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Exogenous Pyocyanin Alters *Pseudomonas aeruginosa* Susceptibility to Ciprofloxacin

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Abstract: This *in vitro* cell-based study identified the contributing role of pyocyanin in the resistance of *Pseudomonas aeruginosa* to ciprofloxacin. **Problem statement:** *P. aeruginosa* is the major pathogen in the Cystic Fibrosis (CF) lung with pyocyanin being a critical component of its virulence. Prevalence is high and, once acquired, chronic infection is difficult to eliminate. Ciprofloxacin remains a crucial oral agent effective against *P. aeruginosa*, but resistance is increasingly reported. **Approach:** Here we examined the extent to which exogenously added pyocyanin affected *P. aeruginosa* susceptibility to ciprofloxacin and the contribution of altered efflux activity and biofilm production with the aim of ultimately increasing sensitivity. **Results:** Metabolic conversion of resazurin to resorufin was used as an index of bacterial cell growth while fluorescent measurement of acriflavine efflux and crystal violet staining was used as markers of efflux activity and biofilm production, respectively. Pyocyanin (100 μ M) added exogenously decreased susceptibility of two *P. aeruginosa* strains, PAO1 and ATCC 27853 to ciprofloxacin at 125 and 500 μ g L⁻¹, respectively. Exogenously added pyocyanin decreased efflux activity in both strains while biofilm production was significantly increased. **Conclusion:** We conclude that increased biofilm production may contribute to the observed decreased susceptibility of *P. aeruginosa* to ciprofloxacin. Ciprofloxacin is a crucial orally effective agent against *P. aeruginosa* with resistance being increasingly reported. This initial study highlights a potential mechanism that may underlie this resistance which may be of clinical interest. Further studies using additional antibiotics and the pyocyanin precursor 1-hydroxyphenazine are required.

Key words: Cystic fibrosis, *Pseudomonas aeruginosa*, pyocyanin, ciprofloxacin, drug resistance, Chlorophenylhydrazone (CCCP), Over-Production Phenotype (OP), hydroxyphenazine, Liverpool Epidemic Strain (LES), Crystal Violet (CV), Analysis Of Variance (ANOVA)

INTRODUCTION

Cystic Fibrosis (CF) is one of the most frequently occurring fatal genetic diseases seen among the Caucasian population (Lau *et al.*, 2004; Grant *et al.*, 2009). Infection with *Pseudomonas aeruginosa* is responsible for the premature death of over 80% of CF patients (Lau *et al.*, 2004) and a large proportion of the morbidity associated with the disease (Rosenfeld *et al.*, 2003). Although current antibiotic therapeutic strategies have decreased the morbidity and mortality associated with CF, *P. aeruginosa* permanently colonises these patients' lungs, with eradication

almost impossible (Lau *et al.*, 2004). Ciprofloxacin is a crucial orally effective agent against *P. aeruginosa* although resistance is increasingly reported (Hodson *et al.*, 1987; Masaadeh and Jaran, 2009).

P. aeruginosa populations in sputum samples taken from CF patients are mixed with respect to both genotype and phenotype, even when isolates represent the same clone (Fothergill *et al.*, 2007). The spread of a drug-resistant strain of *P. aeruginosa* [named the Liverpool Epidemic Strain (LES)] in CF patients has been demonstrated by Cheng *et al.* (1996) whilst Fothergill *et al.* (2007) further showed that some of these isolates express an unusual phenotype

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characterised by the early and over-expression of QS-regulated virulence genes, including those encoding the secretion of the virulence-factor pyocyanin, referred to as the Over-Production Phenotype (OP) (Fothergill *et al.*, 2007). Pyocyanin has been shown to have a number of toxic effects on the respiratory system, with initial research focussed on the pro-oxidant properties of pyocyanin which results in morphological changes culminating in cellular damage (Dietrich *et al.*, 2006). Dietrich *et al.* (2006) recently highlighted the signalling events associated with pyocyanin in *P. aeruginosa* strains PAO-1 and PA14. Pyocyanin was shown to act as a physiological signal for the upregulation of certain Quorum Sensing (QS) controlled genes during stationary phase, including mexGHI-opmD genes involved in efflux, redox process and iron acquisition (Dietrich *et al.*, 2006). Fothergill *et al.* (2007) also found interesting correlations between the OP phenotype and resistance or susceptibility patterns to various antipseudomonal antibiotics (Fothergill *et al.*, 2007). The author suggests that pyocyanin itself may play a direct role in the observed link between antimicrobial susceptibility and the OP phenotype (Fothergill *et al.*, 2007).

This study was conducted to examine the effect of exogenously added pyocyanin in *P. aeruginosa* PAO1 and ATCC 27853 susceptibility to the quinolone antibiotic, ciprofloxacin. Furthermore we have investigated the contribution exogenously added pyocyanin has towards altered efflux activity and biofilm production.

MATERIALS AND METHODS

Pyocyanin was purchased from Sapphire Bioscience (Redfern, NSW, Australia). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Preparation of cultures: Wild-type *Pseudomonas aeruginosa* strains PAO-1 and ATCC 27853 were stored at -70°C in glycerol and subcultured onto Luria-Bertani (LB) agar (Oxoid, Basingstoke, UK) slopes before testing. Overnight cultures of *P. aeruginosa* were prepared in Luria-Bertani (LB) broth and incubated for 18 h at 37°C on a shaker at 200 rpm.

Assessment of bacterial cell growth: In order to detect subtle differences in *P. aeruginosa* PAO1 and ATCC 27853 sensitivity to ciprofloxacin methods were adapted from the method described by Sarker *et al.* (2007) using resazurin in a 96-well microtitre plate format. Briefly, the inoculum was adjusted to a final

density of 5×10^5 CFU mL⁻¹ in a 96-well plate and incubated with 100 µM pyocyanin. Following the addition of 0.5% resazurin ciprofloxacin (31-1000 µg L⁻¹) was added and plates were incubated at 35°C, ambient air. After 20 h resorufin fluorescence was measured (Excitation 535 nm; Emission 590 nm) using a Fluoroskan Ascent microplate fluorometer. Appropriate cell free controls were included to test for potential interaction between resazurin and pyocyanin.

Efflux activity assay: The method for assessing efflux activity was adopted from the protocol of Poole and Srikumar (2001). Briefly, cultures were adjusted to 1×10^8 CFU mL⁻¹ and incubated with 20 µM carbonyl-cyanide-M-Chlorophenylhydrazone (CCCP) and acriflavine. Phenylalanine-arginine b-naphthylamide (PabN) (40 µM) was included as positive controls for the inhibition of efflux. Change in extracellular acriflavin fluorescence, indicative of efflux pump activity was measured using a Fluoroskan Ascent microplate fluorometer (Excitation 450 nm; Emission 500 nm).

Biofilm assay: The colorimetric assay for biofilm quantification with Crystal Violet (CV) staining was adapted from a previously described method (Jackson *et al.*, 2002) and performed under semi-static conditions. Briefly, 1cm lengths of silicone (1.58 mm ID) tubes were used as carriers in the study (Sigma Aldrich, St Louis, MO) and prepared by washing with alcohol-acetone (1:1) and soaking in 70% ethanol for 15min. Following three rinses with sterile distilled water the carriers were transferred to 6 well plates and incubated with adjusted cell cultures (10^6 CFU mL⁻¹) in the presence of pyocyanin (100 µM) for 36h at 37°C. Following incubation the carriers were washed with both water and saline, fixed with methanol (99%) and stained with 0.05% CV in water. Adherent CV was then extracted with 3 mL of 33% acetic acid and absorbance of the extract was read at 550 nm using a Titretek Multiskan MKII microtiter plate reader (Labsystems). Experiments were repeated in triplicate and appropriate controls included.

Data analysis: Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA). Significance was determined by one-way Analysis Of Variance (ANOVA) with Dunnett's multiple comparison test or Student's t-test. Experiments were performed a minimum of three times and the results are presented as the means ± standard deviations. Significance levels were defined as *p<0.05, **p<0.01, ***p<0.001.

RESULTS

Effects of pyocyanin on *P. aeruginosa* susceptibility to Ciprofloxacin: Ciprofloxacin has been demonstrated to have good activity against both PAO-1 and ATCC 27853 with MIC values of 125 and 500 $\mu\text{g L}^{-1}$ respectively. In this study the exogenous addition of PCN (100 μM) did not affect susceptibility of either strain to ciprofloxacin determined visually (not shown). However using metabolic conversion of resazurin to resorufin as a more sensitive measure of bacterial growth we show that PCN (100 μM) decreased susceptibility of both PAO-1 and ATCC27853 to ciprofloxacin at 125 $\mu\text{g L}^{-1}$ ($p < 0.001$) and 500 $\mu\text{g L}^{-1}$ ($p < 0.01$) respectively compared to control cultures (Fig. 1). PCN did not affect the growth of either strain by itself (not shown).

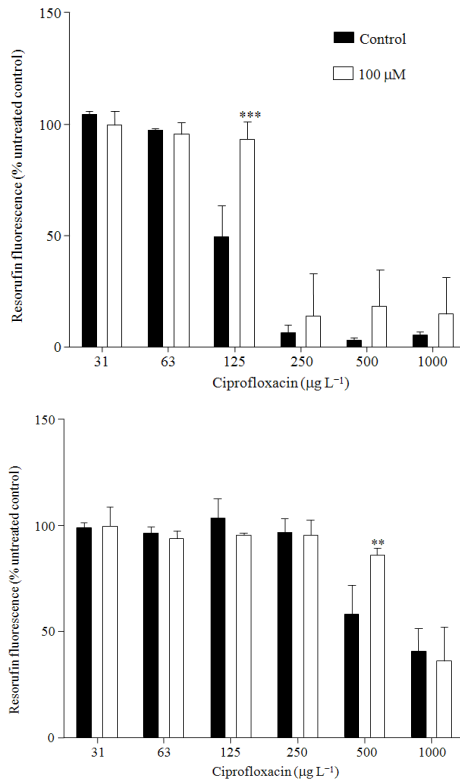


Fig. 1: Pyocyanin decreases susceptibility of *P. aeruginosa* to ciprofloxacin. *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC 27853 were incubated with ciprofloxacin (31-1000 $\mu\text{g L}^{-1}$) in the absence or presence of PCN (100 μM) for 20 h. Cell growth was determined using reassuring reduction to Resorufin by metabolically active bacteria. Data are normalized relative to untreated controls and represent the mean \pm SD of three independent experiments. Comparisons: * treated Vs. untreated

Effects of 100 μM pyocyanin on bacterial efflux activity: Acriflavine is a fluorescent molecule that is exported by a number of multi-drug resistance efflux systems including MexAB-OprM. Export of the compound, via the efflux mechanism, results in increased fluorescence that can then be measured fluorometrically. Here we show that exogenously added PCN (100 μM) significantly inhibits acriflavine efflux in both *Pseudomonas* strains relative to untreated controls, $p < 0.001$ (Fig. 2). This inhibition of acriflavine efflux was largely unaffected in cultures grown continuously in media containing 100 μM PCN (Fig. 2). In both strains the observed inhibition of acriflavine efflux by PCN was less than the positive control PabN (40 μM).

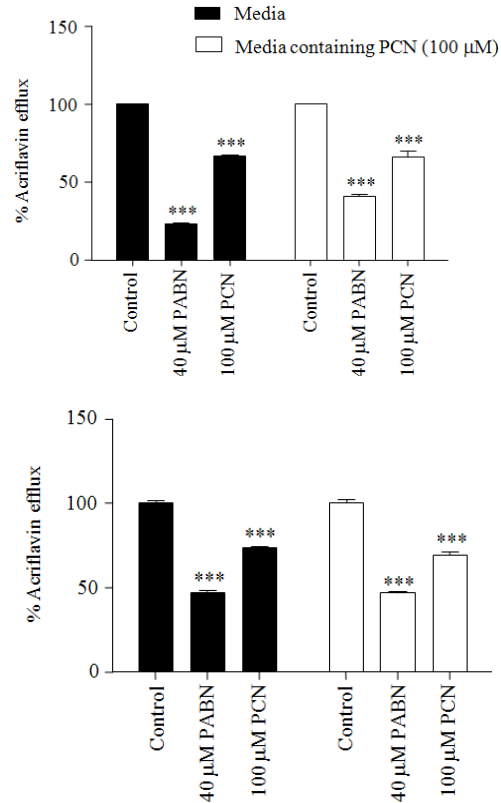


Fig. 2: Pyocyanin decreases acriflavine efflux in *P. aeruginosa*. *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC 27853 were loaded with acriflavine (20 μM) and then treated with PCN (100 μM). PabN (40 μM) was included as the positive control for efflux inhibition. Data are normalised relative to untreated controls and represent the mean \pm SD of three independent experiments. Comparisons: * treated Vs. untreated

Effects of 100 μ M pyocyanin on biofilm production:

Crystal violet staining is widely used to determine biofilm production under static conditions. Here we show that the exogenous addition of PCN (100 μ M) produced a 1.6 ± 0.1 and 3.4 ± 0.1 fold increase in crystal violet absorbance in PAO-1 and ATCC27853 respectively relative to untreated control. Chlorhexidine (0.2%) was included as the negative control and completely inhibited biofilm production (data not shown).

DISCUSSION

Among all current drug treatment options, ciprofloxacin remains a crucial orally effective antibiotic for treating *P. aeruginosa* infections (Hodson *et al.*, 1987). Over-Production Phenotypes (OP) phenotypes were recently described by Fothergill *et al.* (2007) and shown to increase production of exoproducts including pyocyanin. These researchers highlighted interesting correlations between the OP phenotype and patterns of antibiotic resistance. The OP phenotype was shown to have a higher proportion of intermediate antimicrobial susceptibility to ciprofloxacin (Fothergill *et al.*, 2007). Pyocyanin has previously been shown to behave as a signaling molecule influencing the regulation of a number of genes in *P. aeruginosa* (Dietrich *et al.*, 2006).

Exogenous addition of 100 μ M pyocyanin was shown in this study to have a modest inhibitory effect on the susceptibility of *P. aeruginosa* PAO-1 and ATCC27853 to ciprofloxacin when compared to untreated controls. Pyocyanin was shown to have no significant growth inhibitory effects of its own at 100 μ M. Exogenous addition of pyocyanin produced a decrease in susceptibility of both strains tested at concentrations well in excess of the MIC that may be associated with an increase in the persister cell population (Moker *et al.*, 2010). Persister cells constitute a small portion of a culture which is tolerant to killing by lethal doses of bactericidal antibiotics. The phenazine pyocyanin was recently shown to significantly increase the persister numbers in logarithmic *P. aeruginosa* PAO-1 or PA14 cultures (Moker *et al.*, 2010). Although persister cells are believed to contribute to difficulties in treatment, the underlying mechanisms affecting persister formation are not well understood (Moker *et al.*, 2010).

Acriflavine is a known substrate of the MexAB-OprM multidrug resistant pump (Poole and Srikumar, 2001). The MexAB-OprM system of *P. aeruginosa* has

the broadest-spectrum of activity of the efflux pumps described to date and overproduction of this pump, in so called nalB mutants, has been associated with increased MICs for a number of unrelated antibiotics by 8-fold or more (Aeschlimann, 2003). Our results clearly show that exogenously added pyocyanin inhibits acriflavin efflux in both strains when compared to controls. This effect remained unaffected when overnight cultures were grown in the presence of 100 μ M pyocyanin. This would suggest that the decreased susceptibility to ciprofloxacin observed in both strains is unlikely to be related to alterations in efflux activity of MexAB-OprM. This statement is supported by Tohidpour *et al.* (2009) who have demonstrated the association between PabN-inhibition of efflux activity and increased ciprofloxacin activity (Tohidpour *et al.*, 2009). Furthermore our data also suggests that pyocyanin may be a competitive substrate for this system.

Studies have shown that the biofilm producing mucoid phenotype, *P. aeruginosa* can be up to 1,000 times more resistant to antibiotics than their planktonic counterparts (Drenkard, 2003). In our studies, exogenous addition of pyocyanin was shown to significantly increase crystal violet staining for biofilm production in both strains when compared to controls. This would suggest that the observed decrease in ciprofloxacin activity may in part be related to pyocyanin's influence on biofilm production. Interestingly, increased crystal violet stain was not observed for lower concentrations of exogenous pyocyanin (data not shown).

Sputum from CF patients colonized with *P. aeruginosa* has been shown to contain pyocyanin at concentrations up to 130 μ M (Wilson *et al.*, 1988). Our results would suggest that at these concentrations it is likely that pyocyanin may contribute to reduced *P. aeruginosa* susceptibility to ciprofloxacin and therefore may play a role in this organism's ability to evade eradication. However this study needs to be expanded to include other antibiotics including tobramycin and meropenam as well as the pyocyanin precursor 1-hydroxyphenazine.

CONCLUSION

In conclusion, it appears from the results of our initial investigations that the presence of pyocyanin influences biofilm production by a currently undetermined mechanism and that this may contribute to the decreased effectiveness of ciprofloxacin against *P. aeruginosa*.

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