Title: Vitamin D compounds are bactericidal against *Streptococcus mutans* and target the bacitracin-associated efflux system

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Abstract

Vitamin D analogs were identified as compounds that induced lysis of planktonic cultures of *Streptococcus mutans* in a high-throughput screen of FDA-approved drugs. Previous studies have demonstrated that certain derivatives of Vitamin D possess lytic activity against other bacteria, though the mechanism has not yet been established. Through the use of a combinatorial approach, the Vitamin D derivative doxercalciferol was shown to act synergistically with bacitracin, a polypeptide-type drug that is known to interfere with cell wall synthesis, suggesting that doxercalciferol may act in a bacitracin-related pathway. Innate resistance to bacitracin is attributed to efflux by a conserved ABC-type transporter, which in *S. mutans* is encoded by the *mbrABCD* operon. *S. mutans* possesses two characterized resistance mechanisms to bacitracin including the ABC transporter, *S. mutans* bacitracin resistance (Mbr) cassette, consisting of MbrABCD, and the rhamnose-glucose polysaccharide (Rgp) system, RgpABCDEFGHI. Loss of function of the transporter, in *ΔmbrA* or *ΔmbrD* mutants, exacerbated the effect of combination of doxercalciferol and bacitracin. Despite conservation of a transporter homologous to *mbrABCD,* the combination of doxercalciferol and bacitracin appeared to only be synergistic in streptococcal species. We conclude that Vitamin D-derivatives possess lytic activity against *S. mutans* and act through a mechanism dependent on the bacitracin-resistance mechanism of MbrABCD.

Introduction

Streptococcus mutans is the primary etiological agent of dental caries, a disease that affects individuals of all ages, especially those with limited healthcare accessibility and poor socio-economic circumstances (1, 2). *S. mutans* is an early colonizer of the tooth surface that promotes binding of other oral microoragnisms to form a biofilm, known as dental plaque. As one of its primary virulence mechanisms, *S. mutans* is acidogenic, resulting in an environmental pH below that at which tooth demineralization occurs (pH ~5.5) (3-8). The organism is also aciduric, and therefore, can survive the low pH environments that it creates, thereby out-competing other oral organisms to dominate the ecological niche (3-8).

According to the Centers for Disease Control and Prevention, over-usage and incorrectly prescribed antibiotics has led to a dangerous increase in acquired resistance in the US (9), often leaving clinicians with relatively few, and often dangerous, treatment options. Efficient high-throughput strategies for identification of novel antibiotic classes, as well as adjuvants that circumvent resistance mechanisms in combination with thorough characterization of compounds, are essential to successful clinical outcomes. Adjuvants tend to have little-to-no anti-microbial capability, but in combination with specific drugs, can increase potency and block resistance.

Vitamin D is an essential nutrient and hormone that must be obtained either from the diet or from dermal synthesis. It has essential roles in the absorption of calcium, iron, magnesium, phosphate, and zinc — all of which contribute to the formation of hard tissues such as enamel and dentin (10). The health benefits of Vitamin D are wide-ranging and it has been shown to influence various metabolic systems in the body.

Serum levels of Vitamin D vary slightly depending on ethnicity, region, gender, season, and age, with acceptable values reported in the 30-68 ng/mL range, whereas deficiency was characterized as <20 ng/mL (<50 nmol/L) (11). Vitamin D has been suggested to play a role in the etiology of many chronic diseases, such that deficiency is correlated with negative outcome, as in the case of rheumatoid arthritis (12), respiratory infections (13), asthma (11), cancer (14, 15), periodontitis (16), and gingivitis (17).

The link between Vitamin D levels and caries is multifactorial and includes genetic, environmental, nutritional, and socioeconomic factors. There are detectable amounts of Vitamin D in saliva ranging from 105-1000 pg/mL depending on individual, diet, and time of day (18). One study found that increased serum levels of Vitamin D were associated with lower occurrence of dental maladies including caries and hypo-mineralization (19). In combination with calcium supplementation, Vitamin D has been shown to improve overall periodontal health relative to individuals with no supplementation, in addition to reducing the severity of pre-existing cases of periodontitis (20, 21). A review has suggested that increasing serum levels to greater than 40 ng/mL would greatly reduce caries (22), as low serum levels of 25-hydroxyvitamin D (25(OH)D) have been associated with elevated caries (23). Children with severe ECC, a chronic disease of tooth decay, have been found to have significantly lower levels of Vitamin D than caries-free children (24). Moreover, studies have reported that prenatal Vitamin D correlated with reduced occurrence of caries in infants and supported development of healthy dentition (25, 26). Interestingly, the evidence linking improved oral health and Vitamin D has led to additional work

investigating vitamin-coated dental implants to promote surrounding bone mineralization and tissue growth (27, 28).

In addition to contributing to overall well-being, many reports examining the relationship between Vitamin D and infection focus on the direct, significant immunomodulatory role of Vitamin D (reviewed in (29)). Vitamin D alters the innate immune response (30) and, in turn, immune cells differentially regulate Vitamin D-metabolizing enzymes during infection (31). One mechanism for this is that Vitamin D stimulates production of antimicrobial peptides such as cathelicidin and human β-defensin 2 as well as stimulating cell-specific receptors involved in pathogen clearance (32-34).

Single nucleotide polymorphisms of the Vitamin D receptor (VDR) gene have been correlated to patients with and without dental caries (35). Other genetic evidence for a connection between Vitamin D and oral health includes work demonstrating that Vitamin D-associated rickets (also referred to as hypo-phosphatemic rickets) is resistant to Vitamin D supplementation. This condition is, in part, characterized by osteomalacia (defect in the mineralization of bones), caused by mutation of the Vitamin D receptor (36), resulting in severe dental caries (37, 38).

Vitamin D has been associated with bacterial infection clearance and other disease processes. For example, the link between tuberculosis and Vitamin D levels has been investigated since the 1940s and has continued to be the focus of numerous studies (39-41) and reviews (42-44). Vitamin D levels have also been shown to affect the frequency of *S. aureus* infections (45, 46), as well as macrophage clearance of *Pseudomonas aeruginosa* (47). Interestingly, cytomegalovirus (CMV), has been shown

to actively down-regulate expression of the Vitamin D receptor in host cells (48). A Vitamin D decomposition product was shown to have direct bactericidal activity against *Helicobacter pylori* (49). In a monocyte model, Vitamin D inhibits the growth and virulence factor expression of *Porphyromonas gingivalis* (50). In *S. aureus,* both vitamin D and E compounds have been shown to interact with antibiotic efflux (51).

Here, we report the identification of Vitamin D drug derivatives that exhibit direct bactericidal activity against the cariogenic bacteria *Streptococcus mutans*, through a mechanism involving the bacitracin-associated efflux pump MbrA.

Materials and Methods

Strains and growth conditions

Streptococcus mutans strain UA159 (52), was maintained on Brain-Heart Infusion (BHI) 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 media + 1% (w/v) sucrose (TYS). Non-*mutans* bacteria, obtained from laboratory stocks, were maintained on BHI and grown at 37°C (in a 5% (v/v) CO_2/95% air atmosphere, for streptococcal strains).

Adenylate kinase assay

Adenylate kinase assays were performed as previously described (53, 54), 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 Inc., Corning, NY), 10^6 cells/well were combined with test molecule (in DMSO; final concentration of DMSO <0.5%) in a final volume of 100 µL. 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.

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. Drugs were serially diluted (concentrations ranging from 0-64 µg/mL) in fresh TYS and added to wells, followed by incubation for an additional 18h. After 1h equilibration at RT, 50 µl culture supernatant from each well was transferred to opaque 96-well plates with an equal volume of AK reagent (reconstituted according to manufacturer's instructions, see above). The reaction was allowed to proceed for 1h, followed by measurement of luminescence, as described above.

MIC testing

Compounds used in MIC testing were as follows: alfacalcidol and calcitriol (Selleck Chemical, Houston, TX); doxercalciferol (ApexBio, Houston, TX); ciprofloxacin, bacitracin, chloramphenicol, penicillin, streptomycin, and vancomycin (Sigma Aldrich, St. Louis, MO). To determine the MIC of test compounds against *S. mutans* UA159, an overnight culture grown in BHI medium, was diluted 1:50 in fresh TYG medium and grown to exponential phase (OD_{600} ~0.3). A 96-well plate (Corning, Inc., Corning, NY) containing fresh TYG medium was inoculated with 10^5 CFU. A dilution series of test compound (concentrations ranging from 0-64 µg/mL) was added to the plate. Plates were incubated at 37°C in a 5% (v/v) CO_2/95% air atmosphere for 24h. The MIC was considered the lowest compound concentration that inhibited bacterial growth, as measured by OD_{600}.

168 The combinatorial effect of bacitracin and doxercalciferol was determined as
169 follows: a serial dilution of bacitracin was added to a 96-well plate in the presence or
170 absence of sub-inhibitory doxercalciferol (4 µg/mL). Logarithmic phase (OD_{600nm}= ~0.5)
171 cultures were added to a final OD_{600nm} = 0.05. Plates were incubated at 37°C (in a 5%
172 (v/v) CO_2/95% air atmosphere, for streptococcal strains).
173
174 FIC testing
175 Synergy was assessed by identifying the fractional inhibitory concentration (FIC),
176 given by the equation $FIC = \frac{MIC_A}{MIC_{AB}} + \frac{MIC_B}{MIC_{AB}}$, where A and B are the two drugs tested
177 alone, or in combination (AB), and were measured using the standard checkerboard
178 method (55). FICs were interpreted according to standard definitions, where "synergy"
179 is defined as a FICI score ≤0.5, "antagonism" is defined as a FICI score >4.0 and "no
180 interaction" is a score of 0.5 ≤ FICI ≤ 4.0.
181
182 Measurement of biofilm growth by crystal violet
183 Biofilm cultures were assayed with test compounds to examine their ability to
184 prevent biofilm formation. Bacterial cultures grown to logarithmic phase in TYS were
185 added to 96-well plates and incubated at 37°C in a 5% (v/v) CO_2/95% air atmosphere
186 for ~18h. Planktonic cells were removed by washing wells 3 times with distilled water.
187 Plates were dried overnight at 70°C. Biofilms were stained with 100 µl crystal violet
188 (0.1%) for 15min then washed 5 times with distilled water. Adherent crystal violet was
189 reconstituted with acetic acid (500 mM) and plates were read with a BioRad BenchMark
190 Plus Spectrophotometer at 575nm (BioRad, Hercules, CA) (7). The minimum biofilm

inhibitory concentration (MBIC) was defined as the lowest concentration of compound that inhibited *S. mutans* biofilm formation (≥90%), as measured by crystal violet, relative to vehicle control.

Construction of the *mbrA*$^+$ complement strain

The mutant strain *ΔmbrA* was complemented using a single-copy, genomic insertion of the SMU.1006 (*mbrA*) locus, including the intergenic region between *mbrA* and *gtfC,* into the *gtfA* (SMU.881) locus using the Streptococcal integration vector pSUGK-Bgl (56). pSUGKBgl was linearized with the restriction enzyme *Bgl*II. Primers mbrA-comple-F (5'-GAGCTCGAATAGATCTGAAGTCTGAGCTGTAAATTTCTCAGG-3') and mbrA-comple-R (5'-ATTTAAAAATAGATCTTTACTCACCTCCTAACAGCGCTGCC-3') were used to amplify *mbrA* and the intergenic region between *mbrA* and *gtfC.* The resulting amplicon was ligated into the linearized pSUGKBglII using an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA) to produce pSUGKBgl-mbrA. The cloning reaction was transformed into *E. coli* Stellar (Clontech, Mountain View, CA) and positive clones were selected on LB agar medium containing kanamycin. Integrity of the construct was confirmed by sequencing. pSUGKBgl-mbrA was transformed into *S. mutans* Δ*mbrA* and selected on BHI agar medium containing kanamycin. The complemented strain was designated *mbrA*$^+$ and integrity of the complemented locus was confirmed by sequencing with gtfA-Seqkan (5'-GATGTTCAACACTGCCATCTG-3') (57).

Time-kill assay

The kinetics of the bacitracin-doxercalciferol interaction were analyzed by a standard bacterial time-kill assay. *S. mutans* strains MX804, *ΔmbrA*, and *mbrA*$^{+}$ were grown to logarithmic phase (OD_{600nm} ~0.5) and added to TYG (10^6 cells/mL final) containing doxercalciferol (16 µg/mL), bacitracin (32 µg/ml), doxercalciferol & bacitracin, or no drug (DMSO control). Aliquots were taken at 0, 2, 4, 24, 48h after inoculation, serially diluted, and plated on BHI agar medium for enumeration. Data were either normalized to CFU/mL at t=0h and plotted over time, or normalized to CFU/mL of the no drug control for each time point.

Results

Large-scale drug screen revealed that Vitamin D analogs are active against S. mutans

The adenylate kinase (AK) assay was used to screen a library of FDA-approved drugs from Selleck, for activity against *Streptococcus mutans* (54). Of the 853 drugs in the Selleck library, we found 126 drugs that significantly induced lysis of planktonic cells (2-fold above background). Surprisingly, one class of compounds that showed activity against *S. mutans* were various derivatives of the fat-soluble secosteroid, Vitamin D. Analysis of the structure and activity of these compounds revealed that breakage of the steroid ring is required for detection of significant adenylate kinase release, as in the example of lithocholic acid, which did not exhibit bactericidal activity. The three compounds in this class that exhibited the greatest lytic activity against planktonic cells were alfacalcidol, doxercalciferol, and calcitriol (Figure 1), all possessing two hydroxyl-groups on the methylene-cyclohexane ring, implicating a potential role for this structure in activity of the compound. Vitamin D derivatives that do not possess the two-hydroxyl groups did not result in significant lysis relative to background.

All three compounds inhibited growth of WT cells at an MIC of 16 µg/mL. In order to further characterize the activity of the Vitamin D-like compounds, we tested a range of concentrations using the AK assay. *S. mutans* planktonic cultures (OD_{600} ~0.5) were exposed to the test drugs or ciprofloxacin (positive control; MIC: 2 µg/mL) ranging from 0.25X-4X the MIC for 4h followed by read-out with the AK assay. These conditions were chosen to mimic the parameters established in our primary screen, and further validate those results. Treatment with alfacalcidol, calcitriol, or doxercalciferol resulted in at least two-fold higher signal, relative to background (DMSO), in the AK

assay between 1X and 2X MIC (16-32 µg/mL), which is in the range of the concentrations used during the initial drug screen (54). Similar to our initial screen, both alfacalcidol and doxercalciferol exhibited similar activity against planktonic cultures, whereas calcitriol signal was slightly above the 2-fold above background cut-off (54). These results demonstrate that, in agreement with our initial screen, the Vitamin D-analogs tested exhibited lytic activity against *S. mutans.*

Vitamin D analogs have activity against biofilms of S. mutans

S. mutans is found in the oral cavity of humans as part of a multi-species biofilm known as dental plaque. Therefore, it was essential to test whether alfacalcidol, calcitriol, or doxercalciferol had potential to prevent biofilm formation. Despite having similar structures, as well as the ability to lyse planktonic cells, the three Vitamin D analogs exhibited ability to prevent *S. mutans* biofilm formation. The minimum biofilm inhibitory concentration (MBIC) of doxercalciferol and alfacalcidol was 64 µg/mL ($MBIC_{90}$) and 128 µg/mL ($MBIC_{50}$), respectively; whereas, calcitriol did not inhibit biofilm formation at any concentration tested (Figure 2B).

The AK assay also serves as a rapid and sensitive method to detect compounds with activity against pre-formed biofilms. Unlike results from the AK assay using planktonic cultures, only calcitriol and doxercalciferol were able to induce lysis of pre-formed biofilms, suggesting that these similarly structured drugs have different activities under different conditions (Figure 2A vs. 2C). Addition of alfacalcidol did not result in significant signal relative to DMSO control, indicating that it does not possess activity against pre-formed biofilms. Addition of calcitriol resulted in a 40-fold increase in signal

268 relative to DMSO control, making it the most active compound tested under these
269 conditions (Figure 2C). These results demonstrate that analogs of Vitamin D are able to
270 prevent *S. mutans* biofilm formation, as well as have activity against pre-formed
271 biofilms.
272
273 *Doxercalciferol exhibits synergistic activity in combination with bacitracin*
274 Recently, 1,25$(OH)_2D_3$ (Vitamin D) was shown to inhibit the growth of the oral
275 pathogen *Porphyromonas gingivalis* with a MIC between 3.2-6.25 µg/mL (50). In
276 addition to its effect on growth, the Vitamin D derivative was shown to potentially
277 interact with a cell-wall targeting antibiotic. In order to dissect the mechanism of growth
278 inhibition by Vitamin D on *S. mutans*, we used a combinatorial approach to assay a 2-
279 fold dilution series of drugs known to target the bacterial cell membrane. As
280 alfacalcidol, doxercalciferol, and calcitriol possessed different activity in initial
281 characterization of the Vitamin D derivatives, we proceeded with doxercalciferol as it
282 had the most consistent activity in our secondary assays. Two-fold dilution series of the
283 antibiotics bacitracin, chloramphenicol (control), ciprofloxacin (control), penicillin,
284 streptomycin, and vancomycin were all tested in the presence or absence of
285 doxercalciferol (16 µg/mL) (Figure 3). The growth of *S. mutans* in the presence of
286 bacitracin was unaltered relative to the no drug control, which was expected, as
287 bacitracin resistance in *S. mutans* has been characterized (58). However, in the
288 presence of doxercalciferol, bacitracin inhibited the growth of *S. mutans* at
289 concentrations well below bacitracin alone (>256 µg/mL). The MICs of chloramphenicol
290 and ciprofloxacin were not altered in the presence of doxercalciferol, suggesting that the

combinatorial effect of Vitamin D and bacitracin is not a generic phenomenon. Further, the absence of synergy with other tested cell membrane-targeting drugs suggests that the target for Vitamin D-mediated growth inhibition might be analogous to pathways of bacitracin resistance.

The ABC-Transporter, MBR, has a role in the interaction between doxercalciferol and bacitracin

There are two proposed mechanisms for the innate resistance to bacitracin in *S. mutans*, including rhamnose-glucose polysaccharide (RGP)-associated formation within the cell wall or the *S. mutans*-associated bacitracin resistance (MBR) efflux pump (58, 59). In order to distinguish between these mechanisms, we tested the combination of bacitracin and doxercalciferol in the *S. mutans* strains carrying deletions in *rgpF* (SMU.830) and *mbrA* (SMU.1006), which encode key subunits associated with each respective mechanism. We predicted a mutant in the target would be more sensitive to the combination of bacitracin and doxercalciferol than the parent strain, MX804, which is an Erm^R knock-in strain similar to *S. mutans* UA159 (7). The erythromycin selectable marker had no detectable effect on susceptibility to bacitracin or doxercalciferol (data not shown).

Using a checkerboard approach of serial dilution of bacitracin along the x-axis and doxercalciferol along the y-axis, we measured the fractional inhibitory concentration. In the presence of bacitracin alone, the MIC for the parent strain, MX804, was >128 µg/mL, confirming that *S. mutans* exhibits innate resistance to bacitracin (Table 2). Loss of components of the MBR transporter in Δ*mbrA* or Δ*mbrD*,

314 resulted in a significant reduction in the bacitracin MIC to 2 µg/mL and 8 µg/mL,
315 respectively, indicating a loss of bacitracin resistance. Similarly, Δ*rgpF* exhibited a
316 reduction in the MIC of bacitracin confirming its previously documented role in
317 resistance (58). Strains MX804, Δ*mbrA*, and ΔrgpF each exhibited an MIC of 16 µg/mL
318 for doxercalciferol, while Δ*mbrD* was 8 µg/mL. Presence of doxercalciferol significantly
319 reduced the amount of bacitracin that inhibited growth of MX804 (FIC: 0.125), Δ*mbrA*
320 (FIC: 0.25), and Δ*mbrD* (FIC: 0.5), indicating a synergistic interaction, according to
321 standard definitions (55). In contrast, the FIC for the combination of bacitracin and
322 doxercalciferol in Δ*rgpF* was 1.125, indicating no interaction.
323 In order to establish if doxercalciferol was acting as a general efflux pump
324 inhibitor or was specific to bacitracin-associated efflux, we tested the combination of
325 doxercalciferol and bacitracin in a deletion strain lacking a subunit of a well-
326 characterized efflux pump, *pmrA*. The *pmrA* transporter has previously been described
327 in *Streptococcus pneumoniae* and is associated with efflux of fluoroquinolone-class
328 antibiotics (60). Addition of doxercalciferol to Δ*pmrA* in the presence of bacitracin or
329 ciprofloxacin (as a control) resulted in a similar susceptibility pattern as MX804 (Table
330 2). The lack of synergy between doxercalciferol and bacitracin suggests that
331 doxercalciferol is not a general efflux pump inhibitor and may act directly with the MBR
332 efflux pump.
333
334 *Kinetics of doxercalciferol activity in combination with bacitracin*
335 Results from the AK assay demonstrated the bactericidal activity of
336 doxercalciferol, and other vitamin D analogs, against *S. mutans* (Figure 1) and fractional

337 inhibition studies elucidated an interaction between bacitracin and doxercalciferol
338 (Figure 3B). In order to further characterize the efficacy of the combination of bacitracin
339 and doxercalciferol, we examined the killing kinetics via a time-kill assay. Strains
340 MX804, *ΔmbrA,* or *mbrA*$^{+}$ were grown to mid-log and seeded into fresh TYG with and
341 without bacitracin (32 µg/mL) or doxercalciferol (16 µg/mL). Cells were enumerated at
342 0, 2, 4, 24, and 48h after inoculation, as described in Materials and Methods.
343 Addition of doxercalciferol to cultures of MX804, *ΔmbrA,* or *mbrA*$^{+}$ resulted in a 3-
344 log order decrease in survival after 4h of exposure (Figure 4A). This finding is in
345 agreement with results from the AK assay, as significant levels of signal were detected
346 after the 4h incubation period with drug for that assay (Figure 2A). Although, the initial
347 effect of doxercalciferol appears to be bactericidal, cell counts of all strains were
348 recovered between 4 and 24h. Bacitracin alone did not alter the survival of MX804
349 cultures, in contrast to *ΔmbrA*, whose survival decreased 2 log orders within 48h after
350 inoculation (Figure 4B), confirming the previously described role of the MBR-efflux
351 system in bacitracin resistance (58, 61). The viability of the complement strain, *mbrA*$^{+}$,
352 was intermediate to MX804 and *ΔmbrA,* indicating that addition of *mbrA* back into the
353 genome partially restored function with respect to bacitracin resistance.
354 The combination of doxercalciferol and bacitracin resulted in a significant
355 decrease in survival of the MX804 strain at 2, 4, and 48h (Figure 4C). Interestingly, the
356 CFU at 24h in the MX804 cultures appeared elevated, compared to the earlier time
357 points. However, according to Figure 5C, there was no statistically significant increase
358 relative to the no drug control. The *ΔmbrA* strain displayed a similar reduction in
359 viability after 2 and 4h exposure to both drugs; however, unlike MX804, did not recover

360 at 24h and was inviable by 48h. Complementation of *ΔmbrA* resulted in partial
361 restoration of resistance, observed at 24 and 48h in *mbrA*$^{+}$. These results are
362 consistent with an interaction between bacitracin and doxercalciferol and suggest that
363 the MBR-transporter is involved in this mechanism.

364 All of the strains tested (MX804, *ΔmbrA*, and *mbrA*$^{+}$) had similar growth rates and
365 similar reduction in viability between 24-48h (Figure 4D). In order to determine that
366 doxercalciferol and bacitracin, alone or in combination, were responsible for the
367 decrease in cell count, we normalized the time-kill data to a no drug control at each time
368 point, to allow for assessment of relative effects of both drugs at individual time points.

369 After 2h treatment, addition of bacitracin only did not alter CFU recovered from
370 MX804 cultures, but both *ΔmbrA* and *mbrA*$^{+}$ showed a slight decrease (0.53 and 0.33
371 log, respectively), relative to no drug (Figure 5A, compare bars with horizontal lines).
372 While there was no significant decrease in viability in cultures where doxercalciferol was
373 added (Figure 5A, bars with vertical lines), the combination of bacitracin and
374 doxercalciferol resulted in a greater decrease in recovery of MX804 (1 log), *ΔmbrA* (1
375 log), and *mbrA*$^{+}$ (0.44 log), relative to no drug (Figure 5A, compare bars with cross-
376 hatch).

377 All strains showed a reduction in cell viability of approximately 2.5 log orders after
378 exposure to doxercalciferol alone, relative to the no drug control (Figure 4B), confirming
379 results observed with the AK assay (Figure 2A). Addition of bacitracin resulted in a
380 slight, but significant, reduction in survival of all strains at 4h. The combination of
381 bacitracin and doxercalciferol exhibited the greatest reduction in survival of the *ΔmbrA*

strain (3.3 log), compared to either compound alone (cf. Figure 4C vs. Figures 4A & 4B).

Cultures of *S. mutans* MX804 were fully recovered after 24h treatment with all drugs, which may in part be due to resistance to bacitracin, as well as drug concentrations either at or below the MIC used during the time-kill. The *mbrA* mutant and complement strains exhibited a similar phenotype to MX804, with the exception of the combination of doxercalciferol and bacitracin, which exhibited a 4- and 3.5-log decrease in viability at 24h, respectively, relative to the no drug control.

MX804 cultures fully recovered after 48h treatment with all drugs, similar to cultures of *ΔmbrA* and *mbrA*$^{+}$ treated with both compounds (Figure 5D). Cultures of *ΔmbrA* were completely inviable after 48h, whereas the *mbrA*$^{+}$ strain exhibited an approximately 3-log decrease, relative to no drug control. These results support our hypothesis that doxercalciferol possesses bactericidal activity and acts through a bacitracin-associated efflux pump mechanism.

The interaction of doxercalciferol with bacitracin may be specific to streptococci

The *mbrA* membrane component of the bacitracin-associated efflux pump is conserved amongst Gram-positive and Gram-negative bacteria. In fact, many streptococci and lactococci encode genes for multiple ABC-transporter proteins with significant sequence homology to MbrA, many of which have been investigated for their drug efflux-associated functions (62, 63).

Therefore, we investigated whether the presence of MbrA contributes to the mechanism by which doxercalciferol interacts with bacitracin in organisms that encode

genes homologous to *mbrA*. To test this hypothesis, we measured the MIC of bacitracin in the presence or absence of doxercalciferol in a variety of Gram-positive and Gram-negative bacteria, many of which have been used to study mechanisms of bacitracin resistance (64-69). We chose a fixed concentration of doxercalciferol that exhibited a significant effect on *S. mutans* growth in the presence of bacitracin (4 µg/mL).

Results outlined in Table 3 indicate that the presence of *mbrA*-like genes does not necessitate that doxercalciferol exert a significant reduction in bacitracin MIC. The Gram-negative organisms examined as part of this study were not sensitive to bacitracin, and the presence of doxercalciferol did not alter their sensitivity. Of the remaining 10 organisms tested, 5 (50%) exhibited a > 2-fold reduction in the MIC of bacitracin, in the presence of 4 µg/mL doxercalciferol (Table 3). The data suggest that this may be a streptococci-specific phenomenon, as we observed that 5 of the 6 streptococcal species (including the near-neighbor of streptococci, *Enterococcus faecalis*) tested here demonstrated a similar reduction in the MIC of bacitracin when measured in the presence of doxercalciferol. Other Gram-positive bacteria, such as *L. lactis*, *S. aureus,* and *S. sanguinis* exhibited a slight (2-fold) reduction in MIC of bacitracin in the presence of doxercalciferol, possibly due to the doxercalciferol-specific antibacterial activity rather than to potential interaction with an efflux pump.

Discussion

Using the AK assay to screen the Selleck library of 853 FDA-approved drugs, we identified 126 compounds with activity against *S. mutans* planktonic cultures (54). One

428 class of compounds with novel activity against *S. mutans* included several derivatives of
429 the secosteroid Vitamin D. Doxercalciferol is a synthetic vitamin D2 analog used in the
430 treatment of hyperparathyroidism. Although other groups have reported the
431 antibacterial properties of Vitamin D derivatives, this is the first report, to our knowledge,
432 of the interaction with bacitracin as a possible mechanism.

433 Previous reports have demonstrated antibacterial activity of Vitamin D-like
434 compounds against both Gram-positive and Gram-negative bacteria. Treatment with
435 doxercalciferol led to a loss of bacterial viability and cell lysis, similar to the results seen
436 previously with other Vitamin D analogs (49, 50). Interestingly, treatment with
437 doxercalciferol initially led to a significant decrease in cell viability, followed by recovery
438 to levels similar to the no drug control (Figure 4A). These data may suggest that the
439 mechanism of doxercalciferol has different targets that may change throughout time or
440 that cellular machinery needs to be induced in order to overcome the effects of
441 doxercalciferol. In *S. aureus,* combination of Vitamin D and antibiotics in efflux-
442 associated resistant strains resulted in restoration of antibiotic activity, suggesting that
443 Vitamin D may have a role in actively inhibiting antibiotic efflux (51). The activity
444 observed with doxercalciferol is consistent with the definition of an adjuvant, which is a
445 small molecule with little-to-no anti-microbial activity alone that enhances the activity of
446 another drug.

447 Bacitracin resistance has been explored in both Gram-positive (66-71) and, to a
448 lesser extent, in Gram-negative bacteria (64, 65, 72). Although, in *S. mutans* it has
449 been partially attributed to the RGP-associated genes, a commonly recognized
450 mechanism of resistance is the role of ABC-transporters, such as *mbrABCD.* In *S.*

mutans, the *mbrA* gene is located downstream of the glucosyltransferase-encoding genes *gtfB* and *gtfC*. The MBR operon consists of *mbrAB* (SMU.1006 and SMU.1007), the ABC-transporter, and *mbrCD* (SMU.1008 and SMU.1009), a putative two-component system (61).

The *mbrABCD* ABC-transporter is conserved in Gram-positive and Gram-negative bacteria. We tested a variety of bacteria, which all had ABC-transporters with significant homology to *mbrAB* (E<1e-45). We found that despite the presence of transport systems homologous to *SmmbrABCD*, the additive effect of doxercalciferol on bacitracin susceptibility is limited to streptococci (Table 3).

The *mbrAB* transporter is similar to the well-characterized ABC-type lipoprotein and macrolide export system of *E. coli* known as LolCDE (reviewed in (73)). Addition of a LolCDE inhibitor resulted in upregulation of stress response as well as transport, including that of multidrug efflux systems (74). Dysregulation of transport systems in doxercalciferol-treated samples or *mbrABCD* mutant strains, may explain altered antibiotic susceptibilities that were observed (Figure 3 and (58)). In contrast to the effect of doxercalciferol on *S. mutans*, the inhibitor had a low MIC (0.25 µg/mL) likely because components of *Ec*LolCDE are essential (75). While loss of *ΔmbrA* or *ΔmbrB* in *S. mutans* did not exhibit any appreciable phenotypes, relative to the parent strain MX804, under the conditions tested (7), mutants in the *E. coli* LolCDE system are inviable, which results in the accumulation of lipoproteins in the inner membrane (75). Lipoprotein transport and membrane synthesis function has also been attributed both to *mbrABCD* and LolCDE.

473 Bacitracin inhibits dephosphorylation of C55-isoprenyl pyrophosphate, thus
474 interfering with peptidoglycan synthesis. There are several possible mechanisms of
475 action that account for the lytic activity of doxercalciferol (and other Vitamin D analogs)
476 as well as the synergism with bacitracin. Based on the FIC of bacitracin and
477 doxercalciferol in mutants of the bacitracin-associated efflux system (Table 2), it is
478 possible that doxercalciferol circumvents the bacitracin-resistance mechanism in *S.*
479 *mutans* by directly inhibiting bacitracin efflux. However, doxercalciferol failed to inhibit
480 general efflux systems in standard ethidium bromide efflux assays (data not shown). As
481 with many efflux pump inhibitors, doxercalciferol and analogs are lipid-soluble and may
482 interact with the membrane, thus exacerbating the effect of bacitracin. However, other
483 strains carrying deletions in cell wall-related genes were tested and they did not exhibit
484 altered susceptibility to either doxercalciferol or the combination of doxercalciferol and
485 bacitracin (data not shown). Although these mechanisms are not mutually-exclusive,
486 further characterization of the effect of doxercalciferol on *S. mutans* will be necessary to
487 identify specific targets.

488 In conclusion, we found that addition of doxercalciferol a Vitamin D derivative, to
489 *S. mutans* cultures resulted in a time-dependent lytic activity that acts via a bacitracin-
490 resistance-dependent mechanism. Further, this activity is specific to Streptococcal (and
491 closely related) species. Other Vitamin D analogs may prove to be more potent
492 inhibitors of streptococcal species. The broader implications of a compound with robust
493 immunomodulatory roles and growing evidence of antimicrobial activity are exciting.

494

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References

1. **Dye BA, Barker LK, Selwitz RH, Lewis BG, Wu T, Fryar CD, Ostchega Y, Beltran ED, Ley E.** 2007. Overview and quality assurance for the National Health and Nutrition Examination Survey (NHANES) oral health component, 1999-2002. Community Dentistry and Oral Epidemiology **35:**140-151.
2. **Dye BA, Thornton-Evans G.** 2010. Trends in Oral Health by Poverty Status as Measured by Healthy People 2010 Objectives. Public Health Reports **125:**817-830.
3. **Guo L, McLean JS, Lux R, He X, Shi W.** 2015. The well-coordinated linkage between acidogenicity and aciduricity via insoluble glucans on the surface of *Streptococcus mutans*. Sci Rep **5:**18015.
4. **Lemos JA, Burne RA.** 2008. A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology **154:**3247-3255.
5. **Baker JL, Faustoferri RC, Quivey RG, Jr.** 2017. Acid-adaptive mechanisms of *Streptococcus mutans*-the more we know, the more we don't. Mol Oral Microbiol **32:**107-117.
6. **Loesche WJ.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev **50:**353-380.
7. **Quivey RG, Jr., Grayhack EJ, Faustoferri RC, Hubbard CJ, Baldeck JD, Wolf AS, MacGilvray ME, Rosalen PL, Scott-Anne K, Santiago B, Gopal S, Payne J, Marquis RE.** 2015. Functional profiling in *Streptococcus mutans*: construction and examination of a genomic collection of gene deletion mutants. Mol Oral Microbiol **30:**474-495.
8. **Lemos JA, Quivey RG, Jr., Koo H, Abranches J.** 2013. *Streptococcus mutans*: a new Gram-positive paradigm? Microbiology **159:**436-445.
9. **Hampton T.** 2013. Report reveals scope of US antibiotic resistance threat. JAMA **310:**1661-1663.
10. **Berdal A, Papagerakis P, Hotton D, Bailleul-Forestier I, Davideau JL.** 1995. Ameloblasts and odontoblasts, target-cells for 1,25-dihydroxyvitamin D3: a review. Int J Dev Biol **39:**257-262.
11. **Shaikh MN, Malapati BR, Gokani R, Patel B, Chatriwala M.** 2016. Serum magnesium and vitamin D levels as indicators of asthma severity. Pulm Med **2016:**1643717.
12. **Cutolo M, Otsa K, Laas K, Yprus M, Lehtme R, Secchi ME, Sulli A, Paolino S, Seriolo B.** 2006. Circannual vitamin D serum levels and disease activity in rheumatoid arthritis: Northern versus Southern Europe. Clin Exp Rheumatol **24:**702-704.
13. **Miragliotta G, Miragliotta L.** 2014. Vitamin D and infectious diseases. Endocr Metab Immune Disord Drug Targets **14:**267-271.
14. **Yao S, Kwan ML, Ergas IJ, Roh JM, Cheng TD, Hong CC, McCann SE, Tang L, Davis W, Liu S, Quesenberry CP, Jr., Lee MM, Ambrosone CB, Kushi LH.** 2016. Association of serum level of vitamin D at diagnosis with breast cancer survival: A case-cohort analysis in the pathways study. JAMA Oncol doi:10.1001/jamaoncol.2016.4188.

15. **Hatse S, Lambrechts D, Verstuyf A, Smeets A, Brouwers B, Vandorpe T, Brouckaert O, Peuteman G, Laenen A, Verlinden L, Kriebitzsch C, Dieudonne AS, Paridaens R, Neven P, Christiaens MR, Bouillon R, Wildiers H.** 2012. Vitamin D status at breast cancer diagnosis: correlation with tumor characteristics, disease outcome, and genetic determinants of vitamin D insufficiency. Carcinogenesis **33:**1319-1326.
16. **Laky M, Bertl K, Haririan H, Andrukhov O, Seemann R, Volf I, Assinger A, Gruber R, Moritz A, Rausch-Fan X.** 2016. Serum levels of 25-hydroxyvitamin D are associated with periodontal disease. Clin Oral Investig doi:10.1007/s00784-016-1965-2.
17. **Dietrich T, Nunn M, Dawson-Hughes B, Bischoff-Ferrari HA.** 2005. Association between serum concentrations of 25-hydroxyvitamin D and gingival inflammation. Am J Clin Nutr **82:**575-580.
18. **Fairney A, Saphier PW.** 1987. Studies on the measurement of 25-hydroxy vitamin D in human saliva. Br J Nutr **57:**13-25.
19. **Kuhnisch J, Thiering E, Kratzsch J, Heinrich-Weltzien R, Hickel R, Heinrich J, group GIs, group LIs.** 2015. Elevated serum 25(OH)-vitamin D levels are negatively correlated with molar-incisor hypomineralization. J Dent Res **94:**381-387.
20. **Garcia MN, Hildebolt CF, Miley DD, Dixon DA, Couture RA, Spearie CL, Langenwalter EM, Shannon WD, Deych E, Mueller C, Civitelli R.** 2011. One-year effects of vitamin D and calcium supplementation on chronic periodontitis. J Periodontol **82:**25-32.
21. **Miley DD, Garcia MN, Hildebolt CF, Shannon WD, Couture RA, Anderson Spearie CL, Dixon DA, Langenwalter EM, Mueller C, Civitelli R.** 2009. Cross-sectional study of vitamin D and calcium supplementation effects on chronic periodontitis. J Periodontol **80:**1433-1439.
22. **Grant WB.** 2011. A review of the role of solar ultraviolet-B irradiance and vitamin D in reducing risk of dental caries. Dermatoendocrinol **3:**193-198.
23. **Schroth RJ, Rabbani R, Loewen G, Moffatt ME.** 2016. Vitamin D and dental caries in children. J Dent Res **95:**173-179.
24. **Schroth RJ, Levi JA, Sellers EA, Friel J, Kliewer E, Moffatt ME.** 2013. Vitamin D status of children with severe early childhood caries: a case-control study. BMC Pediatr **13:**174.
25. **Schroth RJ, Lavelle C, Tate R, Bruce S, Billings RJ, Moffatt ME.** 2014. Prenatal vitamin D and dental caries in infants. Pediatrics **133:**e1277-1284.
26. **Karras SN, Fakhoury H, Muscogiuri G, Grant WB, van den Ouweland JM, Colao AM, Kotsa K.** 2016. Maternal vitamin D levels during pregnancy and neonatal health: evidence to date and clinical implications. Ther Adv Musculoskelet Dis **8:**124-135.
27. **Satue M, Gomez-Florit M, Monjo M, Ramis JM.** 2016. Improved human gingival fibroblast response to titanium implants coated with ultraviolet-irradiated vitamin D precursor and vitamin E. J Periodontal Res **51:**342-349.
28. **Satue M, Monjo M, Ronold HJ, Lyngstadaas SP, Ramis JM.** 2017. Titanium implants coated with UV-irradiated vitamin D precursor and vitamin E: in vivo performance and coating stability. Clin Oral Implants Res **28:**424-431.

29. **Youssef DA, Miller CW, El-Abbassi AM, Cutchins DC, Cutchins C, Grant WB, Peiris AN.** 2011. Antimicrobial implications of vitamin D. Dermatoendocrinol **3:**220-229.
30. **Kundu R, Theodoraki A, Haas CT, Zhang Y, Chain B, Kriston-Vizi J, Noursadeghi M, Khoo B.** 2016. Cell-type-specific modulation of innate immune signalling by vitamin D in human mononuclear phagocytes. Immunol doi:10.1111/imm.12669.
31. **Stoffels K, Overbergh L, Giulietti A, Verlinden L, Bouillon R, Mathieu C.** 2006. Immune regulation of 25-hydroxyvitamin-D3-1alpha-hydroxylase in human monocytes. J Bone Miner Res **21:**37-47.
32. **Kroner Jde C, Sommer A, Fabri M.** 2015. Vitamin D every day to keep the infection away? Nutrients **7:**4170-4188.
33. **Gombart AF.** 2009. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. Future Microbiol **4:**1151-1165.
34. **Guo C, Gombart AF.** 2014. The antibiotic effects of vitamin D. Endocr Metab Immune Disord Drug Targets **14:**255-266.
35. **Hu XP, Li ZQ, Zhou JY, Yu ZH, Zhang JM, Guo ML.** 2015. Analysis of the association between polymorphisms in the vitamin D receptor (VDR) gene and dental caries in a Chinese population. Genet Mol Res **14:**11631-11638.
36. **Malloy PJ, Zhou Y, Wang J, Hiort O, Feldman D.** 2011. Hereditary vitamin D-resistant rickets (HVDRR) owing to a heterozygous mutation in the vitamin D receptor. J Bone Miner Res **26:**2710-2718.
37. **Hochberg Z, Weisman Y.** 1995. Calcitriol-resistant rickets due to vitamin D receptor defects. Trends Endocrinol Metab **6:**216-220.
38. **Sabandal MM, Robotta P, Burklein S, Schafer E.** 2015. Review of the dental implications of X-linked hypophosphataemic rickets (XLHR). Clin Oral Investig **19:**759-768.
39. **Zeng J, Wu G, Yang W, Gu X, Liang W, Yao Y, Song Y.** 2015. A serum vitamin D level <25nmol/l pose high tuberculosis risk: a meta-analysis. PLoS One **10:**e0126014.
40. **Salamon H, Bruiners N, Lakehal K, Shi L, Ravi J, Yamaguchi KD, Pine R, Gennaro ML.** 2014. Cutting edge: Vitamin D regulates lipid metabolism in *Mycobacterium tuberculosis* infection. J Immunol **193:**30-34.
41. **Tukvadze N, Sanikidze E, Kipiani M, Hebbar G, Easley KA, Shenvi N, Kempker RR, Frediani JK, Mirtskhulava V, Alvarez JA, Lomtadze N, Vashakidze L, Hao L, Del Rio C, Tangpricha V, Blumberg HM, Ziegler TR.** 2015. High-dose vitamin D3 in adults with pulmonary tuberculosis: a double-blind randomized controlled trial. Am J Clin Nutr **102:**1059-1069.
42. **Turnbull ER, Drobniewski F.** 2015. Vitamin D supplementation: a comprehensive review on supplementation for tuberculosis prophylaxis. Expert Rev Respir Med **9:**269-275.
43. **Facchini L, Venturini E, Galli L, de Martino M, Chiappini E.** 2015. Vitamin D and tuberculosis: a review on a hot topic. J Chemother **27:**128-138.
44. **Davies PD, Martineau AR.** 2015. Vitamin D and tuberculosis: more effective in prevention than treatment? Int J Tuberc Lung Dis **19:**876-877.

45. **Wang JW, Hogan PG, Hunstad DA, Fritz SA.** 2015. Vitamin D sufficiency and *Staphylococcus aureus* infection in children. Pediatr Infect Dis J **34:**544-545.
46. **Thomason J, Rentsch C, Stenehjem EA, Hidron AI, Rimland D.** 2015. Association between vitamin D deficiency and methicillin-resistant *Staphylococcus aureus* infection. Infection **43:**715-722.
47. **Nouari W, Ysmail-Dahlouk L, Aribi M.** 2016. Vitamin D3 enhances bactericidal activity of macrophage against *Pseudomonas aeruginosa*. Int Immunopharmacol **30:**94-101.
48. **Rieder FJ, Groschel C, Kastner MT, Kosulin K, Laengle J, Zadnikar R, Marculescu R, Schneider M, Lion T, Bergmann M, Kallay E, Steininger C.** 2016. Human cytomegalovirus infection downregulates vitamin-D receptor in mammalian cells. J Steroid Biochem Mol Biol doi:10.1016/j.jsbmb.2016.08.002.
49. **Hosoda K, Shimomura H, Wanibuchi K, Masui H, Amgalanbaatar A, Hayashi S, Takahashi T, Hirai Y.** 2015. Identification and characterization of a vitamin D(3) decomposition product bactericidal against *Helicobacter pylori*. Sci Rep **5:**8860.
50. **Grenier D, Morin MP, Fournier-Larente J, Chen H.** 2016. Vitamin D inhibits the growth of and virulence factor gene expression by *Porphyromonas gingivalis* and blocks activation of the nuclear factor kappa B transcription factor in monocytes. J Periodontal Res **51:**359-365.
51. **Tintino SR, Morais-Tintino CD, Campina FF, Pereira RL, Costa Mdo S, Braga MF, Limaverde PW, Andrade JC, Siqueira-Junior JP, Coutinho HD, Balbino VQ, Leal-Balbino TC, Ribeiro-Filho J, Quintans-Junior LJ.** 2016. Action of cholecalciferol and alpha-tocopherol on *Staphylococcus aureus* efflux pumps. EXCLI J **15:**315-322.
52. **Ajdic D, McShan WM, McLaughlin RE, Savic G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Qian Y, Li S, Zhu H, Najar F, Lai H, White J, Roe BA, Ferretti JJ.** 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad Sci U S A **99:**14434-14439.
53. **Jacobs AC, Didone L, Jobson J, Sofia MK, Krysan D, Dunman PM.** 2013. Adenylate kinase release as a high-throughput-screening-compatible reporter of bacterial lysis for identification of antibacterial agents. Antimicrob Agents Chemother **57:**26-36.
54. **Saputo S, Faustoferri, R.C. and Quivey Jr., R.G.** 2017. A drug repositioning approach reveals *Streptococcus mutans* is susceptible to a diverse range of established antimicrobials and non-antibiotics. Manuscript submitted.
55. **Odds FC.** 2003. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother **52:**1.
56. **Derr AM, Faustoferri RC, Betzenhauser MJ, Gonzalez K, Marquis RE, Quivey RG, Jr.** 2012. Mutation of the NADH oxidase gene (nox) reveals an overlap of the oxygen- and acid-mediated stress responses in *Streptococcus mutans*. Appl Environ Microbiol **78:**1215-1227.
57. **Gonzalez K, Faustoferri RC, Quivey RG, Jr.** 2012. Role of DNA base excision repair in the mutability and virulence of Streptococcus mutans. Mol Microbiol **85:**361-377.

58. **Tsuda H, Yamashita Y, Shibata Y, Nakano Y, Koga T.** 2002. Genes involved in bacitracin resistance in *Streptococcus mutans*. Antimicrob Agents Chemother **46:**3756-3764.
59. **Kitagawa N, Shiota S, Shibata Y, Takeshita T, Yamashita Y.** 2011. Characterization of MbrC involved in bacitracin resistance in *Streptococcus mutans*. FEMS Microbiol Lett **318:**61-67.
60. **Piddock LJ, Johnson MM, Simjee S, Pumbwe L.** 2002. Expression of efflux pump gene *pmrA* in fluoroquinolone-resistant and -susceptible clinical isolates of *Streptococcus pneumoniae*. Antimicrob Agents Chemother **46:**808-812.
61. **Mikami Y, Suzuki N, Takahashi T, Otsuka K, Tsuda H.** 2011. Bacitracin upregulates *mbrAB* transcription via *mbrCD* to confer bacitracin resistance in *Streptococcus mutans*. J Pharmacol Sci **117:**204-207.
62. **Perreten V, Schwarz FV, Teuber M, Levy SB.** 2001. Mdt(A), a new efflux protein conferring multiple antibiotic resistance in *Lactococcus lactis* and *Escherichia coli*. Antimicrob Agents Chemother **45:**1109-1114.
63. **Becker P, Hakenbeck R, Henrich B.** 2009. An ABC transporter of *Streptococcus pneumoniae* involved in susceptibility to vancoresmycin and bacitracin. Antimicrob Agents Chemother **53:**2034-2041.
64. **Cain BD, Norton PJ, Eubanks W, Nick HS, Allen CM.** 1993. Amplification of the *bacA* gene confers bacitracin resistance to *Escherichia coli*. J Bacteriol **175:**3784-3789.
65. **El Ghachi M, Bouhss A, Blanot D, Mengin-Lecreulx D.** 2004. The bacA gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. J Biol Chem **279:**30106-30113.
66. **Hiron A, Falord M, Valle J, Debarbouille M, Msadek T.** 2011. Bacitracin and nisin resistance in *Staphylococcus aureus*: a novel pathway involving the BraS/BraR two-component system (SA2417/SA2418) and both the BraD/BraE and VraD/VraE ABC transporters. Mol Microbiol **81:**602-622.
67. **Manson JM, Keis S, Smith JM, Cook GM.** 2004. Acquired bacitracin resistance in *Enterococcus faecalis* is mediated by an ABC transporter and a novel regulatory protein, BcrR. Antimicrob Agents Chemother **48:**3743-3748.
68. **Matos R, Pinto VV, Ruivo M, Lopes Mde F.** 2009. Study on the dissemination of the *bcrABDR* cluster in Enterococcus spp. reveals that the BcrAB transporter is sufficient to confer high-level bacitracin resistance. Int J Antimicrob Agents **34:**142-147.
69. **Radeck J, Gebhard S, Orchard PS, Kirchner M, Bauer S, Mascher T, Fritz G.** 2016. Anatomy of the bacitracin resistance network in *Bacillus subtilis*. Mol Microbiol **100:**607-620.
70. **Chen MY, Lira F, Liang HQ, Wu RT, Duan JH, Liao XP, Martinez JL, Liu YH, Sun J.** 2016. Multilevel selection of *bcrABDR*-mediated bacitracin resistance in *Enterococcus faecalis* from chicken farms. Sci Rep **6:**34895.
71. **Zhang S, Li X, Wang X, Li Z, He J.** 2016. The two-component signal transduction system YvcPQ regulates the bacterial resistance to bacitracin in *Bacillus thuringiensis*. Arch Microbiol **198:**773-784.

72. **Harel YM, Bailone A, Bibi E.** 1999. Resistance to bacitracin as modulated by an *Escherichia coli* homologue of the bacitracin ABC transporter BcrC subunit from *Bacillus licheniformis*. J Bacteriol **181:**6176-6178.
73. **Tokuda H, Matsuyama S.** 2004. Sorting of lipoproteins to the outer membrane in *E. coli*. Biochim Biophys Acta **1694:**IN1-9.
74. **Lorenz C, Dougherty TJ, Lory S.** 2016. Transcriptional responses of *Escherichia coli* to a small-molecule inhibitor of LolCDE, an essential component of the lipoprotein transport pathway. J Bacteriol **198:**3162-3175.
75. **Narita S, Tanaka K, Matsuyama S, Tokuda H.** 2002. Disruption of *lolCDE*, encoding an ATP-binding cassette transporter, is lethal for *Escherichia coli* and prevents release of lipoproteins from the inner membrane. J Bacteriol **184:**1417-1422.

745 **Figure Legends**

746 **Table 1: Strains used in this study**

747

748 **Figure 1: Structure of Vitamin D analogs that exhibited activity in the AK assay**

749

750 **Figure 2A: Vitamin D analogs induce adenylate kinase release from *S. mutans***
751 **planktonic cells.** *S. mutans* UA159 cultures were grown in TY medium + 1% (w/v)
752 glucose to exponential phase and used to inoculate fresh medium containing
753 ciprofloxacin (positive control), DMSO (negative control), alfacalcidol, calcitriol, or
754 doxercalciferol at 16, 32, and 64 µg/mL for 4h. Cell lysis was detected using the AK
755 assay as described in Materials and Methods. Data is represented as relative
756 luminescence units (RLU) normalized to background (DMSO) and is representative of
757 three replicate cultures performed in triplicate. (* $p<0.05$; Student's t-Test; two-tailed)

758

759 **Figure 2B: Biofilm formation of *S. mutans* UA159 in the presence of Vitamin D**
760 **analogs.** Cultures of *S. mutans* UA159 were grown in TY medium + 1% (w/v) sucrose
761 to exponential phase and used to inoculate fresh medium containing alfacalcidol,
762 calcitriol, or doxercalciferol at concentrations from 0 – 128 µg/mL. Biofilm formation was
763 quantitated by crystal violet staining as described in the Materials and Methods. Values
764 were normalized to DMSO control (n= 3). (* $p<0.05$; Student's *t*-Test; two tailed)

765

766 **Figure 2C: AK assay of biofilm cultures exposed to Vitamin D analogs.** *S. mutans*
767 UA159 cultures were seeded in 96-well plate format in TY medium + 1% (w/v) sucrose

and grown for 24h to establish biofilms. Wells were washed with PBS followed by addition of ciprofloxacin (positive control), DMSO (negative control), alfacalcidol, calcitriol, or doxercalciferol in fresh TYS at 16, 32, and 64 µg/mL for 18h. Cell lysis was detected using the AK assay as described in Materials and Methods. Data is represented as relative luminescence units (RLU) normalized to background (DMSO) and is representative of three replicates performed in triplicate. The red horizontal line represents the 2-fold cutoff used to classify active compounds (54). (* $p<0.05$; Student's t-Test; two-tailed)

Figure 3. Effect of doxercalciferol on the MIC of cell-wall targeting antibiotics. Cultures of *S. mutans* UA159 were grown in TY medium + 1% (w/v) glucose to exponential phase and used to inoculate fresh medium containing 2-fold serial dilutions of test drugs (bacitracin, chloramphenicol, penicillin, vancomycin, streptomycin) in the presence or absence of doxercalciferol (16 µg/mL). Error bars represent the standard deviation of three independent experiments.

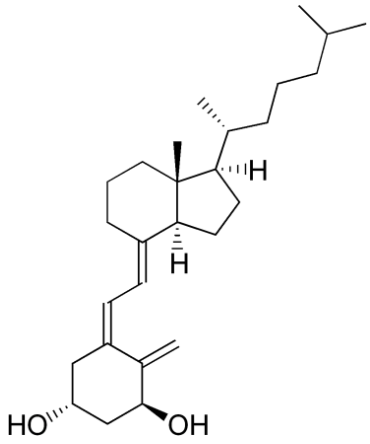
Table 2. Fractional inhibitory concentration of doxercalciferol + bacitracin in planktonic cultures of *S. mutans*. Cultures were grown in TY medium + 1% (w/v) glucose to exponential phase and used to inoculate fresh medium in a 96-well plate. The fractional inhibitory concentrations for doxercalciferol + bacitracin were determined as detailed in Materials and Methods. For bacitracin MICs outside of the tested range (i.e. >128), a value of 256 µg/mL was used to calculate FIC.

Figure 4. Kinetics of doxercalciferol and bacitracin interaction in MX804 (red), *ΔmbrA* (blue), and *mbrA*$^+$ (green). Cultures of *S. mutans* MX804 (red), Δ*mbrA* (blue), and *mbrA*$^+$ (green) were grown in TY medium + 1% (w/v) glucose to exponential phase and 10^5 CFU were used to inoculate fresh medium. Cultures were serially diluted and plated on BHI agar medium. Drugs were then added to the cultures as follows: doxercalciferol (16 µg/mL) (Panel A), bacitracin (32 µg/mL) (Panel B), a combination of doxercalciferol (16 µg/mL) and bacitracin (32 µg/mL) (Panel C), or no drug control (Panel D). Aliquots were removed at 2, 4, 24, 48h following addition of drug and plated for enumeration. Percent survival was calculated by enumeration of CFU/mL at each time point. Data are averages of at least 3 independent replicates and are normalized to CFU/mL at t=0 for each strain. (* $p<0.05$, Student's *t*-test, two-tailed)

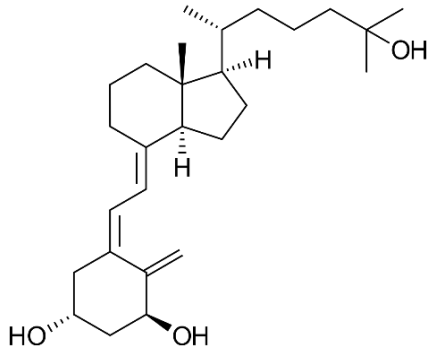
Figure 5. Doxercalciferol/bacitracin interaction occurs through an MbrA-dependent mechanism. Time-kill experiments were performed as described in Figure 4 and Materials and Methods. Aliquots were taken at 2h (Panel A), 4h (Panel B), 24h (Panel C), and 48h (Panel D) after inoculation. Bars represent cultures with doxercalciferol (16 µg/mL, bars with vertical lines), bacitracin (32 µg/mL, bars with horizontal lines), or combination of doxercalciferol and bacitracin (bars with cross hash). Percent survival was calculated by enumeration of CFU/mL at each time point, and normalized to no drug control. Data represent the average and standard deviation of at least three independent replicates. Note, the y-axis is different for each panel. (* $p<0.05$; ** $p<0.001$, Student's *t*-test, two-tailed; ns=not significant.)

814 **Table 3: Effect of doxercalciferol on the MIC of bacitracin for Gram-positive and**
815 **Gram-negative bacteria that encode MbrA-like efflux pumps.** Minimum inhibitory
816 concentrations were determined for bacitracin in select bacterial strains that encode
817 ABC transporters that are homologous to the *S. mutans mbrA* efflux pump subunit. The
818 MIC of bacitracin was also measured in the presence of 4 µg/ml doxercalciferol.
819 Results are the lowest concentrations that inhibited growth in at least two independent
820 experiments, in duplicate. (* indicates species in which addition of doxercalciferol
821 resulted in a significant decrease in bacitracin MIC.)
822

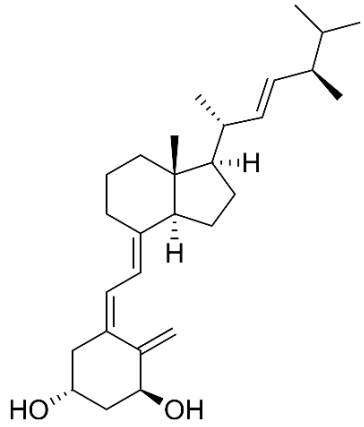
Figure 1



alfacalcidol



calcitriol



doxercalciferol

Figure 2

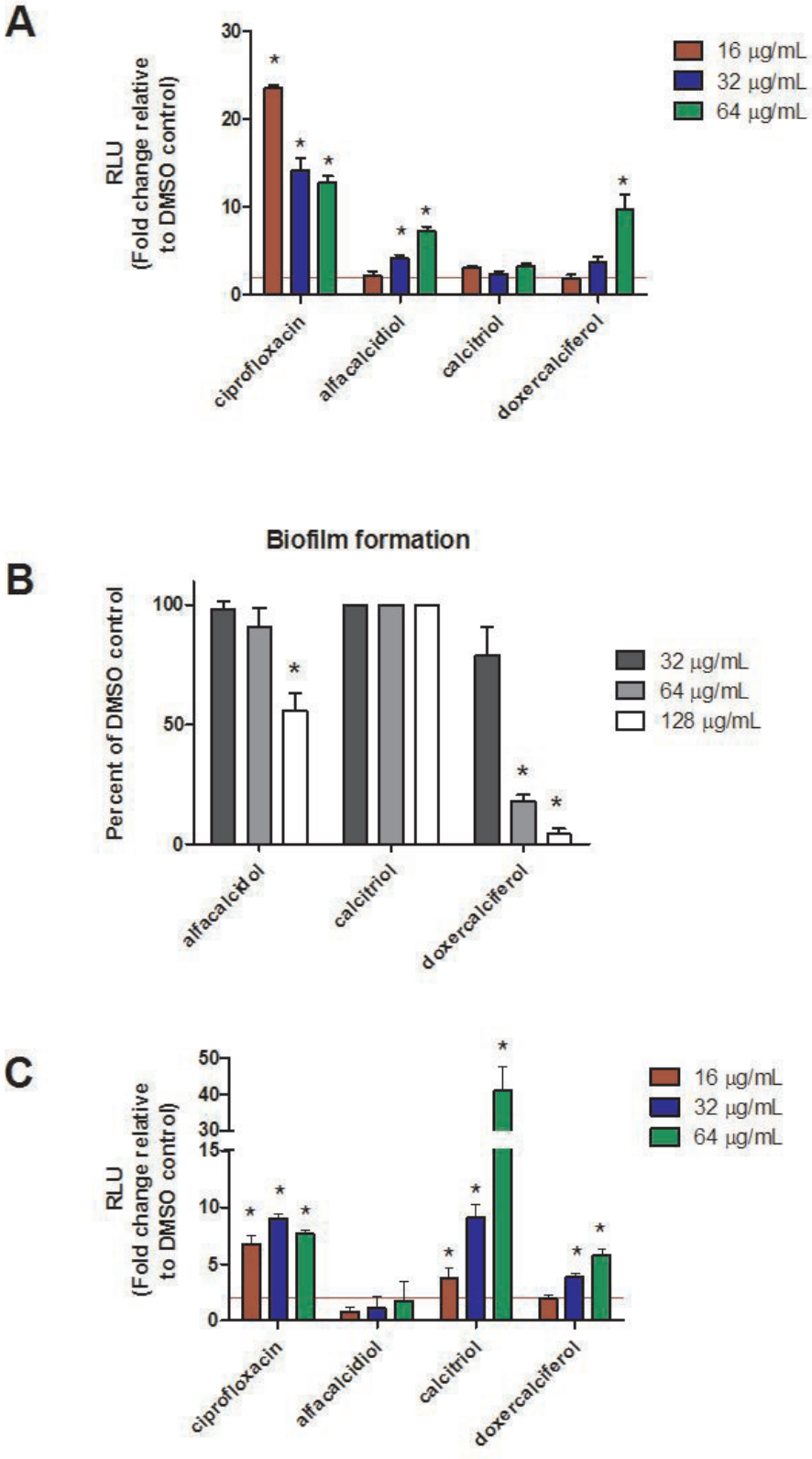


Figure 3

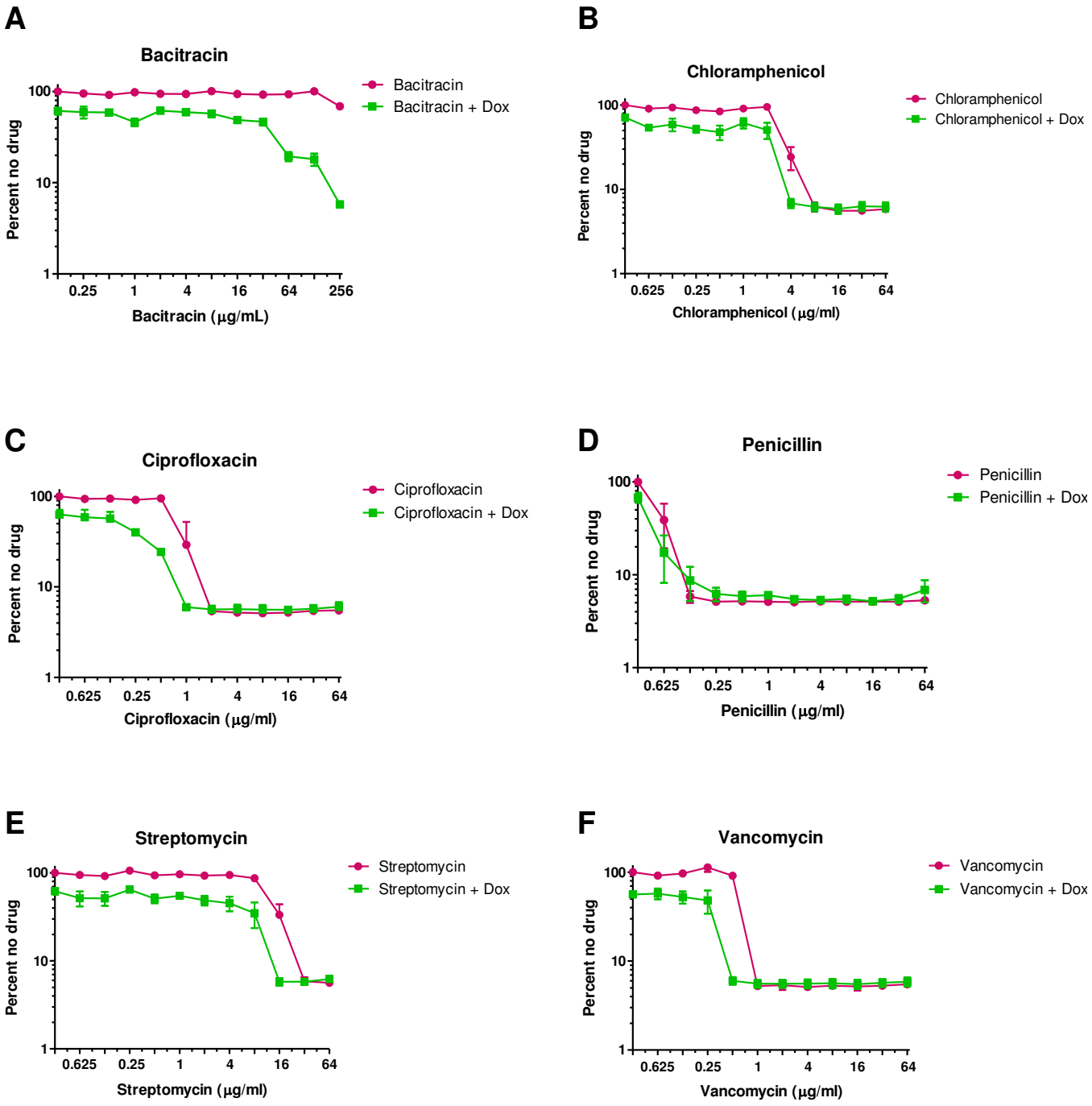


Figure 4

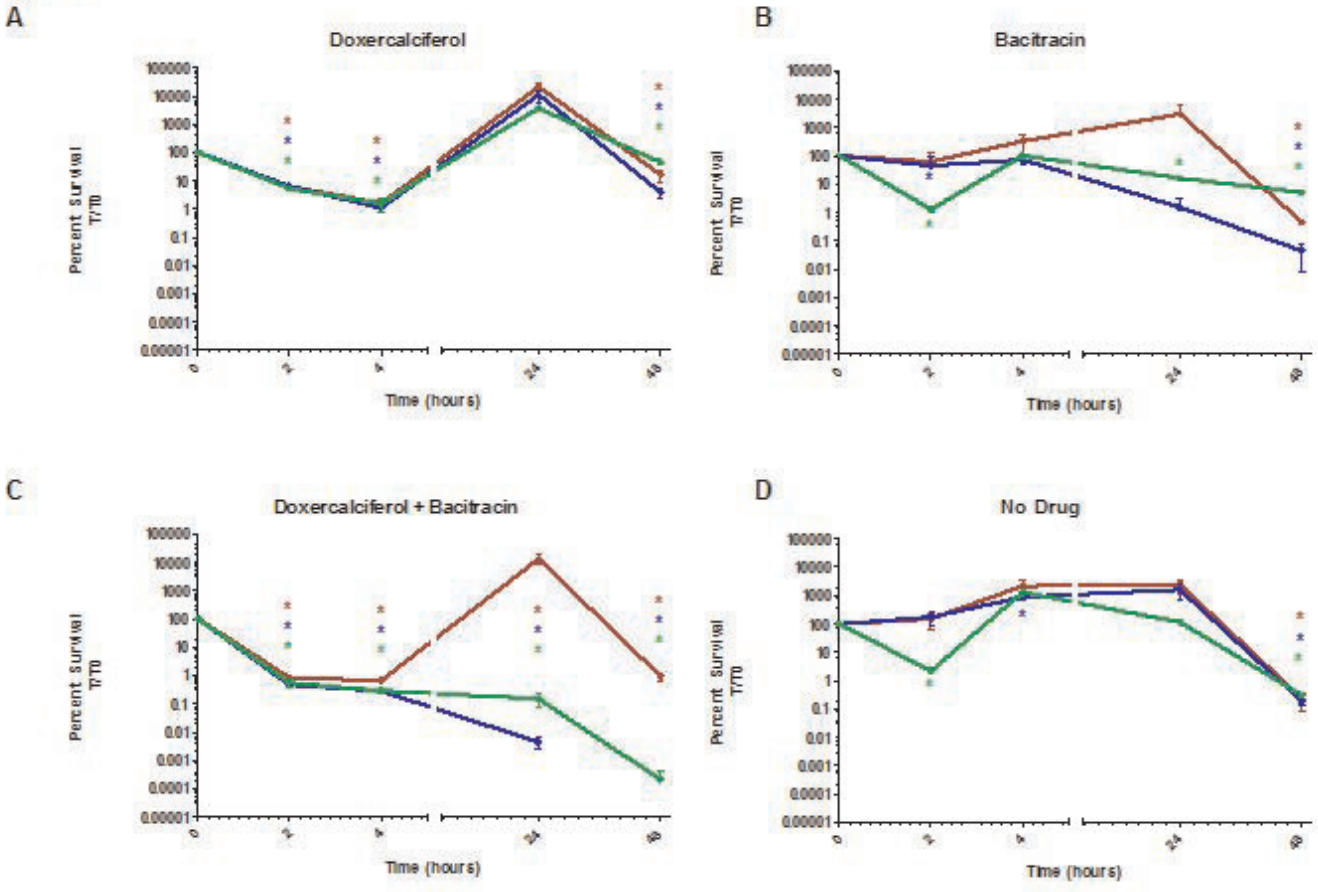


Figure 5

Table 1

Strains	Description
Streptococcus mutans UA159	genomic type strain (Murchison et al. 1986, Adjic et al., 2002)
Streptococcus mutans MX804	Erm^R (Quivey et al. 2015)
Streptococcus mutans ΔmbrA	*mbrA* deletion strain (Quivey et al. 2015)
Streptococcus mutans ΔmbrD	*mbrD* deletion strain (Quivey et al. 2015)
Streptococcus mutans mbrA$^+$	*mbrA* complement strain (this study)
Streptococcus mutans ΔrgpF	*rgpF* deletion strain (Quivey et al. 2015)
Streptococcus mutans ΔpmrA	*pmrA* deletion strain (Quivey et al. 2015)
Bacillus subtilis	*
Enterobacter aerogenes	*
Enterococcus faecalis	*
Escherichia coli	*
Klebisella pneumoniae	*
Lactococcus lactis	*
Mycobacterium smegmatis	*
Pseudomonas aeruginosa	*
Staphylococcus aureus	*
Streptococcus gordonii	*
Streptococcus oralis	*
Streptococcus salivarius	*
Streptococcus sanguinis	*
* Strains were obtained from the UR microbiology laboratory stock collection	

Table 2

	alone		In combination		FIC
	doxercalciferol	bacitracin	doxercalciferol	bacitracin	
MX804	16	>128	4	4	~0.125
Δ*mbrA*	16	2	2	0.25	0.25
Δ*mbrD*	8	4	2	1	0.5
Δ*pmrA*	4	>128	4	32	~1.125
Δ*rgpF*	16	4	2	4	1.125
mbrA^{+}	32	8	4	2	0.375

Table 3

	MIC_{Bac}	
	-Dox	+Dox
Streptococcus mutans *	>128	4
Bacillus subtilis	>128	>128
Enterobacter aerogenes	>128	>128
Enterococcus faecalis *	>128	8
Escherichia coli	>128	>128
Klebisella pneumoniae	>128	>128
Lactococcus lactis	4	2
Mycobacterium smegmatis	>128	>128
Pseudomonas aeruginosa	>128	>128
Staphylococcus aureus	128	64
Streptococcus gordonii *	128	32
Streptococcus oralis *	64	4
Streptococcus salivarius *	4	1
Streptococcus sanguinis	32	16