Inhibitors of ribosome rescue arrest growth of *Francisella tularensis* at all stages of intracellular replication.

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Running Title: Ribosome rescue inhibitors against *F. tularensis*

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Abstract

Bacteria require at least one pathway to rescue ribosomes stalled at the end of mRNAs. The primary pathway for ribosome rescue is *trans*-translation, which is conserved in >99% of sequenced bacterial genomes. Some species also have backup systems, such as ArfA or ArfB, which can rescue ribosomes in the absence of sufficient *trans*-translation activity. Small molecule inhibitors of ribosome rescue have broad-spectrum antimicrobial activity against bacteria grown in liquid culture. These compounds were tested against the Tier 1 Select Agent *Francisella tularensis* to determine if they can limit bacterial proliferation during infection of eukaryotic cells. The inhibitors KKL-10 and KKL-40 exhibited exceptional antimicrobial activity against both attenuated and fully virulent strains of *F. tularensis* in vitro and during ex vivo infection. Addition of KKL-10 or KKL-40 to macrophage or liver cells at any time after infection by *F. tularensis* prevented further bacterial proliferation. When macrophages were stimulated with the pro-inflammatory cytokine interferon-γ before being infected by *F. tularensis*, addition of KKL-10 or KKL-40 reduced intracellular bacteria by >99%, indicating that the combination of cytokine induced stress and a nonfunctional ribosome rescue pathway is fatal to *F. tularensis*. Neither KKL-10 nor KKL-40 were cytotoxic to eukaryotic cells in culture. These results demonstrate that ribosome rescue is required for *F. tularensis* growth at all stages of its infection cycle, and suggest that KKL-10 and KKL-40 will be good lead compounds for antibiotic development.

Introduction

A major challenge associated with the development of effective antibiotics is the variance observed in bacterial physiology under different growth and infection conditions. Antibiotics capable of inhibiting bacterial replication in vitro may be less efficacious ex vivo or in vivo. This potential difference is particularly important when considering the treatment of the pathogen *Francisella tularensis*, which is shielded from the host innate immune response during infection and replication within eukaryotic cells [1-4]. *F. tularensis ssp. tularensis* is a gram-negative, facultative intracellular bacterium responsible for the vector-borne zoonosis tularemia [2]. Human infections can occur through a number of routes, however bites from infected insects are the most common, leading to the ulceroglandular form of the disease [5-8]. Pneumonic tularemia, while less common, is infectious at ≤ 10 colony-forming units (cfu) of aerosolized bacteria, and has a 60% mortality rate if left untreated [5-8]. *F. tularensis* has been classified as a Tier 1 Select Agent by the CDC due to its high infectivity and ease of propagation [9]. Attempts to develop an effective vaccine have been unsuccessful, due in part to the organism's ability to suppress or bypass the host immune response early after infection [1-4]. *F. tularensis* strains resistant to multiple antibiotics are a biowarfare threat [9]. In the absence of an effective vaccine, new antibiotic targets and compounds are needed to ensure biodefense.

During bacterial translation, when the ribosome reaches the 3' end of the mRNA with no stop codon, the ribosome becomes stuck and is unable to translate other proteins [10-13]. Ribosome rescue pathways that release

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ribosomes from non-stop translation complexes have been identified in most bacteria. Ribosome rescue pathways are potential targets for new antibiotics because they are required for virulence or viability in many pathogenic species [14,15]. *Trans*-translation, the primary ribosome rescue pathway, is performed by a ribonucleoprotein complex consisting of a specialized RNA molecule called transfer-messenger RNA (tmRNA), and the small protein SmpB. During *trans*-translation, tmRNA-SmpB recognizes a non-stop ribosome and releases it by inserting a reading frame within tmRNA into the mRNA channel. Translation resumes on tmRNA and terminates at the stop codon at the end of the reading frame. This reaction also targets the nascent polypeptide and mRNA for degradation [10-13]. *F. tularensis* mutants lacking SmpB or tmRNA are attenuated for virulence in mouse models [16]. Some bacteria, such as *E. coli*, can survive without *trans*-translation because they contain ArfA, a protein that allows release factor 2 (RF-2) to terminate translation on ribosomes stalled at the 3' end of an mRNA [17-19]. Other species have ArfB, a protein that recognizes ribosomes at the 3' end of an mRNA and hydrolyzes the peptidyl-tRNA to rescue the ribosome [20-22]. The *F. tularensis* genome does not contain genes encoding ArfA or ArfB, but it may have an unidentified backup system that rescues ribosomes in mutants lacking SmpB or tmRNA [16].

A group of oxadiazole derivatives (Fig. 1) was identified as inhibitors of *trans*-translation using a high throughput screen [23]. These molecules inhibited *trans*-translation in vitro and in vivo, and inhibited growth of *Shigella flexneri*, *Bacillus anthracis*, and *Mycobacterium smegmatis* in liquid culture [23]. Over-expression

of ArfA or addition of a sub-lethal concentration of puromycin relieved growth inhibition by KKL-35, demonstrating that KKL-35 inhibits bacterial growth by blocking ribosome rescue [23]. ArfA over-expression also relieved growth inhibition by KKL-10 and KKL-40, indicating that these compounds also inhibit growth by blocking ribosome rescue (Fig. S1). Here, we describe the activity of these oxadiazoles against *F. tularensis* grown in vitro and during infection of macrophages and liver cells. The results indicate that ribosome rescue is required for all stages of *F. tularensis* proliferation and suggest that molecules similar to the oxadiazoles may be effective antibiotics against *F. tularensis* and other intracellular pathogens.

Materials and Methods

Bacterial culture

Francisella tularensis ssp. tularensis (Schu S4) and *Francisella tularensis ssp. holartica* (LVS) were grown in brain heart infusion (BHI) medium adjusted to pH 6.8 at 37 ° C and 200 rpm. Growth was monitored by performing optical density (OD) readings at 600 nm. For plating assays, bacteria were diluted in 1X phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4 (pH 7.2)) and grown on chocolate agar plates (Mueller Hinton agar supplemented with 1% bovine hemoglobin (Remel, USA) and 1% Isovitalex X Enrichment (Becton Dickinson, France)) at 37 °C in a humidified incubator with 5% CO_2 for 48 h.

MIC determination and in vitro *F. tularensis* enumeration

For MIC assays, triplicate 2-fold serial dilutions of each compound were made in cation-adjusted Mueller-Hinton broth (CAMHB) and added to a 96-well microtiter plate. Stocks of each compound were prepared in 100% dimethyl sulfoxide (DMSO). Overnight cultures of LVS or Schu S4 were diluted to OD_{600} = 0.05 in CAMHB to a final volume of 0.1 ml and added directly to the diluted compounds. The microtiter plates were incubated overnight (~18 hours) at 37 °C in a humidified incubator with 5% CO_2. Bacterial growth was monitored by measuring the optical density at 600 nm. The MIC was determined by observing the lowest concentration at which the compound prevented a significant increase in optical density. To enumerate the *F. tularensis* after exposure to varying concentrations

of KKL-10 or KKL-40, the contents of the MIC assay microtiter plate were removed and plated on chocolate agar at appropriate dilutions. After incubation for 48 hours at 37 °C and 5% CO_2, colonies were counted to calculate cfu/ml.

BMDM Isolation

The hind legs of euthanized C57 mice were skinned, removed at the hip joint, and feet and excess muscle tissue were removed. Marrow was liberated by removing the femur bones proximal to each joint and crushing them in a 70 µm nylon mesh filter in 5 ml PBS using a sterile pestle. Marrow was added to conical tubes and centrifuged at 500 *g* for 10 min at room temperature. The supernatant was discarded, and the pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, USA). The cells were plated in 100 mm petri dishes at a density of 4 x 10^5 cells/ml in 10 ml complete macrophage differentiation medium (DMEM + 20% L929 cell supernatant containing macrophage colony-stimulating factor (M-CSF). The cells were supplemented with and additional 5 ml of media on days 1 and 3, and BMDMs were harvested on day 7.

Invasion assays and enumeration of intracellular *F. tularensis*

RAW 264.7 macrophages (a gift from Dr. James Drake, Albany Medical College), HepG2 human hepatic cells (a gift from Dr. Gary Perdew, The Pennsylvania State University) or BMDMs were seeded onto 12-or 24-well cell culture plates at a density of 2.5 x 10^5 cells/ml in RPMI-1640 medium (ThermoFisher, USA) with 2% FBS, 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 1 mM HEPES. For IFN-γ prestimulation, IFN-γ was added to 50 ng/ml 12 h prior

to infection. *F. tularensis* LVS cultured in BHI broth to mid-exponential growth phase (OD_{600} = 0.5) was used to infect the cells at a multiplicity of 100:1 for macrophages or 1000:1 for HepG2 cells. Infection of the cells was initiated by centrifugation of the culture plates at 300 *g* for 10 min, Cells were incubated for 50 min at 37 °C and 5% CO_2, and extracellular bacteria were killed by removing the medium, washing the wells 3 X with PBS, and incubating the cells in medium containing 100 □g/ml gentamicin for 1 h. KKL-10 and KKL-40 was added directly to the medium to a final concentration of 2.5 µg/ml at various times post infection depending on the assay. Aspirating the medium, washing the wells 3X with PBS, and lysing the cells in 100 µl 0.1% sodium deoxycholate for 5-10 min released the intracellular bacteria. The lysate was diluted and used in plating assays for enumerations as described above.

Cytotoxicity assays

Cytotoxicity assays were performed using RAW 264.7 cells and an LDH release assay kit (Pierce Biochemicals, USA) following the manufacturer's instructions.

Results

Oxadiazole inhibitors KKL-10 and KKL-40 prevent growth of* F. tularensis *in liquid culture.

Broth microdilution assays were used to determine the minimum inhibitory concentration (MIC) for ribosome rescue inhibitors against the attenuated live vaccine strain of *F. tularensis ssp. holartica* (LVS) and the fully virulent Schu S4 strain of *F. tularensis ssp. tularensis* (Table 1). The MICs of KKL-10 and KKL-40

were lower than those of other antibiotics commonly used in the laboratory or as therapeutics. KKL-35 also had a low MIC against LVS, but was insoluble in growth media at concentrations of 7.95 mg/ml or higher. Therefore, In contrast, KKL-10 and KKL-40 showed full solubility at the highest concentrations tested (38 mg/ml and 35 mg/ml, respectively), so these compounds were pursued further. Plating assays showed that KKL-10 and KKL-40 were bacteriostatic at their MICs against both LVS and Schu S4 (Fig. 2). Higher concentrations of KKL-40 resulted in a >5 log decrease in cfu/ml. The bactericidal effect of KKL-10 was less pronounced at the tested concentrations.

KKL-10 and KKL-40 arrest intracellular growth of* F. tularensis *during all stages of infection.

One of the challenges of treating *F. tularensis* infections is that the bacteria are able to enter host cells and proliferate without being detected [1-4]. In a typical infection, *F. tularensis* enters naïve macrophage or dendritic cells by an uncharacterized phagocytic mechanism, escapes the phagosome 1-4 h post infection, and enters the cytoplasm to proliferate rapidly. Within 24 h, the bacterial numbers increase by ~100-fold. Subsequently, *F. tularensis* induces pyroptosis to lyse the host cell and release bacteria capable of infecting new macrophage or dendritic cells [24]. To determine if KKL-10 and KKL-40 arrest growth of LVS inside natural host cells, RAW 264.7 mouse macrophage cells were infected with LVS and after 1 h extracellular bacteria were eliminated by treatment with gentamicin. KKL-40 was added 3 h after infection and cells were

incubated an additional 21 h before the bacteria were enumerated by plating. KKL-40 inhibited growth of LVS in macrophages in a dose-dependent manner, with almost no growth observed at concentrations ≥2.5 μg/ml (Fig. 3A). This result shows that KKL-40 can cross the plasma membrane and inhibit *F. tularensis* growth in the environment of a eukaryotic cell. The increased concentration of KKL-40 required to arrest growth in macrophages compared to growth in liquid culture was due at least in part to the presence of fetal bovine serum, because increasing the serum concentration increased the MIC in vitro and in macrophages (not shown).

As described above, *F. tularensis* passes through multiple cellular compartments during its infection cycle, and the environments of these compartments can be quite different. To determine if KKL-40 and KKL-10 could inhibit growth of *F. tularensis* in all of these compartments, the ex vivo infection experiment was repeated and the inhibitor was added at different times post-infection. At all time points, both KKL-10 and KKL-40 stopped further bacterial replication (Fig. 3B,C). These data suggest that KKL-10 and KKL-40 arrest *F. tularensis* proliferation quickly, regardless of the stage of infection or the intracellular location of the bacteria.

While *F. tularensis* proliferation primarily occurs in macrophages, the bacteria are also capable of infecting other cell types, such as hepatic cells, in mice and humans [25]. To determine if KKL-10 and KKL-40 could arrest *F. tularensis*

growth in different cell types, the ex vivo infection assays were repeated using human hepatic HepG2 cells and primary bone marrow derived macrophage (BMDM) isolated from C57 mouse femurs. For both cell types, KKL-10 and KKL-40 arrested LVS growth (Fig. 4).

KKL-10 and KKL-40 do not affect macrophage viability or function.

Macrophages play a significant role in host immunity and the innate and adaptive immune response, so it was important to determine if KKL-10 or KKL-40 had any negative effects on macrophage viability or function. A lactate dehydrogenase (LDH) release assay was used to determine the cytotoxicity of KKL-10 and KKL-40. Both molecules produced cytotoxic effects <5% at concentrations up to 17.5 µg/ml (Fig. 5A). Likewise, 24 h treatment with 2.5 µg/ml KKL-10 or KKL-40 was cytotoxic for <5% of macrophages (Fig. 5B).

The effect of KKL-10 and KKL-40 on macrophage function was tested by pre-treating macrophages with one of the molecules prior to challenge with *F. tularensis*. If the ability of the macrophages to phagocytose and harbor *F. tularensis* was impaired by KKL-10 or KKL-40, viable counts of *F. tularensis* should be decreased in these assays. RAW 264.7 cells were pretreated with the inhibitors prior to infection for the indicated times shown in Figure 5C and D. The inhibitors were washed from the macrophages, followed by infection with LVS for 24 hours. No significant growth decrease was observed in pretreated infections

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compared to untreated controls, indicating that the inhibitors do not have profound effects on macrophage activity.

***IFN-γ* stimulated macrophages clear *F. tularensis* infections after addition of KKL-10 or KKL-40.**

The pro-inflammatory cytokine IFN-γ is absolutely required for host survival during the course of an *F. tularensis* infection [26]. IFN-γ restricts bacteria to the endosomal compartment, thereby preventing bacterial escape into the cytoplasm. Oxidative stress is induced in the endosome, which aids in the killing of bacteria [26,27]. However, pre-stimulation of macrophages with IFN-γ has been shown to have bacteriostatic effects on *F. tularensis* infections [28]. To evaluate whether macrophages pretreated with IFN-γ were able to clear an LVS infection after ribosome rescue was inhibited, BMDMs were pre-stimulated with IFN-γ for 12 h, and KKL-10 or KKL-40 were added to the macrophage 3 h post-infection. The combination of IFN-γ stimulation and KKL-10 or KKL-40 activity resulted in a reduction of the bacterial load by >99.9% (Fig. 6). An even more significant decrease was observed in IFN-γ stimulated RAW 246.7 macrophage after the addition of KKL-40, which killed 99.99% of bacteria.

Discussion

The data presented here demonstrate that KKL-10 and KKL-40 prevent growth of *F. tularensis* in liquid culture and during ex vivo infection of eukaryotic cells. Using the fully virulent Schu S4 strain, MIC values for KKL-40 (0.4 µg/ml) and

KKL-10 (0.5 μg/ml) were substantially lower than antibiotics used for clinical treatment of tularemia such as tetracycline (2.3 μg/ml) and streptomycin (4.0 μg/ml). KKL-40 and KKL-10 were also able to inhibit growth of *F. tularensis* inside eukaryotic cells, and were effective at all times during the infection cycle. Neither compounds showed toxicity to macrophages nor HepG2 cells. Because the effects of both compounds were enhanced in macrophages that were activated by IFN-γ, their effectiveness might be increased in vivo, where there is a complete innate and adaptive immune system. The only negative indication observed in these experiments was the relatively high concentration of KKL-40 and KKL-10 (2.5 μg/ml) required for activity in the presence of serum, suggesting that these molecules are likely to bind serum protein. Nevertheless, the effective concentrations were still in line with therapeutically useful antibiotics. Overall, these results encourage further development of KKL-40 and KKL-10 as antibiotics for therapeutic use against *F. tularensis* and other intracellular pathogens, particularly for strains that are resistant to existing drugs. Because KKL-40 and KKL-10 have little structural resemblance to other antibiotics and target ribosome rescue, a pathway that is not targeted by other antibiotics, cross-resistance is unlikely to be a problem.

The activity of KKL-10 and KKL-40 also indicates that ribosome rescue is important for *F. tularensis* both in vitro and during infection. Svetlanov, et al., have shown that *F. tularensis* mutants lacking tmRNA or SmpB are viable but grow slowly, are more sensitive to stress, and have attenuated virulence [16]. If

these *F. tularensis* mutants with no *trans*-translation activity can survive, why is growth of wild-type bacteria completely stopped by KKL-10 and KKL-40, which inhibit ribosome rescue? One possibility is that sudden removal of *trans*-translation activity by addition of an inhibitor has more severe consequences than a genetic deletion. The mutant bacteria might survive because other physiological pathways are regulated to compensate for loss of *trans*-translation, whereas after addition of an inhibitor there might not be enough time for a regulatory response before growth ceases. Alternatively, *F. tularensis* may contain an unidentified factor that can rescue ribosomes in the absence of *trans*-translation, and this factor is also inhibited by KKL-10 and KKL-40. In either case, ribosome rescue is clearly important for *F. tularensis* growth in liquid culture and throughout the infection cycle.

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Figure Legends

Fig 1. Chemical structures of small molecule ribosome rescue inhibitors. KKL-10, KKL-22, KKL-35, and KKL-40 are oxadiazoles, and KKL-55 has a related tetrazole structure.

Fig 2. KKL-40 and KKL-10 inhibit growth of *F. tularensis* in culture. *F. tularensis* cells were enumerated after 24 h exposure to KKL-10 or KKL-40. The cfu/ml of the initial bacterial inoculums are represented with a dotted line. (A) LVS treated with KKL-40. (B) LVS treated with KKL-10. (C) SchuS4 treated with KKL-40. (D) SchuS4 treated with KKL-10. Each column indicates the average of 3 biological replicates. Error bars indicate the standard deviation.

Fig 3. KKL-40 and KKL-10 inhibit *F. tularensis* growth at multiples stages of the infection cycle. (A) Intracellular LVS infections were treated with varying concentrations of KKL-40 to determine the minimum inhibitory concentration of the compounds ex vivo. P value determined by one-way ANOVA with Tukey post test. Inhibitory effects of (B) KKL-10 and (C) KKL-40 at various times after intracellular LVS infection were assayed to ensure that neither compound interfered with bacterial proliferation inside the macrophage. Arrows indicate times of inhibitor addition, and dotted lines are growth curves after inhibitor addition. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

Fig 4. KKL-10 and KKL-40 inhibit intracellular growth of LVS in different cell types. Inhibitors were added 3 h post infection of LVS in the indicated cells. (A) KKL-10 treatment in BMDMs. (B) KKL-10 treatment in HepG2 cells. (C) KKL-40 treatment in BMDMs. (D) KKL-40 treatment in HepG2 cells. P values determined by one way ANOVA with Tukey post test. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

Fig 5. KKL-10 and KKL-40 do not show cytotoxic effects on macrophages. LDH release assays using RAW 264.7 cells pre-treated with KKL-10 or KKL-40 for 45 min. (A) or with 2.5 μg/ml of KKL-10 or KKL-40 for 24 h (B). (+) indicates the 1X lysis buffer positive control. Plating assays after LVS infection of RAW 264.7 cells pretreated with KKL-10 (C) or KKL-40 (D) for indicated times before the compound was washed out and LVS was added. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

Fig 6. Effects of KKL-10 and KKL-40 on LVS in macrophage pre-stimulated for 12 h with IFN-γ. Plating assays after LVS infection of the indicated cells with or without IFN-γ pre-treatment. Where indicated, 2.5 μg/ml inhibitor was added 3 h post-infection. (A) RAW 264.7 cells with KKL-40. (B) BMDMs with KKL-10. (C) BMDMs with KKL-40. P values were determined by one-way ANOVA with Tukey

post test. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

Table 1
Antimicrobial activity of ribosome rescue inhibitors and various antibiotics.

Compound	MIC (µg/ml)* LVS	MIC (µg/ml)* SchuS4
KKL-40	0.12	0.44
KKL-10	0.12	0.48
KKL-35	0.10	ND
KKL-22	0.25	ND
KKL-55	0.80	ND
tetracycline	0.82	2.30
kanamycin	0.90	2.08
gentamicin	0.22	1.25
streptomycin	1.46	4.0
ampicillin	>94	>94

ND, not determined.
* Mean values from at least three broth microdilution assays (µg/ml).

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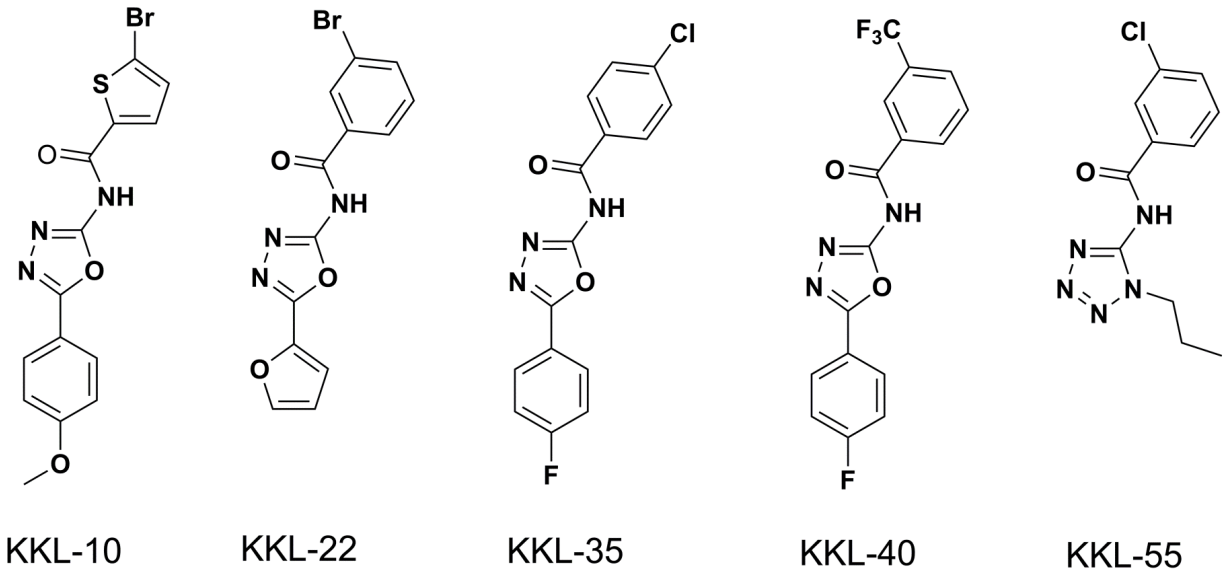

Fig 1. Chemical structures of small molecule ribosome rescue inhibitors. KKL-10, KKL-22, KKL-35, and KKL-40 are oxadiazoles, and KKL-55 has a related tetrazole structure.

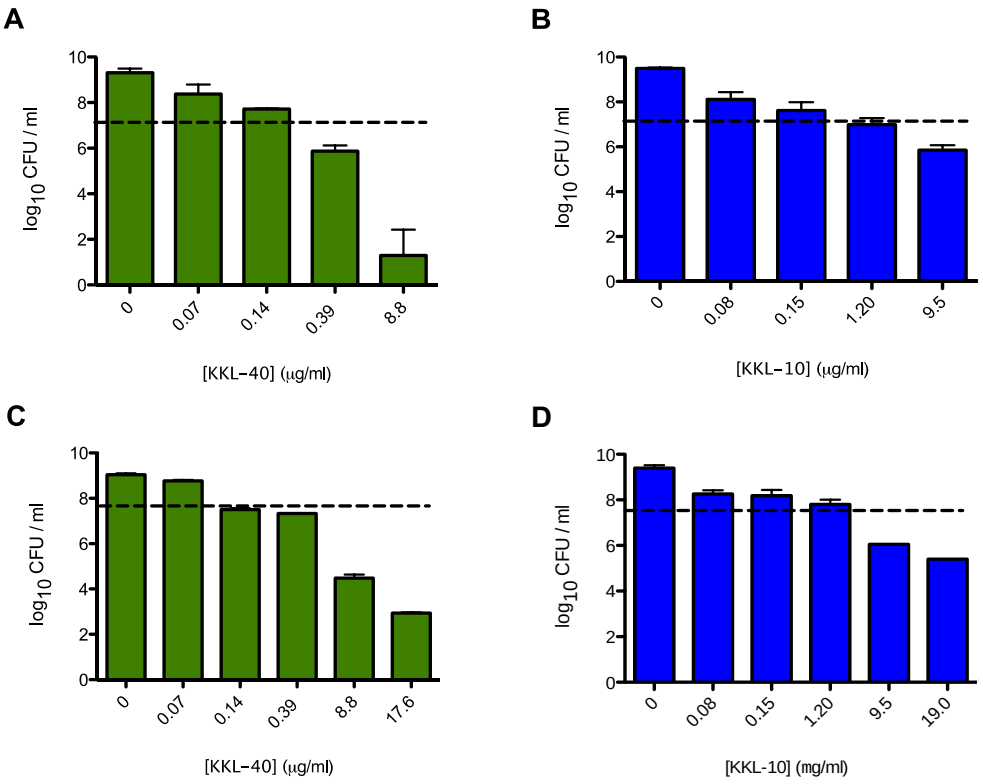

Fig 2. KKL-40 and KKL-10 inhibit growth of *F. tularensis* in culture. *F. tularensis* cells were enumerated after 24 h exposure to KKL-10 or KKL-40. The cfu/ml of the initial bacterial inoculums are represented with a dotted line. (A) LVS treated with KKL-40. (B) LVS treated with KKL-10. (C) SchuS4 treated with KKL-40. (D) SchuS4 treated with KKL-10. Each column indicates the average of 3 biological replicates. Error bars indicate the standard deviation.

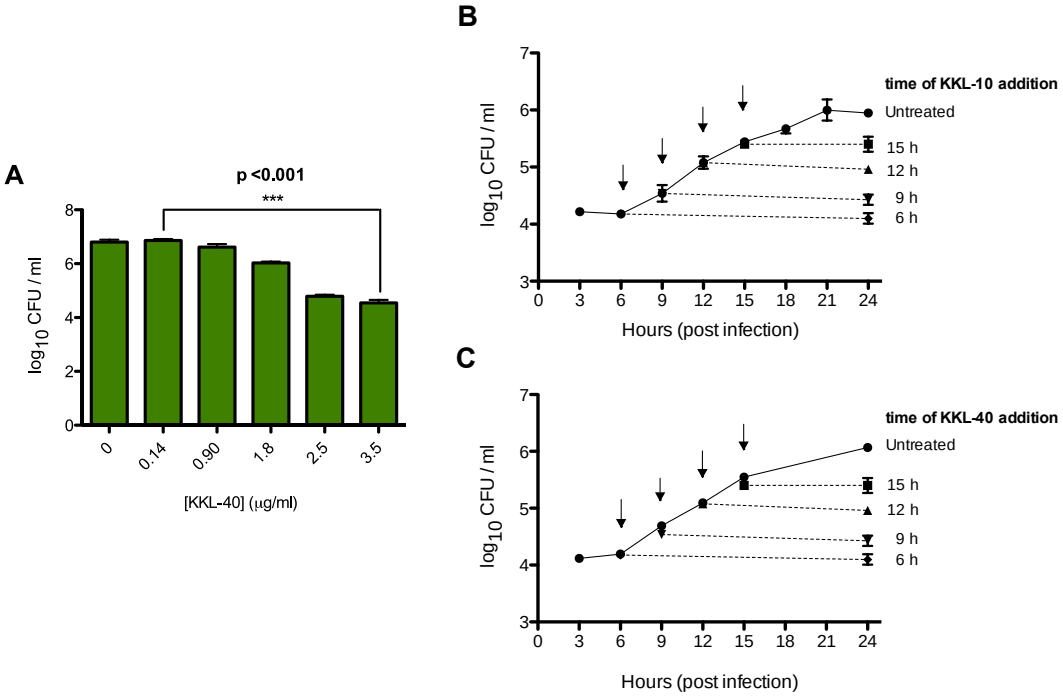

Fig 3. KKL-40 and KKL-10 inhibit *F. tularensis* growth at multiples stages of the infection cycle. (A) Intracellular LVS infections were treated with varying concentrations of KKL-40 to determine the minimum inhibitory concentration of the compounds ex vivo. P value determined by one-way ANOVA with Tukey post test. Inhibitory effects of (B) KKL-10 and (C) KKL-40 at various times after intracellular LVS infection were assayed to ensure that neither compound interfered with bacterial proliferation inside the macrophage. Arrows indicate times of inhibitor addition, and dotted lines are growth curves after inhibitor addition. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

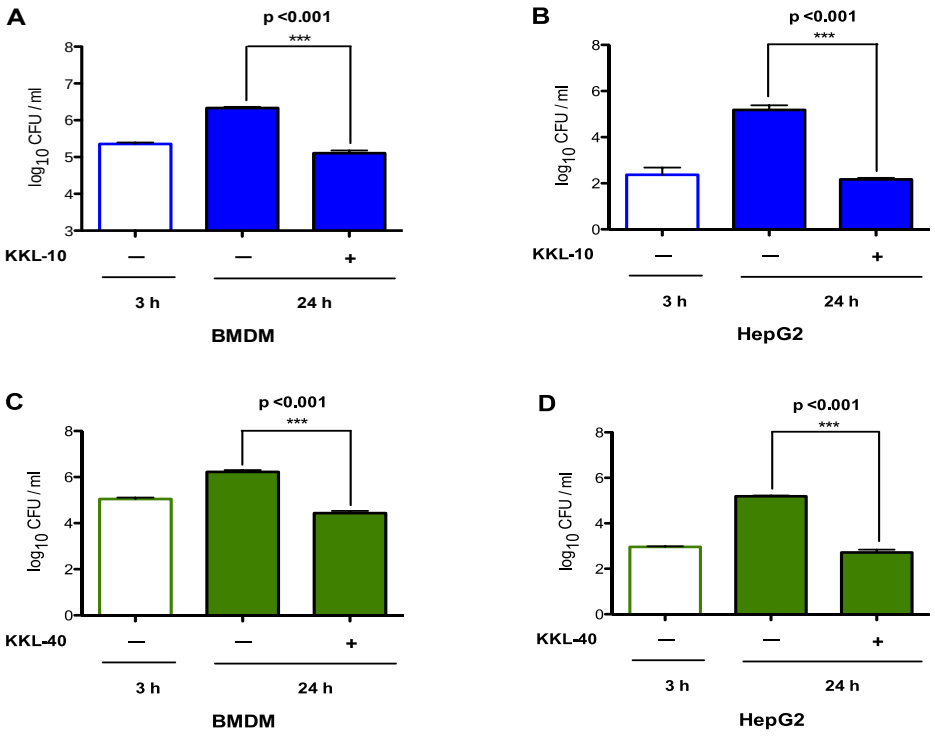

Fig 4. KKL-10 and KKL-40 inhibit intracellular growth of LVS in different cell types. Inhibitors were added 3 h post infection of LVS in the indicated cells. (A) KKL-10 treatment in BMDMs. (B) KKL-10 treatment in HepG2 cells. (C) KKL-40 treatment in BMDMs. (D) KKL-40 treatment in HepG2 cells. P values determined by one way ANOVA with Tukey post test. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

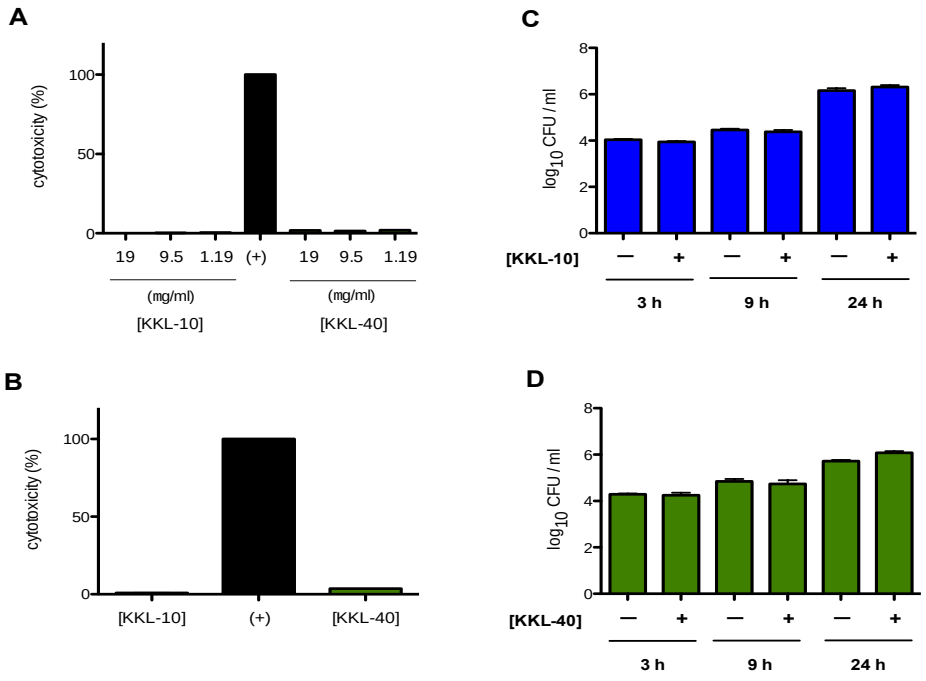

Fig 5. KKL-10 and KKL-40 do not show cytotoxic effects on macrophages. LDH release assays using RAW 264.7 cells pre-treated with KKL-10 or KKL-40 for 45 min. (A) or with 2.5 µg/ml of KKL-10 or KKL-40 for 24 h (B). (+) indicates the 1X lysis buffer positive control. Plating assays after LVS infection of RAW 264.7 cells pretreated with KKL-10 (C) or KKL-40 (D) for indicated times before the compound was washed out and LVS was added. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.

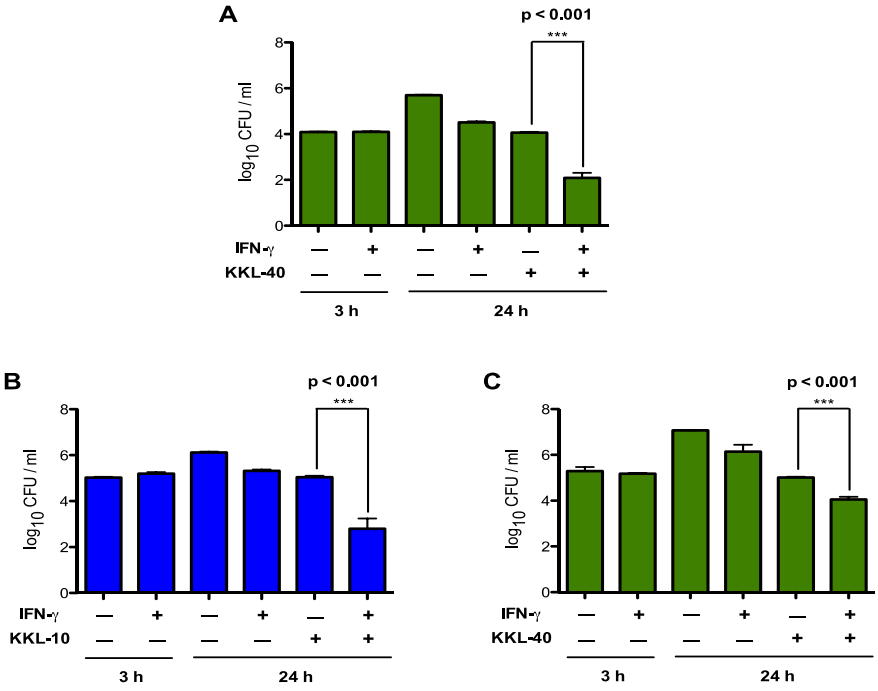

Fig 6. Effects of KKL-10 and KKL-40 on LVS in macrophage pre-stimulated for 12 h with IFN-γ. Plating assays after LVS infection of the indicated cells with or without IFN-γ pre-treatment. Where indicated, 2.5 µg/ml inhibitor was added 3 h post-infection. (A) RAW 264.7 cells with KKL-40. (B) BMDMs with KKL-10. (C) BMDMs with KKL-40. P values were determined by one-way ANOVA with Tukey post test. Each point indicates the average of 3 separate infection experiments performed on the same day, with error bars indicating the standard deviation. The experiments were repeated on at least 3 different days, and data from a representative day are shown.