SMALL MOLECULE INHIBITION OF *TRANS*-TRANSLATION IMPAIRS *STAPHYLOCOCCUS AUREUS* VIABILITY

by

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1. Introduction

Many human bacterial infections in the USA including skin, bloodstream and lower respiratory tract are caused by *S. aureus* (Lowy, 1998; Diekema et al., 2001). Traditionally, the treatment of *S. aureus* infection is antibiotics. However, the development of antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant healthcare challenge (Taylor, 2013; Pottinger, 2013; Otto, 2012). MRSA refers to any strain of *S. aureus* that has developed resistance to beta-lactam antibiotics including penicillin, methicillin, dicloxacillin, etc. As a result, only lower efficiency antibiotics such as vancomycin, linezolid, and daptomycin are used to treat infection caused by MRSA (Taylor, 2013; Pottinger, 2013; Otto, 2012). However, MRSA has already developed resistance even to some of those lower efficiency antibiotics (Mangili et al., 2005; Finks et al., 2009). In the United States, the mortality from MRSA infections is very high, even surpassing that caused by HIV (Heron et al., 2008; Klevens et al., 2007). The direct costs of treating these infections are estimated at $478 million to $2.2 billion annually (Lee et al., 2013).

Although many antibiotics are used in the clinic, the variety of targets that they inhibit has so far been limited. There are four classical targets: 1) Bacterial cell wall synthesis, 2) Protein synthesis, 3) DNA and RNA synthesis, and 4) Folate synthesis (Walsh, 2003). Gram-negative bacteria, for example *Escherichia coli,* and gram-positive bacteria, such as *S. aureus*, require transpeptidase to synthesize peptidoglycan crosslinks, the main components of the bacterial cell wall. Beta-lactam antibiotics are antibiotics that contain a characteristic beta-

lactam ring and directly target and inhibit the transpeptidase enzyme (Spratt & Cromie, 1988). Vancomycin, which is structurally unrelated to beta-lactam antibiotics, also inhibits the peptidoglycan crosslink synthesis by sequestering the substrates away from transpeptidase instead of binding to the enzyme directly (Williams, 1996). Because of structural differences in prokaryotic and eukaryotic ribosomes, a large number of antibiotics selectively target the bacterial ribosome. For example, tetracycline interacts with the conserved sequences of the 16S rRNA of the 30S subunit of the bacterial ribosome and chloramphenicol targets the 23S rRNA of the 50S subunit of bacterial ribosome (Carter et al., 2000; Schlünzen et al., 2001). Other common antibiotics that target ribosome synthesis include linezolid, erythromycin, and doxycycline (Wilson, 2014). DNA replication and transcription are essential for all organisms. While it is difficult to develop a drug that targets DNA/RNA synthesis and shows selective toxicity for prokaryotes, the quinolone family of antibiotics targets prokaryotic DNA gyrase, which regulates DNA supercoiling and rifampicin selectively inhibits prokaryotic RNA polymerase (Maxwell, 1997). Folates are essential for the biosynthesis of a diverse range of components including DNA, RNA and protein in both prokaryotic and eukaryotic cells. For most bacteria, folates must be synthesized from the very beginning through the folate biosynthetic pathway, so inhibition of folate synthesis is toxic (Bermingham & Derrick, 2002). In contrast, mammals utilize the folate transport system to obtain the required folates and so are not affected. Even though we have these many options for treating bacterial infections, we still need new antibiotics due to the inevitable development of antibiotic resistance,

especially in *S. aureus* (Cohen, 2000). Currently, the main strategy in the pharmaceutical industry for developing new antibiotics is to modify the structure scaffolds of existing antibiotics (Walsh, 2003). Although this approach is quite effective, it becomes difficult to meet the increasing demand of new antibiotics due to the dramatic increase in the emergence of antibiotic resistant bacterial strains in recent decades (McDevitt & Rosenberg, 2001).

Since the potential ability of MRSA to develop resistance to our current limited number of antibiotic treatments is high, new pathways that can be targeted for antibiotic development are needed. One pathway that has not been exploited is the *trans*-translation pathway. This pathway is the primary mechanism by which bacterial cells rescue stalled ribosomes and then direct their substrate mRNA and the partially translated peptide for degradation (Keiler, 2008; Shpanchenko et al., 2010; Moore & Sauer, 2007; Keiler & Ramadoss, 2011). There are two ways ribosomes stall, nonstop and no-go translation (Abo et al., 2000). Nonstop translation happens when the mRNA has no in-frame stop codon due to premature termination of transcription, damage to the mRNA or RNase activity. The other situation is frameshifting or read through of the stop codon. As a result, translation of the ribosome occurs to the very 3' end of the mRNA and the ribosome stalls at the end (Keiler, 2008; Doma & Parker, 2007). No-go translation happens when translation reactions are blocked during elongation or termination. Problems during translation elongation are caused by consecutive rare codons as there are a limited number of these anticodon tRNA in the cells and the ribosome stalls during elongation (Hayes et al., 2001; Roche

& Sauer, 1999). During termination, both the stop codon and the nature of the 3' base that follows determine the efficiency of termination (Collier et al., 2002; Poole et al., 1995; Pavlov et al., 1998). A UGA stop codon followed by a C is considered the most insufficient termination sequence, always resulting in nonstop translation (Poole et al., 1995). In most cases, the interactions among the peptidyl-tRNA, the ribosome and the mRNA are so tight that the ribosome cannot dissociate (Schmeing & Ramakrishnan, 2009; Ivanova et al., 2005). These powerful interactions are indispensable for keeping the processivity and fidelity of translation (Schmeing & Ramakrishnan, 2009). Only peptidyl-tRNAs that carry less than six amino acids can exit through ribosomes by a process called drop-off, otherwise they remain associated with the ribosome (de Valdivia & Isaksson, 2005; Cruz-Vera et al., 2004). Without mechanisms like *trans*-translation to rescue the stalled ribosomes, protein synthesis is instantly reduced (Moore & Sauer, 2005; Ramadoss & Keiler, 2013).

The key molecules in *trans*-translation are transfer-messenger RNA (tmRNA) and small protein B (SmpB). tmRNA is a specialized RNA that functions as both tRNA and mRNA (KoMINE et al., 1994). Meanwhile, SmpB is pivotal for keeping tmRNA structure, stability, and activity (Karzai et al., 1999). After tmRNA transcription, its 5' and 3' ends fold together to form a structure like tRNA and then it is charged with alanine (KoMINE et al., 1994; Ushida et al., 1994). During *trans*-translation, alanyl-tmRNA, SmpB and EF-Tu form a complex and then enter to the A site of ribosomes with the acceptor stem of tmRNA in the peptidyl transfer center and SmpB in the anticodon recognition site (Keiler, 2008;

Neubauer et al., 2012). Then as in a normal transpeptidation reaction, the target polypeptide is transferred to alanyl-tmRNA. Therefore, the translational reading frame switches from target mRNA to the reading frame within tmRNA resulting in release and degradation of the original mRNA (Keiler, 2008). After translation of the tmRNA reading frame, the ribosome is released and a peptide tag is added onto the C-terminus of the target polypeptide (Keiler, 2008; Keiler, 1996). This tag can be recognized by multiple intracellular proteases resulting in rapid degradation of the tagged protein (Keiler, 2008; Keiler, 1996; Choy et al., 2007; Flynn et al., 2001; Keiler & Sauer, 1996).

Among all the sequenced bacterial strains, only 22 of them are found without either SmpB or tmRNA in their genomes, which covers less than 1% of the population and suggests the selective advantage of *trans*-translation (Hudson et al., 2014). In *E. coli*, 2-4% of translation initiation reactions terminate in the *trans*-translation pathway. In other words, on average, every ribosome will go through the *trans*-translation pathway 5 times per cell cycle (Ito et al., 2011; Keiler & Feaga, 2014), suggesting that this pathway could be performing a conserved function that is critical for bacterial physiology. Mutations in the components of the *trans*-translation pathway influence viability, stress responses or virulence in a broad range of bacteria. For example, *Shigella flexneri, N. gonorrhoeae, Mycobacterium tuberculosis* and *Helicobacter pylori* require *trans*-translation to maintain their viability and loss of either tmRNA or SmpB is lethal (Ramadoss & Keiler, 2013; Huang et al., 2000; Personne & Parish, 2014; Thibonnier et al., 2008). Mutation of tmRNA increases *E. coli* sensitivity to

antibiotic stress (Li et al., 2013) and *B. subtilis* sensitivity to temperature fluctuations (Shin & Price, 2007). Mutations in *trans*-translation in *Salmonella enterica*, *F. tularensis* and *Yersinia pestis* reduces their virulence (Julio et al., 2000; Svetlanov et al., 2012; Okan et al., 2010). Moreover, there are increasing evidences on the importance of *trans*-translation under stressful conditions, such as high or low temperature, nutrient starvation, cadmium or ethanol treatment, and acid exposure (Okan et al., 2006; Muto et al., 2000; Thibonnier et al., 2008; Abe et al., 2008; Shin & Price, 2007). Under stressful conditions, the intracellular level of tmRNA or SmpB and the total amount of *trans*-translation products are increased (Muto et al., 2000; Fujihara et al., 2002; Rezzonico et al., 2007). Consequently, the *trans*-translation pathway could be a good antibiotic target because it is conserved among a wide range of pathogenic species and its fundamentally important to the proper functioning of the bacterial cell. Because this pathway does not exist in eukaryotes (Keiler, 2008), specific inhibitors are anticipated to have no or limited side effects on host cells.

Recently, several chemical compounds have been identified as inhibitors of *trans*-translation pathway by high throughput screening (Ramadoss et al., 2013). The most potent of these, KKL-35 (Ramadoss et al., 2013) and KKL-40, prevent growth of several bacterial strains including *S. flexneri, B. anthracis, M. smegmatis* and *E. coli ΔtolC*. *Trans*-translation has not been directly studied in *S. aureus* before. However, during saturating transposon mutagenesis studies, no transposon insertion was found in the middle of tmRNA or SmpB gene, suggesting that *trans*-translation may be essential in *S. aureus* (Chaudhuri et al.,

2009). Therefore, we expect *trans*-translation inhibition will impact the viability of *S. aureus* and we believe the *trans*-translation pathway is a potential antibiotic target. To test this, we will use KKL-35 and KKL-40 to determine how inhibition of *trans*-translation influences the viability of *S. aureus* and assess their potential as antibiotics.

2. Methods

2.1 Bacterial strains and growth conditions

S. aureus strains (Newman, Sanger 252, USA300) were grown in Tryptic Soy Broth (TSB, Hardy Diagnostics) at 37°C. *Actinobacter baumannii, Pseudomonas aeruginosa* PA14, and *Streptococcus pyogenes* were grown in Brain-Heart Infusion (BHI, Hardy Diagnostics) medium at 37°C. *E. coli* K-12 were grown in Luria Broth (LB, Hardy Diagnostics) at 37°C. *Klebsiella pneumoniae* were grown in Todd Hewitt Broth (THB, Fluka) at 37°C. All solid bacterial cultures were grown on BHI agar at 37°C. Assays were performed in cation-adjusted Mueller Hinton II Broth (MHBII, BD). Some of them were further supplemented with calcium to a final concentration of 50μg/ml to make CA-MHBII.

2.2 Minimum inhibitory concentration (MIC) assays

Assays were performed in 96 well tissue culture plates (Celltreat). The test medium for most tests was CA-MHBII with the exception of RPMI + 5% LB for LL-37 and THB for *Klebsiella pneumoniae* and *Streptococcus pyogenes*. Bacteria were diluted 1:1000 in assay media for a starting cfu of ~2.0×10^5 cfu/ml for *S. aureus*, ~2.0×10^5 cfu/ml for *E. coli* K-12, ~3.0×10^5 cfu/ml for *Klebsiella pneumoniae,* ~2.5×10^5 cfu/ml for *Actinobacter baumannii,* ~5.0×10^5 cfu/ml for *Pseudomonas aeruginosa* PA14, and ~3.0×10^5 cfu/ml for *Streptococcus pyogenes.* The total volume in each well was 200μl. After 20-24 hours of incubation at 37°C, the MIC was defined as the lowest concentration of antibiotic

with no visible growth and lowest optical density (OD) value at 600nm. Experiments were repeated 3 times and presented as mean +/- SEM.

2.3 Time-dependent killing

An overnight culture of Newman was diluted to an optical density at 600 nm (OD600) of <0.1 in 3mL TSB medium and incubated at 37°C with aeration at 225 rpm to an OD600 of 0.4 (early log phase). Bacteria were centrifuged at 4000 rpm and resuspended in 3 ml of CA-MHBII. Bacteria were then diluted 1:1000 in CA-MHBII for a final concentration around $2.0x10^5$ cfu/ml and challenged separately with KKL-35 and KKL-40 at 1X, 2X and 4X the MIC in a 96 well tissue culture plate at 37°C.

For comparison of killing of early log phase bacteria culture by KKL-40 verses other antibiotics, bacteria were diluted 1:400 in MHBII for a final concentration around $6.0x10^5$ cfu/ml and challenged separately with KKL-40, daptomycin, vancomycin or methicillin at 4X the MIC (8μM, 8μg/ml, 4μg/ml, 16μg/ml respectively) in a 96 well tissue culture plate at 37°C.

For killing of stationary phase cultures overnight cultures were first diluted to OD 600 ≈1.0 in 96 well tissue culture plate and then diluted at 1:1000 in MHBII for a final concentration around $6.0x10^5$ cfu/ml. Diluted bacteria were challenged separately with KKL-40, daptomycin, vancomycin or methicillin at 4X the MIC in a 96 well tissue culture plate at 37°C.

For all assays, 25µl aliquots were removed at indicated time points and plated using serial dilution and grown overnight at 37°C. Colonies were counted and the colony forming units (cfu/ml) were calculated. Experiments were repeated 3 times and presented as mean +/- SEM.

2.4 Generation of resistant strains

10µl of an overnight culture of Newman was diluted in 1 ml of TSB. Cells were incubated at 37°C with agitation, and passaged at 24 hour intervals in the presence of methicillin (2 µg/ml), vancomycin (0.5 µg/ml) or KKL-40 (1µM) at sub-lethal concentrations (approximately 0.5-fold of the original effective MIC). At day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, bacteria were first diluted to OD 600 ≈0.7 in 96 well tissue culture plate and then diluted 1:400 in TSB for MIC assays for a starting concentration of approximately $2.0x10^6$ cfu/ml. Data is plotted as fold change from original MIC (before passage) which was 4µg/ml, 1µg/ml, and 2µM for methicillin, vancomycin and KKL-40 respectively. Experiments were repeated 2 times and presented as mean.

2.5 Synergistic interaction

An overnight culture of Newman was diluted to OD600 <0.1 in 3mL CA-MHBII medium and incubated at 37°C with aeration at 225 rpm to OD 600 of 0.4 (early log phase). Bacteria were then diluted 1:1000 in RPMI + 5% LB (LL-37) or in CA-MHBII (daptomycin, tetracycline, kanamycin, chloramphenicol and erythromycin). They were incubated with either no compound, KKLs, antimicrobials or a combination of KKLs and antimicrobials at 37°C. Bacterial

survival was enumerated at 20 hours by serial dilution and counting colony growth. Experiments were repeated 3 times and presented as mean +/- SEM.

2.6 In vitro cytotoxicity assay in HeLa cell

HeLa cells were fed with Dulbecco's modified Eagle's medium with 10% Fetal Bovine Serum (DMEM – 10%FBS), antibiotic (pen/strep) and L-glutamine were also added to a final concentration of 1%. Trypsin-EDTA solution was used for cell dissociation. Trypan Blue solution was used for cell counting. HeLa cells were plated on tissue culture-treated 96 well tissue culture plates (Celltreat, Shirley, MA). Each well contained $2x10^4$ cells and were incubated for 24 hours at 37°C with 5% CO_2. Media was then removed, cells were washed 1x in PBS, and serum-free DMEM media containing serially diluted KKL compounds up to 100X MIC was added to the cells. Cytotoxicity was assessed at 24 hours after incubation with the compounds by adding 20µl of MTS (CellTiter 96® AQueous non-radioactive cell proliferation assay, Promega, Madison, WI) directly to the culture wells and recording the absorbance at 490nm. Background OD from media alone were subtracted from the OD reading and HeLa cell survival rates were calculated by dividing the OD of KKL treatment cells to non-treated cells. Experiments were repeated 3 times and presented as mean +/- SEM.

2.7 Compatibility with human serum

Early log phase Newman bacteria culture were diluted 1:1000 and incubated with KKL-40 for 24 hours at 37°C. MIC assays were performed in CA-MHBII containing 5% or 20% human serum.

2.8 *Statistics*

All statistical analysis was performed using GraphPad Prism. Specific tests used are described in the figure legends.

3. Results

KKLs inhibit growth of different Staphylococcus aureus strains

Two chemical compounds KKL-35 and KKL-40, which specifically inhibit *trans*-translation, had previously been identified to inhibit the growth of *S. flexneri, B. anthracis, M. smegmatis* and *E. coli ΔtolC* (Ramadoss et al., 2013). These bacterial strains are unrelated to each other, suggesting KKLs may have antibiotic activity against a broad spectrum of bacteria including *S. aureus.* Since the first discovery of *S. aureus* in the 1880s, many subpopulations or strains of *S. aureus* have arisen. These include methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains. The primary difference between them is the sensitivity to beta-lactam antibiotics, although pathogenicity and virulence can also vary (Rozgonyi et al., 2007). In addition, MRSA strains are divided into 2 types: healthcare-associated (HA-MRSA) and community-acquired (CA-MRSA) (Rehm, 2008; Herold et al., 1998; Rehm & Tice, 2010). HA-MRSA is primarily found in healthcare facilities and affects persons with weakened immune systems. In contrast, CA-MRSA is found in the community and affects healthy individuals.

Due to the possibility that MSSA, HA-MRSA and CA-MRSA may have different responses to KKLs, we used Newman, Sanger 252 and USA300 as representatives of these 3 groups for the following reasons. 1) Newman, an MSSA strain, has been used extensively in *S. aureus* research due to its robust virulence phenotypes (Baba et al., 2008). 2) Sanger 252 is a representative of the highly successful HA-MRSA strain isolated in British hospitals and is the most

genetically diverse *S. aureus* strain (Holden et al., 2004). 3) USA300 is the predominant strain of CA-MRSA in the US (Rehm & Tice, 2010). We find that KKLs prevent the growth of all three, MSSA, HA-MRSA and CA-MRSA, at relatively low concentrations (Fig.1). All of them have the same KKL-35 MIC concentration, which is 4μM. The MIC concentrations of KKL-40 for Newman, Sanger 252 and USA 300 are 2μM, 1μM and 1μM, respectively. These results indicate that KKL-35 and KKL-40 can inhibit *trans*-translation and thereby growth in a wide range of *S. aureus* strains.

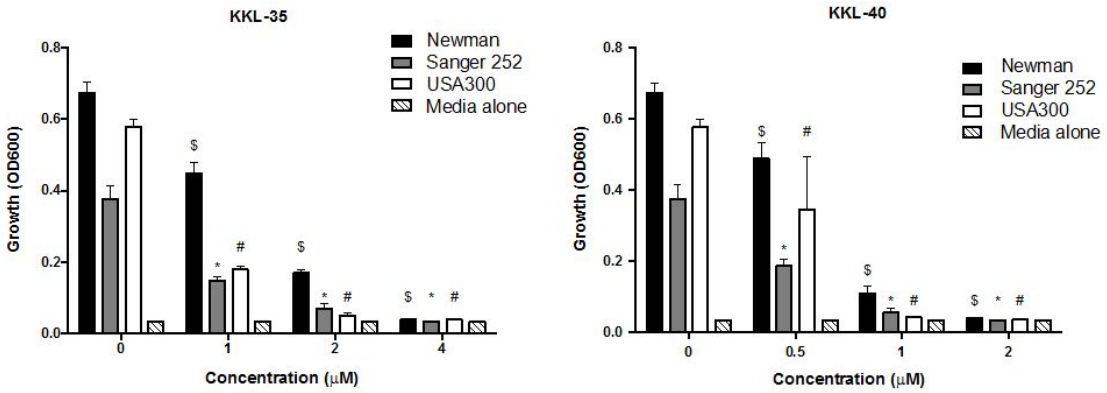

Figure 1. KKL-35 and KKL-40 inhibit growth of different *Staphylococcus aureus* strains. Bacteria were grown to early log phase and diluted 1:1000 in CA-MHBII media and incubated with KKL compounds for 20hrs at the indicated concentration. Optical density was measured at 600 nM. Results from 6 independent experiments are presented as mean +/- SEM. $,*, #, denotes significant growth inhibition in each strain compared to no compound treatment by one-way ANOVA followed by Dunnett's multiple comparisions, $p<0.0001$.

KKLs inhibit growth of S. aureus strains in a time-dependent manner

Current antibiotics are classified into two general categories: bactericidal antibiotics, which kill above 99.9% of the initial bacterial inoculum, and bacteriostatic antibiotics, which inhibit bacterial growth but cannot efficiently kill bacteria (Pankey & Sabath, 2004). Time-kill kinetics is an important factor in evaluation of an antibiotic, including the speed of killing and the

bactericidal/bacteriostatic properties (Norcia et al., 1999; Gallant-Behm et al., 2005). Moreover, among a variety of test techniques, the time-kill kinetic assay is thought to be a relatively good predictor of therapeutic outcome due to positive correlation between the results from time-kill kinetic assay and clinical outcome (Gallant-Behm et al., 2005; Sakoulas et al., 2004; Moise et al., 2007). Our previous experiment showed that both KKL-35 and KKL-40 can inhibit *S. aureus* growth in a similar manner in all 3 strains, therefore we chose just one strain, Newman, which is commonly used as a model strain for *S. aureus*, as a representative strain for further investigation. As expected, both KKL-35 and KKL-40 have the same pattern of time-dependent killing (Fig.2). Increasing the concentrations of KKLs to up to 4 times higher than the MIC concentrations (8 μM for KKL-40 and 16 μM for KKL-35) did not result in a proportional increase in killing indicating that the *trans*-translation has been fully inhibited at 4μM of KKL-35 or 2μM of KKL-40. Except for 4μM of KKL-35 (99.6% killing efficiency), all the others have a more then 99.9% killing efficiency, indicating that the KKLs belong

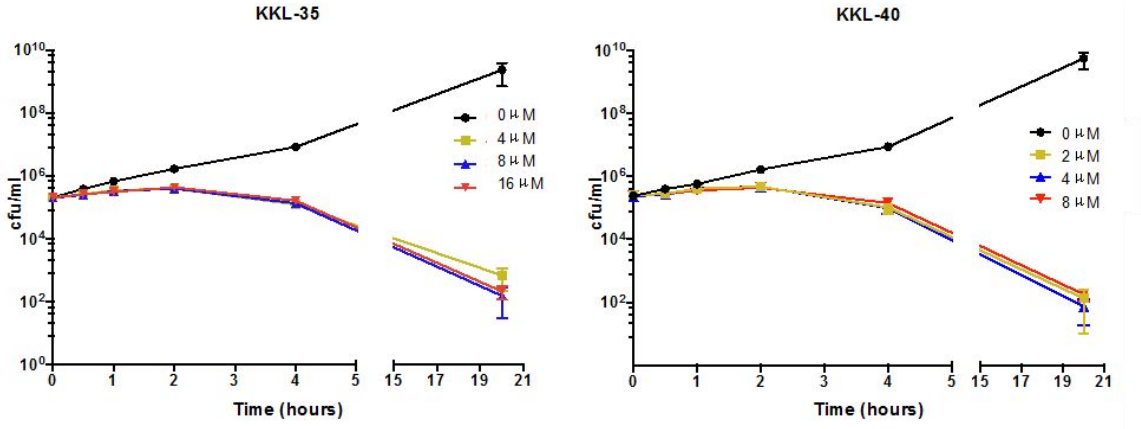

Figure 2. Time-dependent killing of KKL-35 and KKL-40. Newman were grown to early log phase and diluted 1:1000 in CA-MHBII media and incubated with KKL compounds at the indicated concentration. Surviving bacteria were enumerated by serial dilution at the indicated time point up to 20hrs. Results from 3 independent experiments are presented as mean +/- SEM.

to bactericidal antibiotics. Although the KKLs are bactericidal, they have a delayed time to action. It is not until 2 h that KKLs first begin to control growth and killing is not seen until after 4 h.

KKL-40 has a similar killing pattern as vancomycin

During log phase, bacteria are rapidly growing. Their metabolic activity and DNA replication occur at the maximal rate and the number of bacteria increases exponentially. However, in stationary phase, the bacterial growth rate is stable due to the lack of nutrients in the medium. Log phase and stationary phase bacteria do not have identical biological characteristics (Mascio et al., 2007). For example, *S. aureus* enhances its virulence by changing gene expression in stationary phase (Somerville et al., 2002). Stationary phase bacteria are more commonly found in persistent infections and in biofilm-associated infections (Hall-Stoodley et al., 2004; Donlan & Costerton, 2002). Stationary phase bacteria are also often less susceptible to antibiotics (Kim & Anthony,1981). Therefore, it is important to investigate the effectiveness of killing by KKLs for both log phase and stationary bacteria. Due to the great similarity in antibiotic properties between KKL-35 and KKL-40 and the fact that KKL-40 is more effective, we chose to compare KKL-40 to other antibiotics commonly used to treat *S. aureus*, specifically vancomycin, daptomycin and methicillin. Vancomycin is a glycopeptide antibiotic and daptomycin is cyclic lipopeptide antibiotic (Srinivasan et al., 2002; Fowler Jr et al., 2006). Both of them are used as treatments in MRSA infections. Methicillin is a β-lactam antibiotic that was used commonly to treat *S. aureus* infections until the rise of methicillin resistance.

All the antibiotics kill both log phase and stationary phase *S. aureus* growth in a time-dependent manner (Fig.3). Log phase *S. aureus* is slightly more easily killed by daptomycin and methicillin although there is very little difference in killing of log and stationary phase bacteria by vancomycin and KKL-40.

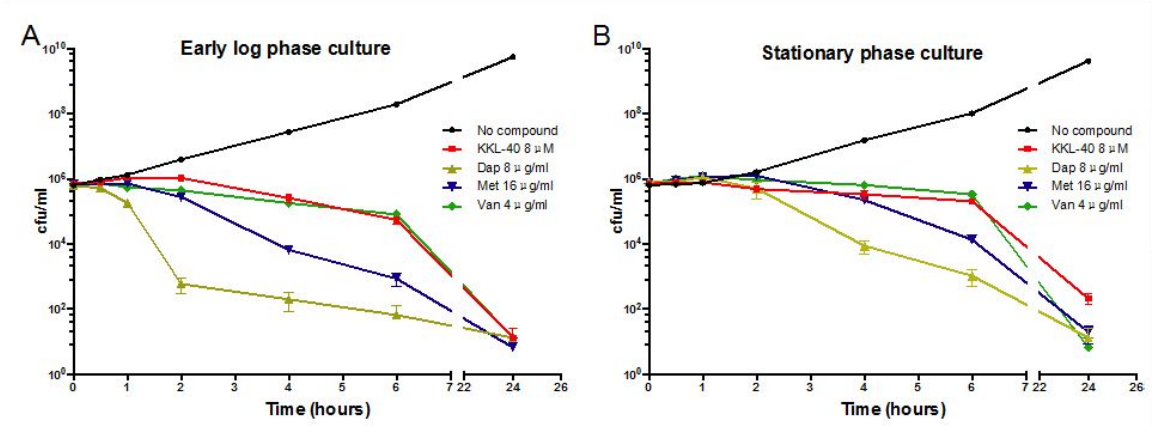

Figure 3. Time-dependent killing of KKL-40 compared to other antibiotics. Newman was challenged separately with KKL-40, daptomycin (Dap), vancomycin (Van) or methicillin (Met) at 4X the MIC. (A).Newman were grown to early log phase and diluted 1:400 in assay media and incubated with antibiotic compounds at the indicated concentration. (b).Overnight Newman bacterial culture were first diluted to OD=1.0 and then diluted 1:400 in assay media and incubated with antibiotic compounds at the indicated concentration. Surviving bacteria were enumerated at the indicated time point up to 24hrs. Results from 3 independent experiments are presented as mean +/- SEM.

KKL-40 inhibits growth of different bacterial strains

Due to the highly successful bactericidal activity of KKLs in *S. aureus*, we next looked at its effectiveness in other common pathogens. *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Streptococcus pyogenes* are well known human pathogens. In addition, all of them have the ability to acquire resistance to multiple antibiotics, leaving few options for treatment of infections (Peleg et al., 2008; Dijkshoorn et al., 2007; Qureshi et al., 2012; Breidenstein et al., 2011; Rubio-López et al., 2012). *E. coli K12* is routinely

used in microbiology studies. Although KKL-40 is able to inhibit growth of all the bacterial strains, it is not equally effective among them (Fig.4). The MIC concentration of KKL-40 in *Acinetobacter baumannii, Klebsiella pneumoniae* and *E. coli K12* is greater than 64μM. For *Pseudomonas aeruginosa,* the effectiveness of KKL-40 is even less with very little antimicrobial activity even at 64 μM. In contrast, KKL-40 maintains its antimicrobial property in *Streptococcus pyogenes* with a MIC concentration at 8μM.

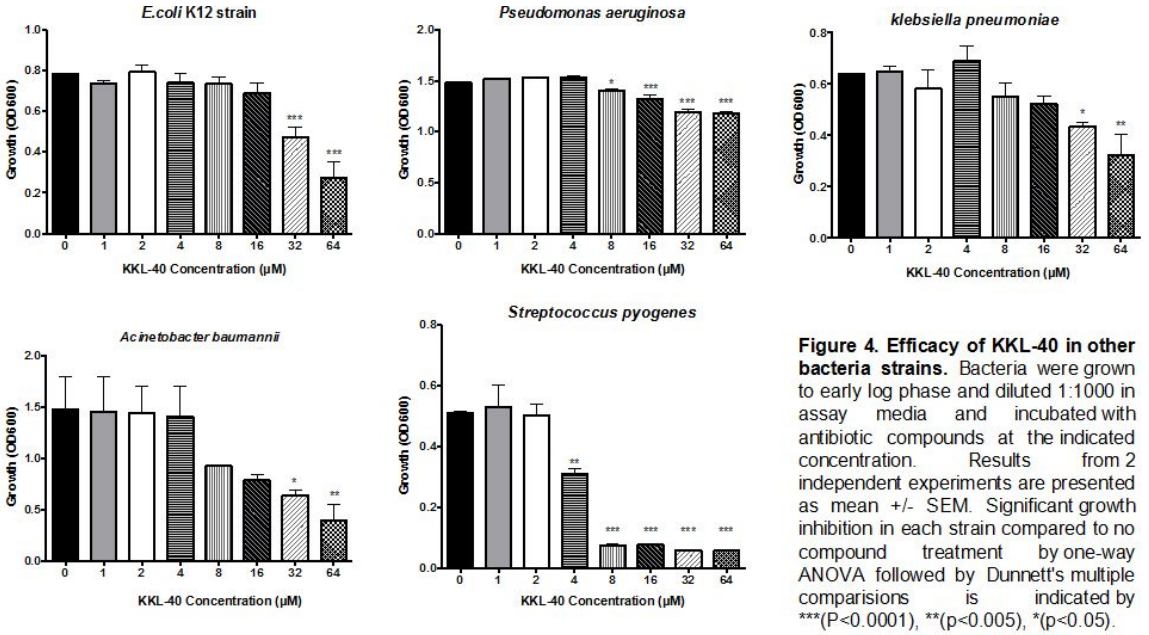

Figure 4. Efficacy of KKL-40 in other bacteria strains. Bacteria were grown to early log phase and diluted 1:1000 in assay media and incubated with antibiotic compounds at the indicated concentration. Results from 2 independent experiments are presented as mean +/- SEM. Significant growth inhibition in each strain compared to no compound treatment by one-way ANOVA followed by Dunnett's multiple comparisions is indicated by ***(P<0.0001), **(p<0.005), *(p<0.05).

Synergistic interactions between KKLs and antimicrobials

The enhanced activity of *trans*-translation under stressful conditions in other bacteria highlights the importance of *trans*-translation for bacterial viability (Muto et al., 2000; Fujihara et al., 2002; Rezzonico et al., 2007). Loss of *trans*-translation during stressful conditions, such as those seen during infection, could significantly impact bacterial survival. We exposed bacteria to stressful conditions

such as exposure to antimicrobials in order to investigate the significance of *trans*-translation in response to these conditions. We treated *S. aureus* with a sub-lethal dose of LL-37, a human antimicrobial peptide that kills bacteria through the formation of membrane pores, and a sub-lethal dose of KKL-35 and KKL-40. We found that although there was no effect on survival by LL-37 or by KKLs alone, however there is a strong synergistic interaction when KKL-35 or KKL-40 are combined with LL-37 (Fig.5).

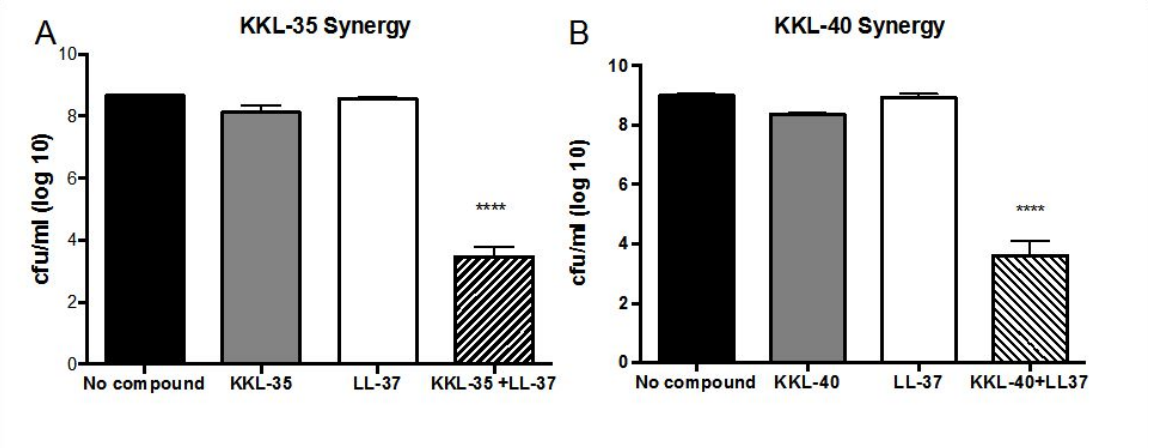

Figure 5. Interaction between KKLs and the human antimicrobial peptide LL-37. Newman were grown to early log phase, diluted 1:1000 in assay media, and incubated with either no compound (black bar), KKL alone (grey bar), LL-37 alone as indicated in figure (white bar) or a combination of KKL and LL-37 (hatched bars) overnight at 37 ℃. (A). Bacteria were incubated with 8 μM KKL-35 and/or 2 μM LL-37 as indicated. (B). Bacteria were incubated with 4 μM KKL-40 and/or 2 μM LL-37 as indicated. Results from 3 independent experiments are presented as mean +/- SEM. Synergistic interaction by two-way ANOVA is indicated by ****(P<0.0001).

Next we tested the antibiotic daptomycin in a similar assay. Daptomycin is cyclic lipopeptide antibiotic. Both daptomycin and LL-37 exert their bactericidal function by disrupting the integrity of the bacterial cell membrane (Rezzonico et al., 2007; Straus & Hancock, 2006; Dürr et al., 2006). Daptomycin and LL-37 also share similar characteristics on mechanism of resistant development. For example, bacteria resistant to daptomycin or LL-37, have different cell membrane

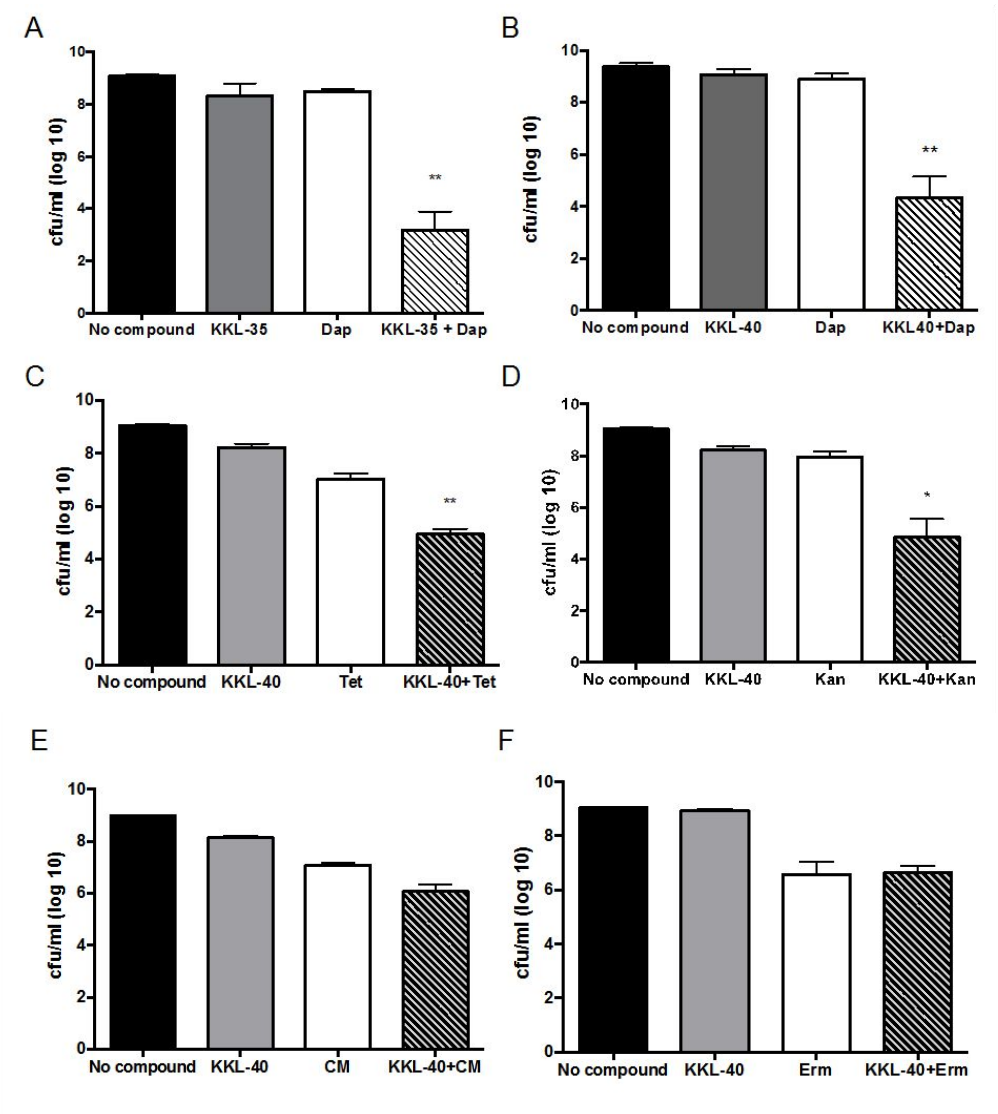

Figure 6. Interaction between KKLs and antibiotics. Newman was grown to early log phase, diluted 1:1000 in CA-MHBII media, and incubated with either no compound (black bar), KKL alone (grey bar), antibiotic alone as indicated in figure (white bar) or a combination of KKL and the indicated antibiotic (hatched bars) overnight at 37 ℃. (A). Bacteria were incubated with 2 μM KKL-35 and/or 0.5 μg/ml daptomycin as indicated. (B) Bacteria were incubated with 0.5 μM KKL-40 and/or 0.5 μg/ml daptomycin as indicated. (C). Bacteria were incubated with 1 μM KKL-40 and/or 2 μg/ml tetracycline as indicated. (D) Bacteria were incubated with 1 μM KKL-40 and/or 2 μg/ml kanamycin as indicated. (E). Bacteria were incubated with 1 μM KKL-40 and/or 6 μg/ml chloramphenicol as indicated. (F). Bacteria were incubated with 0.5 μM KKL-40 and/or 0.25 μg/ml erythromycin as indicated. Results from 3 independent experiments are presented as mean +/- SEM. Synergistic interaction by two-way ANOVA is indicated by ***(P<0.0001), **(p<0.005), *(p<0.05).

surface charges and membrane fluidity (Koprivnjak & Peschel, 2011; Kaatz et al., 2006; Bayer et al., 2013). Similar to the synergy between LL-37 and KKLs, there is a robust synergistic interaction between daptomycin and KKLs although there is little effect on bacterial viability with daptomycin or KKL-35 or KKL-40 alone

(Fig 6A and 6B). Last, we wanted to see whether this phenomenon extended to other antibiotics. We tested tetracycline, kanamycin, chloramphenicol and erythromycin. They all exert their antimicrobial effects by inhibiting the normal function of the ribosome (Nguyen et al., 2014; Faraji et al., 2006). Kanamycin and tetracycline are weakly synergistic with KKLs due to the relatively high cfu/ml compared to the single treatment (Fig. 6C and 6D). However, no synergistic interactions were found between KKLs and chloramphenicol or erythromycin (Fig. 6E-6F).

KKLs are not toxic to human HeLa cells but they lose their activity in human serum

Next we looked at whether KKL-35 and KK-L40 are selectively toxic to bacterial cells by examining their effect on human cells. We incubated either KKL-35 or KKL-40 with human HeLa cells, a cervical cancer cell line, for 24 hours in serum free media. We find that KKLs are not toxic to human HeLa cells even at 100-fold of the effective MIC concentration (Fig.7).

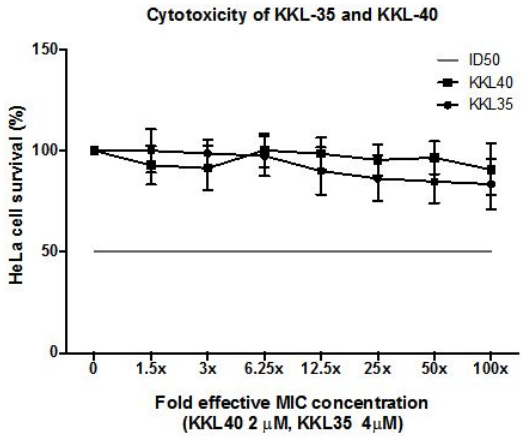

Figure 7. Cytotoxicity in human cells. Hela cells were incubated with KKL compounds for 24hrs and survival was assayed by cell titer 96 ® AQ_{ueous} non-radioactive cell proliferation assay. Results from 3 independent experiments are presented as mean +/- SEM.

Lastly, we tested whether KKLs can function in human serum. Human serum contains thousands of proteins that can bind and inactivate pharmacological agents. Unfortunately, KKL-40 does not maintain its bactericidal activity even in only 5% human serum at concentrations of 8 μM, which is 4-fold its effective MIC (Fig.8).

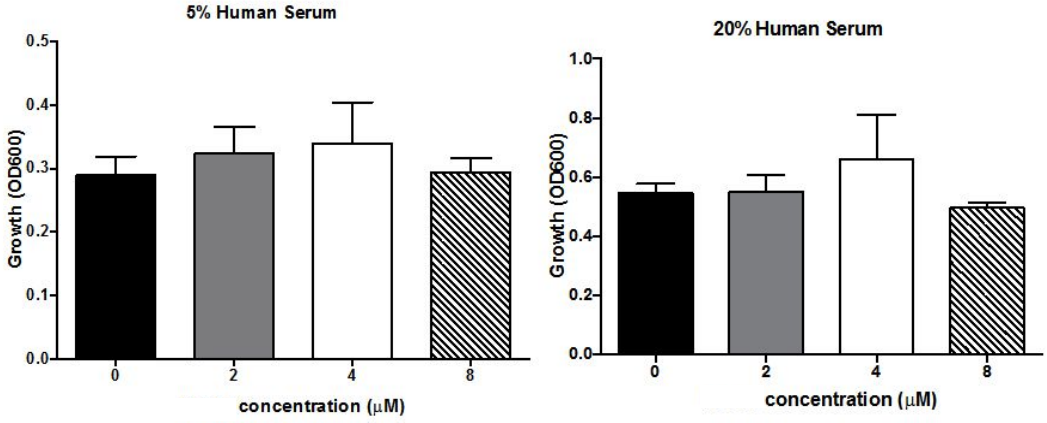

Figure 8. KKL-40 activity in human serum. Newman was grown to early log phase and diluted 1:1000 in assay media with indicated percentage of human serum and incubated with KKL-40 for 24hrs at the indicated concentration. Results from 2 independent experiments are presented as mean +/- SEM.

Generation of S. aureus resistance after antibiotic exposure.

One problem with all antibiotics is the eventual generation of resistance and this is particularly an issue with *S. aureus*. In order to determine how quickly *S. aureus* becomes resistant to KKL-40, we passaged *S. aureus* serially for 30 days in sub-lethal concentrations of KKL-40 and we tested the MIC at each day in the first 10 days and then at every other 5 days to determine whether the effective MIC changed over time. We included the antibiotics methicillin and vancomycin for comparison because these are widely used as treatment for *S. aureus* infection and the generation of antibiotic resistance is an issue for both of

them. Serial passage of *S. aureus* in the presence of sub-MIC levels of methicillin generated resistant mutants within the first passage, however very little change in sensitivity to vancomycin or KKL-40 is seen even after 30 days.

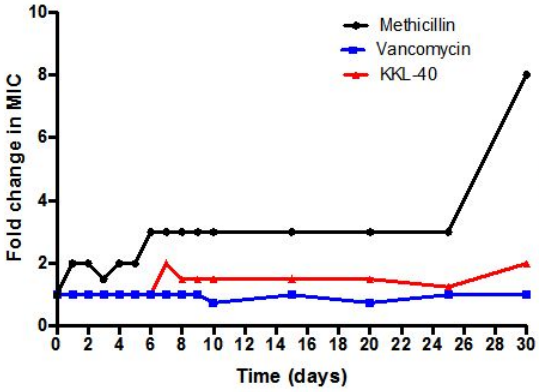

Figure 9. Generation of *S. aureus* resistance after antibiotic exposure. Newman was consecutively incubated for 24 hours at each interval with sub-lethal concentration of antibiotics at 37 ℃ and MIC assays were performed to calculate the fold increase at the indicated time point. Results from 2 independent experiments are presented as mean.

4. Discussion

KKL-35 and KKL-40 were identified as two of the most potent inhibitors of *trans*-translation in a previous screen (Ramadoss et al., 2013). They are able to inhibit growth of MSSA, HA-MRSA and CA-MRSA strains at relatively low concentrations, indicating the importance of *trans*-translation for maintaining the viability of *S.aureus.* This is consistent with the finding in the saturating transposon mutagenesis studies, where no transposon insertion was found in tmRNA or SmpB gene, hinting that these genes may be required for viability (Chaudhuri et al., 2009). The detailed mechanism of action of KKLs is still not clear. For KKL-35, it does not bind to SmpB or tmRNA, suggesting that it exerts its function through inhibiting subsequent steps of *trans*-translation (Ramadoss et al., 2013).

Since an *in vitro* time-kill kinetics assay has the ability to elucidate the speed and extent of bactericidal activity, it is recognized for its ability to characterize the properties of an antibiotic. KKL-35 and KKL-40 share a very similar bactericidal activity against *S. aureus.* This is probably due to the high similarity in structure. KKLs, methicillin and vancomycin have a delayed time to action compared to daptomycin. This may be due to their mechanism of action. Daptomycin exerts its bactericidal effect by inserting and aggregating into the bacterial cell membrane, which creates holes that leak ions (Koprivnjak & Peschel, 2011; Pogliano et al., 2012). Methicillin acts by inhibiting penicillin-binding proteins that are involved in the synthesis of the major structural polymer of the bacterial cell wall, peptidoglycan (Stapleton & Taylor, 2002). Vancomycin

also inhibits the biosynthesis of peptidoglycan by sequestering penicillin-binding proteins from precursor of peptidoglycan. KKLs inhibit *trans*-translation resulting in the accumulation of stalled ribosomes. Because daptomycin has the direct effects to the integrity of bacterial cell membrane the effects of this antibiotic may be seen faster. In comparison, bacterial autolysis through inhibition of cell wall biosynthesis and limitation of functional ribosomes by accumulation of stalled ribosomes on mRNA to achieve bactericidal activity is a relatively slow process. The differences between vancomycin and methicillin may be due to the availability of their targets. There is also a delayed response in the bactericidal activity of daptomycin and methicillin in stationary phase. This may be explained by the relatively low membrane potential in stationary phase *S. aureus* (Kashket, 1981). Except for affecting the integrity of bacterial cell membrane, daptomycin also dissipates the membrane potential to exert its bactericidal activity (Silverman et al., 2003). As a result, a low membrane potential status is more resistant to the change on membrane potential. Moreover, the membrane potential and the proton state of the cell wall have the ability to regulate bacterial cell wall autolysis (Kemper et al., 1993). Therefore, low membrane potential in stationary phase *S. aureus* may impair autolysin activity, causing the delayed response of methicillin. In addition, KKL-40 is capable of inhibiting *S. aureus* growth in both phases and only has a slight difference in antimicrobial activity between log phase and stationary phase, demonstrating it has potent bactericidal activity and the importance of *trans*-translation for *S. aureus* viability in both phases.

KKL-40 shows a diminished bactericidal activity against *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *E. coli K12,* but not against to *S. aureus or S. pygogenes. Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *E. coli K12* are gram-negative bacteria whereas *S. aureus* and *S. pyogenes* are gram-positive. However, KKLs inhibit the growth of *S. flexneri,* another gram-negative bacteria (Ramadoss et al., 2013) so it may not be simply targeting gram-positive verses gram-negative bacteria. One possible explanation for the selective activity is that some bacteria, such as *E. coli* and *B. subtilis,* have backup systems of *trans*-translation. There are two alternative systems that have been found so far. One is the alternative ribosome-rescue factor A (arfA). ArfA recognizes the stalled ribosome complex and then recruits release factor 2 (RF2) to hydrolyse the peptidyl-tRNA bond, which results in the release of the ribosome, the nascent peptide and mRNA (Chadani et al., 2012). The other one is alternative ribosome-rescue factor B (arfB). In contrast to arfA, arfB recognizes the stalled ribosome complex and directly catalyses hydrolysis of the peptidyl-tRNA bond resulting in the release of the ribosome, the nascent peptide and the mRNA (Handa et al., 2010).

trans-translation plays an essential role in maintaining bacterial viability under stressful conditions such as exposure to antimicrobials. LL-37 belongs to the human cathelicidin family and contributes to both innate and adaptive antimicrobial immune defenses (Stapleton & Taylor, 2002; Seil et al., 2010). LL-37 shows enhanced bactericidal activity with KKLs. Inhibition of *trans*-translation

could therefore have a two-part attack on the bacterial infection—first by directly killing the bacteria and second by making the bacteria more susceptible to killing by host antimicrobial peptides. Moreover, KKLs also have synergistic bactericidal effects with the antibiotics daptomycin, tetracycline and kanamycin, but not with chloramphenicol and erythromycin. Both daptomycin and LL-37 have potent and similar synergistic bactericidal effects with KKLs. Because both of them have cell-penetration properties, *trans*-translation may have a significant role in maintaining the integrity of the bacterial cell membrane. Kanamycin and tetracycline have relative smaller synergistic effects with KKLs and no synergy was seen with chloramphenicol and erythromycin. Chloramphenicol, erythromycin, kanamycin and tetracycline all exert their antimicrobial effects through affecting the normal function of ribosome. It is not clear why KKLs only have synergistic interaction with kanamycin and tetracycline. Further investigation is needed to discover which antibiotics synergize with KKLs and the mechanism of action.

The ability of bacteria to develop resistance to antibiotics is a great challenge that we are faced with, especially in *S. aureus*. Although our results show it is not easy for *S. aureus* to generate resistance to KKL-40, resistance could still develop over time. Using a combination of antimicrobials during treatment can weaken this ability and reduce the risk. As a result, using an inhibitor of *trans*-translation and antibiotics at the same time may not only have extraordinary bactericidal effects, but also diminish the possibility of development of resistance.

Although KKL-40 are not toxic to human HeLa cells, it lose its bactericidal activity in human serum. Therefore, KKL-40 itself would not work as potential antibiotic. However, *trans*-translation is still potentially a good antibiotic target due to the fact that inhibition of *trans*-translation exerts excellent bactericidal activity in *S. aureus.* Screening for other compounds that target *trans*-translation and maintain their antimicrobial activity in serum is needed in the future.

In conclusion, inhibition of *trans*-translation is bactericidal to both log and stationary phase *S. aureus.* Inhibition of *trans*-translation makes *S. aureus* much more sensitive to other antimicrobials, including several antibiotics, and could be particularly effective when used in combination treatments. Lastly, inhibitors of *trans*-translation are not toxic to human cells and bacterial resistance to inhibitors of trans-translation may also be slow to develop. Therefore *trans*-translation is a promising target for antibiotic development and further work should be dedicated to identify additional inhibitors that may be of use in the clinic.

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ABSTRACT

SMALL MOLECULE INHIBITION OF *TRANS*-TRANSLATION IMPAIRS *STAPHYLOCOCCUS AUREUS* VIABILITY

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Staphylococcus aureus quickly develops resistance to antibiotics and poses a significant health threat to humans. New antibiotic targets are needed for the development of new antibiotics. *Trans*-translation has important roles in maintaining bacterial viability. Small molecules, KKL-35 and KKL-40, were recently identified as specific inhibitors of *trans*-translation. We have investigated the roles of *trans*-translation on *S. aureus* viability and the potential of KKL-35 and KKL-40 as antibiotics. We find that KKLs show bactericidal activity against multiple *S. aureus* strains at relatively low concentration. We also find that sub-lethal doses of KKLs make *S. aureus* more susceptible to antimicrobials. Neither KKL-35 nor KKL-40 are cytotoxic to human HeLa cells. Unfortunately, KKL-40 is inactivated by human serum. Therefore, new inhibitors will need to be identified in future studies. Notably, the development of resistance by *S.aureus* against KKLs remains at a low level. Therefore *trans*-translation is a promising target for antibiotic development.