Antimicrobial Activity of Ibuprofen Against Cystic Fibrosis Associated Gram–Negative Pathogens


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Running Title: Antimicrobial effects of ibuprofen

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Abstract: Clinical trials have demonstrated the benefits of ibuprofen therapy in cystic fibrosis (CF) patients, an effect that is currently attributed to ibuprofen’s anti-inflammatory properties. Yet, a few previous reports demonstrate an antimicrobial activity of ibuprofen as well, although none investigate its direct effects on the pathogens found in the CF lung, which is the focus of this work. Determination of ibuprofen’s in vitro antimicrobial activity against Pseudomonas aeruginosa and Burkholderia spp. strains through measurements of endpoint colony-forming units (CFU) and growth kinetics showed that ibuprofen reduces the growth rate and bacterial burden of tested strains in a dose-dependent fashion. In an in vitro Pseudomonas biofilm model, a reduction in the rate of biomass accumulation over 8-h of growth with ibuprofen treatment was observed. Next, an acute Pseudomonas pneumonia model was used to test this antimicrobial activity after oral delivery of ibuprofen. Following intranasal inoculation, ibuprofen-treated mice exhibited lower CFU counts and improved survival compared with control animals. Preliminary biodistribution studies performed after aerosolization of ibuprofen to mice demonstrated a rapid accumulation of ibuprofen in serum and minimum retention in lung tissue and bronchoalveolar lavage fluid. Therefore, ibuprofen-encapsulating polymeric nanoparticles (Ibu-NPs) were formulated to improve the pharmacokinetic profile. Ibu-NPs, formulated for aerosol delivery, inhibited the growth of P. aeruginosa in vitro and may provide a convenient dosing method. These results provide an additional explanation for the previously observed therapeutic effects of ibuprofen in CF patients, and further strengthen the argument for its use for these patients.
Chronic infection and inflammation are the hallmarks of cystic fibrosis (CF) lung disease and are responsible for the majority of morbidity and mortality in CF patients (1-3). Infections are polymicrobial with typical pathogens including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Burkholderia* spp., *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans*. The infections elicit an exuberant, acute inflammatory response dominated by neutrophils (4). In the distinctly altered microenvironment of the CF lung, the inflammation fails to clear the infection, causing the lung disease to progress through a self-perpetuating cycle of airway obstruction, chronic endobronchial infection, and excessive airway inflammation (4, 5), which eventually results in bronchiectasis and death (1, 2). Historically, alleviating pulmonary obstruction and bacterial infection have been the mainstays of CF therapy; however, the recent recognition of inflammation as a primary cause of lung destruction has motivated the investigation of therapies directed against the excessive inflammatory response (4, 6, 7). For instance, oral and inhaled corticosteroids, macrolides, as well as non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, have been explored in several *in vivo* models and clinical trials, and have shown an appreciable clinical effect (8-13).

Ibuprofen has appeared particularly advantageous due to its ability to target multiple inflammation pathways and its acceptable safety profile. In both a mouse model of acute *Pseudomonas* pulmonary infection (14) and a rat model of endotoxin-induced alveolitis (15), ibuprofen reduced recruitment of neutrophils into the airways. Similarly, oral ibuprofen treatment in a rat model of chronic *Pseudomonas* endobronchial infection, resulting in a drug plasma concentration of $55 \pm 24 \mu g/mL$, led to a significant reduction in the inflammatory response and improved weight gain compared with placebo treatment (16). At this concentration,
ibuprofen significantly reduced LTB₄ production by stimulated rat neutrophil-rich leukocyte preparations; however, no effect on pulmonary bacterial burden was observed (16). Thus, the beneficial effects of ibuprofen were attributed to inhibition of the lipoxygenase pathway and its downstream effects on neutrophils.

Subsequently, the safety and efficacy of high-dose ibuprofen (50 – 100 μg/mL plasma concentration) was evaluated in CF patients through clinical trials. In a randomized, double-blind, placebo-controlled trial conducted by Konstan et al., high-dose ibuprofen reduced the rate of decline of pulmonary function in patients with cystic fibrosis (CF) with good to excellent pulmonary function (10). A follow-up study analyzed data obtained from the CF registry for patients (6 – 17 years) with baseline Forced Expiratory Volume in one second (FEV₁) > 60% and demonstrated a significantly slower rate of decline of FEV₁ percent predicted for patients using high-dose ibuprofen compared with patients not treated with ibuprofen (17). Lands et al. have also investigated the safety and efficacy of high-dose ibuprofen in children (6 – 18 years) with CF in a randomized, multicenter, double-blinded, placebo-controlled trial (12). Despite the absence of a statistical difference in mean annual rate of decline in FEV₁, a significant decrease in the annual rate of decline of forced vital capacity percent (FVC%) predicted was observed between the ibuprofen and placebo groups. Collectively, these clinical trials document the benefits and relative safety of long-term use of high-dose ibuprofen in CF patients and attribute these findings to the neutrophil-modulating properties of ibuprofen (7, 10, 12, 14, 15, 18-22).

Interestingly, a few reports in the literature document the antibacterial (23-29) and antifungal activity of ibuprofen (23, 30), as well as its synergy with other antimicrobial agents both in vitro and in vivo (29-34). However, the direct effect of ibuprofen on bacterial pathogens prevalent in the CF lung has not yet been investigated in detail. Therefore, these studies aimed to probe the
effects of high-dose ibuprofen (50 – 100 μg/mL) on strains of two important Gram-negative bacterial pathogens responsible for chronic pulmonary infections in CF patients, *Pseudomonas aeruginosa* and *Burkholderia* spp., through *in vitro* and *in vivo* studies. *P. aeruginosa* was chosen due to its prevalence in many CF patients, its ability to form biofilms, develop resistance and develop a hypermutable phenotype, all of which contribute to poor survival outcomes (35-39). On the other hand, while few CF patients harbor *Burkholderia* spp., colonization by this pathogen poses a life-threatening problem because it is inherently antibiotic resistant (40). The results demonstrate a dose-dependent antimicrobial activity of ibuprofen *in vitro* against laboratory and clinical strains of both *P. aeruginosa* and *Burkholderia* spp. Furthermore, oral administration of ibuprofen thrice daily in a mouse model of acute lung infection results in reduced bacterial burden, improved clinical illness scores, and superior survival compared with sham-treatment. Finally, since the lung is the target organ for therapy, aerosolization of ibuprofen into healthy mice was investigated, but the drug was rapidly transported from the lungs into the blood serum. As a solution to this pharmacokinetic problem, aerosolizable polymeric nanoparticle formulations of ibuprofen were developed and their antimicrobial activity was demonstrated *in vitro*.

**MATERIALS AND METHODS**

**Bacterial strains.**

Both CF and non-CF bacterial strains of *Pseudomonas aeruginosa* and *Burkholderia* spp. were utilized for our studies and comprised of laboratory, as well as clinical isolates. *P. aeruginosa* laboratory strain PAO1 was generously donated by Gerald Pier, Ph.D. (Harvard University, Boston, MA); whereas, the CF mucoid clinical isolate PA M57-15 was generously
donated by Thomas Ferkol, M.D. (Washington University, St. Louis, MO) (41). The remaining two *P. aeruginosa* CF clinical isolates (sputum-T63547 and sputum-H25815) were obtained from the American Type Culture Collection. *Burkholderia cenocepacia* K56-2 is a CF clinical isolate, which has been previously characterized (42) and was obtained from BEI Resources (NR-20535; Manassas, VA). The remaining isolates of *Burkholderia* spp., including *Burkholderia cenocepacia* (CF clinical isolate: sputum-HI2477), *Burkholderia cepacia* (clinical isolate-1753), and *Burkholderia multivorans* (CGD clinical isolate: biopsy-SH2) were obtained from the culture collection of the Microbiology Laboratory at the National Institutes of Health (NIH) Clinical Center (Bethesda, MD) and were identified as previously described using 16S ribosomal DNA (rDNA)- and *recA*-targeted polymerase chain reaction (PCR) assays (43, 44).

**In vitro antimicrobial activity of ibuprofen.**

Bacteria were streaked from frozen glycerol stocks on to tryptic-soy agar or tryptic-soy agar with 5% sheep blood plates (Remel) and incubated overnight at 37°C with 5% carbon dioxide (CO₂) until individual colonies formed. A single colony was inoculated in 10 mL MH broth and grown at 37°C in a shaking incubator overnight to stationary phase. The cultures were harvested and washed three times with 150 mM NaCl solution and diluted to a density of 5 x 10⁵ CFU/mL in MH broth. A 10 mg/mL stock solution of ibuprofen (in DMSO) was used to prepare working solutions of ibuprofen at 100, 150, and 200 µg/mL in MH broth containing DMSO (5% v/v). For studies in 96-well plates, 100 µL of bacterial culture was added to ibuprofen working solutions (100 µL), resulting in final ibuprofen concentrations of 50, 75, and 100 µg/mL respectively in a total volume of 200 µL. Un-inoculated MH broth and MH broth with DMSO (5% v/v) without ibuprofen were used as controls for the experiment. The final DMSO concentrations in all samples were 2.5% (v/v). After inoculation, the prepared culture solutions were incubated at...
37°C with CO\(_2\) for 12-h. Subsequently, the number of viable cells was analyzed by plating each culture solution (0.05 mL) after diluting (1:10) with 150 mM NaCl solution, incubating at 37°C with CO\(_2\), and counting CFUs. Antimicrobial activity determination studies were performed in duplicate wells in at least three replicate experiments and the data were pooled for analysis.

**Bacterial spiking with ibuprofen.**

Bacterial strains of *Pseudomonas aeruginosa* (PAO1 and PA M57-15), *Burkholderia cenocepacia* (Bcc K56-2), and *Burkholderia multivorans* were grown and adjusted to a density of 5 x 10\(^5\) CFU/mL in MH broth as previously described. Ibuprofen and control samples were also prepared as described above and incubated with 100 µL of bacterial culture, resulting in final test concentrations of 50, 75, and 100 µg/mL, respectively in a total volume of 200 µL.

After inoculation, the prepared culture solutions were incubated at 37°C with CO\(_2\) for 12-h. At 12-h, the samples were spiked with 10 µL of either MH broth with DMSO (5% v/v) or ibuprofen in DMSO to achieve the initial concentration, and incubated for an additional 6 h (18-h time point). The new volume of 210 µL per well was subsequently used to quantify the CFU/mL.

Growth was performed in duplicate wells in at least four replicate experiments. The number of viable cells was determined by plating and counting CFUs as described in the previous section.

**Activity of ibuprofen in artificial CF sputum.**

*Burkholderia cenocepacia* strain Bcc K56-2 and *Pseudomonas aeruginosa* strain PAO1 were streaked, cultured, and harvested according to the methods described above. The bacteria were diluted to a density of 1 x 10\(^6\) CFU/mL in MH broth or artificial sputum media (ASM), which was prepared per the recipe by Sriramulu *et al.* (45). 200 µL cultures containing bacteria alone in the presence or absence of DMSO (positive controls) or 100 µg/mL ibuprofen (in medium containing DMSO for ibuprofen dissolution) were grown in duplicate in 96-well plates at 37°C.
Blank sample wells with uninoculated media served as negative controls. DMSO was maintained at a final concentration of 2.5% (v/v) in all DMSO-containing samples. The absorbance (OD$_{600}$) was monitored at 1-h intervals over a period of 12-h and 24-h, after which the samples were plated to determine bacterial growth (CFU).

**In vitro biofilm studies.**

Wild-type (WT) *Pseudomonas aeruginosa* PAO1 expressing green fluorescent protein (GFP) on plasmid pMRP9-1 was used for confocal experiments (46). *P. aeruginosa* was streaked from frozen stock onto LB agar (5 g/L yeast extract, 5 g/L casein peptone, 10 g/L sodium chloride, and 15 g/L agar) plates and incubated overnight at 37°C. Three or four colonies were then inoculated into a culture tube containing 4 mL Fastidious Anaerobe Broth (FAB) medium (47) with 3% v/v tryptic soy broth (TSB) and 30 mM D-glucose as the carbon source. Liquid cultures were grown at 37°C on an orbital shaker (Labnet Orbit 1000) operating at 200-250 rpm. LB agar and liquid culture media were supplemented with 150 μg/mL carbenicillin for plasmid maintenance; however, for use in flow cells, the FAB medium was adjusted to contain 3% v/v TSB and 0.3 mM D-glucose without carbenicillin. For ibuprofen exposure experiments, the FAB medium to be used in the flow cell was supplemented with ibuprofen at a final concentration of 100 μg/mL ibuprofen. For control experiments, FAB medium without ibuprofen, but with the appropriate quantity of DMSO, was used. To perform experiments in flow cells, liquid cultures were grown in culture tubes to an optical density at 600 nm (OD$_{600}$) of 0.3 (corresponding to mid-exponential growth phase), as measured by a Thermo Spectronic Genesys 20 spectrophotometer. The culture was then volumetrically diluted to an approximate OD$_{600}$ of 0.0015 for inoculation into the flow cell chambers. Experiments were performed in a standard three-channel flow cell system (48). The flow cell systems were sterilized by autoclaving prior to
mounting the flow cell and bubble trap on the microscope stage. Subsequently, the flow cells were filled with sterile FAB medium and ~1 mL of inoculum was injected via needle and syringe into each chamber of the flow cell. Cells were allowed to remain for one hour in static conditions to facilitate initial attachment, after which FAB medium with or without ibuprofen was perfused through the flow cell at a rate of 65 μL/min using a Watson-Marlow peristaltic pump and image capture was simultaneously begun.

Confocal laser scanning microscopy was performed on an Olympus FV1000 motorized inverted IX81 microscope suite with a 40X air objective. The stage was enclosed with an incubation chamber to allow for temperature control of the sample, and was maintained at 30°C. Image capture was controlled by FV10-ASW version 3.1 software. Confocal z-stacks were taken during two-minute scans in the GFP channel using a 488 nm excitation laser. Images were analyzed using the Fiji distribution of the ImageJ software (49) and voxel counting was performed with custom-written codes in MATLAB as previously described in the articles by Hutchison et al and Kragh et al (50), (51). Here, the voxel counts serve as a measure of biomass accumulation in the biofilm, since bacteria not attached to the surface are transported by the flowing medium and are specifically excluded by the custom-written MATLAB code (50, 51).

Exponential parts of voxel-count curves were analyzed by least-squares regression (Microsoft Excel) and the equation $B = \alpha e^{t/\tau}$ ($B =$ biomass accumulation (measured in voxels), $\alpha =$ initial biomass at the onset of exponential growth (typically 14000 – 22000 voxels), $t =$ time elapsed from the onset of exponential growth, and $\tau =$ a characteristic growth timescale) was used to calculate the doubling time, which is $\tau \ln(2)$. Further refinement of this calculation was performed by fitting an exponential function, $\frac{d^2(B_{\text{voxels}})}{dt^2} = \frac{d^2B}{dt^2} = B_0 e^{t/\tau}$ to the second derivative with respect to time of voxel-counting for the exponential regime of this data, for each biofilm.
The second derivative measures the rate at which biofilm growth rate is increasing and gives a more sensitive indication of the exponential growth regime than the voxel counts or their first derivative. The fit function was extrapolated backward in time to $t = 0$ to determine $\hat{B}_0$, which is equivalent to $\frac{d^2}{dt^2}$. The fit functions were then compared for pairs of control and ibuprofen-treated biofilms that were grown in parallel experiments on the same day.

**Determination of intracellular ATP concentration in bacteria.**

*P. aeruginosa* strain PAO1 was grown as previously described. A planktonic suspension of PAO1 in MH broth containing DMSO ($10^8$ CFU/mL) was incubated in a shaking incubator (37°C, 200 rpm) in the absence (control) or presence of ibuprofen (100 μg/mL) for 30 minutes. After incubation, part of the suspension was plated on agar for CFU determination, while the remainder was immediately frozen at -80°C, to lyse the bacteria, and then thawed at room temperature. Subsequently, ATP was extracted by addition of a solution of 0.5% Triton X-100, 25 mM HEPES, and 2 mM EDTA (pH 7.8) in a 1:1 ratio. ATP content was measured using a spectrophotometer and the Molecular Probes® ATP Determination Kit (Invitrogen), which utilizes firefly luciferase and D-luciferin to generate a bioluminescent signal with an intensity proportional to the ATP concentration.

**Investigation of ibuprofen treatment in a murine acute *P. aeruginosa* pneumonia model.**

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) at 6-8 weeks of age were used for all studies and animals were housed in a barrier facility under pathogen-free conditions until inoculation with bacteria. Studies were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee (IACUC). Ibuprofen (Tokyo Chemical Industry Co. LTD., Tokyo, Japan, Catalog # I0415) was suspended in a 1:1 (v/v) solution of strawberry flavored syrup (Smucker’s®) and DH$_2$O at a concentration of 7.5 mg/ml.
Similarly, sham-treatment solution was prepared by mixing 1:1 (v/v) of strawberry flavored syrup and DH$_2$O. Both treatment solutions were vortexed for 3 minutes and sonicated for 1 minute at 70% amplitude (SONICS Vibra-cell VCX130, Sonics & Materials, Inc., Newtown, CT).

Uninfected C57BL/6J mice were fed via syringe with one dose of 100 µl of either ibuprofen suspension or sham treatment solution (n = 4). One hour post-treatment, the mice were anesthetized, dissected, and euthanized via cardiac puncture. The rationale for sampling blood serum at 1-h post ibuprofen administration was derived from the data previously published by Konstan et al., where they have demonstrated that ibuprofen reaches peak serum concentrations at 1-h and reaches baseline values at approximately 6-h following intraperitoneal and oral administration in rats (16). Similar results have also been observed in several human studies summarized by Davies et al (46). Blood from each mouse was collected into separate serum-separator tubes (Greiner Bio-One, Monroe, NC) and centrifuged at 1,000g for 10 minutes to collect the serum. The ibuprofen concentrations in the serum were determined using an ELISA kit (Neogen$^\text{®}$) according to manufacturer’s instructions.

Acute lung infection studies in mice were performed using a standard laboratory strain of *P. aeruginosa* PAO1. The bacteria were grown on TSA plates as previously described, after which, a single colony was suspended in Luria broth (LB; 10 ml) and grown in a shaking incubator (37°C, 200 rpm) to an OD$_{650}$ of 0.4, corresponding to a bacterial load of ~ $2-3 \times 10^8$ CFU/mL as determined by serial dilution and plating. The first infection model experiment evaluated the ability of ibuprofen to reduce bacterial burden in mice inoculated with PAO1. Following anesthesia, mice were intranasally inoculated with 75 µl of PAO1 in LB broth at an LD$_{50}$ dose of ~5 x $10^5$ CFU per mouse. Subsequently, the mice were weighed and randomly assigned to one of
the two treatment groups. Two hours post-inoculation and every 8 hours thereafter, the mice were fed via syringe with 100 µl of either ibuprofen suspension (0.75 mg ibuprofen) or sham treatment solution. At 36-h post-inoculation, the mice were euthanized (sedation with Avertin® and cervical dislocation), their lungs and spleens harvested and homogenized, and plated onto TSA plates to determine CFU counts. The mice were also weighed and assigned clinical scores at the start of the study, at 12-h, and at 36-h (termination). The clinical scores are a semi-quantitative metric that rates the signs of infection from asymptomatic (0) to moribund (6) based on three parameters: activity, fur, and posture (52). The experiment was performed in triplicate and data from each experiment was pooled. A second experiment was performed to evaluate the effect of ibuprofen on survival of PAO1 infected mice using the same protocol as the previous experiment. However, the mice were monitored for a total of 72 hours after inoculation and time of death for each mouse was recorded immediately. Treatments were also continued at 8-h intervals up to 64 hours post-inoculation. The mice were weighed and assigned a clinical score at the start of the experiment and subsequently at 24-h intervals. The survival experiment was performed in duplicate and the data from each experiment was pooled.

Aerosolization of ibuprofen to mice and determination of preliminary biodistribution.

An aerosolizable formulation of ibuprofen was prepared by dissolving either 25 or 50 mg ibuprofen in a solution containing 1% (v/v) DMSO and phosphate (PO₄) buffer. Healthy mice were randomly distributed into three groups (3 mice per group) and provided with one of the following aerosolized treatment: 25 mg ibuprofen, 50 mg ibuprofen, or vehicle. Aerosolization was achieved using an Aeroneb Lab apparatus (Aerogen Inc., Galway, Ireland) connected to a multi-dosing animal chamber, which is a square Plexiglas™ box with inner dimensions in inches of 8L x 8W x 6H, with the nebulizer mounted in the center of the lid. The Aerogen nebulizer is...
based on a micropump technology that produces droplets (2.5 – 4 μm) in a low velocity aerosol (53). The mice were individually placed into CH-247 restraint tubes (CH Technologies, Westwood, NJ) and then placed inside the multi-dosing chamber to provide aerosolized treatment. Immediately following nebulization, the mice were anesthetized by an intraperitoneal injection of Avertin™, and complete sedation was verified by toe-pinching. The mice were then dissected and euthanized by cardiac puncture, with the blood collected into serum-separator tubes (BD, Franklin Lakes, NJ) and centrifuged at 1,000xg for 10 minutes to collect the serum. Bronchoalveolar lavage fluid (BALF) was collected with 3 mL of sterile PO₄ buffer injected into and aspirated from the lungs via the trachea using a catheter and syringe. Next, the whole lung tissue was harvested and homogenized in 1 mL of sterile PO₄ buffer. The harvested serum, BALF, and lung tissue were analyzed for urea content (BioVision, Inc., Milpitas, CA), which allows for the concentrations of ibuprofen (or other drugs) to be normalized to a true volumetric concentration, since urea concentrations should be constant throughout the body. Finally, the ibuprofen concentrations were determined using an ELISA kit (Neogen®) against a standard of ibuprofen dissolved in the 99% PO₄ buffer/1% DMSO solution used for nebulization.

**Formulation of ibuprofen nanoparticles and determination of preliminary in vitro activity.**

Ibuprofen nanoparticles (NPs) were formulated using the oil-in-water (o/w) emulsion technique from a mixture of two biodegradable polymers: poly (lactide-co-glycolide) (PLGA) and a copolymer of PLGA and poly (ethylene glycol) (PEG) (PLGA-PEG). The polymers were dissolved in 3 mL chloroform (100 mg/mL) along with ibuprofen (10 mg/mL) and the organic solution was emulsified with 100 mL polyvinylpyrrolidone (PVP) solution (10% w/v in water) loaded with or without the sodium salt of ibuprofen, for 3 minutes with high-speed mechanical stirring (~ 2400 rpm), after which the emulsion was gently stirred (~ 300 rpm) at room
temperature for 5 hours to remove the organic solvent. The resulting nanoparticle suspension was centrifuged, washed with sterile distilled, deionized water (DH$_2$O), shell-frozen, and lyophilized to obtain the NPs as a free-flowing powder. Subsequently, the NPs were characterized for surface morphology and size distribution using standard scanning electron microscopy (SEM) techniques using an FEI XL30 ESEM at an accelerating voltage of 15kV. The size distribution of the NPs was determined by analyzing 100 random nanoparticles on SEM images using an appropriate scaling factor (Image J, NIH, Bethesda, MD). Ibuprofen loading within the nanoparticles was determined by dissolving the NPs in chloroform, diluting 50X in sterile DH$_2$O, and measuring ibuprofen content with an ELISA kit (Neogen®).

The antimicrobial activity of ibuprofen nanoparticles was determined using an end-point CFU study. Planktonic suspensions of PAO1 at 5 x 10$^5$ CFU/mL in MH broth were incubated with ibuprofen nanoparticles, empty nanoparticles, or a control solution (MH broth only). The concentration of the nanoparticles used for this study was determined based on the ibuprofen loading (500 μg/mL theoretical ibuprofen concentration) and an equivalent mass of empty nanoparticles was used. The suspensions were placed on a shaking incubator for 6 hours (37°C, 200 rpm), after which the CFU counts were determined by plating. The experiments were performed in duplicate, with three technical replicates for each experiment (total of 6 replicate samples per treatment).

**Statistics.**

Analyses were performed using Prism 6 (GraphPad Software, Inc., San Diego CA). Data are presented as mean ± standard error of the mean (SEM). The end-point differences for growth curves (OD$_{600}$) were analyzed using a two-tailed paired t-test. CFU counts obtained from most *in vitro* studies, differences in ATP/CFU of bacteria, as well as differences in bacterial burden...
between the lungs and spleens, weight differentials, and clinical scores of ibuprofen- and sham-treated mice were analyzed using two-tailed, non-parametric Mann-Whitney test. Serum, lung, and BAL concentrations of ibuprofen following aerosolization of various doses of ibuprofen were compared using two-way ANOVA with Tukey’s multiple comparisons post-hoc test. In the case of CFU counts obtained from the ibuprofen NP experiments, the data were analyzed using ordinary one-way ANOVA with Tukey’s multiple comparisons post-hoc test. The in vivo survival curves in the infection model were compared using a Log-rank Mantel-Cox test. Data were considered to exhibit statistically significant differences when P < 0.05.

RESULTS

Ibuprofen treatment reduces the bacterial burden in a dose-dependent fashion and repeated ibuprofen treatment further extends this effect.

A dose-dependent reduction in the bacterial burden was observed following ibuprofen treatment (50, 75, and 100 µg/mL) for each of the strains of *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Burkholderia cenocepacia*, and *Burkholderia multivorans* over 12-hours (Table 1). For instance, in the case of *Pseudomonas aeruginosa* strain PAO1, exposure to 50 µg/mL of ibuprofen over 12-hours led to approximately 0.5-log₁₀ reduction in bacterial count, which further increased to approximately 1-log₁₀ and 1.5-log₁₀ following exposure to 75 and 100 µg/mL ibuprofen, respectively. Exposure of other strains of *P. aeruginosa* to ibuprofen also resulted in a similar response, where the most significant reduction compared with control was observed at doses of 75 and 100 µg/mL. Of all strains, the greatest reduction in bacterial burden was observed for PA H25815 at an ibuprofen dose of 100 µg/mL leading to approximately a 2-log₁₀ reduction. Similarly, in the case of *Burkholderia cenocepacia* K56-2, exposure to 50
μg/mL of ibuprofen over 12-hours led to approximately a 1/3-log$_{10}$ reduction in bacterial count, which further decreased by approximately 1-2 logs following exposure to 75 μg/mL and 100 μg/mL ibuprofen, respectively. These trends were conserved for other strains of *Burkholderia* spp. as well; the greatest dose-dependent reduction was observed for *B. cenocepacia* HI4277 (~2.5-log$_{10}$ reduction for 100 μg/mL). Separately, the possible inhibitory effect of DMSO on bacterial growth was examined by growing the bacteria in Mueller-Hinton (MH) broth in the absence and presence of DMSO (2.5% v/v), and as expected, no apparent differences were observed between the two treatments (data not shown).

Next, the effects of an additional dose of ibuprofen applied at 12-h were investigated. Four of the previously tested strains (PAO1, PA M57-15, *Bcc* K56-2, and *Burkholderia multivorans* SH-2) were studied and CFU counts were determined for each condition at 12- and 18-h. Regardless of the species, all cultures resulted in lower CFU counts than the control at the 12-h time point. Growth inhibition was significantly influenced by the ibuprofen concentration and exhibited a dose-dependent decrease with increasing ibuprofen concentration (Figure 1). Similar to the observations in Table 1, the effect of ibuprofen on PAO1 and PA M57-15 at 12-h was approximately 1-log$_{10}$ reduction at the highest dose (100 μg/mL) for each strain (Figure 1A and B). After the additional spike and 6-h incubation, ibuprofen continued to impede bacterial growth compared with control treatment. At the highest dose, further growth inhibition was observed for both PAO1 and PA M57-15 leading to lower bacterial burdens than at 12-h. A more robust initial dose-dependent reduction in bacterial burden was observed for both *Bcc* K56-2 and *Burkholderia multivorans* SH-2; the bacterial burden was approximately 3-log$_{10}$ lower than control at 12-h (Figure 1C and D). However, the initial decrease did not appear to be extended by additional spiking in either *Bcc* K56-2 or *Burkholderia multivorans*.
A few molecular mechanisms have been proposed to explain the antimicrobial activity of NSAIDs including ibuprofen (30, 54-59). Based on the evidence regarding the ability of NSAIDs to act as a protonophore, the effects of ibuprofen exposure on ATP production in bacteria was explored as a first step. The preliminary observation documented in Figure 2 shows a significant reduction in ATP content within *Pseudomonas aeruginosa* treated with ibuprofen (100 μg/mL, 30-minute exposure) compared with untreated controls (P < 0.001). In contrast, bacteria treated with the sodium salt of ibuprofen (NaIbu) did not exhibit a significant reduction in ATP content compared with untreated controls (data not shown). The sodium salt of ibuprofen is an FDA-approved formulation found to have a more rapid onset of analgesic action compared with the protonated form of ibuprofen (60). The lack of antimicrobial activity of NaIbu supports the protonophore hypothesis, because this formulation is more polar and hence, less likely to intercalate into the bacterial cell membrane.

**Ibuprofen delays the growth of bacteria in both standard and artificial sputum media.**

Since bacterial growth characteristics are a function of the growth medium, we have compared the growth rate and end-point CFU of two representative Gram-negative pathogen strains (PAO1 and *Bcc* K56-2) in MH broth and artificial sputum medium (ASM). ASM has been designed to mimic the sputum of CF patients and provides nutritional conditions similar to that of CF sputum, as well as synthetic CF sputum medium (45). Further, ASM has gained wide acceptance for assay development requiring conditions that simulate the CF lung (61-64). When starting with a similar inoculum (1 x 10^6 CFU/mL), the growth curves for both PAO1 and *Bcc* K56-2 in MH broth (Figure 3A, 4A) and ASM (Figure 3B, 4B) resemble a typical exponential-type growth curve for bacteria; however, a slightly longer but non-significant lag time was observed for ibuprofen treatment compared with media-only and media-containing-DMSO.
controls. Furthermore, in each instance, the slopes of the growth curves suggest that bacteria subjected to ibuprofen treatment grew at a slower rate compared with controls. Consequently, the final $OD_{600}$ values for ibuprofen-treated bacteria are significantly lower than the $OD_{600}$ values for control bacteria at 12-h in both MH broth and ASM ($P < 0.01$ for PAO1 and $P < 0.05$ for $Bcc$ K56-2). As expected, no differences in the growth rates and final $OD_{600}$ values were observed for bacteria grown in untreated medium-only and medium containing DMSO controls, irrespective of the choice of the medium, suggesting that the presence of DMSO does not hamper bacterial growth over 12 h. Extending the time for growth of PAO1 in MH broth to 24-h continues to demonstrate the difference between ibuprofen in DMSO and MH alone, or MH plus DMSO, controls ($P < 0.0001$ and $P < 0.05$, respectively; Supplementary Figure). Separately, a faster growth of $Bcc$ K56-2 was observed for the two control treatments in ASM compared with MH broth; however, the growth characteristics of control PAO1 remained largely unchanged between the two media. The slower growth rate and lower final $OD_{600}$ values of ibuprofen-treated bacteria in each instance also translated to a significant reduction in bacterial burdens compared with control treatment at 12-h. In the case of PAO1, when using MH broth (Figure 3C), the bacterial burden following ibuprofen treatment was approximately 1.5-$log_{10}$ lower compared with control treatments ($P < 0.01$). This reduction in CFU was somewhat diminished, yet significant when the media was changed to ASM (Figure 3D, $P < 0.05$). Similar results were observed when the effect of ibuprofen on the end-point CFU of $Bcc$ K56-2 in both MH broth and ASM was investigated. Approximately a 1-$log_{10}$ reduction in the mean end-point CFU of $Bcc$ K56-2 was observed in MH broth, which was significantly lower compared with untreated controls (Figure 4C, $P < 0.01$). Again, this reduction in CFU was somewhat diminished when using ASM, yet the
differences between ibuprofen-treatment and untreated controls were significant (Figure 4D, P < 0.01).

Ibuprofen treatment delays the onset of biomass accumulation in *Pseudomonas aeruginosa* biofilms grown *in vitro*.

Figure 5A shows a comparison of biomass accumulation in untreated (control) biofilms and ibuprofen-treated (100 μg/mL) biofilms growing under similar conditions, where voxel count serves as a measure of biomass accumulation. For both untreated and ibuprofen-treated biofilms, the biomass accumulation over a 1.5 to 2-hour window, at the beginning of growth is sub-exponential. The subsequent transition to exponential growth occurs later for ibuprofen-treated biofilms compared with control biofilms, and an initial subjective determination suggests the delay to be 0.5 ± 0.41 hours (mean ± standard deviation). However, since this determination is limited to no better than half an hour in time resolution, further analysis of this data was performed by taking a second derivative with respect to time of the voxel counts, and the original estimation of delay for ibuprofen-treated biofilms was revised to 0.86 ± 0.27 hours (mean ± standard deviation). Once the biofilms reached an exponential growth phase, the bacterial doubling time in IBU-treated biofilms was 1.8 ± 0.29 hours (mean ± standard deviation), which was not significantly different from the doubling time for bacteria in control biofilms (1.5 ± 0.11 hours). Figure 5B documents another representation of this phenomenon, where biomass accumulation in IBU-treated biofilms is normalized to the control biofilms. While the initial biomass in IBU-treated biofilms is slightly higher, a sharp decline in the voxel count ratio (ibuprofen: control) is observed almost immediately. This ratio continues to drop and reaches its lowest value of 0.285 at 8-h with the curve appearing similar to an exponential decay profile. Subsequently, the ratio begins to rise almost linearly, and reaches a value of 1 at approximately
While this observation may suggest a comparable biomass for control and ibuprofen-treated biofilms by 10-h of growth, underestimation of the biomass of the ibuprofen-treated biofilms may occur, when they become confluent, typically at 9-h. The thick ibuprofen-treated biofilms display large volumes that are dim or completely dark; this phenomenon may result from low oxygen concentration deep within the biofilm, which reduces bacterial metabolism and impedes GFP production and folding (Figure 5C). Examination under transmitted light confirms that biofilm is present in these dim or dark regions. However, the measurement of biomass counts only fluorescent regions. Thus, whether the biomass accumulation for both control and ibuprofen-treated biofilms after 9-h is truly equal, or only appears equal due to an imaging artifact, is currently unknown, and is a topic of further investigation.

Oral delivery of ibuprofen achieves therapeutic concentrations in serum, reduces bacterial burden, and improves survival in PAO1 infected mice.

To test the in vivo antimicrobial activity of ibuprofen, a mouse model of acute P. aeruginosa pneumonia was used following preliminary dosing studies in healthy mice. Dosing experiments revealed that an oral dosage of 0.75 mg ibuprofen resulted in a blood-serum concentration of 124.22 ± 15.40 µg/mL at 1 hour post treatment; whereas, negligible amount of ibuprofen was detected in sham-treated animals (0.12 µg/mL). These results provide validation that the ibuprofen concentration necessary to achieve antimicrobial effects (> 50 µg/mL based on in vitro studies) can be realized in the serum of mice following oral delivery. Subsequently, as an initial evaluation of the antimicrobial activity of ibuprofen, bacterial burden in the lungs and spleen of mice infected with an LD_{50} dose of P. aeruginosa strain PAO1 was compared. Following five treatment doses over 36-h post-infection, significant reductions in both lung (Figure 6A) and spleen (Figure 6B) bacterial burdens were observed for the mice treated with ibuprofen. At the
36-h time point, the sham-treated mice had approximately 1-log\textsubscript{10} more bacteria in both their lungs (P = 0.0314) and spleen (P = 0.0096), compared with ibuprofen-treated mice. Concurrent evaluation of other health-associated parameters such as weight loss and clinical scores (Figure 6C, 6D) showed no significant effect of ibuprofen-treatment on weight loss after infection over 36-h (P = 0.6); however, at the same time point, ibuprofen-treated mice exhibited significantly reduced clinical scores compared with sham-treated animals (P = 0.002). During the survival study, the ibuprofen-treated mice exhibited less weight loss compared with sham-treated mice on day 3 (Figure 7B, P = 0.0095) and the clinical scores of ibuprofen-treated mice were significantly lower compared with sham-treated mice on all days (Figure 7C, day 1, P = 0.001; day 2, P = 0.0002; and day 3, P = 0.0045). Further, in these PAO1 infected mice, a significant survival advantage for the ibuprofen-treated mice (92%) was observed compared with sham-treated animals (57%) (P = 0.0386) at 72 hours (Figure 7A).

Nebulization results in a rapid transport of ibuprofen to the serum necessitating the use of controlled delivery nanoparticle formulations.

Next, the possibility of delivering ibuprofen to the lungs of healthy (uninfected) mice via nebulization was explored. Ibuprofen was solubilized in phosphate buffer containing 1% (v/v) DMSO. This formulation was nebulized using an Aerogen Lab apparatus, which is based on the vibrating mesh technology frequently used to deliver antibiotics to the lungs of CF patients. Analysis of ibuprofen concentrations in the blood serum, lung tissue, and bronchoalveolar lavage fluid (BALF) following nebulization of 25 and 50 mg doses demonstrated that the 50 mg dose led to approximately twice the concentration of ibuprofen in each of the tissues compared with the 25 mg does, as expected (Figure 8). Using the serum and tissue ibuprofen concentrations and a predicted blood volume of 1.5 mL for a 25-gram mouse for calculations, approximately 0.8%
of the 25 mg dose and 1.0% of the 50 mg dose were delivered to the mice via nebulization. However, a rapid transport of this small molecule drug into the blood was observed, leading to serum concentrations that were 12 – 15 X higher than BALF concentrations and 4 – 5 X higher than lung tissue concentrations. Additionally, nebulization times for these ibuprofen formulations were unreasonably long (~ 43 minutes and 70 minutes for 25 and 50 mg, respectively). Thus, the attempts to achieve higher lung concentrations of ibuprofen compared with serum through aerosol delivery failed.

As an alternative, nebulizable nanoparticulate formulations encapsulating ibuprofen were prepared. Nanoparticles were prepared using a mixture of PLGA and PLGA-PEG using an o/w emulsion technique, wherein, PLGA forms the core of the nanoparticles and PLGA-PEG primarily concentrates at the surface of the NPs. Scanning electron microscopy (SEM) was used to examine the surface morphology and size distribution of three nanoparticle formulations (Figure 9). Both empty nanoparticles (Figure 9A) and ibuprofen–loaded nanoparticles (Figure 9B) are spherical and exhibit a smooth morphology with minimal defects on the particle surface. On the other hand, SEM images of ibuprofen–loaded nanoparticles formulated with sodium ibuprofen (NaIbu) in the external aqueous phase (Figure 9C) show a mixture of spherical particles, which possess either a smooth surface morphology or rough surface morphology with significant surface defects in the form of pores or divots. Additionally, some incompletely formed or disrupted particles, as well as polymeric debris, are also observed (Figure 9C). While minimal aggregation is observed for both formulations of ibuprofen–loaded nanoparticles, empty nanoparticles demonstrate a larger degree of aggregation. Some empty nanoparticles also appear to fuse during the formulation process. In each instance, the particle size distribution appears to be bimodal with smaller particles exhibiting diameters of the order of a few hundred nanometers.
and larger particles with diameters in the micron range (up to ~ 4 μm). Interestingly, nanoparticles formulated with NaIbu in the external phase had the sharpest size distribution with most particles being sub-micron sized in diameter. Ibuprofen loading studies revealed that addition of NaIbu to the external phase improved the loading of ibuprofen within the nanoparticles significantly. These particles contained 16% ibuprofen by weight (~ 160% encapsulation efficiency) compared with particles formulated without NaIbu in the external phase, which contained ~ 4% ibuprofen by weight (~ 40% encapsulation efficiency). Finally, preliminary investigations of the antimicrobial activity of ibuprofen-loaded nanoparticles using an endpoint CFU study were performed (Figure 10). No differences in CFU counts were observed between bacteria treated with empty nanoparticles and media-only control (P = 0.9998). In contrast, treatment with ibuprofen–loaded nanoparticle formulations revealed a significant reduction in CFU counts of P. aeruginosa strain PAO1 compared with empty nanoparticles and media-only control (p < 0.001), with the highest reduction in bacterial burden observed for Ibu/NaIbu NPs (~0.6-log_{10}).

DISCUSSION

The use of ibuprofen for the treatment of chronic inflammation in cystic fibrosis (CF) lung disease has well-documented beneficial effects, which have been attributed to its ability to act on multiple inflammatory pathways (18-22, 65-68). Yet, a few reports in the literature also document the antimicrobial properties of ibuprofen, ranging from bacteriostatic to bactericidal against several different pathogens, as well as its synergy with other antimicrobials (23-29). These reports are further augmented by additional observations of the antimicrobial and synergistic activity of other NSAIDs, such as salicylate, acetylsalicylic acid, indomethacin,
Collectively, these data raise the possibility that the observed beneficial effects in CF lung disease may be due to a combination of its anti-inflammatory and antimicrobial activity rather than due to the anti-inflammatory activity alone. Because the direct antimicrobial effects of ibuprofen on CF-associated Gram-negative pathogens have not been previously investigated, we sought to systematically explore this effect in two important CF pathogens: *Pseudomonas aeruginosa* and *Burkholderia* spp. Further, our studies were conducted at ibuprofen concentrations ranging from 50 – 100 μg/mL, because doses in this range are clinically achievable and were previously shown to have a beneficial effect in CF patients (10).

Our initial screening studies against several strains of *P. aeruginosa* and *Burkholderia* spp. following exposure to ibuprofen demonstrated a dose-dependent reduction in bacterial burden for all strains at 12-h (Table 1). At the highest ibuprofen dose of 100 μg/mL, the bacterial reduction for *Burkholderia* spp. strains was ~ 2-log₁₀, whereas for *P. aeruginosa* strains the reduction was ~ 1 – 1.5-log₁₀ compared with controls. These results are in partial agreement with other data in the literature documenting antimicrobial effects of ibuprofen ranging from bacteriostatic to bactericidal. For example, Sanyal *et al.* have demonstrated a minimum inhibitory concentration (MIC) of 40 – 80 μg/mL against a *Staphylococcus aureus* strain at pH 5 (23). Similarly, Elvers and Wright observed a concentration- and pH-dependent bacteriostatic effect of ibuprofen on *S. aureus* and *Staphylococcus epidermidis*. Growth suppression occurred at ibuprofen concentrations greater than 150 μg/mL at an initial pH 7, and almost complete growth inhibition was observed at an ibuprofen concentration of 450 μg/mL (24). This effect was further enhanced at pH 6, where almost complete growth inhibition occurred at an ibuprofen concentration of just 125 μg/mL (24). Separately, in the case of *Helicobacter pylori*, complete growth inhibition of
three strains was observed at an ibuprofen concentration ranging from 50 – 125 μg/mL, and an increase in dose to 250 μg/mL or greater led to an onset of bactericidal activity (25).

Additionally, recent findings by Guzman et al. document complete growth inhibition of *Mycobacterium tuberculosis* H₃₇Rv at an ibuprofen concentration of 75 μg/mL, while the MICs against *M. bovis* BCG, *M. aurum*, and *M. neoaurum* were 90, 65, and 65 μg/mL, respectively using a high throughput spot culture growth inhibition (HT-SPOTi) assay *in vitro* (28). These effects also extended to multidrug resistant clinical strains of *M. tuberculosis* 11:139, 11:169, and 11:368 with MICs ranging from 30 – 50 μg/mL (28). When correlated with CFU counts, treatment of *M. bovis* BCG with ibuprofen at 180 μg/mL resulted in ~2-log₁₀ reduction in bacterial burden provided ibuprofen was introduced at the beginning of culture; however, introduction of ibuprofen at the beginning of exponential growth phase led to a minimal CFU reduction. Interestingly, no growth inhibition of bacteria such as *Rhodococcus equi* RHA1, *Pseudomonas putida*, *Escherichia coli* DH5α, and *Staphylococcus aureus* strains ATCC25923 and EMRSA-16 was observed at an ibuprofen concentration of 100 μg/mL when using the HT-SPOTi assay (28). Furthermore, growth suppression of Gram-negative pathogens *Pseudomonas fluorescens* and *E. coli* was not observed even at an ibuprofen concentration of 350 μg/mL by Elvers and Wright (24). These seemingly contradictory results coupled with a lack of explanation regarding the selectivity of ibuprofen in these reports leads us to believe that differences in experimental protocol, such as choice of assay for the determination of antimicrobial activity, ibuprofen formulation, growth conditions, and choice of end-point, plays a considerable role in the observed outcome. As a case-in-point, the HT-SPOTi assay was performed over 16-h; however, in the case of fast-growing bacterial pathogens in our studies, following the initial 8 to
12-h of exposure, we found that the ability of ibuprofen to suppress bacterial growth to be substantially diminished. Thus, we sought to explore whether re-dosing the bacterial cultures with ibuprofen at 12-h could prolong the observed growth suppression. Our rationale for performing these studies was driven by the data available on the formulation-dependent biological half-life of ibuprofen (1.3 – 3 hours) in vivo (46), as well as its dosing regimen at 12 hour intervals both in vivo and in CF patients (10, 12, 16, 66). Furthermore, previous observations suggest the ability of various microorganisms to utilize NSAIDS such as ibuprofen as substrates resulting in their biodegradation, as well as biotransformation (70-73). Indeed, our results demonstrate that the concentration-dependent antimicrobial activity of ibuprofen is extended to 18-h following introduction of fresh ibuprofen to the system at 12-h (Figure 1). Interestingly, the initial growth suppression was more robust for the two *Burkholderia* spp. strains compared with the two *P. aeruginosa* strains, yet at 18-h, the results are more striking in the case of *P. aeruginosa* where an additional 0.5 – 1.0-log$\text{_{10}}$ reduction in CFU counts (compared with 12-h CFU) is observed after spiking the medium at the highest ibuprofen concentration. These data suggest a continued reduction in growth rate and subsequently bacterial burden for *P. aeruginosa* post ibuprofen re-treatment. In contrast, no distinct advantage of spiking with ibuprofen is evident in case of *Burkholderia* strains, except the conservation of the trend pertaining to dose-dependent lowering of CFU counts compared with control treatment. These results coupled with Guzman et al.’s observation that addition of ibuprofen at the start of exponential growth phase failed to achieve CFU reduction point towards interference with a metabolic pathway as one of the targets for the antimicrobial mechanism of action of ibuprofen. Indeed, our preliminary foray in this area has shown significant depletion in intracellular ATP concentration in PAO1 compared with untreated
control (MH broth with DMSO) following exposure for 30 minutes (Figure 2), suggesting that ibuprofen potentially uncouples oxidative phosphorylation in bacteria. Usually weakly acidic, hydrophobic (typically aromatic) compounds exhibit the highest potential to delocalize negative charge in their anionic state, thus acting as uncouplers (56). Most commonly used NSAIDs have these characteristics, and studies have demonstrated that weakly acidic NSAIDs, including ibuprofen, cause uncoupling in mitochondria (54, 55). Note that the sodium salt of ibuprofen (NaIbu) is not a weak acid and thus, as expected, did not demonstrate activity as a uncoupler. Interestingly, the presence of free carboxylic acid moiety was deemed critical for anti-tubercular activity of ibuprofen and its analogs (28), further validating our observation. However, the ability of ibuprofen to induce this disruption in energy generation might depend on the growth stage of the individual bacterial species, thereby possibly explaining species-specific suppression effects we have observed in our spiking experiments. Interestingly, recent chemoinformatics and bioinformatics based studies by Kahlous et al. in combination with bioassays demonstrate structural similarities between ibuprofen and the quinolone and fluoroquinolone class of antimicrobials, such as ciprofloxacin, levofloxacin, and nalidixic acid (59). The authors further report that ibuprofen along with other NSAIDs has the ability to bind the protein target of their similar antibiotics, i.e., DNA gyrase and inhibit bacterial growth (59). Whether the reduction in ATP generation influences DNA gyrase-mediated antimicrobial activity, or other important downstream cellular processes is currently unknown.

All of our experiments were performed using Mueller-Hinton broth, which is a general-purpose medium, used for the culture of a wide variety of microorganisms and microbiological assays. However, the choice of growth medium can have significant effects on the growth and viability, gene expression, and metabolic functions in microorganisms. Additionally, the growth
conditions for bacterial pathogens in the CF lung are markedly different due to the presence of viscous mucus comprising of breakdown products of inflammatory and epithelial cells, including copolymers of DNA and filamentous (F-) actin, bacteria, cell debris, and variable amounts of mucin (74, 75), which also serves as a nutritional source for these pathogens (76). For example, Palmer and coworkers have demonstrated significant differences in Pseudomonas aeruginosa physiology when grown in CF sputum compared with glucose-grown P. aeruginosa (77). Therefore, we investigated the effect of growth medium on ibuprofen’s antimicrobial activity by incubating PAO1 and Bcc K56-2 in the presence and absence of ibuprofen in both MH broth and artificial sputum medium (ASM) (Figures 3 and 4). A slower growth rate and reduction in the end-point OD$_{600}$ (12-h) was observed for both bacteria in MH broth as well as ASM, which translated to approximately a 1-log$_{10}$ reduction in CFU counts compared with controls (untreated bacteria and MH with DMSO). Thus, these results suggest that the growth-suppressive antimicrobial activity of ibuprofen is conserved irrespective of the growth medium.

Next, given the importance of P. aeruginosa biofilms in CF, we investigated the effect of ibuprofen on a GFP expressing WT P. aeruginosa strain (GFP-PAO1) in biofilm growth mode in vitro (Figure 5). Our studies were performed using a flow-cell apparatus and confocal microscopy, which coupled with the choice of GFP-PAO1 as a model biofilm-forming pathogen allows us to monitor these biofilms in real-time and use voxel counts as a measure of biomass accumulation. Our results demonstrate that following an initial sub-exponential biomass accumulation, a transition to exponential growth occurs for both treated and untreated biofilms; however, this transition is delayed in the presence of ibuprofen. This delay likely results from an increase in the surface-associated lag time when PAO1 planktonic cells make the transition to a biofilm growth mode, which is concomitant with the time required to change the expression
levels of a large number of genes responsible for transitioning to the biofilm phenotype (78). Overall, our data match reasonably well with reports in the literature, which document inhibition of biofilm formation by several strains of *S. pneumoniae, E. coli, S. aureus*, and *C. albicans* following ibuprofen exposure (79-82). Mechanistically, the precise pathways by which ibuprofen exerts an anti-biofilm effect are currently unknown, but several clues are available from the literature. For instance, diclofenac, ibuprofen, and salicylic acid have been shown to limit bacterial adhesion to abiotic surfaces (83-85). Exposure of *E. coli* to ibuprofen has been shown to impact bacterial adherence to epithelial cells by inhibition of fimbriae (81, 86) and through alterations to bacterial hydrophobicity and haemolysin production (81, 86), which impacts biofilm formation. Therefore, there is a possibility that ibuprofen may have a similar effect on the ability of *P. aeruginosa* to produce biomolecules and undergo surface modification of the cell wall, thereby limiting biofilm formation (81-84). Additional evidence suggests that NSAIDs including ibuprofen and salicylic acid may be able to directly modulate quorum sensing, particularly through inhibition of the *las* system (87) regulating biofilm formation in *P. aeruginosa*, as well as also decrease the production of virulence factors governed by these systems (88). Separately, since manifestation of these biochemical processes requires ATP, the ability of ibuprofen to curtail ATP generation by acting as a protonophore may also be implicitly responsible for hindering the transition to a biofilm-growth mode.

We examined the *in vivo* antimicrobial activity of ibuprofen in a mouse model of acute *P. aeruginosa* pneumonia. First, we demonstrated that enteral delivery of 0.75 mg ibuprofen resulted in a peak serum ibuprofen concentration of $124.22 \pm 15.40 \mu g/mL$ at 1-h after dosing.

As previously described, our dosing studies were guided by Konstan *et al.*’s work, where they have demonstrated a peak serum concentration of ibuprofen in rats at 1-h after either...
intraperitoneal or oral administration. Thereafter, mice were intranasally infected with an LD_{50} dose of \emph{P. aeruginosa} (5 x 10^5 CFU PAO1) and treated with ibuprofen first at 2-h post-infection and subsequently at 8-h intervals. The effect of ibuprofen on bacterial burden, as well as survival was evaluated. At 36-h post-infection, ibuprofen-treated mice had significantly lower bacterial burden in both the lungs and the spleen (Figure 6A and 6B), as well as improved clinical scores (Figure 6D). Subsequent survival experiments demonstrated not only significant improvement in survival of animals (Figure 7A) but also on weight loss and clinical scores compared with sham-treated animals (Figure 7B, 7C), thus suggesting an overall improved health of ibuprofen-treated animals. In fact, only one out of the thirteen mice in the ibuprofen group died of infection over a period of three days. In contrast, six deaths were recorded among the fourteen sham-treated animals over the same time frame. As far as we are aware, this is the first report documenting reduced bacterial burden, as well as improved health parameters and survival in a \emph{P. aeruginosa} pneumonia model following ibuprofen treatment.

In their seminal work, Konstan \etal observed improved weight gain and reduced inflammation, but no effect on bacterial burden in a rat model of chronic pulmonary \emph{P. aeruginosa} infection (16). While we recognize the close interplay between bacterial infection and the subsequent inflammatory and immune responses and ibuprofen’s ability to modulate both as important factors influencing the \emph{in vivo} outcome in our studies, we suggest an additional yet unrecognized antimicrobial effect of ibuprofen \emph{in vivo} as well, based on recent observations on the direct effect of ibuprofen on bacterial pathogens. We however acknowledge that our studies do not provide incontrovertible proof of this activity \emph{in vivo}. Moreover, we acknowledge that the dissimilarity of our acute \emph{P. aeruginosa} pneumonia model to the chronic, biofilm infections in the cystic fibrosis lung is a limitation of this study. Additional studies are currently
underway to address the challenging task of delineating the individual effect of ibuprofen on each of these systems in vivo to tweeze out the direct antimicrobial effects of ibuprofen.

Unfortunately, despite the advantages of ibuprofen therapy in CF, the utility has been limited due to concerns over safety, particularly GI hemorrhage and nephrotoxicity, even though such occurrences are extremely rare (6). Additionally, the need to establish the dose in each patient based on pharmacokinetic studies has also been reported as an impediment (89). Therefore, developing a novel aerosolizable formulation of ibuprofen would be highly advantageous, since such a formulation could significantly reduce the amount of drug necessary to achieve a clinically relevant outcome. Indeed, a few reports have demonstrated that pulmonary delivery of NSAIDs reduces the dose needed to achieve anti-inflammatory efficacy by 3 – 6 orders of magnitude over oral dosing (90, 91). Therefore, we first aerosolized a water-soluble formulation of ibuprofen containing 1% (v/v) DMSO to healthy mice, which resulted in approximately 1% of the nebulized dose delivered to each mouse; however, we encountered two main challenges. First, the low polarity of the solution resulted in poor aerosolization performance, thereby resulting in excessive aerosolization time. Additionally, the residence time of the drug in the lung was found to be very low as most of the delivered ibuprofen was found in the blood serum rather than in the BALF and lung tissue 1-h post nebulization (Figure 8). Rapid transport of small hydrophobic molecules from the lungs into systemic circulation has been reported, owing to the huge surface area of the lungs, highly dispersed nature of an aerosol, good epithelial permeability, and small aqueous volume at the absorptive surface (92, 93). Therefore, to improve the residence time of ibuprofen in the lung, we have developed nanoparticulate formulations of ibuprofen which can provide a depot delivery to the lung following nebulization. The nanoparticles were formulated using PLGA and PLGA-PEG; two commonly used biodegradable

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and biocompatible FDA-approved polymers with a wide range of applications in human therapy. Furthermore, the incorporation of PEG has been shown to increase the nanoparticle diffusion through human mucoid surfaces (94), which in the case of CF might be particularly advantageous. As expected, all nanoparticles demonstrated spherical particles with a bimodal size distribution, minimal aggregation, and mostly smooth surface morphology with some surface defects (Figure 9), which is a characteristic of nanoparticles formulated using an emulsion method. However, the nanoparticles formulated using NaIbu in the external phase demonstrated more surface defects, which can likely be attributed to the leaching of NaIbu from the particle surface during the washing steps. Separately, these particles also had the tightest size distribution with the largest population of sub-micron sized particles suggesting that the presence of NaIbu in the emulsion modulates the particle diameter by influencing interfacial tension. The addition of NaIbu to the external phase during the formulation process also led to an exceedingly high ibuprofen loading within the nanoparticles by reducing the oil-to-water phase concentration gradient of the drug, thereby minimizing the diffusion of ibuprofen from within the nanoparticles. Lastly, through a preliminary study performed using PAO1, we have demonstrated a reduction in CFU counts compared with controls (empty nanoparticles and media-only) following exposure to ibuprofen nanoparticles, thus validating the antimicrobial activity of these formulations (Figure 10). We acknowledge that the reduction in bacterial burden after exposure to ibuprofen nanoparticles was not as robust as observed for non-formulated ibuprofen (Ibu/Nalbu NPs: ~0.6-\log_{10} vs. 100 µg/mL unformulated ibuprofen: ~1.5-2.0-\log_{10}); however, this preliminary study was performed primarily to demonstrate proof of concept. For the current nanoparticle formulations, the dosing was calculated based on ibuprofen loading alone without factoring in the release rates. Further, these nanoparticles do not possess optimal ibuprofen
loading characteristics and release rates. We are currently optimizing the nanoparticle formulation parameters as well as processes to enhance ibuprofen loading, reduce particle size distribution, modify surface characteristics, and modulate release kinetics as well as pharmacokinetics to obtain the best nanoparticle system for aerosolization. A thorough characterization of these formulations is underway concurrently and will allow us to investigate the antimicrobial activity of the nanoparticles formulations systematically.

In summary, we have demonstrated direct antimicrobial activity of ibuprofen on strains of two important CF-associated Gram-negative bacterial pathogens *in vitro* under a variety of conditions mimicking the environment in the CF lung. We also confirmed the conservation of this activity *in vivo* in a mouse model of acute *P. aeruginosa* pneumonia and demonstrated reduced bacterial burden and an improvement in the overall health of the ibuprofen-treated animals, which led to a superior survival outcome. Based on the conclusions derived from previous literature and our data, we suggest that the beneficial effects of ibuprofen observed in clinical trials in patients with CF are due to a combination of its antimicrobial and anti-inflammatory effects, and not solely the anti-inflammatory effects, as previously believed. Furthermore, to increase the utility of ibuprofen therapy in CF, we have developed an aerosolizable nanoparticle formulation of ibuprofen capable of depot delivery to the lung. This approach may be able to provide the target therapeutic dose locally, thereby preserving the beneficial clinical effects while potentially mitigating toxicity and pharmacokinetic concerns associated with high systemic concentrations resulting from traditional oral therapy. We believe that the robust anti-inflammatory activity of ibuprofen coupled with its mild-to-moderate antimicrobial activity against MDR pathogens makes it an extremely attractive candidate as an
adjunct therapeutic and hope that our findings will provide an impetus for the adoption of this
therapeutic as standard treatment by more CF centers globally.

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REFERENCES:

1. Chmiel JF, Berger M, Konstan MW. 2002. The role of inflammation in the
   Crit Care Med 154:1229-56.
   infection, and pulmonary function in infants and young children with cystic fibrosis. Am
   J Respir Crit Care Med 165:904-10.


Figure 1 – Additional ibuprofen treatment can extend the growth inhibition of gram-negative bacteria. (A) Pseudomonas aeruginosa (PAO1), (B) Pseudomonas aeruginosa (PA M57-15), (C) Burkholderia cenocepacia (Bcc K56-2), and (D) Burkholderia multivorans were grown in the presence of 0, 50, 75, and 100 µg/mL of ibuprofen. Controls included un-inoculated MH broth and MH broth containing DMSO (5% v/v) without ibuprofen (0 µg/mL IBU). At the 12-h time point each sample was spiked with the appropriate treatment and volumetric adjustments were performed to keep concentrations constant. Each column represents the mean ± standard error of
the mean of colony-forming units (CFUs) from at least 4 independent experiments with a total of at least 8 replicate samples per treatment. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to control (two-tailed non-parametric Mann-Whitney test).

**Figure 2** – Ibuprofen exposure (100 μg/mL) leads to a reduction in intracellular ATP in *P. aeruginosa* strain PAO1 compared with untreated control (MH broth with DMSO, 0 μg/mL IBU) bacteria. Data are shown as mean ± standard error from 8 replicate experiments and were analyzed using a two-tailed, non-parametric Mann-Whitney test. *** denotes statistical significance with $P < 0.001$. 

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Figure 3 – Ibuprofen reduces the growth rate and end-point CFU counts of Gram-negative bacteria PAO1 cultured in rich media and artificial CF sputum (ASM). For both experiments, 1x10^6 CFU/mL of PAO1 was grown in duplicate wells of a 96-well plate at 37°C in MH broth and artificial CF sputum in the presence or absence of ibuprofen (IBU) (100 µg/mL) over a 12-hour period. OD_{600} was determined at 1-h intervals to compare the growth rates for PAO1 in (A) MH broth and (B) artificial CF sputum. At 12-h, the bacteria were diluted and plated to determine bacterial burden (CFU/mL) as shown in (C) MH broth and (D) artificial CF sputum. Controls included un-inoculated growth medium (not shown), growth medium with bacteria alone, and growth medium containing DMSO (5% v/v) without ibuprofen (0 µg/mL IBU) inoculated with bacteria. Each dataset represents the mean of 5 independent experiments (total...
10 replicates per treatment) and the data are presented as mean ± standard error. For figures (C, D), starting inoculum of bacteria (empty circle), bacteria alone (filled circle), bacteria treated with ibuprofen (filled square), and bacteria in growth medium containing DMSO but no ibuprofen (filled upright triangles). * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to control, where paired t-test was used to analyze the differences in end-point OD$_{600}$ for growth curves and two-tailed, non-parametric Mann-Whitney test was used to analyze the differences in CFU counts.

**Figure 4** – Ibuprofen reduces the growth rate and end-point CFU counts of Gram-negative bacteria *Bcc K56-2* cultured in rich media and artificial CF sputum (ASM). For both
experiments, $1 \times 10^6$ CFU/mL of Bcc K56-2 was grown in duplicate wells of a 96-well plate at 37°C in MH broth and artificial CF sputum in the presence or absence of ibuprofen (IBU) (100 µg/mL) over a 12-hour period. OD$_{600}$ was determined at 1-h intervals to compare the growth rates for Bcc K56-2 in (A) MH broth and (B) artificial CF sputum. At 12-h, the bacteria were diluted and plated to determine bacterial burden (CFU/mL) as shown in (C) MH broth and (D) artificial CF sputum. Controls included un-inoculated growth medium (not shown), growth medium with bacteria alone, and growth medium containing DMSO (5% v/v) without ibuprofen (0 µg/mL IBU) inoculated with bacteria. Each dataset represents the mean of 5 independent experiments (total 10 replicates per treatment) and the data are presented as mean ± standard error. For figures (C, D), starting inoculum of bacteria (empty circle), bacteria alone (filled circle), bacteria treated with ibuprofen (filled square), and bacteria in growth medium containing DMSO but no ibuprofen (filled upright triangles). * indicates P < 0.05 and ** indicates P < 0.01 compared to control, where paired t-test was used to analyze the differences in end-point OD$_{600}$ for growth curves and two-tailed, non-parametric Mann-Whitney test was used to analyze the differences in CFU counts.
Figure 5 – Ibuprofen at a concentration of 100 μg/mL increases the lag time before bacterial biofilms begin exponential growth. (A) Comparison of growth curves of untreated (control) biofilms and biofilms exposed to ibuprofen demonstrating an increase in the lag time, and a consequent reduction in the biomass accumulation, of *P. aeruginosa* biofilms exposed to 100 μg/mL ibuprofen. (B) Ratios of ibuprofen-treated to untreated biofilm growth over time. All data have been shown as mean ± standard error of the mean for 3 replicate experiments performed in pairs on separate days (total 6 replicates per treatment). Replicated, pairwise comparison of experiments done on the same day allow us to normalize out for the effects of day-to-day experimental variation; we have used a similar approach for other biofilm studies (95). (C) Confocal and z-stack images of *P. aeruginosa* control biofilms or biofilms continuously exposed to 100 μg/mL ibuprofen.
Figure 6 – Ibuprofen reduces bacterial burden in lungs and spleen of PAO1 infected mice and improves clinical scores at 36 hours post-infection. For CFU experiments, male C57BL/6J mice were intranasally inoculated with ~5 x 10^5 CFU P. aeruginosa (PAO1), treated with ibuprofen (n = 17) or vehicle (n = 15) orally at designated time points, and sacrificed at 36 hours. Significant reductions in bacterial burden are observed in (A) the lung and (B) the spleen following ibuprofen treatment. No significant differences in weight-loss were observed between ibuprofen- and sham-treated mice (C) but ibuprofen-treatment significantly reduced the clinical scores of mice at 36 hours compared with sham-treatment (D). Data are displayed as mean ± standard error of the mean and were analyzed using two-tailed, non-parametric Mann-Whitney test.
case of the CFU experiments, each symbol represents an individual mouse. ** denotes statistical significance with $P < 0.01$. 
Figure 7 – Ibuprofen treatment improves survival and reduces weight loss, as well as clinical scores in PAO1 infected mice. Male C57BL/6J mice were intranasally inoculated with \(\sim 5 \times 10^5\) CFU *P. aeruginosa* (PAO1), treated with ibuprofen (n = 13) or vehicle (n = 14) orally at designated time points, and evaluated for 72 hours. (A) Kaplan-Meier survival curves demonstrate greater survival in ibuprofen-treated mice compared with sham-treated mice (92.3% survival in 12/13 ibuprofen-treated mice versus 57.1% in 8/14 sham-treated mice, \(p = 0.0386\) as analyzed using a Log-rank Mantel-Cox test). Ibuprofen treatment also reduces weight-loss on day 3 (B) and improves clinical scores of PAO1 infected mice at each time point (C). Data for weight-loss and clinical scores are displayed as mean ± standard error of the mean and were analyzed using two-tailed, non-parametric Mann-Whitney test. * denotes statistical significance with \(P < 0.05\), ** indicates \(P < 0.01\), and *** indicates \(P < 0.001\).
Figure 8 – Biodistribution studies demonstrating the abundance of ibuprofen in the serum but low concentrations in the lungs and bronchoalveolar lavage fluid (BALF) of mice immediately after nebulization of 25 or 50 mg ibuprofen to healthy C57BL/6J mice. Doses of 25 and 50 mg refer to the amount of ibuprofen placed in the Aerogen Aeroneb™ micro-pump nebulizer. Data are presented as mean ± standard error from 3 replicate mice per treatment with each sample analyzed in duplicate (total 6 replicates per treatment) and are analyzed using two-way ANOVA with Tukey’s multiple comparisons post-hoc test. ** indicates statistical significance with $P < 0.01$ and *** indicates statistical significance with $P < 0.001$ compared to serum ibuprofen concentration for a given dose.
Figure 9 – Scanning electron micrographs demonstrating the surface morphology and size distribution of PLGA–(PLGA-PEG) nanoparticles formulated using an oil-in-water (o/w) emulsion technique (A) Empty nanoparticles, (B) Ibuprofen–loaded nanoparticles, and (C) Ibuprofen–loaded nanoparticles formulated with sodium ibuprofen in the external aqueous phase.

Figure 10 – Nanoparticles encapsulating ibuprofen impede the growth of *P. aeruginosa* (PAO1) compared with controls and empty nanoparticles as demonstrated by endpoint CFU studies over 6 hours. Ibu/NaIbu NPs were formulated using a w/o/w emulsion technique with sodium ibuprofen in the external phase to increase ibuprofen loading within the nanoparticles. A total of 6 replicate samples (2 duplicates x 3 technical replicates) were assessed for each treatment, the
data presented as mean ± standard error, and analyzed using one-way ANOVA with Tukey’s multiple comparisons post-hoc test. ** indicates statistical significance with P < 0.01 and *** indicates statistical significance with P < 0.001 compared to control.
Table 1. Antimicrobial activity (log CFU/mL) in the presence and absence of ibuprofen (IBU) over 12-hours. Controls for the experiment include un-inoculated MH broth and MH broth with DMSO (5% v/v) without ibuprofen (0 µg/mL IBU). Mean CFU counts are from at least three replicate experiments performed in duplicate wells (at least 6 sample replicates per treatment and pathogen) and were analyzed using a two-tailed paired t-test. * indicates significant differences compared to control with P ≤ 0.05.

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