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Research Paper

The antimicrobial effect of CEN1HC-Br against Propionibacterium acnes and its therapeutic and anti-inflammatory effects on acne vulgaris

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ABSTRACT

Propionibacterium acnes is a commensal bacterium, which is involved in acne inflammation. An antimicrobial peptide named CEN1HC-Br, which was isolated and characterized form the green sea urchin, has been shown to possess broad-spectrum antibacterial activity. Little is known concerning the potential effects of its antibacterial and anti-inflammatory properties against P. acnes. To examine the potency of CEN1HC-Br in acne treatment, we conducted experiments to analyze the antibacterial and anti-inflammatory activities of CEN1HC-Br both in vitro and in vivo. The antimicrobial activity of CEN1HC-Br was evaluated by minimal inhibitory concentration (MIC) assays using the broth dilution method. To elucidate the in vitro anti-inflammatory effect, HaCaT cells and human monocytes were treated with different concentration of CEN1HC-Br after stimulation by P. acnes. The expression of TLR2 and the secretion of the pro-inflammatory cytokines IL-6, IL-8, IL-1β, TNF-α, IL-12, respectively, were measured by enzyme immunoassays. An evaluation of P. acnes-induced ear edema in rat ear was conducted to compare the in vivo antibacterial and anti-inflammatory effect of CEN1HC-Br, the expression of IL-8, TNF-α, MMP-2 and TLR2 was evaluated by immunohistochemistry and real time-PCR. CEN1HC-Br showed stronger antimicrobial activity against P. acnes than clindamycin. CEN1HC-Br significantly reduced the expression of interleukin IL-12p40, IL-6, IL-1β, TNF-α and TLR2 in monocytes, but they were not influenced by clindamycin. Both CEN1HC-Br and Clindamycin attenuated P. acnes-induced ear swelling in rat along with pro-inflammatory cytokines IL-8, TNF-α, MMP-2 and TLR2. Our data demonstrates that CEN1HC-Br is bactericidal against P. acnes and that it has an anti-inflammatory effect on monocytes. The anti-inflammatory effect may partially occur through TLR2 down-regulation, triggering an innate immune response and the inhibition of pro-inflammatory cytokines.

1. Introduction

Acne (Acne vulgaris) is a common dermatologic disorder. Approximately 85% of all individuals experience acne to some degree during the adolescence. Acne can have profound psychosocial effects and may undermine self-confidence and self-esteem at a vulnerable time in life [11].

Acne is a multifactorial chronic inflammatory disease of the pilosebaceous duct, including non-inflammatory open and closed comedones and inflammatory papules, pustules, nodules, and cysts. Lesions may be present on the face, neck, chest, or back-areas with the greatest density of pilosebaceous units [4]. Acne has four main pathogenetic mechanisms: abnormal keratinocyte proliferation and desquamation that leads to ductal obstruction, androgen driven increase in sebum production, proliferation of Propionibacterium acnes, and the products of inflammation [1]. The crucial steps for control of acne are to avoid P. acne colonization and inflammation in the pilosebaceous units.

Due to better understanding of the pathogenesis of acne, many anti-acne agents are in use for acne treatment (reviewed by Leyden [28]). Topical treatments (such as benzoyl peroxide and retinoids), topical antibiotics (such as erythromycin and clindamycin), and systemic therapies (such as oral antibiotics, oral retinoids and hormonal therapy) are used for acne treatment today. However, the main limitation is that these agents can induce irritant dermatitis [3,9], induce bacterial resistance [18], or be associated with gastrointestinal disturbance [40] and so on. Although the therapies mentioned above are still commonly

Abbreviations: CEN1HCBr, the brominated heavy chain of centrocin 1; MIC, minimal inhibitory concentration; IL, interleukin; TNF, tumor necrosis factor; TLR, Toll-like receptor; MMP, matrix metalloproteinase; AMPs, antimicrobial peptides; LPS, lipopolysaccharides

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employed to treat acne, safer and more efficient agents are strongly needed.

Recently, more light has been thrown on antimicrobial peptides (AMPs), which can be candidates for acne treatment. AMPs are relatively short amino acid sequences, usually less than 100 amino acids (aa) [12,32]. The sequences commonly contain many positive charged residues such as arginine and lysine, which help to form the net positive charge of cationic AMPs [14,47]. Many antimicrobial peptides fold into an amphipathic structure, which reflects the relative abundance and polarization of hydrophobic and hydrophilic domains in their conformational structures. The hydrophobicity assists water-soluble antimicrobial peptides to interact with the hydrophobic lipid bilayer of the cell membrane. These fundamental features of AMPs also determine their antimicrobial properties. Furthermore, there is evidence that AMPs have multiple roles, not only a direct antimicrobial function but also an indirect modulation of innate immunity, such as modulation of the expression of chemokines/cytokines, and influencing the processes of apoptosis, angiogenesis, and wound healing (reviewed by Hilchie et al. [16]).

Centrocin1 is a heterodimeric peptide which consists of a brominated heavy chain (30 aa, CEN1HC-Br) and a light chain (12 aa, CEN1LC) linked by an internal disulfide bond [30]. It was shown that CEN1HC-Br contributes to the antimicrobial activity while the CEN1LC did not seem to influence activity against microbes. Furthermore, it has been demonstrated that 6.3–12.5 mg/L of the debrominated version of the heavy chain (CEN1HC) was able to kill ≥99% of P. acnes ATCC 6919 in 0.037% BHI broth and 50% heat inactivated simulated wound fluid, respectively [2]. It was also shown that the heavy chain of centrocin1 reduced the release of inflammatory cytokine TNF-α and IL-6 in LPS-stimulated cell line THP-1. The above striking results of CEN1HC-Br motivate us to investigate whether CEN1HC-Br can be used as dermatological agents for treatment of acne disease.

In this study, CEN1HC-Br was examined for its effect against acne related bacterial pathogens including a couple of clinical isolates, especially clindamycin resistant strains. The anti-inflammatory activity of CEN1HC-Br was also studied in two types of cells. It was shown that CEN1HC-Br could inhibit the expression of TLR2 and cytokines such as TNF-α, IL-1β, IL-6. In addition, an animal model was used to investigate anti-inflammatory effects of CEN1HC-Br in vivo. We found that CEN1HC-Br attenuated P. acnes caused tissue swelling, TLR2 expression, and cytokines expression such as IL-8, TNF- α, MMP-2. These results suggested that CEN1HC-Br has drug potency for acne treatment.

2. Materials and methods

2.1. Peptide synthesis and antibiotics

The brominated heavy chain of centrocin 1 (CEN1HC-Br, FKKTFHKVSHAVKSGIHAGQRGCSA LGF) was purchased from BIOMOL International LP (Exeter, UK), which were synthesized by using Fmoc solid phase technology. The content and purity of peptides were determined by high performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis. Clindamycin phosphate, which has been used clinically in acne treatment for a long time [7], served as a positive control.

2.2. Preparation of bacteria

Strains of P. acnes (ATCC 6919), S. epidermidis (ATCC 12228) and S. aureus (ATCC 25913) were ordered from American Type Culture Collection. Fifteen strains of P. acnes were clinically isolated from patients with acne vulgaris at Sir Run Shaw hospital of Zhejiang University. All patients have no history of topical antibiotic treatment. The strains of P. acnes were cultured in Brucella agar supplemented with hemin (5 μg/ml; Sigma, St. Louis, Mo), Vitamin K1 (1 μg/ml; Sigma, St. Louis, Mo), and lysed horse blood (5% v/v; Shanghai, China) at 37 °C under an anaerobic atmosphere using MGC Anaeropack systems (Mitsubishi, Gas Chemical Co., Inc, Japan), respectively. P. acnes was cultured to the exponential phase for 2 days and to the stationary phase for 3–4 days. For antimicrobial testing P. acnes was then grown in Brain Heart Infusion broth (BHI broth; Sigma-Aldrich, USA). S. epidermidis (ATCC12228) and S. aureus (ATCC 25913) were grown in Mueller-Hinton Broth (MHB; Difco Laboratories, Detroit, MI).

2.3. In vitro antimicrobial testing

Minimal inhibitory concentration (MIC) of CEN1HC-Br and clindamycin phosphate was determined as the lowest concentration of test samples that completely inhibit bacterial growth, which was measured by optical density. Briefly, samples were prepared as a stock solution in 0.9% salt water and then twofold serially diluted. Ninety microliter of the broth (BHI broth for P. acnes, MH broth for S. epidermidis, and S. aureus), 10 μl of the bacterial suspension (10^7 CFU/ml) and 10 μl of the test sample were added together into a well of sterile 96-well microtitre plates and incubated under anaerobic conditions at 37 °C for 96 h for P. acnes and under aerobic conditions at 37 °C for 48 h for S. epidermidis and S. aureus. The optical density of cultures was measured at 600 nm by a microplate reader to estimate bacterial growth.

2.4. Cells

Monocytes from healthy donors were obtained from peripheral blood mononuclear cells (PBMC). PBMC were purified from buffy-coats (obtained from Blood Donation Center in Zhejiang, China) using a density gradient (Ficoll-Paque, Pharmacia, Glattbrugg, Switzerland). Monocytes were sorted from PBMC using anti-CD14-labeled magnetic beads (MACS, Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions, and were grown in RPMI medium with 10% fetal bovine serum.

HaCaT cells were grown in DMEM high glucose medium (4.5 g/L, Invitrogen, Basel, Switzerland), supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Invitrogen, Basel, Switzerland).

2.5. Cell viability assay

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) was used to assess the rate of cellular proliferation and quantify cell viability. In brief, HaCaT cells or monocytes were seeded in 96-well plates with 100 μl of medium at a density of 2×10^4 cells per well. After incubation of cells with CEN1HCBr, 10 μl of CCK8 solution was applied to each well and incubated for 1 h at 37 °C. Finally, the absorbance values at 450 nm were determined using a microplate reader (FLX800TBID, BioTek Instruments, Winooski, VT). All experiments were conducted in triplicate.

2.6. In vitro anti-inflammatory effect

Cultured HaCaT cells, which were propagated to at least 70% confluence, or monocytes were challenged with P. acnes (ATCC6919) at 1×10^6 CFU. After the cells were incubated with P. acnes for 1 h, CEN1HC-Br or clindamycin was added to each well at various concentrations (0.5, 1, 5, 10, 50 or 100 mg/L). To determine the expression level of cytokines, supernatants of monocytes or HaCaT cells were collected at 48 h after the addition of CEN1HC-Br or clindamycin. The following cytokines were determined: IL-8, IL-6, IL-1β and TNF-α for monocytes, IL-12p40 for HaCaT cells and monocytes. An enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) for each cytokine was used to determine the expression level of cytokines according to the manufacturer's instructions. All experiments were performed three times independently.

Table 1
Primer list of RT-PCR.

Name	Sequence
TNF-α forward	5′ CGTCAGCCGATTTGCCATTT
TNF-α reverse	5′ TCCCTCAGGGGTGTCCTTAG
MMP-2 forward	5′ AGGGCACCTCTTACAACAGC
MMP-2 reverse	5′ CCCGGTCATAATCCTCGGTG
IL-8 forward	5′ ACTCAAGAATGGTCGCGAGG
IL-8 reverse	5′ ACGCCATCGGTGCAATCTAT
TLR-2 forward	5′ TGGAGGTCTCCAGGTCAAATC
TLR-2 reverse	5′ TGTTTGCTGTGAGTCCCGAG
GAPDH forward	5′ CAAGTTCAACGGCACAGTCA
GAPDH reverse	5′ CACCCCATTTGATGTTAGCG

2.7. In vivo anti-P. acnes experiments

2.7.1. Animal treatment

P. acnes (ATCC6919) was grown to the exponential-phase in brucella agar and then resuspended in 0.9% saline (5×10^8 CFU/ml). Twenty-four female Sprague-Dawley rats (aged 3–5 weeks, weight 100–120 g) were purchased from Zhejiang Experimental Animal Center. The animals were divided into three experimental groups of eight animals each. P. acnes solution (10 μl) was intradermally injected into left ears of the animals. Right ears of the animals received the same volume of 0.9% salt water. The dose of clindamycin was based on previously reported efficacy [13]. 1000 mg/L clindamycin, 500 mg/L CEN1 HC-Br or normal saline was applied on the surface of left ear skin once per day after injection with P. acnes or saline. After bacterial injection for 24 h, ear thickness was measured using a microcaliper.

To investigate the therapeutical effect, the change of ear thickness was determined continuously from day 11 to day 14 using a vernier calliper. The granulomatous inflammation remains constant from the 6th day to 14th day. The rats were maintained at the animal facility at Zhejiang University.All the experimental protocols to use animals were performed under minimized suffering and approved by the Animal Care and Use Committee at Zhejiang University.

2.7.2. Histopathology and immunohistology check

Ears of animals were excised and fixed in neutral-buffered 10% formalin solution and embedded in paraffin. The slides were cut at approximately 4 μm and examined with haematoxylin and eosin staining as well as immunohistochemistry for anti-rat TLR2 antibodies (Rockland Immunochemicals, Gilbertsville, PA), TNF-α and MMP-2 antibodies (Abcam, Cambridge, United Kingdom,). Immunoperoxidase staining sections were acetone fixed and blocked with normal horse serum before incubation with the mAbs for 60 min, followed by biotinylated horse anti-mouse IgG for 30 min. Primary Abs were visualized with the ABC Elite system (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin, and mounted in aqueous dry mounting medium (Crystal Mount; Biomeda). Under a 400 × magnification light microscope, $TLR2^+$ cells, TNF-α^+ cells and $MMP\text{-}2^+$ cells were recorded as cells with brown staining in the cytoplasm. For each slide, five random fields that mainly contained papillary dermis were selected, and the number of positive dermal cells and the total number of dermal infiltration cells in each field were counted. Positive cell density is the ratio of total number of positive cells in five fields divided by total number of cells in five fields.

2.7.3. Real-time polymerase chain reaction (RT-PCR) measurement of inflammatory markers

Ears of rats were injected with live P. acnes (ATCC 6919) as described above. Both ears of the rat were excised on day 15 after bacterial injection respectively, and then homogenized in 0.9% salt water (1 ml per ear biopsy) with a hand tissue grinder. RT-PCR was performed with amplification kits purchased from TAKARA and Applied Biosystems (Life technologies, Sydney, Australia). Total RNA was extracted using TRIzol Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacture's instruction. After initial denaturation at 94 °C for 15 s, amplification was performed for 40 cycles at 95 °C for 5 s, 60 °C for 30 s. All 24 samples were tested for RT-PCR analysis of TNF-α, IL-8, TLR2 and MMP-2 (Primers were listed in Table 1).

2.8. Statistical analysis

One-way ANOVA was used for statistical analyses. Results are expressed as means ± standard errors of the mean. GraphPad (version 5) was used to calculate p values: ***, extremely significant, $p < 0.001$; **, very significant, $p = 0.001$–0.01; *, significant, $p = 0.01$-0.05; and ns, $p > 0.05$.

3. Results

3.1. Antimicrobial activities of CEN1HC-Br against skin bacteria

To determine antibacterial effects of CEN1HC-Br against skin bacteria, threestrains of common skin bacteria and fifteen clinical isolates of P. acnes were chosen for the test. As listed in Table 1, CEN1HC-Br showed strong antimicrobial activities against the tested P. acne strains. Both, peptide and clindamycin, were active against P. acnes ATCC6919 with a MIC value of 4 mg/L. However, CEN1HC-Br, proved more active than clindamycin against fifteen clinically isolated P. acnes strains. The lowest MIC value is 0.125 mg/L. Concentrations of 4–16 mg/L can even inhibit the growth of clindamycin resistant strains (Table 2). This suggests that CEN1HC-Br has stronger anti-P. acne activity than clindamycin in vitro. CEN1HC-Br also showed antimicrobial activity with a MIC of 32 mg/L against Staphylococcus. aureus ATCC 25913, while clindamycin had no activity even at 512 mg/L. In addition, CEN1HC-Br inhibited the growth of S. epidermidis ATCC 12228 at 16 mg/L, while the MIC value of clindamycin was 64 mg/L.

3.2. Cytotoxic assays

Human keratinocytes, which are a major cell type in the epidermis, were used to investigate the cytotoxic effects of CEN1HC-Br. In addition the viability of human monocytes was used to evaluate the cytotoxicity of CEN1HC-Br. We found that monocytes were about 95% viable

Table 2
MIC (mg/L) of CEN1HC-Br and clindamycin against P. acne strains.

	ATCC 6919	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CEN1HC-Br	4	0.125	4	8	2	16	2	8	32	4	16	16	16	16	16	0.125
Clindamycin	4	0.125	*	*	4	*	16	128	4	16	*	*	*	64	64	0.125

MIC was defined as the lowest concentration of test samples that completely inhibit microorganism growth. *MIC value is > 512 mg/L. The clindamycin resistant strains are highlighted with gray.

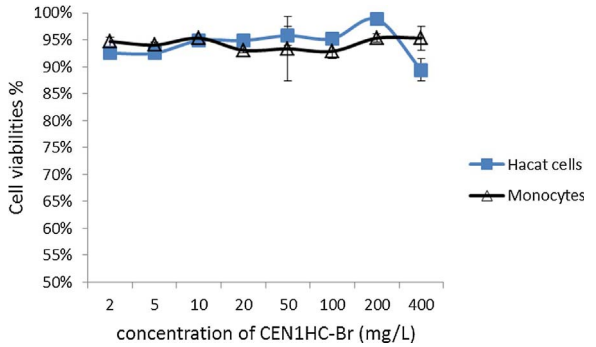

Fig. 1. Cytotoxicity of CEN1HC-Br on human HaCaT cells and monocytes. Each value represents the mean of three individual experiments.

incubated with CEN1HC-Br at concentrations from 2 to 400 mg/L (Fig. 1). Further, although CEN1HC-Br exerted little effects on HaCaT cell viability at 400 mg/L (viability changed from 98.8% to 89.4%), CEN1HC-Br had no impact on cell viabilities from 2 to 200 mg/L. These results indicate that use of CEN1HC-Br does not result in any significant cytotoxicity against HaCaT cells or monocytes even at concentrations more than tenfold higher than necessary for antibacterial activity against P. acnes.

3.3. Effects of CEN1HC-Br on proinflammatory cytokines in vitro

It is known that P. acnes elicit an inflammatory response in acne vulgaris. The inflammation of infected human monocytes by P. acnes results in the activation of TLR2, which leads to the release of a wide variety of proinflammatory cytokines such as IL-6, IL-1β, IL-8, and TNF-α through the NF-κB signaling pathway [22]. To examine whether CEN1HC-Br has an immunomodulatory effect, we examined the expression of TLR2, IL-8, IL-6, IL-1β, TNF-α and IL-12p40 in P. acnes-stimulated monocytes. We found that the expression of TLR2 was increased two-fold in monocytes after P. acnes inoculation for 48 h (data not shown). Importantly, this overexpression of TLR2 was significantly down-regulated by co-treatment with CEN1HC-Br and the effect was dose-dependent (Fig. 2A). Although clindamycin also inhibited the

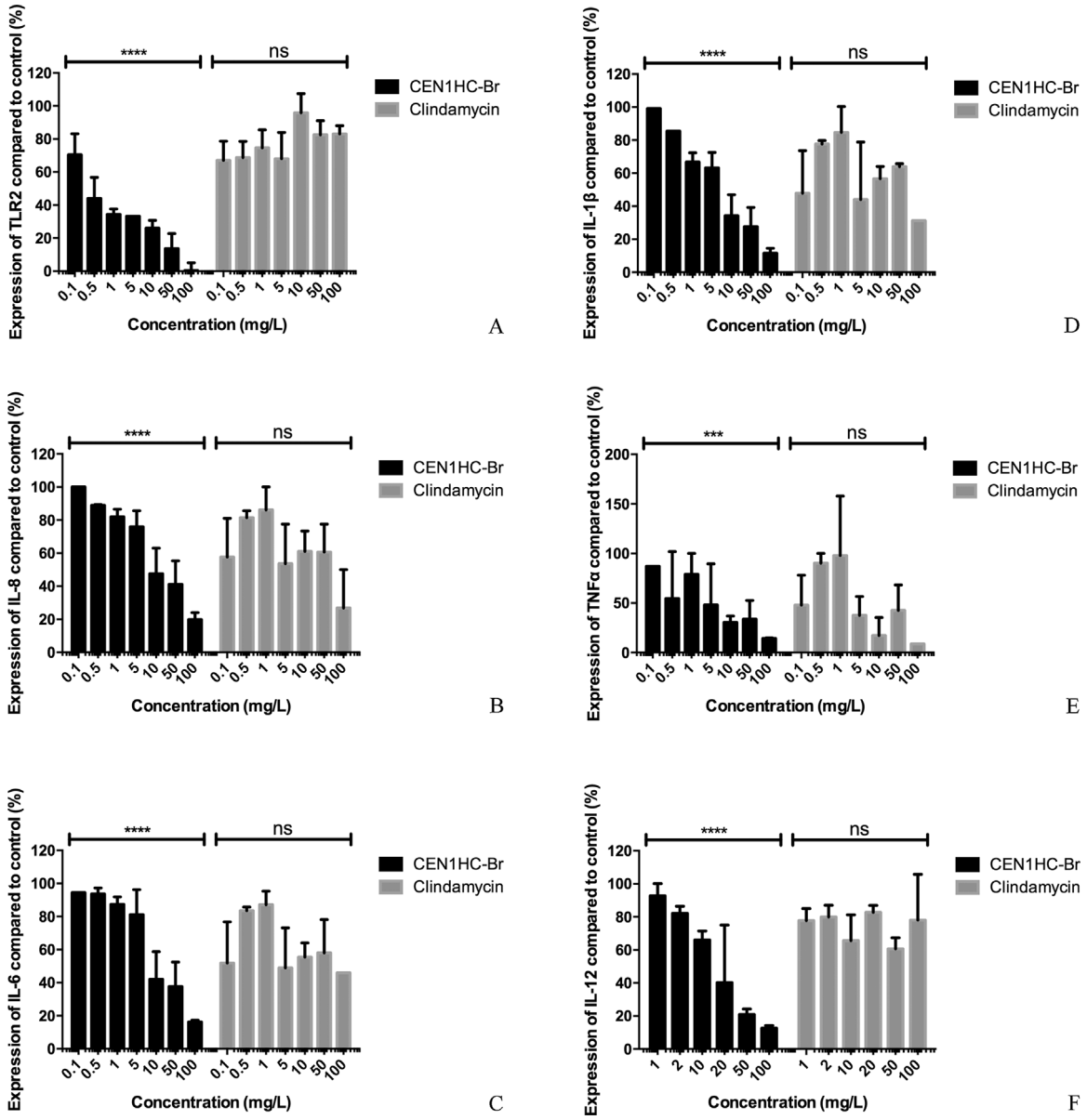

Fig. 2. Inhibitory effects of CEN1HC-Br and clindamycin on TLR2 (A), IL-8 (B), IL-6 (C), IL-1b (D), TNF-α (E) and IL-12p40 (F) secretion in human monocytes. Data represent mean ± SE of three individual experiments.

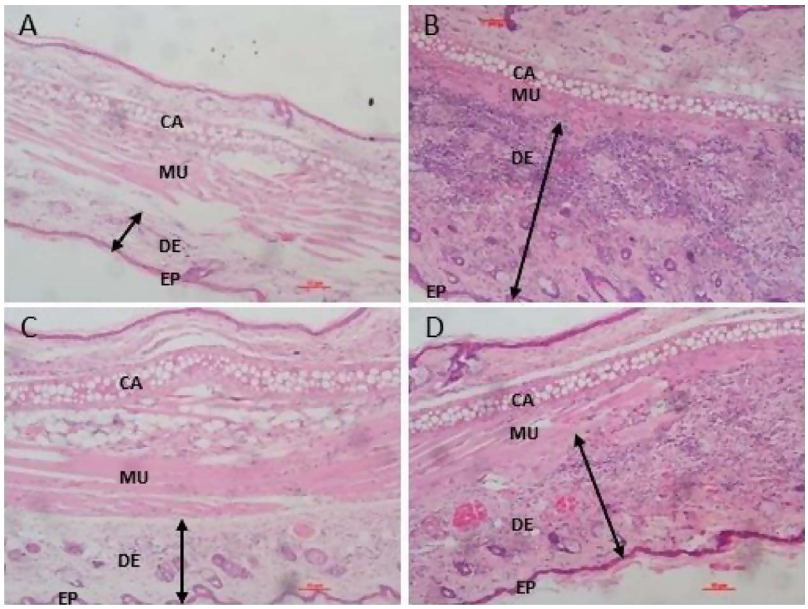

Fig. 3. Histopathology analysis of SD rat ears after epicutaneous application of vehicle (B), CEN1HCBr (C) or clindamycin (D) for 14 days. (A) Rat ear injected with only 0.9% saline. (B, C, D) The ears of experimental rats were intradermally injected with P. acnes. P. acnes significantly induced dermis tissue inflammation and swelling as illustrated in (B). Many infiltrated inflammatory cells could be found in the injection site of P. acnes. After epicutaneous application of 500 mg/L CEN1HCBr solution on the rat ear for 14 days, the inflammation induced by P. acnes was ameliorated effectively, and the number of infiltrated inflammatory cells significantly decreased (C). The positive control 1000 mg/L clindamycin also effectively inhibited ear inflammation induced by P. acnes injection and decreased infiltrated inflammatory cells (D). CA, ear hyaline cartilages; EP, epidermis; DE, dermis; MU, muscle. The arrows in the figure indicate the thickness of the dermis layer. Scale bar = 10 μm.

expression of TLR2, the effect was not dose-dependent. Further, the production of IL-6, IL-1β, IL-10, TNF-α and IL-12p40 in P. acnes-induced monocytes was also inhibited by CEN1HC-Br in a dose-dependent manner (Fig. 2B–F). For example, P. acnes-induced monocytes incubated with 0.5 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, 50 mg/L, and 100 mg/L of CEN1HC-Br resulted in 88% ± 0.3%, 81% ± 3.8%, 75% ± 7.9%, 47% ± 12.5%, 41% ± 11.6%, 19.8% ± 3.4% induced IL-8 secretion compared to the positive control, respectively, while 0.1 mg/L of peptide did not show any inhibition. Although clindamycin exerted a reduction of inflammatory cytokines including IL-8, IL-6, IL-1b, TNF-α and IL12p40 in human monocytes, this effect was not significant.

3.4. Effects of CEN1HC-Br on P. acnes-induced inflammation in vivo

3.4.1. Histopathology and immunohistology check

Ear swelling induced by P. acnes challenge was observed as illustrated in Fig. 4 Seven days post injection (dpi) of P. acnes, visible ear swelling of bacterial challenged rats was three times thicker than the ears challenged with saline (the blank control). No swelling was observed in blank control group. All rats were recovered gradually. At 11 dpi to 14 dpi, the ear thickness of rat only using the vehicle as the treatment control was 279% ± 41.1%, 240% ± 35.8%, 190% ± 32.7%, and 149% ± 37.3% of that of the blank control while that of CEN1HC-Br treated group was 224% ± 34.8%, 197% ± 34.2%, 130% ± 25.5%, and 102% ± 28.1% of that of the blank control, respectively. For the positive group (clindamycin-treated), the thickness of ear was 245% ± 36.9%, 202% ± 40.4%, 153% ± 32.3%, and 114% ± 21.1% of that of the blank control, respectively. Both treatment with CEN1HC-Br and clindamycin exhibited significant reduction of ear swelling ($P < 0.01$), but no significant difference between these two treatments.

To further evaluate the anti-inflammatory effect of CEN1HC-Br in vivo, a histological check was conducted (Fig. 3). Comparison of Fig. 3A and B illustrates that the ear inflammation and swelling in the ear tissue of the experimental rat was induced significantly by intradermally injected P. acnes. Many infiltrated inflammatory cells could be found at the bacterial injection site. After epicutaneous application of CEN1HC-Br solution on the rat ear for 14 days, the inflammation was ameliorated effectively, and the number of infiltrated inflammatory cells decreased markedly (Fig. 3C). However, the treatment control group (with vehicle) did not show a reduction of ear swelling. The positive control treated with clindamycin inhibited the ear inflammation induced by bacterial injection and decreased infiltrated inflammatory cells (Fig. 3D), while its therapeutic potency was less than that of CEN1HC-Br.

To investigate whether P. acnes induces a change of expression of TLR2, TNF-α, and matrix metalloproteinase 2 (MMP-2), the expression of TNF-α, MMP-2 and TLR2 in the challenged rat ear skin was further evaluated by immunohistochemistry. Immunohistochemistry labeling using a specific anti-rat antibody against TNF-α, MMP-2, and TLR2 revealed that the number of TNF-α, MMP-2, and TLR2 positive cells apparently increased in the ear skin of the experimental rat (Fig. 4). After epicutaneous application of CEN1HC-Br or clindamycin solution on the rat ear for 14 days, the expression of TNF-α, MMP-2 and TLR2 was decreased significantly, while there was no significant difference between the CEN1HC-Br and clindamycin group by immunohistochemistry according to the quantitative analysis of stained positive cells (Fig. 5).

3.4.2. Realtime-PCR measurement of inflammatory markers

To determine whether the transcription of the pathogen recognition receptor and proinflammatory cytokines is mediated by CEN1HC-Br, we used real-time PCR to measure the effects of CEN1HC-Br on the expression of TLR2, MMP-2, TNF-α and IL-8. We found that both the expression of MMP-2 and IL-8 in the CEN1HC-Br group is significantly lower than in the clindamycin group and control ($P < 0.05$), while the expression of TNF-α of the CEN1HC-Br group is lower than of the clindamycin and control group but without statistical significance (Fig. 6). The expression of TLR2 in the CEN1 HC-Br and clindamycin group is significantly lower than control group ($P < 0.05$).

4. Discussion

A strong demand motivates research scientists and pharmaceutical companies to seek safer and more effective agents to treat acne, especially agents avoiding antibiotic resistance development. It has been shown that topical erythromycin and clindamycin treatment could lead to a gradual increase in antibiotic resistance [17], which results in the failure of antibiotic treatment for acne. The horizontal gene transfer allows the antibiotic resistant genes to further spread to other potentially pathogenic bacterial species and/or strains [29]. Currently, AMPs as a novel group of antimicrobial agents attract more attention to develop an alternative acne treatment [15]. Several antimicrobial peptides (AMPs) are related with the infection of acne vulgaris. It has been

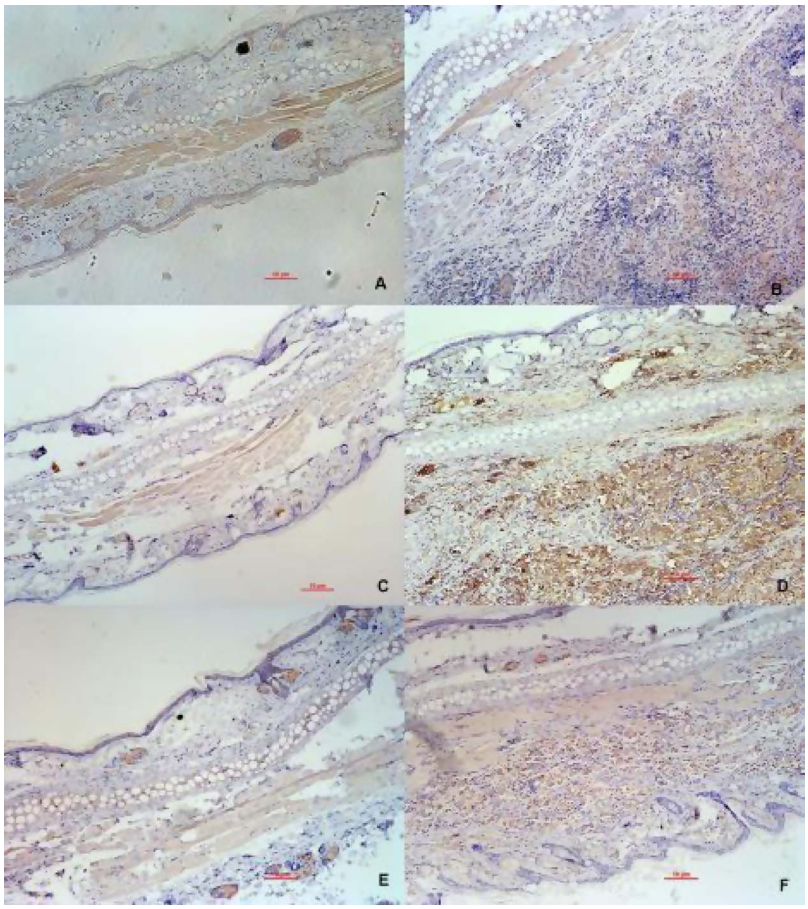

Fig. 4. Immunohistochemistry of the expression of TNF-α, MMP-2, and TLR2 on rat ears. The rat ear injected with only 0.9% saline did not show TNF-α^+ cells (A), MMP-2^+ cells (C), and TLR2^+ cells (E). In contrast, numerous TNF-α^+ cells (B), MMP-2^+ cells (D), and TLR2^+ cells (F) were detected in the inflammatory infiltrate around the perifollicular/peribulbar region 7 days after injection of P. acnes. Original magnification, Scale bar = 10 μm.

reported that the levels of hBD-2, S100A7, HNP1-3 and granulysin increased in acne lesions, suggesting that they may play a protective role in acne. Based upon the expression of AMPs induced by P. acnes, researchers have been attempting to develop hBD-2, LL-37, S100A7, RNase 7 and granulysin as therapeutics for P. acnes-mediated infection, which individually or synergistically display antimicrobial activity against P. acnes. However, hBD-2, LL-37, S100A7 and HNPs may exacerbate inflammation through recruitment and activation of immune cells and release of pro-inflammatory mediators [15]. Only a synthesized granulysin-derived peptide has been reported to suppress P. acnes-mediated cytokine release, regardless of the increase in the number of comedones [31]. Dermcidin (DCD) peptides are the principal

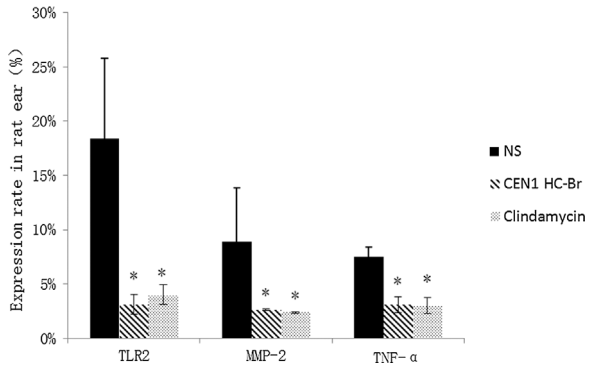

Fig. 5. Immunohistochemistry of TLR2, MMP-2 and TNF-α. After epicutaneous application of CEN1HC-Br, clindamycin or vehicle on the rat ear for 14 days, all the inflammatory markers returned to baseline levels. There is no significant difference between the CEN1HC-Br group and clindamycin group ($P > 0.05$). Data represent mean ± SE of eight rats in each group. NS: vehicle group.

AMPs in sweat. In contrast to the above AMPs, the expression of dermcidin (DCD), a principal AMPs in sweat, is downregulated in the sweat of patients with acne vulgaris. Since DCD possesses antimicrobial activity against P. acnes, its reconstitution may also be a potential candidate [37]. To further augment the therapeutic effect of AMP, various exogenous and synthetic AMPs have been developed recently. Ryu S. et al. examined the role of Helicobacter pylori-derived synthetic antimicrobial peptide HPA3NT3 and an α-helical cationic peptide P5 against P. acnes in the skin, and their ability to block P. acnes-induced inflammation. Both AMPs appear to be highly potential therapeutic agent for the treatment of acne vulgaris by intradermal injection in mice ear [41,42]. Two frog skin-derived antimicrobial peptides [D4k] ascaphin-8 and [T5k]temporin-DRa showed potential activities in inhibiting the growth of P. acnes and the release of pro-inflammatory cytokines, and in stimulating the release of anti-inflammatory cytokines in human peripheral blood mononuclear cells [39].

A Trp–Lys-rich peptide LZ1 was screened and its antimicrobial and anti-inflammation potential was demonstrated by Zhang Z. Furthermore, LZ1 showed little hemolytic activity on human red blood cells and high stability in human plasma. Topical application of LZ1 on mice ear also showed antimicrobial effects and inhibition of the P. acne-induced inflammation [48]. Several synthetic AMPs, such as Omiganan and HB1345 have even entered clinical trials as an alternative treatment of acne vulgaris, but have not yet been approved [38]. Translating AMP activity from in vitro experimentation to clinical application has been difficult due to the cost of goods, their lability to proteolytic degradation, and their unknown toxicology profile when administered systemically. In this context, we try to find a new topical usable AMP for the treatment of acne vulgaris.

CEN1 HC-Br showed strong antimicrobial activities against S. epidermidis (ATCC 12228), S. aureus (ATCC 25913), P. acnes (ATCC

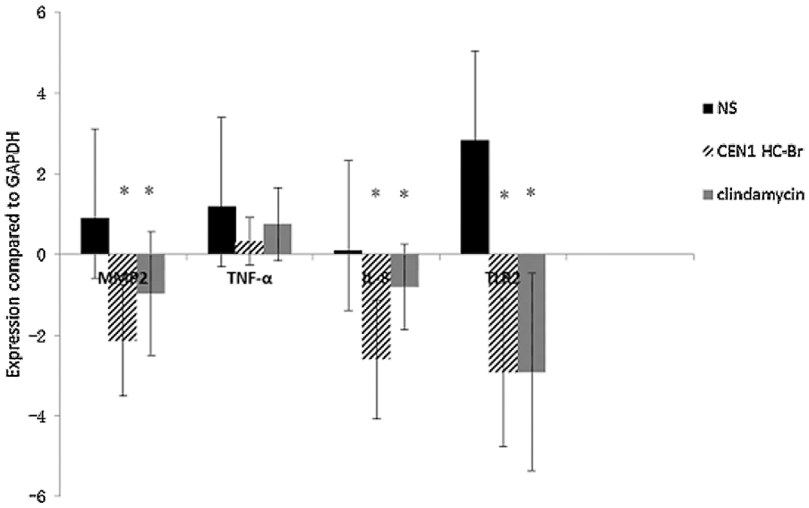

Fig. 6. The expression of TLR2, MMP-2, IL-8, and TNF-α mRNA was measured using real-time RT-PCR and normalized to the expression of GAPDH. All values are expressed as the mean ± SD. *P < 0.05. NS: vehicle group.

6919), and also 15 clinical isolates of P. acnes. This is in agreement with the findings of AMPs' anti-P. acnes activity by other researchers. For examples, 4 μM of synthetic LL-37 killed 100% of P. acnes, while 50 μg/ml of psoriasin alone killed 95% P. acnes [26]. Granulysin is effective against P. acnes at the concentration of 32 μM [33]. In our study, the most striking result is CEN1 HC-Br activity against clindamycin resistant strains. All tested clinical isolates of P. acnes showing clindamycin resistance (MIC > 512 mg/L) were susceptible to CEN1 HC-Br (MIC range from 4 to16 mg/L, Table 1). Although we have no clues for a variety of phenotypes of resistance, the use CEN1 HC-Br and clindamycin to screen clinical isolates and combined with genomic mapping of those isolates might illustrate the target molecules by CEN1 HC-Br and the development of resistance.

Another significant feature of CEN1 HC-Br is that it exerted strong anti-inflammatory effects. Follicular colonization by P. acnes plays a key role in the formation of acne. The proliferation of P. acnes will attract CD4+ lymphocytes and macrophages to the microcomedone [20] and then induce the inflammatory acne lesion with rupture of the follicular wall. As illustrated by Fig. 3B, injection of P. acnes attracted plenty of infiltrated inflammatory cells. After epicutaneous administration of CEN1 HC-Br, the number of infiltrated inflammatory cells decreased markedly (Fig. 3C), and ear swelling induced by P. acnes was inhibited significantly, which suggested a strong anti-inflammatory effect. 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 in late (inflammatory) acne lesions via the activation of TLR2 [8]. Activation of TLR2 on monocytes releases pro-inflammatory cytokines, IL-6, IL-1β, IL-12 and IL-8. IL-8 attracts neutrophils to the site of active lesion, and triggers the release of lysosomal enzymes by neutrophils, which leads to rupture of the follicular epithelium and further inflammation [19,24]. Reduced expression of IL-8 after treatment with CEN1 HC-Br was observed in human monocytes in ELISA (Fig. 2) and in rat ear tissue by immunohistochemistry and RT-PCR respectively (Figs. 4–6). IL-12 is a pivotal cytokine in activating Th1 T cell responses and is one of the major proinflammatory cytokines produced by monocytes in response to Gram-positive organisms [45]. Overproduction of cytokines in Th1 cells such as IL-12 has been implicated in the development of tissue injury in certain autoimmune and inflammatory diseases [21]. The inhibited production of IL-12 by CEN1 HC-Br was shown in human monocytes by ELISA (Fig. 2). Meanwhile, the ELISA results also demonstrated suppressed production of inflammatory cytokines including IL-6 and IL-1β in human monocytes (Fig. 2). TNF-α and MMP-2, mediators and markers of inflammation, were induced by the P. acnes injection, evidenced by an acute rise of their mRNA and protein levels, reflected by the results of the immunohistochemistry and PCR respectively (Figs. 4–6). This is consistent with prior reports that P. acnes can stimulate focal secretion of TNF-α [43]. MMPs can be induced by P. acnes either directly or by P. acnes-induced elevation of TNF-α. [5,27]. P. acnes-derived PGN play an aberrant role due to the enhancement of proMMP-2 production in acne lesions via a TLR2-dependent pathway [44]. In this manner, the activation of TLR2 on monocytes is likely involved in the pathogenesis of acne. The inhibition effect of CEN1 HC-Br on TLR2 may explain the reduced expression of IL-6, IL-1β, IL-12, IL-8, TNF-α and MMP-2 in monocytes.

Furthermore, since MMP-2 has higher substrate specificity to type-IV collagen, a structural element of the basement membrane [46], the acceleration of MMP-2 expression by P. acnes in sebaceous glands in acne lesions is likely to result in the destruction of the basement membrane between the epithelium and dermis, subsequently leading to dermal extracellular matrix degradation for acne scarring. Inhibition of MMP-2 production by CEN1 HC-Br can be effective as an anti-acne agent for the prevention and remission of scar formations.

Clindamycin, a lincosamide antibiotic, was used as a control drug in this study. It also exerted comparative inhibition of the expression of TLR2, IL-8, TNF-α and MMP-2 in rat ear model (Figs. 4–6), but without significant reduction of inflammatory cytokines including IL-8, IL-6, IL-1b, TNF-α and IL-12p40 in human monocytes as shown by ELISA (Fig. 2). It was consistent with previous report that clindamycin has no inhibitory effect against the production of proinflammatory cytokines in mononuclear cells, but down-regulate keratinocyte production of proinflammatory cytokines and chemokines [25]. Thus, it seems to be rational that keratinocytes have a pivotal role in initiating and potentiating inflammation, and that clindamycin can interfere with the keratinocyte activation cycle driven by P. acnes, lymphocyte and keratinocyte-derived cytokines in acne vulgaris [10].

The concentrations of CEN1 HC-Br and clindamycin employed in the present study were 0.1–100 mg/L, being similar to those needed for antibacterial activity against P. acnes. However, drug concentrations in the rat skin measured 4 h after a single application of CEN1HC-Br and clindamycin solution were approximately 20 mg/kg (in wet tissue), similar as the concentration used in the study and under the cytotoxic concentration. Accordingly, the concentration may be achievable in acne lesions after single topical application of these antimicrobials. From the histopathology check of rat ear, no necrosis or abnormal epithelial cells were seen after 15 days application of CEN1HC-Br and clindamycin solution.

Together, our data shows that CEN1 HC-Br has antimicrobial activity against skin bacteria in vitro and inhibits P. acne-induced TLR2 expression and subsequently downregulates inflammatory cytokines both in vitro and in vivo. Therefore, CEN1HC-Br with its dual effects on

acne, might be an outstanding alternative for acne treatment. Especially its activity against antibiotic resistant strains make it stand out from conventional drugs.

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Conflict of interest

The authors have no conflict of interest to declare.

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