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Disruption of heterotypic community development by *Porphyromonas gingivalis* with small molecule inhibitors

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Abstract

Porphyromonas gingivalis is one of the main etiological organisms in periodontal disease. On oral surfaces *P. gingivalis* is a component of multispecies biofilm communities and can modify the pathogenic potential of the community as a whole. Accumulation of *P. gingivalis* in communities is facilitated by interspecies binding and communication with the antecedent colonizer *Streptococcus gordonii*. In this study we screened a library of small molecules to identify structures that could serve as lead compounds for the development of inhibitors of *P. gingivalis* community development. Three small molecules were identified that effectively inhibited accumulation of *P. gingivalis* on a substratum of *S. gordonii*. The structures of the small molecules are derived from the marine alkaloids oroidin and bromoageliferin and contain a 2-aminoimidazole or 2-aminobenzimidazole moiety. The most active compounds reduced expression of *mfa1* and *fimA in P. gingivalis*, genes encoding the minor and major fimbrial subunits respectively. These fimbrial adhesins are necessary for *P. gingivalis* coadhesion with *S. gordonii*. These results demonstrate the potential for a small molecular inhibitor based approach to the prevention of diseases associated with *P. gingivalis*.

Introduction

Bacterial community formation within the oral cavity represents one of the fundamental survival strategies for the more than 700 species (Aas *et al.*, 2005) that can reside in this dynamic environment. The development of complex adherent multispecies microbial communities, otherwise known as biofilmss, occurs in an ordered manner on available surfaces. The attachment of streptococcal species to the salivary pellicle represents a cornerstone in the initial community development on tooth surfaces, and it is to this streptococcal substratum that many subsequent colonizers can attach (Kuboniwa & Lamont, 2010, Wright *et al.*, 2013). In a healthy mouth, the plaque biofilm is controlled by host innate immunity and oral hygiene procedures. Problems arise for the host when this delicate balance is disturbed by such factors as smoking, poor oral hygiene and/or the colonization of

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pathogenic organisms, and the relationship between host and microbial community changes from symbiotic to dysbiotic (Hajishengallis & Lamont, 2012, Hajishengallis & Lamont, 2014).

Periodontitis is one of the more prevalent chronic conditions suffered by adults, with an incidence rate of around 50 % in the adult population of the United States (Albandar, 2005, Eke *et al.*, 2012). *Porphyromonas gingivalis*, a gram-negative anaerobe, is strongly associated with chronic cases of periodontitis (Byrne *et al.*, 2009). However, it worth noting that although *P. gingivalis* can be isolated from a large number of chronic periodontitis cases, it is sometimes present in relatively small numbers compared to other community members. It was proposed that virulence of *P. gingivalis* is expressed through modification of the pathogenicity of the previously commensal biofilm community (Hajishengallis *et al.*, 2011). In addition, communication with oral streptococci can increase the pathogenicity of *P. gingivalis* (Whitmore & Lamont, 2011).

Colonization of *P. gingivalis* is enhanced through coadhesive interactions with a number of oral bacteria (Wright *et al.*, 2013, Kuboniwa & Lamont, 2010, Rosan & Lamont, 2000). One well-studied example of inter-species coadhesion is that of *P. gingivalis* and *S. gordonii*. Binding is effectuated by two pairs of adhesins-receptors: the major fimbriae on the surface of *P. gingivalis* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on the surface of *S. gordonii* cells (Maeda *et al.*, 2004, Lamont *et al.*, 1993); and the minor fimbriae of *P. gingivalis* binding to the SspA/B protein of *S. gordonii*, a member of the antigen I/II family of proteins (Lamont *et al.*, 2002, Park *et al.*, 2005, Daep *et al.*, 2006). The gene encoding the structural subunit of the major fimbriae, *fimA*, is part of a larger operon that responds to a number of environment cues including temperature and hemin (Xie *et al.*, 1997, Park *et al.*, 2007, Nishikawa *et al.*, 2004). Mfa1 is the structural subunit of the minor fimbriae and the *mfa1* gene is regulated by contact with *S. gordonii* through a pathway involving the Ltp1 tyrosine phosphatase and the transcription factor CdhR (Park *et al.*, 2005, Maeda *et al.*, 2008, Chawla *et al.*, 2010, Simionato *et al.*, 2006).

Control of *P. gingivalis* colonization and community development is a potential means to reduce the incidence and severity of periodontitis, and a number of strategies have been explored. One promising approach is based on small peptides representing the binding domain (BAR) of *S. gordonii* SspB, which can inhibit *P. gingivalis*-*S. gordonii* community development and reduce *P. gingivalis* colonization and bone loss in a mouse model (Daep *et al.*, 2011, Daep *et al.*, 2006). In addition, gallium and silver ions can inhibit both the planktonic and biofilm growth of *P. gingivalis* in a mixed species biofilm assay with *S. gordonii* (Valappil et al., 2012). In the current study we focused on a library of small molecule based primarily on the 2-aminoimidazole and 2-aminobenzimidazole scaffolds, and which have been shown to modulate biofilm development in a variety of model systems (Liu *et al.*, 2011, Worthington *et al.*, 2012). Three compounds were identified that specifically inhibited *P. gingivalis* community development with *S. gordonii*, and which regulated expression of *P. gingivalis* adhesins.

Material and Methods

Bacterial strains and growth conditions

P. gingivalis strains ATCC 33277 and A7436 were routinely cultured anaerobically at 37 °C in Trypticase soy broth (TSB) supplemented with 1 g yeast extract, 5 mg hemin and 1 mg menadione (per liter). Solid medium was supplemented with 5 % sheep blood and 1.5% agar. *S. gordonii* was cultured in brain heart infusion broth containing 0.5 % yeast extract.

Screen of small molecule library

An initial screen of the small molecule library of 506 compounds (Liu *et al.*, 2011) for inhibition of *P. gingivalis-S. gordonii* community development utilized a dot blot format as previously described (Kuboniwa *et al.*, 2006). Stock solution of inhibitors were at 10 mM in DMSO. *S. gordonii* cells were washed in PBS and $1x10^8$ cells were applied to a nitrocellulose membrane. The membrane was blocked with 1.5% BSA, in Tris-buffered saline (TBS). *P. gingivalis* cells were labeled with *N*-hydroxysuccinimidobiotin and incubated 10 μM inhibitor (or vehicle alone) for 1 h. *P. gingivalis* cells ($1x10^8$) were reacted with the *S. gordonii* substratum for 12 h with rocking. *P. gingivalis* binding was visualized with alkaline phosphatase (AP)-conjugated streptavidin and AP-specific substrate (BCIP, Sigma).

Confocal laser scanning microscopy (CLSM) of *P. gingivalis-S. gordonii* communities

Mixed species communities of *P. gingivalis* and *S. gordonii* were generated and analyzed essentially as described previously (Kuboniwa *et al.*, 2006). In brief, *S. gordonii* cells were stained with hexidium iodide (15 μg/ml^{-1}, Invitrogen) and 2×10^8 cells were incubated on glass coverslips anaerobically for 16 h at 37 °C. Mid-log cultures of *P. gingivalis* were stained with 5-(and-6)-carboxyfluorescein, succinimidyl ester (4 μg/ml^{-1}, Invitrogen) and 2×10^7 cells were incubated with inhibitors for 5 min before addition to the *S. gordonii* substrate. *P. gingivalis-S. gordonii* communities were incubated anaerobically for 24 h at 37 °C and viewed with an Olympus FV500 confocal microscope. XYZ stacks were digitally reconstructed using the Volocity analysis program (Improvision). Quantitation of the volume of *P. gingivalis* fluorescence was obtained using the Find Objects algorithm in the Volocity program. This process analyzed all *P. gingivalis* fluorescence in the 3D digitally re-created confocal images. To estimate microcolony formation, the Find Objects process was used with a threshold for 3D objects greater than 20 μm^3.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from three independent cultures of *P. gingivalis* in heterotypic communities with *S. gordonii* as described previously (Hirano *et al.*, 2012). RNA was converted to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems) from 20 ng of RNA template. qRT-PCR was performed by StepOne plus by the ΔΔCt method using 16S rRNA as an internal control as described previously (Hirano *et al.*, 2012, Wright *et al.*, 2014). Primers are listed in Table S1.

Statistics

Experiments were carried out in triplicate and Prism 6 (Graphpad) software was used to analyze data displayed as mean ± standard deviation (SD). Multiple data sets were analyzed by ANOVA followed by Tukey post test.

Results

Identification of small molecule inhibitors that inhibit *P. gingivalis* heterotypic community development

The small molecule library of 506 compounds was screened for inhibition of *P. gingivalis* accumulation into heterotypic communities with *S. gordonii* using a semi-quantitative dot blot. Three compounds, 2A4, 2D11 and 2E11 reduced the amount of *P. gingivalis* accumulation to background levels at 10 μM without affecting the integrity of the *S. gordonii* substratum (not shown). The structures of these three compounds are shown in Figure 1.

Characteristics of active compounds

The effects of the three most potent inhibitors on *P. gingivalis* heterotypic community development were visualized and quantified by CLSM. As shown in Fig. 2, over a dose-response range, 2A4 reduced the total biovolume of *P. gingivalis* over 90% ($P < 0.001$) at 20 μM, and 40% at 2.5 μM ($P < 0.05$) with a 50 % inhibitory concentration (IC_{50}) of 3.41 μM ±0.92. Inhibitors 2D11 and 2E11 also exhibited a dose dependent reduction in *P. gingivalis* biovolume with *S. gordonii*, although neither was as effective as 2A4 (Fig. 2). The IC_{50} value for 2E11 was 6.88 μM ± 1.45, while 2D11 gave an IC_{50} of 4.73 μM ± 1.17. As an additional negative control, compound 2B1 which was negative in the initial screen was also tested and found not to inhibit development of the dual species communities (not shown). To ensure that the effects of the inhibitors were not restricted to one strain of *P. gingivalis*, 2A4 was also tested with a disseminating strain of *P. gingivalis*, A7436, originally isolated from a refractory periodontitis patient (Genco *et al.*, 1991). 2A4 inhibited heterotypic community development by A7436 (Fig. 3), although the level of reduction was less than that observed with strain 33277 and the IC_{50} was 4.07 μM ± 2.33. Changes in community architecture were also observed in the presence of inhibitors, with remaining microcolonies developing a greater abundance. The effect was particularly noticeable with A7436. At 5 μM and 2.5 μM concentrations of inhibitor, and an object count revealed a statistically significant increase in *P. gingivalis* A7436 objects over 20 μm^3 (Fig. 4).

Effect of inhibitors on growth of *P. gingivalis*

To verify that the effects of the inhibitors on community development were not due to a decrease in growth rate, 2A4, 2D11 and 2E11 were included in broth cultures of *P. gingivalis*, and growth monitored until stationary phase. When added at the IC_{50} concentration, 2A4, 2D11, and 2E11 did not impact growth of *P. gingivalis* (Fig. S1).

Changes in expression of community associated genes in *P. gingivalis*

Heterotypic community formation by *P. gingivalis* involves the fimbrial adhesins Mfa1 and FimA, intracellular signal transduction through the Ltp1 tyrosine phosphatase, and cell-cell signaling through AI-2. To ascertain if the inhibitors were exerting an effect on these community mediators, quantitative RT-PCR was applied to *P. gingivalis* cells reacted with the inhibitors prior to community formation with *S. gordonii* (Fig. 5). Compared to DMSO vehicle alone, mRNA levels of *fimA* and *mfa1* were reduced by 2A4, 2D11 and 2E11. In contrast expression of *luxS* was increased by all of the inhibitors while *ltp1* was unaffected. Thus, the primary effect of the inhibitors may be on the initial adherence of *P. gingivalis* to *S. gordonii*. An increase in *luxS* expression could be a direct effect on gene transcription of the result of a feedback mechanism as the organism senses and responds to decreased adherence.

Discussion

In this study, a small molecule library was screened for effectiveness against heterotypic community formation by the periodontal pathogen *P. gingivalis*. The library is comprised of a series of molecules derived from marine natural products and structurally based on the 2-aminoimidazole scaffold (Rogers & Melander, 2008), and components have been found to exert anti-biofilm activity against a variety of gram-negative and gram-positive pathogens (Richards *et al.*, 2008, Rogers *et al.*, 2009). In addition, a number of library constituents inhibit the formation of biofilms by the cariogenic organism *Streptococcus mutans* (Liu *et al.*, 2011). While the etiology of periodontitis involves a complex multispecies community (Hajishengallis & Lamont, 2012, Hajishengallis & Lamont, 2014); within this dysbiotic community certain organisms can play a pivotal role. *P. gingivalis* is considered a keystone pathogen as it can elevate the virulence of the community as a whole (Hajishengallis *et al.*, 2011). Other organisms act as accessory pathogens in that they can increase the virulence potential of *P. gingivalis*, but in the absence of overt pathogens usually exist in commensal balance with the host (Whitmore & Lamont, 2011). Hence, interference with the accumulation of *P. gingivalis* into a heterotypic community is an attractive target to disrupt the transition of a balanced periodontal community into a destructive one.

The model system that we utilized involved accumulation of microcolonies of *P. gingivalis* on a substratum of *S. gordonii*. It is well documented that *P. gingivalis* can bind to *S. gordonii* both in vitro and in vivo within supragingival plaque (Slots & Gibbons, 1978, Kuboniwa & Lamont, 2010, Maeda *et al.*, 2008, Chawla *et al.*, 2010, Daep *et al.*, 2011). As oral streptococci such as *S. gordonii* can constitute up to 70% of the early bacterial community on supragingival tooth surfaces (Socransky *et al.*, 1977), their interaction with *P. gingivalis* is considered an important initial colonization mechanism that allows the organism to become established on the tooth surface before spreading to the subgingival area (Kuboniwa & Lamont, 2010). Three small molecules, 2A4, 2D11 and 2E11 were capable of diminishing the biovolume of *P. gingivalis* in dual species communities, without affecting the underlying substratum of *S. gordonii*. Interestingly, two of these, 2A4 and 2D11, were also effective in reducing *S. mutans* accumulations. In the presence of 2A4, production of Antigen I/II and Gtf, two main surface adhesion molecules of *S. mutans* is

significantly reduced, suggesting an effect on the bacterial surface (Liu *et al.*, 2011). Hence, these compounds have potential for use in controlling two of the major diseases of microbial origin: caries and periodontal disease.

In the presence of the inhibitors, the expression of both fimbrial genes *mfa1* and *fimA* was reduced. Lower levels of the major (FimA) and minor (Mfa1) fimbriae compromises the initial attachment of *P. gingivalis* to *S. gordonii* and impedes further development of heterotypic communities (Lamont *et al.*, 1993, Lamont *et al.*, 2002). Both *fimA* and *mfa1* are regulated by the FimS/FimR two component system (TCS) (Hayashi *et al.*, 2000, Wu *et al.*, 2007) and the 2-aminoimidazole or 2-aminobenzimidazole derived compounds can inhibit TCS in other organisms (Worthington *et al.*, 2013). Disruption of TCS control of *fimA* and *mfa1* transcription, therefore, represents a potential mechanism of action of the inhibitors.

As well as observing a decrease in primary binding mechanisms to *S. gordonii*, an alteration in colony architecture was observed in *P. gingivalis. P. gingivalis* microcolonies that successfully developed in the presence of the inhibitors were significantly altered in colony architecture. Explanations for this phenomenon include a decrease in competition with other microcolonies or an increase in autoaggregation of *P. gingivalis* resulting from reduced amounts of FimA or Mfa1 (Kuboniwa *et al.*, 2009). Alternatively, expression of the *luxS* gene was significantly increased by the inhibitors, and the higher degree of AI-2 signaling will increase the size of the microcolonies (Chawla *et al.*, 2010). Moreover, previous reports have established an optimal level of AI-2 for oral microbial community development (Rickard *et al.*, 2006) and disruption of AI-2 dependent communication by the inhibitors could therefore have adverse effects that extend beyond *P. gingivalis-S. gordonii* interactions.

The experimental system adopted for this study represents the first stage in a continuum from in vitro models, to in vivo testing in animals, to human intervention studies. As with all models, in order to be tractable, complexity is reduced. In vivo, the presence of saliva and crevicular fluid, or of other bacteria may modulate the effect of the inhibitors. In addition, bacterial cells in mature biofilms may behave differently to those simple communities. These factors will be investigated in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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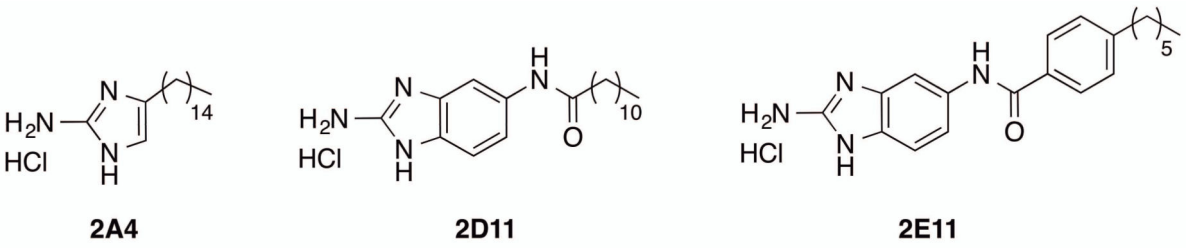

Figure 1. Structures of compounds that inhibited *P. gingivalis*-*S. gordonii* community formation

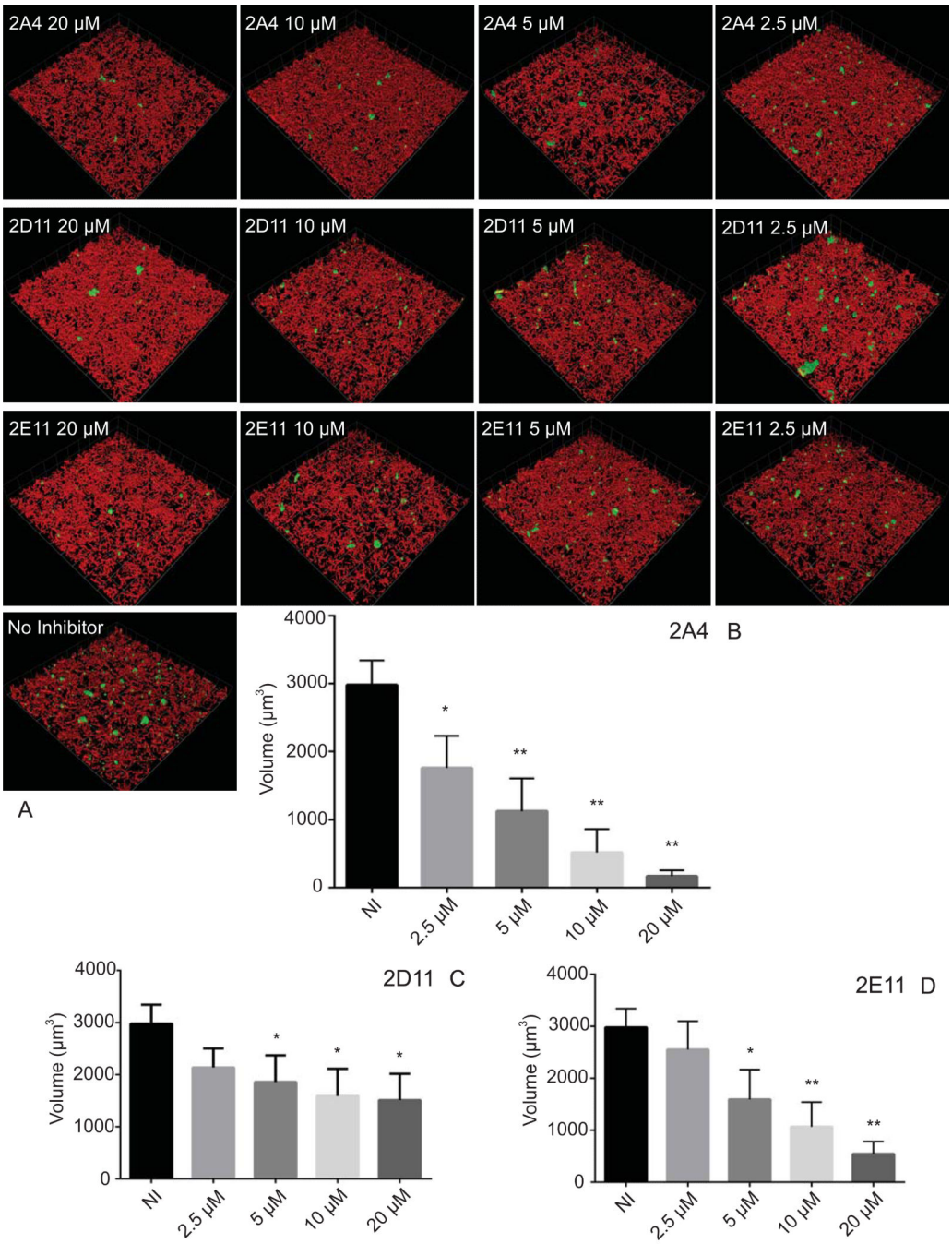

Figure 2. Effects of small molecule inhibitors on heterotypic community development

P. gingivalis 33277 was incubated with the inhibitors at the concentrations indicated, or with vehicle (DMSO) alone, for 18 h. A) Visualization of dual species communities of *P. gingivalis* (green) with *S. gordonii* (red). A series of 20-30 μm-deep optical fluorescent *x-y* sections (213 × 213 μm) were collected to create digitally reconstructed 3D images with Volocity software. B-D) Total *P. gingivalis* biovolume in images represented in A) in the presence of inhibitors or control (NI) measured with Volocity software. Quantitative results

are means with standard deviation of three independent experiments performed in triplicate * – P value of <0.05, ** – P value of <0.01, *** – P value <0.001.

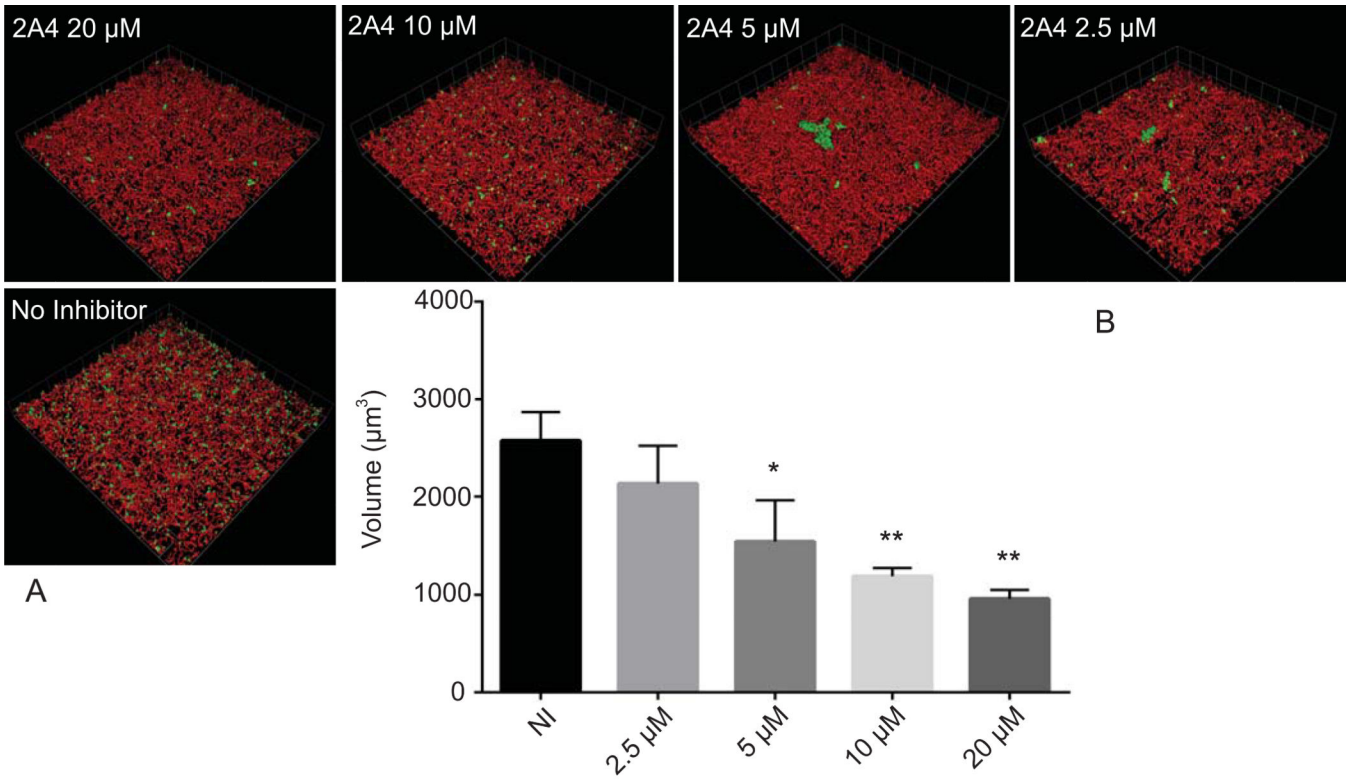

Figure 3. Effect of 2A4 on *P. gingivalis* A7436 community development

P. gingivalis A7436 was incubated with inhibitor 2A4 at the concentrations indicated, or DMSO alone (NI), for 18 h. A) Visualization of dual species communities of *P. gingivalis* (green) with *S. gordonii* (red). A series of 20-30 μm-deep optical fluorescent *x-y* sections (213 × 213 μm) were collected to create digitally reconstructed 3D images with Volocity software. B) Total *P. gingivalis* biovolume in images represented in A) in the presence of inhibitors or DMSO control measured with Volocity software. Results are means with standard deviation of three independent experiments performed in triplicate * – P value of <0.05, ** – P value of <0.01.

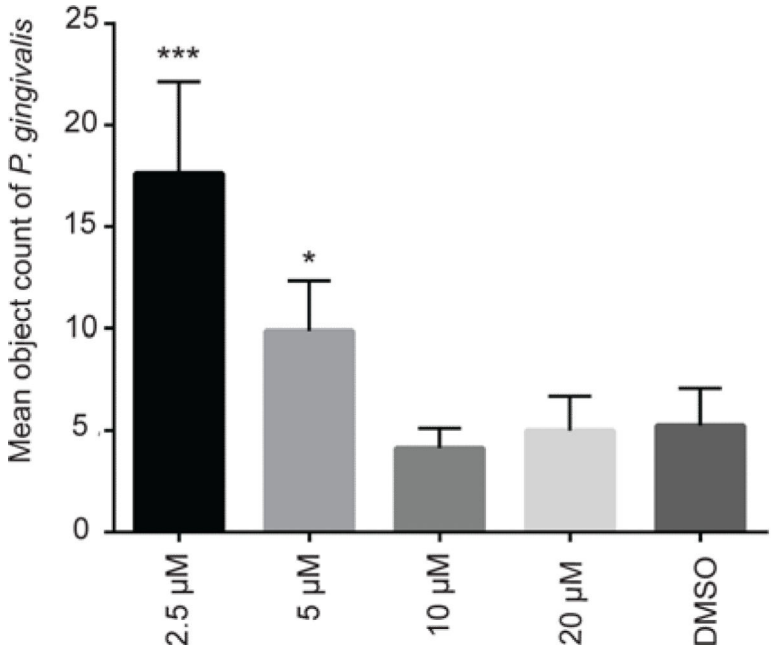

Figure 4. Mean object count of *P. gingivalis* A7436 microcolonies

Volocity software was used to determine number of microcolonies larger than 20 μm^3 from experiments shown in Figure 3. Quantitative results are means with standard deviation of three independent experiments performed in triplicate * – P value of <0.05, *** – P value <0.001.

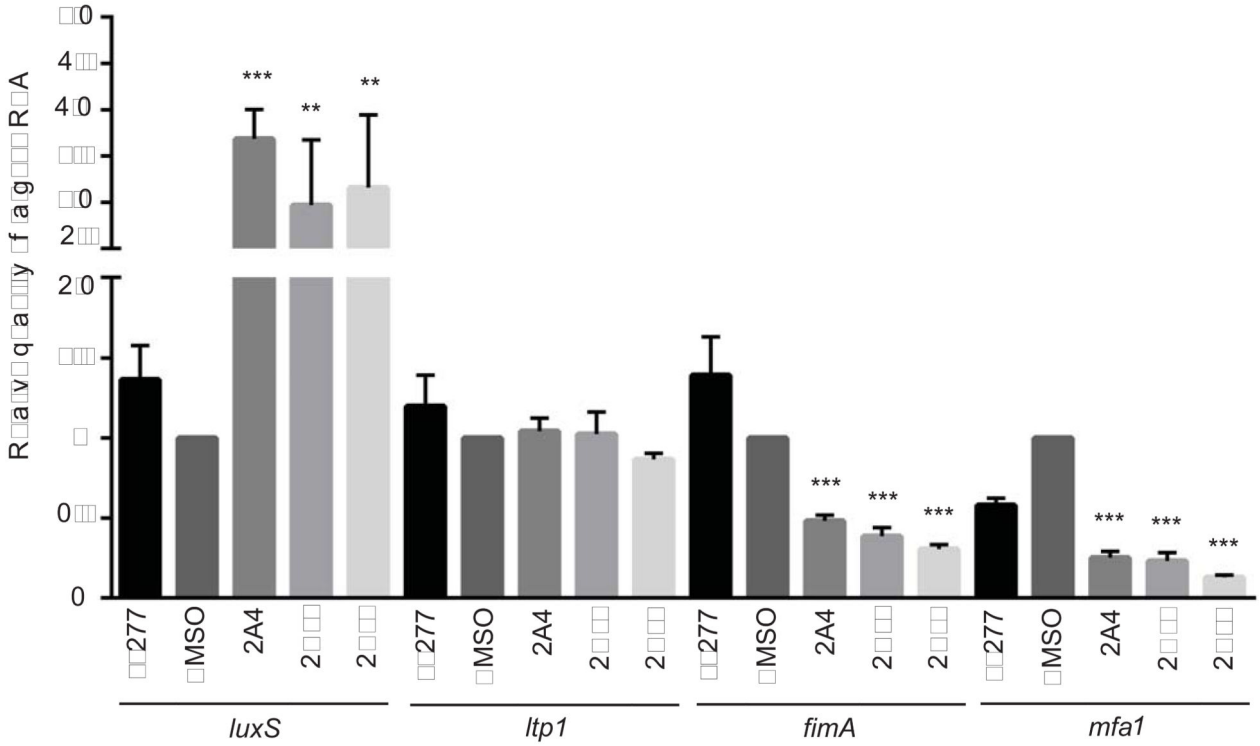

Figure 5. Analysis of differential gene expression by qRT-PCR

mRNA extracted from *P. gingivalis* cells under biofilm conditions incubated with and without inhibitors was analyzed by qRT-PCR. 16s rRNA was used for normalization. ** – P value of <0.01, *** – P value <0.001 compared to DMSO control. Representative data are shown as means with standard deviation of 3 biological replicates.