Effects of Azithromycin on Clinical Isolates of *Pseudomonas aeruginosa* From Cystic Fibrosis Patients*

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**ABSTRACT**

There is considerable interest in the use of azithromycin for the treatment of lung disease in patients with cystic fibrosis (CF). Although its mechanism of action as an inhibitor of bacterial protein synthesis has been well-established, it is less clear how azithromycin ameliorates the lung disease associated with *Pseudomonas aeruginosa*, which is considered to be resistant to the drug. We tested the effects of azithromycin on clinical isolates (CIs) from CF patients and compared them with laboratory reference strains to establish how this drug might interfere with the production of bacterial virulence factors that are relevant to the pathogenesis of airway disease in CF patients. Azithromycin inhibited *P aeruginosa* PAO1 protein synthesis by 80%, inhibiting bacterial growth and the expression of immunostimulatory exoproducts such as pyocyanin, as well as the gene products necessary for biofilm formation. In contrast, the effects of azithromycin on CIs of *P aeruginosa* were much more variable, due in large part to their slow growth and limited exoproduct expression. Culture supernatants for two of three clinical strains induced appreciable CXCL8 expression from cultured epithelial cells. Azithromycin treatment of the organisms inhibited 65 to 70% of this induction; azithromycin had no direct effect on the ability of either normal cells or CF epithelial cells to produce CXCL8. Azithromycin does decrease the *P aeruginosa* synthesis of immunostimulatory exoproducts and is likely to be most effective against planktonic, actively growing bacteria. This effect is less predictable against CIs than the prototypic strain PAO1.

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**Key words:** azithromycin; biofilms; CXCL8; cystic fibrosis; *Pseudomonas aeruginosa*; siderophores

**Abbreviations:** CF = cystic fibrosis; CI = clinical isolate; LB = Luria broth; PMN = polymorphonuclear leukocyte

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** Much of the pathology associated with cystic fibrosis (CF) pulmonary disease is due to excessive airway inflammation, the consequence of chronic infection and the ensuing polymorphonuclear leukocyte (PMN) response. Antimicrobial therapy that reduces the bacterial burden, usually *Pseudomonas aeruginosa* and/or *Staphylococcus aureus* infection, correlates with clinical improvement. Antiinflammatory therapies, such as ibuprofen, are also of benefit but are not widely utilized due to difficulties with administration and side effects.

While the macrolide antibiotics are not usually considered to have significant anti-*P aeruginosa* activity, they have been shown to have beneficial effects in ameliorating CF lung disease. These clinical trials were based on accumulated data from Japan demonstrating that a similar airway infection, panbronchiolitis, responded dramatically to macrolide therapy. One clinical trial has indicated that CF patients who were treated with azithromycin 3 days per week had improvement in FEV$_1$ and fewer pulmonary exacerbations compared with patients who received placebo. The improvement in lung function could not be directly correlated with bacterial eradication, suggesting indirect effects of azithromycin on the immunostimulatory capabilities of the *P aeruginosa* found in the airways of these patients and/or the direct effects on the host immune response.

Data from in vitro experiments have provided insights into the potential mechanism of action of macrolide antibiotics in CF patients. Although not active against *P aeruginosa* by conventional susceptibility testing, azithromycin has been shown to
decrease *P. aeruginosa* growth after prolonged incubation periods. More recent data suggest that azithromycin specifically inhibits the production of the *P. aeruginosa* exoproducts involved in biofilm production.12,13 Since *P. aeruginosa* is thought to adapt to the milieu within the lungs through quorum-sensing systems regulated by lasI and rhlI, this *in vitro* observation could be clinically significant.14 The effect of a macrolide antibiotic on decreasing secreted exoproducts could diminish the immunostimulatory potential of *P. aeruginosa* in the airways, even without cidal activity against the organisms.7 Macrolide drugs taken up by eukaryotic cells, as occurs with azithromycin, could also inhibit the ability of the host to respond to bacterial stimulation.5,15–17

In light of the clinical data supporting the use of azithromycin in CF patients,4–6 we sought to better characterize the effects of the drug against clinical isolates (CIs) of *P. aeruginosa* compared to a genetically defined reference strain, PAO1, and selected isogenic mutants. We selected CIs of *P. aeruginosa* from CF patients who were most likely to be treated with this drug (ie, those with long-standing infection who were no longer responding to conventional therapy).

We postulated that clinically achievable levels of azithromycin could inhibit the expression of *P. aeruginosa* exoproducts, which contribute to infection and the activation of host proinflammatory signaling.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

All strains were grown in Luria broth (LB) or M9 plus casamino acids. *P. aeruginosa* PAO1 was used as a prototype. This strain has been sequenced and is generally used as the reference strain for *P. aeruginosa* genetics. JP2 (PAO1 lasIrhlI), a quorum-sensing mutant, has been previously described.14 A poyoverdin null mutant, PAO1 pvdF, was obtained (M. Vasil, University of Colorado; Denver, CO)18 and was grown under selection as previously noted. *P. aeruginosa* CIs 49, 63, and 78 were multidrug-resistant isolates that were obtained from adolescent/young adult CF patients with longstanding infection during an acute pulmonary exacerbation that was not responding to conventional antibiotic therapy. The organisms were subcultured directly from sputum and were not stored. The patients were from geographically diverse CF centers. One of the three strains was originally mucoid. Azithromycin (Pfizer; Groton, CT) solutions were added to achieve concentrations that were comparable to those measured in the sputum of CF patients.5

For growth curves, bacteria were grown in LB. An overnight culture of bacteria grown in LB using a modification of the method published by Cox.19 The chloroform layer (1 mL) was removed, and an additional 1.5 mL of chloroform was added and extracted with 0.5 mL of 0.025 M HCl-H2O. The 0.5-mL aqueous layer was removed and 5 mL of 10 mM NaOH added. Aliquots were transferred to a 96-well plate, and absorbance at 690 nm was measured.

**Pyocyanin Production**

Pyocyanin was extracted with chloroform from a 5-mL aliquot of supernatant from an overnight culture grown in LB using a modification of the method published by Cox.19 The chloroform layer (1 mL) was removed, and an additional 1.5 mL of chloroform was added and extracted with 0.5 mL of 0.025 M HCl-H2O. The 0.5-mL aqueous layer was removed and 5 mL of 10 mM NaOH added. Aliquots were transferred to a 96-well plate, and absorbance at 690 nm was measured.

**Biofilm Assay**

Biofilm formation was monitored essentially as described by O’Toole et al.20 An overnight culture of bacteria grown in LB with and without 5 μg/mL azithromycin with shaking was diluted 1:100, and 100-μL aliquots were added to a 96-well plate, which was incubated for 72 h at 37°C to allow for the adequate growth of the CIs. After two to three washes with water, crystal violet was added for 15 min followed by three rinses with water then the addition of 95% ethanol. The material was then transferred to a fresh 96-well plate, and absorbance at 540 nm was determined. Each sample was tested in triplicate, and the assay was repeated on three separate occasions. Representative data are shown.

**Epithelial Cell Culture**

1HAEo-cells were grown in minimum essential medium with Earle salts supplemented with 10% fetal calf serum (Invitrogen), as previously described.21 16HBE cells, a human bronchial epithelial cell line stably expressing epoimes encoding CF transmembrane conductance regulator in the sense orientation (normal) or antisense orientation (CF) were obtained (P. Davis; Case Western Reserve University; Cleveland, OH) and grown as previously described.22

**CXCL8 Assays**

Confluent monolayers of 1HAEo-cells, which were weaned from serum overnight, were washed and stimulated with bacteria products were harvested by collecting supernatants from similarly grown cultures followad by centrifugation at 8,000 revolutions per minute for 15 min. The protein concentration of the supernatants was determined using a protein assay kit (Micro BCA Protein Assay Kit; Pierce; Rockford, IL).

**Protein Electrophoresis**

Equal volumes of the supernatant from each culture were used for electrophoresis on a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen; Carlsbad, CA) run in MES buffer. Separated protein was visualized using a stain (Simply Blue Stain; Invitrogen).
(1 × 10⁵ cfu/mL) or bacterial exoproducts (supernatants from 24-h cultures grown with and without 5 μg/mL azithromycin) for 30 min. Fresh minimum essential medium plus gentamicin (100 μg/mL) was added, and, after 3 h, supernatant was removed for CXCL8 enzyme-linked immunosorbent assay (R&D Systems; Minneapolis, MN) performed as previously described. Each data point was performed in quintuplicate and standardized by protein. A mean and SD were calculated, and the statistical significance was determined using a one-way analysis of variance with Bonferroni posttest (Graph Pad Instat, version 3.0: Graphpad Software; San Diego, CA) to test the null hypothesis that there was no difference in the amount of CXCL8 under each test condition, compared to the untreated control. Each experiment was performed at least three times, and a representative study is shown.

**Results**

**The Effects of Azithromycin on *P aeruginosa* Growth**

The effect of clinically achievable amounts of azithromycin (approximately 2.5 to 5 μg/mL) on the growth rates of PAO1, a quorum-sensing mutant, *lasIrhlI*, a pyoverdin mutant, *pvdF*, and three CIs were compared (Fig 1). Azithromycin at a concentration of 5 μg/mL began to affect PAO1 growth at the end of the log phase and similarly inhibited the growth of the mutants lacking either quorum sensing genes or siderophore expression. The CIs behaved differently. All three were noted to be auxotrophs, requiring amino acid supplementation, and none exhibited as rapid a logarithmic growth rate as that observed with the laboratory isolates. There was a modest effect of azithromycin decreasing growth by 0.5 logs, which was noted after 48 h of incubation, for two of the three clinical strains studied. Control experiments were done to document that the optical densities measured correlated with the number of colony-forming units per milliliter (data not shown).

Azithromycin Decreases Exoproduct Production

Many *P aeruginosa* exoproducts are expressed and secreted in late logarithmic and stationary phase of growth when the effects of azithromycin on growth

![Figure 1. The growth of *P. aeruginosa* in the presence of azithromycin. Laboratory strain PAO1, quorum-sensing mutant *lasIrhlI*, pyoverdin null mutant (*pvdF*), and CIs 49, 63, and 78 were grown in media alone (○) and in 5.0 μg/mL azithromycin (■), with the growth rates then assessed by optical density.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22028/)
were noted (Fig 2, top, A). The relative effect of azithromycin on exoproduct expression by the different strains was studied by comparing the proteins from equal aliquots of culture supernatants grown under identical conditions (Fig 2, bottom, B). There was considerable variation in both the amount and the patterns of exoproducts in the culture supernatants of the different strains. PAO1 exoproducts were clearly decreased by azithromycin treatment, and the total exoproduct protein was reduced to 20% of the untreated control cultures. The isogenic quorum-sensing mutant was much less affected. The effect of azithromycin on exoproduct expression by the clinical strains was more variable. CI 49 made few exoproducts to begin with, and these were minimally altered by exposure to azithromycin. In contrast, CI 78 exoproducts were substantially decreased by azithromycin, and CI 63 exhibited an intermediate response as its exported proteins were also inhibited by azithromycin, but to a lesser degree.

**Azithromycin Affects Pigment Production**

A very obvious effect of azithromycin on PAO1 exoproduct expression was a change in pigment production (Fig 3, top, A). Instead of the usual blue-green pigmentation of the liquid cultures, the azithromycin-treated cultures were yellow-brown. Visible pigments of PAO1 included the siderophores pyochelin, pyoverdin, and pyocyanin (N-methyl-1 hydroxyphenazine), which is not a siderophore, but a
redox-active phenazine. The amounts of pyoverdin and pyochelin, the major siderophores of P. aeruginosa, in the control and azithromycin-treated culture supernatants were not affected by azithromycin in either PAO1 or the lasIrhlI mutant. Siderophore production by the CIs was substantially less than that of PAO1, and was only minimally greater than that of the pvdF mutant (Fig 3, middle, B). To account for the notable change in pigment production, the amount of pyocyanin, which is also blue-green, was measured using a modification of the method described by Cox. Pyocyanin production was modestly inhibited by azithromycin in strains PAO1 and the pvdF mutant (Fig 3, bottom, C). There was significant inhibition of pyocyanin expression in one of the three CIs. The one strain that was not blue-green (CI 49) did not make appreciable amounts of pyocyanin.

**Azithromycin and Biofilm Production**

Azithromycin substantially blocked biofilm production by the wild-type strain PAO1 (Fig 4). However, we were surprised to find that none of the three CIs tested, including one that was originally phenotypically mucoid, made significant amounts of biofilm under the standard assay conditions, which made further inhibition by azithromycin undetectable.

**Azithromycin Inhibits CXCL8 Expression**

Many P. aeruginosa gene products evoke the epithelial secretion of CXCL8, the PMN chemokine that is thought to be especially important in the pathogenesis of CF lung disease. By decreasing the expression of exoproducts, azithromycin should also decrease the epithelial expression of CXCL8, which is induced by many P. aeruginosa gene products (Fig 5, top, A). For strain PAO1, there was a dose-response effect observed as increasing amounts of azithromycin resulted in sequentially less CXCL8 stimulation. Many of these gene products appear to be regulated by the lasIrhlI quorum-sensing system. For the clinical strains, the effect of azithromycin was variable. CI 49 was unable to stimulate CXCL8 production, thus any affect of azithromycin could not be measured. In contrast, the immunostimulatory capabilities of both CI 63 and CI 78 were significantly reduced by azithromycin (Fig 5, bottom, B).

**Azithromycin Does Not Inhibit Epithelial CXCL8 Production**

Although we expected that a macrolide antibiotic would inhibit bacterial protein synthesis, we also

![Figure 4. Azithromycin decreases biofilm formation. Biofilm formation by bacteria grown for 72 h with and without azithromycin is shown using the crystal violet assay and a reading absorbance at 540 nm. * = p < 0.001.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22028/)

![Figure 5. Azithromycin decreases CXCL8 production. Culture supernatants, harvested after 24 h of growth under untreated, media-alone conditions or in the presence of azithromycin, were used to stimulate confluent monolayers of iHAEO-human airway epithelial cells. Secreted CXCL8 was quantified by enzyme-linked immunosorbent assay and standardized to media alone control. * = p < 0.001. Top, A: the dose-dependent effect of azithromycin on the ability of PAO1 to stimulate CXCL8. Cells in media alone produced 2.9 pg/μg protein CXCL8. Bottom, B: the effect of 5.0 μg/mL azithromycin on airway epithelial CXCL8 production induced by PAO1, isogenic mutants, and CIs. Cells in media alone produced 0.7 pg/μg protein CXCL8.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22028/)
screened for the possibility that azithromycin accumulates in airway cells and inhibits their signaling capabilities. This may be especially relevant in CF, in which there is an allegedly "hyperstimulatory" phenotype. Airway epithelial cell lines, either normal or CF phenotypes, incubated for up to 10 days in the presence of azithromycin were studied (Fig 6). We did not detect any inhibition in endogenous CXCL8 expression (Fig 6, top, A) or subsequent bacterially induced CXCL8 expression (Fig 6, bottom, B).

Discussion

There have been numerous in vitro and in vivo studies demonstrating that macrolide antibiotics have clinically useful effects in patients with P. aeruginosa pulmonary infection. Randomized, placebo-controlled studies have indicated clinical improvement in CF patients receiving azithromycin without major adverse effects detected, as yet. In the studies reported herein, we have demonstrated that the effects of this macrolide antibiotic on P. aeruginosa PAO1 protein synthesis may help to explain the clinical benefits associated with azithromycin therapy. It has long been appreciated that macrolides have inhibitory effects on P. aeruginosa growth if the growth conditions permit the uptake of the antibiotic into the organism. Azithromycin does not act rapidly enough to stop the early stages of bacterial growth, but can interfere with protein synthesis in the late log to early stationary phases. Thus, the organisms are inhibited in their adaptive responses, such as biofilm formation and siderophore expression, as well as in the production of immunostimulatory exoproducts. As bacteria-induced airway inflammation is a major cause of pulmonary symptoms in CF patients, the ability of azithromycin to inhibit bacterial exoproduct accumulation and the resultant PMN recruitment could be reflected in improved lung function and fewer pulmonary exacerbations.

The predicted effects of azithromycin were most consistently observed with strain PAO1. Azithromycin inhibited the production of bacterial virulence factors that are expressed in late log phase, especially the products of the quorum-sensing system. These results were supported by studies with the lasI/rlhI mutant, specifically lacking the quorum-sensing regulatory genes, which was less immunostimulatory to begin with and was not susceptible to further azithromycin inhibition. Azithromycin decreased P. aeruginosa PAO1 biofilm expression and inhibited exoproduct secretion enough to diminish bacteria-induced epithelial CXCL8 expression.

The effects of azithromycin on unselected CIs of P. aeruginosa from CF patients with established lung disease were much less pronounced than those observed for the laboratory strain PAO1. In general, these clinical strains were "less fit," in that their growth rates were slower than that for PAO1; all were auxotrophs, requiring exogenous amino acids, and their already slow growth rates were only minimally diminished by azithromycin. They were also less metabolically active, secreting fewer exoproducts. Unexpectedly, in contrast to PAO1, none of the CIs was observed to form biofilms in vitro, and siderophore production was also minimal. As the production of siderophores is central to the expression of several P. aeruginosa exoproducts, the lack of siderophore production is consistent with the general attenuation of virulence factor production in these CIs. For the strains that actually synthesized...
exoproducts, azithromycin had the expected effects. However, many of the gene products expected to be clinically important in the pathogenesis of infection in the CF lung, were not made in vitro by these clinical strains. Azithromycin did have a substantial effect on pyocyanin expression, both in PAO1 and in two of the CIs. Pyocyanin is a redox-active compound that contributes to the oxidative stress in the lung and has also been shown to stimulate epithelial CXCL8 production. Thus, the observed effect of azithromycin in decreasing pyocyanin production could contribute to the diminished CXCL8 response observed.

It is important to recognize that the three CIs studied were obtained from typical adolescent/young adult CF patients who were not responding to conventional antimicrobial therapy being given at the time of a pulmonary exacerbation. These are often the patients who are treated with azithromycin in an attempt to provide novel therapy. In these chronically infected patients, much of the bacterial burden is expected to be in the form of biofilms, which are recalcitrant to antimicrobial therapy. However, there is a dynamic equilibrium between organisms within the biofilm and planktonic bacteria that break off and begin to replicate, behaving more like wild-type organisms with the expression of immunostimulatory exoproducts. Although our data suggest that these CIs are less active metabolically, there may be subpopulations of bacteria within the airways that are replicating, particularly during an acute pulmonary exacerbation, and are potential targets for azithromycin. These isolates from patients in geographically diverse areas of the country are unlikely to reflect the properties of all CIs, but they do provide data that are likely to be more clinically relevant than experiments based solely on the behavior of the laboratory standard PAO1. A more extensive analysis of CIs obtained from young CF patients from cultures early in the course of their lung disease may help to establish whether azithromycin should be targeted at patients in younger age groups.

The studies presented provide limited in vitro support for the observed clinical benefits of azithromycin treatment in CF patients, as based on the expected activities of a macrolide antibiotic in inhibiting bacterial protein synthesis. Since we were unable to detect any direct effects of the drug on the epithelial cells themselves, the inhibition of *P. aeruginosa* exoprotein expression and the subsequent induction of CXCL8 production is likely to contribute to the clinical benefits associated with azithromycin therapy in CF patients. There was a substantially greater effect of the drug against the more immunostimulatory strains. This implies that azithromycin would have the greatest effect against early isolates of *P. aeruginosa* in CF, those that are more like PAO1, and environmental strains, than against the strains from patients with longstanding infection, as were studied here. Azithromycin may also target the planktonic organisms that emerge from the bacterial biomass but may not be well-represented in sputum cultures. It remains to be rigorously established whether azithromycin also has clinically relevant activity on immune cells, perhaps affecting the responses of macrophages or leukocytes that are recruited to the lung.

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