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**Research Article**


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# Attenuation of *Porphyromonas gingivali*, an oral infection pathogen by α-amylase and pentamidine

**Dr Atif Amin[1], Dr Muhammad Umar Farooq[2], Alina Ikram[3], Hafiza Sobia Ramzan[4]**

[1]MO RHC 96/12-L Chichawatni, Sahiwal
[2]MO BHU 20/14-L
[3]WMO at RHC Kanjwani
[4]Institute of Molecular Biology and Biotechnology, University of Lahore.
Corresponding author: **Dr Atif Amin,** working at RHC 96/12-L, Tehsil Chichawatni, District Sahiwal.
E-mail: *atifamin8510@gmail.com*

## Abstract

**Purpose:** Oral infections are caused by more than 600 bacterial species; among them the most influential pathogens is *Porphyromonas gingivalis*. In this study, we have implemented a spectrum of antimicrobial activity for these species using a cell growth inhibitor α-amylase, and the bisbenzamidine derivative, pentamidine. **Methods:** The *in vitro* inhibitory activity was investigated with the agar overlay technique. Minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC) were determined. After determining the MBC, we then investigate the differential gene expression by exposing the culture with MBC. Its antimicrobial properties were investigated through microscope analysis, spectrum of activity and general structure. **Results:** The MIC of 6, 100 ng/mL and MBC of 12, 100ng/mL were determined for α-amylase and pentamidine, respectively. Scanning electron microscopy micrograph analysis and bactericidal assay indicated an intriguing possibility that the pentamidine and α-amylase compromises the cell membrane structure of the bacterial cells, which leads to cell death. The positive results with α-amylase and pentamidine led us to explore some mechanisms of action. The expression of the genes encoding hemagglutinins, gingipains, hemin uptake loci, chromosome replication, and energy production was downregulated, while that of the genes related to iron storage and oxidative stress was upregulated by both α-amylase and pentamidine. **Conclusion:** This finding highlights the promising consequence and therapeutic potential of α-amylases and pentamidine. These drugs might be innovative molecules to broaden pharmacological tools for attenuation of oral infections caused by *Porphyromonas gingivalis*.

**Keywords:** *Porphyromonas gingivalis*, antimicrobial, α-amylase, pentamidine

## 1. Introduction

*Porphyromonas gingivalis* (*P. gingivalis*) is a Gram-negative, black-pigmented anaerobe associated with several periodontal diseases (Darveau et al., 1997). The occurrence of periodontitis in over 47% of US population with a prevalence of mild (8.7%), moderate (30%), and severe (8.5%) periodontitis dependents upon oral hygiene, socioeconomic status and other environmental, genetic and metabolic risk factors (Eke et al., 2012). *P. gingivalis* shows a strong positive relationship with diagnostic parameters for periodontitis, including gingival recession, increased sulcular pocket depth and bleeding upon probing (Hutter et al., 2003). In addition, Hajishengallis and colleagues recently demonstrated that although *P. gingivalis* does not independently cause periodontal disease in a germ-free murine model, low numbers of *P. gingivalis* could disrupt host homeostasis through actions requiring both commensal microorganisms and complement, leading to inflammation and periodontal disease (Hajishengallis et al., 2011).

*P. gingivalis* produces multiple virulence factors that allow successful colonization and support evasion of host defenses, many of which contribute to inflammation and destruction of host tissue (Holt et al. 1999). Adhesin molecules (fimbraie, hemagglutinins) promote attachment (Holt et al. 1999, Lamont and Jenkinson 2000) while proteolytic enzymes (cysteine proteinases, hemagglutinins) are capable of degrading multiple substrates in the gingival crevice, facilitating nutrient acquisition and contributing to host tissue degradation (Holt et al., 1999, Lamont and Jenkinson 1998).

Control of oral bacteria is mediated by a diverse array of specific and non-specific innate immune factors present in saliva and on mucosal surfaces (Gorr, 2012). More than 45 antimicrobial proteins and peptides are grouped into functional families that include cationic peptides, metal ion chelators, histatins, defensins, bacterial adhesions and agglutinators, and enzymes directed at the bacterial cell wall. The physiological concentration of most salivary antimicrobial proteins and peptides, however, is lower than the effective concentration in vivo (Gorr, 2012), which suggests that there may be additional immune functions within the saliva.

α-Amylases (E.C.3.2.1.1) are enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose and maltotriose units (Gupta et al., 2003, Rajagopalan and Krishnan 2008). α-Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. Fungal and bacterial amylases could be potentially useful in the pharmaceutical and fine-chemical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries (Gupta et al., 2003, Pandey et al., 2000).

The bisbenzamidine derivative, pentamidine, has been one of the most successful agents against eukaryotic parasites and has been used clinically against trypanosomiasis, leishmananiasis, and Pneumocystis carinii for over 70 years (Burchmore et al., 2002, Wilson et al., 2008). Pentamidine isethionate, discovered to have antiprotozoal activity in 1938, and approved in the United States for the treatment of Pneumocystis carinii pneumonia and other protozoal diseases (Pearson and Hewlett 1985), appears to be an intriguing candidate. In addition to its antiprotozoal activity, pentamidine has been reported to inhibit S100B activity because of its ability to block the interaction at the $Ca^{++}$/p53 site of the protein (Charpentier et al., 2008). Based on this background, the present study was aimed to attenuate *P. gingivalis* mediated oral infection by taking advantage of the antimicrobial activity of α-amylase and pentamidine.

## 2. Materials and Methods

### 2.1. Reagents and chemicals

α-Amylase from porcine pancreas was purchased from Sigma-Aldrich (St. Louis, MO, USA). The bisbenzamidine derivative, Pentamidine was purchased from Sanofi-Aventis (Paris, France). The media (Terrific broth, Luria-Bertani broth) were obtained from Fisher Scientific (Pittsburgh, PA). The modified BacTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) was purchased for the determination of minimum inhibition concentration (MIC). The PCR reagents were obtained from Bio-rad and Invitrogen.

### 2.2. Bacterial culture

*Porphyromonas gingivalis* ATCC 33277 was purchased from the German strain collection.. The cells were cultured in modified Gifu anaerobic medium (GAM) broth (Nissui, Tokyo, Japan), in an aerobic jar (Mitsubishi Gas Chemical, Tokyo, Japan) and in the presence of a deoxygenating reagent (AnaeroPack; Mitsubishi Gas Chemical, Tokyo, Japan) for 48 h at 37°C. Cell concentration was standardized by measuring optical density at 650 nm.

### 2.3.Minimum inhibition and bactericidal concentration assay

MICs were determined for aerobic bacteria as described by Cole et al. (1997), with modifications for each organism as necessary. In brief, the appropriate growth medium for *P.gingivalis* was used to prepare 5-ml overnight cultures to an exponential phase. Bacteria were adjusted to a concentration of $4.5 \times 10^5$ CFU/ml, added to various concentrations of antibiotic in 96-well plates, and incubated at 37°C for a period of 18 to 24 h in an incubator in a humidified container. The MIC was defined as the lowest concentration that prevented 50% growth of cells. Minimum bactericidal concentrations (MBCs) were determined by plating the wells with the half of MIC to double of MIC. After 24 to 48 h of growth, the MBC was determined as the lowest concentration that did not permit visible growth on the surface of the agar. All MIC assays were performed in triplicate.

### 2.4. Scanning electron micrographic examination

*P. gingivalis* cultures were grown to the mid-log phase, and 10 mL of cell suspension ($1 \times 10^4$ cells/mL in modified GAM medium supplemented with α-amylase (12 ng/mL) and pentamidine (100ng/mL), respectively.) was incubated at 37°C for 2 h before collection and fixed in 2.5% glutaraldehyde. The samples were dehydrated with graded ethanol and t-butanol, dried using the critical point method and coated with gold. Cells were observed under a JSM-6510 LV scanning electron microscope (JEOL, Tokyo, Japan).

### 2.5. Determination of differential gene expression

The differential gene expression was determined by quantitative RT-PCR. The bacterial culture (*P. gingivalis*) grown to early exponential phase was adjusted to an OD 600 of 0.1 and divided in half. One half was left untreated, while the other half was treated with α-amylase (12ng/mL) and pentamidine (100ng/mL). After anaerobic incubation for 2 h, the cells were harvested, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with 1 µg of total RNA using the SuperScript II reverse transcriptase (Invitrogen). To identify the expression value of genes related to hemagglutination, hemolysis, proteolysis, hemin uptake, chromosome replication, energy production, and iron storage and oxidative stress, quantitative real-time PCR (QRT-PCR) was performed using specific primers for the selected genes (Table 1). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (gapA) used as control genes in this assay. QRT-PCR was carried out using the MiniOpticon Real-Time PCR Detection System (Bio-Rad) with a reaction mixture containing 10 µl of IQ SYBR Green SuperMix (Bio-Rad), 1 µl of cDNA, and primers to a final concentration of 250 nM in a final volume of 20 µl. To confirm that a single PCR product was amplified, melting curve analysis was performed under the following conditions: 65°C to 95°C, with a heating rate of 0.2°C per s. All quantifications were normalized to the *P. gingivalis* 16S rRNA gene.

### 2.6. Statistical analysis

Statistical analyses were done using SPSS 10.0 (SPSS Inc., U.S.A.) and any differences at $p<0.05$ level were considered as statistically significant. The samples of respective parameters were compared using independent student t-test.

## 3. Results

### 3.1. Minimum inhibition and bactericidal concentration assay

The minimum inhibitory activity against *P. gingivalis* ATCC 33277 cell growth was measured using differential concentrations of α-amylase (2, 4, 6, 8ng/mL) and pentamidine (50, 75, 100, 125ng/mL). The concentration that prevented 50% growth of cells observed for 24 h has been taken as MIC and were determined as 6, 100ng/mL of α-amylase and pentamidine, respectively (Figure 1). The MBC was tested using ± MIC concentration in the cells cultured for 24 to 48 h. The MBC was determined as 12, 100 ng/mL of α-amylase and pentamidine, respectively (Figure 2). Which is the lowest concentration that did not permit visible growth on the surface of the agar, suggesting that *P. gingivalis* cells underwent significant cellular damage.

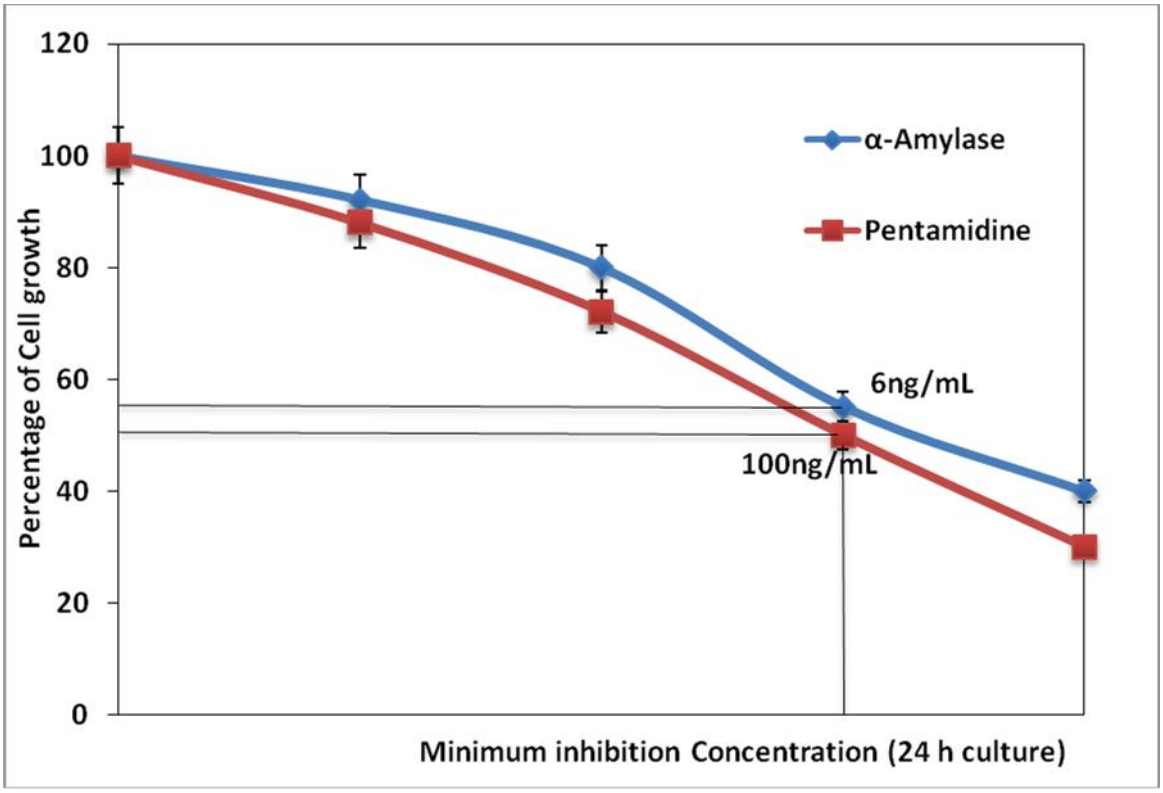


**Figure 1.** The minimum inhibitory concentration required to inhibit 50% growth of *P. gingivalis* was determined as MIC of α-amylase and pentamidine.

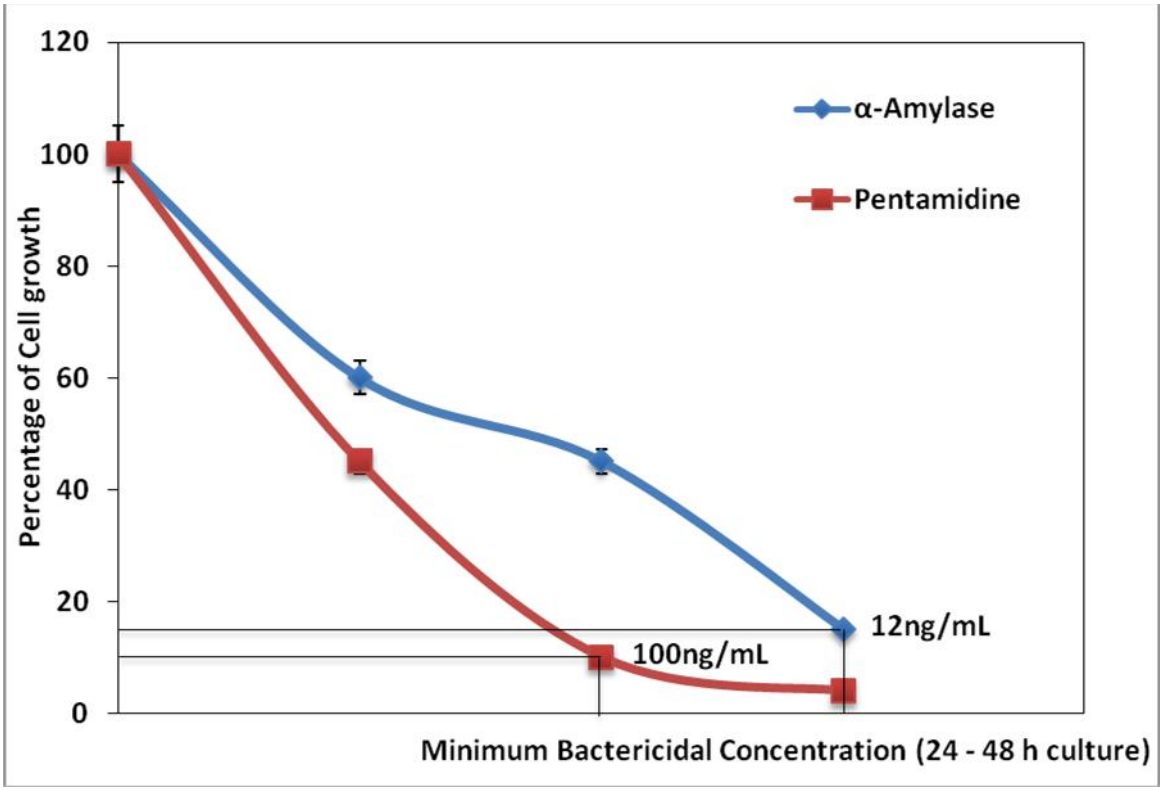


**Figure 2.** The minimum bactericidal concentration required to kill 50% of *P. gingivalis* was determined as MBC of α-amylase and pentamidine.

### 3.2. Scanning electron micrographic examination

Scanning electron micrographs demonstrated that *P. gingivalis* cells treated with α-amylase (Figure 3B) or pentamidine (Figure 3C) showed various stages of lysis. Cellular debris and detached pieces of membrane lay adjacent to the cells. Many cells were distorted with irregular morphology and loss of cellular content. In addition, the cells were more closely aggregated and increased numbers of external blebs were present on and around the bacteria, while compared to that of control (Figure 3B). Similar to α-amylase treated bacteria, pentamidine treated *P. gingivalis* (Figure 3C) also distorted with irregular morphology and was in various stages of lysis with loss of intracellular content. Untreated *P. gingivalis* (Figure 3A) cells exhibited an external structure typical of a healthy Gram-negative coccobacillus7 with multiple blebs present on the cell surface (Figure 3).

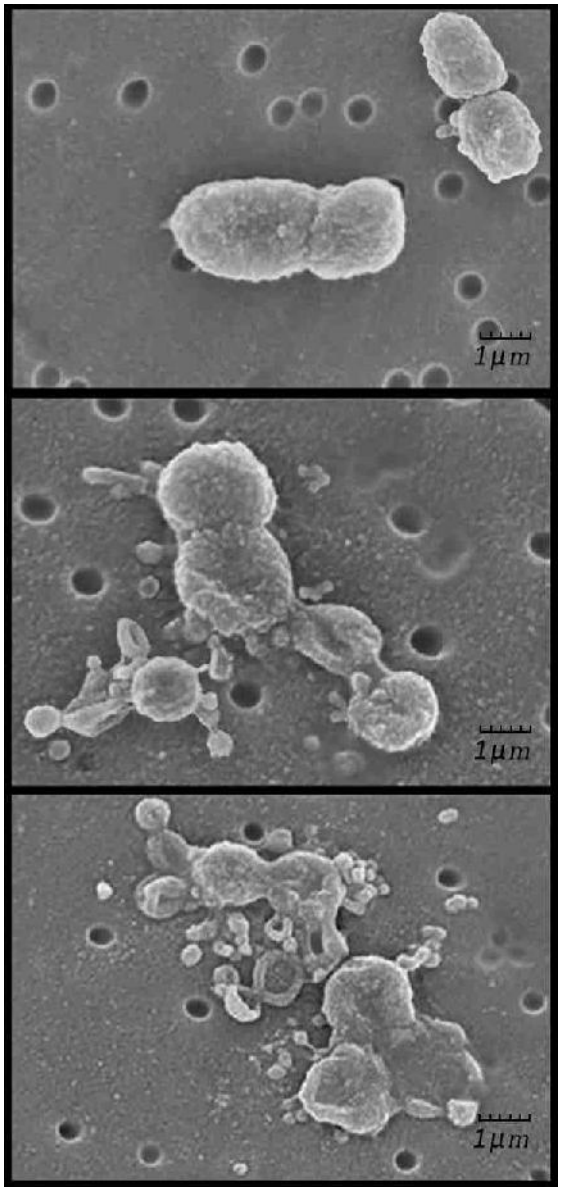


**Figure 3.** Scanning electron microscopy images showing the effects of α-amylase and pentamidine treatments on *P. gingivalis*. Untreated cells (A) exhibit morphology typical of *P. gingivalis* Gram-negative coccobacilli. 1 h treatments of *P. gingivalis* with α-amylase (B), pentamidine (C) resulted in evidence of cellular distortion relative to the untreated bacterium with aggregation of cells and lysis, some with detached pieces of membrane lying adjacent to the cells.

### 3.3. Determination of differential gene expression

After determining the MBC, we then investigate the differential gene expression by exposing the culture with MBC. Expression of the genes related to iron storage and oxidative stress (Dps, rubrerythrin, ferritin, and superoxide dismutase genes) was upregulated, while that of the genes encoding gingipains (RgpA and RgpB) and hemagglutinins (HagA and HagB) were down regulated by 0.03% poly-P75 (Figure 4). Expression of the genes related to energy production (atpA and cydA) and chromosome replication (dnaG) was also down regulated by α-amylase and pentamidine. The expression levels of gapA selected as control genes was not significantly affected. The higher level of expression was found in pentamidine treatment while compare to that of α-amylase (Figure 4).

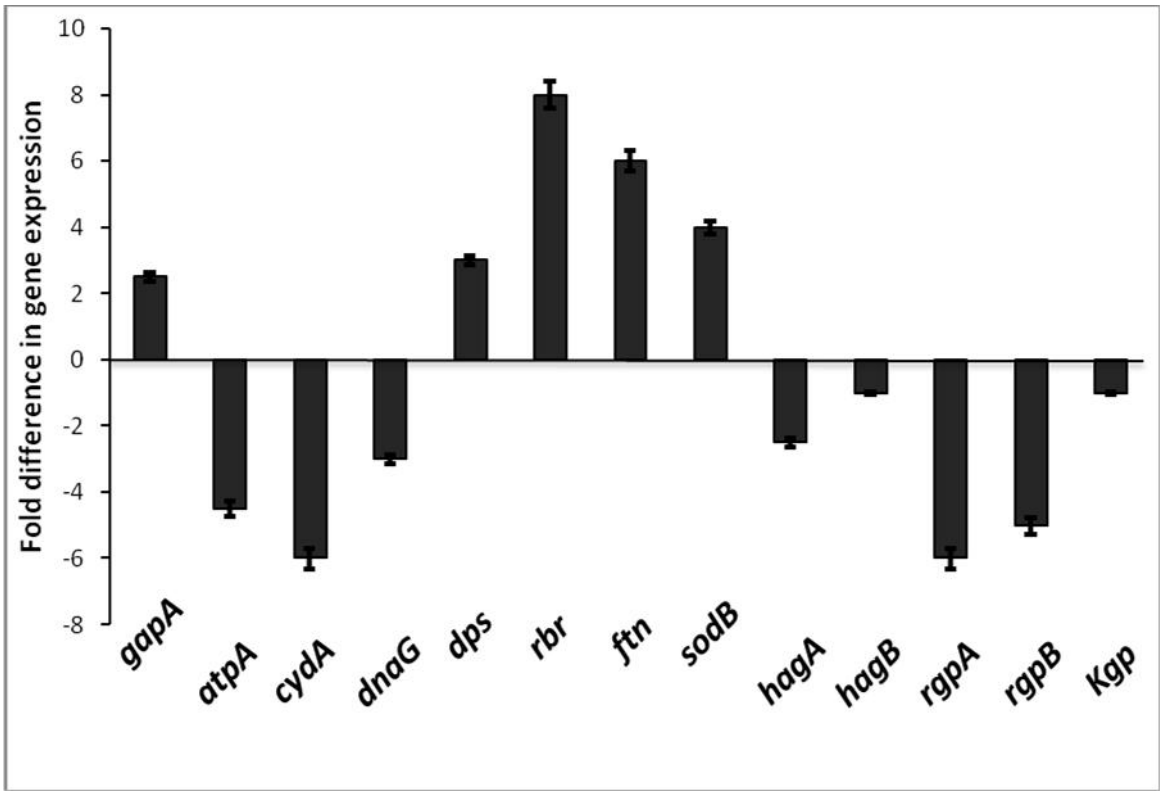


**Figure 4.** Expression levels of the genes related to hemin uptake, hemagglutination, hemolysis, proteolysis, energy production, chromosome replication, and iron storage and oxidative stress in the presence of 0.03% poly-P75. galE, rplD, and gapA were used as control genes. Gene expression was measured by QRT-PCR and normalized to that of the 16S rRNA gene. The expression level of each gene in the absence of inhibitors (α-amylase and pentamidine) was set as 1-fold. The results are presented as the mean SEM of three independent experiments.

## 4. Discussion

In this study, we have investigated two potent inhibitors (α-amylase and pentamidine) against *P. gingivalis* species. The amylases endogenous to saliva and oral mucosa are antimicrobial for *P. gingivalis* and induce structural damage. α-Amylase and pentamidine showed significant inhibitory activity against *P. gingivalis* cell growth with MICs of 6 and 100 ng/mL, respectively. Similarly, the MBC of α-amylase and pentamidine were determined as 12 and 100 ng/mL, respectively. SEM micrograph analysis suggested that the cell membrane structure of bacterial cells was compromised, likely resulting from damage caused by the inhibitors. However, the nature and mechanism of action of the inhibitor is unclear.

Our results are in agreement with growing evidence that pentamidine differentially killing bacteria in a dose-dependent manner and induce cellular damage. For example, E. coli and S. aureus treated with sphingosine, phytosphingosine, or dihydrosphingosine exhibit extensive and differential intracellular and extracellular damage (Fischer et al. 2013). Bibel and colleagues (Bibel et al. 1993) also showed that sphinganine(e.g. dihydrosphingosine) treatment of

*S. aureus* results in ultrastructural damage similar to antibiotic treatment, including lesions of the cell wall, membrane evaginations, and leakage. In addition, treatment of Helicobacter pylori with oleic or linoleic acid exhibits altered morphology with disruption of cellular membranes and cell lysis (Khulusi et al. 1995).

Our work indicates that there may be different mechanisms involved for the activity of different inhibitors. Antimicrobial activity, and ultrastructural damage are all dependent upon the specific lipid treatment. These data, combined with our observation that fatty acids and sphingoid bases exhibit differential activity across bacterial species (Fischer et al. 2012), lead us to believe that the antimicrobial activity of fatty acids and sphingoid bases is a specific interaction that depends upon characteristics of both the bacterium and a particular lipid. We had reported, the mechanisms for the antimicrobial activity of α-amylase and pentamidine against bacteria as membrane disruption by detergent activity and incorporation of lipids into the bacterial plasma membrane.

The surface-accumulated hemin is transported into a bacterial cell to be utilized. To evaluate the effect of α-Amylase and pentamidine on active uptake of hemin by *P. gingivalis*, the selected genes involved in the hemin uptake system, hmuY, hmuR, ihtB and tlr, were downregulated by α-Amylase and pentamidine. Interestingly, unlike DFO, pentamidine increased the amount of cell-associated hemin, which suggests that surplus hemin is accumulated on the bacterial cell surface regardless of energy-driven transport in the presence of pentamidine. The formation of μ-oxo bisheme represents an oxidative buffer mechanism for inducing an anaerobic miroenvironment and protects from hemin-mediated cell damage (Lewis et al., 1999, Smalley et al., 2006). Therefore, excessive accumulation of hemin in the vicinity of the bacterial cell surface without formation of μ-oxo bisheme by the bacterium may cause oxidative stress on *P. gingivalis*. This expectation was confirmed by our QRT-PCR, in which upregulation of the genes involved in oxidative stress, such as dps, rbr, ftn, and sodB, was observed (Figure 4). An oxidative-stress-like phenomenon is one of the shared downstream events leading to bacterial cell death initiated by bactericidal antibiotics (Wright, 2007). Moreover, during bacterial cell death, genes for energy production, chromosome replication, and nucleotide metabolism are inactivated (Asakura and Kobayashi, 2009). Hence, our observation of the oxidative-stress-like response of *P. gingivalis* and depressed expression of the genes for ATP synthesis and chromosome replication of the bacterium grown with α-Amylase and pentamidine may also support the idea that these inhibitors has a bactericidal effect.

This study conclude that using the growth inhibitors α-Amylase and pentamidine for controlling bacterial infection and aiding the innate immune system, as well as promoting gene expressions, seems a attractive strategy for the prevention and treatment of oral cavity infection. Comparing these two inhibitors, pentamidine showed more effective against *P. gingivalis* growth. However, further investigation is required to study its effectiveness in clinical trial.

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