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Human Cathelicidin, LL-37 a potential antiviral therapeutic for Rift Valley Fever Virus in Egypt

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Abstract

Rift Valley Fever Virus (RVFV) is an arbovirus that circulates among animals and can be transmitted to humans. Mosquitoes are the primary vectors that allow RVFV to spread vertically and horizontally. Egypt was exposed to frequent outbreaks with devastating economic consequences. RVFV has a high incidence of worldwide dissemination and no specific vaccine or therapy. Linear Human Cathelicidin (LL-37), is a natural antimicrobial peptide with antiviral activity against numerous viruses. In addition to immunomodulatory effects, LL-37 directly influences viral encapsulation. This study aimed to evaluate the antiviral activity of LL-37 against RVFV in vitro. The post-entry and pre-incubation of LL-37 within Vero cells were assessed in the absence and presence of RVFV. LL-37 activity was assessed using a TCID50 endpoint test, qRT-PCR, and a western blot. When genomic RVFV was quantified, it resulted in a 48% direct inactivation of the viral envelope and a 36% reduction when the virus was pre-incubated with LL-37 before infection. LL-37 decreased viral infection by 75% and protected Vero cells against RVFV infection by 47% at a 1.25 µg/ml dosage. These findings imply that LL-37 exerts antiviral efficacy against RVFV by restricting virus entrance through direct disruption of the virus envelope and indirectly by triggering an immunological response. The effect of LL-37 is time-dependent. As a result, LL-37 may provide rapid and affordable therapies for RVFV infection in Egypt, both during outbreaks and as a preventive strategy.

Keywords: Arboviruses, Rift Valley Fever Virus, Human Cathelicidin, Egypt, *In Vitro*, RT-PCR, Western blot.

1. Introduction

Arthropod-borne viruses' potential to cause significant illness in the number of hosts has lately increased through a variety of pathways and mechanisms. Arboviruses such as West Nile virus (WNV), Japanese encephalitis virus (JEV), and (RVFV) are all carried by mosquito species such as *Aedes aegypti*, *Aedes albopictus, and Culex pipiens* [1]. Because RVFV is a very hazardous emerging disease and is considered a biological weapon, the World Health Organization added RVFV to its list of extremely dangerous emerging pathogens in 2015.

RVFV belongs to the *Phenuiviridae* family, genus *Phlebovirus.* The infectious virion was made up of three segments: M segment encodes two glycoproteins Gn and Gc. The L segment encodes RNA-dependent-RNA polymerase. The N and NS proteins are encoded via an ambisense display in the S segment. The ribonucleoprotein complex is made up of viral RNA encapsidated by nucleoprotein and RNA-dependent RNA polymerase [2]. The M and S segments each encode non–structure proteins NSm and NSs, respectively [3]. Several epizootics have been reported in Egypt, Saudi Arabia, Yemen, Sudan, Kenya, Tanzania, Madagascar, Somalia, Mauritania, and Senegal [4]. Severe epidemics occurred in Turkey and Iran [5, 6]. Severe diseases have lately struck Libya [7]. In humans, infections are acute with a 1-2 % fatality rate and the most common complications include encephalitis, hemorrhagic fever, liver disorders, blindness, and death [8, 9]. RVFV was recently identified as a possible cause of human abortion [10]. RVFV propagation is aided by aerosol transfer and the eating of infected animals or animal products. RVFV survived on mosquito eggs during inter-epizootics due to the existence of RVFV maintenance and amplifying vectors[11]. Worldwide trade, travel, climatic change, and uncontrolled vectors are all considered risk factors for RVFV transmission to new populations such as those in America and Europe, where hosts are vulnerable to mosquito bites [12, 13].

Several outbreaks have been documented in Egypt resulting in devastating morbidity in human and livestock losses [14]. Severe illnesses in cattle and sheep have been observed with substantial fatality rates in young animals. An abortion storm is the main sign of an outbreak explosion [11]. Unfortunately, the locally produced Smithburn

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RVF vaccine failed to introduce immunization against RVFV infection [15].

Natural antimicrobial peptides are produced by both prokaryotes and eukaryotes as part of their innate immune response to effectively protect against bacteria, fungi, viruses, and protozoa [16]. Small antimicrobial peptides (AMPs) have selective action by targeting a wide range of organisms without harming mammalian cells. This is a unique property of AMPs due to their high cholesterol levels and low anionic charge [17], in addition to rapid killing even after initial contact with the membrane [18]. AMPs are a novel and diverse collection of effective molecules categorized according to their amino acids and structure [19, 20]. Cathelicidin is a significant human peptide that may be easily integrated into biological membranes due to the amphipathic α-helical structure [21]. LL-37 exhibits direct microbicidal, immunomodulatory, and inflammatory activity [22]. LL-37 is a solo human cathelicidin that was first identified and isolated from neutrophils in 1995 [23, 24], with the N-terminal domain responsible for proteolytic resistance and chemotaxis activity and the C-terminal domain producing mature peptide upon proteolytic cleavage and mediating antibacterial, antiviral and anticancer activity [25].

The ease with which LL-37 may be manufactured and manipulated makes it one of the most promising therapies. Furthermore, peptide-based therapy has a low molecular weight, fewer adverse effects; is swiftly eliminated after delivery, and is a cost-effective therapeutic [26]. We aimed to investigate the antiviral activity of LL-37 against an RVFV cell culture-adapted strain isolated from an Egyptian sheep at various infection points so that it could be used to treat RVFV infections during outbreaks, benefiting both economics and health.

The LL-37 effect was examined to discover the likely mechanism of action as a starting point for treating and preventing RVFV infections and transmission in Egypt, which may be applied to many other arboviruses.

2. Materials and Methods

2.1. Cells, virus propagation, and titration

Vero E6 cells from African Green Monkeys' kidney cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) from VACSERA, Egypt, with 10% fetal bovine serum (FBS) added as a dietary supplement at 37°C with 5% CO_2. The RVFV cell culture adapted strain from an Egyptian sheep gifted by Dr. Eman Amen, working at VACSERA antiviral research Centre, was propagated on 80% confluent Vero cells for 48 hours to demonstrate the maximum CPE effect (CPE+5). Amounts of the virus were gathered, separated, and kept at -80°C. A ten-fold serial dilution of RVFV was titrated on 80–90% confluent Vero cells using RVFV at MOI of 2 and a 96-well plate. One hour of viral adsorption was permitted. The inoculum was removed, washed with PBS, and a new medium was added. After the cells had been cultured for 48 hours and the CPE effect had been recorded, The TCID 50 was calculated by using the Reed-Muench equation [27].

2.2. Peptide synthesis

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), and scrambled LL-37 (GLKLRFEFSKIKGEFLKTPEVRDIKLKDNRISVQR), reported by [28], were purchased from Genescript (USA). Peptides were synthesized using the Fmoc solid-phase peptide method**.** Scrambling LL-37 was chosen for its size, overall hydrophilicity, and charge resemblance to LL-37, which has the same amino acid content but sequenced differently. Peptides were purified using preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) with ≥ 95 % purity validated by analytical RP-HPLC, and mass spectrometry was used to confirm the masses. The lyophilized powder was reconstituted in endotoxin-free water at a stock concentration of 200 µg/ml and tested for endotoxin using a Limulus Amebocyte Lysate Gel-Clot Endotoxin Kit (Thermo Scientific, UK).

2.3. Viability assay

The colorimetric MTT test was used to assess the maximum non-toxic concentration (MNTC) of LL-37 as a therapeutic that may be administered safely in the Vero cell line. Vero cells were seeded for 24 hours before being treated with LL-37 at two-fold serial dilution. After 24 hours, cells were evaluated for morphological change, washed twice with 200 µl PBS (MP Biomedicals, USA), and incubated for 4 hours in the dark with 50 µl MTT dye (Sigma-Aldrich, Inc. St Loui, MO, USA). To dissolve the formazan crystals, dimethyl sulfoxide (DMSO) was utilized (ADVENT, India), and the optical density (OD) of the surviving cells and control untreated cells were evaluated using a microtiter plate reader HIDEX multimode equipment at 570 nm [29, 30]. The viability % of LL-37 was determined. The colorimetric assay was done in triplicate. Under the same experimental settings, scrambling LL-37 was tested as a negative control.

2.4. Direct inactivation of LL-37 on Vero cell line

An equivalent amount of cell culture-adapted strain of RVFV at MOI=2 was combined once with 5 µg/ml of LL-37 and once with 5 µg/ml of scrambled LL-37 as the negative control, followed by an immediate ten-fold serial dilution in serum-free medium on Vero cells in 96-well plates to test the direct interaction between RVFV and LL-37. After 2 hours, infected Vero cells were washed twice with PBS (MP Biomedicals, USA) and incubated for 24 and 48 hours in a fresh serum-free medium. The CPE effect was assessed daily. In addition to cell controls with medium alone, cells infected solely with RVFV were used as positive viral controls.

To maximize the assessment of LL-37's direct impact on viral entry, 10 µg/ml LL-37 was combined with a concentrated RVFV concentration that produced 100% cell death, and scrambled LL-37 was employed as the negative control. A Virus-positive control, as well as a cell control, were employed. These experiments were employed in three biological replicates. The viral inhibition index (VII) was calculated using the formula VII= B - A, where B is the calculated virus titer in the untreated virus control, and A is the calculated virus titer after the peptide treatment [31]. The difference in viral titer before and after treatment was determined, and the depletion rate % was estimated as virus titer loss. Using the equation below, the results were also reported as a percentage of inhibition [32].

$$PI = 1 - \frac{anti - logarithm\ of\ the\ test\ sample}{anti - logarithm\ of\ control} x100$$

After 24 hours, the supernatant was collected for qRT-PCR analysis to determine the reduction in RVFV mRNA

replication, and samples were collected after 48 hours for western blot examination.

2.5. The viricidal activity of LL-37 on Vero cells

Vero cells were treated with RVFV at MOI=2 equivalently mixed with 5 µg/ml of LL-37, and 5 µg/ml of scrambled LL-37 as the negative control. The mixture was incubated for 1 hour at 37 °C to imitate physiological conditions, followed by ten-fold serial dilution in serum-free medium. Those infected with RVFV at MOI=2 were used as positive viral controls in addition to cells infected with medium alone. Vero cells infected with this mixture were washed twice with PBS (MP Biomedicals, USA) after 2 hours and grown in a fresh serum-free medium for 24 and 48 hours with daily CPE assessment. To maximize the assessment of LL-37 inhibitory activity, 10 µg/ml LL-37 was combined with a concentrated RVFV titer that induced 100% cell death, and scrambled LL-37 was employed as the negative control. A virus-positive control as well as cell control were used. These experiments were employed in three biological replicates. The viral inhibition index (VII) was calculated [31]. The difference in viral titer before and after treatment was determined, and the depletion rate % was estimated as virus titer loss. The results were also provided as a percentage of inhibition [32]. After 24 hours, the supernatant was collected for qRT-PCR analysis to determine suppression of RVFV mRNA replication, and samples were collected after 48 hours for western blot assay.

2.6. Prophylactic activity of LL-37 against RVFV in Vitro

The ability of LL-37 to be maintained and internalized inside Vero cells to mediate prophylactic activity was assessed. Previously seeded Vero cells in 96-well plates were pre-incubated with LL-37 at concentrations of 1.25, 0.625 µg/ml for 4 hours, then washed twice with PBS and infected with RVFV at MOI=2 for 3 hours, with gentle shaking every 15 minutes to ensure uniform viral dispersion. The inoculum was removed and replaced with fresh medium, incubated for 24 and 48 hours at 37°C. Scrambling LL-37 was a negative control, while viral and uninfected cells were virus and cell controls respectively. Cells are examined and inspected daily for morphological change. CPE was detected and recorded. The viral inhibition index (VII) was calculated [31]. The difference in viral titer before and after treatment was determined and the depletion rate percentage was measured as virus titer loss. The findings were expressed as a percentage of inhibition [32]. After 24 hours, the supernatant was collected for qRT-PCR analysis to determine suppression of RVFV mRNA replication, and samples were collected after 48 hours for the western blot test.

2.7. Antiviral activity of LL-37

The post-entry effect of LL-37 was studied in 96-well plates using a confluent monolayer of Vero cells infected with RVFV at MOI=2 for 90 minutes, with gentle shaking every 15 minutes to ensure uniform distribution of infection. The inoculum was removed and different concentrations of LL-37 1.25, 0.625 µg/ml were added to cells. As a negative control, scrambling LL-37 was utilized, and viral and non-infected cells were used as virus and cell controls, respectively. As CPE is found and recorded, cells were monitored daily and scanned for any morphological change. The viral inhibition index (VII) was calculated [31]. The difference in viral titer before and after treatment was determined, and the depletion rate % was estimated as virus titer loss. The results were also provided as a percentage of inhibition [32]. After 24 hours, the supernatant was collected for qRT-PCR analysis to determine the reduction in RVFV mRNA replication, and samples were collected after 48 hours for western blot examination.

2.8. Virus titration

The reduction in virus titer following several LL-37 treatments was used to determine LL-37's potential antiviral effectiveness against RVFV at various stages of the virus life cycle. TCID50 endpoint test is a quantitative test designed to evaluate the morphological changes caused by RVFV infection and represents the CPE effect. Supernatants from LL-37 treated cells were ten-fold serially diluted in 96-well plates and added to wells in triplicate. After 48 hours, the Reed Muench equation calculated the TCID50 values of tested LL-37 and untreated viral control [27]. The difference in TCID50 between the various LL-37 treatments and the RVFV control was shown as a log reduction. Additionally, the percentage of inhibition and depletion rate % were determined.

2.9. Quantification of RVFV by Real-Time Reverse-Transcriptase Polymerase Chain Reaction (q RT-PCR)

The antiviral activity of LL-37 against RVFV at different stages and infection points was evaluated using relative quantitative RT-PCR. Viral RNA was extracted from the supernatant using the Direct-zol RNA Miniprep Plus kit (ZYMO RESEARCH CORP., USA) following the manufacturer's instructions after the virus had infected the cells for 24 hours. The 50 µL of elution buffer used to extract the RNA was then kept at -80°C until usage. Quantitative RT-PCR utilizing SYBR green dye was carried out using the SuperScript IV One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) procedure using the forward primer 5′- GAAGGCAAAGCAACTGTGGA-3′ and the reverse primer 5′- AAGCCACTCACTCAAGACGA-3′. Primers were created for amplifying 147 nucleotides of the conserved N protein of RVFV using the primer3 online tool. Relative quantification (RQ) (2-ΔΔCT), is automatically calculated by the PCR instrument software package.

The following reaction conditions were used, using the applied Biosystem: reverse transcriptase activation at 55 °C for 10 minutes, reverse transcriptase enzyme inactivation at 95 °C for 2 minutes, 40 times 95 °C for 10 seconds, 55 °C for 15 seconds, and 72 °C for 30 seconds, and a final extension step at 72 °C for 5 minutes (Step One Applied Biosystem, Foster City, USA).

2.10. Western blotting

Vero cells were subjected to LL-37 treatment in a variety of applications before being rinsed with new PBS pH 7.4 and then lysed in lysis buffer (PBS pH 7.4 with 1% TritonX-100 and 1X protease inhibitor). SDS-PAGE analysis was carried out by utilizing a 20% denaturing gel and transferring the intracellular lysates and immunoprecipitated materials to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). A measurement of the pH revealed a value of 6.8. Each mixture was heated at 95 °C for 5 minutes before loading on polyacrylamide gel

electrophoresis to ensure protein denaturation. Primary mouse anti-RVFV N (R3-ID8; 1:2,000) (BEIResources, NIH, Manassas, VA, USA) was conjugated with the blots overnights. Anti-beta-actin monoclonal antibodies (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) as beta-actin was used as a loading control after 1 hour of blocking with 0.1% Tween 20 and 3% bovine serum album. The membranes were then cleaned and incubated for 1 hour with a goat anti-mouse-HRP conjugated secondary antibody (1:5,000). Specific reactivity was determined using an enhanced chemiluminescent (ECL) detection system (Santa Cruz Biotechnology, Dallas, TX, USA) (GE Healthcare, Buckinghamshire, UK).

2.11. Statistical analysis

Graph pad Prism 9 was used to evaluate statistical significance to examine the variation in response values between LL-37 treated and untreated controls. A one-way analysis of variance (ANOVA) was utilized to establish the significant difference between the group means of different LL-37 tests, and Bonferroni post hoc comparisons followed a one-way analysis of variance (ANOVA). A statistically significant P - value should be ≤ 0.001.

3. Results

3.1. Viability assessment

Visual examination and tracking of morphological changes in the Vero cell line after treatment with various dosages of LL-37 and scrambling LL-37 revealed that viability varied. The number of metabolically active Vero cells was counted. The maximum non-toxic LL-37 concentration that can be utilized was calculated (Fig. 1). In Vero cells, the computed IC 50% as the half-maximum response at 50 µg/ml as a starting dilution was 34.29 and 31.87 for LL-37 and scrambled LL-37, respectively. To ensure that LL-37 was sequence-specific as a therapy, scrambling LL-37 was utilized as a negative control.

3.2. LL-37 directly inhibited RVFV particles

To determine the maximum inhibitory impact of LL-37 against RVFV, it was evaluated at 5 µg/ml against RVFV TCID50 virus titer and at 10 µg/ml against a concentrated viral stock. Using a TCID50 endpoint assay, LL-37 at 5 µg/ml efficiently reduced RVFV titer resulting in a 2 log reduction after 24 hours and a 1.33 log reduction after 48 hours (Fig. 2a). After 24 and 48 hours, LL-37 at 10 µg/ml reduced the concentrated viral titer by one and 0.5 logs, respectively (Fig. 2b). There was no difference between RVFV-infected viral control cells and Scrambling LL-37-treated cells, indicating that LL-37's inhibitory activity against RVFV in the Vero cell line is sequence-specific. RVFV N mRNA expression was measured by qRT-PCR after 24 hours of viral infection. The RQ of viral expression in LL-37-treated cells was computed and compared to untreated cells and standardized to a reference control. When LL-37 was pre-incubated with TCID50 virus titer, it directly inhibited viral entrance to cells by 48.4% at 2.5 µg/ml, and even at low concentrations of 0.25 and 0.025 µg/ml, it inhibited RVFV by 44% and 40%, respectively ($p < 0.001$) (Fig.2c). LL-37 consistently suppressed the concentrated viral stock by 46 % and 41 % at dosages of 0.5 and 0.05 µg/ml ($p< 0.001$) (Fig. 2d). These findings demonstrated the specificity of LL-37 sequence activity against RVFV and revealed direct contact as the primary mode of action of LL-37. After 48 hours of RVFV infection, whole-cell lysates were examined by western immunoblot (Fig. 5), demonstrating that LL-37 may disrupt the RVFV envelope for an extended period. These findings demonstrated that the efficacy of LL-37 against RVFV declined with time. Time and dose have an inverse relationship with LL-37.

3.3. Simultaneous activity of LL-37 against RVFV

To determine whether LL-37's antiviral activity against RVFV required prior interaction between virus particles

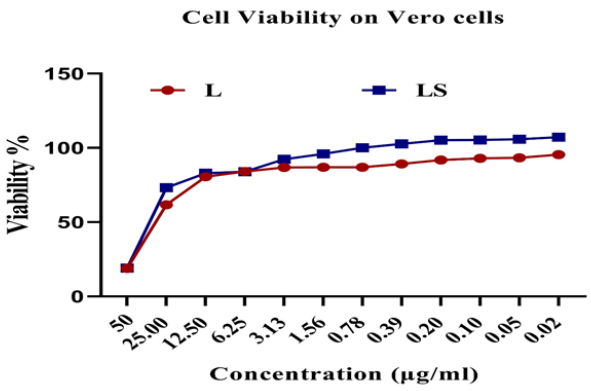

Fig. 1. Viability assay of LL-37, and Scrambling LL-37 by MTT assay on Vero cell line.

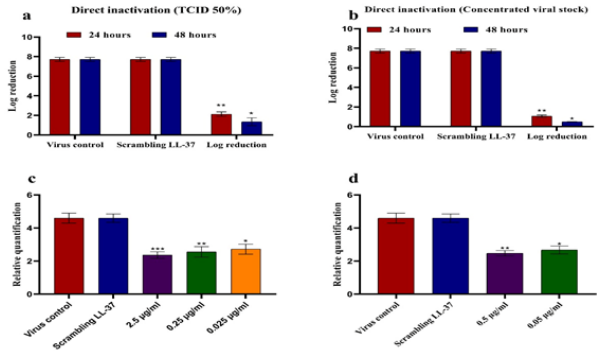

Fig. 2. LL-37 direct inhibitory effect on RVFV particles. (a) Represents the log decrease caused by LL-37 at 5 µg/ml pre-mixed with TCID50 of RVFV at MOI=2, and evaluated by endpoint assay. While (b) represents the log decrease produced by 10 µg/ml LL-37 pre-mixed with a concentrated viral stock of RVFV at MOI=2, and assessed by endpoint assay. Scrambling LL-37 was pre-mixed with RVFV as a control peptide * $P < 0.001$ in comparison to control groups. ** $P < 0.001$ in comparison to groups treated with LL-37 for 24 hours. No statistically significant difference existed between untreated controls and cells treated with scrambled LL-37. (c) Relative Quantification RT-PCR of LL-37 pre-mixed with TCID50 of RVFV administered to Vero cells in triplicate and extracted 24 hours later. *$P < 0.001$ in comparison to the corresponding control groups. ** $P < 0.001$ in comparison to a group treated with LL-37 at a concentration of 2.5 µg/ml. *** $P < 0.001$ compared to a group treated with LL-37 at a 0.25 µg/ml concentration. (d) LL-37 pre-mixed with concentrated RVFV was administered to Vero cells in triplicate and extracted 24 hours later. * $P < 0.001$ in comparison to the corresponding control groups. ** $P < 0.001$ in comparison to group treated with LL-37 at concentration 0.5 µg/ml. Under the same experimental circumstances, there was no significant difference between RVFV control cells and cells infected with RVFV and scrambling LL-37.

and LL-37 under the same physiological conditions during infections, simultaneous exposure to the virus and LL-37 mixture after a 1-hour incubation at 37 °C resulted in a significant reduction ($p < 0.001$) in infectivity. When the TCID 50 endpoint assay measured the virus titer after 24 and 48 hours, LL-37 resulted in 1.5 and 0.5 log decreases, respectively (Fig.3a). LL-37 induced a 0.7 and 0.5 log reduction in concentrated viral stock after 24 and 48 hours, respectively (Fig. 3b). The number of infectious virions in treated Vero cells with LL-37 was compared to non-treated cells and standardized to a reference control using qRT-PCR. Simultaneous exposure of LL-37 with TCID 50 virus titer directly inhibited virus entry to cells by 36 % at concentrations of 2.5 µg/ml. Even at small concentrations such as 0.25 and 0.025 µg/ml, LL-37 inhibited RVFV by 33.7 % and 31 % respectively ($p< 0.001$) compared to virus control (Fig.3d). Compared to virus control, LL-37 consistently suppressed the concentrated virus by 33% and 31% at dosages of 0.5 and 0.05µg/ml ($p< 0.001$) (Fig.3c). These data strengthen the hypothesis that LL-37's primary mode of action is direct interference with the viral envelope. A scrambled LL-37 had no antiviral activity. After 48 hours of RVFV infection, immunoblot examined whole-cell lysates (Fig. 5), and RVFV N protein was expressed. LL-37 is inversely related to time and dosage.

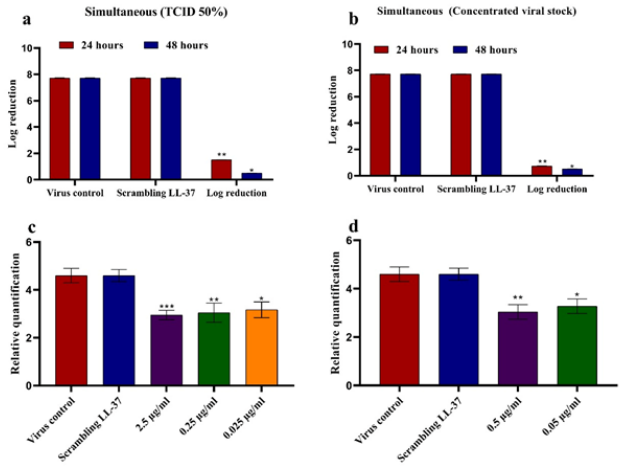

Fig 3. The viricidal activity of LL-37 against RVFV particles. (a) Represents the log reduction caused by LL-37 at 5 µg/ml pre-incubated with TCID50 of RVFV at MOI=2 for 1 hour at 37°C, supernatant was extracted at different time intervals to be determined by TCID50 endpoint assay. While (b) Represents the log reduction caused by LL-37 at 10 µg/ml pre-incubated with a concentrated viral stock of RVFV at MOI=2, for 1 hour at 37°C, the supernatant was extracted at different time intervals to be determined by TCID50 endpoint assay. * $P < 0.001$ in comparison to control groups. * * $P < 0.001$ in comparison to groups treated with LL-37 for 24 hours. No significant difference existed between non-treated controls and cells treated with scrambling LL-37. (c) Relative Quantification of LL-37's Simultaneous effect on RVFV N mRNA expression by RT-PCR, LL-37 was pre-mixed with TCID50 of RVFV and ten-fold serially diluted after a 1-hour incubation at 37°C, added to Vero cells in triplicates, and collected 24 hours later. *$P < 0.001$ in comparison to the corresponding control groups. ** $P < 0.001$ in comparison to a group treated with LL-37 at a concentration of 2.5 µg/ml. *** $P < 0.001$ compared to a group treated with LL-37 at a 0.25 µg/ml concentration. (d) qRT-PCR determination of the simultaneous effect of pre-mixed LL-37 with concentrated RVFV at MOI=2, incubated for 1 hour, applied to Vero cells in triplicates after ten-fold serial dilution, and harvested 24 hours later. * $P < 0.001$ in comparison to the corresponding control groups. ** $P < 0.001$ in comparison to group treated with LL-37 at concentration 0.5 µg/ml. Under the same experimental circumstances, there was no significant difference between RVFV control cells and cells infected with RVFV and scrambling LL-37.

3.4. The protective effect of LL-37 against cell death

Pre-treatment of Vero cells with various dosages of LL-37 may protect against RVFV infection and viral dissemination. Delaying RVFV infection up to 4 hours after LL-37 treatment and after several washing steps indicated the capacity of LL-37 to be maintained in cells, with considerable protection ($P <0.001$). When LL-37 was pre-incubated with cells for 4 hours before viral infection and the supernatant was collected after 24 to measure the decrease in virus titer by TCID50 endpoint assay. LL-37 at concentrations of 1.25 µg/ml and 0.6 µg/ml resulted in 0.5 and 0.3 log reduction, respectively ($P <0.001$) when compared to non-treated control groups (Fig. 4a). Relative quantification of RVFV N mRNA expression by qRT-PCR in pre-incubated cells with LL-37 indicated a significant reduction ($P <0.001$) in virus replication in a dose-dependent manner with about 47 % reduction at 1.25 µg / ml, and about 37 % reduction at 0.6 µg / ml when compared to non-treated

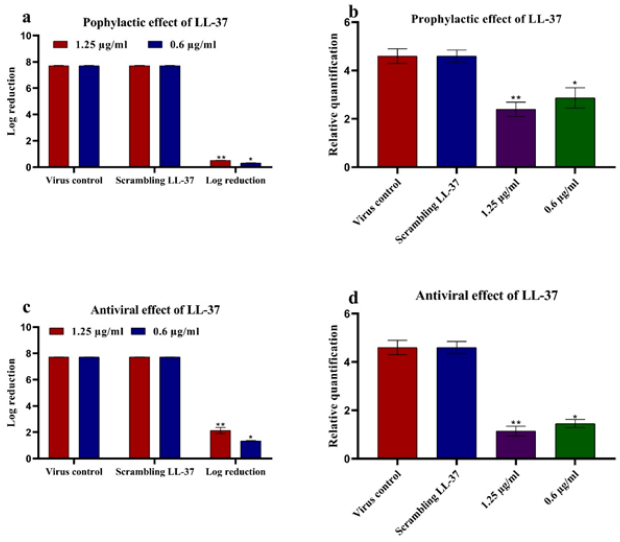

Fig 4. LL-37 protective and antiviral effect against RVFV particles on Vero cell line. (a) Illustration of the protective effect of LL-37 against TCID 50 of RVFV at MOI=2, as a substantial reduction in the virus titer was detected in a dose-dependent manner by TCID50 endpoint assay after 24 hours of RVFV infection. * $P < 0.001$ in comparison to control groups. ** $P < 0.001$ in comparison to groups treated with LL-37 at 1.25 µg/ml. (b) qRT-PCR measured the prophylactic effect mediated by LL-37 in a dose-dependent manner when compared to non-treated control groups. * $P < 0.001$ in comparison to control groups. ** $P < 0.001$ in comparison to groups treated with LL-37 at 1.25 µg/ml. (c) The antiviral impact of LL-37 against TCID 50 RVFV at MOI=2, as a substantial decrease in virus titer was assessed dose-dependently by TCID50 endpoint test after 24 hours of virus infection.* $P < 0.001$ in comparison to control groups. ** $P < 0.001$ in comparison to groups treated with LL-37 at 1.25 µg/ml. (d) Antiviral activity mediated by LL-37 was assessed by qRT-PCR and compared to non-treated control groups. * $P < 0.001$ in comparison to control groups. ** $P < 0.001$ in comparison to groups treated with LL-37 at 1.25 µg/ml. There was no discernible difference between untreated controls and LL-37 scrambled cells.

control groups (Fig. 4b). These findings support the hypothesis that LL-37 can promote protective antiviral activity in Vero cells. The whole-cell lysates were analyzed by western immunoblot after 48 hours of RVFV infection (Fig. 5), with the expression of RVFV N protein showing the incapacity of LL-37 to provide long-term protection against RVFV infection.

3.5. Antiviral effect of LL-37 against RVFV

The capacity of LL-37 to suppress the release of new RVFV infectious particles was assessed by challenging cells with TCID 50 RVFV at MOI =2 and then treating cells with various doses of LL-37. The infectivity of the supernatant collected after 24 hours was determined by TCID50 endpoint test as LL-37 at 1.25 µg/ml and 0.6 µg/ml concentrations, significantly caused 2 and 1.3 log reduction, respectively ($P < 0.001$) (Fig.4c). When compared to non-treated control groups, relative quantification of RVFV N mRNA expression in response to antiviral treatment with LL-37 revealed a significant ($P < 0.001$) reduction in virus replication in a dose-dependent manner, with an approximately 75% reduction in viral mRNA expression obtained at1.25 µg/ml, and approximately 68% reduction obtained at 0.6 µg/ml (Fig. 4d). The whole-cell lysates were examined by western immunoblot after 48 hours of RVFV infection (Fig. 5). The lack of RVFV N protein expression was found as LL-37 showed unique antiviral efficacy against RVFV infections and suppressed the formation of new virus particles.

4. Discussion

RVFV is a serious growing intimidation and etiological agent for humans and livestock which is transmitted by several vectors and exacerbated by animal commerce in particular. Camels influence transmission and introduce the virus to new populations [33]. RVFV was proclaimed as a possible bioterror virus in 2010 [34]. Ribavirin, Favipiravir, and benzavir-1 were tested as antiviral candidates against RVFV infection in cell-based assays and animal models but were not authorized for use [35]. Egypt suffered from many outbreaks. The most severe occurred in 1977 followed by outbreaks in 1993, 1994, 1997, and 2003 [14]. Camel exports to repay the Egyptian market demand continue to introduce RVFV to Egypt. Warm climate conditions, the endemicity of numerous mosquitoes that transmit the virus in Egypt, as well as a lack of immunization, contribute to an increase in RVFV infections [36]. Some circulating antibodies were detected in the absence of a surveillance system or diagnostic instruments, indicating the possibility of future outbreaks. [37].

There are two main families of Antimicrobial peptides (AMPs) in mammalian cells as a part of the neutral immune response [38], defensin and cathelicidin. In humans, there are multiple defensin genes and one cathelicidin gene that is mainly found in neutrophils [39]. The sole human cathelicidin (LL-37) helical structure induces membrane toxicity by electrostatic force and creates a net positive charge, resulting in the quick death of numerous species [40]. Therefore, the competence of LL-37 as a prospective antiviral therapy against RVFV as one member of arboviruses was determined to find the possible mechanism of action and antiviral potentialities. Furthermore, to distinguish between any non-specific impact and to guarantee that the antiviral activity was related to the LL-37 amino

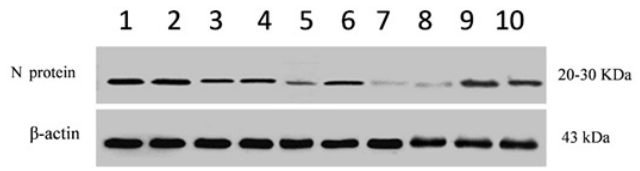

Fig. 5. Western blot analysis of RVFV Nucleoprotein in response to various LL-37 therapies. Lanes 1 and 2 indicate Pre-treatment with 1.25 and 0.6 µg/ml respectively. Lanes 3 and 4 show the Simultaneous incubation of LL-37 with RVFV at a different concentration as lane 4 represents the concentrated viral stock. Lanes 5 and 6 show a direct inactivation effect mediated by LL-37 against different virus concentrations as lane 6 represents the concentrated viral stock. Lanes 7 and 8 represent the post-treatment effect of LL-37 against RVFV at different concentrations as 1.25 and 0.6 µg /ml respectively. Lanes 9 and 10 represent RVFV virus control and scrambling LL-37 respectively. β-actin is used as a loading control.

acid sequence, scrambling LL-37 was employed as a negative peptide control.

Different quantitative assays were used. The genomic RNA of the N protein in the S segment was quantified by RT-PRC in comparison to viral control. The Western blot technique is used to assess the quantity of N protein expression in response to various LL-37 treatment protocols. The TCID50 endpoint test was used to measure the viral CPE impact and to identify the morphological alterations associated with RVFV infections. A titer of RVFV that caused 50% cell death and a concentrated viral stock was utilized to explore the connection between LL-37 and the RVFV envelope. LL-37 exerted a significant antiviral activity at both titers and its effectiveness was negatively related to infection time. It was also observed that the LL-37 effect is dose-dependent. These data show that one of the mechanisms of action of LL-37 against RVFV is interfering with viral entry as an early event. The viricidal effect of LL-37 after incubation in the same physiological conditions supports the hypothesis that LL-37 disrupts the membrane in a single and potent attachment to the virus membrane rather than stepwise degradation in a detergent-like manner, as previously reported [41, 42]. Natural and synthetic LL-37 and LL-37 derivatives were evaluated against the RVFV MP-12 strain. Pre- and post-treatment with LL-37 resulted in a substantial reduction outside and inside the host cell when quantified by PCR [28]. The impact of LL-37 was negatively related to infectious time, and the maximum activity of LL-37 occurred during early viral events interfering with viral entry, which is consistent with the data found here. Our theory was tested against an RVFV strain obtained from Egyptian sheep and adapted to cell culture in BSL-2, besides using 2 different viral concentrations to determine the relation between viral particles and LL-37.

As a generalized antiviral action, the LL-37 effect against VEEV was seen in three distinct cell types. TEM pictures revealed a significant extracellular and intracellular virion aggregation in VEEV particles, indicating that LL-37 selectively interacts with viral particles, interfering with virus entrance [43]. By interacting with the viral membrane, LL-37 inhibits Kaposi sarcoma-associated herpesvirus [44]. LL-37 interacts with the DENV-2 envelope directly [45], but pre-incubating cells with LL-37 alone has little effect on viral activity. Here incubating LL-37 with RVFV for 1 hour before infection or imme-

diately infecting cells reduced RVFV titer and N mRNA expression, suggesting in the light of previous studies that LL-37 directly interacts with RVFV membrane in a dose-dependent manner.

The ability of LL-37 to reduce RVFV replication efficiently in a dose-dependent manner was established after 24 hours and maintained for 48 hours, as evaluated by western blot. The superior antiviral effect is mediated by LL-37 which immunomodulatory properties might explain. According to Weber, Gawanbacht et al. the presence of RNPs strongly activates the antiviral innate immune response by stimulating RIG-I; due to the characteristic panhandle structure of RVFV [46]. Furthermore, LL-37 up-regulated the production of several cytokines such as IL-6 and IL-10 as described in rhinovirus respiratory infection [47].

Prophylaxis activity against RVFV mediated by LL-37 was evaluated on Vero cells by delaying RVFV infection for 4 hours and performing washing steps. Significant protection against RVFV infection was observed for up to 4 hours after LL-37, which might be explained by LL-37 internalizing into cells and inducing protection in epithelial cells as previously reported for RSV [48]. LL-37 was tested against RSV and significantly inhibited RSV entry into HEP-2 cells at 2.5 µg/ml. Internalization of LL-37 into cells resulted in the retention of viral particles and the induction of the anti-RSV effect. [49]. The IVA viral activity was reduced by one log after pre-incubating cells with LL-37 at 10 µg/ml, whereas the greatest activity was observed after virus entry [50], disrupting the virus envelope. As demonstrated in Vaccinia virus [51], and HCV [52], pre-incubation of LL-37 profoundly impacts several viral membrane structures. Both naturally occurring and completely synthesized LL-37 suppressed HIV dose-dependently by decreasing reverse transcriptase activity [53].

Based on previous data and other published studies, it was concluded that LL-37 has direct antiviral activity against RVFV. LL-37 can directly bind to viral membranes and disrupt the viral envelope efficiently and rapidly for an extended period, as well as have indirect action that impacts post-entry events. LL-37 exerts antiviral activity by interfering with later stages in the virus's life cycle by inhibiting RVFV N protein expression and protein synthesis via immunomodulation. While pre-treatment with LL-37 permitted internalization in Vero cells. In all approaches effect of LL-37 was dose-dependent with the effect diminishing over time.

5. Conclusion

This study determined an unprecedented antiviral activity for the sole human cathelicidin LL-37 against RVFV cell culture-adapted strain isolated from sheep in Egypt, an important zoonotic pathogen for which no effective disease-modifying therapies or vaccines exist. It implicated that hCAP-18/LL-37 is an essential, targetable element of innate immunity against RVFV and has a prophylactic modulation. Furthermore, LL-37 can be used at different times and infectious points to prevent RVFV spread and transmission as a promising therapeutic agent, which in turn could apply to other arboviruses. More research will be conducted to assess the antiviral impact of LL-37 in vivo.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Consent for publications: The author read and proved the final manuscript for publication.

Ethics approval and consent to participate

No humans or animals were used in the present research. The study protocol was approved by the Ethics Committee of Suez Canal University no. 201810 PhD1.

Authors' contributions

All authors contributed to the study's conception and design. EM developed, optimized, and applied experiments, and was a major contributor in writing the manuscript. SS, AAB, and ASH reviewed this draft, contributed, and approved the final manuscript.

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