




A Small-Molecule Inhibitor of *trans*-Translation Synergistically Interacts with Cathelicidin Antimicrobial Peptides To Impair Survival of *Staphylococcus aureus*

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ABSTRACT *Staphylococcus aureus* is a leading cause of infection in the United States, and due to the rapid development of resistance, new antibiotics are constantly needed. *trans*-Translation is a particularly promising antibiotic target because it is conserved in many bacterial species, is critical for bacterial survival, and is unique among prokaryotes. We have investigated the potential of KKL-40, a small-molecule inhibitor of *trans*-translation, and find that it inhibits both methicillin-susceptible and methicillin-resistant strains of *S. aureus*. KKL-40 is also effective against Gram-positive pathogens, including a vancomycin-resistant strain of *Enterococcus faecalis*, *Bacillus subtilis*, and *Streptococcus pyogenes*, although its performance with Gram-negative pathogens is mixed. KKL-40 synergistically interacts with the human antimicrobial peptide LL-37, a member of the cathelicidin family, to inhibit *S. aureus* but not other antibiotics tested, including daptomycin, kanamycin, or erythromycin. KKL-40 is not cytotoxic to HeLa cells at concentrations that are 100-fold higher than the effective MIC. We also find that *S. aureus* develops minimal resistance to KKL-40 even after multiday passage at sublethal concentrations. Therefore, *trans*-translation inhibitors could be a particularly promising drug target against *S. aureus*, not only because of their ability to inhibit bacterial growth but also because of their potential to simultaneously render *S. aureus* more susceptible to host antimicrobial peptides.

KEYWORDS LL-37, *Staphylococcus aureus*, *trans*-translation, cathelicidin, oxadiazoles

Infections caused by *Staphylococcus aureus*, particularly strains resistant to existing antibiotics, pose a significant health care challenge. *S. aureus* infections are the leading cause of death by an infectious agent in the United States (1, 2). The direct costs of treating these infections are estimated at \$478 million to \$2.2 billion annually (3). *S. aureus* is notorious for its ability to develop antibiotic resistance, and new antibiotics must be continually developed to treat resistant strains (1, 2). Ribosome rescue is a potential target for new antibiotics. Ribosomes that translate to the 3' end of an mRNA without terminating at a stop codon must be rescued, or the protein synthesis capacity will be lost and the bacteria will die (4). The predominant mechanism for ribosome rescue is *trans*-translation, a pathway in which the transfer-messenger RNA (tmRNA)-SmpB ribonucleoprotein complex tags the nascent polypeptide for proteolysis and releases the stalled ribosome at a stop codon within tmRNA. Genes encoding tmRNA and SmpB have been identified in >99% of bacterial species. In some species, these genes are essential, but other species can survive without them because they have an alternative ribosome rescue factor, such as ArfA or ArfB (4). *S. aureus* does not encode any of the known alternative ribosome rescue factors, and the *ssrA* (encoding tmRNA) and *smpB* genes have been scored as essential in saturating transposon mutagenesis screens (5, 6), suggesting that *trans*-translation is a particularly promising target.

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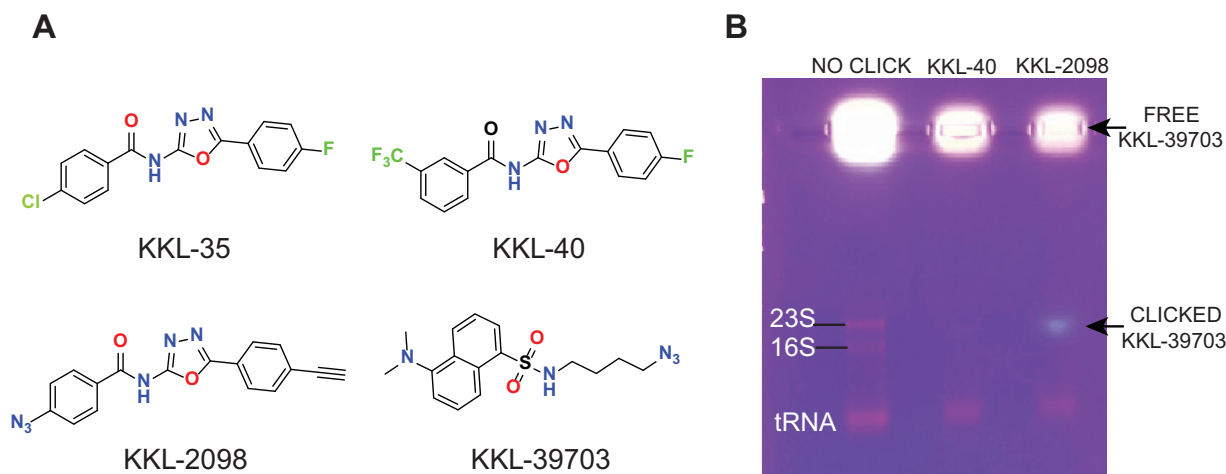


FIG 1 Oxadiazole compounds target 23S rRNA in *S. aureus*. (A) Chemical structures of the oxadiazoles (KKL-35 and KKL-40), a photoreactive analog (KKL-2098), and the fluorescent reporter (KKL-39703) used in the click bioconjugation experiments. (B) Agarose gel analysis of the click conjugation reactions using total RNA preparations from *S. aureus* treated with either KKL-40 or KKL-2098. Click-conjugated KKL-39703 is indicated by the fluorescence band (arrow) comigrating with the 23S rRNA only in the KKL-2098-treated sample. Free, unreacted KKL-39703 remains in the wells. No click, total RNA plus KKL-39703 without reagents for the click reaction; KKL-40, total RNA from cells treated with KKL-40 (which will not cross-link) clicked with KKL-39703; KKL-2098, total RNA from cells treated with KKL-2098 clicked with KKL-39703.

Small-molecule inhibitors of ribosome rescue were identified in a high-throughput screen. A family of oxadiazole benzamides, including KKL-35 and KKL-40 (Fig. 1), was shown to have potent antibiotic activity against *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Francisella tularensis*, *Legionella pneumophila*, and ΔtolC strains of *Escherichia coli* (7–10). Biochemical and cross-linking data indicated that KKL-35 and KKL-40 bind to a site on 23S rRNA that is not bound by other drugs, and this binding inhibits *trans*-translation but not translation (7, 8). Here, we show that these oxadiazole compounds inhibit the growth of *S. aureus* and act synergistically with the human antimicrobial peptide LL-37 *in vitro*. These results suggest that ribosome rescue inhibitors could be developed as antibiotics for treatment of *S. aureus* infections.

RESULTS

Oxadiazoles target 23S rRNA in *S. aureus*. A high-throughput screen to identify small-molecule inhibitors of *trans*-translation yielded several compounds, including the highly active oxadiazole derivatives KKL-35 and KKL-40 (Fig. 1) (8, 10). *In vivo* cross-linking experiments subsequently demonstrated that these oxadiazoles target 23S rRNA in *E. coli* and *Mycobacterium smegmatis* (7). To determine if the compounds have the same activity in *S. aureus*, we performed an intracellular photolabeling experiment using the cross-linkable oxadiazole derivative KKL-2098 (Fig. 1). A log-phase culture of *S. aureus* was divided and treated with either KKL-40, which will not cross-link, or KKL-2098. Cells were irradiated with UV light to activate the azide group of KKL-2098 and initiate cross-linking. The cells were lysed, total RNA was prepared, and the fluorescent reporter KKL-39703 was conjugated to the alkyne moiety of KKL-2098 using a click chemistry reaction (7). Agarose gel analysis showed a fluorescent band that comigrated with 23S rRNA from samples treated with KKL-2098, but this band was absent in the control samples treated with KKL-40 (Fig. 1). These observations were in accord with those of *E. coli* and *M. smegmatis* (7), suggesting that the 23S rRNA target for these oxadiazoles is conserved in *S. aureus*.

***trans*-Translation inhibitors are effective against *S. aureus* and other Gram-positive bacteria.** Broth microdilution assays showed that both KKL-35 and KKL-40 effectively inhibit several strains of *S. aureus*, including strain Newman, a methicillin-susceptible strain that has been used extensively in studying *S. aureus* pathogenesis (11); MRSA252, a globally prevalent genetically diverse health care-associated methicillin-resistant *S. aureus* (MRSA) strain (12); and USA300, the predominant community-associated

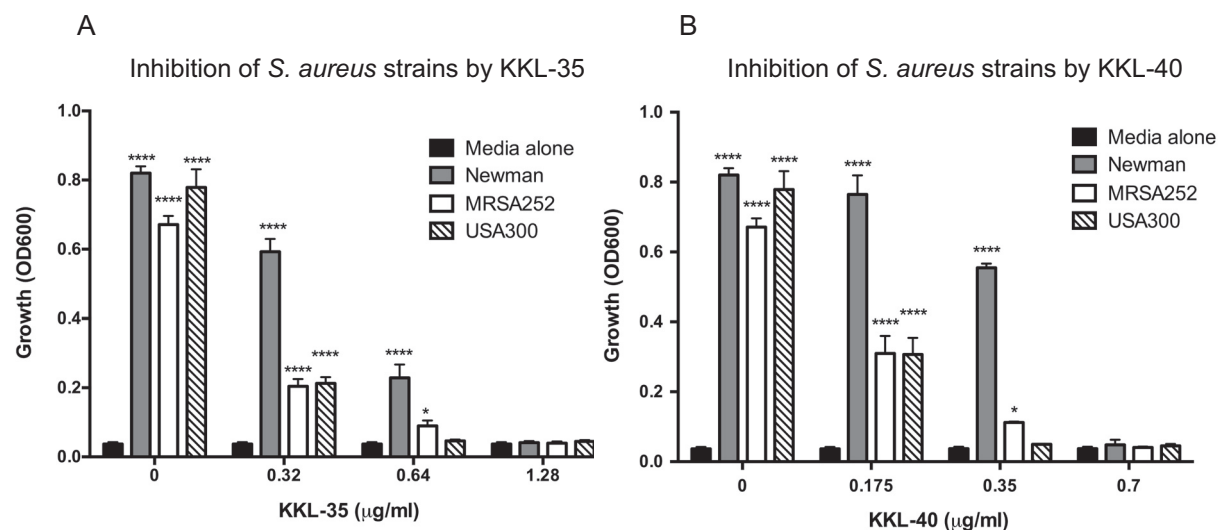


FIG 2 Oxadiazole compounds prevent the growth of *Staphylococcus aureus* strains. Shown are data for growth of the Newman, MRSA252, and USA300 strains of *S. aureus* overnight in the presence of KKL-35 (A) and KKL-40 (B) at the indicated concentrations. Results from 3 independent experiments are presented as means \pm standard deviations (SD). Statistically significant differences from the medium-alone control are presented (*, $P < 0.05$; ****, $P < 0.0001$ [as determined by one-way analysis of variance [ANOVA] followed by Dunnett's multiple-comparison test]).

MRSA strain in the United States (2) (Fig. 2). There were slight differences in susceptibility, with USA300 being the most sensitive, with an MIC of 0.64 $\mu\text{g/ml}$ (KKL-35) or 0.35 $\mu\text{g/ml}$ (KKL-40), while Newman and MRSA252 had the same MIC of 1.28 $\mu\text{g/ml}$ (KKL-35) or 0.7 $\mu\text{g/ml}$ (KKL-40). It should be noted that 2-fold differences in MIC values are within the intrinsic error of the assay method, so these slight differences in MICs between strains may not be biologically significant. Because KKL-40 is slightly more effective, with MIC values that are half those of KKL-35, we chose to focus on KKL-40. In 10% serum, KKL-40 activity was inhibited, and the effective MIC increased to 11.23 $\mu\text{g/ml}$ for *S. aureus* Newman (data not shown). This is in line with results of previous studies (10) but means that either KKL-40 would be limited to a topical antibiotic or oxadiazole derivatives with lower serum binding would need to be developed.

We next looked at the method by which KKL-40 inhibits *S. aureus* and compared it to those of known bactericidal (daptomycin) and bacteriostatic (chloramphenicol) antibiotics. Cultures of *S. aureus* grown overnight were diluted 1:10,000 and grown for 3 h (log phase) or 7 h (early stationary phase) before antibiotics were added at 10 \times or 20 \times their MICs. In both cases, KKL-40 inhibited the growth of *S. aureus* in a manner similar to that of chloramphenicol, with very little change from the starting amount, even though numbers of bacteria in untreated cultures increased by 3 logs (log phase) or 1 log (stationary phase), but it had no bactericidal activity after 24 h (Fig. 3A and B).

In addition to *S. aureus*, KKL-40 was effective against other Gram-positive species, including *Enterococcus faecalis*, *Streptococcus pyogenes*, and *Bacillus subtilis* (Table 1). Our results with Gram-negative species were mixed. Although we saw activity against several species, including *Haemophilus influenzae*, *Yersinia pestis*, *Burkholderia mallei*, and *Burkholderia pseudomallei*, no effective MIC was seen at concentrations of up to 22 $\mu\text{g/ml}$ of KKL-40 against *Escherichia coli* K-12, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, or *Acinetobacter baumannii* (Table 1). MICs were observed using the *E. coli* ΔtolC and IptD4213 mutants, which have defects in efflux and permeability of the outer membrane (13, 14), suggesting that the KKL-40 target is present in *E. coli* but that KKL-40 cannot accumulate in wild-type cells.

KKL-40 synergizes with antimicrobial peptides. In addition to being required for viability in several bacterial species, loss of *trans*-translation leads to increased susceptibility to various stressors, including antibiotics, as well as decreased virulence in bacterial pathogens (4, 15). Therefore, inhibiting *trans*-translation not only may inhibit

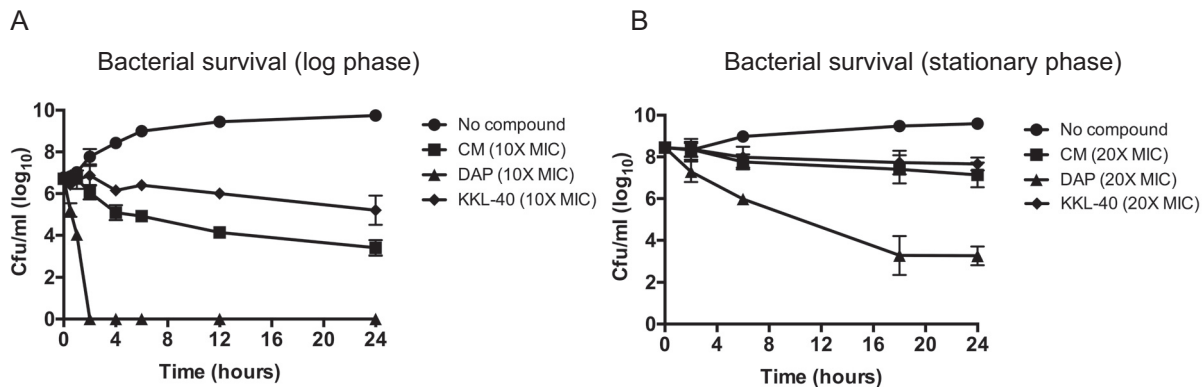


FIG 3 KKL-40 acts in a bacteriostatic manner. Log-phase (A) or stationary-phase (B) *S. aureus* Newman cultures were incubated with KKL-40, daptomycin (DAP), and chloramphenicol (CM) at 10× or 20× their MICs. Surviving CFU per milliliter were enumerated at the indicated time points. Results from 3 independent experiments are presented as means ± SD.

bacterial growth but also could increase susceptibility to host defenses and/or antibiotic treatment. To test this, we performed checkerboard analysis (16), where *S. aureus* Newman was simultaneously treated with 2-fold serial dilutions of the human antimicrobial peptide LL-37, a member of the cathelicidin family of antimicrobial peptides, and KKL-40, arranged in a two-dimensional array. The fractional inhibitory concentration (FIC) index was calculated (Table 2) based on the MIC of the antimicrobials alone and in combination (Table 3), and synergistic inhibition of *S. aureus* growth was seen with cotreatment with LL-37 and KKL-40. We next tried the same assay with the antibiotics daptomycin, kanamycin, and erythromycin. Daptomycin has characteristics similar to those of LL-37, including targeting the bacterial membrane (17), while previous studies have seen an increase in sensitivity to antibiotics, including aminoglycosides, such as kanamycin, and macrolides, such as erythromycin, when components of *trans*-translation are missing (18–21). However, none of these antibiotics exhibited a synergistic interaction with KKL-40 in *S. aureus* using the checkerboard assay (Table 2).

KKL-40 was not effective against many of the Gram-negative species that we tested, including *E. coli*. However, MICs were observed using the *E. coli* Δ tolC and *lptD4213* mutants, which have defects in efflux and permeability of the outer membrane (13, 14). Polymyxin B is a nonribosomal peptide antibiotic that kills Gram-negative bacteria through interaction with lipopolysaccharide (LPS) and disruption of the outer membrane (22). We hypothesized that combining polymyxin B and KKL-40 may increase the

TABLE 1 KKL-40 activity

Species and/or strain	Resistance ^a	KKL-40 MIC (μ g/ml)
<i>Staphylococcus aureus</i> Newman		0.7
<i>Staphylococcus aureus</i> USA300	CA-MRSA	0.35
<i>Staphylococcus aureus</i> MRSA252	HA-MRSA	0.7
<i>Enterococcus faecalis</i> ATCC 51299	VRE	1.4
<i>Streptococcus pyogenes</i> M1 5488		2.8
<i>Bacillus subtilis</i> PY79		0.7
<i>Haemophilus influenzae</i>		0.1
<i>Yersinia pestis</i>		2.3
<i>Burkholderia mallei</i>		1.1
<i>Burkholderia pseudomallei</i>		2.3
<i>Escherichia coli</i> K-12 MG1655		>22
<i>Escherichia coli</i> <i>lptD4213</i>		0.3
<i>Escherichia coli</i> Δ tolC		0.3
<i>Pseudomonas aeruginosa</i> PA14		>22
<i>Klebsiella pneumoniae</i> ATCC 13883		>22
<i>Acinetobacter baumannii</i> ATCC 19609		>22

^aCA-MRSA, community-associated MRSA; HA-MRSA, hospital-associated MRSA; VRE, vancomycin-resistant enterococci.

TABLE 2 Interactions with KKL-40

Bacterial species	Antibiotic A	Antibiotic B	FIC _A index	FIC _B index	FIC _{A+B} index	Synergistic interaction
<i>S. aureus</i> (Newman)	LL-37	KKL-40	0.25	0.25	0.5	Yes
<i>S. aureus</i> (Newman)	Daptomycin	KKL-40	0.5	0.5	1	No
<i>S. aureus</i> (Newman)	Kanamycin	KKL-40	0.5	0.5	1	No
<i>S. aureus</i> (Newman)	Erythromycin	KKL-40	0.5	0.5	1	No
<i>E. coli</i> K-12	Polymyxin B	KKL-40	0.25	0.125	0.375	Yes

effectiveness of KKL-40 against Gram-negative pathogens, as the permeabilization of the outer membrane by polymyxin B would allow KKL-40 to enter the cell. The FIC index was again calculated based on the MIC of the antimicrobials alone and in combination, and synergistic inhibition between KKL-40 and polymyxin was seen (Tables 2 and 3).

KKL-40 is not toxic to human cells and has a low level of resistance development. Next, we looked at whether KKL-40 is selectively toxic to bacterial cells by examining its effect on human cells. We incubated KKL-40 with human HeLa cells, a cervical cancer cell line, for 24 h in serum-free medium. We found that KKL-40 is not toxic to human HeLa cells, even at a concentration of 70 $\mu\text{g/ml}$, which is 100-fold higher than the effective MIC (Fig. 4).

One challenge with all antibiotics is the eventual generation of resistance, and this is particularly an issue with *S. aureus* (1, 2). In order to determine how quickly *S. aureus* becomes resistant to KKL-40, we passaged *S. aureus* serially for up to 30 days with sublethal concentrations of KKL-40 along with sublethal concentrations of either methicillin or daptomycin as a comparison. There was at least an 8-fold change in the effective MIC for both daptomycin and methicillin by days 15 and 30, respectively, whereas only a 2-fold change in MIC was seen for KKL-40 in the same amount of time (Fig. 5).

DISCUSSION

KKL-35 and KKL-40 inhibit the growth of methicillin-susceptible as well as both community- and hospital-associated MRSA strains at relatively low concentrations, indicating the importance of *trans*-translation for maintaining the viability of *S. aureus*. This is consistent with the findings of saturating transposon mutagenesis studies, where no transposon insertion was found in the genes encoding tmRNA or SmpB, indicating that these genes may be required for viability (5, 6). KKL-40 is bacteriostatic and inhibited the growth of both log- and early-stationary-phase *S. aureus*. In addition to *S. aureus*, we find that KKL-40 is effective against several other Gram-positive pathogens, including a vancomycin-resistant strain of *Enterococcus faecalis*, *Bacillus subtilis*, and *Streptococcus pyogenes*. Although activity has been seen against Gram-negative species, including *Shigella flexneri*, *F. tularensis*, and *L. pneumophila* (8, 9), as well as *H. influenzae*, *Y. pestis*, *B. mallei*, and *B. pseudomallei* in this study, we saw little to no activity against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, or *A. baumannii*. However, KKL-40 potently inhibited the growth of two mutant *E. coli* strains that have increased permeability, and coincubation of KKL-40 with polymyxin B, which disrupts the outer membrane of Gram-negative bacteria (22), lowered the effective MIC of KKL-40 in a synergistic manner. This suggests that KKL-40 activity in *E. coli* is limited more by permeability across the outer membrane than by target availability in the cell. The outer membranes of Gram-negative bacteria differ in permeability due to differences in

TABLE 3 MICs from synergistic checkerboard assays

Bacterial species	Assay medium	Antimicrobial	MIC _{alone} ($\mu\text{g/ml}$)	MIC _{combo} ($\mu\text{g/ml}$)
<i>S. aureus</i> (Newman)	RPMI + 5% LB	LL-37	>36	18
		KKL-40	2.8	0.7
<i>E. coli</i> K-12	CA-MHBII	Polymyxin B	0.6	0.15
		KKL-40	>11.2	2.8

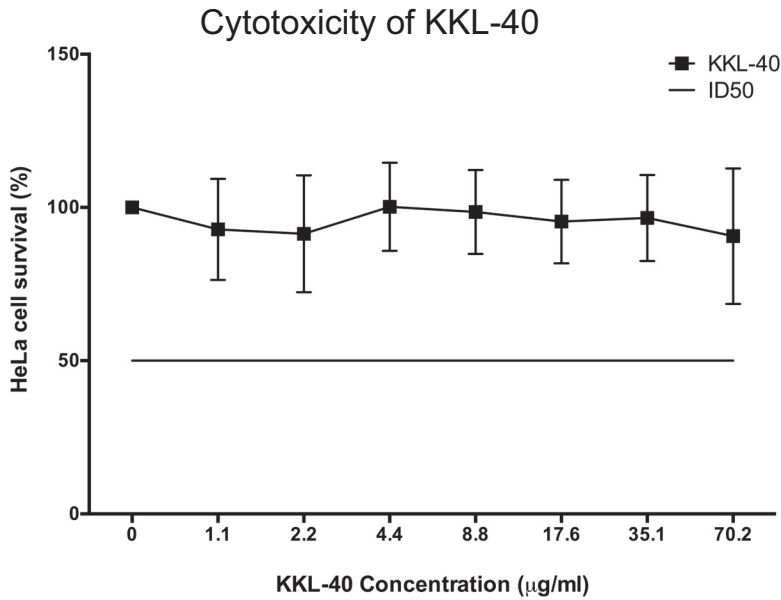


FIG 4 KKL-40 does not exhibit cytotoxicity to mammalian cells. HeLa cells were incubated with KKL-40 at the indicated concentrations for 24 h, and percent cell survival was then calculated. Results from 3 independent experiments are presented as means \pm SD. ID50, 50% infective dose.

porins as well as differences in the chemical structure of LPS (23). It is therefore possible that differences in Gram-negative susceptibility to KKL-40 could be due to differences in outer membrane permeability. While it is likely that KKL-40 will be effective against a wide range of Gram-positive species, Gram-negative species would need to be evaluated on a case-by-case basis.

In addition to acting as a potent antimicrobial on its own, KKL-40 has a synergistic interaction with the human antimicrobial peptide LL-37. The increased effectiveness of KKL-40 in the presence of LL-37 is likely due to membrane damage caused by even sublethal levels of LL-37, which leads to an increased ability of KKL-40 to permeate the cell and increased efficacy at lower concentrations. It is also possible that the inhibition of *trans*-translation causes a maladapted stress response to LL-37, leading to increased susceptibility. Regardless of the exact mechanism, these results further highlight the potential effectiveness of *trans*-translation inhibition. Not only are these compounds

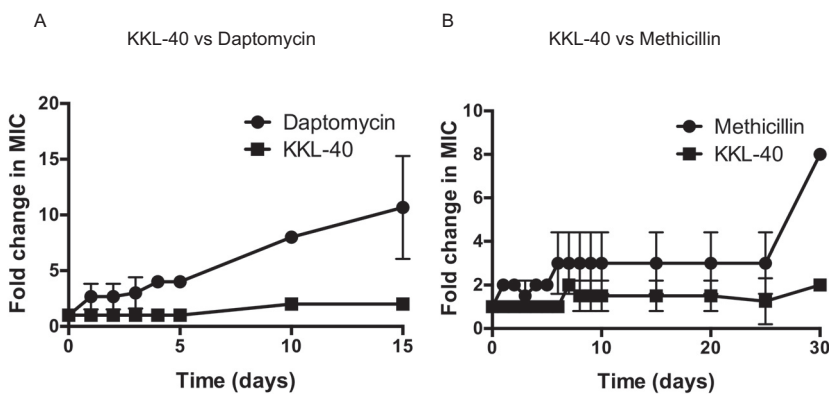


FIG 5 *S. aureus* develops minimal resistance to KKL-40. *S. aureus* Newman was consecutively incubated for 24 h with sublethal concentrations of KKL-40 and daptomycin (A) or methicillin (B), and MIC assays were performed at the indicated days to calculate the fold increase in the MIC relative to the original starting MIC. Results from two (methicillin) or three (daptomycin) independent experiments are presented as means \pm SD.

effective in their own right, they also could increase bacterial susceptibility to host antimicrobial peptides. Furthermore, these compounds are nontoxic in a variety of mammalian cells, including HeLa cells in this study as well as macrophages and a human liver cell line, HepG2 (10). Finally, generation of resistance against KKL-40 is limited, even in *S. aureus*, a pathogen notorious for its ability to acquire antibiotic resistance (1, 2). These results are consistent with those seen in *L. pneumophila*, *E. coli*, and *S. flexneri*, where no resistance to KKL-35 was generated (8, 9). Developing resistance to KKL-40 may be more difficult because mutations that prevent binding to the ribosome are lethal, because mutations would be required in all rRNA genes, or because there is a second, unidentified target for KKL-40. The latter possibility seems unlikely, however, because overexpression of ribosome rescue factors in other bacteria decreases the activity of KKL-40 (10), consistent with ribosome rescue being the target responsible for activity. Unfortunately, KKL-40 is inhibited in the presence of serum, so while it could be used as a topical antibiotic, further development of oxadiazole derivatives with better clinical properties would be needed for more-widespread use. However, given the effectiveness of the KKL compounds against a variety of pathogens, the limited cytotoxicity, and the low propensity for resistance, our results argue for the continued development of inhibitors targeting *trans*-translation as potentially highly effective antibiotics.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics. *S. aureus* strains (Newman, MRSA252, and USA300) and *E. faecalis* ATCC 51299 were grown in tryptic soy broth (TSB; Hardy Diagnostics). *A. baumannii* ATCC 19609, *P. aeruginosa* PA14, and *B. subtilis* PY79 were grown in brain heart infusion (BHI) medium (Hardy Diagnostics). *E. coli* strains and *P. aeruginosa* PA14 were grown in Luria broth (LB; Hardy Diagnostics). *K. pneumoniae* ATCC 13883 and *S. pyogenes* M1 5488 were grown in Todd-Hewitt broth (THB; Fluka). *Burkholderia* species, *Y. pestis*, and *H. influenzae* were grown in Mueller-Hinton II broth (BD) with calcium to a final concentration of 50 $\mu\text{g/ml}$ to make CA-MHBII. All antibiotics were acquired from Sigma, except for daptomycin (Cubist Pharmaceuticals) and LL-37 (Anaspec).

Bioorthogonal photoaffinity labeling and click conjugation. Intracellular photolabeling was performed by adding either KKL-40 or KKL-2098 at 0.02 $\mu\text{g/ml}$ to mid-exponential-phase cultures of *S. aureus*. These cultures were grown for 1 h, and cells were harvested by centrifugation and resuspended in a phosphate-buffered saline (PBS) solution (1.2 g/liter Na_2HPO_4 , 0.22 g/liter NaH_2PO_4 , 8.5 g/liter NaCl [pH 7.5]). Intracellular photoaffinity labeling and click conjugation reactions were performed as previously described (7), with the exception that in this study, KKL-39703 was used as the fluorescent reporter.

Synthesis. KKL-35, KKL-40, and KKL-2098 were synthesized as previously reported (7, 10). For *N*-(4-azidobutyl)-5-(dimethylamino)naphthalene-1-sulfonamide (KKL-39703), dansyl chloride (400 mg; 1.5 mmol) dissolved in 2 ml dichloromethane (DCM) was added dropwise to a round-bottomed flask containing a solution of 4-bromo-1-aminobutane (419 mg; 1.8 mmol) and trimethylamine (453 mg; 4.44 mmol) in 8 ml DCM chilled at 0°C (under an inert atmosphere). This reaction mixture was allowed to warm up gradually to room temperature, and the reaction was then left to proceed for 16 h. The reaction was terminated when thin-layer chromatography (TLC) analysis indicated complete consumption of dansyl chloride. The volatile solvents were evaporated under reduced pressure, and the crude product was purified by flash chromatography over a silica gel eluting with a mixture of 20% ethyl acetate in hexanes, to give the product as a bright-yellow oil (451 mg; 96.7% yield). ^1H NMR (nuclear magnetic resonance) (400 MHz, CDCl_3) δ 1.59 (m, 2H), 1.64 (m, 2H), 2.93 (s, 6H), 2.96 (q, 2H, $J = 6.3$ Hz), 3.22 (t, 2H, $J = 6.3$ Hz), 4.82 (t, 1H, $J = 4.9$ Hz), 7.17 (d, 1H, $J = 7.3$ Hz), 7.54 (m, 2H), 8.24 (m, 2H), 8.53 (d, 1H, $J = 8.5$ Hz). ESI (electrospray ionization) (positive) m/z ; [M + H] $^+$ calculated for $\text{C}_{16}\text{H}_{21}\text{N}_5\text{O}_2\text{S}$, 348.1; observed, 348.1.

MIC assays. Cultures grown overnight were diluted and grown to early log phase at an optical density at 600 nm (OD_{600}) of 0.4. Clinical and Laboratory Standards Institute (CLSI) guidelines were followed, including using at least 5×10^5 CFU/ml in the appropriate medium and subculture volume (24). To facilitate comparisons with daptomycin and polymyxin B, CA-MHBII was used for all assays, with the exceptions that RPMI plus 5% LB was used for assays with LL-37 and THB was used for *Streptococcus pyogenes*. Fetal bovine serum (FBS; Gibco) was used for assays including serum. All assays were carried out in 96-well plates in a total volume of 200 μl . After 16 to 20 h of incubation at 37°C under static conditions, the OD_{600} was read, and the MIC was defined as the lowest concentration of antibiotic with no visible growth. All MIC assays were repeated at least 2 times.

Time-dependent killing. *S. aureus* Newman cultures grown overnight were diluted 1:10,000 into CA-MHBII and grown for 3 h (log phase) or approximately 7 h (early stationary phase with an OD of at least 0.8) at 37°C with shaking. Log- or stationary-phase cultures were then aliquoted into separate culture tubes (2 ml each), antibiotics were added at 10 \times their MICs (KKL-40, 7 $\mu\text{g/ml}$; daptomycin, 10 $\mu\text{g/ml}$; chloramphenicol, 80 $\mu\text{g/ml}$) or 20 \times their MICs (KKL-40, 14 $\mu\text{g/ml}$; daptomycin, 20 $\mu\text{g/ml}$; chloramphenicol, 160 $\mu\text{g/ml}$), and culture tubes were incubated at 37°C with shaking for an additional 24 h. At the indicated time points, 100 μl of culture from each tube was removed, centrifuged, and

resuspended in an equivalent volume of PBS, and 10-fold dilutions were then plated to enumerate surviving CFU per milliliter.

Synergy assays. The checkerboard method was used to determine the MICs for each antibiotic alone and in combination. Both KKL-40 and the paired antimicrobial were 2-fold serially diluted, and 50 μ l containing 4 times the final concentration of both antibiotics was then added to each well, with the final well containing no antibiotic. KKL-40 was added across the columns, and the other antibiotic was added down the rows, resulting in a checkerboard of doubly increasing concentrations of KKL-40 and the paired antibiotic. RPMI plus 5% LB was used for assay mixtures containing LL-37, CA-MHBI was used for all others, and final antibiotic concentrations were 36 μ g/ml to 2.25 μ g/ml for LL-37, 2 μ g/ml to 0.0625 μ g/ml for daptomycin, 2 μ g/ml to 0.0625 μ g/ml for erythromycin, 16 μ g/ml to 0.5 μ g/ml for kanamycin, and 2.4 μ g/ml to 0.0375 μ g/ml for polymyxin B. KKL-40 concentrations for *S. aureus* were 5.6 μ g/ml to 0.176 μ g/ml for assays with RPMI plus 5% LB and 1.4 μ g/ml to 0.044 μ g/ml for assays with CA-MHBI, while KKL-40 concentrations for *E. coli* were 11.23 μ g/ml to 0.7 μ g/ml. Bacterial cultures were grown to log phase (OD of 0.4) in TSB (*S. aureus* Newman) or LB (*E. coli*), washed, and diluted 1:150 in the corresponding medium, and 100 μ l was added per well for a final volume of 200 μ l/well. Plates were incubated at 37°C under static conditions, and the OD was read 22 to 24 h later. The FIC of each antibiotic was determined, where FIC_A is the MIC of antibiotic A in the combination/MIC of antibiotic A alone ($FIC_A = MIC_{A+B}/MIC_A$) and FIC_B is the MIC of antibiotic B in the combination/MIC of antibiotic B alone ($FIC_B = MIC_{B+A}/MIC_B$). The FIC index was calculated by adding FIC_A and FIC_B . When the highest concentration of antibiotic used was not inhibitory (for example, 36 μ g/ml LL-37 with *S. aureus* or 11.2 μ g/ml KKL-40 with *E. coli*), 2 times the highest concentration was used in the FIC calculation (i.e. 72 μ g/ml for LL-37 alone and 22.46 μ g/ml for KKL-40 alone). A synergistic interaction is defined by an FIC index of ≤ 0.5 , no interaction is defined by an FIC index of >0.5 to 4, and an antagonistic interaction is defined by an FIC index of >4.0 (16). All assays were repeated at least 3 independent times.

Mammalian cell cytotoxicity. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% FBS (Gibco), 1% penicillin-streptomycin (Difco) and 1% L-glutamine (HyClone). Cells were plated at 2×10^4 cells per well in 96-well plates and incubated for 24 h at 37°C with 5% CO₂. Medium was then removed, cells were washed once in PBS, and serum-free DMEM containing serially diluted KKL-40 at the indicated concentrations was added to the cells. Cytotoxicity was assessed 24 h after incubation with the compounds by adding 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from the CellTiter 96 AQueous nonradioactive cell proliferation assay kit (Promega, Madison, WI) directly to the culture wells, incubating the mixture for 2 h at 37°C with 5% CO₂, and recording the absorbance at 490 nm. One hundred percent viability was set at the absorbance of the untreated cells, and percent HeLa cell survival was calculated by dividing the OD of KKL-40-treated cells by the OD of untreated cells and multiplying this value by 100.

Generation of resistance. Cells were passaged at 24-h intervals in the presence of sublethal concentrations of methicillin (2 μ g/ml), daptomycin (0.5 μ g/ml), or KKL-40 (0.35 μ g/ml) (approximately 0.5-fold the original effective MIC). In the experiments comparing methicillin and KKL-40, TSB was used for culture medium and MIC assays, and in the experiments comparing daptomycin and KKL-40, CA-MHBI was used for culture medium and MIC assays. MIC assays were conducted at days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30. Bacteria were first diluted to an OD₆₀₀ of ~ 0.7 and then diluted 1:400 in medium (TSB or CA-MHBI), and MIC assays were performed as described above. Data are plotted as fold changes from the original MICs, which were 4 μ g/ml for methicillin, 1 μ g/ml for daptomycin, and 0.7 μ g/ml for KKL-40.

Statistics. All statistical analysis was performed using GraphPad Prism.

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