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Citation	Eller, Chelcie H and Raines, Ronald T. 2020. "Antimicrobial Synergy of a Ribonuclease and a Peptide Secreted by Human Cells." ACS Infectious Diseases, 6 (11).
As Published	10.1021/ACSINFECDIS.0C00594
Publisher	American Chemical Society (ACS)
Version	Author's final manuscript
Citable link	https://hdl.handle.net/1721.1/141233
Terms of Use	Creative Commons Attribution-Noncommercial-Share Alike
Detailed Terms	http://creativecommons.org/licenses/by-nc-sa/4.0/



HHS Public Access

Author manuscript

ACS Infect Dis. Author manuscript; available in PMC 2020 November 17.

Published in final edited form as:

ACS Infect Dis. 2020 November 13; 6(11): 3083–3088. doi:10.1021/acsinfecdis.0c00594.

Antimicrobial Synergy of a Ribonuclease and a Peptide Secreted by Human Cells

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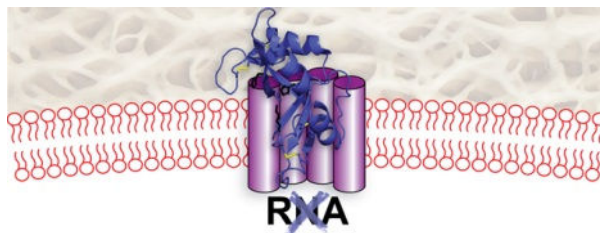
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Abstract

LL-37 is a secretory peptide that has antimicrobial activity. Ribonuclease 1 (RNase 1) is a secretory enzyme that is not cytotoxic. We find that human LL-37 and human RNase 1 can act synergistically to kill Gram-negative bacterial cells. In the presence of nontoxic concentrations of LL-37, RNase 1 is toxic to *Escherichia coli* cells at picomolar levels. Using wild-type RNase 1 and an inactive variant labeled with a fluorophore, we observe the adherence of RNase 1 to *E. coli* cells and its cellular entry in the presence of LL-37. These data suggest a natural means of modulating the human microbiome via the cooperation of an endogenous peptide (37 residues) and small enzyme (128 residues).

Graphical Abstract



Keywords

antimicrobial peptide; cathelicidin; microbiome; ribonuclease 1; synergism

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Accession Code
Human RNase 1 P07998.

The authors declare no competing financial interest.

Skin is the largest organ in humans. While covering ~18 square feet of our bodies,¹ human skin harbors a large and complex microbiome.² This ecosystem is modulated by the cutaneous innate and adaptive immune responses.³ A prominent modulatory role is played by two distinct families of antimicrobial peptides: defensins and cathelicidins.^{4–11} Humans have 10 defensins but only 1 cathelicidin: LL-37.^{12,13} This 37-residue cationic peptide, which has two N-terminal leucine (“L”) residues, forms a pore in microbial membranes to invoke its toxic activity.^{14,15} LL-37 is secreted from epithelial and other cells, and is found in several bodily fluids, including plasma, mucosa, breast milk, and seminal fluid.¹⁶

In addition to secreting antimicrobial peptides, human cells secrete at least eight homologous ribonucleases, RNases 1–8, each expressed from a gene located on chromosome 14q11.2.^{17,18} Of these homologues, RNase 1 (UniProtKB P07998) is the best characterized and most abundant. The expression of the *RNASE1* gene is widespread,¹⁹ and RNase 1 is present in sera at ~0.5 µg/mL (~30 nM).²⁰ This small (14.6 kDa) enzyme is a highly efficient catalyst of RNA cleavage ($k_{\text{cat}}/K_{\text{M}} = 2.8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$)²¹ and a well-known scourge in molecular biology and clinical chemistry laboratories.^{22,23}

RNase 1 has been implicated in physiological processes.^{17,24,25} Human endothelial cells release stored RNase 1 when stimulated with viral RNA agonists of Toll-like receptors.²¹ Its addition to dendritic immune cells stimulates the secretion of cytokines.²⁶ RNase 1 degrades extracellular RNA, which is a natural procoagulant,²⁷ and the plasma of RNase 1 knockout (*Rnase1*^{-/-}) mice clots more rapidly than does wild-type plasma.²⁸ An engineered variant of RNase 1 that evades a cytosolic inhibitor protein^{29,30} exhibits specific toxicity for cancer cells and is in clinical trials as a chemotherapeutic agent.^{31,32} RNase 1 also has demonstrable antiviral activity.^{25,33} Nevertheless, RNase 1 is not known to have antimicrobial activity.¹⁸

RNase 1 gains access to RNA within human cells after binding to the glycocalyx, undergoing endocytosis, and translocating to the cytosol.^{34,35} We reasoned that microbial cells resist the toxicity of RNase 1 by being impenetrable to the enzyme. Given that LL-37 forms a pore in microbial membranes,^{14,15} we suspected that these two denizens of human skin could act in concert to elicit antimicrobial activity. Our findings unveil a natural strategy for modulating the human microbiome.

RESULTS

Production of Proteins and Peptides.

Human RNase 1 and its H12A, P19C, and H12A/P19C variants were produced by heterologous expression in *E. coli*. The H12A substitution excises a key active-site imidazolyl group and thus obviates ribonucleolytic activity.^{21,30,36,37} In contrast, the P19C substitution is inconsequential to the structure and function of RNase 1^{38–40} but provides a sulfhydryl group for the site-specific conjugation of a BODIPY dye to generate RNase 1–BODIPY or H12A RNase 1–BODIPY.

Human LL-37 and Cys-LL-37 were prepared by chemical synthesis. The added N-terminal cysteine of Cys-LL-37 provides a site for the conjugation of a rhodamine dye to generate LL-37-rhodamine without compromising function.¹⁵

Antimicrobial Synergy of RNase 1 and LL-37.

Escherichia coli was chosen as a representative bacterium in this initial study. Under our conditions, we found that LL-37 has a minimum inhibitory concentration (MIC) against *E. coli* of 8.9 μM , that is, 40 $\mu\text{g/mL}$ (Figure 1). This value is similar to those that were determined previously.^{41,42} No growth inhibition was observed at LL-37 concentrations that were 1.7 μM .

In contrast to LL-37, the treatment of *E. coli* cells with RNase 1 did not lead to observable toxicity, even at a concentration of 50 μM (Figure 2). Thus, RNase 1 alone is unlikely to be consequential for *E. coli* in humans.

Remarkably, treating *E. coli* cells with sub-MIC concentrations of LL-37 and concentrations of RNase 1 in the picomolar range halted bacterial growth (Figure 2). This synergistic antimicrobial effect is dependent on ribonucleolytic activity, as treating *E. coli* with LL-37 plus H12A RNase 1 did not lead to toxicity (Figure 2).

Toxicity of RNase 1 and LL-37 for Human Cells.

The cytotoxic synergy of RNase 1 and LL-37 does not extend to human cells. Erythrocytes (that is, red blood cells) do not contain RNA and thus should not be vulnerable to a catalyst of RNA cleavage. They could, however, succumb to agents that disrupt the plasma membrane. Indeed, high concentrations of LL-37 are known to exhibit some toxicity towards human cells.⁴⁴ We found that human erythrocytes were indeed susceptible to LL-37. The addition of RNase 1 did not, however, increase that susceptibility (Figure 3A). Likewise, human endothelial cells (HUVEC) were susceptible to LL-37, but their susceptibility was not increased by RNase 1 (Figure 3B).

Binding of RNase 1 and LL-37 to Bacterial Cells.

We suspected that the antimicrobial activity of RNase 1 requires an affinity for the microbial cell surface. Initially, we probed for binding to the cell surface with a “pull-down” assay. In brief, we incubated fluorescently labeled RNase 1 with *E. coli* cells. We used centrifugation to isolate *E. coli* cells and gel electrophoresis to detect RNase 1–BODIPY that had associated with the cells. The results indicated that RNase 1 does have an affinity for *E. coli* cells (Figure 4A). To obtain quantitative data, we employed fluorescence polarization to titrate RNase 1–BODIPY with *E. coli* cells. Again, the results were consistent with an ability of RNase 1 to bind to *E. coli* cells (Figure 4B). The data indicated that each *E. coli* cell binds 50 nM(N_A/K_d) = 8×10^4 RNase 1–BODIPY molecules at the midpoint of the titration.

Internalization of RNase 1 into Bacterial Cells.

The antimicrobial activity of picomolar concentrations of RNase 1 in the presence of LL-37 suggested to us that the enzyme could enter *E. coli* cells via pores formed by LL-37 in the membrane.^{14,15} We tested this hypothesis by using fluorescently labeled H12A RNase 1,

which lacks the ribonucleolytic activity necessary for cytotoxicity. Confocal microscopy of single *E. coli* cells showed that H12A RNase 1–rhodamine can enter *E. coli* cells in the presence of LL-37–BODIPY (Figures 5, inset). Flow cytometry confirmed this colocalization and indicated that increasing the concentration of LL-37–BODIPY led to a greater uptake of RNase 1–rhodamine (Figure 5).

DISCUSSION

Antimicrobial agents can interact synergistically. Typically, this synergy arises from a combination of different cytotoxic strategies. For example, host-defense and other natural peptides can facilitate the access of antibiotic agents to intracellular targets.^{41,45–48} We have discovered that a well-known human secretory enzyme and a well-known human secretory peptide display extraordinary antimicrobial activity when used in combination. Even though RNase 1 is benign at 50 μM , femtomolar levels of RNase 1 in combination with sublethal concentrations of LL-37 are intolerable to *E. coli* cells (Figure 2).

A putative mechanism for the synergistic antimicrobial activity of RNase 1 and LL-37 is evident. LL-37 is known to create pores in the membranes of bacterial cells.^{14,15} RNase 1 adheres to microbial cells (Figure 4) and is thus poised to enter, given a means to do so. Pores created by LL-37 could provide that means, together with intrinsic gaps in the peptidoglycan layer.^{49,50} Unlike RNA in the human cytosol, which contains a ribonuclease inhibitor protein,^{29,30} microbial RNA has no known defense against the lethal catalytic activity of an invading ribonuclease. Accordingly, the synergy that we observed could extend to other pore-forming peptides, including defensins.^{4–11}

A specific interaction between RNase 1 and LL-37 is not required for their synergistic cytotoxicity. The protein and peptide do not appear to colocalize in *E. coli* cells (Figure 5). The lack of a significant interaction is not surprising, as both RNase 1 and LL-37 are highly cationic, each having a net charge of $Z = +6$. Indeed, we found no evidence for an interaction of LL-37 and RNase 1 by using fluorescence polarization (data not shown).

RNase 1 is known to interact with anionic mammalian glycans such as heparan sulfate and chondroitin sulfate.^{21,34,35,51} In contrast, its affinity for bacterial glycans is unknown. We note, however, that an RNase 1 homologue has been shown to associate with lipopolysaccharides, which pave the outer membrane of Gram-negative bacteria such as *E. coli*.⁵²

Without the implementation of new antimicrobial approaches, infections are expected to cause 10 million deaths annually by 2050.⁵³ Our data indicate that RNase 1 and LL-37 could provide an unappreciated first line of defense against bacterial pathogens. Work to explore the implications of this new-found synergism and exploit its utility is ongoing in our laboratory.

METHODS

General.

Commercial chemicals and biochemicals were of reagent grade or better, and were used without further purification. Measurements of UV-vis absorption were made with an Infinite® M1000 PRO plate reader from Tecan (Männedorf, Switzerland). All procedures were performed at ambient temperature (~22 °C) and pressure (1.0 atm) unless otherwise specified.

Preparation of Peptides, Proteins, and Their Conjugates.

LL-37, which has the amino acid sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES (M_r 4493.3), and Cys-LL-37 (which contains an additional N-terminal cysteine residue) were synthesized by Biomatik (Wilmington, DE). Cys-LL-37 was conjugated to BODIPY® FL *N*-(2-aminoethyl)maleimide from Thermo Fisher Scientific (Waltham, MA) and purified by passage through a desalting column from GE Healthcare (Chicago, IL).

RNase 1 and its H12A, P19C, and H12A/P19C variants were produced in *E. coli* and purified as described previously.⁵¹ H12A/P19C RNase 1 was conjugated to rhodamine red C₂ maleimide from Thermo Fisher Scientific, and P19C RNase 1 was conjugated to BODIPY FL *N*-(2-aminoethyl)maleimide. The resulting conjugates were purified by cation-exchange chromatography.

Toxicity for *E. coli* Cells.

A saturated culture of *E. coli* strain RP437⁵⁴ in Luria-Bertani medium⁵⁵ was diluted by 10000-fold with medium. The diluted cell stock (200 μ L/well) was added to the wells of a 96-well round-bottomed plate from Evergreen Scientific (Vernon, CA). A solution of LL-37 or RNase 1 in phosphate-buffered saline (PBS) was then added to the wells in a serial dilution. In all assays, *E. coli* cells were allowed to grow for 18 h at 37 °C with gentle shaking. Cells were counted by measuring the OD at 600 nm. The fraction of viable cells was determined by normalizing with wells treated with PBS (100%) and cells treated with chloramphenicol (0%). Data with LL-37 alone (Figure 1) and RNase 1 alone (Figure 2) were collected from three independent assays, with each being collected in duplicate ($n = 6$). Values are the mean \pm SEM. Data with mixtures of LL-37 and RNase 1 (Figure 2) are representative of checkerboard assays ($n = 4$).

Toxicity for Human Erythrocytes.

Deidentified human erythrocytes were product SER-PRBC from ZenBio (Research Triangle Park, NC). Cells (2% v/v) were washed three times with Tris-buffered saline (0.01 M Tris-HCl, pH 7.2, containing 0.155 M NaCl) by centrifugation at 3500 rpm. The cells were diluted to 1% in a 96-well plate. Increasing concentrations of LL-37, RNase 1, and solutions of LL-37 in the presence of RNase 1 (500 nM) were added to a final volume of 100 μ L, and the plate was incubated at 37 °C for 1 h. The plate was then subjected to centrifugation at 4000 rpm for 5 min. The supernatant (80 μ L) was transferred to a new plate, and the absorbance of hemoglobin, which is released upon hemolysis, was recorded at 405 nm. The

cell viability was normalized to cells treated with buffer (100%) or Triton X-100 to 4 $\mu\text{g}/\text{mL}$ (0%). Data were collected from three independent assays, with each being performed in duplicate ($n = 6$).

Toxicity for Human Endothelial Cells.

HUVEC cells were product CRL-1730 from the American Type Culture Collection (Manassas, VA). Cells were plated at 5000 cells/well in Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (Carlsbad, CA). After allowing for surface adhesion overnight, cells were treated with LL-37, RNase 1, or H12A RNase 1 as described above for erythrocytes. After 24 h at 37 °C with 5% CO₂, the medium was removed and replaced with 60 μL of Cell Titer assay solution from Promega (Fitchburg, WI) diluted 1:5 in DMEM. After a 2 h incubation, the reduction of the tetrazolium dye was measured at 490 nm. The fraction of cells that were metabolically active was calculated by normalizing with cells treated with PBS (100%) or H₂O₂ to 200 μM (0%). Data were collected from three independent assays, with each being performed in duplicate ($n = 6$).

Microbial Cell Binding by Pull-Down.

Microbial binding was assessed by incubating an aliquot (300 μL) of a culture of *E. coli* cells grown to OD ~0.8 with a solution (10 μL) of RNase 1–BODIPY (final concentration: 1 μM) in 20 mM Tris–HCl buffer, pH 7.2, containing NaCl (50 mM). After a 30 min incubation at 37 °C, cells were pelleted by centrifugation, the supernatant was collected, and the pellet was washed with the buffer. The supernatant and pellet were analyzed with SDS–PAGE. RNase 1–BODIPY was imaged by fluorescence using excitation at 470 nm and emission at 530 nm. Total protein was visualized by staining with Coomassie Brilliant Blue G-250 dye.

Microbial Cell Binding by Fluorescence Polarization.

Fluorescence polarization was employed to assess the affinity of RNase 1 for *E. coli* cells. RNase 1–BODIPY (final concentration: 50 nM) was added to solutions of 20 mM Tris–HCl buffer, pH 7.2, containing NaCl (50 mM) and varied concentrations of bacterial cells. Values of OD at 600 nm were converted to cells/mL by assuming that a solution with OD_{600 nm} = 1.0 contains 8×10^8 cells/mL. After incubation at 37 °C for 30 min, the luorescence polarization was measured by using excitation at 470 nm and emission at 530 nm. Polarization values were fitted by nonlinear regression analysis to the equation

$$Y = \frac{B_{\max}[\text{RNase}]^h}{(K_d^h + [\text{RNase}]^h)}$$

where Y is the relative fluorescence, B_{\max} is the maximum relative fluorescence, and h is the Hill coefficient. Assays were performed in triplicate. Data were collected from five independent assays, with each being performed in duplicate ($n = 10$).

Cellular Internalization of Protein and Peptide.

For cytometry, overnight growths of *E. coli* were diluted with medium until reaching $OD_{600\text{ nm}} = 1.0$. Cells from a 100 μL aliquot of the diluted cell stock were collected by centrifugation at 3000 rpm for 4 min and washed with PBS. Cells were resuspended in PBS and treated with H12A RNase 1–rhodamine (2.5 μM) and LL-37–BODIPY (2.5 μM). After a 2 h incubation, cells were washed (three times) with PBS and collected by centrifugation. Protein internalization was measured with an LSR Fortessa flow cytometer from BD Biosciences (San Jose, CA) at the University of Wisconsin–Madison Flow Cytometry Laboratory.

For visualization, cells (again, a 100 μL aliquot with $OD_{600\text{ nm}} = 1.0$) were treated with H12A RNase 1–rhodamine (2.5 μM) and LL-37–BODIPY (2.5 μM), and the mixture was diluted 4-fold with PBS. An aliquot (2.5 μL) of the resulting solution was spotted between agarose (3% w/v) pads on 24 \times 50 mm coverslips from Thermo Fisher Scientific. Cells were imaged with an Eclipse Ti laser–scanning confocal microscope from Nikon (Tokyo, Japan) equipped with an Axio Camdigital camera from Carl Zeiss (Oberkochen, Germany).

ACKNOWLEDGMENTS

We thank Prof. B. Weisblum (University of Wisconsin-Madison) for help with hemolysis assays, Prof. D. B. Weibel (University of Wisconsin-Madison) for the use of his confocal microscope, and Dr. K. J. Hetrick (Massachusetts Institute of Technology) for comments on the manuscript. This work was supported by grant R01 CA073808 (NIH). The University of Wisconsin-Madison Flow Cytometry Laboratory was supported by grant S10 OD018202.

ABBREVIATIONS

BODIPY	4,4-difluoro-4-bora-3a,4a-diaza- <i>s</i> -indacene
MIC	minimum inhibitory concentration
N_A	Avogadro's number
OD	optical density
PBS	phosphate-buffered saline
RFU	relative fluorescence units
RNase	ribonuclease
SDS–PAGE	polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
Z	net charge = Arg + Lys – Asp – Glu

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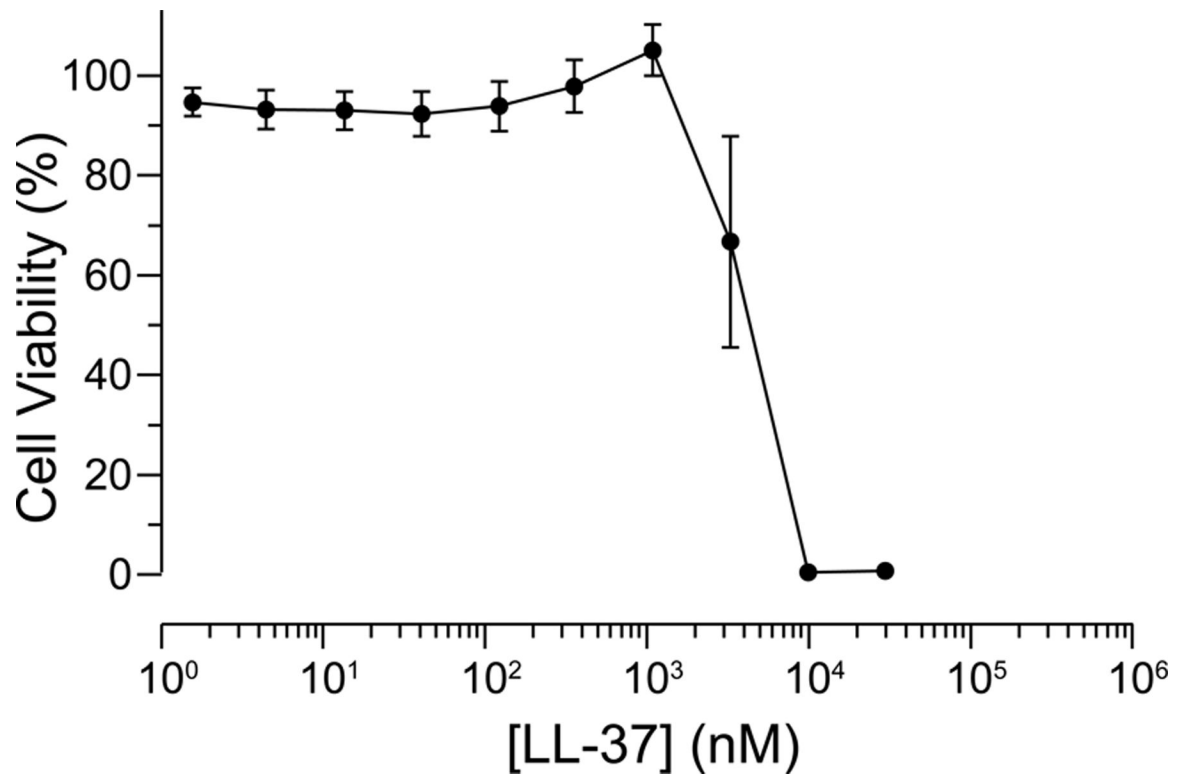


Figure 1. Effect of LL-37 on the viability of *E. coli* cells. Cells were treated with LL-37 for 18 h at 37 °C. Cell viability was assessed via OD_{600 nm}.

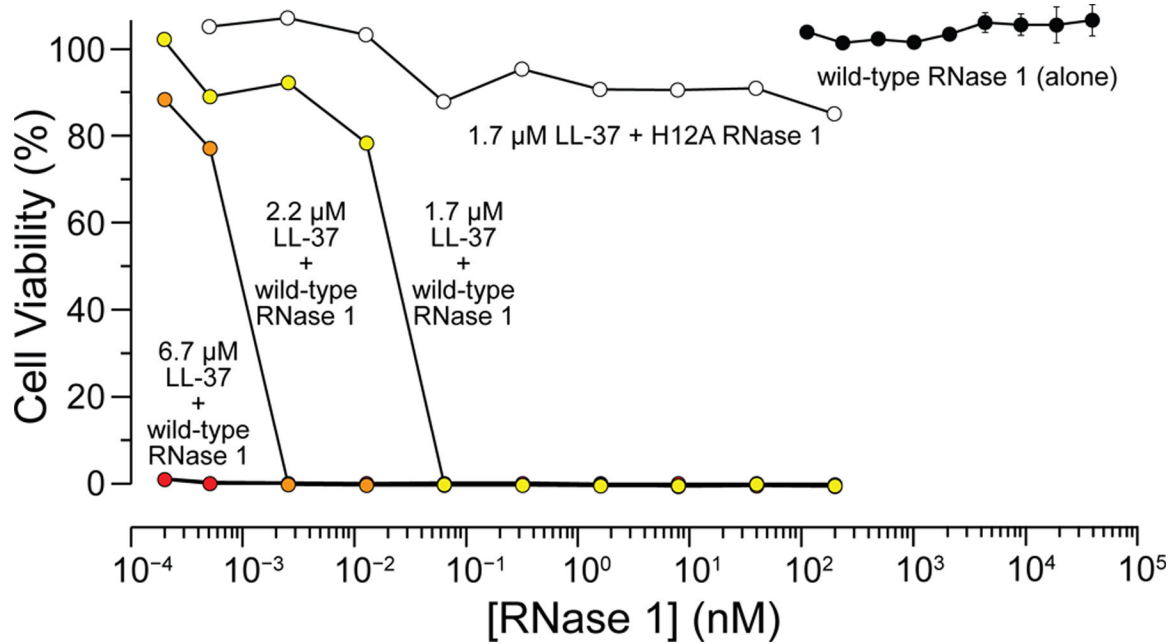


Figure 2. Effect of RNase 1 plus LL-37 on the viability of *E. coli* cells. Cells were treated with the indicated agents for 18 h at 37 °C. Cell viability was assessed via OD_{600 nm}.

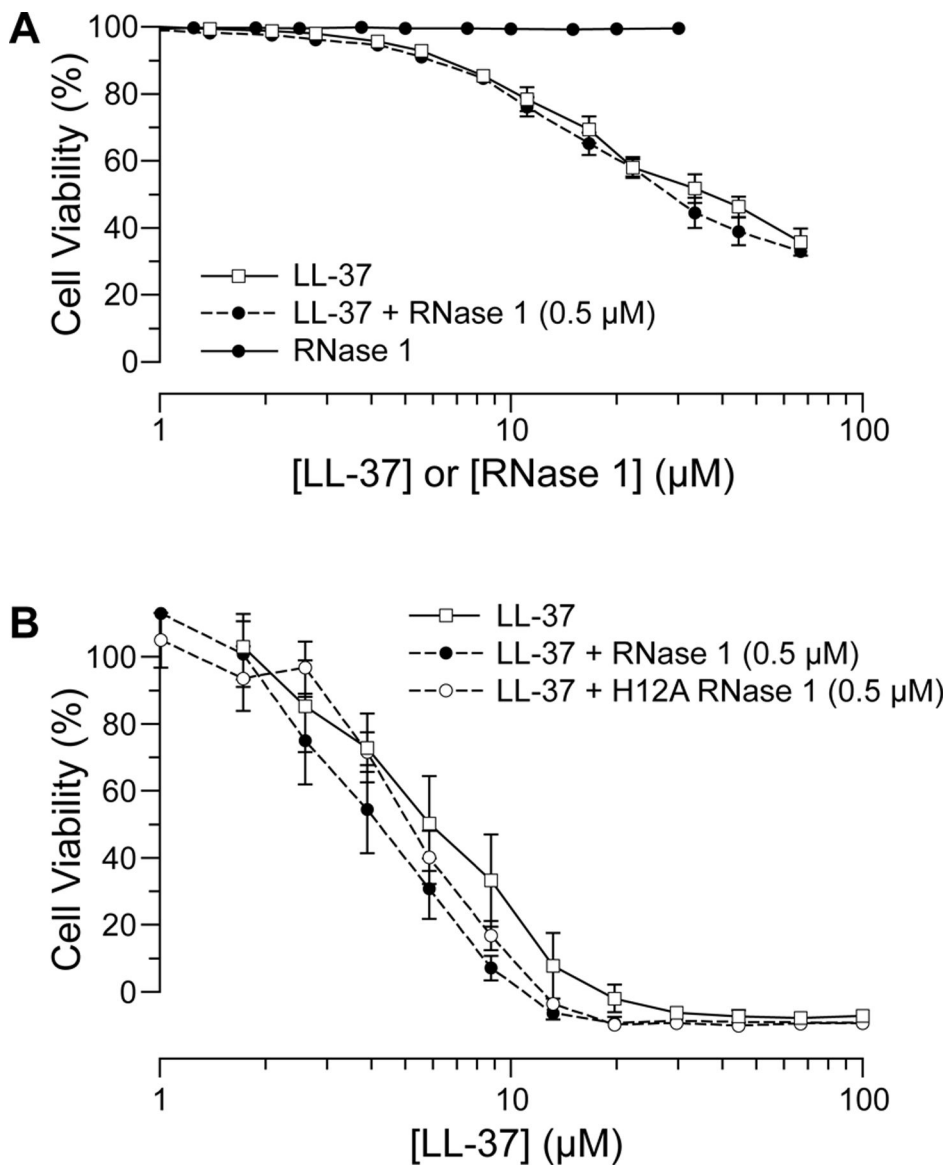


Figure 3. Effect of LL-37 and RNase 1 on the viability of human cells. Cells were treated with a putative toxin. (A) Red blood cells. Cells were treated for 1 h at 37 °C, when the viability was measured by the release of hemoglobin ($A_{405 \text{ nm}}$). (B) HUVEC cells. Cells were treated for 24 h at 37 °C, at which time the viability was assessed with a tetrazolium dye-based assay for metabolic activity.⁴³

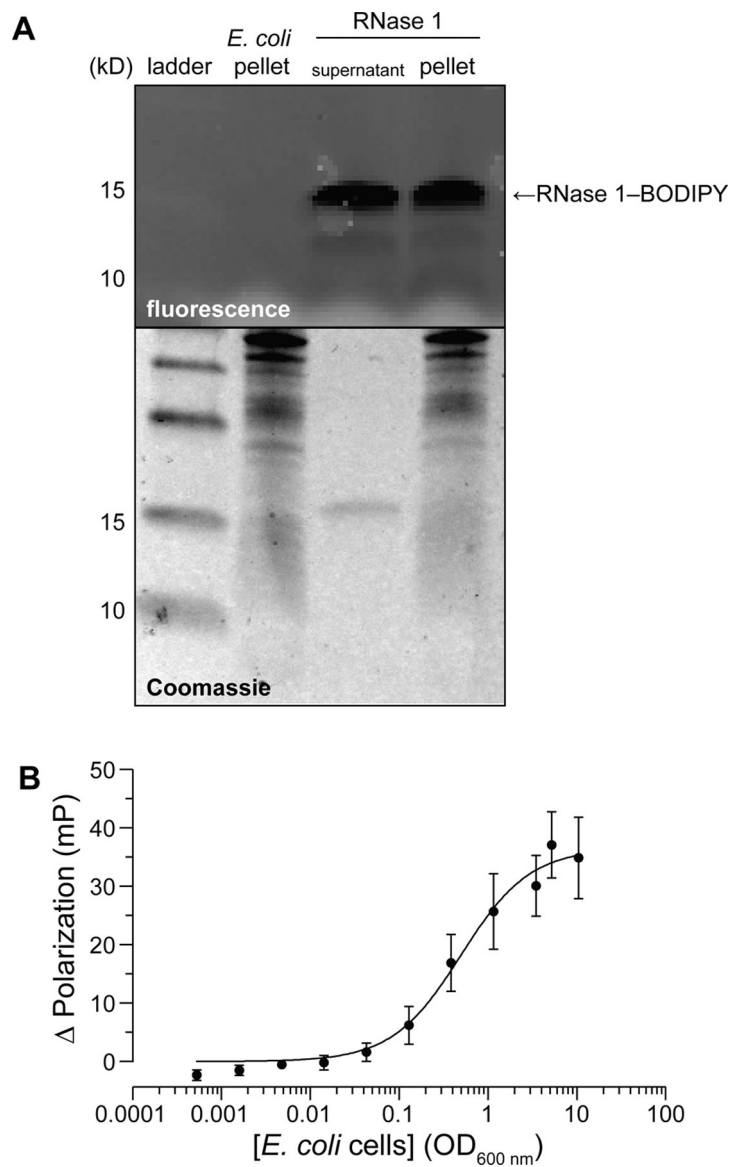


Figure 4. Binding of RNase 1 to *E. coli* cells. (A) SDS-PAGE gel of unbound (supernatant) and bound (pellet) fractions after incubation of RNase 1-BODIPY with *E. coli* cells for 30 min at 37 °C. The gel was imaged by fluorescence (top) or staining with Coomassie Brilliant Blue G-250 dye. (B) Graph showing the fluorescence polarization of RNase 1-BODIPY (50 nM) in the presence of increasing concentrations of *E. coli* cells. Polarization values are relative to that in the absence of *E. coli* cells. $K_d = (0.50 \pm 0.04)OD_{600\text{ nm}} = (0.40 \pm 0.03) \times 10^9$ cells/mL.

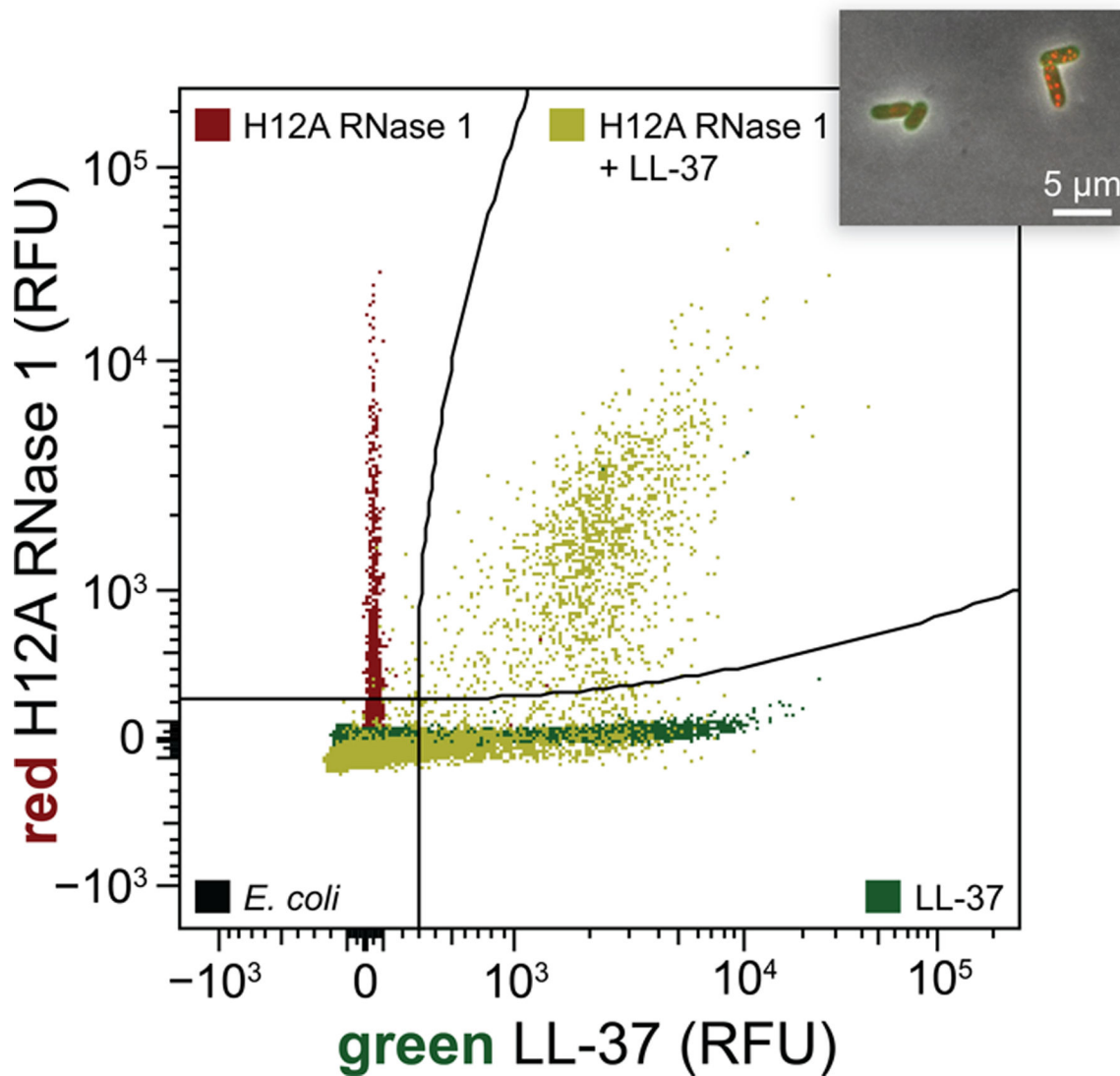


Figure 5. Flow cytogram of *E. coli* cells (8×10^7) incubated with 2.5 μM H12A RNase 1–rhodamine (red), 2.5 μM LL-37–BODIPY (green), or both (yellow) in PBS for 2 h. Inset: Confocal microscopy image of *E. coli* cells incubated with H12A RNase 1–rhodamine and LL-37–BODIPY.