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The ionophore oxyclozanide enhances tobramycin killing of *Pseudomonas aeruginosa* biofilms by permeabilizing cells and depolarizing the membrane potential

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Objectives: To assess the ability of oxyclozanide to enhance tobramycin killing of *Pseudomonas aeruginosa* biofilms and elucidate its mechanism of action.

Methods: Twenty-four hour biofilms formed by the *P. aeruginosa* strain PAO1 and cystic fibrosis (CF) isolates were tested for susceptibility to oxyclozanide and tobramycin killing using BacTiter-Glo™ and cfu. Biofilm dispersal was measured using crystal violet staining. Membrane potential and permeabilization were quantified using DiOC2(3) and TO-PRO-3, respectively.

Results: Here we show that the ionophore anthelmintic oxyclozanide, combined with tobramycin, significantly increased killing of *P. aeruginosa* biofilms over each treatment alone. This combination also significantly accelerated the killing of cells within biofilms and stationary phase cultures and it was effective against 4/6 CF clinical isolates tested, including a tobramycin-resistant strain. Oxyclozanide enhanced the ability of additional aminoglycosides and tetracycline to kill *P. aeruginosa* biofilms. Finally, oxyclozanide permeabilized cells within the biofilm, reduced the membrane potential and increased tobramycin accumulation within cells of mature *P. aeruginosa* biofilms.

Conclusions: Oxyclozanide enhances aminoglycoside and tetracycline activity against *P. aeruginosa* biofilms by reducing membrane potential, permeabilizing cells and enhancing tobramycin accumulation within biofilms. We propose that oxyclozanide counteracts the adaptive resistance response of *P. aeruginosa* to aminoglycosides, increasing both their maximum activity and rate of killing. As oxyclozanide is widely used in veterinary medicine for the treatment of parasitic worm infections, this combination could offer a new approach for the treatment of biofilm-based *P. aeruginosa* infections, repurposing oxyclozanide as an anti-biofilm agent.

Introduction

Cystic fibrosis (CF) is the most common life-shortening genetic disease in Caucasians. It affects 70000 people worldwide and 30000 people in the USA.[1] A mutation in the CF transmembrane conductance regulator gene and subsequent loss of a chloride channel and bicarbonate transport throughout the body causes CF. In the lungs, the loss of coordinated chloride and bicarbonate transport results in the airway mucus becoming thick and dry, hindering the clearance of bacteria and debris.[2,3] This immunological defect makes CF patients prone to recurrent lung infections, including those caused by several members of the MDR 'ESKAPE' pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species).[4] By their mid-to-late teens, the dominant bacterial pathogen colonizing the lungs of CF patients is *P. aeruginosa*.[1] Central to this pathogen's success is its ability to form a biofilm, which is a community of cells embedded in a thick matrix that provides resistance to antibacterial therapies, macrophages and neutrophils.[5,6] Treatment is further hindered by numerous intrinsic antimicrobial resistance mechanisms found in *P. aeruginosa*, including decreased outer membrane permeability, resistance nodulation division (RND) efflux family proteins and chromosomally encoded β-lactamases.[7,8]

Nebulized tobramycin is currently the cornerstone therapy for *Pseudomonas* eradication protocols in CF patients; however, it rarely eradicates this pathogen.[9] Three hundred milligrams of tobramycin is inhaled twice a day for 28 days in on–off cycles, reaching mean sputum concentrations of ∼737 μg/g (∼1576 μmol/g) per dose.[10,11] It has also been found in paediatric CF patients that the mean concentration of bioactive tobramycin within the epithelial

lining fluid is 80 μg/mL per dose ranging from 11 to 265 μg/mL following inhalation.[12] Despite the repeated use of high concentrations of tobramycin, it is estimated that more than half of all CF patients are chronically colonized with *P. aeruginosa* by adulthood.[9] Retrospective studies have shown that the prevention and possible eradication of transient infections by *P. aeruginosa* before a chronic infectious state is underway can extend the lives of CF patients.[13,14] Thus, new therapies that more effectively target this pathogen, especially as it transitions from an acute to a chronic infection, would be of significant clinical benefit for CF patients.

To this end, we previously performed a high throughput screen (HTS) of 6080 compounds from four drug-repurposing libraries (Prestwick, MS2400, LOPAC and focus collection libraries) at the University of Michigan Center for Chemical Genomics to identify novel compounds that enhanced tobramycin killing of mature *P. aeruginosa* biofilms and demonstrated that triclosan exhibits this activity.[15] Here, we report the characterization of another compound identified in the HTS, oxyclozanide. This molecule is a proton ionophore that disrupts proton motive force (PMF) and is approved for the treatment of parasitic worm infections in cattle.[16] We found that oxyclozanide had weak antibacterial activity on its own, while oxyclozanide combined with tobramycin significantly enhanced the rate and degree of aminoglycoside killing of *P. aeruginosa* biofilms. The combination was effective against CF clinical isolates, including a tobramycin-resistant isolate and multiple *S. aureus* isolates growing as biofilms. We further show that oxyclozanide both permeabilized *P. aeruginosa* and functioned as a proton ionophore, reducing the membrane potential ($\Delta\psi$) of *P. aeruginosa*.

Finally, we show that oxyclozanide resulted in increased tobramycin accumulation within cells in biofilms. Our findings suggest that tobramycin combined with oxyclozanide represents a potential new antimicrobial therapy for the treatment of *P. aeruginosa* and *S. aureus* biofilms in CF patients, as well as other biofilm-based infections such as diabetic foot ulcers and burn wounds.[17–19]

Materials and methods

Bacterial strains, culture conditions and compounds

All strains used in this study are listed in Table 1. Bacterial strains were grown in glass test tubes (18×150 mm) at 35°C in CAMHB (Mueller–Hinton Broth II, Sigma–Aldrich) with agitation at 210 rpm. Antibiotics and oxyclozanide were obtained from Sigma–Aldrich. Tobramycin sulphate, gentamicin sulphate and streptomycin sulphate were dissolved in autoclaved deionized water and filter sterilized using 0.22 μm filter membranes (Thomas Scientific). Oxyclozanide and tetracycline were dissolved in 95% and 75% ethanol, respectively.

MICs

MICs were determined as described previously.[20] Briefly, microdilutions were made in a 96-well plate in 10% (v/v) CAMHB diluted in Dulbecco's PBS with magnesium and calcium (DPBS, Sigma–Aldrich). Approximately 1×10^6 cfu/mL were added and incubated for 24 h at 35°C in a humidified chamber with agitation at 150 rpm. MICs were defined as the minimum concentration in which no turbidity greater than background was measured (absorbance at 595 nm), using a SpectraMax M5 microplate spectrophotometer system (Molecular Devices).

Biofilm susceptibility testing using BacTiter-Glo™

We used the Minimum Biofilm Eliminating Concentration (MBEC™, Innovotech) assay to measure antimicrobial susceptibility of biofilms.[21] This assay consists of a lid with 96 polystyrene pegs used to grow biofilms. An overnight culture was washed and diluted to an OD_{600} of 0.001 and seeded into an MBEC™ plate. The plate was then incubated for 24 h at 35°C in a humidified chamber with agitation at 150 rpm. After 24 h, the lid was then washed for 5 min to remove non-adherent cells, transferred to the 96-well treatment plate and incubated for the indicated time at 35°C without agitation. After the incubation, the MBEC™ lid was washed and transferred to a black 96-well ViewPlate (PerkinElmer) filled with 40% (v/v) BacTiter-Glo™ (Promega) diluted in DPBS. The BacTiter-Glo™ microbial cell viability assay is a luminescence assay that determines the number of viable cells present based on quantification of ATP concentration. Cell viability was enumerated using luminescence measured by an EnVison Multilabel Plate Reader (PerkinElmer). Percent killing was calculated as:

$$\%\ reduction = 1 - \left(\frac{luminescence\ of\ treated\ biofilm - background}{luminescence\ of\ untreated\ biofilm - background}\right) \times 100.$$

A calibration curve was previously performed and it was found, using linear regression, that the coefficient of determination was r^2=0.9884 for luminescence versus cfu/mL.[15] Dose–response curves, chequerboard assays and time–kill curves were performed similarly. *S. aureus* biofilms were measured in standard 96-well plates because they do not form biofilms at the air–liquid interface.

Crystal violet staining

To study biofilm dispersal under static conditions, crystal violet staining was performed as previously described.[22] Biofilms formed, as described above, and were stained with crystal violet after a 6 h incubation with treatments.

BacLight™ membrane potential assay and live/dead TO-PRO-3 staining

Twenty-four hour biofilms were formed in glass test tubes (18×150 mm) in 1 mL of 10% (v/v) CAMHB at 35°C and agitated at 150 rpm. Cells were then washed in DPBS to remove non-adherent cells and treated with oxyclozanide and tobramycin for 2 h or 6 h. Following treatment, cells were washed in PBS without magnesium and calcium and the biofilm was disrupted from the air–liquid interface using an autoclaved wooden stick. The cells were stained in 1 mL of PBS for 20 min using the BacLight™ bacterial membrane potential kit in combination with flow cytometry (Thermo Fisher Scientific). This assay uses the dye DiOC2(3), which fluoresces in the FITC channel within all cells. However, greater $\Delta\psi$ drives accumulation and self-association of the dye in the cell cytoplasm, shifting its fluorescence to the phycoerythrin (PE) channel. To this assay we added the TO-PRO-3 iodide live/dead stain that cannot penetrate live cells but can accumulate within cells that have the permeabilized membranes characteristic of dead cells (Thermo Fisher Scientific). Once inside cells with compromised membranes, this dye fluoresces in the allophycocyanin (APC) channel upon intercalating with DNA. Single cell flow cytometry was performed on an LSR II (BD Biosciences), with excitation from 488 mm and 640 mm lasers, and analysed in FITC/PE and APC channels, respectively.

Stationary phase killing assays

Cultures were grown for 20 h and 100 μL/well of stationary phase cells were added to individual wells of a 96-well plate.[23] Treatments were added and the plate was incubated at 35°C without agitation. At hours 2, 4, 6, 8 and 24, aliquots were serially diluted, plated on Dey–Engley neutralizing agar plates, which neutralize the activity of disinfectants and antiseptics

Table 1. Bacterial strains used in this study

Strain	Characteristics	Reference
PAO1	*P. aeruginosa* standard reference strain, isolated in 1954[45]	15
AMT0023_30	*P. aeruginosa* early isolate, patient 6 months old, tobramycin susceptible	46
AMT0023_34	*P. aeruginosa* late isolate, patient 8 years old, tobramycin resistant	46
CF_110_N	*P. aeruginosa* clinical CF isolate, MI, USA	15
CF_110_O	*P. aeruginosa* clinical CF isolate, MI, USA	15
CF_115_J	*P. aeruginosa* clinical CF isolate, MI, USA	15
CF_131_M	*P. aeruginosa* clinical CF isolate, MI, USA	15
USA_300_JE2	MRSA, wound, CA, USA	47
COL	MRSA, Colindale, England	48
Newman (25904)	MSSA, endocarditis, ATCC	49
Wichita (29213)	MSSA, better biofilm former, ATCC	50

(Sigma–Aldrich), and cfu were enumerated. The dilutions that contained 3–30 colonies per 10 μL were used to quantify cfu/mL.

Tobramycin accumulation assay

To measure the accumulation of tobramycin within cells in biofilms, tobramycin was conjugated to Texas Red® (Sigma–Aldrich) using an amine conjugation reaction, as previously described.[24,25] Briefly, conjugated tobramycin was used at a concentration of 250 μg/mL (∼500 μM) alone and in combination with 100 μM oxyclosanide against 24 h biofilms formed in glass test tubes at the air–liquid interface, as described above. Following treatments, biofilms were washed in DPBS for 3 min and then disrupted with autoclaved wooden sticks into 1 mL of 0.2% Triton X-100 to lyse cells (Sigma–Aldrich). Lysed cells were then transferred to spectrophotometer cuvettes (Thermo Fisher Scientific) and read using a SpectraMax M5 microplate spectrophotometer system (λ_{excite}, 595 nm and λ_{emit}, 615 nm).

Results

Oxyclozanide potentiates tobramycin activity against P. aeruginosa and S. aureus biofilms

We previously performed an HTS to identify compounds that enhance tobramycin killing of *P. aeruginosa* biofilms[15] and we report here that the anthelmintic oxyclozanide was identified as a significant hit from that screen. To confirm the results of the screen, *P. aeruginosa* PAO1 biofilms were exposed to 100 μM oxyclozanide or 500 μM tobramycin (∼500-fold the planktonic MIC), alone and in combination, for 6 h and the efficacy was determined using BacTiter-Glo™.[15] Oxyclozanide and tobramycin alone resulted in ∼2-fold fewer viable cells within biofilms compared with untreated controls. However, the combination of oxyclozanide and tobramycin was more effective, eradicating 87% (7.7-fold reduction) of the cells within biofilms (Figure 1). Oxyclozanide combined with the aminoglycosides gentamicin or streptomycin killed 96% (25-fold reduction) and 91% (11.1-fold reduction) of the cells within biofilms, respectively.

We also tested whether oxyclozanide enhanced third- and fourth-generation cephalosporins, β-lactams, fluoroquinolones or tetracycline and determined that in addition to aminoglycosides, oxyclozanide only enhanced tetracycline, increasing killing of the biofilm to 86% (7.1-fold reduction, Figure 1). We also tested the combination against four strains of *S. aureus* and found

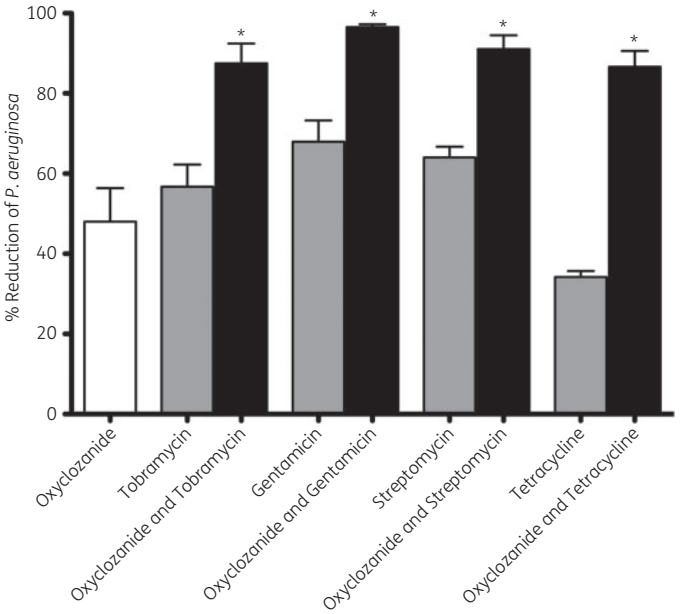

Figure 1. Oxyclozanide enhances aminoglycosides and tetracycline**.** Biofilms (24 h old) were treated for 6 h with oxyclozanide (100 μM), tobramycin (500 μM), gentamicin (100 μM), streptomycin (100 μM) or tetracycline (100 μM), alone and in combination, and the number of viable cells within the biofilms was quantified by BacTiter-Glo™. The assay was performed at least three times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between the combination and each antibiotic alone (*$P<0.05$).

oxyclozanide was effective alone, killing ∼90% of each strain grown as a biofilm (Figure 2). Interestingly, *S. aureus* biofilms were resistant to tobramycin alone and the biofilm increased in cellular number and/or ATP after 6 h of treatment. Supporting our observation, it has been observed clinically that high doses of inhaled tobramycin have no effect on MRSA.[26] This resistance was lost when tobramycin was used in combination with oxyclozanide, resulting in 90% killing, on average, for each strain of *S. aureus*

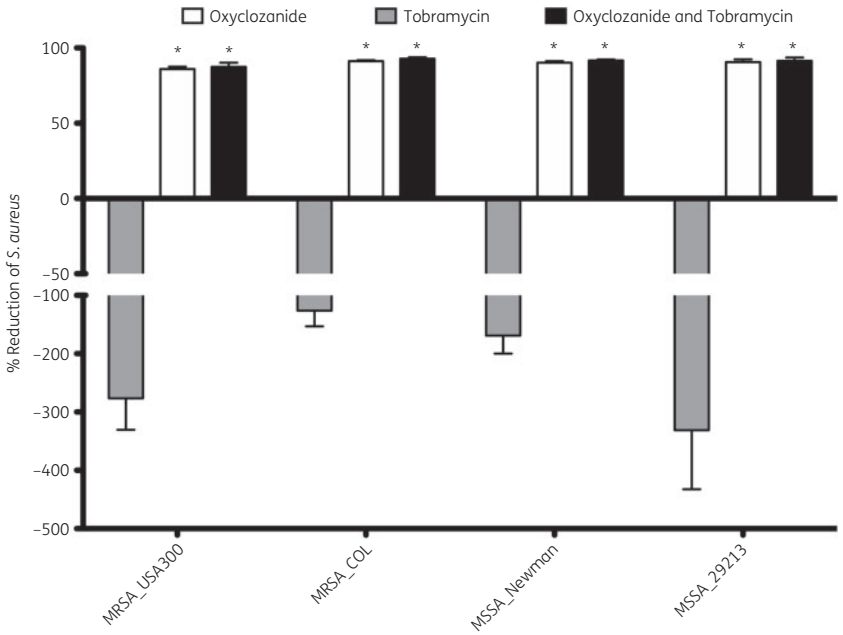

Figure 2. Oxyclozanide kills *S. aureus* biofilms. *S. aureus* biofilms (24 h old) were treated with oxyclozanide (100 μM) or tobramycin (100 μM), alone and in combination, for 6 h. The number of viable cells within the biofilms was quantified by BacTiter-Glo™. The assay was performed twice in triplicate. The results represent means plus the SEM. A two-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between either oxyclozanide alone and tobramycin alone or the combination and tobramycin alone (*$P<0.05$).

tested and a maximum ∼36-fold reduction (MSSA_29213) compared with tobramycin treatment alone.

We also performed MIC assays to determine whether oxyclozanide and these antibiotics were more effective against planktonic cells. The MIC of tobramycin, gentamicin, streptomycin or tetracycline did not change when used in combination with oxyclozanide against PAO1 planktonic cells, suggesting oxyclozanide specifically enhanced the activity of these antibiotics against cells growing as a biofilm (Table S1 available as Supplementary data at *JAC* Online).

Dose–response curves of oxyclozanide and aminoglycosides

To determine the effective ranges of the combinations, we performed dose–response curves. Tobramycin treatment showed modest activity at concentrations between 25 and 400 μM, reducing the number of the cells within biofilms by between 50% and 60% after 6 h of treatment (Figure 3). Similarly, oxyclozanide treatment exhibited modest activity between 25 and 200 μM, killing ∼20%–40% of the cells within biofilms but, at 400 μM, oxyclozanide alone killed ∼80% of the cells within biofilms. The combination of oxyclozanide and tobramycin significantly increased killing of biofilms compared with tobramycin treatment alone between 100 and 400 μM for each compound, with maximum efficacy of 99% killing (100-fold reduction) seen at 200 μM and 400 μM oxyclozanide and tobramycin. Dose–response curves were also performed with gentamicin, streptomycin or tetracycline, alone or in combination with oxyclozanide ranging from 25 to 400 μM (Figure S1). The results were similar with significant killing observed when biofilms were treated with combinations at 100, 200 or 400 μM versus tobramycin alone.

To determine the combinations of lowest possible concentrations of oxyclozanide and tobramycin that resulted in significant killing, we performed chequerboard assays (Figure 4). Biofilms were treated with dilutions of oxyclozanide, ranging from 12.5 to 100 μM, and tobramycin, ranging from 50 to 400 μM. An 86% reduction in cells within the biofilms resulted from 12.5 μM oxyclozanide and 50 μM tobramycin. The greatest reduction was seen at 100 μM oxyclozanide and 400 μM tobramycin, reducing the cells within the biofilms by 92%.

Oxyclozanide accelerates tobramycin killing of cells within biofilms

Another important property of these antimicrobials, which is highly clinically relevant, is the rate at which biofilms are killed. To determine the rate of killing of cells within biofilms by tobramycin, oxyclozanide or the combination, we measured the percentage reduction over the course of 8 h. Oxyclozanide alone did not exhibit significant killing until 6 h (Figure 5). Rather than cell death, tobramycin treatment alone led to an increase in cell number and/or ATP at 2 h, which was lost by ∼4 h. We hypothesize that this increase is due to the mechanism of adaptive resistance that occurs in *P. aeruginosa* in response to aminoglycosides or protein synthesis inhibitors/corruptors. Adaptive resistance, which has been demonstrated in *P. aeruginosa* growing planktonically, in biofilms and in human lungs, is a transient phenotype that occurs within the first 1–2 h of exposure of *P. aeruginosa* to translation inhibitors.[27–29] This phenomenon results in reduced intracellular accumulation of aminoglycosides due to the activation of RND-type efflux pumps such as the MexXY-OprM tripartite efflux pump, among other mechanisms.[28,29] Importantly, this temporary resistance was abolished in the combination treatment as oxyclozanide

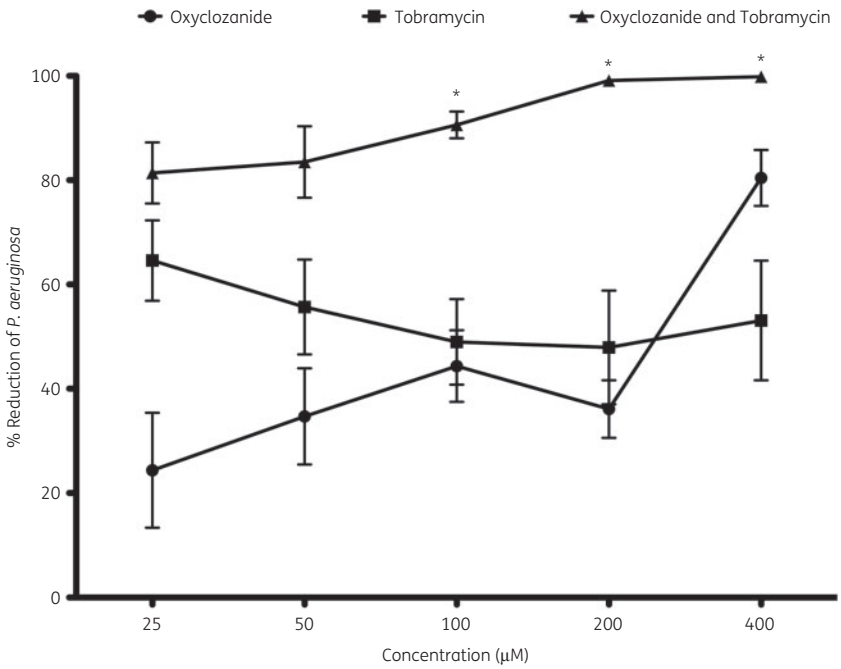

Figure 3. Dose–response curve of oxyclozanide or tobramycin alone and in combination. *P. aeruginosa* biofilms (24 h old) were treated for 6 h with oxyclozanide or tobramycin alone and in combination, in equal molar two-fold dilutions, and the number of viable cells within the biofilms was quantified by BacTiter-Glo™. The assay was performed at least three times in triplicate. The results represent means ± SEM. A one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between the combination and tobramycin alone (*$P<0.05$).

Tobramycin \ Oxyclozanide	100 μM	50 μM	25 μM	12.5 μM	0 μM
400 μM	92% (±4.8)	85% (±5.1)	71% (±3.7)	61% (±3.7)	60% (±1.4)
200 μM	90% (±5.1)	80% (±9.1)	67% (±4.2)	64% (±2.4)	67% (±1.5)
100 μM	88% (±6.9)	84% (±4.2)	80% (±3.9)	77% (±3.8)	76% (±0.46)
50 μM	88% (±7.8)	88% (±5.7)	86% (±5.4)	86% (±2.3)	75% (±1.9)
0 μM	31% (±1.9)	32% (±6.3)	26% (±1.9)	29% (±4.7)	

Figure 4. A chequerboard dilution series of oxyclozanide and tobramycin. Twenty-four hour biofilms were treated with varying combinations of oxyclozanide and tobramycin for 6 h. The number of viable cells within the biofilms was quantified by BacTiter-Glo™. The assay was performed twice in triplicate. The results represent means ± SD. A two-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between the combination and tobramycin alone. Shaded cells indicate significance compared with tobramycin alone ($P<0.05$).

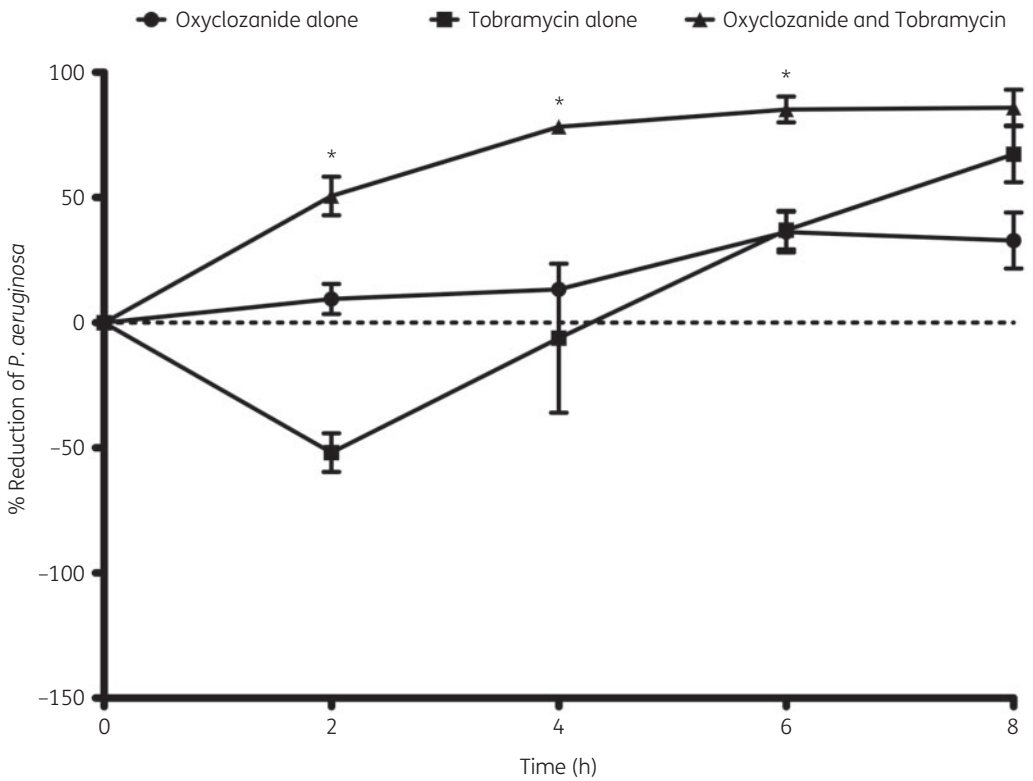

Figure 5. Oxyclozanide accelerates tobramycin killing of *P. aeruginosa* biofilms. Twenty-four hour biofilms were treated with oxyclozanide (100 μM) or tobramycin (500 μM), alone and in combination. At 0, 2, 4, 6, and 8 h, the number of viable cells within the biofilms was determined by BacTiter-Glo™. The assay was performed at least three times in triplicate. The results represent means ± SEM. A one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between the combination and tobramycin alone (*$P<0.05$).

significantly shortened the onset of action of tobramycin from 6 h to 2 h, resulting in 50%, 78% and 85% killing of the cells within the biofilm at 2, 4 and 6 h, respectively (3-, 4- and 6-fold reductions). Thus, at 2 h the combination of oxyclozanide and tobramycin was 100-fold more effective at killing biofilms than tobramycin treatment alone, indicating this combination exhibits both enhanced activity and accelerated action.

Oxyclozanide significantly accelerated the onset of action of gentamicin or tetracycline. However, unlike the other aminoglycosides examined, streptomycin did not exhibit a delay in biofilm killing and minimal enhancement when combined with oxyclozanide was only observed at 6 h (Figure S2B).

Oxyclozanide combined with tobramycin does not increase biofilm dispersal

One explanation for our results thus far is that oxyclozanide induces dispersal of *P. aeruginosa* biofilms. We tested the ability of oxyclozanide to disperse *P. aeruginosa* biofilms using a crystal violet staining assay. Biofilms were treated with oxyclozanide or tobramycin alone and in combination from 50 to 400 μM (Figure 6). Tobramycin significantly reduced biofilm biomass at all concentrations examined. Conversely, oxyclozanide did not cause biofilm dispersal and the combination of tobramycin and oxyclozanide did not cause an increase in biofilm dispersal compared with tobramycin alone. This experiment suggests that oxyclozanide is not acting by inducing biofilm dispersal.

Oxyclozanide and tobramycin are effective against CF isolates of P. aeruginosa

To determine whether the combination of oxyclozanide and tobramycin exhibits widespread activity, we tested its activity against six *P. aeruginosa* CF clinical isolates. Two of the clinical isolates were isolated longitudinally from the same patient at 6 months of age and 8 years of age: AMT0023_30 and AMT0023_34, respectively. In addition, clinical isolates CF_110_N and CF_110_O were isolated longitudinally from the same patient 3 months apart (the strains are described in Table 1). Importantly, these clinical isolates exhibited distinct colony morphologies on blood agar plates. These six clinical isolates were treated with 100 μM oxyclozanide or 500 μM tobramycin, alone or in combination. All isolates displayed no to modest susceptibility to oxyclozanide or tobramycin treatment alone and exhibited the greatest susceptibility to the combination (Figure 7). This increase was statistically significant in 4/6 isolates. Importantly, the combination significantly enhanced killing of strain AMT0023_34, which overexpresses the RND-type MexXY-OprRM tripartite multidrug efflux pump, rendering it resistant to tobramycin,[30] resulting in an ∼16-fold reduction in viable AMT0023_34 cells compared with tobramycin treatment alone (Figure 7).

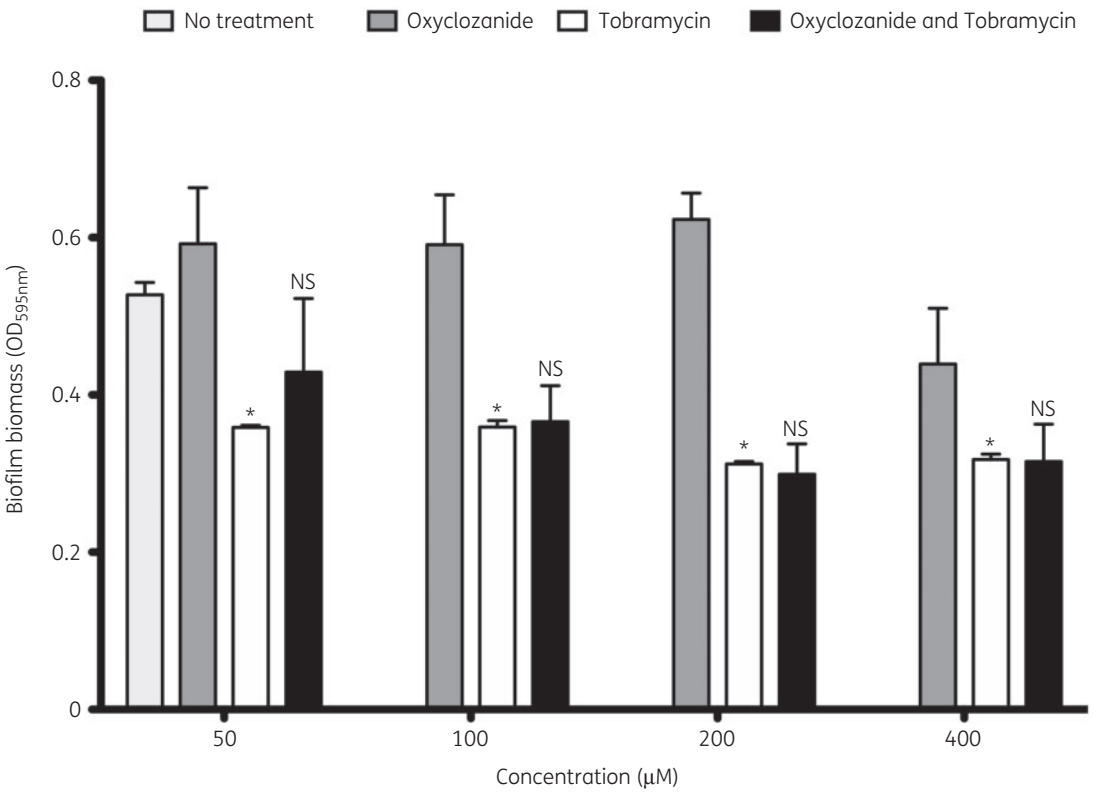

Figure 6. Oxyclozanide does not induce biofilm dispersal. Twenty-four hour biofilms were treated with oxyclozanide, tobramycin or the combination, in equal molar two-fold dilutions, for 6 h. The effect on biofilm biomass was quantified by staining with crystal violet. The experiment was performed twice in triplicate. The results represent the means plus SEM. A one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between tobramycin alone and the untreated control (*$P<0.05$) and between the combination and tobramycin alone. NS, not significant.

Oxyclozanide and tobramycin are more effective against stationary phase cells

We hypothesized that the combination therapy may also be effective against stationary phase cells that mimic cells growing in biofilms as they are in a lower metabolic state and enriched for persister cells. Persister cells are metabolically dormant and thus highly tolerant to antimicrobials that target actively growing cells.[31,32] To test this prediction, we analysed the activity of oxyclozanide (100 μM), tobramycin (50 μM) or the combination on cultures of *P. aeruginosa* grown for 20 h. Similar to our results with biofilms, we found that the combination of oxyclozanide and tobramycin significantly reduced viable cells as measured by quantifying cfu, exhibiting a 10-fold reduction in viable cells compared with tobramycin alone at 8 and 24 h (Figure 8).

Oxyclozanide causes both cellular permeabilization and the loss of a $\Delta\psi$ in P. aeruginosa biofilms

The mode of action of oxyclozanide in parasitic flatworms is the uncoupling of oxidative phosphorylation by the translocation of protons through the inner mitochondrial membrane, disrupting the PMF and eliminating the $\Delta\psi$.[16] Additionally, oxyclozanide has been shown to induce cellular permeabilization in the Gram-positive pathogen *S. aureus*.[33] However, neither ionophore activity nor membrane disruption by oxyclozanide has been demonstrated in Gram-negative bacteria or bacteria growing in a biofilm.

To test whether oxyclozanide exhibits ionophore activity against *P. aeruginosa*, biofilms exposed to tobramycin, oxyclozanide or the combination were assayed with BacLight™, which consists of the dye DiOC2(3). This dye is a marker for cellular $\Delta\psi$ and cells were analysed by flow cytometry (representative plots are shown in Figure S3). Because oxyclozanide has been shown to cause cell permeabilization, we also added to this assay TO-PRO-3 iodide, which stains the DNA of bacteria with disrupted outer membranes, characteristic of dead cells. The combination of these two dyes allowed us to determine both cellular permeabilization and $\Delta\psi$ in the same assay. For those cells that have an intact outer membrane and do not stain with TO-PRO-3, import of DiOC2(3) into the cell cytoplasm, where self-association of this dye shifts its fluorescence spectrum to the PE channel, indicates an intact $\Delta\psi$ (Figure S3).

TO-PRO-3 staining indicated that oxyclozanide caused significant cellular permeabilization and death, increasing the population of permeabilized cells from 10% in untreated biofilms to 27% when treated for 2 h (Figure 9a). Tobramycin treatment alone similarly increased permeabilized cells compared with no treatment, but this increase was not statistically significant compared with oxyclozanide alone (Figure 9a). This is not unexpected, given both oxyclozanide and tobramycin treatment alone caused an ∼50%

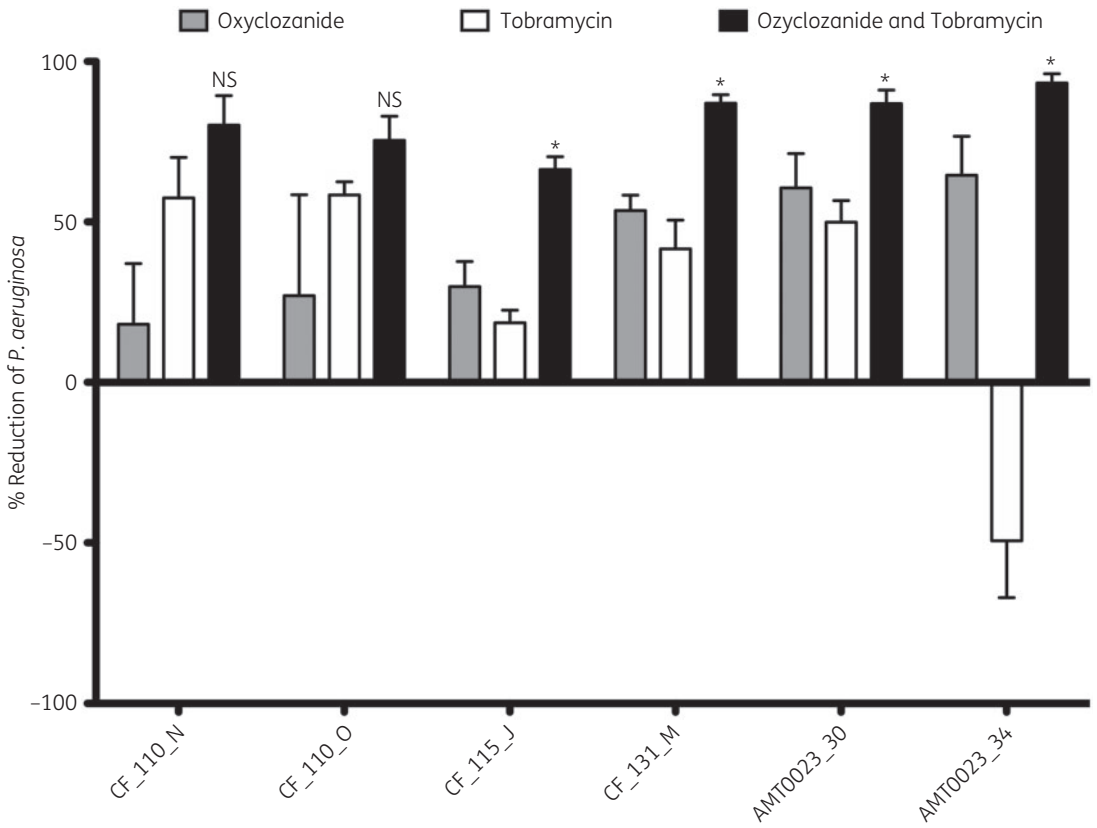

Figure 7. Oxyclozanide is broadly active against *P. aeruginosa*. Twenty-four hour biofilms were treated with oxyclozanide (100 μM) or tobramycin (500 μM), alone and in combination for 6 h. The number of viable cells within the biofilms was quantified by BacTiter-Glo™. The assay was performed three times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to determine statistical significance between the combination and tobramycin alone (*$P<0.05$; NS, not significant).

reduction of viable cells within biofilms (Figure 1). However, oxyclozanide combined with tobramycin significantly increased the population of permeabilized and dead cells compared with tobramycin treatment alone: 59% versus 19%, respectively. Similar results were obtained after 6 h of treatment. TO-PRO-3 staining indicated that oxyclozanide increased the population of permeabilized and dead cells from 2.6% in untreated biofilms to 13% when treated for 6 h (Figure S4A). Tobramycin did not significantly cause permeabilization and death. However, the combination of oxyclozanide and tobramycin significantly increased the population of permeabilized and dead cells to 38% compared with 8% of permeabilized cells with only tobramycin treatment.

Subsequent analysis of the cells that were living (i.e. TO-PRO-3 negative) by DiOC2(3) staining demonstrated that oxyclozanide showed significant ionophore activity against cells within biofilms after 2 h of treatment (Figure 9b). The population of cells maintaining a $\Delta\psi$ was reduced from 39% in untreated biofilms to 19% in oxyclozanide-treated biofilms. Alternatively, tobramycin treatment alone significantly increased the population of cells maintaining a $\Delta\psi$ to 69%. This dramatic increase in the population of cells maintaining a $\Delta\psi$ is, again, indicative of adaptive resistance.[28,29] Oxyclozanide combined with tobramycin abolished this effect, reducing the population of cells maintaining a $\Delta\psi$ to 19%. Similar results were obtained after 6 h of treatment with the exception that tobramycin did not result in a significant increase in the population of cells maintaining a $\Delta\psi$ (Figure S4B). This outcome is expected if the increased $\Delta\psi$ is due to adaptive resistance as this is known to be temporal in nature and a short-lived response.[34] Strikingly, by 6 h virtually no cells exhibited a $\Delta\psi$ when treated with the combination of oxyclozanide and tobramycin.

Dose–response curves were performed with oxyclozanide to further characterize its effects on the $\Delta\psi$ and its ability to permeabilize and kill cells within biofilms. Untreated controls were replotted for comparison. After 2 h of treatment, oxyclozanide caused permeabilization and cell death at 200 μM (Figure S5), resulting in very few cells with a $\Delta\psi$. However, this effect was lost from 100 to 25 μM while a significant reduction of the $\Delta\psi$, compared with no treatment, could be observed. In this experiment, significant permeabilization compared with the untreated control was not observed at 100 μM, which contrasts with our previous results observing permeabilization at this concentration (Figure 9 and Figure S4). However, permeabilization was clear at 200 μM. We interpret these results to indicate that oxyclozanide can permeabilize *P. aeruginosa* biofilms between 100 and 200 μM and at the lower end of these concentrations there can be variability within different experiments.

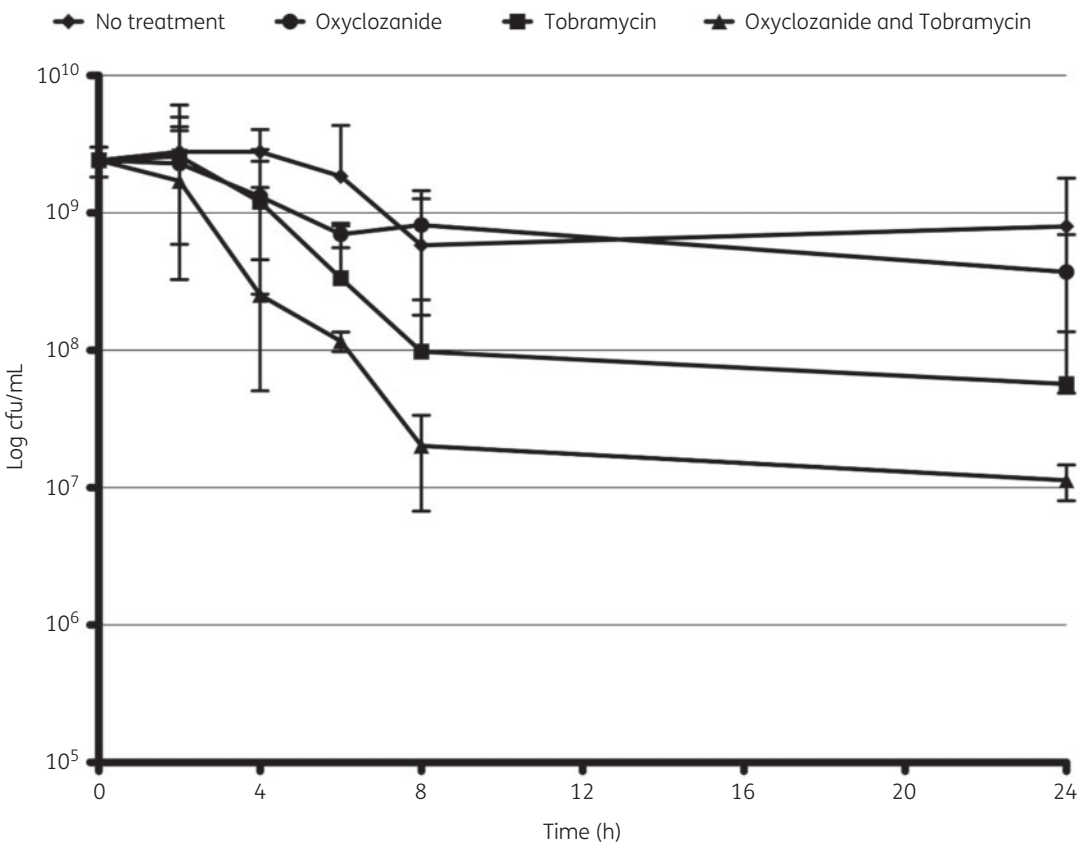

Figure 8. Tobramycin combined with oxyclozanide is more effective against stationary phase cells. Twenty-hour-old stationary phase cells were treated with oxyclozanide (100 μM) or tobramycin (50 μM), alone and in combination. At 0, 2, 4, 6, 8 and 24 h post-treatment, aliquots were enumerated for cfu/mL. The experiment was performed two times in triplicate. The results represent means ±SD.

Oxyclozanide increases cell-associated tobramycin in mature P. aeruginosa biofilms

Since oxyclozanide causes cellular permeabilization, reduces $\Delta\psi$ and accelerates the activity of tobramycin, we sought to determine whether oxyclozanide increased the accumulation of cell-associated tobramycin. To do this, we fluorescently labelled tobramycin with Texas Red® and measured its accumulation within cells of a mature biofilm following 30 min of treatment. We found oxyclosanide in combination with tobramycin resulted in significantly more tobramycin accumulation within cells (Figure 10).

Discussion

Drug repurposing affords many benefits for developing novel antimicrobials, including reduced costs and accelerated deployment.[35] We recently demonstrated that triclosan combined with tobramycin can be repurposed to eradicate biofilms formed by both Gram-negative and Gram-positive bacteria.[14] Here, we show that the anthelmintic drug oxyclozanide enhances the activity of aminoglycosides and tetracycline to eradicate antibiotic-tolerant biofilms. The combination therapy exhibited more rapid killing and was effective against a tobramycin-resistant *P. aeruginosa* clinical isolate. Importantly, we found that the combination had activity against cells within biofilms formed by both Gram-negative and Gram-positive pathogens. Young CF patients are often colonized with *Staphylococcus* organisms, making tobramycin combined with oxyclozanide an applicable therapy for both early and late CF lung pathogens.[1]

Oxyclozanide is given orally at a dose of 10–15 mg/kg (~25–37 μmol/kg).[16] We envisage the use of oxyclozanide in conjunction with tobramycin at a concentration between 50 and 100 μM as an inhaled aerosolized solution for the treatment of CF. This route of administration provides many benefits including reduced side effects and enhanced activity, which could deliver higher concentrations locally while limiting systemic exposure.[36,37] We also imagine the use of tobramycin combined with oxyclozanide as a topical therapy for the treatment of diabetic and burn wounds at concentrations similar to those currently used clinically in animals.

Oxyclozanide has been reported to have two modes of action. It has been shown to function as an ionophore in parasitic worms[16] and it was found to permeabilize the Gram-positive bacterium *S. aureus* growing planktonically.[29] Our results indicate that oxyclozanide exhibits both activities against *P. aeruginosa* biofilms, which could contribute to the observed increase of cellular-associated tobramycin in the presence of oxyclozanide, and we suggest that each activity is important for oxyclozanide and tobramycin enhanced activity (Figure 11).

The PMF is one component of the $\Delta\psi$ that has been shown to contribute to efflux of antibiotics and adaptive resistance in bacteria. A gradient of protons across the inner membrane drives the

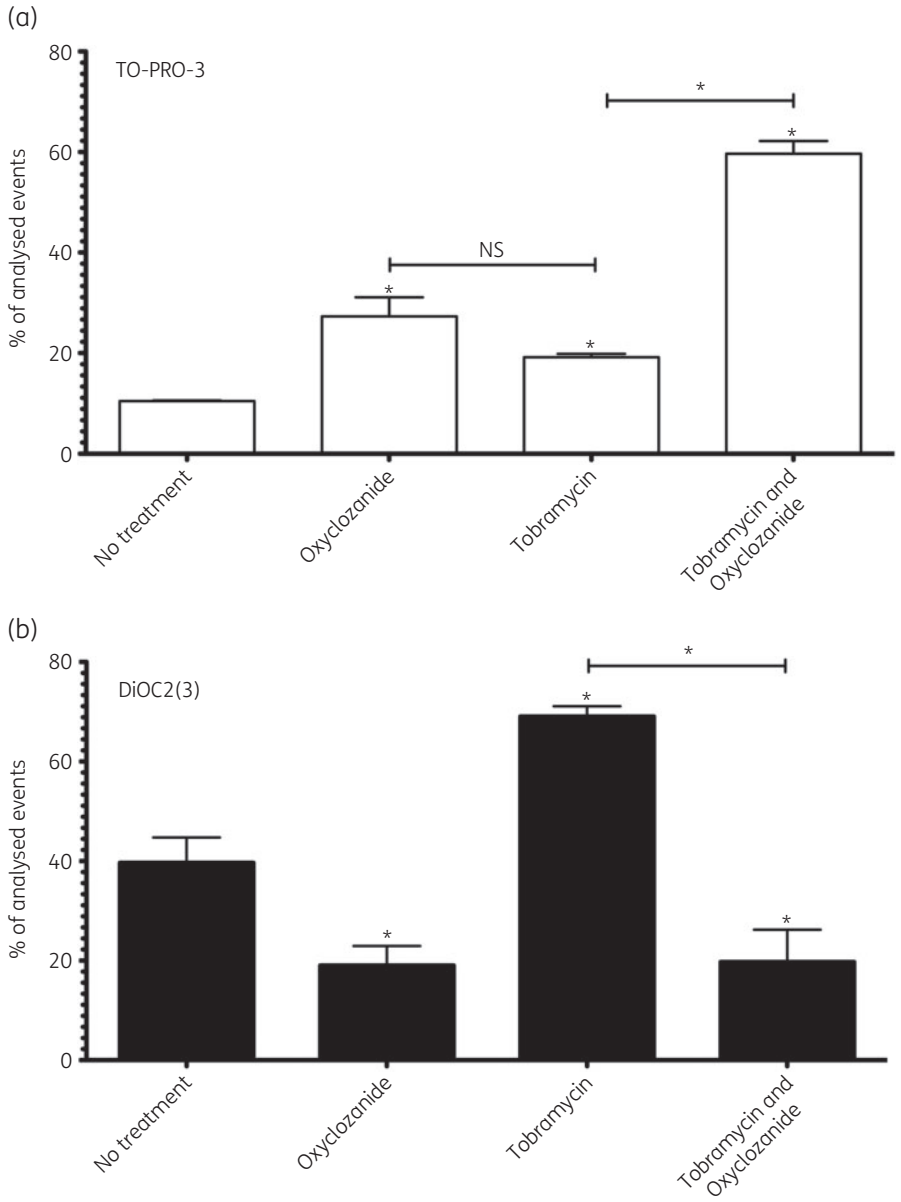

Figure 9. Oxyclozanide induces permeabilization and depolarizes the $\Delta\psi$ of *P. aeruginosa*. Twenty-four hour biofilms were treated with oxyclozanide (100 μM) or tobramycin (500 μM), alone and in combination, for 2 h. (a) Cells were stained with TO-PRO-3 and analysed by flow cytometry to determine the number of cells that were permeabilized. (b) Cells were also stained with DiOC2(3) and analysed by flow cytometry to determine the number of cells maintaining a $\Delta\psi$. Permeabilized cells were excluded from $\Delta\psi$ analysis. The experiment was performed two separate times in duplicate. The results are percentage averages plus the SEM. Percentage values indicate the average relative abundance of events within each gate normalized to the total number of events analysed, excluding artefacts, aggregates and debris. A one-way ANOVA followed by Dunnett's multiple comparison *post hoc* test was used to determine statistical significance between each treatment and the untreated control (*$P<0.05$) and oxyclozanide treatment alone was compared with tobramycin treatment alone in panel (a) (NS, not significant). A one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used to compare tobramycin to the combination in panel (a) and (b) (*$P<0.05$).

production of ATP as they are imported via the phosphorylation of ADP to ATP by ATP synthase.[38] Importantly, the PMF also functions as the energy source for efflux pumps to export antimicrobials, including those in the RND superfamily. The RND efflux superfamily is responsible for MDR and adaptive resistance in *P. aeruginosa*.[28,34,39] Our results are consistent with previous results as we observed that *P. aeruginosa* growing in a biofilm increases the $\Delta\psi$ in response to tobramycin (Figure 9b), which may be responsible for the increased ATP that we observed during the first few hours of tobramycin treatment (Figure 5). Whether uncoupling of the $\Delta\psi$ by oxyclozanide inhibits efflux pump activity remains to be examined. However, one of the most intriguing results presented here is that the combination of oxyclozanide and tobramycin overcomes tobramycin resistance in the well-characterized AMT0023_34

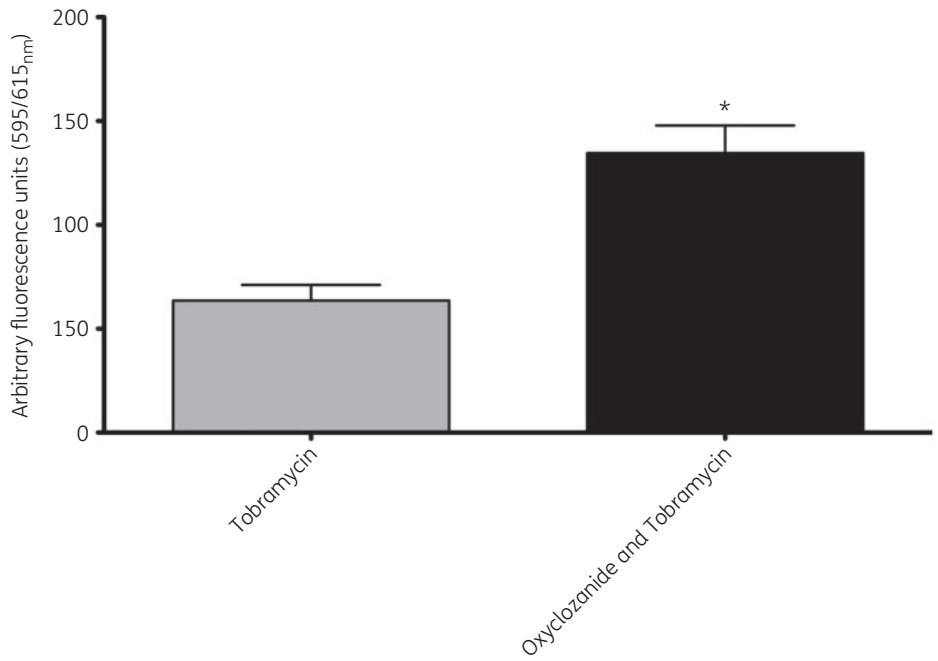

Figure 10. Oxyclozanide results in increased cellular accumulation of tobramycin. Twenty-four hour biofilms grown in glass test tubes were treated for 30 min with oxyclozanide (100 μM) and Texas Red®-labelled tobramycin (250 μg/mL). After washing off the planktonic cells, the biofilms were lysed with 0.2% Triton-X 100® and relative fluorescence units with excitation at 595 nm and emission at 615 nm were measured to quantify labelled tobramycin. The assay was performed twice in duplicate. The results represent means plus the SEM. An unpaired *t*-test was performed comparing tobramycin versus oxyclosanide and tobramycin (*$P<0.05$).

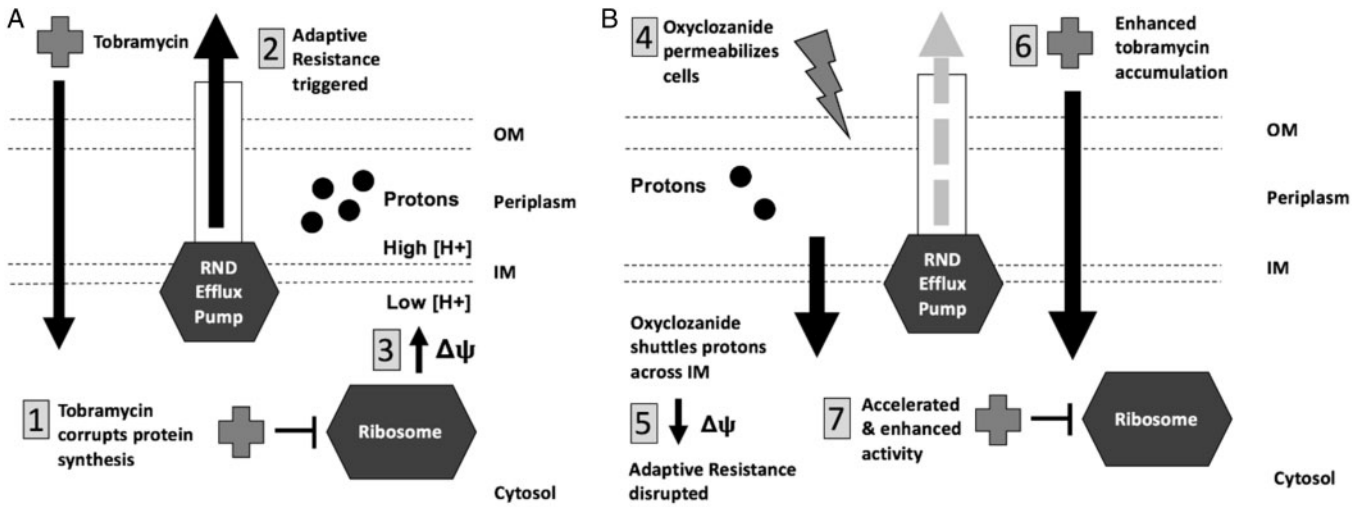

Figure 11. Oxyclozanide sensitizes *P. aeruginosa* to tobramycin by depolarizing the Δψ and further permeabilizing cells. (a) (1) Tobramycin corrupts protein synthesis triggering adaptive resistance, which includes the (2) induction of RND-type efflux pumps such as MexXY-OprM, resulting in reduced accumulation of tobramycin within the cytosol.[42,43] (3) During this time, an increase in Δψ is observed driving RND-type efflux pump activity and providing temporary resistance to tobramycin. (b) (4) Oxyclozanide permeabilizes cells and (5) shuttles protons across the IM, depolarizing the Δψ. We hypothesize that both of these activities interfere with adaptive resistance (6), allowing for greater accumulation of tobramycin within cells resulting in (7) accelerated activity and increased effectiveness. OM, outer membrane; IM, inner membrane.

clinical isolate (Figure 7). Resistance of this isolate is known to be driven by overproduction of efflux pumps.[30] This result is suggestive that the combination negatively influences efflux pump activity in *P. aeruginosa* and, further, it can have clinical efficacy against certain classes of tobramycin-resistant isolates of *P. aeruginosa.*

Based on our data, we propose a model where oxyclozanide enhances tobramycin accumulation in *P. aeruginosa* by abolishing adaptive resistance through permeabilizing the cells and disrupting the Δψ (Figure 11). This causes increased tobramycin accumulation, yielding accelerated activity and increased effectiveness.

In support of this model, oxyclozanide inhibited the large increase in the population of cells maintaining a $\Delta\psi$ that was induced in response to tobramycin (Figure 9b) and significantly increased the rate of killing by tobramycin (Figure 5). Uncoupling the contributions of permeabilization and inhibition of $\Delta\psi$ in the enhancement of tobramycin by oxyclozanide requires further investigation.

Although an intact PMF is considered one of the main mechanisms of aminoglycoside influx into cells, it is well known that, independently of respiration, aminoglycosides can accumulate within cells through a process termed self-promoted uptake.[40] In this process, aminoglycosides interact with the membrane surface by displacing cations, creating 'cracks' or 'fissures' in the outer membrane of cells. This leads to the diffusion of aminoglycosides into the cytosol of cells, which in turn contributes to additional membrane damage by the insertion of misfolded proteins in the outer membrane.[40,41] Thus, at the concentrations we are examining, it is likely that the increased killing in the absence of a $\Delta\psi$ can largely be attributed to the effects of self-promoted uptake combined with the permeabilizing effects of oxyclozanide.

We found that oxyclozanide only enhanced antimicrobials that targeted the ribosome, namely aminoglycosides and tetracycline. We hypothesize that this is due to the fact that only ribosomal inhibitors (and not antimicrobials that target other cellular pathways) trigger the induction of MexXY efflux pumps and adaptive resistance.[42,43] Oxyclozanide overcomes this mechanism by both permeabilizing and disrupting the $\Delta\psi$ of cells within biofilms.

Many bacterial species, including *S. aureus* and *P. aeruginosa*, can be found growing as biofilms in non-healing chronic wounds such as diabetic foot ulcers and burns.[17–19] Previously, it has been shown that oxyclozanide has activity against planktonic *S. aureus* and cancerous cells, indicating broad applicability.[29] The repurposing of veterinary drugs has a proven history of success, notably the ionophore anthelmintic ivermectin, which has been repurposed for the treatment of several diseases in humans.[44] Oxyclozanide combined with tobramycin could be a potential new treatment for *P. aeruginosa* and *S. aureus* infections in CF patients as well as diabetic foot ulcers and burn wounds.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S5 are available as Supplementary data at *JAC* Online.

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