Title: A drug repositioning approach reveals *Streptococcus mutans* is susceptible to a diverse range of established antimicrobials and non-antibiotics

Authors: Saputo, S., Faustoferri, R.C., and Quivey Jr., R.G.[#]

Center for Oral Biology
Box 611
University of Rochester School of Medicine and Dentistry
Rochester, NY 14642

[#]Corresponding author: Robert G. Quivey, Jr.
Department of Microbiology & Immunology
and Center for Oral Biology
University of Rochester School of Medicine & Dentistry,
Rochester, NY 14642
Telephone: 585-275-0382
Email: Robert_Quivey@urmc.rochester.edu

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Abstract

Streptococcus mutans is the primary causative agent of dental caries and contributes to the multispecies biofilm known as dental plaque. An adenylate kinase-based assay was optimized for *S. mutans* to detect cell lysis when exposed to the Selleck library of 853 FDA-approved drugs, in, to our knowledge, the first high-throughput drug screen in *S. mutans*. We found 126 drugs with activity against *S. mutans* planktonic cultures and they were classified into six categories: antibacterials (61), antineoplastics (23), ion channel effectors (9), other antimicrobials (7), antifungals (6), and other (20). These drugs were also tested for activity against *S. mutans* biofilm cultures, and 24 compounds were found to inhibit biofilm formation, 6 killed pre-existing biofilms, 84 exhibited biofilm inhibition and killing activity, and 12 had no activity against biofilms. The activity of 9 selected compounds that exhibited antimicrobial activity were further characterized for their activity against *S. mutans* planktonic and biofilm cultures. Together, our results suggest that *S. mutans* exhibits a susceptibility profile to a diverse array of established and novel antibacterials.

Introduction

Streptococcus mutans is the primary causative agent of dental caries, a disease that affects individuals of all ages, especially those with limited healthcare accessibility and poor socio-economic circumstances. *S. mutans* is amongst the early colonizers of the tooth surface that promotes binding of other oral bacteria to form a biofilm, known as dental plaque. Plaque is a complex biofilm that can consist of over 100 bacterial species in any individual, although, over 700 types of bacteria have been isolated from healthy individuals (1). Dental caries is associated with a decrease in microbial diversity and a predominance of *S. mutans* (2). Even in healthy individuals, the oral microbiome can consist of closely-related commensals, making targeted therapies a challenge. While the most efficient anti-caries therapy is regular preventative treatments consisting of physical removal of plaque, other therapies also exist, such as chemical treatment, including chlorhexidine and fluoride, reduction of dietary sugars, and antibiotics (3). As one of the early colonizers of the plaque biofilm, *S. mutans* is a rational target for anti-caries prophylaxis.

Previous work in targeting *S. mutans* has focused on characterizing the activity of individual drugs, including toothpaste components (4-7), natural products (8-11), or antibiotics (12-15). Large-scale screens for activity of antimicrobial peptides against *S. mutans* have also been reported (16, 17). However, large-scale drug susceptibility assessment of oral microbiome constituents, including mutans streptococci, remains relatively understudied compared to other Gram-positive pathogens, such as *Staphylococcus aureus* ((18-21), for example).

64 The adenylate kinase (AK) assay was originally adapted as a high-throughput
65 method to detect lysis of fungal cells (22). Since then, it has been optimized for use
66 with bacteria in high-throughput screens of the ESKAPE pathogens, as well as *M.*
67 *tuberculosis* (18, 23). Screening for compounds using the AK assay offers numerous
68 advantages: specific detection of bactericidal drugs; increased sensitivity over standard
69 growth-based or Alamar blue assays; detection of bioactive molecules below the
70 minimum inhibitory concentration (MIC) (22); and, importantly, the ability to detect anti-
71 biofilm activity of drugs (18).

72 The Selleck library has been used with other organisms to discover new uses for
73 proven, FDA-approved drugs in an effort to repurpose these compounds for “off-label”
74 uses. Drug repurposing, also known as repositioning, has gained momentum mostly
75 due to its advantages over *de novo* drug discovery (24), including reduced risk to
76 patients due to previously documented clinical trials, lower drug development costs, and
77 faster benchtop-to-clinic transition. Large drug and compound libraries exist and can be
78 screened for new activities and alternate uses, which can aid in rapid identification of
79 new therapies (19, 23, 25). In addition to assigning new activities to previously
80 characterized compounds, compound libraries are also an ideal source of adjuvants,
81 molecules that have little-to-no antibiotic activity alone, but can be used in combination
82 with other drugs to enhance antimicrobial activity and, in some cases, circumvent
83 resistance mechanisms (26-28). For example, we found that the Vitamin D derivative,
84 doxercalciferol, acts synergistically with bacitracin through a mechanism involving the *S.*
85 *mutans* bacitracin resistance transporter, MBR (29).

Herein, we report that the AK assay is an effective method of high-throughput drug screening and detection of *S. mutans* lysis. Our results demonstrate that *S. mutans* exhibits a distinct susceptibility profile to established and novel antibacterials and offers possibilities for new effective anti-caries approaches.

Materials and Methods

Strains and growth conditions

Streptococcus mutans strain UA159 (30), was maintained on Brain-Heart Infusion agar medium (BD/Difco, Franklin Lakes, NJ). Cultures were grown at 37°C in a 5% (v/v) CO_2/95% air atmosphere in either BHI or TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH, 1 mM H_3PO_4) + 1% (w/v) glucose (TYG). For biofilm analyses, cells were grown in TY medium + 1% (w/v) sucrose (TYS).

Chemicals and compound library

We utilized the Selleck library (Selleck Chemical, Houston, TX), a compilation of 853 FDA approved, off-patent compounds that have diverse functions, structures, and cellular targets. The chemicals in the library were at a concentration of 10 mM (stock) in DMSO.

Adenylate kinase assay

Adenylate kinase assays were performed as previously described (18), with minor modifications. Briefly, overnight cultures of *S. mutans* UA159 were diluted 1:50 into 50 mL fresh TYG medium and grown to exponential phase (OD_{600} ~ 0.5). In a 96-well opaque plate (Corning, Corning, NY), 10^6 cells/well were combined with test molecule (solubilized in DMSO; final concentration of DMSO <0.5%) in a final volume of 100 µL (50 µM final concentration of drug). Plates were incubated at 37°C in a 5% (v/v) CO_2/95% air atmosphere for 3h then equilibrated to room temperature for 1h.

Reconstituted adenylate kinase (AK) detection reagent (ToxiLight Non-destructive Cytotoxicity BioAssay Kit, 100 µL; Lonza, Walkersville, MD) was added to each well and the plate was incubated in the dark for 1h at room temperature. Luminescence was measured with an integration time of 1000 ms per well on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Ciprofloxacin (positive control) and DMSO (negative control) were included on all plates. A "hit" was defined as a compound that elicited a 2-fold increase in AK signal when compared to the vehicle (DMSO)-treated cells in two independent screens. Background luminescence was similar across different plates and days. For biofilm cultures, logarithmic phase cells were seeded in flat-bottomed 96-well plates (Corning Inc., Corning, NY) and grown in TYS at 37°C in a 5% (v/v) CO_2/95% air atmosphere for ~18h. Plates were washed 3 times with sterile PBS to remove planktonic cells. Drug (50 µM final concentration) and fresh medium (TYS) were added to wells, and the cultures were incubated for an additional 18h. After 1h equilibration at RT, 50 µl from each well was transferred to opaque 96-well plates with equal volume AK reagent and the reaction was allowed to proceed for 1h, followed by measurement of luminescence, as described above.

Z' score determination

The Z' score for the AK assay was assessed to determine the signal-to-noise ratio, as well as the intra-plate variability (31). A value between 0.5 and 1 indicates that the assay is suitable for high-throughput screening.

S. mutans UA159 was grown to exponential phase (OD_{600} ~ 0.5) in TYG medium. To each well of a 96-well opaque plate, 10^6 CFU were added in fresh media and DMSO

or ciprofloxacin (final drug concentration 20 µg/mL; 10X MIC; Sigma Aldrich, St. Louis, MO) were treated in alternating columns. Total well volume was 100 µL. Plates were treated with the AK assay reagent and luminescence was measured as described above. Z' score was assessed as described previously (31).

MIC testing

To determine the MIC of test compounds against *S. mutans* UA159, a two-fold dilution series of test compounds (biapenem, cefdinir, conivaptan, disulfiram, felodipine, ponatinib, tretinoin, zinc pyrithione (Selleck Chemical, Houston, TX) and simvastatin (Sigma Aldrich, St. Louis, MO)) were added to fresh TYG medium in a 96-well plate (Corning, Inc., Corning, NY) at concentrations ranging from 0-64 µg/mL. An overnight culture of UA159 grown in BHI medium was diluted 1:50 in fresh TYG medium, grown to early exponential phase (OD_{600} ~0.3), and used to inoculate the plate containing fresh medium and drug (10^5 CFU/well). The plate was incubated at 37°C in a 5% (v/v) CO_2/95% air atmosphere for 24h. The MIC was defined as the lowest compound concentration that inhibited ~90% bacterial growth, as measured by OD_{600}.

FIC testing

Synergy was assessed by identifying the fractional inhibitory concentration (FIC), given by the equation: $FIC = \frac{MIC_A}{MIC_{AB}} + \frac{MIC_B}{MIC_{AB}}$, where A and B are the two drugs tested alone, or in combination (AB), and were measured using the standard checkerboard method (32). FICs were interpreted according to standard definitions, where "synergy"

is defined as a FICI score ≤ 0.5, “antagonism” is defined as a FICI score > 4.0 and “no interaction” is a score of 0.5 ≤ FICI ≤ 4.0.

Measurement of biofilm growth by crystal violet

Biofilm formation was measured as previously described (33). Briefly, cultures grown to logarithmic phase (OD_{600} ~0.5) in TYS were added to 96-well plates and incubated at 37°C in a 5% (v/v) CO_2/95% air atmosphere for ~18h in the presence or absence of drug. Planktonic cells were removed by washing wells 3 times with distilled water. Plates were dried overnight at 70°C. Biofilms were stained with 100 µL crystal violet (0.1%) for 15min then washed 5 times with distilled water. Adherent crystal violet was reconstituted with acetic acid (500 mM) and plates were read with a BioRad BenchMark Plus Spectrophotometer at 575nm (BioRad, Hercules, CA). The minimum biofilm inhibitory concentration (MBIC) was defined as the minimum drug concentration that reduced crystal violet staining by 90%.

Results

Optimization of the AK assay for detection of drugs with anti-*Streptococcus mutans* activity

The adenylate kinase (AK) assay has been described as a method to screen bacteria for sensitivity to antimicrobials, or other bioactive compounds, by measuring cell lysis (18, 22). Here, we optimized the AK assay for detection of *S. mutans* adenylate kinase release, indicative of cell lysis. A dilution series of cells was heat-killed and compared to luminescence from live cells, revealing assay sensitivity as low as 10^4 cells (Figure 1A). In order to ensure that the AK assay was suitable for high-throughput screening of *S. mutans*, a Z' score was calculated by comparing ciprofloxacin-treated cells (at 10X MIC) to background signal from DMSO. The Z' score has been described as a method for determining quality and reproducibility of an assay, such that an assay with a Z' score between 0.5 and 1 is suitable for use in high-throughput screening (31). The Z' score measured in our study, using ciprofloxacin as a positive control, was 0.73 (Figure 1B).

Drugs with anti-*Streptococcus mutans* activity are diverse

Using the optimized AK assay parameters, we were then able to screen the Selleck library of 853 off-patent drugs to detect compounds with activity against *S. mutans* grown in planktonic cultures. Drugs that resulted in a greater than 2-fold signal above the negative control (DMSO alone) in two identical, independent screens were considered a "hit" (Figure 2). Of the drugs in the Selleck library, 126 resulted in a signal

that was 2-fold above background luminescence in two independent screens, resulting in an overall hit rate of approx. 15% (Table S1).

Although *S. mutans* is found in saliva, the primary niche is a multi-species biofilm on the tooth surface where it serves as an early colonizer or biofilm initiator (34). Previous studies have shown that sucrose stimulates the production of glucosyltransferases that contribute to the formation of the biofilm matrix (34). Therefore, we used *S. mutans* grown in sucrose to screen the 126 hit compounds found using planktonic cultures, for activity against pre-formed biofilms and for their ability to inhibit biofilm formation.

In order to measure the ability of compounds to lyse established biofilms, the AK assay was adapted, as described previously (18), and optimized for *S. mutans*. Pre-formed biofilms were incubated with compounds (50 µM final concentration) for 18h prior to detection using the AK reagent. Similar to the primary screen, luminescent readouts from biofilms were normalized to DMSO (negative control) and compared to killing by ciprofloxacin (positive control); both DMSO and ciprofloxacin were present in all plates. Of the 126 hits against planktonic cells, 90 compounds induced AK release when exposed to biofilm cultures of *S. mutans*, with a signal at least 2-fold above background, including the internal control, ciprofloxacin (Figure 3A and Table S1).

We then tested the ability of the 126 hit compounds to prevent biofilm formation. Compounds (50 µM final) were incubated with exponential phase cultures (10^6 cells/well) for 18h. Biofilm mass was quantitated by crystal violet staining, relative to DMSO (negative control) and ciprofloxacin (positive control). Drugs that resulted in a >2-fold decrease in signal after crystal violet staining, relative to the negative control

(DMSO alone), were considered "hits." We observed that a large subset of drugs (108 of the 126 hits from the Selleck library) that were effective at inducing AK release when exposed to planktonic cells were also effective at decreasing biofilm mass *in vitro* (Figure 3A).

A majority of the compounds identified in the primary screen (114/126) had some activity against *S. mutans* biofilms (Figure 3A and Table S1). With the exception of 6 drugs (atorvastatin, calcitriol, cephalexin, ivermectin, meclizine, and mitotane), most compounds that induced AK release when exposed to pre-formed biofilms (84) were also able to inhibit *de novo* biofilm formation in our assays. These results confirm that the AK assay is an effective method for detecting compounds with anti-*Streptococcus mutans* activity. Overall, the drugs that displayed activity against *S. mutans* were structurally distinct, were derived from various drug classes, and targeted a diverse array of molecular targets, as well as their original intended uses (Table 1 and S1). Because of the diverse nature of our hits, we chose to confirm one or more drugs from each class (Figure 3B) to validate the AK assay results and to serve as a building block for future work.

Classes and Characterization of hit drugs

Antibacterials

A majority of the 126 hit compounds from the AK screen are classified as antibacterials (approx. 48%), many of which have not been specifically characterized for activity against streptococci. This category also contains a significant portion of common antibiotics, such as ciprofloxacin, ampicillin, and amoxicillin, which served as

internal positive controls for the AK assay. Hit drugs with antibacterial activity were further divided into subclasses: penams, quinolones, tetracycline-derivatives, macrolides, carbapenems, antimycobacterial, cephalosporins, and glycopeptide-derivatives (Figure 3B). Drugs classified as miscellaneous had only one represented drug in the class and included: bacitracin, chloroxine, crystal violet, daptomycin, novobiocin, dequalinium chloride, linezolid, retapamulin, thiamphenicol, and tigecycline.

Carbapenem-class antibiotics are a broad-spectrum class of β-lactam derivatives that have been shown to have potent activity against a range of Gram-negative and Gram-positive bacteria, including anaerobes. We identified 4 carbapenem-class antibiotics as hits in our screen, including biapenem, doripenem, meropenem, and tebipenem. These drugs displayed MICs between 0.5-2 μg/mL, were able to inhibit biofilm formation (Table 1 and Table S1), and also had activity against pre-formed biofilms (Table S1). Follow up assays revealed that biapenem had potent activity against planktonic cultures of *Streptococcus mutans* (MIC = 0.0625 μg/mL), as well as biofilm cultures (MBIC = 0.0625 μg/mL) (Table 1).

Cephalosporins have been shown to have activity in anaerobic conditions as well as enhanced activity in mixed infections (35). Moreover, certain cephalosporins have been found to have variable *in vitro* activity in the presence of glucose or sucrose (36). Cefdinir, one of 6 cephalosporin-class drugs identified in our screen, is a third-generation cephalosporin that has broad-spectrum activity against Gram-positive and Gram-negative bacteria, and is commonly prescribed by dentists, though it has not specifically been shown to have activity against *S. mutans*. As expected, cephalosporin-class drugs had relatively high activity against *S. mutans* (cefdinir

MIC/MBIC: 0.25 µg/mL); however, in contrast to previous reports, we did not observe any significant differences in susceptibility to cefdinir when *S. mutans* was grown in presence of either carbon source.

The retinoid-class drug, tretinoin, is a vitamin A-derivative often used topically to treat skin infection or specific types of leukemia. In combination with erythromycin, tretinoin is used in the treatment of *Propionibacterium acnes* as an alternative to systemic therapies (37). Both tretinoin and its isomer isotretinoin were detected as compounds that significantly induced AK release from *S. mutans* in our screen. However, all concentrations of tretinoin tested were unable to prevent either planktonic (Table 1) or biofilm growth (Figure 4).

Ion channel effectors

One of the acid-tolerance mechanisms used by *S. mutans* is the expulsion of protons from the cell via a membrane-bound F-ATPase, resulting in a more acidic external environment. This decrease in local pH then contributes to the organism's virulence, as other commensal organisms are unable to survive this acidic challenge (38). As such, drugs that target ion channels may be rational, effective drug targets against *S. mutans.* We identified 10 drugs that are known to have activity associated with ion channels, including effectors of calcium channels (6 compounds), potassium channels (1), proton pumps (1), chloride channels (1), and sodium channels (1).

Felodipine is a calcium channel blocker that has been shown, along with analogs, to have weak activity against Gram-positive bacteria and fungi (39). Addition of felodipine to planktonic cultures of *S. mutans* inhibited growth at 32 µg/mL, while

289 concentrations at 0.5X MIC led to a reduction in biofilm formation, relative to the DMSO
290 control (Figure 4).

291 Zinc pyrithione is an anti-seborrheic, often used in topical formulations that has
292 previously been shown to have bacteriostatic properties against streptococci and
293 staphylococci, as well as broad-spectrum antimicrobial activity against other Gram-
294 positive bacteria, Gram-negative bacteria, and fungi (40, 41). The use of zinc pyrithione
295 has been gaining momentum as evidenced by recent reports of its activity against
296 biofilms (42), aminoglycoside resistance (43), and clearance of bacteria from wound
297 sites (40). Mechanistic studies revealed that membrane permeability is increased in
298 zinc pyrithione-treated cells and may also mediate the influx of damaging metal ions into
299 the cell (41, 44). In fungi, pyrithione inhibits membrane transport and specifically targets
300 proton pumps in a pH-dependent manner (45). Its antimicrobial activity has been
301 attributed to its ability to chelate metals and transport them across membranes (41). In
302 *S. mutans,* zinc pyrithione inhibited growth at 1 µg/mL and was bactericidal at 2 µg/mL,
303 while biofilm formation was inhibited at concentrations as low as 0.5 µg/mL (MBIC,
304 Figure 4).

305 Conivaptan is a vasopressin inhibitor that was detected in the AK assay as a
306 compound that induced AK release from *S. mutans*. In humans, vasopressin receptor
307 antagonists, like conivaptan, disrupt water and electrolyte balances by selectively
308 binding to two out of three vasopressin receptors (46). To our knowledge, antimicrobial
309 activity has not been previously attributed to a vasopressin receptor antagonist.
310 Secondary assays showed it did not inhibit growth at the highest concentration tested
311 (128 µg/mL), but reduced biofilm mass when tested at a concentration 64 µg/mL

312 ($p<0.05$; Figure 4). While the genome of *S. mutans* does not possess significant
313 homology to vasopressin receptors, it is possible that conivaptan disrupts osmotic
314 balance through a distinct mechanism.
315
316 *Antineoplastic*
317 Several antineoplastic drugs that exhibited activity against *S. mutans* also
318 possessed activity against other Gram-positive bacteria, including tamoxifen,
319 doxorubicin, and ponatinib, further validating the AK assay (18, 47). Ponatinib is a
320 tyrosine kinase inhibitor that has a similar mechanism of action as the well-known,
321 "rationally designed" drug Gleevec (imatinib), used in the treatment of chronic myeloid
322 leukemia (48-50). To date, no known antimicrobial activity of ponatinib has been
323 reported, though Gleevec has been shown to accentuate the bactericidal activity of
324 macrophages against *Mycobacterium tuberculosis* (51) and inhibit the phosphorylation
325 of CagA, a *Helicobacter pylori* virulence factor (52). While the genome of *S. mutans*
326 does not encode tyrosine kinases or proteins with significant homology to the
327 Helicobacter CagA, it does encode several ABC transporters with significant homology
328 to the human target for leukemia (ABCG2). Secondary assays revealed that ponatinib
329 inhibited growth of *S. mutans* planktonic cultures (MIC = 8 µg/mL) (Table 1), was able to
330 inhibit biofilm formation (MBIC = 4 µg/mL) (Figure 4), as well as induce AK release from
331 pre-formed biofilms. The antibacterial activity of ponatinib against *S. mutans* is a novel
332 finding.
333
334 *Antifungal*

The antibacterial activity of imidazole-derivatives has been previously documented, including evidence that certain azoles can be used to treat oral maladies such as caries and periodontitis (53-55). Consistent with previous work, we detected 6 azole-class drugs that had bactericidal activity against *S. mutans*, including butoconazole, clotrimazole, econazole, fentriconazole, miconazole, and ticonazole. All azole hits displayed a relatively low MIC against *S. mutans* (approx. 8 µg/mL or lower; MIC of fentriconazole was 4 µg/mL). In addition, all six antifungal drugs had the ability to inhibit *S. mutans* biofilm formation, as well as induce AK release when exposed to pre-formed biofilms (Table S1). Interestingly, despite their efficacy and approval for use on mucosal surfaces, many of the azole drugs have not been documented for treatment of bacterial infections.

Statins

Interest in the cholesterol-lowering drugs called statins has been growing since their diverse antimicrobial activity has been elucidated (reviewed in (56)). Statins target the HMG-CoA reductase enzyme in the sterol biosynthetic pathway, a pathway that is conserved in humans, fungi and bacteria. The mevalonate pathway is conserved in Gram-positive bacteria, and has been shown to be essential for growth of *S. pneumoniae* (57). We have previously demonstrated that in *S. mutans,* the loss of HMG-CoA synthase (SMU.943c), HMG-CoA reductase (SMU.942), mevalonate kinase (SMU.181), and mevalonate diphosphate decarboxylase (SMU.937) are lethal events (33). However, interruption of phosphomevalonate kinase (SMU.938) resulted in a viable organism, but with a defect in biofilm formation (33).

Three out of the seven statin-class drugs (lovastatin, simvastatin, and atorvastatin) in the Selleck library had detectable lytic activity against *S. mutans*, as measured by the AK assay (Figure 5A). Secondary assays revealed that statins, including simvastatin, had a minor effect on growth inhibition (Figure 5B), but could significantly inhibit biofilms at concentrations below the MIC (as low as 25 µg/mL) (Figure 5C). However, in the presence of 50 µg/mL simvastatin, biofilm formation was significantly reduced by ~90% relative to control (MBIC = 50 µg/mL) (Figure 5C).

Disulfiram

Disulfiram has previously been shown to have activity against bacteria, such as in the case of growth inhibition of *Staphylococcus aureus* (58) and, recently, acting synergistically with copper to kill *Mycobacterium tuberculosis* (MIC = 16 µg/mL) (59). The purposed mechanism of action from that study was the initial breakage of the disulfide bond (Table 1), followed by coordination with copper, which shuttled ions across the membrane. As a hit in the AK assay screen, disulfiram induced release of AK when exposed to planktonic cultures of *S. mutans*. Therefore, we wanted to examine whether disulfiram had a similar mechanism in *S. mutans* by using copper in the standard fractional inhibition concentration (FIC) assay. In the absence of copper, the MIC of disulfiram against planktonic cells was 16 µg/mL. Combination of disulfiram and copper, resulted in a color change in the growth medium, indicative of breakage of the disulfide bond and coordination of copper, as reported by Dalecki et al. (59). The combination of copper (0.625 mM, 106.6 µg/mL) and disulfiram (4 µg/mL) is synergistic with an FIC of 0.375 (Table 2). When disulfiram was used in conjunction with copper

against biofilm cultures, the combination was also synergistic (FIC 0.313), as 0.625 mM (106.55 µg/mL) copper and 2 µg/mL disulfiram inhibited biofilm formation (MBIC, Table 2). Therefore, it is likely that disulfiram inhibits growth of *S. mutans* with a mechanism similar to the "Trojan horse" mechanism of copper chelation proposed in *M. tuberculosis* (59).

Our data shows that although chelation by copper may play a part, addition of copper did not have as large an impact on *S. mutans* as observed with *M. tuberculosis* (59). Other reports have shown that decomposition products of disulfiram have also been shown to have activity against *Pseudomonas aeruginosa*, where they target *Pa*BADH, an aldehyde dehydrogenase, involved in choline metabolism (60). The genome of *S. mutans* does not encode a protein with significant homology to BADH. Therefore, it is unlikely that disulfiram is specifically targeting the aldehyde dehydrogenase of *S. mutans* (*adhE*) as the primary mechanism.

Discussion

As the primary etiologic agent of dental caries and one of the initiators of dental plaque, *S. mutans* is an ideal target for the prevention and treatment of caries. Here, we describe the first application of the AK assay, as well as, to our knowledge, the first high-throughput drug screen in *S. mutans.* Our data show that AK release from *S. mutans*, indicative of cell lysis, was detectable at approximately 10^4 cells, making the AK assay approximately 10-100X more sensitive than growth-based assays.

Use of the AK assay to screen the Selleck library, containing 853 FDA-approved, off-patent drugs, against *S. mutans* resulted in 126 hits (a rate of ~15%), 90%

of which demonstrated activity against biofilm cultures. Compared to similar screens with other organisms, the hit rate, or number of drugs that exhibited bactericidal activity against *S. mutans*, is relatively high, highlighting the need for bacterial species-specific drug screening and characterization.

One of the goals of this project was to use a repositioning approach to drug discovery in *S. mutans.* Therefore, we chose to further characterize a diverse set of drugs that had not been previously categorized as antibacterial. Functional groups of repurposed drugs can be modified to enhance activity and specificity against an organism. In the age of growing antibiotic resistance, repurposed drugs can also be used in combination therapy or as an adjuvant to boost activity of antibiotics. As part of our secondary assays, we tested antimicrobial activity of all drugs in Table 1 in combination with fluoride, an accepted, preventative dental therapy and potentiator of *S. mutans* (61); though, none of the drugs tested here exhibited synergistic activity in combination with fluoride. However, this does not preclude the possibility of fluoride, or another current oral medication, to synergistically affect the activity of another compound.

Compound libraries have also been used to identify novel antibiotics targeting specific metabolic pathways, such as platensimycin that targets the fatty acid biosynthesis pathway (FASII) (62). Discovering the mechanism of action for such drugs has helped to elucidate the activity of the targeted enzyme (in this case, ß-ketoacyl-acyl-carrier-protein (ACP) synthase). The hit compounds from our study could also be exploited as tools for the discovery of pathway mechanisms in bacteria. Further

experiments are necessary to examine the hits from this study that, despite exhibiting low antibacterial activity (high MIC), resulted in detectable AK release.

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633 **Figure Legends**

634 **Figure 1A: Optimization of the AK assay for detection of AK release from *S.***

635 ***mutans* UA159.** AK activity vs. CFU of *Streptococcus mutans* UA159. Values for live

636 cells and heat-killed cells were statistically significant for each pairwise comparison

637 using Student's *t*-Test (two-tailed); * p<0.001.

638

639 **Figure 1B: Z' score determination for detection of *S. mutans* AK release.** Z' score

640 was calculated according to the equation: $Z' = 1 - 3\left(\frac{\sigma^+ + \sigma^-}{|\mu^+ - \mu^-|}\right)$, where σ and μ are the

641 standard deviation and average, respectively, for the positive (+, ciprofloxacin 20

642 μg/mL) and negative (-, DMSO 0.5%) controls.

643

644 **Figure 2: AK assay data from a representative plate.** Graphical representation of

645 relative luminescence units (RLU, y-axis) vs. plate position. Dotted line shows the cut-

646 off value of 2-fold above background (DMSO). Drugs contained in these wells were

647 used for further analysis.

648

649 **Table 1: Select drugs identified in the AK assay screen of the Selleck library.**

650

651 **Figure 3A: Drugs with activity against biofilm cultures of *S. mutans*.** Of the 126

652 hits with activity against planktonic cells, 24 exclusively inhibited *in vitro* biofilm

653 formation ("inhibits"), 6 exclusively induced AK release in pre-formed biofilms ("kills"),

654 and 84 hits displayed both biofilm inhibition as well as activity against pre-formed

655 biofilms. 12 compounds had no effect on biofilms.

Figure 3B: Classes of hit compounds from the Selleck library with activity against planktonic cultures of *S. mutans* UA159.

Figure 4: Biofilm inhibition in the presence of select hit drugs from the AK assay. Select hit drugs from each class identified from the Selleck screen were assayed at 0.25X, 0.5X, and 1X MIC for their ability to prevent biofilm formation *in vitro*. Data were normalized to DMSO control. * $p<0.05$ (Student's *t*-Test; two-tailed).

Figure 5A: Select statin-class drugs lysed *S. mutans* in AK assay screen of Selleck library. Statins were identified in the screening of the Selleck library against planktonic cultures of *S. mutans* UA159. Data are representative of 2 independent screens.

Figure 5B: Simvastatin inhibits growth of *S. mutans.* Growth of log-phase *S. mutans* UA159 was measured via OD_{600} in the presence of varying amounts of simvastatin. (n=5)

Figure 5C: Simvastatin inhibits biofilm formation of *S. mutans.* Simvastatin was tested at 0.5X and 1X MIC for the ability to prevent *in vitro* biofilm formation of cells grown in TY + 1% (w/v) glucose or TY + 1% (w/v) sucrose, as detected by crystal violet staining (OD_{575}). Data were normalized to DMSO control. * $p<0.05$ (Student's *t*-Test; two-tailed).

679

680 **Table 2: Disulfiram and copper are synergistic against planktonic and biofilm**
681 **cells.** Disulfiram (0-64 µg/mL) and copper (0-10 mM) were arrayed in a checkerboard
682 format in a 96-well plate as described in Materials and Methods. MICs were defined as
683 the lowest concentration that resulted in >90% growth inhibition relative to no drug, as
684 measured by OD_{600} for planktonic cells and crystal violet staining (OD_{575}) for biofilms. #
685 indicates different units used for copper (mM) as compared to disulfiram (µg/mL).

Figure 1A

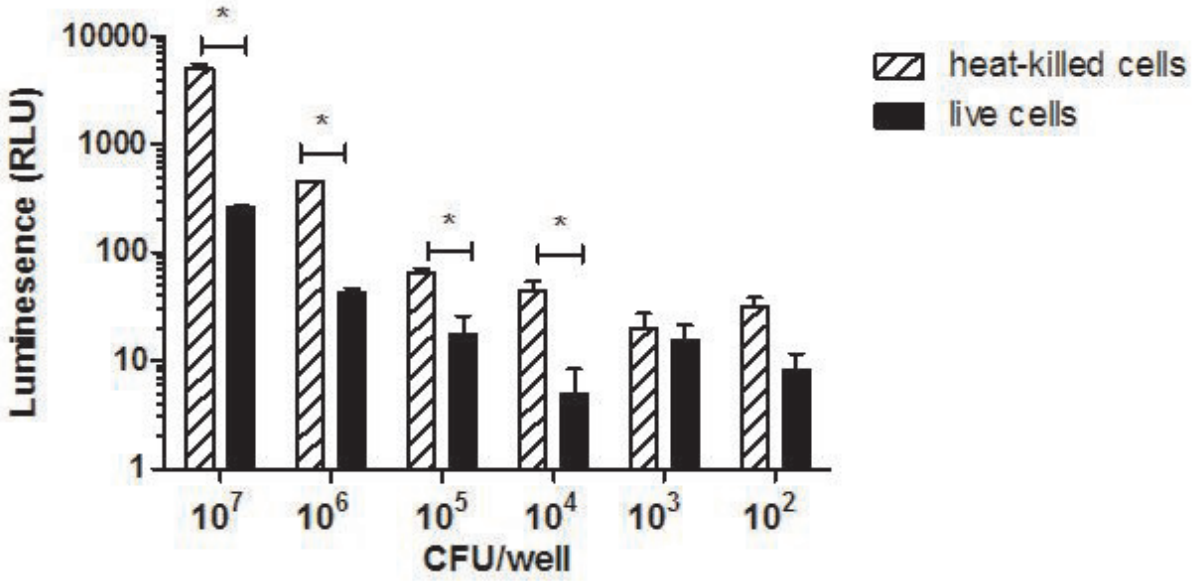


Figure 1B

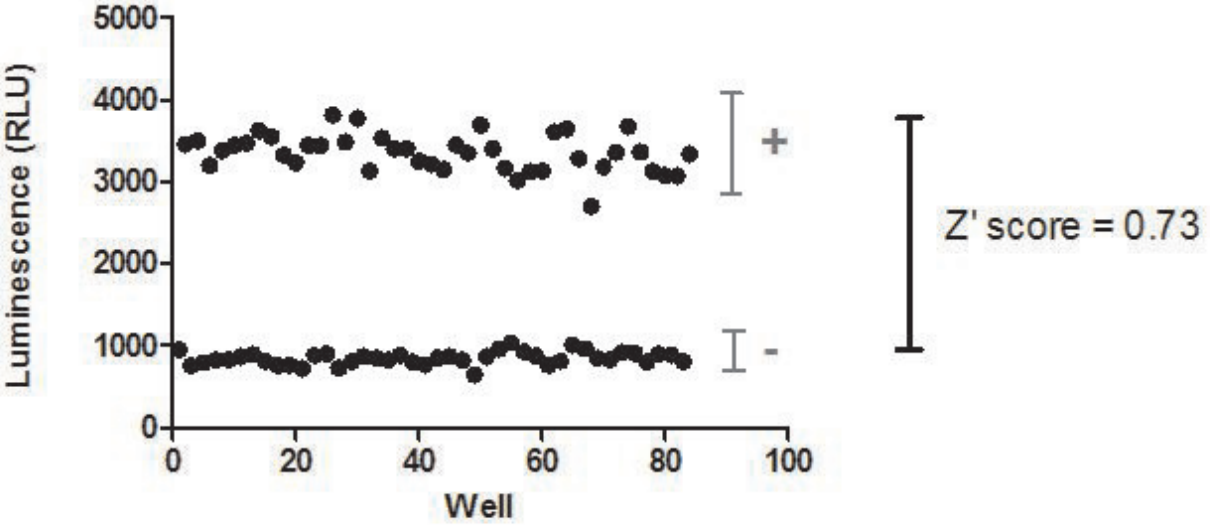


Figure 2

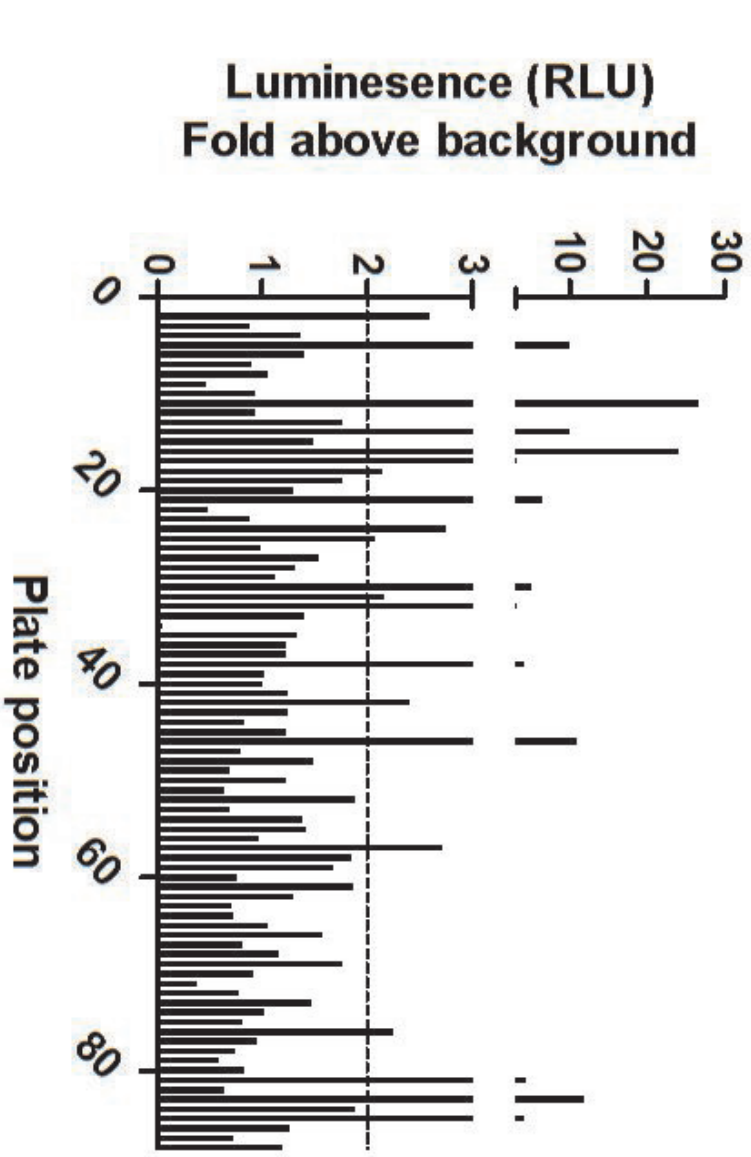


Figure 3A

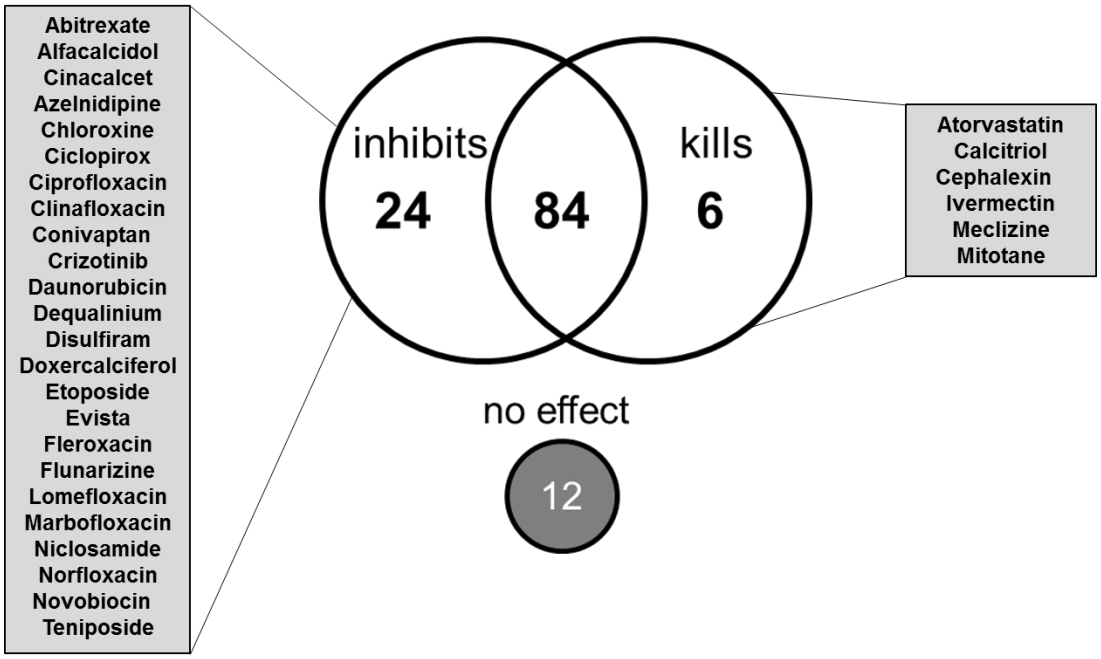
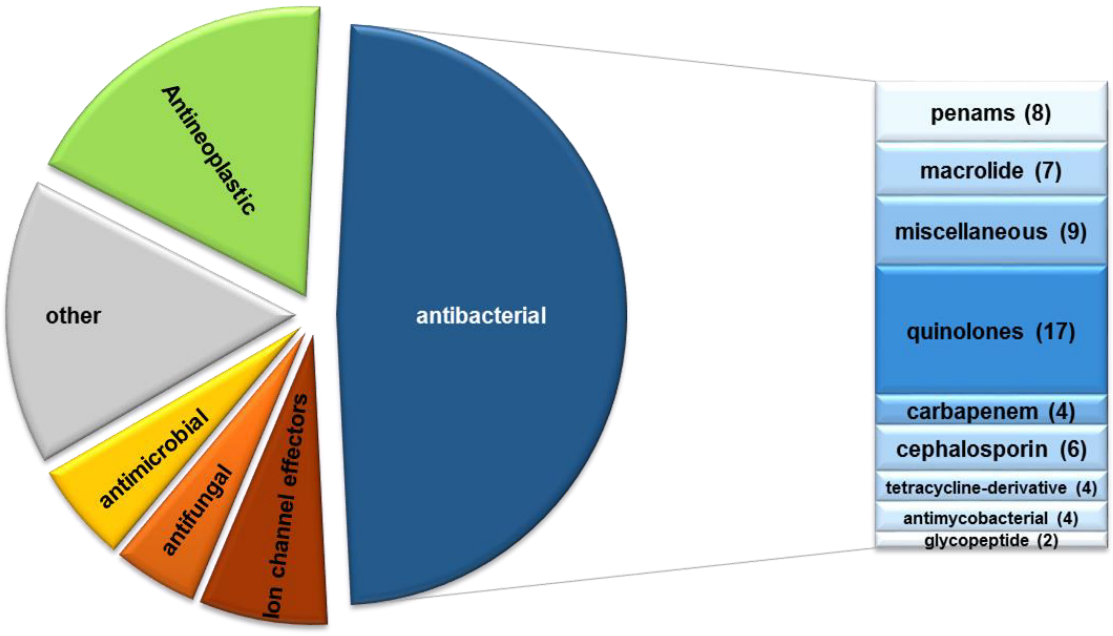


Figure 3B



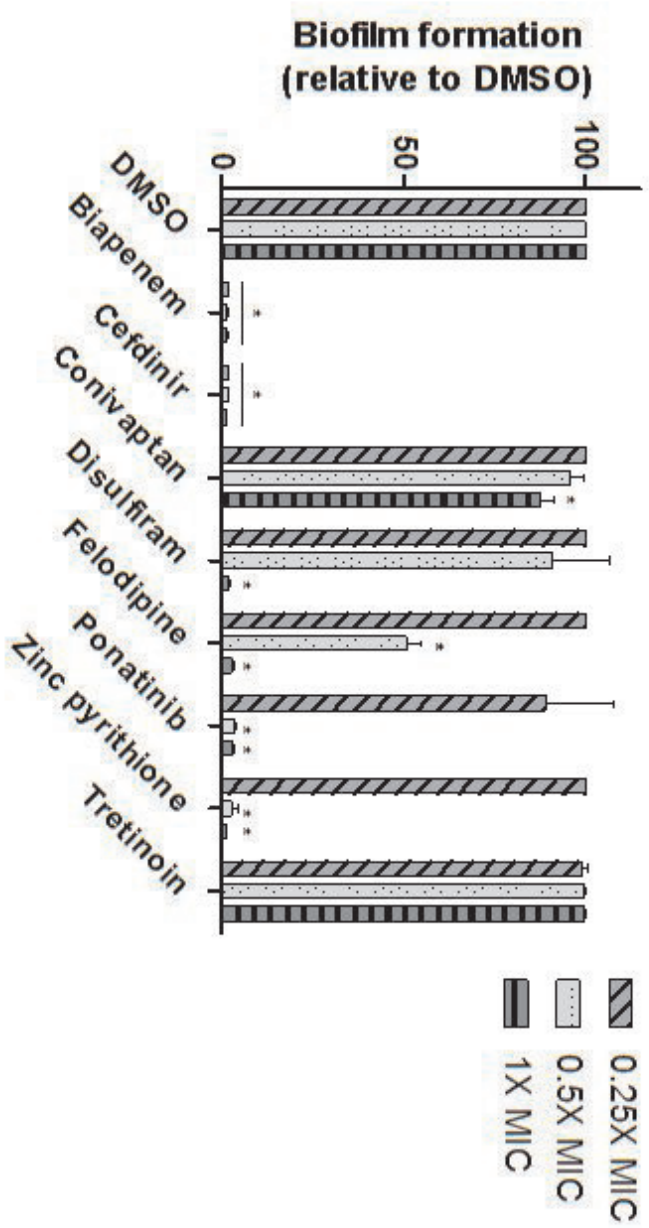


Figure 4

Figure 5A

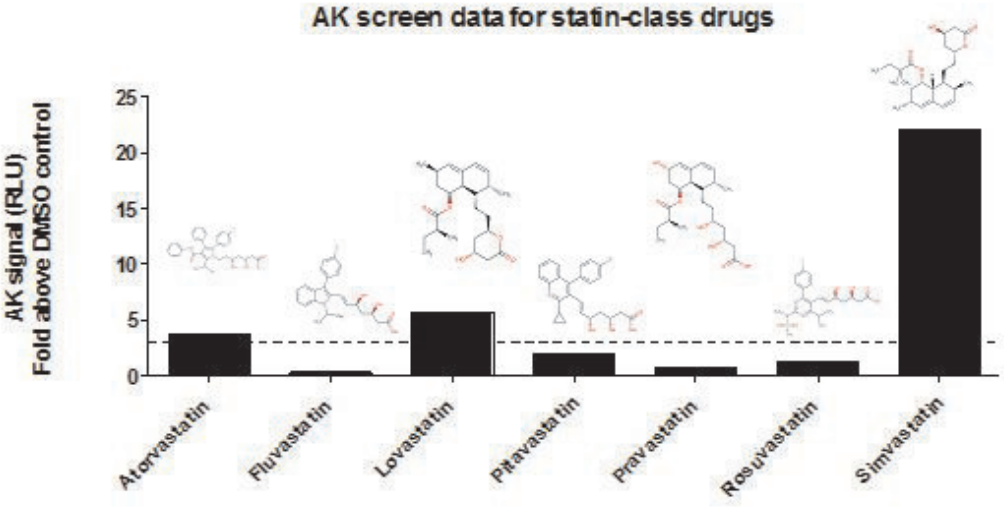


Figure 5B

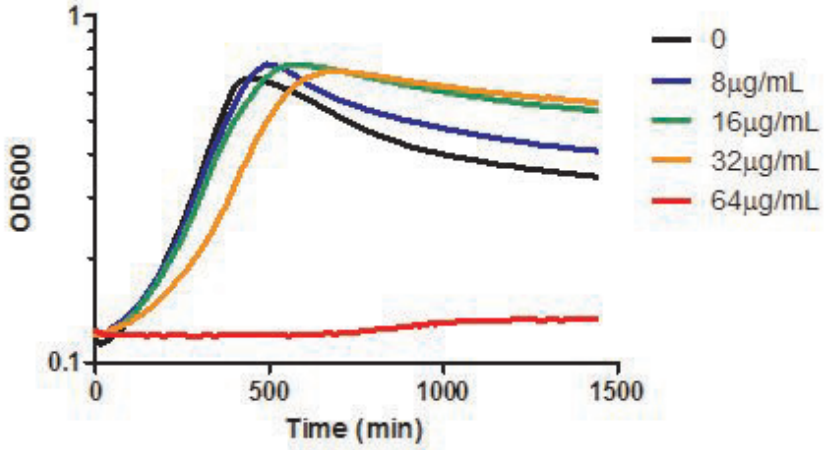


Figure 5C

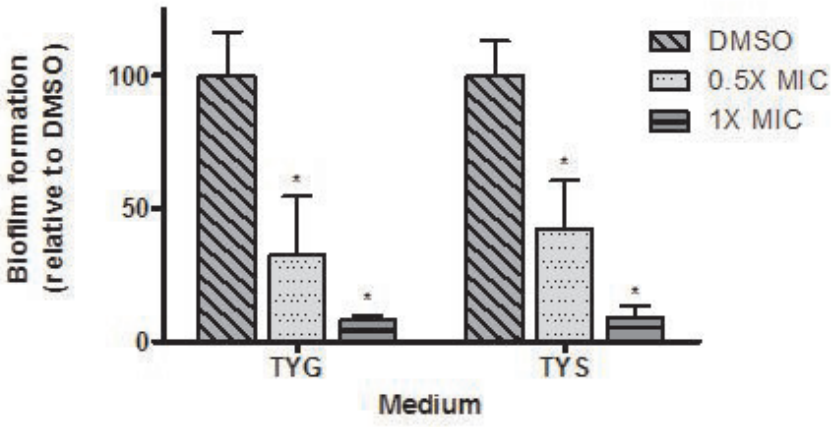


Table 1

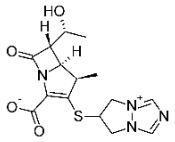
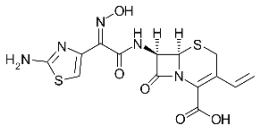
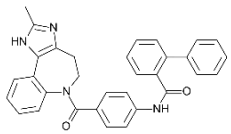
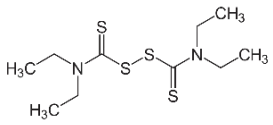
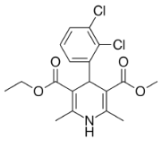
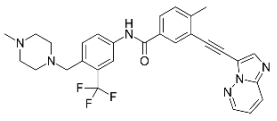
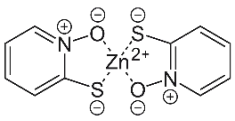
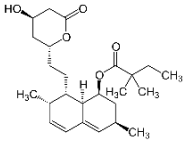
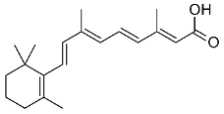
Drug	Structure	[HTS] (µg/mL)	MIC (µg/ml)	
			Planktonic	Biofilm
Biapenem		17.52	0.0625	0.0625
Cefdinir		19.77	0.25	0.25
Conivaptan		26.75	>128	128
Disulfiram		14.83	16	32
Felodipine		19.21	16	16
Ponatinib		26.63	8	4
Zinc pyrithione		15.89	1	1
Simvastatin		20.93	64	50
Tretinoin		15.02	>128	>128

Table 2

	planktonic		biofilm	
	MIC_{alone}	$MIC_{in\ combo}$	MIC_{alone}	$MIC_{in\ combo}$
disulfiram	16	4	32	2
copper	5#	0.625#	2.5#	0.625#
FIC	0.375		0.3125	