Antibacterial activity of peptides derived from envelope glycoproteins of HIV-1

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Abstract **Recent reports have highlighted the anti-HIV-1 activities of defensins, whose structure and charge resemble portions of the HIV-1 transmembrane envelope glycoprotein gp41. The current report explores the obverse, whether peptides derived from HIV-1 envelope glycoproteins can exert antimicrobial activity. Fifteen-residue peptides spanning the entire sequence of HIV-1_{MN} gp120 and gp41 were subjected to radial diffusion assays against laboratory strains of *Escherichia coli* and *Listeria monocytogenes*. Twenty-four active peptides corresponded predominantly to membrane-active domains of gp120 and gp41. Several peptides retained significant activity in higher ionic conditions and may serve as templates for the development of novel peptide antibiotics. The strategies employed herein could uncover additional antimicrobial peptides from envelope proteins of other lytic viruses.**

Key words: Antimicrobial peptide; HIV-1; Glycoprotein; Innate immunity; Env

1. Introduction

Antimicrobial peptides and proteins are evolutionarily among the earliest molecular effectors of innate immunity [1]. A family of antimicrobial peptides, called defensins, are cysteine-rich, cationic antimicrobial peptides expressed by the leukocytes and epithelial cells of birds and mammals [2–5]. Three defensin subfamilies exist in vertebrates: α-defensins, β-defensins, and circular (θ) minidefensins [6]. All derive from an ancestral gene that existed before reptiles and birds diverged [7], contain six cysteines forming three stabilizing intramolecular disulfide bonds, and have primarily β-sheet structures. Another major class of antimicrobial peptides includes those that are linear, cationic, and form amphipathic α-helices upon contact with target membranes. Examples of these peptides include mellitin from bee venom, cecropins from moth hemolymph, and magainins from frog skin (reviewed in [8]).

There are a handful of reported studies on the activity of select antimicrobial peptides against HIV-1. Tachyplesins and polyphemusins are 17–18-residue peptides that are highly abundant in the hemocyte debris of the Japanese (*Tachypleus tridentatus*) and American (*Limulus polyphemus*) horseshoe crabs, respectively [9,10]. T22, T140, and TC14012, analogs of polyphemusin II, are highly specific inhibitors of HIV-1 fusion through the antagonism of the chemokine receptor CXCR4 [11], and thus inhibits the T cell-tropic (T-tropic; 'X4') strains that utilize CXCR4 as a coreceptor for entry. Separate studies have shown that linear [12] and cyclic [13] protegrins, peptides from porcine neutrophils that are structurally similar to protegrins, are active inhibitors of HIV-1. Another study showed that indolicidin, a 13-amino acid peptide isolated from bovine neutrophils, was reproducibly virucidal against HIV-1 only at very high concentrations of peptide [14]. Melittin and cecropin have been found to suppress HIV-1 gene expression [15]. Maximin 3, a linear cationic peptide isolated from the skin secretions of the Chinese red belly toad *Bombina maxima*, possessed modest anti-HIV activity at low micromolar concentrations [16]. A brief report by Nakashima and colleagues suggested that rabbit, rat, and guinea pig defensins can inhibit HIV-1-induced cytopathogenicity of a $CD4^+$ human T-cell line [17]. Although to date the antilentiviral activity of human α-defensins has not been reported, Monell and Strand have shown structural and functional similarities between the looped motifs of α-defensins and peptides derived from the HIV-1 gp41 envelope glycoprotein that may be required for viral fusion and infectivity [18]. Most recently, our group has determined that the primate circular minidefensins are remarkably active inhibitors of HIV-1 infection [19].

Since defensins and certain regions of HIV-1 gp41 share similar characteristics, including size, structure, and cationic charge at neutral pH [18], we have chosen to study whether peptides derived from the HIV-1_{MN} envelope glycoproteins, gp120 and gp41, contain defensin-like antimicrobial activity. Precedents for these studies include several elegant reports revealing a structural correlation between peptides from viral transmembrane proteins and magainins, naturally occurring amphipathic α-helical antimicrobial peptides from frogs [20–22]. Peptides derived from the C-terminal region of gp41, termed lentiviral lytic peptides (LLPs), are amphipathic and cationic, and microbicidal against a broad spectrum of bacteria. In the current study, we test the antimicrobial activity of peptides encompassing the entire sequence of the two envelope proteins from HIV-1_{MN}, gp120 and gp41, and determine

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regions of activity within these membrane glycoproteins that are active against laboratory strains of Gram-positive and Gram-negative bacteria. These studies will allow us to study known membrane-active regions of other viral proteins to determine regions that may be suitable for antimicrobial peptide design and development. Additionally, determining microbicidal domains might better enable us to resolve structural features of viral proteins that are crucial for interactions with the host cell membrane.

2. Materials and methods

2.1. Reagents

A set of 212 peptides derived from HIV-1_{MN} gp160, most of which were 15 amino acids in length with 11-amino acid overlaps between sequential peptides, were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD, USA (Catalog #6451). One hundred and twenty-five peptides that were soluble at 1 mg/ml in acidified water (0.01% acetic acid) were analyzed in subsequent tests of antimicrobial activity. Peptides that span the proteolytic cleavage site between gp120 and gp41 are not representative of the mature envelope glycoproteins and thus are not reported in this study.

2.2. Microbes and culture conditions

Listeria monocytogenes strain EGD and *Escherichia coli* strain ML-35p are laboratory test strains that were treated as described previously [23]. Briefly, liquid nitrogen-frozen stationary cultures of either strain were subcultured immediately prior to use in 50 ml 3% Trypticase Soy Broth (TSB; Becton Dickinson Microbiology Systems, Sparks, MD, USA) for 2.5 h at 37°C in an environmental shaking incubator (250 rpm) to obtain bacteria in mid-logarithmic growth phase. Subcultures were centrifuged at $1400\times g$ for 10 min, washed in Hanks' balanced salt solution (HBSS; Invitrogen Life Technologies, Carlsbad, CA, USA), and resuspended to the desired cell density in HBSS. For each bacterial strain an $OD_{625} = 1.0$ was equivalent to approximately 2.5×10^8 CFU/ml.

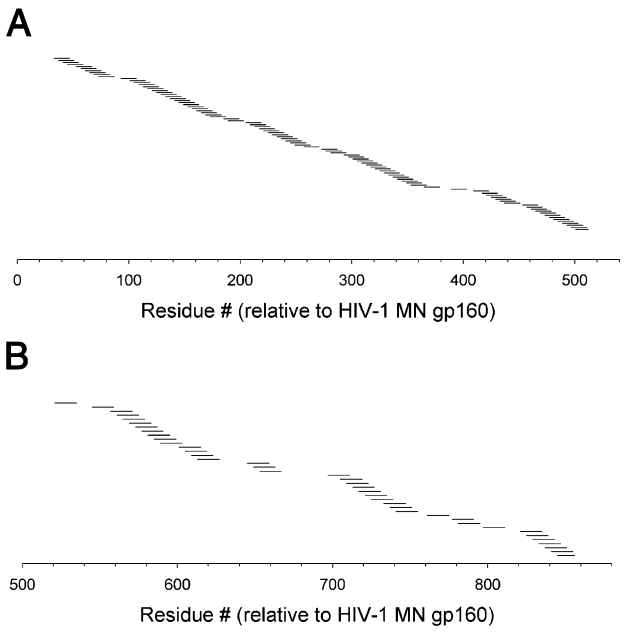

Fig. 1. HIV-1_{MN} env (15-mer) peptides utilized in antimicrobial assays. Peptides were obtained from the NIH AIDS Research and Reference Reagent Program, and those peptides that were soluble in 0.01% acetic acid are given. Shown here are the 86 peptides derived from gp120 (A) and 39 peptides derived from gp41 (B) that were tested for antimicrobial activity in Figs. 2 and 3. Maximum overlap between adjacent peptides is 11 amino acids.

2.3. Antimicrobial assay

Radial diffusion assays (RDAs) were performed as previously described [24]. The underlay consisted of 1% agarose and 1:100 dilution of TSB in either (a) 10 mM sodium phosphate, pH 7.4, (b) 10 mM

Table 1
Antimicrobial activity of HIV-1 peptides derived from gp120 and gp41

Protein	Peptide[a]	Structural motif[b]	Amino acid sequence	Isoelectric point	*E. coli*[c]			*L. monocytogenes*[c]		
					(0)	(50)	(100)	(0)	(50)	(100)
gp120	6238	n.d.	PCVKLTPLCVTLNCT	8.31	33	–	–	–	–	–
	6265	loop A	PAGFAILKCNDKKFS	9.26	53	–	–	19	–	–
	6269	n.d.	KGSCKNVSTVQCTHG	8.90	33	16	–	28	–	–
	6283	V3	VQINCTRPNYNKRKR	11.01	44	53	–	40	–	–
	6284	**V3**	CTRPNYNKRKRIHIG	11.01	70	41	–	29	–	–
	6286	**V3**	RKRIHIGPGRAFYTT	11.72	67	56	28	30	–	–
	6288	V3	GRAFYTTKNIIGTIR	11.00	33	–	–	34	5	–
	6289	V3	YTTKNIIGTIRAQAH	9.99	30	–	–	18	–	–
	6290	V3	NIIGTIRQAHCNISR	10.35	14	–	–	–	–	–
	6291	V3	TIRQAHCNISRAKWN	10.86	36	–	–	–	–	–
	6292	V3	AHCNISRAKWNDTLR	9.51	16	–	–	–	–	–
	6294	n.d.	KWNDTLRQIVSKLKE	9.71	48	32	–	13	–	–
	6295	n.d.	TLRQIVSKLKEQFKN	10.29	38	23	–	17	–	–
	6297	n.d.	LKEQFKNKTIVFNQS	9.70	–	–	–	8	–	–
gp41	6350	HR1	HMLQLTVWGIKQLQA	8.76	38	–	–	–	–	–
	6351	HR1	LTVWGIKQLQARVLA	11.00	33	–	–	34	6	–
	6352	HR1	GIKQLQARVLAVERY	9.99	12	–	–	6	–	–
	6359	n.d.	GKLICTTTVPWNASW	8.22	18	–	–	–	–	–
	6383	**MSD**	RIVFAVLSIVNRVRQ	12.30	37	28	20	34	29	30
	6399	n.d.	LRSLFLFSYHHRDLL	8.76	–	–	–	9	–	–
	6416	LLP-1	VIEVLQRAGRAILHI	9.58	13	–	–	–	–	–
	6417	LLP-1	LQRAGRAILHIPTRI	12.30	44	–	–	–	–	–
	6418	LLP-1	GRAILHIPTRIRQGL	12.30	52	26	–	–	–	–
	6419	LLP-1	LHIPTRIRQGLERAL	11.70	14	–	–	–	–	–

[a]Peptide identification corresponds to reagent numbers in the NIH AIDS Research and Reference Reagent Program.
[b]HR1 = heptad repeat region 1, which also contains a portion of the defensin-like domain; MSD = membrane-spanning domain; LLP-1 = lentiviral lytic peptide-1 domain; n.d. = not determined.
[c]Results are given as RDUs; '–' denotes no activity. All tests were performed in 10 mM sodium phosphate, pH 7.4 either with no added salt '(0)', 50 mM NaCl '(50)', or 100 mM NaCl '(100)'.

sodium phosphate/50 mM NaCl, pH 7.4 or (c) 10 mM sodium phosphate/100 mM NaCl, pH 7.4. Overlay consisted of 6% TSB and 1% agarose in dH_2O. Bacteria (4×10^6) were mixed with 10 ml of underlay gel solution, kept molten at 46–48°C and poured into 100-cm^2 square Petri dishes. After agarose solidified, a series of 3.2 mm diameter wells were punched and 5 μl of peptides (5.6 μg/ml–1 mg/ml) in 0.01% acetic acid were added into designated wells. Plates were incubated for 3 h at 37°C to allow peptide diffusion. The microbe-laden underlay was then covered with 10 ml of molten nutritive overlay and the plates were permitted to harden. Antimicrobial activity was identified as a clear zone around the well after 18 h incubation at 37°C, and represented in radial diffusion units (RDU): [diameter of clear zone in mm minus well diameter]×10. For some peptides, the *x*-intercept of the relationship between zone diameter and $\log_{10}$ peptide concentration was determined by least mean squares regression, and equated to the minimal inhibitory concentration (MIC).

3. Results and discussion

We examined the antimicrobial activity of peptides from the glycoproteins of HIV-1_{MN} envelope because several distinct regions of the glycoproteins are membrane-active and have similar properties to known antimicrobial peptides, such as small size and high local cationic charge at neutral pH. Two hundred and twelve 15-residue peptides with 11-amino acid overlaps between sequential peptides were obtained from the NIH Research and Reference Reagent Program. Peptides that were fully soluble in 0.01% acetic acid (acidified water) are represented in Fig. 1, and span approximately 95.0% of the sequence of gp120 (86 peptides; Fig. 1A) and 79.0% of the sequence of gp41 (39 peptides; Fig. 1B). Peptides that could not be dissolved in acidified water were not tested in subsequent assays, contained a high proportion of hydrophobic amino acids and coincided with regions of minimal exposure to the protein surface.

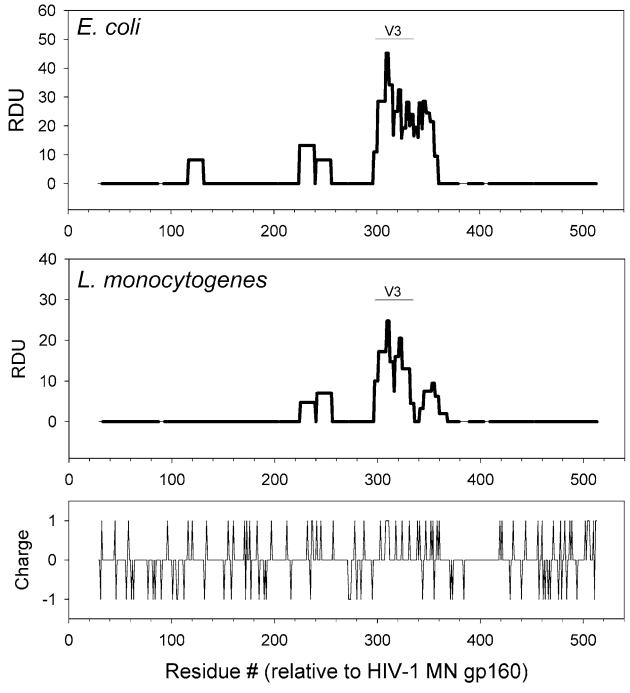

Fig. 2. RDAs demonstrate antimicrobial activity of peptides derived from HIV-1_{MN} gp120. Using RDAs the antimicrobial activity of 5 μg of each 15-residue peptide derived from gp120 was tested separately in 10 mM sodium phosphate, pH 7.4, against *E. coli* (top panel) and *L. monocytogenes* (middle panel). Activity is expressed as RDU, which are a measure of the diffusible peptide activity. The ExPASy program, ProtScale (http://expasy.cbr.nrc.ca/cgi-bin/protscale.pl) was used to plot the charged residue profile of gp120 (bottom panel). Note that peptides in regions of gp120 with a high net positive charge were antimicrobial. Regions of gp120 that were tested by RDA are represented by thick lines, and untested regions are represented by thin lines.

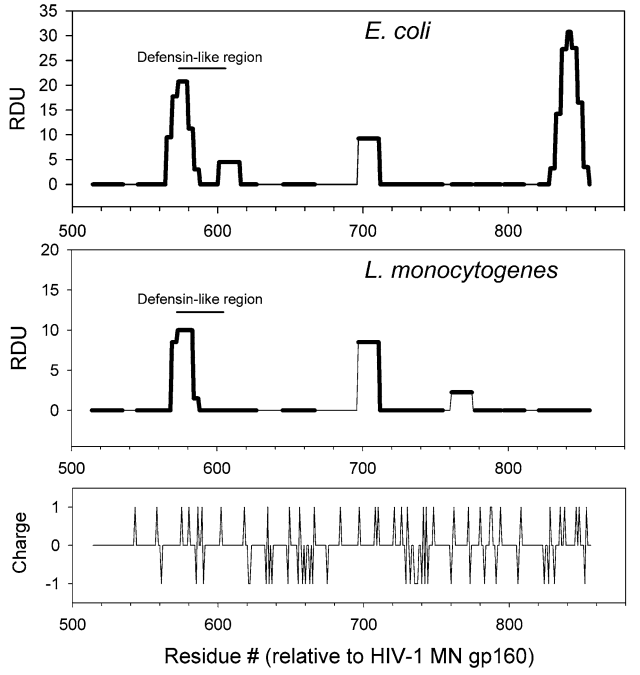

Fig. 3. RDAs demonstrate antimicrobial activity of peptides derived from HIV-1_{MN} gp41. Using RDAs the antimicrobial activity of 5 μg of each 15-residue peptide derived from gp41 was tested separately in 10 mM sodium phosphate, pH 7.4, against *E. coli* (top panel) and *L. monocytogenes* (middle panel). Activity is expressed as RDU, which are a measure of the diffusible peptide activity. ProtScale was used to plot the charged residue profile of gp41 (bottom panel). Peptides in regions of gp41 with a high net positive charge were antimicrobial. Regions of gp41 that were tested by RDA are represented by thick lines, and untested regions are represented by thin lines.

In an initial screen, each 15-residue peptide was subjected to radial diffusion assays in 10 mM sodium phosphate, pH 7.4, against *E. coli* and *L. monocytogenes* and the activity was given as RDU, a measure of diffusible antimicrobial activity (Figs. 2 and 3). The activity of peptides that overlapped a particular residue is expressed as the average RDU for that residue. Fourteen of 86 peptides from gp120 and 10 of 39 peptides from gp41 demonstrated activity against one or both strains of bacteria (Table 1). Peptides that were determined to be antimicrobial in the initial screen were subsequently tested at higher salt concentrations (Table 1). There was a precipitous drop in antimicrobial activity at increased salt concentrations for most peptides tested. At near-physiologic electrolyte conditions (10 mM sodium phosphate, pH 7.4+100 mM NaCl), only two peptides remained active: #6286 and #6383, corresponding to the immunogenic tip of the V3 loop (containing the β-turn GPGR motif) of gp120 and the membrane-spanning domain of gp41, respectively. The MICs for the three most active peptides are given in Table 2.

Most of the peptides determined to exert antimicrobial activity were derived from several areas of gp120 and gp41 that

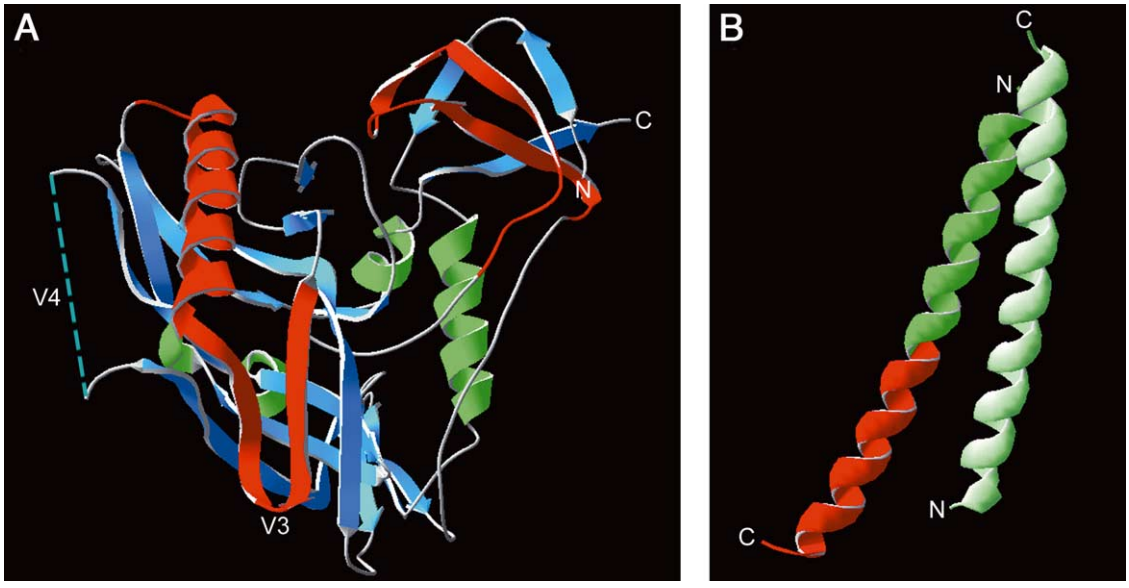

Fig. 4. Structural orientation of antimicrobial regions on the core of gp120 and gp41. A: A model of core gp120 (PDB accession code: 1G9M [40]) was drawn using the Swiss PDB Viewer (http://www.expasy.ch/spdbv/mainpage.html), and colored red to indicate regions of antimicrobial activity. Non-active regions of gp120 are colored gray (connections), green (helices), and blue (β-sheets). The disordered V4 loop is shown by a dashed line. As described in [40], the viral membrane is oriented above gp120, the target membrane is below, and the C-terminal tail of CD4 would be behind the molecule going into the page. B: A model of a monomer of N51 (dark green) and C43 (light green) of the gp41 core (PDB accession code: 1AIK [41]) was drawn using the Swiss PDB Viewer, and colored red to indicated regions of antimicrobial activity. Note that the active region in red corresponds to a segment of the N-terminal heptad repeat region, HR-1.

have been previously described as membrane-active. Within gp120, the entire V3 region, a disulfide-constrained surface-exposed loop encoded by a variable region of *env* [25], and exposed regions flanking V3 (Fig. 4) were potently active. The determinants of HIV-1 usage of chemokine coreceptors (CXCR4 and/or CCR5) for viral attachment and entry primarily reside within the V3 hypervariable region [26–29]. The V3 loop is critical for HIV-1 infection of cells that lack CD4, the primary receptor of gp120, and instead can utilize galactosylceramide on the host cell membrane as an alternative receptor for viral entry [30–32]. Many antimicrobial peptides exert their activity against microbes through a process involving an initial electrostatic binding of peptide to the surface of microbial membranes (reviewed in [33]). Events that interfere with the electrostatic peptide–membrane interaction negatively influence downstream events including peptide insertion, membrane pore formation and microbial lysis. The initial binding step involves interactions between basic residues of the antimicrobial peptide and electronegative regions of bacterial lipopolysaccharide and/or peptidoglycan. Subsequently, insertion will lead to interaction between the peptides and the anionic lipid moieties of the target microbe, and is thus highly dependent on the cationicity of the peptide. It is therefore not surprising that the net cationic charge of the 35-residue V3 loop is highly basic (p*I* 11.01), as were active peptides derived from this region (Table 1). The activity of the V3 loop may be a combination of its cationic character and its ability to bind carbohydrate moieties.

The most active peptide (#6383) originated from the viral membrane-spanning domain of gp41. Indeed, this was the only peptide that retained activity against both *E. coli* and *L. monocytogenes* at the highest salt concentrations tested (10 mM sodium phosphate, pH 7.4+100 mM NaCl). The other two domains of gp41 that were appreciably antimicrobial include the N-terminal heptad repeat region (Fig. 4), which encompasses a portion of the defensin-like domain [18], and the C-terminal tail from which LLPs were derived [20–22]. Synthetic peptides corresponding to either the N-terminal (e.g. T21) or C-terminal (e.g. T20) heptad repeat regions of gp41 are inhibitors of HIV-1 entry [34–36] and are currently under development as therapeutics (reviewed in [37]). Our findings suggest that entry inhibitor therapeutics corresponding to the N-terminal heptad repeat region would have an additional benefit of antibacterial activity.

While the activity of peptide #6383 can be partially explained by its high isoelectric point (p*I* 12.30), other peptides with identical p*I*s, such as #6417 and #6418, were not active at higher salt concentrations and were not active against

Table 2
MICs of peptides derived from gp120 and gp41[a]

Peptide	EC(0) (μg/ml)	EC(50) (μg/ml)	EC(100) (μg/ml)	LM(0) (μg/ml)	LM(50) (μg/ml)	LM(100) (μg/ml)
6284	5.47 ± 2.72	28.7 ± 18.2	n.t.	10.7 ± 0.56	n.t.	n.t.
6286	2.33 ± 0.24	18.1 ± 11.6	92.8 ± 73.8	26.0 ± 14.5	> 250	> 250
6383	**0.50 ± 0.20**	**4.87 ± 1.64**	**2.73 ± 0.56**	**0.66 ± 0.17**	**1.97 ± 0.32**	**3.10 ± 1.20**

[a] $n = 3$ experiments for each condition; n.t. denotes not tested. EC = *E. coli*; LM = *L. monocytogenes*. All tests were performed in 10 mM sodium phosphate, pH 7.4 either with no added salt '(0)', 50 mM NaCl '(50)', or 100 mM NaCl '(100)'. Range of peptides used to extrapolate MIC = 3.9–500 μg/ml.

L. monocytogenes (Table 1). Even though high net positive charge is a primary determinant of microbicidal activity, other structural features also influence activity. Nevertheless, peptides such as #6383 that are active in high ionic conditions would serve as good templates for the design of antimicrobials active in diseases where the salt concentrations have been reportedly elevated, including the airways of patients with cystic fibrosis [38,39]. In addition, the strategy employed herein to determine a protein's antimicrobially active regions should be extended to envelope proteins from other lytic viruses, and might be beneficial in elucidating structures that interact with membrane targets.

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