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Ibuprofen and Antibiotic Co-Delivery via Nanoparticles: A Novel Approach to Treating *Pseudomonas aeruginosa* Infections in Cystic Fibrosis - A Protocol Study

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Abstract

Introduction: Cystic fibrosis (CF) is a genetic disorder that severely affects the lungs, leading to chronic infections primarily by *Pseudomonas aeruginosa (P. aeruginosa)*. This pathogen adapts to the CF lung environment, forming biofilms that contribute to persistent infections and antibiotic resistance. ibuprofen, a non-steroidal anti-inflammatory drug, has shown potential in disrupting biofilms and enhancing antibiotic efficacy against *P. aeruginosa*. Recent studies focus on delivering ibuprofen via nebulization, using nanoparticles to improve targeting and reduce drug dosage.

Methods: This paper explores the *in vitro* and *in vivo* effects of nebulized ibuprofen nanoparticles combined with the antibiotic ceftazidime. The study hypothesizes that this co-delivery system can reduce bacterial load, inflammation, and biofilm formation more effectively than standard treatments.

Results: Results are anticipated to show that ibuprofen enhances antibiotic penetration and disrupts biofilm structure, thereby improving therapeutic outcomes in CF patients.

Discussion: This research suggests a promising direction for CF treatment, leveraging advanced drug delivery systems to combat bacterial resistance and improve patient prognosis.

Keywords: Cystic Fibrosis, P. aeruginosa, biofilms, ibuprofen, nebulization, nanoparticles, antibiotic resistance, ceftazidime

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder which affects mucus and sweat-producing cells, impacting multiple organs, however the lungs are most severely affected. CF stems from mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene, resulting in excessive mucus production. The CFTR protein is a regulated chloride channel which facilitates the passing of chloride through mucus-producing cells along with water which thins the mucus. However, when CFTR is mutated, it results in thick, sticky mucus that obstructs the airways, leading to severe lung infections [1]. This condition prompts a significant influx of neutrophils, which release elastase, overwhelming lung antiproteases and contributing to tissue damage [2]. Additionally, degranulating neutrophils release substantial amounts of nucleic acids and cytosol matrix proteins, further increasing the mucus viscosity [3].

In CF airways, the lungs of patients are often colonized by microorganisms such as bacteria. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative bacterium commonly found in CF patients and associated with life-threatening infections in humans [4]. The bacterium adapts by utilizing oxygen, nitrogen, and potentially other compounds like thiosulfate for energy production. *P. aeruginosa* can also ferment pyruvate and arginine, supporting survival in anaerobic environments without nitrogen, though this allows only limited growth. In nutrient-poor environments, *P. aeruginosa* employs diverse metabolic pathways to utilize complex carbon sources. However, in CF airways, the host provides various nutrients supporting rapid growth but restricts essential factors like iron and zinc. Genes for iron and zinc acquisition are highly expressed, highlighting their importance for bacterial growth. The shift in nutrient availability in CF lungs causes significant metabolic changes until a balance between essential growth functions and secondary functions, including virulence factors, are reached. A critical adaptation involves the pyruvate dehydrogenase (PDH) complex, which links glucose metabolism with the tricarboxylic acid (TCA) cycle and is under negative selective pressure in CF airways. Mutations in PDH complex genes are common and pathoadaptive, leading to reduced PDH expression *in vivo*. This results in a split in central carbon metabolism, utilizing the glyoxylate shunt and amino acid and lactate assimilation instead of glucose, although glucose is abundant. Reduced glycolysis and TCA cycle activity decrease electron donor production, lowering the proton motive force and likely aiding in oxidative stress resistance. These metabolic

adaptations enable *P. aeruginosa* to survive and thrive in the challenging CF lung environment [5].

Chronic infections and inflammation are distinctive features of CF, most caused by biofilm-mediated resistance. These infections elicit multiple courses of antibiotic treatments which lead to adaptation of bacteria to antibiotic-positive environments. This leads to the emergence of antibiotic-resistant bacteria. In CF patients, the biofilm communities prevent the host from effectively clearing the infection, leading to excessive airway inflammation and increased airway obstruction . *P. aeruginosa*, a common CF pathogen, predominantly forms biofilms through alginate biosynthesis genes. Biofilms hinder antibiotic penetration, fostering drug resistance [6].

Despite airway clearance and antibiotics being the primary treatments for CF to combat the effects of inflammation, numerous studies have looked at therapies for targeting inflammatory response. Nebulized antibiotics have emerged to improve airway delivery although they struggle against bacterial biofilm protection. ibuprofen, a low-cost, low-risk, non-steroidal anti-inflammatory drug, disrupts *P. aeruginosa* biofilm by mutating alginate genes, increasing antibiotic effectiveness [5]. In the environment of the CF lung, *P. aeruginosa* undergoes a transformation to a mucoid phenotype. This change is marked by the overproduction of alginate, an exopolysaccharide made up of mannuronic acid and guluronic acid monomers. The mucoid conversion is often linked to inactivating mutations in the *mucoid A* gene, which encodes an anti-sigma factor of alginate biosynthesis transcriptional regulatory protein T (*AlgT*). *AlgT* is essential for the expression of the alginate biosynthetic operon. Alginate is believed to provide protection in the harsh environment of the CF lung, where the bacteria face constant oxidative stress and immune system attacks. This switch to a mucoid form is also thought to help *P. aeruginosa* persist in the airways and typically coincides with a decline in the patient's prognosis [7,8]

Engineered nanomaterials offer considerable potential to enhance disease diagnosis and increase treatment specificity for inflammatory diseases [9]. Recent research has been conducted and advancements made in nanotechnology, with nanoparticles (NPs) being a significant focus [10,11]. Nanoparticles are engineered at the scaled of nanometer [12] and have the potential to improve the stability and solubility of encapsulated cargos, facilitate transport across membranes, and extend circulation times, thereby enhancing safety and efficacy. Therefore, NPs can be employed as more sophisticated systems, such as in nanocarrier-mediated combination therapies, to modulate multiple pathways. This approach can enhance therapeutic efficacy against specific macromolecules, target distinct phases of the cell cycle, and overcome drug resistance mechanisms [10,11].

To maximize pharmacodynamics, some studies suggested the utilization of nebulized ibuprofen nanoparticles for more localized delivery by encapsulating ibuprofen in NPs to be delivered by nebulization. This formulation enhances drug loading and reduces colony forming units (CFU) using *in vivo* models [13,14]

Further studies in nanotechnology have shown that co-drug delivery using NPs has the potential to optimize pharmacokinetics for both drugs by utilizing a single nanocarrier [15]. Reactive oxygen species (ROS) are oxygen-derived chemical species produced by the body, such as hydrogen peroxide, singlet oxygen superoxide, and hydroxyl radicals, which can transform through a series of reactions. Typical stimuli for drug delivery systems (DDSs) include endogenous and exogenous factors with ROS-responsive DDSs being particularly promising for advanced nanomedicines due to their implications in various diseases. ROS-responsive systems can be designed to release drugs, activate sensors, or trigger other responses in the presence of elevated ROS levels, which are often associated with various diseases, inflammation, or tissue damage. These systems can be used for targeted therapy, diagnostic purposes, or as part of smart materials that adapt their properties in response to oxidative conditions [16].

The co-delivery of nebulized ibuprofen nanoparticles with the antibiotic ceftazidime can effectively reduce bacterial load, inflammation, and biofilm formation in *P. aeruginosa* infections associated with CF, compared to standard antibiotic treatments alone. This co-delivery system is hypothesized to enhance antibiotic penetration and disrupt biofilm structure, resulting in improved therapeutic outcomes for CF patients.

To investigate the significance of nebulized ibuprofen in biofilm expression and antibiotic efficacy in *P. aeruginosa* infections, an *P. aeruginosa* biofilm model, an *in vivo* mouse model, and an *in vitro* ROS-responsive polyurethane (PFTU) co-drug delivery model with ibuprofen and ceftazidime will be explored.

Methods

Ibuprofen Treated *P. aeruginosa* Biofilm *in vitro* Model

Artificial sputum medium (ASM) is a class of in vitro bacterial culture medium intended to mimic the nutritional environment of CF pulmonary mucus [17]. Most ASM formulations utilize commercial porcine gastric mucin as their mucin source. Analysis of commercial PGM has revealed it to be a source of contaminating bioavailable metals, lipids, and amino acids. Therefore, to maintain the balanced ionic and amino acid content in ASM, the chelator diethylenetriaminepentaacetic acid is required. Human sputum contains a high concentration of lipids. According to studies mucus production and retention may be influenced by lipid-binding molecules. The interaction between mucus production and lipid content, specifically through 15-keto-prostaglandin-2 (15-keto-PGE2) and its receptor PPAR-γ, plays a significant role in CF pathogenesis [18]. This medium gained acceptance for use in assays which require conditions like a CF lung as opposed to synthetic CF septum medium (SCFM) due to its ability to provide a realistic environment for studying *P.aeruginosa* biofilm formation, as

well as its interactions with host cells and other microbes [18]. Although SCFM provides a consistent environment for studying the physiology and metabolism of *P.aeruginosa* in CF-like conditions, it does not fully replicate the physical and chemical environment of natural CF sputum. The medium is also less effective at supporting biofilm formation compared to ASM [17].

P.aeruginosa will be grown *in vitro* by diluting commercially available PA14 strain. The PA14 strain is a more recent *P.aeruginosa* isolate and is more virulent than other strains which makes it more effective for use in disease models, especially studying biofilm production [19]. An inoculum of OD_{600} 0.05 of an overnight culture of PA14 grown in tryptone soy broth (TSB) will be added to 1 mL of ASM in 24-well cell-culture plates and incubated for 16 hours at 37°C with gentle shaking. Viable counts will be performed with cultures grown for 6 hours to determine growth rate. Cells will be treated for 30 minutes with a solution containing 1 mg/mL cellulase and 400 µg/mL chloramphenicol in 0.05 M citrate buffer (pH 4.6) to dissolve clumps and then plated on agar medium containing either ibuprofen, ceftazidime or both. [20]. Viable and dead bacteria will be discriminated using the LIVE/DEAD BacLight Bacteria Viability Kit (Molecular Probes) according to the manufacturer's instructions. This kit uses the membrane-permeant SYTO 9 dye to label live bacteria with green fluorescence and propidium iodide to label membrane-compromised (non-viable) bacteria with red fluorescence [21]. Susceptibility will be measured by studying the diameter of the zone of growth inhibition [20].

Nebulized Ibuprofen Nanoparticle and Oral Ceftazidime treated *in vivo* Mouse Model

Mice will be handled according to Canadian Council on Animal Care (CCAC) guidelines when being used in study[22]. PA14 will be cultured in MOPS medium on blood agar plates. According to recent studies the localization of ibuprofen delivery can be optimized by the encapsulation of ibuprofen NPs form in order to prevent the rapid transport of nebulized ibuprofen into mouse serum [15]. NPs will be formulated using poly(lactide-co-glycolide) (PLGA), a copolymer of PLGA and polyethylene glycol dissolved in chloroform, followed by emulsification with a polyvinylpyrrolidone solution. After stirring and solvent removal, the nanoparticle suspension is processed to obtain ibuprofen-loaded nanoparticles in powder form [13].

CD1 mice are standardly used in biomedical research [23]. This study will use healthy CD1 male mice aged 5 weeks. Mice will be weighed and randomly assigned to four groups of six. CD1 mice will be anesthetized using isoflurane in a gas chamber prior to the start of the procedure and subsequently intratracheally infected with 40 µl of PA14 in MOPS medium and administered with 50 mg nebulized NPs and 50 mg/kg nebulized ceftazidime every 3 hours [13]. Positive control groups will receive either nebulized ibuprofen or ceftazidime. ceftazidime is one of the standard antibiotics prescribed for CF patients with *P. aeruginosa* infections in nebulized form 25). Negative control group receives empty NPs and medium only. After 72 hours, mice will be anesthetized intranasally using isoflurane and euthanized using cardiac puncture. Lungs will be isolated, homogenized, and tissue bacterial load measured by plating homogenized tissue on blood agar plates and measuring the bacterial colony forming units (CFU) [26].

Co-Delivery of Nebulized Ibuprofen and Ceftazidime Nanoparticle *in vitro* Model

Based on the study by Muhammad *et al*, ibuprofen and ceftazidime will be co-loaded into ROS-responsive polyurethane (PFTU) [14]. PFTU is used in drug localization due to its ability to degrade in response to high ROS levels, making it useful for targeted drug delivery, tissue engineering, and other therapeutic method which use controlled reactions to oxidative stress [27]. ROS-responsive polyurethane will be synthesized from 1,6-hexamethylene diisocyanate, poly(thioketal) dithiol, and poly(propylene fumarate) diol. In this formulation, 50 mg of PFTU, 5 mg of ceftazidime, and 5 mg of ibuprofen will be dissolved in a solvent mixture of 600 µL Dimethyl Sulfoxide, 1200 µL Dimethylformamide and 1200 µL ethanol. The organic phase will be added dropwise to an aqueous phase containing 3 mL of Phosphate Buffered Saline (PBS) (pH 7.4) and 4 mL of 2% Polyvinyl Alcohol solution. Subsequently, 5 mL of PBS will be added, and the mixture will be stirred at room temperature until the solvent evaporates completely. The resulting NPs will be collected by centrifugation at 12,000 rpm for 10 minutes at 4 degrees Celsius. The NPs will be washed with Milli-Q water three times and re-suspended in water. For the preparation of blank NPs, the same procedure will be followed without adding AZN and ibuprofen [14]. The loading contents of AZI and ibuprofen in the NPs will be determined using high-performance liquid chromatography (HPLC). According to Muhammad *et al*, the nanoparticles will be freeze-dried for 24 hours and weighed. After 24 hours they will be re-dispersed in ethanol, sonicated for 1 minute at 4 degrees Celsius, and centrifuged to collect the supernatant. The supernatant will be analyzed using HPLC. To measure the concentrations of ceftazidime and ibuprofen 4 rounds of HPLC injections will be used, with detector wavelengths of around 254 nm for ceftazidime and 220-230 nm for ibuprofen [28]. The quantities of the drugs will be calculated using standard curves for ceftazidime and ibuprofen, respectively [14].

To measure the effect of the NPs on inflammation levels, cytokine levels in RAW264.7 cells after lipopolysaccharides (LPS) stimulation will be measured. A RAW264.7 macrophage cell line are commonly used for investigating immune and inflammatory responses. In this study, RAW264.7 macrophages will be seeded in high-glucose Dulbecco's modified Eagle's medium (DMEM), 96-well plates (2 × 104 cells/well, 100 µL medium/well)

[29]. The cells will be transferred to 12-well plates and treated with ceftazidime (0.5 µg/mL), ibuprofen (0.5 µg/mL), PFTU NPs (5 µg/mL), ceftazidime + ibuprofen NPs (5 µg/mL), or untreated. One hour after treatment, the cells will be treated with LPS (1 µg/mL) and incubated at 37 °C for 24 hours. After incubation, the supernatant will be collected and centrifuged once more at 3000 rpm for 30 minutes to remove cell debris [14]. The levels of Tumor Necrosis Factor-α, Interleukin (IL)-1β, IL-6, IL-4, and IL-10 in the supernatant will be measured using Enzyme-Linked Immunosorbent Assay kits according to the manufacturer's instructions [14].

The antibacterial activity of ceftazidime, ibuprofen, PFTU NPs, and ceftazidime + ibuprofen NPs will be investigated *in vitro* against *P. aeruginosa* bacteria. To prepare the bacterial inoculum, the method described in section will be used. The turbidity of the bacterial culture will be adjusted to match the 0.5 McFarland standard, equivalent to 1 × 10^8 CFU/mL, using a spectrophotometer at 600 nm. A sterile cotton swab will be dipped in the adjusted bacterial solution and used to evenly inoculate tryptone soy agar plates. Cellulose filter discs (6 mm) will be soaked with 100 µL of each drug and nanoparticle solution i.e. ceftazidime (0.5 µg/mL), ibuprofen (0.5 µg/mL), PFTU NPs (5 µg/mL), ceftazidime + ibuprofen NPs (5 µg/mL), or untreated and placed on the inoculated agar plates. MOPS medium soaked with 100 µL of PBS will be used as the negative control, while a disc soaked with ceftazidime will be used as the positive control. The culture plates will be incubated at 37°C for 24 hours, after which the zone of inhibition around each disc will be observed and measured [14]

Anticipated Results

Ibuprofen Treated *P. aeruginosa* Biofilm *in vitro* Model

Study by Shah et al concluded that ibuprofen-treated biofilms experienced a delay of approximately 0.86 hours in transitioning to exponential growth compared to control biofilms. Once in the exponential phase, the doubling time of bacteria was similar for both treated and untreated biofilms. Initial biomass in ibuprofen-treated biofilms was slightly higher, but a decline in biomass ratio occurred, reaching a low at 8 hours before rising again, suggesting comparable biomass by 10 hours [13].

A similar study aimed at exploring the effectiveness of ibuprofen in the expression of alginate genes found that treating *P. aeruginosa* strains with ibuprofen for 48 hours significantly reduced biofilm formation, as measured by the crystal violet staining method. The reduction in biofilm formation ranged from 23% to 49% compared to untreated controls, indicating a notable inhibitory effect of ibuprofen on biofilm development across all tested strains [8].

Similar trends are anticipated after carrying out the protocol described in this paper, characterized by a decrease in biofilm formation by ibuprofen.

Nebulized Ibuprofen treated *in vivo* Mouse Model

Further experiments by Shah *et al*. examined the effect of ibuprofen nanoparticles on reducing CFU counts in an in vivo mouse model. Using sodium ibuprofen (NaIbu) in the external phase improved the ibuprofen loading efficiency to 16%, compared to 4% without NaIbu. The study found that ibuprofen-loaded nanoparticles significantly reduced P. aeruginosa CFU counts, particularly with NaIbu. Additionally, ibuprofen treatment reduced bacterial burdens in the lungs and spleen of infected mice, improved clinical scores, survival rates, weight maintenance, and overall health [13].

No further studies have explored ibuprofen nanoparticles with non-nebulized antibiotics in vivo. However, a study by Konstan *et al*. on rats with chronic P. aeruginosa lung infection showed that ibuprofen significantly reduced lung inflammation without increasing bacterial load and improved weight gain. The effective ibuprofen dose also inhibited leukotriene B4, a pro-inflammatory compound, suggesting ibuprofen could mitigate inflammatory lung damage in cystic fibrosis (CF), supporting its potential as a therapeutic strategy for CF patients [30].

Similar results anticipated, characterized by reduced *Pseudomonas aeruginosa* CFU counts and bacterial burdens in an in vivo mouse model.

Co-Delivery of Nebulized Ibuprofen and Ceftazidime Nanoparticle *in vitro* Model:

Study by Muhammad *et al* evaluated the efficacy of co-delivery of ibuprofen (IBF) with an antibiotic in PFTU nanoparticle form. This study used azithromycin (AZI) instead of ceftazidime for co-delivery. Results showed that treatment with nanoparticles containing AZI and IBF, especially the combined AZI+IBF NPs, significantly reduced these cytokine levels and the expression of inducible nitric oxide synthase (iNOS), indicating reduced inflammation. Furthermore, the AZI+IBF NPs enhanced the release of anti-inflammatory cytokines IL-4 and IL-10 and promoted macrophage polarization to the M2 phenotype, marked by decreased iNOS and increased arginase 1 (Arg-1) expression [14].

In terms of antibacterial properties, the study investigated the antibacterial and anti-inflammatory properties of AZI, IBF, NP formulations against *P.aeruginosa*. AZI alone exhibited strong antibacterial activity, evidenced by a significant inhibition zone, while IBF and PFTU NPs also demonstrated antibacterial effects. Interestingly, blank PFTU NPs showed some antibacterial activity, likely due to their hydrophobic nature disrupting bacterial structures. The combination of AZI and IBF in nanoparticles (AZI+IBF@NPs) showed enhanced antibacterial and anti-inflammatory effects [14].

Similar results are anticipated characterized by enhanced antibacterial and anti-inflammatory effects against *Pseudomonas aeruginosa*, with the ceftazidime+ ibuprofen nanoparticles showing the strongest combined effects.

Discussion

Nebulized Ibuprofen treated *in vivo* Mouse Model

Additional studies should aim at better understanding the specific effects of ibuprofen on these different systems, aiming to isolate and identify its direct antimicrobial properties [13].

Co-Delivery of Nebulized Ibuprofen and Ceftazidime Nanoparticle *in vitro* Model

Limitations include the protocols inability to fully replicate the chronic biofilm infections found in the lungs of CF patients, which are characterized by persistent, biofilm-associated bacteria resistant to treatment. This limitation suggests that the results, particularly regarding the effectiveness of ibuprofen in reducing bacterial load and inflammation, may not directly apply to CF patients. Additionally, the model's complexity makes it difficult to isolate the direct antimicrobial effects of ibuprofen, which is primarily known for its anti-inflammatory properties. Further research with more precise experimental controls is needed to clarify whether ibuprofen's effects are due to its direct antibacterial properties or other indirect mechanisms, crucial for assessing its potential in treating CF and similar chronic conditions [14].

Conclusions

This study highlights the potential of nebulized ibuprofen nanoparticles in conjunction with antibiotics to enhance treatment efficacy against P. aeruginosa infections in cystic fibrosis patients. Current findings suggest promising avenues for advanced drug delivery systems, though further research is needed to address the study's limitations and refine treatment strategies.

List of Abbreviations Used

ASM: artificial sputum medium
CCAC: Canadian Council on Animal Care
CF: cystic fibrosis
CFTR: cystic fibrosis transmembrane conductance regulator
CFU: colony forming units
DDS: drug delivery systems
IL: interleukin
LPS: lipopolysaccharides
NP: nanoparticle
NSAID: non-steroidal anti-inflammatory drug
OD600: optical density at 600 nm
PBS: phosphate buffered saline
PDH: pyruvate dehydrogenase
PFTU: polyurethane
PLGA: poly(lactide-co-glycolide)
P. *aeruginosa*: Pseudomonas *aeruginosa*
ROS: reactive oxygen species
SYTO: Green Fluorescent Nucleic Acid Stains
TCA: tricarboxylic acid (cycle)
TSB: tryptone soy broth

Conflicts of Interest

The author declares that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study did not require ethics approval or participant consent because it is a review of existing literature. As a protocol study, it did not involve the collection of new data from human or animal subjects, nor did it include any direct interaction with participants. The study solely analyzed publicly available information and previously published research, which does not fall under the jurisdiction of ethical review boards.

Authors' Contributions

HT: made substantial contributions to the design of the study, collected, and analyzed data, drafted the manuscript, revised it critically, and gave final approval of the version to be published.

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