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1 Enhancement of the Activity of the Antimicrobial Peptides HNP1 and LL-37 by Bovine
2 Pancreatic Ribonuclease A

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6

7 Abstract

8 Background: HNP1, LL-37, and HBD1 are antimicrobial against *Escherichia coli* ATCC
9 25922 at the standard inoculum but less active at higher inocula.

10 Methods: The virtual colony count (VCC) microbiological assay was adapted for high
11 inocula and the addition of yeast tRNA and bovine pancreatic ribonuclease A (RNase).
12 96-well plates were read for 12 hours in a Tecan Infinite M1000 plate reader and
13 photographed under 10x magnification.

14 Results: Adding tRNA 1:1 to HNP1 at the standard inoculum almost completely
15 abrogated activity. Adding RNase 1:1 to HNP1 at the standard inoculum of $5x10^5$
16 CFU/mL did not enhance activity. Increasing the inoculum to $6.25x10^7$ CFU/mL almost
17 abrogated HNP1 activity. However, adding RNase 25:1 to HNP1 enhanced activity.
18 Adding both tRNA and RNase resulted in enhanced activity, indicating that the
19 enhancement effect of RNase overwhelms the inhibiting effect of tRNA when both are
20 present. HBD1 activity at the standard inoculum was almost completely abrogated by
21 the addition of tRNA, but LL-37 activity was only slightly inhibited by tRNA. At the high
22 inoculum, LL-37 activity was enhanced by RNase. HBD1 activity was not enhanced by
23 RNase. RNase was not antimicrobial in the absence of antimicrobial peptides. Cell
24 clumps were observed at the high inoculum in the presence of all three antimicrobial
25 peptides and at the standard inoculum in the presence of HNP1+tRNA.

26 Conclusions: Antimicrobial peptide-ribonuclease combinations have the potential to be
27 active against high cell concentrations and biofilms, conditions where the antimicrobial
28 agent alone is relatively ineffective.

29

30 Introduction

31

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32 Although cationic antimicrobial peptides (CAPs) have been studied as possible
33 therapeutic agents for many years, few have survived clinical trials to become useful
34 antibiotics. (Mishra 2017) Three CAPs are representative of three different structural
35 classes that contribute to the human innate immune system: human neutrophil peptide
36 1 (HNP1), an alpha defensin; human beta defensin 1 (HBD1); and the human cathelicidin
37 LL-37. (De Smet 2005) One reason why CAP drug candidates have failed to gain
38 approval is a lack of efficacy. (Magana 2020) I demonstrated a pronounced inoculum
39 effect when the defensin HNP1 was assayed against high inocula of *Escherichia coli*
40 ATCC 25922, such that the antimicrobial peptide almost completely lost activity under
41 those conditions. (Ericksen 2020) A pronounced inoculum effect was also observed
42 when HNP1 was assayed against *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus*
43 ATCC 10876. What might cause this decrease in efficacy at high cell concentrations?
44 The molecular basis of the inoculum effect is unclear. However, one possibility is that
45 bacterial cells produce defensin inhibitors that are at a higher concentration when there
46 are more cells present.

47 One possible type of inhibition is that polyanions might bind and inhibit CAPs by
48 electrostatic attraction. Here I hypothesize that the polyanion tRNA might inhibit CAPs,
49 that inhibition by RNA is partially responsible for the inoculum effect, and that the
50 addition of ribonuclease could enhance antimicrobial peptide activity, restoring some of
51 the efficacy lost at high cell concentrations.

52

53 Methods

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55 The VCC assay was adapted for high inocula as previously described (Ericksen
56 2020), and volumes were adjusted to allow for the addition of yeast tRNA (Sigma from
57 *Saccharomyces cerevisiae*) and/or bovine pancreatic ribonuclease (Roche or Macherey-
58 Nagel (MN)). HNP1, LL-37 and HBD1 were synthesized ABI 433A synthesizer using an
59 optimized HBTU activation/DIEA in situ neutralization protocol developed by Kent and
60 coworkers for Boc chemistry solid phase peptide synthesis as previously described.
61 (Zhao 2013)(Pazgier 2013)(Bharucha 2021) Two inocula were studied: the standard
62 inoculum of $5x10^5$ CFU/mL, with cells from a seed culture diluted in 10 mM sodium
63 phosphate pH 7.4, and a high inoculum of $6.25x10^7$ CFU/mL, equivalent to adding
64 undiluted seed culture. Antimicrobial peptides were incubated in 10 mM sodium
65 phosphate pH 7.4 plus 1% tryptic soy broth (TSB) for two hours at 37°C shaking every 5
66 minutes for 3 seconds in a Tecan Infinite M1000 plate reader. An equal volume of

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67 twice-concentrated Mueller Hinton Broth was then added and 96-well plates were read
68 for 12 hours in the plate reader and then some wells containing cell clumps were
69 photographed under 10x magnification. In one experiment, the concentration of TSB
70 present in phosphate buffer was adjusted.

71

72 Results

73

74 Adding tRNA 1:1 to HNP1 at the standard inoculum almost completely abrogated
75 activity (Figure 1). Adding Roche RNase 1:1 to HNP1 at the standard inoculum of $5x10^5$
76 CFU/mL did not enhance activity. Increasing the inoculum to $6.25x10^7$ CFU/mL almost
77 abrogated HNP1 activity. (Figure 2) However, adding RNase 25:1 to HNP1 enhanced
78 activity at the high inoculum. Adding both tRNA and RNase resulted in enhanced
79 activity, indicating that the enhancement effect of RNase overwhelms the inhibiting
80 effect of tRNA when both are present. HBD1 activity at the standard inoculum was
81 almost completely abrogated by the addition of tRNA, but LL-37 activity was only
82 slightly inhibited by tRNA. (Figure 3) At the high inoculum, LL-37 was enhanced, but
83 LL-37 showed greater activity than HNP1 in the absence of RNase. (Figure 4) HBD1
84 activity was not enhanced by RNase. RNase was not active in the absence of
85 antimicrobial peptides. The observations with HNP1 at the high inolculum were
86 repeated using a second RNase manufacturer, Macherey-Nagel. (Figure 5) The
87 experiment with MN RNase was repeated. (Figure 6) 1% TSB was used in most assays,
88 but the %TSB was varied in one experiment, resulting in maximum activity at 4% TSB
89 with either 5x or 25x MN RNase added. (Figure 7) Cell clumps were observed at the
90 high inoculum in the presence of all three antimicrobial peptides with or without RNase
91 and at the standard inoculum in the presence of HNP1+tRNA. (Figure 8) The VCC assays
92 were conducted with TSB added to the 10 mM sodium phosphate incubation buffer. The
93 enhancement of activity caused by RNase was observed with LL-37 but not HNP1 when
94 washed cells were used, indicating that RNase operates by different mechanisms with
95 the two antimicrobial peptides. Although biofilm formation was not directly assayed, it
96 is assumed that the cell clumps photographed at 10x magnification are biofilms.
97 Ribonuclease did not enhance HBD1 activity at the $6.25x10^7$ CFU/mL inoculum,
98 demonstrating a strong inoculum effect with HBD1 vs. *E. coli*. LL-37 had a much lesser
99 inoculum effect against *E. coli*. The effect of ribonuclease on HNP1 is strongest with
100 lowest amounts of TSB present in the phosphate buffer during the 2 hour incubation.
101 The ability of tRNA to abrogate HNP1 and HBD1 activity, and the failure of tRNA to

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102 affect LL-37 activity, at the standard inoculum cannot be explained by net charge.
103 Possibly, hydrophobic interactions play a role in tRNA binding and inhibition. It is also
104 possible that tRNA inducing biofilm formation impacts HNP1 and HBD1 more than LL-
105 37.

106

107 Conclusions

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109 Antimicrobial assays are ordinarily conducted using a single antimicrobial agent,
110 studying its effect in isolation. However, the experiments presented here may offer a
111 glimpse into a more realistic in vivo scenario, in which multiple antimicrobial agents
112 work in concert against infection. Eight RNases are encoded by the human genome,
113 many of which have potent antimicrobial activity, such as RNase 7 expressed in
114 epithelial cells. (Sorrentino 2010) Bovine pancreatic RNase A, on the other hand, has a
115 digestive function degrading RNA and an antimicrobial function has not normally been
116 ascribed to it. RNase A is a basic protein (pI = 9.63). It is unknown whether the RNA-
117 degrading activity of RNase or its cationicity is responsible for the enhancement of
118 HNP1 and LL-37 activity. Product literature suggests assaying RNase A using 100 mM
119 Tris buffer, pH 7.4. Enzymatic activity in 10 mM sodium phosphate buffer was not
120 tested, but RNase A is very stable with four disulfide bonds.

121 The variation in the amount of TSB present in 10 mM phosphate buffer revealed
122 that the increase in activity caused by a small amount of nutrients present, allowing
123 some growth during the two-hour incubation, is counterbalanced by the inhibition of
124 defensin activity at higher TSB concentrations, presumably by the salt content of TSB.
125 This same effect is probably partially responsible for the almost complete abrogation of
126 activity of HNP1 when undiluted seed culture is added to the 96-well plate at the high
127 inoculum in the absence of RNase, since the salt concentration is much higher than in
128 assays at the standard inoculum where the seed culture is diluted in 10 mM sodium
129 phosphate buffer before adding to the 96-well plate.

130 The vast majority of published VCC assays were conducted at the standard
131 inoculum, reflecting a general reliance on the standard inoculum in a wide range of
132 published antimicrobial assays. Under these conditions, cells are predominantly
133 planktonic. However, a high inoculum may be more medically relevant, since high cell
134 concentrations and biofilms can accompany acute infections. This study demonstrates
135 the utility of conducting assays at a high inoculum, revealing details of antimicrobial

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136 activity that would be missed if the antimicrobial agents were studied only at the
137 standard inoculum. Further studies using animal models are necessary to determine
138 whether the enhancement of activity observed at the high inoculum is sufficient to
139 enable the infected host to overcome bacterial infections.

140 It should be emphasized that both RNA and ribonucleases are ubiquitous in vivo.
141 Therefore, these experiments may be more biologically relevant than VCC experiments
142 lacking RNA or ribonuclease. There are several possible sources of bacterial RNA that
143 might be present at the site of a bacterial infection. Firstly, bacteria normally secrete
144 RNA during their growth, which may have a role in the extracellular matrix of biofilms.
145 (Ozoline 2019) The results of the experiments presented here suggest that this secreted
146 RNA may also be a bacterial defense mechanism against antimicrobial peptides.
147 Secondly, once antimicrobial peptides are released at the infection site, cell lysis may
148 result in the release of intracellular RNAs, including mRNA and tRNA. Thirdly, host RNA
149 may be present. Therefore, inhibition by RNA must be regarded as a common obstacle
150 to effective antimicrobial activity that frequently occurs in real world scenarios.

151 The combination of an antimicrobial peptide with a ribonuclease could be
152 regarded as a novel invention that could possibly be used as a therapy to treat bacterial
153 infections. LL-37 and RNase 1 have been shown to act synergistically to kill *E. coli*. (Eller
154 2020) RNases have been tested in clinical trials as chemotherapeutics for the treatment
155 of cancer. (Ardelt 2009)

156 Further studies are warranted to determine whether these results could be
157 generalized to antimicrobial peptide-nuclease combinations, as might be suggested by
158 the presence of DNA in biofilms. A combination of an antimicrobial peptide with both
159 deoxyribonuclease (DNase) and RNase might be expected to be more potent than the
160 combination of the antimicrobial peptide and RNase in the absence of DNase, because
161 DNA is considered a more prevalent structural component of biofilms than RNA. (Gilan
162 2013) DNase is an approved drug, dornase alfa (Pulmozyme), which cuts apart
163 extracellular DNA in the lungs of cystic fibrosis patients, making the mucus thinner and
164 easier to expel. (Wagener 2012) It is possible that DNase in combination with an
165 antimicrobial peptide and RNase would form an effective treatment against acute
166 bacterial infections. A new generation of antimicrobial peptide-nuclease combinations
167 would offer a new hope that peptides that are sometimes defeated by the resistance
168 mechanism of biofilm formation can be repurposed to degrade biofilms instead, with
169 increased activity to fight infections.

170

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171 Acknowledgments

172

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174 are inhibited by RNA, Wuyuan Lu for providing antimicrobial peptides and helpful
175 discussions, and Peprotech, Inc. for funding.

176

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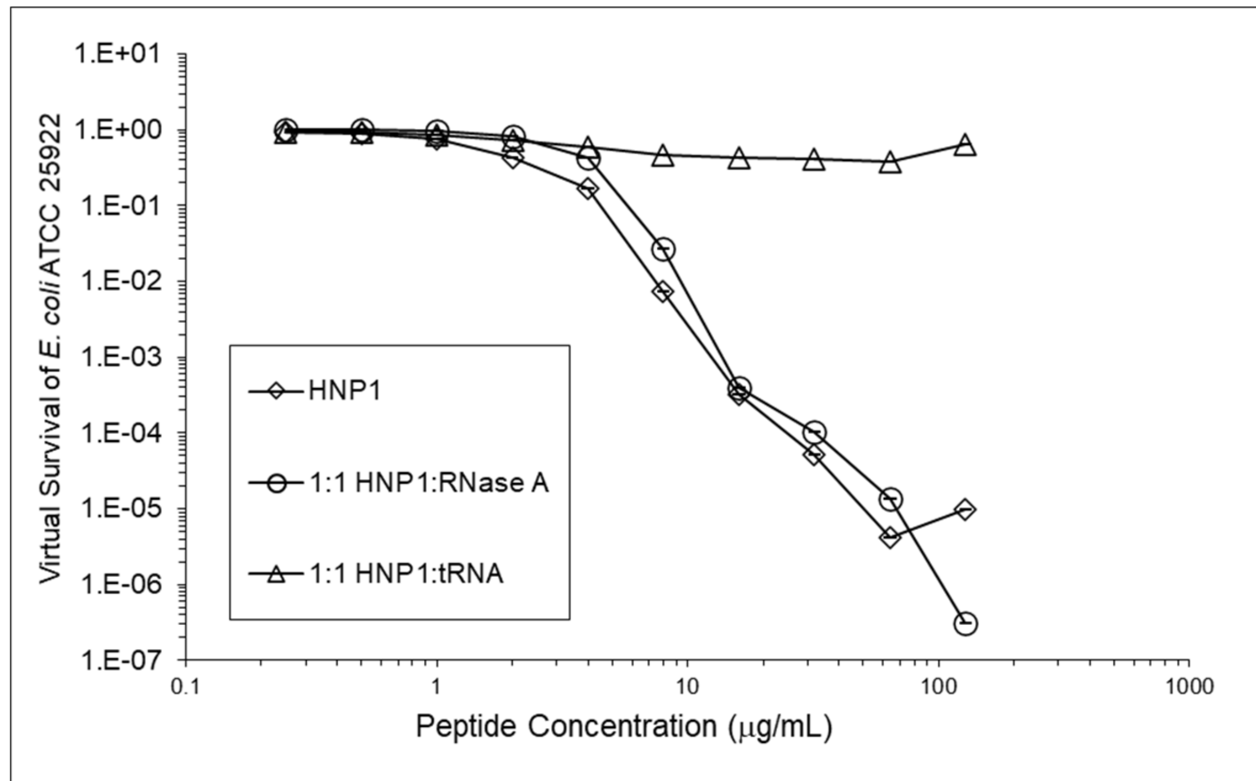


213

214 Figure 1. Activity of HNP1 with or without tRNA and RNase at the standard inoculum.

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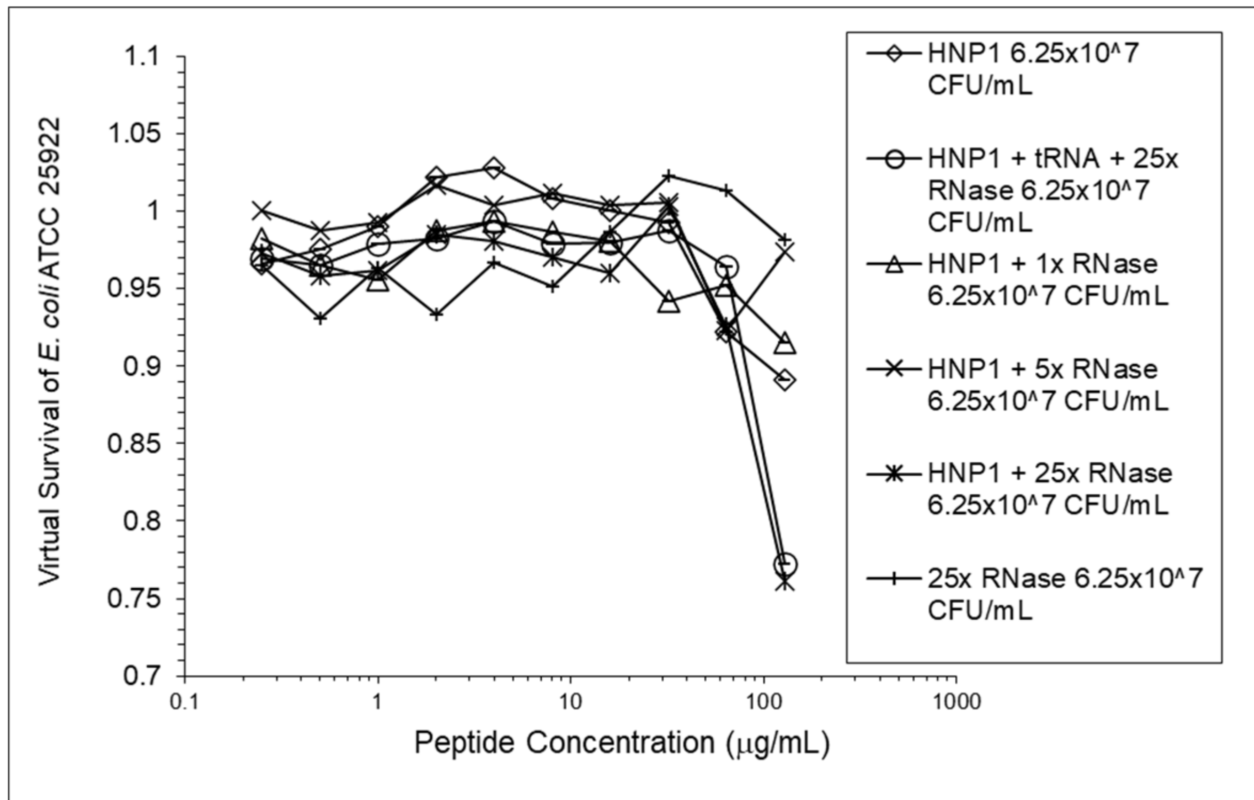


217

218 Figure 2. Activity of HNP1 at the high inoculum with or without tRNA and three
219 concentrations of RNase. Activity with HNP1 and both tRNA and the highest
220 concentration of RNase was essentially the same as HNP1 plus RNase alone, indicating
221 the enhancement of activity overcomes inhibition by tRNA. RNase in the absence of
222 antimicrobial peptides was not antimicrobial.

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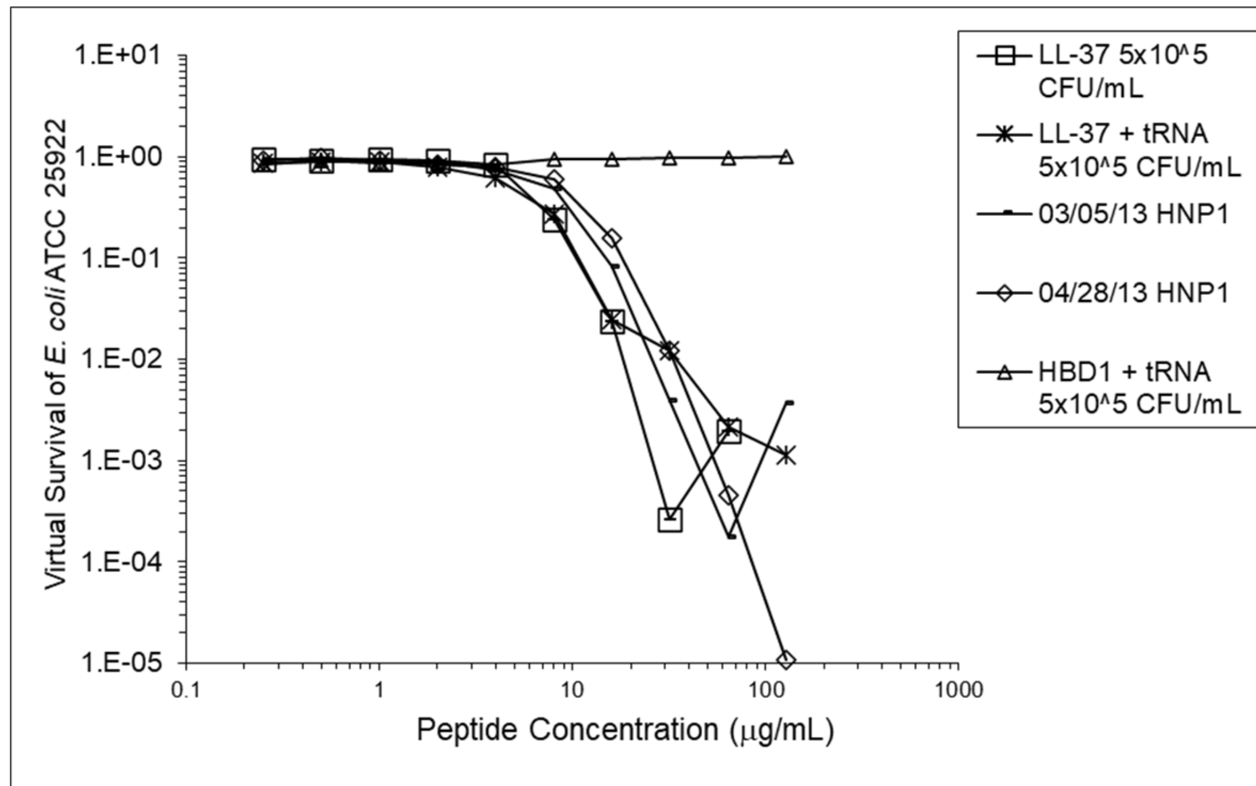


224

225 Figure 3. LL-37 was assayed at the standard inoculum with or without tRNA. HBD1 was
226 assayed at the standard inoculum in the presence of 1:1 tRNA. Two preparations of
227 HNP1 were assayed in the absence of tRNA as positive controls.

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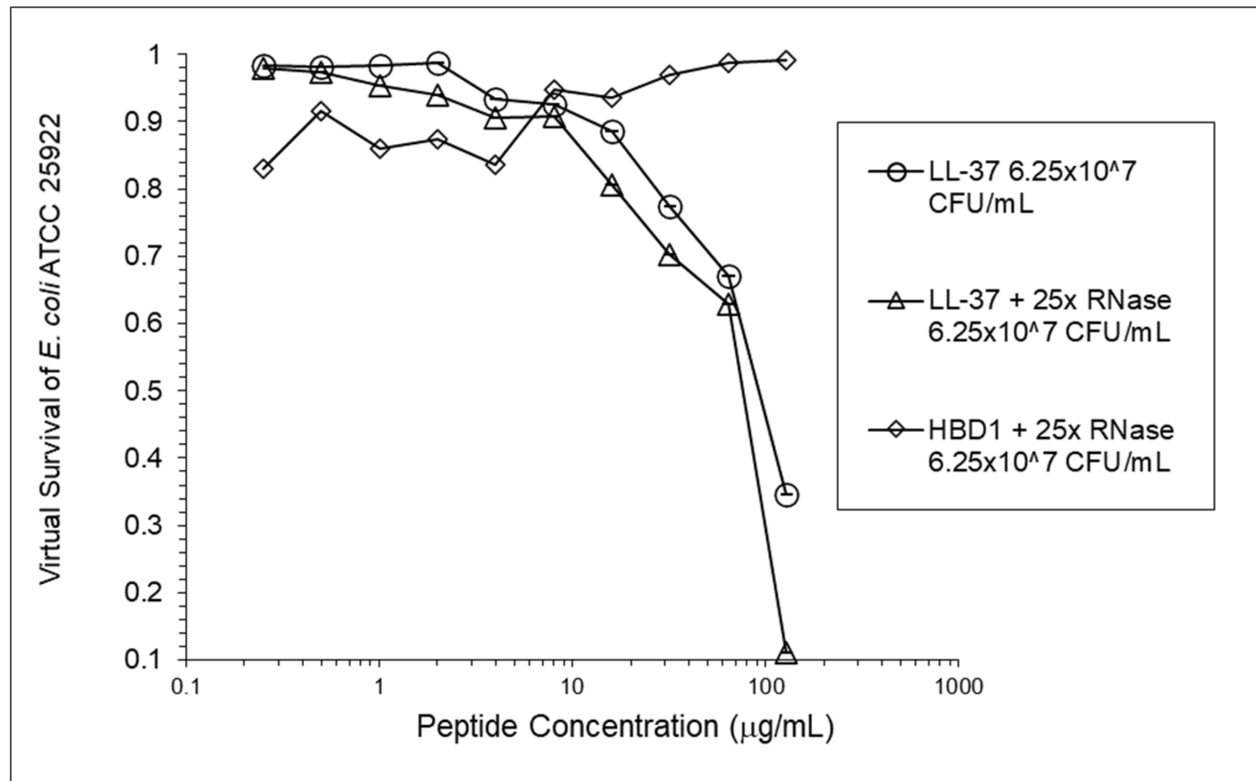


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230

231 Figure 4. LL-37 was assayed at the high inoculum with or without RNase. HBD1 was
232 assayed at the high inoculum with RNase.

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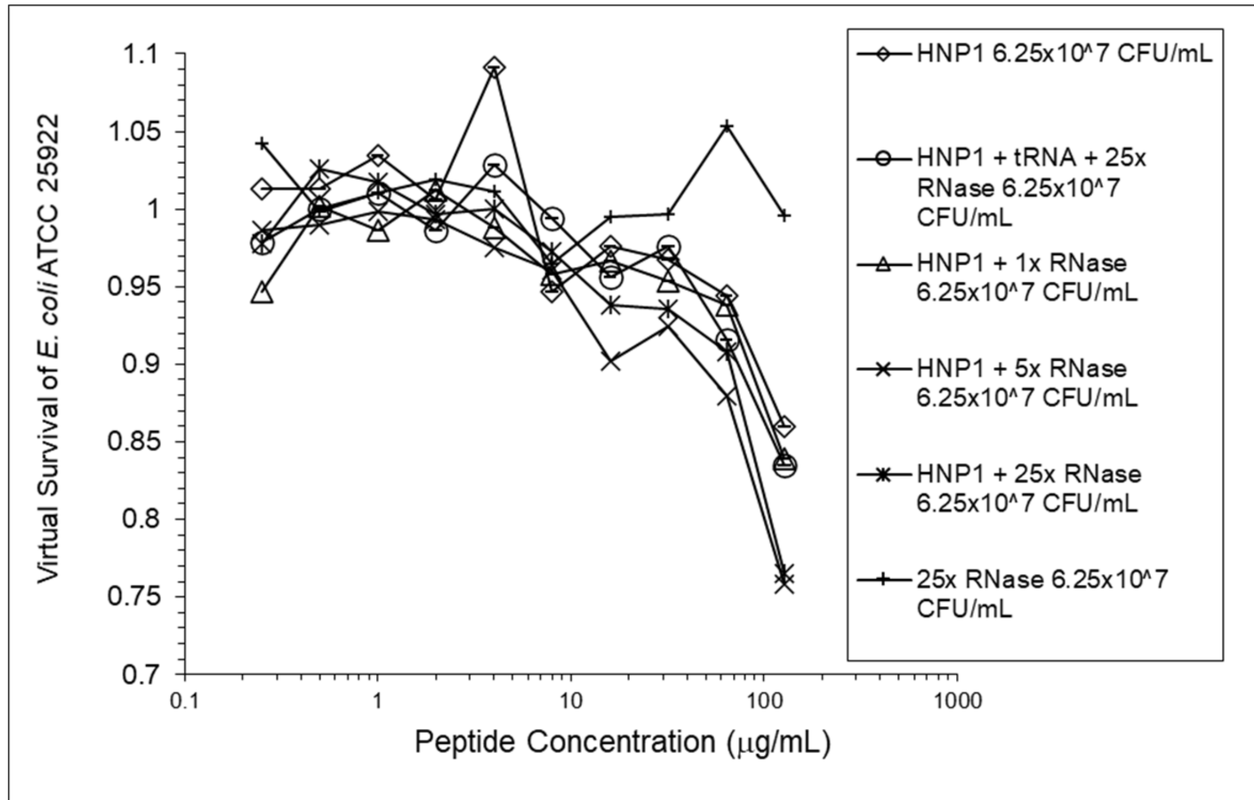


234

235 Figure 5. HNP1 was assayed at the high inoculum in the presence and absence of
236 RNase from a second manufacturer, and in the presence of both tRNA and RNase.

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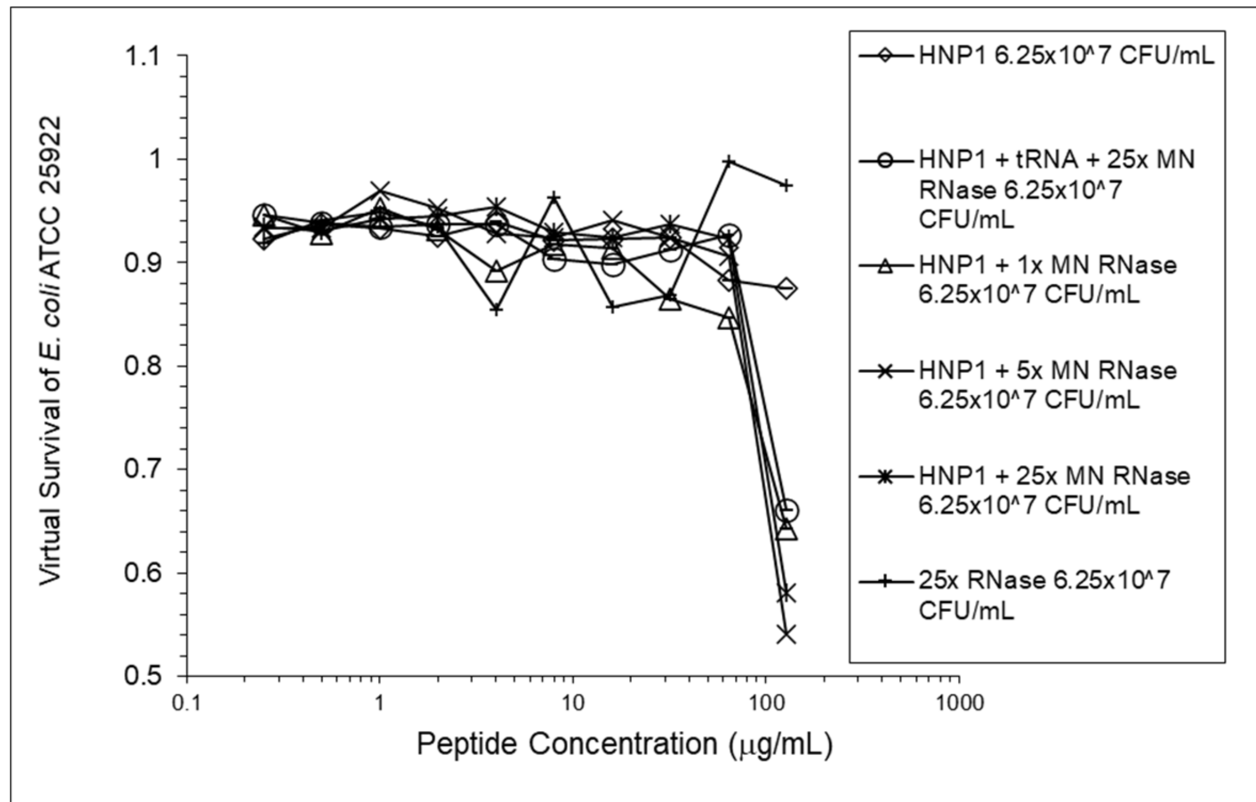


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239 Figure 6. The assay shown in Figure 5 was repeated.

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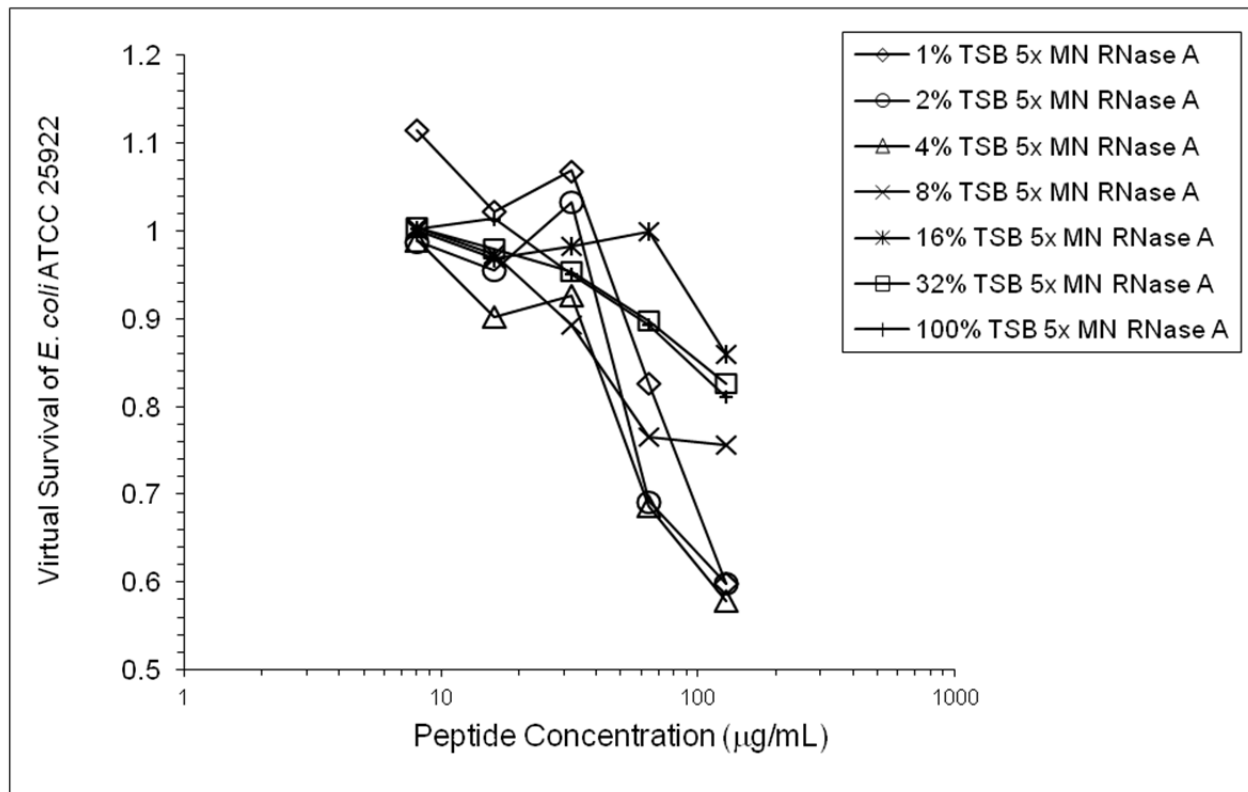


241

242 Figure 7. HNP1 was assayed at the high inoculum with variation in the amount of TSB
243 present during the two-hour incubation in 10 mM sodium phosphate buffer.

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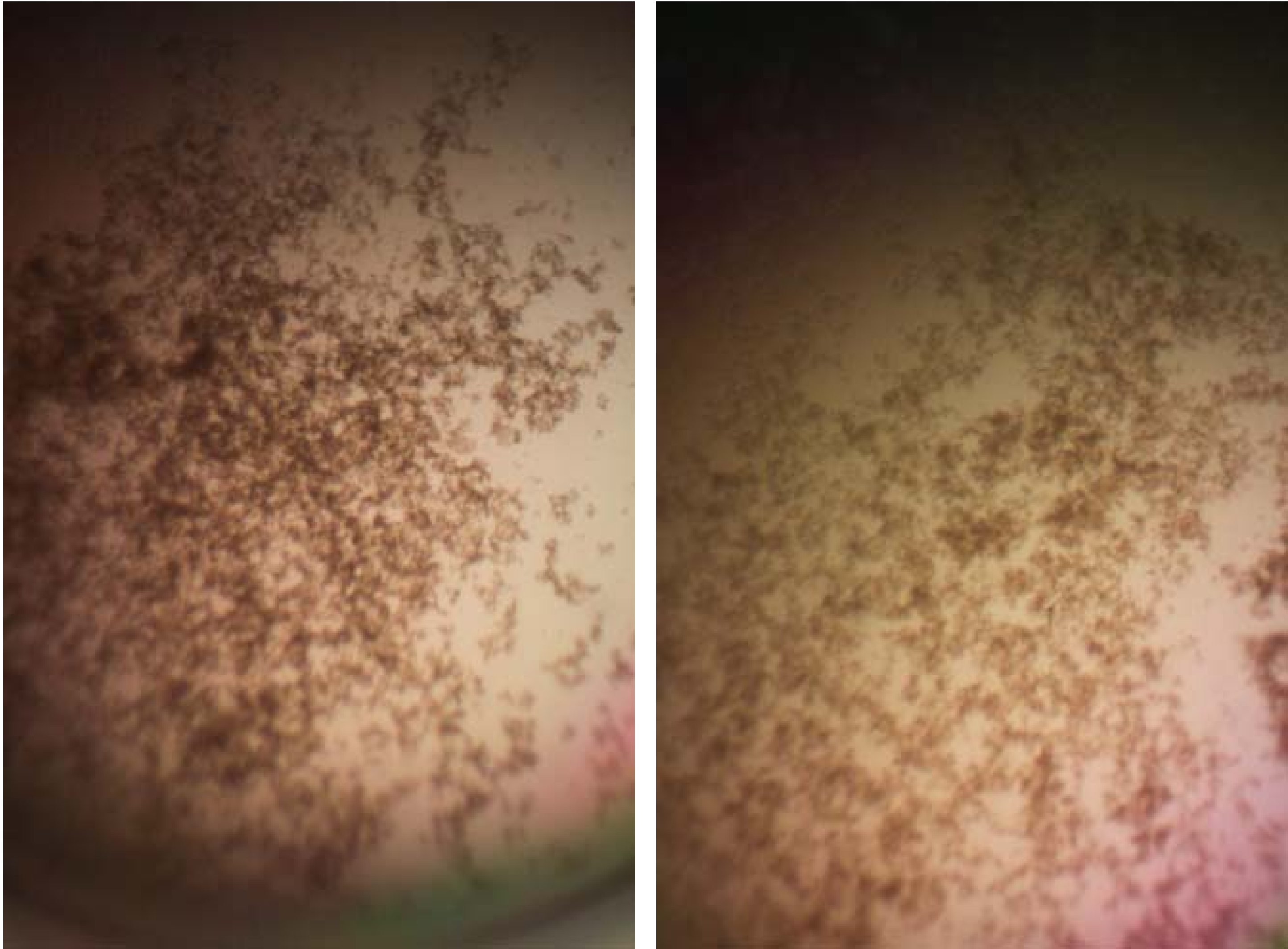

245 Figure 8. Cell clumps photographed at 10x magnification. Left panel: 128 μg/mL HNP1
246 at the high inoculum. Right panel: 128 μg/mL HNP1 + 1:5 RNase at the high inoculum.

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