Antimicrobial activity of solithromycin against clinical isolates of Legionella pneumophila serogroup 1.

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Abstract

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 azithromycin (AZM) using the broth microdilution method. Solithromycin had a MIC$_{50}$ of $\leq 0.015$ µg/ml and a MIC$_{90}$ of 0.031 µg/ml, making its activity at least 8-fold to 32-fold higher than AZM (MIC$_{50}$ and MIC$_{90}$ of 0.125 µg/ml and 1 µg/ml, respectively). Ninety-nine percent of the isolates had MICs for solithromycin ranging from $\leq 0.015$ µg/ml to 0.031 µg/ml whereas 83.6% of the isolates showed MICs for AZM ranging from 0.062 µg/ml to 0.25 µg/ml. Interestingly, 96.7% (30 out of 31 clinical isolates) identified with higher AZM MICs (0.5 µg/ml to 2 µg/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 1X or 8X MICs, displaying bacteriostatic effects, as it would be expected with protein synthesis inhibitors, rather than bactericidal activity. Solithromycin demonstrated the highest in vitro and intracellular potency against all Lp1 isolates when compared to AZM. Given the rapid spread of resistance mechanism 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.

Word count: 245
Introduction

Legionellosis is a major public health concern in industrialized countries. Manifestations of this 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 to 80% in untreated immuno-suppressed 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 the freshwater sites (5). Among more than 54 species of Legionella, L. 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, it has been hypothesized that Lp1 is more virulent and/or more fit for survival in anthropogenic aquatic environments to explain the higher incidence of Lp1 compared to other Legionella species (7). In their 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 rifampicin by L. pneumophila has been reported (11, 12). Although there is 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% – 30% that are usually reported for legionellosis and for fluoroquinolone treatment failures (13). Recently, Lp1 clinical isolates with MICs of 6 µg/ml for azithromycin (AZM) were reported to be outside of the wild type distribution among a large collection of isolates (14). Although there is 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 which has demonstrated high *in vitro* potency against Gram-positive and some Gram-negative pathogens (15, 16) (Figure 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 in a randomized, double-blind Phase 2 study, that 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 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 intracellular activity of solithromycin against a subset of selected isolates internalized within human lung epithelial cells (20).
Material and methods

Bacterial strains

Since 1978, the diagnosis of *Legionella* infections has been centralized at the Public Health Ontario Laboratory (PHOL), 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). 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. Plates were incubated for 3 days at 37°C in 5% CO₂.

Broth microdilution susceptibility testing

Colonies from all isolates were subcultured on BCYE plates for 3 days at 37°C in 5% CO₂ before antimicrobial testing. MICs of solithromycin and AZM were determined by the broth microdilution method in 96-well microtitre plates with minor modifications for *L. pneumophila* according to the Clinical and Laboratory Standards Institute guidelines (21) and Stout *et al* (22). Briefly, suspensions of each isolate were prepared in buffered yeast extract broth (BYEB). The turbidity of the growing broth cultures were adjusted to an optical density equivalent to a 0.5 McFarland standard (~1x10⁵ CFU/ml). The adjusted broth cultures were diluted to approximately 1x10⁷ CFU/ml. Two fold 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 5x10^6 CFU/ml. MICs were read as the first well showing no visible growth after 48 h incubation at 37°C in 5% CO₂. *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 (MICs 1 to 2 µg/ml), the broth microdilution method described above was used in the presence or absence of carbonyl cyanide m-chlorophenylhydrazone (CCCP) at the concentration of 0.031 µg/ml) (23). A reduction of at least two-fold 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 (ST) 1, including 4 isolates with wild type MICs for AZM (≤0.625 µg/ml) and 9 isolates associated with higher MICs for AZM (1-2µg/ml). Genomic DNAs were purified using QIAamp DNA Mini Kit (Qiagen, Mississauga, ON). Ten to fifty ng of DNA were used as a template for each PCR reaction.

Primers for detection of acquired macrolide resistance mechanisms were previously described (24). Moreover, the 3 copies of 23S rRNA *rrl* 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 *rrl* genes copies were performed using Expand Long Range dNTPack (Roche, Montreal, QC) according to the manufacturer instruction, with an annealing temperature (Ta) 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) in standard amplification conditions (Ta= 57°C). Amplification of genes encoding L4 and L22 riboproteins were also performed using Platinum® *Pfx* DNA polymerase High Fidelity (Invitrogen) according to the manufacturer instructions (Ta= 51°C).

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

**Intracellular activity of solithromycin vs. 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 µg/ml to 2 µg/ml (Supplementary Table S1). NCI-H292 cells from human lung mucoepidermoid carcinoma (ATCC, CRL-1848) were grown to 70 – 80% confluence (5x10^5 cells/well) in 24-well plates (Costar 24 well flat bottom plate, Corning Incorporated, Lowell, MA) containing 0.5 ml/well of RPMI 1640 medium (Life...
Technologies, Burlington, ON), supplemented with 10% heat inactivated fetal calf serum and 1% glutamine (Life Technologies), and incubated at 37°C in 5% CO₂ for 24 h. 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% CO₂. Bacterial suspensions were obtained by resuspending colonies in 2 ml of phosphate-buffered saline (PBS). Cell suspensions were adjusted to an OD₆₀₀ of 2 (approximately 1.5 x 10⁹ 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 1.5x10⁷ bacteria/well). Samples were subjected to centrifugation for 5 min at 500 rpm to promote bacterial association with NCI-H292 cells. Cultures were incubated for 3 h at 37°C in 5% CO₂. After 3 washes in serum-free RPMI to remove non-adherent bacteria, gentamicin was added (100 µg/ml final concentration) for 1 h to eradicate extracellular bacteria. Cells were washed three times in serum-free RPMI-1640 (to remove extracellular bacteria and gentamicin) and then incubated with solithromycin or AZM (8X and 1X the MICs of each strain) for 24 h and 48 h at 37°C in 5% CO₂. 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 result from intracellular growth (27). Supernatants of intracellular assays were centrifuged to remove residual antimicrobial agent and resuspended in 0.5 ml of PBS. Lung epithelial cells then were lysed by osmotic shock using ice-cold filter sterilized deionized water for 30 minutes. Lysed cells and their respective supernatant 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% CO₂. The intracellular activity of each antibacterial
gagent was determined by counting CFU/ml on each plate. All assays were performed in
quadruplicate with two independent biological duplicates.

Results were expressed as percentage of viability defined as total *Legionella* CFUs at
each time point (24 h and 48 h) with antibiotics divided by total *Legionella* CFU at 0 h
without antibiotic x 100. Values ≥100% indicate absence of antimicrobial inhibition,
whereas values <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 µg/ml) compared to AZM (≤0.0625 to 2
µg/ml) (Figure 2). In other words, 99% of the isolates had MICs ≤0.031 µg/ml for
solithromycin (only 2 isolates showed MICs of 0.0625 µg/ml) whereas 83.6% of the
isolates had MICs for AZM ranging from ≤0.0625 µg/ml to 0.25 µg/ml (Figure 2).

Solithromycin had an MIC₅₀ of ≤0.015 µg/ml and an MIC₉₀ of 0.031 µg/ml, making its
activity at least 8-fold to 32-fold higher than AZM (MIC₅₀ of 0.125 µg/ml and MIC₉₀ of 1
µg/ml for AZM) (Figure 2).

**Association between sequence types (ST) 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 reported sequence based types of all isolates (19). Categorical clustering analysis of Lp1 based on ST showed that a large clonal group including ST1 and ST52 isolates was associated with the highest AZM MICs from our collection (Figure 3A). Among the isolates of this clonal group, 93.7% of ST1 isolates showed MICs for AZM ranging from 0.5 to 2 µg/ml. In addition, the only isolate with ST52 showed an AZM MIC of 1 µg/ml (Figure 3A). In contrast, isolates from this clonal group did not show solithromycin MICs higher than 0.031 µg/ml (Figure 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 µg/ml to 2 µg/ml (solithromycin MICs of ≤0.015 to 0.031 µg/ml) were compared (Table S1). Following an initial incubation with human NCI-H292 lung epithelial cells, all isolates were internalized with a similar efficiency (data not shown). At 24 h and 48 h post infection, all untreated control assays showed similar amounts of intracellular bacteria (1.62 x 10^{10} to 2.8 x 10^{11} CFU/ml and 4.05 x 10^{12} to 1.17 x 10^{14} CFU/ml respectively; data not shown). Twenty four hours post-treatment, exposures to solithromycin or AZM at 1X of the MIC resulted in the decline of the percentage of CFU/ml relative to the number of internalized bacteria pre-treatment (to 47.2% and 58.8%, respectively) (Figure 4A). Extension of the treatment to 48 hours 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 compared to AZM were used in these assays, no significant difference in
Lp1 viability was observed between the 2 antibiotics’ treatments (Figure 4A). The effect of solithromycin and AZM on intracellular bacteria were next evaluated at 8X times of their respective MICs. Forty eight hours post-treatment, the percentage of viable intracellular bacteria declined to 11.7% (solithromycin) and 23.4% (AZM). Overall, with 8X MICs, solithromycin inhibited significantly more the growth of intracellular Lp1 clinical isolates than AZM (P-value < 0.05).

Analyses of potential mechanisms involved in reduced susceptibility to macrolides.

To investigate the mechanisms involved in reduced susceptibility to AZM (MIC 1-2 µg/ml) compared to the wild type distribution (≤0.0625 to 0.025 µg/ml), the most common targets and acquired mechanisms affecting macrolide susceptibility described in other microorganisms were analyzed (25). First, the ribosomal RNA and riboprotein genes were compared between nine ST1 isolates displaying AZM MICs ranging from 1 to 2 µg/ml vs. four isolates displaying a low susceptibility level 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 mechanism of macrolides resistance (23S rRNA methylases, ermA, ermB, ermC and ermF; efflux pump, mefA/E and erythromycin esterases, 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 concentrations 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 - 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 about 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 solithromycin to the ribosome even in presence of mutations at key positions (i.e. A2058-A2059 per *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 µg/ml to 2 µg/ml (Figure 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 µg/ml) (Figure 3A), but they did not show increased solithromycin MICs (0.031 µg/ml) compared to others sequence types (Figure 3B). ST1 isolates are most commonly associated with Legionnaires’ disease (19) and they are also frequently recovered from environmental samples of 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 therapies (24), increasing the potential of 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 macrolides’ 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, other efflux pumps not affected by CCCP). Further studies are needed to elucidate these mechanisms. It is although interesting to note that the decreased susceptibilities to AZM in Lp1 isolates were not correlated to increased solithromycin MICs suggesting that these unknown potential mechanisms do not affect solithromycin activity.

It is unlikely that these L. pneumophila clinical isolates with reduced susceptibility could re-enter 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 waste water. Some studies have shown that 30 to 40 % of macrolides are excreted in an unchanged active form via urine into waste water, manure and run-off 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 with standard methods such as agar and broth dilutions are only partially relevant. Therefore, we have 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 independent 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 post-treatment against all clinical isolates (Figure 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 when compared to AZM. Interestingly, we show for the first time that solithromycin remains highly potent against isolates with increased MICs to AZM. *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.
Acknowledgements

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Transparency declarations

P.F. is the CEO of Cempra, Inc. All other authors: none to declare.
Figure Captions

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

Figure 2. Distribution of solithromycin and azithromycin MICs for L. pneumophila serogroup 1 isolates. * Lowest concentration of azithromycin that was tested.

Figure 3: Minimum spanning tree of L. pneumophila serogroup 1 (n=196). A categorical clustering was performed based on sequence types (ST). STs sharing the maximum number of single locus variants were connected first. Each circle represents an ST, the size of which is proportional to the number of isolates within that particular type. Colors within circles indicate the MIC ranges for (A) azithromycin and (B) solithromycin. 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 number of differences at a given typing locus. A distance coding of greater than 2 implies a different clonal complex. Angles of the line connections and the overlapping circles have no significance.

Figure 4. Intracellular activity of solithromycin compared to azithromycin (AZM) against Lp1 clinical isolates (n=18) displaying AZM MICs ranging from 0.0625 µg/ml to 2 µg/ml and solithromycin MICs ranging from ≤0.015 to 0.031 µg/ml. Lp1 isolates internalized within NCI-H292 lung epithelial cells were exposed to 2 concentrations of
solithromycin and azithromycin. At indicated time points, samples were harvested and
bacteria were enumerated by CFU assays. 8X, exposure to 8 times the MIC; 1X,
exposure to 1 time the MIC. Percentage of intracellular bacterial viability is calculated as
a total number of Lp1 at each time point with antibiotic divided by the total number of
Lp1 at time zero without antibiotic x100. Student’s t-test * P-value < 0.01, 24h versus
48h; # P-value <0.001, 24h versus 48h, ĭ P-value <0.05, Solithromycin versus AZM.
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Table S1. Lp1 isolates used in the intracellular assay

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<td>ST1</td>
</tr>
<tr>
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REFERENCES


Figure 1: Chemical structure of solithromycin (CEM-101)
Figure 2

Figure 2. Distribution of solithromycin and azithromycin MICs for *L. pneumophila* serogroup 1 isolates. * Lowest concentration of azithromycin that was tested.
Figure 3: Minimum spanning tree of *L. pneumophila* serogroup 1 (n=196).

A categorical clustering was performed based on sequence types (ST). STs sharing the maximum number of single locus variants were connected first. Each circle represents an ST, the size of which is proportional to the number of isolates within that particular type. Colors within circles indicate the minimum inhibitory concentration (MIC) ranges for A. azithromycin and B. solithromycin. 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 number of differences at a given typing locus. A distance coding of greater than 2 implies a different clonal complex. Angles of the line connections and the overlapping circles have no significance.
Figure 4

Intracellular activity of solithromycin (SOLI) compared to azithromycin (AZM) against Lp1 clinical isolates (n=18) displaying AZM MICs ranging from 0.0625 µg/ml to 2 µg/ml and solithromycin MICs ranging from ≤0.015 to 0.031 µg/ml.

Lp1 isolates internalized within NCI-H292 lung epithelial cells were exposed to 2 concentrations of solithromycin and azithromycin. At indicated time points, samples were harvested and bacteria were enumerated by CFU assays. 8X, exposure to 8 times solithromycin MIC; 1X, exposure to 1 time the MIC. Percentage of intracellular bacterial viability is calculated as a total number of Lp1 at each time point with antibiotic divided by the total number of Lp1 at time zero without antibiotic x100.

Student's t-test * P-value < 0.01, 24h versus 48h; # P-value <0.001, 24h versus 48h, Φ P-value < 0.05, SOLI versus AZM.