molecular oral microbiology

molecular oral microbiology

Benzamidine derivatives inhibit the virulence of *Porphyromonas gingivalis*

E. Fröhlich[1], T. Kantyka[2], K. Plaza[2], K.-H. Schmidt[3], W. Pfister[3], J. Potempa[2,4] and S. Eick[5]

1 Department of Experimental Anesthesiology, Clinic for Anesthesiology and Intensive Care Medicine, Jena University Hospital, Jena, Germany
2 Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
3 Institute of Medical Microbiology, Jena University Hospital, Jena, Germany
4 Oral Health and Systemic Diseases Group, School of Dentistry, University of Louisville, Louisville, KY, USA
5 Department of Periodontology, Laboratory of Oral Microbiology, Dental School, University of Bern, Bern, Switzerland

Correspondence: Sigrun Eick, Department of Periodontology, Laboratory of Oral Microbiology, Dental School, University of Bern, Freiburgstrasse 7, CH-3010 Bern, Switzerland Tel.: + 41 31 632 2542; fax: + 41 31 632 8608; E-mail: sigrun.eick@zmk.unibe.ch

Keywords: Arg-gingipains; benzamidine derivatives; fertilized egg model; *Porphyromonas gingivalis*
Accepted 6 November 2012
DOI: 10.1111/omi.12015

SUMMARY

We have previously shown that benzamidine-type compounds can inhibit the activity of arginine-specific cysteine proteinases (gingipains HRgpA and RgpB); well-known virulence factors of *Porphyromonas gingivalis*. They also hinder *in vitro* growth of this important periodontopathogenic bacterium. Apparently growth arrest is not associated with their ability to inhibit these proteases, because pentamidine, which is a 20-fold less efficient inhibitor of gingipain than 2,6-bis-(4-amidinobenzyl)-cyclohexanone (ACH), blocked *P. gingivalis* growth far more effectively. To identify targets for benzamidine-derived compounds other than Arg-gingipains, and to explain their bacteriostatic effects, *P. gingivalis* ATCC 33277 and *P. gingivalis* M5-1-2 (clinical isolate) cell extracts were subjected to affinity chromatography using a benzamidine–Sepharose column to identify proteins interacting with benzamidine. In addition to HRgpA and RgpB the analysis revealed heat-shock protein GroEL as another ligand for benzamidine. To better understand the effect of benzamidine-derived compounds on *P. gingivalis*, bacteria were exposed to benzamidine, pentamidine, ACH and heat, and the expression of gingipains and GroEL was determined. Exposure to heat and benzamidine-derived compounds caused significant increases in GroEL, at both the mRNA and protein levels. Interestingly, despite the fact that gingipains were shown to be the main virulence factors in a fertilized egg model of infection, mortality rates were strongly reduced, not only by ACH, but also by pentamidine, a relatively weak gingipain inhibitor. This effect may depend not only on gingipain inhibition but also on interaction of benzamidine derivatives with GroEL. Therefore these compounds may find use in supportive periodontitis treatment.

INTRODUCTION

A limited number of bacterial species have been associated with periodontitis, among which *Porphyromonas gingivalis*, an anaerobic gram-negative bacterium, is most strongly associated with disease progression (Genco, 1996; Lamont & Jenkinson, 2000; Ezzo & Cutler, 2003). In the last few years a major focus of *P. gingivalis* research has been the link between periodontal and systemic diseases, e.g. atherosclerotic cardiovascular disease, preterm birth, and diabetes (Hayashi *et al.*, 2010). Moreover,

P. gingivalis expresses a peptidyl arginine deiminase that is able to citrullinate proteins, and may provide antigens that drive autoimmune responses in rheumatoid arthritis (Wegner *et al.*, 2010). Among its virulence factors, cysteine proteinases, referred to as gingipains, are considered as major contributors to *P. gingivalis* pathogenic potential (Guo *et al.*, 2010). Indeed, gingipains are responsible for at least 85% of the proteolytic activity of this bacterium (Potempa *et al.*, 1997). While gingipain with arginine-Xaa specificity (Arg-gingipains, Rgps) originate from two different genes, *rgpA* and *rgpB*, the lysine-Xaa-specific gingipain (Lys-gingipain, Kgp) is derived from a single gene, *kgp* (Mikolajczyk-Pawlinska *et al.*, 1998). The gingipains contribute to *P. gingivalis* virulence in multiple ways, for example, by enhancing vascular permeability gingipains generate gingival crevicular fluid (Rubinstein *et al.*, 2001), providing nutrients and essential growth factors for the bacterium. This effect is enhanced by degradation of intracellular adhesion molecule-1, and junction proteins on human epithelial cells (Katz *et al.*, 2002). At the same time, by efficient degradation of multiple components of the complement system, gingipains exert resistance to the antibacterial activity of complement (Popadiak *et al.*, 2007). Simultaneously, the cleavage of immunoglobulin G1 and G3 at the hinge region by Lys-gingipain may abrogate the affects of acquired immunity (Vincents *et al.*, 2011).

Antibiotics are widely used as an adjunct therapy in severe cases of *P. gingivalis*-associated periodontitis. Unfortunately, a side effect of such treatment is the risk of developing antibiotic-resistant isolates. In these circumstances, the application of gingipain-specific inhibitors to reduce *P. gingivalis* virulence seems to be an attractive alternative to antibiotic treatment (Travis & Potempa, 2000). To this end, benzamidine derivatives have previously been found to inhibit the amidolytic activities and growth of *P. gingivalis*. In addition, treatment of the bacterium with these compounds promoted phagocytosis by polymorphonuclear neutrophils. The inhibition of hemagglutination and enhancement of phagocytosis by individual compounds strongly correlated with the potency of the given compound (measured as the K_i-values) to inhibit the amidolytic activity of Rgps. Surprisingly, however, pentamidine, a relatively weak protease inhibitor (high K_i-value), was the most effective compound for growth inhibition (Eick *et al.*, 2003). In this follow-up study we show that benzamidine derivatives bind not only to Arg-gingpains, but also to the chaperone GroEL, and increase expression of the latter protein. Using a fertilized egg model of infection we show that these compounds effectively reduce the lethality of *P. gingivalis* during disease causation.

METHODS

Benzamidine derivatives

Benzamidine (Sigma-Aldrich, Taufkirchen, Germany), pentamidine (Sigma-Aldrich) and 2,6-bis-(4-amidinobenzyl)-cyclohexanone (synthesized by J Stürzebecher, Institute of Vascular Biology and Medicine, University Hospital of Jena) were chosen from a panel of compounds with benzamidine-like structures (Fig. 1). The inhibition constants (K_i) against purified RgpB were 40.3 μM for pentamidine, 0.51 μM for 2,6-bis-(4-amidinobenzyl)-cyclohexanone and 277 μM for benzamidine. K_i-values against HRgpA were 2.9 μM for 2,6-bis-(4-amidinobenzyl)-cyclohexanone, 107 μM for pentamidine and 536 μM for benzamidine. No benzamidine derivative impacted Lys-gingipain

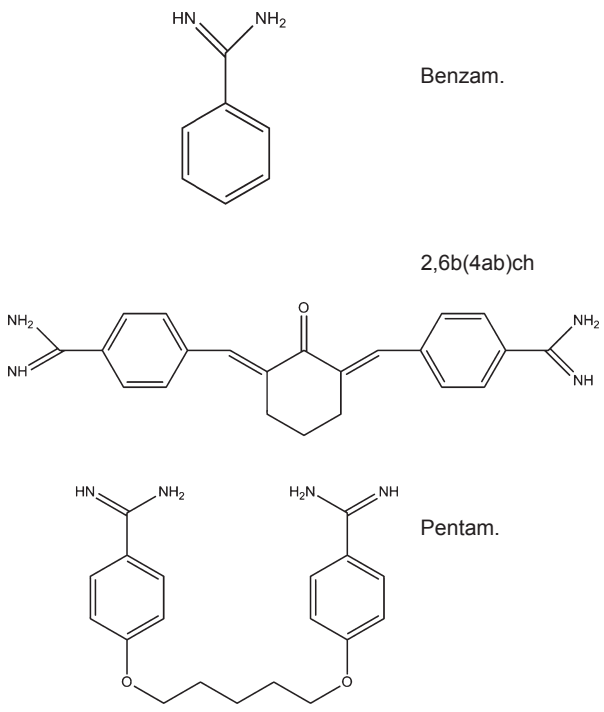

Figure 1 The structures of benzamidine (benzam.), 2,6-bis-(4-amidinobenzyl)-cyclohexanone (2,6b(4ab)ch) and pentamidine (pentam.).

activity ($K_i > 1000$ μM). In all experiments the inhibitors were used at a final concentration of 20 μM.

Bacterial strains

Porphyromonas gingivalis ATCC 33277 was purchased from the German strain collection DSMZ (Braunschweig, Germany). The *P. gingivalis* M5-1-2 strain is a clinical isolate obtained from a patient with severe chronic periodontitis. In the plated sample of subgingival plaque derived from this patient, *P. gingivalis* compromised 64% of the discernable anaerobic microbiota. Bacterial strains were grown anaerobically on Schaedler agar (Oxoid) supplemented with 8% sheep blood, 2.5 μg ml^{-1} menadione; and in Schaedler broth (Oxoid) supplemented with 2.5 μg ml^{-1} menadione.

For growth inhibition experiments, 1×10^6 bacteria ml^{-1} were added to Schaedler broth supplemented with 2.5 μg ml^{-1} menadione and 20 μM inhibitor. After incubation for 24 h and 48 h at 37°C, the number of colony-forming units was determined. These experiments were repeated in triplicate. Statistical analysis was performed using Student's *t*-test.

Purification of Arg-gingipains by means of affinity chromatography on a benzamidine–Sepharose column

p-Aminobenzamidine (375 mg) was ligated with CH-Sepharose 4B (5 g; Pharmacia Fine Chemical, London, UK) using 1.853 g carbodiimide according to manufacturer's protocol. The *P. gingivalis* strains were grown in batch culture (3 l) and harvested by centrifugation (5000 ***g***, 30 min at 4°C). The cell-free culture fluid was precipitated using acetone, and the protein pellet was resuspended in 20 mM Bis–Tris, 150 mM NaCl, 5 mM $CaCl_2$ and 0.02% NaN_3 at pH 6.8. The solution was then dialysed against the same buffer containing 1.5 mM aldrithiole (two changes). Dialysed fractions were clarified by centrifugation (13,000 ***g***, for 15 min at 4°C), concentrated by lyophilization and applied to a column (1.3×13 cm) of *p*-aminobenzamidine-Sepharose equilibrated with the Bis–Tris buffer. After eluting non-binding proteins, a linear NaCl gradient (from 0 to 3 M NaCl), and then a benzamidine gradient from 0 to 2 M was applied at a flow rate of 0.8 ml min^{-1}. Arginine-specific and lysine-specific amidolytic activities in each fraction were assayed at 37°C with 0.5 mM *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) or *N*-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide, respectively, in 1.0 ml of 0.2 M Tris–HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 10 mM cysteine, pH 7.6. Fractions with the highest activity in the *p*-aminobenzamidine–Sepharose flow-through (pool I), NaCl gradient (pool II), and benzamidine elution (pool III) were combined. The collected fractions were desalted using Sephadex G-25-columns equilibrated with 0.05 M Tris–HCl, 1 mM $CaCl_2$, 0.02% (weight/volume) NaN_3 (pH 7.4) and freeze-dried.

Proteins from each pool were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli under non-reduced conditions using 10% polyacrylamide gels. Gels were stained using a silver staining kit (GE Healthcare, Amersham, UK).

For analysis of N-terminal amino acid sequences, protein separated by SDS–PAGE were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. Protein bands were visualized by staining with SYPRO Ruby blot stain (Bio-Rad, Hercules, CA). The stained bands were excised and the N-terminal amino acid sequence was determined by automated Edman degradation at the Leibniz Institute for Age Research, Jena.

Expression of genes encoding GroEL and gingipains

Porphyromonas gingivalis strains were precultivated in Schaedler broth supplemented with 2.5 μg ml^{-1} menadione for 24 h. After this time, the bacterial suspension was divided into five samples in microcentrifuge tubes. Benzamidine, pentamidine and 2,6-bis-(4-amidinobenzyl)-cyclohexanone were added to each sample (to a final concentration of 20 μM). Four samples (three with inhibitors, one without) were incubated at 37°C, while a fifth sample without inhibitor was exposed to 45°C in an anaerobic atmosphere for 1 h.

Total RNA from approximately 2×10^8 bacterial cells was purified using an RNeasy kit (Qiagen GmbH, Hilden, Germany) and cDNA was synthesized from 100 ng total RNA using an Omniscript kit (Qiagen) according to the manufacturer's instructions. Real-time polymerase chain reaction was carried out in a reaction volume of 20 μl, consisting of 2 μl cDNA solution and 18 μl reaction mixture, containing 2 μl

10 × polymerase chain reaction buffer, 2.75 mM $MgCl_2$, 0.2 mM nucleotides, 0.5 μM of each primer, 10^{-4} SybrGreen and 1 U *Taq* polymerase (Fermentas Life Sciences, St Leon-Rot, Germany) using a Rotor-Gene 2000 device (Corbett Research, Sydney, Australia). The oligonucleotide primers were as follows: *rgpA* (accession: U15282) 5′-TAT CCT TCG TGA TGT GCG TG-3′ (forward), 5′-GCT GTA ACG GGA GAA GCA AT-3′ (reverse); *rgpB* (accession: U85038) 5′-CAT TCT CCT CTC TGT TGG GA-3′ (forward), 5′-CGT AGG GGA TTT GAT CAG GA-3′ (reverse); *kgp* (accession: U54691) 5′-TCA AGC AGT TCG ATG CAA GC -3′ (forward), 5′-ACT TGG GTC AGT TCT TGT CC-3′ (reverse); *groEL* (accession: D17342) 5′-TGC CGT TAA AGT TAC CCT CG-3′ (forward), 5′-CAC TTC CTT AGC CAT ACC TG-3′ (reverse). The housekeeping gene *sod* (accession: M60401) 5′-AAT TCC ACC ACG GTA AGC AC-3′ (forward), 5′- TTC TCG ATG GAC AGT TTG CC-3′ (reverse) served as control. All primers were designed using the DNASIS program (Hitachi Software EMEA, Tokyo, Japan). Cycling conditions comprised an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 95°C for 15 s, 58°C for 20 s, 72°C for 20 s. The amount of gingipain and stress gene mRNA was quantified in relation to *sod* mRNA levels. All experiments were performed in triplicate. Statistical analysis was performed using Student's *t*-test for paired samples, samples after exposure to heat or benzamidine derivatives were compared with those without any exposure.

Effect of inhibitors on GroEL protein levels

Porphyromonas gingivalis strains were grown in Schaedler broth supplemented with 2.5 μg ml^{-1} menadione for 24 h, and exposed to inhibitors and stress as described above. Bacteria were collected by centrifugation and pellets containing approximately 2×10^9 bacteria were suspended with 500 μl of sample buffer (0.125 M Tris–HCl, 20% glycerol, 4% SDS), before being subjected to SDS–PAGE (10% polyacrylamide including 0.1% SDS) under non-reduced conditions. To avoid proteolytic degradation of proteins during boiling, all samples were treated with 0.05 mM Phe-Phe-Arg-chloromethylketone (FFRcK). Next, resolved proteins were transferred (Mini Trans-Blot System; Bio-Rad) on to nitrocellulose membranes (GE Healthcare) for immunoblotting. Non-specific binding sites on the membranes were blocked overnight in 5% skimmed milk (BD, Franklin Lakes, NJ). Blots were then probed with a monoclonal mouse antibody against GroEL (Sigma-Aldrich), followed by goat anti-mouse IgG horseradish peroxidase-conjugated antibodies (Dako Deutschland GmbH, Hamburg, Germany). The blots were developed using the ECL Plus (GE Healthcare) substrate kit according to the manufacturer's protocol. Chemiluminescence of immunoreactive bands was recorded on X-ray films (Kodak, Rochester, NY). All experiments were run in quadruplicate.

Testing the effect of benzamidine derivatives on *P. gingivalis* virulence using an egg model of infection

Fertile White Leghorn eggs were set in a humidified, self-turning incubator maintained at 37.8°C. Dose–lethality curves were established by intravenous inoculation through the chorioallantoin vein of 12-day-old chicken embryos with increasing numbers of *P. gingivalis* in Schaedler broth to determine 50 and 90% lethal doses (LD_{50} and LD_{90}). Based on the results of these experiments, fertilized eggs (24 for each inoculum) were inoculated with 2×10^6, 4×10^6, and 1×10^7 of *P. gingivalis* alone (12 eggs), or together with 20 μM of benzamidine derivatives. The viability of chicken embryos was checked hourly for the first 5 h by candling, and then by daily candling for up to 5 days post-infection. At the end of the experiments, randomly selected eggs were checked for the presence of viable bacteria by plating the allantoic fluid on Schaedler agar.

To determine if gingipains by themselves are pathogenic for fertilized eggs in this model, purified HRgpA, RgpB and Kgp at concentrations ranging from 1 nM to 100 nM were injected into eggs in the same manner as live bacteria. Six eggs were used for each group in these experiments.

RESULTS

Growth inhibition by benzamidine inhibitors

We have previously shown that benzamidine-derived compounds affected *P. gingivalis* growth (Eick *et al.*, 2003). Here we have confirmed these data by showing that benzamidine-derived inhibitors at 20 μM concen-

tration reduced the numbers of viable *P. gingivalis* in batch cultures in a time-dependent manner. Specifically, after 24 h, only a slight reduction occurred ($P < 0.05$ for *P. gingivalis* M5-1-2 and both inhibitors); however, by 48 h the number of colony-forming units had decreased by around 20% in the presence of 2,6-bis-(4-amidinobenzyl)-cyclohexanone ($P < 0.05$ for *P. gingivalis* M5-1-2), and by 90% when treated with pentamidine (both strains $P < 0.01$). Interestingly, benzamidine did not notably influence the growth of the bacterial strains tested. Additionally, the effect of inhibitors was apparently strain-independent (Fig. 2).

Separation of proteins on *p*-aminobenzamidine–Sepharose

To identify proteins that interact with benzamidine derivatives, *P. gingivalis* culture medium was subjected to affinity chromatography using *p*-aminobenzamidine–Sepharose. The chromatography resolved arginine-specific amidolytic activity into three peaks, one in the flow-through, a second eluted with 3 M NaCl (pool II) and a third, with the highest activity, eluted at 2 M benzamidine (pool III). In fractions eluted with salt and benzamidine the lysine-specific activity was very low, suggesting that Kgp did not bind to the benzamidine (Fig. 3).

The molecular mass of proteins obtained by chromatography on benzamidine–Sepharose, and estimated by SDS–PAGE, was in the range of 15 to 98 kDa. Proteins were subsequently identified by N-terminal

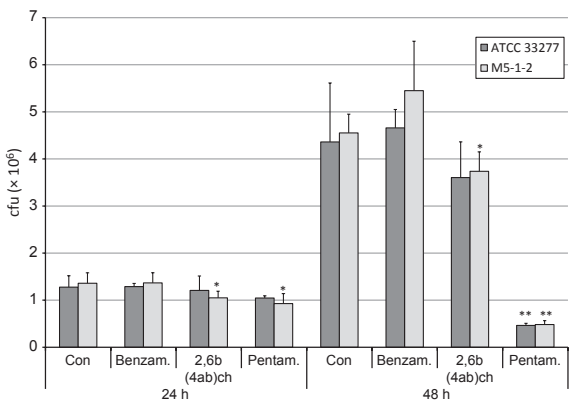

Figure 2 Growth inhibition of *Porphyromonas gingivalis* ATCC 33277 and *P. gingivalis* M5-1-2 (clinical isolate) by 20 μM benzamidine (benzam.), 2,6-bis-(4-amidinobenzyl)-cyclohexanone (2,6b(4ab)ch) and pentamidine (pentam.). *$P < 0.05$, **$P < 0.01$ compared with control.

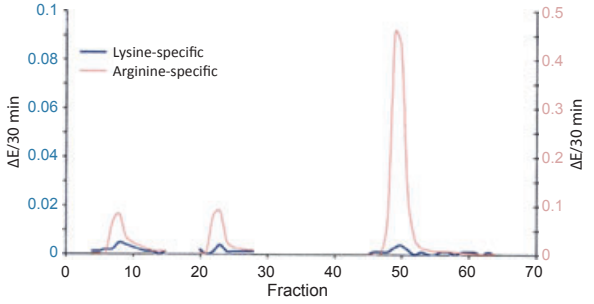

Figure 3 Arginine-specific and lysine-specific amidolytic activities separated by affinity chromatography on a benzamidine–Sepharose column.

sequence analysis, revealing the sequence YTPVEEKQNG in the presence of HRgpA (primary accession number P28784) in a 98-kDa band in pool II. On the other hand, an N-terminal sequence of YTPVEEKENG was determined for 15-kDa, 35-kDa and 48-kDa proteins in pool III, clearly indicating the presence of RgpB (primary accession number P95493). Most interesting, however, was an N-terminal sequence of AKEIKFDME identified by analysis of a 60-kDa protein in pool II that was unambiguously identified as GroEL (primary accession number P42375). GroEL is a ubiquitous protein in bacterial species, and belongs to the heat-shock protein (Hsp) 60 family.

Interaction with GroEL

Interaction of *p*-aminobenzamidine with GroEL suggests that benzamidine-derived compounds may affect expression of GroEL in *P. gingivalis*. To verify this assumption we analysed expression of *groEL* in *P. gingivalis* exposed to heat and benzamidine derivatives. Heat stress (1 h at 45°C) increased the mRNA expression of *groEL* in the M5-1-2 strain of *P. gingivalis* ($P < 0.05$). Similarly, supplementation of cultures with benzamidine and pentamidine led to a significant increase in mRNA levels for *groEL*. This effect was always slightly more pronounced in the clinical isolate M5-1-2 ($P < 0.01$) than in the reference strain ATCC 33277 ($P < 0.05$).

Changes in gingipain gene expression in response to stress and benzamidine derivatives appeared to be strain dependent. Specifically, strain ATCC 33277 responded to heat stress and the presence of benzamidine with increased expression of all gingipain genes (*rgpA* and *rgpB*; $P < 0.05$, *kgp*; $P < 0.05$, $P < 0.01$). Conversely, heat stress and benzamidine

decreased mRNA levels of both *rgpA* ($P < 0.05$, $P < 0.01$) and *kgp* (each $P < 0.01$) in strain M5-1-2. Pentamidine increased the mRNA expression of *rgpA* and *kgp* (each $P < 0.05$; Fig. 4).

Abundance of the GroEL protein in both *P. gingivalis* strains was enhanced by heat stress, 2,6-bis-(4-amidinobenzyl)-cyclohexanone and pentamidine. Densitometry scanning of band intensity allowed calculation of the relative protein contents of individual immunoreactive bands. This analysis revealed that GroEL increased by 100 and 300% in the ATCC 33277 and the M5-1-2 strains after heat exposure and by 50 and 100% after treatment to the benzamidine derivatives. Surprisingly, in the M5-1-2 strain additional bands with a lower molecular mass were immunoreactive with anti-GroEL antibodies, suggesting partial degradation of this protein (Fig. 5).

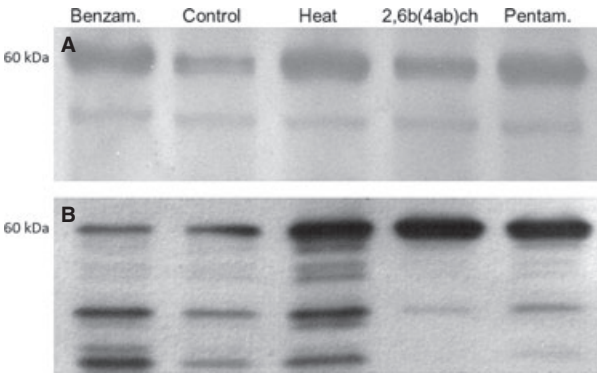

Figure 5 Representative Western blot illustrating the effect of heat shock (1 h) and exposure to benzamidine (benzam.), 2,6-bis-(4-amidinobenzyl)-cyclohexanone (2,6b(4ab)ch) and pentamidine (pentam.) on the abundance of GroEL in strains ATCC 33277 (A) and M5-1-2 (B). Samples were normalized so that the same amount of protein was loaded in each lane.

Virulence in chicken embryos

Gingipains are essential *P. gingivalis* virulence determinants. Therefore we tested if benzamidine-derived compounds are able to attenuate *P. gingivalis* virulence using a fertilized egg model. Most changes in viability of the eggs were observed 3 h after inoculation. Following that, this time-point was chosen for deter-

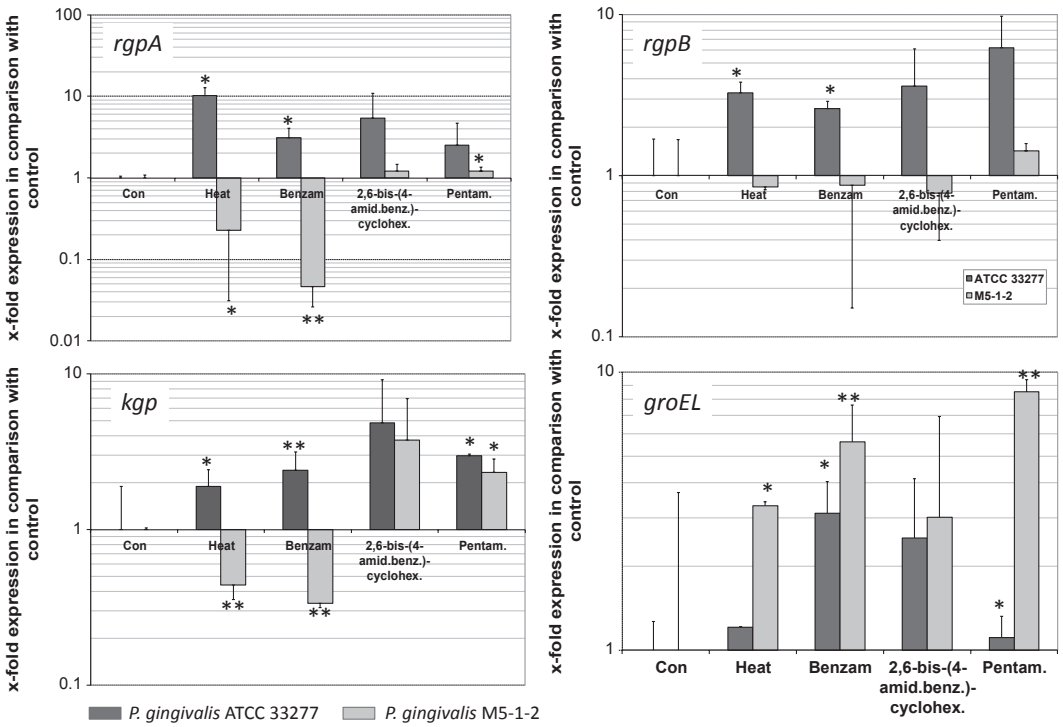

Figure 4 Effect of heat shock (1 h) and exposure to benzamidine (benzam.), 2,6-bis-(4-amidinobenzyl)-cyclohexanone (2,6-bis-(4-amid.-benz.)-cyclohex.) and pentamidine (pentam.) on expression of the gingipains (*rgpA*, *rgpB*, *kgp*) and *groEL* in *Porphyromonas gingivalis* ATCC 33277 and *P. gingivalis* M5-1-2 (clinical isolate); samples normalized to *sod* expression. Results are presented in comparison to *P. gingivalis* that was grown at 37°C without the addition of benzamidine derivatives (control – set to 1). *$P < 0.05$, **$P < 0.01$ compared with control.

mining the LD_{50} and LD_{90}. For both M5-1-2 and ATCC 33277 strains of *P. gingivalis* the LD_{90} of *c.*1×10^7 bacteria was determined.Conversely, the LD_{50} of 4×10^6 bacteria was determined for the ATCC 33277 strain, and of 2×10^6 bacteria for the clinical isolate M5-1-2 (Table 1). *Porphyromonas gingivalis* was cultured from the chorioallantoic fluid of all infected eggs in which embryos were killed, but no *P. gingivalis* cells could be detected from any viable egg at the end of the 5-day incubation.

The addition of benzamidine derivatives reduced the number of eggs killed, whereas benzamidine itself was only slightly effective. Dependent on the time-point, 20 μM 2,6-bis-(4-amidinobenzyl)-cyclohexanone reduced lethality of the ATCC 33277 strain by 38–71% and those of the M5-1-2 strain by 5–71%. The respective values for pentamidine were 13–67% for the ATCC 33277 strain and 17–38% for the M5-1-2 strain (Table 2).

Of note, separate injections of each gingipain at 100 nM concentrations were 100% lethal to inoculated eggs by 2 h. Lower concentrations of Arg-gingipains (1 and 10 nM) did not kill eggs up to 5 h post-inoculum, and only one embryo was dead after 24 h. In contrast, Kgp at 10 nM exerted a strong lethal effect, killing five out of the six inoculated eggs within 2 h after injection (Table 3).

DISCUSSION

The *in vitro* egg model detailed herein has previously been used for the testing of virulence for *Haemophilus influenzae* (Melhus *et al.*, 1998) and *Streptococcus pyogenes* (Schmidt *et al.*, 2001). In this system the antimicrobial activity relies largely on phagocytosis, because the phagocytic system is fully developed in 12-day-old chicken embryos (Schmidt *et al.*, 1993, 2001). At this time, however, lymphocytes are not detectable (Schmidt *et al.*, 1993). Here we show that *P. gingivalis* was lethal to fertilized eggs; however, far greater bacterial counts were needed to kill eggs in comparison to *S. pyogenes* (Schmidt *et al.*, 1997); indicating that *P. gingivalis* is less virulent in this model of infection. Chicken embryos were generally immune to inoculation with up to 1×10^6 bacteria, although interestingly,when eggs were inoculated with higher doses, surviving embryos were free of viable *P. gingivalis* cells, indicating the presence of effective mechanisms for bacterial killing. This result also suggests that the total clearance of infection is necessary for embryo survival. The application of 100 nM of each gingipain resulted in the immediate death of embryos, arguing for high toxicity of these proteolytic enzymes in this model. Using 10 nM gingipains we showed that Lys-gingipain was much more lethal, killing five of six embryos (83%), than Arg-gingipains, which killed only one of six (17%) embryos. It must be kept in mind, however, that in human gingival crevicular fluid, concentrations of Arg-gingipains (up to 1 μM) are much higher than that of Lys-gingipain (up to 10 nM) (Guentsch *et al.*, 2011, 2012). One can only speculate as to how gingipain kills embryos in this infection system. For example, the gingipain lethal effect may be related to cleavage of cell adhesion molecules, exerted by HRgpA, RgpB and Kgp (Sheets *et al.*, 2006), or the degradation of hemoglobin by Kgp (Curtis *et al.*, 2002). Importantly, the time-dependent and dose-dependent lethal effects

Table 1 Percentages of viable eggs (mean and standard deviation) at different times after inoculation with *Porphyromonas gingivalis* ATCC 33277 and *P. gingivalis* M5-1-2 (clinical isolate)

	0 h	1 h	2 h	3 h	4 h	5 h	24 h
Controls	100	100 ± 0	100 ± 0	1002 ± 0	100 ± 0	100 ± 0	100 ± 0
ATCC 33277							
2×10^6	100	95 ± 7	85 ± 14	78 ± 19	77 ± 21	75 ± 23	72 ± 24
4×10^6	100	87 ± 13	70 ± 28	35 ± 28.5	23 ± 34	23 ± 34	23 ± 34
10^7	100	60 ± 40	17 ± 37	0 ± 0	0 ± 0	0 ± 0	0 ± 0
M5-1-2							
2×10^6	100	870 ± 19	75 ± 26	57 ± 38	48 ± 45	48 ± 45	43 ± 66
4×10^6	100	72 ± 18	57 ± 25	27 ± 39	22 ± 40	17 ± 29	13 ± 22
10^7	100	38 ± 40	22 ± 40	12 ± 18	7 ± 9	5 ± 7	3 ± 7

Each group consisted of 12 fertilized eggs. The values represent results from three independent experiments each meaning at all 252 eggs were included in the assays.

Table 2 Effect of benzamidine and benzamidine derivatives (applied at 20 μM concentration) on embryo mortality induced by inoculation of fertilized eggs with *Porphyromonas gingivalis* (4×10^6 CFU/egg)

	Number of dead eggs with/without inhibitor						Total no. of eggs with/without inhibitor
	0 h	1 h	2 h	3 h	5 h	24 h	
2,6-bis-(4-amidinobenzyl)-cyclohexanone[1]							
Controls	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	24/24
ATCC 33277	0/0 (0)	2/4 (50)	2/7 (71)	10/16 (38)	10/18 (45)	11/18 (39)	24/24
M5-1-2	0/0 (0)	2/7 (71)	6/11 (45)	16/17 (6)	17/19 (11)	21/22 (5)	24/24
Pentamidine							
Controls	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	24/24
ATCC 33277	0/0 (0)	1/3 (67)	5/8 (38)	13/15 (13)	14/18 (22)	16/19 (16)	24/24
M5-1-2	0/0 (0)	5/6 (17)	7/10 (30)	12/18 (33)	13/21 (38)	16/21 (24)	24/24
Benzamidine							
Controls	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	24/24
ATCC 33277	0/0 (0)	2/3 (33)	5/7 (24)	15/15 (0)	19/19 (0)	18/18 (0)	24/24
M5-1-2	0/0 (0)	5/7 (29)	10/10 (0)	17/18 (6)	20/20 (0)	19/19 (0)	24/24

Results are shown as: numbers of dead eggs in the presence of inhibitor / number of dead eggs in the absence of inhibitor (% of reduction of dead eggs by addition of an inhibitor).
[1]None of the inhibitors by themselveswere toxic to eggs.

Table 3 Effect of gingipains on the lethality of fertilized eggs

		Dead eggs after:						
Gingipain	Concn (nM)	1 h	2 h	3 h	4 h	5 h	24 h	Total
Control	–	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6
HRgpA	100	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6
	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6
	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	6
RgpB	100	3 (50)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6
	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	6
	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	6
Kgp	100	1 (17)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6
	10	0 (0)	0 (0)	2 (33)	3 (50)	3 (50)	5 (83)	6
	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6

Shown are the cumulative numbers (%) of dead eggs after inoculation with HRgpA, RgpB and Kgp. For each, six eggs were tested.

exerted by gingipains and *P. gingivalis* on chicken embryos were similar. At high concentrations, the embryos had already died after 2 h, whereas at low concentrations this process was extended to 24 h. This underlines once more that gingipains are one of the most essential virulence factors of *P. gingivalis*.

Benzamidine-type inhibitors modulated the virulence properties of *P. gingivalis*, with the chicken embryo model proving to be a suitable system for investigating the influence of inhibitors on bacterial virulence. In this model benzamidine showed no strong protective effect on embryo killing by *P. gingivalis.* Conversely, both derivatives, 2,6-bis-(4-amidinobenzyl)-cyclohexanone and pentamidine, substantially reduced the lethality of *P. gingivalis* strains. Of note, lethality was not completely eliminated, leading to the conclusion that the inhibitors do not block all virulence-related activities, as a number of bacterial cells remained viable. This contention is supported by the fact that inhibitors block the activity of Arg-gingipains, but not Lys-gingipain. Interestingly, the significant difference in K_i-values for Rgps inhibition by 2,6-bis-(4-amidinobenzyl)-cyclohexanone and pentamidine did not corresponded to their protective effect. The latter

compound, although a much weaker inhibitor of Rgps than the 2,6-bis-(4-amidinobenzyl)-cyclohexanone, yielded the same efficient protection of embryo as 2,6-bis-(4-amidinobenzyl)-cyclohexanone. This suggests that the pentamidine-dependent protective effect against *P. gingivalis* in fertilized eggs relies on interaction of this compound with target(s) other than Rgps. The protective effect may be exerted by the ability of benzamidine and benzamidine derivatives to enhance phagocytotic capacity of chicken phagocytes, as was found for *P. gingivalis* clearance by human peripheral blood neutrophils (Eick *et al.*, 2003). The presence of a fully developed phagocytic system in fertilized eggs supports this contention.

The purification of Arg-gingipains by means of a benzamidine–Sepharose column confirmed that these inhibitors have affinity with Arg-gingipains. The binding was very strong, especially for RgpB, which was found in pool III eluted from the column with 2 M benzamidine. In pool II, HRgpA, and surprisingly GroEL (Hsp60), were identified as the main proteins. Hsp60 is a conserved molecular chaperone present in eubacteria as well as in eukaryotic mitochondria, suggesting that eukaryotic Hsp60 has a eubacterial origin (Viale & Arakaki, 1994). Because benzamidine is a polar molecule unable to penetrate lipid membranes we limited our analysis to cell-free culture medium. GroEL of periodontopathogens was purified from cell lysates (Hinode *et al.*, 1995) and in *Aggregatibacter actinomycetemcomitans* GroEL-like protein was found in membrane, cytoplasmic and periplasmic cell fractions (Paju *et al.*, 2000). Conversely, however, in *Helicobacter pylori* an etiological agent in chronic gastritis, a GroEL homolog was found in the extracellular fraction (Vanet & Labigne, 1998). Also, our results indicate a possible extracellular release of GroEL by *P. gingivalis*, which needs to be verified in follow-up studies. GroEL is present in all strains of *P. gingivalis* tested to date (Lu & McBride, 1994; Vayssier *et al.*, 1994), and may play some role in the pathogenesis of periodontitis. For example, *P. gingivalis* GroEL interacts with Toll-like receptors 2 and 4 (Argueta *et al.*, 2006). Additionally, the molecular mimicry between GroEL of *P. gingivalis* and human Hsp60 is apparently responsible for the immunological cross-reactivity of serum antibodies against GroEL with Hsp60 (Tabeta *et al.*, 2000). Recently it was shown that atherosclerotic patients contain serum antibodies cross-reacting with peptide 19 of *P. gingivalis* GroEL, suggesting that the peptide is an immunoreactive epitope in the association of periodontitis and atherosclerosis (Choi *et al.*, 2011). Seroreactivity against this peptide was also predominant in rheumatoid arthritis patients with ongoing periodontitis (Jeong *et al.*, 2012). Moreover, *P. gingivalis* GroEL has been discussed as a candidate for vaccine formulation in preventing periodontal disease (Choi *et al.*, 2005; Lee *et al.*, 2006).

The treatment of *P. gingivalis* with benzamidine and benzamidine-derived inhibitors enhanced the mRNA expression of *groEL* in a compound-dependent and strain-dependent manner. Protein expression was not in exact accordance with the mRNA levels. Weak correlation between mRNA expression and protein level is not uncommon because translation of mRNA is regulated by many factors independently of gene transcription. Moreover, mRNA as well as proteins can be degraded (Picard *et al.*, 2009). Accordingly, we found additional bands in immunoblots of the clinical strain that may indicate a cleavage and or degradation of GroEL. The transcriptional response of gingipain genes in *P. gingivalis* exposed to heat and benzamidine derivatives was seemingly strain-dependent. This finding correlates with previous observations showing a differential response of the gingipain genes to heat-shock in different strains. In the ATCC 33277 strain, heat-shock stimulated the expression of Arg-gingipains (Shelburne *et al.*, 2005), whereas in W50, as with our M5-1-2 strain, this stress negatively impacted levels of Arg-gingpain mRNA (Percival *et al.*, 1999).

Pentamidine is a clinical drug of choice in the treatment of human protozoal infections (Werbovetz, 2006). In addition, in an aerosol form, pentamidine is used to prevent *Pneumocystis jiroveci* pneumonia, which is a serious health risk for immunocompromised patients (Marras *et al.*, 2002). Although pentamidine binds to DNA (Edwards *et al.*, 1992) and RNA (Sun & Zhang, 2008), the precise mechanism of its antimicrobial activity is still unknown. Here we show that pentamidine and 2,6-bis-(4-amidinobenzyl)-cyclohexanone can be used to abrogate *P. gingivalis* virulence. In this case, the protective effect might be exerted by the cumulative effect of inhibition of Arg-gingipains, binding to GroEL, and stimulation of phagocytosis. Yet unknown factors induced by benzamidine derivatives may also contribute to quenching of the *P. gingivalis* pathogenicity. In this context, topical application of pentamidine and

2,6-bis-(4-amidinobenzyl)-cyclohexanone might be a promising development for supportive periodontitis treatment.

ACKNOWLEDGEMENTS

This paper is dedicated to Jörg Stürzebecher (Institute of Vascular Biology and Medicine, University Hospital of Jena) who passed away in 2008. Jörg Stürzebecher encouraged us to investigate the effect of these inhibitors on gingipains. He designed the inhibitors and purified the Arg-gingipains by means of affinity chromatography on a benzamidine–Sepharose column. We are grateful to him for providing the compounds and for all his helpful advice.

The authors would also like to thank Claudia Ranke and Katja Sack for excellent technical assistance. Dr Eckhard Birch-Hirschfeld, University Hospital of Jena, is acknowledged for synthesizing primers. We are indebted to Bernhard Schlott (Leibniz Institute for Age Research, Jena) for N-terminal sequencing. Most of the study was funded by the participating departments, and supported by IUVENTUS Plus (0221/IP1/2011/71, MNiSW, Warsaw, Poland), along with grants from: the National Institutes of Health (Grant DE 09,761, USA), National Science Center (2011/01/B/NZ6/00,268, Kraków, Poland), the European Community (FP7-HEALTH-2010-261,460 *Gums&Joints*), MNiSW (2136/7. PR UE/2011/2, Warsaw, Poland) and the Foundation for Polish Science (TEAM project DPS/424-329/10). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of structural funds from the European Union (grant No: POIG.02.01.00-12-064/08 —*Molecular biotechnology for health*).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this study.

REFERENCES

Argueta, J.G., Shiota, S., Yamaguchi, N., Masuhiro, Y. and Hanazawa, S. (2006) Induction of *Porphyromonas gingivalis* GroEL signaling via binding to Toll-like receptors 2 and 4. *Oral Microbiol Immunol* **21**: 245–251.

Choi J. I., Choi K. S., Yi N. N., Kim U. S., Choi J. S. and Kim S. J. (2005) Recognition and phagocytosis of multiple periodontopathogenic bacteria by anti-*Porphyromonas gingivalis* heat-shock protein 60 antisera. *Oral Microbiol Immunol* **20**: 51–55.

Choi, J., Lee, S.Y., Kim, K. and Choi, B.K. (2011) Identification of immunoreactive epitopes of the *Porphyromonas gingivalis* heat shock protein in periodontitis and atherosclerosis. *J Periodontal Res* **46**: 240–245.

Curtis, M.A., Aduse Opoku, J., Rangarajan, M. *et al.* (2002) Attenuation of the virulence of *Porphyromonas gingivalis* by using a specific synthetic Kgp protease inhibitor. *Infect Immun* **70**: 6968–6975.

Edwards, K.J., Jenkins, T.C. and Neidle, S. (1992) Crystal structure of a pentamidine–oligonucleotide complex: Implications for DNA-binding properties. *Biochemistry* **31**: 7104–7109.

Eick, S., Pfister, W., Sturzebecher, U., Jarema, S. and Sturzebecher, J. (2003) Inhibitors of benzamidine type influence the virulence properties of *Porphyromonas gingivalis* strains. *Acta Biochim Pol* **50**: 725–734.

Ezzo, P.J. and Cutler, C.W. (2003) Microorganisms as risk indicators for periodontal disease. *Periodontol 2000* **32**: 24–35.

Genco, R.J. (1996) Current view of risk factors for periodontal diseases. *J Periodontol* **67**: 1041–1049.

Guentsch, A., Kramesberger, M., Sroka, A., Pfister, W., Potempa, J. and Eick, S. (2011) Comparison of gingival crevicular fluid sampling methods in patients with severe chronic periodontitis. *J Periodontol* **82**: 1051–1060.

Guentsch, A., Hirsch, C. and Pfister, W., et al. (2012) Cleavage of IgG1 in gingival crevicular fluid is associated with the presence of Porphyromonas gingivalis. *J Periodontal Res*. Nov 1. doi: 10.1111/jre.12027. [Epub ahead of print].

Guo, Y., Nguyen, K.A. and Potempa, J. (2010) Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontol 2000* **54**: 15–44.

Hayashi, C., Gudino, C.V., Gibson, F.C. 3rd and Genco, C.A. (2010) Review: pathogen-induced inflammation at sites distant from oral infection: bacterial persistence and induction of cell-specific innate immune inflammatory pathways. *Mol Oral Microbiol* **25**: 305–316.

Hinode, D., Grenier, D. and Mayrand, D. (1995) Purification and characterization of a DnaK-like and a GroEL-like protein from *Porphyromonas gingivalis*. *Anaerobe* **1**: 283–290.

Jeong, E., Lee, J.Y., Kim, S.J. and Choi, J. (2012) Predominant immunoreactivity of Porphyromonas gingivalis

heat shock protein in autoimmune diseases. *J Periodontal Res* **47**: 811–816.

Katz, J., Yang, Q.B., Zhang, P. *et al.* (2002) Hydrolysis of epithelial junctional proteins by Porphyromonas gingivalis gingipains. *Infect Immun* **70**: 2512–2518.

Lamont, R.J. and Jenkinson, H.F. (2000) Subgingival colonization by *Porphyromonas gingivalis*. *Oral Microbiol Immunol* **15**: 341–349.

Lee J. Y., Yi N. N., Kim U. S., Choi J. S., Kim S. J. and Choi, J. I. (2006) *Porphyromonas gingivalis* heat shock protein vaccine reduces the alveolar bone loss induced by multiple periodontopathogenic bacteria. *J Periodontal Res* **41**: 10–14.

Lu, B. and McBride, B.C. (1994) Stress response of *Porphyromonas gingivalis*. *Oral Microbiol Immunol* **9**: 166–173.

Marras T. K., Sanders K., Lipton J. H., Messner H. A., Conly J. and Chan, C. K. (2002) Aerosolized pentamidine prophylaxis for *Pneumocystis carinii* pneumonia after allogeneic marrow transplantation. *Transpl Infect Dis* **4**: 66–74.

Melhus, A., Hermansson, A., Forsgren, A. and Prellner, K. (1998) Intra- and interstrain differences of virulence among nontypeable *Haemophilus influenzae* strains. *APMIS* **106**: 858–868.

Mikolajczyk-Pawlinska, J., Kordula, T., Pavloff, N. *et al.* (1998) Genetic variation of *Porphyromonas gingivalis* genes encoding gingipains, cysteine proteinases with arginine or lysine specificity. *Biol Chem* **379**: 205–211.

Paju S., Goulhen F., Asikainen S., Grenier D., Mayrand D. and Uitto, V. (2000) Localization of heat shock proteins in clinical *Actinobacillus actinomycetemcomitans* strains and their effects on epithelial cell proliferation. *FEMS Microbiol Lett* **182**: 231–235.

Percival, R.S., Marsh, P.D., Devine, D.A. *et al.* (1999) Effect of temperature on growth, hemagglutination, and protease activity of *Porphyromonas gingivalis*. *Infect Immun* **67**: 1917–1921.

Picard, F., Dressaire, C., Girbal, L. and Cocaign-Bousquet, M. (2009) Examination of post-transcriptional regulations in prokaryotes by integrative biology. *C R Biol* **332**: 958–973.

Popadiak, K., Potempa, J., Riesbeck, K. and Blom, A.M. (2007) Biphasic effect of gingipains from *Porphyromonas gingivalis* on the human complement system. *J Immunol* **178**: 7242–7250.

Potempa, J., Pike, R. and Travis, J. (1997) Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. *Biol Chem* **378**: 223–230.

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

Schmidt K. H., Wiesner J., Gerlach D., Reichardt W., Ozegowski J. H. and Kohler, W. (1993) Susceptibility of chicken embryos to group A streptococci: correlation with fibrinogen binding. *FEMS Immunol Med Microbiol* **7**: 231–240.

Schmidt, K.H., Podbielski, A., Reichardt, W., Gubbe, K. and Amberg, C. (1997) Virulence of *Streptococcus pyogenes* for chicken embryos after isogenic inactivation of different streptococcal pathogenicity factors. *Adv Exp Med Biol* **418**: 793–795.

Schmidt, K.H., Gerlach, D., Gubbe, K. *et al.* (2001) Virulence of group A streptococci in fertile hens eggs is mainly effected by M protein and streptolysin O. *Int J Med Microbiol* **291**: 45–56.

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

Shelburne, C.E., Gleason, R.M., Coulter, W.A., Lantz, M.S. and Lopatin, D.E. (2005) Differential display analysis of *Porphyromonas gingivalis* gene activation response to heat and oxidative stress. *Oral Microbiol Immunol* **20**: 233–238.

Sun, T. and Zhang, Y. (2008) Pentamidine binds to tRNA through non-specific hydrophobic interactions and inhibits aminoacylation and translation. *Nucleic Acids Res* **36**: 1654–1664.

Tabeta, K., Yamazaki, K., Hotokezaka, H., Yoshie, H. and Hara, K. (2000) Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. *Clin Exp Immunol* **120**: 285–293.

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

Vanet, A. and Labigne, A. (1998) Evidence for specific secretion rather than autolysis in the release of some *Helicobacter pylori* proteins. *Infect Immun* **66**: 1023–1027.

Vayssier, C., Mayrand, D. and Grenier, D. (1994) Detection of stress proteins in *Porphyromonas gingivalis* and other oral bacteria by western immunoblotting analysis. *FEMS Microbiol Lett* **121**: 303–307.

Viale, A.M. and Arakaki, A.K. (1994) The chaperone connection to the origins of the eukaryotic organelles. *FEBS Lett* **341**: 146–151.

Vincents, B., Guentsch, A., Kostolowska, D. *et al.* (2011) Cleavage of IgG1 and IgG3 by gingipain K from *Porphyromonas gingivalis* may compromise host defense in progressive periodontitis. *FASEB J* **25**: 3741–3750.

Wegner, N., Wait, R., Sroka, A. *et al.* (2010) Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum* **62**: 2662–2672.

Werbovetz, K. (2006) Diamidines as antitrypanosomal, antileishmanial and antimalarial agents. *Curr Opin Investig Drugs* **7**: 147–157.