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Discovery of an adjuvant that resensitizes polymyxin B-resistant bacteria

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ABSTRACT

Infections caused by antibiotic-resistant bacteria are a major threat to health, increasing mortality rates and straining health systems worldwide. Adjuvants targeted to beta-lactamase function are able to resensitize bacteria to beta-lactam antibiotics, but there is comparatively little research into the use of adjuvants against other resistance phenotypes. In this study, we performed a high-throughput screen of 74 natural products to identify adjuvants that synergized with antibiotics to eradicate resistant Gram-negative bacteria. From this, we identified six adjuvant hits which restored growth inhibition when combined with the relevant antibiotic, and pursued a lead candidate, perforone, which possessed selective adjuvant activity in combination with polymyxin B against polymyxin-resistant *Escherichia coli* cells. These results suggest that pairing adjuvants with antibiotics could be a useful general intervention against resistant bacteria, helping to mitigate the effects of antimicrobial resistance.

1. Introduction

Antimicrobial resistance (AMR) is a major threat to global health. Driven by a combination of chromosomal mutations and horizontally transferred resistance elements, resistance has been observed against every antibiotic in current clinical use.[1,2] Infections by antibiotic-resistant bacteria caused approximately 1.27 million deaths across the globe in 2019, and were associated with a further 4.95 million deaths.[3] Some have speculated if left unchecked, antibiotic-resistant bacteria could cause 10 million excess deaths per year by 2050.[4,5]

The rising prevalence of resistance is a natural consequence of antibiotic use, which imposes a selective pressure on pathogenic bacteria.[6] Evolution of resistance over the course of an infection can compromise therapy, and in the case of the developmental candidate AN3365/GSK2251052, even forced an abrupt halt to a phase II clinical trial.[7]. As the development of new drugs has not kept pace with bacterial evolution,[8] methods to restore the efficacy of existing antibiotics are urgently required.

One well-established approach is the use of adjuvants, compounds with little direct activity against bacteria that are able to enhance antibiotic function or restore activity against resistant strains. The beta-lactamase inhibitor clavulanic acid is one such adjuvant, effective against Ambler class A beta-lactamases.[9] As a combination therapy with the beta-lactam amoxicillin, clavulanic acid is on the World Health Organization's list of essential medications, with over six million prescriptions each year in the United States.[10,11] As the evolution of antibiotic resistance often also causes fitness defects,[12] resistant bacteria may also display increased sensitivity to other antibiotics.[13].

In this study we identified adjuvant compounds that were able to synergize with antibiotics and inhibit the growth of resistant bacteria. To do this we performed a high-throughput screen using a small panel of structurally-diverse natural products and a number of antibiotic-resistant bacteria that had been generated in-house.[14] One compound selectively restored killing against the polymyxin-resistant strain used in this work. It was selected for further study, revealing that it allowed the passage of protons across the inner membrane of *E. coli.* All the antibiotic-resistant mutants displayed increased susceptibility to one or more adjuvants, suggesting that adjuvants may be a general strategy against antibiotic-resistant bacteria.

2. Results

2.1. The primary screen

We began by creating a small, diverse natural product library. The 68,000 compounds in the InterBioScreen Natural Compound Collection were passed through ChemMine,[15] sorting them into a hierarchical tree based on chemical similarity. From this tree 99 compounds with molecular masses of 600 Da or less were then chosen, keeping at least two nodes of separation between each compound. 74 of these were soluble in

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DMSO to a concentration of 25 mg/mL, and were carried forward.

Mutants of *E. coli* MG1655 independently resistant to azithromycin, ciprofloxacin, doxycycline, polymyxin B, and the combination of sulfamethoxazole and trimethoprim drawn from prior experiments,[14] or were *de novo* generated by passage of naïve cells through SAGE plates containing the antibiotic of interest at a minimum of 10x its MIC (Table 2). Both naïve and resistant cells were then screened for susceptibility to each compound in the natural product library, either alone or in the presence of each antibiotic/antibiotic pair at half their MIC (Fig. 2). The results of these 17 screens are summarized in Fig. 3.

Five of the compounds inhibited bacterial growth in half or more of the conditions. This includes **1** (well A05, Fig. 3), a derivative of the histological dye hematoxylin known as hematein.[16] Hematein is a known casein kinase II inhibitor able to inhibit cancer growth,[17] and while its activity against bacteria has not been previously reported, the closely related analogue brasilin is a known inhibitor of Gram-positive (but not Gram-negative) bacteria.[18] Other notable antimicrobial compounds include **2** (well B07), an analogue of the well-characterized adjuvant rutin.[19,20] While generally lacking in direct antimicrobial activity, rutin enhances the killing effect of other flavones. Against *E. coli* rutin has been reported to interfere with biofilm formation and virulence factor production, by reducing the secretion of quorum sensors.[21] Well F02 contained the well-known antibiotic doxycycline as a positive control, which as expected inhibited the growth of every bacteria but the doxycycline-resistant strain (Figs. 2 and 3).

Sixteen of the compounds in the screen only had activity against antibiotic-resistant strains, and six of these inhibited growth only in the presence of the antibiotic that the strain had evolved resistance against. This included **3** (ononin, well A01), which inhibited the growth of *E. coli* resistant to azithromycin, ciprofloxacin, and the trimethoprim/sulfamethoxazole combination when paired with the relevant antibiotics, and weakly inhibited doxycycline-resistant cells when combined with doxycycline. Ononin has very weak antimicrobial activity, but like many flavones can rigidify the bacterial membrane at high concentrations.[22] Also of interest was **4** (ugaferin, well C09), which inhibited bacterial growth when combined with doxycycline and weakly inhibited growth with ciprofloxacin, polymyxin B, and the trimethoprim/sulfamethoxazole combination. Originally isolated from the roots of the plant *Ferula ugamica*, ugaferin has no previously reported bioactivity.[23].

2.2. Perforone targets polymyxin-resistant cells

Interested in compounds that could selectively restore activity against antibiotic-resistant bacteria, we focused on an analogue of perforine,[24] compound **5** (well A12). Referred hereafter as perforone, **5** is a hemiketal that weakly inhibited the growth of polymyxin B-resistant *E. coli* when combined with polymyxin B at half its MIC. A checkerboard assay confirmed synergy between the two compounds, with a minimal FIC of 0.19 and a 16-fold reduction in the MIC of polymyxin B. However, this synergy was only present against the polymyxin B-resistant *E. coli.*

Table 1
Bacterial strains and suppliers used in the screen. Speed-selected (SS) strains were passed through antibiotic-free soft agar prior to the evolution of antibiotic resistance, to ensure uniform movement speeds.

Strains	Supplier
Escherichia coli MG1655 SS	Generated in-house[14]
Staphyloccocus aureus ATCC 29213	American Type Culture Collection
E. coli CANWARD 107115	CANWARD ICU Surveillance Studies[30]
Escherichia coli MG1655 PolyB^r	Generated in-house[14]
Escherichia coli MG1655 azithromycin 2	Generated in-house[14]
Escherichia coli MG1655 doxycycline 2–3	This work
Escherichia coli MG1655 trimethoprim/sulfamethoxazole 2	Generated in-house[14]
Escherichia coli MG1655 cipro^r	Generated in-house[14]

Table 2
MICs of naïve and resistant MG1655 *E. coli* cells.

Antibiotics	Naïve MG1655 (mg/L)	Resistant cells MIC (mg/L)
Polymyxin	0.5	256
Ciprofloxacin	0.015	8
Trimethoprim/Sulfamethoxazole	0.0625/1.1875	16/304
Azithromycin	4	128
Doxycycline	0.25	60

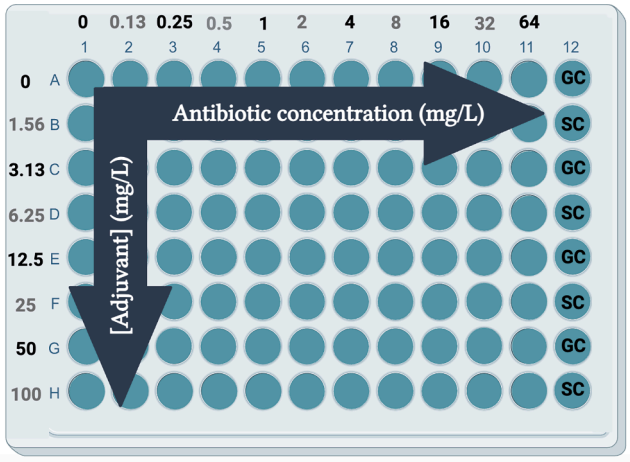

Fig. 1. Checkerboard layout. Antibiotic concentration increases from left side of the plate to the right side. Concentrations doubling from each column to the next. Adjuvant concentration increases from top of the plate to the bottom. Concentrations doubling from each row to the next. Column 12 was used for control wells.

Perforone's effect was additive against *E. coli* MG1655, with a 2-fold reduction in polymyxin's MIC at 100 mg/L of perforone. A similarly small effect was observed with *S. aureus* ATCC 29213 and the antimicrobial peptide melittin. Addition of perforone to the mixture improved the activity of melittin 2-fold, at 75 mg/L of perforone (Table 3).

As polymyxin B is able to destabilize the outer membrane of *E. coli*, potentially allowing compounds entry into the cell,[25] we were concerned that the inhibitory activity we saw might be due to improved access of perforone to the interior of the cell rather than to an improvement in polymyxin B function. We thus repeated the initial screen with polymyxin B nonapeptide (PMBN), an analogue that is still able to permeabilize bacterial membranes but lacks both the original's acyl tail and its antimicrobial activity.[25] No inhibition was observed when perforone and PMBN were applied together (Fig. 4), suggesting that the activity we observed against polymyxin B-resistant *E. coli* was due to resensitization of the bacteria to polymyxin B or to synergy between the two compounds.

2.3. Perforone allows protons to flow across the membrane

Prior sequencing of *E. coli* MG1655 polyB^r revealed a number of mutations in genes responsible for lipopolysaccharide biosynthesis,[14] suggesting that perforone might act via interactions with the bacterial membrane. We thus studied the effect of perforone on membrane integrity, using the fluorogenic probe $DiSC_3(5)$.[26] Under normal conditions $DiSC_3(5)$ accumulates on the inner bacterial membrane, suppressing its own fluorescence. When this membrane is depolarized the dye diffuses into the cell, leading to a measurable increase in fluorescence.[26].

Addition of polymyxin B to *E. coli* MG1655 pretreated with $DiSC_3$ (5) led to the expected increase in fluorescence (data not shown),[27] as did

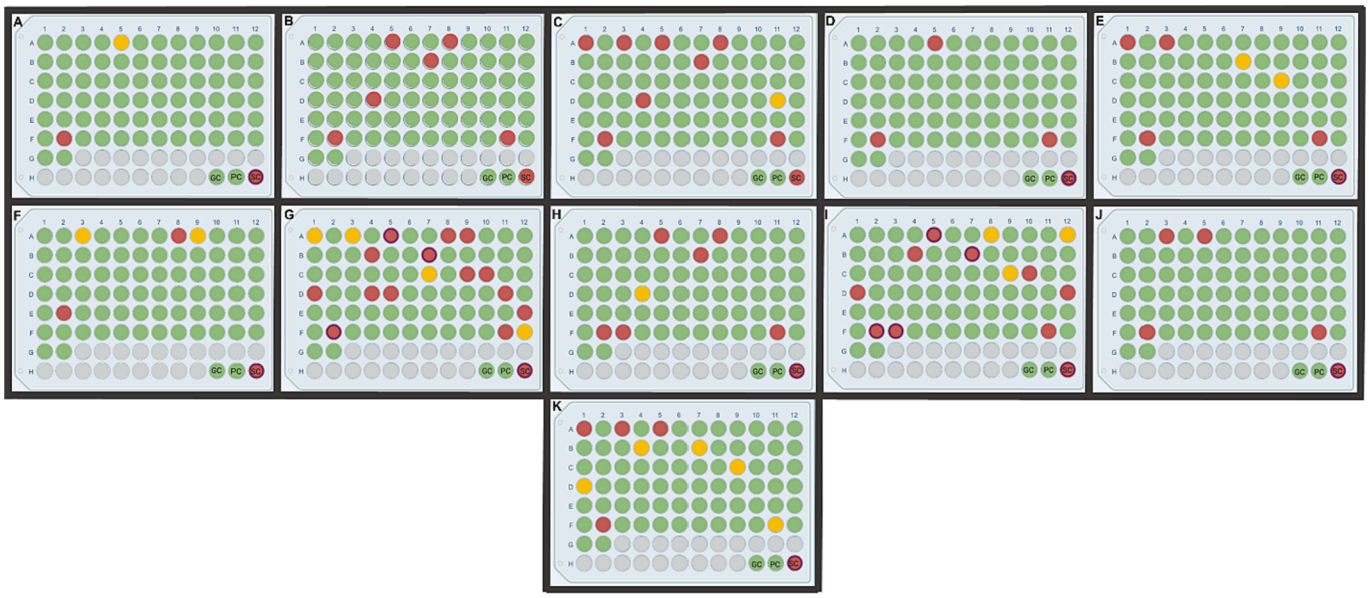

Fig. 2. Primary screen for adjuvant activity. Each plate represents the results of addition of the 74 adjuvants to naïve or antibiotic resistant *E. coli* MG1655 at a concentration of 12.5 mg/L, in the presence or absence of the relevant antibiotic at half its MIC. A) Naïve MG1655 cells, B) azithromycin-resistant cells, C) azithromycin-resistant cells with azithromycin (64 mg/L) and the putative adjuvants, D) ciprofloxacin-resistant cells, E) ciprofloxacin-resistant cells and ciprofloxacin (4 mg/L), F) doxycycline-resistant cells, G) doxycycline-resistant cells and doxycycline (30 mg/L), H) polymyxin B-resistant cells, I) polymyxin B-resistant cells with polymyxin (128 mg/L), J) trimethoprim/sulfamethoxazole-resistant cells, K) trimethoprim/sulfamethoxazole-resistant cells and trimethoprim/sulfamethoxazole (8/152 mg/L). Green wells indicate growth (>2 mm bacterial pellet), yellow wells indicate partial growth (<2 mm bacterial pellet), and red wells indicate no visible growth. Compound positions are conserved across the screens, and every experimental well contains an added adjuvant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

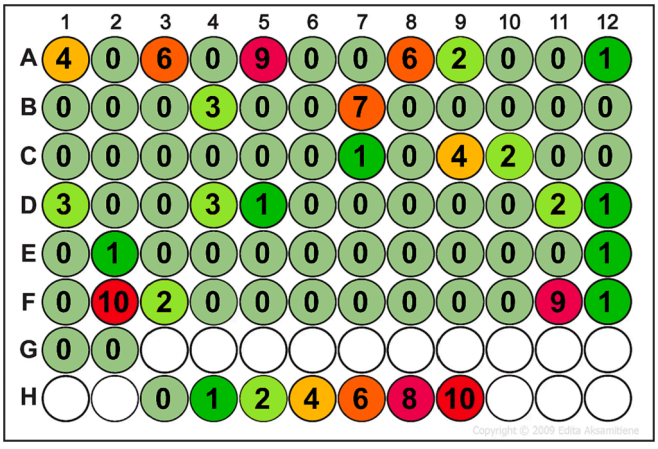

Fig. 3. Summary of the primary screen depicted in Fig. 2. Values indicate the number of plates where the natural product well had reduced growth, while wells at the bottom demonstrate the minimum number of plates for each colour.

Table 3
Perforone-polymyxin B synergy. Checkerboard assays were used to measure the interaction between the two compounds. Perforone synergized with polymyxin B against polymyxin B-resistant *E. coli,* but had little to no effect against wildtype *E. coli* MG1655.

Strain	MIC	MIC with Adjuvant	Increase in activity
E. coli MG1655	4 mg/L	2 mg/L (6.25 mg/L perforone)	2-fold
E. coli MG1655 poly^R	256 mg/L	16 mg/L (50 mg/L perforone)	16 fold

addition of perforone (Fig. 5A). However, attempts to fully permeabilize the membrane of perforone-treated cells with polymyxin B or the surfactant triton X-100 lead to an unexpected and rapid decrease in fluorescence. This appears to be due to direct interaction between perforone and the hydrophobic dye, as mixing of the two in the absence of cells also led to quenching of the dye (Fig. 5B). To ensure that this interaction was not confounding our results, we increased the pH of the media to 8.0 and switched to 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF, AM), a ratiometric pH indicator ideal for measuring changes in the cytosolic pH of cells. Unlike $Disc_3(5)$, which is adsorbed onto the membrane, BCECF, AM localizes to the cytoplasm.[28,29] This was expected to prevent premature quenching of the dye, as permeabilization of the inner membrane will basify the cytoplasm (and increase fluorescence by BCECF, AM) before perforone is able to amass significant intracellular concentrations.

Under these conditions, addition of perforone increased fluorescence from 8.5 to 29.2 arbitrary units (AU), followed by a decrease back to 19.7 AU over a span of 4 min (Fig. 5C). In combination with the $DiSC_3(5)$ results this strongly suggests that perforone is able to permeabilize the inner bacterial membrane, allowing across at a minimum small ions like H^+ and K^+.

3. Discussion

In this study we screened a diverse library of 74 natural products against a small panel of *E. coli* MG1655 mutants that were individually resistant to 6 clinically-relevant antibiotics. From this preliminary screen we identified several compounds that were able to restore killing against the antibiotic-resistant cells, including one, perforone, which was effective in combination with polymyxin B against a polymyxin B-resistant strain. Perforone allowed the passage of protons across the inner membrane of *E. coli,* as measured by changes in the cytosolic pH of treated cells, behaviour that likely synergizes with the pore-forming activity of polymyxin B.[25].

Antimicrobial resistance is a growing global public health crisis, and

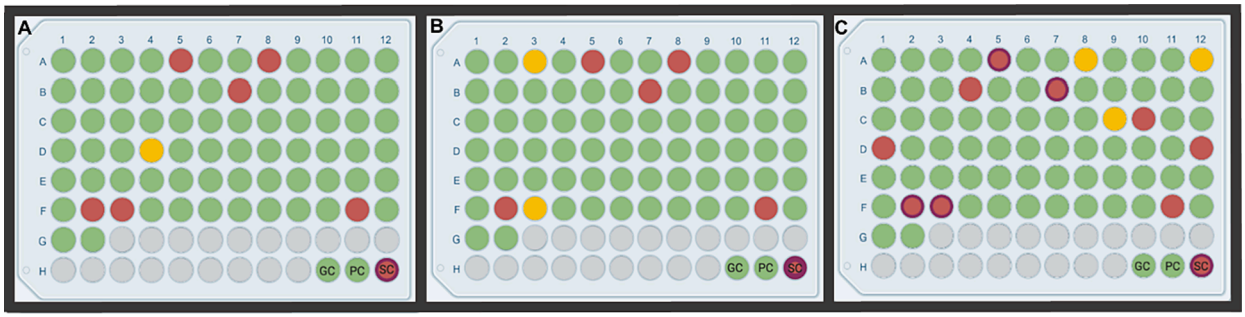

Fig. 4. Comparison of adjuvant activity with PMBN and polymyxin B. A) Polymyxin B-resistant *E. coli* MG1655 the natural product library, B) the same strain with the natural product library and PMBN (8 mg/L), C) the cells in combination with the natural product library and polymyxin B (8 mg/L). Green wells indicate growth (>2 mm bacterial pellet), yellow wells indicate partial growth (<2 mm bacterial pellet), and red wells indicate no visible growth (no bacterial pellet). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

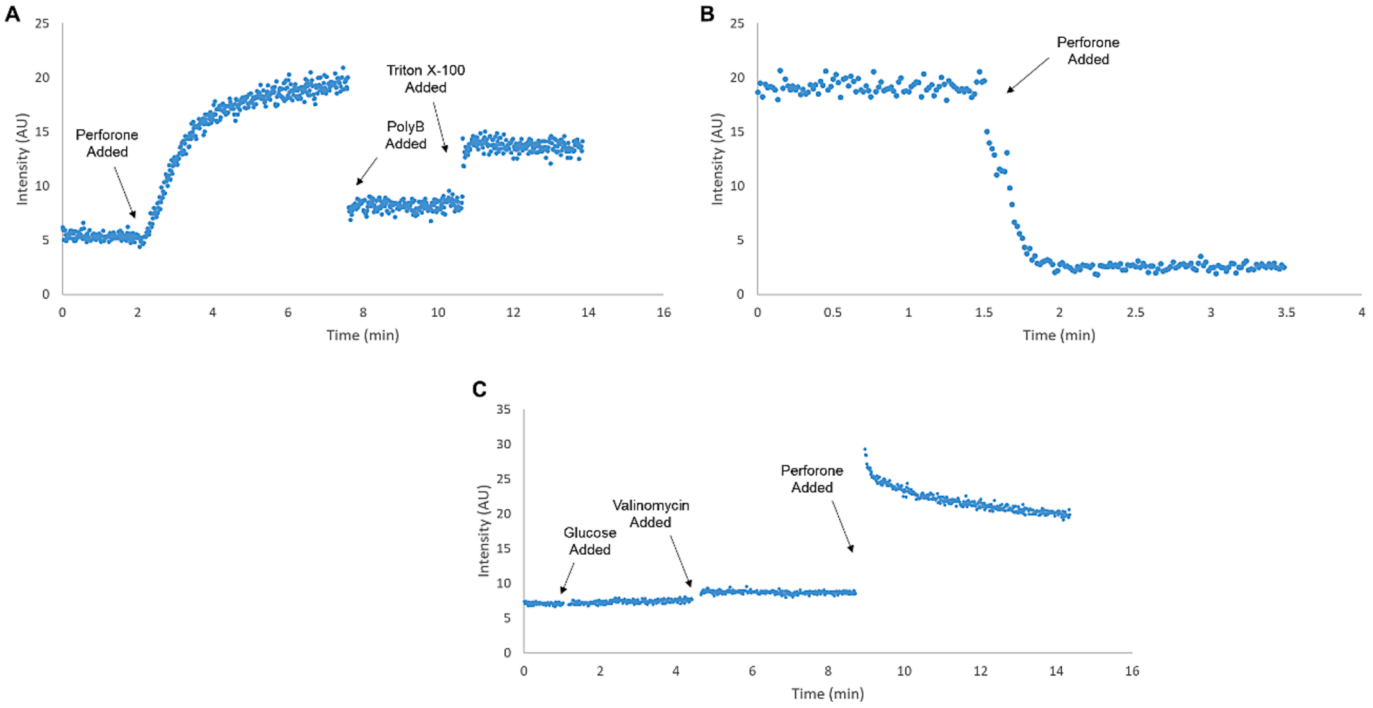

Fig. 5. Measuring changes in membrane integrity with $DiSC_3(5)$ and BCECF, AM. A) Fluorescence slowly increased following the addition of perforone to polymyxin B-resistant *E. coli* MG1655, but decreased to near base levels following the addition of known membrane permeabilizer polymyxin B. Addition of the surfactant triton X-100 only slightly increased fluorescence. B) Addition of perforone to $DiSC_3(5)$ in the absence of cells strongly suppressed fluorescence, indicating that perforone has a significant quenching effect. C) Addition of perforone to cells treated with BCECF, AM and primed with glucose and valinomycin lead to a rapid, significant increase in fluorescence, indicating the flow of protons across the inner bacterial membrane. A slow decrease follows, suggesting the slow quenching of BCECF, AM by perforone. This excitation and emission for $DiSC_3(5)$: 622 nm and 670 nm, excitation and emission for BCECF, AM 500 nm and 522 nm. Data shown is representative of three biological replicates.

new approaches to stem the rising tide of resistant bacteria are urgently needed. Mutations which provide resistance to antibiotics often reduce fitness along other axes,[12] and our results suggest that this vulnerability can be readily exploited. Aside from the positive control doxycycline, only one of the compounds in our panel was able to even partially inhibit the growth of *E. coli* MG1655, but 30 % (22/74) of the compounds inhibited the growth of one or more of the resistant strains. This is far in excess of what would be expected from a normal screen for antibiotic activity, suggesting that it may be possible to significantly prolong the use of common antibiotics against even resistant bacteria, through the discovery of new antibiotic adjuvants.

4. Conclusions

Our findings suggest that antibiotic-resistant mutants of *E. coli* may have their antibiotic sensitivity restored in the presence of adjuvants. Additionally, natural products appear to be an excellent source for the identification of new adjuvants, similar to their well-known potential for antimicrobial activity.

5. Materials & methods

5.1. Chemicals and plasticware

All antibiotics used in this study were purchased from AK Scientific (Union City, USA). 3,3′-Dipropylthiadicarbocyanine Iodide ($Disc_3(5)$) and 2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF, AM) were purchased from Thermo Fisher Scientific (Waltham, USA). Cation-adjusted Muller-Hinton broth (CAMHB) was purchased from MilliporeSigma (Burlington, USA). Sterile 4-well

nunclon treated culture dishes were also purchased from Thermo Fisher Scientific (cat. 167063). Falcon® clear round bottom untreated 96-well polystyrene microplates were used for MIC and checkerboard assays.

5.2. Natural product library

Natural products were sourced from the InterBioScreen Natural Compound collection (Moscow, Russia), an assemblage of natural products weighted towards plant metabolites (60–65 % of the whole). The ChemMine clustering tool was used to arrange the initial library of 68,000 natural products into a Newick hierarchical tree based on structural similarities and physicochemical properties [15]. Natural products were selected from nodes with at least 2 degrees of separation from the highest depth nodes and 2nd highest depth nodes in order to select diverse natural products. The molecular weight cut off was set to 600 Daltons.

5.3. Strains

The source of the strains used in this work are listed in Table 1. *Escherichia coli* MG1655 was a generous gift from Éric Déziel, INRS, Canada. *Staphyloccocus aureus* ATCC 29213 was purchased from Cedarlane (Burlington, Canada). *E. coli* CANWARD 107115 was obtained from the Canadian Antimicrobial Resistance Alliance [30,31].

5.4. Evolution of antibiotic resistance

4-well Nunc-treated plates were raised on one side 8 mm, then molten 0.25 % cation-adjusted Mueller-Hinton agar (CAMHA) was poured to half the height of the well on the lower side (0.45 cm). Once the agar had set (roughly 20 min later), the supports were removed and a second agar solution containing the antibiotic at a concentration 25X the naïve MIC of the respective antibiotic was added to an even depth. Plates were incubated overnight at room temperature to allow diffusion between the two layers. To initiate experiments, up to 75 μL of an overnight bacterial culture was inoculated in a line on the side of the well where the concentration of antibiotic was lowest. The wells were then covered with 3 mL of mineral oil to prevent desiccation and incubated at 37 °C for up to 10 days.

After cells had grown throughout the plate, agar was drawn from the far end of the plate (the area with the highest antibiotic concentration) into a p200 tip. This soft agar was then added to 5 mL of cation-adjusted Mueller-Hinton Broth (CAMHB), which contained the given antibiotic of interest at half its maximal concentration within the SAGE plate. Cells were incubated overnight at 37 °C, then either used directly in subsequent experiments or stored at − 80 °C in 20 % glycerol.

5.5. Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) of all antibiotics was determined by broth microdilution according to Clinical and Laboratory Standards Institute guidelines [32,33]. Briefly, cells were grown overnight in 5 mL CAMHB at 37 °C with shaking at 250 rpm. Cells were then diluted into fresh media to the turbidity of a freshly prepared 0.5 McFarland standard. Cells were further diluted 1:100 in the same media, then mixed 1:1 with media containing the compound of interest in a 96-well plate polystyrene. Growth was evaluated by the naked eye after 16–20 hr of incubation at 37 °C. Growth was ranked based on pellet size as full growth (>2mm pellet), partial growth (≈1mm pellet), minimal growth (<1 mm pellet), and no growth (no pellet).

5.6. High throughput screening

An overnight culture *E. coli* was grown and diluted in MHB as described above for antibiotic susceptibility testing. Cells were then mixed 1:1 with each natural product at a final concentration of 12.5 mg/L. Where indicated the given antibiotic of interest was also added, at half its inhibitory concentration, MIC, a concentration where the cells would still grow in the absence of effective adjuvant. Controls were set in wells *H*10, *H*11, and H12 containing bacteria alone (growth control), the antibiotic alone at half its MIC (positive control), or CAMHB without bacteria (sterility control). Plates were incubated at 37 °C for 16–20 hr, then evaluated as described above for antibiotic susceptibility testing. A natural product was classified as a hit if it caused no inhibition of growth in the absence of antibiotic, but showed no growth, minimal growth, or partial growth in combination with a given antibiotic.

5.7. Checkerboard assays

Bacterial cells were prepared as described above for antibiotic susceptibility testing. 96-well plates were prepared with the antibiotics and their potential adjuvants in increasing concentrations as shown below in Fig. 1. Antibiotic concentrations increased along the x-axis, from 0 to 8x the MIC, while the adjuvant concentration increased along the y-axis from 0 to 100 mg/L. Fractional inhibitory concentration (FIC) indices were calculated according to equation 1 [34]:

$$FIC = FIC_A + FIC_B = \left(\frac{C_A}{MIC_A}\right) + \left(\frac{C_B}{MIC_B}\right)$$

where FIC_A and MIC_A are the fractional inhibitory concentration and minimal inhibitory concentration of compound A, respectively. FIC_B and MIC_B are the fractional inhibitory concentration and minimal inhibitory concentration of compound B. C_A and C_B are the MICs of a compounds A and B in the checkerboard. A FIC value that is less than 0.5 indicates synergism, from 0.5 to 1 indicates additive effects, from 1 to 2 indifference, and greater than 2 antagonism.

5.8. Membrane depolarization assay

Naïve and resistant bacteria were inoculated from −80 °C stocks into 5 mL CAMHB and incubated overnight at 37 °C with shaking at 225 rpm. Cells were washed thrice with 5 mM sodium HEPES buffer, pH 7.4, containing 20 mM glucose, and resuspended in the same buffer plus 0.1 M KCl at an OD of 0.05. The cells were then incubated with 1.2 μM $DiSC_3(5)$ for 10 min in a 3 mL quartz fluorescence cuvette, then placed in a Cary Eclipse fluorescence spectrometer. Fluorescence measurements were taken at excitation $\lambda = 670$ nm and emission $\lambda = 622$ nm. Triton-X was used at a final concentration of 1 % as a positive control.

5.9. BCECF, AM proton permeability assay

Internal bacterial pH was measured with BCECF, AM[29]. Naïve *E. coli* MG1655 cells were inoculated from −80 °C stocks into 5 mL of CAMHB at 37 °C, with shaking at 225 rpm. Cells were spun at 13.3 Kg^{-1} for 1 min, the supernatant was discarded, then the cells were resuspended in 1.5 mL of 50 mM potassium phosphate buffer, pH 8. This process was repeated a total of 3 times, then the cells were suspended in 60 μL of potassium phosphate buffer, pH 8, and stored at room temperature. 5 μL of prepared cells was added to 1 mL of the 5 mM EDTA in the same buffer, alongside 20 μL of 1 mM BCECF, AM. The mixture was then added to a 3 mL quartz fluorimeter cuvette and placed in the fluorimeter. Fluorescence was measured at an excitation wavelength of 504 nm and emission wavelength of 527 nm using the kinetics program on Cary Eclipse fluorescence spectrometer with measurements set every second. After 1 min of measurements 1 μL of 1 M glucose was added. Once the fluorescence intensity stabilized 1 μL of 25 mg/ml valinomycin was then added, followed by either 10 μL of 10 mg/ml perforone or 64 μL of 1 mg/mL polymyxin B. The solution was thoroughly mixed by pipette immediately after each compound was added.

CRediT authorship contribution statement

Michael Mahdavi: Investigation, Writing – review & editing. **Brandon L. Findlay:** Conceptualization, Writing, Acquisition of fundings, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data attached as supplementary information.

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Ethical approval

N/A.

Sequence Information

N/A.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2023.117541.

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