic genes was decreased within soft tissues of residual limb in Palovarotene-treated rats, expression of cartilage catabolic genes was increased, including matrix metalloproteinase-9. Importantly, Palovarotene seemed to exert moderate inhibitory effects on wound healing, raising potential safety concerns related to dosing and timing. Our data show for the first time that Palovarotene significantly inhibits HO triggered by blast injury and associated complications, strongly indicating that it may prevent HO in patients at high risk such as those sustaining combat injuries and other forms of blast trauma.

Keywords

Heterotopic ossification; prophylaxis; animal model; retinoic acid receptor- γ ; agonist; endochondral ossification; traumatic extremity injury; blast overpressure exposure; bioburden

1. Introduction

Heterotopic ossification (HO) consists of ectopic bone formation within non-skeletal tissues and sites and can be classified as genetic, neurogenic or traumatic in origin [1–5]. Post-traumatic HO is most commonly encountered following high-energy trauma, spinal cord and brain injury, severe burns, [6, 7] and in orthopaedic practice following hip or elbow surgery [8, 9]. Recent studies have revealed that HO is actually most prevalent in severe combat-related extremity wounds and within residual limbs after combat-related amputations [3, 10–12]. Blast and other severe polytraumatic injuries elicit complex and often dysregulated inflammatory and physiological responses, and the pathological interactions amongst diverse tissues, cell types, inflammatory mediators and local and systemic signaling pathways provide fertile ground for HO development. The clinical complications of HO include activity-limiting pain, primary (ankylosis) and secondary (muscle-tendon tethering) joint stiffness, skin ulceration and poor tolerance of prosthesis wear, wherein conservative interventions such as multi-modal pain regimens, physiotherapy and prosthetic modification fail to alleviate symptoms in up to 41% of patients [10]. Surgical excision of HO, although the standard of care, often fails to relieve the underlying joint contractures [13], can be fraught with complications and, even when successful, can further delay rehabilitation because of its invasive nature; in some cases, it can even lead to recurrent HO [3, 11, 14]. Surgical excision is also unable to address the chronic symptoms associated with HO including joint contracture, chronic pain, and non-healing wounds. In addition, current prophylaxis strategies used in the general population -such as administration of non-steroidal anti-inflammatory drugs (NSAIDs) and external beam radiotherapy- are generally contraindicated in the setting of multi-system combat trauma because they have deleterious systemic and local side effects and may not be logistically feasible in forward deployed war settings [15]. Furthermore, it is not clear how effective these strategies actually are given that penetrance and severity of HO in the general population vary significantly. Thus, it would be highly desirable to have a simple, effective and safe prophylactic pharmacologic therapy that could be easily administered systemically post-injury, would avoid adverse complications

and outcomes, and would minimize treatment duration. Such treatment would have immense impact on the burden of HO disease and resulting pathogenic sequelae, and the search for such novel and non-invasive means of primary HO prevention continues to attract substantial interest and effort.

Heterotopic ossification, when triggered by traumatic injury or other major insults most often arises via endochondral ossification, one of the two essential processes by which the native skeleton forms and grows during embryogenesis and early postnatal life [16–19]. Skeletal elements including long bones, ribs and vertebrae are initially laid down as entirely cartilaginous structures in early embryogenesis. The cartilaginous tissue then undergoes a process of maturation and hypertrophy, and the resulting hypertrophic cartilage is invaded by vascular and osteopotent cells and is replaced by endochondral bone and marrow. This complex series of events and differentiation steps is governed by equally complex mechanisms that include local and systemic hormones and cytokines, transcription factors and multiple signaling proteins including members of the bone morphogenetic protein (BMP) family. Experimental prophylactic therapies targeting the BMP signaling pathway and its regulatory networks have shown some degree of efficacy against HO in murine models [20, 21]. However, it is not known how effective and safe these treatments would be in patients and in particular those suffering from combat injuries and whether they would have unwanted side effects because of the multiple roles BMPs and BMP signaling have in body physiology and function.

The retinoid signaling pathway and nuclear retinoic acid receptors (RARs) are important regulators of skeletal development and growth [22]. In particular, chondrogenesis was shown to require a steep decrease in endogenous retinoid action during skeletogenesis [23], and exogenous retinoid agonists were in fact found to strongly inhibit chondrogenesis and skeletal development [22, 24]. Because of their ability to block condensation of skeletal progenitor cells and their differentiation into chondrocytes that represent the initial stages of HO, we previously asked whether synthetic retinoid agonists could block HO. We used mouse models of skeletogenic protein- or genetic mutation-induced HO and found that the RAR- γ agonist Palovarotene was amongst the most potent anti-HO agents [25, 26]. Analysis of mechanisms showed that this and other retinoids did, in fact, act by significantly decreasing chondrogenesis and in turn osteogenesis and vascularization. At the cellular level, the drugs dampened canonical BMP signaling -an effect involving ubiquitination and proteasome degradation of Smad proteins- and concurrently increased Wnt β -catenin signaling, a potent endogenous inhibitor of chondrogenesis [27, 28]. While the above studies are quite promising, it remains unclear whether a retinoid-based strategy would be equally effective in the far more complex setting of HO developing after combat injuries in which the affected site is usually large, the wounds are extremely severe, multiple tissues are damaged, and bacterial infections are common.

We recently developed a rat model that incorporates the multiple pathogenic insults sustained by combat casualties with severe orthopaedic extremity injuries which we believe is significantly more representative than non-physiologic models of excess BMP ligand administration or genetic mutations. These insults include exposure to blast overpressure, femur fracture, quadriceps crush injury followed by limb amputation through the zone of

injury, and finally inoculation of the myodesis with a highly virulent isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) obtained from combat wound infections [13, 29]. Previously, we reported on the timing and kinetics of ectopic bone development in this trauma induced HO model. Using molecular and histological tools, we found that chondrogenesis became apparent approximately 3 days following injury and was subsequently followed by cartilage hypertrophy and endochondral bone formation within soft tissues surrounding the amputation site starting around post-injury day 10 [16]. Understanding the timing of these key developmental phases and events was quite important as it now provides a window for prophylactic administration of candidate therapies that could target mechanisms and pathways involved in endochondral ossification. Herein, we sought to determine whether and how Palovarotene may be able to inhibit trauma-induced endochondral HO formation in our rat blast model. To evaluate possible unwanted side effects, we monitored and assessed wound healing as well.

2. Materials and Methods

2.1 Animals

A total of 110 young adult pathogen-free male Sprague Dawley rats (*Rattus norvegicus*; 400–600 g) were purchased from Taconic Farms (Germantown, NY). All animals were housed individually in plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow) and fresh water ad libitum. They were acclimated for at least one week before experimentation. Prior to experimentation, animals were randomized into experimental and control groups. The study protocol (12-OUMD-20S) was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee in compliance with all applicable Federal regulations governing the protection of animals in research.

2.2 Rat HO model and surgical procedures

Exposure to blast and trauma-induced injury was conducted as previously described [12, 22]. Briefly, 106 rats were anesthetized with isoflurane (2–4%) and were exposed to blast overpressure (120 ± 7 kPa) via a pneumatically-driven shock tube. They sustained a controlled femoral fracture followed by a one minute soft tissue crush injury, limb amputation through the zone of injury and inoculation of the local soft tissue with 1×10^6 CFU MRSA. Appropriate débridement of devitalized tissue was performed followed by hamstring and quadriceps myoplasty over the exposed residual femur. The incision was closed with 2 interrupted deep subcutaneous 3-0 Vicryl (Ethicon, Somerville, NJ, USA) sutures and a running 4-0 subcuticular Monocryl (Ethicon, Somerville, NJ, USA). The incision was then covered in Vetbond (3M Animal Care Products, St Paul, MN, USA). Post-surgically, sustained release Buprenorphine (1.2mg/kg) was administered subcutaneously on day of surgery and again on post-operative day 3 for peri-operative pain control. Rats received via oral gavage (100 μ l) either Palovarotene (1.0 mg/kg) or vehicle as control (5% DMSO in corn oil) prepared as previously described [25] every other day for 14 days, starting at postoperative day 1 (POD-1) or POD-5. Rats were euthanized at indicated time points post-injury for *ex vivo* end point analysis by micro-computed CT (μ CT), histology and RT-PCR gene transcript expression.

2.3 Experimental study groups

Study 1: To determine the efficacy of Palovarotene in inhibiting ectopic bone formation, 66 rats were randomized into 1 of 8 treatment groups to monitor the development of HO formation longitudinally and non-invasively by μ CT at 28, 56 and 84 days after injury. All rats were subjected to polytraumatic extremity injury $\pm$ MRSA infection. Group-1 received MRSA + Palovarotene treatment starting at POD-1 (n=8); Group-2 received MRSA + vehicle control starting at POD-1 (n=11); Group-3 received MRSA + Palovarotene treatment starting at POD-5 (n=8); Group-4 received MRSA + vehicle control starting at POD-5 (n=5); Group-5 received Palovarotene treatment starting at POD-1 (n=10); Group-6 received vehicle control starting at POD-1 (n=6); Group-7 received Palovarotene treatment starting at POD-5 (n=10) and Group-8 received vehicle control starting at POD-5 (n=8). Four rats were sacrificed early, two rats each in Group-5 and Group-7 for losing >15% of their body weight, leaving a total of 62 in the observational study (Fig. 1A).

Study 2: In a follow-on study, an additional 40 blast trauma and extremity injured rats with MRSA contaminated wounds were treated with either Palovarotene or vehicle control beginning on POD-1 (Groups 1–2). Rats from each group were randomly euthanized at days 5, 7, 10, 15, and 28 days post injury (n=4 rats per time point/treatment group) (Figure 1B). Soft tissue surrounding but not attached the distal portion of the residual femur was collected for histological and gene expression analysis. Four rats served as sham-treated, uninjured naïve rats to obtain healthy skeletal muscle tissue for gene expression analysis.

2.3 Micro-computed tomography imaging

Progression of ectopic bone development at the site of amputation was tracked by imaging the amputated limb on days 28, 56 and 84 post injury using an *in vivo* high-resolution micro-computed tomography (μ -CT) system (SkyScan 1176; Bruker-MicroCT, Kontich, Belgium) and a standard phantom for normalization. As previously described [13], after the rats were scanned at the mentioned time points, the scanned images from Skyscan software were rendered using NRecon Reconstructions software (Bruker-MicroCT, Kontich, Belgium) to obtain serial flattened longitudinal micro-CT slices. An investigator reviewed all these serial longitudinal micro-CT flattened images at every time point, narrowed the region that encompassed the residual femur and ectopic bone and selected regions of interest (ROI) on every fifth slice to capture all new bone (new bone associated and non-associated with the cortical margins) as well as “soft tissue ectopic bone” (ectopic bone with no association to the cortical bone margins). The three-dimensional image analysis was performed of all the flattened images with ROI yielding a volumetric measurement of total new ectopic bone (Figure 2A) and “soft-tissue” ectopic bone (Figure 2B) based on the selected area of interest.

2.4 Histological analysis

For histological examination, cohorts of rats at the specified time points were euthanized (Fatal Plus 50 mg/kg IP; Patterson Veterinary, Devens, MA) and disarticulation of the hip was performed to collect the residual limb, which was washed with saline thoroughly then

fixed in 10% neutral buffered formalin and subsequently decalcified in 5% formic acid. After complete decalcification, tissues were embedded in paraffin wax, cut into serial 5µm thick longitudinal sections using a microtome and mounted onto glass slides for histology. Sections were used for immunohistochemistry or stained using hematoxylin-eosin (H&E) (Histoserv, Inc., Germantown, MD)[16].

2.5 RNA isolation and gene expression

At indicated time points, skeletal muscle tissue collected from the extremity wound, specifically surrounding the amputation site (not set against the bone), and the corresponding distal quadriceps muscle of the contralateral limb were harvested and stored in RNeasy Lysis Buffer (Qiagen, Crawley, UK) at 4°C. Skeletal muscle samples obtained from age-matched naïve uninjured rats (n=4) served as control tissue. Total RNA was isolated and purified muscle cell lysates using RNeasy columns and DNase-I kits (Qiagen, Crawley, UK) according to the manufacturer's protocols. RNA was stored at -80°C in nuclease-free water (Bio-Rad, Hercules, CA, USA). Total RNA was quantified spectroscopically by using NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA) and RNA integrity/quality was assessed by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to convert 1 µg of RNA to cDNA. mRNA transcripts for 83 key genes involved in early chondrogenic, osteogenic and angiogenic signaling pathways (see Supplementary Table 1 for the complete list of genes and their function) were examined by real-time PCR using a custom low density microarray (Bio-Rad Laboratories, Hercules, CA) [16]. Relative gene transcript expression was calculated using the 2^{-C_t} method and normalized and compared to the gene expression levels of non-injured rat muscle tissue. Gene transcripts values that were significantly differentially expressed between treatment groups are reported.

2.6 Assessment of wound healing

Animals were observed daily by veterinary and research staff for clinical evidence of wound complications to include purulent infection, physiological distress, and superficial (not past the dermis, treated with observation) wound or frank (deep, treated with debridement and secondary surgical closure) dehiscence. We specifically quantified negative effects on wound healing by comparing the proportion of wounds that were not completely healed at 14 days post-injury with Palovarotene *versus* vehicle control and treatment timing, with and without MRSA inoculation. Wounds which required repeat surgery were considered to have delayed healing at 14 days even if the suture line was intact at this time point; however, superficially dehiscent wounds were considered either healed or delayed based upon the presence or absence of complete epithelialization at 14 days.

2.7 Statistical analyses

Non-continuous gene expression data was analyzed with the Mann-Whitney U-test and two-tailed $\alpha < 0.05$ was considered statistically significant. The reliability of the volumetric measurement of ectopic bone by µCT images was assessed using the Intraclass correlation coefficient (ICC). The Welch's two Sample t-test and Fisher exact test were used to determine the statistical differences in ectopic bone volume (mm³) and by correlating the

incidence of postoperative wound dehiscence between the vehicle control and Palovarotene treated rats, respectively, with a statistical significance level of $p < 0.05$. All data are presented as mean $\pm$ the standard error of the mean (SEM) unless otherwise specified.

3. Results

3.1 Palovarotene mitigates post traumatic ectopic bone formation

To test the effectiveness of Palovarotene against blast-induced HO, rats were subjected to whole body blast exposure, femur fracture and crush injury that were followed by amputation of affected limb site and inoculation (or not) of local MRSA. Rats were then administered the drug or vehicle starting every other day for 14 days starting on either postoperative day 1 (POD-1) or POD-5 and were then monitored up to 84 days post-injury. Volumetric assessment of bone formation using μ CT demonstrated significant difference with respect to total new bone volume at the distal end plus ectopic bone outside of cortical margins (Fig. 2A) and soft-tissue ectopic bone volume (Fig. 2B) at 84 days (12-weeks) between the vehicle control group and the Palovarotene group. When treatment started at POD-1 in the absence of MRSA, Palovarotene significantly inhibited the formation of trauma-induced total new bone and soft-tissue ectopic bone formation at 84 days post injury from $16.2 \pm 4.9 \text{ mm}^3$ to $5.3 \pm 1.3 \text{ mm}^3$ ($p=0.02$; 67% mitigation) and from $0.6 \pm 0.4 \text{ mm}^3$ to no detectable ectopic bone formation ($p=0.04$; 100% inhibition) respectively, as revealed by μ CT imaging and quantification (Figs. 2A & 2B). When Palovarotene was administered from POD-5 in the absence of MRSA, it was still effective but less so, causing a decrease of total new bone and soft-tissue ectopic bone from $16.4 \pm 3.4 \text{ mm}^3$ to $8.9 \pm 2.5 \text{ mm}^3$ ($p=0.004$; 46% inhibition) and from $0.7 \pm 0.6 \text{ mm}^3$ to no detectable ectopic bone formation after treatment (100 % inhibition) (Figs. 2A & 2B). MRSA infection is a likely risk factor in combat settings and a known potentiator of trauma-induced HO [13]. In the presence of MRSA infection (Figs. 2B–F) and regardless of when treatment was started, Palovarotene was still able to decrease the volume of total new bone by 84 days post injury by 59–74%. When the Palovarotene treatment initiated at POD-1, the volume of total new bone and soft-tissue ectopic bone decreased from $41.9 \pm 5.6 \text{ mm}^3$ in controls to $16.4 \pm 3.9 \text{ mm}^3$ ($p=0.003$; 61% attenuation) and from $1.68 \pm 0.6 \text{ mm}^3$ to no detectable ectopic bone formation after treatment ($p=0.04$; 100% inhibition), respectively. For the POD-5 treatment, the volume of total new bone and soft-tissue ectopic bone decreased from $38.9 \pm 8.5 \text{ mm}^3$ to $16.0 \pm 6.6 \text{ mm}^3$ ($p=0.004$; 61% attenuation) and from $0.52 \pm 0.19 \text{ mm}^3$ to no detectable ectopic bone formation after treatment ($p=0.004$; 100% inhibition), respectively (Figs. 2A & 2B).

3.2 Palovarotene suppresses post-traumatic chondrogenesis and osteogenesis

Given that the rat blast model causes broader and more encompassing damage and complications compared to the more benign HO mouse models we used previously, it became important to determine the tissue level and phase of HO progression at which Palovarotene was able to inhibit HO in this model. Thus, we collected affected tissues around the amputated area of the limbs at day 15, 28 and 84 from the initial blast injury and processed them for histochemical analyses. In vehicle control samples, the amputated area at days 15 and 28 displayed obvious signs of an ongoing and active endochondral ossification process, with foci of immature and hypertrophying cartilage (Figs 3A–C), numerous

surrounding cells exhibiting progenitor characteristics, acidophilic osteoid matrix and immature woven bone (Figs. 3D–F). These ectopic tissues were all intimately embedded into surrounding tissues and most clearly, skeletal muscles. At 84 days post injury, masses of mature ectopic bone and marrow were present and often became surrounded by fibrous tissue to form a necrotizing pyogranuloma, a sign of ectopic bone resorption/remodeling (Figs. 3G–I). Areas of inflammation with foci of intense bacterial multiplication and necrosis (abscesses) were present in the soft tissue as well. In comparison, specimens obtained from Palovarotene-treated rats showed clear decrease in the amount of detectable immature cartilage and vascularized hypertrophying cartilage at each time point examined (Figs. 3J–L). In place of these skeletogenic tissues, we invariably observed the presence of extensive areas of fibrous connective tissues that were particularly obvious at day 28 post injury (Figs. 3M–O). At the end of the study at 84 days post injury, histological investigation showed that the medullary cavity was largely filled with similar fibrous connective tissue (Figs 3P–R).

3.3 Expression of chondrogenic and osteogenic gene markers is decreased in blast site-tissues from Palovarotene-treated rats

To assess the regulatory levels at which Palovarotene exerted its apparent anti-HO effects, we analyzed the expression of 83 genes associated with early chondrogenic, osteogenic and angiogenic processes related to bone development in blast site-samples collected from vehicle control and Palovarotene-treated rats at day 5, 7, 10, 14 and 28 from injury. As the local skeletal muscles were affected by the blast and were the major site of ectopic bone development; the harvested samples contained soft tissue immediately surrounding the residual femur but not set against the bone. Gene transcript values were normalized to expression levels found in uninjured skeletal muscle obtained from age-matched naïve rats. Samples collected at day 5–14 from injured limbs of vehicle-treated rats displayed significantly higher expression levels of key chondrogenic and osteogenic genes than samples from companion Palovarotene-treated rats (Fig. 4). The chondrogenic genes included *SOX9* ($p = 0.01$ on POD-7 and $p = 0.02$ on POD-10) and collagen 2 (*COL2a1*, $p = 0.01$ on POD-10) (Figs 4A–B), and the osteogenic genes included osteocalcin (*OCN*, $p = 0.02$ on POD7 and POD-10), *RUNX-2* ($p = 0.003$ on POD-5), osteogenic transcription factors *POU5F1* ($p = 0.01$ on POD-10), Osteopontin (*OPN*, $p = 0.049$ on POD-7), bone morphogenetic protein-2 (*BMP-2*, $p = 0.04$ on POD-7) and bone morphogenetic protein-4 (*BMP-4*, $p = 0.02$ on POD-7) (Figs 4C–H). In contrast to the decreases in chondrogenic and osteogenic genes, the samples from Palovarotene-treated animals displayed significantly higher expression of matrix metalloproteinase-9 (*MMP9*; $p = 0.02$ on POD-14), an enzyme involved in the degradation of cartilage extracellular matrix (Fig. 4I)[30].

3.4 Palovarotene may have moderate inhibitory effects on wound healing

As indicated above, bacterial infections are common in combat casualties, but wounded service members also suffer from additional injuries including damaged soft tissues and skin. We did not observe overt differences in common macroscopic wound complications such as surgical site bleeding, skin rashes, erosions, necrosis, abscesses, purulent drainage, and wound infection. However, we also quantified delayed wound healing by determining the proportion of wounds that were not completely healed by 14 days post-injury due to

either persistent superficial dehiscence or frank dehiscence requiring surgical debridement and secondary closure (Fig. 5). Overall, in response to any Palvarotene treatment, irrespective of MRSA contamination, 38% (12/32) of the wounds dehisced versus 23% (7/30) of the wounds in the vehicle control group ($p = 0.28$). However, amongst rats treated with Palvarotene and inoculated with MRSA, 63% (10/16) of rats experienced delayed healing or dehiscence irrespective of treatment timing, whereas 25% (4/16) of MRSA rats treated with vehicle control demonstrated delayed healing or dehiscence ($p = 0.07$).

4. Discussion

Herein, we report for the first time that Palvarotene is a strong inhibitor of HO in blast-associated rat model of combat-related extremity injury. This model is both severe and encompassing and includes not only the initial blast injury, but also compounding complications often occurring in combat situations, including bacterial infections and/or limb amputation. In each permutation, Palvarotene was able to counteract HO and did so in a significant manner, with an average of 50 to nearly 70% inhibition. This result is particularly impressive and promising given that Palvarotene was given every other day for 14 days starting on either POD-1 or POD-5 after blast injury, but HO was measured at later time points, which is critical in order to ensure that late recurrence or maturation of HO does not occur following cessation of treatment. Our data indicate that the developmental step of HO mostly affected by Palvarotene is the early chondrogenic phase. This is in line with the fact that retinoid agonists are particularly effective inhibitors of chondrogenesis [24, 31]. Such effect on an early phase of HO can also explain why Palvarotene was so effective against HO; by inhibition chondrogenesis, the drug was able to inhibit the subsequent osteogenic phases of the process. Our data here are also in agreement with our previous analysis of drug-based HO therapy in mouse models [25, 26]. It is possible that Palvarotene may redirect the commitment and lineage assignment of stem and progenitor cells recruited to the injury blast site, leading them to enter a fibrogenic phenotype to promote and facilitate local tissue healing and regeneration and in turn scar formation. We do detect a robust production of fibrotic connective soft tissue surrounding the amputation site in Palvarotene-treated rats, but it remains unclear whether the drug is able to influence and direct the differentiation of locally-recruited progenitor cells by direct mechanisms or indirect processes and signals occurring within the injured area.

The etiology, pathophysiology and exact mechanism(s) orchestrating trauma-induced HO remain poorly understood, although dysregulated immunological-neuro inflammation, production of local soluble osteogenic mediators, hypoxia and various risk factors have been identified as possible culprits [16, 19, 32]. Induction of chronic bacterial-induced inflammation and ostensibly, indolent infection of the residual limb and surrounding soft tissue are known clinical risk factors and are reported to be potentiators of ectopic bone formation [13]. Non-steroidal anti-inflammatory agents (NSAIDs) are often used to decrease this inflammatory component - specifically inflammatory prostaglandin-E₂- and the ensuing decrease in inflammation may reduce HO formation as well [33]. Our data indicate that the bioburden resulting from MRSA infection does not hamper appreciably the ability of Palvarotene to inhibit HO. This effect was obvious not only by histological inspection, but was also revealed by decreased expression of key chondrogenic (*SOX9* and *COL2a1*) and

osteogenic (*OCN*, *OPN*, *BMP-2*, *BMP-4*, *POU5FL* and *RUNX2*) genes and by increased expression of an important cartilage matrix catabolism gene (*MMP9*) [30].

The safety and tolerability of a candidate HO therapeutic agent must be considered in balance with its clinical efficacy. The ideal agent would not only prevent HO in trauma patients, but would also need to limit its effects on other concomitant injury patterns, such as the early tissue reparative and regenerative processes involved in the healing of complex wounds or fractures. Previous research demonstrates that one potential mode of action of Palovarotene is the dampening of neovascularization during early chondrogenesis [25, 26]. Impaired angiogenesis during the early wound healing process places the healing wound bed at theoretical risk of delayed or incompetent healing, prompting dehiscence. In our study, we observed a trend toward wound complication in the Palovarotene-treated groups, but the difference was not statistically significant. Defining the overall risk-benefit profile for Palovarotene with an in-depth and properly powered study is critical. Wounds that fail to heal often do so in the setting of persistent chronic infection (angiogenesis-dependent processes) [34, 35], which was the case in a large proportion of the MRSA-infected animals in our study. Future work will be directed toward studies assessing whether the therapeutic application of Palovarotene to prevent HO formation might potentially be associated with impaired wound vascularization and tissue repair if given for longer periods of time and/or different doses and frequencies.

There are several questions that the present study did not address. First, we did not test the effects of Palovarotene on the muscle injury or normal healing of long bone fractures, which occurs via endochondral ossification, the known mechanistic target of the drug. The feasibility of using this drug in a model containing concomitant long bone fractures is a focus of an ongoing study. While clarifying the effect on long bone fracture healing is important, we know that there is no ectopic bone attenuation efficacy rebound effect with the use of Palovarotene [25], and therefore a clinically acceptable treatment plan might include initiation of treatment in the early post-injury period to disrupt HO development, and then to stop treatment prior to definitive fracture fixation to permit unimpeded fracture healing. Temporizing fracture fixation could be initiated to maintain length and stability in the interim and portend a clinically acceptable outcome provided the fractures eventually go on to heal within an acceptable timeframe. Nevertheless, patients with bilateral lower extremity amputations, particularly those without upper extremity or spine fractures, could represent a specific cohort that would most markedly benefit from early Palovarotene administration. Second, we have not yet identified the mechanism responsible for the alteration of endochondral ossification caused by Palovarotene administration leading to a fibroblastic rather than osteoblastic endstate. Lastly, we need to further refine the dosing regimen of Palovarotene and determine whether the observed efficacy effects were either due to initiating the treatment earlier at POD-1 or selecting a narrow treatment window (14 days post injury) to specifically target endochondral ossification in the development of ectopic bone. With this knowledge, we can refine our treatment window for desired effect for the attenuation of ectopic bone formation in traumatized tissue without prolonging delays in endochondral ossification elsewhere in the body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMP	Bone morphogenetic proteins
CFU	Colony forming unit
CFU	Colony forming unit
COL1α1	Collagen type I alpha 1
COL2α1	Collagen type 2 alpha 1
DMSO	Dimethylsulfoxide
FGF1	Fibroblast Growth Factor-1
FGF10	Fibroblast Growth Factor-1
H&E	hematoxylin-Eosin
HO	Heterotopic ossification
IP	Intraperitoneal
mCT	Micro-computed tomography
MMP9	Matrix metalloproteinase 9
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NSAIDs	Nonsteroidal anti-inflammatory drugs
OCN	Osteoclastin
POU5F1	POU domain class 5 transcription factor
RAR	Retinoic acid receptor
RT-PCR	Reverse transcription polymer chain reaction
RUNX2	Runt-related transcription factor 2

SOX2	Sex Determining Region Y)-Box2
ROI	Regions of interest

References

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Figure 1. Schematic outline of the experimental studies and outcome measures. The number of rats analyzed in each treatment group is indicated.

Figure 2.

Palovarotene treatment inhibits heterotopic ossification. Analysis and quantification of (A) total new bone and (B) soft tissue ectopic bone volume using the flattened μ -CT images at 28, 56 and 84 days after blast, extremity injury, amputation $\pm$ MRSA (*= $p < 0.05$ using the Welch's two Sample t-test). Representative 3D reconstructed μ CT images of residual amputated limbs from vehicle control (C–D) and Palovarotene (E–F) treated rats at 84 days post injury after blast, extremity injury, and limb amputation with MRSA infection. Red circles indicate soft tissue ectopic bone and yellow circles indicate calcaneal ectopic bone.

Figure 3.

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 day s(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 500µm. Scale bars in images (B), (E), (H), (K), (N) and (Q) corresponds to 200µm. Scale bars in images (C), (F), (I), (L), (O) and (R) correspond to 50µm.

Figure 4.

Effects of Palovarotene treatment (n=4) and vehicle control (n=4) on chondrogenic (A–B) and osteogenic (C–H) related gene targets and matrix metalloproteinase-9 (I) gene expression. The fold change values were calculated by normalizing the values to the samples from non-injured muscle rats. Significantly different changes in gene expression are noted with an asterisk (*) ($p < 0.05$; Mann-Whitney U-test).

Figure 5.
Percentage of wounds that dehisced and were not completely healed by 14 days post-injury.
The number of wounds with delayed healing in each treatment group is indicated.