Thiocillin and Micrococcin Exploit the Ferrioxamine Receptor of *Pseudomonas aeruginosa* for Uptake

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ABSTRACT

Thiopeptides are a class of Gram-positive antibiotics that inhibit protein synthesis. They have been underutilized as therapeutics due to solubility issues, poor bioavailability, and lack of activity against Gram-negative pathogens. We discovered recently that a member of this family, thiostrepton, has activity against *Pseudomonas aeruginosa and Acinetobacter baumannii* under iron-limiting conditions. Thiostrepton uses pyoverdine siderophore receptors to cross the outer membrane, and combining thiostrepton with an iron chelator yielded remarkable synergy, significantly reducing the minimal inhibitory concentration. These results led to the hypothesis that other thiopeptides could also inhibit growth by using siderophore receptors to gain access to the cell. Here, we screened six thiopeptides for synergy with the iron chelator deferasirox against *P. aeruginosa* and a mutant lacking the pyoverdine receptors FpvA and FpvB. Our findings suggest that thiopeptides such as thiocillin cross the outer membrane using FoxA, the ferrioxamine siderophore receptor. Other structurally related thiopeptides did not inhibit growth of *P. aeruginosa*, but had greater potency against methicillin-resistant *Staphylococcus aureus* than thiostrepton and related thiopeptides. These results suggest that thiopeptide structures have evolved with considerations for target affinity and entry into cells.

INTRODUCTION

Thiopeptides are a class of natural products with potent antibacterial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA)[1]. They are characterized chemically by the oxidation state of a central pyridine ring and by the size of a core macrocyclic ring comprised of thiazole moieties[2]. Thiopeptides act on the ribosome to inhibit protein translation but the exact mechanism depends on the number of members in the core macrocyclic ring: 26-membered macrocycles inhibit elongation factor G whereas 29-membered macrocycles block elongation factor Tu binding[3–5]. The exact mechanism of action for 35-membered macrocycles remains unknown, although they are reported to target the 50S ribosome, similar to thiostrepton (TS)[6]. TS is among the best-studied

32 26-membered thiopeptides and has multiple biological properties, including anticancer and antimalarial
33 activities[7,8].

34 In 1948, Su isolated a strain of *Micrococcus* from sewage samples that produced a compound with
35 activity against Gram positive but not Gram negative bacteria[9]. The compound, micrococcin, was the first
36 thiopeptide to be discovered. Since then, the thiopeptide family has expanded to over 100 members of
37 diverse structure and origin[10]. Thiopeptides belong to a larger class of natural products called ribosomally
38 synthesized and post-translationally modified peptides (RiPPs)[11]. These natural products are produced
39 from a peptide precursor whose sequence varies depending on the thiopeptide, and undergo many post-
40 translational modifications to reach their final structure[12]. Most thiopeptides are produced by Gram-
41 positive soil and marine organisms including *Bacillus cereus, Streptomyces* spp., and *Nocardopsis* spp.,
42 although some Gram negatives such as *Serratia marcesens* have biosynthetic gene clusters encoding
43 thiopeptide synthesis[13–17].

44 Due to their poor solubility, challenging synthesis, and limited potency against Gram-negative
45 bacteria, pharmaceutical companies ruled out thiopeptides for development as clinically useful
46 antibiotics. Instead, researchers focused on addressing these issues through total synthesis, discovery of
47 new thiopeptides and structure elucidation, identification of thiopeptide biosynthetic gene clusters, and
48 modification of existing thiopeptides to improve solubility and activity [12,17–25]. However, there is a gap in
49 knowledge regarding their utility against Gram-negative bacteria which likely stems from the assumption
50 that thiopeptides cannot cross the outer membrane.

51 We showed recently that TS hijacks pyoverdine receptors to cross the outer membranes of
52 *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* among the World Health Organization's priority
53 pathogens for new antibiotic development[26 27] *P. aeruginosa* and *A. baumannii* are opportunistic
54 pathogens with intrinsic resistance to many antibiotics and are causative agents of healthcare-acquired
55 infections[28]. Both express receptors for pyoverdine, a siderophore with high affinity for iron. TS uptake
56 through pyoverdine receptors occurred under iron-limited conditions, and deferasirox (DSX), an FDA-
57 approved oral iron chelator, strongly synergized with TS to inhibit the growth of clinical isolates of both
58 species. Combinations of TS with other iron chelators were also effective[29]. These findings showed that
59 thiopeptides can cross the outer membranes of specific Gram-negative bacteria. TS lacks activity against
60 *Escherichia coli* because it does not express pyoverdine receptors. The thiopeptide family has over 100
61 members and recent bioinformatic analyses suggest that there could be hundreds more thiopeptides[30,31].
62 We hypothesized that other thiopeptides may also have activity against Gram-negative pathogens.

63 Here, we screened six thiopeptides (Figure 1, Supplementary Figure 1) for activity against *P.*
64 *aeruginosa* and potential synergy with DSX. Combinatorial assays showed that two of these used the
65 ferrioxamine siderophore receptor, FoxA, to enter the cell, confirming our hypothesis that other
66 thiopeptides could cross the outer membrane. The 26-membered macrocycles are of particular interest

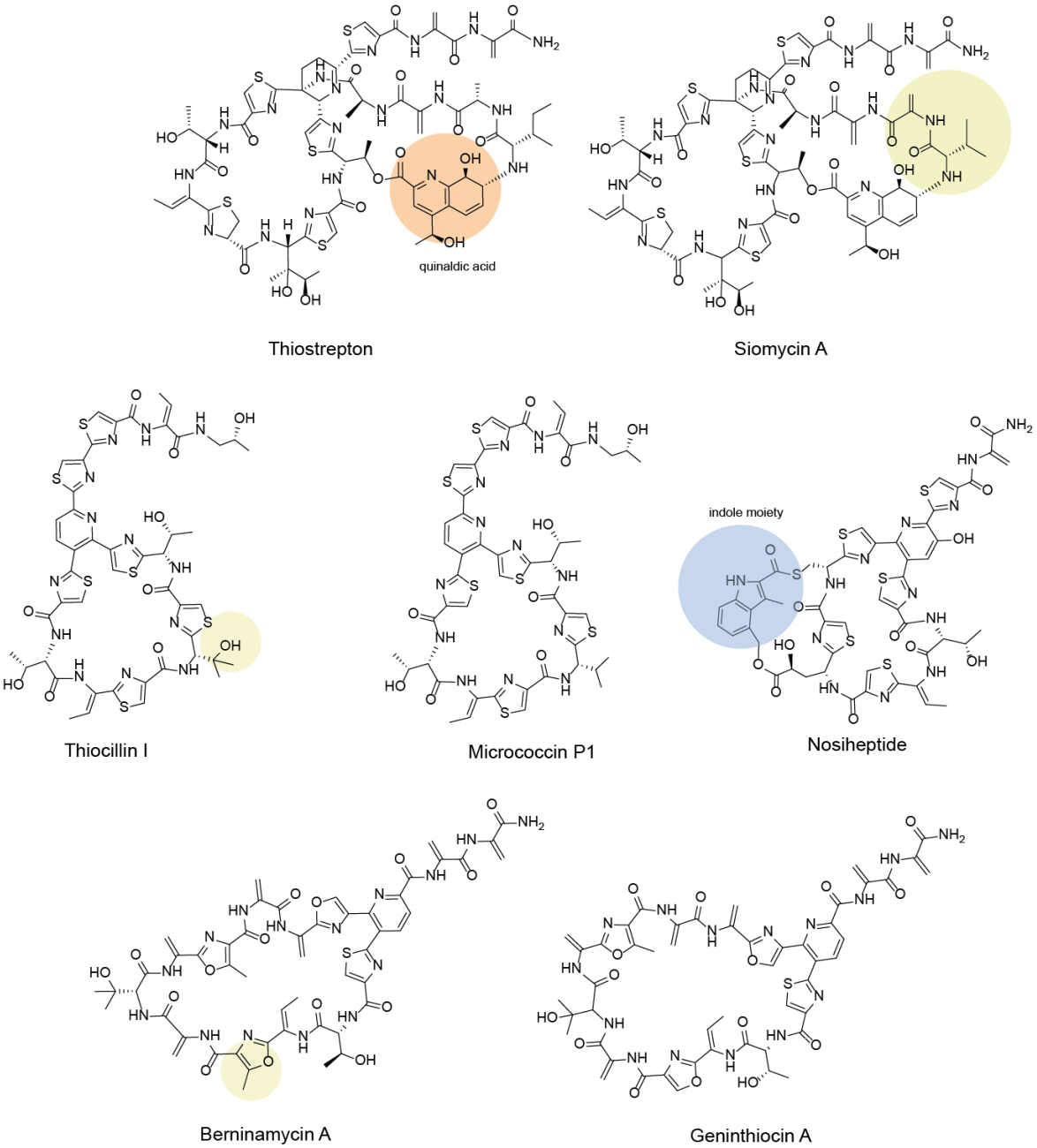

Figure 1. Structures of Thiopeptides Tested for Synergy with DSX. Differences between structural analogues are highlighted in yellow. The following are structural analogues: TS and SM, TC and MC, (26-membered macrocycles) and BER & GEN (35-membered macrocycles). NH is structurally similar to TC and MC, but has a unique indole moiety (blue). SM and TS have a unique quinaldic acid moiety (orange), but SM differs by two amino acids.

67 because modifications to the core macrocyclic ring altered siderophore specificity. Other modifications
68 abolished Gram-negative activity but improved Gram-positive activity. Thiopeptides are an underutilitzed
69 class of antibiotics with potential for development as clinically useful drugs, especially as iron limitation
70 at sites of infection could promote thiopeptide uptake.

71

72 **RESULTS**

73 *Siomycin A Uses the Pyoverdine Receptors for Uptake*

74 We screened the activity of six thiopeptides in combination with DSX against *P. aeruginosa* PA14 using
75 checkerboard assays. We looked for individual thiopeptide activity, synergy, and activity against a mutant
76 lacking pyoverdine receptors FpvA and FpvB, which results in TS resistance. If the combination was active
77 against the wild type but not the *fpvAB* mutant, the thiopeptide likely uses those receptors to cross the
78 membrane. The six thiopeptides in the screen included siomycin A (SM), thiocillin I (TC), micrococcin P1
79 (MC), nosiheptide (NH), berninamycin A (BER), and geninthiocin A (GEN) (Figure 1).

80 SM differs from TS in that the two amino acids adjacent to the quinaldic acid moiety are dehydroalanine
81 and isoleucine instead of alanine and leucine. TC and MC are nearly identical except that TC has a
82 hydroxyvaline and MC has a valine in its macrocyclic ring. BER and GEN differ by a single methyl group on

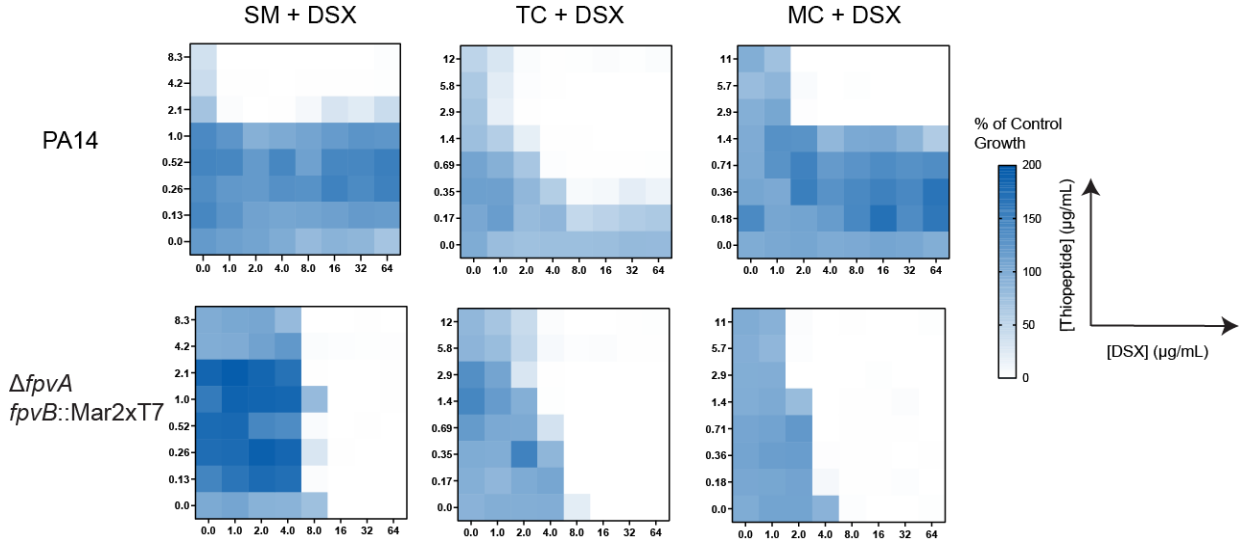

Figure 2. Thiopeptides synergize with DSX against PA14 and PA14 *ΔfpvA fpvB*::Mar2xT7. Checkerboard assays were conducted in 10:90 medium. Top row are checkerboards conducted with each thiopeptide against wild type PA14 whereas bottom row are checkerboards conducted against its pyoverdine receptor-null mutant. The concentration of the thiopeptides are indicated along the column (maximum concentration of 10 µM in µg/mL except for SM which was tested at 5 µM) and the concentration of DSX is indicated along the row (maximum concentration of 64 µg/mL). Growth is proportional to the intensity of blue whereas white indicates no growth. Results are averaged from three independent
83 experiments.

one of the oxazole rings. NH resembles TC and MC but is structurally unique from the other thiopeptides, with an indole moiety linked to the core macrocyclic ring to form a secondary ring. TS, SM, TC, MP, and NH belong to the 26-membered macrocycles whereas BER and GEN have 35-membered macrocycles.

Based on checkerboard assays, BER, GEN, and NH did not synergize with DSX (Supplementary Figure 1). SM synergized with DSX against wild type PA14 but the combination had no effect against the *fpvAB* mutant (Figure 2). Unlike the wild type, growth of the mutant was inhibited by 16 µg/ml DSX alone. These data are similar to those obtained previously with TS + DSX[26] and indicates that SM likely crosses the outer membrane using the pyoverdine receptors. However, the synergistic profile of SM + DSX was less pronounced compared to TS + DSX. There are two potential explanations for its weaker synergistic profile: 1) siomycin has less affinity for the target or 2) siomycin has less affinity for the receptors. To distinguish between these possibilities, we tested each thiopeptdide against the Gram-positive pathogen MRSA USA 300 in a broth dilution assay (Supplementary Figure 2). MRSA lacks the outer membrane; therefore, the main consideration would be differences in affinity for the highly-conserved ribosomal target. No differences between the minimal inhibitory concentration (MIC) of cells treated with TS or SM were observed, suggesting that the differences in susceptibility in *P. aeruginosa* were related to receptor affinity. Further, this result provides some structure-activity relationship (SAR) information regarding interactions between TS and the pyoverdine receptors, specifically that the alanine and leucine adjacent to the quinaldic acid do not affect target activity but improve uptake; however, they are not essential because SM can still use the pyoverdine receptors to cross the outer membrane.

Thiocillin I and Micrococcin P1 Exploit the Ferrioxamine Receptor

In checkerboard assays, TC and MC synergized with DSX against *P. aeruginosa* PA14 (Figure 2). The concentration of DSX was kept constant at 64 µg/mL, while the highest concentration of TC and MC tested due to solubility limitations was 12 µg/mL (10 µM). Remarkably, this combination inhibited growth of the *fpvAB* mutant (Figure 2). This result indicated that that TC and MC likely use a different siderophore receptor to enter the cell. To identify that receptor, we screened a panel of 16 siderophore receptor-deficient transposon mutants of PA14 (Supplementary Table 2). Only a *foxA* transposon mutant, lacking the ferrioxamine receptor, displayed resistance to TC + DSX (Figure 3AB). When the mutant was complemented with *foxA* in trans*,* susceptibility to TC was restored, confirming that *foxA* is necessary for TC uptake (Figure 3C). The susceptibility of the complemented strain was slightly reduced compared to the wild type, likely due to the low copy number of the vector in *P. aeruginosa*.

115

116 *TC and MC inhibit the growth of TS-resistant clinical*
117 *isolates*

118 In our previous studies, we identified *P.*
119 *aeruginosa* clinical isolates resistant to the TS + DSX
120 combination. One isolate, C0379, was resistant due
121 to a 5' truncation of the gene encoding the FpvB
122 receptor[26,29]. We hypothesized that TC or MC might
123 inhibit the growth of this isolate because the only
124 differences in its *foxA* gene compared to that of
125 PAO1 were five silent mutations and a single base
126 pair mutation (D760G) in one beta-strand
127 belonging to a structural component of the
128 receptor (Supplementary Table 3). Consistent with
129 this idea, C0379 was susceptible to combinations
130 of TC + DSX or MC + DSX (Supplementary Figure
131 3A). Bioinformatic analysis showed that *A.*
132 *baumannii* also encodes the FoxA receptor. We
133 tested one multidrug-resistant clinical isolate,
134 C0286, for susceptibility to TC + DSX and found
135 that it was susceptible even at the lowest
136 concentration of TC tested (0.17 µg/mL)
137 (Supplementary Figure 3B). However, the lab
138 strain ATCC 17978 was not susceptible to the
139 combination, suggesting inter-strain differences.
140 This is not unusual as it is well established that lab
141 adapted strains may not behave similarly to
142 clinical isolates[32]. Overall, we conclude that TC +
143 DSX can inhibit the growth of *P. aeruginosa* and *A.*
144 *baumannii* clinical isolates including those
145 resistant to TS.

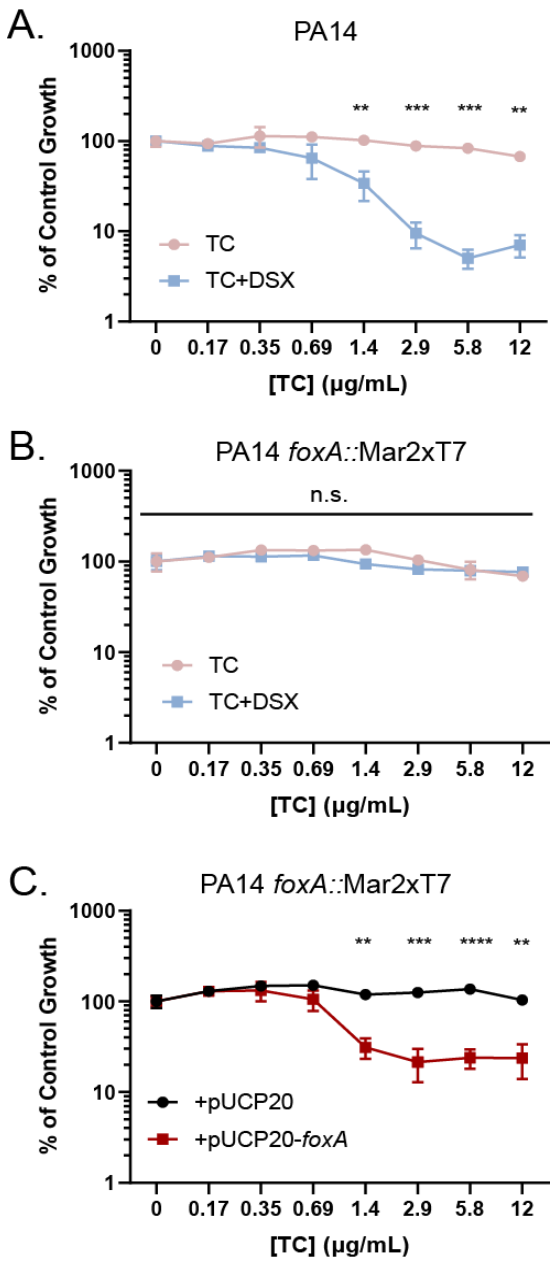

Figure 3. TC crosses the outer membrane through the FoxA siderophore receptor. Wild type PA14 (A) and PA14 foxA::Mar2xT7 (B) were challenged with TC (pink) and TC + DSX (blue). Dose response assays were conducted in 10:90. The transposon mutant was complemented with wild type PA14 foxA (C) and challenged with TC in VBMM. The vector control is in black whereas the complemented mutant is in red. Results were averaged from three independent replicates. n.s, not significant; **, p<0.01; ***, p<0.0005; ****, p<0.0001. Significance was determined using two-way ANOVA followed by Sidak's multiple-comparison test.

146

147 *A cocktail of TS + TC + DSX has an additive effect*

148 Our observation that thiopeptides
149 synergize with DSX against *P. aeruginosa*
150 demonstrated the potential to target gram-
151 negatives expressing either pyoverdine or
152 ferrioxamine receptors using thiopeptide-chelator
153 cocktails. Sub-MIC combinations of TS (0.1 µg/mL)
154 + 64 µg/mL DSX and TC (0.2 µg/mL) + 64 µg/mL
155 DSX, as well as TS + TC + DSX were tested against
156 PA14 in VBMM (Figure 4). Each double
157 combination inhibited growth to ~30% of control;
158 however, the triple combination decreased the
159 growth to only 10% of control. A multiplicative rule
160 approach was used to determine the effect of
161 combining both thiopeptides where the expected
162 growth is the product of the growth inhibition for
163 the individual combinations (i.e. TS + DSX or TC +
164 DSX). For TS + DSX, growth was reduced to 31% of
165 control and for TC + DSX, growth was reduced to
166 29% of control. Therefore, the expected growth for the combination of TC + TS + DSX would be 9% of
167 control. DSX alone had no effect on growth. The experimental value yielded 8.8% of control, suggesting
168 an additive effect of the thiopeptides. These results indicate that thiopeptide cocktails could offer an
169 advantage compared over single thiopeptide treatment by using different siderophore receptors to enter
170 the cell.

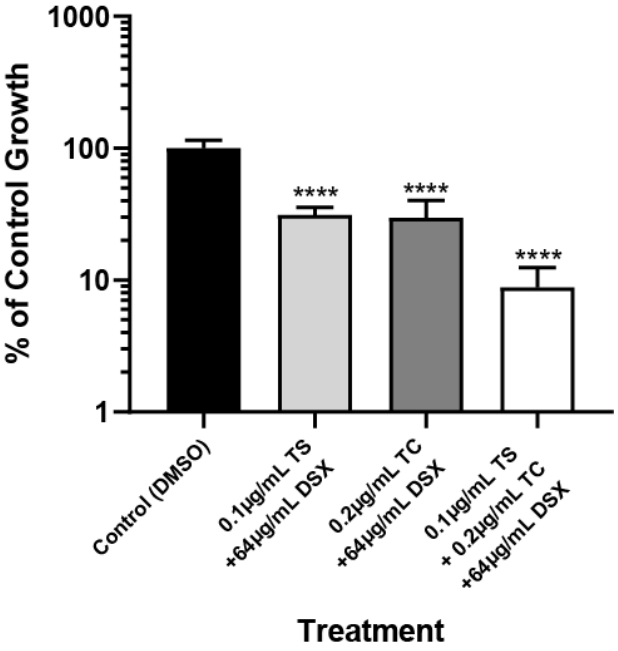

Figure 4. A thiopeptide cocktail significantly reduces PA14 growth. Control growth less than 20% was considered as the MIC cut-off. Assays were conducted in VBMM. Results are averaged from three independent experiments. ****,p<0.0001. Significance was determined using one-way ANOVA followed by Tukey honest significant difference post test.

171

172 *Thiopeptides target specific bacterial genera and species*

173 Unlike the pyoverdine receptors, the ferrioxamine receptor is not unique to Pseudomonads.
174 Therefore, we tested whether other Gram-negative bacteria were susceptible to TC. Bioinformatic
175 analyses of other priority Gram-negative pathogens such as *Escherichia coli, Klebsiella pneumoniae,* and
176 *Salmonella* Typhimurium showed that they carry *foxA* in their genome. However, they were not

177 susceptible to TC or the combination of TC + DSX
178 at the highest concentrations tested, suggesting
179 that the receptors are sufficiently different in
180 sequence to prevent recognition of TC as a
181 ligand. FoxA proteins from *S.* Typhimurium
182 SL1344 and *P. aeruginosa* PAO1 share 40% amino
183 acid sequence identity (Supplementary Figure 4).
184 Residues Y218, H374, and Q441 that interact
185 with the native ferroxamine ligand are conserved
186 in both species, indicating that TC and MC must
187 rely on interactions with other amino acids for
188 uptake into Pseudomonads[33].

189 To determine if there were differences
190 in susceptibilities between different species of
191 *Pseudomonas*, we conducted dose-response
192 assays with TC and TC + DSX against *P. putida*
193 KT2440, *P. marginalis* CVCOB 1152, *P. protegens*
194 Pf-5, and *P. fluorescens* PV5(Supplementary
195 Figure 5. Similar experiments were done with TS
196 and TS + DSX. We defined resistance if the
197 percent of control growth exceeded 80%,
198 susceptible if below 20%, and intermediate if
199 between 20-80%. Interestingly, there was
200 variability in the susceptibility to the
201 combinations between species. *P. putida* was resistant to TS but susceptible to TC (Figure 5,
202 Supplementary Figure 5). *P. protegens* was resistant to all combinations (Figure 5, Supplementary Figure
203 5). Alignments of the FoxA sequences for each species showed 65%, 65%, and 60% similarity for *P.*
204 *fluorescens, P. protegens,* and *P. putida* compared to *P. aeruginosa* PAO1. Despite the differences in
205 susceptibility to TC + DSX, the key residues that interact with the native ligand are conserved, similar to *S.*
206 Typhimurium, suggesting that TC interacts with other amino acids. To focus on possible sites of
207 interaction, we looked for residues shared between *P. aeruginosa*, *P. putida*, and *P. fluorescens*, but not

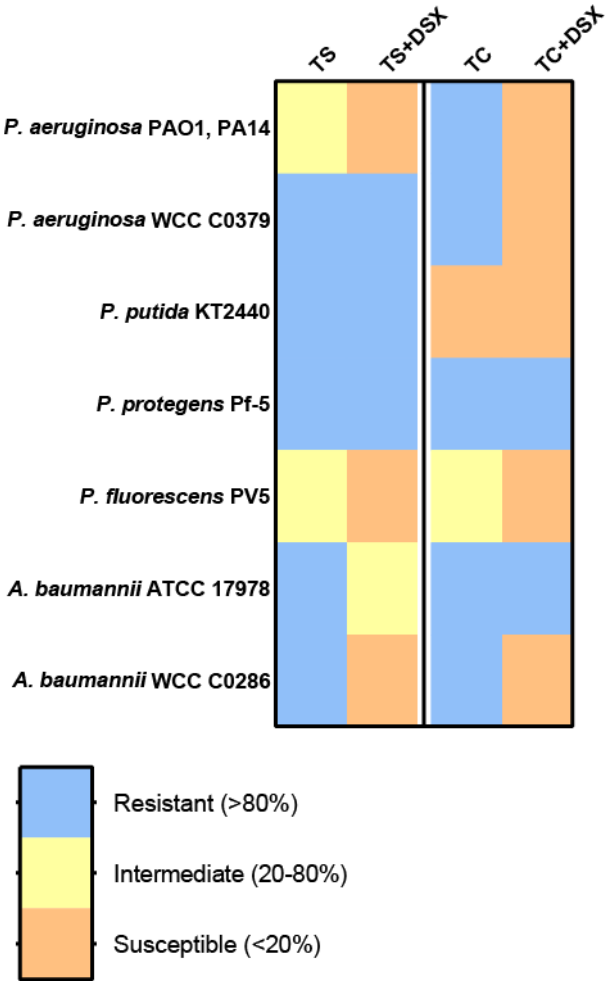

Figure 5. Susceptibility to TS and TC are different between species and strains. Susceptibility of *Pseudomonas* and *Acinetobacter* to TS and TC in combination with DSX. Blue boxes indicate resistance (>80% of control growth). Yellow boxes indicate intermedicate susceptibility (20-80% of control growth). Orange boxes indicate susceptible (<20% of control growth).

208 *P. protegens*, which was resistant to TC and TC + DSX (Figure 5, Supplementary Figure 5). A total of 17
209 residues were identified following this criterion and mapped onto a co-crystal structure of FoxA bound to
210 ferrioxamine (PDB ID: 6I96)[33]. Only three were found in proximity to the native ligand – A620(P), S612(A),
211 and D380(N) (Supplementary Table 4). A629(P) indicates that in the FoxA receptor of *P. aeruginosa*, *P.*
212 *putida,* and *P. fluorescens* at position 620, the amino acid is alanine. However, in *P. protegens* the amino
213 acid at the same position is proline. A620(P) and S612(A) are located in loop 7 of FoxA, which closes upon
214 interaction of the ligand with the active site[33]. Closure of loop 7 prevents the native ligand from
215 dissociation. D380(N) is close to conserved residue H374, which interacts with the native ligand.

216 A BLAST search using the PAO1 FoxA amino acid sequence against *P. aeruginosa, P. putida, P.*
217 *fluorescens* (all TC susceptible under low iron), and *P. protegens* (TC resistant) was conducted to see if
218 A620(P), S612(A), and D380(N) are conserved (Supplementary Figure 6). The top 100 hits were aligned
219 (MUSCLE), which revealed that alanine at position 620 and aspartic acid at position 380 are well conserved
220 in *P. aeruginosa, P. fluorescens,* and *P. putida*. Alanine was the most common amino acid at position 612
221 in *P. fluorescens* rather than serine, suggesting that this residue may not be important for TC interaction
222 with FoxA since this species was susceptible to TC + DSX. In *P. protegens,* which is resistant to TC + DSX,
223 lysine was the most abundant at residue 620. At residue 380, glutamine occurred more frequently. These
224 differences suggest that alanine and aspartic acid at residues 620 and 380 respectively may be involved
225 in TC uptake. These data suggest that TC uptake requires specific amino acid interactions that differ
226 between species.

227

228 *Nosiheptide is a potent inhibitor of MRSA USA 300*

229 The core structure of NH is nearly identical to TC and MC; however, a single indole is linked to the
230 core 26-membered macrocyclic ring (Figure 1). This small change in structure results in loss of synergy
231 with DSX (Supplementary Figure 1). Although synergy was not observed, there was a reduction in growth
232 when NH was combined with DSX. When tested against the *fpvAB* mutant, potentiation was lost
233 (Supplementary Figure 1).

234 There are examples – such as psychotrimine and daptomycin – of natural product antibiotics
235 containing tryptophan or its side chain, indole [34]. Daptomycin is a Gram-positive antibiotic that disrupts
236 the cytoplasmic membrane[35]. Like NH, daptomycin is a bulky molecule whose size prevents it from
237 crossing the outer membrane, making it ineffective against Gram-negative bacteria. Based on its unique
238 indole content, we hypothesized that NH might have improved efficacy against Gram-positive bacteria

239 compared to the other thiopeptides tested. All seven thiopeptides were tested in a dose-response assay
240 against MRSA USA 300, with 260 ng/mL as the highest concentration (Supplementary Figure 2). Consistent
241 with our hypothesis, NH was the most potent against MRSA, with a MIC of <4 ng/mL, followed by TS and
242 SM (32 ng/mL), TC and MC (130 ng/mL), and BER and GEN (>260 ng/mL). These results show that the
243 indole moiety of NH may confer an advantage against Gram-positives compared to other thiopeptides.

244

245 **DISCUSSION**

246 We previously showed that TS hijacked the pyoverdine receptors of *P. aeruginosa* and *A.*
247 *baumannii* to cross the outer membrane. These data informed the hypothesis that other thiopeptides
248 might similarly act as Trojan Horse antibiotics. In this work, we explored the ability of six other
249 thiopeptides to exploit siderophore receptors under iron-limited conditions. We showed that SM – a
250 thiopeptide structurally similar to TS – also uses pyoverdine receptors to enter cells. The differences in
251 structure of the two compounds provided SAR information. The IC_{50} of TS and SM are similar (0.1 µM vs
252 0.25 µM) based on *in vitro* studies of *E. coli* ribosomal activity[36]. This suggests that SM has weaker synergy
253 with DSX because of reduced affinity for the receptor compared to TS. Combinatorial analyses by
254 checkerboard assays suggested that TC and MC used a different siderophore receptor. We screened
255 mutants lacking various receptors in low iron growth media, leading to the discovery that those
256 thiopeptides used FoxA, a xenosiderophore receptor in *P. aeruginosa*. TC was also effective against a TS-
257 resistant *P. aeruginosa* clinical isolate. However, despite widespread expression of FoxA homologues,
258 susceptibility to TC was not universal among Gram-negative bacteria. Even within the genus
259 *Pseudomonas*, there was variability in susceptibility between species. Differences in the levels of
260 siderophore receptor expression could be a factor in the susceptibility profiles as a higher abundance
261 would allow more thiopeptide uptake.

262 This work suggests links between thiopeptide structure and activity against specific Gram-
263 negatives. Thiopeptides were thought to have little or no activity against Gram-negative pathogens due
264 to their inability to cross the outer membrane. However, thiopeptide producers are mainly marine and
265 soil bacteria, which inhabit environments shared by Pseudomonads. We propose that thiopeptide
266 structures evolved to target the siderophore receptors of cohabiting Gram-negatives. This is not
267 unprecedented as many other natural products can use such receptors to cross the outer membrane. For
268 example, *P. aeruginosa* produces pyocins that target the pyoverdine receptors of competing strains[37]..

269 NH failed to synergize with DSX, although the combination reduced growth. Potentiation was lost against
270 the *fpvAB* mutant. This result suggests that NH could have poor affinity for pyoverdine receptors, but
271 further work needs to be done to demonstrate this. We also showed that of the thiopeptides tested, NH
272 had the greatest activity against MRSA USA 300. The exact mechanism remains unknown, but while the
273 core of NH is nearly identical to TC and MC, the indole moiety makes it unique compared to the other
274 thiopeptides. NH does not synergize with DSX which suggests that the indole moiety could interfere with
275 its recognition by the FoxA receptor. Tryptophan and indole increase interactions of peptides with
276 membranes and can act as signaling molecules[38–41]. We speculate that the indole moiety may provide
277 enhanced peptide-membrane interactions, facilitating the ability of NH to cross the membrane and target
278 Gram-positive pathogens like MRSA. We observed a lower MIC with NH (<4 ng/mL) compared to TS (32
279 ng/mL) against MRSA in 10:90 medium, consistent with results from other groups using Mueller-Hinton
280 Broth[1,42]. In contrast, assays using purified ribosomes showed that TS had 40% greater inhibitory activity
281 compared to NH[43]. While NH has weaker inhibitory activity against purified ribosomes, it may compensate
282 with improved membrane permeability. NH has been tested against MRSA in an intraperitoneal mouse
283 infection model[1]. Mice treated with 20 mg/kg NH showed 90% survival over 5 days compared to 40% in
284 the vehicle control group.

285 Comparing TC and TS, it is apparent that the core macrocycle is nearly identical. However, TS has
286 a second macrocyclic ring with a structurally-distinct quinaldic acid moiety. It is likely that this second ring
287 allows TS to interact with the pyoverdine receptors rather than FoxA. In contrast, BER and GEN had no
288 activity against *P. aeruginosa* under iron-limitation. Even against MRSA, these 35-membered macrocycle
289 thiopeptides had no activity at the highest concentration tested (260 ng/mL). This is unsurprising as it was
290 previously reported that BER has weaker inhibitory activity on purified ribosomes compared to TS[6]. Our
291 results do not rule out the potential of 35-membered thiopeptides having other advantages. Furthermore,
292 in this study we did not test 29-membered thiopeptides such as GE2270A, thiomuracin A, and GE37468[44–
293 46]. These molecules could also have potential activity against *P. aeruginosa* and *A. baumannii* under iron-
294 limited conditions.

295 Schwalen et al. recently used a bioinformatics approach to search for enzymes that catalyze non-
296 spontaneous [4+2]-cycloadditions characteristic of thiopeptide biosynthesis. They identified 508 [4+2]-
297 cycloaddition enzymes, 372 of which were unique at the amino acid level[31]. These results suggest that
298 there are many more structurally unique thiopeptides than previously estimated. Diverse thiopeptides
299 may reveal interesting new activities with antimicrobial applications. One example is cyclothioazomycin,

300 a structurally distinct compound with activity against *Bacillus* spp., although weaker relative to other
301 thiopeptides[47]. Interestingly, this thiopeptide has potent antifungal activity via its interactions with
302 chitin[48]. It also was reported to be a human plasma renin inhibitor[49].

303 Trojan horse antibiotics are an interesting avenue for drug development. They have advantages
304 over antibiotics that must simply diffuse through the outer membrane because of their greater specificity
305 and uptake. Moreover, sites of infection are frequently iron-limited, which naturally potentiates such
306 molecules. Hosts produce iron sequestration proteins such as transferrin and lactoferrin that restrict iron,
307 a process called nutritional immunity[50–52].

308 While thiopeptides been overlooked for use in the clinic, we show the potential for these
309 antibiotics to be developed as selective Gram-negative antimicrobials that can target high priority
310 pathogens including *P. aeruginosa* and *A. baumannii*. Our work also supports the future development of
311 NH as an anti-MRSA therapeutic.

312

313 **METHODS**

314 Chemicals

315 Thiopeptides were purchased from Cayman Chemicals and stored at -20°C. Thiopeptide purity was at least
316 ≥95%. All thiopeptides were dissolved in dimethylsulfoxide (DMSO) with no solubility issues ranging from
317 1mg/mL to 20mg/mL. Deferasirox was purchased from AK Scientific (98% purity). A stock solution of 20
318 mg/mL in DMSO was made. Stock solutions were stored at -20°C until use and all serial dilutions were
319 prepared using fresh DMSO at 75x the final concentration.

320

321 Bacterial Strains and Culture Conditions

322 All bacterial strains used in this study are listed in Supplementary Table 1. Bacteria were inoculated from
323 glycerol stocks stored at -80°C and grown overnight in LB at 37°C with shaking (200 rpm). Bacteria were
324 subcultured (1/500 dilution) into fresh 10:90 media or Vogel-Bonner Minimal Media (VBMM) and grown
325 for 16 hours. Optical densities were standardized to OD_{600} 0.1 and diluted 1/500 into fresh 10:90 or
326 VBMM, which was used in microbroth dilution MIC assays or checkerboard assays.

327

328

329 Generation of PA14 f*oxA*::Mar2xT7 pUCP20-*foxA*

330 *foxA* was PCR amplified from wild type PA14 genomic DNA prepared using Qiagen genomic DNA isolation
331 kit. The following forward and reverse primers were used to obtain wild type *foxA* with SacI and XbaI
332 restriction sites respectively (underlined): forward 5'GAAGGAGCTCGTGGACGCTTGCTTTCGT and reverse
333 5'GCGTTCTAGAGACGCCGGCGAATGCCC. The PCR product and pUCP20 were digested with SacI and XbaI
334 (Thermo Scientific) using Thermo Scientific Fast Digest. The products were run on a 1% agarose gel for
335 30mins at 120V, cut out, and purified using GeneJET Extraction Kit (Thermo Scientific). The purified
336 linearized vector and digested *foxA* gene were ligated (1:3 molar ratio) overnight at 4°C using T4 DNA
337 ligase (Thermo Scientific). The construct was transformed into competent *E. coli* DH5α by heat shock at
338 42°C and plated on LB plates with 100 µg/mL ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-
339 galactopyranoside (IPTG) for blue-white screening (BioShop). Plates were incubated at 37°C overnight.
340 White single colonies were streaked on fresh LB plates with ampicillin to confirm that colonies contained
341 the construct. Minipreps were done using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and
342 quantified by NanoDrop. The *foxA* transposon mutant was grown overnight in LB. 1 mL was centrifuged
343 and washed twice with nuclease-free water (Qiagen). The cell pellet was resuspended in 500 µL of
344 nuclease-free water. 100 ng of the construct was added to 100 µL of resuspended cells and electroporated
345 (2.50 V). After a recovery period of 2 hours, cells were plated onto LB agar with 200 µg/mL carbenicillin
346 (Bioshop). Plates were incubated at 37°C overnight. Single colonies were streaked on fresh plates to
347 confirm successful electroporation. A single colony was picked and grown in liquid for MIC assays. The
348 susceptibility of the *foxA* transposon mutant harbouring pUCP20-*foxA* to TC indicated successful
349 complementation. Similarly, the same *foxA* transposon mutant was electroporated with empty pUCP20
350 as vector control.

351

352 Microbroth Dilution and Checkerboard Assays

353 Stock solutions were diluted in DMSO and 2µL of the resulting solutions plus 148µL of a bacterial
354 suspension standardized to an OD600 of ~ 0.1 and diluted 1:500 in 10:90 was added to a 96 well plate
355 (Nunc) in triplicate. Control wells contained 148µL of 10:90 + 2µL DMSO (sterility control) or standardized
356 bacterial suspension + 2µL DMSO (growth control). After incubating at 37°C for 16h, with shaking at 200
357 rpm, the OD600 was read using a plate reader (Thermo Scientific). Assays were performed in triplicate,
358 calculations were done in Microsoft Excel, and results were graphed using Prism (GraphPad) as a

359 percentage of the DMSO control. For wells with 2 compounds, such as increasing TS concentrations with
360 64µg/mL of DSX, 4µL of DMSO was used in the sterility and growth controls.
361
362 Checkerboard assays were set-up using Nunc 96-well plates in an 8-well by 8-well format. Two columns
363 were allocated for vehicle controls and two columns for sterility controls. Vehicle controls contained 4 µL
364 DMSO, in 146 µL of 10:90 inoculated with PA14 as described in Growth Curves. Sterile controls contained
365 the same components in 10:90, without cells. Serial dilutions of each thiopeptide – with 10 µM being the
366 highest final concentration – was added along the ordinate of the checkerboard (increasing concentration
367 from bottom to top) whereas serial dilutions of DSX – with 64 µg/mL being the highest final concentration
368 – was added along the abscissa (increasing concentration from left to right). The final volume of each well
369 was 150 µL and each checkerboard was repeated at least three times. Plates were incubated and the final
370 OD600 determined as detailed above. Checkerboards were graphed as heatmaps in Prism (GraphPad).
371
372 Bioinformatic Analysis
373 MUSCLE alignments were conducted using Unipro UGENE and Geneious Prime. Accession numbers of *P.*
374 *aeruginosa, P. fluorescens, P. protegens, P. putida,* and *S.* Typimurium FoxA are as follows: AAG05854.1,
375 WP_122305244.1, WP_003218248.1, AAY95537.2, NP_742329.1, and CBW16459.1. Genomic analyses for
376 mutations in WCC C0379 were done with breseq by Kara Tsang using *P. aeruginosa* PAO1 as the reference
377 genome.
378
379 **ASSOCIATED CONTENT**
380 **Supporting Information**
381 Table S1, list of strains; Table S2, mutants tested to show that TC uses the FoxA receptor to cross the outer
382 membrane; Table S3, breseq genomic annotations of the FoxA receptor for WCC C0379; Table S4, FoxA
383 amino acid residues that are different in *P. protegens* compared to other *Pseudomonas* spp; Figure S1,
384 checkerboard assays for thiopeptides that did not synergize with DSX; Figure S2, thiopeptide activity
385 against MRSA USA300; Figure S3, thiopeptide activity against *P. aeruginosa* and *A. baumannii* TS-resistant
386 clinical isolates; Figure S4, MUSCLE alignment of *S.* Typhimurium and *P. aeruginosa* FoxA receptors; Figure
387 S5, dose-response assays of thiopeptides against different *Pseudomonas* spp. Figure S6, Sequence logos
388 of FoxA amino acid sequences of *Pseudomonas* spp.

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394 **Author Contributions**

395 DCKC performed all experiments. DCKC and LLB wrote the paper.

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407

408 **ABBREVIATIONS**

409 TS, thiostrepton; TC, thiocillin; MC, micrococcin; NH, nosiheptide; MIC, minimal inhibitory concentration;
410 SM, siomycin; BER, berninamycin; GEN, geninthiocin; DSX, deferasirox; FpvA, ferripyoverdine receptor A;
411 FpvB, ferripyoverdine receptor B; FoxA, ferrioxamine receptor; WCC, Wright Clinical Collection; VBMM,
412 Vogel-Bonner Minimal Media; MRSA, methicillin-resistant *Staphylococcus aureus*; *E. coli*, *Escherichia coli*;
413 *K. pneumoniae, Klebsiella pneumoniae*; *S.* Typhimurium, *Salmonella* Typhimurium; *P. aeruginosa,*
414 *Pseudomonas aeruginosa*; *P. fluorescens*, *Pseudomonas fluorescens*; *P. putida*, *Pseudomonas putida*; *P.*
415 *protegens, Pseudomonas protegens; A. baumannii*, *Acinetobacter baumannii*.

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417

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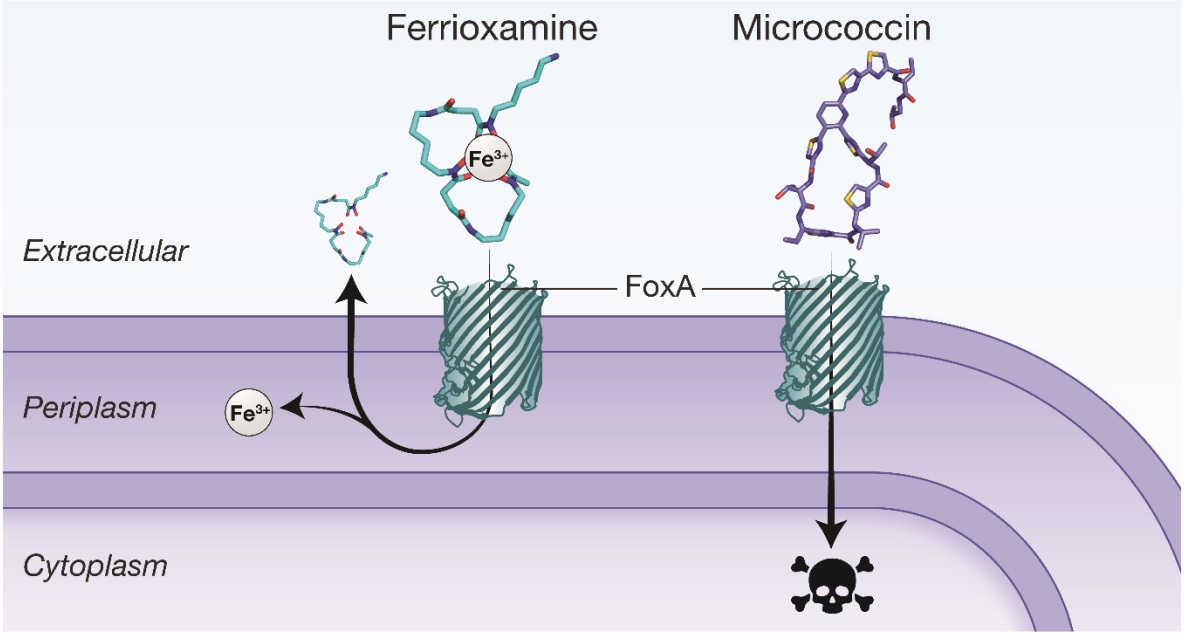
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