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TITLE: Anti-scar Treatment for Deep Partial-thickness Burn Wounds

PRINCIPAL INVESTIGATOR: Dr. Kai Leung

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

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Dr. Kai Leung
email: kai.p.leung.civ@mail.mil

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917 Pacific Ave., Ste. 600
Tacoma, WA 98402

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14. ABSTRACT

The FDA-approved drug pirfenidone is an anti-inflammatory/anti-fibrosis drug indicated for pulmonary fibrosis that we hypothesize can diminish scarring when applied topically to deep partial-thickness burn wounds in two animal models. The long-term objective is to learn to effectively use pirfenidone with regard to dosage, formulation and timing of treatment of burn wounds, such that animal studies will likely translate to the clinic. The objective of this proposal is to evaluate pirfenidone for efficacy in reducing fibrosis and scarring parameters in mouse and porcine models of deep partial-thickness burn wounds. The dosage formulation and schedule of treatment will be optimized and molecular markers of inflammation, angiogenesis, wound healing, and fibrosis will be correlated with scar reduction.

15. SUBJECT TERMS Nothing listed

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Introduction:

Deep Partial-thickness (DPT) burns frequently result in hypertrophic scars that can lead to severe functional impairment, psychological morbidity, and costly long term healthcare. Current treatment options lack effectiveness. The purpose of this research is to identify dosage formulations and treatment schedules for the FDA-approved drug Pf to evaluate it for use as a topical ***prophylactic*** and treatment against fibrotic scarring of DPT-burn wounds. The scope of the research is to evaluate pirfenidone for efficacy in reducing fibrosis and scarring parameters in mouse and porcine models of deep partial-thickness burn wounds. The dosage formulation and schedule of treatment will be optimized and molecular markers of inflammation, angiogenesis, wound healing, and fibrosis will be correlated with scar reduction.

Keywords:

Deep Partial-Thickness Burn; Pirfenidone; Hypertrophic Scar; Fibrosis; Formulations; mouse Burn Model; Porcine Burn Model; Topical; Inflammation; Granulation; Proliferation

Accomplishments:

Major goals of the project

1. Identification of topical formulations and doses that effectively deliver Pf to the dermis of DPT-burn wounds at each phase of healing and mitigate fibrosis of the closed wounds.
2. Optimization of the schedule of topical applications and uses this optimized schedule to determine detailed molecular changes in healing wounds resulting from Pf treatment.
3. Validation of the efficacy of Pf to reduce hypertrophic scarring in the Duroc porcine DPT-burn model.

Major accomplishments of Goal #1:

Identified topical formulations and doses that effectively deliver Pf to the dermis of DPT-burn wounds at each phase of healing and mitigate fibrosis of the closed wounds.

Formulation of Pirfenidone (PF) Ointment

We have completed the formulation of PF at 3 different concentrations (1, 3.5, and 6.5%) in the ointment dosage form. The composition of the ointment formulation at each dosage level is listed in Table 1. The *in vitro* drug release profile of the ointment formulation at each dose is also depicted in Figure 1. Above 90% of the drug was incorporated into the ointment formulations. More than 70% of the drug content was released within 48 hours. Three different batches of the ointment formulation at each dose have been prepared. They showed similar release profiles suggesting that the release profiles of PF in these formulations at different dosing levels are reproducible.

For animal studies, three batches of 40 g of the ointment formulation at each dose plus the placebo formulations without the active have been prepared under aseptic conditions and packaged in sterile aluminum tubes (10 and 5 g) (Figure 2).

Batches of the ointment formulation at different PF dose were also prepared for stability testing (Figure 2). The test includes testing the ointment formulations in simulated wound fluid at 34°C using Franz's cells (cellulose acetate membrane, 0.45µm). The drug content and *in vitro* release profiles will be determined. Other stability study which includes testing the formulations in storage for up to 6 months (26 weeks) at 25˚C ± 2˚C, 60% RH ± 5˚RH will be performed. Furthermore, formulations will be tested for stability under

accelerated conditions at 40˚C ± 2˚C, 75% RH ± 5˚RH (Table 2). At each scheduled time point, the consistency, physical stability, pH and drug content (HPLC) of placebo and PF ointments will be assessed.

Table 1 Composition of ointment formulations containing 1, 3.5 and 6.5% of PF

Batch # [a]	Composition [b]				
	Petrolatum Jelly % (g)	**MO % (g)**	**PEG % (g)**	**BnOH[c] % (g)**	**PF % (g)**
1	78 (31.2)	20 (8)	-	1(0.4)	1 (0.4)
2 (PL [d])	79 (31.6)	20 (8)	-	1(0.4)	-
3	70.5 (28.2)	20 (8)	5 (2)	1 (0.4)	3.5 (1.4)
4 (PL [d])	74 (29.6)	20 (8)	5 (2)	1 (0.4)	
5	90 (36)	2.5 (1)	-	1 (0.4)	6.5 (2.6)
6 (PL [d])	96.5 (38.6)	2.5 (1)	-	1 (0.4)	

[a] Batch size – 40 g
[b] Composition is expressed as percentage and as grams in brackets
[c] Benzyl alcohol
[d] Placebo

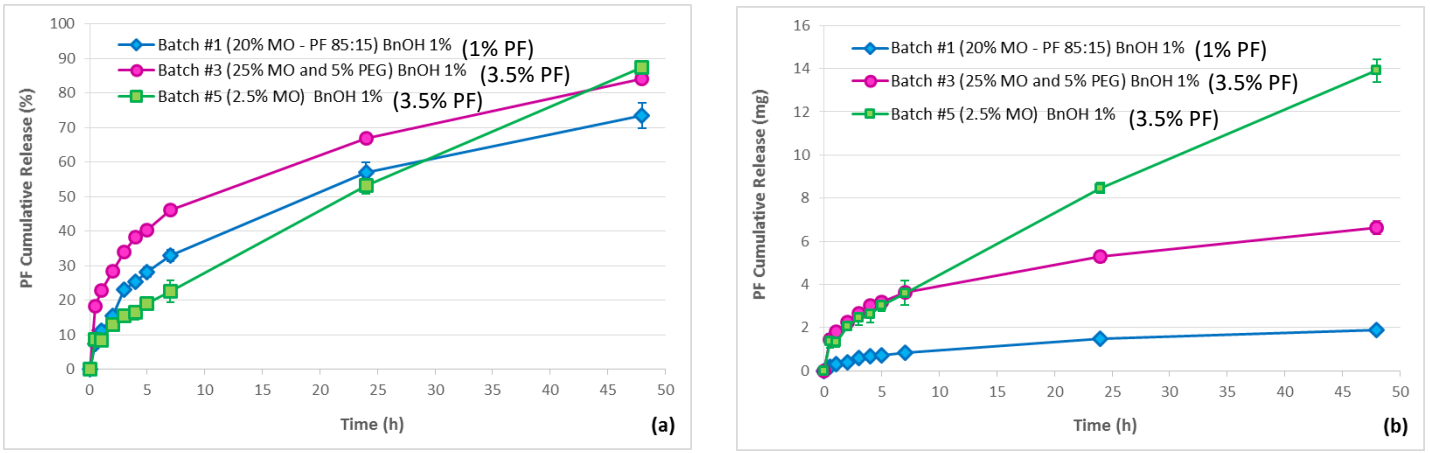


Figure 1. *In vitro* release study of PF ointment formulations, Batch # 1, 3 and 5: (a) % PF cumulative release *vs*. incubation time and (b) mg PF cumulative release *vs*. incubation time. The results were normalized in respect to 250 mg of ointment.

Table 2 Stability study of PF ointment formulations

Samples:	PF as such (PF powder) - (CTR) Placebo Ointments - (CTR) PF ointment (1% w/w) PF ointment (3.5% w/w) PF ointment (6.5% w/w)
Experimental conditions:	25˚C ± 2˚C, 60% RH ± 5˚RH 40˚C ± 2˚C , 75% RH ± 5˚RH (accelerated study)
Scheduled times (weeks):	0, 1, 4, 8, 13 and 26
Assay:	Rheological analysis (consistency) Phase separation phenomena (visual inspection) pH (pH meter) Drug content (HPLC)

Figure 2. PF ointment formulations packaged in 5 and 10 g aluminum tubes for animal and stability studies.

Deep Partial-Thickness Burn Model

The mouse model of deep partial-thickness burns has been established as part of the animal studies portion in the proposal to test the effects of pirfenidone on burn-induced fibrosis. We designed a template with specifications to allow for even contact of bare mouse skin (2×3 cm) with hot water and protect the mouse from further burns (Figure 3A.) To optimize the mouse scald model, we tested a range of temperature (54-60°C) with a scalding time of 10-45 seconds to produce consistent deep partial-thickness burns (Figure 3B). The burns were evaluated by a veterinary pathologist to confirm the depth and damage caused by the scalds (Figure 3C).

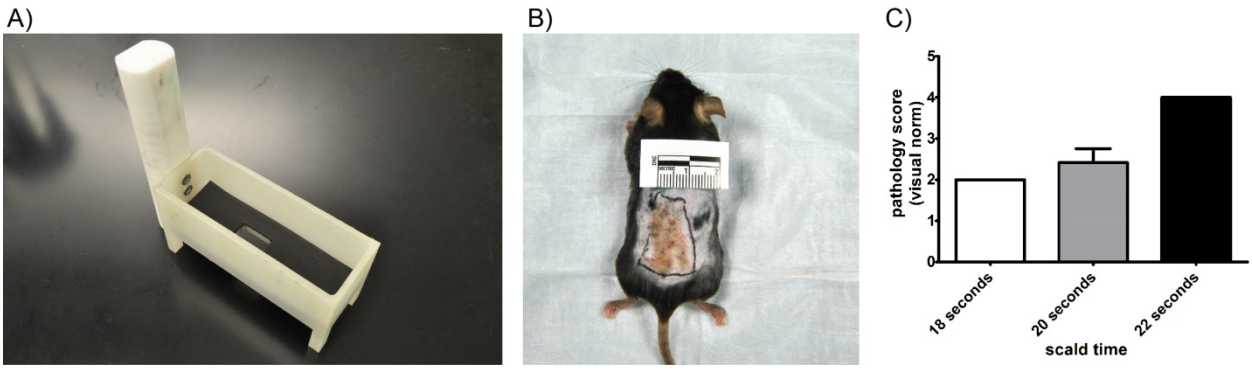


Figure 3. Optimization of partial-thickness burns in mice. A) burn template B) mouse burn wounds C) pathology score.

Additionally, burn wounds were validated using H&E, Masson's trichrome and TUNEL staining to assess the depth of damage and cell death (Figure 4A-C). Our studies indicate that the optimal time and temperature combination to produce a consistent deep partial thickness burn in mice is 20 seconds at 54°C.

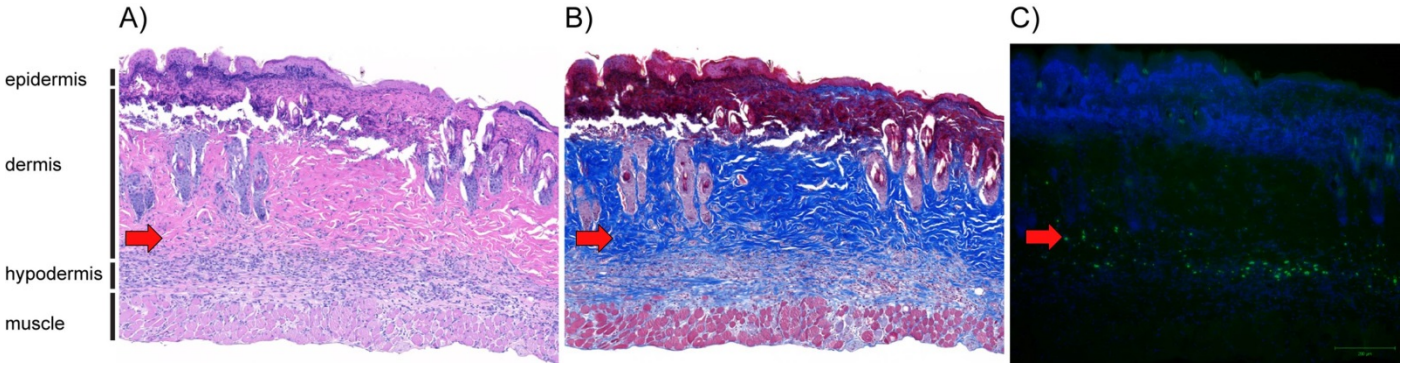


Figure 4. Histological analysis of deep partial-thickness burns in mice. (A) H&E; (B) Masson's trichrome; (C) TUNEL stain. The red arrows indicate the burn depth (A & B) or the boundary between dead (above) and live (below) tissues by TUNEL staining (C).

PF Inhibits Transformation of TGF-β1 Stimulated Human Dermal Fibroblasts (NHDF) into Myofibroblasts

In our earlier report (Q3Y1), we demonstrated that PF caused a significant reduction of α-smooth muscle actin (α-SMA) in TGF-β1 stimulated NHDF. Increased the amount of α-SMA is the hallmark of myofibroblasts. The results collectively indicate that PF could interfere with the transformation of fibroblasts into myofibroblasts in response to TGF-β1 stimulation. In this quarter (Q4Y1), we went on to determine the effects of PF on focal adhesions. Focal adhesions are large macromolecular assembly of over 50 protein components functioned as a mechanical link transmitted between extracellular matrix and the intracellular cytoskeleton. In addition, the focal adhesions serve as a signaling hub mediating multiple pathways that are important in a number of cellular processes such as survival, proliferation, cell shape, and cell migration. We found that PF significantly reduced the size and number of focal adhesions as well as levels of stress fibers in human dermal fibroblasts stimulated with 10 ng/ml of TGF-β1 for 4 days. The effects of PF on focal adhesions and stress fibers are dose dependent (Fig. 5). The results provides some insights that PF could affect the fibroblast migration as focal adhesions serve as traction sites and play a role in regulating cell migration.

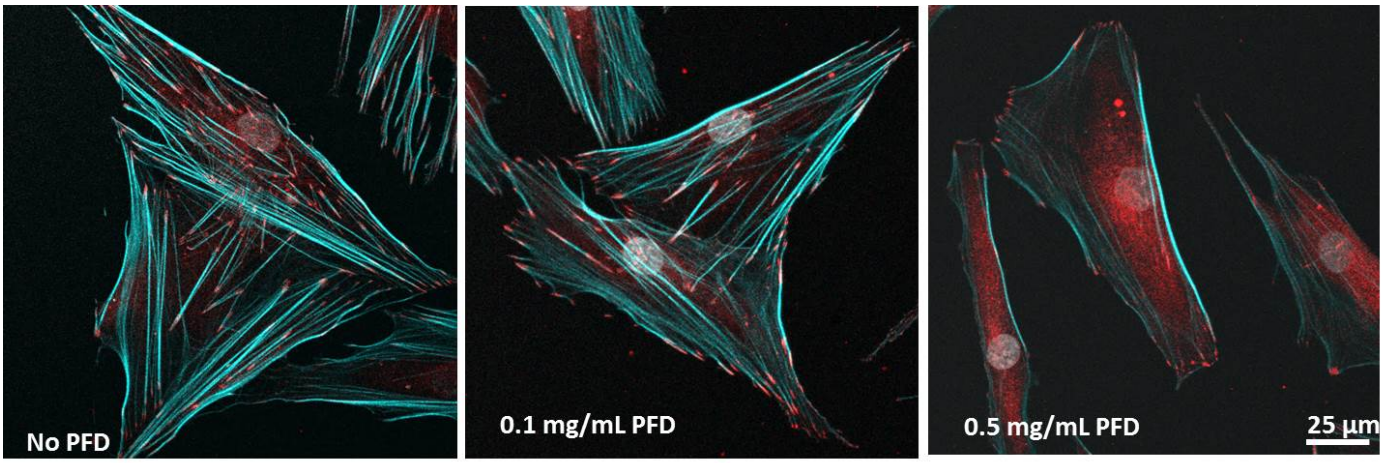


Figure 5. PF alters formation of focal adhesions (red punctate foci) and stress fibers (turquoise) in TGF-β1 stimulated human dermal fibroblasts (NHDF)**.** NHDF seeded at 2,000 cells/cm^2 were fixed after serum starvation and 5 days of treatment in serum-free media (SFM) with 10 ng/mL TGF-β1, TGF-β1 and 0.1 mg/ml or 0.5mg/mL PF, or SFM alone. Cells were fixed in paraformaldehyde and stained with Alexa Fluor 647-conjugated Phalloidin (1:40) for actin filaments, Hoechst 33342 (1:2000) for nuclei (white round bodies), and anti-Vinculin mouse mAb (1:300) detected with Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (1:1000) for focal adhesions. Note the stress fibers (actin filaments containing non-muscle myosin) anchoring into focal adhesions that are located in the specialized regions of the cell membrane.

PF Potentially Targets P38- α Mitogen Activated Protein Kinase (MAPK) in TGF-β1-Stmulated Fibroblasts

To date, the exact mechanism (drug action) of PF in dermal fibroblast cells is not fully understood. Studies aimed at better understanding the mechanism of action of PF in a number of cell types indicated that PF could target kinase activity. In these experiments, we used antibody-based kinase detection arrays to measure phosphorylated kinases as an indicator of kinase activity. Serum starved NHDF were stimulated with TGF-β1 and incubated in the presence or absence of PF for up to 15 minutes at 37^{o}C. Our data suggested that signaling through p38α-MAPK in the stimulated NHDF was reduced by PF (Figure 6). Our data also indicate that p38-δ-MAPK was not targeted by PF treatment. The data also showed that p38-β and –γ might not be expressed in dermal fibroblasts in serum free or fibroblast growth media (Figure 6).

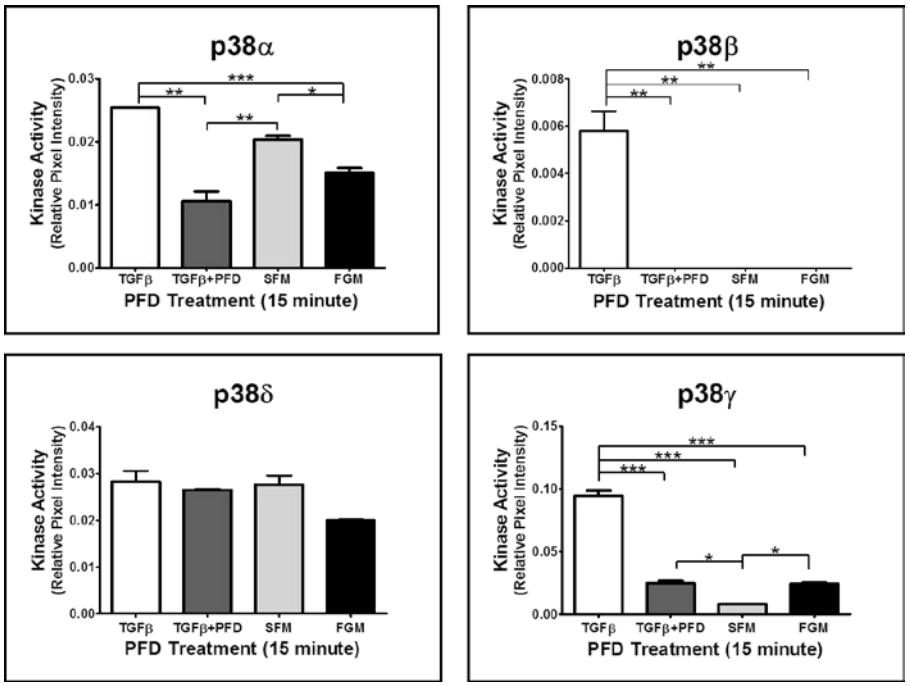


Figure 6. Effects of PF on kinase activity of TGF-β1 (10 ng/mL) stimulated NHDF in the presence or absence of PF. NHDF were serum starved for 24 hours prior stimulation by TGF-β1. PF reduced kinase activity of p38α. SFM, serum starved medium; FGM, fibroblast growth medium (Lonza). **, $p \leq 0.01$; *** $p \leq 0.001$.

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

Nothing to Report.

How were the results disseminated to communities of interest?

We intend to publish the findings in a peer-reviewed journal as a means to disseminate the results to reach the members of research communities who are interested developing therapeutic solutions to reduce fibrosis and scarring.

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

We plan to do the following in the next reporting period:

1. Test the ointment formulation containing PF (1, 3.5, and 6.5%) for reducing fibrosis in mouse deep partial-thickness burn wounds. The ointment will be applied during inflammation, granulation, or proliferation of the healing process.
2. Prepared microspheres encapsulating PF for sustained release of PF for later use in treatments.

Impact:

The successful completion of the first goal produced a lead dosage form that can be further evaluated in the deep partial-thickness mouse model for reducing burn-induced fibrosis.

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

The *in vitro* data obtained provided some insights on how the antifibrotic drug, pirfenidone, could reduce the expression of some of the fibrotic components found in the scar tissue at the cellular level.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

Changes/Problems:

Nothing to report.

Changes in approach and reasons for change

Not applicable.

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

Not applicable.

Changes that had a significant impact on expenditures

Not applicable.

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

Not applicable.

Products:

Publications, conference papers, and presentations

Journal publications

Hall, CL, AR Wells, and KP Leung. Pirfenidone Modulates the TGF-β1-Induced Profibrotic Phenotype in Human Dermal Fibroblasts. Manuscript in Preparation.

Acknowledgement of federal support (yes).

Other publications, conference papers, and presentations.

Two posters were presented in the Wound Healing Society Meeting that was held on April 2016 at Atlanta, GA:

- Medina J. and K. P. Leung. Scald Model of Partial Thickness Burns in C57BL/6 Mice.
- Hall, C. A., A. R. Wells, and K. P. Leung. Pirfenidone Modulates the TGF-β1-Induced Profibrotic Phenotype in Human Dermal Fibroblasts

One abstract was submitted to American Association of Pharmaceutical Scientists to be held on November 13-17 at Denver, CO

- R. Dorati, P.P. DeLuca, and K. P. Leung. Development of an Ointment Formulation as an Antiscarring Product for Treatment of Deep Partial-Thickness Burn

Participants & Other Collaborating Organizations:

What individuals have worked on the project?

Name:	Kai Leung
Project Role:	PI
Nearest person month worked:	3
Contribution to Project:	Dr. Leung will be responsible for insuring compliance with all regulatory requirements. He has chosen the following personnel to assist him in the proposed studies because of their expertise in animal surgery and in the field of molecular biology, histology, histochemistry, and immunohistochemistry, PCR array analysis, as well as wound healing analysis.

Name:	Rodney Chan
Project Role:	Co-PI
Nearest person month worked:	3
Contribution to Project:	Dr. Chan will meet with the laboratory staff regularly to discuss the progress of the project, participate in data analysis, and prepare reports and manuscripts.
Name:	Li-Wu Qian
Project Role:	Research Associate
Nearest person month worked:	10.8
Contribution to Project:	Dr. Qian is responsible for the animal surgical procedures and will plan and execute the animal model required for this proposed research. He will be responsible for the animals for insuring compliance with all regulatory requirements for this project and our institution.
Name:	Rossella Dorati
Project Role:	Visiting Formulation Scientist
Nearest person month worked:	12
Contribution to Project:	Dr. Dorati will be will be participating in the preparation of Pirfenidone-loaded microspheres for controlled drug release used in the treatment of deep partial-thickness burn wounds to reduce fibrosis and hypertrophic scarring in mouse and porcine model, respectively. Dr. Dorati will also prepare pirfenidone is different dosage forms (cream, gel, and ointment) for use in the treatment experiments.
Name:	Jorge Medina
Project Role:	Post-Doctoral Fellow
Nearest person month worked:	9.6
Contribution to Project:	Dr. Medina will be will be participating in developing the deep partial-thickness mouse burn model for use in testing the treatment of burn wounds containing pirfenidone to reduce fibrosis in mouse.

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

None to report.

What other organizations were involved as partners?

Nothing to report.

Provide the following information for each partnership:

Not applicable.

Special Reporting Requirements:

Quad Chart: The Quad Chart (available on https://www.usamraa.army.mil) shall be updated and submitted as an appendix.

Appendices:

Quad Chart is included.

“Anti-scar Treatment for Deep Partial-thickness Burn Wounds”

Log Number: BA150467

Award Number: W81XWH-15-2-0083

PI: Dr. Kai Leung **Org:** The Geneva Foundation/USAISR

Award Amount: $2,177,795

Study/Product Aim(s)

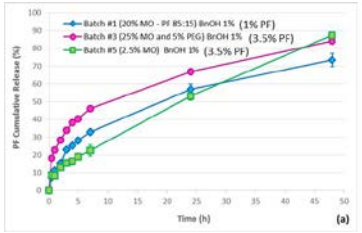
- Aim 1: Identify topical formulations and doses that effectively deliver pirfenidone (Pf) to the dermis of deep partial-thickness (DPT) burn wounds at each phase of healing and mitigate fibrosis of closed wounds.
- Aim 2: Optimize the schedule of topical applications and use this optimized schedule to determine detailed molecular changes in healing wounds resulting from Pf treatment.
- Aim 3: Validate the efficacy of Pf to reduce hypertrophic scarring in the Duroc porcine DPT-burn model.

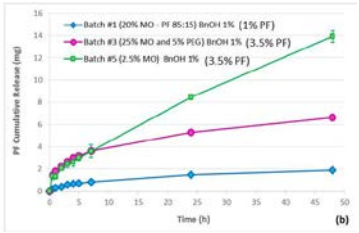
Approach

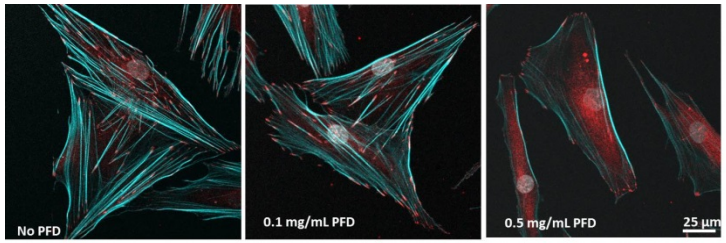
As much as half or more of DPT burn wounds develop hypertrophic scarring, and once formed, treatments are only minimally effective. The proposed research seeks to re-purpose the FDA-approved drug Pf for use as an anti-scarring treatment for DPT-burns. Re-purposing builds upon previous R&D, making the regulatory path shorter. The proposed R&D aims to learn to use Pf with regard to dosage, formulation, and timing of treatment of burn wounds, and molecular markers, such that animal studies will likely translate to the clinic. This information would support advanced development of Pf as an anti-scarring treatment.

Accomplishment

Formulations of Pf and the drug release kinetics are complete (upper panel). Pf showed promising release profiles. Pf attenuated the profibrotic effects of TGF-β1 in human dermal fibroblasts . This is shown by the reduction of focal adhesions in the Pf-treated cells. (lower panel).







Timeline and Cost

Activities CY	15	16	17	18
Identify formulations that penetrate tissue and mitigate fibrosis indicators.			TRL2	
Optimize the schedule of topical applications and identify anti-fibrosis markers.				TRL3
Validate the efficacy of Pf to reduce hypertrophic scarring in the Duroc porcine DPT-burn model.			TRL4	
Estimated Budget ($2.17M)	**171,885**	**702,189**	**745,632**	**558,090**

Updated: 11 OCT 2016

Goals/Milestones

FY16 Goals

☑ Topical dosage formulation of Pf that delivers the drug to the dermis at each phase of healing as determined by pharmacodynamic effects and anti-fibrosis indicators in the mouse DPT-burn model.

FY17 Goals

❑ Optimal formulated dose and schedule of topical application of Pf that best diminishes scarring endpoints in the mouse DPT-burn model.

FY18 Goals

❑ Determination of the effectiveness of the optimal formulated Pf dose and schedule of topical application that reduce hypertrophic scarring in the Duroc porcine DPT-burn model.

Purpose: To provide proof-of concept for the repurposing of the FDA approved anti-fibrotic drug Pirfenidone as a prophylactic to reduce scar caused by DPT burns.

Product: An FDA approved prophylactic anti-scar agent .

Payoff: To improve scarring outcomes of deep partial-thickness burn.

Budget Expenditure as of 9.30.16

Projected Expenditure: $687,541

Actual Expenditure: $41,493