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# Inhibitors of benzamidine type influence the virulence properties of *Porphyromonas gingivalis* strains★

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**Synthetic inhibitors of benzamidine type have been found to have inhibiting effects on arginine specific cysteine proteinases of *P. gingivalis*. The purpose of our study was to assess the effects of these inhibitors on the virulence properties of two *P. gingivalis* strains, the reference strain ATCC 33277 and JH16-1, a clinical isolate obtained from a patient with severe periodontitis. The inhibitors tested were pentamidine, benzamidine, three bis-benzamidine derivatives with a pentamidine-related structure, one bis-benzamidine derivative with another structure, and one arginine derivative as a negative control, each in the concentrations of 2 $\mu$M and 20 $\mu$M. As virulence criteria the following parameters were determined: arginine-specific amidolytic activity, growth inhibition, hemagglutination of sheep erythrocytes, adherence to KB cells and immuno-phagocytosis including intracellular killing. Pentamidine and the bis-benzamidine derivatives with pentamidine-related structure showed the most remarkable effects on reduction of amidolytic activity by 35%, growth inhibition and reduced hemagglutination. Except for the arginine derivative all other inhibitors tested enhanced the phagocytosis capacities of granulocytes. No clear influence of the inhibitors on adherence of *P. gingivalis* to KB cells was seen. Although *in vitro* effects of the synthetic inhibitors of cysteine proteinases on virulence of *P. gingivalis* were observed further *in vitro* tests concerning immunomodulatory effects should be done before these substances are used for therapy in clinically controlled studies.**

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**Abbreviations**: BA*p*NA, *N*-$\alpha$-benzoyl-DL-arginine-*p*-nitroanilide; FBS, foetal bovine serum; MTT, Thiazolyl Blue; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes.

*Porphyromonas gingivalis* has been implicated as an important bacterial species in pathogenesis of severe forms of periodontitis. In the last few years a major focus of research on *P. gingivalis* has been on the cysteine proteinases with arginine and lysine cleavage specificity (Travis *et al.*, 1997; Imamura, 2003). The so-called gingipains as a whole make up 85% of the proteolytic activity of this bacterium, and the molar concentration of Arg-gingipain is consistently 2–3-fold higher than that of Lys-gingipain (Potempa *et al.*, 1997). While the cysteine proteinase activity with arginine specificity originates from two different genes, *rgpA* and *rgpB*, the lysine specific cysteine proteinase activity is derived from a single gene, *kgp* (Mikolajczyk-Pawlinska *et al.*, 1998). *rgpB* mutants possess about 50% of the arginine specific cysteine activity (Aduse-Opoku *et al.*, 1998; Tokuda *et al.*, 1996), a *rgpA* mutant has 40% activity (Tokuda *et al.*, 1998). The gingipains are associated with many of the virulence factors of this species, such as degradation of immunoglobulins G and A (Abe *et al.*, 1998), degradation of complement factors (Grenier, 1992), enhancement of vascular permeability (Rubinstein *et al.*, 2001), and formation of fimbriae (Tokuda *et al.*, 1998).

Antibiotics are widely used as additive therapy in severe cases of *P. gingivalis* associated periodontitis. But this usage has limitations, e.g. because of the development of antibiotic resistance. Most *P. gingivalis* strains are carriers of the *tet*(*Q*) resistance gene (Chung *et al.*, 2002). Therapy with tetracycline fibers is unable to eradicate *P. gingivalis* completely (Mombelli *et al.*, 2002). New ideas to use inhibitors of major virulence properties have been discussed (Potempa *et al.*, 2000). The purpose of this study was to evaluate the effect of inhibitors of benzamidine type on the virulence properties associated with arginine specific cysteine proteinase activity of *P. gingivalis*. Beside the influence on *N*-$\alpha$-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) activity the effect on growth, hemagglutination, adherence to epithelial cells, and immunophagocytosis were determined.

## MATERIALS AND METHODS

***Bacterial strains.*** *P. gingivalis* ATCC 33277 was obtained from the German strain collection DSMZ (Braunschweig, Germany). The JH16-1 strain was a clinical isolate obtained from a female patient with severe chronic periodontitis. Analysis for strain diversity confirmed that the clinical isolate had been persistent for two years in this patient (Eick *et al.*, 2002). The strains were maintained on Schaedler agar enriched with 10% sheep blood and vitamin K. Bacterial cells used in assays were grown to late log phase (36 h), harvested, washed twice and resuspended in Medium 199 (Gibco) to an absorbance at 660 nm corresponding to $10^8$ or $10^9$ bacteria/ml.

***Proteinase inhibitors.*** Seven inhibitors tested were chosen from a spectrum of substances with a benzamidine structure, they were named 1, 2, 3, 4, 6, 7 and 9 (pentamidine). The inhibitors 1, 4, and 6 were bis-benzamidines with a pentamidine related structure. Inhibitor 2 was a bis-benzamidine with another structure. Inhibitor 7 represented benzamidine. "Inhibitor" 3 was an arginine derivative. The tests were carried out as a blind study. At the time of the study the investigators did not know either the group of benzamidines the inhibitors belonged to or that a negative control was included.

The inhibitors were synthesized by the group of J. Stürzebecher (Institute of Vascular Biology and Medicine, University Hospital of Jena, Germany). The inhibitors had been tested against purified Arg-gingipain RgpB (the enzyme was kindly provided by Jan Potempa, Jagiellonian University, (Kraków, Poland) before. The inhibition constant ($K_i$) was up to 0.45 $\mu$M (inhibitor 1).

***Determination of the arginine specific cysteine amidolytic activity.*** Proteinase inhibitors were added to bacterial suspensions

($10^9$/ml) in a final concentration of 2 $\mu$M and 20 $\mu$M. After mixing these suspensions were incubated at 37°C for 1 h. After that each 100 $\mu$l of suspension was placed into a well of a 96 well-microtitre plate. Each well contained 125 $\mu$l of reaction mixture for determination of the arginine specific cysteine amidolytic activity (0.5 mM BA*p*NA, 10 mM L-cysteine, 10 mM $CaCl_2$ and 100 mM Tris/HCl, 1% agar, pH 7.6, according to Grenier & Turgeon (1994)).

The plate was incubated at 37°C for 1 h. Then the bacterial suspension was removed and the plates were washed three times with phosphate-buffered saline (PBS). The arginine specific amidolytic activity was measured by means of a spectrophotometer at 405 nm.

***Growth inhibition by proteinase inhibitors***. Bacteria (100 $\mu$l of a $10^8$/ml suspension) were added to tubes containing 10 ml of Schaedler broth with 10% sheep blood and vitamin K and a proteinase inhibitor (final concentration 0.05–20 $\mu$M), including a negative control without inhibitor. After incubation for 24 h in an anaerobic atmosphere at 37°C colony forming units (cfu) were determined. The $MIC_{50}$ and $MIC_{90}$ of the inhibitor were calculated.

***Hemagglutination***. For hemagglutination assays to bacterial suspensions ($10^9$/ml) proteinase inhibitors were added (final concentration 2 $\mu$M and 20 $\mu$M) and incubated for 1 h. After that the mixtures were diluted in a twofold series with PBS. Aliquots (100 $\mu$l) of each dilution were mixed with an equal volume of a sheep erythrocyte suspension (2% in PBS) and incubated in a round-bottomed microtitre plate at room temperature for 3 h. Hemagglutination was evaluated visually.

***Adherence assay.*** KB cells were seeded into two 24-well tissue culture plates at a density of about $10^4$ cells per well, the cells were grown to confluent monolayer in EMEM (Gibco) enriched with 10% foetal bovine serum (FBS). The cells were washed twice with PBS. Bacterial suspension ($10^8$/ml) in Medium 199 was mixed with EMEM 1:4. Proteinase inhibitors were added to a final concentration of 2 $\mu$M and 20 $\mu$M and each well was inoculated with 1 ml of this suspension. Bacteria were co-cultured with KB cells at 37°C for 1 h. After that the infected monolayers were washed five times with PBS. The number of adherent *P. gingivalis* was counted as cfu after cell lysis.

Additionally the MTT tetrazolium salt colorimetric assay described by Mosmann (1983) was used to measure cytotoxicity. The method is based on the capacity of mitochondrial enzymes of viable cells to reduce the yellow soluble salt MTT to a blue insoluble formazan precipitate, which is quantified after dissolution in an organic solvent. The MTT-formazan production correlates directly with the count of viable cells. Proteinase inhibitors were mixed with EMEM + 10% FCS to a final concentration 2 and 20 $\mu$M and incubated with KB cells for 24 h in a 96-well microtiter plate. Then 10 $\mu$l of a MTT solution (5 mg/ml) was added to 100 $\mu$l of culture media. After incubation for 2 h at 37°C the suspension was removed and 20 $\mu$l of 3% SDS solution and 100 $\mu$l of isopropanole/0.04 M HCl were added and incubated for 1 h at 37°C. Finally the MTT reaction was examined photometrically at 570 nm.

***Immunophagocytosis***. The method used was based on the descriptions by Smith & Rommel (1977) and Pantazis & Kniker (1979). Freshly drawn blood obtained from 10 healthy subjects was layed on a cover slip. The cells adhered at 37°C with 5% $CO_2$ and 100% humidity for 25 min. Thereafter the soft blood clot was removed.

Bacterial suspensions were adjusted photometrically to $10^9$ bacteria per ml with PBS. Bacterial suspension (0.2 ml) was mixed with 0.2 ml human Anti-AB-serum for opsonization for 30 min. These mixtures were centrifuged at 2000 × ***g*** for 10 min and then the pellets were washed and resuspended in 1 ml PBS. Finally, 0.2 ml of foetal bovine serum and the proteinase inhibitor to be tested at a final concentration of 20 $\mu$M were added. The bacterial suspensions were transferred to the cover

slips and the phagocytosis assay was incubated under the conditions mentioned above for 15 min.

Then the slips were stained with acridine orange (2.5 mg/7.5 ml PBS) for 1 min. After decanting the solution they were covered with 0.01% crystal violet in PBS to quench extracellular fluorescence, washed and fixed. A total of 100 polymorphonuclear leukocytes (PMN) were immediately examined by fluorescence microscopy. Viable and killed bacteria in granulocytes were distinguished by their uptake of acridine orange, viable bacteria appeared green and dead bacteria were red. The numbers of PMNs containing bacteria were counted. These phagocytosing cells were separated into groups by the following criteria: (i) cells with less than 10, cells with 10–20, and cells with more than 20 ingested bacteria and (ii) granulocytes with less than 50% and with more than 50% viable bacteria.

***Statistical analysis.*** The statistical analysis was done using SPSS 10.0 software. The significance of the differences between each group with addition of an inhibitor (2 $\mu$M and 20 $\mu$M) and the controls (without inhibitor) was determined by the Mann-Whitney test. The WILCOXON test was used to assess the significance of the results between paired samples in phagocytosis assays.

## RESULTS

### Amidolytic activity

Inhibitor 1 showed the highest reduction of the arginine cysteine amidolytic activity of the two *P. gingivalis* strains, the reduction being 35.5% for the ATCC strain at 2 $\mu$M. Also inhibitor 4, another pentamidine-related inhibitor, reduced the amidolytic activity of the two strains in the lower concentration of 2 $\mu$M. Inhibitor 6 in both concentrations showed effects on the JH16-1 strain, and pentamidine at 20 $\mu$M reduced the amidolytic activity of both strains (Table 1).

### Growth curves

Only the inhibitors of the pentamidine-related group were able to inhibit the growth of the bacteria by 50% and 90% after 24 h. Pentamidine was most effective. No significant differences in growth inhibition was observed between the two strains (Table 2).

**Table 1. Effect of proteases inhibitors on BA*p*NA-reaction activity produced by *P. gingivalis*.**

Activity of the non-treated suspension of bacterial cells was taken as 100%.

| Inhibitor | ATCC 33277 2 μM | ATCC 33277 20 μM | JH16-1 2 μM | JH16-1 20 μM |
|---|---|---|---|---|
| pentamidine-related group | | | | |
| 1 | 64.5 | 72.8 | 90.6 | 91.2 |
| 4 | 86.1 | 98.7 | 87.2 | 90.6 |
| 6 | 106.9 | 96.2 | 90.1 | 90.6 |
| pentamidine | 106.3 | 92.4 | 98.2 | 92.4 |
| 2 | 112.6 | 108.2 | 98.8 | 98.2 |
| 7 | 99.4 | 107.5 | 100.5 | 98.5 |
| 3 | 108.9 | 107.5 | 96.3 | 97.0 |

### Hemagglutination

Without the addition of inhibitors hemagglutination was observed up to a dilution of 1:32 for the ATCC 33277 strain and up to 1:64 for the JH16-1 strain. Inhibitors 3 and 7 did not influence hemagglutination. Both tested concentrations of the pentamidine-related substances (1, 4, 6, and pentamidine) inhibited hemagglutination of the ATCC strain. The effects on the clinical isolate were not so clear. Substance 1 at concentrations of 2 $\mu$M and 20 $\mu$M of the substance 1 reduced hemagglutination of this strain, only the higher concentration of the other pentamidine-related inhibitors had a slight effect on hemagglutination.

### Adherence to KB cells

Following incubation with KB cells in medium $2.2 \times 10^5$ cfu per well of adherent *P. gingivalis* ATCC 33277 and $1.5 \times 10^4$ cfu of adherent *P. gingivalis* JH16-1 were enumerated. Only pentamidine and inhibitor 7 at 20 $\mu$M were able to reduce the adherence of the ATCC 33277 strain, conversely, inhibitor 4 in both concentrations tested enhanced the number of adherent bacteria ($P < 0.05$). A reduced adherence of the clinical isolate JH16-1 strain was observed after addition of inhibitors 1, 2, 3, and 7 in both concentrations and of the pentamidine-related inhibitors 4 and 6 at 20 $\mu$M ($P < 0.05$, Fig. 1).

The MTT values showed no cytotoxicity for the inhibitors with a pentamidine-related structure. Reduced MTT values were measured for inhibitor 2 in both concentrations and the inhibitor 3 in the higher concentration. So it can be concluded that these inhibitors are cytotoxic in the concentrations mentioned above.

### Immunophagocytosis

No differences of phagocytosing capacities of peripheral blood PMNs were observed between the two bacterial strains without inhibitors added. So, in medium 59% of the PMNs had ingested more than 20 *P. gingivalis* ATCC 33277 or JH16-1. Inhibitor 3 (arginine derivative) was the only substance tested with no effect on phagocytosis. All other inhibitors enhanced the percentage of PMNs with more than 20 ingested *P. gingivalis* ATCC 33277 ($P < 0.05$). In contrast, the inhibitor 6 did not influence the phagocytotic capacity of granulocytes to the JH16-1 strain. Pentamidine and inhibitors 1, 2, 4 and 7 promoted the phagocytosis of this clinical isolate ($P < 0.05$). The results are presented in Fig. 2.

In medium, 46% of the phagocytosing PMNs had more than 50% killed *P. gingivalis* ATCC 33277 and 41% of these cells had more than 50% killed *P. gingivalis* JH16-1. The difference between the two strains was significant ($P < 0.05$). Only inhibitor 4 reduced this number to 31% (ATCC 33277) and 24% (JH16-1) ($P < 0.05$). All other inhibitors showed no effects on killing of bacteria (not shown).

**Table 2. Inhibitory concentration of proteases inhibitors with pentamidine-related structure which suppress the growth of *P. gingivalis* strains by 50 and 90%**

| Inhibitor | *P. gingivalis* ATTCC 33277 | | *P. gingivalis* JH16-1 | |
|---|---|---|---|---|
| | $IC_{50}$ (µM) | $IC_{90}$ (µM) | $IC_{50}$ (µM) | $IC_{90}$ (µM) |
| 1 | 9.7 | 11.7 | 8 | 10.4 |
| 4 | 1.2 | 3.1 | 0.30 | 12.4 |
| 6 | 0.50 | 1.1 | 1.1 | 3.2 |
| pentamidine | 0.28 | 0.70 | 0.32 | 0.74 |

## DISCUSSION

The purpose of the study was to determine the effects of inhibitors of benzamidine type

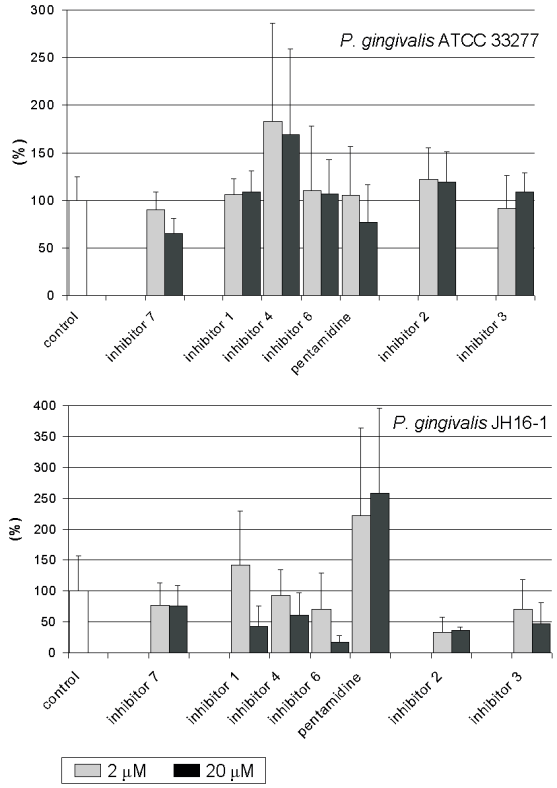


**Figure 1. Influence of 2 $\mu$M and 20 $\mu$M inhibitors of benzamidine type (pentamidine, bis-benzamidine with pentamidine-related structure (inhibitor 1, 4, 6), bis-benzamidine with another structure (inhibitor 2), benzamidine (inhibitor 7) and an arginine derivative (inhibitor 3)) on adhesion of the *P. gingivalis* ATCC 33277 and the JH16-1 strains to KB cells, a permanent epithelial cell line.**

The values are expressed as medians (with the 25 and 75 percentiles) in per cent in relation to each *P. gingivalis* strain without the addition of an inhibitor.

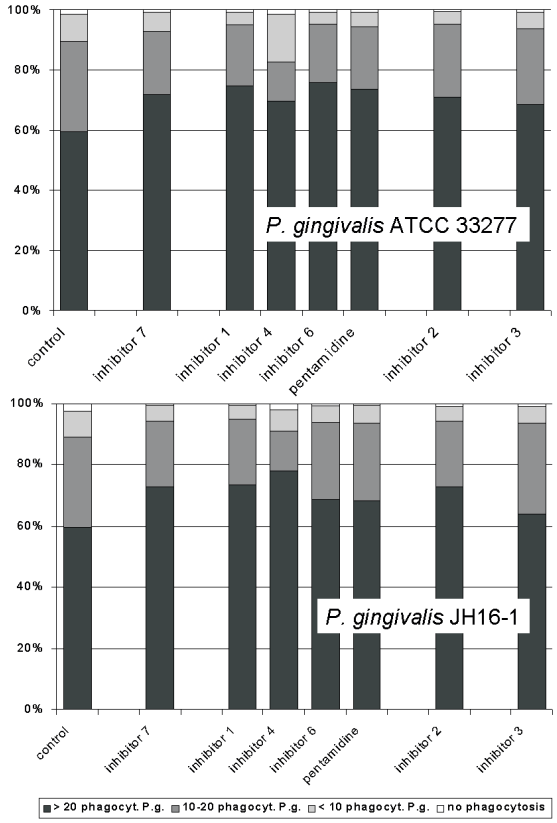


**Figure 2. Influence of 20 $\mu$M of inhibitors of benzamidine type (pentamidine, bis-benzamidine with pentamidine-related structure (inhibitor 1, 4, 6), bis-benzamidine with another structure (inhibitor 2), benzamidine (inhibitor 7) and an arginine derivative (inhibitor 3)) on immune phagocytosis of the *P. gingivalis* ATCC 33277 and the JH16-1 strains by peripheral blood PMNs obtained from healthy subjects.**

The values are expressed as medians in per cent of the granulocytes with no, < 10, 10–20, and > 20 ingested bacteria.

on the bacteria themselves. The $K_i$ values were in the range from 10.4 to 0.45 $\mu$M. The effects were not so clear, if the bacteria as a whole and not the purified enzymes were assayed, but the inhibition of the amidolytic activity of the bacteria showed a good correlation with the $K_i$ values of the different inhibitors. Inhibition of the enzymatic activitity of the ATCC strain by 35% was found, the enzymatic activity of the clinical isolate was only reduced by 13% by one inhibitor.

Several tests have been performed to find an influence of the inhibitors on proteinases associated virulence properties. By cleaving a variety of host proteins, gingipains may provide small peptides and amino acids that meet the nutritional requirements of *P. gingivalis*. Consistently, Arg-gingipains play an important role in growth of this bacterium (Grenier *et al.*, 2001). Bis-benzamidine derivatives, especially pentamidine, suppress the growth of *P. gingivalis* strains. But the effect of the

tested inhibitors on *P. gingivalis* growth was far more significant than inhibition of the bacterium-associated BA*p*NA activity. This suggests a target for these compounds other than Arg-gingipain.

Both expression of fimbriae and hemagglutination are linked to the *rgpA* gene (Tokuda *et al.*, 1998). A constructed *rgpA, rgpB, kgp* triple mutants exhibited no hemagglutinating properties using sheep erythrocytes and an *rgpA rgpB* mutant showed a reduced hemagglutinating activity (Shi *et al.*, 1999). Although the inhibitors block only the arginine specific cysteine proteinases, the inhibition of hemagglutination was in accordance with the inhibition of enzymatic activity.

The results concerning the influence of the inhibitors on adhesion of bacteria to KB cells were contradictory, both an enhancement and a decrease were found. The arginine derivative (substance 3) and the benzamidine inhibitor 2 were cytotoxic to KB cells. In general it is believed that fimbriae represent the adhesins to receptors of epithelial cells. A strain with a double mutation of the *rgp* genes possessed very few fimbriae on the cell surface (Nakayama *et al.*, 1996). Mutants of the *fimA* gene showed remarkably lower adherence and invasion in comparison with the parent strain (Njoroge *et al.*, 1997; Weinberg *et al.*, 1997). Recently it was reported that this mutant possesses only 5% of the Rgp and 13% of the Kgp total activities of the parent strain (Chen *et al.*, 2001). Adherence to epithelial cells can occur in the absence of fimbriae. First, Tokuda *et al.* (1998) reported that an *rgpA* mutant expressed very few fimbriae but attached in high numbers. Later, Chen *et al.* (2001) described that the attachment level of an *rgpA rgpB* mutant was high. Detachment of *P. gingivalis* is caused by the degradation of receptors by Arg-gingipains (Chen *et al.*, 2001). Gingipains are also able to degrade epithelial junctional proteins (Katz *et al.*, 2002). The quantity of adherence to epithelial cells is not crucial for the virulence of *P. gingivalis* strains, as clinical isolates often adhere in a lower number than the reference strain (Eick *et al.*, 2002). Contact of *P. gingivalis* with epithelial cells represses the secretion of Arg-gingipain and Lys-gingipain (Park & Lamont, 1998). But proteases of *P. gingivalis* may be involved in the invasion process (Lamont *et al.*, 1995). *P. gingivalis* persists and multiplies within epithelial cells (Madianos *et al.*, 1996). Persistent *P. gingivalis* strains can trigger immune response by release of proinflammatory interleukins (Eick *et al.*, unpublished). Tests to determine the effects of the inhibitors on invasion, persistence and release of proinflammatory interleukins are in progress in this laboratory.

All the inhibitors tested enhanced the phagocytotic capacity of granulocytes to *P. gingivalis*. We used opsonized bacteria in the assays. So the benzamidines might block the degradation of immunoglobulins and factors of the complement. Gingipains cleave immunoglobulins (Abe *et al.*, 1998). Previously it was decribed that gingipain degrades C3 and in this way eliminates the creation of C3-derived opsonins and renders *P. gingivalis* resistant to phagocytosis (Cutler *et al.*, 1993; Schenkein *et al.*, 1995). Furthermore, *P. gingivalis* alters expression of immunoglobulin G receptors on neutrophiles (Tai *et al.*, 1993). Arg-gingipain reduces the respiratory burst in PMNs (Kadowaki *et al.*, 1998; Abe *et al.*, 1998). In our study the inhibitors did not enhance intracellular killing of *P. gingivalis.* Non-oxidative mechanisms are more important in killing periodontopathogenic bacteria in gingival sulcus (Miyasaki *et al.*, 1994). Interestingly, we found a difference in killing of the strains used, the clinical isolate was more resistant than the ATCC strain.

A strong case has been made for the development of synthetic inhibitors directed against specific pathogen-derived proteinases. Recently a report described an inhibitory effect on arginine-specific gingipains of tetracyclines and their analogues (Imamura *et al.*, 2001). Agents, not in the first place antibiot-

ics, were found to inhibit Arg-gingipains; malabaricone C, isolated from nutmeg, suppress growth of *P. gingivalis* (Shinohara *et al.*, 1999), and protamines obtained from salmon or herring sperm inhibit fimbrial interaction with fibronectin (Kontani *et al.*, 1999). Curtis *et al.* (2002) designed a very effective inhibitor of Lys-gingipains.

We assayed several benzamidine derivatives. Although *in vitro* effects of the synthetic inhibitors of cysteine proteinases on virulence of *P. gingivalis* were observed further *in vitro* tests concerning immunomodulatory effects should be done before these substances are used for therapy in clinically controlled studies. These studies should focus on bis-benzamidine derivatives with pentamidine-related structure, especially inhibitor 1.

## REFERENCES


Abe N, Kadowaki T, Okamoto K, Nakayama K, Ohishi M, Yamamoto K. (1998) Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. *J Biochem.*; **123**: 305–12.

Aduse-Opoku J, Rangarajan M, Young KA, Curtis MA. (1998) Maturation of the arginine-specific proteases of *Porphyromonas gingivalis* W 50 is dependent on a functional prR2-gene. *Infect Immun.*; **66**: 1594–600.

Chen T, Nakayama K, Belliveau L, Duncan MJ. (2001) *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. *Infect Immun.*; **69**: 3048–56.

Chung WO, Gabany J, Persson GR, Roberts MC. (2002) Distribution of erm(F) and tet(Q) genes in 4 oral bacterial species and genotypic variation between resistant and susceptible isolates. *J Clin Periodontol.*; **29**: 152–8.

Curtis MA, Aduse Opoku J, Rangarajan M, Gallagher A, Sterne JA, Reid CR, Evans HE, Samuelsson B. (2002) Attenuation of the virulence of *Porphyromonas gingivalis* by using a specific synthetic Kgp protease inhibitor. *Infect Immun.*; **70**: 6968–75.

Cutler CW, Arnold RR, Schenkein HA. (1993) Inhibition of C3 and IgG proteolysis enhances phagocytosis of *Porphyromonas gingivalis*. *J Immunol.*; **151**: 7016–29.

Eick S, Rödel J, Einax JW, Pfister W (2002). Interaction of *Porphyromonas gingivalis* with KB cells: comparison of different clinical isolates. *Oral Microbiol Immunol.*; **17**: 201–8.

Grenier D. (1992) Inactivation of human serum bactericidal activity by a trypsinlike protease isolated from *Porphyromonas gingivalis*. *Infect Immun.*; **60**: 1854–7.

Grenier D, Turgeon J. (1994) Occurence and identity of proteolytic bacteria in adult periodontitis. *J Periodont Res.*; **29**: 365–70.

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**: 5166–72.

Imamura T. (2003) The role of gingipains in the pathogenesis of periodontal disease. *J Periodontol.*; **74**: 111–8.

Imamura T, Matsushita K, Travis J, Potempa J. (2001) Inhibition of trypsin-like cysteine proteinases (gingipains) from *Porphyromonas gingivalis* by tetracycline and its analogues. *Antimicrob Agents Chemother.*; **45**: 2871–6.

Katz J, Yang QB, Zhang P, Potempa J, Travis J, Michalek SM, Balkovetz DF. (2002) Hydrolysis of epithelial junctional proteins by *Porphyromonas gingivalis* gingipains. *Infect Immun.*; **70**: 2512–8.

Kadowaki T, Nakayama K, Yoshimura F, Okamoto K, Abe N, Yamamoto K. (1998) Arg-gingipain acts as a major processing enzyme for various cell surface proteins in *Porphyromonas gingivalis*. *J Biol Chem.*; **273**: 29072–6.

Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. (1995) *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun.*; **63**: 3878–85.

Kontani M, Amano A, Nakamura T, Nakagawa I, Kawabata S, Hamada S. (1999) Inhibitory effects of protamines on proteolytic and adhesive activities of *Porphyromonas gingivalis*. *Infection Immun.*: **67**: 4917–20.

Madianos PN, Papapanou PN, Nannmark U, Dahlen G, Sandros J. (1996) *Porphyromonas gingivalis* FDC381 multiplies and persists within human oral epithelial cells *in vitro*. *Infect Immun.*; **64**: 660–4.

Mikolajczyk-Pawlinska J, Kordula T, Pavloff N, Pemberton PA, Audrey Chen WC, Travis J, Potempa J. (1998) Genetic variations of *Porphyromonas gingivalis* genes encoding gingipains, cysteine proteinases with arginine or lysine specifity. *Biol Chem.*; **379**: 205–11.

Miyasaki KT, Bodeau AL, Shafer WM, Pohl J, Murthy ARK, Lehrer RI. (1994) New ideas about neutrophil antimicrobial mechanisms: antibiotic peptides, postphagocytic protein processing, and cytosolic defense. In *Molecular pathogenesis of periodontal disease*. Genco R, Hamada S, Lehner T, McGhee J, Mergenhagen S. eds, pp 321–36. ASM Press, Washington DC.

Mombelli A, Schmid B, Rutar A, Lang NP (2002) Local antibiotic therapy guided by microbiological diagnosis. *J Clin Periodontol.*; **29**: 743–9.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.*; **65**: 55–63.

Nakayama K, Yoshimura F, Kadowaki T, Yamamoto K. (1996) Involvement of arginine-specific cysteine proteinase (Arg-gingipain) in fimbriation of *Porphyromonas gingivalis*. *J Bacteriol.*; **178**: 2818–24.

Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA. (1997) A role of fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. *Infect Immun.*; **65**: 1980–84.

Pantazis CG, Kniker WT. (1979) Assessment of blood leukocyte microbial killing by using a new fluorochrome microassay. *J Reticuloendothel Soc.*; **26**: 155–70.

Park Y, Lamont RJ. (1998) Contact-dependent protein secretion in *Porphyromonas gingivalis*. *Infect Immun.*; **66**: 4777–82.

Potempa J, Pike R, Travis J. (1997) Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. *Biol Chem.*; **378**: 223–30.

Rubinstein I, Potempa J, Travis J, Gao XP. (2001) Mechanisms mediating *Porphyromonas gingivalis* gingipain RgpA-induced oral mucosa inflammation *in vivo*. *Infect Immun.*; **69**: 1199–201.

Schenkein HA, Fletcher HM, Bodnar M, Macrina FL. (1995) Increased opsonization of a prH-defective mutant of *Porphyromonas gingivalis* W83 is caused by degradation of complement-derived opsonins. *J Immunol.*; **154**: 5331–7.

Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K. (1999) Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp* and *hag1*. *J Biol Chem.*; **274**: 17955–60.

Shinohara C, Mori S, Ando T, Tsuji T. (1999) Arg-gingipain inhibition and anti-bacterial activity selective for *Porphyromonas gingivalis* by malabaricone C. *Biosci Biotechnol Biochem.*; **63**: 1475–7.

Smith D, Rommel F. (1977) A rapid micro method for the simultaneous determination of phagocytic microbiocidal-activity of human peripheral blood leukocytes in vitro. *J Immunol Methods.*; **17**: 241–7.

Tai H, Kobayashi T, Hara K. (1993) Changes in complement and immunoglobulin G receptor expression on neutrophils associated with *Porphyromonas gingivalis*-induced inhibition of phagocytosis. *Infect Immun.*; **61**: 3533–5.

Tokuda M, Duncan M, Cho MI, Kuramitsu HK. (1996) Role of *Porphyromonas gingivalis* protease activity in colonization of oral surfaces. *Infect Immun.*; **64**: 4067–73.

Tokuda M, Karunakaran T, Duncan M, Hamada N, Kuramitsu H. (1998) Role of Arg-gingipain

A in virulence of *Porphyromonas gingivalis*. *Infect Immun.*; **66**: 1159–63.

Travis J, Potempa J. (2000) Bacterial proteinases as targets for the development of second-generation antibiotics. *Biochim Biophys Acta.*; **1477**: 35–50.

Travis J, Pike R, Imamura T, Potempa J. (1997) *Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis *J Periodontol Res.*; **32**: 120–5.

Weinberg A, Belton CM, Park Y, Lamont RJ. (1997) Role of fimbriae in *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun.*; **65**: 313–316.