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An agonist of the MscL channel affects multiple bacterial species and increases membrane permeability and potency of common antibiotics

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Summary

The bacterial MscL channel normally functions as an emergency release valve discharging cytoplasmic solutes upon osmotic stress. The channel opens and passes molecules up to 30 Å and its pore is the largest of any gated channel. Opening the MscL pore inappropriately is detrimental to the bacterial cell, suggesting MscL as a potential novel drug target. A small-molecule compound, 011A, has been shown to increase sensitivity of the *Escherichia coli* MscL channel, slow growth, and even decrease viability of quiescent cultures. The *mscL* gene is highly conserved and found in the vast majority of bacterial species, including pathogens. Here we test the hypothesis that 011A can influence the growth and viability of other bacterial species, specifically *Staphylococcus aureus* and *Mycobacterium smegmatis*, in a MscL-dependent manner. Furthermore, we demonstrate that the 011A compound can increase potency of other antibiotics, presumably by permeabilizing the membrane and allowing easier access of the antibiotic into the cytoplasm. Thus, MscL activators have potential as novel broad-spectrum antibiotics or adjuvants that work with antibiotics to selectively allow passage across bacterial membranes.

Introduction:

Mechanosensitive (MS) channels serve the physiological role of biological emergency-release valves for bacteria, relieving high cell turgor caused by sudden extracellular decreases in osmolarity (Booth & Blount, 2012). When exposed to high osmolarity, bacteria transport several select molecules such as K^+, glutamate, proline, betaine and other polyamines, and synthesize others such as glutamate, trehalose and proline to keep their

cell turgor high, a requisite for cell growth and division (Walter, 1924, Christian & Scott, 1953, Scott, 1953). When the osmotic environment acutely decreases, water rushes in, turgor increases, and the cell integrity is threatened. Bacteria avoid such catastrophe by activation of membrane-tension-gated MS channels. There are two families of bacterial MS channels, MscS and MscL, and of these, MscL (mechanosensitive channel of large conductance) is the most conserved, the least sensitive to membrane tension, and is the last-ditch effort for bacterial survival. MscL has the largest gated pore of any known channel, about 30 Å in diameter (Cruickshank *et al.*, 1997). MscL is found in the vast majority of bacteria, and a few fungi, but not in mammals. While this channel is primarily selective for size, it has a slight preference for anions (Yang & Blount, 2011) and is expressed in tens to over a thousand copies per cell depending upon the cell conditions and the assay used for the measurements (Bialecka-Fornal *et al.*, 2012). MscL is truly the ultimate release valve – studies in *E. coli* have demonstrated it effects osmotic-dependent permeability to K^+, amino acids, and even some select proteins (Blount *et al.*, 1997, Ou *et al.*, 1998, Ajouz *et al.*, 1998a, Ajouz *et al.*, 1998b, Berrier *et al.*, 2000).

Early studies by us and others demonstrated that if the *E. coli* MscL channel gates inappropriately, it is detrimental to cell growth (Blount *et al.*, 1996, Maurer & Dougherty, 2003, Ou *et al.*, 1998). These and other findings suggested that MscL could be a drug target to generate new antibacterial compounds. More recently, we have found that some common antibiotics appear to use MscL as a major pathway for entry into the bacterial cell (Iscla *et al.*, 2014); the best studied of these is dihydrostreptomycin (DHS), for which the binding site within the pore of the MscL channel has been defined (Wray *et al.*, 2016). These findings further suggested that MscL agonists could increase the potency of antibiotics by permeabilizing the bacterial cell membrane and thus allow easier entry, thus serving as an adjuvant.

Our knowledge of the physiology of the MscL channel leads to predictions for the properties of activators of this channel. Because of the conserved nature of the MscL

protein, we predicted that an agonist of MscL should not be specific for *E. coli*, but also affect other bacterial species, including gram positive organisms. This would include not only decreasing cell growth, but also decreasing viability of stationary/quiescent cultures. However, given differences in bacterial cell walls and membranes, this would need to be tested empirically. In addition, because of its pore size, MscL activators would be predicted to allow a diverse array of antibiotics access to the cytoplasm by permeabilizing the membrane, thus increasing their potency. To date, only one specific MscL modulator that directly binds to and modulates the MscL protein has been found, coined 011A (Wray *et al.*, 2019). This compound gives us a unique tool for assessing the validity of these hypotheses. Here, using 011A we find that other bacterial species are sensitive to the compound in a MscL-dependent manner – showing slowed growth and decreased viability when quiescent cells are treated. In addition, 011A does increase the potency of common antibiotics (see **Fig. S1** for structure of 011A and antibiotics used in this study). These data suggest that MscL is indeed a viable drug target, and compounds that specifically modulate the channel at high potency and efficacy could be candidates for novel antibiotics or adjuvants for increasing potency of other antibiotics.

Results:

Two model Gram-positive organisms, *Staphylococcus aureus* and *Mycobacterium smegmatis* show slowed growth when exposed to the 011A compound

Since MscL is a very conserved protein one of the predictions of an agonist to MscL is that it should work across species, including pathogens and Gram-positive organisms (see (Maurer *et al.*, 2000, Iscla *et al.*, 2008, Iscla *et al.*, 2007) for discussions of domain conservation and see **Fig. S2**). To test this, we initially used a previously characterized strain of *Staphylococcus aureus* (*S. aureus*), R4220, in which a MscL-null strain had been generated and characterized (Kouwen *et al.*, 2009). Beyond availability, we chose this organism

because methicillin-resistant *S. aureus* (MRSA) is a chronic problem in hospital infections and results in almost half of all deaths caused by antibiotic resistant organisms (Siddiqui & Koirala, 2018). As seen in **Fig 1A**, a decrease in overnight growth of cultures treated with 011A was observed in the parental R4220 strain, but not in the MscL-null strain. The efficacy for decreasing growth with expression of endogenous levels of *S. aureus* MscL protein (Sau-MscL) is reminiscent of over-expression of gain-of-function (GOF) mutations of the *E. coli* MscL protein (Eco-MscL) channel in *E. coli* (Ou *et al.*, 1998), and slightly better than that previously published for 011A treatment of an *E. coli* strain overexpressing wild type Eco-MscL (Wray *et al.*, 2018).

We next investigated, *Mycobacterium smegmatis* (*M. smegmatis*), which is within the same genus, and is a model system for, *M. tuberculosis* (Shiloh & Champion, 2010). We thus generated a MscL null strain of the *M. smegmatis* MC2155 strain for these experiments, MC2155 Δ*mscL*. As with the *S. aureus* strain, and as seen in **Fig 1B**, after treatment with 011A, a decrease in growth was observed in the parental MC2155 strain, but not in the MscL-null strain. This decrease was larger than that observed for most Eco-MscL GOF mutations (Ou *et al.*, 1998), that previously published for 011A treatment of an *E. coli* strain overexpressing Eco-MscL (Wray *et al.*, 2018), and even for the *S. aureus* R4220 strain above. These results strongly suggesting that the mechanism of 011A is through MscL activation and that it is effective in different species.

The 011A compound decreases viability of quiescent cells for both *S. aureus* and *M. smegmatis*

The ability to kill quiescent cells could have medical relevance. Essentially all current antibiotics require the cells to be metabolically active to be effective, thus making biofilms like those seen with *S. aureus* infections (Moormeier & Bayles, 2017) and “dormant” infections as seen with *M. tuberculosis* (Russell *et al.*, 2010, Bacon *et al.*, 2014) difficult to

treat. However, MscL is expressed in all phases of growth, and even upregulated in stationary cells (Stokes *et al.*, 2003), and because MscL is a channel, not a pump, it does not require an energy source or metabolic activity to gate – only a stimulus. In a previous study, we demonstrated that 011A decreased the viability of stationary *E. coli* cells expressing Eco-MscL (Wray *et al.*, 2019). We therefore tested the viability of both *S. aureus* and *M. smegmatis* when treated with compound 011A after the cultures achieved a stationary/quiescent phase. As seen in **Fig 2**, both species were sensitive to 011A even after growth ceased. This sensitivity was MscL specific; cells null for MscL were not sensitive to 011A treatment. Consistent with findings for *E. coli* (Wray *et al.*, 2019), re-expression in the null cell re-instated sensitivity to 011A for the *M. smegmatis* strain (**Fig. S3**). Collectively, these data suggest that McsL agonists can be cidal for quiescent cells.

Compound 011A facilitates the entry of dihydrostreptomycin (DHS) into the *E. coli* cytoplasm

MscL opens a very large channel upon activation, and in theory could allow cytoplasmic access for common antibiotics by essentially permeabilizing the membrane. As a first assay to determine if 011A could accelerate the passage of known antibiotics into the cytoplasm, we tested if it could enhance DHS entry into the cell. A sub-threshold concentration (just below the MIC) of DHS was used in the presence of increasing concentrations of 011A; a small but measurable amount of decreased growth was observed (**Fig S4**).

Encouraged by this result, we further tested for the 011A-dependent increased passage of DHS through the MscL pore using molecular dynamic (MD) simulations. We modified a previously used assay (Wray *et al.*, 2019) by reducing the external electric field strength (EEF) by 50% to slow down the process and approach a more physiological condition. We positioned the 011A ligand at its best docking pose, while DHS was positioned in its binding location (Wray *et al.*, 2016). As shown in **Fig. S5A**, the distance between the coordinate

center of DHS and the coordinate center of five K106 residues are applied to describe the passing through process. When distance becomes zero, we assume that DHS passes through the channel. In total, we performed 36 sets of simulations, with and without the 011A ligand bound to Eco-MscL. The distance versus simulation time for a representative MD run is shown in **Fig. S5B**, and the dynamics of the radius of the cycle formed by the five K106 residues along the MD simulation is illustrated in **Fig. S5C**. The radius between these lysines becomes smaller when the MscL channel is partially open, consistent with the tilting of the transmembrane domains and the proposed iris-like opening of the channel (Perozo *et al.*, 2002). We performed statistics on the four outcomes of the virtual passage experiment. As shown in **Fig. 3**, the presence of 011A increased the probability of DHS passing through the pore 3-fold. We also calculated the mean distances of the 36 MD simulation runs as a function of simulation time for both the 011A bound and 011A absent scenarios. The shorter the distance, the closer DHS passes through the channel. As shown in **Fig. S6**, the distances between the coordinate centers of DHS and the five K106 residues decrease faster for the 011A bound scenario (the red curve). Overall, these data strongly suggest 011A enhances passage of DHS through the MscL channel.

Finally, we studied the ability of 011A to stabilize a partially-open Eco-MscL channel. For each scenario (with and without 011A) one MD snapshot was randomly selected as DHS was about to exit the Eco-MscL channel. We then ran 120 nanosecond MD simulations for both cases. The plots of the root mean square deviation (RMSD) of the main chain atoms versus the simulation time are presented in **Fig. S7,** where it is shown that 011A stabilizes the partially open Eco-MscL channel.

Together, these data suggest that the binding of 011A to MscL can increase the probability of DHS entering the *E. coli* cell in an Eco-MscL-dependent manner and predicts that this may be true for other common antibiotics and for other bacterial species.

Compound 011A can be used as an adjuvant to increase the potency of common antibiotics by specifically permeabilizing the bacterial membrane

The MscL channel is one of the primary pathways for entrance of DHS to the bacterial cell; i.e. DHS can open the Eco-MscL channel (Wray *et al.*, 2016), thus complicating the experiments discussed above and shown in **Fig S4**. However, the *Hemophilus influenza* MscL (Hin-MscL) does not bind well to DHS (Wray *et al.*, 2016), thus allowing us to use a higher sub-threshold concentration of DHS (2.25 rather than 0.5 μM) in the experiment. As seen in **Fig 4A**, combinatorial treatment (2.25 μM DHS and varying concentrations of compound 011A) of *E. coli* cells expressing Hin-MscL led to much larger decreases in growth than that observed with treatment of compound 011A alone. Viability was also tested of three conditions: 011A (80 μM), DHS (2.25 μM) and a combination of the two at these concentrations, for both empty plasmid and Hin-MscL. There was no reduction in viability with DHS alone, and a significant but modest reduction for 011A alone. However, the combination of the two showed a greater than 90% reduction in viability for the Hin-MscL expressing bacteria (**Fig S8)**.

We next tested to see if 011A could increase the potency of a sub-threshold concentration of the antibiotic kanamycin (Kan) (**Fig 4B**). Again, when 011A was used in combination with Kan, a much larger reduction in growth was observed in a MscL-dependent manner. Since both DHS and Kan are in the aminoglycoside family of antibiotics, we therefore assayed antibiotics of different classes to evaluate if MscL could grant access to the cell to various types of antibiotics. As seen in **Fig 4C**, a sub-threshold concentration of tetracycline (Tet) also showed increase in efficacy with 011A in this assay. Together, these data suggest that 011A can be used as an adjuvant, permeabilizing the membrane and allowing antibiotics access to the *E. coli* cytoplasm.

Ampicillin (Amp) does not require cytoplasmic access but compromises the cell wall integrity, and thus we speculated that even subthreshold concentrations of Amp could potentially increase 011A efficacy, and we wanted to test this. Since our plasmid, pB10d,

confers resistance to ampicillin (Amp), in order to test this antibiotic, we modified our assay by utilizing null strains, and the endogenously-expressing parental strain. We have previously shown that either expression in trans or endogenous expression of Eco-MscL is necessary and sufficient to observe 011A effects (Wray *et al.*, 2018). Indeed, 011A efficacy was increased, even for endogenous levels of MscL (**Fig 4D**).

Finally, we also looked at whether interactions between 011A and other antibiotics would be plausible with other species by using the *S. aureus* and *M. smegmatis* strains described. Because Tet appeared to be the most tractable of the drugs explored above, we determined appropriate threshold concentrations of Tet for these species and used it in combination with the optimal concentration of 011A (see **Fig 1**). As seen in **Fig 5**, both species showed interactions between compounds, with *S. aureus* showing the best adjuvant properties for the 011A compound. The finding that *M. smegmatis* did not show as much interaction between 011A with Tet may be because of the slow-growth nature of this species. Regardless, collectively, the data demonstrate interactions between the 011A compound MscL-specific compound and common antibiotics for multiple species.

This permeabilization of the cytoplasmic membrane appears to be specific for bacterial strains expressing MscL. The MscL channel is not found in mammals, so unless 011A works through alternative modes of action, it should not be toxic for mammalian cells. As a first test of this, we assayed toxicity of the mammalian cell line HEK293, which showed no indication of decreased viability or membrane permeabilization even with concentrations as high as 100 µM for as long as 48 hours, as assayed using trypan blue (**Fig S9**). Hence, the plasma membrane permeabilization appears to be bacterial-specific, and thus far no secondary or unwanted mechanisms of action have been found, giving promise that obtaining specificity of MscL-modulating compounds is achievable.

Discussion

The gene encoding the MscL channel, *mscL*, has been observed in the vast majority of bacterial species. In addition, when looked for electrophysiologically, either by heterologous expression in *E. coli* (Moe *et al.*, 1998, Folgering *et al.*, 2005, Moe *et al.*, 2000) or in their native environment (Cetiner *et al.*, 2017, Rowe *et al.*, 2013, Szabo *et al.*, 1992, Szabo *et al.*, 1993), MscL activity is invariantly observed. Given the highly conserved nature of the MscL protein and its activity, it seems likely that the newly discovered 011A compound would effect changes in MscL activity in various bacterial species when bound. Indeed, we have previously shown that a variety of MscL orthologues heterologously expressed in a ΔEco-MscL *E. coli* strain effected a 011A-specific slowed growth similar to that observed with Eco-MscL expression. However, different bacterial species have diversity in their membranes, cell walls and compensatory mechanisms that could conceivably lead to resistance to the 011A compound. Here we show that two other unrelated species also show sensitivity to 011A in a MscL-dependent manner, both in their growth rate and their viability when treated in stationary phase. It thus seems likely that 011A, and compounds that may be related, will affect much of the Monera Kingdom, including several pathogens.

While the potency of the 011A compound is not too unreasonable, the efficacy is low for a stand-alone antibiotic. Perhaps this is not too surprising given that previous studies have shown that the bulk of gain-of-function mutations and post-translational modifications of MscL effect relatively weak slow-growth phenotypes (Bartlett *et al.*, 2004, Iscla *et al.*, 2015, Ou *et al.*, 1998). Thus, while it is a worthwhile endeavor to obtain a MscL agonist that is both potent and high efficacy, it may be difficult to perform. On the other hand, while the 011A compound appears to be largely static in growing *E. coli* cells, it has cidal activity when quiescent cells are treated (Wray *et al.*, 2019). We suspect this is because metabolizing cells quickly accommodate cytoplasmic losses, while quiescent cells accumulate these losses to the point where, when environmental conditions are appropriate, they are no longer able to recover to a growth state. In addition to potentially treating non-growing biofilms or nodules,

another viable medical use of MscL agonists could potentially be to serve as adjuvants for conventional antibiotics.

The use of adjuvants is considered a fundamental tool in the mitigation of the antibiotic resistance crises (Wright, 2016, Gill *et al.*, 2015). Interestingly adjuvants have also been shown to reduce the resistance mechanism itself (Gill *et al.*, 2015). Several mechanisms are known including inhibiting the pumping-out or degradation of the antibiotic, interfering with the virulence or host responses, or as in the case of 011A, increasing permeability to allow antibiotics to more easily pass into the bacterial cell.

The MscL channel opens the largest gated pore presently known, serving as an osmotic emergency release valve that normally jettisons osmoprotectants including potassium, glutamate, polyamines, as well as other compounds from the cell (Booth & Blount, 2012). Because of its relative lack of selectivity, except for size, it seemed likely that when open, it could pass non-physiological compounds as well. Indeed, researchers have speculated and studied its potential to be used to generate “smart” contrast agents for MRI, drug delivery devices, and even a way to get compounds or drugs into eukaryotic cells (Yang *et al.*, 2018, Iscla *et al.*, 2013, Kocer, 2010, Pacheco-Torres *et al.*, 2015, Doerner *et al.*, 2012). Thus, in addition to the potential as a new class of antibiotics, compounds that gate MscL may specifically permeabilize bacterial cells, and thus allow compounds, such as conventional antibiotics, to more easily cross the membrane. The data presented here demonstrating interactions between 011A and several conventional antibiotics support such a prospect.

Permeabilization of bacterial membranes by MscL agonists could potentially help with the growing bacterial pathogen drug-resistance crisis. Resistance due to multidrug efflux pumps may be shunted by 011A-like compounds that allow free passage to the cytoplasm. In addition, MscL-modifying compounds could be used with potential antibiotics – e.g. bacterial metabolic inhibitors or activators - that are effective in *in vitro* assays, but do not effectively cross the membrane. On the other hand, antibiotics that cross the membrane

easily but cause significant side effects could be modified so they no longer cross the membrane (e.g. addition of a charge), and used alongside a MscL-opening compound like 011A, and the modified antibiotic would have cytoplasmic access to only bacterial cells, thus potentially decreasing or eliminating side effects. Finally, the observation that they work on quiescent/stationary cells gives promise for treatment of hard-to-treat infections including tuberculosis and biofilms. In summary, MscL agonists are not only potential novel antimicrobials, but they may also serve as adjuvants that work in combination with other antibiotics and be advantageous in otherwise intractable infections.

Experimental Procedures:

Strains and Cell Growth

The *M. smegmatis* MC2155 Δ*mscL* strain was generated *from Mycobacterium smegmatis* MC2155 (ATCC® 700084). Mycobacterial recombineering (van Kessel & Hatfull, 2007) approaches were used to create this strain, with a few modifications. Galactokinase (*galK*) from plasmid pDB88 (Barkan *et al.*, 2011), was inserted 66bp upstream of the levansucrase gene (*SacB*) in plasmid pYUB1471 (Jain *et al.*, 2014) using primers pGK_F and pGK_R to amplfy *galK* and primers pNH01GK_F and pNH01GK_R to PCR amplify the vector backbone, the plasmid was assembled using the Clontech In-Fusion HD kit.

To create the targeting plasmid 600bp regions encoding sequences upstream and downstream of the *mscL* gene in *M. smegmatis* MC2155 were amplified from *M. smegmatis* MC2155 genomic DNA. Using primers pMscLU_F and pMscLU_R to amplify the upstream region and primers pMscLD_F and pMscLD_R to amplify the downstream region (see Table S1 for list of primers used). The targeting plasmid was constructed in two steps: 1) Insertion of the upstream region by digesting the plasmid with NdeI and XhoI and using the Clontech In-Fusion HD kit, 2) Insertion of the downstream region by digesting the result from step 1 with NdeI and BamHI and using the Clontech In-Fusion kit to ligate the plasmid. Linearized

DNA for recombineering was made by digesting the final plasmid with NdeI/NcoI and gel purifying the targeting DNA.

100 ng of targeting DNA were electroporated into *M. smegmatis* MC2155 expressing Che 60 and 61 proteins in the pJV53 plasmid (van Kessel & Hatfull, 2007). The reaction was plated on 7H10 agar containing 150 µg/mL hygromycin. Resulting colonies were plated onto 7H10 agar containing either 150 µg/mL hygromycin (hyg) or 0.25 % 2-deoxy-galactose (dog) + 10 % sucrose (suc). Colonies surviving on hyg plates but not dog + suc were selected for unmarking. Unmarking was completed using bacteriophage pHAe 280 (Jain *et al.*, 2014). After bacteriophage infections, unmarked knockouts survived on dog + suc but not hyg. Colonies were confirmed by colony pCR using primers pCPCR_F and pCPCR_R.

M. smegmatis MscL was expressed in the Δ*mscL* MC2155 strain using plasmid pNH02. pNH02 was built using the pMyNT plasmid backbone (Addgene plasmid #42191), a mycobacterial expression vector with a hygromycin selection cassette an acetamidase promoter. In brief, pMyNT was digested with NcoI at 37 °C for 4 hours and gel purified. *M. smegmatis mscL* was amplified from genomic DNA by PCR using primers MsMscL_F and MsMscL_R, the reactions were gel purified. Following gel purification, the insert was ligated to the plasmid backbone using the Clontech In-Fusion HD kit.

S. aureus R4220 and R4220 Δ*mscL* (Kouwen *et al.*, 2009), *M. smegmatis* MC2155 and MC2155 Δ*mscL*, along with *E. coli* MJF367 (Δ*mscL*::Cam), MJF451 (Δ*mscS*) (Levina *et al.*, 1999) were used; note that only endogenous expression levels (not overexpression) was studied in these strains. *E. coli* MJF455 (Δ*mscL*::Cam, Δ*mscS*) (Levina *et al.*, 1999) cell line was either used alone for endogenous expression or to host the pb10 expression constructs, as noted in text. *E. coli* strains were inoculated from a single colony and were grown in citrate-phosphate-defined media (CphM) pH 7.0, consisting of per liter: 8.57 g of Na_2HPO_4, 0.87 g of K_2HPO_4, 1.34 g of citric acid, 1.0 g NH_4SO_4, 0.001 g of thiamine, 0.1 g of MgSO47H2O, 0.002 g of (NH_4) $2SO_4.FeSO_4.H_2O$, in a shaker incubator at 37 °C, rotated at

250 cycles per minute. Ampicillin was added for strains carrying plasmid constructs (100 µg/ml) and induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Anatrace, Maumee, OH). *S. aureus* strains inoculated from a single colony were grown in Lennox Broth medium (LB) (Fisher Scientific, Pittsburgh, PA) at 37 °C, and rotated at 250 cycles per minute. *M. smegmatis* strains were grown on 7H10 plates (BD Bioscience, Sparks, MD), made per manufacture instructions, for four days at 37 °C, A pre-culture was then started from a single colony into 7H9 media consisting per liter: of 4.7 g 7H9 Difco Middlebrook powder (BD Bioscience, Sparks, MD), 4ml 50% glycerol and 0.05% tween 80, and grown for 72 hours at 37 °C, rotated at 125 cycles per minute. Cultures were then diluted 1:40 and grown overnight for 12-14 hours, in the same media and experiments performed the next day.

In Vivo Assays

Minimal inhibitory concentration curves were performed as previously described (Wray *et al.*, 2018). Briefly, overnight cultures of constructs in MJF455's were diluted 1:50 in CphM and grown until an OD_{600} of 0.2 was reached they were then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 30 minutes. Cultures were diluted 1:200 in pre-warmed CphM, 100 µg/ml ampicillin, 2 mM IPTG and 100 µl added to wells of 96 well plate (Greiner bio-one, Monroe, NC) containing 100ul of CphM with 011A at two times its final concentration solubilized in sterile dimethyl sulfoxide (DMSO) (Sigma,-Aldrich, St. Louis, MO), with a final concentration of DMSO at 0.9%. For endogenous expression of MscL MJF367, MJF451, MJF455, R4220 and MC2155 strains were used without the addition of antibiotics until an OD_{600} of about 0.35 was reached. Cultures were then diluted in the same growth media 1:200 for all strains except *M. smegmatis* MC2155 which was diluted 1:2, with or without the antibiotic being tested at two times their final concentrations. Final concentrations were: Dihydrostreptomycin sesquisulfate at 2.25 µM or 0.5 uM (Sigma

Aldrich St. Louis, MO), kanamycin A at 1 µM (Sigma Aldrich St. Louis, MO), Tetracycline Hydrochloride at 0.5 µM (Thermo Fisher Scientific Waltham, MA) and Ampicillin Sodium Salt at 2 µM (Thermo Fisher Scientific Waltham, MA). 100 µL of these mixtures were immediately added to 100 µL of media with or without compound 011A (2X final concentration), diluted at varying concentrations, and placed in a 96 well plate as described above. Note that antibiotics were added prior to the 011A compound; in the few experiments where this order was reversed with the positively charged DHS or Kan we found that 011A was less effective, possibly because 011A permeated the cells, decreased the membrane potential, and thus also decreased the local concentration of these antibiotics close to the membrane capacitor. The above plates were sealed with a sterile breathable film to prevent evaporation (Axygen, Union City, CA), wrapped in aluminum foil and placed in a 37 °C shaker, rotated at 110 Cycles per minute for 16-17 hours and OD_{620} was then taken with a Multiskan Ascent 354 (Thermo Fisher Scientific Waltham, MA) plate reader.

Growth inhibition at Stationary Phase. For the *S. aureus* R4220 and R4220 Δ*mscL* strains a single colony was picked for each and grown in LB with an argon gas overlay, capped sealed and grown for 24 hours. For the *M. smegmatis* MC2155 and MC2155 Δ*mscL* strains, pre-cultures were grown and diluted 1:40 as described above. After 24 hours, argon was added and tubes were capped and sealed for another 48 hours. All cultures were then divided, and either compound 011A at 40 µM or DMSO only (mock), was added to the tubes, with a final DMSO concentration of 0.9%. The argon overlay was then replaced, capped and sealed in a 1.5 ml tube for 6 hours in a 37 °C shaker. Final OD_{600} was then taken and cultures were diluted 1:20, and then serially diluted six times 1:10 into the same pre-warmed growth media. Liquid drops of 5 µl for each dilution were placed on pre-warmed plates of either LB (*S. aureus* Strains) or 7H10 (*M. smegmatis* strains) and placed in a 37 °C incubator. Colony-forming units were then calculated as soon as colonies could be counted to determine cell viability.

Molecular dynamic (MD) simulations for the passage of DHS through the MscL pore: "Passing through" competition. Molecular docking was conducted with a representative structure of a 150-ns molecular dynamics trajectory of Eco-MscL with a DHS molecule within the pocket formed by the periplasmic loops, as previously described (Wray *et al.*, 2016). The binding pocket for 011A was identified by the SiteID module of the Sybyl-X2.11 software package and flexible-ligand docking was performed with the Glide module of the Schrodinger software package; the sites were as previous (Wray *et al.*, 2016, Wray *et al.*, 2019). To study how well 011A achieves its adjuvant effect by accelerating the passage of known antibiotics into the cytoplasm, we conducted a "passing through" competition with two scenarios considered: the passages of DHS through the unbound and 011A-bound Eco-MscLs. To guarantee the "games" finished within a reasonable time frame, an external electric field (0.1 Volt/Å) was applied along the principal axis of the POPC lipid bilayer (Z-axis) and felt only by DHS. All the simulations started from the same conformation (**Fig. S5A**), the homology model of Eco-MscL, but with different random number seeds for assigning initial velocities. For Scenario 2, 011A was bound at the same binding pocket with the same binding mode of the best docking pose. The competition rules were listed as the follows: (1) the total passing through phase is equally divided into eight sections and each section lasts 5 nanoseconds; (2) the competition is over at the end of a section if the passage of DHS occurs for either of the two scenarios during this section. The passage of DHS through the Eco-MscL was recognized by measuring the distance between the coordinate centers of DHS and five Lys residues (106, 242, 378, 514, 650). Those five Lys residues are in the loops linking the transmembrane domain and the C-terminal domain which forms a cytoplasmic helical bundle. There are four outcomes of each run of competition: (1) Outcome 1 (DHS passing through an Eco-MscL without 011A) wins; (2) Outcome 2 (DHS passing through a 011A bound Eco-MscL) wins; (3) both win and (4) both lose. In total, 36 runs of competition were held to achieve a statistically meaningful result.

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Author contributions

R.W. performed of the whole-cell physiology assays including growth and viability studies. N.H. generated the *M. smegmatis* Δ*mscL* strain and *M. smegmatis* plasmids for this study. J.W. performed the computational analyses including all MD simulations. II helped to analyze, process and present some of the data including the generation of the alignment. II and PB oversaw the whole-cell physiology experiments and orchestrated the project. All authors contributed to the writing of the manuscript.

REFERENCES

Ajouz, B., Berrier, C., Garrigues, A., Besnard, M., and Ghazi, A. (1998a) Release of thioredoxin via the mechanosensitive channel MscL during osmotic downshock of *Escherichia coli* cells. *J. Biol. Chem.* **273**: 26670-26674.

Ajouz, B., Berrier, C., and Ghazi, A. (1998b) Effluxes triggered by osmotic downshock in *E. coli* cells devoid of the mechanosensitive ion channel MscL. *Biophys. J.* **74**: 239a.

Bacon, J., Alderwick, L.J., Allnutt, J.A., Gabasova, E., Watson, R., Hatch, K.A., Clark, S.O., Jeeves, R.E., Marriott, A., Rayner, E., Tolley, H., Pearson, G., Hall, G., Besra, G.S., Wernisch, L., Williams, A., and Marsh, P.D. (2014) Non-replicating Mycobacterium tuberculosis elicits a reduced infectivity profile with corresponding modifications to the cell wall and extracellular matrix. *PloS one* **9**: e87329.

Barkan, D., Stallings, C.L., and Glickman, M.S. (2011) An improved counterselectable marker system for mycobacterial recombination using galK and 2-deoxy-galactose. *Gene* **470**: 31-36.

Bartlett, J.L., Levin, G., and Blount, P. (2004) An *in vivo* assay identifies changes in residue accessibility on mechanosensitive channel gating. *Proc Natl Acad Sci U S A* **101**: 10161-10165.

Berrier, C., Garrigues, A., Richarme, G., and Ghazi, A. (2000) Elongation factor Tu and DnaK are transferred from the cytoplasm to the periplasm of *Escherichia coli* during osmotic downshock presumably via the mechanosensitive channel mscL. *J. Bact.* **182**: 248-251.

Bialecka-Fornal, M., Lee, H.J., DeBerg, H.A., Gandhi, C.S., and Phillips, R. (2012) Single-cell census of mechanosensitive channels in living bacteria. *PloS one* **7**: e33077.

Blount, P., Schroeder, M.J., and Kung, C. (1997) Mutations in a bacterial mechanosensitive channel change the cellular response to osmotic stress. *J. Biol. Chem.* **272**: 32150-32157.

Blount, P., Sukharev, S.I., Schroeder, M.J., Nagle, S.K., and Kung, C. (1996) Single residue substitutions that change the gating properties of a mechanosensitive channel in *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* **93**: 11652-11657.

Booth, I.R., and Blount, P. (2012) Microbial Emergency Release Valves: the MscS and MscL Families of Mechanosensitive Channels. *J Bacteriol* **194**: 4802-4809.

Cetiner, U., Rowe, I., Schams, A., Mayhew, C., Rubin, D., Anishkin, A., and Sukharev, S. (2017) Tension-activated channels in the mechanism of osmotic fitness in Pseudomonas aeruginosa. *J Gen Physiol* **149**: 595-609.

Christian, J.H., and Scott, W.J. (1953) Water relations of Salmonellae at 30 degrees C. *Australian journal of biological sciences* **6**: 565-573.

Cruickshank, C.C., Minchin, R.F., Le Dain, A.C., and Martinac, B. (1997) Estimation of the pore size of the large-conductance mechanosensitive ion channel of *Escherichia coli*. *Biophys. J.* **73**: 1925-1931.

Doerner, J.F., Febvay, S., and Clapham, D.E. (2012) Controlled delivery of bioactive molecules into live cells using the bacterial mechanosensitive channel MscL. *Nature communications* **3**: 990.

Folgering, J.H., Moe, P.C., Schuurman-Wolters, G.K., Blount, P., and Poolman, B. (2005) Lactococcus lactis uses MscL as its principal mechanosensitive channel. *J. Biol. Chem.* **280**: 8784-8792.

Gill, E.E., Franco, O.L., and Hancock, R.E. (2015) Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens. *Chem Biol Drug Des* **85**: 56-78.

Iscla, I., Eaton, C., Parker, J., Wray, R., Kovacs, Z., and Blount, P. (2013) Improving the Design of a MscL-Based Triggered Nanovalve. *Biosensors* **3**: 171-184.

Iscla, I., Levin, G., Wray, R., and Blount, P. (2007) Disulfide Trapping the Mechanosensitive Channel MscL into a Gating-Transition State. *Biophys J* **92**: 1224-1232.

Iscla, I., Wray, R., and Blount, P. (2008) On the structure of the N-terminal domain of the MscL channel: helical bundle or membrane interface. *Biophys J* **95**: 2283-2291.

Iscla, I., Wray, R., Eaton, C., and Blount, P. (2015) Scanning MscL Channels with Targeted Post-Translational Modifications for Functional Alterations. *PloS one* **10**: e0137994.

Iscla, I., Wray, R., Wei, S., Posner, B., and Blount, P. (2014) Streptomycin potency is dependent on MscL channel expression. *Nature communications* **5**: 4891.

Jain, P., Hsu, T., Arai, M., Biermann, K., Thaler, D.S., Nguyen, A., Gonzalez, P.A., Tufariello, J.M., Kriakov, J., Chen, B., Larsen, M.H., and Jacobs, W.R., Jr. (2014) Specialized transduction designed for precise high-throughput unmarked deletions in Mycobacterium tuberculosis. *MBio* **5**: e01245-01214.

Kocer, A. (2010) Functional liposomal membranes for triggered release. *Methods Mol Biol* **605**: 243-255.

Kouwen, T.R., Trip, E.N., Denham, E.L., Sibbald, M.J., Dubois, J.Y., and van Dijl, J.M. (2009) The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. *Antimicrob Agents Chemother* **53**: 4702-4711.

Levina, N., Totemeyer, S., Stokes, N.R., Louis, P., Jones, M.A., and Booth, I.R. (1999) Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. *EMBO J.* **18**: 1730-1737.

Maurer, J., Elmore, D., Lester, H., and Dougherty, D. (2000) Comparing and contrasting Escherichia coli and Mycobacterium tuberculosis mechanosensitive channels (MscL). New gain of function mutations in the loop region. *J. Biol. Chem.* **275**: 22238-22244.

Maurer, J.A., and Dougherty, D.A. (2003) Generation and evaluation of a large mutational library from the *Escherichia coli* mechanosensitive channel of large conductance, MscL - Implications for channel gating and evolutionary design. *J. Biol. Chem.* **278**: 21076-21082.

Moe, P.C., Blount, P., and Kung, C. (1998) Functional and structural conservation in the mechanosensitive channel MscL implicates elements crucial for mechanosensation. *Molec. Microbiol.* **28**: 583-592.

Moe, P.C., Levin, G., and Blount, P. (2000) Correlating a protein structure with function of a bacterial mechanosensitive channel. *J. Biol. Chem.* **275**: 31121-31127.

Moormeier, D.E., and Bayles, K.W. (2017) Staphylococcus aureus biofilm: a complex developmental organism. *Mol Microbiol* **104**: 365-376.

Ou, X., Blount, P., Hoffman, R.J., and Kung, C. (1998) One face of a transmembrane helix is crucial in mechanosensitive channel gating. *Proc. Nat. Acad. Sci. USA* **95**: 11471-11475.

Pacheco-Torres, J., Mukherjee, N., Walko, M., Lopez-Larrubia, P., Ballesteros, P., Cerdan, S., and Kocer, A. (2015) Image guided drug release from pH-sensitive Ion channel-functionalized stealth liposomes into an in vivo glioblastoma model. *Nanomedicine : nanotechnology, biology, and medicine* **11**: 1345-1354.

Perozo, E., Cortes, D.M., Sompornpisut, P., Kloda, A., and Martinac, B. (2002) Open channel structure of MscL and the gating mechanism of mechanosensitive channels. *Nature* **418**: 942-948.

Rowe, I., Elahi, M., Huq, A., and Sukharev, S. (2013) The mechanoelectrical response of the cytoplasmic membrane of Vibrio cholerae. *J Gen Physiol* **142**: 75-85.

Russell, D.G., VanderVen, B.C., Lee, W., Abramovitch, R.B., Kim, M.J., Homolka, S., Niemann, S., and Rohde, K.H. (2010) Mycobacterium tuberculosis wears what it eats. *Cell host & microbe* **8**: 68-76.

Scott, W.J. (1953) Water relations of Staphylococcus aureus at 30 degrees C. *Australian journal of biological sciences* **6**: 549-564.

Shiloh, M.U., and Champion, P.A. (2010) To catch a killer. What can mycobacterial models teach us about Mycobacterium tuberculosis pathogenesis? *Curr Opin Microbiol* **13**: 86-92.

Siddiqui, A.H., and Koirala, J., (2018) Methicillin Resistant Staphylococcus Aureus (MRSA). In: StatPearls. Treasure Island (FL): StatPearls Publishing, pp.

Stokes, N.R., Murray, H.D., Subramaniam, C., Gourse, R.L., Louis, P., Bartlett, W., Miller, S., and Booth, I.R. (2003) A role for mechanosensitive channels in survival of stationary phase: Regulation of channel expression by RpoS. *Proc. Nat. Acad. Sci. USA* **100**: 15959-15964.

Szabo, I., Petronilli, V., and Zoratti, M. (1992) A patch-clamp study of *Bacillus subtilis*. *Biochim. Biophys. Acta* **1112**: 29-38.
Szabo, I., Petronilli, V., and Zoratti, M. (1993) A patch-clamp investigation of the Streptococcus faecalis cell membrane. *The Journal of membrane biology* **131**: 203-218.
van Kessel, J.C., and Hatfull, G.F. (2007) Recombineering in Mycobacterium tuberculosis. *Nat Methods* **4**: 147-152.
Walter, H. (1924) Plasmaquellen und wachstum. *Z. Bot.* **16**: 353–417.
Wray, R., Iscla, I., Gao, Y., Li, H., Wang, J., and Blount, P. (2016) Dihydrostreptomycin Directly Binds to, Modulates, and Passes through the MscL Channel Pore. *PLoS Biol* **14**: e1002473.
Wray, R., Iscla, I., Kovacs, Z., Wang, J., and Blount, P. (2018) Novel compounds that specifically bind and modulate MscL: insights into channel gating mechanisms. *FASEB J*: fj201801628R.
Wray, R., Iscla, I., Kovacs, Z., Wang, J., and Blount, P. (2019) Novel compounds that specifically bind and modulate MscL: insights into channel gating mechanisms. *FASEB J* **33**: 3180-3189.
Wright, G.D. (2016) Antibiotic Adjuvants: Rescuing Antibiotics from Resistance: (Trends in Microbiology 24, 862-871; October 17, 2016). *Trends Microbiol* **24**: 928.
Yang, L.M., and Blount, P. (2011) Manipulating the permeation of charged compounds through the MscL nanovalve. *FASEB J* **25**: 428-434.
Yang, L.M., Zheng, H., Ratnakar, J.S., Adebesin, B.Y., Do, Q.N., Kovacs, Z., and Blount, P. (2018) Engineering a pH-Sensitive Liposomal MRI Agent by Modification of a Bacterial Channel. *Small* **14**: e1704256.

Figure Legends

Fig 1. MscL-dependent effects of compound 011A in two pathogenic bacterial models; percentage of decreased growth (OD_{600}), relative to non-treated are shown.

A. Bacterial strain *Staphylococcus aureus* R4220 WT is shown in red expressing endogenous levels of MscL protein while R4220 Δ*mscL* is shown in black. n=3.

B. Bacterial strain *Mycobacterium smegmatis* MC2155 WT is shown in green expressing endogenous levels of MscL protein while MC2155 Δ*mscL* is shown in black. n=4.

Fig 2. Compound 011A decreases viability of pathogenic bacterial models in stationary phase in a MscL-dependent manner.

The percent reduction in CFU's after treatment with 80 µM of compound 011A after cultures have reached stationary phase. Bacterial strains *S. aureus* R4220 WT and Δ*mscL* R4220 are shown in red, *M. smegmatis* MC2155 WT and MC2155 Δ*mscL* are shown in green as indicated. In the presence of 011A viability decreased in S. aureus WT by ≥40%, while *M. smegmatis* WT showed a ≥50% reduction in viability. Note that WT stains are expressing

endogenous levels of MscL. n=3-4, ** p<0.005, ****p <0.00005 as indicated, 2-tailed, paired T test.

Fig 3. 011A increases the probability of DHS passing through the channel in MD simulations.

36 sets of simulations were performed, and the number of each result plotted. Paired outcome 1 reflects the times when DHS passed through the channel only under the condition where compound 011A was bound, while in outcome 2 DHS passage was observed independent of 011A binding. In a small percentage of sets, DHS passed under both conditions (outcome 3), and under the conditions used, several negatives, where DHS did not pass under either condition, were recorded (outcome 4).

Fig 4. Compound 011A can be used as an adjuvant to increase the potency of antibiotics when MscL is present; values are expressed as a percentage of growth (OD_{600}), relative to non-treated.

A. Curves for Hin-MscL in the MJF455 strain grown in the presence or the absence of 2.25 µM DHS.

B. Curves for Eco-MscL with1 µM kanamycin

C. Curves for Eco-MscL with 0.5 µM tetracycline.

D Curves for Eco-MscL with 2 µM ampicillin; note that MscL channels are expressed at endogenous protein levels using the null mutants indicated.

Fig 5. Compound 011A can be used as an adjuvant to increase the potency of antibiotics for *S. aureus* and *M. smegmatis*. Values are expressed as the percentage of decreased growth (OD_{600}) in the presence of (i) compound 011A (ii) Tetracycline (Tet) (iii) or a combination of both 011A and Tetracycline (011A + Tet); relative to non-treated. A. Bacterial strain *S. aureus* R4220 WT and Δ*mscL* R4220 are shown in the presence of 011A 80 µm, Tet 0.3 µm or the combination of both at the same concentrations as indicated. n=4. ***p< .0005 as

indicated, 2-tailed, paired T test. B. Bacterial strain *M. smegmatis* MC2155 WT and MC2155 Δ*mscL* are shown in the presence of 011A 40 µm, Tet 0.3 µm or the combination of both at the same concentrations as indicated. n=4, *p< .05 as indicated, 2-tailed, paired T test.