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**A dual-mechanism antibiotic targets Gram-negative bacteria and avoids drug resistance**

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26 **ABSTRACT**

27

28 The rise of antibiotic resistance and declining discovery of new antibiotics have created a global  
29 health crisis. Of particular concern, no new antibiotic classes have been approved for treating  
30 Gram-negative pathogens in decades. Here, we characterize a compound, SCH-79797, that  
31 kills both Gram-negative and Gram-positive bacteria through a unique dual-targeting  
32 mechanism of action (MoA) with undetectably low resistance frequencies. In an animal host  
33 model, SCH-79797 reduces pathogenesis of *Acinetobacter baumannii*, a drug-resistant Gram-  
34 negative pathogen. To characterize the MoA of SCH-79797 we combined quantitative imaging,  
35 proteomic, genetic, metabolomic, and cell-based assays. This pipeline shows that SCH-79797  
36 has two independent cellular targets, folate metabolism and bacterial membrane integrity, and  
37 outperforms combination treatments with other antifolates and membrane disruptors in killing  
38 MRSA persisters. Thus, SCH-79797 represents a promising lead antibiotic and suggests that  
39 combining multiple MoAs onto a single chemical scaffold may be an underappreciated approach  
40 to target challenging bacterial pathogens.

41

## 42 INTRODUCTION

43

44 More than twenty unique classes of antibiotics were characterized in the 30 years following the  
45 discovery of penicillin in 1929 (Coates et al., 2011; Davies, 2006). However, a combination of  
46 scientific and economic factors have slowed the discovery and development of these life-saving  
47 molecules to the extent that only six new classes of antibiotics have been approved in the past  
48 20 years, none of which are active against Gram-negative bacteria (Butler et al., 2016). This  
49 decline in the discovery of new antibiotic classes, coupled with the evolution of multi-drug  
50 resistant bacteria and horizontal transfer of resistance mechanisms, has created a public health  
51 crisis that is predicted to only escalate in the coming years (Culyba et al., 2015; Hofer, 2018;  
52 O'Neill, 2014).

53

54 Recent efforts have begun to reinvigorate antibiotics research, but most of this work has  
55 resulted in compounds that function via similar mechanisms to those of traditional antibiotics.  
56 For example, finafloxacin, an exciting new fluoroquinolone antibiotic that was recently approved  
57 to treat ear infections caused by *Pseudomonas aeruginosa*, is more effective than other  
58 fluoroquinolones because it maintains its potency in acidic environments (McKeage, 2015).  
59 However, finafloxacin is still susceptible to the same resistance mechanisms that affect other  
60 fluoroquinolones (Randall et al., 2016). The recent discovery of the natural product teixobactin  
61 suggests that it is possible to find compounds that selectively kill bacteria without being prone to  
62 resistance (Ling et al., 2015). However, teixobactin is only functional against Gram-positive  
63 bacteria and is a large molecule that is difficult to synthesize at commercial scales. Thus, there  
64 is still a strong need for characterizing new classes of antibiotics with distinct mechanisms of  
65 action (MoA).

66

67 An ideal antibiotic would be hard to develop resistance against, able to target Gram-negative  
68 bacteria, and easy to synthesize. It is important to note that while antibiotics that are not prone  
69 to resistance are attractive clinically, selecting for resistant mutants is the most common method  
70 for characterizing MoA, making the characterization of new antibiotic MoAs without resistance  
71 mutants a significant challenge. Phenotypic methods, such as macromolecular synthesis  
72 assays, have been previously used in such cases, as was done for teixobactin (Ling et al.,  
73 2015). However, these assays only allow the classification of molecules with previously-  
74 described MoAs (King and Wu, 2009). Thus, there is also a need for resistance-independent  
75 approaches for the *de novo* characterization of antibiotic MoA.

76  
77 Here, we describe a compound, SCH-79797, that is bactericidal towards both Gram-negative  
78 and Gram-positive bacteria, including clinically important bacteria such as *Staphylococcus*  
79 *aureus* MRSA and *Acinetobacter baumannii*, with no signs of resistance. In an animal host  
80 model, SCH-79797 blocked infection by *A. baumannii* with low toxicity to the host at the dose  
81 required for effective antibiotic activity. To rapidly and efficiently classify the MoA of SCH-79797,  
82 we used a variant of a recently described quantitative imaging-based approach known as  
83 bacterial cytological profiling (BCP) (Nonejuie et al., 2013). This effort showed that SCH-79797  
84 functions through a mechanism distinct from that of any known class of antibiotics. In the  
85 absence of being able to evolve resistance mutants, we used thermal proteome profiling  
86 (Savitski et al., 2014), CRISPRi genetic sensitivity (Peters et al., 2016), and metabolomic  
87 profiling (Kwon et al., 2008, 2010) to characterize the MoA of SCH-79797. Using this multi-  
88 dimensional, systems-level approach, we identified the candidate targets of SCH-79797 as  
89 dihydrofolate reductase and the bacterial membrane. Classical enzymology and membrane  
90 permeability and polarization assays confirmed the targets identified by our high-throughput  
91 approaches. Finally, we used a derivative of SCH-79797 to demonstrate that the two activities  
92 of this compound can be separated. Thus, our findings identify and characterize a promising  
93 new antibiotic and provide a potential roadmap for future antibiotic discovery efforts.

94

## 95 **RESULTS**

96

### 97 ***SCH-79797 is a broad-spectrum, bactericidal antibiotic***

98 With the aim of finding antibiotics with novel mechanisms of action (MoA), we began with an  
99 unbiased, whole-cell screening approach. To include antibiotics that target both Gram-negative  
100 and Gram-positive bacteria, we screened for compounds that inhibited the growth of *E. coli*  
101 *lptD4213*, which has a compromised outer membrane that makes it partially-permeable to  
102 antibiotics that would otherwise have difficulty penetrating the Gram-negative lipopolysaccharide  
103 (Ruiz et al., 2006). We screened a drug library of ~33,000 unique compounds and one of our  
104 most potent hits was SCH-79797, a compound that had been previously reported as a human  
105 PAR-1 antagonist (Ahn et al., 2000). This finding was surprising since there are no PAR-1  
106 homologs in bacteria. A recent report suggested that SCH-79797 increases the ability of  
107 neutrophils to kill bacteria, perhaps by directly functioning as an antibiotic (Gupta et al., 2018).  
108 Given that studies focusing on characterizing its anticoagulant activities suggested that at least  
109 5 mg/kg SCH-79797 can be safely tolerated in animals (Gobbetti et al., 2012; Strande et al.,

110 2007) and its emergence as a potential antimicrobial with no known bacterial target (Gupta et  
111 al., 2018), we decided to further characterize SCH-79797 as a candidate antibiotic.

112

113 To assess the spectrum of bacterial species susceptible to SCH-79797, we measured the  
114 minimal inhibitory concentration (MIC) of SCH-79797 against several clinically-relevant  
115 pathogens. In this study, we define MIC as the concentration of drug that results in no visible  
116 bacterial growth after 14h of growth at 37°C. We found that SCH-79797 significantly hindered  
117 the growth of multiple Gram-negative and Gram-positive pathogens such as *Neisseria*  
118 *gonorrhoeae*, two clinical isolates of *Acinetobacter baumannii*, and methicillin-resistant  
119 *Staphylococcus aureus* (MRSA) (Figure 1A). Using the *E. coli lptD4213* strain from our original  
120 screen, we found that SCH-79797 exhibits potent and rapid bactericidal activity against *E. coli*  
121 *lptD4213* (Figure 1B and C). SCH-79797 also exhibited similar bactericidal activity against a  
122 clinical isolate of *S. aureus* MRSA (USA300) (Tenover and Goering, 2009) suggesting that its  
123 bactericidal activity is not species-specific (Figure S1).

124

### 125 **SCH-79797 is effective in vivo and has a low frequency of resistance**

126 Given SCH-79797's promising ability to kill bacteria, we sought to determine if it can function as  
127 an effective antibiotic *in vivo*. To test its antibiotic activity in the context of an animal host  
128 infection, we focused on *A. baumannii* as it has emerged as an important Gram-negative  
129 pathogen that is targeted by relatively few available antibiotics, and has a well-established host  
130 animal model in the wax worm, *Galleria mellonella* (Gebhardt et al., 2015; Peleg et al., 2009).  
131 We first established that injecting *G. mellonella* with SCH-79797 at concentrations four times  
132 higher than the MIC of SCH-79797 towards *A. baumannii* did not result in higher host toxicity  
133 than the established antibiotics, gentamicin and rifampicin (Fig. S2A-B). We next tested the  
134 ability of SCH-79797 to treat infection of *G. mellonella* with a lethal dose of *A. baumannii*  
135 AB17978. Treatment with SCH-79797 significantly prolonged the survival of *A. baumannii*-  
136 infected *G. mellonella* ( $P < 0.001$ ) (Figure 1D and S1C-D). The survival rate of *G. mellonella*  
137 treated with SCH-79797 exceeded that of gentamicin and rifampicin (Fig. 1D and S2C-D), which  
138 are standard antibiotics used to treat *A. baumannii* infections (Karlowsky et al., 2003; Viehman  
139 et al., 2014).

140

141 To further characterize the promise of SCH-79797 as an antibiotic, we attempted to determine  
142 the frequency with which bacteria develop resistance towards SCH-79797. Because  
143 spontaneous suppressors can restore *E. coli lptD4213*'s membrane barrier functionality, we

144 focused our resistance studies on *S. aureus* MRSA (USA300) (Tenover and Goering, 2009). We  
145 were unable to isolate stable SCH-79797-resistant mutants upon plating  $\sim 10^8$  CFU of MRSA  
146 USA300 onto agar containing 4X MIC of SCH-79797. We were also unable to isolate SCH-  
147 79797-resistant mutants upon plating  $\sim 10^8$  CFU of *B. subtilis*, suggesting that the difficulty in  
148 developing resistant mutants is not species-specific. To address resistance rates more  
149 quantitatively, we serial-passaged 2 biologically independent cultures of *S. aureus* MRSA  
150 USA300 in 0.5X MIC of SCH-79797, as well as three control antibiotics: novobiocin,  
151 trimethoprim, and nisin. Over the course of 25 days, we successfully isolated mutants resistant  
152 to all the control antibiotics while no SCH-79797-resistant mutants emerged (Figure 1E, S3A).  
153 For novobiocin, trimethoprim, and nisin, resistance gradually increased throughout the  
154 experiment, but the resistance level remained constant for SCH-79797 (Figure 1E, S3A),  
155 indicating that these bacteria did not even acquire partial resistance to SCH-79797. To extend  
156 these findings to a Gram-negative species, we repeated our serial passaging study with 2  
157 biologically independent cultures of *A. baumannii* AB17978 (Figure S3B). This study also found  
158 that *A. baumannii* resistance stayed constant for SCH-79797 but increased for all other  
159 antibiotics, supporting the conclusion that the lack of resistance to SCH-79797 is not species-  
160 specific.

161

### 162 ***A variant of Bacterial Cytological Profiling suggests that SCH-79797 has a unique MoA***

163 The inability to isolate SCH-79797-resistant mutants makes SCH-79797 an appealing candidate  
164 antibiotic but poses a challenge for determining its MoA. As a result, we used a quantitative  
165 imaging-based approach to determine if the MoA of SCH-79797 is similar to that of any  
166 previously-characterized antibiotics. Specifically, we modified a single-cell, high-content imaging  
167 methodology, known as Bacterial Cytological Profiling (BCP) (Nonejuie et al., 2013). The logic  
168 of BCP is that antibiotics with similar MoA result in similar death phenotypes such that by  
169 quantifying how bacteria appear upon death, we can gain insight into the cause of death (much  
170 like a bacterial autopsy). Here, we applied our BCP analysis to a training set of 37 distinct  
171 antibiotics with known MoA as well as to SCH-79797. For each compound, we treated *E. coli*  
172 *lptD4213* with 5X MIC of an antibiotic for 2 hours, stained with three dyes that report on nucleoid  
173 morphology (DAPI), membrane morphology (FM4-64), and membrane integrity (SYTOX Green),  
174 and imaged the cells at high resolution (Figure 2A). For each condition we imaged  $\sim 100$  cells  
175 and quantified 14 parameters reflecting various morphological and fluorescence features  
176 (Supplementary Table 2).

177

178 Since we had gold standards of the BCP results of SCH-79797 and of several antibiotics  
179 representing different classes and sub-groups within classes, we applied a machine learning  
180 approach to classify the BCP data. For each treatment group, we populated a 'neighborhood  
181 representation' vector with the one-way Mahalanobis distance as measured from the single-cell  
182 feature mean in question, to the distribution measurement of all other treatment groups. This  
183 distance is normalized by the covariance matrix of the antibiotic treatment group so that  
184 dimensions with large amounts of variance are deemed closer, while distances of dimensions  
185 with less variance are considered farther away. We then used single-linkage clustering to  
186 cluster treatment groups by their neighborhood representation vectors, such that samples  
187 whose neighborhoods were similar would be clustered together. This analysis indicated that  
188 SCH-79797 resulted in a phenotypic death-state that was different from the other antibiotics  
189 tested (Figure 2B), suggesting that SCH-79797 possesses a MoA distinct from that of any of the  
190 antibiotics in our training set.

191

### 192 ***Thermal profiling and CRISPRi genetics demonstrate that SCH-79797 targets*** 193 ***dihydrofolate reductase (DHFR)***

194 In the absence of resistant mutants or similarity to antibiotics with known MoA by BCP, we  
195 turned to a high-throughput proteomics-based approach for *de novo* identification of candidate  
196 SCH-79797 targets. Specifically, we used thermal proteome profiling, an assay that uses mass  
197 spectrometry to compare the thermal stability of the entire proteome with and without drug  
198 treatment (schematized in Figure 3A) (Mateus et al., 2018; Savitski et al., 2014). Briefly, intact  
199 cells or cell lysate samples treated with a range of compound concentrations are heated to a  
200 series of increasing temperatures and the soluble proteins at each temperature are collected  
201 (Becher et al., 2016). Proteins that bind to the drug are thermally stabilized, which leads to a  
202 shift in the temperature at which those proteins precipitate (Figure 3A). Using *E. coli* *lptD4213*,  
203 we treated intact cells and cell lysates with SCH-79797 and found that it significantly shifted the  
204 thermal stability of dihydrofolate reductase (DHFR) (Figure 3B and S4A). The fact that the same  
205 result was observed with both intact cells and cell lysates (Figure 3B and S4A) suggests that  
206 SCH-79797 enters *E. coli* cells and directly binds to *E. coli* DHFR. As a positive control, we  
207 used a well-characterized antibiotic that targets DHFR, trimethoprim, and found that it also  
208 thermally stabilizes its known target, DHFR (Figure 3C and S4B).

209

210 To test both the physiological significance and species-specificity of the suggestion that SCH-  
211 79797 binds to DHFR, we took advantage of a collection of *B. subtilis* essential gene CRISPR-

212 interference (CRISPRi) knockdown mutants (Peters et al., 2016). In each of these mutants, an  
213 essential gene is targeted by CRISPRi to reduce its expression ~3-fold. A strain with reduced  
214 levels of the SCH-79797 target should be sensitized to sub-lethal doses of SCH-79797. Given  
215 the thermal profiling result, we focused on mutants in the folate biosynthesis pathway (Figure  
216 4A). As a negative control, we confirmed that CRISPRi knockdowns of genes unrelated to folate  
217 metabolism are not sensitized to SCH-79797 (Figure S5). As a positive control for our assay, we  
218 used trimethoprim. We confirmed that dihydrofolate reductase (*dfrA*) and dihydrofolate synthase  
219 (*folC*) (an enzyme that acts upstream of DfrA) knockdowns are hypersensitive to trimethoprim,  
220 while enzymes that function downstream of DfrA, *folD* and *glyA*, are not (Figure 4B). SCH-  
221 79797 exhibited the same genetic sensitivity pattern as trimethoprim in that both *dfrA* and *folC*,  
222 but not *folD* and *glyA* knockdowns, were sensitized to SCH-79797 (Figure 4B).

223

### 224 ***SCH-79797 inhibits DHFR activity in cells and in vitro***

225 To determine how SCH-79797 affects folate metabolism in living cells, we used mass  
226 spectrometry to measure the relative abundance of folate metabolite pools in *E. coli* NCM3722  
227 treated with SCH-79797. *E. coli* NCM3722 was used because these bacteria lack mutations that  
228 disrupt primary metabolism in other lab strains of *E. coli* (Soupene et al., 2003). *E. coli*  
229 NCM3722 cells were grown in Gutnick Minimal Media and treated with 1X MIC SCH-79797 for  
230 15 minutes (Kwon et al., 2008, 2010). In response to SCH-79797 treatment, the levels of the  
231 DHFR substrate, 7,8-dihydrofolate (DHF), rose approximately 10-fold compared to untreated  
232 cells, while the levels of folate metabolites downstream of DHF dropped significantly (Figure  
233 4C). This metabolic response is characteristic of DHFR inhibition as we observed a similar  
234 pattern upon treatment with trimethoprim (Figure 4C).

235

236 To determine whether SCH-79797 inhibits DHFR *in vitro*, we obtained purified *E. coli* DHFR  
237 protein and measured its enzymatic activity in the presence of SCH-79797. We found that SCH-  
238 79797 has an IC<sub>50</sub> of  $2.5 \pm 0.6$   $\mu\text{g/mL}$  against DHFR (Figure 4D). We also measured the initial  
239 velocity of DHFR activity at various DHF substrate concentrations in the presence or absence of  
240 SCH-79797 to establish if SCH-79797 acts competitively or non-competitively. Fitting our data  
241 to the Michaelis-Menten equation demonstrated that 1  $\mu\text{g/mL}$  SCH-79797 increases the  $K_m$  from  
242  $29 \pm 9$   $\mu\text{M}$  to  $39 \pm 13$   $\mu\text{M}$  and decreases the  $V_{\text{max}}$  of DHFR from  $3300 \pm 300$  A.U. to  $2700 \pm 300$   
243 A.U.. These results indicate that SCH-79797 functions at least partially as a competitive inhibitor  
244 of DHFR's activity on its DHF substrate (Figure 4E).

245



246 **SCH-79797 also disrupts bacterial membrane potential and permeability**

247 The similarities between SCH-79797 and trimethoprim with respect to DHFR inhibition helped  
248 confirm DHFR as a target of SCH-79797 but were also surprising because these two  
249 compounds did not generate similar profiles in our BCP analysis (Figure 2B). One potential  
250 explanation is that SCH-79797 has additional targets that are not shared with trimethoprim. If  
251 this was the case, we would expect that cells resistant to trimethoprim would still be susceptible  
252 to SCH-79797. Previous studies demonstrated that resistance to trimethoprim can be achieved  
253 by deleting *thyA* and supplementing the media with thymine (Amyes and Smith, 1975). We  
254 confirmed that deleting *thyA* from *E. coli lptD4213* in the presence of excess thymine led to  
255 trimethoprim resistance (Figure 5A). However, these cells showed no change in their sensitivity  
256 to SCH-79797 (Figure 5A), suggesting that SCH-79797 is likely to have a second, folate-  
257 independent MoA.

258  
259 To obtain clues about the potential additional MoA of SCH-79797, we revisited our fluorescent  
260 BCP images of *E. coli lptD4213* cells treated with SCH-79797. We observed SYTOX Green  
261 staining in some of the bacteria (Figure 2A), suggesting that SCH-79797 compromises the  
262 integrity of the bacterial membrane. To directly quantify the effect of SCH-79797 on bacterial  
263 membrane integrity, we used flow cytometry to measure the membrane potential and  
264 permeability of *E. coli lptD4213* in the presence of the fluorescent dyes, DIOC<sub>2</sub>(3) and TO-PRO-  
265 3. DIOC<sub>2</sub>(3) is a cationic dye that accumulates in the cytoplasm of cells with an active  
266 membrane potential and shifts its fluorescence from red to green in these cells, providing a  
267 measure of membrane potential (Figure 5B). TO-PRO-3 is a nucleic acid stain that only  
268 accumulates in cells with compromised membranes, providing an independent measure of  
269 membrane permeability (Figure 5B). As positive controls, we showed that CCCP, a membrane-  
270 decoupler that affects membrane potential but not permeability, and nisin, a pore-forming  
271 antibacterial peptide that disrupts both membrane potential and permeability, caused the  
272 expected shifts in both DIOC<sub>2</sub>(3) and TO-PRO-3 staining (Figure 5C). As negative controls, we  
273 confirmed that antibiotics that do not target the membrane, including ampicillin, rifampicin, and  
274 novobiocin, do not shift DIOC<sub>2</sub>(3) or TO-PRO-3 staining (Figure S6). After 15 minutes of  
275 treatment with SCH-79797 at MIC levels, DIOC<sub>2</sub>(3) and TO-PRO-3 staining revealed significant  
276 defects in both membrane polarization and permeability (Figure 5C). These effects on the  
277 membrane are not secondary consequences of DHFR inhibition, as trimethoprim-treated *E. coli*  
278 showed no significant changes in DIOC<sub>2</sub>(3) and TO-PRO-3 staining (Figure 5C). The  
279 membrane-targeting effect of SCH-79797 is also not species-specific, as similar results were

280 seen with SCH-79797-treated *B. subtilis* W168 (Figure S7). These findings indicate that  
281 independent of its ability to inhibit DHFR activity, SCH-79797 disrupts both membrane potential  
282 and permeability.

283

### 284 **SCH-79797 treatment can kill bacteria in contexts where combination therapy fails**

285 Having established that SCH-79797 disrupts both folate metabolism and membrane integrity,  
286 we sought to determine if these two targets can together explain how SCH-79797 kills bacteria.  
287 To address this question, we used BCP analysis to compare the cell morphology of bacteria  
288 treated with SCH-79797, to that of bacteria treated with trimethoprim and nisin, two different  
289 antibiotics that target DHFR and membrane integrity, respectively (Nonejuie et al., 2013; Wilson  
290 et al., 2016). Qualitative inspection suggested that when stained with DAPI, FM4-64, and  
291 SYTOX Green, SCH-79797 treated *E. coli* appeared similar to *E. coli* *lptD4213* cells treated with  
292 both trimethoprim and nisin (Figure 6A). Quantification of the images confirmed that SCH-79797  
293 closely clusters with the co-treatment of trimethoprim and nisin (Figure 6A). The fact that SCH-  
294 79797 clusters more closely to the co-treatment than to the individual treatments with  
295 trimethoprim or nisin reinforces the conclusion that SCH-79797 kills bacteria by targeting both  
296 DHFR and the membrane. There are no other antibiotics that have been shown to target both  
297 folate metabolism and membrane integrity, indicating that SCH-79797 represents an antibiotic  
298 with a unique MoA. This result also explains why SCH-79797 failed to cluster with any of the  
299 known antibiotics in our BCP analysis (Figure 2B).

300

301 Our findings that SCH-79797 has the same MoA as combined trimethoprim and nisin treatment  
302 raised the question of whether there is a benefit to combining two targeting mechanisms onto a  
303 single molecule. Combination antibiotic therapy has been suggested as a potential means of  
304 circumventing the rise of antibiotic resistance (Tamma et al., 2012; Tyers and Wright, 2019) but  
305 it has remained unclear whether it is better to combine multiple activities on the same molecule.  
306 To probe this issue, we measured the synergy of trimethoprim and nisin against *E. coli* *lptD4213*  
307 and MRSA USA300 persister cells and compared their combined effectiveness to that of SCH-  
308 79797. Interestingly, when *E. coli* *lptD4213* cells are co-treated with trimethoprim and nisin, the  
309 two antibiotics antagonize one another's activity, as measured by viable cell counts after 2  
310 hours of co-treatment (Figure 6B). Examining the ability to kill MRSA USA300 persister cells  
311 yielded an even more striking result in that SCH-79797 could robustly kill these persister cells  
312 while the combination of trimethoprim and nisin could not (Figure 6C). These results suggest  
313 that the combination of two different antibacterial activities on the same molecular scaffold can,

314 at least in the case of SCH-79797, produce a more potent antibacterial effect than co-treating  
315 with two antibiotics with the two separate targeting activities.

316

### 317 ***The chemical basis of the two MoAs of SCH-79797***

318 SCH-79797 consists of a pyrroloquinazolinediamine core that is substituted with an  
319 isopropylphenyl group on one side and a cyclopropyl moiety on the other. In order to test the  
320 function of the pyrroloquinazolinediamine core on the antibiotic activity of SCH-79797, we  
321 synthesized a derivative of SCH-79797 (Two-Headed-Monster-10, or THM-10) that lacks both  
322 side groups (Figure 7A). When compared to the parent molecule SCH-79797, removing the  
323 isopropylphenyl and cyclopropyl groups increased the efficacy against *E. coli* *lptD4213* but  
324 decreased the potency against *B. subtilis* W168, MRSA USA300, and *A. baumannii* AB17978  
325 (Figure 7B). This suggests that while the decorations around the pyrroloquinazolinediamine  
326 core are important, they are not strictly necessary for SCH-79797's antibiotic activity.

327

328 To determine whether the pyrroloquinazolinediamine core of SCH-79797 is specifically involved  
329 in targeting folate metabolism or membrane integrity, we assessed the activity of THM-10 using  
330 the *dfrA* and *folC* CRISPRi hypersensitivity assay and the quantitative flow cytometry membrane  
331 integrity assay. The CRISPRi hypersensitivity assay indicated that THM-10 maintains the ability  
332 to inhibit folate metabolism, suggesting that the pyrroloquinazolinediamine core is sufficient to  
333 target DHFR (Figure 7C). However, unlike SCH-79797, DIOC<sub>2</sub>(3) and TO-PRO-3 staining  
334 showed that THM-10 does not disrupt membrane polarity or permeability (Figure 7D). These  
335 findings suggest that the pyrroloquinazolinediamine core of SCH-79797 targets DHFR while the  
336 isopropylbenzene and/or cyclopropyl side groups help SCH-79797 disrupt membrane  
337 polarization and permeability.

338

339

## 340 **DISCUSSION**

341

342 Due to the rise in resistance to known antibiotics, there is an acute need for new antibiotics with  
343 the key features of having unique MoAs, potency towards Gram-negatives, and reduced  
344 susceptibility to resistance. Here we describe a promising compound, SCH-79797, that is  
345 effective in an animal host and addresses these key criteria: it has a unique dual-targeting MoA,  
346 kills both Gram-negative and Gram-positive pathogens, and exhibits an undetectably low  
347 frequency of resistance. We also describe a systems-level pipeline that combines independent

348 orthogonal approaches to characterize the MoA of SCH-79797 in the absence of resistant  
349 mutants. Specifically, we used Bacterial Cytological Profiling (BCP) classification to categorize  
350 the MoA of SCH-79797 as distinct from those of 37 known antibiotics (Figure 2B). We then used  
351 thermal proteome profiling to identify DHFR as a candidate binding partner of SCH-79797 and  
352 confirmed that SCH-79797 inhibits folate metabolism through metabolomic analysis and  
353 CRISPRi genetic hypersensitivity (Figure 3B, 4B and C). Finally, we confirmed that SCH-79797  
354 directly inhibits DHFR activity *in vitro* by acting competitively towards its DHF substrate (Figure  
355 4D and E). The BCP images also alerted us to a second potential target for SCH-79797, the  
356 bacterial membrane. Quantitative flow cytometry with dyes that report on membrane  
357 permeability and polarity confirmed that SCH-79797 has a folate-independent effect on bacterial  
358 membrane integrity (Figure 5C). Together, these assays constitute a pipeline that can be used  
359 in the future to rapidly characterize antibiotic MoAs *de novo*. Such a pipeline is especially  
360 important for compounds such as SCH-79797 that are not prone to resistance and do not mimic  
361 known MoAs. BCP, thermal proteome profiling, metabolomics, CRISPRi sensitivity, and flow  
362 cytometry are all assays that can be performed in small volumes, such that they can be readily  
363 scaled without the need for synthesizing large amounts of the compound in question. We also  
364 note that the orthogonal nature of the assays enables the independent identification of multiple  
365 MoAs, which may help in the discovery of unique antibiotic classes.

366  
367 Both of the targets of SCH-79797 are relevant for its function as an antibiotic. The CRISPRi and  
368 metabolomic studies demonstrate that SCH-79797 actively disrupts folate metabolism in  
369 multiple bacterial species (Figure 4B and C). Meanwhile, the flow cytometry assay  
370 demonstrates that SCH-79797 simultaneously disrupts membrane integrity even though folate  
371 inhibition itself has no effect on the membrane (Figure 5C). The ability of SCH-79797 to disrupt  
372 membrane integrity is particularly interesting given that similar membrane-disruptors like nisin  
373 are typically selective for Gram-positive bacteria (Zhou et al., 2016), while SCH-79797 also  
374 proved potent against Gram-negative pathogens like *A. baumannii*, *N. gonorrhoeae*, and  
375 pathogenic *E. coli* (Figure 1A). Host toxicity is often a concern for membrane-targeting  
376 antibiotics, but SCH-79797 was well tolerated by *G. mellonella* wax worms at 4 times its MIC  
377 towards *A. baumannii* (Figure S2A-B) and a recent study of retinoid derivatives provided proof-  
378 of-principle that small molecules can preferentially target bacterial membranes (Kim et al.,  
379 2018). While SCH-79797 already has relatively low toxicity in a *G. mellonella* model, future  
380 biophysical characterization and medicinal chemistry will help to further reduce its toxicity.

381 Similarly, the increased potency of the THM-10 derivative towards *E. coli* *lptD4213* suggests  
382 that medicinal chemistry holds promise for improving the potency of SCH-79797.

383  
384 The undetectably low frequency of resistance to SCH-79797 could result from its two distinct  
385 targets. Specifically, we were successful in isolating resistance mutants for mimics of each of its  
386 two individual targets, trimethoprim and nisin, but not for SCH-79797 (Figure 1E). The average  
387 mutation rate in *E. coli* is  $2.1 \times 10^{-7}$  per gene per generation (Chen and Zhang, 2013). If *E. coli*  
388 required 2 mutations to acquire resistance to SCH-79797, the number of bacteria that would be  
389 necessary to find a resistant mutant would be in the range of  $10^{-14}$ . Humans are estimated to  
390 carry roughly  $4 \times 10^{13}$  bacteria in total, so such low frequencies of resistance would be unlikely to  
391 result in resistant mutants in a clinical context.

392  
393 Our studies suggest that SCH-79797 is more potent than combination treatment with antibiotics  
394 that mimic its two activities, the DHFR-inhibitor trimethoprim and the membrane-disruptor nisin.  
395 Co-treatment with trimethoprim and nisin showed antagonistic interactions (Figure 6B), while  
396 MRSA persister cells were killed by SCH-79797 but not by combined treatment with  
397 trimethoprim and nisin (Figure 6C). A potential explanation for the potency of SCH-79797 is that  
398 recruiting a DHFR inhibitor to the membrane could increase its effective concentration or  
399 potentiate its inactivation of DHFR by sequestering it. Permeabilizing the membrane could also  
400 enhance the access of SCH-79797 to its cytoplasmic DHFR target. The difference between  
401 SCH-79797 and the combination of trimethoprim and nisin could also be based on non-primary  
402 target effects such as differences in drug uptake or efflux. Membrane-targeting molecules can  
403 act either synergistically or antagonistically with antibiotics with different MoA's (Brochado et al.,  
404 2018). Since trimethoprim and nisin antagonize one another separately but DHFR inhibition and  
405 membrane disruption synergize in the context of SCH-79797, combining antibiotic activities onto  
406 the same molecule could present a solution for bypassing this antagonistic effect. In any event,  
407 our results suggest that despite the promise of combination antibiotic therapies (Brochado et al.,  
408 2018; Tyers and Wright, 2019), an even more powerful approach could be to combine different  
409 targeting moieties onto the same chemical scaffold.

410

## 411 **MATERIALS AND METHODS**

412

### 413 **Bacterial strains and growth conditions**

414 A complete list of strains and growth medias used to grow each bacterium are listed in  
415 Supplementary Table 1. The *E. coli* strain, *lptD4213*, was derived from *E. coli* MC4100 and  
416 obtained from the lab of Tom Silhavy (Princeton University). Unless otherwise stated, cells were  
417 grown from single colonies grown overnight at 37°C in Luria Broth (LB).

418

#### 419 **Antibiotics**

420 SCH-79797 dihydrochloride was purchased from Tocris Bioscience. All other antibiotics were  
421 purchased from MP Biomedicals at the highest possible purity. All antibiotics except for nisin  
422 and gentamicin were dissolved in sterile 100% DMSO. For enzymatic studies, SCH-79797 was  
423 dissolved in 100% EtOH since DMSO is toxic to DHFR protein. Nisin was dissolved in sterile  
424 0.02N HCl and gentamicin was dissolved in sterile deionized water. The minimum inhibitory  
425 concentration of each antibiotic was defined as the lowest concentration of antibiotic that  
426 resulted in no visible growth. MICs were measured using 2-fold dilutions of each antibiotic in 96-  
427 well plates and cell growth was monitored by measuring the OD600.

428

#### 429 **Compound library**

430 Compounds were sourced from commercial vendors: MicrosourceDiversity, Aldrich,  
431 Sellekchem, Chiromics, and Chembridge. Each compound was screened for antibiotic activity  
432 against *E. coli lptD4213* at 50µM in DMSO. *E. coli lptD4213* were grown in Terrific Broth. After  
433 normalizing for plate-to-plate variation, we used an OD600 of half the median plate OD600 as  
434 our cutoff, below which any compound was assumed to have inhibited the growth of *E. coli*  
435 *lptD4213* and above which compounds were assumed to be ineffective. Compounds that either  
436 had not been previously identified as antibiotics or had unknown or ambiguous MoAs were  
437 chosen for further investigation and their MIC's were measured using the microdilution method  
438 described above.

439

#### 440 ***Galleria mellonella* killing assay**

441 All *Galleria mellonella* larvae were obtained from Vita-Bugs®, distributed through PetCo® (San  
442 Diego, CA), and kept in a 20°C chamber. All injections were administered using a sterile 1 ml  
443 syringe attached to a KD Scientific pump delivered at a rate of 250 µl/min to the fourth leg of the  
444 worm, which was sterilized with EtOH. *A. baumannii* AB17978 (10<sup>5</sup> CFU/larva) and drug  
445 dissolved in DMSO (SCH-79797 at 66.6 µg/larva, gentamicin at 6 µg/larva, rifampicin at 66.6  
446 µg/larva) were pre-mixed prior to injection. The viability of each injected larva was determined  
447 by prodding each larva with a dowel and observing whether there was subsequent movement.

448

#### 449 **Colony Forming Units Assay**

450 Overnight *E. coli* *lptD4213* or *S. aureus* MRSA USA300 cultures were diluted 1:100 in fresh  
451 media and grown to early-mid exponential phase (OD600 = 0.4-0.6). Each culture was then  
452 diluted 1:10 into fresh media and then treated with the desired concentration of each antibiotic.  
453 Each time point was taken by removing 150 $\mu$ L from each treatment condition and diluting 1:10.  
454 6 dilutions of each condition were then plated in the absence of antibiotic and grown at 37°C  
455 overnight. CFU's were measured by counting the resulting number of colonies the next day.

456

#### 457 **Serial passaging Assay to determine the dynamics of resistance emergence**

458 2 independent overnight cultures for each of *S. aureus* MRSA USA300 and *A. baumannii*  
459 AB17978 were diluted 1:150 in LB and treated with 2-fold dilutions of each antibiotic. Bacteria  
460 were grown for 14h in a 96-well plate in LB at 37°C and bacterial growth was measured by  
461 monitoring changed in OD600 in a Tecan microplate reader. Cultures that grew in 0.5X MIC of  
462 each antibiotic were streaked out onto plain LB agar plates and single colonies were used to  
463 create the inoculum of the next passage and 750 $\mu$ L of each inoculum was stored in 25%  
464 glycerol at -80°C. Confirmation of resistance to each antibiotic was determined by re-measuring  
465 the MICs of each glycerol freezer stock.

466

#### 467 **Bacterial Cytological Profiling**

468 Experiments described in Figure 2 were performed as described in (Nonejuie et al., 2013). In  
469 later experiments (Figure 6A), overnight *E. coli* *lptD4213* cultures were diluted 1:100 and grown  
470 to early-mid exponential phase (OD600 = 0.4-0.6). Each culture was then diluted 1:10 into fresh  
471 LB and treated with the desired concentration of antibiotic for 10 minutes. Following antibiotic  
472 treatment, cells were stained with 0.5  $\mu$ M SYTOX Green, 1 $\mu$ g/mL FM4-64, and 2  $\mu$ g/mL DAPI.  
473 Each stained culture was then spotted onto a 1.5% agar pad supplemented with casamino acids  
474 and 20% glucose in M63. The *E. coli* cells were segmented, and single-cell features were  
475 extracted using a custom Matlab code. Principal component analysis was performed using the  
476 `prcomp` function in R and clustering was performed using the single-linkage method.

477

#### 478 **Thermal Proteome Profiling assay**

479 Thermal proteome profiling experiments were performed as described by (Mateus et al., 2018)  
480 Briefly, whole cell samples were treated with 0.6, 1.1, 2.2, 4.4 $\mu$ g/mL SCH-79797 and 0.1, 0.5,  
481 2.3, 11.6 $\mu$ g/mL trimethoprim. Cell lysate samples were treated with 0.4, 1.8, 8.9, 44.4 $\mu$ g/mL

482 SCH-79797 and 0.1, 0.5, 2.3, 11.6µg/mL trimethoprim. The mass spectrometry proteomics data  
483 have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository  
484 with the dataset identifier PXD013673. After heat treatment, the soluble fraction was collected,  
485 digested with trypsin and peptides were labeled with tandem mass tags (Werner et al., 2014).  
486 Samples were subjected to two-dimensional liquid chromatography and analyzed on a Q  
487 Exactive Plus mass spectrometer (Thermo Fisher Scientific). While the data collected is  
488 proteome wide, three different cutoffs were used to define three classes of potential targets. The  
489 color of the point indicates the signal maximal effect size across all temperatures and the largest  
490 change in abundance across all concentrations was selected for continued analysis (Dots,  
491 Figure 3.) A mild effect indicates at least one temperature had a change in abundance of at  
492 least 25% in both whole cell and cell lysate treatments (Triangles, Figure 3) Proteins that had a  
493 change in abundance of at least 25% at three or more temperatures (Squares, Figure 3). To be  
494 considered a consistent effect, the change in abundance of the protein had to show the same  
495 sign at least 90% of the time and have an effect size of at least 1 (2-fold) in either whole cells or  
496 cell lysates.

497

#### 498 ***B. subtilis* CRISPRi hypersensitivity assay**

499 We used the indicated mutants from the CRISPR interference (CRISPRi) library as curated by  
500 (Peters et al., 2016) to measure the sensitivity of mutants involved in folate synthesis to SCH-  
501 79797. MICs were measured using 2-fold dilutions of each antibiotic in 96-well plates and cell  
502 growth was monitored by measuring the OD600 in a Tecan microplate reader.

503

#### 504 **Metabolomics**

505 Overnight *E. coli* NCM3722 cultures were grown and diluted 1:100 in Gutnick Minimal Media  
506 and grown to early-mid exponential phase (OD600 = 0.4-0.6). Cultures were treated with either  
507 1X MIC SCH-79797 (13.89µg/mL) or 1X MIC trimethoprim (0.15µg/mL) for 15 minutes. Folates  
508 were extracted by vacuum filtering 15mL of treated cells using 0.45µm HNWP Millipore nylon  
509 membranes and immediately placing filters into an ice-cold quenching solution containing  
510 40:40:20 MetOH:acetonitrile:25Mm NH<sub>4</sub>OAc + 0.1% sodium Ascorbic in HPLC H<sub>2</sub>O. The  
511 resulting solution was then centrifuged at 16,000×g for 1.5 min at 4°C and the supernatant was  
512 saved for mass spectrometry analysis which was performed as described in (Chen et al., 2017)

513

#### 514 **DHFR Enzymatic Activity assay**



515 *E. coli* DHFR enzyme (FolA) was custom purified by Genscript (Piscataway, NJ). The enzymatic  
516 activity of DHFR with and without SCH-79797 treatment was measured using the Dihydrofolate  
517 Reductase Assay kit from Sigma. Briefly, DHFR activity was measured by monitoring the  
518 change in sample absorbance at 340nm due to DHFR-dependent NADPH consumption.

519

#### 520 **Persister cell assay**

521 Stationary phase *S. aureus* cells have the same antibiotic tolerant properties of persister cells  
522 (Kim et al., 2018). Thus, overnight MRSA USA300 cultures were used to measure the  
523 effectiveness of SCH-79797 against persister cells. An overnight *S. aureus* MRSA USA300  
524 culture was diluted 1:100 in PBS prior and then cell viability was determined using the same  
525 CFU analysis described above.

526

#### 527 **Membrane potential and permeability assay**

528 The BacLight Bacterial Membrane Potential kit from Sigma was used to measure the effect of  
529 SCH-79797 treatment on bacterial membrane potential. This kit uses DiOC2(3) to measure a  
530 cell's membrane potential. This dye concentrates in cells with an active membrane potential  
531 causing the emission of DiOC2(3) to shift from green (488nm excitation, 525/50nm bandpass  
532 filter for emission) to red (488nm ex, 610/20 bp filter). As a result, by measuring the  
533 fluorescence shift from red to green, we can detect changes in a cell's membrane potential. TO-  
534 PRO-3 is a dye that is excluded from cells with a healthy membrane. Thus, we can detect  
535 membrane damage by measuring the far-red fluorescence intensity (640nm ex, 670/30nm bp  
536 filter). The LSRII flow cytometer (BD Biosciences) at the Flow Cytometry Resource Facility,  
537 Princeton University, was used to measure the fluorescent intensities of both dyes in response  
538 to antibiotic treatment. Data was analyzed using FlowJo v10 software as described in (Novo et  
539 al., 1999).

540

#### 541 **FIGURE LEGENDS**

542

543 **Figure 1. SCH-79797 is a broad-spectrum bactericidal antibiotic that is effective in an**  
544 **animal model and has a low frequency of resistance.** A. The MIC of SCH-79797 against  
545 Gram-negative (red) and Gram-positive (black) bacteria. MIC here and in subsequent figures is  
546 defined as the concentration of drug that resulted in no visible bacterial growth. Bacteria were  
547 grown for 14h at 37°C and growth media for each bacterium is specified in table S1. B. The  
548 relative growth of *E. coli* *lptD4213* after treatment with SCH-79797. Bacterial growth was

549 measured for 14h and the final optical density at 600nm (OD600) was plotted against drug  
550 concentration. Each data point represents 2 independent replicates. Mean  $\pm$  s.d. are shown. C.  
551 Colony forming units (CFU ml<sup>-1</sup>) after 3-hour treatment of *E. coli* *lptD4213* with 1% DMSO, 2X  
552 MIC SCH-79797, 2X MIC ampicillin, and 4X MIC novobiocin. Data points at  $1 \times 10^2$  CFU ml<sup>-1</sup> are  
553 below the level of detection. Each data point represents 3 independent samples and 3 technical  
554 replicates. Mean  $\pm$  s.d. are shown. D. The percent survival of *A. baumannii* infected *G.*  
555 *mellonella* larvae after treatment with 2 $\mu$ l/larva of 100% DMSO, 67 $\mu$ g/larva SCH-79797,  
556 6 $\mu$ g/larva gentamicin, and 67 $\mu$ g/larva rifampicin. Data represents a typical cohort (n = 12) from a  
557 biological triplicate. Mantel-cox statistics for the cohort were calculated with PRISM, and the  
558 pooled results are presented in the supplemental material (Figure S2C). E. Fold increase in  
559 resistance of *S. aureus* MRSA USA300 to SCH-79797, novobiocin, trimethoprim, and nisin after  
560 25 days of serial passaging in 0.5X MIC of each drug and plotted on a log<sub>2</sub> scale. Resistance  
561 was confirmed by remeasuring MIC's from aliquots of each passage that were collected and  
562 stored at -80°C. Data represents one biological replicate and the data for the second replicate is  
563 shown in Figure S3A.

564

565 **Figure 2. Bacterial Cytological Profiling indicates that SCH-79797 functions by a**  
566 **mechanism distinct from known classes of antibiotics.** A. Fluorescent images of *E. coli*  
567 *lptD4213* cells treated with antibiotics representative of 5 different antibiotic classes. Cells were  
568 treated for 2h with 5X MIC of each drug. Merged image channels are phase (grey), FM4-64  
569 (red), Dapi (blue), and SYTOX (green). All images are at the same magnification and the scale  
570 bar is 1 $\mu$ m. B. Comparison of cytological profiles of known antibiotics with the cytological profile  
571 of SCH-79797. Single-linkage clustered vectors of Mahalanobis distances from each antibiotic  
572 treatment group were compared to that of all other antibiotic treatment groups. Linkage is  
573 included in the dendrogram.

574

575 **Figure 3. Thermal proteome profiling suggests that SCH-79797 binds DHFR.** A. Schematic  
576 of the thermal shift assay that compares the thermal stability of the entire proteome with and  
577 without drug treatment. Protein samples are aliquoted, and each aliquot is heated to an  
578 increasing temperature. The relative fraction of soluble and insoluble proteins is then  
579 determined for each aliquot by ultracentrifugation and mass spectrometry. B-C. The relative  
580 thermal stability of the soluble *E. coli* *lptD4213* proteome after treatment of whole cell and cell  
581 lysate samples with SCH-79797 and trimethoprim. Changes in thermal stability were determined  
582 by measuring changes in the abundance of soluble protein across 10 different temperatures

583 ranging from 42-72°C and 4 drug concentrations and a vehicle control. For each point, the color  
584 indicates the maximal effect size across all temperatures and the largest change in abundance  
585 across all concentrations. Squares represent the proteins with a change in abundance of at  
586 least 25% at three or more temperatures. To be considered consistent, the change in  
587 abundance of a protein had to show the same sign at least 90% of the time and have an effect  
588 size of at least 2-fold in either whole cells or cell lysates. Triangles represent a milder effect  
589 where at least one temperature had a change in abundance of at least 25% in both whole cell  
590 and cell lysate treatments.

591  
592 **Figure 4. SCH-79797 targets folate metabolism by competitively inhibiting DHFR.** A. A  
593 partial representation of the folate synthesis pathway. B. The growth of CRISPRi *B. subtilis*  
594 knockdown mutants involved in folate synthesis relative to a DMSO-treated control after SCH-  
595 79797 and trimethoprim treatment. Bacterial growth was measured for 14h and the final OD600  
596 of each condition was plotted against drug concentration. Each data point represents 2  
597 independent replicates. Mean  $\pm$  s.d. are shown. C. Metabolomic analysis of *E. coli* NCM3722  
598 cells treated with 0.5X MIC SCH-79797 or trimethoprim. Samples were taken 0, 5, 10, and 15  
599 min. after drug treatment. Folate metabolite abundance at each time point was quantified  
600 relative to the DMSO-treated control samples at the initial timepoint. 3 independent replicates of  
601 this experiment were performed. Each data point represents 3 independent replicates.  
602 Mean  $\pm$  s.d. are shown. D. The enzymatic activity of DHFR upon SCH-79797 treatment, relative  
603 to a vehicle-treated (ethanol) control. A linear-fit was applied to the resulting plot to determine  
604 the IC50 and 3 independent replicates are shown. The shaded region represents the 90%  
605 confidence interval of the fit. E. A Lineweaver-Burk plot of the enzymatic activity of DHFR after  
606 treatment with 1 $\mu$ g/mL SCH-79797 and a 1% ethanol control. Fits to the Michaelis-Menten  
607 equation are shown with shaded regions corresponding to 90% confidence intervals.

608  
609 **Figure 5. SCH-79797 is distinct from other DHFR inhibitors and disrupts membrane**  
610 **integrity.** A. The growth of WT and  $\Delta$ *thyA* *E. coli* *lptD4213* relative to a DMSO-treated control  
611 after SCH-79797 and trimethoprim treatment. Bacterial growth was measured for 14h and the  
612 final OD600 of each condition was plotted against drug concentration. Each data point  
613 represents 2 independent replicates. Mean  $\pm$  s.d. are shown. B. Schematic of flow cytometry  
614 data showing the expected results for each class of polarized, depolarized, permeable and  
615 impermeable bacteria. C. Flow cytometry analysis of the membrane potential and permeability  
616 of *E. coli* *lptD4213* cells after 15 min. incubation with 1% DMSO, 1X MIC CCCP, 1X MIC nisin,

617 1X and 2X MIC SCH-79797, and 10X MIC trimethoprim. CCCP and nisin treatment served as  
618 depolarizing and permeabilizing controls respectively. These controls were used to define the  
619 quadrants outlined in (B).

620

621 **Figure 6. SCH-79797 mimics co-treatment with folate metabolism and membrane integrity**

622 **disruptors but can be more effective than their combination.** A. BCP analysis of *E. coli*

623 *lptD4213* cells after 30 min. of treatment with 1% DMSO, 1X MIC SCH-79797, 10X MIC

624 trimethoprim, 2X MIC nisin, and the combination of 10X MIC trimethoprim and 2X MIC nisin.

625 Cytological profiles were clustered by the first three principal components that account for at

626 least 90% of the variance between samples. Cells were stained with DAPI, FM4-64, and

627 SYTOX Green. Shown here are the merged images of Dapi (blue) and FM4-64 (red) and the

628 scale bar is 1 $\mu$ m. B-C. The viability of (B) *E. coli lptD4213* and (C) *S. aureus* MRSA USA300

629 persister cells measured in CFU mL<sup>-1</sup> after 2 hours of treatment with 1% DMSO, 1X MIC SCH-

630 79797, 10X MIC trimethoprim, 2X MIC nisin, and the combination of 10X MIC trimethoprim and

631 2X MIC nisin. Each bar represents 3 independent samples and 3 technical replicates.

632 Mean  $\pm$  s.d. are shown.

633

634 **Figure 7. A Derivative of SCH-79797 helps elucidate its two MoAs.** A. The structures of

635 SCH-79797 and the pyrroloquinazolinodiamine core lacking the side chains, THM-10. B. The

636 MICs of SCH-79797 and THM-10 against *E. coli lptD4213*, *B. subtilis* W168, *S. aureus* MRSA

637 USA300, and *A. baumannii* AB17978. C. The growth of CRISPRi *B. subtilis* knockdown mutants

638 involved in folate synthesis relative to a DMSO-treated control after THM-10 treatment. Bacterial

639 growth was measured for 14h and the final optical density (OD600) of each condition was

640 plotted against drug concentration. Each data point represents 2 independent replicates.

641 Mean  $\pm$  s.d. are shown. D. Flow cytometry analysis of the membrane potential and permeability

642 of *E. coli lptD4213* cells after 15 min. incubation with 1X MIC THM-10.

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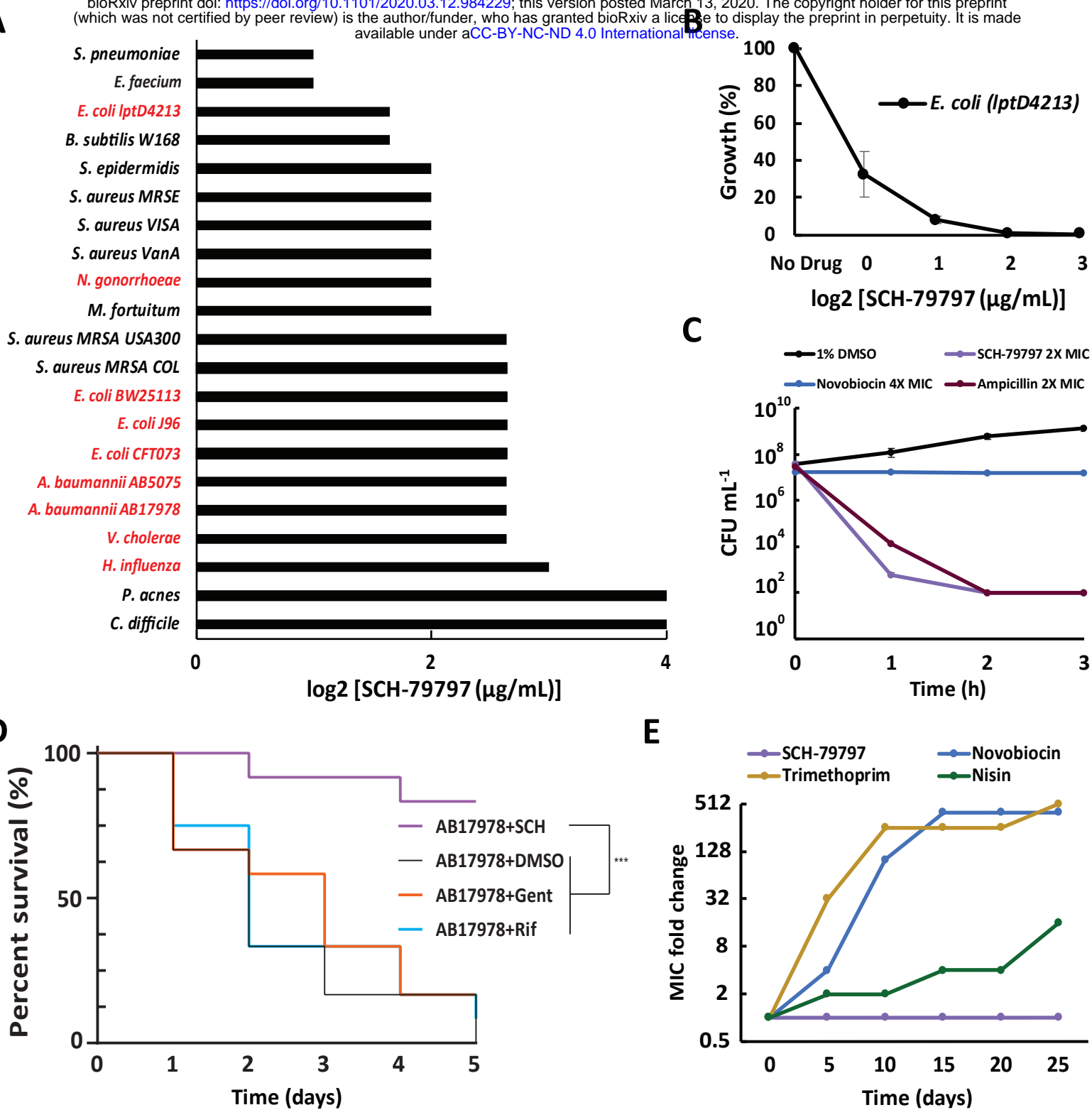
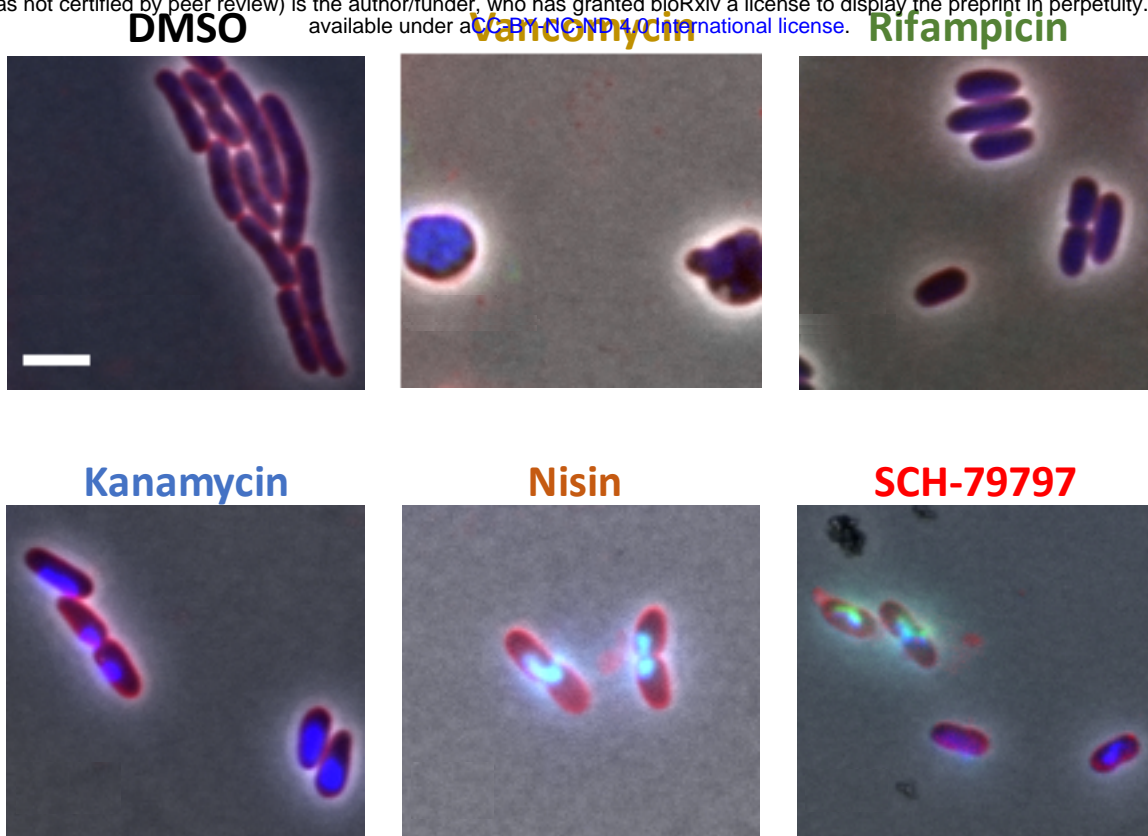
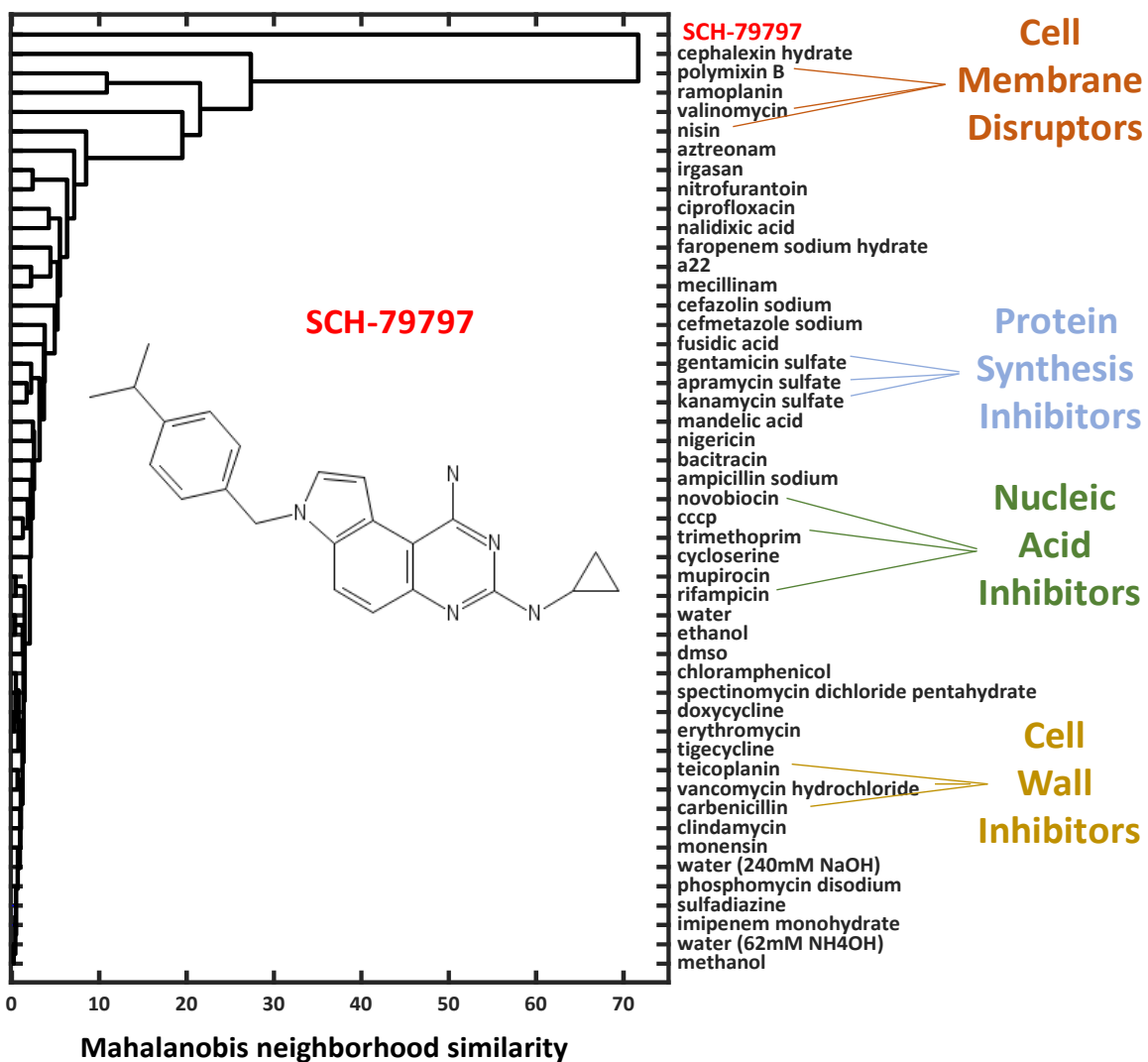


Figure 1

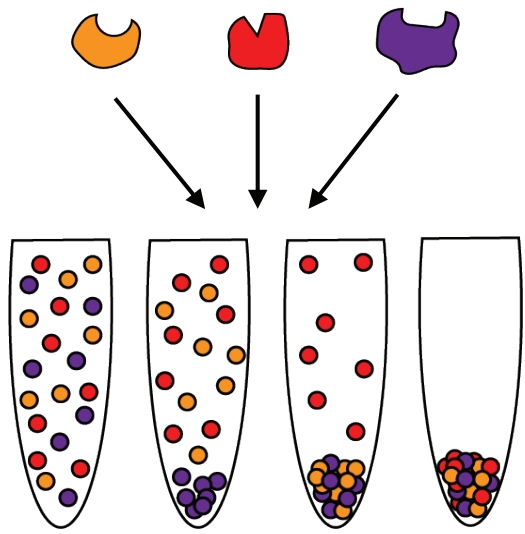


**B**

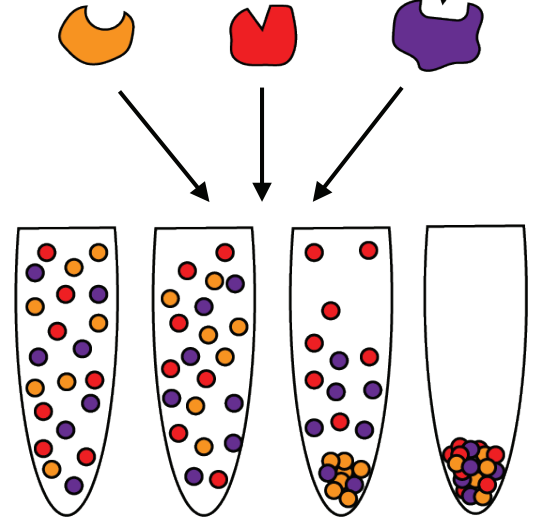


**Figure 2**

**A**



+  
Drug

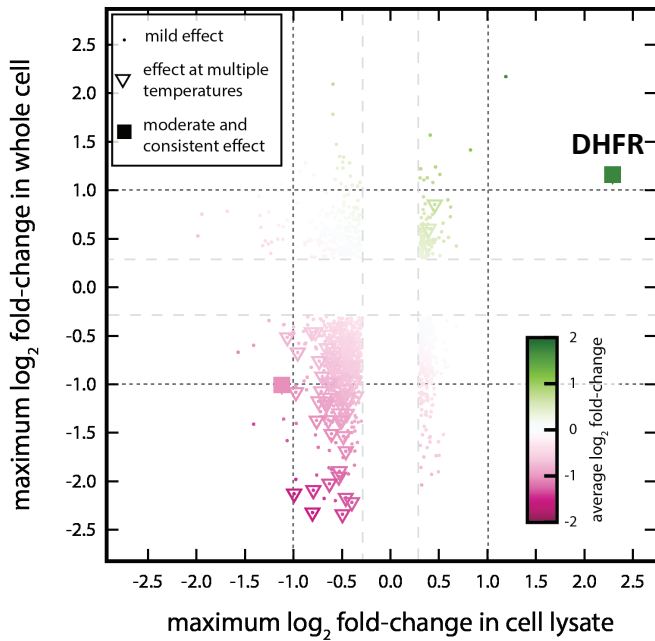


Increasing Temperature

Increasing Temperature

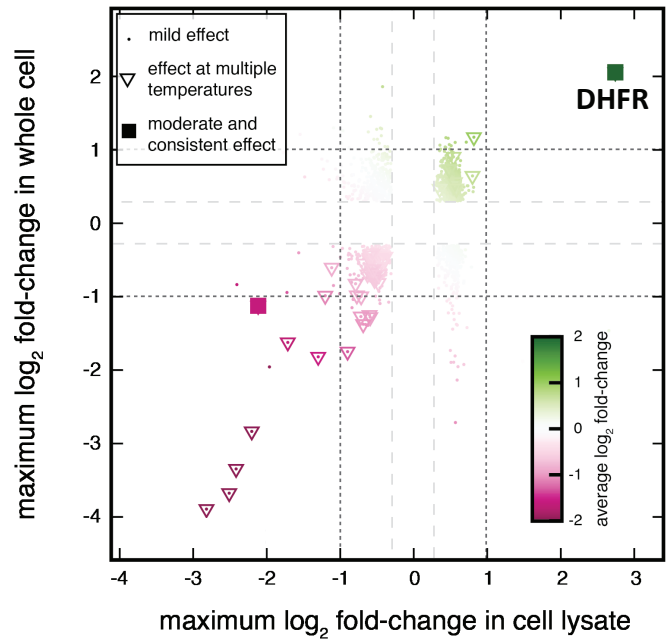
**B**

SCH-79797

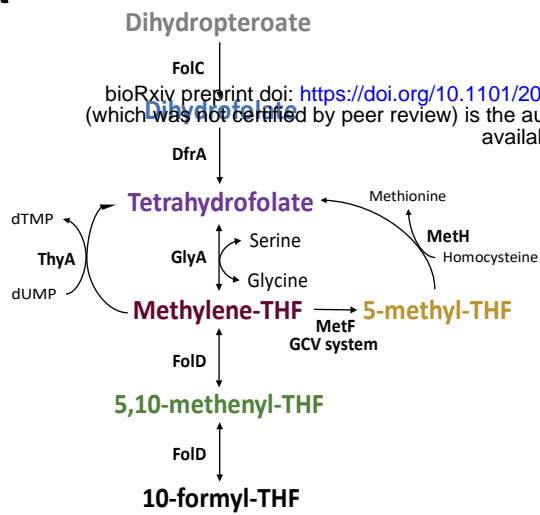


**C**

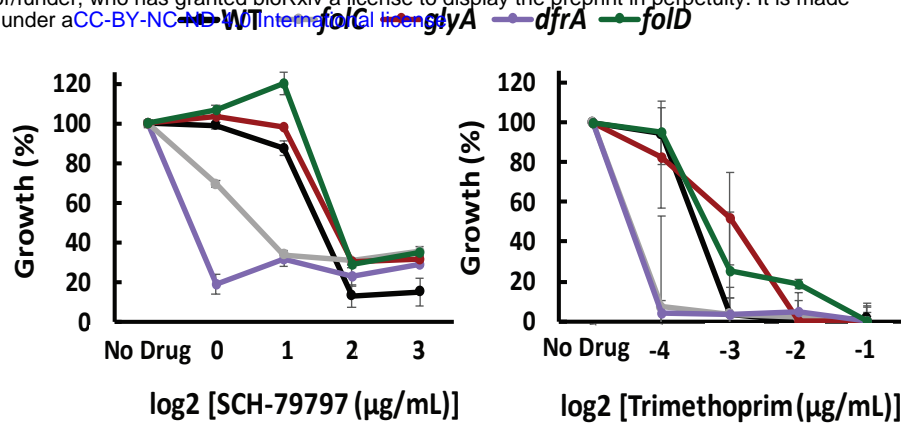
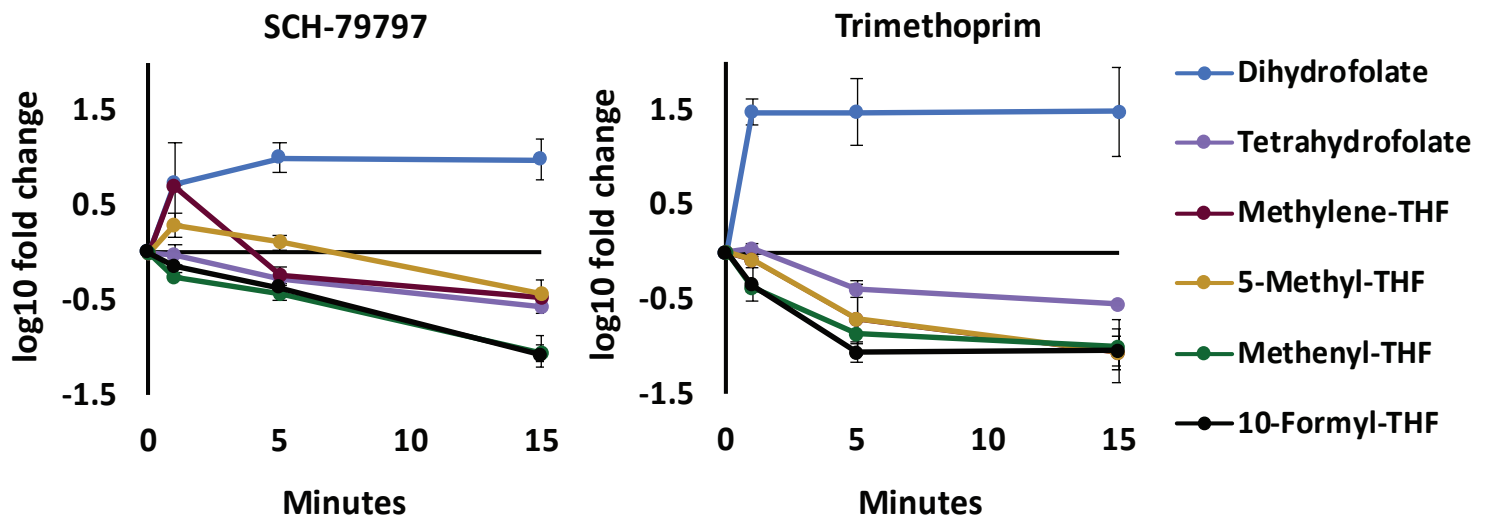
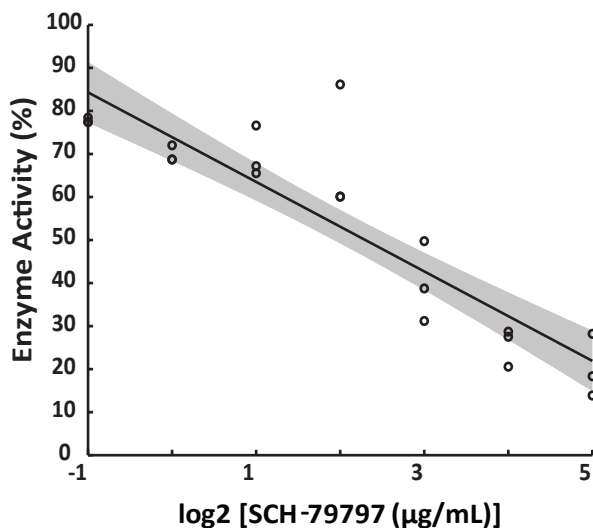
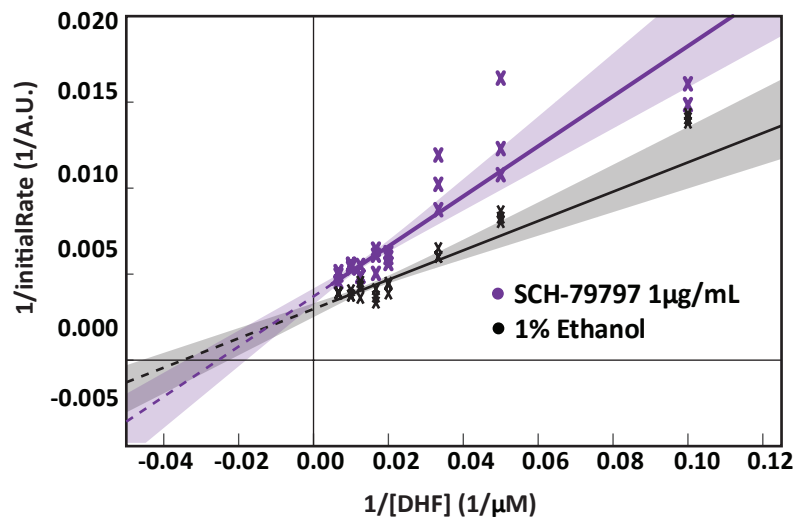
Trimethoprim



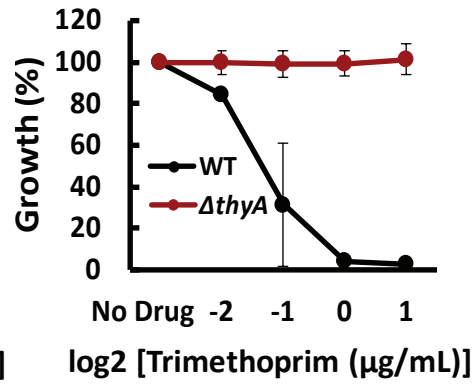
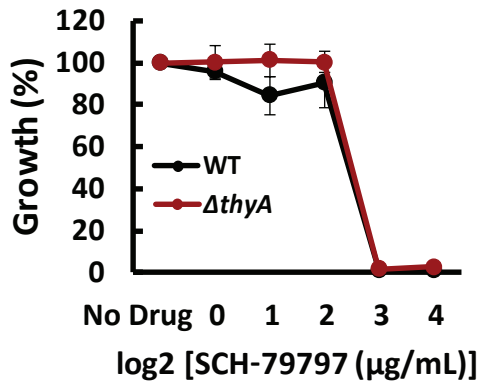
**Figure 3**

**A**

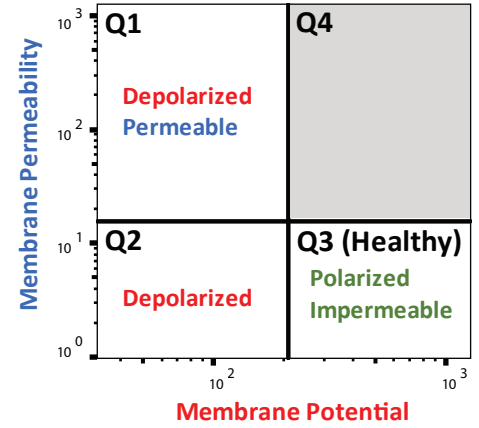
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**B****C****D****E****Figure 4**

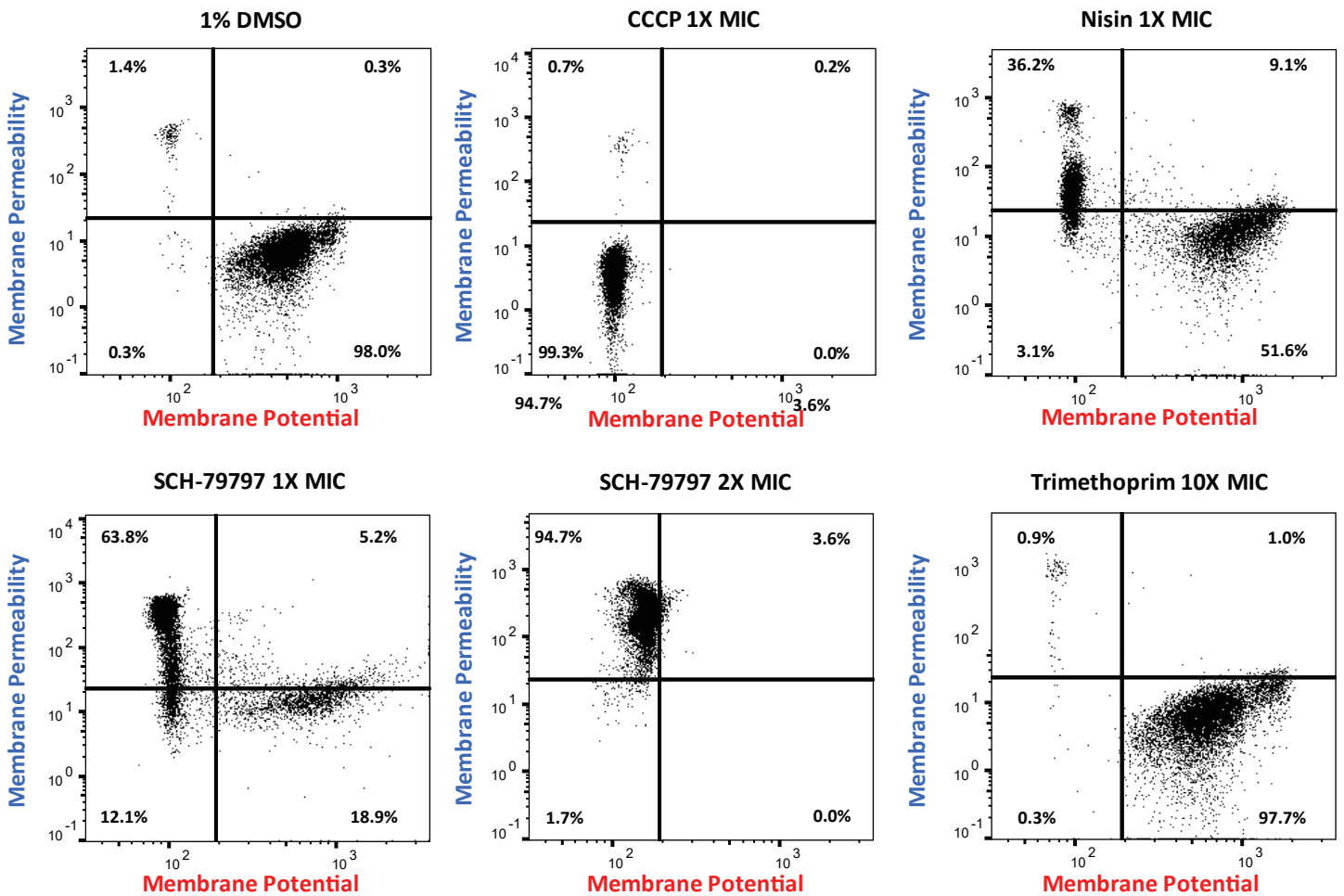
**A**



**B**

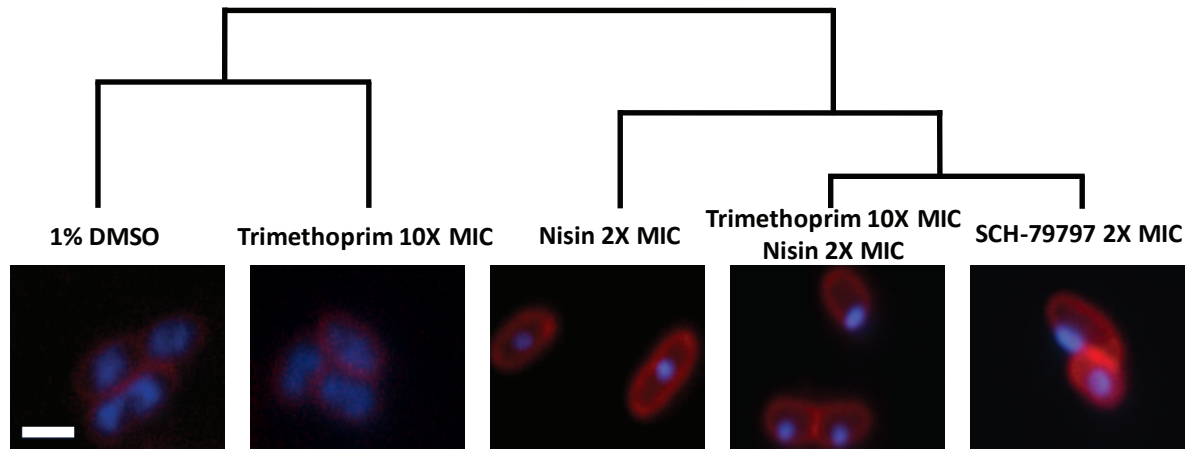


**C**

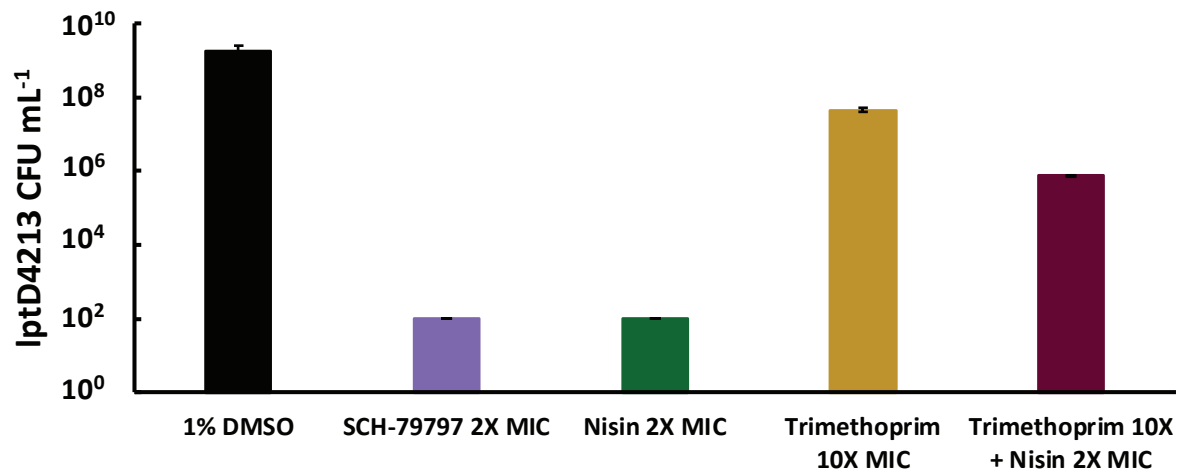


**Figure 5**

A



B



C

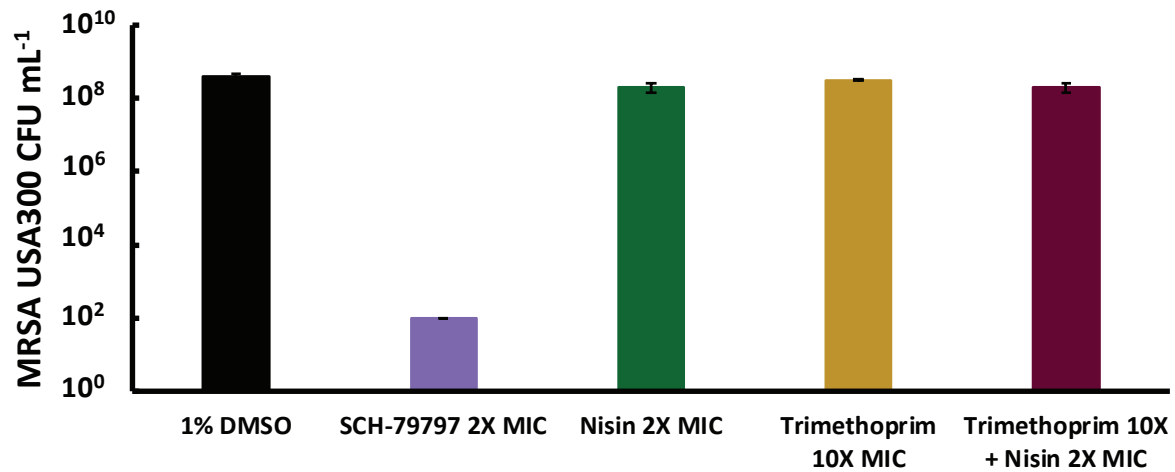
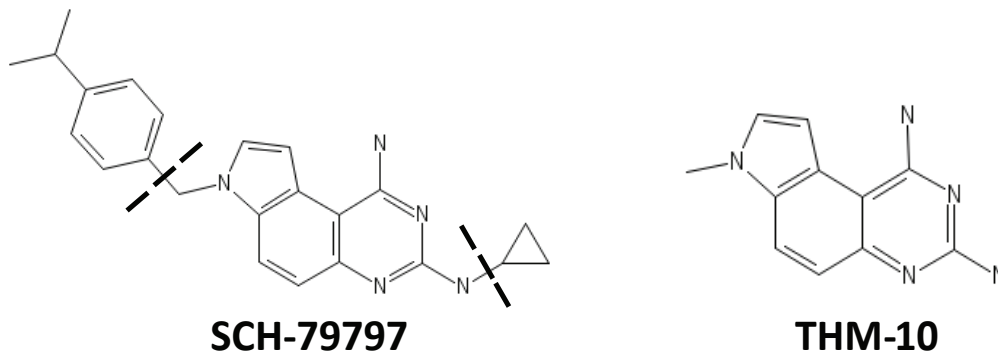


Figure 6

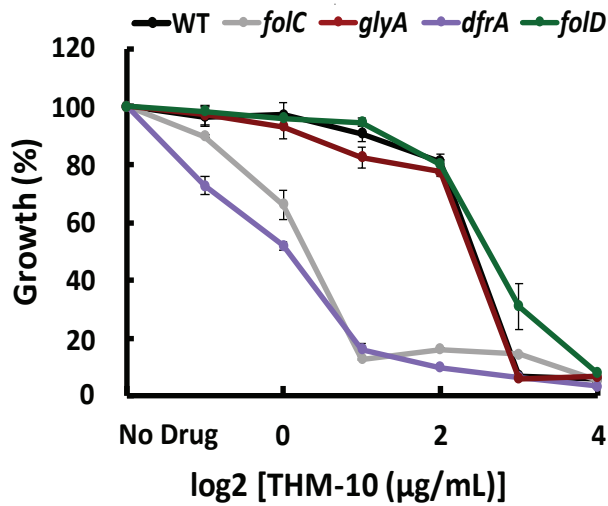
**A**



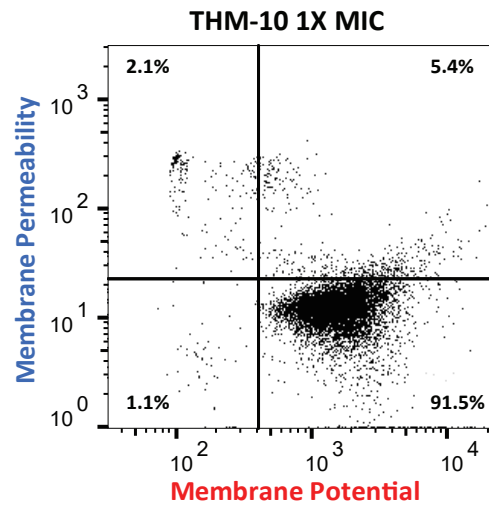
**B**

	Isolate	SCH-79797	THM-10
<b>MIC (<math>\mu\text{g}/\text{mL}</math>)</b>	<i>E. coli</i> <i>lptD4213</i>	3.13	0.78
	<i>B. subtilis</i> <i>W168</i>	3.13	6.25
	<i>S. aureus</i> <i>USA300</i>	6.25	>25
	<i>A. baumannii</i> <i>17978</i>	6.25	>25

**C**



**D**



**Figure 7**