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Inhibition of *Porphyromonas gingivalis* proteinases (gingipains) by chlorhexidine: synergistic effect of Zn(II)

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Background/aims: Gingipains, proteolytic enzymes produced by the periodontal pathogen *Porphyromonas gingivalis*, are regarded as virulence factors in the pathogenesis of periodontitis. Inhibition of gingipain activity therefore may have therapeutic potential, and it has been suggested that chlorhexidine may inhibit the activities of these enzymes. The purposes of the present study were to examine systematically the inhibitory effects of chlorhexidine on three purified gingipains and to determine the effect of Zn(II) on chlorhexidine inhibition.
Methods: The activities of lys-gingipain (Kgp) and two forms of arg-gingipain (RgpB and HRgpA) were measured in the presence of varying concentrations of chlorhexidine and with chlorhexidine supplemented with Zn(II). Inhibition constants (K_i's) were determined for chlorhexidine alone and in the presence of Zn(II). Fractional inhibitory constant indices were calculated to assess the synergy of the chlorhexidine–Zn(II) inhibition.
Results: RgpB, HRgpA, and Kgp were all inhibited by chlorhexidine with K_i's in the micromolar range. For RgpB and HRgpA, the inhibitory effects of chlorhexidine were enhanced 3–30-fold by Zn(II). The chlorhexidine–Zn(II) interaction was synergistic for inhibition of HRgpA and RgpB. For Kgp, the effect of Zn(II) on chlorhexidine inhibition was antagonistic.
Conclusions: Chlorhexidine is an effective inhibitor of gingipains, and the inhibition of R-gingipains is enhanced by Zn(II). A mixture of chlorhexidine and Zn(II) may be useful as an adjunct in the treatment of periodontitis and in the post-treatment maintenance of periodontitis patients.

Key words: chlorhexidine; gingipains; inhibition; synergism; zinc

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Chronic periodontitis is widespread in humans and is the cause of most tooth loss in the adult population. The microbial etiology of chronic adult periodontitis is complex and polymicrobial. Nevertheless, it is known that *Porphyromonas gingivalis*, together with *Tannerella forsythia* and *Treponema denticola*, is a member of the so-called 'red complex', which has been strongly associated with clinical measures of periodontal disease such as pocket depth and bleeding on probing (35). In addition, it has been shown that *P. gingivalis* has great pathogenic potential in an animal model of periodontitis, causing rapid and significant bone loss when implanted in the periodontal microflora of monkeys (13). Thus both clinical and experimental evidence support the contention that this organism plays a major causative role in the development of chronic periodontitis in adults.

P. gingivalis produces and releases high concentrations of a large variety of proteolytic enzymes, including those with 'trypsin-like activity', their presence being related to the virulence of the organism (16, 31). Enzyme purification and gene cloning studies have revealed that the 'trypsin-like' activity of *P. gingivalis* consists of two types of cysteine proteinases with specificity for either Arg-Xaa or Lys-Xaa peptide bonds, commonly referred to

as gingipains R and K, respectively (30, 31). Three genes, *rgpA*, *rgpB* and *kgp*, encode for individual activities known as HRgpA, RgpB, and Kgp. HRgpA is a 95-kDa protein consisting of a catalytic domain in stable non-covalent association with hemagglutinin/adhesin domain or domains, while RgpB (50 kDa) has only the catalytic domain and a small C-terminal fragment of the hemagglutinin/adhesin domain. The predominant form of Kgp in culture fluid is a 105-kDa stable noncovalent complex of a catalytic domain with hemagglutinin/adhesin domains. Taken in concert these enzymes activate or inactivate a number of human host proteins, contributing to the pathogenesis of periodontitis. A partial list includes degradation of cytokines, components of the complement system, and receptors on macrophages and T cells, thus perturbing host gingival host defenses. Mediation of the destruction of periodontal tissue is also known to occur through stimulation of gingival fibroblasts to produce matrix metalloproteinases accompanied by direct activation of secreted but latent matrix metalloproteinases by gingipains (17, 29).

Because of the apparent importance of gingipains in the pathogenesis of periodontal disease, there has been considerable interest in discovering or designing inhibitors of gingipain activity with therapeutic potential (34). Chlorhexidine mouthrinse is widely used as an adjunct in the treatment of periodontitis (19), and early studies showed that chlorhexidine inhibited a number of glycosidic and proteolytic activities of a variety of oral bacteria including *P. gingivalis* (1). More recently, it has been demonstrated that chlorhexidine is an inhibitor of several matrix metalloproteinases and also inhibits the activity of cell-associated 'collagenase' activity of *P. gingivalis* (8, 14). In clinical studies, oral health care products (toothpaste and mouthrinse) containing mixtures of chlorhexidine and Zn(II) appeared to be more effective at controlling plaque, gingivitis, and intraoral volatile sulfur compounds (i.e. oral malodor) than products containing chlorhexidine alone (9, 10, 32, 36). The goals of the present study were to investigate whether chlorhexidine inhibited the purified gingipains HRgpA, RgpB, and Kgp, and if such inhibition was enhanced by Zn(II).

Material and methods

Enzymes and enzyme assays

Gingipains HRgpA, RgpB, and Kgp were purified from culture fluids of *P. gingivalis* H66 and the activities were determined as previously described (4, 30, 31), with stocks of purified enzymes being stored at −80°C. Briefly, all gingipains were assayed in a standard buffer (0.2 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$, pH 7.6, freshly supplemented with 10 mM cysteine; standard assay). In addition, HRgpA and RgpB were assayed in a second system (0.2 M Tris, 0.1 M glycylglycine, 5 mM $CaCl_2$, pH 7.6, freshly supplemented with 10 mM cysteine; gly– gly assay) to take advantage of the specific stimulation of R-gingipain activity by glycylglycine (4). Assays were performed at room temperature (approximately 22°C) using a microplate reader (Spectramax Plus, Molecular Devices) set at 405 nm in the kinetics mode. Reaction mixtures (200 µl per well) containing buffer, enzyme, and inhibitor when required, were mixed and allowed to equilibrate for 10 min, with assays being initiated by addition of substrate in the appropriate buffer. Enzyme concentrations in the assays were 30.0 nM, 17.5 nM, and 52.4 nM for RgpB, HRgpA, and Kgp, respectively. The substrates were benzoyl-arginine *p*-nitroanilide (BApNA) for Rgps and valine-leucine-lysine *p*-nitroanilide (VLK-pNA) for Kgp. All assays were performed in triplicate and results expressed as the mean ± standard deviation.

Inhibitors

Chlorhexidine digluconate (chlorhexidine; 20% aqueous solution; 222.2 mM) and zinc acetate were obtained from Sigma (St. Louis, MO). Stock chlorhexidine solutions were prepared in deionized water and stock zinc acetate solutions in assay buffers. Both were then diluted in the appropriate assay buffer to give concentrations as indicated in figures and tables. Mixtures contained chlorhexidine and Zn(II) in a 1 : 1.1 molar ratio (23).

Determination of inhibition constants

Initial reaction rates were obtained as mOD_{405} min^{-1} at substrate concentrations of 0.500, 0.250, and 0.125 mM. Inhibitor concentrations ranged from 0 to 1111 µM for chlorhexidine and from 0 to 1225 µM for Zn(II). Data were graphed as double reciprocal (Lineweaver-Burk) plots and the slopes determined by first-order linear regression (SigmaPlot). Inhibitor constants (K_i's) were determined by plotting slopes of Lineweaver-Burk plots (vertical axis) against inhibitor concentration (horizontal axis), deriving an equation for each data set by first-order linear regression (SigmaPlot), and solving the regression equation (5).

Fractional inhibitory concentration index

The fractional inhibitory concentration index (FIC index) is a means to determine whether two or more inhibitors, antibiotics, etc., interact synergistically, additively, or antagonistically (2). The FIC index is calculated as

$$\text{FIC Index} = [(A + B)/A] + [(A + B)/B]$$

where A represents the effect of chlorhexidine, B the effect of Zn(II), and A +B the effect of the combination. An FIC index < 1 indicates synergism, FIC index $= 1$ indicates an additive effect, and FIC index > 1 indicates antagonism.

Results

Inhibition of gingipain activity

Figure 1 shows typical results for inhibition of gingipain activity by chlorhexidine and chlorhexidine + Zn(II). In this experiment, RgpB activity was measured in

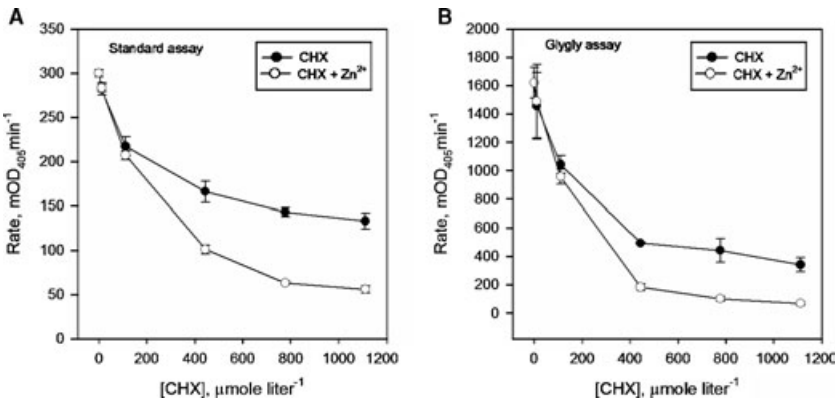

Fig. 1. Inhibition of RgpB as a function of CHX concentration, with and without Zn(II). For each CHX concentration, Zn(II) was fixed at 1.1 [CHX]. A, Standard assay. B, Glycyl–glycine assay. Note differences in scale due to glycylglycine stimulation of RgpB activity.

0.500 mM BApNA using both standard (Fig. 1A) and gly–gly added assays (Fig. 1B) (note differences in rate scales reflecting stimulation of R-gingipain activity by gly–gly). In both assays, RgpB activity was inhibited by chlorhexidine in a concentration-dependent manner, and this inhibition was enhanced by Zn(II). Similar results were obtained in both assays for RgpB at BApNA concentrations of 0.250 and 0.125 mM, both assays for HRgpA activity at all three BApNA concentrations, and Kgp in the standard assay at all three VLK-pNA concentrations (data not shown).

Determination of K_i's

K_i's were determined in a two-step process as shown in Fig. 2 and 3. Figure 2 shows double reciprocal (Lineweaver-Burk) plots of all RgpB data including those shown in Fig. 1. Similar plots were done for all HRgpA data in both assays, and for Kgp in the standard assay (data not shown). All plots were analyzed by linear regression, and the slopes of the regression lines were plotted as a function of chlorhexidine concentration, as shown in Fig. 3. From these plots a value for K_i could then be determined (5). The results of this analysis are summarized in Table 1. Chlorhexidine alone inhibited all forms of gingipain tested with K_i's ranging from 165 to 689 μM, and chlorhexidine + Zn(II) mixtures were also inhibitory with K_i's for chlorhexidine ranging from 11 to 376 μM. Zn(II) enhanced the inhibition of RgpB and HRgpA but not Kgp. For RgpB and HRgpA, K_i's were somewhat elevated in the gly–gly assay as compared to the standard assay. However, relative enhancement of inhibition by Zn(II) was greater in the gly–gly assay. For the R-gingipains, inhibition was 3–30-fold greater with Zn(II) in combination with chlorhexidine.

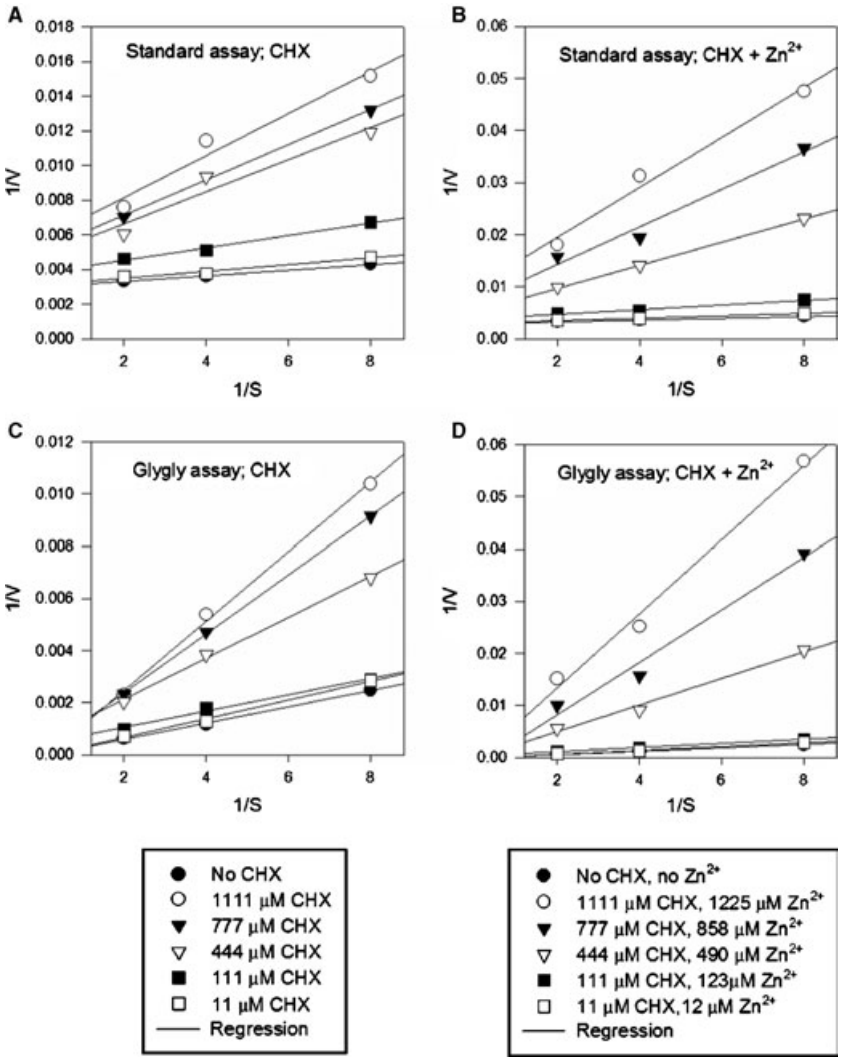

Fig. 2. Lineweaver-Burk plots of inhibition of RgpB by chlorhexidine in the presence or absence of Zn(II). The substrate was BApNA, and substrate concentrations were 0.125, 0.250, and 0.500 mM. Assay conditions and concentrations of chlorhexidine (CHX) and Zn(II) were as indicated. Lines associated with each set of points represent linear regression analysis of the respective data sets.

Determination of FIC index

The determination of FIC index is summarized in Table 2. Reactions were run as indicated, with [substrate] = 0.500 mM, [chlorhexidine] = 1111 μM, and [Zn(II)] = 1225 μM. These concentrations were chosen to maximize the differences between inhibition by chlorhexidine alone and the chlorhexidine + Zn(II) mixture (Fig. 1). Furthermore, Zn(II) is weakly or noninhibitory for RgpB at concentrations of 1 mM or lower (23). Results were expressed as mean ± standard deviation, relative to no-inhibitor controls. Calculations of FIC indices indicated that chlorhexidine and Zn(II) inhibited R-gingipains synergistically and inhibited Kgp somewhat antagonistically. For both HRgpA and RgpB, synergism was markedly stronger in the gly–gly assay.

Discussion

Chlorhexidine has long been valued as an adjunct in the treatment of periodontitis due to its combination of antimicrobial effect, substantivity, and lack of tissue irritation (19). These unique attributes explain the overall effects of chlorhexidine in helping to control gingivitis and the accumulation of dental plaque. In addition to its general antimicrobial effect, chlorhexidine also inhibits bacterial sugar uptake systems (22) as well as extracellular and cell-associated bacterial glycosidases and proteases (1). Human matrix metalloproteinases 2, 8, and 9 are inhibited by chlorhexidine *in vitro* (8). While the inappropriate activity of these enzymes is thought to be important in the destruction of tooth-supporting tissues that is a hallmark of periodontal diseases (8), it seems unlikely that chlorhexidine would penetrate into intratissue locations in which matrix metalloproteinases are made. However, irrigation with chlorhexidine or other agents seems to be of value in periodontal therapy (7, 20), and one can speculate that irrigation with chlorhexidine–Zn(II) mixtures could have both antimicrobial and antiproteolytic effects.

It is known that chlorhexidine directly inhibits the proteolytic activities of a number of periodontal pathogens including *P. gingivalis* (1). This organism is a member of the so-called 'red complex', which is strongly associated with active periodontitis

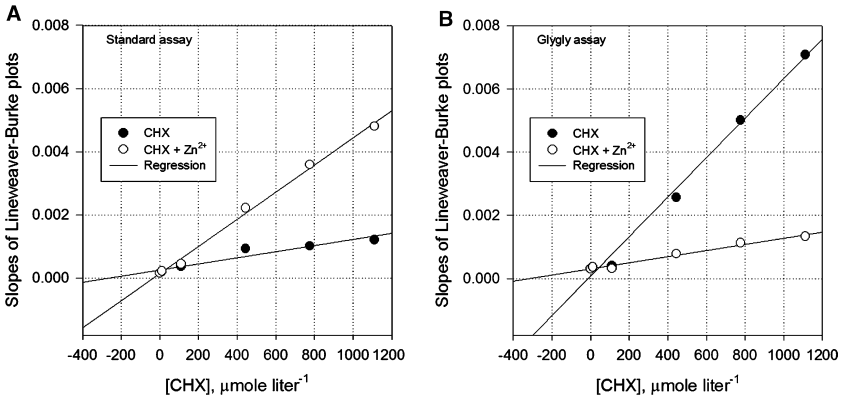

Fig. 3. Estimation of inhibition constant K_i for inhibition of RgpB by chlorhexidine (CHX), with or without Zn(II). Assay conditions were as indicated. Lines associated with each set of points represent linear regression analysis of the respective data sets. K_i's were estimated as the negative abscissa intercepts of the regression lines.

Table 1. Estimated inhibition constant K_i for inhibition of gingipains by chlorhexidine with or without Zn(II)

		K_i, μM	
Enzyme	Assay	Chlorhexidine	Chlorhexidine + Zn(II)
RgpB	Standard	262	36
	Gly–gly	320	11
HRgpA	Standard	387	119
	Gly–gly	689	62
Kgp	Standard	165	376

(35) and has at least 13 extracellular and cell-associated proteolytic activities. Predominant among this myriad of enzymes is a collection of cysteine proteinases known as gingipains (29). Arginine- and lysine-gingipains in concert account for the bulk of *P. gingivalis* proteolytic activity and exhibit a number of pathophysiologically significant actions, as described earlier (17, 29).

The antiproteolytic effects of chlorhexidine on *P. gingivalis* proteinase activities have been examined previously in a number of studies. In a survey of 16 species of dental plaque bacteria including *P. gingivalis*, proteolytic activities were highly sensitive to chlorhexidine, with at least 50% inhibition by 125 μM chlorhexidine (1). In a similar study (11), 0.01% chlorhexidine (111 μM) gave 50% inhibition of trypsin, chymotrypsin, proteinase K, and *P. gingivalis* cell-associated proteolytic activity. At 0.04% (444 μM) chlorhexidine produced essentially complete inhibition of all activities. In a related study the proteolytic activity of a *P. gingivalis* strain producing R- but not K-gingipain activity was completely inhibited by incubation for 2 h at 25°C in 1 mM chlorhexidine (14). In contrast, in chemostat-grown *P. gingivalis* (24) production of trypsin-like proteolytic activity was decreased by the presence of chlorhexidine in the growth medium and was essentially zero with 30 μg/ml chlorhexidine (33.3 μM). These studies all used washed cells, vesicles, or crude culture fluids as sources of proteolytic activity. The present study apparently is the first to examine systematically the effects of chlorhexidine concentration and assay conditions on activities of purified gingipains RgpB, HRgpA, and Kgp.

Estimated inhibition constants (K_i's) for inhibition of gingipains by chlorhexidine range from 262 to 689 μM for R-gingipains and 165 μM for K-gingipain, depending on assay conditions and the form of gingipain (Table 1). The concentration of chlorhexidine digluconate in clinical preparations used in the US is 0.2%, or 2.22 mM. This concentration represents a 3–13-fold molar excess over K_i's during a chlorhexidine oral rinse, assuming similar gingipain reaction conditions and chlorhexidine concentrations *in vitro* and *in vivo*. Although it is difficult to assess the concentration of chlorhexidine in a periodontal pocket *in vivo*, this analysis is consistent with the possibility that chlorhexidine inhibits gingipains *in vivo*. Furthermore, chlorhexidine inhibition of R-gingipains is enhanced 2–15-fold when Zn(II) is present, with K_i's ranging from 11 to 119 μM. For K-gingipain the chlorhexidine K_i is 376 μM when Zn(II) is present. The intraoral concentration after a rinse with 0.2% chlorhexidine has been estimated at 140 μM (3). Thus it is possible that postrinse chlorhexidine inhibition of R-gingipains could continue *in vivo* if Zn(II) was also present.

Combinations of Zn(II) with chlorhexidine or other agents have been used in a variety of oral healthcare preparations, and in general the combinations have been more effective than the single agents in reducing plaque formation, plaque acidogenicity and glycolytic activity, plaque microbial viability, gingivitis, and oral malodor (9, 10, 32, 36). When two or more agents are used simultaneously against the same target, the effects can be additive, synergistic, or antagonistic. In several earlier studies the inhibitory effects of chlorhexidine and Zn(II) were synergistic (9, 10, 36), and this is also the case

Table 2. Fractional inhibitory constant indices to assess synergy of inhibition of gingipains by chlorhexidine with or without Zn(II)

		Relative rate[a]				
Enzyme	Assay	No inhibitor	Zn(II)	Chlorhexidine	Chlorhexidine + Zn(II)	FIC Index[b]
HRgpA	Standard	1.000 ± 0.071[c]	0.743 ± 0.011	0.672 ± 0.032	0.261 ± 0.043	0.72
	Gly-gly	1.000 ± 0.073[d]	0.917 ± 0.085	0.394 ± 0.109	0.103 ± 0.011	0.37
RgpB	Standard	1.000 ± 0.006[e]	0.860 ± 0.008	0.492 ± 0.013	0.238 ± 0.033	0.76
	Gly-gly	1.000 ± 0.132[f]	0.520 ± 0.005	0.228 ± 0.013	0.048 ± 0.002	0.30
Kgp	Standard	1.000 ± 0.048 [g]	1.435 ± 0.058	0.516 ± 0.062	0.554 ± 0.073	1.46

[a]Rate ± standard deviation.
[b]Fractional inhibitory concentration index.
[c] Relative rate 1.000 corresponds to 253 mOD_{405} min^{-1}.
[d]Relative rate 1.000 corresponds to 1108 mOD_{405} min^{-1}.
[e]Relative rate 1.000 corresponds to 257 mOD_{405} min^{-1}.
[f]Relative rate 1.000 corresponds to 1360 mOD_{405} min^{-1}.
[g]Relative rate 1.000 corresponds to 10.6 mOD_{405} min^{-1}.

in the present study (Table 2) at least for R-gingipains. Fractional inhibitory concentration indices (2) for R-gingipains were all less than 1.00 (range 0.30–0.76) (Table 2). Stronger synergism was seen for both RgpB and HRgpA in the glycyl–glycine assay, in which R-gingipain activity is enhanced approximately 5-fold compared to the standard assay (Table 2). In contrast, chlorhexidine and Zn(II) were antagonistic for Kgp (Table 2), with an FIC index greater than 1.00. Nevertheless, even in the presence of Zn(II) the K_i for inhibition of Kgp by chlorhexidine is in the micromolar range (Table 1).

In previous work Zn(II) was only moderately effective as an inhibitor of R-gingipains, with estimated 27% and 48% inhibition of RgpB and HRgpA activities, respectively, by 1 mM Zn(II) (23). In the present study (Table 2), 1.225 mM Zn(II) alone inhibited R-gingipains from 8% to 48%, depending on the form of enzyme and assay conditions. These degrees of inhibition are modest, especially when compared with the effects of Zn(II) and chlorhexidine in combination (Table 2). Zn(II) had little or no inhibitory effect on Kgp, either alone or in combination with chlorhexidine.

Zn(II) enhancement of the potency and selectivity of proteinase inhibitors has been observed for the serine proteinases trypsin, thrombin, factor Xa, and tryptase, and a general paradigm has been presented for Zn(II)-mediated inhibition of serine proteinases (18). In this model, Zn(II) interacts with four chelating heteroatoms (N, O, or S). Two such heteroatoms are supplied by amino acids in the proteinase active site, and two are supplied by the inhibitor. This 'bridging' of the active site and inhibitor can enhance binding by as much as 1000–10,000-fold. This model apparently also holds at least for the R-gingipain family of cysteine proteinases, in which Zn(II) provides a 2–3-fold increase in potency of benzamidine inhibitors especially a bis-benzamidine with a urea linker (23). A proposed binding model has Zn(II) coordinating with the two urea nitrogens of the inhibitor and with the catalytic histidine and cysteine residues of the R-gingipain active site (23). Inspection of the structure of chlorhexidine (1,6-bis-(N^5-[p-chlorophenyl]-N^1-biguanido)hexane (Fig. 4A) suggests that nitrogens could serve to coordinate the chlorhexidine with Zn(II), with coordination to the R-gingipain active site catalytic histidine and cysteine residues as described for bis-benzamidine (Fig. 4B). As measured by decrease in K_i, this leads to

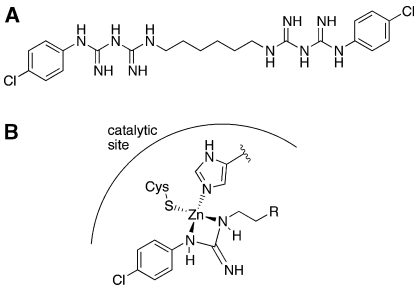

Fig. 4. Model for interaction of chlorhexidine, Zn(II), and R-gingipain. A) Structure of chlorhexidine. B) Model for coordination of Zn(II) to chlorhexidine nitrogens and to catalytic cysteine and histidine in the R-gingipain active site.

an apparent 3–30-fold increase in potency of chlorhexidine as an inhibitor of R-gingipains, depending on enzyme type and assay conditions (Table 1). It is notable that R-gingipains are not appreciably inhibited by Zn(II) concentrations of less than 1 mM (23), even though at higher concentrations Zn(II) is well known as an enzyme activity inhibitor, probably interacting with sulfhydryl groups (28).

K- and R-gingipains are all cysteine proteinases. For that reason it is puzzling that chlorhexidine–Zn(II) mixtures are less effective as Kgp inhibitors than chlorhexidine alone (Table 1), and that the chlorhexidine–Zn(II) interaction apparently is antagonistic for Kgp inhibition (Table 2). Recent work has shown that there are significant differences in the active sites of Kgp and RgpB, particularly with regard to the S1 and S3 substrate binding pockets (A. Białas, J. Grembecka, D. Krowarsch, J. Otlewski, J. Potempa, and A. Mucha, submitted for publication). Furthermore, in Kgp the catalytic cysteine in the active site is immediately adjacent to a noncatalytic cysteine, while in RgpB the catalytic cysteine is flanked by an alanine (26). Coordination of Zn(II) with the noncatalytic cysteine in Kgp could reduce the effectiveness of chlorhexidine–Zn(II) mixtures as synergistic inhibitors. Thus, it is possible that differences with regard to chlorhexidine–Zn(II) inhibition are likely to be related to differences in the active sites of Kgp and R-gingipains, and this should be a topic for future work.

Proteinase inhibitors are of great interest as potential antimicrobial agents (34), and a number of gingipain inhibitors have already been described. *Coptidis rhizoma*, a plant used in traditional Chinese medicine, produces berberine-like alkaloids that inhibit growth and proteolytic activities of a number of oral bacteria including *P. gingivalis* (15), and malabaricone C, a diarylnonanoid from nutmeg (*Myristica malabarica*) is a potent and specific inhibitor of both R-gingipains and the growth of *P. gingivalis* (33). Low microgram levels of green tea catechins inhibit R-gingipains and, to a lesser extent, K-gingipain (27). Salivary histatin 5 has been reported to inhibit both R- and K-gingipains (12), and synthetic inhibitors have been developed based on the histatin cleavage sites of each enzyme (21). These latter inhibitors are very potent, with K_i's in the range 10^{-10}–10^{-11} M. By using active site mapping, together with combinatorial chemistry, a specific K-gingipain inhibitor with a K_i of 0.9 nM has also been developed (6). Tetracycline and similar drugs are frequently used as adjuncts in periodontal therapy, and *in vitro* these compounds at 100 μM totally inhibited the amidolytic activity of R-gingipains (16). However, with the exception of the tetracyclines, none of these inhibitors has been used in clinical trials or approved for use in humans.

Chlorhexidine and Zn(II) both have extensive histories of use in oral health care in humans (19, 25). Chlorhexidine–Zn(II) mixtures have already been shown to be more effective than either component alone in reducing plaque formation, acid production by dental plaque, and oral malodor (9, 10, 32, 36). Gingipains are believed to be significant virulence factors for pathogenesis of periodontitis (17, 29) and a chlorhexidine–Zn(II) combination could be even more valuable than chlorhexidine alone as an adjunct in treatment of periodontitis due to combined antimicrobial and gingipain inhibitory effects. A clinical trial testing this hypothesis is feasible and should be done.

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