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Membrane activity profiling of small molecule *B. subtilis* growth inhibitors utilizing novel dual-dye fluorescence assay

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Small molecule disruption of the bacterial membrane is both a challenge and interest for drug development. While some avoid membrane activity due to toxicity issues, others are interested in leveraging the effects for new treatments. Existing assays are available for measuring disruption of membrane potential or membrane permeability, two key characteristics of the bacterial membrane, however they are limited in their ability to distinguish between these properties. Here, we demonstrate a high throughput assay for detection and characterization of membrane active compounds. The assay distinguishes the effect of small molecules on either the membrane potential or membrane permeability using the fluorescent dyes TO-PRO-3 iodide and DiOC₂(3) without the need for secondary assays. We then applied this assay to a library of 3520 synthetic molecules previously shown to inhibit growth of *B. subtilis* in order to determine the frequency of membrane activity within such a biologically active library. From the library, we found 249 compounds that demonstrated significant membrane activity, suggesting that synthetic libraries of this kind do not contain a plurality of membrane active molecules.

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Introduction

The bacterial membrane is a complex target for antibiotics. There are numerous existing and experimental antibacterial treatments that act through disruptions of the bacterial membrane. Antimicrobial peptides, including nisin,¹ daptomycin,² polymyxin B,³ and colistin,⁴ have long been used to treat various bacterial infections. There are also a number of small molecules under development that are designed to mimic the membrane effects of antimicrobial peptides⁵ as well as inhibit the growth of biofilms.⁶ In addition to using membrane active molecules as stand-alone treatment, these membrane active molecules are being investigated for use as adjuvants to potentiate known molecules against both Gram-negative bacteria such as *E. coli*⁷ and Gram-positive bacteria such as *S. aureus*.⁸

The bacterial membrane is a complex structure. Although the composition can vary from organism to organism, the single phospholipid bilayer of Gram-positive organisms includes large amounts of phosphatidylglycerol and cardiolipin.^{9,10} In *Bacillus* species, phosphatidylethanolamine is also abundant. In addition to the lipid component, the cell membrane consists of the lipid anchor component of lipoteichoic acid as well as numerous transmembrane and li-

poproteins. Along with the peptidoglycan layer, these membranes play a role in maintaining cell shape¹¹ and also provide a surface for cellular respiration, the transport of various ions, toxins, and other solutes in and out of the cell, and cell-cell communication.¹² Molecules that target the membrane generally act through two distinct yet related mechanisms, permeabilization and depolarization. Permeabilization, as induced by compounds such as nisin and daptomycin, is the result of pores or other detrimental disruptions to the structure of the membrane. Depolarization, which is caused by ionophores that move ions against the concentrations gradients imposed by the membrane, targets the proton motive force (PMF). The PMF is composed of the transmembrane electric potential and proton gradient that drive ATP synthesis and the specific transport of various solutes across the cell membrane.¹³

While some are specifically interested in identifying membrane active molecules as potential antibiotics, there are others who are interested in removing membrane active compounds from their hit follow-up. This could be due to concerns over off-target effects, if the intended target is not the membrane, or due to the toxicity challenges of membrane active molecules.^{14,15} To this end, a number of techniques have been created to test compounds for membrane activity. These methods generally use fluorescent dyes to determine the effect of a molecule of interest on membrane permeability or membrane potential. However, testing only for permeability disruption would miss compounds that depolarize without

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impacting the membrane permeability, and testing for depolarization would be unable to distinguish those that only depolarize from those that both depolarize and impact permeability. A combination of two dyes, TO-PRO-3 iodide to measure cell permeability and DiOC₂(3) to measure cell polarity, has been used previously to investigate the mechanism of daptomycin,¹⁶ but not in a high-throughput assay capable of screening large numbers of molecules.

Therefore, we set out to establish and utilize a simple and high-throughput method to quickly characterize the activity of novel bioactive compounds against the bacterial membrane of *B. subtilis*, a model Gram-positive bacterium. The assay utilizes two existing dyes previously used for measuring membrane disruption, TO-PRO-3 iodide and DiOC₂(3), and combines them in a single high-throughput assay. The specificity of the assay is demonstrated using three well-characterized molecules: nisin, a lantibiotic that binds lipid II and creates pores in the cell membrane, CCCP, a proton ionophore, and vancomycin, an antibiotic that inhibits cell wall synthesis while leaving the membrane intact. We also use this assay to screen a collection of 3520 biologically active small molecules to determine the prevalence of membrane activity in a collection of synthetic molecules known to inhibit growth of *B. subtilis*. In performing this screen, we found 448 molecules, or 12.7% of the library, that interfere with the fluorescence of the dyes in the absence of *B. subtilis*; these were removed from further analysis. We found 7.1% of compounds induced a membrane effect in *B. subtilis* at 20 μ M while 5.2% induced an effect at 5 μ M. This suggests that membrane activity may not be as prevalent in synthetic compound libraries, and highlights that care must be taken to ensure no interference between the molecules and the method used to determine membrane activity.

Experimental

Strains and chemicals

B. subtilis 168 was used for all experiments. Both TO-PRO-3 iodide (catalog T3605) and DiOC₂(3) (catalog D14730) were obtained from Thermo Fisher Scientific. Nisin (catalog N5764), vancomycin (catalog V2002), and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (catalog C2759) were obtained from Sigma Aldrich.

Duel-dye membrane disruption assay

An overnight culture of *B. subtilis* 168 was diluted 1/100 in fresh LB media and incubated at 37 °C on a rotating incubator set to 225 rpm until reaching an OD₆₀₀ of 0.4. Cells were pelleted at 3000 g for 10 minutes, the supernatant decanted, and washed twice in PBS+ (0.14 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ at pH 7.4 supplemented with 10 mM glucose and 0.5 mM MgCl₂). The pellet was then resuspended in PBS+ and diluted to an OD₆₀₀ of 0.1. Concurrently, a blank PBS+ sample was also prepared without *B. subtilis*. Dyes were added into both the sample and control to a final concentration of 625 nM TO-PRO-3 iodide (λ_{ex} = 600

nm, λ_{em} = 650 nm) and 10 μ M DiOC₂(3) (λ_{ex} = 450 nm, λ_{em} = 510 nm). Both the culture and dye control solutions were added to 96-well plates and the test compound were added to a final concentration of 5 μ M and 20 μ M. Each plate also contained treatment controls wells for DiOC₂(3) (10 μ M CCCP) and TO-PRO-3 iodide (10 μ g per mL nisin). Samples were incubated at room temperature in the dark for 5 minutes, then treated with the test compound. Fluorescence was read immediately following treatment using a BioTek Synergy H1 plate reader.

Minimum inhibitory concentration

An overnight culture of *B. subtilis* 168 was diluted 1/100 in fresh LB media and incubated at 37 °C on a rotating incubator set to 225 rpm until reaching an OD₆₀₀ of 0.4. The cells were diluted 1/1000 and 198 μ L added to a 96-well plate. 2 μ L of treatment compound was added and the plate incubated for 18 hours in a rotating incubator at 37 °C. After overnight growth, the optical density at 600 nm was read and the MIC determined by the lowest concentration showing less than 10% growth.

Results & discussion

High-throughput assay for determining impact on membrane potential and permeability

Fluorescent dyes have been used to determine the impact of physical and chemical stresses on cell permeability and the membrane potential for over 30 years. These include fluorescein and ethidium bromide,¹⁷ DiSC₃(5),^{8,18} DiSC₂(5),¹⁹ DiOC₂(3),²⁰ SYTO9 and propidium iodide,²¹ TO-PRO-3 iodide,²² and DiOC₂(3).²³ In order to measure the impact on both membrane potential and membrane permeability simultaneously and in a high-throughput manner, we needed to identify dyes having non-overlapping excitation and emission spectra that the signal from one would be independent of the other.

By measuring the absorption and fluorescence emission spectra of four dyes, DiOC₂(3), DiSC₂(5), TO-PRO-3 iodide, and propidium iodide, we selected TO-PRO-3 iodide to measure membrane permeability and DiOC₂(3) to measure changes to the membrane potential. The chemical structures of these molecules are shown in Fig. 1A and the optical separation of their absorbance spectra shown in Fig. 1B. DiOC₂(3) absorbs strongly at 450–500 nm and is strongly fluorescent at 500–550 nm, as shown in Fig. 1C. TO-PRO-3 iodide absorbs strongly at 575–650 nm and is fluorescent around 650–700 nm, as seen in Fig. 1D. In order to achieve good optical separation on our plate reader, we used an λ_{ex} of 600 nm and λ_{em} of 650 nm for TO-PRO-3 iodide and λ_{ex} of 450 nm and λ_{em} of 510 nm for DiOC₂(3).

TO-PRO-3 iodide is a cell impermeant carbocyanine monomer nucleic acid stain that is frequently used in fluorescence and laser confocal microscopy.²⁴ In the presence of an intact membrane, the dye is unable to enter the cytoplasm however when the cell membrane is damaged the dye is able to cross

Fig. 1 Structures and optical properties of TO-PRO-3 iodide and DiOC₂(3). (A) Chemical structures of TO-PRO-3 iodide and DiOC₂(3). (B) Absorbance spectra of TO-PRO-3 iodide (■) and DiOC₂(3) (●) showing the distinct absorbance spectra of the two dyes. (C) Absorbance spectra of DiOC₂(3) (■) with corresponding emission spectra at $\lambda_{\text{ex}} = 450 \text{ nm}$ (●). (D) Absorbance spectra of TO-PRO-3 iodide (■) with corresponding emission spectra at $\lambda_{\text{ex}} = 600 \text{ nm}$ (●).

into the cell and bind DNA, increasing its fluorescence emission 20- to 30-fold. DiOC₂(3) is a cell permeant dye that aggregates in response to a bacterial membrane potential, quenching its fluorescence output. Disruption of the membrane potential reduces this aggregation and significantly increases the fluorescence emission intensity.²⁵

A number of antibiotic molecules with well-characterized mechanisms of action were used to validate the use of these dyes to quantify membrane disruption. Nisin is a lantibiotic known to bind the lipid II component of the peptidoglycan synthesis machinery. It then inserts itself into the bacterial membrane, creating a pore that disrupts the membrane permeability.²⁶ This action not only increases the permeability of the cell membrane but also disrupts the membrane potential. This is because the membrane potential relies on the physical separation of an ion gradient, and disruption to the permeability dissipates this gradient. Treating *B. subtilis* with increasing concentrations of nisin resulted in an increase in the fluorescence emission of both DiOC₂(3) and TO-PRO-3 iodide relative to an untreated control, shown in Fig. 2A. This change in signal took place beginning below $0.1 \mu\text{g mL}^{-1}$ and continued until the minimal inhibitory concentration (MIC) of $4 \mu\text{g mL}^{-1}$. This indicates that both the membrane permeability and membrane potential have been compromised. Treatment with CCCP, a proton ionophore that disrupts the

membrane potential by shuttling protons across the bacterial membrane, increased DiOC₂(3) fluorescence until it plateaued at $2 \mu\text{M}$, near the MIC of $8 \mu\text{M}$, as seen in Fig. 2B. CCCP had no effect on TO-PRO-3 iodide fluorescence. This demonstrated that while the membrane potential was disrupted, there was no impact on the membrane permeability. Finally, vancomycin, which binds the pentapeptide component of peptidoglycan and inhibits cell wall synthesis,²⁷ does not disrupt the cell membrane and thus showed no change in the fluorescence output of either dye. This effect can be seen in Fig. 2C where the fluorescence output was flat even significantly above the concentration required to inhibit growth ($0.5 \mu\text{g mL}^{-1}$). Therefore, the use of DiOC₂(3) and TO-PRO-3 iodide allows us to determine the impact of a molecule on the bacterial cell membrane and distinguish between those that disrupt the membrane potential and the membrane permeability.

Screen for biologically active small molecules against *B. subtilis*

A previous screen on a collection of 141 899 compounds identified 3705 biologically active compounds able to inhibit growth of *B. subtilis*.²⁸ In this screen, $10 \mu\text{M}$ of each compound in the library was added to cultures of *B. subtilis* 168

Fig. 2 Concentration dependent impact of known antibiotics, nisin (A), CCCP (B) and vancomycin (C), on the relative fluorescence of TO-PRO-3 iodide (■) and DiOC₂(3) (▲) in *B. subtilis* cultures. Absorbance spectra of overnight cultures of treated *B. subtilis* was used to compare the fluorescence changes to the molecule's MIC (●). Error bars represent the standard error of three replicate experiments.

and incubated overnight. Growth inhibition was determined by measuring the OD₆₀₀ following overnight incubation and hits were selected using a statistical cutoff of 2 standard deviations below the mean of the full data set. Therefore, molecules that inhibited growth by more than 30% were selected as hits, resulting a hit rate of 2.61% and a total of 3705 *B. subtilis* active molecules. We took advantage of this collection of prioritized bioactives to test the two-dye assay and investigate the frequency of membrane active molecules within the collection.

Membrane disruption by biologically active small molecules

A challenge of using fluorescence as an output for screening is the potential for the screening compounds themselves to directly interfere with the fluorescence emission independent of the effect on a cell. Such inference could include absorption at the excitation frequency, fluorescence quenching by absorbing the emission output, fluorescence of the test compound, or chemical reactivity between the dye and the molecule being tested. In order to remove any such molecules from our dataset, we first screened the sub-library for molecules that alter the dye fluorescence independent of membranes to identify compounds that interfere with TO-PRO-3 and DiOC₂(3) in the absence of *B. subtilis*.

In order to account for plate-to-plate variability in fluorescence emissions, the readings were normalized by taking the log₂ ratio of the emission of the treated dye solution relative to the untreated dyes. The results of this experiment are shown in Fig. 3A and B, with the black dots representing treatment at 20 µM and the grey dots representing treatment at 5 µM. To capture all of the interfering compounds, we used the fluorescence effects of the higher treatment concentration to filter out reactive or optically active compounds. Out of the 3520 compounds tested, 448 had a significant impact on dye fluorescence at 20 µM in the absence of *B. subtilis*. We defined significant as values greater than or less than two standard deviations from the mean of the collection. This cut-off is shown in Fig. 3A and B as a solid horizontal line for 5 µM and the dashed line for 20 µM. Out of interfering molecules, 302 disrupted DiOC₂(3) fluorescence while 196 disrupted TO-PRO-3. This 12.7% of the interfering compounds in the bioactive collection was omitted from further analysis.

To determine the membrane effects of the compounds the collection was screened at two concentrations, 5 µM and 20 µM, against *B. subtilis* in the presence of both TO-PRO-3 iodide and DiOC₂(3). The fluorescence output was processed using the same protocol as the compound controls, with the log₂ ratio of the treated *versus* the untreated emission

Fig. 3 Log₂ ratio of the fluorescence readings from a screen of biologically active compounds at 5 μ M (grey) and 20 μ M (black) against DiOC₂(3) control (A), TO-PRO-3 iodide control (B), membrane potential (C), and membrane permeability (D). The controls consist of the dye in buffer without any *B. subtilis*. Data are represented by a scatter plot (left) as well as a density distribution (right). Solid lines refer to the significance cut-offs for the 5 μ M (solid) and 20 μ M (dashed) concentrations.

intensities used to correct for plate to plate variability (Fig. 3C for DiOC₂(3) and Fig. 3D for TO-PRO-3 iodide). The average *Z'* value of the screening assay was calculated as 0.54

for membrane depolarization through DiOC₂(3) fluorescence using 10 μ M CCCP as a positive control and 0.71 for membrane permeabilization through TO-PRO-3 iodide

Fig. 4 (A) Results summary showing the initial screen for inhibitors of *B. subtilis* growth, the compounds that interfere with the dye fluorescence output, as well as the hits that were determined from the screen. Of the 448 compounds removed from the screen due to fluorescence interference, 302 were for interference with DiOC₂(3) and 196 for interfering with TO-PRO-3 iodide, with 50 interfering with both. (B) Comparing the relative results DiOC₂(3) fluorescence compared to TO-PRO-3 iodide fluorescence at 20 μ M and (C) at 5 μ M. The vertical line denotes the statistical cut-off used for determining activity.

fluorescence using 10 μg per mL nisin as a positive control.²⁹ Using corrected fluorescence output values of greater than two standard deviations from the population mean at 20 μM as our cut-off, 142 compounds were identified as active against membrane potential (cut-off ratio of 0.64) while 151 are active against membrane permeability (cut-off ratio of 0.58). Using the same definition for the lower concentration of 5 μM , we observe 128 hits for membrane potential (cut-off ratio of 0.47) and 88 membrane permeability hits (cut-off ratio of 0.28). This gives an overall rate of active molecules of 7.1% at 20 μM and 5.1% at 5 μM .

We expected that compounds that permeabilized the cells would also depolarize the membrane on the grounds that loss of membrane integrity would allow ions to pass into the cytoplasm, compromising the electrochemical gradient. This effect is evident in the nisin in Fig. 2A where both the TO-PRO-3 iodide and DiOC₂(3) fluorescence increase with increasing concentration of the molecule. To determine whether this was the case, we plotted the data so as to reveal such cross-effects (Fig. 4A). In the 20 μM data: of 249 total hits, 98 acted exclusively on the electrochemical gradient, 44 compromised by the electrochemical gradient and permeabilized the cells while 107 appeared to permeabilize the cells but had no effect on the electrochemical gradient. Similarly, in the 5 μM data: of 183 total hits, 95 compounds compromised the electrochemical gradient exclusively, 33 compromised the electrochemical gradient and permeabilized cells while 55 appeared to permeabilize cells but do not influence the electrochemical gradient.

We reasoned that this apparent paradox may be due to the cut-off values used to select the relative activity. To investigate this further, we plotted the data as shown in Fig. 4B and C showing scatter plots of the relative activity values of DiOC₂(3) against TO-PRO-3 iodide at 20 μM and 5 μM treatment concentrations. The vertical line shows the cut-off value selected for membrane permeabilization while the horizontal line indicates the selected cut-off for activity

against membrane potential. Although there are some compounds defined as membrane permeability hits that are just below the membrane potential cut-off, these are not the majority, suggesting that there are compounds that impact TO-PRO-3 iodide fluorescence while not effecting DiOC₂(3) fluorescence. This may be caused by specific uptake of the TO-PRO-3 iodide dye or temporary permeabilization that allows the dye to enter the cell while not resulting to complete depolarization. Additionally, the compounds may be inducing secretion of extracellular DNA, either by induction of biofilm formation³⁰ or natural competence systems,³¹ where the extracellular TO-PRO-3 iodide is able to bind and increase its fluorescence emission.

As another means of assessing this data, we compared the effects at the two concentrations for each compound to determine whether the fluorescence gave a dose-response with each compound. By plotting the log₂ ratio for the dye fluorescence against the test compound concentration, we found that many of the active compounds induced increased fluorescence output at higher concentration, indicating a dose response, and that the effect of some molecules decreased at higher concentration. This effect is seen in Fig. 5 with the blue solid lines showing compounds with increased effect at higher concentrations while the red dashed lines show decreasing effect. The compounds with reduced effect at higher concentrations were likely due to chemical instability or aggregation at higher concentrations, resulting in lower activity. This highlights the need to assess chemical effects at various concentrations, for compounds that appear to have no effect at high concentrations may be masking an effect visible at lower concentrations.

Finally, we wished to assess the biological relevance of these induced fluorescence changes. Looking at the impact of nisin and CCCP on the dye fluorescence (Fig. 2), both increased the fluorescence output by a log₂ between 1 to 1.5, equivalent to a 2 to 3-fold increase in fluorescence. For the 3705 molecules in the bioactives collection, there are only 17

Fig. 5 Concentration dependent effect on the fluorescence activity for DiOC₂(3) (A) and TO-PRO-3 iodide (B). Lines are shown for compounds that show significant concentration effects, with blue indicating an increase in fluorescence emission with increasing concentration and red indicating a decrease in fluorescence emission.

compounds that induced this magnitude of change in TO-PRO-3 iodide and 37 that induce this magnitude of change in DiOC₂(3). The chemical structures for the compounds are found in Fig. S1 and S2. We calculated the log *P* octanol/water partition coefficient³² for each of the molecules in order to compare these highly active compounds relative to the whole sub-library. In Fig. S3, we show a density plot of the log *P* values for the whole sub-library, the membrane potential hits, and the membrane permeability hits. We observe a slight increase in the log *P* values for the membrane potential hits relative to the full set of molecules and an additional slight increase for the membrane permeability hits. Therefore, the hits show slightly higher lipid solubility than the library as a whole. These data indicate that molecules with significant membrane effects comparable with known membrane disrupting antibiotics are not common within synthetic compound libraries.

Conclusion

We demonstrated the use of a two-dye assay for rapidly identifying and characterizing molecules that permeabilize cells and/or compromise the electrochemical gradient in *B. subtilis*. This assay can be used to test antibacterial candidate compounds for membrane activity to remove them from further consideration or to specifically identify membrane active compounds, as desired. We found that in a collection of 3520 synthetic bioactive compounds, membrane activity was a relatively rare molecular property. With 249 membrane active molecules in the whole collection, and only 54 inducing an effect comparable with known membrane active molecules such as nisin, this suggests that synthetic compound libraries do not contain a plurality of membrane active molecules.

Conflicts of interest

The authors declare no competing interest.

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