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Abstract

There is an urgent need for the discovery of effective new antimicrobial agents to combat the rise of bacterial drug resistance. High-throughput screening (HTS) in whole-animal infection models is a powerful tool for identifying compounds that show antibacterial activity and low host toxicity. In this report, we characterize the activities of four novel antistaphylococcal compounds identified from an HTS campaign conducted using Caenorhabditis elegans nematodes infected with methicillin-resistant Staphylococcus aureus (MRSA). The hit compounds included an Nhydroxy indole-1, a substituted melamine derivative-2, N-substituted indolic alkyl isothiocyanate-3, and pdifluoromethylsulfide analog-4 of the well-known protonophore carbonyl cyanide m-chlorophenyl hydrazone. Minimal inhibitory concentrations (MICs) of the four compounds ranged from 2 to 8 μg/ml against MRSA-MW2 and Enterococcus faecium and all were bacteriostatic. The compounds were mostly inactive against Gram-negative pathogens, with only 1 and 4 showing slight activity (MIC= 32 μg/ml) against Acinetobacter baumanii. Compounds 2 and 3 (but not 1 or 4) were found to perturb MRSA membranes. In phagocytosis assays, compounds 1, 2, and 4 inhibited the growth of internalized MRSA in macrophages, whereas compound 3 showed a remarkable ability to clear intracellular MRSA at its MIC ($p < 0.001$). None of the compounds showed hemolytic activity at concentrations below 64 μg/ml ($p = 0.0021$). Compounds 1, 2, and 4 (but not 3) showed synergistic activity against MRSA with ciprofloxacin, while compound 3 synergized with erythromycin, gentamicin, streptomycin, and vancomycin. In conclusion, we describe four new antistaphylococcal compounds that warrant further study as novel antibacterial agents against Gram-positive organisms.

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Antibacterial Properties of Four Novel Hit Compounds from a Methicillin-Resistant *Staphylococcus aureus-Caenorhabditis elegans* High-Throughput Screen

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Keywords; Antibiotic, bioactive compounds, high-throughput screening, isothiocyanate, melamine, MRSA infection, N-hydroxy indoles, protonophores

Running title: Anti-staphylococcal compounds from HTS

22 ## Abstract

23 There is an urgent need for the discovery of effective new antimicrobial agents to combat the rise
24 of bacterial drug resistance. High-throughput screening (HTS) in whole-animal infection models
25 is a powerful tool for identifying compounds that show antibacterial activity and low host toxicity.
26 In this report, we characterize the activities of four novel anti-staphylococcal compounds identified
27 from a HTS campaign conducted using *Caenorhabditis elegans* nematodes infected with
28 methicillin-resistant *Staphylococci aureus* (MRSA). The hit compounds included: an *N*-hydroxy
29 indole- **1**, a substituted melamine derivative- **2**, an *N*-substituted indolic alkyl isothiocyanate- **3**,
30 and a *p*-difluoromethylsulfide analog- **4** of the well-known protonophore carbonyl cyanide *m*-
31 chlorophenyl hydrazone (CCCP). Minimal inhibitory concentrations (MICs) of the four
32 compounds ranged from 2-8 µg/mL against MRSA-MW2 and *Enterococcus faecium* and all were
33 bacteriostatic. The compounds were mostly inactive against Gram-negative pathogens, with only
34 **1** and **4** showing slight activity (MIC = 32 µg/mL) against *Acinetobacter baumanii*. Compounds **2**
35 and **3** (but not **1** or **4**) were found to perturb MRSA membranes. In phagocytosis assays,
36 compounds **1**, **2** and **4** inhibited the growth of internalized MRSA in macrophages, whereas
37 compound **3** showed a remarkable ability to clear intracellular MRSA at its MIC (p<0.001). None
38 of the compounds showed hemolytic activity at concentrations below 64 µg/mL (p=0.0021).
39 Compounds **1, 2** and **4** (but not **3**) showed synergistic activity against MRSA with ciprofloxacin,
40 while compound **3** synergized with erythromycin, gentamicin, streptomycin and vancomycin. In
41 conclusion, we describe four new anti-staphylococcal compounds that warrant further study as
42 novel antibacterial agents against Gram-positive organisms.

43 # Introduction

44 Antibiotic resistance is a major current and future threat to the global population, and new
45 antibiotics are urgently needed to combat the inexorable rise of multi-drug resistant bacteria.
46 Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen[1] that can
47 cause localized and systemic infections [2]. Drug resistance in MRSA occurs primarily through the
48 production of β-lactamases or altered penicillin binding proteins [3]. According to the Center for
49 Disease Control and Prevention (CDC), in the U.S. there are more than 11,000 deaths and 80,000
50 severe cases of MRSA infection each year [4]. Vancomycin has typically been the choice of
51 antibiotic against serious multi-drug resistant Gram-positive bacterial infections but reports of
52 vancomycin-resistant *S. aureus* are now common [5]. Combination antimicrobial treatment is a
53 promising strategy [6].

54 Development of new antimicrobial agents has significantly declined in the past two
55 decades due to challenging regulatory guidelines, perceptions around poor financial returns and
56 difficulties in discovering the mechanism of action of new compounds [7]. But, the whole animal
57 *Caenorhabditis elegans*-based high throughput screening provides a powerful tool for identifying
58 new antimicrobial agents, anti-virulence agents and immunomodulators. To identify novel
59 antibacterial leads, we have employed *C. elegans* as a simple whole-animal host for studying
60 infections of human pathogens [8]. We recently completed a *C. elegans* high-throughput screen
61 (HTS) to identify small molecules that are active against MRSA and show low host toxicity [9]. This
62 report details the broader antibacterial properties of four novel anti-staphylococcal hit compounds
63 discovered during an MRSA-*C. elegans* high-throughput screening campaign.

64

65 # Materials and Methods

66 **Bacterial and nematode strains**. Bacteria were all from the Mylonakis laboratory collection
67 (Table 1). *S. aureus* MW2 and *Enterococcus faecium* ATCC E007 were grown in tryptic soy broth
68 (TSB) (BD Biosciences, Franklin Lakes, NJ, USA); *Klebsiella pneumoniae* ATCC 77326,
69 *Acinetobacter baumannii* ATCC 17978*, Pseudomonas aeruginosa* PA14 and *Enterobacter*
70 *aerogenes* EAE 2625 strains were grown in Luria-Bertani broth (LB) (BD Biosciences). All strains
71 were grown at 37 °C. The *C. elegans glp-4(bn2);sek-1(km4)* double mutant strain was maintained
72 at 15 °C on lawns of *Escherichia coli* HB101 on 10 cm plates [9]. The *glp-4(bn2)* mutation renders
73 the strain unable of producing progeny at 25 °C [10], and the *sek-1(km4)* mutation increases
74 sensitivity to pathogens [11], reducing assay time.

75 ***C. elegans*-MRSA liquid infection assays**. The *C. elegans*-MRSA infection assay has been
76 described previously [9]. In brief, *C. elegans glp-4(bn2);sek-1(km4)* worms were grown at 25 °C and
77 harvested with M9 buffer. MRSA-MW2 was grown overnight at 37 °C in TSB under aerobic
78 conditions and then transferred to anaerobic conditions at 37 °C. Bacteria were added at a final
79 OD_{600} of 0.04 to 384-well assay plates (Corning, Corning, NY, USA) containing test compounds
80 at a final concentration of 2.86 µg/mL. Adult sterile worms (15 were then added to each well using
81 a Complex Object Parameter Analyzer and Sorter (COPAS, Union Biometrica, Holliston, MA,
82 USA). After 5-days of incubation at 25 °C, the plates were washed (to remove bacteria) with a
83 microplate washer and Sytox Orange (Life Technologies, Carlsbad, CA, USA) was added to
84 selectively stain dead worms. After overnight incubation at 25 °C, the wells were imaged using an
85 Image Xpress Micro automated microscope (Molecular Devices, Sunnyvale, CA, USA), capturing
86 both transmitted light and TRITC (535 nm excitation, 610 nm emission) fluorescent images with
87 a 2X objective. Images were processed using the open source image analysis software CellProfiler

88 (http://www.cellprofiler.org/). The ratio of Sytox worm area to bright field worm area and the
89 resultant percentage survival data were calculated by the software for each well [9]. Assays were
90 completed in duplicate.

91 **Hit compounds**. The compounds were an *N*-hydroxy indole (NHI) **1**, a melamine derivative **2**,
92 indole isothiocyanate (ITC) **3** and a protonophore **4** related to carbonyl cyanide *m*-chlorophenyl
93 hydrazone (CCCP). Compounds **1** (6-hydroxy-7,8,9,10-tetrahydro-[1,2,5]oxadiazolo[3,4-
94 c]carbazole) and **2** (2-*N*,4-*N*-ditert-butyl-6-hydrazinyl-1,3,5-triazine-2,4-diamine) were purchased
95 from Asinex (Winston-Salem, NC, USA). Compound **3** (1-(2-isothiocyanatoethyl)-1H-indole)
96 was purchased from Lifechemicals (Burlington, Canada) and compound **4** (3, 2-[[4-
97 (difluoromethylsulfanyl)phenyl] hydrazinylidene]propanedinitrile) was purchased from Enamine
98 (Monmouth, NJ, USA). All compounds were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO,
99 USA) to obtain 10 mg/mL stock solutions that were diluted for experiments.

100 **Antibacterial susceptibility assays**. *In vitro* antibacterial activities were tested using the broth
101 microdilution method [12]. Assays were carried out in triplicate using Müller-Hinton broth (BD
102 Biosciences, Franklin Lakes, NJ, USA) in 96-well plates (BD Biosciences) with a total assay
103 volume of 100 µL. Two-fold serial dilutions were prepared over the concentration range 0.01−64
104 µg/mL. An initial bacterial inoculum was adjusted to OD_{600} = 0.06 and incubated with test
105 compounds at 35 °C for 18 hours. OD_{600} was measured and the lowest concentration of compound
106 that suppressed bacterial growth was reported as its MIC[13]. Broth cultures (10 µL) from the MIC
107 assays were plated onto Müller-Hinton agar (BD Biosciences) and after overnight incubation at 37
108 °C the lowest concentration at which colonies were not observed was reported as the minimal
109 bactericidal concentration (MBC).

110 **Time to kill assays**. The antibacterial properties of **1-4** against MRSA-MW2 were further
111 examined using time to kill assays, as previously described [14]. Briefly, overnight cultures of *S.*
112 *aureus* MW2 were diluted in fresh TSB to a density of 10^8 cells/mL and placed into 10 mL tubes
113 (BD Biosciences). Test compounds at 4x MIC were added and the tubes incubated at 37 °C, with
114 agitation. Aliquots were periodically drawn from the tubes over a 4 h period, serially diluted with
115 TSB and plated onto tryptic soy agar (TSA; BD Biosciences). CFUs were then enumerated after
116 overnight incubation at 37 °C. Assays were carried out in triplicate.

117 **Membrane permeabilization assays**. Sytox Green (Life Technologies, Carlsbad, CA, USA) was
118 used to probe the effects of **1-4** on MRSA-MW2 membrane permeabilization, as previously
119 described [15]. Assays were carried out in duplicate in 96 wells plates (Corning). Bacterial cells were
120 harvested from logarithmically growing cultures by centrifugation at 3724 g for 5 minutes, washed
121 twice with phosphate buffered saline (PBS, pH 7.4) and resuspended in PBS to OD_{595} nm = 0.2.
122 Sytox Green was added at a final concentration of 5 μM and cells were incubated in the dark for
123 30 min. Cell suspensions (50 μL) were added to 50 μL of compound (64 μg/mL in PBS), and the
124 fluorescence intensity was measured (excitation 485 nm, emission 530 nm) periodically over 60
125 minutes. DMSO was included as the vehicle control. Membrane effects of compounds were
126 indicated by an increase in cellular fluorescence caused by enhanced permeability of the DNA
127 staining, membrane impermeable dye.

128 **Intracellular MRSA killing assays.** RAW 264.7 macrophages were used to examine intracellular
129 killing of MRSA-MW2 by **1-4**, as described by Schmitt et al [16]. Macrophages were grown in
130 Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented
131 with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) and
132 maintained at 37 °C in 5% CO_2 [17,18]. Cells (50,000) in antibiotic and serum free DMEM were

seeded in 12-well plates 24 h prior to infection. MRSA-MW2 (multiplicity of infection (MOI) = 50) were added to macrophages and phagocytosis allowed to proceed. Planktonic bacteria were removed after 2 h and DMEM supplemented with 200 μg/mL gentamicin was added for 2 h to eliminate extracellular bacteria. Antibiotic and serum-free DMEM with and without test compounds was added and the cells incubated in a 5% CO_2. After 4, 8, 12, or 24 h SDS was added to a final concentration of 0.02 % to lyse the macrophages only (i.e. not ingested bacteria). Cell lysates were diluted serially with TSB, plated onto TSA plates and CFUs enumerated. Vancomycin (8 μg/mL) was used as a positive control and DMSO 0.1% as the negative control. Assays were carried out in triplicate [19].

Human blood cell (RBC) hemolysis assays. Human erythrocytes (Rockland Immunochemicals, Limerick, PA, USA) were used to measure the hemolytic activity of the compounds, as described by Isnansetyo et al [20]. Briefly, human erythrocytes (4%, in PBS, 50 μL) were added to 50 μL of serially diluted test compounds in PBS in 96-well plates. After incubating at 37 °C for 1 h, the plates were centrifuged at 500 x g for 5 min and 50 μL of the supernatant from each well was transferred to a second 96-well plate. Absorbance (540 nm) was used as a measure of hemolytic activity. Assays were carried out in triplicate.

Cytotoxicity assay. Mammalian cell lines HepG2 (hepatic cell line), MKN-28 (gastric cell line), HKC-8 (renal cell line) were used to determine the cytotoxicity of the compound, as detailed elsewhere [17,21,22]. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) and maintained at 37°C in 5% CO_2. Cells were harvested and suspended in DMEM, and 100 μl of cells were added to each well at a final concentration of 5 x 10^4 cells. The compound was serially diluted in serum and antibiotic-free DMEM and added to the

156 monolayer and incubated at 37°C in 5% CO_2 for 24 h. For the last 4 h of this 24 h incubation
157 period, 10 µl of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium (WST-
158 1) solution (Roche, Mannheim, Germany) was added to each well. The WST-1 reduction was
159 measured at 450 nm using Vmax microplate reader (Molecular Device Sunnyvale, CA, USA). This
160 assay was done in triplicate, and the percentage of survival was calculated by comparing with
161 DMSO-treated vehicle control.

162 **Checkerboard assays.** Antibacterial synergy for combinations of compounds **1**-**4** with each other
163 and clinical antibiotics from various class of antibacterial agents such as fluoroquinolone,
164 tetracycline, aminoglycosides, macrolides and glycopeptides (ciprofloxacin, doxycycline,
165 erythromycin, gentamicin, streptomycin and vancomycin) was tested for using checker board
166 assays. Cultures of MRSA-MW2 were adjusted to $OD_{600} = 0.06$ and added to compound pairs that
167 had been serially diluted in the same 96 well plates, vertically for one compound and horizontally
168 for the other. Assays were carried out in triplicate as described for antibacterial susceptibility
169 assays. The combinatorial inhibitory concentration was indicated by fractional inhibitory
170 concentration index (FICI) was calculated using the formula: $MIC_{\mathbf{A}}$ combination / $MIC_{\mathbf{A}}$ alone +
171 $MIC_{\mathbf{B}}$ combination / $MIC_{\mathbf{B}}$ alone [23].

172 **Statistical analysis.** Statistical analysis (Two-way ANOVA followed by Bonfererroni post-test)
173 was carried out using GraphPad Prism version 6.04 (GraphPad Software, La Jolla CA, USA) and
174 *p* values of <0.05 were considered significant.

175

176 ## Results

177 **HTS assay.** We previously reported a *C. elegans* HTS assay for the identification of novel
178 antibacterial hits against MRSA [9] and screened 3,930 compounds in Asinex 1 library and 3,892
179 compounds in Life chemicals library [9,15]. During the screening, we identified that compounds **1-4**
180 (**Figure 1**) prolonged the survival of *C. elegans* infected with MRSA-MW2 at a concentration of
181 2.86 μg/mL compared to the DMSO control (**Figure 2 A-D**).

182 **Antibacterial susceptibility.** The antibacterial activity of the four hits was evaluated against a
183 panel of ESKAPE pathogens. All four compounds were found to inhibit the growth of the Gram-
184 positives MRSA-MW2 and *E. faecium* (MICs 2-8 mg/mL, **Table 1**). Compounds **1** and **4** were
185 slightly active against *A. baumannii* (MIC = 32 μg/mL) but no other activity was observed against
186 Gram-negatives. The MIC of vancomycin was 4 μg/mL against Gram-positives and polymyxin B
187 was 2-8 μg/mL against Gram-negatives in the ESKAPE panel (**Table 1**). The minimum
188 bactericidal concentrations (MBC) of **1** and **4** against MRSA-MW2 were 64 and 32 μg/mL,
189 respectively, while the MBC of compounds **2** and **3** was > 64 μg/mL. The MIC of oxacillin,
190 vancomycin, polymyxin B, was tested with various clinical *S. aureus* strains. All the clinical strains
191 were resistant to oxacillin. The MICs of compounds **1-4** were listed in **Table 2**. Time to kill assays
192 were used to further confirm the bactericidal/bacteriostatic properties of **1-4** against MRSA-MW2.
193 When cells were exposed to the compounds at 4X MIC all showed only bacteriostatic activity
194 relative to DMSO controls (**Figure 3**). While compounds **1**, **2** and **4** inhibited bacterial growth,
195 ITC derivative **3** was able to reduce CFU/mL counts by 2-$\log_{10}$.

196 **Membrane permeabilization.** To evaluate the membrane effects of **1-4**, uptake of the membrane-
197 impermeable DNA-binding fluorescent dye Sytox Green into MRSA-MW2 cells was monitored

198 in the presence/absence of the compounds. Exposure of cells to the compounds at 64 μg/mL
199 identified that only **2** and **3** show effects on MRSA membranes, as indicated by increases in cellular
200 fluorescence (**Figure 4**). Observing membrane effects with **2** and **3** was in agreement with previous
201 reports on members from the melamine [24] and ITC classes [25]. In contrast, compounds **1** and **4**
202 showed no changes in cellular fluorescence (**Figure 4**), indicating that they do not elicit their
203 antibacterial effects through action on membranes.

204 **Killing of intracellular MRSA in macrophages.** It is known that *S. aureus* can act as an
205 intracellular pathogen [26]. To explore the effects of **1-4** on intracellular MRSA, RAW 264.7
206 macrophages were exposed to MRSA-MW2 cells and treated with test compounds at 1x MIC,
207 vancomycin (positive control, 8 μg/mL, 2x MIC) and 0.1 % DMSO (negative control).
208 Compounds **1**, **2** and **4** were found to significantly inhibit the growth of intracellular MRSA
209 relative to DMSO ($p<0.001$). While vancomycin was able to produce a slight reduction in bacterial
210 counts, compound **3** completely cleared intracellular MRSA after 8 hours of treatment (**Figure**
211 **5A**). The difference observed between the time to kill kinetics and intracellular killing of MRSA
212 when treated with compound **3**, may due to limited duration (only 4 hours) of compound exposure
213 to bacterial cells in time to kill kinetics. However, we treated MRSA-MW2 cells with compounds
214 **1-4**, and incubated as indicated in the macrophage assay and we observed that compound **3** killed
215 the planktonic bacteria after prolonged incubation (**Figure 5B**).

216 **Human red blood cell lysis assays and cytotoxicity.** Serial dilutions of **1-4** were added to human
217 red blood cells to establish whether they show hemolytic activity. It was found that none of the
218 compounds showed hemolysis at concentrations up to 64 μg/mL. Serially diluted triton-X (0.001
219 to 1%) as a positive control were added to human RBCs caused substantial lysis (**Figure 6A**).
220 Hepatotoxicity of the test compounds **1-4** was evaluated using the liver cell line HepG2, commonly

221 used to test the toxicity of compounds [14]. In this series of experiments, the IC_{50} of the compound
222 **1-4** against HepG2 was 32, 16, 8, and 1 μg/mL respectively (**Figure 6B**). Also, we tested the
223 cytotoxicity with gastric and renal cell lines and we observed similar results with hepatic cell lines.
224 The IC_{50} of compounds **1-4** was against MKN-28 was 64, 32, 4 and 4 μg/mL respectively (**Figure**
225 **6C**); and against HKC-8 was 64, 32, 2, 2 μg/mL respectively (**Figure 6D**). The IC_{50} of compounds
226 **3** and **4** were high in mammalian cell lines, however, we are working on the analogues to eliminate
227 the cytotoxicity as well as sustain potent antimicrobial ability. In addition, we monitored the
228 survival of macrophages in the presence of test compounds **1-4** at MIC level and observed that the
229 compound **3** was harmful to macrophages (**Figure 6E**) and bacteria (**Figure 5B**).

230 **Antibacterial synergy.** Use of paired combinations of drugs can reduce bacterial resistance and
231 even restore clinical efficacy of some antibiotics [27]. Checkerboard assays were performed to
232 establish whether compounds **1-4** act synergistically against MRSA-MW2 when paired with one
233 another and five clinical antibiotics from different class of antibacterials (i.e. ciprofloxacin,
234 doxycycline, erythromycin, gentamicin, streptomycin and vancomycin). Paired combinations of
235 compounds and their observed fractional inhibitory concentration indices (FICI) are listed in **Table**
236 **3**. Synergistic effects, where the combined antibacterial activity of the two agents is more than the
237 sum of their effects alone, were identified by FICI ≤ 0.5, antagonism by FICI > 4.0 and 'no
238 interaction' by FICI > 0.5 - 4.0 [28].

239 Antagonism was not observed for any of the compound combinations. Compounds **1-4**
240 showed no interactions when paired with one another but all four compounds showed synergy with
241 at least one antibiotic. Ciprofloxacin was synergistic with compounds **1**, **2** and **4**, with compound
242 **4** also showing synergy with doxycycline. Compound **3** showed no synergy with ciprofloxacin or
243 doxycycline but synergized with all four of the antibiotics. Previous studies have reported that the

244 activity of natural ITCs is enhanced by clinical antibiotics [29-31], in agreement with our observations

245 with **3**.

246

247 ## Discussion

248 Bacterial resistance to antibiotics has become a major global public health threat, with drug-
249 resistant bacteria causing significant and increasing mortality and morbidity [32]. There is an urgent
250 need to develop new antibiotics, ideally with novel mechanisms of action to slow the onset of
251 resistance. Lead antibacterials are usually either synthesized chemically or isolated from natural
252 products that exhibits antibacterial activity [33,34]. We completed a *C. elegans*-MRSA HTS study
253 and identified four small molecules that rescued nematodes from MRSA infection at 2.86 µg/mL
254 [9].

255 Compound **1** represents a [1,2,5]oxadiazolo derivative from the NHI class, which are
256 known to have antibacterial activity against Gram-positive organisms [35]. Natural products bearing
257 the NHI group, such as the nocathiacins and thiazomycins and their semi-synthetic analogues,
258 exhibit activity against Gram-positive bacteria by inhibiting protein synthesis through direct
259 interactions with the bacterial 50s ribosome [35]. The related 7-hydroxy indole reportedly shows
260 anti-virulence effects against *P. aeruginosa* [36].

261 Compound **2** was a derivative from the widely-studied melamine class, whose examples
262 have found use in antimicrobial polymers [37] and as water and food disinfectants [38]. Melamine
263 derivatives related to compound **2** have found applications in biocidal polymers, in food industries,
264 as water disinfectants and as additives in livestock feeds [37,39]. Reports have described the
265 antibacterial activity of melamine [40] and Weaver AJ Jr, *et al*. reported that melamine derivatives
266 target the bacterial membrane *via* non-specific interactions [24].

267 Compound **3** contained an alkyl ITC attached to an indole nitrogen *via* a 2-carbon linker.
268 ITC derivatives are known to show activity against Gram-positive and Gram-negative bacteria [41].
269 ITCs are also present in several plant natural products [42] and can produce both bactericidal and

270 bacteriostatic activities against a range of bacterial pathogens [43]. ITCs are known to react with
271 amines and alcohols due to their highly electrophilic character [44], suggesting non-target specific
272 mechanisms for compound **3**. However, Breier *et al*., reported that ITCs can selectively inhibit the
273 ATP binding sites of P-ATPase in bacteria via reaction with a cysteine residue, suggesting the
274 possibility of target-specific activity [45]. Also, Sofrata *et al*., reported that benzyl isothiocyanate
275 promotes outer membrane penetration in Gram-negative bacteria, leading to effects similar to those
276 observed with cationic antimicrobial peptides [46].

277 Compound **4** was a diarylacylhydrazone and close structural analog of the protonophore
278 CCCP. Protonophores are molecules that dissipate the proton motive force in bacterial membranes
279 leading to growth inhibition [27]. Compounds of this type were recently shown to exert non-
280 specific (protonophoric) antibacterial effects against the Gram-positive bacterium *Clostridium*
281 *difficile* [47]. Clinically used protonophores include the salicylanilide anthelmintics niclosamide,
282 oxyclozanide and closantel, which are also known to show anti-staphylococcal activity [9,14].

283 Characterization of the antibacterial properties of **1-4** here confirmed that they each show
284 direct activity against two Gram-positives, inhibit intracellular growth of MRSA in macrophages,
285 are non-hemolytic and synergize with clinical antibiotics. Future work focusing on the specific
286 characteristics of each compound would likely provide further insights into their mechanisms of
287 action. For example, studies exploring the effects of compound **1** on bacterial 50s ribosomes and
288 its anti-virulence activity against *S. aureus* would be informative. Indole ITC **3** showed the most
289 interesting activity of the four compounds, being able to clear intracellular MRSA from
290 macrophages and synergizing with multiple antibiotics against MRSA, possibly due to its
291 membrane permeabilizing properties (Figure 4). While it is unlikely that **3** could be developed into
292 a drug for systemic MRSA infections due to the reactive ITC group, it would be interesting to

293 study its activity against skin and other body-surface MRSA infections in mammalian models,
294 particularly in combination with the antibiotics it was shown here to synergize with.

295 In conclusion, screening for novel antibacterial compounds using a whole-animal HTS
296 identified novel small molecule hits with anti-staphylococcal activity. Combinatorial activity with
297 clinical antibiotics might decrease the chances of emerging antimicrobial resistance and absence
298 of antagonism with other compounds can be a valid credential of the hit compounds. Validation
299 of the activity of the compounds here suggest further investigations and warrant further evaluation
300 in mammalian models.

301 Financial & competing interest disclosure

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306

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444

445 **Figure Legends**

446 **Figure 1. Chemical structures of compounds 1-4.**

447 **Figure 2. Images from *C. elegans*-MRSA HTS**. Worms observed in light microscope (left) are
448 outlined in red and dead worms were identified in Sytox Orange-stained images (right) and were
449 marked in green. Compounds were classified as hits based on extension of survival of MRSA-
450 MW2 infected worms. **A** compound **1**; **B** compound **2**; **C** compound **3**; **D** compound **4**; **E**
451 Vancomycin; **F** DMSO.

452 **Figure 3. Time to kill assay.** MRSA-MW2 cells were exposed to compounds **1-4** at 4X MIC and
453 cell viability was monitored over 4 h. Data represent the mean ± SEM (n = 3).

454 **Figure 4. Bacterial membrane permeabilization assay.** Cellular fluorescence of MRSA-MW2
455 cells treated with Sytox Green and compounds **1-4** (64 μg/mL) was monitored over a 1 h period.

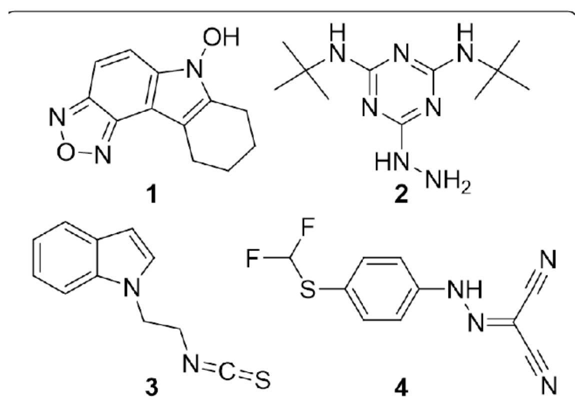
456 **Figure 5**. **A. Killing of intracellular MRSA-MW2 in macrophages.** MRSA-MW2 cells were
457 exposed to RAW 264.7 macrophages, treated with test compounds **1-4** at 1X MIC and the killing
458 of internalized bacteria was measured by CFU enumeration. Vancomycin (8 μg/mL) was used as
459 a positive control and DMSO 0.1% as the negative control. Data represent the mean ± SEM (n =
460 3). ***p<0.001, two-way ANOVA with Bonfererroni post-test comparing DMSO control at 24
461 hour time point. **B. Killing of planktonic MRSA-MW2.** MRSA-MW2 cells were exposed test
462 compounds **1-4** at 1X MIC and the CFU was measured.

463 **Figure 6. Cytotoxicity of compounds 1-4. A. Hemolytic activity.** Human RBCs were exposed
464 to 2-fold serial dilutions of compounds and hemolysis was measured after 1 hour. Serially diluted
465 triton-X (0.001 to 1%) was included as a positive control. **B-D**. **Cytotoxicity**. Mammalian cells
466 (HepG2, MKN-28, HKC-8) were treated with 2-fold serial dilutions of compounds and the

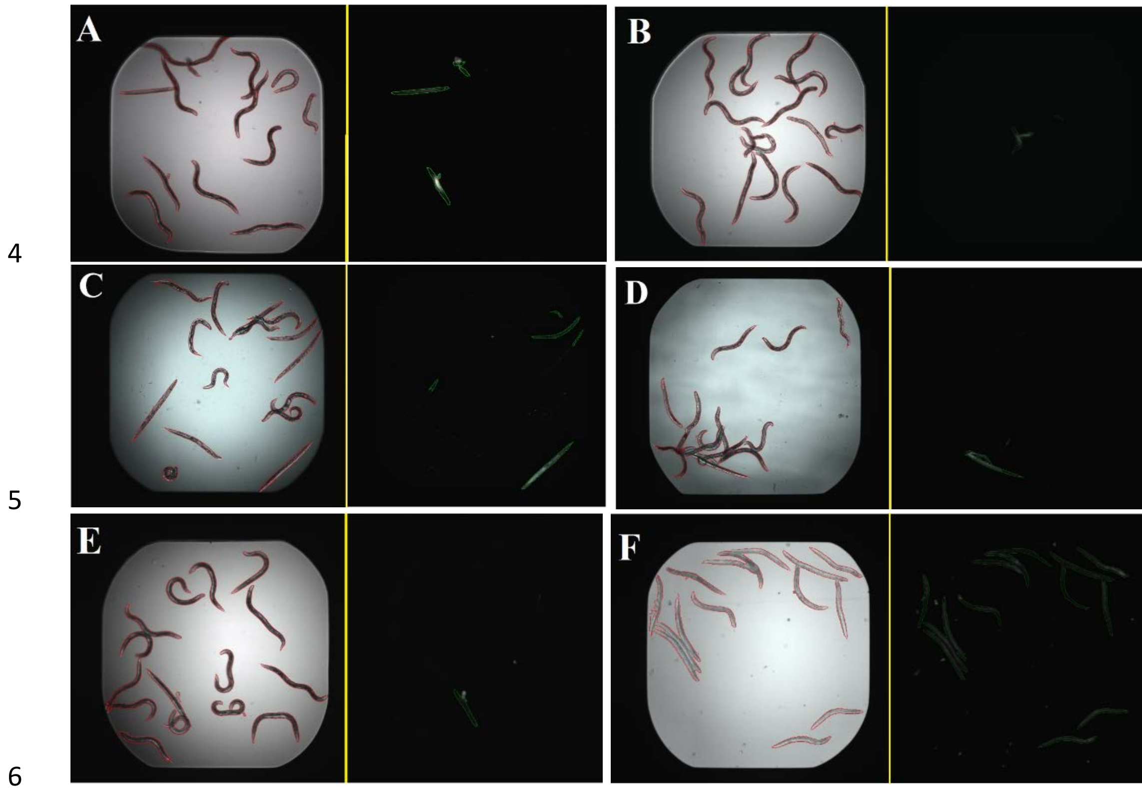
467 cytotoxicity was measured after 24 h by WST-1. **B**. HepG2 cells; **C**. MKN-28; **D**. HKC-8. Data

468 represent the mean ± SEM (n = 3).

Figure 1. Chemical structures of compounds 1-4.



3 **Figure 2. Images from *C. elegans*-MRSA HTS**.



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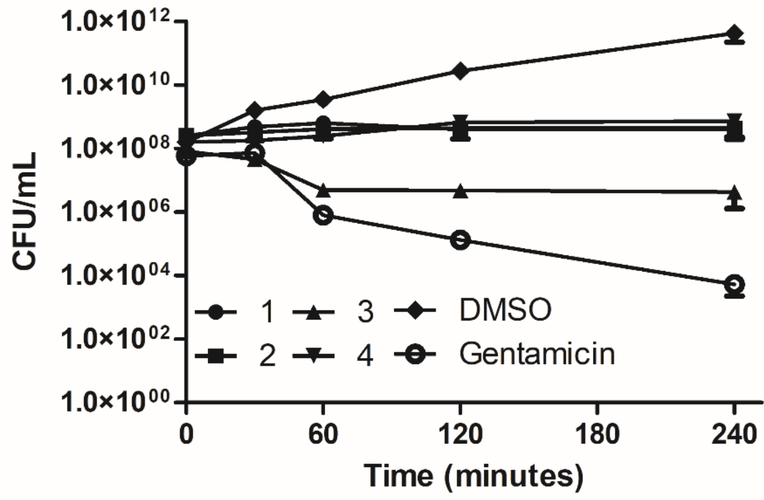
7 **Figure 3. Time to kill assay.**



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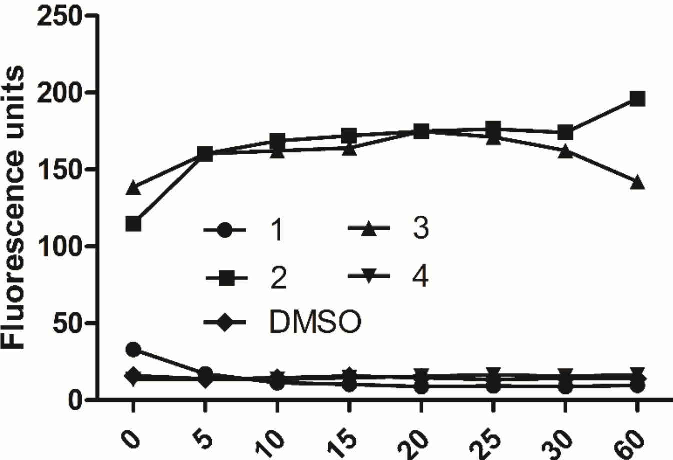
9 **Figure 4. Bacterial membrane permeabilization assay.**



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11 **Figure 5**. **Killing of intracellular MRSA-MW2 in macrophages.**

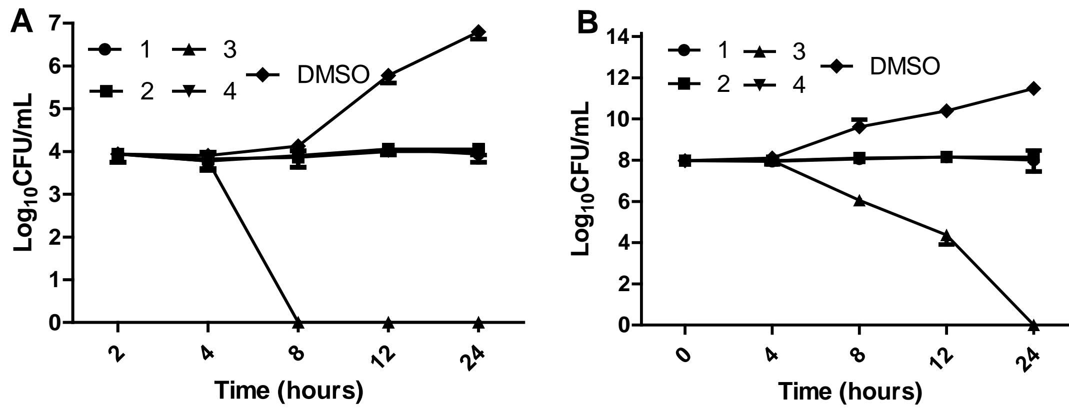

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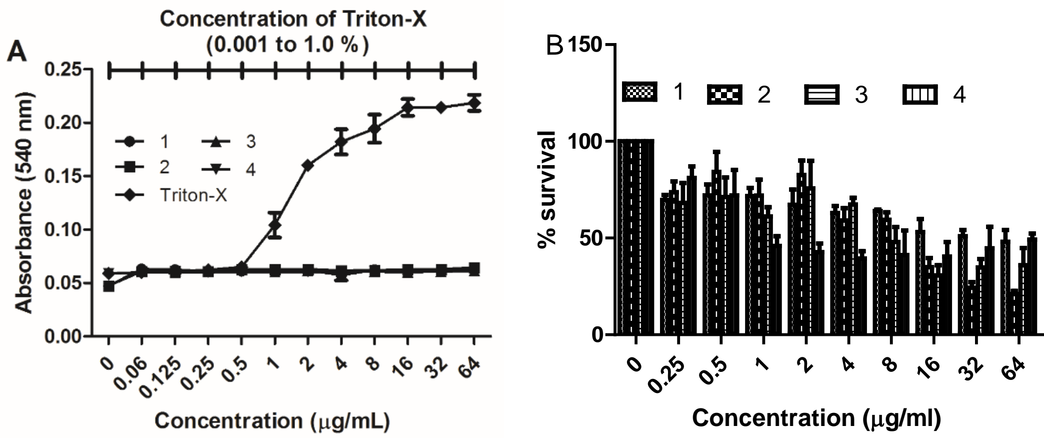
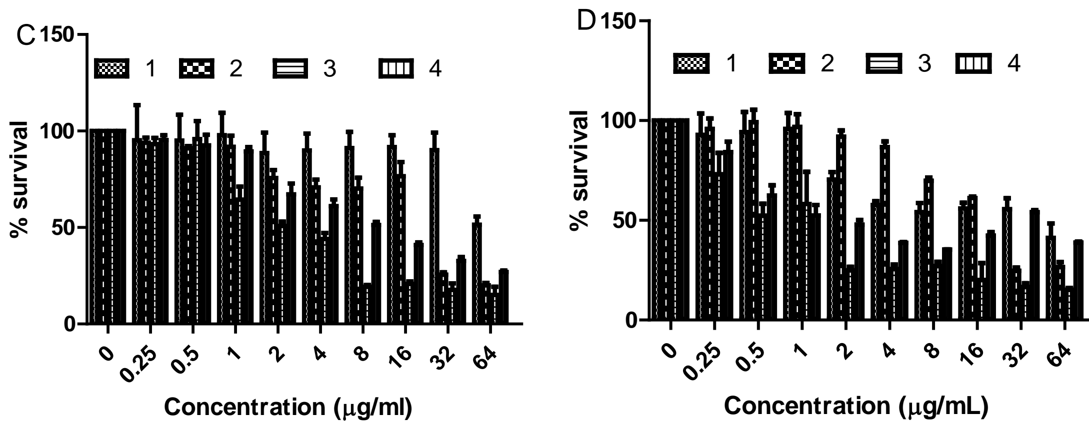
Figure 6. Cytotoxicity of compounds 1-4.





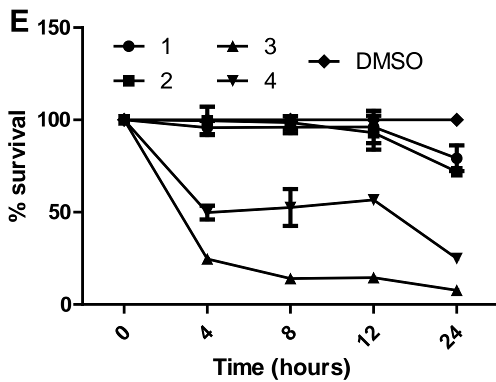


Table 1. Antibacterial activity (μg/mL) of compounds 1-4 against ESKAPE pathogens.

	1		**2**		**3**		**4**		**Vancomycin**		**Polymyxin B**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Staphylococcus aureus	4	64	8	>64	8	>64	2	32	4.0	16	>64	>64
Enterococcus faecium	8	ND	8	>64	8	>64	2	>64	4.0	64	>64	>64
Acinetobacter baumanii	32	>64	>64	>64	>64	>64	32	>64	>64	>64	4	8
Enterobacter aerogens	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	8	8
Klebsiella pneumoniae	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	8	8
Pseudomonas aeruginosa	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	2	4

Table 2. Antibacterial activity (μg/mL) of compounds 1-4 against clinical *S. aureus* pathogens.

	MIC (μg/mL)						
	1	**2**	**3**	**4**	**Vancomycin**	**PolymyxinB**	**Oxacillin**
***S. aureus* BF1**	4	8	8	2	2	>64	>64
***S. aureus* BF2**	4	8	8	2	2	>64	>64
***S. aureus* BF3**	4	8	8	2	2	>64	32
***S. aureus* BF4**	4	8	8	2	2	>64	16
***S. aureus* BF5**	4	8	8	2	2	>64	>64

Table 3. Fractional inhibitory concentration index (FICI) of compounds 1-4 used in paired combinations with each other and with antibiotics.

Compound	FICI									
	Compound				Clinical antibiotics					
	1	2	3	4	CIP	DOX	EMN	GMN	STN	VAN
1		0.75	1.0	0.75	0.5	1.0	0.75	0.75	2.0	2.0
2	0.75		1.0	0.75	0.5	1.0	1.0	1.0	0.625	1.0
3	1.0	1.0		1.0	1.0	0.75	0.5	0.5	0.5	0.5
4	0.75	0.75	1.0		0.5	0.5	0.75	1.0	1.0	1.0

CIP- Ciprofloxacin; DOX- Doxycycline; EMN- Erythromycin; GMN-Gentamicin; STN- Streptomycin; Van- Vancomycin.

Synergy FICI ≤ 0.5, antagonism FICI > 4.0, no interaction 0.5 > FICI ≤ 4.0 [28].