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KKL-35 exhibits potent antibiotic activity against *Legionella* species independently of trans-translation inhibition

Running title: Antibiotic activity of KKL-35 on *Legionella pneumophila*

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Abstract

Trans-translation is a ribosome rescue system that is ubiquitous in bacteria. A new family of oxadiazole compounds that inhibit trans-translation have been found to have broad-spectrum antibiotic activity. We sought to determine the activity of KKL-35, a potent member of the oxadiazole family, against the human pathogen *Legionella pneumophila* and other related species that can also cause Legionnaire's disease (LD). Consistent with the essential nature of trans-translation in *L. pneumophila*, KKL-35 inhibits growth of all tested *L. pneumophila* trains at sub-micromolar concentrations, and is active against other LD-causing *Legionella* species. KKL-35 is also active against *L. pneumophila* mutants that have evolved resistance to macrolides. KKL-35 exhibits bactericidal activity at the minimal inhibitory concentration (MIC) on all tested strains. KKL-35 inhibits multiplication of *L. pneumophila* in human macrophages at several stages of infection. No resistant mutants could be obtained, even during extended and chronic exposure, suggesting that resistance is not easily acquirable. Surprisingly, KKL-35 is not synergistic with other ribosome-targeting antibiotics, and remains active against *L. pneumophila* mutants lacking tmRNA, the essential component of trans-translation. These results indicate that the antibiotic activity of KKL-35 is not related to the specific inhibition of trans-translation and its mode of action remains to be identified. In conclusion, KKL-35 displays strong antibiotic activity against the human pathogen *L. pneumophila*, including in an intracellular infection model and with no detectable resistance.

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Introduction

Legionella pneumophila is a ubiquitous freshwater bacterium that infects a wide spectrum of environmental protozoans. Human-made systems such as sanitary water networks and air-cooling towers can disseminate contaminated water through aerosolization. Breathing microscopic droplets contaminated with *L. pneumophila* can lead to infection of alveolar macrophages and development of a life-threatening pneumonia called Legionnaire's disease (LD) or Legionellosis. LD remains an important cause of both morbidity and mortality in Europe with over 6900 cases reported in 2014[1]. Guidelines for the management of LD recommend the use of macrolides (with a preference for azithromycin) or fluoroquinolones (levofloxacin/moxifloxacin) to treat the infection[2,3]. Despite a rapid diagnosis and the correct administration of antibiotics, death rate of LD is over 10%[4]. *L. pneumophila* isolates are considered susceptible to macrolides and fluoroquinolones[5] but mutants resistant to both antibiotic families can be easily obtained *in vitro*, suggesting that resistant strains may emerge during treatment[6–8]. Indeed, resistance to fluoroquinolones acquired in the course of a fluoroquinolone therapy has been recently reported[9,10]. New compounds active against *L. pneumophila* resistant to fluoroquinolones and macrolides or that could potentiate these existing treatments may improve the outcome of the disease.

Trans-translation has recently been proposed as a novel target for the development of a new class of antibiotics[11]. Trans-translation is the primary bacterial mechanism to resolve ribosome stalling in bacteria[12–14]. Ribosome stalling can be induced by ribosome-targeting antibiotics, lack of necessary tRNAs, or translation of an mRNA lacking a stop codon (non-stop mRNA) and is a life-threatening issue in metabolically active bacteria[15,16]. Trans-translation is operated by a highly conserved nucleoprotein complex[17] encoded by two genes: *ssrA* encoding a highly expressed and structured RNA called tmRNA[18,19], and *smpB* encoding a small protein involved in specific recognition and loading of tmRNA in stalled ribosomes[20–22]. Once the complex is loaded in the free A site, translation resumes using the coding section of the tmRNA as template. This messenger section of tmRNA encodes a degradation tag that is appended to the unfinished polypeptide, targeting it to different proteases[23–25]. The coding section of tmRNA ends with a stop codon, allowing normal termination of translation and dissociation of the ribosomal subunits. In addition, the tmRNA-SmpB complex interacts with RNAse R to degrade the faulty mRNA[26,27]. Thus, in addition to resolving ribosome stalling, the trans-translation

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system prevents the rise of further problems by promoting the degradation of both the problematic mRNA and the aborted polypeptide[28].

Alternative ribosome rescue systems have been identified in *Escherichia coli* and named ArfA and ArfB (Alternative rescue factors A and B)[29,30]. Both ArfA and ArfB can partially complement the loss of trans-translation by promoting dissociation of the stalled ribosome but lack mechanisms to trigger degradation of the aborted polypeptide and faulty mRNA[12]. These appear less conserved than the tmRNA-SmpB system[15]. Trans-translation is essential in species lacking alternative mechanisms[16]. In agreement with these observations, alternative ribosome-rescue systems are absent in members of the *Legionellaceae* genus and we indeed found that trans-translation is essential for *L. pneumophila* growth and infection of its cellular host[31]. In *L. pneumophila*, expressing the alternate rescue factor ArfA from *E. coli* can compensate for the loss of trans-translation activity indicating that the ribosome-dissociating activity of the trans-translation system is the sole function required for viability[31]. Because it is essential for viability in multiple pathogens, the trans-translation system has been proposed as a valid, yet-unexplored target for a new class of antibiotics[11].

A high-throughput screen using an *in vivo* assay of trans-translation recently identified a family of small molecule able to inhibit trans-translation at micromolar concentrations[32]. One of the most active compounds, KKL-35, was found to exhibit a bactericidal activity against several pathogenic bacterial species in which trans-translation was known to be essential[32]. KKL-35 and two related compounds KKL-10 and KKL-40 display antibiotic activity against the intracellular pathogen *Francisella tularensis* during infection of its host[33]. However, the specificity of action of the molecules has not been confirmed in this species. The present study assessed KKL-35 activity *in vitro* against the intracellular pathogen, *L. pneumophila*. MIC and MBC values were determined for a set of *Legionella* species and strains. We report that KKL-35 exhibits potent bactericidal activity against *L. pneumophila in vitro* at very low concentrations and is able to stop bacterial multiplication in a model of infection of human macrophages. Yet, multiple evidence indicates that KKL-35 does not target trans-translation and, as such, its true target(s) remains to be identified.

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Materials and methods

Strains, growth media and antibiotics used

Strains used in this study included clinical isolates of *L. pneumophila* of strains Paris (CIP 107629), Lens (CIP 108286), Philadelphia-1, Lorraine (CIP 108729) and 130b, as well as isolates of *L. longbeachae*, *L. dumoffii*, *L. micdadei*. *L. pneumophila* str. Paris resistant to erythromycin or azithromycin were obtained from a previous work[8]. *L. pneumophila* Paris was transformed with the plasmid pX5, a pMMB207C derivative harboring the *gfp*+ gene under a strong constitutive promoter and used for live monitoring of intracellular multiplication by fluorescence reading. The tmRNA mutant strains $ssrA^{ind}$ and $ssrA^{ind}$/pArfA were previously described[31]. ACES-Yeast Extract broth medium (AYE) was prepared with 10 g/L ACES (*N*-(2-acetamido)-2-aminoethanesulfonic acid), 12g/L yeast extract, 0.3 g/L iron III pyrophosphate and 0.5 g/L L-cysteine. pH was adjusted at 6.9 with KOH and the solution was filter-sterilized and kept away from light, at 4°C. ACES-buffered charcoal yeast extract (CYE) plates were prepared with 10g/L ACES and 10g/L granulated yeast extract autoclaved together, 30 g/L agar and 4 g/L charcoal autoclaved together, and complemented with 0.25 g/L filtered iron III nitrate and 0.4 g/L L-cysteine. Unless indicated otherwise, cultures on CYE were incubated for 72h at 37°C in air, then patched onto CYE again for 24h to obtain fresh cultures before experiments were performed. When appropriate, chloramphenicol (5 µg/mL) was added to the medium. A stock solution of KKL-35 (Ambinter, Orléans, France) was prepared at 10 mM (3.2 g/L) in dimethylsulfoxide (DMSO) and stored at -20°C.

Time-kill assay, determination of MIC and MBC

For the time-kill assay, *L. pneumophila* strain Paris was resuspended in AYE medium at 3.10^6 CFU/mL with a range of two-fold dilutions of KKL-35. Tubes were then incubated at 37°C in air, with shaking. Every 24h, serial dilutions were plated on CYE agar and CFUs were counted. For MIC determination, no CSLI guidelines are available for testing antibiotic susceptibility of *Legionella* strains. EUCAST guidelines were recently published but are based on the gradient strip test. KKL-35 strip tests are not commercially available, and we found the charcoal of CYE medium to seriously impede KKL-35 activity. Therefore, we used the previously described AYE broth microdilution method for MIC and MBC determination[34]. Briefly, strains were resuspended in AYE medium and placed into

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the wells of a 96-well polystyrene plate and a range of twofold dilutions of KKL-35 was added to the cultures. The inoculum (10^6 CFU/mL) was verified by plating and counting of serial dilutions of the cultures at the beginning of the experiment. The 96-well plate was sealed with a Breathe-Easy® membrane (Sigma-Aldrich) to prevent evaporation and was incubated for 48h at 37°C in air with no agitation. At 48h, MICs were determined visually as the lowest concentrations inhibiting bacterial growth. Cultures from all wells with concentration higher or equal the MIC were collected, serial diluted and plated onto CYE agar for counting. MBC was defined at the minimal concentration at which 99.9% of bacteria were killed. A bactericidal effect was defined by a MBC/MIC ratio ≤ 4.

Evaluation of synergistic activity

The chequerboard broth microdilution method was used to evaluate a possible synergistic activity between KKL-35 and chloramphenicol and erythromycin on *L. pneumophila* strain Paris. Bacteria were inoculated in AYE medium in a 96-well polystyrene plate containing a twofold range of concentration of KKL-35 in columns, crossing a range of another antibiotic in rows. The plate was then incubated for 48h in a Tecan Infinite M200Pro Reader at 37°C, with both agitation and absorbance reading at 600nm every 10 minutes. Growth value was defined as the highest absorbance reading recorded during the growth kinetic. Compared to the classic qualitative evaluation of growth by visual observation, this method allowed us to obtain a quantitative measure of growth. Growth inhibition was defined as a maximal absorbance value <10% the value of the positive control. Fractional Inhibitory Concentration Index (FICI) were interpreted in the following way: FICI<=0.5 = synergy; FICI>4.0 = antagonism; FICI>0.5-4 = no interaction[35].

Activity of KKL-35 on intracellular growth

U937 cells grown in RPMI 1640 containing 10% fetal calf serum (FCS) were differentiated into human macrophages by addition of phorbol 12-myristate 13-acetate (PMA) at 100 ng/mL, then seeded into 96-well polystyrene plates for 3 days (10^6 cells/well). 4 hours before infection the medium was replaced with fresh medium + 10% FCS. *L. pneumophila* str. Paris was plated from a glycerol stock at -80°C onto CYE and incubated at 37°C in air for 72h, then plated again onto CYE plates for 24h to obtain a fresh culture. 4 hours before infection, bacteria were resuspended in RPMI 1640 and incubated at 37°C. Infection of macrophages was performed by replacing their medium by RPMI 1640 + 2% FCS containing *L. pneumophila* at a multiplicity of infection of 10. Plates were centrifuged 10 min at 1000 g then incubated at 37°C with 5% CO_2 for 72h. Micrographs were taken with an inverted microscope (Nikon Eclipse TS100). Live monitoring of infection of U937 macrophages was performed as

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described above, except that the GFP-producing *L. pneumophila* str. Paris pX5 was used, and that the infection was performed in CO_2-independent medium after differentiation, and was monitored by a Tecan Infinite M200Pro plate reader. The plate was incubated in the reader at 37°C and GFP fluorescence levels were automatically monitored every hour for 72h at an excitation wavelength of 470nm, and emission wavelength of 520nm.

Selection of resistant mutants by serial passages

Two different lineages were founded from *L. pneumophila* str. Paris and propagated by serial passages in the presence of KKL-35 or norfloxacin, as previously described[7,8]. Briefly, a suspension of *L. pneumophila* str. Paris in AYE was added to a concentration of 10^8 CFU/mL in a 24-well polystyrene plate with twofold KKL-35 or norfloxacin concentrations ranging from 0.5 times to 8 times the MIC that was determined for the parental strain (norfloxacin: 0.25 mg/L, KKL-35: 0.04 mg/L). Plates were sealed with a Breathe-Easy® membrane (Sigma-Aldrich) and incubated for four days at 37°C in air without agitation, after which the minimum inhibitory concentration was noted for each antibiotic. Bacteria from the well with the highest antibiotic concentration in which growth was observable were transferred using a 1:40 dilution to a new plate containing twofold KKL-35 or norfloxacin concentrations ranging from 0.5 to 8 times the MIC of the previous cycle. Serial passages were repeated 10 times, and the experiment was performed twice independently.

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Results

KKL-35 inhibits *Legionella* growth *in vitro*

MIC and MBC of KKL-35 were determined *in vitro* on five *L. pneumophila* strains and three non-*pneumophila* species causing LD (Table 1). KKL-35 strongly inhibited growth of all tested species, with the highest observed MIC of 5 mg/L for *Legionella micdadei*. KKL-35 was particularly potent against the species *Legionella pneumophila*, with all tested strains exhibiting a MIC around 0.04 mg/L. MBC ranged from one time to two times the MIC for all tested strains, indicating a bactericidal activity. A time-kill assay on *L. pneumophila* str. Paris showed a decrease in viability at 24h after addition of KKL-35 at concentrations equal or higher than MIC (Figure 1). At 72h following addition of KKL-35 at the MIC, the viable count was reduced by four orders of magnitude. Exposure to a half-MIC led to transient bacteriostatic activity for 48h, but then followed by growth suggesting that KKL-35 degrades and looses activity under those conditions. We also tested KKL-35 against twelve *L. pneumophila* mutants that were evolved from the Paris strain to become highly resistant to erythromycin and azithromycin (4000-fold increase in MIC)[8]. The MIC of KKL-35 on these mutants was identical to that of the parent strain (0.04 mg/L) and thus unaffected by ribosomal mutations involved in macrolide resistance (23S rRNA, L4 and L22 proteins mutations).

KKL-35 inhibits intracellular growth of *L. pneumophila*

L. pneumophila can infect human macrophages and replicate extensively within a membrane-bound compartment until cell lysis. Two molecules, KKL-10 and KKL-40, structurally related to KKL-35 were found to be non-toxic to macrophages at concentrations up to 19 mg/L[33]. Indeed, we found that KKL-35 at 10 mg/L protected monocyte-derived macrophages from killing by *L. pneumophila* at a multiplicity of infection (MOI) of 10 (Figure 2, panel A). In order to better characterize the inhibitory activity of KKL-35, we followed the replication of GFP-expressing *L. pneumophila* in monocyte-derived macrophages[36]. Within minutes of forced contact with macrophages, *L. pneumophila* is internalized in a vacuolar compartment that escapes fusion with lysosomes[37,38]. Addition of KKL-35 1h after infection, when bacteria are intracellular but not yet multiplying, prevented *L. pneumophila* replication at concentrations above 1 mg/L (Figure 2, panel B). Moreover, addition of KKL-35 at later timepoints (18 or 24h), when bacteria are actively dividing, stopped bacterial replication (Figure 2, panel B). Interestingly, KKL-35 appeared more potent when added to actively multiplying intracellular bacteria (Figure 2, panel B). This may indicate that either KKL-35 is more active against actively

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212 dividing cells or that the active fraction of KKL-35 gradually decreases over time. In any case, the data
213 show that KKL-35 inhibits replication of *L. pneumophila* within macrophages.

214 KKL-35 does not induce phenotypes associated with loss of trans-translation

215 Lack of trans-translation increases the sensitivity to ribosome-targeting antibiotics in *E. coli*[39,40] and in
216 *L. pneumophila*[31]. The *L. pneumophila* strains *ssrA*^{ind} carrying an IPTG-inducible allele of the tmRNA-
217 encoding gene *ssrA* is unable to grow if IPTG is not supplied in the medium[31]. Low levels of IPTG
218 allow growth with artificially reduced levels of tmRNA, resulting in increased susceptibility to
219 erythromycin and chloramphenicol[31]. Complete lack of trans-translation may further increase the
220 sensitivity of *L. pneumophila* to these antibiotics. Thus, we anticipated that KKL-35 could be
221 synergistic with erythromycin and chloramphenicol. To determine a potential synergy we performed a
222 chequerboard analysis[35]. Interestingly, the MIC of erythromycin (0.125 mg/L) and chloramphenicol (1
223 mg/L) were not affected by KKL-35, indicating the absence of synergy (FICI=2). Thus, unlike the
224 genetic alteration of trans-translation, KKL-35 does not potentiate activity of ribosome-targeting
225 antibiotics. Another phenotype of *L. pneumophila* cells genetically deprived of tmRNA is extended
226 filamentation, indicating that trans-translation is required for cell division[31]. In contrast to *L.*
227 *pneumophila* cells defective for trans-translation, *L. pneumophila* cells treated with KKL-35 at, below,
228 or above the MIC, still display normal morphology (Figure 3, panel A). The inability of KKL-35 to
229 reproduce the phenotypes associated with loss of trans-translation suggests that its potent antibiotic
230 activity is not primarily linked to inhibition of trans-translation.

231 KKL-35 is equally active on *L. pneumophila* lacking trans-translation

232 To test whether the antibiotic activity of KKL-35 was linked to the inhibition of trans-translation, we
233 tested KKL-35 on the *L. pneumophila* strains *ssrA*^{ind}. When IPTG is supplied at high concentrations,
234 tmRNA is expressed at near normal levels, and the strain grows like the wild-type strain. Expectedly, in
235 the presence of IPTG this strain is equally sensitive to KKL-35 (MIC=0.04 mg/L) (Figure 3, panel B).
236 In the absence of IPTG, this strain is strongly impaired for growth. Yet, despite its low levels of
237 tmRNA, the strain is not more sensitive to KKL-35. Ectopic expression of the alternate ribosome-
238 rescue system ArfA from *E. coli* can restore growth of the *ssrA*^{ind} strain in the absence of IPTG. Under
239 these conditions, the strain does not produce tmRNA and is therefore deficient for trans-translation[31].
240 Despite not requiring trans-translation for growth, the MIC of KKL-35 on this strain remained identical
241 to that on the wild-type strain (Figure 3, panel B).

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L. pneumophila does not acquire resistance to KKL-35

In vitro selection of resistance is a common way to identify and characterize potential resistance determinants. Plating of large number of bacteria on solid medium containing antibiotic above the MIC often allows isolation of resistant mutants when resistance is conferred by a single mutation (i.e., rifampicin, streptomycin). This strategy failed to produce mutants resistant to KKL-35. Continuous culture of a bacterial population in increasing concentrations of antibiotics represents an alternate approach when several mutations are required to confer resistance. In *L. pneumophila*, this method has been used to characterize the mutational path to resistance to fluoroquinolones and macrolides[7,8]. In agreement with previous reports, in two independent experiments, we here observed a 500-fold increase in the MIC of norfloxacin in only six passages (about 30 generations) (Figure 4). In contrast, no significant increase in the MIC of KKL-35 was obtained, even after 10 passages (over 60 generations) (Figure 4). Thus, in the tested experimental setup, *L. pneumophila* could not acquire resistance to KKL-35.

Discussion

We investigated the effect of KKL-35 *in vitro* on several *Legionella* species, and found it to be bactericidal on all tested strains. KKL-35 was especially potent on the different tested strains of *L. pneumophila*, the species responsible for more than 90% of cases of LD, with a MIC of 0.04 mg/L (0.125 µM). The bactericidal effect of KKL-35 on *L. pneumophila* was observed at one or two times the MIC and led to a progressive decline in cell viability over time. KKL-35 was found to retain a normal activity on different tested strains of erythromycin-resistant *L. pneumophila*. There is apparently no cross-resistance between macrolides and KKL-35, potentially providing an alternative treatment option in case of the development of macrolide resistance in clinical isolates. In addition, and in contrast to fluoroquinolones and macrolides, *L. pneumophila* did not develop resistance *in vitro*. This result suggests that mutations of the gene encoding the target of KKL-35 may be highly detrimental for the bacteria, and/or that several cellular components are targeted.

Related oxadiazoles (KKL-10 and KKL-40) were recently found to be able to stop intracellular multiplication of *F. tularensis*[33]. In this report, KKL-35 was not tested on intracellular bacteria during infection of human cells because of its lower solubility at efficient concentrations in the tested conditions; we did not encounter solubility problems in our conditions at tested concentrations and up to 10 times the *in vivo* MIC. KKL-35 stops multiplication of *L. pneumophila* str. Paris in monocyte-derived human macrophages when added to the medium at T+1h, 18h or 24h after the beginning of the

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infection. Interestingly, KKL-35 was more potent on actively-multiplying *L. pneumophila* at later stages of infection. This indicates that KKL-35 is able to cross the biological membranes of the macrophage to reach intracellular *L. pneumophila*. Macrophages exposed to KKL-35 for 72h at up to 10 times the *in vivo* MIC were protected from infection by *L. pneumophila*.

As trans-translation activity has been described to be involved in bacterial resistance to ribosome-targeting antibiotics[31,39,40], we tested KKL-35 in combination with other antibiotics to assess their possible synergy. Contrary to what was expected, no synergy was found with ribosome-targeting antibiotics, whereas we previously found that a reduction in tmRNA levels led to an increased susceptibility of *L. pneumophila* to such antibiotics[31]. Similarly, *L. pneumophila* cells treated with KKL-35 for up to 24h did not display the filamentation phenotype observed in cells deprived of tmRNA. These results led to us to question the specific inhibition of trans-translation by KKL-35. KKL-35 was first identified in a high-throughput screen that aimed to identify molecules able to inhibit trans-translation activity *in vitro*[32]. Its antibacterial activity was discovered subsequently and is assumed to result from the inhibition of that pathway. However, we found evidence suggesting that trans-translation may not be the target of KKL-35 in *L. pneumophila*, or at least not its only target. Indeed, MICs were identical when tested on a wild-type strain of *L. pneumophila*, on an inducible mutant of the tmRNA-encoding gene exhibiting different levels of expression of that gene, or when the inhibition of tmRNA expression was complemented by an alternative ribosome rescue system (ArfA from *E. coli*). The molecular target of KKL-35 has not yet been identified. It is possible that in *L. pneumophila*, this target is not prone to support viable mutations. Normal translation may also be inhibited by KKL-35 in *L. pneumophila*, explaining why trans-translation does not seem involved. Alternatively, it could be that other important cellular mechanisms are inhibited in addition or instead of trans-translation, or that KKL-35 targets multiple cellular mechanisms. Other oxadiazoles of the same class seem to inhibit the degradation of unfinished proteins released from ribosomes rescued by trans-translation[32]. KKL-35 does not seem to inhibit this activity in *E. coli*, but it could be different in *L. pneumophila*. Additional studies are needed to better understand the mechanism of action of KKL-35, and to assess further the potential of oxadiazoles in treatment.

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Transparency declarations

None to declare

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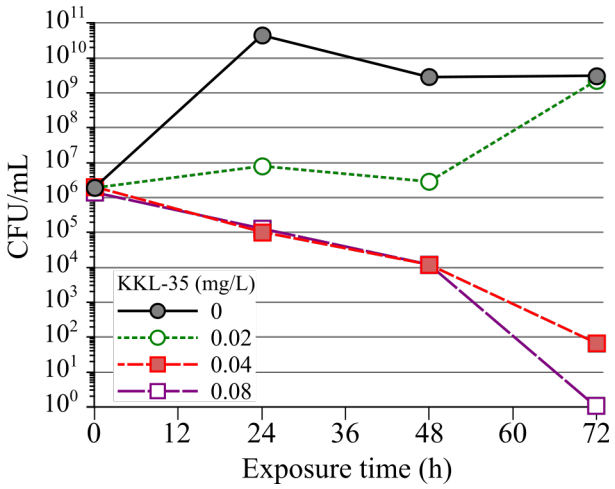
Figures and Table

Figure 1.



Time-kill analysis of KKL-35 on *L. pneumophila*. *L. pneumophila* strain Paris was resuspended in AYE medium at 3.10^6 CFU/mL with a range of two-fold dilutions of KKL-35. Tubes were then incubated at 37°C. Every 24h, serial dilutions were plated on CYE agar and CFUs were counted. Presented data are average of triplicate samples. Black filled circle, no KKL-35; green open circles, KKL-35 at 0.02 mg/L; red filled squared KKL-35 at 0.04 mg/L; purple open squares KKL-35 at 0.08 mg/L.

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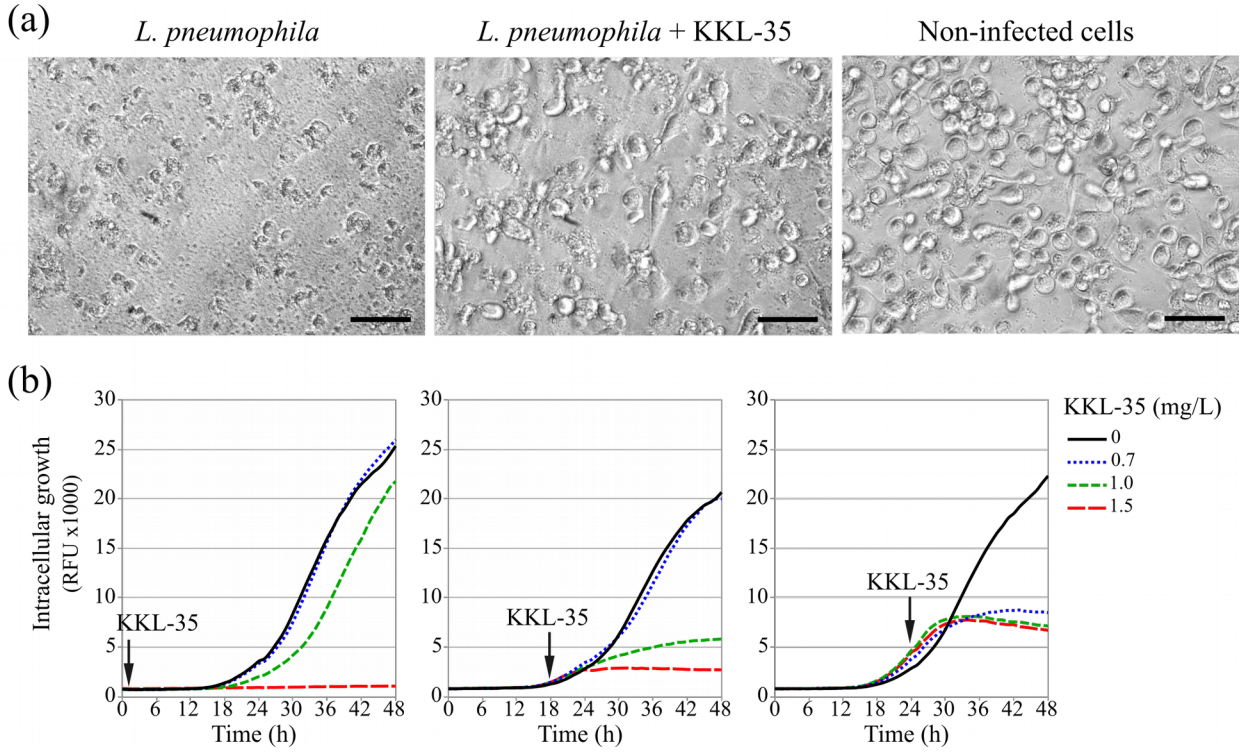
Figure 2.



Activity of KKL-35 on *L. pneumophila* in an intracellular infection model. (a) Bright light microscopy imaging of U937-derived macrophages infected with *L. pneumophila* (MOI=10) for 72h in the presence of absence of KKL-35 at 10 mg/L. Black scale bar represents 50 µm. (b) Live monitoring of infection of U937-derived macrophages by GFP-producing *L. pneumophila* str. Paris carrying plasmid pX5. KKL-35 was added at 1h, 18h or 24h post infection. GFP fluorescence levels were automatically monitored every hour for 48h. Black solid line, no KKL-35; blue short dashed line , KKL-35 at 0.7 mg/L; green intermediate dashed line, KKL-35 at 1mg/L; red long dashed line, KKL-35 at 1.5 mg/L. RFU, relative fluorescence units.

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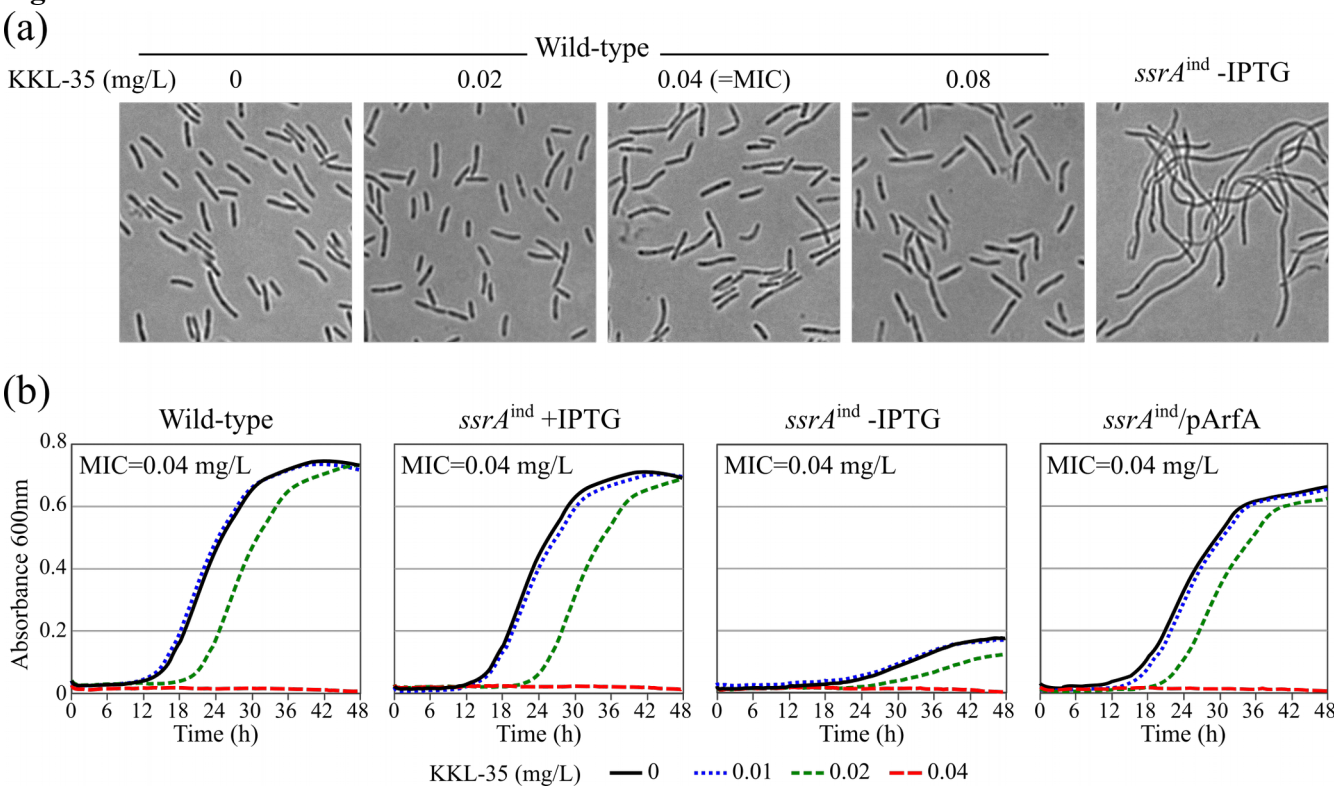
Figure 3.



KKL-35 does not primarily target trans-translation in *L. pneumophila*. (a) Phase contrast light microscopy of wild-type *L. pneumophila* treated with KKL-35 for 24h and of the trans-translation deficient *ssrA*^{ind} mutant deprived of IPTG for 24h. (b) Activity of KKL-35 on *L. pneumophila* strains deficient for trans-translation. Growth curve of the wild-type, *ssrA*^{ind} mutant in the presence or absence of IPTG and of *ssrA*^{ind} mutant rescued by expression of the *E. coli* ArfA. MIC were determined on the basis of absorbance reading. Black solid line, no KKL-35; blue short dashed line, KKL-35 at 0.01 mg/L; green intermediate dashed line, KKL-35 at 0.02 mg/L; red long dashed line, KKL-35 at 0.04 mg/L.

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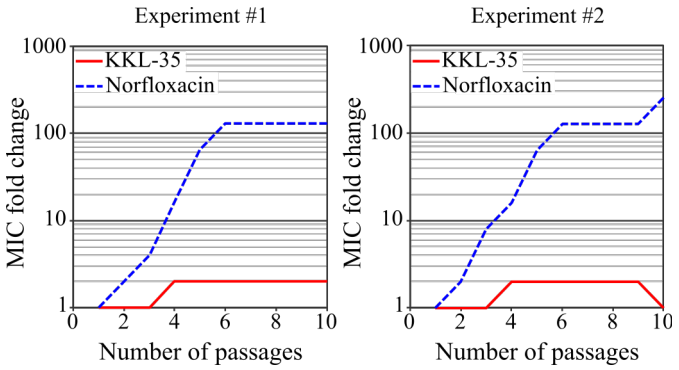
Figure 4.



***L. pneumophila* does not acquire resistance to KKL-35.** Two different lineages were founded from *L. pneumophila* str. Paris and propagated by serial passages in the presence of KKL-35 (red solid line) or norfloxacin (blue dashed line). MIC was determined at each passage and presented relative to the initial MIC (norfloxacin: 0.25 mg/L, KKL-35: 0.04 mg/L).

Table 1. MIC and MBC of KKL-35 on several *Legionella* species *in vitro* (average and standard deviation from three independent determinations).

Strain	Average MIC (mg/L)	Average MBC (mg/L)
L. pneumophila str. Paris	0.04 (±0)	0.04 (±0)
L. pneumophila str. Lens	0.04 (±0)	0.08 (±0)
L. pneumophila str. Lorraine	0.04 (±0)	0.04 (±0)
L. pneumophila str. Philadelphia	0.067 (±0.062)	0.067 (±0.023)
L. pneumophila str. 130b	0.04 (±0)	0.04 (±0)
Legionella longbeachae	0.32 (±0)	0.533 (±0.185)
Legionella micdadei	5.12 (±0)	10.24 (±0)
Legionella dumoffii	2.56 (±0)	5.12 (±0)