Inhibitory and anti-inflammatory effects of Helicobacter pylori-derived antimicrobial peptide HPA3NT3 against Propionibacterium acnes in the skin

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Conflict of interest disclosures

None declared
What’s known/what’s new statements:

What’s already known about this topic?

- The cutaneous inflammation associated with acne vulgaris can be triggered by *Propionibacterium acnes* (*P. acnes*) through activation of the innate immune system in the skin
- There is interest in developing new therapeutic agents for acne with mechanisms of action that block *P. acnes*-induced inflammation

What does this study add?

- A synthetic peptide derived from *Helicobacter pylori* has direct antimicrobial effects on *Propionibacterium acnes* (*P. acnes*)
- HPA3NT3 is a customized α-helical cationic peptide shown to inhibit inflammatory effects triggered by *P. acnes* through TLR activation in cultured primary human keratinocytes
- This antimicrobial peptide also significantly reduces *P. acnes*-induced skin inflammation in a murine model
- As a treatment for acne, this peptide offers the ability to decrease the population of *P. acnes* and to inhibit skin inflammation triggered by this organism
ABSTRACT

Background: An effective treatment strategy for acne vulgaris is the reduction of Propionibacterium acnes (P. acnes) in the skin. Helicobacter pylori (H. pylori)-derived synthetic antimicrobial peptide HPA3NT3 is a customized α-helical cationic peptide with antibacterial and anti-inflammatory activity.

Objectives: To examine the role of HPA3NT3 as a treatment against P. acnes induced skin inflammation.

Methods: Morphological alteration of individual P. acnes cells by HPA3NT3 was visualized by scanning electron microscopy. Modulation by HPA3NT3 of a number of P. acnes-induced innate immune responses was analyzed in vitro using cultured normal human keratinocytes (HK) and in vivo using the ICR mouse, a well-established model for P. acnes-induced skin inflammation.

Results: The minimal inhibitory concentration (MIC) of HPA3NT3 against P. acnes was low (0.4 µM). HPA3NT3 showed no cytotoxicity to HK cells at concentrations used in our in vitro and in vivo studies. Treatment with HPA3NT3 in vitro induced morphological disruptions in P. acnes cells suggestive of a bactericidal effect. HPA3NT3 significantly decreased P. acnes-induced IL-8 expression and intracellular calcium mobilization in HK cells by inhibiting P. acnes-activated TLR2-mediated NF-κB signaling pathways. Intradermal injection of HPA3NT3 in vivo effectively decreased viable P. acnes as well as erythema, swelling and inflammatory cell infiltration in ICR mouse ears inoculated with P. acnes.
Conclusions: Our data suggest that HPA3NT3 has potential as a therapeutic agent for acne vulgaris due to its antimicrobial effects on *P. acnes* and its ability to block *P. acnes*-induced inflammation.
INTRODUCTION

Acne vulgaris is a multifactorial inflammatory skin disease that can result in significant scarring and disfigurement of the face and upper trunk due to follicular inflammation that leads to comedones, papules, pustules, nodules, and cysts\(^1\). Key factors involved in the pathophysiology of acne are hyperkeratinization, sebum production, inflammatory mediators, androgens, and the presence of *Propionibacterium acnes* (*P. acnes*). This Gram-positive, anaerobic, and micro-aerobic bacterium is a skin commensal in healthy hosts but triggers inflammation and tissue injury originating in the sebaceous follicle in people with acne through both innate and adaptive immune responses\(^2-5\). Many existing treatments for acne, including topical antibiotics and benzoyl peroxide, reduce the number of *P. acnes* of affected skin, which has been shown to correlate with clinical improvement\(^6,7\). With increasing understanding of the inflammation induced by *P. acnes*-activation of the innate immune receptor TLR2 on keratinocytes and infiltrating monocytes and macrophages in the pathogenesis of acne\(^3,8\), there is opportunity for the design of new therapeutic agents for the treatment of acne.

Endogenous antimicrobial peptides (AMPs) such as defensins and cathelicidins are able to directly kill bacteria and modulate interactions between the innate and adaptive immune systems\(^4,9\). While endogenous AMPs can be upregulated by *P. acnes* *in vitro*\(^10,11\), they do not appear to be capable of reducing this organism sufficiently to prevent its role in acne pathogenesis in many individuals with acne even though beta-defensin is bactericidal for *P. acnes* and inhibits host inflammatory responses to this bacteria\(^3,9,12\). Exogenous and synthetic AMPs are being developed with the goal of
augmenting and focusing AMP effects. In this study we examine the role of the exogenous synthetic peptide HPA3NT3 against *P. acnes* in the skin. HPA3NT3 is synthesized from HP(2-20), the peptide formed by amino acids 2-20 of *Helicobacter pylori*-derived ribosomal protein L1 (RpL1). HPA3NT3 and HP(2-20) are cationic peptides with previously demonstrated potent bactericidal activity but with low hemolytic or cytotoxic effects on normal eukaryotic cells\textsuperscript{13}. In this study we demonstrate the potential of HPA3NT3 as a therapeutic agent for the treatment of acne vulgaris due to its antimicrobial effects on *P. acnes* and its ability to block inflammatory activities triggered by *P. acnes* in the skin without cytotoxic effects on skin cells.
MATERIALS AND METHODS

Reagents and cells

P. acnes (ATCC11828 and ATCC6919: American Type Culture Collection, Manassas, VA) was cultured in Reinforced Clostridial Medium (BD: Franklin Lakes, NJ) under anaerobic conditions using Gas-Pak at 37 oC, harvested by centrifugation at 2,000 x g for 10 minutes at 4 oC, and resuspended in starvation medium at 1x10^8 colony-forming units (CFU)/mL. Normal human keratinocytes (HK) from foreskin were purchased from PromoCell (Heidelberg, Germany) and cultured in supplemented keratinocyte growth medium at 37 oC in 5% CO_2.

Cultured HK cells were propagated to at least 70% confluence, were inoculated with P. acnes at 1x10^6 CFU, then were incubated for various time periods as indicated in the results. HPA3NT3 or HPN3 was added to each well at various concentrations (0.8, 1.6, or 3.2 µM) 24 hours after P. acnes infection. HP(2-20) (AKKVKFRLFLEKLFSKIQNDK), HPA3NT3 (FKRLKLFKKIWNWK), and HPN3 (RLEKLFSKIQNDK) were synthesized as previously described^{13}.

MIC test by microdilution assays.

P. acnes was suspended in its liquid media to a concentration of 1x10^8 CFU/mL. Two-fold serial dilutions of each of the peptides (0.39 to 100 µM) were plated into sterile 96-well microtitre plates. The suspension of P. acnes was then added to each well and incubated overnight at 37 oC under anaerobic conditions. The suspension of P. acnes plus each peptide was collected from the wells, aliquotted, plated onto agar plates, incubated at 37 oC for 1 to 2 days, and colony counts were obtained. The MIC was
defined as the lowest concentration of peptide that yielded no visible growth on agar plates\textsuperscript{14}.

**Scanning electron microscopy (SEM) analysis**

SEM analyses were performed as previously described\textsuperscript{15}. Briefly, cultured *P. acnes* cells (5x10\textsuperscript{5}) were incubated with each of the peptides (50% of MIC) at 37°C for 20 minutes in 10 mM sodium phosphate buffer (pH 5.5). The cells were then fixed with 4% glutaraldehyde and dehydrated with 50 to 100% ethanol followed by incubation for 10 minutes at 37°C. All samples were then coated with gold and visualized with field emission-scanning electron microscopy (FE-SEM, JSM-7100F, Jeol, Japan) under 20,000x magnification at 15.0 kV.

**MTT assay**

A standard colorimetric assay for assessing cell viability based on measuring the activity of reducing enzymes for MTT (yellow tetrazolium salt: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed according to the manufacturer’s instructions (Molecular Probes, Inc., Eugene, OR) using HK cells (5x10\textsuperscript{3} per 200 \textmu L culture media) in the presence or absence of HPA3NT3 or HPN3 at concentrations ranging from 1.6 to 6.4 \textmu M. Data are presented as the percentage of viable HK cells compared to the percentage of viable cells after treatment with 2% Triton X-100, the positive control for cell cytotoxicity.
Determination of the expression of IL-8 and TLR2 mRNA by real-time RT-PCR and protein by ELISA

HK cells were incubated with *P. acnes* ATCC11828 (1x10^8 CFU/ml) for 24 hours in the presence or absence of 3.2 µM HPA3NT3 or HPN3. Total cellular RNA was isolated using an Rneasy Mini Kit (Qiagen; Maryland, MD) then reverse-transcribed to cDNA using an M-MLV reverse transcription kit (Promega; Madison, WI) according to the manufacturer’s instructions. Target gene mRNA expression was analyzed by real-time RT-PCR as described in the manufacturer’s protocol (ABI 7500 real-time PCR system using SYBR Green master mix; Applied Biosystems, Foster City, CA).

Oligonucleotide primers used to amplify human IL-8 and TLR2 cDNA were designed using the manufacturer’s software (Primer Express 3.0; Applied Biosystems) based on published sequences\textsuperscript{16,17}. Quantification of target gene expression was normalized using an internal control gene, 18S rRNA\textsuperscript{18}. The IL-8 primer sequences used were 5'-GCAGTTTTGCCAAGGAGTGCT-3'(sense) and 5'-TTTCTGTGTGTCGCGAGCAGTGGTG-3'(antisense). The TLR2 primer sequences used were 5'-TGTTGTGTGACCGCAATGGT-3'(sense) and 5'-TGTTGGACAGGTCAAGGCTTT-3'(antisense). The 18S rRNA primer sequences used were 5'-CGGCTACATCCAAGGAA-3'(sense) and 5'-GCTGGAATTACCGCGGCTT-3'(antisense).

To quantitatively measure IL-8 protein, HK cell supernatants were tested by ELISA using the Quantikine human IL-8 immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. All experiments were performed in triplicate.
Determination of NF-κB nuclear translocation and TLR2 cellular localization by immunofluorescence staining

Immunofluorescence analyses for NF-κB localization and TLR2 cellular localization were performed as previously described\textsuperscript{19}. Human keratinocytes were grown to about 70% confluence in chamber slides (154526, Nalgene, Rochester, NY) and then treated with \textit{P. acnes} (1×10\textsuperscript{8} CFU/mL) for 30 minutes in the presence or absence of 3.2 μM HPA3NT3 or HPN3. The cells were then incubated with rabbit anti-human NF-κB p65 polyclonal antibody (Rel A) or rabbit anti-human TLR2 antibodies (Rockland, Gilbertsville, PA), which was diluted 1:3000 in blocking buffer (ImmPRESS kit; Vector Laboratories, Burlingame, CA), and subsequently incubated for 1 hour at room temperature in the dark with FITC-conjugated affinity-purified goat anti-rabbit IgG (H+L; Jackson ImmunoResearch Laboratories, INC., West Grove, GA), which was diluted 1:300. The cells were visualized with a microscope (Olympus EX51; Center Valley, PA). Images were acquired with the QICAM fast 1394 camera (Westmont, IL).

Analysis of HK intracellular calcium mobilization

Intracellular calcium mobilization was determined as previously described using the InCyt Basic IM Fluorescence Imaging System (Intracellular Imaging INC, Cincinnati, OH) and 2 μM of the fluorescent calcium probe fura-2/acetylmethyl (AM) ester (Invitrogen, Carlsbad, CA)\textsuperscript{20}. Cultured HK cells were pretreated on a glass coverslip with or without 3.2 μM HPA3NT3 or HPN3, and then \textit{P. acnes} was added during the active measurement of intracellular calcium mobilization.
Determination of \textit{P. acnes} viability in ear tissue and \textit{P. acnes}-induced \textit{in vivo} inflammation

HPA3NT3 (6.4 \text{\textmu}M, 20 \text{\textmu}L), clindamycin (0.2 \text{\textmu}M, 20 \text{\textmu}L), or PBS (20 \text{\textmu}L) were injected intradermally into the right ears of ICR mice (Harlan, Indianapolis, IN) 24 hours after \textit{P. acnes} (1x10^8 CFU per 20 \text{\textmu}L in PBS) inoculation at the same site. Left ears of the same mice were injected with 20 \text{\textmu}L of PBS. In negative control mice, right ears were untreated with AMPs while left ears received intradermal injections of PBS. The \textit{in vivo} dose of 6.4 \text{\textmu}M HPA3NT3 was selected from pilot study results and is one-fold higher than the dose used in \textit{in vitro} studies. The dose of clindamycin was based on previously reported efficacy\textsuperscript{21,22}. Ear thickness over time was measured using a micro caliper (Mitutoyo 547-400S; MSI Viking Gage, Charleston, SC) prior to injection and at 24, 48, and 72 hours after injection (10 mice per group). In separate groups of mice 10 mg of tissue from 8 mm punch biopsies taken from ears 24 hours after peptide injection was homogenized in 250 \text{\textmu}L of sterile PBS using a tissue grinder. \textit{P. acnes} was quantified by plating serial dilutions of the homogenate on agar plates and incubating under anaerobic conditions for 48 hours. Additionally, ear tissue was collected at various time points for staining with hematoxylin and eosin (Sigma) and visualized using a Zeiss Axioskop2 plus microscope (Carl Zeiss).

\textbf{Statistical analysis}

Results are expressed as mean \pm SD. ANOVA with probabilities was performed for both overall significance and pairwise comparison. \textit{P}<0.05 was considered to be statistically significant.
RESULTS

HPA3NT3 has significant antibacterial activity against *P. acnes* with minimal cytotoxicity to HK cells.

To determine antibacterial activity against *P. acnes*, we tested the MIC of HPA3NT3, HP(2-20), and HPN3 for two commonly utilized *P. acnes* strains; ATCC11828 and ATCC6919. The MIC values of HPA3NT3 and HP(2-20) against both strains were 0.4 and 0.8 µM, respectively (Table 1a). Because HPN3 had a 25 times higher MIC value (>12.8 µM) than HPA3NT3, we elected to use this inactive peptide as a control in future studies. The MIC values of both HPA3NT3 and HP(2-20) were similar to the reported MIC value for clindamycin (MIC <0.2 µM), a common topical treatment for acne vulgaris\(^\text{21,22}\).

The MTT assay was used to determine the cytotoxicity of AMPs to human keratinocytes within the dose ranges used in these studies. HK cells were 100% viable 24 hours after treatment with 1.6, 3.2, or 6.4 µM of HPA3NT3 or HPN3 (Table 1b). These results demonstrate that treatment with these peptides does not result in any significant cytotoxic effect on HK cells even at concentrations several fold higher than necessary for antibacterial activity against *P. acnes*.

HPA3NT3 induces morphological disruption in *P. acnes* suggestive of bactericidal effect.

*P. acnes* morphology following treatment with AMPs was visualized by scanning electron microscopy. HPA3NT3 induced morphological perturbation and blebs of *P. acnes* cell walls at 50% of MIC (Fig. 1b), whereas HP(2-20) (Fig. 1c) and HPN3 (Fig.
1d) induced minimal or no morphologic changes of the cell wall, respectively. These results suggest that the antimicrobial activity of HPA3NT3 against *P. acnes* may be bactericidal rather than bacteriostatic\(^\text{13}\).

**HPA3NT3 inhibits the *P. acnes*-induced production of IL-8 in HK cells.**

IL-8 is a strong chemotactic cytokine that recruits neutrophils and lymphocytes to sites of pathogenic infection in the skin\(^\text{3}\). We first examined *P. acnes*-induced HK IL-8 expression *in vitro* in the presence or absence of HPA3NT3, HP(2-20), or HPN3. IL-8 mRNA expression was 34-fold higher in *P. acnes*-treated keratinocytes compared to untreated control cells (Fig. 2a), which corresponds to published studies\(^\text{23}\). HPA3NT3 down-regulated (75%) *P. acnes*-induced IL-8 mRNA expression more than did HP(2-20) (19%) or the negative control peptide HPN3 (0%). Furthermore, HPA3NT3 caused a greater reduction (56%) in *P. acnes*-induced IL-8 protein secretion compared to HP(2-20) (35%) or HPN3 (0%) 24 hours after peptide treatment. None of the tested AMPs alone induced IL-8 protein secretion by HK (Fig. 2b). Since HPA3NT3 had a more profound ability to block *P. acnes*-induced HK IL-8 production than HP(2-20), we focused on this AMP in additional studies.

**HPA3NT3 inhibits *P. acnes*-induced HK NF-κB nuclear translocation and abrogates the associated rapid intracellular calcium mobilization in HK cells.**

NF-κB is a key transcriptional regulator of multiple genes including IL-8 and TNFα, which are cytokines that participate in inflammatory responses in the skin\(^\text{24}\). We tested the role of HPA3NT3 in mediating NF-κB responses to *P. acnes* by examining
cellular NF-κB localization. In untreated keratinocytes NF-κB staining was observed primarily in the cytoplasm (Fig. 3a). NF-κB nuclear translocation was rapidly induced by the addition of *P. acnes* (Fig. 3b). Co-incubation of HK cells with *P. acnes* plus HPA3NT3 effectively blocked *P. acnes*-induced NF-κB nuclear translocation (Fig. 3c). In contrast, the negative control peptide HPN3 did not block *P. acnes*-induced NF-κB nuclear translocation (Fig. 3d). Addition of AMPs alone caused no NF-κB nuclear translocation (data not shown).

Since the *P. acnes* culture supernatant induces intracellular calcium signaling in human keratinocytes via proteinase-activated receptor-2\(^2\), we examined whether HPA3NT3 modulates *P. acnes*-induced HK intracellular calcium mobilization. Addition of *P. acnes* to HK cells *in vitro* resulted in rapid intracellular calcium mobilization (Fig. 4a), which was abrogated by pre-incubation with 3.2 µM HPA3NT3 but not with the negative control peptide HPN3 (Fig. 4b). Stimulation with the peptides alone resulted in almost no intracellular calcium mobilization (Fig. 4c).

**HPA3NT3 significantly inhibits *P. acnes*-induced TLR2 expression in HK cells.**

*P. acnes* contributes to inflammation in acne through activation of TLR2, which leads to the release of pro-inflammatory cytokines such as IL-8 and TNF\(\alpha\) through the NF-κB signaling pathway\(^3\). Thus, we examined whether HPA3NT3 modulates *P. acnes*-induced HK TLR2 expression. TLR2 mRNA expression was increased 2 fold in keratinocytes 24 hours after *P. acnes* inoculation, and this overexpression was significantly down-regulated by co-treatment with HPA3NT3 (*P*<0.001) but not HPN3 (Fig. 5a). HPA3NT3 inhibited *P. acnes*-induced HK TLR2 protein expression 24 hours
after treatment (Fig. 5b, ii and iii) compared to the negative control peptide HPN3 (Fig. 5b, iv). Treatment with HPA3NT3 alone (Fig. 5b, v) did not significantly alter baseline TLR2 protein expression in HK cells.

Intradermal injection of HPA3NT3 significantly reduced viable P. acnes and P. acnes-induced inflammation in vivo.

In ICR mouse ears 24 to 48 hours after intradermal inoculation with P. acnes, a significant increase in cutaneous erythema (Fig. 6a), ear swelling (Fig. 6b), and inflammatory infiltrate (Fig. 6d) was elicited as compared to PBS-injected negative control ears. We observed that intradermal injection of HPA3NT3 (6.4 µM) 24 hours after P. acnes inoculation reduced both visible erythema (Fig. 6a) and ear swelling over time as compared to P. acnes-inoculated ears treated with either HPN3 or clindamycin (0.2 µM) (Fig. 6b). Furthermore, HPA3NT3 significantly reduced viable P. acnes colony-forming units (CFUs) retrieved from the ear tissue (Fig. 6c). HPA3NT3 reduced histologic P. acnes-induced mouse ear edema and inflammatory infiltrate 48 hours after P. acnes injection as visualized by H&E staining as shown in this representative data (Fig. 6d). HPA3NT3 alone induced no inflammation when injected into mouse ears.

These results demonstrate that intradermal HPA3NT3 injection exerts anti-inflammatory effects in response to P. acnes inoculation and also has antibacterial activity against P. acnes in vivo.
DISCUSSION

There is considerable interest in the development of new treatment approaches for acne vulgaris. These range from vaccinations against *P. acnes* to hand-held infrared medical devices to development of novel pharmacologic agents. As our understanding of the cutaneous microbiome and the role of commensal bacteria in driving skin inflammation increases, therapeutic targeting of *P. acnes* in the treatment of acne vulgaris is an area of active research. Furthermore, different *P. acnes* strains have recently been identified in the microbiome of healthy patients and those with acne which could be relevant to some types of anti-acne therapies.

We determined that a 15 amino acid cationic synthetic antimicrobial peptide, HPA3NT3, derived from Helicobacter pylori-derived ribosomal protein L1 (RPL1) was more effective at killing 2 strains of *P. acnes* than its parent peptide called HP(2-20) that is composed of 19 amino acids. In order to kill *P. acnes*, HPA3NT3 may reach the bacterial membrane as highly ordered oligomers, whereas HP(2-20) aggregates on the negatively-charged bacterial membrane predominately in the form of monomers. Importantly, we also found that HPA3NT3 had no cytotoxic effects on keratinocytes in vitro or on ear tissue in vivo at the doses used to kill *P. acnes* and block inflammation in these studies. Furthermore, we detected no induction of inflammatory mediators by this peptide when tested alone. Our studies again demonstrate that *P. acnes* induces inflammatory mediators in keratinocytes through activation of the innate immune system. The ability of the exogenous antimicrobial peptide HPA3NT3 to attenuate *P. acnes*-induced keratinocyte IL-8 production and TLR2 pathway activation as well as to
reduce in vivo inflammation induced by \textit{P. acnes} suggest that this peptide has potential in the treatment of acne vulgaris.

There are clear obstacles to the topical use of peptides when the pharmacologic target requires penetration through the epidermis. However, there is increasing evidence that \textit{P. acnes} forms biofilms in sebaceous follicles of patients with acne\textsuperscript{33-35}. Therefore, a topical formulation of an exogenous AMP such as HPA3NT3 could be highly effective in reducing viable \textit{P. acnes} in many types of acne lesions without the need to penetrate into the dermis. In addition, a bactericidal AMP may be able to overcome mechanisms of resistance found in bacteria in biofilms such as decreased growth rate and expression of resistance genes. While few studies to date have studied the role of \textit{P. acnes} biofilms in inflammatory nodules and cystic acne lesions, we are exploring the development of microneedle delivery of exogenous AMPs as a treatment modality for these types of lesions.

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REFERENCES


FIGURE LEGENDS:

Figure 1. Morphological perturbation and blebs of *P. acnes* induced by HPA3NT3.

*P. acnes* cells in the absence (a) and in the presence of 0.2 µM HPA3NT3 (b), HP(2-20) (c), and HPN3 (d) antimicrobial peptides. The red arrows indicate morphological perturbations and blebs, which are mainly apparent following treatment only with HPA3NT3 but not HP(2-20) or HPN3.

Figure 2. Inhibitory effect of HPA3NT3 on *P. acnes*-induced HK IL-8 production.

The expression of HK IL-8 mRNA was measured by real-time RT-PCR (a). The relative intensity was normalized using expression of 18S rRNA as an internal control. *P. acnes*-induced HK IL-8 secretion was measured by ELISA (b). HK treatment with either 3.2 µM HPA3NT3, HP(2-20), or HPN3 in the absence of *P. acnes* served as negative controls. The data shown are representative of triplicate experiments. All values are expressed as mean ± SD. Statistically significant differences in the expression of HK IL-8 were determined by ANOVA with probabilities shown for both the overall significance and the pairwise comparison (*P*<0.001).

Figure 3. Inhibition of *P. acnes*-induced NF-κB nuclear translocation by HPA3NT3 in HK cells. Immunolocalization of NF-κB was determined by immunofluorescent staining of cellular NF-κB as described in “Materials and Methods”. NF-κB was detected using specific anti-NF-κB p65 polyclonal antibodies (anti-NF-κB pAb), and its intracellular localization (green) was compared with Hoechst-stained nuclei (blue).
Untreated HK cells (a); HK cells treated with *P. acnes* (b); HK cells treated with *P. acnes* plus HPA3NT3 (c); HK cells treated with *P. acnes* plus HPN3 (d). Bars=20 µm.

**Figure 4.** *P. acnes*-induced intracellular calcium mobilization in HK cells inhibited by HPA3NT3. HK cells were treated with *P. acnes* (1x10⁸ CFU/150 µL) in the absence (a) or presence of pretreatment with 3.2 µM HPA3NT3 or the inactive control peptide HPN3 (b). As negative controls, HK cells were also treated with 3.2 µM HPA3NT3 or HPN3 alone without the addition of *P. acnes* (c). Intracellular free calcium concentration (nM) was determined by measuring the ratio of fluorescence at excitation wavelengths of 340 and 380 nm. Arrow heads (▲) precisely indicate the timing of treatment with *P. acnes* (a, b) or with HPA3NT3 or HPN3 (c).

**Figure 5.** *P. acnes*-induced TLR2 expression inhibited by HPA3NT3. Expression of HK TLR2 mRNA was measured by real-time RT-PCR (a). The relative intensity was normalized using expression of 18S rRNA as an internal control. All values are expressed as mean ± SD. Statistically significant differences in the expression of TLR2 mRNA were determined by ANOVA (*P<0.001). Localization of TLR2 was determined by immunofluorescent staining (b). FITC-labeled TLR2 (green) was shown in untreated HK cells (i), HK cells treated with *P. acnes* (ii), HK cells treated with *P. acnes* plus HPA3NT3 (iii), and HK cells treated with *P. acnes* plus HPN3 (iv). Treatment of HK cells with HPA3NT3 alone without *P. acnes* served as a negative control (v). Bars=20 µm.
Figure 6. *P. acnes* growth and *P. acnes*-induced inflammatory response were both inhibited by HPA3NT3 *in vivo*. Inflammation-associated erythema was visualized 24 hours after injection with *P. acnes*, *P. acnes* plus HPA3NT3, and HPA3NT3 alone, and compared to untreated control ICR mouse ears (a). The percent differences (right vs. left ear) in ear edema were compared among treatment groups every 24 hours over 96 hours (b). The total number (CFUs) of *P. acnes* recovered from ears treated with HPA3NT3 was significantly reduced compared to untreated ears inoculated with *P. acnes*. All values represent mean ± SD of three individual experiments (*P*<0.001) (c). Hematoxylin and eosin staining of paraffin-embedded ear sections demonstrated that HPA3NT3 dramatically reduced the inflammatory infiltrate associated with *P. acnes* infection (d). Staining of an untreated and non-inoculated ear served as a negative control. Bars=0.2 mm.
# TABLES AND FIGURES

**Table 1.**

a. The MIC values of synthetic AMPs against *P. acnes*

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<td>HPA3NT3</td>
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<td>HPN3 (inactive control)</td>
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b. HK cell viability measured by MTT assay in the presence of synthetic AMPs

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<tr>
<td>1.6</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>3.2</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>6.4</td>
<td>100 %</td>
<td>100 %</td>
</tr>
</tbody>
</table>

Treatment with 2% Triton X-100 as a positive control resulted in less than 2% HK cell viability; MIC, Minimal inhibitory concentration; AMPs, antimicrobial peptides; *P. acnes*, *Propionibacterium acnes*; HK, normal human keratinocytes.
Fig. 1.
Fig. 2.

(a) Relative Fold Increase: HKE8 mRNA

(b) HK IL-8 (pg ml⁻¹)

- Untreated
- P. acnes only
- P. acnes+3.2 μM AMP
Fig. 3.
Fig. 4.
Fig. 5.

(a) Relative Fold Increase: HK TLR2 mRNA

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK: Untreated</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>P. acnes only</td>
<td>2.1 ± 0.2 *</td>
</tr>
<tr>
<td>P. acnes + HPA3NT3</td>
<td>2.0 ± 0.1 *</td>
</tr>
<tr>
<td>P. acnes + HPN3</td>
<td>2.1 ± 0.1 *</td>
</tr>
</tbody>
</table>

(b) Immunofluorescence images:

(i) HK TLR2: control
(ii) HK TLR2 treated with P. acnes
(iii) HK TLR2 treated with P. acnes + HPA3NT3
(iv) HK TLR2 treated with P. acnes + HPN3
(v) HK TLR2 treated with HPA3NT3 alone
Fig. 6.

(a) Right Ear  Left Ear
Untreated Control  PBS
P. acnes only  PBS
P. acnes + HPA3NT3  PBS
HPA3NT3 alone  PBS

(b) Induced ear swelling ratio:
% difference of experiment

0 24 48 72 96
Time (hours) after treatment

P. acnes+HPN3
P. acnes only
P. acnes+Clindamycin
P. acnes+HPA3NT3

(c) P. acnes (CFU/g ear tissue)

Untreated control  P. acnes only
P. acnes + HPA3NT3  HPA3NT3 only

(d) Untreated control  HPA3NT3 only
P. acnes only  P. acnes+HPA3NT3