

In Vitro Activity of Ro 23-9424 against Clinical Isolates of *Legionella* Species

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Agar and broth microdilution MICs of Ro 23-9424 that inhibited 90% of 22 *Legionella* clinical isolates tested were 0.64 and 0.08 µg/ml, respectively; respective erythromycin values were 1.0 and 0.12 µg/ml. Ro 23-9424 (1 µg/ml) was slightly more active than the same erythromycin concentration in a macrophage system, for both *Legionella pneumophila* strains studied.

Ro 23-9424 is a dual-action antibacterial agent in which desacetyl cefotaxime is covalently linked to fleroxacin (8). The drug is hydrolyzed to a limited extent in vivo and in vitro, with release of free fleroxacin (2, 8). Ro 23-9424 is broadly active against both gram-positive and some gram-negative bacteria (1, 9). It is unknown whether Ro 23-9424 is concentrated in cells. Fleroxacin, but not cefotaxime, is active for intracellular *Legionella pneumophila* and in a guinea pig model of *L. pneumophila* pneumonia (6, 13, 14). We tested *Legionella* spp. with Ro 23-9424 by using a variety of susceptibility testing methods, designed to determine whether the drug is active for intracellular and extracellular *Legionella* spp. Two different extracellular susceptibility testing methods were used to allow comparison of results with those previously published by us and others using the two different methods (5-7, 10-13).

All legionellae studied were clinical isolates. These strains were identical to those used in prior studies and were composed of two strains each of *L. dumoffii*, *L. longbeachae*, and *L. micdadei*, one strain of *L. bozemanii*, and 15 strains of *L. pneumophila* (5, 7). *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as control organisms for susceptibility testing. Legionellae were grown on locally made buffered charcoal-yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEα) (3). Incubation of all media was done at 35°C in humidified air. Standard powders of Ro 23-9424 and fleroxacin were obtained from Hoffmann-LaRoche, Inc., Nutley, N.J.; erythromycin powder was obtained from Abbott Laboratories, North Chicago, Ill.

Agar dilution susceptibility testing was performed as described previously (5). Briefly, antimicrobial agent-containing BCYEα agar plates were inoculated with ≈10⁵ CFU of bacteria. The control *S. aureus* and *E. coli* strains were inoculated onto antimicrobial agent-containing Mueller-Hinton agar plates, as well as BCYEα plates, to determine whether BCYEα medium inhibited antimicrobial agent activity. The plates were incubated for either 24 h (nonlegionellae) or 48 h (legionellae), at which time MICs were determined. Broth microdilution susceptibility testing was performed by using buffered yeast extract broth supplemented with 0.1% α-ketoglutarate (BYEα) (legionellae) or with Mueller-Hinton broth (nonlegionellae), with a final volume of 200 µl and a final bacterial concentration of 5 ×

10⁵ CFU/ml (3). Otherwise, the microdilution method was performed exactly as described previously for a macrodilution method (5). All testing was done in duplicate, and results were expressed as geometric means. A MIC found to be less than or equal to the lowest antimicrobial agent concentration tested was arbitrarily defined to be the lowest concentration tested. Erythromycin was included as a control; data for the activity of this drug for the *Legionella* strains we tested, as measured by agar dilution susceptibility testing, have been presented previously (5).

Guinea pig pulmonary alveolar macrophages were harvested and purified as described previously (5). The final concentration of macrophages was approximately 10⁵ cells per well. Antimicrobial susceptibility testing of intracellular *L. pneumophila* was performed as described previously (5). Briefly, ≈10⁴ CFU of washed BCYEα plate-grown *L. pneumophila* was added to the purified alveolar macrophages. The bacteria and macrophages were incubated for 1 day after 1 h of incubation with shaking. Antimicrobial agents were added to the respective wells, after the wells had been washed three times to remove nonadherent bacteria. Sonic extracts of two replicate, non-antimicrobial agent-containing wells were quantitatively cultured for use as the day 1 bacterial count. Non-antimicrobial agent-containing wells were used as growth controls. After 2 more days of incubation, the supernatants were sampled and quantitatively cultured; all wells were then washed to remove antimicrobial agents. Bacterial counts in the supernatant of each well were determined for another 4 days. To check for antimicrobial agent toxicity, uninfected macrophages were incubated with the highest concentration of antimicrobial agent tested and observed microscopically daily. The relative activities of Ro 23-9424 and fleroxacin were determined for one *L. pneumophila* strain; the activity of fleroxacin alone (0.5 µg/ml) was contrasted to that of the combination drug (1 µg/ml). The fleroxacin extracellular broth microdilution MIC for *L. pneumophila* strain F2111 is 0.02 µg/ml (6). All experiments were carried out in triplicate, and quantitative plating was carried out in duplicate.

Ro 23-9424 and erythromycin agar and broth microdilution MICs for the 22 *Legionella* strains tested are shown in Table 1. A single *L. micdadei* strain did not grow well enough in BYEα broth for broth microdilution testing to be performed, allowing only 21 *Legionella* strains to be tested by this method. The erythromycin broth microdilution MIC for another *L. micdadei* strain (0.5 µg/ml) was much higher than that for any other strain tested. Erythromycin broth micro-

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TABLE 1. Agar and broth microdilution susceptibilities of 22 *Legionella* sp. strains

Drug	MIC ($\mu\text{g/ml}$) with:					
	BCYE α agar			BYE α broth		
	MIC ₅₀	MIC ₉₀	Range	MIC ₅₀	MIC ₉₀	Range
Ro 23-9424	0.64	0.64	0.16–1.28	0.08	0.08	0.04–0.08
Erythromycin	0.12	1.0	≤ 0.06 –1.0	0.06	0.12	≤ 0.06 –0.5

dilution MICs for strains F889 and F2111 were ≤ 0.06 and $0.125 \mu\text{g/ml}$, respectively. Ro 23-9424 broth microdilution MICs for the same strains were $0.08 \mu\text{g/ml}$.

Erythromycin, but not Ro 23-9424, was slightly inhibited by BCYE α agar, as determined by its MIC for the control *S. aureus* strain by using BCYE α and Mueller-Hinton agar media. The erythromycin MIC for *S. aureus* was 1 log₂ dilution greater with BCYE α agar. However, BCYE α agar inhibition of Ro 23-9424 was observed when MICs for *E. coli* were determined; the Ro 23-9424 MIC for the *E. coli* control strain was 2 log₂ dilutions greater with BCYE α agar than with Mueller-Hinton agar. Ro 23-9424 and erythromycin MICs for the non-*Legionella* sp. control strains were both 1 log₂ dilution greater with BYE α broth than with Mueller-Hinton broth.

The major differences in MICs with agar and broth dilutions that were detected in this study have been noted by us and others previously (5–7, 11). Many feel that use of non-charcoal-containing test media gives the most accurate results because of decreased antibiotic inhibition by these media, in comparison to results obtained by using BCYE α medium. However, there exist no clinical data which would determine which method is more accurate. The additional complication of sometimes very poor correlation between in vitro susceptibility of *L. pneumophila* and results of clinical and animal studies makes such in vitro testing only a very rough guide to potential clinical utility of antimicrobial agents for the treatment of Legionnaires' disease (reviewed in reference 4). No previous studies of the activity of Ro 23-9424 against *Legionella* spp. have been done, making comparison of these results with those of other studies impossible. Both cefotaxime and feroxacin alone are active against extracellular *L. pneumophila*, with feroxacin MICs for 90% of strains tested (MIC₉₀s) of 0.64 and $0.04 \mu\text{g/ml}$ by agar and broth microdilutions, respectively, for the same strains used in this study (6) and cefotaxime MIC₉₀s of 4 and $\leq 0.12 \mu\text{g/ml}$ by agar and broth microdilutions, respectively, for different *L. pneumophila* strains (10, 12). Since we did not test equimolar concentrations of the two Ro 23-9424 components, it is unknown how similar the activity of the combination drug is to that of the combined free components.

The intracellular activity of Ro 23-9424 and erythromycin for two *L. pneumophila* serogroup 1 strains grown in guinea pig alveolar macrophages is shown in Fig. 1 and 2. For *L. pneumophila* strain F889 (Fig. 1), both drugs were only inhibitory in their activity, although there was a significantly longer postantibiotic effect observed for Ro 23-9424. Both erythromycin and Ro 23-9424 were similar in their inhibitory activity for *L. pneumophila* F2111 (Fig. 2). Fleroxacin alone ($0.5 \mu\text{g/ml}$) was more active than Ro 23-9424 ($1.0 \mu\text{g/ml}$), although the feroxacin concentration was the same in both the single and combination drugs. The slight decrease in viable counts of strain F2111 observed on day 5, for eryth-

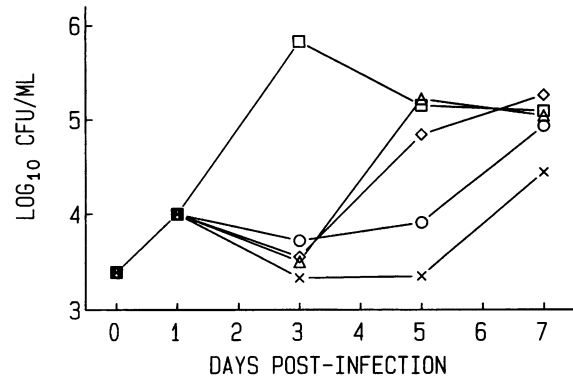


FIG. 1. Growth of *L. pneumophila* F889 serogroup 1 in guinea pig alveolar macrophages at postinfection days 1 to 7. Symbols: \square , growth control; Δ and \diamond , 0.25 and $1.0 \mu\text{g}$ of erythromycin per ml, respectively; \circ and \times , 0.25 and $1.0 \mu\text{g}$ of Ro 23-9424 per ml, respectively.

romycin and Ro 23-9424 (both $0.25 \mu\text{g/ml}$), is artificial because of macrophage killing by the bacteria by day 3; many of the bacteria in these tissue culture wells were washed out with the damaged macrophages. Macrophage toxicity caused by antimicrobial agents alone was not observed.

These studies show that Ro 23-9424 is about as active as erythromycin for both extracellular and intracellular *Legionella* spp., with neither drug being bactericidal. The significantly greater activity of feroxacin than that of the combination drug is probably related to limited extracellular hydrolysis of the combination drug, which releases free feroxacin. The free feroxacin is probably more concentrated in macrophages than is the combination drug. Because *L. pneumophila* is protected from extracellular antimicrobial agents by its intracellular location in the lungs and elsewhere in the body, the in vitro activity of drugs against intracellular *L. pneumophila* is generally a good predictor of their clinical activity. Ro 23-9424 has potential use for the treatment of Legionnaires' disease, and possibly other intracellular infections, pending animal model treatment studies and clinical trials. Whether the combination drug would be more or less

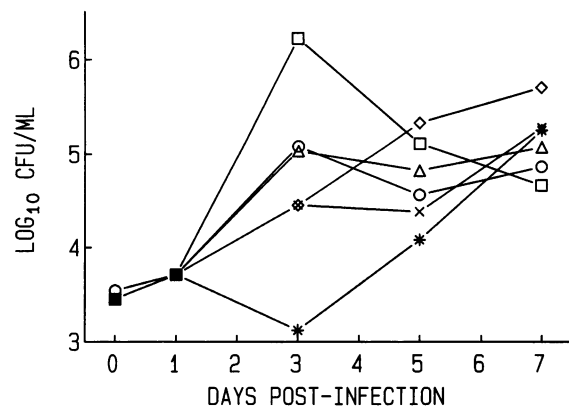


FIG. 2. Growth of *L. pneumophila* F2111 serogroup 1 in guinea pig alveolar macrophages at postinfection days 1 to 7. Symbols: \square , growth control; Δ and \diamond , 0.25 and $1.0 \mu\text{g}$ of erythromycin per ml, respectively; \circ and \times , 0.25 and $1.0 \mu\text{g}$ of Ro 23-9424 per ml, respectively; *, $0.5 \mu\text{g}$ of feroxacin per ml.

clinically effective than erythromycin can only be determined by comparative clinical studies.

This study was funded in part by Hoffmann-LaRoche, Inc. Minxia Liu provided excellent technical assistance.

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