A novel small molecule ZY354 inhibits dental caries-associated oral biofilms

Chenzi Zhang[a], Xinyi Kuang[a], Yuanzheng Zhou[b], Xian Peng[a], Qiang Guo[a], Tao Yang[b, c], Xuedong Zhou[a], Youfu Luo[b#] and Xin Xu[a#]

[a]State Key Laboratory of Oral Diseases & Department of Operative Dentistry and Endodontics, West China Hospital of Stomatology, Sichuan University, Chengdu, China.

[b]State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China.

[c]Laboratory of Human Diseases and Immunotherapies, West China Hospital, Sichuan University, Chengdu, China.

Running Head: Effect of compound ZY354 on caries-associated biofilms

#Address correspondence to Youfu Luo, luo_youfu@scu.edu.cn, and Xin Xu, xin.xu@scu.edu.cn,

Abstract

Biofilm control is a critical approach to the better management of dental caries. Antimicrobial small molecules have shown their potential in the disruption of oral biofilm and control of dental caries. The objectives of this study were to examine the antimicrobial activity and cytotoxicity of a newly designed small molecule compound ZY354. ZY354 was synthesized and its cytotoxicity was evaluated in human oral keratinocytes (HOK), human gingival epithelial cells (HGE) and macrophages (RAW) by CCK-8 assays. Minimal inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), minimum biofilm inhibition concentrations (MBICs) and minimum biofilm reduction concentrations (MBRCs) of ZY354 against common oral streptococci (i.e. *Streptococcus mutans*, *Streptococcus gordonii*, *Streptococcus sanguinis*) were determined by micro-dilution method. The exopolysaccharides (EPS)/bacteria and the dead/live ratio in the ZY354-treated multispecies biofilms were determined by confocal laser scanning microscopy, and the microbial composition was visualized and quantified by fluorescent in situ hybridization and qPCR. The demineralizing activity of ZY354-treated biofilms was evaluated by transverse microradiography. The results showed that ZY354 exhibited low cytotoxicity against HOK, HGE and RAW, and exhibited potent antimicrobial activity against common oral streptococci. The EPS and the abundance of *S. mutans* were significantly reduced after ZY354 treatment, along with an increased dead/live microbial ratio in multispecies biofilms compared with the

non-treated control. The ZY354-treated multispecies biofilms exhibited a reduced demineralizing activity at the biofilm/enamel interface. In conclusion, small molecule compound ZY354 exhibits low cytotoxicity and remarkable antimicrobial activity against oral streptococci, and it may have a great potential in the anti-caries clinical applications.

Introduction

Dental caries is a multifactorial infectious disease dependent on diet, nutrition, resident oral flora, and host response (1-3). Mechanical removal of dental plaque by tooth brushing is utmost important to control dental caries. However, difficulties in maintaining adequate plaque control, particularly at interproximal sites, necessitate the use of chemotherapeutic agents for plaque control (4).

Among the chemotherapeutic agents used in mouthwashes, chlorhexidine (CHX) is the 'gold-standard' or positive control in comparison with other substances due to its proven efficiency (5-8). Though effective, it has certain side-effects including tooth discoloration, oral mucosal erosion, and bitter taste. One possible drawback of CHX is its cytotoxicity on alveolar bone cells and gingival epithelial cells (9, 10). Hence, there is a need for alternative mouth rinse that could negate the side-effects of chlorhexidine, but yet exhibit equivalent effectiveness.

A novel strategy to control oral biofilm is to disrupt its formation (11). Small molecules are promising for controlling biofilm formation due to their good stability, activity at low concentrations, and low toxicity (12). Previous studies showed that molecules with nitroimidazole pharmacophore possessed biological activities against various infectious diseases, especially antibacterial potency (13). Our previous work has shown that 3-(substitutedmethylene)indolin-2-ones, such as compound 5c (Fig. 1), exhibited excellent bactericidal activity against both clinical related Gram-positive and

Gram-negative bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli* and *Pseudomonas aeruginosa* (14). Antibiotics featuring nitrofuran, another antibacterial pharmacophore with similar mode of action of nitroimidazole (13, 15), have shown inhibitory activities against oral bacteria such as *S. mutans* and *Enterococcus faecalis* (16-18), implying the good potential of this motif in the treatment of oral diseases such as dental caries. Hence, we combined indole-2-one and nirtofuran motifs to get potent pharmacophore against oral bacteria (Fig. 1). However, this type of hybrid usually has poor aqueous solubility, which may limit its pharmaceutical characterization. Thus, in this study, we designed and synthesized a novel water-soluble hybrid of indolin-2-one and nitrofuran ZY354 (Fig. 1), in which a hydrophilic side chain, 3-(piperidin-1-yl) propenamide, was introduced at the C-5 position.

The objectives of this study were to examine the cytotoxicity and antimicrobial effects of a newly designed small molecule compound ZY354 against oral microbial biofilms. We hypothesize that ZY354 exhibits low cytotoxicity but good antimicrobial activity against oral streptococcal biofilms. In addition, ZY354 can alter the microbial composition and consequently suppress the demineralizing activity of the oral biofilms.

Results

Chemical Synthesis of compound ZY354

As shown in Figure 1, the synthesis of the target compound ZY354 has been accomplished in three steps: 1) a reduction of nitro group of the starting material, 5-nitroindolin-2-one, was performed to afford intermediate 2. 2) 3-(piperidin-1-yl)propanoic acid was then attached to intermediate 2 through amide condensation to afford intermediate 3. 3) finally, the target compound ZY354 was obtained from an aldol condensation of intermediate 3 with 5-nitrofuran-2-carbaldehyde (Fig. 1). Compound ZY354 exists as an *E* isomer exclusively (Fig. S1).

ZY354 exhibits a good antimicrobial activity against oral streptococci

ZY354 was bactericidal against *S. mutans*, *S. gordonii* and *S. sanguinis* planktonic cultures, with MICs ranging from 0.12 to 0.49 μg/mL and MBCs ranging from 0.24 to 1.95 μg/mL (Table 1). More importantly, ZY354 exhibited a good antibiofilm activity against oral streptococcal biofilms, with MBICs against *S. mutans*, *S. gordonii* and *S. sanguinis* biofilms ranging from 0.24 to 0.49 μg/mL, and MBRCs ranging from 0.12 to 31.25 μg/mL (Table 1).

ZY354 exhibits low cytotoxicity against human oral cells

The cytotoxicity of ZY354 was evaluated by testing the viability of HOK, HGE and

RAW after treatment. Both ZY354 and CHX exhibited low cytotoxicity against the tested cells in an exposure duration of 5-min (IC50 > 62.5 μg/mL) (Fig. 2A, 2B and 2C). It is noticeable that ZY354 was even less cytotoxic relative to CHX, as the viability of ZY354-treated cells were significantly higher at the dose of 31.25 μg/mL and 62.50 μg/mL for HOK, 62.50 μg/mL for HGE, and ≥1.95 μg/mL for RAW compared to the CHX-treated cells.

ZY354 suppresses the development of oral streptococcal biofilms

The development of oral streptococcal biofilm is a concerted process of bacterial accumulation and EPS generation. We further examined the effect of compound ZY354 on the bacterial cell viability and EPS production in a multispecies consortium. The microarchitecture of the multispecies biofilms treated with ZY354 and CHX were significantly disrupted as compared with the negative control (Fig. 3A). Either bacterial viability or EPS production of the biofilms was significantly reduced after ZY354 treatment compared with the other two groups ($P < 0.05$; Fig. 3B). Specifically, the dead/live fluorescent staining of the multispecies biofilms showed a comparable inhibitory effect of ZY354 against the development of multispecies biofilms compared with non-treated controls (Fig. 3D and 3E), and both ZY354 and CHX treatment did not alter the EPS/bacteria ratio of the biofilms as compared to the negative control (Fig. 3C), indicating that the reduction of EPS is parallel to the elimination of bacteria within the biofilms.

The effects of ZY354 on the microbial composition of multispecies biofilms were further investigated with species-specific FISH and qPCR. Both ZY354 and CHX altered the microbial composition of the oral streptococcal biofilms. The abundance of *S. mutans* was significantly reduced after ZY354 treatment (Fig. 4A and 4B) compared to the non-treated negative control. Conversely, the abundance of *S. sanguinis* and *S. gordonii* increased after ZY354 treatment (Fig. 4A and 4B). Of note, although CHX treatment suppressed *S. mutans* in the multispecies biofilms, it also depleted the commensal *S. sanguinis* in the biofilms.

ZY354 halts the biofilm-mediated demineralization process of tooth enamel

The demineralizing activity of oral biofilm is closely related to its cariogenicity. Hence, we developed saliva-derived biofilm on the tooth enamel and used transverse microradiography to further evaluate the effect of ZY354 on the biofilm-mediated demineralization process of tooth enamel. Either the depth of biofilm-induced lesion or the mineral loss of the tooth enamel was significantly reduced after ZY354 treatment as compared to the negative control. More importantly, the ZY354 treatment showed a superior inhibitory effect against biofilm-mediated demineralization as compared to the CHX treatment (Fig. 5A, 5B and 5C).

Discussion

Dental caries has a polymicrobial infection etiology (3). Effective plaque biofilm control is the utmost important measure for the management of dental caries. Along with mechanical removal, chemotherapeutic is an indispensable supplementary to dental plaque control (4, 19). Here we developed a small molecule compound ZY354, which showed potent antimicrobial activity against streptococcal biofilms and low cytotoxicity against human oral keratinocytes, human gingival epithelial cells and macrophages, indicating its great translational potential in the clinical control of dental caries.

Our previous data have demonstrated that small molecules with nitroimidazole pharmacophore (14) possesses biological activities against various infectious diseases with good antibacterial potency (13). Antibiotics with nitrofuran (13, 15) also show inhibitory activities against oral bacteria such as *S. mutans* and *E. faecalis* (16-18). Here we designed and synthesized compound ZY354, a hybrid of indolin-2-one and nitrofuran. We observed a good antimicrobial activity of this compound against oral streptococci in both planktonic cultures and biofilms.

Oral biofilm formation is a well concerted process of bacterial adhesion, EPS production and biomass accumulation. EPS produced by oral streptococci, particularly by the cariogenic *S. mutans*, functions as scaffold for biofilm growth and maturation, and it is also closely associated with biofilm properties including surface adhesion, spatial and

chemical heterogeneities, synergistic/competitive interactions (20, 21, 36-38). Hence, EPS has been well recognized as the key virulence factor of oral biofilm regarding caries development, and to inhibit EPS generation is a promising approach to the disruption of oral biofilms (20, 22, 39). In the current study, we found that compound ZY354 could significantly reduce the EPS production by the oral biofilms, and this is likely attributed to its great bactericidal effect against oral streptococci. The reduced live bacterial cells within the oral biofilms after ZY354 treatment further led to a decreased production of EPS, and ultimately disrupt the microarchitecture and formation of the matured biofilms.

Microbial equilibrium is a major determinant of biofilm cariogenicity (1-3, 23). An oral biofilm with well-balanced acidogenic streptococci such as *S. mutans* and peroxidase- and alkaline-generating competitors such as *S. sanguinis* is not cariogenic (1, 2, 24). The emergence of *S. mutans* in the multispecies biofilms usually imply an enhanced acidogenicity and may consequently result in dental caries (25-27). Here we developed a 3-species biofilm consisting of *S. mutans*, *S. gordonii*, and *S. sanguinis*, and we found that ZY354 treatment could selectively inhibit *S. mutans* but enrich competing streptococci within the biofilms. Interestingly, although exact mechanism still needs further investigation, we found that as compared to CHX which suppressed both *S. mutans* and alkaline-generating *S. sanguinis*, ZY354 treatment kept the abundance of *S. sanguinis* intact and in the meantime enriched the peroxidase-generating *S. gordonii* within the multispecies biofilms, leading to a microbial consortium with a better

competitive edge against the opportunistic overgrowth of *S. mutans*, thus being beneficial to caries management.

The biocompatibility of newly synthesized material is the bottle neck for its clinical translation. This study tested the cytotoxicity of ZY354 against common human oral cells that might be exposed to a mouth rinse, including HOK, HGE and RAW. Intriguingly, although its exact activities on human cells are still unclear, the compound ZY354 exhibited lower cytotoxicity compared to CHX, one of the most commonly used antimicrobial mouth rinse in the dental clinic (5-8), at the comparable tested concentrations. These data demonstrate that short-duration exposure to ZY354 is not cytotoxic to human oral cells, and this compound may have great clinical translational potential with comparable anti-plaque efficacy as CHX.

In conclusion, this study investigated the antimicrobial effects and cytotoxicity of a novel small molecule ZY354 against selected oral streptococci in planktonic cultures and multispecies biofilms for the first time. The ZY354 has low cytotoxicity against common human oral cells and possesses potent antimicrobial effect against oral biofilms. It can also inhibit the key cariogenic factor of oral biofilm and alter the microbial composition, leading to a biofilm with lessened demineralizing activity. The small molecule ZY354 may have a great potential in the development of novel anti-plaque and anti-caries oral hygiene products.

Materials and Methods

Synthesis of ZY354 and Specimen Preparation

Synthesis of intermediate 2. Activated carbon (1.0 g) and $FeCl_3$ (1.0 g) were added to a suspension of 5-nitroindolin-2-one (5.0 g, 1.0 eq.) in EtOH (50 mL). The mixture was heated to 78 °C and stirred for 10 minutes. Then 80% aqueous solution of hydrazine hydrate (8.0 eq.) was added dropwise into the reaction mixture in 5 minutes, the resulting mixture was stirred at 78 °C for 8-10 hours, then cooled to room temperature. The mixture was filtered to remove residue of activated carbon, and the filtrate was concentrated under vacuum to afford crude product that was purified by recrystallization from EtOH (about 15 mL) to give 5-aminoindolin-2-one (intermediate **2**) as a pale yellow solid (yield 91.9%).

Synthesis of intermediate 3. Triethylamine (1.4 eq.) and 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) (1.3 eq.) were added to a solution of 3-(piperidin-1-yl)propanoic acid (600 mg, 1.0 eq.) in dichloromethane (DCM) (7 mL). The reaction mixture was stirred at room temperature for 20-30 minutes, then 5-aminoindolin-2-one (1.1 eq.) was added into the reaction mixture. The resulting mixture was stirred at room temperature for 6 hours. After the reaction was finished, the solvent was evaporated under vacuum to give the crude product which was purified by

column chromatography to afford intermediate 3 as a colorless oil (yield 33.4%).

Synthesis of the target compound ZY354, (*E*)-N-(3-((5-nitrofuran-2-yl)methylene)-2-oxoindolin-5-yl)-3-(piperidin-1-yl) propanamide. To a solution of the intermediate 3 (200 mg, 1.0 eq.) in MeOH (5 mL), piperidine (1.5 eq.) and 5-nitrofuran-2-carbaldehyde (1.2 eq.) were added. The reaction mixture was stirred at room temperature for 30 minutes. After the reaction was finished, water (15 mL) was added to quench the reaction. The resulting mixture was extracted with DCM (10 mL) for three times. The combined organic layer was evaporated under vacuum, then purified by column chromatography to afford compound ZY354 as a dark brown solid. The product exists as an *E* isomer (yield 48.6 %). ^{1}H NMR (400 MHz, Pyr-d_5) δ 12.03 (s, 1H), 10.86 (s, 1H), 8.98 (s, 1H), 7.84 (d, J = 6.0 Hz, 1H), 7.63 (s, 1H), 7.56 (m, 1H), 7.10 (s, 1H), 7.02 (d, J = 7.6 Hz, 1H), 2.79 (m, 4H), 2.39 (m, 4H), 1.48 (m, 4H), 1.28 (m, 2H) (Fig. S2). 13C NMR (101 MHz, Pyr-d_5) δ 170.89, 169.70, 152.92, 152.56, 141.57, 133.90, 130.05, 126.04, 121.55, 120.72, 119.40, 117.19, 113.98, 110.20, 55.01, 53.98 (2C), 34.26, 26.14 (2C), 24.39 (Fig. S3). HRMS (Q-TOF): calculated for C21H22N4O5 [M] :410.1590. Found [M+H]+ : 411.1665 (Fig. S4).

Bacterial Strains and Growth Media

Streptococcus mutans UA159, *Streptococcus gordonii* DL1, and *Streptococcus sanguinis* ATCC 10556 were kindly provided by the State Key Laboratory of Oral

Diseases (Sichuan University, Chengdu, China). *S. mutans*, *S. gordonii* and *S. sanguinis* were routinely grown at 37°C under aerobic condition (5% CO_2) in brain heart infusion broth (BHI; Difco, Sparks, MD).

Bacteria Inoculation and Biofilm Formation

Inoculum for the experiment was adjusted to 1×10^7 CFU/mL for *S. mutans*, *S. gordonii* and *S. sanguinis* based on the OD_{600} nm versus CFU/mL graph of each bacterium. When needed, medium was supplemented with 1% sucrose (designated BHIS), or 1% sucrose and ZY354 at the same time, and the pH value was adjusted to 7.0 before experiment. For multispecies biofilm formation, 300 μL of bacterial inoculum consisting of *S. mutans* (1×10^5 CFUs/mL), *S. gordonii* (1×10^5 CFUs/mL), and *S. sanguinis* (1×10^5 CFUs/mL) in BHIS was added in the chemotaxis chamber (μ-slide 8 Well, 80826, Ibidi) as described by Arthur *et al* (28). For single-species biofilm formation, bacteria were inoculated at a concentration of 1×10^7 CFUs/mL in 100 μL of BHIS in 96-well plate. The bacteria culture medium was changed every 24h.

Bacterial susceptibility assays

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ZY354 against *S. mutans*, *S. gordonii* and *S. sanguinis* were determined by a micro-dilution method as described previously (27) and by Nudera *et al*

(29). Microtiter plates contained ZY354, their respective solvent controls, chlorhexidine (CHX) as a positive control, cell control (test bacteria and growth medium) as a negative control, blank well (sterile ddH_2O), and blank medium (containing sterile ddH_2O and growth medium). All plates were then placed in aerobic condition (5% CO_2) at 37.0°C for 24 hours for *S. mutans*, *S. gordonii* and *S. sanguinis*. OD_{600nm} values were obtained with a microplate reader (Power Wave 200 Microplate Scanning Spectrophotometer; Bio-TeK Instruments Inc, Winooski, VT) and the Windows-based computer program KC4 Data Analysis Software (Bio-TeK Instruments Inc). The MIC for test solutions was the concentration of test solution allowing <0.05 A600 nm. The MBC of the test solutions was determined by inoculating the solution mixture in wells representing the MIC and the next 3 higher concentration wells onto the BHI agar plates (1.5% agar; Difco, Sparks, MD). The inoculated agar plates were placed in an aerobic condition (5% CO_2) at 37.0°C for 24 hours, and the MBC was determined as the lowest concentration of test solution that exhibited no growth.

Biofilm susceptibility assays

The effects of ZY354 on *S. mutans*, *S. gordonii* and *S. sanguinis* biofilm formation were examined by the micro-dilution method as described by Wei *et al* (30). *S. mutans* UA159, *S. gordonii* or *S. sanguinis* (1×10^7 CFU/mL) was grown in BHIS and ZY354 (0.10 to 125 μg/mL) at 37°C for 24 h. The culture supernatant from each well was then

decanted, and the adherent biofilm was washed three times with PBS, fixed with methanol for 15 min, and stained with 0.1% (wt/vol) crystal violet (Sigma) for 5 min. Subsequently, the wells were rinsed with deionized water until the blank wells appeared colorless; 200 µl of 95% ethanol was added. The plates were shaken at room temperature for 30 min, and the absorbance at 595 nm was recorded. The minimum biofilm inhibition concentration (MBIC) was defined as the lowest ZY354 concentration that resulted in at least 90% ($MBIC_{90}$) inhibition of the formation of biofilms compared with that in the untreated control. A parallel study was also performed with BHIS.

The effect of ZY354 on the removal of *S. mutans, S. gordonii* and *S. sanguinis* biofilms was determined by the micro-dilution method modified from that of Ramage *et al* (31). A 200 µl of bacterial cell suspension (1×10^7 CFU/mL) in BHIS was added to the wells of a 96-well microtiter plate for biofilm formation. After anaerobic incubation at 37°C for 24 h, the growth medium was removed without disrupting the integrity of the biofilms. The formed biofilms were then washed three times with PBS to remove nonadherent cells. BHIS supplemented with ZY354 (0.10 to 125 µg/mL) was added to wells containing biofilm and incubated at 37°C for 24 h. The control wells contained BHIS without ZY354. The treated biofilms were then stained and quantified with the method described above, and the minimum biofilm reduction concentration (MBRC) was defined as the lowest ZY354 concentration that resulted in at least 90% ($MBRC_{90}$) reduction of biofilms compared with that in the untreated control.

294 **In vitro cytotoxicity/viability assay**

295 The cell viability was determined colorimetrically with CCK-8 as described by Diab
296 *et al* (32) and Tsukatani *et al* (33). Cytotoxicity was evaluated in human oral
297 keratinocytes (HOK), human gingival epithelial cells (HGE) and macrophages (RAW).
298 HOK, HGE and RAW cells (50×10^3 cell/100 µl/ well in a 96 well plate) were grown in
299 medium for 24h, then treated with medium containing different concentrations of ZY354
300 (0.12 to 62.5 µg/mL) for 5min, and the positive control was treated with CHX at the same
301 time. After incubation, a volume of 10 µl of CCK-8 was added per well, and the plate was
302 incubated in the CO_2 incubator for 3 h. The samples absorbance was measured at the
303 wavelength of 450 nm against blank which containing medium only. The cell viability
304 was calculated according to the following formula: (%) = (OD of sample - OD of blank)/
305 (OD of control - OD of blank) × 100%.

306

307 **Biofilm Imaging**

308 Oral multispecies biofilms were cultured in accordance with a previous study (28).
309 Biofilms were then exposed to PBS, 62.5 µg/mL ZY354, 0.2% CHX for 3 days (5 min
310 three times per day).

311 For EPS staining, 2.5 µM Alexa Fluor 647-labeled dextran conjugate (Molecular
312 Probes) was added at the beginning of biofilm formation and the bacteria were stained
313 with 2.5 µM SYTO9 (Molecular Probes) for 15 min after biofilms formed (34). The

biofilms were imaged with a Leica DMIRE2 confocal laser scanning microscope equipped with a 60× oil immersion objective lens as dead/live imaging.

For dead/live imaging, biofilms were stained following the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Briefly, the biofilms were stained with 2.5 μM SYTO9 (Molecular Probes, Invitrogen) and propidium iodide (Molecular Probes) for 15 min. The labeled biofilms were imaged with a Leica DMIRE2 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with a 60× oil immersion objective lens (34).

For fluorescent in situ hybridization imaging, biofilms were fixed in 4% paraformaldehyde overnight and investigated by species-specific probes (34). The biofilms were imaged with a Leica DMIRE2 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with a 60× oil immersion objective lens.

All 3-dimensional reconstructions of the biofilms were performed with Imaris 7.0.0 (Bitplane, Zürich, Switzerland), and the quantification of dead/live and EPS/bacteria volume ratio was performed with Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) and COMSTAT (http://www.image-analysis.dk) (21), respectively.

DNA Isolation and Real-time Polymerase Chain Reaction

Total DNA of biofilms were isolated and purified using a TIANamp Bacteria DNA kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The

bacteria were lysed using enzymatic lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM sodium EDTA and 1.2% Triton X-100) containing 25 mg/mL of lysozyme at 37 °C for 1.5 h. The purity and concentration of DNA were detected by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The extracts were stored at −20 °C until use. TaqMan real-time polymerase chain reaction (Life Technologies, Carlsbad, CA, USA) was used to quantify the absolute number of *S. mutans*, *S. gordonii* and *S. sanguinis* as described by the manufacturer (Takara, Dalian, China).

Transverse Microradiography

This experiment was performed on extracted human teeth. Collection of extracted human teeth was approved by the Ethics Committee of West China Hospital of Stomatology, Sichuan University. All efforts were made to minimize suffering and ensure the highest ethical and humane standards.

The labial dental crown of extracted tooth was cut into sections measuring 5 mm × 5 mm × 2 mm by using a diamond-coated band saw with continuous water cooling (Struers Minitom; Struers, Copenhagen, Denmark). Enamel blocks were embedded in polymethylmethacrylate and painted with two layers of acid-resistant nail varnish, leaving a 4 mm × 4 mm window exposed on the labial enamel surface. These surfaces were then ground flat with water-cooled carborundum discs of waterproof silicon carbide paper (Struers) of various grits (1000, 1200, 2400, 3000 and 4000). All of the polished

354 samples were individually sonicated in distilled water for 5 min to remove the residual
355 abrasives.

356 A 2 mL of bacterial inoculum consisting of *S. mutans*, *S. gordonii*, and *S. sanguinis*
357 (1×10^7 CFUs/mL for each bacterium) in BHIS was added to each well with enamel disc,
358 and then was cultured anaerobically in BHIS at 37°C (5% CO_2). Then discs with biofilms
359 were exposed to PBS, 62.5 μg/mL ZY354, 0.2% CHX for 5 min three times per day. To
360 minimize the variation in baseline mineral level, enamel discs obtained from the same
361 tooth were evenly distributed to each test group. The pH of all experimental solutions was
362 adjusted to 7.0 prior to treatment. After exposure, specimens were washed with PBS and
363 repositioned in the plate. BHIS was refreshed after the third exposure every day. After
364 5-day incubation, biofilms were detached by sonification and discs were repeatedly
365 washed by PBS. The result enamel discs were prepared as described by Eversole *et al*
366 (35). X-ray films of experimental lesions were acquired by an X-ray generator (Softex,
367 Japan) equipped with a microradiography camera, and then were further examined using
368 Zeiss AXIO Imager A2 microscope (Carl Zeiss, Germany). Quantitative data was
369 acquired by a calibrated analysis system TMR2006 (Inspektor Research Systems BV,
370 Netherlands). Data are obtained as the mean of 10 separate samples.
371

372 **Statistical Analysis**

373 All the experiments repeated at least 3 times independently. One-way analysis of

variance was performed to detect the significant effects of variables, followed by the Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$. Statistical analysis was performed with the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA).

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Conflict of interest

The authors declare no competing financial interests.

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484

Figure legends

Figure 1.

(A) Chemical structures of compound 5c and ZY354; (B) Synthesis of compound ZY354. Reagents and conditions: (i) 80% aqueous solution of hydrazine hydrate, $FeCl_3$, activated carbon, EtOH, 78°C, 91.9 %; (ii) HATU, Et_3N, DCM, 25°C, 33.4 %; (iii) piperidine, MeOH, 25°C, 48.6 %.

Figure 2. Cytotoxicity of ZY354 on human oral keratinocytes, human gingival epithelial cells and macrophages.

Viability of (A) HOK, (B) HGE, and (C) RAW treated with ZY354 was evaluated by CCK-8 assay. Data are represented as mean % ± SD. HOK, human oral keratinocytes; HGE, human gingival epithelial cells; RAW, macrophages; CHX, chlorhexidine.

Figure 3. The antimicrobial effects of ZY354 against oral streptococcal multispecies biofilms.

(A) Representative images multispecies biofilms treated by ZY354. Green, bacteria (SYTO 9); red, extracellular polysaccharides (EPS); (B) quantitative analysis of EPS and bacteria within the biofilms; (C) the ratio of EPS/ bacteria within the biofilms; (D) Representative image of dead/live bacteria within the multispecies biofilms after treatment; Green, live bacteria, red, dead bacteria; (E) quantitative ratio of dead and live bacteria after treatment. Data are presented as mean ± standard deviation. *$P < 0.05$.

504

505 **Figure 4. The composition shift of multispecies biofilms.**

506 (A) Representative Fluorescent in situ hybridization images of multispecies biofilms; *S. mutans* (*S. m*,

507 green), *S. gordonii* (*S. g*, blue) and *S. sanguinis* (*S. s*, red); (B) The ratio of *S. mutans*, *S. gordonii*, and

508 *S. sanguinis* in multispecies biofilms quantified by qPCR. Data are presented as mean ± standard

509 deviation. *P < 0.05.

510

511 **Figure 5. The anti-demineralization effect of ZY354 against multispecies biofilms.**

512 (A) Representative transverse microradiography images of human enamel discs exposed to 5-day

513 biofilm-induced experimental demineralization. The high-density regions represent the sound enamel

514 tissues, while the low-density shadows indicate the caries-like lesions. (B) Lesion depth and (C)

515 mineral loss were calculated. Data are presented as mean ± standard deviation. *P < 0.05.

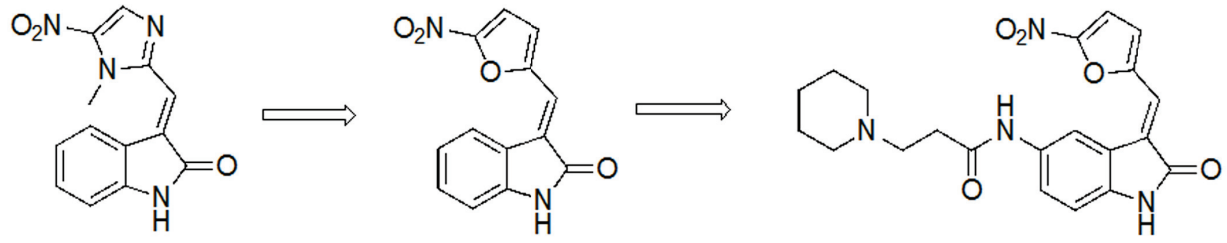
516

Table 1. Antimicrobial effect of ZY354 against *S. mutans*, *S. gordonii* and *S. sanguinis* planktonic cultures and biofilms in BHI medium.

Bacterial Strain	Planktonic cells		Biofilm	
	MIC (μg/ml)	MBC (μg/ml)	MBI (μg/ml)	MBRC (μg/ml)
S.mutans	0.24	1.95	0.24	31.25
S.gordonii	0.49	0.98	0.24	15.63
S. sanguinis	0.12	0.24	0.49	0.12

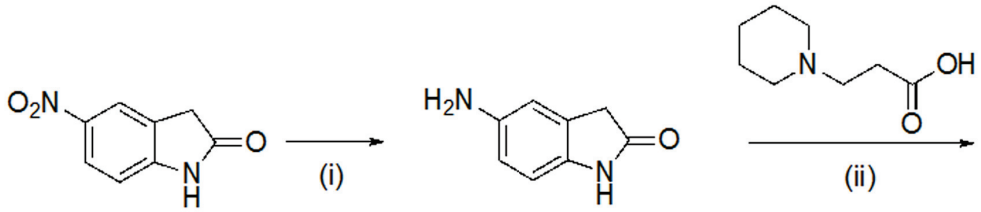
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBIC, minimum biofilm inhibition concentrations; MBRC, minimum biofilm reduction concentrations.

A



5c | Newly desighed pharmacophore | ZY354

B



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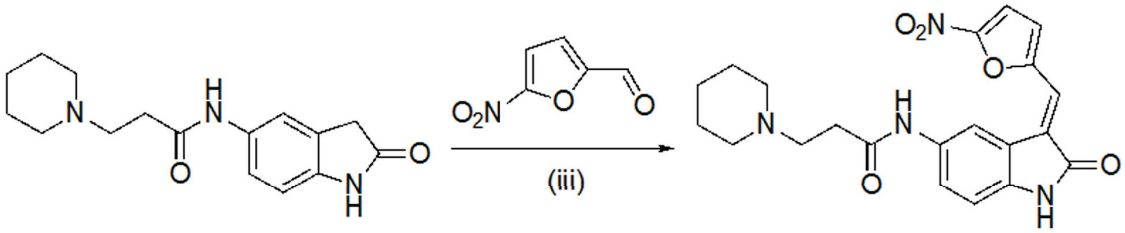

3 | ZY354