*Journal of Antimicrobial Chemotherapy* (1998) **41**, 289–291

JAC

# Activity of azithromycin, clarithromycin, roxithromycin, dirithromycin, quinupristin/dalfopristin and erythromycin against *Legionella* species by intracellular susceptibility testing in HL-60 cells

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**We evaluated a human monocyte cell line (HL-60) as a model for testing the intracellular activity of anti-*Legionella* antibiotics; 1.5 x $10^6$ HL-60 cells/well were differentiated into adherent cells and infected with 1.5 x $10^7$ cfu of *Legionella pneumophila*. The most active agents against *L. pneumophila* as judged by broth dilution MICs were (in order of activity) azithromycin, clarithromycin, roxithromycin, quinupristin/dalfopristin, erythromycin and dirithromycin. The most active inhibitors of *L. pneumophila* intracellular multiplication were (in order of activity) azithromycin, erythromycin, quinupristin/dalfopristin, roxithromycin, dirithromycin and clarithromycin. All the agents were highly active against *Legionella micdadei* and *Legionella bozemanii* when compared with *L. pneumophila*.**

## Introduction

Erythromycin has been considered to be the drug of choice for the treatment of Legionnaires' disease,[1] but major drawbacks have become apparent with accumulated clinical experience, including a requirement for a large volume of fluid for parenteral administration, phlebitis, gastrointestinal intolerance and ototoxicity. We compared the in-vitro susceptibility of *Legionella pneumophila* serogroup 1 and other *Legionella* species to erythromycin and newer antimicrobial agents by broth dilution and the HL-60 intracellular model.

## Materials and methods

### *Bacterial strains*

Isolates of *Legionella* spp. used for broth dilution susceptibility testing included *L. pneumophila* serogroup 1 ATCC 33152 and nine patient isolates obtained from our Special Pathogens Laboratory: *Legionella micdadei* ATCC 33218 and *Legionella bozemanii* serogroup 1 ATCC 33217 and serogroup 2 ATCC 35545. A patient isolate of *L. pneumophila* serogroup 1 (VA no. 1074), *L. micdadei* (ATCC 33218) and *L. bozemanii* (ATCC 33217) were used in the HL-60 intracellular assay.

### *Broth microdilution susceptibility testing*

MIC determinations were made in 96-well microtitre plates as previously described.[2] Control strains were *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

### *Infection and intracellular susceptibility testing in HL-60 cells*

HL-60 cells were obtained from the American Type Culture Collection, Rockville, MD and were maintained and differentiated into macrophage-like cells as previously described.[3] Cells were added to 24-well tissue culture plates and incubated for 48 h at 37°C in 5% $CO_2$. *Legionella* was added to the monolayer at a bacteria:cell ratio of 10:1. Cells were incubated for 6 h and the extracellular bacteria were removed by four washes in Hanks' balanced salt solution. Medium with antibiotic at 1 × MIC and 8 × MIC was added and the cells were incubated for

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48 h. Cells were removed with 0.5% trypsin–0.53 mM EDTA and cell-associated bacteria were counted from duplicate wells by hypotonic lysis of the cells with sterile distilled water followed by serial dilution and plate count on buffered charcoal yeast extract agar.[2]

Each macrolide was tested at 1 × MIC and 8 ×MIC, in duplicate, within the same experiment, on three separate days. The 1 × MIC concentrations used in the intracellular assay were (i) for *L. pneumophila*: erythromycin, 0.25 mg/L; azithromycin, 0.25 mg/L; quinupristin/dalfopristin, 0.25 mg/L; roxithromycin, 0.25 mg/L; dirithromycin, 1.0 mg/L; clarithromycin, 0.25 mg/L; (ii) for *L. micdadei*: erythromycin, 1.0 mg/L; azithromycin, 0.5 mg/L; quinupristin/dalfopristin, 0.25 mg/L; roxithromycin, 1.0 mg/L; dirithromycin, 4.0 mg/L; clarithromycin, 0.125 mg/L; (iii) for *L. bozemanii*: erythromycin, 0.125 mg/L; azithromycin, 0.125 mg/L; quinupristin/dalfopristin, 0.06 mg/L; roxithromycin, 0.125 mg/L; dirithromycin, 16.0 mg/L; clarithromycin, 0.03 mg/L. Results were expressed as percentage inhibition, defined as total *Legionella* at 48 h with agent divided by total *Legionella* at 48 h without agent × 100. Thus, values of >100% indicated an absence of inhibition by the agent, whereas values of <100% indicated an inhibitory effect.

### *Statistical analysis*

Analysis of variance (ANOVA) was used to determine significant ($P = 0.05$) differences in mean percent change of control from a minimum of three separate experiments. Dunnett's test was used for pairwise comparisons with erythromycin. Data were transformed from total cfu/mL to $\log_{10}$ cfu for this analysis.

## Results

### *Broth microdilution susceptibility testing*

Greater in-vitro activity in terms of MIC was shown for azithromycin (0.06–1.0 mg/L), clarithromycin (0.03–0.25 mg/L), roxithromycin (0.06–0.25 mg/L) and quinupristin/dalfopristin (0.125–0.25 mg/L) compared with erythromycin (0.125–1.0 mg/L) against *L. pneumophila.* For *L. micdadei*, clarithromycin (0.125 mg/L), azithromycin (0.5 mg/L) and quinupristin/dalfopristin (0.25 mg/L) were more active than erythromycin (1.0 mg/L); roxithromycin (1.0 mg/L) was equally active, and dirithromycin (4.0 mg/L) was less active. For *L. bozemanii*, clarithromycin (0.03 mg/L) was more active than erythromycin (0.125 mg/L); azithromycin, roxithromycin and quinupristin/dalfopristin were equally active (0.125 mg/L); and dirithromycin was less active (8.0 mg/L). The non-*Legionella* control organisms had MICs within the published ranges of the NCCLS.

### *HL-60 intracellular model*

The percentage increases in $\log_{10}$ cfu within HL-60 cells at 48 h post-infection compared with the inoculum for each species for this series of experiments were as follows: *L. pneumophila*: 113% (4.8 ± 0.3 vs 4.2 ± 0.4; six experiments); *L. micdadei*: 126% (5.66 ± 0.3 vs 4.49 ± 0.3; four experiments), *L. bozemanii*: 130% (5.47 ± 0.6 vs 4.11 ± 0.4; four experiments).

At 1 × MIC, the mean percentage reduction for azithromycin against *L. pneumophila* was significantly grater than that for erythromycin (43.4% vs 16.8%; $P < 0.05$, Dunnett's test). The mean percentage reduction ± S.D. for *L. pneumophila* against each agent at 1 × MIC and 8 × MIC was as follows: azithromycin, 43.4 ± 4.7 and 60.9 ± 11.4; erythromycin, 16.8 ± 12.1 and 44.9 ± 9.9; quinupristin/dalfopristin −11.4 ± 35.1 and 39.6 ± 26.6; roxithromycin, 16.44 ± 22.5 and 28.7 ± 11.8; dirithromycin, −6.2 ± 36.7 and 4.88 ± 44.4; clarithromycin, −11.4 ± 35.1 and −4.07 ± 37.3. *L. micdadei* and *L. bozemanii* were very susceptible to all the agents tested, especially at 8 × MIC (Table).

## Discussion

Various in-vitro and in-vivo models have been used to study the intracellular activity of antimicrobial agents against *L. pneumophila*,[1] but no one model has emerged as the 'gold standard'. U937 and HL-60 cells are human-derived cell lines of the monocyte–macrophage lineage (the host cells in which *Legionella* replication occurs in the human body) and have been used as models for the study of *Legionella* intracellular parasitism and pathogenesis.[4–6]

We selected the HL-60 promyelocytic leukaemic cell line as a model for our studies because: (i) intracellular multiplication of *L. pneumophila* in differentiated HL-60 cells has been shown to be similar to that in normal human monocyte–macrophages,[3,5] and (ii) γ-interferon activation of HL-60 cells inhibits intracellular growth of *Legionella* in a similar manner to that in normal human monocyte–macrophages.[5] We also found that rifampicin and erythromycin, two established antimicrobial agents for the treatment of Legionnaires' disease, proved to be active in this cell line in contrast to cefamandole, an antibiotic that is not effective in the treatment of Legionnaires' disease (data not shown). In addition, the HL-60 cell culture model allowed us to compare the activity of up to six antimicrobial agents under the same experimental conditions.

Our MIC results for *L. pneumophila* were similar to those from other in-vitro broth dilution studies and were used in determining intracellular activity.[7,8] Except for clarithromycin, each of the agents inhibited *L. pneumophila* intracellular multiplication at 8 × MIC (Table). Only azithromycin demonstrated superior activity in the intracellular assay when compared with erythromycin ($P < 0.05$). This finding is consistent with two other studies

**Table.** Effects of macrolide antibiotics and quinupristin/dalfopristin on the intracellular multiplication of *Legionella* species at 1 × MIC and 8 × MIC

| Antimicrobial agent | MIC | Percentage inhibition[a] ± S.D. | | |
|---|---|---|---|---|
| | | *L. pneumophila* | *L. micdadei* | *L. bozemanii* |
| Azithromycin | 1× | 56.6 ± 4.1 | 14.4 ± 15.2 | 1.1 ± 0.1 |
| | 8× | 42.1 ± 7.3 | 5.9 ± 4.2 | 0.6 ± 0.3 |
| Erythromycin | 1× | 83.2 ± 10.3 | 28.2 ± 3.2 | 15 ± 14.7 |
| | 8× | 55.0 ± 8.6 | 4.9 ± 2.6 | 0.2 ± 0.1 |
| Quinupristin/dalfopristin | 1× | 111.7 ± 31 | 78.7 ± 9.5 | 83.1 ± 15.1 |
| | 8× | 60.4 ± 23.1 | 17.5 ± 9.0 | 33.2 ± 9.0 |
| Roxithromycin | 1× | 83.6 ± 19.5 | 9.11 ± 0.5 | 8.0 ± 2.2 |
| | 8× | 71.4 ± 10.4 | 7.8 ± 0.12 | 4.1 ± 0.4 |
| Dirithromycin | 1× | 106.1 ± 31.8 | 11.0 ± 0.7 | 0.9 ± 0.8 |
| | 8× | 95.1 ± 38.4 | 7.0 ± 6.0 | 0.07 ± 0.9 |
| Clarithromycin | 1× | 102.2 ± 26.9 | 8.3 ± 6.7 | 1.1 ± 1.1 |
| | 8× | 104.0 ± 32.3 | 6.2 ± 5.3 | 0.2 ± 0.2 |

[a]Percentage inhibition is calculated as total number of *Legionellae* at 48 h with agent/total number of *Legionellae* at 48 h without agent × 100. Values are the mean of a minimum of three experiments. Lower ratios imply greater activity.

where azithromycin demonstrated better activity than erythromycin *in vitro* and in a guinea pig model.[9,10] *L. micdadei* and *L. bozemanii* were more susceptible than *L. pneumophila* to all of the agents tested (Table).

Our method of using multiples of the MIC as the concentration for intracellular testing may have underestimated the activity of clarithromycin since it can achieve high tissue and serum concentrations. We repeated the experiments by performing limited testing using concentrations that are achievable in tissue for clarithromycin, erythromycin and azithromycin (2.0–20.0 mg/L). The percentage inhibition of *Legionella* intracellular multiplication by clarithromycin was comparable to that by erythromycin (data not shown), but azithromycin still demonstrated the greatest inhibitory activity.

Studies to assess the applicability of the HL-60 model for intracellular susceptibility testing of *Legionella* with other classes of antimicrobial agents are continuing. The agents that performed well in this in-vitro study warrant clinical evaluation for the treatment of Legionnaires' disease.

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*Received 10 September 1996; returned 27 November 1996; revised 26 February 1997; accepted 18 September 1997*