Progress of Antimicrobial Discovery Against the Major Cariogenic Pathogen *Streptococcus mutans*

Tao Cui[1,2]*, Wenfu Luo[1], Letong Xu[1], Baoqiang Yang[1], Wen Zhao[1], Huaixing Cang[2*]

[1]Research & Development Institute of Northwestern Polytechnical University in Shenzhen, Shenzhen 518057, Guangdong, P. R. China.

[2]School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, Shaanxi, P. R. China.

*Corresponding authors: cuitao@nwpu.edu.cn, hxcang@nwpu.edu.cn

Abstract

Dental caries, also known as tooth decay, or cavities, remains a global public health issue. *Streptococcus mutans* is considered the most significant contributor to dental caries. Its cariogenicity typically depends on its unique metabolic activity and lifestyle, including acid production, acid tolerance and biofilm formation. Currently used anti-caries therapies, such as fluoride and chlorhexidine, are characterized by side-effects and drug resistance. Therefore, the development of alternative inhibitors against *S. mutans* growth is urgently needed. In the last decade, a larger number of natural products and their derivatives from plants, marine organisms and microorganisms were studied to evaluate their antibacterial activity against *S. mutans.* In addition,

drug-repositioning base screening and target based high-throughput screening were employed, resulting in inspiring progresses in recent years. In this review, we summarized the available evidences regarding the inhibition of *S. mutans* growth. We focus on the sources, structures and potential mechanism of action of these inhibitors. Beside small molecular compounds, we also considered antibacterial peptides and protein inhibitors developed in this field.

Introduction

Tooth decay, also known as dental caries or cavities, is a significant public health problem worldwide (Pitts *et al*., 2017). Approximately 2.43 billion people (36% of the population) have dental caries. Tooth decay is a result of demineralization of the tooth by acids made by oral microorganisms (Figure 1). Food debris and sugars on the surface of the tooth are metabolized by microorganisms, which produce acids and lead to tooth decay. The oral cavity is inhabited by over 600 different bacterial species (Dewhirst *et al*., 2010; Kilian *et al*., 2016), among which *S. mutans* is the most prosperous colonizer causing dental caries. During carbohydrate metabolism, *S. mutans* produce lactic acid, leading to a drop of pH in the environment, which subsequently inhibits the growth of other microbial competitors, making *S. mutans* a predominant species in the oral cavity.

Unlike other pathogens that live in the blood, cells or tissues that are of difficult access, *S. mutans* lives in the tooth surface, which is easily and mechanically accessible, thus, mechanical approaches for removing *S. mutans* can be considered. However, *S. mutans* can tightly adhere to the tooth surface and its efficient removal is difficult by mechanical method due to the formation of a

biofilm. Dental plaque biofilm is made by a community of oral microorganisms, which adhere to the tooth surface, and *S. mutans* is the most predominant species (Ajdić *et al*., 2002; Mitchell, 2003; Nobbs *et al*., 2009; Flemming *et al*., 2010; Kolenbrander *et al*., 2010; Avilés-Reyes *et al*., 2017).

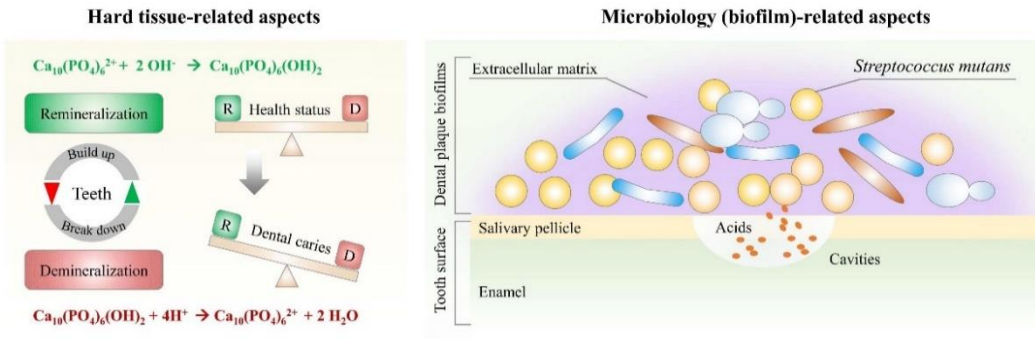

Figure 1. Dental caries and *S. mutans*. Dental caries is a result of tooth demineralization by acids produced by microorganisms in the dental plaque. Dental plaque is a multi-species and cross-kingdom biofilm in which *S. mutants* is the predominant species. $Ca_{10}(PO_4)_6(OH)_2$ is the formula of hydroxyapatite, which is the primary mineral of tooth enamel.

Taken together, at least three indispensable factors play essential roles in the cariogenicity of *S. mutans*. The first one is the acid production. Lactic acid produced by *S. mutans* metabolic activity breakdowns the tooth enamel and causes a pH decline of the surrounding environment, inhibiting the growth of other species and making *S. mutans* the dominant species in the oral cavity. The second one is the acid tolerance (Matsui *et al*., 2010; Smith *et al*., 2012; Baker *et al*., 2017). Acid production results in a drop of pH in the local environment. Therefore, *S. mutans* should be (and it actually is) able to respond and adapt to this acid environment to survive. The third one is the biofilm formation. The formation of a biofilm results in multiple benefits for *S. mutans*.

Indeed, biofilm formation allows *S. mutans* to tightly adhere to the tooth surface, thus avoiding being removed. Moreover, its formation prevents the dilution or/and neutralization of acids produced in the biofilm by water or saliva. Furthermore, biofilm is helpful for adsorbing nutrition. Finally, biofilm formation reduces the access of antibiotics to *S. mutans,* thus increasing antibiotic resistance.

In this review, we summarized the available evidences regarding the inhibition of *S. mutans* growth. We focus on the sources, structures and potential mechanisms of action of these inhibitors. Beside small molecular compounds, we also considered antibacterial peptides and protein inhibitors developed in this field.

1. Small molecules currently used in dental cavity treatment

Currently, widely used anti-caries compounds include fluoride, chlorhexidine, and xylitol (Figure 2). Most toothpastes contain between 0.22% and 0.312% fluoride, usually in the form of sodium fluoride or sodium monofluorophosphate (Buzalaf *et al*., 2011; Pandit *et al*., 2011; Takahashi *et al*., 2011; Liu *et al*., 2012; Nóbrega *et al*., 2016; Demonte *et al*., 2017; Liao *et al*., 2017). Sodium fluoride came into use to prevent tooth decay in the 1940s. The effects of fluoride on dental caries can be divided into the following two: 1. Fluoride reduces tooth enamel decay by the formation of fluorapatite and its incorporation into it; 2. Fluoride exhibits an inhibitory action on *S. mutans* growth. The glycolytic pathway contributes to the acid production by *S. mutans*. Fluoride inhibits the glycolytic pathway by directly targeting the enolase enzyme (Takahashi *et al*., 2011). The accumulation of

internal polysaccharide when sugars are in excess helps *S. mutans* to respond to starvation stress, thus contributing to cariogenicity by *S. mutans*. A recent study showed that sodium monofluorophosphate inhibits internal polysaccharide biosynthesis in *S. mutans* by targeting the *S. mutans* ADP-glucose pyrophosphorylase (Demonte *et al*., 2017). Chlorhexidine is a broad-spectrum antibiotic, came into medical use in the 1950s and it is the most widely used antibacterial agent against *S. mutans*. Chlorhexidine is positively charged in solution and can bind to the bacterial cell wall, damaging its cell membrane (Liu *et al*., 2012; Coelho *et al*., 2017). Xylitol is a sugar alcohol used as a sweetener and it attracted popular attention because evidence suggests that it reduces the incidence of cavities. Several potential mechanisms of action are proposed for its anti-caries activity. Xylitol is non-fermentable and cannot be metabolized into lactic acid by *S. mutans*, as well as it cannot be used as an energy source although being taken up into the cell to interfere with bacterial growth and reproduction (Takahashi *et al*., 2011). Xylitol can promote the remineralization of the tooth enamel by coordinating with and transporting polyvalent cations, such as Ca^{2+}.

Current anti-caries compounds mainly contribute to the prevention and reduction of the incidence of dental caries. However, they have very limited success in the treatment of already formed dental caries. In addition, they also face the challenges of side-effects and drug resistance. These compounds possess low selectivity, which may destroy the homeostasis of oral microbiome. Due to the extensive use of fluoride, many microorganisms developed a resistance against its effects. For example, fluoride antiporter protein mediates microbial fluoride

resistance by exporting fluoride ions (F^-) to maintain a low F^- level in the cell (Liao *et al*., 2017). Therefore, the development of alternative inhibitors against *S. mutans* growth is expected. In this review, we summarized current efforts on the development of inhibitors against *S. mutans* growth.

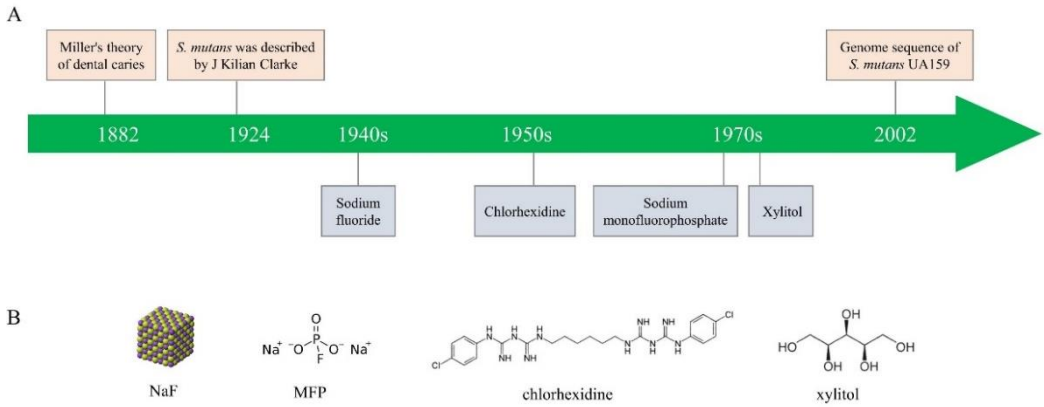

Figure 2. Timeline of our understanding on the pathology of dental caries and development of anti-caries agents. Current treatments for dental caries have limited success and face the problems of having side-effects and developing drug resistance. Alternative antimicrobials against *S. mutans* are required.

2. Natural products from plants

Natural products are rich sources of compounds with potential powers against several diseases, including caries. Plants are the important source of natural products (Koehn *et al*., 2005; Harvey *et al*., 2015). Numerous plant natural products exhibit inhibitory activities against *S. mutans* growth. According to their chemical structures, these compounds mainly include polyphenolic compounds, especially flavonoids (Figure 3). However, their mechanism of action against *S. mutans* survival is not very clear. The potential mechanisms include a specific action on cell membrane and a modification of its permeability, leading to cell death. At the transcriptional level, some compounds can induce the change in

the expression of genes involved in biofilm formation. Some compounds can inhibit enzyme activity involved in acid production pathways. Interestingly, many of these compounds can be found in tea, thus suggesting its potential anti-caries effect (Jeon *et al*., 2011; Cheng *et al*., 2012; Abachi *et al*., 2016).

Earlier studies suggested that flavonoids exhibit inhibitory activities against *S. mutans* growth. Flavonoids have the general structure of a 15-carbon skeleton, which consists of two phenyl rings and heterocyclic ring. This carbon structure can be abbreviated C6-C3-C6. Compounds in this category include apigenin (Koo *et al*., 2003; Koo *et al*., 2005; Jeon *et al*., 2009), myricetin (Falsetta *et al*., 2012), quercetin, kaempferol (Guan *et al*., 2012) and epigallocatechin gallate (EGCG) (Xu *et al*., 2011; Bai *et al*., 2016). Apigenin affects the accumulation and polysaccharide content of *S. mutans* biofilms without major impact on bacterial viability (Koo *et al*., 2003; Koo *et al*., 2005; Jeon *et al*., 2009). Myricetin inhibits the ability of *S. mutans* to assemble biofilms without altering cell viability. It inhibits glucosyltransferase (Gtf) activity and reduces the expression of the *gtfBC* gene cluster, which are involved in the synthesis of glucans (Falsetta *et al*., 2012). Quercetin and kaempferol inhibit the growth of *S. mutans* and several other oral bacteria. In addition, They also significantly inhibit acidogenicity, acidurity and F-ATPase activity of *S. mutans* (Guan *et al*., 2012). EGCG inhibits various cariogenic virulent factors of *S. mutans* at the transcriptional and enzymatic levels, leading to reduced acidogenicity and compromised stress tolerance (especially acid tolerance). Real-time PCR results demonstrated that EGCG significantly suppresses the *ldh*, *eno*, *atpD*, and *aguD* genes, which encode virulence factors that are involved in acid production and stress response of *S. mutans*. Inhibition of the enzymatic activity of F_1F_o-ATPase and lactate dehydrogenase is also observed (Xu et al., 2011).

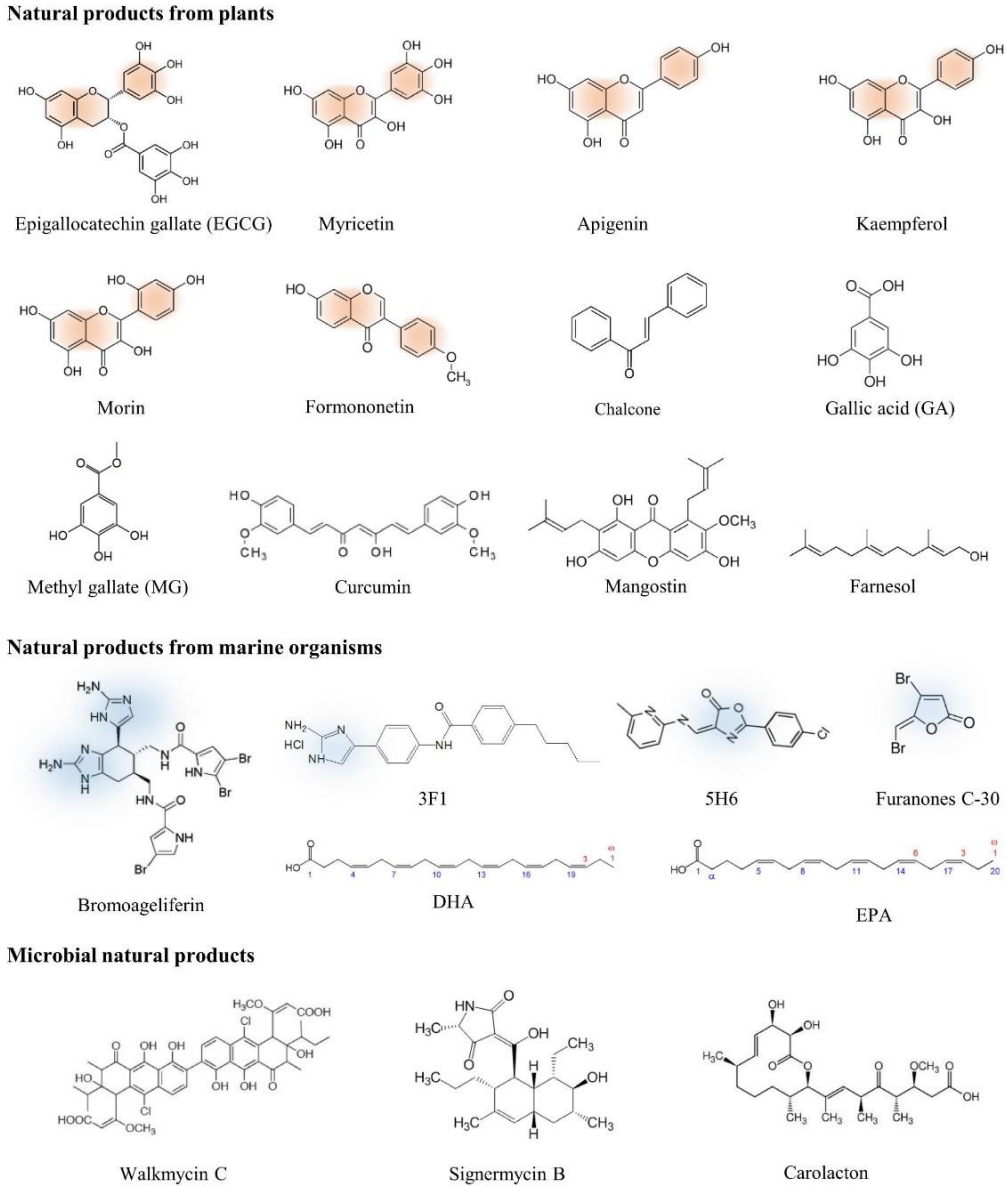

Figure 3. *S. mutans* growth inhibitors from plant natural products, marine natural products and microbial natural products. Natural products from plants mainly include polyphenolic compounds, especially flavonoids (EGCG, myricetin, apigenin, kaempferol, morin, and formononetin). Marine natural products mainly include heterocyclic compounds (imidazole ring in bromoageliferin and 3F1; oxazole ring in 5H6; furanone ring in furanones C-30) and fatty acids (DHA and EPA).

Several flavonoids, such as morin (Huang *et al*., 2014) and formononetin (Park *et al*., 2017) inhibit the activity of Sortase A (SrtA). SrtA is a membrane-associated transpeptidase responsible for the anchoring of surface-exposed proteins to the cell wall envelope of gram-positive bacteria (Hendrickx *et al*., 2011), including *S. mutans*. A mutant *S. mutans* strain lacking *srtA* exhibits a decreased ability to colonize oral mucosa and teeth, consequently reducing caries formation. Therefore, this enzyme represents a potential target in the treatment and/or prevention of dental caries (Park *et al*., 2017). Morin is another natural plant extract that inhibits *S. aureus* and *S. mutans* SrtA activity, the latter inhibited by an IC_{50} of 27.2 ± 2.6 μM. *S. mutans* adheres to the tooth surface through the sortase A (SrtA)-mediated cell wall anchored protein Pac. Western blotting demonstrated that 30 μM morin induces Pac protein partial release into the supernatant (Huang *et al*., 2014). *trans*-chalcone is the precursor molecule of many flavonoids, and it inhibits *S. mutans* SrtA *in vitro* and biofilm formation. Mass spectrometry revealed that *trans*-chalcone forms a Michael addition adduct with the active site on SrtA cysteine (Wallock-Richards *et al*., 2015). Recently, two new flavonoids, 7-hydroxy-6-methoxyflavanone and formononetin, were found as strong inhibitors against *S. mutans* SrtA activity, with IC_{50} values of 46.1 and 41.8 μM, respectively (Park *et al*., 2017).

Other polyphenols with inhibitory activity against *S. mutans* survival include tannic acid (Sendamangalam *et al*., 2011), gallic acid (GA) (Kang *et al*., 2008, Sendamangalam *et al*., 2011; Shao *et al*., 2015) and methyl gallate (MG) (Kang *et al*., 2008; Kacergius *et al*., 2017). Tannic acid inhibits *S. mutans* biofilm

formation. Enzyme activity assay indicated that tannic acid exhibits an inhibitory effect on two enzymes, glucosyltransferase and fructosyltransferase. These two enzymes are involved in the synthesis of glucans and fructans, the important components of extracellular polymers (Sendamangalam *et al*., 2011). Tannic acid can be hydrolyzed into GA. GA can be found in many plants including tea leaves. It has inhibitory effects on the growth of cariogenic and periodontopathic bacteria. Moreover, GA inhibits the *in vitro* formation of *S. mutans* biofilms (Kang *et al*., 2008; Sendamangalam *et al*., 2011; Shao *et al*., 2015). Methyl gallate is the methyl ester of gallic acid. It has antibacterial and antibiofilm effects on *S. mutans*. (Kang *et al*., 2008; Kacergius *et al*., 2017). In addition, MG inhibits the acidogenicity of *S. mutans* (Kacergius *et al*., 2017).

Carvacrol and thymol are natural monoterpene phenols and can be found in the plant *Origanum vulgare* L. Carvacrol and thymol IC_{50} values against *S. mutans* were 65 and 54 μg/ml, respectively. The mechanism of action may be their involvement in the permeabilization and depolarization of the cytoplasmic membrane. Deformed and lysed cells as observed under scanning electron microscope further confirm that thymol and carvacrol treatment results in the lysis of the cells (Khan *et al*., 2017).

Other natural products from plants include curcumin (Hu *et al*., 2013a; Hu *et al*., 2013b), α-mangostin (Nguyen *et al*., 2011; Nguyen *et al*., 2014) and *trans-trans* farnesol (Koo *et al*., 2003; Jeon *et al*., 2011). Curcumin is an inhibitor of *S. aureus* and *S. mutans* SrtA activity (Hu *et al*., 2013a) and it inhibits *S. mutans* biofilm formation (Hu *et al*., 2013b). Curcumin exerts a strong inhibitory activity

against SrtA with a half maximal inhibitory concentration (IC_{50}) of 10.2±0.7 μM. α-mangostin is a xanthone purified from ethanolic extracts of the tropical plant *Garcinia mangostana* L. α-Mangostin is a potent inhibitor of acid production by *S. mutans* UA159. Studies on the mechanism of action suggest that α-mangostin is a multitarget inhibitor. α-Mangostin is active against membrane enzymes, including the F(H+)-ATPase and the phosphoenolpyruvate - sugar phosphotransferase system. It also inhibits the glycolytic enzymes aldolase, glyceraldehyde-3-phosphate dehydrogenase, and lactic dehydrogenase. Glycolysis in intact cells in suspensions or biofilm formation is inhibited by α-mangostin at concentrations of 12 and 120 μM, respectively. Other α-mangostin inhibitory effects include (i) malolactic fermentation, involved in alkali production from malate, and (ii) NADH oxidase, the major respiratory enzyme for *S. mutans* (Nguyen *et al*., 2011; Nguyen *et al*., 2014). *tt*-farnesol is a bioactive sesquiterpene alcohol commonly found in propolis (a beehive product) and citrus fruits. It disrupts the ability of *S. mutans* to form biofilms. In general, *tt*-farnesol significantly increases the membrane proton permeability and reduces the glycolytic activity of *S. mutans* in the planktonic state and in biofilms. It may also affect the competitiveness of *S. mutans* in a mixed-species environment by primarily disrupting the membrane function and physiology of this bacterium (Koo *et al*., 2003; Jeon *et al*., 2011). In addition, *tt*-farnesol inhibits acid production, acid tolerance, and polysaccharide synthesis in *S. mutans* (Falsetta *et al*., 2012).

Crude extracts and essential oils

Plants from traditional medicine, folk medicine, and herbal medicine represent

valuable resources for identifying bioactive compounds. The discovery of artemisinin as anti-malaria drug from traditional Chinese medicine is one of the most successful examples (Tu, 2011). Beside pure compounds, crude extracts and the essential oils from plants are also investigated to identify alternative antimicrobial agents against *S. mutans* (Morimoto-Yamashita *et al*., 2011; Swadas *et al*., 2016; Gulube *et al*., 2016; Yadav *et al*., 2016; Tofiño-Rivera *et al*., 2016; Lall *et al*., 2017; Janardhanan *et al*., 2017; Liu *et al*., 2017; Tardugno *et al*., 2018). The constituents are unknown for most extracts and require further activity-based fractionation to identify the active constituents. It is worthy of attention that extracts from fruit, seed, fruit peel, plant leaves for tea beverages and plants used as condiments in cooking exhibit an inhibitory action against *S. mutans* growth.

3. Natural products from marine organisms

Marine organisms are another important source of natural products (Molinski *et al*., 2009). Many marine natural products possess antibacterial and antibiofilm activity on a wide range of bacteria. For example, marine sponge-derived natural product bromoageliferin is a potent and versatile antibiofilm agent. Therefore, it attracts the interest of researchers in their aim of discovering potential inhibitors against *S. mutants* growth from marine natural products (Figure 3). Current reported marine natural products with antibacterial activity against *S. mutans* include oxazole derivatives (Chen *et al*., 2016), 2-Aminoimidazole (2-AI) derivatives (Rogers *et al*., 2008; Liu *et al*., 2011; Pan *et al*., 2015; Garcia *et al*., 2017), free fatty acids (Huang *et al*., 2010; Sun *et al*., 2017) and furanones (He *et al*., 2012).

Oxazole derivatives

Oxazole and its natural product-related derivatives, especially those from marine environment, possess significant biological activities, including anti-tumor, antibacterial, anti-viral, antimalaria, and anthelmintic activities. Recently, an oxazole derivative, named 5H6, was identified as a new inhibitor against *S. mutans* survival. 5H6 inhibits *S. mutans* biofilm formation and adhesion without affecting bacterial growth. *In vivo* study using a rat caries model showed that 5H6 disrupts the development of smooth-surface caries and sulcal-surface carious lesions to the same extent as treatment with fluoride at 250 μg/mL. A study on the mechanism of action suggested that 5H6 prevents the synthesis of extracellular polysaccharides by antagonizing Gtfs (Chen *et al*., 2016).

2-AI derivatives

As mentioned above, marine sponge–derived bromoageliferin is a broad spectrum antibiofilm agent. The 2-AI functional group plays key roles in its antibiofilm activity. Therefore, many 2-AI derivatives were synthesized to identify more effective and specific biofilm inhibitors. Encouragingly, numerous 2-AI derivatives exhibit inhibitory activity against *S. mutans* biofilm formation (Liu *et al*., 2011; Pan *et al*., 2015; Garcia *et al*., 2017). For example, 2-AI derivative 3F1 disperses mature *S. mutans* biofilm. However, 3F1 do not disperse biofilms formed by the commensal species *Streptococcus sanguinis* or *Streptococcus gordonii*. In addition, 3F1 treatment effectively prevents dental caries by controlling *S. mutans* in a rat caries model without perturbing the oral microbiota. These results suggest the specificity of 3F1 against *S. mutans* survival. The

mechanism of 3F1 action is not dependent on well-characterized biofilm factors, including AgI/II, Gtfs, glucans, and eDNA, since they are often associated with the initial development of *S. mutans* biofilm, while proteins and factors involved in biofilm maintenance and maturation are largely unknown. Thus, 3F1 may represent a target as a novel biofilm-related protein potentially inhibiting a crucial interaction between *S. mutans* and the biofilm matrix *in vitro* (Garcia *et al*., 2017).

Free fatty acids

Unsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) display antibacterial effects on mature *S. mutans* biofilm (Huang *et al*., 2010; Sun *et al*., 2017). Free fatty acids are found naturally at high levels in many marine organisms. DHA and EPA possess highly inhibitory effects on various Gram-positive and Gram-negative bacteria survival. DHA and EPA mainly affect the integrity of the bacterial plasma membrane, thereby leading to bacteria damage and death. In addition, DHA or EPA may target extracellular polysaccharides (EPS) synthesis, since an expression profile showed the downregulation of *gtfB* and *ftf* gene, which encode enzymes that catalyze the cleavage of sucrose to synthesize extracellular glucan and fructan polysaccharides, facilitating bacteria adherence and aggregation.

Furanones

Furanones are natural inhibitors of quorum sensing (QS) signaling system, which regulate biofilm formation in many species. The red marine alga *D. pulchra* developed a defense mechanism to protect itself from extensive bacterial colonization by producing brominated furanones as inhibitors. Synthetic

furanone C-30 inhibits *S. mutans* biofilm formation. The mechanism of action of furanones is that these molecules, whose structures are similar to autoinducers of QS signaling system, can bind to QS response regulators but fail to activate them, thus inhibiting the QS system (He *et al.*, 2012).

4. Microbial natural products

Microorganisms usually secrete molecules to antagonize competitors. Therefore, many antibiotics were and are currently isolated from microbial natural products. Several microbial natural products are inhibitors of *S. mutans* survival (Figure 3). These compounds include walkmycin C (Eguchi *et al*., 2011), signermycin B (Watanabe *et al*., 2012), carolacton (Kunze *et al*., 2010; Reck *et al*., 2011; Li *et al*., 2013; Sudhakar *et al*., 2014) and vizantin (Takenaka *et al*., 2016).

The distinguishing feature of these compounds is that they target signaling system of *S. mutans*. Walkmycin C inhibited the *in vitro* autophosphorylation activity of three purified *S. mutans* histidine kinases, including VicK, CiaH, and LiaS. Walkmycin C treatment represses biofilm formation, acid tolerance, and competence of *S. mutans* (Eguchi *et al*., 2011). Signermycin B is identified as an inhibitor of histidine kinase WalK. It targets the conserved dimerization domain of WalK to inhibit autophosphorylation (Watanabe *et al*., 2012).

Carolacton is a secondary metabolite isolated from *Sorangium cellulosum*. It has high activity against *S. mutans* biofilm formation. At a concentration of 10 nM carolacton, biofilm damage is already at 35% under anaerobic conditions. Planktonic growth of bacteria is only slightly impaired and no acute cytotoxicity

against mouse fibroblasts is observed (Kunze *et al*., 2010). A knock-out mutant for *comD*, encoding a histidine kinase specific for the competence stimulating peptide (CSP) is slightly less sensitive to carolacton than the wildtype (Kunze *et al*., 2010). Competence related alternate sigma factor ComX expression is strongly reduced by carolacton. A comparative time series microarray analysis identified VicKRX and ComDE as two-component signal transduction system and genes involved in cell wall metabolism, playing essential roles in the response to carolacton treatment. A sensitivity testing of mutants with deletion of all the 13 viable histidine kinases and the serine/threonine protein kinase PknB of *S. mutans* identified only the ΔpknB deletion mutant as being insensitive to carolacton treatment. A strong overlap between the regulon of PknB in *S. mutans* and the genes affected by carolacton treatment was found. The results suggested that carolacton acts by interfering with PknB-mediated signaling in growing cells. The resulting altered cell wall morphology causes membrane damage and cell death at low pH (Reck *et al*., 2011). Comparative proteome analysis identified 192 proteins whose expression is affected by carolacton. Function analysis of these proteins indicated that carolacton exerts its inhibitory effects by disturbing peptidoglycan biosynthesis and degradation, thereby causing damages to the integrity of the cell envelope, leading ultimately to cell death (Li *et al*., 2013). A regulatory network analysis identified several regulators involved in the response of *S. mutans* to carolacton (Sudhakar *et al*., 2014). It was recently found that carolacton targets FolD, a key enzyme from the folate-dependent C1 metabolism in *S. pneumonia* (Fu *et al*., 2017).

Vizantin is a molecule derived from trehalose-6,6′-dimycolate, a major

constituent of the outer surface membrane of the human pathogen *Mycobacterium tuberculosis*. Vizantin was originally developed as a safe immune-stimulating compound (Oda *et al*., 2014). Recently, vizantin has also been found to have antibiofilm activity against *S. mutans*. Notably, supplementation with sulfated vizantin up to 50 μM do not affect either bacterial growth or biofilm formation, whereas 50 μM sulfated vizantin causes the detachment of the biofilm from the surface. Sulfated vizantin at the concentration of 50 μM upregulates *gtfB* and *gtfC* gene expression, but downregulates *gtfD* gene expression, suggesting an altered architecture in the biofilm (Takenaka *et al*., 2016).

5. Drug repositioning

Drug repositioning is a drug development strategy that tries to find new applications of existing drugs (Ashburn and Thor, 2004). For example, a recent study found the anti-cancer activity of the alcohol-abuse drug disulfiram (Skrott *et al*., 2017). Drug repositioning strategy has been used to investigate inhibitors against *S. mutans* growth in recent years (Figure 4). Several small-scale drug-repositioning studies identified trimetrexate analogues (Zhang *et al*., 2015), saxagliptin (De *et al*., 2016), zafirlukast (Gerits *et al*., 2017), clotrimazole, econazole (Qiu *et al*., 2017) and reserpine (Zeng *et al*., 2017) as inhibitors against *S. mutans* growth. Particularly, a latest high-throughput drug-repositioning screening of 853 FDA-approved drugs identified 126 drugs with inhibitory activity against planktonic *S. mutans* growth. These drugs were further tested for activity against *S. mutans* biofilm and the results showed that 24 drugs inhibit biofilm formation, 6 drugs kill preexisting biofilms, 84 exhibit both

biofilm inhibition and killing activity, and 12 has no activity against biofilms (Saputo *et al*., 2017a).

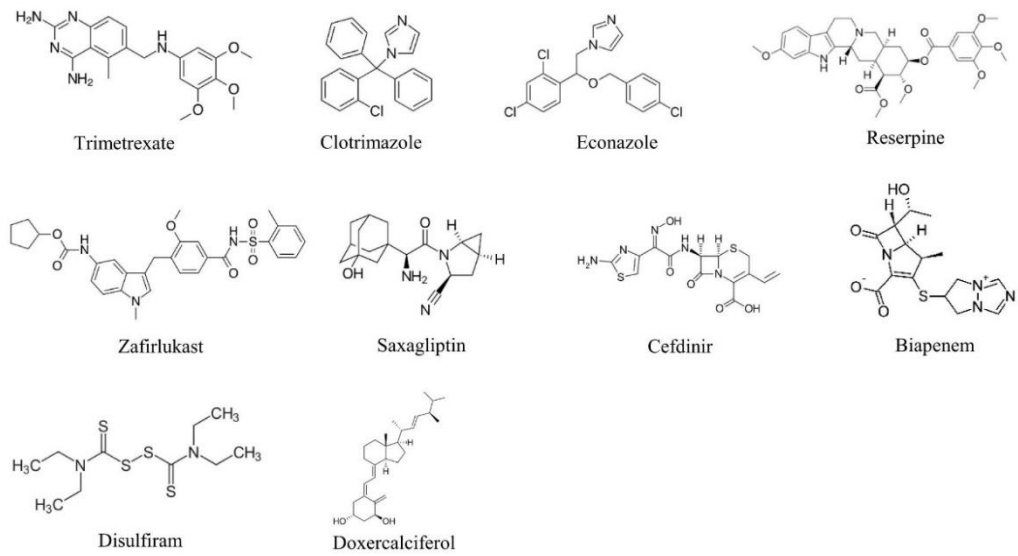

Figure 4. Examples of *S. mutans* growth inhibitors from drug-repositioning studies.

Trimetrexate analogs

Trimetrexate is a quinazoline derivative and targets human dihydrofolate reductase (DHFR). A library of analogs based on trimetrexate was screened and three new analogs that selectively inhibits *S. mutans* survival were identified. The most potent inhibitor has an IC_{50} of 454.0±10.2 nM related to the biofilm formation and 8.7±1.9 nM associated to DHFR activity in *S. mutans*. In contrast, the IC_{50} of this compound for human DHFR is approximately 1000 nM, a >100-fold decreased potency, demonstrating the high selectivity of the analog. An analog that exhibited the least potency against *S. mutans* biofilm formation has also the lowest activity towards *S. mutans* DHFR inhibition, further indicating that inhibition of biofilms is related to a reduced DHFR activity (Zhang *et al*., 2005).

Saxagliptin

Saxagliptin is used in the treatment of type 2 diabetes. It targets human dipeptidyl peptidase-4 (DPP-4). *S. mutans* encodes an intracellular Xaa-Pro dipeptidyl aminopeptidase (XPDAP) which is similar to the human DPP-4 enzyme. XPDAP is a narrow-range serine protease, which cleaves oligopeptides with a proline as the penultimate residue from the N-terminus. Several studies have suggested importance of XPDAP in utilization of proline-rich salivary peptides as well as virulence in *S. mutans* (De *et al*., 2016). The inhibition constants (K_i) of anti-human DPP-4 drugs sitagliptin, vildagliptin, and saxagliptin against *S. mutans* XPDAP activity were determined, showing that saxagliptin inhibits the activity of *S. mutans* XPDAP (K_i = 129 ± 16 μM) (De *et al*., 2016).

Zafirlukast

Zafirlukast is used for the chronic treatment of asthma. It targets human leukotriene receptor antagonist (LTRA). Zafirlukast inhibits *S. mutans* growth (MIC: 25 μM), biofilm formation (MBIC: 50 μM) and reduces preformed biofilm (MBRC: 12.5 μM). In addition, zafirlukast exerts no toxicity on human osteoblasts (Gerits *et al*., 2017).

Clotrimazole and Econazole

Dental plaque is not only a multi-species biofilm but also a cross-kingdom biofilm. For example, *C. albicans*, a fungal microorganism, is frequently detected with heavy infection by *S. mutans* in plaque-biofilms from children with

early-childhood caries. Furthermore, several recent studies suggested the synergistic effects between *S. mutans* and *C. albicans* on biofilm formation (Hwang *et al*., 2017; Ellepola *et al*., 2017). Therefore, compounds with inhibitory activity against both *S. mutans* and *C. albicans* survival may have more effective anti-caries activity. Based on this hypothesis, inhibitory effects of eight antifungal drugs on *S. mutans* survival were determined in a recent study. Two antifungal drugs, clotrimazole and econazole show an antibacterial activity on *S. mutans* UA159 and *S. mutans* clinical isolates at 12.5 and 25 mg/L, respectively. Clotrimazole and econazole can also inhibit *S. mutans* biofilm formation and reduce the viability of preformed biofilm. In addition, they inhibit pH drop, lactate acid production and acid tolerance in *S. mutans*. The antifungal effects of clotrimazole and econazole are similar. They kill individual fungal cells by altering the permeability of their cell wall. They bind to phospholipids in the cell membrane and inhibit the biosynthesis of ergosterol and other sterols required for cell membrane formation, leading to cell death via loss of intracellular elements. As regard *S. mutans*, clotrimazole and econazole inhibit the expression of *S. mutans ldh* gene, which is involved in the acid production. However, their specific targets in *S. mutans* and their mechanism of action underlining multiple inhibitory activities on *S. mutans* are not very clear and require further analysis. Anyhow, this study provided a starting point in the development of inhibitors that target two relevant species that contribute in the formation of dental plaque biofilm (Qiu *et al*., 2017).

Reserpine

Bacterial efflux pumps are membrane proteins that can extrude substrates out of

the cells, including drugs, antibiotics, chemicals and signal molecules. Efflux pumps play important roles in bacterial pathogenicity, antibiotic resistance and biofilm formation. Two predicted ABC efflux pumps encoded by *rcrP* and *rcrQ* are associated with cellular physiology, genetic competence, and acid and oxidative stress tolerance of *S. mutans*. Therefore, inhibiting the efflux pump with its inhibitors may suppress the virulence properties of *S. mutans*. Inhibitory activities of several efflux pump inhibitors, including reserpine, verapamil hydrochloride, thioridazine hydrochloride and sodium orthovanadate against *S. mutans* growth were tested. Results indicated that reserpine suppresses acid tolerance, mutacin production and transformation efficiency in *S. mutans*, and modifies biofilm architecture and extracellular polysaccharide distribution. Studies on the mechanism of action suggested that reserpine inhibits glycosyltransferase activity in *S. mutans*. Results from quantitative real-time PCR demonstrated that reserpine significantly alters QS expression profile and virulence-associated genes (Zeng *et al*., 2017)

Doxercalciferol

Bacitracin is a polypeptide-type drug that interferes with cell wall synthesis. Innate resistance to bacitracin is attributed to efflux by a conserved ABC-type transporter, which is encoded by the mbrABCD operon in *S. mutans*. Doxercalciferol is a vitamin D drug derivative that exhibits direct bactericidal activity against the cariogenic *S. mutans*. In addition, through the use of a combinatorial approach, doxercalciferol was shown to act synergistically with bacitracin, suggesting that doxercalciferol may act in a bacitracin-related pathway (Saputo *et al*., 2017b).

6. Target-based screening

Target-based screening strategy firstly identifies essential proteins involved in the pathogenicity of bacteria. Secondly, the inhibitory activity of compounds against target proteins was tested by an enzyme activity experiment or another protein function experiment. If the three-dimensional structure of the target protein is available, a structure-based screening is usually performed before the experimental test. Afterwards, compounds with activity on target proteins are evaluated by phenotype assays (Terstappen *et al*., 2007; Swinney and Anthony, 2011; Eder *et al*., 2014). One advantage of target-based screening is that the screening can be easily performed in a high-throughput manner. In addition, target proteins selection provides the opportunity for selecting unique proteins of pathogens as candidate targets for screening, which is important for the discovery of inhibitors with high specificity. This is particularly important for inhibitory screening against *S. mutans*. The expected ideal inhibitor needs to be antibacterial to *S. mutans* but without destroying of oral microbiome. Two proteins, GtfC (Ren *et al*., 2015; Ren *et al*., 2016; Zhang *et al*., 2017) and QS signaling protein ComA (Ishii *et al*., 2017) have been used in target-based inhibitor screening against *S. mutans*.

Glucosyltransferase

Biofilm formation in *S. mutans* is promoted by major virulence factors known as Gtfs, which synthesize adhesive EPS. A structure-based virtual screening of approximately 150,000 commercially available compounds was performed against the crystal structure of the glucosyltransferase domain of the GtfC

protein from *S. mutans*. Then, 51 chemically diverse lead compounds were further screened for their inhibitory activities on EPS synthesis *in vitro*, resulting in the identification of a quinoxaline derivative as a potential Gtf inhibitor. *In vitro* assays showed that this compound is capable of inhibiting EPS synthesis and biofilm formation in *S. mutans* by selectively antagonizing Gtfs instead of killing the bacteria directly. Moreover, the *in vivo* anti-caries efficacy of the compound was evaluated in a rat model. The results showed that the compound significantly reduces the incidence and severity of smooth and sulcal surface caries *in vivo* with a concomitant reduction in the percentage of *S. mutans* in the animals' dental plaque (Ren *et al*., 2016). In another study, a small molecule library of 500,000 small molecule compounds was screened *in silico* against the GtfC catalytic domain. A total of 90 compounds with diverse scaffolds which vary in their functional groups, hydrophobicity, and H-bond accepting/donating capacity were then purchased and subjected to *in vitro* biofilm assays using *S. mutans*. Seven potent low micromolar inhibitors were identified. Two of these compounds were the most potent, as they inhibit more than 85% of *S. mutans* biofilms at 12.5 μM. Compounds #G16 and #G43 share several functional groups including a nitro group, heterocyclic rings, and a polar carbonyl functional property (Zhang *et al*., 2017).

S. mutans expresses 3 genetically distinct Gtfs, including GtfB, GtfC, and GtfD. Recently, it was found that GtfB plays key roles in cross-kingdom biofilm formation of *S. mutans* and *C. albicans* (Hwang *et al*., 2017; Ellepola *et al*., 2017). This property makes GtfB a potential candidate target for a future inhibitor screening.

QS signaling system protein ComA

The QS system is a bacterial cell-cell signal communication system mediated by an inherent signal molecule called autoinducer. The ComABCDE pathway is a QS system in *S. mutans*. Autoinducer peptides are processed from the precursor ComC and concomitantly exported to the extracellular space by ComA and ComB. The accumulated autoinducers bind to the membrane-bound receptor kinase ComD, which subsequently phosphorylates the response regulator ComE to activate the transcription of a specific set of genes, such as those essential for the competence development and biofilm formation in *S. mutans*. ComA is a bi-functional ATP-binding cassette (ABC) transporter that comprises three domains: a N-terminal peptidase domain (PEP), a transmembrane domain, and a C-terminal nucleotide-binding domain. A high-throughput screening system using a fluorescence-labeled substrate was performed to identify inhibitors of PEP in *S. mutans*. The first screening of 164,514 compounds yielded 951 hits (0.58%) that inhibits PEP in *S. mutans* activity by >50% at a compound concentration of 20 μM. These compounds were further subjected to screening that examined inhibition against *S. mutans* biofilm formation. Finally, six compounds were found to inhibit biofilm formation without inhibiting cell growth. Two of the six compounds are quinuclidine derivatives, and the other four compounds have no primary chemical structure in common (Ishii *et al*., 2017).

7. Antibacterial peptides and proteins

Besides small molecule inhibitors, antibacterial peptides were also investigated for its activity against *S. mutans* survival. Compared to small molecule antibiotics,

the advantages of antibacterial peptides include broad-spectrum antimicrobial activity, low toxicity to mammalian cell and less selection stress. Less selection stress decreases the risk of antibiotic-resistant bacteria development, which is one of the biggest challenges in the treatment of bacterial infection. In addition, antibacterial peptides are encoded by genes and thus they can be easily modified with genetic engineering techniques to obtain more derivatives compared to small molecule derivatives obtained by chemical synthesis. Several antibacterial peptides, such as nisin (Tong *et al*, 2010; Yamakami *et al*., 2013), IMB-2 (Mai *et al*., 2011), decapeptide KSL (Liu *et al*., 2011), cyclic lipopeptide 4 (CLP-4) (Min *et al*., 2017) and lipopeptide C14KKc12K (Meir *et al*., 2017), possess antimicrobial activity against *S. mutans*.

As we mentioned above, antimicrobial peptides are usually broad-spectrum. The use of antimicrobial peptides in the oral cavity may disturb the oral microbiome and cause unexpected problems. Therefore, it is better to improve the specificity of antimicrobial peptides against *S. mutans*. The development of the antimicrobial peptide IMB-2 is an enlightening work for this purpose. IMB-2 is modified from a marine-derived, broad-spectrum antimicrobial peptide pleurocidin. ComC signaling peptide pheromone (CSP) can bind to *S. mutans* ComC. The foundation for creating IMB-2 is based on the addition of a targeting domain of *S. mutans* ComC CSP to a killing domain of pleurocidin to generate a target-specific antimicrobial peptide. The results showed that nearly 95% of *S. mutans* bacteria lost their viability following exposure to fusion peptide IMB-2 (5.65 μM) for 15 min. In contrast, only 20% of *S. sanguinis* or *S. gordonii* bacteria were killed following the same exposure. A *S. mutans* mutant defective in the

CSP receptor resulted in 60% survival following exposure to IMB-2, suggesting that the targeted peptide predominantly bound to the CSP receptor to mediate the killing of the wild-type strain (Mai *et al*., 2011).

The stability of the antimicrobial peptide in oral environment is another potential problem for antimicrobial peptide inhibitors against *S. mutans***.** Nisin, produced by *Lactococcus lactis*, is an antibiotic peptide to effectively antagonize a broad spectrum of Gram-positive bacteria. Nisin targets the membrane-bound peptidoglycan precursor lipid II to inhibit peptidoglycan biosynthesis, leading to membrane pore formation. Nisin displays an antimicrobial activity against multiple oral microorganisms including *S. mutans* (Tong *et al*. 2010). The naked form of nisin is sensitive to proteolytic degradation and oxidation. To improve its stability, an encapsulated form of nisin within liposomes has been developed. The concentration of nisin-liposome required for an efficacious inhibition of glucan-biofilm synthesis is four times lower than that of naked nisin (Yamakami *et al*., 2013).

Recently, two studies also tried to develop protein inhibitors against *S. mutans* (Yang *et al*., 2016; Ito *et al*., 2017). Bacteriophage-encoded lysins are peptidoglycan hydrolases that digest the bacterial cell wall, leading to the rapid lysis and death of bacteria. ClyR, an engineered lysin, is active against all clinical *S. mutans* isolates tested and *S. mutans* biofilms (Yang *et al*., 2016). Another protein inhibitor against *S. mutans* is agaricus bisporus agglutinin (ABA), which belongs to lectin. The mechanism of action of ABA is largely different from the one of ClyR. Oral bacteria initiate biofilm formation by adhering to the tooth

surface through an interaction of a lectin-like bacterial protein with carbohydrate chains on the pellicle. ABA binds to the carbohydrate chains and thus competitively inhibits the attachment of *S. mutans* to carbohydrate chains on the pellicle (Ito *et al*., 2017).

Conclusion and future perspectives

Dental caries remains a worldwide public health problem. *S. mutans* is the primary pathogen in dental caries. Current anti-caries agents face the challenge of side-effects and drug resistance. Therefore, alternative therapies are expected. Recently years, many inhibitors against *S. mutans* were identified by phenotypic or target-based screening. Most of these inhibitors come from natural products and their derivatives. These compounds provide an important basis for the development of new anti-caries therapies in the future. However, several works also need to be addressed in the future. First, the inhibitory effects of many compounds were only tested by *in vitro* assay. Their anti-caries ability in animal model, influence on the oral microbiome and cytotoxicity need to be further evaluated. Second, inhibitory mechanisms at the molecular level are largely unknown for many of these compounds. Target identification of these inhibitors is required for further understanding their mechanism of action. Last, many of the currently proposed inhibitors are broad spectrum antimicrobials. Identifying essential and unique proteins in *S. mutans* is required for uncovering more candidate targets for a future target-base screening of inhibitors with high specificity.

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