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Short Communication

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In vitro leishmanicidal activity of antimicrobial peptide KDEL against *Leishmania tarentolae*

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Abstract

Leishmaniasis, caused by the intracellular protozoan parasite *Leishmania,* remains an important neglected tropical infectious disease. Infection may be lethal if untreated. Currently, the available drugs for the disease are limited by high toxicity and drug resistance. There is an urgent need to develop novel anti-leishmanial strategies. Antimicrobial peptides (AMPs) have been described as the first-line immune defense against pathogenic microbes and are being developed as emerging anti-parasitic therapies. In the present study, we showed the anti-leishmanial activity of the synthetic 4-amino acid peptide lysine, aspartic acid, glutamic acid, and leucine (KDEL), the endoplasmic reticulum retention sequence, against *Leishmania tarentolae* promastigote and amastigote. Different concentrations of KDEL peptides were incubated with promastigotes, MTT viability assay, and promastigote assay were carried out. Macrophages infected with GFP-transfected *L. tarentolae* promastigotes were incubated with KDEL peptides, and the anti-amastigote activity of the KDEL peptides was measured by fluorescence microscopy. The damage of *L. tarentolae* was observed by light microscopy and electron microscopy. The cell apoptosis was analyzed using the Annexin V-FITC/PI apoptosis detection kit and mitochondrial membrane potential assay kit and by flow cytometry. Results showed that *L. tarentolae* was susceptible to KDEL peptides in a dose-dependent manner, and KDEL peptides disrupted the surface membrane integrity and caused cell apoptosis. In our study, we found for the first time an AMP KDEL from *Pseudomonas aeruginosa* and proved its significant therapeutic potential as a novel anti-leishmanial drug.

Key words: antimicrobial peptide, KDEL, *Leishmania* tarentolae, Leishmaniasis

Introduction

Leishmaniasis is a family of vector-borne diseases caused by the intracellular protozoan parasite *Leishmania* with diverse clinical manifestations, including cutaneous, mucocutaneous, diffuse cutaneous, viscera, and post-kala-azar dermal leishmaniasis [1]. It is one of the most prevalent causes of death and animal morbidity especially in undeveloped countries [2]. *Leishmania* parasites have two developmental forms: motile, rod-shaped promastigote in the vector sandflies, and non-motile spherical amastigote in mammalian hosts. Promastigotes are transmitted to the mammalian animal during the course of a blood meal and transformed into amastigotes after being phagocytosed by macrophages, which can survive inside parasitophorous vacuoles and escape from host immune responses [3]. An effective vaccine against leishmaniasis is not available, and chemotherapy is the only way to choose. However, current

treatments, including pentavalent antimonials, liposomal amphoteric B, paromomycin, and miltefosine, are toxic and expensive, and drug resistance has been emerging, making the search for new anti-leishmanial agents necessary. [4].

Antimicrobial peptides (AMPs) are low-molecular-weight amphiphilic peptides with activity against bacteria, fungi, and viruses. Previous studies have found various types of AMPs with different structures, including defensin, cecropins, magainins, and cathelicidins. They are naturally occurring compounds produced by all prokaryotic and eukaryotic cells and considered as essential components of the innate immune system against pathogenic microbes [5,6]. The mechanisms of AMPs are related to the disruption of the normal mycobacterial cell membranes, interaction with the intracellular targets, modulation of the innate immunity, and promotion of the adaptive immunity [7,8].

Pseudomonas exotoxin (PE) is a protein toxin that consists of three functional domains, including the receptor binding, translocation, and enzymatic domains. The receptor-binding domain can be genetically modified to create an immunotoxin by substituting the binding domain with antibodies specific for various cancers, leading to the killing of target cells by inhibition of protein synthesis via ADP-ribosylation of elongation factor 2 and induction of apoptosis [9]. By changing the amino acids at the COOH terminus of PE from REDLK to KDEL, the endoplasmic reticulum retention sequence, the cytotoxic activity of PE was found to be enhanced [10], prompting us to synthesize the short peptide KDEL and evaluate its bioactivities as an AMP.

In the present study, we used non-pathogenic *Leishmania tarentolae*, a parasite isolated from the gecko *Tarentolae annularis*, as an *in vitro* model for evaluation of the specific leishmanicidal activity and action mechanism of the peptide KDEL [11]. The present research demonstrates that KDEL derived from *Pseudomonas aeruginosa* is a promising candidate as a novel anti-leishmanial drug against *L. tarentolae* and lays a foundation for further studies on anti-leishmanial drugs.

Materials and Methods

Parasites

The UC strain of *L. tarentolae* promastigote preserved in our own laboratory was cultured in BHI medium supplemented with 1% penicillin–streptomycin and 5 μg/ml hemin (Sigma, St Louis, USA) at 26°C. GFP-transfected *L. tarentolae* was constructed using *Leishmania* expression vector pLEXSY-neo2 (Jena Bioscience, Thuringia, Germany), as previously described [12]. The GFP expression cassette was integrated into the chromosomal *ssu* locus of *L. tarentolae* via homologous recombination. GFP can be stably expressed in the life-cycle of *Leishmania* species, including promastigote and amastigote.

Peptide synthesis

KDEL, a 4-amino acid peptide (lysine, aspartic acid, glutamic acid, and leucine) with a molecular mass of 503.26 Da, was synthesized with a Symphony Multiplex Synthesizer (Rainin, Woburn, USA) and characterized by mass spectroscopy (**Supplementary Fig. S1**) and amino acid analysis. Purified peptides were lyophilized and dissolved in sterile PBS at a concentration of 1 mg/ml and stored at 4°C until use.

MTT viability assay

The viability of KDEL-treated *L. tarentolae* was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma), as previously described [13]. Briefly, promastigotes were seeded at 3×10^6 cells per well into a 96-well flat-bottom microtiter plate and exposed to 25, 50, and 100 μg/ml KDEL at 26°C for 72 h. PBS was used as the control. The plate was centrifuged at 3500 *g* for 15 min, resuspended in 100 μl fresh medium, and treated with 10 μl of 5 mg/ml MTT at 26°C for 4 h. Each group was repeated three times. Absorbance was read at 570 nm to determine the amount of formazan produced, which correlates with relative cell viability. Values were normalized to the untreated control. Cell viability (%) = ($A_{570\ nm}$ of treated cells/$A_{570\ nm}$ of control)×100%.

Promastigote assay

Parasites were seeded into a 96-well flat-bottom microtiter plate at 5×10^6/well in the presence of 25, 50, and 100 μg/ml KDEL or PBS as a control. Assays were performed in triplicate wells per concentration. After 72 h, viable parasite counts were determined using a hemocytometer following trypan blue staining.

Amastigote assay

Peritoneal macrophages, which were isolated from 4- to 6-week-old BALB/c mice by peritoneal lavage, were seeded into a 24-well flat-bottom microplate at 2×10^5 cells/well and cultured in RPMI medium (Thermo Fisher, Waltham, USA) overnight at 37°C in 5% CO_2 [6]. Macrophages were infected with stationary phase GFP-transfected *L. tarentolae* promastigotes at a ratio of one parasite per cell for 4 h and washed three times with RPMI medium. The cells were incubated with 25, 50, and 100 μg/ml KDEL or PBS for 72 h. The amastigote counts were determined by analyzing 20 microscopic fields under an inverted fluorescence microscope.

Light and electron microscopy

Leishmania tarentolae promastigotes were treated with different concentrations (25, 50, and 100 μg/ml) of KDEL or PBS at 26°C for 72 h. For light microscopy, 10 μl of each sample was loaded onto a glass slide, fixed with methanol, and stained with Giemsa. For electron microscopy, the ultrathin sections were prepared as previously described [14] and observed using a transmission electron microscope.

Cell apoptosis assays

After *L. tarentolae* promastigotes were treated with different concentrations of KDEL, the apoptotic cells were identified using the Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen, Nanjing, China) and a Mitochondrial Membrane Potential Assay Kit (JC-1) (Beyotime, Nantong, China) according to the manufacturer's protocols [15]. *Leishmania tarentolae* promastigotes were cultured in BHI medium with 25, 50, and 100 μg/ml KDEL, or PBS for 72 h. Carbonylcyanide-p-chlorophenol hydrazone (CCCP), which can cause quick mitochondrial membrane depolarization, was used as the positive control [16]. Cells were stained with JC-1 dye and analyzed by flow cytometry. Flow cytometry analysis was performed in a FACSCalibur Flow Cytometer (BD Bioscience, San Diego, USA), and the data were analyzed using BD CellQuest Pro software.

Statistical analysis

A one-way analysis of variance (ANOVA) was used for multiple group comparisons. Differences between the two groups were deter-

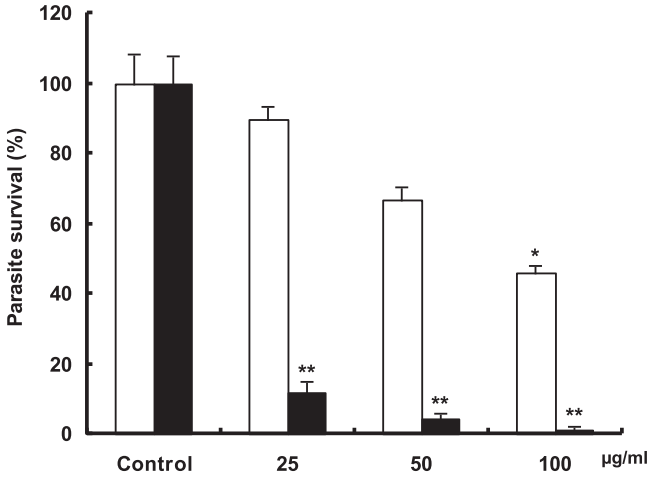

Figure 1. KDEL-mediated killing of promastigotes and amastigotes of *L. tarentolae* *Leishmania tarentolae* promastigotes and peritoneal-derived mouse macrophages infected with GPF-expressing promastigotes were incubated with different concentrations of KDEL at 26°C for 72 h. The promastigote (clear bars) and amastigote (black bars) counts were determined using a hemocytometer after trypan blue staining and analyzed using 20 microscopic fields under an inverted fluorescence microscope. Data were presented as the mean ± SEM of four replicates. $^{*}P < 0.05$, $^{**}P < 0.01$.

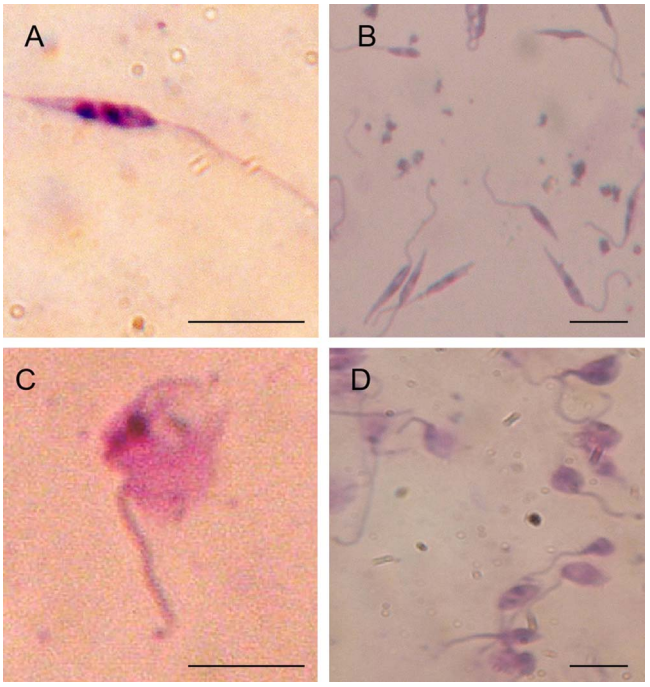

Figure 2. Morphologic changes of *L. tarentolae* promastigotes induced by KDEL Untreated (A,B) or peptide-treated (C,D) *L. tarentolae* promastigotes were Giemsa-stained after 72 h of exposure to 100 μg/ml KDEL. Magnification is ×1000 for the left row, ×400 for the right row (scale bar = 10 μm).

mined using a Student's *t*-test. ANOVA was used for multiple group comparisons. *P*-values of less than 0.05 were considered statistically significant.

Results

KDEL inhibited the growth of *L. tarentolae* promastigote and amastigote *in vitro*

KDEL was shown to have antibacteria activity with no cytotoxicity (Supplementary Fig. S2). We investigated the effects of the peptide KDEL on the growth of *L. tarentolae* promastigote. *Leishmania tarentolae* promastigotes were cultured in BHI medium with different concentrations of the peptide at 26°C for 72 h. Promastigote assay results showed that KDEL could inhibit the growth of *L. tarentolae* promastigotes by 10.4%, 33.3%, and 54.2% at the concentration of 25, 50, and 100 μg/ml, respectively (Fig. 1). MTT viability assay produced similar results (data not shown).

Transmission electron microscopy of KDEL-treated parasites presented a severe disruption of the membrane structure and a nearly complete loss of electron dense cytoplasmatic components (Fig. 3). This is consistent with the loss of membrane integrity in bacteria and in parasite incubated with AMPs and the action mechanisms of these peptides [6].

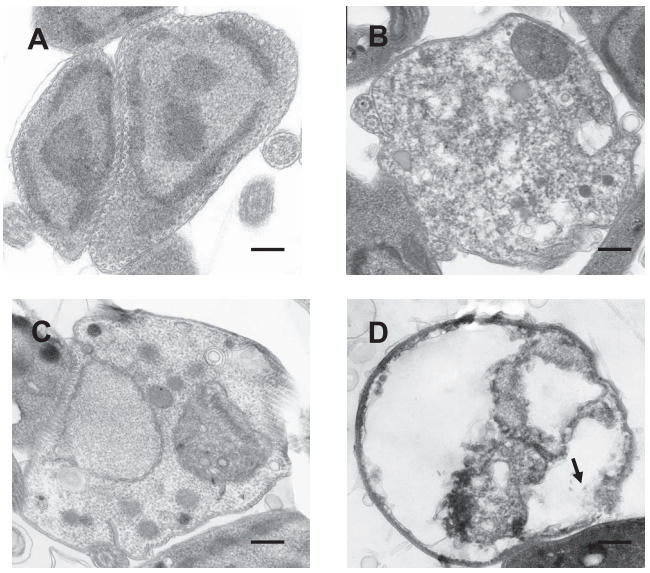

Figure 3. Transmission electron microscopy of KDEL-treated *L. tarentolae* promastigotes Parasites were cultured in media with PBS (A) or 25 μg/ml (B), 50 μg/ml (C), and 100 μg/ml (D) KDEL for 72 h. Membrane disruption (indicated by arrow), cytoplasm blebbing, and membrane breakages and depletion of electron-dense cytoplasmic material were observed (scale bar = 1 μm).

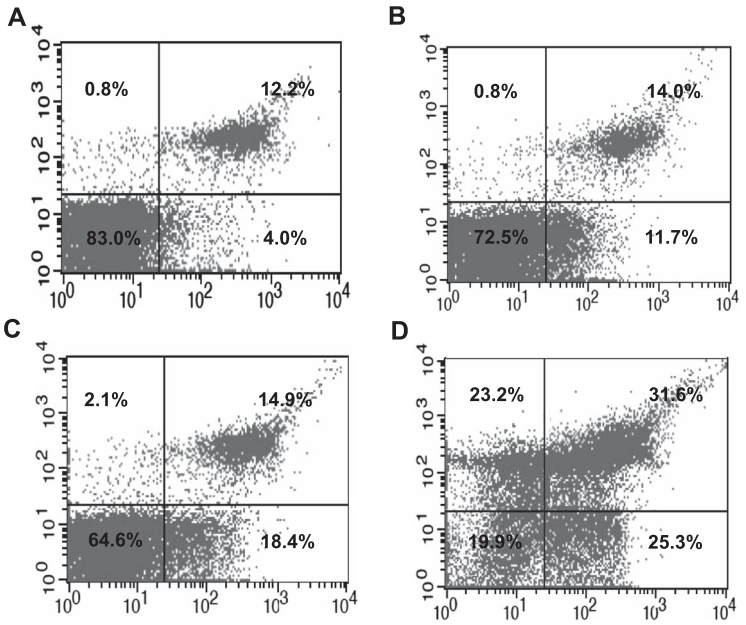

Figure 4. Cell membrane permeability and phosphatidylserine exposure induced by KDEL in *L. tarentolae* promastigotes Parasites were cultured in media with PBS (A), 25 μg/ml (B), 50 μg/ml (C), or 100 μg/ml (D) KDEL for 72 h. The cells were stained with Annexin V-FITC and PI and then analyzed by flow cytometry. The results are representative of at least three independent experiments.

To evaluate the anti-amastigote activity of KDEL, peritoneal-derived mouse macrophages were infected with GPF-expressing promastigotes and subsequently incubated with KDEL. The amastigote counts were then determined by analyzing 20 microscopic fields under an inverted fluorescence microscope and typical images were shown in Supplementary Fig. S3. The results showed that amastigote burdens in infected macrophages were inhibited by 88.4%, 95.9%, and 98.7% at 25, 50, and 100 μg/ml, respectively (Fig. 1). These data demonstrated that promastigotes and amastigotes were killed by KDEL in a dose-dependent manner, and amastigotes were more susceptible to KDEL than promastigotes.

KDEL induced morphologic changes of *L. tarentolae* promastigotes

Microscopic observation showed that KDEL can cause dramatic changes in the structural integrity of the parasite. Untreated promastigotes showed thin, elongated cells with a unipolar flagellum and a clearly visible nucleus and kinetoplast. However, KDEL-treated promastigotes presented rounding and swelling of cell bodies, with clear disruption of the cellular membrane accompanied by release of cytoplasmic contents into the medium. After 72 h of exposure, nearly all of the treated promastigotes lost their elongated form and appeared in various states of disintegration (Fig. 2).

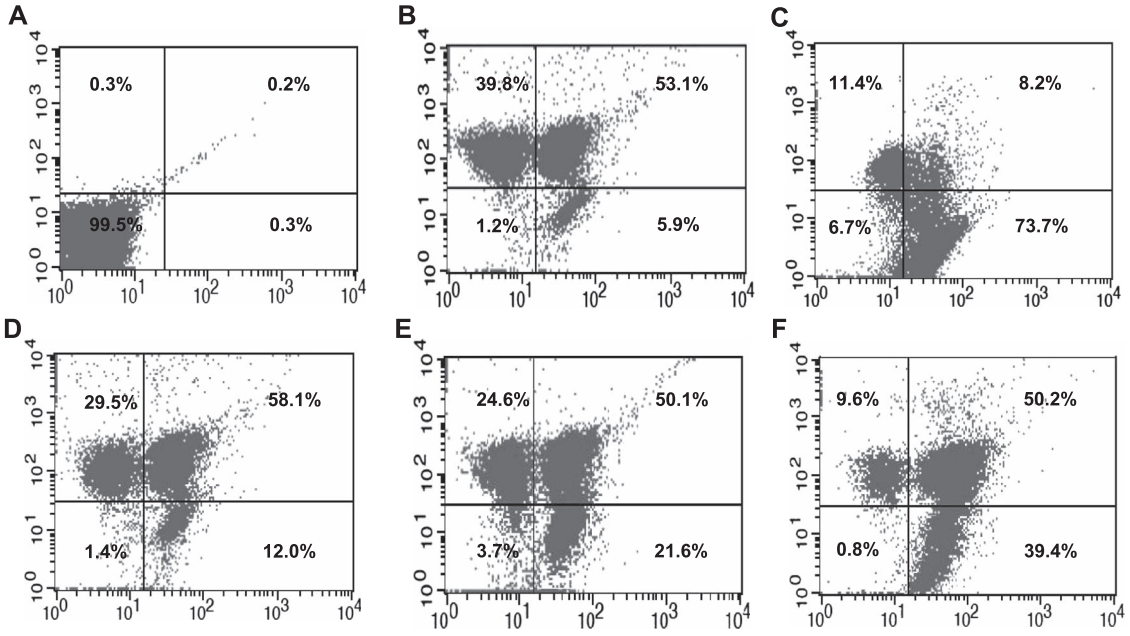

Figure 5. Depolarization of the mitochondrial membrane induced by KDEL To explore the mitochondrial membrane induced by KDEL, parasites were treated in different ways. (A) Untreated promastigotes not stained with JC-1. (B) Untreated promastigotes stained with JC-1. (C) CCCP-treated promastigotes stained with JC-1. (D–F) Promastigotes were treated with 25, 50, and 100 μg/ml KDEL, respectively, and stained with JC-1. Results are representative of three independent experiments.

KDEL induced membrane perturbation in *L. tarentolae* promastigotes

Cell apoptosis, caused by phosphatidylserine (PS) exposure and mitochondrial membrane depolarization, can also occur in unicellular protozoa. The externalization of PS from the inner side to the outer layer of the membrane is an early step of cell apoptosis. We co-stained KDEL-treated promastigotes with propidium iodide (PI) and Annexin V-FITC that is a Ca^{2+}-dependent phospholipid-binding protein with an affinity for PS. PI can permeate cells and bind to cellular DNA only if a loss of plasma membrane integrity occurs at the same time as necrosis or late-stage apoptosis.

The number of parasites staining positive for PI but negative for Annexin V (upper left quadrant) was significantly increased following exposure to 50 and 100 μg/ml KDEL, compared with that exposed to 25 μg/ml KDEL (Fig. 4), suggesting that the peptide-induced late-stage apoptosis or necrosis of the parasite is dose-dependent. The peptide could also induce early-stage apoptosis (lower right quadrant; Fig. 4). These results demonstrated that KDEL has distinct effects on promastigotes in terms of membrane permeability and PS accessibility.

The loss of mitochondrial membrane potential is a key indicator for the initiation of programmed cell death. After exposure to different concentrations of KDEL, JC-1-stained parasites led to a decrease in red fluorescence intensity (upper left quadrant) and increase in green fluorescence intensity (lower right quadrant), compared to untreated parasites. These results suggest that KDEL can induce the loss of membrane electrochemical gradient in the mitochondria (Fig. 5).

Discussion

AMPs are small molecules with a broad spectrum of antibiotic activities against antibiotic-resistant bacteria, protozoa, yeasts, fungi, and other pathogenic microbes and take part in the anti-inflammatory and immunomodulatory activities [17]. With the rapid increase in drug-resistant pathogenic microorganisms, AMPs, produced synthetic and natural sources, have been developed as novel therapeutic agents. AMPs are grouped into several families and share general features, such as hydrophobic and hydrophilic structures, and present in a soluble form. Therefore, AMPs could interact with biological membranes, inactivate intracellular targets, and modulate the host immune responses [10,18]. Several amphibian AMPs, such as magainin, temporin, dermaseptin, bombinin, and cecropin–melittin hybrid peptide, could kill a large number of pathogens, including bacteria, viruses, fungi, and parasites [19,20]. AMPs of DRS 01 were reported to possess anti-leishmanial activity [21]. In our study, we first demonstrated the leishmanicidal activity of a short peptide KDEL, the endoplasmic reticulum retention sequence, changing the amino acids at the COOH terminus of PE from REDLK to KDEL and enhancing the cytotoxic activity of PE.

Leishmaniasis is a tropical diseases caused by various leishmania species, posing a serious threat to millions of people worldwide every year. This disease ranges from self-healing lesions of cutaneous leishmaniasis to potentially lethal visceral leishmaniasis. Leishmania parasites infect vertebrate hosts through the bite of infected female phlebotomine sandflies and alternates between two life forms in its lifecycle: an extracellular promastigote form living in the digestive tract of female sandflies and an intracellular amastigote form residing in vertebrate macrophages [18]. *Leishmania tarentolae* belongs to genus *Leishmania* and shares over 90% homology with *Leishmania* species [22]. In the present study, we choose *L. tarentolae* as an experimental model for the following considerations. Firstly, *L. tarentolae* does not cause pathology in humans and mice [23] and could ensure the researchers safety. Secondly, *L. tarentolae* promastigote can differentiate into amastigote *in vitro* and can also infect macrophages *in vitro* [24]. The last, *L. tarentolae* amastigotes present similar sensitivity to pentavalent antimony and amphotericin B as other

Leishmania species that are pathogenic in humans [11]. Therefore, the non-pathogenic species *L. tarentolae* is a promising experimental model to evaluate the anti-leishmanial activities of AMPs.

In the present study, KDEL showed obviously leishmanicidal activity against *L. tarentolae* promastigotes and amastigotes in a concentration-dependent manner. Moreover, amastigotes were more susceptible to KDEL comparing with promastigotes. Metalloprotease gp63 [*Leishmania* donovani gp63 (Ldgp63)] was a critical virulence factor secreted by *Leishmania* and located on the surface of promastigotes, attached through a GPI anchor [25]. Ldgp63 knockout mutants are more susceptible to some types of AMPs and undergo cellular apoptosis more readily than wild-type parasites. Ldgp63 is significantly downregulated in amastigotes, which decreases the prevention ability of the peptide activity. Therefore, KDEL was more effective against *L. tarentolae* amastigotes than promastigotes [26,27].

There are at least two major mechanisms responsible for AMP-mediated antimicrobial effects, including apoptotic and non-apoptotic mechanisms [28]. In this study, the number of parasites positive for PI, but negative for Annexin V, was significantly increased following exposure to 50 and 100 μg/ml KDEL, compared to 25 μg/ml, suggesting that late-stage apoptosis of parasites induced by KDEL is dose-dependent. KDEL can cause significant disruption of membrane integrity in *L. tarentolae* promastigotes and ultimately result in osmotic instability, vacuolar swelling, loss of cytosolic contents, and eventual cell death. KDEL-treated parasites presented positive for PI, but negative for Annexin V, which resembled typical early apoptotic stage, in which PS exposure occurred, but the membrane was not permeable. The early apoptosis in promastigotes was also confirmed by depolarization of mitochondrial membrane potentials.

In conclusion, we evaluated the synthetic 4-amino acid peptide KDEL as a novel anti-leishmanial agent against *L. tarentolae* promastigote and amastigote. The action mechanisms of the peptide against *L. tarentolae* are related to disruption of surface membrane integrity and cellular apoptosis. Results suggest that KDEL, the endoplasmic reticulum retention sequence, has significant therapeutic potential as a novel anti-leishmanial drug, which should further be validated in parasites pathogenic for humans or animals.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Funding

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