Attenuation of *Porphyromonas gingivalis* oral infection by α-amylase and pentamidine

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Abstract. The *Porphyromonas gingivalis* bacterium is one of the most influential pathogens in oral infections. In the current study, the antimicrobial activity of α-amylase and pentamidine against *Porphyromonas gingivalis* was evaluated. Their *in vitro* inhibitory activity was investigated with the agar overlay technique, and the minimal inhibitory and bactericidal concentrations were determined. Using the bactericidal concentration, the antimicrobial actions of the inhibitors were investigated. In the present study, multiple techniques were utilized, including scanning electron microscopy (SEM), general structural analysis and differential gene expression analysis. The results obtained from SEM and bactericidal analysis indicated a notable observation; the pentamidine and α-amylase treatment destroyed the structure of the bacterial cell membranes, which led to cell death. These results were used to further explore these inhibitors and the mechanisms by which they act. Downregulated expression levels were observed for a number of genes coding for hemagglutinins and gingipains, and various genes involved in hemin uptake, chromosome replication and energy production. However, the expression levels of genes associated with iron storage and oxidative stress were upregulated by α-amylase and pentamidine. A greater effect was noted in response to pentamidine treatment. The results of the present study demonstrate promising therapeutic potential for α-amylases and pentamidine. These molecules have the potential to be used to develop novel drugs and broaden the availability of pharmacological tools for the attenuation of oral infections caused by *Porphyromonas gingivalis*.

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Introduction

Porphyromonas gingivalis (*P. gingivalis*) is a Gram-negative, rod-shaped, anaerobic pathogenic bacterium associated with several periodontal diseases (1). The occurrence of periodontitis in >47% of the US population, with a high prevalence of mild (8.7%), moderate (30%) and severe (8.5%) cases, is due to variables, such as oral hygiene, socioeconomic status and other environmental, genetic and metabolic risk factors (2). *P. gingivalis* exhibits a strong positive association with the diagnostic parameters of periodontitis, including gingival recession, increased sulcular pocket depth and bleeding upon probing (3). In addition, Hajishengallis *et al* (4) demonstrated that although *P. gingivalis* does not independently cause periodontal disease in a germ-free murine model, low numbers of *P. gingivalis* are able to disrupt host homeostasis through actions involving commensal microorganisms and complement, leading to inflammation and periodontal disease (4).

P. gingivalis produces multiple virulence factors that allow successful colonization and support evasion of host defenses, a number of which contribute to the inflammation and destruction of host tissues (5). Adhesins, such as fimbraie and hemagglutinins, promote attachment (5,6) and proteolytic enzymes, such as cysteine proteinases and hemagglutinins, are capable of degrading multiple substrates in the gingival crevice, facilitating nutrient acquisition and contributing to host tissue degradation (5,6).

The control of oral bacteria is mediated by a diverse array of specific and non-specific innate immune molecules present in saliva and on mucosal surfaces (7). There are number of functional families consisting of >45 antimicrobial proteins and peptides, including cationic peptides, metal ion chelators, histatins, defensins, bacterial adhesions and agglutinators and enzymes directed at the bacterial cell wall. However, the physiological concentration of the majority of salivary antimicrobial proteins and peptides is lower than the effective concentration *in vivo* (7), which suggests that there may be additional immune functions within the saliva.

The enzyme α-amylase catalyzes the hydrolysis of internal α-1,4-glycosidic linkages within carbohydrate moieties, including glucose, maltose and maltotriose units (8,9). α-amylases are used in a number of industrial processes in the food, fermentation, textiles, paper, detergent and pharmaceutical industries. Fungal and bacterial amylases may have

the potential for use in the pharmaceutical and fine-chemical industries. Advances in biotechnology have led to the expansion of amylase application in numerous fields, such as biomedical and analytical chemistry, and also textiles, food, brewing and distilling industries (8,10).

The bisbenzamidine derivative, pentamidine, has proved one of the most successful agents for targeting eukaryotic parasites, and has been used clinically for >70 years (11,12). In 1938, pentamidine isethionate was identified to have antiprotozoal activity, and was approved in the United States for the treatment of *Pneumocystis carinii* pneumonia and other protozoal diseases (13). Pentamidine has the ability to inhibit interaction at the Ca^{2+}/p53 site of the protein, and has been reported to inhibit S100B activity (14). On the basis of the previous studies, the present study aimed to take advantage of the antimicrobial activity of α-amylase and pentamidine to attenuate oral infection of *P. gingivalis*.

Materials and methods

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 S.A. (Paris, France). The media (Terrific broth and Luria-Bertani broth) were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). The modified BacTiter-Glo Microbial Cell Viability Assay kit (Promega Corporation, Madison, WI, USA) was purchased for the determination of minimum inhibition concentration (MIC). The polymerase chain reaction (PCR) reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA), and Invitrogen Life Technologies (Carlsbad, CA, USA).

Bacterial culture. *Porphyromonas gingivalis* ATCC 33277 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). The cells were cultured in modified Gifu anaerobic medium (GAM) broth (Nissui, Tokyo, Japan), in an aerobic jar and in the presence of a deoxygenating reagent (AnaeroPack; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) for 48 h at 37˚C. Cell concentration was standardized by measuring optical density at 650 nm using a Lumetron colorimeter (Photovolt Corp., Indianapolis, IN, USA).

MIC and minimum bactericidal concentration (MBC) assay. MICs were determined with modifications for each organism according to methods described by Cole *et al* (15). 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 $4.5x10^5$ colony-forming units/ml, added to various concentrations of antibiotic in 96-well plates, and incubated at 37˚C for a period of 18-24 h in a humidified container. The MIC was defined as the lowest concentration that prevented 50% growth of cells. MBCs were determined by plating the wells with concentrations of 50-200% MIC. Following 24-48-h 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.

Scanning electron microscopy (SEM). *P. gingivalis* cultures were grown to the mid-log phase, and 10 ml cell suspension [$1x10^4$ cells/ml in modified GAM supplemented with α-amylase (12 ng/ml) or pentamidine (100 ng/ml)] was incubated at 37˚C for 2 h prior to 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, Ltd., Tokyo, Japan).

Determination of differential gene expression. The differential gene expression was determined by quantitative (q) PCR. The bacterial culture (*P. gingivalis*) grown to early exponential phase was adjusted to an optical density 600 of 0.1 and split into two groups. One half was left untreated, while the other half was treated with α-amylase (12 ng/ml) and pentamidine (100 ng/ml). Following anaerobic incubation for 2 h, the cells were harvested, and total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA was synthesized with 1 μg total RNA using the SuperScript II reverse transcriptase (Invitrogen Life Technologies). To identify the expression value of genes associated with hemagglutination, hemolysis, proteolysis, hemin uptake, chromosome replication, energy production, iron storage and oxidative stress, qPCR was performed using specific primers for the selected genes (Table I). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (gapA) was used as a control gene. qPCR was conducted using the MiniOpticon Real-Time PCR Detection system (Bio-Rad Laboratories) with a reaction mixture containing 10 μl iQ SYBR Green Supermix (Bio-Rad Laboratories), 1 μl 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, a melting curve analysis was performed under the following conditions: 65˚C to 95˚C, with a heating rate of 0.2˚C/sec. All quantifications were normalized to the *P. gingivalis* 16S ribosomal RNA gene.

Results

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 and 8 ng/ml) and pentamidine (50, 75, 100 and 125 ng/ml). The concentrations that prevented 50% growth of cells observed for 24 h were considered to be the MIC and were determined as 6 and 100 ng/ml α-amylase and pentamidine, respectively (Fig. 1). The MBC was tested using concentrations of 50-200% MIC and cells were cultured for 24-48 h. The MBCs were determined as 12 and 100 ng/ml for α-amylase and pentamidine, respectively (Fig. 2). This 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.

SEM analysis. SEM results demonstrated that *P. gingivalis* cells treated with α-amylase (Fig. 3A) or pentamidine (Fig. 3B) exhibited various stages of lysis. Cellular debris and detached pieces of membrane lay adjacent to the cells. A number of cells were distorted with irregular morphology and loss of cellular

Table I. Primer sequences used in the current study.

Target gene	Gene identification	Primer sequence (5'-3')
16S ribosomal RNA		F:TGTTACAATGGGAGGGACAAAGGG
		R:TTACTAGCGAATCCAGCTTCACGG
gapA	GAPDH, type I	F:GGCAAACTGACGGGTATGTC
		R:ATGAAGTCGGAGGAAACCAC
atpA	ATP synthase subunit A	F:ATCAGGACGGGAAAGACCAC
		R:ACGATGGGGTTGAAAGTGTC
cydA	Cytochrome d ubiquinol oxidase, subunit I	F:TGGATTCTTATCGCCAATGC
		R:ATACGCCCAAAGCAAATACG
dnaG	DNA primase	F:GACACAGGGCTTTCCATCC
		R:GCGAGCAATCTCTTTCTTGG
dps	Dps family protein	F:CAGAAGTGAAGGAAGAGCACGAA
		R:GTAGGCAGACAGCATCCAAACG
rbr	Rubrerythrin	F:TCCACGGCTGAGAACTTGCG
		R:TGCTCGGCTTCCACCTTTGC
ftn	Ferritin	F:CGTGGCGGCGAGGTGAAG
		R:CGGAAGCAGCCCTTACGACAG
sodB	Superoxide dismutase, Fe-Mn	F:GCCAAACCCTCAACCACAATCTC
		R:GCCATACCCAGCCCGAACC
hagA	Hemagglutinin protein HagA	F:ACAGCATCAGCCGATATTCC
		R:CGAATTCATTGCCACCTTCT
hagB	Hemagglutinin protein HagB	F:TGTCACTTGACACTGCTACCAA
		R:ATTCAGAGCCAAATCCTCCA
rgpA	Arginine-specific cysteine proteinase	F:GCCGAGATTGTTCTTGAAGC
		R:AGGAGCAGCAATTGCAAAGT
rgpB	Arginine-specific cysteine proteinase	F:CGCTGATGAAACGAACTTGA
		R:CTTCGAATACCATGCGGTTT
kgp	Lysine-specific cysteine proteinase	F:GCTTGATGCTCCGACTACTC
		R:GCACAGCAATCAACTTCCTAAC

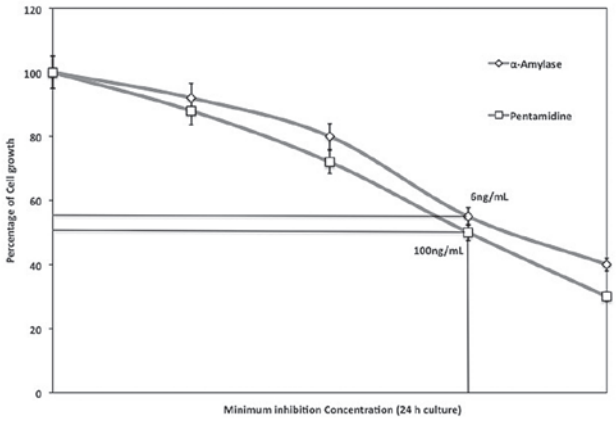

Figure 1. Concentration required to inhibit 50% growth of *P. gingivalis* indicated the minimum inhibitory concentration of α-amylase and pentamidine treatment.

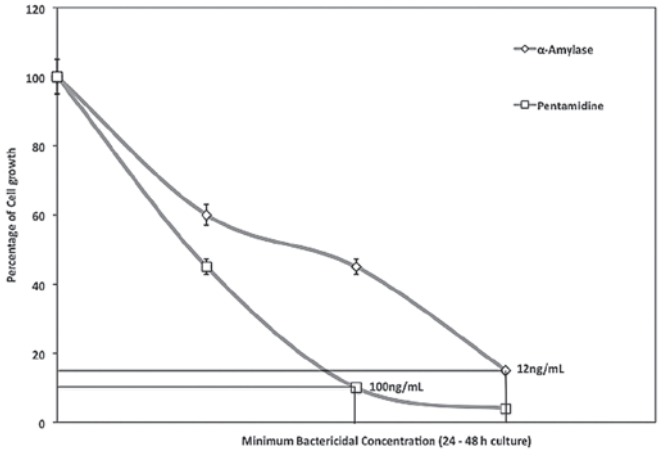

Figure 2. Concentration required to kill 50% *P. gingivalis* was denoted as the minimum bacterial concentration of α-amylase and pentamidine treatment.

content. In addition, the cells were more closely aggregated and increased numbers of external blebs were present on and around the bacteria compared with those in controls (Fig. 3A). Similar to α-amylase-treated bacteria, pentamidine-treated *P. gingivalis* (Fig. 3B) was also distorted with irregular morphology and presented various stages of lysis with loss

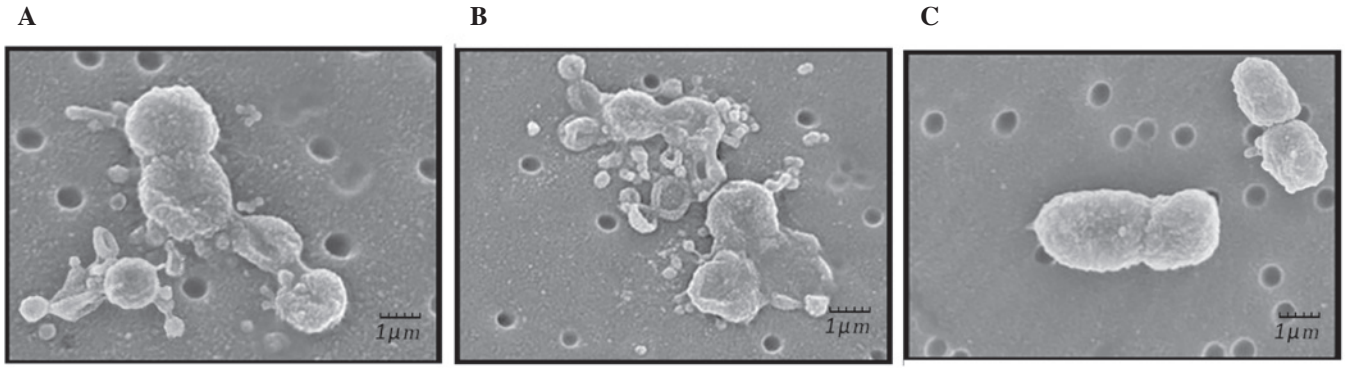

Figure 3. Scanning electron microscopy images presenting the effects of α-amylase and pentamidine on *P. gingivalis*. Treatment of *P. gingivalis* with (A) α-amylase or (B) pentamidine for 1 h resulted in evidence of cellular distortion with aggregation of cells and lysis, and detached pieces of membrane lying adjacent to the cells. (C) Untreated cells exhibited a morphology typical of healthy *P. gingivalis* Gram-negative coccobacilli.

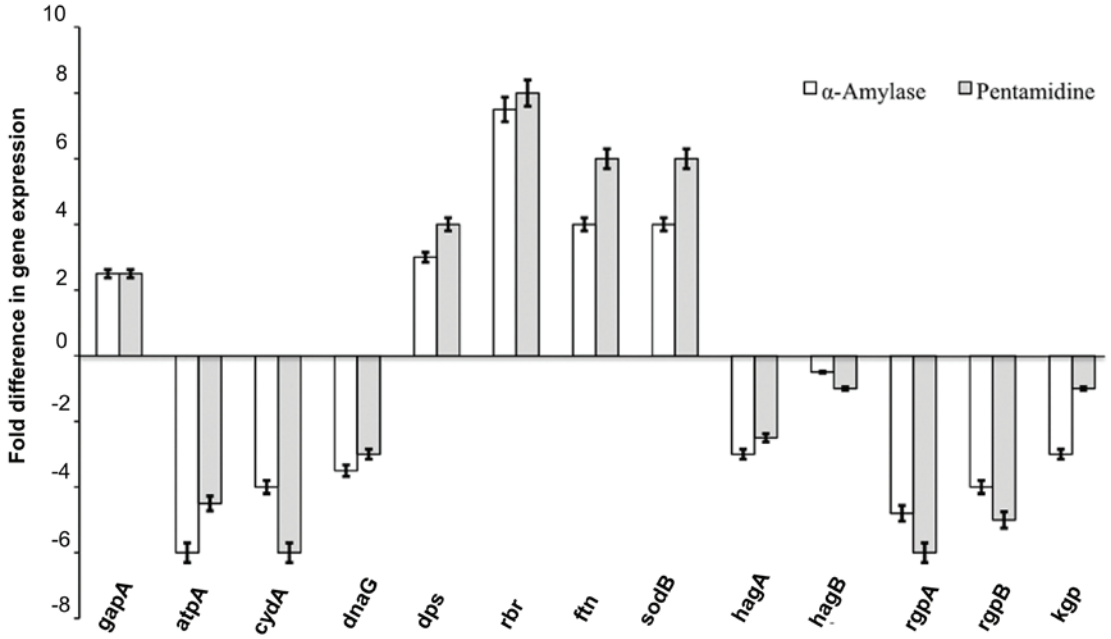

Figure 4. Expression levels of genes associated with hemin uptake, hemagglutination, hemolysis, proteolysis, energy production, chromosome replication, iron storage and oxidative stress. GapA was used as the control gene. Gene expression was measured by quantitative-polymerase chain reaction and normalized to that of the 16S ribosomal RNA 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 ± standard error of three independent experiments.

of intracellular content. Untreated *P. gingivalis* (Fig. 3C) cells exhibited an external structure typical of a healthy Gram-negative coccobacillus 7 bacterium with multiple blebs present on the cell surface.

Determination of differential gene expression. Following determination of the MBC, differential gene expression was investigated by exposing the culture to the MBC. The genes associated with iron storage (dps, rbr and ftn) and oxidative stress (sodB) were indicated to have upregulated expression levels, while levels of genes encoding gingipains (rgpA, rgpB and kgp) and hemagglutinins (hagA and hagB) were downregulated by α-amylase and pentamidine (Fig. 4). The inhibitors also downregulated the expression of genes associated with energy production (atpA and cydA) and chromosome replication (dnaG). The expression levels of the control gene gapA were not significantly affected.

Discussion

In the current study, two potent inhibitors of the *P. gingivalis* species (α-amylase and pentamidine) were investigated. These amylases, which are endogenous to saliva and oral mucosa are antimicrobial for *P. gingivalis*, and induce structural damage. α-amylase and pentamidine demonstrated significant inhibitory activity against *P. gingivalis* cell growth, and had MICs of 6 and 100 ng/ml, respectively. Similarly, the MBCs of α-amylase and pentamidine were determined as 12 and 100 ng/ml, respectively. SEM analysis suggested that the cell membrane structure of bacterial cells was compromised, likely

resulting from damage caused by the inhibitors. However, the nature and mechanisms of action of the inhibitors remain unclear.

The results of the present study are in agreement with growing evidence that pentamidine kills bacteria in a dose-dependent manner and induces cellular damage. For example, *E. coli* and *S. aureus* treated with sphingosine, phytosphingosine or dihydrosphingosine exhibit extensive and differential intracellular and extracellular damage (16). Bibel *et al* (17) also demonstrated that sphinganine (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 results in altered morphology, with a disruption of cellular membranes and cell lysis (18). The present study indicates that there may be different mechanisms underlying the actions of different inhibitors. Antimicrobial activity and ultrastructural damage are dependent upon the specific lipid treatment. These data, combined with a previous observation that fatty acids and sphingoid bases exhibit differential activity across bacterial species (19), suggest that the antimicrobial activity of fatty acids and sphingoid bases is a specific interaction that depends upon characteristics of the bacterium and a particular lipid. The current study indicates that the mechanisms for the antimicrobial activity of α-amylase and pentamidine against bacteria involve membrane disruption by detergent activity and incorporation of lipids into the bacterial plasma membrane.

Surface-accumulated hemin is transported into bacterial cells so that it can be utilized. To evaluate the effect of α-amylase and pentamidine on *P. gingivalis*, the expression levels of selected genes were analyzed to determine whether they were up- or downregulated. The genes associated with iron storage (dps, rbr and ftn) and oxidative stress (sodB) presented upregulated expression levels. Notably, pentamidine increased the level 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. A small decrease in the level of kgp was observed, the suppressed formation of μ-oxo bisheme may be explained by the fact that RgpA or RgpB, or the two together, with kgp activity are required by *P. gingivalis* to produce μ-oxo bisheme (20). The formation of μ-oxo bisheme represents an oxidative buffer mechanism for inducing an anaerobic miroenvironment and protects from hemin-mediated cell damage (20,21). 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 qPCR, which indicated upregulation of the genes involved in oxidative stress, such as dps, rbr, ftn and sodB. An oxidative-stress-like phenomenon is one of the shared downstream events leading to bacterial cell death initiated by bactericidal antibiotics (22). Additionally, during bacterial cell death, genes for energy production, chromosome replication and nucleotide metabolism have been demonstrated to be inactivated (23). Therefore, the observation of the oxidative-stress-like response of *P. gingivalis* and decreased expression of the genes required for ATP synthesis and chromosome replication of the bacterium grown with α-amylase and pentamidine in the current study may also support the idea that these inhibitors have a bactericidal effect.

In conclusion, the present study demonstrated that the use of the growth inhibitors α-amylase and pentamidine for controlling bacterial infection and aiding the innate immune system, in addition to promoting gene expression, may be an effective strategy for the prevention and treatment of oral cavity infection. Following comparison of these two inhibitors, pentamidine was demonstrated to be more effective at inhibiting the growth of *P. gingivalis* cells. However, further investigation is required to investigate its suitability for use in humans.

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