國立臺灣大學醫學院微生物學研究所

碩士論文

Graduate Institute of Microbiology
College of Medicine
National Taiwan University

Master Thesis

小分子合成物對於會引起心內膜炎的鏈球菌的生物膜形成之抑制作用

Inhibition of endocarditis-inducing streptococcal biofilm formation by synthetic small molecules

陳宜婷

Yi-Ting Chen

指導教授：賈景山 博士

Advisor: Jean-San Chia, Ph.D.

中華民國 101 年 7 月

July, 2012

國立臺灣大學碩士學位論文
口試委員會審定書

小分子合成物對於會引起心內膜炎的鏈球菌的生物膜形成之抑制作用

Inhibition of endocarditis-inducing streptococcal biofilm formation by synthetic small molecules

本論文係 陳宜婷 君（學號 R99445132 ）在國立臺灣大學醫學院微生物學研究所所完成之碩士學位論文，於民國 101 年 7 月 26 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

（簽名）

（指導教授）

系主任、所長 （簽名）

i

誌謝

兩年的碩士生活，真得是一轉眼就過了呢！這段日子，雖然走得跌跌撞撞，但很幸運地，身旁有許多人陪伴著我、鼓勵著我，並隨時給予我必要的協助，讓我得以完成這本碩士論文。首先，真的很感謝這兩年來所有指導我論文的老師們。謝謝賈老師在平日的教導，無論是 seminar 的準備或實驗設計方面，總是給我很多想法，讓我的眼界因此拓展了許多。另外，非常謝謝忻老師提供我小分子合成物作為篩選材料，並一再地幫我補充所需要的藥量，讓我得以完成論文後半部的動物實驗。也很感謝符老師、鄧老師和楊老師在 committee 時提供我許多寶貴意見，讓我在研究上有改正和進步的機會。

實驗室的學姐們及夥伴們更是我完成論文的推力之一。感謝惠婷學姐、秋月學姐和筱菁學姐在這段期間的大力幫助，不論在儀器的操作上、實驗的設計或是報告前的 rehearsal，總是提供我許多協助。另外感謝鴻偉學長、李泱和學儒，因為你們，我才得以學會動物實驗及許多跟細菌相關的基本操作。還有，一起打拼的三位夥伴：杜杜、佳儒、昱璇，這兩年來咱們同甘共苦的時光將是我難忘的回憶之一。謝謝小幫手佩青，最後這幾個月的動物實驗真是辛苦妳了！也謝謝 R1509 的其他夥伴：鏡文、派派、英哲、阿翔、Lori、高醫師和高高，因為你們，整個實驗室總是洋溢著歡樂的氣氛。除此之外，感謝大學的好友們(冰箱、雨蓉、志鴻)、杏林管弦的好夥伴們(恭仰、淑涵等)及微生物所上的好友群(婉倫、漢堡、喬巴等)的大力相挺，你們的鼓勵，讓我更有力量面對每一天的挑戰。

最後，非常謝謝家人們的全力支持。感謝父母體諒我沒辦法長時間的陪伴，也謝謝妹妹容忍我有時沒來由的任性。因為你們的支持和鼓勵，讓我這兩年可以無後顧之憂的完成碩士學位。

中文摘要

感染性心內膜炎（infective endocarditis）是一種高致死率、高復發率的心血管感染疾病，主要由一些口腔中的鏈球菌例如:轉糖鏈球菌(*Streptococcus mutans*)所引發。這些會引起心內膜炎的致病株可在受傷的瓣膜上形成生物膜(biofilm)，並與血小板、纖維蛋白(fibrin)及發炎細胞堆疊成贅疣（vegetation）。目前治療的潛在問題是這些在贅疣中形成生物膜的致病株具有高抗藥性。先前實驗室曾利用體外篩選模式(*in vitro*)模擬體內產生贅疣的狀況，證實血小板對於轉糖鏈球菌生成生物膜是重要的、以及所生成的生物膜對於抗生素具有更高的抗性，因此有必要進一步尋找一些小分子合成物來輔助抗生素抑制生物膜形成。本篇研究藉由體外生成生物膜的方法，並搭配結晶紫(crystal violet)的染色，篩選出 CYY-X022, 47, 48, 58, 60 五種小分子合成物能夠抑制細菌形成生物膜，而 CYY-X011, 12, 22, 47, 52, 55, 56 則可以干擾由細菌和血小板所形成的生物膜的生成。進一步測定細菌生長曲線，發現 CYY-X022, 47, 48, 58, 60 能有效地抑制細菌生長。利用血小板凝集計(aggregometer) 分析血小板活性以及測定細菌和血小板之間交互作用的狀況，發現 CYY-X011, 12, 22, 47, 52, 55, 56 能夠藉由干擾細菌和血小板之間的接觸來抑制血小板凝集，進而抑制由細菌和血小板所形成的生物膜。在活體 (*in vivo*)實驗中，利用實驗室先前所建立的感染性心內膜炎大鼠模式(experimental streptococcal endocarditis rat model)，將 CYY-X022, 47, 52, 55, 56 分別注入大鼠體內，發現能夠降低贅疣中生物膜生成的狀況。除此之外，當抗生素搭配小分子一同使用，利用共軛焦顯微鏡的觀察以及菌落的計數，發現抑制效果比給予單一抗生素處理還要好。本篇研究結果篩選出具有抑制生物膜生成的小分子合成物，希望對於未來在感染性心內膜炎的治療上能夠提供重要的資訊，並且能夠在其他臨床的研究上也有更多的應用。

Abstract

Infective endocarditis (IE) is a cardiovascular disease with high mortality rate and usually caused by oral streptococci (such as *Streptococcus mutans*) infection. The characteristic of IE is the formation of vegetations, fibrin-platelet clots with the embedded bacteria forming biofilm, which is refractory to routine antibiotic treatment. Previously, our data reported that platelets play important roles in vegetation formation and could enhance the resistance of streptococcal biofilm to antibiotics. Therefore, to search novel prophylactic agents that specifically target the platelet-associated biofilm will provide effective strategy for the successful control of IE. In this study, 76 different synthetic molecules have been screened *in vitro* by using biofilm formation assay and crystal violet staining. We found that CYY-X022, 47, 48, 58 and 60 could eliminate homotypic bacterial biofilm formation, and CYY-X011, 12, 22, 47, 52, 55 and 56 could interfere with platelet-associated biofilm formation. Among these effective synthetic molecules, CYY-X022, 47, 48, 58 and 60 could inhibit the bacterial growth , and CYY-X011, 12, 22, 47, 52, 55 and 56, alternatively, could interfere with the binding of *S. mutans* and platelets, as well as inhibiting the streptococci-induced platelet aggregation according to the results of platelet aggregation test. Consistent with the *in vitro* data, the vegetation size was decreased by intravenous administration of CYY-X022, 47, 52, 55 and 56 in the experimental streptococcal endocarditis rat model. Moreover, antibiotics combined with the targeted small molecules *in vitro* could effectively inhibit the biofilm formation based on the results of confocal laser scanning microscope observation and the measurement of survival bacteria. Taken together, the data suggest that these synthetic small molecules could eliminate streptococcal biofilm formation by inhibiting the bacterial

growth or the binding of *S. mutans* and platelets. For clinical treatment on infective endocarditis, these targeted small molecules may have promising applications and offer another strategy.

Table of Contents

Chapter 1: Introduction

1.1 *Streptococcus mutans*

S. mutans was first identified by J Kilian Clarke in 1924[1]and also completely sequenced in 2002. It is a Gram-positive coccus-shaped organism, belonged into viridans streptococci, and usually lives in facultatively anaerobic environment such as the niche of teeth in oral cavity. They are also an important contributor to tooth decay. By metabolizing sucrose to lactate, they create an acidic environment in mouth. As time progresses, the acid makes the portions of the mineral content at the surface of teeth dissolved and then causes dental caries[2].

These oral streptococci must adapt to the adverse circumstances in oral cavity. By equipping some specialized surface proteins, these bacterial could adhere onto the teeth and aggregate as biofilm to form plaque. Antigen I/II and GTF, a glucosyltransferase, the two proteins play an important role in adhesion and biofilm formation by *S. mutans*. In the absence of sucrose, they use Antigen I/II, a cell surface fibrillar protein, to anchor the surface of teeth at the initial adhesion[3, 4]. On the other hand, GTF is responsible for synthesizing the glucan polymers which is related to binding and biofilm formation in the present of sucrose[5].

It has been reported that biofilms are involved in many microbial infection. Biofilms are the structure that bacteria aggregate layer by layer, encased in an extracellular matrix such like exopolysaccharides, proteins, extracellular DNA and amyloid fibers, etc[6-8]. By forming biofilms, *S. mutans* could resist phagocytosis from host immune cells and the threats from antibiotics. It makes difficulties in treatment[9]. As many clinical reports, it is also known that biofilm formation by *S. mutans* is strongly associated with dental plaque and infectious endocarditis[2, 4, 5, 10]. Although a lot of studies about the prevention or therapy have been published, the efficacy still needs to be improved.

1.2 Infective endocarditis (IE)

Infective endocarditis (IE) is a form of cardiovascular disease. As a subacute type that progresses more slowly than acute IE, it is usually caused by oral commensal streptococci. In Taiwan, *S. mutans* is one of the prevalent infectious agents. These oral microorganisms gain access to the vascular system through trauma by surgery or other physics forces and cause the transient bacteremia[11]. If the endocardial surface is abnormally roughened, it may offer a foothold for the adherence. By the binding of fibrin-platelet clots and embedded bacteria layer by

layer, the vegetation with characterized by a firm architecture is made up[10] (Appendix. 1). Bacteria in vegetation have higher resistance to antibiotics and could escape the phagocytosis since that the dense nature of vegetation may restrict the migration of phagocytes. Although IE is uncommon, it may have serious complications such like stroke due to the embolism caused by dissection of vegetation[12, 13]. According to the clinical reports, the frequency in recurrence and mortality rate are even up to 40% and 30% respectively. Unfortunately, there is still no appropriate way on prophylaxis and diagnosis. .

1.3 The role of platelets in IE model

In IE model, the pathogenesis is complex. Briefly, the whole process could be dissected into three parts: the oral bacteria accessing the circulation, escaping the surveillance of immune cells and colonizing on valves to form vegetation[10, 14]. In our previous study, we found that *S. mutans* would change the gene expression and phenotype when exposure to human plasma at a low concentration (< 10%)[15]. They can even bind to the components in plasma such as fibronectin to help them resist phagocytosis[14]. Furthermore, the microbes can also interact with platelets through their surface proteins or plasma components to induce platelet aggregation[16].

When platelets (PLTs) are activated, they undergo aggregation and form thrombus to plug the damaged endothelium. Bacteria take the advantage of that. By interacting with specific surface receptors of PLTs to induce aggregation, they promote the colonization at host tissue[17] (Appendix. 3). In the case of staphylococci, *S. aureus* could use fibrinogen to bridge between ClfA (clumping factor A) and GPIIb/IIIa combined with immunoglobulin binding and induce the signal transduction to stimulate platelets aggregation[18] (Appendix. 2)[16]. In addition to fibrinogen, a fibronectin bridge may also take a part in *S. aureus*-induced platelet aggregation (Appendix. 2). Similar observation could be made in streptococci. In the presence of specific immunoglobulin to rhamnose-glucose polymers, *S. mutans* could trigger PLTs activation by using these cell wall polysaccharides[19]. Moreover, in *S. mutans*, it was also reported that protein antigen c (PAc) could both participate in extracellular matrix binding and PLTs aggregation[20]. In *S. gordonii*, SspA/SspB belonged to Antigen I/II family proteins could have the effect on PLTs adhesion and aggregation[21]. Also, results obtained from our previous report suggest that activated platelets could enhance biofilm formation of IE-inducing streptococci[22]. These PLTs-bacteria biofilms are more highly resistant to antibiotics and refractory to routine antibiotics treatment. Collectively, activated PLTs with some plasma components can offer

circulating bacteria adherence and enhance the pathogenesis.

1.4 Prophylaxis treatment

The history of prophylaxis treatment for streptococci infections could be back to the 1950s[23]. At that time, penicillin was used in prophylaxis strategies for preventing the diseases caused by streptococci such as rheumatic fever and infectious endocarditis. People who may have a risk of bacteremia would be administered high dosage of penicillin in oral or intramuscular manner to maintain the effective level of drug in blood. In 2007, American Heart Association (AHA) provided the recent guidelines for prophylaxis treatment of IE[24]. They recommended that: patients with the high risk situation (i.e. those have predisposing cardiac condition such like complex cyanotic congenital heart disease or equipping prosthetic valves or those procedures may cause magnitude of bacteremia) would need the prophylaxis treatment. With regard to antibiotics choice, amoxicillin (2g) is more often used in the prophylaxis since that it is absorbed well in gastrointestinal tracks. If patients would be allergic to β-lactams, they can take clindamycin (600mg), azithromycin (500mg) or other cephalexin family drugs instead[9, 25]. Also, the guidelines recommended that patients should be administered these antibiotics in an hour before the surgery or other procedure at the high risk[26].

Although many guidelines about IE prevention have been established, there are some arguments about prophylaxis[27]. First, in consideration of ethic issue, the effectiveness of prophylaxis treatment has only been shown in animal model, lack of actual scientific evidence in human beings. Furthermore, it is known that the level of bacteremia is one of essential factors, however, our daily activities, such like tooth brushing, may offer a great entry for microbe to cause transient bacteremia. So, it seems that the prophylaxis should not only focus on occasional procedure, but also take personal background into consideration, for example, the general hygiene. What is more, the factors associated with high risk of IE death should need more studies so that it could be applied into prophylactic strategies in the future.

1.5 Biofilm dispersal

Biofilms are the structure whicht bacteria aggregate layer by layer, encased in an extracellular matrix. These compact three-dimensional communities have the ability to resist phagocytosis and antibiotics treatment. The processes of biofilm formation can be divided into three steps: microbe attachment, population growth and biofilm maturation. However, scientists found that mature biofilm would undergo dispersal stage and then release planktonic cells which could migrate to new environment and

build up another communities, as a “biofilm life cycle”. Every step in biofilm life cycle is highly regulated. Recently, there are many studies focusing on the final dispersal stage, especially the mechanisms of regulation. A range of signal cues or effects, from environment and even bacteria oneself, have been found to participate in biofilm dispersal[28] (Appendix. 4). In addition, there are also more and more small molecules or products derived from nature for targeting bacterial biofilm. But their effectiveness needs more demonstrations

1.5.1 Dispersal factors

Nutrients, one of the effectors from environment, could regulate the biofilm dispersal, correlated with increase or decrease concentration. Some bacterial species, like *Pseudomonas aeruginosa*, may trigger biofilm dispersal in response to carbon limitation[29]; however, *Acinetobacter* sp. str. GJ12 would be packed when encountering starvation[30]. Similarly, changes in temperature or the level of oxygen and nitric oxide may also take part in the regulation of biofilm dispersal. For example, in response to hypoxia and low concentration of nitric oxide, in *P. aeruginosa*, biofilm dispersal would be triggered and sequencely release sessile cells[31].

Quorum sensing, well known in regulation of bacteria biofilm formation, also plays an important role in dispersal. AHLs (acyl-homoserine lactones) and AIP

(autoinducing peptide), secreted by Gram-negative and Gram positive bacteria respectively, has been reported that could induce biofilm dispersal by activating the signal transduction (i.e. cyclic di-GMP signaling) and producing the enzymes or surfactants to degrade the hard biofilm structure. Previously, quorum sensing peptides were applied in the inhibition against biofilms. They used CSP (competence stimulating peptides, belonged to quorum sensing systems)[32] hybridized with activate antimicrobial peptides to serve as specifically targeted antimicrobial peptides (STAMPs)[33]against *S. mutans* biofilm. Other dispersal factors such like polysaccharide-degrading enzymes and rhamnolipids n *P. aeruginosa* could trigger biofilm disassembly under the certain stimuli[34, 35]. Interestingly, bacteriophages are also linked to biofilm dispersal. They may provide degrading enzymes by inducing the related genes expression[36].

1.5.2 D-amino acids

Recently, it is shown that D-amino acids also participate in biofilm disassembly. D-amino acids, produced in bacteria stationary phase, can influences peptidoglycan composition to adapt to changing environmental conditions[37, 38]. In *Bacillus subtilis*, these amino acids incorporate into the anchored site of TasA fiber (TasA, subunits of amyloid fibers) to cell wall and consequently govern the cell wall even triggering

biofilm disassembly. To further prove the importance of D-amino acids, Ilana Kolodskin-Gal and her coworkers carried out liquid chromatography-mass spectrometry followed by L-FDAA to identify the composition of cultured medium. They found that D-tyrosine, D-leucine, and D-methionine were present and effective in regulation of dispersal. Moreover, they also used racemases mutant strains, which could not produce D-amino acids, and the bacteria failed to drive biofilm disassembly. These phenomena could be observed in other species, such as *P. aeruginosa* and *S. aureus*. It seems to be a general strategy in bacteria population for regulating the biofilm disassembly. In addition to D-amino acids, norspermidine, another dispersal factor, could interact with exopolysaccharides directly and together with D-amino acids on biofilm-inhibiting effect[39].

1.5.3 Small molecules

Biofilms are correlated with many diseases, such as whooping cough, cystic fibrosis or endocarditis, etc. Therefore, more and more small molecules have been developed for targeting biofilm-associated disease or for prophylaxis.

Chang Liu et al. screened a focus library of nitrogen-dense marine alkaloid compounds, about 506 small molecules, and they found that eight of them had inhibition against *S. mutans* biofilm formation[3]. The inhibiting effect is mainly on

biofilm-associated genes, such like *pac* (adherence-associated protein), *gtfB* (glucosyltransferase), *gbpB* (glucan-binding protein) and *comDE* (quorum-sensing associated protein). Genetic evidence showed that these molecules could interfere with the genes expression and protein production level at 0.94 uM. Also, these small molecules could have effect on planktonic cell growth at 2 uM.

In Gram-negative bacteria, curli and type I pili are important in mediating biofilm formation. Lynette Cegelski and her groups discovered that dihydro thiazolo ring-fused 2-pyridone is pilicides[40, 41]. These molecules structurally interfere with the *Escherichia coli* pilus chaperon-subunits complex and consequently inhibit the fibers assembly. Next, they modified some functional groups, such as the exchange of cyclopropyl group, and generated another compound, which is not only pilicidal ability but also curlicidal that can inhibit CsgA (curli subunit) polymerization[42].

1.6 Specific aim

The characteristic of IE is the formation of vegetations which is refractory to routine antibiotic treatment. Our data reported that platelets could promote vegetation formation and enhance the resistance of streptococcal biofilm to antibiotics[22]. Therefore, to search prophylactic agents that specifically target the platelet-associated

biofilm will provide effective strategy for the successful control of IE.

Previously, many studies made an effort toward inhibiting bacterial biofilm. They used D-amino acids[38], modified small molecules[3, 42] or STAMPs[33] to eliminate homotypic biofilm formation. However, there is no report about the inhibition against streptococci-platelet biofilm formation. Therefore, the objectives in my study are that:

1. To achieve the goal of searching the ideal candidates which could target platelet-bacterial biofilm formation by screening a range of small molecules *in vitro* (provided from Dr. Ling-Wei Hsin);
2. To investigate the potential inhibiting mechanisms of these targeted small molecules;
3. To examine the effect of targeted small molecules in rat endocarditis model;
4. To treat bacteria with antibiotics addition to targeted small molecules and examine the synergistic effect *in vitro*.

These results suggest that these synthetic small molecules may offer another strategy for clinical treatment on infective endocarditis.

Chapter 2: Materials and Methods

2.1 Bacteria strains and culture

S. mutans GS5 strains were used in this study. Bacteria were grown and maintained in brain-heart infusion (BHI) broth (BD, Bacto) and agar plate (BD, Difco). Strains were cultured at 37 ℃ under an anaerobic atmosphere with 95% N_2 and 5% CO_2 for 16-18 hours. For confocal laser scanning microscopy observation, GFPuv-tagged GS5 strains were generated by transformed with the GFPuv-contained shuttle plasmid (pPDGFPuv) and selected by spectinomycin (500 μg ml^{-1}).

2.2 Preparation of human platelets

Whole blood was collected from health donors in the laboratory by using venous blood collection tubes containing sodium citrate 3.2 % (BD Vacutainer® citrate tube).

2.2.1 Platelet-rich plasma (PRP)

For preparation of platelet-rich plasma (PRP), whole blood was manipulated by centrifugation at 1200 rpm for 12 min at 25℃ and collected the upper layer. This PRP would be used in biofilm formation and bacteria-platelet interacting assay to mimic the condition *in vivo*.

2.2.2 Platelet-poor plasma (PPP)

The platelet-poor plasma (PPP), used as blank in platelet aggregation assay, was collected by centrifugation at 3000 rpm for 10 min at 25℃. The upper layer would be clearer than PRP.

2.2.3 Platelet suspension (PS)

The platelets suspension (PS) would be prepared for analyzing the direct interaction between platelets and bacteria. Briefly, PRP was added with heparin (10 U ml^{-1}; B. Braun Melsungen AG) and Prostaglandin E1 (1 μM; Sigma-Aldrich) and centrifuged at 3000 rpm for 6-8 min. Collect the pellet and resuspended gently with Tyrode solution (11.2 mM glucose, 136.8 mM NaCl, 11.9 mM $NaHCO_3$, 2.8mM KCl, 1.1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 1.0 mM $CaCl_2$, and 3.5 mg bovine serum albumin per ml; pH 7.35~7.4). Heparin (6.4 U ml^{-1}) and PGE1 (1 μM) were further added to the suspension, and again, centrifuged at 2500 rpm for 5-7 min. After that, the pellet was repeatedly resuspended with Tyrode solution. The concentration of PS would be suggested at 3×10^8 ~5×10^8 platelets per ml.

2.3 Preparation of small molecules

A range of small molecules were kindly provided from Associate Professor

Ling-Wei Hsin (Graduate institute of Pharmaceutical Sciences, College of Medicine, NTU). These 76 different small molecules were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 10 mg ml^{-1}. The test concentrations *in vitro* were 100, 20, 10 μg ml^{-1} diluted with double-distilled water. For experiment in animal model, the concentration was used at 6 mg kg^{-1}.

2.4 Biofilm formation assay

To screen the targeted small molecules, the inhibiting effect would be test by using bacterial biofilm formation assay in 96-well U-bottom polystyrene microtiter plates (Greiner bio-one, NO. 650101) and condition was set in normal nutrient broth (BHI) or PRP. *S. mutans* GS5 strain, cultured overnight, was centrifuged (3000rpm, 10 minutes) for removing the upper cultured medium. Pellet was washed by 1X phosphate buffer saline (PBS) and suspended by sonication (40W) for 5 minutes. After that, bacteria suspension was prepared as the concentration of 10^9 CFU ml^{-1} (optical density: A_{550} adjusted to 1.5) in 1X PBS and then seeded into wells as a ratio of 1 to 100 in BHI supplemented 1 % glucose or 1 to 10 in PRP. Simultaneously, small molecules with (or without) antibiotics (gentamicin 20 μg ml^{-1}; penicillin 0.5 μg ml^{-1}) were added with. The incubation time was 18-20 hours at 37℃. After incubation,

the upper liquid was removed and washed twice with distilled water. The 96-well plates were dried for 45 minutes and then stained with crystal violet (0.05%) for 5 minutes. After rinsed twice, the plates were distained with acetone-ethanol (ratio of 1 to 4) for 1 hour. By detecting the absorbance at 550 nm using a MicroELISA reader (Dynatech Corp), the inhibition ability on biofilm formation would be semi-quantified. Each experiment would be repeated three times in triplicate. For the confocal microscopy observation, the biofilms would be cultured in 24-well plate which a round glass coverslip was put in individual wells. After incubation, the coverslips were washed by 1X PBS and fixed with 2 % paraformaldehyde.

2.5 Bacteriostatic analysis

To examine whether targeted small molecules could inhibit bacterial growth, it was necessary to quantify the bacterial population size and plot the values as a growth curve. As mentioned before, bacteria suspension was prepared and inoculated into BHI at 10^7 CFU ml^{-1}. The inoculation was set as the first timepoint ("0 hour"), and optical density was determined by measuring the absorbance at 550 nm. Repeatedly, record the absorbance every hour until the 8^{th} timepoint, and the 9^{th} data was measured at the 24^{th} hour. All the records would be display as a curve by using

GraphPad Prism 5.

2.6 Platelet aggregation test

Platelets aggregation test could examine the ability of platelets activation. After adding the agonist such as adenosine diphosphate (ADP) into PRP, platelets were activated and sequencely underwent aggregation. Once platelets aggregated, the level of light transmission would be increased. By measuring the difference of light transmission level between pre- and post-stimulation, it could determine the ability of platelets activation.

In this study, both *S. mutans* GS5 strain and ADP were used as agonists. The concentration of bacterial treatment was 10^{10} CFU ml^{-1} and ADP was 10 μM. Un-stimulated PRP was set as 0 % of light transmission and PPP was as 100 %. The analysis was performed as following. PRP incubated with small molecules (100 and 20 μg ml^{-1}) was pre-warmed for 3 minutes and stirred at 900 rpm in a test cuvette at 37 ℃. After adding the agonist (bacteria or ADP), the level of platelet activation would be monitored continually by the photocell in Lumi-Aggregometer (Payton Scientific)[43]. If targeted small molecules have the inhibiting effect on bacterial-inducing aggregation, light transmission level would not change for 25-30

minutes at least after addition of bacteria.

2.7 Bacteria-platelet interacting assay

For investigating whether targeted small molecules could interfere with the interaction between bacteria and platelets, the experiment would be used as a quantification assay. Bacterial cultured overnight were centrifuged at 3000 rpm for 10 minutes to remove the cultured medium, and the pellet was washed by 1X PBS. Again, bacterial suspension was centrifuged and suspended by sonication (40 W) with ELISA coating buffer instead. Next, bacteria were prepared as the concentration of 10^9 CFU ml^{-1} (optical density: A_{550} adjusted to 1.5) and then coated onto the 96-well plates (Nunc MaxiSorp® flat-bottom 96 well plate) at 4 ℃ overnight. The upper liquid part was removed and the plats were rinsed 1X PBST (1X PBS supplemented with 1% Tween-20) once. Additionally, the wells were blocked by 1% bovine serum albumin (BSA). The targeted small molecules were diluted with PRP (indirect-binding assay) or PS (direct-binding assay), and then these samples were added into wells which had coated with bacteria. The plates were incubated at 37℃ for 2 hours and sequencely washed by 1X PBST once again. Next, phosphatase substrate (1 tablet per 5 ml buffer; Sigma-Aldrich) dissolved in specific buffer (0.1M

Na-acetate addition of 0.1% Triton-X100; pH 5.5) was added into wells, and the plates were incubated at 37℃. If platelets bind to bacteria, phosphatase substrate would interact with phosphatase, which located inside platelets, and undergo chromogenic reaction (**Fig. 6**). By detecting the absorbance at 405 nm, the inhibition effect on the bacteria-platelet interaction would be quantified.

2.8 Experimental streptococcal endocarditis rat model

All animal experiments in this study were approved by National Taiwan University Institutional Animal Care and Use Committee.

2.8.1 Experimental schedule design

At the first day, rats were operated with cardiac catheterization. After 24 hour, small molecules (I.V.) or Aspirin (I.P.) would be injected into these animals. 30 minutes later, these rats were treated with bacteria. For Aspirin (25mg kg^{-1}; Sigma-Aldrich treatment), there would be two more injections at 30 minutes and 3 hours after bacterial infection. To analyze the effect of targeted small molecules on bacteria survive in circulation, blood was collected at three timepoints: 30 minutes, 3 hours and 24 hours after bacteria treatment. At the third day, these rats were sacrificed and their hearts were removed (**Fig. 8A**).

2.8.2 Preparation for bacteria, small molecules and anesthetic

Preparation for bacteria (GFP-tagged or normal GS5 strain) and small molecules were described as previously. The amount of bacterial infection was 10^9 CFU and the concentration of targeted small molecules was 6 mg kg^{-1}. The anesthetic ("Zoletil 50", Virbac) were supplemented with muscle relaxant ("Rompun", Bayer HealthCare) and water (for injection only) as a ratio of 500:280:220 respectively.

2.8.3 Cardiac catheterization

8-week-old male rats (Wistar) were used as ideal endocarditis rat model. A stainless steel (10 cm) embedded into a polyethylene tube (8 cm; I.D. 0.28mm and O.D. 0.61mm; Becton Dickinson, PE10) as a catheter which was further bent as 1/4 of circle by a tweezers. This catheter was then inserted into carotid artery through an incision on the chest and moved on to the left ventricle along the vessel. The catheter would tremble more and more intensely with heartbeat when it was closed to the aortic valves. When it no longer went forward by the blood flow resistance, the catheter remained right there and was fixed by sutures.

2.8.4 Quantification and observation

For quantification of the effect on bacteria survive in circulation, blood was drew and lysed by 1% Triton-X100 (in 1X PBS). The cell lysate was plated on BHI agar

with optimized dilutions. To determine the density of bacteria colonized in vegetations, the vegetations were removed and their masses were weighed. By sonication in 1X PBS, the homogenous suspension was further plated on Mitis Salivarius agar (MS agar; BD, Difco) added with 20% sucrose. Both BHI and MS agars were incubated at 37 ℃ for 2 days. The biofilms inside vegetations were also visualized by confocal microscopy and their thicknesses were measured by vertical section quantification.

2.8 Confocal laser scanning microscopy (CLSM) analysis

By using confocal laser scanning microscopy (CLSM; Leica TCS SP5), the detailed composition of biofilm formation in wells and inside vegetation would be observed. Biofilms cultured in wells were washed with 1X PBS and fixed with 2 % paraformaldehyde. GFP-tagged bacteria were visualized by detecting emission spectra of GFP. For platelets observation, on the other hand, samples which were incubated with 1% Triton-X100 for 5 minutes and stained with rhodamine-conjugated phalloidin (1 to 500 dilution; Invitrogen) were traced by detecting the emission wavelength at 565 nm. For the vegetation observation, it was performed as describes previously.

Chapter 3: Results

3.1 Identification of streptococci-platelet biofilm specific inhibitors

For searching the ideal candidates which target streptococci-platelet biofilm formation, 76 different small molecules, provided from Associate Professor Ling-Wei Hsin, were screened *in vitro*. By using crystal violet, biofilms were stained and the formation activity was semi-quantified by detecting the absorbance at 550 nm. Among these compounds, CYY-X022, 47, 48, 58, and 60 could obviously inhibit the homotypic biofilm formation in BHI (**Fig. 1A** and **1C**); as regards the platelet-associated biofilm, CYY-X011, 12, 22, 47, 52, 55 and 56 had an inhibiting effect on that (**Fig. 1B** and **1D**). DMSO, the solvent for small molecules, would not have inhibition against biofilm formation (**Fig. 1E** and **1F**). CYY-X058 and 60, having an effect on normal biofilm formation (**Fig. 1C**), could also have partial inhibition against platelet-bacterial biofilm (**Fig. 1D**). The absorbance upon the treatment of CYY-X022 or 52 at 100 μg ml^{-1} was much higher than others (**Fig. 1B** and **1D**) since these compounds may precipitate with plasma components and cause the pseudo-positive results (**Fig. 1F**).

The inhibition effect of these targeted small molecules was assessed by CLSM. Compared with the controls (“no treatment” and “DMSO”) (**Fig. 2A**), the bacteria

aggregates in the groups treated with the targeted molecules were much smaller and randomly distributed (**Fig. 2B-D**). The architectures were not compact anymore, either. As the concentration decreased, the inhibition effect was diminished, with the bacterial cluster becoming thicker and thicker. The biofilm thicknesses upon 100 μg ml^{-1} of treatment was less than 10 μm in most treated groups except CYY-X022 (**Fig. 2B-D**) which may cause the precipitation with platelets and plasma components as aggregates. These data indicate that some of small molecules may have ability to inhibit biofilm formation, and, most importantly, seven of them could even target platelet-associated biofilms.

3.2 The correlation between small molecules concentration and bacterial number in inhibition effect

To further find out the effective dosage of the targeted molecules, we analyzed the correlation between molecules concentration and bacterial number. The bacterial inoculation was manipulated by serial 10-fold dilutions (from 10^8 to 10^4 CFU) and targeted small molecules, CYY-X047, 52, 55, and 56, were examined at five different dosage: 100, 20, 10, 5, 1 and 0 μg ml^{-1}. In BHI, CYY-X047 (100 μg ml^{-1}) had broad inhibiting effect under all different amount of inoculation (**Fig. 3A**); CYY-X052 and

56 (100 μg ml^{-1}) would eliminate biofilm formation only when bacterial number was below 10^5 CFU (**Fig. 3B** and **3D**); CYY-X055 would still have no inhibition against normal biofilm formation upon all of different bacterial amount treatment even at 100 μg ml^{-1} (**Fig. 3C**). In PRP, when bacterial amount was below 10^7 CFU, these four molecules could inhibit the platelet-associated biofilm formation at five different dosage (**Fig. 3E-3H**); however, when bacterial number was up to 10^8 CFU , the only effective concentration would be 100 μg ml^{-1} (**Fig. 3E-3H**). These results indicated that these molecules could inhibit biofilm formation with bacterial number below 10^7 CFU, and the most effective concentration would be 100μg ml^{-1}. It suggested a correlation between the molecules dosage and bacterial number for effective treatment.

3.3 The effect of targeted small molecules on bacteria growth

At the first screening experiments, it was found that some of targeted small molecules at 100 μg ml^{-1} could eliminate normal biofilm formation in BHI condition. Therefore, we hypothesized that these molecules may have ability to inhibit bacteria growth. To examine this hypothesis, bacteria were treated with the targeted small molecules at 100μg ml^{-1} and the population of survival bacteria was quantified by

analyzing the changes in turbidity every hour. CYY-X055 had no inhibition against cell growth (**Fig. 4B**). CYY-X011, 12, 52, and 56 partially interfered with the bacteria growth; after 8 hours incubation, however, the inhibition would be compensated (**Fig. 4A** and **4B**). The other targeted small molecules, CYY-X022, 47, 48, 58 and 60 had an obvious inhibiting effect on bacteria growth (**Fig. 4B**), and this inhibition could maintain for 24 hours (data not shown). These results were further analyzed by colony counting. Compared with positive control (DMSO 1%), there was a significant difference in the groups treated with CYY-X022, 47, 48, 58 and 60 at 100 μg ml^{-1} (**Fig. 4C** and **4D**). Interestingly, these five molecules also inhibited homotypic biofilm formation in BHI. The results revealed that these five molecules could interfere with biofilm formation due to their ability to inhibit bacteria growth.

3.4 Inhibition effect of targeted small molecules on bacteria-induced platelet aggregation

In addition to having ability to inhibit homotypic biofilm formation, some of them, such as CYY-X011, 12, 52, 55 and 56, could prevent the streptococci-platelet biofilm forming (**Fig. 1B** and **5D**), and some may even have dual abilities, like CYY-X022 and 47 (**Fig. 1A-1D**). According to our previous results, platelets played

an important role in PRP biofilm formation[22]; moreover, the aggregation by induced by bacteria was also another essential factor. Therefore, we supposed that these seven molecules may inhibit PRP biofilm by interfering with bacteria-induced aggregation. To prove the hypothesis, PRP incubated with targeted small molecules (100 and 20 μg ml^{-1}) for 20-30 minutes was added with agonist (GS5 strain and ADP) and then examined the aggregation ability. Compared with control groups (**Fig. 5A and 5B**), most PRP treated with the targeted molecules at 100 μg ml^{-1} did not aggregate within 25 minutes (**Fig. 5C-E** and **5I-L**); however, as the concentration decreased to 20 μg ml^{-1}, the inhibition would be compensated, except CYY-X022 (**Fig. 5F-H** and **5M-P**). Considering these compounds may block all the physiological functions of platelets, ADP stimulation was as a control (**Fig. 5Q-S**). After added with ADP, it was found that the platelets supplemented with these molecules could aggregate as usual while the magnitude upon the treatment of CYY-X022 (100 μg ml^{-1}) was partially weaker. According to above data, it indicated that CYY-X011, 12, 22, 47, 52, 55 and 56 could inhibit platelet-associated biofilm through interfering with bacteria-induced aggregation.

3.5 Inhibition effect of targeted small molecules on bacteria

adherence to platelets

In previous studies, it had been pointed out that *S. mutans* could induce platelet aggregation through its specialized polysaccharides, proteins[19, 20] or the components in plasma. To further explore the underlying mechanism of the inhibition against platelets aggregation, it was assessed by the bacteria-platelet interacting assay (**Fig. 6**). Compared with control groups, most PRP treated with the targeted molecules at 100 μg ml^{-1}could not bind to bacteria significantly except CYY-X052 and 56, which represented a partial inhibiting effect (**Fig. 7A** and **7B**).With the dosage of treated molecules decreased, the inhibiting effect would diminish. These data exhibited that in PRP which was abundant in plasma proteins these targeted small molecules could interfere with the indirect binding of streptococci and platelets, and the inhibiting effect was dose-dependent. To analyze the effect on direct binding, PS purified from PRP was pretreated with these small molecules and added into the wells coated with bacteria. As the results revealed, CYY-X012, 47 and 52 at 100 μg ml^{-1} could interfere with the direct binding while others had a slightly inhibiting effect (**Fig. 7C** and **7D**). The above results indicated that these small molecules had ability to inhibit bacteria adherence to platelets.

3.6 Inhibition effect of targeted small molecules on biofilm formation in rat endocarditis model

Next, the inhibiting effect of these targeted small molecules on biofilm formation was verified by using the experimental streptococcal endocarditis rat model. Rats were infected with bacteria after pretreated with the targeted molecules (CYY-X012, 47, 52, 55 and 56) or Aspirin (**Fig. 8A**). According to the CLSM images, it was found that compared with the controls ("no treatment" and "DMSO 1 %") the biofilm thickness of the treated groups decreased obviously (less than 40 nm) and the structure also lost the multi-layer characteristic in vertical section (**Fig. 8B**). The density of colonized bacteria in the groups treated with the targeted molecules, however, decreased slightly (**Fig. 8D**) and the biomass of vegetation had no significantly difference, either (**Fig. 8E)**. To further investigate the effect on bacterial survival in bloodstream, the blood was collected at the 0.5^{th}, 3^{rd} and 24^{th} hour. The data revealed that these targeted small molecules had no obvious inhibition against bacteria survival in circulation (**Fig. 8D)**.Taken together, CYY-X012, 47, 52, 55 and 56 could interfere with the biofilm formation in injured valves but have limited inhibition on bacteremia and the density of colonized bacteria inside vegetations.

3.7 Synergistic effect with antibiotics

An attempt was made to further analyze whether antibiotics combined with these targeted molecules could have more effective inhibition against platelet-associated biofilm formation. Bacteria treated with antibiotics (penicillin and gentamicin) and the small molecules (CYY-X011, 12 and 22) were seeded into 24-well plates. In the CLSM images, it was found that the biofilms architecture in the treated groups became more loosed and small-aggregated (**Fig. 9B)** compared with the untreated and antibiotic-treated-only groups (**Fig. 9A)**. With the dosage of the small molecules decreased to 20 μg ml^{-1}, the inhibiting effect would be diminished. For the effect on bacterial survival in PRP biofilm, the results showed that CYY-X022 (100 μg ml^{-1}) addition to penicillin and gentamicin respectively could significantly inhibit the bacterial survival (**Fig. 9C** and **9D)**, and the inhibiting effect would sustain even the treated dosage reduced to 20 μg ml^{-1} (gentamicin treated group). These data revealed that CYY-X022 could assist antibiotics with inhibiting both the streptococci-platelet biofilm formation and the bacterial survival in PRP biofilm, suggesting the role of synergistic effect.

Chapter 4: Discussion

4.1 Summary

IE, an infectious disease with a high mortality rate, is characterized by the formation of vegetations, fibrin-platelet clots with the embedded bacterial biofilm. This firm architecture exhibiting highly resistant to antibiotics treatment makes it difficult on the clinical management. Additionally, the prophylactic treatment to prevent IE is also controversial. Previously, our data reported that platelets could promote the vegetation formation and enhance the resistance of streptococcal biofilm to antibiotics. Therefore, searching novel prophylactic agents that specifically target the platelet-associated biofilm will provide effective strategy for the successful control of IE.

In this study, 76 synthetic small molecules were screened and examined. These results are summarized as following:

1. To achieve the goal of searching the ideal candidates which could target platelet-bacterial biofilm formation by screening a range of small molecules *in vitro* (provided from Dr. Ling-Wei Hsin);

   Among the 76 synthetic molecules, it was found that CYY-X022, 47, 48, 58 and 60 could eliminate homotypic bacterial biofilm formation, and CYY-X011, 12,

22, 47, 52, 55 and 56, alternatively, could interfere with platelet-associated biofilm formation. And 100 μg ml^{-1} was suggested as an effective concentration.

2. To investigate the potential inhibiting mechanisms of these targeted small molecules;

   By interfering with the bacterial growth, CYY-X048, 58 and 60 were able to inhibit biofilm formation. On the other hand, CYY-X011, 12, 52, 55 and 56 may reduce the platelet-associated biofilm formation through interfering with the binding of streptococci and platelets. CYY-X022 and CYY-X047 had dual abilities to inhibit both the bacterial growth and the interaction between bacterial and platelets.

3. To examine the effect of targeted small molecules in rat endocarditis model;

   Consistent with the *in vitro* data, the vegetation size was decreased by intravenous administration of CYY-X022, 47, 52, 55 and 56 in the experimental streptococcal endocarditis rat model; however, the inhibiting effect on bacteremia and the colonized bacteria inside vegetations was limited.

4. To treat bacteria with antibiotics addition to targeted small molecules and examine the synergistic effect *in vitro*;

   Antibiotics combined with CYY-X011, 12 and 22 respectively could effectively

target the platelet-associated biofilm, suggesting that the role of synergistic effect *in vitro*.

Taken together, these results indicated that these targeted synthetic small molecules could eliminate streptococcal biofilm formation by inhibiting the bacterial growth or the binding of bacteria and platelets.

4.2 The inhibiting effect of the targeted small molecules on biofilm formation

In this study, we found that some of small molecules could inhibit the biofilm formation through different mechanisms, such as interfering with bacterial growth or the indirect (or direct) binding of bacteria and platelets. However, some side effects need to be solved:

1. In the *in vitro* biofilm formation assay, CCY-X022 and CYY-X052 precipitated in plasma as small aggregates surrounded by rhodamine-tagged platelets (**Fig. 1F** and **2B**). It indicated that the two molecules were slightly hydrophobic and may precipitate with plasma components in PRP. The functional group of these molecules will be modified additionally for more effective inhibition against PRP biofilm formation.

2. In the animal model, the thickness of the biofilms inside vegetation was decreased after prophylactic treatment of targeted small molecules. However, after pretreated with CYY-X047, 52, 55 and 56 by intravenous injection, these rats performed hematuria within 30 minutes. Also, CYY-X022 could interfere with the magnitude of normal platelet aggregation slightly in response to ADP (**Fig. 5Q**). The cytotoxicity-associated issue would need further investigation.

The effective concentration of these targeted molecules was 100μg ml^{-1} according to the i*n vitro* and *in vivo* experiments (**Fig. 1, 2** and **8**). Based on the data in this study, it was shown that when bacterial number below 10^7 CFU, these targeted molecules, even at 1 μg ml^{-1}, could perform effective inhibition against streptococci-platelet biofilm formation (**Fig**. **3E-3H**). However, in biofilm formation assay and animal experiments, the number of the bacteria treatment was up to 10^8-10^{10} CFU, for the GFP tracing in CLSM observation. In regard to clinical cases, the amount of bacterial infection would be less than 10^8 CFU per exposure. Therefore, the effective concentration of the molecules could be adjusted to a lower dosage.

According to the result, it indicated that CYY-X048 could effectively inhibit the bacterial growth. Nevertheless, it still could not eliminate the platelet-associated biofilm formation. On the other hand, CYY-X022 and CYY-X047 had more effective

inhibition against biofilm formation owing to equipping with dual abilities to inhibit both bacterial growth and the interaction between bacteria and platelets. Together, it revealed that uni-target of inhibition was not enough to eliminate the firm architecture of the complex biofilm.

Aspirin, an anti-platelet drug[44], was reported to be applied in pre-treatment of IE patients before the disease onset[45]. This strategy would be associated with a lower risk of embolism. Consistent with our published data, we also found that Aspirin could inhibit *S. mutans*-induced platelet aggregation and reduce the platelet-biofilm formation in rat experimental model and *in vitro* assay[22]. Similar results in animal model were observed in this study (**Fig**. **8B**). Compared with Aspirin, the targeted small molecules had comparable inhibiting effect on platelet-associated biofilm formation (**Fig**. **8B**) since that both of Aspirin and those targeted molecules have the ability to eliminate the bacteria-induced platelet activation (**Fig**. **5**).

Besides making an effort on searching effective prophylactic agents, we also attempted to explore the potential mechanisms. We found that some of the molecules (CYY-X048, 58 and 60) could target the bacterial growth, and some (CYY-X011, 12, 52, 55 and 56) may alternatively have inhibiting effect on the interaction between bacteria and platelets. What's more, some of them (CYY-X022 and 47) could have

dual abilities to target both bacterial growth and platelet aggregation. However, the detail about the inhibiting mechanism remains unclear. There may be a range of possible targets for eliminating biofilm formation. In genomic level, the molecules could inhibit the biofilm-associated genes expression in planktonic cell, such as *gtf*B, *gbp*B or *com*DE and then cause the inhibition against the bacterial biofilm formation; or, in proteomic level, the production of adhesion-associated proteins, such like GTF and Antigen I/II, may be blocked by these small molecules so that bacteria could not adhere to platelets and build a firm biofilm anymore. Others like metabolic or signalling-associated proteins, i.e. DAG (diacylglycerol), may be the potential targets. DAG and DAG kinase, ubiquitous in proeukaryotic and eukaryotic cells, participate in many essential signaling pathways, such like stress response and lipid recycling[46-48], etc. In proeukaryote, if these proteins have defect, there would be a severe impact on bacterial survival. In platelets, DAG plays an important role in activation. After priming, DAGs are generated through Gq protein-PKC (protein kinase C) pathway and sequencely induce the secondary aggregation[49-51]. Defect in DAG would fail to secretion and aggregation in response to ADP and thrombin[49]. Therefore, we hypothesis that : after cells uptake CYY-X022 and 47 through some membrane transporters, these molecules may inhibit both bacterial growth and platelet

aggregation by targeting bacterial and cellular DAG. The additional studies may shed light on the underlying mechanisms.

4.3 Application of small molecules as biofilm dispersal factors

In perspective of biological evolution, biofilm dispersal is beneficial for the whole population maintenance. In response to the changes of nutrients level or the competition, microcolonies in biofilm would be driven from mature to dispersal stage through different signaling regulation, and these dispersal cells would move away from the parent colonies to a new environment and form another communities. Taking advantage of this characteristic, nowadays, scientists put it in use. They modified the nature dispersal factors, trying to regulate biofilm life cycle instead of killing biofilm. Previously, many studies made an effort toward the application of biofilm dispersal factors. They used D-amino acids[38, 39], which were produced in bacteria stationary phase, to trigger biofilm disassembly. Other examples like synthesizing some molecules, which could targeted some biofilm-associated genes[3] or adhesion-associated protein[3, 42], to eliminate homotypic bacterial biofilm formation. Based on the previous studies, bacteria-induced platelet aggregation was benefit for bacterial survival[16, 17]. Moreover, according to our prior study, platelet could promote

the vegetation formation and enhance the resistance of streptococcal biofilm to antibiotics in IE[22]. Therefore, the attempt of this study is to search some novel agents that target the platelet-associated biofilm, especially the interaction between bacteria and platelets. By interfering with the biofilm formation, bacteria, as planktonic-like population, would become more sensitive to antibiotics. According to the data in this study, we found that CYY-X022, which has multi-ring-fused structure with two short chains, has better inhibition against platelet-associated biofilm. This may reveal that the characteristic structure may play an important role in the inhibition and have potential therapeutic value. However, for the clinical application, many aspects need to be considered, such as solubility, cytotoxicity and immunogenic properties, etc. Accordingly, the future studies are required to further probe into these important issues.

Chapter 5: References

1. Clarke, J.K. On the bacterial factor in the aetiology of dental caries. *Br J Exp Pathol* **5**, 141-147 (1924).
2. Hamada S, S.H. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* **44**, 331-384 (1980).
3. Liu, C., Worthington, R.J., Melander, C. & Wu, H. A new small molecule specifically inhibits the cariogenic bacterium *Streptococcus mutans* in multispecies biofilms. *Antimicrob Agents Chemother* **55**, 2679-87 (2011).
4. Mitchell, T.J. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol* **1**, 219-30 (2003).
5. Kazuhiko Nakano, R.N., Takashi Ooshima. *Streptococcus mutans* and cardiovascular diseases. *Japanese Dental Science Review* **44**, 29-37 (2008).
6. Flemming, H.C. & Wingender, J. The biofilm matrix. *Nat Rev Microbiol* **8**, 623-33 (2010).
7. Hall-Stoodley, L., Costerton, J.W. & Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**, 95-108 (2004).
8. Abee, T., Kovacs, A.T., Kuipers, O.P. & van der Veen, S. Biofilm formation and dispersal in Gram-positive bacteria. *Curr Opin Biotechnol* **22**, 172-9 (2011).
9. Que, Y.A. & Moreillon, P. Infective endocarditis. *Nat Rev Cardiol* **8**, 322-36 (2011).
10. Moreillon, P. & Que, Y.A. Infective endocarditis. *Lancet* **363**, 139-49 (2004).

11. Fekete, T. Controversies in the prevention of infective endocarditis related to dental procedures. *Dent Clin North Am* **34**, 79-90 (1990).

12. Hetzer, R. & Hummell, M. Thromboembolism and endocarditis--it's not simply the vegetation. *Eur Heart J* **22**, 811-2 (2001).

13. Cabell, C.H., Abrutyn, E. & Karchmer, A.W. Cardiology patient page. Bacterial endocarditis: the disease, treatment, and prevention. *Circulation* **107**, e185-7 (2003).

14. Jung, C.J., Zheng, Q.H., Shieh, Y.H., Lin, C.S. & Chia, J.S. *Streptococcus mutans* autolysin AtlA is a fibronectin-binding protein and contributes to bacterial survival in the bloodstream and virulence for infective endocarditis. *Mol Microbiol* **74**, 888-902 (2009).

15. Chia, J.S., Lee, Y.Y., Huang, P.T. & Chen, J.Y. Identification of stress-responsive genes in *Streptococcus mutans* by differential display reverse transcription-PCR. *Infect Immun* **69**, 2493-501 (2001).

16. Fitzgerald, J.R., Foster, T.J. & Cox, D. The interaction of bacterial pathogens with platelets. *Nat Rev Microbiol* **4**, 445-57 (2006).

17. McNicol, A. & Israels, S.J. Mechanisms of oral bacteria-induced platelet activation. *Can J Physiol Pharmacol* **88**, 510-24 (2010).

18. Moreillon, P. et al. Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infect Immun* **63**, 4738-43 (1995).

19. Chia, J.S., Lin, Y.L., Lien, H.T. & Chen, J.Y. Platelet aggregation induced by serotype polysaccharides from *Streptococcus mutans*. *Infect Immun* **72**, 2605-17 (2004).

20. Matsumoto-Nakano, M. et al. Contribution of cell surface protein antigen c of *Streptococcus mutans* to platelet aggregation. *Oral Microbiol Immunol* **24**, 427-30 (2009).

21. Kerrigan, S.W. et al. Role of *Streptococcus gordonii* surface proteins SspA/SspB and Hsa in platelet function. *Infect Immun* **75**, 5740-7 (2007).

22. Jung, C.J. et al. Platelets enhance biofilm formation and resistance of endocarditis-inducing streptococci on the injured heart valve. *J Infect Dis* **205**, 1066-75 (2012).

23. Rammelkamp, C.H. Prevention of rheumatic fever and bacterial endocarditis through control of streptococcal Infections. *Circulation* **15** (1957).

24. Wilson, W. et al. Prevention of infective endocarditis: guidelines from the American Heart Association: a guideline from the American Heart Association Rheumatic Fever, Endocarditis, and Kawasaki Disease Committee, Council on Cardiovascular Disease in the Young, and the Council on Clinical Cardiology, Council on Cardiovascular Surgery and Anesthesia, and the Quality of Care and Outcomes Research Interdisciplinary Working Group. *Circulation* **116**, 1736-54 (2007).

25. Khan, I.A. & Ijaz, N. Prophylaxis of infective endocarditis: current perspectives. *Int J Cardiol* **134**, e105-6 (2009).

26. Oliver, R., Roberts, G.J., Hooper, L. & Worthington, H.V. Antibiotics for the prophylaxis of bacterial endocarditis in dentistry. *Cochrane Database Syst Rev*, CD003813 (2008).

27. Duval, X. & Leport, C. Prophylaxis of infective endocarditis: current tendencies, continuing controversies. *Lancet Infect Dis* **8**, 225-32 (2008).

28. McDougald, D., Rice, S.A., Barraud, N., Steinberg, P.D. & Kjelleberg, S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol* **10**, 39-50 (2012).

29. Sawyer, L.K. & Hermanowicz, S.W. Detachment of *Aeromonas hydrophila* and *Pseudomonas aeruginosa* due to variations in nutrient supply. *Water Science and Technology* **41**, 139-145 (2000).

30. James, G.A., Korber, D.R., Caldwell, D.E. & Costerton, J.W. Digital image analysis of growth and starvation responses of a surface-colonizing *Acinetobacter* sp. *J Bacteriol* **177**, 907-15 (1995).

31. An, S., Wu, J. & Zhang, L.H. Modulation of *Pseudomonas aeruginosa* biofilm dispersal by a cyclic-Di-GMP phosphodiesterase with a putative hypoxia-sensing domain. *Appl Environ Microbiol* **76**, 8160-73 (2010).

32. Qi, F. et al. Peptide pheromone induced cell death of *Streptococcus mutans*. *FEMS Microbiol Lett* **251**, 321-6 (2005).

33. Eckert, R. et al. Targeted killing of *Streptococcus mutans* by a pheromone-guided "smart" antimicrobial peptide. *Antimicrob Agents Chemother* **50**, 3651-7 (2006).

34. Allison, D.G., Ruiz, B., SanJose, C., Jaspe, A. & Gilbert, P. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiol Lett* **167**, 179-184 (1998).

35. Schooling S, C.U., Allison DG, Gilbert P. A role for rhamnolipid in biofilm dispersion. *Biofilms* **1** (2004).

36. Sillankorva, S., Neubauer, P. & Azeredo, J. Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*. *Biofouling*

26, 567-75 (2010).

37. Lam, H. et al. D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science* **325**, 1552-5 (2009).

38. Kolodkin-Gal, I. et al. D-amino acids trigger biofilm disassembly. *Science* **328**, 627-9 (2010).

39. Kolodkin-Gal, I. et al. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. *Cell* **149**, 684-692 (2012).

40. Aberg, V. & Almqvist, F. Pilicides-small molecules targeting bacterial virulence. *Org Biomol Chem* **5**, 1827-34 (2007).

41. Pinkner, J.S. et al. Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. *Proc Natl Acad Sci U S A* **103**, 17897-902 (2006).

42. Cegelski, L. et al. Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nat Chem Biol* **5**, 913-9 (2009).

43. Born, G.V. & Cross, M.J. The aggregation of blood platelets. *J Physiol* **168**, 178-95 (1963).

44. Michelson, A.D. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov* **9**, 154-69 (2010).

45. Pepin, J. et al. Chronic antiplatelet therapy and mortality among patients with infective endocarditis. *Clinical Microbiology and Infection* **15**, 193-199 (2009).

46. Shibata, Y. et al. *Streptococcus mutans* diacylglycerol kinase homologue: a potential target for anti-caries chemotherapy. *J Med Microbiol* **60**, 625-30 (2011).

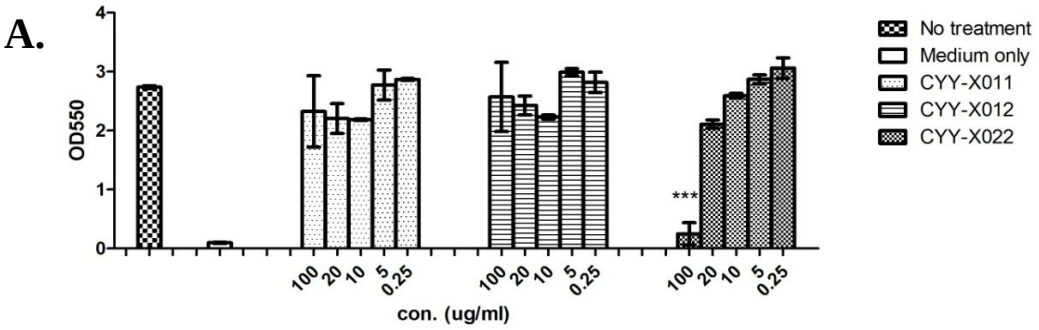
47. Van Horn, W.D. & Sanders, C.R. Prokaryotic diacylglycerol kinase and undecaprenol kinase. *Annu Rev Biophys* **41**, 81-101 (2012).

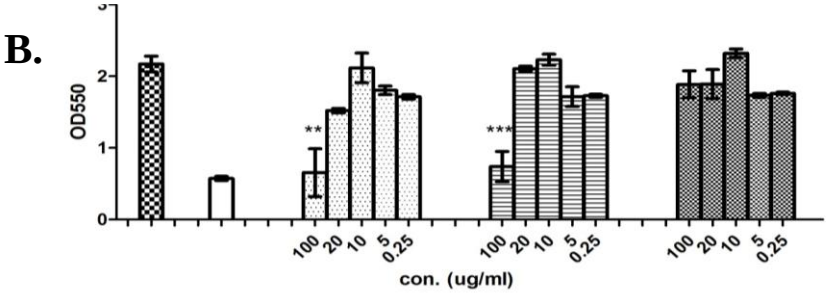
48. Yamashita, Y., Takehara, T. & Kuramitsu, H.K. Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. *J Bacteriol* **175**, 6220-8 (1993).

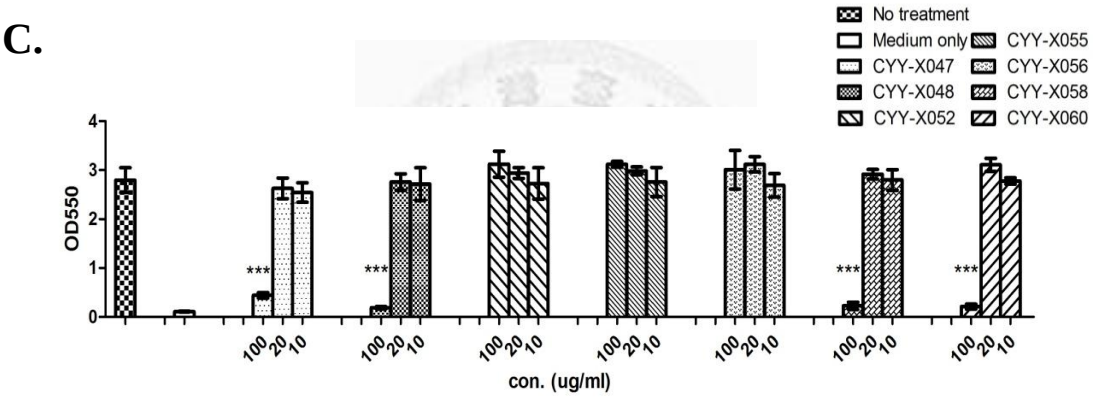
49. Li, Z., Delaney, M.K., O'Brien, K.A. & Du, X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* **30**, 2341-9 (2010).

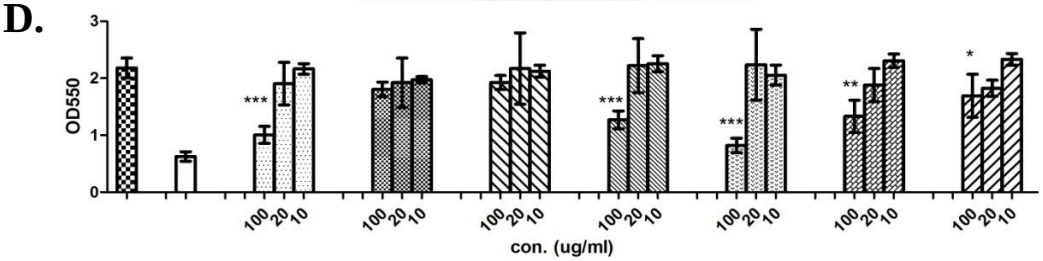
50. Werner, M.H., Senzel, L., Bielawska, A., Khan, W. & Hannun, Y.A. Diacylglycerol overcomes aspirin inhibition of platelets: evidence for a necessary role for diacylglycerol accumulation in platelet activation. *Mol Pharmacol* **39**, 547-56 (1991).

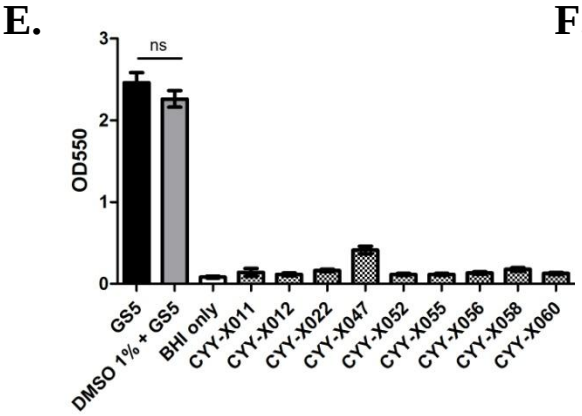
51. Yoshida, K., Asaoka, Y. & Nishizuka, Y. Platelet activation by simultaneous actions of diacylglycerol and unsaturated fatty acids. *Proc Natl Acad Sci U S A* **89**, 6443-6 (1992).


A.
No treatment
Medium only
CYY-X011
CYY-X012
CYY-X022
OD550
con. (ug/ml)


B.
OD550
con. (ug/ml)


C.
No treatment
Medium only
CYY-X047
CYY-X048
CYY-X052
CYY-X055
CYY-X056
CYY-X058
CYY-X060
OD550
con. (ug/ml)


D.
OD550
con. (ug/ml)


E.
ns
OD550
GS5
DMSO 1% + GS5
BHI only
CYY-X011
CYY-X012
CYY-X022
CYY-X047
CYY-X052
CYY-X055
CYY-X056
CYY-X058
CYY-X060

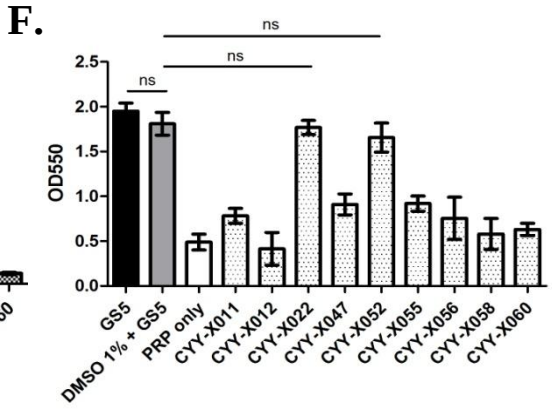
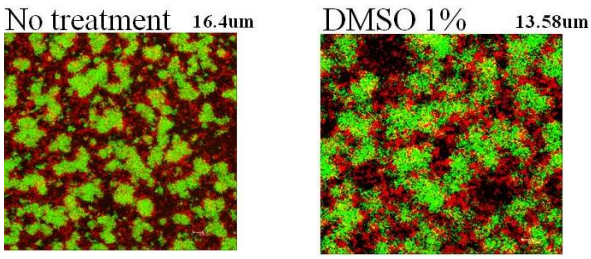

F.
ns
OD550
GS5
DMSO 1% + GS5
PRP only
CYY-X011
CYY-X012
CYY-X022
CYY-X047
CYY-X052
CYY-X055
CYY-X056
CYY-X058
CYY-X060

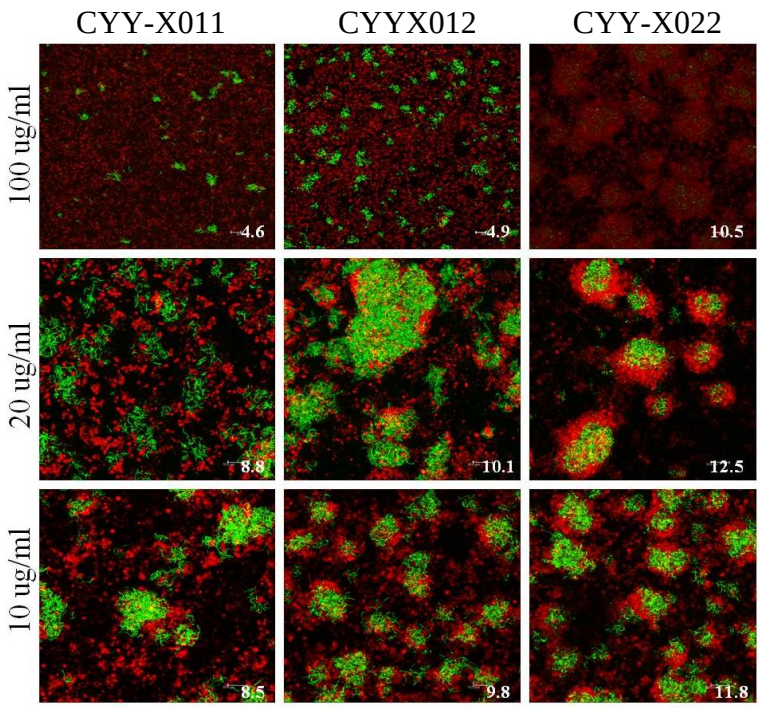
Fig. 1. Identification of streptococci-platelet biofilm specific inhibitors by crystal violet staining.

For biofilm formation, *S. mutans* GS5 strain was inoculated into 96-well plates for 18-20 hours. Biofilms were then stained with 0.05% crystal violet and quantified by measuring the absorbance at 550 nm. The culture conditions were nutrient broth (BHI supplemented with 1% glucose**;** **A**, **C**, and **E**) and PRP (**B**, **D**, and **F**). (**A** and **C**), the quantification of CYY-X011, 12, and 22 treated groups; (**B** and **D**), the results of groups treated with CYY-X047, 52, 55, 56. DMSO, the solvent of small molecules, was the control group (**E** and **F**). For the solubility test, the small molecules were added into BHI (**E**) and PRP (**F**) without bacteria inoculation. The data were expressed as the mean ± SD. Each experiment was triplicate. ***P < 0.001, **P < 0.005, *P < 0.05, Student *t* test.

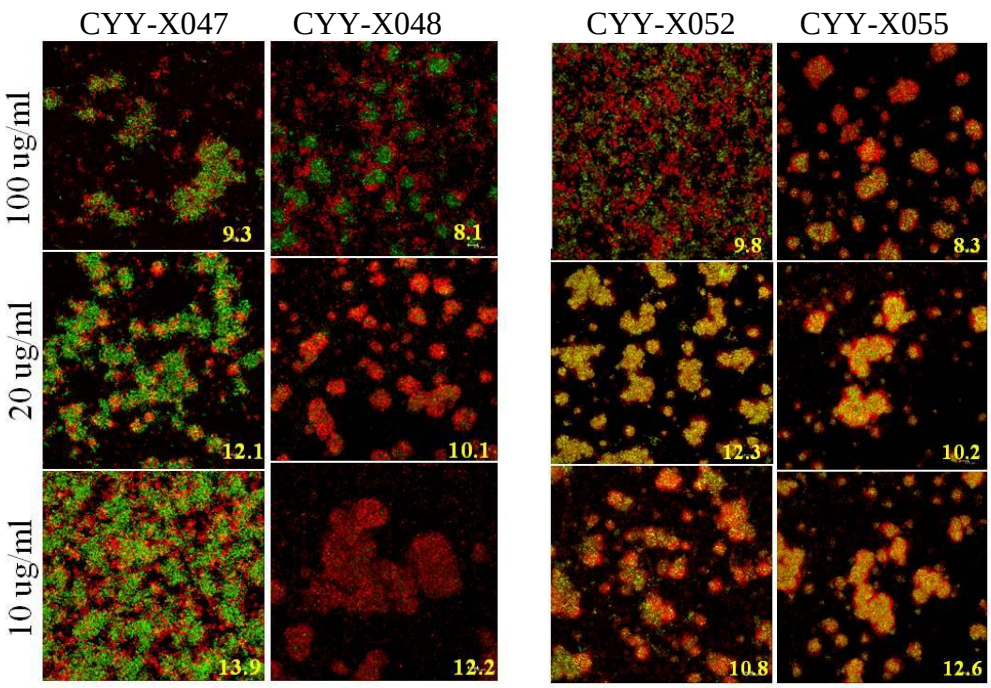
A.


No treatment 16.4um
DMSO 1% 13.58um

B.


CYY-X011
CYYX012
CYY-X022
100 ug/ml
20 ug/ml
10 ug/ml
4.6
4.9
10.5
8.8
10.1
12.5
8.5
9.8
11.8

C.


CYY-X047
CYY-X048
CYY-X052
CYY-X055
100 ug/ml
20 ug/ml
10 ug/ml
9.3
8.1
9.8
8.3
12.1
10.1
12.3
10.2
13.9
12.2
10.8
12.6

D.

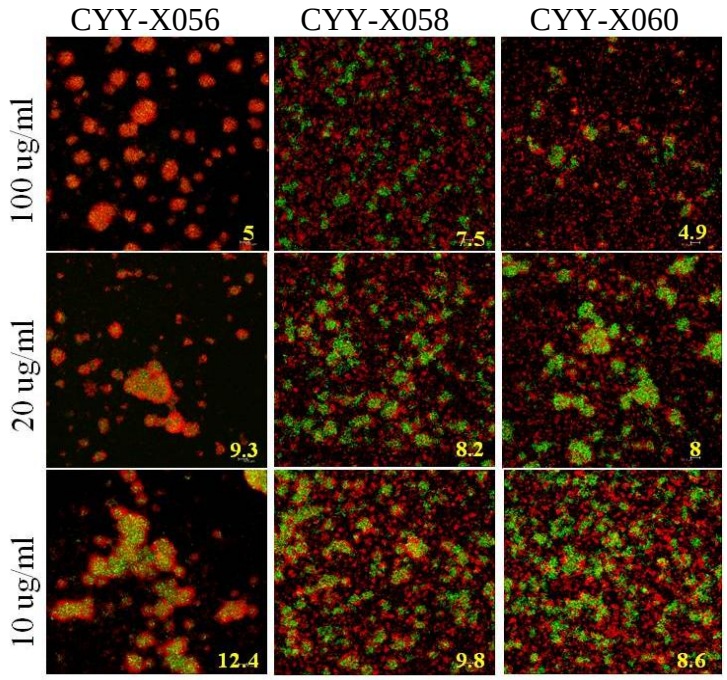
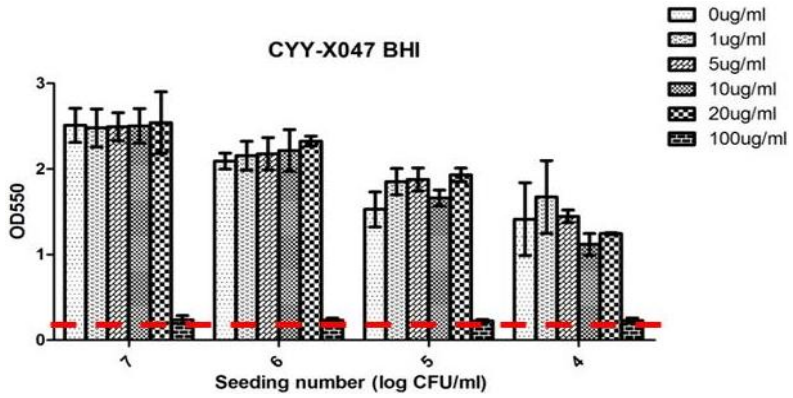


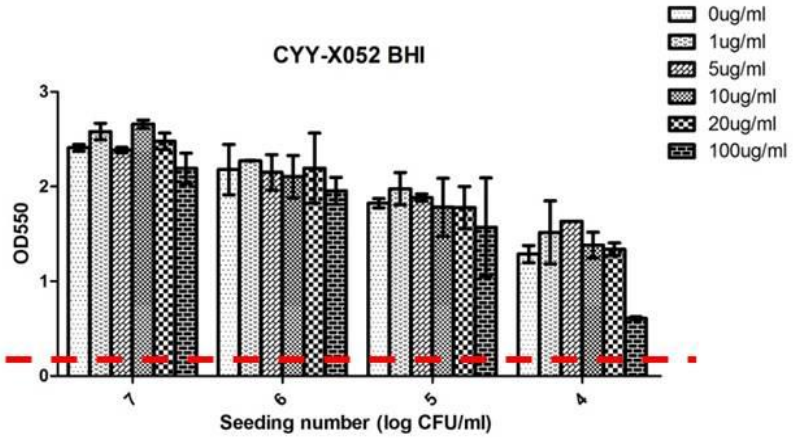
Fig. 2. Identification of streptococci-platelet biofilm specific inhibitors by confocal laser scanning microscopy

PRP biofilms were cultured in 24-well plates which a round glass coverslip was put in individual wells. The inhibiting effect was assessed by observation of CLSM (magnification X 630). Bacteria were traced by detecting the spectrum of GFP; platelets, stained by rhodamine-conjugated phalloidin (1:500), were visualized as red spots. (**A**), the control groups ("no treatment" and "DMSO 1 %"). (**B**), the groups treated with CYY-X011, 12, and 22; (**C**), the results of CYY-X047, 48, 52, and 55 treatment; (**D**), the observation of CYY-X056, 58, and 60 treatment. Each molecule was used at three different concentrations (100, 20, and 10 μg ml^{-1}). On the lower right corner of individual figures was the thickness of biofilm.

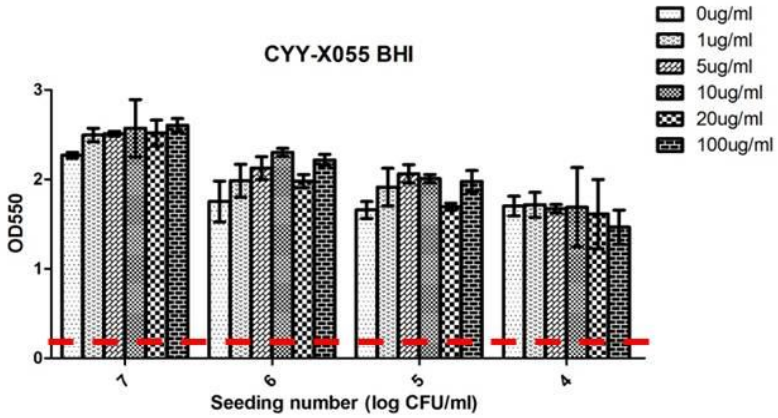
A.


CYY-X047 BHI
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
Seeding number (log CFU/ml)

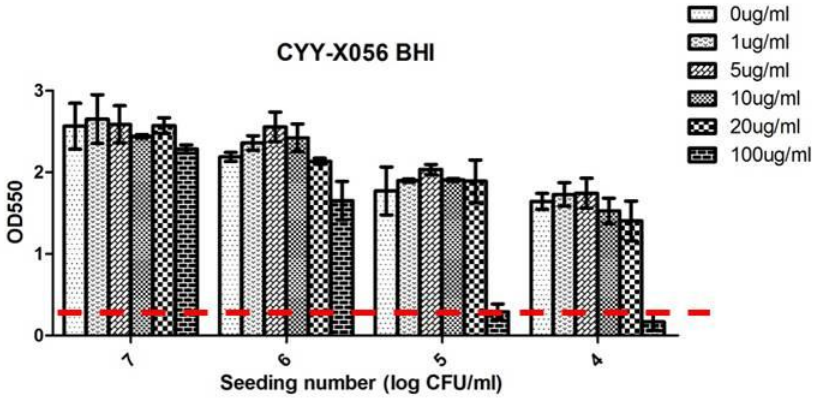
B.


CYY-X052 BHI
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
Seeding number (log CFU/ml)

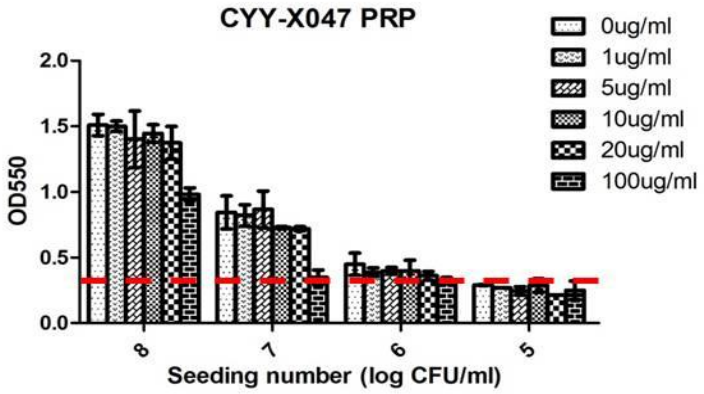
C.


CYY-X055 BHI
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
Seeding number (log CFU/ml)

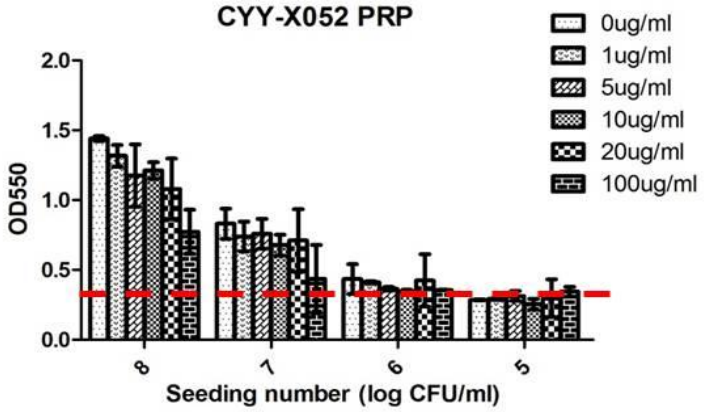
D.


CYY-X056 BHI
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
Seeding number (log CFU/ml)

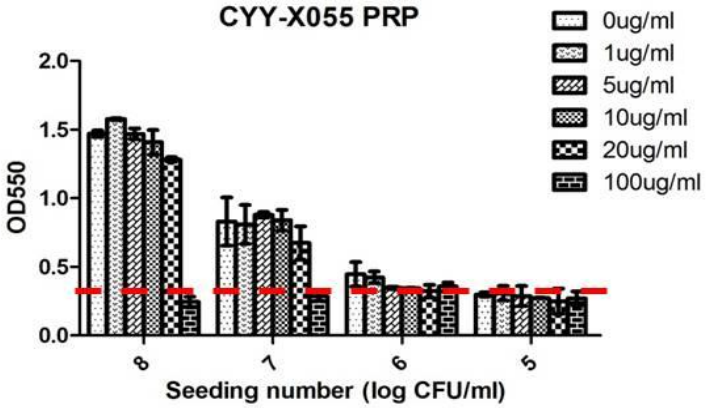
E.


CYY-X047 PRP
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
2.0
1.5
1.0
0.5
0.0
8
7
6
5
Seeding number (log CFU/ml)

F.


CYY-X052 PRP
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
2.0
1.5
1.0
0.5
0.0
8
7
6
5
Seeding number (log CFU/ml)

G.


CYY-X055 PRP
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
2.0
1.5
1.0
0.5
0.0
8
7
6
5
Seeding number (log CFU/ml)

H.

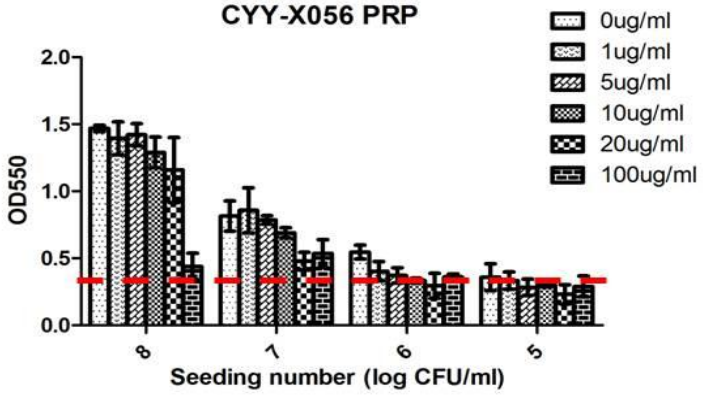

CYY-X056 PRP
0ug/ml
1ug/ml
5ug/ml
10ug/ml
20ug/ml
100ug/ml
OD550
2.0
1.5
1.0
0.5
0.0
8
7
6
5
Seeding number (log CFU/ml)

Fig. 3. The correlation between small molecules concentration and bacterial number in inhibition effect

Biofilms were cultured in BHI (**A**-**D**) and PRP (**E**-**H**) by using 96-well plates and stained with crystal violet. The bacterial inoculation was diluted serially by 10-fold, and the small molecules (CYY-X047, 52, 55, 56) were used at 100, 20, 10, 5, 1 and 0 μg ml^{-1}. Red line: the value of NC. The data were expressed as the mean ± SD.

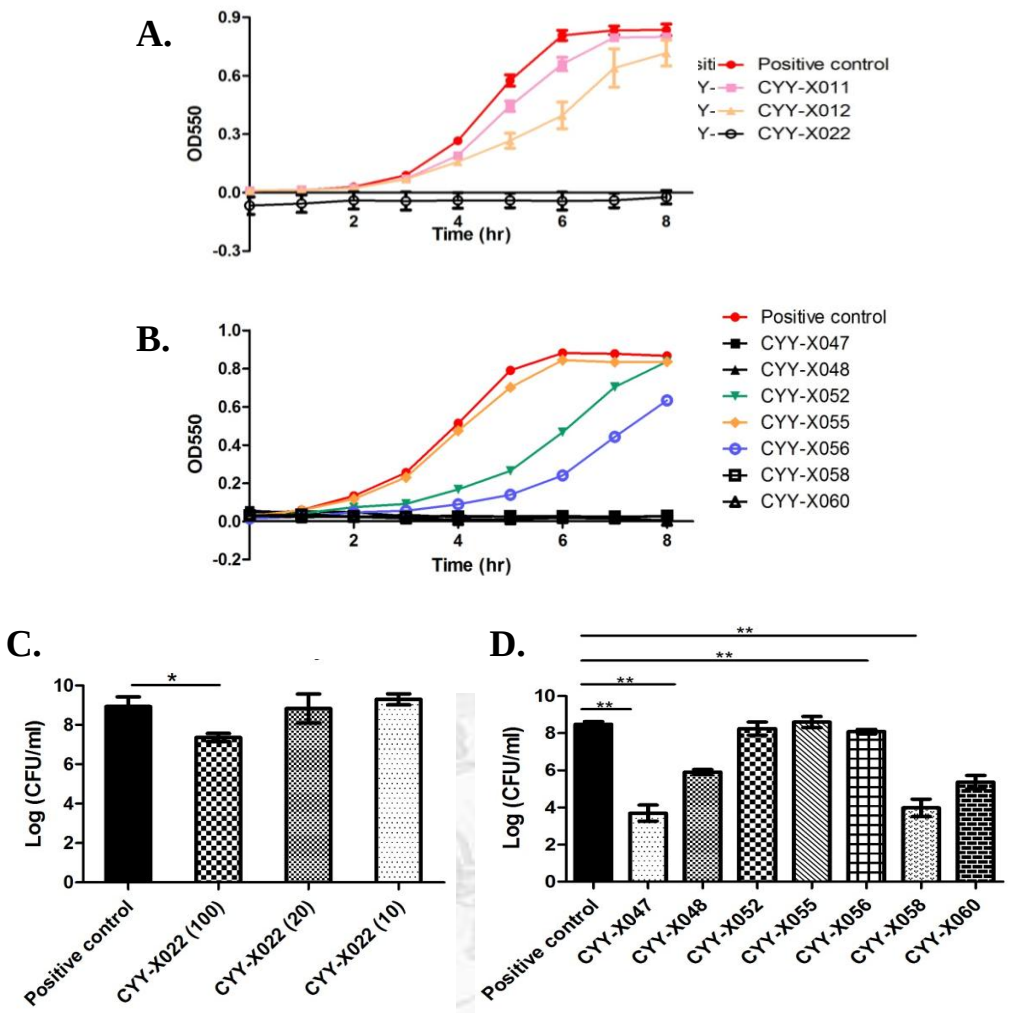
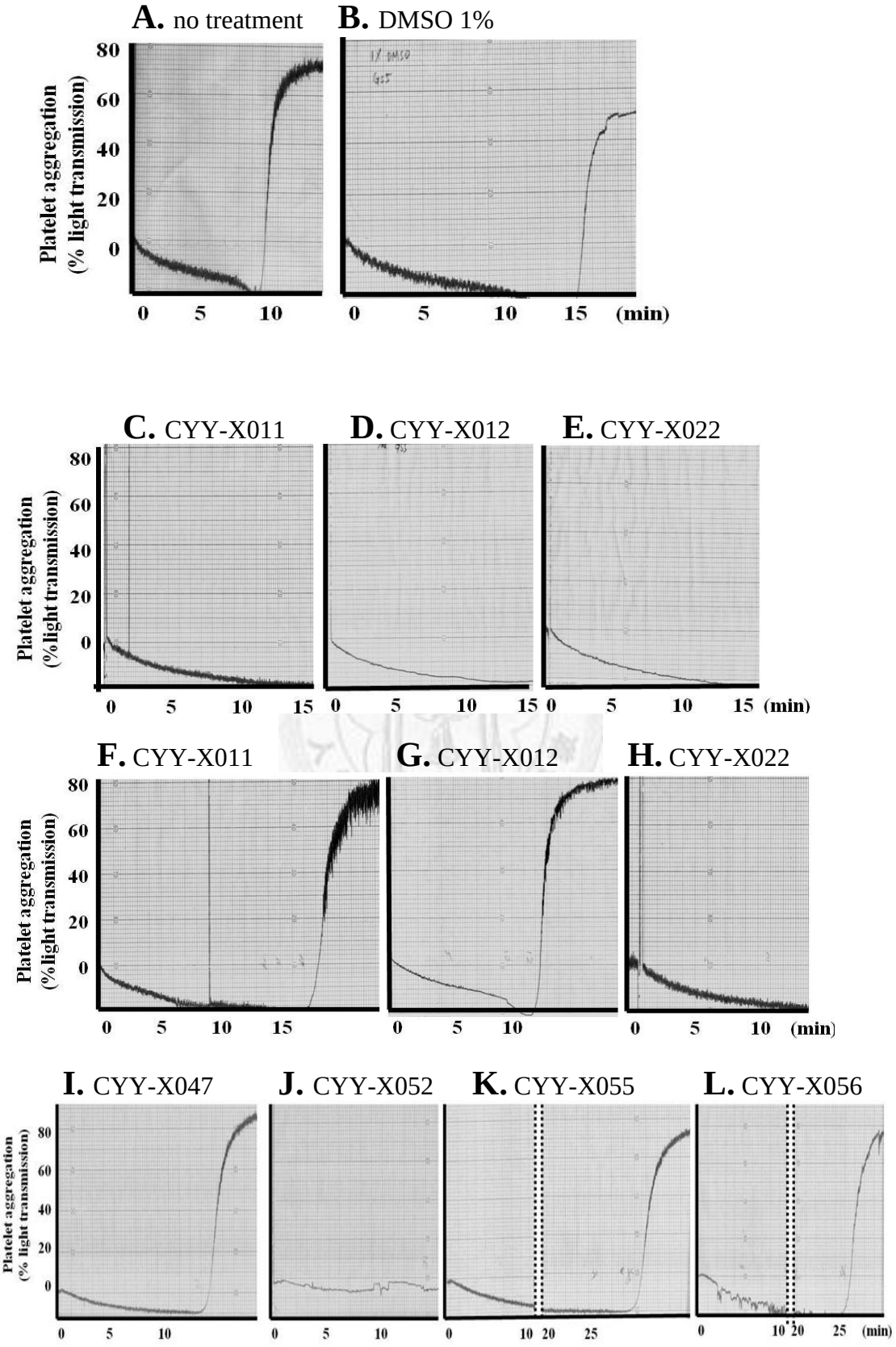


Fig. 4. The effect of targeted small molecules on bacteria growth

Bacteria were inoculated into BHI at 10^7 CFU ml^{-1} and treated with the targeted small molecules at 100μg ml^{-1}. The inoculation was set as the 1st timepoint ("0 hour"), and the absorbance at 550 nm was measured every hour until the 8th timepoint. (**A** and **B**), the growth curves of bacteria treated with the targeted molecules; (**C** and **D**), the results of colony quantification. Positive control: DMSO 1%. The data were expressed as the mean ± SD. Each experiment was triplicate. **P < 0.005, *P < 0.05, Student *t* test.


A. no treatment
B. DMSO 1%
C. CYY-X011
D. CYY-X012
E. CYY-X022
F. CYY-X011
G. CYY-X012
H. CYY-X022
I. CYY-X047
J. CYY-X052
K. CYY-X055
L. CYY-X056
Platelet aggregation (% light transmission)
(min)

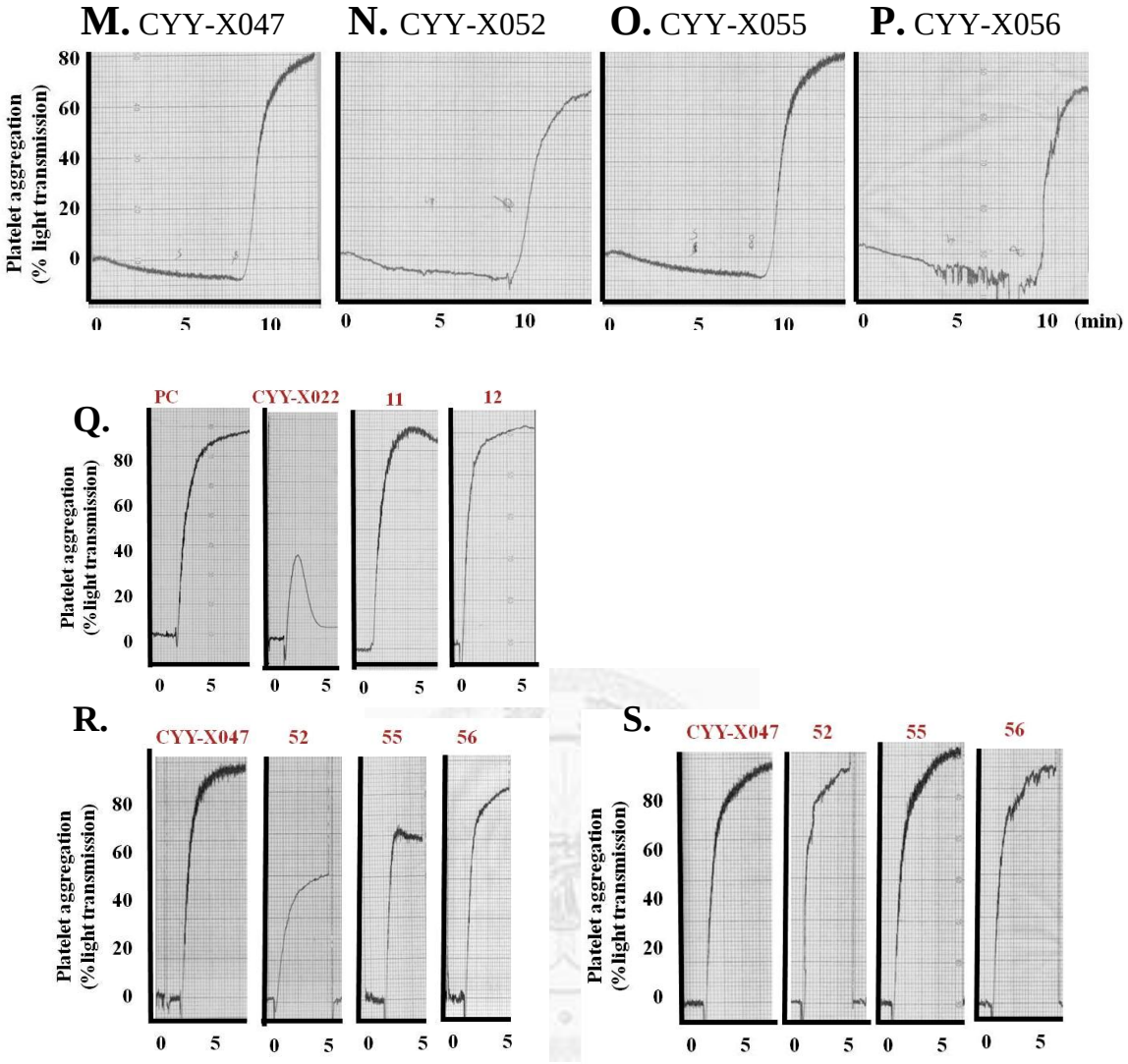


Fig. 5. Inhibition effect of targeted small molecules on bacteria-induced platelet aggregation

Both GS5 strain (10^{10} CFU ml^{-1}) and ADP (10 μM) were used as agonists. PRP was set as 0 % of light transmission and PPP was as 100 %. (**A** and **B**), control groups ("no treatment" and "DMSO 1 %"); PRP would be treated with CYY-X011, 12, 22 (**C**-**E**, 100 μg ml^{-1}**; F**-**H**, 20 μg ml^{-1}), 47, 52, 55 and 56 (**I**-**L**, 100 μg ml^{-1}**; M**-**P**, 20 μg ml^{-1}). ADP stimulation was the control (**Q and R,** 100 μg ml^{-1}**; S,** 20 μg ml^{-1}**)**.

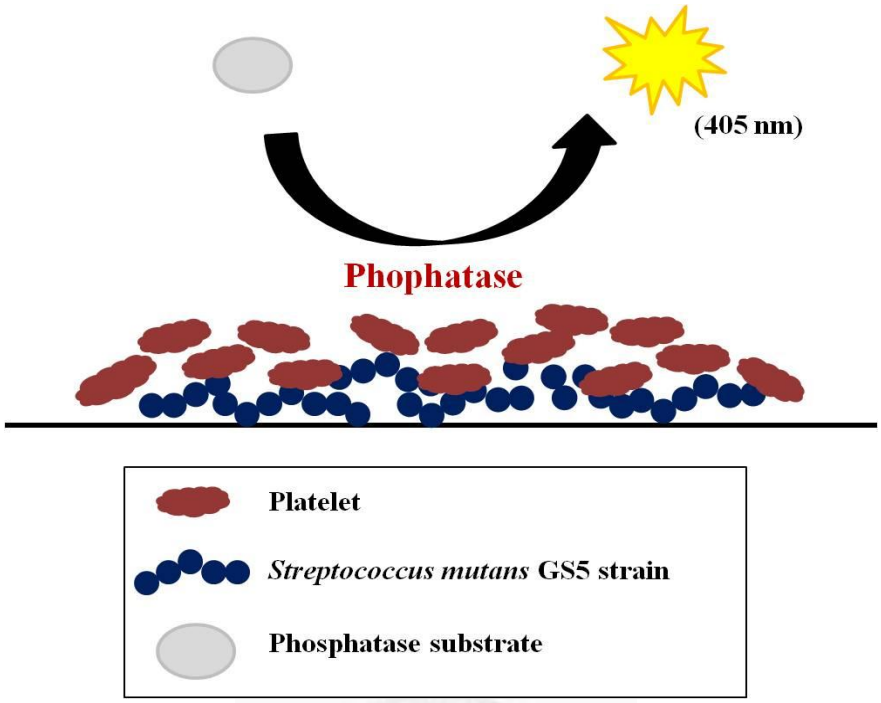
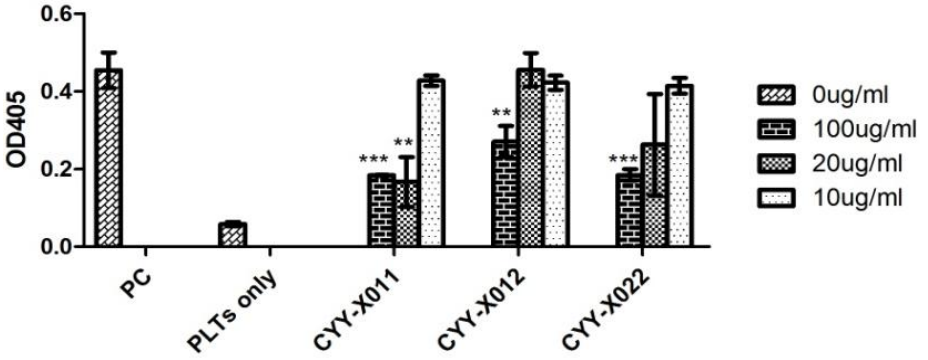


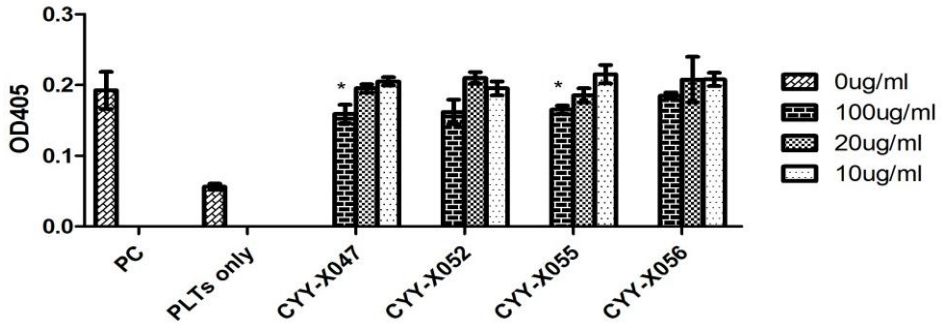
Fig. 6. The diagram of bacteria-platelet interacting assay

When platelets bind to bacteria, phosphatase substrate would be catalyzed by phosphatase, which located inside platelets, and undergo chromogenic reaction. By detecting the absorbance at 405 nm, the inhibition effect on the bacteria-platelet interaction would be quantified.

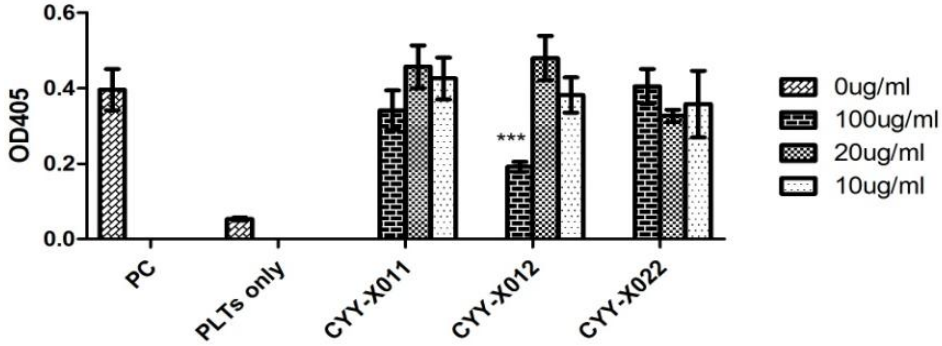
A.


OD405
0.6
0.4
0.2
0.0
0ug/ml
100ug/ml
20ug/ml
10ug/ml
PC
PLTs only
CYY-X011
CYY-X012
CYY-X022

B.


OD405
0.3
0.2
0.1
0.0
0ug/ml
100ug/ml
20ug/ml
10ug/ml
PC
PLTs only
CYY-X047
CYY-X052
CYY-X055
CYY-X056

C.


OD405
0.6
0.4
0.2
0.0
0ug/ml
100ug/ml
20ug/ml
10ug/ml
PC
PLTs only
CYY-X011
CYY-X012
CYY-X022

D.

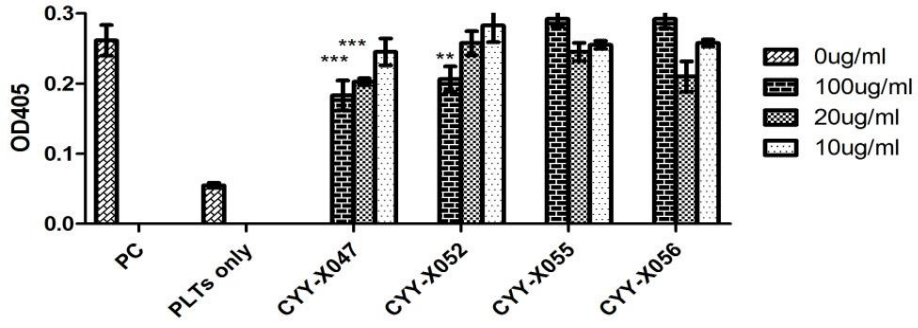
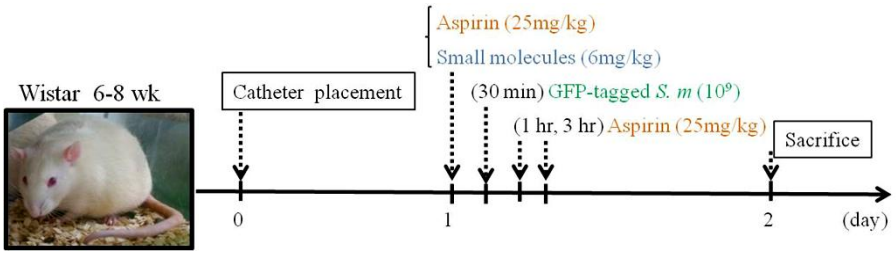

OD405
0.3
0.2
0.1
0.0
0ug/ml
100ug/ml
20ug/ml
10ug/ml
PC
PLTs only
CYY-X047
CYY-X052
CYY-X055
CYY-X056

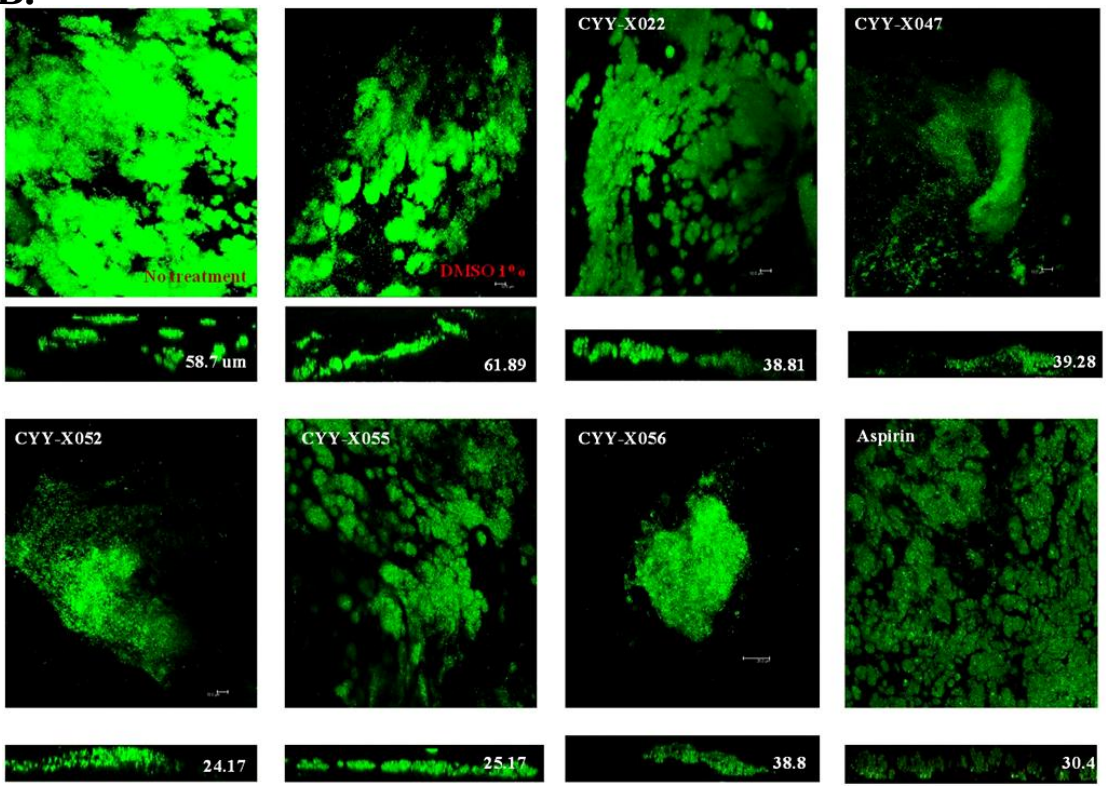
Fig. 7. Inhibition effect of targeted small molecules on bacteria adherence to platelets

Indirect binding of streptococci and platelets was examined in PRP (**A** and **B**), and direct binding was analyzed in PS (**C** and **D**). Test concentrations of molecules were 100, 20, 10 μg ml^{-1}. DMSO 1% was positive control (PC). The data were expressed as the mean ± SD. Each experiment was triplicate. ***P < 0.001, **P < 0.005, *P < 0.05, Student *t* test.

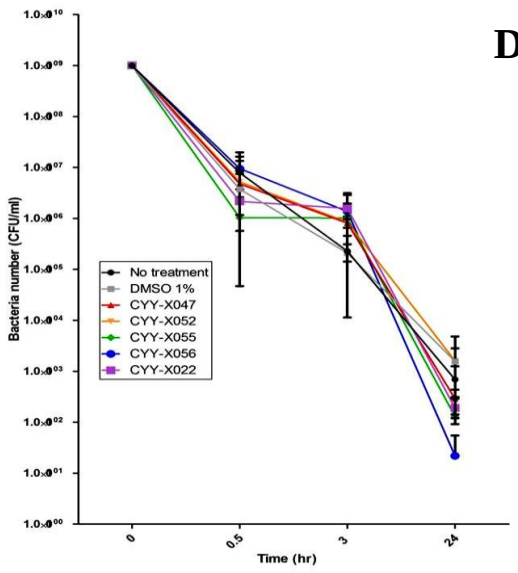
A.


Aspirin (25mg/kg)
Small molecules (6mg/kg)
Wistar 6-8 wk
Catheter placement
(30 min) GFP-tagged S. m (10^9)
(1 hr, 3 hr) Aspirin (25mg/kg)
Sacrifice
0
1
2
(day)

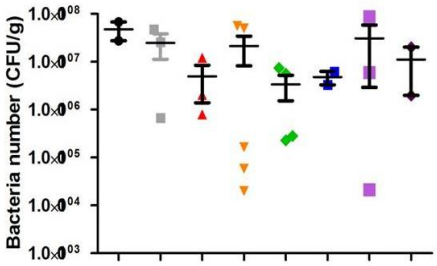
B.


No treatment
DMSO 1%
CYY-X022
CYY-X047
58.7 um
61.89
38.81
39.28
CYY-X052
CYY-X055
CYY-X056
Aspirin
24.17
25.17
38.8
30.4

C.


Bacteria number (CFU/ml)
No treatment
DMSO 1%
CYY-X047
CYY-X052
CYY-X055
CYY-X056
CYY-X022
Time (hr)
0
0.5
3
24

D.


Bacteria number (CFU/g)

E.

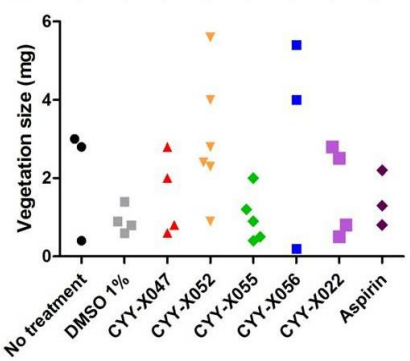
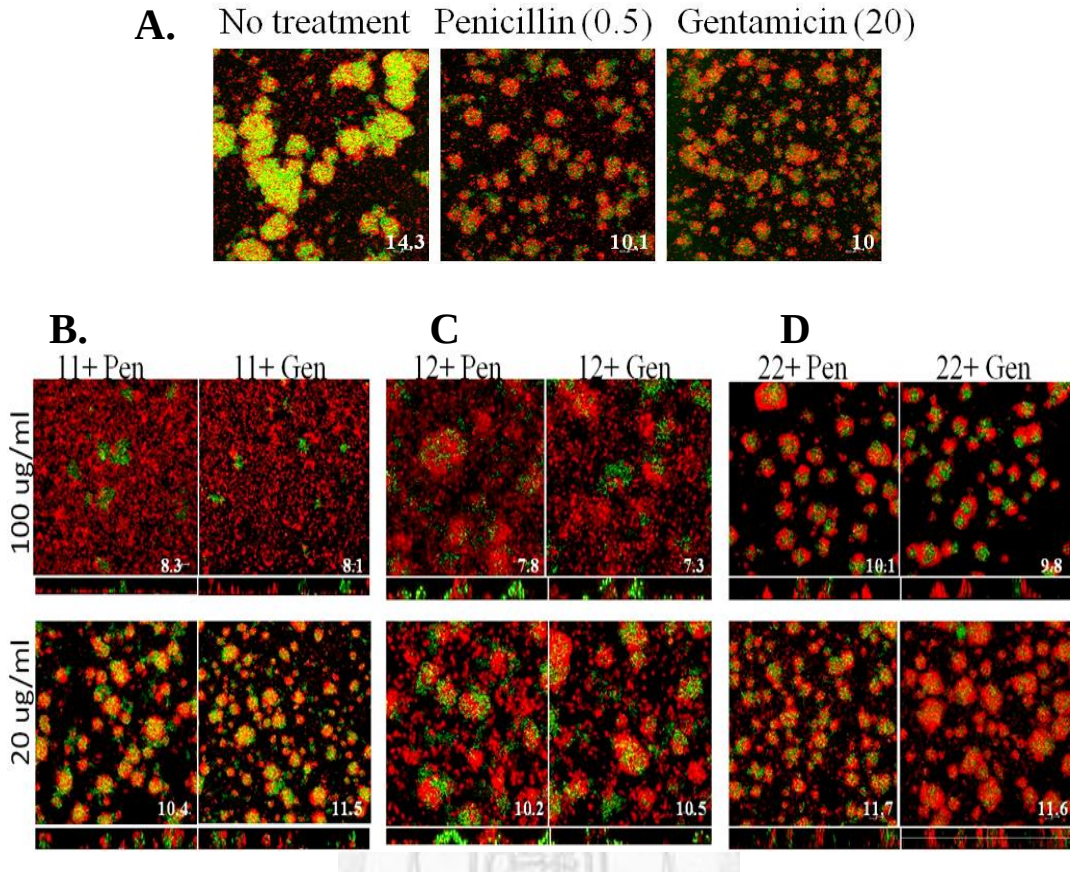
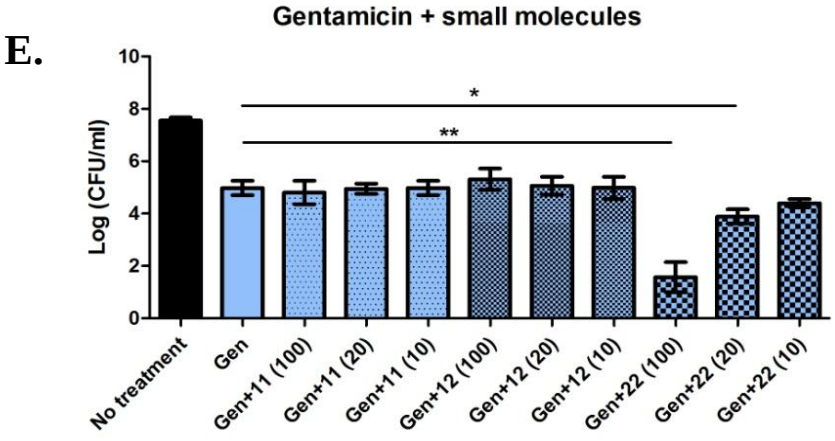

Vegetation size (mg)
No treatment
DMSO 1%
CYY-X047
CYY-X052
CYY-X055
CYY-X056
CYY-X022
Aspirin

Fig. 8. Inhibition effect of targeted small molecules on biofilm formation in rat endocarditis model

CYY-X022, 47, 52, 55 and 56 were administrated by intravenous injection in the experimental streptococcal endocarditis rat model. (**A**), the Wistar rats were infected with GFP-tagged GS5 strain 10^{10} CFU after prophylactic treatment with the targeted molecules and Aspirin. (**B**), the CLSM images (magnification X630) of *in situ* biofilm formation on the valves (the biofilm thickness shown in lower figure in white number); (**C**) the quantification of survival bacteria in circulation; (**D**), the density of colonized bacteria inside the vegetation; (**E**), the biomass of vegetation. The data were expressed as the mean ± SD. Each experiment was repeated triplicate at least.


A.
No treatment
Penicillin (0.5)
Gentamicin (20)
14.3
10.1
10
B.
C
D
11+ Pen
11+ Gen
12+ Pen
12+ Gen
22+ Pen
22+ Gen
100 ug/ml
20 ug/ml
8.3
8.1
7.8
7.3
10.1
9.8
10.4
11.5
10.2
10.5
11.7
11.6


E.
Gentamicin + small molecules
Log (CFU/ml)
*
**
No treatment
Gen
Gen+11 (100)
Gen+11 (20)
Gen+11 (10)
Gen+12 (100)
Gen+12 (20)
Gen+12 (10)
Gen+22 (100)
Gen+22 (20)
Gen+22 (10)

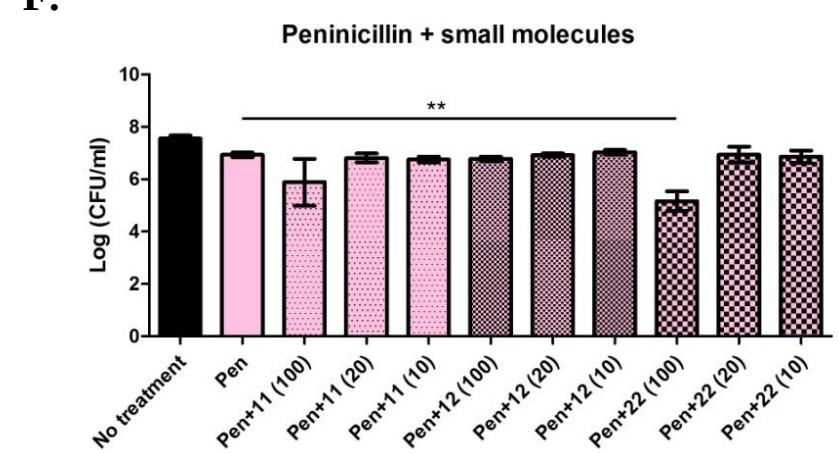
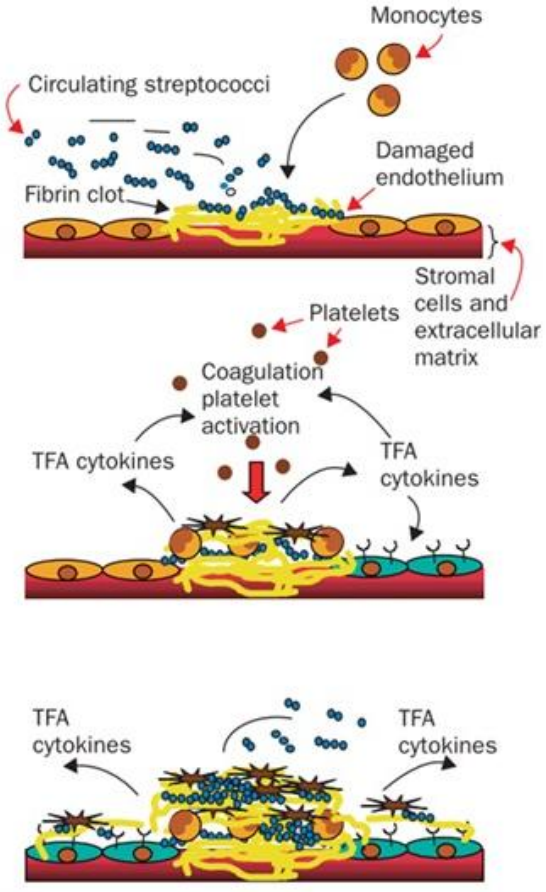

F.
Peninicillin + small molecules
Log (CFU/ml)
**
No treatment
Pen
Pen+11 (100)
Pen+11 (20)
Pen+11 (10)
Pen+12 (100)
Pen+12 (20)
Pen+12 (10)
Pen+22 (100)
Pen+22 (20)
Pen+22 (10)

Fig. 9. Synergistic effect with antibiotics

GFP-tagged bacteria were cultured in PRP after pre-treated with targeted molecules or supplemented with antibiotics. The biofilms were then stained with rhodamine-conjugated phalloidin (1:500 dilutions; red) and observed by using CLSM (magnification X630). (**A-D**), the CLSM images of control groups ("no treatment"," penicillin 0.5 μg ml^{-1}" and "gentamicin 20 μg ml^{-1}") and treatment groups (antibiotics combined with targeted molecules); (**E** and **F**), the quantification of survival bacteria in biofilm. The data were expressed as the mean ± SD. Each experiment was triplicate. **P < 0.005, *P < 0.05, Student *t* test.

Appendix. 1. The pathogenesis of streptococcal-induced infective endocarditis.

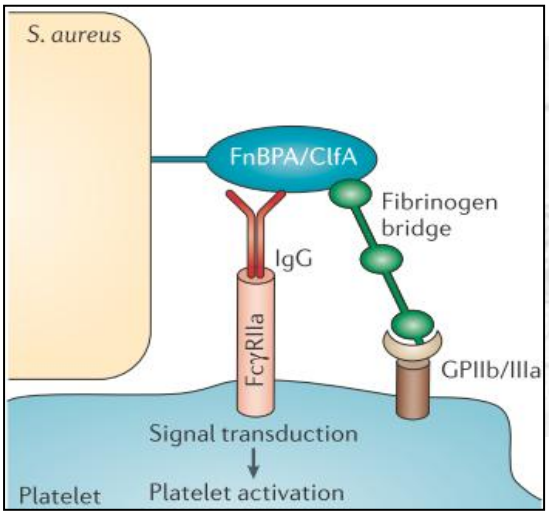


***Lancet* 363, 139-149 (2004)**

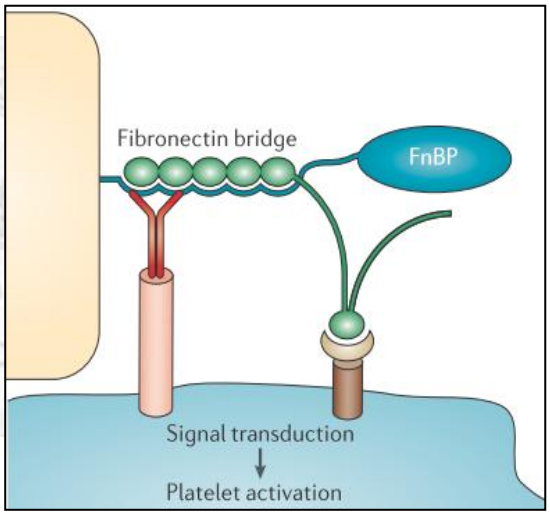
Appendix. 2. The mechanism of *S. aureus*-mediated platelet activation

S. *aureus* could use Fg (or Fn) to bridge between ClfA (or FnBP) and GPIIb/IIIa combined with immunoglobulin binding and induce the signal transduction to stimulate platelets aggregation.

A. Fibrinogen (Fg) bridge



B. Fibronectin (Fn) bridge



***Nature reviews. Microbiology* 4, 445-457 (2006)**

Appendix. 3. The mechanisms of oral bacteria-induced platelet activation

Bacteria	Bacterial protein	Intermediate	Platelet protein
Streptococcus sanguinis	PAAP	—	α2β1
	Unknown	IgG	FcγRIIA
	SrpA	—	GPIb–IX–V
	VWF	—	GPIb–IX–V
	Unknown	C1q	gC1q-R
	ATPase	ADP	$P2Y_1/P2Y_{12}$
Streptococcus gordonii	Hsa and GspB	—	GPIb–IX–V
		IgG	FcγRIIA
	PadA	—	αIIbβ3
Streptococcus mitis	PblA and PblB	—	GPIb–IX–V
Streptococcus mutans	Rhamnose–glucose polymer	IgG	FcγRIIA
	PAc	Unknown	Unknown
Streptococcus agalactiae	Unknown	IgG_2, IgM, and IgA	FcγRIIA
Streptococcus pneumonia	Unknown	IgG	FcγRIIA
Streptococcus pyogenes	Unknown	Unknown	FcγRIIA
Staphylococcus epidermidis	SdrG	Fibrinogen	FcγRIIA and αIIbβ3
Porphyromonas gingivalis	RgpB	—	PAR-1 and PAR-4
	HRgpA	—	PAR-1 and PAR-4

Canadian journal of physiology and pharmacology **88, 510-524 (2010)**

Appendix. 4. The dispersal factors

Signals	Nutrients	Effectors	Physiology cues
AHLs	Nutrients level	EPS-degrading enzymes	D-amino acids
AIP	carbon	chitinase	NO
DSF	nitrogen	nuclease	
AL-2	oxygen	dispersin	
	Iron		

AHLs, acyl-homoserine lactones**; AIP,** autoinducing peptide**; AI-2,** autoinducer 2**; DSF,** diffusible signal factor**; EPS,** extracellular polymeric substances**; NO,** nitric oxide

Nature reviews. Microbiology **10, 39-50 (2012)**