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Azithromycin and ciprofloxacin: A possible synergistic combination against *Pseudomonas aeruginosa* biofilm-associated urinary tract infections

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ABSTRACT

Biofilm formation is becoming a predominant feature in nosocomial infections. Since biofilms are increasingly resistant to antibiotics, making monotherapy ineffective, combination therapy appears to be relevant for their eradication. This study assessed the potential of azithromycin (AZM) and ciprofloxacin (CIP) alone and in combination in vitro and in a mouse model of urinary tract infection (UTI) induced with biofilm cells of *Pseudomonas aeruginosa*. In vitro antibacterial and antibiofilm activities of antibiotics alone and in combination were assessed using the fractional inhibitory concentration index (FICI), time–kill analysis and confocal laser scanning microscopy (CLSM). In vivo efficacy was evaluated in a UTI model by quantitation of bacterial burden in kidney and bladder tissue, renal histopathology, pathology index factors (MDA and NO), and pro-inflammatory (MIP-2 and IL-6) and anti-inflammatory (IL-10) cytokines. MICs of AZM and CIP for strain PAO1 were 256 and 0.5 μg/mL, respectively; MBECs were 4096 and 1024 μg/mL. Synergistic interaction was observed between AZM and CIP both against planktonic and biofilm bacteria (FICI < 0.5). The combination was also able to inhibit biofilm formation (at MIC levels) as observed with CLSM. Oral therapy with AZM (500 mg/kg) and CIP (30 mg/kg) combination in mice for 4 days showed accelerated clearance of bacteria from kidney and bladder tissue, improved renal histopathology, decreased levels of MDA and NO, significant decline in MIP-2 and IL-6, and increased IL-10 in the kidney ($P < 0.0001$). We conclude that AZM + CIP therapy holds promise against biofilm-associated UTIs as it confers antibacterial, immunomodulatory and anti-inflammatory effects.

1. Introduction

Biofilm-associated infections are creating havoc in healthcare facilities globally. Biofilms either formed on natural tissues (skin, mucosa, teeth, bones, etc.) or artificial devices (contact lenses, central venous catheters, endotracheal tubes, intrauterine devices, urinary catheters, etc.) collectively contribute to 60–85% of all microbial infections [1]. *Pseudomonas aeruginosa* is a prominent human pathogen that primarily target patients in nosocomial settings suffering from cystic fibrosis, catheterised urinary tract infections (UTIs) and other immunocompromised situations, and forms biofilms that become difficult to eradicate. Bacteria within a biofilm are >1000-fold more resistant to antibiotics compared with their planktonic counterparts and are shielded from the host immune response, leading to chronic or recurrent infections [2].

One of the novel approaches to treat biofilm-associated infections is the use of an antibiofilm agent that aids conventional antibiotic penetration of the biofilm by acting synergistically and resulting in its eradication. Azithromycin (AZM), an azalide (a subclass of macrolide antibiotics), is one such agent that possesses antibiofilm activity but does not show significant bactericidal activity against *P. aeruginosa* at therapeutic concentrations [3]. Several studies have suggested that AZM positively influences the clinical outcome in patients suffering from chronic *P. aeruginosa* infections as seen in diffuse panbronchiolitis, cystic fibrosis and chronic pulmonary disorders [3,4]. The antibiofilm activity of AZM in vitro and its therapeutic potential against *P. aeruginosa* experimental UTI induced with planktonic cells has also been reported [5]. Since AZM can disrupt biofilm and may revert bacteria to a planktonic state, combining it with an antipseudomonal antibiotic might be more effective than using antibiotics alone for the treatment of biofilm-associated infections [6]. The present study aimed to identify synergy between ciprofloxacin (CIP), one of the most effective antibiotics against *P. aeruginosa*, in combination with AZM in vitro and to further evaluate their in vivo efficacy in a mouse

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model of acute pyelonephritis established with biofilm cells of *P. aeruginosa*.

2. Materials and methods

2.1. Bacterial strains

Fifteen clinical isolates of *P. aeruginosa* isolated from patients having catheter-associated UTI attending Government Medical College and Hospital (Chandigarh, India) as well as the standard strain of *P. aeruginosa* PAO1 (obtained from Barbara H. Iglewski, University of Rochester, Rochester, NY) were employed in the study. All strains were maintained as 50% glycerol stocks and were stored at −20 °C.

2.2. Antibiotics used

AZM was obtained from Cipla Pharmaceuticals (Goa, India) and all other antibiotics used in susceptibility studies were from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Antibiotics were stored according to the manufacturer's recommendations.

2.3. Experimental animals

Female LACA (Swiss Webster) mice (6–8 weeks old, weight 20–30 g) obtained from Central Animal House, Panjab University (Chandigarh, India) were used in the study. Animals were housed in clean polypropylene cages and were fed on standard antibiotic-free synthetic diet (Hindustan Levers Ltd., Mumbai, India) and water ad libitum.

2.4. Antimicrobial susceptibility testing

Clinical isolates from catheter-associated UTI patients as well as the standard strain of *P. aeruginosa* PAO1 were tested for their susceptibility to various antibiotics by the Kirby–Bauer disk diffusion method following the criteria of the Clinical and Laboratory Standards Institute (CLSI) [7,8]. Minimum inhibitory concentrations (MICs) of chosen antibiotics against *P. aeruginosa* strains were determined using the broth microdilution technique [7].

2.5. In vitro synergy testing

2.5.1. Determination of the fractional inhibitory concentration index (FICI)

The effect of AZM and CIP in combination was assessed using the broth microdilution chequerboard technique [9,10]. Concentrations used for both the antimicrobials ranged between 1/64× MIC to 2× MIC.

2.5.2. Time–kill analysis

The bactericidal action of the antibiotics alone and in combination was established by time–kill assay [9]. Concentrations of AZM and CIP showing synergism as determined by the chequerboard assay were used.

2.6. Inhibition of biofilm formation

P. aeruginosa PAO1 strain (1×10^5 CFU/200 μL) in Luria broth (HiMedia Laboratories Pvt. Ltd.) was incubated at 37 °C for 24 h with AZM and CIP alone and in combination at 1× MIC, 1/10× MIC and 1/100× MIC in 96-well microtitre plates (Laxbro Manufacturing Co., Pune, India) and the optical density of biofilm cells was measured at 595 nm after staining with crystal violet (HiMedia Laboratories Pvt. Ltd.) (0.1%, w/v) [10].

2.7. Confocal laser scanning microscopy (CLSM)

P. aeruginosa 24-h-old biofilms grown on coverslips with no antibiotic and with AZM and CIP alone as well as in combination at MIC levels were stained with SYTO® 9 and propidium iodide using a LIVE/DEAD *Bac*Light™ Kit (Molecular Probes Inc., Eugene, OR). Coverslips were examined using an Olympus 1X81 + FV1000 microscope (Olympus Corp., Tokyo, Japan).

2.8. Determination of minimum biofilm eradication concentration (MBEC)

The antimicrobial susceptibility of *P. aeruginosa* PAO1 biofilm towards AZM and CIP was determined as described by Mataraci and Dosler [10]. Serial two-fold dilutions ranging from 8192 μg/mL to 8 μg/mL for both the antibiotics were used against 24-h-old biofilms.

2.9. Antibiofilm activity

The antibiofilm effect of AZM and CIP in combination was determined using the modified broth microdilution chequerboard technique [11]. Combinations of antibiotics were tested over suprainhibitory (starting from 2× MIC) and inhibitory (MIC) concentrations.

2.10. Therapeutic efficacy of azithromycin and ciprofloxacin in vivo

2.10.1. Generation and estimation of biofilm cells

Biofilm cells were generated in vitro on a Foley catheter following the method of Bala et al. [5]. Then, 50 μL of bacterial inoculum (10^8 CFU/mL) of 4-day-old biofilm cells scraped from the catheter surface were used for in vivo experiments.

2.10.2. Establishment of experimental acute pyelonephritis

An experimental model of acute pyelonephritis was established in female LACA mice as described by Mittal et al. [12]. Briefly, a soft Intramedic™ polyethylene tubing (PE-10) catheter (0.28 mm ID × 0.61 mm OD) (BD Biosciences, San Jose, CA) was inserted in the bladder through the urethra and 50 μL of bacterial inoculum (10^8 CFU/mL) consisting of biofilm cells of *P. aeruginosa* PAO1 was slowly injected into the bladder to avoid leakage and reflux, kept in place for 10 min and then withdrawn carefully. No obstruction or further manipulation of the urinary tract was done. Five groups consisting of 12 animals each were used in the study: (i) infection control group: infection was given with biofilm cells; (ii) saline control group: infection was given with biofilm cells and normal saline was given orally to infected animals 24 h post infection (p.i.) until the 5th post-infection day (p.i.d.); (iii) AZM-treated group: infected animals were treated therapeutically with an oral dose of AZM (500 mg/kg body weight) 24 h p.i. until the 5th p.i.d.; (iv) CIP-treated group: infected animals were given an oral dose of CIP (30 mg/kg body weight) 24 h p.i. until the 5th p.i.d.; and (v) AZM + CIP-treated group: infected animals were given oral doses of AZM 500 mg/kg and CIP 30 mg/kg 24 h p.i. until the 5th p.i.d.

2.10.3. Bacteriological studies

Animals were sacrificed on the 3rd and 5th p.i.d. Kidney and bladder tissues were excised aseptically, weighed and homogenised in 1 mL of phosphate-buffered saline. The quantitative bacterial count (CFU) per gram of renal and bladder tissue was calculated as reported by Harjai et al. [13].

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