Synergy of AcpP PPMO and Piperacillin/Tazobactam in the Breakdown of *Pseudomonas aeruginosa* PA01 Biofilms

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Abstract

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium and one of the most common causes of hospital-acquired infection, especially in immunocompromised patients. It is particularly pathogenic because of its ability to form biofilm, an extracellular matrix that makes it more resistant to host defenses and antibiotic therapies. Combination therapies have proven to be more effective at clearing biofilms because they target different processes or cell populations, but concerns about toxicity and antibiotic resistance have led to the exploration of alternative therapies that target biofilm formation at a genetic level. One such alternative is the use of peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs), which are sequence-specific antisense oligomers that target the mRNA of bacterial genes and prevent translation of particular proteins.

We hypothesize that PPMOs targeting the essential gene AcpP will act synergistically with the antibiotic Piperacillin/Tazobactam (Pip/Tazo) in the breakdown of *Pseudomonas aeruginosa* PA01 biofilms *in vitro*.

To test synergy between the PPMO and antibiotic, PA01 biofilms were grown in filtered Mueller Hinton broth II (MHII) on minimum biofilm eradication concentration (MBEC) plates. MBEC plates are 96-well plates that have pegs attached to the lid to provide additional surface area for biofilm growth. After 24 hours of growth, the biofilm-covered pegs were switched to another 96-well plate containing fresh media with different combinations of antibiotic and PPMO in each well. Three such doses were administered every 8 hours, and at 48 hours of total growth, the biofilm remaining on the pegs was analyzed by one of four methods: 1) crystal violet assay, 2) resazurin assay, 3) colony forming unit (CFU) count , and 4) confocal microscopy.

Crystal violet and resazurin assays demonstrated that Pip/Tazo and AcpP PPMO were potentially synergistic in biofilm breakdown for Pip/Tazo 0.5-0.0625 ug/mL and AcpP 5-0.625 uM. For several combinations, CFU measurements yielded 2-log or 3-log reduction in CFU compared to the control and synergistic effects on biofilm breakdown, particularly in cases where the antibiotic alone had no effect. While confocal microscopy demonstrated a decrease in viable bacteria with antibiotic treatment and PPMO treatment, the most significant eradication of biofilm occurred with combined treatment.

In conclusion, the synergy demonstrated *in vitro* between AcpP PPMO and Pip/Tazo in the breakdown of *P. aeruginosa* PA01 biofilms is promising for *in vivo* studies as well, since PPMOs have the potential to increase biofilm sensitivity to lower doses of traditional antibiotics.

Introduction

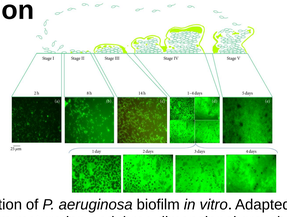


Figure 1: Formation of *P. aeruginosa* biofilm *in vitro*. Adapted from Rasamiravaka *et al.* 2015. Biofilm maturation and three dimensional growth occurs over days 1-4.

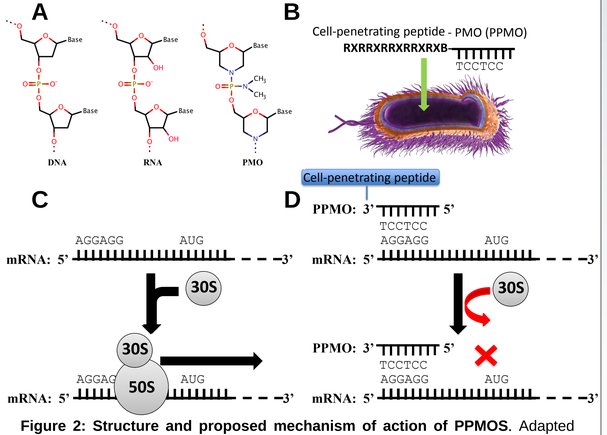


Figure 2: Structure and proposed mechanism of action of PPMOS. Adapted from Daly *et al*. 2017. **(A)** DNA and RNA structures compared to phosphorodiamidate morpholino oligomers (PMOs). **(B)** Conjugation of PMOs to cell-penetrating peptides (PPMOs) at the 3' or 5' end enhances bacterial cell entry. **(C)** The 30S ribosomal subunit recognizes the ribosomal binding site on mRNA to begin translation, but **(D)** in the presence of PPMO the subunit is sterically blocked, inhibiting translation of the target mRNA.

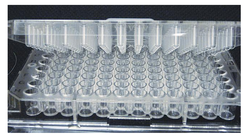
Introduction (continued)

Piperacillin-Tazobactam (Pip/Tazo) targets bacterial cell wall synthesis. This combination antibiotic consists of a beta lactam, which binds penicillin-binding proteins (PBPs) to prevent crosslinking, and a beta lactamase inhibitor, which helps prevent the breakdown of the beta lactam.

The PPMO AcpP specifically targets the mRNA of the essential gene for acyl carrier protein, which is responsible for membrane synthesis.

The combination of Pip/Tazo and 5' $(RXR)_4$XB AcpP PPMO was previously shown to be synergistic in biofilm prevention assays, laying a foundation for the use of Pip/Tazo + 3' $(RXR)_4$XB AcpP PPMO in this biofilm breakdown experiment.

Methods



Day 0: Inoculate MBEC plates

Biofilm Growth
Grow for 24 hours in MHII
(37°C and 110 rpm)

Day 1: Begin dosing

Dosing x3
Transfer pegs to media with
desired dose of Abx or PPMO

Day 2: Measure Biofilm

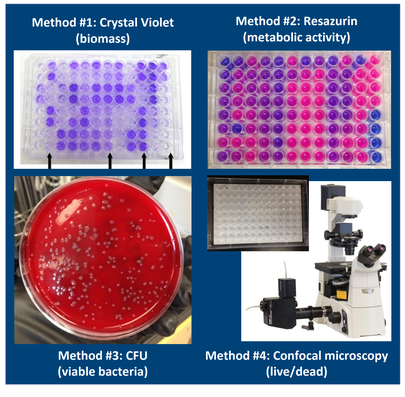


Figure 3: Overview of Methods.
A) Biofilm Growth and Treatment.
On day 0, MBEC plates were inoculated with 5E5 CFU/mL PA01. Bacteria were grown for 24 hours at 37°C and 110 rpm.
On day 1, one dose of Pip/Tazo and/or AcpP PPMO was administered every 8 hours by transferring pegs to fresh media containing desired dose.
On day 2, biofilm was measured by one of 4 methods.
B) Four methods of Biofilm Quantification.
Method #1: Crystal violet dye stains both bacteria and extracellular matrix, thus serving as a measure of total biomass in a biofilm. Differences in staining were quantified on the spectrophotometer.
Method #2: The resazurin assay is a viability assay based on the reduction of resazurin (blue) to resorufin (red/pink) in the presence of living cells, thus serving as a measure of metabolic activity of biofilms. Pegs were transferred to media containing resazurin dye and developed until positive controls reached maximum fluorescence, about 6.5 hours. Changes were quantified on the spectrophotometer.
Method #3: CFU counts are commonly used to assess the number of living bacteria, thus serving as a measure of viable bacteria within the biofilm. Pegs were removed from the lid, placed in a tube of DPBS, and sonicated in the water bath for 20 minutes at room temperature. Serially diluted samples were plated on sheep blood agar plates, and colonies were counted after 18 hours of incubation at 37°C.
Method #4: Confocal microscopy employs two stains, propidium iodide (red) and syto9 (green), to visualize live (green) versus dead (red) bacteria.

Results

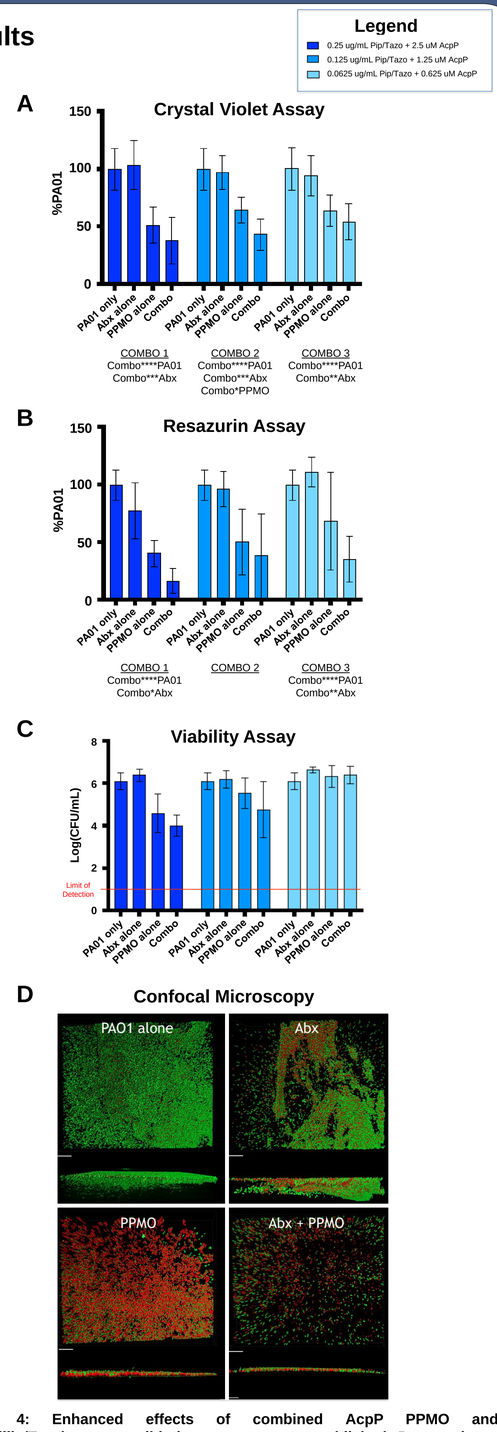


Figure 4: Enhanced effects of combined AcpP PPMO and Pipericillin/Tazobactam antibiotic treatment on established *P. aeruginosa* strain PA01 biofilm. PA01 biofilms were established on MBEC pegs for 24 h, then either treated with 0.25, 0.125, or 0.0625 µg/ml Pip/Tazo; 2.5, 1.25, or 0.625 µM AcpP; or a combination of both, at 24-, 32-, and 40 h post-inoculation. At 48 h, the MBEC pegs were processed by A) crystal violet staining (total biomass, n=5), B) resazurin assay (viability and metabolism, n=3), C) sonication and dilution for plate count (viability, n=5), or D) Live/Dead staining (red- propidium iodide, green – syto9) and spinning disk confocal microscopy (40X/1.3 NA) for COMBO 1. Significance for each pair of conditions was determined by paired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Error bars represent standard deviation.

Discussion

This experiment builds upon the lab's previous research (Howard *et al*. 2017), using the 3' $(RXR)_4$XB PPMO rather than the 5' $(RXR)_4$XB PPMO and focusing primarily on the breakdown of existing biofilm. The 3' $(RXR)_4$XB PPMO was selected for this experiment because of the promising results of its 5' $(RXR)_4$XB counterpart and the fact that the 3' $(RXR)_4$XB PPMOs have been shown to be more effective in mice.

The combination of Pip/Tazo and AcpP PPMO is interesting because the antibiotic and PPMO target separate processes within the bacterial cell (cell wall synthesis and cell membrane synthesis, respectively). This finding supports previous conclusions that synergy can occur between antibiotics and PPMOs with similar or dissimilar processes, most likely due to the PPMO's effect on membrane permeability (Howard *et al.* 2017).

The quantification of biofilm was a particularly important element of this experiment. Due to the heterogeneous nature of biofilm and decreased metabolic state, some of the traditional methods for quantifying bacteria, such as crystal violet, provide an incomplete picture of how treatment affects biofilm.

The crystal violet assay shows that there is a significant decrease in biomass in the combination compared to the antibiotic alone. However, it is unclear whether this is due to a decrease in bacteria or matrix, and by this method alone, it appears that the combination provides little/no benefit over PPMO alone.

The resazurin assay elucidates this finding by providing a measure of metabolic activity. Particularly in COMBO 1, there is a striking decrease in bacteria when treated with the combination.

However, because of the decreased metabolic activity and structural heterogeneity that occurs in biofilm, it is also important to check the viability of the bacteria by plating for CFU. In previous papers, a bacteriocidal treatment is defined as one that causes a 3-log reduction in CFU. Though the aggregated results show only a 2-log reduction from antibiotic alone to combination for COMBO 1, several experiments showed 3-log reduction when examined separately.

Confocal microscopy also informs our understanding of the effects of each treatment on both bacteria and matrix. The antibiotic causes a slight decrease in living material (green), while the PPMO eliminates almost all of the living material, leaving only dead material (red). The combination of antibiotic and PPMO demonstrates a significant decrease in both living and dead material, pointing to the additive or even synergistic effects of Pip/Tazo and AcpP PPMO in breaking down existing biofilm.

Conclusions

With increasing antibiotic resistance, novel treatments are needed to target bacteria with high potential for resistance, such as *P. aeruginosa*. Previous experiments have demonstrated that PPMOs are non-toxic and effective agents for rescuing sub-inhibitory concentrations of antibiotics used to treat *P. aeruginosa*, and the present examination of Pip/Tazo + AcpP PPMO is highly suggestive of synergy between these two agents. The methods and findings in this experiment strengthen the foundation for studying this combination in the breakdown of difficult-to-treat biofilms *in vivo*.

References

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