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Lipoxin A_4 (LxA_4) Promotes Reduction and Antibiotic Efficacy Against *Pseudomonas aeruginosa* Biofilm

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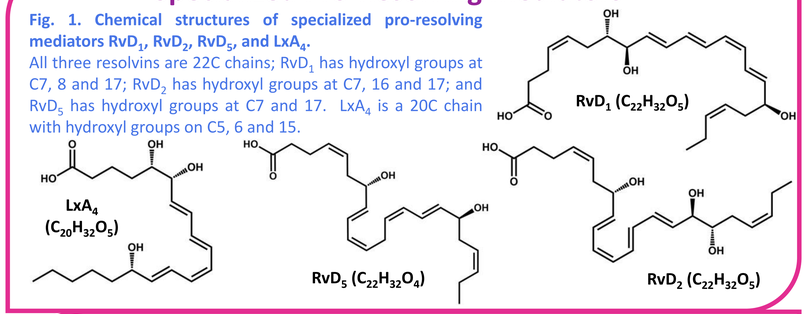
Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic bacterium commonly found in wound infections and airways of cystic fibrosis patients. *P. aeruginosa* readily forms biofilms which can reduce the efficacy of antibiotics used to eradicate the pathogen. We have previously shown that a Specialized Pro-resolving Mediator (SPM), Lipoxin A4 (LxA_4) is a quorum sensing inhibitor which can reduce *P. aeruginosa* virulence. In this study, we examined the direct actions of LxA_4 and RvD_2 on *P. aeruginosa* biofilm formation and virulence gene expression. The influence of LxA_4 on antibiotic efficacy and the combined effects on biofilm formation were also investigated. LxA_4 and RvD_2 reduced *P. aeruginosa* biofilm formation and virulence gene expression. LxA_4 increased ciprofloxacin inhibition on biofilm formation but did not affect ciprofloxacin's action on non-adherent bacteria. On the other hand, LxA_4 increased bacterial killing action of imipenem but did not affect imipenem's action on biofilm. We also found that LxA_4 can increase ciprofloxacin's bacterial killing ability in established biofilm. Together these results suggest that LxA_4 has direct effects on *P. aeruginosa* biofilm formation and can increase antibiotic efficacy directly.

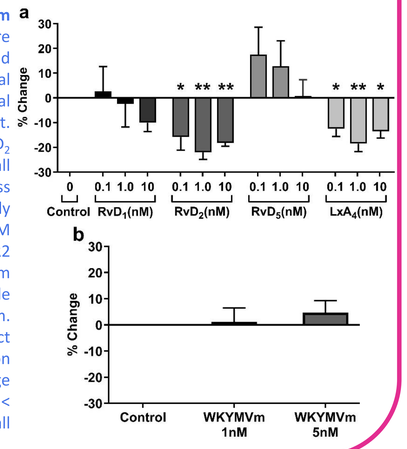
Specialized Pro-Resolving Mediators

Fig. 1. Chemical structures of specialized pro-resolving mediators RvD_1, RvD_2, RvD_5, and LxA_4.
All three resolvins are 22C chains; RvD_1 has hydroxyl groups at C7, 8 and 17; RvD_2 has hydroxyl groups at C7, 16 and 17; and RvD_5 has hydroxyl groups at C7 and 17. LxA_4 is a 20C chain with hydroxyl groups on C5, 6 and 15.



RvD_2 and LxA_4 Reduce *P. aeruginosa* Biofilm Formation

Fig. 2. RvD_2 and LxA_4 reduce *P. aeruginosa* biofilm formation. (a) In a 96-well plate, cultures were treated with multiple concentrations of each SPM and incubated at 37°C overnight. The cultures and apical biofilm were washed away, and the remaining apical biofilm rings were stained with 0.1% crystal violet. Absorbance was measured at 600 nm. RvD_2 significantly reduced biofilm formation at all concentrations, with treatment at 0.1 nM less effective than at 1 nM or 10 nM. LxA_4 significantly reduced biofilm formation, though 0.1 nM and 10 nM were less effective than 1 nM. (b) Effects of FPR2 receptor peptide agonist on *P. aeruginosa* biofilm formation. Cultures were treated with multiple concentrations of the peptide agonist WKYMVm. Peptide agonist WKYMVm did not significantly affect the formation of biofilm at either concentration tested. Data are mean ± s.e.m. of percent change from control adjusted to zero. * = $p < 0.05$; ** = $p < 0.01$; n = 3 independent experiments for all treatments.



Non-adherent cell growth of *P. aeruginosa* was unaffected by LxA_4 or RvD_2

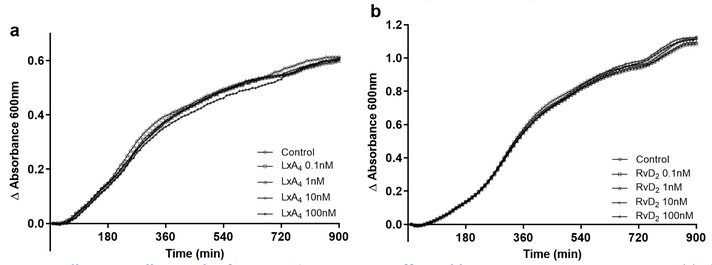
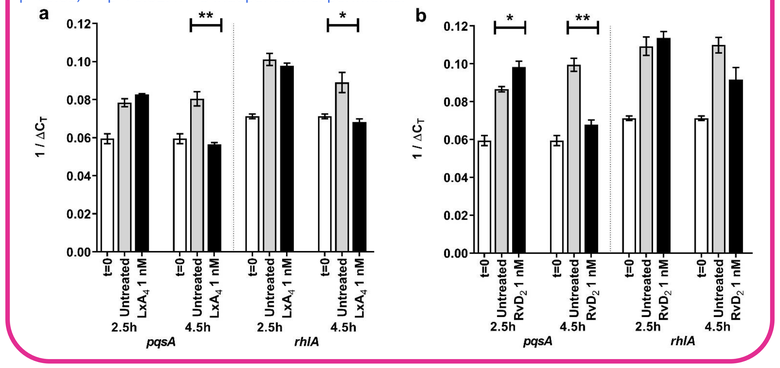


Fig. 3. Non-adherent cell growth of *P. aeruginosa* was unaffected by LxA_4 or RvD_2. SPMs were added to *P. aeruginosa* cultures in 96-well plates and incubated with orbital shaking at 37°C in a microplate reader for 15 hr. Absorbance was measured every 10 min. Neither LxA_4 (a) nor RvD_2 (b) showed any significant effect on non-adherent cell growth in minimal media. n = 3 (LxA_4) and n = 4 (RvD_2) independent experiments.

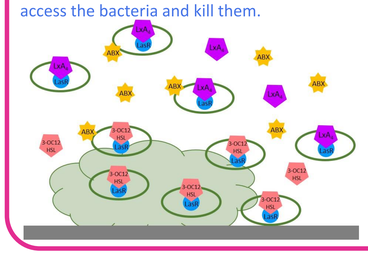
LxA_4 and RvD_2 Reduce Two Quorum-sensing Genes' Expression

Fig. 4. Effects of SPMs on *Pseudomonas aeruginosa* quorum-sensing virulence gene expression. (a) 1/ΔCT of two virulence genes' expression when treated with 1 nM LxA_4. LxA_4 significantly reduced both genes' expression at 4.5 hr. (b) 1/ΔCT of two virulence genes' expression when treated with 1 nM RvD_2. RvD_2 significantly reduced *pqsA* gene expression at 4.5 hr. Data are mean ± s.e.m., with p-values determined by unpaired t-test comparing untreated control to treatment group of same timepoint. * $p < 0.05$, ** $p < 0.01$. n = 3 independent experiments.

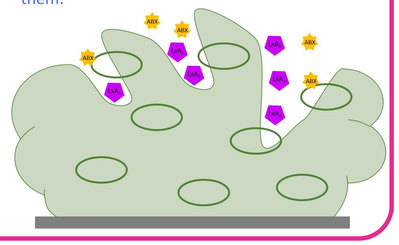


Hypotheses: How LxA_4 Can Assist Antibiotics Against Biofilms

Biofilm Formation: LxA_4 antagonizes the LasR receptor, blocking the quorum-sensing auto-inducer 3-OC12 HSL, reducing biofilm formation. Antibiotics (ABX) are then able to access the bacteria and kill them.



Established Biofilm: LxA_4, as a lipid, can integrate with the biofilm matrix, weakening its integrity. This provides an opportunity for antibiotics (ABX) to access the bacteria within the biofilm and kill them.



LxA_4 Enhances Ciprofloxacin Effects on Biofilm Formation But Has No Affect on Planktonic Cell Growth

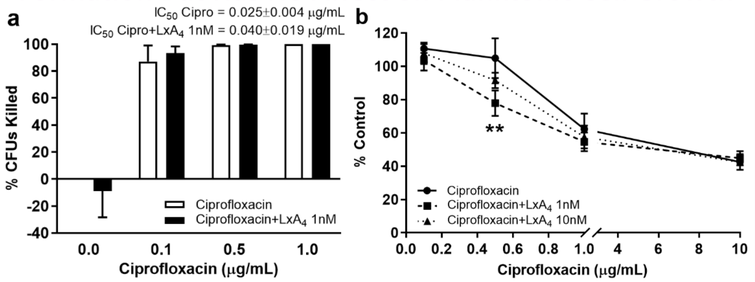
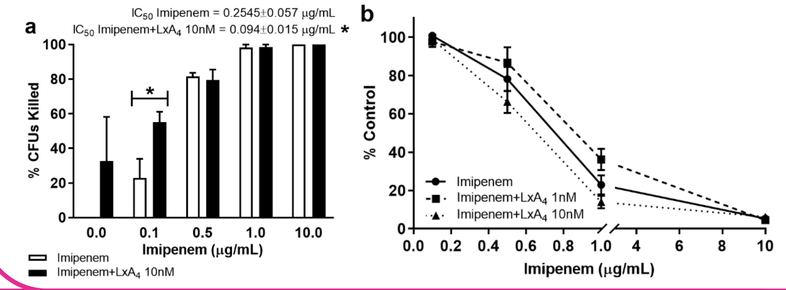


Fig. 5. LxA_4 treatment can aid the efficacy of the antibiotic ciprofloxacin in reducing biofilm formation. Cultures were treated in a 96-well plate overnight with multiple concentrations of ciprofloxacin and LxA_4. The cultures and apical biofilm were washed away, and the remaining apical biofilm rings were stained with 0.1% crystal violet. Absorbance was measured at 600 nm. Some cultures were recovered from the 96-well plate, diluted and spread on tryptic soy agar plates to incubate overnight at 37°C. Colonies were counted the next day. (a) LxA_4 does not have an effect when combined with ciprofloxacin on non-adherent cell growth, determined by colony forming units (CFUs). (b) When combined with LxA_4, biofilm formation is significantly reduced in ciprofloxacin treatments at and above bactericidal doses (0.5 μg/mL). CFU data are mean ± s.e.m. of percent change from control adjusted to zero. Biofilm data are mean ± s.e.m. percent of control. ** = $p < 0.01$; CFUs n = 4 independent experiments; biofilm n = 6 (1 nM) and n = 3 (10 nM) independent experiments.

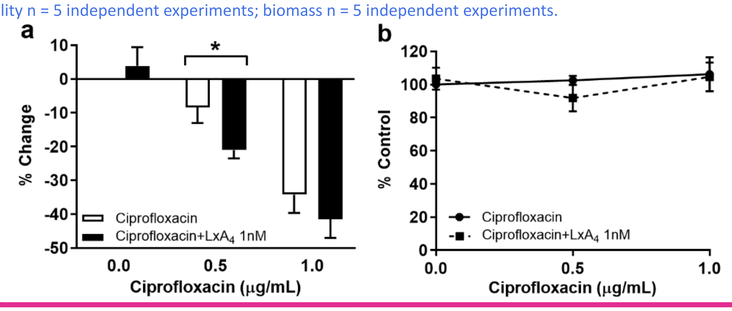
LxA_4 Does Not Affect Imipenem Efficacy Against Biofilm Formation But Enhances Suppression of Planktonic Growth

Fig. 6. LxA_4 in combination with imipenem significantly decreased non-adherent cell growth but not biofilm formation. Cultures were treated in a 96-well plate overnight with multiple concentrations of imipenem and LxA_4. The cultures and apical biofilm were washed away, and the remaining apical biofilm rings were stained with 0.1% crystal violet. Absorbance was measured at 600 nm. Some cultures were recovered from a 96-well plate, diluted and spread on tryptic soy agar plates to incubate overnight at 37°C. Colonies were counted the next day. (a) LxA_4 (10 nM) in combination with imipenem (0.1 μg/mL) significantly decreased non-adherent cell growth. (b) LxA_4 does not significantly affect the efficacy of imipenem against biofilm formation. These data combined with the ciprofloxacin data (Fig. 4) suggests the synergistic action of LxA_4 with antibiotics is dependent on the class of antibiotics and their mechanism of action. CFU data are mean ± s.e.m. of percent change from control adjusted to zero. Biofilm data are mean ± s.e.m. percent of control. * = $p < 0.05$; CFUs n = 4 independent experiments; biofilm n = 3 (LxA_4 1 nM) and n = 4 (LxA_4 10 nM) independent experiments.



LxA_4 Can Increase the Efficacy of Ciprofloxacin Against Bacteria of an Established Biofilm

Fig. 7. Effects of LxA_4 on ciprofloxacin action in pre-formed biofilms. (a) LxA_4 (1 nM) significantly increased the efficacy of ciprofloxacin (0.5 μg/mL) to reduce the amount of metabolically active bacterial cells associated with the biofilm, determined by MTT assay. (b) LxA_4 does not significantly alter the efficacy of ciprofloxacin to reduce biofilm biomass, determined by crystal violet staining. This suggests that LxA_4 treatment can aid the ciprofloxacin in accessing the bacteria associated with a pre-formed biofilm. Viability data are mean ± s.e.m. of percent change from control adjusted to zero. Biofilm data are mean ± s.e.m. percent of control. * = $p < 0.05$; viability n = 5 independent experiments; biomass n = 5 independent experiments.



Conclusions

In summary, our results show that LxA_4 and RvD_2 can directly reduce *P. aeruginosa* biofilm formation. LxA_4 and RvD_2 can also downregulate virulence gene expression. LxA_4 enhances the efficacy of antibiotics directly against *P. aeruginosa* biofilm formation and bacterial cells within existing biofilm. These studies also provide evidence that further investigation into the antimicrobial mechanisms of RvD_2 is warranted. The results suggest that there is relative selectivity in SPM inhibition of biofilm formation.

Future Directions

- SPM treatment of *P. aeruginosa* to evaluate the effects of SPMs on quorum-sensing gene expression and regulation
- 2-hit treatments of SPMs ± antibiotics on established biofilms to assess the effects of SPMs on antibiotic ability to kill biofilm-associated persister cells
- Pre-treatment of THP-1 monocytes with SPMs against established biofilm
- Co-incubation of THP-1 monocytes with SPMs against established biofilm to assess the effects of SPMs on immune cell ability to phagocytose biofilm

Acknowledgments

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