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Infections of Severe Burns and Wounds

PRINCIPAL INVESTIGATOR: Jerry A. Nick, MD

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**14. ABSTRACT**

Findings to date support the hypothesis that a dual therapeutic approach of targeted anti-inflammation and a biofilm specific antibiotic will significantly limit severe *Pseudomonas aeruginosa* infection associated with serious burns and wounds. A 12-mer N2 peptide was synthesized to bind and competitively inhibit $IgM^{CM-22}$ that recognizes self antigens on the nonmuscle myosin heavy chain II (NMHC-II) that are exposed at the time of injury. An optimal dosing strategy was determined that utilizes the combination of topical administration at the burn site and intravenous tail vein injection. The effect of N2 peptide in limiting neutrophil influx to the injury site was dramatic at 4 hours, however, the effect was no longer present by 48 hours, despite repeated N2 dosing. Nonetheless, the initial disruption of neutrophil influx was sufficient to significantly reduce inflammatory changes at the histological level, and gross tissue injury 48 hours later. When *P. aeruginosa* was applied to the burn site 2 hours after injury, the N2 peptide demonstrated a similar effect in reducing early inflammation, but not in reducing later neutrophil influx despite repeated dosing. However, this effect was sufficient to result in a significant reduction in *P. aeruginosa* infection of the burn. These results prove that by briefly stopping the influx of neutrophils to the site of thermal injury, the subsequent wound severity is decreased and growth of *P. aeruginosa* delayed. With the addition of azithromycin into this model, we fully expect that this infection reducing strategy will be substantially improved.

**15. SUBJECT TERMS**

Burn, Wound, *Pseudomonas aeruginosa*, neutrophil, biofilm

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**Year 1 Progress Report**
**DR080371: Targeted Prevention or Treatment of Bacterial Biofilm Infections of Severe Burns and Wounds**

**Introduction:** Persistent infection of severe wounds, and burns in particular, represents a significant cause of deployment-related morbidity and mortality. Inability to successfully treat wound and burn infections relates to the capacity of the bacteria to form a **biofilm**[1]. In patients with severe burns, 75% of deaths will occur from sepsis or infectious complications, with *P. aeruginosa* accounting for over half of all burn infections. In the setting of thermal injury, an intense inflammatory response is universally present, culminating in massive recruitment of **neutrophils** to the tissue[1]. In both thermal and reperfusion injury, vascular spasm and impaired blood flow to the site is followed by reperfusion and vascular leak. Importantly, this tissue injury uncovers **self antigens** on the nonmuscle myosin heavy chain II (NMHC-II) that are recognized by a specific natural IgM subclass (IgM$^{CM-22}$), as an early response of the innate immunity (**Figure 1**). Binding of IgM$^{CM-22}$ to NMHC-II triggers the complement cascade, and the subsequent recruitment of neutrophils to the site of injury[2]. Systemic or local administration of a 12-mer synthetic peptide (N2) analogous to NMHC-II is capable of binding and competitively inhibiting IgM$^{CM-22}$, thus greatly reducing inflammation at the site of the wound[2]. Excessive neutrophil accumulation, combined with impaired clearance of the dead and dying cells, is clearly linked to tissue damage. However, recent reports have demonstrated that neutrophil products can accelerate *P. aeruginosa* biofilm formation[3]. As neutrophils undergo necrosis, long strands of DNA and F-actin are released into the inflammatory milieu, and polymerize through covalent attraction. Recently it was reported that *P. aeruginosa* can exploit the neutrophil-rich environment by utilizing these polymers as a scaffolding, significantly enhancing early biofilm formation[3]. A strategy to limit local neutrophil influx, without systemically suppressing the immune system, represent as novel approach to both **prevent** biofilm formation, and possibly as an adjuvant to medically **treat** an early biofilm infection. Concurrent with reduced tissue inflammation, a different approach to antibiotic therapy is proposed. Macrolide antibitoics such as azithromycin have now been demonstrated to have a potent antimicrobial effect when *P. aeruginosa* is allowed to form a biofilm[4]. Azithromycin has excellent penetration into tissues, as well as potent intrinsic anti-inflammatory properties independent of its function as an antimicrobial. Therefore, we will test the effect of a dual therapeutic approach combining a targeted anti-inflammatory with a biofilm specific antibiotic to significantly reduce local and systemic infection associated with serious burns and wounds.
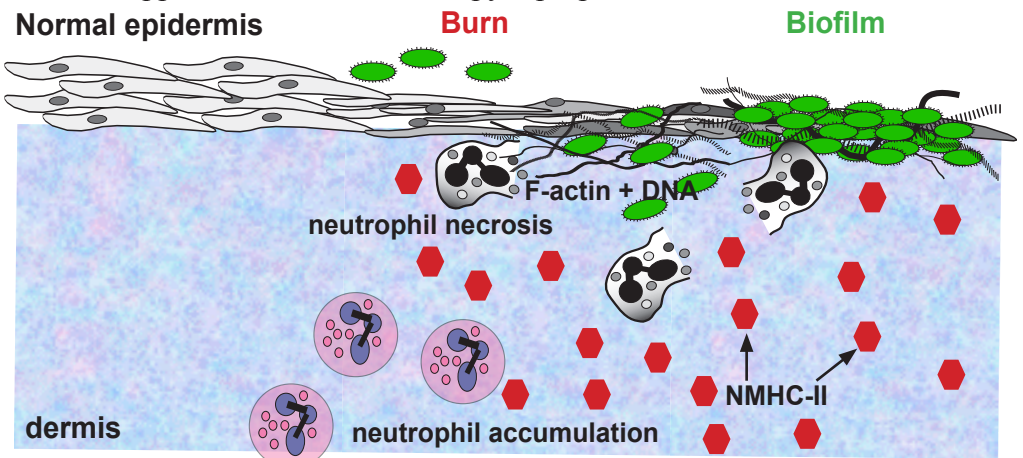


**Figure 1: Excessive neutrophil accumulation enhances *Pseudomonas* biofilm formation in burns and wounds.** Tissue damage exposes the self-antigen NMHC-II (red polygons), which is bound by IgM$^{CM-22}$, resulting in activation of the complement cascade. The ensuing recruitment of massive quantities of neutrophils overwhelms mechanisms to clear dying cells from the tissues. DNA and F-actin released from necrotic neutrophils form polymers, which serve as a scaffolding for *P. aeruginosa* and accelerates the formation of biofilms.

**Body:**

Over the first 12 months of this proposal, we have made significant progress towards the completion of all stated Aims within the approved Statement of Work. Results of the experiments to date have supported the validity of the Central Hypothesis. Important findings towards the completion of this project are presented below, and organized as they relate to the proposed Tasks and Milestones within the Statement of Work, which are unchanged from the original application.

**Task 1:** Test the capacity of N2 peptide with and without azithromycin to <u>prevent</u> *P. aeruginosa* wound infection. (**Timeframe 1-10 months**)

> *The purpose of this task is to assess the capacity of an anti-inflammatory strategy, combined with a biofilm targeting antibiotic, to prevent P. aeruginosa biofilm formation.*

**Milestone 1:** Synthesis of N2 peptide and 12-mer scrambled peptide (control). (**Timeframe 1 month**)
**Progress:** The N2 peptide and 12-mer scrambled control peptide have been successfully synthesized in sufficient quantities to complete the entire proposal.

**Milestone 2:** Institutional Review of modification to existing animal protocols. (**Timeframe 1-2 month**).
**Progress:** All required amendments to our existing protocol (AS2751_04_10) were approved by the National Jewish Health IACUC in December 2009, and the corresponding approval by the ACURO was completed in January 2010. In April 2010, the original protocol was required to undergo a routine Triennial Review, which mandated the entire protocol be rewritten and re-reviewed. The new protocol (AS2751_03_13) was approved by the NJH IACUC in April 2010. As a result of this Triennial Review, the protocol was then required to be re-reviewed by ACURO. The current protocol received re-approval in June 2010. Given the sensitive nature of inflicting a burn, followed by infection with *P. aeruginosa*, we were aware that the procedure would received considerable scrutiny. Nonetheless, the entire time involved in meeting regulatory requirements resulted in some delays in meeting proposed milestones. Currently all proposed experiment have been approved by the NJH IACUC and the ACURO, and we do not anticipate further regulatory review between now and the completion of the Project.

**Milestone 3:** Test the effect of <u>pre-administration</u> of N2 peptide (i.v.) and/or (topical) to reduce post-burn *P. aeruginosa* wound infection. (**Timeframe 2-6 months**)
**Progress:** The purpose of these proposed experiments was to define the conditions by which N2 peptide evokes the greatest anti-inflammatory effect. Comparisons were made between i.v. and topical administration, as well as the potential for an additive effect of the two administration methods combined. **The goal** was determination of
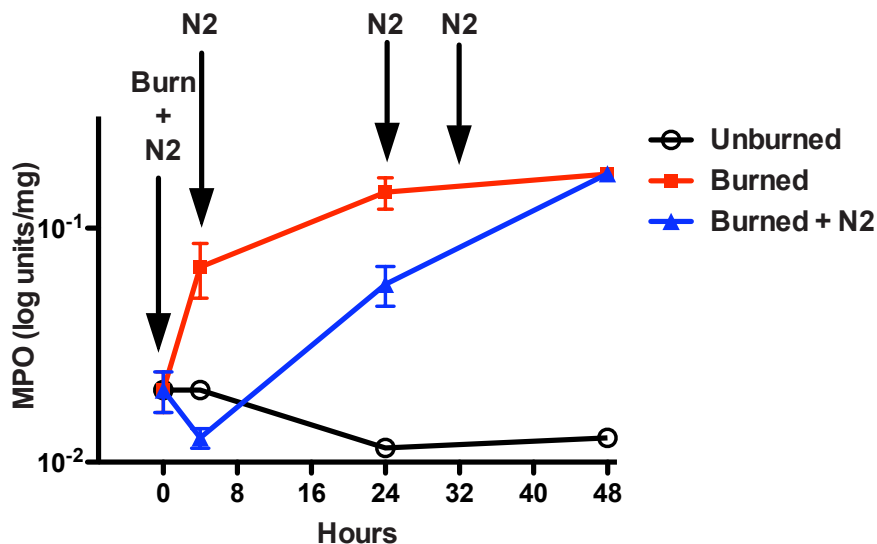
**Figure 2: Effect of N2 peptide in reducing neutrophil accumulation to the skin following thermal injury.** Animals burned at time 0 received N2 peptide administered IV through tail vein injection and topically at the site of the burn. The agent was administered at the times depicted by arrows. Neutrophil accumulation was assessed by MPO activity in skin homogenates at the burn site. After 4 hours, the N2 peptide dramatically reduced neutrophil recruitment to the burn, but this effect was lost over time, despite repeated administration of the compound. The effect of the N2 administration over the entire 48-hour period studied was significant, with $p=0.0023$ by two-way ANOVA.
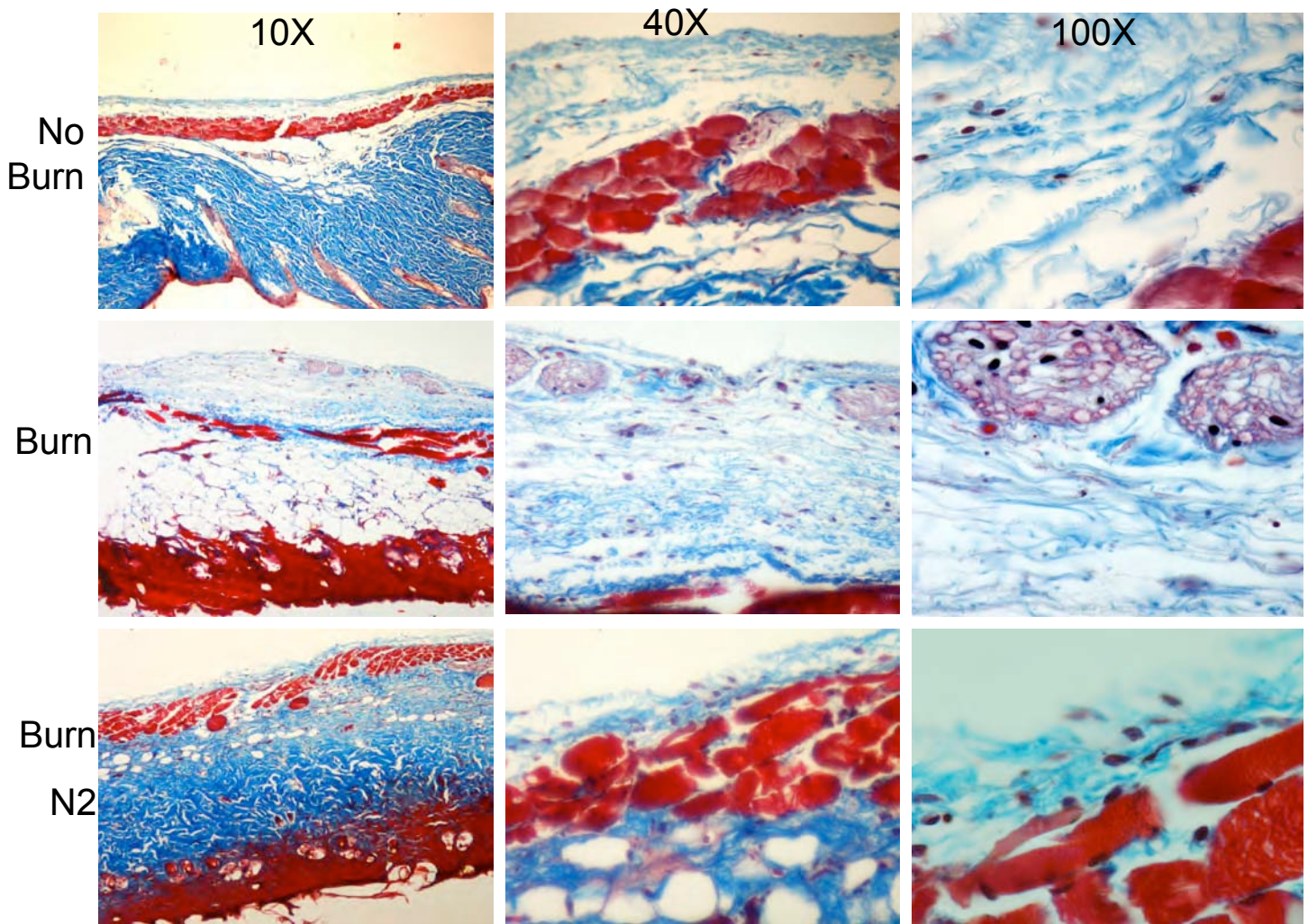
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**Figure 3: Effect of N2 peptide in reducing subcutaneous inflammation following thermal injury.** Animals burned at time 0 received N2 peptide administered IV through tail vein injection and topically at the site of the burn. Photomicrographs depict histological changes 4 hours following the burn for a representative animal that was shaved but not burned (top row), burned but received no compound (middle row), or burned and received N2 at the time of the burn. Magnification is as labeled for each column. Results validate the findings of the MPO assay (**Fig. 2**), as neutrophil accumulation is not present in the N2 treated animals. In addition, these results indicate that N2 also results in nearly complete elimination of tissue edema and other morphologic changes of the burn, suggesting that these early events are neutrophil-mediated. Figures representative of 3 animals in each group, with areas of injury that were typical.

the optimal method of N2 administration associated with suppression of inflammation of neutrophil accumulation to the skin (assayed by MPO and histology), with the most effective dose and delivery of N2 peptide to be utilized in subsequent tasks of this proposal.

N2 peptide works to bind and competitively inhibit IgM$^{CM-22}$ that recognizes self antigens on the nonmuscle myosin heavy chain II (NMHC-II) that are exposed at the time of injury. In pilot experiments, we determined that administration of the N2 peptide prior to the burn afforded no protective advantage, as the injury had not yet occurred, and IgM$^{CM-22}$ was not yet present. Thus, N2 peptide can be administered at the time of the injury with maximal effectiveness, which would actually represent a more plausible use of the agent in the setting of combat.

As proposed, a series of time courses and dose response assessments of I.V. and/or topical application of N2 were conducted. We determined that when administered in the setting of the thermal injury alone, the optimal delivery of N2 was the combination of both the I.V. (200ul of 40uM solution) and topical administration (200ul of 40uM). Best results were seen when the topical N2 was combined with DMSO to allow for rapid subcutaneous absorption. With these doses, the animals tolerate the agent without apparent toxicity. However, at higher doses we observed that the ears of the animals underwent necrotic changes. In response to the thermal injury alone, the anti-neutrophil effect of N2 is profound at 4 hours, but even with repeated dosing this effect is no longer present

No Burn     Burn     Burn+ N2

**Figure 4: Effect of N2 peptide in reducing gross tissue damage following thermal injury.** Animals burned at time 0 received N2 peptide administered IV through tail vein injection and topically at the site of the burn. Photographs demonstrate gross tissue injury 48 hours the burn for cohorts of four animals that were shaved but not burned (left), burned but received no compound (middle), or burned and received N2 at the time of the burn (right). While significant ulceration and eschar formation is evident in the burned animals, the extent of gross injury is clearly less in the group receiving N2, despite the relatively short-duration of anti-neutrophil effects at the site of the injury.

by 48 hours (**Figure 2**). However, this early anti-neutrophil is also associated with a broader anti-inflammatory effect at 4 hours, which is sufficient to prevent nearly all evidence of thermal injury by histology (**Figure 3**). By 48 hours, animals that were burned but did not receive N2 demonstrated significant tissue injury, ulceration and eschar formation (**Figure 4**). In contrast the animals that had received N2 at the time of the burn demonstrated much less severe gross tissue damage (**Figure 4**). Together, the results of these experiments indicated that N2 could be successfully administered at the time of the burn, and that a single administration is sufficient to evoke a profound anti-neutrophil effect early after injury, that results in significant reduction in tissue damage at later timepoints.

When the experimental model was expanded to include both a thermal injury followed by coetaneous infection with *P. aeruginosa*, the administration of N2 also resulted in a significant reduction in inflammation at early timepoints (**Figure 5**). However, as seen in the absence of infection, this effect is not present at a later timepoint.

Of greatest importance to this proposal is the capacity of N2-mediated neutrophil inhibition to afford protection against *P. aeruginosa* infection. At the 8 hour timepoint, we found a dramatic reduction in P. aeruginosa infection in the skin (**Figure 6**). At the 24 hour timepoint, partial protection remained, which generally correlates to the decreasing anti-inflammatory effect of the N2 peptide. However, overall the effect was significant ($p$=0.01 by two-way ANOVA). Together, these results support the validity of our Central Hypothesis, and represent completion of Milestone 3.
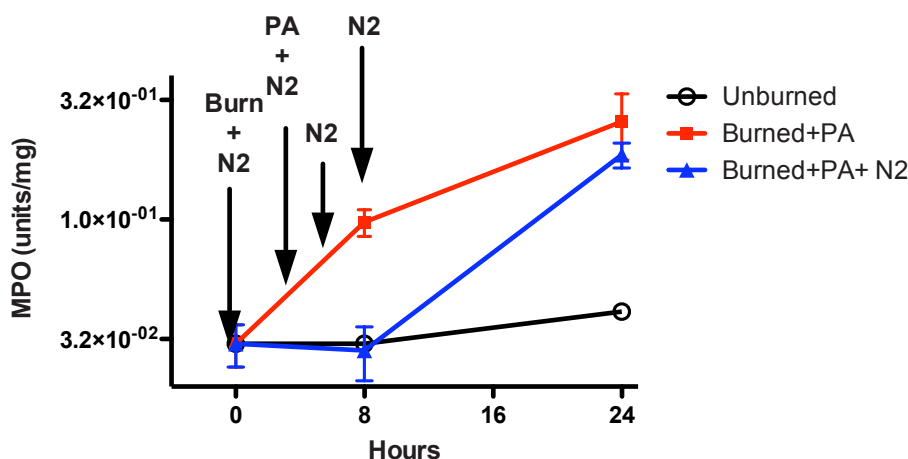


**Figure 5: Effect of N2 peptide in reducing neutrophil accumulation to the skin following thermal injury associated with *P. aeruginosa* infection.** Animals burned at time 0 received N2 peptide administered IV through tail vein injection and topically at the site of the burn. Two hours after the thermal injury, the site was infected with *P. aeruginosa*, and N2 was administered again. The agent was given twice more in the first 8 hours, as depicted by arrows. Neutrophil accumulation was assessed by MPO activity in skin homogenates at the burn site. As seen in the absence of infection, the N2 peptide dramatically reduced neutrophil recruitment to the burn, but this effect was lost over time, despite repeated administration of the compound. The effect of the N2 administration over the time studied was significant, with $p$=0.004 by two-way ANOVA.
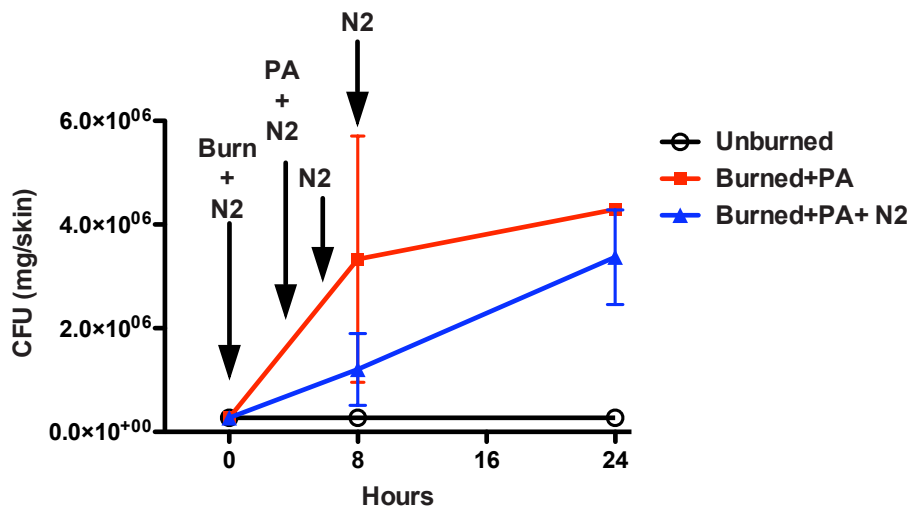
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**Figure 6: Effect of N2 peptide in reducing bacterial burden associated with *P. aeruginosa* infection in the burn model.** Quantity of bacteria present under the condition described for **Figure 5** was analyzed by standard methods. Associated with decreased neutrophil accumulation to the dermis was a decrease in burden of *P. aeruginosa*. This effect was greatest at the earlier timepoint, corresponding to the pattern of neutrophil influx. Effect of treatment over the time studied was significant, with $p=0.01$ by two-way ANOVA.

**1.4.** Test the effect of <u>pre-administration</u> of N2 peptide combined with azithromycin to reduce post-burn *P. aeruginosa* wound infection. (**Timeframe 7-9 months**)

*Using the most efficacious dose and delivery of N2 peptide defined from subtask 1.1, the additive effect of azithromycin to prevent P. aeruginosa biofilm development will be tested. **Milestone 4** will be the determination if azithromycin reduces wound infection beyond what is achieved by N2 peptide alone.*

Work is now underway on this subaim, with the determination of dose, delivery and timing of N2 administration representing the most time-consuming portion of this project. A maximum dose of AZM for the mouse model has been chosen from the literature, and we anticipate completion of this Milestone within the next month.

**Task 2**: Test the capacity of N2 peptide with and without azithromycin to <u>treat</u> established *P. aeruginosa* wound infection, or to augment standard antibiotic treatment. (**Timeframe 9-18 months**)

**2.1.** Test the effect of N2 peptide to reduce an established *P. aeruginosa* wound infection. (**Timeframe 9-11 months**) **Milestone 5** will be to prove or disprove the utility of the N2 peptide as an effective local anti-inflammatory in the setting of an ongoing wound infection (assayed by MPO and histology).

**2.2.** Test the effect of N2 peptide combined with azithromycin to reduce an established *P. aeruginosa* wound infection. (**Timeframe 11-12 months**) **Milestone 6** will be to determine if the N2 peptide combined with azithromycin reduces the bacterial burden of an ongoing wound infection (assayed by cfu's per gram of tissue). <u>168 animals</u> are projected.

**2.3.** Test the effect of the optimal combination of N2 peptide and azithromycin to augment conventional antibiotic treatment with tobramycin (i.v.) of a post-burn *P. aeruginosa* wound infection. (**Timeframe 13-16 months**) This subtask address the likelihood that the strategy tested in 2.1 and 2.2 will significantly reduce the bacterial burden, but not eradicate an already established infection. However, a well-established principal of infectious disease management is that antibiotic effect can be greater in the setting of lower bacterial density. Thus, the test strategy will be combined with a conventional anti-*Pseudomonal* antibiotic (tobramycin), to assess if the capacity of tobramycin to clear the post-wound infection can be augmented. **Milestone 7** will be to determine if N2 peptide with azithromycin enhances the efficacy of tobramycin (assayed by cfu's per gram of tissue). <u>168 animals</u> are projected.

Work on Milestones 5-7 has not yet started. However, these subaims represent a relatively minor modification of the current protocol, and do not pose any significant technical obstacles. Based on the results to date, it seems likely that the earlier administration of N2, within the first hour of the burn as opposed to following establishment of the chronic infection, will prove most efficacious.

**2.4. Milestone 8:** will be the preparation and initial submission of the manuscript **(<u>Timeframe 16-18 months</u>).**

We have initiated work on the first of two manuscript that will describe this work. In particular, we have mastered the histological techniques needed to clearly illustrate the effect at the tissue level (**Figure 3**).

**Key Research Accomplishments**
- Synthesis of the N2 peptide and scrambled 12-mer control
- Identification of the optimal dose and delivery method of the N2 peptide
- Definition of the maximum duration of effect of N2 on reducing neutrophil accumulation to the skin following thermal injury
- Identification of broader anti-inflammatory effects in reducing tissue swelling and injury following thermal injury
- Demonstration of longer-term effects in reducing gross tissue damage following thermal injury
- Demonstration of the effectiveness of N2 in reducing neutrophil accumulation in the setting of post-burn *P. aeruginosa* infection
- Demonstration of the association between decreased neutrophil-mediated inflammation and decreased infectious burden by *P. aeruginosa*.

**Reportable Outcomes**
None to date, although we expect that two manuscripts will be generated directly from this proposal, and that this work will serve as the basis for a R01 research grant submission to the NIH.

**Conclusion**
Our findings to date support the central hypothesis of this grant, that a dual therapeutic approach of targeted anti-inflammation and a biofilm specific antibiotic will significantly limit severe local and systemic infection associated with serious burns and wounds. We have essentially proven that by briefly stopping the influx of neutrophils to the site of thermal injury, the subsequent wound severity is decreased. More importantly, this also results in decreased early *P. aeruginosa* wound infection. With the addition of azithromycin into this model, we fully expect that this infection reducing strategy will be substantially improved. To date our findings underscore the importance of early and aggressive treatment to prevent *P. aeruginosa* biofilm formation. As proposed we will test this method in the setting of a established post-burn infection, although based on our initial results, it appears the effectiveness of the N2 peptide is largely confined to the first hours after the injury.

We believe that the importance and utility of this research to the combat situation is clear, as it is entirely feasible that a topical and/or IV agent be administered within the first hours of a severe wound or burn, with the goal of reducing the currently high rate of secondary infection by *P. aeruginosa*. In a larger sense, we believe that this work relates to a number of other medical conditions, as biofilm-mediated infection by *P. aeruginosa* occurs in highly predictable settings, thus lending it to preventive strategies.

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## Appendices
None

## Supporting Data
None

## Acronyms and Symbol Definitions

| | |
|---|---|
| Azm: | azithromycin |
| C57BL/6J: | standard laboratory mouse strain |
| cfu: | colony-forming unit |
| DNA: | Deoxyribonucleic acid |
| F-actin: | filamentous actin |
| IgM: | Immunoglobulin M |
| i.p.: | intraperitoneal |
| i.v.: | intravenous |
| MPO: | myeloperoxidase |
| N2 peptide: | 12 mer peptide sequence LMKNMDPLNDNV with homology to NMHC-II |
| NMHC-II: | nonmuscle myosin heavy chain II |
| PA: | *Pseudomonas aeruginosa* |
| Tob: | tobramycin |
| tp: | topical |