ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1989, p. 2132–2136
0066-4804/89/122132-05$02.00/0

Vol. 33, No. 12

# WIN 57273 Is Bactericidal for *Legionella pneumophila* Grown in Alveolar Macrophages

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Received 28 July 1989/Accepted 11 September 1989

**The in vitro antimicrobial activity of WIN 57273, a new quinolone antimicrobial agent, was determined for 21 *Legionella* strains, using broth macrodilution and agar dilution testing methods; ciprofloxacin and erythromycin were tested as well. Three different buffered yeast extract media were used for the agar dilution studies, two of which were made with starch rather than charcoal. Broth macrodilution susceptibility testing was performed with buffered yeast extract broth and two *Legionella pneumophila* strains. Antimicrobial inhibition of *L. pneumophila* growth in guinea pig alveolar macrophages was also studied, using a method able to detect bacterial killing. The MICs for 90% of the 21 strains of *Legionella* spp. grown on buffered charcoal yeast extract medium were 0.125 μg/ml for WIN 57273, 0.25 μg/ml for ciprofloxacin, and 1.0 μg/ml for erythromycin. These MICs were falsely high, because of inhibition of drug activity by the medium used. Use of less drug-antagonistic, starch-containing media did not support good growth of the test strains. The broth macrodilution MICs for two strains of *L. pneumophila* serogroup 1 were ≤0.03 μg/ml for WIN 57273 and ciprofloxacin and 0.125 μg/ml for erythromycin. WIN 57273, ciprofloxacin, and erythromycin all inhibited growth of *L. pneumophila* in guinea pig alveolar macrophages at concentrations of 1 μg/ml, but only WIN 57273 prevented regrowth or killed *L. pneumophila* after removal of extracellular antimicrobial agent.**

Erythromycin is considered the treatment of choice for Legionnaires disease on the basis of retrospective studies. However, a major limitation of erythromycin therapy is that a long treatment course is required to prevent disease relapse (14). The search for alternatives to erythromycin therapy is compromised by the poor correlation of results of classical in vitro antimicrobial susceptibility testing with clinical experience (7, 11, 14). This discrepancy is likely due to antimicrobial antagonism by components of the media and to the protected intracellular site in which legionellae exist. Some investigators have claimed the superiority of one medium over another for the purposes of susceptibility testing, mainly in terms of limited inhibition of antimicrobial agents, but few comparative studies have been performed (2, 8, 13, 20).

Antimicrobial treatment of an animal model of Legionnaires disease has demonstrated good correlation with clinical observations, but is too expensive for screening purposes (3, 7, 10, 18–20). To address this, several investigators have validated the use of monocyte or macrophage growth inhibition tests to predict the effectiveness of antimicrobial agents in animal models (1, 9, 11, 20–22). However, reversible inhibition of bacterial growth has been detected in these cellular systems (12, 21).

We performed susceptibility testing of legionellae with erythromycin, ciprofloxacin, and WIN 57273, a new quinolone antimicrobial agent, to determine whether use of different media influenced susceptibility results and inhibition of antimicrobial agents. We also studied the effects of these antimicrobial agents on *Legionella* spp. growing in macrophages, with an assay capable of demonstrating nonreversible inhibition (killing). We demonstrate that WIN 57273 was able to inhibit irreversibly the growth of *Legionella pneumophila* grown in guinea pig alveolar macrophages and that there is a considerable medium effect on in vitro susceptibility results.

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## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All legionellae studied were clinical strains isolated by us with the exceptions of TEX-KL, Toronto 3, and Knoxville, which were obtained from William Bibb, Sandu Toma, and William Cherry, respectively (Table 1). All of the non-"named" strains had been passed fewer than three times previously; *L. pneumophila* serogroup 1 strain F889 has been described previously as Wadsworth strain 82-063A (7). *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 29522 were used as controls. To obtain inocula for susceptibility testing, legionellae were grown on locally made buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEα) (4), and nonlegionellae were grown on commercially made Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.). Incubation of all media was at 35°C in humidified air.

**Agar and broth dilution susceptibility testing.** Agar dilution susceptibility testing was performed with antibiotic-containing basal media. The basal media included locally made BCYEα, Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.), and locally made buffered yeast extract medium made with 1.5% starch, rather than charcoal, and supplemented with either 0.1% α-ketoglutarate (BSYEα) or 0.5% L-glutamate (BSYEγ) (4, 5, 21). All media were made with 10% less water to accommodate the antibiotic volumes added. Broth macrodilution susceptibility testing was performed with Mueller-Hinton broth (Difco) or locally made buffered yeast extract broth supplemented with 0.1% α-ketoglutarate (BYEα) (5). The pH of the buffered yeast extract media was 6.90 ± 0.05, and that of the Mueller-Hinton media was 7.3 ± 0.1. Standard powders of ciprofloxacin, WIN 57273, and erythromycin were obtained from

TABLE 1. Identity of the 21 clinical *Legionella* isolates tested

| Strain | *Legionella* species | Serogroup | Name |
|---|---|---|---|
| F1591 | *L. bozemanii* | 2 | Toronto 3 |
| F1478 | *L. dumoffii* | 1 | TEX-KL |
| F1717 | *L. dumoffii* | 1 | |
| F850 | *L. longbeachae* | 1 | |
| F1548 | *L. longbeachae* | 1 | |
| F774 | *L. micdadei* | 1 | |
| F1438 | *L. micdadei* | 1 | |
| F237 | *L. pneumophila* | 1 | |
| F736 | *L. pneumophila* | 1 | |
| F889 | *L. pneumophila* | 1 | |
| F1380 | *L. pneumophila* | 1 | |
| F1344 | *L. pneumophila* | 1 | |
| F1527 | *L. pneumophila* | 1 | Knoxville |
| F1821 | *L. pneumophila* | 1 | |
| F1924 | *L. pneumophila* | 1 | |
| F1948 | *L. pneumophila* | 1 | |
| F2111 | *L. pneumophila* | 1 | |
| F2127 | *L. pneumophila* | 1 | |
| F624 | *L. pneumophila* | 4 | LA 1 |
| F1807 | *L. pneumophila* | 6 | |
| F1864 | *L. pneumophila* | 9 | |

Miles Laboratories, Inc., West Haven, Conn.; Sterling Drug Inc., Rensselaer, N.Y.; and Abbott Laboratories, North Chicago, Ill., respectively.

Bacteria were grown on either BCYEα (legionellae) or Trypticase soy agar containing 5% sheep blood (nonlegionellae) agar overnight and then harvested to BYEα or Mueller-Hinton broth to approximate the turbidity of a no. 0.5 McFarland standard. To inoculate agar dilution plates, the bacterial suspensions were inoculated onto test media with a Steers replicator (Melrose Machine Shop, Chester, Pa.), which delivered approximately $10^5$ CFU per inoculum spot (8). Controls included non-antibiotic-containing media inoculated before and after stamping of each set of antimicrobial agent-containing media and subcultures of inoculum suspensions to assure purity. To determine the degree of antimicrobial inhibition by the buffered yeast extract-containing media, control strains were inoculated identically onto the buffered yeast extract-containing media and Mueller-Hinton agar. Results were recorded after 24 h of incubation for the nonlegionella bacteria and after 48 h of incubation for the legionellae. The presence of more than one colony was considered evidence of lack of antibiotic inhibition.

Broth macrodilution susceptibility testing was performed as described previously (7) for *L. pneumophila* F889 and F2111 only. The bacteria were grown, harvested, and diluted as described above. An additional 1:200 dilution of the bacterial suspension matching the no. 0.5 McFarland standard was made in the respective test media, and then the bacterial suspension (1 ml) was added to an equal volume of the test medium. The final concentration of bacteria was approximately $10^5$ CFU/ml. The control strains were tested in both BYEα and Mueller-Hinton broths to determine antimicrobial inhibition by the media. Broths were incubated without shaking for the same time periods as for the agar dilution studies. Any broth turbidity was considered evidence of growth.

**Growth of *L. pneumophila* in alveolar macrophages.** Pulmonary alveolar macrophages were obtained by bronchoalveolar lavage of 350- to 500-g male Hartley strain guinea pigs (VAF Plus; Charles River Laboratories, Wilmington, Mass.) as described previously, except that medium 199 (J&R Scientific, Woodland, Calif.) rather than RPMI 1640 was used (6). The macrophages were purified by centrifugation, washing, and plating in 22-mm-diameter Linbro tissue culture plates (Flow Laboratories, Inc., McLean, Va.) (6). The final concentration of macrophages was approximately $10^5$ cells per well. Macrophages were used on the day of harvest. Incubation conditions for all studies were 5% $CO_2$ in air and 37°C.

*L. pneumophila* F889 and F2111 grown overnight on BCYEα were used to infect the macrophages. Approximately $10^4$ bacteria were added to each well, although this varied in some experiments.

**Intracellular growth inhibition by antimicrobial agents.** Bacteria were incubated with macrophages for 2 h in a shaking incubator as described previously (6). Supernatant samples (100 μl) were then quantitatively cultured on BCYEα, after which antimicrobial agents were added to appropriate wells. Two days later, determinations of *L. pneumophila* concentrations in each well were performed by culturing supernatant samples on BCYEα. Some experiments were continued after this point by washing out the antimicrobial agents and then determining changes in bacterial count. To do this, the wells were washed (500 μl) three times with fresh tissue culture medium, and bacterial counts in the supernatant of each well were determined daily for another 5 days. All experiments were carried out in duplicate or triplicate, and quantitative plating was done in duplicate. All wells were observed microscopically daily to detect macrophage infection and to quantitate roughly the numbers of macrophages in the wells. In this system there is no extracellular growth of *L. pneumophila*, so all increases in supernatant bacterial concentration are the result of intracellular growth (6).

In an additional experiment designed to maximize intracellular bacteria prior to adding antimicrobial agents, the bacteria were incubated in stationary culture with the macrophages for 1 day after 1 h of shaking incubation. One set of replicate wells was then washed (500 μl) three times with tissue culture medium and sonicated by using a 13-mm-diameter sterile tip at a setting of 2.5 with 50% duty cycle for 10 s (model W225; Heat Systems Ultrasonics, Farmingdale, N.Y.). These sonication conditions do not affect bacterial counts (unpublished data). The sonic extract was cultured quantitatively as above, and the resulting bacterial count was used as the 1-day count. Antimicrobial agents were then added to the nonsonicated wells; to serve as growth controls, several wells had no antimicrobial agent added. The infected tissue cultures were then incubated for 2 days, after which supernatant samples were taken for quantitative culture. The antimicrobial agents were then removed by washing, and the experiment was continued as described in the preceding paragraph.

## RESULTS

**Agar dilution susceptibility.** All 21 *Legionella* strains tested were susceptible to all three antimicrobial agents tested with BCYEα medium base. There was no evidence of antimicrobial carry-over or bacterial contamination. Growth of *Legionella* spp. on antibiotic-free BSYEα and BSYEγ media was sparse in comparison to that observed on BCYEα medium, precluding accurate determination of MICs on these media; only about half of the *Legionella* strains tested grew at all on the starch-containing media during the 48-h incubation time. The MICs required to inhibit 90% of strains

TABLE 2. Agar dilution MICs for control strains of *S. aureus* ATCC 29213 and *E. coli* ATCC 29522 tested on BCYEα and Mueller-Hinton (MH) agars

| Drug | MIC (μg/ml) for: | | | |
|---|---|---|---|---|
| | *S. aureus* | | *E. coli* | |
| | BCYEα | MH | BCYEα | MH |
| WIN 57273 | 0.5 | ≤0.0625 | 2.0 | 0.125 |
| Ciprofloxacin | >2.0 | 0.5 | 0.25 | ≤0.0625 |
| Erythromycin | 2.0 | 0.5 | >2.0 | >2.0 |

cultured on BCYEα medium were 0.125, 0.25, and 1.0 μg/ml for WIN 57273, ciprofloxacin, and erythromycin, respectively. MIC ranges were ≤0.0625 to 0.25, 0.125 to 1.0, and 0.25 to 1.0 μg/ml for WIN 57273, ciprofloxacin, and erythromycin, respectively, with respective modal values of ≤0.0625, 0.25, and 0.25 μg/ml. *L. pneumophila* F889 and F2111 had MICs of WIN 57273 and ciprofloxacin of ≤0.0625 and 0.25 μg/ml, respectively; erythromycin MICs were 0.25 and 1.0 μg/ml for F889 and F2111, respectively.

All three antimicrobial agents were inhibited by BCYEα medium, (Table 2), with average fourfold-greater MICs when the *S. aureus* control strain was grown on BCYEα medium, as opposed to growth on Mueller-Hinton agar. There was, in general, one twofold dilution less antimicrobial inhibition when BSYEα and BSYEγ media were used as opposed to BCYEα (data not shown).

**Broth dilution susceptibility.** Both *L. pneumophila* strains tested, F889 and F2111, were more susceptible to all three antimicrobial agents when tested in broth rather than on agar. The MICs of both WIN 57273 and ciprofloxacin were ≤0.03 μg/ml for both bacterial strains, and that of erythromycin was 0.125 μg/ml for both strains. In contrast to BCYEα agar inhibition of antimicrobial agents, there was no difference in the MIC for the control *S. aureus* strain when tested with ciprofloxacin or erythromycin in either BYEα or Mueller-Hinton broth; MICs were within specified ranges (17). The WIN 57273 MICs for the control *E. coli* and *P. aeruginosa* strains were one twofold dilution lower in BYEα broth than in Mueller-Hinton broth and were within the limits specified by the manufacturer. The only exception to this good agreement was a fourfold-higher ciprofloxacin MIC for *P. aeruginosa* ATCC 27853 when tested in BYEα instead of Mueller-Hinton broth.

**Antimicrobial inhibition of intracellular growth.** Both *L. pneumophila* serogroup 1 strains grown in guinea pig alveolar macrophages were significantly inhibited after 48 h of incubation with erythromycin, WIN 57273, or ciprofloxacin, but not cefoxitin (Table 3).

While erythromycin, ciprofloxacin, and WIN 57273 were all inhibitory to *L. pneumophila* F889 grown in alveolar macrophages, only 1 μg of WIN 57273 per ml prevented bacterial regrowth after washing out of the antimicrobial agents (Fig. 1). This was true for the entire 5 days that cultures were taken after antimicrobial washout. Because this experiment only studied ciprofloxacin at a concentration of 0.25 μg/ml, the experiment was repeated with 1-μg/ml concentrations of ciprofloxacin and WIN 57273; both antimicrobial agents at this concentration prevented bacterial regrowth after antimicrobial washout on day 2 postinfection (data not shown).

A fourth experiment studied bacterial regrowth after first allowing macrophage infection to take place for 1 day before addition of antimicrobial agents (Fig. 2). Before antimicrobial agents were added, the macrophages were washed to remove nonadherent bacteria, and representative wells were sonicated to determine the number of intracellular bacteria. This experiment showed that only 1 μg of WIN 57273 per ml prevented bacterial regrowth after antimicrobial removal. In none of the four macrophage experiments did macrophage monolayers become detached before onset of grossly evident infection with *L. pneumophila*.

TABLE 3. Change in viable count of *L. pneumophila* F889 and F2111 in macrophage supernatants after 48 h of incubation with antimicrobial agents

| Antibiotic | Concn (μg/ml) | Change in viable count ($log_{10}$ CFU/ml) | |
|---|---|---|---|
| | | F889 | F2111 |
| None (control) | | +2.6[a] | +2.9[a] |
| Erythromycin | 1 | −0.2 | −0.5 |
| Cefoxitin | 5 | +2.5 | +2.7 |
| WIN 57273 | 0.25 | −1.4 | >−1.8 |
| | 1 | >−2.0 | >−1.8 |
| Ciprofloxacin | 0.25 | −0.8 | >−1.8 |

[a] Starting counts before antimicrobial addition were $10^{3.7}$ and $10^{3.5}$ CFU/ml for *L. pneumophila* F889 and F2111, respectively. All values are the means of triplicate experiments.

## DISCUSSION

WIN 57273, like other quinolone antimicrobial agents, is very active in vitro against *Legionella* spp. (3, 9, 11, 13, 16, 19–21). The agar dilution MICs obtained with BCYEα medium are probably falsely high, as judged by the behavior of control strains on Mueller-Hinton and BCYEα media. Unfortunately, less antibiotic-antagonistic solid media for testing *Legionella* spp. did not allow adequate growth of these organisms. The modified media we used behaved differently from that used by Saito and colleagues and Liebers and colleagues (13, 19, 20). We and others have previously reported on the failure of modified BCYEα media to support adequate growth of a broad range of *Legionella* strains (2, 8). A possible explanation for these discrepancies is strain variability; we used chiefly low-passage isolates, as opposed to the studies showing better results with modified BCYEα media. Regardless, the importance of using adequate growth and antibiotic activity controls is stressed.

Use of BYEα broth for susceptibility testing appears attractive based on our studies and those of others (11, 13, 16). The lack of antimicrobial antagonism and good growth of the strains we tested may make this a preferred method for susceptibility testing. We tested only two *L. pneumophila* strains with this medium, but others have successfully tested more strains (11, 13, 16); whether its use would be suitable for susceptibility testing of more fastidious *Legionella* strains is unknown.

We have demonstrated that WIN 57273 is highly inhibitory and apparently bactericidal for one strain of *L. pneumophila* grown in macrophages. This effect is undoubtedly because of intracellular, and not extracellular, inhibition or killing on the basis of several lines of evidence. First, *L. pneumophila* does not multiply extracellularly in the system studied. Second, cefoxitin, which is highly active against *L. pneumophila* in BYEα broth (MIC, ≤0.25 μg/ml), was unable to inhibit the growth of *L. pneumophila* in these experiments. Thus, these experiments truly studied the ability of antimicrobial agents to inhibit intracellular growth.

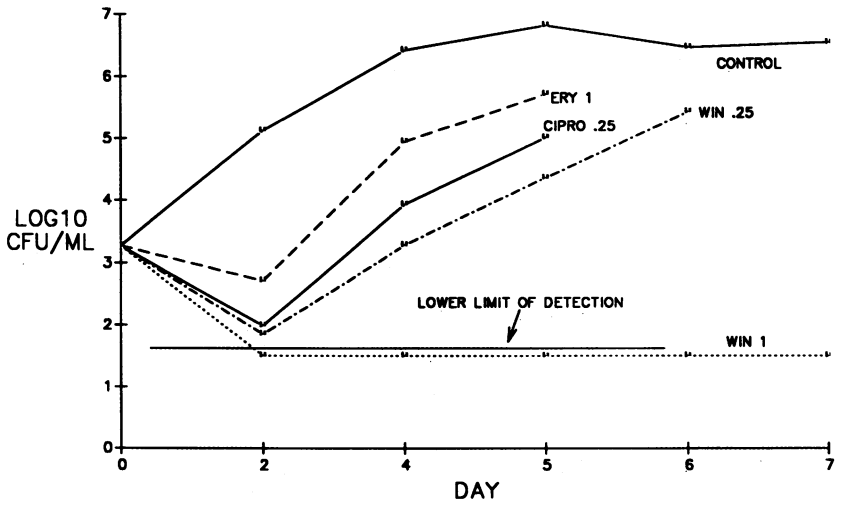


FIG. 1. Growth of *L. pneumophila* serogroup 1 strain F889 in guinea pig alveolar macrophages versus day of incubation after initiation of infection. Antibiotics were added to infected macrophages 2 h after infection. Viable counts of *L. pneumophila* were determined from the culture supernatant on the specified days. Bacterial counts of <50 CFU/ml could not be detected (designated by solid horizontal line). Control wells contained no antibiotics, well ERY 1 contained 1 μg of erythromycin per ml, well CIPRO .25 contained 0.25 μg of ciprofloxacin per ml, and wells WIN .25 and WIN 1 contained 0.25 and 1.0 μg of WIN 57273 per ml, respectively. All points represent the means of triplicate experiments.

Using a system similar to ours, Horwitz and Silverstein (12) reported that neither erythromycin (1 μg/ml) nor rifampin was cidal for intracellular *L. pneumophila*. Our findings with erythromycin confirm their studies and disagree with the results of studies performed by Fitzgeorge, Yoshida and Mizuguchi, and Miller and colleagues (9, 15, 22). Strain variation, definitions of killing, and differences in methods may account for these discrepancies, including the lack of studies of bacterial regrowth after antimicrobial removal. Vildé and colleagues demonstrated that pefloxacin prevented *L. pneumophila* regrowth after antimicrobial washout from a monocyte culture, but regrowth eventually occurred after 4 days of incubation in antibiotic-free medium (21). Our methods differed from those of Vildé and colleagues, making direct comparison difficult.

It is unclear whether failure of *L. pneumophila* intracellular regrowth after WIN 57273 removal is peculiar to that drug. It is possible that higher concentrations of ciprofloxacin or erythromycin could produce the same results.

An alternative and unlikely explanation of the ability of WIN 57273 to prevent bacterial regrowth is macrophage cytotoxicity, resulting in fewer macrophages available for intracellular growth of *L. pneumophila*. This was not the case in these experiments, based on careful microscopic

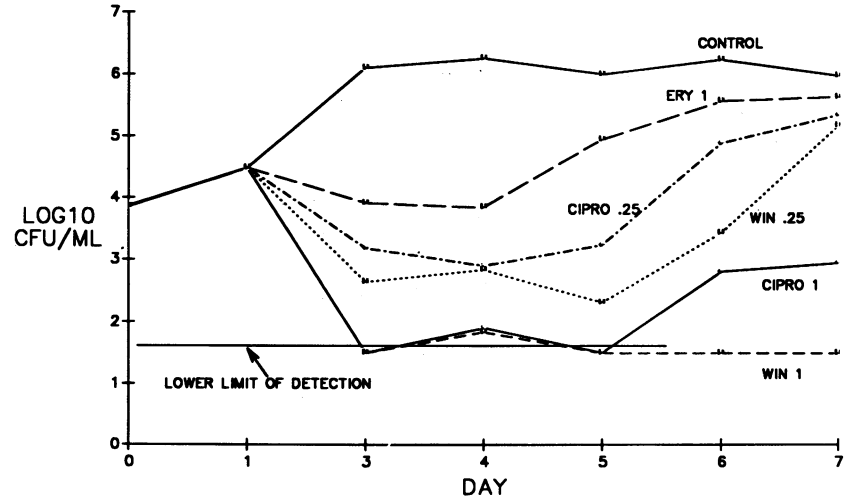


FIG. 2. Growth of *L. pneumophila* serogroup 1 strain F889 in guinea pig alveolar macrophages versus day of incubation after initiation of infection. Antibiotics were added to infected macrophages 1 day after infection and after wells were washed to remove extracellular bacteria. Two days later (day 3), after supernatant sampling, wells were washed to remove antibiotics. Viable counts of *L. pneumophila* were determined from the culture supernatant on the specified days, except on day 1, when the absolute concentration of intracellular bacteria was determined by sonication and culture of replicate wells. All points represent the means of triplicate experiments. See legend to Fig. 1 for explanation of symbols and contents of wells.

examination of macrophage monolayers. In fact, since there was no observable infection of the macrophages treated with 1 μg of WIN 57273 per ml, these monolayers were better preserved than those incubated with other antimicrobial agents.

Because of the nature of these types of studies, it is unclear what definitions should be used for the term "killing." Failure to regrow after antimicrobial washout seems a reasonable definition, although others might be content with a several-log decrease in bacterial count. This effect appears to be inoculum dependent, as judged by the different effectiveness of ciprofloxacin when added to recently infected macrophages versus its addition to macrophages 1 day postinfection. Extrapolating these results to the treatment of human infection is difficult because our cellular system lacks the ability to inhibit or kill *L. pneumophila* in the absence of antimicrobial agents, which is not true in human infection. As a general measure of intracellular growth inhibition, this model system correlates well with results of human and other animal studies.

The clinical meaning of in vitro bacterial killing is unclear, as erythromycin, which is strictly inhibitory to *L. pneumophila* in this system, is thought to be an effective drug for the treatment of Legionnaires disease on the basis of retrospective studies. In one animal model study, it was clear that pefloxacin, which may be bactericidal in a cellular system, was much more active than erythromycin (3). It is possible that short-course therapy with WIN 57273, and perhaps other quinolone antimicrobial agents, may be just as effective or more effective than longer courses of erythromycin, without danger of relapse.

The failure of *L. pneumophila* to regrow after exposure to 1 μg of WIN 57273 per ml could be due to either killing of the bacterium or prolonged persistence of WIN 57273 in the macrophage. The kinetics of intracellular concentration of this drug have not been established, but it is possible that the drug persists in its intracellular location for days after removal of extracellular drug. If so, this property could be useful for intermittent treatment of some chronic intracellular infections.

## ACKNOWLEDGMENTS

Joel Weidenfeld provided excellent technical assistance.
This work was supported in part by Sterling Drug, Inc.

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