AZITHROMYCIN SELECTIVELY REDUCES TUMOR NECROSIS FACTOR α IN CYSTIC FIBROSIS AIRWAY EPITHELIAL CELLS.

Running title: Azithromycin reduces TNF-α in cystic fibrosis cells.

Cristina Cigana, Baroukh Maurice Assael and Paola Melotti*

Cystic Fibrosis Center, Azienda Ospedaliera di Verona, Verona, ITALY.

*Corresponding author. Mailing address: Cystic Fibrosis Center - Azienda Ospedaliera di Verona, piazzale Stefani, 1 - 37126 Verona, Italy. Phone: +39 045 8123419. Fax: +39 045 8122042. E-mail: paolamelotti@libero.it.
Abstract

Azithromycin (AZM) ameliorates lung function in cystic fibrosis (CF) patients. This macrolide has been suggested to have anti-inflammatory properties as well as other effects potentially relevant for therapy of CF. In this study, we utilized three CF (IB3-1, 16HBE14o-AS3 and 2CFSMEo-) and two isogenic non-CF airway epithelial cell lines (C38 and 16HBE14o-S1) to investigate whether AZM could reduce Tumor Necrosis Factor alpha (TNF-α) both at mRNA and protein level by real time quantitative PCR analysis and an ELISA-based assay respectively. We studied effects on the DNA binding of Nuclear Factor-kB (NF-kB) and Specificity protein 1 (Sp1) by an ELISA-based assay. Non-CF cells express significantly lower TNF-α mRNA and protein levels when compared with an isogenic CF cell line. In CF cells AZM treatment causes a 30% reduction of TNF-α mRNA levels (p<0.05) and a 45% decrease in TNF-α secretion (p<0.05), reaching approximately the levels of the untreated isogenic non-CF cells. In CF cells NF-kB and Sp1 DNA binding were also significantly decreased (about 45 and 60% respectively, p < 0.05) after AZM treatment. Josamycin (JM), a macrolide lacking clinically described anti-inflammatory effects, was ineffective. Finally, AZM did not alter the mRNA expression levels of interleukin 6, a pro-inflammatory molecule not differentially expressed in CF and isogenic non-CF cells. The results of our study support the anti-inflammatory activities of this macrolide, since we show that AZM reduced the levels of TNF-α, and propose inhibitions of NF-kB and Sp1 DNA binding as possible mechanisms of this effect.
Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene encoding a transmembrane chloride channel.

Neutrophil-dominated inflammation is a hallmark of the airway disease in CF. Uncontrolled release of neutrophilic cytotoxic contents leads to mucus hypersecretion and progressive lung damage, ultimately contributing to the morbidity and mortality in CF patients.

It remains controversial whether there is an intrinsic hyper-inflammatory state arising directly from a lack of functional CFTR (10, 24). The hypothesis of primary inflammation preceding detectable infection is based firstly on clinical observations of inflammation in CF neonates and young children (25, 34), but also on experimental reports (12, 40). Other evidences suggest that CF airways inflammatory response to infectious agents is exaggerated and/or prolonged (33). CF patients have been shown to exhibit greater amounts of neutrophils and interleukin (IL)-8 in bronchoalveolar lavage (BAL) than non-CF subjects in response to similar levels of infection (28). Furthermore CF airway epithelial cell lines produced higher quantities of IL-8 in comparison with CFTR-corrected cells in response to IL-1β and tumor necrosis factor (TNF)-α (1, 43) and to bacterial stimulation (17, 43).

Clinical studies have shown that macrolides have beneficial effects on lung function in CF patients (18, 36). CF patients treated with azithromycin (AZM) experienced improvement in the viscoelasticity of the sputum (4), decreased content of mucoid Pseudomonas (P.) aeruginosa in sputum samples (19), decline in the number of pulmonary exacerbations (30, 35) and significant increases in respiratory function parameters such as FEV1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) (19, 30, 35).

High concentrations of TNF-α (8, 43), a pro-inflammatory cytokine able to induce the
production of secondary mediators by epithelial cells, including cytokines (e.g. IL-8), have been reported in CF airways fluids.

The aim of this work was to evaluate whether AZM could reduce TNF-α levels in CF cells and whether CF cells express higher amounts of this cytokine than non-CF cells. Finally, we investigated the molecular mechanisms of AZM effect on these cells studying the transcriptional activity of Nuclear Factor-kB (NF-kB) and Specificity protein 1 (Sp1) transcription factors before and after incubation with AZM, as both these proteins are involved in the regulation of TNF-α gene expression.

Materials and methods

Cell cultures.

IB3-1 and isogenic C-38 cells, with CF and non-CF phenotype respectively, obtained from P. Zeitlin (Johns Hopkins University, MD, USA) (46), were grown in LHC-8 media (Biosource, Rockville, MD) supplemented with 5% foetal bovine serum (FBS) (Cambrex Bio Science, Verviers, Belgium). IB3-1 cells were derived from bronchial epithelium of a CF patient and the isogenic rescued cell lines C38 express plasmid encoded functional CFTR (46).

16HBE14o- AS3 and the isogenic 16HBE14o- S1 cell lines with CF and non-CF phenotype, respectively (32), obtained from P. Davis (Case Western Reserve University, OH, USA), were grown in Eagle's MEM (Cambrex Bio Science) supplemented with 10% FBS, 1% L-glutamine (Cambrex Bio Science) and 0.4% G418 sulfate (Calbiochem, CN biosciences, La Jolla, CA, USA). 16HBE14o- AS3 cells are lacking of CFTR expression following transfection with an antisense CFTR sequence while isogenic 16HBE14o- S1 cells transfected with a sense CFTR sequence express wild type CFTR (32).
The CF cell line 2CFSMEO-, kind gift of D. Gruenert, (University of California, CA, USA) (13) was derived from sub mucosal tracheobronchial glands of a CF patient and grown in Eagle's MEM supplemented with 10% FBS and 1% L-glutamine.

Epithelial respiratory cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂. Cells were seeded in a concentration of 1.5 x 10⁵ cells/cm² and, after 24 hours, they were exposed up to 8 µg/ml AZM (Pfizer, Italy) and/or JM (Yamanouchi Pharmaceutical, Japan) for 24 hours. These concentrations, which are in the sub-MIC range for *P. aeruginosa*, are consistent with those described in lungs of patients treated with AZM (16, 23).

In the presence of AZM for 24 hrs at 8 µg/ml the cell viability was >95% as determined by Tripan Blue exclusion test while at higher concentrations starting from 16 µg/ml the viability was <95%.

Serial dilutions (0, 0.125, 0.5, 2 and 8 µg/ml) of the macrolides were utilized in dose-response experiments. We observed no statistically significant effects on TNF-α expression after AZM treatment with concentrations lower than 8 µg/ml (data not shown).

**RNA isolation, quantification and reverse transcription.**

Cells were lysed. Total RNA was extracted with the Total RNA Isolation kit (Roche, Germany) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, USA). The reaction was then incubated at 25°C for 10 min and at 37°C for 2 hrs.

**RNA quantification.**

Relative quantification of gene expression was performed by real time quantitative PCR analysis as described by the manufacturer (Applied Biosystems User Bulletin 2). The cDNA (2 µl) was amplified using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Grand Island, NY, USA) in the ABI Prism 5700 sequence detection system. The Primer Express Software
(Applied Biosystems) was used to select the specific primers. Primer sets (Sigma-Genosys, St. Louis, MO, USA) are shown in Table 1. The PCR thermal protocol consisted of 2 min at 50°C, a denaturation step at 95°C for 2 min followed by 50 cycles of a 15 s 95°C denaturation step and a 30 s annealing/extension step at 60°C. The real-time PCR reactions were performed in duplicates for both target and normalizer gene. Changes in mRNA expression level were calculated following normalization to GAPDH. The degree of variation of the ratio target gene / normalizer gene in the experiments was less than 8 % in each cell line. Results were expressed as mean ± standard deviation (SD).

**Protein quantification.**

TNF-α secretion was determined in supernatants from the cell cultures above described by Enzyme Amplified Sensitivity Immunoassay using the TNF-α EASIA kit (Bender MedSystems, Austria) according to the manufacturer’s instructions. The limit of detection was 3.83 pg/ml. Measurements were performed at least in duplicate. Values were normalized to 10⁶ cells; results were expressed as mean ± SD.

**DNA binding activity studies.**

DNA binding activity of NF-kB and Sp1 was measured in nuclear extracts using TransAM kits (Active Motif, Belgium) and Mercury Transfactor Profile kit (Clontech, USA), respectively, according to the manufacturer’s instructions. Measurements were performed at least in duplicate; results were expressed as mean ± SD.
Statistical analysis.

Statistical calculations and tests were performed using Friedman test for comparison between non-CF to CF cells and Mann-Whitney test for evaluation of treatments with macrolides of the CF cell line.

The limit of statistical significance was defined as \( p \leq 0.05 \). All data were expressed as mean ± standard deviation (SD).

Results

Regulation of TNF-\( \alpha \) mRNA expression by AZM treatment.

In this study, first of all, we measured the expression levels of TNF-\( \alpha \) gene. All cell lines constitutively expressed TNF-\( \alpha \) mRNA, however the level of basal expression in CF cells was significantly higher than in isogenic non-CF cells (see Fig 1). We confirmed this differential TNF-\( \alpha \) expression using cells at different passages as well as after 96 hours from sedimentation (data not shown). Following exposure of CF cell lines to 8 \( \mu \)g of AZM for 24 hours we found that this macrolide reduced TNF-\( \alpha \) mRNA of about 35\% and 25\% in IB3-1 and 16HBE 14o-AS3 respectively (\( n=5 \), \( p<0.01 \), Fig 1A e 1B), approximately to the levels of untreated isogenic non-CF cells C38 and 16HBE 14o- S1 respectively. A 30\% reduction of TNF-\( \alpha \) mRNA was detected in 2CFSMEo- cells after AZM treatment (\( n=5 \), \( p<0.05 \), Fig 1C). The macrolide josamycin (JM), known to lack clinical anti-inflammatory properties, had no significant effects on TNF-\( \alpha \) mRNA expression in all cell lines (data not shown).

Effects of AZM treatment on IL-6 mRNA expression.
In terms of IL-6 mRNA expression we found no statistically significant differences between CF cell lines and isogenic non-CF cells (see Fig 2). We then exposed CF cell lines to 8 µg of AZM for 24 hours. AZM had no statistically significant effects on IL-6 mRNA expression in IB3-1, 16HBE 14o- AS3 and 2CFSMEo- cells (Fig 2A, 2B and 2C respectively).

Regulation of TNF-α protein levels by AZM treatment.

We confirmed the higher TNF-α expression in CF cells in comparison to isogenic non-CF cells also at protein level (see Fig 3). Treatment with 8 µg of AZM was effective in reducing TNF-α protein levels of 45% and 50% in IB3-1 and 16HBE 14o- AS3 treated for 24 hours respectively (n=3, p<0.05, Fig 3A and 3B), to the levels of untreated isogenic non-CF cells.

Furthermore AZM reduced TNF-α protein expression of 40% in 2CFSMEo- (n=3, p<0.05, Fig 3C).

Effects of AZM on NF-kB DNA binding activity.

We found that IB3-1 and 16HBE 14o- AS3 cells showed 2 folds higher constitutive NF-kB DNA binding levels than isogenic non-CF cell lines C38 and 16HBE 14o- S1 cells (n=3, p<0.01 and p<0.05 respectively, Fig 4A). Following exposure of CF cell lines to 8 µg/ml AZM for 24 hours the NF-kB DNA binding activity in IB3-1 and 16HBE 14o- AS3 was reduced of 40% and 45% respectively, nearly to the levels of untreated C38 and 16HBE 14o- S1. A 45% reduction of NF-kB DNA binding activity was detected in 2CFSMEo- cells after AZM treatment (n=3, p<0.05, Fig 4B). Furthermore, JM had no effects on NF-kB DNA binding activity (data not shown).

Effects of AZM on Sp1 DNA binding activity.

We decided to evaluate whether AZM could affect the levels of Sp1 DNA binding. We did not detect statistically significant differences in the constitutive Sp1 DNA binding levels in IB3-1
and 16HBE 14o- AS3 cells versus isogenic non-CF cell lines C38 and 16HBE 14o- S1 cells respectively, as shown in Fig. 5A. After 24 hours of treatment with 8 µg of AZM, 60%, 64% and 65% Sp1 DNA binding activity reductions were detected in IB3-1, 16HBE 14o- AS3 and 2CFSMEo- cells respectively (n=3, p<0.01, p<0.01 and p<0.05 respectively, Fig 5B). JM was not effective also on Sp1 DNA binding activity (data not shown).

Discussion
Defective expression or function of the CFTR channel in airway epithelial cells leads to persistent and overwhelming infection and inflammation. Several studies indicate that inflammation occurs very early in the lungs of CF patients and often seems to precede clear signs of infection (25, 34, 40). Moreover, CF airway inflammation may manifest as disproportionately increased or prolonged in relation to the level of stimuli (1, 17, 28, 43). However whether a dysregulation of inflammation exists in CF patients is debated (2, 15).

Data from literature regarding of AZM effects on TNF-α expression are scarce and contradictory. Although AZM has been reported to inhibit TNF-α expression in both in vivo in animal models and in vitro (20, 21), in healthy human subjects sera TNF-α protein concentration was unaffected by a 24 hours-long treatment with AZM (14). On this regard the experimental model seems to be critical and in particular differences between CF and non-CF models are relevant.

In this work we firstly aimed to establish whether differential expression of a relevant inflammatory marker as TNF-α could be detected in our CF cell models since it plays a relevant role in the pathogenesis of CF. We showed not only that its constitutive expression was significantly higher in CF versus isogenic non-CF cells both at mRNA and protein level, but also
that this finding was not dependent on cells passage or period from sedimentation. Moreover, we found no differential IL-6 mRNA expression between CF and non-CF cell lines. We determined that exposition to LPS derived from *P. aeruginosa* for 24 hours induced higher expression of TNF-α mRNA in CF cells in comparison to non-CF cells in our models (data not shown), confirming an exaggerated inflammatory response in CF cells in the presence as well in the absence of stimuli.

The association between increased inflammatory markers and CFTR mutations is however controversial. Aldallal et al (1) found higher IL-8 expression in CF versus non-CF cells in cell line models but not in primary cultures, revealing a considerable variability in airway epithelial cell inflammation among different individuals and cell models. Becker MN et al (5) found no differential IL-8 and IL-6 expression between CF and non-CF cell lines and primary cultures respectively. These contradictory results could be due not only to the choice of the cell model and its origin but also to different culture conditions. Our experimental model consists of several human CF cell lines derived from different airway cell types and two of them have been compared to their isogenic non-CF cell lines. This experimental model was appropriate for reproducing anti-inflammatory effects of AZM described *in vivo* (41, 45).

Macrolide antibiotics are receiving increasing attention for their possible therapeutic benefits in the treatment of CF. AZM was chosen over other macrolides because of ease of administration and its accumulation in sputum and tissues. Its plasma half-life is considerably longer than that of other macrolides. It also accumulates in alveolar macrophages, which could represent a delivery vehicle to transport it to affected sites. Finally, the results of several clinical trials (19, 30, 35) encourage clinicians to subject CF patients to long-term treatment with AZM, although certain heterogeneity in the response has been reported. However, the mechanisms of its efficacy...
are still unclear. Clinical beneficial effects of AZM might derive from the synergism of different effects, including inhibition of *P. aeruginosa* bacterial growth (29, 44), decreased expression of bacterial virulence factors (39, 44), modulation of inflammatory response (41), ion transport (31) and tight junctions (3).

We found that AZM reduced TNF-α expression as both transcript and protein levels in all our CF cell lines, bringing it to the levels of untreated isogenic non-CF cells. Conversely, IL-6 mRNA expression was not significantly affected by AZM treatment. As we found higher expression of TNF-α, but not of IL-6, in CF versus non-CF cells, we can speculate that AZM may be effective towards those proinflammatory molecules induced in the constitutive inflammation. The specificity of the results is warranted by the observation that JM, a macrolide known to lack clinical anti-inflammatory properties (38), was ineffective.

The possibility that AZM may act at the transcriptional level was tested by measuring the DNA binding activity of two transcription factors relevant in the regulation of TNF-α gene, NF-kB (27) and Sp1 (42).

We found that in the presence of AZM NF-kB DNA binding activity in CF cells was reduced approximately to the levels detected in isogenic non-CF cells. Also Sp1 DNA binding was reduced following treatment with AZM, while activity of this transcription factor was not significantly different in CF and non-CF cells. Once again the inhibitory effect was peculiar to AZM as JM had no effect on NF-kB and Sp1 DNA binding activity. Assays of transcription factors binding to DNA do not rule out an effect on their activation. This approach has been utilized in order to establish whether NF-kB and Sp1 could be considered as targets of AZM potentially involved in the regulation of TNF-α transcription by this macrolide. We are focusing on the ability of AZM to affect activation of NF-kB, Sp1 and AP-1 at different levels (11, 12).
Increased NF-κB activation in CF versus isogenic non-CF specimens was observed in several studies, both in absence and in presence of stimulation (9, 17, 22, 43) in different experimental models. Furthermore, our results are consistent with previous studies describing higher NF-κB activation in CF versus non-CF cells in a cellular model utilized in this study (17, 43). Furthermore, it is of note that therapeutic inhibition of NF-κB has been proposed for treatment of inflammatory and immune diseases (7, 37). Decreased levels of TNF-α and IL-8, two NF-κB-regulated genes, could reduce the recruitment of neutrophils which are considered responsible for epithelial damage in CF airways (41).

Sp1 can functionally cooperate with NF-κB to elicit maximal promoter activation of inflammatory genes (26). Investigating the effects of AZM on Sp1 was considered relevant as this transcription factor has been described to regulate several inflammatory genes including the chemokine Macrophage Inflammatory Protein-2, heparanase and TNF-α (42) and therefore its inhibition could influence inflammatory responses. This approach was considered appropriate for investigating a possible mechanism of regulation of TNF-α by AZM. Inhibition of Sp1 activity by AZM seems to be a novel effect of this macrolide.

At present no satisfactory anti-inflammatory treatments are available for clinical use in CF because of the limited efficacy and/or undesired effects (6, 24). Therefore the identification of novel therapeutic targets are required to develop novel strategies for treatment of lung inflammation in CF. Our results indicate that the antibiotic AZM has the features of an anti-inflammatory drug and that NF-κB and Sp1 transcription factors are relevant targets whose inhibition might contribute to ameliorate the excessive inflammatory response in CF.
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References


relative IL-6 mRNA expression

A

C

IB3-1 untreated
IB3-1 treated with AZM
C38 untreated

relative IL-6 mRNA expression

B

16HBE 14o untreated
16HBE 14o AS3 untreated
16HBE 14o AS3 treated with AZM

relative IL-6 mRNA expression

2 CFSE untreated
2 CFSE treated with AZM
Figure 1. TNF-α mRNA expression. Expression of TNF-α mRNA in (A) non-CF C38 cells and isogenic CF IB3-1 cell line, (B) non-CF 16HBE 140- S1 cells and isogenic CF 16HBE 140- AS3 cell line and (C) 2CFSMeo- cells at constitutive level and after treatment with AZM. Total RNA was extracted and retrotranscribed. The values of TNF-α mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) IB3-1, (B) 16HBE 140- AS3 and (C) 2CFSMeo- cells (means ± SD). The experiment was repeated five times. *p<0.05, **p<0.01, ***p<0.001.

Figure 2. IL-6 mRNA expression. Expression of IL-6 mRNA in (A) non-CF C38 cells and isogenic CF IB3-1 cell line, (B) non-CF 16HBE 140- S1 cells and isogenic CF 16HBE 140- AS3 cell line and (C) 2CFSMeo- cells at constitutive level and after treatment with AZM. Total RNA was extracted and retrotranscribed. The values of IL-6 mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) IB3-1, (B) 16HBE 140- AS3 and (C) 2CFSMeo- cells (means ± SD). The experiment was repeated five times.

Figure 3. TNF-α protein release. Secretion of TNF-α in (A) non-CF C38 cells and isogenic CF IB3-1 cell line, (B) non-CF 16HBE 140- S1 cells and isogenic CF 16HBE 140- AS3 cell line and (C) 2CFSMeo- cells at constitutive level and after treatment with AZM and JM. TNF-α protein levels were measured by commercial ELISA kit. The values represent the secretion levels relative to untreated (A) IB3-1, (B) 16HBE 140- AS3 and (C) 2CFSMeo- cells (means ± SD). The experiment was repeated three times. *p<0.05, **p<0.01.
Figure 4. DNA binding of NF-kB. (A) Constitutive binding to the DNA of NF-kB in non-CF C38 cells and isogenic CF IB3-1 cell line and in non-CF 16HBE 140- S1 cells and isogenic CF 16HBE 140- AS3 cell line. (B) Effect of the treatment with AZM on the DNA binding of NF-kB in CF cells (IB3-1, 16HBE 140- AS3 and 2CFSMeo-). NF-kB DNA binding activity was analysed using a commercial kit following the manufacturer’s instructions. The experiment was repeated three times (means ± SD). *p<0.05, **p<0.01.

Figure 5. DNA binding of Sp1. (A) Constitutive binding to the DNA of Sp1 in non-CF C38 cells and isogenic CF IB3-1 cell line and in non-CF 16HBE 140- S1 cells and isogenic CF 16HBE 140- AS3 cell line. (B) Effect of the treatment with AZM on the DNA binding of Sp1 in CF cells (IB3-1, 16HBE 140- AS3 and 2CFSMeo-). Sp1 DNA binding activity was analysed using a commercial kit following the manufacturer’s instructions. The experiment was repeated three times (means ± SD). *p<0.05, **p<0.01.
Table 1. Primer sequences utilized in the quantitative PCR analysis

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*concentration used for FW/RV primers (nM)