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The effect of autoinducer 2 quorum sensing inhibitors against biofilm formation of periodontopathogens

치주병인균의 생물막 형성에 대한

Autoinducer 2 쿼럼센싱 억제제의 효과

2017년 2월

서울대학교 치의학대학원

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류은주
Abstract

The effect of autoinducer 2 quorum sensing inhibitors against biofilm formation of periodontopathogens

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Objectives

Bacterial behaviors which are critical for survival such as virulence factor secretion, biofilm formation are performed effectively through quorum
sensing, to which bacteria respond to control gene expression in response to changes in cell density and species complexity. Quorum sensing involves the production, release, and subsequent detection of chemical signal molecules called autoinducers. Autoinducer 2 (AI-2) is an universal quorum sensing molecule that mediates intergeneric signaling in multispecies bacterial communities. Especially, AI-2 of *F. nucleatum* plays an important role in formation of subgingival biofilm composed mostly of Gram-negative anaerobic bacteria in oral environment. The aim of this study was to design and synthesize new quorum sensing inhibitors (QSIs) and evaluate their inhibitory effect on AI-2 activity and thus reduce biofilm formation of pathogenic oral bacteria.

**Methods**

D-Galactose and newly synthesized brominated furanone analogs were used as quorum sensing inhibitors (QSIs). Also, reported QSIs including D-ribose and (Z-)-4-Bromo-5-bromomethylene-2(5H)-furanone compound were used as reference compounds to compare the relative efficacy of the QSIs. To evaluate the effect on AI-2 activity, AI-2 reporter strain *Vibrio harveyi BB170* and semi-purified *Fusobacterium nucleatum* AI-2 was used and the
bioluminescence of *Vibrio harveyi BB170* was assessed. The effect on biofilm formation of periodontopathogens was evaluated by crystal violet staining and confocal laser scanning microscopy after culturing each bacteria with *F. nucleatum* AI-2 and the QSIs. As QSIs should attenuate bacterial pathogenicity rather than bacterial growth, the bacterial growth was monitored in the presence of the each QSI without *F. nucleatum* AI-2. After that, to determine whether the QSIs are toxic or induce inflammatory response in host cell, the effect of the QSIs on host cells response were assessed by cytotoxicity test and detection of gene expression level of pro-inflammatory cytokines using real time RT-PCR.

**Results**

Designed QSIs remarkably inhibited AI-2 activity of *F. nucleatum* and biofilm formation of major periodontopathogens (*F. nucleatum, Porphyromonas gingivalis, and Tannerella forsythia*) which was induced by *F. nucleatum* AI-2 without bacteriocidal effect. Furthermore, the synthesized QSIs did not have cytotoxicity and induce expression of host inflammatory cytokines in host cells (THP-1, HGFs and HOK-16B).
Conclusion

Our results demonstrate that newly designed QSIs may be used as a preventive agent against biofilm formation of pathogenic bacteria by targeting QS signaling. Especially, the widespread use of antibiotics has resulted in bacterial resistance such as multiple drug resistance, to control the pathogens by QS inhibition appears to be a promising strategy to prevent periodontitis and other bacterial infectious diseases caused by biofilms.

Keywords: Periodontopathogens, Biofilm, Quorum Sensing, AI-2 inhibitor, Periodontitis

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Contents

Abstract

I. Introduction 1

II. Materials and Methods 5

2.1. Quorum Sensing Inhibitors 5

2.2. Bacteria culture 6

2.3. Purification of AI-2 7

2.4. Determination of AI-2 activity 7

2.5. Effect of QSIs on bacterial biofilm formation 8

2.6. Biofilm formation assay using a transwell system 9

2.7. Effect of QSIs on planktonic bacterial growth 10

2.8. Cytotoxicity test 10

2.9. Evaluation the effect of QSIs on host immune response 11

2.10. Statistical analysis 13

III. Results 14

3.1. Inhibitory effect on *F. nucleatum* AI-2 activity 14

3.2. Inhibitory effect of D-Galactose on biofilm formation of periodontopathogens induced by *F. nucleatum* AI-2 16

3.3. Inhibitory effect of new synthesized furanone analogs on biofilm formation of major periodontopathogens 21

3.4. Effect of QSIs on planktonic growth of bacteria 28

3.5. Effect of QSIs on host cell viability 31

3.6. Effect of QSIs on host immune response 33

IV. Discussion 36
V. Conclusion 41
VI. References 42
국문초록 49
I. Introduction

Quorum sensing (QS) is the process of cell-to-cell communication in bacteria mediated by small signaling molecules, known as autoinducers, that are secreted from bacteria [1, 2]. Autoinducers above a threshold level so called quorum can affect various phenotypes including bioluminescence, virulence factor secretion, adhesion and biofilm formation [2]. Specifically, biofilm formation is not only enhances the resistance of bacteria to antibiotics, but also leads to chronic inflammatory diseases including periodontitis. Periodontitis is caused by subgingival biofilms, which are mostly composed of various anaerobic bacteria including *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Over-aggressive immune and inflammatory responses against these microorganisms can lead to tissue damage, often accompanied by tooth loss.

Autoinducer 2 (AI-2) is a universal QS molecule secreted from both Gram-negative and Gram-positive bacteria [3-5]. It plays a critical role in biofilm formation and the virulence of pathogenic bacteria [6]. Therefore, AI-2 can be a suitable target in the control of periodontal infection, and AI-2 inhibitors are ideal for inhibiting oral biofilm formation [7, 8]. In the oral environment, *Fusobacterium nucleatum* is the major coaggregation bridge
organism that links early colonizers and late pathogenic colonizers in dental biofilm [9-13]. Furthermore, it has been demonstrated that *F. nucleatum* plays a key role in dental plaque formation by coaggregation with pathogenic bacterial species in the oral cavity, thus leading to periodontitis [14, 15]. Recently, QS inhibitors (QSIs) including D-ribose and (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone compound have been found to inhibit the biofilm formation of major periodontopathogens such as *F. nucleatum, P. gingivalis, T. forsythia, and T. denticola* by inhibiting AI-2 [16].

Since QS is responsible for virulence in clinically relevant bacteria [17], inhibition of QS appears to be a promising strategy for controlling pathogens. Notably, QSIs do not threaten bacteria with life-or-death situations, whereas traditional antibacterial agents are based on compounds that aim to kill or inhibit bacterial growth. The widespread use of antibiotics has resulted in bacterial resistance such as multiple drug resistance. Since QSIs are less likely to promote drug-resistant phenotypes in bacteria, they are regarded as a potential target for the development of antibacterial agents [11, 18-21]. Indeed, there has been an increase in research or reports on QS [22-25].

Based on previous QS studies [16, 26], we focused on the development of new QSIs by searching for ligands of the AI-2 receptors of
periodontopathogens or designing new structures of furanone analogs that can reduce toxicity and retain biofilm inhibitory activities. In the search for AI-2 receptors in periodontopathogens and AI-2 inhibitors, we focused on the 36-kDa galactose-binding protein (Gbp36) of *F. nucleatum*, which has high structural similarity to the RbsB of *A. actinomycetemcomitans* and *E. coli*. A galactose-binding adhesin (30-kDa) on the surface of *F. nucleatum* has been demonstrated to be involved in coaggregation with other periodontopathogens including *P. gingivalis* and *A. actinomycetemcomitans* [27, 28]. However, the roles of the galactose-binding protein of *F. nucleatum* as an AI-2 receptor and galactose as an AI-2 inhibitor have not been assessed. Brominated furanones, produced by the macro algae *Delisea pulchra* is known to prevent microbial colonization [29, 30]. In addition, bacterial biofilm formation can be inhibited by brominated furanones, which are covalently modified and inactivate the AI-2 producing enzyme LuxS [21, 31-33]. However, some furanone compounds have been reported to be toxic in either bacteria or eukaryotes [34-36]. Recently, a bicyclic version of brominated furanones has been demonstrated to have lower toxicity [37, 38]. Therefore, we designed new structures of brominated furanone analogs to improve their antagonistic activity in biofilm inhibition with reduced toxicity.
In this study, we investigated whether the newly designed QSIs including D-galactose and furanone analogs can act as AI-2 inhibitors by inhibiting the AI-2 activity and biofilm formation of periodontopathogens. We demonstrated that the QSIs showed high inhibitory activity against AI-2 activity of *F. nucleatum* and biofilm formation of major periodontopathogens in the presence of semi-purified AI-2 or secreted molecules of *F. nucleatum*. 
II. Materials and Methods

2.1. Quorum Sensing Inhibitors

Two different kinds of quorum sensing inhibitors were evaluated in this study. One is D-galactose, a ligand of D-galactose-binding protein (Gbp) of \textit{F. nucleatum}. As there have been no previously identified AI-2 receptors of \textit{F. nucleatum}, we searched for a protein in \textit{F. nucleatum} that had a sequence similarity with AI-2 receptors of other oral bacteria using a BLAST (\textit{Basic Local Alignment Search Tool}) search performed by Department of Bioinformatics in Soongsil University. As a result, we found that D-galactose-binding protein (Gbp36) of \textit{F. nucleatum} showed high level of amino acid sequence similarity with the RbsB of \textit{A. actinomycetemcomitans} [39]. Therefore, we evaluated D-galactose for the competitive binding of AI-2 to Gbp and thus its feasibility as a quorum sensing inhibitor for further analysis. D-Ribose and D-galactose were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and Sigama Aldrich Co. (St. Louis, MO, USA) respectively.

The other kind of QSIs are furanone analogs which were newly designed and synthesized by Department of Chemistry in Seoul National University. These new structures of brominated furanone analogs (BMK-Q106:Q106,
BMK-Q111:Q111 and BMK-Q131:Q131) were devised to improve their biological efficacy and reduce toxicity. Reference furanone compound, (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone, was purchased from Sigama Aldrich Co. (St. Louis, MO, USA)

2.2. Bacteria culture

AI-2 reporter strain, *Vibrio harveyi* BB170 (ATCC BAA-1117) was cultured aerobically at 30°C in autoinducer bioassay (AB) medium consisted of 0.3 M sodium chloride, 0.05 M magnesium sulfate, 0.2% casamino acids, 10 mM potassium phosphate (pH 7.0), 1 mM L-arginine and 2% glycerol with shaking. *F. nucleatum* (ATCC 25586) and *P. gingivalis* (ATCC 33277) were cultured anaerobically (10% H2, 10% CO2, 80% N2) in brain heart infusion broth supplemented with vitamin K (0.2 mg/ml) and hemin (10 mg/ml) at 37°C. *T. forsythia* (ATCC 43037) was cultured anaerobically in new oral spirochete (NOS) broth (ATCC medium 1494) supplemented with N-acetylmuramic acid (0.01 µg/ml) and vitamin K (0.2 mg/ml) at 37°C.
2.3. Purification of AI-2

AI-2 of *F. nucleatum* was partially purified as described previously[16]. In brief, *F. nucleatum* which was cultured overnight was diluted 1:20 with fresh culture medium. After incubating the culture mixture until the late logarithmic phase (OD$_{660}$ nm = 0.7), the culture supernatants were collected by centrifugation at 10,000 x g at 4°C. The culture supernatants were passed through 0.2 μm pore-size membrane filters (Sartorius Stedium Biotech, Goettingen, Germany) and the filtrates were subsequently passed through a Centricon YM-3 3-kDa exclusion filter (Millipore, Bedford, MA). Then the filtrates were chromatographed on a C18 Sep-Pak reverse-phase column (Waters Co., Milford, MA) according to the manufacturer’s instructions.

2.4. Determination of AI-2 activity

As QS molecules stimulates the luciferase operon to express luciferase of *V. harveyi* BB170, an AI-2 reporter strain, bioluminescence of *V. harveyi* BB170 was determined as AI-2 activity. *V. harveyi* BB170 was diluted to a concentration of 1 x 10$^6$ bacteria/ml in fresh AB medium. Then the bacterial suspension was mixed with 10% (vol/vol) of partially purified AI-2 of *F. nucleatum* in the presence or absence of QSIs at various concentrations, and
incubated for 1-6 h under aerobic condition with shaking at 30°C. The bioluminescence was measured using a luminometer (GloMax-Multi detection system, Promega, Madison, WI, USA) and the value was converted into a percentage out of untreated control value.

2.5. Effect of QSIs on bacterial biofilm formation

Biofilm formation assay was performed by crystal violet staining and confocal laser scanning microscopy. Bacterial suspensions were added to 24-well plates with round glass slips (12 mm radius) in the presence of \textit{F. nucleatum} AI-2 (10% vol/vol) and the QSIs at various concentrations. The initial number of \textit{F. nucleatum}, \textit{P. gingivalis}, and \textit{T. forsythia} was 2 x 10^7 bacteria/ml, 2 x 10^8 bacteria/ml, and 2 x 10^8 bacteria/ml, respectively and incubated for 48 h under anaerobic condition (10% H\(_2\), 10% CO\(_2\), 80% N\(_2\)). After 48 h, biofilms formed on the glass slips were washed with phosphate buffered saline three times and stained with 1% crystal violet for 10 min and destained with 1 ml of acetone–alcohol (20:80,vol/vol). The absorbance at 595 nm of the destaining solution containing crystal violet was measured using a microplate reader (Wallac Victor3 microtiter, PerkinElmer Life Sciences, Waltham, MA). Biofilm formed on the glass slips was stained by
the Live/Dead-BacLight bacterial viability kit (Invitrogen, Grand Island, NY) and then observed using a confocal scanning laser microscope (Carl Zeiss LSM 700, Germany) at 1000 x magnification and quantified by measuring fluorescence intensity and average thickness of the biofilm using Carl Zeiss LSM 700 program. Biomass was determined by dividing total intensity by the area where the fluorescence was obtained.

2.6. Biofilm formation assay using a transwell system

A two-compartment separated co-culture system was used to evaluate the effect of secreted molecules of *F. nucleatum* on biofilm growth of *P. gingivalis* and *T. forsythia*. *P. gingivalis* (1 x 10^7 bacteria/ml) or *T. forsythia* (1 x 10^7 bacteria/ml) was suspended in co-culture medium and placed in the bottom wells of Transwell plates (BD FalconTM, Franklin Lakes, NJ) and *F. nucleatum* (1 x 10^7 bacteria/ml) cells were placed in the upper wells which were separated by a membrane with a 0.4 µm pore size. Co-culture medium was composed of one part of *F. nucleatum* culture medium and one part of culture medium of other partner bacterium. Bacterial growth for each species in co-culture medium was confirmed to be indistinguishable from the growth in their own culture medium. After anaerobic incubation at 37°C for 72 h,
biofilms formed on the glass slips in the bottom wells were stained with crystal violet and measured as described above.

2.7 Effect of QSIs on planktonic bacterial growth

_F. nucleatum, P. gingivalis, and T. forsythia_ were grown anaerobically at 37°C in the presence or absence of QSIs without addition of _F. nucleatum_ AI-2 for 48 h. Bacterial growth was monitored every 24 h by measuring absorbance at 600 nm using a spectrophotometer.

2.8. Cytotoxicity test

Human monocytic cell line (THP-1), human gingival fibroblasts (HGFs) and Human oral keratinocyte cell line (HOK-16B) were used to evaluate the cytotoxicity of the QSIs on host cells. THP-1 cells were cultured in RPMI 1640 media (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Waltham, MA, USA), 2.05 mM L-glutamate and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). HGFs were cultured in DMEM media (Hyclone, Waltham, MA, USA) supplemented with 4 mM L-glutamine, 4500 mg/L Glucose, sodium pyruvate, antibiotics

(100 units/ml penicillin, 100 μg/ml streptomycin) and 10% fetal bovine serum. HOK-16B cells were cultured in Keratinocyte Basal Medium (Lonza, Walkersville, MD, USA) supplemented with insulin, epidermal growth factor, bovine pituitary extract, hydrocortisone and gentamicin sulfate amphotericin. Cultured THP-1 cells (1 x 10^5 cells/well) were seeded in 96 well microtiter plate and incubated in the presence of the compounds for 24 h. HGFs (2 x 10^4 cells/well) and HOK-16B (5 x 10^4 cells/well) were seeded in 96 well plates and grown until the confluence of 85%. The cells were then treated with the compounds and incubated for 24 h. The cell viability was evaluated using the Cell Counting Kit-8 (CCK-8, DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol.

2.9. Evaluation the effect of QSIs on host immune response

Effects of the QSIs on host immune response was evaluated using real-time RT PCR. THP-1 cells (1 x 10^6 cells/well) and HGFs (2 x 10^5 cells/well) and HOK-16B (5 x 10^5 cells/well) in 6 well plates were incubated at 37°C in the presence or absence of QSIs or lipopolysaccharides (LPS, 1 μg/ml) known as endotoxin of gram-negative bacteria that elicits strong
immune responses in host cells. After 24 h incubation, RNA from THP-1 cells and HGFs cells was extracted using Easy-Blue total RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's protocol. In case of HOK-16B cells, RNA was extracted after 12 h incubation. cDNA samples (2 µl) synthesized from the extracted RNA (1 µg) using a M-MLV Reverse Transcription kit (Promega, Madison, WI) were mixed with each primer pairs (10 pM) and Power SYBR Green Master Mix (Applied Biosystems, Warrington, UK) in a 20 µl reaction volume. The mixtures were subjected to ABI PRISM 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the following thermocycling: amplification for 40 cycles composed of a denaturation step at 95°C for 15 sec, an annealing and extension step at 60°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, the housekeeping gene, was used as a reference gene for normalization of gene expression level of inflammatory cytokines including interleukin 6 (IL-6) and interleukin 8 (IL-8). The sequences of the primers used in this study were as follows: 5´-GTG GCC AGC CGA GCC-3´ and 5´-TGA AGG GGT TGA TGG CA-3´ for GAPDH; 5´-GAT TCA ATG AGG AGA CTT GCC TGG-3´, 5´-GCA GAA CTG GAT CAG GAC TTT-3´ for IL-6 ; 5´-CTG TGT GAA GGT GCA GTT TTG C-3´ and 5´- AAC TTC TCC ACA ACC
CTC TGC-3’ for IL-8.

2.10. **Statistical analysis**

Statistical analysis was performed using Student’s t test. Statistically significant differences between the control and AI-2 or between AI-2 and QSI-treated groups were analyzed. A $p$ value of $<0.05$ was considered statistically significant.
III. Results

3.1. Inhibitory effect of QSIs on *F. nucleatum* AI-2 activity

The effect of designed QSIs on *F. nucleatum* AI-2 activity was evaluated and its inhibitory effect was compared with those of D-ribose and (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone compound at the same concentrations. As shown in Fig. 1, D-galactose and newly synthesized furanone analogs (Q106, Q111 and Q131) significantly inhibited bioluminescence of *V. harveyi* BB170, that these molecules have inhibitory effects on AI-2 activity of *F. nucleatum* in a dose dependent manner. The inhibitory activity of D-galactose against *F. nucleatum* AI-2 was higher at the low concentration (2 mM and 20 mM) than that of D-ribose, while it was lower at 200 mM than that of D-ribose (Fig. 1 A). Furanone analogs (Q106, Q111 and Q131) significantly inhibited the bioluminescence of *V. harveyi* BB170 induced by *F. nucleatum* AI-2 at a concentration of 2 μM, but their inhibitory activity was less than the inhibitory efficacy of the reference furanone (Fig. 1B). Through additional bacterial growth test on *V. harveyi* BB170, we confirmed that the reference furanone significantly inhibited the growth of *V. harveyi* BB170, resulting in the reduction of bioluminescence. However, the newly synthesized furanone analogs did not
affect the planktonic growth of *V. harveyi* BB170.

**Fig. 1. Inhibitory effect of QSIs on AI-2 activity.** AI-2 activity was assessed by measuring bioluminescence using AI-2 reporter strain *V. harveyi* BB170. *V. harveyi* BB170 (1 x 10^6 bacteria/ml) was incubated for 6 h with 10% of semi-purified *F. nucleatum* AI-2 in the absence or presence of D-galactose (A) and furanone analogs (B). The bioluminescence of *V. harveyi* BB170 was measured using a luminometer and the value was converted into a percentage out of the control value. The experiments were performed three times in triplicate, and representative data are shown. *p < 0.05* compared to the control value (none), only *F. nucleatum* AI-2 treated value in the absence of the QSIs.
3.2. Inhibitory effect of D-galactose on biofilm formation of periodontopathogens

As semi-purified *F. nucleatum* AI-2 induced biofilm formation of periodontopathogens (Jang et al., 2013), the inhibitory effect of D-galactose and D-ribose on biofilm formation of major periodontopathogens was evaluated in the presence of *F. nucleatum* AI-2. The biofilm formed was assessed by confocal laser scanning microscopy and crystal violet staining. Fig. 2A-C shows that biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* induced by *F. nucleatum* AI-2 was remarkably reduced by D-galactose and D-ribose. When the biofilm was visualized by confocal image analysis, D-galactose significantly reduced the biofilm formation to a similar degree as that observed with D-ribose at 200 mM. In addition, D-galactose inhibited the biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* in the presence of *F. nucleatum* AI-2 in a dose-dependent manner when measured by crystal violet staining. Its inhibitory effect was comparable to or better than that of D-ribose at the same concentrations. Although D-ribose did not inhibit AI-2 activity at 2 mM during 6 h of treatment (Fig. 1A), a biofilm formed over 48 h was inhibited by D-ribose at this concentration (Fig. 2).
Next, the inhibitory effect of D-galactose on biofilm formation of periodontopathogens was tested using transwell culture system and compared with that of D-ribose, where secreted molecules including AI-2 of *F. nucleatum* in the upper well could affect biofilm formation of *P. gingivalis* or *T. forsythia* in the bottom well without physical contact of two bacterial pairs. As shown in Fig. 3, secreted molecules from *F. nucleatum* in the upper well significantly induced the biofilm formation of *P. gingivalis* and *T. forsythia* in the bottom wells and D-galactose significantly inhibited their biofilm formation at all of the concentrations used when measured by crystal violet staining (Fig. 3).
Fig. 2. Inhibitory effect of QSIs on biofilm formation of periodontopathogens. *F. nucleatum (Fn, \(2 \times 10^7\) bacteria/ml), *P. gingivalis
(Pg, $2 \times 10^8$ bacteria/ml) and *T. forsythia (Tf, $2 \times 10^8$ bacteria/ml) were cultured with 10% partially purified *F. nucleatum* AI-2 in the presence of D-galactose (D-Gal) or D-ribose (D-Rib) for 48 h under anaerobic conditions at 37°C. Biofilms formed were stained by a Live/Dead-BacLight bacterial viability kit and observed using a confocal laser scanning microscope at 1,000 × magnification. Biofilm formation of the bacteria treated with various concentration (2 mM, 20 mM and 200 mM) of D-galactose (D-Gal) or D-ribose (D-Rib) was assessed by crystal violet staining. The experiments were performed three times in triplicate, and representative data are shown.*$P < 0.05$ compared to the untreated control value; #$P < 0.05$ compared to the biofilm formation of the *F. nucleatum* AI-2- treated value in the absence of the QSIs.
Fig. 3. Inhibitory effect of D-galactose on biofilm formation of *P. gingivalis* and *T. forsythia* induced by secreted molecules of *F. nucleatum*. *F. nucleatum* (1 × 10^7^ bacteria/ml) was cultured in the upper well, while *P. gingivalis* (A, 1 × 10^7^ bacteria/ml) or *T. forsythia* (B, 1 × 10^7^ bacteria/ml) was cultured in the bottom wells in the presence of D-ribose (D-Rib) or D-galactose (D-Gal) for 72 h under anaerobic conditions at 37°C. Biofilm formation was assessed by crystal violet staining. The experiments were performed three times in triplicate, and representative data are shown. *P < 0.05 compared to the untreated control value; #P < 0.05 compared to the biofilm formation in the absence of D-ribose or D-galactose.
3.3. Inhibitory effect of new synthesized furanone analogs on biofilm formation of periodontopathogens

The effect of the reference furanone and newly synthesized furanone analogs were tested whether they have inhibitory activity against biofilm formation of periodontopathogens. The biofilm formation was assessed by crystal violet staining and confocal image analysis. As shown in Fig. 4, the reference furanone and new furanone analogs significantly reduced biofilm formation of *F. nucleatum*, *P. gingivalis*, and *T. forsythia* in the presence of *F. nucleatum* AI-2. New furanone analogs (Q106, Q111 and Q131) showed similar or better inhibitory effect when compared to the reference furanone. All of the analogs significantly inhibited the biofilm formation of *F. nucleatum* and their inhibitory activity was higher at all concentrations ranged between 0.002 - 2μM than that of the reference furanone. In case of *P. gingivalis*, Q131 showed higher inhibitory effect on the biofilm formation than the reference compound at all concentrations tested, whereas Q106 and Q111 showed higher inhibitory activity at the concentration of 0.02 μM and 0.2 μM. Compared to the reference furanone, higher inhibitory activity on biofilm formation of *T. forsythia* was shown by Q131 at 0.002 -0.2 μM and by Q106 at 0.2 μM, and by Q111 at 0.2 μM and 2 μM. In case of Q106 and Q131, the inhibitory activity did not follow dose-dependent tendency, which...
varied according to the bacterial species. The reduced biofilms by the QSIs were visualized by confocal image analysis (Fig. 5) and biofilm of each group was quantified by measuring fluorescence intensity and average thickness of the biofilm (Table. 1).
Fig. 4. Inhibitory effect of new furanone analogs on biofilm formation of periodontopathogens. F. nucleatum (2 x 10^7 bacteria/ml), P. gingivalis (2 x 10^8 bacteria/ml) and T. forsythia (2 x 10^8 bacteria/ml) were cultured with
10% of semi-purified *F. nucleatum* AI-2 in the presence of the reference furanone (Furanone) or new furanone analogs (Q106, Q111 and Q131) at various concentrations for 48 h under anaerobic condition at 37 °C. Biofilm formed was assessed by crystal violet staining. The experiments were performed three times in triplicate, and representative data are shown. *p < 0.05 compared to the untreated control value, and #p < 0.05 compared to biofilm formation of the *F. nucleatum* AI-2 treated value in the absence of the QSIs.
Fig. 5. Biofilm images showing the inhibitory effect of new furanone analogs on biofilm formation of *F. nucleatum, P. gingivalis* and *T. forsythia*. *F. nucleatum* (A, $2 \times 10^7$ bacteria/ml), *P. gingivalis* (B, $2 \times 10^8$ bacteria/ml), and *T. forsythia* (C, $2 \times 10^6$ bacteria/ml).
bacteria/ml) and *T. forsythia* (C, 2 x 10^8 bacteria/ml) were cultured with semi-purified *F. nucleatum* AI-2 in the reference furanone (Fur) or new furanone analogs (Q106, Q111 and Q131) at 2 µM under anaerobic condition at 37°C. After 48 h, each biofilm formed was stained by live/dead-BacLight bacterial viability kit and observed by a confocal laser scanning microscope at 1000 x magnification. The control group is the biofilm formation of each bacteria which was cultured without *F. nucleatum* AI-2 and the QSIs.
Tabel 1. Biomass and depth of biofilm of periodontopathogens.

<table>
<thead>
<tr>
<th>bacteria and treatment</th>
<th>F. nucleatum</th>
<th>P. gingivalis</th>
<th>T. forsythia</th>
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<tbody>
<tr>
<td></td>
<td>Biomass (µm³/µm²)</td>
<td>Average Depth (µm)</td>
<td>Biomass (µm³/µm²)</td>
</tr>
<tr>
<td>Control</td>
<td>0.43</td>
<td>2.11</td>
<td>0.37</td>
</tr>
<tr>
<td>Fn AI-2</td>
<td>2.99*</td>
<td>5.37*</td>
<td>3.73*</td>
</tr>
<tr>
<td>Fn AI-2/ Furanone</td>
<td>0.42*</td>
<td>2.02*</td>
<td>0.30*</td>
</tr>
<tr>
<td>Fn AI-2/ Q106</td>
<td>0.39*</td>
<td>1.82*</td>
<td>0.31*</td>
</tr>
<tr>
<td>Fn AI-2/ Q111</td>
<td>0.38*</td>
<td>1.87*</td>
<td>0.29*</td>
</tr>
<tr>
<td>Fn AI-2/ Q131</td>
<td>0.32*</td>
<td>1.72*</td>
<td>0.27*</td>
</tr>
</tbody>
</table>

Biomass and average depth of biofilm were analyzed using Carl Zeiss LSM 700 program. \(*p < 0.05\) compared to the untreated control value, and \(\#p < 0.05\) compared to biofilm formation of the *F. nucleatum* AI-2 treated value in the absence of the QSIs.
3.4. Effect of QSIs on planktonic growth of periodontopathogens

As *F. nucleatum* plays the central role in biofilm formation of periodontopathogens, we tested whether the inhibitory effect of D-galactose on biofilm formation is attributed to *F. nucleatum* growth inhibition. D-Galactose did not affect the planktonic growth of the bacterium until 48 h incubation (Fig. 6A). In case of D-Ribose, it did not affect the planktonic bacterial growth at the concentrations of 2 and 20 mM, but slightly reduced growth was observed at 24 h incubation with 200 mM (Fig. 6B).

The effects of the reference furanone and new furanone analogs on bacterial growth were also assessed in major periodontopathogens (*F. nucleatum, P. gingivalis* and *T. forsythia*) at the highest concentration (2 µM). As a result, all compounds did not have inhibitory effect directly on bacterial growth (Fig. 7).
Fig. 6. Effect of D-galactose and D-ribose on planktonic *F. nucleatum* growth. *F. nucleatum* was grown in brain-heart infusion medium for 48 h under anaerobic conditions at 37°C in the presence of D-galactose (D-Gal) or D-ribose (D-Rib). Bacterial growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer. *P < 0.05* compared to the untreated control value. The experiments were performed three times in triplicate, and representative data are shown.
Fig. 7. Effect of furanone compound and new furanone analogs on planktonic bacterial growth. *F. nucleatum, P. gingivalis* and *T. forsythia* were grown for 48 h under anaerobic condition at 37°C in the presence of the reference furanone (Fur) or new furanone analogs (Q106, Q111 and Q131) at 2 µM concentration. Each bacterial growth was monitored by measuring absorbance at 600nm using a spectrophotometer every 24 h. The experiments were performed three times in triplicate, and representative data are shown.
3.5. Effect of QSIs on host cell viability

To check whether the QSIs are toxic to the eukaryotic host cell, cytotoxicity test was performed in three types of human cells including human monocytic cell line (THP-1), human gingival fibroblasts (HGFs) and human oral keratinocytes (HOK-16B). As shown in Fig. 8, new furanone analogs as well as the reference furanone did not reduce host cell viability (Fig. 8A-C). In case of D-galactose, it did not showed cytotoxicity at lower concentration (2 mM and 20 mM) but reduced cell viability at concentration of 200 mM. (Fig. 8D-F)
**Fig. 8. Effect of QSIs on host cell viability.** THP-1 cells, HGFs and HOK-16B cells were incubated with the reference furanone (Fur), new furanone analogs (Q106, Q111 and Q131), D-ribose (D-Rib) and D-galactose (D-Gal) for 24 h to evaluate cytotoxicity using Cell Counting Kit-8 assay. Control means none treated group. The experiments were performed three times in triplicate, and representative data are shown. *p < 0.05 compared to none treated control value.
3.6. Effect of furanone analogs on host immune response

Using real-time RT-PCR, mRNA level of IL-6 and IL-8 in human cells (THP-1 and HGFs) treated with the reference furanone analogs or new furanone analogs was analyzed to confirm whether they induce inflammatory response in host cells. New furanone analogs (Q106, Q111 and Q131) did not induce inflammatory response while the reference furanone or LPS (1 µg/ml) as a positive control significantly induced gene expression level of IL-6 and IL-8 in THP-1 cells (Fig. 9A and 9B). In HGFs, they did not induce gene expression level of IL-6 and IL-8 (Fig. 9C and 9D), while the reference furanone induced gene expression level of IL-8 and LPS (1 µg/ml) induced gene expression level of both cytokines. In HOK-16B cells, mRNA levels of IL-6 and IL-8 were not induced by Q111 and Q131. However, the expression level of IL-6 was induced by LPS, the reference furanone or Q106 (Fig. 9E) and IL-8 was induced by LPS and Q106 (Fig. 9F).
Fig. 9. Effect of furanone analogs on host cell immune response. THP-1 cells (1 x 10^6 cells/well, A and B), HGFs (2 x 10^5 cells /well, C and D) and
HOK-16B (5 x 10^5 cells/well, E and F) in 6 well plates were treated with the reference furanone (Fur) and new furanone analogs (Q106, Q111 and Q131). The expression of IL-6 and IL-8 mRNA was analyzed by real-time RT-PCR. *p < 0.05 compared to the untreated control value. The experiments were performed three times in triplicate, and representative data are shown.
IV. Discussion

This study demonstrated that the newly designed QSIIs including D-galactose and furanone analogs can be used as AI-2 inhibitors to prevent the biofilm growth of periodontopathogens. Since QS enhances biofilm formation and virulence expression, it is considered as a potential target against bacterial infections [21, 40, 41].

Periodontopathogens including *F. nucleatum, P. gingivalis, Prevotella intermedia*, and *A. actinomycetemcomitans* are known to produce AI-2 [20, 42]. In addition, the AI-2 of *F. nucleatum* has been demonstrated to induce biofilm formation and surface adhesins of ‘the red complex’ *P. gingivalis, T. denticola* and *T. forsythia* [14, 16, 43, 44]. Since AI-2 plays an important role in biofilm formation of periodontopathogens [16, 42, 45], antagonists of AI-2 can be good candidates for inhibiting biofilm formation.

AI-2 receptors or transporters have been identified in several species of bacteria: LsrB and LsrR in *E. coli* and *S. typhimurium*; AgrC in *S. aureus*; TlpB in *H. pylori*; and LuxP/Q in *Vibrio* species [46]. However, the AI-2 receptors of periodontopathogens have been reported only in *A. actinomycetemcomitans* [45, 47]. *A. actinomycetemcomitans* expresses two periplasmic proteins, RbsB and LsrB, which can interact with AI-2. *F.
*F. nucleatum* connects early colonizers and late pathogenic colonizers including *P. gingivalis* and *T. forsythia*, so called ‘red-complex’, which are highly proteolytic and present in the subgingival biofilm adjacent to the pocket epithelium [9, 44]; thus, identifying the AI-2 receptor of *F. nucleatum* and blocking the receptor would be an approach to prevent the development of biofilms composed of periodontal pathogens. The high structural similarity of the Gbp36 of *F. nucleatum* to the AI-2 receptor RbsB of *A. actinomycetemcomitans* and *E. coli* prompted us to analyze the role of D-galactose as an AI-2 inhibitor. As D-galactose inhibited the AI-2 acitivity of *F. nucleatum*, Gbp may be an AI-2 receptor.

A galactose-binding adhesin (30-kDa), located on the outer membrane of *F. nucleatum* PK1594 has been reported to coaggregate with periodontopathogens such as *A. actinomycetemcomitans* and *P. gingivalis* and mediate hemagglutination [27, 28, 48]. Furthermore, it has been shown to mediate the interaction of *F. nucleatum* with epithelial cells, fibroblasts, lymphocytes, and erythrocytes, and these interactions can be inhibited by D-galactose. However, it is unlikely that the galactose-binding adhesin described refers to the Gbp36 identified in our study. In addition to different molecular weights, the subcellular locations of these two molecules are different; galactose-binding adhesin is located on the bacterial surface[28],
whereas Gbp36 is thought to localize in the periplasm. According to sequence homology, Gbp36 is a galactose/glucose ABC transporter. *F. nucleatum* can utilize sugars and amino acids as carbon and energy sources, whereby amino acids are the preferred energy source [49]. *F. nucleatum* takes up galactose by the galactose/glucose ABC transporter, which is a member of pentose/hexose sugar-binding protein family of the type I periplasmic binding protein superfamily in various bacterial species. The ABC transporter is involved in chemotaxis towards galactose and glucose, and active transport of the sugars. *F. nucleatum* possesses genes encoding the enzymes involved in galactose utilization [49]. Galactose is converted to glucose-6-phosphate which can enter the glycolysis pathway. In our study, D-galactose did not affect the bacterial growth at the concentrations used in the experiments, thus suggesting its role as an AI-2 inhibitor instead of an energy source. *F. nucleatum* requires sugars including glucose, galactose, and fructose for the synthesis of intracellular polymers, which can support survival under conditions of amino acid deprivation [50].

In this study, D-galactose and the new furanone analogs did not affect bacterial growth at the concentrations used in the experiments. The inhibition of biofilm formation by these molecules could be attributed to coaggregation inhibition by competitive binding to adhesin instead of AI-2 activity.
inhibition. However, they also inhibited the AI-2 activity of *F. nucleatum* as revealed by bioluminescence assay, indicating their function as AI-2 inhibitors. These results indicate that the inhibitors can reduce the biofilm formation of periodontopathogens by competitive binding to the AI-2 receptor with AI-2. The inhibitory activities of new furanone analogs (Q106 and Q131) did not follow a dose-dependent tendency, which varied according to the bacterial species. Although AI-2 is a universal signaling molecule, the structure of the AI-2 receptor may be slightly different among bacterial species; this may be because these analogs can act as partial agonists or antagonists depending on their concentration level in accordance with the different structures of AI-2 receptors. In addition to the direct effect of the inhibitors, it is also possible that they may inhibit other adhesins of periodontopathogens induced by AI-2 [16]. However, this hypothesis remains to be investigated. Although we demonstrated that the newly designed QSIs can act as effective AI-2 inhibitors and function as preventive agents against the biofilm formation of pathogenic bacteria by targeting QS signaling, other aspects should be further investigated. Because of the diversity of oral bacteria, it is difficult to selectively eliminate only periodontopathogens without affecting other commensal bacteria. The effect of the QSIs on biofilm formation and growth of commensal bacteria should
be evaluated for maintaining a balanced healthy oral environment [51, 52].
Furthermore, the stability and safety of these inhibitors should be evaluated for commercialization as preventive agents.
V. Conclusion

Our results demonstrate that newly designed furanone analogs and D-galactose significantly inhibited *F. nucleatum* AI-2 activity and biofilm formation of *F. nucleatum*, *P. gingivalis* and *T. forsythia* without bactericidal effect or cytotoxicity on host cell. And they did not induce expression of host inflammatory cytokines such as IL-6 and IL-8 in human cells. Therefore, these compounds may be used as a preventive agent against pathogenic biofilm formation by targeting QS signaling. Especially, as the widespread use of antibiotics has resulted in many bacteria acquiring resistance, QS inhibition appears to be a promising strategy to prevent bacterial infectious diseases caused by biofilms.
VI. References


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국문초록

치주병인균의 생물막 형성에 대한

**Autoinducer 2 쿼럼센싱 억제제의 효과**

류 은 주

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(지도교수 최 봉 규)

1. 목적

세균들은 쿼럼센싱이라고 하는 세균종 간의 신호전달체계를 통해 병독력 획득, 생물막 형성과 같은 다양한 표현형들을 효율적으로 조절한다. Autoinducer는 이러한 쿼럼센싱을 매개하는 신호전달물질이며, 그 중에서도 Autoinducer 2 (AI-2)는 동종 및 이종간의 쿼럼센싱에 사용되는 보편적인 쿼럼센싱 신호분자이다. 특히 구강 세균막 형성에서 다양한 세균종의 가교역할을 하는
Fusobacterium nucleatum의 AI-2는 후기집락군인 치주병인균(F. nucleatum, Porphyromonas gingivalis, Tannerella forsythia)의 세균막 형성 및 발달에 중요한 역할을 하여 결국 치주질환이 유발된다. 본 논문은 이러한 AI-2 활성을 억제하기 위해 새롭게 고안된 쿼럼센싱 억제제의 유효성과 안전성을 평가하고, 그로 인해 치주병인균의 생물막 형성을 억제할 수 있는지에 대해 연구하였다.

2. 방 법

AI-2에 대한 억제제로는 기존의 쿼럼센싱 억제제로 알려진 퓨라논과 새롭게 합성한 퓨라논 계열의 구조적 유사체를 사용하였다. 또한 다른 치주질환균(Aggregatibacter actinomycetemcomitans)의 AI-2 수용체인 리보오스 결합단백질(Rbs, Ribose binding protein)과 높은 아미노산 서열 유사성을 갖는 F. nucleatum의 갈락토스 결합단백질(Gbp, Galactose binding protein)의 리간드인 D-galactose를 사용하였다. 각각의 쿼럼센싱 억제제가 AI-2활성을 억제하는지 보기 위해, F. nucleatum AI-2 및 쿼럼센싱 억제제가 있는 조건에서 AI-2 reporter strain인 Vibrio harveyi BB170의 생물발광도를 측정하였다. 측정된 생물발광도는 쿼럼센싱 억제제를 처리 하지 않은 대조군에 대비하여 백분율로 환산한 뒤, AI-2
활성도(%)로 나타내어 비교 분석하였다. AI-2 퀴럼센싱 억제제가 AI-2 에 의해 매개되는 주요 치주병인균( F. nucleatum, P. gingivalis, T. forsythia )의 생물막 형성에 미치는 영향을 평가하기 위해 각각의 세균을 F. nucleatum AI-2 및 퀴럼센싱 억제제와 함께 배양하였다. 형성된 세균막은 크리스탈 바이올렛 염색법과 공초점 현미경 관찰을 통해 정량분석과 이미지 분석을 하였다. 또 세균의 성장에 대한 퀴럼센싱 억제제의 영향을 보기위해 배양액의 흡광도를 측정하여 관찰하였다. 그리고 사용한 퀴럼센싱 억제제가 숙주세포에 미치는 영향을 평가하기 위해 사람 단핵구 세포주인 THP-1 세포에 퀴럼센싱 억제제를 처리한 후 세포독성과 염증성 사이토카인 발현 여부를 확인하였다.

3. 결 과
본 연구에서 사용한 퓌라논 계열 유사체와 D-galactose 는 AI-2 활성과, 첨가된 F. nucleatum AI-2 에 의해 증가된 F. nucleatum, P. gingivalis 그리고 T. forsythia 의 세균막 형성을 유의하게 감소시켰다. 그러나 세균의 성장에 대해서는 직접적인 항균효과를 보이지 않았다. 또한 사용한 퀴럼센싱 억제제는 숙주세포에
세포독성을 보이지 않았으며 염증성 사이토카인 발현을 유도하지 않았다.

4. 결 론

쿼럼센싱 억제제가 구강 내 세균과 숙주세포에 미치는 영향을 확인해 본 결과, 본 연구에 사용된 쿼럼센싱 억제제는 AI-2로 매개되는 여러 가지 병인균의 생물막 형성과 발달을 억제함으로써 이로 인해 기인하는 치주질환을 예방할 수 있을 것이다. 특히 이는 직접적으로 세균을 죽이는 기존의 항생제와 달리, 세균이 내성기작을 획득할 가능성이 적으며, 숙주세포에 대한 쿼럼센싱 억제제의 안전성이 더욱 검증된다면 구강질환 뿐 아니라 다른 세균성 감염질환에 대한 차세대 예방제로도 기여하게 될 것이다.

주요어 : 치주병인균, 생물막, 쿼럼센싱, AI-2 억제제, 치주질환
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