Effect of Lanthanides on *Porphyromonas Gingivalis* Proteases

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Abstract

Host and bacterial proteases play a vital role in periodontitis. Inhibitors of these proteases are necessary for control of this disease. The purpose of this study was to evaluate the effect of lanthanides on proteins from *Porphyromonas gingivalis*, a major pathogen in periodontitis. Benzoyl-L-Arg-p-nitroanilide (BAPNA); H-Gly-Pro-pNA · HCl and gelatin were used to evaluate the activity of *P. gingivalis* proteins in the presence of lanthanides. Proteins extracted from cell surfaces and culture media of *P. gingivalis* were assessed for activity in the presence of different lanthanides by BAPNA assay. Only gadolinium chloride was used for H-Gly-Pro-pNA · HCl assay and gelatin-zymography. Concentration-dependent reduction of absorbance was observed in the presence of lanthanides with BAPNA and a similar observation was made with gadolinium chloride using H-Gly-Pro-pNa. Collagenolytic activity in cell surface extracts and culture media-precipitated proteins was absent in the presence of gadolinium chloride. These results suggest that the lanthanide gadolinium can be a potential inhibitor of *P. gingivalis* proteases.

Key words: Gadolinium chloride, P. gingivalis, antibacterial, BAPNA, H-Gly-Pro-pNa· HCl, zymography

Introduction

Oral bacterial pathogens, more specifically *P. gingivalis,* are one of the major etiological agents involved in gingivitis and adult periodontitis (Moore *et al.*, 1982; White and Mayrand, 1981). Virulence factors, such as lipopolysaccharide, fimbriae, haemagglutinins, vesicles and bacterial proteases, have been well characterized (Slots and Genco, 1984). Of the various virulence factors listed above, there is a great emphasis on bacterial proteases (Grenier and Mayrand, 1987; Marsh *et al.*, 1989; Smalley *et al.*, 1989). The cell extracts, extracellular culture fluid, the cell envelope and vesicles have been shown to possess proteolytic activity. With a major proportion of the activity being associated with the cell envelope (Grenier and Mayrand, 1993) and extracellular vesicle-associated trypsin-like activity [increased in older cultures (72 h vs. 48 h) whereas the cell-associated activity decreased for the same culture] we studied both these proteases. *P. gingivalis* depends upon proteases for growth. The role of a protease depends upon its location: cell-associated proteinases are likely to provide nutrients for the organism. They attach to oral surfaces and other bacteria by releasing outer membrane vesicles (Whittaker *et al.*, 1996). Their adherence would prevent removal of proteolytic activity by the mechanical action of crevicular fluid. It has been shown that trypsin-like enzymes modify the receptors for bacteria on oral surfaces, thus modulating bacterial attachment and colonization of subgingival sites. The collagenolytic activity and trypsin-like activity of *P. gingivalis* has been well documented and has been correlated with its tendency to cause lesions in experimental models (Grenier and Mayrand, 1993). Trypsin-like and thiol proteases characterized from *P. gingivalis* strain 381 degrade BAPNA when used as substrates. Trypsin-like protease and collagenase from the same strain also degrade gelatin. Infectious strains of *P. gingivalis* have a tendency to be more collagenolytic than those that failed to cause lesions in guinea pigs (Grenier and Mayrand, 1993). It has been suggested that the very high proteolytic activity of *P. gingivalis* enables invasion of the deeper structures of connective tissues by degrading epithelial cell-cell junction complexes, thus allowing systemic spread of the bacteria (Katz *et al.*, 2000).

Because antibiotics used clinically have a similar mechanism of action, resistance to these agents represents a serious problem, especially in the oral cavity where colonization of many species of bacteria occurs. Therefore, resistance represents a serious problem that might be resolved by using a new generation of antibiotics possessing a different mechanism of action. In a situation such as periodontitis, where a wide range of proteases from different organisms (Potempa *et al.*, 2000) has effects, it is possible that bacterial protease inhibitors constitute an interesting potential for treat-

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ment. Also, it is possible that their efficacy is not only attributed to their effect as antimicrobials but also their non-antimicrobial mechanisms such as host and bacterial protease inhibition, as has been demonstrated for tetracycline, for example (Suomalainen *et al.*, 1992). This demonstrates that protease inhibition has a role in treatment of periodontal diseases. Further, proteases are excellent templates for rational design of drugs, with a potential to cure and prevent periodontitis.

Calcium has been known for its role in protease activity (Mellgren, 1987). Lanthanide ion sizes, bonding, coordinating geometry, and donor atom preference are remarkably similar to calcium (Turro *et al.*, 2003). Certain enzymes that require Ca^{2+} are inhibited or activated by lanthanide ions through the formation of an abortive enzyme-substrate complex (Evans, 1981). Darnall and his colleagues demonstrated the usefulness of lanthanides as functional replacements for Ca^{2+} in enzymatic reactions (Darnall and Birnbaum, 1970). Carboxyl and hydroxyl oxygen on proteins also act as major ligands for lanthanide ions (Wang *et al.*, 2001). They can activate the conversion of trypsinogen into trypsin, but inhibit staphylococcal nuclease (Furie *et al.*, 1973). However, in spite of the data, the effect of lanthanides on periodontal pathogens is still unknown. Use of lanthanides may help to specifically target virulence factors like bacterial proteases rather than the whole microbial flora. Therefore, we investigated the effect of lanthanides on trypsin-like and collagenolytic activity of *P. gingivalis*.

Material and Methods

Bacterial strains and growth conditions

P. gingivalis 381 was grown in brain heart infusion broth (EMD Chemicals, Inc., Gibbstown, NJ) supplemented with 5μg/ml of hemin and 0.5% vitamin K (Sigma-Aldrich, Milwaukee, WI), pH 7.4, at 37°C for two days in a Forma anaerobic chamber (85% N_2, 10% H, 5% CO_2). All steps in the isolation of *P. gingivalis* proteins were carried out at 4^0C. Proteins were isolated from two-day-old cultures of *P. gingivalis* strain 381. The cells and culture media were separated, by centrifugation at 10,000 x *g* for 10 min in a Sorvall centrifuge (Sojar *et al.*, 1993).

Extraction of cell surface proteins

P. gingivalis cells collected after centrifugation were washed three times with 50 mM HEPES buffer, pH 7.0. The washed cells were stirred with 1% Triton X-100 (Fisher Scientific, Pittsburgh, PA) dissolved in HEPES at 4°C for 15 min to solubilize cell surface proteins. The cells were centrifuged at 10,000 x g in a Sorvall centrifuge for 10 min, and the supernatant was collected (Sojar *et al.*, 1993). The amount of protein (1 mg/ml) was estimated using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), by absorbance at 280 nm.

Precipitation of proteins from bacterial culture media

Ammonium sulfate was added to the culture supernatant to achieve 80% saturation in a volume of 400 ml. After 4 h, the precipitate was collected by centrifugation at 11,000 x *g* for 45 min. It was then dissolved in HEPES and dialyzed against 4.0 L of HEPES buffer. The precipitate formed on dialysis was removed by centrifugation at 10,000 x *g* for 30 min, the supernatant was collected and the precipitated material was discarded (Sojar *et al.*, 1993). The amount of protein was estimated using NanoDrop ND-1000 (2 mg/ml), by absorbance at 280 nm.

Lanthanide compounds

Lanthanide group elements (Sigma-Aldrich) were selected to assess their role on cell surface-extracted and culture media-precipitated proteins from *P. gingivalis*. These included cerium chloride heptahydrate ($CeCl_3 \cdot 7H_2O$), 99.9%; lutetium chloride hexahydrate ($LuCl_3 \cdot 6H_2O$), 99.9%; samarium chloride hexahydrate ($SmCl_3 \cdot 6H_2O$), 99%; gadolinium chloride hexahydrate (GdCl3 $\cdot 6H_2O$), 99% and gadolinium nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$), 99.9%.

Enzyme assays

Trypsin-like and collagenolytic activity of *P. gingivalis* 381 was assessed using BAPNA and H-Gly-Pro-pNa·HCl (H-glycyl-proline p-nitroanilide hydrochloride) as substrates.

BAPNA assay

Trypsin only hydrolyses peptide bonds in which the carbonyl function comes from a lysine or arginine residue. *P. gingivalis* proteases with trypsin-like activity were shown to act on BAPNA, which contains an arginine residue (Grenier and Maryand, 1993). The hydrolytic cleavage of the peptide BAPNA by trypsin liberates p-nitroaniline, which has a yellow color, and a spectrum that is different from BAPNA. Therefore, the difference was used to measure activity of proteases in the presence and absence of lanthanides. More activity was related to more liberation of p-nitroanailine, which was measured by absorbance at 405 nm. Activity of cell surface-extracted and of culture-precipitated proteins was assayed by using BAPNA (Erlanger *et al.*, 1961).

Hydrolysis of BAPNA with culture media-precipitated proteins

The reaction mixture with 100 mM substrate in 50 mM HEPES buffer (pH 7.0) contained 25 μl of protein and 5% 2-mercaptoethanol. Various lanthanide concentrations from 2 mM to 10 mM were used. After incubation at 37°C for 1 h, 100 μl of 5 N acetic acid were added to stop the reaction. The total volume was brought up to 1

ml by addition of water. The p-nitroaniline released was determined by measuring absorbance at 405 nm.

Hydrolysis of BAPNA with cell surface-extracted proteins

The reaction mixture with 100 mM substrate in 50 mM HEPES buffer (pH 7.0) contained 50 µl of protein and 5% 2-mercaptoethanol. Various lanthanide concentrations of 0.4 mM to 2.4 mM were used. After incubation at 37°C for 1 h, 100 µl of 5 N acetic acid were added to stop the reaction. The total volume was brought up to 1 ml by addition of water. The p-nitroaniline released was determined by measuring absorbance at 405 nm.

H-Gly-Pro-pNa HCl assay

As collagen is made up of high levels of Gly-Pro we assessed the collagenolytic activity by using H-Gly-Pro-pNa · HCl. The hydrolytic cleavage of the peptide H-Gly-Pro-pNa· HCl by substances that can act on Gly-Pro (for example, collagenase) liberates p-nitroaniline. The hydrolytic cleavage of cell surface-extracted proteins was assayed by using H-Gly-Pro-pNa as substrate.

Hydrolysis of H-Gly-Pro-pNa with cell surface-extracted proteins

Collagenolytic activity of cell surface-extracted protease was measured using the substrate H-Gly-Pro-pNa (1 mM final concentration) in assay buffer (50 mM HEPES [pH 7.0]) at 37°C containin 25 µl of protein and 5% 2-mercaptoethanol. Gadolinium chloride concentrations from 1.875 mM to 30 mM were used. Under identical experimental conditions, another experiment was carried out in the presence of calcium chloride (Fisher Scientific). Calcium chloride concentration was ten times higher than gadolinium chloride. In these experiments the activity of cell surface-extracted proteins was measured spectrophotometrically at 405 nm by the amount of p-nitroaniline liberated from H-Gly-Pro-p-nitroanilide.

Zymography

Since *P. gingivalis* proteases cleave gelatin effectively, the gelatin-cleaving activity of the extracted proteins was checked with zymography as described by Heussen and Dowdle (Heussen and Dowdle, 1980) using 10% Zymogen (Gelatin) Gel; 1.0 mm x 10 well (Invitrogen Corporation, Carlsbad, CA). The extracted protein, with the reducing sample buffer containing 5% 2-mercaptoethanol, was electrophoresed. After electrophoresis, the gel was washed in 50 mM HEPES, pH 7.0, containing 2% Triton X-100 for 30 min and rinsed twice with the same buffer without Triton X-100. The gel was then cut into two parts before transferring to a developing buffer containing 50 mM HEPES and 5% 2- mercaptoethanol, pH 7.0. One part was incubated with developing buffer whereas the other part was incubated with 5 mM of gadolinium chloride. The gel was incubated at 37°C overnight on a rocker platform. The incubated gel was then fixed with 50% methanol and 12% acetic acid for 30 min and stained with 0.1% Coomassie blue (Reisner, 1984) in 50% methanol and 12% acetic acid to visualize lytic bands.

Results

Influence of lanthanides on BAPNA hydrolysis

In the absence of lanthanides BAPNA was hydrolyzed with cell surface-extracted and culture media-precipitated proteins. Concentration dependent reduction of BAPNA hydrolysis was observed in the presence of lanthanides. When cell culture-precipitated proteins were incubated with lanthanide compounds gadolinium nitrate, gadolinium chloride, samarium chloride, cerium chloride, and lutetium chloride, absorbance values were reduced by 78, 70, 75, 11 and 59% (*Figure 1*) in comparison with the controls. A similar reduction in absorbance values was observed with cell surface extracts: 56, 53, 59, 32, and 60% (*Figure 2*). Calcium, at a concentration 10-fold in excess compared to the lanthanides, precipitated in this reaction mixture. Statistical analysis (by ANOVA) of results (obtained with BAPNA as substrate) with compounds of the same cation but a different anion (gadolinium chloride vs gadolinium nitrate) showed that there was no statistically significant difference between those compounds (*Figure 3 A and B)* for both cell surface and secretory proteins.

Influence of gadolinium and calcium on H-Gly-Pro-pNa hydrolysis

In the absence of gadolinium chloride, H-Gly-Pro-pNa was hydrolyzed with cell surface-extracted proteins. A concentration-dependent reduction of H-Gly-Pro-pNa hydrolysis was observed in the presence of gadolinium chloride. When cell surface-extracted proteins were incubated with gadolinium chloride, absorbance values were reduced (*Figure 4*) in comparison with the controls.

Gelatin zymography

Proteins extracted from cell surfaces and those precipitated from cell culture medium were subjected to zymography. High proteolytic activity was detected on the gelatin zymogram in the absence of gadolinium chloride (*Figures 5A, 6A*). However, negative staining bands were absent in the presence of gadolinium chloride at a concentration of 5 mM (*Figures 5B, 6B*). The apparent size of cell surface proteins inhibited in the presence of gadolinium chloride was around 50 kDa. With cell culture media-precipitated proteins the sizes of the proteins that were inhibited were inferred to be around 50 kDa and 30 kDa.

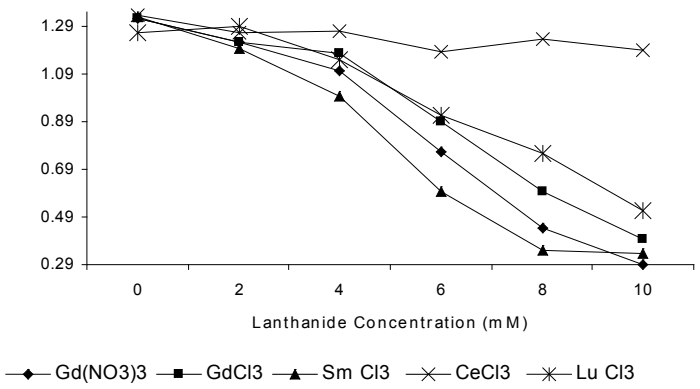


Figure 1. Hydrolysis of BAPNA with cell culture proteins in the presence of lanthanides.

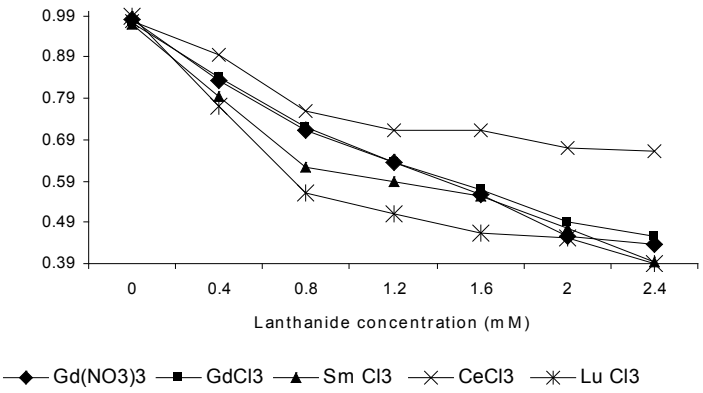
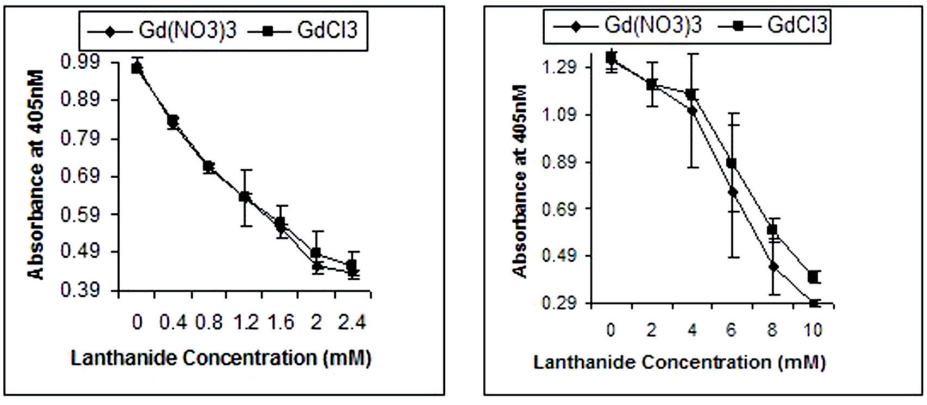


Figure 2. Hydrolysis of BAPNA with cell surface proteins in the presence of lanthanides.



A **B**

Figure 3. (A) Proteins extracted from cell surfaces; (B) Proteins precipitated from cell culture medium.

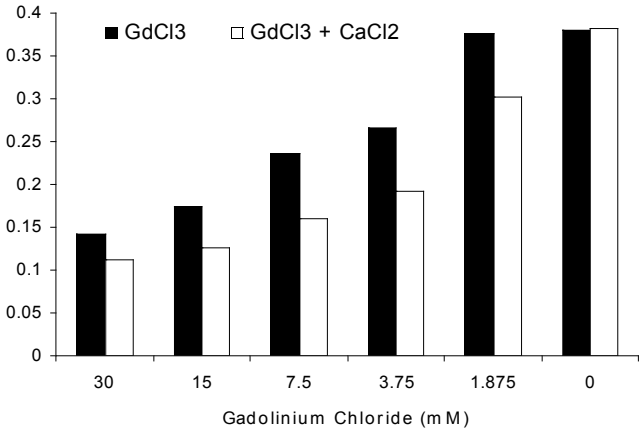


Figure 4. Gadolinium and calcium effect on H-Gly-Pro-pNa hydrolysis.

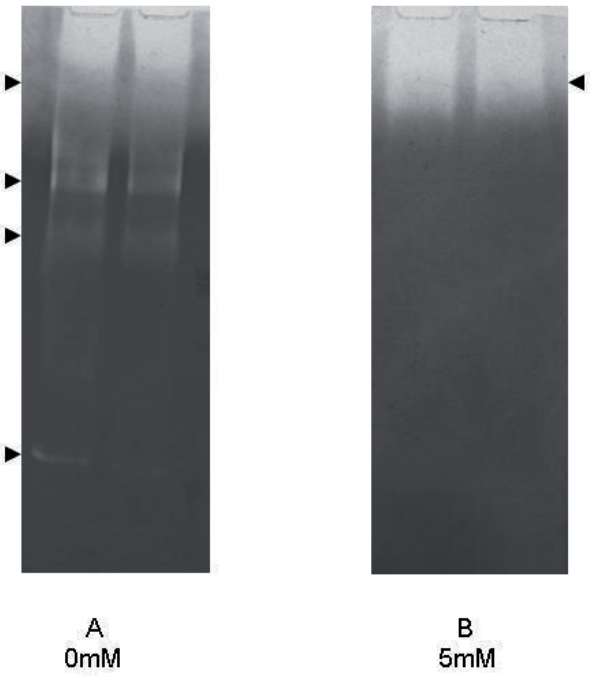


Figure 5. Effect of gadolinium on proteins precipitated from cell culture medium. The arrow points to the area of protease activity.

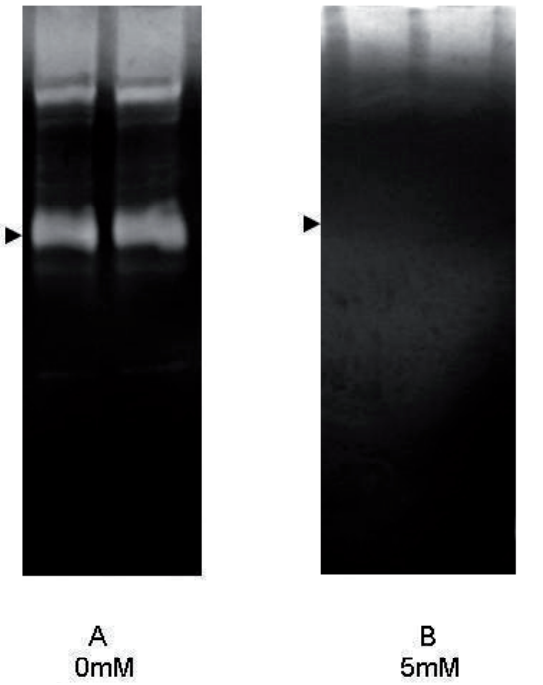


Figure 6. Effect of gadolinium on proteins extracted from cell surfaces. The arrow points to the area of protease activity.

Discussion

As described in the introduction, the importance of development/identification of agents that can inhibit proteases and their role in periodontitis was investigated. Our work has demonstrated inhibition of *P. gingivalis* protease activity by use of lanthanides.

These results showed reduced absorbance when cell envelope proteins acted on BAPNA and H-Gly-Pro-pNa in the presence of lanthanides. We also observed an absence of protease activity on gelatin zymogram in the presence of gadolinium chloride. These observations can be explained by the fact that some of the proteases for which these peptides and gelatin were used as substrates were unable to act to their greatest potential in the presence of lanthanides and gadolinium chloride. A similar inference was made when lanthanides were used with culture media proteins in the presence of substrates such as BAPNA and gelatin.

With BAPNA, there was no statistical significance among anions in affecting the activity over cell surface-extracted proteins ($p = 0.32$) and culture media-precipitated proteins ($p = 0.1$) as shown in *Figure 3A* and *3B*. Because chelated forms of gadolinium are being

widely used in humans for many therapeutic purposes, and oral administration in rats has had no noticeable effect in concentrations up to 5000 mg/kg (Bruce *et al.*, 1963), we continued using a gadolinium compound (gadolinium chloride) in our experiments with H-Gly-Pro-pNa and gelatin.

Calcium plays a role in stabilization of the *P. gingivalis* proteases (Bhogal *et al.*, 1997; Eichinger *et al.*, 1999; Nakayama, 1997). To assess the influence of calcium in our experiment we added 10-fold excess of calcium chloride with H-Gly-Pro-pNa as substrate. We observed that calcium has little influence in reversing the inhibitory effect of gadolinium. Rather, the presence of calcium along with gadolinium reduced the chromogenic activity by 70%, compared to 62% with gadolinium alone (*Figure 4*).

We inferred that trypsin-like and collagenolytic activities of cell surface and culture media were inhibited by comparing analysis of the gels with previous work done in our laboratory as well as by others (Grenier *et al.*, 1989), which showed that the apparent size of proteins inhibited in the presence of gadolinium chloride is around 50 kDa. Most of the proteases that act on periodontal tissues (Potempa *et al.*, 2000) that have trypsin-like and collagenolytic activity are around 50 kDa (Grenier and Maryand, 1993). So, the use of gadolinium may represent an approach in controlling the role of oral pathogens in pathogenesis of periodontitis.

However, this is the first time that lanthanides, and in particular gadolinium chloride, have been assessed for effects on a pathogen associated with periodontitis. Continued work with this group of elements is important to determine that lanthanides have effects only on pathogenic organisms but not on normal oral microbial flora.

Literature related to the safety of gadolinium shows that it has no significant adverse effects through oral administration (Bruce *et al.*, 1963) in female rats. As mentioned in the introduction, lanthanides act on proteases and other enzymes, which suggests that they may also affect host-derived enzymes. Also, gadolinium was known to have cytotoxic effects on phagocytes (Pendino *et al.*, 1995). Future investigation is focused towards understanding these mechanisms to achieve optimal clinical treatment conditions and their safety. Because initial *in vitro* studies with tetracyclines and their derivatives have proven to cause monocyte/macrophage cell apoptosis (Bettany and Wolowacz, 1998), it would be interesting to investigate if gadolinium has an effect on macrophages relative to periodontitis.

Because gadolinium has an inhibitory effect on protease activities, as shown by this present study, and was evaluated *in vitro* at neutral pH, in the future several parameters should be addressed for *in vivo* application. Our initial promising results, plus the demand for development of agents other than antibiotics for maintenance of routine oral hygiene, broaden the scope of the future role of gadolinium and lanthanides in routine oral hygiene as well as in periodontal therapy.

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