Review Article

An Emerging Role of Cathelicidin Antimicrobial Peptides in Bone Biology

Suttichai Krisanaprakornkit[1]

[1]Center of Excellence in Oral and Maxillofacial Biology, Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand

Correspondence to:

Suttichai Krisanaprakornkit. Center of Excellence in Oral and Maxillofacial Biology, Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200 Thailand Tel: 053-944451 Fax: 053-222844 Email: suttichaikris@yahoo.com; suttichai.k@cmu.ac.th

Abstract

Cathelicidins are small cationic antimicrobial peptides, whose essential roles in several distinct pathophysiological processes, including antimicrobial activity, cell proliferation and migration, immune-regulatory function, angiogenesis and wound healing, have been thoroughly scrutinized in the past two decades. In this review, several lines of new evidence are provided to show another novel function of cathelicidins, including LL-37 in humans and a mouse ortholog of LL-37, *i.e.*, murine cathelicidin-related antimicrobial peptide (mCRAMP), in the inhibition of osteoclast formation and function. Moreover, LL-37 can function to accelerate bone regeneration in a model of rat calvarial bone defect. All of these latest findings point toward an emerging role of cathelicidins in bone biology. With the implication of cathelicidins in bone metabolism, it is hoped that the cathelicidins will be therapeutically beneficial for future clinical uses in dentistry, especially for bone regeneration in a common bone-resorbing disease like periodontitis.

Key words: Antimicrobial peptide; Cathelicidin; LL-37; Nuclear factor of activated T-cells; Osteoblast; Osteoclast

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Introduction

To mark a special occasion for the 75th anniversary of dentistry in Thailand in 2015, the author was invited by the Editor of Journal of the Dental Association of Thailand to write a special review article in order to provide the latest information and knowledge gained from research and studies in the field of oral biosciences in Thailand. In this review, the author presented an interesting research topic on a novel role of cathelicidin antimicrobial peptides in bone biology, which has received attention from several groups of scientists around the world during the past five years. This review will be useful for Thai dentists to learn some new scientific knowledge that can potentially be applied for future clinical uses.

Host immune responses are largely categorized into two major systems, including innate and acquired immune systems. Innate immunity is primitive and evolutionarily conserved but it is very essential for human life, since it acts as the first line of the host defense mechanisms against invading pathogens by recognition of various pathogen-associated molecular patterns with a number of host pattern-recognition receptors, such as Toll-like receptors (TLRs).[1] A myriad of innate effector molecules that are produced and released from innate immune cells, subsequent to microbial challenges, are critical for full activation of the second immune system, acquired immunity, to efficiently cope with an imminent danger from invading pathogens. One of these well-studied effectors is a family of small cationic antimicrobial peptides that are reported to exert a broad spectrum of antimicrobial activities by a direct interaction with the microbial membrane.[2] In addition, it has been subsequently demonstrated in the scientific literature that the antimicrobial peptides are, indeed, multifunctional molecules, which can play several other roles in the modulation of inflammation by synthesis of pro-inflammatory cytokines and chemokines, enhancement of cell proliferation and migration, and induction of new blood vessel formation, a biological process known as angiogenesis.[3] All of these roles are essential for wound healing processes, especially in the oral cavity. There are two main families of antimicrobial peptides that have been well characterized in humans to date, including **defensin** and **cathelicidin**. However, due to the space limitation of this review, only the cathelicidin family and its newly emerging role in bone biology, which has recently drawn attention from several groups of researchers worldwide, are described. Some basic background in osteoclast biology is also reviewed in this article for readers to better understand this novel function of the cathelicidin peptides in bone biology.

Cathelicidin antimicrobial peptides

Cathelicidin is a family of small cationic antimicrobial peptides that are evolutionarily conserved host defense molecules against infections and contain a highly conserved N-terminal cathelin domain and a highly variable C-terminal antimicrobial domain (Fig. 1).[4] The conserved cathelin domain aids in the discovery of new cathelicidins from diverse animal species, whereas large variations in amino acid sequence of the antimicrobial domain account for various molecular structures of mature cathelicidins. They are classified into four different structural groups, including α-helices (A) with an amphipathic structure containing both hydrophobic and hydrophilic residues, β-hairpins (B), extended structures (C), and cyclic peptides (D) (Fig. 1).[5] It is suggested that various molecular structures of cathelicidins probably reflect the nature of microbial diversity. The first cathelicidin antimicrobial peptide was isolated from bovine neutrophils.[6] Several cathelicidin peptides were subsequently identified in many other mammalian species, including mice and humans. The only cathelicidin in humans is an alpha-helical LL-37 peptide without intrinsic disulfide bonds that is derived from proteolytic cleavage of the 18-kDa propeptide, namely human cationic antimicrobial peptide 18 (hCAP18), by serine proteases of the kallikrein family in keratinocytes[7] and proteinase 3 in neutrophils (Fig. 1).[8] LL-37 consists of

37 amino acid residues with two leucine (L) residues at its N-terminal end,[9] and its isoelectric point (pI) value is around 10.6.[10] A mouse ortholog of LL-37 was later found and named murine cathelicidin-related antimicrobial peptide (mCRAMP). LL-37 is constitutively synthesized by neutrophils and stored as a precursor hCAP18 in the secondary granules in the amount of 0.627 micrograms per one million neutrophils.[11] In addition to neutrophils, LL-37 is widely expressed in several other cell types, including macrophages, natural killer cells, mast cells, lymphocytes, epithelial cells lining the oral cavity, the skin, the respiratory and the gastrointestinal tracts, and the ocular surface.[12] A recent study has shown a hierarchy of expression of hCAP18 in human peripheral blood-derived cells with low levels in lymphocytes, medium levels in monocytes, and high levels in neutrophils.[13] LL-37 expression in skin keratinocytes and gastric epithelial cells is induced by *Staphylococcus aureus* and *Helicobacter pyroli*, respectively.[14,15] LL-37 expression can also be up-regulated by active vitamin D hormone, *i.e.*, 1, 25-dihydroxyvitamin D, whose functions are known to control calcium homeostasis and to regulate host innate immunity.[16] We now learn that when macrophages sense a bacterial infection, they can convert circulating vitamin D into 1, 25-dihydroxyvitamin D, which is a potent inducer of hCAP18/LL-37 expression.[13,17] The induction of hCAP18/LL-37 expression is thus demonstrated to protect mice from tuberculosis infection.[18]

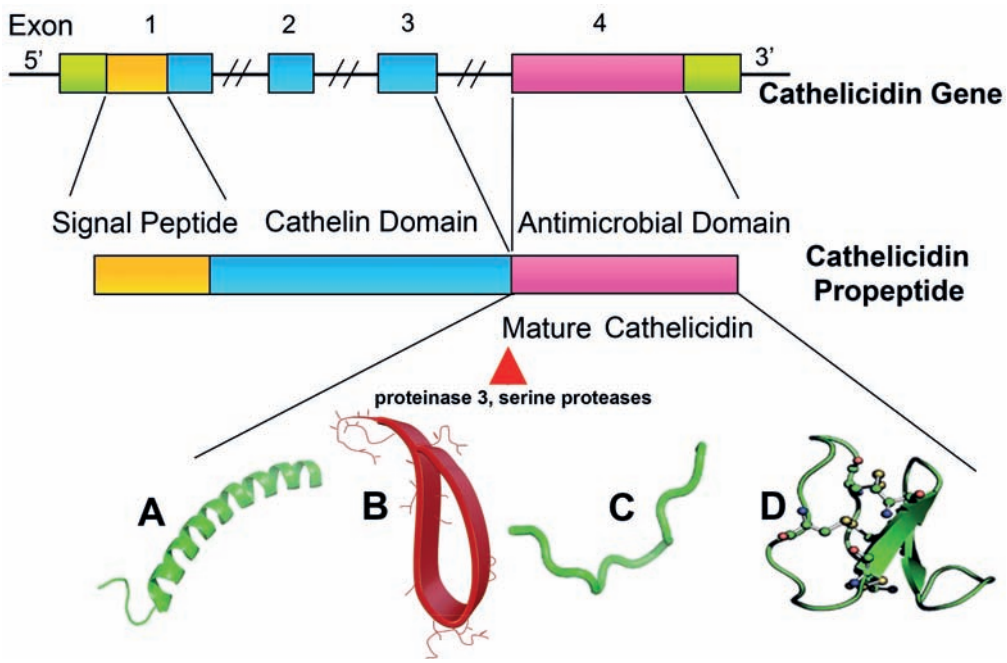

Figure 1 *The structure of the cathelicidin gene and protein*

The cathelicidin gene contains four exons, in which the first exon encodes the signal peptide domain (orange), the second and the third encode the cathelin domain (light blue), while the fourth encodes the antimicrobial domain, or mature cathelicidin (purple), whose various molecular structures can be categorized into four main groups; A. helices, B. hairpins, C. extended structures and D. cyclic peptides. The green regions of the cathelicidin gene represent 5'-untranslated and 3'-untranslated regions. A red triangle indicates the cleavage site by proteinase 3 and serine proteases.

In the oral cavity, LL-37 expression is up-regulated in inflamed gingival tissues and the concentrations of LL-37 in the gingival tissues correlate positively with the depth of the gingival crevice, suggesting that the LL-37 levels may be used as a diagnostic biomarker in inflammatory periodontal diseases.[19] In addition, LL-37 peptide is detected in saliva samples of adults and children[20,21] and in gingival crevicular fluid (GCF),[22] and LL-37 levels in GCF are significantly elevated in patients with chronic periodontitis as compared with those in patients with gingivitis or in healthy volunteers.[23] Raised LL-37 levels in GCF samples from patients with chronic periodontitis are also strongly associated with enhanced alveolar bone destruction, as demonstrated by increased levels of chondroitin sulfate, one of the catabolic bone markers.[24] It is likely that LL-37 present in saliva and GCF originates mostly from the secondary granules of neutrophils.[25] In this review, the author discusses the widely-characterized biological activities of LL-37 and mCRAMP, particularly the antimicrobial action and the immune-regulatory function, as well as the recently-recognized role in bone biology.

Antimicrobial action of cathelicidins

In the skin, both LL-37 and mCRAMP can prevent invasive bacterial infections.[26] Mice deficient in mCRAMP are more susceptible to gastrointestinal and urinary tract infections.[27,28] In the lung, CRAMP-deficient mice demonstrate impaired bacterial clearance, increased bacterial dissemination, and reduced survival in response to intratracheal administration of Gram-negative bacterial pneumonia.[29] With regard to the antimicrobial action of LL-37 against oral bacteria, it was initially demonstrated that concentrations of LL-37 less than 12 μg/ml already killed different strains of *Aggregatibacter actinomycetemcomitans* and *Capnocytophaga* species, which are implicated in the pathogenesis of aggressive periodontitis and gingivitis, respectively, by 99 %.[30] Subsequently, under a more detailed investigation into the antimicrobial effect of LL-37 against different kinds of periodontal bacteria, involved with various stages of dental plaque formation, it was demonstrated that the early colonizing yellow-complex bacteria, such as oral *Streptococci, Actinomyces,* etc., and the bridging orange-complex bacterium, *Fusobacterium nucleatum,* are susceptible to the bactericidal activity of LL-37 with low minimum inhibitory concentrations in μg/ml.[31,32] In contrast, the red-complex periodontal pathogens, including *Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola,* are more resistant to LL-37 than are other bacteria,[32] suggesting their strong implication with periodontitis.

Cathelicidins: multifunctional peptides

In addition to the antimicrobial action, it has been shown that LL-37 can either abrogate or enhance immune responses depending on the cell type and the timing or the context within which cells are exposed to LL-37. For example, LL-37 can inhibit the binding of endotoxin lipopolysaccharide to its receptor complex, comprising TLRs and CD14, which results in prevention of sepsis[33,34] and suppression of the synthesis of nitric oxide,[35] tumor necrosis factor- α (TNF- α), and prostaglandin E_2 (PGE_2).[36] On the contrary, LL-37 can, indeed, activate immune responses by enhancing the migration of monocytes, neutrophils, CD4 T lymphocytes, and eosinophils along its concentration gradient.[37,38] Moreover, LL-37 promotes the migration of rat mast cells and human corneal epithelial cells,[39,40] and induces histamine release from mast cell granules,[41] leading to enhanced phagocytosis of opsonized microorganisms. LL-37 also induces dendritic cell differentiation, which can then activate cell-mediated acquired immunity through a Th1 profile.[42]

Several studies have shown an inducible effect of LL-37 on the expression of several cytokines and chemokines. For instance, LL-37 induces expression of chemokines and chemokine receptors[43] and that of intercellular adhesion molecule-1,[44] implying an indirect role of LL-37 in chemotaxis in addition to its direct role as mentioned above. LL-37 up-regulates interleukin-8 (IL-8) expression and increases cell proliferation in human bronchial epithelial cells.[45] Similarly, LL-37 enhances

IL-8 expression and release by human airway smooth muscle cells and human gingival fibroblasts.[46,47] In addition, LL-37 induces cyclooxygenase-2 (COX-2) expression, and thus PGE_2 secretion, in human gingival fibroblasts.[48] LL-37 treatment also activates expression of interleukin-1β, interleukin-6, IL-8, and TNF-α in human corneal epithelial cells, gingival epithelial cells and gingival fibroblasts.[40,49]

Besides the immune-regulatory effects, LL-37 plays a role in tissue repair by stimulating airway epithelial cell proliferation and wound closure,[50] and by activating keratinocyte proliferation and migration in a process known as re-epithelialization.[51,52] Consistent with these *in vitro* studies, the levels of LL-37 decrease in chronic ulcer epithelium,[51] whereas adenoviral transfer of LL-37 to the wound in mice results in a significant improvement of wound healing by enhanced re-epithelialization and granulation tissue formation.[53] Furthermore, it has been shown that LL-37 can suppress keratinocyte apoptosis via a COX-2-dependent mechanism,[54] which is in agreement with the function of LL-37 in promoting cell proliferation and tissue repair as indicated above.Interestingly, adding LL-37 exogenously into the wound induces angiogenesis, as shown in an *in vitro* study,[55] which demonstrates endothelial cell proliferation and increased numbers of new blood vessel formation in cultured endothelial cells in response to LL-37 treatment. In dentistry, it has been demonstrated that LL-37 enhances migration of human pulp cells, and this effect may be involved with pulp-dentin complex regeneration.[56] With regard to periodontitis, the deficiency of LL-37 shows a direct link to the pathogenesis of some certain types of periodontitis, including morbus Kostmann-associated periodontitis and aggressive periodontitis. Therefore, it is of great interest for the author to further examine the effect of LL-37 on alveolar bone resorption in periodontitis, which is principally carried out by osteoclasts. In the following heading, the author reviews some basic knowledge in the osteoclast biology before providing readers some evidence for the novel role of cathelicidins in bone biology.

Biology of osteoclasts

In the normal healthy state, although it seems that bone mass is constant or unchanged throughout an entire human lifespan, bone remodeling, a coupling process between bone resorption and formation, continuously takes place by resorbing old bone and instantly forming new bone in order to maintain bone mass and to keep essential mineral ions, like calcium ions, in the blood circulation in balance. It is estimated that approximately 10 per cent of old bone in the human body is replaced with formation of new bone annually. The process of bone formation is carried out by osteoblasts that are derived from undifferentiated mesenchymal cells, which can differentiate into many cell types, such as fibroblasts, adipocytes, muscle cells, osteoblasts and chondroblasts, whereas osteoclasts are an important cell type to function in the process of bone resorption. Osteoclasts stem from a monocytic lineage of hematopoietic stem cells that can also develop into other cell types, including macrophages and dendritic cells. Several proteins are involved and tightly modulate the bone remodeling process between osteoblasts and osteoclasts. For example, it is well known that osteoblasts can control osteoclastogenesis, or generation of osteoclasts, by production of two main factors, including macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B ligand (RANKL).[57] On the other hand, osteoclasts can, in turn, regulate the formation of osteoblasts by resorbing bone, which then releases several growth factors stored in bone matrix that induce osteoblast formation. These factors belong to two major growth factor families, including bone morphogenetic protein and transforming growth factor-β. Clinically, aberrations in the balance between osteoblast and osteoclast formation and function can lead to several skeletal disorders, either bone-resorbing diseases, *e.g.,* osteoporosis, rheumatoid arthritis, periodontitis, or an overwhelming

bone-forming pathological state like osteopetrosis. However, since periodontitis is a common bone-resorbing disease found in the oral cavity and because of the recently-discovered new role of cathelicidin antimicrobial peptides in regulating osteoclast formation and function by the author and others, only the biology of osteoclasts is focused in this section.

Osteoclasts are multinucleated cells resulting from a fusion process of several monocytes. They possess efficient machinery for dissolving crystalline hydroxyapatite and degrading organic bone matrix rich in collagen fibers.[58] Within osteoclasts, there are a number of mitochondria, an organelle necessary for energy generation in a form of adenosine triphosphate required for various cell functions, several vesicles containing hydrochloric acid and proteinases, and vacuoles and lysosomes that are loaded with degraded bone products of collagen and other matrix components, taken up into osteoclasts, transported through the cells, and expulsed into the environment on the other side (Fig. 2).[58] This transcytotic route allows osteoclasts to remove large amounts of matrix-degradation products without losing their tight attachment to underlying bone. Other typical characteristics of osteoclasts include a ruffled border, a specialized highly folded membrane at the bone interface known as the apical membrane, and a sealing zone that contains thick bundles of actin cytoskeleton, which help osteoclasts to tightly adhere to the bone surface and to separate the resorption lacuna, or Howship's lacuna, from the extracellular environment (Fig. 2).[57] This separation thus creates a restricted tiny compartment for bone resorption on the bone surface.

On the ruffled border, there are a number of proton pumps that push protons into the resorption lacuna against their concentration gradient, making the environment in the lacuna more acidic in order to demineralize inorganic components in bone matrix (Fig. 2).[57] The functional part of these pumps is encoded by the *TCIRG1* gene, which stands for "T-cell, immune regulator 1, ATPase, H^+-transporting, lysosomal V0 subunit A3." The A3 subunit is one part of a large protein complex known as a vacuolar H^+-ATPase (V-ATPase), which acts as a pump to move positively charged hydrogen atoms across membranes.[59] Furthermore, the acidic milieu in the lacuna can activate the proteolytic function of some proteases, like cathepsin K and matrix metalloproteinase-9, encoded by the *CTSK* and *MMP-9* genes, respectively, to degrade organic matrix in the bone, especially collagen type I. In addition to the proton pumps, osteoclasts possess chloride channels, encoded by the gene chloride channel 7 *(CLCN7)*, on their ruffled border to transport chloride ions for neutralizing the positive charge of protons in the lacuna (Fig. 2). It has been demonstrated that loss-of-function mutations of either *TCIRG1* or *CLCN7* account for more than 50 per cent of osteopetrotic cases in humans, which result from impairment or failure of osteoclast function.[59] On the opposite side of the ruffled border, several types of receptors can be found on the so-called basolateral membrane of osteoclasts.[60] Some of these proteins include a calcitonin receptor (CALCR) specific for binding to calcitonin, a hormone known to suppress the osteoclast function and thus bone resorption, and a receptor activator of nuclear factor-kappa B (RANK) that can specifically bind RANKL to promote osteoclastogenesis (Fig. 2).

In summary, all of the aforementioned proteins can be regarded as osteoclastic markers, generally used in *in vitro* studies in the field of osteoclast biology. In addition to a prominent feature of osteoclasts as multinucleated cells (containing ≥ 3 nuclei), the expression of tartrate-resistant acid phosphatase (TRAcP) and the appearance of an F-actin ring around the cell periphery are other common hallmarks for mature osteoclasts. Finally, apart from these hallmarks, it is also necessary to determine the function of osteoclasts by examining resorption of dentin slices *in vitro.* The readers will appreciate utilization of all of these markers in the author's research in the next heading.

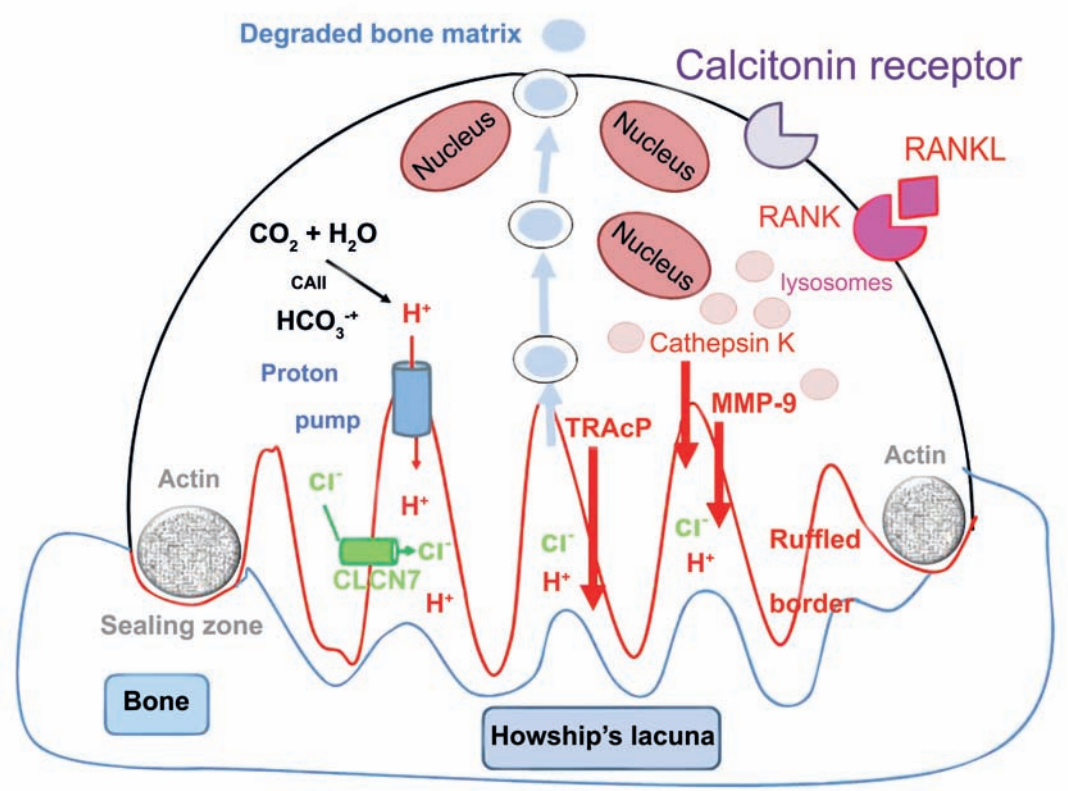

Figure 2 *The unique morphology of osteoclasts*

Osteoclasts contain multiple nuclei together with a ruffled border (red) and a sealing zone, which contains thick bundles of actin cytoskeleton that seal a tiny compartment, called Howship's lacuna. On the ruffled apical membrane, there are numerous proton pumps that drive protons (H^+) against their concentration gradient into the resorption lacuna and the chloride channels 7 (CLCN7) to release chloride ions (Cl^-). Protons are generated inside the osteoclast by an enzymatic reaction, catalyzed by the carbonic anhydrase II (CAII) enzyme. Other enzymes that are implicated in bone matrix degradation include cathepsin K, matrix metalloproteinase-9 (MMP-9) and tartrate-resistant acid phosphatase (TRAcP). On the basolateral membrane (black), there are various types of receptors, some of which are calcitonin receptor and receptor activator of nuclear factor-kappa B (RANK).

Nuclear factor of activated T-cells 2, a master transcription factor for osteoclastogenesis

Nuclear factor of activated T-cells 2 (NFAT2), belonging to the family of NFAT transcription factor that consists of five members, including NFAT1 - 5, was first discovered for its action on activation of T-lymphocyte functions almost 30 years ago.[61] Nevertheless, it was not until 2002 that Ishida and co-workers[62] demonstrated a pivotal role of NFAT2 in osteoclastogenesis in a RAW264.7 cell line. It was found in that study that after RANKL stimulation, NFAT2 was dephosphorylated by a group of phosphatase enzymes, collectively called calcineurin,[63] changing from an inactive hyperphosphorylated state to an active hypophosphorylated state, and then moved from the cytoplasm into the nucleus, in a process commonly known as nuclear translocation, to activate gene transcription of many osteoclast-specific genes, including the *NFAT2* gene by itself.[62] They

are mainly divided into three groups according to the dependence on NFAT2 activity. In the first group, the gene transcription relies solely on the activity of NFAT2. An example of this group is *CALCR* that encodes expression of the calcitonin receptor.[64] However, in the second group, other transcription factors, such as microphthalmia-associated transcription factor (MITF) and a tissue-specific transcription factor PU.1 found in hematopoietic cells, are required to activate gene transcription in conjunction with the activity of NFAT2. Examples of this group are *CTSK, MMP-9,* and *ACP5,* that encode expression of cathepsin K, MMP-9, and TRAcP, respectively.[65] For the last group, NFAT2 does not activate gene transcription, but instead suppresses gene transcription of *TNFRSF11B* that encodes expression of osteoprotegerin, a decoy receptor, whose suppressive effect on osteoclastogenesis is opposite to the promoting effect of RANKL.[66]

There are two major domains within the molecular structure of NFAT. The first domain at the N-terminal end is called an NFAT homology region, a region that is involved in the regulation of NFAT activity, since it contains a nuclear localization signal (NLS) sequence and has at least 18 serine residues that can potentially be the sites for phosphorylation.[67] The second domain at the central region is called a Rel homology region, which is a DNA-binding region that can interact with several other transcription factors.[68] It has been demonstrated that stimulating osteoclast precursor cells with RANKL can induce oscillatory changes in intracellular Ca^{2+} concentration, leading to Ca^{2+}/calcineurin-dependent dephosphorylation, or removal of phosphate groups, in the NFAT homology region of NFAT2. Therefore, the cryptic NLS sequence is free from the obstruction by phosphate groups, resulting in nuclear translocation of NFAT2 and in activated transcription of many osteoclast-specific genes to allow complete osteoclast differentiation.[69] The role of calcineurin in promoting osteoclast formation is verified by a few studies that use inhibitors to block the calcineurin activity, like cyclosporine, FK506, or antisense oligonucleotide, which can lead to suppression of osteoclast formation.[63,70] Moreover, osteoclast precursors that do not express NFAT2 cannot differentiate into mature osteoclasts,[71,72] whereas overexpression of NFAT2 in these cells increases osteoclast formation.[63] Collectively, the findings from these studies clearly demonstrate the significance of Ca^{2+}/calcineurin/NFAT2 signaling pathway in promoting osteoclastogenesis. The involvement of this signaling pathway in the inhibitory effect of LL-37 on osteoclastogenesis was, therefore, tested in the author's study,[73] and the published results are illustrated and explained in details in the following section. Several new ideas for future *in vitro* and *in vivo* studies are put forward in the next heading as well.

Evidence for a novel role of cathelicidins in bone biology

In this section, several lines of evidence from the author's laboratory and others for the involvement of cathelicidins in bone biology are extensively reviewed so that the readers are convinced of an emerging role of these small cationic antimicrobial peptides in bone biology. It is anticipated that dental practitioners in Thailand will become more familiar with these peptides and with their significant role in bone biology. In 2010, the author began a pilot study to investigate the inhibitory effect of LL-37 on osteoclastogenesis after the author came across some articles, written by Pütsep and co-workers,[74] Carlsson and co-workers,[75] and Puklo and co-workers,[22] which reported an absence or a decrease of LL-37 in GCF samples from patients with morbus Kost mann that exhibited severe alveolar bone loss and with aggressive periodontitis. The author thought that LL-37 might exert a suppressive effect on osteoclast formation, so the LL-37 deficiency in these patients might result in an excessive number of osteoclasts that could then lead to severe alveolar bone resorption. At that time, the author happened to meet Dr. Chayarop Supanchart, a talented researcher at the Faculty of Dentistry, Chiang Mai University, who just received his Ph.D. from the Max Planck Institutes, Berlin, Germany, in the field of osteoclast

biology. Thereafter, the author began to collaborate in a new and exciting research project to examine the inhibitory effect of LL-37 on *in vitro* osteoclastogenesis and had one paper published in the Journal of Dental Research.[73] However, that publication was just the beginning of some very complex studies that might eventually be translated into clinical applications in the future! The author is currently conducting several *in vitro* and *in vivo* experiments to determine the effects of both mCRAMP and LL-37 on osteoclast formation and function in both osteoblast-osteoclast co-cultures and animal models and to further dissect the molecular mechanism(s) underlying the suppressive effect in greater details.

In the collaborative study, the author found a novel inhibitory effect of LL-37 on osteoclastogenesis. When human peripheral blood mononuclear cells (PBMCs) cultured for eight days in the presence of M-CSF and RANKL, a number of osteoclast-like cells stained positively with TRAcP (red) were seen in the culture (arrows in Fig. 3A). Surprisingly, when 4 μM of LL-37 was exogenously added into the culture in the presence of both M-CSF and RANKL, no osteoclast-like cells were observed (Fig.3B). As anticipated, when PBMCs were cultured without M-CSF, cells underwent apoptosis, or programmed cell death (Fig. 3C). Interestingly, when PBMCs were cultured with 4 μM of LL-37 alone, cells could survive and proliferate even without the presence of M-CSF, but did not differentiate into osteoclast-like cells (Fig. 3D). With these unexpected and surprising findings, the author was very enthusiastic to continue additional studies into the characterization of these osteoclast-like cells by several techniques in molecular biology and into the intracellular signaling mechanisms that mediate the inhibitory effect of LL-37 on osteoclastogenesis.

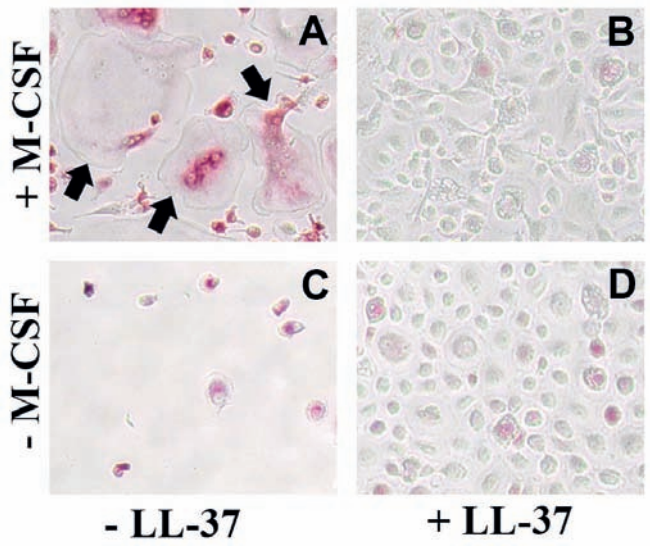

Figure 3 *The inhibitory effect of LL-37 on in vitro osteoclastogenesis*

Peripheral blood mononuclear cells were incubated

A. in the presence of M-CSF and RANKL

B. in the presence of M-CSF, RANKL and 4 μM of LL-37

C. in the absence of M-CSF and RANKL

and D. in the presence of LL-37 and RANKL without the presence of M-CSF

After that, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAcP). Arrows in A indicate large multinucleated cells with TRAcP-positive staining (red). Note a marked decrease in the number of cells in C. due to cell death. Magnification power = 200X.

The inhibitory effect of LL-37 on *in vitro* osteoclastogenesis was repeatedly confirmed by subsequent experiments. The findings showed a gradual decrease in TRAcP staining (red) upon treatment with increasing concentrations of LL-37 from 2 to 8 μM, while an increase in expression of proliferative cell nuclear antigen (brown), one of the proliferative markers, was observed in cells that were treated with LL-37 (Fig. 4A). This finding suggests that LL-37 treatment not only inhibits osteoclast differentiation but also promotes cellular proliferation. In addition to TRAcP staining, LL-37 treatment inhibited an F-actin ring formation (green rings at the cell periphery in Fig. 4B). To quantify the inhibitory effect on osteoclastogenesis, the number of osteoclast-like cells that contained ≥ 3 nuclei and were positively stained with TRAcP (dark gray bars) and the number of TRAcP-positive mononuclear cells (light gray bars) were counted. It was found that LL-37 treatment resulted in a significant reduction in the numbers of osteoclast-like cells and of TRAcP-positive mononuclear cells in a dose-dependent manner (Fig. 4C).

To determine the inhibitory effect on osteoclastogenesis at the molecular level, real-time polymerase chain reaction (PCR) analyses for mRNA expression of several osteoclast-specific genes were conducted. It was shown that treatment with 4 and 8 μM of LL-37 significantly abolished expression of several osteoclast-specific genes, including *MMP-9, TCIRG1, NFAT2, RANK, CALCR* and *CTSK* (Fig. 4D). Conversely, mRNA expression of the M-CSF gene (*CSF1*) was instead induced by treatment with 4 and 8 μM of LL-37 (Fig. 4D), consistent with a known proliferative effect of M-CSF on hematopoietic cells. The expression of chloride channel 7 (CLCN7), which is an essential characteristic of functional osteoclasts, was examined. It was demonstrated that LL-37 inhibited expression of CLCN7 protein in a dose-dependent fashion (Fig. 4E). Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a housekeeping gene control, was equivalent among different samples (Fig. 4E). To determine whether LL-37 treatment could inhibit osteoclast function in addition to osteoclast formation, PBMCs grown on dentin slices were incubated with various doses of LL-37. It was demonstrated that LL-37 significantly inhibited the resorptive function of the osteoclasts on the dentin slices in a dose-dependent fashion ($p < 0.05$).[73]

To explore the molecular mechanisms underlying the inhibitory effect on osteoclast formation, the suppressive effect of LL-37 on NFAT2, a master transcription factor for osteoclastogenesis, was first determined. By an immunofluorescence study, both 4 and 8 μM of LL-37 blocked nuclear translocation of NFAT2 (red) (upper panels in Fig. 5A). Staining with 4', 6-diamidino-2-phenylindole (DAPI) showed the location of nuclei (purple blue in lower panels; Fig. 5A). Cells that were treated without RANKL showed a low basal level of NFAT2 expression (Fig. 5A). Correspondingly, by an immunoblot analysis, LL-37 treatment caused the absence of NFAT2 in the nuclear extract, whereas the expression of NFAT2 in the cytosolic extract was equal among different samples (Fig. 5B). Cells that were not stimulated with RANKL served as a negative control and showed no NFAT2 expression, either in the nuclear or in the cytosolic extract (Fig. 5B). Since it has been previously demonstrated that NFAT2 can up-regulate its own expression at the transcriptional level, *i.e.,* auto-amplification, the author, therefore, sought to determine the inhibitory effect of LL-37 on the function of NFAT2 by investigating mRNA expression of NFAT2 upon LL-37 treatment. As expected, LL-37 treatment down-regulated NFAT2 mRNA expression in a dose-dependent manner, whereas GAPDH expression was equal among different samples (Fig. 5C). By a real-time PCR analysis, LL-37 treatment significantly decreased NFAT2 mRNA expression (Fig. 5D). Taken together, all of these findings indicate that LL-37 blocks nuclear translocation of NFAT2, resulting in down-regulation of NFAT2 expression. In order to prevent nuclear translocation of NFAT2 from the cytoplasm, NFAT2 needs to stay in the hyperphosphorylated inactive state, and this can be achieved by inhibiting the activity of calcineurin enzymes. Therefore, the levels of calcineurin activity were measured by a

calcineurin cellular activity assay kit in the presence of various concentrations of LL-37. It was demonstrated that LL-37 treatment could significantly decrease the calcineurin activity by approximately 50 % as compared with the untreated control sample (Fig. 5E).

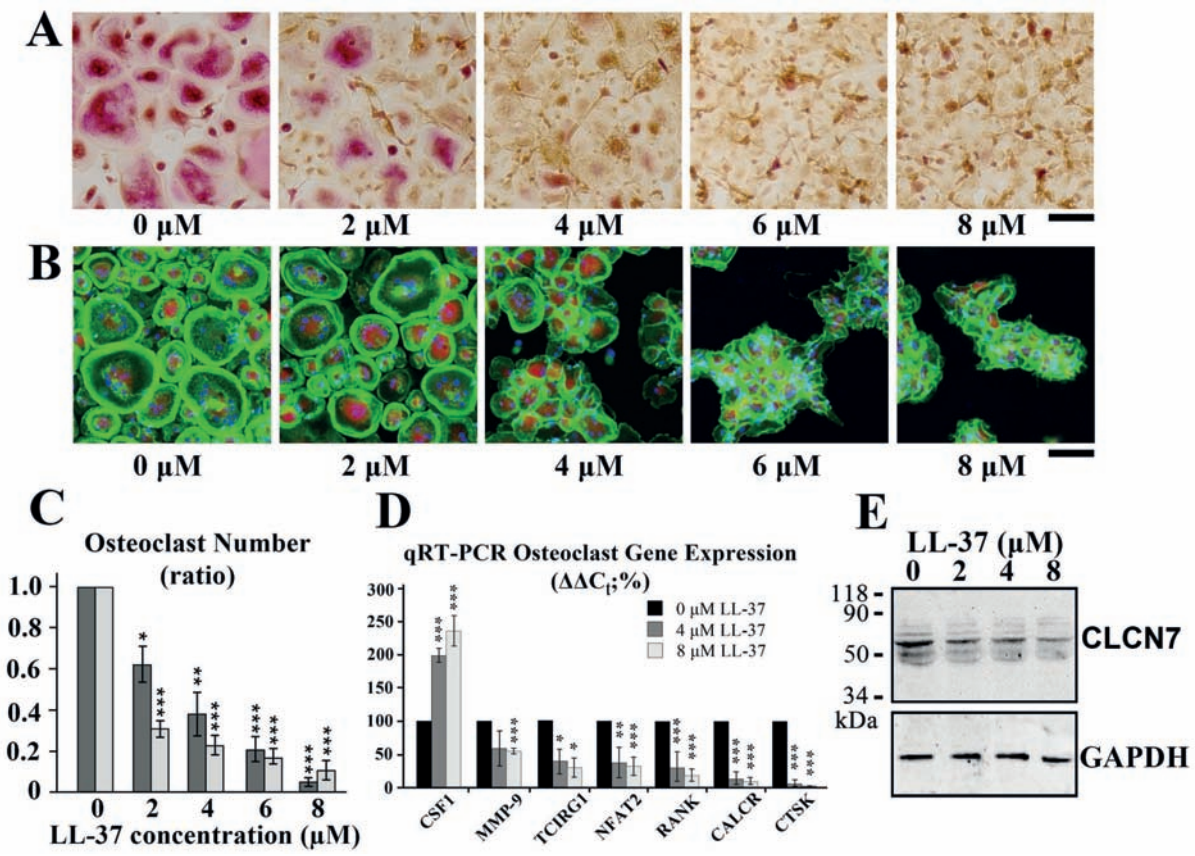

Figure 4 *The inhibition of in vitro osteoclastogenesis at the molecular level*

Peripheral blood mononuclear cells (PBMCs) incubated with M-CSF and RANKL in the presence of indicated doses of LL-37 and observed by

A. TRAcP staining (red) and immunocytochemistry of proliferative cell nuclear antigen (brown)

B. staining with TRAcP (red), F-actin (green), and DAPI (blue). Bar = 100 μm.

*C. The bar graph demonstrates the ratios of multinuclear (dark gray bars) and of mononuclear (light gray bars) TRAcP-positive cells in LL-37-treated samples relative to the untreated sample, whose ratio was set to 1 (error bars = SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).*

*D. The bar graph shows the percentages of CSF1, MMP-9, TCIRG1, NFAT2, RANK, CALCR, and CTSK mRNA expression in LL-37-treated samples (dark gray and light gray bars for 4 and 8 μM of LL-37, respectively) relative to the percentage of gene expression in the untreated sample (black bars), set to 100 % (error bars = SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).*

E. Immunoblot detection of CLCN7 and GAPDH in PBMC lysates during osteoclast induction in the presence of indicated doses of LL-37.

Reproduce from Supanchart and co-workers[73] (DOI:10.1177/0022034512460402).

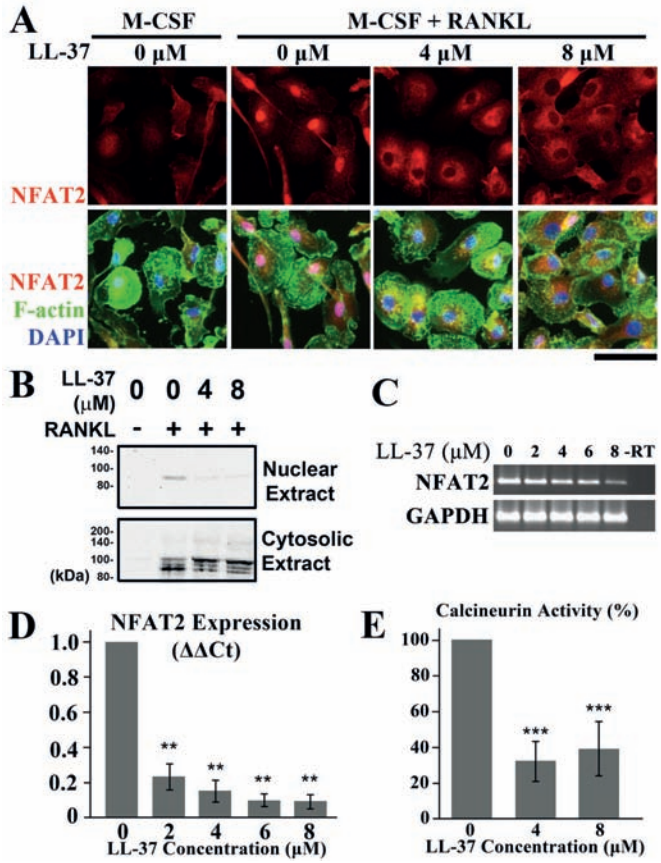

Figure 5 *The blockade of nuclear translocation of NFAT2 by LL-37*

Peripheral blood mononuclear cells (PBMCs) were cultured in osteoclast medium, containing M-CSF with or without RANKL, as a negative control, in the presence of indicated doses of LL-37.

A. Cells were stained with NFAT2 (red), F-actin (green) and DAPI (blue), and observed under a fluorescence microscope. Note weak staining of NFAT2 in the culture without RANKL activation (the leftmost column) and the punctate staining of F-actin. Bar = 50 μm.

B. The nuclear and cytosolic protein fractions were extracted, subjected to immunoblotting, and probed with anti-NFAT2 antibody. Note the absence of bands in the nuclear and the cytosolic extracts of PBMCs without RANKL activation (the leftmost lane), consistent with the finding in A. The presence of several bands in the cytosolic extract may suggest post-translational modification of NFAT2.

C. Total RNA was extracted from PBMCs, cultured in osteoclast medium in the presence of indicated doses of LL-37. –RT is a sample where the reverse transcriptase enzyme was omitted in the reaction.

*D. A real-time PCR analysis of NFAT2 expression was conducted using complementary DNA from C. The bar graph illustrates the relative ratios (ΔΔCt) of LL-37-treated samples to that of the untreated sample, set to 1 (error bars = SD, ** $p < 0.01$).*

*E. The calcineurin activity assay. PBMCs were cultured in osteoclast medium, containing 0, 4, or 8 μM of LL-37, and then lysed. The bar graph shows the percentages of calcineurin activity in LL-37-treated samples relative to that of the untreated sample, set to 100 % (error bars = SD, *** $p < 0.005$).*

Reproduce from Supanchart and co-workers[73] (DOI:10.1177/0022034512460402).

The suppressive effect of LL-37 on osteoclastogenesis discovered in the author's study[73] was later confirmed by a group of Japanese investigators who found that both mCRAMP and LL-37 blocked osteoclast formation in human PBMC cultures, but not in mouse bone marrow macrophage cultures, suggesting distinct responses to LL-37 treatment by two different cell types.[76] Furthermore, mCRAMP inhibits osteoclast formation in mouse osteoblast-osteoclast co-cultures treated with lipopolysaccharide.[76] In addition to the inhibitory effect of LL-37 on osteoclast precursor cells, LL-37 can promote bone regeneration in a rat calvarial bone defect by accelerating angiogenesis and subsequent recruitment of mesenchymal stem cells into the bone defect to induce bone regeneration.[77] Therefore, it is suggested in that study that LL-37 may be clinically applied for a therapeutic use in bone-resorbing disorders in addition to its well-known action as a broad spectrum antibiotic. It is interesting to note that LL-37 not only inhibits osteoclast formation but also triggers the differentiation of human blood-derived monocytes into a novel bone forming cell that can function both *in vitro* and in an animal model of bone injury.[78,79] In summary, all of the findings from the author's laboratory and others unequivocally show an essential role of the cathelicidin peptides, including LL-37 in humans and mCRAMP, in the regulation of bone metabolism in both osteoclasts and osteoblasts.

With respect to the author's future *in vitro* studies, it is interesting to further dissect the molecular mechanisms of LL-37 for the inhibition of osteoclastogenesis, particularly the significance of intracellular calcium rise and candidate receptor(s) for the inhibitory effect of LL-37. It has been shown that extracellular adenosine facilitates fusion of monocytes by activating the purinergic $P2X_7$ receptor.[80] Therefore, the $P2X_7$ receptor may be a potential target for LL-37. Otherwise, TLR9 may be another candidate receptor, since osteoclast formation is blocked by TLR9 ligand,[81] and the author's preliminary result has shown the co-localization between LL-37 and TLR9 in the cytoplasm of monocytes (unpublished observation). Lastly, GAPDH has been shown to be an intracellular receptor for LL-37 in human monocytes,[82] and GAPDH can, in fact, form a complex with inositol 1, 4, 5-triphosphate receptor (IP_3R) to enhance calcium ion release from its storage in the endoplasmic reticulum, leading to an increase in intracellular calcium concentration.[83] Consequently, it is plausible that GAPDH may bind LL-37 and then form a complex with the IP_3R to open up the channel for calcium ion release. This warrants further investigations. Meanwhile, several *in vivo* experiments are going to be conducted to determine the inhibitory effect of LL-37 and mCRAMP on osteoclast formation in animal models.

Conclusion

The main purposes of this invited review article are firstly to introduce a family of small cationic antimicrobial peptides, namely cathelicidins, and their multiple biological functions, critical for several pathophysiological processes in humans, so that readers become familiar with these peptides and their clinical relevance. Secondly, a novel and amazing role of these peptides in bone biology is thoroughly explained for the readers so that they will be more aware of the potential therapeutic uses of these peptides in dentistry, especially for the management of bone-resorbing diseases, like periodontitis. It is very likely that more and more scientific papers and knowledge regarding the role of cathelicidins in bone biology will be distributed in the near future and our main duty is, therefore, to try to catch up with this fast growing field. It is hopeful that this review article will be useful for Thai general dentists and personnel from other medical professions. Finally, the invitation from Journal of the Dental Association of Thailand is greatly appreciated.

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