HHS Public Access

Author manuscript

Prostaglandins Other Lipid Mediat. Author manuscript; available in PMC 2022 February 01.

Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2021 February ; 152: 106505. doi:10.1016/j.prostaglandins.2020.106505.

Lipoxin A4 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 Journal 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.

Keywords

Lipoxin A4; Resolvin; *Pseudomonas aeruginosa*; biofilm; antibiotics; Pro-resolving mediators

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative bacterium which is a major cause of nosocomial infections and secondary infections in immunocompromised patients

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AUTHOR CONTRIBUTIONS

J. M. Thornton designed and performed the studies, analyzed data, and wrote the paper. J. M. Walker helped perform the biofilm studies, designed the gene expression studies, and helped write the paper. P. Y. Kadiyam Sundarasivarao performed the gene expression studies. B. W. Spur and A. Rodriguez synthesized the SPMs. K. Yin designed the studies, analyzed data, and wrote the paper.

(1). Biofilms are found to be present in burns and chronic open wounds, in infections involving indwelling catheters, and surgical implants (2). The ability of *P. aeruginosa* to form cooperative biofilm communities adds to the difficulty in treating and eradicating these infections. *P. aeruginosa* virulence is controlled by a bacterial population density-mediated signaling network called quorum sensing (QS) (3). QS signaling in *P. aeruginosa* involves four major interconnected signaling pathways (3). The signaling molecules for these pathways are: N-(3-oxododeconoyl)-L-homoserine lactone for the *las* pathway, N-butanoyl-L-homoserine lactone (BHL) for the *rhl* pathway, 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* Quinolone Signal, PQS) pathway and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing, IQS) (3–5). When a critical population density is reached, *P. aeruginosa* secretes these molecules which then act on their respective receptors to trigger coordinated expression of virulence genes (5). With respect to the signaling hierarchy, the *las* signaling system appears to sit upstream of the PQS, IQS and *rhl* pathways, with PQS having influence over *rhl* as well (5). Biofilm production is initiated, exotoxins such as pyocyanin and elastase are produced, and antibiotic resistance genes are activated (6–8).

Biofilms are bacterial extracellular matrices containing populations which adhere to each other and often to solid surfaces. These biofilms encapsulate bacterial cells with proteins and nuclear material using exopolysaccharides (9). This increases bacterial virulence by reducing the ability of antibiotics or immune cells to penetrate the biofilm and clear the infection. Intrinsic and acquired antibiotic resistance causes *P. aeruginosa* to be increasingly challenging to eradicate.

Specialized Pro-Resolving Mediators (SPMs) are lipids, endogenously produced by the host as part of inflammation resolution (10, 11). During inflammation or infection, there is transcellular biosynthesis of lipoxins from arachidonic acid and resolvins from omega-3 fatty acids (11). The transcellular biosynthesis may occur between neutrophils, monocyte/macrophages, and epithelial cells (12). Lipoxin A_4 (LxA_4) has been reported to decrease neutrophil activation, increase monocyte/macrophage recruitment, and attenuate proinflammatory cytokine production in models of infection and sepsis (12–14). Furthermore, previous studies have shown that LxA_4 can directly increase the phagocytic ability of neutrophils isolated from septic mice (14) and promote macrophage phagocytosis of apoptotic neutrophils (15, 16). Similarly, resolvins of the D-series derived from docosahexaenoic acid (22:6) and E-series derived from eicosapentaenoic acid (20:5) reduced inflammation and decreased bacterial load in different models of inflammation and infection (17–21). Furthermore, RvD_2 decreased antibiotic requirements in an *in vivo* model of infection (20). Although there have been many studies which have focused on SPM action on host response in inflammation and infection, little work has been conducted to examine if SPMs have direct effects on bacteria growth or virulence.

Many studies have targeted synthetic inhibitory molecules to antagonize QS signaling (22). We have previously shown that LxA_4 is a potent inhibitor of QS signaling by acting as a strong antagonist of the LasR receptor (23). Furthermore, *P. aeruginosa* secretes an arachidonate 15-lipoxygenase enzyme (LoxA) which may alter the local environment by synthesizing SPMs such as LxA_4 (24). On the other hand, there is evidence that *P.*

aeruginosa inhibits production of 15-epi-LxA_4, suggesting that the bacteria possesses a mechanism to reduce resolution in the local environment (25). Therefore, we reasoned that LxA_4 may directly reduce *P. aeruginosa* biofilm formation.

Two common antibiotics used in the treatment of *P. aeruginosa* infections are ciprofloxacin and imipenem (2). *P. aeruginosa* has been shown to develop resistance to ciprofloxacin and imipenem (26–28). Ciprofloxacin (a topoisomerase inhibitor) (29) at sub-inhibitory concentrations may cause increased formation of biofilm (30). Ciprofloxacin has been shown to increase biofilm-associated bacterial cells' antibiotic resistance above that of non-adherent cells, only recovering susceptibility after 5–10 passages (31). Ciprofloxacin and imipenem (a cell wall inhibitor) (29) have both shown QS-inhibitory action on *P. aeruginosa* (32, 33). Ciprofloxacin decreases expression for a range of QS-regulated virulence factors, suggesting the mechanism of QS inhibition may be mediated by alterations to membrane permeability and the flux of the QS autoinducer signaling molecule (33, 34). It is therefore plausible that a known QS inhibitor such as LxA_4 may augment the actions of these antibiotics.

In this study, we used an established *in vitro* system to examine the effects of LxA_4 and other SPMs on *P. aeruginosa* biofilm formation. We also examined the effects of SPMs on virulence gene expression. We investigated the potential synergistic action of LxA_4 with antibiotics to reduce bacterial biofilm formation and/or increase antibiotic bactericidal activity.

2. Materials and methods

2.1 Culture preparation and biofilm growth:

P. aeruginosa ATCC 27853™ (American Type Culture Collection, Manassas, VA, USA) was streaked on a Tryptic soy agar plate (TSA; Ward's Scientific, Rochester, NY, USA) and incubated overnight at 37°C. From the streaked plate, liquid cultures were made by swabbing *P. aeruginosa* with a wooden applicator stick and depositing into glass culture tubes with 3 mL LB broth (Luria-Bertani broth; Gibco: Gaithersburg, MD, USA). The cultures were incubated for 5 hr at 37°C with shaking (180 rpm) and pelleted by centrifuging for 6 min at 9100 g. LB broth was removed, and pellets were washed 3 times with M63 minimal media (Amresco, Cleveland, OH, USA) supplemented with 1 mM $MgSO_4$, 0.2% glucose, and 0.5% casamino acids (Fisher BioReagents, Pittsburgh, PA, USA). Cultures were diluted in M63 to an OD_{600} between 0.05 – 0.10. 125 μL of the cultures were plated into each well of U-bottom 96-well microplates. 0.1 – 10 nM of SPM (obtained by organic synthesis, see section 2.9), FPR2 peptide agonist WKYMVm (Tocris, Minneapolis, MN, USA) or saline (Molecular Biologicals International, Irvine, CA, USA) was added to the wells. The OD_{600} was measured to verify consistent plating of the culture (Biotek Synergy H1 plate reader; Biotek, Winooski, VT, USA). The plates were incubated overnight (19 – 20 hr) at 37°C. As lowered pH is found in airways of cystic fibrosis patients (35), these studies were conducted at lowered pH to better simulate the clinically relevant conditions, which are environments *P. aeruginosa* may be prevalent.

2.2 Biofilm assay:

To assay the biofilm biomass, plates were gently submerged in ddH_2O three times to rinse off the non-adherent bacteria. 155 μL of 0.1% crystal violet in ddH_2O (Sigma, St. Louis, MO, USA.) was added to each well for 15 min. The plates were washed in ddH_2O three times to remove excess crystal violet stain and dried upside down overnight. 175 μL of modified biofilm dissolving solution (36) (10% SDS dissolved in 80% ethanol) was added to each well and incubated for 15 min. The solution was transferred to flat-bottom microtiter plates and OD_{600} was measured to quantify the crystal violet staining.

2.3 Bacteria colonies:

To estimate non-adherent colony forming units (CFUs), cultures from 2–3 wells of each treatment group were recovered prior to washing the plate in ddH_2O. These cultures were diluted in saline, 100 μL were spread on TSA plates, incubated overnight at 37°C, and colonies counted.

2.4 Antibiotic experiments:

For experiments using antibiotics, dilutions were made such that 0.1 – 10 μg/mL of antibiotic was added to treatment wells. LxA_4 (1, 10 nM) or vehicle saline was added to the 96-well microplates first, followed by ciprofloxacin (Enzo, Farmingdale, NY, USA), imipenem (Alfa Aesar, Tewksbury, MA, USA) or saline with gentle swirling. In these studies, culture was plated at densities at which LxA_4 alone did not affect biofilm formation. The plates were incubated at 37°C for 20 hr. Biofilm assays or CFU counts of non-adherent bacteria were performed as described in 2.2 and 2.3.

2.5 Pre-formed biofilm assay:

Untreated biofilm was grown for 20 hr before addition of antibiotics and LxA_4 for 6 hr. At the end of treatment incubation time, biofilm biomass was quantitated as described in 2.2.

2.6 MTT assay:

Based on methods previously published (37–39) with modifications as follows. In brief, 100 μL untreated biofilm was grown for 20 hr. The plates were gently submerged in 1X PBS (Molecular Biologicals International, Irvine, CA, USA) three times to rinse off the culture and non-adherent bacteria. Ciprofloxacin with or without LxA_4 in M63 media was added (125 μL) and incubated with pre-formed biofilm for 6 hr, then rinsed in 1X PBS three times. Thiazolyl Blue tetrazolium bromide (MTT) (Alfa Aesar, Tewksbury, MA, USA) stock solution was made to a concentration of 5 mg/mL in 1X PBS, and a working solution was made in M63 to a final concentration of 0.45 mg/mL. 150 μL of this MTT working solution was plated and incubated for 4 hr at 37°C. The solution was pipetted from the plate, and 175 μL of solubilization solution (alkaline DMSO: 0.5mM NaOH in DMSO) was incubated for 30m at 37°C. The solubilized formazan was transferred to flat-bottom microtiter plates and OD_{570} was measured to quantify the formazan, reflecting the relative amount of metabolically active bacterial cells in the biofilm.

2.7 Growth curves:

Cultures were prepared as in 2.1. 200 μL of the cultures were plated into flat bottom 96-well microtiter plates. 0.01 – 100 nM of LxA_4 or RvD_2 or saline solution was added to control wells. Empty wells were filled with 200 μL ddH_2O to serve as a moisture reservoir. The plates were covered and shaken at 37°C, and OD_{600} was measured every 10 min for 15 hr in a microplate reader.

2.8 Gene expression via RT-PCR and qRT-PCR:

Cultures were prepared as in section 2.1 and incubated for 8 hr at 37°C with shaking (180 rpm). 1 mL of the cultures were aliquoted, and 1 nM of each treatment or saline was added to the tubes. Treatments were incubated for designated time points before RNA extraction. RNA was extracted using Trizol Max Bacterial RNA isolation Kit (Thermo Fisher Scientific, Waltham, MA). RNA purity (Absorbance ratio A260/A280, A260/A230) and RNA concentration were measured using a nanophotometer (IMPLEN P330; Implen, Los Angeles, CA, USA). Extracted RNA was stored at −70°C until further use.

Approximately 1 μg of RNA was reverse transcribed using High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). RNase Inhibitor (Thermo Fisher Scientific Waltham, MA, USA) was used in all reactions to prevent RNA degradation. Reverse Transcription was performed using kit protocol and recommended conditions. cDNA purity (Absorbance ratio A260/A280, A260/A230) and concentration was measured using a nanophotometer. Samples were stored at −20°C until further use.

Quantitative real-time PCR (qRT-PCR) was performed by SYBR green method using Fast SYBR green master mix (Thermo Fisher Scientific, Waltham, MA, USA) and primers (Table 1). Primer sequences for genes *pqsA*, r*hlA*, and 16S rRNA were obtained from published work (40). 16S rRNA was used as the endogenous control. The program used for qRT-PCR was adapted from recommended thermal cycler conditions with modifications according to primers used (Table 2) and carried out in an Eppendorf Realplex Mastercycler (Eppendorf, Westbury, NY, USA).

2.9 SPM preparation:

All SPMs (LxA_4, RvD_1, RvD_2, RvD_5) were prepared by total organic synthesis (41–44). Purity of the compounds was measured by HPLC-Mass Spectrometry and was determined to be > 98%. The SPMs were diluted in saline and bubbled with argon to displace oxygen. Structures of SPMs used are shown in Figure 1.

2.10 Statistical analyses:

All analyses ware performed using GraphPad Prism (San Diego, CA, USA). Biofilm data were subjected to one-way ANOVA. Dunnett's test for multiple groups was then used to test for significance compared to controls. For growth curves, areas under the curve were calculated and then subjected to one-way ANOVA. Student's t-test was used to test significance between antibiotic and antibiotic + LxA_4 at each concentration of antibiotic. IC_{50} was measured after drawing individual cubic spline plots for each treatment. The average IC_{50} for each treatment was then calculated and compared using t-test. In all

analyses, $P < 0.05$ was taken as significant. All data is expressed as mean ± s.e.m. For gene expression, relative gene quantification was done by ΔC_T method (45). Relative quantification was performed by calculating average of triplicate C_T values. $1/\Delta C_T$ values were used to measure statistical significance between treated and untreated controls of the same time point.

3. Results

3.1 Biofilm formation:

P. aeruginosa cultures were incubated with saline vehicle or SPM (LxA_4, RvD_1, RvD_2, RvD_5) in 96 well plates for 20 hr. At the end of the incubation period, wells were washed and stained with crystal violet. After drying, the stained biofilm mass was removed with detergent solution containing SDS and ethanol. LxA_4 and RvD_2 (0.1 – 10 nM) significantly reduced biofilm formation, but RvD_1 and RvD_5 did not (Figure 2a). The results suggest that there is SPM selectivity in biofilm reduction. To further examine other receptor-mediated effects, we performed studies with a FPR2 receptor peptide agonist, WKYMVm. The FPR2 peptide had no significant effect on biofilm formation (Figure 2b).

3.2 Growth curves:

In these studies, we examined if LxA_4 and RvD_2 had any effect on non-adherent *P. aeruginosa* growth rate. Neither LxA_4 nor RvD_2 had a significant effect on non-adherent *P. aeruginosa* growth, suggesting that the reduction in biofilm biomass was not a result of an inhibitory effect on cell growth (Figure 3).

3.3 Effects of SPMs on quorum-sensing gene expression:

P. aeruginosa was incubated with either saline or 1 nM SPMs (LxA_4 or RvD_2). At 2.5 hr, *Pseudomonas aeruginosa* untreated controls increased expression of the two virulence genes investigated (*pqsA, rhlA*) compared to expression at t=0 (Figure 4). LxA_4 did not significantly affect gene expression at 2.5 hr (Figure 4a). At 4.5 hr, LxA_4 reduced expression of *pqsA* and *rhlA* compared to saline controls at the same time point (Figure 4a). On the other hand, RvD_2 increased expression of *pqsA* at 2.5 hr, but at 4.5 hr, RvD_2 significantly reduced expression of *pqsA* (Figure 4b). There was a strong tendency for RvD_2 to inhibit *rhlA* expression at 4.5 hr, but the inhibition did not reach significance (Figure 4b). These results suggest that both LxA_4 and RvD_2 can inhibit expression of virulence genes at 4.5 hr after treatment. The results also suggest that at these initial time points of biofilm formation, LxA_4 has a greater effect on downregulation of virulence genes compared to RvD_2. Therefore, only LxA_4 was used in further experiments.

3.4 Interactions of LxA_4 with ciprofloxacin:

LxA_4 (1, 10 nM) or saline was incubated with ciprofloxacin (0.1 – 50 μg/mL) for 20h. Biofilm was then measured. The number of non-adherent bacteria (CFUs) was measured in parallel. At 0.1 and 0.5 μg/mL concentrations, ciprofloxacin did not affect *P. aeruginosa* biofilm formation (Figure 5b). It should be noted that 0.5 μg/mL ciprofloxacin was bactericidal i.e. causing 99% inhibition of non-adherent *P. aeruginosa* growth (Figure 5a). Interestingly, LxA_4 (1 nM) significantly reduced biofilm formation when incubated with

ciprofloxacin (0.5 μg/mL) (Figure 5b), providing evidence that any effects of LxA_4 on antibiotic efficacy were synergistic. LxA_4 (1 nM), however, did not have any effect on ciprofloxacin-induced inhibition of bacterial growth (Figure 5a). These results suggest that a bactericidal concentration of ciprofloxacin did not affect biofilm formation without concomitant addition of LxA_4.

3.5 Interactions of LxA_4 with imipenem:

LxA_4 (1, 10 nM) or saline was incubated with imipenem (0.1 – 10 μg/mL) for 20 hr before biofilm mass measurement. As in studies with ciprofloxacin, the number of non-adherent bacteria (CFUs) was measured in parallel. Imipenem dose-dependently reduced biofilm formation (Figure 6b). LxA_4 did not affect imipenem induced biofilm reduction. Imipenem dose-dependently reduced non-adherent bacterial growth (Figure 6a). Unexpectedly, LxA_4 (10 nM) enhanced the bacterial killing action of imipenem at 1 μg/mL (Figure 6a). Overall, IC_{50} of imipenem was significantly lowered by LxA_4 (Figure 6a). These findings suggest that LxA_4 increased the efficacy of imipenem's bactericidal activity *in vitro*.

3.6 Interaction of LxA_4 with ciprofloxacin in pre-formed biofilm:

In these studies, *P. aeruginosa* biofilm was grown for 20 hr before addition of ciprofloxacin (0.5, 1 μg/mL) with or without LxA_4. Bacterial cell viability within the biofilm was then assessed by the MTT assay. Our results show that ciprofloxacin alone at 0.5 μg/mL had no effect on *P. aeruginosa* viability within the biofilm, but addition of LxA_4 increased the bactericidal activity of ciprofloxacin (Figure 7a). LxA_4 had no effect on the action of ciprofloxacin on pre-formed *P. aeruginosa* biofilm biomass (Figure 7b).

4. Discussion

This study provides evidence that LxA_4 and RvD_2 have beneficial effects to reduce *P. aeruginosa* biofilm formation directly and reduce virulence gene expression. LxA4 improved the efficacy of antibiotics (ciprofloxacin and imipenem). With respect to its effects in concert with antibiotics, LxA_4 increased efficacy of ciprofloxacin (0.5 μg/mL) to reduce biofilm formation and increased killing of biofilm-associated *P. aeruginosa* in pre-formed biofilm. This action did not affect ciprofloxacin's bacterial killing of non-adherent cells. On the other hand, LxA_4 did not affect imipenem action on biofilm formation, but unexpectedly increased imipenem's bacterial killing of non-adherent cells. LxA_4 alone had no effect on *P. aeruginosa* growth.

The inflammation resolution actions of SPMs have been documented in various models of *in vivo* inflammation and infection (12, 13, 17, 18, 21, 46). With respect to infection, SPMs have been shown to reduce systemic inflammation as well as bacterial load (13, 18, 21). In mammalian cells, these actions are mediated by specific G-protein receptors. Specifically, LxA_4 binds to the formyl peptide receptor 2 (FPR2) while RvD_1 binds both FPR2 and GPR 32 (47, 48). RvD_5 binds only GPR 32 while RvD_2 binds only GPR 18 (18, 20). The direct actions of these SPMs on bacterial cells, however, have not been examined extensively.

LxA_4 and RvD_2, but not RvD_1 or RvD_5, reduced *P. aeruginosa* biofilm formation. As LxA_4 shares a similar receptor with RvD_1, while RvD_2 does not share a similar receptor with

either SPM, the mechanism of biofilm inhibition is likely not related to structural binding to a common receptor, such as FPR2. In our experiment, using an FPR2 peptide agonist did not have any significant effect on biofilm formation. This is consistent with other studies showing that the formyl peptide receptors are mammalian receptors and thus *P. aeruginosa* is unlikely to be affected by FPR agonists (49). We have shown that LxA_4 binds to the lasR receptor and inhibits the quorum sensing pathway (23). Taken together, our results suggest that LxA_4 reduces biofilm formation through a QS inhibitory mechanism. This conclusion is supported by our results showing that neither LxA_4 nor RvD_2 given alone, altered non-adherent cell growth in any way. Additionally, there was no significant dose-dependent effect of LxA_4 or RvD_2 at the concentrations tested. This would suggest that the biofilm inhibitory mechanism is easily saturated. We have previously shown similar results when looking at the effects of LxA_4 in reducing pyocyanin release from *P. aeruginosa* (23). These data have significant clinical relevance because diminishing biofilm formation should augment immune cell clearance of the pathogen. This possibility is a subject of ongoing studies in our lab.

Virulence gene *pqsA* is indispensable for biofilm formation in *Pseudomonas aeruginosa* (50). PqsA is required to produce metabolites such as 2,4-dihydroxyquinoline (DHQ) and 4-hydroxy-2-alkylquinolines (HAQs) which act as signals for cell-cell communication (51). As both LxA_4 and RvD_2 downregulated *pqsA* expression, it suggests that the mechanism by which LxA_4 and RvD_2 inhibit biofilm formation is at least partially through down regulation of *pqsA* expression. *pqsA* is downstream of lasR and therefore from other data published by our lab (23), the effect seen on *pqsA* could be a result of LxA_4 affecting lasR. *rhlA* is required for biosynthesis of rhamnolipids, which are dimers of 3-hydroxy fatty acids linked to a mono- or di-rhamnose moiety by an O-glycosidic link (52). Importantly, rhamnolipids are vital to maintaining the architecture of biofilms and protect the biofilms against host neutrophils (52–54). Our results showing that LxA_4 significantly reduced both *pqsA* and *rhlA* expression further supports the notion that LxA_4 can directly suppress *P. aeruginosa* virulence. As RvD_2 increased *pqsA* at 2.5 hr and had a smaller reducing effect at 4.5 hr than LxA_4, it suggests that RvD_2 is less potent than LxA_4 in decreasing virulence at these time points. Interestingly, neither LxA_4 nor RvD_2 had any effect on reducing virulence gene expression at 2.5 hr. This result suggests that early virulence (including early biofilm formation) is not inhibited by either SPM and would therefore account for the modest decreases in biofilm formation with both SPMs. This result also suggests that SPM downregulation of virulence genes is not through early inhibition of transcription, but a later suppression of these genes.

P. aeruginosa biofilm is an exopolysaccharide matrix that encapsulates the bacterial cells and provides a protection against antibiotics (26, 28). The mechanism for this resistance is now believed to be dependent on the type of antibiotic used. Fluoroquinolone antibiotics such as ciprofloxacin can penetrate *P. aeruginosa* biofilm, while aminoglycoside penetration is limited (26). However, penetration of ciprofloxacin into the biofilm does not affect the biofilm-associated *P. aeruginosa* viability. It is thought that one mechanism of ciprofloxacin resistance in biofilm is due to a subpopulation switch to the persister phenotype, which contributes to a failure in biofilm eradication (55).

It is important to note that we performed counts of non-adherent bacteria in wells side by side to wells used for biofilm biomass determination. By employing this method, we were able to ascertain the bacteria killing activity of the antibiotic in a physiologically relevant setting where *P. aeruginosa* actively forms biofilm. The results strongly suggest that a ciprofloxacin concentration (0.5 μg/mL) that is bactericidal to non-adherent bacteria does not reduce biofilm formation to any significant degree. This concentration is consistent with published work showing that it is indeed bactericidal for *P. aeruginosa* (56), supporting our methodology. We show that LxA_4 can increase efficacy of ciprofloxacin to reduce biofilm formation. As there is evidence that LxA_4 can inhibit quorum sensing (23), it is possible that LxA_4 can reduce the ciprofloxacin-mediated switch of *P. aeruginosa* to the persister phenotype and reduce *P. aeruginosa* biofilm. It is also possible that by reducing rhamnolipid production, ciprofloxacin can more easily destroy biofilm architecture.

With respect to pre-formed or existing biofilm, our results suggest that ciprofloxacin at 0.5 μg/mL did not significantly kill biofilm associated *P. aeruginosa*. We show for the first time that LxA_4 promoted the bactericidal actions of ciprofloxacin in pre-formed biofilm matrix, which supports the plausibility of LxA_4 as adjunctive treatment for *P. aeruginosa* infections. There was no effect of ciprofloxacin on biofilm mass nor did LxA_4 have any effect on this. The results suggest that neither ciprofloxacin at bactericidal concentrations nor LxA_4 have any effect on destroying pre-formed biofilm structure. If combined treatments of SPMs can help make classically used antibiotics effective against multi-drug resistant bacterial infections, we have a method to combat the growing public health crisis of multi-drug resistant pathogens.

Imipenem is an antibiotic which works by inhibiting cell wall synthesis of gram negative and gram positive bacteria, where it inactivates specific proteins involved in the last stages of cell wall synthesis (29). Imipenem alone dose-dependently inhibited biofilm formation and LxA_4 did not affect this action. Others have shown that imipenem works on the las QS system, just as LxA_4 (57). It is unlikely that LxA_4 affected imipenem's action through the *las* QS system because there was no effect on biofilm formation. On the other hand, LxA_4 increased bactericidal efficacy of imipenem on non-adherent bacteria at the low dose of 0.1 μg/mL imipenem but not at higher doses. At higher doses (≥ 0.5 μg/mL), imipenem caused ≥ 80% bacterial death, making it difficult to increase the bactericidal action of imipenem any further. The effect of LxA_4 on the action of low dose imipenem was unexpected because LxA_4 does not have any effect on *P. aeruginosa* growth. We are currently investigating if this effect with imipenem is specific to *P. aeruginosa* or whether it extends to other bacterial pathogens.

The beneficial effects of combining an SPM such as LxA_4 with an antibiotic may be of great significance in the topical treatment of wound infections. This is because wounds infected with *P. aeruginosa* have decreased healing due to excessive neutrophil infiltration and increased inflammatory cytokine release (58, 59). It is plausible that adjunctive or concomitant use of antibiotic and LxA_4 may have beneficial actions to increase efficacy of the antibiotic but also to increase wound healing through inflammation resolution. However, the therapeutic window for LxA_4's effects on antibiotic efficacy is small, between 1 – 10 nM

LxA_4, and is similar to LxA_4's effect on biofilm formation (Figure 2). The small therapeutic window may be due to the rapid saturation of its actions.

P. aeruginosa lung infections are common in cystic fibrosis patients. The bacteria use mechanisms such as biofilm formation to evade antibiotic killing or host defense mechanisms. These persistent infections cause chronic inflammation. Studies have shown that SPM production is reduced in cystic fibrosis (60, 61). Importantly, use of an SPM such as LxA_4 has been reported to decrease both bacterial burden and disease severity *in vivo* (61). *In vitro* studies showing that LxA_4 delayed *P. aeruginosa* colonization of airway epithelial cells (62) and augmented airway tissue repair (63) provide possible mechanisms for the beneficial actions of LxA_4 in cystic fibrosis. Our study adds to current mechanistic knowledge by showing that LxA_4 can directly reduce *P. aeruginosa* biofilm formation and importantly increases the efficacy of two antibiotics commonly used against *P. aeruginosa* infections.

4.1 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.

ACKNOWLEDGEMENTS

The work was supported by NIH RO1 AI128202 and a grant from the New Jersey Health Foundation. We thank Rachael Wilson for helping with completion of this work.

Source of support: This work was supported by the NIH (RO1 AI128202) and the New Jersey Health Foundation

Abbreviations:

SPMs	Specialized Pro-resolving Mediators
LxA_4	Lipoxin A4
Rv	Resolvin
CFUs	Colony forming units
PQS	*Pseudomonas* Quinolone Signal
IQS	Integrated Quorum Sensing Signal

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HIGHLIGHTS

- Lipoxin A_4 (LxA_4) and Resolvin D2 (RvD_2) directly reduce *Pseudomonas aeruginosa* biofilm formation.
- LxA_4 and RvD_2 reduce expression of *Pseudomonas aeruginosa* virulence genes.
- LxA_4 augments ciprofloxacin's effects on biofilm reduction but does not affect ciprofloxacin's bactericidal action on non-adherent cells.
- LxA_4 augments imipenem's bactericidal action on non-adherent cells but does not affect imipenem's action on *Pseudomonas* biofilm formation.
- LxA_4 augments ciprofloxacin's antimicrobial action within pre-formed *Pseudomonas* biofilm

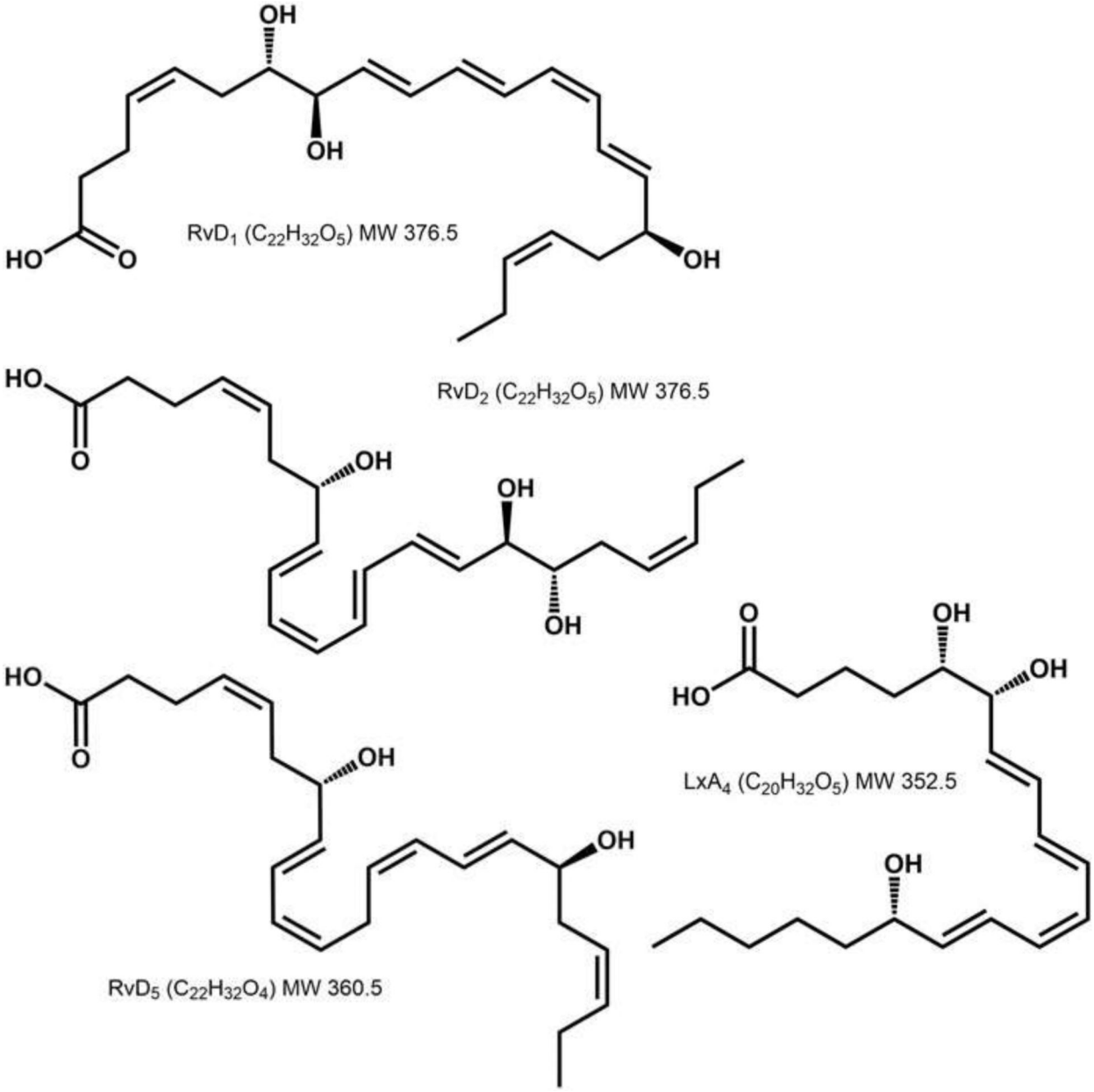

Figure 1.

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 (21, 42–44, 64). LxA_4 is a 20C chain with hydroxyl groups on C5, 6 and 15 (41).

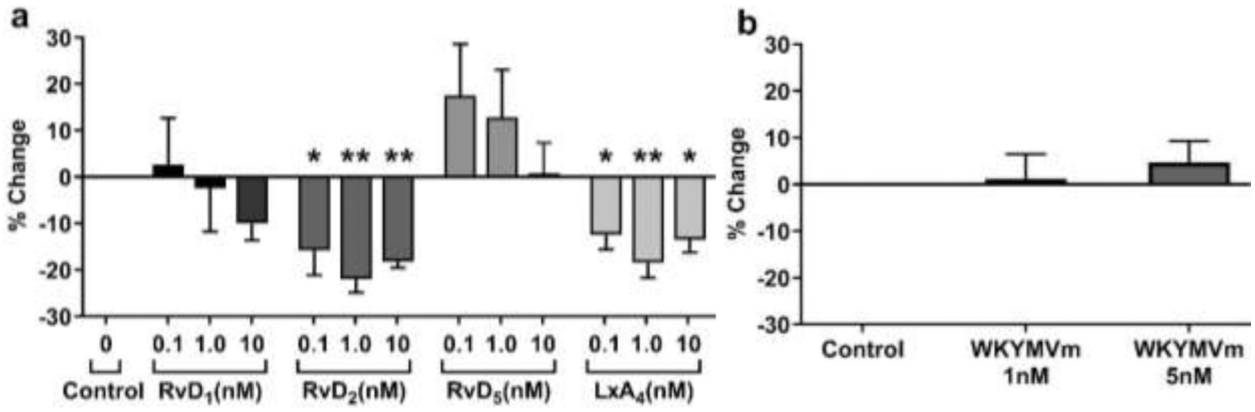

Figure 2.

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 three 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.

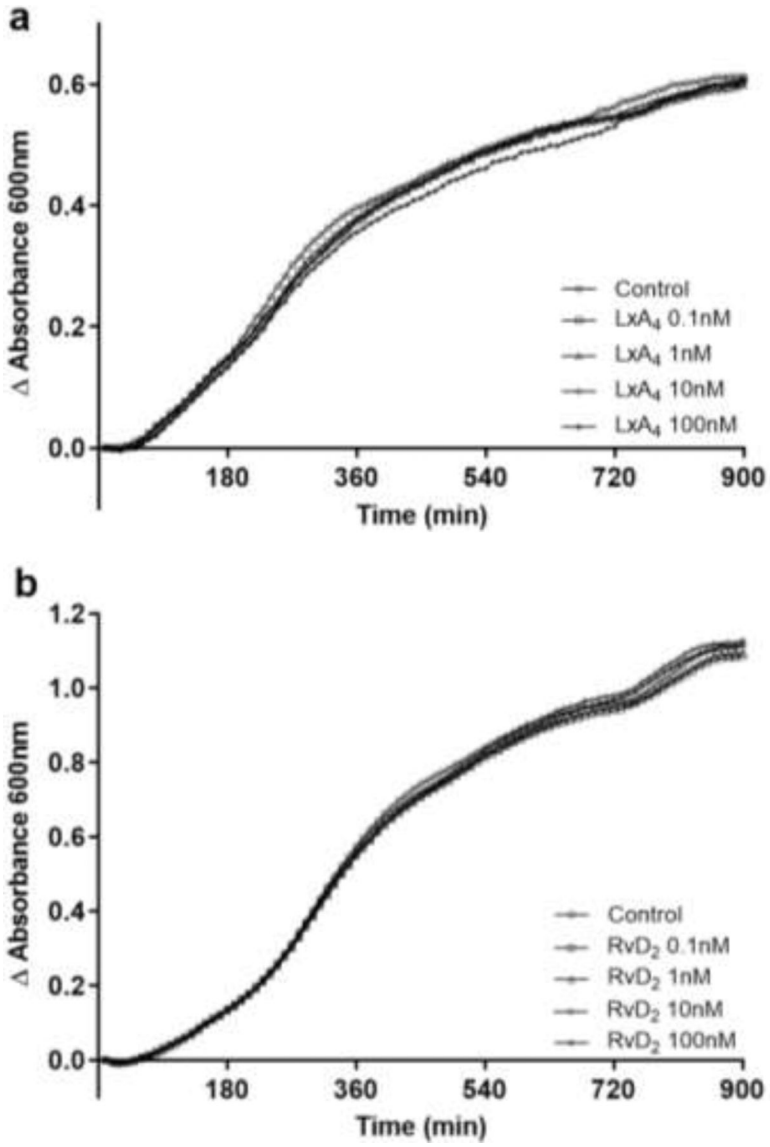

Figure 3.

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.

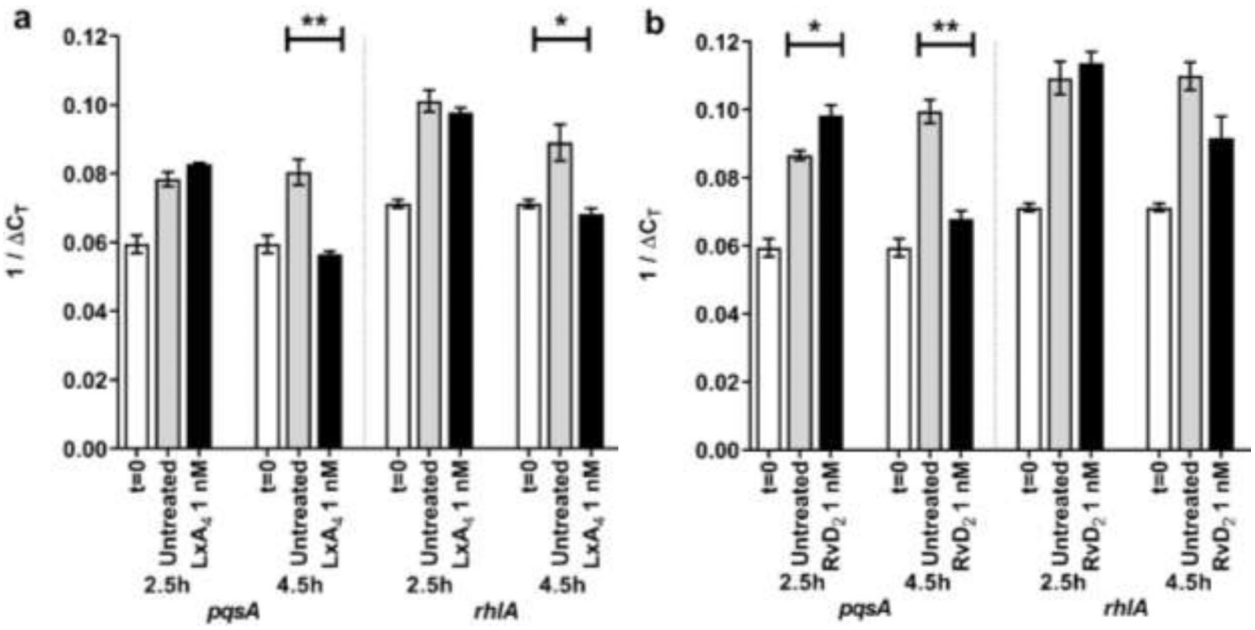

Figure 4.

Effects of SPMs on *Pseudomonas aeruginosa* quorum-sensing virulence gene expression. **(a)** 1/ΔCT of two virulence genes' expression when treated with 1 nM LxA4. LxA4 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.

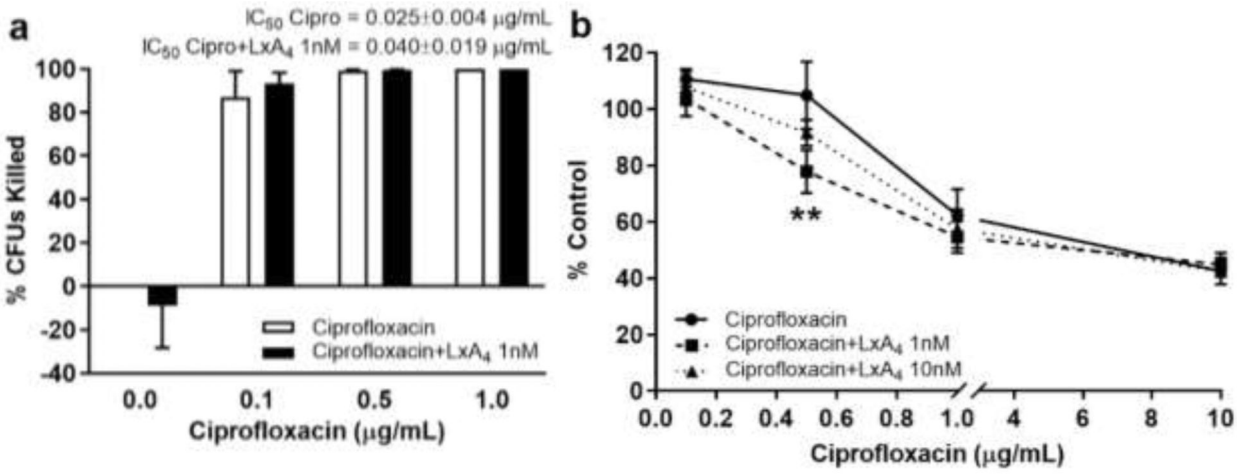

Figure 5.

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.

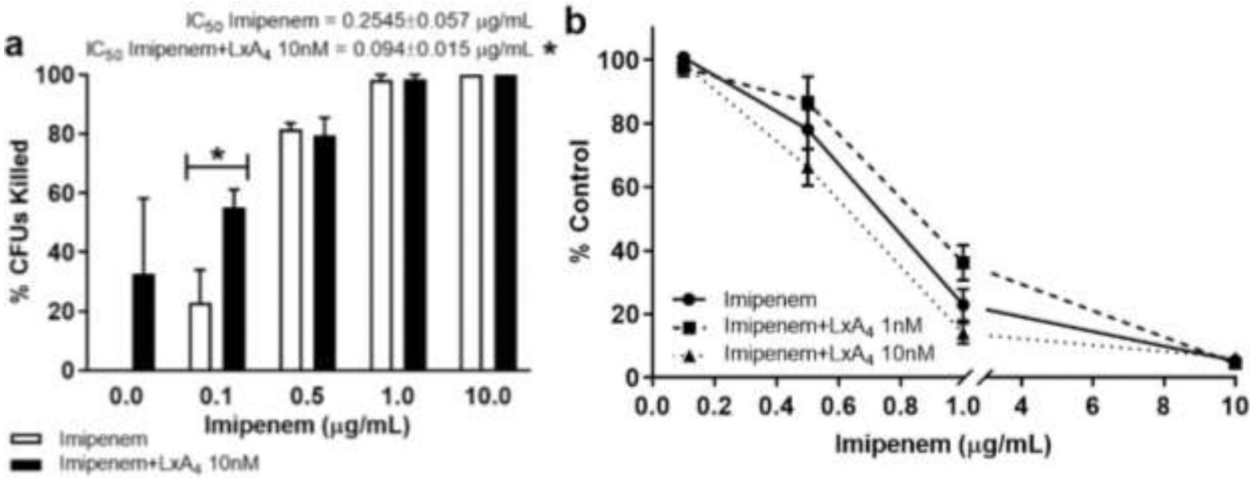

Figure 6.

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.

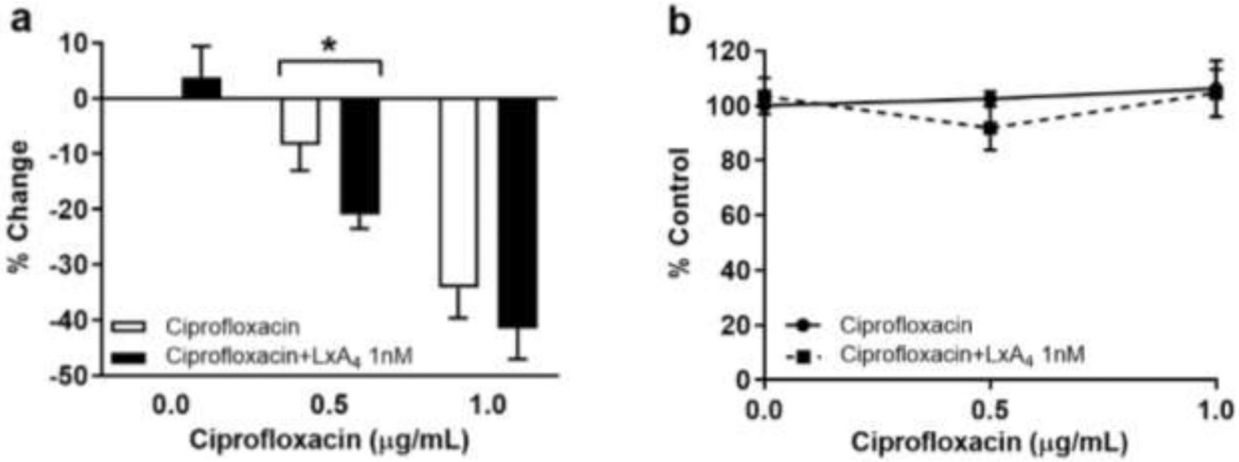

Figure 7.

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.

Table 1.

Primer sequences used for virulence gene expression qRT-PCR.

GENE NAME	PRIMER SEQUENCES	AMPLICON LENGTH
16S rRNA	Forward: 5'-GGAGAAAGTGGGGGATCTTC-3' Reverse: 5'-CCGGTGCTTATTCTGTTGGT-3'	316
rhlA	Forward: 5'-GCGCGAAAGTCTGTTGGTAT-3' Reverse: 5'-ATTTCCACCTCGTCGTCCTT-3'	249
pqsA	Forward: 5'-ACCGCGAAGGACACACTATC-3' Reverse: 5'-GGCAGGTAGGAACCAGAACC-3'	297

Table 2.

Thermal cycler program optimized for virulence gene expression qRT-PCR.

STEP	NAME	Temp (°C)	Duration (sec)	Number of Cycles
1	Initiation	95	120	1
2	DNA polymerase activation	95	20	1
3	Denaturation	95	3	50
4	Anneal and Extend	60	30	50
5	Endpoint	60	120	1