Mechanism of Action and Efficacy of Antibacterial ChemBridge Compounds

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Abstract

Current efforts to develop antibiotics are not keeping up with the need for new antimicrobials due to the increasing prevalence of antibiotic resistance. This is especially problematic for infections with Gram-negative and intracellular bacteria, which are harder for antimicrobials to access due to the structure of the double membrane envelope. One way to combat this issue is to find antibiotics that synergize with the immune insults found within host cells. A screen designed in this manner has uncovered three structurally related antibacterial molecules termed "the Class 1 compounds."

Elucidation of the mechanism of action of these compounds could provide valuable insights about potential new antibiotic targets. Previous work has shown that the Class 1 compounds likely affect the inner membrane. Further investigation has revealed that the compounds disrupt bacterial membrane voltage without permeabilizing the inner membrane, but not to an extent that causes voltage-dependent proteins to be mislocalized. Additionally, the compounds are not effective in a *Galleria mellonella* waxworm infection model and do not affect macrophage cytokine release.

1. Introduction

There is a substantial need for the discovery of new antibiotics that current development efforts are not keeping up with. Early investigators looked to nature to find preexisting antibacterial molecules with great success (Durand et al., 2019). Unfortunately, the utility of this method has been mostly exhausted. New antibiotics developed after the 1980s were mainly analogs of those that already exist, causing resistance to the new drug to develop faster because it has already been selected for due to the use of the parent compound (Durand et al., 2019). Initially, it was believed that there were already enough antibiotics available that resistance would not become a problem; however, the development of antibiotic resistance has been accelerated by overuse in both humans and animals (Årdal et al., 2020). Current estimates predict that antibiotic-resistant infections will cause 10 million deaths in the year 2050 (Kamaruzzaman et al., 2017).

Despite the serious need for new antibiotics, only moderate effort has been put into developing new drugs due to financial and regulatory barriers (Årdal et al., 2020). A single new drug costs hundreds of millions of dollars to develop but has only a 5% chance of succeeding in becoming a clinical antibiotic (Årdal et al., 2018; Payne et al., 2007; Sertkaya et al., 2014). Even those antibiotics that do make it into the clinic are not very profitable due to short treatment courses (Årdal et al., 2020; Holmes et al., 2016). Additionally, new drugs are only used as a last resort in order to slow the development of resistance. Therefore, most large pharmaceutical companies have abandoned antibiotic development altogether, leaving the burden to smaller labs with fewer resources (Årdal et al., 2020).

Treatment of infections caused by intracellular and Gram-negative bacteria poses even greater problems. Bacteria within host cells are harder to treat because the antibiotic must be able to

accumulate to the effective concentration inside the host cells and then inside the bacterial cells, which is inhibited by both host and bacterial efflux pumps and exocytosis (Kamaruzzaman et al., 2017). More than two-thirds of current antibiotics are ineffective against intracellular bacteria (Abed & Couvreur, 2014). This problem is compounded in Gram-negative bacteria due to the second bacterial membrane providing an additional permeability barrier (Zgurskaya & Rybenkov, 2020).

The Gram-negative bacterium *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) serves as a model organism of intracellular infection. This pathogen causes gastroenteritis in most humans and systemic infection in immunocompromised individuals and many animals (Ohl & Miller, 2001). During systemic infection, *S.* Typhimurium often infects macrophages and resides within phagocytic vesicles (Gorvel & Méresse, 2001). This provides an opportunity for antibiotics to synergize with host immune insults encountered within the macrophage, such as antimicrobial peptides, reactive oxygen and nitrogen species, low pH, and nutrient limitation (Pucciarelli & García-Del Portillo, 2017).

An effective way to screen for new antibiotics that synergize with the host immune system is through a Screen for Anti-infectives using Fluorescence microscopy of IntracellulaR Enterobacteriaceae (SAFIRE) (Reens et al., 2018). This assay examines the combined effect of the drug and host immune insults by monitoring *S.* Typhimurium replication within RAW 264.7 macrophage-like cells during compound treatment. In broth, the immune insults encountered within the macrophage phagosome can be mimicked using the cationic antimicrobial peptide polymyxin B (PMB), which increases outer membrane permeability (Pucciarelli & García-Del Portillo, 2017; Sahalan & Dixon, 2008).

A screen of 20,000 molecules from ChemBridge's CombiSet library revealed 445 compounds that reduced *S.* Typhimurium growth in conjunction with PMB but not in broth alone (Ewing, 2023). 83 of the compounds were effective in SAFIRE and minimally toxic to the host cells. These molecules were sorted into 12 classes by chemical structure. Class 1 consists of three lipophilic compounds designated 1.11, 1.12, and 1.13.

Previous work has provided multiple insights into the mode of action of the Class 1 compounds (Ewing, 2023). Notably, it has been shown that the compounds do not permeabilize the outer membrane because they do not enable the membrane-impermeable antibiotic novobiocin to inhibit bacterial growth. Additionally, *E. coli* mutants with increased outer membrane permeability are susceptible to 1.11 and 1.12 in the absence of PMB, and broth conditions that destabilize the outer membrane increase *S.* Typhimurium sensitivity to the Class 1 compounds in the absence of PMB. Bacterial strains with decreased efflux pump capacity are also more susceptible to all three compounds in the absence of PMB. In addition, the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* are sensitive to 1.11 alone. Together, these results suggest that the Class 1 compounds are able to cross the outer membrane unassisted, but not quickly enough to overcome the rate of export via efflux pumps and reach an effective concentration within the bacterial cell. Finally, it has been shown that 1.11 and 1.12 disrupt voltage across the bacterial membrane in the presence of PMB, suggesting that the Class 1 compounds target the inner membrane.

While these past results have unveiled useful information, further investigation is necessary to uncover the mechanism of action of the Class 1 compounds. To this end, the effects of the compounds on bacterial inner membrane permeability and voltage-dependent protein

localization were investigated, revealing that 1.11 and 1.12 disrupt bacterial membrane voltage without permeabilizing the inner membrane, but not to an extent that voltage-dependent proteins are mislocalized. Additionally, it was shown that 1.11 is not effective in a *Galleria mellonella* waxworm infection model and that it does not affect macrophage cytokine release.

2. Materials and Methods

Bacterial strains, growth conditions, and reagents. *Salmonella enterica* serovar Typhimurium strain ATCC SL1344 was used for the propidium iodide, *Galleria mellonella* infection, and cytokine release assays. *Bacillus subtilis* strain DK5092 was used for fluorescence microscopy and the $DiSC_3(5)$ fluorescence assay (Yu et al., 2021). Overnight cultures were grown in LB liquid media at 37 °C. 1.11, the inactive 1.11 isomer, 1.12, and JD1 were purchased from ChemBridge Corporation and resuspended in DMSO. In *S*. Typhimurium with 1 μg/mL PMB, the MIC_{95} of 1.11 is 13.9 μM, the MIC_{95} of 1.12 is 15.1 μM, and the MIC_{95} of JD1 is 14.1 μM. In *B. subtilis*, the MIC_{95} of 1.11 is 50 μM.

Propidium Iodide Assay. Overnight cultures of *S.* Typhimurium were diluted to an OD_{600} of 0.1 and then grown to an OD_{600} of 0.3-0.5 at 37 °C in the presence of 0.75 μg/mL PMB. 1% DMSO, 0.01% SDS, or 0.5X, 1X, or 2X MIC_{95} JD1, 1.11, or 1.12 was then added. Propidium iodide (Life Technologies) was added to 10 μg/mL five minutes before measurements. 5, 10, 15, 30, or 60 minutes after compound treatment, cells were washed twice in 1X PBS and resuspended in PBS. Fluorescence was measured in a 96-well plate using a Synergy H1 plate reader at a 535 nm excitation / 617 nm emission.

Fluorescence Microscopy. Overnight cultures of *B. subtilis* were treated with 0.25% DMSO, 100 μM CCCP, or 50 μM 1.11 for 15 minutes and then washed once in 1X PBS and resuspended to 3X the original density in 1X PBS. Resuspended cultures were mounted on a pad of 1% agarose. Slides were imaged within 30 minutes using an Olympus IX81 widefield microscope with Olympus cellSens Dimension V3.2 64-bit software.

***Galleria mellonella* husbandry.** *G. mellonella* (Carolina catalog number 143928) were kept at 28 °C in the dark (Jorjão et al., 2018; Pereira et al., 2020). They were fed a diet of oats, bran flakes, and honey. Eggs were transferred into a new container once each week.

***Galleria Mellonella* Infection Assay.** Larvae weighing 200±50 mg were selected for testing. *S.* Typhimurium overnight cultures were diluted in phosphate-buffered saline (PBS) to a concentration of approximately 2.5×10^6 bacteria/mL. The right last proleg of each larva was sterilized with 70% ethanol and injected with 10 µL PBS or *S.* Typhimurium. Larvae were incubated at 37 °C in the dark for 2 hours. Then, the left last proleg of each larva was sterilized with 70% ethanol and injected with 1X PBS, 2.3 mg/mL spectinomycin, or 50 mg/kg 1.11 using a 10 µL volume. Larval volume was calculated as previously described (Andrea et al., 2019). 1.11 was diluted in PBS from a 20 µM stock in DMSO. Larvae were incubated at 37 °C in the dark for five days and monitored every 24 hours for survival. Larvae were considered dead when dark brown in color and unresponsive to touch (Ignasiak & Maxwell, 2017; Mil-Homens et al., 2018; Silva et al., 2021; Viegas et al., 2013; Zeng et al., 2020).

Cytokine Release Assay. RAW 264.7 macrophage-like cells in complete DMEM were seeded in a 24-well plate at 500 cells per well and incubated overnight. 0, 5, 25, 50, or 100 µM 1.11 was then added. 2 or 18 hours after compound treatment, the media was aspirated and sent to the University of Colorado Anschutz Human Immunology & Immunotherapy Initiative for analysis using a V-PLEX Proinflammatory Panel 1 Mouse Kit.

$DiSC_3(5)$ Fluorescence Assay. Overnight cultures of *B. subtilis* were diluted to an OD_{600} of 0.1 and then grown to an OD_{600} of 0.3-0.5 at 37 °C. Cultures were then diluted to an OD_{600} of 0.3 and $DiSC_3(5)$ (Invitrogen) was added to a final concentration of 2 µM. Cultures were incubated at 37

°C for 5-10 minutes and then transferred to a 96-well plate. Fluorescence was measured using a Synergy H1 plate reader at 650 nm excitation / 680 nm emission every 50 seconds for 5 minutes, and then 0.25% DMSO, 32 µg/mL gramicidin, or 25 µM, 50 µM, or 100 µM inactive isomer was added. Fluorescence was read at 650 nm excitation / 680 nm emission every 50 seconds for 50 minutes.

3. Results

3.1 Effects of the Class 1 compounds on the bacterial membrane

To further investigate the effect of the Class 1 compounds on the bacterial membrane, permeabilization of the inner membrane during compound treatment was monitored in *S.* Typhimurium with PMB-induced outer membrane permeabilization (Figure 1). Inner membrane permeabilization was quantified using propidium iodide, a cell-impermeable dye that fluoresces upon intercalation with DNA (López-Amorós et al., 1995). As expected, treatment with the detergent SDS resulted in a large increase in the fluorescent signal within 5 minutes, indicating cell lysis. Treatment with 1X and 2X MIC_{95} (minimum inhibitory concentration, the concentration at which 95% of bacterial growth is inhibited) JD1 increased the average fluorescent signal, although these changes were not statistically significant. JD1 is a small molecule previously demonstrated to permeabilize the inner membrane and increase the propidium iodide signal within 30 minutes of treatment with 1X MIC_{95} and 10 minutes of treatment with 2X MIC_{95} (Dombach et al., 2020). Both 1.11 and 1.12 showed smaller average fluorescent signals than JD1, with large increases in fluorescence only occurring at later timepoints with 2X MIC_{95} treatments. 15 minutes of treatment with 0.5X MIC_{95} 1.11 caused a statistically significant increase in the propidium iodide signal; however, this increase was of a smaller magnitude than JD1 and not sustained at the 30- and 60-minute timepoints. Increases in the fluorescent signal did not occur during treatment with 1X MIC_{95} 1.11 or 1.12, showing that the compounds are able to inhibit *S.* Typhimurium growth at concentrations that do not cause inner membrane permeabilization. Therefore, the Class 1 compounds can inhibit *S.* Typhimurium growth through a mechanism other than inner membrane permeabilization.

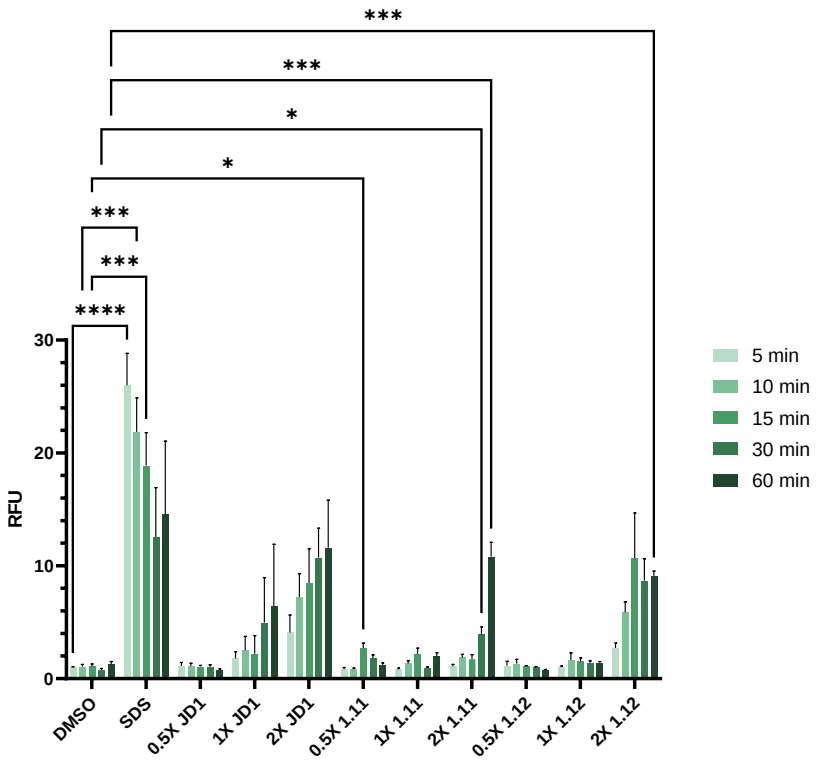

Figure 1 Inner membrane permeabilization is not the primary mechanism of action of the Class 1 compounds. To quantify inner membrane permeability, propidium iodide fluorescence was monitored in the presence of 1% DMSO, 0.01% SDS, and 0.5X, 1X, and 2X MIC_{95} JD1, 1.11, and 1.12. Mean +/- SEM of 3-4 biological replicates normalized to DMSO at 5 minutes. * adjusted $P < .05$, *** adjusted $P < 0.001$, **** adjusted $P < .0001$, two-way ANOVA and Dunnett's posttest, DMSO control versus indicated treatment at the same timepoint. Data collected in collaboration with Dr. Samual Allgood; 1.11 data collected by Dr. Donald Evans.

In order to explore the possibility that the membrane depolarization caused by 1.11 could be sufficient to inhibit bacterial growth, localization of the voltage-dependent cell cycle protein FtsZ was observed during treatment of *B. subtilis* with 1.11. FtsZ depends on membrane voltage to localize to the cell septum, where it polymerizes into a ring structure (the Z-ring) and initiates cytokinesis (Strahl & Hamoen, 2010). Mislocalization of the Z-ring results in defects in daughter cell size and chromosome segregation. *B. subtilis* was used for observation of FtsZ localization instead of *S.* Typhimurium because *B. subtilis* is more amenable to imaging due to its large size (Graumann, 2012). Additionally, *B. subtilis* is Gram-positive, allowing the Class 1 compounds to cross the membrane without the assistance of PMB. *B. subtilis* cells with a chromosomal FtsZ-

mNeonGreen fusion were imaged after 15 minutes of treatment with DMSO, CCCP, or 1X MIC_{95} 1.11 (Figure 2). While most CCCP-treated cells contained mislocalized FtsZ, as expected, normal Z-rings were observed in the majority of cells in both the DMSO and the 1.11 conditions, indicating that 1.11 does not cause defects in Z-ring localization.

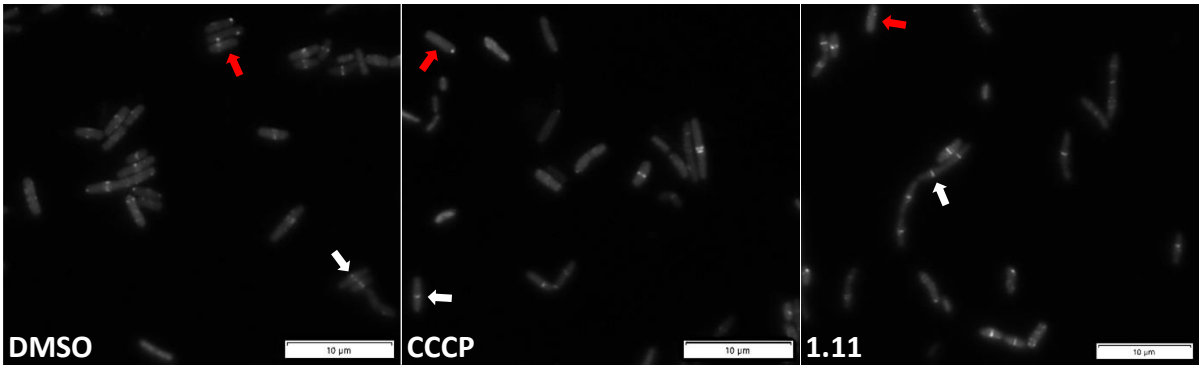

Figure 2 Treatment with 1.11 does not cause voltage-dependent FtsZ mislocalization. Representative images for treatment with 0.25% DMSO (n = 405 cells), 100 µM CCCP (n = 368 cells), or 1X MIC_{95} 1.11 (n = 458 cells). White arrows indicate examples of bacteria with intact Z-rings; red arrows indicate examples of bacteria with disrupted Z-rings.

3.2 Effects of the Class 1 compounds on the host

In order to investigate the efficacy of 1.11 *in vivo*, *Galleria mellonella* (greater wax moth) larvae were used as an animal infection model because the *Galleria mellonella* and human innate immune responses are highly similar (Pereira et al., 2020). Larvae were infected with *S.* Typhimurium (infected) or a mock infection control (uninfected) and then treated with PBS, spectinomycin, or 1.11 2 hours post-infection (Figure 3). As expected, all uninfected larvae treated with PBS or spectinomycin as well as all infected larvae treated with spectinomycin survived for all 5 days of observation. Additionally, infected larvae treated with PBS showed the expected die-off over the course of observation. 5 of the 6 uninfected larvae treated with 1.11 survived the entire observation period, indicating that 1.11 is minimally toxic to the host;

however, only 1 of the 6 infected larvae treated with 1.11 survived to day 5, indicating that 1.11 does not increase survival of *S*. Typhimurium infection.

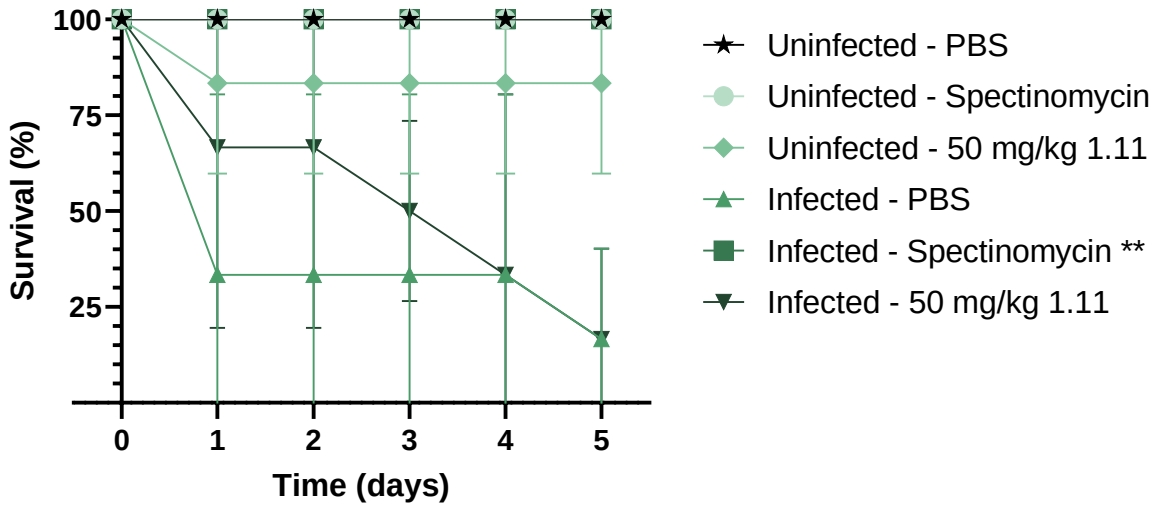

Figure 3 1.11 does not increase *Galleria mellonella* larvae survival of *S.* Typhimurium infection. Larvae were infected with 2.5×10^4 *S*. Typhimurium (infected) or a PBS control (uninfected) and then treated with PBS, spectinomycin, or 50 mg/kg 1.11 2 hours post-infection. n = 6 larvae per condition. ** $P < 0.01$, log-rank (Mantel-Cox) test, uninfected - PBS versus uninfected - indicated treatment or infected - PBS versus infected - indicated treatment.

To determine whether the failure of 1.11 to rescue *Galleria mellonella* survival was due to differences in the responses to 1.11 between mammalian phagocytes, such as macrophages, and the analogous cells in *Galleria mellonella* (hemocytes), the effect of 1.11 on RAW 264.7 macrophage-like cells was examined. The extracellular levels of 10 cytokines were quantified during 1.11 treatment in order to determine if 1.11 activates the cells (Figure 4). None of the extracellular cytokine levels were altered to a biologically relevant degree, suggesting that 1.11 does not affect the activation state of the cells.

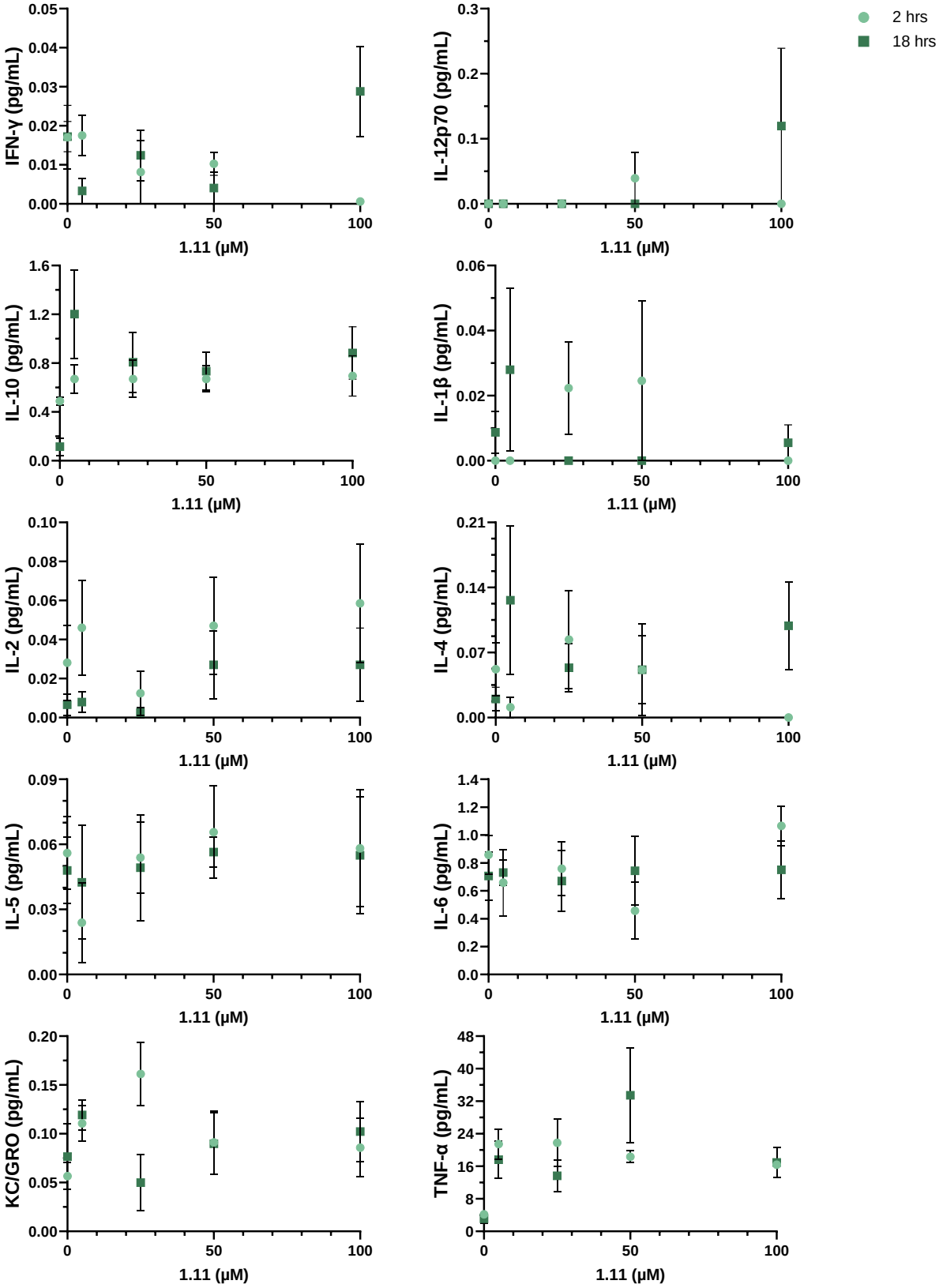
2 hrs
18 hrs
IFN-γ (pg/mL)
IL-12p70 (pg/mL)
IL-10 (pg/mL)
IL-1β (pg/mL)
IL-2 (pg/mL)
IL-4 (pg/mL)
IL-5 (pg/mL)
IL-6 (pg/mL)
KC/GRO (pg/mL)
TNF-α (pg/mL)
1.11 (μM)

Figure 4 1.11 does not activate RAW 264.7 macrophage-like cells. Extracellular cytokine levels were quantified after 2 or 18 hours of treatment with 1.11. Mean +/- SEM of three biological replicates with two technical replicates. ns, one-way ANOVA and Dunnett's posttest, 0 μM 1.11 versus 100 μM 1.11 at 18 hrs. Data collected in collaboration with Dr. Samual Allgood.

3.3 An inactive isomer of 1.11 disrupts bacterial membrane voltage

A recent shipment of what was thought to be 1.11 demonstrated no activity against *B. subtilis* and *E. coli* strains previously shown to be susceptible to treatment with 1.11 (Ewing, 2023). However, this compound retained the ability to disrupt voltage across the bacterial membrane at high concentrations. Further investigation into this compound revealed that it is an isomer of 1.11 (Figure 5A). In order to quantify whether the inactive 1.11 isomer depolarizes the bacterial cell membrane of *B. subtilis*, 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) fluorescence was quantified during treatment of *B. subtilis* with the inactive isomer (Figure 5B). $DiSC_3(5)$ is a self-quenching fluorescent dye that accumulates in the cell membrane when the voltage across the membrane is normal (Singh & Nicholls, 1985). When membrane voltage is disrupted, $DiSC_3(5)$ is released, allowing it to fluoresce. Treatment of *B. subtilis* with gramicidin in the presence of $DiSC_3(5)$ caused an immediate increase in $DiSC_3(5)$ fluorescence, as expected. Treatment with 0.5X or 1X $MIC_{95\ (1.11)}$[1] inactive isomer did not cause an increase in fluorescence greater than that of DMSO treatment; however, an increase was observed in the 2X $MIC_{95\ (1.11)}$ inactive isomer treatment. Previous results in *S.* Typhimurium demonstrated that 1.11 increases $DiSC_3(5)$ fluorescence at 1X and 2X MIC_{95}, but not 0.5X MIC_{95} (Ewing, 2023). The similarity between the abilities of the inactive isomer and 1.11 to disrupt membrane voltage suggests that 1.11 has a mode of action beyond this mechanism.

[1] MIC_{95} of 1.11 in *B. subtilis*

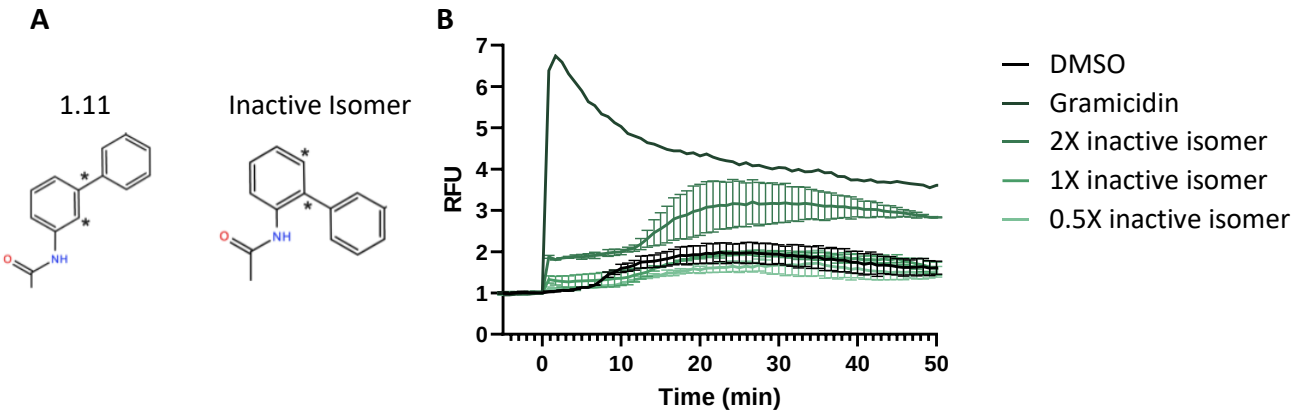

Figure 5 An inactive isomer of 1.11 disrupts membrane voltage. **(A)** Structures of the altered portion 1.11 and the inactive 1.11 isomer. Asterisks indicate altered carbons. **(B)** Treatment with the inactive isomer causes an increase in $DiSC_3(5)$ fluorescence. To monitor membrane voltage, $DiSC_3(5)$ fluorescence was measured for 50 minutes of treatment with 0.25% DMSO, 32 µg/mL gramicidin, or 25 µM, 50 µM, or 100 µM (0.5X, 1X, or 2X $MIC_{95\ (1.11)}$) inactive isomer. Compounds were added at Time 0. DMSO and inactive isomer, mean +/- SEM of 2 biological replicates; gramicidin, 1 biological replicate.

Discussion

Although 1.11 was found to be ineffective at increasing *Galleria mellonella* larvae survival of *S.* Typhimurium infection, determining the mechanism of action of the Class 1 compounds will still be useful in the effort to develop new antibiotics. If the Class 1 compounds are found to have a mechanism of action distinct from antibiotics currently available, targeted screens for other molecules with this mechanism of action could be conducted. Additionally, medicinal chemistry could be used to create a 1.11 analog that has efficacy in the host.

In order for these possibilities to be explored, the mechanism of action of the Class 1 compounds must first be determined. Previous work showing that 1.11 and 1.12 disrupt voltage across the inner membrane, along with the lipophilicity of these compounds, suggests that the Class 1 compounds target the cell membrane (Ewing, 2023). Because the Class 1 compounds only inhibit *S.* Typhimurium growth under conditions that permeabilize the outer membrane, the compounds likely target the inner membrane. The results of the propidium iodide assay showing that 1.11 and 1.12 do not permeabilize the bacterial inner membrane at concentrations that inhibit bacterial growth suggest that the Class 1 compounds have a more subtle mechanism of action than permeabilization. It is possible that the disruption of membrane voltage is sufficient to inhibit bacterial growth; however, this is unlikely because the voltage disruption was not found to be sufficient to disrupt FtsZ localization and the inactive isomer causes voltage disruption without inhibiting bacterial growth. The observation that a highly similar isomer of 1.11 does not retain any antibacterial activity suggests that 1.11 binds to a specific target, as opposed to the possibility that it nonspecifically interacts with the inner membrane. Recent work showing that

it is difficult to generate mutants resistant to 1.11 suggests that this target is a lipid or an essential protein, as these are unlikely to become mutated (Ewing, 2023).

More experiments are necessary in order to determine the target of the Class 1 compounds. First, the inactive 1.11 isomer will be examined using SAFIRE. It is possible that this isomer will have an effect within the host cells, despite its ineffectiveness in broth conditions used to imitate the macrophage environment. If the isomer does not decrease the bacterial load in SAFIRE, it will suggest that 1.11 has a target that the isomer cannot bind, but if the isomer decreases bacterial load in SAFIRE, it will invalidate this hypothesis and suggest that 1.11 is more nonspecific. Another avenue currently being explored is a library of essential gene knockdowns in *B. subtilis* (Ewing, 2023). If any of the strains in this library are sensitive or resistant to the Class 1 compounds, it would suggest a possible target protein or pathway. Examinations of strains with lipid knockouts or depletions could be used to complement this screen (Schäfer & Wenzel, 2020). Finally, transcriptomics or proteomics could be used to analyze the bacterial response to the Class 1 compounds, but the tendency of these methods to reveal changes in the gene expression of a large volume of pathways that may not be related to the primary effect of the compound decreases their utility.

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