Bioengineered lysozyme in combination therapies for *Pseudomonas aeruginosa* lung infections

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There is increasing urgency in the battle against drug-resistant bacterial pathogens, and this public health crisis has created a desperate need for novel antimicrobial agents. Recombinant human lysozyme represents one interesting candidate for treating pulmonary infections, but the wild type enzyme is subject to electrostatic mediated inhibition by anionic biopolymers that accumulate in the infected lung. We have redesigned lysozyme's electrostatic potential field, creating a genetically engineered variant that is less susceptible to polyanion inhibition, yet retains potent bactericidal activity. A recent publication demonstrated that the engineered enzyme outperforms wild type lysozyme in a murine model of *Pseudomonas aeruginosa* lung infection. Here, we expand upon our initial studies and consider dual therapies that combine lysozymes with an antimicrobial peptide. Consistent with our earlier results, the charge modified lysozyme combination outperformed its wild type counterpart, yielding more than an order-of-magnitude reduction in bacterial burden following treatment with a single dose.

In the developed world, healthcare consumers have become inured to the fact that much of modern medicine is predicated on the availability of efficacious antibiotics, but the spread of drug-resistant bacterial pathogens is now undermining this cornerstone of medical practice.1, 2 Patients infected with drug-resistant strains suffer from longer hospital stays, increased mortality rates, and dramatically higher healthcare costs.3 In the United States alone, current data indicates that drug-resistant bacteria cause more than 2 million infections each year, with a conservative estimate of 23,000 annual deaths directly attributable to these infections.4 Seventy years of experience has demonstrated that the clinical utility of small molecule antibiotics is inevitably limited by the rapid emergence and subsequent spread of resistance elements.2 Thus, there is an increasingly urgent need to access and engineer novel drug candidates that break the mold of conventional antibacterial chemotherapies.

Lytic enzymes have been gaining attention as prospective therapeutic agents.5-7 These bactericidal proteins could prove particularly powerful, as they act through mechanisms orthogonal to those of conventional antibacterial drugs. Namely, their capacity to catalytically degrade cell wall peptidoglycan underlies a long list of advantages including (1) efficacy against strains resistant to conventional drugs; (2) exquisite specificity for bacteria and minimal off-target effects; (3) efficacy against cells exhibiting low metabolic activity, such as those within biofilms; (4) potential for low effective dosages due to catalytic mechanism of action; and (5) putative reduced rates of resistance development. This latter point is related

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to the nature of the enzymes’ subcellular target. Peptidoglycan exhibits a highly conserved core structure among virtually all bacterial genera, and the peptidoglycan molecule is not directly encoded by genetic information. Consequently, the bacterial cell wall exhibits a lower degree of evolutionary plasticity than the protein and nucleic acid targets of most conventional antibiotics. In aggregate, the cell wall is an attractive target for antibiotic development, and lytic biocatalysts are the clear front runners with respect to direct degradation of peptidoglycan itself.

To date phage endolysins and catalytic bacteriocins have dominated the ranks of therapeutic candidates, but recombinant human \( \text{C-type lysozyme (hLYS)} \) has also seen recent preclinical development. Human lysozyme is highly expressed and broadly distributed throughout a variety of secretions and biological fluids, and it has been shown to be the most powerful cationic antimicrobial in human nasal secretions. In addition to its ability to enzymatically degrade peptidoglycan, lysozyme exhibits catalysis-independent modes of action related to membrane disruption and dysregulation of bacterial autolysins. While its broad-spectrum activity and endogenous role in human immunity make hLYS an interesting drug candidate, the wild type enzyme has limitations in therapeutic applications. For example, there is extensive evidence that hLYS is subject to electrostatic mediated inhibition by anionic biopolymers, which are known to accumulate in the infected and inflamed lung. Thus, reengineering and optimization of hLYS might provide for greater therapeutic benefit in the treatment of pulmonary infections.

To realize this goal, we have leveraged combinatorial library construction and high throughput screening to generate an hLYS variant that was less prone to anionic biopolymer inhibition. This engineered enzyme, variant 2-3-7, bore only two mutations relative to wild type hLYS (R101D and R115H). In in vitro experiments, 2-3-7 effectively evaded inhibition by the clinically relevant biopolymers DNA, alginate, mucin, and F-actin. At the same time, the variant’s bactericidal activity toward \( \text{Micrococcus luteus} \) and \( \text{Pseudomonas aeruginosa} \) equaled or exceeded that of wild type hLYS. Analysis of the 2-3-7 crystal structure revealed that the R101D and R115H substitutions caused negligible structural perturbation (RMSD 0.42 Å main chain atoms; 1.17 Å overall) but manifested a substantially remodeled electrostatic potential field (Fig. 1). Presumably, the reduced magnitude of the variant’s electropositive potential disrupts Coulombic attractions with anionic biopolymers, yet the geometry of the electrostatic field remains consistent with the enzyme’s inherent antibacterial function. While the charge engineered enzyme clearly outperformed wild type hLYS in vitro, the infected and inflamed lung is a vastly more complex environment. Therefore, it was not immediately clear that the variant’s enhanced in vitro performance would translate into greater in vivo efficacy.

We have recently conducted a series of in vivo studies to more rigorously assess the therapeutic potential of enzyme 2-3-7. We employed a murine model of acute lung infection based on a mucoid (i.e., alginate producing) isolate of \( \text{P. aeruginosa} \), an opportunistic pathogen responsible for 8% of all hospital acquired infections in the United States. Our strain, FRD1, was isolated from the lungs of a cystic fibrosis patient, a cohort that invariably suffers from chronic \( \text{P. aeruginosa} \) infection and...
would benefit from the availability of novel antibacterial therapies. We infected the lungs of C57BL/6 mice (age, 8 to 12 wk; Jackson Laboratories) with a suspension containing 5 \times 10^7 colony forming units (CFU) of FRD1. One hour after infection, the mice were treated by oropharyngeal aspiration of an isotonic solution of wild type hLYS, engineered variant 2-3-7, or a phosphate buffered saline control (PBS). Twenty-three hours later, mice were sacrificed and the serum, liver, lung lavage, and lung tissue were collected for analysis. The studies showed that the engineered biotherapeutic caused neither acute toxicity nor allergic hypersensitivity, that it reduced bacterial burden in a dose-response fashion, and that it outperformed wild type hLYS with respect to numerous clinically relevant measures (lung bacterial burden, lung immune cell infiltration, lavage protein concentration, and lavage cytokine concentration). These results provided preliminary evidence that the engineered enzyme might indeed represent a performance-enhanced biotherapeutic.

In addition to analysis of 2-3-7 and wild type hLYS as single agent treatments, both enzymes were examined as combination therapies with tobramycin, a frontline antibiotic for cystic fibrosis patients (Fig. 2). At a clinically relevant dose (75 \mu g per mouse), tobramycin alone reduced bacterial burden to 13\% of the PBS control group. Simultaneous treatment with tobramycin and 100 \mu g of variant 2-3-7 showed a weak trend toward improved bacterial clearance (10\% of the control). On the other hand, combining tobramycin with wild type hLYS caused a disadvantageous increase in bacterial burden (106\% of control). In this model, therefore, wild type hLYS and tobramycin are antagonistic at concentrations effective for each single agent, whereas analogous combinations of engineered variant 2-3-7 and tobramycin trend toward increased efficacy. The mechanism for the observed wild type lysozyme antagonism remains unknown, but the result further supports the use of a charge modified lysozyme when considering human clinical trials, particularly for cystic fibrosis patients who are frequently on concomitant tobramycin therapy.

Intrigued by our unexpected results with lysozyme-tobramycin combinations, we contemplated whether similar trends might be observed in combinations with an alternative antibiotic whose mechanism of action was entirely orthogonal to that of tobramycin. Antimicrobial peptides (AMPs) are bactericidal agents best known for their membrane disrupting and immune modulating activities,\textsuperscript{23,24} and both natural and synthetic AMPs are under development as biotherapeutic agents.\textsuperscript{25} The membrane-disrupting action of AMPs would be expected to enhance lysozymes’ access to the peptidoglycan, which normally resides beneath the outer membrane lipid bilayer of Gram-negative bacteria. We identified from
In aggregate, these initial in vivo efficacy studies demonstrate that the objectives of our original molecular engineering efforts have been achieved. Specifically, variant 2-3-7 appears to outperform wild type hLYS in combating lung infections by a mucoid clinical isolate of *P. aeruginosa*, and the evidence suggests that this enhanced efficacy derives from the variant’s reduced susceptibility to electrostatic mediated aggregation with, and inhibition by, anionic biopolymers in the infected lung. Furthermore, the redesigned lysozyme exhibits an unanticipated advantage when used in combination with either tobramycin or a synthetic AMP. Namely, we have observed that co-administration with wild type hLYS tended to reduce efficacy relative to one or both single agents, whereas 2-3-7 combinations showed small gains in bacterial clearance. As with our earlier tobramycin studies, the differential mechanisms for combined action of the AMP with wild type vs. engineered lysozyme are not immediately obvious. We note, however, that both of the examined auxiliary antibiotics are cationic in nature (tobramycin +3 to +5 at neutral pH,17 and Tet009 +5 to +6, based on theoretical calculations with Vector NTI Advanced v11.0, Invitrogen Corp). Hen egg white lysozyme, a close homolog of hLYS, has been shown to complex with the antibiotic triclosan, improving its efficacy via delivery to bacterial targets.28 Although highly speculative, it is interesting to consider the possibility that the expanded electronegative potential of 2-3-7, relative to wild type hLYS, provides for similarly productive and synergistic interactions with cationic antibacterial agents. Proof of this or other alternative mechanisms awaits further analysis, but the current data provides convincing evidence that our charge engineered lysozyme outperforms wild type hLYS in combination therapies.

**Materials and Methods**

All experiments were performed as described in detail elsewhere.20 Lysozyme and Tet009 combination treatments were administered by oropharyngeal aspiration of a 40 μl volume containing both agents. The protocol for animal infection and lysozyme administration was approved by the Institutional Animal Care and Use Committee of the University of Vermont, in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All surgeries were performed under pentobarbital anesthesia, and all efforts were made to minimize animal suffering. Tet009 was purchased from Genscript at >95% purity.

**Disclosure of Potential Conflicts of Interest**

K.E.G. and T.C.S. hold a patent pending on charge engineered human lysozymes and their therapeutic applications. Both authors hereby affirm that this potential conflict of interest has been appropriately managed and that the data presented here are accurate and free of bias.

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