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Probing the Mechanism of LAL-32, a Gold Nanoparticle-Based Antibiotic Discovered through Small Molecule Variable Ligand Display

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Abstract

The unrelenting rise of antimicrobial-resistant bacteria has necessitated the search for novel antibiotic solutions. Herein we describe further mechanistic studies on a 2.0-nm-diameter gold nanoparticle-based antibiotic (designated LAL-32). This antibiotic exhibits bactericidal activity against the Gram-negative bacterium *Escherichia coli* at 1.0 μ M, a concentration significantly lower than several clinically available antibiotics (such as ampicillin and gentamicin), and acute treatment with LAL-32 does not give rise to spontaneous resistant mutants. LAL-32 treatment inhibits cellular division, daughter cell separation, and twin-arginine translocation (Tat) pathway dependent shuttling of proteins to the periplasm. Furthermore, we have found that the *cedA* gene imparts increased resistance to LAL-32, and shown that an *E. coli cedA* transposon mutant exhibits increased susceptibility to LAL-32. Taken together, these studies further implicate cell division pathways as the target for this nanoparticle-based antibiotic and demonstrate that there may be inherently higher barriers for resistance evolution against nanoscale antibiotics in comparison to their small molecule counterparts.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00199.

Synthesis of gold nanoparticles; LAL-32 place-exchange reactions; bacterial growth inhibition and viability assays; microscopy; genomic overexpression library construction and testing (PDF)

The authors declare no competing financial interest.

The emergence of resistance to multiple antimicrobial agents by pathogenic bacteria has become a significant global health issue that is seriously threatening the vast medical advancements made possible by antibiotics over the past 70 years.¹ Many medical interventions, including surgery, premature infant care, cancer chemotherapy, care of the critically ill, and transplantation medicine, are feasible only with the existence of effective antibiotic therapy. The need for novel antibiotics is further accentuated by recent reports of a *Klebsiella pneumoniae* infection that was recalcitrant to treatment by every clinically available antibiotic in the U.S., ultimately leading to patient mortality.²

Of all the potential bacterial threats, the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) represent some of the greatest unmet needs for therapeutic intervention.³ Despite the clear need for new antibiotic development, there have been only two novel classes of antibiotics that have reached the clinic over the past two decades, both being Gram-positive selective, leaving four of the six ESKAPE pathogens with rapidly dwindling therapeutic options.⁴ To address the pressing need for antibiotics that target Gram-negative ESKAPE pathogens and are potentially less susceptible to the resistance mechanisms that compromise small-molecule antibiotics, our laboratories and others have been exploring the use of gold nanoparticle-based antibiotics.^{5–10}

Our approach utilizes a drug discovery paradigm termed small molecule variable ligand display (SMVLD),^{5–7} in which combinations of small organothiol ligands are covalently bound to gold nanoparticles to create libraries of mixed-ligand modified nanoparticle conjugates that are subsequently screened for bacterial growth inhibition. We have previously reported that a SMVLD screen yielded nanoparticles with in vitro bacterial growth inhibition activity against *Escherichia coli* and one of the ESKAPE pathogens, *K. pneumoniae*.⁶ The activities of the nanoparticles depend on the specific combination of ligands, with the most potent nanoparticle discovered being a 2.0-nm-diameter gold cluster modified with *p*-mercaptobenzoic acid (pMBA), glutathione, cysteamine, and 3-mercapto-1-propanesulfonic acid (mPSA) (designated LAL-32, ligands depicted in Figure 1). Solid-state, cross-polarization magic angle spinning (CPMAS) ¹³C NMR, IR spectroscopy, and ion mobility mass spectrometry data reported previously confirmed the presence of the thiol ligands on the surface of LAL-32 in average mole ratios per nanoparticle of 23 glutathiones, 19 cysteamines, 10 3-mercapto-1-propane sulfuric acids, and 8 *p*-mercaptobenzoic acids.^{5,6}

LAL-32 exhibits 99.9% growth inhibition (which we define as MIC_{99.9}) at 250 nM (7.5 mg/mL) for *E. coli* and 625 nM (18.75 mg/mL) for *K. pneumoniae*. Both the combination of ligands and their conjugation to the nanoparticles are important for activity. For instance, the ligands shown in Figure 1 were 360 times more active when conjugated to gold

nanoparticles than the same concentration of free ligands mixed in solution. LAL-32 has previously been shown to be nontoxic to Hep G2/2.2.1 cells at concentrations of up to 800 nM, while in a murine toxicology study it was shown to cause some renal complications at higher concentrations that could be abated by incorporating a thiolated oligoethylene glycol ligand without loss of antibiotic activity.⁶ The preliminary mechanism of action studies through analysis of RNA-seq data suggested that processes involved in cell division are most likely the target for LAL-32.

Herein we report further delineation of the mechanism of action of LAL-32. Using *E. coli* as our model organism, we have established that LAL-32 is bactericidal, that bacteria do not develop resistance to acute doses of LAL-32, and that LAL-32 inhibits daughter cell separation. We have also found that the presence of the *cedA* gene, which encodes for the cell division activator, Ceda,¹¹ imparts resistance against LAL-32, and a *cedA* transposon mutant exhibits increased susceptibility to LAL-32, further implicating cell division as the target for this nanoparticle-based antibiotic.

Studies were initiated by determining the growth kinetics of bacteria in escalating concentrations of LAL-32 (Figure 2). At 250 nM (the MIC_{99.9}), we noted a biphasic growth curve where bacterial viability dropped by 2 orders of magnitude in the first 6 h (10⁶ to 10⁴ CFU/mL), followed by a recovery phase where bacterial viability increased by 4 orders of magnitude (to 10⁸ CFU/mL). Such growth kinetics mirror those of bacteria treated with certain classes of bactericidal small molecule antibiotics such as β -lactams, vancomycin, and daptomycin at their MIC.^{12–14} This is in contrast to untreated bacteria whose viability rapidly increased to 10⁹ CFU/mL in 6 h before entering stationary phase. At higher concentrations of LAL-32 (1000 nM), we observed similar time kill kinetics as treatment at 250 nM (10⁶ to 10⁴ CFU/mL) over the first 6 h, followed by sustained bactericidal activity such that bacterial numbers were driven below the limit of detection (<10² CFU/mL) with no growth rebound observed out to 24 h. As an antibiotic is defined as bactericidal when it reduces viable bacteria by 99.9% in comparison to the starting inoculum at no more than four times its MIC,¹⁵ LAL-32 is bactericidal, reducing viable bacteria by >99.99% after 24 h.

Given the rebound growth with the 250 nM treatment, we next determined whether this was due to resistance evolution by reculturing surviving *E. coli* in the presence of LAL-32 (again at 250 nM). Identical growth kinetics to the original culture were observed, indicating that the surviving bacteria had not acquired resistance to an acute, single dose nanoparticle treatment (data not shown).

In an effort to understand the molecular basis of the mode of action of LAL-32, we turned to previously published RNA-seq data, which indicated that genes involved in cell division were up-regulated in *E. coli* treated with LAL-32.⁶ This suggests that cell division could be a target of nanoparticle treatment. To determine if cell division was indeed affected by LAL-32, we analyzed *E. coli* treated with LAL-32 by phase contrast microscopy (Figure 3). Treated cells grew in long chains of up to 14 cells. Additionally, by measuring the lengths of 150 cells per image in Figure 3, we found that LAL-32-treated cells were rounder than untreated cells. Indeed, on average the treated cells were 58% shorter along the long axis of

the bacteria (and thus rounder in appearance by optical microscopy) compared to the rods observed in the nontreated control, suggesting that in addition to cell separation, cell shape was also affected.

Next, given that the final stages of cell separation are orchestrated by a suite of cellular amidases that are translocated to the periplasm by the twin-arginine translocation (Tat) pathway, and that strains lacking genes encoding essential Tat components have been reported to be defective in cell separation and to form long chains of cells similar to those observed upon treatment with LAL-32,¹⁶ we investigated the effect that LAL-32 had upon Tat-dependent protein translocation (Figure 4). We used an arabinose-inducible reporter construct that contained green fluorescent protein (GFP) fused to a TorA leader peptide that signals for periplasm translocation and an SsrA peptide that signals for ClpXP degradation if the fusion protein is not translocated out of the cytoplasm (annotated pTGS).¹⁷ High fluorescence levels observed in cells encoding pTGS are indicative of an active Tat pathway.¹⁷ This is supported by the fact that high fluorescence levels were observed when wt *E. coli* was transformed with the pTGS construct and induced with arabinose, while minimal to no fluorescence was observed when *E. coli* *tatABCD* (*tat*- strain) was transformed with pTGS. When wt *E. coli* cells transformed with pTGS were treated with 1.0 μ M LAL-32, the level of fluorescence was decreased by 60%, suggesting that the nanoparticles inhibit the activity of the Tat secretion system. When *E. coli* *tatABCD* (*tat*- strain) was transformed with pTGS and treated with LAL-32 (1.0 μ M), no difference in fluorescence was noted, further supporting the hypothesis that LAL-32 targets the Tat secretion pathway.

Finally, we determined which pathways might impart resistance to LAL-32 treatment through an overexpression screen. A library consisting of 1–8 kB genomic fragments was cloned into pBR322 and transformed into *E. coli*. The resulting bacterial population was then plated on agar plates supplemented with 1.0 μ M LAL-32. After 24 h, of the ca. 33 000 unique transformants, 178 colonies grew. Each colony was then individually inoculated into MHB and tested for sensitivity to LAL-32 (1.0 μ M). Of the 178 colonies, 17 resistant colonies were identified, while the other 161 colonies were false positives. The genomic DNA inserts from all 17 of these colonies were sequenced and all 17 were found to encode for *cedA*, a gene whose expression activates cellular division.¹¹ To confirm the role of *cedA* in nanoparticle resistance, we measured the activity of LAL-32 against an *E. coli* *cedA* transposon mutant from the Keio collection.¹⁸ This strain exhibited an MIC of 78.1 nM as compared to 312.5 nM for the Keio parent strain. The MIC of LAL-32 against the unrelated *pmrD* Keio mutant was determined in the presence of kanamycin, to rule out any contribution of the selection antibiotic to the increased susceptibility to the nanoparticle, and found to be identical to that of the parent strain.

In conclusion, we have demonstrated that LAL-32, a 2.0-nm-diameter gold nanoparticle modified with pMBA, glutathione, cysteamine, and mPSA, is potently bactericidal, reducing viable bacteria from a starting inoculum of 10⁶ CFU/mL to below the detectable limit (<10²). Phase contrast microscopy revealed that LAL-32 affects both cellular division and shape, while studies using GFP fusions demonstrated that Tat-dependent protein translation is disrupted. Finally, we have shown that overexpression of *cedA*, which encodes for a cell division regulatory protein, abrogates LAL-32 activity, and that the disruption of *cedA*

results in increased susceptibility to LAL-32. Taken together, these studies further establish that the antibacterial activity of LAL-32 is a result of disruption of cell division. Studies to determine the exact molecular target of LAL-32 and to evaluate its activity in vivo are currently underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
LAL-32 ligand set.

Figure 2. Growth curve and CFU quantification for *E. coli* treated with LAL-32. Blue, untreated control; red, 250 nM treatment; green 500 nM treatment; purple, 1000 nM treatment.

Figure 3.
Phase contrast microscopy of (A) untreated *E. coli* 25922 and (B) *E. coli* 25922 treated with 1.0 μ M LAL-32.

Figure 4.

Fluorescence profile of transformed *E. coli*. A. Arabinose induced fluorescence in cells transformed with a pTG plasmid, a protein construct lacking the SsrA fusion. B. Arabinose induced fluorescence in pTGS transformed *E. coli*. C. Effects of LAL-32 treatment in absence or presence of arabinose. D. *E. coli* Δ tatABCD ($\mp$ arabinose) control.