Effects of Biphenyl Sulfonlamido Methyl Bisphosphonic Acids on Porphyromonas Gingivalis and Cytokine Secretion by Oral Epithelial Cells

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Abstract: Bisphosphonate drugs are well known to inhibit osteoclastic activity and have been proposed for the management of bone diseases, including periodontitis which is associated with alveolar bone destruction. In this study, we evaluated the effects of four arylsulfonamide bisphosphonates on growth of the periodontopathogenic bacterium Porphyromonas gingivalis as well as their capacity to reduce cytokine secretion by lipopolysaccharide (LPS)-stimulated oral epithelial cells. The growth of P. gingivalis was inhibited by (4’-Chloro-biphenyl-4-sulfonlamino)methyl-1,1-bisphosphonic acid while the three other arylsulfonamide bisphosphonates ((4-Methoxy-phenylsulfonlamino)methyl-1,1-bisphosphonic acid, (4-Nitro-phenylsulfonlamino)methyl-1,1-bisphosphonic acid, and (Biphenyl-4-sulfonlamino)methyl-1,1-bisphosphonic acid) had no effect. Growth inhibition was more pronounced under an iron-restricted condition. All four arylsulfonamide bisphosphonates decreased the production of the pro-inflammatory cytokines IL-6 and IL-8 by Aggregatibacter actinomycetemcomitans LPS-stimulated oral epithelial cells. In conclusion, we uncovered additional properties of bisphosphonates that may be beneficial for the treatment of periodontal diseases. In particular, (4’-Chloro-biphenyl-4-sulfonlamino)methyl-1,1-bisphosphonic acid combines the already disclosed antiresorptive activity with anti-inflammatory and antibacterial properties.

Keywords: Bisphosphonate, periodontal disease, antibacterial, anti-inflammatory.

INTRODUCTION

Periodontal diseases include a group of inflammatory diseases of bacterial origin affecting the tooth-supporting tissues. While gingivitis is a reversible inflammation of the gingiva, periodontitis is characterized by loss of periodontal attachment, periodontal pocket formation and alveolar bone destruction. Several Gram negative bacterial species, called periodontopathogens, have been associated with chronic and aggressive forms of periodontitis. More specifically, Porphyromonas gingivalis, along with Treponema denticola and Tannerella forsythia, are more prevalent in periodontal progressive sites [1-3]. These periodontopathogens colonizing the subgingival sites can mediate inflammation and periodontal tissue destruction through activation of mucosal and immune cells resulting in the expression of inflammatory mediators and matrix metalloproteinases [4, 5]. Mostly on the basis of epidemiological studies, chronic periodontitis has been linked to several systemic disorders, including cardiovascular and respiratory diseases, diabetes, and preterm low birth weight [6].

Bisphosphonates, characterized by the P-C-P bond, are analogues of inorganic pyrophosphates (P-O-P) and are resistant to chemical and enzymatic hydrolysis [7]. They are able to chelate metal cations, such as Fe³⁺, Al³⁺, Zn²⁺ and Ca²⁺ [8, 9]. Because of this property, they strongly bind hydroxyapatite crystals, targeting the bone tissue. Bisphosphonates are widely used in the treatment of diseases involving osteoclast-mediated bone resorption, such as osteoporosis and Paget’s disease [10]. Moreover, bisphosphonate drugs have been proposed for the management of periodontitis which is associated with alveolar bone destruction [11]. Simple bisphosphonates (clodronate, etidronate and tiludronate) are intracellularly metabolized to cytotoxic ATP analogues, while the more potent amino-bisphosphonates (alendronate, zolendronate, risedronate) act by blocking the mevalonate pathway inhibiting the farnesylpyrophosphate (FPP) synthase in the HMG-CoA reductase route. Thereby they prevent the prenylation of lipid-anchored proteins, necessary for the normal function and survival of osteoclasts [12].

The phosphonate groups of bisphosphonates can inhibit the proteolytic activity of MMPs through chelation of the catalytic zinc ion [13, 14]. This evidence prompted the synthesis and biological evaluation of a series of inhibitors characterized by a bisphosphonic moiety as the zinc binding group, in line with our previous work on MMP inhibitor [15-18]. More specifically, compounds 1-4 (Fig. 1), which are chemically stable arylsulfonamido bisphosphonates, were synthesized in our laboratory and found to be active on
MMPs and osteoclasts [19]. Their average IC50s toward MMP-2 are in the nanomolar range and they are selective toward MMP-9 [19]. Compounds 1, 3 and 4 demonstrated micromolar activity against macrophage J774 cells, a model system to screen the inhibitory effects on osteoclasts, while they have no effects on HepG2 cells showing good safety profile [19]. Osteoclasts were efficiently killed by treatment with the same compounds, that were also able to abolish the formation of actin rings, the functional structure typical of resorbing osteoclasts, and inhibited bone resorption [19].

The activity demonstrated by compounds 1-4 toward MMPs and osteoclasts prompted us to investigate additional properties that may be beneficial against periodontitis. More specifically, we evaluated their effect on growth of the periodontopathogenic bacterium P. gingivalis as well as their capacity to reduce cytokine secretion by lipopolysaccharide (LPS)-stimulated oral epithelial cells, in order to assess if arylsulfonamide bisphosphonates may be of interest for the treatment of inflammation associated with this disease.

**MATERIALS AND METHODS**

**Arylsulfonamide Bisphosphonic Acids**

(4-Methoxy-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (1), (4-Nitro-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (2), (Biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonic acid (3), and (4-Chloro-biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonic acid (4) were synthesized in accordance with our previously reported procedure [19]. Elemental analysis for the each compound is as follows: (1) Anal. calcd for C8H11NO3P2S·2H2O: C 43.19%; H 3.53%; found: C 43.59%; H 4.37%; N 3.59%; (2) Anal. calcd for C8H10N2O3P3S·2H2O: C 21.33%; H 3.07%; N 7.11%; found: C 21.05%; H 3.30%; N 6.76%; (3) Anal. calcd for C13H15NO3S·1.5H2O: C 35.95%; H 4.18%; N 3.23%; found: C 35.81%; H 4.29%; N 3.25%; (4) Anal. calcd for C13H14ClNO3P3S·1.5H2O: C 33.31%; H 3.66%; N 2.99%; found: C 33.09%; H 3.67%; N 3.16%. Stock solutions at 10 mM were prepared in dimethyl sulfoxide and stored at 4°C protected from light.

**Growth Inhibitory Effect**

P. gingivalis ATCC 33277 was routinely grown in Todd-Hewitt broth (BBL Microbiology Systems, Mississauga, ON, Canada) supplemented with 20 μM hemin and 0.0001% vitamin K (THB-HK) at 37°C under anaerobic conditions (80% N2/10% H2/10% CO2) for 24 h. The effect of compounds 1-4 on P. gingivalis growth was assessed in two different culture media using a microplate dilution assay. THB-HK contained excess iron, while Mycoplasma broth base (MBB; BBL Microbiology Systems) supplemented with 10 μM hemin (MBB-H) represented an iron-restricted condition. Briefly, 24-h cultures of P. gingivalis in THB-HK or MBB-H were diluted in fresh broth medium to obtain an optical density of 0.2 at 660 nm (OD660). Equal volumes (100 μL) of P. gingivalis suspension and bisphosphonate derivatives 1-4 (50, 25, 12.5, 6.25, and 3.12 μM) in THB-HK or MBB-H were mixed in the wells of 96-well plates (Sarstedt, Newton, NC, USA). Wells with no P. gingivalis or compounds were used as controls. After a 48-h incubation at 37°C under anaerobic conditions, bacterial growth was determined by measuring the OD660 using a microplate reader. Penicillin G was used as a positive control for growth inhibition.

**Inhibition of Cytokine Secretion by LPS-Stimulated Oral Epithelial Cells**

The oral epithelial cell line GMSM-K, kindly provided by Dr. Valerie Murrah (Department of Diagnostics Sciences and General Dentistry, University of North Carolina, Chapel Hill, NC, USA), was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/mL of penicillin G/streptomycin at 37°C in a 5% CO2 atmosphere. Epithelial cells were harvested by gentle trypsinization (0.05% trypsin-EDTA; Gibco-BRL, Grand Island, NY, USA), washed once in DMEM-FBS, and suspended at a density of 4 × 104 cells per mL in DMEM with 1% heat-inactivated FBS. Cells were seeded in a 12-well plate (4 × 105 cells/well in 1 mL) and cultured overnight at 37°C in a
5% CO₂ atmosphere to allow cell adhesion prior to the stimulation with *P. gingivalis*. The epithelial cells were pre-treated with increasing concentrations of bisphosphonate derivatives 1-4 (0, 1, 5, 20, and 50 µM) and were incubated at 37°C in 5% CO₂ for 2 h before the stimulation with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) at 1 µg/mL. After a 24-h incubation at 37°C in 5% CO₂, cell-free supernatants were collected and stored at -20°C until used. Commercial enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN, USA) were used to quantify IL-6 and IL-8 levels in the cell-free supernatants according to the manufacturer’s protocols. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The rated sensitivities of the commercial ELISA kits were 9.3 pg/mL for IL-6 and 31.2 pg/mL for IL-8. An MTT (3-[4, 5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay performed according to the manufacturer’s protocol (Roche Diagnostics, Mannheim) was used to exclude that decreased cytokine secretion may result from loss of viability of oral epithelial cells treated with bisphosphonate derivatives.

**Statistical Analysis**

Data are expressed as the means ± standard deviations of at least three assays. The statistical analysis was conducted using the Student t-test with Bonferroni corrections. A *p* < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

Compounds 1-3 had no effect on growth of *P. gingivalis* in both culture medium (Fig. 2). On the contrary, the growth of *P. gingivalis* was inhibited by compound 4. In THB-HK medium (iron-rich condition), the growth was almost completely inhibited at 50 µM, while in MBB-H medium (iron-poor condition), a similar inhibition was obtained at 6.25 µM (Fig. 2). Penicillin G used a positive control of growth inhibition showed a minimal inhibitory concentration (MIC) of 0.125 µg/mL. To the best of our knowledge, there is only report in the literature on the anti-bacterial properties of bisphosphonates [20]. The fact that compound 4 inhibited growth more efficiently under an iron-limiting condition, requiring much lower concentrations to significantly reduce the growth of *P. gingivalis*, suggests that the mechanism of growth inhibition may be related to a chelating activity. This is in agreement with previous studies reporting the iron-chelating property of bisphosphonates [8, 21].

All molecules under consideration showed the same chelating function, and different substitutions on the aromatic ring do not affect their ability to chelate the iron. In fact the substituent in para position does not modify the
chelating geometry. Moreover, calculating the pKa of molecules under study, using the Calculator Plugin in Marvin 5.11.1, 2012, ChemAxon (http://www.chemaxon.com), the same values for all ligands were obtained, demonstrating that the substitution occurs too far from the bisphosphonate function to influence the acidity of this group and its ionization state at physiological pH. Analysis of their lipophilic nature (AlogP) using the software Canvas v 1.4.112 (New York, NY, USA: Schrodinger, LLC; 2011) showed differences between compounds. In fact, compound 4 is more lipophilic (calculated AlogP = 0.71) with respect to the other analogues (calculated AlogP = -1.49, -1.58, and 0.05 respectively for compounds 1, 2, and 3). This property could be responsible for the higher activity observed for compound 4 able to pass the outer cell membrane of P. gingivalis [22].

The cytotoxicity of the four bisphosphonate derivatives towards oral epithelial cells was then evaluated. None of the compound reduced cell viability even at a concentration of 100 μM, confirming their safety for this cell type. The ability of bisphosphonate derivatives 1-4 to inhibit the secretion of IL-6 and IL-8 by oral epithelial cells stimulated with the LPS of A. actinomycetemcomitans was then tested. Treating epithelial cells with the LPS increased IL-6 and IL-8 secretion by 3.2-, and 6.2-fold, respectively, compared to control cells (Figs. 3 and 4). Compounds 1-4 inhibited the secretion of both cytokines; the inhibition of IL-6 was more pronounced. At the lowest concentration tested (1 μM), bisphosphonate derivatives 1, 2, 3, and 4 reduced IL-6 secretion by 58, 25, 52, and 74%, respectively (Fig. 3). As reported in (Fig. 4), compounds 1-4 also decreased IL-8 but to a lesser extent. To cause a reduction ≥ 50%, a concentration of 20 μM of the compounds had to be used.

The reduction of IL-6 production by clodronate has been reported for macrophages [23, 24]. On the other hand, many articles pointed out the pro-inflammatory activity of amino-bisphosphonates, such as alendronate [25], zolendronate [26]. It seems the pro- or anti-inflammatory activity of bisphosphonic acids depends on the structural class [27]. In this respect, our compounds seem to work as the clodronate, showing a marked anti-inflammatory activity. The observed reduction of cytokine production improves the safety profile of tested compounds with respect to well known clinically used amino-bisphosphonates.

Bisphosphonate drugs are well known to inhibit osteoclastic activity and have been proposed for the management of bone diseases, including periodontitis which is associated with alveolar bone destruction. In this study, we uncovered additional properties of bisphosphonates that may be beneficial for the treatment of these diseases. In particular, com-
pound 4 combines the already disclosed antiresopitive activity with anti-inflammatory and anti-bacterial properties, resulting in a promising tool for the treatment of periodontitis.

CONFLICT OF INTEREST

None of the authors report any conflicts of interest related to this study.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FPP</td>
<td>Farnesylpyrophosphate</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MBB-H</td>
<td>Mycoplasma broth base-hemin</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MTT</td>
<td>3-[4, 5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>THB-HK</td>
<td>Todd-Hewitt broth-hemin-vitamin K</td>
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REFERENCES


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