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

Nagendran Tharmalingam
Brown University

Rajmohan Rajmuthiah
Brown University

Wooseong Kim
Brown University

Beth Fuchs
Brown University

Elamparithi Jeyamani
Massachusetts General Hospital

See next page for additional authors

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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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Authors

Nagendran Tharmalingam, Rajmohan Rajmuthiah, Wooseong Kim, Beth Fuchs, Elamparithi Jeyamani, Michael J. Kelso, and Eleftherios Mylonakis

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

Nagendran Tharmalingam[1], Rajmohan Rajmuthiah[1], Wooseong Kim[1], Beth Burgwyn Fuchs[1], Elamparithi Jeyamani[2], Michael J. Kelso[3], Eleftherios Mylonakis[1,*]

[1]Infectious Diseases Division, Warren Alpert Medical School of Brown University, Rhode Island Hospital, Providence, RI, 02903, USA.

[2]Massachusetts General Hospital, Harvard Medical School, Boston, MA, 02114, USA.

[3]Illawarra Health and Medical Research Institute and School of Chemistry, University of Wollongong, Wollongong, NSW 2522, Australia

***Corresponding author:** Eleftherios Mylonakis, M.D., Ph.D., FIDSA,

Rhode Island Hospital

593 Eddy Street, POB, 3rd Floor, Suite 328/330

Providence, RI, 02903

Tel: 401-444-7856

Fax: 401-444-8179

Email: emylonakis@lifespan.org

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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 anti-staphylococcal compounds identified from a HTS campaign conducted using *Caenorhabditis elegans* nematodes infected with methicillin-resistant *Staphylococci aureus* (MRSA). The hit compounds included: an *N*-hydroxy indole- **1**, a substituted melamine derivative- **2**, an *N*-substituted indolic alkyl isothiocyanate- **3**, and a *p*-difluoromethylsulfide analog- **4** of the well-known protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). Minimal inhibitory concentrations (MICs) of the four compounds ranged from 2-8 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 anti-staphylococcal compounds that warrant further study as novel antibacterial agents against Gram-positive organisms.

Introduction

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

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

Materials and Methods

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

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

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

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

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

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

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

Intracellular MRSA killing assays. RAW 264.7 macrophages were used to examine intracellular killing of MRSA-MW2 by **1**-**4**, as described by Schmitt et al [16]. Macrophages 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 [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

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

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

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

Results

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

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

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

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

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

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

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

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

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

activity of natural ITCs is enhanced by clinical antibiotics [29-31], in agreement with our observations with **3**.

Discussion

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

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

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

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

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

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

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

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

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

Financial & competing interest disclosure

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References

1. **Waness A.** 2010. Revisiting methicillin-resistant Staphylococcus aureus infections. Journal of global infectious diseases 2: 49.
2. **Weinstein R.A., Gaynes R., Edwards J.R., and System N.N.I.S.** 2005. Overview of nosocomial infections caused by gram-negative bacilli. Clinical Infectious Diseases 41: 848-854.
3. **Stryjewski M.E., and Corey G.R.** 2014. Methicillin-resistant Staphylococcus aureus: an evolving pathogen. Clin Infect Dis 58 Suppl 1: S10-9.
4. **CDC**. 2016. Biggest Threats.
5. **Gardete S., and Tomasz A.** 2014. Mechanisms of vancomycin resistance in Staphylococcus aureus. The Journal of clinical investigation 124: 2836-2840.
6. **Aktas G., and Derbentli S.** In vitro activity of daptomycin combinations with rifampicin, gentamicin, fosfomycin and fusidic acid against MRSA strains. Journal of Global Antimicrobial Resistance.
7. **Rodvold K.A., and McConeghy K.W.** 2014. Methicillin-resistant Staphylococcus aureus therapy: past, present, and future. Clinical infectious diseases 58: S20-S27.
8. **Ewbank J.J.** 2002. Tackling both sides of the host–pathogen equation with Caenorhabditis elegans. Microbes and Infection 4: 247-256.
9. **Rajamuthiah R., Fuchs B.B., Jayamani E., Kim Y., Larkins-Ford J., Conery A., Ausubel F.M., and Mylonakis E.** 2014. Whole animal automated platform for drug discovery against multi-drug resistant Staphylococcus aureus. PLoS One 9: e89189.
10. **Beanan M.J., and Strome S.** 1992. Characterization of a germ-line proliferation mutation in C. elegans. Development 116: 755-66.

11. **Tanaka-Hino M., Sagasti A., Hisamoto N., Kawasaki M., Nakano S., Ninomiya-Tsuji J., Bargmann C.I., and Matsumoto K.** 2002. SEK-1 MAPKK mediates Ca2+ signaling to determine neuronal asymmetric development in Caenorhabditis elegans. EMBO Rep 3: 56-62.

12. **Wikler M.A.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. Clinical and Laboratory Standards Institute.

13. **Gwisai T., Hollingsworth N., Cowles S., Tharmalingam N., Mylonakis E., Fuchs B., and Shukla A.** 2017. Repurposing niclosamide as a versatile antimicrobial surface coating against device-associated, hospital-acquired bacterial infections. Biomedical Materials.

14. **Rajamuthiah R., Fuchs B.B., Conery A.L., Kim W., Jayamani E., Kwon B., Ausubel F.M., and Mylonakis E.** 2015. Repurposing salicylanilide anthelmintic drugs to combat drug resistant Staphylococcus aureus. PLoS One 10: e0124595.

15. **Kim W., Conery A.L., Rajamuthiah R., Fuchs B.B., Ausubel F.M., and Mylonakis E.** 2015. Identification of an Antimicrobial Agent Effective against Methicillin-Resistant Staphylococcus aureus Persisters Using a Fluorescence-Based Screening Strategy. PLoS One 10: e0127640.

16. **Schmitt D.M., O'Dee D.M., Cowan B.N., Birch J.W., Mazzella L.K., Nau G.J., and Horzempa J.** 2013. The use of resazurin as a novel antimicrobial agent against Francisella tularensis. Front Cell Infect Microbiol 3: 93.

17. **Tharmalingam N., Park M., Lee M.H., Woo H.J., Kim H.W., Yang J.Y., Rhee K.-J., and Kim J.-B.** 2016. Piperine treatment suppresses Helicobacter pylori toxin entry in to

gastric epithelium and minimizes β-catenin mediated oncogenesis and IL-8 secretion in vitro. American journal of translational research 8: 885.

18. **Lee M.H., Cho Y., Do Hyun Kim H.J.W., Yang J.Y., Kwon H.J., Yeon M.J., Park M., Kim S.-H., Moon C., and Tharmalingam N.** 2016. Menadione induces G2/M arrest in gastric cancer cells by down-regulation of CDC25C and proteasome mediated degradation of CDK1 and cyclin B1. American journal of translational research 8: 5246.

19. **Jayamani E., Tharmalingam N., Rajamuthiah R., Coleman J.J., Kim W., Okoli I., Hernandez A.M., Lee K., Nau G.J., Ausubel F.M. and others**. 2017. Characterization of a Francisella tularensis-Caenorhabditis elegans pathosystem for the evaluation of therapeutic compounds. Antimicrob Agents Chemother 00310.

20. **Isnansetyo A., and Kamei Y.** 2003. MC21-A, a bactericidal antibiotic produced by a new marine bacterium, Pseudoalteromonas phenolica sp. nov. O-BC30(T), against methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 47: 480-8.

21. **Tharmalingam N., Jayamani E., Rajamuthiah R., Castillo D., Fuchs B.B., Kelso M.J., and Mylonakis E.** 2017. Activity of a novel protonophore against methicillin-resistant Staphylococcus aureus. Future Medicinal Chemistry 9: 1401-1411.

22. **Kim S.-H., Lee M.H., Park M., Woo H.J., Kim Y.S., Tharmalingam N., Seo W.-D., and Kim J.-B.** Regulatory Effects of Black Rice Extract on Helicobacter pylori Infection-Induced Apoptosis. Molecular Nutrition & Food Research: 1700586-n/a.

23. **Zheng Z., Tharmalingam N., Liu Q., Jayamani E., Kim W., Fuchs B., Zhang R., Vilcinskas A., and Mylonakis E.** 2017. Synergistic efficacy of Aedes aegypti antimicrobial peptide cecropin A2 and tetracycline against Pseudomonas aeruginosa. Antimicrob Agents Chemother 61.

24. **Weaver Jr A.J., Shepard J.B., Wilkinson R.A., Watkins R.L., Walton S.K., Radke A.R., Wright T.J., Awel M.B., Cooper C., and Erikson E.** 2014. Antibacterial activity of THAM Trisphenylguanide against methicillin-resistant Staphylococcus aureus. PloS one 9: e97742.

25. **Borges A., Abreu A.C., Ferreira C., Saavedra M.J., Simões L.C., and Simões M.** 2015. Antibacterial activity and mode of action of selected glucosinolate hydrolysis products against bacterial pathogens. Journal of food science and technology 52: 4737-4748.

26. **Brouillette E., Grondin G., Shkreta L., Lacasse P., and Talbot B.G.** 2003. In vivo and in vitro demonstration that Staphylococcus aureus is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. Microb Pathog 35: 159-68.

27. **Torella J.P., Chait R., and Kishony R.** 2010. Optimal drug synergy in antimicrobial treatments. PLoS Comput Biol 6: e1000796.

28. **Odds F.C.** 2003. Synergy, antagonism, and what the chequerboard puts between them. Br Soc Antimicrob Chemo.

29. **Kaiser S.J., Mutters N.T., Blessing B., and Günther F.** 2017. Natural isothiocyanates express antimicrobial activity against developing and mature biofilms of Pseudomonas aeruginosa. Fitoterapia 119: 57-63.

30. **Palaniappan K., and Holley R.A.** 2010. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International journal of food microbiology 140: 164-168.

31. **Tajima H., Kimoto H., and Taketo A.** 2001. Specific antimicrobial synergism of synthetic hydroxy isothiocyanates with aminoglycoside antibiotics. Bioscience, biotechnology, and biochemistry 65: 1886-1888.

32. **Davies J., and Davies D.** 2010. Origins and evolution of antibiotic resistance. Microbiology and molecular biology reviews 74: 417-433.

33. **Tharmalingam N., Kim S.-H., Park M., Woo H.J., Kim H.W., Yang J.Y., Rhee K.-J., and Kim J.B.** 2014. Inhibitory effect of piperine on Helicobacter pylori growth and adhesion to gastric adenocarcinoma cells. Infectious agents and cancer 9: 43.

34. **Kim S.-H., Park M., Woo H., Tharmalingam N., Lee G., Rhee K.-J., Eom Y.B., Han S.I., Seo W.D., and Kim J.B.** 2012. Inhibitory effects of anthocyanins on secretion of Helicobacter pylori CagA and VacA toxins. Int J Med Sci 9: 838-842.

35. **Rani R., and Granchi C.** 2015. Bioactive heterocycles containing endocyclic N-hydroxy groups. European journal of medicinal chemistry 97: 505-524.

36. **Lee J., Attila C., Cirillo S.L., Cirillo J.D., and Wood T.K.** 2009. Indole and 7-hydroxyindole diminish Pseudomonas aeruginosa virulence. Microbial biotechnology 2: 75-90.

37. **Chen Z., and Sun Y.** 2005. Antimicrobial polymers containing melamine derivatives. II. Biocidal polymers derived from 2-vinyl-4,6-diamino-1,3,5-triazine. Journal of Polymer Science Part A: Polymer Chemistry 43: 4089-4098.

38. **Stewart M.L., Bueno G.J., Baliani A., Klenke B., Brun R., Brock J.M., Gilbert I.H., and Barrett M.P.** 2004. Trypanocidal activity of melamine-based nitroheterocycles. Antimicrobial agents and chemotherapy 48: 1733-1738.

418 39. **Kandelbauer A., and Widsten P.** 2009. Antibacterial melamine resin surfaces for wood-
419 based furniture and flooring. Progress in organic coatings 65: 305-313.
420 40. **Sun X., Cao Z., Porteous N., and Sun Y.** 2010. Amine, melamine, and amide N-
421 halamines as antimicrobial additives for polymers. Industrial & engineering chemistry
422 research 49: 11206-11213.
423 41. **Zou Y., Jung L.S., Lee S.H., Kim S., Cho Y., and Ahn J.** 2013. Enhanced
424 antimicrobial activity of nisin in combination with allyl isothiocyanate against Listeria
425 monocytogenes, Staphylococcus aureus, Salmonella Typhimurium and Shigella boydii.
426 International Journal of Food Science & Technology 48: 324-333.
427 42. **Kim M., and Lee H.** 2009. Growth-Inhibiting Activities of Phenethyl Isothiocyanate and
428 Its Derivatives against Intestinal Bacteria. Journal of food science 74: M467-M471.
429 43. **Tajima H., Kimoto H., Taketo Y., and Taketo A.** 1998. Effects of synthetic hydroxy
430 isothiocyanates on microbial systems. Bioscience, biotechnology, and biochemistry 62:
431 491-495.
432 44. **Dufour V., Stahl M., and Baysse C.** 2015. The antibacterial properties of
433 isothiocyanates. Microbiology 161: 229-243.
434 45. **Breier A., and Ziegelhoffer A.** 2000. " Lysine is the Lord", thought some scientists in
435 regard to the group interacting with fluorescein isothiocyanate in ATP-binding sites of P-
436 type ATPases. But, is it not cysteine?(Minireview). General physiology and biophysics
437 19: 253-264.
438 46. **Sofrata A., Santangelo E.M., Azeem M., Borg-Karlson A.-K., Gustafsson A., and**
439 **Pütsep K.** 2011. Benzyl isothiocyanate, a major component from the roots of Salvadora
440 persica is highly active against Gram-negative bacteria. PLoS One 6: e23045.

47. **Chen C., Dolla N.K., Casadei G., Bremner J.B., Lewis K., and Kelso M.J.** 2014. Diarylacylhydrazones: Clostridium-selective antibacterials with activity against stationary-phase cells. Bioorg Med Chem Lett 24: 595-600.

Figure Legends

Figure 1. Chemical structures of compounds 1-4.

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

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

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

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

Figure 6. Cytotoxicity of compounds 1-4. A. Hemolytic activity. Human RBCs were exposed to 2-fold serial dilutions of compounds and hemolysis was measured after 1 hour. Serially diluted triton-X (0.001 to 1%) was included as a positive control. **B-D**. **Cytotoxicity**. Mammalian cells (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.

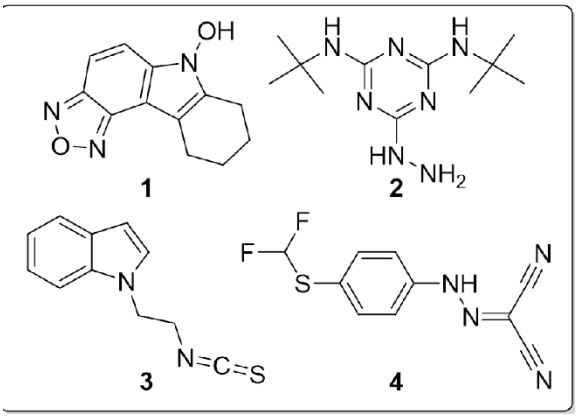

Figure 2. Images from *C. elegans*-MRSA HTS.

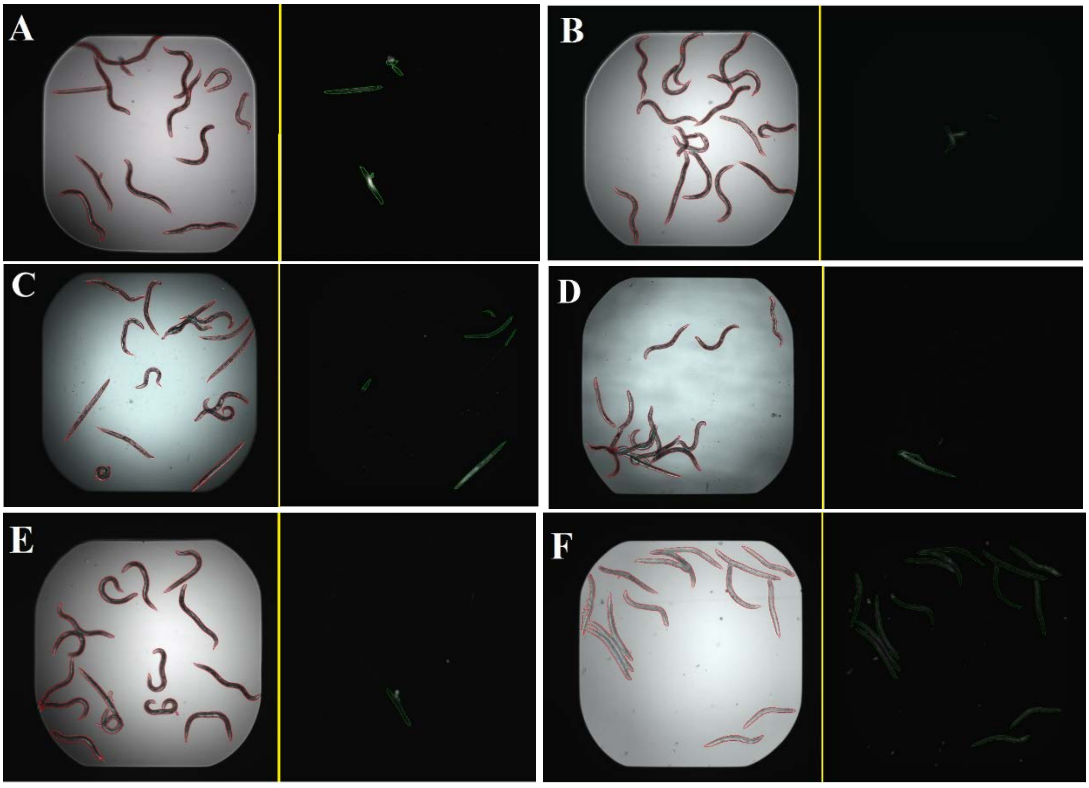

Figure 3. Time to kill assay.

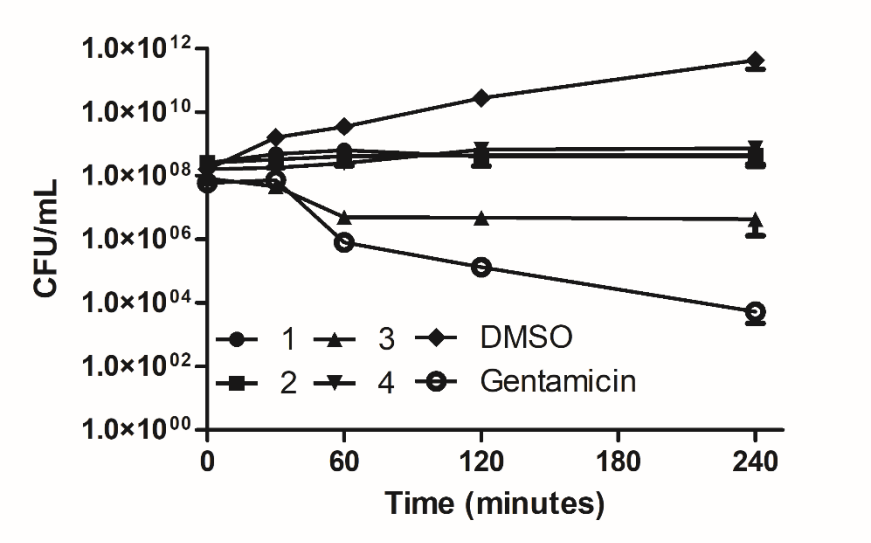

Figure 4. Bacterial membrane permeabilization assay.

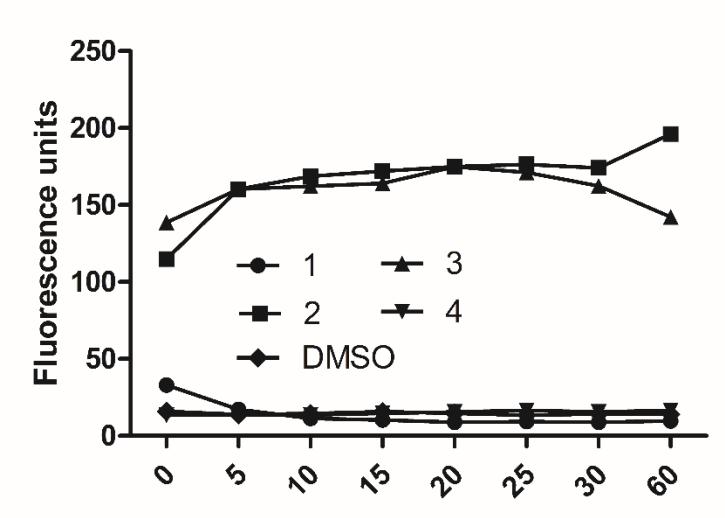

Figure 5. Killing of intracellular MRSA-MW2 in macrophages.

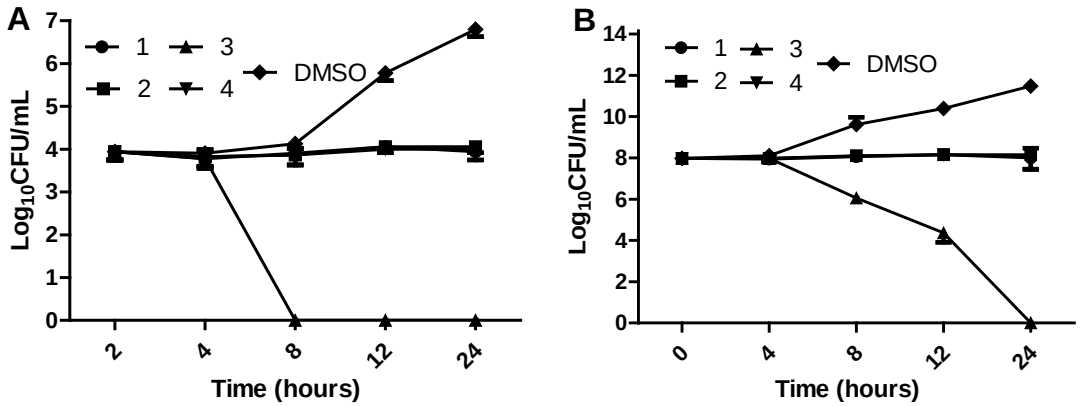

Figure 6. Cytotoxicity of compounds 1-4.

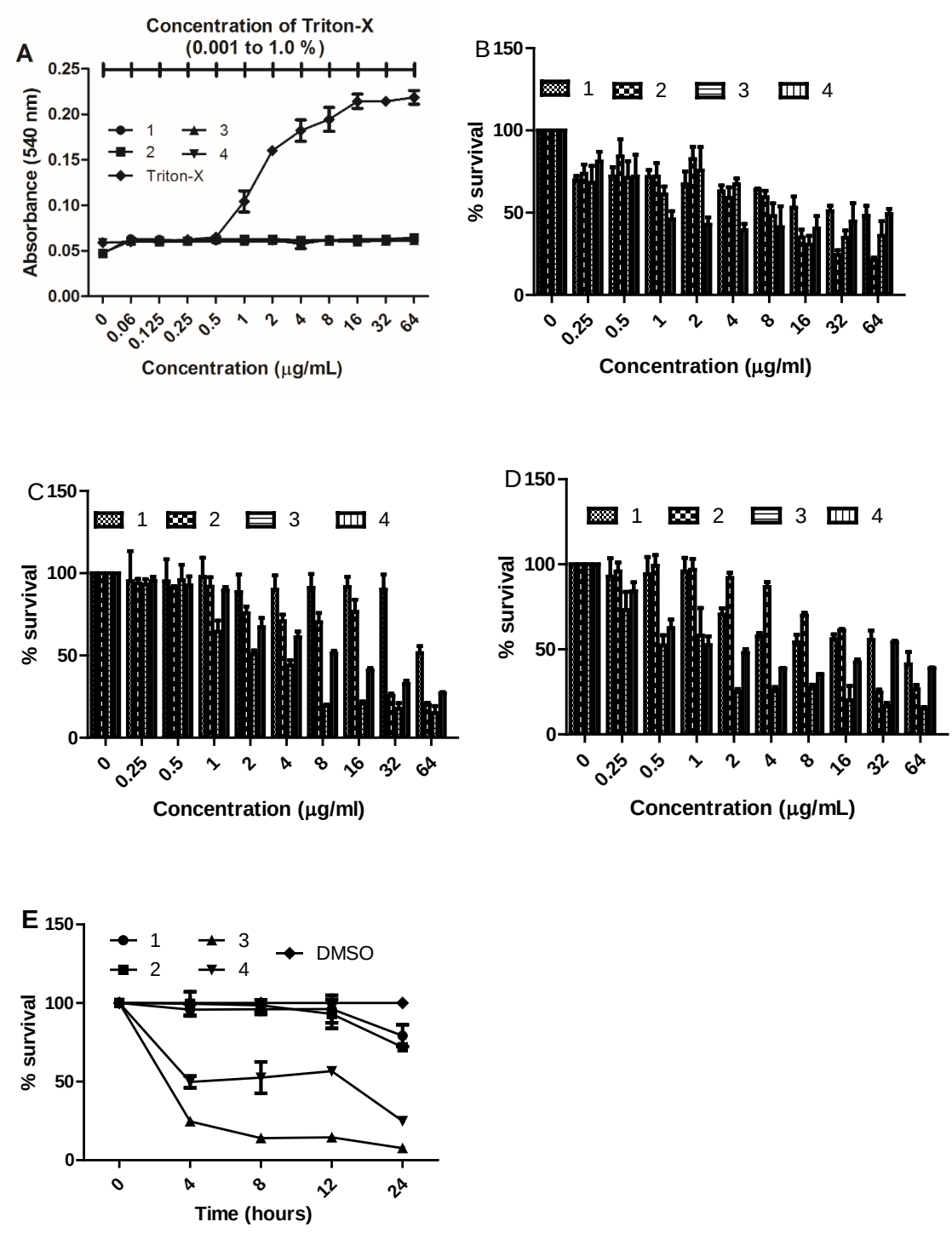

Table 1. Antibacterial activity (μg/mL) of compounds 1-4 against ESKAPE pathogens.

	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 $\leq$ 0.5, antagonism FICI > 4.0, no interaction 0.5 > FICI $\leq$ 4.0 [28].