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

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14. ABSTRACT

Studies have shown that the rat dorsal skin and hind limb burn injury models of 2nd degree burns are appropriate for testing the analgesic and healing rate efficacy of test drugs. These models were used to test Ibuprofen (Ibu), Ibuprofen associated with phosphatidylcholine (Ibu-PC), Indomethacin (Indo), and Indo-PC. The experimental drug Indo-PC showed analgesic and anti-inflammatory efficacy over Indo alone or the Ibu drugs, notably when administered parenterally without notable side-effects. These studies support a rationale for continued development of parenteral Indo-PC for treatment of 2nd 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, as an alternative to drugs acting on the CNS. 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, PC-NSAIDs, which have been shown in other experimental systems to reduce inflammation and pain and promote healing with reduced gastrointestinal (GI) toxicity. 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 PC-NSAID treatment in the burn model and the effect of test drugs on clotting time.
3) Determine the mechanism of action of PC-NSAIDs in the treatment of burn pain/healing.

Milestones and target dates:

1) Complete testing for topical and iv Indo, Indo-PC, Ibu and Ibu-PC in hind limb burn injury model – target was month 6 – 100% complete
2) Complete testing for topical and iv 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 iv NSAID-PCs in hind limb burn injury model – target was month 15 – 100% complete
4) Complete testing for topical vs iv 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 – 100% 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 PC-NSAIDs on thrombus formation and clotting time – target was month 18 – 100% complete

8) Assessment of COX inhibition and effects on inflammation as a mechanism for PC-NSAID efficacy – target was month 24 – 100% complete
9) Assessment of skin hydrophobicity and histology as a mechanism for PC-NSAID efficacy – target was month 24 – 100% complete

Accomplishments:

1) Major activities: During this project period, the efficacy of Indo, Indo-PC, Ibu and Ibu-PC were evaluated in the dorsal skin burn model and the hind limb burn model. Indo-PC produced significant analgesia in both models, notably when administered parenterally. Biochemical measures and histology supported the finding with Indo-PC.
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.
3) Significant results:
   There were two 2nd degree burn injury models used in this grant, both of which are described separately below, along with pertinent results.
   Dorsal skin burn injury model. This animal (rat) protocol consisted of initially performing baseline behavioral testing of rat hind paw withdrawal to von Frey filament stimulation (explained below). Then the animals were administered the dorsal burn wound (1 cm glass surface heated to 99°C for 45 seconds) under anesthesia and were maintained for two days with treatments of buprenorphine for acute pain. Experimental drugs treatments were begun two hours after initiation of the burn wound, and continued daily until the termination of the study. At days 3, 5, 10 and 15 post-burn, the animals were again tested for sensitivity to a stimulus (von Frey hair of varying stiffness) on a hind paw. This test was to determine whether any treatment could reverse burn-induced hyperalgesia (hypersensitivity to pain). Also at days 3, 5, 10 and 15 post-burn, the burn wound size was measured by caliper to determine the rate of healing of the wound. In addition at these times during the 15 day treatment period, samples of fecal pellets were collected for determination of hemoglobin as a measure of gastrointestinal (GI) bleeding into the intestinal lumen which is a potential side effect of NSAIDs. Finally, at euthanasia, the stomach, intestines and colon were examined macroscopically for signs of lesions or adhesions which are GI manifestations of NSAID-induced injury, and samples of the burn wound were processed for histological examination. This model causes hyperalgesia on day 3 and day 5 (a reduction in hind limb withdrawal threshold), so to simplify presentation of results, only data for those two days are shown.

   For behavioral testing of pain with the dorsal skin burn model, animals were placed in a special cages so that one hind paw was exposed to stimulations with up to eight different force von Frey hairs using the Dixon up/down method. The mechanical threshold of force that the animal responds to 50% of the time was used to calculate the percentage of pre-injury value. Thus in the data graphs, a lower score after injury (less than 100%) means more sensitivity to pain

(hyperalgesia) and a higher score (greater than 100%) means less sensitivity to pain (analgesia).

In the first series of animal studies, Indomethacin or Indo-PC at 2 mg/kg (Figure 1), or Ibuprofen or Ibu-PC at 20 mg/kg (Figure 2) was administered *parenterally (subcutaneous)*. Only Indo-PC significantly provided analgesia compared to PBS-treated. Neither Indo nor the Ibuprofen drugs were able to reduce hyperalgesia. It should be noted, in this and subsequent figures we focused on behavioral and biochemical measurements taken on day-3 and day-5 post-burn injury, as the animals showed no evidence of burn-induced pain (hyperalgesia) at day-10 and day-15.

Figure 1. Indo/Indo-PC administered sc.

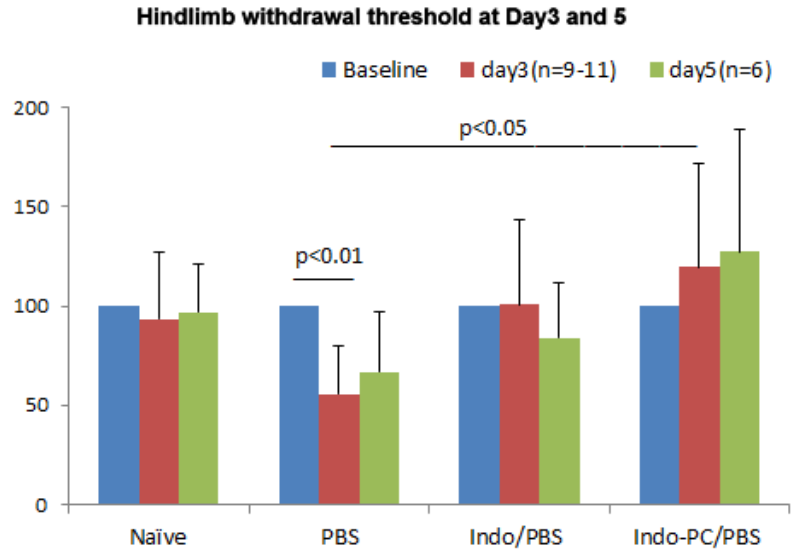
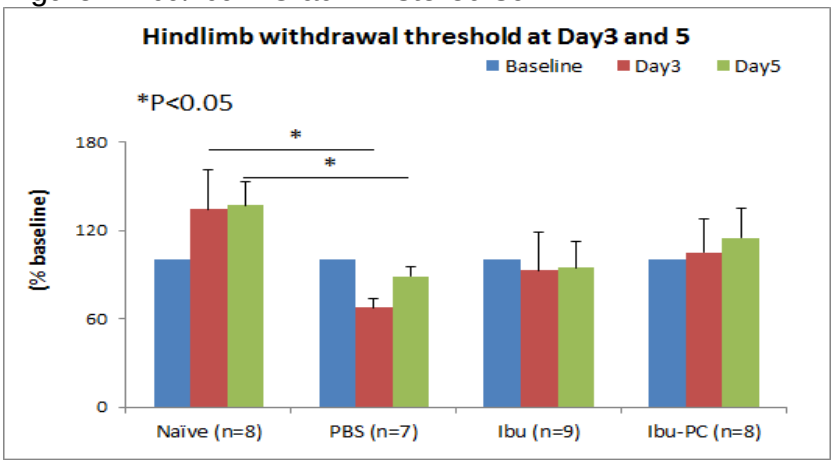


Figure 2. Ibu/Ibu-PC administered sc.



In a second series of animal studies, Indomethacin or Indo-PC at 2 mg/kg (Figure 3), or Ibuprofen or Ibu-PC at 20 mg/kg (Figure 4) were administered *topically*. Topical oil was used as a control. None of the Indomethacin or the Ibuprofen drugs were able to provide consistent analgesia through the topical route.

Figure 3. Indo/Indo-PC administered topically.

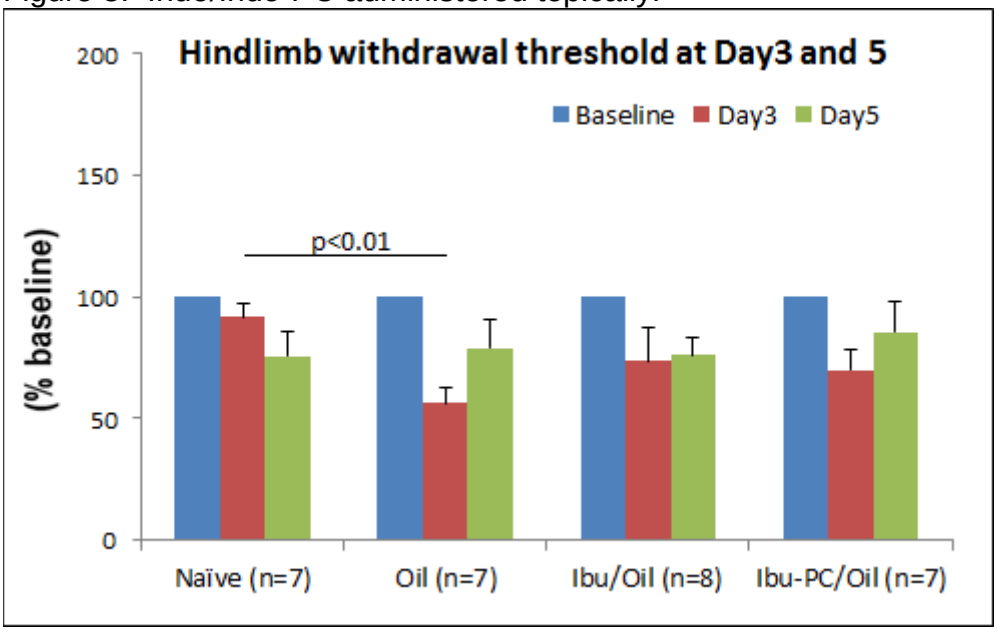
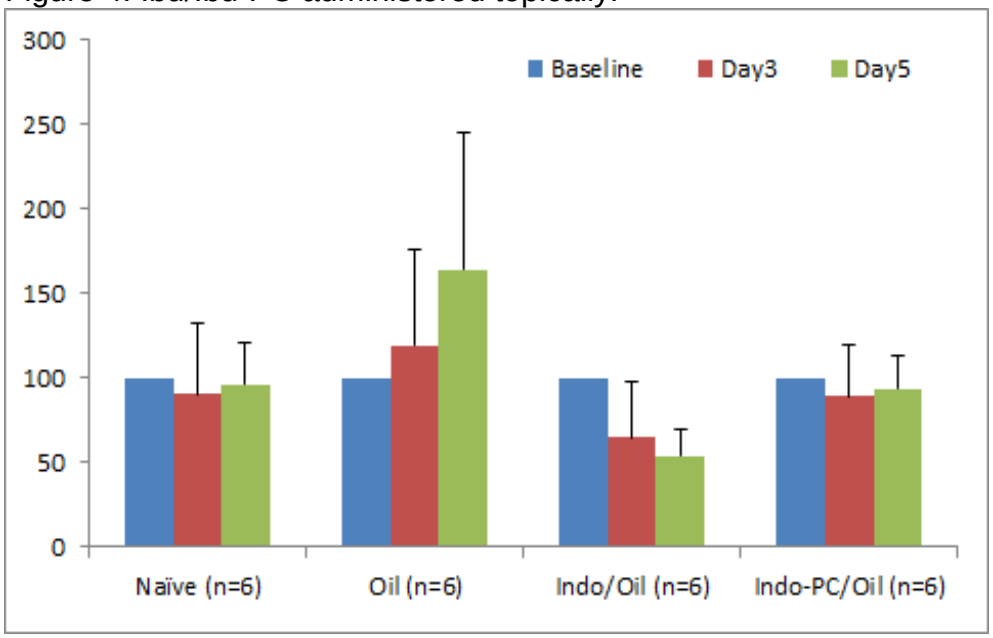
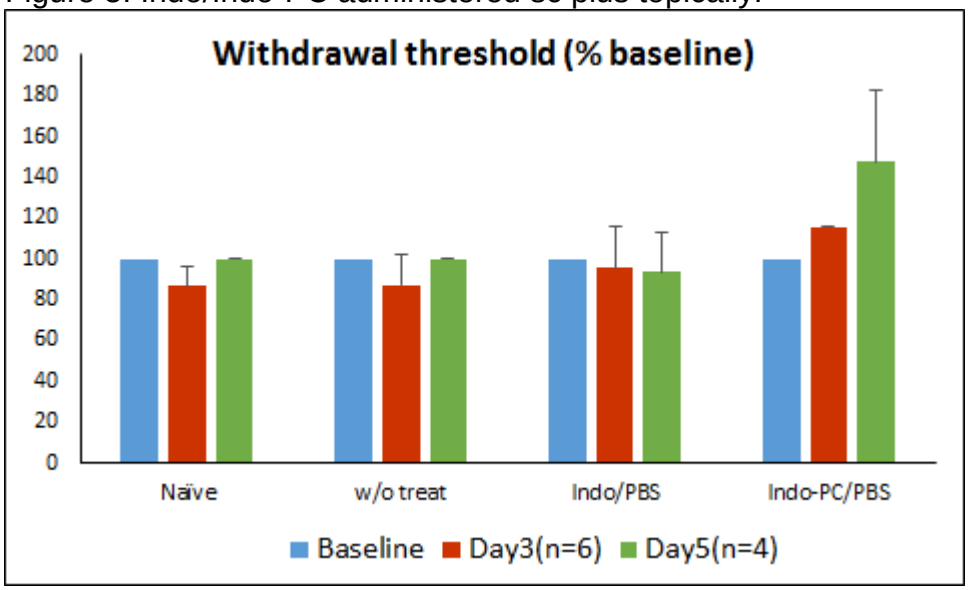


Figure 4. Ibu/Ibu-PC administered topically.



In a third series of animal studies, to determine whether there was a benefit from administering the test drugs by a combination route of *topical plus subcutaneous*, only Indo and Indo-PC were tested (Figure 5). Neither of the Indo drugs gave significant improvement by this combination route, although Indo-PC had a tendency for analgesia.

Figure 5. Indo/Indo-PC administered sc plus topically.



To further investigate the burn injury process through a biochemical measure, we examined the effectiveness of both Ibu/Ibu-PC and Indo/Indo-PC on 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 (Figure 6), MPO activity tended to be elevated over naïve (pre-burn) controls and suppressed in both Ibu-PC and Indo-PC treatments. This result confirms the anti-inflammatory activity of these PC-NSAID test drugs.

Figure 6. Ibu/Ibu-PC and Indo/Indo-PC on MPO activity.

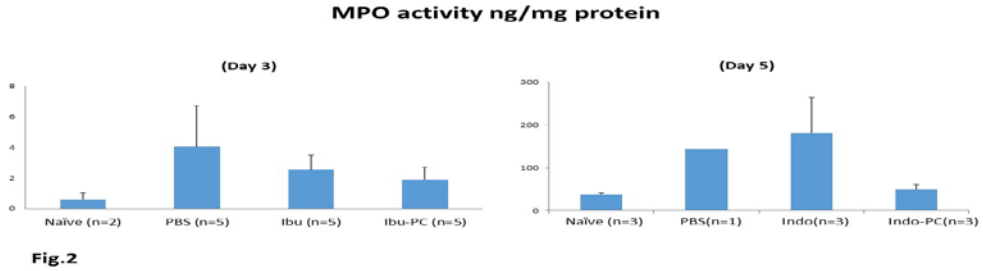
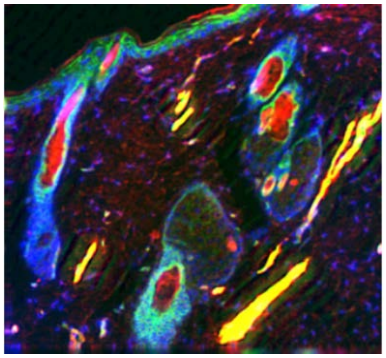


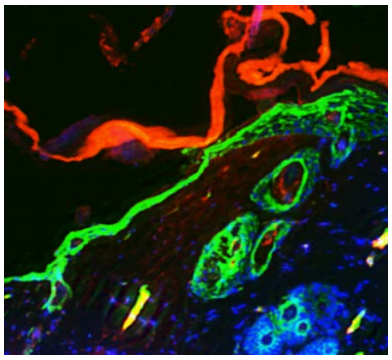
Fig.2

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), Indomethacin (2 mg/kg, topical) and Indomethacin-PC (2 mg/kg, topical). His analysis (Figure 7) shows that both Indomethacin drugs have some capacity to protect or promote healing of the burned tissue, as indicated by the maintenance of the surface stratum corneal layer.

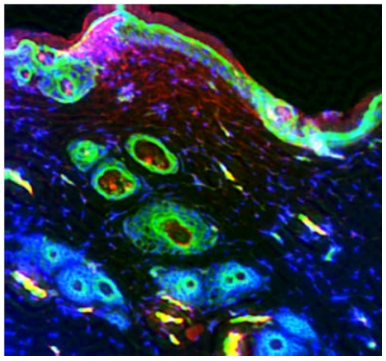
Figure 7. Fluorescent images of 2nd degree burn injury.



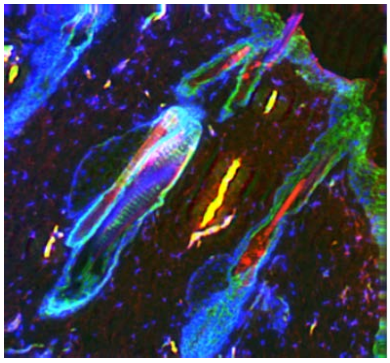
Normal Skin



Burned - PBS



Burned -Indo (topical)



Burned -Indo-PC (topical)

Green = f-actin (phallocidin);

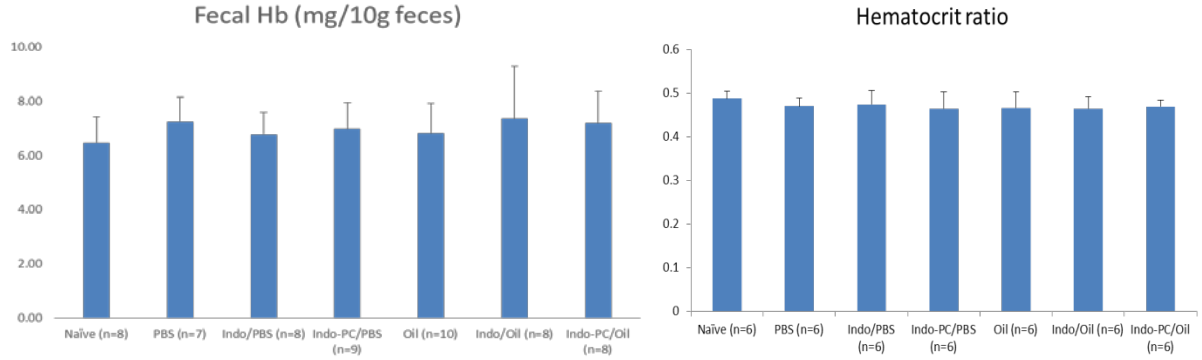
Red/Orange = smooth muscle actin (Texas Red);

Yellow = overlap, Texas Red+f-actin;

Blue = nuclei (DAPI)

The potential for NSAID-induced GI bleeding in the dorsal skin burn model was evaluated and is shown in two parts of Figure 8. The left figure shows bleeding into fecal pellets collected just prior to euthanasia at day 15, where no differences among groups are evident. The right figure shows the blood hematocrit at that time, where again, no differences among groups are seen. These results show that the doses of Ibu and Indo used, did not produce any clinically significant GI bleeding. These results are consistent with the finding of no lesions, adhesions or perforations upon examination of stomach and intestines at euthanasia in the animals administered the test NSAIDs either parenterally (graph bars 2-4) or topically (graph bars 5-7). Very similar negative results were obtained with the hind limb burn model.

Figure 8. Measures of potential GI bleeding.



<u>Hind limb skin burn injury model.</u> Using a second burn model to induce 2nd degree injury, rats were subjected to a 20 second exposure of their left hind paw to water at 57°C. Treatments and sample collections were as described for the previous burn model. Also, inflammation of the affected area was analyzed by use of a fluorescent probe as described below.

Because the location of the burn was on the hindpaw, for behavior testing we used a Girdle testing method (lumbar region) to evaluate the spontaneous pain/hyperalgesia reaction. Rats were placed in a Plexiglass restraining device to be acclimated, then 2G and 26G of Von Frey filament was pressed on their lumbar site. Results were determined by counting the vocalization number. The data is presented as average number of reactions to the stimulations. Thus, a higher value means more sensitivity to perceived pain, and a lower value means less sensitivity (analgesia).

In the first series of animal studies on this hind limb burn model, Indomethacin or Indo-PC at 2 mg/kg (Figure 9), or Ibuprofen or Ibu-PC at 20 mg/kg (Figure 10) was administered *parenterally (subcutaneous)*. Both Indo and Indo-PC significantly provided analgesia compared to PBS-treated, which was most clearly seen when we used the thicker 26G fiber. The Ibuprofen drugs were not able to consistently reduce hyperalgesia.

Figure 9. Indo/Indo-PC administered sc.

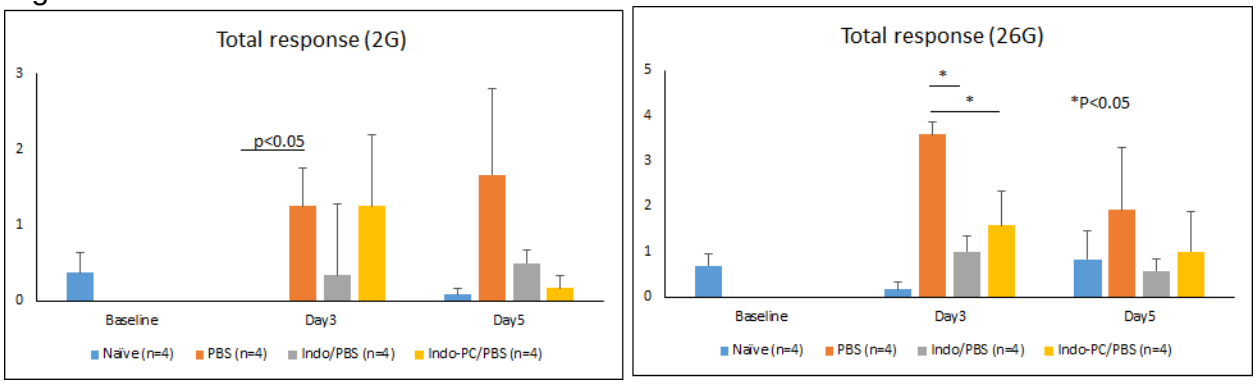
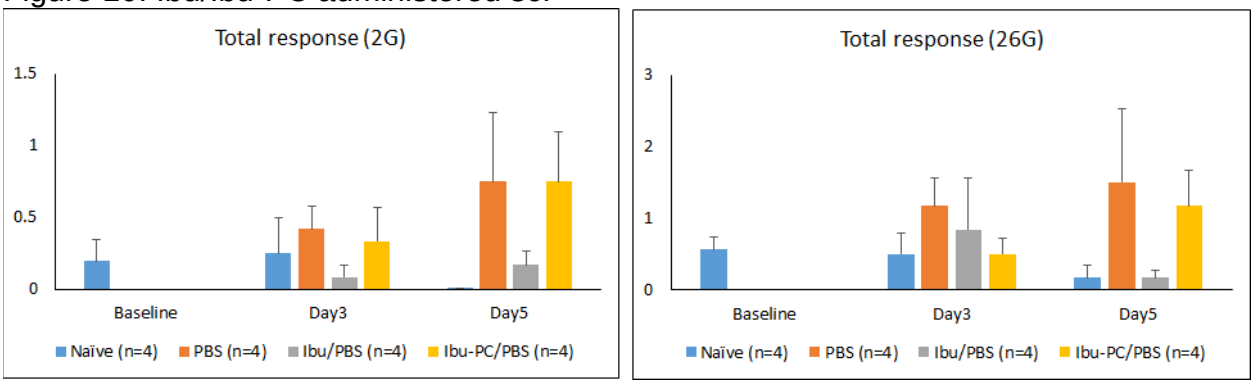


Figure 10. Ibu/Ibu-PC administered sc.



In a second series of animal studies using the hind limb burn model, Indomethacin or Indo-PC at 2 mg/kg (Figure 11), or Ibuprofen or Ibu-PC at 20 mg/kg (Figure 12) were administered *topically*. Topical oil was used as a control. While there was a tendency for the Indo-PC to reduce pain perception, none of the Indomethacin or the Ibuprofen drugs were able to provide a statistically significant analgesic response through the topical route.

Figure 11. Indo/Indo-PC administered topically.

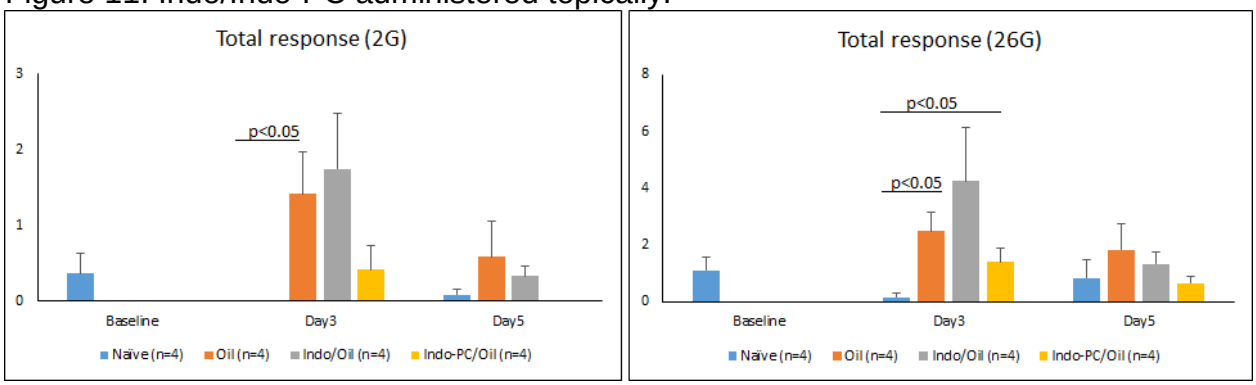
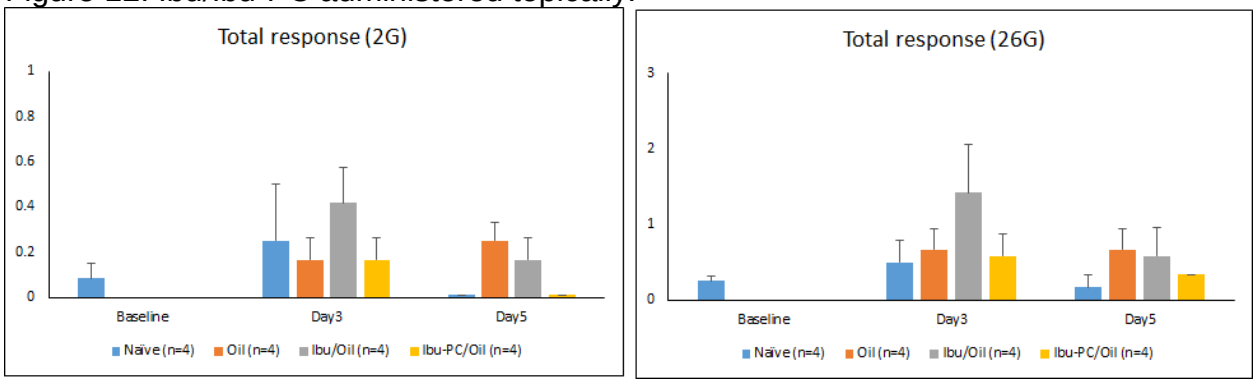
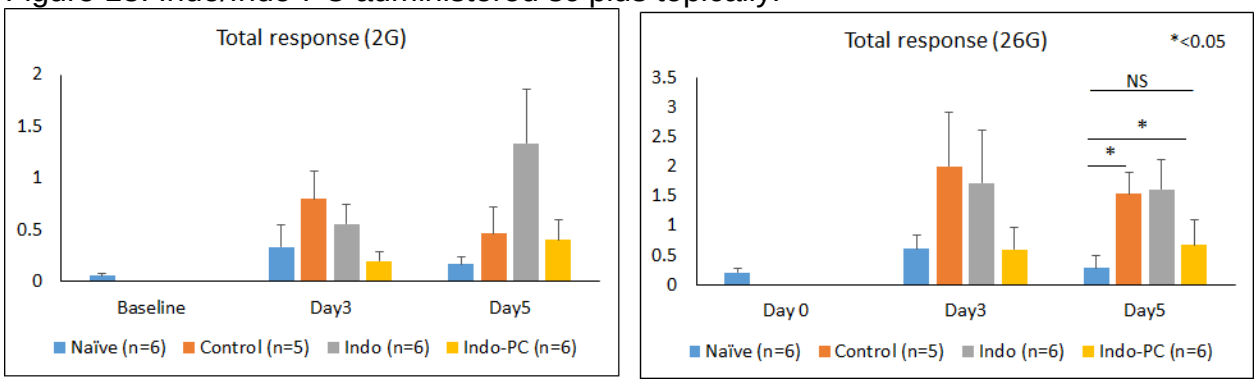


Figure 12. Ibu/Ibu-PC administered topically.



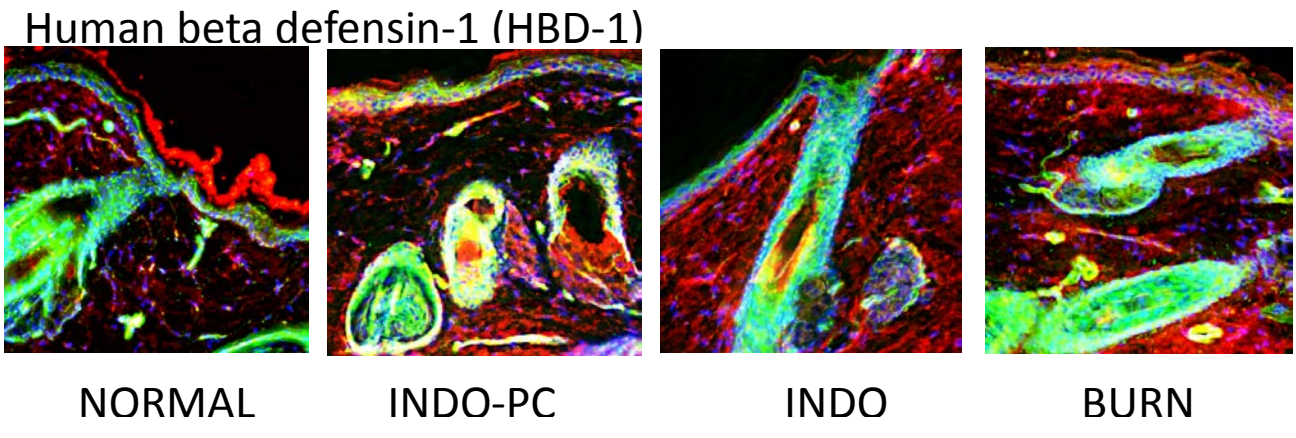
In a third series of animal studies using the hind limb model, to determine whether there was a benefit from administering the test drugs by a combination route of *topical plus subcutaneous*, only Indo and Indo-PC were tested (Figure 13). Neither of the Indo drugs gave significant improvement by this combination route, although Indo-PC had a tendency for analgesia.

Figure 13. Indo/Indo-PC administered sc plus topically.



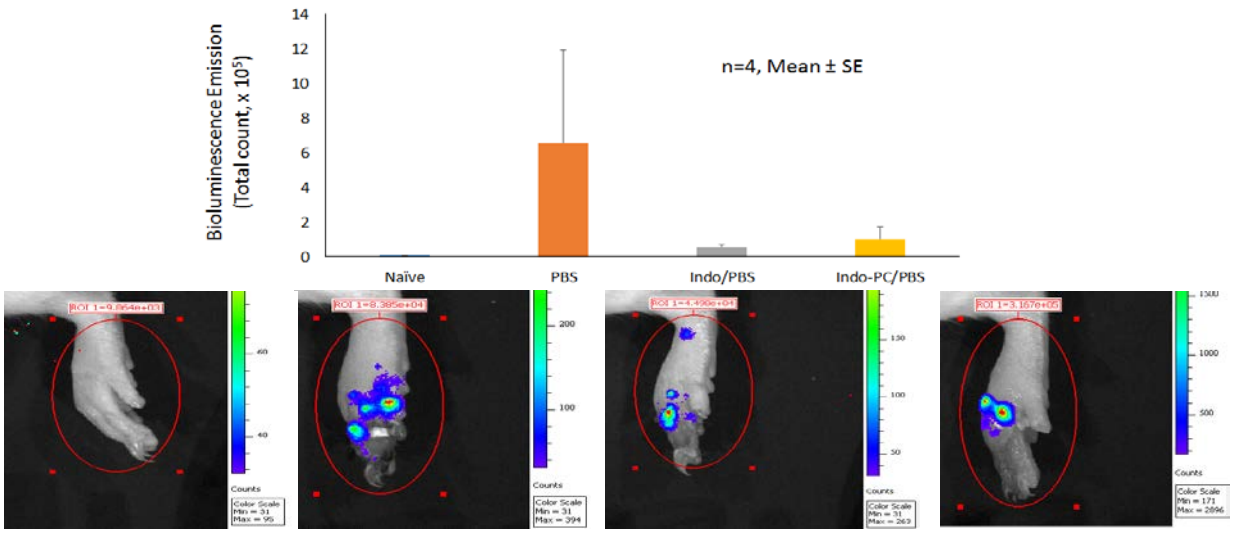
A possible cellular mechanism for Indo/Indo-PC benefits was investigated by imaging the presence of several endogenous antimicrobial proteins (AMPs) that have been linked to recovery of dermal injury. Human beta defensin-1 and -3 (also found in rodents) were evaluated by immunohistochemistry and a sample of results is seen in Figure 14. In brief, the Indo-PC treatment maintained the dermis most similar to the normal tissue. A more detailed description is that in non-burned skin there is the presence of HBD-1 (red-orange) in the corneum (dense), the germinativum, the spinosum and in diverse papillary dermis cells. This is again the pattern with INDO-PC, although there is a reduction of surface HBD-1. With INDO alone, there is a definite lack of surface defensin, but a much greater amount in the dermal layers. Skin tissue dissected from animals in the untreated burn group shows an intermittent loss of epidermis and disrupted and broken dermal structures such as capillaries, some hair shafts, and ducts, This observation of increased sub dermal HBD-1 has been reported previously as a result of burn injury, and supports our experimental findings with this approach. These results suggest a role for HBDs in recovery from burn injury and treatment with Indo-PC.

Figure 14. Immunohistochemical analysis of HBD-1 in hind paw burn injury.



In order to evaluate 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 Figure 15 where yellow and red indicate greater inflammation and blue is the least. It is apparent that both Indo and Indo-PC were able to reduce paw inflammation, although statistical significance was not reached.

Figure 15. Fluorescent analysis of inflammation in hind paw burn injury.



To assess the possibility that either Indo or Indo-PC may have an effect on bleeding time due to the drugs' anti-platelet activity - which could be a limiting side effect, measures of bleeding-time were made at euthanasia on the rats in both the burn models. The time (in seconds) was determined for bleeding to stop following a small tail-cut. The results in Figure 16 (left) show that the times varied over a small range (300-400 sec) and were not different between groups for the hind limb model. The dorsal skin model (right) showed a slight increase in bleeding time for both Indo drugs, although the values are within the same range as the hind limb model and was not considered relevant.

Figure 16. Bleeding times for Indo/Indo-PC.

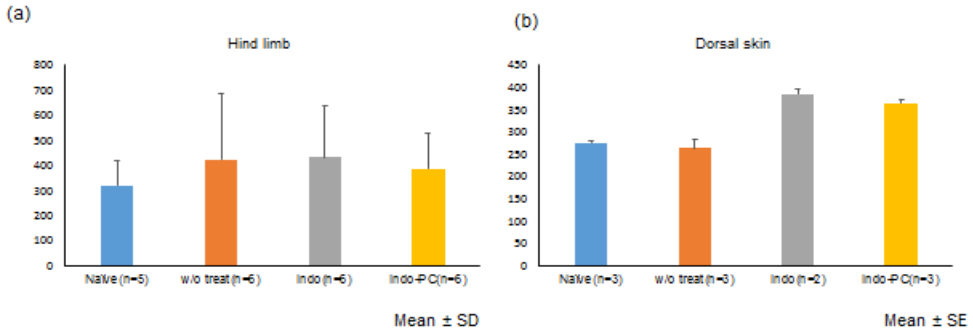


Fig 4. Bleeding time (sec.)

Summary: Of the four drugs tested in the two 2nd degree burn rodent model systems, only Indo-PC showed consistent significant efficacy at reducing pain perception and inflammation when administered subcutaneously, without causing significant GI bleeding or affecting clotting time. Indo-PC is therefore, a good candidate for further development in the treatment of this burn injury.

Opportunities for training and professional development

Nothing to report.

Results dissemination

Nothing to report.

Goals for next reporting period

Nothing to report (end of grant).

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 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:

Changes in use of vertebrate animals

There was one request for a change in route of drug administration and several requests for the use of more animals than originally anticipated. All of these requests were within the scope of the original application and were approved by the institutional IACUC and by ACURO. The dates of these approvals are as follows.

- IACUC approval for grant project 22-Oct-2014
- ACURO approval for grant project 19-Nov-2014
- IACUC amendment (use of sc injections) approval 12-Nov-2015
- ACURO amendment approval 12-Nov-2015
- IACUC amendment (addition of animals) approval 4-Apr-2016
- ACURO amendment approval 13 Apr 2016
- IACUC amendment (addition of animals) approval 9-Dec-2016
- ACURO amendment approval 17-Jan-2017
- IACUC amendment (addition of animals) approval 31-Mar-2017
- ACURO amendment approval 5-Apr-2017

6. PRODUCTS:

Publications, conference papers, and presentations

In order to report the research results obtained from this grant, a presentation was made by the grant Principal Investigator, Dr. Lichtenberger, at the JCP-8 Pain Management In-Progress Review in Ft. Detrick MD on Oct 13, 2016. The title of the presentation was “Use of Topical PC-NSAIDs to Treat Burn Injury and Pain”.

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

Change in other support

Nothing to report.

Other organizations as partners

Nothing to report

SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS:

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

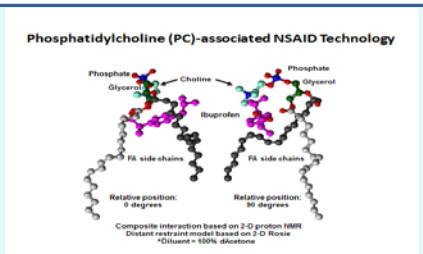
PI: Lenard M. Lichtenberger, PhD **Org: The University of Texas Health Science Center at Houston** **Award Amount: $449,470**

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.



Product: To evaluate therapeutic ability of PC-NSAIDs to treat 2nd degree burns to promote healing and analgesia.

Accomplishments: Studies compared single and combination topical and parenteral treatments of INDO, INDO-PC, IBU and IBU-PC on dorsal skin and hind limb burn models . INDO-PC had the most beneficial effects on both models..

Timeline and Cost

Activities CY	14	15	16	17
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: Oct 2017

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 – Project completion
☐ Select optimal burn formulation
Comments/Challenges/Issues/Concerns
- Studies are completed.

Budget Expenditure to Date
Projected Expenditure: $449K
Actual Expenditure: $449K

8. APPENDICES: None