Front. Biol. 2017, 12(3): 192–198
DOI 10.1007/s11515-017-1442-6

REVIEW

Inhibition and attenuation of pathogenicity of *Porphyromonas gingivalis* by leupeptin: A review

Hansa Jain (✉)

Harsarn Dass Dental College, India

BACKGROUND: *Porphyromonas gingivalis* is a periodontal pathogen, which is considered to be a keystone pathogen for periodontitis. A diverse conglomerate of *P. gingivalis* virulence factors including lipopolysaccharide, fimbriae, capsular polysaccharide, haemagglutinin and cysteine proteases (Arg-gingipains and Lys-gingipain) are considered to be involved in the pathogenesis of periodontitis. Leupeptin is a cysteine protease inhibitor which is specific for Arg gingipains. The present review focuses on action of leupeptin on Arg gingipains.
METHOD: A search was carried out systematically from the start till September, 2016. The search was made in Medline database via PubMed. The keywords enlisted were "leupeptin"; "gingipains"; "periodontitis" using Boolean operator "and."
RESULTS: The result was selection of 58 articles which linked leupeptin to periodontitis and gingipains; pathogenesis of periodontitis, pathogenicity of gingipains and role of leupeptin.
CONCLUSION: It was concluded that leupeptin inhibits and attenuates a number of destructive activities of Arg gingipains including inhibition of platelet aggregation; inhibit degradation of LL-37, which is an antimicrobial peptide; blocking inhibition of monocyte chemoattractant protein; restoring level of interleukin-2; inhibiting degradation of collagen type I and IV to name a few.

Keywords *Porphyromonas gingivalis*, gingipains, leupeptin, cysteine protease inhibitor, periodontitis

Introduction

Periodontitis is a long lasting inflammatory disease of tooth supporting tissues. It leads to gradual degradation of connective tissue and bone enveloping the root portion of the teeth (Islam et al., 2015; Kanakdande et al., 2015). It is primarily a bacterial infection that involves the dental biofilm and dental plaque. It is observed that biofilms causing gingivitis and periodontitis are site-specific and the complex polymicrobial communities are usually resistant to antimicrobial agents and host-defense mechanisms (Marsh, 2005). It has also been observed that some of the low-abundance microbial pathogens cause induction of the inflammation by bringing about transformation of a normally benign microbiota to a dysbiotic organism (Reynolds, 2014).

There are over 700 different species found in oral bacterial microbiome. The subgingival plaque contains about 400 species and almost half of the phytotypes may be present at one point of time in an individual. The periodontopathogens can be segregated into five color complexes including red, orange, yellow, purple and green (Ximenez-Fyvie et al., 2000; Socransky et al., 2000). The microorganisms were subdivided on the basis of closely related species. Red and orange complexes were related to pocket depth (Socransky et al., 2000). They were observed to be higher in subgingival plaque. It was examined that red complex species proportion in subgingival plaque was twice that observed in supragingival plaque whereas orange complex species comprised approximately 18%–28% of the total count in supra and subgingival plaque respectively (Ximenez-Fyvie et al., 2000).

Porphyromonas gingivalis, *Treponema denticola* and *Tannerella forsythia*: the red complex. The red complex is expressed as part of the climax communities in the biofilms present at the sites that express chronic periodontitis. Literature also suggests that two members of the red complex including *P. gingivalis* and *T. denticola* are considered prime candidates for clinical destruction of the periodontium. They have been observed to occur collectively at the sites of periodontal destruction and have been associated

Received September 4, 2016; accepted January 15, 2017
Correspondence: Hansa Jain
E-mail: drhansajain25@gmail.com

topologically in the developing biofilm. Other studies related *P. gingivalis* with *T. forsythia* at different pocket depths in subgingival plaque and even stated that *P. gingivalis* was never detected in the absence of *T. forsythia* (Holt and Ebersole, 2005). Till date surfeit number of studies have stated about the pathogenicity of *P. gingivalis*, the following review focuses on pathogenicity of *P. gingivalis* and role of leupeptin, a protease inhibitor, in impeding the destructive effect of this red complex specie. It is the first review to list various aspects of leupeptin as an inhibitor of virulence of *P. gingivalis*.

Role of *P. gingivalis* in periodontitis

May be due to the reason that *P. gingivalis* was easiest of red complex to grow and manipulate genetically, it was most widely studied (Holt and Ebersole, 2005). It is a gram-negative anaerobic bacterium producing black-brown colonies on blood agar (Scheres et al., 2010). It is rod-shaped, immobile and an asaccharolytic bacterium (Gamboa et al., 2014). *P. gingivalis* is observed to invade the gingival epithelial cells, periodontal ligament fibroblasts and alveolar osteoblasts. The infection caused by it leads to modulation of host-immune inflammatory responses thus disturbing the balance of the normal cell cycle and apoptosis (Scheres et al., 2010). Hajishengallis and Lamont (2012) stated that *P. gingivalis* cause impairment of the innate immunity thus enhancing the growth and causing alteration of the periodontal microbiota. It causes inhibition of gingival interleukin (IL)-8-like chemokines thus delaying the recruitment of neutrophils. This facilitates the initial colonization and promotes breeding of other microorganisms. The immune response is observed to be mediated by extracellular signal-related kinase (ERK), C-Jun NH2-terminal protein kinase and p38 pathways in macrophages (Lv et al., 2015).

Virulence of this periodontopathogen was dependent on a varied number of molecules including colonization factors like fimbriae and hemagglutinins, proteolytic enzymes like gingipains, outer membrane vesicles and lipopolysaccharides (Holt and Ebersole, 2005; Hajishengallis and Lamont, 2012). These factors can initiate an inflammatory cascade which involves reactive oxygen species, proinflammatory cytokines and matrix metalloproteinases (MMP) (Kuula et al., 2009). Fimbriae of *P. gingivalis* are filamentous appendages that intervene the adherence of bacteria to host cells, host macromolecules and other bacteria (Baek et al., 2015). Reactive oxygen species can cause degradation of a number of structurally and metabolically functional macromolecules including free and conjugated proteins, lipids and carbohydrate thus leading to cellular damage (Waddington et al., 2000). Literature suggests absence of proinflammatory cytokines demonstrate decreased bone loss (Baker et al., 1999). Kesavalu et al. stated that the proinflammatory cytokines initiate connective tissue inflammation and alveolar bone resorption (Kesavalu et al., 2002). MMPs also play a pivotal role in bringing about periodontal destruction. They initiate of digestion of type 1 collagen, the most abundant collagen present in periodontium thus leading to destruction of tissues to a major extent (Kuula et al., 2009).

Gingipains

There are three cysteine proteinases including arginine-specific gingipains A and B (RgpA and RgpB) and lysine-specific gingipains (Kgp). Gingipains are considered to be the most eminent virulence factors that contribute to the pathogenesis of periodontal disease (Nakayama et al., 2015). The cysteine proteinases RgpA, RgpB and Kgp have a broad spectrum due to which they play a pivotal role in host colonization, inactivation of host defenses, tissue destruction, and modulation of the host immune system. Along with being crucial in the pathogenic process, gingipains is important in controlling expression of virulence factors, stability and processing of extracellular or cell surface protein of *P. gingivalis* (Grenier et al., 2003).

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is an important regulator of a varied number of host cell signal transductions including proliferation, cell survival, differentiation, metabolism, endocytosis and vesicular trafficking and host inflammatory responses. Nakayama et al. stated that gingipains led to dysfunctioning of PI3K (upstream of Akt) and even brought about changes in Akt signaling pathway during *P. gingivalis* infection thus disarraying cell functions including cell survival and growth, apoptosis, endocytosis, and metabolism. *P. gingivalis* also causes decrease in the phosphorylation downstream proteins GSK3, mTOR and Bad present in the gingival epithelium. The effect on Bad results in apoptosis during *P. gingivalis* infection, there is dysregulation of glucogen synthesis due to effects on GSK3 and disrupted signaling pathway in endocytosis due to effects on mTOR (Nakayama et al., 2015).

It has been observed that Rgps contribute to the modulation of the hemoglobin binding receptor protein domain and the hemagglutinating activity of the hapA gene product. It also processes an immunogenic 75-kDa cell surface protein, prifimbrillin and pro-Kgp (Grenier et al., 2003). Thus, these multidomain gingipains are pivotal in causing hemagglutination, hemolysis, capture and degradation of hemoglobin. They are also instrumental in mediating adherence to extracellular matrix proteins and oral epithelial cells thus enhancing survival and growth of the microorganisms in oral environment (Nadkarni et al., 2014).

Peptidases cause nutrient acquisition, cleavage of host cell surface receptors, signaling via protease-activated receptors and inactivation of cytokines and components of the complement system. Kgp leads to cleavage of human connective tissue and plasma proteins including immunoglobulins, fibronectin, plasma kallikrein, fibrinogen, iron and peptidase inhibitors (de Diego et al., 2014). *P. gingivalis* causes decrease in cytokine response and this process is

mainly carried out by proteases (Choi et al., 2014).

Brief on leupeptin

Leupeptin is a proteases inhibitor, specifically targeting Arg-gingipains (Rubinstein et al., 2001). It is a tripeptide of bacterial origin and it's structure is acetyl-orpropianyl-L-leucyl-L-leucyl-DL-argininal. It's different analogs might have isoleucine or valine instead of two leucine (Maeda et al., 1971; Freeman and Lloyd, 1983). A study stated that leupeptin which was produced in a synthetic medium gave leucine on amino acid analysis whereas leupeptin produced in an organic medium consisted of isoleucine and valine along with leucine (Kondo et al., 1969).

Leupeptin has antiplasmin activity and brings about inhibition of trypsin, papain and kallikrein. It causes inhibition of coagulation of rabbit and human blood. It also inhibits carrageenan inflammation in rats and also exhibit anti-inflammatory activity (Aoyagi et al., 1969a). When leupeptin was administered orally it had an anti-inflammatory effect on edema (Aoyagi et al., 1969b). It also decreases edema forming effects of RgpA and downregulates the increased influx of macromolecules (Rubinstein et al., 2001). Maeda et al. (1971) reported that different analogs of leupeptin might show different biological activities. The leupeptin having terminal carboxylic acid, alcohol or di-n-butyl acetyl group in place of aldehyde had no effect on fibrinolysis by plasmin. Then few analogs were observed to inhibit proteolysis by plasmin more strongly than others.

The aldehyde group of leupeptin which is present at the C-terminal position is considered to be necessary for its potent inhibitory activity. The aldehyde group of other protease inhibitor including antipain, chymostatin, elastatinal were also found to consist of the inhibitory activity like these protease (Kuramochi et al., 1979). It promotes synapse formation and brings about improvement in neuromuscular recovery during nerve trauma (McConnell et al., 1993).

Cysteine proteases are proteolytic enzymes that are involved in the degradation of proteins. They can be subdivided into groups of sequence homology. They can be categorized into three structurally distinct groups: papain-like (clan CA), ICE-like (clan CD) and picornaim-like (clan PACC) (Table 1) (Santos and Moreira, 2007). The cysteine protease inhibitor can be divided into three families, the stefins, the cystatins and kininogens. It has been observed that all of them are stable at high temperature and pH and are specific for their cysteine proteases (Otto and Schirmeister, 1997).

Table 1 Classification of cysteine proteases

Sr. No.	Papain-like (clan CA)	ICE-like (clan CD)	Picornain-like (clan PA(C))
1)	Calpains	Caspases	Human Rhinoviruses
2)	Parasite Cysteine Proteases	Legumain	
3)	Cathepsins	Clostripain	
4)		Gingipains	
5)		Separase	

Virulence of gingipains and role of leupeptin (Table 2)

Gingipains activate protease activity receptors (PAR) which result in platelet aggregation thus linking periodontitis with cardiovascular diseases. In a study, it was observed that leupeptin decreases interaction between epinephrine and *P. gingivalis*. Epinephrine, a circulating hormone participates in platelet activation and even has interaction with *P. gingivalis* thus increasing platelet aggregation. Along with this, leupeptin affects activity of PAR-4 and PAR thus affecting platelet aggregation (Nylander et al., 2008). *P. gingivalis* infection causes increase in the area of atherosclerosis lesions. It along with its outer membrane vesicles can cause aggregation of platelets thus playing a pivotal role in the development of atherosclerotic plaque. *P. gingivalis* even causes stimulation of murine macrophages thus accumulating low density lipoprotein to form foam cells. Leupeptin along with TLCK (another protease inhibitor) was observed to partially inhibit LDL aggregation induced outer membrane vesicles, attenuated the increase in LDL mobility (Miyakawa et al., 2004).

Gingipains inhibit production of monocyte chemoattractant protein-1 (MCP-1) which is one of the most effective chemo-attractants for monocytes and its level increases significantly in gingival crevicular fluid, saliva and serum in patients of chronic periodontitis. It has been observed that leupeptin blocks the inhibition of MCP-1 mRNA expression (Choi et al., 2014). In a study by McCrudden et al. (2013) levels of an antimicrobial peptide LL-37 which is observed to play a therapeutic role in periodontitis where analyzed using leupeptin. It was observed that LL-37 is degraded by cysteine proteases including Arg and Lys gingipains. Leupeptin which is a protease inhibitor also inhibited degradation of LL-37. Thus both leupeptin and LL-37 combined can be used as therapeutic for treating chronic form of periodontitis.

Lamont et al. presented that *P. gingivalis* had the caliber to invade and translocate into the cytosol of gingival epithelial cells thus establishing itself by replicating in host cells and evading the host immune system. It also affects immune system by inhibiting CXCL-8 expression thus impairing immune cell recruitment. Khalaf and Bengtsson (2012) stated that *P. gingivalis* downregulates expression of IL-2 at the protein level. It affects and reduces AP-1 and NF-κB activity below the basal levels. AP-1 on the other hand regulates IL-2 expression. It is also mentioned that this is carried out by gingipains. They observed that leupeptin restored the levels of IL-2 thus regulating the inflammatory response and improving the deteriorated condition of tissue.

Table 2 Role of gingipains and leupeptin in various biological mechanisms

Sr. No.	Biological site or mechanism	Role of gingipains	Role of leupeptin
1)	Platelet aggregation	Activates protease activity receptors resulting in platelet aggregation	Decreases interaction between epinephrine and *Porphyromonas gingivalis* thus decreasing platelet aggregation
2)	Increasing area of atherosclerotic plaque and conversion of low density lipoprotein to foam cells	Stimulates the situation	Inhibits the process
3)	Production of monocyte chemoattractant protein-1	Inhibits the process	Prevents inhibition
4)	Degradation of antimicrobial peptide LL-37	Stimulates the process	Inhibits the process
5)	Reduction of expression of interleukin-2	Stimulates the process	Inhibits the process, restores the level of interleukin-2
6)	Activation of Matrix Metalloproteinases which causes degradation of collagen	Stimulates the process	Inhibits the process
7)	Degradation of laminin, fibronectin, type IV collagen and Matri protein	Stimulates the process	Inhibits the process
8)	Direct Collagen degradation	Stimulates the process	Inhibits the process, along with this, it causes thirty fold increase in the volume fraction of cross-banded collagen fibrils.
9)	Cell detachment	Stimulates the process	Inhibits the process
10)	Inhibition of hydrolysis of α1-antitrypsin, α2-macroglobulin, apotransferrin, benzoyl-L-arginin-ρ-nitroanilide, benzoyl-D3L-arginine-β-naphthylamide and tosyl-L-arginine methyl ester, cleavage of LPS receptor CD14 from the surface of human U937 macrophage like cells	Stimulates the process	Inhibits the process
11)	Degradation of transferrin for acquisition of iron	Stimulates the process	Inhibits the process

MMP as mentioned before are pivotal in causing periodontal destruction, they are endopeptidases family which are dependent on zinc and bring about degradation of multiple extracellular matrix components. The proteinases expressed by *P. gingivalis* can cause activation of latent MMP thus accelerating degradation of extracellular matrix. It has been observed that they can activate latent MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 and catalyze the super-activation of MMP-1 by MMP-3. MMP-8 causes degradation of type I collagen whereas MMP-9 causes denaturation of collagen, mainly type IV collagen which helps in monocyte migration. Zhou et al. carried out a trial and observed that leupeptin led to decrease in migratory activity of monocyte and inhibited activity of cysteine proteinases (Kuula et al., 2009; Zhou et al., 2012).

Andrian et al. stated that leupeptin inhibited degradation of laminin, fibronectin, type IV collagen and Matri protein constituents caused by gingipains. Matri is an in vitro reconstituted basement membrane model (Andrian et al., 2004). Among collagen, type 1 collagen is the predominant collagen present in the periodontal tissue. Gingipains cause degradation of type 1 collagen. In a study inactivation of gingipains completely reduced the capability of *P. gingivalis* collagenase to cause cleavage of type 1 collagen suggesting role of gingipains in bringing about this action. Houle and colleagues reported that leupeptin and other protease inhibitors almost completely inhibited collagen degradation caused by *P. gingivalis* (Houle et al., 2003). Everts et al. (1985) evaluated the digestion of collagen and the role of leupeptin in its inhibition. It was observed that leupeptin caused a 30 fold increase in the volume fraction of cross-banded collagen fibrils contained in lysosomal structure.

In a study by Curtis et al. (2002) Rgp were pretreated with 2 mM leupeptin and it was observed that there was no Rgp activity following the leupeptin treatment. At the concentration of 0.16 and 0.3 μM leupeptin was able to inhibit the activity of Arg gingipain by 27 and 42% respectably. Another study evaluated role of leupeptin on pathogenicity of *P. gingivalis* and stated that it attenuated hydrolysation of interconnecting adherens junction E-cadherin molecules. E-cadherin mediated cell-cell adhesins, which is the major structural component of the adherens junctions (Katz et al., 2002).

Apoptosis is defined as genetically programmed form of cell death. It activates caspases which causes cellular shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation, plasma membrane changes signaling phagolytic update and mitochondrial membrane permeabilization. Literature suggests role of gingipains in endothelial cell-caspase dependent apoptosis. RgpB was observed to have structural similarities with caspase 1 and 3 and it was stated that it gets activated in a similar manner as caspases. In

a study it was observed that pretreatment with leupeptin produced inhibition in cell detachment produced by gingipains (Sheets et al., 2006).

Grenier and colleagues (2001) stated that leupeptin decreased the degradation of serum albumin by resting *P. gingivalis*. It was also observed that leupeptin was more effective than other protease inhibitors tested. Kitano et al. (2001) carried out a clinical trial to evaluate the effects of leupeptin on the suppression of gingival inflammation induced by *P. gingivalis*. They divided the rats into three groups, group A was administered only *P. gingivalis*, group B bacteria along with leupeptin whereas group C was administered leupeptin for 6 weeks after bacterial inoculation. It was observed that leupeptin inhibited degrading effects of *P. gingivalis* on periodontal tissue. It was also noted that leupeptin was more effective in the later stages of the disease.

Arg gingipain and lys gingipain was observed to inhibit expression of class II major histocompatibility complex proteins as a response to the stimulation of endothelial cells with human gamma interferon which plays a pivotal role in the regulation of variety of immune functions. It was observed that addition of leupeptin prohibited the hydrolysis of human gamma interferon, thus decreasing inflammation and periodontitis (Yun et al., 1999).

Lactoferrin is a glycoprotein belonging to innate immune system. It is also found in gingival crevicular fluid and possess a number of anti-microbial properties including being a bactericidal agent. Lactoferrin also inhibits adhesion of periodontopathogen to human plasma protein, connective tissue components, epithelial cells and fibroblasts. In a study it was observed that leupeptin inhibited degradation of lactoferrin caused by *P. gingivalis* (Alugupalli and Kalfas, 1996). As mentioned before *P. gingivalis* possesses fimbriae on its cell surface. When the cultured fibroblasts were treated with the proteases, the binding of the *P. gingivalis* was significantly increased. On addition of leupeptin the enhancing effect was diminished (Kontani et al., 1996).

Leupeptin inhibits hydrolysis of α1-antitrypsin, α2-macroglobulin, apotransferrin, benzoyl-L-arginin-ρ-nitroanilide, benzoyl-D3L-arginine-β-naphthylamide and tosyl-L-arginine methyl ester, cleavage of LPS receptor CD14 from the surface of human U937 macrophage like cells, all of which is caused by proteases (Yoshimura et al., 1984; Bedi and Williams, 1994; Duncan et al., 2004). *P. gingivalis* contains a black haem-pigment which is composed of μ-oxo bishaem of iron (III) protoporphyrin IX (Fe(III)PPIX)2O which is derived from hemoglobin. The (Fe(III)PPIX)2O complex gets deposited on the surface of *P. gingivalis* cells protects it from hydrogen peroxide. It is generated by two routes, first proteolytically released from oxyhemoglobin and deoxyhemoglobin and secondly it is generated from OH bearing haem group derived from methemoglobin which is oxidized form of hemoglobin. Leupeptin inhibits production of methemoglobin resulting in formation of hemoglobin haemichrome (Smalley et al., 2007).

Gingipains even has the capacity to degrade hemoglobin, cleave haptoglobin and transferrin for acquisition of iron which is essential for growth of *P. gingivalis*. Transferrin is reported to be a major source of iron for periodontal pathogens in the subgingival sites. Leupeptin inhibited the growth of *P. gingivalis* when it was grown in a medium containing transferrin as a source of iron but was unable to cause inhibition when hemin was the source of iron (Brochu et al., 2001). Peptidase which is a result of degradation of host proteins by proteases, plays a significant role in the nutrition and sustainability of *P. gingivalis*. One of the peptidase i.e. arginine amino peptidase play a significant role in growth of *P. gingivalis*. It is also useful in the identification of *P. gingivalis* in the clinical samples thus can aid in the diagnosis of periodontal disease. Arginine amino peptidase was observed to be inhibited by leupeptin (Suido et al., 1986; Grenier et al., 2001).

Literature suggests a correlation between bleeding tendency of gingiva and *P. gingivalis* which was suggested to be through fibrinogen as *P. gingivalis* degrades fibrinogen. It makes us conclude that it has fibrinogenolytic and fibrinolytic activities are attributed to gingipains. In a study it was stated that gingipains prolongs thrombim time in a dose dependent manner which was reduced on addition of leupeptin (Imamura et al., 1995).

Leupeptin and treatment

Leupeptin has been used in the treatment of motoneuron degeneration. Kieran and Greensmith exposed rat motoneurons to alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid, leupeptin improved motoneuron survival following exposure thus inhibiting motoneuron cell death and brought about significant improvement in muscle function (Kieran and Greensmith, 2004). In an experiment leupeptin treatment prevented/delayed the onset of muscular dystrophy in mice (Sher et al., 1981). Leupeptin decreases protein degradation in rat skeletal and cardiac muscle and downregulates protein breakdown in denervated rat muscles and in mice suffering from hereditary muscular dystrophy. It was also mentioned that leupeptin can be used therapeutically as it is non-toxic and is absorbed when given orally (Libby and Goldberg, 1978).

Conclusion

There is abundant literature which elaborates on various inhibitory activity of leupeptin on pathogenicity of *P. gingivalis* or to be precise of gingipains. But we lack studies where leupeptin was directly used as a therapeutic agent in the treatment of chronic periodontitis. Therefore there is need for experimental studies evaluating role of leupeptin for treating periodontal inflammation.

Acknowledgements

The author would like to thank Mr. Salil Jain and Mrs. Parul Jain. It was a self-funded research.

Compliance with ethics guidelines

As it is a narrative review there was no requirement of ethical approval or consent letter. There was no reported conflict of interest.

References

Alugupalli K R, Kalfas S (1996). Degradation of lactoferrin by periodontitis-associated bacteria. FEMS Microbiol Lett, 145(2): 209–214

Andrian E, Grenier D, Rouabhia M (2004). *In vitro* models of tissue penetration and destruction by *Porphyromonas gingivalis*. Infect Immun, 72(8): 4689–4698

Aoyagi T, Miyata S, Nanbo M, Kojima F, Matsuzaki M, Ishizuka M, Takeuchi T, Umezawa H (1969a). Biological activities of leupeptins. J Antibiot (Tokyo), 22(11): 558–568

Aoyagi T, Takeuchi T, Matsuzaki A, Kawamura K, Kondo S, Hamada M, Maeda K, Umezawa H (1969b). Leupeptins, new protease inhibitors from Actinomycetes. J Antibiot (Tokyo), 22(6): 283–286

Baek K J, Ji S, Kim Y C, Choi Y (2015). Association of the invasion ability of *Porphyromonas gingivalis* with the severity of periodontitis. Virulence, 6(3): 274–281

Baker P J, Dixon M, Evans R T, Dufour L, Johnson E, Roopenian D C (1999). CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. Infect Immun, 67: 2804–2809

Bedi G S, Williams T (1994). Purification and characterization of a collagen-degrading protease from *Porphyromonas gingivalis*. J Biol Chem, 269: 599–606

Brochu V, Grenier D, Nakayama K, Mayrand D (2001). Acquisition of iron from human transferrin by *Porphyromonas gingivalis*: a role for Arg- and Lys-gingipain activities. Oral Microbiol Immunol, 16(2): 79–87

Choi E K, Kim S Y, Kim S H, Paek Y W, Kang I C (2014). Proteolytic activity of *Porphyromonas gingivalis* attenuates MCP-1 mRNA expression in LPS-stimulated THP-1 cells. Microb Pathog, 73: 13–18

Curtis M A, Aduse Opoku J, Rangarajan M, Gallagher A, Sterne J A, Reid C R, Evans H E, Samuelsson B (2002). Attenuation of the virulence of *Porphyromonas gingivalis* by using a specific synthetic Kgp protease inhibitor. Infect Immun, 70(12): 6968–6975

de Diego I, Veillard F, Sztukowska M N, Guevara T, Potempa B, Pomowski A, Huntington J A, Potempa J, Gomis-Ruth F X (2014). Structure and mechanism of cysteine peptidase gingipain K (Kgp), a major virulence factor of *Porphyromonas gingivalis* in periodontitis. J Biol Chem, 289(46): 32291–32302

Duncan L, Yoshioka M, Chandad F, Grenier D (2004). Loss of lipopolysaccharide receptor CD14 from the surface of human macrophage-like cells mediated by *Porphyromonas gingivalis* outer membrane vesicles. Microb Pathog, 36(6): 319–325

Everts V, Beertsen W, Tigchelaar-Gutter W (1985). The digestion of phagocytosed collagen is inhibited by the proteinase inhibitors leupeptin and E-64. Coll Relat Res, 5(4): 315–336

Freeman S J, Lloyd J B (1983). Inhibition of proteolysis in rat yolk sac as a cause of teratogenesis. Effects of leupeptin *in vitro* and *in vivo*. J Embryol Exp Morphol, 78: 183–193

Gamboa F, Acosta A, Garcia D A, Velosa J, Araya N, Ledergerber R (2014). Occurrence of porphyromonas gingivalis and its antibacterial susceptibility to metronidazole and tetracycline in patients with chronic periodontitis. Acta Odontol Latinoam, 27: 137–144

Grenier D, Gauthier P, Plamondon P, Nakayama K, Mayrand D (2001). Studies on the aminopeptidase activities of *Porphyromonas gingivalis*. Oral Microbiol Immunol, 16(4): 212–217

Grenier D, Imbeault S, Plamondon P, Grenier G, Nakayama K, Mayrand D (2001). Role of gingipains in growth of *Porphyromonas gingivalis* in the presence of human serum albumin. Infect Immun, 69(8): 5166–5172

Grenier D, Roy S, Chandad F, Plamondon P, Yoshioka M, Nakayama K, Mayrand D (2003). Effect of inactivation of the Arg- and/or Lys-gingipain gene on selected virulence and physiological properties of *Porphyromonas gingivalis*. Infect Immun, 71(8): 4742–4748

Hajishengallis G, Lamont R J (2012). Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol, 27(6): 409–419

Holt S C, Ebersole J L (2005). *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototypepoly bacterial pathogenic consortium in periodontitis. Periodontol 2000, 38(1): 72–122

Houle M A, Grenier D, Plamondon P, Nakayama K (2003). The collagenase activity of *Porphyromonas gingivalis* is due to Arg-gingipain. FEMS Microbiol Lett, 221(2): 181–185

Imamura T, Potempa J, Pike R N, Moore J N, Barton M H, Travis J (1995). Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation: implications for bleeding tendency at periodontitis sites. Infect Immun, 63: 4877–4882

Islam S A, Seo M, Lee Y S, Moon S S (2015). Association of periodontitis with insulin resistance, β-cell function, and impaired fasting glucose before onset of diabetes. Endocr J, 2015(62): 981–989

Kanakdande V, Patil K P, Nayyar A S (2015). Comparative evaluation of clinical hematological and systemic inflammatory markers in smokers and non-smokers with chronic periodontitis. Contemp Clin Dent, 6(3): 348–357

Katz J, Yang Q B, Zhang P, Potempa J, Travis J, Michalek S M, Balkovetz D F (2002). Hydrolysis of epithelial junctional proteins by *Porphyromonas gingivalis* gingipains. Infect Immun, 70(5): 2512–2518

Kesavalu L, Chandrasekar B, Ebersole J L (2002). *In vivo* induction of proinflammatory cytokines in mouse tissue by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol, 17(3): 177–180

Khalaf H, Bengtsson T (2012). Altered T-cell responses by the periodontal pathogen *Porphyromonas gingivalis*. PLoS One, 7(9): e45192

Kieran D, Greensmith L (2004). Inhibition of calpains, by treatment with leupeptin, improves motoneuron survival and muscle function in models of motoneuron degeneration. Neuroscience, 125(2): 427–439

Kitano S, Irimura K, Sasaki T, Abe N, Baba A, Miyake Y, Katunuma N, Yamamoto K (2001). Suppression of gingival inflammation induced by *Porphyromonas gingivalis* in rats by leupeptin. Jpn J Pharmacol, 85(1): 84–91

Kondo S I, Kawamura K, Iwanaga J, Hamada M, Aoyagi T, Maeda K, Takeuchi T, Umezawa H (1969). Isolation and characterization of leupeptins produced by Actinomycetes. Chem Pharm Bull (Tokyo), 17(9): 1896–1901

Kontani M, Ono H, Shibata H, Okamura Y, Tanaka T, Fujiwara T, Kimura S, Hamada S (1996). Cysteine protease of *Porphyromonas gingivalis* 381 enhances binding of fimbriae to cultured human fibroblasts and matrix proteins. Infect Immun, 64: 756–762

Kuramochi H, Nakata H, Ishii S (1979). Mechanism of association of a specific aldehyde inhibitor, leupeptin, with bovine trypsin. J Biochem, 86: 1403–1410

Kuula H, Salo T, Pirila E, Tuomainen A M, Jauhiainen M, Uitto V J, Tjaderhane L, Pussinen P J, Sorsa T (2009). Local and systemic responses in matrix metalloproteinase 8-deficient mice during *Porphyromonas gingivalis*-induced periodontitis. Infect Immun, 77 (2): 850–859

Libby P, Goldberg A L (1978). Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. Science, 199 (4328): 534–536

Lv J, Zhu Y X, Liu Y Q, Xue X (2015). Distinctive pathways characterize *A. actinomycetemcomitans* and *P. gingivalis*. Mol Biol Rep, 42(2): 441–449

Maeda K, Kawamura K, Kondo S I, Aoyagi T, Takeuchi T, Umezawa H (1971). The structure and activity of leupeptins and related analogs. J Antibiot (Tokyo), 24(6): 402–404

Marsh P D (2005). Dental plaque: biological significance of a biofilm and community life-style. J Clin Periodontol, 32(s6 Suppl 6): 7–15

McConnell R M, York J L, Frizzell D, Ezell C (1993). Inhibition studies of some serine and thiol proteinases by new leupeptin analogues. J Med Chem, 36(8): 1084–1089

McCrudden M T, Orr D F, Yu Y, Coulter W A, Manning G, Irwin C R, Lundy F T (2013). LL-37 in periodontal health and disease and its susceptibility to degradation by proteinases present in gingival crevicular fluid. J Clin Periodontol, 40(10): 933–941

Miyakawa H, Honma K, Qi M, Kuramitsu H K (2004). Interaction of *Porphyromonas gingivalis* with low-density lipoproteins: implications for a role for periodontitis in atherosclerosis. J Periodontal Res, 39(1): 1–9

Nadkarni M A, Chhour K L, Chapple C C, Nguyen K A, Hunter N (2014). The profile of *Porphyromonas gingivalis* kgp biotype and fimA genotype mosaic in subgingival plaque samples. FEMS Microbiol Lett, 361(2): 190–194

Nakayama M, Inoue T, Naito M, Nakayama K, Ohara N (2015). Attenuation of the phosphatidylinositol 3-kinase/Akt signaling pathway by *Porphyromonas gingivalis* gingipains RgpA, RgpB, and Kgp. J Biol Chem, 290(8): 5190–5202

Nylander M, Lindahl T L, Bengtsson T, Grenegard M (2008). The periodontal pathogen *Porphyromonas gingivalis* sensitises human blood platelets to epinephrine. Platelets, 19(5): 352–358

Otto H H, Schirmeister T (1997). Cysteine Proteases and Their Inhibitors. Chem Rev, 97(1): 133–172

Reynolds M A (2014). Modifiable risk factors in periodontitis: at the intersection of aging and disease. Periodontol 2000, 64(1): 7–19

Rubinstein I, Potempa J, Travis J, Gao X P (2001). Mechanisms mediating *Porphyromonas gingivalis* gingipain RgpA-induced oral mucosa inflammation in vivo. Infect Immun, 69(2): 1199–1201

Santos M M, Moreira R (2007). Michael acceptors as cysteine protease inhibitors. Mini Rev Med Chem, 7(10): 1040–1050

Scheres N, Laine M L, de Vries T J, Everts V, van Winkelhoff A J (2010). Gingival and periodontal ligament fibroblasts differ in their inflammatory response to viable *Porphyromonas gingivalis*. J Periodontal Res, 45(2): 262–270

Sheets S M, Potempa J, Travis J, Fletcher H M, Casiano C A (2006). Gingipains from *Porphyromonas gingivalis* W83 synergistically disrupt endothelial cell adhesion and can induce caspase-independent apoptosis. Infect Immun, 74(10): 5667–5678

Sher J H, Stracher A, Shafiq S A, Hardy-Stashin J (1981). Successful treatment of murine muscular dystrophy with the proteinase inhibitor leupeptin. Proc Natl Acad Sci USA, 78(12): 7742–7744

Smalley J W, Birss A J, Szmigielski B, Potempa J (2007). Sequential action of R- and K-specific gingipains of *Porphyromonas gingivalis* in the generation of the haem-containing pigment from oxyhaemoglobin. Arch Biochem Biophys, 465(1): 44–49

Socransky S S, Haffajee A D, Smith C, Duff G W (2000). Microbiological parameters associated with IL-1 gene polymorphisms in periodontitis patients. J Clin Periodontol, 27(11): 810–818

Suido H, Nakamura M, Mashimo P A, Zambon J J, Genco R J (1986). Arylaminopeptidase activities of oral bacteria. J Dent Res, 65(11): 1335–1340

Waddington R J, Moseley R, Embery G (2000). Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. Oral Dis, 6(3): 138–151

Ximenez-Fyvie L A, Haffajee A D, Socransky S S (2000). Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. J Clin Periodontol, 27(10): 722–732

Yoshimura F, Nishikata M, Suzuki T, Hoover C I, Newbrun E (1984). Characterization of a trypsin-like protease from the bacterium Bacteroides gingivalis isolated from human dental plaque. Arch Oral Biol, 29(7): 559–564

Yun P L, DeCarlo A A, Hunter N (1999). Modulation of major histocompatibility complex protein expression by human gamma interferon mediated by cysteine proteinase-adhesin polyproteins of *Porphyromonas gingivalis*. Infect Immun, 67: 2986–2995

Zhou J, Zhang J, Chao J (2012). *Porphyromonas gingivalis* promotes monocyte migration by activating MMP-9. J Periodontal Res, 47(2): 236–242