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Exploring the mechanisms of macrolides in cystic fibrosis

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Summary Several studies have reported clinical improvements in cystic fibrosis (CF) patients on macrolide antibiotics although the mechanism of action remains unclear. We conducted an open-label study of azithromycin (500 mg daily for 2 weeks) in 9 adult CF patients to explore 3 possible mechanisms: up-regulation of the multi-drug resistance (MDR) or cystic fibrosis transmembrane regulator (CFTR) proteins, correction of epithelial ion transport and reduced bacterial adherence. End-points included nasal potential difference (PD) measurements, nasal epithelial MDR and CFTR mRNA levels and *Pseudomonas aeruginosa* adherence to nasal epithelium. Forced expiratory volume in the 1st second (FEV_1) increased significantly after 2 weeks of azithromycin (pre- 41.1 [5.0]%; post- 44.6 [5.8]%; $P < 0.05$), although improvements in forced vital capacity (FVC) did not reach significance (pre- 61.3 [4.0]%; post- 67.1 [5.4]%, NS). Before treatment all subjects had nasal PD measurements characteristic of CF. Treatment led to no significant group differences in any measures of either sodium absorption or chloride secretion. Neither CFTR nor MDR mRNA levels had altered significantly and the adherence of *P. aeruginosa* did not decrease. We conclude that these are unlikely to be significant contributing mechanisms accounting for the consistent beneficial results observed in clinical trials of macrolides in CF.

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Introduction

Despite recent increases in survival, cystic fibrosis (CF) is still associated with significant morbidity and mortality, leading to the search for new therapies. Following success with the macrolide group of antibiotics in panbronchiolits,[1] a disease with many similarities to CF, we and others have reported that this group of drugs leads to clinical benefit in CF patients.[2–4] Data from clinical and laboratory studies suggest that these agents are not acting as conventional antibiotics in vivo, which has led to alternative hypotheses regarding their mechanisms of action.

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes an apically-situated epithelial ion channel.[5] Mutations lead to impaired secretion of chloride and hyperabsorption of sodium across the cell membrane.[6] It is thought that these ion transport defects lead to a dehydrated airway surface liquid and subsequent impairment of pulmonary mucociliary clearance.[7] Chronic infection and an exaggerated inflammatory response lead to irreversible airway damage and death from respiratory failure in the majority of patients.[8] One proposed mechanism of action of macrolides is a direct effect on either CFTR or related ion transport proteins. CFTR belongs to the family of ATP binding cassette (ABC) proteins along with the multi drug resistance (*MDR*) gene product, P-glycoprotein. The two share 80% sequence homology and possess several common functions including chloride transport.[9] Reports of CF patients treated with chemotherapy for co-existing malignancies, have demonstrated pulmonary improvements thought to be due to the upregulated MDR proteins complementing deficient CFTR function.[10] Macrolides have also been shown to upregulate the expression of MDR in epithelial cells.[11] In CF this could either improve ion transport or affect other functions of these proteins. Alternatively, normalisation of ion transport, either via improved trafficking or function of the CFTR protein or effects on other ion channels could lead to clinical benefit. There have also been suggestions that macrolides may exert an indirect anti-bacterial effect, in the absence of effective bacterial killing. Adherence of bacteria to cell surfaces is thought to be an essential prerequisite to established infection and is increased to CF epithelial cells.[12,13] Macrolides have been shown to reduce adherence in both an animal model[14] and a human study on the buccal mucosa.[15] Alternative hypotheses for the benefits of the drugs include the inhibition or breakdown of biofilm matrix,[16,17] a direct bactericidal effect on the slow-growing organisms within a biofilm, or an anti-inflammatory effect.[18] However, none of the recent clinical trials have confirmed either significant antibacterial or anti-inflammatory effects, despite clinical improvement.[2–4]

An increased understanding of the mechanism(s) of action of macrolides in CF could facilitate the development of compounds with superior efficacy. In this clinical study we assessed the effects of 2 weeks of azithromycin (AZM) on adult subjects with CF, focussing on three possible mechanisms: *CFTR* and *MDR* expression, epithelial ion transport and *Pseudomonas aeruginosa* adherence.

Materials and methods

Subjects

Adult CF subjects were recruited from the outpatient clinic at the Royal Brompton Hospital. CF had been diagnosed on standard criteria (positive sweat test or mutation analysis in the context of a suggestive clinical presentation). All subjects were infected with *P. aeruginosa* with at least 3 positive cultures of the organism from sputum over the previous year. Due to the potential adverse effects of azithromycin, exclusion criteria included: (a) deafness (in either the subject or a first degree relative at less than 40 years of age), (b) liver disease (aspartate transaminase (AST) >3 times the upper limit of normal, abnormal coagulation or treatment with ursodeoxycholic acid), (c) any macrolide therapy during the 2 months prior to trial entry, (d) any positive sputum culture of *Burkholderia cepacia*, (e) bilateral nasal polyps (which can affect nasal PD measurements), (f) upper respiratory tract infection within 3 weeks of commencing the study and (g) breast feeding. The study was approved by the Ethics Committee of the Royal Brompton and Harefield NHS Trust and informed consent was obtained from all patients.

Study protocol

The study protocol involved 6 visits over a 9-week period as illustrated in Table 1. Following 3 pre-treatment visits (V1–V3), patients received 500 mg oral AZM (Pfizer, UK) once daily for 14 days. They were reviewed at the end of this 2-week period (V4), 1 week later (V5, when tissue AZM levels would still have been high), and finally, a further 3 weeks later (V6). PD measurements at V5 were made in an attempt to confirm any drug effects

Table 1 Flow chart of trial visits and investigations.

Week	1	2	3	4	5	6	7	8	9
Visit	V1	V2	V3		V4	V5			V6
Spirometry	X	X	X		X	X			X
AZM			Daily, 2 weeks						
PD	X	X	X		X	X			X
Nasal brushing			X		X				X

Patients attended on 6 occasions over a 9-week time period for the investigations indicated. Immediately after V3, patients were commenced on AZM 500 mg od for a total of 14 days.

observed at V4 and to limit the chances of missing a delayed effect. All assays were performed at V6 in order to demonstrate that any changes observed at V4 or V5 had reversed, in support of a plausible pharmacological effect of the drug. Lung function was measured on a Vitalograph® 2120 spirometer and data are expressed as percent predicted for age, sex and height.

Nasal PD

These measurements were performed using a conventional protocol as previously decribed.[19] A reference electrode was taped to the abraded skin of the forearm. An initial skin PD of between −35 and −50 mV was obtained in order to confirm the integrity of the circuit. The exploring catheter was then inserted below the inferior turbinate of one nostril to the point of maximal (most negative) PD and held by the patient for the duration of the measurements. Infusions were perfused at a constant rate of 4 ml/min. The protocol included a baseline measurement (Hepes) followed by responses to the sodium channel blocking agent, amiloride (10^{-4} M for 3 min), low chloride solution and isoproterenol (10^{-5} M) for a total of 8 min. Unless otherwise stated, all chemicals and solutions were obtained from Sigma Aldrich, UK, and were of Analar grade or best available. Three recordings were obtained prior to commencing AZM treatment. Subsequent measurements were obtained at the end of 14 days AZM, 1 week after stopping treatment and again 4 weeks later. Traces were assessed by an investigator blinded as to the timing of the measurements.

CFTR and MDR mRNA levels

Nasal epithelial cells were obtained by brushing (3 mm cytology brush, Diagmed, Thirsk, UK) the inferior surface of the inferior turbinate of the opposite nostril to that used for PD measurements at the time points indicated (Table 1). Previous studies have shown that this method yields approximately $4–10 \times 10^5$ cells.[13] Cells were suspended in phosphate-buffered saline (PBS) and divided into two equal aliquots. One aliquot was processed for reverse transcription-polymerase chain reaction RT-PCR. Cells were suspended in Dulbecco's Modified Eagle Medium in RNase-free microfuge tubes and split into 2 aliquots. One sample was centrifuged for 10 s at 20,000*g* and the pelleted cells were resuspended in 350 μl of RLT buffer (Qiagen, Crawley, UK). The sample was immediately placed on crushed dry ice before storage at −80 °C. Total RNA was prepared using RNeasy mini protocols (Qiagen). Levels of CFTR and MDR were quantified in separate real-time quantitative multiplex TaqMan RT-PCR reactions using ABI PRISM 7700 Sequence Detection System and Sequence Detector v1.6.3 software (Applied Biosystems, Warrington, Cheshire, UK). The oligonucleotide primer and fluorogenic probe sequences were designed using Primer Express Software version 1.5 (Applied Biosystems). CFTR (ABCC7) mRNA was quantified using forward CFTR primer (5′-ggaaaaggccagcgttgtc-3′), reverse CFTR primer (5′-ccaggcgctgtctgtatcct-3′) and fluorogenic CFTR probe (5′-FAM-ccaaacttttttcagctggaccagaccaa-TAMRA-3′) encompassing bases 150–229 of the CFTR cDNA sequence (NCBI:NM_000492) and spanning intron 1 of the CFTR gene. MDR (ABCB1) mRNA was quantified using forward MDR primer (5′-tggttcaggtggctctg-3′), reverse MDR primer (5′-ctgtagacaaacgatgagctatcaca-3′) and fluorogenic MDR probe (5′-FAM-aggccagaaaaggtcggaccacca-TAMRA-3′) encompassing bases 2124–2195 of the MDR cDNA sequence (NCBI:NM_000927) and spanning intron 14 of the MDR gene. Levels of 18 s ribosomal RNA (rRNA) were simultaneously quantified when determining the levels of CFTR or MDR mRNA using 18 s Ribosomal RNA Control Reagents (Applied Biosystems, Warrington, UK). Total RNA

was heated to 75 °C for 5 min and then reverse transcribed with TaqMan RT reagents (Applied Biosystems). The RT-reaction mix (5 μl) consisted of 1 × TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTPs, 0.4 U/μl RNase inhibitor, 1.25 U/μl Multiscribe Reverse Transcriptase, 0.4 μM CFTR or MDR reverse primer, 0.4 μM reverse rRNA primer, and approximately 5 ng total RNA. Reactions were incubated at 48 °C for 30 min followed by at 95 °C for 5 min. Subsequently, triplicate 25 μl PCR reactions were performed for each sample. Each 25 μl reaction consisted of 1 × TaqMan Universal PCR Mastermix (Applied Biosystems), 300 nM of the appropriate forward and reverse primers, 100 nM of the appropriate probe, 50 nM forward and reverse rRNA primer, 50 nM rRNA probe and 5 μl reverse-transcribed template. Reactions were incubated at 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Controls included omission of reverse-transcribed template and omission of reverse transcriptase and RNase inhibitor. Relative levels of CFTR and MDR mRNA in each sample were determined using the $\Delta\Delta C_t$ method after adjustment for total RNA content by normalisation to experimentally determined levels of rRNA.[20] mRNA levels of all experimental samples are expressed relative to the level of expression observed at V3 in arbitrary units termed Relative mRNA Expression (RME).

Pseudomonas aeruginosa adherence

The other aliquot of nasal cells was used to assess *P. aeruginosa* adherence as previously described.[13] Cells were washed twice in PBS by centrifugation to remove mucus and resuspended in 250 μl of PBS. A laboratory reference strain of *P. aeruginosa*, International Antigenic Typing Scheme (IATS) serotype 0:1 (NCTC 11440, ATCC 3348) was used. This strain was chosen as it possesses pili, believed largely to mediate adherence, and because in our previous studies, adherence of this strain could be reduced either by *CFTR* gene transfer[13] or by blocking the putative receptor, asialoGM1.[21] The bacteria were maintained at 4 °C on soft agar slants, plated onto blood agar, and cultured overnight in Brain Heart Infusion broth (Oxoid, Basingstoke, UK). Bacteria were pelleted, resuspended in PBS and concentration adjusted by photospectrometry to approximately 5×10^9 CFU/ml (previously confirmed on serial dilution and colony counting). Cells were incubated with 50 μl of bacterial suspension at 37 °C for 1 h, and then spun through 50% percoll (Pharmacia, St. Albans, UK) to remove non-adherent bacteria (30 min; g_{av} 20,000; 4 °C). The cell layer was removed, cytospun onto a thermanox coverslip (Emitech, Ashford, Kent, UK) and fixed overnight in 2.5% glutaraldehyde. Slides were washed in sodium cacodolyte buffer (Merck, Poole, UK), and dehydrated with serial concentrations of ethanol followed by HMDS (TAAB, Aldermaston, UK). Slides were mounted on aluminium stubs (Agar Scientific, Stanstead, UK), sputtered with gold and coded for the purposes of blinding. Samples were viewed under the scanning electron microscope and bacteria quantified on each single or small group of cells within a field of view, when adhesion was clearly seen to be directly to the cell surface rather than related to mucus. Adherence was separately quantified for the apical (ciliated) surface and the basolateral surfaces of ciliated cells. Data are presented as an adherence index based on the numbers of bacteria binding to 10 cells.

Statistical analysis

Changes pre (mean of V1–3 or V3 as stated) and post AZM (V4, V5) were analysed using the Wilcoxon signed rank sum test using SPSS v10. The null hypothesis was rejected at $P<0.05$. For convenience, data are expressed as mean [SEM].

Results

Nine patients (7 male) completed the six trial visits. Mean age was 29.3 [2.5] years. Four patients were homozygous for the ΔF508 mutation, 4 were compound heterozygotes (ΔF508/ R347P, ΔF508/ G551D, ΔF508/−, ΔF508/−) and one patient had no identifiable mutations. Lung function data for each individual over the 3 pre-treatment assessments (V1–V3) were pooled to give a single mean baseline value for each subject. Mean values for the whole group were FEV_1 41.1 [5.0]% and FVC 61.3 [4.0]%. Following 2 weeks of treatment with AZM, FEV_1 had increased significantly (44.6 [5.8]%; $P<0.05$) but change in FVC was not significant (67.1 [5.4]%) (Table 2).

Nasal PD results

All patients had characteristic CF PD recordings at the start of the study. The 3 measurements made at weekly intervals before commencing treatment were meaned to give a single pre-treatment value (V1–V3; Table 1). Neither V4 nor V5 measurements were significantly different from those at baseline for any parameter of nasal PD (Fig. 1).

Table 2 Clinical data of study subjects.

Patient (M/F)	Age (years)	FEV_1/FVC(%)		*CFTR* genotype
		Pre-treatment (mean V1–V3)	Post-treatment (V4)	
1 (M)	42	26/63	26/63	ΔF508/ R347P
2 (M)	26	54.5/79	61/95	ΔF508/ ΔF508
3 (F)	25	61.5/66	66/72	ΔF508/G551D
4 (M)	19	27/48	29/50	ΔF508/–
5 (M)	30	31/49	31/46	ΔF508/ ΔF508
6 (M)	30	38.5/72	46/83	ΔF508/ ΔF508
7 (F)	21	45/63	48/71	ΔF508/-
8 (M)	37	61/89	68/-	ΔF508/ ΔF508
9 (M)	34	25/51	26/52	–/–

CFTR and MDR mRNA level

Brushings of nasal epithelial cells were taken for RT-PCR analysis of CFTR and MDR mRNA levels at 3 time points: immediately before commencing AZM (V3), after the 14-day course of treatment (V4) and 4 weeks after treatment had been completed (V6). Total RNA preparation and CFTR and MDR mRNA analysis was successful in 26 of the 27 available samples. Both MDR and CFTR mRNA were detected in all pre-treatment (V3) samples, but no significant differences were observed after treatment, values remaining essentially unchanged throughout the study (Fig. 2). Three patients demonstrated greater than 6-fold increases in MDR mRNA, of whom 2 also showed a similar increase in CFTR mRNA. However, these patients did not show significant changes in either nasal PD or bacterial adherence.

Bacterial adherence

Samples were obtained at V3, V4 and V6. Samples were of sufficient quality to be analysed in 7 patients at V3, 6 at V4 and 7 at V6. Pre-treatment, the adherence index for the apical and basolateral surfaces were similar to our previously published values for CF subjects (8.37 [0.83] and 11.4 [1.40], respectively). At the end of 2 weeks of AZM, adherence to neither surfaces had changed (apical: 9.62 [1.72]) and baslolateral 9.93 [4.93]; Fig. 3).

Discussion

Three placebo-controlled clinical trials have now reported consistent clinical benefit from macrolides in patients with CF. In a double-blind placebo controlled study in adult patients, Wolter et al. reported significant effects on lung function, requirement for antibiotics and systemic inflammation as measured by C-reactive protein levels.[3] We conducted a placebo-controlled cross-over trial in children and found a significant improvement FEV_1, but no effect on either bacterial density or sputum inflammatory markers.[2] Most recently, Saiman et al. reported on a multi-centre study also demonstrating significant lung function improvements and fewer intravenous antibiotic courses in patients on treatment.[4] Long-term treatment with macrolides has become part of routine management for certain groups of patients in many CF centres. Despite this, the mechanism(s) of action of these drugs in CF remain unclear.

The aim of this study was to explore 3 possible mechanisms: (a) upregulation of either CFTR or MDR proteins; (b) improved ion transport in the absence of protein upregulation, for example, by enhanced CFTR trafficking or function; (c) reduced adherence of *P. aeruginosa*. From this study, we conclude that none of these mechanisms provides an explanation for the consistent benefits observed in several clinical trials.

The first hypothesis to be explored was that AZM upregulated either CFTR or MDR expression. Lallemand et al. reported a patient with CF who demonstrated a dramatic improvement in lung function after treatment for a fibrosarcoma.[10] The authors hypothesised that combination chemotherapy led to upregulation of MDR protein, which complemented the functions of CFTR. In support of this, levels of MDR mRNA were increased in this patient's nasal epithelium, whereas they were undetectable in another CF patient who had not received chemotherapy. In view of the patient's persistently raised sweat electrolytes, the authors concluded that this effect was not related to normalisation of chloride ion transport, but suggested that an alternative function of CFTR, such as glutathione transport, had been complemented.

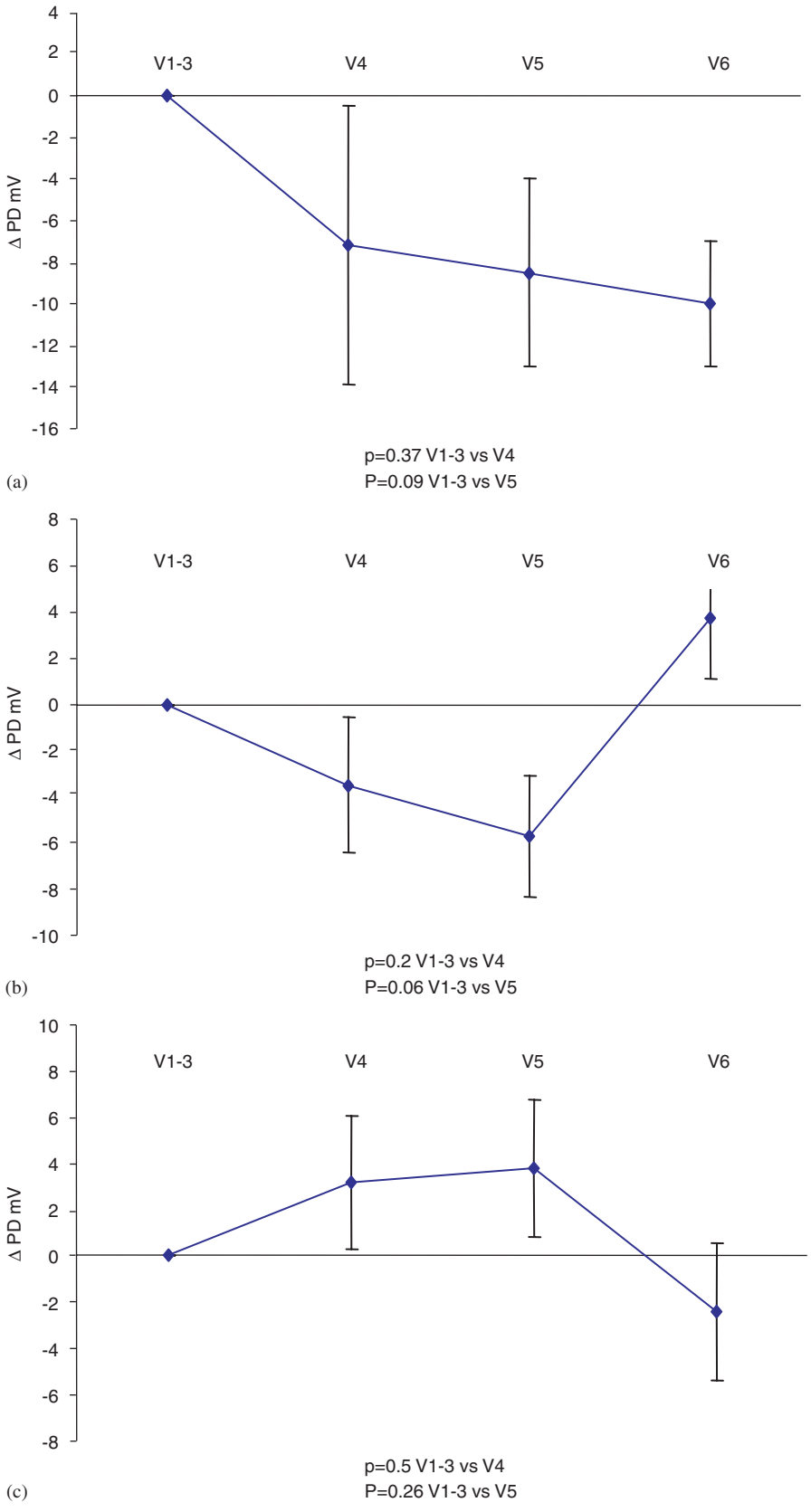

Figure 1 Nasal PD (a) Baseline measurements, (b) responses to amiloride, (c) responses to low chloride solution and isoproterenol. For each of the measurements, values for individuals obtained on visits 1–3 were meaned and are represented as zero for ease of visualisation. All post-treatment values are presented as changes from baseline. Data points represent group means and bars SEM. No significant changes were observed in any parameter after 2 weeks of AZM treatment (V4) or 1 week later (V5).

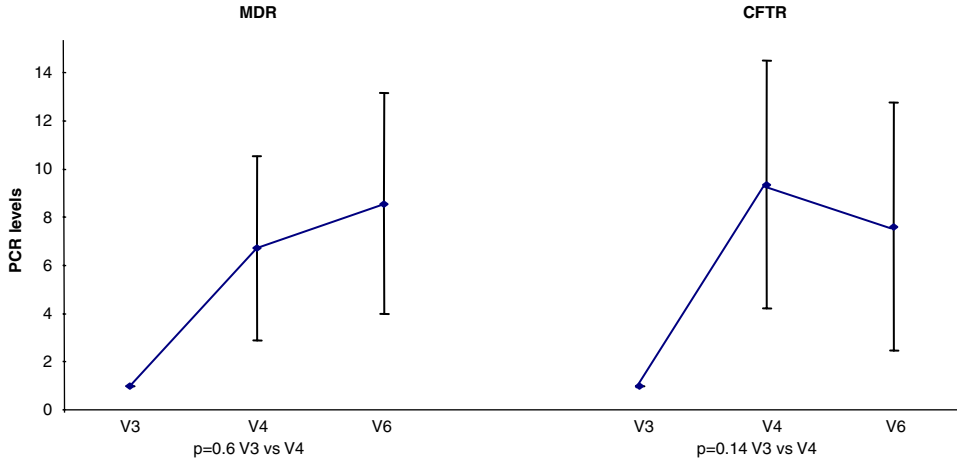

Figure 2 CFTR and MDR mRNA levels: The levels of CFTR (a) and MDR (b) mRNA were determined in nasal brushings taken from each study subject on visits 3,4 and 6. Post-treatment data are presented as changes from baseline (anchored at 1 RME). Data points represent group means and bars SEM. No differences were observed in either parameter when comparing values pre (V3)-and post (V4)-AZM treatment.

Erythromycin, a member of the macrolide group of antibiotics has also been reported to upregulate expression of MDR protein in epithelial cells,[11] leading to the suggestion that this could be the mechanism of action of azithromycin in CF patients.[22] We have previously designed protocols capable of sensitive detection of even small amounts of mRNA with real-time RT-PCR,[23] and have applied this TaqMan technology to measure levels of CFTR and MDR mRNA in the current study. For the group as a whole, there were no significant changes in levels of either mRNA. However, 3 of the 9 patients demonstrated large ($>$6-fold) increases in MDR mRNA, and in 2 of these (ΔF508/ΔF508 and ΔF508/G551D), similar increases were also observed for CFTR mRNA. These subjects did not demonstrate any significant changes in nasal PD, making it unlikely that improved ion transport resulted from this upregulation. Furthermore, as only mRNA was measured, the effect of this change on CFTR or MDR protein levels is unknown. It is possible that other, unmeasured, functions of these ABC proteins were complemented, but given the lack of change in the group as a whole, we consider that this mechanism is an unlikely explanation for the consistent benefits in clinical trials.

The second hypothesis to be tested was that AZM improved ion transport. Correction of abnormal ion transport could result from altered expression of relevant proteins as discussed above, or alternatively, trafficking of mutant CFTR to the apical cell surface, enhanced CFTR function, or indirect effects on interactions with other proteins, such as the epithelial sodium channel, ENaC. Pradal et al. have reported in abstract form, that AZM treatment led to normalisation of the nasal PD response to zero chloride perfusion in 6 of 10 CF patients receiving the drug for a month.[24] In their study, the underlying mechanism was not further explored, although interestingly, subsequent perfusion with isoproterenol did not result in further chloride secretion, and baseline PD was not altered. Baseline PD is largely a measure of sodium absorption, and is increased in CF due to loss of the inhibitory action of normal CFTR on ENaC. Thus, the observation that chloride secretion was improved in the absence of changes in sodium transport might be explained by a drug effect on MDR rather than CFTR. However, both proteins are regulated by cAMP, so the lack of response to the cAMP agonist, isoproterenol is more difficult to explain. We performed repeated nasal PD measurements using a conventional protocol[19] to assess both sodium transport (baseline and amiloride responses) and chloride secretion (response to low chloride solution and isoproterenol). Overall, there were no significant changes in any of these parameters. Despite the lack of significance, it is apparent from Fig. 1 that for each of the parameters, the trend was in the direction of normalisation, ie lower baseline and amiloride responses and greater LCI responses. Post hoc power calculations determined that for the changes observed at V4 to have reached significance ($P<0.05$ with 80% power) the number of subjects would have to have been increased to either 44 (for LCI) or 55 (baseline). Interestingly, the trend towards normalisation appears to continue out to V5. We cannot rule out the possibility therefore, that had we included greater patient numbers or

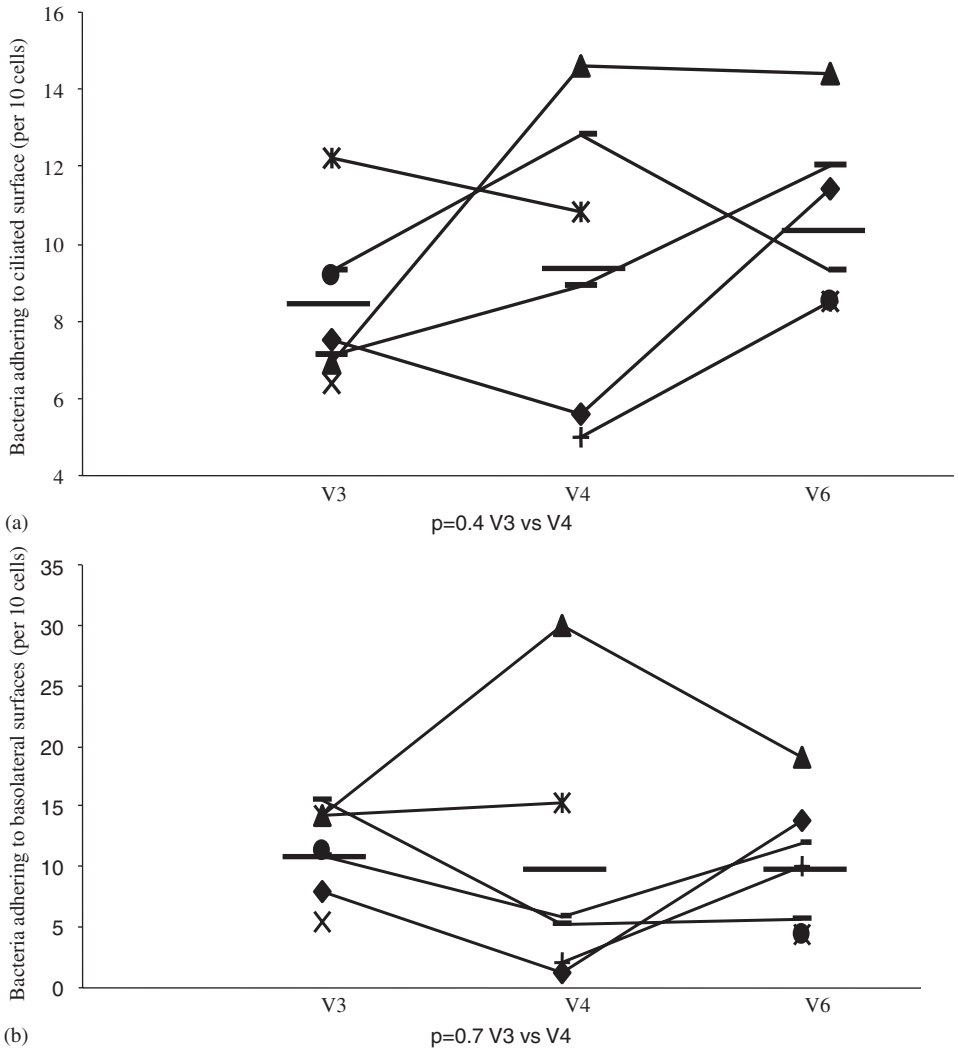

Figure 3 *P. aeruginosa* adherence: Bacteria were quantified separately on the apical, ciliated (a) surface and basolateral (b) surfaces of cells obtained by nasal brushing. Symbols represent individual patients and are linked when data from consecutive time points were available. Long bars represent group means. No reduction in adherence to either surface was observed following AZM treatment (V4).

continued treatment for longer, we might have seen a significant effect on ion transport. However, these relatively modest effects seem unlikely to account for the much more dramatic effects on lung function observed in clinical macrolide trials.

Finally, we attempted to address the possibility that AZM was exerting a therapeutic effect by reducing adherence of bacteria to the cell surface. Macrolides do not appear to have a direct antibacterial effect in CF; although these agents may be bactericidal in vitro, particularly in synergy with other drugs,[25] clinical studies have reported no change in sputum bacterial density. This led us to consider other infection-related mechanisms. Many groups have demonstrated increased binding of *P. aeruginosa* to CF respiratory epithelial cells, most likely related to an increased abundance of receptors.[12,13,26] However, it is not at all clear whether this increased adherence is relevant in disease pathogenesis. Where tissue has been available, for example, at post-mortem or transplantation, bacteria are clearly seen to be in mucus in the airway lumen, as opposed to directly adhering to the epithelial surfaces.[27] These tissues were obtained in the late stages of disease, whereas adherence to mucosal surfaces may be important only in the early, acquisition stage of the process. Alternatively, adherence of bacterial components such as pili or flagellae may be more important than that of the whole organism. Ligation of receptors on the cell surface by such bacterial components has been shown to lead to a brisk inflammatory response, which is potentially detrimental to the host.[28] Baumann et al. demonstrated a significant

reduction in *P. aeruginosa* adherence to buccal epithelial cells from children treated with AZM for 3 months,[15] leading us to believe that this could be a potential mechanism for clinical benefit. Even if adherence of whole bacteria were not a relevant pathogenic mechanism in the lower airway, a reduction in available binding sites could be beneficial by reducing inflammation rather than aiding clearance of organisms. We assessed *P. aeruginosa* adherence to nasal epithelial cells obtained by brushing. Using this technique we have previously reported that the increased binding to CF cells is directly related to mutant *CFTR*, in that cationic liposome-mediated *CFTR* gene transfer *ex vivo* significantly reduces adherence.[13] In this study, we were unable to demonstrate any effect of 2 weeks' AZM on adherence levels. One limitation of interpretation is that analysable samples were not available on all subjects at each of the 3 time points. Also, the assay is noisy, adherence most likely being affected by other factors such as upper respiratory tract infection or inflammation in addition to the underlying CFTR defect. We assessed a single set of experimental conditions, based on previous studies and cannot rule out the small possibility that we may have observed changes under other conditions, for example, longer incubation periods or different concentrations. However, no trends towards a reduction were apparent, and we therefore consider this an unlikely explanation for the clinical benefits seen in other studies.

We have explored 3 mechanisms which we considered might account for the clinical efficacy of macrolides in CF patients, and conclude that these are unlikely to be significant contributory factors. Limitations of this study include its size and duration. We prospectively designed this study on the basis of small numbers, based on the increased power of paired statistical analysis and the relatively invasive and burdensome assays required of the patients. For both nasal PD and bacterial adherence, data from our own studies[29] and those of others[7,30] have demonstrated significant changes in response to treatments, even on unpaired statistical analysis. We also deliberately elected to make this study of short duration as, based on available pharmacokinetic data,[31,32] we considered that changes in gene expression or protein function would be likely to occur quickly, whereas clinical changes used as measures of success in previous trials may take longer. We consider that the changes in spirometry observed over this 2 week period led some support to this design. However, this reasoning may be flawed, and it certainly may not be the case for other mechanisms, which remain to be thoroughly explored. Alternative mechanisms of action of this class of drugs include a wide variety of anti-inflammatory effects including impairment of neutrophil chemotaxis,[33] reduced levels of the pro-inflammatory cytokine, IL-8[34] and inhibition of superoxide generation,[35] all potentially beneficial to the CF airway. However, no studies in CF have demonstrated changes in local inflammatory status; although we examined sputum inflammatory markers in our longer-term paediatric study,[2] we were unable to provide any evidence in support of an anti-inflammatory effect. Bacterial biofilms have become a major area of interest lately, with recent evidence that *P. aeruginosa* exists in the biofilm state within the CF airway once chronic infection has been established.[36] In the early stages of infection, bacteria exist in the free-floating, planktonic state. Once a critical number of bacteria has been reached, and if environmental conditions are favourable, these bacteria settle down and produce a matrix which surrounds them and protects from the host immune response. Investigators have demonstrated that macrolides are effective in biofilm models,[37,38] although the underlying mechanisms, for example, breakdown of matrix components, potentially facilitating penetration of antimicrobial factors and inflammatory cells, are undetermined. All of our patients were infected with *P. aeruginosa*, but currently assessment of biofilm in vivo is difficult and further studies will be required to confirm whether this is the mechanism leading to clinical benefit in previous trials. Finally, these agents may be reducing the viscosity of sputum,[39,40] promoting airway clearance and improving lung function in a similar way to other mucolytic agents. This possibility could also be addressed in future studies.

In conclusion, in the context of CF, the beneficial effects of macrolides do not appear to relate to the ability of these agents to upregulate MDR or CFTR proteins, correct ion transport, or to reduce *P. aeruginosa* adherence. Future studies will explore other mechanisms, a further understanding of which may lead to the development of similar classes of drugs with more potent effects.

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