Journal of Antimicrobial Chemotherapy (2007) **60**, 1155–1158
doi:10.1093/jac/dkm338
Advance Access publication 10 September 2007

JAC

Comparison of the effects of macrolides, amoxicillin, ceftriaxone, doxycycline, tobramycin and fluoroquinolones, on the production of pneumolysin by *Streptococcus pneumoniae in vitro*

R. Anderson[1]*, H. C. Steel[1], R. Cockeran[1], A. von Gottberg[2], L. de Gouveia[2], K. P. Klugman[2,3], T. J. Mitchell[4] and C. Feldman[5]

[1]Medical Research Council (MRC) Unit for Inflammation and Immunity, Department of Immunology, Faculty of Health Sciences, University of Pretoria, and Tshwane Academic Division of the National Health Laboratory Service, South Africa; [2]MRC/NICD/WITS Respiratory and Meningeal Pathogens Research Unit, National Institute for Communicable Diseases, Johannesburg, South Africa; [3]Hubert Department of Global Health, Rollins School of Public Health, and Division of Infectious Diseases, School of Medicine, Emory University, Atlanta, GA, USA; [4]Division of Infection and Immunity, Glasgow Biomedical Research Centre, University of Glasgow, Scotland, UK; [5]Division of Pulmonology, Department of Medicine, Johannesburg Hospital and University of the Witwatersrand, Johannesburg, South Africa

Received 4 June 2007; returned 22 July 2007; revised 7 August 2007; accepted 8 August 2007

***Objectives*: To compare the effects of subinhibitory concentrations of amoxicillin, ceftriaxone, azithromycin, clarithromycin, erythromycin, telithromycin, clindamycin, ciprofloxacin, moxifloxacin, tobramycin and doxycycline on pneumolysin production by a macrolide-susceptible strain and two macrolide-resistant strains [*erm*(B) or *mef*(A)] of *Streptococcus pneumoniae*.**

***Methods*: Pneumolysin was assayed using a functional procedure based on the influx of Ca^{2+} into human neutrophils.**

***Results*: Only the macrolides/macrolide-like agents caused significant attenuation of the production of pneumolysin, which was evident with all three strains of the pneumococcus.**

***Conclusions*: Macrolides, at sub-MICs, but not other classes of antibiotic, subvert the production of pneumolysin, even in the presence of (and irrespective of the mechanism of) macrolide resistance in *S. pneumoniae*.**

Keywords: macrolide resistance, pneumococcus, protein synthesis

Introduction

We and others have reported that macrolide antibiotics, at therapeutically relevant concentrations, inhibit the production of the pneumococcal toxin, pneumolysin, by macrolide-resistant strains of *Streptococcus pneumoniae in vitro* and *in vivo*.[1–3] Pneumolysin is believed to cause bacteraemic disease by promoting extra-pulmonary dissemination of the pneumococcus.[4] Clarithromycin-mediated inhibition of the production of pneumolysin by macrolide-resistant strains of the pneumococcus was evident at sub-MICs of this antimicrobial agent and was independent of the type of macrolide resistance expressed (*erm* or *mef* genes).[3] However, relatively little is known about the comparative effects of different types of macrolides and macrolide-like agents at sub-MICs on the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of *S. pneumoniae*, as well as the effects on production of the toxin of other classes of antibiotics that may be used in the treatment of pneumococcal infection.

In the current study, we have compared the effects of macrolides (clarithromycin, erythromycin, azithromycin, telithromycin and clindamycin) with those of amoxicillin, ceftriaxone, ciprofloxacin, moxifloxacin, tobramycin and doxycycline, all at a fixed final concentration of 0.1 mg/L, on the production of

*Correspondence address. Department of Immunology, University of Pretoria, PO Box 2034, Pretoria 0001, South Africa. Tel: +27-12-319-2425; Fax: +27-12-323-0732; E-mail: ronald.anderson@up.ac.za

pneumolysin by a macrolide-susceptible strain of *S. pneumoniae*, as well as two resistant strains expressing either the *erm* or *mef* genes. The concentration of 0.1 mg/L was either subinhibitory or close to the MIC value for most of the tested antibiotics.

Materials and methods

Antimicrobial agents

Pure substances of amoxicillin, clindamycin, doxycycline and tobramycin were purchased from Sigma Chemical Co, St Louis, MO, USA, whereas the other antimicrobial agents (azithromycin, clarithromycin, erythromycin, telithromycin, ceftriaxone, ciprofloxacin and moxifloxacin) were kindly supplied by the relevant pharmaceutical manufacturers. All agents were made to stock solutions of 1 g/L in distilled water.

Bacteria

One clinical macrolide-susceptible strain of *S. pneumoniae* (strain 172) and two macrolide-resistant strains, 2507 and 521, which express the *erm*(B) and *mef*(A) genes, respectively, all isolated in South Africa and known to be serotype 23F, were used in this study, and the molecular/microbiological procedures used to confirm the identity of these strains are described in detail elsewhere.[3] Currently, 23F is an important clonal, clinical isolate that has spread to many countries, and for which we had a well-characterized laboratory strain, as well as genetically identical clinical isolates with varying degrees of macrolide resistance.[3]

Effects of the antimicrobial agents on pneumolysin production

To investigate the effects of the test antimicrobial agents on pneumolysin production, uncomplicated by their inhibitory effects on bacterial proliferation, the macrolide-susceptible and macrolide-resistant strains of *S. pneumoniae* were cultured in tryptone soy broth (TSB; Biolab Diagnostics, Johannesburg, South Africa), for 6 h at 37°C in an atmosphere of 5% CO_2, after which they were harvested by centrifugation, then transferred to and washed in indicator-free tissue culture medium RPMI 1640 [Highveld Biological (Pty) Ltd, Johannesburg, South Africa]. Bacterial suspensions were then adjusted to give concentrations of $\sim 0.5-3 \times 10^8$ cfu/mL, depending on the strain. The bacteria were incubated for 1 h at 37°C in an atmosphere of 5% CO_2 with the 11 different test antimicrobial agents at a fixed, final concentration of 0.1 mg/L. Following the 1 h incubation period, bovine serum albumin (5 g/L final; Sigma Chemical Co) was added to each tube, followed by a further incubation period of 16 h, after which pneumolysin was assayed both in the bacteria-free supernatants and in the lysates (sonicates), as described below. Pneumolysin in supernatants/sonicates was also measured at the outset, immediately before exposure of the bacteria to the antibiotics.

Pneumolysin assay

Pneumolysin in the bacteria-free supernatants and sonicates was measured using a fura-2/AM (Sigma Chemical Co)-based spectrofluorimetric procedure that detects toxin-mediated influx of Ca^{2+} into isolated human neutrophils, as described in detail elsewhere.[3]

Effects of clarithromycin on bacterial protein synthesis

Each of the three strains of the pneumococcus was cultured in the absence or presence of 0.1 mg/L clarithromycin, as mentioned earlier, in RPMI 1640 supplemented with 0.5 mCi/L of a radiolabelled amino acid mixture (L-amino acid mixture ^{14}C[U], 37 MBq, Du Pont-NEN Products, Boston, MA, USA) and incubated at 37°C/5% CO_2. After 6 h of incubation, the bacteria were pelleted by centrifugation and washed, followed by the addition of warm 5% trichloroacetic acid to lyse the bacteria and release proteins. Radioactivity in the lysates was measured using liquid scintillation spectrometry.

Statistical analysis

The results of each series of experiments are presented as mean values $\pm$ SEMs. Levels of statistical significance were calculated using the Student's *t*-test (unpaired *t* statistic).

Results

MIC values

MIC values of each strain are shown in Table 1. As expected, strains 2507 and 521 were resistant to azithromycin, clarithromycin and erythromycin, whereas strain 2507 was resistant to clindamycin, and both strains were susceptible to telithromycin. Strain 172 was susceptible to all macrolides/macrolide-like agents. All three test strains of *S. pneumoniae* were susceptible to the remaining antibiotics. For all of the strains tested, 0.1 mg/L represented either a sub-MIC or was close to the MIC value; doxycycline and telithromycin were the exceptions with MIC values for each strain being higher and lower, respectively, than 0.1 mg/L. Strain 521 was particularly susceptible to amoxicillin, whereas strain 2507 was resistant to clindamycin.

Table 1. MICs of the test antimicrobial agents for the macrolide-susceptible and macrolide-resistant strains of *S. pneumoniae*

	MIC (mg/L)		
Antimicrobial agents	172[a]	2507[b]	521[b]
Azithromycin	0.5	>256	8
Clarithromycin	0.064	>256	2
Erythromycin	0.094	>256	4
Telithromycin	0.015	0.03	0.015
Clindamycin	0.06	>256	0.06
Amoxicillin	0.75	0.25	0.023
Ceftriaxone	1	0.5	0.06
Ciprofloxacin	0.19	0.38	0.38
Moxifloxacin	0.05	0.094	0.064
Tobramycin	0.125	0.19	0.125
Doxycycline	4	4	8

[a]Macrolide-susceptible.
[b]Macrolide-resistant.

Pneumolysin

The effects of the test antibiotics on the production of total (intracellular + extracellular) pneumolysin by the macrolide-susceptible and two macrolide-resistant strains of *S. pneumoniae* are shown in Figure 1. Exposure of all three strains to clarithromycin, erythromycin, telithromycin and clindamycin was accompanied by significant decreases in the levels of pneumolysin. The effects of azithromycin, although similar to those of the other macrolides/macrolide-like agents, were of a lesser magnitude, and, in several instances, did not achieve statistical significance. With the exception of amoxicillin, which suppressed the production of pneumolysin by strain 521, probably due to the high level of sensitivity of strain 521 to this antibiotic, none of the other antimicrobial agents affected the production of the toxin. The inhibitory effects of the antibiotics on the concentration of extracellular and total pneumolysin were comparable (data not shown).

Because of the relatively high MIC values of doxycycline for all three strains of the pneumococcus, we also investigated the effect of this agent at a concentration of 2 mg/L on toxin production. At this higher concentration, doxycycline caused significant inhibition of pneumolysin production by all three strains of the pneumococcus; the mean percentages of inhibition (± SEMs) are 63 ± 5, 34 ± 5 and 67 ± 3 ($P < 0.05$ for each value) for strains 172, 521 and 2507, respectively.

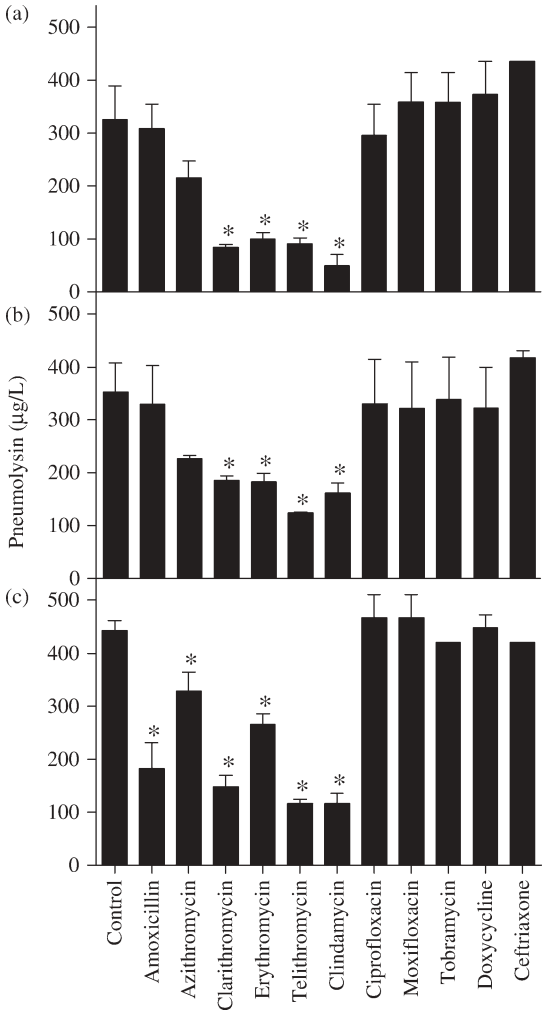

Figure 1. Effects of the test antimicrobial agents on the production of pneumolysin by (a) strain 172 (macrolide-susceptible) and strains (b) 2507 and (c) 521 (macrolide-resistant, *erm* and *mef*, respectively) of *S. pneumoniae*. The results of four experiments are expressed as the mean values ± SEM for total pneumolysin; $*P < 0.05$ for comparison with the corresponding antibiotic-free control systems.

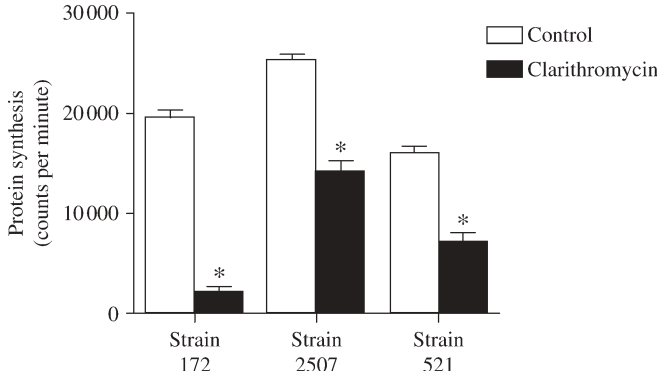

Figure 2. Effects of clarithromycin (0.1 mg/L) on protein synthesis by strain 172 (macrolide-susceptible) and strains 2507 and 521 (macrolide-resistant, *erm* and *mef*, respectively) of *S. pneumoniae*. The results of three different experiments with three to five replicates for each system are expressed as the mean values ± SEM for total protein synthesis; $*P < 0.05$ for comparison with the corresponding clarithromycin-free control systems.

The numbers of viable bacteria (cfu) were 2.1 ± 0.64, 1.5 ± 0.4 and 1.2 ± 0.5 × 10^8 cfu/mL for strains 172, 2507 and 521, respectively, at the outset, whereas the corresponding values after 16 h of incubation in RPMI were 1.3 ± 0.8, 2.3 ± 0.5 and 0.9 ± 0.2 × 10^6 cfu/mL. With the exception of significantly ($P < 0.05$) decreased numbers of viable bacteria following the 16 h exposure of strain 521 to amoxicillin (0.9 ± 0.2 × 10^6 versus 0.2 ± 0.1 × 10^6 cfu/mL), there were no significant differences between the numbers of viable bacteria in systems without and with the antibiotics after 16 h of incubation.

Protein synthesis

The effects of clarithromycin (0.1 mg/L) on bacterial protein synthesis are shown in Figure 2. Significant ($P < 0.05$) inhibition of protein synthesis was observed with all three strains of the pneumococcus.

Discussion

Only the macrolides and macrolide-like antibiotics were found to inhibit the production of pneumolysin by all three strains of the pneumococcus, with clarithromycin, erythromycin, telithromycin and clindamycin exhibiting comparable activities, whereas azithromycin was generally somewhat less active. Importantly, macrolides, at the concentration used in the current study, do not interfere with the pneumolysin assay system.[3] Neither doxycycline nor tobramycin, both inhibitors of bacterial protein synthesis, affected the production of pneumolysin by any of the test strains of the pneumococcus. However, increasing the concentration of doxycycline to 2 mg/L resulted in significant inhibition of the synthesis of pneumolysin by all three strains of the pneumococcus.

Inhibition of protein synthesis, as opposed to possible non-ribosomal mechanisms of antimicrobial activity, appears to be involved in the macrolide-mediated inhibition of synthesis of pneumolysin by macrolide-resistant strains of the pneumococcus. This contention is based on the observation that clarithromycin (0.1 mg/mL) inhibited protein synthesis by macrolide-resistant strains of the pneumococcus, albeit to a lesser extent than that observed with the macrolide-susceptible strain. Macrolides and clindamycin, at subinhibitory concentrations, have also been reported to interfere with several virulence-related activities of *Pseudomonas aeruginosa*, including biofilm formation, twitching motility and quorum sensing.[5–8] Interestingly, these unusual effects of macrolides on *P. aeruginosa*, as well as those on pneumolysin production by macrolide-resistant strains of the pneumococcus described in the current study, are unlikely to be detected by conventional assays of *in vitro* antibiotic susceptibility testing.

In comparison with the other classes of antibiotic tested, macrolides at sub-MICs effectively antagonize the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of the pneumococcus, compatible with a role for these agents as adjuncts to β-lactams in the treatment of severe pneumococcal disease.[9,10]

Funding

The study was supported in part by a grant awarded to R. C. by the National Health Laboratory Service of South Africa Research Trust. R. A. has received a research grant from Abbott Laboratories in partial support of the current study.

Transparency declarations

C. F. has acted on the advisory board of pharmaceutical companies manufacturing and/or marketing antibiotics (MSD, Abbott, Pfizer and Sanofi-Aventis) and has received honoraria for lectures from pharmaceutical companies manufacturing and/or marketing antibiotics (Abbott, MSD, GlaxoSmithKline, Pfizer and Sanofi-Aventis) and has also received assistance for congress attendance from pharmaceutical companies manufacturing antibiotics (Abbott, GlaxoSmithKline and Sanofi-Aventis). K. P. K. is in receipt of research funding from Bayer and Sanofi-Aventis and has received consultant fees from Bayer, Sanofi-Aventis and GlaxoSmithKline in the last year.

References

1. Lagrou K, Peetermans WA, Jorrisen M *et al.* Subinhibitory concentrations of erythromycin reduce pneumococcal adherence to respiratory epithelial cells *in vitro*. *J Antimicrob Chemother* 2000; **46**: 717–23.

2. Fukuda Y, Yanagihara K, Higashiyama Y *et al.* Effects of macrolides against pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. *Eur Respir J* 2006; **27**: 1020–5.

3. Anderson R, Steel HC, Cockeran R *et al.* Clarithromycin alone and in combination with ceftriaxone inhibits the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of *Streptococcus pneumoniae*. *J Antimicrob Chemother* 2007; **59**: 224–9.

4. Musher DM, Phan HM, Baughn RE. Protection against bacteremic pneumococcal infection by antibody to pneumolysin. *J Infect Dis* 2001; **183**: 827–30.

5. Ichimiya T, Yamasaki T, Nasu M. *In-vitro* effects of antimicrobial agents on *Pseudomonas aeruginosa* biofilm formation. *J Antimicrob Chemother* 1994; **34**: 331–41.

6. Wozniak DJ, Keyser R. Effects of subinhibitory concentrations of macrolide antibiotics on *Pseudomonas aeruginosa*. *Chest* 2004; **125** Suppl 2: 62S–9S.

7. Tateda K, Standiford TJ, Pechere JC *et al.* Regulatory effects of macrolides on bacterial virulence. *Curr Pharm Res* 2004; **10**: 3055–65.

8. Naka Y, Jansch L, Bredenbruch F *et al.* Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PA01: a global approach. *Antimicrob Agents Chemother* 2006; **50**: 1680–8.

9. MacFarlane JT, Boswell T, Douglas G *et al.* British Thoracic Society guidelines for the management of community-acquired pneumonia in adults. *Thorax* 2001; **56**: 1–64.

10. Waterer GW. Monotherapy versus combination therapy for pneumococcal pneumonia. *Curr Opin Infect Dis* 2005; **18**: 157–63.