The Inhibitory Effect of combined human cathelicidin with antibiotics on antibiotic-resistant *Cutibacterium acnes*

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ผลของ HUMAN CATHELICIDIN เมื่อใช้ร่วมกับ ANTIBIOTICS ต่อการยับยั้งเชื้อดื้อยา CUTIBACTERIUM ACNES

น.ส.รักวารี สีหราช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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รักวารี สีหราช : ผลของ HUMAN CATHELICIDIN เมื่อใช้ร่วมกับ ANTIBIOTICS ต่อการยับยั้งเชื้อดื้อยา CUTIBACTERIUM ACNES. (The Inhibitory Effect of combined human cathelicidin with antibiotics on antibiotic-resistant *Cutibacterium acnes*) อ.ที่ปรึกษาหลัก : รศ.ดร. นพ.ดิเรกฤทธิ์ เชี่ยวเชิงชล, อ.ที่ปรึกษาร่วม : ดร. ธนิษฐา ฉัตรสุวรรณ

Cutibacterium acnes เป็นหนึ่งในปัจจัยสำคัญที่เกี่ยวข้องกับการเกิดสิวในผู้ป่วยโดยพบว่าเชื้อ *C. acnes* มักดื้อต่อยาปฏิชีวนะที่ใช้ในการรักษาสิว ได้แก่ clindamycin และ doxycycline ซึ่งการเพิ่มขึ้นของเชื้อ *C. acnes* ดื้อยาปฏิชีวนะนั้นเป็นสิ่งที่น่ากังวล สำหรับการศึกษาในครั้งนี้ผู้วิจัยได้พยายามค้นหาแนวทางการรักษาใหม่ๆ โดยใช้สารที่มีฤทธิ์ต้านแบคทีเรียจากธรรมชาติที่ผลิตได้จากร่างกายมนุษย์ คือ เปปไทด์ ชนิด human cathelicidin หรือ LL-37 ซึ่งมีความสามารถในการต้านจุลชีพได้หลากหลายรวมถึงเชื้อแบคทีเรีย นำมาศึกษาความสามารถในการยับยั้งเชื้อ *C. acnes* ที่ได้จากผู้ป่วยสิวของโรงพยาบาลจุฬาลงกรณ์ ด้วยวิธี broth microdilution assay และความสามารถในการออกฤทธิ์ร่วมกับยา clindamycin และ doxycycline ด้วยวิธี checkerboard assay และ time-killing assay ควบคู่ไปกับการทดสอบหาความเป็นพิษต่อเซลล์ด้วยวิธี MTT assay จากการศึกษาพบว่า LL-37 สามารถยับยั้งการเจริญเติบโตของ *C. acnes* ที่ดื้อต่อยาทั้งหมดที่แยกได้มาจากผู้ป่วยด้วยความเข้มข้นระหว่าง 25-50 μg/ml และยังสามารถเสริมฤทธิ์ร่วมกันกับยา doxycycline ได้ โดยมีค่าดัชนีชี้วัดประสิทธิภาพร่วม (FICI) เท่ากับ 0.5 ที่ความเข้มข้นของ LL-37 ที่ 0.1 μg/ml และความเข้มข้นของ doxycycline ที่ 8 μg/ml อย่างไรก็ดีสารทั้งสองชนิดที่สามารถเสริมฤทธิ์กันนี้ ตรวจพบว่ามีความเป็นพิษต่อเซลล์ผิวหนังของมนุษย์ จึงต้องมีการศึกษาเพิ่มเติมต่อไปในอนาคตเพื่อลดความเป็นพิษต่อเซลล์ รวมไปถึงความสามารถของ LL-37 ในการออกฤทธิ์ร่วมกับยาที่เป็น topical agent ชนิดอื่นๆ

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Cutibacterium acnes is one of the major factors involved in the pathogenesis of acne vulgaris which sometimes develops antibiotic resistance particularly clindamycin- and doxycycline-resistant *C. acnes*. In this study, human cathelicidin (LL-37) which is a type of human antimicrobial peptide against a wide range of microorganisms was used. We investigated the antimicrobial activity of LL-37 on *C. acnes* isolated from patients with acne at the King Chulalongkorn Hospital using broth microdilution. Moreover, the synergistic effects of LL-37 with clindamycin and doxycycline were investigated using checkerboard microdilution, and time-killing assays. The cytotoxicity of LL-37 was also evaluated by MTT assay. The results demonstrated that LL-37 had an inhibitory effect against all clinical isolates of a *C. acnes* including antibiotic-resistant strain at concentrations ranging from 25 to 50 µg/ml. Furthermore, LL-37 at the concentration of 0.1µg/ml showed a synergistic effect with doxycycline at the concentration of 8 µg/ml with the fractional inhibitory concentration index (FICI) of 0.50. However, the combination of LL-37 and doxycycline showed a significant cytotoxic effect on human keratinocytes. Therefore, better understandings how to reduce the cytotoxicity of this drug combination are required, and the synergistic effect of LL-37 with other topical antibiotics is probably needed.

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CHAPTER I

INTRODUCTION

BACKGROUND INFORMATION AND RATIONALE

Acne is a common chronic inflammatory skin disease found approximately 95% in adolescents and 25% in adults without gender preference (1). Patients with acne typically manifests with comedones, inflammatory papules, pustules, painful nodules, or cysts at seborrheic area such as face, upper chest and back. Scarring is the most concerned outcome of acne (2), and this condition not only affects physical appearance of the patients but also lowers their self-esteem causing mental health problems (3). In clinical practice, patients with acne are initially treated with topical therapies (e.g., antibiotics, retinoids and hydroxy acids) and/or systemic treatment (e.g., antibiotics, hormones and isotretinoin). The aim of these agents is to reduce skin inflammation and decrease colonization of *Cutibacterium acnes* which is involved in the pathogenesis of acne (4).

Cutibacterium acnes or *C. acnes* is an anaerobic, rod-shaped, gram-positive bacteria (5) which is a type of skin microbiota and this microorganism is commonly found inside sebaceous glands (anaerobic condition). However, *C. acnes* is associated with other conditions including eye

complications, opportunistic infections, herniated disc, and sarcoidosis (6) . Importantly, *C. acnes* is one of the main four factors involved in the pathogenesis of acne. The other main factors are a) increased sebum production, b) keratinocyte proliferation leading to obstruction of pilosebaceous unit (hair follicle and sebaceous gland) that promotes formation of comedones, and c) skin inflammation. The obstruction and comedone formation provides pilosebaceous unit with an anaerobic condition which favors the growth of *C. acnes*. This organism consumes epidermal lipids and produces metabolites that trigger skin inflammation (5).

Topical and systemic therapies are very effective in reduction of *C. acnes* in most patients with acne, but the side effects of these agents are common such as skin peeling, irritation, dryness, photosensitivity, and systemic symptoms (7). As elimination of *C. acnes* colonization is a major goal of acne treatment, there are a variety of commonly used in patients such as clindamycin, erythromycin, tetracycline, doxycycline, etc. However, a recent study has shown that antibiotic-resistant *C. acnes* is increasing tremendously due to prolonged and improper use of antibiotics (8). In fact, the antibiotic-resistant *C. acnes* was firstly reported in 1979 and it was found that clindamycin- and erythromycin-resistant strains could be frequently isolated from

inflammatory lesions of patients with acne. Moreover, other antibiotic-resistant *C. acnes* (e.g., tetracycline resistance) were also observed. Recently, it has been estimated that the incidence of antibiotic-resistant *C. acnes* has been increasing up to 64% (8), particularly erythromycin- and clindamycin-resistance (9). For this reason, novel treatments for *C. acnes* have become the major interest in many studies (10).

One of the most potential antimicrobial agents for acne treatment is human antimicrobial peptides (AMPs). Human AMPs are short cationic amphipathic peptides with diverse sequences produced by various cells and tissues (11). AMPs, also known as host defense peptides (HDPs), because they protect the host cells from invading microorganisms, and play essential roles in signaling pathways during immune responses (12). HDPs directly destroy bacteria through initial interactions with the bacterial membrane by electrostatic interaction and form multiple pores on the bacterial cell membrane leading to cell rupture (13).

One of the most abundant AMPs on human skin is cathelicidin. This peptide is a highly effective AMPs that shows an the inhibitory effect on different microorganisms (14). A previous study showed that active form of cathelicidin (or LL-37) inhibited growth of *Pseudomonas*

aeruginosa, Salmonella typhimurium, Escherichia coli, Listeria monocytogenes, Staphylococcus epidermidis, Staphylococcus aureus, and vancomycin-resistant *Enterococci* (15). Moreover, it was reported that cathelicidin was a non-toxic substance to human cells (16). Although the inhibitory effects of cathelicidin on many pathogens have been demonstrated, this effect on *C. acnes* has never been explored.

Therefore, this study aimed to investigate the inhibitory effect of LL-37 on *C. acnes* and the resistant strains. Clindamycin- and doxycycline-resistant *C. acnes* isolated from patients with acne vulgaris were used in this study. Moreover, the synergistic effect of LL-37 and the most common systemic antibacterial agent (e.g., clindamycin and doxycycline) were determined. The results from this study could provide new insights on alternative treatment for patients with acne vulgaris particularly *C. acnes* resistant strains.

RESEARCH OBJECTIVES

1. To investigate the inhibitory effect of human cathelicidin on a standard strain of *C. acnes* (ATCC strain) and antibiotic-resistant *C. acnes* from clinical isolates of patients with acne.

2. To investigate the synergistic effect of human cathelicidin and antibiotics (e.g., doxycycline, clindamycin) on the inhibition of *C. acnes* ATCC strain and antibiotic-resistant *C. acnes* from clinical isolates of patients with acne

3. To investigate the cytotoxicity of human cathelicidin on human cell lines (e.g., human keratinocytes)

CHAPTER II

LITERATURE REVIEW

A. HUMAN CATHELICIDIN

Human cathelicidin is a short cationic amphipathic peptide and classified as a type of antimicrobial peptides (AMPs) that shows a broad spectrum of antimicrobial property. There is only one cathelicidin gene (*CAMP*) found in humans and located at locus p21 of chromosome 3 and "LL-37" is the only peptide encoded by this *CAMP* gene (17). The LL-37 peptide forms into an alpha-helical structure and it has a positive net charge (+6), that binds to negative charge of pathogen cell membranes using electrostatic interaction, leading to pathogen destruction (11, 18).

LL-37 is consecutively produced by various cell types such as keratinocytes, neutrophils, macrophages, NK cells, epithelial cells of eccrine ducts, and mast cells (12, 19). The expression of LL-37 in these cells is regulated by different stimuli such as pathogens (e.g. bacteria and fungi), inflammatory cytokines (e.g. IL-1), and certain essential vitamins (e.g. vitamin D3) (20). The production of LL-37 starts at *CAMP* gene transcription to protein translation. First, *CAMP* gene encodes a preform peptide (cathelicidin pre-propeptide). This preform is thereafter cleaved by protease enzyme into hCAP-18 sequence which has molecular weight of 18 KDa. The C-

terminal antimicrobial domain of the hCAP-18 sequence is cleaved into LL-37 that contains 37 amino acids including two leucines at the N-terminal (**Figure 1**) (21). Human LL-37 is eventually cleaved by proteinase 3 and elastase enzymes which are finally eventually formed into different short segments (e.g., KR-20, KS-30, and RK-31) (22).

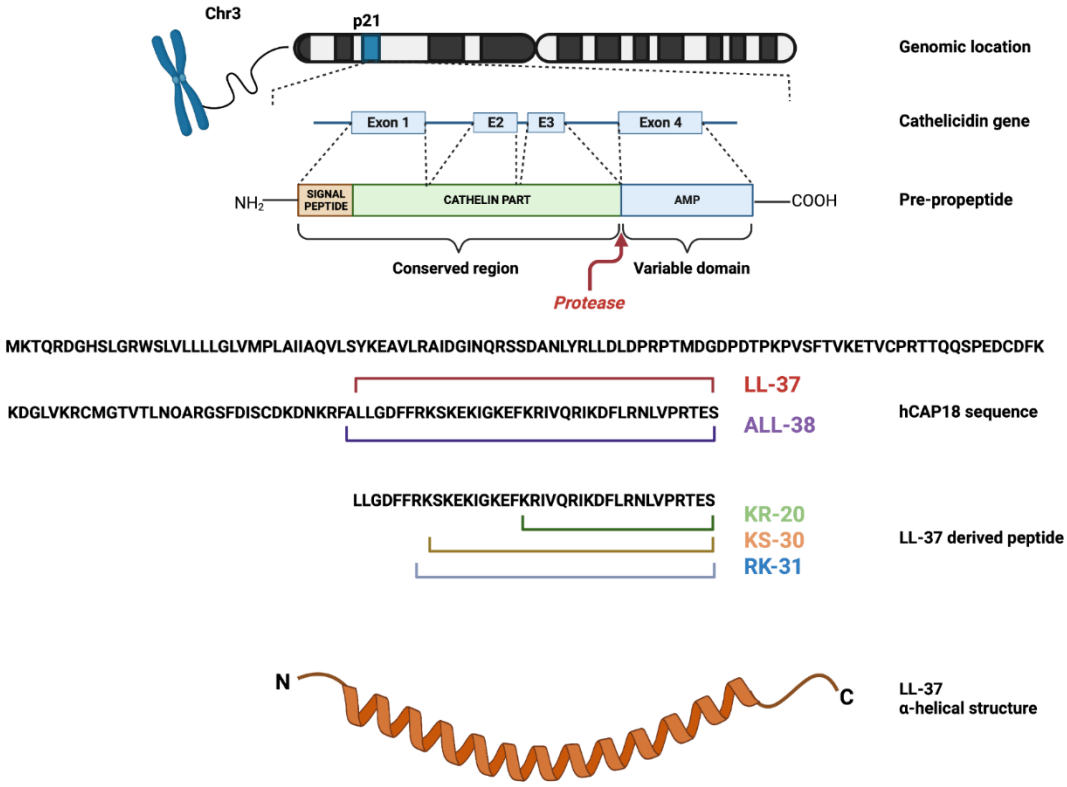


Figure 1: Molecular structure of cathelicidin.

The structure of CAMP gene on human chromosome 3. The pre-propeptide is cleaved by protease enzyme into hCAP-18 sequence. LL-37 is a C-terminal segment of hCAP-18 and eventually cleaved by proteinase 3 and elastase into various fragments (e.g. KR-20, KS-30, and RK-31)(23)

1. Functions of human cathelicidin (LL-37)

Human cathelicidin or LL-37 is a natural innate immune defense which is one of the first chemical barriers against a wide range of pathogens such as bacteria, fungi, viruses, and parasites (24). As mentioned earlier, LL-37 possesses highly positive charge and disrupts cell membrane of

the pathogens using electrostatic interaction (25). The mechanisms of action of LL-37 are demonstrated in three different models: a) carpet model; b) barrel-stave model; and c) toroidal pore model (**Figure 2**) (11, 26-28).

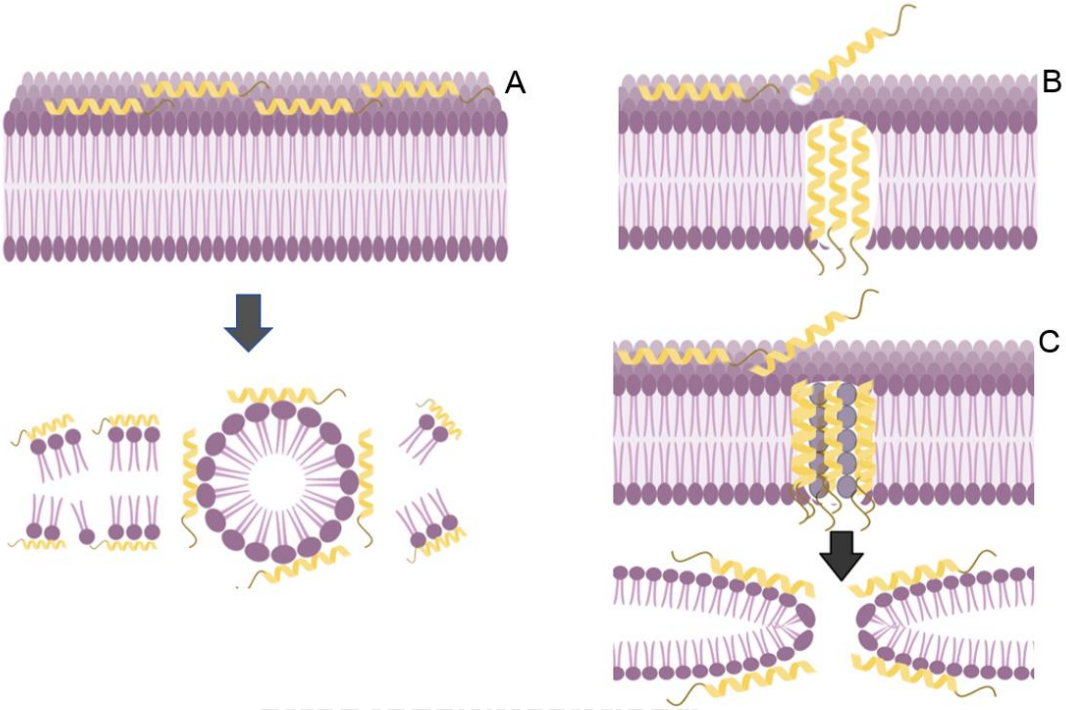


Figure 2: Mechanisms of human cathelicidin (LL-37)

***(A)** Carpet model; an accumulation of LL-37 (yellow) on the surface of pathogen cell membrane (purple) causing pathogen destruction. **(B)** Barrel stave model; LL-37 aggregation and insertion in parallel into the lipid bilayer of the pathogen cell membrane, leading to a pore formation. **(C)** Toroidal pore model; accumulation of LL-37 vertically embedded into the cell membrane resulting in a formation of a ring hole (11, 26-28).*

1.1 The carpet model

The carpet model is a typical interaction between LL-37 cationic peptide and anionic lipid bilayer of the target cell membrane. LL-37 line horizontally in parallel with cell membrane of the pathogen and bind to the phospholipid head groups on the cell membrane using electrostatic interaction. Finally, LL-37 cover entire membrane surface in a pattern of carpet and disrupt cell membrane causing micellization (**Figure 2A**) (27, 29).

1.2 The barrel stave model

In this model, LL-37 are aggregated onto the surface of the target cell membrane using their peptide monomers. After complete aggregation, the lipid bilayer of cell membrane is inserted and formed multiple channels that cause cell membrane leakage leading to pathogen destruction (**Figure 2B**) (26, 30).

1.3 The toroidal pore model

LL-37 horizontally attach onto the cell membrane of the pathogen and penetrate the hydrophobic region of the lipid bilayer. The gap between cell membrane causes a curve inward of each end leading to a pore formation and irreversible rupture of the plasma membrane (26, 30, 31).

2. Antimicrobial properties of human cathelicidin (LL-37)

2.1 Antibacterial activity

Many previous studies showed that LL-37 inhibited growth of gram-positive and gram-negative bacteria (11). For example, LL-37 inhibited growth of *Staphylococcus* spp. (e.g. *S. aureus* and *S. epidermidis*) and rapidly reduced biofilm formation (32). Moreover, LL-37 showed an inhibitory effect on *P. aeruginosa* isolated from the sputum of cystic fibrosis patients (33), and inhibited growth of *L. pneumophila* which is a waterborne intracellular pathogenic bacterium causing pneumonia(34). In addition, LL-37 was also able to inhibit gram-negative pathogenic bacteria. For example, administration of LL-37 into mice infected with *E. coli* intraperitoneally injection significantly reduced bacterial colony count in blood and tissues (35). In addition, LL-37 showed an inhibitory effect on facultative and obligate anaerobic bacteria and reduced biofilm formation. For example, LL-37 inhibited the growth of facultative anaerobic bacteria: *Streptococcus mutans* (ATCC 25174), *Streptococcus sanguinis* (ATCC 10556), and *Actinomyces naeslundii* (ATCC 19039), and obligate anaerobic bacteria: *Veillonella parvula* (ATCC 17745), *Parvimonas micra* (ATCC 49256) and *Fusobacterium nucleatum* (ATCC 33270) at the concentrations of 25 to 100 µg/ml. Moreover, LL-37 significantly reduced biofilm formation of

these microorganisms at the concentration of 250 μg/ml (36). It was reported that LL-37 in a combination with meropenem and moxifloxacin synergistically inhibited antibiotic-resistant *C. difficile* (37). Furthermore, LL-37 inhibited growth of *Helicobacter pylori* which is a microaerophilic bacterium (38).

2.2 Antifungal activity

Antifungal property of LL-37 was reported in many previous studies. It was found that LL-37 inhibited biofilm formation of *Candida sp.* isolated from patients with candidiasis (39). For example, LL-37 inhibited *Candida auris*, a multidrug-resistant pathogenic yeast that caused severe invasive fungal infections. In a previous study, it was demonstrated that LL-37 at concentrations of 25-100 μg/ml significantly inhibited growth of *C. auris* and the concentration of LL-37 at 50-200 μg/ml showed fungicidal activity. Moreover, synergistic effect LL-37 in a combination with antifungal drugs such as fluconazole, amphotericin B and caspofungin was reported (40). In addition, *C. albicans* and *C. krusei* that caused vulvovaginal candidiasis were inhibited by LL-37 at the concentrations of 2-64 μg/ml and biofilm formation of the pathogens were significantly reduced by LL-37 at these concentrations (41).

2.3 Antiviral activity

The inhibitory effect of LL-37 on viruses was demonstrated in previous studies. It was shown that LL-37 activated epithelial cells to produce interferons which could inhibited herpes simplex virus type 1 (HSV-1) replication (42). Moreover, the active form of LL-37 significantly protected HEp-2 cells from RSV (43). Another study demonstrated that LL-37 inhibited Kaposi's sarcoma associated herpesvirus (KSHV) by blocking viral envelope (44) In addition, LL-37 inhibited viral replication of Venezuelan equine encephalitis virus (VEEV) (45), dengue virus type 2 (46), influenza A viruses (47) and HIV-1 (48).

In recent studies, LL-37 has shown efficiently binding to the SARS-CoV-2 viral receptor binding domain (49). It has been hypothesized that LL-37 blocks ACE2 receptor that allows SARS-CoV-2 spike viral protein entry into the cells (50, 51).

2.4 Antiparasitic activity

A few studies demonstrated inhibitory effects of LL-37 on protozoa and parasites (e.g., *Leishmania* sp.) (52). LL-37 showed leishmanicidal activity against *Leishmania donovani* and *Leishmania major* (53). It was reported that LL-37 also inhibited *Entamoeba trophozoites*, which caused amebiasis (54).

3. Other properties of human cathelicidin (LL-37)

3.1 Anticancer activity

The inhibitory effect of LL-37 on the development of colon cancer has been studied. In vivo studies, It has been reported that LL-37 shows indirect anticancer activity through epithelial-mesenchymal transition (EMT) inhibition and fibroblast-mediated colon cancer proliferation. LL-37 binds to tubulin protein and disrupts cytoskeletal tubulin distribution in CCD-18Co fibroblasts that support colon cell proliferation (55). Moreover, C-terminal segment of LL-37 inhibits human oral squamous cell carcinoma (SAS-H1 cells) by an induction of cell apoptosis and mitochondrial depolarization (56).

Anti-cancer effect of LL-37 was demonstrated in hematologic malignancies. In previous studies, it was reported that LL-37 activated cell apoptosis of malignant human T cell line (Jurkat cell) by an induction of cell death via loss mitochondrial transmembrane potential (57-59).

3.2 Immunomodulatory effects

LL-37 exhibits a wide range of immunomodulatory functions such as stimulation of pro- and anti-inflammatory mediator production, induction of cell migration, increased cell

proliferation/differentiation, regulation of epithelial cell and neutrophil apoptosis, and enhanced wound healing process (60, 61).

3.2.1 Cell migration

In migration assay, human dermal fibroblasts (HDFs) cultured in conditioned medium (CM) isolated from adipose stem cells (ASCs) treated with LL-37, showed significantly increased cell migration when compared with the CM isolated from the ASCs without, as measured by the expression of *CXCR4* mRNA and protein expression (62). Moreover, LL-37 induced migration of airway epithelial cells via epidermal growth factor receptor (EGFR), G protein-coupled receptor (GPCR), and mitogen-activated protein kinase (MAPK) (63). In addition, LL-37 induced keratinocyte migration via heparin-binding-EGF-mediated transactivation of epidermal growth factor receptor (64).

LL-37 induced cell migration and proliferation of tumor cells as found in malignant melanoma cell lines that showed increased expression of Y-box binding protein (65). In addition, LL-37 promoted cell migration in breast cancer via interaction with CXCR4 which was associated tumor progression (66).

3.2.2 Cell proliferation

Many previous studies demonstrated that LL-37 promoted cell proliferation. For example, LL-37 promoted bone marrow stromal cell proliferation (67) and fibroblast cell lines (68). Moreover, LL-37 promoted cell proliferation in hepatocellular carcinoma through EGFR/HER2/Akt signaling pathway (69).

3.2.3 Cell differentiation

LL-37 induced dendritic cell differentiation as shown in monocyte-derived dendritic cells (70) and osteoblasts (67).

3.2.4 Healing process

Enhanced wound healing was found in human corneal epithelial cells (HCECs) treated with LL-37. It was demonstrated that Heparin-binding epidermal-like growth factor alkaline phosphatase (HB-EGF-AP) was released, and it activated epidermal growth factor receptor (EGFR) (61). Moreover, culture with medium containing LL-37 promoted keratinocyte migration leading to the induction process of wound healing (64). Interestingly, LL-37 in a combination with chitosan hydrogel was administered into the hip of mice with ulcer significantly promoted wound healing determined by histological analysis and size of the ulcer (71).

Table 1.Antimicrobial properties of LL-37 against various microbes

Antimicrobial activity	Aerobe	*S. aureus, S. epidermidis, P. aeruginosa, L. pneumophila, E. coli, N. gonorrhoeae*
	Anaerobe	*Veillonella parvula* (ATCC 17745), *Parvimonas micra* (ATCC 49256), *Fusobacterium nucleatum* (ATCC 33270),*C. difficile*
Antifungal activity	*C. auris, C. albicans, C. krusei*	
Antiviral activity	Herpes simplex virus type 1, Kaposi's sarcoma associated herpesvirus (KSHV), RSV, Venezuelan equine encephalitis virus (VEEV), HIV-1, dengue virus type 2, Influenza A Viruses, SARS-CoV-2	
Antiparasitic activity	*Entamoeba spp, Leishmania major parasites*	

Table 2. Other properties of human cathelicidin LL-37

Anticancer activity	colon carcinoma, squamous cell carcinoma, hematologic malignancy	
Immunomodulatory effects	migration	human dermal fibroblast, melanoma cell lines, airway epithelial cells, keratinocyte
	proliferation	bone marrow stromal cell, hepatocellular carcinoma cells, fibroblast cells
	differentiation	dendritic cell, osteogenic, Th17, human monocyte
	healing process	human corneal epithelial cells (HCECs) and human keratinocytes by inducing HB-EGF-AP release and EGFR activation, healing of pressure ulcers, healing skin wounds by the induction of keratinocyte migration

4. Clinical application and future perspectives

As mentioned previously, LL-37 shows multiple properties including antimicrobial and anticancer activities including immunomodulatory effects on many cell types. Therefore, it is possible that LL-37 could potentially be used as a treatment for many conditions. Recent studies have shown that LL-37 as a topical agent (e.g., gel) is applied to the venous leg ulcers (VLUs) of patients and shows a clinical improvement without side effects (72). Moreover, LL-37 in a combination with magnetic nanoparticles used to treat patients with drug-resistant microorganisms shows remarkable clinical improvements (73).

5. LL-37 and Cutibacterium acnes

The expression of LL-37 in healthy skin is physiologically low but the production of LL-37 is increased during skin inflammation and infection such as acne (74). In particular, the expression of LL-37 in sebaceous glands has been studied and it has been found that LL-37 mRNA expression is detected in human sebaceous glands especially after co-incubation with *C. acnes*. Moreover, LL-37 inhibited *C. acnes* (75, 76), but the studies of an interaction between LL-37 and *C. acnes* are limited especially C. acnes antibiotic-resistant strains isolated from acne patients in Thailand.

B. CUTIBACTERIUM ACNES

1. Morphology

Cutibacterium acnes (formerly known as *Propionibacterium acnes*) is a gram-positive non-spore forming, anaerobic or microaerophilic bacteria. It has a rod shape and slightly curved with 0.4 to 0.7 µm width and 3 to 5 µm length (**Figure 3A**). The colonies of *C. acnes* on brucella agar are opaque white or gray and the size of colony is approximately 0.5 mm in diameter with a zone of hemolysis (**Figure 3B**) (5, 77). As genomic and metagenomic studies lead to the new definition of genus for cutaneous bacteria, the name of *P. acnes* has been replaced by *C. acnes* based on its specific feature and phylotype that colonizes onto the skin (5).

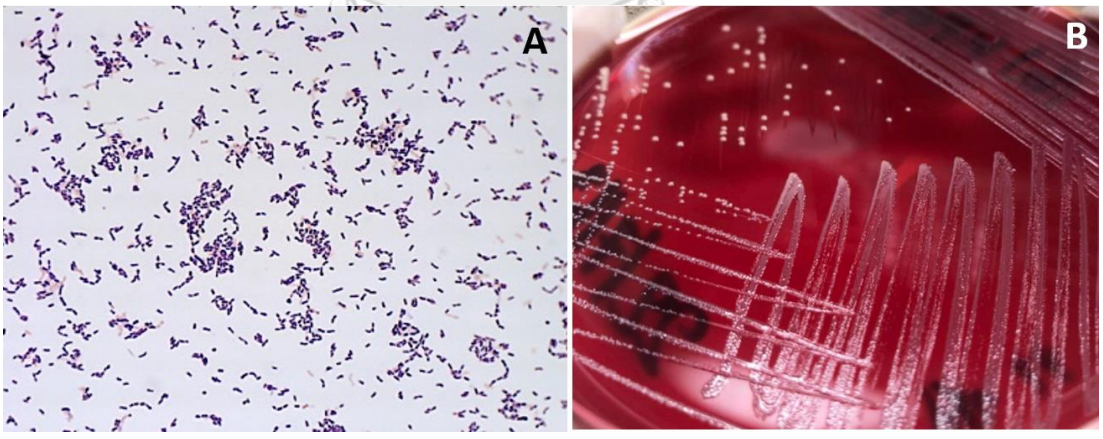


Figure 3.C. acnes morphology

Gram staining picture of C. acnes (gram-positive rod shape) under light microscopy with a magnification of 1000X (A), and bacterial colonies of C. acnes on brucella agar (B)

C. acnes is classified as a member of Propionibacteriaceae family and the new genus "*Cutibacterium"* is composed of four species that are *C. acnes, C. avidum, C. granulosum*, and *C. humerusii* (5). In the species of *C. acnes*, it has been divided into 3 subspecies which are *C. acnes* subsp. acnes, *C. acnes* subsp. *defendens*, and *C. acnes* subsp. *elongatum* (78-80). The classification of *C. acnes* is shown in Figure 4.

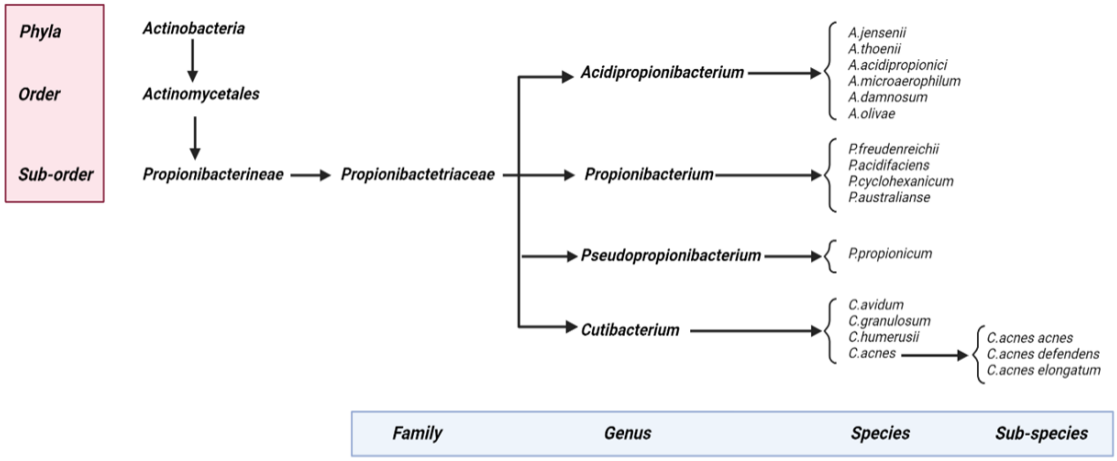


Figure 4. The classification of C. acnes (5)

C. acnes differs from other gram-positive bacteria because the composition of cell wall contains phosphatidylinositol, triacylglycerol, and other common lipids (81). Moreover, *C. acnes* produces superoxide dismutase, catalase and peroxidase enzymes which allow it to survive in low

oxygen condition because these enzymes degrade oxygen free radicals such as hydrogen peroxide (H_2O_2), superoxide anion (O^-_2), hydroxyl radical ($OH^•$), and singlet oxygen ($O_2^•$) that are toxic to *C. acnes* (82). However, the division time of *C. acnes* is approximately 5 hours, and it grows very slowly (5-7 days) in anaerobic condition, and it stops growing in aerobic condition (5, 83).

The strains of *C. acnes* are currently classified into three phylotypes (I, II, III). Phylotype I or *C. acnes* subsp. *acnes* is divided into 5 clades IA-1, IA-2, IB-1, IB-2, IB-3 whereas phylotype II or *C. acnes* subsp. *defendens* and phylotype III or *C. acnes* subsp. *elongatum* have no clades. The IA-1 and IA-2 clades of *C. acnes* subsp. *acnes* preferentially found in acne patients whilst phylotypes II and III are commonly found in deep skin infections (84).

2. Virulence factors

Numerous virulence factors of *C. acnes* have been reported (**Table 3**). For example, Christie, Atkins, Munch-Peterson (CAMP) factors are membrane pore-forming toxins that induce cytotoxicity and activate skin inflammation in acne (85). Porphyrins are another virulence factor that contributes to the perifollicular inflammatory reaction in the pathogenesis of acne. Moreover, porphyrins stimulate the expression of keratinocyte-derived interleukin-8 and prostaglandin E2

which are common inflammatory mediators in acne pathology (86, 87). Another virulence factor produced by *C. acnes* is hyaluronate lyase. This enzyme degrades dermal and epidermal extracellular matrix (e.g. hyaluronic acid and glycosaminoglycans) that increases skin inflammation in acne (88).

2.1 CAMP factors

CAMP factors are toxic proteins that generate pores on the host cell membrane causing cell disruption. Five genes (*CAMP1 - 5*) of CAMP factors have been identified in *C. acnes*. *CAMP1* is strongly expressed by phylotype IB and II whereas *CAMP2* is mostly expressed by phylotype IA. Moreover, *CAMP1* is one of the most important proteins that is highly expressed in the pilosebaceous unit (5, 89).

2.2 Porphyrins

Porphyrins are fluorescent molecules that absorb ultraviolet and visible light. Porphyrins produced by *C. acnes* contribute to the perifollicular inflammatory reaction as they induce expression of proinflammatory mediators such as IL-8 and prostaglandin E2. Phylotype I of *C. acnes* strains produce more porphyrins than other phylotypes (5, 89).

2.3 Hyaluronate lyase

Hyaluronate lyase (HYL) is an enzyme that degrades hyaluronic acid (HA) and glycosaminoglycans (GAG) that are present in the extracellular matrix of epidermis and dermis. HYL is a virulence factor of *C. acnes* as it facilitates bacterial invasion into the skin. HYL digests the upper layer of the skin and promotes skin inflammation. In addition, the end products of HA degradation are a source of nutrients for *C. acnes* (5, 89).

2.4 Lipase

Glycerol-ester hydrolase A(GehA) is a type of lipase enzyme produced by *C. acnes* and this enzyme hydrolyzes sebum triacylglycerides in the pilosebaceous unit of hair follicle. The final product of sebum degradation is glycerol and free fatty acids (FFAs) (90). Glycerol is a source of nutrients for *C. acnes* whereas free fatty acids promote skin inflammation (91). In addition, FFAs enhance cell adhesion between *C. acnes* and keratinocytes inside hair follicle which favors *C. acnes* colonization (5, 92)

2.5 RoxP

RoxP (Radical oxygenase of *Propionibacterium acnes*) is an enzyme secreted by *C. acnes* (93). It reduces oxygen free radicals such as superoxide anion (O^-_2), hydroxyl

radical ($OH^{\bullet}$) and singlet oxygen ($O_2^{\bullet}$), which are toxic to *C. acnes*. RoxP is produced by *C. acnes* phylotypes IA, IB, II, and III, but phylotype I of *C. acnes* shows higher expression levels of *RoxP* than other phylotypes. Therefore, RoxP promotes *C. acnes* survival in oxygen-rich environment such as skin surface (93-95).

2.6 Other virulence factors

Endoglycoceramidase catalyses glycosidic linkage between oligosaccharides and ceramides that are major lipid components of the skin (96). Sialidase cleaves sialoglycoconjugates into sialic acids and these products are used as carbon and energy sources for *C. acnes* (97, 98). Dermatan sulphate adhesin (DsA) is a bacterial surface protein of *C. acnes* and it adheres specifically with extracellular matrix of the host cell which enhances *C. acnes* colonization and infection (99).

Biofilm formation is produced by glycosyltransferases, uridine diphosphate N-acetyl glucosamine 2-epimerase. These enzymes induce polysaccharide biosynthesis of biofilm which is resistant to antibacterial agents as the extracellular polymeric substance acts as a barrier and delays penetration of antimicrobial agents into *C. acnes* (89, 100).

Table 3 Virulence factors of C. acnes

Virulence factors	Functions
Christie, Atkins, Munch-Peterson factor	Immunoglobulin binding, pore-forming toxins
Porphyrins	Tissue damage and skin inflammation
Hyaluronate lyase	Extracellular matrix of connective tissue degradation
Lipase	Hydrolysis of sebum triglycerides
RoxP	Reduction of free radicals
Endoglycoceramidase	Cell surface disruption
Sialidases	Tissue degradation
Dermatan sulphate adhesins	Putative adhesion

3. *C. acnes* and Inflammation

Keratinocytes, monocytes, sebocytes, and fibroblasts interact with *C. acnes* during skin inflammation. These cells are stimulated by *C. acnes* via pattern recognition receptors (PRRs) particularly Toll-like receptors (TLRs) which play an important role in acne pathogenesis (5). For example, *C. acnes* are recognized by TLR 2, TLR 4, NOD-like receptor protein 3 (NLRP3) and caspase 1 that are expressed on monocytes, macrophages and sebocytes. Once the cells have become activated via these cell receptors, they produce pro-inflammatory cytokines such as IL-1β, IL-1α, IL-6, IL-8, IL-12, TNF-α. In addition, *C. acnes* also

stimulate the release of antimicrobial peptides such as β-defensin-2 and metalloproteinases (MMP) causing tissue inflammation.

C. acnes also stimulate adaptive immune cells including T helper 1 (Th1) and Th17 cells causing secretion of IFN-γ and IL-17A and other pro-inflammatory cytokines (e.g., IL-1β and IL-6). Activation of Th17 induces IL-17 production that is an important cytokine for the recruitment and activation of neutrophils. However, IL-17 also activates other cells such as keratinocytes, endothelial cells, monocytes, and fibroblasts that cause further secretions of pro-inflammatory cytokines. Overall, *C. acnes* can induce inflammatory response by activating several signaling pathways which may involve several virulence factors (5, 101-104).

C. ACNE VULGARIS

Acne vulgaris (AV) is a chronic inflammatory skin disorder of the pilosebaceous unit (PSU) caused by 4 main factors: hyperkeratinization of the hair follicle, increased sebum production, *C. acnes* colonization, and skin inflammation. AV occurs particularly in the areas that are composed of numerous sebaceous glands such as face, neck, upper chest, shoulders, and upper back. AV affects psychological problems in most cases as it often leaves disfiguring scars causing

low self-esteem that is associated with anxiety and depression (105-109). As *C. acnes* is one the main factors involved in the pathogenies of acne, this pathogen therefore is an important target for acne treatment.

1. Disease prevalence

AV is the one of eight most common skin diseases affecting approximately 9.38% in population across that world and shows the highest prevalence in adolescents (108). It was found approximately 95% in adolescents and 25% in adults without gender preference (1). The prevalence of acne varies in different countries and among different age groups (110, 111).

In Thailand, the prevalence of acne in patients with mild symptoms was 52%, with moderate acne was 22%, and with severe acne was 8% (112). In another study, it was found that the prevalence of moderate to severe acne was 32.7% in patients between aged 14-44 years old and it was comparable between rural and urban areas (113). The previous studies reported a prevalence of acne in Thailand. However, the prevalence of acne may differ in each country due to different factors such as ethnicity, climate, diet, etc.

2. Disease pathogenesis

There are 4 major factors involved in the pathogenesis of acne

2.1 Increased sebum production

Increased rate of sebum production from sebaceous glands is one of the most important factors involved in the development of acne. It has been reported that patients with acne have higher secretion of sebum than patients without, and androgen (male sex hormone) mainly regulates size and growth of the sebaceous glands. Evidence shows that patients with acne who have larger sebaceous glands are prone to have high numbers of acne in the sebaceous area of the skin (114, 115).

2.2 Follicular hyperkeratinization

Follicular hyperkeratinization is an excessive shedding of epidermal cells at the opening of the hair follicles. Accumulation of keratin causes occlusion of the opening pores of the hair follicles. A combination of sebum and hyperkeratinization of epidermal cells leads to the formation of microcomedones (102, 116, 117). These microcomedones are finally formed and become mature as open (blackhead) or closed (whitehead) comedones (**Figure 6B and 6C**).

Importantly, comedones favor growth of *C. acnes* because of an anaerobic condition in the PSU and source of nutrients especially lipid substrates for *C. acnes* (118).

2.3 *C. acnes* colonization

Hyperkeratinization of epidermal cells in a combination with increased sebum production which is a major source of nutrient for *C. acnes* colonization onto the skin and hair follicles. Moreover, triglycerides in the PSU are hydrolyzed into free fatty acids by lipase secreted by *C. acnes*. These free fatty acids further induce skin inflammation (89, 119).

2.4 Skin inflammation of PSU

Triglycerides are hydrolyzed into free fatty acids and glycerol by lipase produced by *C. acnes* inside the PSU. The FFAs such as palmitic, lauric, oleic, acetic, propionic, isobutyric, and isovaleric acid induce an inflammatory reaction and recruit inflammatory cells into the PSU. Pro-inflammatory cytokines (e.g., IL-8, and IL-12) are produced by the recruited inflammatory cells leading to the formation of papules, pustules, and cysts in acne (109, 120-123).

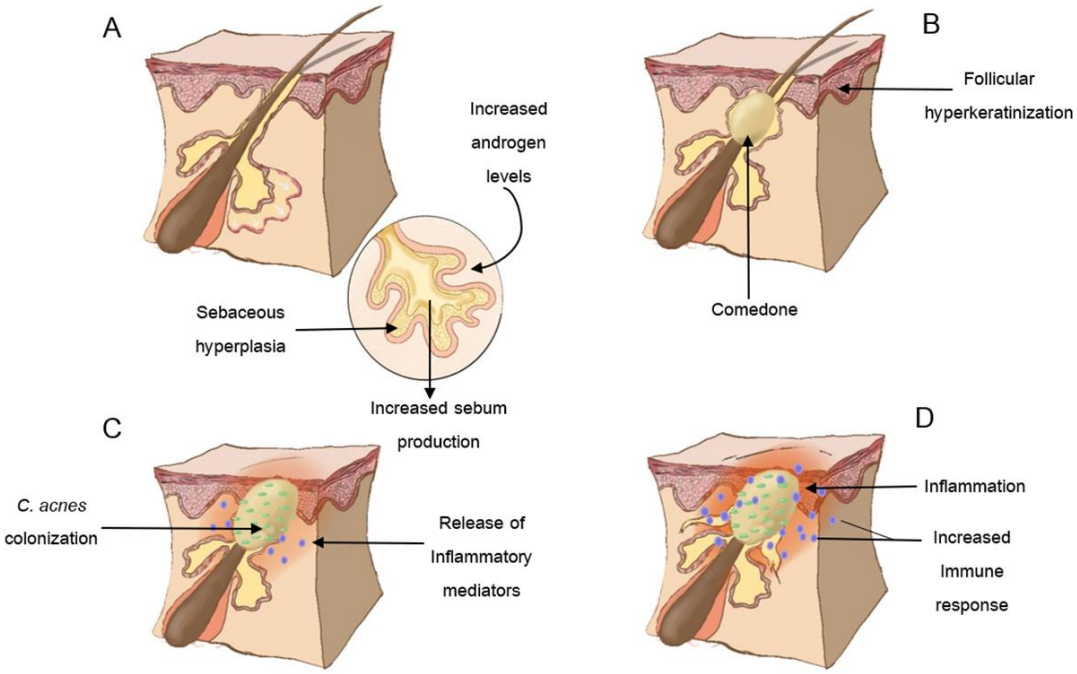


Figure 5. Pathogenesis of acne vulgaris

A) increased sebum production under the influence of androgens, B) Follicular hyperkeratinization causing comedones, C) Favor of C. acnes colonization due to anaerobic condition and D) Skin inflammation in pilosebaceous unit (modify from (124))

3. Clinical manifestations

3.1 Type of acne

3.1.1 Non-inflammatory acne is a type of acne that is caused by a clogging of the hair follicles called comedones (ductal hypercornification). There are 2 types of comedones.

- Closed comedone (or whitehead) are caused by clogging within the hair follicles due to keratin and sebum. A closed comedone appears as a small, white-colored papule without visible central pore.

- Open comedone (or blackhead) is a small flat or slightly raised black papule with a central pore cause by accumulation of sebum and keratin. The black color of the open comedone is caused by oxidation of lipid and melanin.

3.1.2 Inflammatory acne is caused by clogged hair follicles and skin inflammation because of influx of inflammatory cells around the PSU. *C. acnes* colonization stimulates epidermal cells that recruit inflammatory cells leading to skin inflammation.

- Inflammatory papule is a small raised reddish papule that is an early stage of inflammatory acne with or without pain.

- Pustule is a small vesicle containing pus and it is usually surrounded by red skin which is painful and tenderness.

- Nodule/cyst is a large deep painful and more solid lesion that contains blood or pus.

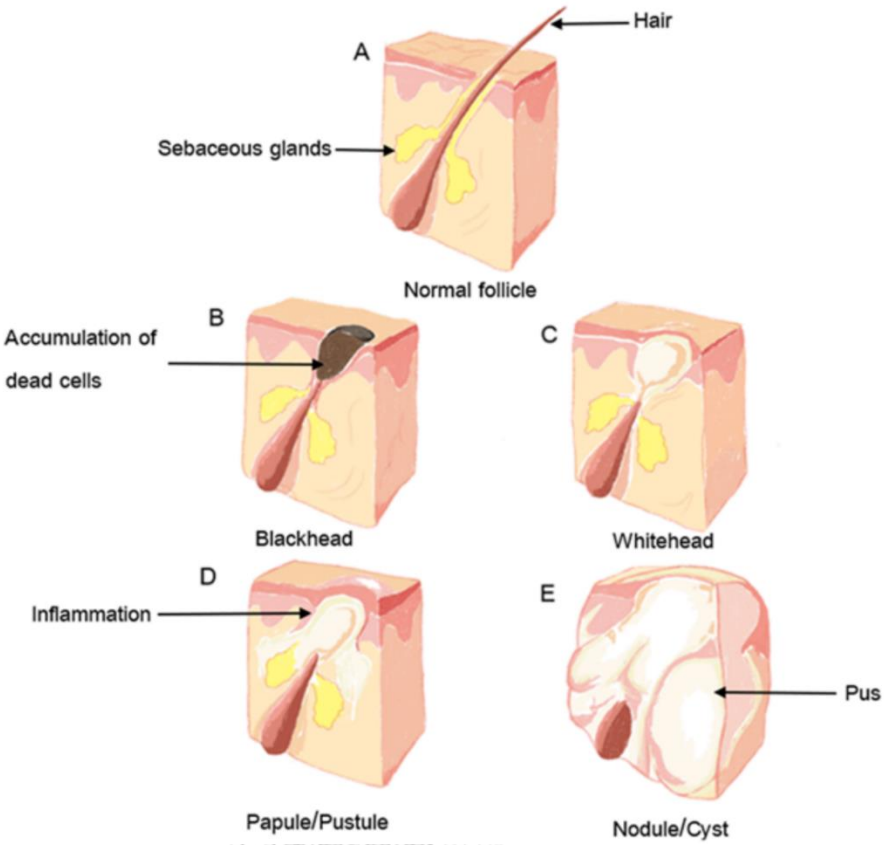


Figure 6. Types of acne:

A) Normal hair follicle; B) blackhead; C) whitehead; D) papule or pustule and E) nodule or cyst (modify from(125))

3.2 Acne grading

Investigator's Global Assessment (IGA) severity scale is the most well-known grading system to categorize patients with acne from mild to severe forms (126-128).

Table 4. IGA scale for acne vulgaris (126-128).

Score	Grade	Description
0	Clear	Normal, clear skin with no inflammatory or non-inflammatory lesions
1	Almost clear	Rare non-inflammatory lesions, with rare non-inflammatory papules
2	Mild	Some non-inflammatory lesions, with few inflammatory lesions (papules/pustules only, no nodulocystic lesions)
3	Moderate	Many non-inflammatory lesions. Multiple inflammatory lesions evident with several to many papules/pustules, and there may be 1 small nodulocystic lesion
4	Severe	Inflammatory lesions are more apparent, many comedones and papules/pustules, there may be a few nodulocystic lesions
5	Very severe	Highly inflammatory lesions predominate, variable number of comedones, many papules/ pustules and many nodulocystic lesions

4. Diagnosis

The diagnosis is based on the typical lesions as described previously (e.g., whitehead, blackhead, pustules, nodules, or cysts) in the distribution of sebaceous areas (e.g., face, back, neck and chest). Laboratory investigations are usually unnecessary unless it is indicated (109, 129). For example, hyperandrogenism such as obese women with hirsutism, abnormal menstruation, male pattern balding or having deep voice. Other differential diagnoses are gram

negative folliculitis and pityrosporum folliculitis which need gram staining and culture to make a diagnosis.

5. Treatments

5.1 Topical treatments

5.1.1 Topical retinoid

Topical retinoids are vitamin A derivatives that include tretinoin, adapalene and isotretinoin. Tretinoin (trans-retinoic acid) is the first generation of retinoid that shows comedolytic effect and reduces skin inflammation. Adapalene (naphthoic) is the third generation of retinoid that shows the same effects. These two agents are commonly used in clinical practice, and they come in different preparations such as lotion, gel and cream. They are photolabile and need to be applied on the affected skin at bedtime. The common side effects of topical retinoids are skin peeling and irritation. Moreover, breastfeeding and pregnant women should be avoided using these agent (115, 130, 131).

5.1.2 Benzoyl peroxide (BP)

BP is a common topical antibiotic (usually in gel formulation) used to treat acne and to eliminate *C. acnes* colonization. This agent produces superoxide which intracellularly oxidizes

cytoplasmic proteins of *C. acnes* leading to cell death. The most common side effects are skin irritation and peeling (115, 130, 131).

5.1.3 Topical antibiotic

Clindamycin and erythromycin are commonly used as topical antibiotics of acne. Both medications inhibit *C. acnes* growth, neutrophil chemotaxis and show anti-comedogenic effect. In clinical practice, 1% clindamycin phosphate or 1-2% erythromycin (lotion or gel) is usually prescribed by general practitioners. The most common side effects are skin burning and irritation (115, 130, 131).

5.1.4 Azelaic acid

Azelaic acid is another form of topical treatment in patients with acne. This acid eliminates *C. acnes* colonization and reduces skin inflammation. Moreover, azelaic acid shows mild comedolytic effect but the most common side effects are skin burning and peeling(115, 130, 131).

5.2 Systemic treatment

5.2.1 Oral antibiotic

Oral tetracycline inhibits *C. acnes* colonization and neutrophil chemotaxis. However, many side effects including drug allergy may occur in some patients. Anti-inflammatory properties such as inhibition of matrix metalloproteinase activity, reduced inflammatory cytokine

production, and decreased inflammatory cell chemotaxis are the main mechanism of this agent. Erythromycin in oral preparation displays a strong bacteriostatic activity against *C. acnes* as this agent selectively binds to 50s ribosomal subunit of the bacterial rRNA complex and inhibits protein synthesis of *C. acnes*. Oral doxycycline displays a strong bacteriostatic activity against *C. acnes* as this agent selectively binds to 30s ribosomal subunit of the bacterial rRNA complex and inhibits protein synthesis of *C. acnes*.

Oral sulfamethoxazole/trimethoprim is sometimes used in patients with acne as it shows a bacteriostatic activity against *C. acnes* by inhibition of bacterial dihydrofolate. The side effects are skin rash, hepatitis, and severe drug allergy (SJS/TEN) (115, 130, 131).

5.2.2 Oral isotretinoin

Isotretinoin (13-cis-retinoic acid) is the only systemic vitamin A acid used in patients with acne. Importantly, this agent is one of the most powerful systemic treatments as it effectively reduces size of the sebaceous glands and shows a strong anti-inflammatory effect. However, the most concerned side effects are teratogenicity and psychiatric problems (e.g., major depression).

Dry skin and mucous membranes (e.g., eyes and mouth), hyperlipidemia and GI disturbance are also mild common side effects of oral isotretinoin (115, 130, 131).

5.3 Other treatments

Oral contraceptive pills (e.g., progesterone and estrogen) are sometimes used in women with acne as they inhibit androgen hormones, but side effects are GI irritation and increased body weight. LASER treatment and light therapy may be useful in some cases with acne(115, 130, 131).

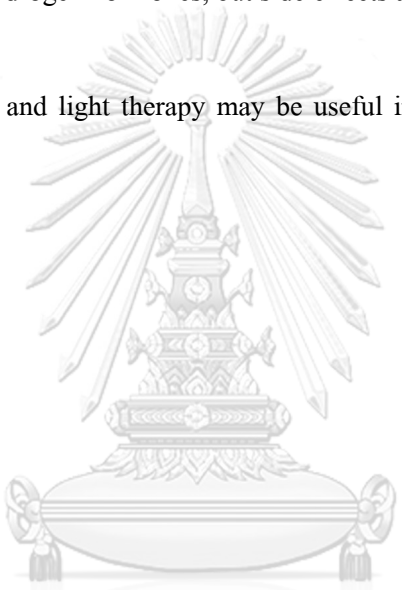


Table 5 Treatment algorithm for the management of acne vulgaris in adolescents and young adults(127).

	Mild	Moderate	severe
First-Line Treatment	BP or Topical Retinoid -or- Topical Combination Therapy** BP + Antibiotic or Retinoid + BP or Retinoid + BP + Antibiotic	Topical Combination Therapy** BP + Antibiotic or Retinoid + BP or Retinoid + BP + Antibiotic -or- Oral Antibiotic + Topical Retinoid + BP -or- Oral Antibiotic + Topical Retinoid + BP + Topical Antibiotic	Oral Antibiotic + Topical Combination Therapy** BP + Antibiotic or Retinoid + BP or Retinoid + BP + Antibiotic -or- Oral Isotretinoin
Alternative Treatment	Add Topical Retinoid or BP (if not on already) -or- Consider Alternate Retinoid -or- Consider Topical Dapsone	Consider Alternate Combination Therapy -or- Consider Change in Oral Antibiotic -or- Add Combined Oral Contraceptive or Oral Spirolactone (Females) -or- Consider Oral Isotretinoin	Consider Change in Oral Antibiotic -or- Add Combined Oral Contraceptive or Oral Spirolactone (Females) -or- Consider Oral Isotretinoin

The double asterisks (**) indicate that the drug may be prescribed as a fixed combination product or as separate component. BP, Benzoyl peroxide.

D. ANTIMICROBIAL-RESISTANT *CUTIBACTERIUM ACNES*

1. Prevalence of Antibiotic Resistance

In Thailand, antibiotic-resistant *C. acnes* has been reported in only a few studies. In 2001, it was firstly reported that the prevalence of antibiotic-resistant *C. acnes* was approximately 6.15% for erythromycin and clindamycin. In contrast, there was no strains that were resistant to doxycycline and tetracycline (132). Another previous study reported that the prevalence of antibiotic-resistant *C. acnes* was approximately 64% for erythromycin and 62.66% for clindamycin while 1.33% for tetracycline. However, doxycycline and amoxicillin-resistant *C. acnes* were found in this study (133). Therefore, the prevalence of antibiotic-resistant *C. acnes* was frequently resistant to erythromycin and clindamycin. For this reason, it is now recommended that proper use of antibiotics for treatment of *C. acnes* may reduce the incidence of antibiotic resistance. However, most general practitioners routinely prescribe these antibiotics to treat patients in clinics. Therefore, current studies are searching for new strategies to minimize antibiotic-resistant *C. acnes* in these patients.

2. Mechanisms of Antibiotic Resistance

The emergence of antibiotic-resistant *C. acnes* is firstly reported in 1983 (134). It was demonstrated that oral antibiotics used to treat patients with acne was less effective than previous years. Moreover, it was also found that *C. acnes* isolated from lesions of patients with acne were highly resistant to erythromycin and clindamycin (21-70%), and tetracycline (4-30%) (135). However, there were only a few studies demonstrating the mechanism of antibiotic resistance in *C. acnes* (89). In previous studies, it was shown that chromosomal point mutations of *C. acnes* (136) were associated with 23S rRNA gene for erythromycin resistance, and 16S rRNA gene for tetracycline resistance. In addition, transposon containing *erm(X)* gene was also found in *C. acnes* resistant to clindamycin and erythromycin. Doxycycline-resistant *C. acnes* was rarely observed in patients with acne, but it was demonstrated that amino acid substitution in the ribosomal S10 protein was associated with the resistance mechanism of this agent.

Another important factor that causes antibiotic-resistant *C. acnes* is biofilm formation. Biofilm naturally consists of extracellular polymeric substance (EPS), polysaccharide, protein, lipid, and extracellular DNA. Biofilm formation is one of the bacterial defenses for survival that provides protection from environmental factors, promotes communications among

microorganisms, and contains source of nutrients. In addition, biofilm coats outside the bacteria which can reduce penetration of antibiotics. For this reason, the reduction of antibiotic concentration due to less diffusion-reaction causes antimicrobial-resistant *C. acnes* (89).

The mechanisms of antimicrobial resistance of *C. acnes* mainly involve genetic mutations in ribosomal RNA (rRNA) and biofilm formation, leading to higher virulence and transmission of resistant strains due to altered efflux pumps, and enzymatic inactivation of antibiotics (137).

3. Treatments of antibiotics-resistant *C. acnes*

As mentioned previously, the prevalence of antibiotic-resistant *C. acnes* has been increasing. The Global Alliance to Improve Outcomes in Acne Group has recommended that topical and oral antibiotics and/or topical retinoids should be co-prescribed because these agents are the most effective treatment, and they also decrease the development of antibiotic resistance in patients with acne. In general, a proper course of antibiotic treatment for patient with acne is approximately 2-3 months and it is crucial to choose a combination of topical antibiotics with

topical benzoyl peroxide (138) as the combination significantly reduces the incidence of antibiotic-resistant *C. acnes*.

Nevertheless, common adverse effects (e.g., skin dryness, skin peeling and irritation, and photosensitivity) usually occur in patients treated with these agents. Therefore, new acne treatments have been now under investigations such as antimicrobial peptides (AMPs), bacteriophage therapy, skin probiotics, and vaccine development which are probably future trends of treatment in acne patients (139).

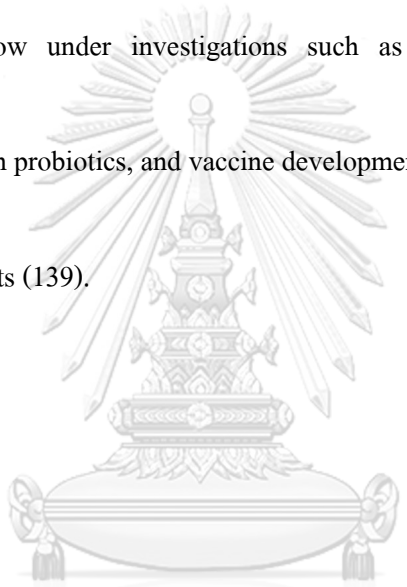


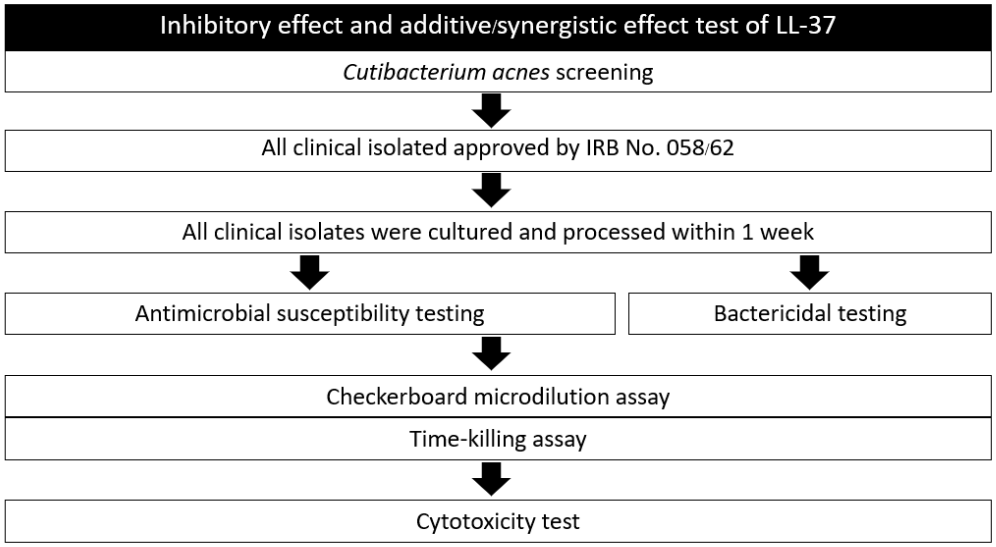
Table 6. The mechanism of antibiotic resistance in C. acnes

Antibiotics	Examples	Mode(s) of resistance
Tetracyclines	Tetracycline, Doxycycline	point mutation on 16S rRNA, point mutation on ribosomal S10 protein, efflux pumps, and enzymatic inactivation
Macrolides	Erythromycin	point mutation on 23S rRNA and macrolide inactivation by phosphotransferases and esterases
Lincosamides	Clindamycin	genetic mutations involving ribosomal proteins, including amino acid exchange, deletion and insertion, and rRNA modification by rRNA methyltransferases encoded by *erm* genes
Co-trimoxazole	Trimethoprim/Sulfamethoxazole	plasmid modification of dihydrofolate reductase, decreased bacterial permeability, decreased enzyme affinity inactivation of trimethoprim/sulfamethoxazole, increased P-aminobenzoic acid

CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL DESIGN



SAMPLESIZE CALCULATION

The expected incidence of antibiotic-resistant *C. acnes* in our Thai population was approximately 10% (134, 140). Sample size was calculated using the formula below.

Z statistic: For the level of confidence of 95% = 1.96

Proportion = 10%

Error (d) = 0.05

$$n = \frac{z^2_{1-\frac{\propto}{2}} p(1-p)}{d^2}$$

$$n = \frac{(1.96)^2 0.1(1-0.1)}{(0.05)^2}$$

Sample size (n) = 139 and allowance for another 10% = 13.9

Therefore, the total sample size was 139+13.9 = 153

MATERIALS

The following reagents were used in this study: Cathelicidin LL-37 (Peptide Institute, Japan); Doxycycline hyclate (Sigma-Aldrich, USA); Clindamycin hydrochloride (Bio Basic Inc, CAN); Sufamethoxazole (Sigma-Aldrich, USA); Trimethoprim (Sigma-Aldrich, USA); Erythromycin (Sigma-Aldrich, USA); Tetracycline hydrochloride (Sigma-Aldrich, USA); Brucella agar (BD BBLTM, USA); Brucella broth (BD BBLTM, USA); Hemin (Sigma-Aldrich, USA); Phytomenadione (Neon Healthcare Ltd, UK); Defibrinated sheep blood (CLINAG CO.,Ltd.THA); Laked horse blood (Thermo Fisher Scientific Inc.,Swed); *Bacteroides fragilis*

ATCC 25285, *Cutibacterium acnes* ATCC 6919 (ATCC, USA); Primary epidermal keratinocytes (HEKn) (Kurabo Industries, Japan)

BACTERIAL CULTURE

Bacteroides fragilis ATCC 25285 were used as laboratory quality controls (QC) for testing the antibacterial drug susceptibility of *C. acnes* clinical isolates. The *C. acnes* 45VPOP, 106KKCL and 71PYIP drug-resistant isolates collected from patients with acne vulgaris were randomly chosen for this study. The *C. acnes* ATCC 6919 strain was used as a standard species. All strains and isolates were grown on brucella agar (BR; 0.1% v/v vitamin K, 0.1% v/v hemin, 2.5% v/v sheep blood, 1.5% w/v agar) at 37ºC in an incubator for 72 h prior to perform any experiments.

ANTIBACTERIAL AGENTS

Stock solutions of antibiotics (clindamycin and doxycycline) and LL-37 were prepared in sterile water and frozen at -20ºC freezer before use. The final concentrations of clindamycin and doxycycline ranged from 0.5 to 512 µg/ml, 0.0625-64 µg/ml, respectively. The final concentration of LL-37 ranged from 0.049 to 50 µg/ml, diluted by brucella broth media, based on the Clinical and Laboratory Standards Institute (CLSI) broth microdilution M11-A7 guidelines for anaerobic bacteria(141).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Stock solutions of *C. acnes* isolates were streaked onto brucella agar plates and incubated for three days to allow mature and active *C. acnes* growth. The Minimum Inhibitory Concentration (MICs) of clindamycin and doxycycline were determined using the agar dilution method, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines for anaerobic bacteria (141). Inocula were prepared colonies from three day growth were collected and suspended as necessary in 0.9% sterile saline solution to an optical density of 0.5 McFarland scale (Remel Microbiology Products, Lenexa, KS, USA) to obtain a suspension of 1×10^8 colony forming units (CFU/ml) according to the manufacturer's instructions, to produce a final concentration of bacterial inoculum approximately 1×10^6 CFU/ml. The concentrations of clindamycin and doxycycline ranged from 0.5 – 512 µg/ml and 0.0625 - 64 µg/ml, respectively. MICs results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) M11-A7 guidelines for anaerobic bacteria to identify *C. acnes* resistant strain. The MICs breakpoints used for interpretation as resistance of clindamycin and doxycycline are $\geq$ 8 µg/ml and MIC $\geq$ 4 µg/ml, respectively. All experiments were performed in biological triplicates.

BACTERICIDAL TESTING

Minimum bactericidal concentrations (MBCs) of LL-37 were determined using the spread plate technique. Cell suspension of each concentration that showed no visible growth from the MIC determination were plated onto brucella agar plates and incubated at 37°C in an incubator for 72 h. The concentrations of the peptide ranged from 0.1-50 ug/ml and the concentration that demonstrated no viable colonies were recorded as MBCs. All experiments were performed in biological duplicates.

CHECKERBOARD MICRODILUTION ASSAY

The fractional inhibitory concentrations (FICs) between doxycycline, clindamycin and LL-37 assessed by checkerboard assay were recorded. The FIC index (FICI) was calculated using the sum of the FICs of each drug tested. The FIC of each drug was determined using the MIC of the drug when used in combination, and divided by the MIC of that drug when used alone at 48-72 h. The FICI values were interpreted as follows: FICI $\leq$ 0.5, synergistic; 0.5 < FICI $\leq$ 1, additive; 1 < FICI $\leq$ 4, no interaction (indifferent); FICI > 4, antagonistic (142) . All experiments were performed in biological triplicates.

FICA + FICB = FICI

- FICA is the MIC of drug A in combination divided by the MIC of drug A alone

$$FICA = \frac{MIC(A)\ incombination}{MIC\ (A)alone}$$

- FICB is the MIC of drug B in combination divided by the MIC of drug B alone

$$FICB = \frac{MIC(B)\ incombination}{MIC\ (B)alone}$$

TIME-KILLING ASSAY

An aliquot of the suspension *C. acnes* 45VPOP (100 µl) of initial inoculum (equivalent to 0.5 McFarland scale) were incubated in 10 ml of brucella broth with 5% LHB alone (growth control), or medium with MIC of doxycycline alone (8 µg/ml), MIC of LL-37 alone (0.01 µg/ml), or a combination between 8 µg/ml of doxycycline and 0.001 µg/ml of LL-37. The final concentration of bacterial inoculum was approximately 1 $x10^6$ CFU/ml. The bacterial cell suspensions were incubated at 37°C in an incubator. At 0, 6, 12, 24, 30, 48, 72, 96 and 120 h incubation, aliquots of 200 µl from each tube were serially diluted and 100 µl of each dilution was plated onto BR plates. Colony counts were determined after 72-120 h incubation at 37ºC (143-145).

CELL LINE AND CULTURE

Primary epidermal keratinocytes (HEKn) were cultured in serum-free keratinocyte growth medium, HuMediaKG2 containing human epidermal growth factor (0.1 ng/ml insulin, 10 µg/ml hydrocortisone, 0.5 µg/ml gentamycin, 50 µg/ml and 0.4% vol/vol bovine brain pituitary extract). The cells were cultured at 37ºC in a humidified atmosphere containing 5% CO_2 incubator and serially passaged before reaching confluence, experiments were conducted with subconfluent cells at 70–80% confluence.

CYTOTOXICITY TEST

Cytotoxicity of LL-37 was measured using the MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay. Briefly, 10^4 cells of Primary Normal Human Epidermal Keratinocytes (HEKn) were seeded with 50 µl of DMEM in a precoating 96-well plate. The different concentrations of LL-37 at 0.1, 25, 50, 75, and 100 µg/ml and doxycycline 8 µg/ml, and combination of LL-37 and doxycycline were added into the cells and incubated in a 5% CO_2 incubator at 37ºC for 24 h. The medium cultures were then collected and transferred into 96 well-plate and incubated with MTT solution and incubated for 4 h at room temperature. The absorbance of all controls and samples was measured with a 570 nm filter and calculated the

percentage of cytotoxicity by the following formula using cell supernatant in culture medium alone as low control and supernatant from cells treated with lysis buffer as high control:

$$\textbf{Cell viability (\%)} = \frac{\text{absorbance of treated cell}}{\text{absorbance of untreated cell}} \times 100$$

Cell cytotoxic (%) = 100 – Cell viability

STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism software version 9. The comparison performed with student's t-test. Data wrer expressed as mean ± SEM, and differences with a p-value of <0.05 were considered statistically significant.

ETHICAL CONSIDERATION

This study has been approved by the Institutional Review Board (IRB No. 058/62), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

CHAPTER IV

RESULTS

1. ANTIMICROBIAL SUSCEPTIBILITY TESTING

The antimicrobial susceptibility of *C. acnes* clinical isolates was evaluated to determine the minimum inhibitory concentration (MIC) values of clindamycin, doxycycline, erythromycin, tetracycline, and trimethoprim/sulfamethoxazole using the Clinical and Laboratory Standards Institute (CLSI) anaerobe broth microdilution M11-A7 method (1). The results showed that one hundred forty-three (100%) strains were resistant to trimethoprim/sulfamethoxazole, while one hundred-five (73.34%) strains were resistant to clindamycin and one hundred-five (73.34%) strains were resistant to erythromycin. Moreover, seventy-three (51.05%) strains were resistant to doxycycline and sixty-five (45.45%) strains were resistant to tetracycline (**Table 7**).

Table 7 Antibiotics susceptibility of C. acnes strain

Antibiotics	MIC(µg/ml) min-max	MIC breakpoints (µg/ml)	*C. acnes* resistance n(%)
Doxycycline	0.125 - 32	≥ 4	73(51.05)
Tetracycline	0.25 - 128	≥ 16	65(45.45)
Erythromycin	0.03 - >512	≥ 2	105(73.43)
Clindamycin	0.25 - >512	≥ 8	105(73.43)
trimethoprim/sulfamethoxazole	2 - >512	≥ 1	143(100)

(N = 143), MIC; Minimun Inhibitory Concentration

Next, *C. acnes* clinical isolates were randomly chosen to determine the minimum inhibitory concentration (MIC) values of clindamycin and doxycycline using the Clinical and Laboratory Standards Institute (CLSI) anaerobe broth microdilution M11-A7 method (1). There were 3 clinical isolates: *C. acnes* 45VPOP, *C. acnes* 71PYIP and *C. acnes* 106KKCL. The MICs to each *C. acnes* clinical isolates were measured and compared with the MICs to a standard strain, *C. acnes* ATCC6919. The results showed that the MICs of both clindamycin and doxycycline to the standard strain, *C. acnes*

ATCC6919 were at 0.125 µg/ml. The MICs of clindamycin to *C. acnes* 71PYIP and *C. acnes* 45VPOP were at 512 µg/ml whilst the MIC of clindamycin to *C. acnes* 106KKCL was at 2 µg/ml. The MICs of doxycycline to *C. acnes* 71PYIP, 106KKCL, and 45VPOP were at 0.5, 8, and 16 µg/ml, respectively. As mentioned previously in the Method section, the MICs breakpoints used for interpretation as resistance of clindamycin and doxycycline were $\geq$ 8 µg/ml and MIC $\geq$ 4 µg/ml, respectively. Therefore, *C. acnes* 45VPOP represented a clindamycin- and doxycycline-resistant strain whilst *C. acnes* 71PYIP represented a clindamycin-resistant strain and *C. acnes* 106KKCL represented a doxycycline-resistant strain (**Table 8**).

Table 8. Antimicrobial susceptibility tests of clindamycin and doxycycline against Cutibacterium acnes clinical isolates and a standard strain (ATCC6919)

Bacterial isolates	MICs (µg/ml)	
	Clindamycin	**Doxycycline**
Cutibacterium acnes ATCC6919	0.125 (S)	0.125 (S)
Cutibacterium acnes 45VPOP	512 (R)	16 (R)
Cutibacterium acnes 71PYIP	512 (R)	0.5 (S)
Cutibacterium acnes 106KKCL	2 (S)	8 (R)

S; susceptible, R; resistant

2. THE SYNERGISTIC EFFECT OF HUMAN CATHELICIDIN LL-37 ON ANTIBIOTIC-RESISTANT *CUTIBACTERIUM ACNES*

In our previous experiment, it was found that LL-37 inhibited the growth of all antibiotic-resistant clinical isolates of *C. acnes* at the concentrations of 25-50 μg/ml, we therefore performed checkerboard assays to determine the synergistic effect of LL-37 and clindamycin, and the synergistic effect of LL-37 and doxycycline, on antibiotic-resistant *C. acnes*. In this experiment, *Cutibacterium acnes* 45VPOP that represented clindamycin- and doxycycline-resistant *C. acnes* was chosen to determine the synergistic effect of LL-37.

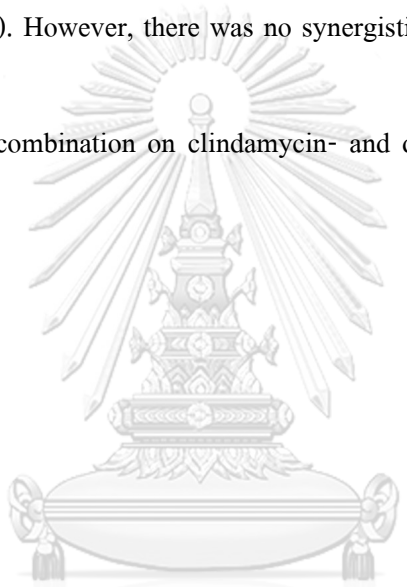
3.1 THE SYNERGISTIC EFFECT OF HUMAN CATHELICIDIN LL-37 AND CLINDAMYCIN

The checkerboard assay of LL-37 and clindamycin was performed, and the results showed that the MIC of clindamycin was decreased from 512 to 128 μg/ml and the MIC of LL-37 was decreased from 50 to 25 μg/ml (**Table 9**).

Table 9. Individual and combined agents between LL-37 and clindamycin against C. acnes 45VPOP

Bacterial isolate	Individual agents (μg/ml)		Combined agents (μg/ml)		
	Clindamycin	LL-37	Clindamycin	LL-37	FICI
Cutibacterium acnes 45VPOP	512	50	128	25	0.75

Moreover, The fractional inhibitory concentration index (FICI) of LL-37 and clindamycin value was calculated as mentioned in the Method section. The result demonstrated that FICI of combined agents ranged from 0.75 to 1.5 (**Figure 7**). According to this FICI value, it was suggested that both LL-37 and clindamycin showed additive effects ($0.5 < FICI \leq 1$) and no interaction ($1 < FICI \leq 4$). However, there was no synergistic effects (≤ 0.5) and antagonistic effects (>4) of this drug combination on clindamycin- and doxycycline-resistant *C. acnes* (*C. acnes* 45VPOP).



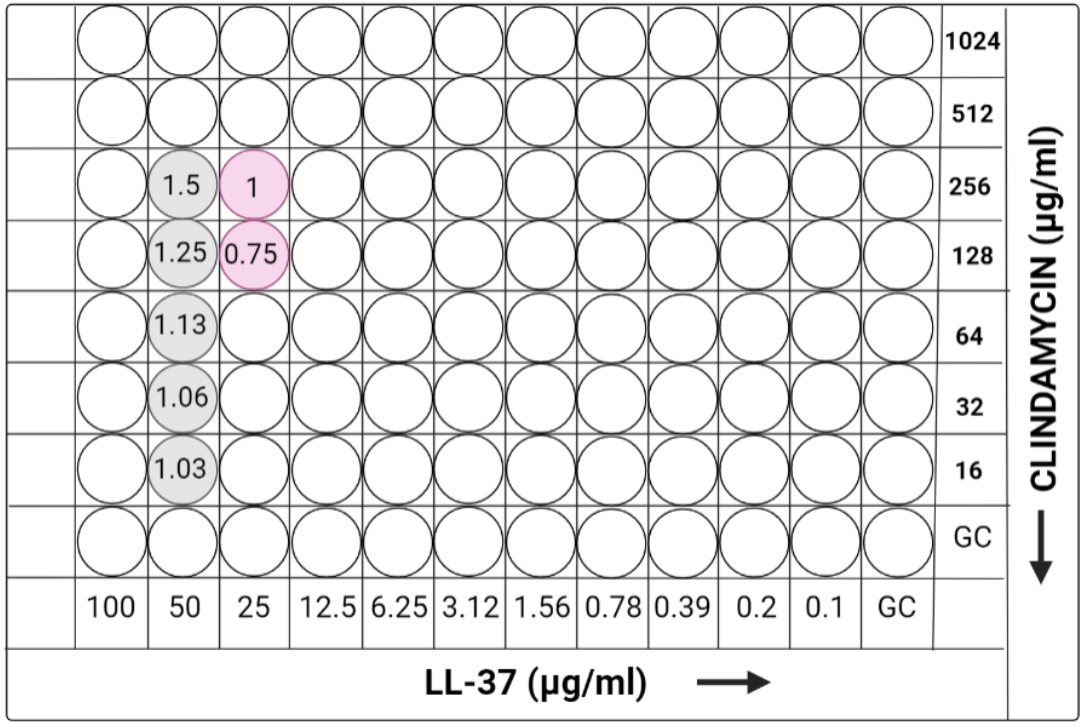


Figure 7. Checkerboard microdilution assay of LL-37 and clindamycin

Checkerboard microdilution assay of LL-37 and clindamycin against clindamycin- and doxycycline-resistant C. acnes isolate (C. acnes 45VPOP). C. acnes were incubated in combinations between clindamycin (16-1024 µg/ml) and LL-37 (0.1-100 µg/ml) for 24 h. FICI $\leq$ 0.5 showed synergistic effect, 0.5 < FICI $\leq$ 1 showed additive effect (pink), 1 < FICI $\leq$ 4 showed no interaction (light grey), and FICI > 4 showed antagonistic on C. acnes growth (n=3).

3.2 THE SYNERGISTIC EFFECT OF HUMAN CATHELICIDIN LL-37 AND DOXYCYCLINE

The checkerboard assay of LL-37 and doxycycline was performed, and the results showed that the MIC of doxycycline was decreased from 16 to 8 μg/ml and the MIC of LL-37 was decreased from 50 to 0.1 μg/ml (**Table 10**).

Table 10. Individual and combined agents between LL-37 and doxycycline against C. acnes 45VPOP

Bacterial isolate	Individual agents (μg/ml)		Combined agents (μg/ml)		
	Doxycycline	LL-37	Doxycycline	LL-37	FICI
Cutibacterium acnes 45VPOP	16	50	8	0.1	0.50

Moreover, the fractional inhibitory concentration index (FICI) of LL-37 and doxycycline value ranged from 0.5 to 1.5 (Figure 8). According to this FICI value, it was suggested that both LL-37 and doxycycline showed synergistic effects ($\leq$ 0.5), additive effects (0.5 < FICI $\leq$ 1), no interaction (1 < FICI $\leq$ 4) without antagonistic effects (>4) on clindamycin- and doxycycline-resistant *C. acnes* (*C. acnes* 45VPOP) isolate.

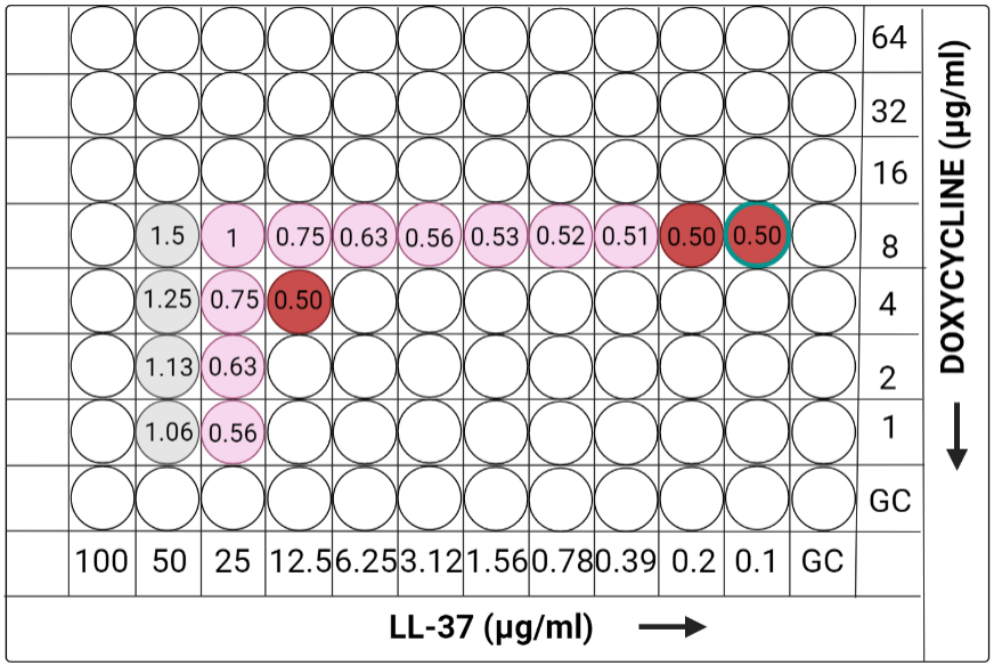


Figure 8. Checkerboard microdilution assay of LL-37 and doxycycline

Checkerboard microdilution assay of LL-37 and doxycycline against clindamycin- and doxycycline-resistant C. acnes (C. acnes 45VPOP). C. acnes were incubated in combinations between doxycycline (1-64 µg/ml) and LL-37 (0.1-100 µg/ml) for 24 h. FICI $\leq$ 0.5 showed synergistic effect (red); 0.5 < FICI $\leq$ 1 showed additive effect (pink), 1 < FICI $\leq$ 4 showed no interaction (light grey), and FICI > 4, antagonistic effect on C. acnes growth (n=3).

4. TIME KILLING ASSAY OF LL-37 ALONE AND IN A COMBINATION WITH DOXYCYCLINE AGAINST ANTIBIOTIC-RESISTANT *CUTIBACTERIUM ACNES*

As the synergistic effect between LL-37 and clindamycin was not found in our study, we therefore only performed time-killing assays to confirm the synergistic effect of LL-37 and doxycycline against clindamycin- and doxycycline-resistant *C. acnes*. The concentrations of doxycycline and LL-37 for time-killing assays were chosen from the MICs of checkerboard assays, which were at 0.1 µg/ml of LL-37 and at 8 µg/ml of doxycycline. The time-killing assays at 120 h showed that the growth of clindamycin- and doxycycline-resistant *C. acnes* in drug combination treatment (5.14 log10 ±0.33 CFU/ml) was significantly decreased at 2.92 log10 in CFU/ml compared to doxycycline alone (8.05 log10 ±0.68 CFU/ml), and significantly decreased at 6.52 log10 in CFU/ml compared to LL-37 alone (11.66 log10 ±0.53 CFU/ml). Moreover, the growth of resistant strain in drug combination treatment was significantly decreased at 7.29 log10 in CFU/ml compared to untreated group (12.43 log10 ±0.01 CFU/ml) (**Figure 9**). These results supported that LL-37 showed a synergistic effect with doxycycline in inhibition of growth of clindamycin- and doxycycline-resistant *C. acnes* as the *C. acnes* growth was significantly reduced by more than 2 log10 CFU/ml.

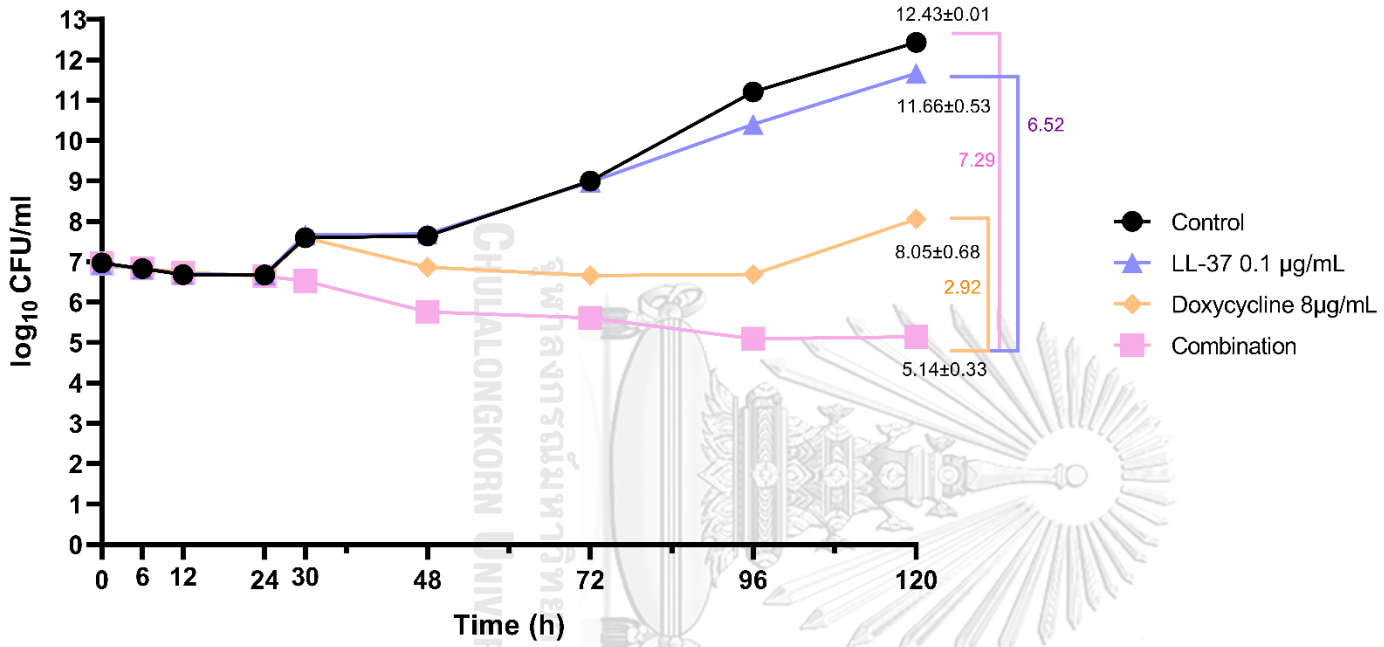


Figure 9.Time-killing assay of LL-37 alone and in a combination with doxycycline

Time-killing assay of LL-37 alone and in a combination with doxycycline against clindamycin- and doxycycline-resistant C. acnes (C. acnes 45VPOP). C. acnes cells were incubated with doxycycline at 8 µg/ml (orange), LL-37 at 0.1 µg/ml (purple), a combination between doxycycline 8 µg/ml and LL-37 0.1 µg/ml (pink), and control group (black) for 120 h. (n=3)

5. CATHELICIDIN LL-37 CYTOTOXICITY TOWARDS HUMAN CELLS

To determine the cytotoxic effect of LL-37 on human cells, MTT assay was performed on primary normal human epidermal keratinocytes (HEKn) treated with different concentrations of LL-37. The result demonstrated that LL-37 showed a significant cytotoxicity at the concentrations of 25 ug/ml (71.61 ± 7.63%), 50 ug/ml (92.21 ± 2.18%), 75 ug/ml (93.91 ±0.43%), and 100 ug/ml (93.78 ± 0.33 %) when compared with untreated cells.

The cytotoxic effect was also found in doxycycline alone (27.35 ± 4.98 %) and in a combination between LL-37 and doxycycline (34.73 ± 8.51 %) on HEKn when compared with untreated control cells (100.00 ± 6.01 %) (**Figure 10a and 10b**). However, the cytotoxicity of LL-37 at concentration of 0.1 ug/ml (16.31 ± 8.40 %) on HEKn was not significantly different from untreated cells (**Figure 10b**).

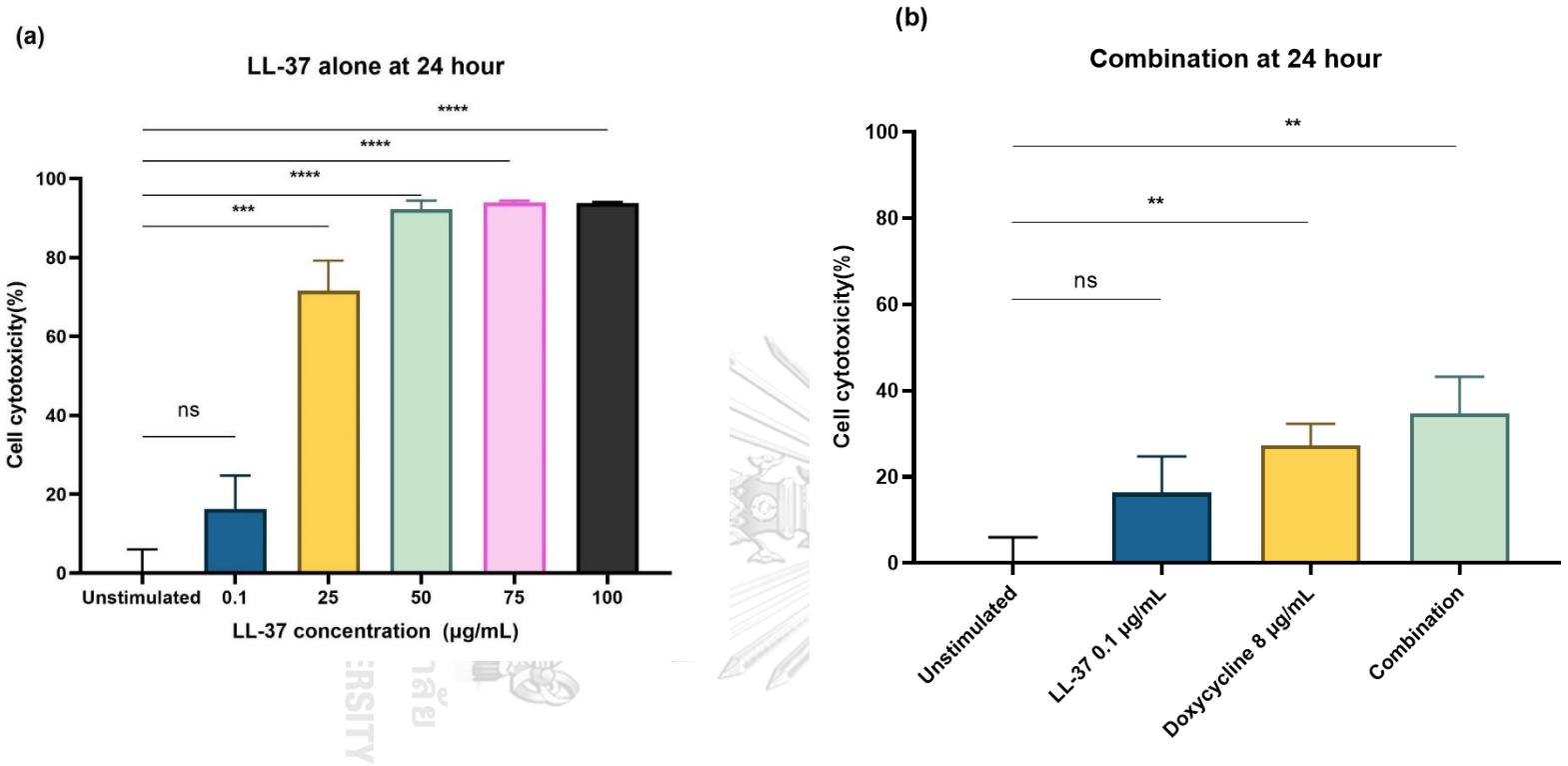


Figure 10. Cytotoxicity of LL-37 alone or in a combination with doxycycline on HEKn cells.

*HEKn cells were co-cultured with different concentrations of LL-37 (0.1, 25, 50, 75, 100 ug/ml) (a) or in a combination with doxycycline (8 ug/ml) (b) at 37°C in a CO_2 incubator for 24 h. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, Student's t-test. ns: not significance. (n=3).*

CHAPTER V

DISCUSSION AND CONCLUSION

Human cathelicidin is one of the most powerful antimicrobial peptides of the innate immune system. This peptide is naturally produced by leukocytes such as neutrophils, macrophages, mast cells and dendritic cells, and epithelial cells such as human keratinocytes(1, 2). The main function of human cathelicidin is antimicrobial activity against different microorganisms including bacteria, virus, and fungi(3). However, human cathelicidin also plays role in the activation of cell proliferation and migration which supports human homeostasis and tissue regeneration, and involves in the anti-tumor immune response(4-10).

LL-37 is the only type of cathelicidin peptide found in human and it shows a strong antibacterial activity against gram-positive bacteria including *C. acnes* which is the main pathogen involved in the pathogenesis of acne (11, 12). Although the inhibitory effect of LL-37 on the growth of *C. acnes* has been reported (12), this effect on *C. acnes* clinical isolates particularly antibiotic-resistant strains from Thai patients with acne has never been explored. In our study, it was found that 143 clinical isolates of *C. acnes* from patients with acne vulgaris were

highly resistant to trimethoprim/sulfamethoxazole (100%), clindamycin (73.34%) and erythromycin (73.34%). Moreover, half of clinical isolates were resistant to doxycycline (51.05%) and tetracycline (45.45%). This finding suggests that the prevalence of antibiotic-resistant *C. acnes* has been increasing in our Thai patients (17), probably due to improper use of antibiotics.

This study first aimed to investigate whether LL-37 inhibited the growth of *C. acnes* standard strain and antibiotic-resistant strains from patients with acne. In addition, *C. acnes* often resists to clindamycin and doxycycline which are commonly used topical and oral antibiotics in patients with acne (13-16), this study therefore determined the effect of LL-37 on clindamycin- and doxycycline-resistant strains of *C. acnes* from clinical isolates. The *C. acnes* 71PYIP, 106KKCL and 45VPOP clinical isolates were randomly chosen for antimicrobial susceptibility testing for LL-37. These clinical isolates represented clindamycin-resistant, doxycycline-resistant and both clindamycin- and doxycycline-resistant strains, respectively. The results showed that LL-37 inhibited *C. acnes* standard strain (ATCC6919) and all antibiotic-resistant strains at the MICs of

25 to 50 μg/ml and at the MBCs of 25 to more than 50 μg/ml. These findings suggest that LL-37 could potentially be used as an antimicrobial agent for treatment of patients with acne.

We further investigated the synergistic effect of LL-37 and clindamycin or LL-37 and doxycycline on antibiotic-resistant *C. acnes*. In this study, *C. acnes* 45VPOP that represented clindamycin- and doxycycline-resistant *C. acnes* was chosen as it showed higher MICs (50 μg/ml) and MBCs (>50 μg/ml) than other strains. The result demonstrated that LL-37 and clindamycin showed no synergistic effect but only additive effect or no interaction was found. We hypothesized that *C. acnes* 45VPOP clinical isolate that showed very high MIC (512 μg/ml) for clindamycin probably had multiple mechanisms such as efflux pump, drug inactivation and target-site modification to resist clindamycin as shown in previous studies (18). For example, it was demonstrated that a combination of LL-37 and vancomycin could not inhibit the growth of vancomycin-resistant *S. aureus* with high MIC of (512 μg/ml) (19). Therefore, these multiple resistant mechanisms of clindamycin plus the action of LL-37 alone were perhaps inefficient to inhibit *C. acnes* 45VPOP clinical isolate so this drug combination was unable to show the synergistic effect in this study.

Next, we investigated the effect of LL-37 and doxycycline on antibiotic-resistant *C. acnes* 45VPOP. Interestingly, the result demonstrated the synergistic effect of this drug combination using a checkerboard assay (20, 21), which was confirmed by time-killing assays at 120h (**Figure 9**) with the concentrations of LL-37 at 0.1 μg/ml and doxycycline at 8 μg/ml. We hypothesized that this synergism reflects the mechanism of LL-37 that induces pore formation or micellization on the membrane of *C. acnes*, which promotes doxycycline intracellular uptake (22, 23). Increased influx of doxycycline thereby inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit, leading to bacterial cell death. These findings suggest that this drug combination could potentially be used for treatment of patients with acne especially those who are poorly responsive to antibiotic therapy or are evidently colonized with clindamycin- and doxycycline-resistant *C. acnes*. However, the route of drug administration of LL-37 and doxycycline needs to be further explored as there is no topical preparation of doxycycline available in current treatment of acne. Nevertheless, it was reported that patients with atopic dermatitis were clinically improved after topical doxycycline treatment. Therefore, the

combination of LL-37 and doxycycline in a topical preparation could possibly be made for treatment of patients with acne.

Finally, the cytotoxicity of LL-37 alone and the combination of LL-37 and doxycycline which showed the synergistic effect was investigated. The results demonstrated that LL-37 alone at the concentrations of >25 μg/ml showed cytotoxicity to primary human keratinocytes (HEKn) in a dose-dependent manner. These cell lines were chosen because they are usually affected by *C. acnes* leading to skin inflammation causing acne in patients. We found that LL-37 at the concentration of 0.1 μg/ml was not toxic to these cells which was the same concentration of LL-37 that showed synergistic effect with doxycycline. Unfortunately, this concentration of LL-37 (0.1 μg/ml) plus doxycycline at the concentration of 8 μg/ml showed cytotoxic effect on human keratinocytes in our study (**Figure 10b**). In a previous study, it was reported the cytotoxicity of doxycycline at the concentrations 4-6 μg/ml on human peripheral blood lymphocytes due to inhibition of DNA synthesis or G2 phase of cell cycle(24). Therefore, we assumed that doxycycline at the concentration of 8 μg/ml were also toxic to human keratinocytes. These findings suggest that the cytotoxic effect of this drug combination was probably caused by

doxycycline toxicity alone, and the combination of LL-37 and doxycycline at these concentrations were probably not appropriate for the treatment of *C. acnes*. However, it was demonstrated that the concentration of doxycycline was 1.5–3.0 μg/ml in plasma of patients after oral administration of 100–200 mg/day which is the standard dose for acne treatment (25). Thus, the concentrations of LL-37 (0.1-12.5 μg/ml) and doxycycline (less than 4 μg/ml) need to be further investigated for cytotoxicity (24, 26).

In conclusion, this study showed the inhibitory effect of LL-37 on *C. acnes* clinical isolates and all antibiotic-resistant strains from Thai patients with acne. We found that the combination of LL-37 and doxycycline showed synergistic effect on clindamycin- and doxycycline-resistant *C. acnes*. However, the concentrations of this drug combination were toxic to human keratinocytes.

Future plans:

1. To optimize the concentration of LL-37 (0.1-12.5 μg/ml) and doxycycline (<4 μg/ml) on human keratinocytes that show no cytotoxicity.

2. To investigate the cytotoxicity of LL-37 and doxycycline on dermal fibroblasts.

3. The synergistic effect of LL-37 with other topical antibiotics such as erythromycin, benzoyl peroxide, etc.

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