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Alarin but not its alternative-splicing form, GALP (Galanin-like peptide) has antimicrobial activity

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ABSTRACT

Alarin is an alternative-splicing form of GALP (galanin-like peptide). It shares only 5 conserved amino acids at the N-terminal region with GALP which is involved in a diverse range of normal brain functions. This study seeks to investigate whether alarin has additional functions due to its differences from GALP. Here, we have shown using a radial diffusion assay that alarin but not GALP inhibited the growth of *Escherichia coli* (strain ML-35). The conserved N-terminal region, however, remained essential for the antimicrobial activity of alarin as truncated peptides showed reduced killing effect. Moreover, alarin inhibited the growth of *E. coli* in a similar potency as human cathelicidin LL-37, a well-studied antimicrobial peptide. Electron microscopy further showed that alarin induced bacterial membrane blebbing but unlike LL-37, it did not cause hemolysis of erythrocytes. In addition, alarin is only active against the gram-negative bacteria, *E. coli* but not the gram-positive bacteria, *Staphylococcus aureus*. Thus, these data suggest that alarin has potentials as an antimicrobial and should be considered for the development in human therapeutics.

1. Introduction

In recent years, research in the field of novel antimicrobial peptides has intensified due to the need for newer effective antibiotics to overcome resistance issues of conventional antibiotics. Antimicrobial peptides, isolated from various bacteria, fungi, plants, invertebrates and vertebrates are important components of natural defenses of most living organisms [1]. These molecules have the added advantages of being very small in size, amphipathic and positively charged which allow them to bind and disrupt microbial membranes [2]. Some effective antimicrobial peptides reported thus far include human LL37 which is active against *Staphylococcus aureus* [3,4] and *Escherichia coli* [5] and β-defensins against *Enterococcus faecalis* and *Helicobacter pylori* [6–9].

The galarin family of neuropeptides consists of galanin, a galanin-like peptide (GALP) and a newer member called alarin. Human galanin consists of 30 amino acids and is encoded by the *GAL* gene. GALP consists of 60 amino acids and it can activate galanin receptors (GalRs) because residues 9–21 of GALP are identical to the first 13 amino acids of galanin [10]. Alarin consists of 25 amino acids and is derived from an alternative-splicing of the *GALP* gene that excludes exon 3. Its precursor consists of the signal sequence of the prepro-GALP, the first 5 amino acids of the mature GALP peptide and another 20 amino acids that are not identical to any other peptides. Unlike galanin and GALP, alarin does not bind to GalRs [11,12].

Galanin and GALP mRNAs are widely distributed in CNS as well as in the periphery in GIT, heart, dermis, epidermis, nerves, bone and joint tissues [13]. Alarin mRNA was first detected in ganglionic cells of neuroblastonic tumors [11] and it has a much wider CNS distribution than GALP [14]. It can also be found localized around blood vessels in the skin [11]. Galanin and GALP are involved in a diverse range of normal brain functions such as feeding and

Abbreviations: GALP, galanin-like peptide; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*.

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metabolism, osmotic regulation and water intake, nociception, neuronal injury, survival, regeneration, and neuroprotection, learning and memory and anxiety-related behaviors [13]. Like galanin and GALP, alarin is involved in feeding behavior [12,15,16], food intake and reproductive hormone secretion [12,16,17]. It also has vasoactive and anti-inflammatory activities [15]. The relationship between alarin and GALP is evolutionally and functionally interesting. Alarin shares only 5 conserved amino acids (APAHR) of the N-terminal region with GALP, while all other residues of the C-terminal region are very different. Hence, it is interesting to uncover other novel and specific functions of alarin arising from the differences in amino acid sequence from GALP. This paper investigates the antimicrobial activity of alarin and GALP against *E. coli* and *S. aureus*.

2. Materials and methods

2.1. Materials

Human LL-37, human GALP (galanin-like peptide), and human alarin were purchased from the Peptide Institute, Inc (Japan). Truncated peptides of human alarin [alarin(6–25), alarin(11–25) and alarin(16–25)] were synthesized and purified by IBL co. (Japan). Trypto-Soya Broth was purchased from Nissui (Japan).

2.2. Radial diffusion assay

The antibacterial activities of human LL-37, human GALP (galanin-like peptide), human alarin, and its truncated peptides were all evaluated by radial diffusion assay [18,19], a modification of the sensitive assay for antimicrobial peptides described by Lehrer and colleagues [20,21]. Briefly, to obtain bacteria growth in the mid-logarithmic-phase, an overnight bacterial culture was diluted 1:1000 in Tryptic soy broth (TSB) and was incubated at 37 °C until the optical density of the aliquot reached an absorbance value of 0.4 measured at 620 nm wavelength. The bacteria suspension was centrifuged at 900*g* for 10 min at 4 °C, washed once with ice-cold 10 mM sodium phosphate buffer (SPB; pH 7.4), and was re-suspended in ice-cold SPB. Based on previously prepared standards of the optical density at 620 nm wavelength, a volume containing 1×10^6 bacterial CFU was added to 10 ml of previously autoclaved 10 mM SPB containing 3.0 mg of TSB medium, 1% low-electroendosmosis-type agarose (Sigma), and 0.02% Tween 20.

After rapid dispersion of the bacteria, the agar was poured into an agar plate to form a uniform layer of approximately 2 mm deep and was punched with a 3-mm-diameter gel punch to make evenly spaced wells. Following the addition of 5 μl of antimicrobial agents to each well, the plates were incubated for 3 h at 37 °C and were then overlaid with 10 ml of sterile agar consisting of 6% TSB (double-strength solution) and 1% agarose. Antibacterial activity is identified as a clear zone around the well following incubation for 18–24 h at 37 °C and is measured as the difference in the diameters of the clear zone around the wells containing the antimicrobial peptides and buffer control (3 mm). These experiments were repeated four times.

2.3. Electron microscopy

The effect of LL-37 and alarin on the morphology of *E. coli* was evaluated by scanning electron microscopy. Briefly, the bacteria were treated for 2 h with 20 μM LL-37 or alarin on a MAS coated slide glass (Matsunami co., Japan), air-dried and then fixed with 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4). Specimens were further fixed in 1% osmium tetroxide and dehydrated through a graded ethanol series (50%, 70%, 80%, 95%, and 100%) and isopentyl acetate. The dehydrated specimens were then mounted on steel stubs, sputter-coated with a mixture of gold/palladium (Joel JFC-1100), and imaged using a Jeol JSM-840A electron microscope (Jeol, Japan) at 8 kV.

2.4. Hemolysis

Horse blood was purchased from Nihon Bio-Test co. (Japan). Horse blood erythrocytes were rinsed three times in phosphate buffer saline (PBS) by centrifugation at 1000*g* for 10 min and then re-suspended in 8% (vol/vol) PBS. Next, a 50 μl erythrocyte suspension was mixed with 50 μl LL-37, alarin, or GALP and incubated for 1 h at 37 °C. Tween 20 at 2% served as a positive control. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm wavelength and this is expressed as a percentage of the value of Tween 20-induced hemolysis.

2.5. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using the GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA), with p values < 0.05 considered as significant. Statistical significance is expressed as $^{*}p < 0.05$ from data ($n = 4$).

3. Results

The antimicrobial activity of alarin (200 pmol) was compared to its alternative splicing form, GALP and a well-studied antimicrobial peptide human cathelicidin, LL-37. Alarin inhibited the growth of *E. coli* (ML-35), with an increase of ~3.5 mm in the clear zone diameter in comparison to the buffer control (Fig. 1A and B). This inhibitory activity of alarin was also comparable to LL-37 at 200 pmol where its clear zone diameter had an increase of 4 mm when compared to the PBS buffer control (Fig. 1A and B). Its alternative-splicing form, GALP, however, did not show any antimicrobial activity against *E. coli* where the clear zone diameter remained the same as that of the buffer control (Fig. 1A and B). To examine further the dose–response of the antimicrobial activity of alarin; 25, 50, 100 and 200 pmol alarin were used for a radial diffusion assay. Alarin showed significant dose-dependent increases in antimicrobial activity against the gram-negative bacteria, *E. coli* (Fig. 1C). However, a dose up to 200 pmol alarin did not show any antimicrobial activity against the gram-positive bacteria, *S. aureus* (Fig. 1D). LL-37 in micromolar concentrations have been reported to induce bacterial membrane blebbing, leading to a leaky membrane and cell death in *Burkholderia pseudomallei* and *Burkholderia thailandensis* [22,23]. Using electron microscopy, we showed that 20 μM LL-37 induced extensive membrane blebbing on *E. coli* (Fig. 2B) compared to the buffer control (Fig. 2A). Membrane blebbing was also observed on *E. coli* incubated with alarin at 20 μM but is less extensive than those caused by LL-37 (Fig. 2C).

To explore the role of the N-terminal region of alarin, antimicrobial activity of truncated peptides of alarin, alarin (6–25), alarin (11–25), and alarin (16–25) were examined by a radial diffusion assay (Fig. 3). Alarin (6–25) and alarin (11–25) showed weak antimicrobial activity, while the antimicrobial activity of alarin (16–25) was completely abolished (Fig. 3). These results suggest that both the C-terminal and N-terminal regions of alarin are essential for its strongest killing effect.

It was reported that LL-37 has hemolytic activity on erythrocytes [22]. To determine the hemolytic activity of alarin and GALP, 10 μM of each peptide were incubated with horse erythrocytes for

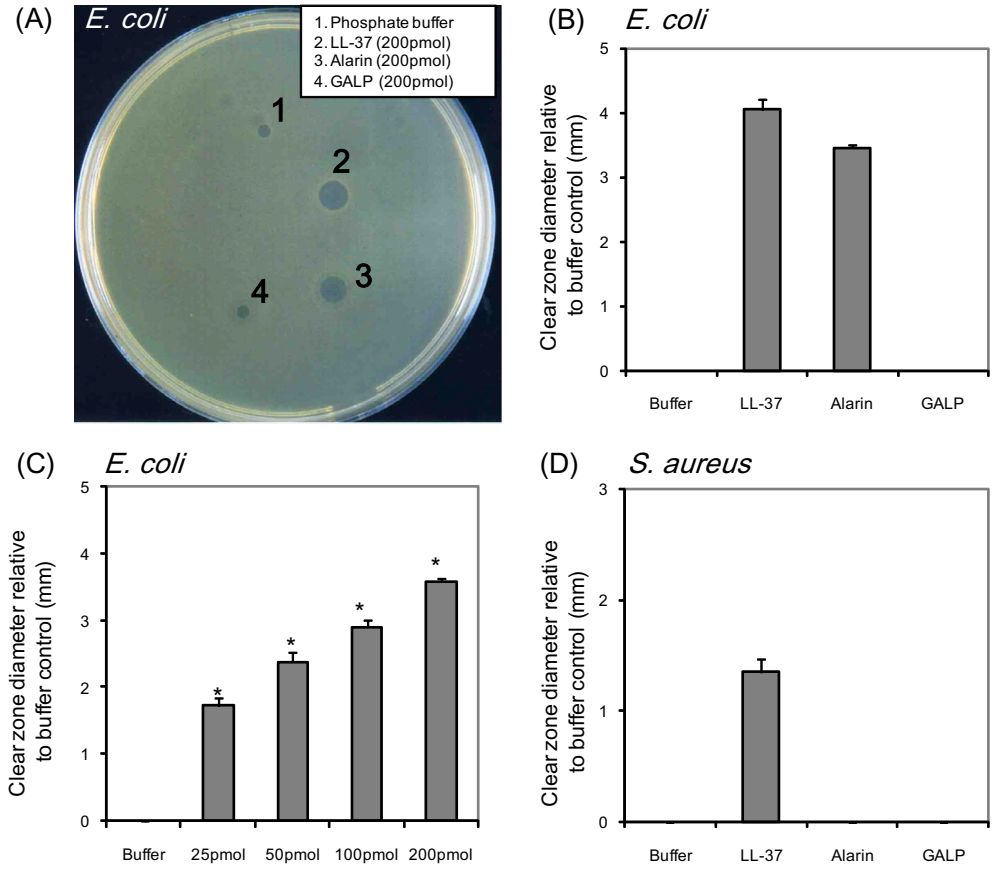

Fig. 1. Antimicrobial activities of LL-37, alarin and GALP against *E. coli* ATCC43827 (ML-35) and *S. aureus* ATCC25923 by radial diffusion assay. (A) Bacterial culture plate used for radial diffusion assay of LL-37, alarin and GALP. (B) Clear zone diameter (mm) depicting antimicrobial activity of LL-37, alarin and GALP at 200 pmol. (C) Dose-dependent antimicrobial activities of alarin against *E. coli* ATCC43827 (ML-35) and (D) dose-dependent antimicrobial activities of alarin against *S. aureus* ATCC25923. Zone diameter (mm) represents the antimicrobial activity of various concentrations of alarin relative to buffer control. An increase in the zone size caused by alarin is obtained by subtracting the diameter of the buffer control well (3 mm). Statistical significance is expressed as $^{*}p < 0.05$ (one-way ANOVA with Dunnett's Multiple Comparison Test) versus buffer control. Each bar represents the mean ± SD of data ($n = 4$).

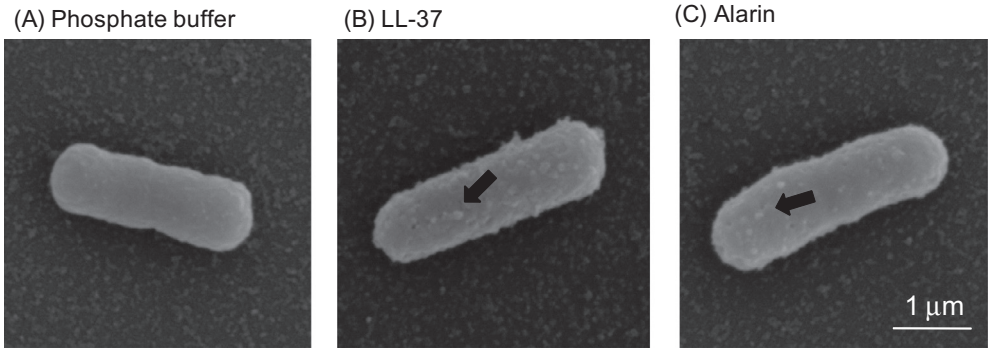

Fig. 2. Electron micrographs of *E. coli* ATCC43827 (ML-35) cells. (A) *E. coli* incubated with phosphate buffer showed normal surface of membrane; bacteria incubated with (B) 20 μM LL-37 and (C) 20 μM alarin showed blebs (black arrows) on the membranes. Scale bar, 1 μM.

1 h at 37 °C. LL-37 showed hemolytic activity to horse erythrocytes but not to alarin and GALP (Fig. 4).

4. Discussion

Alarin is differentiated from its alternative splicing form, galanin-like peptide (GALP) at the C-terminal region. Although alarin shares some similar functions with GALP, it is unknown if alarin has other more specific roles. Antimicrobial peptides are characterized as amphiphilic molecules and consist of basic amino acids. Because the C-terminal region of alarin contains some basic amino acids such as Arg and Lys, we postulated that alarin may have antimicrobial activity.

Results from the present study showed that alarin has antimicrobial activity but unlike the human cathelicidin, LL-37 which has a broader spectrum of antimicrobial activity, the antimicrobial activity of alarin is limited to the gram-negative bacteria, *E. coli*. This is not surprising as different susceptibilities of bacterial species against a particular peptide have been observed and this can be attributed to various reasons such as bacterial cell surface

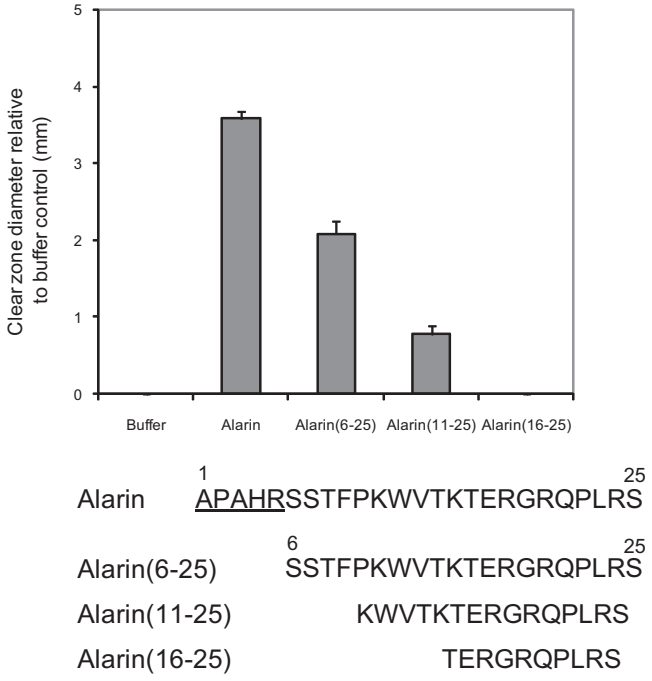

Fig. 3. Antimicrobial activities of alarin and its truncated peptides against *E. coli* ATCC43827 (ML-35) by radial diffusion assay. Clear zone diameter (mm) depicting antimicrobial activity of alarin and its truncated peptides at 200 pmol (upper panel) and amino acid sequences of the truncated peptides (lower panel). Conserved sequences of alarin and GALP are underlined.

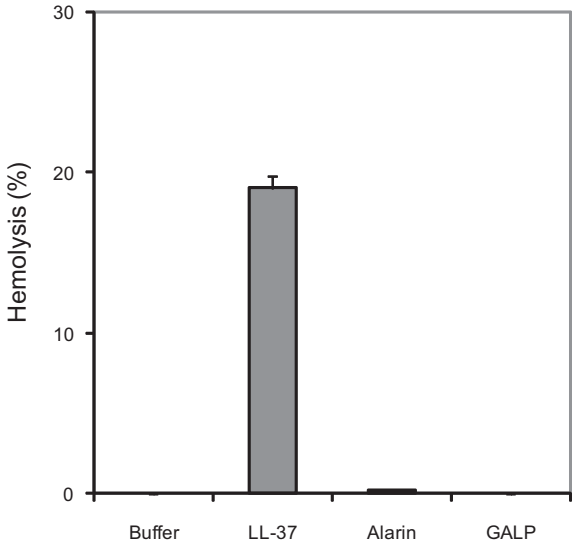

Fig. 4. Hemolytic activity of LL-37, alarin and GALP. Horse erythrocytes were incubated with each peptide (10 μM) for 1 h at 37 °C. Hemolytic activity is a measurement of the percentage of Tween 20-induced hemolysis. LL-37 showed hemolytic activity against horse erythrocytes but not alarin and GALP.

charge [24], genetic diversity [25], host ionic conditions [26] and peptide-to-lipid ratios [27]. The antimicrobial activity of alarin, however, is specific as alarin exhibited dose-dependent inhibition of the growth of *E. coli* and demonstrated potency equal to that of LL-37. We further showed by electron microscopy that alarin is capable of causing bacterial membrane blebbing, suggesting that membrane disruption could be one of its killing mechanisms. An effective antimicrobial peptide should be cytolytic and cell-penetrating but not hemolytic [28]. In general, hydrophobic interactions with eukaryotic cell membranes increase hemolytic activity [29]. Our results showed that alarin does not induce hemolysis of erythrocytes, suggesting that its hydrophobic interactions with the erythrocyte membranes are minimal. Hence, alarin can fulfill the desirable features of an effective antimicrobial peptide.

More interestingly, GALP, an alternative splicing form of alarin demonstrated no antimicrobial activity. However, deletion of the conserved amino acid sequence (APAHR) at the N-terminal region of alarin reduced its antimicrobial activity suggesting that APAHR remain essential for its antimicrobial activity. The C-terminal original sequence of alarin (6–25), which contains some basic amino acids are important for the antimicrobial activity of alarin as successive deletions reduced and eventually abrogated the antimicrobial activity of alarin. These results, hence, suggest that even though the C-terminal region of alarin confers its antimicrobial activity, the conserved N-terminal of alarin and GALP remains crucial for its maximum antimicrobial activity.

LL-37, defensins and histatins are some of the well-studied antimicrobial peptides which contain 37, 29–42 and ~32 amino acids, respectively [30]. With the discovery of its antimicrobial activity, alarin with a length of 25 amino acids is now one of the shortest antimicrobial peptides known. Results from this report show that alarin has antimicrobial potentials and should be considered for the development as a human therapeutic.

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