A new small-molecule specifically inhibits the cariogenic Streptococcus mutans in multispecies biofilms

Chang Liu¹, Roberta J. Worthington², Christian Melander² and Hui Wu¹

¹Department of Pediatric Dentistry, UAB School of Dentistry, Birmingham, AL 35294
²Department of Chemistry, North Carolina State University, Raleigh, NC 27695

Running title: Small-molecule inhibitors against S. mutans

Corresponding Author: Hui Wu, Department of Pediatric Dentistry, UAB School of Dentistry, Birmingham, AL 35294, Email: hwu@uab.edu; Phone number: 205-996-2392 and Fax number: 205-975-4430
Streptococcus mutans is a major cariogenic bacterium. It has adapted to the biofilm lifestyle, which is essential for pathogenesis of dental caries. We aimed to identify small-molecules that can inhibit cariogenic *S. mutans* and to discover lead structures that could give rise to therapeutics for dental caries. In this study, we screened a focused small-molecule library of 506 compounds. Eight small-molecules were identified which inhibited *S. mutans* at a concentration of 4 µM or less, but did not affect cell growth or biofilm formation of commensal bacteria, represented by *Streptococcus sanguinis* and *Streptococcus gordonii*, in monospecies biofilms. The active compounds share similar structural properties, which are characterized by a 2-aminoimidazole (2-AI) or a 2-aminobenzimidazole (2-ABI) subunit. In multispecies biofilm models, the most active compound also inhibited cell survival and biofilm formation of *S. mutans* but did not affect commensal streptococci. This inhibitor down-regulated the expression of six biofilm-associated genes: *ftf, pac, relA, comDE, gbpB* and *gtfB* in planktonic *S. mutans* cells, while it only down-regulated the expression of *ftf, pac* and *relA* in the biofilm cells of *S. mutans*. The most potent compound also inhibited production of two key adhesins, antigen I/II and GTF of *S. mutans*. However, the compound did not alter the expression of the corresponding genes in both *S. sanguinis* and *S. gordonii*, indicating that it possesses a selective inhibitory activity against *S. mutans*. 
INTRODUCTION

Bacterial biofilms are defined as surface-attached bacterial communities encased in an extracellular matrix of polysaccharides, proteins, and DNA. About 75% of infectious diseases are associated with biofilms (4). As sessile bacteria grown in biofilms inherently withstand host immune responses, and are more resistant to antibiotics, biocides, and hydrodynamic shear forces than their planktonic counterparts, there is a significant hurdle to treating biofilm-mediated infections (30). Therefore, there is an urgent need to develop new and effective therapeutics for biofilm-associated diseases.

Dental caries is one of the most common infectious diseases in humans, and is initiated by the formation of dental plaque biofilms. Although different bacteria have been found to be associated with pathogenesis of dental caries, the mutans streptococcal group represented by Streptococcus mutans is considered to be a major etiologic agent in the pathogenesis of dental caries. Currently, dental plaque is eradicated mainly through non-specific mechanical removal or treatment with broad-spectrum antibiotics. In addition, a number of derivatives from natural products, such as cranberry constituents, plant lectins, crude extracts of Morus alba leaves, and fractions of barley coffee have been shown to be effective against biofilm formation of S. mutans. These substances can regulate the activities of surface-anchored virulence factors, glucosyltransferase and fructosyltransferase (5, 7, 9, 14, 34). Numerous small-molecules, including anthraquinones, apigenin, tt-farnesol, chitosan, and 7-epiclusianone, have been characterized and shown to have anti-biofilm activity towards S. mutans (2, 17, 25, 26). However, none of them are reported to possess selectivity against S. mutans biofilms. The prevention and treatment approaches based on these existing methods...
tend to disturb the ecological balance between pathogens and commensal residents in the oral cavity, which may lead to more severe infections. Therefore, it is necessary to develop a new approach which can selectively inhibit pathogenic bacteria and biofilms.

To achieve this selectivity goal, we elected to pursue small-molecules based upon nitrogen-dense marine alkaloids. We have designed numerous derivatives of marine natural products based primarily on the 2-aminoimidazole (2-AI) scaffold and have shown that these compounds are potent inhibitors of biofilm formation by both Gram-positive and Gram-negative bacteria (12), albeit the underlying mechanisms of biofilm inhibition/dispersion are still under investigation. One of the outcomes of these studies is that we have assembled a focused library of molecules (28) whose activity is biased towards modulating biofilm development. Therefore, screening this library represents a convenient platform for rapid identification of small-molecules that modulate biofilm formation of target bacterial species. In this study, we screened the focused small-molecule library and identified eight small-molecules that selectively inhibited cariogenic biofilms of \textit{S. mutans}. We further evaluated how the inhibitors regulated expression of known biofilm-associated genes and adhesion molecules by \textit{S. mutans}.
MATERIALS AND METHODS

Bacterial strains, culture conditions, and chemicals. Bacterial strains including S. mutans UA159, S. sanguinis SK36, and S. gordonii DL1 were used in this study (3, 8, 39). Strains were grown statically at 37 °C on Todd-Hewitt broth (THB) or THB agar plates under an aerobic atmosphere with 5% CO₂. A single colony of each strain was inoculated into 3 ml THB, and incubated for 24 h without agitation. The overnight cultures were then inoculated into fresh THB to allow bacteria to grow until they reached exponential growth phase at OD_{470}=1. The exponentially grown bacteria were then inoculated for biofilm assays. The media used to grow bacterial biofilms is a chemically defined media-BM media containing 1% sucrose (22). A library of 506 small-molecules dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration was arrayed in 96-well format and used for screening.

Biofilm formation and inhibition assays in a monospecies biofilm model. The inhibitory effects of the library compounds on bacterial biofilm formation were examined using 96-well flat-bottom polystyrene microtiter plates (Nalge Nunc International, Rochester, USA). Lead compounds identified from the screen were then re-synthesized and analyzed in static biofilm inhibition assays by utilizing crystal violet staining to confirm activity. In brief, each bacterial strain, grown at exponential phase, was inoculated into wells of a 96-well plate with optimized dilutions. The final concentration of DMSO in each well was 1%. S. mutans and S. gordonii were inoculated at 1:100 dilution while S. sanguinis was inoculated at 1:40 to obtain reproducible and compatible biofilms. The incubation time was 16 h for S. mutans and S. sanguinis, and 12 h for S. gordonii. Bacterial growth was measured at 470 nm using a microplate reader (BioTek ELx, USA). Crystal violet staining was used to monitor biofilm
formation as described previously (42). Each assay was done with duplicate samples and replicated three times. Compounds that inhibited more than 50% of biofilm formation of *S. mutans* at 20 µM in the initial screen were selected and subsequently examined for their effects on the cell growth and biofilm formation of *S. sanguinis* and *S. gordonii*. Moreover, the concentration of a given compound that inhibits biofilm formation by 50% (MIC<sub>50</sub>) was determined by serial dilutions. The most active compound isolated from the library was further analyzed.

**Evaluation of inhibitory effects of small-molecule compounds in a multispecies biofilm model.** To examine the precise effects of these selected compounds on biofilm formation by more than one species, we used GFP tagged *S. mutans UA159*, that carries kanamycin resistance gene, to co-culture with *S. sanguinis SK36* and/or *S. gordonii DL1* in BM media. Equal cell numbers of exponentially grown *S. mutans* and partner strains were inoculated into wells of a 96-well microtiter plate that contained variable concentrations of anti-biofilm compounds, and incubated under 5% CO<sub>2</sub> at 37 °C for 16 h. Biofilm mass was quantified by crystal violet staining, and total bacteria in the biofilms were scraped from the bottom of wells and rinsed with PBS to make sure all of the cells were collected. The cells from the biofilm were mechanically disrupted by vigorous pipetting and vortexing. The cells were series diluted, streaked onto plate, and then enumerated by determining colony forming units (CFU). The THB plates supplemented with or without kanamycin were used to assess the numbers of *S. mutans* cells and the entire biofilm cells, respectively. The survival percentage was calculated based on bacterial CFU of the compound treated group and the total CFU from the DMSO treated group (control).
Confocal Laser Scanning Microscope (CLSM) analysis of biofilms. *S. mutans UA159, S. sanguinis SK36* or *S. gordonii DL1* were grown in BM medium with 1 µM of compound 2A4 or with DMSO control on glass coverslips placed in wells of a sterile 6-well cell culture plate (Corning Costar Corp., Cambridge, MA, USA) under 5% CO₂ at 37 °C for 16 h. The biofilm samples were gently rinsed with PBS three times to remove unattached cells, and dried for 5 min, then stained with SYTO 9 (Molecular Probes, Invitrogen, Carlsbad, USA). The stained samples were then examined by Confocal Laser Scanning Microscopy (CLSM, LSM 710, Zeiss) with 63× oil immersion objective. Images were obtained from serial optical sections and captured at 488 nm. The z section was used to record the biofilm thickness. Vertical lines were chosen randomly for analysis of each image.

Expression of biofilm-associated genes by *S. mutans, S. sanguinis* and *S. gordonii*. Overnight cultures of *S. mutans UA159, S. sanguinis* and *S. gordonii* were inoculated as described above into BM medium with control DMSO or compound 2A4 at 1 µM, and then grown on a sterile 6-well cell culture plate to obtain biofilm cells, or in test tubes to obtain planktonic cells, at 37 °C for 16 h. In the 6-well plate, each well was gently rinsed with PBS to remove loose bacteria, and firmly attached cells were scraped from the substrate by pipetting and rinsed with PBS to make sure all cells were collected. In the test tubes, floating cells were aspirated and collected by centrifugation at 6,000 g at 4 °C for 10 min, and used as planktonic cells. Total RNA was extracted from the same numbers of cells from control and test groups respectively. All harvested cells were digested by N-acetylmuramidase (mutanolysin, Sigma–Aldrich, St. Louis, USA) at 20 µg/ml and lysozyme at 10 µg/ml at 37 °C for 60 min. Total RNA samples of the biofilm cells or planktonic cells were then
extracted with Trizol (Invitrogen, Carlsbad, USA), and further digested by RNase-free DNase (Promega, Madison, USA) to remove trace amounts of contaminated DNA. The isolated RNA was reverse transcribed into cDNA using random primers (Promega, Madison, USA). cDNA samples were then quantified by real-time PCR using iQ SYBR Green supermix kit (BioRad, Madison, USA). PCR primers are listed in Table 1. The PCR cycle was set up as follows: 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. After the last cycle, the reactions were kept at 95 °C and then 55 °C for 1 min each, and followed by a slow ramp from 55 °C to 95 °C for 10 sec. A standard curve was generated for each gene, and then plotted by amplification of a series of diluted cDNA samples. RNA samples without reverse transcription were used as negative controls to ensure no contamination by genomic DNA. The expression levels of all selected genes were normalized using 16S rRNA as an internal standard.

**Western blotting analysis of antigen I/II and GTF.** Planktonic and biofilm cells were harvested as described above. The same cell numbers of each bacterial sample were digested by 100 µl of mutanolysin and lysozyme mixture in lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5 % (v/v) NP-40) for 10 min at room temperature. Cell supernatants were harvested by centrifugation at 13,000 rpm for 5 min after the removal of cell debris and used as protein extracts. The concentrations of protein extracts were determined with a BCA Protein Assay kit (Pierce, Rockford, USA). Protein samples were then dissolved into 5×SDS loading buffer, and subjected to SDS-PAGE analysis followed by western blotting analysis using anti-SBR (1:20,000 dilution) or anti-GLU (1:10,000 dilution) antibody. Heat shock protein 70 was used as an internal control.
Statistical analysis. Experimental data were analyzed by SPSS10.0 software (SPSS Inc., Chicago, IL, USA) and presented as mean±standard deviation (SD). Two-group comparisons were performed using Student’s t-test. P<0.05 was considered to be statistically significant.

RESULTS

Identification of small-molecule compounds that selectively inhibit homotypic biofilm formation by cariogenic bacterium S. mutans. We screened our library of 506 compounds and identified eight potent small-molecules that inhibited S. mutans biofilm formation by at least 50% at concentrations ≤ 4 µM, while follow up dose-response studies determined the IC\textsubscript{50} value for each compound (Table 2). Compounds 2A4, 2B1, 2B5, 2B7, 3H5, and 4B9 (designated by plate and well numbers) all contain the 2-aminoimidazole (2-AI) subunit. 2D2 and 2D11 are functionalized 2-aminobenzimidazoles (2-ABI). Among these compounds, 2A4 exhibited the most potent activity, with an IC\textsubscript{50} value of 0.94±0.02 µM. This compound also inhibited cell growth, eliciting 50% reduction in cell growth (planktonic cell-IC\textsubscript{50}) at 2.0±0.5 µM (two-fold greater than the biofilm IC\textsubscript{50}). In addition, over 87% of the planktonic cells survived when grown in the presence of the IC\textsubscript{50} of 2-A4 (0.94 µM) when compared to the control. These data indicate that the compound had a modest selectivity towards inhibiting biofilm formation despite its ability to inhibit cell growth.

The identified compounds did not inhibit cell growth (Fig.1A) or biofilm formation (Fig.1B) of the commensal colonizers, S. sanguinis and S. gordonii at the concentration at which the cell growth and biofilm formation of S. mutans was significantly inhibited (P<0.03). We also increased the concentrations of each compound to assess the safe dose for S. sanguinis and S. gordonii. For S. sanguinis biofilm formation (Fig.2A), compounds 2A4 or
4B9 did not inhibit *S. sanguinis* (*P*>0.05) at 10 µM. Compound 3H5 at 5 µM, also had no inhibition against *S. sanguinis* (*P*>0.05). For *S. gordonii* biofilm formation, the compounds 2A4 or 4B9 at 5 µM did not affect *S. gordonii*, and compounds 2B7 and 3H5 at 10 µM also did not exhibit any inhibition against *S. gordonii* (*P*>0.05) (Fig. 2B). In addition, other selected compounds exhibited no inhibitory effect on both *S. sanguinis* and *S. gordonii* even at 20 µM (Fig. 2A and B).

**Evaluation of effects of the most active compound on cell growth and biofilm formation in a heterotypic biofilm model.** As 2A4 was the most potent inhibitor, we examined its efficacy in a multispecies biofilm model (Fig. 3). Up to 10 µM, 2A4 inhibited 34±3.0% of the cell growth of bacteria co-cultured with *S. mutans* (*P*<0.05) (Fig. 3A), and also inhibited 45±1.0% of the co-cultured biofilms (*P*<0.01) (Fig. 3B). The effect is likely due to inhibition of *S. mutans*. To verify this, we determined the survival rate of *S. mutans*, and the co-cultured bacteria respectively. In the dual-species biofilm community of *S. mutans* and *S. sanguinis*, 2A4 at 10 µM inhibited 94.9±2.6% of *S. mutans* (*P*<0.01) (Fig. 3C), whereas 60±9.0% of *S. sanguinis* remained in the biofilm (Fig. 3D). In the dual-species biofilm community of *S. mutans* and *S. gordonii*, only 4.2±3.8% and 11±5% of *S. mutans* survived at 10 µM, and 5 µM, respectively (*P*<0.05) (Fig. 3C). Over 33±10% of *S. gordonii* survived at 10 µM, and the survival rate reached 80±5% at 5 µM (Fig. 3D). In a three-species biofilm, the inhibitory profile was similar to that of the dual-species biofilms (Fig. 3C, D), supporting the idea that the compound has modest selectivity toward the cariogenic bacterium *S. mutans*.

**The most active compound altered cariogenic biofilm structure.** The effect of the most active compound 2A4 on biofilm structures of cariogenic bacteria was assessed by
confocal laser scanning microscopy. Upon treatment with 2A4 at 1 µM, *S. mutans* randomly distributed over the surface without large aggregates, and vertically elongated colonies were sporadically scattered on the biofilm substrate (Fig. 4A). In the control group (treated with DMSO), *S. mutans* cells clustered together and formed thick and firm typical biofilms (Fig. 4B). Vertical section (Fig. 4C, D) revealed that the biofilm in the DMSO treated group (14.15±0.81 µm) was at least twice as thick as that in the 2A4 treated group (5.85±0.72 µm). These data suggested that the structure of *S. mutans* biofilm was disrupted by the active small-molecule. There were no significant differences between 2A4 treated and untreated biofilms from *S. sanguinis* (Fig. 4E, F) and *S. gordonii* (Fig. 4I, J). In biofilms formed by *S. sanguinis*, the thickness for the compound treated group and DMSO treated group was 7.98±0.54 µm and 8.20±0.57 µm respectively (Fig. 4G, H). In biofilms of *S. gordonii*, the thickness for the two groups was 4.76±0.10 µm and 5.19±0.10 µm respectively (Fig. 4K, L).

Interestingly, biofilm architecture of the three strains in the control groups was quite different from each other albeit their biomass was similar. The biofilm formed by *S. mutans* was much thicker than that formed by the other two strains. The biofilm of *S. gordonii* appeared compacted and lacked elaborated architecture. The z section image revealed that the biofilm of *S. gordonii* was continuous (Fig. 4K, L).

The small-molecule compound altered expression profiles of biofilm-associated genes. To explore potential mechanisms of the inhibition, we examined the effect of the most potent small-molecule on the expression of a number of biofilm-associated genes. The relative expression level of the chosen genes was evaluated by real-time quantitative RT-PCR. Compared with the DMSO treated group, six biofilm-associated genes were significantly
down-regulated after treatment with compound 2A4 in planktonic cells ($P<0.05$) (Fig. 5A). These genes were *ftf*, *pac*, *comDE*, *relA*, *gbpB* and *gtfB*. Only three biofilm-associated genes: *ftf*, *pac* and *relA*, were significantly affected upon treatment of the biofilm cells ($P<0.05$) (Fig. 5B). However, no significant difference in the expression of these altered genes was observed between the treated and untreated groups ($P<0.05$) from both *S. gordonii* (Fig. 6A) and *S. sanguinis* (Fig. 6B).

The small-molecule reduced production of two biofilm-associated adhesins, antigen I/II and GTF. Two cell surface proteins are differentially involved in adhesion and biofilm formation by *S. mutans*. Antigen I/II, a surface-anchored protein is responsible for the binding of *S. mutans* onto tooth surface in the absence of sucrose. GTF, a glucosyltransferase, synthesizes glucan polymers in the presence of sucrose. We evaluated the effect of 2A4 on the production of these two adhesins. Compared with the DMSO treated group (Fig. 7A, lanes 1 and 3), the production of the two putative virulence determinants in both planktonic and biofilm cells was reduced significantly in the 2A4 treated cells (Fig. 7A, lanes 2 and 4). Since anti-SBR antibody recognizes antigen I/II homologs in *S. sanguinis* and *S. gordonii*, we also evaluated the effect on this homolog. The compound had no effect on production of antigen I/II homologs from *S. sanguinis* (Fig. 7B, lanes 1 and 2) and *S. gordonii* (Fig. 7B, lanes 3 and 4).
DISCUSSION

A series of small-molecules derived from marine natural products have been shown to inhibit biofilm formation of diverse bacteria (13, 29). These compounds demonstrated anti-biofilm activity against Gram-negative *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Bordetella bronchiseptica*, and Gram-positive *Staphylococcus aureus* biofilms through a non-microbicidal mechanism. In this study, we screened a focused library and identified small-molecules that inhibited the cariogenic bacterium *S. mutans* but did not inhibit two commensal colonizers, *S. sanguinis* and *S. gordonii*. The active compounds possess similar chemical structures, suggesting there is a correlation between structure and activity. It is well known that biofilm cells are often more resistant to antimicrobial compounds than planktonic cells. In the contrary, more biofilms of *S. mutans* were inhibited (50%) than the planktonic cells (27%) when the most potent compound 2A4 was used, indicating that the compound has a modest selectivity towards inhibiting biofilms albeit it has microbicidal activity. Importantly, the compound not only inhibited biofilm formation of *S. mutans* in a monospecies model but also selectively inhibited *S. mutans* when it was co-cultured with commensal *S. sanguinis* and/or *S. gordonii* in multispecies models. As oral biofilms are multispecies communities that develop through a variety of coadhesive, nutritional, metabolic and signaling interactions among diverse constituent organisms (32), the identification of a selective small-molecule inhibitor will help to specifically inhibit pathogenic bacteria. A similar approach of "targeted killing" of cariogenic *S. mutans* in both planktonic and biofilm cells has been devised using a hybrid peptide that combined an *S. mutans*-specific binding peptide and an active antimicrobial peptide (STAMPs) (6, 19). Such
a strategy is desirable to achieve effective therapies. Together, these studies have demonstrated that antibiotics play a role in fighting against susceptible bacteria as well as regulating microbial biofilm communities (21). Furthermore, the identified small-molecule inhibitor will be a useful probe to explore the molecular mechanism underlying how *S. mutans* responds to the inhibitor while the commensal streptococci avoid the inhibition. The small molecules we identified may functionally resemble analogues identified as quorum sensing molecules which possess antibacterial activity via their ability to lyse bacterial cells (15). The active small molecules we report here may interact with the cell membrane of *S. mutans*, and alter the pH gradient of the membrane, which explains why they have bactericidal effects. Additional studies are required to further delineate these potential mechanisms.

In the present study, the effect of the compound on the structures of *S. mutans* biofilms was also assessed using a non-invasive analytical method, CLSM (23). It was worth noting that untreated *S. mutans* formed a thicker biofilm with an elaborated architecture, while the biofilms formed by commensal streptococci *S. sanguinis* and *S. gordonii* were much thinner and compacted. Although biofilm mass derived from *S. mutans*, *S. gordonii* and *S. sanguinis* was comparable, the effects of 2A4 on the elaborated biofilms formed by *S. mutans*, but not on the biofilms formed by commensal streptococci were evident, supporting the concept that the small-molecule compound is selective against *S. mutans*. The molecular mechanism underlying this phenotype response is not clear. The biofilms formed by *S. mutans* possessed putative water channels, which were embedded into the thick biomass as an integral part of the biofilm structure. The water channels are a potential target for the selective compound, as
such channels were not evident in the treated biofilms formed by *S. mutans*, a phenotype that has been observed in *Pseudomonas aeruginosa* biofilms grown in the presence of quorum sensing inhibitors (33). It should also be noted that the water channel has been linked to low efficacy of anti-biofoulant agents (27, 37). The precise impact of the small-molecule compound on the water channels of *S. mutans* biofilms awaits further studies.

Alternatively, 2A4 could selectively target bacterial adhesion, the first step in the formation of biofilms. Indeed, 2A4 reduced production of two important adhesion molecules that are involved in surface binding of *S. mutans*. Concurrent with the reduced biofilm formation in the presence of 2A4, we observed decreased production of antigen I/II and GTF by *S. mutans*. Antigen I/II is a cell surface fibrillar protein, which is related to the initial adherence of *S. mutans* to salivary pellicle. By contrast, the compound had no impact on production of antigen I/II homologs from *S. sanguinis* and *S. gordonii*, indicating it indeed has a selectivity toward antigen I/II produced by *S. mutans*. Glucosyltransferase (GTF) is another important adhesion molecule from *S. mutans*. It can synthesize water-soluble and water-insoluble glucans, which play a major role in plaque biofilm formation and bacterial pathogenesis (45). Consistent with this, expression of two genes, *pac* and *gtfB*, coding for antigen I/II and GTF of *S. mutans* were significantly down-regulated by compound 2A4. However the compound failed to inhibit expression of the homologous genes from *S. sanguinis* and *S. gordonii*, supporting the idea that it possesses the selectivity towards *S. mutans*.

To adapt to a community biofilm lifestyle, biofilm cells undergo extensive phenotypic changes, in which an array of genes are up or down-regulated. Our studies revealed that 2A4
altered expression of six biofilm-associated genes in planktonic cells of *S. mutans*. These genes included adherence associated gene *pac* (24), glucosyltransferase gene *gtfB* (10, 42), glucan binding protein encoding gene *gbpB* (36), acid producing gene *ftf* (16, 38), *relA*, and regulatory gene *comDE*. They have been linked to different steps of biofilm formation. The *relA* gene encodes guanosine tetra (penta)-phosphate synthetase and is also involved in acid tolerance of *S. mutans*, another key virulence property of its cariogenesis (18). The regulatory gene *comDE* is a part of the quorum-sensing cascade of *S. mutans* (20). However, expression of other biofilm-associated genes including *smu630* (1), *brpA* (41), *secA* (11, 40) and *luxS* (31) were not affected by treatment with the small-molecule. These results support the notion that the compound selectively targets certain biofilm-related pathways. Indeed, only three biofilm-associated genes (*ftf, pac* and *relA*) were down-regulated in the biofilm cells of *S. mutans*. Differential effects of the small-molecule inhibitor on biofilm and planktonic cells further support the idea that the small-molecule compound possesses selectivity against biofilms. As we only tested a small number of biofilm-associated genes from *S. mutans*, our studies may not represent the complete picture of how the small-molecule inhibitor interacts with the biofilm genetic network. Therefore, future studies using genomic or proteomic approaches may shed more light on the effect of 2A4 on biofilm-associated signaling regulation (35).

In conclusion, our studies have identified small-molecules that have selectivity against *S. mutans*. Furthermore, our studies have revealed the differential effects of the small-molecule on expression of biofilm-associated genes, suggesting a selective mechanism is linked to the mode of action of the small-molecule inhibitor. As biofilm formation is a dynamic process...
that is associated with metabolic and signaling networks (43), the impact of the small-molecule on biofilm biology requires further investigations.

ACKNOWLEDGEMENTS

We also thank Dr Tom Wen from LSU provided us with GFP-tagged S. mutans UA159. We thank Dr Noel Childers from UAB for anti-SBR and anti-GLU antibodies. This work was partially supported by NIH/NIDCR011000 (HW), as well as the North Carolina Biotechnology Center and Keenan Institute (CM).

REFERENCES


FIGURE LEGENDS

Fig.1. Effect of small-molecule compounds on cell growth and biofilm formation in a monospecies biofilm. S. mutans, S. sanguinis or S. gordonii was treated with DMSO or selected small-molecule compounds. Cell growth (A) and biofilm formation (B) were determined respectively. The percentage of cell growth or biofilm formation in compound treated groups was calculated based on the DMSO control groups (100%). Values represent the mean±standard deviation from three independent experiments. *indicates that the cell growth between DMSO- and compound-treated groups was significantly different. # indicates that the biofilm formation between DMSO- and compound-treated groups was significantly different. 2-AI: 2-aminoimidazoles, 2-AI+: 2-aminoimidazoles and functionalized amides, 2-ABI: 2-aminobenzimidazoles, 2-ABI+: 2-aminobenzimidazoles and functionalized amides.

Fig.2. Effects of selected small-molecule compounds on cell growth and biofilm formation of commensal streptococci in a monospecies biofilm. S. sanguinis (A) and S. gordonii (B) were treated with described small-molecule compounds at different concentrations, and cell growth and biofilm formation of each strain were determined. The percentage of cell growth or biofilm formation in compound treated groups was calculated based on the DMSO control groups (100%). Values represent the mean±standard deviation from three independent experiments.

Fig.3. Effects of the most potent small-molecule compound 2A4 on S. mutans in multispecies biofilms. S. mutans was grown with S. sanguinis or S. gordonii or both in biofilm media, and treated with DMSO or 2A4. Cell growth (A) and biofilm formation (B) in the multispecies biofilms were determined. Survival percentage of S. mutans (C) and each commensal streptococcus (D) treated with 2A4 were calculated. The percentage of cell growth or biofilm formation in 2A4 treated groups was calculated based on the DMSO control groups (100%). Values represent the mean±standard deviation from three independent experiments.

Fig.4. Biofilm structures analyzed by confocal laser scanning microscopy. The left panels represent biofilms treated with 2A4, and the right panels represent control biofilms treated with DMSO. The top view (A, B, E, F, I and J) and vertical section (C, D, G, H, K and L) of the biofilms by S. mutans (A, B, C and D), S. sanguinis (E, F, G and H), and S. gordonii (I, J, K and L) are depicted. The biofilms were stained by Syto9 and examined with confocal laser scanning microscopy. The scale bar is 10 µm.

Fig.5. Expression of biofilm-associated genes by S. mutans. Planktonic (A) and biofilm cells (B) treated with 2A4 were harvested and used to extract RNA; expression of biofilm-associated genes was examined by real-time RT-PCR. # represents significant difference observed from comparison of the DMSO treated group with the 2A4 treated groups. The mRNA expression levels were calibrated using 16S rRNA. Values represent mean±standard deviation.

Fig.6. Expression of biofilm-associated genes in commensal streptococci. Planktonic and
biofilm cells from *S. sanguinis* (A) and *S. gordonii* (B) were treated with 2A4, harvested and used to extract RNA. The expression of biofilm-associated genes was examined by real-time RT-PCR. The mRNA expression levels were calibrated using 16S rRNA. Values represent mean±standard deviation.

**Fig. 7.** Effect of the small-molecule compound 2A4 on production of bacterial adhesins. Planktonic and biofilm cells from *S. mutans* (A), *S. sanguinis* and *S. gordonii* (B) treated with DMSO (control) and 2A4 were harvested, and protein samples were extracted and subjected to western blotting analysis for antigen I/II and GTF using anti-SBR and anti-Glu antibodies. Heat shock protein 70 (HSP) was used as an internal protein reference to control protein loading in each sample.
<table>
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<th>Reverse primer</th>
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Primer sequences were derived from published DNA sequences. The primers for ftf and 16S rRNA were adapted from Tam (36), and the primers for pac and secA were derived from Huang (11), the primers for gtfB were derived from Yoshida (42), the primers for brpA, comDE, relA and smu630 were derived from Steinberg (33), and the primers for vicX, gbpB were derived from Senadheera (28), Primers for gtfG and comD of S. gordonii were derived from Gilmore (8). Other genes primers were designed in this study.
Table 2. IC₅₀ and structures of selected compounds

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Fig. 2.
Fig. 3.

A) Cocultured cell growth with 2A4

B) Cocultured biofilm formation with 2A4

C) *S. mutans* survival with 2A4

D) Commensal bacteria survival with 2A4
Fig. 6.

A

B

Relative expression

Genes

sspC sspG stp7 comD staE relA

Relative expression

Genes

sspA gtfC comD ghpB relA

Legend:
- Biofilm 2A4
- Planktonic 2A4
- Biofilm DMSO
- Planktonic DMSO
Fig. 7.

**A**

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**B**

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