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Resistance to the quorum quenching compounds brominated furanone C-30 and 5-fluorouracil in *Pseudomonas aeruginosa* clinical isolates

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Abstract

The quorum quenching compounds brominated furanone C-30 and 5-fluorouracil inhibit the pathogenicity of the *Pseudomonas aeruginosa* laboratory strains PA01 and PA14; however, there is no report studying the effectiveness of these compounds for clinical isolates. Therefore, the effect of both quorum quenchers on the production of pyocyanin, elastase and alkaline protease of eight clinical strains from children was evaluated. Although both compounds were in general effective for the attenuation of these factors, three strains resistant to C-30 were found. For 5-fluorouracil, PA01 and some clinical isolates showed resistance for at least one phenotype.

Introduction

Pseudomonas aeruginosa, one of the main pathogens responsible for nosocomial infections (Jarvis & Martone, 1992), has a remarkable resistance against antimicrobials and produces recalcitrant biofilms (Poole, 2011). Therefore new therapies to treat it are needed. Among them, bacterial cell-cell communication (quorum sensing or QS) has been proposed as a target (Rasko & Sperandio, 2010), since several *P. aeruginosa* virulence factors are activated by QS (Winzer & Williams, 2001). In contrast to classical antimicrobials, quorum quenchers inhibit virulence rather than bacterial growth, minimizing

the chance of generating resistance (Bjarnsholt, *et al.*, 2010). Nevertheless, recently we demonstrated that one of the best characterized quorum quenchers, brominated furanone C-30, is effluxed by the MexAB-OpmR pump and that mutants in the transcriptional repressors *mexR* and *nalC*, which overexpress this efflux pump, are resistant to C-30 (Maeda, *et al.*, 2012). Brominated furanones interfere with QS systems based on acyl homoserine lactones (AHLs) by interfering with the AHL receptors (Defoirdt, *et al.*, 2007); hence, brominated furanones inhibit the QS-controlled production of pyoverdine, exo-protease, chitinase, and biofilm formation (Hentzer, *et al.*, 2002, Manefield, *et al.*, 2002, Hentzer, *et al.*, 2003) and promote the clearance of pulmonary infections in mice (Wu, *et al.*, 2004).

The pyrimidine analog, 5-flourouracil (5-FU), is a novel QS inhibitor that attenuates the production of pyocyanin, elastase, rhamnolipids, swarming and biofilms (Ueda, *et al.*, 2009). The mechanism of QS inhibition by 5-FU is not yet known but its effect on gene expression is indicative of a global repressor of QS (Ueda, *et al.*, 2009). An attractive feature of this compound is that it is currently used in the treatment of cancer.

Since to date the anti-virulence effects of both compounds have been demonstrated only in laboratory strains, and their effectiveness against clinical isolates has not yet been studied extensively, in this work, the effect of C-30 and 5-FU on the production of pyocyanin, LasB elastase and alkaline protease was evaluated in eight *P. aeruginosa* clinical strains collected in the Hospital Infantil de Mexico from pediatric patients with infectious processes during 2007-2010. They were obtained from urine, blood and catheter tips. Their antimicrobial susceptibility was evaluated by determining the minimum inhibitory concentration, employing the agar dilution method in Mueller-Hinton medium, as recommended by the Clinical and Laboratory Standards Institute. Six isolates showed a multidrug resistance profile to ciprofloxacin, meropenem, cefepime and amikacin (CI 1-3 and CI 6-8) and two isolates (CI4 and CI5) were sensitive to all the above listed antibiotics. All isolates were susceptible to colistin.

Results and Discussion

To compare the effect of the quorum quenchers, the laboratory strains PA01 and PA14 were used. 5-FU was purchased from SIGMA and C-30 was synthesized by bromination of levulinic acid (SIGMA) (Manny, *et al.*, 1997). Cultures were grown in LB at 37°C and 200

rpm until O.D. 600 ~ 1.0, then C-30 or 5-FU, were added at 30 and 60 μM (for simplicity, only results with the highest concentrations are shown in the main text). Since C-30 was dissolved in ethanol, negative controls with this solvent were done. After adding the compounds, bacteria were cultivated for 4 h and the selected phenotypes measured. This protocol was adapted from (Hentzer*, et al.*, 2002) in which the effect of furanones in PA01 was tested. Elastase and pyocyanin were determined as in Maeda et al. (2012) and alkaline protease as in Howe and Iglewski (1984). The production of the main autoinducer N-(3-oxo-dodecanoyl) HSL was determined using the cross-feeding bioassay with *Agrobacterium tumefaciens* NTL4 (Schaber*, et al.*, 2004).

As shown in Figure-1A, both C-30 and 5-FU attenuated the production of elastase by more than 50% in both laboratory strains and in the clinical isolates CI-1 to CI-4. In addition, 5-FU also clearly inhibited elastase production in CI-5. In contrast, C-30 only slightly inhibited CI-5 elastase, and both quorum quenchers were unable to significantly inhibit the elastase of CI-6 to CI-8. Figure 1B shows that as for elastase, C-30 was able to inhibit pyocyanin production of the laboratory strains and of the clinical isolates CI-1 to CI-4 and CI-8 by greater than 50%, while pyocyanin of CI-5 was inhibited only by 36% and pyocyanin CI-6 was not inhibited. The other quorum quencher, 5-FU, inhibited pyocyanin production greater than 50% for CI-4, CI-6 and CI-8, slightly inhibited PA14, CI-2 and CI-3 and was unable to inhibit PA01 and CI-1 and even promoted CI-5 pyocyanin production by 85%. CI-7 produced very low levels of pyocyanin and therefore it was not used for the analysis of this virulence factor. Figure 1C shows that alkaline protease was inhibited greater than 50% by both C-30 and 5-FU in PA14 and in clinical isolates CI-4, CI-6, and CI-8 and by 5-FU but not by C-30 in CI-7. Iin addition, 5-FU and C-30 both inhibited CI-1 and CI-2 alkaline protease by ~ 45% but both quenchers were unable to inhibit PA01, CI-3 and CI-5 alkaline protease. For all phenotypes and strains, both compounds had a dose response effect when 30 μM instead of 60 μM were used (Supplemental Figure S1). Both laboratory strains and all isolates except CI-4 and CI-6 produced similar amounts of N-(3-oxo-dodecanoyl) autoinducer, suggesting the presence of functional HSL dependant quorum sensing systems in most of the strains (Figure 1D).

Our results show that C-30 is in general effective to attenuate the three virulence factors tested in the clinical isolates; nevertheless, the susceptibility of the isolates towards this quorum quencher was variable, and indeed three out of eight isolates (CI-5, CI-6 and CI-7)

presented higher levels of resistance than the rest of the strains. Of these three isolates, two of them, CI-6 and CI-7, are resistant to multiple antibiotics, while CI-5 is sensitive. Since the only C-30 resistance mechanism known to date is the efflux of the compound by the pump MexAB-OmpR (Maeda*, et al.*, 2012), co-resistance between antibiotics and C-30 in strains with an active pump is expected. This was observed previously in PA14 *mexR* and *nalC* mutants, and in two clinical C-30 resistant strains from cystic fibrosis patients; however, for those isolates, the resistance was tested only by their ability to grow using adenosine as sole carbon source in the presence of C-30 (Maeda*, et al.*, 2012) and not by their production of QS dependant virulence factors. In this study, two isolates resistant to multiple antibiotics that are also resistant to C-30 were identified (CI-6 and CI-7); of those two strains, CI-7 produced very low levels of N-(3-oxo-dodecanoyl), suggesting that its AHL-mediated QS may be disrupted, rendering C-30 treatment ineffective. Nevertheless, 5-FU was able to inhibit its pyocyanin production by 90%. In contrast, CI-5 and CI-6 produced normal levels of the autoinducer. For CI-6, the mechanism responsible for C-30 resistance may be efflux. In contrast, for CI-5, its resistance is likely not due to efflux, since it is sensitive to β-lactams, quinolones and aminoglycosides, that are effluxed by MexAB-OmpR and other multidrug resistance pumps. Hence this strain should possess a novel C-30 resistance mechanism and its elucidation will be the subject of further research.

Our study shows that although generally effective, these two quorum quenching compounds are not able to inhibit the virulence factors of all clinical strains and in some cases they may increase their production. Therefore, the application of quorum quenchers to treat *P. aeruginosa* infections may be not always adequate and more extensive studies including larger numbers of clinical isolates and experiments with animals are required to better evaluate their effectiveness. Our study also points out that resistance against C-30 without a concomitant multi-antibiotic resistance exists and adds evidence demonstrating that quorum quenching resistance is already an existing phenomenon in some strains infecting humans even without pre-exposition to quorum sensing inhibitors (Maeda*, et al.*, 2012).

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