

Antimicrobial Activity of Solithromycin against Clinical Isolates of *Legionella pneumophila* Serogroup 1

Julia Mallegol,^a Prabhavathi Fernandes,^b Roberto G. Melano,^{a,c,d} Cyril Guyard^{a,c,d}

Public Health Ontario Laboratory, Toronto, Ontario, Canada^a; Cembra Inc., Chapel Hill, North Carolina, USA^b; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada^c; Mount Sinai Hospital, Toronto, Ontario, Canada^d

The activity of solithromycin was evaluated against clinical *Legionella pneumophila* serogroup 1 (Lp1) isolates ($n = 196$) collected in Ontario, Canada, from 1980 to 2011. Its *in vitro* activity was compared to that of azithromycin (AZM) using the broth microdilution method. Solithromycin had a MIC₅₀ of ≤ 0.015 $\mu\text{g/ml}$ and a MIC₉₀ of 0.031 $\mu\text{g/ml}$, making its activity at least 8-fold to 32-fold higher than that of AZM (MIC₅₀ and MIC₉₀, 0.125 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$, respectively). Ninety-nine percent of the isolates had MICs for solithromycin ranging from ≤ 0.015 $\mu\text{g/ml}$ to 0.031 $\mu\text{g/ml}$, whereas 83.6% of the isolates showed MICs for AZM ranging from 0.062 $\mu\text{g/ml}$ to 0.25 $\mu\text{g/ml}$. Interestingly, 96.7% (30 out of 31 clinical isolates) identified with higher AZM MICs (0.5 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$) belonged to the clinically prevalent sequence type 1. To investigate the intracellular activity of solithromycin, *in vitro* invasion assays were also performed against a subset of representative Lp1 isolates internalized within human lung epithelial cells. Solithromycin and AZM both inhibited growth of all intracellular Lp1 isolates at $1\times$ or $8\times$ MICs, displaying bacteriostatic effects, as would be expected with protein synthesis inhibitor rather than bactericidal activity. Solithromycin demonstrated the highest *in vitro* and intracellular potency against all Lp1 isolates compared to AZM. Given the rapid spread of resistance mechanisms among respiratory pathogens and the reported treatment failures in legionellosis, the development of this new fluoroketolide, already in phase 3 oral clinical studies, constitutes a promising alternative option for the treatment of legionellosis.

Legionellosis is a major public health concern in industrialized countries. Manifestations of the disease range from a mild respiratory illness (Pontiac fever) to a severe and rapidly fatal pneumonia (Legionnaires' disease) (1). The case fatality rate of legionellosis ranges between 40 and 80% in untreated immunosuppressed patients but can be reduced from 5 to 30% with appropriate case management (2). Macrolides and fluoroquinolones have become the preferred and recommended therapeutic agents to treat these infections (3). Legionellosis is acquired by inhaling airborne water droplets contaminated with *Legionella* bacteria (4). These bacterial species are found worldwide and are detected in up to 80% of freshwater sites (5). Among more than 54 species of *Legionella*, *Legionella pneumophila* is the major cause of outbreaks (91.5%), and serogroup 1 (Lp1) is the predominant serotype isolated from patients (84.2%) (6). Since human-to-human transfer of these bacteria has not been reported, to explain the higher incidence of Lp1 than of other *Legionella* species, it has been hypothesized that Lp1 is more virulent and/or more fit for survival in anthropogenic aquatic environments (7). In its natural environments, *Legionella* is presumably exposed to residual concentrations of antibiotics used in medical or veterinary practice (8). This could constitute a potential selective pressure for the acquisition of antimicrobial resistance or decreased susceptibility to antibiotics for *L. pneumophila*. Indeed, it has been shown that the environmental exposure of bacteria to low antibiotic concentrations may promote the selection of resistance mechanisms (9, 10). The *in vitro* acquisition of resistance to erythromycin, ciprofloxacin, and rifampin by *L. pneumophila* has been reported (11, 12). Although there are no data available suggesting the *in vivo* emergence of resistance against commonly used antimicrobial agents in patients, the hypothesis of resistance emergence *in vivo* would be consistent with the mortality rates of 10% to 30% that are usually reported for legionellosis and for fluoroquinolone treat-

ment failures (13). Recently, Lp1 clinical isolates with MICs of 6 $\mu\text{g/ml}$ for azithromycin (AZM) were reported to be outside the wild-type distribution among a large collection of isolates (14). Although there are no established macrolide CLSI breakpoints for *Legionella*, these isolates with reduced susceptibility to AZM represent a potential risk for patient treatment.

Solithromycin is a fluoroketolide that has demonstrated high *in vitro* potency against Gram-positive and some Gram-negative pathogens (15, 16) (Fig. 1). It is the first fluoroketolide under clinical development that has been well tolerated in phase I studies, with high plasma, tissue, and intracellular concentrations (17). Moreover, Oldach et al. reported recently that in a randomized, double-blind, phase 2 study, solithromycin had comparable efficacy and favorable safety relative to levofloxacin in the treatment of patients with community-acquired bacterial pneumonia (CABP) (18). The objective of our study was to evaluate the *in vitro* activity of solithromycin compared to that of AZM against a large, well-characterized population-based collection of clinical *L. pneumophila* isolates that was collected between 1980 and 2011 in Ontario, Canada (19). In addition, we also investigated the intra-

Received 26 July 2013 Returned for modification 30 September 2013

Accepted 15 November 2013

Published ahead of print 25 November 2013

Address correspondence to Roberto G. Melano, roberto.melano@oahpp.ca, or Cyril Guyard, cyril.guyard@gmail.ca.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.01639-13>.

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doi:10.1128/AAC.01639-13

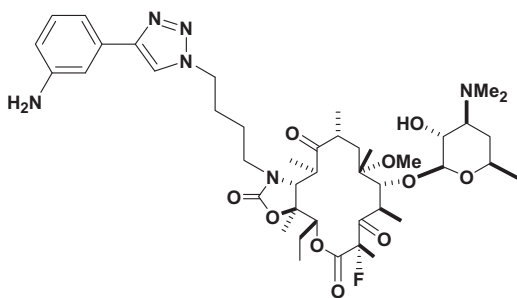


FIG 1 Chemical structure of solithromycin (CEM-101).

cellular activity of solithromycin against a subset of selected isolates internalized within human lung epithelial cells (20).

(Part of this study was presented at the 23rd European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany, 27 to 30 April 2013 [poster 1640].)

MATERIALS AND METHODS

Bacterial strains. Since 1978, the diagnosis of *Legionella* infections has been centralized at the Public Health Ontario Laboratory (PHOL), Toronto, Ontario, Canada, which serves as the reference laboratory and performs all testing for outbreak investigations and most testing of clinical specimens in Ontario. A total of 196 Lp1 clinical isolates collected from 1980 to 2011, representative of the strains isolated in Ontario in the past 3 decades and previously sequence base typed at the PHOL, were analyzed in this study (19). The isolates were stored in Trypticase soy broth supplemented with 5% horse blood at -80°C . Cultures from the frozen stock were prepared by inoculating buffered charcoal yeast extract (BCYE) plates, which were incubated for 3 days at 37°C in 5% CO_2 .

Broth microdilution susceptibility testing. Colonies of all isolates were subcultured on BCYE plates for 3 days at 37°C in 5% CO_2 before antimicrobial testing. The MICs of solithromycin and AZM were determined by the broth microdilution method in 96-well microtiter plates with minor modifications for *L. pneumophila* according to the Clinical and Laboratory Standards Institute guidelines (21) and the methods of Stout et al. (22). Briefly, suspensions of each isolate were prepared in buffered yeast extract broth (BYEB). The turbidities of the growing broth cultures were adjusted to an optical density equivalent to 0.5 McFarland standard ($\sim 1 \times 10^8$ CFU/ml). The adjusted broth cultures were diluted to approximately 1×10^7 CFU/ml. Twofold serial dilutions of antibiotics were prepared in broth, and the wells were filled with a 50- μl antimicrobial solution. Then, the bacterial suspension was added to an equal volume (50 μl) in each well to reach a final volume of 100 μl per well and a bacterial concentration of approximately 5×10^6 CFU/ml. MICs were read as the first well showing no visible growth after 48 h of incubation at 37°C in 5% CO_2 . *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as quality control strains. No susceptibility-testing breakpoints are available for *L. pneumophila*. Correlation analysis between MICs and sequence-based molecular types was performed using BioNumerics software.

To determine if efflux pumps were involved in isolates with reduced susceptibility to AZM (MIC, 1 to 2 $\mu\text{g}/\text{ml}$), the broth microdilution method described above was used in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at a concentration of 0.031 $\mu\text{g}/\text{ml}$ (23). A reduction of at least 2-fold in the AZM MICs in the presence of CCCP was considered an indicator of efflux activity.

Analysis of macrolide targets in *L. pneumophila*. The main targets involved in macrolide resistance were analyzed in 13 isolates belonging to sequence type 1 (ST1), including 4 isolates with wild-type MICs of AZM (≤ 0.625 $\mu\text{g}/\text{ml}$) and 9 isolates associated with higher MICs of AZM (1 to 2 $\mu\text{g}/\text{ml}$). Genomic DNAs were purified using a QIAamp DNA Mini Kit

(Qiagen, Mississauga, ON, Canada). Ten to 50 ng of DNA was used as a template for each PCR.

Primers for detection of acquired macrolide resistance mechanisms were previously described (24). Moreover, the 3 copies of 23S rRNA *rml* genes identified in Lp1 were analyzed by PCR amplification and DNA sequencing, using an approach previously described (25). Primers were designed from the genome of *L. pneumophila* Philadelphia (GenBank accession number NC_002942.5) (Table 1) (26). Long PCR amplifications of a 4-kb region from each of the three *rml* gene copies were performed using Expand Long Range dNTPack (Roche, Montreal, QC, Canada) according to the manufacturer's instructions, with an annealing temperature (T_a) of 52°C . Each 4-kb amplicon was used as a DNA template for nested PCRs to specifically amplify domains V (the peptidyl-transferase loop) and II (hairpin 35, involved in the binding of solithromycin, but not AZM), using the primers listed in Table 1. Nested PCRs were performed by using the Platinum *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada) under standard amplification conditions ($T_a = 57^{\circ}\text{C}$). Amplification of genes encoding the L4 and L22 riboproteins were also performed using Platinum *Pfx* DNA polymerase High Fidelity (Invitrogen) according to the manufacturer's instructions ($T_a = 51^{\circ}\text{C}$).

Sequencing of all of the amplicons was performed using the same primers for amplification on a 3130xl Genetic Analyzer (Applied Biosystems, Streetsville, ON, Canada).

Intracellular activity of solithromycin versus that of AZM. *In vitro* invasion assays were performed using monolayers of NCI-H292 human lung epithelial cells and 18 Lp1 clinical isolates displaying AZM susceptibility levels ranging from 0.0625 $\mu\text{g}/\text{ml}$ to 2 $\mu\text{g}/\text{ml}$ (see Table S1 in the supplemental material). NCI-H292 cells from human lung mucoepidermoid carcinoma (ATCC CRL-1848) were grown to 70 to 80% confluence (5×10^5 cells/well) in 24-well plates (Costar 24-well flat bottom plate; Corning Inc., Lowell, MA) containing 0.5 ml/well of RPMI 1640 medium (Life Technologies, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal calf serum and 1% glutamine (Life Technologies) and incubated at 37°C in 5% CO_2 for 24 h. The cells were washed three times in serum-free RPMI 1640 prior to infection. Lp1 isolates were grown on BCYE agar plates and incubated for 4 days at 37°C in 5% CO_2 . Bacterial suspensions were obtained by resuspending colonies in 2 ml of phosphate-buffered saline (PBS). Cell suspensions were adjusted to an OD_{600} of 2 (approximately 1.5×10^9 CFU/ml) and added to the epithelial cells at a multiplicity of infection (MOI) of 30:1 in a volume of 0.5 ml per well (approximately 1.5×10^7 bacteria/well). Samples were subjected to centrifugation for 5 min at 500 rpm to promote bacterial association with the NCI-H292 cells. The cultures were incubated for 3 h at 37°C in 5% CO_2 . After 3 washes in serum-free RPMI to remove nonadherent bacteria, gentamicin was added (100- $\mu\text{g}/\text{ml}$ final concentration) for 1 h to eradicate extracellular bacteria. The cells were washed three times in serum-free RPMI 1640 (to remove extracellular bacteria and gentamicin) and then incubated with solithromycin or AZM (8 \times and 1 \times the MIC for each strain) for 24 h and 48 h at 37°C in 5% CO_2 . Wells containing no antibiotic were used as growth controls at each time point (0, 24, and 48 h). RPMI 1640 does not sustain the extracellular growth of *L. pneumophila*, and all bacteria collected from the extracellular milieu resulted from intracellular growth (27). The supernatants of the intracellular assays were centrifuged to remove residual antimicrobial agent and resuspended in 0.5 ml of PBS. The lung epithelial cells were then lysed by osmotic shock using ice-cold filter-sterilized deionized water for 30 min. The lysed cells and their respective supernatants were pooled and homogenized by pipetting. Serial dilutions of bacterial suspension were plated on BCYE agar and incubated for 3 days at 37°C in 5% CO_2 . The intracellular activity of each antibacterial agent was determined by counting the CFU per milliliter on each plate. All assays were performed in quadruplicate with two independent biological duplicates.

The results were expressed as percent viability, defined as the total *Legionella* CFU at each time point (24 h and 48 h) with antibiotic divided by the total *Legionella* CFU at 0 h without antibiotic $\times 100$. Values of

TABLE 1 Oligonucleotides used for amplification and sequencing of domains II and V of the 23S rRNA gene and riboproteins L4 and L22

PCR and primer designation	Sequence (5' to 3')	Amplicon size
Primary allele PCR		
Internal common primer		
23S common 741-744	CTTACTGACCGATAGTGAACCAGTACCGTG	
External specific primer		
23S1- <i>tufA</i> 1	CGTGCCCACGTTTACGTGCGGCTTCTTACG	4 kb
23S2- <i>lpl0613</i> -R	TCATGATGGTCCTTATACTCCAGGCGGTAG	4 kb
23S3- <i>lpl2670</i> -R	AGATAATCACACTCTGAGTCAGCAATCCAG	4 kb
Nested PCR 23S rRNA, domain V		
23S 2058 region		
23S common 2302-2332-F	CCGTAACTATAACGGTCCTAAGGTAGCGAA	423 bp
23S 3201-3350-R	CTTTACTACTCTTGGTACGATGTCCGACCGT	
23S 2611 region		
23S common 2302-2332-F	CCGTAACTATAACGGTCCTAAGGTAGCGAA	928 bp
23S 2604-2605-R	CTTCCACACCTTGCTATCAACGTCGTAGT	
Nested PCR 23S rRNA, domain II		
23S 752 region		
23S region 741-744-F	CAGCGAGTTACTTTCAGTGGCGAGGTTAAC	688 bp
23S region741-744-R	CGTTACTCATGTCAGCATTTCGCACTTCTGA	
L4 and L22 riboproteins		
L4-F		
L4-R	GCAATTCTGGTGCTCCAGGTTCTAGAGTA	757 bp
L22-F	CTGTTAAACTTATCGGCCATGACAGTTGC	
L22-R	ATGGTTGGTCATAAATTAGGTGAGTTTGCC	463 bp
L22-R	CGTATACCTATTGGGTTTACTTTTTGTCCC	

≥100% indicate absence of antimicrobial inhibition, whereas values of <100% indicate an inhibitory effect.

RESULTS

Susceptibility testing. Solithromycin demonstrated the highest potency against all *L. pneumophila* isolates, displaying lower MIC values (≤ 0.015 to 0.0625 $\mu\text{g/ml}$) than AZM (≤ 0.0625 to 2 $\mu\text{g/ml}$) (Fig. 2). In other words, 99% of the isolates had solithromycin MICs of ≤ 0.031 $\mu\text{g/ml}$ (only 2 isolates showed MICs of 0.0625 $\mu\text{g/ml}$), whereas 83.6% of the isolates had AZM MICs ranging from ≤ 0.0625 $\mu\text{g/ml}$ to 0.25 $\mu\text{g/ml}$ (Fig. 2). Solithromycin had an MIC₅₀ of ≤ 0.015 $\mu\text{g/ml}$ and an MIC₉₀ of 0.031 $\mu\text{g/ml}$, making its activity at least 8-fold to 32-fold higher than that of AZM (MIC₅₀, 0.125 $\mu\text{g/ml}$, and MIC₉₀, 1 $\mu\text{g/ml}$) (Fig. 2).

Association between STs and MICs. To evaluate a possible correlation between the MICs and the molecular-typing profiles of the Ontario Lp1 isolates, we performed a phylogenetic cluster analysis combining the susceptibility profiles with the previously

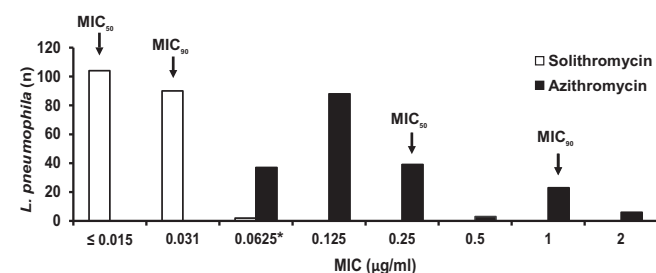


FIG 2 Distribution of solithromycin and azithromycin MICs for *L. pneumophila* serogroup 1 isolates. *, lowest concentration of azithromycin that was tested.

reported sequence-based types of all isolates (19). Categorical clustering analysis of Lp1 based on STs showed that a large clonal group, including ST1 and ST52 isolates, was associated with the highest AZM MICs from our collection (Fig. 3A). Among the isolates of this clonal group, 93.7% of ST1 isolates showed MICs for AZM ranging from 0.5 to 2 $\mu\text{g/ml}$. In addition, the only isolate with ST52 showed an AZM MIC of 1 $\mu\text{g/ml}$ (Fig. 3A). In contrast, isolates from this clonal group did not show solithromycin MICs higher than 0.031 $\mu\text{g/ml}$ (Fig. 3B).

Antimicrobial inhibition of intracellular growth of *L. pneumophila*. The intracellular susceptibilities to solithromycin and AZM of 18 selected Lp1 isolates displaying AZM MICs ranging from 0.0625 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ (solithromycin MICs of ≤ 0.015 to 0.031 $\mu\text{g/ml}$) were compared (see Table S1 in the supplemental material). Following an initial incubation with human NCI-H292 lung epithelial cells, all the isolates were internalized with similar efficiencies (data not shown). At 24 h and 48 h postinfection, all untreated control assays showed similar numbers of intracellular bacteria (1.62×10^{10} to 2.8×10^{11} CFU/ml and 4.05×10^{12} to 1.17×10^{14} CFU/ml, respectively [data not shown]). Twenty-four hours posttreatment, exposure to solithromycin or AZM at $1 \times$ the MIC resulted in the decline of the percentages of CFU per milliliter relative to the number of internalized bacteria pretreatment (to 47.2% and 58.8%, respectively) (Fig. 4A). Extension of the treatment to 48 h significantly reduced the viability of the Lp1 isolates exposed to solithromycin (to 24.7%) and AZM (to 26.6%). Although 4 to 64 times lower concentrations of solithromycin than of AZM were used in these assays, no significant difference in Lp1 viability was observed between the 2 antibiotic treatments (Fig. 4A). The effects of solithromycin and AZM on intracellular bacteria were next evaluated at $8 \times$ their respective MICs. Forty-eight hours posttreatment, the percentages of viable

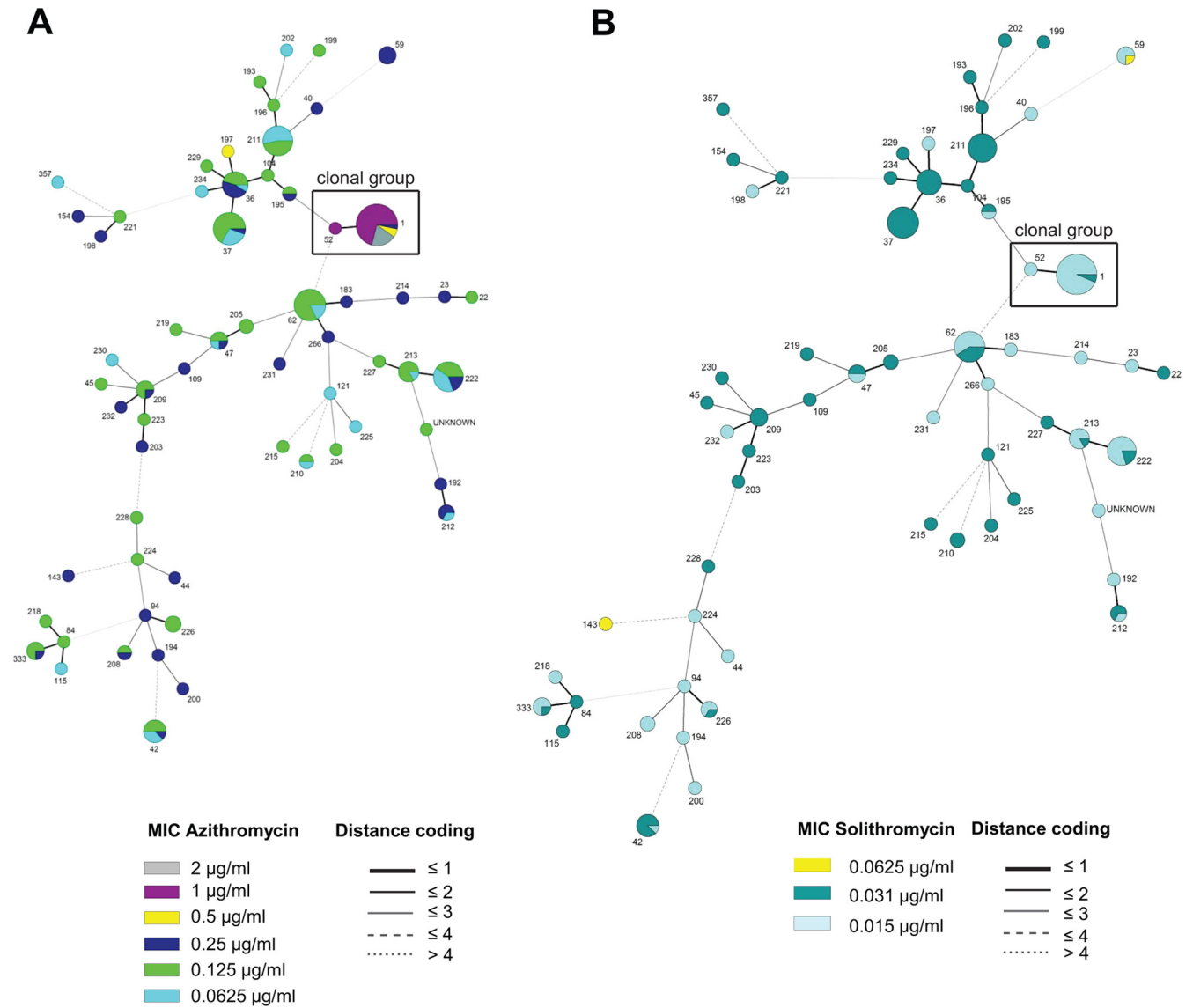


FIG 3 Minimum spanning tree of *L. pneumophila* serogroup 1 ($n = 196$). A categorical clustering was performed based on STs. STs sharing the maximum number of single-locus variants were connected first. Each circle represents an ST, and its size is proportional to the number of isolates within that particular type. The colors of the circles indicate the MIC ranges for azithromycin (A) and solithromycin (B). Relationships between the STs are depicted by the lines connecting the STs and the relative lengths of the branches linking them. Distance coding enumerates the differences at a given typing locus. A distance code of greater than 2 implies a different clonal complex. The angles of the line connections and the overlapping circles have no significance.

intracellular bacteria declined to 11.7% (solithromycin) and 23.4% (AZM). Overall, with $8\times$ MICs, solithromycin inhibited the growth of intracellular Lp1 clinical isolates significantly more than AZM ($P < 0.05$).

Analyses of potential mechanisms involved in reduced susceptibility to macrolides. To investigate the mechanisms involved in reduced susceptibility to AZM (MIC, 1 to 2 µg/ml) compared to the wild-type distribution (MIC, ≤ 0.0625 to 0.25 µg/ml), the most common targets and acquired mechanisms affecting macrolide susceptibility described in other microorganisms were analyzed (25). First, the rRNA and riboprotein genes were compared between nine ST1 isolates displaying AZM MICs ranging from 1 to 2 µg/ml versus four isolates displaying a low level of susceptibility to AZM (≤ 0.0625 µg/ml). The nine isolates

with reduced susceptibility showed no difference in domains V and II in the *3 rrl* gene copies or in the *rplD* and *rplV* genes (riboproteins L4 and L22) compared to the wild-type isolates. Acquired mechanisms of macrolide resistance (*23S* rRNA methylase genes, *ermA*, *ermB*, *ermC*, and *ermF*; efflux pump genes, *mefA* and *mefE*; and erythromycin esterase genes, *ereA* and *ereB*) were not detected in these isolates. Next, the role of efflux pumps in reduced susceptibility to AZM was evaluated using an inhibitor. None of the tested isolates showed a change in their AZM MIC levels in the presence of CCCP (data not shown).

DISCUSSION

L. pneumophila is a facultative intracellular pathogen. Hence, antimicrobial agents capable of achieving intracellular concentra-

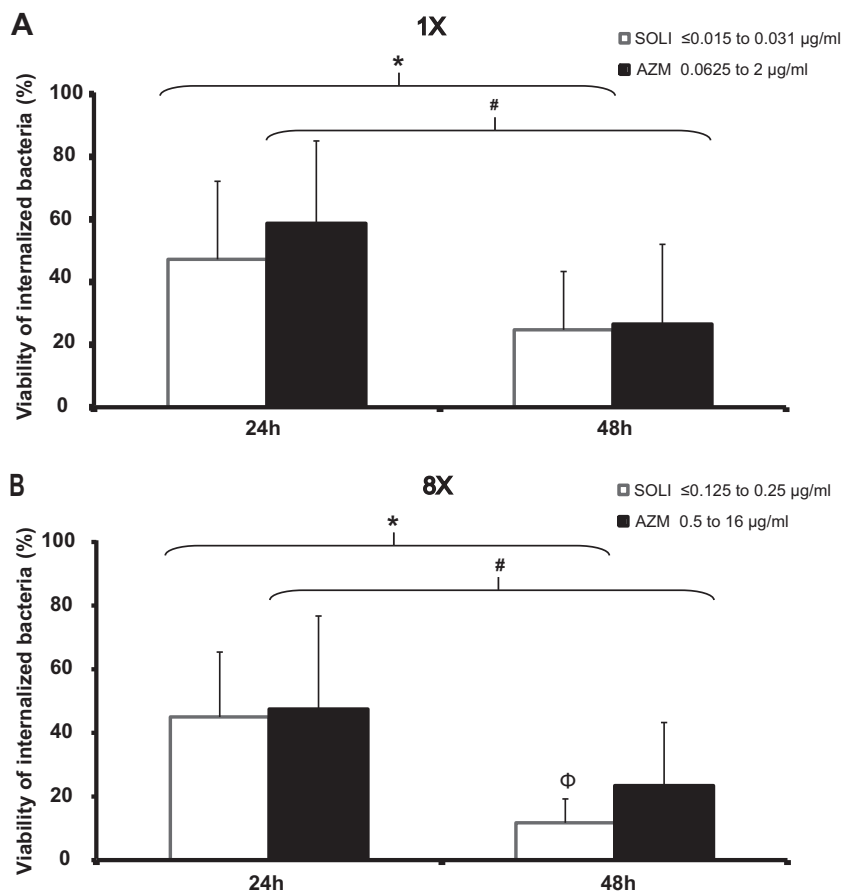


FIG 4 Intracellular activity of solithromycin compared to that of AZM against Lp1 clinical isolates ($n = 18$) displaying AZM MICs ranging from 0.0625 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ and solithromycin MICs ranging from ≤ 0.015 to 0.031 $\mu\text{g/ml}$. Lp1 isolates internalized within NCI-H292 lung epithelial cells were exposed to 2 concentrations of solithromycin and azithromycin. At the indicated time points, samples were harvested, and bacteria were enumerated by CFU assays. 8 \times , exposure to 8 times the MIC; 1 \times , exposure to 1 \times the MIC. The percent intracellular bacterial viability was calculated as the total number of Lp1 organisms at each time point with antibiotic divided by the total number of Lp1 organisms at time zero without antibiotic $\times 100$. *, $P < 0.01$ at 24 h versus 48 h; #, $P < 0.001$ at 24 h versus 48 h; Φ , $P < 0.05$ for solithromycin versus AZM (Student's t test). The error bars indicate standard deviations.

tions higher than the *in vitro* bacterial MICs (e.g., tetracyclines, macrolides, rifampin, and fluoroquinolones) are usually recommended for the treatment of legionellosis, rather than antibiotics with poor intracellular penetration (28). Despite the use of these antimicrobial agents and the improvement of diagnostic methods allowing early detection of infections, the mortality rate among legionellosis patients remains elevated (10 to 30%) (13). This situation is worsened by the fact that there are no *in vitro* antimicrobial breakpoints for *L. pneumophila* to define reduced susceptibility or resistance. Case reports of initial empirical treatment failures showed that final cures were achieved after changing the antimicrobial class during the treatment (29), pointing out the potential emergence of isolates with reduced susceptibility and the need for more careful use of the available antibiotics. In the present study, we evaluated the activity and potency of solithromycin against a collection of clinical Lp1 isolates. Solithromycin is a fluoroketolide with high affinity for bacterial ribosomes. In contrast to AZM, which binds only one site on the 23S ribosomal subunit, evidence suggests that solithromycin has three sites of interaction with the 23S ribosomal subunit (30), thereby enhancing its activity due to the higher number of binding sites. These multiple interactions may explain the better anchoring of soli-

thromycin to the ribosome even in the presence of mutations at key positions (i.e., A2058-A2059 [*E. coli* numbering]) (31).

Our study shows that solithromycin has better *in vitro* activity than AZM against a variety of *L. pneumophila* clinical isolates, displaying AZM susceptibility levels ranging from ≤ 0.0625 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ (Fig. 2). This range of AZM MICs is consistent with previous studies reporting MIC distributions in clinical *L. pneumophila* serogroup 1 isolates using broth dilution and intracellular susceptibility assays (22, 32).

A possible correlation between the MICs and the molecular typing profiles of the Ontario Lp1 isolates was evaluated, and our data showed that most ST1-related isolates were associated with higher AZM MICs (0.5 to 2 $\mu\text{g/ml}$) (Fig. 3A), but they did not show increased solithromycin MICs (0.031 $\mu\text{g/ml}$) compared to other sequence types (Fig. 3B). ST1 isolates are most commonly associated with Legionnaires' disease (19), and they are also frequently recovered from environmental samples from various countries, including the United States and Canada (33, 34). This suggests that this prevalent *L. pneumophila* clonal group may have evolved to become less susceptible to macrolides such as AZM. No human-to-human transmission has been recorded to date for legionellosis patients, but other pathogens can become resistant

during the course of antimicrobial therapy (24), increasing the potential for horizontal gene transfer between bacterial genera. Mechanisms of macrolide resistance remain unknown in *L. pneumophila*, but three different mechanisms have been well documented in other bacteria. These mechanisms include modifications of the ribosomal target by methylases (35), ribosomal modifications by point mutations in the macrolide targets (25, 36), and the overexpression of chromosomal or acquired efflux pump systems (37). The reduced AZM susceptibility observed in some isolates was investigated by addressing the possible acquisition of resistance mechanisms from other pathogens and by searching for mutations in macrolide targets. However, none of the acquired mechanisms screened were detected in the isolates, nor were the mutations in riboproteins L4 and L22 and the *rrl* gene (domains II and V, related to antimicrobial resistance in other species). Additionally, no MIC differences were observed in the presence of the efflux pump inhibitor CCCP. These results indicate that there are other mechanisms involved in the reduced susceptibility to AZM in *L. pneumophila* (e.g., permeability or other efflux pumps not affected by CCCP). Further studies are needed to elucidate these mechanisms. It is interesting, though, to note that the decreased susceptibilities to AZM in Lp1 isolates were not correlated with increased solithromycin MICs, suggesting that these unknown potential mechanisms do not affect solithromycin activity.

It is unlikely that the *L. pneumophila* clinical isolates with reduced susceptibility could reenter their natural aquatic reservoirs and spread to other human hosts. However, a growing concern for environmental pathogens is the possible selection and emergence of decreased antimicrobial susceptibilities due to the presence of residual macrolides in wastewater. It has been shown that 30 to 40% of macrolides are excreted in an unchanged active form via urine into wastewater, manure, and runoff water (38). Although these macrolides end up in a diluted form in various aquatic environments and/or soil, they could still be of great importance for the evolution of resistance (38).

Due to the intracellular location of *L. pneumophila* in alveolar macrophages, lung epithelial cells, and monocytes (39), antimicrobial susceptibility assays by standard methods, such as agar and broth dilutions, are only partially relevant. Therefore, we evaluated the activity of solithromycin against *L. pneumophila* isolates using an established intracellular model of infection. We showed that solithromycin and AZM inhibit the growth of Lp1 clinical strains independently of their sequence type. Solithromycin maintained the same efficiency as AZM after 24 h of exposure. Interestingly, solithromycin showed a much higher relative intracellular potency 48 h posttreatment against all clinical isolates (Fig. 4). This finding is in agreement with the study of Lemaire et al., who showed first that solithromycin has high intracellular activity against reference laboratory strains, such as *S. aureus* ATCC 25923, *Listeria monocytogenes* strain EGD, and *L. pneumophila* ATCC 33153, due to high intracellular accumulation (40). Here, in agreement with this preliminary study performed with one reference laboratory strain with a low AZM MIC, solithromycin demonstrated the highest *in vitro* and intracellular potency against a large population-based collection of clinical isolates compared to AZM. Interestingly, we show for the first time that solithromycin remains highly potent against isolates with increased AZM MICs. *Legionella* has also been recognized as a significant pathogen in CABP (41), and given the reported treatment failure in

legionellosis, our findings suggest that solithromycin could be used in the treatment of legionellosis. In a phase 2 study, solithromycin was well tolerated, with efficacy comparable to that of levofloxacin in patients with CABP (18). Altogether, these results support the current development of this new fluoroketolide in phase 3 studies in patients with CABP.

ACKNOWLEDGMENTS

We thank Patrick Tang (Public Health Ontario) for providing the *L. pneumophila* isolates used in this study.

This work was supported by Cempra, Inc., Chapel Hill, NC.

P.F. is the CEO of Cempra, Inc. We have no other conflicts of interest to declare.

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