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2017

Roizman, D., Vidaillac, C., Givskov, M., & Yang, L. (2017). In Vitro Evaluation of Biofilm Dispersal as a Therapeutic Strategy To Restore Antimicrobial Efficacy. Antimicrobial Agents and Chemotherapy, 61(10), e01088-17-.

https://hdl.handle.net/10356/87235

https://doi.org/10.1128/AAC.01088-17

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In Vitro Evaluation of Biofilm Dispersal as a Therapeutic Strategy To Restore Antimicrobial Efficacy

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ABSTRACT As a proof-of-concept study, the direct impact of biofilm dispersal on the *in vitro* efficacy of imipenem and tobramycin was evaluated against 3-day-old biofilms of *Pseudomonas aeruginosa*. Arabinose induction of biofilm dispersal via activation of the phosphodiesterase YhjH in the *P. aeruginosa* engineered strain PAO1/p_{BAD}-*yhjH* resulted in increased antimicrobial efficacy and synergy of the imipenem-tobramycin combination. These results support the use of biofilm dispersal to enhance antimicrobial efficacy in the treatment of biofilm-associated infections, representing a promising therapeutic strategy.

KEYWORDS antibiotic resistance, biofilms, cyclic di-GMP, dispersal, synergism

Received 26 May 2017 **Returned for modification** 19 June 2017 **Accepted** 19 July 2017

Accepted manuscript posted online 31 July 2017

Citation Roizman D, Vidaillac C, Givskov M, Yang L. 2017. *In vitro* evaluation of biofilm dispersal as a therapeutic strategy to restore antimicrobial efficacy. Antimicrob Agents Chemother 61:e01088-17. https://doi.org/10.1128/AAC.01088-17.

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The current strategy to eradicate chronic bacterial biofilm infections involves long-term combinatorial antibiotic treatments at high dosages and the surgical removal of the infected tissue/foreign body (1). Recently, there has been growing interest in combining chemical agents that interfere with bacterial communication/signaling pathways with broad-spectrum antibiotics to treat biofilm-associated infections. Thus, through attenuation of biofilm formation, these quorum-sensing inhibitors restore the killing efficiency of antimicrobial treatments (2). Cyclic di-GMP (c-di-GMP) is a signaling nucleotide with multiple regulatory functions, including a central role in controlling biofilm formation. Higher intracellular levels of c-di-GMP enhance biofilm formation, whereas lower levels lead to biofilm dispersal with the potential to at least partially restore antimicrobial susceptibility (Fig. 1). Although this phenomenon is widely recognized, proof-of-concept studies to support this principle have been lacking. The present study tested the hypothesis that biofilm dispersal has the potential to enhance antimicrobial synergy and ultimately improve antimicrobial killing efficacy compared with mature biofilms.

(This work was presented at the 27th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID 2017], Vienna, Austria, 22 to 25 April 2017; and the 15th International Union of Microbiological Societies [IUMS 2017], Singapore, 17 to 21 July 2017.)

To test our hypothesis, we evaluated the combinatory potential of tobramycin and imipenem to control *Pseudomonas aeruginosa* biofilms. Strains of *P. aeruginosa* used in this study consisted of the previously described constructs, PAO1/pJN105 (vector control carrying the *araBAD* promoter and gentamicin resistance for selection) and PAO1/p_{BAD}-*yhjH* (a plasmid carrying *yhjH* under the *araBAD* promoter) (3). *P. aeruginosa* strains were cultured in Mueller-Hinton broth II or agar (MHB or MHA) (Difco) unless stated otherwise. The potential for the antimicrobial synergy of tobramycin plus imipenem (Sigma-Aldrich) and the effect of biofilm dispersal on antimicrobial efficacy were further assessed using *in vitro* experiments that included checkerboard and

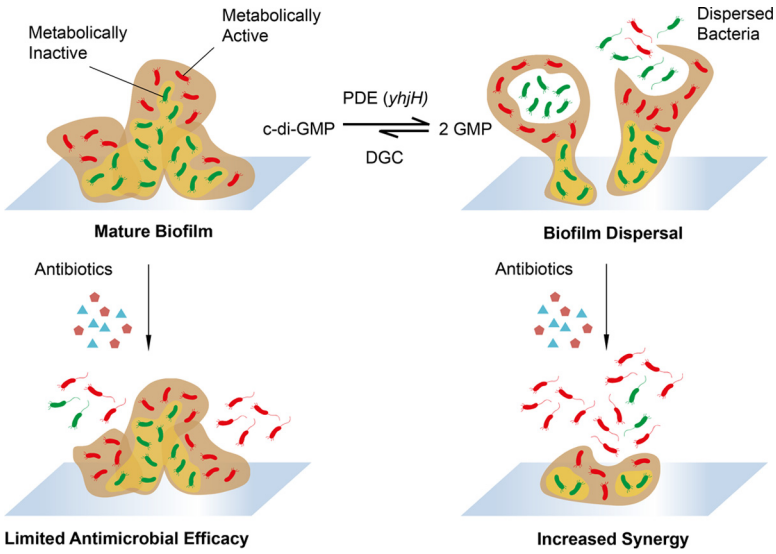

FIG 1 Reduction of intracellular c-di-GMP before antimicrobial treatment has the potential to restore drug efficacy by increasing the synergistic effect of antimicrobial combinations. PDE, phosphodiesterase; DGC, diguanylate cyclase.

time-kill assays (4). In all experiments, before challenging biofilms with antimicrobial agents, samples were washed twice with 0.9% NaCl and exposed to fresh MHB containing 1% (wt/vol) arabinose for 4 h to induce biofilm dispersal. Control experiments were performed in parallel using an arabinose-free medium (5). Minimum biofilm eradication concentration (MBEC) values and fractional biofilm eradication concentration (FBEC) indices were determined on 3-day-old biofilms using a modified version of the Calgary biofilm pin lid device (CBD) (Nunc, Thermo-Fischer) (6). The concentrations of imipenem and tobramycin tested were 0.25 to 64 and 2,048 μg/ml, respectively. After 24 h of incubation at 37°C, samples were washed twice in 0.9% NaCl and stained with resazurin, as previously described (7, 8). MBEC values were defined as the lowest drug concentrations resulting in fluorescence values with a magnitude similar to that of the negative control. FBEC and ΣFBEC values were calculated as previously described (9). As per *Antimicrobial Agents and Chemotherapy* guidelines (June 2017), combinations with a ΣFBEC value of $\leq$0.5 were considered synergistic. Biofilm time-kill kinetics were assessed on 3-day-old biofilms formed on the

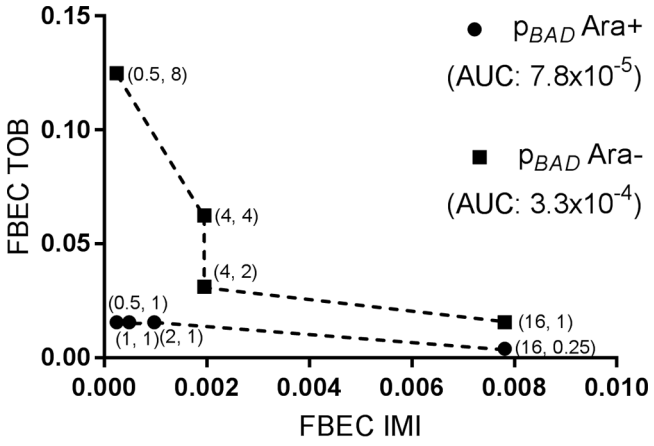

FIG 2 Isobolograms illustrating the relative fractional biofilm eradication concentration (FBEC) values for imipenem (IMI) and tobramycin (TOB) used in combination against 3-day-old biofilms of *P. aeruginosa* strain PAO1/p_{BAD}-*yhjH* following or not following biofilm dispersal (p_{BAD} Ara+ or p_{BAD} Ara−, respectively). Results from five independent experiments were used to plot the isobolograms.

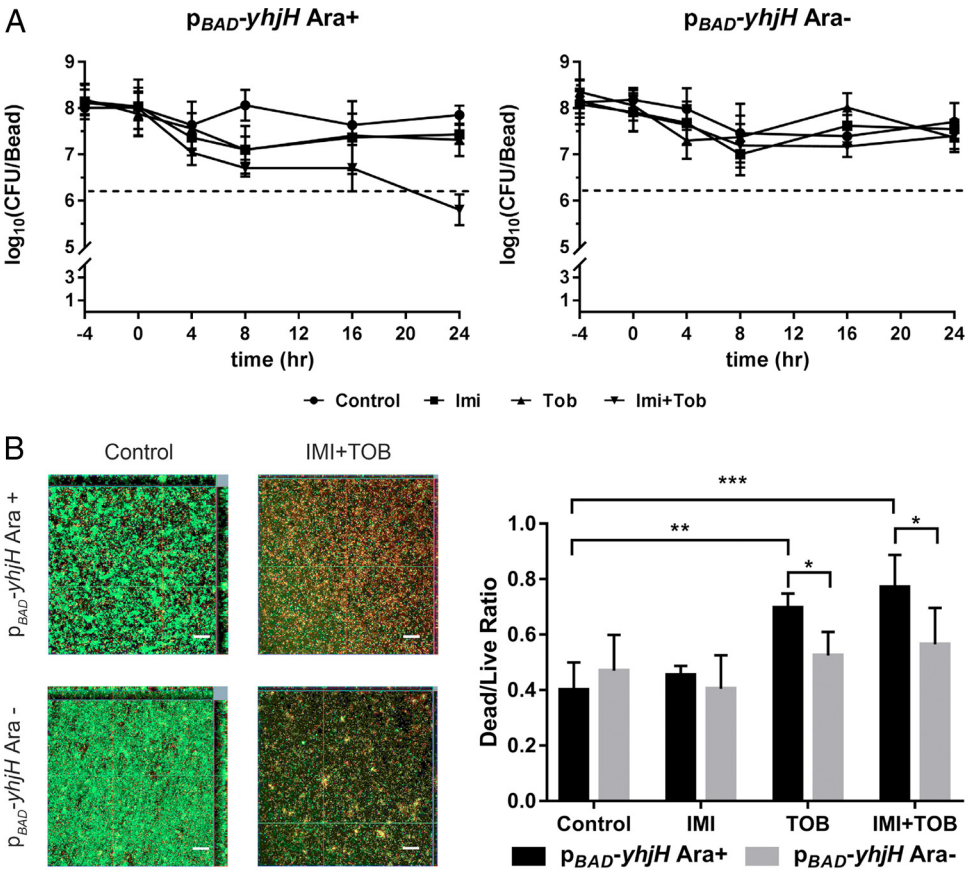

FIG 3 Biofilm dispersal induced by arabinose enhances the antimicrobial synergistic effect of tobramycin (TOB) combined with imipenem (IMI) against *P. aeruginosa* PAO1/p_{BAD}-*yhjH*. (A) Residual bacterial load (CFU/bead) after exposing a 3-day-old biofilm of *P. aeruginosa* PAO1/p_{BAD}-*yhjH* with (p_{BAD} Ara+) or without (p_{BAD} Ara−) arabinose 1% to IMI and TOB alone or in combination at 8 μg/ml. Dashed line represents 2-$\log_{10}$ reductions relative to untreated controls, i.e., synergy. (B) Left: CLSM images (20× objective) of 3-day-old biofilms of *P. aeruginosa* PAO1/p_{BAD}-*yhjH* treated with arabinose 1% (p_{BAD} Ara+) or arabinose free (p_{BAD} Ara−) for 4 h, followed by 24 h exposure to IMI, TOB, or IMI-TOB combination at 8 μg/ml each. Biofilms were stained with Syto9 and propidium iodide before imaging. Dead and live cells are red and green, respectively; scale bar is 20 μm. Right: bacterial load after 24 h antibiotic exposure reported as dead/live ratio. Images from five independent experiments were analyzed to determine dead/live ratios for each condition. Results were analyzed using two-way ANOVA. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

surface of 5-mm glass beads, as described by Konrat et al. (10). Two beads (representing two technical replicates) were placed into each well of a 24-well microtiter plate (Nunc, Thermo-Fischer). Biofilms were next exposed to fresh MHB containing 1% (wt/vol) arabinose or arabinose-free medium (control samples). After 4 h, samples were treated with imipenem and tobramycin (8 μg/ml) alone or in combination. Beads were collected at indicated intervals, subjected to a series of 4 × 10 s sonication (at 37 kHz) and 10 s vortex. Bacterial suspensions were subsequently serially diluted in 0.9% NaCl before being drop-plated onto lysogeny broth agar plates (Difco). After 24 h of incubation at 37°C, the residual biofilm was quantified as CFU/bead and plotted against time. The efficacy of the combined antibiotic therapy was finally examined using confocal laser scanning microscopy (CLSM) (Zeiss 780; Germany). Biofilm samples were grown on ibidi 8-well glass-bottom μ-slides for 3 days before exposure to imipenem and tobramycin (at 8 μg/ml) alone or in combination. After the 24-h antibiotic challenge, samples were stained for 10 min, using the dead (propidium iodide)/live (Syto-9) BacLight bacterial viability kit. Biofilms were further assessed by CLSM. Five images were acquired in Z stacks for each sample. Image sets were subsequently analyzed for biovolume ($\mu m^3/\mu m^2$) using the COMSTAT 2 ImageJ plugin (11), with each corresponding channel set to a constant threshold of 50. Dead/live

ratios were analyzed in Excel 2010; statistical significance (using two-way analysis of variance [ANOVA]) and final plotting were conducted in Prism GraphPad 7.

Arabinose induction of biofilm dispersal had no effect on imipenem and tobramycin MBEC values (2,048 and 64 μg/ml for imipenem and tobramycin, respectively) for PAO1/pJN105 and PAO1/p_{BAD}-*yhjH*. The imipenem-tobramycin combination resulted in a greater killing effect against PAO1/p_{BAD}-*yhjH* after induction of biofilm dispersal (ΣFBEC ranged from 0.012 to 0.017 versus 0.024 to 0.033 for nondispersed biofilms) (Fig. 2). This effect was not observed in biofilms formed by control strain PAO1/pJN105 (see Fig. S1 in the supplemental material). Imipenem and tobramycin MBEC values were significantly lowered after dispersal induction (up to 7- and 3-fold $\log_2$ decrease, respectively), reaching clinically achievable concentrations after biofilm dispersal, compared with arabinose-free PAO1/p_{BAD}-*yhjH* biofilms (Fig. 2). The area under the curve (AUC) for each isobologram was 7.8×10^{-5} AU after dispersal versus 3.3×10^{-4} AU in the absence of dispersal. The stronger synergistic effect following induced biofilm dispersal was further confirmed by time-kill assessments (Fig. 3A). At 24 h, a 2-$\log_{10}$ reduction difference was observed between dispersed and nondispersed PAO1/p_{BAD}-*yhjH*, suggesting a synergistic effect. This effect was not seen with control strain PAO1/pJN105 (difference, <0.5 $\log_{10}$ reduction at 24 h) (see Fig. S2 in the supplemental material). This observation was confirmed with confocal microscopy and the dead/live cell ratio (Fig. 3B; see also Fig. S3 and S4 in the supplemental material). After exposure to the imipenem-tobramycin combination, a significant increase ($P < 0.05$) in the dead/live cell ratio was observed for PAO1/p_{BAD}-*yhjH* treated with arabinose (dispersed) versus the arabinose-free (nondispersed) biofilm.

In conclusion, using a *P. aeruginosa* construct PAO1/p_{BAD}-*yhjH*, we demonstrated that biofilm dispersal induced by arabinose enhanced the killing efficacy of a combined antibiotic treatment. Our results suggest that induction of biofilm dispersal has the potential to result in higher antimicrobial efficacy (Fig. 1). Our findings are in agreement with the literature for the combination of carbapenem agents with tobramycin (12–15), although after induced biofilm dispersal, values decreased further to clinically achievable concentrations. This model serves as a proof of concept for future development of antimicrobial treatments, including dispersal agents.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01088-17.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

ACKNOWLEDGMENTS

This research was supported by the National Research Foundation and the Ministry of Education of Singapore under its Research Centre of Excellence Programme (AcRF Tier 2 – MOE2014-T2-2-172).

We thank Sharon Longford (SCELSE, Singapore) for her input and proofreading the manuscript and Kathrin Koch from Hochschule der Medien (HdM, Stuttgart, Germany) for her contribution and input on graphic design.

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