Moxifloxacin but not ciprofloxacin or azithromycin selectively inhibits IL-8, IL-6, ERK1/2, JNK, and NF-κB activation in a cystic fibrosis epithelial cell line

Hannah Blau, Keren Klein, Itamar Shalit, Drora Halperin, and Ina Fabian

1Pulmonary Unit and Grub Cystic Fibrosis Center, Schneider Children’s Medical Center of Israel, Petah Tikva; 2Department of Cell and Developmental Biology, Sackler School of Medicine, Tel Aviv University, Tel Aviv; 3Infectious Disease Unit, Schneider Children’s Medical Center of Israel, Petah Tikva; and 4Pediatric Department, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

Submitted 21 January 2006; accepted in final form 18 July 2006

Blau H, Klein K, Shalit I, Halperin D, Fabian I. Moxifloxacin but not ciprofloxacin or azithromycin selectively inhibits IL-8, IL-6, ERK1/2, JNK, and NF-κB activation in a cystic fibrosis epithelial cell line. Am J Physiol Lung Cell Mol Physiol 292: L343–L352, 2007.—Cystic fibrosis (CF) is associated with severe neutrophilic airway inflammation. We showed that moxifloxacin (MXF) inhibits IL-8 and MAPK activation in monocytic and respiratory epithelial cells. Azithromycin (AZM) and ciprofloxacin (CIP) are used clinically in CF. Thus we now examined effects of MXF, CIP, and AZM directly on CF cells. IB3, a CF bronchial cell line, and corrected C38 cells were treated with TNF-α, IL-1β, or LPS with or without 5–50 μg/ml MXF, CIP, or AZM. IL-6 and IL-8 secretion (ELISA), MAPKs ERK1/2, JNK, p38, and p65 NF-κB (Western blot) activation were measured. Baseline IL-6 was sixfold higher in IB3 than C38 cells but IL-8 was similar. TNF-α and IL-1β increased IL-6 and IL-8 12- to 67-fold with higher levels in IB3 than C38 cells post-TNF-α (P < 0.05). Levels were unchanged following LPS. Baseline phosphorylated form of ERK1/2 (p-ERK1/2), JNK, and NF-κB p65 were higher in IB3 than C38 cells (5-, 1.4-, and 1.4-fold), and following TNF-α increased, as did the p-p38, by 1.6- to 2-fold. MXF (5–50 μg/ml) and CIP (50 μg/ml), but not AZM, suppressed IL-6 and IL-8 secretion by up to 69%. MXF inhibited TNF-α-stimulated MAPKs ERK1/2, 46-kDa JNK, and NF-κB up to 60%, 40%, and 40%, respectively. In contrast, MXF did not inhibit p38 activation, implying a highly selective pretranslational effect. In conclusion, TNF-α and IL-1β induce an exaggerated inflammatory response in CF airway cells, inhibited by MXF more than by CIP or AZM. Clinical trials are recommended to assess efficacy in CF and other chronic lung diseases.

inflammation; quinolones; immunomodulation; airway epithelium

Cystic Fibrosis (CF) is the most common life-shortening genetic disease in Caucasian populations. It is due to a mutation in the CF transmembrane regulator protein (CFTR), which is responsible for regulation of chloride and sodium transport across epithelial membranes (33). CF is characterized by chronic neutrophilic inflammation with resultant production of oxidants and proteases (17). This has been demonstrated in vivo (7, 9, 24, 26) as well as in studies on CF epithelial cells in culture (4, 6, 28). The ongoing inflammation causes destruction of the connective tissue in airway walls and lung parenchyma with cystic bronchiectases and eventual respiratory failure (17). The inflammatory response in CF may be initiated by chronic infection, particularly with Pseudomonas aeruginosa (28), which occurs within small airways, occasionally even in infancy (24).

Central to this inflammatory response is an increased level of chemotactic cytokines, particularly IL-8. It is associated with an increase in activation of intracellular signaling molecules, including the mitogen-activated protein kinase (MAPK) ERK1/2, as well as the transcription factor, NF-κB (30, 47, 49).

There is ongoing controversy regarding the possibility of an intrinsically exaggerated inflammatory response in CF. Recent studies (29, 42) suggest that this is directly associated with the mutation in CFTR, although the exact mechanism remains unknown. Others (2) show considerable variability and that CF airway epithelial cells in primary culture do not exhibit significant dysregulation of inflammation. Possible reasons for these inconsistencies include different culture conditions, variations between cell lines and primary cultures, different stimuli, and differing measures of the inflammatory response.

Clearly, however, there is an urgent need for anti-inflammatory therapeutic strategies in CF. Clinical studies suggest that macrodilates, and in particular azithromycin (AZM), may have anti-inflammatory activities beyond their known antimicrobial effects (3, 37, 40, 45). The mechanisms of these anti-inflammatory effects are not known. Although sputum and lavage specimens have decreased levels of inflammatory cytokines, no in vivo studies have shown the mechanism whereby proinflammatory cytokines are downregulated during treatment.

Quinolones, and particularly ciprofloxacin (CIP), have long been used as antimicrobial therapy in CF, aiming to suppress infection with P. aeruginosa (35). Several studies (10, 15, 39) have shown that fluoroquinolones also possess immunomodulatory properties beyond their antimicrobial effects.

We (38, 50) previously showed that the quinolone moxifloxacin (MXF) inhibits IL-8 and TNF-α, preventing neutrophilic pneumonitis in immuno suppressed mice as well as inhibiting IL-8 secretion by activated human monocytes. In addition, we (51) showed that MXF inhibits ERK1/2 activation and NF-κB nuclear translocation in a lung epithelial cell line.

In the present study, we therefore compared the anti-inflammatory effects of MXF, CIP, and AZM directly on CF bron-
chial epithelial cells (IB3) and a stably rescued bronchial cell line (C38).

MATERIALS AND METHODS

IB3 and C38 Cell Lines

The IB3 human CF bronchial epithelial cell line (mutations ΔF508/ W128X) and the stably rescued cell line C38 (American Type Culture Collection; P. Zeitlin, Johns Hopkins University) containing functional CFTR were cultured in Laboratory of Human Carcinogenesis basal medium #8 (LHC-8) (BioSource, Camarillo, CA) with 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂.

IL-8 and IL-6 Production Assays

IB3 and C38 cells were plated at a concentration of 1 × 10⁶/ml for 24 h, then serum-starved for 24 h, followed by addition of either TNF-α (PeproTech, Rocky Hill, NJ), IL-1β (R&D Systems, Minneapolis, MN), or LPS from P. aeruginosa (Sigma Chemical, St. Louis, MO). Cells were then incubated for a further 48 h. The following drugs were added at concentrations of 5–50 μg/ml MXF or CIP (both from Bayer, Wuppertal, Germany) dissolved in H₂O or AZM (Pfizer, Groton, CT) dissolved in DMSO. In all studies of AZM, effects were compared with control cells with DMSO vehicle alone. Cells were then incubated for another 48 h.

Cell-free supernatants were recovered, and the concentrations of IL-8 and IL-6 were determined using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems). The sensitivity of the assay for IL-8 is >10 pg/ml and for IL-6 is >5 pg/ml.

Western Blot Analysis of ERK1/2, JNK, and p38

IB3 and C38 cells were incubated with 50 ng/ml TNF-α in the presence or absence of 5–20 μg/ml MXF, CIP, and AZM for 5–60 min. Following incubation, the cells were collected on ice, washed twice with ice-cold phosphate buffered saline (PBS), and suspended in 40 μl of the lysis buffer; 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.6% Nonidet P-40, 1 mM Na₃VO₄, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM p-nitrophenyl phosphate, and 1:25 complete mini protease inhibitor cocktail (Boehringer, Mannheim, Germany), as previously described by us (50). After being kept on ice for 30 min, the lysates were subjected to centrifugation (20,000 g) at 4°C for 15 min to obtain a cytosolic fraction. The protein concentration was determined by a Bradford assay (Bio-Rad, Munich, Germany) before storage at −70°C. An aliquot of the cytosol fraction containing 35 μg of protein for ERK1/2 and 50 μg of protein for JNK and p38 was resolved by 10% SDS-polyacrylamide gel. After electrophoresis and electrophoretic transfer of proteins to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), the membranes were blocked with 3% nonfat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween (TBST) for 1 h. Membranes were then rinsed three times in TBST and incubated at room temperature (RT) with mouse monoclonal anti-MAPK, activated diphosphorylated ERK-1/2, phospho-p44/42 MAPK antibody (Ab) (Sigma Chemical), anti-phospho-JNK (Ab detects JNK only when activated by phosphorylation at Thr183/Tyr185; 1:20,000 and 1:1,000 dilution, respectively; New England Biolabs, Beverly, MA), and anti-phospho-p38 Ab (Ab detects p38 MAPK only when activated by dual phosphorylation at Thr180/Tyr182; 1:700 dilution) for 1 h. As positive and negative controls for JNK phosphorylation we used 10 μl of phosphorylated and nonphosphorylated SAPK/JNK Control Cell Extracts (Cell Signaling Technology, Danvers, MA). Actin levels were also assessed as a loading control using an Ab (Santa Cruz Biotechnology, Santa Cruz, CA) that reacts with a broad range of actin isoforms. The blots were then incubated with a secondary Ab, horseradish peroxidase-linked anti-mouse IgG (Santa Cruz Biotechnology) for 1 h. After 1 h at RT and three washes in TBST, blots were incubated in enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech). Relative density values of ERK1/2, JNK, and p38 were determined by densitometric analysis followed by photographing the specific bands (Kodak XLS-1 film).

Western Blot of Nuclear Extracts for the p65 Fraction of NF-κB

To assess activation of NF-κB, nuclear extracts were analyzed by Western blot for the p65 fraction of NF-κB. Following incubation, the cells were collected on ice before isolation of nuclear extracts as previously described by us (50). The samples were subjected to three cycles of freezing and thawing, and the suspended nuclei were centrifuged (12,000 g) for 10 min at 4°C. The supernatants were stored at −20°C after measurement of their protein content using the Bio-Rad protein assay kit. An aliquot of the nuclear fraction containing 45 μg of protein for NF-κB was resolved by 10% SDS-polyacrylamide gel, and NF-κB was detected with anti-NF-κB p65 rabbit polyclonal Ab (diluted 1:500; Santa Cruz Biotechnology). Actin levels were assessed as a loading control. The blots were then incubated with a secondary Ab, horseradish peroxidase-linked anti-rabbit IgG (Santa Cruz Biotechnology).

Statistics

Results of ELISA studies and of densitometric quantification of various Western blots before and after stimulation with TNF-α, as well as of blots stimulated with TNF-α with or without MXF, CIP, or AZM, were compared using the two-tailed Student’s t-test analysis for paired differences (P ≤ 0.05 level of significance).

RESULTS

IL-8 Secretion

Stimulation with TNF-α. In preliminary experiments, there was a dose-dependent increase of IL-8 secretion using TNF-α of 30 and 50 ng/ml with a slight decrease at higher concentrations. Therefore, 50 ng/ml TNF-α was used in further experiments. IL-8 secretion by unstimulated IB3 and C38 cells was 54 ± 19 pg/ml and 52 ± 20 pg/ml, respectively (means ± SE; n = 8). Following incubation of the cells with 50 ng/ml TNF-α, IL-8 secretion increased to 3,154 ± 451 pg/ml and 1,850 ± 321 pg/ml by IB3 and C38 cells, respectively (Fig. 1A), thus indicating that IL-8 level following TNF-α was 1.7-fold higher in IB3 cells compared with C38 cells (P < 0.001). Cells exposed to MXF showed a dose-dependent inhibition of IL-8 secretion of up to 64% ± 3% in IB3 cells (means ± SE; n = 13; P < 0.009) (Fig. 1B) and 69% ± 5% in C38 cells (means ± SE; n = 11; P < 0.03) (Fig. 1C). In both IB3 and C38 cells, CIP caused significant inhibition of IL-8 secretion only at the highest dose of 50 μg/ml (Fig. 1, B and C). There was no significant inhibition by AZM in IB3 (Fig. 1B) or C38 cells (Fig. 1C).

Stimulation with IL-1β or LPS. We chose to use 5 ng/ml IL-1β, a commonly used concentration (4) shown in preliminary dose-response experiments using 0.1–10 ng/ml to be just beyond the beginning of the plateau response level in both IB3 and C38 cells (data not shown). IL-8 secretion by unstimulated IB3 and C38 cells was 199 ± 25 pg/ml and 245 ± 142 pg/ml, respectively (means ± SE; n = 3). Following incubation of the cells with 5 ng/ml IL-1β, IL-8 secretion increased to 5,011 ± 2,191 pg/ml and 2,838 ± 994 pg/ml by IB3 and C38 cells, respectively, thus indicating that IL-8 level following IL-1β
was 1.8-fold higher in IB3 cells compared with C38 cells (P < 0.03). Addition of MXF, CIP, or AZM at doses of 10–50 
mg/ml did not cause inhibition of IL-8 secretion.

There was no change in levels of IL-8 secretion in either IB3 or C38 cells following exposure to 100 or 1,000 ng/ml LPS, which are commonly used concentrations of this inflammatory stimulus (31).

IL-6 Secretion

Stimulation with TNF-α. IL-6 secretion by unstimulated IB3 and C38 cells was 825 ± 223 pg/ml and 129 ± 25 pg/ml, respectively (means ± SE; n = 3). Following incubation of the cells with 50 ng/ml TNF-α, IL-6 secretion increased to 13,284 ± 1,250 pg/ml and 5,679 ± 812 pg/ml by IB3 and C38 cells, respectively, (Fig. 2A), thus indicating that IL-6 level following TNF-α was 2.3-fold higher in IB3 cells compared with C38 cells (P < 0.005). Cells exposed to MXF showed a dose-dependent inhibition of IL-6 secretion of up to 55% in IB3 cells (means ± SE; n = 3) and 21% in C38 cells (means ± SE; n = 3) (Fig. 2B). Incubation with CIP resulted in inhibition of IL-6 secretion in IB3 cells (means ± SE; n = 3) and no significant inhibition of IL-6 secretion in C38 cells (Fig. 2C).

Fig. 1. IL-8 secretion by IB3 [a cystic fibrosis (CF) bronchial cell line] and C38 cells stimulated with TNF-α. A: effect of TNF-α stimulation. IL-8 secretion by IB3 and C38 cells cultured in Laboratory of Human Carcinogenesis basal medium #8 (LHC-8), serum-starved for 24 h, and incubated with 50 ng/ml TNF-α for a further 48 h. Results are expressed as means ± SE of 3 experiments performed in duplicate. *P < 0.001, TNF-α-stimulated IB3 cells vs. C38 cells. B: effect of drugs on IL-8 secretion. IB3 (B) and C38 (C) cells were incubated with TNF-α together with 5–50 μg/ml moxifloxacin (MXF), ciprofloxacin (CIP), or azithromycin (AZM) for 48 h, and percent inhibition of IL-8 secretion was determined by ELISA. Results are expressed as means ± SE of 3 experiments performed in duplicate. **P < 0.05, cells treated with TNF-α and drugs vs. TNF-α-stimulated cells.

Fig. 2. IL-6 secretion by IB3 and C38 cells stimulated with TNF-α. A: effect of TNF-α stimulation. IL-6 secretion by IB3 and C38 cells cultured in LHC-8 medium, serum-starved for 24 h, and incubated with 50 ng/ml TNF-α for a further 48 h. Results are expressed as means ± SE of 4 experiments performed in duplicate. *P < 0.05, TNF-α-stimulated IB3 cells vs. C38 cells. B: effect of drugs on IL-6 secretion. IB3 (B) and C38 (C) cells were incubated with TNF-α together with 5–50 μg/ml MXF, CIP, or AZM for 48 h, and percent inhibition of IL-6 secretion was determined by ELISA. Results are expressed as means ± SE of 3 experiments performed in duplicate. **P < 0.05, cells treated with TNF-α and drugs vs. TNF-α-stimulated cells.
cells to 5, 10, and 20 μg/ml MXF inhibited TNF-α-stimulated ERK phosphorylation by 58% ± 9% (P < 0.01), 36% ± 4% (P < 0.01), and 25% ± 6% (NS), respectively (n = 3) (Fig. 5, C and D; lanes 3, 4, and 5). As seen in the same figure, 5 and 10 μg/ml CIP significantly inhibited ERK phosphorylation by 41% ± 6% and 31% ± 6% (lanes 6 and 7) (P < 0.05), but inhibition was not significant at 20 μg/ml (lane 8). Inhibition was not dose dependent in either cell line. AZM did not inhibit ERK phosphorylation in IB3 cells (Fig. 5, E and F) or C38 at any dose (data not shown).

**JNK Phosphorylation Induced by TNF-α and the Effect of MXF, CIP, and AZM**

Preliminary studies showed that exposure of IB3 and C38 cells to TNF-α resulted in a time-dependent increase in phosphorylation of both the p46-kDa (JNK1) and p54-kDa (JNK2) isoforms of JNK, which was maximal at 15 min (data not shown). Thereafter, experiments on JNK activation were conducted following 15 min of exposure to TNF-α.

In unstimulated cells, JNK1 was not significantly different in IB3 compared with C38 cells, whereas phosphorylation of JNK2 was 2.3-fold higher in IB3 cells than C38 cells (P < 0.05) (Fig. 6, A and B). Following exposure to TNF-α, phosphorylation of the JNK1 increased by 2- and 2.5-fold in IB3 and C38 cells, respectively; effect of drugs vs. IL-1stimulated cells.

**ERK1/2 Phosphorylation Induced by TNF-α and Effect of MXF, CIP, and AZM**

We first performed time-dependent studies. Exposure of IB3 cells to 50 ng/ml TNF-α resulted in a time-dependent increase in ERK1/2 phosphorylation, which was maximal at 15 min (Fig. 4A). Findings were similar for C38 cells (data not shown). Therefore, experiments on ERK activation were conducted following 15 min of exposure to TNF-α.

ERK phosphorylation was fivefold higher in unstimulated IB3 cells compared with C38 cells (Fig. 4, B and C; lane 1 compared with 3, respectively; P < 0.05). Following exposure to TNF-α, ERK activation increased 1.4-fold in IB3 cells (Fig. 4, B and C; lane 2 compared with 1; P < 0.05) and 1.7-fold in C38 cells (Fig. 4, B and C; lane 4 compared with 3; P < 0.05). TNF-α-stimulated ERK phosphorylation remained markedly greater in IB3 than C38 cells (P < 0.005). Next we examined the effect of MXF, CIP, and AZM on TNF-α-induced ERK phosphorylation. Exposure of IB3 cells to 5, 10, and 20 μg/ml MXF inhibited TNF-α-stimulated ERK phosphorylation by 62% ± 12% (P < 0.05), 39% ± 10% (P < 0.05), and 21% ± 12% [not significant (NS)], respectively (n = 3) (Fig. 5, A and B; lanes 3, 4, and 5).

CIP (5 μg/ml) inhibited ERK phosphorylation by 31% ± 11% (lane 6), but inhibition was not significant at this or higher doses (lanes 7 and 8). Figure 5C shows that exposure of C38 cells to 5, 10, and 20 μg/ml MXF inhibited TNF-α-stimulated ERK phosphorylation by 58% ± 9% (P < 0.01), 36% ± 4% (P < 0.01), and 25% ± 6% (NS), respectively (n = 3) (Fig. 5, C and D; lanes 3, 4, and 5). As seen in the same figure, 5 and 10 μg/ml CIP significantly inhibited ERK phosphorylation by 41% ± 6% and 31% ± 6% (lanes 6 and 7) (P < 0.05), but inhibition was not significant at 20 μg/ml (lane 8). Inhibition was not dose dependent in either cell line. AZM did not inhibit ERK phosphorylation in IB3 cells (Fig. 5, E and F) or C38 at any dose (data not shown).

**Fig. 3.** IL-6 secretion by IB3 and C38 cells stimulated with IL-1.

(A) Effect of IL-1β stimulation. IL-6 secretion by IB3 and C38 cells cultured in LHC-8 medium, serum-starved for 24 h, and incubated with IL-1β for a further 48 h. Results are expressed as means ± SE of 3 experiments performed in duplicate. *P < 0.05. IL-1β-stimulated IB3 cells vs. C38 cells. B and C: effect of drugs on IL-6 secretion. IB3 (B) and C38 (C) cells were incubated with IL-1β together with 5–50 μg/ml MXF, CIP, or AZM for 48 h, and percent inhibition of IL-6 secretion was determined by ELISA. Results are expressed as means ± SE of 3 experiments performed in duplicate. **P < 0.05, cells treated with IL-1β and drugs vs. IL-1β-stimulated cells.

**Fig. 4.** Effect of TNF-α on the phosphorylation of ERK1/2. A: time-dependent studies. IB3 cells were preincubated for 24 h in serum-free medium followed by the addition of TNF-α (50 ng/ml) for the indicated times. Cytoplasmic extracts were prepared and subjected to Western blot (WB) using an antibody (Ab) specific for phosphorylated forms of ERK1/2 (p-ERK) and for total ERK (ERK).

B: a representative blot comparing ERK1/2 phosphorylation in IB3 (lanes 1 and 2) and C38 cells (lanes 3 and 4). Cells were preincubated for 24 h in serum-free medium followed by the addition of TNF-α (50 ng/ml) for 15 min (lanes 2 and 4) compared with control (lanes 1 and 3). C: densitometric analysis of p-ERK1/2 shown in B. Means ± SE of 3 experiments performed in duplicate. *P < 0.05 for each cell type following TNF-α stimulation.
Incubation of IB3 cells with 5 and 20 μg/ml MXF resulted in 24% ± 2% and 37% ± 1% inhibition of JNK1, respectively (Fig. 6, C and D; lanes 5 and 4; P < 0.05; n = 3). Phosphorylation of JNK2 was unchanged. Five and 20 μg/ml CIP (Fig. 6, C and D; lanes 5 and 6) and AZM (Fig. 6, E and F) did not inhibit JNK activation in IB3 cells.

Exposure of C38 cells to 5 and 20 μg/ml MXF inhibited TNF-α-stimulated JNK phosphorylation by 40.8% ± 3% and 27% ± 6%, respectively (P < 0.05; n = 3). Five and 20 μg/ml CIP 5 and 20 μg/ml AZM did not significantly inhibit JNK activation (results for C38 cells not shown).

p38 Phosphorylation Induced by TNF-α and Effect of MXF, CIP, and AZM

Preliminary studies showed that exposure to TNF-α resulted in an increase in p38 phosphorylation, which was maximal at 15 min (data not shown). Thereafter, experiments on p38 activation were conducted following 15 min of exposure to TNF-α. Figure 7B shows that p38 phosphorylation was not significantly different in unstimulated IB3 compared with C38 cells (lanes 1 and 3) and that TNF-α induced a twofold increase in both IB3 and C38 cells (Fig. 7, A and B; P < 0.05; n = 4).

Exposure of IB3 cells to MXF or CIP at doses of 5, 10, and 20 μg/ml did not inhibit TNF-α-stimulated p38 phosphorylation in IB3 cells (Fig. 7, C and D). Similarly, 5, 10, and 20 μg/ml AZM did not inhibit p38 phosphorylation in IB3 cells (Fig. 7, E and F). Neither the quinolones (MXF and CIP) nor AZM inhibited TNF-α-stimulated p38 phosphorylation in C38 cells (data not shown).

Activation of NF-κB p65 Induced by TNF-α and the Effect of MXF, CIP, and AZM

Incubation of IB3 cells with TNF-α significantly induced NF-κB nuclear translocation in a time-dependent manner peaking at 30–60 min (Fig. 8A). Thereafter, experiments were performed following exposure of the cells to TNF-α for 60 min. Similar kinetics were found for C38 cells.

We compared baseline levels of p65 NF-κB protein in IB3 and C38 cells. Figure 8, B and C (lanes 1 and 3), indicates that the level of NF-κB p65 protein was 1.5-fold higher in IB3 cells compared with C38 cells (P < 0.05; n = 3). Exposure to TNF-α resulted in a twofold increase in NF-κB p65 protein within nuclear extracts in IB3 cells and 1.7-fold in C38 cells. Thus nuclear p65 expression was significantly higher in IB3 cells compared with C38 cells (lanes 1 and 3).

Figure 8D shows that when IB3 cells were treated with MXF, there was a dose-dependent inhibition of expression of the p65 NF-κB protein within nuclear extracts. Figure 8E shows this to be 20% ± 7% (NS) and 46% ± 4% (P < 0.01) for 5 and 20 μg/ml MXF, respectively (lanes 3 and 4). However, neither CIP nor AZM inhibited expression of NF-κB.
p65 protein (lanes 5–8; n = 3). Similar results were found for C38 cells (data not shown).

**DISCUSSION**

Our present study demonstrates for the first time that MXF, but not CIP, except at the highest dose, or AZM significantly inhibited IL-6 and IL-8 secretion by the TNF-α/H9251-stimulated CF epithelial cell line IB3 and the CFTR-corrected C38 cell line. Similarly, MXF inhibited IL-6 secretion by IL-1β/H9252-stimulated IB3 and C38 cells. These findings have possible therapeutic implications for the inflammatory aspects of CF and other chronic lung diseases. As found by others in IB3 (42, 47) and other CF cell lines (48), we found that both IL-6 and IL-8 secretion was markedly increased following stimulation by TNF-α and IL-1β but not by LPS in both cell lines and that this was exaggerated significantly in IB3 cells compared with C38 cells.

Cytokine activation in CF epithelial cell lines as demonstrated by us and others (30, 47) can explain in part the exaggerated neutrophilic inflammatory response found within CF airways (26). Increased levels of IL-6, IL-8, TNF-α, and IL-1β are also found within CF airway fluids (7, 32). It may be that dysregulation of inflammation is intrinsic and not dependent on adherence of *P. aeruginosa* (29). This is supported by in vitro studies linking the phenomenon to the CFTR mutation (42, 49). However, the issue remains controversial. These studies were performed in cell lines and intrinsic dysregulation was not always demonstrated in primary CF epithelial cultures (2).

Both IL-6 and IL-8 are likely to play pivotal roles in amplifying or regulating the inflammatory response in CF. IL-8 is one of the most potent neutrophil chemoattractants, a founding member of the chemokine superfamily and a key cytokine in the pathogenesis of the neutrophil-dominated lung disease of CF (43). Although barely detectable in healthy tissues, it is rapidly induced by 10- to 100-fold in response to proinflammatory cytokines such as TNF-α or bacterial products (8). IL-6 is a proximal trigger, upregulating expression of the transcription factor NF-IL-6, which can enhance, maintain, or terminate the inflammatory response (18). IL-6 also increases neutrophil survival time (14).

In our study, IB3 and C38 cells were activated by TNF-α and IL-1β but not LPS. The lack of stimulation by LPS in airway epithelial cells is consistent with the findings of others (46) showing that there was no surface expression of Toll-like receptor 4 (TLR4) on airway epithelial cells, resulting in a lack of LPS responsiveness unless transfected with the TLR4 coreceptor MD2 (22). Only following an additional stimulus, such
as respiratory syncytial virus, was TLR4 upregulated and airway epithelial cells sensitized to endotoxin (31).

In initial experiments on signal transduction, we found that ERK1/2 and JNK phosphorylation as well as NF-κB were increased at baseline in IB3 cells compared with C38 cells, and these as well as p38 phosphorylation rose markedly following stimulation with TNF-α in both cell lines.

Recent advances indicate that several sites are involved in IL-8 and IL-6 gene regulation following inflammatory stimuli, including TNF-α. Induction of IL-8 requires derepression of the gene promoter and transcriptional activation by both the NF-κB and the activator protein-1 (AP-1) pathways. Finally, stabilization of IL-8 mRNA occurs by the p38 MAPK pathway (19). The MAPK JNK binds to the AP-1 site on the IL-8 promoter, whereas ERK1/2 independently stimulates both NF-κB and AP-1 pathways (20). This intricate mechanism enables both a rapid increase and fine-tuning of IL-8 secretion and subsequent attraction of leukocytes to the site of inflammation. Similarly, both NF-κB and JNK are essential for IL-8 expression (11, 27).

Consistent with our findings, MAPKs, including ERK1/2, JNK, and p38, are directly implicated in increased IL-8 expression in human airway epithelial cells exposed to Pseudomonas pyocyanea (16) or Burkholderia cepacia (36). In IB3 cells, NF-κB activation and ERK phosphorylation are independently associated with the exaggerated IL-8 response (30).

The key finding of this paper is that in both IB3 and C38 cells there was significant, consistent inhibition by MXF of IL-1β-stimulated IL-6 secretion and of TNF-α-stimulated IL-8 and IL-6 secretion as well as ERK1/2 and JNK phosphorylation and NF-κB expression. In contrast, there was no inhibition by MXF of p38 phosphorylation in either cell line, implying a highly selective effect of MXF on certain pathways of cytokine activation.

Incubation with CIP resulted in inhibition of IL-8 and IL-6 secretion only at the highest concentration of 50 μg/ml. However, it should be noted that this concentration is unlikely to be attained clinically and is far beyond usual serum concentrations of up to 5 μg/ml (15). In addition, CIP caused some inhibition of ERK1/2, JNK, and NF-κB activation, generally not reaching significance, but no inhibition of p38 phosphorylation in either cell line. AZM had no inhibitory effect at all on cytokine secretion, ERK1/2, JNK, p38, or NF-κB activation.

Others have shown that whereas MXF serum levels are ~3–5 μg/ml, the concentration within lung biopsies is ~20 μg/ml and within alveolar macrophages just above 50 μg/ml (41). Similar levels are achieved for AZM (12). In contrast, CIP levels within lung tissue are only ~1.4 times higher than serum levels (21). Therefore, although inhibition of cytokine secretion by MXF did not reach a plateau even at 50 μg/ml, we felt that higher doses within the culture medium were not clinically relevant for this or the other drugs.

The fact that both MXF and CIP are fluoroquinolones, possessing a cyclopropyl moiety at the position N1 of the quinolone core structure, appears to be associated with their immunomodulatory effects compared with other quinolones (15). However, MXF possesses a methoxy group (−CHO) on carbon 8 and a bulky C7 side chain, which differentiate it chemically from other fluoroquinolones, including CIP, and may be associated with MXF’s wider antibacterial spectrum as well as increased tissue penetration (23). These structural...
features and the higher concentration of MXF compared with CIP within cells may explain the enhanced immunomodulation by MXF shown in this study.

New therapies that downregulate the massive neutrophilic inflammation within CF airways as well as other inflammatory lung diseases are urgently needed. Therapeutic targeting of the production of IL-8, IL-6, and other inflammatory proteins may most effectively be achieved by inhibiting key intracellular signaling molecules. Our present study demonstrates that MXF, and to some extent CIP, function in this way and might be new modalities to prevent progressive destruction of lung parenchyma in these diseases.

This work extends our previous findings showing that MXF prevented murine neutrophilic pneumonitis following *Candida albicans* infection, by inhibiting lung IL-8 production (38) and NF-κB nuclear translocation (5), and that MXF inhibited inducible nitric oxide synthase (iNOS) expression, nitric oxide secretion, and intracellular signaling in the A549 pulmonary epithelial cell line (51). Consistent with these findings, our current study showed inhibition of cytokine secretion and intracellular signaling in both the IB3 cells and the corrected C38 cell line, suggesting possible clinical relevance for both CF and other inflammatory lung diseases.

We (50) previously found that MXF prevented nuclear translocation of NF-κB as well as inhibiting IκB degradation, which is required for NF-κB-dependent transcription of IL-8. Similarly, the present study shows significant decrease in NF-κB expression. As this and our previous studies show, MXF also inhibited TNF-α-activated phosphorylation of JNK, required for activation of the IL-8 promoter by AP-1, as well as of ERK1/2, which can act independently on both the NF-κB and AP-1 transcription pathways of IL-8 induction. In contrast, the p38 MAPK pathway, which activates IL-8 production by a posttranscriptional step, stabilizing mRNA (19), was completely unaffected by MXF. We, therefore, suggest that inhibition is likely to lie upstream of or near the NF-κB/AP-1 signaling bifurcation. Alternatively, it could lie downstream at the site of chromatin remodeling, associated with histone phosphorylation or acetylation, which are important in allowing access for transcription factor DNA binding and gene transcription (34).

The fact that MXF inhibited both TNF-α- and IL-1β-induced IL-6 secretion would support the sites of action suggested above. Interestingly, however, inhibition of IL-8 secretion by MXF occurred only following stimulation with TNF-α and not following IL-1β, suggesting a more upstream site of action. Future studies are needed to further investigate these possible mechanisms.

A limitation of this study is that it did not analyze the pathway of downregulation by MXF more directly. Although we assume that the inhibition of IL-6 and IL-8 secretion by MXF is due to the downregulation demonstrated in JNK and ERK phosphorylation and NF-κB translocation, future studies should examine the direct effect of MXF on the activation of the IL-8 promoter and determine whether this is inhibited or whether the effect is at the level of cytokine secretion.

A further limitation is that we studied only the effect of MXF as pretreatment at the time of the inflammatory stimulus. Since most patients with CF and other chronic lung diseases have ongoing neutrophilic airway inflammation, it would be
clinically relevant to test the effect of MXF added after stimulation of epithelial cells with cytokines.

This study did not demonstrate inhibition by AZM of chemokines or their intracellular signaling molecules within either the CF or corrected airway epithelial cells. This is surprising in view of the remarkable protective anti-inflammatory effects of AZM and other macrolides observed in vivo in CF and other pulmonary diseases (3, 37) as well as in a murine model of lung inflammation (45). However, a recent study (40) of in vitro modulation of IL-8 and ERK phosphorylation by macrolides showed that IL-8 secretion was markedly increased at 24 and 48 h following exposure to AZM as part of a polyphasic response, partly due to ERK phosphorylation. Conversely, some macrolide antibiotics decrease IL-8 release and inhibit nuclear transcription factors in vitro but usually after prolonged exposure (1). The scope of our present study did not include an extended time of exposure to AZM, and this might be of interest in future studies. Possibly, the suppression of inflammation by macrolides in vivo is related to direct modulation of neutrophil function (13), cytokine production by neutrophils or other host immune cells (25), or due to disruption of the biofilm that protects P. aeruginosa from host defenses within airways (37, 44).

In conclusion, we have demonstrated that quinolones and particularly MXF have remarkable immunomodulatory effects on both a CF and a corrected bronchial epithelial cell line. Thus quinolones may be important anti-inflammatory therapies for CF and other chronic lung diseases. Future studies are needed to assess their clinical immunomodulatory efficacy.

ACKNOWLEDGMENTS

This work was presented in part at the American Thoracic Society 2005 San Diego International Conference.

GRANTS

K. Klein acknowledges the Joan and Jaime Constantiner Institute for Molecular Genetics for a travel grant to present the results described in this publication.

REFERENCES

ANTI-INFLAMMATORY EFFECTS OF MOXIFLOXACIN IN CF CELLS


