Antipseudomonal agents exhibit differential pharmacodynamic interactions with human polymorphonuclear leukocytes against established biofilms of *Pseudomonas aeruginosa*

Athanasiros Chatzimoschou\textsuperscript{1}, Maria Simitsopoulou\textsuperscript{1}, Charalampous Antachopoulos\textsuperscript{1}, Thomas J. Walsh\textsuperscript{2} and Emmanuel Roilides\textsuperscript{1*}

\textsuperscript{1}Infectious Diseases Laboratory, 3\textsuperscript{rd} Department of Pediatrics, Faculty of Medicine, Aristotle University School of Health Sciences, Hippokration Hospital, 54642 Thessaloniki, Greece

\textsuperscript{2}Transplantation-Oncology Infectious Diseases Program, Departments of Medicine, Pediatrics, and Microbiology & Immunology, Weill Cornell Medical Center of Cornell University, New York, NY

**Running title:** Antibiotic-PMN interactions vs P. aeruginosa biofilms

**Corresponding address:** Emmanuel Roilides, MD, PhD

3\textsuperscript{rd} Department of Pediatrics, Hippokration Hospital

Konstantinoupolos 49, GR-54642, Thessaloniki, Greece

Tel: +30-2310-892444, FAX: +30-2310-992981

E-mail: roilides@med.auth.gr
Abstract

*Pseudomonas aeruginosa* is the most common pathogen infecting the lower respiratory tract of cystic fibrosis (CF) patients, where it forms tracheobronchial biofilms. *Pseudomonas* biofilms are refractory to antibacterials and to phagocytic cells of innate immunity, leading to refractory infection. Little is known about the interaction between antipseudomonal agents and phagocytic cells in eradication of *P. aeruginosa* biofilms. Herein, we investigated the capacity of three antipseudomonal agents, amikacin (AMK), ceftazidime (CAZ) and ciprofloxacin (CIP), to interact with human polymorphonuclear leukocytes (PMNs) against biofilms and planktonic cells of *P. aeruginosa* isolates recovered from sputa of CF patients. Three of the isolates were resistant and three were susceptible to each of these antibiotics. Concentrations 2, 8 and 32mg/l studied were subinhibitory for biofilms of resistant isolates; whereas, for biofilms of susceptible isolates they ranged between sub-MIC to 2xMIC values. The activity of each antibiotic alone or in combination with human PMNs against 48-hour mature biofilms or planktonic cells was determined by XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay. All combinations of AMK with PMNs resulted in synergistic or additive effects against planktonic cells and biofilms of *P. aeruginosa* isolates as compared to each component alone. More than 75% of CAZ combinations exhibited additive interactions against biofilms of *P. aeruginosa* isolates; whereas, CIP had mostly antagonistic or no interaction with PMNs against biofilms of *P. aeruginosa*. Our findings demonstrate a greater positive interaction between AMK with PMNs than that observed for CAZ and especially CIP against isolates of *P. aeruginosa* from the respiratory tract of CF patients.

**Key words:** biofilms; *Pseudomonas aeruginosa*; antimicrobial agents; neutrophils; synergism
Pseudomonas aeruginosa causes acute and chronic infections predominantly associated with compromised innate host defenses (1, 2). In patients with cystic fibrosis (CF), chronic P. aeruginosa infection of the lower respiratory tract is associated with excessive morbidity and mortality, as more than 80% of these patients succumb to respiratory failure (3, 4). A critical virulence trait of this pathogen that dominates in the CF lung is its capacity to form biofilms, a characteristic that has been linked to antimicrobial resistance and host defense evasion (5, 6). Chronic pulmonary infections arise because host responses are ineffective against biofilms (7). However, virulence factors that depend on the cell-to-cell communication system of P. aeruginosa are shown to have a major role as a defense against polymorphonuclear neutrophils (PMNs) (8-10).

Biofilms are surface-attached structured networks of aggregated bacteria, embedded in a self-produced matrix composed of polysaccharides, protein and DNA. These aggregates can resist high concentrations of antimicrobial agents that would efficiently eliminate the single cell planktonic phenotype (11-13).

Exposure of bacteria to subinhibitory antibiotic concentrations (sub-MICs) results in regression of certain virulence factors that control cell morphology, adherence or enzymatic secretion, eventually leading to alterations in the biofilm architecture. Such alterations could interfere with the ability of the pathogens to colonize susceptible hosts and develop an infectious process (14-17).

To date, several studies have investigated the effects of antimicrobial agents on P. aeruginosa biofilm formation or the response of the innate immune cells to P. aeruginosa biofilms (6, 18). However, to our knowledge, no study has yet addressed the combined effect of antibiotics with PMNs against established biofilms of P. aeruginosa. We hypothesized that anti-pseudomonal antimicrobial agents could interact with PMNs to enhance biofilm destruction. To this end, we evaluated the pharmacodynamic effects of representative...
antibiotics of three classes of antimicrobial agents: an aminoglycoside [amikacin (AMK)], cephalosporin [ceftazidime (CAZ)] and fluoroquinolone [ciprofloxacin (CIP)], alone or in combination with PMNs, against established biofilms and planktonic cells of susceptible or resistant *P. aeruginosa* strains isolated from patients chronically infected with CF.

**Materials and Methods**

**Bacterial isolates and growth conditions.** Six clinical isolates of *P. aeruginosa* were collected from sputa of six CF patients followed at Hippokration Hospital of Thessaloniki from 2007 to 2012. Isolate identity was determined using a Vitek II automated system (Biomerieux, Marcy l’ Etoile, France) according to the manufacturer’s instructions. Stock cultures were divided into small portions and maintained at -35°C in a solution containing 25% glycerol and 75% peptone. All isolates were revived from frozen stock cultures on cation-adjusted (50 mg/l Ca$^{2+}$, 25 mg/l Mg$^{2+}$, pH 7.2) Mueller Hinton (MH; Scharlau Chemie, S.A., Barcelona, Spain) agar plates after growth at 37°C for 12 h. Five to ten colonies from each isolate were subsequently transferred to 20 ml of cation-adjusted MH broth and incubated at 37°C overnight on a rocking table. The grown cultures were harvested by centrifugation at 2,000 rpm for 20 min, washed twice with 10 ml of phosphate-buffered saline solution pH 7.2 (PBS; 0.02 M phosphate, 0.15 M NaCl) and resuspended in RPMI-1640 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) buffered to pH 7.2 with 3-(*N*-morpholino) propanesulfonic acid (Sigma-Aldrich Chemie GmbH). Three of the clinical isolates were resistant to each of amikacin (AMK-r), ceftazidime (CAZ-r) and ciprofloxacin (CIP-r), while the other three isolates were susceptible to these antibiotics (AMK-s, CAZ-s and CIP-s). The isolates studied were classified as resistant or susceptible following the CLSI interpretive standards of breakpoints for resistance: $\geq 32$=R, $\leq 16$=S for AMK; $\geq 32$=R, $\leq 8$=S.
for CAZ; ≥4=R, ≤1=S for CIP (19). For all experiments, the organisms were used at a final concentration of $10^6$/ml.

**Antimicrobial agents.** AMK was obtained from Vianex S.A. (Athens, Greece), CAZ from GlaxoSmithKline S.A. (Athens, Greece) and CIP from Bayer Hellas S.A. (Athens, Greece). Each antibiotic was dissolved in distilled water to a final concentration of 1,000 mg/ml for AMK and CAZ and 2,000 mg/ml for CIP. They were then maintained as a stock solution at -35°C for up to one month.

**Biofilm formation.** Bacterial biofilms were produced in RPMI-1640 growth medium using 96-well microtiter polystyrene plates as described previously with minor modifications (20). Briefly, for each isolate, 100 µl of $10^6$ cells/ml suspension were added into each well and incubated at 37°C for 48 h under constant linear shaking in order to promote biofilm formation. Mature biofilm production was evaluated by staining the polysaccharide structure of the extracellular matrix of biofilms with safranin. Mature biofilms were first washed twice with PBS in order to remove non-adherent cells and then stained with 200 µl of 0.1% safranin for 5 min. The optical absorbance at 492 nm was measured using a microplate reader (ChroMate 4300; Awareness Technology, Inc., Palm City, FL). All six isolates were strong biofilm producers.

**Planktonic and biofilm drug susceptibility.** Minimum inhibitory concentrations (MICs) of the three antibiotics for planktonic cells were determined using both the broth microdilution method, according to Clinical Laboratory Standards Institute (CLSI) guidelines and the XTT colorimetric assay (21). Antibiotic susceptibility of mature biofilms was also determined by the XTT assay. Briefly, antimicrobial agents were added to corresponding wells of biofilms and free-living planktonic cell cultures at serially 2-fold dilutions with concentrations ranging from 0.5 to 512 mg/l. The microtiter plates were incubated at 37°C for 24 h with shaking. Drug-free wells containing only growth medium served as controls. At the
end of the incubation period, antimicrobial susceptibility was determined by the XTT assay, as described below. MICs were determined as the lowest antibiotic concentration at which a prominent decrease in turbidity was observed either microscopically or colorimetrically, corresponding to 50% bacterial damage as compared to untreated controls.

Isolation of human PMNs. Heparinized whole blood was obtained from healthy adult volunteers and PMNs were isolated by dextran sedimentation and Ficoll centrifugation as previously described (22). Following hypotonic lysis of red blood cells, the purified PMNs were resuspended in Hanks’ balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\) (Gibco, BRL, Life Technologies Ltd., Paisley, Scotland, UK) and counted with a hemocytometer. The cell concentration was adjusted to 10\(^6\) cells/ml.

Combined treatment of *P. aeruginosa* isolates by antibiotics and PMNs. Biofilms and planktonic cells of resistant or susceptible *P. aeruginosa* isolates were incubated in the presence of each antipseudomonal agent alone or in combination with human PMNs at effector cell-to-target (E:T) ratios of 1:20 and 1:10 at 37°C in a humidified 5% CO\(_2\) incubator for 24 h. Based on the MICs of each antibiotic, the sub-MIC concentrations chosen were 2, 8 and 32 mg/l. Due to susceptibility differences between resistant and susceptible isolates, the above concentrations ranged between sub-MIC to 2x MIC values.

Assessment of biofilm and planktonic cell damage. Bacterial damage induced by phagocytes and/or antimicrobial agents was assessed by using an XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay as previously described with minor modifications (23, 24). Briefly, 150 µl of XTT (0.25 g/l; Sigma) containing coenzyme Q (40 mg/l; Sigma) was added to microtiter plates after a washing step with PBS to remove antibacterial agents, PMNs, or growth medium. The plates were then incubated at 37°C for 20-30 min, and the change in color, indicating the degree of bacterial damage, was measured in a microtiter plate reader at 450 nm with a reference wavelength of 690 nm.
Antibacterial activity was calculated according to the following formula: % bacterial damage

\[ \text{% bacterial damage} = \frac{1 - (X/C)}{100} \]

where \( X \) is the absorbance of experimental wells and \( C \) is the absorbance of control wells. The MIC for biofilms and planktonic cells was defined as the least antimicrobial concentration causing \( \geq 50\% \) bacterial damage compared to that for untreated controls.

**Statistical analysis.** Each concentration of AMK, CAZ, and CIP or PMNs for every clinical isolate was tested in quadruplicate per experiment. Each experiment was performed with the cells of one PMN donor, and four independent experiments were done in total. The means of the replicate wells from each experiment were used in the data analysis to calculate the mean±SE of all the experiments at the same conditions. The differences in the mean values of three or more groups were evaluated by repeated measures analysis of variance (ANOVA) with Bonferroni post-test analysis. The damage induced by the PMNs alone and the antibiotic alone was calculated and compared with the effect of the combined treatment by PMNs and antibiotic.

Synergism (SYN) was defined as an antimicrobial effect (damage) caused by the combination that was significantly greater than the effect of PMNs alone plus the effect of the antibiotic alone. An additive effect (ADD) was defined as an antimicrobial effect of the combination that was significantly greater than the effect produced by either PMNs alone or antibiotic alone but that did not reach synergism. Antagonism (ANT) was defined as an antimicrobial effect of the combination that was significantly less than the effect produced by either PMNs or antibiotic alone. Differences between biofilm and planktonic growth forms were analyzed by Student's t-test after Kolmogorov–Smirnov test of normality showed a normal distribution of data. Data were analyzed using Instat (version 3) biostatistics software (GraphPad Inc., San Diego CA). A \( P \) value of <0.05 indicated statistical significance.
Results

Activity of antibiotics on *P. aeruginosa* biofilms and planktonic cells. Planktonic MIC values obtained by the CLSI method were comparable to those obtained by the XTT assay (data not shown). As shown in Table 1, MICs of AMK, CAZ and CIP for biofilms of resistant *P. aeruginosa* isolates were almost equal to MICs observed for planktonic cells. By comparison, MICs of AMK, CAZ and CIP against biofilms of the susceptible isolates were 1x-2x dilutions higher than those against planktonic cells (Table 1).

The maximum biofilm damage caused by AMK and CIP was evidenced at 512 mg/l against *P. aeruginosa* isolates and ranged within 92%-94%. By comparison, the maximum biofilm damage caused by CAZ was 78% at 128 mg/l for the CAZ-r isolate and 97% at 256 mg/l for the CAZ-s isolate. Overall, the antibiotics, when used at subinhibitory concentrations, appeared to cause similar damage to biofilms and planktonic cells that ranged between 10% and 62% (Fig. 1-3). However, significant differences in damage were observed between biofilms and planktonic cells mostly at higher antibiotic concentrations (8 or 32 mg/l, Fig. 1A and 1B, Fig. 2A, Fig. 3A and 3B, respectively; *P*<0.01).

PMN activity on *P. aeruginosa* biofilms and planktonic cells. Overall, the activity of PMNs against *P. aeruginosa* isolates was limited. With the exception of AMK-r and CAZ-s isolates, the damage induced by PMNs against mature biofilms was significantly less than that induced against their corresponding planktonic cells [from 9%±1.4% vs 11%±2.1% for AMK-r isolate (1:20; ns, Fig. 1A) to 13.4%±1.8% vs 26.5%±2.9% for AMK-s isolate (1:10; *P*<0.01, Fig. 1B); from 9.3%±0.5% vs 16.5%±1.6% for CAZ-r isolate (1:20; *P*<0.01, Fig. 2A) to 18%±4.1% vs 22%±2.5% for CAZ-s isolate (1:10; ns, Fig. 2B); from 14%±2.6% vs 10%±1.6% for CIP-r isolate (1:20; ns, Fig. 3A) to 10%±1.1% vs 22%±5.4% for CIP-s isolate (1:10; *P*<0.01, Fig. 3B)]. In general, for both resistant and susceptible isolates, the biofilm damage effected by PMNs ranged from 9% to 14% at 1:20 E:T ratio and from 10% to 18% at...
1:10 E:T ratio (Fig. 1A-3B). By comparison, the damage effected by PMNs against planktonic cells ranged from 11% to 23% at 1:20 ratio and from 20% to 32% at 1:10 ratio (Fig. 1-3).

Damage of *P. aeruginosa* isolates caused by the combined treatment with PMNs and antimicrobial agents.

a) Combined effect of PMNs with AMK. The simultaneous treatment of AMK-r biofilms and planktonic cells with the combination of PMNs and AMK exhibited several synergistic effects (biofilms: 1:20 with 2 mg/l and 1:10 with 8-32 mg/l; planktonic cells: 1:20 with 2 mg/l and 8 mg/l and 1:10 with 8 mg/l; P<0.01, Fig. 1). Likewise, the effects of PMNs combined with AMK against biofilms (1:20 and 1:10 with 8 mg/l) or planktonic cells (1:10 with 2 mg/l) of the AMK-s isolate showed synergism (P<0.01; Fig. 1B). Additivity was observed against biofilms and planktonic cells of AMK-r and AMK-s isolates (P<0.01; Fig. 1A and 1B); whereas, no antagonistic interactions were shown for AMK.

b) Combined effect of PMNs with CAZ. This antibiotic showed additive interactions with most combinational treatments against both cell forms of *P. aeruginosa* CAZ-r and CAZ-s isolates (P<0.01; Fig. 2A and 2B). However, under biofilm conditions, the combination of CAZ (2 and 8 mg/l) with PMNs (1:20) was antagonistic for CAZ-r and CAZ-s isolates (Fig. 2A and 2B). Similarly, for planktonic cells, the combinations of CAZ (8 and 32 mg/l) with PMNs (1:20) showed antagonism for the corresponding susceptible isolates (Fig. 2B).

c) Combined effect of PMNs with CIP. Synergistic effects were observed when planktonic cells of CIP-r isolate were treated with the combination of PMNs (1:20 and 1:10) with CIP (2 mg/l; P<0.01, Fig. 3A). CIP demonstrated additive effects with PMNs against planktonic cells (P<0.01; Fig. 3A and 3B), whereas biofilms were resistant to most combinational treatments. Additionally, for both cell forms antagonistic interactions were
observed. Under biofilm conditions, the combination of CIP (32 mg/l for CIP-r isolate and 2-8 mg/l for CIP-s isolate) with PMNs (1:20 and 1:10 for CIP-r and CIP-s isolates was antagonistic (Fig. 3A and 3B). Under planktonic conditions, the combination treatment of CIP (2 mg/l) with PMNs (1:20) showed antagonism for the susceptible isolate (Fig. 3B). Taken together, statistically significant antagonism leading to bacterial growth was shown for selective combinations of CAZ and CIP with PMNs, but not for AMK.

Results of the combined studies showed that all combinations of AMK with PMNs produced either synergism or additivity at damaging biofilms and planktonic cells of the corresponding isolates. In contrast, 83% and 58% of CAZ and CIP combinations, respectively, showed synergistic or additive effects at damaging cells of *P. aeruginosa* isolates. The maximum biofilm damage of the combinations tested was observed with PMNs at 1:10 E:T ratio combined with 32 mg/l of the following antibiotics: 48% for AMK against AMK-r and AMK-s isolates (Fig. 1A and 1B); 31% and 60% for CAZ against CAZ-r and CAZ-s isolates, respectively (Fig. 2A and 2B); 41% and 69% for CIP against CIP-r and CIP-s isolates, respectively (Fig. 3A and 3B). Of the three *P. aeruginosa* resistant isolates studied, high-level of resistance was exhibited by biofilms and planktonic cells of AMK-r and CAZ-r isolates to corresponding antibiotics (128 mg/l), followed by the CIP-r isolate to CIP (64 and 32 mg/l, respectively).

**Discussion**

In this study we demonstrated that all combinations of AMK with PMNs resulted in synergistic or additive effects against both planktonic cells and biofilms of *P. aeruginosa* isolates as compared to each component alone. More than 75% of CAZ combinations exhibited additivity against biofilms of *P. aeruginosa* isolates; whereas, CIP was mostly either antagonistic or not interactive with PMNs against biofilms of *P. aeruginosa*. To the
best of our knowledge, this is the first report on the simultaneous interactions of antipseudomonal antibiotics with human PMNs against resistant and susceptible *P. aeruginosa* isolates collected from sputa of CF patients.

Of note, the concentrations shown to have an interactive effect in our study (2-32 mg/l, Figures 1-3) are below or around the ranges of concentrations achieved by iv administration and especially by inhalation of these antipseudomonal agents to CF or non-CF patients. For example, after iv infusion of 300 mg/m$^2$ AMK to healthy adults the peak concentration was 52.4 mg/l (25). In CF patients doses of oral CIP ranging from 500-1000 mg yielded peak serum concentrations between 2.8, and 4.6 mg/l (26). In addition, iv dosing of 200 mg CIP bid resulted in peak serum level of 4.9±2.9 and in bronchial mucosa 21.6±5.6 mg/l (27). AMK 30 mg/kg/day iv once daily has given peak serum concentrations 116±37 mg/l and sputum concentrations 5.9±2.7 mg/l; whereas, ceftazidime 200 mg/kg/day as a continuous infusion has given steady state serum levels 56±23 mg/l (28). On the other hand, during inhalation of antibiotics high concentrations are achieved in respiratory tract alveolar macrophages and epithelial lining fluid (ELF). For example, during administration of nebulized AMK 400 mg bid to mechanically ventilated patients, a median concentration of 976 mg/l of ELF was achieved (29). In addition, when liposomal CIP was given intranasally to rats with pneumonia 387±11 mg/l was achieved in alveolar macrophages and 55±3 mg/l in ELF; whereas, when CIP alone was administered by the same route the respective concentrations were 44±3 and 20±1 mg/l (30).

Microorganisms in the form of biofilms are generally resistant to antimicrobial agents. The same is true for *P. aeruginosa*. The intrinsic resistance of biofilm lifestyle to antipseudomonal chemotherapy maybe due to several factors including the secretion of extracellular matrix that compromises antibiotic penetration, neutralizes antimicrobial agents through metal chelation or limits oxygen and nutrient availability (18, 31).
In the antibiotic-pathogen-host interaction, antibacterial agents may affect pathogen viability either directly or indirectly by passive diffusion or modulation of the phagocytes’ antibacterial responses. Sub-inhibitory concentrations of certain antibiotics have been shown to alter the cell wall morphology and the expression of structural and soluble virulence factors in such a manner that the pathogen becomes more susceptible to the action of phagocytes (26, 27). In the context of the triple interaction, phagocyte-mediated alterations of pathogen metabolism or cell structure may lead to increased or decreased susceptibility of the pathogen to the antibacterial effect of the antibiotic (32, 33).

As demonstrated in this study, after 24 h of incubation, PMNs exhibited minimal damage against biofilms of *P. aeruginosa* isolates. Moreover, PMN-induced damage was E:T ratio-dependent. Our results are consistent with previous reports that the presence of PMNs triggers biofilms to release toxic components that compromise the phagocytic activity of PMNs, resulting in failure of immune cells to protect against bacterial biofilms (9, 10, 34). Nevertheless, several reports have shown that, after PMNs come in contact with biofilm cells, they may not exhibit significant motility but they are able to mount a respiratory burst, degranulate and engage in phagocytosis (8, 35, 36).

A previous study on the effect of AMK on its killing capacity of planktonic *P. aeruginosa* cells has reported that sub-inhibitory (1/4x MIC and 1/2x MIC) concentrations of aminoglycosides decrease growth of *P. aeruginosa* (37). In addition to their inhibitory effect on protein synthesis, aminoglycosides can disturb the normal structure of *P. aeruginosa* lipid bilayer displacing anionic lipopolysaccharides by means of their electropositivity. In this way, these cells are killed by a combination of aberrant protein production and cell lysis (38, 39).

In our study, AMK showed concentration-dependent killing for planktonic and biofilm-grown *P. aeruginosa* cells. That the combination of AMK with PMNs produced synergistic or additive effects suggests that both AMK and PMNs contributed to such an effect. Although
the relative contributions of each component cannot be inferred, the pro-oxidant activity of AMK has been demonstrated in time-dependent cellular experiments in which low concentrations of the drug caused enhanced hydrogen peroxide release by human PMNs (40). Nevertheless, the maximum biofilm damage induced by the combined action of amikacin (32 mg/l) with PMNs (1:10) was not higher than 48%, confirming the resistant phenotype of P. aeruginosa biofilms. However, an additional point that is a contributing factor in resilience of P. aeruginosa biofilms is the low permeability of aminoglycosides through the mucoid exopolysaccharide matrix (41, 42). The high concentrations of aerosolized aminoglycoside in treatment of P. aeruginosa infections in CF patients may overcome this low impermeability.

Several antibiotics with different chemical structures and mechanisms of action have the capacity to activate or repress a great number of genes, including those involved in virulence and metabolic processes, when P. aeruginosa cells are exposed to low concentrations of these antibiotics; such capacity is distinct from their known inhibitory activity (43). Sub-inhibitory levels of antibiotics may even enhance biofilm formation, but this does not pertain to this study, as each antibiotic was added after mature biofilm was formed. Investigations have shown that sub-inhibitory concentrations of CAZ, apart from blocking cell wall synthesis, exhibit strong quorum sensing inhibitory activity that leads to decreased production of several virulence factors. CAZ inhibits the production of protease, elastase, chitinase and rhamnolipids released by P. aeruginosa to protect biofilms against the action of antibiotics and the oxidative metabolites of PMNs (44-46). Furthermore, Labro et al., showed that sub-inhibitory concentrations of CAZ induced higher PMN oxidative burst against P. aeruginosa than untreated bacteria and this was due to alterations in bacterial structure (47). Findings of previous investigations could partially explain the additive interactions we observed by most CAZ concentrations in combination with PMNs against biofilm and planktonic cells of P. aeruginosa isolates.
In this study, CIP was found to interact synergistically or additively with both E:T ratios of PMNs against planktonic cells of *P. aeruginosa* isolates. The significant interactions we observed between CIP and PMNs are consistent with earlier reports shown that quinolones and in particular CIP kill *P. aeruginosa* either directly, by disrupting the regulatory mechanisms that control cell morphology and production of certain virulence factors (14, 48), or indirectly by entering PMNs and killing the bacteria intracellularly (49). However, biofilm-grown cells have shown variable results in this study and other reports: although we observed a concentration-dependent effect for *P. aeruginosa* biofilm cells exposed to CIP, the majority of the combinations of CIP and PMNs produced either non-significant or antagonistic interactions. Contradicting results on CIP activity against biofilms of *P. aeruginosa* have been reported by two studies on the secretion of proteases by these bacteria exposed to CIP. Oldak et al., (50) found that secretion of proteases continued even after exposure to CIP for 4 days, supporting bacterial growth through the supply of nutrients, whereas Skindersoe et al., (45) reported that CIP decrease the expression of several virulence factors, including proteases, in *P. aeruginosa* with the concomitant decrease in bacterial growth. Furthermore, a recent study showed that CIP induced the production and release of hydroxyl radicals by *P. aeruginosa* biofilms, which may be a contributing factor to the killing of cells embedded in biofilm (51). Although the exact mechanisms for the observed antagonism cannot be concluded from our study, we hypothesize that the oxidative burst of PMNs as they come in contact with bacteria may interfere with the release of hydroxyl radicals induced by CIP or with other functions of the antibiotic causing an antagonistic effect or non-significant interaction to take place. As ciprofloxacin acts on topoisomerase and DNA gyrase of replicating bacteria, the lower replication rate of *P. aeruginosa* within biofilms also may contribute to the lack of synergistic or additive interaction.
In conclusion, sub-inhibitory concentrations of AMK combined with PMNs significantly damage biofilms of *P. aeruginosa* resistant and susceptible strains, while combination of ceftazidime with polymorphonuclear leukocytes follow in order of efficacy. However, CIP interacts either antagonistically with PMNs or shows an insignificant result for most of the combined treatments against the corresponding biofilm cells. Further animal studies are needed to extend these *in vitro* findings and test the impact of biofilm production by *P. aeruginosa* on AMK susceptibility and explore host-antibiotic interactions *in vivo*. 
Acknowledgements

Potential conflicts of interest. E.R. has received research grant support from Pfizer, Gilead, Enzon, Schering, Wyeth, has served as consultant to Schering, Gilead, Astellas Gilead, Cephalon, Pfizer and has been in the speakers’ bureau of Wyeth, Schering, Merck, Aventis, Astellas. T.J.W is a Scholar of the Henry Schueler Foundation, and a Scholar of Pediatric Infectious Diseases of the Sharp Family Foundation; he receives support from the Save Our Sick Kids Foundation, as well as research grants for experimental and clinical antimicrobial pharmacotherapeutics from Astellas, Cubist, Novartis, Merck, ContraFect, and Pfizer, and has served as consultant to Astellas, ContraFect, Drais, iCo, Novartis, Pfizer, Methylgene, SigmaTau, and Trius. The remaining authors have no relevant disclosures.
References


tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.


Figure legends

Figure 1

Bacterial damage of *P. aeruginosa* AMK-r (A) and AMK-s (B) isolates induced by the co-incubation with PMNs and AMK for 24 h. Bacterial damage of planktonic cells and biofilms was assessed by XTT assay. Data are presented as means±SE of % damage of planktonic cells or biofilms for all experiments of each of the six clinical isolates. Asterisks demonstrate significant differences between planktonic cells and biofilms for the indicated concentrations of PMNs alone, drug alone or their combination (*, *P*<0.05; **, *P*<0.01). The letter symbols show significant differences for the indicated combination of PMNs with AMK (s, synergism; a, additivity).

Figure 2

Bacterial damage of *P. aeruginosa* CAZ-r (A) and CAZ-s (B) isolates induced by the co-incubation with PMNs and CAZ for 24 h. Bacterial damage of planktonic cells and biofilms was assessed by XTT assay. Data are presented as means±SE of % damage of planktonic cells or biofilms for all experiments of each of the six clinical isolates. Asterisks demonstrate significant differences between planktonic cells and biofilms for the indicated concentrations of PMNs alone, drug alone or their combination (*, *P*<0.05; **, *P*<0.01). The letter symbols show significant differences for the indicated combination of PMNs with CAZ (s, synergism; a, additivity; n, antagonism).

Figure 3

Bacterial damage of *P. aeruginosa* CIP-r (A) and CIP-s (B) isolates induced by the co-incubation with PMNs and CIP for 24 h. Bacterial damage of planktonic cells and biofilms was assessed by XTT assay. Data are presented as means±SE of % damage of
planktonic cells or biofilms for all experiments of each of the six clinical isolates. Asterisks demonstrate significant differences between planktonic cells and biofilms for the indicated concentrations of PMNs alone, drug alone or their combination (**, P<0.01). The letter symbols show significant differences for the indicated combination of PMNs with CIP (s, synergism; a, additivity; n, antagonism).
aMICs (mg/l) for both planktonic cells and biofilms were determined as the lowest antibiotic concentration at which a prominent decrease in turbidity was observed colorimetrically, corresponding to 50% bacterial damage as compared to untreated controls.

b: resistant *P. aeruginosa* strain; s: susceptible *P. aeruginosa* strain.

### Table 1. MICs of antibiotics for planktonic cells and biofilms of *P. aeruginosa* isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Antibiotic</th>
<th>Amikacin</th>
<th>Ceftazidime</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMK-r(^b)</td>
<td>AMK-s(^b)</td>
<td>CAZ-r</td>
<td>CAZ-s</td>
</tr>
<tr>
<td>Planktonic cells</td>
<td>128</td>
<td>8</td>
<td>128</td>
<td>4</td>
</tr>
<tr>
<td>Biofilms</td>
<td>128</td>
<td>32</td>
<td>128</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\)MICs (mg/l) for both planktonic cells and biofilms were determined as the lowest antibiotic concentration at which a prominent decrease in turbidity was observed colorimetrically, corresponding to 50% bacterial damage as compared to untreated controls.

\(^b\): resistant *P. aeruginosa* strain; s: susceptible *P. aeruginosa* strain.
Figure 1.
Figure 2

A. CAZ-r

B. CAZ-s

% Bacterial Damage

PMN (E/T ratio)  PMN+CAZ (mg/L)  CAZ (mg/L)
Figure 3

A. CIP-r

% Bacterial Damage

PMN (E:T ratio) 1:20 1:10 2 8 32 1:20+2 1:20+32 1:10+2 1:10+8 1:10+32
CIP (mg/L) PL BF

B. CIP-s

PMN (E:T ratio) 1:20 1:10 2 8 32 1:20+2 1:20+32 1:10+2 1:10+8 1:10+32
CIP (mg/L) PL BF