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Accessibility
Repurposing Salicylanilide Anthelmintic Drugs to Combat Drug Resistant Staphylococcus aureus

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Abstract

Staphylococcus aureus is a Gram-positive bacterium that has become the leading cause of hospital acquired infections in the US. Repurposing Food and Drug Administration (FDA) approved drugs for antimicrobial therapy involves lower risks and costs compared to de novo development of novel antimicrobial agents. In this study, we examined the antimicrobial properties of two commercially available anthelmintic drugs. The FDA approved drug niclosamide and the veterinary drug oxyclozanide displayed strong in vivo and in vitro activity against methicillin resistant S. aureus (minimum inhibitory concentration (MIC): 0.125 and 0.5 μg/ml respectively; minimum effective concentration: ≤ 0.78 μg/ml for both drugs). The two drugs were also effective against another Gram-positive bacteria Enterococcus faecium (MIC 0.25 and 2 μg/ml respectively), but not against the Gram-negative species Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter aerogenes. The in vitro antimicrobial activity of niclosamide and oxyclozanide were determined against methicillin, vancomycin, linezolid or daptomycin resistant S. aureus (minimum inhibitory concentration (MIC): 0.125 and 0.5 μg/ml respectively), but not against the Gram-negative species Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter aerogenes. The in vitro antimicrobial activity of niclosamide and oxyclozanide were determined against methicillin, vancomycin, linezolid or daptomycin resistant S. aureus clinical isolates, with MICs at 0.0625-0.5 and 0.125-2 μg/ml for niclosamide and oxyclozanide respectively. A time-kill study demonstrated that niclosamide is bacteriostatic, whereas oxyclozanide is bactericidal. Interestingly, oxyclozanide permeabilized the bacterial membrane but neither of the anthelmintic drugs exhibited demonstrable toxicity to sheep erythrocytes. Oxyclozanide was non-toxic to HepG2 human liver carcinoma cells within the range of its in vitro MICs but niclosamide displayed toxicity even at low concentrations. These data show that the salicylanilide anthelmintic drugs niclosamide and oxyclozanide are suitable candidates for mechanism of action studies and further clinical evaluation for treatment of staphylococcal infections.
Introduction

*Staphylococcus aureus* has emerged as a primary cause of hospital and community-acquired infections [1, 2], often leading to bacteremia and sepsis [3, 4]. The virulence of this pathogen is manifested through its wide spectrum of virulence factors and strategies to bypass host defense mechanisms [5, 6]. Unfortunately, the rising tide of antibiotic resistance, especially against β-lactam antibiotics, such as methicillin, has severely constrained treatment options. In 2011, the Centers for Disease Control and Prevention (CDC) estimated that methicillin resistant *S. aureus* (MRSA) was associated with 80,461 life-threatening infections, resulting in 11,285 fatalities in the US alone and that the yearly mortality rate surpassed that of Acquired immunodeficiency syndrome (AIDS) [7, 8]. Therefore, novel anti-infectives and treatment strategies are urgently needed against *S. aureus*.

Classical antimicrobial drug discovery involves *in vitro* screening for antimicrobial candidates, Structure Activity Relationship (SAR) analysis, followed by *in vivo* testing for toxicity [9]. Bringing drugs from the bench to the bedside involves huge expenditures in time and resources. This, along with the relatively short window of therapeutic application for antibiotics attributed to the rapid emergence of drug resistance, has, at least until recently, resulted in a waning interest in antibiotic discovery among pharmaceutical companies [10]. In this environment, “repurposing” (defined as investigating new uses for existing drugs) has gained renewed interest, as reflected by several recent studies [11–13].

The free living nematode *C. elegans* has recently become a popular model organism for studying pathogenesis of many microbes [14–17], including *S. aureus* [18, 19]. Using *C. elegans* as a platform for antimicrobial drug discovery enables simultaneous assessment of toxicity and efficacy of the test compounds, which lowers the burden for further animal testing [20]. We have developed a high throughput *C. elegans*-MRSA liquid infection assay and screened a compound library consisting of bioactives and FDA approved drugs for compounds that prolong survival of *C. elegans* infected with MRSA [21]. An unexpected finding from that study was that, although a nematode was used as a model host, the anthelmintic drug closantel was identified as a hit that prolonged survival of the infected worms. Moreover, the *in vivo* and *in vitro* activity of closantel was comparable to that of vancomycin, a drug of last resort that is widely used to combat MRSA infections [22].

The identification of closantel as a potent anti-staphylococcal drug [21, 23] motivated us to determine the anti-staphylococcal properties of niclosamide and oxyclozanide, two analogs of closantel. All three compounds belong to the salicylanilide family of anthelmintic drugs. Niclosamide is an FDA approved drug widely used for treating tape worm infection in humans and has lately been shown to possess anti-cancer and anti-diabetic activities [24–26]. Niclosamide is also listed in the World Health Organization (WHO) model list of essential medicines [27]. Oxyclozanide is used in veterinary medicine, primarily for ruminants such as cattle, goat and sheep to control parasitic flatworms called flukes [28, 29]. The antimicrobial properties of salicylanilide derivatives have recently been described [30–33]. Cheng et al. [30] used a high-throughput screening strategy to identify a salicylanilide derivative that inhibits MRSA cell wall synthesis and Kratky et al. [31, 33] and Pauk et al. [32] described the synthesis of salicylanilide analogues that exhibited antimicrobial activity against a variety of bacteria including MRSA and mycobacteria. However, the studies mentioned above did not involve salicylanilide drugs that are currently in use, such as niclosamide and oxyclozanide.

We report here that niclosamide and oxyclozanide were effective against MRSA both *in vitro* and *in vivo* in the *C. elegans* infection model, and that the mode of action of oxyclozanide may involve bacterial membrane disruption. Niclosamide displayed toxicity to mammalian cells even at low concentrations whereas oxyclozanide displayed toxicity only at concentrations
higher than the effective concentration against MRSA. The findings from this study suggest the advisability of further studies on the antibacterial mechanism of action of salicylanilide anthelminthic drugs and their clinical potential for treating staphylococcal infections.

Materials and Methods

Bacterial and nematode strains

All bacterial strains tested in this study were selected from an existing strain collection maintained in our laboratory. The following strains (Tables 1 and 2) were used to evaluate the antibacterial activities of anthelmintic drugs: *S. aureus* (MRSA MW2, Newman, RN4220, RN6390, USA100, USA300, USA400), *S. epidermidis* (9142), *Enterococcus faecium* (E007), *Klebsiella pneumoniae* (ATCC 77326), *Acinetobacter baumannii* (ATCC 17978), *Pseudomonas aeruginosa* (PA14), *Enterobacter aerogenes* (EAE 2625) and 44 *S. aureus* clinical isolates. All bacterial strains were grown at 37°C. Staphylococcal strains were grown in tryptic soy broth (TSB); *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. aerogenes* were grown in Luria-Bertani broth (LB) and *E. faecium* was grown in Brain-heart infusion medium (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

The *C. elegans* glp-4(bn2);sek-1(km4) double mutant strain were maintained at 15°C on a lawn of *E. coli* strain HB101 on 10 cm plates as described [34]. The glp-4(bn2) mutation renders the strain incapable of producing progeny at 25°C [35] and the sek-1(km4) mutation enhances sensitivity to various pathogens [36], reducing assay time.

C. elegans-MRSA liquid infection assay

The *C. elegans*-MRSA liquid infection assay has been described [21, 37]. In brief, 4,500 glp-4 (bn2);sek-1(km4) L1 hatchlings were grown on SK-HB101 agar plates for 52 hours at the restrictive temperature of 25°C until animals became sterile young adults and later harvested with M9 buffer. *S. aureus* MW2 was grown overnight at 37°C in TSB, first under aerobic conditions and later shifted to anaerobic growth conditions at 37°C. The infection assay was performed in standard 384-well assay plates (Corning no. 3712; Corning, Corning, NY, USA), in the presence of the compound being tested or 1% dimethyl sulfoxide (DMSO) as control. Bacteria were added to the wells at a final OD_{600} of 0.04, followed by the use of a Complex Object Parametric Analyzer and Sorter (COPAS) large particle sorter (Union Biometrica, Holliston, MA, USA) to transfer 15 adult worms to each well of an assay plate. After 5 days of incubation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Niclosamide (μg/ml)</th>
<th>Oxyclozanide (μg/ml)</th>
<th>Vancomycin (μg/ml)</th>
<th>Polymyxin B (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td><em>E. faecium</em> (E007) [65]</td>
<td>0.25</td>
<td>&gt;64</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td><em>S. aureus</em> (MW2) [66]</td>
<td>0.125</td>
<td>32</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (ATCC 77326)</td>
<td>&gt;64</td>
<td>ND</td>
<td>&gt;64</td>
<td>ND</td>
</tr>
<tr>
<td><em>A. baumannii</em> (ATCC 17978)</td>
<td>&gt;64</td>
<td>ND</td>
<td>&gt;64</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (PA14) [67]</td>
<td>&gt;64</td>
<td>ND</td>
<td>&gt;64</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. aerogenes</em> (EAE 2625)</td>
<td>&gt;64</td>
<td>ND</td>
<td>&gt;64</td>
<td>ND</td>
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doi:10.1371/journal.pone.0124595.t001
in a humidified chamber at 25°C, the bacteria and other debris were washed from the wells with a microplate washer and the worms were stained with the vital dye Sytox Orange (Life Technologies, Carlsbad, CA, USA). After overnight incubation at 25°C in a humidified chamber, the plates 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.

The images from the infection assay were processed using the open source image analysis software CellProfiler ([http://www.cellprofiler.org/](http://www.cellprofiler.org/)) and analysis modules as described previously [38, 39]. The ratio of Sytox worm area to bright field worm area, and the resultant percentage survival data, is calculated by the software for each well of the assay plates. The entire assay was performed in duplicate.

### Antibacterial susceptibility testing

Compounds (5 mg/ml stock solution in DMSO) were tested by broth microdilution and disc diffusion assays in triplicate, adapted from established protocols [40]. Broth microdilution was performed in triplicate in 96-well plates using Müller-Hinton broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA). For testing MIC with daptomycin, the media was supplemented with CaCl₂ at a final concentration of 50 μg/ml. The assay volume was 100 μl and two-fold serial dilutions were carried out to get compounds in the concentration range 0.0625–64 μg/ml. The bacterial concentration was adjusted to an initial OD₆₀₀ of 0.03. After overnight incubation at 35°C, the absorbance was measured to determine antimicrobial activity. The minimum bactericidal concentration (MBC) was determined by plating 10 μl of culture volume from the MIC assay on Müller-Hinton agar and colony formation was examined after overnight incubation at 35°C. The lowest concentration at which colonies were not observed was regarded as the MBC.

The disc diffusion test was carried out in triplicate on Müller-Hinton agar (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Discs made from Whatman filter paper (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using a paper punch were impregnated with 25 μg of test compounds and air-dried. Three hundred microliters of an overnight bacterial culture was spread on an agar plate and air-dried. The antimicrobial discs were overlaid on the plate and incubated at 35°C for 20 hrs. Antimicrobial susceptibility was determined by measuring the diameter of the zone of inhibition.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Niclosamide (μg/ml)</th>
<th>Oxyclozanide (μg/ml)</th>
<th>Oxacillin (μg/ml)</th>
<th>Vancomycin (μg/ml)</th>
<th>Daptomycin (μg/ml)</th>
<th>Telavancin (μg/ml)</th>
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<tbody>
<tr>
<td>MW2 [66]</td>
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<td>0.5</td>
<td>64</td>
<td>4</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Newman [68]</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
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<tr>
<td>RN4220 [69]</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>2</td>
<td>4</td>
<td>0.125</td>
</tr>
<tr>
<td>RN6930 [70]</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>2</td>
<td>4</td>
<td>&lt;= 0.0625</td>
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<tr>
<td>USA100 [71]</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
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<tr>
<td>USA300 [71]</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>&gt;64</td>
<td>4</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>USA400 [71]</td>
<td>&lt;= 0.0625</td>
<td>&lt;= 0.0625</td>
<td>&gt;64</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>9142^ [72]</td>
<td>0.5</td>
<td>0.125</td>
<td>&gt;64</td>
<td>4</td>
<td>2</td>
<td>&lt;= 0.0625</td>
</tr>
</tbody>
</table>

* is an *S. epidermidis* strain, rest are *S. aureus* strains.

doi:10.1371/journal.pone.0124595.t002

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC Daptomycin (μg/ml)</th>
<th>MBC Daptomycin (μg/ml)</th>
<th>MIC Telavancin (μg/ml)</th>
<th>MBC Telavancin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2 [66]</td>
<td>0.125</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>Newman [68]</td>
<td>&lt;= 0.0625</td>
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<td>RN4220 [69]</td>
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<td>RN6930 [70]</td>
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<td>USA100 [71]</td>
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<tr>
<td>9142^ [72]</td>
<td>0.5</td>
<td>0.125</td>
<td>0.5</td>
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* is an *S. epidermidis* strain, rest are *S. aureus* strains.

doi:10.1371/journal.pone.0124595.t002

Antimicrobial susceptibility testing

Compounds (5 mg/ml stock solution in DMSO) were tested by broth microdilution and disc diffusion assays in triplicate, adapted from established protocols [40]. Broth microdilution was performed in triplicate in 96-well plates using Müller-Hinton broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA). For testing MIC with daptomycin, the media was supplemented with CaCl₂ at a final concentration of 50 μg/ml. The assay volume was 100 μl and two-fold serial dilutions were carried out to get compounds in the concentration range 0.0625–64 μg/ml. The bacterial concentration was adjusted to an initial OD₆₀₀ of 0.03. After overnight incubation at 35°C, the absorbance was measured to determine antimicrobial activity. The minimum bactericidal concentration (MBC) was determined by plating 10 μl of culture volume from the MIC assay on Müller-Hinton agar and colony formation was examined after overnight incubation at 35°C. The lowest concentration at which colonies were not observed was regarded as the MBC.

The disc diffusion test was carried out in triplicate on Müller-Hinton agar (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Discs made from Whatman filter paper (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using a paper punch were impregnated with 25 μg of test compounds and air-dried. Three hundred microliters of an overnight bacterial culture was spread on an agar plate and air-dried. The antimicrobial discs were overlaid on the plate and incubated at 35°C for 20 hrs. Antimicrobial susceptibility was determined by measuring the diameter of the zone of inhibition.
Time-kill study

The bactericidal properties of test compounds were assessed using a time-kill study, adapted from a protocol described earlier [41]. The assay was conducted in 5 ml round bottomed tubes (BD Biosciences no. 352235; BD Biosciences, San Jose, CA, USA). An overnight culture of MRSA (MW2) was diluted in fresh TSB to a density of $10^6$ cells/ml. Test compounds, at 4x their MIC concentration, were added to each tube and incubated with shaking at 37°C. At periodic intervals, aliquots sampled from the tubes were serially diluted and plated on tryptic soy agar (TSA; Becton Dickinson and Company, Franklin Lakes, NJ, USA) plates. The plates were incubated overnight at 37°C and the colonies were counted to measure viability. The assay was done in duplicate.

Bacterial cell membrane permeabilization assay

Permeabilization of bacterial membranes was determined by Sytox Green (Life Technologies, Carlsbad, CA, USA) uptake by bacterial cells in 96 well plates (Corning CLS3300; Corning, Corning, NY, USA) as described in an earlier study [42]. The assay was done in triplicate. MRSA strain MW2 cells in logarithmic growth phase were harvested by centrifugation at 4000 rpm for 5 minutes, the pellet was washed twice in phosphate-buffered saline (PBS), and resuspended in PBS to an absorbance of 0.5 at 595 nm. Sytox Green was added to the cells at a final concentration of 5 μM and incubated in the dark for 30 minutes. Fifty microliters of cell suspension was added to 50 μl of compounds serially diluted in PBS. The fluorescence intensities were measured at different time points, with the excitation and emission at 485 nm and 530 nm respectively. The assay was repeated twice.

Bacterial lysis assay

The assay for testing the ability of the antimicrobial compounds to induce lysis of bacterial cells was adapted from a protocol described in [43]. Briefly, logarithmically growing MRSA (MW2) cells were harvested by centrifugation at 4000 rpm for 5 minutes, washed twice with PBS, and resuspended in PBS to an absorbance of 0.5 at 595 nm. The assay was carried out in triplicate in a 96 well plate by adding 50 μl of cell suspension to 50 μl of compounds serially diluted in PBS, and incubating at 37°C. The absorbance at 595 nm was measured at periodic time points and the relative absorbance was calculated by dividing the absorbance of each well with that of the negative control (DMSO). All assays were performed in triplicate.

Sheep blood hemolysis

The protocol to test the ability of compounds to cause hemolysis of sheep erythrocytes (Rockland Immunochemicals, Limerick, PA, USA) was adapted from Rosch et al. [44]. In a 96 well plate, 50 μl of 2% sheep erythrocytes suspended in PBS was added to 50 μl of compounds serially diluted in PBS and incubated at 37°C for 1 hour. The plate was then centrifuged at 500 G for 5 minutes and 50 μl of the supernatant from each well of the assay plate was transferred to a fresh 96 well plate. Hemolysis was confirmed by both visual observation and measuring absorbance at 540 nm. Treatment was conducted in triplicates.

Cytotoxicity assay

The protocol for measuring cytotoxicity was described in Kwon et al. [45]. HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum, 25 mM D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin and maintained at 37°C in 5% CO₂. For the toxicity
test, HepG2 (ATCC HB 8065; ATCC, Manassas, VA, USA) were cultured at 70–80% confluence in 96-well plates in a volume of 100 μl/well culture medium. Serially diluted chemical compounds were incubated with the cells at 37°C in 5% CO2 for 24 hours. Ten microliters of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (WST-1) solution (Roche, Mannheim, Germany) were added per well for the last 4 hours of the 24 hours period. WST-1 reduction was detected using absorbance at 490 nm by a Vmax microplate reader (Molecular Device, Sunnyvale, CA, USA). The percent fluorescence relative to that of the no-treatment control was calculated. The assay was done in triplicate.

Results

Salicylanilide anthelmintic drugs prolong worm survival in the C. elegans-MRSA liquid infection assay

In a previous study, we established a C. elegans-MRSA whole animal liquid infection assay for high throughput screening of chemical libraries to identify anti-infectives that prolong survival of infected worms [21]. The Biomol 4 library comprising of 640 FDA-approved drugs was screened and one of the hits identified was closantel, a salicylanilide veterinary anthelmintic drug used to control nematode and trematode infection of the intestines and liver [46]. The Biomol 4 library also included 13 other non-salicylanilide anthelmintic drugs (S1 Fig), none of which prolonged survival of C. elegans infected with MRSA. Closantel demonstrated good in vitro and in vivo activity against MRSA at a low concentration of 0.78 μg/ml and was only mildly toxic to C. elegans even at a high concentrations (>50 μg/ml). These findings motivated us to investigate other commercially available salicylanilide anthelmintic drugs, such as niclosamide and oxyclozanide (Fig 1) for potential antimicrobial properties. The in vivo antimicrobial activity of niclosamide and oxyclozanide were compared with vancomycin in the C. elegans-MRSA liquid infection assay. Niclosamide and oxyclozanide behaved similarly to vancomycin in prolonging survival of infected worms at the lowest tested concentration of 0.78 μg/ml (Fig 2).

Salicylanilide anthelmintic drugs inhibit Gram-positive bacteria in the ESKAPE panel

The ESKAPE pathogens E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa and Enterobacter spp. are the main causes of nosocomial infections, which are often difficult to treat due to development of antimicrobial resistance [47]. The in vitro antimicrobial activity of niclosamide and oxyclozanide were evaluated using agar disc diffusion and broth microdilution assays (Fig 3 and Table 1). In the case of the S. aureus (MRSA strain MW2), niclosamide

![Chemical structure of the salicylanilide anthelmintic drugs niclosamide and oxyclozanide.](https://doi.org/10.1371/journal.pone.0124595.g001)
Fig 2. Salicylanilide anthelmintic drugs prolong survival of *C. elegans* infected with MRSA. A 384 well assay plate was co-inoculated with nematodes, bacteria and either DMSO (negative control), vancomycin (positive control) or the salicylanilide anthelmintic drugs. Serial dilutions of the drugs were tested to adjust the concentration between 0.78–50 μg/ml. (A) Sytox Orange stained and bright field images of assay wells containing a gradient of the tested drugs. Dead worms take up the vital dye Sytox Orange and fluoresce. (B) Percent survival of infected worms was calculated from intensity of fluorescence measured in each well of the assay plate.

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and oxyclozanide formed large clear zones of growth inhibition and the MICs of niclosamide and oxyclozanide were 0.125 and 0.5 μg/ml respectively and the MBCs for the two drugs were 32 and 2 μg/ml respectively (S2B Fig). For \textit{E}. \textit{faecium}, growth inhibition zones for niclosamide and oxyclozanide appeared somewhat diffused. The MICs of niclosamide and oxyclozanide against \textit{E}. \textit{faecium} were 0.25 and 2 μg/ml respectively and the MBCs of the same drugs were >64 and 32 μg/ml respectively (S2A Fig). The 2 salicylanilide anthelmintic drugs displayed no zones of growth inhibition against \textit{K}. \textit{pneumoniae}, \textit{A}. \textit{baumannii}, \textit{P}. \textit{aeruginosa} and \textit{E}. \textit{aerogenes} and did not inhibit bacterial growth in the broth microdilution assays even at the maximum tested concentration of 64 μg/ml.

\textbf{Niclosamide is bacteriostatic and oxyclozanide is bactericidal against \textit{S}. \textit{aureus}}

An antibacterial compound is generally regarded as bactericidal if the MBC is no more than four times the MIC and it is regarded as bacteriostatic for MBC levels beyond this range [48]. Based on the data presented in the previous section, the MBC/MIC ratios of niclosamide, and oxyclozanide against \textit{S}. \textit{aureus} (MRSA-MW2) were 256 and 4 respectively suggesting that niclosamide is bacteriostatic, whereas oxyclozanide is bactericidal. To further explore the nature of action against MRSA-MW2, a time-kill assay was performed with niclosamide and oxyclozanide at a concentration of 4xMIC. Oxyclozanide was able to kill MRSA completely during a 4 hour exposure period. During the same exposure period, niclosamide only caused a six fold drop in colony forming units (CFU) (Fig 4), showing that niclosamide is bacteriostatic whereas oxyclozanide is bactericidal against \textit{S}. \textit{aureus}.
Oxyclozanide disrupts the S. aureus cell envelope but does not induce cell lysis

In order to investigate the antibacterial mode of action of the 2 salicylanilide anthelmintic drugs, the potential of these drugs to cause disruption of the bacterial cell envelope was examined. MRSA-MW2 cells were exposed to niclosamide or oxyclozanide at concentrations between 1–64 μg/ml and bacterial membrane disruption was evaluated by studying uptake of the DNA staining dye Sytox Green over a period of 30 minutes. In cells treated with oxyclozanide, a dose dependent uptake of Sytox Green was observed as represented by the increase in cellular fluorescence caused by binding of the dye with the bacterial DNA (Fig 5B). However, cells treated with niclosamide showed no uptake of the dye even at a high concentration of 64 μg/ml (Fig 5A), suggesting that the cell envelope remained intact.

To determine if oxyclozanide also induces lysis of MRSA cells apart from causing membrane damage, a bacterial lysis assay was performed according to the protocol detailed in the Methods section. Niclosamide and oxyclozanide were tested at concentrations between 1–64 μg/ml. Cells treated with lysostaphin, a known membrane disrupting compound that causes cell lysis, at the same concentration range were used as positive control, and DMSO treated cells were the negative control. Lysostaphin caused near complete lysis of bacterial cells as early as 15 minutes after the start of the assay as reflected by the relative absorbance dropping nearly to 0. However, the anthelmintic drugs did not cause any reduction of the absorbance of MRSA even after 8 hours of incubation at the maximum tested concentration of
Fig 5. Permeabilization of MRSA cells by niclosamide and oxyclozanide. Time course of membrane permeabilization by oxyclozanide (A) and niclosamide (B) was measured by following the increase in Sytox Green fluorescence ($\lambda_{exc} = 485$ nm; $\lambda_{em} = 530$ nm). The drugs were tested at serially diluted concentrations between 1 to 64 $\mu$g/ml.

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64 μg/ml (data not shown), suggesting that oxyclonanide disrupts the bacterial cell envelope without causing lysis of the cell.

Niclosamide and oxyclonanide do not cause hemolysis

The ability of oxyclonanide to cause disruption of the bacterial cell envelope raises the possibility of a similar activity against mammalian cell membranes. To test this possibility, the ability of niclosamide and oxyclonanide to induce hemolysis of sheep erythrocytes was examined. Sheep red blood cells (RBCs) were treated with serial dilutions of the anthelmintic drugs (0.063–64 μg/ml) for 1 hour. Cells treated with serial dilutions of triton X-100 (0.001–1% solution) were the positive control. Neither of the anthelmintic drugs caused any RBC hemolysis even at the maximum tested concentration of 64 μg/ml (Fig 6), which was also confirmed by measuring absorbance at 540 nm (data not shown). The positive control triton X-100 caused hemolysis at all concentrations starting from 0.008%.

Cytotoxicity of niclosamide and oxyclonanide against HepG2 human liver carcinoma cells

Niclosamide is an FDA approved drug and has been in clinical use for several decades. In the case of oxyclonanide, though the drug has been extensively tested in animals, no information is available on effects in humans. The cytotoxicity of niclosamide and oxyclonanide was evaluated against HepG2, a cell line derived from human liver carcinoma cells. HepG2 cells were treated with serial dilutions of the anthelmintic drug between the concentration range 1–64 μg/ml and cellular viability was measured. The mitochondrial toxin rotenone, which interferes with electron transport chain in mitochondria, was used as the positive control. Cells treated with oxyclonanide were almost 100% viable for drug concentrations up to 8 μg/ml and viability dropped with further increase in concentration. Niclosamide displayed toxicity, 40% of the drug treated cells were viable at the lowest tested concentration of 1 μg/ml and viability dropped even further with increasing drug concentrations (Fig 7).

![Concentration](image-url)
Salicylanilide anthelmintic drugs are effective against drug resistant S. aureus clinical isolates and against staphylococci of different genetic backgrounds.

The antimicrobial activity of niclosamide and oxyclozanide was evaluated against the following drug-resistant clinical staphylococcal isolates: 26 MRSA isolates (methicillin MICs ≥ 16 μg/ml and vancomycin MICs between 1–2 μg/ml), 3 vancomycin resistant isolates (vancomycin MICs ≥ 8 μg/ml), 4 linezolid resistant isolates (linezolid MICs ≥8 μg/ml) and 11 daptomycin resistant isolates (daptomycin MICs ≥4 μg/ml). Niclosamide and oxyclozanide displayed MICs between 0.0625–0.5 and 0.5–2 μg/ml respectively against all tested clinical isolates (data not shown). The antimicrobial activity of niclosamide and oxyclozanide was also evaluated against staphylococci of diverse genetic backgrounds and their in vitro activity was compared with commercially available anti-staphylococcal drugs such as telavancin, oxacillin, vancomycin and daptomycin. Both niclosamide and oxyclozanide were as effective as the commercial anti-staphylococcal drugs and displayed MICs between 0.0625–0.5 μg/ml for both drugs (Table 2).

Discussion and Conclusions

The aim of this study was to investigate the antimicrobial properties of niclosamide and oxyclozanide, which are anthelmintic drugs belonging to the salicylanilide structural family [49]. Niclosamide is primarily used for treating tape worm infection in humans and oxyclozanide is a veterinary drug used for treating fluke infection in ruminants. To our knowledge this is the first study to systematically study the antimicrobial properties of salicylanilide anthelmintic drugs against nosocomial pathogens, including clinical isolates, and also test them in an in vivo infection model. The 2 anthelmintic drugs act specifically against Gram-positive bacteria and were as effective as vancomycin in inhibiting MRSA both in vitro and in the C. elegans whole...
animal model. Though the mode of action of niclosamide against MRSA is unclear, the antimicrobial activity of oxyclozanide potentially involves causing disruption to the bacterial cell envelope. Neither of the anthelmintic drugs was hemolytic against sheep RBCs. Niclosamide and oxyclozanide were further tested for toxicity against mammalian cells. Oxyclozanide was found to be non-toxic within the range of the drug MICs whereas niclosamide displayed some toxicity even at the lowest tested concentration of 1 μg/ml.

Earlier studies have documented the antimicrobial properties of the anthelmintic drug closantel [21, 23]. Macielag et al. demonstrated that closantel inhibits the KinA/Spo0F two-component regulatory system in Bacillus subtilis. In the same study the authors determined the MIC of closantel against S. aureus, E. faecalis and E. faecium to be in the range 0.12–1 μg/ml. The in vivo antimicrobial activity of closantel was also shown by our group in a previous study where we established a C. elegans-MRSA liquid infection assay for high throughput screening of small molecule libraries [21]. In both studies mentioned above, the MIC of closantel was well below that of vancomycin, attesting to the potential importance of this class of anthelmintic drugs as anti-staphylococcal agents, especially in light of the paucity of drugs with novel structural features for treating Gram-positive bacterial infections.

Salicylanilide anthelmintic drugs are in most circumstances consumed orally. There is very little absorption or metabolism of these drugs in the intestine and the systemic circulation of these drugs and their metabolites is also minimal [50, 51]. Since oxyclozanide is used in veterinary medicine, earlier studies have focused mostly on the drug’s pharmacology in ruminants [50]. Niclosamide on the other hand has been used in humans for several decades and detailed studies on the pharmacological profile of this drug are available [51]. In both humans and animals, salicylanilide anthelmintic drugs are extensively plasma bound which would explain the poor distribution in tissues. Human volunteers given an oral dose of 2,000 mg niclosamide eliminated the drug within 2 days. The fraction eliminated in urine was up to 25% and the rest was eliminated with feces. It was also found that the maximal serum concentration of niclosamide was 0.25–6 μg/ml, which is within the concentration range at which niclosamide inhibited growth of S. aureus as determined from the MIC assay (Table 2). However, the low level of systemic circulation coupled with the rapid elimination profile of niclosamide suggests the necessity for further testing of the potential of niclosamide and oxyclozanide for treating systemic infections. Further studies should include the evaluation of these compounds in systemic and localized infection models in rodents and the evaluation of derivative compounds.

Both anthelmintic drugs behaved similarly to vancomycin in prolonging survival of C. elegans infected with MRSA (Fig 2). The C. elegans-S. aureus infection model has been widely used to study staphylococcal virulence and pathogenesis [18, 19, 52–55]. Key virulence factors that are important for staphylococcal pathogenesis in the nematode model are also involved in pathogenesis in humans [18]. Apart from studying pathogen virulence and host response, the nematode infection model can also be used for high throughput screening of chemical libraries, as demonstrated by recent studies carried out with P. aeruginosa and S. aureus [21, 56]. One of the important advantages in the C. elegans infection model is that while testing the antimicrobial properties of a compound, toxicity can be assessed simultaneously since toxic compounds will kill the nematodes [57]. Although it may appear surprising that the anthelmintic drugs being tested were non-toxic to C. elegans, a recent study which compared the effect of different classes of anthelmintic drugs, such as albendazole, ivermectin, pyrantel and nitazoxanide (analogue of niclosamide) on diverse nematodes, reported that only pyrantel was slightly toxic to C. elegans [58].

The anthelmintic drugs demonstrated activity specifically against Gram-positive species from the ESKAPE pathogen panel (Fig 3 and Table 1). Obvious reasons for the difference in activity could be due to structural and molecular differences between the two classes of bacteria.
The action of efflux pumps in transporting the drugs out of Gram-negative bacteria cannot also be ruled out. For example, efflux pumps have been implicated in the resistance of *P. aeruginosa* against tetracycline, chloramphenicol and norfloxacin [59, 60], agents that are quite effective against Gram-positive bacteria such as *S. aureus*. Moreover, the time-kill experiment data together with the MIC and MBC activity against MRSA suggest that niclosamide is bacteriostatic whereas oxyclozanide is bactericidal (Fig 4). Therefore even within susceptible bacteria, the drugs might have different modes of action.

In order to further investigate the mechanism of killing of MRSA by the anthelmintic drugs, their effect on the integrity of the bacterial cell envelope was examined. Oxyclozanide rapidly permeabilized the MRSA cell membrane after a short exposure as seen by the dose dependent increase in fluorescence caused by binding of Sytox Green dye with bacterial nucleic acid (Fig 5). No such observation was made for niclosamide. Although oxyclozanide targets the bacterial cell membrane, it does not cause disintegration of the bacterial cell since treatment of bacterial cells with these drugs resulted in no change in the bacterial density (data not shown). These findings suggest that further studies are needed to identify the exact mechanism of action of salicylanilide anthelmintic drugs against *S. aureus*. It is reasonable to assume that apart from targeting the bacterial cell envelope, oxyclozanide might also have other cellular targets which might also be the targets of niclosamide.

The activity of niclosamide and oxyclozanide against mammalian cell membranes was investigated by studying the hemolytic effect of these drugs on sheep erythrocytes. The positive control Triton X-100 displayed a dose dependent lysis of sheep RBCs, which was confirmed by both visual and spectrophotometric observation (Fig 6). In contrast, neither anthelmintic drug caused hemolysis. These findings are in agreement with earlier studies which indicate that it is not uncommon that compounds targeting bacterial cell membrane need not cause similar damage to mammalian cell membranes [42, 43]. The cytotoxicity of niclosamide and oxyclozanide were evaluated against HepG2 human liver carcinoma cells. Both drugs caused a dose dependent loss in cell viability but the toxicity was greater for niclosamide than oxyclozanide especially at lower concentrations (Fig 7). Similar results were obtained when the toxicity of niclosamide and oxyclozanide were evaluated against HEK293 human embryonic kidney cells (data not shown). It is not surprising that niclosamide appears to be toxic against HepG2 and HEK293 cells as similar observations have been made by other investigators [24, 61, 62]. Niclosamide was shown to block Wnt signaling in HEK293 cells [61]. The same study also reported that niclosamide inhibits Wnt signaling in prostate and breast cancer cells, directly inhibiting cancer cell proliferation and also promoting cancer cell apoptosis at concentrations as low as 0.4 μg/ml. Another study by Tinghong Ye et al. reported the potency of niclosamide against breast cancer both *in vitro* and *in vivo* [24]. Similar to these findings, Minoru Tomizawa et al. reported that niclosamide at a concentration of 0.3 μg/ml inhibited hepatic cancer cell proliferation through inhibition of the Wnt signaling pathway [62]. The findings from our study and the studies mentioned above suggest that niclosamide displays general toxicity to cancer cells and also blocks signaling pathways in other cell lines such as HEK293, which may lead to cytotoxicity. The results of the toxicity assay for oxyclozanide appear to be more interesting since the veterinary drug displayed lesser toxicity than niclosamide when tested on mammalian cells. Toxic effect of oxyclozanide was only seen starting at concentrations 16 fold higher than the MIC range against MRSA (Fig 7). These results do not necessarily indicate the relative safety of oxyclozanide versus niclosamide but suggests that it is worth initiating further studies to explore the possibility of repurposing oxyclozanide as a human drug. The results from this study suggest that there are both similarities and differences in the activities and toxicity profiles of niclosamide and oxyclozanide, not only between the two, but also with structural analogues reported in earlier studies. For example, Hilliard et al. [63] described a salicylanilide analogue...
that had good \textit{in vitro} activity against \textit{S. aureus} and was also capable of causing damage to both bacterial and erythrocyte membranes. However, the same study reported other salicylanilide analogues that were equally effective against \textit{S. aureus} but did not have any effect on bacterial or erythrocyte membranes. The authors of another study \cite{64} tested the effects of 32 salicylanilide derivatives on HepG2 cells and observed varying degrees of toxicities. The findings from our study and earlier studies suggest that niclosamide and oxyclozanide may have more than one mechanism. The relatively low toxicities of niclosamide and oxyclozanide that we observed is consistent with the fact that have been approved as human and veterinary therapeutics, respectively.

In conclusion, the salicylanilide anthelmintic drugs niclosamide and oxyclozanide inhibit growth of the Gram-positive bacteria \textit{S. aureus} and \textit{E. faecium}. Moreover, since oxyclozanide targets the bacterial cell envelope, there is a low propensity of emergence of antimicrobial resistance against this drug. Further studies are needed to study the exact mode of action of these drugs and their pharmacokinetic and pharmacodynamics profiles. Since niclosamide is FDA approved and all of the salicylanilide anthelmintic drugs are already out of patent, they are attractive candidates for drug repurposing and warrant further clinical investigation for treating staphylococcal infections.

**Supporting Information**

\textbf{S1 Fig.} Chemical structures of the non-antimicrobial anthelmintic drugs from the Biomol 4 library. These anthelmintic drugs were present in the Biomol 4 library and they did not prolong survival of infected worms in the \textit{C. elegans}-MRSA liquid infection assay. (TIF)

\textbf{S2 Fig.} Estimation of Minimal bactericidal concentration (MBC) of niclosamide and oxyclozanide. MBC against \textit{E. faecium} (A) and \textit{S. aureus} (B) was estimated by plating 10 \(\mu\)l of bacterial samples from the overnight MIC plates and observing bacterial growth on Müller-Hinton agar plates. (TIF)

**Author Contributions**

Conceived and designed the experiments: RR EM. Performed the experiments: RR WK EJ BK. Analyzed the data: RR BBF ALC FMA EM. Contributed reagents/materials/analysis tools: BBF. Wrote the paper: RR ALC FMA EM.

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