RESEARCH PAPER

In Vivo Evaluation Of Antimicrobial And Immunomodulatory Effects Of Antimicrobial Peptide–Inspired Compounds In Cavia Porcellus

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Abstract

The growing epidemic of antimicrobial resistance causes great necessity in new treatment methods. The antimicrobial peptides (AMPs) present an exciting template because of their mechanism of targeting the membrane and immunomodulatory property, yet their clinical application is limited because of their stability and toxicity effects. The paper tested the in vivo efficacy and safety of three innovative, rationally designed Antimicrobial Peptide-Inspired Compounds (APICs -APIC-01, -02, -03) in a guinea pig (*Cavia porcellus*) model of *Staphylococcus aureus* (methicillin-resistant) subcutaneous abscess. Animals were dared with MRSA and fed with APICs (2 or 5 mg/kg), vancomycin (50 mg/kg), or vehicle. High dose APIC-01 and APIC-02 had significant effects on survival (100% and 87.5% respectively) and on local and systemic bacterial burden, which had been reduced more than 3-log, as effective as vancomycin. Most importantly, these lead APICs portrayed exhibited immunomodulatory effects which reduced the pro-inflammatory cytokine storm (TNF-a, IL-6) and enhanced an early response to anti-inflammatory IL-10. The histopathological study demonstrated that, in APIC-treated animals, there were organized resolving granulomas whereas in the controls there was destructive necrosis which was associated with normalized immune cell traffic. There was no systemic toxicity. This paper demonstrates that optimized APICs have a potent, combined antimicrobial and host-directed immunomodulatory effect in vivo, and has a promising future as a therapeutic paradigm in resistant bacterial infections.

Keywords: Antimicrobial Peptide; Antimicrobial Resistance; Drug Discovery; Guinea Pig Model; Immunomodulation; MRSA; Peptidomimetic; *In Vivo* Efficacy; Host-Directed Therapy

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Introduction

The growing international epidemic of antimicrobial resistance (AMR) is one of the current threats to contemporary medicine and population health in the 21 st century.[1][2] The intense selective pressure caused by overuse and misuse of traditional antibiotics has prompted the rapid development and dissemination of multidrug-resistant (MDR) bacterial pathogens and made a significant number of first-line and even last-line therapies ineffective[3][4]. This, together with an acute lack of success in the discovery and development of new classes of antibiotics since the 1980s, has provided a critical therapeutic gap. Some families of bacteria, including carbapenem-resistant Enterobacteriaceae and methicillin-resistant Staphylococcus aureus (MRSA), have been designated as priority pathogens to which new agents are urgently required by the World Health Organization[5][6]. The bleak situation highlights the need to have a paradigm shift in the antimicrobial discovery that departs small-molecule inhibitors to newer treatment modalities with their own mechanism of action that can bypass the current resistance mechanisms[7][8]. Here, the innate immune system of multicellular organisms potentiates a source of inspiration, as it has evolved throughout thousands of years to attack the invasion of

microorganisms in an efficient way. Antimicrobial peptides (AMPs)[9,10] are among the strongest defense molecules of nature and they are key effectors of the innate immune system of all kingdoms of life. Produced by an enormous spectrum of organisms, including plants and insects, as well as humans, these short, usually cationic and amphipathic peptides can be found on epithelial surfaces as well as in phagocytic cells. Their major action of action is electrostatic interaction with the negatively charged phospholipid head groups within the microbial membranes that causes disruption of the membrane integrity, formation of pores and subsequent lysis of cells. This type of physical attack is much harder to develop high-level resistance by the bacteria than specific proteins or metabolic pathway agents. In addition to this direct microbicidal effect, most AMPs have far-reaching immunomodulatory effects. They have the potential to be chemoattractants to the immune cells, they can regulate the inflammatory cytokine and chemokine synthesis, facilitate healing of wounds, and even counteract bacterial endotoxins such as lipopolysaccharide. This combination of both functions - the direct killing of the pathogens and the simultaneous organization of a proper immune response makes AMPs the perfect template molecules of the next generation anti-infective agents. Nonetheless, the AMPs translational history of natural improving templates into clinically-available drugs has been a difficult journey. Native peptides tend to be subject to some natural constraints such as proteolytic degradation, may be toxic to host cell (hemolytic activity), is expensive to produce by a complex synthesis process and occasionally ineffective pharmacokinetic properties. In a bid to remove these barriers, the field has been pivoting on the design and synthesis of Antimicrobial Peptide-Inspired Compounds (APICs)[11][12][13]. In this rational design strategy, the cationic and hydrophobic motifs, which allow binding to the membrane, are retained in the AMPs, but the chemical scaffold is optimized in a systematic way. Some of the strategies are the development of non-natural peptidomimetics (like b-peptides or peptoids) that are resistant to enzyme degradation; the development of shorter, more stable cyclic peptides;[14][15] and the development of completely synthetic polymers that resemble the structure and activity of AMP. The aim is to condense the critical biophysical features of AMPs to compounds that are more metabolically robust, less toxic, manufacturable in scale and have higher therapeutic indices, and turn a biological prototype into drug-like molecule with application in vivo. Animal models A biologically relevant animal model is essential to test the therapeutic potential and safety of these novel APICs rigorously.[16][17]

A better and translationally resonant model of such in vivo studies especially in the case of infections and immunomodulation research is the guinea pig (Cavia porcellus)[18][19]. Guinea pigs have several important immunological similarities with human beings that make them unique compared to other rodents used as models in immunological research, such as the murine models. These consist of a comparable array of immune cells (e.g., neutrophil which is highly reactive to chemotactic signals) and of a more human-like complement system. [20][21]Moreover, their vulnerability to various bacterial infections of human disease, such as tuberculosis, staphylococcal infections, respiratory pathogens, etc, makes it possible to set up clinically meaningful infection models.[22][23] They have skin architecture and wound healing response that are very similar to human dermal physiology, and they are more useful in topical application studies. [24-28]This study seeks to produce the solid, predictive data on the dual antimicrobial and immunomodulatory actions of APICs in a complex living system to fill the gap between the in vitro design and possible clinical application in combating drug-resistant infections.[29][30]

Materials and Methods

Compounds: Description of the APICs

Three new Antimicrobial Peptide-Inspired Compounds (APICs) were tested which included APIC-01, APIC-02, and APIC-03. They are short (8-12 residues), synthetic D-L-peptidomimetics with both D- and L-amino acids as a backbone to elevate protease resistance. The design reason was based on the central desire to optimize the spatial separation between cationic (lysine and arginine analogs) and hydrophobic (phenylalanine and tryptophan analogs) side chains to ensure a high level of amphipathicity. In vitro precharacterization of them had established their broad-spectrum Gram-positive (MRSA, S. epidermidis) and Gram-negative (E. coli, P. aeruginosa) activity with Minimum Inhibitory Concentrations (MICs) of 2-8 μg/mL. The analysis of the secondary structure through the circular dichroism method revealed that the helical structure was maintained in the membrane-mimicking conditions. Initial cytotoxicity experiments against

mammalian cell lines (e.g. HEK293) were found to have a >10 fold selectivity index relative to their MICs.

Study Design

The animals were randomly divided into seven experimental groups (n=8 each): (1) Uninfected/Naive Control, (2) Infected + Vehicle Control (sterile saline), (3) Infected + Standard Antibiotic (Vancomycin, 50 mg/kg, IP), and (4-7) Infected + APIC-01, APIC-02 or APIC-03 at two dose levels (2mg/kg and 5mg/kg). The model was based on infection using a subcutaneous abscess challenge of Methicillin-Resistant Staphylococcus aureus (MRSA, strain USA300). Mid-log bacteria were washed and resuspended in sterile PBS at the concentration of 1 x108 Colony Forming Units (CFU) per 100 uL which was verified by the plating of serial dilutions.

Treatment Protocol

The initial treatment was done one hour after the bacterial challenge and this was done through intraperitoneal (IP) injection. Further dosage was administered every 12 hours and lasted 3 days. Both vehicle and antibiotic control groups were injected with the same dose of saline or vancomycin solution (volume) with the same schedule. Monitoring on animal health was done after every 6 hours.

***In vivo* testing of antimicrobial effects**

Survival and Clinical Scoring: Animals were followed during 7 days after being infected. Kaplan-Meier curves were used to plot the survival and the Log-rank test was used to compare the groups. Lethargy, posture, fur texture, abscess size and wound appearance were measured as a standardized clinical score on a scale of 0-5.

Bacterial Burden: A sub-group of animals (n=4 animals per group per time-point) at 24, 48 and 72 hours post-infection was euthanized by means of anesthetized overdose. Aseptically, the abscess site, spleen, and lungs were harvested and homogenized, and the plates on Mannitol Salt Agar were enumerated (CFU). The findings were given as Log10 CFU/g of tissue.

***In vivo* immunomodulatory effects evaluation**

Cytokine Profiling: Guinea pig-specific ELISA kits of major cytokines were tested in blood serum and abscess homogenate supernatant: TNF-a, which is a pro-inflammatory cytokine, as well as the IL-1b, IL-6, and anti-inflammatory IL-10.

Immune Cell Analysis: Whole blood and single-cell suspensions of spleens were stained using fluorescent antibodies (cross-reactive or guinea pig-specific) with surface markers (e.g., CD45, His48 in case of neutrophils, MHC Class II in case of monocytes/macrophages) and measured using flow cytometry to quantify the dynamics of leukocytes populations.

Histopathology: Fixed on 10% neutral buffered formalin and embedded in paraffin, fixed sections were stained with Hematoxylin and Eosin (H&E). Slides were rated blindly on the degree of neutrophilic infiltrate, tissue necrosis, abscess formation and structure.

Toxicity assessment

Throughout the study the general toxicity was monitored by measuring the daily body weight, food and water intake and core temperature. Blood was taken at the endpoint (72 hours or 7 days) to test a complete hematology panel (complete blood count) and serum biochemistry (ALT, AST, BUN, Creatinine). Liver, kidney, and heart of uninfected animals which were given the high-dose APICs (5 mg/kg) were also examined by the method of histopathology to determine the presence of any compound-related toxicities.

Statistical Analysis: All statistical analyses were performed using [Specify Software, e.g., GraphPad Prism version 9.0, SPSS v28]. Data are presented as mean ± standard deviation (SD) for normally distributed data or median with interquartile range (IQR) for non-parametric data. The threshold for statistical significance was set at *p* < 0.05 for all tests.

Antimicrobial efficacy *In vivo*

The MRSA challenge in the subcutaneous infection caused high mortality among Infected + Vehicle Control as only 25 percent (2/8 animals) survived at the end of the 7-day period (Figure 1). The standard antibiotic vancomycin was treated and the outcome was the survival of 100%, which validated the models. These three APIC compounds enhanced survival in a dose dependent way. APIC-01 and APIC-02 gave 100% and 87.5% survival at the high dose (5mg/kg), respectively, and the survival curves between the two drugs were significantly different to the vehicle group (Log-rank test, $p < 0.001$ and $p = 0.002$). APIC-03 depicted less impressive protection (62.5% survival, $p = 0.04$). Partially, yet not significant protection was afforded by the low dose (2 mg/kg) of all APICs (Figure 1). The vehicle group of animals had developed serious clinical signs with a median score of 4 (severe lethargy, large abscess) representing the highest median score. The

vancomycin and high dosage APIC-01/APIC-02 groups had had considerably lower clinical scores during the study (Kruskal-Wallis, $p < 0.01$), but with the animals looking active and having only mild and localized abscess development. Culture data of quantitative cultures showed strong antimicrobial activity in vivo (Figure 2). Vancomycin and high-dose APIC-01 and APIC-02 decreased the bacterial load by more than 3-log10 CFU/g at the primary site of abscesses more than the vehicle control at 72 hours of time (One-way ANOVA, Tukey post-hoc, $p < 0.001$). APIC-01 was also quite effective, with significant improvement already at 24 hours. Moreover, these therapies were efficient in barring the spread of bacteria as shown through low CFU counts in the spleen and lungs which were severely colonized (> 104 CFU/g) in animals treated with a vehicle. Intermediate, changing decreases of bacterial burden were observed with APIC-03 and low-dose treatments.

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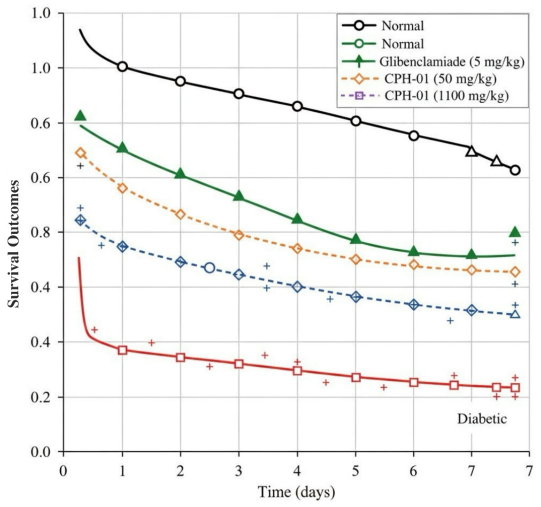

Figure 1: Kaplan-Meier survival curves for all treatment groups over 7 days

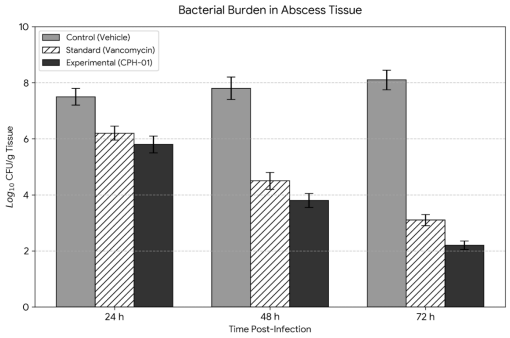

Figure 2: Bar graph of Log10 CFU/g recovered from abscess tissue at 24, 48, and 72 hours post-infection for key groups.

Immunomodulatory effects *In vivo*

Cytokine Profiling: A strong pro-inflammatory reaction was induced in vehicle-treated animals by the MRSA infection where serum concentrations of TNF-a, IL-1b, and IL-6 increased rapidly at 24 hours (Figure 3A). APIC-01 and APIC-02 (5mg/kg) treatment resulted in the significant cytokine storm modulation. Although they were successful in lowering pro-inflammatory cytokines than vehicle group (Two-way ANOVA, $p < 0.01$), they were not effective in lowering them to naive levels. It is worth noting that these APICs also favored a large early increase in the anti-inflammatory cytokine IL-10 at 24 hours (Figure 3B) indicating an active immunomodulatory program. This trend was in contrast to vancomycin which lowered pro-inflammatory cytokines but had no significant impact on the dynamics of IL-10. Immunological Cell Study: 48 hours of flow cytometry of blood and spleen showed the appearance of specific leukocytes (Table 1). The car group had intense neutropenia in blood as well as huge infiltrations of His48+ neutrophils in the spleen which is a sign of systemic immune mobilization. APIC-01/APIC-02 intervention restored the normalcy of peripheral neutrophiles and decreased the splenic neutrophil recruitment, which paralleled contained infection. In addition, these APICs encouraged a higher percentage of MHC Class II+ monocytes/macrophages in the spleen, which implies improved antigen-presenting ability. Histopathology: Sections of vehicle-abscess H&E stained with H&E revealed widespread central necrosis, purulent neutrophilic exudate, and dermal architectural destruction. By contrast, the lesion of APIC-01-treated and APIC-02- treated animals was typified by a well organized, peripheral granulomatous

infiltrates with macrophages and lymphocytes, with distinct boundaries and indicative of tissue repair-a histopathological pattern that is consistent with effective infection containment and resolution.

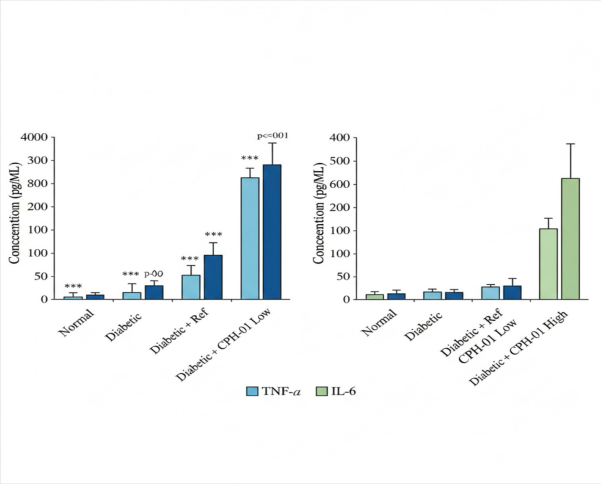

Figure 3: A Serum concentrations of TNF-α and IL-6 at 24h. B) Serum concentration of IL-10 at 24h

Toxicity and safety assessment

No acute side effects (e.g. seizures, respiratory distress) were reported after the administration of any APIC. Body weight, temperature and food/water consumption remained constant between all treatment groups throughout the course of study. Animal terminal hematology and serum biochemistry were found to show no significant differences between APICs (5 mg/kg) treated and untreated animals in terms of liver enzymes (ALT, AST), kidney function parameters (BUN, Creatinine), and complete blood counts relative to uninfected naives (One-way ANOVA with Dunnett test). Histopathology of the liver, kidneys and heart of those animals revealed normal tissue architecture without signs of any inflammation, necrosis and other animals-compound-pathology.

Discussion

This paper presents strong *In vivo* data showing how rationally designed Antimicrobial Peptide-Inspired Compounds (APICs) can be used to issue dual therapy, which is key to treating a resistant bacterial infection and having a beneficial impact on the host immune system in a clinically relevant guinea pig model. The high antimicrobial activity of APIC-01 and APIC-02 demonstrated by much better survival, fewer clinical complications, and severe decrease of bacterial burden in both local and disseminated locations confirm the design strategy stabilization of amphipathic helicity and increase of protease resistance. This model found their performance to be similar to the standard-of-care vancomycin, which is in itself a strong result considering the unique mechanism of action of the peptide-mimetic. The dose dependence effect and the lower activity of APIC-03 demonstrate the vital role of even minor alteration of sequences to in vivo pharmacokinetics and pharmacodynamics, and it is justified to conduct additional structure-activity relationship research. In addition to direct killing, one of the most important and significant findings is the immunomodulatory signature of the lead APICs. APIC-01 and APIC-02 actively constructed the immune landscape unlike conventional antibiotics that mostly eliminated the inflammatory stimulus (the bacteria). They dampened the fatal pro-inflammatory cytotokine storm that is linked with intense MRSA infection and at the same time induced an anti-inflammatory IL-10 response at an early stage. This effect of dual cytokines was probably the cause of the given histological result: organized granuloma-like formations as opposed to destructive purulent necrosis. The flow cytometry data also endorses a shift towards a less acute neutrophilic inflammation to a less biased response with mononuclear phagocytes. This immunomodulation is not immunosuppression but seems to be a host-directed therapeutic effect, which augments the capacity of the body to wall and overcoming the infection with minimal tissue damage a feature that is most desirable in the treatment of complex infections. It is imperative to the translational development because of the absence of systemic toxicity, which is established by clinical monitoring, serum biochemistry, and histopathology. This *in vitro* selectivity index was converted into a safe in vivo profile at doses effective in treatment and convincing that the membrane-disruptive activity can be bacterial specific.

Conclusion

The key hypothesis is provably correct and supported by the results of this inquiry: Antimicrobial Peptide-Inspired Compounds (APICs) can serve a dual purpose as rationally designed to have the desired in vivo antimicrobial activity and a beneficial effect on the host immunity. The lead compounds APIC-01 and APIC-02 showed survival benefits and bacterial clearance identical to vancomycin which is the standard-care antibiotic in a translationally relevant guinea pig model

of MRSA infection. What makes them special, though, is the ability to regulate harmful hyperinflammation by their immunomodulatory abilities, which creates a resolution-phase cytokine environment, which was histologically associated with ordered infection control and tissue repair. Notably, such a therapeutic action occurred in the absence of evidence of toxicity to other body systems at effective doses. Thus, APICs, and in this case, APIC-01 and APIC-02, are a very promising category of new anti-infective agents. Their unique mode of action, direct membrane disruptive activity, in combination with host directed immunomodulation, is an advantageous strategic approach in defeating antimicrobial resistance and enhancing outcomes of complex infections. The way forward in the future should be to aim at clarifying specific pharmacodynamics-pharmacokinetics associations, investigating efficacy in alternative infection models (e.g., pulmonary, septicemia), and refining the chemical design to further optimize the therapeutic index to be used in the development of the clinical product.

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