In vitro and in vivo activity of peptidomimetic compounds that target the periodontal pathogen Porphyromonas gingivalis

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Running Head: Peptidomimetic compounds targeting P. gingivalis

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Abstract

The interaction of the periodontal pathogen *Porphyromonas gingivalis* with oral streptococci is important for initial colonization of the oral cavity by *P. gingivalis* and is mediated by a discrete motif of the streptococcal antigen I/II protein. A synthetic peptide encompassing this motif functions as a potent inhibitor of *P. gingivalis* adherence but the use of peptides as topically applied therapeutic agents in the oral cavity has limitations arising from the relatively high cost of peptide synthesis and their susceptibility to degradation by proteases expressed by oral organisms. In this study, we demonstrate the *in vitro* and *in vivo* activity of five small molecule mimetics of the streptococcal peptide. Using a three species biofilm model, all five compounds were shown to effectively inhibit the incorporation of *P. gingivalis* into *in vitro* biofilms and exhibited IC$_{50}$ values of 10 to 20 μM. Four of the five compounds also significantly reduced maxillary alveolar bone resorption induced by *P. gingivalis* infection in a mouse model of periodontitis. All of the compounds were non-toxic towards a human telomerase immortalized gingival keratinocyte cell line. Three compounds exhibited slight toxicity against the murine macrophage J774A.1 cell line at the highest concentration tested. Compound PCP-III-201 was non-toxic to both cell lines and the most potent inhibitor of *P. gingivalis* virulence and thus may represent a novel potential therapeutic agent that targets *P. gingivalis* by preventing its colonization of the oral cavity.
Periodontitis is a widespread inflammatory disease that is caused by a consortium of anaerobic bacteria including *Porphyromonas gingivalis*, *Tannerella forsythsis* and *Treponema denticola* (1-5). Of these organisms, *P. gingivalis* has been the most extensively studied and has been suggested to represent a keystone pathogen that targets the host innate immune response leading to disruption of normal host-microbe homeostasis (dysbiosis). This results in increased microbial biomass and significant population shifts in the oral microbiome leading to chronic inflammation (6-8). Periodontitis has also been associated with a variety of systemic disorders such as cardiovascular disease, diabetes mellitus and rheumatoid arthritis (9-13). Current methods to treat periodontitis involves removing the microbial biofilm by scaling and root planing and in more severe cases, surgery may be required to reduce gingival pocket depth. In general, therapeutic approaches that specifically target periodontal pathogens like *P. gingivalis* are lacking and methods that prevent or limit the re-colonization of the oral cavity by *P. gingivalis* after clinical treatment of diseased sites are not available. Thus, the disease commonly recurs and requires additional treatment.

The primary niche for *P. gingivalis* is in a mixed community of bacterial species that reside in the subgingival pocket, however, upon initial entry into the oral cavity *P. gingivalis* initially colonizes supragingival plaque and interacts with oral streptococci (14). Our previous results suggested that the interaction of *P. gingivalis* with oral streptococci is important for this early colonization event (15,16) and thus represents an ideal point for therapeutic intervention to control the initial colonization or re-colonization of oral tissues by *P. gingivalis*. Adherence of *P. gingivalis* to streptococci is species specific and is driven by a protein-protein interaction that occurs between the minor fimbrial antigen (Mfa) of *P. gingivalis* and the antigen I/II (Ag I/II) polypeptide of streptococci (17-19). Daep et al. identified a discrete domain in Ag I/II protein that mediates its interaction with Mfa and showed that this region...
resembles the eukaryotic nuclear receptor (NR) box protein-protein interaction domain (17,18). Within the NR box-like domain are two functional peptide motifs, VXXLL and NITVK, that are essential for P. gingivalis adherence to streptococci. Deap et al. also showed that a synthetic peptide encompassing both motifs functioned as a potent inhibitor of P. gingivalis adherence and significantly reduced P. gingivalis virulence in vivo (18,19). These studies suggest that P. gingivalis colonization of the oral cavity may be controlled by preventing its initial association with streptococci and that inhibitors of the Mfa-Ag I/II interaction represent potential therapeutic agents to treat or prevent recurrence of periodontitis.

The use of peptides as topically applied therapeutic agents in the oral cavity has limitations arising from the relatively high cost of peptide synthesis and their susceptibility to degradation by proteases expressed by oral organisms, including P. gingivalis itself. To address these limitations, Patil et al. (20) designed and synthesized potent and stable small molecule inhibitors that mimic the natural peptide substrate recognized by Mfa by employing a strategy that joined mimics of VXXLL and NITVK together via the “click” reaction (21,22). Within the expansive area of nitrogen/oxygen heterocycles, the 2,4,5-trisubstituted oxazole framework was selected as a starting point for the NITVK-associated inhibitors of Mfa/Ag I/II interaction (23,24) and several of these compounds potently blocked P. gingivalis adherence to streptococci in vitro when click-coupled with substituted arylalkynes. In this study, we show that five small molecule mimetics inhibit the incorporation of P. gingivalis into a microbial biofilm and reduce P. gingivalis virulence in a mouse model of periodontitis when administered simultaneously with P. gingivalis oral infection. The most potent compounds do not exhibit significant toxicity against human gingival epithelial or mouse macrophage cell lines at concentrations that inhibit biofilm formation or virulence and thus may represent novel therapeutics to limit P. gingivalis colonization of the oral cavity.
Results

Inhibition of *P. gingivalis* biofilm formation. Fifty peptidomimetic compounds were previously tested for inhibition of *P. gingivalis* adherence to *S. gordonii* (20) and the five most potent inhibitors were selected for further analysis in this study. The IC₅₀ values for inhibition of interspecies adherence by these compounds ranged from 5 – 15 μM (20). In the oral cavity however, the microbiome is more complex and *P. gingivalis* and *S. gordonii* form biofilms in the presence of other bridging organisms that can independently adhere to both *P. gingivalis* and *S. gordonii*. To determine if the peptidomimetic compounds inhibit *P. gingivalis* biofilm formation in the presence of a bridging organism, a three species biofilm model was employed in which *P. gingivalis* and *S. gordonii* were incubated in the presence of a bridging organism, *F. nucleatum*. As shown in Table 1, all five compounds inhibited the incorporation of *P. gingivalis* into the three species biofilm and exhibited IC₅₀ values between 10 and 20 μM, similar to the IC₅₀ values previously reported for inhibition of *P. gingivalis* adherence to streptococci (20). A representative image showing the three species biofilms obtained in the presence of compound PCP-III-201 is shown in Figure 1. Interestingly, in addition to reducing the levels of *P. gingivalis*, each compound also reduced the level of *F. nucleatum* adhered to streptococci. Thus the peptidomimetic compounds prevented the interaction of *P. gingivalis* with *S. gordonii* and inhibited biofilm formation in the presence of a bridging species, suggesting that the compounds may be effective in reducing *P. gingivalis* colonization of more complex microbial communities.

Compound PCP-III-201 was also tested for activity against a pre-formed three species biofilm. Representative images of three species biofilms treated with PCP-III-201 are shown in Supplemental Figure S1. As shown in Figure S1 and Table 2, a dose and time dependent inhibition was observed. This indicates that in addition to inhibiting the formation of a three species biofilm, the compound was able to disrupt a pre-formed biofilm.
Inhibition of *P. gingivalis* virulence by peptidomimetic compounds. Next, to determine if the peptidomimetic compounds affect *P. gingivalis* virulence *in vivo*, each compound was examined in a mouse model of periodontitis that was previously described by Deap et al. (19). Since a primary clinical outcome of periodontitis in humans is the resorption of alveolar bone supporting the teeth, virulence was assessed in this model by measuring the extent of alveolar bone loss around the maxillary molars that was induced by *P. gingivalis* infection. Representative images of the murine maxilla obtained from sham-infected, *P. gingivalis*-infected, and treated animals are shown in Figure 2. Sham-infected (Fig. 2A) and PCP-III-201-treated (Fig. 2C) animals exhibited a smooth alveolar bone crest (ABC) whereas *P. gingivalis*-infected animals (Fig. 2B) exhibited significantly increased alveolar bone resorption and an irregular ABC (indicated by arrows). Quantification of alveolar bone loss for all animal groups is shown in Figure 3. After establishing *S. gordonii* in the oral cavity of mice, infection of animals with *P. gingivalis* (group SgPg) induced a significant increase in alveolar bone loss relative to sham infected animals (*p* < 0.001). In contrast, infection of mice with *P. gingivalis* in the presence of four of the five peptidomimetic compounds significantly reduced the amount of alveolar bone loss relative to *P. gingivalis* infected animals without compound (i.e., group SgPg). For mice treated with compound PCP-III-201, the extent of bone loss observed was not significantly different from sham-infected animals (*p* < 0.10). Although compounds PCP-III-206, PCP-III-212 and PCP-IV-20 significantly reduced bone loss relative to untreated *P. gingivalis* infected animals (*p* < 0.01), the mice in these groups exhibited more bone loss than sham infected animals (*p* < 0.05). The extent of bone loss after treatment with compound PCP-III-293 was not significantly reduced relative to the SgPg group (*p* < 0.16). Thus, four of the five compounds tested inhibited or partially inhibited *P. gingivalis* virulence.

Toxicity of peptidomimetic compounds. Although none of the mice exhibited visible detrimental effects after administration of the peptidomimetic compounds, four additional in vitro approaches were
employed to assess compound toxicity against telomerase immortalized human gingival keratinocytes (TIGK) and the murine J774.A1 macrophage cell line. First, to determine if compounds induce cell lysis or compromise cell membrane integrity, lactate dehydrogenase (LDH) activity released into the cell culture medium was measured after 18 h exposure of cells to each of the compounds. As shown in Figure 4A, none of the compounds induced a significant increase in the release of LDH from TIGK cells over the range of concentrations that were tested. With J774A.1 cells, a small but statistically significant increase in LDH release was observed when cells were exposed to compounds PCP-III-206 (40 μM), PCP-III-293 (60 μM) or PCP-IV-20 (60 μM) at the highest concentrations tested. However, under these conditions the level of LDH release was still significantly less than the lysis control, suggesting that these three compounds may only induce low levels of cell lysis at high concentration. The effect of the compounds on TIGK and J774A.1 cells was also assessed by determining ATP levels, an indicator of metabolic activity. As shown in Figure 5A, no significant reduction in ATP levels relative to the medium/DMSO control was observed in TIGK cells, although the values obtained for compounds PCP-III-293 and PCP-IV-20 at 60 μM approached statistical significance. In contrast, treatment of J774A.1 cells with PCP-III-206 (at 20 μM and 40 μM) and PCP-IV-20 (at 60 μM) resulted in significant reductions in ATP levels (see Fig. 5B) indicating that these compounds may impair the metabolic activity of J774A.1 cells. Interestingly, treatment of J774A.1 cells with PCP-III-201, PCP-III-212, PCP-III-293 and PCP-IV-20 at 5 μM increased the levels of ATP (see Figure 5B), suggesting that these compounds at this concentration may stimulate metabolic activity of J774A.1 cells.

To determine if exposure of cells to the peptidomimetics induced cell apoptosis, TIGK and J774A.1 cells were labeled with Sytox and Alexa488nm-labeled annexin V and analyzed by flow cytometry. A representative series of images obtained from flow cytometry using TIGK cells is shown in Supplemental Figure S2 and a complete summary of the populations of live cells (lower left quadrant),
early apoptosis (lower right quadrant) and late apoptosis/necrosis (upper right quadrant) for each cell line and compound tested is provided in Table 3. As shown, none of the compounds induced a significant increase in apoptotic cells relative to the control reactions for either of the cell lines, with the exception of TIGK cells exposed to PCP-III-293 at 60 μM. Finally, we also tested each of the compounds for hemolytic activity and as shown in Figure 6, none of the compounds induced hemolysis of sheep red blood cells. Similar results were observed using human red blood cells (data not shown). Together, these results indicate that the peptidomimetic compounds exhibited no toxicity towards human TIGK cells and minimal toxicity against J774A.1 cells at the concentrations that were previously shown to be effective in inhibiting P. gingivalis adherence to S. gordonii.

Discussion

Interspecies adherence of P. gingivalis and commensal oral streptococci is important for the initial colonization of the oral cavity by P. gingivalis and thus represents a viable target for therapeutic intervention to limit pathogen colonization. Adherence is driven by a protein-protein interaction between the streptococcal antigen I/II polypeptide and the P. gingivalis minor fimbrial, antigen (Mfa) and mediated by a discrete structural motif in antigen I/II (16, 25). Daep et al. showed that a synthetic peptide representing this motif functioned as a potent competitive inhibitor of adherence to streptococci and significantly reduced P. gingivalis virulence in a mouse model of periodontitis (17, 19). However, the use of peptides as therapeutic agents has limitations arising from the relatively high cost of peptide synthesis and their susceptibility to degradation by proteases expressed by oral organisms, including P. gingivalis itself. To address these limitations, Patil et al. designed and synthesized compounds that mimic the natural peptide substrate recognized by Mfa and identified several that were potent and stable inhibitors of P. gingivalis adherence to streptococci (20). The dental biofilm is very complex and for these compounds to be effective in the oral cavity, they must be capable of reducing P.
gingivalis adherence in the presence of other organisms that may independently interact with both P.
gingivalis and streptococci. Our results show that the five compounds tested here prevent P. gingivalis
biofilm formation even in the presence F. nucleatum, a bridging organism that interacts with both P.
gingivalis and streptococci independently of antigen I/II and Mfa. Furthermore, the compounds exhibit
IC_{50} values that are similar to those that were previously reported for inhibition of P. gingivalis
adherence to streptococci, suggesting that the presence of the bridging organism does not substantially
reduce their effectiveness. Under the conditions tested, the active compounds prevented the formation
of the biofilm by inhibiting P. gingivalis adherence to streptococci. Thus, these compounds represent
potential therapeutics that may be effective in preventing P. gingivalis colonization of the oral biofilm as
it re-forms after a professional prophylaxis. However, compound PCP-III-201 also disrupted a pre-formed
three species biofilm, suggesting that active compounds may also potentially be effective in treating
periodontitis by reducing biofilm load in the oral cavity. Unexpectedly, the compounds also inhibited
the accumulation of F. nucleatum in a dose dependent manner even though the mimetics are specifically
targeted to block the P. gingivalis/streptococcal interaction. One possible explanation for this is that the
interaction of P. gingivalis with streptococci induces a complex adaptive response that involves changes
in P. gingivalis gene expression and metabolic crosstalk that promotes P. gingivalis biofilm formation
and virulence (26-28). It is possible that this adaptive response in P. gingivalis may in turn also benefit
other members of the microbial community and thus, when P. gingivalis adherence to streptococci is
prevented, the stability and persistence of the entire community is affected.

Four of the five compounds also significantly inhibited P. gingivalis-induced alveolar bone
resorption in a mouse model of periodontitis. Bone loss that occurred in animals treated with the most
potent compound, PCP-III-201, was not significantly greater than sham-infected mice. The remaining
active compounds reduced bone loss but not the extent of PCP-III-201, indicating that these compounds
only partially inhibit *P. gingivalis* virulence. PCP-III-293 exhibited little effect on *P. gingivalis* virulence which was unexpected since this compound was a potent inhibitor of in vitro adherence to streptococci (20) and the formation of three species biofilms. Given the gross structural similarities between the five inhibitors, such as the 2,4,5-trisubstituted oxazole, terminal aromatic groups and the internal triazoles, the individual substituent atoms and overall molecular shape or conformation likely accounts for the differences in in vivo activity. The relationship of the aromatic methoxy groups of PCP-III-212 to the aromatic fluorines on PCP-III-201 is bioisosteric (29-31) but while the methoxynaphthalene rings common to PCP-III-212, PCP-III-293 and PCP-IV-20 represent a high degree of hydrophobicity, the steric demand of the naphthalenes is much greater than the ortho-methoxyphenyls of the more potent PCP-III-201 and PCP-III-206. The most active compounds represent compact molecules whereby the maximal intra-atomic distances between the fluorine and methoxy groups is 18.5Å for PCP-III-201 and 18.4Å for PCP-III-206. In contrast, the other compounds assume more bent conformations whereby the interatomic distances between each oxazole methoxy group and the naphthalenyl methoxy group are more variable (up to 24.9Å). Thus, the in vivo activity of the inhibitors appears to be favored by a more planar and more linear conformation with the halogenated aryl oxazoles bearing fluorine and bromine in both positions.

Since the peptidomimetics were delivered simultaneously with *P. gingivalis* during the infection procedure, animals were only transiently exposed to the compounds. Under these conditions, none of the mice exhibited overt adverse reactions to the compounds. Furthermore, none of the compounds exhibited toxicity to human immortalized gingival keratinocytes. However, several compounds affected J774A.1 cells, albeit at relatively low levels and at the highest concentration tested. PCP-III-201 was not toxic to either cell type and was also the most potent inhibitor of *P. gingivalis* virulence, suggesting that
it may represent a novel potential therapeutic agent that targets *P. gingivalis* and can serve as a lead compound to develop the next generation inhibitors.
Materials and Methods

Bacterial strains and culture conditions. *S. gordonii* ATCC DL-1 was cultured in brain heart infusion broth (Difco) supplemented with 5% (wt/vol) yeast extract (BHIY) at 37°C for 16 hr. *P. gingivalis* ATCC 33277 was grown in TSBY medium, which consists of 30 g/liter Trypticase soy broth (Difco) supplemented with 5% (wt/vol) yeast extract, 5 mg/Liter hemin and 1mg/Liter menadione under anaerobic conditions (10% CO$_2$, 10% H$_2$, and 80% N$_2$) at 37°C for 48 hr. Prior to inoculation, TSBY medium was reduced by incubation under anaerobic conditions for 24 hr at 37°C. *F. nucleatum* ATCC 25586 was grown in reduced brain-heart infusion (BHI) broth supplemented with 5 mg/Liter hemin and 1mg/Liter menadione under anaerobic conditions (10% CO$_2$, 10% H$_2$, and 80% N$_2$) at 37°C for 48 hr.

Formation of *P. gingivalis* biofilms. To prepare bacterial cells for biofilm formation, 10 ml of an overnight *S. gordonii* culture was centrifuged at 3700 x g for 5 min and the cell pellet was suspended in 1 mL sterile phosphate buffered saline (10 mM Na$_2$HPO$_4$, 18 mM KH$_2$PO$_4$, 1.37 mM NaCl, and 2.7 mM KCl, pH 7.2; [PBS]). Subsequently, 10 µL of 5 mg/ml hexidium iodide (Molecular Probes) was added to the labeled cells and incubated for 15 min with gentle shaking at room temperature in the dark. The labeled cells were centrifuged as described above, washed with PBS and the cell pellet was suspended in PBS at a final O.D$_{600nm}$ of 0.8. Similarly, 10 mL of 48 hr cultures of *P. gingivalis* or *F. nucleatum* were centrifuged at 3700 x g for 15 min and the cell pellets were suspended in 1 mL reduced PBS. To label *P. gingivalis* or *F. nucleatum*, 20 µl of 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (4 mg/ml, ThermoFisher) or 20 µl of 2 mM Cell Trace™ Far Red dye (DDAO-SE, ThermoFisher) was added to the cell suspension, respectively. After incubation for 30 min with gentle shaking at room temperature in the dark, each suspension was centrifuged, washed as described above and suspended in PBS at a final O.D$_{600nm}$ of 0.4 for *P. gingivalis* or 2.0 for *F. nucleatum*. For biofilm formation, 1 mL of labeled *S. gordonii* cells was added to each well of a 12-well microtiter plate (Greiner Bio-one) containing a circular
coverslip (ThermoFisher) and incubated under anaerobic conditions on a rotary shaker for 24 hr at 37°C. Unbound cells were removed by aspiration and 1 ml each of labeled *P. gingivalis* and *F. nucleatum* cells containing the desired concentration of test compound was added and incubated under anaerobic conditions for 24 hr at 37°C. Test compounds were initially dissolved in dimethylsulfoxide (DMSO) to generate 1000x stock solutions and were routinely tested over a final concentration range of 0 – 60 µM. 1 µL of the appropriate stock solution was added to each 1 mL aliquot of labeled *P. gingivalis* or *F. nucleatum* cells prior to adding the suspension to the microtiter plate wells. For control biofilms, 1 µL of DMSO was added to the *P. gingivalis*/*F. nucleatum* cell suspension and incubated as described above.

To test for activity against pre-formed biofilms, cells were grown and labeled as described above and biofilms were formed in buffer alone for 24 hr at 37°C. After removing unbound cells by aspiration, biofilms were incubated in 1 ml buffer containing the desired concentration of the test compounds and incubated for 1 to 3 hr under anaerobic conditions.

**Visualization of *P. gingivalis* – *S. gordonii* Biofilms.** To visualize *P. gingivalis* biofilms, unbound cells were removed by aspiration and coverslips were washed once with PBS. Biofilms were fixed by incubating the coverslips with 1 mL of 4% paraformaldehyde for 5 min followed by two washes with PBS. The coverslips were then removed, placed face down on a glass microscope slide containing a drop of Prolong Gold anti-fade reagent (Life Technology) and sealed with nail polish. Visualization of biofilms was carried out by laser scanning confocal microscopy with a Leica SP8 confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) using a 488 nm laser to detect labeled *P. gingivalis*, a 552 nm laser to detect *S. gordonii*, and a 633 nm laser to detect *F. nucleatum*. Z-plane scans of 25 µm in depth were collected at three randomly chosen frames on each coverslip using a z-step thickness of 0.7 µm. Background noise was minimized using software provided with the Leica SP8 and three dimensional reconstruction of the Z-plane scans and quantification of total green, red and far red fluorescence was
conducted using Volocity 6.3 Image analysis software (Perkin Elmer, Akron, Ohio). For biofilm images, far red fluorescence was arbitrarily assigned the color blue. The extent of *P. gingivalis* binding and accumulation was expressed as the ratio of total green (*P. gingivalis*) to red (*S. gordonii*) fluorescence and the IC₅₀ for each compound was defined as the concentration that reduced the ratio of green to red fluorescence by 50%. Experiments were carried out in triplicate for each concentration of test compound and three independent experiments were conducted for each compound. GraphPad InStat3 software was used for data analysis and statistical significance was defined as *p* < 0.05.

**In vivo model of periodontitis.** This protocol (#16486) used for this study was approved on May 3, 2016 by the Institutional Animal Care and Use Committee at the University of Louisville as described in the Federal guidelines for the care and use of laboratory animals. Specific pathogen-free BALBc/ByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 10-weeks old and housed in the University of Louisville Research Resource Center animal facility. The mice were fed with Lab Diet 5001 meal form (Purina Mills, LLC, Gray Summit, MO) during the entire experiment.

Oral infection of mice was performed essentially as previously described by Daep et al (19). *S. gordonii* was first established in the oral cavity of mice prior to infection with *P. gingivalis*. A total of 7 mice per group were used per experiment. Animals were initially treated with mixture of sulfamethoxazole (MP Biomedical, Solon, OH) at a final concentration of 800 μg/ml and trimethoprim (Sigma, St. Louis, MO) at a final concentration of 400 μg/ml *ad libitum* for 10 days. Four days after the last antibiotic treatment, the mice were orally infected with 10⁹ cfu *S. gordonii* cells suspended in 1 ml 2% carboxymethylcellulose (CMC; MP Biomedical, Solon, OH) in sterile PBS using a 2.25 mm feeding needle (Popper and Sons, Inc, New Hyde Park, NY). Subsequently, animals were infected 5 times with 10⁷ cfu *P. gingivalis* at two day intervals over a ten day period. *P. gingivalis* cells were suspended in 1 ml 2% CMC containing either the peptidomimetic compound or buffer. The concentration of compound
administered was as follows: PCP-III-201 (20 \mu M); PCP-III-206 (40 \mu M); PCP-III-212 (20 \mu M); PCP-III-293 (15 \mu M) and PCP-IV-20 (60 \mu M) and was approximately 3-fold higher than the IC_{50} value previously reported for inhibition of *P. gingivalis* adherence to *S. gordonii* (20). After infection, animals were rested for 47 days and then euthanized via CO_{2} asphyxiation. The total duration of the experiment was 80 days.

Mouse skulls were defleshed by autoclaving for 15 min, then immersed in 3% hydrogen peroxide overnight at room temperature to remove any remaining musculature and washed with deionized water. Skulls were then soaked in 1% bleach solution for 30 seconds, sonicated at 14 volts for 1 min and then washed with water. To remove any remaining bacteria and tissues, the skulls were brushed with toothpaste and sonicated in 1% bleach solution for an additional 30 seconds at 14 volts. The cleaned skulls were stained with 1% methylene blue for 15 seconds and rinsed with deionized water to remove excess dye. The stained skulls were allowed to air dry prior to measurement for alveolar bone loss.

Bone loss was assessed by measuring the distance between the alveolar bone crest (ABC) and the cemento-enamel junction (CEJ) at 7 sites on the buccal side of the right and left maxillary molars for a total of 14 measurements. This was accomplished using a dissecting microscope fitted with a video imaging marker measurement system (model VIA-170K; Fryer) at a total magnification of 40x (19). Measurements were taken in millimeters. The average of the total bone loss for each mouse group was assessed and subtracted from the baseline bone loss observed in sham-infected mice.

Statistical differences in bone loss was analyzed by ANOVA using GraphPad Instat (La Jolla, CA). A pair-wise, parametric analysis of variance using a Bonferroni multiple comparison post-test was used to determine the statistical difference among the individual mouse groups. A p value of \leq 0.05 was considered to be statistically significant.
Cell culture. Human telomerase immortalized gingival keratinocytes (TIGKs) were provided by Dr. Richard Lamont (University of Louisville) and were authenticated by comparison to primary gingival epithelial cells for cell morphology, growth, cytokeratin expression and the expression of toll-like receptors. TIGKs were cultured at 37°C in an atmosphere of 5% CO₂ in Basal Medium Dermalife K complete kit with Supplements (LifeLine, Frederick, MD). Cultures were incubated for 5 days and attained >95% confluence. The mouse macrophage cell line J774A.1 was obtained from the American Type Culture Collection and grown in DMEM (Thermo Fisher Scientific) supplemented with 4.5 g/ml glucose, 10% Fetal Bovine Serum and 100 U/ml penicillin/streptomycin (Sigma). Cultures were incubated at 37°C in an atmosphere of 5% CO₂ for 4 days and reached >95% confluence.

Measurement of Lactate Dehydrogenase (LDH) Activity. Lactate dehydrogenase (LDH) activity was determined using the CytoTox 96 non-radioactive Cytotoxicity Assay (Promega). TIGK and J774A.1 cells were inoculated in a 96 well microtiter plate at a density of 4000 cells per well and grown for 24 hr. The medium was then removed and replaced with fresh medium containing the desired concentration of peptidomimetic compound. The cells were further cultured for 18 hr in the presence of the peptidomimetic compounds, centrifuged for 4 min at 250 x g and 50 µl of supernatant was transferred to a fresh 96 well microtiter plate. Subsequently, 50 µl of LDH substrate was added per well and plates were incubated at room temperature for 30 min. Reactions were terminated by the addition of 50 µl of stop solution provided in the CytoTox 96 kit. LDH activity was determined by measuring the optical density at a wavelength of 490 nm. For positive control reactions, 15 µl of lysis buffer provided in the CytoTox 96 kit was added to the cells and incubated for 1 hr. Negative control reactions comprised cells that were incubated with media alone. All samples were assayed in triplicate.
**Determination of cell metabolic activity.** Cell metabolic activity was assessed by quantifying total ATP levels in cell culture samples, using CellTiterGlo reagent (Promega). TIGK and J774A.1 cells were cultured and incubated with compounds as described above, washed three times with sterile PBS and incubated with 100 μl of CellTiterGlo substrate for 2 min with shaking and for an additional 10 min without shaking. Total light production was measured using a Victor 3 multi-label plate reader (PerkinElmer) in luminometer mode. All samples were assayed in triplicate.

**Measurement of cell apoptosis.** The degree to which the peptidomimetic compounds induced apoptosis in TIGK and J774A.1 cells was determined using the PE Annexin V/Dead Cell Apoptosis Kit with SYTOX® Green for Flow cytometry (Invitrogen). TIGK and J774A.1 cells were cultured in 12 well microtiter plates with an initial density 2 X 10^5 cells in 1.5 ml media. After 24 hr at 37°C, the medium was decanted, replaced with fresh medium containing the desired concentration of peptidomimetic compound and incubated for an additional 18 hr. The cells were washed with PBS, trypsinized and centrifuged at 250 x g. The cell pellet was suspended in 100ul of binding buffer supplemented with 1 μl Sytox and 5 μl Annexin florescent dye and incubated for 15 min at 37°C. Samples were then diluted by addition of 400 μl binding buffer and analyzed by flow cytometry using a FACScalibur flow cytometer (Becton Dickinson), measuring the fluorescence emission at 530 nm and 575 nm. Medium only served as control.

**Determination of hemolytic activity.** A sample of 100 μl of 1% sheep or human erythrocytes (BioclaimationIVT, MD) was suspended in 1ml sterile PBS containing 5% fetal bovine serum (FBS) and the desired concentration of peptidomimetic compound was subsequently added. The suspension was incubated at 37°C for 3 hr, centrifuged at 3500 x g for 5 min and 200 μl of the each supernatant were transferred into each well of a 96 well microtiter plate. Hemoglobin release was recorded by spectrophotometry using a Victor 3 multi-label plate reader (Perkin Elmer) at a wavelength 538 nm. All
samples were assayed in triplicate. Erythrocytes suspended in PBS/5% FBS served as the negative control and erythrocytes that were lysed by suspension in \( \text{dH}_2\text{O} \) served as a positive control.

Statistical analysis. Data from each of the toxicity tests were analyzed by ANOVA using a Bonferroni multiple comparison post-test to compare each experimental sample to the control sample. A p value of \( \leq 0.05 \) was considered to be statistically significant.
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1. Socransky, SS, Haffajee AD, Cugini MA, Smith C, and Kent RL, Jr. 1998. Microbial complexes in subgingival plaque. J. Clin. Periodontol. 25:134-144.

2. Sheiham, A, and Netuveli, GS. 2002. Periodontal diseases in Europe. Periodontol 2000. 29:104-121.

3. Corbet, EF, Zee, KY, and Lo, E. 2002. Periodontal diseases in Asia and Oceania. Periodontol 2000. 29:122-152.

4. Dye, BA. 2012. Global periodontal disease epidemiology. Periodontol 2000. 58:10-25.

5. Eke, PI, Dye, BA, Wei, L, Slade, GD, Thornton-Evans, GO, Borgnakke, WS, and Genco, RJ. 2015. Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. Journal of Periodontal. 86:611-622.

6. Hajishengallis G, Liang S, Payne, MA, Hashim, A, Jotwani, R, Eskan, MA, McIntosh, ML, Alsam, A, Kirkwood, KL, Lambris, JD, Darveau, RP and Curtis, MA. 2011. Low-abundance biofilm species orchestrate inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe. 10:497-506.

7. Hajishengallis, G, Darveau, RP, and Curtis, MA. 2012. The keystone-pathogen hypothesis. Nat Rev Microbiol 10:717-725.

8. Lamont, RJ, and Hajishengallis, G. 2015. Polymicrobial synergy and dysbiosis in inflammatory disease. Trends Mol Med. 21:172-183.

9. Kim, J, and Amar, S. 2006. Periodontal disease and systemic conditions: a bidirectional relationship. Odontology, 94(1), 10-21.

10. Scannapieco, FA. 2013. The oral microbiome: Its role in health and in oral and systemic infections. Clinical Microbiology Newsletter 35: 163-169.

11. Kaur, S, Bright, R, Proudman, SM, and Bartold, PM. 2014. Does periodontal treatment influence clinical and biochemical measures for rheumatoid arthritis? A systematic review and meta-analysis. Semin Arthritis Rheum 44:1133-122.

13. Genco, RJ, and Borgnakke, WS. 2013. Risk factors for periodontal disease. Periodontol 2000, 62:59-94.

14. Wright, CJ, Burns, LH, Jack, AA, Back, CR, Dutton, LC, Nobbs, AH, Lamont, RJ, and Jenkinson, HF. 2013. Microbial interactions in building of communities. Mol. Oral Microbiol. 28:83-101.

15. Lamont, RJ, El-Sabaeny, A, Park, Y, Cook, GS, Costerton, JW, and Demuth, DR. 2002. Role of the Streptococcus gordonii SspB protein in the development of Porphyromonas gingivalis biofilms on streptococcal substrates. Microbiol. 148:1627-1636.
16. Park, Y, Simionato, MR, Sekiya, K, Murakami, Y, James, D, Chen, W, Hackett, M, Demuth, DR and Lamont, RJ. 2005. Short fimbriae of Porphyromonas gingivalis and their role in coadhesion with Streptococcus gordonii. Infect. Immun. 73:3983-3989.

17. Daep, CA, James, DM, Lamont, RJ, and Demuth, DR. 2006. Structural characterization of peptide-mediated inhibition of Porphyromonas gingivalis biofilm formation. Infect. Immun. 74:4055-4062.

18. Daep, CA, Lamont RJ, and Demuth, DR. 2008. Interaction of Porphyromonas gingivalis with oral streptococci requires a motif that resembles the eukaryotic nuclear receptor box protein-protein interaction domain. Infect. Immun. 76:3272-3280.

19. Daep, CA, Novak, EA, Lamont, RJ, and Demuth, DR. 2011. Structural dissection and in vivo effectiveness of a peptide inhibitor of Porphyromonas gingivalis adherence to Streptococcus gordonii. Infect. Immun. 79:67-74.

20. Patil, PC, Tan, J, Demuth, DR, and Luzzio, FA. 2016. 1,2,3-Triazole-based inhibitors of Porphyromonas gingivalis adherence to streptococci and biofilm formation. Bioorg. Med. Chem. 24:5410-5417.

21. Mocharla, VP, Colasson, B, Lee, LV, Roper, S, Sharpless, KB, Wong, CH, and Kolb, HC. 2005. In situ click chemistry: enzyme-generated inhibitors of carbonic anhydrase II. Angew. Chem. Int Ed. 44:116-120.

22. Borman, S. 2002. In situ click chemistry. Chem Eng. News. 80:29-34.

23. Patil, PC, Luzzio, FA, and Demuth, DR. 2015. Oxazoles for click chemistry II: synthesis of extended heterocyclic scaffolds. Tetrahedron Lett. 56:3039-3041.

24. Loner, CM, Luzzio, FA and Demuth, DR. 2012. Preparation of azidoaryl- and azidoalkyloxazoles for click chemistry. Tetrahedron Lett. 53:5641-5644.

25. Brooks, W, Demuth, DR, Gil, S and Lamont, RJ. 1997. Identification of a Streptococcus gordonii SspB domain that mediates adhesion to Porphyromonas gingivalis. Infect. Immun. 65:3753-3758.

26. Hendrickson, EL, Beck, DA, Miller, DP, Wang, Q, Whiteley, M, Lamont, RJ and Hackett, M. 2017. Insights into dynamic polymicrobial synergy revealed by time-coursed RNA-Seq. Front Microbiol. 8:261. Doi: 10.3389/fmicb.2017.00261

27. Kuboniwa, M, Houser, JR, Henrickson, EL, Wang, Q, Alghamdi, SA, Sakanaka, A, Miller, DP, Hutcherson, JA, Wang T, Beck, DAC, Whiteley, M., Amano, A., Wang, H., Marcotte, EM, Hackett, M and Lamont RJ. 2017. Metabolic crosstalk regulates Porphyromonas gingivalis colonization and virulence during oral polymicrobial infection. Nat Microbiol. 2:1493-1499.

28. Liu, C, Miller, DP, Wang, Y, Merchant, M and Lamont RJ. 2017. Structure-function aspects of the Porphyromonas gingivalis tyrosine kinase Ptk1. Mol Oral Microbiol 32:314-323.

29. Clader, JW. 2004. The Discovery of Ezetimibe: A View from Outside the Receptor. J. Med. Chem. 47:1-9.
30. Meanwell, NA. 2018. Fluorine and Fluorinated Motifs in the Design and Application of Bioisosteres for Drug Design. J. Med. Chem. DOI10.1021/acsjmedchem7b011788.

31. Meanwell, NA. 2015. Applications of Fluorine in Medicinal Chemistry. J. Med. Chem. 58:8315-8359.
**Figure 1.** Representative images of a three species biofilm comprising *S. gordonii* (red), *F. nucleatum* (blue) and *P. gingivalis* (green). (A) Control biofilm formed in buffer alone, (B, C, D) biofilms formed in buffer containing 5 μM, 10 μM or 20 μM PCP-III-201, respectively.

**Figure 2.** Representative images of the maxillary molars of sham-infected animals (A), *P. gingivalis*-infected animals (B), and animals infected with *P. gingivalis* in the presence of 20 μM PCP-III-201 (C).

**Figure 3.** Alveolar bone loss of untreated *P. gingivalis*-infected mice (SgPg) and animals infected with *P. gingivalis* in the presence of peptidomimetic compounds PCP-III-201, PCP-III-206, PCP-III-212, PCP-III-293 or PCP-IV-20. Asterisks indicate a significant increase in bone loss relative to sham infected animals; ** - *p*<0.01, *** - *p*<0.001. Ampersands indicate a significant reduction in bone loss relative to untreated mice (group SgPg); & - *p*<0.05, &&& - *p*<0.001.

**Figure 4.** Release of lactate dehydrogenase (LDH) activity from TIGK (A) and J774A.1 (B) cells after 18 hr exposure to peptidomimetic compounds. Concentrations of compounds used is as follows: PCP-III-201 (5 μM, 10 μM, 20 μM), PCP-III-206 (5 μM, 20 μM, 40 μM), PCP-III-212 (5 μM, 10 μM, 20 μM), PCP-III-293 (5 μM, 20 μM, 60 μM), and PCP-IV-20 (5 μM, 20 μM, 60 μM). Asterisks indicate a significant increase in LDH activity relative to cells exposed to medium containing 0.1% DMSO; * * * - *p*<0.001.

**Figure 5.** Quantification of ATP levels in TIGK (A) and J774A.1 (B) cells after 18 hr exposure to peptidomimetic compounds. Concentrations of compounds used is as follows: PCP-III-201 (5 μM, 10
μM, 20 μM), PCP-III-206 (5 μM, 20 μM, 40 μM), PCP-III-212 (5 μM, 10 μM, 20 μM), PCP-III-293 (5 μM, 20 μM, 60 μM), and PCP-IV-20 (5 μM, 20 μM, 60 μM). Asterisks indicate a significant decrease in ATP relative to cells exposed to medium containing 0.1% DMSO; * p<0.05, *** - p<0.001.

Figure 6. Hemolytic activity of peptidomimetic compounds against sheep red blood cells. Concentrations of compounds used is as follows: PCP-III-201 (5 μM, 10 μM, 20 μM), PCP-III-206 (5 μM, 20 μM, 40 μM), PCP-III-212 (5 μM, 10 μM, 20 μM), PCP-III-293 (5 μM, 20 μM, 60 μM), and PCP-IV-20 (5 μM, 20 μM, 60 μM).
Table 1. Three species biofilm inhibition by peptidomimetic compounds.

| Compound | Structure | IC$_{50}$ (µM) |
|----------|-----------|----------------|
| PCP-III-201 | ![Structure](image1) | 15 |
| PCP-III-206 | ![Structure](image2) | 15 |
| PCP-III-212 | ![Structure](image3) | 17 |
| PCP-III-293 | ![Structure](image4) | 10 |
| PCP-IV-20 | ![Structure](image5) | 20 |
Table 2. Activity of PCP-III-201 against a pre-formed biofilm.

| Treatment time (hr) | 5 µM | 10 µM | 20 µM |
|---------------------|------|-------|-------|
| 1                   | 3.3  | 22.3  | 49.5  |
| 2                   | 2.5  | 35.4  | 58.7  |
| 3                   | 29.5 | 41.4  | 60.5  |
Table 3. TIGK and J774A.1 cell apoptosis induced by peptidomimetic compounds

| Treatment conc (µM) | Live cells (%) (TIGK / J774A.1) | Early apoptosis (%) (TIGK / J774A.1) | Late apoptosis/ necrosis (%) (TIGK / J774A.1) |
|---------------------|----------------------------------|--------------------------------------|-----------------------------------------------|
| Medium              | 91.8 / 96.7                      | 2.1 / 0.4                            | 0.9 / 0.6                                     |
| Medium/DMSO         | 96.9 / 97.2                      | 1.6 / 0.3                            | 0.6 / 0.3                                     |
| H₂O₂ (5 mM)         | 50.0 / 21.3                      | 25.6 / 7.1                           | 10.9 / 21.4                                  |
| 201 5               | 94.7 / 97.4                      | 0.9 / 0.3                            | 0.7 / 0.5                                     |
| 10 94.9 / 94.7      | 0.6 / 0.4                         | 0.9 / 0.4                            |
| 20 95.7 / 93.9      | 0.9 / 0.5                         | 0.5 / 0.5                            |
| 206 5               | 96.9 / 97.1                      | 0.9 / 0.3                            | 0.5 / 0.5                                     |
| 20 95.7 / 94.0      | 1.1 / 0.5                         | 0.8 / 0.6                            |
| 40 95.9 / 89.7      | 0.7 / 0.4                         | 0.6 / 0.6                            |
| 212 5               | 95.0 / 98.4                      | 0.7 / 0.2                            | 2.3 / 0.1                                     |
| 10 95.6 / 96.7      | 0.8 / 0.4                         | 1.8 / 0.2                            |
| 20 95.3/94.8        | 0.8 / 0.2                         | 1.9 / 0.2                            |
| 293 5               | 92.2 / 93.2                      | 1.2 / 0.5                            | 4.4 / 0.2                                     |
| 20 96.7 / 97.2      | 0.9 / 0.3                         | 1.2 / 0.1                            |
| 60 78.3* / 96.6     | 4.2 / 0.4                         | 15.2* / 0.2                          |
| 20 5                | 98.5 / 96.4                      | 0.3 / 0.4                            | 0.4 / 0.2                                     |
| 20 94.4 / 96.0      | 1.1 / 0.3                         | 3.2 / 0.1                            |
| 60 91.5 / 96.2      | 2.4 / 0.3                         | 4.4 / 0.1                            |

* p < 0.05
Figure 1. Representative images of a three species biofilm comprising *S. gordonii* (red), *F. nucleatum* (blue) and *P. gingivalis* (green). (A) Control biofilm exposed to buffer alone, (B, C, D) biofilms exposed to buffer containing 5uM, 10uM or 20uM PCP-III, respectively.
Figure 2. Representative images of the maxillary molars of sham-infected (A), *P. gingivalis*-infected (B), and animals infected with *P. gingivalis* in the presence of 20 μM PCP-III-201 (C).
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