

AWARD NUMBER: W81XWH-14-2-0016

TITLE: Use of Topical PC-NSAIDs to Treat Burn Injury and Pain

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14. ABSTRACT Studies in year two have shown that the rat dorsal skin and hind limb burn injury models are appropriate for testing the analgesic and healing rate efficacy of test drugs. These models were used to test Ibuprofen (Ibu), Ibu-PC, Indomethacin (Indo), and Indo-PC. The experimental drug Indo-PC showed a tendency for analgesic efficacy over Indo alone or the Ibu drugs, notably when administered parenterally. These studies support a rationale for continued development of parenteral Indo-PC for treatment of 2 nd degree burn wounds.					
15. SUBJECT TERMS Burn, phospholipid, NSAID, topical, pain, inflammation					
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1. INTRODUCTION:

The major problem under study is the development of a topical or parenteral treatment for pain due to 2nd degree burn injury. The experimental drug treatment consists of the use of nonsteroidal anti-inflammatory drugs (NSAIDs) that are complexed with the phospholipid phosphatidylcholine (PC) to produce a new class of drugs, NSAID-PCs, which have been shown in other experimental systems to reduce inflammation and pain and promote healing. This grant will utilize rodent models of burn injury to test the efficacy of indomethacin-PC (Indo-PC) and ibuprofen-PC (Ibu-PC) versus unmodified indomethacin (Indo) and ibuprofen (Ibu) when administered either topically or subcutaneously. Measurements of pain and wound healing will be made. After these drugs are fully tested in preclinical systems and shown to be beneficial, they can be further developed for clinical use.

2. KEYWORDS:

Burn, Phospholipid, NSAID, Topical, Pain, Inflammation

3. ACCOMPLISHMENTS:

Major goals of the project:

- 1) Compare the efficacy of topical Indomethacin-PC and Ibuprofen-PC in rodent models of 2nd degree burn injury, and determine any additional benefit of combined topical and parenteral administrations.
- 2) Evaluate the GI side effects of NSAID-PC treatment in the burn model and the effect of test drugs on clotting time.
- 3) Determine the mechanism of action of NSAID-PCs in the treatment of burn pain/healing.

Milestones and target dates:

- 1) Complete testing for topical and sc Indo, Indo-PC, Ibu and Ibu-PC in hind limb burn injury model – target was month 6 – 70% complete
- 2) Complete testing for topical and sc Indo, Indo-PC, Ibu and Ibu-PC in dorsal skin burn injury model – target was month 12 – 100% complete
- 3) Complete testing for topical vs sc NSAID-PCs in hind limb burn injury model – target was month 15 – 70% complete
- 4) Complete testing for topical vs sc NSAID-PCs in dorsal skin burn injury model – target was month 18 – 100% complete
- 5) Determination of NSAID-induced GI side effects in hind limb burn injury model – target was month 15 – 70% complete
- 6) Determination of NSAID-induced GI side effects in dorsal skin burn injury model – target was month 18 – 100% complete
- 7) Determination of effects of NSAID-PCs on thrombus formation and clotting time – target was month 18 – 0% complete
- 8) Assessment of COX inhibition and effects on inflammation as a mechanism for PC-NSAID efficacy – target was month 24 – 0% complete
- 9) Assessment of skin hydrophobicity and histology as a mechanism for NSAID-PC efficacy – target was month 24 – 70% complete

Accomplishments:

- 1) Major activities: During this second grant year, the efficacy of Indo, Indo-PC, Ibu and Ibu-PC continued to be evaluated in the dorsal skin burn model. Ibu, Ibu-PC and Indo tended to show analgesia, while Indo-PC produced significant analgesia, notably when administered parenterally. Biochemical measures and histology supported the finding with Indo-PC. A second burn injury model, that of hind limb skin injury, was optimized and then used to test Indo and Indo-PC efficacy. Both Indo drugs tended to provide analgesia.
- 2) Specific objectives: To test topically and parenterally administered Ibu, Ibu-PC, Indo and Indo-PC vs appropriate controls in the dorsal skin and hind limb burn injury models for ability to relieve pain and promote wound healing.
- 3) Significant results:

Dorsal skin burn injury model. This model causes hyperalgesia on day 3 and day 5 (a reduction in hind limb withdrawal threshold (comparing naïve with PBS-treated rats, $p=0.041$, $p=0.021$ respectively, Fig 1 left), with a tendency for both Ibu and Ibu-PC, at the NSAID dose tested (20 mg/kg) when administered parenterally to exhibit a longer time to withdrawal of the paw from mechanical pressure applied to the base of the paw. For indo and indo-PC treatment, indo-PC significantly reduced hyperalgesia compared to PBS-treated (Fig.1 right).

Hind limb withdrawal threshold at Day3 and 5

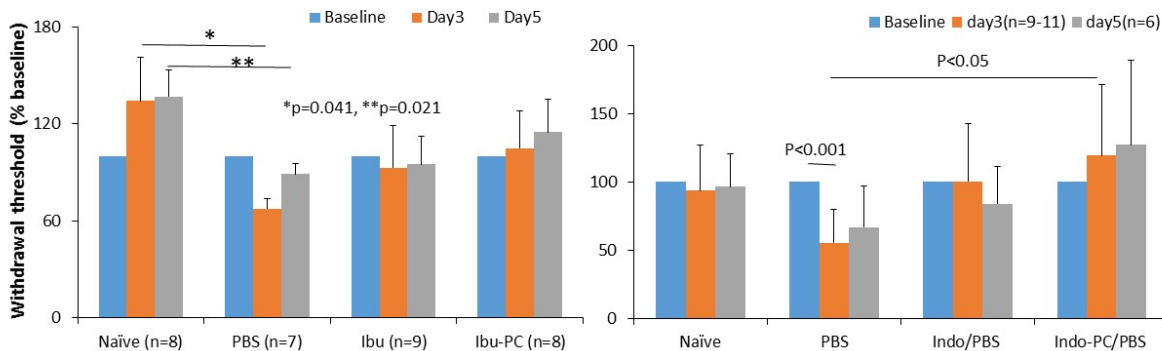


Fig. 1

To investigate the effectiveness of both Ibu/Ibu-PC and Indo/Indo-PC on burn injury, biochemical changes which occur during the burn injury process was analyzed with myeloperoxidase (MPO) activity. MPO is an enzyme known to be elevated in tissue after injury due to the presence of inflammatory cells (neutrophils contain MPO) that migrate to the injury. In dorsal skin burn injury, MPO activity tended to be suppressed in both Ibu/Ibu-PC and Indo/Indo-PC treatments (Fig.2). It was noted that both the NSAID-PC drugs appeared to reduce this inflammatory index to a somewhat greater extent than the parent NSAID.

MPO activity ng/mg protein

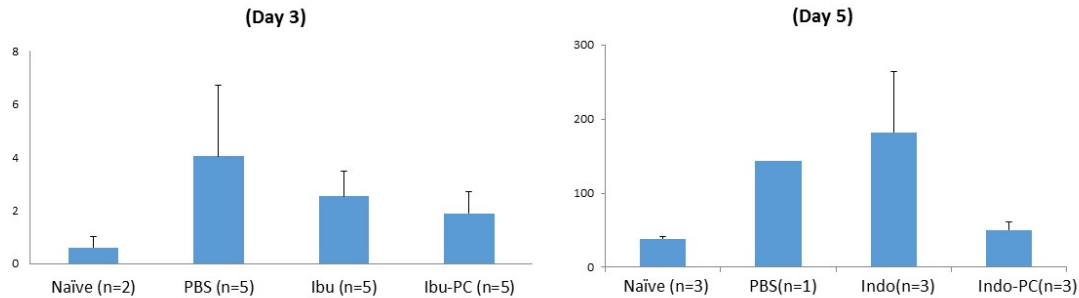


Fig.2

To examine the histologic burn injury changes that occur with or without drug treatments, our collaborator Dr. Roger Bick examined normal skin tissue and burn tissue from three groups at 3-5 days post burn: PBS (control burn), Indo (20mg/kg, topical) and Indo-PC (20mg/kg, topical). His analysis below shows that both indomethacin drugs have some capacity to protect or promote healing of the burned tissue. More tissue analysis is needed to confirm these findings.

(Fig. 3 left) Normal control skin is shown with hematoxylin and eosin staining (H&E). All internal structures are intact.

(Fig. 3 right) Fluorescent image of normal skin shows intact surface and structures. In this and the subsequent figures, the color code is as follows: Green = f-actin (phalloidin); Red/Orange = smooth muscle actin (Texas Red); Blue = nuclei (DAPI); Yellow = overlap, Texas Red+f-actin

(Fig.4 left) The full section H&E image of PBS-treated burned skin shows disruption and loss of the stratum corneum, severe damage to the stratum spinosum and, even though staining for mitosis was not performed, it can be assumed that the stratum basale, Merkel Cells and Langerhans cells will all be damaged or killed. This will greatly affect protection via loss of lamellar bodies and immune functions, as well as compromised sensory and mechanical function; Meissner's plexus and Merkel cells, respectively. (Fig.4 right) The fluorescence image confirms these findings as a detached keratin layer can be seen, and a clumped actin network, unlike the web-like distribution in the right of the image. The image also shows that the included hair root and follicle is intact, though the exit is in the less damaged portion of the skin sample. Papillary and reticular dermis structures appear intact, such as the blood vessel, duct and eccrine gland.

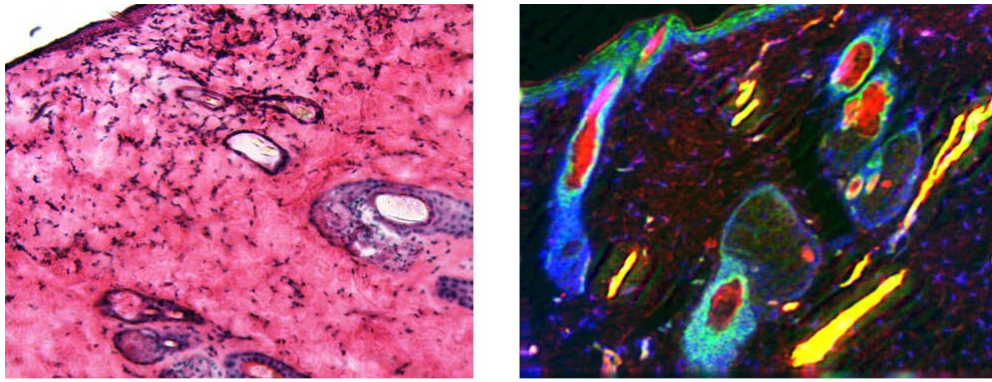


Fig.3 Normal skin tissue

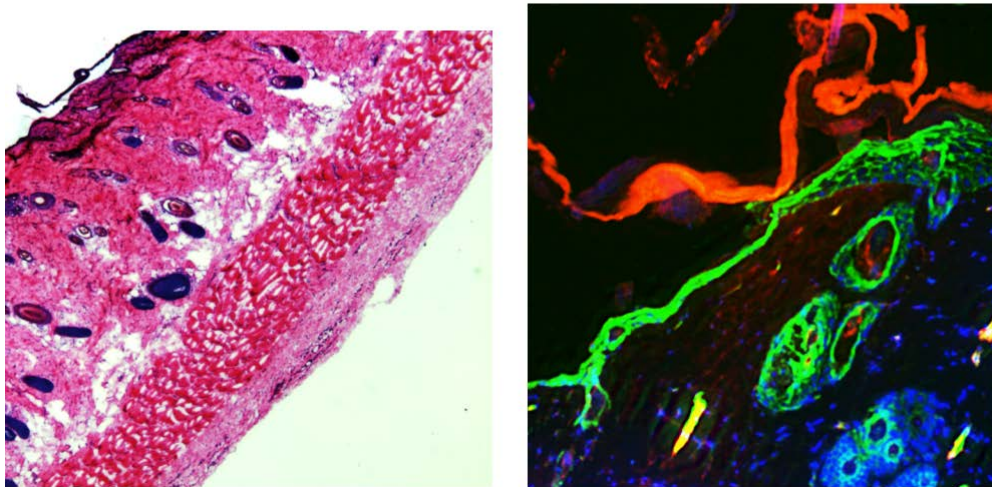


Fig.4 PBS treated burned tissue 3 days post injury

(Fig.5 left) H&E image of skin from topically-treated indomethacin group shows a fairly healthy skin sample with hair follicles and connective tissue intact throughout down to the deeper dermis. There is thinning of the upper epidermal keratin, and some of the collagen looks denatured but overall, it is quite healthy.

(Fig.5 right) Fluorescence probing also reveals healthy looking cells and structures. Hair follicles, vessels, smooth muscle and papillary plexus look fine. There might be a little bleeding into the epidermis, but damage is slight.

(Fig.6 left) The H&E image of skin from topically-treated Indo-PC group demonstrates a loss of keratin from the corneum, but the papillary dermis and reticular dermis appear in good shape with intact hair shafts, follicles, sebaceous glands and arrector pili muscle. It also appears that there is at least some continuity of the spinosum and some areas of stratum basal integrity. This suggests that mitosis, melanin synthesis and transfer and sensory mechanisms, might all be functioning, at least to some extent.

(Fig.6 right) Fluorescence image shows structural integrity, and therefore, skin viability is seen.

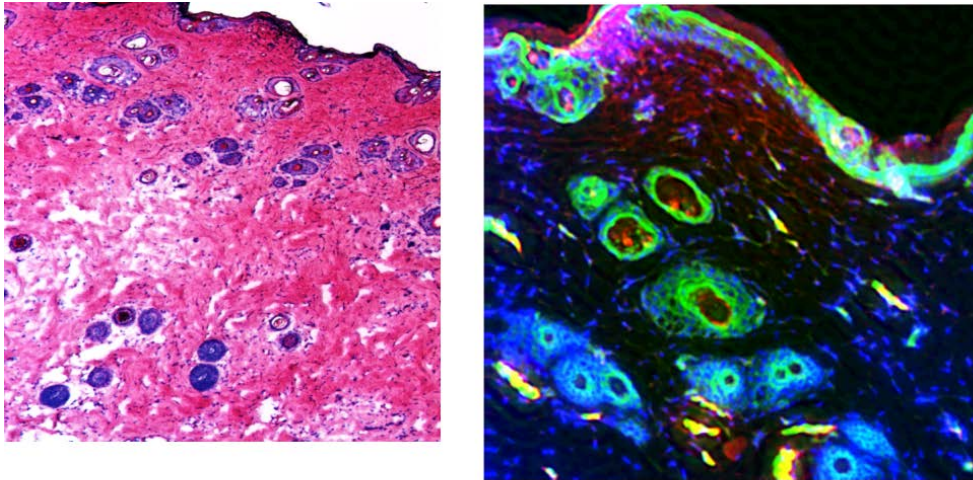


Fig.5 Topical indomethacin, 5days post injury

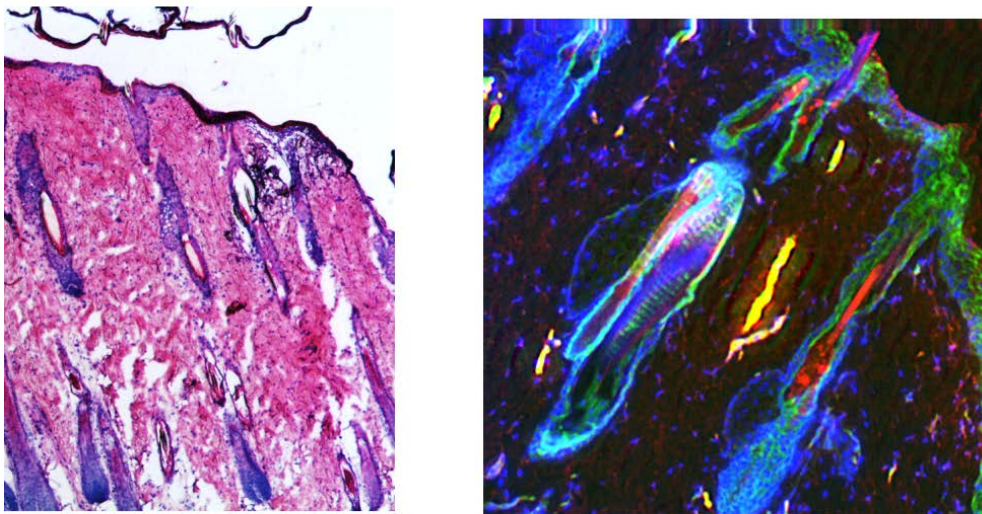
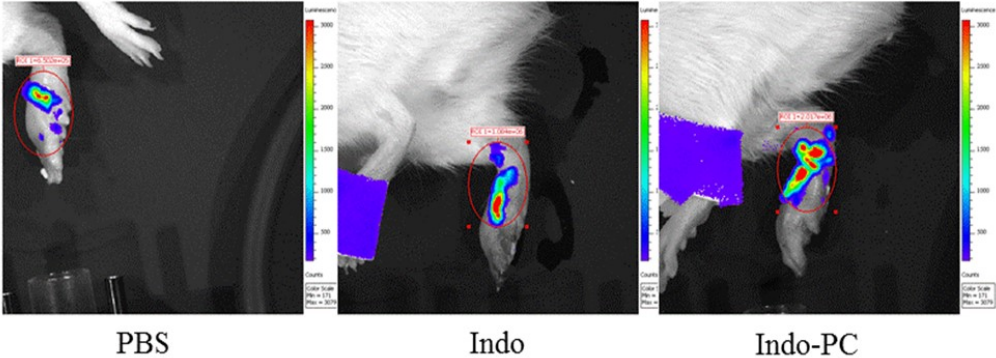


Fig.6 Topical indomethacin-PC, 5 days post injury

Hind limb skin burn injury model. Using a published burn model to induce second-degree injury, rats were subjected to a 25 second exposure of their left hind paw to water at 57-58°C. Treatment groups consisted of burn control (PBS), burn + Indo and burn + Indo-PC, sc. It should be noted that this model was first evaluated by parenteral administration of the drug, as that was more effective than topical in the earlier dorsal skin model of 2nd degree burn injury. In order to estimate the inflammation status following hind limb burn injury, a live animal image analysis system (IVIS Lumina XR) was used. A probe that produces a chemiluminescent signal, luminol sodium salt (200 mg/kg body weight), was intraperitoneally injected into test burned animals on day 3, and the injured site images were captured at a standard 20 minutes after the injection. Luminol sodium salt induces neutrophil chemiluminescence as a measure of myeloperoxidase activity (MPO) without disturbance to the animals, so we can continue behavior analyses until the end point of the experiment. Results can be analyzed based on the colors produced as shown in Fig.7 where yellow and red indicate greater inflammation and blue is the least. In this initial experimental series, the burn injury in all animals was more severe than expected, producing somewhere between

second-and third-degree injury. It was decided to limit the burn conditions in subsequent studies to those which would produce only a second degree injury.



Luminol (200 mg/kg BW, i.p.) and imaged 20 min after inj.

Fig.7

A study was performed to optimize conditions under which a consistent second degree burn to a hind paw was obtained. An experiment was conducted with varied time of burn (10-25 seconds) and temperature (51-57°C). The measure of injury was by analysis of burn skin MPO. From the results shown in Fig.8, it was decided to use 57°C for 20 sec as our experimental method.

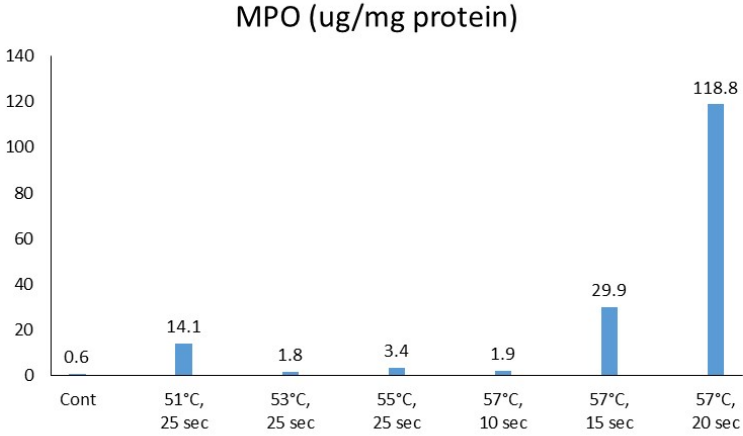


Fig. 8

The hind limb skin burn model tested Indo and Indo-PC treatment with subcutaneous as well as topical application (in oil). Control groups included non-burn (Cont), burn+PBS, and burn+oil. For behavior testing we used a Girdle testing method to evaluate the spontaneous pain/hyperalgesia reaction for the hind limb burn injury animals. Rats were placed in a Plexiglass restraining device to be acclimated, then 2G (4.31) and 26G (5.46) of Von Frey filament was pressed on their lumbar site. Results were determined by counting the vocalization number. The data is presented as actual number of the total reaction of the stimulations. The behavior results (Fig 9) indicated that the effects of subcutaneously administered Indo drugs tend to suppress hyperalgesia compared to PBS treatment. In contrast, topically applied Indo oils produced a more variable analgesic response with some suggestion that Indo-PC oils were better than Indo oils at 3- and 5-days after burn injury. From the result of a live animal image analysis system (Bioluminescence emission from an IVIS Lumina XR), there was not much effect of the test drugs (Fig 10). We also measured paw thickness by caliper after hind limb burn injury, expressed as a percentage of pre-injured thickness (Fig 11). All injured rats exhibited edema on day 3 and were showing some recovery on day 5. The indo drugs tended to show slight improvement.

Total response with 2 and 26 grams Force at Day3 and 5

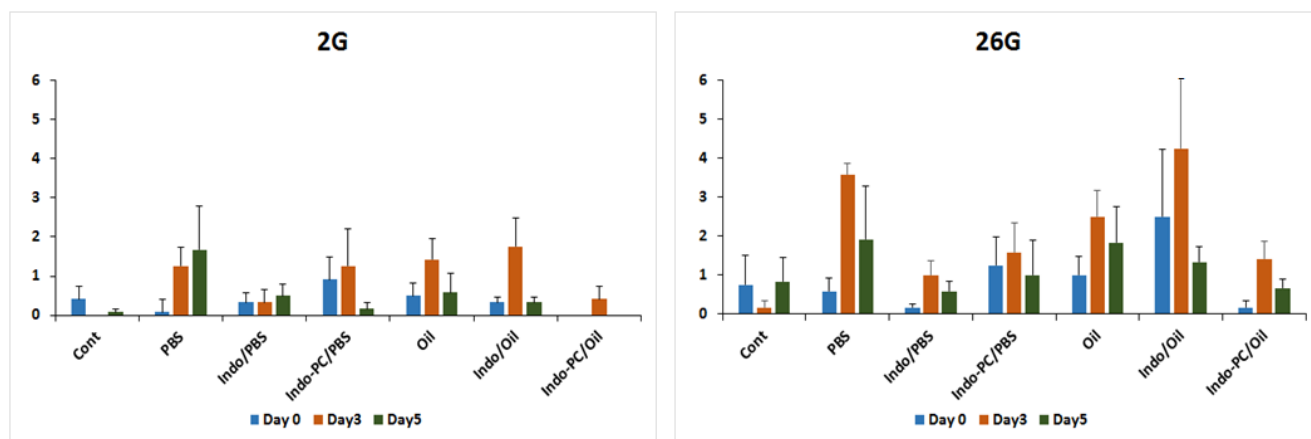


Fig. 9

Bioluminescence Emission (Total count)

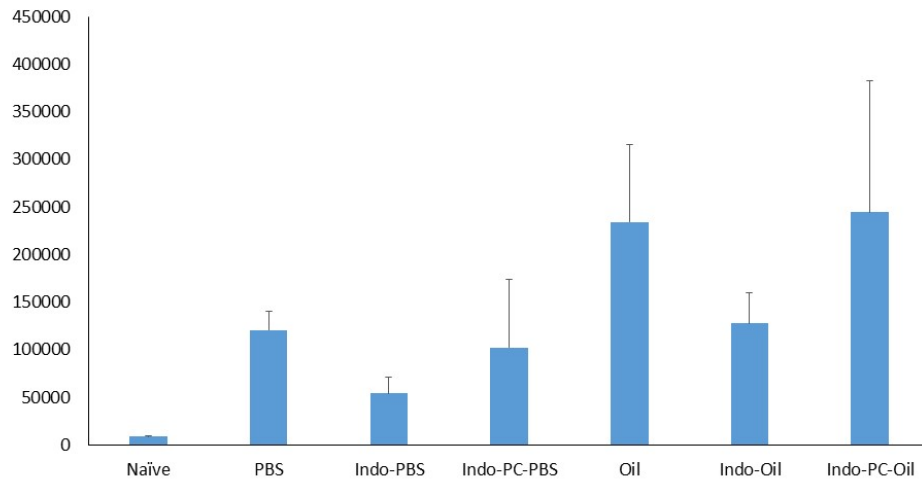


Fig. 10

N=4, mean \pm SE

% paw thickness from preinjury

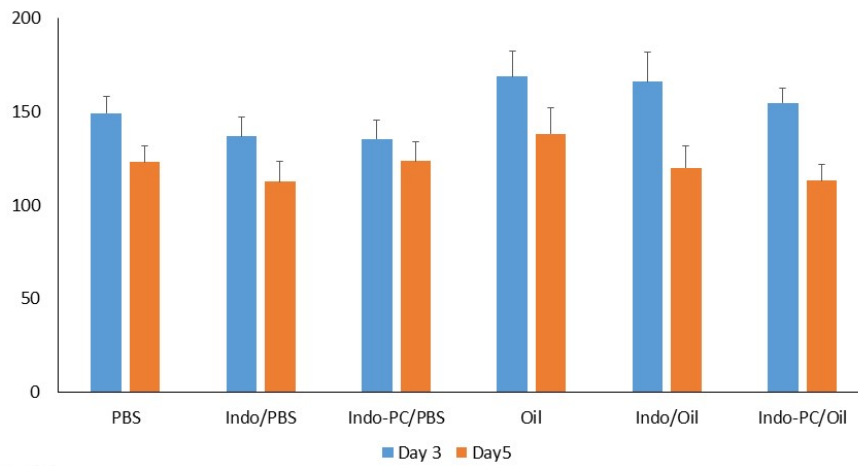


Fig. 11

Opportunities for training and professional development

Nothing to report.

Results dissemination

Nothing to report.

Goals for next reporting period

Studies will continue with the rodent hind limb burn injury model to test the efficacy of Indo, Indo-PC, Ibu, and Ibu-PC at relieving pain and promoting healing.

4. IMPACT:

Impact on principal discipline (pharmacological treatment of 2nd degree burns)

Results to date using animal models support further research into the use of parenteral and topical Indo-PC for treating 2nd degree burns. This product may provide analgesia while burn wounds are healing.

Impact on other disciplines

The topical and parenteral products under study may find applications in other areas of (non-burn) wound pain suppression or healing.

Impact on technology transfer

The topical and parenteral products under study are covered in patents held jointly by Dr. Lichtenberger and The University of Texas Health Science Center at Houston, and licensed to PLx Pharma LLC of Houston TX. PLx is in a position to develop the products for the US and global markets.

Impact on society

Nothing to report.

5. CHANGES/PROBLEMS:

We requested and received a 9 month no-cost extension on the ending date of this study period, which will allow us to complete all project tasks.

6. PRODUCTS:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Lenard Lichtenberger	Dexing Fang	Kaori Ono	Tri Phan
Project role	Principal Investigator	Senior Research Scientist	Research Associate	Senior Research Assistant
Researcher identifier				
Person-month worked	1.2	0.45	8.4	1.95
Contribution to project	Planned and directed all studies; wrote reports	Supervised staff; analyzed data	Performed animal studies including wound induction, drug dosing, behavioral testing and histological preparation	Assisted with burn wound induction and analyzed hematocrit and hemoglobin in fecal pellets
Funding support	This grant; NIH grants; state of Texas funds	This grant; NIH grants; state of Texas funds	This grant; NIH grants; non-profit grants	This grant; NIH grants; state of Texas funds

Lenard M. Lichtenberger

Closed Support

MDACC Moon Shot (PI, Lichtenberger) 10/22/2014-10/21/2015
0.24 calendar months \$25,000/yr

Evaluation of the chemopreventive activity of Aspirin-PC, using in vitro and in vivo models of colorectal cancer (CRC)

The major goal of this small grant is to evaluate Aspirin-PC for activity against colorectal cancer.

Overlap: There is no scientific or budgetary overlap with the current proposal.

MDACC Ovarian SPORE (PI, Lichtenberger) 09/01/2014-08/31/2015
0.24 calendar months \$23,100/yr

Use of Aspirin-PC alone and in combination with chemotherapeutic agents to treat ovarian cancer

The major goal of this small grant is to evaluate Aspirin-PC in combination with other chemotherapeutic agents for activity against ovarian cancer.

Overlap: There is no scientific or budgetary overlap with the current proposal.

New Active Support

R21 CA202751	(PI, Lichtenberger)	12/01/2015-11/30/2017
NIH/NCI	1.2 calendar months	\$125,000/yr

Phospholipids in saliva of cancer patients

The major goal of this R21 grant is to examine the hypothesis that cancer patients undergoing radiation therapy may sustain injury to the salivary glands resulting in a decrease in the concentration of salivary phosphatidylcholine (PC) and related surface active phospholipids below a critical level, and possibly an increase break-down products such as lyso-PC, which is a potent cytotoxic agent.

Overlap: There is no scientific or budgetary overlap with the current proposal.

Other organizations as partners

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS:

Use of Topical PC-NSAIDs to Treat Burn Injury and Pain USARMC 11203006; Award W81XWH-14-2-0016

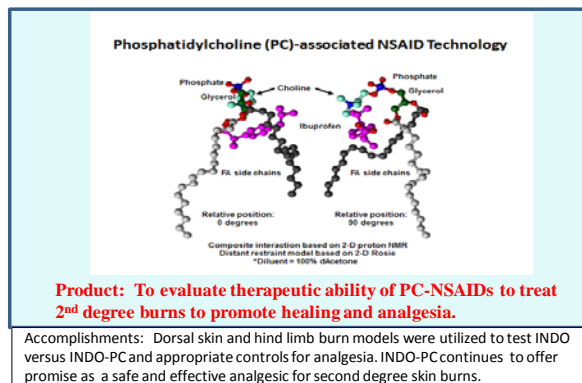
PI: Lenard M. Lichtenberger, PhD Org: The University of Texas Health Science Center at Houston Award Amount: \$449,470

Study/Product Aim(s)

- Compare the efficacy of topical Indomethacin-PC and Ibuprofen-PC (vs the respective unmodified NSAID and vehicle) in rodent models of 2nd degree burn injury, and determine any additional benefit of combined topical and parenteral administrations.
- Evaluate the GI side effects of PC-NSAID treatment in the burn model and the effect of our test drugs on clotting time
- Determine the mechanism of action of PC-NSAIDs in the treatment of burn pain/healing

Approach

This proposal will utilize two rodent models of burn injury in which animals are subjected to a controlled 2nd degree burn injury under general anesthetic, and the pain response, rate of healing, and biochemical factors can be followed using an array of tests. The two PC-associated NSAIDs, which will be administered either as a sterile topical or parenteral are Ibuprofen (IBU)-PC and Indomethacin (INDO)-PC.



Timeline and Cost

Activities	CY	13	14	15	16
Hind limb burn - test topical & parenteral PC-NSAIDs					
Dorsal skin burn-test topical and parenteral PC-NSAIDs					
Hind limb burn - optimal forms					
Dorsal skin burn - optimal forms					
Estimated Budget (\$K)		\$000	\$110	\$225	\$114

Updated: July 2016

Goals/Milestones

CY14 Goals – Hind limb/dorsal skin burn models

- Test topical and parenteral formulations of Indomethacin-PC
- Test topical and parenteral formulations of Ibuprofen-PC

CY15 Goal – Hind limb/dorsal skin burn models

- Optimize formulations

CY16 Goal – Continue studies using hind limb burn model; Project completion

- Select optimal burn formulation

Comments/Challenges/Issues/Concerns

- Animal studies are ongoing.

Budget Expenditure to Date

Projected Expenditure: \$449K

Actual Expenditure: \$263.2K

9. APPENDICES: None